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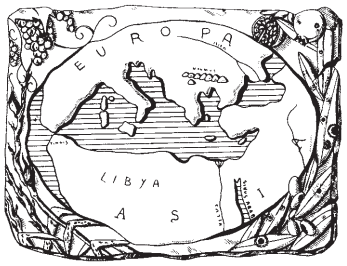

PHYTOPATHOLOGIA MEDITERRANEA

Plant health and food safety

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Research Papers

Molecular analysis of grapevine Pinot gris virus and its association with grapevine leaf mottling and deformation on 'Vermentino' grapevines in Sardinia

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Summary. In 2017–2018, grapevines of cultivar 'Vermentino' infected with grapevine Pinot gris virus (GPGV) in Sardinia, Italy, exhibited leaf symptoms of mosaic, chlorotic mottling, and curling, and stunted shoots. Disease incidence assessed in 2018 was greater (67%, 103 symptomatic plants out of 153 monitored) than in 2017 (26%, 40 of 153 plants). All symptomatic samples tested by RT-PCR were positive for GPGV in both years, while 70% (53 of 76) of the asymptomatic samples in 2017, and 42% (19 of 45) in 2018, were also positive for GPGV. Characterizing six GPGV isolates from 'Vermentino' by RT-PCR and sequencing of a genomic fragment covering the movement and coat protein genes showed high conservation at the nucleotide level (98.7% to 100.0%) among Sardinian isolates and isolates for which sequence information is available in GenBank. Phylogenetic analysis indicated that most Sardinian GPGV isolates grouped with other European isolates. This is the first characterization of GPGV in a Sardinian vineyard.

Keywords. GLMD, GPGV, symptoms, sequencing.

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INTRODUCTION

Grapevine Pinot gris virus (GPGV, Giampetruzzi *et al*., 2012) (*Trichovirus*, *Betaflexiviridae*) was identified by small RNA sequencing on the grapevine 'Pinot gris' in northern Italy. Since its discovery, GPGV has been reported in Korea (Cho *et al*., 2013), Slovakia and the Czech Republic (Glasa *et al*., 2014), Slovenia (Pleško *et al*., 2014), Greece (Martelli, 2014), France (Beuve *et al*., 2015), Turkey (Gazel *et al*., 2016), Georgia (Casati *et al*., 2015), Germany (Reynard *et al*., 2016), Canada (Xiao and Meng 2016), the United States

of America (Al Rwahnih *et al*., 2016), China (Fan *et al*., 2016b), Spain (Ruiz-García and Olmos 2017), Pakistan (Rasool *et al*., 2017), Brazil (Fajardo *et al*., 2017), Croatia (Vončina *et al*., 2017), Australia (Wu and Habili 2017), the United Kingdom (Silva *et al*., 2018), Chile (Medina *et al*., 2018), Ukraine (Eichmeier *et al*., 2018), Armenia (Eichmeier *et al*., 2019), Iran (Tokhmechi and Koolivand 2019), and Argentina (Debat *et al*., 2020). Plants infected by GPGV show a variety of symptoms that may include stunting, chlorotic mottling and leaf deformations (Giampetruzzi *et al*., 2012; Tarquini *et al*., 2018). Additionally, an association between GPGV and grapevine leaf mottling and deformation disease (GLMD) has been reported (Babini *et al*., 2018).

The GPGV genome is a single-stranded positive RNA of length 7,259 bp, excluding the 3' poly-A tail. The 5' untranslated region (UTR) is composed of 104 nucleotides in the SK13, SK01 and SK30 isolates, as determined by RT-PCR 5'-RACE. Furthermore, the 3' UTR is composed of 82 nucleotides in isolates ZA505-1A and SK30. GPGV is phylogenetically related to grapevine berry inner necrosis virus (GINV). The 5' UTR and 3' UTR of Slovak GPGV isolates share identities of 78 and 85%, respectively, with those of GINV (Giampetruzzi *et al*., 2012; Glasa *et al*., 2014). By comparing the genome of GPGV and other trichoviruses, identities of 69.0% with GINV (NC_015220), 49.0% with apple chlorotic leafspot virus (ACLSV, X99752), 48.7% with apricot pseudo-chlorotic leaf spot virus (APCLSV, AY713379), 47.7% with cherry mottle leaf virus (CMLV, NC_002500), and 48.6% with peach mosaic virus (PcMV, NC_011552), have been reported (Saldarelli *et al*., 2017). The GPGV genome is composed of three open reading frames regions (ORFs): ORF1 (214 kDa) encodes an RNA-dependent RNA polymerase (RdRP); ORF2 (42 kDa) encodes the movement protein (MP); and ORF3 (22 kDa) encodes the coat protein (CP) (Giampetruzzi *et al*., 2012). Different primer pairs were designed in the MP/CP (588 bp, Saldarelli *et al*., 2015), MP (302 bp, Glasa *et al*., 2014; 770 bp, Beuve *et al*., 2015) and CP (412 bp, Glasa *et al*., 2014; 430 bp, Bertazzon *et al*., 2016) genomic regions, and these are used for RT-PCR based diagnoses of GPGV.

Symptoms of stunting, leaf chlorotic mottling and deformation were observed on cv 'Vermentino' grapevines in Sardinia by Gentili *et al.* (2017). The aims of the present study were to monitor progression of GPGV and GLMD in a vineyard of 'Vermentino', the second most cultivated variety in Sardinia covering an overall area of about 4,200 hectares (http://www.sardegnavinitaly.it/), and to characterize Sardinian GPGV isolates.

MATERIALS AND METHODS

Study vineyard

The study was conducted in a 15-year-old, 1 ha 'Vermentino' vineyard located in northern Sardinia (Italy), near Olmedo (SS, 40°36'57"N, 8°23'05"E). The vines were in 26 rows with plant spacings of 1.8 × 0.8 m.

Monitoring and sampling

A total of 153 vines were monitored in the vineyard in the spring of 2017 and spring 2018 for typical GLMD symptoms. These included leaf deformation and chlorotic mottling, and shoot stunting. Symptoms of other grapevine virus diseases were also assessed. Five to six leaves per plant were collected to examine the presence of GPGV and other viruses based on molecular analyses.

RNA extraction and polymerase chain reaction (PCR)

For each sample, total RNA was extracted from 100 mg of petioles using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) following the manufacturer's protocol. A partial movement protein (MP) and coat protein (CP) genomic regions were amplified in RT-PCR with primers DET-Fow and DET-Rev (Saldarelli *et al*., 2015). The MP and CP genomic regions were also amplified in RT-PCR with primers 5637F and 5939R for MP, and 6609F and 7020R for CP (Glasa *et al*., 2014). PCRs were each performed in 50 µL containing: 1× Green buffer, 10-25 µg of DNA template, 0.2 mM of dNTPs, 0.5 µM of each primer, and 1.25 U of *Taq* polymerase (G2-Go *Taq* polymerase, Promega). The PCR programme included one cycle at 94°C for 1 min, 35 cycles each at 94°C for 20 s, 56°C for 20 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min. Reactions were carried out in a T100 ™ Thermal Cycler (BioRad). In addition, all samples were tested by multiplex RT-PCR to simultaneously test for grapevine leafroll associated virus 1 (GLRaV-1), grapevine leafroll-associated virus 2 (GLRaV-2), grapevine leafroll-associated virus 3 (GLRaV-3), grapevine virus A (GVA), grapevine virus B (GVB), arabis mosaic virus (ArMV), grapevine fanleaf virus (GFLV), and grapevine fleck virus (GFkV), according to published protocols (Gambino and Gribaudo 2006; Faggioli *et al*., 2012).

GPGV sequence analysis

The CP and MP gene sequences of six Sardinian GPGV isolates were analysed with the Vector software

Table 1. Sequences used in this study to generate phylogenetic trees. All the sequences were used to construct the coat protein tree (see Figure 3 a), and the sequences in bold font were used for construction of the movement protein dendrogram (Figure 3 b).

Accession number	Isolate	Country	Reference
KF686810	**SK 30-01**	**Slovakia**	**Glasa *et al.* (2014)**
KF134123	**SK-30**	**Slovakia**	**Glasa *et al.* (2014)**
KM491305	**Mer FR**	**France**	**Beuve *et al.* (2015)**
KR528581	**Tannat UY**	**Korea**	**Direct submission[a]**
KT894101	**TN US**	**California**	**Al Rwahnih *et al.* (2016)**
KX522755	**25-3 DE**	**Germany**	**Reynard *et al.* (2016)**
KU312039	**FEM01**	**Italy**	**Gualandri *et al.* (2017)**
KT345219	BJ-MGX	China	Fan *et al.* (2016b)
KF134125	**SK13**	**Slovakia**	**Glasa *et al.* (2014)**
FR877530	**IT**	**Italy**	**Giampetruzzi *et al.* (2012)**
KT345218	BJ-MLZ	China	Fan *et al.* (2016b)
KT345217	LN-HDQ	China	Fan *et al.* (2016b)
AB731567	KR	Korea	Direct submission[a]
KT345221	LN-MGX	China	Fan *et al.* (2016b)
KT345222	LN-PLZ	China	Fan *et al.* (2016b)
KU234316	**LN-Beta-RS**	**China**	**Fan *et al.* (2016a)**

[a] Sequences available in Genbank.

(Invitrogen). A CP phylogenetic tree was created using the sequences of 16 GPGV isolates and one GINV isolate (Table 1). An MP phylogenetic tree was constructed using the sequences of nine GPGV isolates and one GINV isolate (Wu and Habili 2017).

Two phylogenetic trees (Figure 3) were constructed from pairwise distance matrix by the Neighbor Joining method, applying MEGA version X software. Bootstraps analysis with 1000 replicates was carried out to estimate the statistical support of different tree branches. In addition, GPGV MP and CP sequence identities were compared to other sequences deposited in GenBank, using BLAST (NCBI).

Statistical analyses

All statistical analyses were carried out using R statistical software version 3.10 (R Development Core Team, 2016). A Chi-square test was used to assess differences in distribution of symptoms between the two years of monitoring. Pearson's standardized residuals were calculated to test observed *vs* expected values. Logistic regression model was used to test the probability that plants with specific symptoms was related to the presence of virus infections. Generalized linear models (GLMs) with binomial error distribution were considered to assess differences in probability of viral infection among plants showing different levels of symptoms (i.e. symptomatic, asymptomatic, or other symptoms). GLMs were carried out separately in 2017 and 2018 considering implicit and explicit bias reduction method suggested by Kosmidis and colleagues (2019) and using the brglmFit function in the "brglm2" package in R (Kosmidis 2020). Significance of predictor (i.e. symptoms) was tested using Wald test followed by multiple pairwise comparison (at $P = 0.05$) for means separation.

RESULTS

The main symptoms detected on 'Vermentino' leaves were typical of GLMD. These included mosaics and chlorotic mottling, reduced vein distension inducing leaf curling and folding of the margins. Stunted shoots with short internodes and apical shoot necrosis were also observed (Figure 1). Symptoms appeared in May-July in both years, and sometimes negatively affected plant growth and final grape yields.

To infer associations between symptoms and virus entities, 153 'Vermentino' vines were monitored and grouped into three symptom classes, of asymptomatic, symptomatic for GLMD or other symptoms (i.e. symptoms of other virus diseases). Symptom expression was different between the two years of vineyard monitoring ($\chi^2 = 60.08$, df = 2, $P < 0.01$). The numbers of asymptomatic vines and vines with other symptoms were significantly less in 2018 than in 2017. In contrast, the number of vines with specific GLMD symptoms was greater in 2018 than in 2017 (Table 2).

All vines exhibiting GLMD symptoms tested positive for GPGV in 2017 (40 of 40) and in 2018 (103 of 103), but 70% (53 of 76) of the asymptomatic plants also tested positive for GPGV in 2017, and 42% (19 of 45) of asymptomatic plants also tested positive for GPGV in 2018. The numbers of GPGV positive samples from symptomatic plants was greater ($P < 0.05$) in 2018 than in 2017 (Figure 2). The number of asymptomatic plants and plants with symptoms of other virus diseases decreased in 2018. Among all samples tested by multiplex RT-PCR, 20 were infected by GVA and 52 by grapevine GFkV. No samples were positive for GLRaV-1, GLRaV-2, GLRaV-3, GVB, GFLV or ArMV.

Results from GLM analyses showed that the probabilities of a vine testing positive for GPGV significantly differed among plants with or without GLMD symptoms, both in 2017 ($\chi^2 = 9.2$, $P < 0.01$) and 2018 ($\chi^2 = 44.1$, $P < 0.01$). The probability of GPGV infection in asymptomatic plants was 69.53 ± 5.2% in 2017, but was

Figure 1. Typical symptoms of chlorotic leaf mottling and deformation on a GPGV-infected 'Vermentino' grapevine. Stunted shoots with short internodes (a), mosaics, chlorotic pitting and curling of leaves (b).

Table 2. Occurrence of grapevines without (asymptomatic) or with GLMD symptoms (symptomatic), or with symptoms of other virus diseases (other symptoms) in 2017 and 2018. Results of Pearson's standardized residuals, indicating statistical differences between observed and expected values, are indicated in parentheses.

Plant appearance	2017	2018	Total
Asymptomatic	76 (+)	45 (−)	121
Symptomatic	40 (−)	103 (+)	143
Other symptoms	37 (+)	5 (−)	42
Total	153	153	306

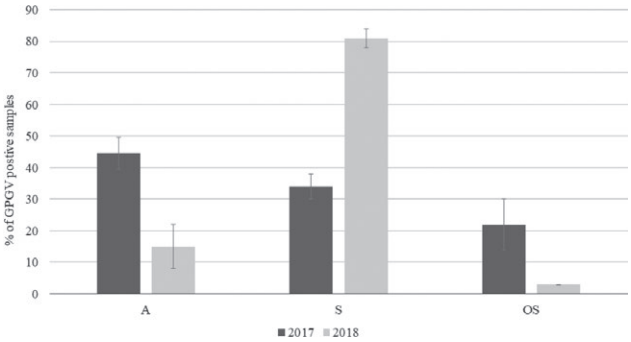

Figure 2. Mean proportions (%) of GPGV infected plants in 2017 and 2018 in different symptom classes of asymptomatic (A), symptomatic for GLMD (S) or with symptoms of other virus diseases (OS). Percentages were calculated for total numbers of positive samples in the two years of survey.

less in 2018 at 42.4 ± 7.4%. In contrast, the probability of a vine with symptoms of other viruses testing positive for GPGV was slightly less in 2017 (69.8 ± 7.5%) than in 2018 (75.9 ± 19.1%), but this was influenced by the very few plants with other symptoms detected in 2018 (Table 2). The probability of symptomatic plants testing positive for GPGV was high both in 2017 (98.9 ± 1.6%) and 2018 (97.7 ± 1.5%).

The GPGV MP and CP gene sequence analyses showed slight differences between the Sardinian sequences and sequences available in GenBank. The sequence identity ranged from 98.7% (VRM 4 MP) to 100.0% (VRM 9 MP). Both phylogenetic trees indicated that most Sardinian GPGV CP and MP gene sequences grouped with European isolates in a separate clade from sequences of Korean or Chinese isolates (Figure 3). The only exception was VRM5 MP, which grouped with isolates from Korea and the United States of America.

Sardinian GPGV isolates were 98.0% identical at the nucleotide level to most GPGV sequences available in GenBank. The identities of Sardinian GPGV CP and MP sequences were, respectively, approx. 70.0% and 82.0% with those of GINV.

DISCUSSION

GLMD symptoms were observed for the first time in 2001 in Slovenia (Pleško *et al.*, 2014) and then in

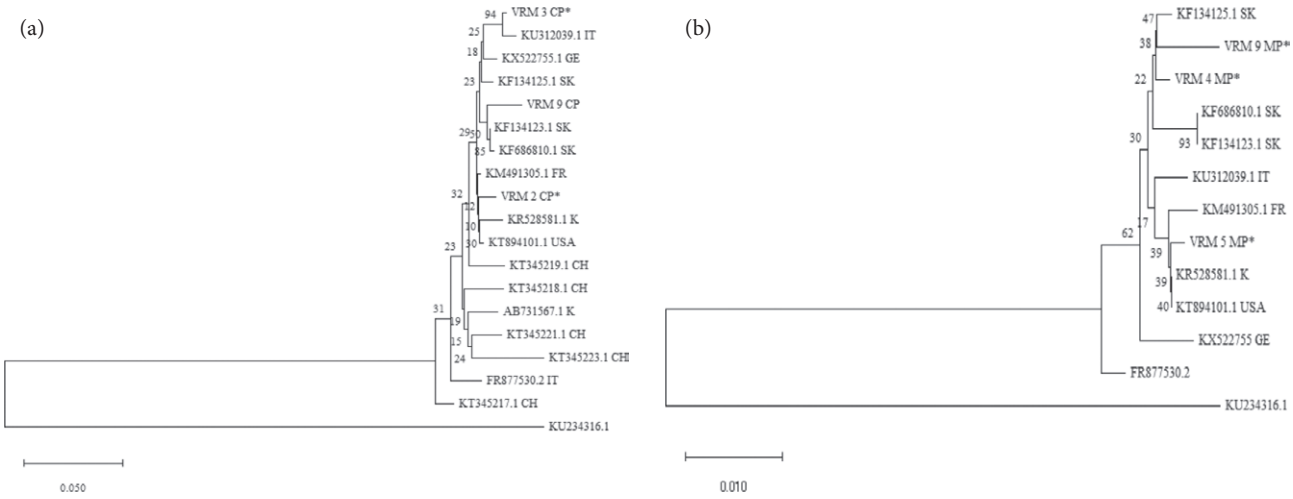

Figure 3. Phylogenetic trees generated from partial nucleotide genome sequences of the coat protein region (a) and movement protein region (b) of GPGV isolates. Bootstrap values were obtained using 1000 replicates. CP and MP sequences of GPGV isolates determined in this study are indicated with an asterisk. The phylogenetic trees were reconstructed using the neighbor joining algorithm implemented in MEGA X. Sixteen isolates from China (CH), France (FR), Italy (IT), Korea (K), Slovakia (SK), Germany (GE) or California (USA) were selected from GenBank database and used to construct the CP dendrogram (a). Nine isolates from Korea (K), France (FR), Italy (IT) or California (USA) were used for the MP dendrogram (b). The GINV sequence (KU234316) was used as the outgroup. The number on each tree node represents the bootstrap value.

2003 in Trentino (Giampetruzzi *et al*., 2012). In agreement with the observations by Giampetruzzi *et al.* (2012), GPGV on 'Vermentino' was also previously associated with GLMD in Sardinia (Gentile *et al*., 2017). In the 'Vermentino' vineyard selected for the present study, GLMD symptoms were observed for the first time in 2014. The present study confirmed the correlation between GLMD symptoms and the presence of GPGV in 'Vermentino' plants. Nonetheless, GPGV was also found in symptomless grapevines, as has been reported by Glasa *et al.* (2014). Several studies have suggested the existence of two GPGV strains, one causing disease symptoms and the other not associated with symptoms (Bianchi *et al*., 2015; Saldarelli *et al*., 2015; Bertazzon *et al*., 2017; Spilmont *et al*., 2018; Bertazzon *et al.,* 2020). In the present survey, several plants that were asymptomatic in 2017 became symptomatic in the following year. This may have been because of an increase of virus concentrations in the plant tissues from one year to the next, or due to the incubation period between mite-transmitted inoculation of GPGV and onset of host symptoms.

It would therefore be interesting to test samples by quantitative real time PCR to check whether clear differences in virus titre can be detected between symptomatic and asymptomatic vines that are infected by GPGV. In addition, virus isolates from asymptomatic samples should be sequenced to check for any difference in sequence composition.

Phylogenetic analyses indicated that Sardinian GPGV isolates mostly grouped in the clade of European strains, suggesting that the use of infected propagation material could be involved in the spread of the virus.

The emergence of new virus diseases is often associated with factors related to ecological changes or intensive agronomic practices (Elena *et al*., 2014), which are commonly and frequently occurring in some premium wine production regions where GPGV has been found. To reduce the expansion of GPGV, collaborative efforts are needed to clarify disease biology and epidemiology, and to include this virus in grapevine certification programmes.

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Research Papers

Candidate marker genes and enzymes for selection of potato with resistance to early blight, caused by *Alternaria alternata*

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Summary. Early blight, caused by *Alternaria alternata* (Fr.) Keissler is a serious disease of potato and other cultivated *Solanum* species. The molecular components defining defense responses to *A. alternata* in potato are limited. Host transcript accumulation after *A. alternata* inoculation of six potato genotypes (10/33/R1, 3/33/R2 and 21/33/R2, resistant to the pathogen, and 8707/106, 8703/804 and 8707/112, susceptible) was examined to develop understanding of mechanisms of their responses to *A. alternata* genotypes. The marker genes *PR-2*, *ChtA*, *PR-5*, *PR1-b*, *PIN2*, *ERF3*, *PAL* and *LOX*, activity of catalase (CAT), superoxide dismutase (SOD), peroxidase (POX), polyphenol oxidase (PPOs) and phenylalanine ammonia-lyase (PAL), as well as biomass growth parameters, were analysed. Expression of *PR-2*, *ChtA*, *PR-5*, *PR1-b* and *PAL* genes was greatly increased in the inoculated resistant genotypes compared to the susceptible genotypes and un-inoculated controls. Transcription levels of *PIN2*, *ERF3* and *LOX* genes were decreased in resistant inoculated plants. Simultaneously, activities of POX, SOD and PPOs were greatly increased in the inoculated resistant host genotypes, compared to the susceptible and non-inoculated controls. CAT activity in genotype 21/33/R2 and PAL activity in resistant genotypes 21/33/R2 and 10/33/R1 increased in the susceptible and non-inoculated. Host growth parameters of inoculated plants decreased compared to un-inoculated controls. Knowledge of changes in gene expression levels and enzyme production in defense processes in infected potato plants can inform future studies to identify the defense mechanisms, and assist generation of potato cultivars resistant to early blight.

Keywords. Antioxidant enzymes, biomass, defense mechanism, *Solanum tuberosum*.

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INTRODUCTION

Potato (*Solanum tuberosum* L., *Solanaceae*) an important human source of carbohydrates, protein and vitamin C (Hussain, 2016; Rajiv and Kawar, 2016; Kumari *et al*., 2018). Potato crops can also be grown in different envi-

ronmental conditions, and these provide important staple food in many regions (Hussain, 2016).

Among the diseases impacting potato crops, early blight is considered the most devastating. This disease is caused by several species of *Alternaria*, including *A. solani* and *A. alternata* (Nasr-Esfahani, 2018; Zhai *et al*., 2018). *Alternaria alternata* (Fr.) Keissler (*Dothideomycetes*) is capable of infecting *Solanaceae* crops, including potato, at all stages of plant development, causing considerable yield losses (Pourarian *et al*., 2018; Moghaddam *et al*., 2019). Severe impacts of *Alternaria* species on potato production occur in Iran, with the potato pathotype of *A. alternata* being the dominant pathogen species, causing significant yield and nutritive value losses (Nasr-Esfahani, 2018; Moghaddam *et al*., 2019). This pathogen causes brown spots with concentric rings on potato leaves, and ultimately leaf senescence (Esfahani, 2018a; Pourarian *et al*., 2018; Nasr-Esfahani, 2019). Early blight has the potential to reduce the tuber production by more than 20% in susceptible cultivars under favourable environmental conditions (Raimo *et al*., 2018; Ding *et al*., 2019).

Alternaria alternata causes necrotic lesions on potato leaves and tubers, although these lesions are smaller than those caused by *A. solani* (Ding, 2018; Ding *et al*., 2019). Field symptoms of early blight are often confused with physiological damage caused by ozone or nutrient deficiencies (Evenhuis *et al*., 2020). Although applications of fungicides have been recommended for control of early blight, indiscriminate use of pesticides may increase hazards to humans and the environment (Meier *et al*., 2015; Nasr-Esfahani, 2018). Therefore, effective, economical, and harmless, disease management is to develop and/or select disease resistant potatoes capable of producing satisfactory tuber yields, even within heavily pathogen-infested environments (Nasr-Esfahani *et al*., 2018; Moghaddam *et al*., 2020). Three potato clones (BR3, BR5, and BR85) incorporating combined resistances to early blight from *S. palustre* and late blight from *S. bulbocastanum* into a *S. tuberosum* background yield well in temperate climate conditions. In addition to possessing heritable resistance to early and late blights, these clones have other desirable agronomic traits, are fertile, and readily cross to established potato cultivars (Meier *et al*., 2015). Similar results were obtained through screening of several potato genotypes for early blight disease resistance (Odilbekov *et al*. 2014; Xue *et al*., 2019; Ding, 2021).

Pathogenesis-related (PR) protein synthesis genes have provided resistance to pathogens in various crops (McNeece *et al*., 2019). The studies of Wang et al. (2008), Derksen *et al*. (2013), Moghaddam *et al*. (2019) and Hoegen *et al*. (2002) emphasize on this hypothesis, supporting the role of *PR* genes in enhancing resistance to biotic stresses and providing a strategy for development of disease-resistant transgenic food crops (Ali *et al*., 2018). Zhai *et al*. (2018) used RNAi technology to silence the tomato *PR5* gene for resistance to *A. alternata*, and Toufiq *et al*. (2018) isolated the *chitinase* (*ChtA*) gene from *Hordeum vulgare* L., which could inhibit important pathogenic fungi. In a similar study, Khan *et al*. (2017) generated transgenic potato, which overexpressed the *H. vulgare endo-chitinase* gene, indicating high resistance of transgenic potato plants to *A. solani*. Thus, knowledge of changes in expression levels of *PR* genes in resistant and susceptible potato genotypes to *A. alternata* could indicate how *PR* genes play roles in potato resistance to this pathogen (Moghaddam *et al*., 2019; Bagheri *et al*., 2020). However, knowledge is scarce of the molecular defense responses with systemic (leaf) defenses before and after *A. alternata* inoculation of potato. So the present study aimed to provides information on leaf expression levels of the *PR-2, ChtA, PR-5, PR1-b, PIN2, ERF3, PAL and LOX* analysis genes in six contrasting potato genotypes after inoculation with *A. alternata.* In addition to pathogenesis-related (PR) protein synthesis genes, plant hormone mediated signalling pathways may also play important roles in plant disease resistance.

Proteinase inhibitors (PIN) in plants are small proteins involved in defense mechanisms against pathogenic microorganisms, that may imperil the plant integrity (antimicrobial properties) (Rehman *et al.,* 2017). Therefore, studying the changes in enzymes involved in defense processes in response to biotic and abiotic stresses can identify biochemical pathways for creation of resistant crop varieties (Bektas and Eulgem, 2015; Zhang and Liu, 2019; Isah, 2019). Activity of stress-related enzymes (PPO, POX, SOD, PAL and CAT) has been reported in pepper by Bagheri *et al*. (2021), tomato by Moghaddam et al. (2020), and apple by Huang et al. (2016), in response to *A. alternata,* with increases in activity of the related enzymes. These findings were supported by Yang *et al*. (2017) for tomato fruit, with activating and increased expression of corresponding genes for resistance to *A. alternata*. Although defense mechanisms vary across different cultivars, the antifungal effects of chitinases and other hydrolytic enzymes have been determined against *A. solani* and other biotic stresses (Moghaddam *et al.,* 2019). Further research also showed that the genes responsible for the production of pathogenesis-related proteins increased resistance against various pathogens in different crops (McNeece *et al.,* 2019). Furthermore, the role of *Glomus mosseae* and *Trichoderma harzianum* in the protection of cucum-

ber (*Cucumis sativus*) against *A. alternata* indicated increased activity of catalase and peroxidase enzymes was associated with increased resistance to this pathogen (Matrood *et al*., 2020).

In addition to a increased activity of antioxidant enzymes at different host growth stages, overexpression of pathogenesis-related (PR) proteins is a common and widely distributed defense mechanism in plants that minimize disease in non-infected plant organs (Zhang *et al*., 2012a, b; Ali *et al*., 2018). PR proteins are produced in plants after pathogen attack, and are induced as part of systemic acquired resistance (Moghaddam *et al*., 2019; Bagheri *et al*., 2020; Tehrani *et al*., 2020). Several studies have shown overexpression of related genes induced by pathogens encoding host PR proteins, Increase expression of PR1, PR2 and PR3 genes was observed in inoculated tomato genotypes resistant to *A. alternata* (Moghaddam *et al*., 2019; 2020). Furthermore, activation of PR-1 and PR-5 genes was affected by *Phytophthora infestans* in potato cultivars and proximity to the inoculation sites (Wang *et al*., 2008), and high accumulation of mRNA and protein of PR1-b occurred in response infection by this pathogen in potato leaves (Hoegen *et al*., 2002). Intact salicylic acid signalling is required for potato defense against the necrotroph *A. solani* with increases in expression of PAL1, PAL2, PR-1 and PR-2 genes in moderately resistant potatoes. This indicates the role of the salicylic acid pathway in plant defense response (Derksen *et al*., 2013; Brouwer *et al*., 2020). Hormone signalling pathways were induced in four potato genotypes by a concentrated culture filtrates of *P. infestans*. The genotypes were ranked according to their levels of resistance to *P. infestans*, and discriminant analysis of gene expression profiles separated the most resistant genotype from the three others, particularly because of a strong induction of the salicylic acid (SA) pathway. In this genotype, transcripts (*EDS1*, *WRKY1*, *PR-1* and *PR-2*) involved in the SA pathway were induced by concentrated culture filtrate. SA pathway involvement was confirmed by a peak of SA accumulation 12 h after elicitation and by the induction of *jasmonate Zim domain protein 1* transcripts, which inhibit defense responses mediated by jasmonic acid (JA) (Saubeau *et al*., 2016).

Studies of the molecular components defining defense responses to *A. alternata* in potato are limited. The present study aimed to provide knowledge to identify the genes involved in the resistance genes effective against this pathogen. Marker genes for phytohormones and defense-related enzymes, not all of which exclusively indicate defense responses, were examined after *A. alternata* inoculations to identify the genes involved in the resistance

MATERIALS AND METHODS

Plant material

Forty nine potato genotypes were obtained from the Potato and Onion Research Department, Seed and Plant Improvement Institute, Karaj, Alborz Province, Iran. These genotypes were from crosses between Lotta ♀*♂ Kaiser; Agria ♀*♂ Savalan; Agria ♀*♂ Kaiser; Kaiser ♀*♂ Savalan. Of these, the main parents were; Agria – Quarta ♀*♂ Semlo; Kaiser – Monalisa ♀*♂ Rop B 1178, and Savalan – 91/6122 ♀*♂ 88/05, and these were provided by the Plant Improvement Institute (PORD/SPII), Karaj, Alborz Province, Iran. Primary screening of the resistance levels of the potato genotypes to the potato pathotype of *A. alternata*, based on development of brown spot symptoms on leaves and tubers, were performed in the field under natural infection conditions. After this field screening, six genotypes, including three (10/33/R1, 3/33/R2 and 21/33/R2) resistant to the pathogen and three susceptible (8707/106, 8703/804 and 8707/112) were selected (Table 1). To confirm the resistance levels of these genotypes to *A. alternata*, re-screening experiments were carried out in a greenhouse in a completely randomized design experiment, with five replications for the inoculated and un-inoculated (control) host genotypes. These greenhouse experiments were carried out at the Isfahan Agriculture and Natural Resources Research Center, Isfahan, Iran (Esfahani, 2018a).

Fungus cultures, inoculum preparation and pathogenicity assessments

To prepare inoculum of the potato pathotype of *A. alternata*, active cultures previously isolated from infected potatoes sed. d. Comparisons with isolates available in the plant protection department of Isfahan Agriculture and Natural Resources Research Center, Isfahan, Iran were used to confirm identity of the prepared isolates. The isolates were sub-cultured on potato dextrose agar and maintained at 25°C for 10 d (Esfahani, 2018a; Ding *et al*., 2021).

Pathogenicity experiments were carried out by planting potato seed tubers in plastic pots (30 cm diam.) containing soil and perlite (1:1), in greenhouse conditions (18 to 25°C, 14 h of light). A potato seed tuber (approx. 50 g) treated with thiabendazole was planted in each pot. The experiments were arranged in completely randomized designs with five replicates (each of one potato tuber), for all the genotypes, and for experimental controls (non-inoculated tubers). The pots were irrigated each day (Nasr-Esfahani *et al*., 2017; Nasr

Esfahani, 2018b). Resulting 1-month-old potato plants were inoculated with sprayed conidium suspensions (10^3 conidia mL^{-1}), and each pot was then with aplastic bag for 48 h (Esfahani, 2018a). Seven days after inoculation, symptomatic leaves were harvested, and youngest newly emerged leaves were collected into aluminum foils and stored at −20°C for enzyme evaluations, and at −80°C for RNA isolation (Pourarian *et al*., 2018; Naderi *et al*., 2020; Yang *et al*., 2020).

Plant biomass growth parameters

Biomass growth parameters, including root fresh weight (RFW), root dry weight (RDW), stem diameter (SD), stem length (SL), stem fresh weight (SFW), stem dry weight (SDW), root diameter (RD), root length (RL), root volume (RV) and leaf length (LL), were measured for all plants. (Bagheri *et al*., 2020; Hashemi *et al*., 2020; Li *et al*., 2020). These parameters were measured 2 weeks after inoculation, by gently up-rooting each plant. Root volumes were measured using changes in the water volumes (mm^3). Each main root from the point of first secondary root initiation, and root collar diameter (mm) were measured using a digital caliper (accuracy = 0.01 mm). In addition, stem, root and the sixth leaf lengths were measured for each plant. Plant part dry weights were measured after drying at 80°C until constant weight (Hashemi *et al*., 2020).

RNA extraction and cDNA synthesis

The IRaizol kit (RNA Biotech Co.) was used for RNA extractions. For each sample, approx. 100 mg of fresh tip leaf tissue was ground to a fine powder in liquid nitrogen, and was the homogenized with 1 mL of extraction buffer containing 4 M guanidium thiocyanate, 25 mM sodium citrate/pH 7.0, 0.5% (w/v) *N*-lauroylsarcosine and 0.1 M 2-mercaptoethanol, and was then held at room temperature for 5 min. Chloroform (300 µL) was then added and mixed, the mixture was centrifuged at 13,000 g for 5 min. After transferring the supernatant, 1 mL of absolute ethanol was added and nucleic acid was precipitated (Moghaddam *et al*., 2019). After determining the quality and quantity of the extraction product using electrophoresis and nanodrop (NanoDrop 2000, Thermo Scientific), cDNA was synthesized with an RB M-MLV Reverse Transcriptase Kit (RNA Biotech Co.). Initially, extracted RNA was treated with DNase I (RNA Biotech, Co.), and cDNA synthesis was performed using the RB MMLV Reverse Transcriptase Kit (RNA Biotech, Co,) according to the manufacturer's instructions. First, 0.5 µg of treated RNA was mixed with 2 µM Oligo (dT) Primer and 1 mM dNTPs; this mix was then heated at 65°C for 10 min and then immediately placed on ice for 8–10 min. Then, RT buffer (5×; 4 µL) and 1 µL (200 units) of reverse transcriptase were added to each tube, and the tubes were incubated at 50˚C for 50 min followed by 15 min incubation at 72˚C to stop cDNA synthesis (McNeece *et al*., 2019; (Moghaddam *et al*., 2020).

Primer design

The primer sequences of pathogenesis-related protein 2 (*PR-2*), acidic endochitinase (*ChtA*), pathogenesis-related protein 5 (*PR-5*), pathogenesis-related protein 1b (*PR-1b*), proteinase inhibitor II (*PIN2*), ethylene-responsive transcription factor 3 (*ERF3*), phenylalanine ammonia-lyase (*PAL*) and lipoxygenase (*LOX*) genes were selected from the study of Arseneault *et al*. (2014), and elongation factor 1-alpha (*ef1-α*) by that of Gangadhar *et al*. (2016). The sequence of primers were evaluated and approved by Oligo Primer Analysis Software (ver. 7, Molecular Biology Insights). These primers reproduce fragments in the range of 60 to 66 bp (Supplementary Table 1).

Real-time PCR conditions

Real-time PCR reactions were carried out an RB Sybr qRT-PCR 2X Master Mix (RNA Biotech, Co.,) and in a StepOne Real-Time PCR instrument (Thermo Fisher Scientific). The reaction mixtures were each prepared in 0.1 mL qPCR 8-Strip Tubes (Gunster Biotech) as follows: 250 ng of cDNA, 12.5 µL of RB Sybr qRT-PCR 2X Master Mix and 0.25 µM of each primer in a final volume of 25 µL. The reaction temperature program was set as follows: 4 min at 94°C, then 40 cycles each at 94°C for 20 sec, annealing temperature (specified for each primer pair) for 20 sec and 72°C for 40 sec (Sohrabipour *et al*., 2018)., two technical repetitions were used for each sample. After the qPCR reaction was complete, the threshold cycle (Ct) values for each cDNA were calculated using StepOne Software (ver. 2.3, Thermo Fisher Scientific), and the equation was used to determine the relative expression levels of the evaluated genes (Wan *et al*., 2020). The *ef1-α* housekeeping gene was used for data normalization (Lekota *et al*., 2019; Liu *et al*., 2020).

Evaluation of defense-related enzyme activities

Protein extract preparation

Sodium phosphate buffer (0.1 M, pH 6.8) was mixed with 200 mg of each potato leaf sample, and then homogenized. The mixture was then centrifuged at 13,000 g for 10 min, the supernatant (protein-extract) was separated, and the protein concentration was determined using the Bradford method with a known concentration of Bovine serum albumin (A8806, Sigma) (Bradford, 1976; Nasr-Esfahani *et al*., 2020; Bagheri *et al*., 2021).

Superoxide dismutase (SOD) activity

For each sample, approx. 2 mL of reaction buffer (phosphate buffer 50 mM, methionine 13 mM, EDTA 0.1 µM, riboflavin 2 µM) was mixed with 100 µL of protein extract. This was then placed in the light for 15 minutes. For experimental control samples, the reaction buffer without protein extract was placed in darkness. Mixture absorption was measured at 560 nm. SOD activity was expressed units per mg protein (Giannopolitis and Ries, 1977).

Catalase (CAT) activity

For each sample, approx. 2 mL of reaction buffer (phosphate buffer 50 mM, pH 7, hydrogen peroxide 15 mM) was mixed with 100 µL of protein extract, and a mixture absorption change curve was recorded at 240 nm for 3 min. The enzyme activity was measured based on unit changes at 1 min mg^{-1} protein (Dazy *et al*., 2009).

Phenylalanine ammonia-lyase (PAL) activity

For each sample, approx. 2 mL of reaction buffer (Tris-hydrochloric acid 0.5 mM, pH 8, Phenylalanine 6 µmol) was mixed with 100 µL of protein extract and then held at 40°C for 1 h. To inhibit the reaction of cinnamic acid production from phenylalanine, 50 µL of hydrochloric acid (5 N) was added to the mixture, and absorption was measured at 290 nm. Enzyme activity was based on nanomoles of cinnamic acid production min^{-1} mg^{-1} protein (Beaudoin-Eagan and Thorpe, 1985; Kroner *et al*., 2011).

Peroxidase (POX) activity

For each sample, approx. 2 mL of reaction buffer (phosphate buffer 25 mM, pH 7, guaiacol 5 mM) was mixed with 100 µL of protein extract, and the spectrophotometer was zeroed with this mixture at 470 nm. Then, 5 µL of 30% hydrogen peroxide was added to the mixture and absorption was immediately measured at 10 sec intervals for 1 min. Enzyme activity was based on absorption changes min^{-1} mg^{-1} protein (Radotić *et al*., 2000).

Polyphenol oxidase (PPO) activity

For each sample, approx. 2 mL of reaction buffer (phosphate buffer 200 mM, pH 6, pyrogallol 20 mM) was held at 40°C, and 100 µL of protein extract was then added, mixture absorption changes in the mixture were measured at 430 nm (Raymond *et al*., 1993).

Statistical analyses

All the experiments were carried out with completely randomized designs, each with three replications, and with two technical replications for gene expression analyses. The qPCR data were analyzed using StepOne software and two-way analyses of variance (ANOVA). Enzyme activity data were analyzed using one-way ANOVA, and the LSD method was used for comparisons of means. The statistical analyses were carried out using SPSS software (ver. 16.0) (Li *et al*., 2018).

RESULTS

Disease severity evaluations of potato genotypes inoculated with Alternaria alternata

Analysis of variance of data from potato genotype reactions to *A. alternata* indicated different effects ($P \leq 0.01$) of inoculations on the host genotypes (Table 1). The greatest mean proportions of infection were 80% for genotype 8707/112, 78% for 8707/106, and 77% for 8703/804. The least mean infection proportions were 12% for 10/33/R1, 18% for both the 3/33/R2, and 21/33/R2 genotypes.

Effects of inoculations on biomass growth parameters

Variance analyses of potato plant biomass growth parameter data (Figure 3, A to J) showed that host geno-

Table 1. The impacts of the potato genotypes[a] evaluated in this study, including genotype numbers, registered name, .origin, scientific name, company, disease severity (%) and reaction to disease to leaf spot disease, *Alternaria alternata.*

S/No	Genotype No	Registered name	Origin	Scientific name	Company	Disease severity*, ** (%)	Reaction
1	10.25	10/33/R1	Karaj-Iran	*Solanum tuberosum* L.	PORD/SPII[a]	11.66[b] ± 2.88	Resistance
2	3.25	3/33/R2	Karaj-Iran	*Solanum tuberosum* L.	PORD/SPII	18.33[b] ± 5.77	Resistance
3	21.25	21/33/R2	Karaj-Iran	*Solanum tuberosum* L.	PORD/SPII	18.33[b] ± 5.77	Resistance
4	4.23	8707/106	Karaj-Iran	*Solanum tuberosum* L.	PORD/SPII	76.66[a] ± 5.77	Susceptible
5	10.23	8703/804	Karaj-Iran	*Solanum tuberosum* L.	PORD/SPII	78.33[a] ± 5.77	Susceptible
6	7.23	8707/112	Karaj-Iran	*Solanum tuberosum* L.	PORD/SPII	80.00[a] ± 10.00	Susceptible

*, ** significant at 5 or 1% probability level.

[a] Potato and Onion Research Department, Seed and Plant Improvement Institute, Karaj, Alborz Province, Iran.

Pedigree/Properties ♀*♂: These lines are the outcome of the cross between Lotta ♀*♂ Kaiser; Agria ♀*♂ Savalan; Agria ♀*♂ Kaiser; Kaiser ♀*♂ Savalan. The main parents are: Agria- Quarta ♀*♂ Semlo; Kaiser – Monalisa ♀*♂ Rop B 1178, and Savalan- 91/6122 ♀*♂ 88/05.

The experiments were performed in a completely randomized design (CRD) with 3 replications and 2 technical replications for gene expression analysis.

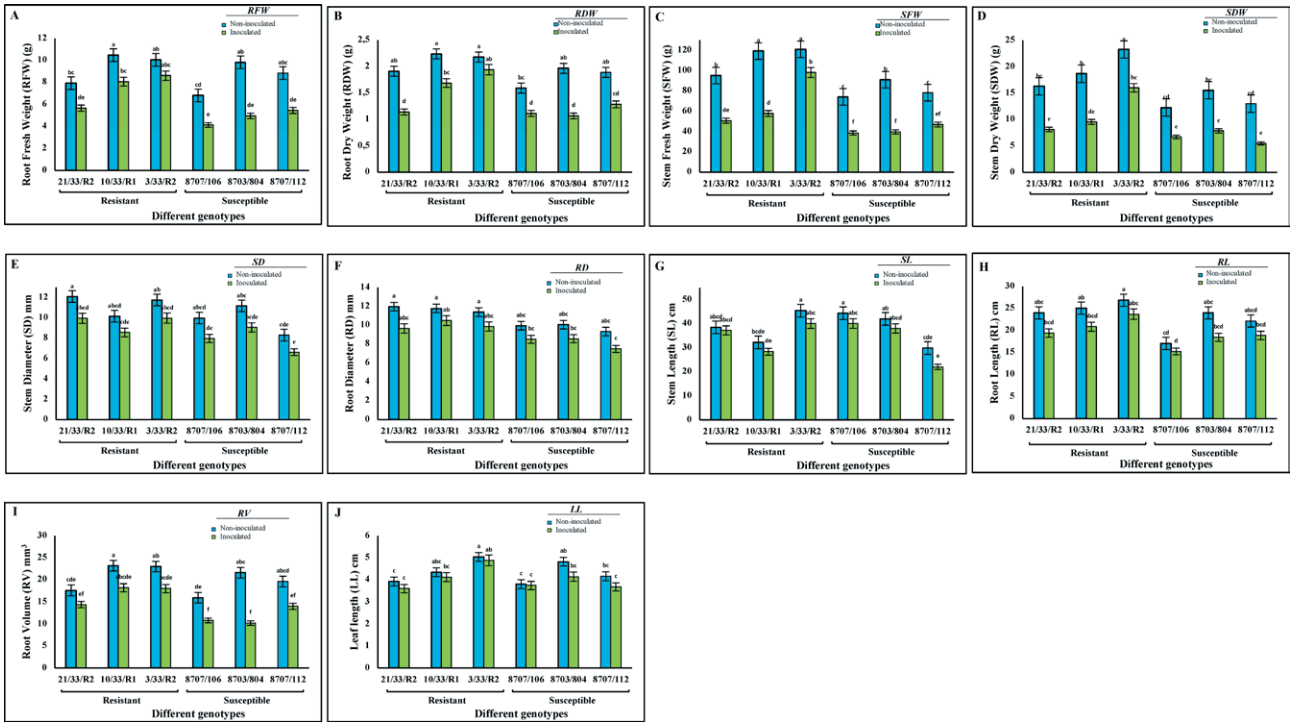

Figure 1. Variance analysis of potato plant biomass growth parameters, and comparison of the mean individual effects of inoculation treatment and host genotype in *Alternaria alternata* inoculated resistant and susceptible potato genotypes as compared to non-inoculated controls.

type and inoculation treatment affected ($P \leq 0.01$) plant growth (Supplementary Table 2). However, only the interaction of these two factors was statistically significant for SFW (Figure 1 C). Comparison of the individual means for the inoculation treatment effects showed reductions in parameters for inoculated samples compared to uninoculated controls. Individual effects of genotype were also statistically significant except SL (Figure 1 G), and LL, between the resistant and susceptible genotypes. Overall, the mean growth parameters were greater in non-inoculated resistant genotypes than in the inoculated plants. Greatest mean RFW was 10.5 g and mean dry

Table 2. Mean squares of analysis of variance for the relative expression level of defense genes, for susceptible and resistant potato genotypes to leaf spot disease, *Alternaria alternata.*

S.O.V	df	*PR-2*[a]	*ChtA*	*PR-5*	*PR-1b*	*PIN2*	ERF3	PAL	LOX
Inoculation treatment (I)	1	60.723**	22.040**	7.327**	22.070**	3.958**	0.014**	0.591**	10.731**
Genotype (G)	5	12.483**	4.237**	5.620**	6.257**	7.413**	0.833**	0.942**	2.173**
Interaction I × G	5	11.213**	3.681**	1.791**	6.734**	0.815**	1.158**	0.206**	0.834**
Error	11	0.280	0.139	0.061	0.087	0.067	0.034	0.036	0.076

Ns, *, ** not significant or significant at 5 or 1% probability level, [a]*PR-2* (pathogenesis-related protein 2), *ChtA* (acidic endochitinase), *PR-5* (pathogenesis-related protein 5), PR-*1b* (pathogenesis-related protein 1b), *PIN2* (proteinase inhibitor II), *ERF3* (ethylene-responsive transcription factor 3), *PAL* (phenylalanine ammonia-lyase) and *LOX* (lipoxygenase).

weight was 2.2 g for the non-inoculated resistant genotype 10/33/R1, and greatest mean SFW was 120.9 g and SDW was 23.3 g for 3/33/R2. Genotype 3/33/R2 had the greatest mean SD (11.8 cm), mean RD (11.4 cm), mean SL (45.5 cm), mean RL 26.9 cm), mean LL (23.0 cm), and mean RV (5.1 cm^3) (Figure 1). Correlation between plant growth parameters were statistically significant for all the evaluated factors, except for the relationship between SL with RFW, RDW, RL) RV (Figure 1).

Expression pattern analyses for defense genes

Analysis of variance of changes in expression levels of marker genes *PR-2*, *ChtA*, *PR-5*, *PR1-b*, *PIN2*, *ERF3*, *PAL* and *LOX* are presented in Table 2. These showed a significant ($P \leq 0.01$) effects of the inoculation treatment and host genotypes and their interactions on the transcription rates of the evaluated genes.

Expression of marker genes increased after inoculations with *A. alternata* in the resistant host genotypes (10/33/R1, 3/33/R2 and 21/33/R2), which had the lowest disease severities in the primary field resistance screening tests. The susceptible genotypes (8707/106, 8703/804 and 8707/112) and the non-inoculated controls genotypes had the lowest gene expression values (Figure 2, A, B, C, D and G). Up-regulation of *PR-2* in the host genotypes 10/33/R1, 3/33/R2 and 21/33/R2 was 4.95, 2.95, and 4.59 fold greater than that of controls. Up-regulation of *ChtA* was, respectively, 3.34, 3.29 and 4.46 fold greater in these three genotypes, and for *PR-5* was, respectively, 2.33, 1.87 and 1.73 fold greater than for the susceptible genotypes and the non-inoculated controls. Transcription of the *PR1-b* gene in these genotypes was, respectively, 2.46, 3.48, and 2.35-fold greater compared with non-inoculated controls. For the *PAL* gene, this increase was 1.49 and 1.30-fold, respectively, in genotypes 10/33/R1 and 3/33/R2. In genotype 21/33/R2, however, there was no change in *PAL* expression. Expression levels of these genes in the susceptible genotypes (8707/106, 8703/804 and 8707/112) decreased or were un-changed (Figure 2, A, B, C, D, and G). In genotype 8707/106, transcription changes of all the five genes assessed were not statistically significant. In genotype 8707/112, expression of *PR-2*, *ChtA* and *PR1-b* genes were un-changed, but increased by 1.58-fold got *PR-5* and 1.42-fold for *PAL* compared to non-inoculated controls. In genotype 8703/804, expression of *PR-2* and *PAL* genes remained un-unchanged, *ChtA* was up-regulated (1.69-fold), and *PR-5* and *PR1-b* genes were down-regulated (respectively, 1.58 and 1.88-fold) compared to susceptible genotypes and the non-inoculated controls genotypes.

Changes in the transcription levels of *PIN2*, *ERF3* and *LOX* genes in resistant and susceptible genotypes showed decreasing trends (Figure 2, E, F and H). For *PIN2* the relative expression level was 1.79-fold less in genotype 3/33/R2, 1.27-fold less in 21/33/R2 and 3.43-fold less in 8707/106 than for the non-inoculated controls. In genotypes 10/33/R1 8707/112 and 8703/804 expression of *PIN2* was un-changed. Reductions in expression of *ERF3* in the three resistant and 8703/804 genotypes were, respectively, 1.82, 2.34, 1.91 and 1.74-fold. Down-regulations in *LOX* expression were 1.49-fold in genotype 3/33/R2, 3.35-fold in 21/33/R2, 7.07-fold in 8707/106, and 4.65-fold in genotype 8707/112, compared with susceptible genotypes and the non-inoculated controls.

Comparison of gene expression level changes between inoculated resistant and susceptible potato genotypes indicated expression of *PR-2*, *ChtA*, *PR-5*, *PR1-b* and *PAL* genes in the resistant genotypes increased more than in susceptible genotypes, and that gene expression was 8.31-fold greater for *PR-2*, 5.72-fold greater for *ChtA*, 2.47-fold greater for *PR-5*, 8.61-fold greater for *PR1-b*, and 2.36 fold greater for *PAL*. For the *PIN2* and *LOX* genes, expression decreased in the *A. alternata* inoculated plants, and this decrease in *PIN2* and *ERF3* genes in resistant genotypes was greater than in the susceptible plants, with 1.24-fold decrease for *PIN2* and 1.87 fold decrease for *ERF3*. In the *LOX* gene, reductions in

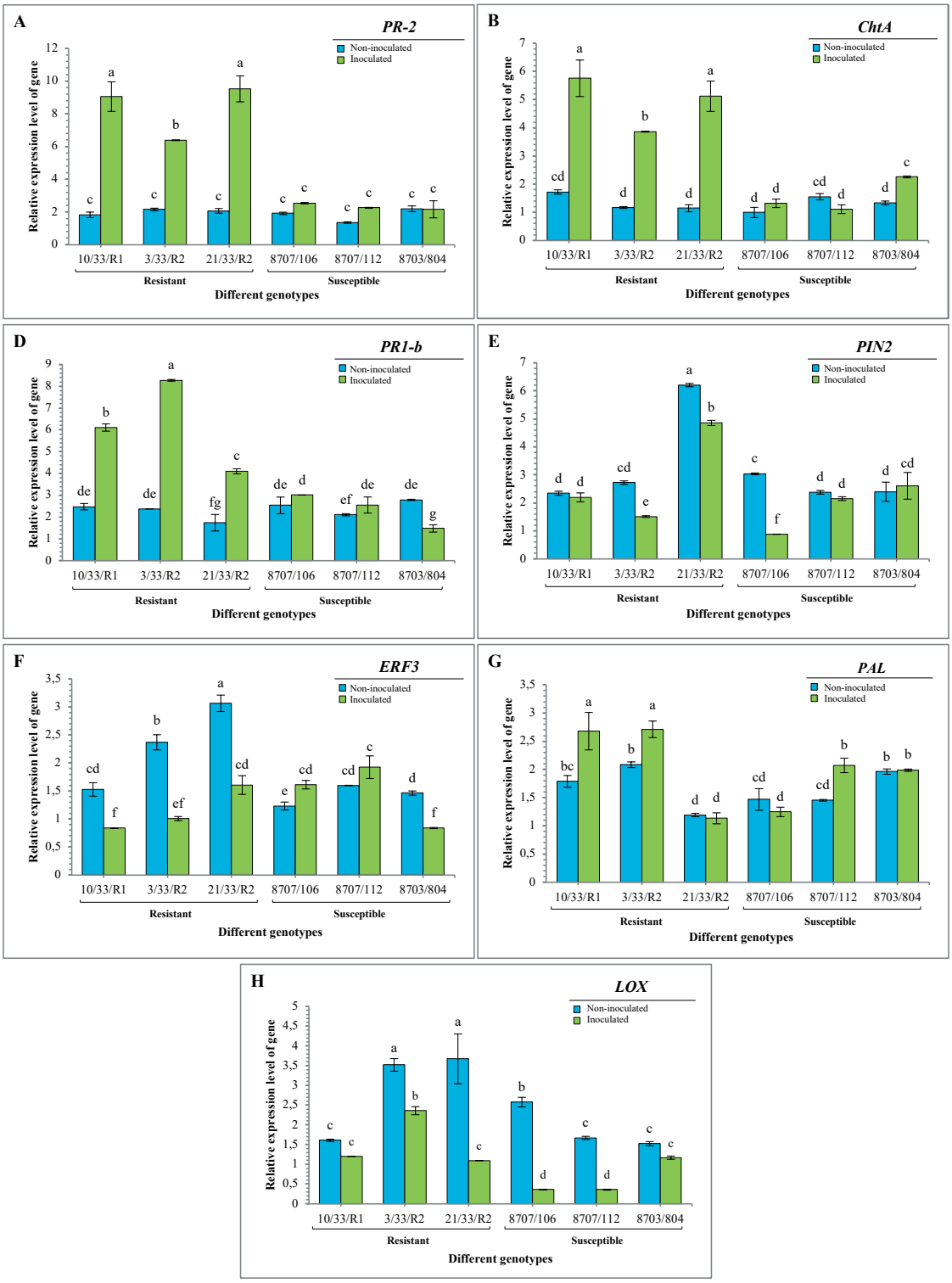

Figure 2. Relative expression levels of the genes for pathogenesis-related protein 2 (*PR-2*), acidic endochitinase (*ChtA*), pathogenesis-related protein 5 (*PR-5*), pathogenesis-related protein 1b (*PR-1b*), proteinase inhibitor II (*PIN2*), ethylene-responsive transcription factor 3 (*ERF3*), phenylalanine ammonia-lyase (*PAL*), and lipoxygenase (LOX), for *Alternaria alternata* susceptible (4.23, 7.23, 10.23) and resistant (10.25, 3.25, 21.25) potato genotypes. Normalization of the data obtained from the Real-time PCR reactions was carried out using the EF1α housekeeping gene. The experiment was carried out with three biological replicates for each sample and two technical replicates. Different letters indicate statistically significant differences ($P \leq 0.05$).

Table 3. Variance analysis of quantification of enzymes activities in inoculated resistant and susceptible potato genotypes as compared to controls, un-inoculated ones to leaf spot disease, *Alternaria alternata*.

S.O.V	df	Total protein	POX		SOD		PPOs		CAT		PAL	
			Activity	Specific activity	Activity	Specific activity	Activity	Specific activity	Activity	Specific activity	Activity	Specific activity
Inoculation treatment (I)	1	0.258**	6.352**	2.054**	0.605**	0.198**	4231.5**	1982.3**	98.3**	25.3**	1.021**	0.325**
Genotype (G)	5	0.316**	7.032**	3.004**	0.706**	0.252**	5054.4**	2007.6**	109.6**	31.1**	1.124**	0.354**
Interaction I × G	5	0.148**	5.264**	1.985**	0.352**	0.158**	3826.5**	2145.3**	85.3**	21.3**	1.251**	0.425**
Error	11	0.014	0.032	0.012	0.024	0.001	38.2	1.3	5.9	0.139	0.021	0.001

Enzymes activity of, superoxide dismutase (SOD), peroxidase (POX), polyphenol oxidase (PPOs), catalase (CAT), and phenylalanine ammonia-lyase (PAL).
** = Significant at 1% probability level.

expression in susceptible and resistant genotypes were very similar (Figure 2).

Changes in defense enzyme activities

Variance analyses of enzyme activity quantification data for inoculated resistant and susceptible potato genotypes compared to non-inoculated controls showed that host genotype factor affected ($P \leq 0.01$) enzyme activities in *A. alternata*-inoculated plants (Table 3). The greatest increase in activity was detected for the POX and PPO enzymes in genotype 10/33/R1, with 7.4-fold increase in POX and 4.7-fold increase in PPO (Figure 3, A, B, C, D and E). Changes in the specific activity of POX, SOD and PPOs enzymes (Figure 3, A, B and C) in inoculated resistant genotypes (10/33/R1, 3/33/R2 and 21/33/R2) showed significant ($P \leq 0.05$) incremental trends (Table 4) in all the three genotypes compared to non-inoculated controls. Specific activity of PAL was up-regulated 3.3 and 2.0-fold, compared to the controls. In genotype 3/33/R2, specific activity of CAT was un-changed, while that of PAL was 1.5-fold less compared with control samples. The activities of all the enzymes (POX, SOD, PPOs, CAT and PAL) in the susceptible genotypes (8707/106, 8703/804 and 8707/112) did not change greatly, with the greatest increase of 1.3-fold in comparison to resistant genotyped, and 7.4-fold increase after inoculation compared to non-inoculated controls (Figure 3).

Relationships between of defense gene expression, enzyme activities and biomass growth parameters

The results in the Table 5 showed statistically significant positive correlations between *PR-2*, *ChtA*, *PR-5* and *PR1-b* genes with POX, SOD and PPOs enzyme activities; *ChtA*, *PR-5*, *PR1-b* and *PAL* genes with PAL enzyme; *PAL* gene with POX, PPOs, CAT and PAL enzymes; and *PIN2* gene with CAT enzyme activities. In addition, changes in expression levels of these genes also showed positive significant correlations with some of the potato plant parameters, such as *PIN2*, *ERF3* and *LOX* genes with RFW, RDW, SFW SDW and RL; *ERF3*-SD; *LOX*-RD; *PAL*-SL; *PIN2* and *LOX* with RV; and PIN2, *ERF3* and *PAL* with LL, respectively (Table 5). For the *PAL* gene, a significant positive correlation was recorded with PPOs and PAL enzymes and SL parameter. The *LOX* gene did not show any correlation with changes in defense enzymes, and a significant positive correlation was found with the parameters SFW, RDW, SDW, RD, RL or RV. No statistically significant correlations were detected between the activities of defense enzymes and plant growth parameters, except in CAT enzyme with SL (r = .617*) (Table 5).

DISCUSSION

There were considerable changes in biomass growth parameters, with decreasing trends in plants inoculated with *A. alternata*, compared to the non-inoculated plants. The reductions in these parameters in susceptible genotypes were greater than in the resistant plants. Similar effects of *A. alternata* have been demonstrated in cotton seedlings (Le and Gregson, 2019), tomato (Moghaddam *et al*., 2019), *Cucumis sativus* (Matrood *et al*., 2020), and American ginseng (Neils *et al*., 2021). Reductions in growth of potato due to the pathogen in the present study confirms the deleterious effects of *A. alternata* on host growth.

The present study has shown significant increases in relative expression of marker genes in all the inoculated resistant potato genotypes. McNeece *et al*. (2019) stated that pathogenesis-related (PR) protein synthesis

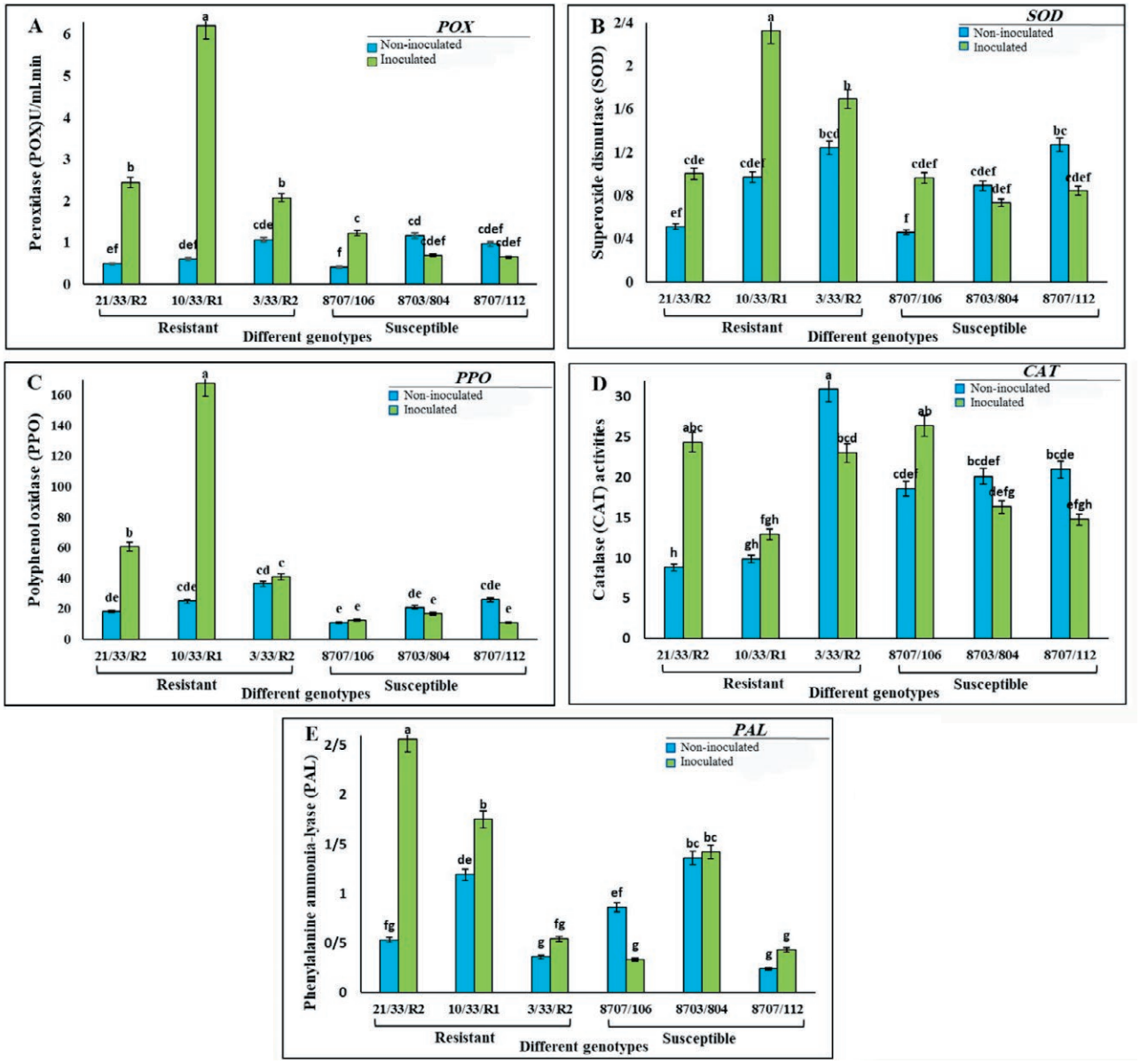

Figure 3. Activities of peroxidase (A), superoxide dismutase SOD (B), polyphenol oxidase PPO (C), catalase CAT (D), and phenylalanine ammonia lyase PAL (E) in *Alternaria alternata* inoculated potato genotypes that are resistant or susceptible to leaf spot.

genes provided resistance to pathogens in several crop plant types. Changes in *PR-2*, *ChtA*, *PR-5* and *PR1-b* in resistant potato genotypes indicate important roles of these genes in the direct defense mechanism of potatoes against *A. alternata*. Differences in resistance between the six potato genotypes can be partly explained by the potential for expression of defense-related proteins. Studies by Hoegen *et al.* (2002), Wang *et al.* (2008), Derksen, *et al.* (2013) and Moghaddam *et al.* (2019) have emphasized this hypothesis, supporting the role of *PR* genes in enhancing resistance to biotic stresses and providing an approach for development of disease-resistant transgenic products (Ali *et al.*, 2018). Zhai *et al.* (2018) used RNAi technology to silence the tomato *PR5* gene to *A. alternata,* and Toufiq *et al.* (2018) isolated *ChtA* gene from *Hordeum vulgare* L., which could inhibit important pathogenic fungi. Khan *et al.* (2017) generated transgenic potato which overexpressed the *H. vulgare endo-chitinase* gene, which gave high resistance of transgenic potato plants to *A. solani*. Thus, changes in expression levels of *PR* genes in resistant and susceptible potato genotypes to *A. alternata* detected in the present study,

Table 4. Quantification of enzymes activity in inoculated resistant and susceptible potato genotypes as compared to controls, non-inoculated ones to *Alternaria alternata*.

Resistance	Treatment	POX	SOD	PPOs	CAT	PAL
Resistant	Control	15.33b	0.225c	34.74b	7.32ab	0.717c
	Inoculated	3052a	0.885a	76.10a	8.26a	1.583a
Susceptible	Control	15.11b	0.239c	29.78b	4.79c	0.750b
	Inoculated	17.11b	0.409b	27.12b	5.57bc	0.667d

Enzymes activity of Superoxide dismutase (SOD), peroxidase (POX), polyphenol oxidase (PPOs), catalase (CAT), and phenylalanine ammonia-lyase (PAL).
Means in each column having same letter are not significantly different according to LSD test (P≤0.05).

Table 5. Correlation between expression of defense genes, enzymes activities and biomass growth parameters in inoculated resistant and susceptible potato genotypes as compared to controls, un-inoculated ones to leaf spot disease, *Alternaria alternata.*

Genes	POX[a]	SOD	PPOs	CAT	PAL	RFW	RDW	SFW	SDW	SD	RD	SL	RL	RV	LL
PR-2[b]	0.694*	0.943**	0.588*	0.320	0.471	-0.099	-0.154	-0.133	-0.161	-0.055	-0.033	-0.046	-0.021	-0.117	0.013
ChtA	0.693*	0.933**	0.596*	0.233	0.515*	-0.116	-0.184	-0.176	-0.224	-0.065	-0.066	-0.152	-0.009	-0.110	-0.067
PR-5	0.706*	0.904**	0.658*	0.173	0.661*	0.110	0.014	0.053	0.069	0.244	0.274	0.033	0.248	0.078	0.153
PR-1b	0.948**	0.828**	0.925**	-0.237	0.787**	-0.105	-0.194	-0.306	-0.301	-0.142	0.077	-0.212	-0.156	-0.103	-0.189
PIN2	-0.161	0.108	-0.180	0.604*	-0.365	0.528*	0.562*	0.686*	0.729**	0.474	0.350	0.401	0.635*	0.520*	0.712**
ERF3	-0.394	-0.344	-0.335	0.296	-0.326	0.521*	0.553*	0.667*	0.766**	0.516*	0.498	0.475	0.594*	0.487	0.689*
PAL	0.514*	0.461	0.590*	-0.562*	0.766**	-0.170	-0.303	-0.391	-0.440	-0.290	-0.032	-0.621*	-0.158	-0.141	-0.506*
LOX	0.030	0.036	0.154	0.014	0.040	0.750**	0.750**	0.755**	0.736**	0.466	0.727**	0.125	0.617*	0.813**	0.408

*, **: Correlation is significant at the 0.05 or 0.01 level.
[a] POD = Peroxidase, superoxide dismutase (SOD), polyphenol oxidase (PPOs), catalase (CAT), phenylalanine ammonia-lyase (PAL), root fresh weight (RFW), root dry weight (RDW), stem fresh weight (SFW), stem dry weight (SDW), stem diameter (SD), root diameter (RD), stem length (SL), root length (RL), root volume (RV) and leaf length (LL).
[b] *PR-2* (pathogenesis-related protein 2), *ChtA* (acidic endochitinase), *PR-5* (pathogenesis-related protein 5), PR-*1b* (pathogenesis-related protein 1b), *PIN2* (proteinase inhibitor II), *ERF3* (ethylene-responsive transcription factor 3), *PAL* (phenylalanine ammonia-lyase) and *LOX* (lipoxygenase).The bolded numbers show the significant correlations between the defense genes, enzymes activities and biomass growth parameters in inoculated resistant and susceptible potato genotypes as compared to controls, un-inoculated ones to leaf spot disease, *Alternaria alternata.*

and consistency of the results with the other reports, indicates that *PR* genes play important roles in creating resistance in potato genotypes against this pathogen (Moghaddam *et al*., 2019; Bagheri *et al*., 2020). However, knowledge of the molecular defense responses with systemic (leaf) defenses before and after *A. alternata* inoculation in potato is scarce. The present study is the first to provide information on leaf gene expression levels of *PR-2, ChtA, PR-5, PR1-b, PIN2, ERF3, PAL* and *LOX* in six contrasting genotypes of potato after inoculation with *A. alternata*.

In addition to pathogenesis-related (PR) protein synthesis genes, plant hormone mediated signaling pathways also play an important roles in plant disease resistance. Up-regulation of PR protein synthesis genes, *PR-2*, *ChtA*, *PR-5*, *PR1-b* and *PAL* was a reaction to the presence of *A. alternata* infections. These genes likely encode key enzymes in the salicylic acid (SA) biosynthesis pathway (Tian *et al*., 2020). Potato plants exhibit increased resistance to *A. alternata* infections utilizing both salicylic acid (SA) and jasmonic acid (JA) signaling pathways (Derksen *et al*., 2013; Brouwer *et al*., 2020). Additionally, it is becoming increasingly evident that not only the JA and SA pathways are important in the host defense against necrotrophs, but that the plant hormones abscisic acid and indole acetic acid can also modulate host defense against necrotrophs, including *A. alternata* (Derksen *et al*., 2013; Brouwer *et al*., 2020). However, the mechanism was demonstrated by Tian *et al*. (2020), where the two defense-related hormones, salicylic acid and jasmonic acid signaling transduction pathways have antagonistic effects. In general, the jasmonic acid signaling pathway enhances resistance to hemi-biotrophic and necrotrophic pathogens, while resistance to biotrophic pathogens mainly depends

on salicylic acid signaling pathways. The present study confirms that up-regulation of PR protein synthesis genes and antioxidant enzymes occurs in pathogen-resistant potato genotypes (Hu *et al*., 2018).

Analysis of the expression level of the *PIN2* gene in resistant and susceptible potato genotypes in response to *A. alternata* indicated expression did not change or decreased in inoculated genotypes. Arseneault *et al*. (2015) reported no changes in *PIN2* expression in potato leaves to inoculated with *Pseudomonas fluorescens* LBUM223 or *Streptomyces scabies* (Arseneault *et al*., 2014). In the present study, changes in *PIN2* gene transcripts did not affect resistance in the potato genotypes to *A. alternata*. The effects of *PIN* genes are considered as not suitable for genetic engineering for the resistant plants against this pathogen. (Turra and Lorito, 2011).Previous and the present study therefore confirm that *PIN* gene expression is little-affected by the fungal pathogens.

Ethylene-response factors (ERFs) are transcription factors binding to specific motifs on DNA and regulate ethylene-dependent resistance responses (Debbarma *et al*., 2019). Kim *et al*. (2012) showed overexpression levels of the genes *ERF I* and *ERF II* in sweet potato leaves in response to *Pectobacterium chrysanthemi*. Ogata *et al*. (2012) showed similar responses in tobacco to tobacco mosaic virus , and potato also similarly responded to *P. infestans* (Chen *et al*., 2008; Gallou *et al*., 2011). RNA-Seq analysis in apples inoculated to the *A. alternata* apple pathotype showed induction of subfamilies of *ERF* and *DREB* genes (Huang *et al*., 2016). A model to explain the response of chrysanthemums to *A. alternata* based on RNA sequencing information showed that the products of genes for abscisic acid signalling, salicylic acid, EDS1, ethylene metabolism (ERF2) and extrusion compounds (MATE) could play important roles in defending against *A. alternata* (Li *et al*., 2020). Contrary to these studies, the present study found that expression of *ERF3* in infected resistant potato genotypes decreased compared to non-inoculated controls. This suggests that the *ERF3* protective function is indirect, and changes in *ERF3* patterns are likely to lead to expression of defense genes that may enhance resistance to *A. alternata*. This hypothesis could be confirmed by functional studies, such as where gene silencing was confirmed by application of *Streptomyces scabies* in infection of potato (Arseneault *et al*., 2014), and by *A. solani* (Tian *et al*., 2020), where reductive changes in *ERF3* gene were also reported.

Activity of lipoxygenases has also been identified in pathogenic defense response processes, confirmed by Kolomiets *et al*. (2000) for accumulation of *POTLX-3* mRNA in the leaves of potatoes infected by *P. infestans*, and by Hu *et al*. (2015) for susceptibility of a transgenic host to a *Cladosporium fulvum* was increased. Hou *et al*. (2018) also generated transgenic *Arabidopsis* which overexpressed the persimmon *9-LOX* gene, indicating responses of increased salicylic acid content and bacterial mortality, and decreased cell death occurred in *Arabidopsis* to *Pseudomonas syringae* pv. *tomato*. Increasing changes in *LOX* gene expression levels have also been reported in *Fusarium oxysporum*-inoculated *Iris* (Tehrani *et al*., 2020) and *P. melonis*-inoculated cucumbers (Hashemi *et al*., 2020). In the present study, expression of *LOX* in resistant and susceptible potato genotypes decreased after inoculation with *A. alternata*, which was not consistent with the results of Kolomiets *et al*. (2000) for *P. infestans*, Hu *et al*. (2015) for *Cladosporium fulvum* or Hou *et al*. (2018) for to *P. syringae* pv. *tomato*. This could be due to decreased expression of the *ERF3* gene, or to increased reactive oxygen species, or to weakening of the lipoxygenase pathway. Results of the present study indicated that the defense response induced by *A. alternata* inoculation was different in the six selected potato genotypes. Expression of marker genes *PR-2*, *ChtA*, *PR-5* and *PR1-b* was significantly increased in resistant infected plants, indicating that these genes are involved in the defense response. Decreasing expression of *PIN2*, *ERF3* and *LOX* genes may indicate the lack, or indirect, effect of these genes in the defense processes against to *A. alternata*.

The present study also revealed changes in the activity of candidate enzymes in all the resistant potato genotypes, where activity of CAT enzyme in 21/33/R2 genotype and PAL in 21/33/R2 and 10/33/R was significantly increased. In similar studies, the resistance-inducing substances, salicylic acid, abscisic acid and *Pseudomonas fluorescens* increased activity of POX, PPO and PAL enzymes in potato infected by *P. infestans* and tomato infected by *P. atrosepticum* (Kroner *et al*., 2011), tomato infected by *A. alternata* (Moghaddam *et al*., 2020), and in cucumber (Hashemi 2020; Nasr-Esfahani *et al*., 2020). Results of the present study are consistent with this research, which showed increasing activity of the enzymes created resistance in potato genotypes. Induction of oxidation reactions to *A. alternata* and production of free radicals leads to the formation of chain reactions that damage cells (Moghaddam *et al*., 2019; 2020). Increasing activity levels of POX, SOD, PPOs and PAL enzymes terminate chain reactions and create oxidative balance. As a result, increasing the activity of antioxidant enzymes will lead to the biochemical response of resistant in potato genotypes and other crops (Kroner *et al*., 2011; Nasr-Esfahani *et al*., 2020; Bagheri *et al*., 2021). There is a critical need for understanding of the genetic population and biochemical response of resistant and

susceptible potato to *A. alternata*, and for incorporating this knowledge into plant breeding strategies to develop *A. alternata*-resistant crops.

There are no previous reports of marker genes, protein profiles and changes of host growth parameters in response to of *A. alternata* infection of potato, using transcriptomics-proteomics-biomass approaches. The results presented here are a basis for future studies, to design efficient disease management strategies against early blight of potato.

CONCLUSIONS

From forty-nine potato genotypes screened for resistance to *A. alternata*, lowest infection percentages were recorded for the three genotypes 10/33/R1, 3/33/R2, and 21/33/R2 which are possible sources of resistance to this pathogen. Decreasing trends in biomass growth parameters were also recorded for plants inoculated with *A. alternata* compared to un-inoculated controls, and these decreases were greater in the susceptible than in the resistant genotypes. Molecular analyses of eight genes and five enzymes potentially involved in host resistance have demonstrated that inoculation of potato plants with *A. alternata* increased expression of marker genes and activity of enzymes in inoculated resistant potato genotypes compared to non-inoculated controls. Studies to evaluate genes and enzymes involved in defense processes in different potato genotypes can increase knowledge of the roles of these factors in plant defense processes. This knowledge can assist in identifying and selecting resistant genotypes. The use of resistance resources in breeding programmes will lead to production of new cultivars with high performance and resistance to biotic stresses.

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Research Papers

Efficacy of chemical and biological spray seed treatments in preventing garlic dry rot

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Summary. Garlic dry rot caused by *Fusarium proliferatum* is an emerging postharvest disease that has resulted in severe economic losses, necessitating design and implementation of efficient disease control strategies. Sanitation of planting cloves is critical for preventing garlic dry rot. This study evaluated the efficacy of commercial chemicals and biocontrol agents, applied at planting as spray treatments, for reducing disease severity and the occurrence of *Fusarium* spp. in garlic, from the field stage then through 9 months of postharvest storage. Tebuconazole was the most effective for reducing disease severity, giving 26.5% reduction of basal plate rots and 44% reduction of bulb rots, and 33.4% reduction in visible symptoms on cloves relative to the untreated controls. Comparable results were obtained by applying *B. subtilis* and *S. griseoviridis*. However, none of the active ingredients tested in this study reduced the incidence of *F. oxysporum* and *F. proliferatum* on basal plates, although tebuconazole reduced the postharvest incidence of *F. proliferatum* on cloves by nearly 50%. Incidence of *F. proliferatum* increased by 37% in bulbs transferred from storage to room temperature (25°C) for 15 days, simulating storage in consumers' homes. These results demonstrate that spray seed clove treatments have inhibitory effects on postharvest garlic dry rot, although further research is required to determine the persistence of these treatments during prolonged storage, especially without low temperatures.

Keywords. *Allium sativum*, *Fusarium* spp.,fungicide, biocontrol agent, disease management, garlic dry rot.

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INTRODUCTION

Garlic (*Allium sativum*) is an important horticultural crop grown in temperate regions (Lopez-Bellido *et al.*, 2016). Garlic produced in Europe accounts for 3.4% of annual global production (FAO-STAT, 2018), and is of high quality, making a significant contribution to local economies (Spagnoli, 2014).

In northern Italy, garlic production starts with planting in mid-October to early November, with harvest in July of the following year. Bulbs develop during springtime starting from the end of April. After harvest, garlic bulbs are sun-dried for 15 to 30 d; some are selected as planting material for subsequent production, while others are stored for up to 9 to 10 months in cold chambers at −4°C until they are delivered to market. Consumers purchase

and store garlic at room temperature until consumption, which can be for 10 to 20 d or longer.

Postharvest decay of garlic, in which bulbs are partially or completely empty, was first reported by consumers. Upon close observation, centrally depressed polygonal brown spots were observed under clove sheaths during the drying process, with white mycelia visible in severe cases. These symptoms were mostly recorded postharvest and resulted in yield losses of up to 30% (Tonti *et al*., 2012). In 2002, garlic dry rot was first described as an emerging global disease caused by *Fusarium proliferatum* (Dugan *et al*., 2003), which was later confirmed by several authors in different geographic areas (Stankovic *et al*., 2007; Palmero *et al*., 2010; Sankar and Babu, 2012; Tonti *et al*., 2012; Salvalaggio and Ridao, 2013; Quesada-Ocampo *et al*., 2014; Leyronas *et al*., 2018). In the field, *Fusarium* spp. cause symptoms on garlic roots or remain latent between leaves and cloves (Stankovic *et al*., 2007; Mondani *et al.,* 2021b). In the only systematic studies to date carried out in Italy, the two main species isolated from asymptomatic and symptomatic cloves at field and postharvest stages were *F. proliferatum* and *F. oxysporum,* with *F. proliferatum* also confirmed as a fumonisin producer (Mondani *et al*., 2021a, 2021b), in agreement with Seefelder *et al*. (2002). These studies confirmed the relevance of infected seed cloves for dry rot outbreak, even if it is primarily a postharvest disease.

A study conducted in North America showed that the incidence of bulbs infected with *F. proliferatum* at harvest ranged from 25% to 50%, and that up to 77% of cloves were symptomatic when peeled after 9 to 16 months of storage, even if they appeared healthy and firm at the time of harvest (Dugan *et al.,* 2019).

As garlic is propagated vegetatively, selection and use of healthy seed cloves is critical for reducing fungus dissemination and dry rot severity. There are no reports of appropriate methods for selecting healthy garlic seed cloves on industrial scale, or on control strategies to prevent/reduce *Fusarium* incidence along the garlic production chain. Some studies have evaluated the efficacy of chemical or biological treatments against *Fusarium* spp. in garlic. The efficacy of the benomyl in wounded bulbs was previously demonstrated (Dugan *et al*., 2007), but this fungicide has since been withdrawn in Europe. Due to the EU Directive on Sustainable Use of Pesticides (2009/128/EC), the number of approved chemical active ingredients has decreased, and this trend is expected to continue. It is therefore important that alternative ingredients and control methods are considered, especially those with greater sustainability than pesticides (Lamichhane *et al*., 2016; 2020).

Gálvez Patón *et al*. (2017), in Spain, evaluated the commercial fungicides Cabrio® Duo (dimetomorph + pyraclostrobin), Luna® Experience (fluopyram + tebuconazole, and Flint® Max (tebuconazole + trifloxystrobin), with promising *in vitro* results. In these experiments, *F. proliferatum* was grown on potato dextrose agar (PDA) plates containing low amounts of the fungicides. However, the same products applied in the field as foliage sprays during crop development (May and June) failed to prevent postharvest garlic rot,

Thermotherapy at 50°C has been reported to greatly decrease the viability of *F. proliferatum* conidia grown *in vitro*, but the treatment has yet to be tested on garlic seed cloves (Palmero Llamas *et al*., 2013). Other studies have also reported that *Fusarium* spp. was controlled *in vitro* with biocontrol agents (BCAs) (Ju *et al*., 2013; Evangelista-Martínez, 2014; Ghanbarzadeh et al., 2014; Samsudin and Magan, 2016), but these agents have not been tested on garlic crops in the field. *Trichoderma* spp. and *Bacillus subtilis* were the most promising BCAs, with laboratory trials (Mondani *et al*., 2021c) demonstrating their efficacy against *Fusarium* spp. isolated from garlic.

Garlic seed clove treatments have not been tested, but this disease management strategy may ensure protection in early stages of plant development and improve yield quality and postharvest product preservability. Attention has been paid to this approach, although detailed knowledge is lacking (Lamichhane *et al*., 2016; Pedrini *et al*., 2016; Hitaj *et al*., 2020). The lack of efficacy of foliar applied fungicides suggests that intervention on propagation material could prevent pathogen activity from early crop growth stages, particularly as *Fusarium* spp. have been confirmed in seed cloves.

The aim of the present study was to assess the efficacy of chemical and biological active ingredients, applied as spray pre-planting seed clove treatments, for preserving garlic bulbs during crop growth and postharvest storage. Disease incidence and severity, caused by *Fusarium* spp., in garlic bulb basal plates and tissues, were used as measures of the effectiveness of the seed clove treatments.

MATERIALS AND METHODS

Media

Water agar (WA) was prepared by dissolving 20 g of agar (2%; Oxoid) in 1 L of double-distilled water. PDA was prepared by mixing 15 g of agar (2%; Oxoid) and 10 g of dextrose with 1 L of potato broth (200 g of potato per litre of water).

Table 1. Commercial plant protection products (chemicals or biocontrol agents) used in field trials.

Product	Active ingredients	Manufacturer	Product label use rates
Chemicals			
MYSTIC 430SC	Tebuconazole, 250 g L^{-1}	Nufarm Italia, Bologna, Italy	580 mL ha^{-1}
BUMPER P	Prochloraz, 400 g L^{-1}/l + propiconazole 90 g L^{-1}/l	ADAMA Italia, Grassobio, Italy	1100 mL ha^{-1}
Biocontrol agents			
Serenade Max	*Bacillus subtilis* (5.13×10^{10} CFU g^{-1})	Bayer Crop Science, Milan, Italy	2500 g in 500 L, as seed clove treatment
Mycostop	*Streptomyces griseoviridis* K61 (5×10^{8} CFU g^{-1})	Verdera Oy, Kurjenkellontie, Finland	10 g in 100 L, as seed clove treatment
Remedier	*Trichoderma harzianum* + *T. gamsii* (3×10^{7} CFU g^{-1})	Isagro, Milan, Italy	10 g L^{-1} as seed clove treatment + 2500 g ha^{-1} as soil treatment
Lifestrong Vam L	*Mycorrhiza* (100 spores g^{-1}) + *B. subtilis*, *Pseudomonas fluorescens*, *Azospirillum lipoferum* (3×10^{8} CFU g^{-1}) + *Trichoderma* (1×10^{8} CFU g^{-1})	Fertilidea Srl, Pompei, Italy	2 to 4 L ha^{-1}

Seed treatments, field location, and experimental design

The experiment field was located in San Pietro in Cerro, Piacenza (northern Italy; 45.01 N, 9.94 E). The research was conducted over two consecutive garlic growing seasons (2017–2018 and 2018–2019). Six commercial plant protection products were evaluated, including two chemicals and four BCAs. These had been shown to be effective in preliminary *in vitro* experiments (Mondani *et al.*, 2021c). All the products were used at the doses indicated on the product labels (Table 1). At the end of October each growing season, spray seed clove treatments were applied through the planting machinery (four row bulb planter; JJ Broch), by wetting the cloves and spraying the products into planning furrows. The trials were each arranged in a strip plot design with four replicates, with each strip covering an area of 810 m^2, with interrow distance of 30 to 40 cm and seed clove density of 27 cloves m^{-2}.

Monitoring of seed clove treatment efficacy throughout the garlic production process

Garlic plants (locally developed variety Ottolini) were sampled over the 2 years of this study, both from the field and postharvest, as shown in Table 2. Three crop growth stages were selected to monitor disease severity and fungal incidence in bulb basal plates and tissues: BBCH 15 (5th leaf visible; mid-April), BBCH 45 (half bulb final diameter; end of May), and BBCH 49 (harvest; end of June) (Lopez-Bellido *et al.* 2016; Mondani *et al.*, 2021b). Harvested bulbs were sun-dried for 1 month until the beginning of August in an open field (with coverage in case of rain) and were then stored in cold chambers at −4°C for 9 months, according to the locally used management practices. Inspections of symptomatic bulbs postharvest were carried out four times: after 3 months (end October), 6 months (early February of the following year), and 9 months (mid-May of the following year) of cold chamber storage, and then 15 d after the bulbs were transferred from the cold chamber to room temperature (≈ 25°C), simulating the period after delivery to consumers. After 9 months of storage and 15 d at room temperature, symptomatic cloves and bulbs were counted, and fungi were isolated from bulb basal plates and cloves. Basal plates and bulbs were separately analyzed as previously described (Mondani *et al.*, 2021a). Bulb water activity (a_w) was measured at four critical steps of the production process: at BBCH49, at the end of natural drying, after 9 months of cold storage, and after 15 d at room temperature.

Inspections and fungus isolations from bulb basal plates

Four replicates of five plants per seed clove treatment (total of 20 plants) were sampled at three field growth stages (BBCH15, BBCH45, and BBCH49). Based on visual inspections, symptom severity on each bulb was scored using five categories (Mondani *et al.*, 2021b): 0%, asymptomatic; 10%, small brown spots near the basal plate (base of radicles); 35%, brown spots on one-half of the basal plate; 65%, brown spots on the entire perimeter of the basal plate, with or without white mycelia on the inner cloves and violet pigmentation on the radicles; or 90%, brown spots on the basal plate and bulb, necrotic radicles, and visible white mycelia. Basal plate severity index (BPSI) was calculated for each sampling time point by multiplying the number of plants in

Table 2. Experimental scheme of garlic plant/bulb sampling time points in the production chain, from growth in the field through postharvest cold storage and delivery[a].

Data collection time point	Field				Cold storage			Room temperature
	BBCH15	BBCH45	BBCH49	Drying	3 months	6 months	9 months	15 d
	April	May	June	July	October	February	May	May
Basal plates								
Inspection of bulb basal plates (BPSI)	20 plants	20 plants	20 plants					
Fungus isolations from bulb basal plates	20 plants	20 plants	20 plants				24 bulbs	24 bulbs
Bulbs								
Water activity[b]			five bulbs	five bulbs			five bulbs	five bulbs
Visual inspection of symptomatic bulbs (BSI)					120 bulbs	120 bulbs	120 bulbs	120 bulbs
Count of symptomatic cloves/bulb							24 bulbs	24 bulbs
Fungus isolations from bulbs							24 bulbs	24 bulbs

[a]The number of plants/bulbs reported at each sampling time point is the total number sampled for each seed clove treatment.
[b]Water activity was measured at key steps in the chain.
Abbreviations: BBCH15, 5th leaf visible (mid-April); BBCH45, half of bulb final diameter (31 May); BBCH 49 (ripening; 30 June); BPSI, basal plate severity index; BSI, bulb severity index.

each disease severity category with the corresponding disease severity value and dividing by the number of plants collected per sampling time point (20).

Direct fungus isolation was performed by sampling a portion of basal plates from each bulb (whether it was symptomatic or asymptomatic), during field growth stages and postharvest. The samples were washed with tap water for 20 min, surface-disinfected with 1% NaOCl for 1 min, and plated on WA as previously described (Mondani *et al.*,2021b). Resulting colonies were transferred to PDA and identified at the genus level (Schwartz and Mohan, 2016), and *Fusarium* colonies were identified at the species level based on morphological features (Leslie and Summerell, 2006). One isolate per morphological group was confirmed by molecular identification (Mondani *et al.,* 2021b) using established protocols (Nicolaisen *et al.*, 2009; Mbofung and Pryor, 2010).

Inspection and fungus isolation from bulbs

To simulate product selection by garlic producers, bulbs were inspected at four time points during storage (Table 2). Bulb disease severity was determined by touching the cloves and estimating the number that were emptied (0–100% emptiness per bulb), without removing the or disrupting the bulb sheath. As the mean number of cloves per bulb was 10, each empty clove represented a 10% increment in severity. Bulb severity index (BSI) was calculated with the following formula:

$$BSI = ([10 \times n_1] + [20 \times n_2] + [\ldots] + [100 \times n_{10}]) / \text{no. of plants sampled},$$

where numbers from 10 to 100 represent the percentages of cloves emptied by the fungus, and n_1 to n_{10} are the number of bulbs in each severity category.

At the 9-month and 15-d time points, six bulbs were randomly selected from the four trial replicates based on selling quality category (bulb diam. = 35–46 mm, or >46 mm), for a total of 24 bulbs per seed bulb treatment. To verify disease severity, all sampled bulbs were peeled and the percentage of symptomatic cloves in each bulb was calculated with the following formula:

% symptomatic cloves = (no. of cloves with brown spots / total no. of cloves in bulb) × 100.

Direct fungus isolation was carried out from cloves at 9 months and 15 d. Asymptomatic and symptomatic cloves (20 each) were randomly selected for each quality category and seed clove treatment (20 cloves × seven seed clove treatments × two categories × two sampling time points × 2 years = total of 1120 asymptomatic and 1120 symptomatic cloves). Clove portions were washed with tap water for 20 min and plated, and resulting fungus colonies were identified as reported above.

Determination of bulb water activities (a_w)

At BBCH 49, after 1 month of natural drying, at 9 months of cold storage, and after 15 d of room tem-

perature storage, a_w was measured using an Aqualab Pre instrument (Meter Group). Five bulbs were sampled from each seed clove treatment. Central cloves of each bulb were removed (two replicates) and used for analysis. Each measurement was repeated 3 times.

Statistical analyses

BPSI, BSI, % symptomatic cloves/bulb, and incidence of fungus species (*Fusarium* spp.) were arcsine-transformed to homogenize means (Clewer and Scarisbrick, 2001). Transformed data and a_w values were subjected to analyses of variance. Tukey's test was used to compare means ($P < 0.01$). Statistical analyses were carried out using PASW Statistics v25 (SPSS Inc.).

RESULTS

Basal plate inspections and fungus isolations

Basal plates were divided into severity categories, and BPSI was calculated at three growth stages (BBCH15, BBCH45, and BBCH49) during crop development, and at two postharvest time points (9 months and 15 d). During the postharvest period, all bulb basal plates were of severity category 3 (brown spots visible on the entire basal plate perimeter, with growing mycelia occasionally visible). This category corresponded to a severity value of 65%, and these data were excluded from the statistical analyses.

Statistically significant differences were detected between treatments ($P < 0.01$) based on the ANOVA. Untreated control plants had the greatest mean disease severity (43.7%), along with those treated with the commercial BCA product including *Trichoderma* and *B. subtilis* as active ingredients. Greatest disease reductions resulted from the chemicals tebuconazole (mean severity = 32.1%) and prochloraz + propiconazole (35.0%). Chemicals (mean BPSI = 33.6%) gave greater bulb rot reductions than the BCAs (mean BPSI = 37.1%), but *B. subtilis* and *S. griseoviridis* as single active ingredients gave similar results (mean BPSI = 35.6%) to the chemical treatments. Statistically significant differences were observed between the 2 years of the study, with a 12.9% greater mean disease severity in year 2 compared to year 1. Disease severity on bulb basal plates increased during the cropping seasons, from 4.1% at BBCH15 to 55.9% at BBCH49), and to 65% postharvest (Table 3).

The main fungus genus isolated from basal plates was *Fusarium*. Other genera were sporadically isolated, including *Rhizopus* (mean of 3.2% for all treatments and years) analyzed), *Trichoderma* (1.0%), *Alternaria* (0.82%), and *Penicillium* (0.42%). Data for these other genera were excluded from the statistical analyses.

Two species of *Fusarium* were identified, namely, *F. oxysporum* and *F. proliferatum*. Active ingredients applied as seed clove treatments were not effective in controlling these two fungi on bulb basal plates (Table 3). Both *F. oxysporum* and *F. proliferatum* were detected at incidences comparable to those in the untreated control samples. Mean incidence of *F. oxysporum* was greater in year 1 (28.5%) than in year 2 (21.3%) ($P < 0.01$) (Table 3). The rates of isolation of the two species also varied according to the time point of sampling. Greatest incidence of *F. oxysporum* was at BBCH15 (mean = 38.2%) and BBCH49 (29.6%) ($P < 0.01$). *Fusarium proliferatum* showed a similar trend, with least incidence at BBCH45 (mean = 50.7%) and greatest at the time of harvest (BBCH49, 63.2%) ($P < 0.01$) (Table 3). After the cold storage period, when bulbs were stored at ≈ 25°C for 15 ds, the two species showed opposite trends: *F. oxysporum* mean incidence on bulb basal plates decreased by 7.5% from 26.5% at 9 months, and to 19.0% at 15 d, whereas that of *F. proliferatum* increased by 12.5% from 52.1% to 64.6% ($P < 0.01$) (Table 3).

Bulb inspections and fungus isolations

Bulbs were first inspected by touch to estimate the percentage of cloves emptied by fungus pathogens, which is the method commonly used by garlic producers. Among the treatments, tebuconazole and bacterium BCAs gave the least bulb disease severity (mean BSI = 10.6%), followed by prochloraz + propiconazole (13.5%) and the fungus BCA (mean = 14.7%). All the treatments resulted in significant differences in disease severity ($P < 0.01$) relative to the untreated controls (16.6%) (Table 4).

After 9 months of cold storage, bulbs were peeled and the number of symptomatic cloves per bulb was counted. Tebuconazole was the most effective treatment for reducing disease symptoms (mean = 30.5% symptomatic cloves/bulb), and this treatment differed ($P < 0.01$) the untreated control (45.8% symptomatic cloves/bulb). All the other treatments showed comparable performance, with *B. subtilis* (35.4%) and *Trichoderma* + *B. subtilis* (36.8%) giving less disease than the untreated control. Differences in BSI were observed between the years of study (6.0% in year 1 and 20.1% in year 2; $P < 0.01$) (Table 4). BSI increased during bulb storage, but the number of symptomatic cloves in the bulbs was the same at 9 months and after 15 d at room temperature. However, bulbs with large diameters (>46 mm) had greater mean proportion (41.2%) of symptomatic

Table 3. Mean garlic bulb basal plate severity indices (BPSI), and mean incidence of fungi isolated from basal plates, for different field trial treatments, two growing seasons (Year 1, 2017–2018, and Year 2, 2018–2019), and field crop and postharvest sampling time points[a].

Parameter	BPSI (%)	Incidence (%)	
		F. oxysporum	*F. proliferatum*
A Treatment[b]	**	n.s.	n.s.
Control	43.7 d[c]	22.6	55.8
Trichoderma + Bacillus subtilis	39.8 cd	24.9	55.8
T. harzianum + T. gamsii	37.9 bc	25.9	55.1
S. griseoviridis	34.9 abc	25.9	57.0
B. subtilis	35.8 abc	24.0	59.0
Prochloraz + propiconazole	35.0 ab	23.4	60.6
Tebuconazole	32.1 a	27.5	55.0
B Year	**	**	n.s.
Year 1	34.5 a	28.5 b	56.2
Year 2	39.6 b	21.3 a	57.6
C Sampling time point	**	**	**
BBCH15	4.1 a	38.2 d	53.9 abc
BBCH45	51.0 b	11.1 a	50.7 a
BBCH49	55.9 c	29.6 cd	63.2 bc
9 months	§	26.5 bc	52.1 ab
15 days	§	19.0 ab	64.6 c
Interactions			
A × B	n.s.	n.s.	n.s.
A × C	n.s.	n.s.	n.s.
B × C	*	**	n.s.
A × B × C	n.s.	n.s.	n.s.

[a]Garlic seed cloves were subjected to seven treatments. Experimental factor statistical significance (*, P <0.05; **, P < 0.01; n.s., not significant P > 0.05) is indicated.
[b]Treatment (A), year (B), and sampling time point (C) were factors in the analysis of variance of data.
[c]Means accompanied by the same letters are not different (Tukey's test, P < 0.01).
§ Indicates that data were not included in statistical analysis (see text).

cloves than small (36–46 mm) bulbs (34.0%; P < 0.01) (Table 4).

Fusarium was the main genus isolated from cloves (mean incidence = 31.3%), followed by *Penicillium*. (7.2%). Other genera, including *Rhizopus*, *Aspergillus*, and *Alternaria*, were isolated sporadically (mean incidence <2% across treatments and years), and were excluded from statistical analyses. Tebuconazole reduced *F. proliferatum* incidence on bulbs mean = 16.6%) relative to the untreated control (31.7%) (P < 0.05), whereas occurrence of *F. oxysporum* was unaffected by the applied treatments (Table 4). Incidence of *Fusarium* spp. on bulbs was comparable between the 2 years of this study. However, only *F. proliferatum* showed significant differences between sampling time points, with incidence almost doubled after bulbs were removed from the cold chambers and stored at room temperature for 15 d (19.7% after 9 months and 31.3% after the subsequent 15 days; P < 0.01). Large bulbs had greater incidence of *F. oxysporum* (7.2%) than smaller bulbs (3.8%; P < 0.05) (Table 4). Mean incidence of *Penicillium* spp. was also reduced by tebuconazole (mean = 1.9%) compared to the untreated control (7.9%), and these fungi were isolated at a greater rate in year 2 (7.6%) than in year 1 (0.6%; P<0.05).

Water potential of garlic cloves

No statistically significant differences (P > 0.05) in a_w values were detected during the four time points after harvest during storage, nor were there differences in these measurements between the applied treatments. However, differences were observed between the 2 years of this study, with greater overall a_w in year 1 (mean = 0.927) than in year 2 (0.947). Similarly, there were differences in this parameter between the sampling times, with the greatest mean a_w at harvest (0.976), which decreased after the natural drying process (to 0.874), before increasing during cold storage (to 0.940 at 9 months), and then in storage for 15 d at room temperature (to 0.959) (Table 5).

DISCUSSION

Garlic is propagated vegetatively, and the selection of healthy planting material is critical to ensure high quantity, quality, and consumer safety in bulb production. *Fusarium proliferatum*, the main causative agent of dry rot (Palmero *et al.,* 2012; Tonti *et al*., 2012; Mondani *et al*., 2021a; Mondani *et al.,* 2021b), is detected in significant proportions of apparently healthy garlic cloves (Dugan *et al.,* 2019), so selection of pathogen-free seed cloves on an industrial scale is difficult and usually not achievable.

In the present study, spray seed clove treatments with fungicides were assessed as a measure for reducing bulb dry rot severity and latent infections in symptomless bulbs. Efficacy of chemicals and BCAs was assessed for control of *F. proliferatum* and garlic dry rot, from early crop stages and through prolonged storage periods.

The tested active ingredients were selected according to published *in vitro* efficacy data and preliminary

Table 4. Mean garlic disease severity indices (BSI), percentages of symptomatic cloves/bulbs and mean incidence of fungi isolated garlic cloves, for different field trial treatments, two growing seasons (Year 1, 2017–2018, and Year 2, 2018–2019), and field crop and postharvest sampling time points[a]. [†].

