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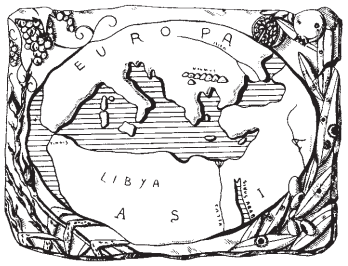

PHYTOPATHOLOGIA MEDITERRANEA

Plant health and food safety

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founded by A. Ciccarone and G. Goidànich

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Research Papers

Characterization of *Pyrenophora tritici-repentis* (tan spot of wheat) races in Kazakhstan

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Summary. Tan spot, caused by *Pyrenophora tritici-repentis*, is an economically important foliar disease of wheat in Kazakhstan. Population structure of the pathogen changes every year due to climate change. This study aimed to characterize the race structure of *P. tritici-repentis* isolates recovered from wheat in south and north Kazakhstan, and identify tan spot resistance in host genotypes based on disease phenotypes and molecular screening. Virulence profiles were determined within 40 isolates of the pathogen from wheat crops during the 2020 growing season. Seven races, (1, 3, 4, 5, 6, 7 and 8) were identified. A collection of 80 wheat accessions, including promising lines and cultivars from Kazakhstan and Russia, were evaluated for their reactions to races 1 and 5 of the pathogen, and to Ptr ToxA and Ptr ToxB, using greenhouse assessments and molecular markers diagnostic for the *Tsn1* and *Tsc2* genes. From a practical viewpoint, 18 wheat genotypes were insensitive to the two races and the two Ptr toxins. This resistant germplasm can be used in breeding programmes aiming to develop wheat varieties resistant to *P. tritici-repentis*.

Keywords. Tan spot, Ptr ToxA, Ptr ToxB, Race 1, Race 5.

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INTRODUCTION

Wheat is grown in many countries as the main source of nutrition for almost 40% of the global population, and provides 20% of dietary protein and calories (Giraldo *et al*., 2019). Global wheat use was projected to increase by 1.5 million tons in 2019-2020 compared to 2018-2019, mainly due to a 3.5% increase in feed demand (FAO, 2019). However, climate change and the onset of severe plant disease epidemics will probably reduce wheat yields and grain quality (Gurung *et al*., 2014). Between 5 and 14% of wheat yields are lost each year due to diseases Tan spot is a major wheat disease, which

occurs in temperate and warm wheat growing areas, including Kazakhstan (Duveiller *et al*., 1998; Phuke *et al*., 2020). This country suffers from crop losses due to common bunt, and yellow, leaf and stem rusts, but in recent years tan spot has been causing increased damage (Kokhmetova *et al*., 2016a; Kokhmetova *et al*., 2017; Kokhmetova *et al*., 2018a; Kokhmetova *et al*., 2019a; Kokhmetova *et al*., 2020a; Kokhmetova and Atishova, 2020; Gultyaeva *et al*., 2020; Madenova *et al*., 2021).

Tan spot is caused by the necrotrophic fungus *Pyrenophora tritici-repentis* (d.) Dreches (anamorph *Dreschslera tritici-repentis* (d.) Shoemaker). The tan spot pathogen was first described in 1823 (Hosford, 1982), and subsequently outbreaks of this disease were reported in Europe, USA, and Japan in early 1900, where the pathogen was considered to be a saprophyte causing minor to severe spotting in wheat (Wegulo, 2011). Tan spot epidemics were first reported in 1970s in Canada, the United States, Australia, and South Africa (Hosford, 1971; Tekauz, 1976; Rees and Platz, 1992; Lamari *et al*., 2005a), and then spread throughout Central Asia. The tan spot pathogen infects entire plants, but is usually most noticeable on leaves, as well as stems and head tissues. These infections lead to reductions in photosynthesis and ultimately to decreased crop yields and deterioration of grain quality. In severe cases, crop losses can exceed 50% (Wegulo, 2011). In recent years, this necrotrophic pathogen has caused increased wheat crop losses, which have been associated with reduction in tillage practices, as *P. tritici-repentis* overwinters in wheat stubble (Cotuna *et al*., 2015).

Pyrenophora tritici-repentis infects susceptible host germplasm due to host-selective toxins produced by different races, which induce necrotic or chlorotic symptoms (Lamari and Bernier, 1991) (Lamari and Barnier, 1989; Strelkov *et al*., 1999; Lamari *et al*., 2003). Three host-specific toxins, Ptr ToxA, Ptr ToxB and Ptr ToxC, have been identified and characterized in the eighth known pathogen races, while race 4 does not produce any known toxins and are non-pathogenic (Lamari and Strelkov, 2010). Ptr ToxA induces necrosis on sensitive wheat cultivars (Balance *et al*., 1989; Toma's *et al*., 1990; Zhang *et al*., 1997), and is produced by races 1, 2, 7 and 8 (Lamari *et al*., 2003). Ptr ToxB causes chlorosis in sensitive wheat genotypes, and was identified in isolates of races 5 (Oralaza *et al*., 1995), 6, 7 and 8 (Strelkov and Lamari, 2003). Ptr ToxC, causes extensive host chlorosis and was found to be produced by races 1, 3, 6 and 8 (Strelkov and Lamari, 2003).

There are three known effector-dominant susceptibility gene interactions: ToxA-*Tsn1*, which induces necrotic symptoms, ToxB-*Tsc2* and ToxC-*Tsc1*, both causing chloroses (Faris *et al*., 2013). The *Tsn1*-ToxA interaction in development of tan spot is dependent on the host genetic background, and the wheat *Tsn1* gene is a major determinant for susceptibility to the disease (Mofat *et al*., 2014). Lamari *et al*. (2003) noted that this interaction follows the inverse gene-for-gene model. Genotypes without the *Tsn1* gene are insensitive to the toxin (Lamari and Barnier, 1991; Faris *et al*., 1996; Gamba *et al*., 1998; Anderson *et al*., 1999; Friesen *et al*., 2003). However, Adhikari *et al*. (2009) proposed that recognition of ToxA through *Tsn1* may activate important genes involved in host defense response and signaling pathways. The Ptr ToxB-*Tsc2* interaction has accounts for up to 69% of the phenotypic variation in disease caused by race 5 (Friesen and Faris, 2004), so a compatible Ptr ToxB-*Tsc2* interaction plays a major role in tan spot development (Abeysekara *et al*., 2010).

Surveys of wheat fields in Central Asia and Kazakhstan in 2003 showed that tan spot was most common on winter wheat, with the severity that could reach 50% to 100% (Koyshybayev, 2002; Lamari *et al*., 2005b). Analysis of the available studies indicates a widespread pathogen in Kazakhstan (Kokhmetova *et al*., 2016b; Kokhmetova *et al*., 2017). Investigation of *P. tritici-repentis* Ptr population structure in Kazakhstan have drawn attention since the beginning of 2000s, and continued in recent years (Zhanarbekova *et al*., 2005; Maraite *et al*., 2006; Kokhmetova *et al*., 2016b; Kokhmetova *et al*., 2017). As previous varies in different years in Kazakhstan by geographical and climatic zones, and in recent years it has become more widespread globally. The races 1, 3, 4, 6 and 8 were identified in 2013–2015 (Kokhmetova *et al*., 2016b; Kokhmetova *et al*., 2017), and races 1, 2, 3, 7 and 8 in 2018 (Kokhmetova *et al*., 2020b). In both years, races 1 and 8 were dominant. In these years, races 1 and 8 were dominant (Table 1).

Previous study of germplasm resistance (Kokhmetova *et al.,* 2019b) allowed identification of high-yielding wheat genotypes resistant to *P. tritici-repentis*. In 2018, 27 genotypes (42% of those assessed) were insensitive to ToxA, and showed field resistance to the pathogen. In 2020, 20 advanced wheat lines (18% of those assessed) showed moderate to high levels of field resistance to tan spot, and these were selected and recommended for use in the resistance breeding (Kokhmetova and Atishova, 2020c). In 2021, 48 entries (27% of those assessed) with the lowest field assessed tan spot severities were confirmed to be insensitive o Ptr ToxA in the molecular screening. Entries which were resistant under field conditions had similar levels of seedling resistance. Of the 103 host entries evaluated, 28 can be directly used in breeding programmes to improve

Table 1. The frequency of occurrence of *P. tritici-repentis* races in Kazakhstan.

Years	Race								References
	1	2	3	4	5	6	7	8	
2001	+	+	-	-	-	-	-	-	Lamari *et al*., 2005b
2003–2004	+	+	+	+	-	-	-	-	Maraite *et al*., 2006
2013–2015	+	-	+	+	-	+	-	+	Kokhmetova *et al*., 2016b
2018	+	+	+	-	-	-	+	+	Kokhmetova *et al*., 2020b

tan spot resistance and productivity of winter wheat (Kokhmetova *et al*., 2021b).

Integrated plant disease management requires a combination of several strategies to effectively combat disease. For tan spot, the use of resistant wheat varieties is the best option to sustainably manage the disease. In addition, utilizing host resistance it is the most cost-effective and environmentally friendly method for disease control. To this end, the breeding of resistant wheat varieties should be a major objective for tan spot control, which should include assessment of germplasm disease susceptibility (Engle *et al*., 2006).

The objectives of the present study were; 1) to characterize race structure of *P. tritici-repentis* isolates recovered from wheat in south and north Kazakhstan, and 2) to identify the tan spot resistance in wheat cultivars based on disease phenotypes and molecular screening. The results of this study will provide knowledge for regional wheat breeders and plant pathologists involved in development of tan spot management strategies.

MATERIALS AND METHODS

Plant material and field disease phenotyping

This study assessed 80 winter wheat genotypes. These included: 13 cultivars (Almaly, Daulet, Egemen 20, Dana, Diana, Dinara, Krasnovodopadskaya 25, Krasnovodopadskaya 210, 2 Matay, President, Zhadyra, Zhetisu Pirotrix 50), 47 elite lines (10204_1KSI, 10204_2KSI, 10204_3KSI, 10205_2KSI, 10205_3KSI, 601_SP2, 605_SP2, 612_SP2, 620_SP2, 621_SP2, 624_SP2, 630_SP2, 631_SP2, 632_SP2, 634_SP2, 636_SP2, 637_SP2, 638_SP2, 640_SP2, GF_1_CP, GF_2_CP, GF_3_CP, GF_4_CP, GF_5_CP, GF_6_CP, GF_7_CP, GF_8_CP, GF_9_CP, GF_10_CP, 4_PSI, 9_PSI, 1_PSI, 2_PSI, 3_PSI, 5_PSI, 6_PSI, 7_PSI, 8_PSI, 602_SP2, 607_SP2, 609_SP2, 613_SP2, 618_SP2, 635_SP2, 633_SP2, 639_SP2, 10205_1KSI) from Kazakhstan, and 20 cultivars (Aragella, Priirtyshskaya, Danaya, Obskaya ozimaya, Veselka, Povolzhskaya-Niva, Darina, Bazis, Leonida, Turanus, Clavdiya 2, Italmas, Voronezhskaya 18, Kalixo, Streletskaya 12, Universiya, Likamero, Sonett Rima, Obskaya ozimaya) from Russia (Table 3).