Parameter	BSI (%)	% Symptomatic cloves/ bulb[d]	Incidence, %	
			F. oxysporum	*F. proliferatum*
A Treatment[b]	**	**	n.s.	*
Control	16.6 c[c]	45.8 c	7.0	31.7 b
Trichoderma + B. subtilis	15.3 bc	36.8 ab	7.7	24.0 ab
T. harzianum + T. gamsii	14.1 bc	37.2 abc	5.3	28.4 ab
S. griseoviridis	11.1 a	40.2 bc	6.3	29.7 b
B. subtilis	11.4 a	35.4 ab	2.8	20.9 ab
Prochloraz + Propiconazole	13.5 b	37.5 abc	4.7	27.2 ab
Tebuconazole	9.3 a	30.5 a	4.7	16.6 a
B Year	**	n.s.	n.s	n.s.
Year 1	6.0 a	38.8	6.7	27.1
Year 2	20.1 b	36.4	4.3	23.8
C Sampling time point	**	n.s.	n.s.	**
3 months	10.1 a			
6 months	11.8 b			
9 months	13.3 c	36.3	5.9	19.7 a
15 days	17.0 d	38.9	5.1	31.3 b
D Category		**	*	n.s.
Bulb diameter 36–46 mm		34.0 a	3.8 a	23.9
Bulb diameter ≥46 mm		41.2 b	7.2 b	27.1
Interactions				
A×B	**	*	**	*
A×C	n.s.	n.s.	*	*
A×D	n.s.	n.s.	n.s.	n.s.
B×C	**	n.s.	*	*
B×D	n.s.	**	**	n.s.
C×D	n.s.	n.s.	n.s.	n.s.
A×B×C	n.s.	n.s.	n.s.	n.s.
A×B×D	n.s.	n.s.	n.s.	n.s.
A×C×D	n.s.	n.s.	n.s.	n.s.
B×C×D	n.s.	n.s.	n.s.	n.s.
A×B×C×D	n.s.	n.s.	n.s.	n.s.

[a]Garlic seed cloves were subjected to seven treatments. Experimental factor statistical significance (*, P <0.05; **, P < 0.01; n.s., not significant P > 0.05) is indicated.
[b]Treatment (A), year (B), sampling time point (C), and category (D) were factors in the analysis of variance of data.
[d] % of symptomatic cloves/bulb = (No. of cloves showing brown spots / total No. of cloves in bulb) × 100.
[c]Different lowercase letters indicate significant differences with Tukey's test (P<0.01).

experiments conducted *in vitro* and in pots (Mondani *et al.,* 2021c). Triazoles are fungicides that block the demethylation step of sterol biosynthesis in cell membranes (Osborne and Scott, 2018), and these compounds inhibit *Fusarium* growth, with 50% lethal doses (LD_{50}s) ranging from 0.24 to 6.5 mg L^{-1} and LD_{90} of 10 mg L^{-1}/l (Müllenborn *et al.*, 2008; Marín *et al.*, 2013). BCAs were selected based on their modes of action, which likely reflect different mechanisms of interaction with *Fusarium* under field conditions. Bacterial BCAs produce compounds that inhibit plant pathogen growth and stimulate systemic immune responses in crops (Stein, 2005). *Bacillus subtilis* was shown to suppress *F. oxysporum* and *F. graminearum* mycelium growth and sporulation (Kim and Knudsen, 2013; Zhao *et al.*, 2014). *Streptomyces griseoviridis* can produce antibiotics and hydrolytic

Table 5. Mean water activity (a_w) in garlic cloves, for different field trial treatments, two growing seasons (Year 1, 2017–2018, and Year 2, 2018–2019), and field crop and postharvest sampling time points[a].

Parameter	a_w	
A Treatment[b]	n.s.	
Control	0.939	
Trichoderma + B. subtilis	0.941	
T. harzianum + T. gamsii	0.923	
S. griseoviridis	0.942	
B. subtilis	0.936	
Prochloraz + propiconazole	0.934	
Tebuconazole	0.945	
B Year	**	
Year 1	0.927	a[c]
Year 2	0.947	b
C Sampling time	**	
BBCH49	0.976	d
Natural drying	0.874	a
9 months	0.940	b
15 days	0.959	c
Interactions		
A×B	n.s.	
A×C	*	
B×C	**	
A×B×C	n.s.	

[a]Garlic seed cloves were subjected to seven treatments. Experimental factor statistical significance (*, P <0.05; **, P < 0.01; n.s., not significant P > 0.05) is indicated.
[b]Treatment (A), year (B), and sampling time point (C) were factors in the analysis of variance of data.
[c]Different lowercase letters indicate significant differences with Tukey's test (P<0.01).

enzymes that affect cell membranes in *F. oxysporum* and *F. proliferatum* isolated from cucurbit plants (Zhao *et al.*, 2013). In contrast, fungus BCAs such as *Trichoderma* spp. compete with pathogenic microbes for space and nutrients (Kubicek *et al.*, 2001; Kavitha and Nelson, 2013). Some *Trichoderma* spp. isolates were reported to rapidly colonize substrates and parasitize *F. oxysporum* mycelia within 48 h after inoculation (Sharma, 2011).

The present study assessed the efficacy of fungicides based on visible symptoms and incidence of *Fusarium* on garlic plant basal plates and bulbs. The effects of seed clove treatments on disease severity in basal bulb plates and plant roots (as measured by BPSI) confirmed previous results from trials conducted with plants grown in pots under conditions (sowing period and environment) comparable to open field cultivation (Mondani *et al.*, 2021c). Tebuconazole was the most effective active ingredient (mean BPSI = 32.1%) and gave similar disease levels as prochloraz + propiconazole (35.0%) and bacterial BCAs (35.4%).

The treatments did not affect incidence of *F. oxysporum* or *F. proliferatum* on bulb basal plates, despite reducing disease severity. Additional analysis is required to determine the sites of *Fusarium* spp. colonization of the cloves, given that *F. proliferatum* has been detected in 25 to 50% of apparently healthy garlic bulbs (Dugan *et al.*, 2019). In preliminary studies in our laboratory, *F. proliferatum* was isolated from garlic cloves, which may explain the low efficacy of the seed clove treatments.

Differences in BPSI were observed between the 2 years of this study, with mean disease severity increasing from 34.5% in year 1 (2017–2018) to 39.6% in year 2 (2018–2019). Weather conditions differed over the 2 years (Mondani, *et al.*, 2021b): more rainfall and lower temperatures were recorded in the spring (mid-April until end of May) of year 2 than in year 1, when bulbs were between BBCH15 and BBCH45 growth stages. In year 1, spring rainfall was 258.3 mm and mean temperature was 14.6°C, while in year 2 spring rainfall was 82.8 mm and mean temperature was 18.7°C). This difference may have increased the impact of *Fusarium* on garlic bulbs in year 2. However, the active ingredient × year interaction effect was not significant, so irrespective of disease severity, the seed clove treatment reduced BPSI, but only by 9% to 27% relative to the untreated controls.

Tebuconazole and the BCAs *B. subtilis* and *S. griseoviridis* controlled dry rot in the garlic bulbs. A maximum of one or two cloves per bulb were emptied by the fungus (14% less BSI compared to the controls). After bulb peeling, tebuconazole was confirmed as the most effective active ingredient, with fewer visible symptoms on the cloves (35% lower) relative to the controls. *Bacillus subtilis* as single active ingredient, *Trichoderma + B. subtilis*, and *T. harzianum + T. gamsii,* so as the chemicals prochloraz + propiconazole, were less effective for reducing visible symptoms, although these treatments did not differ significantly from tebuconazole. The treatments had no effects on the incidence of *F. oxysporum* and very limited effects on *F. proliferatum* in cloves, with only tebuconazole (mean BSI = 16.6%) giving a statistically significant reduction in the abundance of these fungi compared to the controls (BSI = 31.7%). Other fungal genera (*Rhizopus*, *Aspergillus*, and *Alternaria*) were isolated sporadically and their occurrence was unaffected by the treatments applied to seed cloves.

The seed clove treatments did not affect water activity (a_w) in garlic cloves. Values above 0.90 (the threshold for *Fusarium* growth and metabolic activity; Marín *et al.*, 1996) were recorded during cold storage, and

these increased after the bulbs were removed from cold chambers (mean a_w = 0.959), and were then stored at a temperature (≈25°C) that is favorable for *F. proliferatum* activity, as found in markets or after sale consumer homes. The increase in a_w is consistent with previous results (Mondani *et al*., 2021a) and was expected because of the high relative humidity (≈90%) set inside cold chambers to prevent garlic dehydration.

Application of fungicides or BCAs as sprayed seed treatments generally yielded positive results for controlling postharvest rot in garlic bulbs, and reduced visible symptoms, from the field stage through prolonged storage. This is unlike fungicides sprayed on garlic leaves, which was shown to be ineffectual (Gálvez Patón *et al.,* 2017). Tebuconazole was the most effective seed clove treatment, giving a 44% reduction in BSI, 33% reduction in visible rot symptoms, and 48% reduction of incidence of *F. proliferatum*. BCAs, and particularly *B. subtilis*, also performed well, giving 31% reduction of BSI, 23% reduction of visible symptoms, and 34% reduction of incidence of *F. proliferatum*. Therefore, promising BCAs are available for use in organic farming.

The impacts of these treatments on bulb dry rot cannot be considered as satisfactory, because 30-35% of bulbs were symptomatic, with incidence of *F. proliferatum* 16% recorded from the most effective treatment. The treatments had limited impacts on the incidence of both *F. proliferatum* and *F. oxysporum*, especially once the bulbs were transferred to room temperature. The a_w >0.94 during cold storage, which increased in the last step of the garlic production chain (room temperature storage), probably promoted growth of the pathogenic fungi. One option to enhance garlic clove protection during germination and seedling development is to apply treatments as film coatings instead of sprays (Rocha *et al*., 2019; Afzal *et al*., 2020). On the other hand, the isolation of *F. proliferatum* from cloves is a further concern. Future studies should also investigate whether treatment of cloves can minimize the presence of fungi while preserving clove germinability. Persistence of treatments should also be determined, especially in the case of BCAs that can potentially survive cold storage and be reactivated once treated material is moved to room temperature.

This study contributes to the management of garlic dry, rot as it has demonstrated positive effects achieved by seed clove treatments, although they can probably be improved. The study has also identified weak points in the garlic production chain as topics for future research. Further effort is required to provide stakeholders with safe and highly effective solutions for preventing and managing garlic dry rot.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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Research Papers

Characterization of *Stemphylium* spp. associated with tomato foliar diseases in Algeria

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Summary. Leaf blight and spot caused by *Stemphylium* spp. and *Alternaria* spp. are the most common destructive tomato diseases in north-western Algeria. During 2018 growing seasons, more than 30% of samples collected from plants grown in greenhouses or open fields were infected with *Stemphylium*. Initial symptoms were small, multiple, irregular to oval, yellow leaf spots, which enlarged to brown lesions later. In these lesions, *Stemphylium* mostly co-occurred with *Alternaria* spp. Twenty nine *Stemphylium* isolates were characterized based on morphological features, and multi-locus phylogenies using *ITS*, *gpd*, and *cmdA* genomic loci. Five *Stemphylium* species (*S. lycopersici*, *S. gracilariae*, *S. eturmiunum*, *S. vesicarium*, *S. lycii*) were associated with tomato leaf spot, of which *S. lycii* is a new report for tomato. Pathogenicity tests on healthy 2-months-old tomato seedlings reproduced symptoms similar to those observed in tomato crops. The tested fungus isolates differed in pathogenicity. Two isolates of *S. lycopersici* were more aggressive than those of the other species, causing major lesions on tomato plants. The five identified *Stemphylium* species are reported for the first time as new pathogens for tomato in Algeria, and *S. lycopersici*, *S. gracilariae, S. eturmiunum,* and *S. lycii* as new species of Algerian mycoflora.

Keywords. Tomato leaf spot, disease complex, pathogenicity test.

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INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is the most common temperate vegetable crop cultivated in Mediterranean countries. In Algeria, demand for tomato is increasing as a result of human population growth, where tomato is the fourth most commonly grown crop, after potato, watermelon and onion (Messak, 2014). Tomato crops covered 24,996 ha and yielded was 59,124 kg ha^{-1} in 2019 (FAO, 2019). However, heavy crop losses are caused by pests and fungus diseases every year. Leaf blight caused by *Stemphylium* spp. and *Alternaria* spp. has become of increasing importance in recent years. These fungi are serious pathogens on tomatoes and other *Solanaceae* in the north-western growing regions of Algeria, viz. Mostaganem, Oran, Mascara,

Tlemcen and Ain Témouchent (Bessadat *et al*., 2016; Bessadat *et al*., 2019).

Stemphylium (*Pleosporaceae*) was first described in 1833 by Wallroth and then by Wiltshire (1938). This genus includes saprophytic and pathogenic fungi (Inderbitzin *et al*., 2009). These fungi reproduce asexually through production of conidia, and in some species through *Pleospora* teleomorphic stages (Simmons, 2007; Inderbitzin *et al*., 2009). *Stemphylium* is morphologically similar to the related genus *Alternaria*. The conidia of both genera are multiseptated and pigmented, and form on conidiophores generating from hyphae in the mycelium. *Stemphylium* is distinguished from *Alternaria* based on apically percurrent conidiophores which produce successive conidia (Simmons, 2007). Because of its widespread distribution and pathogenicity, *Stemphylium* is an important and destructive pathogen, causing leaf spot diseases in several crop hosts (Ellis, 1971). Leaf spot has the potential of becoming a threat to tomato production, especially in regions where susceptible cultivars are grown. Incidence of *Stemphylium* has been reported to reach as high as 100% (Cedeño and Carrero, 1997).

Symptoms are often first seen on young tomato seedlings. Conidia of the pathogen invade leaves primarily through stomata, and a vesicles developed inside the substomatal cavities (Benets and Matsuoka, 2005). Symptoms appear as small brown or grayish spots with yellow halos that later expand into necrotic lesions, sometimes with gray centres and dark brown borders. As the disease progresses, affected leaves become chlorotic, and the lesions dry out and usually crack. Severely infected leaves die and fall off (Blancard *et al*., 2012). The pathogen can persist on dead or dying plant material on alternative hosts (pepper, eggplant and other solanaceous crops or weeds) where pesudothecia can form (Cerkauskas, 2005). Conidia and ascospores are the primary inocula in the following season (Basallote-Ureba *et al*., 1999; Brahamanage *et al*., 2018), and these are disseminated by wind or rain (Rossi *et al*., 2005; Cerkauskas, 2005). The disease is favoured by extended periods of leaf wetness from rains or dew, and by moderate to warm temperatures (20–30°C), all of which characterize the temperate climate of Mediterranean basin regions.

These environmental conditions also enhance the appearance and development of other diseases on tomato, including *Alternaria* (Chaerani and Voorrips, 2006) and bacterial leaf spots (Huang and Tsai, 2017). *Stemphylium* and *Alternaria* have been shown to cause similar symptoms in the field (Fernández and Rivera-Vargas, 2008). *Alternaria* spp. were associated with *Stemphylium* spp. in more than 25% of analyzed samples from tomato producing areas of Algeria between 2011 and 2013 (Bessadat *et al*., 2016). *Stemphylium* disease of tomato may be confused with symptoms of bacterial leaf spot or late blight (Huang and Tsai, 2017). Thus, it is often difficult to accurately diagnose the disease caused by *Stemphylium* through visual observations.

Stemphylium spp. have wide host ranges, with cases of multiple pathogens on individual hosts, as well as single species on diverse hosts (Ellis and Gibson, 1975; Köhl *et al*., 2009; Brahmanage *et al.,* 2018; Farr and Rossman, 2021). Many *Stemphylium* species have been reported on tomato, including *S. solani* (Weber, 1930; Cedeño and Carrero, 1997), *S. floridanum* syn. *lycopersici* (Rotem and Bashi, 1977; Blancard *et al*., 1986), *S. vesicarium* (Blancard *et al*., 1986), *S. lycopersici* (Enjoji, 1931; Al-Amri *et al.,* 2016; Huang and Tsai, 2017), *S. botryosum* (Dickens and Evans, 1973; Rotem and Bashi, 1977), *S. tomatonis* syn. *vesicarium* and *S. eturmiunum* (Simmons, 2001; Andersen and Frisvad 2004).

Characterization and taxonomic determination of *Stemphylium* spp. has relied mainly on morphological characteristics including conidium shape, size, septation, length/width ratio and ornamentation (Simmons, 1969). However, high variability of species in culture and plasticity in host preference for these fungi have made it difficult to identify isolates to species (Chowdhury *et al*., 2015; Poursafar *et al*., 2016; Subash and Saraswati, 2016). Molecular tools have been widely implemented for accurate differentiation between *Stemphylium* spp., with species identification relying on sequence analyses of various DNA regions (Câmara *et al*., 2002; Inderbitzin *et al*., 2009; Woudenberg *et al*., 2017) and metabolite profiling (Andersen *et al*., 1995; Olsen *et al*., 2018). Several molecular-based studies have confirmed that combining the internal transcribed spacers (*ITS*) and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) regions could help resolve *Stemphylium* to species level (Câmara *et al*., 2002; Wang *et al*., 2010). More than 90 *Stemphylium* species have been isolated from different host plants (Poursafar *et al.* 2016). However, understanding of phylogenetic relationships within the genus has evolved. Woudenberg *et al*. (2017) revised *Stemphylium* taxonomy based on multi-gene phylogeny, and showed that only 28 species can be distinguished based on combined DNA sequences of the *ITS*, *gpd* and calmodulin (*cmdA*) gene regions. In that study, 22 species names were synonymized, and two new combinations were proposed. More recently, five new species were described based on morphological and on multi loci phylogeny (Brahmanage *et al.,* 2019; Marin-Felix *et al*., 2019; Crous *et al*., 2020).

Stemphylium species associated with gray leaf spot symptoms of tomato have been reported in several countries of the Mediterranean basin (Blancard *et al*., 1986

2012), but distribution of many species has not been thoroughly investigated in Algeria. The main purpose of the present study was to evaluate the composition of *Stemphylium* spp. on leaves and fruits of tomato collected in the northwest regions of the country. Specific objectives were: (1) to identify *Stemphylium* species using sequence analyses of combined datasets of the *ITS*, *gpd*, and *cmdA* gene regions, because morphological criteria were not reliable enough for this purpose; and (2) to test and compare the pathogenicity of different isolates under experimental conditions. Knowledge obtained will provide a basis for establishing effective management strategies for leaf spot diseases of tomato.

MATERIALS AND METHODS

Sample collection and isolation

Samples with characteristic symptoms of leaf spot were collected from different tomato fields in Mostaganem and Oran regions of Algeria during the 2018 growing season. To isolate the causal agent, pieces of diseased leaf or fruit samples collected randomly from the prospected fields were disinfected with 2% NaOCl for 2 min, rinsed with sterile distilled water three times, plated on potato carrot agar (PCA), and then incubated at room temperature (18–25°C). After 1 to 3 weeks, mycelium of growing fungi with typical characteristics of *Stemphylium* was transferred with a fine sterile needle onto new PCA plates. The incidence of fungi associated with the diseased tomato material was summarized as isolation frequency (number of diseased leaves from which a species was isolated compared to the total number of leaves incubated × 100). Pure cultures of the resulting fungi were obtained transferring single conidia onto 2% water agar or hypha tips onto potato dextrose agar (PDA). Purified isolates were subcultured onto PDA slants and kept at 4°C for further examination. Pure cultures of all identified species were deposited in fungal culture collection COMIC (SFR QUASAV, Angers, France) (Table 1).

Morphological assessments

For macroscopic descriptions (colony colour, shape, texture and diameter), purified cultures were grown on PDA and incubated for 7 d at 25°C in continuous darkness. Microscopic characters were assessed based on the standardized conditions suggested by Simmons (2007). Isolates were incubated at room temperature on PCA under a light/dark photoperiod for 5 to 7 d. The standard technique of adhesive tape preparation (Scotch tape prep or cellophane tape prep) was used for slide preparation (Forbes *et al*., 2002), with lactic acid solution as mounting fluid. Morphological observations were made with a light microscope (Optika 190 B) and a stereomicroscope. Microscopic features recorded were compared with available literature.

DNA extractions and PCR amplifications

Genomic DNA of each *Stemphylium* isolate was extracted using a microwave mini-prep extraction method (Goodwin and Lee, 1993). The primers ITS1/ITS4 (White *et al*., 1990) were used for amplification of the internal transcribed spacer regions of ribosomal DNA (*ITS rDNA*), primers gpd1/gpd2 (Berbee *et al*., 1999) for the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene region, and CALDF1 /CALDR1 (Lawrence *et al*., 2013) for the calmodulin (*cmdA*) gene region. Polymerase chain reaction (PCR) amplification was carried out using the primer and PCR protocols described in Woudenberg *et al*. (2017). Each PCR amplification was carried out in a total volume of 25 µL, containing 75mMTris-HCl pH 9.0, 20 mM $(NH_4)_2SO4$, 0.01 % (w/v) Tween 20, 1.5 mM $MgCl_2$, 200 µM desoxyribonucleotide triphosphate, 1 unit of thermostable DNA polymerase (GoTaq, Promega), and 400 nM of each relevant oligonucleotide primer. PCR products were sent to GATC lab for sequencing. The newly generated sequences of *ITS, gpd* and *cmdA* were submitted to GenBank (Table 1).

Phylogenetic analyses

The resulting sequences from each locus were concatenated and aligned through ClustalW algorithm and refined manually using MEGA 7 (Kumar *et al*., 2016). Sequences of the type isolates were retrieved from GenBank and included in the analysis. *Alternaria alternata* isolate GV14-634a1 was used as an outgroup. Phylogenetic analyses were carried out using the maximum likelihood (ML) under IQTree v.1.6.11 (Nguyen *et al*., 2015), and Bayesian inference (BI) with MrBayes v.3.2.1 (Ronquist and Huelsenbeck, 2003). The best-fit evolutionary models for each dataset calculated by ModelFinder (Kalyaanamoorthy *et al*., 2017) under the Bayesian Information Criterion (BIC) selection procedure were K2P + G4 for *ITS* and *gpd*, and HKY + F + G4 for *cmdA*. The ML analysis was carried out with 1000 ultrafast bootstrap replicates, and only values above 70% were considered significant. BI analyses were carried out to estimate the posterior probabilities (PP) of tree topologies based on the Markov Chain Monte

Table 1. *Stemphylium* isolates from tomato characterized in this study, their geographical origins, and the GenBank accession numbers of their internal transcribed spacer (*ITS*), glyceraldehyde-3-phosphate dehydrogenase (*gpd*) and calmodulin marker (*cmdA*) genomic sequences.

Isolate	Location	Species	*ITS*	*gpd*	*cmdA*
NB644	Mostaganem	*Stemphylium gracilariae.*	MZ093115	MZ152654	MZ152682
NB646	Mostaganem	*Stemphylium gracilariae*	MZ093112	MZ152649	MZ152677
NB649	Mostaganem	*Stemphylium gracilariae*	MZ093113	MZ152653	MZ152681
NB650	Mostaganem	*Stemphylium gracilariae*	MZ093115	MZ152652	MZ152680
NB651	Mostaganem	*Stemphylium vesicarium.*	MZ099818	MZ152669	MZ152697
NB654	Mostaganem	*Stemphylium vesicarium.*	MZ099817	MZ152668	MZ152696
NB682	Mostaganem	*Stemphylium vesicarium*	MZ099816	MZ152667	MZ152695
NB683	Mostaganem	*Stemphylium lycii*	MZ090945	MZ130945	MZ152704
NB690	Oran	*Stemphylium gracilariae*	MZ093117	MZ152651	MZ152679
NB696	Oran	*Stemphylium gracilariae*	MZ090945	MZ152648	MZ152676
NB709[1]	Mostaganem	*Stemphylium eturmiunum*	MZ093122	MZ152670	MZ152698
NB710[1]	Mostaganem	*Stemphylium lycopersici*	MZ093124	MZ152657	MZ152685
NB711[2]	Mostaganem	*Stemphylium lycopersici*	MZ093125	MZ152658	MZ152686
NB712[2]	Mostaganem	*Stemphylium eturmiunum*	MZ093119	MZ152671	MZ152699
NB713[3]	Mostaganem	*Stemphylium vesicarium*	MZ099814	MZ152663	MZ152691
NB714[3]	Mostaganem	*Stemphylium eturmiunum*	MZ093118	MZ152672	MZ152700
NB715	Mostaganem	*Stemphylium vesicarium*	MZ099812	MZ152666	MZ152694
NB717	Mostaganem	*Stemphylium gracilariae.*	MZ093114	MZ152650	MZ152678
NB719	Mostaganem	*Stemphylium lycopersici*	MZ093126	MZ152659	MZ152687
NB720	Mostaganem	*Stemphylium vesicarium*	MZ099811	MZ152665	MZ152693
NB731	Mostaganem	*Stemphylium eturmiunum*	MZ093121	MZ152675	MZ152703
NB735	Mostaganem	*Stemphylium eturmiunum*	MZ093123	MZ152674	MZ152702
NB736	Mostaganem	*Stemphylium eturmiunum*	MZ093120	MZ152673	MZ152701
NB737	Mostaganem	*Stemphylium vesicarium*	MZ099815	MZ152662	MZ152690
NB744	Mostaganem	*Stemphylium lycopersici*	MZ093129	MZ152661	MZ152689
NB747	Mostaganem	*Stemphylium lycopersici*	MZ093127	MZ152655	MZ152683
NB748	Mostaganem	*Stemphylium lycopersici*	MZ093130	MZ152660	MZ152688
NB751	Mostaganem	*Stemphylium lycopersici*	MZ093128	MZ152656	MZ152684
NB754	Mostaganem	*Stemphylium vesicarium*	MZ099813	MZ152664	MZ152692

[1], [2] and [3]: Isolates recovered from a same leaf necrosis.

Carlo (MCMC) analysis with four chains, 1M generations, sampled every 1000 generations. Burn-in was set to 25% and only PP values greater than 0.95 were considered significant.

Pathogenicity tests

To confirm the disease and its causal agents, a series of pathogenicity tests was conducted under greenhouse conditions. These used 2-month-old seedlings of tomato var. 'Saint Pierre' planted in pots (two seedlings per pot) containing 3:1 sterilized potting media:sand. Inoculum was prepared of each of the 28 representative isolates as described (Bessadat *et al*., 2016; 2019). For each trial, three plants were inoculated with 10 mL of a 1×10^4 conidia mL^{-1} suspension by spraying foliage with a hand atomizer. Three plants were also sprayed with sterile distilled water as experimental controls. Plants were then covered with polyethylene bags for 2 d to maintain a high relative humidity. Leaf spot progression was evaluated at 7, 14 and 21 d after inoculation (dai). The area under disease progress curves (AUDPC) was calculated using the open source image analysis software ImageJ (https://imagej.nih.gov/ij/plugins/). Symptoms expressed were studied and compared with those that occurred on the infected leaves collected from field-grown plants. Isolations were made from diseased inoculated plants for assessing fulfilment of Koch's postulates. Mean percentage of necrotic leaf area (n.l.a) and AUDPCs were determined for three replicates. Statistical analyses of obtained data were carried out using R software for ANOVA and Tukey post-hoc tests.

RESULTS

Pathogen isolation and disease incidence

During the 2018 growing season, severe leaf chlorosis and necrosis, characterized by brown to dark brown colour (Figure 1, a to f), followed by defoliation, were observed on tomato plants in the Mostaganem and Oran regions of northwest Algeria. In many cases, the symptoms were also associated with *Alternaria* diseases (leaf spot and blight) and other pests including *Tuta absoluta*. Field observers had difficulty distinguishing *Alternaria* leaf blight and spot symptoms from those caused by *Stemphylium* spp., because symptoms caused by fungi of both genera strongly resemble each other. However, the typical symptoms of *Stemphylium* on ripe and unripe fruit (brown specks, Figure 1, g and h) were easily distinguishable from the black mould symptoms caused by *Alternaria* spp. Fruit with physiological conditions allowing secondary infection by fungi, such as blossom end rot pathogens (Figure 1, i), were mainly colonized by *Stemphylium* and *Alternaria* species with small conidia. The brown lesions observed on tomato stems and petioles were induced by *Alternaria* sp. only.

Figure 1. Symptoms caused by *Stemphylium* spp. on tomato leaves and field-grown fruit. Developing lesions on tomato leaves (**a** and **b**). Darkening lesions on a tomato leaflet (**c**). Advanced necroses on tomato leaves (**d**, **e**, and **f**). Brown speck on ripe (**g**) and unripe tomato fruit (**h**). Dead tissues of a tomato fruit, colonized by *Stemphylium* with black felting corresponding to conidium production (**i**).

After 1 to 3 weeks, a total of 949 colonies were obtained from 370 repetition pieces of symptomatic tomato tissues. We frequently observed morphologically distinct fungal colonies on PCA plates originating from a single lesion; these primarily consisted of *Stemphylium* and *Alternaria* species. *Stemphylium* sp. were associated with *Alternaria* spp. in more than 29% of the samples. Simultaneous isolation of the two fungi from symptomatic tomato leaves in Algeria has been previously reported (Bessadat *et al*., 2019), but co-infection of individual lesions has not. *Stemphylium* sp. were recovered from 20 to 47% of lesions on tomato (average = 34%), whereas *Alternaria* spp. were isolated at a frequency of approx. 41%. Secondary leaf invaders were isolated at an average frequency of 25%, and these included *Penicillium* sp., *Cladosporium* sp. and *Chaetomium* sp.

A total of 93 isolates with *Stemphylium* characteristics were obtained by single conidium or hyphal tip methods. Cultural and macro-morphological features of strains were characterised by mycelium colour (white, beige, orange, olivaceous, grey, pink or greyish green) and texture. Diffusible pigmentation into culture medium produced by some isolates was also observed (Figure 2). Twenty-nine isolates were selected for further characterization (Table 1). Preliminary identification made by ITS sequencing confirmed that all these isolates were *Stemphylium*.

Species identification

A multi-gene phylogeny approach was used to identify *Stemphylium* isolates at the species level, using currently accepted methods to differentiate *Stemphylium* species (Woudenberg *et al*., 2017). The combined dataset of *ITS*, *gpd,* and *cmdA* sequences from 29 tomato isolates and from 68 representative strains of 31 recognized species of *Stemphylium* were compared. The dataset had a total length of 1462 bp (*ITS*: 451 bp, *gpd*: 394 bp, *cmdA*: 617 bp) of which 265 bp were phylogenetically informative (*ITS*: 20 bp, *gpd*: 89 bp, *cmdA*: 156 bp). The topologies of the trees inferred by the two phylogenetic methods (ML and BI) were similar. The ML phylogenetic tree with high bootstrap support (99%) and posterior probability values (1.0) indicated that the 29 tomato isolates grouped into five well-supported phylogenetic species (Figure 3). Additional analysis of morphological characteristics was carried out to describe the isolates from tomato.

Stemphylium eturmiunum

Six isolates (NB709, NB712, NB714, NB731, NB735, NB736), all from Mostaganem, were attributed to *S. eturmiunum*. Colonies were cottony to sub-aerial, dark green with greyish surfaces and white regular margins, reaching 56 to 76 mm diam., and occasionally producing yellow pigment on PDA after 7 d at 25°C in the dark (Figure 2, a to e). Conidium production was moderate on PCA after 7 d. Conidiophores were swollen at their tips, brown in colour, cylindrical, occasionally branched, and of short to moderate length, measuring 25–120 × 5–8 µm, and with two to eight transverse septa. Conidiophore each generally produced a single apical conidium. Conidia were ovoid to oblong, ellipsoid, occasionally rectangular, muriform upon aging, light to dark brown in colour (Figure 4 a), and measuring 22–38 × 10–22 µm. Mean conidium length/width ratio was 1.7 ± 0.3, and conidia had one to four transverse septa and zero to five longitudinal or oblique septae per transverse segment. The conidia were mostly constricted at the median transverse septae. Short conidium chains of two to four conidia, and lateral or apical secondary conidiophores were frequently observed at the centres of colonies. Ascomata observed on agar were spherical, subspherical, and dark brown, with dark hyphal outgrowths (Figure 5 a), and were single or aggregated, ranging from 200 to 980 µm in length. Asci and ascospores were observed on PCA after 4 to 5 weeks incubation. Asci were cylindrical with bitunicate and broadly rounded apices, gradually narrowed near the bases (Figure 5 e), and measured 145–221 × 21–37 µm. Ascospores were oblong, ellipsoid, with obtusely rounded ends, yellowish brown with darkened septae, constricted mostly at the 2nd transverse septae, and had zero to three longitudinal septa per transverse segment. Ascospores had dimensions of 27–46 × 10–20 µm.

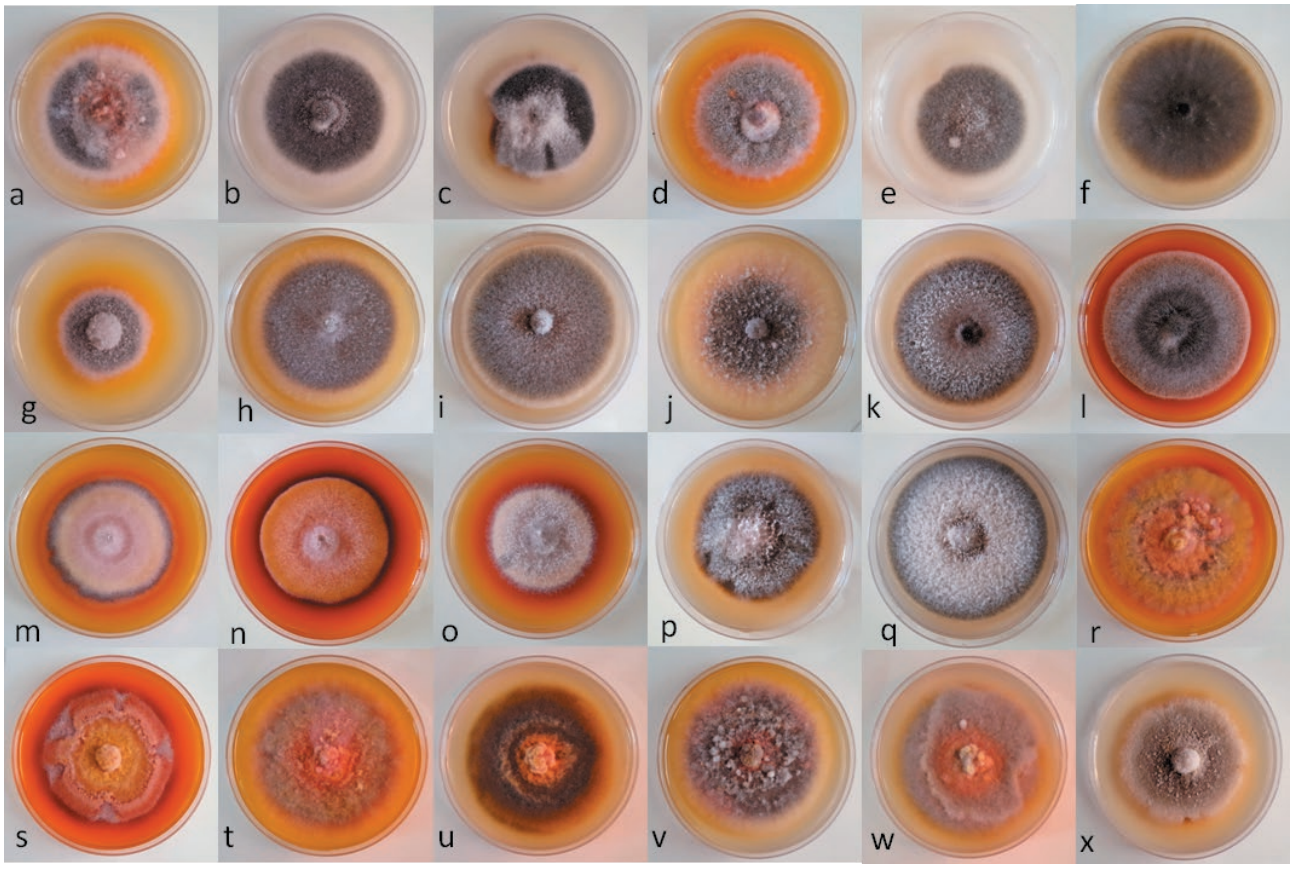

Figure 2. Colonies of 21 field isolates of *Stemphylium* spp. on potato dextrose agar after 7 d incubation at 25°C in the dark. *Stemphylium eturmiunum*, NB709 (a); NB712 (b), NB714 (c), NB731 (d), NB736 (e): *S. vesicarium,* NB654 (f), NB713 (g), NB715 (h), NB720 (i), NB737 (j): *S. gracilariae*, NB644 (k), NB646 (l), NB649 (m), NB650 (n), NB690 (o), NB696 (p), NB717 (q): *S. lycopersici*: NB710 (r), NB711 (s), NB719 (t), NB744 (u), NB747 (v), NB748 (w): *S. lycii*: NB683 (x).

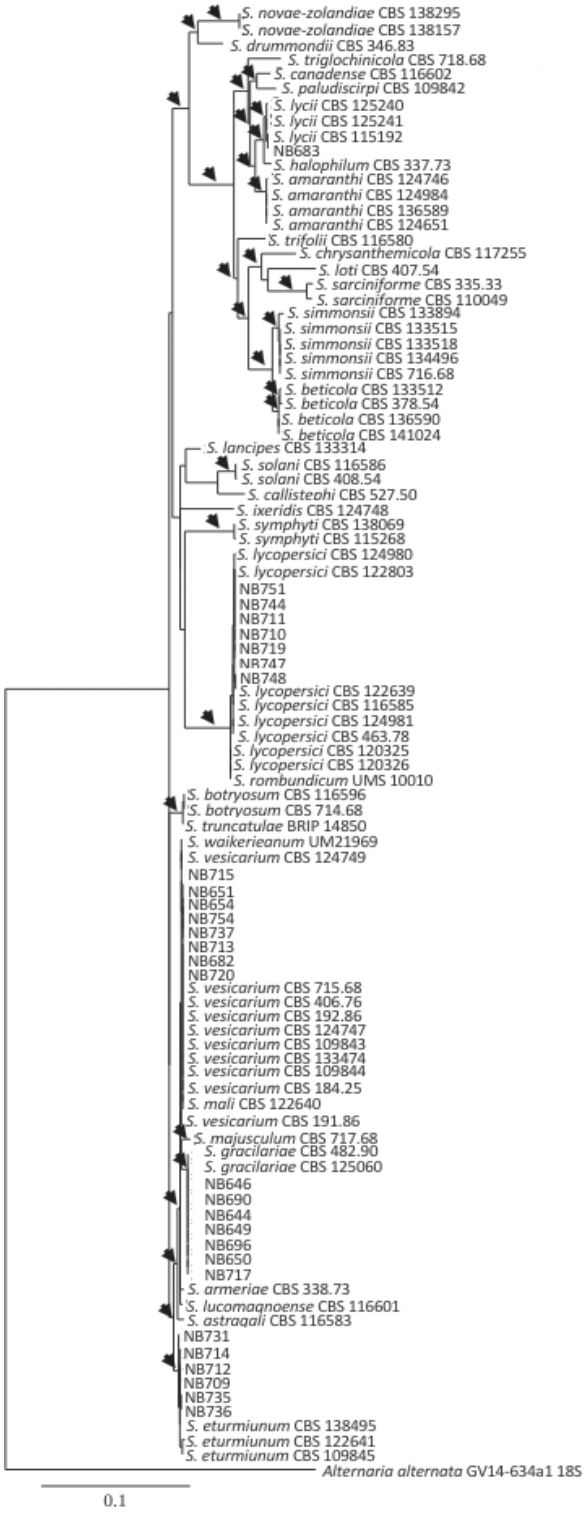

Figure 3. Phylogenetic tree reconstructed using the maximum likelihood method, from the alignment of *ITS*, *gpd* and *cmdA* sequences of *Stempylium* isolates. The tree was rooted with *Alternaria alternata* isolate GV14-634a1. Bootstrap support values greater than 0.7 are indicated by arrows near nodes. The scale bar indicates the expected number of substitutions per position.

Stemphylium vesicarium

Eight isolates (NB651, NB654, NB682, NB713, NB715, NB720, NB737, NB754), all from Mostaganem, were attributed to *S. vesicarium*. These isolates developed cottony colonies 49 to 80 mm diam. white regular margins on PDA after 7 d at 25°C in the dark (Figure 2, f to j). Colonies were olivaceous to greyish green with yellow pigmentation of the medium. Conidium production was moderate on PCA after 7 d. Conidiophores were straight or occasionally branched, each with one or two swollen apices and two to 11 septae, and were of short to moderate lengths (27–163 µm) and widths of 5–10 µm. Conidia were medium to dark drown, oblong, ellipsoid to muriform, with One to three (four) transverse and one to four longitudinal septae per transverse segment. The conidia were each constricted at one to three of the major transverse septae (Figure 4, b). Conidium dimensions were from 21–45 × 12–25 µm, with mean length/width ratio of 1.7 ± 0.3. Ascomata were formed after 7 d, and matured after 5 to 6 weeks. They were dark brown, thick-walled, spherical to sub-spherical, ranging from 356 to 700 µm in length (Figure 5, b). Asci were cylindrical to clavate, 138–243 × 22–32 µm with each containing eight ascospores. Mature ascospores were yellowish brown, oblong-ovoid, rounded at the bases and with conical apices (Figure 5 f), and measured 30–55 × 11–21 µm, with up to three transverse eusepta and zero to three longitudinal septae per transverse segment, and more or less constricted at the transverse septae. However, no fully mature ascospores could be found with NB713 and NB737 isolates in culture.

Stemphylium gracilariae

Seven isolates (NB644, NB646, NB649, NB650, NB690, NB696, NB717), from Mostaganem and Oran, were assigned to *S. gracilariae*. Colonies on PDA were cottony, sub-aerial, and olivaceous, with greyish or whitish surfaces and white to beige regular margins, reaching 59 to 78 mm diam. after 7 d at 25°C in the dark. Pigmentation of the growth media varied from pale yellow to dark orange (Figure 2, k to q). Abundant conidium production was observed on PCA. Conidiophores were brown, swollen, cylindrical, short (25–75 × 4–9 µm), with one to seven transverse septae, each bearing one or two conidia. Conidia were brown to dark drown, muriform, ovoid to oblong, ellipsoid, with one to three transverse and one to three (four) longitudinal septae per transverse segment. The conidia were constricted at the median transverse septae (Figure 4 c). Conidium dimensions were 20–37 ×

10–22 µm, with mean length/width ratio of 1.8 ± 0.3. Ascomata were abundantly formed, and were dark to black, covered with hyphal outgrows, and were spherical or irregular (Figure 5 c), broadly to narrowly oblong, single or aggregated, and ranged from 200 to 900 µm diam. Asci were cylindrical, bitunicate, hyaline (Figure 5, g), and measured 90–251 × 25–37 µm, and each ascus contained eight ascospores. Ascospores were 30–55 × 12–20 µm, yellowish brown in colour, each three main transverse septae which were thickened, darkened and constricted.

Stemphylium lycopersici

Seven isolates (NB710, NB711, NB719, NB744, NB747, NB748, NB751), all from Mostaganem, were identified as *S. lycopersici*. Colonies on PDA after 7 d at 25°C in the dark were 66 to 81 mm diam., cottony, compact, with sparse floccose aerial mycelium, and had regular to irregular velvety margins. Isolates were characterised by heterogeneous mycelium pigmentation (beige, orange and greyish pink to red), and each produced a yellow or a strong orange pigment that diffused into the culture medium (Figure 2, r to w). Conidium production was moderate to abundant on PCA after 7 d. Conidiophores were mostly solitary, straight, three to 17 septate, measuring 75–381 × 5–11 µm, with swollen apical conidiogenous cells and nodded appearance, sometimes due to successive periods of growth. Isolates did not form ascomata. Conidia were solitary, yellowish to brown, smooth or with a punctate ornamentation, mostly 42–87 × 13–22 µm, and with mean length/width ratio of 3.6 ± 0.5. On PCA, apical secondary conidiophores were occasionally observed. Conidia were oblong, cylindrical with conical apices and rounded bases, divided by three to eight transverse spetae and two to three longitudinal or oblique septae per transverse segment (Figure 4, d). Each conidium was constricted at the transversal septum. Isolates of this species did not form ascomata. This species can be distinguished its large conidia, long conidiophores and strong pigmentation of culture media.

Stemphylium lycii

Isolate NB683 was attributed to *S. lycii*. Colony diameter in 7-d-old PDA cultures was 62 to 68 mm. Colonies were cottony and compact with white irregular margins, and were grey with whitish centres and orange mycelial tufts (Figure 2x), and developed pale-yellow pigmentation. Conidium production was abundant on PCA after 7 d. Conidiophores had apically swollen conidiogenous cells, and were straight, cylindrical, unbranched, measuring 45–208 × 5–8 µm with three to 14 transverse septae, each bearing one to four conidiogenous sites. Conidia were solitary, brown, oblong to ellipsoid become muriform with age (Figure 4, e), measuring 26–45 × 13–20 µm, and with mean length/width ratio of 2.1 ± 0.4. Conidia had one to four transverse septae and zero to six longitudinal or oblique septae per transverse segment, and were usually constricted at the first or second major transverse septae. The sexual morph formed after 2 months. Ascomata were spherical to ovoid (Figure 5, d), single, ranging in size from 336–772 µm. Cylindrical to clavate asci measured 170–246 × 20–30 µm, and each contained eight ellipsoidal, muri-

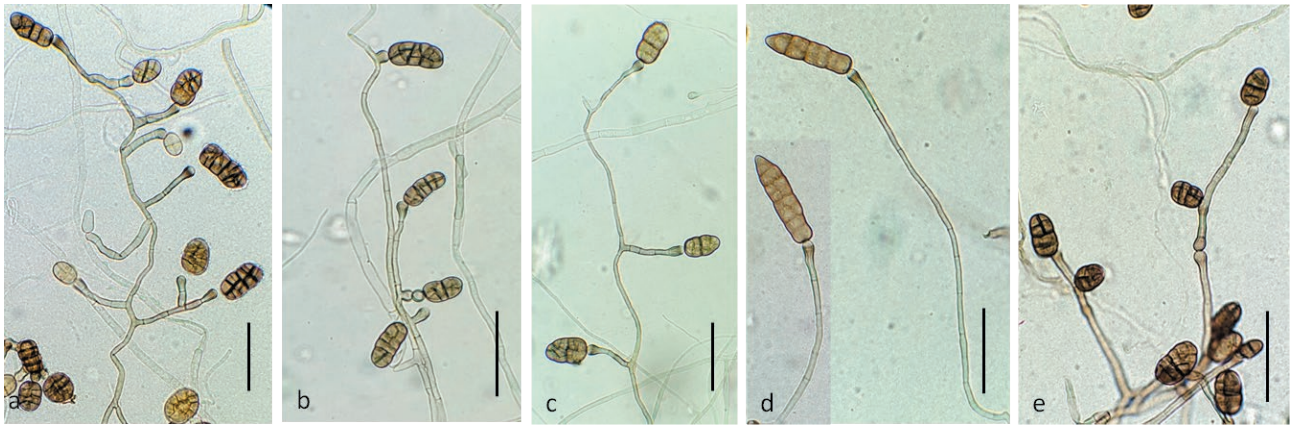

Figure 4. *Stemphylium* spp. on potato carrot agar, after 7 d incubation at room temperature; conidia and conidiophores with swollen apical cells. *Stemphylium eturmiunum* NB731 (a), and NB712 (b); *S. vesicarium* NB737 (c), NB713 (d), and NB654 (e)€; *S. gracilariae*, NB690 (f), NB696 (g), and NB644 (h); *S. lycopersici* NB711 (i); and *S. lycii* NB683 (j). Bars = 50 µm.

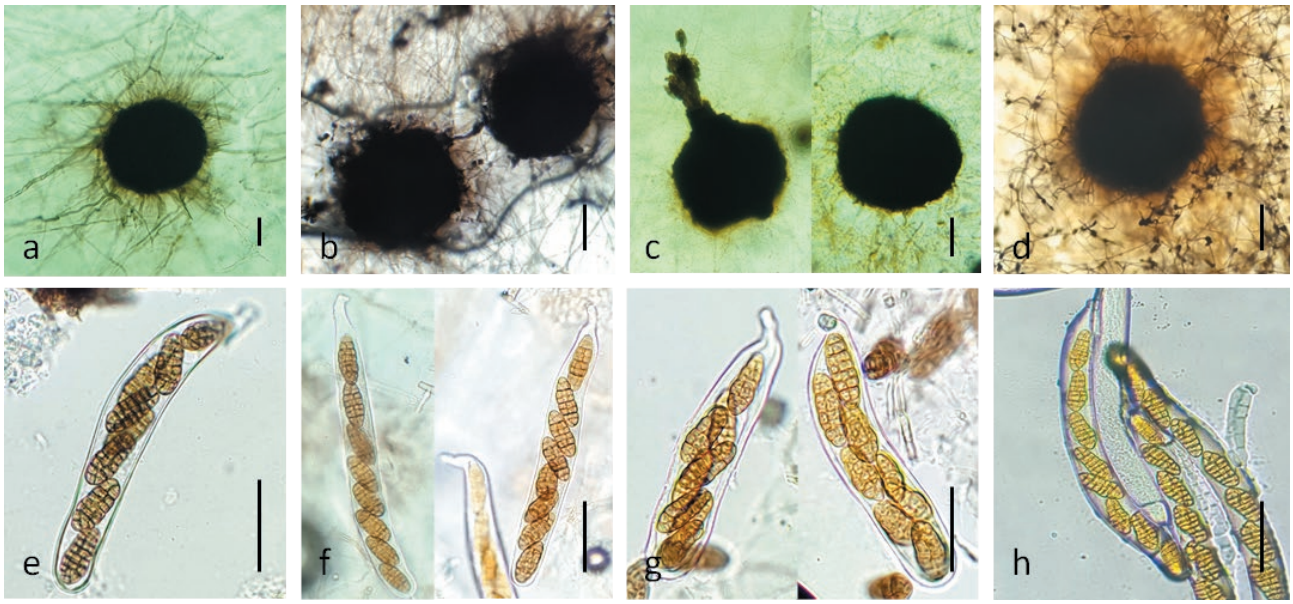

Figure 5. *Stemphylium* spp. ascoma, asci and ascospores of teleomorph stages developed on potato carrot agar after 1 to 5 weeks incubation at room temperature. *Stemphylium eturmiunum (Pleospora eturmiuna)* NB714 (a and e); *S. vesicarium (Pleospora herbarium)* NB654 (b and f); *S. gracilariae* NB696 (c and g); and *S. lycii* NB683 (d and h). Bars = 100 µm (a–d) and 50 µm (e–h).

form ascospores. Ascospores were yellowish brown and measured 32–42 × 12–17 µm, with three (four) transverse septae and one to three septae per transverse segment (Figure 5, h). This species is distinguished from *S. vesicarium* and *S. eturmiunum* by its long conidia and conidiophores formed on PCA, and colony characteristics on PDA.

Pathogenicity tests

All isolates produced symptoms on inoculated tomato plants. After 5 to 7 d, leaf spot symptoms appeared on plants, and the lesions increased progressively with time. Isolates differed in the severity of disease caused, as shown by proportions of leaf necrotic area (% l.n.a) and AUDPC values (Table 2; Figure 6). ANOVA of AUDPC data confirmed a strong "strain" effect ($P = 0.00448$) and no "repeat" effect ($P = 0.471$). Two isolates of *S. lycopersici* (NB744, NB747) were classified as highly virulent, with average AUDPCs >30 000. Aggressive isolates produced necrotic leaf lesions on the inoculated leaves which coalesced to encompass the entire leaves. Proportions of leaf area affected were >80% at 21 dai. No other statistically supported ($P > 0.05$) class of aggressiveness could be defined for all the other isolates, based on AUDPC data, and they were moderately virulent. However, three isolates, *S. gracilariae* NB649, *S. lycopersici* NB711 and *S. eturmiunum* NB714, produced small necrotic leaf lesions on leaves, which did not expand. These isolates were classified as minor pathogens for tomato, and the affected leaf areas for these isolates were less than 11% at 21 dai.

In general, *S. lycopersici* produced characteristic symptoms of dark brown spots on host leaves, which were most severe at 21 dai (Figure 7, a, b and c). Small and longitudinal, brown lesions were also observed on host stems. When plants were exposed to high relative humidity, *Stemphylium*-type conidia were produced on the lesions (Figure 7, l).

All isolates were re-isolated from 90 ± 15% of the inoculated infected leaves. The re-isolated fungi formed typical conidia on PCA and on diseased host leaflets from the artificial inoculations. No symptom development was observed on inoculation control leaves treated with sterilized water.

DISCUSSION

This study is the first report of presence and pathogenicity of five *Stemphylium* species on tomato in Algeria, highlighting the risk of these pathogens causing disease in the future. These pathogens were often recovered from tomato plant lesions along with the closely related *Alternaria* on *Solanaceae* (Bessadat *et al.*, 2016; 2019). Diseased materials collected in northwestern regions, during 2018 growing seasons, were infected by *Stemphy-*

lium spp. and *Alternaria* spp. The high isolation frequency of both genera from foliar lesions was irrespective of symptom morphology, but was most commonly associated with asymmetric, necrotic lesions.

Focusing on the *Stemphylium* spp. component, the polyphasic approach based on morphological and molecular analyses permitted the identification of five species among 29 tested isolates. Molecular data showed that species considered closely related based on morphological traits were phylogenetically distinct. Therefore, initial identifications on the basis of conidium dimensions alone were unreliable, as molecular data indicated that strains were *S. eturmiunum* while morphologically resembling *S. gracilariae* or *S. vesicarium*, and *vice versa*. Therefore, relying on morphological characters to identify species is not recommended (Köhl *et al*., 2009; Han *et al*., 2019; Jayawardena *et al*., 2020). Nevertheless, observations based on conidium size, shape and presence/absence of sexual states on PCA were reliable to distinguish *S. lycopersici* isolates from other species.

None of the isolates studied here were *S. botryosum*, although previous reports have indicated that this species was pathogenic for tomato (Dickens and Evans, 1973; Rotem and Bashi, 1977; Blancard *et al*., 2012; Blancard, 2017). This could be due to misidentifications with the closely related species *S. vesicarium* (Köhl *et al*., 2009; Woudenberg *et al*., 2017). Similarly, neither *S. solani* nor the newly described *S. simmonsii* that have been previously isolated from tomato (Woudenberg *et al*., 2017) were detected in the present study.

Several *Stemphylium* species have been identified as causal agents of tomato diseases in regions with differing climates, such as: South America (Venezuela, Argentina) (Cedeño and Carrero, 1997; Franco *et al*., 2017a, b), Europe (England, Russia) (Dickens and Evans,

Table 2. Mean percentages of leaf necrotic area (l.n.a) recorded on tomato plants inoculated with different *Stemphylium* isolates at 21 d after inoculations.

Isolate	Species	Mean*	Std dev
NB644	*S. gracilariae*	38.5	3.8
NB646	*S. gracilariae*	46.2	4.6
NB649	*S. gracilariae*	9.1	10.5
NB650	*S. gracilariae*	25.9	4.6
NB651	*S. vesicarium*	43.8	14.2
NB654	*S. vesicarium*	35.5	1.2
NB682	*S. vesicarium*	55.8	8.0
NB683	*S. lycii*	53.4	8.3
NB690	*S. gracilariae*	38.8	13.0
NB696	*S. gracilariae*	44.6	6.3
NB709	*S. eturmiunum*	29.0	3.5
NB710	*S. lycopersici*	30.7	7.2
NB711	*S. lycopersici*	10.4	7.9
NB712	*S. eturmiunum*	36.4	10.0
NB713	*S. vesicarium*	26.9	1.0
NB714	*S. eturmiunum*	6.4	2.3
NB715	*S. vesicarium*	50.8	6.1
NB717	*S. gracilariae*	44.3	0.5
NB719	*S. lycopersici*	40.8	2.3
NB720	*S. vesicarium*	32.6	6.1
NB731	*S. eturmiunum*	44.9	7.3
NB735	*S. eturmiunum*	45.9	4.9
NB737	*S. vesicarium*	58.7	5.4
NB744	*S. lycopersici*	89.9	8.8
NB747	*S. lycopersici*	86.5	6.4
NB748	*S. lycopersici*	63.1	5.7
NB751	*S. lycopersici*	38.9	5.0
NB754	*S. vesicarium*	49.4	11.6

* Each value is the mean of three repeats.

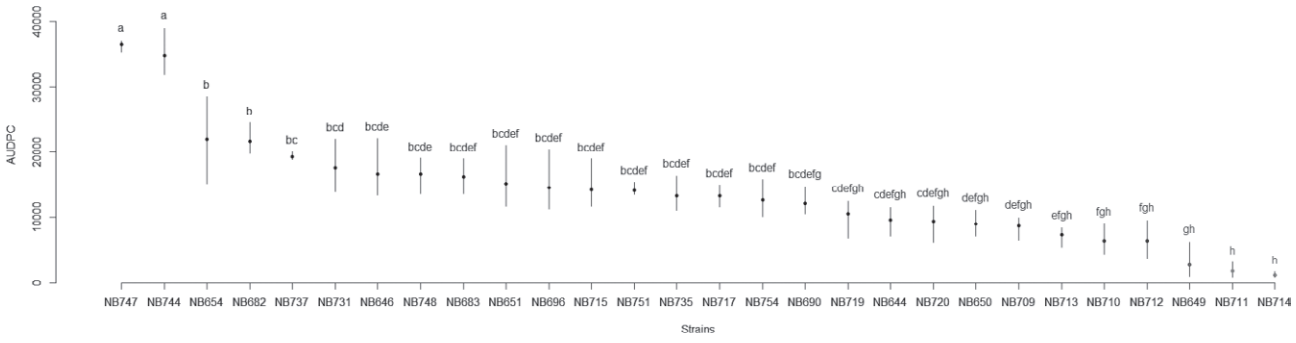

Figure 6. Mean areas under disease progress curves (AUDPCs) for disease progression on tomato plants inoculated with the different *Stemphylium* spp. isolates. The data were determined at 7, 14 and 21 d after inoculation for three replicates for each isolate. Means accompanied by the same letter are not different ($P > 0.05$).

Figure 7. Lesions caused by *Stemphylium* spp. on tomato leaves 21 d after inoculation (dai). *S. lycopersici*: NB744 (a), NB748 (b), NB747 (c); *S. vesicarium*; NB731 (d), NB720 (e), NB737 (f); *S. gracilariae*: NB644 (g), NB646 (h); *S. lycii*: NB683 (i); *S. eturmiunum*: NB709 (j), NB712 (k). Stereomicroscope view (×40 magnification) of an inoculated tomato leaflet at 21 dai (l).

1973; Gannibal, 2012), Asia (Oman, Taiwan, Malaysia, China) (Gannibal, 2012; Nasehi *et al*., 2012; Al-Amri *et al*., 2016; Sun *et al*., 2016; Huang and Tsai, 2017), Oceania (New Zealand) (Simmons, 2001) and Africa (Ivory Coast, Gambia, Tanzania, Tunisia, Morocco) (Blancard *et al*., 1986; 2012). All the isolates tested in the present study produced brown to dark necrotic lesions similar to those observed in fields, on tomato leaves under greenhouse conditions. This indicates the potential of these pathogens to cause disease in Algerian production areas. Huang and Tsai (2017) also reported that *S. lycopersici* was more likely to cause black/dark brown rather than gray lesions, suggesting that the disease name "gray leaf spot of tomato" should not be used for *Stemphylium* leaf spots. Rotem and Bashi (1977) suggested combining the diseases caused by three different *Stemphylium* species, including *S. lycopersici*, under the disease name, "*Stemphylium complex*" on tomatoes.

Stemphylium lycopersici is known to be pathogenic to tomato, and in the present study the most aggressive isolates all belonged to this species. The species was also isolated from all surveyed sites in Algeria. The sexual state of this species was not observed, which agrees with what other researchers have reported (Inderbitzin *et al*., 2009; Al Amri *et al*., 2016). This species is known to cause plant cell death through synthesis and release of phytotoxic secondary metabolites (Medina *et al*., 2019). Also, Zeng *et al*. (2018) predicted 511 secreted proteins putatively related to pathogenesis from *S. lycopersici*. This fungus can also infect a variety of host families, including ornamental and cultivated plants in the *Scrophulariaceae, Asteraceae, Caryophyllaceae, Juncaceae,*

Lamiaceae, Liliaceae, Araceae, Fabaceae, Malvaceae, Plantaginaceae, Rosaceae and *Solanaceae* (Nishijima, 1993; Nishi *et al*., 2009; Gannibal, 2012; Nasehi *et al*., 2014; Woudenberg *et al*., 2017; Kee *et al*., 2018).

Stemphylium eturmiunum was described on tomato fruit by Simmons (2001), with *Pleospora eturmiuna* as its teleomorph. This species has also been reported as a causal agent of postharvest mold in tomato (Andersen and Frisvad, 2004). *Stemphylium eturmiumum* was isolated from hosts in other families, including *Asphodelaceae* and *Amaryllidaceae* (Woudenberg *et al*., 2017). Similarly, the diverse host range of *S. vesicarium* and *S. gracilariae*, which includes solanaceous and non-solanaceous crops in different parts of the world, indicates the adaptability of these fungi to different environments.

Isolate NB683 from the present collection was identified as *S. lycii* and was found to be pathogenic on tomato. This species was described for the first time on leaves of *Lycinum chinense* leaves (Pei *et al*., 2011), and then identified on *Cucurbita moschata, Apium graveolens, Pistacia vera, Protea cynaroides*, *Triticum* sp. and *Hordeum vulgare* (Poursafar *et al*., 2016; Woudenberg *et al*., 2017), but not previously on tomato. The present report is therefore the first for *S. lycii* infecting tomato.

Symptoms observed in the field, such as brown/dark brown leaf necroses and defoliation, could be the result of interactions between different *Pleosporaceae* species with different virulence levels. *Stemphylium* and *Alternaria* spp. were always present and dominant in surveyed tomato fields from 2011 to 2017 (Bessadat *et al*., 2016; 2019). This indicates that these fungi may infect and develop on plant tissue causing a mixture of symptoms difficult to distinguish. *Stemphylium* and *Alternaria* spp. have been previously reported to simultaneously infect cultivated crops (Falloon *et al*., 1987; Hahuly *et al*., 2018; Das *et al*., 2019).

Abiotic factors are also likely to affect disease development. Temperature requirements for conidium germination of *Stemphylium* spp. vary within a range from 20°C to above 30°C (Bakr, 1991; Sinha and Singh, 1993; Mwakutuya and Banniza, 2010). These temperatures commonly occur in Algeria for extended periods from spring to autumn, due to global warming. *Alternaria* spp. have also been reported to be affected by temperature and incubation time in a similar manner to *Stemphylium* spp. (Montesinos and Vilardell, 1992; Mwakutuya and Banniza, 2010). Other factors could also be related to disease development, such as lack of crop rotation, type of fungicides used, dew periods, and host susceptibility. *Alternaria* and *Stemphylium* have capacity to produce very large amounts of secondary inoculum in short periods under favourable environmental conditions, which make tomato leaf blight and spot difficult to manage. Factors affecting disease development, and interactions with different hosts and pathogens have been little studied, so these factors require further study over long time periods.

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Research Papers

Genotype variation of citrus tristeza virus after passage on different hosts, and changes in the virus genotype populations by the vector *Aphis gossypii*

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Summary. Phylogenetic analyses categorize seven genotypes of citrus tristeza virus (CTV). The symptoms caused by this pathogen, their expression and severity are influenced by CTV genotypes, host species, cultivars, and infected host rootstocks. This study aimed to verify how populations of Chilean CTV isolates changed following inoculation from infected sweet orange to Mexican lime trees, and to determine if CTV genotype populations influenced transmission efficiency *via Aphis gossypii*. Reverse transcription polymerase chain reaction showed variation in genotypes of populations of CTV in Mexican lime, after graft inoculations using infected sweet orange chip-buds. Severe genotypes (VT) were detected after inoculation of mild isolate CTV populations (T30). The T30 donor populations also reduced transmissibility *via A. gossypii*; however, these results may not be conclusive due to mixture with the VT genotype. There is evidence of high rates of virus acquisition by this aphid species, but also low transmission efficiency, which may partially explain the historical absence of *tristeza* epidemics in Chile.

Keywords. *Citrus sinensis*, *Citrus aurantifolia*, mild CTV isolates, VT genotype.

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INTRODUCTION

Citrus tristeza virus (CTV) is the causal agent of one of the most important and destructive citrus diseases in the world (Bar-Joseph *et al.*, 1989; Moreno *et al.*, 2008; Dawson *et al.*, 2015), with three major host syndromes grouped into *tristeza*: decline inducing (DI); stem pitting (SP); and – exclusively under greenhouse conditions – seedling yellows (SY) (Rocha-Peña *et al.*, 1995; Moreno *et al.*, 2008). The symptoms and severity of disease expres-

sion depend on three factors: species or cultivar; species of rootstock; and CTV isolate genotype (Moreno *et al*., 2008; Harper *et al*., 2016).

CTV has a positive-sense single-stranded RNA genome of approx. 19,250 bp (Pappu *et al*., 1993). Phylogenetic analyses of the most variable regions of the CTV genome have distinguished seven genotypes of this virus (Melzer *et al*., 2010; Harper, 2013; Yokomi *et al*., 2018), including T36 and T30 from Florida, United States of America (Albiach-Martí *et al*., 2000; Karasev, 2000); VT from Israel (Mawassi *et al*., 1993); T68, T3, and RB, from New Zealand (Harper *et al*., 2010); and HA, from Hawaii, United States of America (Melzer *et al*., 2010). Sequence analyses of CTV isolates have shown that the 5' and 3' termini of the genomes are highly heterogeneous making it difficult to sequence complete segments (Satyanarayana *et al*., 2002; Albiach-Martí *et al*., 2010; Chen *et al*., 2018). This knowledge has led to development of molecular markers, proposed by Hilf *et al.,* (2005), to discriminate the different CTV genotypes. Among the isolates, T30 is the only one characterized as less virulent, described here as "mild".

Besides grafting of infected tissues, transmission of CTV occurs naturally by semi-persistent transmission by aphids (Bar-Joseph *et al*., 1989). Although aphid vectors play fundamental roles in the virus infection cycle, additional epidemiological information is needed, especially concerning the relationships between horizontal transmission efficiency and genetic the CTV variants that may be involved (Harper *et al*., 2016; Harper *et al*., 2018). The main reported CTV vectors are *Toxoptera citricida* (Kirkaldy) and *Aphis gossypii* Glober (Hemiptera: Aphididae). *Aphis gossypii* is the only species reported in Chile (Nieto *et al*., 2016; Blackman and Eastop, 2006). Although *T. citricida* is the most efficient vector of CTV isolates, epidemics of *tristeza* have been attributed to *A. gossypii* activity in both Spain and the United States of America (California) (Moreno *et al*., 2008).

CTV was first reported in central Chile, the largest citrus growing area of this country, during the 1960s. The virus was found in Meyer lemons (Weathers *et al*., 1969). Although apparently infected with the CTV T30 genotype, no DI epidemics were reported in orchards of sweet oranges grafted on sour orange rootstocks; however, SP was observed in grapefruit orchards in the northern Chile – i.e., the Pica and Matilla oases - in plants infected with VT genotype, in single or mix infections with the T30 genotype (Besoain, 2008; Besoain *et al*., 2015). In California and Israel, lag periods of 30 to 50 years occurred between the introductions of trees infected with CTV and natural dispersion of severe strains of the virus *via A. gossypii* (Rocha-Peña *et al*., 1995). Bar-Joseph (1978) described a natural cross-protection hypothetically exerted by mild CTV isolates during this latency period, in which isolates not transmissible by aphids may affect the horizontal transmission of the most efficient isolates from being transmitted by *A. gossypii*. They suggested that this protection would break down after 30 years, resulting in increased spread of severe CTV isolates.

Studies of formation and modelling of virus populations in plants recognize the role of aphid vectors (D'Urso *et al*., 2000; Schnieder and Roossinck, 2001). No horizontal transmission of virus isolates from infected plants to nearby trees was observed in several lemon orchards in Chile over a period of 5 years (Besoain, 2008). CTV isolates are always naturally found in mixed populations (Brlansky *et al*., 2003; Hilf *et al*., 2005; Harper *et al*., 2014). Thus, the composition of CTV genotype populations is important for understanding how disease develops in a given location, how these populations vary between hosts, and what is the role of vectors in this process.

The serological characteristics of CTV isolates are also. For example, Permar *et al.* (1990) developed a monoclonal antibody (MCA13) to detect virulent isolates of CTV from Florida. However, CTV isolates not reacting with MCA13 induced symptoms in Mexican lime, but did not cause *tristeza* symptoms in sweet orange grafted onto sour orange, or SY or SP (grooves in the wood) in grapefruit. Additionally, research by Brlansky *et al.* (2003) on CTV population untangled by *T. citricida* transmission showed that field isolates contain mild and severe CTV sub-isolates, as shown by different responses to the MCA13 test between the sub-isolates and their parent field samples. Another study with Chilean CTVs showed that all isolates considered severe tested MCA13 positive, while isolates considered mild were MCA13 negative (Besoain *et al*., 2015).

The populations of CTV genotypes present in potential donor plants are likely to be influenced by the host species, and the presence of mild genotypes probably affects the transmission *via Aphis gossypii*. The objectives of the present study were: (i) to confirm the variability of genotypic composition of 20 Chilean CTV populations in sweet orange plants, and determine the respective reactions after patch grafting onto Mexican limes plants (including MCA13 reaction); and (ii) to determine if genotype populations present in inoculated Mexican limes influence acquisition and transmission *via A. gossypii*.

MATERIALS AND METHODS

Transfer of CTV isolates to Mexican lime

Twenty CTV isolates from previously characterized Chilean populations (Besoain, 2008) were collected from different localities and hosts (Table 1). These populations were previously transferred from their original hosts into sweet orange (*Citrus sinensis* 'Madame Vinous'), and were characterized by Hilf *et al.* (2005) with eleven pairs of multiple molecular markers. In the present study, these isolates were transferred by chip-budding onto Mexican lime seedlings (*C. aurantiifolia*), and were maintained under screenhouse conditions, free of insects. Shoots from donor plants were radially cut and printed on a nitrocellulose membrane from a commercial Direct Tissue Blot Immunoassay-ELISA kit (DTBIA; Plant Print, Diagnostic) to confirm the presence of CTV. After confirmation of presence of the virus, Mexican lime plants were newly characterized following Hilf *et al.* (2005). Detection of the MCA13 epitope was achieved using the MagicDAS-ELISA (MCA13 monoclonal antibody) Plant Print Diagnostics® kit, following supplier instructions and using 1 g of leaf petioles and veins for each sample. The results obtained from the Mexican limes and their responses to MCA13 were compared with those previously obtained by Besoain (2008) in sweet orange (Table 2).

Transmission of CTV to Mexican lime plants via Aphis gossypii

Five Chilean CTV isolates present in Mexican lime (106, 231, 305-7, 334, and 366; Table 1) were used. To determine the ability of *A. gossypii* to transmit Chilean CTV isolates, only Mexican limes grown from seeds of certified plants from Lyn Citrus Seed, Inc. (disease free) were used as CTV isolate recipient plants. Collected in central Chile (Quillota, Valparaíso Region), aphid species was determined following the taxonomic keys of Simbaqueba *et al.* (2014). To provide sufficient avirulent apterous adults for the transmission assays, aphids were reared for 6 weeks placed on cotton plants (*Gossypium hirsutum* L.) before inoculation, and were maintained in greenhouse conditions. In parallel, five Mexican lime plants were inoculated with CTV isolates using chip-bud grafting. For virus acquisition by aphids, ten new shoots from each donor plant received a cotton plant shoot with 20 apterous adult aphids, which were confined inside a plastic tube covered with anti-aphid mesh to prevent escape. To acquire CTV isolate, aphids were maintained on the donor plants for a 24 h virus acquisition access period (AAP). For the transmission tests, ten replicates of recipient plants were used per isolate exposed (one repetition for each donor plant shoot), with the aphids fed on donor plants for a 24 h inoculation access period (IAP). Another ten replicates were exposed to aphids fed on ten shoots of a CTV-free Mexican lime plant for the same periods, as experimental controls. After the IAP, aphids were killed using Confidor® 350 SC. The ten aphids from each donor plant shoot, with ten replicates, and the ten aphids present on the control plants were then transferred into Eppendorf tubes and stored at -20°C for subsequent RNA extraction to determine the presence or absence of CTV, and virus acquisition. In the case of positive CTV identification, the genotypes present in the aphids were then determined.

Recipient plants were kept in a greenhouse for 6 months under controlled conditions with temperatures varying between 20 and 28°C. After this period, the plants were evaluated for symptoms, and checked using ELISA tests (BIOREBA) and qPCR following Bertolini *et al.* (2008) to verify the presence of CTV, as well as detection of the MCA13 epitope for CTV positive plants as described above. Virus transmission efficiency *via A. gossypii* was calculated from this information.

CTV genotype evaluation

The CTV isolate genotypes were assessed before the transmission tests (Mexican lime donor plants), in the vector (*A. gossypii*), and 6 months after the transmission test (CTV-positive Mexican lime recipient plants). Extractions of virus RNA from the donor plants, aphids, and positive recipient plants were performed with the commercial RNeasy Plant Mini Kit (QIAGEN) following the manufacturer's instructions. For plants, 0.1 g of petioles and leaf veins were used, and ten aphids were used for each isolate transmission test and replicate.

Characterization of the CTV followed Hilf *et al.* (2005), using the RT-PCR and a set of 11 primer pairs. Briefly, each synthesis of the cDNA was achieved using 2 µL of RNA, 1 µL of 10 µM of reverse primers, 10× M-MLV buffer (Promega), 10mM of dNTP, 40 U of RNase Inhibitor, and 1 µL of M-MLV (Promega). The mix was incubated at 50°C for 1 h and then 10 min at 72°C. Each PCR reaction included 3 µL of cDNA, 5 U of Taq DNA polymerase (Promega), 10 mM of dNTP, 2.5 mM of $MgCl_2$, 1× of Taq DNA polymerase buffer, and 10 mM of each primer. The parameters in the thermal cycler were: 94°C for 2 min, 30 cycles each of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and a final cycle of 72°C for 5 min. The RT-PCR products were visualized in 1% agarose gel with GelRed™ stain (Biotium).

Table 1. Biological characterization of Chilean CTV populations used in this study.

Isolate	Locality	Field host	Stem pitting		Seedling yellows		DI SwO/SO[b]	Biological group[c]
			Grapefruit	Sweet orange	Grapefruit	Sour orange		
42-4	Pica	Grapefruit	+	-	+	-	-	12
106[a]	Pica	Mexican Lime	-	-	-	+	+	4
231[a]	Azapa	Orange	-	-	-	+	ND	ND
281-1	Vallenar	Sweet Orange	-	-	-	-	+	7
301-34	Comiña	Orange	-	+	+	-	+	5'
305-1	Limache	Orange	-	-	+	-	+	7
305-2	Limache	Orange	-	-	-	-	-	13
305-4	Limache	Orange	-	-	-	-	-	13
305-7[a]	Limache	Orange	+	-	-	-	-	7
334[a]	Pica	Orange	+	-	-	+	+	3
351	Melipilla	Orange	-	-	-	-	-	13
366[a]	Buin	Lemon	-	-	-	-	-	13
369	Paine	Orange	-	-	-	-	-	13
389	Peumo	Orange	-	-	+	-	+	7
496	Ovalle	Lemon	-	-	+	-	+	5
497	Ovalle	Lemon	+	-	+	+	-	9
502	Vallenar	Orange	-	-	+	-	-	13
508	Pica	Grapefruit	-	+	-	-	+	5'
509	Pica	Grapefruit	+	-	+	-	+	5
510	Pica	Grapefruit	+	-	+	-	-	12

Source: Besoain, (2008); Besoain *et al.* (2015).
[a] Isolates used for inoculation via *A. gossypii*; ND: not determined.
[b] SwO, Sweet orange; SO Sour orange.
[c] Biological characterization by Besoain *et al.* (2015) using methodology of Garnsey *et al.* (1987). Genotype analyses of these isolates was based on Hilf *et al.* (2005) methodology, and is presented in Table 2.

Real-Time RT-PCR 6 months post CTV transmission

For the detection of CTV in the transmission trial, petioles and veins from recipient plants were tested 6 months post CTV transmission. The primers, probes and methodology used were based on those of Bertolini *et al.* (2008). The reaction cocktail contained 1× Taqman Universal PCR Master Mix (Applied Biosystems), 1× MultiScribe and RNase Inhibitor Mix (Applied Biosystems), 1 µM primer 3'UTR1 (5' CGT ATC CTC TCG TTG GTC TAA GC 3'), 1 µM primer 3'UTR2 (5' ACA ACA CAC ACT CTA AGG AGA ACT TCT T 3'), 150 nM TaqMan probe 181T (5' TGG TTC ACG CAT ACG TTA AGC CTC ACT TG 3') and 5 µL of purified RNA.

The real-time PCR protocol consisted of one step at 48°C for 30 min, 95°C for 10 min, followed by 45 cycles of amplification (each at 95°C for 15 s and 60°C for 1 min). Real-time PCR was carried out using the StepOnePlus™ PCR system (Applied Biosystems) and the StepOne Software v2.2.2.

RESULTS

Transfer of CTV populations to Mexican lime plants

The change of host from sweet orange to Mexican lime for each of the 20 Chilean CTV isolates resulted in variations of presence and predominance of the detected genotypes (Table 2). In Mexican lime, more VT-type isolates were detected than in sweet orange, especially in those where only the mild T30 genotype had been detected. Only three isolates, 106, 231 and 496, did not change genotype when changing host. For 14 of the CTV isolates in Mexican lime (isolates 301-34, 334, 305-1, 305-2, 305-7, 351, 366, 369, 389, 497, 502, 508, 509, and 510), more genotypes were shown. In twelve of these plants (281-1, 305-1, 305-2, 305-7, 351, 366, 369, 389, 497, 502, 509, and 510) all of the markers associated with the VT genotype were amplified. In previous studies on sweet orange (Besoain *et al.*, 2015), all of these isolates were characterized as mild, and ten (305-1; 305-2, 305-7, 351, 366, 369, 389, 496, 497, and 502) were collected

Table 2. Characterization of Chilean CTV genotypes based on marker patterns according to Hilf *et al.* (2005), and serological reaction to MCA13 present in sweet orange and transmitted to Mexican lime through chip-bud grafting.

Isolate	Genotype and/or detected marker in sweet orange	MCA13 Sweet orange	Genotype and/or detected marker in Mexican lime	MCA 13 Mexican lime
42-4	T30K17, VT 5' VTK17	+	T30 5'	-
106[a]	VT	+	VT	-
231[a]	VT+T30K17	+	VT+T30K17	+
281-1	T30K17, VT 5' VTK17+T30Pol	-	VT, T30	-
301-34	VT	+	VT+T36 5'	-
334[a]	VT	+	VT+T30K17	-
305-1	T30K17	+	VT+T30K17+T36 5'	-
305-2	T30K17	-	VT, T30	-
305-4	T30K17	-	Only T36CP	-
305-7[a]	T30K17	-	VT, T30	-
351	T30K17	-	VT, T30+T36 5'	-
366[a]	T30K17	-	VT, T30	-
369	Only T36CP	-	VT 5', VTK17, VTPol	-
389	Only T36CP	-	VT, T30	-
496	Only T36CP	-	Only T36CP	-
497	Only T36CP	+	T30K17, VTK17,VTPol	-
502	Only T36CP	-	VT, T30+T36 5'	-
508	VT	+	VT, T30+T36 5'	+
509	Only T36CP	+	VT+T36 5', T30K17, T30Pol, VT 5', VTK17	-
510	VTK17	+	VT, T30 5', T30K17, VT 5' VTK17+T36 5'	+

[a] Isolates used in inoculation with *A. gossypii*.

from the central zone of Chile. In addition, in one isolate (42-4) the VT genotype (partial) was initially detected in sweet orange, and when transferred to Mexican lime only the T30 5' marker was detected. The MCA13 epitope analysis also showed a difference following host change. Of the ten MCA13 positive isolates in sweet orange, only three continued to be MCA13 positive in Mexican lime, seven underwent loss or variation of the MCA13 epitope, causing them to become undetectable by this technique (Table 2).

Transmission of CTV isolates by Aphis gossypii

Six months after CTV transmission by *A. gossypii*, only two plants were positive for CTV as indicated by RT-qPCR. The positive plants were numbers 231 (replicate 4) and 334 (replicate 1) (Table 3). All other plants were negative. For CTV-positive plants used to transmit all six CTV isolates by aphids, all ten shoots used were previously found to be CTV-positive by DTPA tests, denoting systemic distribution. All ten shoots of the control plants were CTV-negative. The recipient plants positive for CTV after 6 months showed few symptoms. However, the average Ct values obtained by RT-qPCR for both CTV-positive plants were adequate titre levels, of 28.3 for isolate 334-1 and 30.3 for isolate 231-4. The tests were repeated 6 months later, and similar Ct values were obtained.

Table 3 presents the results obtained from amplification of each pair of the primers proposed by Hilf *et al.* (2005) in the donor and recipient plants. Genotypes transmitted from the donor plants did not amplify the complete marker pattern associated with the T30 genotype. In the two CTV positive recipient plants, the genotypes were found to be similar to those also present in the donor plants. From the MCA13 epitope analysis, no positive results were obtained in recipient plants, although one of the donor plants was MCA13 positive before transmission.

Of all the aphids analyzed in the transmission tests (Table 4), CTV was not detected in the case of isolate 366, nor when the T36CP genotype primers, considered universal for all CTV isolates, were used. This showed that no aphids were able to acquire measurable quantities of CTV particles from isolate 366 after the 24 h acquisition period. Excluding the isolate 366, the CTV markers T36CP, VT5', and VTPOL were detected in all the pools of *A. gossypii* used in the transmission trials. \

Table 3. Genotypes determination based on marker patterns obtained using the Hilf *et al.* (2005) methodology. Genotypes in infecting donor plants and CTV-positive recipient plants after transmission through *Aphis gossypii*.

Isolate[a]	RT-PCR markers											CTV marker	
Donor plants	T36 CP	T36 5'	T36 K17	T36 Pol	T3 K17	T30 5'	T30 K17	T30 Pol	VT 5'	VT K17	VT Pol	Genotypes or CTV markers	MCA13
106	1[b]	0[b]	0	0	0	0	0	0	1	1	1	VT	-
231	1	0	0	0	0	0	1	0	1	1	1	VT +T30K17	+
305-7	1	0	0	0	0	1	1	1	1	1	1	VT + T30	-
334	1	0	0	0	0	0	1	0	1	1	1	VT +T30K17	-
366	1	0	0	0	0	1	1	1	1	1	1	VT + T30	-
NC	0	0	0	0	0	0	0	0	0	0	0	0	-
Recipient plants	T36 CP	T36 5'	T36 K17	T36 Pol	T3 K17	T30 5'	T30 K17	T30 Pol	VT 5'	VT K17	VT Pol	Genotypes or CTV markers	MCA13
231.4[c]	1	0	0	0	0	0	1	0	0	1	1	T30K17+VTK17+ VTPOL	-
334.1[c]	1	0	0	0	0	0	1	0	0	1	1	T36 CP +T30K17 +VTK17+VTPOL	-

NC, negative control.
[a] Isolate in donor or recipient plants.
[b] 1 indicates band presence in electrophoresis, 0 indicates absence for each of the primers.
[c] Number of replicate plant detected positive to CTV.

Table 4. Frequency of amplified markers patterns in groups of ten *Aphis gossypii* individuals during the transmission assays of Chilean CTV isolates.

Isolate in 10 aphids	RT-PCR markers										
	T36 CP	T36 5'	T36 K17	T36 Pol	T3 K17	T30 5'	T30 K17	T30 Pol	VT 5'	VT K17	VT Pol
106	9	0	0	0	0	0	2	0	2	0	10
231	7	0	0	0	0	0	3	0	4	1	8
305-7	6	0	0	0	0	0	0	3	1	0	7
334	6	0	0	0	0	0	0	0	2	0	9
366	0	0	0	0	0	0	0	0	0	0	0
NC	0	0	0	0	0	0	0	0	0	0	0
Total	28/60	0/60	0/60	0/60	0/60	0/60	5/60	3/60	9/60	1/60	34/60

NC, negative control.

Table 5. Efficiency of CTV acquisition and transmission by *Aphis gossypii* in lots of ten aphids per isolate, with ten replicates per isolate and 24 h inoculation access periods and 24 h acquisition access periods.

Isolate transmitted	Acquisition efficiency (%)	Transmission efficiency (%)
106	100	0
231	80	10
305-7	70	0
334	90	10
366	0	0
NC	-	-
Average	68%	4%

NC, negative control.

The marker T30K17 was detected only in pools 106 and 231, while T30Pol was detected in 305-7 and VTK17 in 231. The transmission efficiency of CTV by *A. gossypii* was lower than the CTV acquisition efficiency (Table 5). The isolate most acquired by *A. gossypii* (isolate 106) was not transmitted to the recipient plants.

DISCUSSION

This study has demonstrated that the CTV genotype population of the same isolates can change in two different hosts. Transmission of CTV from sweet orange to Mexican lime changed the presence and predominance of population genotypes, which coincides with results

presented by Harper *et al.* (2015). The present study also demonstrated the existence of genotype population modification of an individual isolate after passage through two different hosts, showing that the presence and predominance of population genotypes were modified by transmission from sweet orange to Mexican lime. This also agrees with the results from Harper *et al.* (2015).

Previous studies on sweet orange (Besoain *et al.*, 2015) have indicated the predominance of VT genotype isolates in the north of Chile, and T30 type isolates in the central area of this country. In the present study, the same CTV isolates previously characterized as mild in sweet orange hosts showed high presence of the VT genotype once transferred into Mexican lime (Table 2). It is possible that the VT genotype was present in sweet orange, but at such a low concentration or genotype titre as to be undetected by endpoint RT-PCR techniques. It may also be that the T30 isolate interfered with the replication of VT genotype isolates when present in sweet orange, but not in Mexican lime. This may explain why cross-protection is not always effective, and may depend on the type of host and/or virus isolate used. The present study has shown that a mild isolate may hide additional subisolates, and that further evaluations in different hosts are required for complete identification of the population before declaring them as truly mild isolates.

Regarding *A. gossypii* transmission efficiency, the present results differ from what is reported the literature. For example, Hermoso de Mendoza *et al.* (1984) found 78% CTV transmission *via A. gossypii* using a T30 isolate from Spain, later characterized as a VT type isolate by Rubio *et al.* (2001). *Aphis gossypii* has proven to be an efficient CTV vector and a threat to citrus production areas including California, Israel, and Spain (Cambra *et al.*, 2000; Marroquín *et al.*, 2004; Yokomi and De Borde, 2005). Fuller *et al.* (1999) argued that population differences in *A. gossypii* could be affected by climatic variations, leading to the selection of biotypes, which could also affect CTV epidemiology. For example, the contrasting results of the efficiency of the proportions of vector acquisition and transmission of viruses show that the virus particles are retained by *A. gossypii.* For example, the isolate 106 (VT-type) showed a 100% virus acquisition by the aphids, but no transmission (Table 5). Notwithstanding, the ten *A. gossypii* in this trial probably had less transmission efficiency than that reported from a single *T. citricida* aphid (Yokomi *et al.*, 1994; Tsai *et al.*, 2000).

Two CTV isolates (231 and 334) transferred from donor Mexican lime plants had VT + T30 K17 genotype (Table 2), while the non-transferred isolates only had the VT or VT plus T30 genotypes. This raises the question of the role of the T30K17 region in the transmission by *A. gossypii*. In order to address this, a transmission study using a greater numbers and types of CTV isolates than used here should be conducted.

Genotype population almost certainly affects transmission efficiency. In northern Chile, citrus orchards are small, and mostly grow Mexican lime plants. In contrast, the orchards of central Chile almost exclusively contain sweet orange and lemon trees (*Citrus limon* L.).

The non-detection of MCA13 in *A. gossypii*-inoculated plants from MCA-13 positive donor plants suggests that some populations of CTV genotypes can be transmitted more efficiently than others. This is consistent with the low prevalence of MCA13-positive isolates in central Chile (Besoain *et al.* 2015). Pappu *et al.* (1993) demonstrated that a nucleotide mutation in the MCA13 epitope prevents the antibody from recognizing CTV. This may be because the CTV genotypes with epitope modifications (MCA13 negative) are transmitted by *A. gossypii* more frequently than those without epitope modifications (MCA13 positive). Further research is required to determine which characteristics of the vector allow "selection" of different molecular variants of viruses.

Although the presence of SP was reported in northern Chile, where severe isolates (type VT and MCA13+) were found, absence of the *tristeza* syndrome in central Chile may be associated with virus populations present in the Chilean CTV genotypes, and the low transmission efficiency of the vector. However, previously collected data (Besoain *et al.*, 2015) suggest that the development of SP syndrome in grapefruit trees in the locations Pica and Matilla oases could be influenced by the appearance of severe VT-type strains present in Mexican lime, which in turn emerged from orange trees that were brought from central Chile. It is important to recognize that Mexican lime is the main species grown in these oases. Mexican lime, known in Chile as "*limón de Pica*", may have been a donor of severe CTV isolates transmitted by *A. gossypii* and causing SP in grapefruit trees.

The present study contributes to knowledge on virus genotype variation after the passage through different hosts, and how CTV genotype populations may influence the success of virus transmission by the vector *Aphis gossypii.*

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Research Papers

Rutin-stevioside and related conjugates for potential control of grapevine trunk diseases

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Summary. Flavonoids and phenolic acids play roles in grapevine defence against pathogens causing grapevine trunk diseases (GTDs). Rutin is a major flavonoid in vegetative organs of the grapevines, and this compound, unlike other flavonoids, is non-toxic and non-oxidizable. Rutin was assayed *in vitro* and *in vivo* against two *Botryosphaeriaceae* taxa. The limited bioavailability of this compound was circumvented by conjugation with stevioside, a glycoside obtained from *Stevia rebaudiana*. Clear synergistic effects were observed for the stevioside-rutin adduct, resulting in EC_{50} and EC_{90} values of 306.0 and 714.9 μg·mL^{-1} against *Neofusicoccum parvum* and 241.6 and 457.8 μg·mL^{-1} against *Dothiorella viticola*. In greenhouse experiments, moderate inhibition of *N. parvum* growth and complete inhibition of *D. viticola* were observed. These inhibitory effects were greater than those of ferulic acid, which has been considered the most effective phenolic acid against GTDs. Conjugation with stevioside provided solubility enhancement of rutin, paving the way to the design of glycopesticides based on rutin-rich plant extracts as promising antifungals against GTDs.

Keywords. Antifungal, Botryosphaeria dieback, candyleaf, GTDs, rutoside.

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INTRODUCTION

Grapevine (*Vitis vinifera* L.) cultivars can be severely affected by many pathogens, including fungi, bacteria, oomycetes, and viruses. Grapevine trunk diseases (GTDs) have long been responsible for significant economic losses, with some of these diseases being well-known for more than 100 years. The interest of the viticulture sector in this group of diseases has increased in the last three decades, due to, firstly, increased mortality of young nursery-produced grapevine plants, especially 1–3 years after planting in the field, and secondly, the progressive suspension of the use of chemical

fungicides, which has resulted in progressive increases in the incidence and losses due to these diseases.

Current agricultural policies are provoking interest in the development of alternative, naturally-derived antifungal products for the sustainable management of grapevine diseases. However, there are other factors directly or indirectly involved in the expansion of GTDs, in young and mature vineyards. Some factors are related to changes in cultural practices, such as reduced protection of pruning wounds or reductions in sanitary control measures in certified propagation material (Graniti *et al.*, 2000). It is also commonly accepted that a correlation exists between the increase in the incidence of decay of young grapevine plants and increased demand for new plantings or replacements in the different world production areas. This has led to the advocation of a system where infected propagation material from nurseries is considered the primary source of inoculum causing young vine decline. Numerous studies (see Surico (2001) and Fourie and Halleen (2004)) have correlated the presence of particular fungi causing propagation material decay in nurseries with the dead of grapevine plants in the very first years after vineyard planting.

Natural compounds that have been tested against the three main GTDs (Botryosphaeria dieback, Esca complex, and Eutypa dieback) include chitosan, garlic extract, tea tree (*Melaleuca alternifolia* (Maiden & Betche) Cheel) oil, green coffee extract, lemon peel extract, honey, propolis, seaweed extract, and saponins (Mondello *et al.*, 2018). Nonetheless, few studies have focused on the specific bioactive phytochemicals associated with these natural extracts and compounds, which could provide increased efficacy for products with variable phytochemical composition, resulting from genetic variability, and/or environmental variability influenced by weather or soil fertility.

Phytoalexins, which in grapevines are phenolic compounds including tannins, phenolic acids, flavonoids, and stilbenes, are involved in grapevine defence, increasing host resistance to pathogens (Del Río *et al.*, 2004). The progression of fungal pathogens along grapevine wood is inhibited by polyphenol-rich reaction zones (Fontaine *et al.*, 2016). The possible roles of phenolics in defence against GTD casual agents were studied by Lambert *et al.* (2012a), who analysed the *in vitro* effects of 24 grapevine compounds (eight phenolic acids, three flavan-3-ols, two flavonols, and 11 stilbenoids) on six *Botryosphaeriaceae* taxa. They showed that these pathogens were differentially susceptible to phenolics, and concluded that ferulic acid (*trans*-4-hydroxy-3-methoxycinnamic acid) was one of the most active compounds, causing an inhibition comparable to that from the stilbenoids ε-viniferin, vitisin A and B, or *trans*-pterostilbene. However, Lambert *et al.* (2012a) detected no inhibitory activity on any wood disease fungi for flavonols (kaempferol and quercitin) and flavan-3-ols [(+)-catechin, (–)-epicatechin, and epicatechin-3-*O*-gallate]. In some cases, these compounds enhanced the growth of some of the assayed fungi. In contrast, other authors have reported that catechin inhibited fungi involved in Petri disease, and other GTDs (caused by *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams, *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & L. Mugnai, *Eutypa lata* (Pers.) Tul & C. Tul, and *Stereum hirsutum* (Willd.) Pers.) (Del Río *et al.*, 2004). Furthermore, the antifungal efficacy of flavonoids is well-recognised (Jin, 2019; Al Aboody and Mickymaray, 2020). To gain further insight into these conflicting results, the present study compared the effectiveness of the flavonoid-3-*O*-glycoside rutin (also known as rutoside, phytomelin, or quercetin 3-*O*-rutinoside) with that of ferulic acid. Rutin is one of the most abundant polyphenols (excluding stilbenes) in the vegetative organs of grapevine plants, found at mean concentrations of 257 $mg \cdot kg^{-1}$ (Goufo *et al.*, 2020), and has significant antimicrobial activity (Ganeshpurkar and Saluja, 2017). The use of rutin is advantageous compared with other flavonoids as it is non-toxic and non-oxidizable (Sharma *et al.*, 2013).

Low water-solubility of phenolic acids, e.g., ferulic acid (Shakeel *et al.*, 2017) and flavonoids (Chebil *et al.*, 2007), can limit their bioavailability and applicability (Hussain *et al.*, 2017). This may be circumvented through the formation of inclusion compounds or conjugate complexes with terpene glycosides (Nguyen *et al.*, 2017). Stevioside (the major constituent of *Stevia rebaudiana* (Bertoni) Bertoni extract) may be a suitable option to form conjugate complexes, resulting in enhanced antifungal activity. Clear synergistic effects have been reported from the conjugation of phenolic acids with stevioside against *Fusarium culmorum* (Wm.G.Sm.) Sacc. (Buzón-Durán *et al.*, 2020) and *Phytophthora cinnamomi* Rands (Matei *et al.*, 2018b).

The goal of the present study was to assess the *in vitro* and *in vivo* antifungal activities of rutin against the two most important *Botryosphaeriaceae* taxa that cause GTDs, especially of young grapevines. Rutin was assessed alone and in a conjugate complex with stevioside, and was compared with ferulic acid as a reference substance. This information should be useful for selecting promising plant sources of natural antifungal products for use in organic or integrative viticulture.

MATERIALS AND METHODS

Reagents

Rutin hydrate (CAS 207671-50-9, ≥94%), ferulic acid (CAS 537-98-4, European Pharmacopoeia reference standard), sodium alginate (CAS 9005-38-3), calcium carbonate (CAS 471-34-1, ≥99.0%), and methanol (CAS 67-56-1, UHPLC, suitable for MS) were supplied by Sigma-Aldrich/Merck KGaA (Darmstadt, Germany). Stevioside (CAS 57817-89-7, 99%) was purchased from Wako Chemicals GmbH (Neuss, Germany). Potato dextrose agar (PDA) was supplied by Becton, Dickinson & Company (Franklin Lakes, NJ, USA).

Fungal isolates

Neofusicoccum parvum (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips (code ITACYL_F111; isolate Y-091-03-01c; isolated from 'Verdejo' cultivar grapevines in a nursery in Navarra, Spain, in 2006) and *Dothiorella viticola* A.J.L. Phillips & J. Luque (code ITACYL_F118; isolate Y-103-08-01; isolated from grapevines in Extremadura, Spain, in 2004) were supplied as lyophilized vial cultures (later reconstituted and refreshed as PDA subcultures) by the Agricultural Technological Institute of Castilla and Leon (ITACYL, Valladolid, Spain) (Martin and Cobos, 2007).

Preparation of bioactive formulations

Treatments based on pure stevioside, rutin, or ferulic acid were prepared by dissolving the respective compounds in Milli-Q water (stevioside) or methanol (for rutin and ferulic acid), without further purification.

Ultrasonication-assisted aqueous biphasic system separation was used to prepare the stevioside-polyphenol conjugate complexes in a 1:1 molar ratio. 50 mL of an aqueous solution of stevioside (126 mg, MW = 804.87 g·mol^{-1}, 0.156 mM) were mixed with a 50 mL methanol solution of either ferulic acid (95.2 mg, MW = 610.517 g·mol^{-1}, 0.156 mM) or rutin (75.3 mg, MW = 482.44 g·mol^{-1}, 0.156 mM). The solutions were sonicated with a probe-type UIP1000hdT ultrasonicator (Hielscher, Teltow, Germany; 1000 W, 20 kHz) for 15 min, keeping the temperature below 60°C.

For *in vivo* experiments, the conjugate complexes were dispersed in a calcium alginate matrix, in the form of hydrogel beads. The beads were prepared as follows: each bioactive product was added to a 3% sodium alginate solution in a 2:8 ratio (20 mL bioactive product:80 mL sodium alginate), and this solution was then dispensed drop by drop onto a 3% calcium carbonate solution to spherify (polymerize) the solution. Beads of ⌀ = 0.4–0.6 cm containing the different treatments were obtained.

In vitro *tests of mycelium growth inhibition*

The biological activity of the different treatments was determined using the agar dilution method, incorporating aliquots of stock solutions into PDA medium to provide final concentrations of 62.5, 93.75, 125, 187.5, 250, 375, 500, 750, 1000, and 1500 μg·mL^{-1}. Mycelium plugs (⌀ = 5 mm) of each pathogen from the edges of 7-day-old cultures were transferred to plates filled with amended media (three plates per treatment and concentration). Plates containing only PDA without amendment were used as experimental controls. The experiment was carried out twice.

Radial mycelium growth was determined by calculating the average of two perpendicular colony diameters for each replicate. Mycelium growth inhibition, after 7 days of incubation at 25°C in the dark for each treatment and concentration, was calculated according to the formula: $((d_c\text{-}d_t)/d_c) \times 100$, where d_c is the average fungal colony diameter in the experimental control and d_t is the average colony diameter treated with composite.

Fitting the radial growth inhibition values (%) with a DoseResp function, using an orthogonal distance regression (ODR) algorithm, allowed expression of the results as 50% (EC_{50}) and 90% (EC_{90}) effective concentrations.

For treatment interactions, synergy factors (SF) were estimated using Wadley's method (Levy *et al.*, 1986). This method assumes that one component of a mixture can substitute at a constant proportion for the other component. The expected effectiveness of the mixture is then directly predictable from the effectiveness of the constituents if the relative proportions are known (as in this case). The synergy factor (SF) was estimated as:

$$SF = \frac{ED(exp)}{ED(obs)} = \frac{\left(\frac{a+b}{\frac{a}{ED_A}+\frac{b}{ED_B}}\right)}{ED(obs)}$$

where a and b are the proportions of the products A and B in the mixture and $a + b = 1$, ED_A and ED_B are their equally effective doses, *ED(exp)* is the expected equally effective dose and *ED(obs)* is the equally effective dose observed in the experiment.

If SF = 1, the hypothesis of similar joint action (i.e., additivity) can be accepted; if SF > 1, there is synergistic action; and if SF < 1, there is antagonistic action between the two fungicide products.

Greenhouse bioassays on grafted plants

The protective capabilities of the most promising formulations and dosages, as indicated from the *in vitro* mycelium growth inhibition experiments, were further assayed in grafted plants against the two selected *Botryosphaeriaceae*, using the method described by Buzón-Durán *et al.* (2021). Briefly, 68 plants were used, half of which were 2-year-old 'Tempranillo' vines (CL. 32 clone) grafted on 775P rootstock, and the rest were 1-year-old 'Garnacha' vines (VCR3 clone) grafted on 110R rootstock. The two cultivars were tested to assess potential differences in their sensitivity to the pathogens under study. The cultivar choice was guided by the relevance of 'Tempranillo' and 'Garnacha' in the protected designations of origin in Aragón, Spain.

Plants were grown on 3.5 L capacity plastic pots containing a mixed substrate of moss peat and sterilized natural soil (75:25), to which a slow-release fertilizer was incorporated when needed. The plants were kept in a greenhouse with drip irrigation and anti-weed ground cover for 6 months (from June to December 2020).

One week after placing in the greenhouse, the grapevine plants were artificially inoculated with the two pathogens (*N. parvum* and *D. viticola*) and simultaneously treated with either the stevioside–rutin or the stevioside–ferulic acid treatment. Inoculations of both pathogens and bioactive products were carried out directly on the trunks of the living plants at two sites on each plant, at least 5 cm apart from each other, and below the grafting point (and not reaching the root crown). For the pathogens, agar plugs from 5-day-old fresh PDA cultures of each species were used as the fungal inoculum. At the two inoculation points, slits (⌀ ≈ 15 mm, 5 mm deep) were made with a scalpel. Agar plugs (⌀ = 5 mm) were then placed so that the mycelium was in contact with the stem vascular tissue. The beads containing the bioactive product were then placed at both sides of the agar plug, and the agar plug and beads were covered with cotton soaked in sterile double distilled water and sealed with Parafilm™ tape.

Five repetitions were arranged for each pathogen/bioactive product and plant (cultivar/rootstock) combination. Four positive controls/(pathogen*cultivar) and three negative controls (only the bioactive product) for each treatment were used (Table 1).

During the assay period, cuprous oxide (75%) was applied in mid-July to control downy mildew outbreaks, together with a first sprouting (followed by periodic sprouting). *Amblyseius* (*Typhlodromips*) *swirskii* Athias-Henriot was used for the biological control of whitefly, thrips, and spider mite; *Encarsia formosa* Gahan/*Eretmocerus eremicus* Rose & Zolnerowich for whitefly; and *Aphelinus abdominalis* Dalman for aphids at the end of July (Biobest Group NV, Almería, Spain). The grapevine plants were visually examined each week throughout the assay period for the presence of foliar symptoms (including interveinal and veinal necroses).

At the end of the experiment, plants were removed and two transversal sections of each inoculated stem, between the grafting point and the root crown, were prepared and sectioned longitudinally. The effects of the inoculated fungi were evaluated by measuring the lengths of longitudinal vascular necroses in each direction from the inoculation point.