Evaluation of adult plant resistance to *P. tritici-repentis* was carried out under field conditions at the Kazakh Research Institute of Agriculture and Plant Growing (KRIAPG), Almalybak (43°13'N, 76°36'E, 789 masl), Almaty Region in southeast Kazakhstan, during the growing seasons of 2019 and 2020. The experiments were completely randomized with three replicate plots. Individual plot size was 3 m^2 (3 m by 7 rows at 15 cm spacings). The source of infection within the field experiments was from naturally colonized wheat straw. In October, before sowing, the infected straw (1 kg m^{-2}) was incorporated into the soil. The growing seasons were favourable for pathogen infection and disease development. Mean daily temperature and relative humidity measurements showed similar trends in both years, although average temperatures were lower in the 2020 than in 2019 growing season. The average maximum air temperature for mid-May in 2019 was 18.6°C and in 2020 was 14.5°C. From April, May and June 2019, mean daily temperatures were, respectively, 11.4°C, 16.9°C and 22.3°C, and in 2020 were 14.0°C, 16.7°C and 21.6°C. For April, May and June 2019 the monthly rainfalls were, respectively, 168, 39 and 72 mm, and mean relative humidity (RH) was 84.13%. In April, May and June 2020, monthly rainfalls were, respectively, 140, 74 and 30 mm, and mean RH was 81.52%, (www. pogodaiklimat.ru/monitor.php, accessed 15 June, 2021). These climatic conditions were highly conducive for tan spot infection and development. Disease was assessed three times at ZGS 75–80 (Zadoks *et al.,* 1974), until maximum disease development was reached. The amounts of plant damage were evaluated as a percentage of leaf area occupied by tan spot. The foliar disease intensity scale of Saari and Prescott (1975), as modified for tan spot (Kremneva and Volkova, 2007) was used for these assessments. Wheat germplasm lines were classified into five groups according to tan spot severity as follows: resistant (R), 5–10%; moderately resistant (MR), 11–20%; moderately susceptible (MS), 21–30%; susceptible (S), 31%+, or immune (I),

0%. The cultivars Salamouni and Glenlea were included as, respectively, susceptible and resistant controls.

Wheat differential lines

Four hexaploid wheat genotypes (Glenlea, 6B662, 6B365, and Salamouni) were included as a differential set, which is effective for the differentiation of eight currently known races of *P. tritici-repentis* (Lamari *et al*., 2003). Seeds of each genotype were sown in 10 cm diam. plastic pots filled with the potting mix at six seeds per pot. The resulting seedlings were maintained in a growth cabinet at 20°C/18°C (day/night) with a 16 h daily photoperiod at 180 mmol m^{-2} s^{-1}, until they were inoculated at the two- to three-leaf stage. Seedlings were assessed 6 d after inoculation and were evaluated based on the development of necrosis or chlorosis or absence of symptoms.

Survey and fungal isolations

Surveys were carried out in the main wheat-growing regions of Kazakhstan during 2019 and 2020 cropping seasons. Each survey sample consisted of 40 leaves exhibiting typical tan spot symptoms, and these were collected randomly from wheat fields in south and north Kazakhstan. Several different wheat fields were surveyed in each region. In south Kazakhstan, 16 fields were surveyed (including disease monitoring in the Karasai, Talgar and Zhambyl regions), while in north Kazakhstan, six fields were surveyed (Karabalyk region). Wheat growth stages at the time of the survey ranged from the beginning of stem elongation (ZGS 30) to the milk stage (ZGS77) (Zadoks *et al*., 1974). Leaves showing symptoms of tan spot were carefully cut and placed in paper envelopes, which were left to air dry at room temperature. Fungal isolations and inoculum production were carried out as described by Lamari and Bernier (1989). Leaves were cut into 1 to 2 cm pieces, surface-sterilized with 30% alcohol for 20 sec then 1% sodium hypochlorite solution for 2 min, and then washed three times (1 min each), with sterile distilled water (Gilchrist-Saavedra *et al*., 2006). The tissue pieces were then placed in Petri dishes, each containing two layers of sterile filter paper moistened with sterile distilled water to maintain high humidity. The dishes were then kept in the dark and incubated for 24 h at 15°C to induce the formation of conidia on the tips of the conidiophores (Lamari and Bernier, 1989). After incubation, the leaf tissue pieces were examined using ×40 binocular magnifiers, and individual conidia identified as *P. tritici-repentis* were placed onto V8-PDA medium (150 mL of V8 juice, 10 g of Potato Dextrose Agar, 3 g of CaCO3, 10 g of water agar, and 850 mL of distilled water) and incubated at 20°C until colonies reached approx. 4 cm diam. A total of 186 single-conidium isolates of *P. tritici-repentis* were obtained, with 122 isolates recovered from south Kazakhstan and 64 from north Kazakhstan. These isolates were subsequently phenotypically characterized on the wheat differential set. A subset of 40 isolates was selected for further characterization (Table 2).

Inoculum production, inoculation, disease assessments and toxin infiltration

The *P. tritici-repentis* cultures were incubated on V8-PDA medium in the dark for 7 to 8 d at 20°C, until colonies reached approx. 4 cm diam. The cultures were then incubated for 24 h under light at room temperature (20–22°C), followed by 24 h at 15°C in the dark. Mycelium plugs (0.5 cm diam.) were then excised from the colonies and transferred singly to 9 cm diam. Petri dishes each containing 25 mL of V8-PDA. Conidia were then harvested by flooding the Petri dishes with sterile distilled water and dislodging the conidia with a wire loop. The inoculum concentration was adjusted to 3,000 conidia mL^{-1} (assessed with a hemocytometer), and a drop of Tween 20 was added per 100 mL to reduce surface tension in the conidium suspensions (Lamari and Barnier, 1989).

Wheat seedlings at the two-leaf stage were sprayed with conidium suspensions to run off, using a hand sprayer. Precautions were taken to avoid cross-infection of isolates. The inoculated seedlings were incubated in a dew chamber for 24 h at 20°C (day) and 18°C (night) with a 16 h daily photoperiod, and 90% relative humidity (Lamari *et al*., 2005b). The seedlings were evaluated for symptom development 7 d after inoculation. Tan spot severity was assessed using the 1 to 5 scale developed by Lamari and Bernier (1989), where: 1 = small, dark-brown to black spots, without any surrounding chlorosis or tan necrosis; 2 = small dark-brown to black spots, with very little chloroses or tan necroses; 3 = small, dark-brown to black spots, completely surrounded by distinct chlorotic or tan necrotic rings, not coalescing; 4 = small, dark-brown to black spots, completely surrounded by tanned chlorotic or necrotic zones, sometimes coalesced; and 5 = most lesions consisting of coalescing chlorotic or tan necrotic tissue. Seedlings with lesion types 1 to 2 were considered to be resistant (–), whereas those with scores of 3 to 5 were classified as susceptible to a given trait (+). For analyses, the seedlings were assigned the following binomials (+,–), (+,+), (–),

and (–,+) to indicate, respectively, the presence (+) or absence (–) of necrosis and chlorosis (Lamari and Bernier, 1991).

Infiltration with toxins was carried out on wheat seedlings at the two-leaf stage (Oralaza *et al*., 1995; Faris *et al*., 1996), which were grown in the conditions described above. The second leaf of each plant (three plants from each genotype) was infiltrated with 25 µL of the purified toxins Ptr ToxA or Ptr ToxB, using a 1 mL capacity syringe. Four leaves of each genotype were treated twice with the culture filtrate of each of the two toxins. The infiltrated plants were then placed in a growth chamber set at 21°C and 16 h daily photoperiod. Plants were evaluated 4 d after infiltration. The leaves of experimental control plants were each infiltrated with 25 µL of sterile distilled water. The leaves were evaluated as sensitive or insensitive to ToxA as presence/absence of necroses, or as sensitive or insensitive to ToxB as presence/absence of chlorosis, on the infiltrated side of each leaf (Faris *et al*., 1996).

Virulence was determined for 186 single conidium *P. tritici-repentis* isolates, which were obtained from infected plants collected from Kazakhstan wheat fields during the 2020 growing season. A total of 40 single conidium isolates were recovered and characterized (Table 2).

Identication of Tsn1 *and* Tsc2 *genes in wheat genotypes*

Genomic DNA was extracted from 5-d-old wheat seedlings using the CTAB method (Riede and Anderson, 1996). To identify the carriers of resistance genes, PCR protocols were used, with primers flanking diagnostic gene markers and DNA samples from the 80 wheat genotypes. Leaf samples from all entries, including the two reference cultivars, were genotyped with the SSR marker *Xfcp623* designed to detect alleles of the *Tsn1* gene. The primers and PCR conditions corresponded to those of Faris *et al*. (2010). The marker had two alleles: 380 bp (the dominant allele of the *Tsn1* gene linked to sensitivity) and the null allele (the recessive allele of the *tsn1* gene linked to insensitivity to Ptr ToxA) (Zhang *et al*., 2009). The sequence of primers for the *Xfcp623* marker (5'–3') were F – CTATTCGTAATCGTGCCTTCCG; R - CCTTCTCTCTCACCGCTATCTCATC (Faris *et al*., 2010), and the *XBE444541* – STS marker for the *Tsc2* locus sensitive to Ptr ToxB. The marker has two alleles: 340 bp (the dominant allele of the *Tsc2* gene linked to sensitivity to the Ptr ToxA) and 505 bp (recessive allele of the *tsc2* gene linked to resistance to the Ptr ToxB). Sequence of primers for marker *XBE444541* (5'–3') were F – TGGACCAGTATGAGA; R – TTCTGGAGGATGTTGAGCAC (Abeysekara *et al*., 2010). PCR reactions were carried out in a T100TM Thermal Cycler (Bio-Rad). Each PCR mixture (25 µL) contained 2.5 µL of genomic DNA (30 ng), 1 µL of each primer (1 pM μL^{-1}) (Sigma Aldrich), 2.5 µL of dNTP mixture (2.5 mM, dCTP, dGTP, dTTP and dATP aqueous solution) (ZAO), 2.5 µL $MgCl_2$ (25 mM), 0.2 µL Taq polymerase (5 units μL^{-1}) (ZAO), 2.5 µL 10× PCR buffer and 12.8 µL ddH_20. PCR amplification was performed with a Mastercycler (Eppendorf), with initial denaturation at 94°C for 3 min, 45 cycles: 94°C for 1 min, annealing at 60°C for 1 min, 72°C for 2 min, and final elongation at 72°C for 10 min. The amplification products were separated on 2% agarose gel in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8) (Chen *et al*., 1998) with the addition of ethidium bromide. A 100-bp DNA ladder (Fermentas) was included to determine amplification lengths. Results were visualized using the Gel Documentation System (Gel Doc XR, BioRad).