Samples from the assayed plants were further processed to re-isolate the previously inoculated fungi, and to fulfill Koch's postulates. Wood chips (length = 5 mm) exhibiting vascular necroses (1–2 cm around the wounds) were washed, surface sterilized, and placed in PDA plates amended with streptomycin sulphate (to avoid bacterial contamination). The plates were incubated at 26°C in the dark for 2–3 days in a culture chamber. Emerging colonies were identified based on their morphological characters. A selection of the isolates recovered from vascular lesions was identified by comparing ribosomal ITS sequences with those from the inoculated isolates.

Table 1. Details of plant/treatment combinations for the greenhouse bioassay. Each grafted plant was inoculated at two sites below the grafting point.

Plant	Treatment	Pathogen	Number of replicates
'Tempranillo' (CL. 32 clone) on 775P rootstock	Stevioside-ferulic acid	*N. parvum*	5
		D. viticola	5
		Nil (negative control)	3
	Stevioside-rutin	*N. parvum*	5
		D. viticola	5
		Nil (negative control)	3
	Nil (positive control)	*N. parvum*	4
		D. viticola	4
'Garnacha' (VCR3 clone) on 110R rootstock	Stevioside-ferulic acid	*N. parvum*	5
		D. viticola	5
		Nil (negative control)	3
	Stevioside-rutin	*N. parvum*	5
		D. viticola	5
		Nil (negative control)	3
	Nil (positive control)	*N. parvum*	4
		D. viticola	4

Statistical analyses

Given that the homogeneity and homoscedasticity requirements were satisfied, according to Shapiro–Wilk and Levene tests, the results of the *in vitro* mycelium growth inhibition experiments were statistically analyzed using one-way analysis of variance (ANOVA), followed by *post hoc* comparison of means through Tukey's test at $P < 0.05$. For the greenhouse assays, since normality and homoscedasticity requirements were not met, Kruskal-Wallis non-parametric test was used, with Conover-Iman test for *post hoc* multiple pairwise comparisons. R statistical software was used for all the statistical analyses (R Core Team, 2020).

RESULTS

In vitro *tests of mycelium growth inhibition*

From *in vitro* tests (Figure 1 and Figure S1), greater antifungal activity was recorded from stevioside or rutin alone than for ferulic acid against both *Botryosphaeriaceae* taxa, especially against *N. parvum* (Table S1). Statistically significant increases in antifungal activity were observed for the stevioside-rutin and stevioside-ferulic acid conjugate complexes. For stevioside-rutin, almost complete inhibition of *N. parvum* occurred at 1000 µg·mL^{-1}, and for *D. viticola* complete inhibition was observed at 750 µg·mL^{-1}, compared with 1500 µg·mL^{-1} for the non-conjugated compounds against both fungi. For the ferulic acid adduct, efficacy was also slightly lower than that of the rutin adduct: concentrations of 1500 and 1000 µg·mL^{-1} were required for complete inhibition of *N. parvum* and *D. viticola*, respectively. Inhibition of 64% for *N. parvum* and 74% for *D. viticola* resulted from the 1500 µg·mL^{-1} ferulic acid treatment.

Comparison of EC_{50}s and EC_{90}s (Table 2) for colony diameter measurements corroborated the lower EC values for rutin than for ferulic acid, especially against *D. viticola*. On the basis of the EC_{90} values, synergism was observed for the two complexes, with SF values close to 1.45 for *N. parvum*, and ranging from 1.6 to 2.2 for *D. viticola*.

Greenhouse bioassays with grafted plants

After removing, cutting, and measuring vascular necroses present in the different treated plants, no statistically significant differences were detected between cultivar/rootstock combinations, as shown in Table S2.

Statistically significant differences were detected for mean lengths of vascular necroses between treated and non-treated plants for both pathogens. Against *N. parvum* (Figure 2), the two assayed formulations gave similar results (Table 3). Against *D. viticola*, the stevioside-rutin treatments gave a greater reduction in lesion lengths than the stevioside-ferulic acid treatment, as it occurred in the *in vitro* tests (Table 4). Mean lengths of necroses for the treated plants were not significantly different from those of the negative controls (i.e., those from plants treated with conjugate complexes without pathogens), indicating strong inhibition of *D. viticola*. Lesions from the two negative controls were similar.

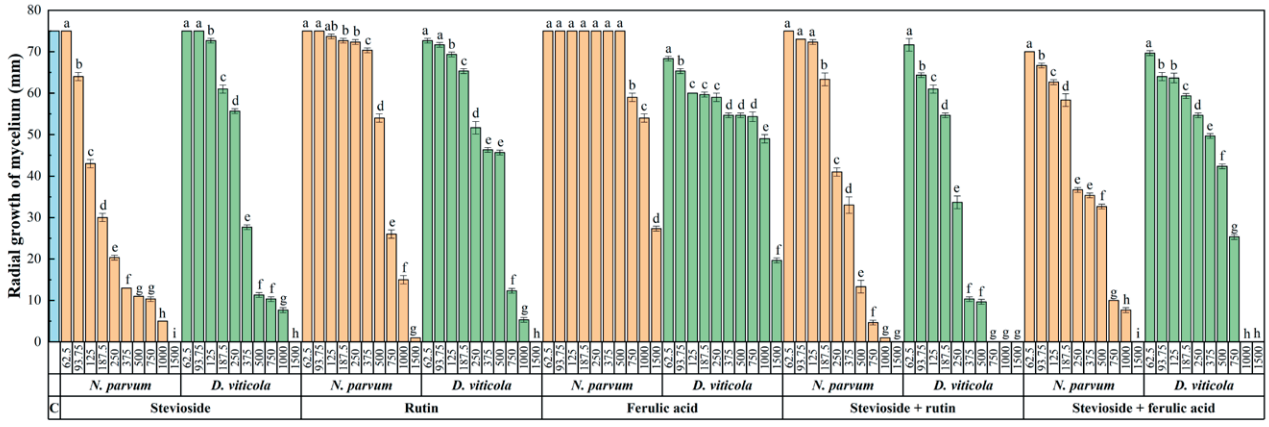

Figure 1. Mean colony radial diameters for *Neofusicoccum parvum* and *Dothiorella viticola* strains when cultured in PDA plates containing the different treatments) at concentrations ranging from 62.5 to 1500 µg·mL^{-1}. Means accompanied by the same letters are not significantly different ($P < 0.05$), and bars indicate standard deviations.

Table 2. Mean EC_{50}s and EC_{90}s ($\mu g \cdot mL^{-1}$; ± standard errors), and calculated synergy factors (S.F.).

Pathogen	Effective Concentration	Stevioside	Rutin	Ferulic acid	Stevioside–rutin	S.F.	Stevioside–ferulic acid	S.F.
N. parvum	EC_{50}	154.9±13.5	656.9±25.4	1394.4±63.0	306.0±23.6	0.82	435.6±66.8	0.64
	EC_{90}	923.8±56.7	1156.5±72.2	4121.3±313.5	714.9±31.9	1.44	1032.2±43.1	1.46
D. viticola	EC_{50}	309.6±16.6	575.1±34.9	1287.2±51.3	241.6±12.8	1.67	574.4±46.3	0.87
	EC_{90}	1007.1±66.0	981.1±58.6	2948.6±168.0	457.8±21.7	2.17	921.8±72.5	1.63

Figure 2. Foliar symptoms and vascular necroses observed in grapevine plants artificially inoculated with *Neofusicoccum parvum* and treated with two conjugate complexes of natural products. Top row, left to right; general aspect of plants treated with *N. parvum* (positive control), stevioside-ferulic acid, stevioside-rutin, *N. parvum* + stevioside-ferulic acid and *N. parvum* + stevioside-rutin. Bottom row; vascular lesions after sectioning the stems of the grapevine plants in the top row.

Table 3. Mean lengths of the vascular necroses in grapevine plants after inoculations with *Neofusicoccum parvum*. Mean of rank values accompanied by the same letters are not significantly different ($P < 0.05$; Kruskal-Wallis test, and multiple pairwise comparisons using the Conover-Iman procedure).

Treatment	Mean of ranks	Groups		
Stevioside-rutin negative control	42.573	A		
Stevioside-ferulic acid negative control	48.188	A		
Stevioside-rutin	145.656		B	
Stevioside-ferulic acid	155.638		B	
Positive control	183.313			C

Table 4. Mean lengths of vascular necroses in grapevine plants after inoculations with *Dothiorella viticola*. Mean of rank values accompanied by the same letters are not significantly different ($P < 0.05$; Kruskal-Wallis test, and multiple pairwise comparisons using the Conover-Iman procedure).

Treatment	Mean of ranks	Groups			
Stevioside-rutin	90.472	A			
Stevioside-rutin negative control	110.813	A	B		
Stevioside-ferulic acid negative control	123.713		B	C	
Stevioside-ferulic acid	145.632			C	
Positive control	260.766				D

DISCUSSION

Comparison with reported antifungal efficacies for bioactive substances

When comparing results of sensitivity of fungal pathogens to exposure to fungicidal compounds, susceptibility profiles in these microorganisms are usually species, and isolate-dependent, so comparisons of effective concentrations discussed below should be taken with caution.

Previous research has advocated ferulic acid as the phenolic acid having the strongest anti-fungal activity (Lambert *et al.*, 2012a; Sabel *et al.*, 2017; Zabka and Pavela, 2013). In assessments of the efficacy of ferulic acid against GTDs, Lambert *et al.* (2012b) assayed a concentration of 500 µM (97 µg·mL^{-1}), and measured *in vitro* growth inhibitions of 23% against *N. parvum* strain PER20 and 35% against *N. parvum* strain Bp0014. However, these authors did not report MIC or EC values for ferulic acid against these fungi. Gómez *et al.* (2016) reported EC_{50} values of 3530 µg·mL^{-1} for ferulic acid against several *Botryosphaeriaceae* species and 4740 µg·mL^{-1} against *Phaeoacremonium minimum* (Tul. & C. Tul.) Gramaje, L. Mostert & Crous. Dekker *et al.* (2002) reported 62% inhibition for ferulic acid against *Botryosphaeria* Ces. & de Not. species at 25 mM (4855 µg·mL^{-1}), with an EC_{50} value of 15 mM (2913 µg·mL^{-1}). In general terms, EC_{50}s in these reports were 2 to 3 times greater than those recorded in the present study (1340 and 1454 µg·mL^{-1}). Srivastava *et al.* (2013) tested ten naturally occurring phenolic compounds from plants against isolates from different *Botryosphaeriaceae* genera (viz. *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *B. obtusa* (Schwein.) Shoemaker, and *Neofusicoccum ribis* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips)). They reported that ferulic acid at 25 mM (4855 µg·mL^{-1}) gave ca. 80% mycelium growth inhibition of *L. theobromae* and ca. 70% inhibition of *N. ribis*, while 100% inhibition was attained at 20 mM (3885 µg·mL^{-1}) for *B. obtusa*. These concentrations are similar to the EC_{90} values obtained in the present study against *N. parvum* (3230 µg·mL^{-1}) and *D. viticola* (3921 µg·mL^{-1}).

Zabka and Pavela (2013) assessed the efficacy of 21 phenolic components of essential oils and plant substances against several toxicogenic filamentous fungi. They reported MIC values >1000 µg·mL^{-1} for ferulic acid against *Fusarium oxysporum* Schltdl., *F. verticillioides* (Sacc.) Nirenberg, *Penicillium brevicompactum* Dierckx, *P. expansum* Link, *Aspergillus flavus* Link, and *A. fumigatus* Fresen. EC_{50} values ranged from 411 (*P. expansum*) to 895 µg·mL^{-1} (*A. flavus*). Wu *et al.* (2010) found that ferulic acid inhibited conidium germination of the watermelon soil-borne pathogen *F. oxysporum* f. sp. *niveum* (E.F. Sm.) W.C. Snyder & H.N. Hansen at concentrations of 800 µg·mL^{-1}. Asiegbu *et al.* (1996) reported that ferulic acid at 5000 µg·mL^{-1} severely repressed growth of the lignocellulolytic fungi *Trichoderma harzianum* Rifai, *Chaetomium cellulolyticum* Chahal & D. Hawksw., *Phanerochaete chrysosporium* Burds., *Trametes versicolor* (L.) Lloyd and *Pleurotus sajor-caju* (Fr.) Singer. Ferulic acid or ferulic acid-rich extracts have also been suggested as natural alternatives for reducing post-harvest fruit losses. Hernández *et al.* (2021) reported almost 100% inhibition of *Monilinia fructicola* (G. Winter) Honey at a dose of 2 mM (390 µg·mL^{-1}), and 90% inhibition of *Alternaria alternata* (Fr.) Keissl. at 3 mM (583 µg·mL^{-1}), and of *Botrytis cinerea* Pers. at 7.5 mM (1457 µg·mL^{-1}).

There have been no previous reports of the antifungal activity of rutin against GTDs. To date, more than 70 plant species have been shown to contain rutin (Gullón *et al.*, 2017), and different plant extracts with high contents of this compound have shown inhibitory effects on the growth of fungi. Devi *et al.* (2007) found significant growth inhibition from extracts of *Eupatorium birmanicum* DC [*Eupatorium cannabinum* subsp. *cannabinum*]

against *F. oxysporum* (at 1000 ppm), *Curvularia lunata* Boedijn (at 500 ppm), and *Trichoderma viride* Pers. (at 100 ppm). Salvador *et al.* (2004) showed that ethanolic extracts from *Alternanthera maritima* (Mart.) St. Hil gave moderate inhibition of *Candida albicans* (C.P. Robin) Berkhout, *C. tropicalis* (Castell.) Berkhout, *C. glabrata* (H.W. Anderson) S.A. Mey. & Yarrow, *C. parapsilosis* (Ashford) Langeron & Talice, *Trichophyton mentagrophytes* C.P. Robin) R. Blanch. and *T. rubrum* (Castell.) Sabour. Pure rutin against the same fungi gave MIC values of 500 $\mu g \cdot mL^{-1}$.

Parvu *et al.* (2015) found rutin contents of 130 $\mu g \cdot mL^{-1}$ in flower extracts of *Hedera helix* L. (ivy), and 170 $\mu g \cdot mL^{-1}$ in fruit extracts of the same plant, and assayed these against *Aspergillus niger* Tiegh., *B. cinerea, F. oxysporum* f. sp. *tulipae* Apt., *Penicillium gladioli* L. McCulloch & Thom, and *Sclerotinia sclerotiorum* (Lib.) Korf & Dumont. Full inhibition of mycelium growth of these fungi was attained at concentrations of 8 to 12% for flower extracts, and of 10 to 14% for fruit extracts.

Elansary *et al.* (2020a) assayed the stem extracts of six *Ferocactus* species (*F. gracilis, F. pottsii, F. herrerae, F. horridus, F. glaucescens,* and *F. emoryi*), with rutin contents of up to 108 mg per 100 g DW, against several bacteria and fungi. They found strong antifungal effects against *A. flavus, A. ochraceus, A. niger, C. albicans, Penicillium funiculosum,* and *P. ochrochloron* (with MICs from 100 to 730 $\mu g \cdot mL^{-1}$). They found slightly greater rutin concentrations (139 mg/100 g DW) in *Ocimum basilicum* L. (basil), giving MIC values from 290 to 560 $\mu g \cdot mL^{-1}$ against these fungal pathogens (Elansary *et al.*, 2020d). Much greater rutin concentrations (1533 and 1010 mg/100 g DW) were found in leaves of *Acacia saligna* L. and *Ruta graveolens* L. leaves by Elansary *et al.* (2020b; 2020c). In the associated *in vitro* assays, conducted for the leaf methanolic extracts and several pure bioactive compounds detected by high-performance liquid chromatography–diode array detection (HPLC-DAD), these authors found MIC values against the different fungi ranging from 180 to 300 $\mu g \cdot mL^{-1}$ for pure rutin, from 300 to 580 $\mu g \cdot mL^{-1}$ for *A. saligna* extract, and from 330 to 780 $\mu g \cdot mL^{-1}$ for *R. graveolens* extract.

Concerning the antifungal activity of stevioside (*Stevia rebaudiana* extracts), Ghosh *et al.* (2008) and Abou-Arab and Abu-Salem (2010) reported growth inhibition effects against *A. solani, Helminthosporium solani, A. niger, A. ochraceus* K. Wilh., *A. parasiticus* Speare, *A. flavus*, and *Penicillium chrysogenum* Thom, but MIC values were not determined in these studies. Arya *et al.* (2012) also demonstrated the antifungal activity of stevioside (at 50000 $\mu g \cdot mL^{-1}$) against *A. flavus, A. fumigatus, A. niger,* and *Fusarium oxysporum*. Abdel-Fatt *et al.* (2018) measured MIC values of 250 to 300 $\mu g \cdot mL^{-1}$ for stevioside against *A. flavus, A. ochraceus, A. niger,* and *Fusarium moniliforme* Sheldon. Shukla *et al.* (2013) reported stevioside MIC values of 3 $mg \cdot mL^{-1}$ against *B. cinerea* and 2 $mg \cdot mL^{-1}$ against *F. oxysporum*, and Guerra Ramírez *et al.* (2020) found that the hexane extract of stevioside at 833 ppm inhibited mycelium growth of *F. oxysporum* by up to 50%.

No data are available on the antifungal activity of stevioside-polyphenol conjugate complexes against fungi associated with GTDs. Buzón-Durán *et al.* (2020) reported an EC_{50} of 123 $\mu g \cdot mL^{-1}$ and an EC_{90} of 160 $\mu g \cdot mL^{-1}$ against *F. culmorum* for conjugate complexes based on a 1:1 mixture of stevioside with polyphenols present in milk thistle seeds (*Silybum marianum* (L.) Gaertn). Composites based on stevioside:ferulic acid inclusion compounds (in a 5:1 molar ratio), combined with chitosan oligomers in hydroalcoholic solution or in choline chloride:urea deep eutectic solvent media, were assayed against *F. culmorum* by Matei *et al.* (2018a), obtaining EC_{50}s ranging from 175 to 292 $\mu g \cdot mL^{-1}$ and EC_{90}s in the 377 to 713 $\mu g \cdot mL^{-1}$ interval, depending on the dispersion medium. Inclusion compounds from stevioside and ferulic acid in 6:1 ratio, dispersed in a hydroalcoholic solution of chitosan oligomers, were also assayed against *P. cinnamomi* by Matei *et al.* (2018b) and Matei *et al.* (2020), obtaining EC_{50}s of 171 to 229 $\mu g \cdot mL^{-1}$ and EC_{90}s of 446 to 450 $\mu g \cdot mL^{-1}$, depending on the presence or absence of silver nanoparticles.

Mechanisms of action

The antimicrobial activity of ferulic acid can involve different modes of action, mainly related to the destabilization and permeabilization of cytoplasmatic membranes and to enzyme inhibition by the oxidized products (Borges *et al.*, 2013). Phenolic acids also affect the physicochemical surface properties of microbial cells, given that these compounds are electrophilic and change hydrophobicity. Due to their partially lipophilic character, it is assumed that ferulic acid crosses cell membrane by passive diffusion in undissociated form, disturbing cell membrane structure by localized hyperacidification, and possibly acidifying the cytoplasm and causing protein denaturation. The alteration of cell membrane potential makes it more permeable and causes leakage of cell constituents, including proteins and nucleic acids. Additionally, ferulic acid (like p-coumaric acid and caffeic acid) affects the cell membrane structure and rigidity, and alters phospholipid chain dynamics (Ota *et al.*, 2011). Shi *et al.* (2016) also noted that ferulic acid causes changes in intracellular ATP concentrations.

The antifungal mechanism of action of flavonoids was comprehensively reviewed by Al Aboody and Mickymaray (2020). Flavonoids inhibit fungal growth via various underlying mechanisms, including disruption of plasma membranes, induction of mitochondrial dysfunction, and inhibition of efflux mediated pumping, cell division, cell wall formation, and protein and RNA syntheses. For rutin, the mechanisms of pharmacological action were summarized by Koval'skii *et al.* (2014), who also noted that this compound can interact with various structures at molecular levels (including free radicals, protein systems, and enzymes).

In addition to direct effects, which cause reductions of fungal growth by altering hyphal morphology, grapevine phenolic compounds also exert their actions against GTD fungi through indirect effects, via inhibition of the extracellular fungal manganese peroxidase (MnP) involved in lignin degradation (Gómez *et al*., 2016). A decrease of laccase production and pectinase activity of *Botryosphaeria* isolates resulting from phenolic compounds was also reported by Srivastava *et al.* (2013).

Khan *et al.* (2017) suggested that the antimycotic activity of phytoglycosides is mediated through different and multiple targets that are not fully understood. However, there is a consensus that the main antimycotic mechanism is related to their ability to complex with sterols of fungal membranes. This produces spore-like structures that cause pore formations in membranes, losses in membrane integrity, and even membrane rupture, leading to fungal cell death.

Solubility and synergistic behaviour

A major disadvantage associated with rutin is its poor bioavailability, mainly caused by its low aqueous solubility and poor stability. Gullón *et al.* (2017) indicated that this hinders the *in vivo* biological effects of rutin, although the compound may have detectable bioactivity in different *in vitro* systems. Common approaches used to enhance rutin bioavailability include particle diminution to the submicron range and complex formation with cyclodextrins and various metals. Also, various carrier systems have been proposed for rutin delivery, including micro- and nano-emulsions, nanocrystals and nanosuspensions (Sharma *et al*., 2013).

An alternative approach is to use steviol glycosides as natural solubilizers, an approach that has been successfully tested for several natural phenols, such as curcumin (a diarylheptanoid) (Zhang *et al*., 2011; Nguyen *et al*., 2017), liquiritin (the 4'-*O*-glucoside of the flavanone liquiritigenin) (Nguyen *et al*., 2014), and betulinic acid (a pentacyclic triterpenoid) (Zhang *et al*., 2016). For rutin, Ko *et al.* (2016) optimized its solubility by the Box-Behnken design with the aid of microwave treatment (instead of ultrasonic treatment, as in the present study). Nguyen *et al.* (2015) attained similar results for quercetin, by complexation with rubusoside and rebaudioside, finding that as the glycoside concentration increased, the solubility of quercetin in water increased, without reducing its biological functions. Solubility optimization could be responsible for synergistic effects of conjugates of the two glycodrugs against GTD pathogens. The same rationale may be applied to tentatively explain the synergy observed for ferulic acid (although this was weaker than that attained for rutin).

Opportunities for future GTDs treatments

Levels of phenolic compounds have been reported to increase in the discoloured wood of Esca-affected grapevines (Agrelli *et al*., 2009; Amalfitano *et al*., 2011), while phenolic compounds have also been shown to limit fungal development in grapevine vascular tissues (Lambert *et al*., 2012a; Lima *et al*., 2011). Spagnolo *et al.* (2014) found the greatest levels of total phenolics in the brown striped wood of three grapevine cultivars infected with *N. parvum* and *Diplodia seriata* De Not. Martin *et al.* (2009) showed that stilbene polyphenols such as resveratrol and ε-viniferin increased in the wood of vines artificially inoculated with *Phaeomoniella chlamydospora*. Quercetin-3-*O*-glucoside and *trans*-caffeoyltartaric acid (analogous to rutin and ferulic acid studied here) were associated with resistance to *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni in grapevine leaves (Ali *et al*., 2012), and increases in quercetin-3-*O*-galactoside and kaempferol-3-*O*-glucoside have been found in asymptomatic leaves of plants infected with the Bois noir phytoplasma (Rusjan *et al*., 2012).

The approach presented here, based on mimicking the grapevine response via intrinsic phenolic compounds, along with solubility and bioavailability enhancements, is a "natural" and effective way to control the development of particular GTD pathogens. Although pure reagents were assayed in this study, the results attained indicate that selection of natural antifungal compounds could show promise. In the case of rutin, extracts from *Echinodorus grandiflorus* (Cham. & Schltdl.) Micheli, *Sambucus nigra* L., *Drimys winteri* J.R.Forst. or *Taraxacum officinale* Weber ex Wiggins (Meinhart *et al*., 2020) may deserve further attention for large-scale field experiments. If plants rich in rutin and ferulic were preferred, the activity of the extracts from sea buckthorn (*Hippophae Rhamnoides* L.) (Criste *et al*., 2020), *Rhinacanthus nasutus* (L.) Kurz (Huang *et al*., 2015), *Artemisia absinthium* L., *Achil-*

lea millefolium L., *Sambucus nigra* L. or *Salvia officinalis* L. (Bljajić *et al*., 2021) could be explored.

CONCLUSIONS

In an attempt to mimic grapevine defence responses against GTDs, the anti-fungal efficacy of rutin, either alone or in conjugate complexes with stevioside, was assayed against two *Botryosphaeriaceae* taxa. While the *in vitro* performance of the pure flavonoid-3-*O*-glycoside was moderate, with EC_{90}s of 1157 μg·mL^{-1} against *N. parvum* and 981 μg·mL^{-1} against *D. viticola*, considerable increases in activity (EC_{90}s of 715 and 458 μg·mL^{-1}, respectively) were attained for stevioside-rutin. The synergistic behaviour (with SFs of 1.44 and 2.17) may be due to solubility and bioavailability optimization. Testing of the formulations in greenhouse *in vivo* conditions showed moderate inhibition of *N. parvum* and full inhibition against *D. viticola* for the stevioside-rutin treatments. These EC_{90}s and *in vivo* results were consistently better than those found for ferulic acid and stevioside-ferulic acid, used as references. The promising results attained with this approach provide guidance for the selection of new plant extracts that could be utilized as antifungal agents in organic viticulture.

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Research Papers

Identity and pathogenicity of *Botryosphaeriaceae* and *Diaporthaceae* from *Juglans regia* in Chile

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Summary. English walnut (*Juglans regia*) has become an important crop in Chile, representing 11.5% of the total area of fruit trees, surpassed only by grapevine. As the Chilean walnut industry rapidly expands, young orchards are at risk from the emergence of new fungal diseases. *Botryosphaeriaceae* and *Diaporthaceae* fungi have been recognized as main causes of wood diseases in walnut, with symptoms of dieback, canker, and blight. In winter 2017, samples were collected from different orchards in Valparaíso and Maule regions. Fungal isolates recovered were cultured, characterized morphologically, and identified using DNA sequence analyses. Three species (*Neofusicoccum parvum*, *Diplodia mutila*, *Diplodia seriata*) were characterized in *Botryosphaeriaceae* and two (*Diaporthe cynaroidis*, *Diaporthe australafricana*) in *Diaporthaceae*. Pathogenicity tests showed that *N. parvum* was the most aggressive species to walnut. This study confirmed the presence of pathogenic *Botryosphaeriaceae* and *Diaporthaceae* in *J. regia* that should be considered an increasing risk for the growing Chilean walnut industry.

Keywords. *Diaporthe*, *Diplodia, Neofusicoccum,* walnut, wood canker, host range.

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INTRODUCTION

During the last two decades, production of English walnut (*Juglans regia* L.) has rapidly increased, with China as the main producer (369,000 metric tons), followed by the United States of America (USA) (250,389 metric tons) (www.nutfruit.org). Chile has also become a major producer, with approx. 49,000 planted ha mainly of cultivar 'Chandler' and 150,000 metric tons (Muñóz, 2017), positioning Chile as the third walnut exporting country worldwide.

Botryosphaeriaceae is one of the major fungal groups adversely affecting walnut production, including in California, USA (Chen *et al*., 2014),

Spain (López-Moral *et al*., 2020), China (Li *et al*., 2015) and Iran (Abdollahzadeh *et al*., 2013). Symptoms caused by these fungi include canker on trunks and scaffold branches and dieback of spurs and shoots resulting from previous infections of fruit peduncles and leaf scars (Moral *et al*., 2019b). Fungi reported as pathogenic to walnut include *Botryosphaeria dothidea*, *Diplodia mutila*, *Dip. seriata*, *Dothiorella iberica*, *Dot. omnivora*, *Dot. sarmentorum*, *Lasiodiplodia citricola*, *Las. pseudotheobromae*, *Las. theobromae*, *Neofusicoccum mediterraneum*, *Neof. nonquaesitum*, *Neof. parvum*, *Neof. ribis*, *Neof. vitifusiforme*, and *Neoscytalidium dimidiatum* (Haggag *et al*., 2007; Rumbos 2007; Chen *et al*., 2013a, 2014, 2019; Li *et al*., 2015; Eichmeier *et al*., 2020; Gusella *et al*., 2020; López-Moral *et al*., 2020). *Lasiodiplodia citricola* and *Neof. parvum* (Chen *et al*., 2013a, 2014) have been determined to be highly aggressive to English walnut, while *D. seriata* and *Dot. sarmentorum* are considered less aggressive (López-Moral *et al*., 2020). Furthermore, *Neof. parvum* has been reported to be widely distributed in over 90 hosts in more than 29 countries on six continents (Sakalidis *et al*., 2013; Gusella *et al*., 2020).

In contrast, fungi in the *Diaporthaceae* are less aggressive to English walnut than *Botryosphaeriaceae* species (Chen *et al*., 2014). Symptoms include stem dieback and branch canker, shoot blight, leaf spot and fruit rot (Chen *et al*., 2014). *Diaporthe amygdali, D. bicincta*, *D. eres*, *D. euonymi*, *D. juglandis*, *D. neotheicola*, *D. rhusicola*, *D. rostrata*, *D. rudis*, *Phomopsis albobestita*, and *P. arnoldiae* have been associated with *Juglans* spp. in America, Europe, and Asia, (Uecker 1988; Anagnostakis 2007; Udayanga *et al*., 2011, 2014; Gomes *et al*., 2013; Chen *et al*., 2014; Fan *et al*., 2015, 2018; López-Moral *et al*., 2020). *Diaporthe neotheicola* has been reported as the most widespread pathogen in several hosts including walnut (Chen *et al*., 2014; López-Moral *et al*., 2020).

In Chile, *Bot. dothidea*, *Dip. mutila*, *D. australafricana* and *D. cynaroidis* have been described as pathogens of walnut (Rina 2010; Díaz *et al*., 2018a; Jiménez Luna *et al*., 2020;). Additionally, several studies have documented the presence of *Botryosphaeriaceae* and *Diaporthaceae* species on other cultivated crops or tree hosts, including grapevine (*Vitis vinifera*) (Auger *et al*., 2004; Morales *et al*., 2012; Valencia *et al*., 2015; Larach *et al*., 2020), apple (*Malus domestica*) (Diaz *et al*., 2018b, 2018c), avocado (*Persea americana*) (Valencia *et al*., 2019), highbush blueberry (*Vaccinium corymbosum*) (Guerrero *et al*., 1987; Espinoza *et al*., 2008, 2009; Elfar *et al*., 2013), kiwifruit (*Actinidia deliciosa*) (Díaz *et al*., 2017; Palma *et al*., 2000), hazelnut (*Corylus avellana*) (Guerrero and Pérez, 2013), and native forest trees including *Araucaria araucana*, *Drimys winter*, and *Aristotelia chilensis* (Besoain *et al*., 2019; Zapata *et al*., 2020).

A common avenue for species in both *Botryosphaeriaceae* and *Diaporthaceae* to infect trees is through spores depositing on tree wounds caused by pruning, mechanical trunk shakers and wind injuries (Agustí-Brisach *et al*., 2019; Moral *et al*., 2019a, 2019b). Implementing cost-effective preventative practices that limit the incidence of these pathogens is key to long-term profitability of walnut orchards. The Chilean Institute of Agricultural Research (INIA) has begun a research program to identify fungi involved in walnut dieback and canker, and conduct fungicide tests to develop disease management strategies (Gamalier and Valeria, 2019). Species in *Botryosphaeriaceae* and *Diaporthaceae* have been shown to be threats to walnut production in several countries, and many species have already been found on other crops in Chile. The goal of this study is to establish the baseline of infection through an extensive survey in new walnut production areas in Chile and identify the taxonomic names of the species associated with walnut wood diseases using phylogenetic analysis and confirm pathogenicity with standard plant bioassays.

MATERIALS AND METHODS

Sampling locations and collection of fungi

In the winter of 2017, 13 walnut orchards (5 to 15 years old) from the major Chilean production regions were surveyed. These were in the central zone of Chile, including two orchards in the Valparaíso Region, four in the O'Higgins Region and seven in the Maule Region. Only five of these orchards (all cultivar 'Chandler') showed symptoms of dieback, with four orchards in the Maule Region and one in the Valparaíso Region. Twenty-five symptomatic wood samples were collected (five symptomatic trees × five samples/tree) from each orchard. Fungal isolates obtained were from trees with diseased branches and twigs showing necrotic brown discolorations in the cortical and vascular tissues.

Symptomatic wood samples were disinfected with 1% sodium hypochlorite for 30 s and then rinsed three times in sterile water. Five wood chips (≈3 × 3 ×3 mm) were placed in Petri dishes containing 2% potato dextrose agar (PDA; Difco Laboratories) supplemented with 0.2 g per L of tetracycline to suppress bacterial growth (Morales *et al*., 2012; Chen *et al*., 2014). Pure fungal cultures were grown on 2% agar with the addition of sterile grape leaves to stimulate formation of conidia so that cultures could be examined morphologically. Identification of morphotype isolates was based on morphological

characters as described by Phillips *et al.* (2013) for the *Botryosphaeriaceae*, and Udayanga *et al.* (2011) for the *Diaporthaceae*.

DNA extraction, and PCR amplification and sequencing

Young mycelium covering an area of approx. 2 cm^2 from each pure culture was removed for DNA extraction. DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Each PCR amplification was performed in a 25 µL reaction volume on a T100 thermal cycler (BioRad). Each reaction consisted of 17.4 µL of sterile H_2O, 2.5 µL of PCR buffer, 1 µL dNTPs (10 mM), 0.5 µL of each primer (4 µM), 2 µL $MgCl_2$ (25 mM), and 0.1 µL of *Taq* DNA polymerase (5 u μL^{-1}), with DNA added at 1–2 µL (10-20 ng DNA μL^{-1}). The thermocycler setting consisted of initial denaturation at 94°C for 2 min, then 35 cycles of the following three steps: 1 min at 94°C for strand separation, 1 min at 58 to 65°C for primer annealing and 1 min at 72°C for amplification. The final extension step was for 3 min at 72°C. Amplicons were run on a 1% agarose gel using gel electrophoresis and were then stained with Gel Red dye (Biotium Inc.). The DNA regions amplified were the nuclear ribosomal internal transcribed spacer (ITS) region using the ITS1-ITS4 primer pair at 58°C annealing temperature (White *et al.*, 1990), the *translation elongation factor 1-α* (EF) gene using primers EF1-728F and EF1-986R at 58°C annealing temperature (Carbone *et al.*, 1999), and the *β-tubulin* (TUB) gene using primers Bt2a and Bt2b at 65°C annealing temperature (Glass and Donaldson, 1995). Resulting bands were visualized under UV light using a Gel Doc Imager (Bio-Rad), and PCR products were purified using the QiaQuick PCR Purification Kit (Qiagen). Forward and reverse reads were generated by Sanger sequencing, carried out at the UCR Institute of Integrative Genome Biology.

Phylogenetic analyses

Forward and reverse reads of each DNA sample were edited and combined into a consensus sequence using Sequencher v. 5.0.1 (Gene Codes Corporation). Sequences from each region were concatenated using Geneious v. 2020.1.1 (Biomatters Ltd) and aligned using ClustalW implemented in MEGA 7 (Kumar *et al.*, 2016) with manual adjustments. Sequence alignments and phylogenetic analyses were performed separately for *Botryosphaeriaceae* and *Diaporthaceae*. CBS and CMW type specimens and taxa identified from walnut and cultivated and wild plant hosts in Chile were used as reference sequences for phylogenetic reconstructions. Reference taxa were obtained from fungal culture collections, including the Westerdijk Institute/Centraalbureau voor Schimmelcultures, CBS-KNAW, Utrecht, The Netherlands, and the CMW collection of the Forestry and Agricultural Biotechnology Institute, Pretoria, South Africa. The nucleotide sequences of reference taxa were downloaded from the GenBank sequence database maintained by the National Center for Biotechnology Information (NCBI). All accession numbers are listed in Table 1.

The complete dataset of *Botryosphaeriaceae* consisted of three novel sequences and 28 reference sequences. The outgroup was *Cophinforma atrovirens* (Zhang *et al.*, 2021). The *Diaporthaceae* dataset consisted of 2 novel sequences and 35 reference sequences. The outgroup was *Diaporthella corylina* (Gomes *et al.*, 2013). Sequences were aligned using ClustalW implemented in MEGA 7 (Kumar *et al.*, 2016), with manual adjustments. Phylogenetic trees for the *Botryosphaeriaceae* and *Diaporthaceae* were constructed using Maximum Likelihood, with the optimal nucleotide substitution model determined by the corrected Akaike Information Criterion (AICc; Akaike, 1974; Hurvich and Tsai, 1989). Nodal support consisted of nonparametric bootstrapping with 1000 replicates. All positions containing gaps and missing data were eliminated. Single-gene phylogenies from each of the three gene partitions were also examined for the *Botryosphaeriaceae* and *Diaporthaceae* datasets to check for incongruence. Constructed trees are presented in Figure S1. Bootstrap support values for clades containing isolates obtained in the present study are shown in Table S1.

Separate phylogenetic analyses were performed for all fungal pathogens reported from alternative hosts (tree crops and wild hosts) in Chile for which DNA sequences were available in GenBank. These analyses were based on the ITS region alone because sequences for EF and TUB were not available for all the taxa in the NCBI database.

In planta *pathogenicity tests*

Although fungal isolates were collected in winter 2017, pathogenicity tests were carried out in winter 2019, when a completely randomized experimental design was set up for the morphotypes of each identified fungal species: *Neof. parvum*, *Dip. mutila*, *Dip. seriata*, *D. australafricana* and *D. cynaroidis*. The test was conducted on trees planted under field conditions at the Escuela de Agronomía, Pontificia Universidad Católica de Valparaíso, Chile. The cultivar used for inoculation was a 1.5-year-old 'Chandler' walnut

Table 1. GenBank accession numbers, hosts and species identity for all *Botryosphaeriaceae* and *Diaporthaceae* taxa used for phylogenetic analyses.

Identity	Collection code	Host	Country of Origin	ITS	EF	TUB
Botryosphaeriaceae						
Botryosphaeria dothidea	CMW 8000	*Prunus* sp.	Switzerland	AY236949	AY236898	AY236927
Bot. dothidea	CMW 7780	*Fraxinus excelsior*	Switzerland	AY236947	AY236896	AY236925
Cophinforma atrovirens	CBS 117451	*Eucalyptus* sp.	Venezuela	KX464086	KX464556	KX464782
Diplodia africana	RGM 2718	*Araucaria araucana*	Chile	MN046380	-	-
Dip. africana	CBS 120835	-	South Africa	MH863094	-	-
Dip. mutila	CBS 112553	*Vitis vinifera*	Portugal	AY259093	AY573219	DQ458850
Dip. mutila	CBS 230.30	*Phoenix dactylifera*	USA	DQ458886	DQ458869	DQ458849
Dip. mutila	4D33	*Persea americana*	CA, USA	KF778789	KF778979	KF778884
Dip. mutila	PALUC1M	*Persea americana*	Chile	MF568683	-	-
Dip. mutila	DMnog4	*Juglans regia*	Chile	MG386824	-	-
Dip. mutila	Mz-F22	*Malus domestica*	Chile	MG450386	-	-
Dip. mutila	Sample 301	*Juglans regia*	Chile	MW412902	MW574125	MW596891
Dip. pinea	CMW 39341	*Cedrus deodara*	Montenegro	KF574998	KF575028	KF575094
Dip. pinea	CMW 39338	*Cedrus atlantica*	Serbia	KF574999	KF575029	KF575095
Dip. sapinea	CMW 190	*Pinus resinosa*	USA	KF766159	AY624251	AY624256
Dip. seriata	CBS 112555	*Vitis vinifera*	Portugal	AY259094	AY573220	DQ458856
Dip. seriata	CBS 119049	*Vitis* sp.	Italy	DQ458889	DQ458874	DQ458857
Dip. seriata	PALUC14M	*Persea americana*	Chile	MF578223	-	-
Dip. seriata	KJ 93.56	*Vitis vinifera*	Chile	AF027759	-	-
Dip. seriata	Mz-F1	*Malus domestica*	Chile	KU942427	-	-
Dip. seriata	Sample 105	*Juglans regia*	Chile	MW412901	MW574124	MW596890
Dip. scrobiculata	CBS 109944	*Pinus greggii*	Mexico	DQ458899	DQ458884	DQ458867
Dip. scrobiculata	CBS 113423	*Pinus greggii*	Mexico	DQ458900	DQ458885	DQ458868
Dothiorella iberica	CBS 115041	*Quercus ilex*	Spain	AY573202	AY573222	EU673096
Dot. iberica	CBS 113188	*Quercus suber*	Spain	AY573198	EU673278	EU673097
Dot. iberica	PALUC3M	*Persea americana*	Chile	MF578225	-	-
Dot. sarmentorum	CBS 115038	*Malus pumila*	The Netherlands	AY573206	AY573223	EU673101
Lasiodiplodia citricola	6I34	*Juglans regia*	CA, USA	KF778809	KF778999	KF778904
Las. citricola	CBS 124707	*Citrus* sp.	Iran	GU945354	GU945340	KU887505
Las. citricola	IRNKB3	*Juglans regia*	Iran	MN634040	MN633994	MN633442
Las. pseudotheobromae	CBS 116459	*Gmelina arborea*	Costa Rica	EF622077	EF622057	EU673111
Las. theobromae	CBS 164.96	Fruit along coral reef coast	Papua New Guinea	AY640258	AY640255	EU673110
Las. theobromae	PALUC449F	*Persea americana*	Chile	MF578754	-	-
Neofusicoccum arbuti	B03-07	Blueberry 'Aurora'	Chile	EU856061	-	-
Neof. arbuti	UW01	*Arbutus menziesii*	WA, USA	AY819720	-	-
Neof. australe	CMW 6837	*Acacia* sp.	Australia	AY339262	-	-
Neof. australe	CAP258	*Olea europaea*	Italy	EF638778	-	-
Neof. australe	PALUC439F	*Persea americana*	Chile	MF578755	-	-
Neof. australe	B1-05	Blueberry 'Duke'	Chile	EU856059	-	-
Neof. australe	vid-1559	*Vitis vinifera*	Chile	JX290091	-	-
Neof. mediterraneum	6I29	*Juglans regia*	CA, USA	KF778849	KF779039	KF778944
Neof. nonquaesitum	UCR2733	*Persea americana*	USA	KT965281		-
Neof. nonquaesitum	PALUC4M	*Persea americana*	Chile	MF578228		-
Neof. nonquaesitum	CABI IMI-500168	*Vaccinium corymbosum*	Chile	JX217819	-	-
Neof. nonquaesitum	4L78	*Juglans regia*	CA, USA	KF778851	KF779041	KF778946
Neof. nonquaesitum	PD90	*Prunus dulcis*	CA, USA	GU251157	GU251289	GU251817
Neof. parvum	CBS 110301	*Vitis vinifera*	Portugal	AY259098	AY573221	EU673095

(Continued)

Table 1. (Continued).

Identity	Collection code	Host	Country of Origin	ITS	EF	TUB
Neof. parvum	CMW9080	*Populus nigra*	New Zealand	AY236942	-	-
Neof. parvum	CMW 9081	*Populus nigra*	New Zealand	AY236943	AY236888	AY236917
Neof. parvum	PALUC16M	*Persea americana*	Chile	MF578229	-	-
Neof. parvum	B1-06	Blueberry 'Mistry'	Chile	EU856063	-	-
Neof. parvum	Sample 172	*Juglans regia*	Chile	MW412903	MW574126	MW596892
Neof. vitifusiforme	5H02	*Juglans regia*	CA, USA	KF778868	KF779058	KF778963
Neof. vitifusiforme	CBS 110881	*Vitis vinifera*	South Africa	AY343383	AY343343	KX465061
Neoscytalidium dimidiatum	CBS 499.66	*Mangifera indica*	Mali	FM211432	EU144063	FM211167
Diaporthaceae						
Diaporthe ambigua	CBS 114015	*Pyrus communis*	South Africa	KC343010	-	-
D. ambigua	6-KF	*Actinidia deliciosa*	Chile	KJ210025	-	-
D. ambigua	5.5.4r1(2)	*Vaccinium* sp.	Chile	KC143171	-	-
D. ampelina	CBS 111888	*Vitis vinifera*	USA	KC343016	KC343742	KC343984
D. amygdali	CBS 115620	*Prunus persica*	GA, USA	KC343020	KC343746	KC343988
D. amygdali	CBS 126679	*Prunus dulcis*	Portugal	KC343022	KC343748	KC343990
D. amygdali	ColPat-533	*Juglans regia* 'Chandler'	Spain	MK447999	MK490937	MK522117
D. araucanorum	RGM 2472	*Araucaria araucana*	Chile	MN509709	-	-
D. asheicola	CBS 136968	*Vaccinium ashei*	Chile	KJ160563	KJ160595	KJ160519
D. asheicola	CBS 136967	*Vaccinium ashei*	Chile	KJ160562	KJ160594	KJ160518
D. australafricana	CBS 111886	*Vitis vinifera*	Australia	KC343038	KC343764	KC344006
D. australafricana	CBS 113487	*Vitis vinifera*	South Africa	KC343039	KC343765	KC344007
D. australafricana	16-KF	*Actinidia deliciosa*	Chile	KX999702	-	-
D. australafricana	Pho73-07	*Vaccinium* sp.	Chile	KC143190	-	-
D. australafricana	15.2.2(4)	*Vaccinium* sp.	Chile	KC143175	-	-
D. australafricana	Sample 302	*Juglans regia*	Chile	MW407063	MW574121	MW574123
D. beckhausii	CBS 138.27	*Viburnum* sp.	-	KC343041	KC343767	KC344009
D. chamaeropis	CBS 454.81	*Chamaerops humilis*	Greece	KC343048	KC343774	KC344016
D. chamaeropis	CBS 753.70	*Spartium junceum*	Croatia	KC343049	KC343775	KC344017
D. cynaroidis	CBS 122676	*Protea cynaroidis*	South Africa	KC343058	KC343784	KC344026
D. cynaroidis	Sample 102	*Juglans regia*	Chile	MW407062	MW574120	MW574122
D. eres	CBS 101742	*Fraxinus* sp.	The Netherlands	KC343073	KC343799	KC344041
D. eres	CPC 16510	*Vaccinium corymbosum*	Chile	KJ160572	-	-
D. foeniculina	CBS 117166	*Aspalathus linearis*	South Africa	DQ286286	-	-
D. foikelawen	RGM 2539	*Drimys winteri*	Chile	MN509713	-	-
D. neotheicola	CBS 123208	*Foeniculum vulgare*	Portugal	EU814480	GQ250315	JX275464
D. neotheicola	6I30	*Juglans regia*	CA, USA	KF778871	KF779061	KF778966
D. neotheicola	3.4.4r1(1)	*Vaccinium* sp.	Chile	KC143192	-	-
D. neotheicola	ColPat-445	*Juglans regia* 'Tulare'	Spain	MK447993	MK490932	MK522106
D. neotheicola	ColPat-448	*Juglans regia* 'Serr'	Spain	MK447994	MK490939	MK522107
D. neotheicola	ColPat-450	*Juglans regia* 'Vina'	Spain	MK447996	MK490934	MK522109
D. neotheicola	ColPat-532	*Juglans regia* 'Chandler'	Spain	MK447998	MK490936	MK522111
D. neotheicola	ColPat-551	*Juglans regia* 'Hartley'	Spain	MK448000	MK490940	MK522112
D. nobilis	CBS 200.39	*Laurus nobilis*	Germany	KC343151	KC343877	KC344119
D. novem	CBS 127271	*Glycine max*	Croatia	KC343157	-	-
D. novem	1-KF	*Actinidia deliciosa*	Chile	KJ210020	-	-
D. passiflorae	CPC 19183	*Passiflora edulis*	South America	JX069860	-	-
D. passiflorae	15.3.1r1	*Vaccinium* sp.	Chile	KC143196	-	-

(Continued)

Table 1. (Continued).

Identity	Collection code	Host	Country of Origin	ITS	EF	TUB
D. rudis	10-KF	*Actinidia deliciosa*	Chile	KJ210029	-	-
D. rudis	CBS 449.82	*Lupinus* sp.	The Netherlands	KC343240	KC343966	KC344208
D. rudis	CBS 100170	*Fraxinus excelsior*	The Netherlands	KC343230	KC343956	KC344198
D. rudis	CBS 114011	*Vitis vinifera*	Portugal	KC343235	KC343961	KC344203
D. rudis	CBS 113201	*Vitis vinifera*	Portugal	KC343234	KC343960	KC344202
D. rhusicola	CBS 129528	*Rhus pendulina*	South Africa	JF951146	KC843100	KC843205
D. rhusicola	6I14	*Prunus dulcis*	CA, USA	KF778872	KF779062	KF778967
D. rhusicola	6I31	*Juglans regia*	CA, USA	KF778874	KF779064	KF778969
D. rhusicola	ColPat-444	*Juglans regia* 'Tulare'	Spain	MK447992	MK490931	MK522105
D. sterilis	CBS 136969	*Vaccinium corymbosum*	Italy	KJ160579	KJ160611	KJ160528
D. sterilis	CBS 136970	*Vaccinium corymbosum*	Italy	KJ160580	KJ160612	KJ160529
D. toxica	CBS 534.93	*Lupinus angustifolius*	Western Australia	KC343220	KC343946	kC344188
D. toxica	CBS 546.93	*Lupinus* sp.	Western Australia	KC343222	KC343948	KC344190
D. vaccinia	CBS 160.32	*Vaccinium macrocarpon*	USA	KC343228	KC343954	KC344196
D. amygdali	ColPat-533	*Juglans regia* 'Chandler'	Spain	MK447999	MK490937	MK522117
D. amygdali	CBS 126679	*Prunus dulcis*	Portugal	KC343022	KC343748	KC343990
D. amygdali	CBS 115620	*Prunus persica*	GA, USA	KC343020	KC343746	KC343988
D. cf. heveae	CBS 852.97	*Hevea brasiliensis*	Brazil	KC343116	KC343842	KC344084
Diaporthella corylina	CBS 121124	*Corylus* sp.	China	KC343004	KC343730	KC343972

scion grafted to a Vlach clonal rootstock. Pathogenicity tests were conducted on two sets of plants inoculated at different times for logistical reasons, with one set used for the three *Botryosphaeriaceae* species and one set for the two *Diaporthe* species. A total of 70 trees were inoculated, with ten trees for each treatment. The experiment was repeated twice. Inoculations were each conducted by using 3 mm diam. mycelium/PDA plugs from a 7-d-old pure culture. Each stem wound was produced 30 cm above ground (half-way up the stem), and a 3 mm diam. hole was produced with a cork borer to insert an agar plug bearing mycelia. Negative controls were inoculated with sterile 2% PDA plugs. After inoculation, the wounds were wrapped with parafilm. Data were recorded 3 months after inoculation by measuring canker lengths in the host xylem tissues. To complete Koch's postulates, pathogens were re-isolated and cultured in 2% PDA medium, and presence of each pathogen was confirmed morphologically.

Statistical analyses

The data collected were analyzed using R studio and depicted as box and whisker plots. The data were subjected to analysis of variance, and treatment means were compared using Tukey's least significant difference test at $P \leq 0.05$.

RESULTS

Five samples collected from one orchard in the Valparaíso Region were infected with *Botryosphaeriaceae* fungi. All these isolates developed gray mycelium which then became dark green with fusoid, hyaline and thin-walled conidia. The isolates were keyed as *Neofusicoccum* according to Phillips *et al.* (2013). Of the 20 samples collected from the four orchards in the Maule Region, 14 samples were infected with *Botryosphaeriaceae* fungi, and six samples were infected with *Diaporthaceae* fungi. *Botryosphaeriaceae* isolates had abundant aerial and initially white to white-gray fast-growing mycelium that turned dark green with time. Conidia were thick-walled and aseptate. Isolates keyed as *Diplodia* according to Phillips *et al.* (2013), were of two morphotypes which were separated on the basis on conidium pigmentation, one with hyaline conidia and the other with brown conidia (Phillips *et al.*, 2013). *Diaporthe* isolates were characterized by production of black conidiomata with alpha conidia in cultures (Udayanga *et al.*, 2011). Two morphotypes were separated on the basis of production of beta conidiospores with only one morphotype producing these conidiophores. Three DNA loci (ITS, TUB, and EF) were sequenced for species identification of the five selected morphotypes, including three *Botryosphaeriaceae* (one *Neofusicoccum* sp. and two *Diplodia* spp.) and two *Diaporthaceae*.

Alignment of 32 DNA sequences from species in the *Botryosphaeriaceae* resulted in a dataset of 1308 nucleotide positions (557 positions in the ITS partition, 330 in the EF partition and 421 in the TUB partition). These included 871 conserved sites (ITS = 436, EF = 108, TUB = 327), 425 variable sites (ITS = 121, EF = 201, TUB = 103), 371 parsimony-informative sites (ITS = 106, EF = 182, TUB = 83), and 54 singleton sites (ITS = 15, EF = 19, TUB = 20). The optimum model of nucleotide substitution inferred using the AICc was the Tamura-Nei model (Tamura and Nei, 1993), with a discrete Gamma distribution and a proportion of invariant sites (TN93 + G + I). The tree with the greatest log likelihood (-3242.47) is shown in Figure 1. The phylogenetic analyses supported with strong bootstrap values the placement of the three *Botryosphaeriaceae* samples 301, 105 and 172 in, respectively, the *Dip. mutila*, *Dip. seriata* and *Neof. parvum* clades.

A separate phylogenetic analysis including ITS sequences of fungal samples previously identified from alternate hosts in Chile was also generated (Figure 2). Alignment of 54 DNA sequences of species in *Botryosphaeriaceae* resulted in a dataset of 1211 nucleotide positions. These included 408 conserved sites, 139 variable sites, 120 parsimony-informative sites, and 19 singleton sites. The optimum model of nucleotide substitution inferred using the AICc was the Tamura-Nei model (Tamura and Nei, 1993), with a discrete Gamma distri-

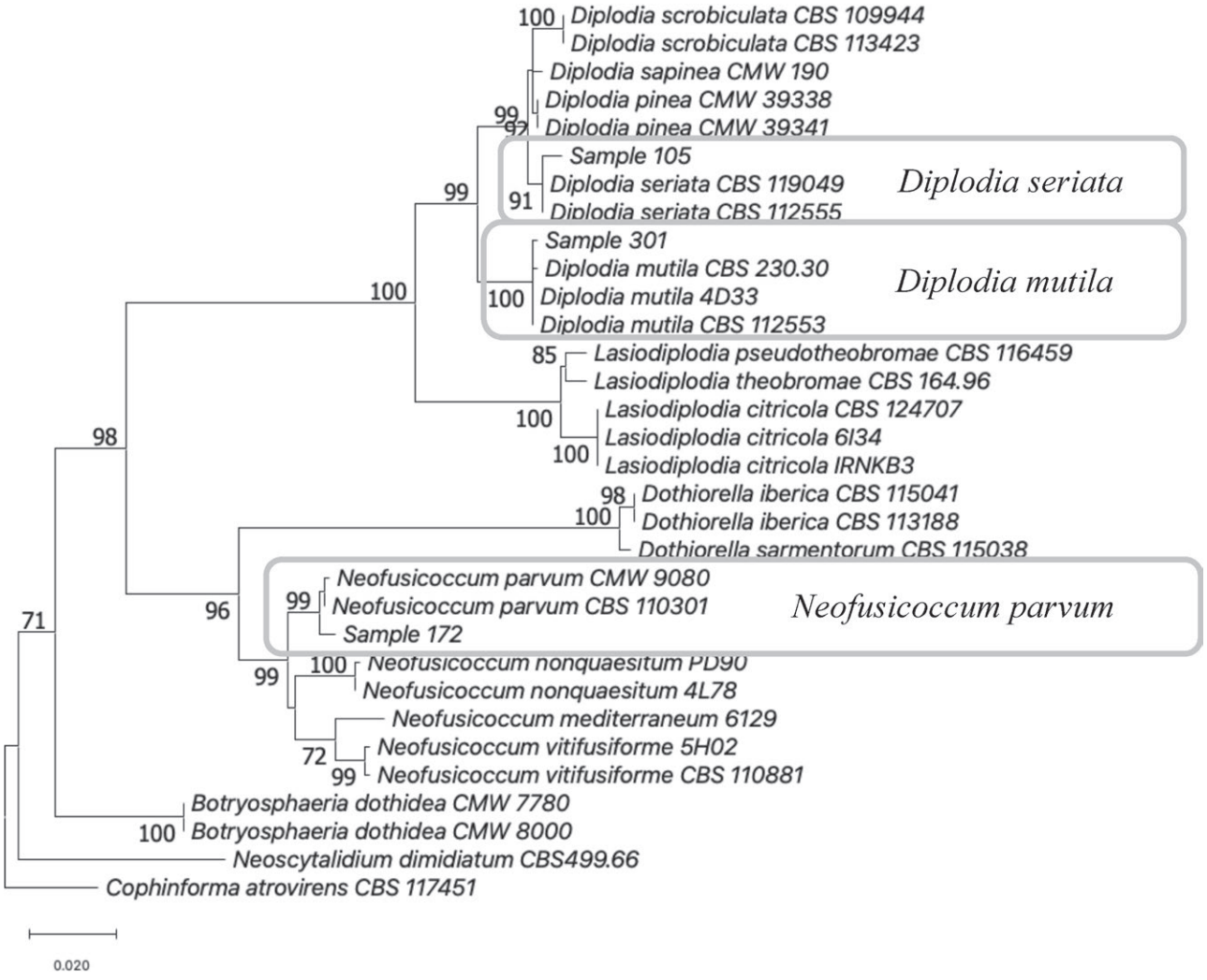

Figure 1. Phylogenetic tree reconstructed by maximum likelihood analysis from the concatenated sequences of the nuclear ribosomal internal transcribed spacer, translation elongation factor and beta-tubulin for three *Botryosphaeriaceae* species isolated from three walnut orchards in Chile and 29 *Botryosphaeriaceae* reference sequences retrieved from the GenBank database. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers on branches are bootstrap support values.

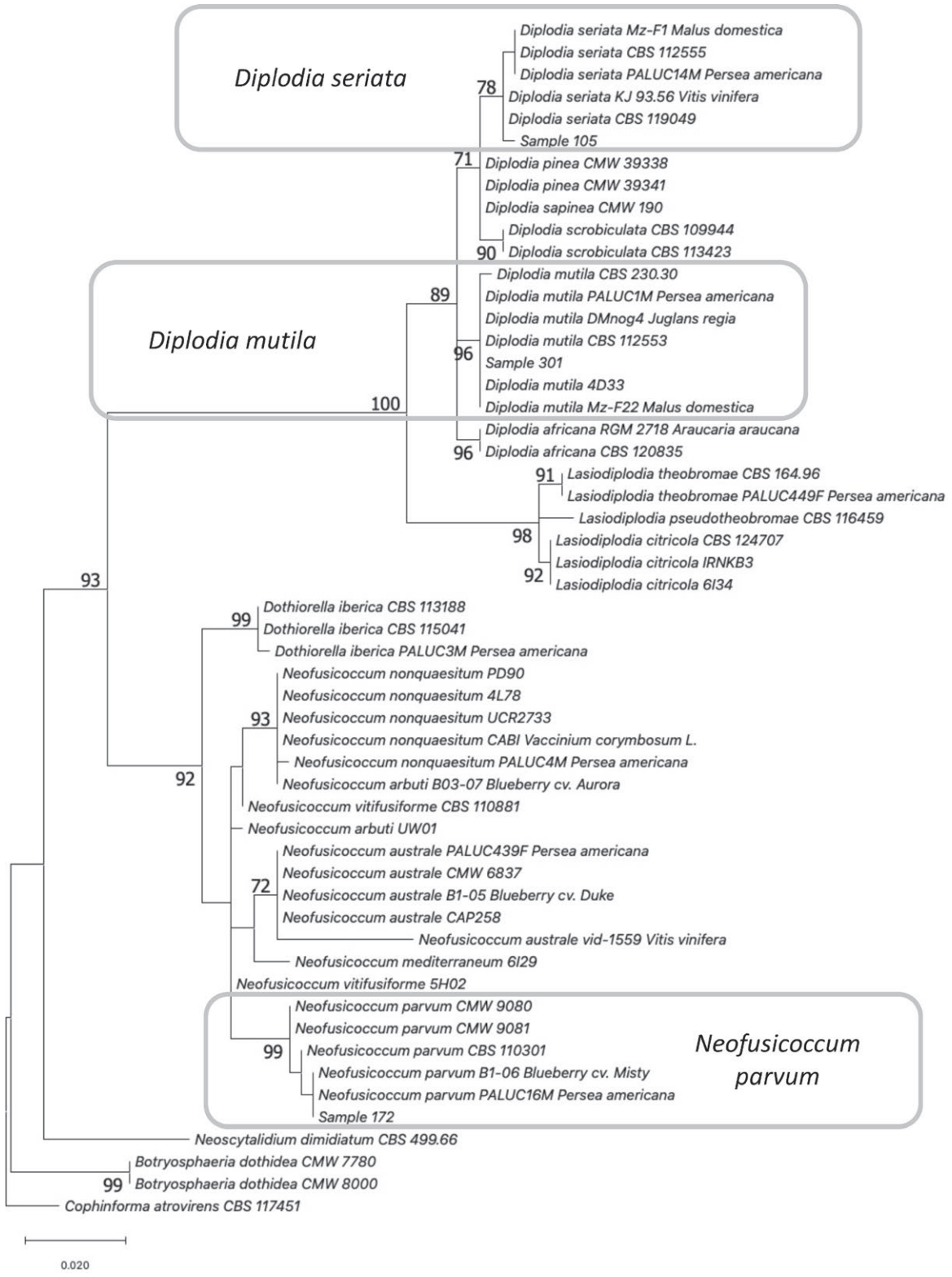

Figure 2. Phylogenetic tree reconstructed by maximum likelihood analysis from the sequences of the nuclear ribosomal internal transcribed spacer. The dataset included three novel *Botryosphaeriaceae* taxa isolated from three walnut orchards in Chile and 51 GenBank sequences of *Botryosphaeriaceae* that included reference sequences as well as sequences of previously reported Chilean walnut pathogens. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers on branches are bootstrap support values.

bution and a proportion of invariant sites (TN93 + G + I). The tree with the greatest log likelihood (-1363.49) is shown in Figure 2. The results illustrated the diversity of pathogens in *Botryosphaeriaceae* present in Chile and the range of crops affected by these pathogens. Sample 301 was one of four *Dip. mutila* isolates reported in Chile, with the three others originating from apple, avocado and walnut (Díaz *et al*., 2018b; Valencia *et al*., 2019). Sample 105 was one of four *Dip. seriata* isolates, and the other three were reported from apple, avocado, and grapevine (Morales *et al*., 2012; Díaz *et al*., 2018c). Sample 172 was one of three *Neof. parvum* isolates, the

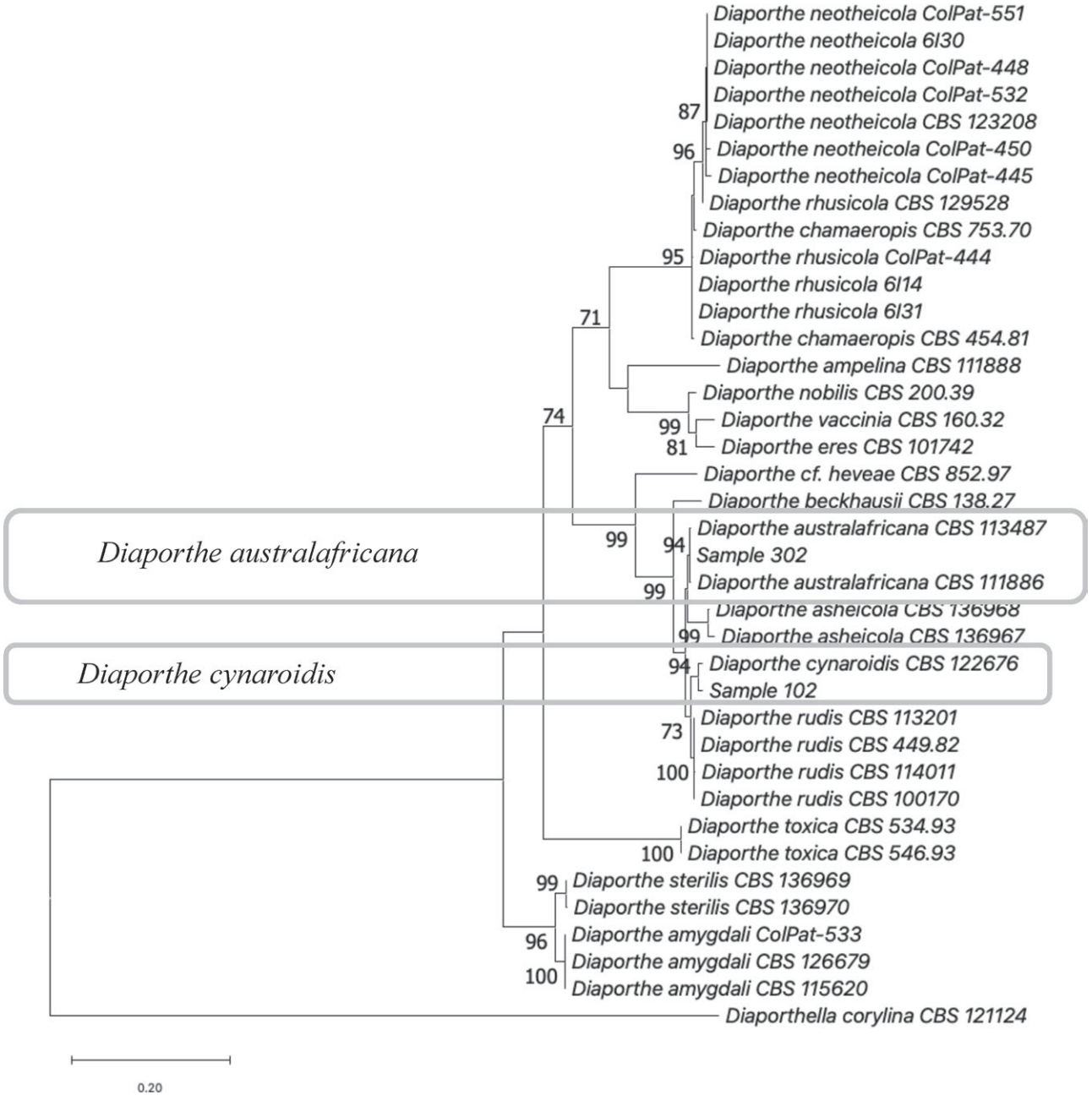

Figure 3. Phylogenetic tree reconstructed by maximum likelihood analysis from the concatenated sequences of the nuclear ribosomal internal transcribed spacer, translation elongation factor and beta-tubulin for two *Diaporthaceae* taxa recovered from two walnut orchards in Chile, and 36 *Diaporthe* reference sequences retrieved from the GenBank database. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers on branches are bootstrap support values.

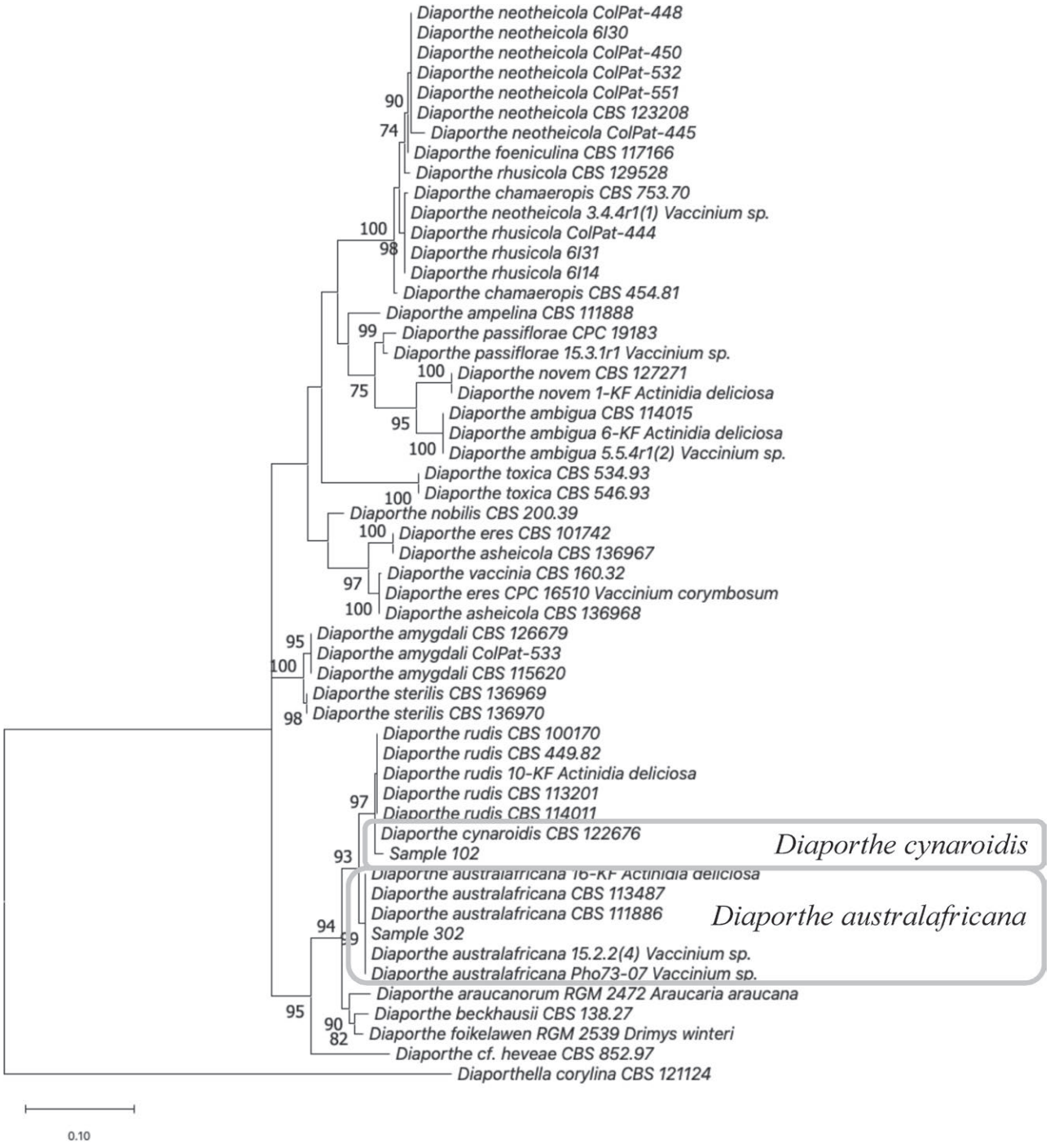

Figure 4. Phylogenetic tree reconstructed by maximum likelihood analysis from the sequences of the nuclear ribosomal internal transcribed spacer for two novel *Diaporthaceae* taxa recovered from two walnut orchards in Chile, and 51 GenBank sequences of *Diaporthaceae* that included reference sequences as well as sequences of previously reported Chilean walnut pathogens. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers on branches are bootstrap support values

other two were from avocado and blueberry (Espinoza *et al*., 2008, 2009; Valencia *et al*., 2019).

The alignment of 38 *Diaporthaceae* DNA sequences comprised 1791 nucleotide positions (610 ITS, 403 EF and 778 TUB), of which 794 were conserved (ITS = 403, EF = 81, TUB = 310), 872 were variable (ITS = 176, EF =

287, TUB = 409), 575 were parsimony informative (ITS = 93, EF = 224, TUB = 258), and 292 were singleton sites (ITS = 83, EF = 62, TUB = 147). The AICc-inferred optimal model of nucleotide substitution was a General Time Reversible model (Nei and Kumar 2000) with a discrete Gamma distribution (GTR + G). The tree with the greatest log likelihood (-6640.99) is shown in Figure 3. Phylogenetic reconstruction placed our samples 302 and 102 in clades of, respectively, *Diaporthe australafricana* and *D. cynaroidis*, with strong bootstrap support.

A separate phylogenetic analysis including ITS sequences of *Diaporthaceae* previously identified from alternative hosts in Chile was also generated because the sequences for all three loci were not available in the NCBI database (Figure 4). This analysis was based on an alignment of 54 *Diaporthaceae* DNA sequences comprising 564 nucleotide positions in the ITS region, of which 351 were conserved, 181 were variable, 117 were parsimony-informative, and 64 were singleton sites. The AICc-inferred optimal model of nucleotide substitution was a General Time Reversible model (Nei and Kumar 2000) with a discrete Gamma distribution and a proportion of invariant sites (TN93 + G + I). The tree with the greatest log likelihood (-2291.02) is shown in Figure 4. Sample 102 was the only *D. cynaroidis* isolate reported in Chilean walnut at this time, and sample 302 was one of four *D. australafricana* isolates, the other three originating from blueberry and kiwifruit (Espinoza *et al*., 2008; Elfar *et al*., 2013; Díaz *et al*., 2017).

Phylogenies derived from the individual gene partitions did not reveal incongruence, although bootstrap support was less than in the concatenated trees (Table S1). Of the three partitions, the ITS region provided the strongest support for clades harboring four of the novel samples. Only Sample 102 received strongest support from EF for its affiliation with the *D. cynaroidis* clade. This is consistent with the results of Santos *et al*. (2010), who recommended the EF region for use in *Diaporthe*.

All five species caused wood lesions on inoculated walnut stems compared to the mock-inoculated control plants, but there were some differences in virulence (Figure 5) ($P < 0.05$; One-way ANOVA followed by Tukey test for multiple comparison of means). *Neofusicoccum parvum* was the most aggressive species causing larger lesions ($P = 0.0045$) than *Dip. mutila*. *Diplodia seriata* gave intermediate lesion lengths, but these were not significantly different from either *Neof. parvum* or *Dip. mutila* ($P = 0.0764$) (Figure 5A). In addition, mean lesion lengths were not significantly different ($P > 0.05$) between *D. australafricana* and *D. cynaroidis* but were greater than those from the mock-inoculated controls (Figure 5B).

DISCUSSION

The survey indicated that incidence of wood diseases in Chilean walnut orchards was low, as only five of the thirteen assessed orchards were symptomatic. This is probably because that commercial walnut production is a young industry in Chile, coupled with the long incubation period required for wood pathogens to cause wood dieback (Duthie *et al*., 1991). The industry was first established in the late 1970's with approx. 4000 ha planted in the Valparaíso Region. After 2000, Chile walnut production grew ten-fold to over 43,000 ha (INC, 2021), but the bulk of the new planted area was further south in the O'Higgins and Maule Regions that have wetter and cooler weather patterns than the Valparaíso Region. The dryer Valparaíso Region displayed low wood disease incidence, and *Neof. parvum* was the only pathogen isolated. In contrast, a broader diversity of pathogenic species and greater disease incidence was recorded in the two major walnut producing regions in central Chile, where two *Diplodia* and two *Diaport*he species were identified. These results were similar to those of Larach *et al*. (2020), who also identified greater incidence of disease in wetter and cooler Chilean vineyards in comparison to dryer areas. This suggests that pathogenic fungi causing wood disease may become increasingly problematic as orchards age, as plantation size expands, and also possibly because environmental conditions in wetter areas are more suitable for the pathogens to spread and cause wood diseases. Overall, this study has confirmed the results of Díaz *et al*. (2018a) and Jiménez Luna *et al*. (2020), who found *Dip. mutila*, *D. australafricana* and *D. cynaroidis* in walnut orchards. The present results also expand the host range of *Dip. seriata* and *Neof. parvum* to walnut in Chile.

Infection of young orchards with both *Botryosphaeriaceae* and *Diaporthe* species could be attributed to different infection routes for these fungi. Infections may have initially come from plant nursery materials, as has been reported for several crop types (Smit *et al*., 1996; Espinoza *et al*., 2009; Chen *et al*., 2013b; Whitelaw-Weckert *et al*., 2013; Tennakoon *et al*., 2017), including walnut (Chen *et al*., 2013a). However, inoculum originated most likely from alternative hosts grown in proximity to walnut orchards, either following wet events (rainfall or sprinkler or furrow irrigation), which aid pathogen infection by dispersing inoculum (Valencia et al., 2019) to exposed tree wounds caused by pruning or mechanical harvesters (Michailides and Morgan 1993; Luo *et al*., 2020). In Chile, all three *Botryosphaeriaceae* species isolated from walnut have also been shown to cause branch canker and dieback in avocado trees, and walnuts are

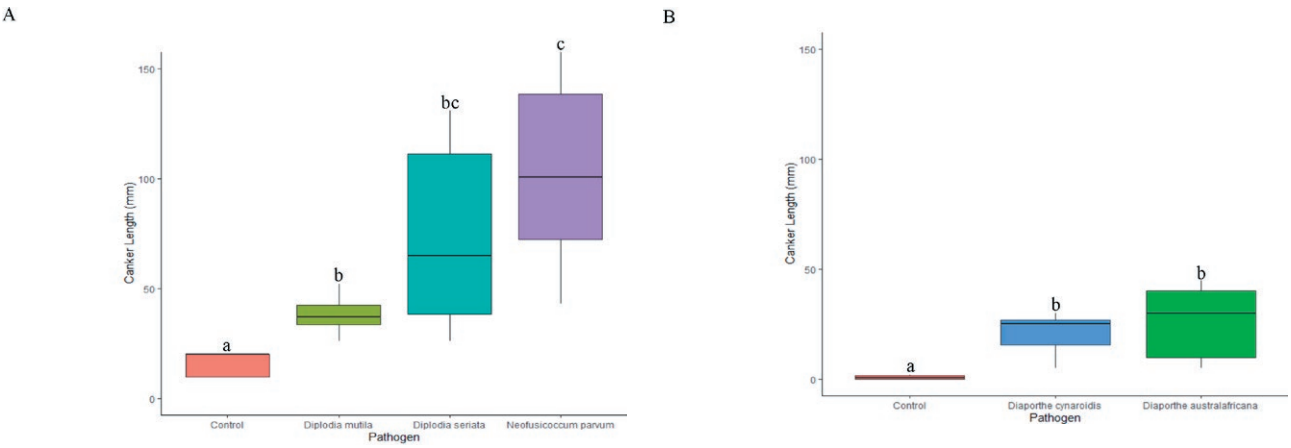

Figure 5. Average canker length (mm) after 12 weeks caused by three *Botryosphaeriaceae* species (**A**) and two *Diaporthe* species (**B**) on walnut stems inoculated with mycelial plugs. Bars topped with different letters indicate treatment means that are significantly different for *Botryosphaeriaceae* and *Diaportheaceae* ($P < 0.05$) species.

often planted near avocado orchards in the regions surveyed (Valencia *et al*., 2019). The pathogens have also been found on apple, blueberry, and grapevine (Auger *et al*., 2004; Espinoza *et al*., 2009; Morales *et al*., 2012; Díaz *et al*., 2018b; Larach *et al*., 2020), all of which have been grown locally, and infected hosts could become the inoculum sources for neighboring walnut orchards. Similarly, *D. australafricana* and *D. cynaroidis* have been identified in European hazelnut, blueberry, and kiwifruit (Elfar *et al*., 2013; Guerrero *et al*., 2013; Díaz *et al*., 2017). The phylogenetic analyses of the present study indicated that several additional *Botryosphaeriaceae* and *Diaporthe* species known to be pathogenic to walnut (*Las. theobromae*, *Dot. iberica*, or *D. neotheicola*; Chen *et al*., 2014; Lopez-Moral *et al*., 2020; Sohrabi *et al*., 2020) have been reported in Chile on avocado (Valencia *et al*., 2019) and blueberry (Espinoza *et al*., 2008), and these inoculum sources could potentially become threats to local walnut production.

The present study has demonstrated that *Neof. parvum*, *Dip. seriata*, *Dip. mutila*, *D. australafricana* and *D. cynaroidis* isolated from walnut wood diseases were all pathogenic. This study gave similar results to previous studies indicating that *Neof. parvum* is one of the most aggressive wood pathogens to many crops in addition to English walnut (Chen *et al*., 2014; López-Moral *et al*., 2020), including almond (Inderbitzin *et al*., 2010; Holland *et al*., 2021), avocado (McDonald *et al*., 2009), citrus (Adesemoye and Eskalen, 2011), and grapevine (Úrbez-Torres *et al*., 2009). The broad incidence and high virulence of *Neof. parvum* indicates that this fungus is one of the main pathogens of walnut in Chile causing trunk and limb cankers, eventually resulting in decline of affected trees. *Diplodia. seriata* and *Dip. mutila* were weakly virulent with respect to wood lesions caused to walnut branches compared to *Neof. parvum*, and these results are similar to those in other reports (Chen *et al*., 2014; López-Moral *et al*., 2020). *Diaporthe australafricana* and *D. cynaroidis* were also in the same range of virulence as *Dip. seriata* and *Dip. Mutila*, and were comparable to previous reports of mild aggressiveness of species in the *Diaporthe* group including *D. rhusicola* and *D. neotheicola* (Chen *et al*., 2014; López-Moral *et al*., 2020). Fungus genomics have showed that *Diaporthe* species and *Diplodia* species have limited enzymatic capabilities to colonize woody tissues and break down cell wall lignin (Morales-Cruz *et al*., 2015; Garcia *et al*., 2021), and that these fungi may be more responsible for shoot/fruit blights and twig dieback symptoms than capable of causing cankers on tree trunks and scaffolds, as reported with *Neof. parvum*.

Protecting host wounds with fungicide applications is the best strategy for preventing fungal infections, as has been demonstrated in other pathosystems (Rolshausen *et al*., 2010; Díaz and Latorre, 2013). In Chile, applications of lime sulfur are currently used in walnut to control the development of *Botryosphaeriaceae* and *Diaporthaceae* (http://www.sag.cl/ambitos-de-accion/plaguicidas-y-fertilizantes). Integrated disease management remains effective for control of fungi causing wood diseases. Pruning in dry weather, managing canopy size allowing ventilation and sunlight exposure, and maintaining low tree planting densities are recommended practices to minimize the risks and severity of infections (Moral *et al*., 2019a, 2019b). In addition, pruning and removal of dead and infected tissues, and avoiding excessive wetting of host trunks or canopies is strongly encouraged,

to limit the build-up and spread of pathogen inoculum and extend crop longevity and productivity, as has been shown in pistachio orchards and vineyards (Michailides and Morgan 1993; Gispert *et al*., 2020). To date there are no walnut cultivars known to be resistant to the causal agents of wood disease, although cultivar 'Chandler' has been reported to be more tolerant to infections, followed by 'Tulare' and 'Vina' (Chen *et al*., 2014). As Chile looks to expand walnut production to meet global market demand, management of these diseases will be key to sustaining the longevity and productivity of walnut orchards.

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Research Papers

Activity of bacterial seed endophytes of landrace durum wheat for control of Fusarium foot rot

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Summary. Five bacterial endophytic isolates obtained from durum wheat seeds (landrace "Timilia reste nere") and identified as belonging to *Pantoea* (isolates A1, F7, F15 and G1) and *Paenibacillus* (isolate B) genera on the basis of 16S rDNA gene sequences, were assayed *in vitro* and *in vivo* for their ability to inhibit *Fusarium culmorum* growth and the disease (Fusarium foot rot) it causes in durum wheat. All isolates significantly reduced *in vitro* growth of *F. culmorum* in comparison with the control. After 120 hours of incubation, isolates B and G1 showed the greatest mycelial growth inhibition, i.e., respectively, 76 and 74%. When durum wheat "Simeto" seeds were treated with bacterial isolates singly or in combinations and then inoculated with *F. culmorum*, all treatments with endophytes showed increased, but not statistically significant, seed germination. Except for isolate A1, all bacterial isolates stimulated vegetative parameters of durum wheat seedlings. Mixture of isolates F7 + F15 was the most effective in improving shoot height (+94%), root length (+47%) and vigour index (+81%). Mixture of isolates A1 + B reduced Fusarium foot rot incidence (-21%) and severity (-30%), and isolate A1 reduced incidence (-15%) and severity (-16%) of the disease. These results indicate potential of bacterial seed endophytes, identified in this study, for control of Fusarium foot rot and suggest that bacterial seed endophytes may provide a new biocontrol agent for an environmentally sustainable durum wheat disease management programme.

Keywords. Landraces, biological control, *Fusarium culmorum*, *Pantoea* spp., *Paenibacillus* spp.

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INTRODUCTION

Fusarium foot rot is a widespread disease of wheat crops, (Scherm *et al*., 2013), particularly in Italy where wheat cultivation is one the most important field crops with 1,755 ha of production area (FAOSTAT, 2019). Fusarium foot rot is caused by several *Fusarium* species, among which *F. culmorum* (W.G.

Smith) Saccardo is one of the main soil-borne pathogens in the Mediterranean area. This pathogen causes Fusarium root rot and Fusarium head blight of wheat (Campanella and Miceli, 2009; Chekali *et al*., 2011; Scherm *et al*., 2013; Tok and Arslan, 2016). The importance of *F. culmorum* is also due to its capacity to produce mycotoxins such as deoxynivalenol (DON), which inhibits genes involved in host defense responses in wheat (Wagacha and Muthomi, 2007; Scherm *et al*., 2013), and type B trichothecenes (Scherm *et al*., 2011). In cereals, *F. culmorum* can cause pre-emergence seedling blight, root and foot rot, head blight and white heads (Bockus *et al*., 2010).

Several agronomic practices have been adopted to control diseases caused by *F. culmorum*, including as tillage, crop rotation with non-intermediate-host crops (e.g., brassicas; Campanella *et al*., 2020), use of healthy seeds coated with fungicides, and crop treatments (Ghosh *et al*., 2018). However, these approaches are often inefficient due to inappropriate cultural practices and unfavourable environmental conditions. The use of resistant varieties is also a possible strategy for controlling pathogen and disease, but there are still no known durum wheat varieties resistant to Fusarium foot rot.

In recent decades, the use of beneficial root-colonizing micro-organisms (i.e., *Bacillus* sp., *Pseudomonas* sp., *Streptomyces* spp., *Trichoderma* spp., *Glomus* spp.) for control of plant pathogens, has been extensively studied as an alternative control strategy, which is likely to be safe for humans and the environment (Kanini *et al*., 2013; Campanella and Miceli, 2021). More recently, bacterial endophytes of seeds of gramineous plants have demonstrated inhibitory effects against phytopathogenic fungi. Cottyn *et al*. (2001) highlighted the inhibitory activity of strains of *Pantoea* sp., *Bacillus subtilis*, *Enterobacter cloacae*, *Xanthomonas* sp., *Paenibacillus macerans* and *B. subtilis*, towards *Rhizoctonia solani* and *Pyricularia grisea*. Ruiza *et al*. (2011) found antifungal activity of *Pantoea* and *Paenibacillus* strains towards *Curvularia* sp., *F. oxysporum* var. *radices-lycopersici* and *Phytium ultimum*, while Herrera *et al*. (2016) reported ability of *Paenibacillus* isolates to restrain growth of *F. graminearum*, suggesting future agricultural applications (Verma *et al*., 2014).

"Timilia reste nere" is one of 32 ancient durum wheat landraces (Perrino and Hammer, 1983), still cultivated in several areas in Sicily, because it is deemed healthier than modern varieties (Sciacca *et al*., 2018; Ficco *et al*., 2019). Timilia landrace is also used to produce typical black bread, the best known of which is named "black bread of Castelvetrano" for the production specification developed by artisans of Castelvetrano (Visioli *et al*., 2021).

In recent years, much research has addressed the extraction and isolation of bacterial endophytes from cereal seeds (Rijavec *et al*., 2017; Ruiza *et al*., 2011; Orole *et al*., 2011; Herrera *et al*., 2016; Liu *et al*., 2017; Celador-Lera *et al*., 2018; Krishnamoorthy *et al*., 2020), but there have been no similar reports regarding Sicilian durum wheat landraces such as "Timilia reste nere".

Due to the yield-limiting importance of Fusarium foot rot of durum wheat and the necessity of implementing environmentally sustainable agricultural practices, the objectives of the present study were: i) to isolate and identify bacterial endophytes from durum wheat seeds; ii) to verify, the *in vitro* inhibitory effects of these bacterial endophytes toward growth of *F. culmorum*; and iii) to evaluate the use of these endophytes for control of Fusarium foot rot.

MATERIALS AND METHODS

Seed samples

Seeds of durum wheat landrace "Timilia reste nere" seeds originated from an organic farm in the Palermo Province, and were provided by the Assessorato Agricoltura of Regione Siciliana of Palermo (Italy). "Simeto" durum wheat seeds produced from a conventional farm in Enna Province were provided by the Research Centre for Plant Protection and Certification (CREA-DC) of Palermo (Italy). All durum wheat seed specimens were collected in 2019 and stored in paper packages at 4°C until analysis.

Surface sterilization of seeds

"Timilia reste nere" seeds were surface sterilized by stepwise immersion in 70% ethanol for 1 min, in sodium hypochlorite solution (2.5% NaClO) for 2 min, and again in 70% ethanol for 1 min. Subsequently, while stirring at 100 rpm they underwent two 30 min rinses in sterile distilled water at 25°C. To confirm seed surface sterilization, 1 mL of water used for the first rinse was placed on two solid media: tryptone soya broth agar (TSA) (17.0 g pancreatic digest casein, 3.0 g enzymatic digest soya bean, 5.0 g NaCl, 2.5 g K_2HPO_4, 2.5 g glucose, 17.0 g agar, 1 L H_2O), and L-B (Luria-Bertani) agar (20.0 g agar, 10.0 g NaCl, 10.0 g tryptone, 5.0 g yeast extract, 1 L H_2O, adjusted to pH = 7.0 with NaOH). Agar plates were incubated at 30°C for 4-5 d, then examined for growth. Effectiveness of the sterilization treatment was confirmed by absence of any microorganism development on the solid media.

Isolation of endophytic bacteria from "Timilia reste nere" seeds

Endophytic bacteria were isolated as described by Alibrandi *et al.* (2017). Individual surface sterilized seeds, were placed in separate test tubes, soaked for 1 h in sterile distilled water, then ground with a Potter-Elvehjem Tissue Grinder. Each resulting homogenate was resuspended in 50 mL of phosphate buffer saline (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH = 7.4) per 7.5 g of seeds, and then stirred at 150 rpm for 1 h. One-hundred µL of this suspension were each plated on L-B and TSA media. The plates were then incubated in the dark at 30°C until appearance of bacterial colonies.

Morpho-physiological characterization of isolated bacteria

Colonies from L-B and TSA were repeatedly subcultured on NSA (1.0 g beef extract, 2.0 g yeast extract, 5.0 g peptone, 5.0 g NaCl, 5.0 g sucrose, 15.0 g agar, in 1 L H_2O) to obtain pure cultures. These were subsequently grown using different media for morpho-physiological characterization as reported by Scortichini (1995) and Schaad *et al.* (2001). YDC (10.0 g yeast extract, 20.0 g dextrose, 20.0 g $CaCO_3$, 15.0 g agar, in 1 L H_2O) was used to determine pigmentation production and colony consistency, NSA was used for determining colony morphology, and King's B medium (20.0 g peptone, 15 mL glycerol, 1,5 g K_2HPO_4, 1.5 g $MgSO_4 \times 7H_2O$, 15.0 g agar, in 1 L H_2O, pH = 7.2) was used to assess production of fluorescent pigment. Bacterial isolates were assessed for Gram reaction.

Each isolate obtained was sequentially labelled with a capital letter and a number.

Molecular identification of isolated bacteria

All isolates were incubated at 28°C on NSA, and fresh colonies were each picked and suspended in 25 µL of TE buffer (Tris-HCl 10 mM pH = 8.0, EDTA 1 mM pH = 8.0). The lysate was then centrifuged (13,000 rpm for 5 min), the supernatant collected and diluted 1:10 with sterile distilled water, then used as the DNA template for colony PCR. Bacterial endophytes were taxonomically characterized by PCR as reported by Gallo *et al.* (2012) and Milanesi *et al.* (2015), based on their 16S rDNA gene sequence, using the universal bacterial primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT).

PCR reactions were each carried out in a total volume of 30 µL, containing 10 ng of genomic DNA, 10 mM Tris-HCl (pH = 8), 50 mM KCl, 0.2 µM dNTPs, 1.5 Mm $MgCl_2$, 1.5 units of Taq DNA polymerase (0.5 U μL^{-1} recombinant, Invitrogen Life Technologies), and 2 µM of 27F and 1492R primers. To increase specificity of amplification, 5% DMSO (1.5 µL) was added to the total volume of the PCR reaction. The reaction mixture was incubated in a thermal cycler (Biometra T-Personal Thermal Cycler), and amplification steps were carried out for 40 cycles, starting with a 5 min denaturation at 95°C, followed by 39 cycles at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, and a final extension for 10 min at 72°C. A negative control (no template DNA present in the PCR reaction) and a positive control (DNA template of *Streptomyces coelicolor* M145) were included in each experiment. Amplicons were analyzed by electrophoresis in 1% agarose gel on TAE buffer (Sambrook *et al.*, 1989), and were visualized by staining with 4 µL mL^{-1} Gel Red® (Molecular Acid Gel Stain, Biotium). Gel images were recorded using a camera system connected to an image processing workstation (GelDOC, Bio-Rad).

The PCR products were purified according to the manufacturer's instructions, using Quick Gel Extraction & PCR Purification Combo (Thermo Fischer Scientific). Sequencing was carried out by BMR Genomics. Paired raw forward and reverse sequences were checked for quality with the Geospiza's FinchTV software (PerkinElmer Inc.; www.geospiza.com/Products/finchtv.shtml), and were used to reconstruct the 16S rDNA sequences. Bacterial sequences were deposited in GenBank and compared to related available taxa using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). For each isolate, the best hits were compared to attribute species names with the relative percentage of identity ≥97% of sequence similarities. Purified DNA fragments obtained by PCR amplification were sequenced without any cloning step. After alignment of a number of amplicons, five were selected without duplications. In order to analyze data and obtain homogeneous results, only common regions were chosen where nucleotides were unequivocally readable (containing 16S V1-V4 hypervariable regions in the present case), thus comparing fragments of the same/similar length/position. Priority at this stage was to identify isolated endophytes focusing on genera, while characterizing their functional features.

Inoculum production for in vitro *assays*

The Sicilian isolate 162 of *Fusarium culmorum* from the collection of the Research Centre for Plant Protection and Certification (CREA-DC) of Palermo (Italy) was used. Once characterized morpho-physiologically

and molecularly (Campanella *et al*., 2020), the isolate was tested for pathogenicity to durum wheat. It was grown on plates of PDA (39 g L^{-1}; Oxoid, Ltd) incubated at 20±2°C for 7 d under NUV light (Sylvania 36W/BLB-T8), alternating light/darkness on 12 h cycles.

Bacterial seed endophytes were grown on Nutrient Broth (NB) (13.0 g L^{-1}; Difco, Becton Dickinson) in 100 mL flasks and maintained in the dark in agitation for 96 h at 20±1°C. The concentration of resulting bacterial suspensions was estimated using decimal dilution and colony counting on Nutrient Agar (NA) (3.0 g beef extract, 5.0 g peptone, 15.0 g agar in 1 L H_2O). The bacterial suspensions were adjusted to the final concentration of 1 × 10^6 CFU mL^{-1} by dilution with sterile distilled water containing 0.1% Tween 20.

In vitro *assays*

One mL aliquots of bacterial suspensions were spread onto PDA plates (90 mm diam.) and left to dry under a laminar flow hood. A 5 mm diam. plug of *F. culmorum*, taken from an actively growing colony, was centrally seeded onto each amended PDA plate. Five replicates were prepared for each bacterial isolate, using PDA amended with distilled sterile water as controls. Plates were incubated at 18±2°C under NUV light (Sylvania 36W/BLB-T8) alternating light/darkness on 12 h cycles. Every 24 h, mycelial growth was assessed by measuring two colony diameters at right angles to each other until pathogen growth in the control plates reached the outer edge of the plates. Mycelial growth inhibition (MGI) was calculated according to the formula MGI = [(D1-D2) / D1] × 100 (Kaiser *et al*., 2011), where D1 = growth of the pathogen in the absence of antagonist, and D2 = growth of the pathogen in the presence of antagonist.

Plant material and inoculum production for in vivo *assays*

Seeds of the durum wheat "Simeto" were surface disinfected by dipping them in a sodium hypochlorite solution (2.0% NaClO) for 3 min, rinsing three times with sterile distilled water (Montorsi *et al*., 1991), and then drying at room temperature on sterile absorbent paper.

Fifteen-day-old cultures of *F. culmorum* grown on PDA plates were each flooded with 10 mL of sterile distilled water. Conidia were removed by scraping the surface of mycelium with a sterile bacteriology loop. Conidium concentration of the *F. culmorum* suspension was determined using a haemocytometer and adjusted to 1 × 10^4 conidia mL^{-1} by dilution with sterile distilled water (Imathiu *et al*., 2010).

Bacterial endophyte inoculum was prepared for *in vitro* assays as described above. Inoculum concentrations were the same (1 × 10^6 CFU mL^{-1}) whether endophyte strains were applied individually or as mixtures.

In vivo *assays*

Surface disinfected durum wheat "Simeto" seeds were soaked for 3 min in *F. culmorum* suspensions (1 × 10^4 conidia mL^{-1}), then left to dry under a laminar flow hood. Twenty-five inoculated seeds were distributed on sterile filter paper (Whatman N°1, 110 mm diam.) within 120 mm diam. glass Petri plates, and 10 mL of each bacterial suspension was added to the paper in each plate. Four replicates for each bacterial isolate were set up. Seeds inoculated with *F. culmorum* were used as positive controls, and seeds soaked with sterile distilled water were used as negative controls. Ten mL of sterile distilled water were added on the filter paper of each control. Plates were then incubated for 48 h at 5°C (first step), followed by 5 d in the dark at 20°C (second step) and then for 7 d at 22±1°C (third step), alternating light/darkness on 12 h cycles. All resulting seedlings received 10 mL of sterile distilled water on day 2 of the third incubation step. The following parameters were recorded: seed germination and emergence, shoot height, number and length of roots, vigour index (VI), and incidence (I) and severity (S) of disease. Seed germination was expressed as percentages. Germination was assessed at the end of the second incubation step, and seeds were considered germinated when radicles and hypocotyls were >10 mm. Seed/seedling emergence expressed as proportions were the numbers of symptomatic and asymptomatic seed/seedlings detected at the end of the third incubation step divided by the total number of seeds. Shoot height and root length of plants were expressed for each bacterial endophyte treatment.

Shoot height of seedlings was measured from shoot insertion points to the tips of the primary leaves. Seminal root lengths were assessed from root insertion points to the apices of roots. Vigour index (VI) (Maisuria and Patel, 2009), was calculated for each seedling as follows: VI = (∑root length + shoot length) × % seed germination. Presented vigour indices are means of all seedlings for each treatment. Shoot height, root length, vigour index, disease incidence and severity were evaluated at the end of the third incubation step.

Disease severity (S) was assessed using an empirical scale ranging from 0 to 4, where 0 = absence of symptoms/healthy; 1 = slight browning at the base of the culm/roots; 2 = browning of approx. 50% of the culm/roots; 3 = culm/root browning > 50%; 4 = com-

plete browning of culm/roots. Disease severity was evaluated according to the Mc Kinney index: $\Sigma [(v \times n) / (N \times V)] \times 100$, where v = numeric value of the class; n = number of observed cases for each class; N = total number of observed cases; and V = numeric value of the greatest class.

Statistical analyses

In vitro and *in vivo* experiments were arranged in completely randomized designs. All experiments were repeated at least twice, obtaining similar results. The reported data are from representative experiments and are expressed as overall averages of the replicates. To identify statistically significant differences following endophyte treatments, all data were tested for homogeneity and normality according to Bartlett's test, and the data were then submitted to analysis of variance (ANOVA), and means were compared using Duncan's Multiple Range test. Analyses were carried out using the Statistical Analysis System software XLSTAT (Addinsoft, 2021). Data of the *in vitro* assessment of antagonistic activity of bacterial seed endophytes were recorded only after 120 and 216 h of incubation. Data of seed germination, disease incidence and severity are expressed as percentages, and were transformed to Bliss angular values (arcsine √%) prior to analyses.

RESULTS

Isolation, morpho-physiological characterization and molecular identification of bacterial endophytes from "Timilia reste nere" seeds

Overall, eight endophytic bacterial isolates were obtained from seeds of durum wheat landrace "Timilia reste nere". However, only five of these isolates inhibited growth of *F. culmorum* in preliminary *in vitro* tests.

Table 1. Morpho-physiological characteristics of wheat bacterial endophyte isolates.

Isolates	Gram staining[a]	Colony colour on YDC	Fluorescence[b]
A1	-	Milky white	-
B	+	Cream yellow	-
F7	-	Light yellow	-
F15	-	Light yellow	-
G1	-	Light beige	-

[a] Gram positive bacteria +; Gram negative bacteria -.
[b] Fluorescence reaction positive (+), or negative (-).

Colonies were grouped by colony morphology (size, shape, colour, margin, opacity, elevation and consistency) and physiologic traits (Table 1).

Subsequent 16S rRNA gene molecular analyses revealed that isolates A1, F7, F15 and G1 belonged to *Pantoea*, and isolate B was *Paenibacillus*. Isolates A1, F7, F15 and G1, were the best fits to *Pan. agglomerans*, all with similarity >99.0%. Isolate B fit to *Pae. polymyxa* (Table 2). All sequences were deposited in the GenBank database with the following accession numbers: A1, MW 925116; B, MW 925117; F7, MW 925114; F15, MW 925115; and G1, MW 925118 (Table 2). No data on incidence of the different bacterial endophyte strains were collected during this investigation.

In vitro *assays*

All the bacterial seed endophytes inhibited ($P \leq 0.001$) growth of *F. culmorum* in comparison to controls (Table. 3). The greatest mycelial growth inhibition

Table 2. BLAST analysis results for different bacterial endophytes isolated from seeds of durum wheat landrace "Timilia reste nere".

Isolate	Genbank accession No.	Bacterial genus	16S rRNA gene sequence length (nts)	Nearest phylogenetic neighbour	Sequence similarity (%)
A1	MW925116	*Pantoea*	928	*Pantoea agglomerans* (ATCC 27155)	99.03
B	MW925117	*Paenibacillus*	899	*Paenibacillus polymyxa* (DSM 36)	99.78
F7	MW925114	*Pantoea*	968	*Pantoea agglomerans* (DSM 3493)	99.69
F15	MW925115	*Pantoea*	968	*Pantoea agglomerans* (DSM 3493)	99.38
G1	MW925118	*Pantoea*	950	*Pantoea agglomerans* (ATCC 27155)	99.47

Table 3. Activities of bacterial seed endophytes on growth of *Fusarium culmorum* mycelium, after 120 and 240 h incubation.