RESULTS

Race characterization

The majority of the tested isolates from south Kazakhstan (21.2%) induced chlorosis on the wheat differential line 6B365, but lines 6B662 and "Glenlea" each exhibited a resistant reaction, symptoms typical for race 3 of *P. tritici-repentis*. In these isolates, race 1 (15.1%), race 4 (12.1%), race 5 (6.1%), race 6 (10.0%), race 7 (18.2%) and race 8 (18.2%) were also identified in 2020. Analysis of the virulence of isolates from north Kazakhstan in 2020 showed that they belonged to two races, race 4 (71.4%) and race 7 (28.6%) (Table 2).

Fifty-six wheat genotypes, representing 70% of those assessed, showed insensitivity to both race 1 and race 5 of *P. tritici-repentis*. The most interesting were the 15 entries GF_1_CP, GF_2_CP, GF_5_SP2, GF_6_SP2, GF_7_SP2, GF_10_CP, 10204_3_KSI, 10205_1_KSI, 10205_2_KSI, 601_SP2, 620_SP2, 624_SP2 and 640_SP2 from the Kazakhstan collection and the three entries Danaya, Povolozhskaya Niva, and Darina) from the Russian collection, which were insensitive to the pathogen (severity scores 1 to 1.6), to two races, and to the two toxins (Ptr ToxA and Ptr ToxB).

The purpose of genotyping wheat genotypes using a molecular marker was to identify carriers of genes that control sensitivity to the toxins. The *Xfcp623* marker amplified a fragment of 380 bp associated with the *Tsn1* gene, which demonstrates host sensitivity to the toxin. The results of genotyping with marker *Xfcp623* are presented in Table 3.

Table 2. Reactions of differential *Triticum aestivum* genotypes to inoculation with 40 *Pyrenophora tritici-repentis* isolates collected from Kazakhstan in 2020.

Isolate	Geographic origin	Reaction of differential genotypes to the PTR inoculation			Race number
		Glenlea	6B365	6B662	
KZ-29-S-2020	Almalybak, Almaty oblast.	N	C	R	1
KZ-30-S-2020	Almalybak, Almaty oblast.	N	C	R	1
KZ-7-S-2020	Almalybak, Almaty oblast.	N	C	R	1
KZ-28-S-2020	Almalybak, Almaty oblast.	N	C	R	1
KZ-40-S-2020	Almalybak, Almaty oblast.	N	C	R	1
KZ-23-S-2020	Almalybak, Almaty oblast.	R	C	R	3
KZ-24-S-2020	Almalybak, Almaty oblast.	R	C	R	3
KZ-26-S-2020	Almalybak, Almaty oblast.	R	C	R	3
KZ-31-S-2020	Almalybak, Almaty oblast.	R	C	R	3
KZ-32-S-2020	Almalybak, Almaty oblast.	R	C	R	3
KZ-33-S-2020	Almalybak, Almaty oblast.	R	C	R	3
KZ-34-S-2020	Almalybak, Almaty oblast.	R	C	R	3
KZ-5-S-2020	Almalybak, Almaty oblast.	R	R	R	4
KZ-1-N-2020	Karabalyk agricultural experimental station, Kostanay oblast	R	R	R	4
KZ-2-N-2020	Karabalyk agricultural experimental station, Kostanay oblast	R	R	R	4
KZ-5-N-2020	Karabalyk agricultural experimental station, Kostanay oblast	R	R	R	4
KZ-6-N-2020	Karabalyk agricultural experimental station, Kostanay oblast	R	R	R	4
KZ-8-N-2020	Karabalyk agricultural experimental station, Kostanay oblast	R	R	R	4
KZ-21-S-2020	Almalybak, Almaty oblast.	R	R	R	4
KZ-22-S-2020	Almalybak, Almaty oblast.	R	R	R	4
KZ-27-S-2020	Almalybak, Almaty oblast.	R	R	R	4
KZ-3-S-2020	Almalybak, Almaty oblast.	R	R	C	5
KZ-41-S-2020	Almalybak, Almaty oblast.	R	R	C	5
KZ-4-S-2020	Almalybak, Almaty oblast.	R	C	C	6
KZ-25-S-2020	Almalybak, Almaty oblast.	R	C	C	6
KZ-39-S-2020	Almalybak, Almaty oblast.	R	C	C	6
KZ-1-S-2020	Almalybak, Almaty oblast.	N	R	C	7
KZ-2-S-2020	Almalybak, Almaty oblast.	N	R	C	7
KZ-6-S-2020	Almalybak, Almaty oblast.	N	R	C	7
KZ-47-S-2020	Almalybak, Almaty oblast.	N	R	C	7
KZ-8-S-2020	Almalybak, Almaty oblast.	N	R	C	7
KZ-9-S-2020	Almalybak, Almaty oblast.	N	R	C	7
KZ-3-N-2020	Karabalyk agricultural experimental station, Kostanay oblast	N	R	C	7
KZ-4-N-2020	Karabalyk agricultural experimental station, Kostanay oblast	N	R	C	7
KZ-11-S-2020	Almalybak, Almaty oblast.	N	C	C	8
KZ-12-S-2020	Almalybak, Almaty oblast.	N	C	C	8
KZ-35-S-2020	Almalybak, Almaty oblast.	N	C	C	8
KZ-36-S-2020	Almalybak, Almaty oblast.	N	C	C	8
KZ-37-S-2020	Almalybak, Almaty oblast.	N	C	C	8
KZ-38-S-2020	Almalybak, Almaty oblast.	N	C	C	8

The field evaluations of resistance of 80 wheat genotypes tan spot showed that five lines or cultivars were immune (severity = 0%), and 44 (55%) were resistant (severity = 5–10%). wheat genotypes. The five genotypes identified with immunity to tan spot were GF_2_CP, GF_10_CP, 637_SP2, Matay and President. Table 3 presents average field assessment data for 2018, 2019 and 2020. These field tan spot evaluation results allowed the genotype levels of resistance to be assessed.

Table 3. Reactions of wheat genotypes to *Pyrenophora tritici-repentis* races 1 and 5, and the toxins ToxA and ToxB, in molecular screening and field evaluations.

Wheat genotype	Geographic origin	*Xfcp623, Tsn1*	*XBE444541, Tsc2*	Response to isolates of races and HST toxins				Tan spot field evaluation %
				Race 1 #KZ-7-S-6	Ptr ToxA	Race 5 #KZ-41-N-2019	Ptr ToxB	
GF_2_CP	KZ	*tsn1*	*tsc2*	1.0	I	1.3	I	0
GF_10_CP	KZ	*tsn1*	*tsc2*	1.0	I	1.1	I	0
637_SP2	KZ	*tsn1*	*tsc2*	2.3	I	2.3	I	0
Matay	KZ	*tsn1*	*tsc2*	2.3	I	2.3	I	0
President	KZ	*Tsn1*	*tsc2*	3.3	S	1.0	I	0
GF_6_CP	KZ	*tsn1*	*tsc2*	1.0	I	1.2	I	5
GF_7_CP	KZ	*tsn1*	*tsc2*	1.0	I	1.1	I	5
GF_9_CP	KZ	*Tsn1*	*tsc2*	3.2	S	1.0	I	5
10204_2KSI	KZ	*tsn1*	*tsc2*	2.0	I	2.2	I	5
10204_3KSI	KZ	*tsn1*	*tsc2*	1.0	I	1.1	I	5
601_SP2	KZ	*tsn1*	*tsc2*	1.0	I	1.0	I	5
624_SP2	KZ	*tsn1*	*tsc2*	1.0	I	1.2	I	5
630_SP2	KZ	*tsn1*	*tsc2*	2.2	I	2.0	I	5
631_SP2	KZ	*Tsn1*	*tsc2*	3.5	S	2.3	I	5
Zhadyra	KZ	*Tsn1*	*tsc2*	3.2	S	1.0	I	5
Zhetisu	KZ	*tsn1*	*tsc2*	2.2	I	2.3	I	5
GF_1_CP	KZ	*tsn1*	*tsc2*	1.0	I	1.0	I	10
GF_4_CP	KZ	*tsn1*	*Tsc2*	2.0	I	3.5	S	10
GF_5_CP	KZ	*tsn1*	*tsc2*	1.0	I	1.1	I	10
GF_8_CP	KZ	*tsn1*	*tsc2*	2.1	I	2.1	I	10
4_PSI	KZ	*tsn1*	*tsc2*	2.2	I	2.1	I	10
9_PSI	KZ	*Tsn1*	*tsc2*	3.3	S	1.2	I	10
10204_1KSI	KZ	*tsn1*	*tsc2*	2.1	I	2.2	I	10
10205_2KSI	KZ	*tsn1*	*tsc2*	1.0	I	1.1	I	10
10205_3KSI	KZ	*Tsn1*	*tsc2*	3.2	S	1.1	I	10
605_SP2	KZ	*tsn1*	*Tsc2*	2.2	I	2.5	I	10
612_SP2	KZ	*tsn1*	*Tsc2*	2.2	I	3.3	S	10
620_SP2	KZ	*tsn1*	*tsc2*	1.0	I	1.2	I	10
621_SP2	KZ	*tsn1*	*tsc2*	2.1	I	2.2	I	10
632_SP2	KZ	*tsn1*	*tsc2*	2.1	I	2.3	I	10
634_SP2	KZ	*Tsn1*	*tsc2*	3.2	S	1.0	I	10
636_SP2	KZ	*tsn1*	*tsc2*	2.2	I	2.2	I	10
638_SP2	KZ	*tsn1*	*tsc2*	2.2	I	2.4	I	10
640_SP2	KZ	*tsn1*	*tsc2*	1.0	I	1.1	I	10
Dana	KZ	*tsn1*	*tsc2*	2.2	I	2.2	I	10
Diana	KZ	*tsn1*	*tsc2*	2.2	I	2.2	I	10
Krasnovodopadskaya 210	KZ	*tsn1*	*tsc2*	1.0	I	1.1	I	10
Aragella	RU	*tsn1*	*tsc2*	2.3	I	2.5	I	10
Priirtyshskaya	RU	*tsn1*	*tsc2*	2.1	I	2.1	I	10
Danaya	RU	*tsn1*	*tsc2*	1.0	I	1.6	I	10
Obskaya ozimaya	RU	*Tsn1*	*tsc2*	3.2	S	1.3	I	10
Veselka	RU	*tsn1*	*Tsc2*	2.1	I	3.2	S	10
Povolzhskaya-Niva	RU	*tsn1*	*tsc2*	1.0	I	1.2	I	10
Darina	RU	*tsn1*	*tsc2*	1.0	I	1.5	I	10
Bazis	RU	*Tsn1*	*tsc2*	3.6	S	1.6	I	10
Leonida	RU	*tsn1*	*tsc2*	2.3	I	2.3	I	10

(Continued)

Table 3. (Continued).