Treatment[a]	Mean colony diameter (mm)[b]		Mycelium growth inhibition (%)	
	120 H	216 H	120 H	216 H
Control	37.8±1.7 A	75.3±3.2 A	- -	- -
A1	25.0±2.2 B	56.9±4.6 B	33.8 C	24.4 D
F7	20.3±2.7 BC	29.3±4.5 C	46.3 B	61.1 C
F15	13.8±1.3 D	22.4±1.1 D	63.5 B	70.3 B
G1	9.9±0.9 E	20.3±0.5 DE	73.8 A	73.1 AB
B	9.0±1.5 E	15.6±1.7 E	76.2 B	79.3 A

[a] Treatment: A1 (*Pan. agglomerans*); F7 (*Pan. agglomerans*); F15 (*Pan. agglomerans*.); G1 (*Pan. agglomerans*); B (*Pae. polymyxa*); Control (*F. culmorum*).
[b] Mean of five replicates ± standard deviation.
Means in each column accompanied by the same letters are not statistically different ($P \leq 0.01$; Duncan's Multiple Range Test).

occurred with isolate B (*Pae. polymyxa*) with an average reduction of 76% after 120 h of incubation and 79% at 216 h (Figure 1). Overlapping mycelial growth inhibition values were also detected for isolate G1 (*Pan. agglomerans*). Isolate A1 (*Pan. agglomerans*) showed the least mycelial growth inhibition, with average values of 34% (120 h) and 24% (216 h).

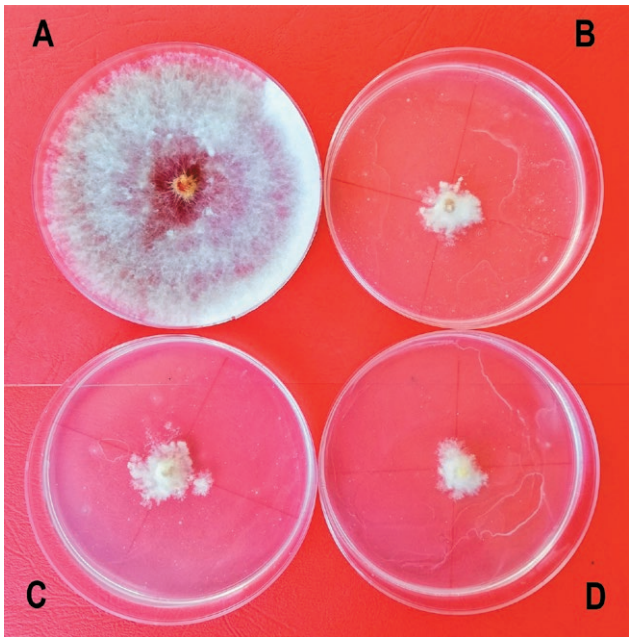

Figure 1. Colonies of *Fusarium culmorum* after 216 h incubation with different bacterial strains applied at 1×10^6 CFU mL^{-1} in agar medium. The plates were each centrally seeded with a 5 mm diam. plug of *F. culmorum*. Letters represent: A, *F. culmorum* alone; B, the pathogen with isolate G1 (*Pan. agglomerans*); C, with isolate F15 (*Pan. agglomerans*); D, with isolate B (*Pae. polymyxa*).

In vivo *assays*

Although all bacterial seed endophytes had no statistically significant effects on normal seed germination of durum wheat seeds in comparison to seeds inoculated with *F. culmorum* alone (positive control; Table 4), all treatments with the different bacterial strains gave greater seed germination rates than positive controls, ranging from an 8% increase from isolate A1 (*Pan. agglomerans*), to 2% increase from isolate G1 (*Pan. agglomerans*).

After application of bacterial endophytes, no differences were observed in the number of durum wheat roots (data not presented).

With the exception of isolate A1, all the bacterial endophytes, whether alone or in combinations, increased ($P \leq 0.05$) mean seedling shoot height in comparison to the positive control (Table 4). The greatest growth occurred with the isolate mixture F7 + F15 (both *Pan. agglomerans*) with an average increase of 95%, followed by A1 + B (*Pan. agglomerans* + *Pae. polymyxa*) at 87%. When applied individually, isolate G1 (*Pan. agglomerans*), gave the greatest shoot development, with an average increase of 69%, while isolate F15 (*Pan. agglomerans*) showed the least increase at 46%.

Table 4. Mean wheat seed germination and seedling heights from different bacterial endophyte isolate treatments applied to "Simeto" seeds artificially inoculated with *Fusarium culmorum*.

Treatment[a]	Germination (%)[b]	Treatment	Shoot height (mm)[c]
Control -	93±3.0 a	Control -	57.7 bc
Control +	90±5.2 a	Control +	35.4 d
A1	97±3.8 a	F7 + F15	68.9 a
F7 + F15	95±3.8 a	A1 + B	66.2 ab
A1 + B	95±2.0 a	G1	59.8 bc
B	95±6.0 a	B	58.7 bc
F15	94±2.3 a	F7	51.9 c
F7	93±6.0 a	F15	51.7 c
G1	92±7.3 a	A1	34.0 d

[a] Treatments: A1 (*Pan. agglomerans*); B (*Pae. polymyxa*); F7 (*Pan. agglomerans*); F15 (*Pan. agglomerans*); G1 (*Pan. agglomerans*); A1 + B; F7 + F15; Control -, (no pathogen, no endophytes); Control + (*F. culmorum* alone).
[b] Means of five replicates (± standard deviations).
[c] Means of five replicates.
Means in each column accompanied by the same letters are not statistically different ($P \leq 0.05$; Duncan's Multiple Range Test).

Table 5. Mean "Simeto" seedling root lengths and seedling vigour indices (VI) for seedlings were grown from seeds treated with different bacterial endophyte treatments and artificially inoculated with *Fusarium culmorum*.

Treatment[a]	Root length[b] (mm)	VI
Control -	71.5 a	40304.0 a
Control +	47.6 c	21686.0 c
F7 + F15	70.1 a	39194.5 a
B	64.9 a	36955.6 a
A1 + B	64.8 a	36629.9 a
G1	57.4 b	30745.9 b
F15	55.6 b	29199.2 b
F7	54.2 bc	28999.6 b
A1	32.7 d	18496.4 c

[a] Treatments: A1, *Pan. agglomerans*; B, *Pae. polymyxa*; F7, *Pan. agglomerans*; F15, *Pan. agglomerans*; G1, *Pan. agglomerans*; A1 + B; F7 + F15; Control -, (no pathogen, no endophytes); Control +, *F. culmorum* alone.
[b] Mean of five replicates.
Means in each column accompanied by the same letters are not statistically different ($P \leq 0.05$; Duncan's Multiple Range Test).

Table 6. Mean incidence (I) and severity (S) of Fusarium foot rot (*Fusarium culmorum*) on wheat "Simento" seedlings after seed treatments with different bacterial endophyte strains.

Treatment[a]	I (%)	Treatment	S (%)
Control -	0 -	Control -	0 -
Control +	93.1 a	Control +	76.9 a
F15	93.6 a	F15	74.4 ab
G1	90.0 ab	G1	73.7 ab
B	83.5 abc	B	66.1 abc
F7	82.7 abc	A1	64.3 abc
F7 + F15	80.9 bc	F7	64.1 abc
A1	79.5 bc	F7 + F15	59.2 bc
A1 + B	73.6 c	A1 + B	54.2 c

[a] Treatments: A1, *Pan. agglomerans*; B, *Pae. polymyxa*; F7, *Pan. agglomerans*; F15, *Pan. agglomerans*; G1, *Pan. agglomerans*; A1 + B; F7 + F15; Control –, (no pathogen, no endophytes); Control + *F. culmorum* alone.
Means in each column accompanied by the same letters are not statistically different ($P \leq 0.05$; Duncan's Multiple Range Test).

Root length was increased by most of the endophyte isolates in comparison to the positive controls (Table 5). The greatest root length increase 47% was from the mixture F7 + F15 (both *Pan. agglomerans*), followed by isolate B (*Pae. polymyxa*) (+36%), the isolate mixture A1 + B (*Pan. agglomerans* + *Pae. polymyxa*) (36%), G1 (*Pan. agglomerans*) (21%) and F15 (*Pan. agglomerans*) (17%). Although the increase in root length from isolate F7 (*Pan. agglomerans*) was 14%, this was not statistically significant. Only isolate A1 (*Pan. agglomerans*) produced shorter root length than the positive control.

Most of the endophytes influenced mean seedling vigour index. The statistically significant increases were (in decreasing order): the mixture F7 + F15 (both *Pan. agglomerans*), 81%; isolate B (*Pae. polymyxa*), 70%; mixture A1 + B (*Pan. agglomerans* + *Pae. polymyxa*), 69%; isolate G1 (*Pan. agglomerans*), 42%; isolate F15 (*Pan. agglomerans*), 35%; and isolate F7 (*Pan. agglomerans*) 34% (Table 5). The influence of isolate A1 (*Pan. agglomerans*) was not statistically significant.

The isolate mixture A1 + B (*Pan. agglomerans* + *Pae. polymyxa*), isolate A1 (*Pan. agglomerans*) and the mixture F7 + F15 (both *Pan. agglomerans*) reduced incidence of Fusarium foot rot in comparison to the positive control (Table 6), with average decreases of 21% from A1 + B, 15% from A1, and 13 % from F7 + F15. No statistically significant responses were detected from isolates F7 (*Pan. agglomerans*), B (*Pae. polymyxa*) or G1 (*Pan. agglomerans*), despite respective disease index reductions of 11%, 10% and 3%. Only the mixtures F7 + F15 (both *Pan. agglomerans*) and A1 + B (*Pan. agglomerans* + *Pae. polymyxa*) significantly reduced severity of disease in comparison to the positive control, with average reductions of 30% from F7 + F15 and 23% from A1 + B (Table 6). There were no statistically significant differences between isolates F7 (*Pan. agglomerans*), A1 (*Pan. agglomerans*), B (*Pae. polymyxa*), G1 (*Pan. agglomerans*), or F15 (*Pan. agglomerans*) and positive control. Nevertheless, decreases in disease severity were 17% from isolate F7 (*Pan. agglomerans*) and 3% from F15 (*Pan. agglomerans*).

DISCUSSION

In this study isolations of bacterial strains inhabiting "Timilia reste nere" seeds were performed using culture-dependent methods. This approach allowed isolation and characterization of five isolates (A1, B, F7, F15 and G1). Subsequent BLAST analyses showed that isolates A1, F7, F15 and G1, if compared to their nearest phylogenic neighbours (ATCC 27155, DSM 3493; DSM 3493 and ATCC 27155), belonged to *Pantoea*, specifically *Pantoea agglomerans*, with similarities to the four neighbours from 99.03 to 99.69%. The same analysis for isolate B when compared to DSM 36 gave best fit to *Paenibacillus*, specifically to *Paenibacillus polymyxa* with 99.78% similarity. Similar results have been obtained in studies of rice, maize, wheat (*Triticum aestivum*) and switch-

grass (Ruiza *et al*., 2011; Verma *et al*., 2014; Truyens *et al*., 2015; Grady *et al*., 2016).

Although the present results were obtained by testing only one isolate of *F. culmorum*, they are indications for further research. All the bacterial endophytes studied reduced mycelial *in vitro* growth of *F. culmorum*. Isolate B (*Pae. polymyxa*) was the most effective for inhibition of mycelium growth of the fungus. Next most effective was isolate G1 (*Pan. agglomerans*) with similar growth inhibition, while *Pan. agglomerans* isolates were less inhibitory. Similar results were reported by Herrera *et al.* (2016) for isolates of *Paenibacillus* sp. obtained from seeds of *Triticum aestivum* used to control *F. graminearum*, and by Lounaci *et al.* (2016) using a strain of *P. polymyxa* for management of diseases caused by *F. graminearum*, *F. culmorum*, *F. verticillioides, Microdochium nivale* and *Rhizoctonia solani*. Several authors have also reported results similar to those of the present study, using strains of *P. agglomerans* to reduce charcoal root rot caused by *Macrophomina phaseolina* (Vasebi *et al*., 2013), or to manage *R. solani* responsible of root and crown rot of sugar beet (Nabrdalik *et al*., 2018), and *Phytophthora capsici* and *Pythium aphanidermatum* of *Cucumis* spp. (Khalaf and Raizada, 2020). The present study results also highlight fungistatic rather than fungitoxic activity the bacterial endophyte strains.

Endophytes probably exert antifungal activity through several mechanisms. In a study on antagonistic activity of *Paenibacillus* strains, Selim *et al.* (2005) reported that a polymyxin-related peptide was responsible for antifungal activity against *Fusarium* spp. In research where *P. agglomerans* was used to control *M. phaseolina*, Vasebi *et al.* (2013) showed that inhibition of mycelial growth was related to production of antibiotics. Antifungal substances capable of inhibiting growth of *F. graminearum* were also found from *Paenibacillus* isolates (Herrera *et al*., 2016). Therefore, inhibition of *F. culmorum* mycelial growth observed in the present study was likely attributable to release by the bacteria of similar antifungal compounds.

The *in vivo* experiment showed that none of the bacterial endophytes had negative effects on "Simeto" seed germination. Although no statistically significant differences in seed emergence were detected for any of the bacterial endophyte compared to positive controls, an average increase of 5% (range 2 to 8%) in seed emergence was measured. Similar results were obtained by Hsieh *et al.* (2005), where an isolate of *P. agglomerans* reduced bacterial wilt of bean caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*.

All vegetative parameters of "Simeto" seedlings were increased after treatment with most of the bacterial endophytes. In particular, the isolate mixture F7 + F15 (both *Pan. agglomerans*) gave the greatest increases in on seedling shoot height, root length and vigour index, with average increases in these parameters from 47 to 95%. Similar effects on shoot and root development were also measured for the isolate mixture A1 + B (*Pan. agglomerans* + *Pae. polymyxa*). Individual endobacterial applications also increased specific seedling growth parameters. Similar results using strains of *P. agglomerans* were reported by Feng *et al.* (2006) treating rice seeds, by Xie *et al.* (2017) for mulberry seeds, and Quecine *et al.* (2012) for sugarcane. In addition, results comparable to those of the present study were also obtained by Quyet-Tien *et al.* (2010) with isolates of *P. polymyxa* used to treat pepper roots. Several studies have also reported increased plant growth from bacterial production of growth stimulants, including auxin, cytokinin, or indole-3-acetic acid (Ryu *et al*., 2006; Quyet-Tien *et al*., 2010; Vasebi *et al*., 2013; Xie *et al*., 2017). The increases in seedling vegetative parameters observed here are likely to be direct consequences of production of similar compounds. However, the possible roles of factors other than these cannot be excluded, i.e., competition for nutrients and sites, or production of siderophores, chitinases, or antibiotics, as reported by Lacava and Azevedo, (2013).

In the present study, isolate A1 (*Pan. agglomerans*) was the outlier. Although this isolate reduced *in vitro* pathogen growth and gave the greatest "Simeto" seed germination rate (97%), it failed to increase any seedling vegetative parameters, compared to positive controls. These results could be explained by limitations in conditions essential for antagonistic activity, such as competition and/or direct physical contact with the pathogen, as was demonstrated by Poppe *et al*. (2003) and Pusey *et al.* (2011).

The present study has also demonstrated reductions of both incidence and severity of Fusarium foot rot following application of the bacterial isolate mixture A1 + B (*Pan. agglomerans* + *Pae. polymyxa*). Suppressive effects on disease incidence were also found for bacterial isolate A1 (*Pan. agglomerans*), while the mixture F7 + F15 (both *Pan. agglomerans*) reduced foot rot incidence and severity. Several studies have reported similar reductions in disease parameters, using *P. agglomerans* strains to reduce fire blight caused by *Erwinia amylovora* (Kearns and Hale, 1995), reduce *Penicillium expansum*, *Botrytis cinerea* and *Rhizopus stolonifer* infections on pears in postharvest (Nunes *et al*., 2001), control soybean charcoal root rot caused by *M. phaseolina* (Vasebi *et al*., 2013), and bacterial blight caused by *Pseudomonas syringae* pv. *mori* (Xie *et al*., 2017). Similar results were

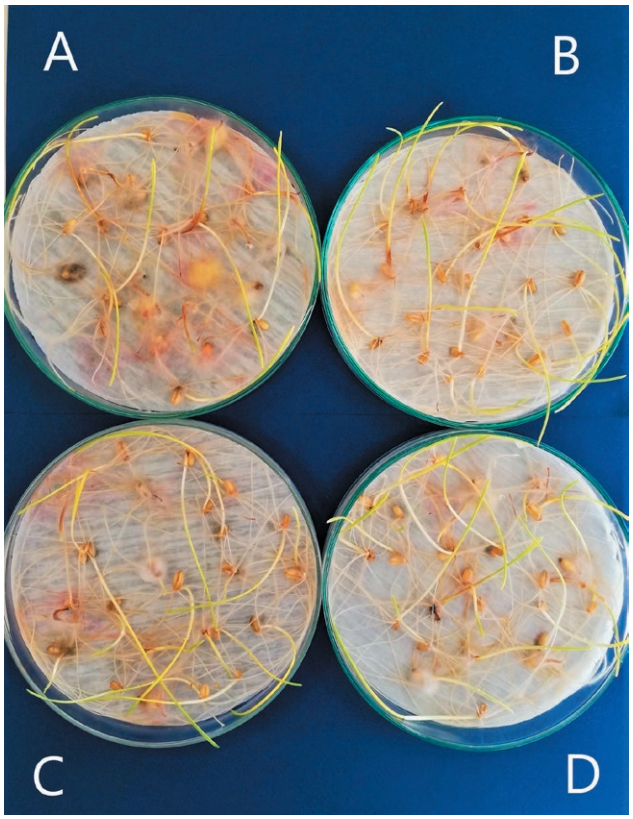

Figure 2. Wheat "Simeto" seedlings grown from seeds artificially inoculated with *Fusarium culmorum* and then treated with different bacterial endophyte strains. Suspensions of bacterial strains were applied at 1×10^6 CFU mL^{-1} on filter paper, onto which the seeds artificially inoculated with the pathogen were distributed. A, *F. culmorum* alone; B, F7 (*Pan. agglomerans*) + *F. culmorum*; C, F15 (*Pan. agglomerans*) + *F. culmorum*; D, A1 (*Pan. agglomerans*) + *F. culmorum*.

also obtained by Xu and Kim (2014), using strains of *Paenibacillus* (including *P. polymyxa*) to manage Fusarium crown and root rots of tomato.

Conversely, no statistically significant, but minimal, decreases in incidence of disease was obtained for isolates F7 (*Pan. agglomerans*), B (*Pae. polymyxa*) and G1 (*Pan. agglomerans*). The partial reduction of incidence of disease observed from these bacterial isolates could be explained by a suboptimal bacterial inoculum (1×10^6 CFU), because greater inoculum concentrations (1×10^9 CFU) are conventionally used in commercial products. Similarly, isolates F7, A1, B, G1 and F15 all gave small non-significant reductions in disease severity.

The present results are the first on presence of bacterial endophytes inhabiting seeds of durum wheat "Timilia reste nere". Four of five strains found were *P. agglomerans* and the fifth was *P. polymyxa*.

The results of this study support the efficacy of these endobacteria to reduce mycelial growth of *F. culmorum*. Furthermore, they highlight the specific abilities of the bacteria to enhance seedling emergence (Figure 2), improve seedling growth and reduce disease. This study supports the conclusion that mixtures of bacterial endophytes outperform individual isolates for enhancing wheat seedling growth (as from isolate mixture F7 + F15), and reducing incidence and severity of disease (from mixture A1 + B).

The expanding interests in use of beneficial microorganisms as alternatives to chemical pesticides and fertilizers has made it possible to produce and market several products. Cedomon®, Cerall® and Cedress®, BioAgri (containing *Pseudomonas chlororaphis*), Micosat F®, CCS Aosta (*Bacillus subtilis, Paenibacillus durum*, *Streptomyces* spp.), Nitroguard®, Mapleton AgriBiotec Pty, (*Azospirillum brasiliense*, *Azorhizobium caulinodens*, *Azoarcus indigens*, *Bacillus spigens*), are examples of many commercially available products containing active biological agents for use in cereal seed treatments (O'Callaghan, 2016; Le Mire *et al*., 2016). Therefore, endobacteria from durum a wheat landrace could be useful tools for improving yields of wheat, and to manage Fusarium foot rot in the Mediterranean areas. However, further research is required to confirm the effectiveness of these bacterial seed endophytes, to provide environmentally-friendly wheat diseases management methods, possibly using greater bacterial inoculum (e.g., 1×10^9 CFU g^{-1} seed) as has been used in commercial products.

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Research Papers

A cryptic powdery mildew (*Golovinomyces hieraciorum* sp. nov.) on *Hieracium* and *Pilosella* (*Compositae*)

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Summary. The *Golovinomyces cichoracearum* complex is morphologically highly variable and causes powdery mildew on a wide range of *Compositae*, including *Hieracium* spp. and *Lapsana communis*. A comprehensive phylogenetic analysis of *Golovinomyces*, published in 2013, revealed that *G. cichoracearum s. str.* was confined to *Scorzonera* and *Tragopogon* spp. as hosts (*Compositae*, tribe *Lactuceae*, subtribe *Scorzonerinae*). To clarify the phylogeny and taxonomy of *Golovinomyces* on *Hieracium* spp. (*Compositae*, tribe *Lactuceae*, subtribe *Hieraciinae*), which is common in Asia and Europe, morphology and phylogenetic analyses of ITS + 28S rDNA sequences were conducted for 57 powdery mildew specimens of *Hieracium* spp. and *Lapsana communis*. *Golovinomyces* on *Hieracium* spp. in Asia and Europe, previously referred to as *G. cichoracearum*, is a previously undescribed, cryptic species, which is described here as *Golovinomyces hieraciorum* sp. nov.. Since a sequence retrieved from a powdery mildew on *Lapsana communis* in Switzerland clustered in sister position to the *G. hieraciorum* clade, numerous German specimens identified as *G. cichoracearum* were included in the present examinations. All the re-examined collections of "*G. cichoracearum*" on *L. communis* were misidentified, and were shown to belong to *Neoërysiphe nevoi* or *Podosphaera ergerontis-canadensis*.

Keywords. *Golovinomyces cichoracearum*, *Erysiphaceae*, *Ascomycota*, taxonomy, phylogenetic analysis.

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INTRODUCTION

Hieracium L. (*Compositae*, tribe *Lactuceae*; hawkweed) is a taxonomically challenging genus of herbaceous perennial herbs, with ligulate flowers and milky latex in stems and leaves. This genus comprises terrestrial perennial plants, native to Africa, Asia, Europe and North to South America, which are used as forage for livestock (Wilson *et al.*, 2006). *Hieracium* comprises ca.

770 sexually producing species, and ca. 5200 apomictic microspecies, according to The International *Compositae* Alliance (TICA) (CWG, 2021). *Hieracium* is divided into three subgenera, viz. *Pilosella*, *Hieracium*, and *Chionoracium* (Wilson *et al*., 2006). Subgenus *Pilosella* is considered a separate genus, *Pilosella*, i.e., the former subgenus *Hieracium* is treated as *Hieracium s. str.*, and subgenus *Chionoracium* is assigned to the genus *Crepis* (Gaskin and Wilson, 2007). Members of the genera *Hieracium* and *Pilosella* are model plants that are used to study the mechanisms of apomixis (not involving the fusion of male and female gametes in reproduction) (Hand *et al*., 2015; Płachno *et al*., 2017; Chrtek *et al*., 2020). Apomixis is a key evolutionary mechanism in certain angiosperms, providing reproductive assurance and isolation (Mráz and Zdvořák, 2019).

Hieracium spp. are susceptible to powdery mildews. Six species, viz. *Erysiphe lamprocarpa*, *Golovinomyces cichoracearum*, *Leveillula taurica*, *Podosphaera fusca, Po. fuliginea*, and *Po. macularis*, have been reported on *Hieracium* from many countries (Farr and Rossman, 2021). However, these names do not reflect the current taxonomy, i.e., they were based on outdated broad species concepts. *Golovinomyces cichoracearum* (basionym: *Erysiphe cichoracearum*) was originally described on *Scorzonera hispanica* and *Tragopogon porrifolius* (de Lamarck and de Candolle, 1805). Salmon (1900) widened the concept of *G. cichoracearum* by assigning powdery mildew on numerous hosts of various plant families, including *Boraginaceae* and *Plantaginaceae*, to this species. Braun (1987) confined *G. cichoracearum* to a wide range of *Compositae* hosts. Braun and Cook (2012) further reduced its circumscription to *Golovinomyces* on hosts in the subfamily *Cichorioideae* of *Compositae*, but emphasized that this circumscription still represented a heterogenic complex species. This is demonstrated by molecular sequence analyses (Matsuda and Takamatsu, 2003; Lebeda and Mieslerová, 2011), which supported this compound species comprising at least several *formae speciales* or possibly cryptic species.

The morphology of *G. cichoracearum s. lat.* is not uniform on various hosts, i.e., somewhat lobed hyphal appressoria and conidiophores with curved foot-cells are common on *Lactuca*, *Scorzonera*, and *Tragopogon*, whereas most appressoria are nipple-shaped and the foot-cells are mostly straight on *Hieracium* and *Picris* (Lebeda and Mieslerová, 2011; Braun and Cook, 2012). Takamatsu *et al.* (2013) showed in comprehensive phylogenetic analyses that *G. cichoracearum s. str.* is further confined to host species of the genera *Scorzonera* and *Tragopogon* (*Compositae*, *Cichorioideae*, tribe *Lactuceae*, subtribe *Scorzonerinae*), since the powdery mildews on hosts of these genera morphologically coincide and are a clade which is separate from all other clades within *Golovinomyces*.

Golovinomyces cichoracearum (or *E. cichoracearum*) on *Hieracium* (tribe *Lactuceae*, subtribe *Hieraciinae*) and *L. communis* (tribe *Lactuceae*, subtribe *Crepidinae*) is common throughout Europe, North America, and in parts of Asia (Zeller and Levy, 1995; Braun and Cook, 2012; Farr and Rossman, 2021). Takamatsu *et al.* (2013) showed a sequence retrieved from *Golovinomyces* on *L. communis* in Switzerland was genetically distinct from *G. cichoracearum s. str.*, and forms a separate lineage. Additional sequences and morphological observations are required to clarify the relationship between *G. cichoracearum s. str.*, *Golovinomyces* collections on *Hieracium*, and *Golovinomyces* collections on *L. communis*.

In 2014 and 2017, *Golovinomyces* sp. was found on *H. umbellatum* in Heihe City, Heilongjiang Province, China. To identify the powdery mildew species involved, morphological and molecular phylogenetic analyses (based on ITS and 28S rDNA sequences) were conducted. Morphologically, this fungus is close to *G. cichoracearum* as circumscribed in Braun and Cook (2012), but genetically it clusters far away from *G. cichoracearum s. str.* and forms a clade of its own in sister position to a sequence obtained from *Golovinomyces* on *L. communis* in Europe. Therefore, the present study aimed to: (i) clarify the identity of the causal agent of the *Hieracium* powdery mildew based on morphological re-examinations and molecular analyses, and determine if this pathogen is distributed and common in Asia and Europe; and (ii) identify the *Golovinomyces* on *L. communis* in Europe.

MATERIALS AND METHODS

Fungal material

A total of 57 specimens were examined from four countries. Twenty-eight specimens of *Hieracium* (including 14 species) with *Golovinomyces* were examined, including two samples collected in China, one specimen from Russia, one from Slovakia, and 24 additional collections from Germany. One specimen on *Pilosella procera* from China was included, and 27 *Lapsana communis* specimens from Germany were also examined.

Morphological examination

For morphological observations, dried samples from herbarium collections were mounted in lactic acid and

examined using a light microscope (Zeiss Axio Scope A1, Germany). Dimensions of at least 30 different fungus anamorph and teleomorph structures were measured for each sample.

DNA extractions, PCR amplification and sequencing

Whole-cell DNA was extracted from chasmothecia or conidia and mycelia using the Chelex-100 method (Walsh *et al*., 1991; Hirata and Takamatsu, 1996). The rDNA internal transcribed spacer (ITS) regions including the 5.8S rDNA were amplified with primers ITS5 and ITS4 (White *et al*., 1990), or PM10 and PM2 (Bradshaw and Tobin, 2020). And the partial 28S rDNA, including D1 and D2 domains, was amplified with primers LSU1 and LSU2 (Scholin *et al*., 1994; Mori *et al*., 2000), or PM28F and PM28R (Bradshaw and Tobin, 2020). The DNA amplification and sequencing were carried out as outlined in Qiu *et al.* (2018).

Molecular phylogenetic analyses

The obtained sequences, including complete ITS and partial 28S rDNA, were deposited in GenBank. The combined dataset of ITS and 28S rDNA sequences was aligned with closely related sequences of the genus *Golovinomyces* (Table S1) (Kiss *et al*., 2001; Matsuda and Takamatsu, 2003; Takamatsu *et al*., 2006; 2008; 2009; 2013; Park *et al*., 2010; Scholler *et al*., 2016; Bradshaw *et al*., 2017; Braun *et al*., 2018; 2019; Meeboon *et al*., 2018; Ellingham *et al*., 2019; Qiu *et al*., 2020). *Golovinomyces inulae* was selected as the outgroup taxon (Takamatsu *et al*., 2013). Multiple sequence alignments were carried out using MUSCLE implemented in the MEGA X (Kumar *et al*., 2018). Subsequently, the alignments were manually refined with MEGA X and deposited in TreeBASE (Piel *et al*., 2009) under the accession number of S28413. Phylogenetic trees were obtained from the generated dataset using the maximum parsimony (MP), maximum likelihood (ML) and Bayesian Inference (BI) methods. MP analysis was implemented in PAUP* 4.0 (Swofford, 2002) with the heuristic search option using the "tree-bisection-reconnection" (TBR) algorithm with 100 random sequence additions to find the global optimum tree. All sites were treated as unordered and unweighted, with gaps treated as missing data. The strength of the internal branches of the resulting trees was tested with bootstrap (BS) analysis using 1000 replications with the step-wise addition option set as simple and maximum tree number as 100 to save analysis time. Tree scores, including tree length (TL), consistency index (CI), retention index (RI), and rescaled consistency index (RC), were also calculated. ML analyses were carried out in raxmlGUI 2.0 beta (Edler *et al*., 2021) under a GTRGAMMA model. The BS supports and trees were obtained by running rapid bootstrap analyses of 1000 replicates followed by a search for the tree with the greatest likelihood. Bootstrap supports below 75% were discarded for both analyses. In BI analyses, the best-fit substitution models for different datasets were estimated by MrModeltest ver. 2.3 based on the implementation of the Akaike information criterion (AIC) (Nylander, 2004). Four Markov Chain Monte Carlo (MCMC) were run from random starting trees for 2,000,000 generations, and trees were sampled every 100 generations by MrBayes v. 3.2.7 (Ronquist and Huelsenbeck, 2003). The runs ended when the standard deviation of split frequencies reached below 0.01. The first 25% of all generations were discarded as burn-in and a majority rule consensus tree of all remaining trees was calculated to determine the posterior probabilities for individual branches. The resulting trees were visualized using FigTree 1.3.1 (Rambaut, 2009).

RESULTS

Morphological description

Golovinomyces hieraciorum G.-X. Guan & S.-Y. Liu **sp. nov.** (Figure 1).

MycoBank Number: MB10001660

Etymology. Epithet derived from the name of the host genus, *Hieracium*, using the plural genitive, meaning "*Golovinomyces* of *Hieracium* spp."

Typification. on *Hieracium umbellatum*, China. Heilongjiang Province, Heihe City, Sunwu County, the south coast of Xunbiela River, 20 Sep. 2014, Feng-Yun Zhao, Jian Liu, Shu-Rong Tang, Peng-Lei Qiu (holotype, HMJAU-PM91858; isotype, HMAS249776); GenBank number: MZ420204 (ITS+28S). Paratype: Heilongjiang Province, Heihe City, Sunwu County, 2 Sep. 2017, Feng-Yun Zhao, Van-Ninh Nguyen, Jia-Ni Li, Jing-Sheng Lu (HMJAU-PM91859), GenBank number: MZ420205 (ITS+28S).

Description. Mycelium on leaves, amphigenous, and stems, forming irregular white patches or effuse, finally covering whole leaf surfaces, persistent. Hyphae straight to sinuous-geniculate, hyaline, thin-walled, septate, hyphal cells 60–90 µm long and 4–10 µm wide. Later in the season hyphae becoming pigmented, above and all around chasmothecia (secondary hyphae). Hyphal appressoria nipple-shaped, solitary or in opposite pairs, 4–8 µm diam. Conidiophores on the upper surfaces of hyphal mother cells, erect, position usually towards

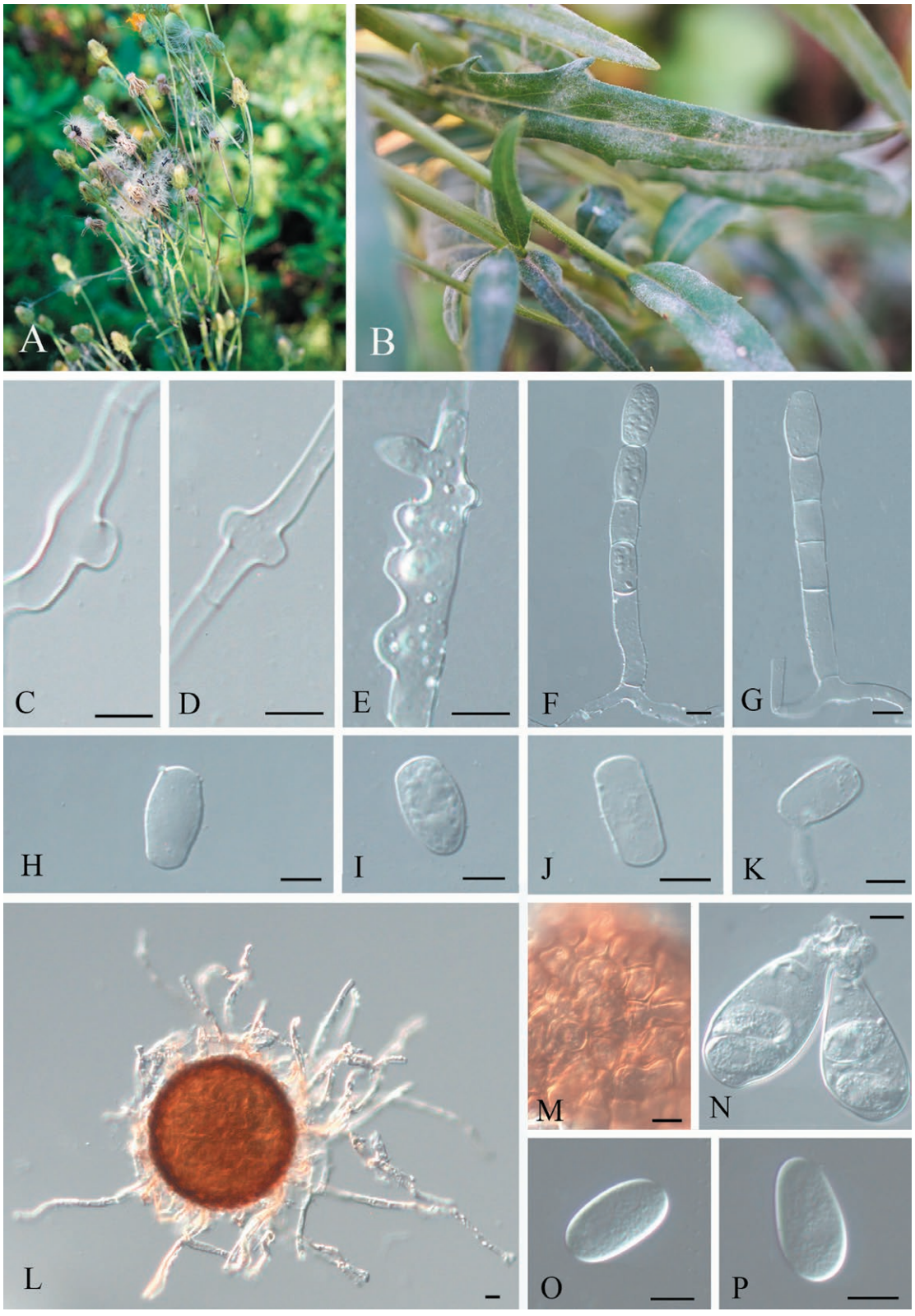

Figure 1. *Golovinomyces hieraciorum* on *Hieracium umbellatum* (HMJAU-PM91858). A and B, symptoms on host plants. C to E, hyphal appressoria. F and G, conidiophores. H to J, conidia. K, conidium with germ tube. L, chasmothecium. M, peridium cells. N, asci. O and P, ascospores. Bars = 10 μm.

one septum of each mother cell, rarely in the middle or almost so, (40–)47–90(–120) × 9–15 µm. Conidiophores foot-cells cylindrical or slightly increasing in width from bases to tops, straight, occasionally somewhat curved at the bases, with basal septae at the junctions (or almost so) with the hyphal mother cells, occasionally slightly elevated (5–10 µm), 22–80 × 8–15 µm, followed by 1–3 short cells. Conidia formation catenescent, outline of conidium chains distinctly sinuate. Conidia ellipsoid-ovoid to doliiform-cylindrical, 22–35(–40) × 11–20 µm, length/width ratio (1.3–)1.5–2.1(–2.7). Germ tubes club-shaped, produced in perihilar positions, short, without swollen tips. *Euoidium type*. Chasmothecia, amphigenous, scattered to gregarious, when mature often surrounded by secondary (pigmented) hyphae, 80–156 µm diam. Peridium cells with irregularly curved-sinuous walls, (7.4–)10–25 µm diam. Appendages numerous, in the lower half of the ascomata, sometimes also in the upper half, mycelioid, simple but rarely irregularly branched and interlaced with each other, 0.2–2.3 times as long as the chasmothecial diam., 3–9 µm wide, septate, walls thin, smooth or somewhat rough, at first hyaline, later brown, paler towards the apices or colourless near the apices, shorter appendages may be brown throughout (differentiation between appendages and secondary hyphae often difficult). Asci 6–16 per chasmothecium, clavate to saccate, (28–)45–75(–80) × (18–)20–40(–45) µm, length/width ratio (1.2–)1.5–2.5(–3.3), usually with numerous small to moderately large oil droplets, sessile to short-stalked, stalks 5–15 µm long, rarely longer, to 25 µm, walls thin, 1–2 µm, terminal oculi inconspicuous, 7–15 µm diam., 2-spored. Ascospores ellipsoid-ovoid, (11–)15–20(–31) × 11–18 µm, length/width ratio 1.0–1.9(–2.3), colourless.

Host range and distribution. *Compositae* (tribe *Lactuceae*, subtribe *Hieraciinae*): On *Hieracium* (*albiflorum*, *alpinum*, *amplexicaule*, *anchusoides*, *argillaceum*, *aurantiacum*, *auricula*, *belonodontum*, *bifidum*, *bombycinum*, *boreale*, *bupleuroides*, *caesium*, *caespitosum*, *canadense*, *carpathicum*, *carpaticum*, *cinerascens*, *cymosum*, *danubiale*, *dentatum*, *echioides*, *fallax*, *flagellare*, *florentinum*, ×*floribundum*, *gentile*, *glaucinum*, *glaucum*, *groenlandicum*, *hryniawiense*, *humile*, *incisum*, *integrifolium*, *jankae*, *juranum*, *kotschyanum*, *lachenalii*, *laevicaule*, *laevigatum*, *lanatum*, *lanceolatum*, *lehbertii*, *leucophaeum*, *longifolium*, *lycopifolium*, *murorum*, *oistophyllum*, *onosmoides*, *pallidum*, *paniculatum*, *pictum*, *pilosella*, *piloselloides*, *pleiotrichum*, *porrifolium*, *praealtum*, *praecox*, *pratense*, *prenanthoides*, *pulmonarioides*, *quercetorum*, *racemosum*, *rapunculoides*, *regelianum*, *robustum*, *rotundatum*, *rubrum*, *sabaudum*, *scabrum*, *schmidtii*, *semisylvaticum*, sp., *staticifolium*, *subinuloides*, *submurorum*, *sylvaticum*, *sylvularum*, *tenuiflorum*, *trachselianum*, *transylvanicum*, *tridentatum*, *tritum*, *umbellatum*, *vagum*, *villosum*, *virosum*, *viscosum*, *vulgatum*), and *Pilosella* (*aurantiaca*, *procera*). Asia (Armenia, China), Europe (Belarus, Czechoslovakia, Denmark, Estonia, Finland, France, Germany, Hungary, Iceland, Italy, Netherlands, Norway, Poland, Romania, Russia, Slovakia, Spain, Sweden, Switzerland, UK, Ukraine, Yugoslavia), and North America (Amano, 1986; Braun, 1995; Farr and Rossman, 2021).

Additional material examined. on *H. amplexicaule*, Germany, Sachsen-Anhalt, Landkreis Jerichower Land, Burg, 10 June 1997, H. Jage (GLM-F-50151); on *H. bombycinum*, Germany, Sachsen, Dresden, Großer Garten, 27 Sep. 1994, F. Klenke (GLM-F-102977); on *H. fallax*, Germany, Sachsen-Anhalt, Landkreis Wittenberg, Kemberg, 25 Aug. 2009, H. Jage (GLM-F-97335); on *H. glaucinum*, Germany, Sachsen, Görlitz, 1 Nov. 2009, H. Boyle (GLM-F-99602); Germany, Sachsen-Anhalt, Landkreis Mansfeld-Südharz, between Questenberg and Hainrode, 15 May 2009, A. Hoch (GLM-F-97576); on *H. lachenalii*, Germany, Sachsen-Anhalt, Landkreis Anhalt-Bitterfeld, Gröbzig, 10 Oct. 2006, H. Zimmermann (GLM-F-95815); on *H. laevigatum*, Germany, Sachsen-Anhalt, Sandersdorf, Stakendorfer Busch, 13 Sep. 1977, U. Braun (HAL 946 F); Germany, Sachsen, landkreis Görlitz, Boxberg, OT Bärwalde, 26 Aug. 2004, H. Boyle (GLM-F-53711); Germany, Sachsen-Anhalt, Eisleben, Steinmetzgrund, 18 Oct. 2001, H. John (GLM-F-54365); Germany, Thüringen, Landkreis Gotha, Friedrichroda, 25 Sep. 2004, H. Jage & H. Boyle (GLM-F-64230); on *H. murorum*, Germany, Sachsen-Anhalt, Landkreis Wittenberg, north west of Oranienbaum, 20 May 2003, H. Jage (GLM-F-63253); Germany, Sachsen-Anhalt, Landkreis Mansfeld-Südharz, Grillenberg, 9 Nov. 2003, H. Jage (GLM-F-63260); on *H. porrifolium*, Slovakia, Žilinský kraj, Okres Bytča, Súľovské vrchy, 26 Jul. 2011, F. Klenke (GLM-F-105347); on *H. racemosum*, Germany, Nordrhein-Westfalen, Mönchengladbach, Schloß Dyk, 6 Sep. 2003, U. Raabe (GLM-F-63139); on *H. sabaudum*, Germany, Sachsen-Anhalt, Dübener Heide, Rotta, 09 Oct. 1976, U. Braun (HAL 953 F), GenBank number: MZ420213 (28S rDNA); Germany, Sachsen, Bautzen, 15 Oct. 2003, H. Boyle (GLM-F-51367); Germany, Sachsen, Landkreis Bautzen, Bischofwerda, 12 Jul. 2007, H. Boyle (GLM-F-81076); Germany, Sachsen, Görlitz, 2 Sep. 2003, H. Boyle (GLM-F- 51329); Germany, Sachsen, Görlitz, 9 Sep. 2003, S. Hoeflich & H. Boyle (GLM-F-51429); Germany, Sachsen, Landkreis Görlitz, Niesky, 16 Nov. 2006, H. Boyle (GLM-F-78755); Germany, Sachsen, Bautzen, 19 Sep. 2006, H. Boyle (GLM-F-74935); Germany, Sachsen, Landkreis Görlitz, Herrenhut, 28 June 2007, H. Boyle

(GLM-F-79431); Germany, Sachsen, Landkreis Görlitz, Seifhennersdorf, 28 June 2007, H. Boyle & G. Zschieschang (GLM-F-79445); Germany, Sachsen, Landkreis Bautzen, Großröhrsdorf, 23 Sep. 2009, H. Boyle (GLM-F-99681); *H. schmidtii*, Germany, Sachsen-Anhalt, Saalekreis, Gimritzer Porphyrlandschaft, 21 Nov. 2007, H. John (GLM-F-94444); on *Hieracium* sp., Germany, Thüringen, Greiz, Cossengrün, 5 Sep. 1999, H. Jage (GLM-F-47535); on *H. virosum*, Russia, Bashkortostan Tujmazinskij Rayon, south west of Tujmazy, Kandrikul Lake, 12 Jul. 1977, U. Braun (HAL 859 F), GenBank number: MZ420206 (ITS+28S); on *Pilosella procera*, China, Xinjiang, Altay, Fuhai, 6 Aug. 1975, Z.Y. Zhao (HMAS39956).

Notes. *Golovinomyces cichoracearum s. lat.* (syn. *Erysiphe cichoracearum*) on *Hieracium* and *Pilosella* spp. is common and widespread in Europe, North America and parts of Asia. *Golovinomyces cichoracearum s. str.* does not occur on *Hieracium* spp. Based on comprehensive phylogenetic examinations, Takamatsu *et al.* (2013) demonstrated that *G. cichoracearum s. str.* is confined to host species of *Scorzonera* and *Tragopogon*. A single re-examined collection on *H. sabaudum* (HAL 953 F) was a mixed infection with *G. hieraciorum* and *Neoërysiphe nevoi*. This is the first report of the *N. nevoi* on *Hieracium*. The asexual stage in this collection was *N. nevoi*, confirmed by ITS rDNA sequence data retrieved from mycelia and conidia, whereas the chasmothecia in this collection were of *G. hieraciorum*, which was also confirmed by ITS rDNA sequences data obtained from chasmothecia.

Golovinomyces cichoracearum on *L. communis* has been reported from numerous European countries (Amano, 1986; Braun, 1995; Farr and Rossman, 2021). In order to clarify the phylogenetic and taxonomic position of this taxon and to find appropriate material for additional sequence analyses, all German powdery mildew collections on *L. communis*, deposited at GLM and HAL, have been re-examined. All of these collections were shown to be either misidentified or were powdery mildew species other than *G. cichoracearum s. lat.*

The following re-examined collections, including some originally misidentified as *G. cichoracearum*, pertain to *Podosphaera ergerontis-canadensis* on *L. communis*. Germany, Hessen, Schwalm-Eder-Kreis, Bad Zwesten, 14 Jul. 2007, C. Klencke (GLM-F-104902). Germany, Sachsen, Landkreis Nordsachsen, Zschepplin, OT Krippehna, 29 Sep. 1997, H. Jage (GLM-F-48602). Germany, Sachsen, Landkreis Nordsachen, Laußig, OT Pressel, 22 Oct. 1998, H. Jage (GLM-F-50206). Germany, Sachsen, Oschatz, park, 8 Oct. 2006, H. Jage (GLM-F-86559). Germany, Sachsen, Landkreis Görlitz, Oberoderwitz, Kirchweg, 19 Jul. 2007, H. Boyle (GLM-F-80873). Germany, Sachsen, Landkreis Görlitz, Niesky, Stannewisch, 5 Jul. 2007, H. Boyle & S. Hoeflich (GLM-F-79525). Germany, Sachsen, Landkreis Görlitz, Boxberg, Uhyst, 20 Oct. 2005, H. Boyle (GLM-F-70244). Germany, Sachsen, Görlitz, 26 Aug. 2005, H. Boyle (GLM-F-70145). Germany, Sachsen, Zittau, Westpark, 9 Aug. 2007, H. Boyle (GLM-F-80893). Germany, Sachsen-Anhalt, Landkreis Börde, north east of Samswegen, Heidberg, 16 Oct. 2006, H. Jage (GLM-F-86402). Germany, Sachsen-Anhalt, Landkreis Wittenberg, Wörlitz, Wörlitzer Park, 16 Nov. 2000, H. Jage (GLM-F-47950). Germany, Sachsen-Anhalt, Burgenlandkreis, Burg Saaleck, 29 Sep. 2003, H. Jage (GLM-F-66171). Germany, Sachsen-Anhalt, Dessau, Kühnauer Park, 13 Sep. 2001, H. Jage (GLM-F-54331). Germany, Sachsen-Anhalt, Burgenlandkreis, Osterfeld, east of OT Waldau, 27 Aug. 2003, H. Jage (GLM-F-63279). Germany, Sachsen-Anhalt, Landkreis Wittenberg, Bleddin, west of Bleddiner Riß, 31 Oct. 2000, H. Jage (GLM-F-48009). Germany, Sachsen-Anhalt, Halle (Saale), Herrmann-Street, 1 Jun. 2020, U. Braun (HAL 3350 F). Other collections (only asexual morphs), mostly identified as *G. cichoracearum*, proved to be *N. nevoi* on *L. communis* [Germany, Mecklenburg-Vorpommern, Usedon, east of Ückeritz, 9 Aug. 1988, H.Jage (GLM-F-58821). Germany, Rheinland-Pfalz, Kreis Cochem, Zell, OT Tellig, 12 Jul. 1999, H. Jage (GLM-F-46819). Germany, Sachsen, Landkreis Nordsachsen, Laußig, OT Görschlitz, 22 June 1997, H. Jage (GLM-F-50126). Germany, Sachsen, Landkreis Nordsachen, Zscheppelin, OT Hohenprießnitz, 10 May 1998, H. Jage *et al.* (GLM-F-49966). Germany, Sachsen, Landkreis Görlitz, Bertsdorf, 31 May 2007, H. Boyle (GLM-F-79245). Germany, Sachsen, Landkreis Görlitz, Johnsdorf, Hänischmühe, 21 June 2007, H. Boyle & S. Hoeflich (GLM-F-79470). Germany, Sachsen-Anhalt, Landkreis Wittenberg, Kemberg, 3 Apr. 1999, H. Jage (GLM-F-46829). Germany, Sachsen-Anhalt, Salzlandkreis, Calbe, 5 May 2001, H. Jage (GLM-F-57042). Germany, Sachsen-Anhalt, Landkreis Börde, Oscherleben, OT Hadmersleben, 12 June 2002, H. Jage (GLM-F-57288). Germany, Sachsen-Anhalt, Halle (Saale), Gustav-Anlauf-Street, 2 Jun. 2020, U. Braun (HAL 3349 F). Germany, Thüringen, Ilm-Kreis, Arnstadt, 5 June 1997, H. Jage (GLM-F-50147)].

Therefore, reports of *G. cichoracearum s. lat.* (syn. *Erysiphe cichoracearum*) on *L. communis* from various countries [Europe (Belarus, England, France, Hungary, Italy, Netherlands, Norway, Poland, Romania, Sweden, Switzerland, Ukraine, United Kingdom, Yugoslavia), Asia (Armenia)] are doubtful, and need to be reviewed and confirmed.

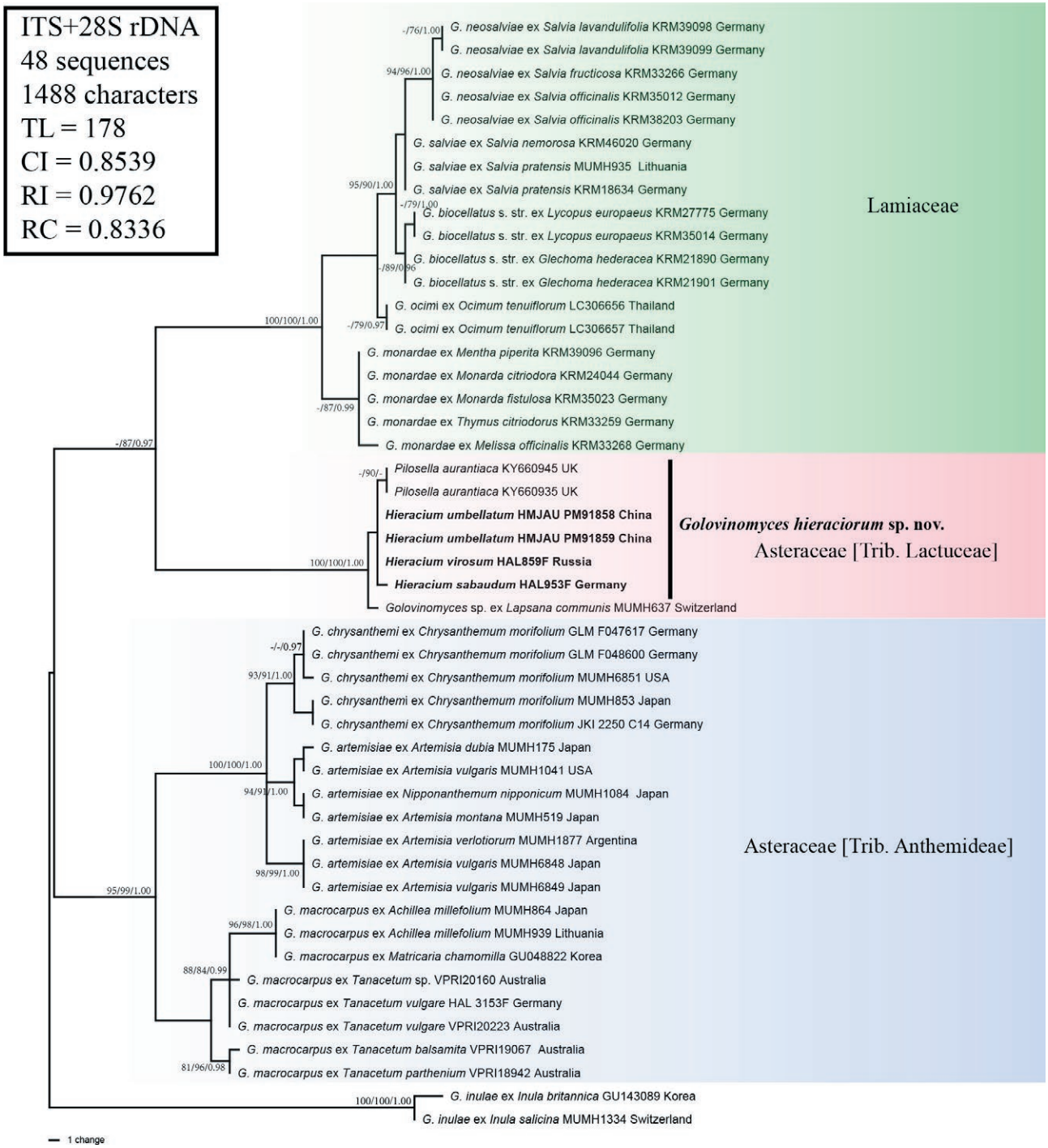

Figure 2. Phylogenetic analysis of the ITS and 28S rDNA regions. The bootstrap support values greater than 75% for maximum parsimony (MP) and maximum likelihood (ML) are displayed followed by posterior probabilities greater than 0.90 for Bayesian Inference (BI). The sequences determined in this study were shown in bold font and pink shade. *Golovinomyces inulae* was used as the outgroup taxon.

Phylogenetic analyses

ITS and 28S rDNA sequences were generated from four *Hieracium* spp. specimens (highlighted in bold font in Figure 2). Newly determined sequences were aligned with other closely related sequences that were retrieved from DNA databases, and were based on Scholler *et al.* (2016), Bradshaw *et al.* (2017), and Meeboon *et al.* (2018).

Golovinomyces inulae was used as the outgroup taxon. The combined dataset of ITS and 28S rDNA sequences consisted of 48 sequences including 1488 characters, of which 108 (7.3%) were parsimony informative and 14 (0.9%) were parsimony-uninformative. The maximum parsimony tree (TL = 178, CI = 0.8539, RI = 0.9762, RC = 0.8336) with the greatest likelihood value is shown in Figure 2. A phylogenetic tree generated from ML and BI analysis was almost identical to the MP tree, so this tree is not presented here.

The tree comprises three groups with high reliability, one comprising isolates from *Compositae* (tribe *Anthemideae*) (MP = 95%, ML = 99%, BI = 1.00), the second representing the *G. biocellatus* complex clade, comprising isolates from *Lamiaceae* with high reliability (MP =100%, ML = 100%, BI = 1.00), and the third was a cluster composed of isolates from *Compositae* (tribe *Lactuceae*) also with high reliability (MP =100%, ML = 100%, BI = 1.00). The second and third groups formed a big clade with high reliability (ML = 87%, BI = 0.97).

Sequences from *Hieracium* spp. and *Pilosella aurantiaca* as hosts are situated in lineages IX, comprising isolates from *Compositae* (tribe *Lactuceae*) with strong reliability (Figure S1). Two sequences from powdery mildew on *Pilosella aurantiaca* formed a subclade with strong BS supports (ML = 90%) (Figure 2).

DISCUSSION

Golovinomyces, with *Euoidium* as the synonymous anamorph genus, currently comprises 71 species and five varieties (Braun and Cook, 2012; Liu and Wen, 2013; Bradshaw *et al.*, 2017; Meeboon *et al.*, 2018; Braun *et al.*, 2019; Qiu *et al.*, 2020). Takamatsu *et al.* (2013) published comprehensive phylogenetic analyses of 33 *Golovinomyces* spp., which were split into 11 genetically distinct lineages. Sequences obtained from seven collections from hosts in tribe *Lactuceae* of the *Compositae* were in two genetically distinct lineages. One was composed of sequences from collections on *Scorzonera* and *Tragopogon* (tribe *Lactuceae*, subtribe *Scorzonerinae*), viz. *G. cichoracearum s. str.*. The second lineage consisted of a single sequence from a collection on *L. communis* (tribe *Lactuceae*, subtribe *Crepidinae*) from Switzerland, which can currently only be referred to as *Golovinomyces* sp. New sequences retrieved from *Golovinomyces* collections on *Hieracium* spp. and *Pilosella aurantiaca* (tribe *Lactuceae*, subtribe *Hieraciinae*) have been generated and added to the current phylogenetic ITS and 28S rDNA analyses (Figure S1, including 43 species of *Golovinomyces*), which significantly increased the *Golovinomyces* lineage. This lineage, with high reliability (MP = 100%, ML = 100%, BI = 1.00), is distant from all other clades within *Golovinomyces*, and remains divided into two clades, viz., the *Hieracium* clades (including *Pilosella aurantiaca*) and a *L. communis* clade. The present study supports previous presumptions of a close co-evolution between *Golovinomyces* species and particular host tribes and subtribes of the *Compositae* (Matsuda and Takamatsu, 2003; Takamatsu *et al.*, 2013; Qiu *et al.*, 2020).

The *Hieracium* clade, represents a new, hitherto undescribed species of *Golovinomyces*, previously hidden within *G. cichoracearum s. lat.*, which is described here as *G. hieraciorum*. The new species is confined to *Hieracium* and *Pilosella* spp. (tribe *Lactuceae*, subtribe *Hieraciinae*), which reflects the close co-evolution of *Golovinomyces* with host tribes and subtribes within the *Compositae*. *Pilosella* is phylogenetically close to *Hieracium* and was previously often treated as a subgenus of *Hieracium*. Fehrer *et al.* (2007) and Krak *et al.* (2013) conducted comprehensive phylogenetic analyses, indicating that *Hieracium* and *Pilosella* should be two distinct plant genera. The phylogenetically proven occurrence of *G. hieraciorum* on *Hieracium* and *Pilosella* spp. reflects the close affinity of these two host genera.

Golovinomyces on *Hieracium* and *Pilosella* spp. is widespread in Europe, North America, and parts of Asia. Besides *G. hieraciorum*, two additional powdery mildews may occur on *Hieracium* spp., viz. *Neoërysiphe nevoi* and *Podosphaera erigerontis-canadensis*. Mixed infections with these two pathogens have to be previously taken into consideration. One collection on *H. sabaudum* from Germany was such a mixed infection of *G. hieraciorum* and *N. nevoi*. This is the first report of *N. nevoi* on *Hieracium*. *Neoërysiphe nevoi* and *G. hieraciorum*, two powdery mildew species on *Compositae*, are easily confusable and not easily distinguishable based on morphology. However, the asci of *Neoërysiphe* spp. remain immature, and ascospores do not develop in 2–8-spored asci until after overwintering. In *Golovinomyces* spp., including *G. hieraciorum*, ascospores mature in the current season and usually develop in 2-spored asci. Furthermore, the hyphal appressoria in *N. nevoi* are lobed, in contrast to nipple-shaped hyphal appressoria on *G. hieraciorum*.

The *L. communis* clade within *Golovinomyces* posed a special problem. *Lapsana* belongs to subtribe *Crepidinae* of tribe *Lactuceae* and is now confined to a single species, *L. communis* (Pak and Bremer, 1995). Attempts failed to solve this problem by tracing additional collections for detailed morphological and phylogenetical examinations of this powdery mildew. Numerous collections on *L. communis* from the herbaria GLM and HAL,

identified as *Erysiphe cichoracearum* or *G. cichoracearum*, have been re-examined, but all were misidentified. Most collections (without chasmothecia) were shown to belong to *N. nevoi* (hyphal appressoria lobed, foot-cells of the conidiophores characteristically short, 20–50 × 9–15 µm, basal septae of conidiophores foot-cells often conspicuously constricted, conidia with striate surfaces [as shown by scanning electron microscopy]). The occurrence of *N. nevoi* on *L. communis* has also been confirmed by sequence analyses and affirmed in the first report of this species on this host from Germany (Schmidt and Braun, 2020). There were even some collections with chasmothecia, originally identified as "*Golovinomyces cichoracearum*", which turned out to be *Po. erigerontis-canadensis* (chasmothecia with single asci). *Podosphaera erigerontis-canadensis* is already known on *L. communis* in Germany (reported in Jage *et al.* (2010), under the name *Po. fusca*). In summary, all collections on *L. communis* assigned to *G. cichoracearum* or *E. cichoracearum*, including those reported in Jage *et al.* (2010), were here shown to be misidentified. Therefore, the identity of the true *Golovinomyces* on *L. communis* could not be clarified in the course of the present study. Records of *G. cichoracearum* and *E. cichoracearum*, respectively, on *Lapsana* from various countries in Europe (Belarus, England, France, Hungary, Italy, Netherlands, Norway, Poland, Romania, Sweden, Switzerland, Ukraine, United Kingdom, former Yugoslavia) and Near East-Caucasus (Armenia) remain unclear and need to be verified.

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Research Papers

Mechanisms of resistance to powdery mildew in cucumber

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Summary. *Podosphaera xanthii* causes powdery mildew of cucumber, and is associated with significant yield and quality losses. Development of resistant or tolerant varieties is the most effective and eco-friendly strategy for powdery mildew management. An important host resistance mechanism is based on the recognition of conserved resistance genes, resulting in durable resistance. To determine powdery mildew resistance mechanisms in cucumber, total RNAs were isolated from the powdery mildew resistant cultivar Meltem, the tolerant line VT18, and the susceptible local variety Camlica. Expression levels of nine genes in these plants were analysed by Reverse Transcription Polymerase Chain Reaction (RT-PCR). The host reactions were assessed using microscope observations of stained specimens. *Serine/threonine* (*STN7*), *transcription factor* (*WRKY22*), *serine/threonine-protein kinase* (*D6PKL1*), and *serine/threonine receptor kinase* (*NFP*) genes were induced, as positive regulators in defence mechanisms against powdery mildew. *Polygalacturonase Inhibitor (PGIP*) did not express after *P. xanthii* inoculation of Camlica, resulting in susceptibility. After inoculation, *callose synthase (CALLOSE)* and *cinnamyl alcohol dehydrogenase* (*CAD*) gene expression levels were increased in resistant Meltem, but Hypersensitive Reaction (HR) and ROS formation were only linked in the tolerant VT18. Powdery mildew development was less in Meltem than in VT18, indicating that cell wall thickening and HR play separate roles in resistance to this disease.

Keywords. Signaling pathway genes, *Podosphaera xanthii.*

INTRODUCTION

Cucumber (*Cucumis sativus* L.) is an economically important vegetable crop belonging to *Cucumis* genus and *Cucurbitaceae* family. The world's annual cucumber production is concentrated in Asia, where Turkey ranks second in production with 1.9 million tons of produce after China contributing about million tons of cucumber production in 2019 (FAO, 2019). Cucumber plants with broader leaves contain a high-water content resulting in their susceptibility to diseases and pests that damage green parts reducing yields. Powdery mildew is caused by the obligate biotrophic pathogen *Podosphaera xanthii*, which is the most common fungal pathogen of cucurbits (Zitter *et al.*, 1996). The most effective and eco-friendly strategy for disease management is the development of resistant host varieties. It is important to under-

stand cucumber genetic and molecular defence mechanisms to develop resistant varieties to powdery mildew.

Characterization of powdery mildew resistant genotypes began with cucumber lines Porto Riko 37 (Kooistra, 1968), PI 197087 (Barnes, 1961), PI 279465 (Fugieda and Akiya, 1962), PI 200815 and PI 200818 (Kooistra, 1968). Six temperature-dependent quantitative trait loci (QTLs) have been identified for resistance to the disease, with a population established using susceptible Santou and resistant PI 197088-1 cucumber lines (Sakata *et al*., 2006). The QTLs *pm5.1*, *pm5.2*, *pm5.3*, and *pm6.1* have been defined using F_2 and F_3 populations of line K8 (Zhang *et al*., 2011), and six QTLs have been mapped on chromosome 4 and *pm5.1* and *pm5.2* major QTLs on chromosome 5 also using F_2 and F_3 populations of line WI2757 (He *et al*., 2013). A total of nine different QTLs were identified for powdery mildew resistance on Recombinant Inbred Lines (RILs) derived from CS-PMR1 × Santou (Fukino *et al*., 2013). Among these, the loci *pm3.1*, *pm5.1*, *pm5.2,* and *pm5.3* were confirmed, and associated with the powdery mildew resistance. Additionally, four different QTLs were found in a RIL population from the PI197088 (resistant) × 'Coolgreen' (susceptible) (Wang *et al*., 2018).

Many genes have been identified for genetic control of powdery mildew resistance in different cucumber cultivars, but the identified QTLs in these studies are inconsistent and uncertain (Chen *et al*., 2020), and the molecular mechanisms underlying powdery mildew resistance are still not clearly understood (Nie *et al*., 2015a). Research on the mechanism of resistance to powdery mildew in cucumber has revealed the host defences at the molecular level (Liu *et al*., 2008). Resistance is associated with cell wall thickening (Nie *et al*., 2015b), reactive oxygen species (ROS) and programmed cell death (PCD) with the expression of pathogen defence proteins (Xu *et al*., 2019). Resistance to powdery mildew in cucumber is genetically complex, cucumber plants can contain many genes and have signal transduction pathways for resistance (Chen *et al*., 2020).

In the present study, responses to powdery mildew in susceptible, tolerant, and resistant cucumber plants were analyzed. For investigation of cucumber-*P. xanthii* interactions, *WRKY*22 transcription factor, *D6PKL*1, *NFP*, and *STN*7 receptors were selected from the Plant-Pathogen Interaction pathway associated with fungal effectors in the Kyoto Encyclopedia of Genes and Genomes (KEGG) Database. Other genes in this study were selected from different plant homologous genes that provide resistance against powdery mildew. After inoculations, target gene expression levels were compared in resistant, tolerant, and susceptible cucumber plants. Additionally, 3, 3'-diaminobenzidine (DAB, Sigma Germany) and trypan blue staining were performed after inoculation to visualize hypersensitive reaction (HR) and ROS accumulation.

MATERIALS AND METHODS

Plant material and Podosphaera xanthii *inoculation*

Previously described powdery mildew resistant *Cucumis sativus* 'Meltem' (resistance score 0.66), tolerant line VT18 (score 1.00), and susceptible local variety Camlica (score 2.75) were used (Yuceson *et al*., 2020). The resistance of these phenotypes was previously scored on a 0 to 4 scale, where 0 is resistant, and 4 is susceptible (Adam and Somerville, 1996). Six seeds from each variety (Meltem, VT18, or Camlica) were germinated and planted separately in pots containing a 1:1 mixture of sterile soil and perlite. Two plants from each variety were inoculated with the *P. xanthii*; and non-inoculated control plants were also used for RT-PCR analyses. The other two inoculated plants of each variety were also assessed visually and microscopically. Also, the remaining plants from each group were used as healthy plants for comparisons with disease symptoms. The *P. xanthii* conidia used for inoculations were collected from infected leaves, and suspended in water 10^6 conidia mL^{-1} (Zhang *et al*., 2021). Plants of the different varieties with first true leaves were inoculated by spraying with conidium suspensions. The experiment was replicated three times.

Trypan Blue, DAB staining and microscope observations

Following the procedures of Thordal-Christensen *et al*. (1997), DAB staining was carried out to determine HR and ROS, and Trypan Blue staining was used to observe of *P. xanthii* structures after inoculation of plant leaves. Inoculated leaves were harvested at 7 dpi; each leaf from each plant group was boiled in 96% ethanol to remove chlorophyll. Approximately 100 µL of trypan blue solution (250 mg L^{-1}) was spread on each microscope slide, and the leaf preparations were viewed with a light microscope (Leica DM 500).

RT-PCR primers

The *Clathrin adaptor complex subunit* (*CACS*) gene and its specific primers (Miao *et al*., 2019) have been used as a housekeeping control gene. For *CACS* prim-

ers, annealing temperature was optimized for RT-PCR. *WRKY*22 transcription factor, *D6PKL*, *NFP*, and *STN7* receptors were selected from Plant-Pathogen Interaction pathway in KEGG Database associated with fungal pathogens. The *PGI*, *CALLOSE*, *GLYK*, and *CAD* genes were selected because homologs of the genes play a role in powdery mildew resistance in different plants. Sequence information of genes were downloaded from Phytozome (https://phytozome-next.jgi.doe.gov). Gene-specific primers were designed using Primer-BLAST (NCBI) software (Table 1).

RNA Isolation and RT-PCR amplification

A 100 mg sample of leaf tissue was collected from the un-inoculated plants before inoculation (0 dpi). At l and 2 dpi, leaves were harvested separately from each host variety (Meltem, VT18, and Camlica). The samples were quickly crushed in liquid nitrogen with a pre-chilled mortar and pestle. Total RNAs were isolated using the GeneJET Plant RNA Purification Kit (Thermo Fisher Scientific), using the manufacturer's protocol. The isolated RNA was quantified with a spectrophotometer, and Verso 1-Step RT-PCR Kit ReddyMix (Thermo Fisher Scientific) was used for RT-PCR amplification. The reaction mixtures were each prepared using 0.25 µL of enzyme mix, 7.5 µL PCR Reddy mix, 7.5 µL distilled nuclease-free water, 0.75 µl RT-enhancer, 1 µL each of forward and reverse *CACS* primers (10 µM), and 20 ng RNA. The RT-PCR has performed with cDNA synthesis at 50°C for 15 min, RT inactivation at 95°C for 2 min and 36 cycles of denaturation at 95°C for 45 sec, annealing at 56°C for 50 sec, and extension at 72°C for 50 sec.

Relative expression assay

After RT-PCR amplification, the amplified products were run on 2% agarose gel using electrophoresis, and were visualized by the gel imaging system, and photographed. The gel electrophoresis analysis was performed by scanning densitometry in GelAnalyzer 19.1 (Lazar *et al*., 2010). The raw data were obtained from band intensity, and the data value was calibrated as an expression unit for calculating relative expression (RE) level. After the calibrations for individual genes, the RE values were calculated using the following equation (Martiansyah *et al*., 2018):

RE = expression unit of target genes/expression unit of *CACS*.

The RE value was accepted as 1 for the housekeeping *CACS* gene, and if the target gene was not amplified in any band, the target gene RE value was accepted as 0.

Table 1. Genes and their primers, associated with host resistance that were used in RT-PCR analyses.

Gene and accession number	Forward and reverse sequence 5'-3'	Product size (bp)
Clathrin adaptor complex subunit CACS (Cucsa.313280)	TGGGAAGATTCTTATGAAGTGC CTCGTCAAATTTACACATTGGT	171
WRKY22 transcription factor WRKY22 (Cucsa.106960)	TAGCCTCTCCGATCCCGAAG CTTCCCCATCTGTCACACCT	218
Thioredoxin F1 THXF1 (Cucsa.356300)	TCGAAGAGTGGCTGCTTCAG GGGTGTACATGTCGAGCACA	211
Polygalacturonase Inhibitor PG1 (Cucsa.038100)	CAATCCCACCCTTTGTGGGT GGTTGGCAAGAGAGCTAGGG	243
Cinnamyl alcohol dehydrogenase CAD (Cucsa.219440)	CACATGGGGGTGAAATTGGC GGATGACCATGGGAGAGACG	289
Serine/threonine-protein kinase STN7 (Cucsa.033920)	AATCCCTGGTGCCATTCTGG GCTATTAGCACAAGCACGCC	310
Callose synthase CALLOSE (Cucsa.249900)	ATAGAGCTACCGGTCGTGGT TGGTTGCAGGCACACCAATA	318
Glycerol kinase GLYK (Cucsa.105620)	CGAGAAATTGGCATCGCAGG CCAAGGGCAGTTGTCTCGAT	363
Serine/threonine receptor kinase NFP (Cucsa.101780)	GGGCAGCTTCTGTTCATTCC AAACCAACACCCAAAGCCAC	509
Serine/threonine-protein kinase D6PKL1 (Cucsa.083190)	TTGCAGCATGGGGTCCTTAG CAGGTAGAAACCTGGGGCTG	631

RESULTS AND DISCUSSION

Inoculations and symptom development

Typical powdery mildew symptoms appeared on cotyledons and leaf surfaces of the susceptible variety Camlica. Each plant was covered by conidia and white mycelia (Figure 1a). Symptoms of the disease were not common in the tolerant line VT18 compared to Camlica (Figure 1 b). The disease symptoms were not observed on the resistant Meltem (Figure 1 c). The inoculated plants developed the same powdery mildew symptoms as previously reported by Yuceson *et al.* (2020).

Microscope observations and hypersensitive response (HR)

After inoculations, HR-associated superoxide accumulated only in the tolerant line VT18 (Figure 2, d e and f). However, powdery mildew growth and sporulation continued on the host leaf surfaces. The HR was not observed in resistant Meltem plants after inoculations (Figure 2, g, h and i). Typical powdery mildew symptoms appeared on Camlica cotyledons and leaves, which were covered with powdery mildew (Figure 1 b). The disease symptoms were not common on the tolerant line VT18 (Figure 1 f), and powdery mildew was not observed on plants of the resistant variety Meltem (Fig-

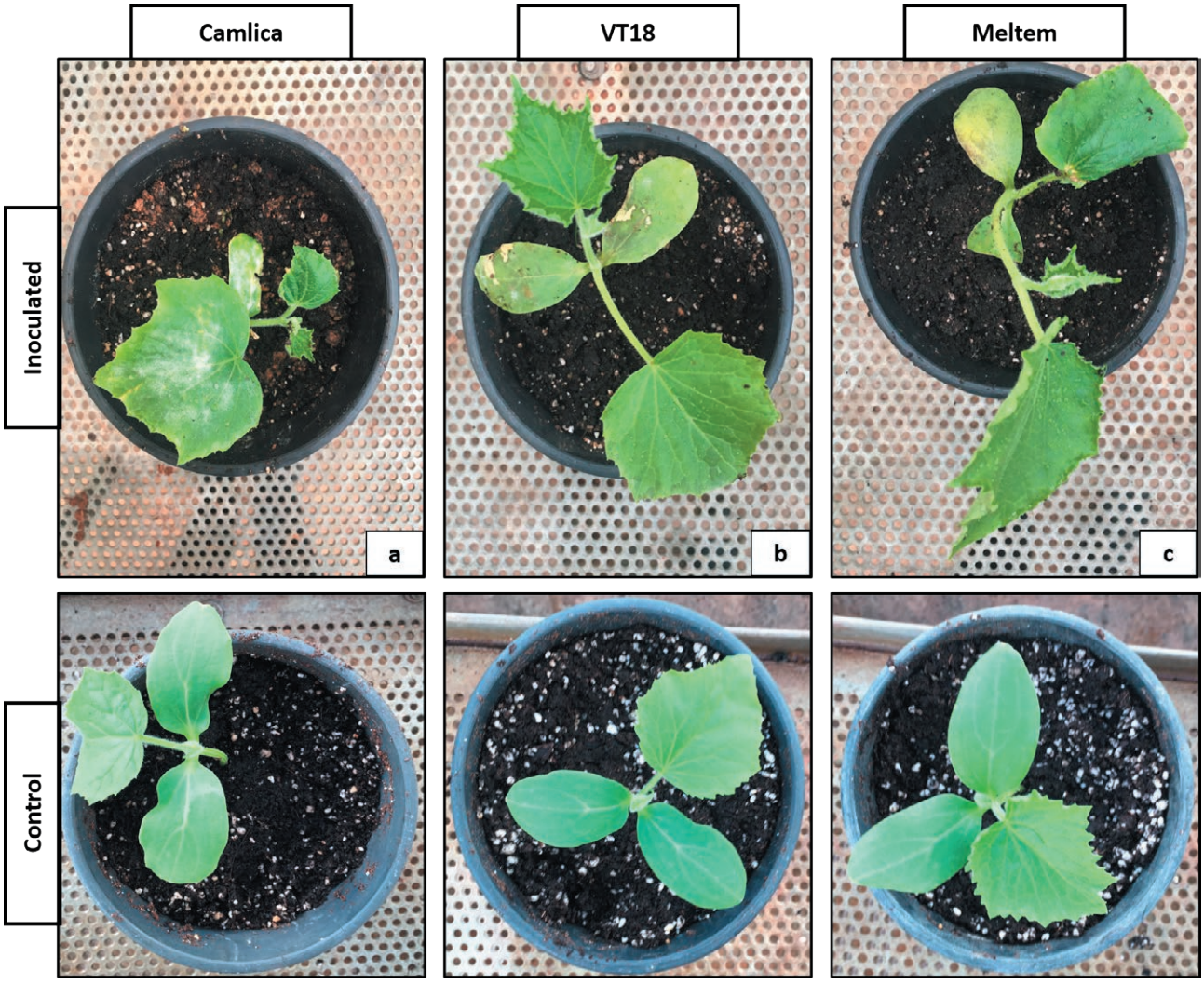

Figure 1. Phenotypes of powdery mildew for inoculated and un-inoculated (Control) cucumber plants of three varieties at 15 days post inoculation.

ure 1 j). These inoculation results were similar to those previously reported by Yüceson *et al.* (2020). The microscope observations with DAB and trypan blue staining showed no association with HR (Figure 2, g, h and i).

RT-PCR and relative expression

The housekeeping *CACS* gene was similarly expressed at the different inoculation time points (0, 1 and 2 dpi) in Meltem, VT18, and Camlica. However, *PGI*, *CALLOSE*, *ThxF1*, *STN7*, *GLYK*, *WRKY22*, *D6PKL1*, *NFP,* and *CAD* genes accumulated in different amounts at these time points for the three host groups (Figure 3). The relative expression levels of the *CACS* gene were obtained after RT-PCR analysis using the GelAnalyzer program (Figure 4).

Transcription factors play regulatory roles in response to different stress conditions (Gao et al., 2020). The *WRKY22* gene has been identified as one of the transcription factors in KEGG database, which affect plant-pathogen interactions. The *WRKY22* showed increased gene expression levels in resistant Meltem plants at 1 dpi (Figure 4 a), but levels decreased after 2 dpi. In the susceptible Camlica plants, the *WRKY22* gene was not expressed (Figure 4g), indicating that *WRKY22* is a candidate gene in the regulation of defense signalling pathways in early stages to powdery mildew infections.

In cucumber plants, the *Thioredoxin* (*ThxF1*) gene regulates accumulation of ROS and controls changing ROS levels, where accumulation of *ThxF1* was previously reported in powdery mildew resistant and susceptible plants (Xu *et al*., 2019). In the present study, the expression level of the *ThxF10* was very high in non-inoculated plants (Figure 4, a, d and g). However, expression of *ThxF10* was not detected in tolerant VT18 plants after 2 dpi (Figure 4, e and f).

The *Polygalacturonase* (*PG1*) gene is a fungus pathogenicity factor allowing decomposition of plant cell wall polygalacturone at early stages of fungal penetration. Conversely, cucumber plants employ *cell wall-binding polygalacturonase inhibitory* (*PGIP*) to limit fungal PG1 enzyme activity (De Lorenzo and Ferrari, 2002). There was no *PGIP* expression in the susceptible Camlica plants at 1 dpi (Figure 4, g and h), but resistant Meltem and tolerant VT18 showed a greater expression at 1 dpi (Figure 4, a and d).

Cinnamyl alcohol dehydrogenase (*CAD*) is synthesized in plants for physical fortification of cell walls

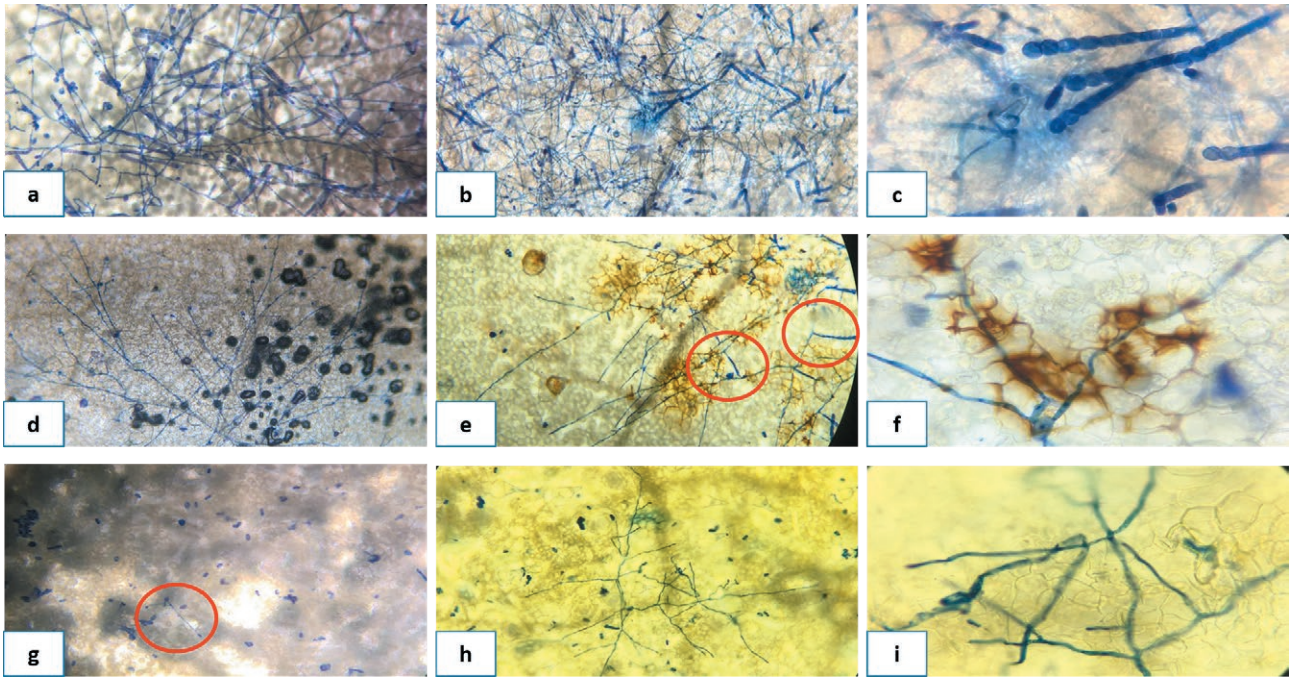

Figure 2. Micrographs of powdery mildew inoculated cucumber leaves with trypan blue and 3,3'-diaminobenzidine (DAB) staining. a, b and c: Growth of mycelia, conidiophores and conidia on Camlica (susceptible) leaf surfaces. d, e and f: Accumulation of superoxide and conidium germination tubes revealed after DAB and trypan blue staining in VT18. Superoxide production was visible as brown-reddish colour in the plant cells. Although conidia germinated and penetrated on the leaf surfaces of resistant Meltem (g, h and j), no new conidiophores or conidia developed, and no superoxide production was associated with conidia (red circle).

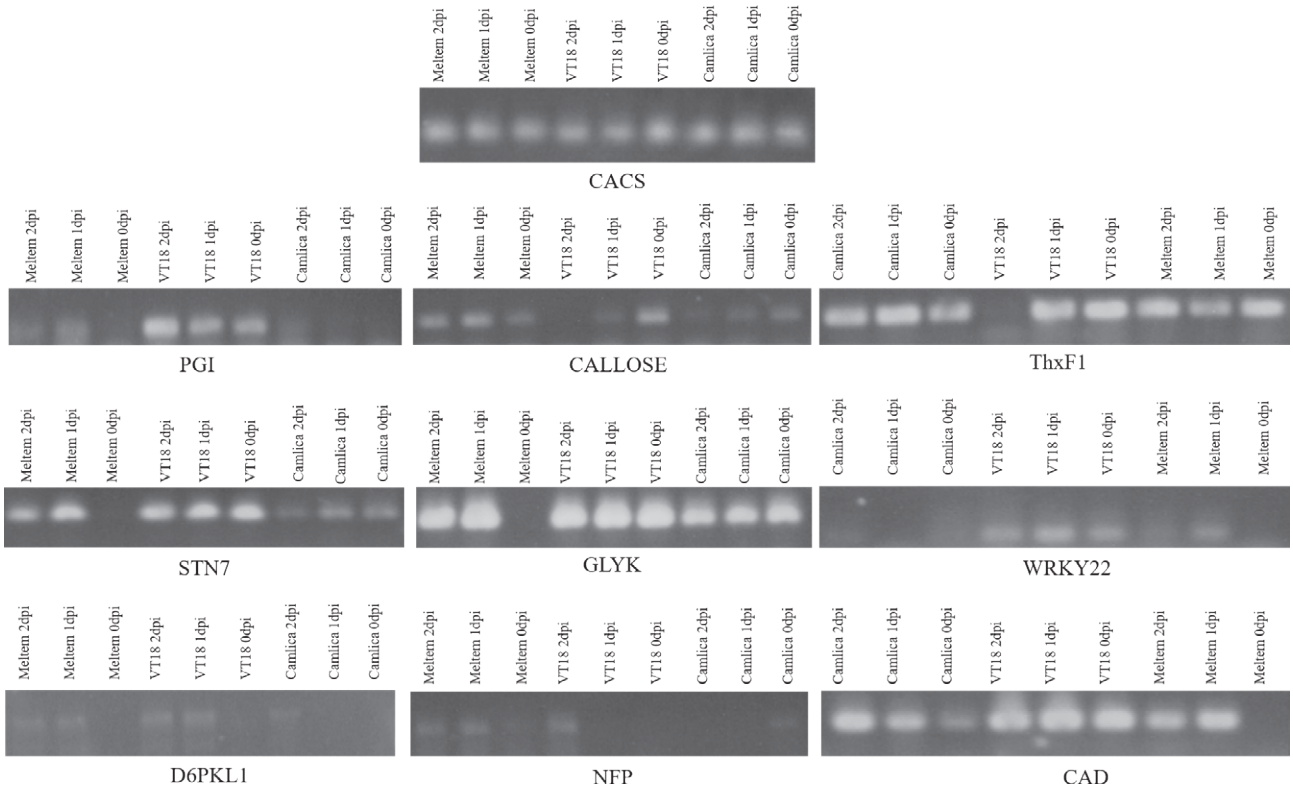

Figure 3. Semi-quantitative RT-PCR analysis results for powdery mildew resistant Meltem, tolerant VT18 and susceptible Camlica cucumber varieties, at 0, 1 or 2 dpi. CACS: *Clathrin adapter complex subunit gene* used as housekeeping gene, PG1: *Polygalacturonase Inhibitor* gene, CALLOSE: *Callose synthase* gene, ThxF1: *Thioredoxin F1* gene, STN7: *Serine/threonine-protein kinase* gene, GLYK: *Glycerol kinase* gene, WRKY22: *WRKY22 transcription factor* gene, D6PKL1: *Serine/threonine-protein kinase* gene, NFP: *Serine/threonine receptor kinase* gene, and CAD: *Cinnamyl alcohol dehydrogenase* gene.

and preventing pathogen penetration. *CAD* has been involved in the synthesis of monolignol, a chemical precursor for lignin biosynthesis (Kim *et al*., 2004). This gene also plays a crucial role in defense mechanisms against bacterial and fungal pathogens (Rong et al., 2016). *CAD* was highly induced in resistant Meltem plants at 1 dpi (Figure 4 a, b), but induction of this gene was reduced in tolerant VT18 at 1 dpi (Figure 4, d and e). The *CAD* expression levels increased in susceptible Camlica (Figure 4, g and h) after powdery mildew inoculations, and expression of this gene was high in susceptible Camlica, tolerant VT18, and resistant Meltem plants after inoculation (Figure 4). Expression of *CAD* in susceptible Camlica, and tolerant VT18 plants showed that this gene did not play a role of in cell wall thickening. However, induction of *CAD* after the inoculation of Meltem could be induced by different genes in other pathways for cell wall thickening, and result in powdery mildew resistance.

Another important gene is *CALLOSE,* usually accumulating during plant growth and development, and in response to different stress conditions. The callose protein plays an essential role in defense against plant pathogens. It is involved in cell wall synthesis and thickening, acting as a physical barrier to slow and prevent pathogen penetration. *CALLOSE* accumulation provides resistance in *Arabidopsis* against powdery mildew (Nauman *et al*., 2013). As in *Arabidopsis*, powdery mildew resistant Meltem plants produced more *CALLOSE* in the inoculated plants (Figure 4, a and b) compared to the susceptible/tolerant plants (Figure 4, d, e, g and h). This indicates that callose expression is an important resistance response in resistant/tolerant cucumber plants.

Serine/Threonine (*STN7*) is another important gene involved in plant-pathogen interactions. Increased expression of *STN7* was measured in resistant Meltem plants at 1 and 2 dpi (Figure 4, a and b) compared to susceptible Camlica plants (Figure 4, g, h and i). There was no difference in the expression of *STN7* between 0 dpi and post-inoculation in VT18 (Figure 4, d, e and f). However, high expression of *STN7* was found in VT18 plants. Thus, *STN7* in resistant Meltem and tolerant

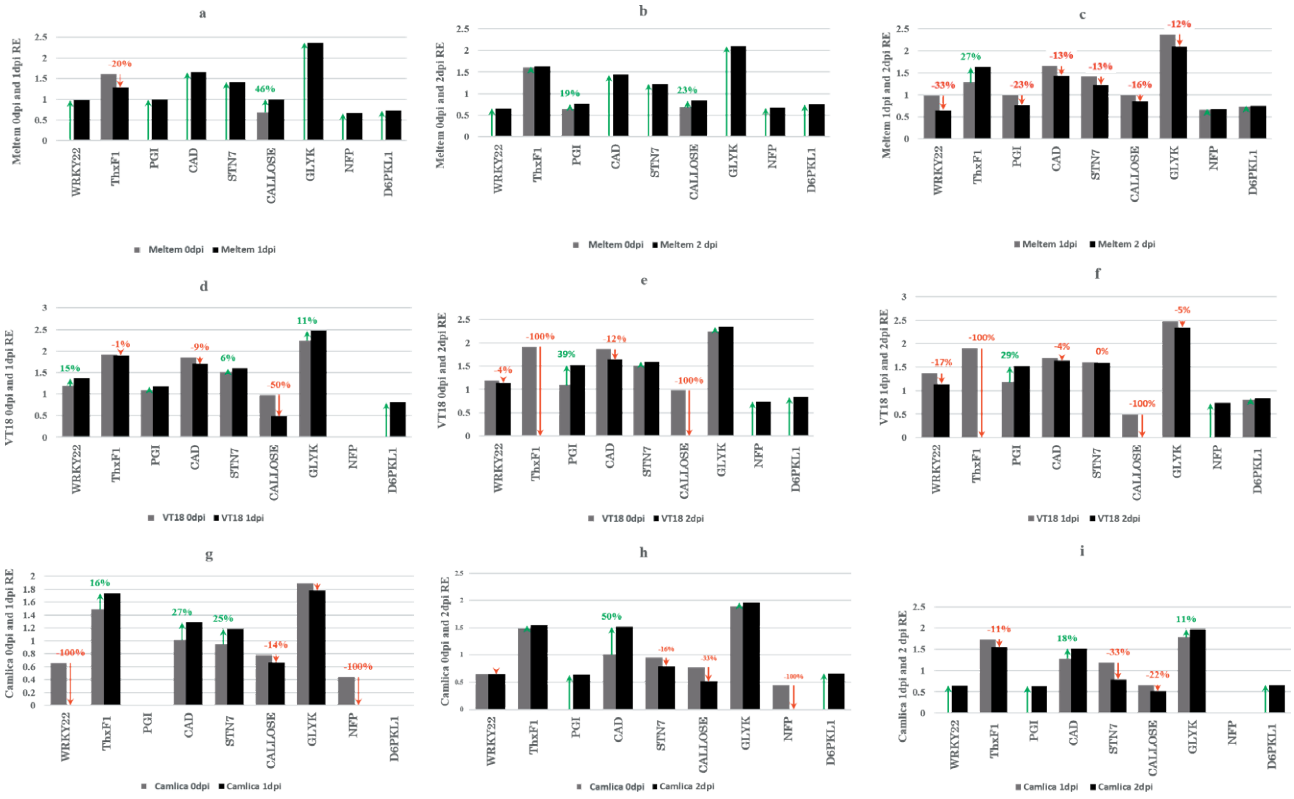

Figure 4. Relative expression values for nine genes, obtained from RT-PCR results using the GelAnalyzer 19.1 program.

VT18 plants indicated that this gene may control resistance in these plants (Figure 4, a and d).

Glycerol kinase (*GLYK*) converts glycerol to glycerol-3-phosphate, and controls resistance in *Arabidopsis thaliana* ecotypes to *Pseudomonas syringae* (Kang *et al.*, 2003). Additionally, glycerol applications on wheat plants induced resistance to the wheat powdery mildew pathogen *Blumeria graminis* f. sp. *tritici* (Li *et al.*, 2020). In the present study, *GLYK* was highly induced in resistant Meltem plants after powdery mildew inoculations (Figure 4, a and b). Expression of *GLYK* was similar at 0 dpi and in inoculated tolerant VT18 and susceptible Camlica plants at 1 dpi (Figure 4, d, e, g and h). These results indicate that *GLYK* could be involved in the functional differences in resistance of the three host groups.

The other key receptor in the KEGG pathway database involved in plant-pathogen interactions is *serine/threonine receptor-like kinase* (*NFP*). This gene was at a greater level in resistant Meltem and tolerant VT18 plants at 1 dpi (Figure 4, a, b, d, and e) than in Camlica. The susceptible plants did not accumulate *NFP* at 1 dpi (Figure 4, g and h). This indicates that *NFP* may have a specific receptor for powdery mildew recognition in resistant plants.

Expressions of the *Serine/Threonine-protein kinase* (*D6PKL1*) gene was increased in Meltem and VT18 plants at 1 dpi (Figure 4, a, b, d, and e). However, there was also a little expression of *D6PKL1* in susceptible Camlica plants. Although expression of this gene increased in Camlica at 2 dpi (Figure 4 h), it was slightly lower than in resistant and tolerant plants. This indicates that *D6PKL1* was a receptor at the penetration stage of *P. xanthii*.

In the present study, powdery mildew resistant Meltem, tolerant VT18, and susceptible Camlica plants were used to provide understanding of the cucumber resistance mechanisms to *P. xanthii*. DAB and trypan blue staining clearly showed that resistance in VT18 plants depends on HR, but this resistance is not associated with HR in resistant Meltem. Instead, the RT-PCR results showed that cell wall thickening associated with *Callose* played an essential role in resistance in Meltem. The study also demonstrated that *PG1*, *NFP*, *STN7* and *D6PKL1* are involved with varying levels of expressions in resistant Meltem, tolerant VT18, and susceptible Camlica plants. *PGI* did not express in susceptible Camlica, indicating that *PGI* expression is crucial for limiting powdery mildew. The cucumber receptors *NFP*, *STN7*,

and *D6PKL*1 can recognize powdery mildew. After recognition, *PGIP* is triggered and restricted pathogen penetration. *PGIP* is essential for host resistance. When this gene was not triggered, powdery mildew developed rapidly, as occurred in susceptible Camlica. Two different host defence mechanisms against powdery mildew were identified; HR and cell-wall thickening. The HR restricted powdery mildew in VT18. However, cell-wall thickening associated *Callose* and *CAD* trigged by the pathogen and highly was expressed in resistant Meltem. Microscope observations showed that *P. xanthii* could not develop conidia although there was no HR in resistant Meltem.

Understanding the different powdery mildew resistance mechanisms in cucumber is important for development of resistant cultivars. Development of new resistant cultivars is difficult because the resistance is complex and polygenic. The present study also showed that cell wall thickening is a more effective defence mechanism in cucumber against *P. xanthii* than HR. Although HR is generally known as a dominant pathogen resistance mechanism, different defence mechanisms could provide resistance against powdery mildew pathogens. Studies are continuing to focus on these HR mechanisms, with the aim of using alternative mechanisms for development of new powdery mildew resistant cucumber cultivars.

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Review

Copper resistance mechanisms in plant pathogenic bacteria

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Summary. Copper is an essential element for microbes as it is involved in many redox reactions. Numerous resistance systems have been evolved in microbes to maintain copper homeostasis under copper stress conditions. These systems are responsible for the influx and efflux of copper ions in the cells. In phytopathogenic bacteria, copper ions play essential roles during disease development in plants. Copper-based chemicals are extensively used for control of diseases caused by bacteria, which leads to induced pathogen resistance derived from various copper resistance systems. Previous studies have shown that copper ions are harnessed by host plants to protect against bacterial infections, triggering immune responses through activation of defence signalling pathways. Thus, it was anticipated that bacterial copper resistance could play an alternative role in adaptation to plant immunity. This review summarizes current knowledge of copper resistance systems in plant pathogenic bacteria, which may provide a new perspective of molecular mechanisms associated with bacterial adaptation in host plants.

Keywords. Resistance systems, stress conditions, signalling pathways, bacterial copper resistance, plant immunity.

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INTRODUCTION

In nature, plants are always under threats from pests and pathogens. Pathogenic bacteria are a major cause of diseases in diverse plants, resulting in negative effects on plant growth and crop yields.

Utilization of copper is essential in living organisms. Due to easy conversion process between reduced Cu(I) and oxidized Cu(II) with low energy consumption, copper serves as a cofactor for many key enzymes that are involved in essential biochemical and physiological processes, including electron transport, oxidative stress response, denitrification, respiration and photosynthesis (Arredondo and Nunez, 2005; Turski and Thiele, 2009; Festa and Thiele, 2011; Argüello *et al*., 2013; Rensing and McDevitt, 2013;). However, copper ions are toxic when exceeding a threshold value within cells (Adrees *et al*., 2015; Husak *et al*., 2018; Kalita *et al*., 2018). The toxicity mechanisms have been attributed to generation of highly reactive hydroxyl radicals via Fenton and Haber-Weiss reactions (Liochev and Fridovich, 2002), affecting

biomolecules such as peptides, DNA, and lipids (Freinbichler *et al*., 2011). Excess copper can also bind to adventitious sites in proteins, disrupting protein structure and inactivating function through displacement of native metal ions (Keyer and Imlay, 1996; Macomber and Imlay, 2009). Organisms have developed complex resistance mechanisms to deal with deleterious copper-induced reactions while satisfying supply for intracellular copper-requiring biological processes.

The copper (Cu) resistance system was initially discovered in *E. coli* and has been widely studied. *Escherichia coli* has evolved two chromosomal encoded *cue* and *cus* systems and a plasmid-encoded *pco* system to resist copper stress (Argüello *et al*., 2013; Bondarczuk and Piotrowska-Seget, 2013; Solioz, 2018). Many copper based bactericides and fungicides have been used in agriculture over a period of time such as Bordeaux mixture, which is the sixth highest selling product in this regard (Cha and Cooksey, 1991). Cu^{2+} was found to be an integral component that impairs protein activity by damaging nucleic acids ultimately, leading to the suppression of microbial activity (Zhang *et al*., 2018). Experimental pieces of evidence also showed that a low concentration of copper ions could effectively protect the plants against bacterial infection by activating defense signaling pathways (Liu *et al*., 2015). For instance, the ethylene (ET) biosynthesis pathway, which is involved in plant immunity, is induced by Cu^{2+} in Arabidopsis (Liu and Zhang, 2004). Cu^{2+} repressed the expression of genes *StABA1* and *StNCED1* for abscisic acid (ABA) biosynthesis, eliciting ET-dependent immunity against bacterial and fungal pathogens (Liu *et al*., 2020). Additionally, copper composites have been used as an effective treatment against bacterial spot disease, as copper composites improve the efficacy of metallic copper by reducing particle aggregation providing a strong shield against bacterial speck (Strayer-Scherer *et al*., 2018).

However, copper resistance has evolved in phytopathogenic bacteria due to extensive use of copper-based bactericides for plant disease control. Since the first description of the copper-inducible system in *Pseudomonas syringae* pv. tomato (Pst) (Cooksey, 1987), many copper resistance systems have been identified in numerous plant-pathogenic species of *Pseudomonas* (Cazorla *et al*., 2002; Gutiérrez-Barranquero *et al*., 2013; Colombi *et al*., 2016), *Xanthomonas* (Lee *et al*., 1994; Behlau *et al*., 2011, 2012, 2013), *Pantoea* (Nischwitz *et al*., 2007) and *Erwinia* (Al-Daoude *et al*., 2009; Águila-Clares *et al*., 2018). Although there are some homologous copper resistance genes between *E. coli* and plant pathogenic bacteria, they differ in gene size, genetic organization and molecular regulation. Thus, plant-pathogenic bacteria have evolved different copper response mechanisms due to diverse living conditions, host stresses, and adopted ecological niches. In the present review, the molecular mechanisms related to copper resistance developed by plant-pathogenic bacteria are summarized, with emphasis on *Pseudomonas* and *Xanthomonas* species. It suggested that some mechanisms are unique in plant pathogenic bacteria, and some occur in *E. coli* and plant pathogens.

COPPER RESISTANCE SYSTEMS IN *PSEUDOMONAS SYRINGAE*

Pathovars of *P. syringae* are plant pathogens that can severely damage their hosts. Long-term utilization of copper compounds for control of these pathogens has resulted in the generation of copper resistant strains, compromising the efficacy of copper (Sundin *et al*., 1989; Zhang *et al*., 2017). In Pst, four copper response genes, namely, *copABCD* are localized within a 35-kb pPT23D plasmid controlled by one promoter which is specifically induced by copper ions (Cooksey, 1987; Cha and Cooksey, 1991). These genes have similarities to corresponding *pco* genes in *E. coli* (Silver and Walderhaug, 1992).

CopA, a 72 kDa periplasmic protein, shares similarity with multicopper oxidase CueO from *E. coli* (Arnesano *et al*., 2002). The particular motifs rich of methionine, histidine, and aspartic acid have enabled CopA protein to bind up to 11 copper ions (Cha and Cooksey, 1991; Cooksey, 1993). Compared with CueO in *E. coli* combined with five copper ions, high copper-binding capacity and oxidase activity give CopA with major role in sequestration and detoxification in copper resistance.

CopB is an outer membrane protein containing numerous methionine residues that can combine copper ions, but the copper binding ability of CopB has not yet been proven (Arnesano *et al*., 2002; Puig *et al*., 2002; Zhang *et al*., 2006).