Wheat genotype	Geographic origin	*Xfcp623, Tsn1*	*XBE444541, Tsc2*	Response to isolates of races and HST toxins				Tan spot field evaluation %
				Race 1 #KZ-7-S-6	Ptr ToxA	Race 5 #KZ-41-N-2019	Ptr ToxB	
Turanus	RU	*tsn1*	*tsc2*	2.2	I	2.5	I	10
Clavdiya 2	RU	*tsn1*	*tsc2*	2.3	I	2.0	I	10
Italmas	RU	*tsn1*	*Tsc2*	1.0	I	3.5	S	10
Voronezhskaya 18	RU	*Tsn1*	*tsc2*	3.3	S	1.3	I	15
Kalixo	RU	*Tsn1*	*tsc2*	3.4	S	1.1	I	15
Streletskaya 12	RU	*tsn1*	*tsc2*	2.2	I	2.1	I	15
Universiya	RU	*tsn1*	*tsc2*	1.0	I	2.0	I	15
7_PSI	KZ	*tsn1*	*tsc2*	2.2	I	2.1	I	20
633_SP2	KZ	*Tsn1*	*tsc2*	3.4	S	1.3	I	20
Dinara	KZ	*tsn1*	*tsc2*	2.1	I	2.1	I	20
Krasnovodopadskaya 25	KZ	*tsn1*	*tsc2*	1.0	I	2.3	I	20
Pirotrix 50	KZ	*tsn1*	*tsc2*	2.2	I	2.3	I	20
GF_3_CP	KZ	*tsn1*	*tsc2*	1.0	I	2.2	I	25
3_PSI	KZ	*tsn1*	*tsc2*	2.4	I	2.0	I	25
5_PSI	KZ	*tsn1*	*tsc2*	2.2	I	2.1	I	25
6_PSI	KZ	*tsn1*	*tsc2*	2.1	I	2.1	I	25
8_PSI	KZ	*tsn1*	*tsc2*	2.3	I	2.0	I	25
10205_1KSI	KZ	*tsn1*	*tsc2*	1.0	I	1.1	I	25
609_SP2	KZ	*tsn1*	*tsc2*	1.0	I	2.2	I	25
613_SP2	KZ	*tsn1*	*tsc2*	2.3	I	2.2	I	25
635_SP2	KZ	*tsn1*	*tsc2*	2.2	I	2.0	I	25
Likamero	RU	*Tsn1*	*tsc2*	3.4	S	2.2	I	25
Sonett	RU	*Tsn1*	*tsc2*	3.3	S	2.1	I	25
1_PSI	KZ	*tsn1*	*tsc2*	2.3	I	2.2	I	30
2_PSI	KZ	*tsn1*	*tsc2*	2.5	I	2.0	I	30
602_SP2	KZ	*Tsn1*	*tsc2*	2.8	S	2.3	I	30
607_SP2	KZ	*tsn1*	*Tsc2*	2.1	I	2.2	I	30
618_SP2	KZ	*Tsn1*	*tsc2*	3.0	S	1.1	I	30
639_SP2	KZ	*Tsn1*	*Tsc2*	3.3	S	3.4	S	30
Daulet	KZ	*tsn1*	*tsc2*	1.0	I	1.2	I	30
Almaly	KZ	*Tsn1*	*tsc2*	2.3	I	2.2	I	30
Rima	RU	*Tsn1*	*Tsc2*	3.5	S	2.0	I	30
Obskaya ozimaya 2	RU	*Tsn1*	*tsc2*	3.6	S	1.4	I	30
Egemen 20	KZ	*Tsn1*	*tsc2*	3.3	S	2.2	I	40
Salamouni	Lebanon	*tsn1*	*tsc2*	1.0	I	1.0	I	5
Glenlea	Canada	*Tsn1*	-	3.8	S	1.0	I	40
6B662	Unknow	-	*Tsc2*	-	-	3.8	S	35

Notes: KZ: Kazakhstan; RU: Russia; *Xfcp623* is the SSR marker to the *Tsn1* locus sensitive to Ptr ToxA, amplifies a 380 bp DNA fragment; *XBE444541,* the STS marker to the *Tsc2* locus, amplifies a 340 bp DNA fragment in wheat entries sensitive to ToxB and 505 bp in insensitive; Salamouni, the insensitive control for races 1 and 5, toxins Ptr ToxA, and Ptr ToxB, carrier of the recessive genes *tsn1* and *tsc2*; Glenlea, the susceptible control for race 1 and Ptr ToxA, carrier of the dominant *Tsn1* gene; 6B662, susceptible control for race 5 and Ptr ToxB, carrier of the dominant *Tsc2* gene. Lesion types 1–5 based on the Lamari and Bernier's scale (1989); 1–2 indicates resistance, and 3–5, susceptibility. The reaction to toxin infiltration: I, insensitivity; S, susceptibility. Tan spot field evaluation Ptr, % based on the intensity scale of Kremneva and Volkova, 2007. Wheat germplasm was classified into five groups according to tan spot severity as follows: resistant (R): 5–10%, moderately resistant (MR): 11–20%, moderately susceptible (MS): 21–30%, susceptible (S): 31%+ and Immune (I):0%.

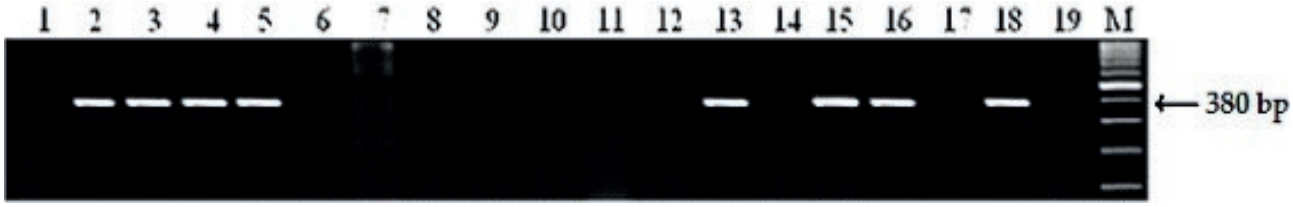

Figure 1. DNA amplification products for wheat cultivars and elite lines obtained with diagnostic marker *Xfcp623* linked to the *Tsn1* gene sensitive to Ptr ToxA. Lane: 1, GF_3_CP; 2, GF_9_CP; 3, 10205_3KSI; 4, 631_SP2; 5, 634_SP2; 6, 1_PSI; 7, Daulet; 8, Dinara; 9, Streletskaya 12; 10, Pirotrix 50; 11, Zhetisu; 12, Aragella; 13, President; 14, Matay; 15, Voronezhskaya 18; 16, Sonett; 17, Salamouni (resistant reference cultivar for race 1, insensitive to Ptr ToxA, with recessive gene *tsnl*); 18, Glenlea (susceptible reference cultivar for race 1, sensitive to Ptr ToxA, with dominant gene *Tsnl*); 19, ddH_2O; M, molecular weight marker (Gen-RulerTM; 100 bp DNA Ladder). Fragments amplified by *Xfcp623* were separated in 2% agarose gels. The bands are 380 bp for the *Tsn1* allele (lanes 2, 3, 4, 5, 13, 15, 16 and 18, control), sensitive to Ptr ToxA; and null allele for the *tsn1* allele, insensitive to Ptr ToxA (lanes 1, 6, 7, 8, 9, 10, 11, 12, 14 and 17 control).

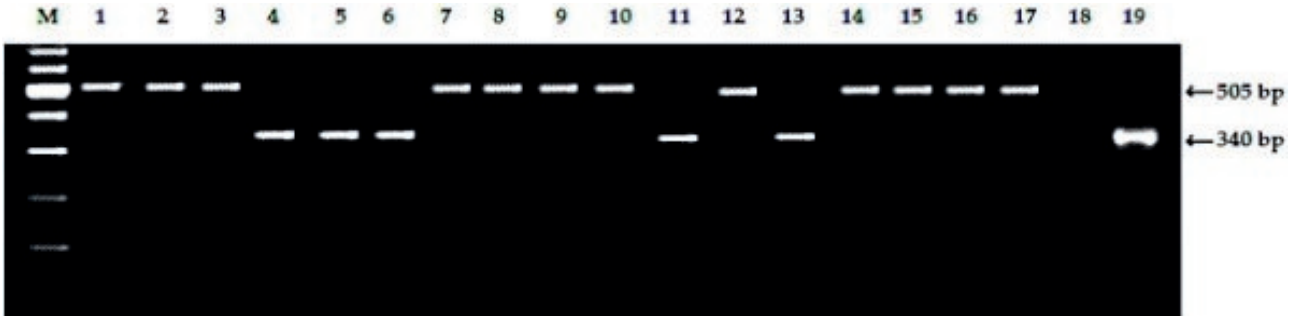

Figure 2. DNA amplification products for wheat cultivars and elite lines obtained with diagnostic marker *XEE444541* linked to the *Tsc2* gene sensitive to Ptr ToxB. Lane: M, molecular weight marker (Gen-RulerTM; 100 bp DNA Ladder), 1, GF_1_CP; 2, GF_10_CP; 3, 609_SP2; 4, GF_4_CP; 5, 612_SP2; 6, 639_SP2; 7, Egemen 20; 8, Almaly; 9, Danaya; 10, 601_SP2; 11, Veselka; 12, Dinara; 13, Italmas; 14, Zhadyra; 15, Pirotrix 50; 16, 640_SP2; 17, 10204_1KSI; 18, ddH_2O; 19, 6B662 (susceptible reference genotype for race 5, sensitive to Ptr ToxB with dominant gene *Tsc2*). Fragments amplified by *XEE444541* were separated in 2% agarose gels. The bands are 340 bp for the *Tsc2* allele (lanes 4, 5, 6, 11, 13 and 19), sensitive to Ptr ToxB and null allele for the *tsc2* allele, insensitive to Ptr ToxB (lanes 1, 2, 3, 7, 8, 9, 10, 12, 14, 15, 16 and 17).

The proportion of genotypes insensitive to Ptr ToxA (*tsn1*) was high, with 59 of the 80 tested genotypes insensitive to the toxin. The genotypes included 47 from Kazakhstan (78%) and 12 from Russia (60%) (Table 3). Examples of PCR results for 18 host genotypes are shown in Figure 1. Seven genotypes (GF_9_CP, 10205_3KSI, 631_SP2, 634_SP2, President, Voronezhskaya 18, and Sonett) had 380 bp fragments, indicative of the dominant *Tsn1* allele conferring toxin Ptr ToxA sensitivity. Nine genotypes (GF_3_CP, 1_PSI, Daulet, Dinara, Streletskaya 12, Pirotrix 50, Zhetisu, Aragella, and Matay) gave no amplification products (null allele), indicative of the recessive tsn1 allele conferring insensitivity to the toxin Ptr ToxA (Figure 1).

The *XBE444541* marker amplified a 340 bp fragment linked to the *Tsc2* allele, which controls sensitivity to the toxin in eight wheat entries (GF_4_CP, 605_SP2, 607_SP2, 612_SP2, 639_SP2, Rima, Veselka, Italmas and in control 6B662). Twelve host genotypes (GF_1_CP, GF_10_SP, 609_SP2, Egemen, Almaly, Danaya, 601_SP2, Dinara, Zhadyra, Pirotrix 50, 640_SP2, 10204_1KSI) had the amplification product (505 bp) indicative of the recessive *tsc2* allele, conferring toxin Ptr ToxB insensitivity (Figure 2). These 12 genotypes all showed insensitivity to race 5 and Ptr ToxB toxin when screened using the race 5 isolate and HST Ptr ToxB infiltrate. In general, the proportion of the examined genotypes insensitive to Ptr ToxB was high, at 72 of the 80 tested genotypes. The degree of linkage of the marker *XBE444541* with insensitivity to race 5 and Ptr ToxB was 90% (Table 3).

Identification of genotypes resistant to *P. tritici-repentis* was based on the results of molecular analyses, screening of the wheat genotypes for reaction to races 1 and 5 of the pathogen, as well as reactions to the toxins Ptr ToxA and Ptr ToxB. The reactions of wheat genotypes to isolates of races and Ptr ToxA and Ptr ToxB are presented in Table 3. In general, the frequency of genotypes resistant to race 1 and race 5 in the wheat collection was high at 70%. Eighteen wheat entries presented the were the most resistant. These were: GF_1_CP,

GF_2_CP, GF_5_CP, GF_6_CP, GF_7_CP, GF_10_CP, 10204_3KSI, 10205_1KSI, 10205_2KSI, 601_SP2, 620_SP2, 624_SP2, 640_SP2, Daulet, Krasnovodopadskaya 210, Danaya, Povolzhskaya-Niva, and Darina. These host lines showed insensitivity (scores 1–1.6 point) to both races of the pathogen and to two toxins (Ptr ToxA and Ptr ToxB), and were also insensitive to the toxins as indicated in the molecular screening (Table 3). A moderate degree of insensitivity to pathogen races and the toxins was observed in 38 wheat entries. Susceptibility to race 1 and ToxA was found in twenty host genotypes (25%), including twelve Kazakh lines and eight Russian cultivars. Susceptibility to race 5 and ToxB was detected in only five of the host genotypes.

DISCUSSION

The race population structure of *P. tritici-repentis* in Kazakhstan has had large fluctuations in recent years (Kokhmetova *et al*., 2016b., Kokhmetova *et al*., 2020b). Population structure and race composition of the pathogen has been studied in many geographic regions in the world. In North American pathogen collections, Lamari *et al*. (1995) first identified races 1 to 4, with prevailing races 1 and 2 (Lamari *et al.,*1998). Later, these races were identified in mainly wheat growing regions. Race 1 was identified in Azerbaijan, Kyrgyzstan, Kazakhstan, Uzbekistan and Syria, and race 2 was found in Azerbaijan and Kazakhstan and in South America (Lamari *et al.,* 2005a, Kokhmetova *et al.,* 2018b, Kokhmetova *et al.,* 2019a, Gamba *et al.,* 2012). Studies conducted in 2016 to determine the racial composition of the pathogen in Kazakhstan showed that races 1 and 8 were dominant (Kokhnetova *et al*., 2016b). Benslimane *et al.* (2011) showed that six PTR races were identified in Algeria (races 1, 4, 5, 6, 7 and 8). Four of these (races 1, 4, 7 and 8) are described in Algeria for the first time. Lamari *et al.* (1998) were the first to report race 5 in Algeria, and this race has since been reported in Canada (Strelkov *et al*., 2002), the United States of America (Ali *et al*.,1999), Syria and Azerbaijan (Lamari *et al*., 2005b). In contrast, race 6 was found in Algeria and Morocco (Strelkov *et al*., 2002; Benslimane, 2018; Gamba *et al*., 2017), while races 7 and 8 were found only in the Middle East, Caucasus and Algeria, and Kazakhstan in 2018 and 2020 (Kokhmetova *et al*., 2020b; Benslimane, 2018, Ouaar *et al*., 2022). In 2021, in the North Caucasus region of Russia, races 1, 3 and 4 were identified (Kremneva *et al*., 2021). Races 2, 4, 5 and 7 were found in Tunisia by Kamel *et al*. (2019).

Studies carried out in 2018 on reaction of wheat germplasm to inoculation and toxin infiltration made it possible to identify more than 78% of entries that are simultaneously resistant to *P. tritici-repentis* races 1 and 5 and to the Ptr ToxA and Ptr ToxB (Kokhmetova *et al*., 2018b). In previous studies in Kazakhstan in 2019, the present authors found positive correlations between seedling and field scores (Kokhmetova *et al.,* 2019b).

Races 1 and 8 were predominant in 2016 in isolates from southeast Kazakhstan. (Kokhmetova *et al.,* 2016b). In 2018, five races of *P. tritici-repentis* were identified in Kazakhstan, including races 1, 2, 3, 7 and 8 (Kokhmetova *et al.,* 2020b). The results from the present study indicate the presence of seven races, 1, 3, 4, 5, 6, 7 and 8 in this country. Race 2, found in the 2018 studies, was absent, but additional races 4, 5 and 6 were found. These differences in *P. tritici-repentis* population structure in Kazakhstan indicate the need for annual monitoring, and study of the distribution of tan spot. This would enhance understanding of the dynamics of variability and distribution of *P. tritici-repentis* and the disease this pathogen causes.

In 2020 most of the wheat cultivars from Kazakhstan (72.6%) showed sensitive responses to race 1 of *P. tritici-repentis*, while 67.5% of the lines were resistant to race 5. As a result of this study, 25 lines with the best combinations of SNP alleles associated with resistance to races 1 and 5 were identified, for use as candidates for future wheat variety selection and release (Kokhmetova *et al.,*2021a).

In the present study, a collection of 80 common wheat accessions, including promising lines and cultivars from Kazakhstan and Russia, were evaluated for reaction to race 1 and 5 of *P. tritici-repentis*, and to Ptr ToxA, and Ptr ToxB, and were characterized using the *Xfcp623* and *XBE444541* molecular markers diagnostic for the *Tsn1* and *Tsc2* genes. The *XBE444541* marker amplified a 340 bp fragment linked to the *Tsc2* allele, which controls sensitivity to the toxin in eight wheat entries. However, the race 5 isolate did not always cause chlorosis in wheat genotypes, for which the presence of a dominant allele of the *Tsc2* gene, sensitive to Ptr ToxB, was assumed. Thus, a resistant reaction to race 5 and the Ptr ToxB, instead of the expected susceptible reaction, was found in the wheat lines 605_SP2 and 607_SP2, and in Rima. This is consistent with the results of a number of studies on the interaction of genes *Tsn1* and *Tsc2* and toxins of *P. tritici-repentis*, where it has been shown that sensitivity to toxins does not always determine sensitivity to tan spot and depends on the genetic background of the host, i.e., on a specific wheat genotype (Chu *et al.,* 2008, Kariyawasam *et al*., 2016). Zhang *et al.* (2009) have also observed differential responses to toxins and conidium inoculations. Durum and common wheat breed-

ers alike should strive to remove both Tsc1 and Tsc2 from their materials, using marker-assisted selection to achieve disease resistance (Virdi *et al.,* 2016).

From a disease management point of view, 18 wheat entries were shown to have resistance to races 1 and 5 of *P. tritici-repentis*, and confirmed resistance to Ptr ToxA in molecular screening. These include fifteen wheat cultivars from Kazakhstan and three from Russia. Susceptibility to Ptr ToxA did not always correlate with susceptibility to race 1 of the pathogen, and depended on the genetic background of the hosts. In the previous study, 19 winter wheat entries were highly resistant to race 1 and resistant under field conditions, so it is recommended that these genotypes are used to deploy resistance genes in wheat breeding programmes (Kokhmetova *et al*., 2021b). Evolution of virulence involves the generation of genetic variation, followed by selection. Genetic variation arises by mutation, chromosomal rearrangement, recombination, and inter- and intra-species hybridization (Burnett, 2003).

The present study has shown the prevalence of a diverse population of *P. tritici-repentis* in regions of Kazakhstan. Differences in results in regions may depend on wheat varietal characteristics and climatic conditions, which differed in each region. The obtained data indicate that annual studies should continue to recognize the population dynamics of *P. tritici-repentis*, as well race distribution areas. The pathogen should also be periodically monitored for any virulence changes. The identification of six *P. tritici-repentis* races on wheat demonstrates high diversity of the pathogen population in Kazakhstan, which requires further in-depth characterization. The results of genotyping and screening of wheat entries for resistance to the most common races of *P. tritici-repentis* in Kazakhstan will increase efficiency of breeding, based on the elimination of carriers of dominant alleles of the *Tsn1* gene, which provides sensitivity to the aggressive toxin Ptr ToxA toxin from breeding material. Carriers of the identified *tsn1* gene for resistance to Ptr ToxA can be used in breeding programmes for pyramiding of genes for resistance to wheat diseases.

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AUTHOR CONTRIBUTIONS

AK and MK conceived the manuscript and designed the research. MK and NK analyzed the data and wrote the manuscript. MA, KA, NM and ZhK generated the phenotypic and genotyping data. All authors reviewed the manuscript.