CopC is a 10.5 kDa soluble molecule with a β-barrel structure. This protein comprises two completely different but interdependent binding sites for, respectively, reduced Cu(I) and oxidized Cu(II). In periplasmic space, copper ions probably substitute two sites due to change in oxidation state (Zhang *et al*., 2006). CopC has been proposed to function as a redox switch to maintain free copper ion concentrations at sub-picomolar levels. When a Cu(II) site is empty, the Cu(I) ion is oxidized by air, but when both binding sites are occupied, no oxidation occurs, showing that CopC acts as a Cu chaperone in oxidizing periplasm, potentially interacting with its neighbor proteins (Zhang *et al*., 2006). A hypotheti-

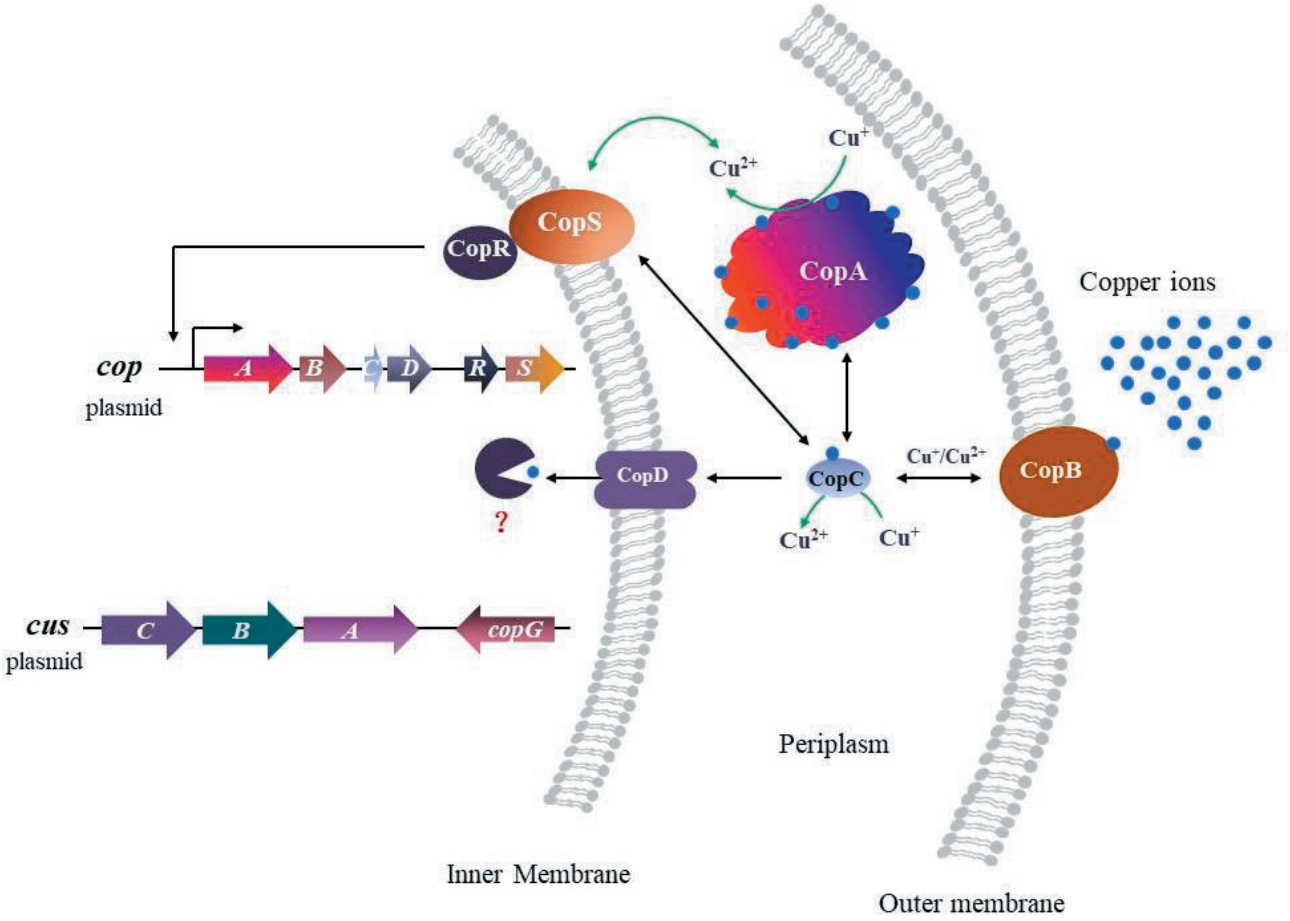

Figure. 1. Proposed model of encoded proteins involved in copper resistance in *Pseudomonas syringae*. Arrows indicate interactions between proteins. CopR induces the expression of *copABCD* via CopS which detects excess periplasmic copper. CopA sequesters excess periplasmic copper due to its strong binding ability; CopB combines with copper; CopC transfers copper from CopA to CopD, and CopD then transports copper into bacterium cytoplasm. CopC also functions as a redox switch to maintain free copper ion concentrations in solution at sub-picomolar levels. The structure of the *cus* operon and *copG* in plasmids of *P. syringae* pv. syringae is a novel efflux system, but the location of these proteins in cell has not been identified.

cal model has been suggested that CopC interacts with CopA and/or CopB to detoxify excess copper (Arnesano *et al.*, 2002) (Figure 1).

CopD is a 33 kDa protein located in plasma membranes, and contains eight predicted transmembranous helices and some conserved histidine residues (Arnesano *et al.*, 2002). CopD transports essential copper ions delivered by CopC through inner cell membranes into the cytoplasm to balance the abundant periplasmic copper sequestered from CopA and CopB (Cooksey, 1993; Arnesano *et al.*, 2002). CopD and CopC are mutually involved in copper uptake in cytoplasm resulting in increased copper accumulation and copper sensitivity (Arnesano *et al.*, 2002).

Except for an induction by high levels of copper ions, transcription of the *copABCD* operon requires a two-component regulatory system CopRS. *CopRS* genes are the downstream components of *copABCD* operon with similar transcriptional orientation but constitutive expression (Mills *et al.*, 1993). CopS acts as a sensor kinase that traverses cytoplasmic membrane and detects copper concentration in the cell periplasm. When the copper ion binds to CopS, a conserved histidine residue is autophosphorylated. Upon phosphorylation of conserved aspartic acid residue, CopR consecutively activates the expression of *copABCD* (Cooksey, 1993; Mills *et al.*, 1993).

Although copper resistance genes in *P. syringae* pv. syringae (Pss) isolated from mango trees can hybridized to *copABCD* DNA, the homologues to *copABCD* are present in a 62-kb plasmid, showing rich diversity (Cazorla *et al.*, 2002). Further studies detected a novel plasmid

structure located between *copABCD* and *copRS* has been detected, that encoded the efflux system *cusCBA* and a putative metal transporting P-type ATPase *copG* in 62-kb plasmid (Gutiérrez-Barranquero *et al.*, 2013). This arrangement has also been observed in other *P. syringae* pathovars affecting different hosts in several countries, and with high sequence similarity (Renick *et al.*, 2008; Studholme *et al.*, 2009; Cai *et al.*, 2011). The genetic organization is involved in the increase of copper resistance in Pss strain (Gutiérrez-Barranquero *et al.*, 2013). In a recent study, a new Tn*7* transposon containing copper resistance genes (COARS Tn*7*-like) has been localized in the chromosome of Pss strains from mango trees. This new COARS Tn*7*-like was found to confer high levels of resistance against copper sulphate that could probably be due to the continuous application of copper. *P. fluorescens* and *Pseudomonas syringae* pv. actinidiae (Psa) also possess the same genomic sequence of COARS Tn*7*-like transposon (Aprile *et al.*, 2020). In addition, the model Pss strain B728a, responsible for brown spot of bean, contains the *copABCD* operon in its chromosome. Pss strain B728a is an epiphyte that feeds on the surface of leaves, from where it can colonize the plant and behave as a pathogen (Vaughn and Gross, 2016).

Zhang *et al.*, (2017) assessed copper resistance in *P. syringae* pv. phaseolicola (Psp) responsible for halo blight disease in beans. Bacterial populations on liquid NB media indicated that 28 out of 35 (80%) strains of this pathogen were resistant to copper, and the bacterial population was similar to that grown on casitone-yeast extract (CYE) agar. Both types of strains have an adequate rate of copper i.e., 161 mg mL^{-1} $CuSO_4$, indicating that CYE agar containing copper can be used for rapid evaluation of copper resistance in this pathogen. Further experiments showed that addition of mancozeb enhanced the effectiveness of copper hydroxide against Psp strain, as mancozeb elevates the solubility of fixed copper.

Psa, causal agent of kiwifruit canker disease, was found to be resistant against copper through integrative conjugative elements (ICEs) and plasmids. Further analyses showed that Psa strains containing genes *czc/cusABC* and *copABCD* were not only resistant to copper but also resistant against arsenic and cadmium. Out of seven strains examined, five showed resistance to copper encoded by ICEs lying at different positions in the Psa genome (Colombi *et al.*, 2017). In general, *P. syringae* pathovars respond to copper stress mainly through sequestration and compartmentalization of the element in cell periplasm and outer membranes (Cha and Cooksey, 1991; Cooksey, 1994). This mechanism is different from the *cue* and *cus* system in *E. coli*, which exhibits resistance by pumping and reducing cellular accumulation of copper (Rensing and Grass, 2003; Bondarczuk and Piotrowska-Seget, 2013). However, the *cus* system in *P. syringae* has an additional efflux mechanism (Gutiérrez-Barranquero *et al.*, 2013). Pathovars of *P. syringae* have evolved a complex response and detoxification system to deal with copper stress in natural environments.

COPPER RESISTANCE SYSTEMS IN *XANTHOMONAS*

Three distinct copper resistance systems have been detected in plant pathogenic—*Xanthomonas*, including a well-known copper-inducible chromosomal *cohABCD* system and a plasmid-born *copLAB* system. The *cusAB/smmD* system similar to that of *Stenotrophomonas maltophilia*, has been discovered from the plasmids of *Xanthomonas* strains, including *X. citri* subsp. *citri*, *X. gardneri,* and *X. euvesicatoria* (Richard *et al.*, 2017). The plasmid-encoded *cop* genes play dominant roles due to the presence of chromosomal copper resistance genes in copper sensitive *Xanthomonas* and *Pseudomonas* strains (Cooksey *et al.*, 1990; Lim and Cooksey, 1993; Behlau *et al.*, 2011). Since copper resistance systems in xanthomonads vary among different species and strains, current understandings of *X. arboricola* pv. juglandis, *X. axonopodis* pv. vesicatoria, and *X. citri* subsp. *citri* is summarized below.

Xanthomonas arboricola *pv.* juglandis

The chromosomal *cohABCD* operon has been fully elucidated in *X. arboricola* pv. juglandis C5, which is the homologous system of *copABCD* in *P. syringae*. CohA protein shares 65% similarity with CopA from Pst, and contains three highly conserved regions of multicopper oxidase. Similarly to CopA, CohA has been proposed to bind four copper ions due to the presence of only one tandem repeat of MX_2MXHX_2M (Lee *et al.*, 1994). Although CohB and CopB share 45% similarity of amino acid sequences, the N terminus of CohB has a hydrophilic region while, CopB contains a hydrophobic region. CohA is a cytosolic protein and CohB has been detected only in cytoplasmic membranes, showing distinctive differences from their homologues in Pst (Teixeira *et al.*, 2008). *CohAB* are essential for copper resistance, while *cohABCD* are required for complete resistance to copper. Inactivation of *cohAB* in other *Xanthomonas* strains supported this conclusion (Teixeira *et al.*, 2008).

The plasmid-borne *copLAB* gene cluster has also been identified from Italian strains of *X. arboricola* pv. juglandis by PCR amplification (Giovanardi *et al.*, 2016).

The sequence of *copA* shares 78% similarity with *cohA* in the chromosomal *cohABCD* operon. In contrast, neither *copL* nor *copB* exhibited sequence similarity with any gene member of the *cohABCD* operon. This variance in copper resistance gene organization within one same species indicated that the genetic basis for copper resistance varies at the intraspecific level.

Xanthomonas axonopodis *pv.* vesicatoria

The *copLAB* resistance system was first detected in the plasmid of *X. axonopodis* pv. vesicatoria 7882. The gene cluster (based on DNA or amino acid sequence) was different from common copper resistance systems in pseudomonads and *E. coli*. CopL, a 122 amino acid protein, exhibited a regulatory role required for *copA* induction under copper stress, since knock-out *copL* resulted in a complete loss of copper-dependent transcription of *copA* (Voloudakis *et al*., 2005). CopL is rich in histidine and cysteine residues that can bind to copper ions. However, expression of *copL* is copper-independent, and is transcribed at the lowest level dominated by a constitutive promoter lacking strong ribosome binding sites (Voloudakis *et al*., 2005). *CopLAB* is widely distributed in the plasmids of *Xanthomonas* from different world regions (Behlau *et al*., 2011; Richard *et al*., 2017), and has also been found on the chromosome of a few *Xanthomonas* strains and *Xylella fastidiosa*, regardless of their copper sensitivity or resistance (Simpson *et al*., 2000; da Silva *et al*., 2002; Potnis *et al*., 2011; Kong *et al*., 2018). *CopL* has been found to be the least conserved *cop* gene in previously sequenced xanthomonads (Behlau *et al*., 2013). However, *copA* is the most conserved copper resistance gene in xanthomonads that have been extensively studied. Several amino acids deletion mutation could cause copper sensitivity (Kong *et al*., 2018). *CopB* is also probably not as important as *copA*, because disruption of *copB* did not result into complete removal of copper resistance (Behlau *et al*., 2011).

Copper resistance in *X. axonopodis* pv. vesicatoria was regarded as only plasmid-born (Bender *et al*., 1990; Garde and Bender, 1991), until a unique chromosomal copper resistance gene cluster was identified in *X. axonopodis* pv. vesicatoria strain XvP26 (Basim *et al*., 2005). Five open reading frames (ORFs), ORF5, ORF4, ORF3, CopR, and CopS are sequentially arranged in this cluster. Total genomic DNA digests of XvP26 could not be hybridized by the *cop* gene cluster in *X. campestris* pv. vesicatoria as indicated using Southern hybridization analysis. CopR, ORF3, and ORF4 are major determinants for complete resistance to copper. CopR contains a conserved palindrome copper box motif, which is essential for copper-inducible activity at the *pcoA* promoters in *E. coli* (Rouch and Brown, 1997). Although defect in the *copS* gene showed no effect on copper resistance, ORF3 failed to respond to copper induction (Basim *et al*., 2005). Integrity of *copS* played an essential role in completing the two-component signal transduction system in *X. campestris* pv. vesicatoria. The CopRS two component regulatory system, has only been found in *X. axonopodis* pv. vesicatoria XvP26, and not in other *Xanthomonas* strains (Basim *et al*., 2005).

Xanthomonas citri *subsp.* citri

A more complicated *copLAB* gene cluster was identified in the *X. citri* subsp. *citri* A44 plasmid (Behlau *et al*., 2011). Compared the *copLAB* operon in *X. arboricola* pv. juglandis and *X. axonopodis* pv. vesicatoria, *copMGCDF* have been identified as the downstream of *copLAB* genes in *X. citri* subsp. *citri* A44. *CopLAB* are the most important genes essential for copper resistance in *X. citri* subsp. *citri* A44, while *copMGCDF* displayed a dose-dependent effect (Behlau *et al*., 2011). CopL possibly regulated *cop* gene expression by interacting with the intergenic region between *copL* and *copA* (Behlau *et al*., 2012). It has been assumed that CopM is a cytochrome c oxidase involved in electron transport, CopG is a hypothetical export protein, CopC and CopD are transmembrane transporter proteins, and CopF is a putative copper-transporting p-type ATPase (Behlau *et al*., 2011). However, elucidation of the specific functions requires more experimental evidence.

In *X. citri* subsp. *citri* strain LM199, the *copLAB* amplicon could not be detected by PCR. The *copABCD* copper resistance system is plasmid-derived, showing more than 97% similarity with the chromosomal *cohABCD* system of *X. arboricola* pv. juglandis (Pereira *et al*., 2015). Although it lacks the two-component regulator *copRS*, a MerR transcriptional regulator, which controls the transcription of proteins CopA and CueO in *E. coli* (Stoyanov *et al*., 2001; Sameach *et al*., 2017), was found close to the *copABCD* cluster (Richard *et al*., 2017). The genetic arrangement and composition of homologues of these plasmidic and chromosomal copper resistance genes in different *Xanthomonas* strains is illustrated in Figure 2.

An HME-RND system *cusAB/smmD* was identified in the plasmid of several *X. citri* subsp. *citri* strains from Réunion, Martinique and Argentina, which showed more than 95% amino acid similarity to RND efflux pumps of *S. maltophilia* isolated from the citrus phyllosphere (Crossman *et al*., 2008). The components of the HME-RND system in *X. citri* subsp. *citri* contain a CusA inner membrane pump, a CusB periplasmic protein

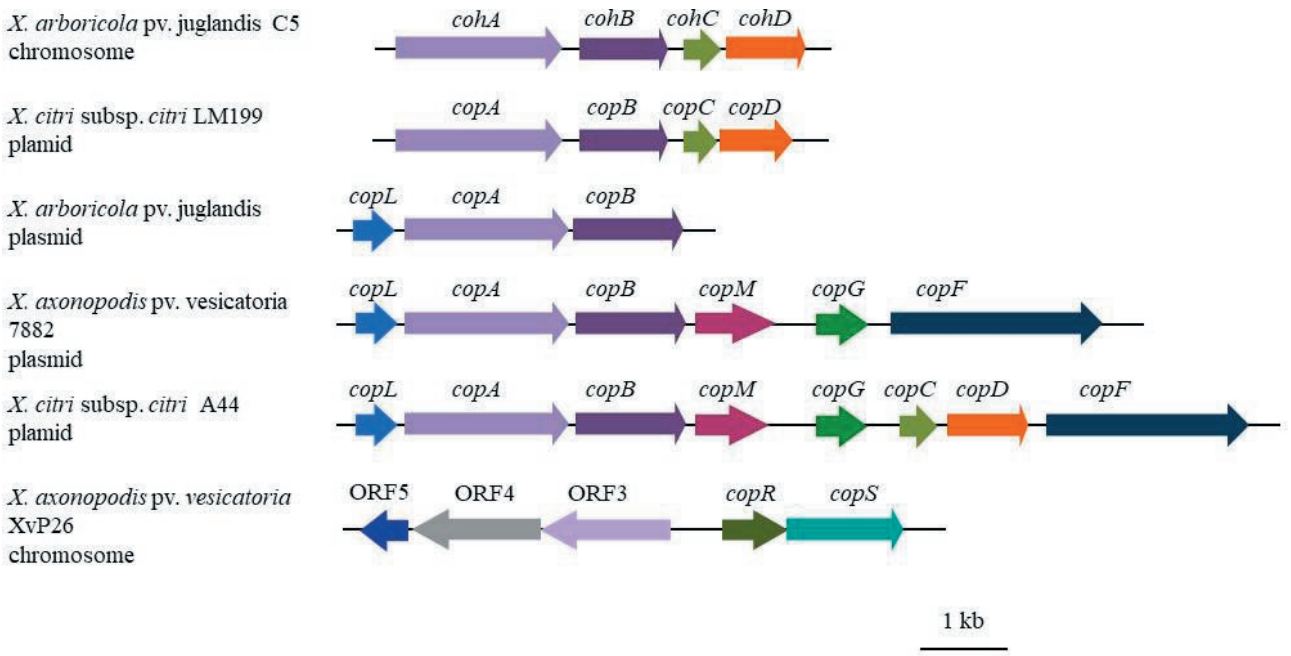

Figure. 2. The genetic arrangement and composition of copper resistance genes in *Xanthomonas* strains. Areas with the same colour indicate homologous genes among the strains. Chromosome indicates the corresponding gene cluster located in the chromosome. Plasmid represents the gene cluster present in a plasmid.

and an SmmD outer membrane protein (Richard *et al.*, 2017). However, *cusAB*/*smmD* is not widely distributed among xanthomonads, and only exists in copper resistance *X. citri* susp. *citri*, *X. gardneri, X. euvesicatoria* and *X. vesicatoria* ATCC 35937 (Richard *et al.*, 2017).

In a Chinese *X. citri* subsp. *citri* strain 29-1, deletion mutation of the conserved membrane protein gene *XAC1347* led to reduced resistance to copper ions (Guo *et al.*, 2015; Fan *et al.*, 2018). *XAC1347* is low in cysteine and methionine residues with no histidine residue, implying that *XAC1347* has little ability to bind copper ions. This indicates this gene may play a role in maintaining cell integrity and osmotic balance (Cybulski and de Mendoza, 2011; Kar *et al.*, 2017). The two-component regulator *colRS* regulates the expression of *XAC1347*, and is involved in copper resistance in *X. citri* subsp. *citri* (Yan and Wang, 2011; Fan *et al.*, 2018). As it is distributed in all known *X. citri* subsp. *citri* strains, this gene could be a universal mechanism required for copper osmotic balance by this pathogen.

ACQUISITION OF COPPER RESISTANCE GENES BY PLANT PATHOGENIC BACTERIA

As a result of continuous application of copper-based chemicals, there has been widespread emergence of copper resistant pathogens (Sundin *et al.*, 1989; Behlau *et al.*, 2013; Colombi *et al.*, 2016). In Florida, nearly 100% of *X. euvesicatoria* and *X. perforans* strains were found to be resistant to copper, due to 50 years of application of copper-based chemicals (Pohronezny *et al.*, 1992). Eighty percent of Psp populations in commercial snap bean fields have become copper resistant (Zhang *et al.*, 2017). In contrast, all *X. campestris* pv. vitians strains causing foliar disease in lettuce were sensitive to copper due to less use of pesticides based on the element (Pernezny *et al.*, 1995). Comprehensive research is required on the origins of copper resistance acquired by plant pathogenic bacteria.

Under selection pressure caused by extensive use of copper-based chemicals, plasmid-born copper resistance genes are responsible for developing copper-resistant bacteria in the field. This can be attributed to horizontal gene transfer (HGT), which usually occurs through plasmids conjugation and bacteriophages transduction (Popa and Dagan, 2011; Achtman, 2012; Sen *et al.*, 2013; Hobman and Crossman, 2015). Comparative genomics and phylogenetic network analyses support the acquisition of copper resistance systems through plasmid incorporation by *X. citri* subsp. *citri* populations (Richard *et al.*, 2017; Gochez *et al.*, 2018). *In vitro* conjugation studies of copper resistance determinants that substituted intra- and inter-specifically within plant-pathogenic bacteria

confirmed this conclusion (Sundin *et al*., 1989; Behlau *et al*., 2012). In addition, copper resistance genes from plasmids of phyllosphere microorganisms can be expressed in *Xanthomonas*, although they have not been found in *Xanthomonas* species. This showed that a broad range of copper resistance gene sources is available for HGT in nature.

In addition to plasmid conjugation, copper resistance genes are possibly acquired through the uptake of integrative conjugative elements (ICEs). In the kiwifruit pathogen *P. syringae* pv. actinidiae, acquisition of Psa NZ45ICE_Cu by a copper sensitive strain Psa NZ13 was detected *in vitro* and *in planta* (Colombi *et al*., 2016). As well, several genomic islands, including genes of plasmid origin, were detected on the chromosome of *X. citri* subsp. *citri* (Gordon *et al*., 2015). This evidence supports the conclusion that HGT is the most important process for copper resistance evolution.

CONCLUSIONS AND PERSPECTIVES

Long-term use of copper-based bactericides has led to the increased populations of copper resistant phytopathogenic bacteria. Detailed studies on structure and function of copper resistance systems may allow rational development of new bactericides that inhibit these systems. However, extensive research is required to achieve this goal. The cytoplasmic copper chaperone responsible for the transportation and detoxification of copper ions has yet to be identified. The present has attempted to encapsulate research progress on copper resistance systems in model bacteria, including *Pseudomonas* and *Xanthomonas*. Although the systems have some similarities with those in *E. coli*, biochemical characteristics and crystal structures of various proteins, and the regulatory networks that control the expression are different in both *Pseudomonas* and *Xanthomonas*.

Formulations of copper complexed with heptagluconic acid induce innate plant immunity, and could be used as an alternative treatment against bacterial attack (González-Hernández *et al*., 2018). Considering the existence of plasmid-borne copper resistance systems, the most effective chemical disease control method could be strict adherence to appropriate dosage and frequency of copper sprays, to reduce the probability of transferring copper resistance genes within and between phytopathogenic bacteria. Use of copper composites against copper tolerant strains can also lessen the chances of bacterial resistance as they do not accumulate in soil or water and exhibit higher antimicrobial activity. Following best cultural practices and incorporating biopesticides in copper composite mixture can minimize the chances of bacterial resistance. Overall, rational studies on evolution of copper resistant phytopathogenic bacteria can lead to design more effective formulations of copper-based chemicals and control strategies that could limit the resistance to copper.

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Research Papers

Characterization of *Colletotrichum* strains associated with olive anthracnose in Sicily

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Summary. Anthracnose caused by *Colletotrichum* spp. is the most damaging olive fruit disease in many countries, including Italy. This disease has been sporadically detected in Sicily, but new agronomic practices can increase risk of olive anthracnose in this region. An etiological study of the disease focused on local olive cultivars growing at the International Olive Germplasm Collection (IOGC) in Villa Zagaria, Enna, Sicily has been undertaken. During 2018 and 2019, 137 *Colletotrichum* strains were isolated from olives. Colony morphology, conidium characteristics, and multilocus sequence analyses aided identification of three species: *C. acutatum* (affecting 70% of symptomatic olives), *C. gloeosporioides,* and *C. cigarro*. Three *C. acutatum* strains (B13-16, P77, and P185), and one strain of each *C. gloeosporioides* (C2.1) and *C. cigarro* (Perg6B) were evaluated for pathogenicity on olive fruits from 11 Sicilian cultivars, known for their high-quality oil. Differences in virulence were detected among strains and their pathogenicity to the cultivars. The *C. acutatum* isolates were more virulent than those of *C. gloeosporioides* or *C. cigarro*. The Sicilian olive cultivars Cavaliera, Carolea, Calatina, and Nocellara del Belice were the most susceptible to the pathogen, while the cultivars Biancolilla and Nocellara Etnea were the most tolerant. Cultivar response under field conditions showed that anthracnose severity and fruit-rot incidence were positively correlated. This is the first report of *C. acutatum* and *C. cigarro* affecting olive trees in Sicily. Control measures for anthracnose depend on accurate characterization of the etiological agents and host cultivar resistance.

Keywords. Pathogenicity, multilocus analyses, cultivar susceptibility, phylogenetic tree.

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INTRODUCTION

Italy ranks second (after Spain) in olive oil production, with average production of 400,000 tonnes per year (ISTAT, 2021). Olive oil production in

Sicily was 16% of the total Italian yield in 2020 (ISMEA, 2020). In this region, olive trees (*Olea europaea* subsp. *europaea* L.) have been widely cultivated since ancient times, and wild olives are important components of the Mediterranean scrub vegetation. In this part of Italy, due to favorable growing conditions for olives, high intracultivar variations have been accumulated (Muzzalupo *et al.*, 2011). Most olive oils produced in Sicily are extra virgin, with eight recognized as Protected Designation of Origin (PDO) and 28 certified as Protected Geographical Indication (PGI).

Olive anthracnose, caused by *Colletotrichum* spp., is the most damaging olive fruit disease (Moral *et al.,* 2021). These pathogens cause rot and drop of mature drupes, chlorosis and necrosis of leaves, and dieback of twigs and branches (Cacciola *et al.*, 2012). Olive anthracnose epidemics are affected by the autumn rainfall, inoculum density, fruit maturity, and susceptibility of host varieties (Moral and Trapero, 2012). Oil extracted from olives affected by *Colletotrichum* spp. has off-flavours associated with reddish colour and high acidity (Moral *et al.*, 2014; Peres *et al.*, 2021).

Eighteen *Colletotrichum* species have been associated with olive trees (Talhinhas *et al.,* 2018; Moral *et al.,* 2021). These fungi have endophytic to necrotrophic lifestyles and are characterized by a high phenotypic and genotypic diversity (Moral *et al.*, 2009; Talhinhas *et al.*, 2011; Cacciola *et al.*, 2012; Talhinhas and Baroncelli, 2021). The *Colletotrichum* species associated with olive were primarily classified as *C. acutatum* and *C. gloeosporioides* species complexes (Talhinhas *et al.*, 2018). Subsequently, phylogenetic studies have identified numerous species in the *C. acutatum* complex associated with the disease. These include *C. acutatum sensu stricto* (hereafter *C. acutatum*), *C. fioriniae, C. nymphaeae*, *C. simmondsii, C. godetiae,* and *C. rhombiforme* (Mosca *et al.*, 2014; Chattaoui *et al.*, 2016; Talhinhas *et al.*, 2018). Among these species, *C. acutatum* has been described in many Mediterranean countries, including Portugal, Italy, Morocco, Greece, Tunisia, and Egypt (Talhinhas *et al.*, 2005; Rhouma *et al.*, 2010; Embaby *et al.*, 2014; Chattaoui *et al.*, 2016; Msairi *et al.*, 2017; Iliadi *et al.*, 2018).

The *C. gloeosporioides* species complex includes 22 species and one subspecies, although there is little information on the identity of isolates obtained from olive fruit (Weir *et al.*, 2012). Although *C. gloeosporioides* and *C. theobromicola* species complexes have been reported as causal agents of olive anthracnose, they have not been associated with disease outbreaks (Agosteo *et al.*, 2002; Talhinhas *et al.*, 2009). Five additional species (*C. aenigma, C. kahawae* subsp. *cigarro, C. karstii, C. queenslandiicum*, and *C. siamense*) in the *C. boninense* and *C. gloeosporioides* species complexes have also been identified as pathogenic to artificially inoculated ripe olive fruit (Mosca *et al.*, 2014; Schena *et al.*, 2014). *Colletotrichum kahawae* subsp. *kahawae* and subsp. *cigarro* have been separated into two different species, respectively *C. kahawae* and *C. cigarro comb. et stat. nov.* (hereafter *C. cigarro*). This was based on morphological, biochemical, and molecular data (Cabral *et al.*, 2020). Similarly, *C. helleniense* has been described as a new species, phylogenetically close but clearly differentiated from *C. kahawae* (Guarnaccia *et al.,* 2017).

Colletotrichum species have caused several olive anthracnose epidemics in Italy since the 1940s. During the last two decades, the disease has been reported in new olive production areas in southern Italy, mainly caused by *C. godetiae* (syn. *C. clavatum*) (Agosteo *et al.*, 2002; Faedda *et al.*, 2011; Mosca *et al.*, 2014; Schena *et al.*, 2014). Olive anthracnose was first reported in Sicily by Graniti *et al.* (1954). Many years later, *C. gloeosporioides* was associated with the disease in this region (Cacciola *et al.*, 2012). Although many *Colletotrichum* spp. have been identified affecting different plants in Sicily (Faedda *et al.*, 2011; Polizzi *et al.,* 2011, Aiello *et al.*, 2015; Ismail *et al.*, 2015; Guarnaccia *et al.*, 2017), knowledge of the *Colletotrichum* spp. in Sicilian olives is inconsistent and unclear. As well, susceptibility of the local varieties to these fungi is unknown. Increased risk of olive anthracnose is expected because new olive plantations include susceptible foreign cultivars (e.g., 'Arbequina'), and intensive agronomic practices (high plant density and irrigation) (Moral and Trapero, 2012) are increasingly used. It is therefore important to gain knowledge of the main *Colletotrichum* species present in olives in Sicily, and of the susceptibility to anthracnose of local olive varieties.

The objectives of the present study were: i) to accurately identify the main species of *Colletotrichum* causing anthracnose of olive in Sicily, using multilocus phylogenetic analyses; and ii) to characterize the virulence of these fungi, and determine the susceptibility to them of 11 traditional Sicilian olive cultivars.

MATERIALS AND METHODS

Sampling and isolation of fungi

During December 2018 and 2019 anthracnose-affected fruits were collected in the International Olive Germplasm Collection (IOGC) at Villa Zagaria (lat. 37°30'52''N; long. 14°17'46''E), where the incidence of anthracnose was approx. 10%. The IOGC is located close to the Pergusa Lake (c. 650 m above sea level), and

includes 400 olive accessions distributed in four plots: 53 accessions of local (Enna Province) cultivars, 45 accessions from other Sicilian Provinces, 180 from different regions of Italy, and 126 international cultivars. The Enna Province has a dry sub-humid Mediterranean climate (Thornthwaite index) with most rainfall occurring from September to February each year. The olive trees were planted in 2004 and are occasionally treated with copper-based fungicides.

Isolations of *Colletotrichum* spp. were made from olive fruit showing symptoms of anthracnose. Small portions of the mesocarp were removed from affected fruit areas, and were then surface sterilized in a sodium hypochlorite solution (10%) for 30 s. Following three rinses with sterile water, the pieces were plated onto Potato Dextrose Agar (PDA) amended with 100 μg mL^{-1} streptomycin, and were then incubated at 25°C for 10 d. Cultures were also established from fruit surface acervuli onto Petri plates containing PDA.

Colletotrichum cultures on PDA were visually classified into different groups according to their morphological characteristics (Table 1). Single conidium cultures were used for sequencing target genes and pathogenicity tests. All the strains were stored in the CREA - Centro di Ricerca di Olivicoltura, Frutticoltura e Agrumicoltura (Italy, Acireale) culture collection.

Amplification and sequencing of target genes

Partial regions of four loci from eight isolates were amplified from lysed mycelium after heat treatment (100°C for 10 min) (Supplementary Table S1). The primers ITS1 and ITS4 were used to amplify the internal transcribed spacer region (ITS) of the nuclear ribosomal RNA operon, including the 3' end of the first internal transcribed spacer region, the 5.8S rRNA gene, the second ITS region, and the 5' end of the 28S rRNA gene. For each isolate, the partial glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was amplified using primers GDF1 and GDR1 (Guerber *et al.,* 2003). The primers ACT-512F and ACT-783R were used to amplify part of the actin gene (*ACT*), while the partial beta-tubulin (*TUB2*) gene was amplified with primers T1 and Bt-2b (Guarnaccia *et al.*, 2017). As well, a 900 bp intron of the glutamine synthetase (GS) gene was amplified and sequenced from isolate Perg6B using primers GSF1 and GSR1 (Guerber *et al.*, 2003), and submitted to GenBank under the accession number MW053386.

The PCR amplification mixtures and cycling conditions for the four loci were as described by Damm *et al.* (2012). Briefly, the PCRs were performed in a Veriti 96 wells Thermal Cycler (Applied Biosystems) in total volumes each of 25 μL. The ITS, GAPDH, ACT and TUB2 PCR mixtures each contained 1 μL of lysed mycelium, 0.2 μM of each primer, 1 × PCR DreamTaq Green PCR Master Mix (Thermo Scientific Inc.), and 1.26 μL DMSO (not added in the ITS mixture). Amplification conditions constituted an initial denaturation step of 5 min at 94°C, followed by 40 cycles each of 30 s at 94°C, then 30 s at 52°C for GADPH, 55°C for TUB2, 58°C for ACT, or 56°C for ITS, and 30 s at 72°C, and a final elongation step of 7 min at 72°C. The PCR products were enzymatically purified by Exosap-IT™ PCR product clean-up Reagent (Thermofisher Scientific Inc.), and were sequenced in both directions by Microsynth®. Generated DNA sequences were analysed, and consensus sequences were computed using the software MEGA X (Kumar *et al.*, 2018).

Table 1. Strains of *Colletotrichum* from different Sicilian olive cultivars used in the present study.

Isolate	Species	Host cultivar	Isolation matrix
P77	*Colletotrichum acutatum*	Giarraffa	Mesocarp
P185	*C. acutatum*	Vaddarica	Single acervuli
B13-16	*C. acutatum*	Biancolilla nana	Mesocarp
33B	*C. acutatum*	Brandofino	Mesocarp
177B	*C. acutatum*	Zaituna	Single acervuli
237-5	*C. acutatum*	Cipressino	Mesocarp
105-6B	*C. acutatum*	Nocellara del Belice	Mesocarp
105-AC4	*C. acutatum*	Nocellara del Belice	Single acervuli
Perg6B	*C. cigarro*	Biancolilla	Mesocarp
C2.1	*C. gloeosporioides*	Carolea	Mesocarp

Phylogenetic analyses

The new sequences generated in this study were blasted against the NCBI's GenBank nucleotide database to determine the closest sequences for a taxonomic framework. The sequences were aligned using MEGA X (Kumar *et al.*, 2018) and trimmed according to nucleotide length with sequences retrieved from GenBank based on recent studies (Damm *et al.*, 2012; Weir *et al.*, 2012; Guarnaccia *et al.*, 2017; Baroncelli *et al.*, 2017) (Supplementary Table S1). Phylogenetic analyses were carried out individually for each locus (data not shown), and the four loci combined using Bioedit v7.0 to establish isolates at the species level. Two separate analyses were carried out, one for strains belonging to the *C. acutatum* species complex and another for the *C. gloeosporioides* species complex. The multigene analysis of the *C. acutatum* species complex consisted of 47 sequences,

including the outgroup sequence of *C. orchidophilum* (CBS 632.80). Isolates belonging to the *C. gloeosporioides* complex were grouped in a combined phylogeny consisting of 35 sequences, including the outgroup sequence of *C. orchidophilum* (CBS 632.80). A maximum parsimony analysis was performed for each gene, and the multilocus alignment (ITS, *TUB2, GAPDH,* and *ACT*), using MEGA X. Tree length (TL), consistency index (CI), composite index (CI), and Retention Index (RI) were calculated for parsimony. The bootstrap analysis was based on 1000 replications. Sequences generated in this study were deposited in GenBank (Supplementary Table S1).

Pathogenicity and cultivar susceptibility tests on fruit

Pathogenicity tests were carried out using olive fruit of 11 different Sicilian cultivars, and 'Frantoio' used as a resistant control cultivar (Tables 2 and 3). Fruit samples collected at the onset of ripening from healthy olive trees were used, since unripe fruits are unhelpful to discriminate the phenotypic reaction of the olive cultivars against the pathogen (Moral *et al.*, 2008). The fruits were washed, surface sterilized with a 10% solution of commercial bleach (50 g L^{-1} Cl) in sterile water for 1 min. They were then rinsed twice with distilled water, allowed to air dry, and were stored at 4°C in plastic containers until use (less than 7 d). Fruits were inoculated by spraying conidium suspensions of five representative strains of *Colletotrichum* using the methods described by Moral *et al.* (2008). The conidium suspensions were each adjusted to 10^5 conidia mL^{-1}, and sterile water was used as experimental controls. Inoculated and control fruits were incubated in moist chambers (plastic containers, each 22 × 16 × 10 cm, with 100% RH) at 23 ± 2°C under fluorescent lights (12 h photoperiod of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$). There were three replicates (moist chambers) per treatment and ten fruits per replicate. The experiment was conducted twice. Disease severity was assessed every 5 d for 20 d, using a 0 to 5 scale based on symptoms affecting the fruit surfaces where 0 = no visible symptoms, 1 = symptoms affecting less than 25% of the fruit surface, 2 = 25 to 50%, 3 = 51 to 75%, 4 = 76 to 100% of surface affected but with little pathogen sporulation, and 5 = fruit completely rotted showing abundant conidia in gelatinous matrices (soapy fruit) (Moral *et al.*, 2008). Disease index (DI) was calculated using the following formula:

$$DI = (\Sigma n_i \times i)/N$$

where i is the severity score (0 to 5), n_i is the number of fruits with the severity of i, and N is the total number of inoculated fruits.

For each treatment and repetition, the Area Under Disease Progress Curve (AUDPC) was calculated as the area under the DI curve over time, according to Moral *et al.* (2008). Fungi were re-isolated from inoculated fruits onto PDA, and the resulting colonies were compared with the inoculated isolates.

Cultivar responses under field conditions

Fruit-rot incidences of 20 olive cultivars maintained in the IOG Collection at Villa Zagaria were assessed in December 2019. Eighteen cultivars were selected among the IPG Sicilian varieties, and the cultivars Cipressino (susceptible) and Frantoio (resistant) were used as comparisons (Moral *et al.,* 2017). Each cultivar included four replicated olive trees. Olive fruits were harvested at different ripening stages, from green (colour class = 0) to black (colour class = 4), according to Moral *et al.* (2008). The incidence (%) of fruit with unequivocal anthracnose symptoms was quantified in a sample of 300 fruits per cultivar, under laboratory conditions. Disease severity was assessed using the 0 to 5 severity scale described above.

Data analyses

Two-way and one-way ANOVA were performed on AUDPC data, and cultivar means were compared using the Student-Newman-Keuls test at $P \leq 0.01$. A heatmap with dendrograms was displayed using the severity of the disease as dependent variability, to examine similarities between olive cultivars or *Colletotrichum* strains. Disease incidence (%) for each olive cultivar in the orchard trial was compared with the response of the same cultivar in artificial inoculations using the Pearson's correlation test. In addition, a Zar's test of multiple comparisons of these proportions was carried out to examine the effect of the cultivar on the fruit rot incidence (Zar, 2010). Relationships between fruit rot incidence and ripening were analyzed using Pearson's correlation coefficient. Relationship between fruit rot incidence and disease index were assessed using different linear and non-linear models using exponential and potential equations (Campbell and Madden, 1990). The best model was selected based on the statistical significance of the estimated parameters ($P \leq 0.05$), Mallow's Cp statistic, Akaike's information criterion, and the coefficient of determination (R^2). Data were analyzed using Statistix 10 (Analytical Software) and R Studio.

RESULTS

Morphological characteristics of Colletotrichum *spp. colonies*

A total 137 *Colletotrichum*-like colonies were isolated from symptomatic olive fruits and were divided into two groups according to their morphological traits. Ninety-six of the strains (70%) had colonies with regular margins and dense cottony and white or pale salmon aerial mycelium with pinkish spore masses. Within this group, the isolates P77, P185, B13-16, 105B, 177B, 33B, 105-AC4, and 237-5 had conidia that were aseptate, hyaline, and with fusiform ends (Table 1, Figure 1), and these isolates were classified as *C. acutatum* species complex. The second group included 15 strains (10%) characterized by colonies with dark olive-grey mycelium, many orange and black acervuli, and concentric dark circles on the reverse sides (Table 1, Figure 1). Conidia of these strains were aseptate, hyaline, and each slightly constricted in the middle and rounded at each end, and the strains were classified as *C. gloeosporioides* species complex. Among this species complex, the strains Perg6B and C2.1 were selected for further studies. In addition, colonies of *Fusarium lateritium*, *Alternaria* spp., *Phoma foliofoma*, and *Septoria protearum* were occasionally isolated from diseased fruits.

Phylogenetic analyses

Eight representative strains of the two *Colletotrichum* species complexes were selected for further characterization by phylogenetic analyses and taxonomy (Supplementary Table S1). The four phylogenetic trees, derived from single gene sequence alignment for *C. acutatum* and *C. gloeosporioides* species complexes, were topographically similar to those previously reported (Damn *et al.*, 2012; Weir *et al.*, 2012; Baroncelli *et al.*, 2017). These trees confirmed that strains P77, P185, B13-16, 105B, 177B, and 237-5 belong to the *C. acutatum* species complex, while strains Perg6B and C2.1 belong to the *C. gloeosporioides* species complex.

A total of 1469 characters (ITS: 1–469, TUB2: 477–980, GAPDH: 988–1217, ACT: 1225–1469) were analyzed to assess phylogeny of the *C. gloeosporioides* species complex. Of these, 291 were parsimony-informative, 507 were variable and parsimony-uninformative, and 916 were conserved. A maximum of 1000 equally most parsimonious trees were saved (TL = 866, CI = 0.6348, RI = 0.8052, CI = 0.5988). Strain Perg6B clustered within the *C. kahawae/C. cigarro* phylogenetic group. According to nucleotide sequence analysis of the glutamine synthetase gene, isolate Perg6B unequivocally belonged to *C. cigarro*, due to the presence of a 22 bp insertion, which was

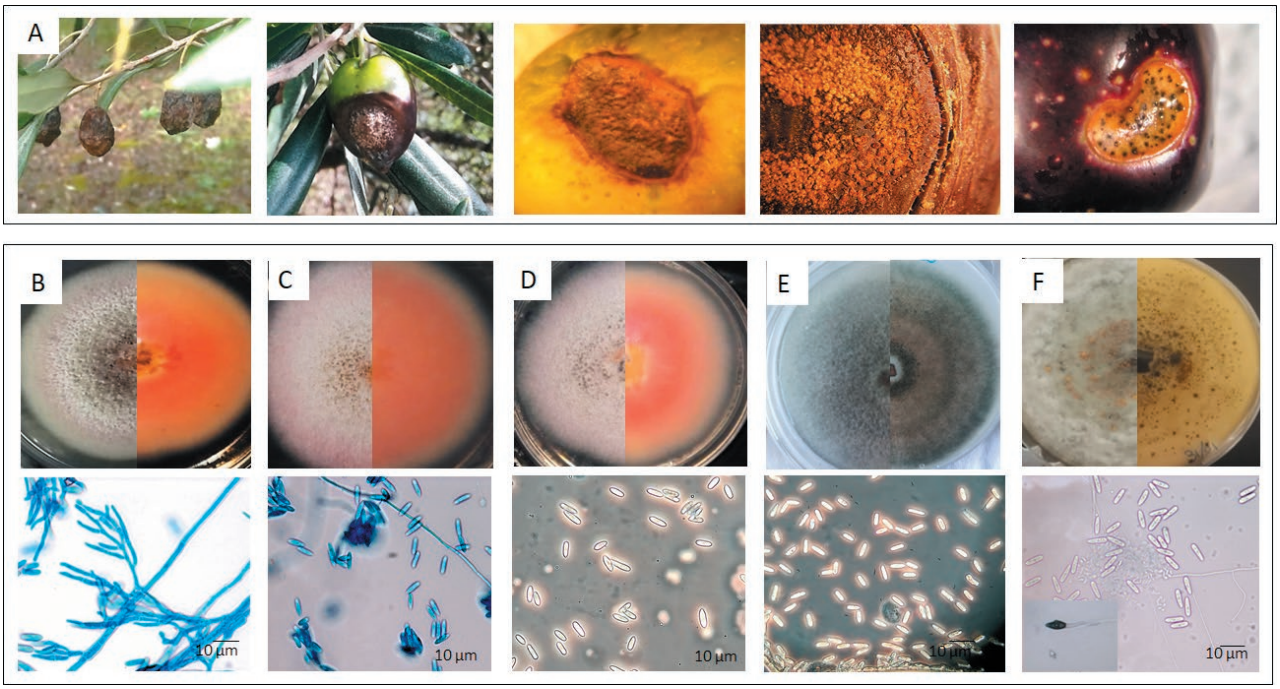

Figure 1. Symptoms of anthracnose (i.e. mummified fruits on the tree, rot, circular sunken lesions) found in olive fruits of the International Olive Germplasm Collection (IOGC) at Villa Zagaria (Sicily, Italy) (A). Above and below views of colony morphology of PDA cultures (top), and respective conidia or conidiophores (bottom) of *Colletotrichum acutatum* P185 (B), *C. acutatum* B13-16 (C), *C. acutatum* P77 (D), *C. gloeosporioides* C2.1 (E), and *C. cigarro* Perg6B (F).

absent in *C. kahawae* isolates. Strain C2.1 was classified as *C. gloeosporioides* (Figure 2).

Regarding the *C. acutatum* species complex, a total of 1321 positions (ITS: 1-407, TUB2: 414-906, GAPDH: 913-1112, and ACT: 1119-1321) were included in the analysis. A total of 163 were parsimony-informative, 318 were variable and parsimony-uninformative, and 978 characters were conserved. A maximum of 1000 equally parsimonious trees were saved (TL = 501; CI = 0.6047; RI = 0.8881; CI = 0.6541). The strains P77, B13-16, P185, 105-6B, 177B, and 237-5 clustered with reference strains of *C. acutatum* (CBS 112996, CBS 112759, CBS 129952) and two Sicilian strains (CBS 142407 and CPC 26987) obtained from *Citrus* (Figure 3).

Pathogenicity on detached fruits

All the inoculated olive fruits developed typical anthracnose symptoms (soapy rot). Two-way ANOVA showed statistically significant differences ($P < 0.001$) between *Colletotrichum* strains and olive cultivars and their interactions. For this reason, differences in cultivar susceptibility were examined for each pathogen strain (Table 2). Fruits of the resistant 'Frantoio' showed slight symptoms on few fruits. In contrast, the first anthracnose symptoms were observed in the fruits of 'Calatina' and 'Carolea' at 5 d after inoculation with the *C. acutatum* isolates.

Fruits of 'Frantoio' were almost completely resistant to the five *Colletotrichum* strains, while among all the other tested cultivars 'Biancolilla', 'Ogliarola messinese', and 'Nocellara messinese' were the most resistant. However, 'Nocellara messinese' developed greater disease severity than 'Vaddarica' when inoculated with isolate Perg6B of *C. cigarro* (Table 2). In contrast, the cultivars Carolea, Cavaliera, and Calatina were the most susceptible, in all the cases showing average AUDPCs > 42. The dendrogram of the heatmap (Figure 4), indicated that the cultivars formed three groups: resistant ('Frantoio'), susceptible ('Nocellara messinese', 'Ogliarola messinese', 'Biancolilla', and 'Vaddarica', 'Santagatese') and highly susceptible ('Nocellara del Belice', 'Carolea', 'Calatina', 'Nocellara etnea', 'Zaituna', 'Cavaliera').

Regarding virulence of the strains, the three *C. acutatum* strains caused a similar severity of symptoms on all the tested cultivars (AUDPC ≈ 40), but greater severity than those caused by *C. gloeosporioides* (AUDPC = 23.1) and *C. cigarro* (AUDPC = 21.7). The three *C. acutatum* strains were grouped according to the heatmap dendrogram, while the other two species were in the same clade (Figure 4). *Colletotrichum* isolates re-isolated on PDA medium from inoculated fruits showed morphological characteristics the same as the inoculated isolates.

Cultivar responses under field conditions

Symptoms on mature fruits occurred as extensive dark necroses, with large orange conidial masses and emerging black acervuli (Figure 1), and these symptoms were also evident in some immature fruits of susceptible cultivars. In green fruits, the rots appeared as small brown sunken lesions. Mummified fruits were also detected, although in small proportion (6%). The correlation between the fruit-rot incidence (%) and the ripening-scale [from 0 (green) to 4 (black)] was statistically significant (Spearman coefficient $r = 0.909$).

Since the disease severity and fruit rot were greater in the ripening fruits comparisons were made between olive cultivars showing the same ripening stage (Table 3). Fruit rot incidence and DI were low in the cultivars with green fruits (ripening class 0) with mean incidence of 0.48% and mean DI of 0.01. In the cultivars with green-yellowish fruits (ripening class = 1), there were significant differences among the cultivars for fruit rot incidence, but not for DI. For the cultivars with fruits at ripening class = 2, the cultivar Nocellara Etnea was more susceptible to *Colletotrichum* spp. than 'Moresca', 'Turdunazza', or 'Zaituna'. For the cultivars showing fruit at ripening class = 3, the cultivar Nocellara del Belice was the most resistant (Table 3). For the cultivars with ripe fruit (colour class = 4), 'Tonda iblea' was more resistant ($P < 0.05$) to the pathogen than 'Cipressino', but only for DI. DI and the fruit rot incidence (%) were significantly correlated ($r = 0.855$; $P < 0.001$).

Although different nonlinear regression models were evaluated for describing the relationship between disease severity and incidence, the best (and selected) model was a linear regression. This regression was forced through the origin because of the biological meaning (Figure 5).

A comparison of results from the pathogenicity tests in controlled conditions and the natural incidence of fruit rots, conducted for the cultivars Santagatese, Ogliarola messinese, Frantoio, Vaddarica, Nocellara messinese, and Calatina which all had the same maturation levels (value of 0-1), showed that there was a statistically significant correlation between these two parameters (Pearson coefficient $r = 0.689$; $P < 0.001$).

DISCUSSION

Olive anthracnose has been rarely reported in Sicily in traditional olive groves, suggesting that agronomic (e.g., cultivar resistance) or climatic conditions are not suitable for disease epidemics (Cacciola *et al.,* 2012). New agronomic practices, including high-density plantations with non-local olive cultivars or new irrigation systems,

can increase the risk of anthracnose in this region of Italy. In the present study, anthracnose incidence was assessed in the Olive Germplasm Collection of Villa Zagaria, which is located in a subhumid area in Enna Province, and which is managed according to traditional agronomic practices.

Ten representative *Colletotrichum* isolates were recovered from approx. 80% of symptomatic olive drupes, and the isolates were identified by phylogenetic and taxonomic analyses, and their pathogenicity on detached fruits was assessed.

For the isolates, colony morphology, conidium characteristics, and multigene phylogenetic analyses identified three main species. *Colletotrichum acutatum* was the most frequently isolated fungus from symptomatic olive fruits, along with a fewer isolates of *C. gloeospori-*

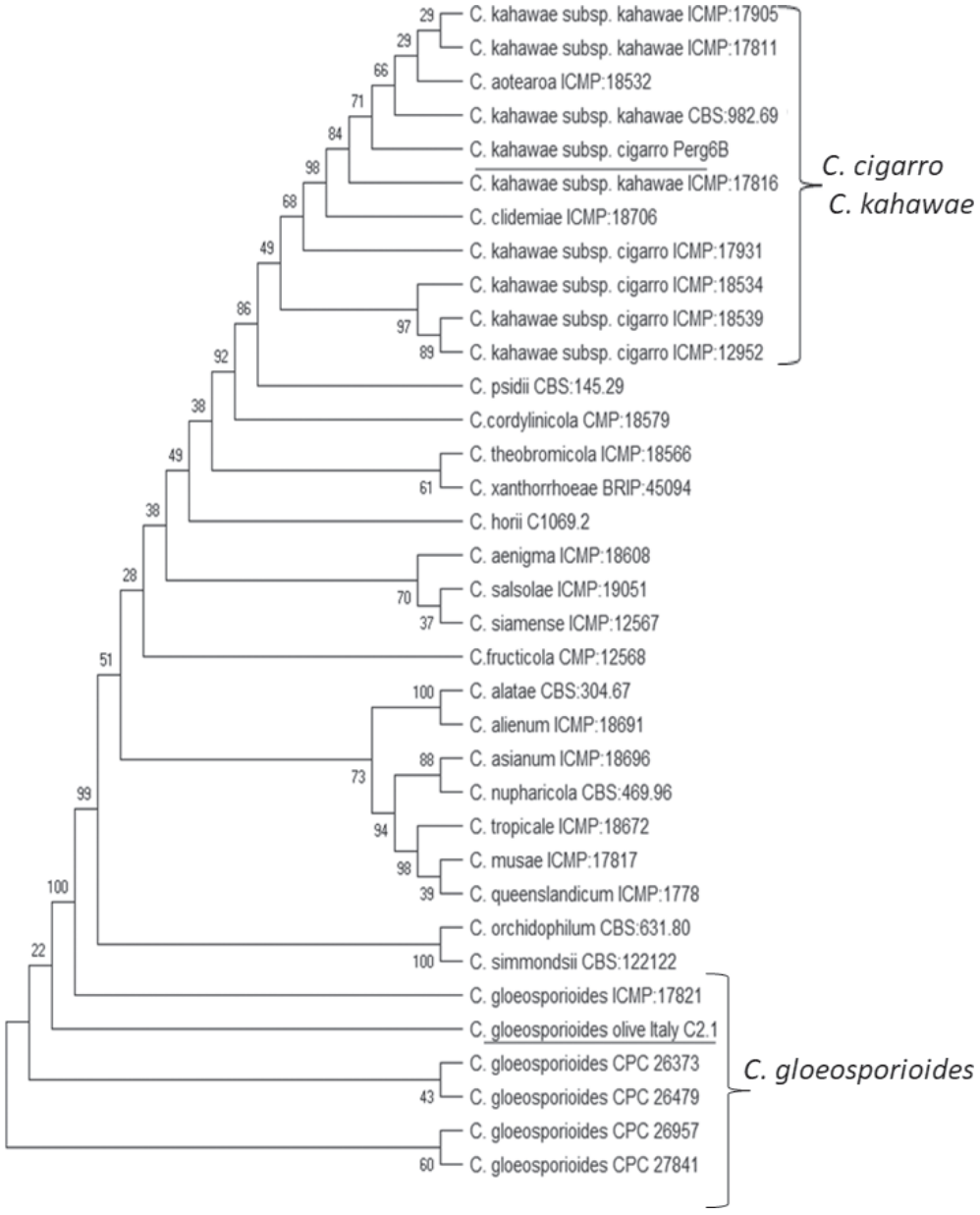

Figure 2. Phylogenetic analysis of the 35 *Colletotrichum gloeosporioides* species complex strains listed in Supplementary Table S1, based on a multilocus concatenated alignment of the ITS, *GAPDH, ACT*, and *TUB2* genes. The evolutionary history was inferred using the Maximum Parsimony method. *Colletotrichum orchidophilum* CBS 632.80 was used as an outgroup. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Strains described for the first time are underlined.

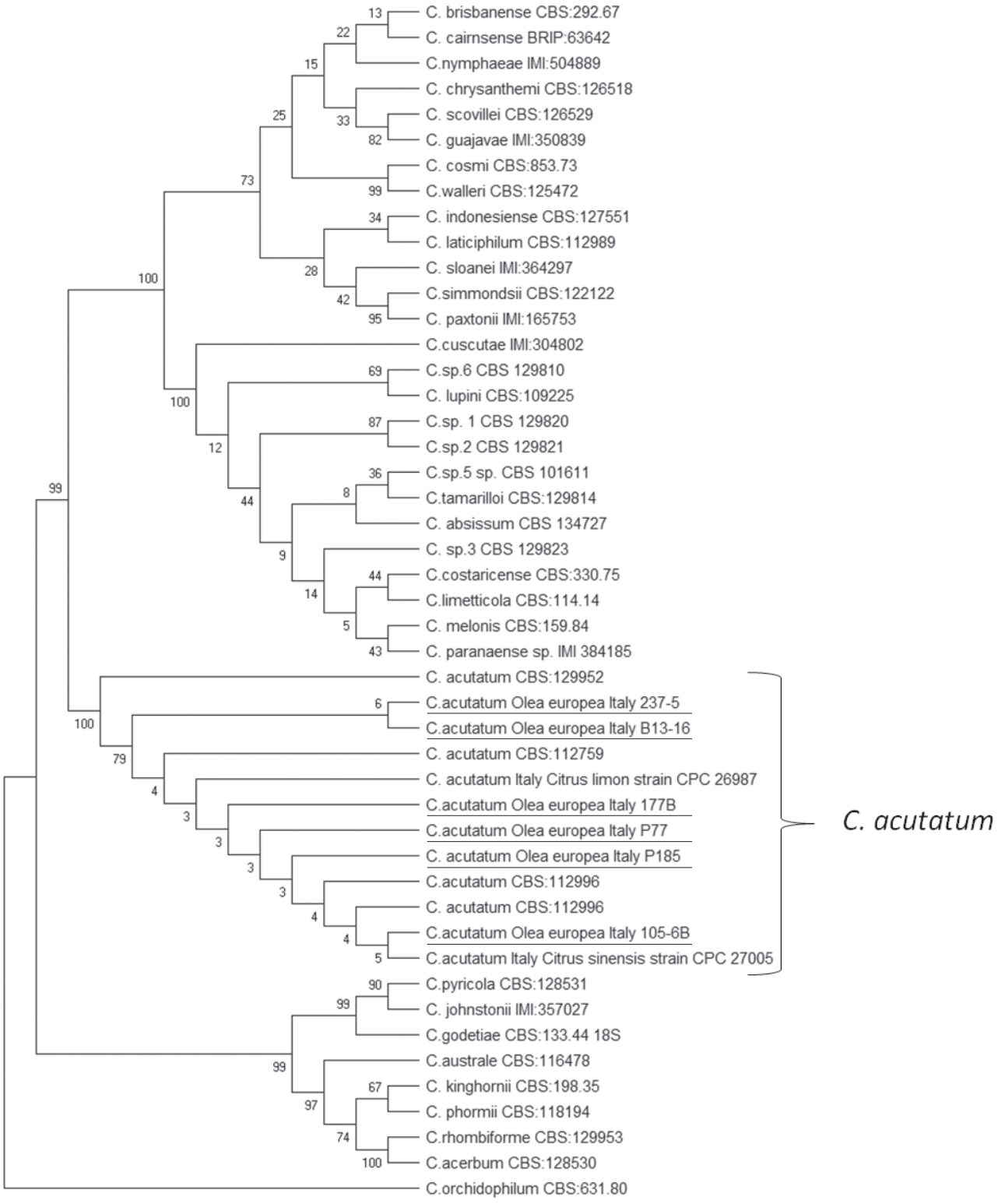

Figure 3. Phylogenetic analysis of the 47 *Colletotrichum acutatum* species complex strains listed in Supplementary Table S1, based on a multilocus concatenated alignment of the ITS, *GAPDH, ACT,* and *TUB2* genes. The evolutionary history was inferred using the Maximum Parsimony method. *Colletotrichum orchidophilum* CBS 632.80 was used as an outgroup. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Strains described for the first time in this study are underlined. For strains originating from Italy, the hosts are indicated.

Table 2. Mean Area Under Disease Progress Curves (AUDPCs) for 11 traditional Sicilian olive cultivars and 'Frantoio' (used as resistant control) inoculated with three isolates of *Colletotrichum acutatum* (P185, P77 or B13-16), or one isolate each of *C. gloeosporioides* (C2.1), or *C. cigarro* (Perg6B).

Cultivar	*C. acutatum*			*C. gloeosporioides*	*C. cigarro*	Average
	P185	P77	B13-16	C2.1	Perg6B	
Frantoio	0.8 a*	0.6 a	0.7 a	0.5 a	0.4 a	0.6
Biancolilla	35.8 bc	32.5 b	38.5 cd	14.3 b	14.0 b	27.0
Nocellara messinese	38.0 bc	32.0 b	33.1 b	25.1 c	21.3 cd	29.9
Ogliarola messinese	40.1 c	30.8 b	34.2 bc	22.3 c	17.5 bc	28,9
Santagatese	41.3 c	41.6 c	33.6 bc	23.3 c	13.6 b	30.7
Vaddarica	32.2 b	43.6 c	33.9 bc	29.3 d	14.7 b	30.7
Nocellara etnea	50.2 d	45.2 c	41.4 d	17.0 b	15.1 b	33.7
Nocellara Belice	38.0 bc	41.3 c	32.1 b	35.4 e	34.8 f	36.3
Zaituna	51.2 d	45.2 c	48.2 e	17.0 b	28.0 e	37.9
Cavaliera	53.5 d	52.1 d	50.3 ef	25.0 c	31.9 f	42.5
Carolea	54.1 d	40.3 c	53.5 fg	37.9 e	44.1 g	45.9
Calatina	61.1 e	59.3 e	57.5 g	30.1 d	25.1 de	46.6
Average	41.3	38.7	38.1	23.1	21.7	

*One way ANOVA was performed on AUDPC values. Means within each column accompanied by the same letter are not significantly different ($P < 0.01$), Student-Newman-Keuls test.

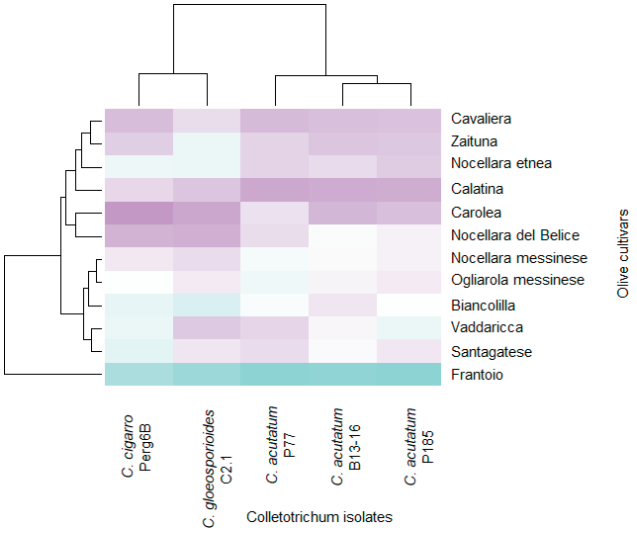

Figure 4. Heatmap and hierarchical clustering of olive fruit susceptibility to anthracnose in 11 Sicilian cultivars, using severity of the disease as the dependent variability to indicate similarity between olive cultivars or *Colletotrichum* strains. The clustering analysis showed three main groups for cultivar susceptibility and two main groups for pathogen strain virulence.

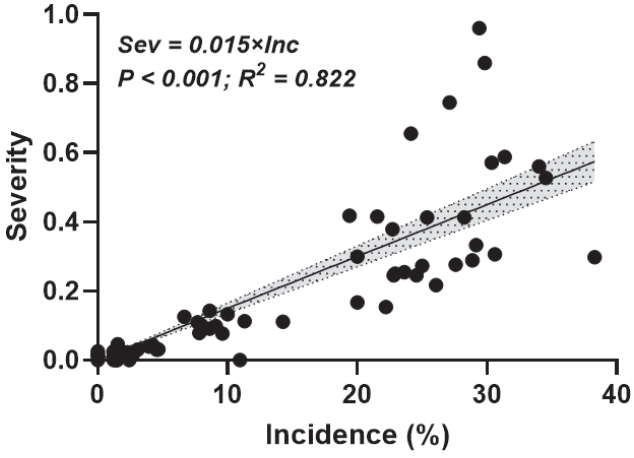

Figure 5. Relationship between fruit rot severity indices and fruit rot incidence (%) for olive fruit affected by *Colletotrichum* spp. in olive trees growing at the International Olive Germplasm Collection (IOGC), Villa Zagaria, Enna, Italy.

oides and *C. cigarro*. While the presence of *C. gloeosporioides* species complex affecting olive fruits was reported by Agosteo *et al.* (2002) in Sicily, the present report is the first for *C. cigarro* and *C. acutatum* associated with olive anthracnose in this region.

Based on ITS sequence analyses, the six strains of *C. acutatum* obtained in the present study are homogenous, and belong to the genotype Acu1, which was prevalent in Calabria, where three genotypes of *C. acutatum* were identified (Mosca *et al.*, 2014). Multilocus sequence analysis showed high homology levels between the isolates and those obtained from *Citrus* in Sicily (Guarnaccia *et al.*, 2017), indicating low diversity and little specificity within the isolates of *C. acutatum*. The data are also

Table 3. Olive fruit ripening classes and disease reactions (mean fruit rot incidence and mean disease index) for 20 selected olive cultivars, naturally infected by *Colletotrichum* spp., in the International Olive Germplasm Collection (IOGC), Villa Zagaria, Enna, Italy.

Cultivar	Ripening class[a]	Fruit-rot (%)[b]	Disease Index[c]
Santagatese	0	0.0 a	0.00 a
Ogliarola Messinese	0	0.0 a	0.00 a
Frantoio Corsini	0	0.0 a	0.00 a
Aitana	0	0.6 a	0.01 a
Minuta	0	1.05 a	0.01 a
Vaddarica	0	1.22 a	0.01 a
Nocellara messinese	1	0.37 a	0.00 a
Calatina	1	1.41 a	0.01 a
Nerba Catanese	1	2.14 b	0.02 a
Cerasuola	1	2.32 b	0.02 a
Moresca	2	9.19 a	0.10 a
Turdunazza	2	9.43 ab	0.10 a
Zaituna	2	9.56 a	0.11 a
Nocellara Etnea	2	25.72 b	0.26 b
Giarraffa	3	31.58 b	0.34 b
Biancolilla	3	24.64 b	0.26 a
Nocellara del Belice	3	22.26 a	0.41 c
Brandofino	3	32.56 b	0.56 d
Tonda iblea	4	21.86 a	0.22 a
Cipressino	4	27.62 a	0.81 b

[a] Fruit ripening was evaluated using a 0 to 4 rating scale (from green to black) (Moral *et al.,* 2017).
[b] Fruit rot incidence was calculated from numbers of infected fruit/total number of fruit. For each ripening scale, means in each column accompanied by different letters are significantly different, Zar's Test for multiple comparisons of proportions (Zar, 2010).
[c] For each ripening scale, cultivar means were compared using the Least Significant Differences (LSD) test at $P = 0.05$.

consistent with the results based on rDNA-ITS sequences, which showed variability up to 4% among *C. acutatum* isolates and indicated they belong to a monophyletic group (Damm *et al.,* 2012; Baroncelli *et al.,* 2017). It is likely that *C. acutatum* was recently introduced in Sicily, as occurred in Portugal and Tunisia (Talhinhas *et al.*, 2009; Chattaoui *et al.*, 2016).

Available data on the diffusion of the *Colletotrichum* population in Italy indicates that *C. godetiae* is dominant. In contrast, the *C. gloeosporioides* species complex was rare (Faedda *et al.*, 2011; Cacciola *et al.*, 2012).

In Portugal, *C. acutatum* is restricted to the Algarve region, whereas *C. nymphaeae*, *C. simmondsii*, and *C. godetiae* were prevalent in other olive-growing regions (Talhinhas *et al.,* 2009). Presence of *C. acutatum* has been described in most of the Mediterranean countries, including Tunisia (Chattaoui *et al.*, 2016), Greece (Iliadi *et al.*, 2018), Morocco (Msairi *et al.*, 2017), and–Egypt (Embaby *et al.*, 2014).

In the present study, analysis of the glutamine synthetase gene allowed the first identification in Sicily of *C. cigarro comb. et stat. nov.* (Cabral *et al.,* 2020) affecting olive fruits. Previously, this species has been detected in Calabria on olive fruits (Schena *et al.,* 2014; Mosca *et al.*, 2014) and mandarin leaves (Perrone *et al.*, 2016), and in Piedmont (northern Italy) from symptomatic leaves and stems of the ornamental plant *Liquidambar styraciflua* (Guarnaccia *et al.*, 2021).

The sporadic detection of *C. gloeosporioides* in the present study confirms the occasional presence of this fungus in Sicilian olives, but it has not been associated with severe disease (Agosteo *et al.*, 2002; Talhinhas *et al.*, 2009; Faedda *et al.*, 2011; Cacciola *et al.*, 2012). However, this species was previously reported affecting sweet orange (*Citrus sinensis*) (Aiello *et al.*, 2015) and mango (*Mangifera indica*) trees in this region (Ismail *et al.*, 2015).

The spread of *C. gloeosporioides* in the Mediterranean region has been at low levels. In Portugal, this fungus occurred sporadically associated with olive anthracnose (3% incidence) in the Algarve region. Only two isolates were identified during a large-scale survey in Northern Tunisia (Chattaoui *et al.*, 2016). However, olive fruits infected by *C. gloeosporioides* species complex have been sporadically reported in Tunisia (Rhouma *et al.*, 2010) and in Morocco (Achbani *et al.*, 2013) and Iran (Sanei and Razavi, 2012).

In Portugal, ITS DNA analysis allowed distinction between two groups of *C. gloeosporioides* isolates, CG-1 and CG-2, differing for only one nucleotide, but with CG-2 isolates associated with greater disease severity than the CG-1 isolates (Talhinhas *et al.*, 2009). The strain C2.1 of *C. gloeosporioides* had the same ITS sequence of other CG-2 olive strains of this species previously described by Schena *et al.* (2014).

Pathogenicity of the *Colletotrichum* strains assessed in the present study was separately evaluated by inoculating detached green fruits of 11 olive cultivars (selected among the most relevant in Sicily) with conidia of isolates of *C. acutatum*, *C. gloeosporioides* and *C. cigarro*. The three strains of *C. acutatum* were the most virulent, and less virulence was demonstrated in the other two species, as has been previously observed (Talhinhas *et al.*, 2011; 2018; Schena *et al.*, 2014; 2017).

The *C. cigarro* isolate was able to infect green detached drupes, reproducing late anthracnose symptoms after 10-15 d (data not shown). Previous inoculation tests on the cultivar Coratina gave no rots on green

fruits and noticeable development of rot on ripening olives (Schena *et al*., 2014), probably due to low susceptibility of this cultivar.

Analysis of symptom evolution under laboratory conditions revealed strong interactions among cultivars and isolates. These require further study. The high susceptibility of the cultivar Nocellara del Belice is also important, as olives of this cultivar were severely diseased when inoculated with *Colletotrichum* strains of low virulence.

Under field evaluations in December, most Sicilian cultivars did not show anthracnose symptoms as previously described for Italian cultivars (Moral *et al*., 2017). The cultivar Cipressino was susceptible in these evaluations, while 'Frantoio' showed well-recognized resistance to the disease (Moral and Trapero, 2009). In contrast, olives of the cultivars Nocellara del Belice, Vaddarica, Santagatese and Oglialora were not affected by the pathogen. This could have been due more to an escape (the fruits were less ripe) than innate resistance (Moral *et al*., 2017). In contrast, all the cultivars, which were evaluated when the fruits were violet-black, developed diseased fruits. This reinforces the recommendation of early harvesting as an effective disease control strategy (Cacciola *et al*., 2012; Moral and Trapero, 2012). Some inconsistencies between the field and laboratory evaluations were observed on the cultivars Calatina and Zaituna, which could be due to the pathogen population or to fruit maturity. The present study is the first to examine the relationship between disease severity and olive fruit rot incidence. In general, both variables were positive and highly correlated, reinforcing the concept that cultivar resistance can be evaluated only according to the incidence of affected fruit (Moral and Trapero, 2012).

In conclusion, the present study has shown that *C. acutatum* is present in olive groves in Sicily. According to DNA sequence analyses, the population is likely to be homogenous, and does not differ from strains isolated from other hosts (i.e., *Citrus*). Since climatic conditions in some olive producing areas in Sicily are not favourable for anthracnose development, in the short term, the risk of disease remains low for most cultivars as they are not susceptible until they ripen. Furthermore, other pests, such as the olive fruit fly (*Bactrocera oleae*), could affect quality of Sicilian olive oils. Climate changes, adaptation to new hosts, or cross-infection events by *Colletotrichum* among different hosts, could lead to re-emergence of olive anthracnose in Sicily, as has occurred for diffusion of these pathogens in the northern hemisphere (Talhinhas *et al*., 2009; Mosca *et al*., 2014). Further surveys are required to classify the field performance of Sicilian cultivars, and to determine interactions among cultivars and isolates, and define pathogen phenotype dynamics.

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New and Opinion

Understanding the control strategies effective against the esca leaf stripe symptom: the edge hypothesis

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Summary. A peculiar symptom that may develop in grapevines affected by wood pathogens involved in the esca complex of diseases is the leaf stripe symptom, which also gives the name to the Grapevine Leaf Stripe Disease. Multiple studies have revealed strong links between fungal presence, wood symptomatology and expression of the leaf stripe symptom. However, numerous other factors have been shown to play roles in symptom onset, incidence, severity and yearly fluctuation of this disease. While the factors triggering the leaf stripe symptom are still under investigation, three control strategies have been proven effective for substantially reducing its expression, namely trunk surgery, and applications of sodium arsenite or a fertilizer mixture. These control strategies are examined here, including their (putative or confirmed) modes of action, and how they may influence the leaf stripe symptom development. In this article, we also propose the 'edge' hypothesis to tentatively explain symptoms onset, keeping in consideration past knowledge and recent advances in the understanding of the esca leaf stripe symptom. Ultimately, it is our intention to offer food-for-thought and stimulate debate within the phytopathological community.

Keywords. Grapevine trunk diseases, interveinal necrosis, grapevine, Fomitiporia mediterranea, trunk surgery.

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INTRODUCTION

Esca is a complex of wood diseases that belongs to the Grapevine Trunk Diseases (GTD) cluster, i.e., fungal diseases that affect perennial and annual tissues of grapevine. The role of numerous fungi as causal agents of internal wood symptoms, including brown wood streaking, necrotic lesions and white rot, has been widely demonstrated (Mugnai *et al*., 1999; Bertsch *et al*., 2013; Gramaje *et al*., 2018). However, the relationship among fungal pathogens, wood symptoms and the leaf stripe symptom remain to be fully validated. This uncertainty has led to the proposal of a separate disease, namely Grape-

vine Leaf Stripe Disease (GLSD), characterized by permanent wood symptoms (i.e., brown wood streaking and necrotic lesions) and a discontinuous leaf stripe symptom. Nevertheless, the leaf stripe symptom is also found in esca proper-affected vines, the difference between GLSD and esca-proper being the presence of white rot in the latter (Surico, 2009).

Since the review by Mugnai *et al.* (1999), three main theories have been proposed, and partly explored, to identify the triggering factor(s) implicated in the onset of the leaf stripe symptom.

(a) The toxins hypothesis. Several studies support the involvement of toxic metabolites of fungal origin (Bruno and Sparapano, 2006; Bruno *et al*., 2007; Andolfi *et al*., 2011; Schilling *et al*., 2021), a well-known mechanism in two major GTDs (i.e., Eutypa dieback and Botryosphaeria dieback; Colrat *et al*., 1999; Masi *et al*., 2018; Trotel-Aziz *et al*., 2019; Cobos *et al*., 2019; Schilling et al., 2021). Still, the evidence provided to explain the leaf stripe symptom development and yearly fluctuation in esca-affected vines remains unsatisfactory.

(b) The by-products of wood degradation hypothesis. 'By-products of wood degradation' is an umbrella term that groups all the molecules that may develop as a consequence of the plant-pathogens interaction (Mugnai *et al*., 1999). In their recent articles, Moretti *et al.* (2021), Schilling *et al.* (2021) and Pacetti *et al.* (2022) explored a number of pathways that lead to potential triggering factors. Among them, we find (a) wood extractives, such as tannins and polyphenols, (b) lignocellulose-degrading enzymes, (c) fungal or plant derived non-enzymatic proteins, and (d) low molecular weight diffusible compounds generated during the process of cellulose and lignin degradation (e.g., DAMPs; Héloir *et al.,* 2019). It remains to verify empirically if any of them, alone or in combination, is the primary responsible for the leaf stripe symptom expression.

(c) The competition-induced metabolites hypothesis. Brought forward by Bruez *et al.* (2020), this recent hypothesis suggests that the microbe-microbe interaction among esca-associated fungi and/or other components of the endophytic microbiome (e.g., bacteria) may trigger the release of phytotoxic secondary metabolites. While certainly plausible, in light of multiple examples in the literature (Hardoim *et al*., 2015), to date, no experimental evidence supports this hypothesis.

Despite these different hypotheses, it is commonly agreed that the translocation of fungal-derived or plant-derived molecules from wood to leaves is a key component in the leaf stripe symptom onset (Bruno *et al*., 2007). In the following text, to simplify communication, we have grouped all possible fungal toxins, byproducts of wood degradation and competition-induced metabolites, into a single category, namely Leaf Stripe Symptoms-Inducing Molecules (LSSIM).

Since the early 1990ies, numerous studies have shown that multiple biotic and abiotic factors, as well as agronomic practices, are correlated to onset, discontinuity, incidence and severity of the leaf stripe symptom (Table 1; Fischer and Ashnaei, 2019; Claverie *et al*., 2020; Del Frari *et al*., 2021). Among these, meteorological conditions (e.g., rainfall, drought, temperature, soil moisture) play major roles in yearly symptom fluctuations (Surico *et al*., 2000; Marchi *et al*., 2006; Bruez *et al*., 2013; Andreini *et al*., 2014; Calzarano *et al*., 2018;

Table 1. Biotic and abiotic factors and agronomic practices correlated to esca leaf stripe symptoms in grapevine.

Biotic factors	Abiotic factors	Agronomic practices
Plant vigour [a]	Climatic conditions [h]	Pruning and training system [m]
Plant physiology [b]	Plant protection products [i]	Trunk surgery [n]
Xylem vessels characteristics [c]	Soil [j]	Grafting type [o]
Scion and rootstock genotype [d]	Nutrients [k]	
Grapevine age [e]	Water availability [l]	
Fungal endophytes (wood pathogens and/or biocontrol agents) [f]		
Rhizosphere microbiome [g]		

[a]Lecomte *et al.*, 2011; Fischer and Ashnaei, 2019
[b]Christen *et al.*, 2007; Magnin-Robert *et al.*, 2011; Fontaine *et al.*, 2016a; Goufo and Cortez, 2020; Calvo-Garrido *et al*., 2021
[c]Pouzoulet *et al.*, 2014
[d]Marchi, 2001; Bruez *et al.*, 2013; Andreini *et al.*, 2014; Murolo and Romanazzi, 2014; Pouzoulet *et al.*, 2014; Borgo *et al*., 2016
[e]Mugnai *et al*., 1999; Graniti *et al*., 2000; Surico *et al*., 2008
[f]Larignon and Dubos, 1997; Peros *et al*., 2008; Bigot *et al.*, 2020; Nerva *et al*., 2019; Del Frari *et al.*, 2021; Fotios *et al.*, 2021; Di Marco *et al*., 2022
[g]Landi *et al.*, 2021
[h]Surico *et al.*, 2000; Marchi *et al.*, 2006; Bruez *et al.*, 2013; Calvo-Garrido *et al*., 2021
[i]Di Marco *et al.*, 2011; Songy *et al.*, 2019; Bruez *et al.*, 2021
[j]Surico *et al.*, 2000; Guérin-Dubrana *et al.*, 2005; Fischer and Ashnaei, 2019; Nerva *et al.*, 2019
[k]Calzarano *et al*., 2007; Calzarano *et al.*, 2014; Calzarano and Di Marco, 2018
[l]Marchi *et al.*, 2006; Andreini *et al.*, 2014; Calzarano *et al.*, 2018; Serra *et al.*, 2018; Bortolami *et al.*, 2021; Calvo-Garrido *et al*., 2021
[m]Lecomte *et al.*, 2018
[n]Cholet *et al.*, 2021; Pacetti *et al.*, 2021
[o]Mary *et al.*, 2017

Serra *et al*., 2018; Bortolami *et al*., 2021; Calvo-Garrido *et al*., 2021).

Several studies demonstrated that grapevines infected and symptomatic in the wood may remain fully asymptomatic in the leaves for several consecutive years (Surico *et al*., 2000; Calzarano *et al*., 2018), despite an apparently unaltered wood infection and symptomatology. As wood infections and related symptomatology tend to build up with time, leaf symptoms also become more frequent as vines age. This may be why studies have often correlated the presence of white rot with leaf symptoms manifestation (Pollastro *et al*., 2000; Peros *et al*., 2008; Kuntzmann *et al*., 2010; Maher *et al*., 2012; Bruez *et al*., 2014; Elena *et al*., 2018; Del Frari *et al*., 2021). Indeed, the older the vine, the greater is the chance of it being infected by white rot agents. On the contrary, less frequent are the studies dedicated to young or adult vines showing the leaf stripe symptom in plants seemingly unaffected by white rot (Edwards and Pascoe, 2004; Calzarano and Di Marco, 2007; Romanazzi *et al*., 2009; Hofstetter *et al*., 2012; Raimondo *et al*., 2019).

Interestingly, esca-associated fungi are occasionally found in asymptomatic wood, suggesting – under some circumstances – a non-pathogenic behaviour (e.g., Graniti *et al*., 2000; Hofstetter *et al*., 2012; Del Frari *et al*., 2019a). This confirms that the microbiological aspect of the wood of leaf symptomatic vines is only one of the factors of a bigger equation. For example, even the grapevine rhizosphere, in particular arbuscular mycorrhizal fungi, may alter their abundance in leaf symptomatic vines (Landi *et al*., 2021).

Regardless of this multiplicity of factors, which complicates the achievement of clear etiological and epidemiological patterns, three control strategies strongly influence the expression of the esca leaf stripe symptom. These are trunk surgery (Cholet *et al*., 2021; Pacetti *et al*., 2021) and sodium arsenite (Songy *et al*., 2019; Bruez *et al*., 2021), which decrease symptoms for multiple sequential years after a single treatment, and a fertilizer mixture, which reduces symptoms incidence and severity, when sprayed on vine canopies, in the year of application (Calzarano *et al*., 2014; Calzarano and Di Marco, 2018). A fourth method, namely the application of *Trichoderma* products for pruning wound protection (e.g., Di Marco *et al*., 2004), prevents fungal infection, and it was recently validated as a means of reducing the leaf stripe symptom incidence (Bigot *et al*., 2020; Di Marco *et al*., 2022).

In the following section, we have examined the putative or confirmed modes of action of the first three control strategies mentioned, and we elaborate a hypothesis that joins LSSIM, the grapevines health status and the leaf stripe symptom expression.

WHY DO DIFFERENT TREATMENTS REDUCE LEAF SYMPTOMS?

Trunk surgery. The earliest version of trunk surgery has ancient origins, being employed in Greece and Asia Minor since Roman times (Mugnai *et al*., 1999). This consisted in mechanically splitting the trunk of esca leaf symptomatic vines and placing a stone between the two trunk halves (Figure 1). This technique was used sporadically over the years, and revived in North-East Italy about 30 years ago, where it was named '*Metodo Armano*', after the agronomist who spread its use. Allegedly, treated vines remained asymptomatic in the leaves for several years, regardless of the presence of wood symptoms. However, no scientific survey was conducted to assess the validity of these claims. Another similar and recently re-discovered technique is the *slupatura* (in Italian), also known as *curettage* (in French) or trunk surgery (in English), which is commonly used on olive trees in the Mediterranean region. This consists in removing all decayed tissue (*i.e*., the white rot) from the vine trunk, using a small chainsaw (or a tool like the one called *malepeggio*, in Italy). Albeit white rot being associated with vine apoplexy since the report of Ravaz (1909), and only later with the leaf stripe symptom by Viala (1926), Fischer (2002) identified *Fomitiporia mediterranea* as the main causal agent of white rot in grapevines in the Mediterranean region – up to Iran (Moretti *et al*., 2021). Trunk surgery has been recently validated as an effective control strategy to reduce the leaf stripe symptom relapse and to improve several physiological parameters in treated vines (Cholet *et al*., 2021; Pacetti *et al*., 2021).

Why is trunk surgery effective?

According to several authors (Cholet *et al*., 2021; Pacetti *et al*., 2021; Del Frari *et al*., 2021), the success of this technique lies in its drastic effect on the presence of white rot and, as a consequence, on the abundance of *F. mediterranea*. In their study, Pacetti *et al*. (2021) detected significant alterations in the fungal and bacterial microbiota of vines treated with trunk surgery. With concern to fungi, the authors report a massive decrease in *F. mediterranea* abundance, while esca-associated pathogens *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp., and other GTD-associated fungi (e.g., *Eutypa* spp. and *Botryosphaeriaceae*, causal agents of Eutypa dieback and Botryosphaeria dieback) remained unaltered by the treatment. The bacterial genera most affected by trunk surgery are *Burkholderia*, *Massilia* and *Pantoea* (Pacetti *et al*., 2021). Some of the putative driv-

Figure 1. 'Low-tech' grapevine trunk surgery (known in some Italian regions as '*Metodo Armano*'). It consists of splitting the trunk of a leaf symptomatic vine and fitting a stone in between the two trunk halves. These two treated vines were photographed in December 2016 (A) and in June 2017 (B), in two vineyards near Spessa, Friuli, Italy.

ers of these microbial alterations may be biotic factors, such as the fungus-fungus interaction resulting from variations in niches composition, or competition with wood colonizers; abiotic factors, such as the oxygenation levels in grapevine wood, or increased temperature fluctuations in the, now thin, trunk. In Pacetti *et al.* (2021), the authors speculate that the strong reduction in the leaf stripe symptom is linked to the second theory of symptoms manifestation, i.e. the byproducts of wood degradation hypothesis, despite concluding that more evidence is required to validate this assumption.

In addition to the microbiological aspect, trunk surgery may trigger strong defense responses in vines, such as release of phenolic compounds and PR proteins, which normally occur after host wounding (e.g., during annual pruning) (Blanchette and Biggs, 1992; Ferreira *et al.*, 2007; Pouzoulet *et al.*, 2013). Future research may investigate their putative role in contributing to LSSIM mitigation at leaves level.

Sodium arsenite. During the 20th century, sodium arsenite ($NaAsO_2$) was the only known chemical treatment capable of greatly reducing the leaf stripe symptom manifestation in grapevines. Its use in vineyards was discontinued in 2003, due to its highly toxic effects on humans, animals, plants and the environment in general (Carbonell-Barrachina *et al.*, 1997a; Larignon *et al.*, 2008; Songy *et al.*, 2019), even if symptoms incidence had been increasing in some European regions, France included (Fontaine *et al.*, 2016b). The grapevine endophytic microbiota is known to be affected by the use of several fungicides (Del Frari *et al.*, 2019b) and, unsurprisingly, $NaAsO_2$is among them (Larignon and Fontaine, 2018; Bruez *et al.*, 2021). In their article, Larignon and Fontaine (2018) explored a century of knowledge over the use of $NaAsO_2$ in viticulture, especially interesting for French-speaking readers.

Recently, Songy *et al.* (2019) studied the effects of $NaAsO_2$ on the physiology of young grapevines, following an application on the stem during plant dormancy, and located the presence of this chemical in all vine organs, including leaves, confirming some results obtained in earlier studies on adult vines (Carbonell-Barrachina *et al.*, 1997b; Larignon *et al.*, 2008). Songy *et al.* (2019) also detected a number of physiological alterations in response to $NaAsO_2$ application, including photosynthesis-related parameters and activation of a plant defence response (up-regulation of genes *SOD*, *GST1* and *CHV5*, in different plant organs). Sodium arsenite has *in vitro* fungicidal activity, also affecting some wood pathogens at different concentrations (Santos *et al.*, 2006; Larignon *et al.*, 2008; Bruez *et al.*, 2021). For example, *F. mediterranea* is strongly affected, even at low $NaAsO_2$ concentrations (IC_{50}= 0.4 mg L^{-1}), while tracheomycotic fungi and other endophytes assessed were relatively tolerant to the chemical (e.g., *P. chlamydospora,* IC_{50}= 6 mg L^{-1}; *Trichoderma* sp., IC_{50}= 29 mg L^{-1}; *Penicillium* sp., IC_{50}= 139 mg L^{-1}) (Bruez *et al.*, 2021). In a recent study, Bruez *et al.* (2021) examined the effects of $NaAsO_2$ on adult vines, confirming its fungicidal activity *in planta*. The most striking observation was the collapse of *F. mediterranea* abundance in white rot (-90%), a tissue known to accumulate $NaAsO_2$ following application on trunks (Larignon *et al.*, 2008). Other known wood pathogens, including *P. chlamydospora*, were not affected by the chemical, or even increased in relative abundance, depending on the woody tissue analyzed. Similarly, no major changes occurred in the endophytic bacterial microbiome, despite $NaAsO_2$ bactericidal properties (Larignon and Fontaine, 2018). Bruez *et al.* (2021) confirmed the treatment efficacy, noting that none of the treated plants manifested leaf stripe symptoms during the following growing season, while all untreated vines did.

Why is sodium arsenite effective?

Available evidence suggest that the most likely explanation to the this question lies in the drastic effect of $NaAsO_2$ on *F. mediterranea* abundance (Bruez *et al.*,

2021), in agreement with the trunk surgery technique. *F. mediterranea* is highly sensitive to this chemical, which also accumulates in *F. mediterranea* preferred niche. Consequently, important changes occur in the microbial composition within vine trunks due to $NaAsO_2$ fungicidal activity, such as the creation of pathogen-free host tissues, and newly available nutrients for other components of the endophytic microbiota. Following this reasoning, the wood affected by white rot would not be degraded by *F. mediterranea*, but – hypothetically – by other organisms (e.g., bacteria; Haidar *et al*., 2021), which do not contribute to the production of LSSIM, altogether suggesting a role of the microbiota as a whole. Before its ban, the application of sodium arsenite in vineyards used to be carried out following a 2 years on – 2 years off regime (Di Marco *et al*., 2000), and symptoms reappeared if the treatment was discontinued as the chemical gradually lost activity via plant detoxification (Larignon and Fontaine, 2018) and/or it is expelled via the root system (Carbonell-Barrachina *et al*., 1997b). The leaf stripe symptom relapse suggests that *F. mediterranea* may reclaim its original niche or infest asymptomatic wood, but more research is necessary to clarify this point.

However striking the effect on *F. mediterranea* may be, the involvement of $NaAsO_2$ at different levels should not be excluded. For example, the up-regulation of genes implicated in detoxification and stress tolerance may be partly involved in the mitigation of negative LSSIM effects. Another hypothesis worth exploring concerns the presence of $NaAsO_2$ in leaves throughout the growing season (Carbonell-Barrachina *et al*., 1997b) and its possible interference with some biochemical and/or physiological processes that mediate the vine-LSSIM interaction (Carbonell-Barrachina *et al*., 1997a; Larignon and Fontaine, 2018).

Fertilizer mixture. In multiple studies, Calzarano *et al*. reported that a mixture of nutrients and seaweed extract significantly reduced the incidence of the esca leaf stripe symptom in grapevine (Calzarano *et al*., 2014; Calzarano and Di Marco, 2018; Calzarano *et al*., 2021). This mixture, based on $CaCl_2$, $Mg(NO_3)_2$, and *Fucales* seaweed extract (Algescar®, Natural Development Group, Castelmaggiore, Bologna, Italy), was developed following observations of high calcium contents in leaves of diseased but leaf-asymptomatic grapevines, when compared to never symptomatic and leaf-symptomatic plants (Calzarano *et al*., 2009). Magnesium was added to the mixture to avoid possible imbalances following Ca applications, while the seaweed extract acts as a carrier of Ca and Mg, thereby improving their activity on the leaf stripe symptom reduction, which was confirmed by the increased druses in the cells of treated leaves (Calzarano *et al*., 2014).

After five applications of the fertilizer mixture, at fruit set and berries pea-sized growth stages, Calzarano *et al*. (2021) recorded greater Ca and Mg contents in asymptomatic diseased vine leaves, compared to leaves from untreated never symptomatic and from symptomatic diseased vines. Instead, at berries pea-sized stage, leaves of pre-symptomatic shoots had very low contents of Ca and Mg, and contents of *trans*-resveratrol and flavonoids also increased in leaves of treated vines (Calzarano *et al*., 2014). Until the pre-bunch closure stage, contents of *trans*-resveratrol, *trans*-ε-viniferin and *trans*-δ-viniferin were greater in treated asymptomatic leaves compared to those untreated asymptomatic leaves (Calzarano *et al*., 2017a). Calzarano *et al*. (2021) also recorded high values of normalized difference vegetation index (NDVI) and green normalized difference vegetation index (GNDVI) at fruit set, in treated asymptomatic leaves from diseased vines. From full flowering to fruit set, the same leaves also had greater water indices (WIs) compared to untreated leaves.

Why is the fertilizer mixture effective?

The above-mentioned findings highlight the role of Ca and Mg in reducing the leaf stripe symptom development and severity. According to some authors, these symptoms are caused by the oxidative burst generated by the plant response, rather than by a direct effect of LSSIM on leaf tissues (Petit *et al*., 2006; Andolfi *et al*., 2009; Magnin-Robert *et al*., 2011). The hypothesis that necrosis formation is comparable to a hypersensitive reaction (HR) is reinforced by the high levels of phytoalexins in symptomatic leaves. Indeed, high levels of phytoalexins are usually synthesized after the HR (Heath, 2000; Lima *et al*., 2012; Calzarano *et al*., 2016).

The calcium ion is an intracellular second messenger involved in plant defense responses. Variations in the cytosolic concentration of Ca^{2+} ($[Ca^{2+}]cyt$) are linked to the triggering of such responses. Salicylic acid, a key molecule in signal transduction, is also required for these responses, and it can cause a strong oxidative response, as it happens in the HR. Availability of Ca may reduce host oxidative responses and HR necrosis. Calcium, penetrating into the leaves, can increase the synthesis of calmodulin, which regulates salicylic acid and reduces the HR (Lecourieux *et al*., 2006; Du *et al*., 2009). High numbers of druses in treated vine leaves also suggest that Ca accumulation in extracellular spaces may increase phytoalexin synthesis and/or strengthen the cell walls (Conway *et al*., 1991; Tavernier *et al*., 1995; Lecouriex *et al*., 2006; Calzarano *et al*., 2014). The role of Mg in the control of the leaf stripe symptom could result from general improve-

ments of host plant health, and in delay of chlorophyll degradation (Datnoff *et al*., 2007). However, Mg could also act directly, detoxifying toxic metabolites produced by esca fungi, as has been observed for *Eutypa lata* infections, where eutypin was detoxified to eutypinol by Mg^{++} and Mn^{++} (Colrat *et al*., 1999).

In untreated vines, phytoalexin synthesis was probably not involved in the reduction of the leaf stripe symptom (Calzarano *et al*., 2017b). Instead, in the leaves of asymptomatic vines, following applications of the fertilizer mixture, early and high phytoalexin synthesis does not exclude their possible contribution to the control of the leaf stripe symptom (Calzarano *et al*., 2017a). The effects of Ca and Mg may be boosted by accumulation of *trans*-resveratrol and other phytoalexins, at berries pea-sized and until pre-bunch closure. In addition, the improved physiological efficiency, demonstrated in treated vines by leaf reflectance measurements (NDVI and GNDVI), and from increased WI, in the first growth stages until fruit set, could indirectly modulate the host responses (Calzarano *et al*., 2021).

In summary, reductions in the leaf stripe symptom, achieved from fertilizer mixture applications, could depend on several complex effects that interfere with mechanisms of symptom development, which operate in the early growth stages of grapevine growing seasons, and lead to the manifestation of symptoms in later stages. The findings discussed above do not bring about evidence in support of any of the three theories for the leaf stripe symptom expression; nevertheless, it further confirms the earliest observation that metabolites brought to the leaves cause leaf reactions and symptoms development (Mugnai *et al*., 1999).

THE 'EDGE' HYPOTHESIS

Considering the different modes of action of the three techniques described above, we here propose the 'edge' hypothesis as a possible explanation for the leaf stripe symptom onset and yearly fluctuation. According to this hypothesis, all grapevines affected by esca-associated pathogens, and related internal symptoms, are constantly under the influence of LSSIM, which are transported in the host vascular system and reach the canopies. However, as long as the concentration of these molecules remains below an hypothetical edge (i.e., a multi-factor threshold), the triggering of the leaf stripe symptom does not occur. The edge concept aims at identifying the current health status of each vine, which is influenced by the biotic and abiotic factors, and agronomic practices listed in Table 1.

To clarify this hypothesis and the concept of edge, four hypothetical case scenarios could apply (Figure 2). These are:

1. A vine with a fungal infection and wood symptoms, such as those found in GLSD or esca proper, does not exhibit leaf symptoms if the abundance of LSSIM remains below the edge (Figure 2A). This represents a condition favourable to the vine, which may occur when environmental factors positively affect the plant health status, raising the edge and, therefore, the tolerance to LSSIM; or during an early fungal infection, when the fungal load, wood symptoms and LSSIM are present in relatively low abundance.

2. If one or more environmental factors negatively affect the vine, for example adverse climatic conditions and/or poor nutritional status, the edge lowers. Consequently, a similar abundance of LSSIM, as in Figure 2A, is sufficient to trigger leaf symptoms (Figure 2B). Nevertheless, since the factors that are known to be involved in this process are numerous, current predictors of leaf symptoms manifestation remain uncertain.

3. As vines age and fungal infections progress, leading to increased pathogen abundance and species richness, along with wood symptomatology extent, the plants increasingly reach a stage where LSSIM levels surpass the edge (Figure 2C). This may be due to increased LSSIM abundance, unfavourable environmental conditions, and/or protracted decrease in the edge, due to prolonged stress conditions (i.e., several years of fungal pathogens activity in the wood). At this stage, leaf symptom development, year after year, becomes more probable, when compared to plants like those in Figure 2B).

4. Treating plants with $NaAsO_2$ or trunk surgery drastically affects the abundance of LSSIM, by targeting *F. mediterranea* (Figure 2D); and may improve the vines tolerance to LSSIM, by acting on vines physiology and defense response. In these cases, the edge may or may not be lowered, based on the possible direct negative effects of the treatments on host plants. Nevertheless, the abundance of LSSIM remains below the edge for multiple years post-treatment.

The fertilizer mixture may improve host nutritional status and defenses, thereby increasing the edge, instead of lowering LSSIM abundance (e.g., Figure 2A).

Every year, numerous factors play roles in tweaking the edge, which may fluctuate throughout the growing season, based on the environment and human interventions (Table 1). This may explain yearly fluctuations as well as the differences in leaf symptoms manifestation during each growing season (e.g., plants manifesting symptoms during late spring – early summer – midsummer – late summer). Environmental changes may have a

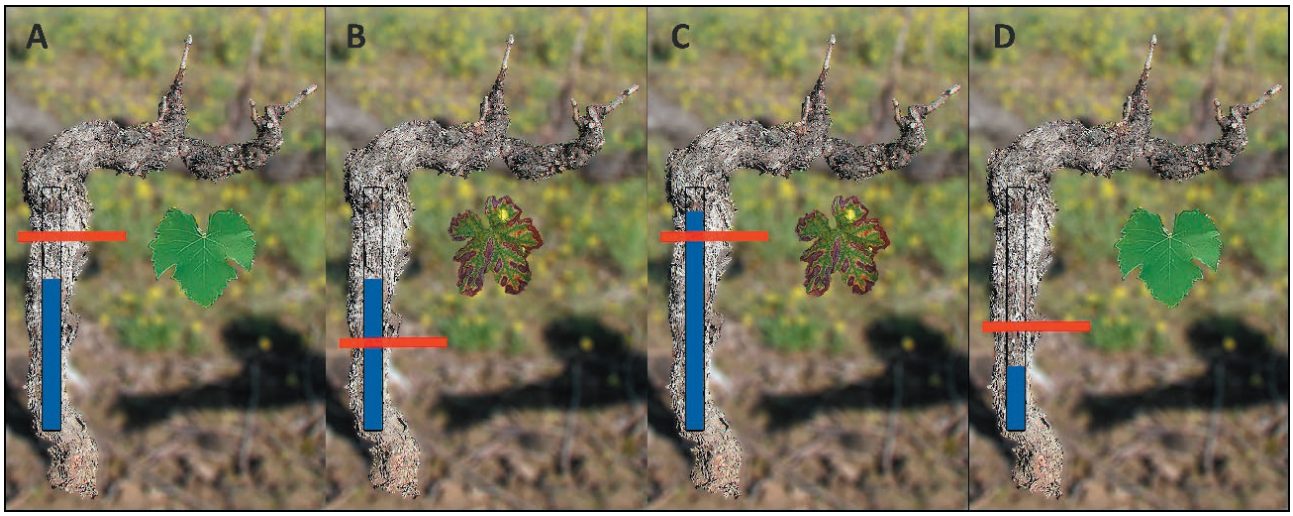

Figure 2. The results of joint effects of different factors on the edge hypothesis. Blue bars represent the hypothetical abundance of LSSIM in grapevine perennial and/or annual organs. Red bars represent the hypothetical edge, above which hosts manifest leaf symptoms. In leaf asymptomatic vines (A and D), LSSIM abundance remains below the threshold. In leaf symptomatic plants (B and C), the threshold is surpassed. Biotic and abiotic factors may act as stressors, contributing to lowering the threshold (B and D).

direct effect on the host plant and/or on the endophytic microbiome, the latter being of increasing interest in grapevine health and GTD research (Del Frari *et al*., 2019a; Bruez *et al*., 2020; Bettenfeld *et al*., 2021; Fotios *et al*., 2021; Geiger *et al*., 2021).