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Research Papers

A real time loop-mediated isothermal amplification (RealAmp) assay for rapid detection of *Pleurostoma richardsiae* in declining olive plants

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Summary. *Pleurostoma richardsiae* is associated with host trunk diseases, known to cause dieback, cankers and wilting of woody trees, and human infections. This fungus was isolated from wood tissues of declining olive trees and grapevines showing esca disease symptoms, in the Apulia region of Italy. Fungus detection has been based on morphological and molecular features, which are time-consuming to identify and require well-trained personnel. Improvement of *Pl. richardsiae* detection in olive was achieved through development of real time loop-mediated isothermal amplification targeting the intergenic spacer (IGS) region of the fungus. Specificity of the assay was confirmed using ten *Pl. richardsiae* strains and 36 other fungus strains of species usually isolated from declining olive trees. The achieved limit of detection was 7.5×10^{-2} ng μL^{-1} of *Pl. richardsiae* genomic DNA. A preliminary validation of RealAmp was also performed using material from infected olive plants artificially inoculated in a greenhouse.

Keywords. IGS, molecular assay, olive decline, fungi.

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INTRODUCTION

General decline of olive trees, similar to olive quick decline caused by *Xylella fastidiosa* (Saponari *et al.*, 2013), was observed in olive orchards in Barletta-Andria-Trani (BAT) and Foggia provinces of the Apulia region of Southern Italy, which is not in the demarcated area infected by *X. fastidiosa* (Commission Implementing Decision EU 2017/2352 https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32017D2352&from=IT). Typical symptoms were wilting of apical shoots, dieback of twigs and branches, and

brown streaking under the bark of the trunks of affected trees. According to Carlucci *et al.* (2013), three fungal species, *Pleurostoma richardsiae, Neofusicoccum parvum* and *Phaeoacremonium minimum*, were strongly associated with these symptoms, and were diagnosed through morphological, molecular and pathogenicity tests. *Phaeoacremonium* spp. and *Botryosphaeriaceae* spp. are known to cause dieback, cankers and wilting on olive and grapevine plants (Carlucci *et al*., 2015c; 2015b). *Pleurostoma richardsiae* was isolated and associated for the first time in Italy from brown wood streaking and canker of olive (Carlucci *et al.,* 2013) and grapevine plants (Carlucci *et al.,* 2015a), confirming its pathogenicity. Further reports have associated this pathogen with olive and grapevine plants. Pintos Varela *et al.* (2016), Özben *et al.* (2017) and Canale *et al.* (2019) confirmed *Pl. richardsiae* as a grapevine pathogen in Spain, Turkey and Brazil, while Ivic *et al.* (2018), Canale *et al.* (2019), Agustì-Brisiach *et al.* (2021), Lawrence *et al.* (2021) and Van Dyk *et al.* (2021) confirmed this fungus as an olive pathogen in Croatia, Brazil, Spain, California and South Africa.

Pleurostoma richardsiae is also known as an uncommon cause of human infections, usually through traumatic skin inoculations with contaminated vegetable matter, causing subcutaneous Phaeohyphomycosis (Pitrak *et al.,* 1988; Vijaykrishna *et al*., 2004).

Identification of *Pl. richardsiae* has been based on micromorphological and cultural features (Schol-Schwarz, 1970; Domsch *et al*., 1980; De Hoog *et al*., 2000; Vijaykrishna *et al*., 2004). Molecular tools targeting the 5.8S rDNA gene flanking internal transcribed spacers 1 and 2 (ITS) have more recently been used (Carlucci *et al*., 2015c; Vijaykrishna *et al.,* 2015; Spies *et al*., 2020). The polymerase chain reaction is an efficient technique for the detection of *Pl. richardsiae*, although the DNA extraction phase and the thermal cycle requirements for the Taq polymerase function are constraints in terms of costs and time for the application of this technique at large scale. These limitations can be overcome by the loop-mediated isothermal amplification (LAMP) assay, which relies on partial DNA extraction and isothermal polymerase to amplify the DNA (Notomi *et al.,* 2000). LAMP is a technique that uses six primers that are complementary to eight regions of genomic DNA, and therefore ensures high specificity and allows results to be obtained more rapidly than PCR or qPCR. In addition, the isothermal character of the reaction requires only a heat block or water bath at the target temperature to successfully amplify DNA. Bst polymerase used in LAMP assays allows a full reaction to take place in less than 1 h in an isothermal environment (ranging from 60 to 70°C) depending on the type of enzyme and the optimal temperature for the primer characteristics (Notomi *et al*., 2000).

LAMP reaction can be detected in end point, as resolving amplified products on agarose gel electrophoresis (Notomi *et al*., 2000), by eye observation of precipitate (Mori *et al*., 2001), or employing DNA-binding dyes (Le *et al*., 2012) or colorimetric indicators (Parida *et al*., 2008; Tomita *et al*., 2008). End point detection strategies do not allow real-time diagnoses. They may result in false positives detections due to contamination of samples (e.g., reaction tube opening procedure). In addition, determination of the LAMP reaction by visual observation is subjective, because it is based on individual perception of colour (Bista *et al*., 2007). These problems can be overcome by real-time LAMP reactions (RealAmp), that can also be performed *in situ* using Smart-DARTTM (Diagenetix), a portable device equipped with D&A software that can analyze eight samples each time. This is a major advantage of RealAmp for the rapid detection of pathogens in plant material, in the field and at entry points and nurseries, avoiding risks of pathogen spread through the movement of infected material (Yaseen *et al*., 2015). This technique has also been applied in the field and laboratory for diagnoses of human and animal diseases and food safety control (Abdulmawjood *et al*., 2014).

LAMP assays have been extensively developed for the detection of several plant pathogens, including *Candidatus Liberibacter solanacearum* (Ravindran *et al.,* 2012), *Pythium aphanidermatum* (Fukuta *et al.,* 2013), *Sclerotinia sclerotiorum* (Duan *et al.,* 2014), *Xylella fastidiosa* (Yaseen *et al*., 2015), *Erysiphe necator* (Thiessen *et al.,* 2016), and *Xanthomonas fragariae* (Gètaz *et al.,* 2017).

Considering the importance of *Pl. richardsiae* as a vascular pathogen of economically important woody plant species, and the high performance offered by the RealAmp assay, the present study aimed to develop a RealAmp assay specific for *Pl. richardsiae*.

MATERIALS AND METHODS

Fungus strains

All fungus strains used in this study are reported in Table 1. The *Pl. richardsiae* strains were obtained from olive or grapevine plants showing symptoms of decline. Eight of the strains were from the north of Apulia region (Southern Italy), and two were of Algerian origin. One strain each of *Pl. ootheca* and *Pl. repens* from Algeria, were also included to assess the specificity of the RealAmp. Another 36 fungal strains of *Phaeoacremonium* and *Neofusicoccum* spp. from different countries and hosts were included in this study. All fungal strains were

Table 1. List of fungal strains isolated from different sources used in this study.

Fungus species	Isolate code/collection[a]	Location; Source
Pleurostoma richardsiae	Pl31/ Dept. DAFNE	Italy; olive tree
	Pl4/ Dept. DAFNE	Italy; olive tree
	Pl_M 1/ CIHEAM of Bari	Italy; olive tree
	Pl_M 2/ CIHEAM of Bari	Italy; olive tree
	Pl_M 3/ CIHEAM of Bari	Italy; grapevine
	Pl_M 4/ CIHEAM of Bari	Italy; grapevine
	Pl_M 5/ CIHEAM of Bari	Italy; grapevine
	Pl_M 6/ CIHEAM of Bari	Italy; olive tree
	Pl_G1/ ENSA	Algeria; grapevine
	Pl_G2/ ENSA	Algeria; olive tree
Pl. ootheca	CBS 115329/ ENSA	British Columbia; degrading wood
Pl. repens	CBS 294.39/ ENSA	USA; pine lumber
Phaeoacremonium alvesii	CBS 408.78/ Dept. DAFNE	USA; humans
	CBS 729.97/ Dept. DAFNE	USA; humans
P. amygdalinum	Pm10/ Dept. DAFNE	Italy; almond
P. griseorubrum	CBS 111657/ Dept. DAFNE	South Africa; humans
P. hispanicum	CBS 123910 Dept. DAFNE	Spain; grapevine
P. iranianum	Pm121/ Dept. DAFNE	Italy; grapevine
P. italicum	Pm17/ Dept. DAFNE	Italy; olive tree
	Pm31M/ Dept. DAFNE	Italy; almond
	CBS 137763/ Dept. DAFNE	Italy; grapevine
	Pm45/ Dept. DAFNE	Italy; olive tree
	Pm50M/ Dept. DAFNE	Italy; olive tree
P. minimum	Pm41/ Dept. DAFNE	Italy; olive tree
	Pm67/ Dept. DAFNE	Italy; olive tree
	Pm68/ Dept. DAFNE	Italy; grapevine
P. oleae	Pm14/ Dept. DAFNE	Italy; olive tree
P. parasiticum	Pm88/ Dept. DAFNE	Italy; olive tree
P. rubrigenum	CBS 498.94/ Dept. DAFNE	USA; humans
	CBS 112046/ Dept. DAFNE	USA; humans
P. scolyti	Pm92A/ Dept. DAFNE	Italy; olive tree
	Pm73M/ Dept. DAFNE	Italy; olive tree
	P.sc1/ Dept. DAFNE	Spain; unknown
	PMM2242 / Dept. DAFNE	South Africa; pomegranate
	CSN 1081/ Dept. DAFNE	South Africa; quince
	CSN55 / Dept. DAFNE	South Africa; plum
	CBS 121755/ Dept. DAFNE	South Africa; peach
	PMM2442/ Dept. DAFNE	South Africa; loquat
	PMM2270 / Dept. DAFNE	South Africa; rose
	CSN 1471/ Dept. DAFNE	South Africa; guava
	CBS 121439/ Dept. DAFNE	South Africa; japanese plum
	CBS 121756/ Dept. DAFNE	South Africa; peach
P. sicilianum	Pm65/ Dept. DAFNE	Italy; olive tree
Neofusicoccum luteum	B531/ Dept. DAFNE	Italy; olive tree
N. mediterraneum	B604/ Dept. DAFNE	Italy; olive tree
N. parvum	B651/ Dept. DAFNE	Italy; olive tree

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Table 2. LAMP primers used for the detection of *Pleurostoma richardsiae*.