While it is generally accepted, but with little experimental evidence, that the triggering of the leaf stripe symptom depends on the concentration of LSSIM and the health status of each grapevine plant, the edge hypothesis aims to consolidate this correlation by giving it a name (i.e., edge) and, possibly in the future, a numerical value to the so-called health status. The edge hypothesis may be validated by identifying, qualitatively and quantitatively, the disease triggering factors (i.e., LSSIM; blue bars in Figure 2), and by giving a numerical value to the hypothetical edge (red bars in Figure 2). While the former may be determined with current technology and targeted experimental approaches (e.g., sampling pre-symptomatic leaves and other plant organs); the latter, being still an abstract concept, may require assistance from advanced computing technology (e.g., machine learning and mathematical models), that integrates information of the multiple factors known to influence the leaf stripe symptom expression (Table 1).

CONCLUSION

In this paper, we offer an overview of the three control strategies known to strongly reduce the esca leaf stripe symptom manifestation in grapevines, acknowledging an effective fourth one, that acts on the origin of the wood infection, reducing wood colonization by protecting pruning wounds (Di Marco *et al*., 2022). For obvious reasons, sodium arsenite was banned from vineyards and it is not a viable option anymore, while trunk surgery is an expensive technique, not suitable for all vine growers. Nevertheless, understanding their mode of action, seemingly very different – yet quite similar, may help us shed some light over some key features of the esca leaf stripe symptom.

Trunk surgery and sodium arsenite mostly affect the wood pathogen *Fomitiporia mediterranea* and/or related wood symptomatology, i.e., white rot. This suggests key roles of this fungus, or of the complex biological phenomena that precede or follow its degrading activity, towards leaf stripe development. Disease management strategies that heavily affect *F. mediterranea* have been shown to be an interesting medium-term solution, however, one question remains: can we control the leaf stripe symptom with $NaAsO_2$ or the Metodo Armano, treating vines apparently unaffected by *F. mediterranea* and white rot? If the answer was positive, we could certainly exclude *F. mediterranea* as key component of the leaf stripes symptom riddle. Instead, treatments effectiveness may be a result of the mitigation of LSSIM negative effects by acting on the vine physiological and biochemical processes, and defense response.

These different modes of action underline the relevance of an edge hypothesis, whereby a combination of

LSSIM abundance and multiple environmental factors that affect grapevine health status are responsible for the expression of the esca leaf stripe symptom. This hypothesis tentatively answers some questions relating to the causes of symptoms, such as symptoms onset and yearly fluctuations, yet, some of the most basic questions, e.g., the qualitative and quantitative influence of the triggering factors, remain unanswered. The esca leaf stripe symptom will certainly keep researchers busy in the years to come.

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Short Notes

Potato (*Solanum tuberosum*) - a new host for the root-knot nematode *Meloidogyne inornata*

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Summary. The tropical root-knot nematode (RKN) *Meloidogyne inornata* infects soybean, tobacco, yacon, common bean and *Anthurium andreanum*. This species was reported as the cause of damage in commercial yacon production in Brazil, and has potential to cause losses to common bean crops. Potato (*Solanum tuberosum* L.) cv. Désirée infected with *M. inornata* exhibited typical RKN galls on roots and tuber surfaces, similar to those caused by the two quarantine pathogens *M. chitwoodi* and *M. fallax,* and the non-quarantine *M. luci*. This study has indicated that *M. inornata* has considerable potential to cause severe damage to potato tubers. The potential spread of this pathogen into new areas should be assessed, as it can damage potato tubers and could be a problem for economically important crops. Ensuring pest-free seed potatoes is important to prevent dissemination and establishment of *M. inornata* in uninfested areas. Phytosanitary measures and monitoring programmes developed to prevent spread of this pest in Europe may be warranted.

Keywords. Pathogenicity, plant-parasitic nematodes.

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INTRODUCTION

Species of tropical root-knot nematodes (RKN), clade I of genus *Meloidogyne*, cause significant economic losses in several agricultural crops, including vegetables, and field and fruit crops. *Meloidogyne inornata* Lordello, 1956 was first described from soybean in Brazil (Lordello, 1956; Carneiro *et al.*, 2008), and was later detected on tobacco *Nicotiana tabacum* L., yacon *Smallanthus sonchifolius* (Poepp.) H. Rob., common bean *Phaseolus vulgaris* L. and *Anthurium andreanum* Linden ex André (Whitehead, 1968; Carneiro *et al.*, 2008; Machado *et al.*, 2013; Camara *et al.*, 2020). This nematode, reported by Camara *et al.* (2020) as the cause of damage in commercial yacon production in Brazil, also has potential to cause losses in common bean crops (Dadazio *et al.*, 2016).

Meloidogyne inornata is closely related and very similar to *M. ethiopica* and *M. luci*, and the three species are collectively referred to as the *M. ethiopica* group (Gerič Stare *et al.*, 2019). While the broad host range of *M. ethiopica* and *M. luci* overlaps (Gerič Stare *et al.*, 2017), few hosts have been reported for *M. inornata*. Given the close relationship and similarities

between the three species, it is plausible that hosts of *M. ethiopica* and *M. luci*, including potato, could also be parasitized by *M. inornata*.

Meloidogyne luci has already been found in potato fields in Portugal, and reproduced in 16 commercial cultivars with high reproduction factor values (Maleita *et al.*, 2018; Rusinque *et al.*, 2021; Şen and Aydınlı, 2021). Severe damage caused by RKN species of the tropical group had been recorded in Europe (Bačič *et al.*, 2016), and due to climate change and increased temperatures, these pests may become increasing problems in potato production in temperate regions (Širca *et al.*, 2021). Potato is an important crop in Europe and was cultivated on 1.7 million hectares (ha) in the EU-27 in 2020 (Eurostat, 2021), which corresponds to an estimated 1.7% of all arable land in the EU. The harvested production of potato in the EU was 55.3 million tonnes in 2020. The value at basic prices of raw potatoes and seed potatoes produced in the EU in 2020 was an estimated EUR 12.3 billion, which was 3.1% of the value of total EU agricultural output, but which varied among Member States (Eurostat, 2021).

The potato–*M. inornata* relationship was evaluated, due to the importance of potato production in Europe, and as a first step in assessing whether *M. inornata* is a threat in Europe. Three plants of potato Désirée were infected with 42,000 *M. inornata* eggs/plant, and uninfected plants were used as experimental controls. Plants were grown in individual 5 L pots filled with autoclaved 2:2:1 substrate containing fine-grain (MP1/G) and coarse-grain (MP4) quartz sand (Termit), and peat substrate Potgrond P (Klasmann-Deilmann). The substrate was supplemented with 5 g L^{-1} slow release fertilizer Osmocote® Exact Standard [ICL Specialty Fertilizers; N-P-K(+Mg) = 15-9-12(+2)]. The experiment was conducted from May to September 2021 in a greenhouse in Ljubljana, Slovenia (natural daylight, 19.7±2.4°C, 67.0±11.9% relative humidity). The *M. inornata* isolate used in this study was first isolated from tomato, *Solanum lycopersicum* L., in Chile and was provided by Dr. Gerrit Karssen, National Plant Protection Organization, The Netherlands.

The above ground plant parts did not show specific symptoms, but typical RKN galls were detected on the roots (Figure 1). The majority (>90%) of the tubers had characteristic symptoms of RKN infections, i.e. surface galls which were small, pimple-like quality-reducing

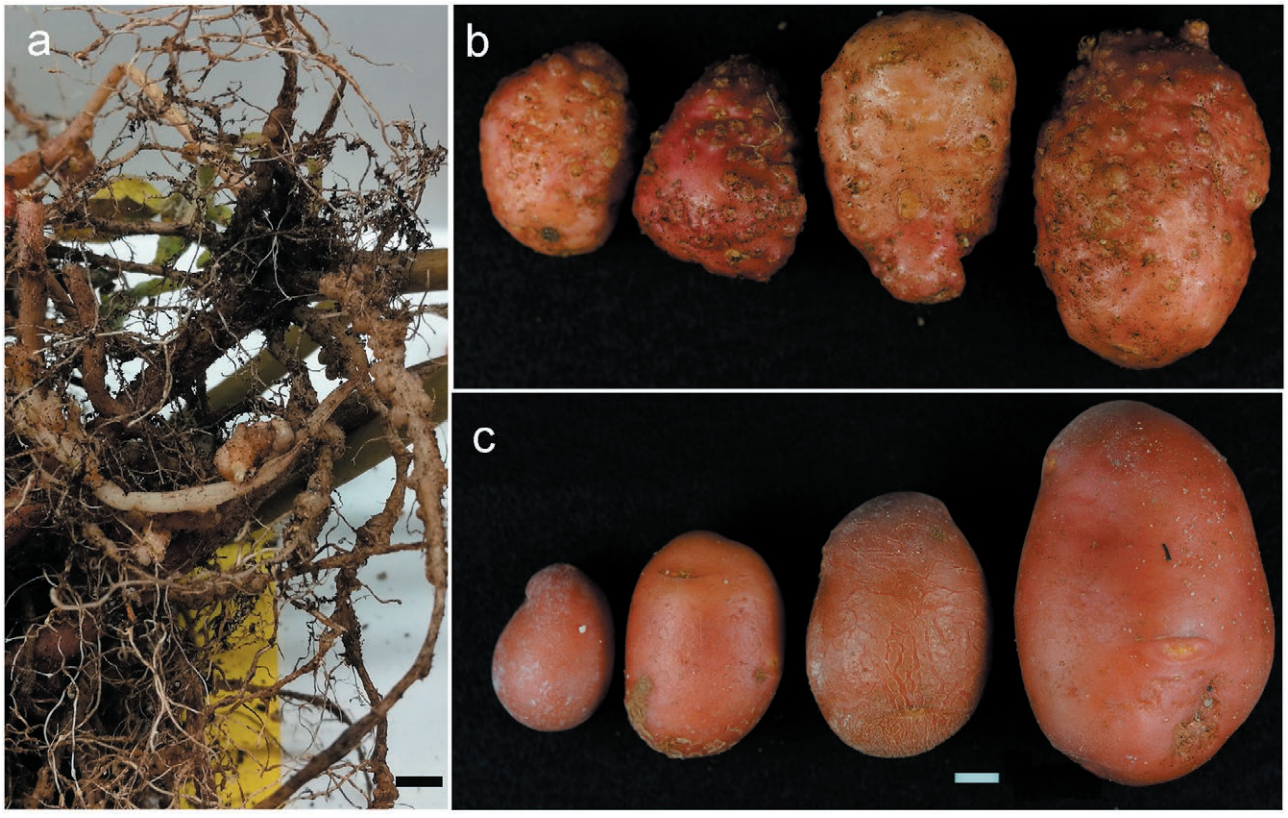

Figure 1. Symptoms of infection by the root-knot nematode *Meloidogyne inornata* on *Solanum tuberosum* 'Désirée'. a) Roots with galls. b) Tubers with surface galls. **c)** Uninfected potato tubers. Scale bars = 1 cm.

swellings on the tuber surfaces (Figure 1). A few tubers had no visible symptoms on the surfaces, although female nematodes were detected under the skins of the potato tubers. These were defined as latent infections. The infection symptoms by *M. inornata* were identical to those caused by *M. luci* (Žibrat *et al*., 2021), and by the two quarantine species *M. chitwoodi* and *M. fallax* (EPPO, 2016).

Species identification of the nematodes in the infected tubers was based on morphology of females, males and second-stage juveniles from galls and infected roots, and was confirmed by isozyme analysis of malate dehydrogenase (MDH) and esterase (EST) of individual females (Gerič Stare *et al*., 2017). The morphometric analyses found characters typical for *M. inornata*. In females, the stylets were robust, slightly curved dorsally with well-developed knobs. Perineal patterns of the females were variable (oval to squarish), with dorsal arches moderately high to high and rounded to squarish. Lateral fields were without distinct incisures, phasmids were small and located posterior to the anuses. The morphometric characters of females, males and second-stage juveniles were in agreement with previously published data for the *M. inornata* Chile isolate (Gerič Stare *et al*., 2019).

Determination of morphological characters alone and comparison with previous reports by Carneiro *et al*. (2008) and Gerič Stare *et al*. (2019) did not provide unambiguous identification, as several morphological characteristics were overlapping between sister species of the *M. ethiopica* group (Gerič Stare *et al.,* 2019). The *M. inornata* species-specific isozyme esterase pattern I3, and a non-specific malate dehydrogenase N1 were observed (Figure 2). A real-time PCR-based assay was performed to detect *M. inornata* directly in secondary tuber peels, using the *M. ethiopica* group primers (Žibrat *et al*., 2021). *Meloidogyne inornata* was detected in infected tubers with a low detection limit and high analytical sensitivity. One peel of a symptomatic tuber added to 99 uninfected peels gave Ct = 32.26 ± 0.24, and ten isolated females with attached egg mass added to a sample of 100 uninfected peels gave a Ct = 27.21 ± 0.06. Addition of one or three female(s) with attached egg mass to a sample of 100 uninfected peels weren't reliably detected across all biological and technical replicates tested. Melting temperature (Tm: 68.2–68.7°C) indicated specific DNA amplification.

The pathogenicity assay identified potato as a new experimental host (i.e. identified by artificial inoculation) of *M. inornata*. Although *M. inornata* was not detected on potato in natural field conditions in a specific geographic location, the results indicate that this nematode has considerable potential to cause severe damage to potato crops. Further studies to determine *M. inornata* reproduction factor and susceptibility of different potato cultivars are needed. The potential spread of this pathogen into new areas should be determined, as it is able to damage potato tubers and could be a threat to economically important crops. Ensuring pest-free seed potatoes is critical to prevent the dissemination and establishment of *M. inornata* to uninfested areas of Europe and elsewhere. Phytosanitary measures and monitoring programmes to prevent the introduction and spread of this pest in Europe may be warranted. Additionally, as *M. ethiopica* and *M. luci*, which are pests on the EPPO Alert List, pose a similar threat, a real-time PCR-based test to detect the *M. ethiopica* group in potatoes could be a useful tool for phytosanitary monitoring programmes.

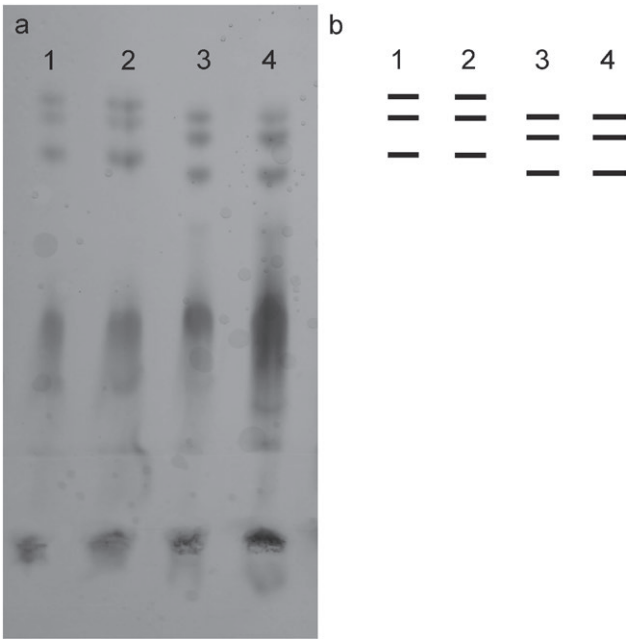

Figure 2. a) Esterase and malate dehydrogenase phenotypes, and b) schematic representation of *Meloidogyne inornata* (lanes 3 & 4). Lanes 1 & 2: *M. javanica* (reference isolate).

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Research Papers

Susceptibility of crop plants to the root-knot nematode *Meloidogyne luci*, a threat to agricultural productivity

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Summary. The root-knot nematode (RKN) *Meloidogyne luci* is included in the Alert List of the European Plant Protection Organization, because it has potential negative impacts on economically important crops. Identification of plant species/cultivars resistant to *M. luci* is important for its management. Susceptibility of 35 commercial plant species/cultivars, from nine families to a *M. luci* isolate from Portugal was evaluated in pot assays, assessing root gall index (GI) and reproduction factor (Rf) 60 d after inoculation, with tomato 'Coração-de-Boi' used as the positive susceptible experimental control. Presence/absence of RKN resistance genes was also determined in the tomato and pepper cultivars. One cultivar of cabbage, three of lettuce, ten of pepper, one of sugar beet, and all the cultivars of Cucurbitaceae (five), Fabaceae (two) and Poaceae (one) were susceptible to *M. luci* (GI = 4-5; Rf = 2.1-152.3). One cultivar each of carrot, passion fruit, lettuce 'Cocktail', cabbage 'Bacalan', 'Coração' and 'Lombarda', and spinach 'Tayto' were resistant/hypersensitive (Rf < 1; GI > 2). The tomato 'Actimino', 'Briomino', 'Veinal' and 'Vimeiro', which carried at least one copy of the *Mi-1.2* gene, were resistant to the nematode (GI = 1-2; 0.0 < Rf < 0.1). These results indicate that the tomato cultivars have potential to contribute to reduction of *M. luci* populations in agro-ecosystems and improve the crop yields.

Keywords. Gall index, pathogenicity, plant-parasitic nematodes, reproduction factor.

INTRODUCTION

Root-knot nematodes (RKN, *Meloidogyne* spp.) are plant parasites responsible for significant economic crop losses (Nicol *et al*., 2011). *Meloidogyne* includes 98 described species, which are obligate parasites of almost all vascular plants (Jones *et al*., 2013; Subbotin *et al*., 2021). Although four species (*M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica*) are considered the most common, many others have been gaining importance due as poten-

tial causes of damage to economically important crops (Elling, 2013).

Meloidogyne luci is a damaging and polyphagous RKN included in the European and Mediterranean Plant Protection Organization Alert List since 2017 (EPPO, 2017). This species has a wide host range and is a threat to agricultural productivity and ecosystem sustainability (Şen and Aydınlı, 2021). Additionally, *M. luci* shares some morphological and biochemical similarities with *M. ethiopica* and *M. inornata* (Gerič Stare *et al*., 2019), fact that has led to the misidentification of several *M. luci* populations in Europe (Gerič Stare *et al*., 2017b). In recent years, molecular information on *M. luci* has been attained, and molecular diagnostic methods for the accurate detection/discrimination of this RKN species were developed (Gerič Stare *et al*., 2019; Susič *et al*., 2020a; Maleita *et al*., 2021; Žibrat *et al*., 2021).

Meloidogyne luci was first described in 2014 from isolates originally collected from roots of lavender (*Lavandula spica* L.) in Brazil, and was maintained by periodically culturing on tomato (*Solanum lycopersicum* L. 'Santa Clara') (Carneiro *et al*., 2014). In this country, the nematode was found parasitizing broccoli (*Brassica oleracea* L.), common bean (*Phaseolus vulgaris* L.), cucumber (*Cucumis sativus* L.), kiwifruit [*Actinidia deliciosa* (A. Chev.) C.F. Liang & A.R. Ferguson], lettuce (*Lactuca sativa* L.), loofah [*Luffa cylindrica* (L.) Roem.], okra [*Abelmoschus esculentus* (L.) Moench], soybean [*Glycine max* (L.) Merr.], and yacón (*Polymnia sonchifolia* Poepp) (Carneiro *et al*., 2014; Machado *et al*., 2016; Bellé *et al*., 2016, 2019a, 2019b). Apart from Brazil, *M. luci* has been identified in Argentina, Bolivia, Chile, Ecuador, Greece, Guatemala, Iran, Italy, Slovenia, and Turkey, associated with economically important crops, ornamentals, herbs and weeds. The recorded host include aubergine (*Solanum melongena* L.), barley (*Hordeum vulgare* L.), beetroot (*Beta vulgaris* L.), broccoli, buckwheat (*Fagopyrum esculentum* Moench), carrot (*Daucus carota* L.), cauliflower (*Brassica oleracea* var. *botrytis* L.), celery (*Apium graveolens* L.), chicory (*Cichorium intybus* L.), common bean, endive (*Cichorium endivia* L.), Florence fennel (*Foeniculum vulgare* Mill.), grapevine (*Vitis vinifera* L.), groundsel (*Senecio vulgaris* L.), herb curled dock (*Rumex patientia* L.), kohlrabi (*Brassica oleracea* L.), lucerne (*Medicago sativa* L.), melon (*Cucumis melo* L.), morning glory (*Ipomoea* spp.), onion (*Allium cepa* L.), pea *(Pisum sativum* L.*),* peach [*Prunus persica* (L.) Batsch], pepper (*Capsicum annuum* L.), potato (*Solanum tuberosum* L.), pumpkin (*Cucurbita moschata* Duchesne ex Poir.), radish (*Raphanus sativus* L.), rice (*Oryza sativa* L.), rose (*Rosa* sp. L.), sedum [*Hylotelephium spectabile* (Boreau) H. Ohba], snapdragon (*Antirrhinum majus* L.), spinach (*Spinacia oleracea* L.), sunflower (*Helianthus annuus* L.), sweet corn (*Zea mays* L.), tobacco (*Nicotiana tabacum* L.), tomato, and watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) (Širca *et al*., 2004; Strajnar *et al*., 2009, 2011; Conceição *et al*., 2012; Aydınlı *et al*., 2013; Carneiro *et al*., 2014; Aydınlı and Mennan, 2016; EPPO, 2017; Gerič Stare *et al*., 2017a, 2017b; Aydınlı, 2018; Santos *et al*., 2019; Gonçalves *et al*., 2020; Susič *et al*., 2020b; Kaspary *et al*., 2021; Žibrat *et al*., 2021).

In Portugal, *M. luci* was first detected in a potato field near Coimbra, in 2013 (Maleita *et al*., 2018). Since then, it was found parasitizing roots of ornamental cabbage trees [*Cordyline australis* (G.Forst.) Endl.], the weed yellow wood sorrel (*Oxalis corniculata* L.), and tomato in subsistence farms of Coimbra district, and potato in Pico Island, Azores (Santos *et al*., 2019; Rusinque *et al*., 2021).

Although the application of pesticides may be effective for RKN control, increasing environmental and human health concerns about pesticide use have stimulated development of alternative management strategies (Wesemael *et al*., 2011). Crop rotation and use of resistant host cultivars are known to be effective on managing RKN populations. Knowledge of host response of crops and cultivars to RKN is important for successful implementation of these nematode management methods (Nyczepir and Thomas, 2009; Rashidifard *et al*., 2021).

Presence of host plant genes conferring resistance to RKN constitutes an important strategy for integrated nematode pest management. Several resistance genes have been identified from various plant sources, including the tomato gene *Mi-1.2* (Williamson *et al*., 2009). This nematode resistance gene is a well characterized example, and is effective against various *Meloidogyne* species including *M. arenaria*, *M. ethiopica*, *M. incognita*, *M. javanica* and *M. luci* (Williamson, 1998; Aydınlı and Mennan, 2019; Santos *et al*., 2020). Several studies have also demonstrated that the *Mi-1.2* gene has greater effectiveness in homozygous (MiMi) host genotypes than on heterozygous (Mimi) genotypes (Jacquet *et al*., 2005; Maleita *et al*., 2012; Santos *et al*., 2020). However, when soil temperatures exceed 28°C, resistance conferred by this gene is overcome (Dropkin, 1969; Ammati *et al*., 1986; Tzortzakakis *et al*., 2014).

In pepper, the *Me* genes *Me1*, *Me3* and *Me7*, and the dominant *N* gene, were found to be effective against *M. arenaria*, *M. incognita* and *M. javanica* (Djian-Caporalino *et al*., 2011; Wang *et al*., 2009), but their effectiveness has been shown to decrease when used intensively (Djian-Caporalino *et al*., 2011). Laboratory assays showed that resistance conferred by *Me1* could not be overcome

by virulent isolates, although virulent variants overcame *Me3* gene resistance (Castagnone-Sereno *et al*., 2001).

The primary objective of the present study was to evaluate the ability of *M. luci* to reproduce on 35 cultivated plants which are commonly cropped on subsistence farms in Portugal, and some of which are grown in rotations with potato. In addition, the presence of RKN resistance genes was investigated in cultivars of tomato (*Mi-1.2* gene) and pepper (*Me*1, *Me*3, *Me7* and *N* genes), which were assessed for their host status to *M. luci*, to evaluate whether resistance in these plants was related to presence of these gene markers.

MATERIALS AND METHODS

Nematode isolate

An isolate of *M. luci*, originally obtained from a potato field in Coimbra, Portugal (Maleita *et al*., 2018), was maintained on tomato 'Coração-de-Boi', in a temperature-controlled growth chamber (23 ± 2°C) with 12 h daily light periods. The species identification of the isolate was confirmed by esterase phenotype analysis (Maleita *et al*., 2018).

Host status

The responses to *M. luci* of 35 commercial plant cultivars (Table 1), representing 15 species from nine botanical families, were evaluated under controlled conditions (23 ± 2ºC; 12 h daily light periods). Plants were grown from seeds in Petri dishes containing water-soaked filter paper, at 25ºC in the dark. After germination, seedlings were individually transplanted into 5 cm diam. plastic pots filled with a mixture of sterilised sandy loam soil, sand and substrate (1:1:2). After four weeks, the seedlings were transplanted into 10 cm diam. pots containing a mixture of sterilised sandy loam soil, sand and substrate (1:1:1).

Nematode inoculum was obtained from infected tomato 'Coração-de-Boi' roots, through extraction of eggs using a 0.52% sodium hypochlorite (NaOCl) solution (Hussey and Barker, 1973). Five plants from each plant species/cultivar were inoculated with 5000 *M. luci* eggs (initial population density, Pi). Tomato 'Coração-de-Boi' was included as susceptible control of inoculum viability, and non-inoculated plants of each cultivar were included as negative controls. The pots were arranged in a completely randomized design in a growth chamber, which was set at 23 ± 2°C, 12 h daily photoperiod, and ±60% relative humidity, and the plants were watered each day.

Sixty days after inoculation, the plants were harvested and the root systems washed free of soil substrate. Numbers of galls/plant were recorded, and gall indices (GI) were assessed using a 0–5 scale (0 = no galls, 1 = 1–2, 2 = 3–10, 3 = 11–30, 4 = 31–100, 5 ≥ 100 galls) (Taylor and Sasser, 1978). Nematode eggs were extracted from each plant, as described above (Hussey and Barker, 1973), to determine the final population density (Pf), and the reproduction factor (Rf = Pf/Pi) was calculated. Host susceptibility to *M. luci* was assessed based on GI and Rf (Sasser *et al*., 1984).

DNA analyses for the Mi-1.2*,* Me1*,* Me3*,* Me7 *and* N *genes*

Plant material

Roots of four tomato cultivars ('Actimino', 'Briomino', 'Veinal' and 'Vimeiro') and ten pepper cultivars ('Amarelo', 'Celta', 'Claudio', 'Rainbom', 'Rialto', 'Solero', 'Tauro', 'Torpedo', 'Vermelho' and 'Yoacali') were assessed for *Mi*-mediated resistance in tomato and *Me*-mediated resistance in pepper. The pepper accessions 'Yolo Wonder', lacking the RKN resistance genes *Me1*, *Me3*, *Me7* and *N*, and the double haploid pepper lines 'DH149' (with the *Me3* resistance gene) and 'DH330' (carrying the *Me1* resistance gene), were included in this analysis as experimental controls. No positive control for the *N* gene was included.

DNA extractions from plants

DNA from tomato and pepper plants was extracted using the kit DNeasy Plant Mini Kit (Qiagen) for purification of total DNA from plant tissues, with some modifications. Instead of using liquid nitrogen, plant roots were ground on ice, after being frozen overnight at -80°C. Genomic DNA concentration was determined in a Nanodrop 2000c spectrophotometer (ThermoScientific), and the samples were stored at -20°C until PCR analyses.

For the host accessions Yolo Wonder and DH149, plant DNA extraction was carried out from leaf tissue, and for the accession DH330 from seeds, for both tissue types using the protocol of Maleita *et al*. (2012).

Detection of the *Mi-1.2* gene

DNA amplification was carried out using the Mi23 marker to assess the present/absence of the *Mi* gene on tomato plants. DNA amplification was carried out as described by Seah *et al*. (2007), using the primers Mi23F

(5'-TGG AAA AAT GTT GAA TTT CTT TTG-3') and Mi23R (5'-GCA TAC TAT ATG GCT TGT TTA CCC-3'). PCR reactions were carried out as described in Maleita *et al.* (2012), and were analysed using 1.5% agarose gel electrophoresis with 1× TBE buffer and staining with GreenSafe (NZYTech).

Detection of *Me1*, *Me3*, *Me7* and *N* genes

Amplifications of the markers linked to the *Me1* and *Me7* genes, and the *Me3* gene as a SCAR, were carried out as described by Djian-Caporalino *et al.* (2007), using the primers CD-F/R (5'-GAA GCT TAT GTG GTA MCC-3' and 5'-GCA AAG TAA TTA TAT GCA AGA GT-3') for *Me1* and *Me7*, and B94-F/R (5'-GCT TAT CAT GGC TAG TAG GG-3' and 5'-CGG ACC ATA CTG GGA CGA TC-3') for *Me3*. Amplification of the marker linked to the *N* gene as a SCAR (forward 5'-AAT TCA GAA AAA GAC TTG GAA GG-3' and reverse 5'-TAA AGG GAT TCA TTT TAT GCA TAC-3') was carried out as described by Wang *et al.* (2009). The PCR products were analysed on 1.5-3% agarose gels for the *Me1*, *Me3* and *Me7* genes, or on a 15% polyacrylamide gel in 1× TBE buffer for the *N* gene, which were stained with GreenSafe.

Data analyses

Statistical significance between the different plant species/cultivars was obtained, for each parameter, using Analysis of Variance (ANOVA), after checking assumptions of normality (Shapiro–Wilk test) and equality (Levene's test). *Post-hoc* Fisher's Least Significant Differences (LSD) test was applied to test for differences between the plant species/cultivars. Statistical analyses were carried out using Statsoft Statistica version 7 for Windows.

RESULTS

Nematode reproduction

Meloidogyne luci reproduced (Rf > 1) on 24 of 35 the plant species/cultivars, although considerable variation was found among replicates (Table 1). Cabbage 'Kale', faba bean, maize, lettuce ('Batavia', 'Butterhead' and 'Folha-de-Carvalho'), pea 'Maravilha D'América', pepper ('Amarelo', 'Celta', 'Cláudio', 'Rainbom', 'Rialto', 'Solero', 'Tauro', 'Torpedo', 'Vermelho' and 'Yoacali'), pumpkin 'Havana F1', beetroot, sweet melon 'Galia F1', tomato 'Coração-de-Boi', watermelon 'Sugar Baby', and zucchini ('Black Beauty' and 'Nova Zelândia') were classified as susceptible hosts, with $2.1 \leq Rf \leq 152.3$ and $GI \geq 4$ (Table 1). Additionally, seven cultivars were classified as resistant/hypersensitive, including cabbage ('Bacalan', 'Coração' and 'Lombarda'), carrot, lettuce 'Cocktail', spinach 'Tayto', and passion fruit, with $0.0 \leq Rf \leq 0.9$ and $GI \geq 4$ (Table 1). Four tomato cultivars ('Actimino', 'Briomino, 'Veinal' and 'Vimeiro') were classified as resistant ($0.0 \leq Rf \leq 0.1$ and $1 \leq GI \leq 2$; Table 1).

Statistically significant differences in *M. luci* reproduction were detected between the different plant species/cultivars ($P \leq 0.05$, Fisher's LSD test). Calculation of Rf across species/cultivars showed that the most susceptible hosts were (in order of susceptibility; Table 1): tomato 'Coração-de-Boi' > zucchini 'Nova Zelândia' > sweet melon 'Galia F1' > pepper 'Cláudio' > pepper 'Torpedo' > Pepper 'Vermelho' > Zucchini 'Black Beauty' > Pepper 'Tauro' > Pepper 'Amarelo' > Pea 'Maravilha d'América' > Lettuce 'Flor-de-Carvalho' > Pumpkin 'Havana F1' > Pepper 'Solero' > Pepper 'Rialto' > Faba bean > Pepper 'Celta' > Sugarbeet > Pepper 'Raimbom' > Lettuce 'Butterhead' > Maize > Lettuce 'Batavia' > Watermelon 'Sugar Baby' > Pepper 'Yoacali' > Cabbage 'Kale' > Lettuce 'Cocktail' > Spinach 'Tayto' > Cabbage 'Lombarda' > Cabbage 'Coração' > Carrot > Passion Fruit > Tomato 'Actimino' > Cabbage 'Bacalan' > Tomato 'Briomino' > Tomato 'Vimeiro' > Tomato 'Veinal'.

Detection of Mi-1.2 *gene in tomato*

Amplification of the Mi23 marker was carried out using DNA from the tomato genotypes, resulting in one band of approx. 380 bp, associated with homozygous resistant genotypes (MiMi), for 'Actimino' and 'Briomino'. This confirmed the presence of the *Mi-1.2* gene. Two bands of 430 bp and 380 bp were associated with the heterozygous Mimi genotypes in 'Veinal' and 'Vimeiro' (Mimi) (Figure 1).

Detection of Me1, Me3, Me7 *and* N *genes in pepper*

Amplification of the SCAR_B94 marker using DNA from pepper cultivars resulted in a single DNA band of approx. 240 bp for the pepper accession 'DH149', indicating the presence of the *Me3* gene, and a band of approx. 220 bp for the remaining pepper cultivars ('Amarelo', 'Celta', 'Cláudio', 'Raimbom', 'Rialto', 'Solero', 'Tauro', 'Torpedo', 'Vermelho', 'Yoacali' and 'Yolo Wonder'), representing the absence of this gene in these cultivars (Figure 2).

Table 1. Means of GI, Pf and Rf (see footnote) for different cultivated plants inoculated with the root-knot nematode *Meloidogyne luci,* 60 d after inoculation with 5000 eggs/plant in a pot assay conducted in a growth chamber (23 ± 2°C, 12 h daily photoperiod, ±60% relative humidity).

Family Species (Common name)	Cultivar	GI[a]	Pf[b]	Rf[c]	Host status[d]
Apiaceae					
Daucus carota L. (Carrot)	-	4	1740 ± 3098	0.4^{p,q}	RH
Asteraceae					
Lactuca sativa L. (Lettuce)	Batavia	5	28867 ± 10753	5.8^{l,m}	S
	Butterhead	5	45720 ± 3293	9.1^{l}	S
	Cocktail	5	4531 ± 2075	0.9^{n,o,p}	RH
	Folha-de-Carvalho	5	167067 ± 44537	33.4^{f,g,h}	S
Brassicaceae					
Brassica oleracea L. (Cabbage)	Bacalan	5	82 ± 168	0.1^{p,q}	RH
	Coração	5	805 ± 683	0.1^{p,q}	RH
	Lombarda	5	713 ± 160	0.2^{p,q}	RH
	Kale	5	10587 ± 7377	2.1^{n,o}	S
Chenopodiaceae					
Beta vulgaris L. (Beetroot)	-	5	77680 ± 21389	15.5^{j,k}	S
Spinacia oleracea L. (Spinach)	Tayto	5	2437 ± 3646	0.5^{o,p,q}	RH
Cucurbitaceae					
Citrullus lanatus (Thunb.) Matsum & Nakai (Watermelon)	Sugar Baby	5	13387 ± 3530	2.7^{m,n}	S
Cucumis melo L. (Sweet melon)	Galia F1	5	313080 ± 50659	62.6^{c}	S
Cucurbita moschata Duchesne ex Poir. (Pumpkin)	Havana F1	5	162667 ± 24057	32.5^{f,g,h}	S
C. pepo L. (Zucchini)	Black Beauty	5	214320 ± 20273	42.9^{d,e,f}	S
	Nova Zelândia	5	425520 ± 51288	85.1^{b}	S
Fabaceae					
Pisum sativum L. (Pea)	Maravilha D'América	5	172267 ± 47378	34.5^{f,g,h}	S
Vicia faba L. (Faba bean)	-	4	155296 ± 93023	31.1^{h,i}	S
Passifloraceae					
Passiflora edulis Sims (Passion Fruit)	-	4	4 ± 4	0.0^{p,q}	RH
Poaceae					
Zea mays L. (Maize)	-	4	42400 ± 9879	8.5^{l}	S
Solanaceae					
Capsicum annuum L. (Pepper)	Amarelo	5	175680 ± 41311	35.1^{f,g}	S
	Celta	5	94720 ± 9545	18.9^{i,j}	S
	Cláudio	5	272147 ± 37839	54.4^{c,d}	S
	Raimbom	5	55527 ± 49571	11.1^{k,l}	S
	Rialto	5	160960 ± 26478	32.2^{g,h}	S
	Solero	5	161920 ± 25911	32.4^{f,g,h}	S
	Tauro	5	194480 ± 63049	38.9^{e,f,g}	S
	Torpedo	5	260160 ± 30145	52.0^{c,d}	S
	Vermelho	5	240533 ± 57283	48.1^{d,e}	S
	Yoacali	5	11653 ± 2699	2.3^{m,n}	S
Solanum lycopersicum L. (Tomato)	Actimino	2	197 ± 107	0.1^{p,q}	R
	Briomino	1	19 ± 29	0.0^{q}	R
	Coração-de-Boi	5	761616 ± 86004	152.3^{a}	S
	Veinal	1	0 ± 0	0.0^{q}	R
	Vimeiro	1	20 ± 44	0.0^{q}	R

[a] GI = Gall Index (0-5): 0 = no galls, 1 = 1-2, 2 = 3-10, 3 = 11-30, 4 = 31-100, 5 ≥ 100 galls/root system.
[b] Pf = final population density. Data are means of five replicates (except for *Passiflora edulis*, four replicates) ± standard deviation. Means in this column followed by the same combination of letters do not differ ($P > 0.05$), according to the *Post hoc* Fisher's Least Significant Differences test.
[c] Rf (Reproduction factor) = Pf/initial population density (5000 eggs).
[d] Host status categories: S = susceptible (GI > 2, Rf > 1), RH = resistant/hypersensitive (GI > 2, Rf ≤ 1), R = resistant (GI ≤ 2, Rf ≤ 1) (Sasser *et al*., 1984).

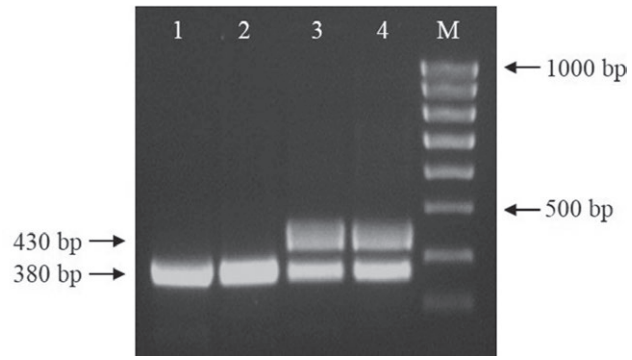

Figure 1. DNA amplification products of tomato (*Solanum lycopersicum*) using the Mi23 markers linked to the *Mi-1.2* gene. Bands 1, 'Actimino'; 2, 'Briomino'; 3, 'Veinal'; 4, 'Vimeiro'; M, DNA marker; (Hyper-Ladder IV, Bioline).

Similarly, amplification of the SCAR_CD produced a single DNA fragment of approx. 160 bp, implying absence of the *Me1* and *Me7* genes in all the pepper cultivars, with the exception of pepper 'DH330'. This accession was used as a positive control of the *Me1* gene, and displayed a band of approx. 100 bp, confirming the presence of the gene (Figure 2).

Amplification of the SCAR marker linked to the *N* gene resulted in a band of approx. 330 bp similar to that expected in the negative control 'Yolo Wonder'. This indicated absence of the *N* gene in all the assessed pepper cultivars (data not shown for 'Yoacali' and 'Raimbom'; Figure 3).

DISCUSSION

In this study, the *M. luci* isolate reproduced (Rf > 1) on 24 (69%) of the 35 plant species/cultivars assessed. *Cucurbitaceae* and *Fabaceae* species/cultivars were susceptible to *M. luci*. However, watermelon 'Sugar Baby' displayed a lower reproduction factor (Rf = 2.7) that was less than the other cucurbitaceous cultivars. Aydınlı *et al.* (2019) assessed the susceptibility of five pumpkin genotypes to *M. luci*, measuring nematode reproduction as a percentage of the most susceptible genotype, and found that two genotypes were moderately resistant. All genotypes allowed significant egg production, which was 3.1 to 6.9 fold greater than the initial population density (5000 eggs), contributing to the build-up of the *M. luci* populations (Aydınlı *et al.*, 2019). Sen and Aydınlı (2021) showed that watermelon 'Charleston Gray' was a good host for *M. luci* reproduction (Rf = 2.5), while 'Crimson Sweet' was a poor host (Rf = 0.52). Gerič Stare *et al.* (2017b) reported that watermelon 'Charleston Gray' was

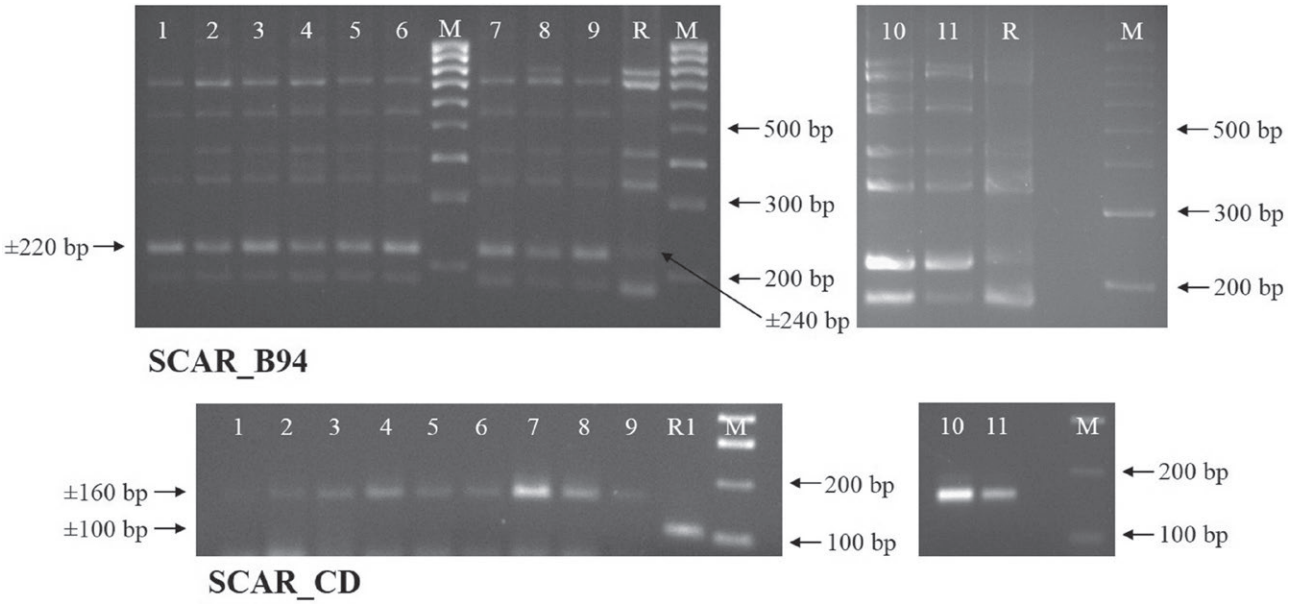

Figure 2. DNA amplification products from pepper cultivars (*Capsicum annuum*), using SCAR_B94 linked to the *Me3* gene and SCAR_CD linked to *Me1* and *Me7*. Band 1, 'Yolo Wonder'; 2, 'Amarelo'; 3, 'Vermelho'; 4, 'Celta'; 5, 'Cláudio'; 6, 'Rialto'; 7, 'Solero'; 8, 'Tauro'; 9, 'Torpedo'; 10, 'Yoacali'; 11, 'Raimbom'; R, 'DH149'; R1, 'DH330'; M, DNA marker (HyperLadder IV, Bioline).

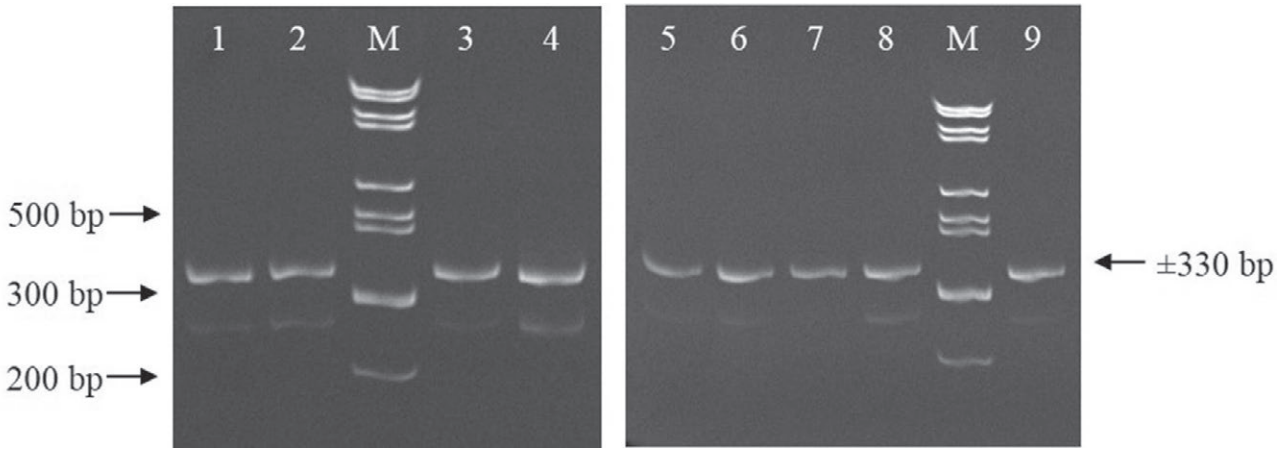

Figure 3. DNA amplification products from pepper cultivars (*Capsicum annuum*), using SCAR linked to the *N* gene. Band 1, 'Cláudio'; 2, 'Celta'; 3, 'Amarelo'; 4, 'Yolo Wonder'; 5, 'Tauro'; 6, 'Solero'; 7, 'Vermelho'; 8, 'Rialto'; 9, 'Torpedo'; M, DNA marker (HyperLadder IV, Bioline).

a poor host for two populations of *M. ethiopica* respectively from Brazil and Africa, and a Slovenian population of *M. luci* (Rf < 1), while Carneiro *et al*. (2003) reported that watermelon 'Charleston Gray' was a good host for *M. ethiopica* and a non-host of *M. luci*. Watermelon cultivars have been described as poorer hosts of RKN, such as *M. incognita* and *M. javanica*, than their Cucurbitaceae counterparts (López-Gómez *et al*., 2016). On the other hand, plant species such as *Luffa cylindrica* (L.) Roem. (Cucurbitaceae) (Bellé *et al*., 2019a) or *Phaseolus vulgaris* L. (Fabaceae) (Bellé *et al*., 2016; 2019b; Sen and Aydınlı, 2021) have been described as highly susceptible to *M. luci*, and displayed considerably high Rf values. Other *Meloidogyne* spp., such as *M. arenaria*, *M. hispanica*, *M. incognita* and *M. javanica,* were also found parasitizing cucurbitaceous and fabaceous plants, so these plants can be generally classified as good hosts of RKN (Hillocks *et al*., 1995; Anwar and McKenry, 2010; Maleita *et al*., 2012; López-Gómez and Verdejo-Lucas, 2014; Aydınlı *et al*., 2019). Conceição *et al*. (2012) demonstrated that maize (Poaceae) was susceptible to *M. luci* (Rf = 8.5), although the cultivars 'Apex' and 'Merit' were considered poor hosts (Rf = 0.44 and 0.09, respectively), whereas 'Otello' and 'Sy Lucroso' were found to be good hosts (Sen and Aydınlı, 2021). Other poaceous crops have also been reported as suitable hosts for other *Meloidogyne* spp., including *M. graminicola, M. hispanica* and *M. kikuyensis* (Maleita *et al*., 2012; Onkendi *et al*., 2014).

Carrot (Apiaceae) and passion fruit (Passifloraceae) have been referred to as good hosts for RKN reproduction, including for *M. incognita* (Anwar and McKenry, 2010; Khan *et al*., 2017). In the present study, however, low reproduction of *M. luci* was obtained in carrot (Rf = 0.4) and passion fruit (Rf = 0.0). Similarly, carrot and parsley (Apiaceae) were classified as poor hosts by Sen and Aydınlı (2021), because they supported low levels of *M. luci*. The susceptibility of a greater number of plants within these two families should be evaluated, to further elucidate host suitability of Apiaceae and Passifloraceae plants to *M. luci*. In addition, brassicas are either poor or average hosts for *Meloidogyne* spp. (Anwar and McKenry, 2010; Carneiro *et al*., 2000; Maleita *et al*., 2012; Sen and Aydınlı, 2021), including broccoli (*Brassica oleracea* L. 'Italica'), which was previously identified as a host for *M. luci* (Carneiro *et al*., 2014). In the present study, Brassicaceae cultivars, except for 'Kale', were resistant/hypersensitive, indicating that they are non-efficient hosts that endure significant nematode damage (GI > 2) despite the nematode not actually reproduced (Rf<1) (Canto-Sáenz, 1985). Although some brassicas may not be good hosts for *M. luci*, caution should be taken when using cabbage as cover crops, as some cultivars may support nematode reproduction, and their use must be limited to non-infested soils or soils with low nematode population densities (Sen and Aydınlı, 2021).

Substantial Rf values variation occurred among hosts in the Chenopodiaceae, Asteraceae and Solanaceae. Solanaceous plants, including pepper, potato and tomato, are considered good or very good *M. luci* hosts (Širca *et al*., 2004; Carneiro *et al*., 2014; Maleita *et al*., 2018; Santos *et al*., 2019; Sen and Aydınlı, 2021). In the present study, the assessed pepper cultivars displayed mostly high Rf values, and were all classified as susceptible to *M. luci*, which is in accordance with the results obtained with SCAR_CD and SCAR_B94 markers and the marker linked to the *N* gene. These results indicated that the *Me1*, *Me3*, *Me7* and *N* genes were absent from these pepper cultivars.

The tomato 'Coração-de-Boi', used as the susceptible control, displayed the greatest Rf (152.3) among all the plant species/cultivars assessed. In contrast, the other tomato cultivars ('Actimino', 'Briomino', 'Veinal' and 'Vimeiro') exhibited low Rf values, and were classified as resistant (GI ≤ 2, Rf ≤ 1). The molecular assays indicated that these plants carried at least one copy of the *Mi-1.2* gene. Although this resistance has been described as more efficient in the presence of two copies of the gene (MiMi), as opposed to one copy (Mimi) (Jacquet *et al*., 2005; Maleita *et al*., 2012; Santos *et al*., 2020), the Rf values did not vary significantly according to the genotype displayed (Rf = 0.0 to 0.1). These resistant tomato cultivars could be used to inhibit nematode population increase and reduce losses due to *M. luci*. The cultivars may have potential for inclusion in integrated nematode management programme. However, the duration of resistance can be limited due to selection to virulence in nematode populations by continuous exposure to resistant plants or changes in the environmental conditions (Dropkin, 1969). Additionally, assessment of *Mi*-tomato plants for susceptibility to local populations before their field use is advisable, because natural virulent nematode population may be present (Maleita *et al*., 2012; Aydınlı and Mennan, 2019).

The present study has confirmed that *M. luci* is a polyphagous species with a wide host range which includes plants from different families. This indicates that control strategies based on crop rotations could be ineffective against this RKN, if susceptible plants are among rotation candidates. Although the pot experiment in this study was not repeated, due to the high number of plant species/cultivars assessed, the results show that most of the plants were susceptible to *M. luci*, while 11 were resistant or resistant/hypersensitive. Variability was observed among replicates of each cultivar, but host status was consistent among these replicates. Inoculum viability was also confirmed by the high Rf values obtained on tomato 'Coração de Boi'.

Brassicas prevented nematode reproduction in three of four cultivars, with low Rf values. Cabbage 'Bacalan', 'Coração' and 'Lombarda' may be suitable as rotation crops since they are widely used in Portugal. Likewise, the resistant tomato 'Actimino, 'Briomino', 'Vimeiro' and 'Veinal' can also be recommended for the management of *M. luci* populations. Knowledge on the susceptibility of local cultivars to RKN, along with the use of these resistant cultivars in fields where susceptible hosts are grown, could reduce *M. luci* population densities and increase crop yields.

This study has highlighted the importance of identification of *Meloidogyne* resistant local cultivars, to be used in crop rotations as an efficient strategy to maintain agricultural sustainability.

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Abstract

Abstracts of invited, oral and poster papers presented at the 16th Congress of the Mediterranean Phytopathological Union, April 4–8, 2022, in Limassol, Cyprus

The 16th Congress of the Mediterranean Phytopathological Union (MPU2022) took place in Limassol, Cyprus from 4–8 April, 2022. Plant pathologists from the region and beyond celebrated the 60th anniversary of the Union and the 62 years of publication of *Phytopathologia Mediterranea* as a top level plant pathology journal.
MPU2022 entitled "Safeguarding Mediterranean Plant Health" promoted dissemination of the latest scientific advances, and facilitated dialogue and collaboration between researchers interested in all aspects of Phytopathology. This conference also addressed wider perspectives in scientific fields not previously explored at MPU Congresses, including abiotic stresses, biopesticides, forest pathology, smart agriculture and gender equality in research, funding, teaching and professional development.
Papers presented at the Congress included 83 oral presentations, including 15 keynote presentations from leading scientists, and 63 poster presentations, covering all areas of plant pathology and plant stress research, from 130 participants. Five special sessions in cutting edge subjects were organised. A special session was organised jointly with the Arab Society of Plant Protection. All these scientific contributions are part of this issue.

KEYNOTE LECTURES AND INVITED TALKS

Euphresco: a platform to link research and policy in the Mediterranean area and beyond. B. GIOVANI, I. AL-JBOORY, M. CHOUIBANI, N. HORN, L. MUGNAI and A.M. D'ONGHIA. *Euphresco network for phytosanitary research coordination and funding. E-mail: bgiovani@euphresco.net*

Mediterranean agriculture, forests and other environments are threatened by numerous quarantine and emerging pests. The negative impacts caused by these pests are expected to increase due to acceleration of global trade and climate change, that respectively favour movement of these organisms over long distances and facilitate their adaptation to and establishment in new environments. In the face of these challenges, the Mediterranean region is particularly vulnerable, due to the shortcomings in national quarantine systems, limited expertise and phytosanitary infrastructures, and the lack of funds for research activities in support of statutory plant health. The strengthening of research in the field of plant health is one of the main challenges that countries in the Mediterranean region have to address. The diversity of priorities, both for pests and infrastructures and skills, has reduced the impacts of national efforts, but plant health challenges require rethinking of the organization of research activities in all countries and their coordination, to increase efficiency and impacts. Coordination at the Mediterranean level will reduce fragmentation of actions; it will promote convergence of national programmes; it will build critical mass. Several Mediterranean organizations and initiatives have joined forces to improve international collaboration in, and coordination of, research efforts on plant health and plant protection for Mediterranean countries. The various activities undertaken to date, and the results of discussions with Mediterranean country representatives, will be presented.

CIHEAM policy in plant health to enhance food security in the Mediterranean region. A.M. D'ONGHIA. *Centre International des Hautes Etudes Agronomiques Méditerranéennes – Mediterranean Agronomic Institute of Bari, Via Ceglie 9, 70010 Valenzano (BA), Italy. E-mail: donghia@iamb.it*

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Most pests and diseases affecting Mediterranean crops are seriously compromising food security, and the sustainability of rural populations in several countries in the Mediterranean region. The importance of Plant Health has been clearly highlighted by the United Nations, that declared 2020 as the International Year of Plant Health. CIHEAM, an intergovernmental Organization of 13 Member States, has a long experience in plant health, which is one of the main pillars of the CIHEAM Strategic Agenda 2025 – adopted in 2016 by the Ministries of Agriculture of CIHEAM Member States. This is approached through: training of researchers, officers, professionals; applied research linking local scientists with the international research communities; participatory governance that encourages discussions and interactions among scientific, institutional and private stakeholders; and cooperational development of programmes enhancing country capacity building and raising of awareness. The CIHEAM Institute of Bari, in particular, has more than 30 years of experience in plant health, implementing and financing numerous international research initiatives in the Mediterranean region and neighbouring countries (e.g. Iran, Sultanate of Oman, Iraq). It is also engaged in initiatives funded by the Italian Cooperation, addressing plant health in several countries (in the Mediterranean, Balkan, African, Near eastern and Middle eastern regions). These initiatives have delivered institutional capacity building and technical assistance to many officials of national and local entities. Furthermore, in line with the statutory mission of CIHEAM Bari (higher education, research and development cooperation), since 1985 the Institute has delivered specialized and post-graduate diplomas on Integrated Pest Management to thousands of trainees, from over 50 countries. Particular attention of CIHEAM is on early surveillance, detection and control of transboundary pests and diseases which may seriously threaten crops, environments and sustainability of rural populations (e.g. *Xylella fastidiosa*). The achieved results have demonstrated that it is possible to ensure movement towards sustainable production if quarantine measures are strengthened and comprehensive phytosanitary management is applied. CIHEAM initiatives are therefore focusing on harmonizing national rules with the EU technical/ phytosanitary/legal standards, in order to safeguard agriculture and promote improvement in domestic and export trading of safer food in conformity with Governments' strategic plans.

Plant biology and climate emergency. D. INZE. *VIB-UGent Center for Plant Systems Biology, Belgium. E-mail: dirk.inze@psb.vib-ugent.be*

The world is experiencing an unprecedented climate crisis that requires urgent action at all fronts. Plant biology offers great opportunities for adapting to climate change and to mitigate accumulation of greenhouse gasses. Higher temperatures and longer drought episodes will greatly reduce crop productivity in many areas, including the Mediterranean basin. Understanding how plants adjust their growth and development in response of environmental cues such as drought is the first step towards breeding and/or engineering stress tolerant crops. This paper illustrated the challenges of achieving this ambitious goal, by research on drought responses in maize. The molecular and phenotypic comparison of drought response of maize plants cultivated in growth chamber, greenhouse and field conditions highlighted the importance of applying an experimental 'lab to field to lab' approach. Furthermore, drought responses involve many interacting genes. Genome editing offers new perspectives to tackle complex multi-genic traits such as drought and to engineer drought tolerant crops. Plants have evolved to efficiently use CO_2 for growth and development. Selection of plants with improved CO_2 sequestration capabilities above ground and below ground is likely to become a valuable tool to combat climate change. These plants should also be resilient to other environmental stresses, and show a low dependency on fertilizers. Various approaches to develop such plants for climate emergency were outlined.

Gender in plant health and pathology: Exploring the research agenda. M. VAN DER BURG. *Gender Studies, Social Sciences, Wageningen University, NL. E-mail: margreet.vanderburg@wur.nl*

This presentation takes the audience on a short journey to explore gender in the plant health and pathology research agenda. First explained is the importance of the EU efforts to promote gender integration in research organisations in both their operational functioning (culture, recruitment and career, work-life balance, and governance) as well as their research and educational content. Since 2022 the EU made having a Gender Equality Plan (GEP) conditional to EU funding. Second, it is illustrated with historical and contemporary examples how gender differences are deeply engrained in plant health and pathology. These examples clearly under-

line the awareness of the societal importance of this research. Though, it also has traditionally overlooked its bias in campaigns which unconsciously reflected and reinforced stereotypes of gender behaviour and white supremacy; it also did not anticipate in preventing harmful social impacts. Third, a transgression from sole component approaches to socially connected system approaches (e.g., pest and weed integrated management) are providing opportunities to include social context and impacts into the research design and research circle. It helps to reckon with that wherever interventions on farm or natural resources environment are suggested, all other parts in the system from the micro to the macro level will be influenced. This certainly includes the people who are directly or indirectly affected, often differently according to gender or other social axes of inequality. To contribute to equality is definitely mandatory to address social impact and people as actors to anticipate on contributing to inequality and transform research accordingly.

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Molecular host-microbe interactions in plants: from microbes to host innate immunity. P.F. SARRIS. *Department of Biology, University of Crete, 714 09 Heraklion, Crete, Greece; Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology-Hellas, Heraklion, Crete, Greece; Biosciences, University of Exeter, Exeter, United Kingdom. E-mail: p.sarris@imbb.forth.gr*

In their long history of interspecific interaction, plants and invading microorganisms – beneficial or pathogenic – are constantly involved in complex co-evolutionary dynamics. Several plant-associated microorganisms have acquired sophisticated colonization mechanisms to manipulate the physiological processes of their hosts. These mechanisms also include specialised proteins, known as "effectors", that are utilized by microbes to promote colonization. However, their molecular function(s) and their targets in host cells, are largely unknown for many of these effectors. This is a major question in Molecular Plant-Microbe Interactions (MPMI) research. However, effector activities/functions can be used as tools to identify important components of plant innate immunity and physiology, that could lead to innovative strategies for crop improvement. Plants have also evolved well-organized and complex innate immunity systems, to recognize pathogen invasion. The recognition occurs through perception of microbial structural patterns, and the secreted effector proteins, by specialized transmembrane or intracellular receptors of host cells. The microbial effectors are mainly recognised, in resistant hosts, by members of the (Nucleotide-binding Leucine-rich Repeat (NLR) receptors. The elucidation of plant NLR molecular functions will assist future development of novel and/or synthetic immune receptors with new recognition capacities in crop plants.

Enabling genome editing for European agriculture: will scientific evidence prevail? D. INZE. *VIB-UGent Center for Plant Systems Biology, Belgium. E-mail: dirk.inze@psb.vib-ugent.be*

Agriculture and food production must become more sustainable in a world facing growing population under changing climate conditions and environmental degradation. All possible approaches, including improved plant breeding technologies, are essential to address these challenges. To develop new crop varieties, scientists and plant breeders need to have access to the array of breeding tools. The most recent addition is precision breeding, also known as genome editing, with a preference for CRISPR. This technology allows scientists and breeders to develop desired crop varieties in rapid, relatively simple and directed ways compared to older breeding technologies. However, there are legal and procedural uncertainties regarding the status of genome edited crops in Europe. While CRISPR technology is being increasingly adopted, the current regulatory framework remains outdated. Moreover, the European Court of Justice (ECJ) ruling from 2018 (C-528/16) brought increased confusion, because of the interpretation that crops obtained by precision breeding are subject to the GMO regulatory provisions. This regulatory burden even applies to crops with small, specific CRISPR-mediated DNA alterations, that can also occur spontaneously in nature. The ruling leads to inconsistency in the legislation, because conventional mutagenesis - which results in thousands of random DNA alterations - is exempt from the GMO regulatory provisions. The legislation no longer correctly reflects the current state of scientific knowledge. Subjecting genome-edited crops to the current EU GMO regulation will also delay development of climate-resilient crops, hinder progress in sustainable agriculture, reduce the EU competitiveness and hamper global trade. The ruling hampers cultivation of genome edited crops with beneficial traits for human or environmental health, as improved nutritional composition, increased digestibility, reduced contents of anti-nutritional components, reduced allergenicity

or requiring reduced production inputs. The scientific community in Europe responded unitedly, and published a position paper and an open statement to call upon the European Commission and the European Parliament. With an increasing number of signatories, currently 129, European research institutions and organizations, from 21 Member States and the United Kingdom, the European Sustainable Agriculture through Genome Editing EU-SAGE network was launched. This aims to provide information about genome editing, and promote development of European and EU member state policies that enable the use of genome editing for sustainable agriculture and food production. An update of the ongoing discussions with the European Commission was presented. Hopefully, scientific evidence will prevail.

The plant inside as a diverse microhabitat: communities, niches, colonization behaviour of beneficial endophytes and relation to plant growth and health. S. COMPANT. *Austrian Institute of Technology, Center for Health and Bioresources, Bioresources Unit, Tulln, Austria. E-mail: stephane.compant@ait.ac.at*

Plants host different fungi and bacteria in their roots, stems, leaves, flowers, fruits and seeds. Most of these microbes are derived from the soil and rhizosphere, as recognized since the 19th century, but other sources like the anthosphere, carposphere, phyllosphere, laimosphere and caulosphere can also provide endophytes inside plant tissues. Other microbes can also be derived from animals or from plants growing near each other. Thorough understanding of the communities, routes and colonization niches of endophytes in the phytobiome has led to a better knowledge on how to use specific microbes for stimulating plant growth and increase host health, and to increase understanding of how plants shape their microbiomes. Most of the research has been done on fungi or bacteria, but recent studies further show that some bacteria can internally colonize beneficial fungi, and increase benefits to plants, leading further to a strategy of using multipartite interactions to boost plant protection.

Current situation of *Xylella fastidiosa* impacts in Spain: ongoing research initiatives to understand and tackle this pathogen. B.B. LANDA. *Instituto de Agricultura Sostenible (IAS), Consejo Superior de Investigaciones Científicas (CSIC), Córdoba, Spain. E-mail: blanca.landa@ias.csic.es*

Xylella fastidiosa (*Xf*) was first reported in 2016 in Majorca in the Balearic Islands, and in 2017 in Alicante province, Mainland Spain. Currently, three *Xf* subspecies and four STs have been detected in the Balearic Islands (subsp. *fastidiosa* ST1, subsp. *multiplex* ST7 and ST81, and subsp. *pauca* ST80). In contrast, only *Xf* subsp. multiplex ST6 has been detected in the Valencian Community, where (as at November 2021) the Demarcated Area covered an extension of >136,200 ha, of which >1,100 ha, 12,500 orchards and 90,000 trees were eradicated. The main research initiatives in Spain were outlined, which aimed at understanding *Xf* epidemics and mitigating its impact in Spain and Europe. These initiatives have contributed to filling knowledge gaps on *Xf* in Europe, gathering fundamental information on several aspects including: i) Characterizing the genetic population structures of *Xf* in the different EU outbreaks, including Spain, and linking the genetics of the bacterium with its pathways of entry; ii) testing and developing new diagnostic tools based on molecular and proximal- and remote-sensing approaches; iii) searching for new control tools targeting *Xf* in hosts or searching for host resistance; and iv) understanding the epidemiology and modelling disease development and developing risk analyses. These results have contributed knowledge to inform Spanish and EU policy related to *Xf* management at different spatial scales (from regional to EU levels) and socio-economic contexts.

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The sanitary crisis caused by *Xylella fastidiosa*, a plant pathogenic bacterium recently discovered in Europe, with a focus on the situation in France. M-A JACQUES, E DUPAS, and S CESBRON. *University of Angers, Institut Agro, INRAE, IRHS, SFR QUASAV, F-49000 Angers, France. E-mail: marie-agnes.jacques@inrae.fr*

Emergence of plant pathogenic bacteria have important effects when they cause diseases with major socio-economic consequences or that threaten valuable plant production and the environment. This is the case for diseases caused by *Xylella fastidiosa* recently discovered in Europe. Ability to anticipate, prevent and mitigate this type of emerging plant pathogen depends on increased understanding of current pathogen distribution over space and time, invasion routes, conditions

favouring their emergence, and population reservoirs. Development and testing of several detection/identification methods (multiplex qPCR assays, nested MLST, metagenomics) has allowed reconstruction of the invasion scenario of *X. fastidiosa* subsp. *multiplex* ST6 and ST7 in France. These studies have relied on genome sequence analyses and tip-dating to date the divergence of French *X. fastidiosa* subsp. *multiplex* strains from their American relatives. A MultiLocus Variable Number of Tandem repeat Analysis (MLVA) on infected plant samples was developed to take advantage of both a large sample collection and the capacity of these markers to monitor recent evolutionary events. The divergence time between French strains and their American relatives suggests that previous introductions have remained unnoticed, probably because of the unspecific symptoms affecting diverse ornamental and native species in a diversified landscape. Bayesian methodologies were used to infer the number of introductions and the most probable scenario of population evolution and spread in the French regions of Corsica and PACA.

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Fungicide resistance in *Botrytis cinerea* populations from protected crops in the Mediterranean basin: Current status and implications with its management, fungicide sensitivity and genetic diversity of *Botrytis cinerea* populations from conventional and organic tomato and strawberry fields in Cyprus and Greece
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Botrytis cinerea is one of the most destructive pathogens infecting numerous crops, including several important crops cultivated in the Mediterranean basin such greenhouse grown vegetables or strawberries. Resistance of the pathogen to fungicides used for disease management is widespread throughout the Mediterranean basin in crops heavily treated, including those cultivated in greenhouses, and resistance is associated with reduction in product performance and control failures. The current status was reviewed of resistance to major botryticide groups, including such as SDHIs, QoIs, hydroxyanilides or anilinopyrimidines, in *Botrytis* spp. populations, and detailed information was presented on methodologies for resistance detection, variability of mutations conferring resistance to fungicides, their effects on the fungal sensitivity to the different active ingredients, and the fitness of resistant strains. Implications of cross-resistance pattern complexity and fitness cost for the resistant mutants were discussed in relation to resistance management in greenhouse grown vegetables, taking into account the recent introduction of new active ingredients into the agricultural practice, such as new SDHI molecules. Future prospects and challenges were presented, with emphasis on the roles of alternative control methods, such as the integration of Biological Control Agents (BCAs) with conventional fungicides, for reducing fungicide selection pressure on fungal populations, and thus minimizing the risk for resistance development.

From Myco-key to myco-twin: mycotoxin management along food/feed chains.
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Fungal diseases are important contributors to the occurrence and severity of mycotoxin contamination of crop plants. Phenotypic and metabolic plasticity has enabled mycotoxigenic fungi (MF) to colonize many agriculturally important crops, and to adapt to different environmental conditions. New mycotoxin-commodity combinations provide evidence for the ability of fungi to adapt to changing conditions, and the emergence of genotypes with enhanced aggressiveness towards plants and/or altered mycotoxin production profiles. Among diseases caused by MF, the most important are from species complexes. Examples are the Fusarium ear rot of maize, Fusarium head blight (e.g. of wheat, barley, and oat), black point of wheat kernels caused by the *Alternaria alternata* species complex and related species; and various rots caused by Aspergilli. Mycotoxins in plant products and in processed food and feed have significant economic impacts and pose serious problems for animal and human health. Good pre-harvest management practices are important for minimizing risks of mycotoxin accumulation in the crops before harvest. These practices can involve crop rotation, tillage, appropriate fertilization, and fungicide or biological controls, host variety selection, timely planting and harvests, and control of insects which facilitate infections by toxigenic fungi. It is also important to prevent post-harvest contamination, and develop practical and effective post-harvest procedures to reduce mycotoxins in food supply chains,

and provide alternative and safe use options for contaminated products. An update review was presented on integrated management of pre- and post-harvest practices aiming to minimize risks of mycotoxin contamination along production chains, and main effective solutions developed by EU projects MycoKey (http://www.mycokey.eu/) and MycoTwin (https://www.mycotwin.eu/project).

Fungal pathogens of wood: are they threats to Mediterranean fruit crops? V. GUARNACCIA. *Dept. Agricultural, Forestry and Food Sciences (DISAFA), University of Torino, Largo Braccini 2, 10095 Grugliasco (TO), Italy. E-mail: vladimiro.guarnaccia@unito.it*

Intensification, diversification and globalization cause collateral effects on agriculture. The risks of disease dissemination via trade and the movement of goods and people are increasing. Climate change is also affecting plant health by modifying interactions between host plants, pathogens and the environment. Fruit tree plantations are increasingly threatened by fungal diseases. How fruit trees share fungal pathogens with woody plants not considered as trees has also been observed. Primary and opportunistic pathogens can cause cankers, twig blight and wood rotting. *Diaporthe* and *Neofusicoccum* spp. are examples of pathogens able to infect broad ranges of fruit trees. There are also numerous latent pathogens such as *Diatrypaceae* or *Cytospora* and *Neocosmospora,* and many others. Recent knowledge of fungus/host combinations was presented, with emphasis on connections between species identification development of modern diagnostic tools. Cultivar diversity, propagation material health and rootstock selection, also should be investigated in relation to the pathogens, to provide support for producers of the economically important agriculture sector.

Plant and seed priming for improved growth and abiotic stress protection under a changing climate. V. FOTOPOULOS. *Cyprus University of Technology. E-mail: vassilis.fotopoulos@cut.ac.cy*

Increased frequency of extreme environmental events resulting from global climatic changes influence plant growth and development. Examination of plant-to-plant communication in nature has revealed the development of unique strategies from plants for responding to abiotic stresses, including priming for improved defence responses. The process of priming involves prior exposure to biotic or abiotic stress factors making plants more tolerant to future exposures. Priming can also be achieved by applying natural or synthetic compounds, which act as signalling transducers 'activating' host defence systems. An up-to-date overview was presented describing research carried out at the Cyprus University of Technology, using priming agents towards induced acclimation of plants to environmental challenges. Recent findings were also presented on evaluation of chemical compounds and nanoparticles that potentially have growth promoting properties in plants. These studies were related to existing expertise and previous observations in priming against stress.

Cypress Canker: a model pathosystem for study of fungal invasions. M. GARBELOTTO[1], G. DELLA ROCCA[2], C. EYRE[1], T. OSMUNDSON[1] and R. DANTI[2]. *[1]Department of ESPM, 54 Mulford Hall, University of California, Berkeley, CA 94720, United States of America. [2]IPSP, National Research Council CNR, Via Madonna del Piano 10, 50127 Sesto Fiorentino, FI, Italy. E-mail: matteog@berkeley.edu*

Understanding of invasions by plant pathogens lags behind that of other biological invasions. Cypress Canker Disease (CCD), caused by *Seiridium cardinale (Sc)*, is an excellent pathosystem to provide increased understanding of fungal invasions. By using a population genetics approach, *Sc* has been shown to be endemic to the interior of California, where the pathogen occurs exclusively on exotic, off-site or artificially-bred cypress species. The early 1920s outbreak in the interior of California was responsible for introductions of the pathogen into Italy and New Zealand. Coalescent analyses showed that the Italian population of *Sc* played a major role in the diffusion of the pathogen throughout the Mediterranean. Phenotypic traits in Mediterranean invasive *Sc* populations indicate that small conidium size, high phenotypic plasticity and high sporulation potential are key traits for a successful invasion, while high virulence and high growth rate are not necessary. Comparative analyses indicate that geographically isolated *Sc* populations are now genetically and phenotypically distinct, and inoculating a range of California *Sc* genotypes on Italian cypress has shown that resistance to CCD can be eroded by Californian genotypes. A recent study has shown that this has occurred within the last 20 years. the Mediterranean and California populations, respectively, were sources for new introductions into New Zealand and Morocco, indicating that additional introductions are possible. These data show that invasive populations can-

not be regarded analogous to the source populations that generated them.

Innovative smart technologies for agricultural production and plant health. T. CAFFI. *DIPROVES - Sustainable Crop and Food Protection Facoltà di Scienze Agrarie, Alimentari e Ambientali, Università Cattolica del Sacro Cuore, via Emilia Parmense, 84, I-29122 Piacenza, Italy. E-mail: tito.caffi@unicatt.it*

Pests are defined by FAO and IPPC as "any species, strain or biotype of plant, animal or pathogenic agent injurious to plants or plant products". They can cause sever enormous crop losses with damage that can occur in the field, from sowing to harvesting, and also in the product storage. Secure food supply for future generations requires environmentally safe and sustainable production. A goal of the modern agriculture is to reduce intensive fertilizer and pesticide use and decrease heavy exploitation of natural resources (water, soil, energy). Precision agriculture (PA) includes agricultural practices that focus on specific areas of a field at particular times. The main goal of PA can be summarized in doing "the right thing, at the right time, in the right place"). With recent scientific advancements, technological innovations and legislatives tools, it is now possible to achieve these strategic goals and to increase agricultural sustainability. Use of Smart Technologies can contribute to enhance plant health, reduce hunger and poverty, protect the environment, and boost economic development, goals highlighted in 2020, the International Year of Plant Health. The actual availability of innovative tools and data management techniques, also leading to big data management and analysis requirement, allow consideration of integrated systems that provide phytosanitary monitoring for pilot crops. This is effective, rapid, objective, repeatable in the most varied environmental contexts, and suitable to provide appropriate support to the various phytosanitary control needs in particular regions or areas. It is possible to integrate this monitoring into modern decision support systems information, protocols, and guidance, to allow trained personnel to carry out surveys and data collection. This can be achieved through information, alerts and guidelines (photographic or video supported) provided by the systems. This approach could lead to complementary monitoring and enhancement to the forecast models, which will allow to optimization of the information and alerts provided to operators, guaranteeing clearaware pictures of the phytosanitary and physiological states of host plants and crops.

Integrated pest management smart technologies to precisely detect and control plant diseases. D.I. TSITSIGIANNIS. *Department of Crop Science, Laboratory of Plant Pathology, Agricultural University of Athens. E-mail: dimtsi@aua.gr*

The ever-increasing demands of international markets for safe food have led to development of integrated plant protection strategies for efficient and sustainable agriculture, and robust certification and control systems for agricultural products. Novel integrated management systems (IPMs) of serious plant diseases and mycotoxin contamination of plant products are being developed using innovative smart agricultural systems. The purposes are to: (a) accelerate the prognosis of disease outbreaks through prediction models; (b) develop advanced methods of artificially intelligent diagnoses using spectral imaging techniques or mass spectrometry sensors for accurate detection and assessment of disease severity; (c) evaluate novel biocontrol and chemical plant protection products to effectively control diseases; and (d) develop innovative prototype sprayers actuating different nozzle types and adopting variable rate control based on canopy characteristics, pathogen dispersal and disease development. We also develop and validate Decision Support Systems (DSS) based on computer-based knowledge systems that enable disease prediction and monitoring by combining epidemiological data, biological and chemical control strategies and precision farming tools. These systems determine the critical stages of the various plant protection spray interventions, taking into account: (a) the environmental conditions (temperature, rainfall, relative air humidity and leaf wetness obtained from local meteorological stations); (b) the developmental stage of the host; (c) the cultivation practices; and (d) the microbiological load and other parameters. The ultimate goal of the smart technologies is to reduce the European reliance on agrochemicals, resulting in reduced residues and impacts on human health.

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Applications of remote sensing and information technology in the surveillance of quarantine diseases of fruit tree crops. A.M. D'ONGHIA. *Centre International des Hautes Etudes Agronomiques Méditerranéennes – Mediterranean Agronomic Institute of Bari, Via Ceglie, 9 - 70010 Valenzano (BA), Italy. E-mail: donghia@iamb.it*

Early and accurate detection systems for quarantine diseases of fruit trees are essential for efficient large-scale surveillance, rapid implementation of control measures and evaluation of their effects. New technologies can also provide solutions in plant health, facilitating operators to implement efficient, accurate, timely and cost-effective real-time and large-scale surveillance programmes. Large amounts of data are required (e.g. climatic, geographical, satellite imaging, diagnostic), accurately captured at distance and on the ground, using applications, sensors, forecasting models, and specific diagnostic methods. Remote sensing (RS) can help to identify infections on large scales, even when symptoms are not yet visible. There are many platforms and sensors designed for the acquisition of remote sensing data, including from satellites, aircraft, and, more recently, Unmanned Aircraft Systems (UAS, drones).. The availability of high-resolution time series of images, such as Sentinel-1 and Sentinel-2 introduced with the European Copernicus programme, has facilitated a scale-step in the use of Earth Observation (EO) data for agricultural and phytosanitary applications. In addition to RS, Information Technology (IT) provides other tools for plant protection programmes, allowing accurate storage, retrieval, transmission and manipulation of monitoring data. IT tools can be used for this purpose, including diagnostic devices, Applications (accurate on-site data acquisition), and DSS. An overview on RS and IT systems was provided, with particular reference to those already available and applied in official pathogen monitoring programmes (Citrus tristeza virus, *Xylella fastidiosa* in Apulia region, Italy) These systems include: (i) an automatic procedure for tree counting, using GeoEye-1 multispectral images; (ii) prediction maps using WorldView-2 satellite images in GIS environments for early detection of CTV-suspected trees; (iii) photointerpretation of high-resolution aerial images for identification of OQDS trees in the demarcated area of *X. fastidiosa*; (iv) applications for accurate field data acquisition by NPPOs (e.g. Apps for *Xylella fastidiosa*, XylApp, XylApp^{EU}, XylApp^{NENA}) and citizens (XylApp^{citizens}); and (v) integrated DSS made of databases, forecasting models and users interfaces.

COMMUNICATIONS

Session organized by the Arab Society for Plant Protection

Pathogenic viability of wheat rust diseases in the southern and eastern Mediterranean region: current status, challenges, and regional collaboration. K. NAZARI. *ICARDA, Izmir, Turkey. E-mail K.Nazari@cgiar.org*

Wheat yellow, stem and leaf rust diseases are continued threats to wheat production in Central West Asia and North Africa (CWANA), and the Mediterranean region. The rust pathogens are capable of rapidly developing new virulence to resistance genes, following the "boom and bust" disease cycle. Considering the transboundary nature of the rust pathogens, regional monitoring of these pathogens and information exchange are key requirements in disease resistance mitigation strategies. In response to a call for regional rust research collaboration, the Turkey-ICARDA Regional Cereal Rust Research Center (RCRRC) was established in Izmir in 2013. The Center has coordinated regional rust surveillance and Trap Nurseries, and since 2018, regional race analysis of yellow rust and stem rust was carried out using a Cereal Rust Biosafety Laboratory at RCRRC. A regional precision wheat rust phenotyping platform was also established within the RCRRC, which has provided precise field phenotyping data for more than 60,000 wheat accessions from the international and national breeding programmes. The RCRRC has received more than more than 1000 yellow rust and Stem rust samples from the Middle east and Mediterranean regions and East Africa since 2018. Using differential varieties and molecular marker approaches, it was evident that most of the the samples from these regions have common virulence structures with the European races, indicating similarity of the races and free movements of the pathogens. Regional race analysis and Trap Nurseries, and regional collaboration for precision phenotyping capacity were described.

Epidemiology and management of legume and cereal viruses in Arab and Mediterranean regions. S.G. KUMARI[1], A. NAJAR[2], N. ASAAD[3] and A. RAHMAN MOUKAHEL[1]. *[1]International Center for Agricultural Research in the Dry Areas (ICARDA), Terbol Station, Zahle, Lebanon. [2]National Agricultural Research Institute of Tunisia (INRAT), Ariana, Tunisia. [3]General Commission for Scientific Agricultural Research (GCSAR), Al-Ghab, Hama, Syria. E-mail: s.kumari@cgiar.org*

In the Arab and Mediterranean regions, cool-season food legumes (chickpea, faba bean, lentil and pea) are infected naturally by many viruses, and the number of viruses involved continues to increase. However, at any specific location only a few of these pathogens are of economic concern. Yield losses resulting from virus

attack vary widely, from little, as in the case of *Broad bean mottle virus* in Tunisia and Morocco, to complete crop failure when conditions permit widespread virus infection at the vulnerable early plant growth stage, as with *Faba bean necrotic yellows virus* in Egypt and Syria. Cereal crops (wheat, barley and oat) in Arab and Mediterranean regions are also affected by viruses, mainly *Barley yellow dwarf virus-PAV*, and this virus is reported to occur at epidemic levels in only Algeria, Morocco and Tunisia. Epidemic spread of most viral diseases was always associated with high vector populations and activity. Although virus disease management can be achieved through the combined effects of several approaches, development of resistant genotypes is one of the most promising control components. Experience over the last few decades has clearly showed that no single method of virus disease control reduces yield losses in legume crops. Some progress was made on the disease management of some legume and cereal viruses using combinations of healthy seed, host resistance, cultural practices (such as adjustments of planting date and plant density, and roguing of infected plants early in the season) and chemical control virus vectors.

Phytoplasma and virus diseases of fruit crops in East Mediterranean countries. E. CHOUEIRI. *Department of Plant Protection, Lebanese Agricultural Research Institute, Tal Amara, P.O. Box 287, Zahlé, Lebanon. E-mail: echoueiri@lari.gov.lb*

Crops of stone and pome fruits and grapes are important in the eastern Mediterranean region. Quality and quantity losses in these crops due to virus and phytoplasma infections are reported from these countries. Almond witches' broom associated with '*Candidatus* Phytoplasma phoenicium' is responsible for death of >100,000 almond and peach trees in Lebanon. In Egypt, serious economic losses due to infections with European stone fruit yellows (ESFY) on apricots and peaches have been reported. In Turkey, ESFY was mostly detected in symptomatic apricot and plum. In Jordan, aster yellows phytoplasma (16SrI) affecting peach trees was reported, in addition to recent incidence of '*Candidatus* Phytoplasma solani' infecting plum. On pome fruits, pear decline induced by *Ca* P. pyri has been observed on pear cultivars in Lebanon and in pear and quince orchards in Turkey. Some apple varieties were infected with apple proliferation disease in Turkey. *Ca*. Phytoplasma solani, the causal agent of bois noir in grapevine has been recorded in Lebanon, Syria, Turkey and Jordan associated with typical grapevine yellows, whereas *Ca*. Phytoplasma omanense' was detected in a grapevine sample from cultivar Syrah in Lebanon. Many viruses were recorded on fruit trees such as Prunus necrotic ringspot virus, Prune dwarf virus, Apple mosaic virus in several countries, while American plum line pattern virus and plum pox virus were detected only in some countries. On grapes, grapevine leafroll associated viruses, vitiviruses and nepoviruses (GLRaV-1, GLRaV-2, GLRaV-3, GVA, GVB, GFLV) were the most common virus pathogens.

Rapid risk appraisal for potential entry, establishment and spread of *Xylella fastidiosa* in NENA countries. M. DIGIARO[1], K. DJELOUAH[1], M. FREM[2], H. EL BILALI[1], G. CARDONE[1] and T. YASEEN[3]. *[1]International Centre for Advanced Mediterranean Agronomic Studies (CIHEAM-Bari), Via Ceglie 9, 70010 Valenzano (Bari), Italy. [2]External consultant of CIHEAM-Bari. [3]Food and Agriculture Organization of the United Nations (FAO)/ Regional Office for the Near East and North Africa Region (RNE) 11 Al Eslah El Zerai St., Dokki, Cairo, Egypt. E-mail: Thaer.Yaseen@fao.org; digiaro@iamb.it*

Potential risks were assessed of introduction, establishment and spread of *Xylella fastidiosa* (*Xf*) on the host crops olive, vine, citrus, stone fruit and ornamental plants in NENA countries, by examining the cases of Algeria, Egypt, Jordan, Lebanon, Libya, Morocco, Palestine, Syria and Tunisia. The study was based on data retrieved directly from official public sources and questions to experts. Questions aimed to assess the risk of entry of *Xf* (volume of potential host commodities imported from officially infected countries, number of entry points, existing technical readiness, legislation for prevention), or the risks of *Xf* establishment and spread (existing surveillance programmes and certification programmes of plant propagation material, presence of vectors, favourable climatic conditions, abundance of main crops and alternative hosts). For each parameter, scores ranged from 1 (low risk) to 6 (high risk). Results showed levels of *Xf* entry risk from medium to high. Morocco resulted in the most vulnerable country, followed by Palestine, Lebanon, Syria and Egypt. The risks of *Xf*-establishment and spread were instead greatest for Syria and Lebanon, followed by Palestine, Morocco and Jordan. Combining these results, Morocco, Lebanon, Palestine and Syria were the most exposed to *Xf* entry and establishment risks, with scores (from 2 to 12) between 6.95 and 7.75, above 6.0 set as a high-risk threshold. Other countries were in intermediate and low risk classes. The prompt and effective implementation of legislative,

financial, technical and scientific measures against *Xf* is necessary in each country.

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Fusarium head blight and crown rot of wheat in Algeria and other southern Mediterranean countries: distribution, identification and pathogenicity of associated species. H. BOUREGHDA. *Laboratory of Phytopathology and Molecular Biology, Department of Botany, The National Higher School of Agronomy (ENSA), El Harrach, Algiers, Algeria. E- mail: hou.boureghda@gmail.com*

Fusarium head blight (FHB) and crown rot (CR) of wheat are serious diseases which may affect yields and grain contamination by mycotoxins. FHB occurs when prolonged wet weather coincides with host anthesis. CR is a chronic problem in dry climatic conditions and where continuous wheat cropping is adopted. In the southern Mediterranean countries, climatic conditions are conducive for both diseases, that can coexist. In Algeria, CR is more widespread because wheat is commonly grown in arid and semi-arid regions, whereas FHB is restricted to humid and sub-humid areas where CR is also present. In Tunisia, FHB was reported only in the sub-humid and semi- arid upper areas. Based on published data, in Algeria, *Fusarium culmorum* was reported as the dominant species associated with both diseases, with *F. pseudograminearum* as the second causal agent. In Tunisia, *F. culmorum* was the main pathogen causing CR, but for FHB, the dominant species was *Microdochium nivale* followed by *F. culmorum*. In Morroco and Egypt, *F. culmorum* and *Bipolaris sorokiniana* were reported as majors species associated with CR. In addition, *Rhizoctonia oryzea* was also associated with CR and *F. graminearum* with FHB in Egypt. Pathogenicity assessments have shown that in Algeria, *F. culmorum* was the most aggressive on wheat seedlings and heads, while *F. pseudograminearum* was the most aggressive on host crowns. In Tunisia, *F. culmorum* and *F. pseudograminearum* were the most aggressive on crowns, and in Egypt *F. culmorum* was the most aggressive pathogen.

Evaluation of the susceptibility of improved and landrace durum wheat genotypes to *Zymoseptoria tritici* under nitrogen supply. M. HASSINE and S. AYADI. *University of Carthage, National Agronomic Institute of Tunisia, LR14AGR01, Laboratory of Genetics and Cereal Breeding, Avenue Charles Nicolle 43, 1082 Tunis, Tunisia. E-mail: marwa.hassine1@gmail.com*

Appropriate nitrogen nutrition is a limiting factor for durum wheat production. This nutrient is important in agriculture practices and causes significant environmental and production costs. The fungal pathogen *Zymoseptoria tritici* is responsible for significant yield losses on durum wheat (*Triticum turgidum* ssp. *durum*) in Tunisia. The present study was conducted to evaluate effects of different nitrogen rates on development of Septoria leaf blotch (STB) and durum wheat yield components. Different levels of nitrogen (0, 50, 75, 150, 200 and 250 kg N ha^{-1}) were applied under field conditions, to evaluate the degree of susceptibility of the improved wheat varieties Karim and Salim,) and the landrace genotypes Chili and Mahmoudi. Disease severity increased from 9% without nitrogen supply to 40% under the high rate (250 kg N ha^{-1}). The iImproved genotypes were more sensitive to STB (Karim 35% and Salim 51%) than the landrace genotypes (Mahmoudi 17% and Chili 12%). Decreases in thousand kernel weights (TKW) were measured with the increasing disease severity and nitrogen rates, from means of 48.58 g from 0N to 41.66 g from 250 kg N ha^{-1}. These results provide new insights into the implications and efficiency of the use of different sources of nitrogen fertilizers in field performance of durum wheat and disease control.

This research was financially supported by National Institute of Field Crops (INGC) Boussalem, Tunisia.

Determination of antibiotic residues in the endemic spurge honey (*Euphorbia resinifera* O. Berg) from Morocco, using biochip multi array technology and LC-MS/MS. R. BENJAMAA[1], A.K. ESSAMADI[1], A. MOUJANNI[2], B. NASSER[1]. *[1]Hassan First University of Settat, Faculty of Sciences and Technologies, Laboratory of Biochemistry, Neurosciences, Natural Resources and Environment. [2]National Office of Food Safety (ONSSA), Avenue Hadj Ahmed Cherkaoui, Agdal, Rabat, Maroc. E-mail: essamadi@uhp.ac.ma*

Antibiotic residues in honey present risk to the health of consumers, because they could be sources of allergic reactions and can lead to obtaining bacterial resistant strains to antibiotics after consumption of honey. Screening methods are the first step in controlling antibiotic residues in foods. These procedures can detect the presence of an antibiotic or group of antibiotics,

and usually provide qualitative results. A second step includes testing of positive samples for quantification, mostly by quantitative confirmation methods such as high performance liquid chromatography associated with a mass detector (HPLC-MS/MS). In this study, a total of 37 *Euphorbia resinifera* honey samples were analysed, using a screening test: Evidence Investigator™, an immuno-enzymatic method for detection of 27 antibiotic residues; followed by liquid chromatography-tandem mass spectrometry (LC- MS/MS) for confirmation of suspect samples. In all tested samples, no antibiotic residues were detected, except for one showing Trimethoprim at 6.48 μg kg $^{-1}$.

Molecular host-microbe interactions in plants: from microbes to host innate immunity

Uncovering the mechanisms involved in *Pinus pinaster* resistance to pine wilt, by analysis of coding and non-coding transcriptomes .I. MODESTO[1,2,3], L. STERCK[3,4], V. ARBONA[5], A. GÓMEZ-CADENAS[5], I. CARRASQUINHO[6,7], Y. VAN DE PEER[3,4,8] and C.M. MIGUEL[2,9]. *[1]ITQB NOVA, Universidade Nova de Lisboa, Oeiras, Portugal. [2]iBET, Oeiras, Portugal. [3]Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent, Belgium. [4]VIB-UGent Center for Plant Systems Biology, Ghent, Belgium. [5]Departament de Ciències Agràries i del Medi Natural. Universitat Jaume I, Castelló de la Plana, Spain. [6]INIAV, Oeiras, Portugal. [7]LEAF – Linking Landscape, Environment, Agriculture and Food, Instituto Superior de Agronomia, Universidade de Lisboa. [8]Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Pretoria, South Africa. [9]Biosystems & Integrative Sciences Institute, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal. E-mail: cmmiguel@fc.ul.pt*

Pine wilt disease (PWD), caused by the nematode *Bursaphelenchus xylophilus* (pinewood nematode (PWN)), is a threat to conifer forests in Europe and Asia. *Pinus pinaster* is one of the most affected hosts due to its high susceptibility to PWN. Heritable resistance has been reported in *P. pinaster* trees, opening the possibility for host selection and breeding for this trait. To uncover the mechanisms underlying resistance, transcriptional responses were analysed after inoculation with PWN to plants showing resistant and susceptible phenotypes. Strong reprograming of gene expression was demonstrated, particularly in resistant plants. Differential gene expression analysis indicated roles for lignin synthesis and jasmonic acid defence pathways in resistant phenotypes, confirmed by biochemical analyses of cell wall composition and hormone pathways. Additionally, secondary metabolism, oxidative stress response and resistance genes were also probably relevant to overcome PWD. Approximately 100 miRNAs were responsive to PWN inoculation, some of which had predicted targets associated with roles in the jasmonate-response pathway, ROS detoxification and terpenoid biosynthesis. Several *P. pinaster* genes putatively targeted by PWN miRNAs were also identified, which was supported by degradome analysis. Targets for *P. pinaster* miRNAs were also predicted in PWN, suggesting a role for trans-kingdom miRNA transfer and gene silencing, both in PWN parasitism and in *P. pinaster* resistance to PWD. These results provide novel insights into transcriptional and post-transcriptional regulatory mechanisms which may be useful for the development of new strategies to protect *P. pinaster* forests from PWD.

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The necrosis and ethylene inducing gene *VdNEP* as a molecular marker for differentiation between *Verticillium dahliae* pathotypes. A. TRIANTAFYLLOPOULOU[1], A.K. TZIMA[1], A. TZANAKI[1], O.I. BALOMENOU[1], I. TSOUTSOS[1], R.M. JIMÉNEZ-DÍAZ[2] and E.J. PAPLOMATAS[1]. *[1]Laboratory of Phytopathology, Agricultural University of Athens, Iera Odos 75, Athens, Greece. [2]Universidad de Córdoba, College of Agriculture and Forestry (ETSIAM), Departamento de Agronomía, Campus de Excelencia Internacional Agroalimentario, Edificio C-4 Celestino Mutis, Campus Rabanales, Córdoba, 14071, Spain. E-mail: epaplom@aua.gr*

Verticillium dahliae is a cosmopolitan soilborne pathogen with a wide host range, leading to major agricultural loses. Isolates of the pathogen are categorized as defoliating (D) and non- defoliating (ND) pathotypes, with the former being highly virulent causing distinct defoliation in cotton, okra and olive. To differentiate isolates, a new molecular marker based on the necrosis and ethylene inducing *VdNEP* gene has been developed. Southern blots probed with *VdNEP* showed fragment polymorphisms between isolates of the two pathotypes. To isolate regions flanking the gene, inverse PCR was performed on genomic DNA from D and ND reference strains of *V. dahliae*. Sequencing detected differences between the

two pathotypes in the 3′ untranslated region of *VdNEP*. Based on these findings, specific primers were designed for PCR detection. The primers were evaluated by screening of a collection of *V. dahliae* isolates from olive and cotton from Greece, as well as a limited number of isolates from different parts of the world. Primer efficiency was compared with already available markers. For the D isolates, pathogenicity experiments were conducted with cotton plants to verify the molecular findings. The results indicated that the new primers are a robust and reliable tool for the differentiation of pathotypes, leading to subsequent increased chances for successful management of the pathogen.

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Biological control of *Aspergillus carbonarius* and *Botrytis cinerea* in grapevine berries, and transcriptomic changes of genes encoding pathogenesis-related (PR) proteins. D. GKIZI[1], E. POULAKI[2] and S.E. TJAMOS[2]. *[1]School of Food Sciences, University of West Attica, Ag. Spyridonos str Egaleo, 12243, Athens, Greece. [2]School of Plant Sciences, Laboratory of Plant Pathology, Agricultural University of Athens, 75 Iera Odos Str., 11855, Athens, Greece. E-mail: dgkizi@uniwa.gr*

Botrytis cinerea and *Aspergillus carbonarius* are among the most important widespread pathogens causing bunch rot of grapevine berries, resulting in significant economic losses. Additionally, *A. carbonarius*, although a secondary invader, is the main source of ochratoxin A in grapes. The plant protective activity of the biological control agents, *Bacillus velezensis* (previously *Paenibacillus alvei*) K165, *Blastobotrys* sp. FP12 and *Arthrobacter* sp. FP15 was examined against *B. cinerea* and *A. carbonarius* on grapes. Preliminary dual culture experiments showed reductions of fungus growth by K165, while FP15 restricted only the growth of *A. carbonarius*, and FP12 was ineffective. *Ex vivo* experiments on grape berries showed that K165, FP12 and FP15 reduced *A. carbonarius* rot severity by, respectively, 81%, 57% and 37%, compared to controls. Growth of *B. cinerea* in berries was reduced only by K165 (by 75%). Gene expression analyses of pathogenesis-related proteins PR2, PR3, PR4 and PR5, using real-time PCR, indicated activation of multiple defence responses involved in the biocontrol activity of the assessed biocontrol agents.

Comparative proteome analysis provides new insights into the complex responses of *Citrus aurantium* grafted with *C. sinensis* and infected with Citrus tristeza virus. M. TRINDADE[1,§], S.A. DANDLEN[2,§], L. ANJOS[1], A. DUARTE[2], D.M. POWER[1], N.T. MARQUES[3]. *[1]Comparative Molecular and Integrative Biology, Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal. [2]Mediterranean Institute for Agriculture, Environment and Development (MED), Faculdade de Ciências e Tecnologia, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal. [3]Center for Electronics, Optoelectronics and Telecommunications (CEOT), Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal. E-mail: nmarques@ualg.pt; nmarques@ualg.pt. § Both authors contributed equally to this work*

Sour orange (*Citrus aurantium* L.; CA) has low susceptibility to Citrus tristeza virus (CTV). When sweet orange is grafted onto CA and is infected with a quick decline CTV isolate, a quick decline syndrome may develop. This syndrome is related to obliteration and necrosis of the sieve tubes in rootstocks close to the bud unions, which culminate in dieback symptoms. To investigate the molecular response of CA to CTV, CA stem tissues below bud unions were analysed by SWATH-MS in 2-year-old plants, infected or uninfected with the quick decline isolate T36, in the following conditions: i) CA grafted with CA infected or not; or ii) CA grafted with sweet orange [*C. sinensis* 'Valencia Late' (VL)] infected or not. A Library of 2689 proteins was generated for CA. Differentially expressed proteins (DEPs, for a $P < 0.05$ and FD = 1.5x) between CA infected and uninfected were 352, from which 31 were modified in common with VL infected and uninfected. Enriched terms (KEGG pathways) of modified proteins common to CA grafted with uninfected or infected CA and CA grafted with uninfected or infected VL, were mainly assigned to glycolysis and glyoxylate and dicarboxylate metabolism. Specific modified terms in CA grafted with VL, infected and uninfected, were mainly in alpha-linolenic acid metabolism, tyrosine and carbon fixation metabolism. In summary, VL grafted scions promote considerable modifications in the stem CA proteomes. Furthermore, CTV infection differently affects the CA stem proteome when the grafted scion is CA or VL.

***Bacillus velezensis* K165 mediated resistance against *Verticillium dahliae*, *Botrytis cinerea* and *Hyaloperonospora arabidopsidis*, and the role of histone acetyltransferases in biocontrol.** D. GKIZI[1], M. MALAI[2], K.

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The use of microbes for the biological control of plant diseases has been well studied, along with the molecular aspects of plant-biocontrol agent (BCA) interactions. Nevertheless, epigenetic effects of the BCAs on plants is a relatively new research field. We have shown that treatment of *Arabidopsis thaliana* with the BCA *Bacillus velezensis* (previously *Paenibacillus alvei*) K165 confers inherited resistance to the soilborne pathogen *Verticillium dahliae* in K165-treated plants and their offspring. The observed plant protection was attributed to histone acetylation of genes participating in lignin biosynthesis and immune responses, resulting in lignin accumulation and induction of the jasmonate/ethylene pathway. In the present study, the role was examined of K165 in the *Arabidopsis-Botrytis cinerea/Hyaloperonospora arabidopsidis/Pseudomonas syringae* pv. *tomato* (Pst) interactions, in K165-treated wild type plants and their offspring. This showed that K165 protected the plants against *B. cinerea* and *H. arabidopsidis*; while the offspring of the K165-treated plants were as susceptible as the controls. Pathogenicity experiments with *Arabidopsis* mutants showed the role of histone acetyltrasferases (HAT) of GNAT-MYST (HAG) and CBP (HAC) families in the K165 mediated disease resistance.

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Enriched epigenetic marks at Pm-0 locus genes prime courgette and induce SAR responses against powdery mildew. T. MARGARITOPOULOU[1], D. KIZIS[1], D. KOTOPOULIS[1], I.E. PAPADAKIS[2], C. ANAGNOSTOPOULOS[3], E. BAIRA[3], A. TERMENTZI[3], A.-E. VICHOU[1], C. LEIFERT[4,5] and E. MARKELLOU[1]. *[1]Scientific Directorate of Phytopathology, Benaki Phytopathological Institute, Athens, 14561, Greece. [2]Faculty of Crop Science, Agricultural University of Athens, Athens, 11855, Greece. [3]Scientific Directorate of Pesticides' Assessment & Phytopharmacy, Benaki Phytopathological Institute, 14561, Greece. [4]SCU Plant Science, Southern Cross University, Military Rd., Lismore, NSW, Australia. [5]Department of Nutrition, IMB, University of Oslo, 0372 Oslo, Norway. E-mail: th.margaritopoulou@bpi.gr*

Powdery mildew, caused by *Podosphaera xanthii*, is an important disease of cultivated *Cucurbita* species. Recently, the appearance of highly aggressive *P. xanthii* strains has led to powdery mildew outbreaks even in resistant crops, making disease management difficult. Plant breeders rely on host genetic characteristics for powdery mildew control. Analysis of commercially available intermediate resistance courgette varieties, using cytological, molecular, and biochemical approaches, showed that the plants were under a primed state and an induced Systemic Acquired Resistance status. Plants exhibited enhanced callose production, upregulation of Salicylic Acid (SA) defence signalling pathway genes and accumulation of SA and defence metabolites. The intermediate resistant varieties also showed an altered epigenetic landscape in histone marks that affect transcriptional activation. Courgette plants were shown to have enriched H3K4me3 marks on SALICYLIC ACID-BINDING PROTEIN 2 (SABP2) and YODA (YDA) genes of the Pm-0 interval introgression, a genomic region that confers resistance in Cucurbits against *P. xanthii*. The open chromatin state of SABP2 and YDA genes was consistent with gene differential expression, induced SA pathway, altered stomata characteristics and activated SAR responses. These results indicate that the Pm-0 SABP2 and YDA genes modulate resistance against *P. xanthii* by setting plants in a primed state, and that the epigenetic background of courgette varieties has an important regulatory role in defence and induced SAR responses. This background could be further explored for production of varieties with enhanced resistance to the pathogen.

Invasive pathogens and emerging diseases

Potential insect vectors of *Xylella fastidiosa* in Morocco: the case of spittlebug. N. HADDAD[1,2], I. MRABTI[1,2], M. AFECHTAL[1], K. EL HANDI[3], R. BENKIRANE[2] and M.C. SMAILI[1]. *[1]National Institute for Agricultural Research (INRA), Regional Center for Agricultural Research of Kenitra, Box: 257, Kenitra, Morocco. [2]Faculty of Sciences, Laboratory of Plant, Animal, and Agro-Industry Productions, University Ibn Toufail, Kenitra, Morocco. [3]Laboratoire de Biotechnologie Végétale et Valorisation des Bio-Ressources, Faculté des Sciences,*

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Xylella fastidiosa is a potential risk to the Moroccan flora. Thorough understanding of the distribution and ecology of potential vectors in wine-producing systems in Morocco is important for development of successful control of this pathogen. A sweeping net was used to collect insects (2604 specimens), and to assess *X. fastidiosa* occurrence, and to establish its potential insect vectors during 2019 and 2020. Five potential vectors were recorded, and these were mainly located in the northern part of Morocco. The vectors were; *Philaenus tesselatus*, *P. maghresignus*, *Philaenus* sp., *Neophilaenus campestris* and *N. lineatus*. *Philaenus tesselatus* was the most important spittlebug recorded, with uneven occurrence throughout the country. *Philaenus spumarius* was not found in the surveys, which indicates that it has been replaced by *P. tesselatus*. Therefore, *P. tesselatus* is probably the main potential insect vector of *X. fastidiosa* in Morocco.

Modelling temperature response of *Xylella fastidiosa* strains, and xylem vessel temperature in woody plants M. ROMÁN-ÉCIJA, B.B. LANDA, L. TESTI and J.A. NAVAS-CORTÉS. *Instituto de Agricultura Sostenible (IAS), Consejo Superior de Investigaciones Científicas (CSIC), Córdoba, Spain. E-mail: mromanecija@ias.csic.es*

Temperature is a key factor affecting *Xylella fastidiosa* (*Xf*) biology and epidemiology. Knowledge of the response of *Xf* to temperature is needed to assess the potential establishment of *Xf* strains occurring in the European outbreaks of the pathogen, and to develop regionalised risk models. Furthermore, as this is a xylem-inhabiting microorganism, it is relevant to determine whether air temperature, used to develop these models, is related to that in xylem vessels of host plants. This study aimed to determine the effects of temperature on *in vitro* cell cultures of *Xf* strains, and to determine xylem vessel temperature dynamics in an olive orchard. growth, biofilm formation and survival were determined of 37 *Xf* strains representative of five subspecies and 13 STs from a wide geographic origin, and host plants were evaluated in a temperature range from 4 to 40°C. Several thermocouples were installed within plant branches and trunks at 1 and 4 cm depths, to measure air, soil and xylem temperature at 10-min intervals for 31 months. Results indicated that *Xf* strains showed differential responses to temperature. When grouped by subspecies, the widest optimal growth range was estimated for *Xf* subsp. *fastidiosa* (19 to 33°C) and subsp. *multiplex* (20 to 31°C), while *Xf* subsp. *pauca* strains had lower optimal ranges (19 to 27°C). Similarly, extreme temperatures differentially affected cell survival. Temperatures between 4 to 10°C did not affect cell survival, while incubation at 36 and 40°C were lethal. The relationship between air and xylem or soil temperatures estimated by regression models indicated buffer effects of trunk tissues and soil, especially for maximum temperatures occurring during summer.

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Spread and current situation of *Fusarium oxysporum* f. sp. *cubense* tropical race 4 affecting banana in Israel and the Middle East. M. MAYMON[1], N. SELA[1], U. SHPATZ[1,2], N. GALPAZ[2] and S. FREEMAN[1]. *[1]Department of Plant Pathology and Weed Research, ARO, The Volcani Institute, Rishon LeZion, 7505101 Israel. [2]R & D, Kiryat Shmona 11016, Israel. E-mail: freeman@volcani.agri.gov.il*

Fusarium oxysporum f. sp. *cubense* tropical race 4 (TR4) is an important soilborne pathogen of banana, causing mortality to Cavendish group bananas. The pathogen was first detected in South East Asia, spread to the greater Mekong subregion, Australia and was disseminated to India, Pakistan, Oman, Turkey and Mozambique (Africa), and recently was identified in Colombia and Peru. TR4 was discovered in the Middle East (Jordan and Lebanon in 2014, and Israel in 2016). In Israel, typical TR4 symptoms of leaf-yellowing and wilting, accompanied by internal vascular discolorations of rhizomes and pseudostems, were observed in mature 'Grand Naine' Cavendish plants from Shfeya, the Carmel coastal plain and locations along the eastern shores of Lake Galilee. TR4 representative isolates from symptomatic plants were tested for pathogenicity, and identification was reconfirmed by PCR. Sequenced genomes of five representative TR4 isolates (two from Israel, and one each from Jordan, the Philippines, and Indonesia), and 11 additional isolates from other countries, were compared by single nucleotide polymorphisms (SNPs) analysis, to determine the origin of the Israeli isolates. SNP detection and phylogeographical analyses indicated that the Middle Eastern isolates are closely related, implying that Jordan was the origin of the pathogen in Israel, while isolates from Colombia were related to a representative isolate from

Indonesia. Host range, susceptibility/tolerance of germplasm to TR4, and epidemiological studies and survival of the pathogen are currently being investigated.

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New and emerging fungal diseases of super-high-density olive trees in California. F.P. TROUILLAS, M.T NOURI, R. TRAVADON and D.P. LAWRENCE. *Department of Plant Pathology, University of California, Davis, 95616 Davis, CA, USA. E-mail: flotrouillas@ucanr.edu*

California produces 95 percent of the olives grown in the United States with a production area of approx. 14,700 ha. During the winter of 2016, Neofabraea leaf and twig lesions were first detected in super-high-density (SHD) oil olive orchards in California. Affected trees showed leaf and shoot lesions, and cankers in branches, which developed at wounds caused by mechanical harvesters. *Phlyctema vagabunda* and *Neofabraea kienholzii* were consistently associated with the disease, and Koch's postulates for these fungi were completed. The cultivar 'Arbosana' was very susceptible to the disease, whereas 'Arbequina' and 'Koroneiki' were tolerant. Field trials indicated that several fungicides can reduce disease incidence, and management strategy guidelines were implemented to limit further spread of the disease. Pleurostoma decline of olive trees caused by *Pleurostoma richardsiae* was also recently detected in SHD olive orchards. Symptoms of Pleurostoma decline in olive trees included leaf yellowing and browning, leaf drop, and wilting and dieback of twigs and branches, and brown to dark discolouration of the wood, while severely affected trees died. Field observations suggested that infections by *P. richardsiae* initiate at wounds caused by field equipment in trunks and branches. Following a California -wide survey of olive orchards, olive anthracnose was not detected in SHD orchards. However, the disease was observed in an orchard of Gordal-Sevillana olives, located at the Kearney Agricultural Research and Extension Center. Fungal isolates obtained from olive fruits in this orchard were identified as *Colletotrichum fioriniae*.

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A new disease complex threatening fig (*Ficus carica* L.) in Southern Italy. W. HABIB[1], CAVALIERI[1,2], M. CARLUCCI[3], C. DONGIOVANNI[1] and F. NIGRO[1,3]. *[1]Centro di Ricerca, Sperimentazione e Formazione in Agricoltura – Basile Caramia (CRSFA), Via Cisternino 281, Locorotondo, 70010 Bari, Italy. [2]Istituto per la Protezione Sostenibile delle Piante – CNR, Via Amendola 122/D, 70126, Bari, Italy. [3]Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti, Università degli Studi di Bari - Aldo Moro, Via Amendola 165A, 70126 Bari, Italy. E-mail: whabib@lari.gov.lb*

Symptoms of severe decline of fig (*Ficus carica*) have been recently detected in Salento area of the Apulia region (southeastern Italy). Symptoms begin with leaf chlorosis and evolve into necroses, twig defoliation and death of lateral branches. Cankers are often observed, associated with extensive wood discolouration under the dead bark and together with bark beetle galleries. In September 2021, wood sections and adult insect specimens were collected from symptomatic trees in several orchards. Bark beetles were morphologically and molecularly identified as *Cryphalus dilutus* (*Curculionidae*, *Scolytinae*). Fungal isolations were performed on PDA amended with 0.5 g L^{-1} streptomycin sulfate, from 200 wood fragments and 52 *C. dilutus* adults. Three groups of potential pathogens were recovered from the plant tissues and insects, including *Botryosphaeriaceae* (38.5%), *Fusarium solani* species complex FSSC (36.9%), and Ophiostomatoid fungi (6.7%). Mixed infections with at least two pathogens were common (67%). A set of 47 single spore isolates were identified using multilocus phylogenetic analyses based on the sequences of three informative genes. Preliminary results showed that almost 50% of the FSSC isolates belong to the newly described species *Neocosmospora perseae* causing trunk cankers on avocado in Sicily. At least three species of *Botryosphaeriaceae* (*Neofusicoccum parvum*, *Lasiodiplodia theobromae*, *Botryosphaeria dothidea*) and two genera of Ophiostomatoid fungi (*Graphium* and *Ceratocystis*) were also identified. Phylogenetic analyses and pathogenicity tests are ongoing to determine the role of each fungus in the aetiology of the observed decline syndrome, and verify the potential role of the *C. dilutus* as a pathogen vector.

Changes in the xylem microbiota associated to infection by *Xylella fastidiosa* in Brazilian olive groves. M. ANGUITA-MAESO[1], J.A. NAVAS-CORTÉS[1], H.D. COLETTA-FILHO[2] and B.B. LANDA[1]. *[1]Institute for Sustainable Agriculture (IAS), Spanish National Research Council (CSIC), Avenida Menéndez Pidal s/n, 14080, Córdoba, Spain. [2]Centro de Citricultura Sylvio Moreira, Instituto Agronômico-IAC, Rod. Anhanguera, km 158 -*

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The health of olive groves is under threat due to an increase of diseases caused by vascular pathogens, including *Xylella fastidiosa* and soil-borne *Verticillium dahliae*. Both pathogens may compromise olive production due to their capacity to reduce olive plant growth by colonizing and blocking host xylem vessels causing desiccation of leaves and branches and ultimately death of trees. Knowledge of the xylem-inhabiting microbiota is important to provide understanding of the resistance response observed in some olive cultivars under high inoculum pressure, and to select potential biocontrol microorganisms. Xylem-inhabiting bacterial communities were investigated in stem and root tissues of "Grappolo" olive trees at five field locations (two in the state of Sao Paulo and three in Minas Gerais State) in Brazil. Changes occurring on trees infected and non-infected by *Xylella fastidiosa* were also compared. Sequencing data resulted in a total of 925 bacteria ASVs, distributed in 15 phyla and 421 genera. Proteobacteria was the most abundant bacterial phylum (91.4%) followed by Firmicutes (4.2%), whereas *Pseudomonas* (31.2%) and *Methylobacterium* (24.2%) were the dominant genera. Microbial diversity was firstly determined by field location, followed by ecological niche and in a lesser extent by *X. fastidiosa* infection. Network analysis identified 32 keystone species with positive and negative associations with *X. fastidiosa*. *1174-901-12* and *Pseudomonas* showed the greatest number of negative ASVs associated with *X. fastidiosa*, whereas *Acidiphilium* and *Corynebacterium* co-occurred with the pathogen. This research increases understanding of the contribution of the plant microbiome to host health status, to promote and maintain sustainable olive agroecosystems.

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Etiology and management of Phytophthora crown and root rot of pistachio in California. F.P. TROUILLAS, A.I. HERNANDEZ, M.T NOURI, R.J FRIAS and T.B. BOURRET. *Department of Plant Pathology, University of California, Davis, 95616 Davis, CA, USA. E-mail: flotrouillas@ucanr.edu*

Pistachio is a widely cultivated nut crop in California, with approx. 115,000 ha of bearing pistachio trees. Several orchards have been identified with declining trees leading to substantial tree losses. Symptoms included trees with poor vigour, yellowing and wilting of leaves, crown rot and profuse gumming on the lower portions of trunks. Thirty-seven *Phytophthora*-like isolates were obtained from crown rot tissues of rootstocks of grafted pistachio trees, and were characterized using multilocus phylogeny comprising ITS rDNA, beta- tubulin and mt cox1 sequence data. This identified *Phytophthora niederhauserii, P. mediterranea* and *P.* taxon walnut associated with declining pistachio trees. Pathogenicity studies in potted UCBI rootstocks confirmed that all three *Phytophthora* species can cause crown and root rot of pistachio, thus fulfilling Koch's postulates. The widespread occurrence of Phytophthora crown rot in recently planted pistachio orchards and the susceptibility of UCBI rootstocks suggest this disease is an emerging threat to the Californian industry. Experiments have been conducted to determine the relative tolerance of UCBI (*P. atlantica* × *P. integerrima*), PGI (*P. integerrima*) and Platinum (*P. integerrima* × *P. atlantica*) commercial rootstocks to Phytophthora, and identify tolerant/resistant rootstocks that can be used to sustainably managed this soil-borne disease. Experiments using mycelium plugs for stem inoculation, or inoculated rice grain or zoospores for soil/root inoculations, of the various commercial rootstocks have indicated that Platinum is the most tolerant rootstock to crown rot diseases, compared to PGI and clonal UCBI rootstocks.

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First report of Glomerella leaf spot in South Tyrolean (Italy) apple orchards. E. DELTEDESCO[1], J. CHRISTANELL[2] and S. OETTL[1]. *[1]Research Centre Laimburg, Laimburg 6, 39040 Auer/Ora (BZ), Italy. [2]South Tyrolean Extension Service for Fruit and Wine Growing, Andreas-Hofer- Str. 9/1, 39011 Lana (BZ), Italy. E-mail: evi.deltedesco@laimburg.it*

Glomerella leaf spot (GLS) of apple is an emerging disease, caused by several *Colletotrichum* spp. GLS was restricted to apple growing areas with humid and subtropical climates, but had not been reported from European apple growing areas. Extreme weather conditions, including heavy rainfall and warm temperatures at the end of August 2020, led to an unknown symptomatology in South Tyrolean (Italy) apple orchards. This included leaves with necrotic lesions, proceeding rapidly to extended chloroses. Affected leaves dropped prematurely and resulted in complete defoliation after

approx. 1 month. A few days after the appearance of the first leaf lesions, brownish spots, often surrounded by purple halos, started to develop on fruit, progressing to affect up to 100% of individual crops. Fungal isolates were recovered from symptomatic leaves and fruit spots, and morphological analysis identified these isolates as *Colletotrichum*. A multi-locus sequence analysis based on the ITS region and fragments of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin (ACT) and beta-tubulin (TUB2) genes, assigned all isolates to *Colletotrichum fructicola*. Pathogenicity assays on apples were carried out, and Koch's postulates were confirmed for this pathogen. Climatic conditions, such as increasing air temperatures and frequency and intensity of precipitation, may lead to a further spread of this pathogen, resulting in notable yield losses in commercially managed orchards. Targeted plant protection procedures and containment strategies should be implemented to control and prevent the spread of this disease.

***Sclerotinia sclerotiorum*: a new pathogen of sugar beet in the USA.** M.F.R. KHAN[1,2] and M.Z.R. BHUIYAN[1]. *[1]Department of Plant Pathology, North Dakota State University, Fargo, ND 58108. [2]University of Minnesota, St. Paul, MN, USA. E-mail: Mohamed.khan@ndsu.edu*

Sclerotinia sclerotiorum (Lib.) DeBary adversely affects a wide range of crops. This fungus may exist as mycelia and survive as resting sclerotia. The pathogen was reported for the first time on sugar beet (*Beta vulgaris*) in North Dakota, USA in 2019, and was confirmed in several beet producing states areas in 2020, causing foliar blight and root rot. Radial growth of mycelium and development of sclerotia was evaluated on PDA media. Co-cultivation experiments with commercial sugar beet seeds with mycelium plugs and sclerotia of the pathogen were carried out to determine the pathogenicity of inocula. Inoculated seeds showed partial emergence and/or complete seedling death, whereas non-inoculated controls produced well-developed seedlings. The sensitivity of *S. sclerotiorum* was determined on PDA amended with 0, 0.01, 0.1, 1, or 10 ppm of Proline® (prothioconazole, DMI), Priaxor® (pyraclostrobin, QoI + fluxapyroxad, SDHI), or triphenyltin hydroxide. Priaxor® gave the greatest reductions of radial mycelium growth. Commercial varieties from three seed companies were evaluated for sensitivity to *S. sclerotiorum*. All the varieties were susceptible to the pathogen, indicating no resistance to the pathogen. Presence of this new pathogen is important for the sugar beet industry which is economically important in sugar beet producing states. Field research will be implemented to develop strategies for managing disease caused by *S. sclerotiorum*.

Biocontrol, natural compounds and plant defence stimulants

Physiological, cellular, and molecular responses of *Cucurbita pepo* genotypes infected by *Podosphaera xanthii* and treated with *Reynoutria sachalinensis* plant extract. T. MARGARITOPOULOU[1], D. KIZIS[1], I. THEOLOGIDIS[2], A. TERMENTZI[2], E. BAIRA[2], M. MAKRIDAKIS[3], J. ZOIDAKIS[3], N. VAKIRLIS[2], E. TOUFEXI[1], G. BALAYIANNIS[4], C. ANAGNOSTOPOULOS[5], A.-E. VICHOU[1], L. REMPELOS[6], C. LEIFERT[7] and E. MARKELLOU[1]. *[1]Laboratory of Mycology, Scientific Directorate of Phytopathology, Benaki Phytopathological Institute, 8 Stefanou Delta Street, 14561, Kifissia, Athens, Greece. [2]Laboratory of Toxicological Control of Pesticides, Scientific Directorate of Pesticides' Control & Phytopharmacy, Benaki Phytopathological Institute. [3]Proteomics Laboratory, Foundation of Biomedical Research of the Academy of Athens, 4 Soranou Ephessiou Street, 11527, Athens, Greece. [4]Laboratory of Chemical Control of Pesticides, Scientific Directorate of Pesticides' Control & Phytopharmacy, Benaki Phytopathological Institute. [5]Laboratory of Pesticides Residues, Scientific Directorate of Pesticides' Control & Phytopharmacy, Benaki Phytopathological Institute. [6]School of Natural and Environmental Sciences, Newcastle University, Newcastle upon Tyne, NE1 7RU, UK. [7]Centre for Organics Research, Southern Cross University, Military Rd., Lismore, NSW, Australia. E-mail: e.markellou@bpi.gr; d.kizis@bpi.gr*

Powdery mildew (PM), caused by *Podosphaera xanthii*, is an important courgette disease, causing high yield losses. The diseases and is mainly controlled using fungicides, biocontrol agents, botanicals, and sulphur applications in conventional and organic courgetti production. Giant knotweed (*Reynoutria sachalinensis*; RS) extract is a known elicitor of plant defences, but its mode of action remains unknown. This study investigated the mechanisms of foliar RS applications and how these affect PM severity and crop performance when used alone or in combination with genetic resistance. Physiological, cellular, and molecular approaches showed that RS can have lasting effects on PM progress on courgette leaves, by enhancing plant defence responses at different levels. RS foliar treatments reduced PM severity on

both an intermediate resistance (IR) and a susceptible (S) host genotype. RS induced the formation of callose papillae, hydrogen peroxide accumulation, and expression of the Salicylic Acid (SA)-regulated *NPR1*, *PR1*, *PR2* and *PAL* genes, which corelated with callose deposits and ROS production. Increased SA production was also detected, along with increased p-coumaric and caffeic acid concentrations. These results show that RS elicits plant defences through the SA pathway. To gain molecular level insights, transcriptomic (Illumina sequencing), proteomic (nanoHPLC-HRMS/MS) and metabolomic (UHPLC-HRMS/MS) analyses were carried out on the S genotype inoculated with *P. xanthii* and treated with *R. sachalinensis* prior to inoculation. Functional classifications of the DEGs showed enriched GO-terms, including hormone-mediated responses, oxidation-reduction processes, lipid biosynthesis and metabolism, and defence responses. Glycerophospholipids were also detected, which are chemical signals facilitating signalling during defence responses.

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Bacterium mixtures; combining compatible endophytic *Bacillus* strains with strong biological control potential *in vitro* and *ex vivo*. P.C. TSALGATIDOU[1,2], E.-E. THOMLOUDI[1], E. BAIRA[3], P. KATINAKIS[1] and A. VENIERAKI[4]. [1]*Laboratory of General and Agricultural Microbiology, Crop Science Department, Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece.* [2]*Department of Food Science and Technology, University of the Peloponnese, 24100 Kalamata, Greece.* [3]*Laboratory of Toxicological Control of Pesticides, Scientific Directorate of Pesticides' Control and Phyto-pharmacy, Benaki Phytopathological Institute (BPI), Kifissia, 14561 Athens, Greece.* [4]*Laboratory of Plant Pathology, Crop Science Department, Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece. E-mail: venieraki@aua.gr*

Excessive use of chemical plant protection products has caused significant adverse effects on the environment. In agriculture, Use of selective beneficial bacterial biological control agents (BCAs) in agriculture can effectively support plant protection. Due to their characteristic compositions, medicinal plants from the Mediterranean basin are potential sources of efficient endophytic bacterial BCA candidates. Bacterial endophytes occur within host plant without causing visible pathogenic symptoms. Endophytic *Bacillus* isolated from the native medicinal plants *Calendula officinalis* and *Hypericum hircinum* showed strong antagonism to phytopathogenic fungi, including *Botrytis cinerea* and *Colletotrichum acutatum* under *in vitro* and *ex vivo* conditions. All the *Bacillus* strains studied successfully colonized plant tissues and competed with the phytopathogenic fungi for nutrients and space. An integrative approach was applied, coupling genome mining and metabolic profiling to indicate potential of these endophytes as BCAs against pathogenic fungi. Chemical analyses of the bacterium extracts indicated that these *Bacillus* endophytes secreted several bioactive secondary metabolites, and the bacteria were investigated for fungal growth suppression, either as individual strains or as bacterium mixtures. Formulation of compatible bacterial strains resulted in some mixtures with strong BCA ability.

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Effects of volatile organic compounds emitted by the biocontrol agent *Paenibacillus alvei* K165 against *Verticillium dahliae*. E.G. POULAKI[1], F. L'HARIDON[2], R. CARRON[2], L. WEISSKOPF[2] and S.E. TJAMOS[1]. [1]*Agricultural University of Athens, Crop Science Department, Lab of Phytopathology.* [2]*Department of Biology, University of Fribourg, Fribourg, Switzerland. E-mail: sotiris@aua.gr*

Effect of the volatile organic compounds (VOCs) emitted by *Paenibacillus alvei* K165 grown on Luria Broth (LB) medium were assessed against *Verticillium dahliae*, under *in vitro* and *in planta* conditions. GC/MS analysis showed that K165 grown on LB produced several volatile compounds, including 2,3 butanediol and tropone. Pure 2,3 butanediol and tropone inhibited the growth of *in vitro V. dahliae* by, respectively, 10 and 20 %. The *in planta* VOC emitting effect of the K165 against *V. dahliae* was examined in a dual compartment device, where the plants were placed in soil in the upper part of the device and the bacterial cells are applied in the lower part, ensuring spatial separation of plant roots from K165 cells. K165 was inoculated on LB medium in the lower part of the devise. The endophytic presence of *V. dahliae* was examined in the plants of the different treatments at 3 and 7 dpi, by Real Time PCR analyses. The K165 treated plants were less colonized by *V. dahliae* compared to controls, at both sampling time points. In agreement with the Real Time PCR results, disease

assessments showed that the symptoms, mainly wilting, were less in the plants exposed to the K165 VOCs compared to controls.

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Valorization of *Gelidium sesquipedale* residue in the control of Ascochyta blight of chickpea. H. ERRATI[1,2,3], S. LEBBAR[3], K. DARI[1], L. HILALI[1] and S. KRIMI BENCHEQROUN[2]. *[1]University Hassan 1st, Faculty of Science and Techniques, Laboratory of Agro Alimentary & Health, P.O. Box 577, Settat, Morocco. [2]National Institute of Agriculture Research (INRA, CRRA-Settat), P.O. Box 589, Settat, Morocco. [3]Setexam Company for the Study and Exploitation of Algae and Maritime Products, Kenitra - 14000 Morocco. E-mail: sanae.krimibencheqroun@inra.ma*

The use of biological products as alternatives to chemical fungicides has become important due to potentially adverse health and environmental impacts. The red alga *Gelidium sesquipedale* is mainly used for extraction of Agar-Agar. However, large amounts of by- product residues are also produced and are unused. Bioactive compounds in *G. sesquipedale* residues were evaluated for their antifungal activity against *Ascochyta rabiei* which causes Ascochyta blight of chickpea. The alga residues extraction were carried out using water, dichloromethane, or 1:1 (v:v) dichloromethane:ethanol as solvents. Antifungal activity against the pathogen was first tested *in vitro* on PDA medium amended with different extracts, using seven concentrations from 0 to 8 mg mL^{-1}. Phytotoxicity of these products was also evaluated on chickpea seeds. *In vivo* experiments were carried out in a greenhouse to evaluate the efficacy of aqueous residue extracts (as seed of foliar treatments) for control of Ascochyta blight. The chemical fungicide azoxystrobin (at 250g L^{-1}) was used for comparison. Aqueous residue extract at 8 mg L^{-1} was the most effective at inhibiting mycelium growth of *A. rabiei* by 80%. No phytotoxic effects of aqueous extract was observed on chickpea seed germination at all tested concentrations. Applications of aqueous residue extract as foliar treatments controlled the disease, reducing disease severity by 73%, similar to azoxystrobin. However, no effect was observed from the seed treatments. Aqueous extract from *G. sesquipedale* residues, used as foliar treatments, could be further investigated as a potential a biological antifungal product.

***Trichoderma atroviride* SC1: a biocontrol solution for pathogens of grapevine and other important crops.** M. KAIAFA, A. NESLER, J. GOOSSENS and A. VERMAETE. *Bi-PA nv* (*Biological Products for Agriculture), Technologielaan, 7. 1840 Londerzeel, Belgium. E-mail: maria.kaiafa@bi-pa.com*

Biological Products for Agriculture (Bi-PA nv) is working to develop and register biological plant protection products. A success for the company is Vintec®, a biofungicide based on active conidia of *Trichoderma atroviride* strain SC1. Vintec® was initially developed and registered for protection of grapevines against trunk diseases (Esca, *Eutypa lata)*. The product is applied preventively on pruning wounds. The mode of action is mainly based on prevention of pathogen colonization of pruning wounds, through creation of barrier against the pathogens and competition for space and nutrients. After registration of Vintec®, Bi-PA nv has continued to develop the product. Vintec® has obtained label extensions for the use against *Botrytis cinerea* on grapes and tomatoes, and against the fungal pathogens of stone fruit, *Coryneum beijerinckii, Monilinia laxa* and *Taphrina deformans*. Protection of tomato plants against *Botrytis cinerea* was confirmed by efficacy trials in protected conditions in the Netherlands, Italy and Spain. Greatest efficacy resulted from Vintec® applied at 5 to 10 g 100 L^{-1}. Efficacy was greater than, or comparable to, the reference products, when applied as preventive applications. For protection of stone fruits against major pathogens, Vintec® performed well in efficacy trials in open field conditions in Italy, Spain, Greece and Portugal. Optimal dose was 200 g ha^{-1}, which gave greater than or comparable efficacy to reference fungicide products.

Evaluation of the biocontrol capabilities of *Clonostachys rosea* against grapevine trunk diseases. A. GEIGER[1,2], Z. KARÁCSONY[1], J. GEML[3], K.Z. VÁCZY[1]. *[1]Eszterházy Károly Catholic University, Food and Wine Research Center, Leányka utca 6, H-3300, Eger, Hungary. [2]Doctoral School of Environmental Sciences, Hungarian University of Agriculture and Life Sciences, Páter Károly u. 1. H-2100, Gödöllő, Hungary. [3]ELKH-EKKE Lendület Environmental Microbiome Research Group, Eszterhazy Károly Catholic University, Leányka u. 6. H-3300, Eger, Hungary. E-mail: geiger.adrienn@uni-eszterhazy.hu*

The biocontrol agent *Clonostachys rosea* is asoil borne, and is known for its antagonistic abilities against numerous plant pathogenic fungi, nematodes and insects. This

fungus has not been investigated for antagonism to grapevine trunk disease pathogens. This study characterized the biocontrol capabilites of *C. rosea* against the pathogens causing GTDs. Confrontation tests were carried out with five *C. rosea* isolates and six GTD pathogens. The greatest growth inhibition was against the *Phaeomoniella chlamydospora* (50% reduction) and *Eutypa lata* (30%). Mycoparasitism was observed against *Botryosphaeria* and *Phomopsis* species. Elicitor and biomass production and the sporulation of the *C. rosea* isolates were also compared. The 19b1 of *C rosea* was selected for *in planta* assessments. Cabernet sauvignon cuttings were grown under greenhouse conditions, and inoculated with the patoghens *P. chlamydospora, E. lata and Botryosphaeria dothidea*. Plants were further grown in untreated soil or a soil amended with 10^4 spores g^{-1} of isolate 19b1. After 3 months incubation, symptom development was assessed. Lesion lengths caused by *P. chlamydospora* and *E. lata* were decreased in the presence of the isolate, while virulence of *B. dothidea* was unaffected. The biocontrol fungus was frequently re-isolated from the cutting bases, colony forming units were increased from 10^4 to 10^5 g^{-1} of treated soil. These results indicate that *C. rosea* can be used as a biocontrol agent against GTDs.

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Evaluation of biological and synthetic plant protection products for the management of downy mildew in grapevines. C.K. KAVROUMATZI, M.K. ILIADI, M. VARVERI, D. AKRIVOPOULOU and D.I. TSITSIGIANNIS. *Department of Crop Science, Laboratory of Plant Pathology, Agricultural University of Athens, Greece. E-mail: dimtsi@aua.gr*

Downy mildew, caused by *Plasmopara viticola*, is the most serious threat to grapevines in most viticulture regions where rainfall occurs regularly during the late spring and summer. The pathogen can destroy a vineyard if no control measures are taken. The main strategies to restrict the pathogen are have been based on fungicides that result in soil accumulation and potential environmental hazards. Large amounts of fungicides must be applied many times through each production season to keep the pathogen under control. Present strategies for grapevine downy mildew control mainly use preventive copper or mancozeb treatments, from the beginning of periods when plants are susceptible to infections. The efficacy of different plant protection products (PPPs) was assessed in grapevine leaves after artificial infection with *P. viticola* zoosporangia. Nine commercial bio-PPPs and five synthetic PPPs were tested against the pathogen, using susceptible host cultivars Moschato and Agiorgitiko. The most effective bio-PPPs for both cultivars were Amylo-X®, Remedier®, Trianum® and Vacciplant®, but these were not as effective as copper formulations. All the tested synthetic PPPs controlled the disease, The most efficient was Bion-MX® which combines the biostimulant acibenzolar-S-methyl and a chemical metalaxyl.

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Evaluation of biological control agents for the protection of almond pruning wounds against fungal canker pathogens. R. TRAVADON, D.P. LAWRENCE, S. LI and F.P. TROUILLAS. *Department of Plant Pathology, University of California, Davis, 95616 Davis, CA, USA. E-mail: rtravadon@ucdavis.edu*

Fungal cankers are ubiquitous in perennial crops grown for fruit and nut production in regions with a Mediterranean climate. In almond, these diseases decrease yields and severely limit the productive lifespan of orchards. The fungal pathogens infect almond trees through pruning wounds, as pruning is a commonly used to establish tree architecture and invigorate fruitwood. Biological control agents (BCAs) have gained increased interests for pruning wound protection because of increasing restrictions for pesticide use. The present study evaluated the biocontrol potential of commercial and experimental BCAs against common fungal canker pathogens of almond. Initial screening of nine BCAs in dual cultures with seven pathogens allowed the selection of the four most promising BCAs for further testing of their antagonistic activities *in planta*. The four BCAs were evaluated against a subset of four pathogens (*Cytospora plurivora*, *Eutypa lata*, *Neofusicoccum parvum* and *Neoscytalidium dimidiatum*). This showed that some BCAs reduced almond pruning wound infections by canker pathogens. The four BCAs were further evaluated in the field in two almond orchards planted with two distinct cultivars, Sonora and Nonpareil. Based on 2 years of field data, two BCAs provided levels of disease control similar to those reached with thiophanate-methyl). These BCAs are promising candidates for use in integrated pest management strategies for almond production.

Mineral oils against powdery mildew: paraffin oil induces resistance in grapevine against *Erysiphe necator*, and is applicable in disease management. X. PÁLFI[1], M. LOVAS[1], Z. KARÁCSONY[1], J. KÁTAI[3], K.Z. VÁCZY[1] and ZS. ZSÓFI[2]. *[1]Food and Wine Research Institute, Eszterházy Károly Catholic University, H3300 Eger, Hungary. [2]Institute for Viticulture and Enology, Centre for Research and Development, Eszterházy Károly Catholic University, H3300 Eger, Hungary. [3]Faculty of Agricultural and Food Sciences and Environmental Management Institute of Agrochemie and Soil Sciences, University of Debrecen, H4032, Debrecen, Hungary. E-mail: palfi.xenia@uni-eszterhazy.hu*

Petroleum-derived spray oils (PDSOs) have been used widely and for a long time in pest management of several crops. They are mostly used as washing sprays and adjuvants, but they also have antifungal effects. Good results with these oils were observed in field experiments against powdery mildew (GPM) of grapevine (*Vitis vinifera* L.), although the mode of action of the antifungal property is poorly understood. The possible direct fungicidal activity against GPM and the stress-inducing capability of 2% v:v paraffin oil (PFO) on grapevine were examined using "Kékfrankos" cuttings and *Erysiphe necator*. No direct fungicide activity was detected on *E. necator*. However, PFO induced significant physiological changes in grapevines. Several stress-related processes were observed, including: increased H_2O_2 and salicylic acid production; secondary thickening of cell walls through lignin deposition and accumulation of phenolic compounds. Changes in some enzyme activities related to oxidative stress or metabolism of phenolics were also measured, and were in accordance with the physiological changes. These results indicate that PFO could induce systemic acquired resistance in grapevine plants through the elicited stress responses, which leads to reduced susceptibility to GPM. The potential benefit of PFO to plant immunity was also experienced in a field spraying experiment in 2015–2016, where combining PFO with conventionally used fungicides increased efficacy against GPM. In addition to its elicitor role, PFO can increase adherence and absorption of spray agents, so application of PDSOs in disease management can be rewarding.

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Towards Nutrition-Sensitive Agriculture: an evaluation of biocontrol effects, nutritional value, and ecological impacts of bacterial inoculants. G.-I-R. SHAZHAD[1], A. PASSERA[1], V. VACCHINI[3], G. COCETTA[1], A.A. ARPANAHI[1,2], P. CASATI[1], A. FERRANTE[1] and L. PIAZZA[3]. *[1]Department of Agricultural and Environmental Sciences – Production, Landscape, Agroenergy, Università degli Studi di Milano, Milan, Italy. [2]Present address: Soil Sciences Department, Agriculture Faculty, Lorestan University, Khorramabad, Iran. [3]Department of Environmental Science and Policy – Production, Landscape, Agroenergy, Università degli Studi di Milano, Milan, Italy. E-mail: gul.shahzad@unimi.it; paola.casati@unimi.it*

The integration of Nutrition Sensitive Agriculture with plant protection is a promising domain, which gave new insights to balanced agriculture production by filling the gap of nutritional and ecological aspects alongside food safety. Based on this concept, microbial-based products can be alternatives to synthetic products, without compromising health-related properties, sustainable production, while maintaining biodiversity in agroecosystems. Performance of romaine lettuce plants (*Lactuca sativa*) was evaluated in pots under optimized greenhouse conditions, with or without treatments of potential plant-beneficial bacterial strains *Paenibacillus pasadenesis* 'R16', *Pseudomonas syringae* '260-02' and *Bacillus amyloliquefaciens* 'CC2' against the soil-borne pathogens *Rhizoctonia solani* and *Pythium ultimum*. considered factors included the biocontrol efficacy against pathogens, microbial biodiversity in the bulk soil, rhizosphere and root endosphere, and nutraceutical and plant growth promotion parameters. Strain R16 reduced symptom severity caused by both pathogens, while '260-02' and 'CC2', as well as a *Trichoderma*-based commercial product, showed less efficient biocontrol ability. Among all treatments, radical variation was observed in the microbial composition of rhizospheres and root endospheres, but not in the bulk soil, showing no substantial ecological side effects. Most plant nutraceutical parameters, including photosynthetic efficiency, carotenoid content, and phenolic content remained uninfluenced by the treatments. Chlorophyll content was also greater in R16-treated leaves challenged with *Rhizoctonia solani,* demonstrating a positive physiological effect on carbon fixation. None of the treatments had negative effects on plant growth, showing the suitability of these strains.

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Integrated disease management

SDHI fungicide has potential to reduce storage rot in sugar beet caused by *Botrytis cinerea* in USA. M.F.R. KHAN[1,2] and M.Z.R. BHUIYAN[1]. [1]*Department of Plant Pathology, North Dakota State University, Fargo, ND 58108, USA.* [2]*University of Minnesota, St. Paul, MN, USA. E-mail: Mohamed.khan@ndsu.edu*

Botrytis cinerea Pers. affects more than 200 plant species, and causes significant economic losses to sugar beet industries in regions where beet roots are stored for up to 9 months. The pathogen is typically present as a characteristic gray mold on roots, causing increased respiration rates that reduce recoverable sucrose by up to 60 to 80%. There are many fungicides that are extensively used for sugar beet production, but relatively few have been evaluated for control of storage rot of sugar beet. Penthiopyrad, a new SDHI fungicide labelled for sugar beet, was evaluated for reducing the activity of *B. cinerea* on sugar beet roots in storage conditions. Harvested roots were washed, dried and sprayed or dipped in different concentrations of the fungicide. Harvested sugar beet roots were washed, dried, and sprayed and dipped in different concentrations (10, 20, 40 or 80 mL L^{-1}), followed by inoculation with mycelium plugs of *B. cinerea*. The treated beets were then kept at 4°C and their respiration rates were measured at 0, 30, 60, 90 and 120 days post-inoculation. Penthiopyrad at 20 mL L^{-1} or greater reduced respiration rate (CO_2) in treated beets, and has potential for use to reduce storage losses caused by *B. cinerea* on sugar beet in long term storage.

Multilocus-sequencing–based genetic composition and DMI fungicide resistance in *Erysiphe necator* populations in Hungary. M.Z. NÉMETH[1], A. PINTYE[1], O. MOLNÁR[1], F. MATOLCSI[1,2], Á.N. HORVÁTH[1], V. BÓKONY[1], ZS. SPITZMÜLLER[3], K.Z. VÁCZY[3], L. KISS[4] and G.M. KOVÁCS[1,2]. [1]*Plant Protection Institute, Centre for Agricultural Research, ELKH, Herman Ottó út 15., 1022 Budapest, Hungary.* [2]*Department of Plant Anatomy, Institute of Biology, Eötvös Loránd University, Pázmány Péter sétány 1/C, 1117 Budapest, Hungary.* [3]*Food and Wine Research Centre, Eszterházy Károly Catholic University, Leányka utca 6., 3300 Eger, Hungary.* [4]*Centre for Crop Health, Institute for Life Sciences and the Environment, University of Southern Queensland, 487-535 West Street, 4350 Toowoomba, Queensland, Australia. E-mail: matolcsi.fruzsina@atk.hu (presenter), nemeth.mark@atk.hu (corresponding author)*

Grapevine powdery mildew (GPM) caused by *Erysiphe necator* is usually controlled by sterol demethylation inhibitor (DMI) fungicides, but long-term use of these agents has resulted in resistance in GPM to these fungicides. A common marker of resistance is the A495T nucleotide substitution in the *CYP51* gene. The GPM populations in Europe can be divided into two groups, which may differ in their seasonality and fungicide resistance. We sampled GPM in Hungary to describe genetic composition of these populations, and investigate the prevalence of the A495T-marker, and the relationships between its occurrence, fungicide treatments, sampling sites and years, and *E. necator* genetic groups. Sampling was conducted in six wine regions for 3 years. Fragments of four genes were sequenced. Occurrence of A495T was determined by sequencing or real-time PCR, and the probability of A495T occurrence was analysed with a generalized linear model. Fourteen haplotypes of *E. necator* were obtained, of which eight were previously unknown. These included haplotypes which represented SNPs characteristic to both genetic groups, and might represent recombinants. A495T was detected in all wine regions, in ≈16% of the samples. Differences in occurrence of A495T were found among several wine region and cultivar combinations, and between study years. Occurrence of A495T was not significantly different between treated and untreated sites, neither between seasons nor between genetic groups. These results indicate that in Hungary, *E. necator* populations consist of diverse haplotypes, including recombinants. These frequently harbour the A495T mutation, occurrence of which is mainly influenced by wine region and cultivar, and the sampling year.

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Development of cost-effective methods for detection of the DMI fungicide resistance marker A495T of the grapevine powdery mildew fungus *Erysiphe necator*. M.Z. NÉMETH[1], A. PINTYE[1], O. MOLNÁR[1], F. MATOLCSI[1,2], Á.N. HORVÁTH[1], ZS. SPITZMÜLLER[3], K.Z. VÁCZY[3] and G.M. KOVÁCS[1,2]. [1]*Plant Protection Institute, Centre for Agricultural Research, ELKH, Herman Ottó út 15., 1022 Budapest, Hungary.* [2]*Department of Plant Anatomy, Institute of Biology, Eötvös Loránd University, Pázmány Péter sétány 1/C, 1117 Budapest,*

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Demethylase inhibitor (DMI) fungicides applied to control *Erysiphe necator*, which causes grapevine powdery mildew, are integral for disease management in grapevine cultivation. Intensive application of DMIs resulted in fungicide resistance in some *E. necator* populations. Resistance is most commonly caused by a point mutation, A495T in the *CYP51* gene, resulting in an amino acid change in the CYP51 protein, rendering the fungus resistant to DMIs. Monitoring fungicide resistance is essential for effective disease control. This study aimed to develop cost-effective methods for detection of the A495T point mutation. A simple protocol was optimized for DNA extraction from *E. necator*. This is based on crushing single chasmothecia in extraction medium, and the resulting extract is directly used for direct PCR amplification of the gene region containing A495T. A quantitative real-time PCR (qPCR) assay was also adapted to detect A495T. To further simplify diagnostics, utility of loop mediated isothermal amplification (LAMP) for detection of A495T was tested. Primers were designed for detection of A495T. The developed qPCR and LAMP protocols combined with the rapid DNA extraction are suitable for cost-effective genotyping of *E. necator* for the A495T mutation.

This research was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences, and by the Széchenyi 2020 Programme, the European Regional Development Fund and the Hungarian Government (GINOP-2.3.2-15-2016-00061) and partly by the ELTE Thematic Excellence Program 2020 (TKP2020-IKA-05) of the National Research, Development and Innovation Office.

Role of early season control on Botrytis bunch rot epidemics in vineyards. G. FEDELE[1], E. GONZÁLEZ-DOMÍNGUEZ[2] and V. ROSSI[1]. *[1]Department of Sustainable Crop Production (DIPROVES), Università Cattolica del Sacro Cuore, via Emilia Parmense, 84, 29122, Piacenza, Italy. [2]Horta srl, via Egidio Gorra, 55, 29122, Piacenza, Italy. E-mail: vittorio.rossi@unicatt.it*

Botrytis bunch rot (BBR) is an important disease affecting grapevines, which requires adequate control, often based on routine application of fungicides at the end of flowering (A), pre- bunch closure (B), veraison (C), and before harvest (D). This simple, calendar-based scheduling of fungicides is at odds with the complexity of the *Botrytis cinerea* life cycle and existence of different infection pathways. A mechanistic model has shown that early-season development of *B. cinerea* determines the severity of BBR on ripe bunches, because of latent infection of young berries and saprophytic colonisation of bunch floral debris (or bunch trash). A meta-analysis of 116 studies confirmed that BBR control at growth stage A is more effective than at B, with control at B being useful only under high disease pressure when the full A, B, C and D strategy is needed for effective BBR control. The combination of control at A, C and D provides good and additive control of the early-season infection pathways and the multiple infection events during berry ripening. Following an integrated BBR management approach, early-season use of alternative products (e.g., biological control agents and botanicals) based on model predictions provides effective BBR management in vineyards.

Development and evaluation of a weather-driven, mechanistic model for predicting blossom blight caused by *Monilinia laxa* and *M. fructicola* on stone fruits. V. ALTIERI, I. SALOTTI and V. ROSSI. *Department of Sustainable Crop Production (DIPROVES), Università Cattolica del Sacro Cuore, via Emilia Parmense, 84, 29122, Piacenza, Italy. E-mail: vittorio.rossi@unicatt.it*

Monilinia laxa, M. fructicola, and *M. fructigena* are closely related fungi that affect blossoms, twigs and fruit, and can cause substantial economic damage to stone fruit production. Blossom blight is mainly caused by *M. laxa* and *M. fructicola*. Disease control generally relies on fungicide sprays, but spray programmes often provide inconsistent disease control. This may be due to poor spray timing, or may result in unjustified sprays when the conditions are not suitable for infection. To improve decision making in disease control, a mechanistic, weather-driven model was developed that is based on the available knowledge on processes leading to blossom infection by *M. laxa* and *M. fructicola*. A systematic literature review and systems analysis was used to retieve, analyze and synthesize a relational diagram, which considers the following compartments: i) production of conidia on mummified fruits; ii) release and deposition of the conidia produced on mummies; iii) infection caused by conidia on stone fruit blossoms; and iv) incubation and disease onset. The model is driven by weather-dependent mathematical equations developed based on literature information. Model validation is ongoing by comparing model predictions *vs.* independent (i.e., not used in model development) observed data. Different cropping systems and stone fruit hosts are being consid-

ered to evaluate the model's accuracy and robustness in a wide range of conditions.

Evaluation of epiphytic grape yeasts for the control of *Aspergillus carbonarius* and ochratoxins in grapes. M.K. ILIADI, E.G. POULAKI, C.K. KAVROUMATZI, M.F. VARVERI, D.N. PERIVOLARIS, S.E. TJAMOS, E.J. PAPLOMATAS and D.I. TSITSIGIANNIS. *Department of Crop Science, Laboratory of Plant Pathology, Agricultural University of Athens, Greece. E-mail: dimtsi@aua.gr*

Yeasts are considered ideal biological control agents, as they are able to survive in a wide range of environmental conditions, grow rapidly with simple nutritional requirements, can colonize plant surfaces even under prolonged dry conditions, and do not secrete toxic substances for humans. These fungi have been widely used against plant pathogens at pre- or post-harvest levels. This study aimed to discover effective endemic yeasts as biocontrol agents against the black rot of grapes, caused by *Aspergillus carbonarius*. This pathogen adversely affects the organoleptic characteristics of wine, and produces carcinogenic ochratoxins. Grapevine yeasts were assessed from the collection of Laboratory of Phytopathology (AUA), as well as new isolates from different grapevine varieties and regions in Greece. Several strains were tested *in vitro* for inhibition of conidium production by the ochratoxigenic strain *A. carbonarius* Ac-29 on Yeast Malt Agar. Antagonism and ochratoxin inhibition bioassays were also carried out on detached grape berries. Yeasts with high antagonistic properties against *A. carbonarius* were selected for field trials on the Greek white grape varieties Savatiano and Asyrtiko. Results of these experiments were presented. Discovery of endemic yeast strains that inhibit ochratoxin production and are acclimated and adapted to local physical and biological environments may lead to development of novel biocontrol products.

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Modelling potential climatic suitability for olive vascular diseases in southern Spain. L.F. ARIAS-GIRALDO, B.B. LANDA and J.A. NAVAS-CORTÉS. *Department of Crop Protection, Institute for Sustainable Agriculture (IAS), Spanish National Research Council (CSIC), Alameda del Obispo s/n, 14004 Córdoba, Spain. E-mail: lfarias@ias.csic.es*

Andalusia in Southern Spain is the main world olive oil producing region, 1.6 million ha of olive trees. Verticillium wilt (VW) caused by soil-borne *Verticillium dahliae* (*Vd*) and the olive quick decline syndrome, caused by *Xylella fastidiosa* (*Xf*) are currently the main threats for olive production. *Vd* occurs widely in Southern Spain, causing severe yield losses in some areas. In contrast, *Xf* is not known to be present in this region, but represents a threat since this pathogen has been detected in Spain. This study carried out a risk analysis that may help to prevent the spread of *Vd* to avoid the establishment of *Xf* in Andalusia. Species distribution modelling was used to determine relationships between sample location for the two pathogens with associated environmental variables, and estimate the ecological requirements for particular species. Bio5 and Bio15 were identified as the main climatic factors that determine the potential distribution of *Vd* in Andalusia, with the areas along the Guadalquivir River showing the greatest suitability, which agrees with the current known *Vd* distribution in the region. The model also identified extensive areas with moderate to high suitability in regions where olive plantations are currently expanding. Suitable areas for *Xf* are mainly characterized by warm temperatures during summer-dry periods, mild winters, and a well-defined rainy seasons, with the eastern part of Andalusia being at greatest risk. The developed models would allow better management of VW by identifying the risk levels for established and new plantations, and for prioritizing surveillance programmes for *Xf*, based on the level of risk from this pathogen.

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Strategic management of fungicide resistant *Cercospora beticola* in sugar beet using a holistic management strategy in the climate change era. M.F.R. KHAN. *Department of Plant Pathology, North Dakota State University, Fargo, ND 58108; and University of Minnesota, St. Paul, MN, USA. E-mail: Mohamed.khan@ndsu.edu*

Cercospora beticola causes Cercospora leaf spot (CLS), a damaging foliar disease of sugar beet grown in warm and humid regions. Since the 1970s, growers have become dependent on fungicides for effective control of

CLS. Fentin acetate and triphenyltin hydroxide, benzimidazoles, demethylation inhibitors (DMIs), and quinone outside inhibitors (QoIs) have been used with varying levels of success for control of *C. beticola*. The pathogen produces large numbers of conidia and multiple generations during each host growing season, and has developed resistance to most of the fungicides used for its control. In 2016, growers in the United States of America lost >$200 million because of QoI resistance and a CLS epidemic. Few fungicides currently provide effective control of *C. beticola*, when used alone, and there are very few effective alternating partners. Recently developed varieties (CR+) with improved resistance to *C. beticola* had better resistance to the pathogen than the best currently approved varieties. Fungicides were applied on a calendar basis, and only when required when symptoms and favourable environmental conditions occurred. CR+ varieties with and without fungicide applications resulted in similar or increased higher recoverable sucrose compared to similarly treated older varieties. Strategies including incorporating infected crop debris, crop rotation, planting away from previously infected fields, use of CR+ varieties, and judicious use of fungicide mixtures will be necessary for sustainable and economic production of sugar beet.

Modelling the airborne inoculum of *Polystigma amygdalinum*, which causes red leaf blotch of almond in Catalonia, Spain. G. PONS-SOLÉ[1], E. LÁZARO[2], A. VICENT[2] and J. LUQUE[1]. *[1]Sustainable Plant Protection Program, Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Ctra. de Cabrils km 2, 08348 Cabrils, Spain. [2]Institut Valencià d'Investigacions Agràries (IVIA), Ctra. CV-315 km 10.7, 46113 Moncada, Spain. E-mail: gemma.pons@irta.cat*

Red leaf blotch (RLB) of almond, caused by *Polystigma amygdalinum*, is the main foliar disease affecting almond orchards in the Mediterranean Basin. The pathogen overwinters in leaf litter, and ascospores are released from perithecia in spring to infect new almond leaves. RLB is characterized by a long incubation period, and fungicide sprays should be scheduled during the period of ascospore release. Airborne *P. amygdalinum* ascospores were monitored from 2019 to 2021 (February to September each year), by placing a 7-day volumetric spore trap (Hirst type) in two RLB-affected almond orchards in Catalonia. Exposed tapes were collected weekly, and were analysed with real-time qPCR to quantify *P. amygdalinum* trapped ascospores on a daily basis. Hierarchical Bayesian beta regression models were developed to fit the dynamics of accumulated ascospore catches to several environmental variables for both locations. The best model included accumulated degree-days (ADDs) and ADDs considering both rainfall and vapour pressure deficit (ADDwet) as fixed factors, and year as the random factor. For this model, the linear regression of the median posterior predictive distribution against observed values accounted for 78% of the total variance, with a mean absolute error of 0.1415 and a root mean square error of 0.1799. A Decision Support System using this model is currently under development to schedule fungicide sprays and optimize control of almond RLB.

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The consequences of co-infection by *Cucumber green mottle mosaic virus* and *Pythium* species under different environmental conditions. O. FRENKEL[1], A.M. PHILOSOPH[1,2], Y. ELAD[1], A. KOREN[3], N. MOR[4] and A. DOMBROVSKY[1]. *[1]Department of Plant Pathology and Weed Research, ARO, Volcani Center, Rishon LeZion POB 15159, Israel. [2]Department of Plant Pathology and Microbiology, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, POB 12, Rehovot, 76100, Israel. [3]Hishtil Nurseries, Moshav Nehalim, 4995000, Israel. [4]Extension Service, Ministry of Agriculture and Rural Development Rishon LeZion, IL 7505101, Israel; E-mail: omerf@volcani.agri.gov.il*

Plant pathology studies have mainly focused on the one host/one pathogen paradigm, but pathosystems can also involve co-infections by several pathogen species. Co-infections may produce symptoms dissimilar to infections by each pathogen alone, and may increase host damage. This study described the synergistic effects of co-infection by *Cucumber green mottle mosaic virus* (CGMMV) and *Pythium* spp. Late-wilting has increased in cucumber greenhouses during CGMMV outbreaks. As wilting occurs in defined patches accompanied by root rot, we hypothesized that the disease is caused by co-infections of soilborne pathogen/s and CGMMV. A field survey showed that 69% of the wilting plants were colonized simultaneously by *Pythium* spp. and CGMMV, whereas only 20% of the wilting plants were colonized only by *Pythium* spp. and 6.6% were infected with CGMMV. Artificial inoculations of cucumber plants

showed that co-infection with *P. spinosum* and CGMMV gave a synergistic wilting effect and reduced host growth parameters. This synergism was detected under a wide range of (optimal and suboptimal) temperatures, and *P. spinosum*, Which mostly prevails in mild temperatures, caused high mortality at an extended temperature range and even at 32°C**.** This study has demonstrated the complexity of pathosystems involving co-infections by two pathogens, and encourages a broader perspective of the complexity of agricultural diseases so appropriate disease management can be applied.

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A mechanistic weather-driven model for *Ascochyta rabiei* infection and disease development in chickpea. I. SALOTTI and V. ROSSI. *Department of Sustainable Crop Production (DIPROVES), Università Cattolica del Sacro Cuore, via Emilia Parmense, 84, 29122, Piacenza, Italy. E-mail: vittorio.rossi@unicatt.it*

Ascochyta blight, caused by *Ascochyta rabiei*, is an important disease of chickpea (*Cicer arietinum* L.). The available knowledge on *A. rabiei* was retrieved from literature, analysed and used to develop a mechanistic, weather-driven model for the prediction of Ascochyta blight epidemics. Using systems analysis, the pathogen life cycle was used to draw the model structure, and published quantitative information was used to develop algorithms driving model variables. The model was validated using data from literature and a field trial performed in Southern Italy in 2019. The ability of the model to predict primary infections was evaluated using data from Washington (United States of America) in 2004 and 2005, Israel in 1996 and 1998, and in Spain from 1988 to 1992. The model showed good accuracy and specificity. The probability of correctly predicting infections was 0.838, and that there was no infection when not predicted was 0.776. The model failed to predict some real infections, which caused very low amounts (8.1%) of total disease. Model capability to predict the disease progress during a growing season was evaluated using data collected in Australia from 1996 to 1998 and in Southern Italy in 2019. Significant linear regression (R^2 = 0.904, P<0.001) between predicted and observed data was obtained, indicating that the model is accurate and robust for predicting infections and dynamics of Ascochyta blight epidemics. The model could then be used to support control of this disease.

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Microbiomes and their role in plant health

Rhizosphere-enriched microbes as a pool to design synthetic communities with beneficial effects on plant fitness and health. M.-D. TSOLAKIDOU[1], I.A. STRINGLIS[2], N. FANEGA-SLEZIAK[1], S. PAPAGEORGIOU[1], A. TSALAKOU[1] and I.S. PANTELIDES[1]. *[1]Department of Agricultural Sciences, Biotechnology and Food Science, Cyprus University of Technology, Lemesos, Cyprus. [2]Plant-Microbe Interactions, Department of Biology, Science4Life, Utrecht University, Utrecht, the Netherlands. E-mail: iakovos.pantelides@cut.ac.cy*

Suppressive composts are an environmentally friendly approach to combat soilborne plant pathogens and improve plant growth. In a previous study, the microbial nature of the suppressiveness of a compost was demonstrated. The compost-derived microbial communities enriched in the rhizosphere of plants were identified and characterized for *in vitro* antifungal activity against soilborne fungal pathogens, and for their potential to change plant growth parameters. In the present study, two simplified microbial synthetic communities (SynComs) were designed with different composition, using representative bacteria from the rhizosphere community, to provide evidence of their beneficial effects on plant fitness and health. SynCom1, consisting of bacterial strains reflecting their relative abundance in the rhizosphere community, displayed a negative effect on *Arabidopsis in vitro*, but promoted tomato growth in pot experiments. SynCom2, consisting only of *Bacillus* strains, did not affect *Arabidopsis* growth but increased tomato growth and suppressed disease symptoms caused by *Fusarium oxysporum* f. sp. *lycopersici*. These results demonstrate that the composition of complex microbial communities can affect plant phenotypes, and these effects can be host-dependent. Identification and characterization of their traits could facilitate design of novel synthetic microbial communities, that could be used as inoculants with defined and controllable properties, conferring consistent beneficial effects towards plants.

Detailed survey of fungal communities of four vineyards containing different cultivars, located in the

Palava region (Czech Republic). A. BERRAF-TEBBAL[1], D. TEKIELSKA[1], J. PECENKA[1], M. SPETIK[1], A.E. MAHAMEDI[2], K. STUSKOVA[1], J. WOHLMUTH[1], D. CERNOHORSKA[3], E. PENAZOVA[1], J. CECHOVA[1], M. BARANEK[1], R. POKLUDA[1], D. GRAMAJE[4] and A. EICHMEIER[1]. *[1]Faculty of Horticulture, Mendeleum-Institute of Genetics, Mendel University in Brno, Valticka 334, 69144 Lednice, Czech Republic. [2]Laboratoire de Biologie des Systèmes Microbiens (LBSM), Département des Sciences Naturelles, Ecole Normale Supérieure de Kouba, Alger BP 92, Vieux-Kouba, Alger, Algeria. [3]Vinařství Plenér s.r.o., třída 1. máje 3414/11c, 690 02 Břeclav, Czech Republic. [4]Instituto de Ciencias de la Vid y del Vino (ICVV), Consejo Superior de Investigaciones Científicas, Universidad de la Rioja-Gobierno de La Rioja, Ctra. de Burgos Km. 6, 26007 Logroño, Spain. E-mail: ales.eichmeier@mendelu.cz*

Grapevine cultivars are differentially vulnerable to infections caused by fungal trunk disease (TD) pathogens. This study examined the total mycoflora (including TD pathogens) in four vineyards containing different cultivars located in the Palava region, the most famous wine-growing region in the Czech Republic. Ten plants each of cultivar Blaufränkisch, Palava, Pinot Noir and Welschriesling, showing symptoms of TD, were collected in each vineyard in July 2019. Fungal communities were examined using traditional isolation on artificial media and high-throughput amplicon sequencing (HTAS) of the internal transcribed spacer (ITS2) region. HTAS increased the resolution of the fungal community analysis and revealed a highly diverse and complex mycoflora from grapevine wood, compared to the classical isolation approach. According to symptom severity evaluated in 2020 and 2021, Pinot Noir was the most sensitive cultivar to TD pathogens. This observation correlated with the results of fungal detection assays. The most prevalent fungi detected by the classical approach were *Diaporthe* and *Phaeoacremonium*, and this was comparable with the HTAS results. This study provides important and practically useful insights into fungal communities among the four cultivars, and is the first to examine fungal communities on grapevine plants by using MiniSeq, 2- channel sequencer combined with classical isolation. The study also provides the most comprehensive survey of TD pathogens in Czech vineyards, and of grapevine tolerance against TD pathogens in the Palava region.

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Changes in grapevine-associated plant pathogenic fungal communities are greater among intraindividual microhabitats and terroirs than among healthy and Esca-diseased plants. A. GEIGER[1,2], Z. KARÁCSONY[1], R. GOLEN[1], K.Z. VÁCZY[1] and J. GEML[1,3]. *[1]Food and Wine Research Centre, Eszterházy Károly Catholic University, Leányka u. 6. H-3300, Eger, Hungary. [2]Doctoral School of environmental Sciences, Hungarian University of Agriculture and Life Sciences, Páter Károly u. 1. , H-2100, Gödöllő, Hungary. [3]ELKH-EKKE Lendület Environmental Microbiome Research Group, Eszterhazy Károly Catholic University, Leányka u. 6. H-3300, Eger, Hungary. E-mail: geml.jozsef@uni-eszterhazy.hu*

Grapevine is vulnerable to numerous diseases including grapevine trunk disease (GTD), which is a threat in wine industries, causing serious losses due to the premature vine decline and yield losses. Several studies have examined causes of GTD, but key questions on emergence and severity of the disease remain unanswered, including possible differences in plant pathogenic fungal communities of asymptomatic and symptomatic grapevines. Fungal DNA metabarcoding data were generated from bark, perennial wood, and soil samples from symptomatic and asymptomatic grapevines from three terroirs. Larger compositional differences in plant pathogenic fungi were found among different plant parts within, than among, grapevine plants. GTD-related fungi were dominant in perennial woody tissues, while non-GTD pathogens were found in soil. Asymptomatic plants did not differ from symptomatic plants. These result indicate that fungi generally associated with Esca disease belong to the core grapevine microbiome, and are likely to be commensal endophytes and/or latent saprotrophs, some of which can act as opportunistic pathogens on stressed plants. In addition, compositional differences were found among sampling sites, particularly in soil, which suggest influence of local edaphic and mesoclimatic factors on communities of plant pathogenic fungi. Differences among terroirs in plant pathogenic fungal communities were also detected in grapevine woody parts, which indicates that environmental factors are important for the development of Esca disease. Further research is required to determine effects of abiotic conditions on fungal compositional dynamics in Esca-affected plants.

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emy of Sciences) to KZV, and the Széchenyi 2020 Programme GINOP-2.3.2-15-2016-00061 (the European Regional Development Fund and the Hungarian Government).

Fungus-bacterium interactions in grapevine wood: consequences for plant health

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Viticulture is confronted with heavy economic losses caused by Grapevine Trunk Diseases (GTDs). Fungi have been described as the main causes of GTDs, but bacterium diversity is very high in grapevine wood. The function of these wood-inhabiting bacteria is relatively unknown, with only limited established knowledge of their interactions with grapevines or their wood-inhabiting fungi. Using different bacterial strains isolated from wood of Bordeaux grapevines, and three major GTD pathogens, bacterial-fungal interactions were shown to range from synergism to antagonism, depending on the species of bacteria and fungi involved. Screening of bacterial strains against *Neofusicoccum parvum*, *Phaeomoniella chlamydospore (Pch)* and *Fomitiporia mediterranea*, showed that some strains inhibited the growth of these fungi, and reduced severity of the wood necroses caused by *N. parvum* and *Pch* in young grapevines. In contrast, synergistic relationships were observed for other bacterial strains and *N. parvum* or *F. mediterranea*. Some strains independently destroyed grapevine wood components (cellulose and hemicellulose) and fungus ability to degrade wood structures was strongly influenced by bacteria inhabiting wood. A newly described bacterium *Paenibacillus xylinteritus* displayed synergism with *F. mediterranea*, and promoted wood degradation compared to that caused by *F. mediterranea* alone. These results raise question the functional diversity of the bacterial communities colonizing grapevine wood, and their direct or indirect involvement in GTDs.

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Characterization of endophytic *Alternaria* species isolated from grapevine (*Vitis vinifera*) shoots.

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Grapevine is associated with diverse endophytic fungal communities affecting the health status and productivity of the host plants. Previous research has shown that several *Alternaria* spp. are common endophytes inhabiting different grapevine varieties, and these fungi have been studied primarily because of their challenging taxonomy and abilities to produce a variety of secondary metabolites. However, data are limited on well-identified species in Hungarian vineyards, and their secondary metabolites. Molecular identification was determined for endophytic *Alternaria* species colonizing aboveground grapevine tissues, and the metabolite profiles of the *Alternaria* isolates were assessed. Based on the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA, the *Alternaria* species in *Alternaria* sect. *Alternaria* predominated in isolates from asymptomatic leaves and clusters. Since species-level discrimination within the *Alternaria* sect. *Alternaria* is not adequate using only the ITS region, the isolates were subjected to combined analyses of five additional genomic loci (RPB2, ALTA1, endoPG, OPA10-2 and KOG1058). The metabolite profiling was carried out using ultra-high performance liquid chromatography (UHPLC)-high-resolution mass spectrometry (HRMS). This indicated that the endophytic fungi from healthy grapevine shoots of were of two distinct lineages, the *A. alternata* and *A. arborescens* species complexes, and that numerous compounds characteristic to the genus occur in the different *Alternaria* lineages.

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Fungal diversity in necrotic wood of *Prunus* trees in Germany. U. DAMM[1] and S. BIEN[2]. [1]*Senckenberg Museum of Natural History Görlitz, PF 300 154, 02806 Görlitz, Germany.* [2]*Department of Forest Protection, Northwest German Forest Research Institute, 37079 Göttingen, Germany. E-mail: ulrike.damm@senckenberg.de*

Wood diseases of fruit trees have far-reaching ecological and economic consequences. Lack of knowledge of the diversity and taxonomy of wood disease fungi impedes effective and targeted control measures in cases of sudden disease outbreaks. A survey was conducted in orchards of *Prunus avium*, *P. cerasus* and *P. domestica* in three important fruit production areas in Germany. More than 1000 fungus isolates were obtained from symptomatic host wood, and at least 172 species (mostly *Ascomycota*) were identified, based on ITS and LSU sequence data. *Aposphaeria corallinolutea* (*Dothideomycetes*) and *Pallidophorina paarla* (*Leotiomycetes*) were most frequently detected, from all three host species and from all sampling regions. Based on the number of nucleotide differences to reliable reference sequences, species were identified with high certainty and and 20 species with low certainty. The first group included 14 taxa that were analysed in-depth within this project, of which eight species (*Arboricolonus simplex*, *Collophorina badensis*, *C. germanica*, *C. neorubra*, *Cadophora prunicola*, *Ca. ramosa*, *Minutiella pruni-avium*, *M. simplex* and *Proliferodiscus ingens*) and two genera (*Arboricolonus* and *Pallidophorina*) were newly described. A further 69 taxa could not be assigned to any species, and were regarded as potential new species. Reduced conidiogenous cells and a yeast-like phases were frequently observed (e.g. in *P. paarla*, and *Collophorina* and *Minutiella* species), and are likely to adaptations to living within host wood.

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Mycotoxins: prevention and control

***Fusarium tricinctum* species complex members are emerging pathogens in several crops: the case of apple.** M.T. SENATORE[1], R. SOLDESTI[1], M. CALI'[1], E. CAPPELLETTI[1], M. SULYOK[2] and A. PRODI[1]. [1]*Department of Agricultural and Food Sciences, Alma Mater Studiorum University of Bologna, Viale Fanin, 44, 40127 Bologna, Italy.* [2]*Department of Agrobiotechnology (IFA-Tulln), University of Natural Resources and Applied Life Sciences, Vienna (BOKU), Konrad Lorenz Strasse, 20, A-3430, Tulln. E-mail: antonio.prodi@unibo.it*

Members of the *Fusarium tricinctum* species complex (FTSC) are polyphagous and widespread pathogens, although some are considered of secondary importance on several crops. However, their presence has increased in crops such as wheat, barley, ryegrass, redcurrant, box and apple, causing yield losses, quality reduction and mycotoxin contamination. FTSC members have been reported as emerging pathogens on apple fruits and wood. This study analysed FTSC fungi isolated from Italian apple wood compared with isolates from different crops. The abilities of the FTSC isolates to produce mycotoxins such as Enniatins and Beauvericin (ENNs and BEA) was also assessed, through molecular detection of *Esy1n* gene, and this was confirmed in all isolates examined. Phylogenetic analysis based on the TEF-1α gene was conducted, and this assigned the the analysed isolates into four phylospecies: FTSC 2 (*F. acuminatum*), FTSC 3 (*F. tricinctum*), FTSC 4 (*F. avenaceum*) and FTSC 14. One isolate did not cluster with any of the reference sequences representing a potential novel species in the population. Pathogenicity and mycotoxin production of *F. acuminatum* and *F. avenaceum* strains was evaluated by artificial fruit inoculation on the apple cultivars Golden Delicious and Fuji. Although patulin has been the main mycotoxin problem for apple production, with *Fusarium* threatening wet apple cores, the problem is extended to emerging mycotoxins such as ENNs and BEA.

Biocontrol by atoxigenic *Aspergillus* strains and *Trichoderma* spp. C. ALTOMARE and A. LOGRIECO. *Istituto di Scienze delle Produzioni Alimentari, Consiglio Nazionale delle Ricerche, Via Amendola 122/O, 70126 Bari, Italy. E-mail: claudio.altomare@ispa.cnr.it*

Aflatoxins are produced *Aspergillus* from infections of agricultural crops and spoilage of stored food and feeds. These compounds are carcinogenic, mutagenic and hepatotoxic, and are found in cereals, oilseeds, spices, tree nuts, maize, peanuts, pistachios, dried fruit and figs, and their derived products. Studies of genetic and aflatoxigenic diversity in *A. flavus* have led to development of a biological control strategy based on competitive exclusion, which relies on the inundative introduction of

non-toxigenic strains, that displace native aflatoxigenic genotypes. This has been effective for reducing aflatoxin contamination by more than 90% in maize, peanuts, pistachios and cottonseed. However this strategy limits and drawbacks. Non-aflatoxigenic *A. flavus* strains may not be non-pathogenic, and can crop cause diseases and yield losses. Capability to produce aflatoxins may be transferred to progeny of non-toxigenic strains by mating with aflatoxigenic strains. Other toxic metabolites beside aflatoxins may be still produced by non-aflatoxigenic *A. flavus* strains. The capability of members the biocontrol genus *Trichoderma* to control *A. flavus* and reduce aflatoxins biosynthesis has been recently highlighted, is raising increasing interest. Using *Trichoderma* spp. for biocontrol of aflatoxigenic fungi may overcome some limits, and provide advantages compared to use of non-aflatoxigenic *A. flavus* strains. As broad-spectrum biocontrol agents, *Trichoderma* can also protect plants from other pathogens other than *A. flavus*. *Trichoderma* strains also have indirect favourable effects of enhancement of plant resilience to drought stress and prevention of insect pest damage, which are factors that facilitate aflatoxin occurrence, particularly in climate change and global warming scenarios. An overview was presented of current technologies based on non-aflatoxigenic strains, and of prospects for the use of *Trichoderma* spp. for biological control of *A. flavus* and mitigation of aflatoxins risk.

Wood diseases in fruit crops

Fungal pathogens associated with grapevine trunk diseases in Cyprus. G. MAKRIS, M. CHRISTODOULOU, S. SOLONOS, L.I. KANETIS and M. CHRISTOFOROU. *Department of Agricultural Sciences, Biotechnology, and Food Science, Cyprus University of Technology, Limassol, 3036, Cyprus. E-mail: loukas.kanetis@cut.ac.cy*

Grapevine trunk diseases (GTDs) are major concerns for grape industries. In Cyprus, there has been no comprehensive study of the pathogen population structure and aetiology of this important disease complex. During 2017, 182 fields were surveyed in the main grape-producing areas of the country (Limassol and Paphos). Wood pieces from three to five selected vines exhibiting typical GTDs symptoms per field, were excised for fungal isolations. Samples were collected from of 24 grapevine cultivars, although 62% were from the two most prevalent, indigenous wine cultivars Mavro and Xinisteri. More than 600 samples were processed and approx. 750 fungal strains were isolated. Molecular identification based on ITS sequences identified more than 60 species or genera. GTD-related pathogenic species genera identified were *Phaeomoniella*, *Phaeoacremonium*, *Neofusicoccum*, *Botryosphaeria*, *Diplodia*, *Eutypa*, *Eutypella*, *Diaporthe*, *Cryptovalsa*, *Cytospora*, *Neofabraeae*, *Seimatosporium*, *Kalmusia*, and *Paraconiothyrium*. Phylogenetic analyses of these, using species-specificmarkers, identified *P. chlamydospora* (14.5% od isolates), *Phaeoacremonium minimum* (4.4%), *Diplodia seriata* (4%), *Botryosphaeria dothidea* (3.5%), and *Eutypa lata* (3.2%) as the predominant GTD-related species. Isolates of *Alternaria*, *Cladosporium, Fusarium*, *Neosetophoma*, *Ulocladium*, *Paecilomyces,* and *Penicillium*, commonly associated with grapevine microbiomes, were also detected. Pathogenicity tests were carried out using wood inoculation of young potted grapevine plants. Symptoms were recorded and re-isolations were confirmed, based on morphological characteristics, thus fulfilling Koch's postulates. This is the first systematic study of GTDs in Cyprus.

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***In vitro* effects of cell density on development and metabolism of *Phaeomoniella chlamydospra*, a pathogen causing Esca disease of grapevine.** Z. KARÁCSONY and K.Z. VÁCZY. *Food and Wine Research Institute, Eszterházy Károly Catholic University, Leányka str. 6/G, Eger, H3300, Hungary. E-mail: karacsony.zoltan@uni-eszterhazy.hu*

Phaeomoniella chlamydospora is an important pathogen causing esca disease of *Vitis vinifera*. Esca belongs to the group of grapevine trunk diseases (GTDs) caused by fungal infections of vascular tissues, leading to symptoms both perennial (wood necroses) and annual tissues (stunted shoots, foliar chloroses and necroses) of host plants. The pathogenesis processes of GTDs have still to be fully defined. Uneven expression of external symptoms on infected plants and the lack of correlation between pathogen abundance in hosts and occurrence of symptoms suggest that external factors and unique events can affect disease development. Critical pathogen cell population density within hosts, which shift otherwise harmless fungi to pathogenic behaviour, can be important. Microorganisms perceive cell populations by the "quorum sensing" mechanism, which affects the virulence of several bacterial and fungal pathogens. Results in this study have indicated that cell population density is important for development and metabo-

lism *P. chlamydospora*. Experiments in solid medium cultures indicate large cell populations promote germination of conidia and later the formation of polysaccharide-rich mycelium patches, which are indicators of biofilm formation. High cell population densities promoted colony pigmentation and synthesis of antibacterial compound(s), indicating a shift towards secondary metabolism. These phenomena may also occur in infected plants, leading to disease development.

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Aetiology and management of trunk and scaffold canker diseases of almond in California. L.A. HOLLAND, F.P. TROUILLAS, M.T NOURI, D.P. LAWRENCE and R. TRAVADON. *Department of Plant Pathology, University of California, Davis, 95616 Davis, CA, USA. E-mail: flotrouillas@ucanr.edu*

Trunk and scaffold canker diseases (TSCD) caused by fungal pathogens are destructive diseases of almond trees. Common TSCD symptoms include sunken bark lesions, wood discolouration and gummosis in trunks and branches. Recent surveys showed the occurrence of four major canker diseases in California, including *Botryosphaeriaceae* cankers, Ceratocystis canker, and Cytospora and Eutypa cankers. Up to 21 fungal pathogens were found to be associated with TSCD of almond. Pruning wounds made for scaffold selections and maintenance pruning were common entry sites for canker pathogens. Strategies were investigated for prevention of pruning wound infections. Fungicide trials were carried out to determine the best products for the protection of pruning wounds. These indicated superior efficacy of thiophanate-methyl against several canker pathogens. Additionally, the biocontrol agent *Trichoderma atroviride* SC1 provided excellent pruning wound protection, allowing sustainable control solutions against canker diseases. Additional studies were investigated seasonal susceptibility of pruning wounds and duration of pruning wound susceptibility, in relation to time (month) of pruning. These showed that duration of pruning wound susceptibility was least when pruning is carried out in January. Overall wound susceptibility declined substantially after 1 to 2 weeks following pruning. This research has indicated that one application of pruning wound protectant such as with the conventional fungicide thiophanate-methyl or the biocontrol agent *T. atroviride* SC1, following late pruning in January, can reduce risks of infection of pruning wounds by canker pathogens.

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***Botryosphaeriaceae* species as fungal pathogens associated with olive trunk diseases in southern Italy.** A. CARLUCCI, F. LOPS and M.L. RAIMONDO. *Department of Agriculture, Food, Natural resources and Engineering (DAFNE), University of Foggia, Via Napoli 25, 71122 Foggia, Italy. E-mail: antonia.carlucci@unifg.it*

Botryosphaeriaceae fungi have cosmopolitan distribution and a wide plant host range, including olive trees. Olive cultivation in southern Italy is important, with large areas and high yields. Different *Botryosphaeriaceae* have been associated with olive trunk diseases, causing cankers, dieback and olive decline. Several wood samples collected over 15 years from olive trees showing symptoms yielded a collection of fungal isolates, many of which were in this family. A phylogenetic study, carried out on representative strains and combining sequences from internal transcribed spacer region and translation elongation factor 1-α gene, allowed identification of four *Neofusicoccum* species such as *N. luteum*, *N. mediterraneum*, *N. parvum* and *N. vitisifusiforme*, and *Botryosphaeria dothidea*. Conidium size, colour and shape confirmed the molecular identifications. This study highlighted that the symptomatic olive wood analyzed was mainly infected by *Neofusicoccum* species. In particular, *Neofusicoccum* species above mentioned resulted all to be severe pathogens for olive by pathogenecity test carried out in vivo conditions.

Effects of extreme weather conditions on incidence and spread of grapevine trunk diseases. A. CSÓTÓ[1], P. BALLING[2], N. RAKONCZÁS[1], C.S. KOVÁCS[3], A. NAGY[1] and E. SÁNDOR[1]. *[1]University of Debrecen, Faculty of Agricultural and Food Sciences and Environmental Management, Debrecen, Hungary. [2]Research Institute for Viticulture and Oenology, Tokaj, Hungary. [3]National Agricultural Research and Innovation Centre Fruitculture Research Institute Development Institute of Újfehértó, Hungary. E-mail: csoto.andras@agr.unideb.hu*

Grapevine trunk diseases (GTDs) are important in viticulture. This disease complex may cause decay and death of grapevine arms and whole trunks with a wide range of symptoms. Losses of vines year by year accelerate the economic aging, so vineyards become prema-

turely unproductive. There are no effective preventative or curative treatments or agronomic practices against these diseases, because of the diversity of GTD pathogens, their protected niches within host woody tissues, which are difficult to reach with chemicals, and effects of abiotic factors on GTD incidence and symptom development. The present study aimed to determine the environmental parameters that may have significant influences on GTD development and spread. Determination of incidence patterns of different diseases within plantations, and the effects of climate and other environmental factors, require long time data collection. More than 3-year surveys were carried out in the Tarcal and in Pallag regions of Hungary, to detect incidence of GTDs and isolate fungi from cordon woody tissues. Results indicate that uneven rainfall, late spring frosts, high groundwater levels, and soil erosion have all increased GTD incidence. Species of *Botryosphaeriaceae* were the most common GTD pathogens in the examined plantations, based on isolations and isolate morphology and molecular identifications.

Exploration of the non-enzymatic wood degradation pathway for *Fomitiporia mediterranea*, the historical Esca agent. S. MORETTI[1], M.L. GODDARD[1,2], J. LALEVÉE[3], S. DI MARCO[4], L. MUGNAI[5], C. BERTSCH[1] and S. FARINE[1]. *[1]Laboratoire Vigne Biotechnologies et Environnement UPR-3991, Université de Haute Alsace, 33 rue de Herrlisheim, 68000 Colmar, France. [2]Laboratoire d'Innovation Moléculaire et Applications, Université de Haute-Alsace, Université de Strasbourg, CNRS, LIMA, UMR 7042, 68093 Mulhouse cedex, France. [3]Université de Haute-Alsace, CNRS, IS2M UMR 7361, F-68100 Mulhouse, France. [4]Institute of BioEconomy, National Research Council, Bologna, Italy. [5]Plant Pathology and Entomology Section, Department of Agricultural, Food, Environmental and Forestry Science and Technology (DAGRI), University of Florence, Florence, Italy. E-mail: samuele.moretti@uha.fr*

Fomitiporia mediterranea (Fmed) is a white rot agent that has been associated with esca in grapevines, but it's the biomolecular mechanisms of wood degradation by this fungus are not fully understood. Like all white rot agents, Fmed has an enzymatic pool (laccases, manganese peroxidases, endoglucanases and β-glucosidases) that can attack and depolymerizing cellulose, hemicellulose and lignin components of lignocellulosic biomass. Comparative genomics studies and experimental observations on the importance of iron in Fmed pathogenesis, retrieved from the literature, allowed formulation of the hypothesis that Fmed could utilize non-enzymatic and enzymatic mechanisms for wood degradation in grapevine. This hypothesis is based on the Chelator Mediated Fenton (CMF) model, proposed in the late 1990s for brown rot fungi. Present study results, under appropriate experimental conditions and as close as possible to the physiological conditions of grapevine wood, have demonstrated the *in vitro* ability of Fmed to: *i*) produce low molecular weight chelating metabolites, *ii*) reduce ferric iron to ferrous iron, and *iii*) produce radical species such as the hydroxyl radical, thus satisfying all the conditions supporting non-enzymatic wood degradation mechanisms. The CMF model is also likely to be strain-dependent in Fmed. Further research is ongoing to study this model *in lignum* and *in planta*.

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Induction of grapevine defence mechanisms by the oomycete *Pythium oligandrum*, against *Neofusicoccum parvum*, a pathogenic fungus involved in Esca. A. YACOUB[1,2], R. HAIDAR[1,2], J. GERBORE[3], M.C. DUFOUR[1,2] and P. REY[1,2]. *[1]Université de Pau et des Pays de l'Adour/E2S UPPA/ CNRS, Institut des Sciences Analytiques et de Physicochimie pour l'Environnement et les Matériaux - UMR 5254, IBEAS Avenue de l'Université, Pau, 64013, France. [2]Bordeaux Sciences Agro, UMR1065 SAVE, Université de Bordeaux, ISVV, F-33140 Villenave d'Ornon, France. [3]Université de Bordeaux, ISVV, Unité de recherche* Œnologie *EA 4577, USC 1366 INRA, Bordeaux INP, F-33140 Villenave d'Ornon, France. 3BIOVITIS, 15400 Saint Etienne de Chomeil, France. E-mail: amira.yacoub@univ-pau.fr*

Grapevine trunk diseases (GTDs) are increasing in vineyards. Many pathogens (e.g. *Phaeomoniella chlamydospora* and *Neofusiccoccum parvum*) are involved in these diseases, and there are no chemical treatments available for their control. Biocontrol of GTD pathogens using microorganisms has been developed. These include the oomycete *Pythium oligandrum*, strains of which naturally colonized grapevine roots in vineyards in several countries. The ability of *P. oligandrum* to induce grapevine resistance against *N. parvum* was evaluated. Two greenhouse assays showed that necrosis of Cabernet Sauvignon cuttings caused by *N. parvum* was reduced by 65% when *P. oligandrum* colonized the root systems of young vines. Expression levels of a set of 96 genes ("NeoViGen96"chip) involved in grapevine defence pathways were assessed by real-time PCR in grapevine trunks, to determine plant responses after inoculation

by *P. oligandrum* and/or *N. parvum*, at three different sampling time points. This showed that sampling time affected gene expression for all the treatments. At each sampling time, specific host responses to the different treatments (control, *P. oligandrum*, *N. parvum*, *P. oligandrum* + *N. parvum*) were also differentiated. When *P. oligandrum* colonizes grapevine root systems, infection with this pathogen is associated with intense up-regulation of PR protein and signalling pathway genes. A priming effect of the host defence system was induced in presence of *P. oligandrum*.

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Structure analysis of the ribosomal intergenic spacer (IGS) region as a putative marker for *Phaeoacremonium* phylogeny. M.L. RAIMONDO, F. LOPS and A. CARLUCCI. *Department of Agriculture, Food, Natural resources and Engineering (DAFNE), University of Foggia, Via Napoli 25, 71122 Foggia, Italy. E-mail: marialuisa.raimondo@unifg.it*

Most species of *Phaeoacremonium* are associated with wood diseases of various plants, including *Vitis vinifera*, *Olea europaea* and *Prunus* species. The increasing recognition of novel *Phaeoacremonium* species (to date 63), and their recent taxonomic reassignment through phylogeny based on the β-tubulin and actin genes, have highlighted the presence of paraphyly, intraspecific variation, and incongruence of some *Phaeoacremonium* species. The entire IGS region of a collection of 57 *Phaeoacremonium* strains was amplified, sequenced and subjected to phylogenetic analysis. A detailed analysis of the structure of IGS region was carried out for *Phaeoacremonium italicum* strains as a model, and compared with those of the closest related species, *P. alvesii* and *P. rubrigenum*. This showed five categories of repeat elements that were organized into distinct patterns. The comparison of the trees (IGS, and β- tubulin and actin) indicated that the intergenic spacer rDNA region distinguished intraspecific and interspecific variations. Preliminary studies of phylogenetic informativeness suggested that IGS could be a useful marker to resolve shallow divergence in *Phaeoacremonium* genus. Further molecular studies are required to determine if intergenic spacer sequences can improve precision in defining *Phaeoacremonium* phylogeny, and prevent misidentification and the introduction of vague species boundaries for the genus.

POSTER SESSIONS

Pathogenesis-related proteins of *Arabidopsis thaliana* in response to combination of abiotic (salinity) and biotic (fungus gnats and dodder) stresses. L. ZAGORCHEV, D. TEOFANOVA, K. GEORGIEVA and A. ATANASOVA. *Department of Biochemistry, Faculty of Biology, Sofia University "St. Kliment Ohridski", 1164, 8 Dragan Tsankov blvd., Sofia, Bulgaria E-mail: lzagorchev@biofac.uni- sofia.bg*

This study was assessed the response of *Arabidopsis thaliana* L. to a combination of abiotic and biotic stresses, including: 1) salinity; 2) fungus gnat (Sciaridae) herbivores; and 3) parasitic plants – *Cuscuta campestris* Yunck. (dodder), as well as the indirect effect on the dodder. The major pathogenesis-related protein classes chitinases and proteases were profiled under single, double or triple stresses in directly and indirectly affected host organs. Enzymes were studied by zymography analyses after separation on polyacrylamide gel electrophoreses, and data were analyzed by ANOVA and principal component analysis. All stress factors affected the number of isoforms and the relative activity of both enzyme groups, with the effect of fungus gnats being the most pronounced. The combination of stresses leads to mostly antagonistic effects in roots and cumulative effects in leaves. Salt stress caused similar effects to parasitism, a differential effect in comparison to herbivores, and did not interact with biotic stresses. Herbivores and parasitism affected the PR-proteins differentially, but interacted strongly. Salinity and herbivores, applied to the host plants, also affected the parasitic plants.

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Genome-wide characterization of WD40 protein family in *Monilinia fructigena*. A. ZAMBOUNIS and A. XANTHOPOULOU. *Institute of Plant Breeding and Genetic Resources, HAO 'Demeter', Thermi, Thessaloniki, 57001, Greece. E-mail: antonios.zamb@gmail.com*

The ascomycete *Monilinia fructigena* is among the most serious causal agents of brown rot in deciduous fruit trees. The pathogen genome is available, providing novel genomic resources for thorough characterization of particular gene families. WD40 proteins are scaffolding molecules in protein interactions, and although these proteins were extensively characterized in other organ-

isms, little is known in phytopathogenic fungi of their expansion and structural patterns, as well evolutionary selective pressures acting upon their WD40 repeats. Sixty-two WD40 proteins were identified in the *M. fructigena* genome (MfWD40s), and based on their phylogenetic classification and domain architectures they were categorized into five clusters and 17 classes, indicating their diverse expansion. Gene ontology analysis revealed that MfWD40s participate in protein binding and are involved in various biological processes. RNAseq data showed that the greatest number of MfWD40s genes had stage-specific expression profiles, with most being highly expressed during germination of conidia. The evolutionary signatures acting upon their WD40 repeats were accurately assessed. These results indicate existence of purifying selection acting across their phylogenies. However, a majority of amino acid residue sites were positively selected and were localized widely across the WD40 repeats, putatively affecting their ligand-binding specificities. These results will allow further deciphering of the diverse functions of the WD40 gene family in *M. fructigena*.

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RNA sequencing-based transcriptional profiling of kiwifruit during infection by *Botrytis cinerea*. A. ZAMBOUNIS[1], I. GANOPOULOS[1], D. VALASIADIS[2], L. KARAPETSI[3] and P. MADESIS[3]. *[1]Institute of Plant Breeding and Genetic Resources, HAO 'Demeter' Thermi, Thessaloniki, 57001, Greece. [2]Laboratory of Pomology, Department of Agriculture, Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece. [3]Institute of Applied Biosciences, CERTH, Thermi, Thessaloniki, 57001, Greece. E-mail: antonios.zamb@gmail.com*

Botrytis cinerea is a widespread plant pathogen, which causes gray mould, and infects many economically important hosts, including kiwifruit. To decipher the induced defence mechanisms upon infection in kiwifruit, an RNA sequencing (RNA-Seq) approach was used to explore the transcriptome of mature affected fruits at 12, 24, and 48 h after infection (HAI). Eighteen cDNA libraries were produced generating 978,286,445 base pair-end reads. A total of 2,795 differentially expressed genes (DEGs) were identified, with most being up-regulated at late stages across a broad and time-dependent transcriptional reprogramming during infection. Suppression of photosynthesis-related pathways was detected at 12 h HAI, whereas DEGs involved in specific cell wall modification processes may have facilitated early pathogen colonization. A strong shift towards defence responses and an induced transcriptional reprogramming was triggered but not earlier than 24 HAI. The majority of DEGs were up-regulated mainly at 48 HAI, and were predominately involved in the induction of biosynthesis of secondary metabolites, pattern-triggered immunity and signalling transduction cascades, activation of pathogenesis-related proteins, membrane-localized receptor-like kinases, transcription factors, and various transporters. A subset of *B. cinerea* genes that are linked to pathogen growth or manipulation of particular plant defence responses were also induced. These data provide novel insights into kiwifruit-*B. cinerea* compatible interactions, providing new knowledge for efficient management of this pathogen.

Infection and colonization of grapevine propagation material by pathogens associated with young grapevine decline in Greece. C. TSOUKAS, A.K. TZIMA, A. TRIANTAFYLLOPOULOU and E.J. PAPLOMATAS. *Laboratory of Plant Pathology, Agricultural University of Athens, Iera Odos 75, Athens, Greece. E-mail: epaplom@aua.gr*

Colonization of grapevine propagation material by young grapevine decline-associated pathogens, was investigated in eighty canes sampled (in July 2018) from four different varieties of mature mother plants ("Ksinomavro", "Agiorgitiko", "Asirtiko", or "Roditis"). In December 2018, forty 4-month-old vines ("Soultanina") grafted on rooted rootstocks (R110 or 41B) were also collected. Nested PCR reactions based on amplifications of the ITS region and species-specific primers showed that grafted young vines were heavily infected by the young grapevine decline-associated pathogens. Fourteen (35%) of 40 grafted cuttings were infected by *Phaeomoniella chlamydospora* and 35 (88%) were infected by Black Foot disease- associated pathogens. Multiplex PCR assays revealed that the dominant species was *Dactylonectria macrodidyma*. In July, pathogens could not be detected by PCR at the bases of growing season canes used for grafting. For the colonization studies, a *P. chlamydospora* isolate was transformed with the green fluorescent protein gene (*gfp*) using *Agrobacterium tumefaciens*-mediated transformation. Twenty four 4-month-old grafted vines were inoculated with either with the GFP *P. chlamydospora* strain or the wild type, and then kept in a greenhouse for 3 months. Fluorescence microscopy showed that, at 3 months post inoculation, the pathogen had colonized the cells around the

xylem vessels, parenchyma cells and primary xylem, but had not advanced more than 2 cm above or below the inoculation points.

Investigation of the disease-associated role, cellular localization and secretion of the thermo unstable translation elongation factor (Ef-tu) encoded by the vascular wilt fungus *Verticillium dahliae*. G. PATSIS[1], A. TRIANTAFYLLOPOULOU[1], D. GKIZI[2], S. KANG[3], A. TZIMA[1] and E. PAPLOMATAS[1]. *[1]Laboratory of Plant Pathology, Agricultural University of Athens, 75 Iera Odos, 11855 Athens, Greece. [2]Department of Food Science and Technology, University of West Attica, 28 Agioy Spiridonos, 12243 Aigaleo, Greece. [3]Department of Plant Pathology and Environmental Microbiology, Pennsylvania State University, University Park, PA 16802, United States. E-mail: aliki@aua.gr*

Besides its crucial role in protein synthesis, thermo unstable translation elongation factor (Ef-tu) plays other multiple other roles (moonlighting effect) in procaryotes and eucaryotes. The phytopathogenic bacterial Ef-tu triggers plant immune systems Pathogen-Associated-Molecular-Pattern (PAMP), recognized by the specific receptor EFR. Subcellular localization of the orthologous protein was investigated in *V. dahliae* (Vtu) to determine whether it is translocated to cell membranes or is extracellular to interact with host plants. The vtu gene was fused with the enhanced green fluorescent protein (*egfp*) gene under the control of a strong fungal promoter. This construct was used to transform *V. dahliae* race 1 strain 70V via *Agrobacterium tumefaciens*-mediated transformation. Localization of Vtu-Egfp was detected using confocal microscopy, which showed that the Vtu protein was localized in the host cytoplasm and vacuoles. Measurement of the vtu transcript level in various transformants with qPCR showed that integration of additional copies of the vtu gene in *V. dahliae* may have activated the RNA silencing mechanism in some transformants. Selected *V. dahliae* transformants expressing Vtu-Egfp will be tagged with a different fluorescent protein gene to investigate whether the Vtu-Egfp protein is secreted during host infection.

***Bactrocera oleae* as a transmitting agent of olive drupes anthracnose caused by *Colletotrichum acutatum*.** P. ADAMI[2], P. VELAETI[1], C. TSOUKAS[1], S. DERVISOGLOU[2], E.J. PAPLOMATAS[1] and D. PERDIKIS[2]. *[1]Laboratory of Plant Pathology, Agricultural University of Athens, Iera Odos 75, Athens, Greece. [2]Laboratory of Agricultural Zoology and Entomology, Agricultural University of Athens, Iera Odos 75, Athens, Greece. E-mail: epaplom@aua.gr*

To investigate possible transmission of *Colletotrichum acutatum* by the olive fruit fly *Bactrocera oleae*, thus contributing to disease spread, pupae and healthy olive drupes were collected in October 2021 from olive orchards in Greece. The drupes were artificially inoculated by an aqueous suspension of 10^6 conidia mL^{-1} of the pathogen, while control drupes were sprayed with ddH_2O. Seven days later after incubation under appropriate conditions, the drupes were transferred for 2 d into separate entomological cages containing adult flies that had emerged from the collected pupae. All drupes were subsequently discarded and replaced by new sterilized drupes that remained for 2 days with the flies. Thereafter, molecular detection of *C. acutatum*, on flies and on drupes, was performed using Real-Time PCR assays based on TaqMan chemistry, with *C. acutatum*-specific primers targeting the HIS-3 gene. Pathogen DNA was detected in all artificially inoculated drupes and in some of the flies. Fungus DNA could not be detected in negative control drupes, while pathogen DNA was detected in drupes that remained for 2 d with adult flies previously caged with artificially inoculated fruits. To investigate the possibility that fungal spores were carried passively by the flies, adult insects were superficially sterilized using either hexane or water. Besides detection in non-sterilized flies, DNA of the pathogen was also detected in surface sterilized flies. These results indicate that *B. oleae* could be a vector of *C. acutatum* anthracnose on olive drupes.

Manipulation of ACC (1-aminocyclopropane-1-carboxylic acid) deaminase gene in *Verticillium dahliae* revealed a binary role for ACC in regulating virulence and plant defence: two sides of the same coin. M.-D. TSOLAKIDOU[1], I.S. PANTELIDES[1], A.K. TZIMA[2], S. KANG[3], E.J. PAPLOMATAS[2] and D. TSALTAS[1]. *[1]Cyprus University of Technology, Department of Agricultural Sciences, Biotechnology and Food Science, 3603 Limassol, Cyprus. [2]Agricultural University of Athens, Laboratory of Plant Pathology, 75 Iera Odos, 11855, Athens, Greece. 3Department of Plant Pathology & Environmental Microbiology, The Pennsylvania State University, University Park, PA 16802, USA. E-mail: maria.tsolakidou@cut.ac.cy*

Some microorganisms that promote plant growth can manipulate the level of ethylene in plants by decreasing ACC, an ethylene precursor, using ACC deaminase

(ACCd), Increasing host resistance to abiotic and biotic stresses. Despite the beneficial effect of ACCd-producing microorganisms, the role of ACCd in phytopathogens has been little studied. whether ACCd of the soil-borne pathogen *Verticillium dahliae* was involved in pathogenesis and ethylene production was investigated. Overexpression and deletion mutants of the *V. dahliae* gene (ACCd) encoding this enzyme produced more ethylene than the wild type strain, with deletion mutants producing more ethylene than overexpression strains at most time points of observation. However, overexpression of the ACCd gene increased virulence of the pathogen in tomato, while disruption of ACCd resulted in reduced disease severity. ACC holds a key position in many plant physiological processes, with its main role as direct precursor of ethylene. Recent studies have shown that ACC may act as a potential signalling molecule independent from ethylene. To investigate whether the pathogenicity phenotype observed was due to ACC regulation, roots of WT and Never Ripe (Nr) tomato plants and Col-0 and etr1-1 *Arabidopsis* plants were treated with ACC prior to *V. dahliae* inoculation. Plants pre-treated with ACC displayed less severe symptoms than untreated controls. ACC application to roots of Col-0 and etr1-1 plants *in vitro* triggered root hair formation and induced hormone-dependent defence responses. These results suggest a novel role of ACC as a regulator of plant defence and pathogen virulence.

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Phenotypic and molecular responses of potato genotypes to infection by pathotype 18(T1) of *Synchytrium endobioticum* (Schilb.) Perc. T. MARGARITOPOULOU, I. THEOLOGIDIS, D. KIZIS, N. VAKIRLIS, C. KRITIKOS, D. TSIROGIANNIS and I. VLOUTOGLOU. *Benaki Phytopathological Institute, Scientific Department of Phytopathology, Laboratory of Mycology, 8 St. Delta Street, 145 61 Kifissia, Attica, Greece. E-mail: i.vloutoglou@bpi.gr*

The obligate biotrophic pathogen *Synchytrium endobioticum* causes potato wart, and si an important quarantine pest of cultivated potato (*Solanum tuberosum*. As chemical control is not effective in eradicating the pathogen, strict phytosanitary measures combined with the use of resistant potato varieties are the only successful strategies to prevent the disease from spreading. The pathogen can overcome host resistance by developing new pathotypes. More than 40 pathotypes have been reported, of which pathotypes 2(G1), 6(O1) and 18(T1) are the most aggressive and widespread in Europe. Previous studies conducted under natural and controlled environment conditions have shown that only two of 50 tested European potato genotypes exhibited phenotypic resistance to pathotype 18(T1), which occurs in Greece. The potato varieties Kuba (resistant), Novano (tolerant) and Spunta (very susceptible) were selected to further study their molecular response to infection by pathotype 18(T1), using RNA-Seq transcriptomic analysis. Raw data genome-guided transcriptome assembly was carried out using STAR2.5.3.a and HTSeq. Differential Gene [removed]DGE) was used in R using DESeq2 1.32.0, followed by functional DEGs classifications using topGO in R and G-profiler. Many genes related to cell division, lipid catabolism, hormone transportation and peroxidase function were identified. GO-term analysis revealed enrichment regarding terms related to pathogen resistance, membrane bound organelle, cell cycle, defence response, structural molecule activity and response to external biotic stimulus, in intra- and inter-variety (infected or not) comparisons.

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***BAM3* plays a significant role in host resistance against *Fusarium oxysporum*.** E.N. KALOGEROPOULOU[1], K.A. ALIFERIS[2], M.D. LYKOGIANNI[1,2], S.E. TJAMOS[3], I. VLOUTOGLOU[1] and E.I. PAPLOMATAS[3]. *[1]Benaki Phytopathological Institute, Scientific Directorate of Phytopathology, Laboratory of Mycology, 8 St. Delta str., GR-145 61 Kifissia, Athens, Greece. [2]Agricultural University of Athens, Laboratory of Pesticide Science, 75 Iera Odos, GR-118 55 Athens, Greece. [3]Agricultural University of Athens, Laboratory of Plant Pathology, 75 Iera Odos, GR-118 55 Athens, Greece. E-mail: e.kalogeropoulou@bpi.gr*

Fusarium wilt, caused by phytopathogenic strains of the soil-borne *Fusa-rium oxysporum* (*Fo*) species complex, is responsible for serious yield losses in >120 economically important crops. Because of the soil-borne nature

and the wide host range of the pathogen, Fusarium wilt management is difficult. Breeding for resistance has shown positive results in reducing the pathogen spread and increasing crop yields. The role of β-amylase (*BAM*) genes in host resistance to the *F. oxysporum* complex was investigated *in planta* using *Arabidopsis thaliana* ecotype Columbia-0 (wild-type, wt) and its mutants *bam1*, *bam2*, *bam3* and *bam4*, and their combinations, inoculated with *F. oxysporum* f. sp. *raphani*. The pathogenicity tests showed that *bam3* plants were less susceptible to infection than the wt plants. Disease intensity and fungal biomass in the host vascular system, as quantified by qPCR, were lower in *bam3* mutants compared to the wt plants. Transcriptomic and metabolomic analyses carried out using, respectively, DNA microarrays and gas chromatography-mass spectrometry (GC-MS), showed that the resistant phenotype of bam3 plants is associated with differential gene expression and differential production of metabolites related to the cell wall integrity, carbohydrate metabolism, amino acids, and plant hormones.