Primer	Primer sequences 5'→3'	Length	Tm°
F3	TGCCCTGCCGTCGTTG	16	60.87
B3	GGAACCGATTCGCCTCGA	18	60.99
FIP	TCACACACCTCCCGCAGCCTTTTCCCACTAGAGGTGCTGTCG	42	> 75
BIP	TGGGCTGTCCTGCTTCGCTTTTTAGGCGGCGCATCCC	38	> 75
LoopF	ACCGCTCCAGGTGCTGA	17	60.40
LoopB	GGCACCTTAGGGTCGCTCA	19	63.44

previously identified using morphological and molecular tools.

DNA extraction and IGS amplification

Genomic DNA of each strain reported in Table 1 was extracted from 200 mg of fresh mycelium, that was grown on potato dextrose agar plates (PDA, Oxoid Ltd) at 25°C for 14 d. according to the protocol optimized for vascular fungi by Carlucci *et al.* (2013). DNA quality and concentrations were determined with a Beckman Coulter DU-800 UV/Vis spectrophotometer.

PCR reactions to amplify IGS regions of *Pl. richardsiae* and *Phaeoacremonium* strains were carried out using the universal primers LR12R (5'-GAACGCCTCTAAGTCAGAATCC-3'; anchored in the 3' of the LSU gene) and invSR1R (5'-ACTGGCAGAATCAACCAGGTA-3'; anchored in the 5' of SSU of the RNA gene), according to Laidani *et al.* (2021).

Design of LAMP primers

As for the design of LAMP primers for *Pl. richardsiae* detection, the IGS regions of *Pl. richardsiae* and *Phaeoacremonium* sequences were aligned using BioEdit version 7.0.9 (http://www.mbio.ncsu.edu/BioEdit) to find conserved regions in the target fungi. Primers were designed using PrimerExplorer v. 5 (http://primerexplorer.jp) and were checked by BLAST in the GenBank to ensure their specificity. Three sets of LAMP primers were generated, with each set made of six LAMP primers (external primers F3 and B3, internal primers FIP and BIP, and loop primers LoopF and LoopB). All primers were analyzed to check for hairpin, self-dimer, and heterodimer production, using Integrated DNA Technologies (Coralville) OligoAnalyzer, software v. 3.1 (Owczarzy *et al.*, 2008). The best performing LAMP primer set was selected, and primer sequences (Table 2) were synthesized by Eurofins MWG Operon.

The designed primers were the outer primers F3 and B3, the loop primers LoopF and LoopB, and the inner primers BIP and FIP that are complementary, respectively, to the sequences of the regions B1C and B2, F1C and F2 (Figure 1).

RealAmp assay

RealAmp reactions were each carried out in a final volume of 25 µL, containing; 15 µL of OptiGene master mix, 2.5 µL of primer mix (final concentrations of 1.6 µM FIP and BIP, 0.2 µM F3, B3 and 0.4 µM LoopF, LoopB), 2 µL of DNA template (30 ng) and 5.5 µL of water. RealAmp fluorescence signal (RFU) was plotted automatically using the CFX96™ real-time PCR detection system (Bio-Rad) collecting fluorescence signals at

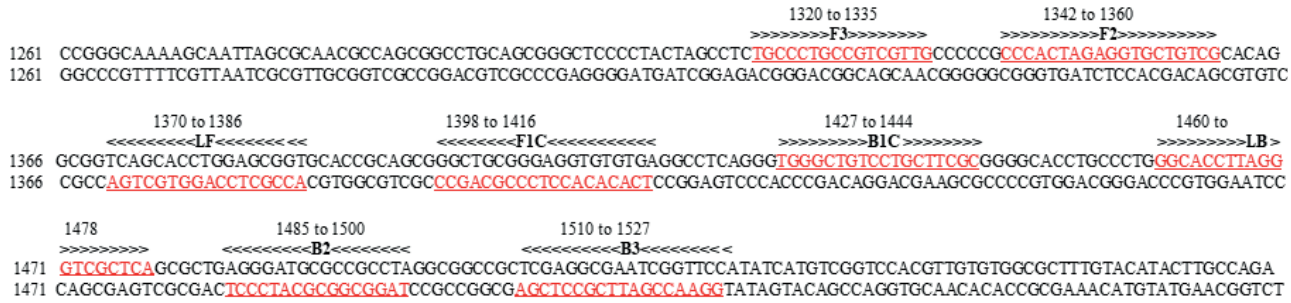

Figure 1. Positioning and orientation of LAMP primers within the nucleotide sequence of the IGS region of *Pleurostoma richardsiae*.

30 sec intervals at 70°C for 45 min. For all reactions, a result was considered positive when an amplification curve was obtained, while a linear or slightly oblique amplification curve indicated a negative result. All reactions were carried out three times, and the negative controls contained nuclease-free water.

Specificity and sensitivity of RealAmp

To assess the specificity of the LAMP primer dataset, assays were carried out using DNA extracted from ten strains of *Pl. richardsiae*, two strains phylogenetically related to *Pleurostoma* species, and other strains of *Phaeoacremonium* and *Neofusicoccum* commonly associated with olive trunk disease (Table 1).

The limit of detection (LoD) of the RealAmp assay was determined through two successive experiments using pure genomic DNA obtained from *Pl. richardsiae* cultures. In the first experiment, a ten-fold dilution series was prepared, from 3 ng μL^{-1} to 3×10^{-6} ng μL^{-1} (3 fg μL^{-1}). Each dilution was analyzed in ten replicates to obtain an initial estimate of LoD. Results of the first experiment were used to narrow the range of target concentrations and make a more precise estimate. In the second experiment, a two-fold dilution series was prepared, from 3×10^{-1} ng μL^{-1} to 7.5×10^{-2} ng μL^{-1}. Each dilution was tested in 20 replicates. In both experiments, nuclease-free water was used as negative controls (NTC).

RealAmp detection of Pleurostoma richardsiae *in inoculated olive plants*

As part of an ongoing experiment on olive plants ('Ogliarola salentina') artificially inoculated in a greenhouse with the *Pl. richardsiae* strain Pl31 (Table 1), six symptomatic plants were used for a preliminary validation of the *in vivo* RealAmp assay. The tested plants showed clear symptoms of necrotic wood lesions and vascular discolourations after bark removal. To ascertain the presence of the fungus, isolations were made from the stem portions at the inoculation points, according to Carlucci *et al.* (2013), and each sample was also used for DNA extraction using the DNA secure Plant Kit (Tiangen), according to the manufacturer's protocol. DNA used as negative controls was extracted from three uninoculated olive plants, three olive plants inoculated with *Neofusicoccum parvum* and three olive plants inoculated with *Phaeoacremonium italicum*. Each assay was carried out using 30 ng of genomic DNA from olive plants, and reactions were carried out using RealAmp.

RESULTS AND DISCUSSION

This is the first reported application of RealAmp as a rapid, specific and sensitive method for detection of *Pl. richardsiae* using specific primers. To screen for optimal LAMP primers, the intergenic spacer (IGS) region was employed as targets for primer design. All tested *Pl. richardsiae* strains showed positive reactions in the RealAmp assays, as reported in Figure 2. Optimized conditions in this study, at 70°C for 45 min, enabled amplification of the target sequence giving clear curves. The rapidity of this technique in pathogen detection was also reported by Besuschio *et al.* (2017). Unlike other nucleic acid amplification techniques, RealAmp can be carried out in 30 min, rather than at least 90 min (e.g. for PCR). For specificity of the RealAmp assay using DNAs of other relative fungi, the reaction curves indicated that the *Pl. richardsiae* primer dataset specifically amplified the target DNA sequence of this fungus (Figure 2), the DNA isolated from inoculated olive plants (Figure 3), but not DNA of the other fungi assayed (Figure 2; Table 1). The RealAmp products of *Pleurostoma*

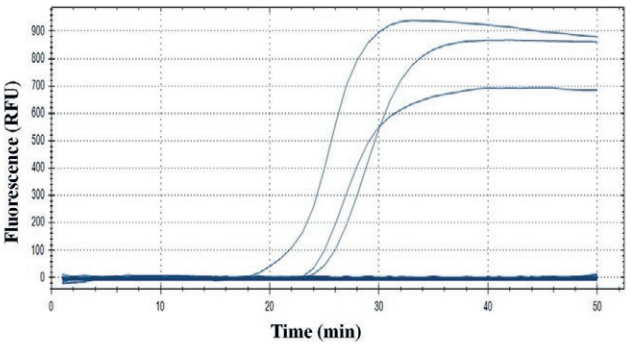

Figure 2. The specificity of the *Pleurostoma richardsiae* RealAmp method. The amplification plots (RFU) of three representative isolates of *Pl. richardsiae* and other fungus strains. Negative samples showed linear curves.

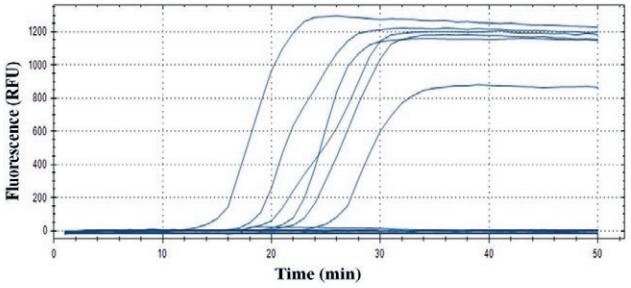

Figure 3. Detection of *Pleurostoma richardsiae* in inoculated olive plants, using RealAmp assay. Samples used as negative controls showed linear curves.

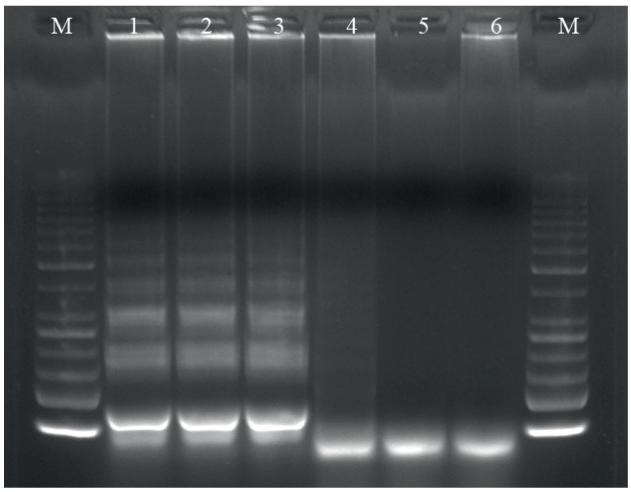

Figure 4. Detection of *Pleurostoma richardsiae* using the RealAmp products, which were electrophoresed in a 2% agarose gel and inspected under UV light. M: marker; 1, 2, and 3: *Pl. richardsiae*; 4: *Pl. repens*; 5: *Pl. ootheca*; 6: water control.

were also observed in the agarose gel electrophoresis, which confirmed the specificity of the RealAmp assay (Figure 4).