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Evaluation of foliar resistance of Greek wine grape varieties to downy mildew by phenotyping methods and comparative transcriptomic and proteomic analyses. E.N. KALOGEROPOULOU[1], D. TSIROGIANNIS[1], G. TSIOLAS[3], D. KIZIS[1], E. BAIRA[2], I. THEOLOGIDIS[2], E. MARGARITIS[1], C. KRITIKOS[1], P. BATAKIS[2], A. ARGIRIOU[3], E. MARKELLOU[2] and I. VLOUTOGLOU[1]. *[1]Benaki Phytopathological Institute, Scientific Directorate of Phytopathology, Laboratory of Mycology, 8 St. Delta Street, 145 61 Kifissia, Attica, Greece. [2]Benaki Phytopathological Institute, Scientific Directorate of Pesticides' Control & Phytopharmacy, Laboratory of Toxicological Control of Pesticides, 7 Ekalis Street, 145 61 Kifissia, Attica, Greece. [3]Centre for Research and Technology Hellas, Institute of Applied Biosciences, 6th km Charilaou-Thermi Road, P.O. Box 60361, Thessaloniki, Greece. E-mail: i.vloutoglou@bpi.gr; e.markellou@bpi.gr; d.kizis@bpi.gr*

The obligate biotrophic oomycete *Plasmopara viticola* (Berk & Curt.) Berl. & de Toni, the causal agent of grapevine downy mildew (DM), is a serious threat to viticulture. Sustainable and environmentally friendly DM management strategies rely mainly on host resistance. No information is available on the response of Greek winegrape varieties to *P. viticola* infections. The resistance level of 11 native wine grape varieties was assessed using leaf disc bioassays. The responses of the grape varieties to DM was evaluated based on the host necrotic reactions and the intensity of pathogen sporulation during 15 d post-inoculation (dpi). The host varieties exhibited different responses to DM, while none were resistant. The least susceptible and the most severely affected varieties were selected for transcriptomic and proteomic analyses, to gain an insight into the molecular aspects of the host-pathogen interactions. Libraries prepared from total RNA and protein extracts from grapevine leaf disc samples (inoculated and non-inoculated) collected 5 dpi were analyzed, respectively, with the Illumina Nexteq500 and the Thermo Q-Exactive Orbitrap nanoHPLC-HRMS/MS platforms. Transcriptomic analysis was performed according to the "New Tuxedo" pipeline. Differential expression and functional classifications of the DEGs revealed genes involved in disease resistance, modulation of pathogen defence and leaf cell death, downy mildew-specific resistance, and plant hormone regulation. Proteomic analysis conducted using R programming revealed significant differences between the two varieties.

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Exploring the selective signatures upon LRR-containing genes towards their functional diversification… the cases of cherries and mulberries. A. ZAMBOUNIS[1], I. GANOPOULOS[1], A. TSAFTARIS[2] and P. MADESIS[3]. *[1]Institute of Plant Breeding and Genetic Resources, HAO 'Demeter', Thermi, Thessaloniki, 57001, Greece. [2]Perrotis College, American Farm School. Thessaloniki, 57001, Greece. [3]Institute of Applied Biosciences, CERTH, Thermi, Thessaloniki, 57001, Greece. E-mail: antonios.zamb@gmail.com*

Cherry (*Prunus avium*) and mulberry (*Morus notabilis*) crops are affected by several phytopathogenic fungi. Gene families that contain leucine-rich repeat (LRR) domains are key components of plant immune responses. The subfamily of LRR receptor-like serine threonine kinases plays a pivotal role in defence responses against phytopathogenic fungi. Resistance gene analogues (RGAs), which are closely related to the resistance (R) genes, are widely employed as functional molecular markers in breeding programmes towards host disease resistance against pathogens. The evolutionary profiles of these genes (173 RGAs of cherry and the 142 LRR receptor-like serine threonine kinases of mulberry) were

assessed to determine if successive episodes of diversifying selection contributed to acquisition of novel pathogen recognition repertoires. These genes were subjected to strong positive selection, while the majority of the positively selected amino acid residues were localized widely across gene sequences. These residues could have originally conferred specificity to a hypothetical ligand which was repeatedly altered to provide novel binding functions. The clustered distribution of these genes could give high birth and death rates, with diversifying episodes acting on their functional domains, putatively affecting their ligand-binding specificities. These evolutionary insights indicate how these genes are evolving, making them the foremost surveillance mechanism for durable resistance against rapidly evolving pathogens.

This research was financially supported by the European Union (ESF) and the Hellenic Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF).

Genetic and structural diversity of disease resistance genes through whole genome re-sequencing of sweet cherry (*Prunus avium* L.) cultivars. A. ZAMBOUNIS[1], I. GANOPOULOS[1], A. TSAFTARIS[2], P. MADESIS[3], A. MOLASSIOTIS[4] and A. XANTHOPOULOU[1]. *[1]Institute of Plant Breeding and Genetic Resources, HAO 'Demeter' Thermi, Thessaloniki, 57001, Greece. [2]Perrotis College, American Farm School. Thessaloniki, 57001, Greece. [3]Institute of Applied Biosciences, CERTH, Thermi, Thessaloniki, 57001, Greece. [4]Laboratory of Pomology, Department of Agriculture, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece. E-mail: antonios.zamb@gmail.com*

Sweet cherry is affected by many pathogens that are major threats to fruit production. As climate change affects the susceptibility of sweet cherry cultivars to rapidly evolved and emergent pathogens, it is important to thoroughly investigate the repertoire of high impact structural variations in disease resistance genes among cultivars, to facilitate selection of superior host genotypes. Whole-genome resequencing (WGRS) of 21 sweet cherry cultivars, representing the majority of wild and cultivated Greek germplasm, was employed to characterize genetic and structural variation among 119 defence-related genes (such as RPM1, RPP13, RGA2 homologues). A total of 2,468 structural variants were mapped on 107 disease resistance genes. The majority of the variants were heterozygous and were assigned as missense or synonymous variants. Forty-four NBS-LRR genes had single nucleotide polymorphism mutations in their coding sequences, with high impacts across the sweet cherry genotypes. These data of this genome-wide analysis provide promising resources for expanding knowledge of diversity and evolution of variations in disease resistance genes across in different sweet cherry cultivars. As well, screening and genetic analyses of these genes would facilitate identification of functional variations contributing to variations in host resistance. This approach would facilitate evaluation of natural resistance resources, towards increasing durable disease resistance against sweet cherry pathogens.

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Transcriptome analysis and gene expression profile in response to drought stress in *Citrus macrophylla* stem tissues. M. SILVA[1], P.I.S. PINTO[1], A. DUARTE[2], S. DANDLEN[2], R. GUERRA[3], D. POWER[1] and N.T. MARQUES[3]. *[1]Comparative Molecular and Integrative Biology, Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal. [2]Mediterranean Institute for Agriculture, Environment and Development (MED), Faculdade de Ciências e Tecnologia, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal. [3]Center for Electronics, Optoelectronics and Telecommunications (CEOT), Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal. E-mail: nmarques@ualg.pt.*

Drought is a major abiotic stress with adverse effects on citrus. In grafted plants, drought tolerance is mainly determined by the rootstock. *Citrus macrophylla* Wester, a rootstock used in the Mediterranean region, is known to confer greater drought tolerance than other rootstocks. Transcriptional changes of *C. macrophylla* under water deficit was studied by Illumina RNA-seq technology, and responsive genes were identified. A total of 2745 differentially expressed transcripts (DETs, fold changes >2), were identified, of which 631 (23%) were up-regulated and 2114 (77%) were down-regulated. Gene ontology analysis identified "phenylpropanoid biosynthesis", "amino sugar and nucleotide sugar metabolism" and "photosynthesis" as greatly down-regulated in response to drought stress. Up-regulated processes were soluble carbohydrates and amino acids, which contribute to osmotic balance and protect against oxidative damage. Glycolysis and photorespiration were also strongly up-regulated in *C. macrophylla* as well as transcripts for low molecular weight proteins such as late embryogenesis abundant protein. Citrus plants face abiotic and

biotic stresses. To assess the interplay of drought stress and Citrus tristeza virus (CTV) infection, twelve genes were profiled by quantitative PCR (qPCR) analysis, in control and CTV-infected *C. macrophylla* plants, with or without drought stress. The twelve analysed transcripts were correlated ($r = 0.82$; $P < 0.001$) with the RNA-Seq results, and gave insight into the response of *C. macrophylla* to drought and/or infection with CTV. Transcriptome results showed highly responsive genes to drought stress in stem tissues, indicating potential candidates for genetic selection of highly drought tolerant *C. macrophylla* plants.

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Efficiency of different proactive measures in the management of Verticillium wilt of potato in Lebanon. F. BAROUDY[1], L. SAADÉ[2], Z. MAHFOUZ[3], C. SAAB[1], E. GERGES[1] and W. HABIB[1,*]. *[1]Laboratory of Mycology, Department of Plant Protection, Lebanese Agricultural Research Institute, P.O. Box 90-1965, Fanar, Lebanon. [2]Faculty of Agricultural Engineering and Veterinary Medicine, Lebanese University, P.O. Box 55530, Dekwaneh, Lebanon. [3]Faculty of Agricultural and Food Sciences, Holy Spirit University of Kaslik, P.O. Box 446, Jounieh, Lebanon. *W. HABIB current affiliation: Centro di Ricerca, Sperimentazione e Formazione in Agricoltura – Basile Caramia (CRSFA), Via Cisternino 281, Locorotondo, 70010 Bari, Italy. E-mail: whabib@lari.gov.lb*

Verticillium wilt, caused by *Verticillium dahliae* Kleb., is an economically important disease producing severe losses in Lebanese potato crops. Management of the disease is difficult, and requires integration of several methods because the pathogen can survive in soil as microsclerotia for up to 15 years. Two field experiments were carried in artificially inoculated soil over 2 years, to evaluate effects of different control methods on wilt incidence and severity, soil inoculum density, tuber yields and plant height. In the first trial, the fumigant allyl-isothiocyanate reduced microsclerotium population by 41% and wilt incidence by 18%. Application of fungicides (azoxystrobin + mefenoxam) at planting reduced wilt incidence by 20%. In the second trial, inoculum population density was reduced (58%) by soil solarization and integration of solarization and incorporation of broccoli residues (57%). Soil solarization decreased disease severity by 25%, and integration of fresh broccoli residues and soil solarization reduced disease incidence by 23%. Although incorporation of fresh broccoli residues before sowing did not significantly affect the disease parameters in both trials, this promoted average total tuber yield by 15.1 t ha^{-1} compared to 7.9 t ha^{-1} in the untreated controls. The results from both experiments indicate that integration of control methods, particularly soil solarization and incorporation of fresh broccoli residues are promising for management of Verticillium wilt of potato in Lebanon.

Endophytic bacteria as potential biocontrol agents against *Phaeomoniella chlamydospora*, the dominant causal agent of Petri disease in grapevines. C. TSOUKAS, A.K. TZIMA and E.J. PAPLOMATAS. *Department of Crop Science, Laboratory of Plant Pathology, Agricultural University of Athens, Greece. E-mail: epaplom@aua.gr*

Petri disease is most destructive disease of grapevines, and its occurrence in grapevine nurseries is widely reported. Due to the lack of agrochemicals to control the disease, utilization of effective biological control agents (BCAs) becomes important. The grapevine root bacteriome was assessed for biocontrol potential against the fungal pathogen *Phaeomoniella chlamydospora*, to further develop microbial communities capable of suppressing the disease. Bacteria were isolated from roots derived from vines of different ages (5 to >50 years) with no symptoms of trunk diseases. ITwenty-three bacterial isolates were selected, based on colony morphology. The antagonistic abilities of the strains was assessed in *in vitro* experiments, using a modified dual culture technique. Inhibition was estimated as proportion (%) of mycelial growth compared with experimental controls. Four isolates significantly inhibited growth of *P. chlamydospora*, compared to the rest of the isolates. Sequencing of the 16S rRNA region showed that one strain was *Bacillus halotolerans* while the other three were *Bacillus sp.* To develop efficient microbial communities, interaction assays between the most effective bacterial strains based on antagonism is underway. These preliminary results have shown that, the endophytic microbiome has the potential to be utilized in control of grapevine trunk disease.

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Monitoring of Copper persistence on Plants by Active Thermography. M. RIPPA[1], V. BATTAGLIA[2], M. CERMOLA[2], P. MORMILE[1] and E. LAHOZ[2]. *[1]Institute of Applied Sciences and Intelligent Systems "E. Caianiello" of CNR, 80072 Pozzuoli (Na), Italy. [2]Research center for Cereal and Industrial Crops - Council for Agricultural Research and Economics (CREA-CI) Via Torino 3, 81100 Caserta (CE). E- mail: valerio.battaglia@crea.gov.it*

Optimization of agricultural practices is important. Copper (Cu) has been used as a fungicide in agriculture for more than a century, playing a key role in integrated disease management. Because of Cu accumulation in soil, use needs to be monitored and controlled, using modern technologies to minimize Cu use. We propose a novel approach based on the Active Thermography (AT) to monitor the persistence of Cu on leaf surfaces, and the use of a new polysaccharide-based adjuvant to prolong the persistence of Cu fungicides. Thermal responses were monitored after different treatments of grapevine and tobacco plants over a 3-week period. Spatial maps of leaf thermal recovery times were calculated and compared. These assessments showed that the adjuvant prolonged persistence of the fungicide on the leaves. The measurements were used to estimate the amount of the Cu on the leaves. Maximum difference in the amount of Cu between the plants treated with Cu alone or with Cu + adjuvant (after 2 weeks) was approx. 29% for grapevine and 32% for tobacco. These results demonstrate that an approach based on AT can effectively evaluate in real-time the presence of Cu on treated plants, allowing optimization of use of these fungicides in the agricultural practice.

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Fungicide resistance of *Botrytis fabae* population isolated from faba bean in Morocco. S. AOUZAL[1,2], S. TOFFOLATTI[3], H. ERRATI[1,2], R. MENTAG[4], H. HOUMAIRI[2] and S. KRIMI BENCHEQROUN[1]. *[1]National Institute of Agricultural Research, (INRA, CRRA- Settat), P.O. Box 589, Settat, Morocco. [2]University of Hassan 1st, Faculty of Science and Technology, P.O. Box 577, Settat, Morocco. [3]Department of Agricultural and Environmental Sciences, Milan, Italy. [4]Biotechnology Research Unit, National Institute of Agricultural Research, (INRA, CRRA-Rabat), Morocco. E-mail: sanae.krimibencheqroun@inra.ma*

Chocolate spot, caused by *Botrytis spp.*, is one of the most economically important diseases of faba bean. Management of this disease in Morocco largely depends on chemical treatments using several classes of fungicides, especially with pathogen site-specific fungicides. *Botrytis spp.* have high risks for the development of resistance to fungicides. Sensitivity was assessed of *Botrytis fabae* populations to fludioxonil, fenhexamid and boscalid, which are currently used for disease control in Morocco. Thirty-one isolates of *B. fabae* were collected from seven bioclimatic regions, where faba bean production is important. The sensitivity of the isolates to fludioxonil, fenhexamid and boscalid was evaluated with an automated quantitative test, using 96-well microtitre plates. Growth inhibition percentage (GIP) of pathogen was determined, using absorbance data (at 492 mm) after 72h of incubation, to determine sensitive isolates to each fungicide. The results showed that isolates with resistance to fludioxonil and boscalid were widespread. Seven resistant profiles were determined in the population (FluRFenRBosR, FluSFenSBosS, FluRFenRBosS, FluRFenSBosR, FluRFenSBosS, FluSFenSBosR and FluSFenRBosR). Among these, FluRFenRBosR was widely distributed and present in 58% of the isolates, in almost all of the assessed locations. The prevalence of resistant profiles found in this study will be useful in designing appropriate management of chocolate spot of faba bean in Morocco.

Integrated management of *Aspergillus carbonarius* and ochratoxins in vineyards in Greece. M.K. ILIADI, C.S. LAGOGIANNI, M.D. KAMINIARIS, E-F.N. VARVOUNI, M.N. VARVERI, E.G. POULAKI, C.K. KAVROUMATZI, A.X. VARYMPOPI, E.I. MARGARITIS, N.S. MASTRODIMOS, K.E. POLITIS and D.I. TSITSIGIANNIS. *Department of Crop Science, Laboratory of Plant Pathology, Agricultural University of Athens, Athens, Greece. E-mail: dimtsi@aua.gr*

Aspergillus spp. can cause severe pre- and post-harvest berry rots in grapevines, including sour rot of grapes. *Aspergillus carbonarius* is responsible for ochratoxin A production, a carcinogenic mycotoxin which affects humans and animals. Control of *Aspergillus* spp. is difficult, as application of many fungicides, including systemic compounds, is required in vineyards. The increasing use of pesticides in grapevine cultivation during the last decade, combined with low maximum residue levels, requires development of sustainable methods to control sour rots and ochratoxin A contamination in vineyards. This study aimed to develop an integrated

Aspergillus sour rot and ochratoxin A production. Biological and chemical plant protection products were evaluated for control of sour rot and OTA production. These were: 1) Grape yeasts (isolated from Greek vineyards) from the collection of the Laboratory of Plant Pathology of Agricultural University of Athens, 2) Botector®, 3) Trianum®, 4) Tusal®, 5) Serenade Max®, 6) Vacciplant®, 7) EM-Effective Microorganisms®, 8) Remedier®, 9) Mevalone®, 10) Switch®, 11) Quadris®, 12) Geoxe®, 13) Chorus®, 14) Cantus®, and 15) Scala®. Experiments were carried out in commercial vineyards for four years (2016–2019) on the two Greek grapevine varieties Malagousia and Savvatiano. A successful integrated ochratoxin management system combining biological and synthetic fungicides was developed.

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IKOPROTECTA - Agricultural composted products as plant protection and growth regulators. M. VARVERI[1], A. BAKOS[1], P. GIANNIOTIS[2] and D.I. TSITSIGIANNIS[1]. *[1]Department of Crop Science, Laboratory of Plant Pathology, Agricultural University of Athens, Greece. [2]IKORGANIC, Greece. E-MAIL: dimtsi@aua.gr*

Composts are natural products known for beneficial properties in plant nutrition by enriching soil with organic matter and nutrients. Composts contain high microbe populations, that are mainly non-pathogenic, and studies have highlighted their capacity to suppress or control plant diseases. This study assessed the biopotential of two composted products, (IKORGANIC), using disease assessments in greenhouse and *in vitro* experiments. The bacterial/fungal strains that were isolated from the composted products were evaluated for their ability to inhibit growth of phytopathogenic fungi and bacteria, including *Verticillium dahliae*, *Colletotrichum acutatum* and *Pseudomonas syringae* pv. *tomato*. The *in vitro* antagonistic activity of the isolates was investigated using a dual culture technique. Subsequently, the plant growth promotion and disease severity reduction efficiency of the composted products against the three pathogens was evaluated in a greenhouse on tomato plants, and in planta on olive fruits, according to the host of each pathogen. Results of the *in vitro* experiments and the comparative study of the two composted products for ability to control foliar and soil-borne plant diseases was presented.

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Investigation of DMI-fungicide sensitivity and resistance in grape powdery mildew (*Erysiphe necator*) populations in Hungary. Z. SPITZMÜLLER[1], X. KARÁCSONY-PÁLFI[1], A. PINTYE[2], O., MOLNÁR[2], M. Z. NÉMETH[2], Á.N. HORVÁTH[2], L. KISS[2,3], G.M. KOVÁCS[2,4] and K.Z. VÁCZY[1]. *[1]Eszterházy Károly Catholic University, Food and Wine Research Institute, Leányka str. 6., Eger H3300, Hungary. [2]Centre for Agricultural Research, Plant Protection Institute, P.O. Box 102, Budapest H-1525, Hungary. [3]Centre for Crop Health, University of Southern Queensland, Toowoomba, QLD 4350, Australia. [4]Eötvös Loránd University, Institute of Biology, Department of Plant Anatomy, Pázmány Péter sétány 1/C. 1117 Budapest, Hungary. E-mail: spitzmuller.zsolt@uni-eszterhazy.hu*

Powdery mildew caused by *Erysiphe necator* is a widespread disease of grapevine (*Vitis vinifera* L.). Continuous and intensive use of sterol demethylation inhibitor (DMI) fungicides has resulted in gradual development of insensitivity to these fungicides by alterations in several genes. Occurrence of resistant *E. necator* populations is generally low, and pathogens with reduced sensitivity to one DMI compound be sensitive to other DMIs. This study aimed to detect single nucleotide polymorphisms (SNPs) in genes causing DMIs resistance, and to determine fungicide sensitivity of Hungarian populations of *E. necator*. A commonly used method was adapted to determine sensitivity to propiconazole, tebuconazole, myclobutanil or penconazole of *E. necator* conidia collected from the vineyard of Eszterházy Károly Catholic University. . The fungicides were tested at different concentrations as triplicates. The ratios of the germinating and non-germinating conidia and their germtube lengths were determined. Reduced sensitivity was detected to propiconazole, tebuconazole and myclobutanil, but not to penconazole. With gene sequence analysis, conserved nucleotide variation was detected in the coding region of 14 alfa-demethylase (CYP51), the target of the commonly used DMI fungicides. SNP in CYP51 causing tyrosine to phenylalanine substitution at amino acid position 136 (Y136F) of the protein is associated with DMI resistance in *E. necator*. Further studies will test fungicides with other mechanism of action.

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Markers of resistance to succinate dehydrogenase inhibitor fungicides in *E. necator* populations in Hungary. D. SERESS[1], F. MATOLCSI[1,2], O. MOLNÁR[1], A. PINTYE[1], Á.N. HORVÁTH[1], G.M. KOVÁCS[1,2] and M.Z. NÉMETH[1]. *[1]Plant Protection Institute, Centre for Agricultural Research, ELKH, Herman Ottó út 15, 1022 Budapest, Hungary. [2]Department of Plant Anatomy, Institute of Biology, Eötvös Loránd University, Pázmány Péter sétány 1/C, 1117 Budapest, Hungary. E-mail: seress.diana@atk.hu (presenter), nemeth.mark@atk.hu (corresponding author)*

Succinate dehydrogenase inhibitor fungicides (SDHIs) are widely used to control grapevine powdery mildew, caused by *Erysiphe necator*. Resistance to SDHIs was observed in *E. necator*, and is often caused by mutations in the genes encoding subunits of the succinate dehydrogenase enzyme (*sdhB*, *sdhC*, *sdhD*). To effectively manage downy mildew, knowledge of fungicide resistance is crucial. This study assessed whether markers of SDHI resistance were present in *E. necator* populations in Hungary. Powdery mildew infected grapevine leaves were collected from two Hungarian wine regions (Eger and Szekszárd) in 2018 and 2019. DNA was extracted from field samples, and from newly established *in vitro E. necator* isolates, and the sdhB and sdhC genes were amplified and sequenced. The presence of two mutations indicating resistance to SDHIs was confirmed. An A-G nucleotide change (A794G) was identified at position 794 of the coding region of the sdhB gene, which results in an amino acid substitution in the protein (H242R). This mutation is the marker of resistance to boscalid, a commonly used SDHI. A G-A nucleotide change (G724A) was also identified in nucleotide position 724 of the coding region of the sdhC gene, resulting in an amino acid substitution (G169D). This mutation has not been reported previously from Hungary. This mutation leads to reduced efficacy of several different SDHIs (e.g., boscalid, fluopyram, fluxapyroxad). The monitoring of fungicide resistance is fundamental for the successful management of grapevine powdery mildew, and for long-term maintenance of SDHI effectiveness.

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Expression of *Tomato spotted wilt virus* genes in antisense orientation affects virus progression in *Nicotiana benthamiana*. V. PIRES[1,§], S.A. DANDLEN[2,§], G. NOLASCO[2], M.R. FÉLIX[3], P. MATERATSKI[4], C. VARANDA[4] and N. MARQUES[1]. *[1]CEOT Centro de Eletrónica, Optoeletrónica e Telecomunicações, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro. [2]MED Instituto Mediterrâneo para a Agricultura, Ambiente e Desenvolvimento, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal. [3]MED-Instituto Mediterrâneo para a Agricultura, Ambiente e Desenvolvimento & Departamento de Fitotecnia, Escola de Ciências e Tecnologia, Universidade de Évora, Pólo da Mitra, Ap. 94, 7006-554 Évora, Portugal. [4]MED - Instituto Mediterrâneo para a Agricultura, Ambiente e Desenvolvimento, Instituto de Investigação e Formação Avançada, Universidade de Évora, Pólo da Mitra, Ap. 94, 7006-554 Évora, Portugal. E-MAIL: nmarques@ualg.pt. [§]Both authors contributed equally to this work*

Tomato spotted wilt virus (TSWV) (Tospoviridae) infects a wide range of hosts. The TSWV genome has three linear negative-sense or ambisense RNA segments. Segment S RNA encodes the silencing suppressor NSs, and the nucleocapsid protein N. Segment mRNA encodes the cell-to-cell movement protein NSm and two glycoproteins (Gn and Gc). Antisense transcripts of the N, NSs and M viral genes were expressed in leaves of *Nicotiana benthamiana* infected with TSWV to assay for their ability to silence virus progression. Constructs in the binary vector pK7WG2 were transiently co-expressed with p7WG2-GFP in *N. benthamiana* leaves through agroinfiltration, followed by inoculation with TSWV after 48h. Inoculated leaves were harvested 5 d after agroinfiltration for RNA extraction. Antisense transcripts of partial sequences of the three genes were also expressed throughout the host tissues, using the Tobacco rattle virus viral vector (pTRV). New leaves were harvested 10 d after agroinfiltration of the pTRV viral vector. TSWV detection and absolute quantification was carried out using a TaqMan real-time RT-PCR assay. Leaves inoculated only with TSWV and new leaves showed had low virus titre, indicating host resistance to TSWV infection. In both assays, antiviral M mRNA transcripts limited TSWV genomic RNA accumulation and propagation compared to antiviral transcripts of N or NSs genes. These results indicate that the M gene transcripts in the antisense orientation is a suitable target for limiting virus progression in *N. benthamiana* plants.

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Polysaccharides and plant protection against *Verticillium dahliae*. P. NEOFYTOU, D. GKIZI, and S.E. TJAMOS. *Agricultural University of Athens, School of Crop Science, Laboratory of Plant Pathology. Iera Odos 75, 11855, Athens, Greece. E-mail:* sotiris@aua.gr

Verticillium dahliae is a widely distributed and destructive soil fungus, infecting many economically important crops. The lack of efficient chemically based disease management strategies has focused research on the development of resistant cultivars or root stocks, biological control agents, or compounds to induce host plant resistance. Plant protective activity of starch and cellulose were investigated against Verticillium wilt of eggplants. Both polysaccharides reduced symptom development compared to controls. In a split root system experiments, starch and cellulose treated plants were less infected than controls. Transcriptomic analysis showed greater expression levels of PR1 and PR4 in the cellulose treated plants compared to the controls at 5 d post inoculation (dpi) with *V. dahliae*. Starch treated plants exhibited greater PR4 expression levels than controls at 10 dpi. Pathogenicity experiments on plants grown in sterilised soil demonstrated the efficacy of starch and cellulose to protect plants against *V. dahliae*, while the polysaccharides failed to protect plants in a split root system. These results indicate that starch and maltose may positively affect microbial population that trigger ISR in plants, and that starch and cellulose could direct effects against *V. dahliae*.

Soil solarisation and biological control of soil borne pathogens in strawberry plantations. A. TZIONIS[1], I. KEFALOGIANNI[2], I. CHATZIPAVLIDIS[2] and S.E. TJAMOS[1]. *[1]Laboratory of Plant Pathology, Agricultural University of Athens, School of Crop Science, Iera Odos 75, 11855, Athens, Greece. [2]Laboratory of General & Agricultural Microbiology, Agricultural University of Athens, School of Crop Science, Iera Odos 75, 11855, Athens, Greece. E-mail: sotiris@aua.gr*

Soil solarization is a suitable method for control of soilborne pathogens in Mediterranean countries. Strawberry is a high value crop that is susceptible to fungus and oomycete pathogens. Soil fumigants have low efficacy against soilborne pathogens. Next generation polyethylene foils for soil solarization were assessed in strawberry plantations under greenhouse conditions. A totally impermeable 7-layer film (KRITIFIL® TIF, Plastika Kritis SA) was the most efficacious among the tested soil disinfection films. The main soil pathogens were *Verticillium dahliae*, *Fusarium oxysporum* f. sp. *fragariae, Phytophthora* sp., *Rhizoctonia solani* and *Macrophomina phaseolina*. In the TIF covered soil there were 4–10 times fewer diseased plants less than in metam sodium treated soil. Analysis of the microbial community of the different treatments showed the presence of 18 prevailing and distinct bacteria. Dual culture tests showed that six of the isolated bacteria reduced growth of *V. dahliae* and *Fusarium oxysporum*. The six isolates were tested as single and mixed treatments against Verticillium wilt of eggplants. The consortium of the isolates was the most efficacious treatment. Further analysis of the six isolates showed that they had biocontrol and plant growth promoting traits, including cellulolytic activity, indole acetic acid and siderophore production, phosphorus dissolving activity and swarming motility.

The multiple effects of the biocontrol agent *Pseudomonas putida* Z13 against *Botrytis cinerea* in tomato fruits. L. AMPNTELNOUR, E.G. POULAKI and S.E. TJAMOS. *Agricultural University of Athens, School of Crop Science, Laboratory of Plant Pathology. Iera Odos 75, 11855, Athens, Greece. E-mail: litsaabd@gmail.com*

Gray mold, caused by *Botrytis cinerea*, is an important postharvest disease of fresh-market tomatoes. Although fungicide treatments have been a major method for controlling gray mold, there is increasing international concern over the heavy use of fungicides on crops because of the possible harmful effects on human health and the emergence of pathogen resistance to fungicides. Therefore, there is requirement for alternative disease control methods, including the use of beneficial microorganisms. *Pseudomonas* includes species with biocontrol activity against various plant pathogens. Recently, we reported the isolation and identification of strain *Pseudomonas putida* Z13, a potent biocontrol agent against *Verticillium dahliae*. *Pseudomonas putida* is commonly used in environmental studies because it can degrade many aromatic compounds, cellulose and chitin, and also produces many antibiotics. The biocontrol activ-

ity of strain Z13 was assessed against *Botrytis cinerea* in tomato fruits. Application of Z13 to tomato fruits reduced disease severity and incidence by 50% compared to controls. This protective activity can be attributed to antibiotic production, since Z13 reduced *in vitro* growth of *B. cinerea* and also primed plant defence responses. qPCR analyses showed that expression of the defence associated genes PR1 and WRKY70 was upregulated in the Z13 treated fruit, and the upregulation was most prominent after *B. cinerea* infection. These results indicate that Z13 was a potential biocontrol agent targeting multiple pathogens in different host plants, as experiments have shown activity against *V. dahliae* and *B. cinerea* in, respectively, fruits of eggplant and tomato.

Characterization of rhizobacteria from Cyprus indigenous wine grape cultivars bearing antagonistic traits against grapevine trunk pathogens. C. OPLOS, S. EFSTATHIOU and L. I. KANETIS. *Department of Agricultural Sciences, Biotechnology, and Food Science, Cyprus University of Technology, Limassol, 3036, Cyprus. E-mail: loukas.kanetis@cut.ac.cy*

The Cyprus wine industry has increasingly focused on exploitation of indigenous grapevine varieties. Grapevine trunk diseases (GTDs) are the most destructive biotic complex, limiting vineyard productivity and longevity. Based on recent data, Cyprus vineyards are seriously affected by GTDs. The present study was the first attempt to describe rhizobacteria communities from the indigenous varieties, *Vitis vinifera* "Giannoudi", "Maratheftiko", "Promara", and "Xinisteri", to select effective biocontrol agents (BCAs) that could be used for GTD management. Antagonistic activities of 499 bacterial isolates were assessed *in vitro* against the important GTD pathogens *Phaeomoniella chlamydospora, Diplodia seriata, Neofusicoccum parvum, Eutypa lata, Ilyonectria liriodendri, Dactylonectria alcacerensis*, and *Dactylonectria torresensis*. Eighty-two isolates exhibiting ≥ 40% fungal inhibition were selected and further characterized. Based on 16S sequencing, isolates were assigned to *Acinetobacter* (1.2%), *Bacillus* (17.1%), *Bordetella* (2.4%), *Chryseobacterium* (3.7%), *Paenibacillus* (1.2%), *Pantoea* (6.1%), *Pseudomonas* (19.5%), *Olivibacter* (2.4%), *Rhizobium* (14,6%), *Serratia* (22%), *Streptomyces* (2.4%) and *Variovorax* (7.4%). Enzymatic traits of the selected isolates related to antifungal activities were also examined. Activities were detected for lipase (in 42% of isolates), esterase (63%), protease (78%), amylase (37%), xylanase (37%), pectinase (37%), cellulase (46%), chitinase (32%), HCN activity (11%), and biofilm formation (34% of isolates). Assessments for antibiotic production, VOC activity and growth promotion traits are underway to complete characterization of a pool of promising novel, indigenous BCAs for GTD management.

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A newly reported bacteriophage against *Pseudomonas syringae* pv. tomato and its plant protective activity. P. PAPAZOGLOU[1], D. SKLIROS[1], E.G. PARASKEVOPOULOU[1], D. GKIZI[4], D.E. GOUMAS[3], S.E. TJAMOS[2] and E. FLEMETAKIS[1]. *[1]Agricultural University of Athens, Biotechnology Department, Lab of Molecular Biology. [2]Agricultural University of Athens, Crop Science Department, Lab of Phytopathology. [3]Hellenic Mediterranean University, School of Agricultural Sciences, Department of Agriculture, Lab of Phytopathology. [4]University of West Attica, Department of Wine, Vine and Beverage Sciences. E-mail: polyxenipapazoglou@gmail.com*

Bacteriophages have been extensively studied to explore new and environmentally friendly methods for managing phytopathogenic bacteria. *Pseudomonas syringae* pv. *tomato* (*Pst*) causes bacterial speck in tomato plants, with symptoms of black or brown spots with chlorotic margins on leaves and fruits. The most common disease management strategy is applications of copper-based pesticides. Biological control of *Pst* with bacteriophages could be an alternative environmentally-friendly approach to reduce damage from *PSt* in tomato crops. Potential preventive efficacy of the lysogenic bacteriophage Medea1 was investigated in Medea1 has been isolated from a tomato crop and has been characterized. This is a ds-DNA bacteriophage with a genome of 58,919 bp, having strong lytic activity against many strains of *Pst*, and ability to suppress bacterial populations *in vitro* for at least 10 h. The efficacy of Medea1 against *Pst* was further evaluated *in planta*, under greenhouse conditions. The *Pst* and Medea1 populations were monitored at 1 and 3 d post-infection, using RT qPCR. Host gene expression patterns involved in defence against *Pst* were also assessed. The bacterial population was reduced by up to 85%, and disease symptoms were delayed by up to 6-fold. These results indicate that the Medea1 phage could be an effective alternative control for *Pst* in greenhouse tomato crops. Further isolation of bacteriophages against *Pst*, and design of a phage-based cocktail, could provide an efficient bacterial speck management strategy.

Evaluation of biological and synthetic plant protection products for the management of Alternaria leaf blight in carrots. C.K KAVROUMATZI, M.K. ILIADI, D. AKRIVOPOULOU, E.G. POULAKI and D.I. TSITSIGIANNIS. *Department of Crop Science, Laboratory of Plant Pathology, Agricultural University of Athens, Greece. E-mail: dimtsi@aua.gr*

Alternaria dauci causes Alternaria leaf blight, an important foliar disease in carrot production. The disease commonly occurs when carrots are cultivated during moderate temperatures, and the leaves are exposed to prolonged periods of wetness due to rainfall, dew, or sprinkler irrigation. Severe epidemics have been reported to reduce carrot yields by 40–60%. Under high disease pressure, no single control measure is sufficient to adequately manage the disease. Disease management relies on the combination of applications of synthetic plant protection products (PPPs), use of partially resistant varieties, and monitored disease prediction models. Efficacy of several commercial biopesticides, plant resistance inducers and synthetic PPPs against Alternaria leaf blight in carrots was evaluated under greenhouse conditions in two experiments. For the pathogenicity trials, an intermediately resistant and a susceptible variety were used, and these were inoculated with conidium suspensions of *A. dauci* 2 d after the application of PPPs. All the tested PPPs successfully controlled Alternaria leaf blight. Among the synthetic PPPs, *Luna Sensation*®, *Signum*® and *Dagonis*® consistently decreased disease severity on both cultivars. *LBG-01F34*®, *Trianum*® and *Sonata*® were the most efficient bio-PPPs on both hybrids. Results from the comparative study of the different PPPs were presented.

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Antifungal and phytotoxic properties of essential oil from three spontaneous *Lamiaceae* species from Morocco, against the main chickpea pathogens. A. ENNOURI[1,2], L. ET-TAZY[1,2], H. ERRATI[1,2], A. LAMIRI[2] and S. KRIMI BENCHEQROUN[1]. *[1]National Institute of Agriculture Research (INRA, CRRA-Settat), P.O. Box 589, Settat, Morocco. [2]University Hassan 1st, Faculty of Science and Techniques, Laboratory of Applied Chemistry and Environment, P.O. Box 577, Settat, Morocco. E-mail: sanae.krimibencheqroun@inra.ma*

Development of biological products is important for health and environmental reasons. This study determined the chemical composition of some aromatic *Lamiaceae*, and their antifungal activity against the causal pathogens of Ascochyta blight and Fusarium wilt of chickpea. Three plant species that grow spontaneously in Morocco were selected: *Origanum compactum*, *Thymus vulgaris,* and *Mentha pulegium*. Oregano was the most effective against *Fusarium oxysporum* (MIC = 0.5 μL mL^{-1}) for inhibiting mycelium growth and conidium germination, followed by thyme and pennyroyal (MIC = 1.5 μL mL^{-1}). Complete inhibition of *Ascochyta rabiei* was also observed using oregano at 0.15 μL mL^{-1}. No phytotoxic effects of the oregano and thyme oils at low doses were observed on chickpea seed germination, but the pennyroyal oil reduced seed germination. Thymol and carvacrol were the major compounds of oregano and thyme oil as indicated by GC/MS analyses, and these compounds are likely to be the main active ingredient for pathogens control. Greenhouse experiments were carried out to evaluate the efficacy of oregano and thyme oils against *A. rabiei* as a protective or curative treatments. In curative treatments, 0.15 μL mL^{-1} of oregano oil decreased ($P < 0.05$) Ascochyta blight severity by 66%, similar to the fungicide azoxystrobin at 250 g L^{-1}. The thyme oil at 0.5 μL L^{-1} also reduced disease severity by 53%. These essential oils or their major components could be investigated as natural antifungal products against chickpea pathogens.

Effects of the leafy liverwort extract on plant pathogenic fungi causing olive fruit rot and gray mold of strawberry. J. LATINOVIC[1], M. SABOVLJEVIC[2], M. VUJICIC[2], N. LATINOVIC[1] and A. SABOVLJEVIC[2]. *[1]University of Montenegro, Biotechnical Faculty, Mihaila Lalica 1, 81000 Podgorica, Montenegro. [2]University of Belgrade, Institute of Botany and Botanical Garden, Faculty of Biology, Takovska 43, 11000 Belgrade, Serbia. E-mail: jelenalat@ucg.ac.me*

Bryophytes were among the first land plants and they faced harsh terrestrial environments, and have interacted with different organisms during their long evolutionary history. They cope well with fungal cohabitants in mutualistic, parasitic or neutral relationships. Due to interesting chemical contents, they are assumed not readily infected by fungi. This feature was used to assess effects of selected bryophyte extracts on plant pathogenic fungi. Methanol extract of leafy liverwort (*Scapania nemorea* (L.) Grolle) was assessed for growth inhibition of *Botryosphaeria dothidea* isolated from rotted olive fruits and *Botrytis cinerea* isolated from diseased strawberries. Extract treatments containing 5, 10 or 15 μL

were assessed, while distilled water served as experimental controls. The bryophyte extracts reduced growth of *B. dothidea* and *B. cinerea*. The extract applied at 15 µL inhibited mycelium growth ($P < 0.01$) of *B. cinerea*, and at 10 or 15 µL Inhibited growth of *B. dothidea*. These inhibitory effects indicate potential for bryophytes as sources of natural active substances that could be used for development of environmentally-friendly alternatives as controls for important plant diseases.

This research was financially supported by the Serbia-Montenegro bilateral project "From Plantation to Table: Improving the Process of Berry and Stone Fruit Production by Biotreatment with Bryo-Extracts to Reduce Risk of Pesticide Use and Obtain a Healthy Product" 2019–2020 and by the project "Study on biological efficacy of newly synthesized compounds and plant extract to the most important diseases of grapevine in Montenegro (BIOEXTRA) 2018–2020.

The phenolic responses in callus to scion sanitary status and disinfectants in biological grapevine nurseries in Slovenia. D. RUSJAN, S. GAČNIK and M. MIKULIČ PETKOVŠEK. *Chair for fruit growing, viticulture and vegetable, Department of Agronomy, Univeristy of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia. E-mail: denis.rusjan@bf.uni-lj.si*

Effects were assessed of sanitary status of the scions infected by grapevine trunk diseases (GTD) and five different disinfectants (one combined with thermotherapy (TT) on the phenolics in callus during grapevine propagation. The study included scions from healthy vines (HLT), GTD-infected asymptomatic vines (ASYM) and GTD-infected symptomatic vines (SYM), of Cabernet Sauvignon. Scions were treated separately with Beltanol, Serenade, sodium bicarbonate, Remedier, BioAction or with Beltanol plus TT. Calluses were assessed soon after callusing and later after classification in autumn. Different sanitary status of the scions affected the content of phenolic compounds in callus. After callusing the greatest contents of TAP (363–378 µg g^{-1} FW), flavanols (FLA; 297–310 µg g^{-1} FW) and stilbenes (STB; 25.7–27.0 µg g^{-1} FW) in callus was measured from SYM and ZDR scions, while in autumn the greatest contents of TAP, FLA and STB were measured in callus from ASYM scions. However, the greatest content of TAP (599–783 µg g^{-1} FW) was measured in callus from ZDR and SYM scions treated with BioAction. In autumn, the contents of TAP (6951–7110 µg g^{-1} FW) were measured in callus from ASYM scions treated with Remedier or Beltanol, from ZDR scions (3231–3262 µg g^{-1} FW) treated with Remedier and sodium bicarbonate, and from SYM scions (5024 ± 40 µg g^{-1} FW) treated with sodium bicarbonate. This study contributes to the knowledge of the impacts of biocontrol agents on alteration of phenolics in the callus from scions with different GTD status.

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***Melia azedarach* induces the expression of marker genes involved in tomato defence responses against nematodes.** A. KROKIDA[1], D. KIZIS[2], M. SAMARA[3] and N. NTALLI[3]. *[1]Scientific Directorate of Phytopathology, Benaki Phytopathological Institute, 8 Stefanou Delta Street, 14561, Kifissia, Athens, Greece. [2]Laboratory of Mycology, Scientific Directorate of Phytopathology, Benaki Phytopathological Institute, 8 Stefanou Delta Street, 14561, Kifissia, Athens, Greece. [3]Laboratory of Biological Control of Pesticides, Scientific Directorate of Pesticides' Control & Phytopharmacy, Benaki Phytopathological Institute, 8 Stefanou Delta Street, 14561, Kifissia, Athens, Greece. E-mail: n.ntalli@bpi.gr*

Tomato is an important food crop, Root-Knot Nematodes (RKN) are important tomato pests. Environmental and health concerns influence the use of plant secondary metabolites as ecofriendly alternatives to the conventional nematicides. Previous research showed that ripe fruits of *Melia azedarach* yielded nematicidal extracts against *Meloidogyne* sp., with substantial activity under field conditions, and that these extracts triggered plant defence mechanisms in cucumber. However, the molecular processes by which these extracts activate plant defence against RKN remain largely unclear. qRT-PCR was used to monitor the expression of selected key defence genes and the H_2O_2 degrading enzymes of catalase and ascorbate peroxidase in tomato rootsat 5 and 30 d after RKN inoculation. *Melia azedarach* aqueous extract induced the expression of salicylic acid pathway genes (*PR-1*, *PR-5*), the *Mi1* gene that confers resistance against RKNs, and the proteinase inhibitor *Pi1*. Overexpression of *ACO* and *RAB18* genes, markers of, respectively, the ethylene and ABA signalling pathways, was also observed. Low induction of jasmonic acid pathway genes (*LOXD* and *SAM*) was detected, and expression of *CCD7*, involved in strigolactones biosynthesis, was reduced at 30 d after RKN inoculation. The expression profiles of catalase and ascorbate peroxidase genes were altered 30 d after RKN inoculation. These results provide evidence that *M. azedarach* extract can modulate

the expression of genes and metabolites associated with plant defence responses in tomato.

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***In vitro* and *in vivo* antifungal properties of thymol against *Ascochyta rabiei*.** F.Z. IBN EL MOKHTAR[1,2], H. ERRATI[1], H. HOUMAIRI[1] and S. KRIMI BENCHEQROUN[2]. *[1]University Hassan 1st, Faculty of Science and Techniques, P.O. Box 577, Settat, Morocco. [2]National Institute of Agriculture Research (INRA, CRRA-Settat), P.O. Box 589, Settat, Morocco. E-mail: sanae.krimibencheqroun@inra.ma*

Ascochyta blight, caused by *Ascochyta rabiei* (Pass.) Labr., is an economically important disease of chickpea. With increasingly used organic farming practices, there is requirement for reduced chemical inputs in agricultural systems, and to develop new effective biological products. Thymol is a natural monoterpenoid phenol, found mainly in the essential oil from thyme *(Thymus vulgaris*) and other aromatic and medicinal plants. The antifungal properties of thymol against *A. rabiei* were evaluated *in vitro* and *in vivo*. The *in vitro* test was carried out on chickpea malt extract medium amended with six concentrations of thymol (0 to 0.3 µL mL^{-1}). Minimum inhibitory concentrations (MIC) were estimated by calculating the percentage of inhibition of mycelium growth (MGI). The fungistatic or fungicidal properties of thymol were determined using the Thompson method. Greenhouse experiments were also carried out to evaluate the efficacy of thymol as a curative or preventive treatment for Ascochyta blight control. Radial growth of the pathogen was completely inhibited by thymol at low concentration (MIC = 0.15 µL mL^{-1}). Thymol was fungicidal against *A. rabiei* at >0.3 µL mL^{-1}. Application of thymol to plants was effective for control of the disease as preventive or curative treatments at 0.4 µL mL^{-1} by reducing the mean disease index by 39% (preventive treatment) and 32% (curative). Thymol can be investigated as a natural antifungal product for management of Ascochyta blight.

Potential of microbial agents for biocontrol of early blight of potato and tomato. Ž. IVANOVIĆ, T. ŽIVKOVIĆ and D. ŽEŽELJ. *Institute for Plant Protection and Environmnet, Teodora Drajzera 9, 11010 Belgrade, Serbia. E-mail: zarko.ivanovic@yahoo.com*

Biological control for management of plant diseases has been extensively investigated as an alternative to chemical pesticides. Use of antagonistic microorganisms could reduce environmental pollution avoid pesticide resistant plant pathogens. Early blight of *Solanaceae*, caused by *Alternaria* pathogens, is economically important, and is responsible for severe damage of production from these crops. Although the disease can be partially controlled by fungicides, *Alternaria* strains resistant to the compounds cause problems for early blight management. Thirty-nine bacterial isolates were obtained from rhizospheres and phyllospheres of field-grown potato and tomato plants. All the isolates displayed the antagonistic activity towards *Alternaria solani* and *A. tomatophila*, producing inhibition zones in dual culture assays of at least 5 mm. Based on 16S rDNA sequencing, the isolates with greatest antagonistic potential were identified as *Bacillus velezensis, Bacillus safensis, Bacillus subtilis* or *Bacillus amyloliquefaciens.* Although potential for these bacteria as biological control agents has been demonstrated, they need to be further studied using *in planta* experiments.

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BIOVEXO: a BBI-JU-H2020 project on biocontrol of *Xylella* and its vector in olive trees, for integrated pest management. S. COMPANT[1] and BIOVEXO Consortium[2]. *[1]AIT Austrian Institute of Technology, Center for Health and Bioresources, Bioresources Unit, Tulln, Austria. [2]BIOVEXO Consortium: RTDS Group (Austria), AIT Austrian Institute of Technology (Austria), CNR – Istituto per la protezione sostenibile delle piante (Italy), Centro di Ricerca, Sperimentazione e Formazione in Agricoltura "Basile Caramia" (Italy), Universidad de Sevilla (Spain), Universiteit Antwerpen (Belgio), Globachem NV (Belgium), Domca SA (Spain), Acies Bio (Slovenia), Aimerit SL (Spain) and ASAJA (Spain). E-mail: stephane.compant@ait.ac.at*

Xylella fastidiosa is increasingly causing diseases on olive trees and other crops in the Mediterranean region. This pathogen destroyed a number of olive groves in Italy and Spain in only a few years. The climate of the southern

European Union is ideal for *Xylella*. Due to rapid transmission across cultivation areas, *X. fastidiosa* is projected to cause yield losses of 35–70% in olive harvests and 13% in almond harvests. There are no products available against *X. fastidiosa*, which is spread by xylem-feeding insects, particularly the spittlebug *Philaenus spumarius* which occurs in many countries and in different climatic conditions. Only chemical insecticides are authorized to control xylem sap-feeding insects, but these are not permitted in organic crop production. In the BBI JU-H2020 project BIOVEXO, 11 partners from five countries, and belonging to industries, SMEs, RTOs and universities, aim to develop environmentally sustainable and economically viable plant protection solutions that can be used for integrated management of *X. fastidiosa* and its spittlebug vector. At least two best performing solutions will be progressed closer to market at the end of the project (Technical Readiness Level 7-8). Integrated pest management measures will be applied to existing and the newly established orchards during large-scale pilots in Apulia (Italy) and Mallorca (Spain), two most important *Xylella* outbreak regions of Europe. An overview of BIOVEXO was presented, including objectives and research plans. For more information about the BIOVEXO project: www.biovexo.eu.

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An emerging threat to chestnut nuts: *Gnomoniopsis castanea* in the Northern Apennines. E. SCALI[1], C. AGLIETTI[1], S. PINI[1], A. GUIDOTTI[3], P. CAPRETTI[1] and L. GHELARDINI[1,2]. *[1]Department of Agriculture, Food, Environment and Forestry (DAGRI), Plant Pathology and Entomology section, University of Florence, Piazzale delle Cascine 28, I-50144, Firenze, Italy. [2]Institute for Sustainable Plant Protection (IPSP), National Research Council of Italy (CNR), Via Madonna del Piano 10, 50019 Sesto Fiorentino, Italy. [3]Regione Toscana, Servizio Fitosanitario, Via Alessandro Manzoni 16, 50121 Firenze, Italy E-mail: luisa.ghelardini@unifi.it*

Gnomoniopsis castanea (syn. *Gnomoniopsis smitholgivyi*) is an emerging pathogen that damages nuts of sweet chestnut (*Castanea sativa*) nuts, and may cause twig and leaf necroses on sweet chestnut and hazelnut (*Corylus avellana*). Nut rot of sweet chestnut has long been observed in Northern Italy, but was only recently associated with presence of *G. castanea*, which is considered responsible for heavy pre- and post-harvest nut losses that chestnut growers are experiencing in the Northern Appenines. Little is known of the epidemiology of this fungus, which is often reported as a natural endophyte in chestnut and was found associated with galls of the Asian gall wasp *Dryocosmus kuriphilus*. Presence of *G. castanea* was investigated in the Northern Apennines (Liguria and Toscana regions), where sweet chestnut has historically been grown for nut production and as coppices. Nuts and wasp galls were sampled, and *G. castanea* was isolated with high frequency from all sites including those where symptoms were moderate, revealing common and broad presence of the fungus. Temperature responses for mycelium growth and spore germination of the pathogen were assessed to assist modelling of disease spread and incidence at sites with different climatic conditions. Since application of management strategies relies on accurate and rapid identification of the pathogen before occurrence of symptoms, a specific diagnostic assay is also being developed based on Loop mediated isothermal AMPlification (LAMP) technology. This will be used as a field-deployable tool for helping risk assessment, damage prevention and disease control.

This research received funding from the Agreement for Scientific Cooperation between Regione Toscana and the University of Florence – DAGRI, on quarantine organisms harmful to agriculture and forestry.

Wheat rust monitoring in Tuscany and Sicily: re-emergence of stem rust on durum and common wheat cultivars. M. NOCENTINI[1], B. RANDAZZO[2], M.S. HOVMOLLER[3], M. PATPOUR[3], A.F. JUSTESEN[3] and L. MUGNAI[1]. *[1]Department of Agriculture, Food, Environment and Forestry (DAGRI), University of Florence, Piazzale delle Cascine 28, I-50144, Firenze, Italy. [2]As.A.R. – Ciminna, Palermo, Italy. 3Department of Agroecology, Aarhus University, Flakkebjerg 4200, Denmark. E-mail: marco.nocentini@unifi.it*

Wheat stem rust had not been reported in Western Europe for several decades, but in the last 10 years an outbreak occurred in central Europe, first, mainly in Germany, and then sporadically, in Denmark, Sweden and the United Kingdom. The presence of *Puccinia graminis* f. sp. *tritici*, which causes wheat stem rust, was also recorded in Sicily with an epidemic outbreak in 2016. Stem rust symptoms were also reported in Tuscany during the last 2019, 2020 and 2021, and during the 2021 crop season all monitored wheat varieties showed symptoms. In Sicily, stem rust was found in 13 of 14 monitored sites. Monitoring and sampling wheat varieties is important to provide understanding of epidemics

and detect the rust races present in the different countries and regions. For this purpose, some varieties were sown in Tuscany and Sicily to: 1) follow the appearance of rust symptoms in different climatic and soil conditions; 2) evaluate an early warning system coordinated between Tuscany and Sicily, where symptom appearance occurs well ahead of the central-northern regions; and 3) assess the races present on the same varieties in different regions. First results from molecular analyses of collected samples from both regions showed that clades III-B and IV-F occurred in both regions, and that race TTRTF (clade III-B) and race TKKTF (clade IV-F) were detected in Sicily.

Diversity of dangerous fungus and fungus-like pathogens of fruit and berry crops in Russia. YU. TSVETKOVA[1,2], D. SHUKHIN[1] and A. KUZNETCOVA[1]. *[1]FGBU All-Russian Plant Quarantine Centre. Moscow region, Ramensky district, Bykovo, Pogranichnaya st. 32, 140150 Russian Federation. [2]Lomonosov Moscow State University, 119234 Moscow, Russi*a. *E-mail: yutska@mail.ru*

Production of fruit and berry crops is important in Russian agriculture, but production of planting material in Russia is not sufficient to meet demands. Import of propagation material of horticultural crops in 2018 was estimated to be 19.8 million pieces (excluding grape). Fruit and berry crops are susceptible to fungal diseases, especially in humid conditions within the main zones of fruit production. Pathogen diversity in Russia is influenced by pathogens imported with the planting material. This study provided an overview of quarantine and other fungal pathogens found in imported berry fruit production material and fungal pathogens that are widespread in Russia. Anthracnose of strawberry, caused by *Colletotrichum acutatum*, was detected on strawberries samples in 2018–2019. The species *C. acutatum* species complex affecting strawberries, and species found on other fruit crops, were separated. Species of the *Colletotrichum gloesporioides* complex have also been studied. Several harmful prevailing pathogens have been identified on strawberries, including: *Pilidium concavum*, *Pestalotia longisetula*, *Diplocarpon earliana*, *Phytophthora cactorum*, and *Phytophthora nicotianae*. Bown rot, caused by *Monilinia* spp., was also important. *Monilinia fructigena* and *M. laxa* are present in the Russian Federation. *Monilinia laxa* causes damage to stone fruits, while *M. fructigena* damages pome fruits. Plant samples were collected in different regions of the European part of Russia and from the Far East. Fungi were isolated on PDA, and identifications were carried using cultural and morphological characters, and analyses of nucleotide sequences of ITS regions.

Characterization and distribution of *Pseudomonas syringae* pv. *syrinae* on wheat in Syria. M. KASSEM[1], N. ASAAD[2], S.G. KUMARI[3] and A.R. MOUKAHEL[3]. *[1]Plant Protection Department, Faculty of Agriculture, University of Aleppo, Aleppo, Syria. [2]General Commission for Scientific Agricultural Research (GCSAR), Al-Ghab, Hama, Syria. [3]International Center for Agricultural Research in the Dry Areas (ICARDA), Terbol Station, Beqa'a, Zahle, Lebanon. E-mail: s.kumari@cgiar.org*

Bacterial leaf blight, caused by *Pseudomonas syringae*, threaten wheat production in many regions. Approximately 500 wheat leaf samples showing symptoms suggestive of bacterial infections were collected from 107 fields in north, central and coastal areas of Syria during the 2021 growing season. Disease symptoms consisted of water-soaked dark green to brown lesions on expanding leaves which became necrotic and turned from grey-green to tan-white. A total of 56 isolates were selected using a semi-selective medium, and were characterized further using morphological, biochemical (LOPAT), and pathogenicity tests to identify the virulent properties of isolates, and molecular characterization with PCR using specific primers for the syringomycin production gene. Field observations showed that leaf blight severity was approx. 54%, whereas the coastal area isolates gave high disease severity (61.5%), followed by the central area isolates (56%). The pathogenicity tests showed that all the isolates were pathogenic to wheat under greenhouse conditions, with 63% of ranked as highly virulent. The PCR results confirmed that the isolates were *P. syringae* pv. *syringae*. This study increased understanding of the ecology, distribution and severity of the microbial wheat communities in Syria.

***Diatrype stigma* and *D. whitmanensis* associated with canker and dieback of Russian olive (*Elaeagnus angustifolia*) trees in Iran.** F. AHMADYOUSEFI-SARHADI, H. MOHAMMADI and S. PANAHANDEH. *Department of Plant Protection, Faculty of Agriculture, Shahid Bahonar University of Kerman, P.O.Box: 76169-14111, Kerman, Iran. E-mail: saleh.panahandeh@yahoo.com*

Russian olive (*Elaeagnus angustifolia*, *Elaeagnaceae*) is a shrub or small deciduous tree that can grow in various provinces of Iran. During 2018–19, surveys for fungi associated with canker and dieback of Russian olive trees were conducted in Southern provinces

of Iran. Samples were collected from branches of trees showing disease symptoms, and fungal isolations were made from necrotic wood tissues onto potato dextrose agar (PDA) supplemented with streptomycin sulphate. Fifteen isolates of Diatrypaceous fungi were obtained from the symptomatic branches. Isolates were placed onto 2% water agar amended with autoclaved pine needles and incubated under a 12 h daily photoperiod for 3–5 weeks to induce sporulation. Based on morphological characteristics and phylogenetic analysis of the internal transcribed spacer (ITS) nrDNA and a partial sequence of the β-tubulin gene, isolates were identified as *Diatrype stigma* (eight isolates) and *D. whitmanensis* (seven isolates). Pathogenicity of these species was assessed by inoculation of detached shoots of Russian olive trees under controlled conditions. Both species were pathogenic and caused significant wood discolourations on inoculated shoots 40 d post-inoculation. *Diatrype stigma* was more virulent than *D. whitmanensis*, based on the mean lengths of wood discolourations. This is the first report on the occurrence and pathogenicity of *D. stigma* and *D. whitmanensis* on Russian olive trees.

Identification of *Venturia asperata* on scab-resistant apple cultivars in South Tyrol, Italy. S. OETTL. *Research Centre Laimburg I-39040 Auer/Ora (BZ), Italy. E-mail: sabine.oettl@laimburg.it*

Reports of atypical fruit spots on *Vf*-resistant cultivars from South Tyrolean apple orchards increased in August 2020. Symptoms were similar to apple scab, caused by *Venturia inaequalis*, but the spots were less pronounced and light grey. Microscope examination of mycelia scraped off the spots from the fruit peels revealed rounded-tip conidia of 10–14 µM length, and were morphologically assigned to *Venturia asperata*. Molecular analyses of the ITS regions using species-specific primers resulted in amplicons only for the atypical scab lesions, but not for typical leaf or fruit spots caused by *V. inaequalis*. Sequence analysis of the amplicons confirmed the presence of *V. asperata*. To date, the atypical scab symptoms were observed only on *Vf*-resistant cultivars, such as Bonita, Topaz, CIVM49 and Ipador, where low-impact plant protection strategies were applied. Further monitoring of the occurrence of *V. asperata* is necessary to develop targeted fungicide management, and identification of scab-resistant cultivars, to prevent emergence of new fungal diseases.

Occurrence of *Monilinia* species in South Tyrolean (Italy) sweet cherry orchards. U. SPITALER, A. PFEIFER, S. HAUPTKORN, E. DELTEDESCO and S. OETTL. *Research Centre Laimburg, Laimburg 6, 39040 Auer/Ora (BZ), Italy. E-mail: urban.spitaler@laimburg.it*

Sweet cherry could be a niche crop for small farmers in the mountainous province of South Tyrol (Italy), and commercial orchards have increased during the decade to 2020. The most important fungal pathogens of stone fruit are *Monilinia fructigena*, *M. laxa* and *M. fructicola,* causing brown rot. However, these species have not been found in South Tyrol. Thus, in 2020 a collection of fruit mummies from 17 orchards in the main cherry growing areas was analyzed using modified real-time PCR. On 30% of the mummified fruit samples at least one *Monilinia* sp. was detected. *Monilinia laxa* was the most common species, detected on 22 % of the samples, while *M. fructigena* as the single species was identified only on 4 % of the samples. Mixed infections with these species were identified on 6% of the samples. *Monilinia fructicola* was found on four samples from two bordering cherry orchards. This is the first detection of the former quarantine organism *M. fructicola* in South Tyrolean sweet cherry orchards.

Chemical management of *Colletotrichum acutatum* causing olive anthracnose. M. VARVERI, M.K. ILIADI, C.K. KAVROUMATZI and D.I. TSITSIGIANNIS. *Department of Crop Science, Laboratory of Plant Pathology, Agricultural University of Athens, Greece. E-mail: dimtsi@aua.gr*

Infections of olive fruit (*Olea europaea*) by *Colletotrichum* are very frequent and destructive. *Colletotrichum acutatum* is considered to be the causal agent of olive anthracnose (OA) in Greece. This fruit rot leads to fruit drop or mummification, resulting in significant yield losses and degradation of oil quality. Control of olive anthracnose is difficult, and is usually achieved through a combination of methods including chemical control. Several chemical plant protection products (PPPs) were evaluated for the control of *C. acutatum* in the two Greek olive varieties, "Koroneiki" and "Kalamon", using 12 commercial fungicides. Fruits were surface sterilized and then immersed in a solution of each PPP according to the maximum certified dose for each fungicide. Two days later, treated fruits were sprayed with a conidium suspensions of a Greek *C. acutatum* isolate. Several of the PPPs inhibited colonization and sporulation of *C. acutatum* on olive fruits. Nine *C. acutatum* strains from

different regions of Greece were characterized, *in vitro*, for sensitivity/resistance to the active ingredients of the most effective fungicides. All isolates were sensitive to the selected sterol-inhibiting fungicides (SBIs), while most of the isolates were resistant to copper compounds.

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Fungicide sensitivity and genetic diversity of *Botrytis cinerea* populations from conventional and organic tomato and strawberry fields in Cyprus and Greece. G. MAKRIS[1], A. SAMARAS[2], N. NIKOLOUDAKIS[?], G.S. KARAOGLANIDIS[2] and L.I. KANETIS[1]. [1]*Department of Agricultural Sciences, Biotechnology, and Food Science, Cyprus University of Technology, Limassol, 3036, Cyprus.* [2]*Plant Pathology Laboratory, Faculty of Agriculture, Forestry and Natural Environment, Aristotelian University of Thessaloniki, POB 269, 54124, Thessaloniki, Greece. E-mail: loukas.kanetis@cut.ac.cy*

Botrytis cinerea is a complex species prone to fungicide resistance and characterized by broad genetic diversity. Unravelling pathogen population diversity facilitates development of efficient disease management schemes. During 2016–2017, 360 *B. cinerea* isolates were collected from conventional and organic tomato or strawberry farms in Greece and Cyprus, to analyze the genetic diversity and population structure of *B. cinerea* from different hosts and farming systems, in terms of agrochemical-inputs. Results highlighted widespread fungicide resistance, with 61.9% of the isolates found to be resistant to at least one botryticide, while 15.5% of isolates from conventional farms, and 85.1% from organic farms, were sensitive to all the tested active ingredients. Resistance frequencies of the isolates collected from conventional and organic fields were, respectively, 67.7 and 8.2% to boscalid, 65.9 and 29.1% to cyprodinil, 7.3 and 0% to fludioxonil, 18.6 and 3% to fenhexamid, 18.6 and 0% to iprodione, 67.3 and 9.7% to pyraclostrobin, and 61.4 and 15.7% to thiophanate-methyl. Fludioxonil and fenhexamid resistance were not detected in any of the tomato farms or countries, while resistance to iprodione was not recorded from organic fields. Multiple fungicide resistance was common, with fungicide sensitivity classes ranging from >3 for conventional farms and <1 for organic farms. Lambda measure of association showed showed a symmetric value (0.563 ± 0.065; $P < 0.001$), indicating that farming systems correlated with fungicide resistance, across crops and countries. The *B. cinerea* populations were also genotyped using a set of seven microsatellite markers. *Botrytis pseudocinerea* was not detected, while the populations exhibited high genetic diversity and mixed modes of reproduction. Genetic variance among strawberry and tomato populations was evident, ranking host specificity higher than other selection forces. AMOVA suggested significant associations of population structure and resistance to thiophanate-methyl, pyraclostrobin, boscalid, and cyprodinil.

Protection of grapevine pruning wounds against natural infections by trunk disease fungi. R. BUJANDA[1], B. LÓPEZ-MANZANARES[1], S. OJEDA[1], O. ONEKA[2], L.G. SANTESTEBAN[2], J. PALACIOS[3] and D. GRAMAJE[1]. [1]*Instituto de Ciencias de la Vid y del Vino (ICVV), Consejo Superior de Investigaciones Científicas, Universidad de la Rioja - Gobierno de La Rioja, Ctra. LO-20 Salida 13, Finca La Grajera, 26071 Logroño, Spain.* [2]*Dpto de Agronomía, Biotecnología y Alimentación, Universidad Pública de Navarra (UPNA), Campus Arrosadia, 31006 Pamplona, Spain. 3Viticultura Viva S.L., Cabmesado 4, 31390 Olite, Spain. E-mail: david.gramaje@icvv.es*

Infection of grapevines by grapevine trunk disease (GTD) fungal pathogens primarily occurs through annual pruning wounds made during the host dormant season. Efficacy was evaluated (in two field trials over one growing season) of liquid formulation fungicide (pyraclostrobin + boscalid) and paste treatments, as well as biological control agents (*Trichoderma atroviride* SC1, *T. atroviride* I- 1237, or *T. asperellum* ICC012 + *T. gamsii* ICC080), for their potential to prevent natural infections of grapevine pruning wounds by trunk disease fungi. The vineyards were in Samaniego, Northern Spain (19-years-old; "Tempranillo") and Madiran, Southern France (24-years-old; "Cabernet Franc"). Wound treatments were applied immediately after pruning in February 2020. Untreated controls were treated with sterile distilled water. In February 2021, canes were harvested from vines and brought to the laboratory for *Trichoderma* spp. and fungal trunk pathogen assessments. A total of 1,848 isolates were collected from the Samaniego vineyards, and 1,179 were from Madiran vineyards, associated with five GTDs. In some cases, it was not possible to establish statistically significant differences between treatments due to the low incidence of some GTDs in the untreated control treatments. The efficacy of each product varied according to the GTD fungi and the grape production region.

Trichoderma recovery percentages ranged from 16.7 to 67.5% from Samaniego, and from 32.5 to 89.6% from Madiran. The experiment will be repeated during the 2021–2022 season.

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Fungal trunk pathogens associated with *Juglans regia* in the Czech Republic. J. PECENKA[1], M. SPETIK[1], T. NECAS[2], I. ONDRASEK[2], J. ARMENGOL[3], M. LEÓN[3], C. BERLANAS[4], D. GRAMAJE and A. EICHMEIER[1]. [1]*Mendel University in Brno, Faculty of Horticulture, Mendeleum - Institute of Genetics, Valticka 334, 69144, Lednice, Czech Republic.* [2]*Mendel University in Brno, Faculty of Horticulture, Department of Fruit Growing, Valticka 337, 69144, Lednice, Czech Republic.* [3]*Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Camino de Vera S/N, 46022 Valencia, Spain.* [4]*Instituto de Ciencias de la Vid y del Vino (ICVV), Consejo Superior de Investigaciones Científicas, Universidad de la Rioja-Gobierno de La Rioja, Ctra. de Burgos Km. 6, 26007 Logroño, Spain. E-mail: jakub.pecenka@mendelu.cz*

The spectra of fungal trunk pathogens associated with wood necrosis of the English walnut trees in the Czech Republic was assessed in field surveys during 2016, in Moravia region. Branches of the walnut trees showing the typical symptoms of dieback were selected for the sampling. In total, 138 of the fungal isolates were obtained from six orchards. Based on the morphology and sequencing data, the isolates were identified in 10 species of trunk pathogens including: *Cadophora novi-eboraci*, *Cadophora spadicis*, *Cryptovalsa ampelina*, *Diaporthe eres*, *Diplodia seriata*, *Dothiorella omnivora*, *Eutypa lata*, *Eutypella* sp., *Peroneutypa scoparia*, and *Phaeoacremonium sicilianum*. The most predominant fungal taxon was *Cadophora* followed species of *Botryosphaeriaceae* and *Diatrypaceae*. Pathogenicity tests showed that isolates of *Cadophora* spp. and *Eutypa lata* were virulent to the walnut branches.

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Preliminary observations on the interaction among *Neocosmospora solani* (syn. *Fusarium solani*), *Neofusicoccum batangarum* and *Opuntia ficus-indica*. S. BURRUANO[1], S. GIAMBRA[1], G. GUSELLA[2], G. CONIGLIARO[1] and G. SURICO[3]. [1]*Dipartimento di Scienze Agrarie, Alimentari Ambientali (SAAF), Università degli Studi di Palermo, Viale delle Scienze 2, 90128 Palermo, Italy.* [2]*Dipartimento di Agricoltura, Alimentazione e Ambiente (Di3A), Università degli Studi di Catania, Via S. Sofia 100, 95123 Catania, Italy.* [3]*Department of Agricultural, Food, Environmental and Forestry Science and Technology (DAGRI), Plant Pathology and Entomology Section, Università degli Studi di Firenze, Piazzale delle Cascine, 28, 50144 Firenze, Italy. E-mail: santella.burruano@unipa.it*

Opuntia ficus-indica (L.) Mill. (*Cactaceae*) is a xerophilous plant native to Mexico and naturalized throughout the Mediterranean Basin, where it is a common landscape species. This plant *O. ficus-indica* is also cultivated on many continents. In Italy, production is mostly concentrated in some hilly Sicilian areas. A severe disease of cactus pear was reported on the minor Sicilian islands of Lampedusa and Linosa (Pelagie archipelago), Favignana (Aegadian archipelago), and Ustica. The disease was named "scabby canker", and its causal agent was identified as *Neofusicoccum batangarum*, (*Botryosphaeriaceae*, *Ascomycetes*). Scabby cankers appear as radially expanding, concentric, necrotic cankers on cladodes. They first appear in April each year, following first rains, and continue to develop during summer. Occasionally, *Neocosmospora solani* isolates have been associated with symptomatic cladodes. Interactions between *N. solani* and *N. batangarum*, and between the two fungi and *O. ficus-indica*, were studied *in vitro* and *in planta*. Dual growth *in vitro* assays showed *N. solani* overgrowing *N. batangarum*, and dark reaction zones were observed at points of contact of the two colonies, suggesting antagonism. *In planta* assays carried out by inoculating cladodes with *N. solani*, individually or in association with *N. batangarum* (which was applied after 30 d) never showed lesions. Lesions were clearly visible on the cladodes after inoculations *N. batangarum* only, or simultaneously with *N. solani*. *In vitro* and *in planta* observations indicated that *N. solani* has a neutral interaction with *O. ficus-indica,* and that it has antagonistic activity towards *N. solani*. Furthermore *N. batangarum* is confirmed as the only causal agent of scabby cankers.

This research is dedicated to the memory of Dr Gaetano Conigliaro.

An innovative protocol for the monitoring and onsite detection of *Erwinia amylovora* in Sicily. F. VALENTINI, F. SANTORO, M. GALLO, L. OUALGUIRAH and A. M. D'ONGHIA. *Centre International des Hautes Etudes Agronomiques Méditerranéennes – Mediterranean Agronomic Institute of Bari, Via Ceglie 9, 70010 Valenzano (BA), Italy. E-mail: valentini@iamb.it*

Erwinia amylovora (*Ea*), which causes fire blight of pomefruits, remains a quarantine agent (list A2), despite widespread occurrence in the European Union. Effective control of the bacterium is based on the use of resistant varieties, the application of reliable tests for pathogen detection, knowledge of climatic conditions that can promote infections, and adoption of specific agronomic practices (e.g. pruning) and/or chemical treatments to reduce the inoculum of the pathogen. This study aimed to develop an effective protocol for monitoring *Ea*, to detect infections early before onset of symptoms, without moving infected plant material from infection outbreak areas. Infections were monitored in pomefruit groves in the *Ea* outbreak of Sicily. Visual inspections for fire blight symptoms and detection of the pathogen using real-time LAMP with an onsite device were carried out during two vegetative seasons (March-October, 2018 and 2019). The results were correlated to parameters measured at an agroclimatic station located in the study area, thus implementing the Maryblight model. The combination of the forecasting model and onsite pathogen detection by real time LAMP is an innovative approach for official monitoring of this pathogen.

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Development of reagent kits for the identification of *Candidatus* Phytoplasma mali and *Candidatus* Phytoplasma pyri using Real-time PCR. I.G. BASHKIROVA[1,5], G.N. BONDARENKO[1,5], A.A. SHVARTSEV[2], Ya.I. ALEXEEV[2,3,4] and S.A. BLINOVA[2]. *[1]All-Russian Plant Quarantine Center, 140150 Bykovo, Ramenskoe, Moscow Region, Russia. [2]LLC SYNTOL, 127434 Moscow, Russia. [3]Federal State Institution of Science Russian National Research Institute Viticulture and Winemaking "Magarach", 298600 Yalta, the Republic of Crimea, Russia. [4]Institute for Analytical Instrumentation of the Russian Academy of Sciences, 198095 St. Petersburg, Russia. [5]Peoples' Friendship University of Russia, 117198, Moscow, Russia. [6]Russian Timiryazev State Agrarian University, Moscow Timiryazev Agricultural Academy, 127434, Moscow, Russia. E-mail: bashkirovaid@mail.ru, syntol@syntol.ru.*

The apple proliferation group phytoplasmas (16SrX) were included in the List of Quarantine Objects A2 of the European and Mediterranean Plant Protection Organization (EPPO), as well as in The Uniform list of quarantine objects of the Eurasian Economic Union. Kits of reagents were required to enable fast and accurate identification of these pathogens in plant material. The main and most susceptible hosts of *Candidatus* Phytoplasma mali and *Candidatus* Phytoplasma pyri are *Rosaceae* plants, species of *Pyrus*, *Malus*, *Cydonia* and *Prunus*. Phytoplasma DNA extraction was carried out using methods recommended by the EPPO, with modifications. Real-time PCR was carried out using four instruments: CFX-96 (BIO-RAD, USA), Rotor-Gene (QiaGen, USA), ANK (IAI RAS, Russia), and DTprime (DNA-Technology, Russia). The mplification programme was: hot start at 95°C for 5 min, then 50 cycles each of 95°C for 15 sec and 60°C for 40 sec. Primers and probes were designed for the *dihydrolipoamide acetyltransferase* (*aceF*) gene. Specificity of the reagent kits was tested on 37 samples of related objects, and DNA cultures were obtained from the DSMZ-GmbH, CIRM-CFBP, All-Russian Plant Quarantine Center and other collections. There were no false-negative results of analyses of plant samples containing phytoplasma DNA, nor false-positive results of analyses of plant DNA and bacterial DNA. Analytical sensitivity of the developed reagent kit for Candidatus *Phytoplasma* mali was ≤14.0 × 103 copies of DNA per milliliter of sample, and for the *Candidatus* Phytoplasma pyri kit was ≤6.0 × 103 copies of DNA mL^{-1}.

Development of an algorithm of recognition for automatic reading of nitrocellulose membranes processed by Direct Tissue Blot Immunoassay. S. GUALANO, E. PANTALEO, F. SANTORO, D. FRASHERI and A. M. D'ONGHIA1. *Centre International des Hautes Etudes Agronomiques Méditerranéennes – Mediterranean Agronomic Institute of Bari, Via Ceglie 9, 70010 Valenzano (BA), Italy. E-mail: gualano@iamb.it*

Serological tests are simple and rapid methods of detecting plant pathogens. Enzyme- Linked ImmunoSorbent Assay (ELISA) and Direct Tissue Blot ImmunoAssay (DTBIA) are commonly used for screening large numbers of plant samples with efficiency for most pathogens. Compared to ELISA, DTBIA is easy to use, shorter and

cheaper, and can also be used as on-site tests. However, the reading phase of the DTBIA under stereomicroscope is longer and manual, while an automatic optical reader is available for the ELISA plates. In this study, the first approach was, therefore, to develop an automatic reading procedure for the DTBIA. The main components of variability of the processed membranes (e.g. the membrane commercial brand, the storage period of the processed membrane) were analysed for print colour and membrane background using plant material infected by Citrus tristeza virus or *Xylella fastidiosa*. Significant reading variability correlated to some components was observed, confirmed by the optical characteristics. On the basis of these results, an algorithm was developed for recognition of positive prints using normalized high-resolution images.

This research was financially supported by the Project Multitrace - Advanced Information Technologies and Systems for Traceability in Phytopathology (PON MISE H2020).

Phenometabolomics of olive quick decline syndrome, using nuclear magnetic resonance, hyperspectral reflectance and integrative chemometrics analysis. F. SANTORO[2], A. ELHUSSEIN M.F.M.H.[1,2], S. GUALANO[2], B. MUSIO[1], A.M. D'ONGHIA[2] and V. GALLO[1,3]. *[1]Department of Civil, Environmental, Land, Building Engineering and Chemistry (DICATECh), Polytechnic University of Bari, Via Orabona, 4, I-70125, Bari, Italy. [2]International Centre for Advanced Mediterranean Agronomic Studies of Bari (CIHEAM Bari), Via Ceglie 9, 70010, Valenzano (BA), Italy. [3]Innovative Solutions S.r.l. – Spin-off company of Polytechnic University of Bari, Zona H 150/B, 70015, Noci (BA), Italy. E-mail: fsantoro@iamb.it*

Xylella fastidiosa (*Xf*) subsp. *pauca* (sequence type ST53) has severely affected olive groves in the Apulia region of Southern Italy, infecting approx. 11 million olive trees in more than 50,000 ha. Disease caused by *Xf* (olive rapid decline syndrome (OQDS)) causes phenotypic and metabolomic changes in host plants. The control strategy currently uses destruction of infected plants, that are detected on the basis of symptoms and positive diagnostic tests. Detection of infections before symptoms develop can provide an advantage against spread of the disease, allowing preventive action. The combination of hyperspectral reflectance (HSR) and nuclear magnetic resonance (NMR) can offer a non-destructive method to detect the host phenotypic and metabolic fluctuations caused by *Xf*. Young plants of the susceptible olive variety Cellina di Nardò, artificially infected with *Xf*, were grown in a temperature-controlled environment, and were co-inoculated with additional xylem-inhabiting fungi. Asymptomatic leaves were exposed to HSR acquisition, and their extracts were subjected to an untargeted metabolomic study, to select diagnostic signals and wavelengths associated with *Xf*-infected plants. Covariance matrices between these methods were then used assess linkage between HSR spectral features and NMR and HRMS diagnostic signals. These matrices showed that different wavelength ranges were associated with different levels of association with particular metabolites, with a few regions within the visual range of 420–520 nm, 570–720 nm, and more within the near-infrared range of 1000–1830 nm.

Evaluation of resistance of grape varieties to *Aspergillus carbonarius* and ochratoxin contamination. M.K. ILIADI and D.I. TSITSIGIANNIS. *Laboratory of Plant Pathology, Department of Crop Science, Agricultural University of Athens, Iera Odos 75, 118 55 Athens, Greece. E-mail: dimtsi@aua.gr*

Aspergillus carbonarius causes grape berry rots in vineyards, and is responsible for production of the mycotoxin ochratoxin A (OTA), which affects organoleptic characteristics of wine and grape and wine quality and safety. OTA poses a serious risk to consumer safety because it is nephrotoxic, hepatotoxic and carcinogenic. The present study assessed 21 Greek grapevine varieties (Razaki, Mandilaria, Roditis, Malagouzia, Savatiano, Fraoula, Athiri, Sideritis, Mavrodafni, Asyrtiko, Xinomavro, Debina, Agianiwtiko, Pavlos, Agiorgitiko, Aidini, Lagorthi, Limnio, Georgiana, Kydonitsa and Stafida) for their resistance to *A. carbonarius* rots and OTA contamination. *In vitro* artificial inoculations were carried out on grape berries from each of the varieties, using a mixture of four ochratoxigenic strains of *A. carbonarius* isolated from Crete, Peloponnese, Attica or Macedonia. Evaluations of resistance were by assessment of *A. carbonarius* grape rot spots, conidim production and OTA production in berries. The results showed significant variation in these parameters among the grape varieties.

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Characterization of *Alternaria* species associated with black point of wheat kernels in Lebanon. M. MASIELLO[1], W. HABIB[2,*], R. EL GHORAYEB[3], E. GERGES[2], C. SAAB[2], A. SUSCA[1], G. MECA[4], J.M. QUILES[4], A.F. LOGRIECO[2] and A. MORETTI[2]. *[1]Institute of Science of Food Production - ISPA, Research National Council – CNR, Via Amendola, 122/O, 70126 Bari, Italy. [2]Laboratory of Mycology, Department of Plant Protection, Lebanese Agricultural Research Institute, P.O. Box 90-1965, Fanar, Lebanon. [3]Faculty of Agricultural and Food Sciences, Holy Spirit University of Kaslik, P.O. Box 446, Jounieh, Lebanon. [4]Department of Preventive Medicine, Nutrition and Food Science Area, University of Valencia, Avenida Vicent Andres Estelles s/n, 46100 Burjassot, Valencia, Spain. *W. HABIB current affiliation: Centro di Ricerca, Sperimentazione e Formazione in Agricoltura – Basile Caramia (CRSFA), Via Cisternino 281, Locorotondo, 70010 Bari, Italy. E-mail: mario.masiello@ispa.cnr.it*

Alternaria commonly infects wheat crops, pre- and post-harvest decay of wheat grains and symptoms on heads, such as dark brown discolouration (black point disease) of grain embryos. Several *Alternaria* species can produce a range of mycotoxins, associated with different toxicities to humans and animals. The aims of the present study were to: i) assess the incidence of black point disease of wheat in Lebanon and isolate the *Alternaria* species causing the disease; ii) evaluate the levels of contamination by *Alternaria* mycotoxins in 33 grain samples; and iii) to identify the main occurring *Alternaria* species, using a molecular approach, and study their phylogenetic relatedness. The disease occurred at the majority (97%) of sampled sites, with the greatest average incidence in Akkar district (55%) and least (2%) in Baalbeck. Chemical analyses, using HPLC-DAD, showed that altenuene, alternariol, alternariol monomethyl ether, and tenuazonic acid were not detected in any sample. The phylogenetic analyses, based on DNA sequences of *β-tubulin*, *glyceraldehyde-3-phosphate dehydrogenase*, *allergen alt-a1*, and *calmodulin* genes, showed that isolates were in two main clades: 37 strains belonged to section *Infectoriae* and 42 strains to section *Alternaria*. This study showed that although black point disease of wheat kernels is widespread in Lebanon, the risks of contamination by *Alternaria* mycotoxins remains low.

Cultivar-dependent differences in the phyllosphere-associated mycobiome of grapevine (*Vitis vinifera*). A. MOLNÁR[1], J. GEML[1,4], A. GEIGER[1,3], C. M. LEAL[3,4], G.M. KGOBE[3,4], A.M. TÓTH[2], S.Z. VILLANGÓ[2], L. MÉZES[1], A.M. CZEGLÉDI[1] ans Z.S. ZSÓFI[2]. *[1]Food and Wine Research Centre, Eszterházy Károly Catholic University, Leányka u. 6, Eger 3300, Hungary. [2]Institute for Viticulture and Enology, Eszterházy Károly Catholic University, Leányka u. 6, Eger 3300, Hungary. [3]Doctoral School of Environmental Sciences, Hungarian University of Agricultural and Life Sciences, Páter K. u. 1, Gödöllő 2100, Hungary. [4]ELKH – EKKE Lendület Environmental Microbiome Research Group, Eszterházy Károly Catholic University, Leányka u. 6, Eger 3300, Hungary. E-mail: molnar.anna@uni-eszterhazy.hu*

Grapevines naturally host reservoirs of fungi affecting grape production and quality, and plant health. Above-ground host tissues, the phyllosphere, is a dynamic and harsh habitat for microbial colonizers, due to exposure to environmental factors. An important question is, what drivers shape the phyllosphere fungal composition, thereby contributing to plant health and possibly to crop quality and quantity? This study investigated potential differences of phyllosphere-associated mycobiomes inhabiting healthy leaves and berries of the three grapevine cultivars *V. vinifera* "Furmint", "Kadarka" and "Syrah", grown under very similar environmental conditions in the Eger wine region. The study assessed whether the grapevine cultivars affected richness, relative abundance and composition of the fungal phyllosphere communities, using high-throughput DNA sequencing. To better clarify the background community differences, host physiological parameters, macro- and micro-element composition of leaf samples, and sugar content of berries were also assessed. While richness and relative abundance of fungal functional groups did not statistically differ among the cultivars, strong compositional differences were detected among the cultivars. In contrast, The cultivar berry samples differed in community richness and relative abundance, but not in community composition, suggesting the existence of a core grape microbiome at least at terroir level.

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Selecting strawberry and melon germplasm tolerant to *Macrophomina phaseolina*. S.A. YOUSSEF[1], N. DAI[3], R. COHEN[4], S. FREEMAN[2] and A.A. SHALABY[1]. *[1]Plant Pathology Research Institute, Agricultural Research Center, 9 El-Gamma St., Giza, Egypt. [2]Department of Plant Pathology and Weed Research, ARO, Volcani Cent-*

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Macrophomina phaseolina is a soilborne pathogen causing crown and root rot of strawberry and vine decline of melons, is a destructive pathogen of strawberry and melon in the Mediterranean region. This fungus was isolated from different cultivars of strawberry showing crown rot, foliage wilting, charcoal rot and plant death, as well as vine wilting and death of of melon. Similar disease symptoms were recorded in melon and strawberry cultivation areas in Egypt and Israel. Effective management of these diseases on resistant host germplasm selected using reliable techniques. Pathogenicity tests of representative *M. phaseolina* isolates were carried out to determine virulence and viability using the toothpick method, whereby microsclerotia are produced aseptically on toothpicks that are subsequently used to inoculate stem and crown tissues of tested plants. In addition, artificial soil inoculation was also assessed by inserting plants in pots containing a soil mixed with 2.5 × 103 sclerotia mL^{-1}. All inoculated plants were grown at 30°C in greenhouse conditions. In strawberry, complete plant mortality was observed approx. 3 weeks after inoculation. However, microsclerotium inoculation was gave more rapid development of disease symptoms at 2 weeks after inoculation. Using the microsclerotia method, disease symptoms progressed more rapidly and faster in the susceptible cultivar `Festival` compared to the more resistant `Fortuna`, which had the least disease progress. In melon, disease symptoms following toothpick inoculations in the greenhouse and field produced similar symptoms to those for plants growing in naturally infested soils. The toothpick method was reliable for differentiating between susceptible and resistant melon germplasm in but not for strawberry. Disease screening of host germplasm resistant *M. phaseolina* should rely on an accurate and reliable inoculation technique.

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Identification and characterization of *Fusarium solani* and *Dematophora necatrix* on hemp (*Cannabis sativa*) in southern Italy. R. SORRENTINO[1], V. BATTAGLIA[1], F. RAIMO[1], D. CERRATO[1], G. PICCIRILLO[2], A. MEROLA[1] and E. LAHOZ[1]. *[1]Research center for Cereal and Industrial Crops - Council for Agricultural Research and Economics (CREA-CI) Via Torrino 3, 81100 Caserta, Italy. [2]Servizi di saggio di Agrotecniche (SESAT) Via IX Novembre, 81055 Santa Maria Capua Vetere (CE) Italy. E-mail: roberto.sorrentino@crea.gov.it*

Since 2016, cultivation of industrial hemp is allowed in Italy according to Law no. 242/2016. Industrial hemp can be grown for production of fibre, seed, oil, or cannabinoids. In a survey commencing in 2018, two new diseases were highlighted as affecting hemp crops. The soil-borne pathogens *Fusarium solani* in indoor crops, and *Dematophora necatrix* in outdoor crops were identified. *Fusarium solani* caused development, at soil line, of brownish to dark brown areas along the main host stems, followed by wilting and shedding of leaves. In correspondence with the lesions, the stems swelled to create cankers. Removal of the bark of affected plants revealed that the inner cortices were also affected. *Dematophora necatrix* caused root rot followed by host death that generally occurred within 2 to 3 weeks after symptoms appeared, with approx. 10% of plants showing yellowing, canopy wilt, and roots covered with white fan-like mycelium under the bark. These were the first records for both pathogens in Europe. Since interest in hemp production is increasing, appropriate management tools are required for these two pathogens.

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Behaviour characterization of durum wheat varieties to distinct strains of *Pyrenophora tritici- repentis* (tan spot) in field and controlled conditions. S. TISSAOUI[1], A. MOUGOU-HAMDANE[1], N. OMRI-BENYOUSSEF[2] and B. NASRAOUI[1]. *[1]LR14AGR02 Laboratory of Bioaggressors and Integrated Pest Management in Agriculture, National Agronomic Institute of Tunisia, University of Carthage, Tunis, Tunisia. [2]Field Crop Laboratory, National Agronomic Research Institute of Tunisia, Tunisia. E-mail: salmatissaoui2@gmail.com*

Tan spot of wheat, caused by *Pyrenophora tritici-repentis,* is of economic importance, and an emerging disease in Tunisia. Necrosis with chlorosis, and chlorosis on wheat leaves were distinct symptoms induced. The necrotrophic pathogen produces host-specific toxins responsible for symptom variation on susceptible genotypes. The reaction of 14 durum wheat varieties to *P. tritici-repentis* was evaluated in field and controlled con-

ditions, by inoculating with three characterized pathotypes. Susceptibility of commonly grown varieties was determined by percentage of leaf area infections. Tan spot lesions developed on all the inoculated wheat genotypes, which showed response variations in the two trial conditions. Differences ($P<0.05$) in strain virulence were recorded. The varieties also differed disease reaction to different pathogen strains. The most virulent was strain I1 induced chlorosis on varieties which showed reaction variation. The varieties Khiar, Sculture and Monastir were moderately resistant in controlled conditions, but susceptible in the field. A statistically significant isolate × variety interaction was detected, which demonstrated specificity between strains and varieties. The overall reaction of varieties indicated that the most resistant was Monastir, while the most susceptible varieties were Maali and Razzak. The use of distinct pathotypes could assist evaluation wheat varieties in order to select resistant lines for develop tan spot management.

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Effects of rootstock and viroid infections on yield and composition of essential oil from skins of Tunisian sweet orange (*Citrus sinensis*) "Maltaise". G. ZOUAGHI[1], A. NAJAR[2], A. AYDI[3], C CLAUMANN[4], A. ZIBETTI[5], A JEMMALI[2], F. MOUSSA[6], M ABDERRABBA[1] and N. CHAMMEM[7]. *[1]Laboratory materials, molecules, applications, IPEST (Preparatory Institute for Scientific and Technical Studies), University of Carthage, La Marsa 2070, Tunis, Tunisia. [2]Laboratory of plant protection, National Institute of Agronomic Research of Tunisia, University of Carthage Rue Hédi Karray, 1004 El Menzah, Tunisia. [3]Department of Chemical and Materials Engineering, College of Engineering, Northern Border University, P.O. Box 1321, Saudi Arabia. [4]Laboratorio de Controle de Processos, Departments of Chemical Engineering and Food Engineering, Universidade Federal de Santa Catarina (UFSC), P.O. Box 476, Florianópolis, SC,Brazil. [5]Department of Informatics and Statistics (INE), Universidade Federal de Santa Catarina (UFSC), P.O. Box 476, Florianópolis, SC, Brazil. [6]Laboratory for the study of molecular analysis techniques and instruments, IUT d'Orsay - Moulon plateau - Building 602 - 91400 ORSAY. [7]Laboratory of Microbial Ecology and Technology, Department of Biological and Chemical Engineering, National Institute of Applied Sciences and Technology (INSAT), Carthage University, B.P. 676, 1080 Tunis, Tunisia. E-mail: asmanajara@yahoo.fr*

Beside agronomic performance, attention is given to yield and composition of essential oils (EO) from citrus peels by some industries. Citrus bioactive components are reported to be influenced by environmental factors. Effects of host rootstocks were assessed in presence or absence of viroid infections for yield or peel EO composition differences in the the Tunisian sweet orange Maltaise. *Citrus exocortis viroid* (CEVd) and *Hop stunt viroid* (HSVd) or Cachexia viroid were inoculated to eight host rootstocks. Preliminary results showed that yields varied between 0.4 and 0.8%. However, infections with CEVd and Cachexia reduced by 50% for *Citrumelo swingle* but did not affect other rootstocks. Regarding the composition of EO, healthy *Citrus macrophylla* gave the 17% more oxygenated monoterpenes than infected plants. CEVd infections enhanced this class of monoterpenes in *Citrus macrophylla* by 32%, in *C. volkameriana* by 11%, and in *Citrange carrizo* by 22%, while infections by Cachexia infection in *C. macrophylla* caused monoterpene reductions of 17 to 3%.

This research is part of PRF (Federated Research Project) on Citrus, entitled "Principal Citrus Viruses: Characterization and Integrated Management" and was financially supported by Ministry of Higher Education and Scientific Research of Tunisia (MESRST.

Decision Support Systems to improve crop protection, productivity and sustainability: piloting and internationalization. M. RUGGERI[1], P. MERIGGI[1], A. KARTSIAFLEKIS[2] and S.E. LEGLER[1]. *[1]Horta srl, Spin-off Università Cattolica Sacro Cuore, Piacenza, Italy. [2]Fondagro, Larissa, Greece. E-mail: s.legler@horta-srl.com*

Since 2008, Horta has implemented Decision Support Systems (DSSs) for sustainable crop management, following the "super consultant" approach for achieving acceptance by agricultural stakeholders. Horta's DSSs are web-based platforms that collect real-time site-specific weather and crop data, through remote and proximal sensors, organize these data in cloud systems and analyse them via advanced modelling and big data techniques, provide automated agronomic interpretation of model outputs, and produce alerts and decision supports. Process-based mathematical models are the core of the DSSs. These provide advice to decision makers for overall crop management including: pest/disease management, weed control, fertilization, and irrigation. The DSSs support (but not replace) farmers in practical implementation of Integrated Crop Management. Benefits are related to all sustainability pillars including: economic (e.g., reduction of directs costs); environ-

mental (e.g., reduction of GHG emissions); and social (e.g., reduction of exposure to pesticides, workers' satisfaction). To date, DSSs are available for wheat, barley, wine and table grapes, olives, legumes, tomatoes, corn, potatoes, while others are in-progress. Users are farmers, technicians, private/public advisors, producer organizations, and agri-food companies. The DSSs are used in Italy and, increasingly, in other countries. For instance, vite.net® (grapevine) is widespread in Spain, grano.net® (wheat) and orzo.net® (barley) in Greece and Bulgaria, and mais.net® (corn) in Hungary. To quantify, the DSS for wheat and barley in Europe was used by more than 6,300 farms, yielding more than 575,100 tons of grains in 2019. Internationalization of DSSs is a resources-demanding process, which includes customization for each country and demonstration of the multifold benefits to local stakeholders from DSSs in relevant agricultural contexts.

Metagenomics analyses of fungal communities associated with postharvest diseases in pear fruits. A. ZAMBOUNIS[1], I. GANOPOULOS[1] and P. MADESIS[2]. *[1]Institute of Plant Breeding and Genetic Resources, HAO 'Demeter', Thermi, Thessaloniki, 57001, Greece. [2]Institute of Applied Biosciences, CERTH, Thermi, Thessaloniki, 57001, Greece. E-mail: antonios.zamb@gmail.com*

An amplicon metagenomic approach was employed to identify the composition of fungal communities associated with postharvest diseases of pear fruits. Fruits were harvested at an orchard using routine management practices involving different fungicides, and were transferred to a storage packinghouse. Effective tag sequences clustered into OTUs and Ascomycota were the dominant phyla (83.4%), followed by Basidiomycota (15.8%). Pear fruits supported a high diversity of microbes, but *Penicillium*, *Rhodotorula*, *Alternaria* and *Cladosporium* were the most abundant, representing 59–95% of the relative abundance of all effective sequences. Pear fruits displayed different fungal communities, according to diversity analyses among untreated and treated samples. Interruption of chemical treatments for 1 month before harvest gave significant modifications in the structure of the fruit fungal communities. It is assumed that various antagonistic episodes occurred among the detected fungal genera in the fruit carpospheres, and relative abundances were affected by fungicide treatments.

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DNA-based comparisons of plant pathogenic fungi between grapevine and wild woody *Rosaceae* with, a focus on trunk diseases. L.A. LEPRES[1,2], J. GEML[1,3], Z. KARÁCSONY[1], A. GEIGER[1,2], A. TÁNCSICS[2] and K.Z. VÁCZY[1]. *[1]Food and Wine Research Center, Eszterházy Károly Catholic University, 3300 Eger, Leányka u. 6., Hungary. [2]Doctoral School of Environmental Sciences, Hungarian University of Agriculture and Life Sciences, 2100 Gödöllő, Páter Károly u. 1., Hungary. [3]ELKH-EKKE Lendület Environmental Microbiome Research Group, Eszterházy Károly Catholic University, 3300 Eger, Leányka u. 6., Hungary. E-mail: lucalepres.agrar@gmail.com*

Grapevine (*Vitis vinifera*) is a major cultivated plant, which is naturally colonized by commensal, beneficial or pathogenic microorganisms. Many of these can influence plant health and disease incidence and severity. Grapevine microbiome studies have primarily focused on plant parts and rhizosphere, while the influence of semi-natural ecosystems on the composition of the grapevine microbiome is practically unknown. This study focused on trunk diseases that can be caused by a variety of fungi, e.g., species of *Phaeoacremonium*, *Phaeomoniella*, *Eutypa*, or *Diplodia*. Many fungi in these genera can also infect wild and cultivated fruit-producing hosts, particularly *Rosaceae*, and can cause symptoms that are similar to those of grapevine trunk diseases. Possible connections were explored between the grapevine microbiome and that of wild woody *Rosaceae* present in surrounding landscapes. The compositional overlap was assessed for pathogenic fungi associated with grapevine and nearby wild *Rosaceae* shrubs and trees. Plant samples were taken from living trunks of the studied plants, with or without symptoms of trunk diseases. Several pathogens were present in all studied plants, including species of *Botryosphaeria*, *Phaeoacremonium*, and *Phaeomoniella* that are known to cause trunk diseases in vineyards. This overlap suggests that wild *Rosaceae* fruit species living near vineyards may be inoculum sources of plant pathogenic fungi capable of infecting grapevines.

This research was financially supported by the Lendület Programme No. 96049 (Eötvös Loránd Research Network and Hungarian Academy of Sciences) to JG, PhD. scholarships (Hungarian University of Agriculture and Life Sciences) to LL and AG, the European Regional Development Fund (ERDF) János Bolyai Research Scholarship (Hungarian Academy of Sciences) to KZV, and the Széchenyi 2020 Programme GINOP-2.3.2-15-2016-00061 (the European Regional Development Fund and the Hungarian Government).

Grapevine environmental DNA provides insights into temporal succession of plant pathogenic fungi, under organic and conventional vineyard management. C.M. LEAL[1,3], A. GEIGER[2,3] and J. GEML[1,2]. *[1]ELKH – EKKE Lendület Environmental Microbiome Research Group, Eszterházy Károly Catholic University, Leányka u. 6, Eger 3300, Hungary. [2]Food and Wine Research Centre, Eszterházy Károly Catholic University, Leányka u. 6, Eger 3300, Hungary. [3]Doctoral School of Environmental Sciences, Hungarian University of Agricultural and Life Sciences, Páter K. u. 1, Gödöllő 2100, Hungary. E-mail: lmota.carla@gmail.com*

Plant growth and health are dependent on plant-associated microbes. Despite recent advances, there is still no systematic overview of the diversity of grapevine-associated fungi, and how the grapevine microbiome is influenced by crop cultivation methods. This study described the diversity and composition of plant pathogenic fungal communities occurring in grapevines under an organic or conventional management. The type of plant protection used in conventional and organic vineyards was hypothesized to greatly influence the microbial, especially fungal, community structure associated with grapevine plants. Leaves of the cultivar Bianka at the Eszterházy vineyard, in Eger, Hungary, were sampled in 2020. After DNA extractions and Illumina NextSeq sequencing, the quality-filtered and rarefied dataset reads were assigned to functional groups, of which 911 ASVs were considered plant pathogens. *Phaeomoniella* showed the greatest number of ASVs, followed by *Alternaria*, *Epicoccum* and *Diplodia*, from the 88 genera found (ASV richness). Differences in fungal richness were apparent among months, but not between organic and conventional crop management types. Similarly, community structure was primarily influenced by sampling month, as shown by NMDS and PERMANOVA analyses. Beside the strong temporal turnover of leaf- associated fungi, the organic vs. conventional management comparison did not significantly affect leaf fungal communities. It is unclear how much of this community turnover was driven by periodic fungicide applications or seasonality. The lack of significant differences among cultivation types suggests that leaf-associated fungi undergo a seasonal succession.

This research was supported by the Lendület Program (award no. 96049) of the Hungarian Academy of Sciences and the Eötvös Lóránd Research Network, awarded to József Geml.

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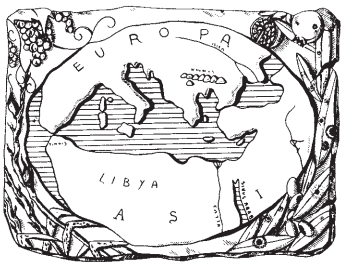
Founded by Antonio Ciccarone



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