Figure 5 shows the results of experiments conducted to determine the LoD assay. The analysis carried out on the ten-fold dilution series of *Pl. richardsiae* genomic DNA resulted in an initial estimate of the LoD. Positive reactions were obtained for target concentrations ranging from 3 ng μL^{-1} to 3×10^{-2} ng μL^{-1}, with frequencies, respectively, of 100% and 50% (ten replicates for each dilution were analysed). No amplification signal was observed in subsequent dilutions from 3×10^{-3} ng μL^{-1} to 3×10^{-6} ng μL^{-1}, or in the NTC (Figure 5A). To define the target concentration that produced at least 95% positive replicates (LoD at 95%) (Forootan *et al.*, 2017), the assay was repeated over a narrower range of concentrations. On the basis of the results obtained, a two-fold dilution series was prepared starting from 3×10^{-1} ng μL^{-1}, corresponding to the lowest concentration of targets with 100% positives replicates, to 7.5×10^{-2} ng μL^{-1}.

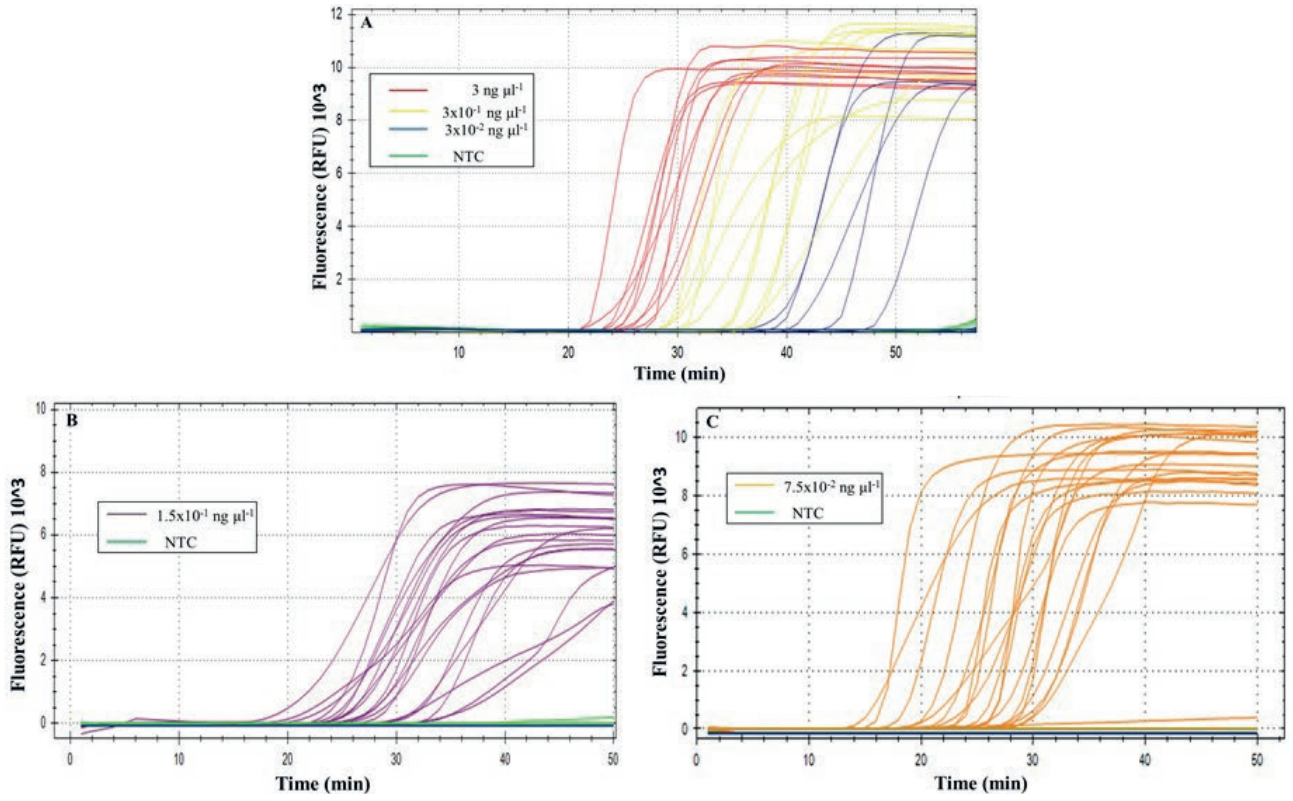

Figure 5. Sensitivity of the RealAmp assay for *Pleurostoma richardsiae* detection. (A) Results for the preliminary estimate of the LoD: 100% of positive reactions were obtained for target concentrations of 3 ng μL^{-1} (red curves) and 3×10^{-1} ng μL^{-1} (yellow curves); 50% of positive reactions for 3×10^{-2} ng μL^{-1} (blue curves); no reaction was observed from 3×10^{-3} ng μL^{-1} to 3×10^{-6} ng μL^{-1} or in the NTC (green curves). (B and C). Results for the estimate of LoD at 95%: positive reactions with frequency of 100% were obtained analysing target concentration of 1.5×10^{-1} ng μL^{-1} (purple curves), and 95% (orange curves) analysing 7.5×10^{-2} ng μL^{-1} (C).

Twenty replicates for each dilution were analysed for accurate estimation. Positive reactions with 100% frequency were obtained analysing target concentration of 1.5×10^{-1} ng μL^{-1} (Figure 5B), and 95% frequency analysing 7.5×10^{-2} ng μL^{-1} (Figure 5C). No amplification was observed in the NTC. Results of these analyses indicated 7.5×10^{-2} ng μL^{-1} as the LoD at 95% of the assay.

For the artificially inoculated olive plants, results from isolations indicated that all the plants were infected by *Pl. richardsiae*. These results were confirmed by RealAmp, which showed amplification for all inoculated olive plants and no amplification from the negative controls (Figure 3). Similar RealAmp results were obtained by Lee *et al.* (2020) for detection of Peach latent mosaic viroid in peach pollen.

The developed RealAmp assay is specific, sensitive, and rapid, for detecting *Pl. richardsiae* from DNA extracted from pure strains of the fungus and from infected olive plants.

Three of the six species of *Pleurostoma* were used to validate the developed assay in this study: *Pl. richardsiae* (ten strains), the ex-type of *Pl. repens*, and the ex-type of *Pl. ootheca*. However, full RealAmp validation with the remaining three *Pleurostoma* species could not be accomplished, due to lack of data (for *Pl. candollei*) and funding limitations (for *Pl. ochraceum* and *Pl. hongkongense*). In addition, further studies are required to evaluate the field performance of this test for grapevine and other hosts of this pathogen. These promising results are in accordance with the successful application of this technique for reliable and rapid detection of several plant pathogens, including *X. fastidiosa* in olive trees (Yaseen *et al*., 2015). Among the several features that make RealAmp an excellent diagnostic tool, its use, both with a real time instrument for processing numerous samples and with portable devices for few samples, are strengths compared to other PCR techniques.

In the last two decades, infections caused by *Pl. richardsiae* have been widespread on olive groves, often with severe symptoms leading to general tree decline, such as browning and leaf drop, wilting and decay of twigs and branches, and dark streaks and necroses in the inner tissues of host wood (Hallen *et al*., 2007; Carlucci *et al*., 2013; Carlucci *et al*., 2015a; Canale *et al*., 2019). RealAmp could become a useful tool in large-scale monitoring programmes for this pathogen, and in the phytosanitary controls with certified pathogen-free propagating material of olive. In addition, as *Pl. richardsiae* is an uncommon agent of human infection, it may also be of interest to further investigate the application of RealAmp assay in the medical field.

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Short Notes

Fusarium annulatum causes Fusarium rot of cantaloupe melons in Spain

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Summary. During the summer of 2018, there was high incidence of fruit rots of cantaloupe melons (*Cucumis melo* var. *cantalupensis*) in Murcia province, south-eastern Spain. The fruits showed development of whitish mycelium and pulp softening. Morphological and molecular analysis of the internal transcribed spacer (ITS), translation elongation factor 1-α (*TEF1-α*) and the second largest subunit of RNA polymerase (*RPB2*) genes confirmed *Fusarium annulatum* as the causal agent of the disease. A phylogenetic study indicated that *F. annulatum* is in the *Fusarium fujikuroi* species complex (FFSC). Pathogenicity of the isolate was determined on healthy fruit verifying Koch's postulates. The first symptoms of fruit rot were observed 3 d after inoculations at 28°C. Fruit infections only occurred in artificially wounded melons, and *F. annulatum* was re-isolated from the wounds. This disease appeared after fruit harvesting, and could generate substantial economic losses mainly in fruit destined for foreign markets due to long transportation times. This is the first report of melon fruit rot caused by *F. annulatum* in Spain.

Keywords. *Cucumis melo*, fungal pathogen, pathogenicity test, first report, postharvest disease.

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INTRODUCTION

Melon (*Cucumis melo* L.) is widely cultivated in south-eastern Spain. Total melon production for Spain during 2018 was 664,353 t, the Region of Murcia being the largest producer, with 220,768 t obtained from the 5,576 ha dedicated to melon cultivation (MAPA, 2018).

Fusarium species are important cucurbit pathogens, causing diseases in host fruits, stems and roots (Chehri *et al*., 2011), and several *Fusarium* species have been reported as causal agents of fruit rot diseases. These include; *F. equiseti* (Corda) Sacc., *F. pallidoroseum* syn. *F. semisectum* (Cooke) Sacc., *F. solani* (Mart.) Sacc. (Snowdon, 1991); *F. graminum* Ces., *F. acuminatum* Ellis & Everh, *F. culmorum* (W.G. Smith) Sacc., *F. moniliforme* J. Sheld, *F. pallido-*

roseum syn. *F. incarnatum* (Roberge) Saccardo, *F. scirpi* syn. *F. longipes* Wollenweber & Reinking, *F. oxysporum* f. sp. *melonis* Snyder & Hansen (Zitter *et al*., 1998), *F. graminearum* Schwabe, *F. proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg, and *F. roseum* syn. *F. sambucinum* Fuckel (Kim and Kim, 2004).

Regarding the *Fusarium* species affecting cucurbits in Spain, the following species have become particularly important: *F. oxysporum* (Martínez *et al*., 2003), and *F. solani* f. sp. *cucurbitae* Snyder & Hansen on watermelon grafted to pumpkin (Armengol *et al*., 2000), zucchini (Gómez *et al*., 2008) and melon (Gómez *et al*., 2014). In many studies only Fusarium rots caused by *Fusarium* sp. are mentioned, without specifying the pathogenic specifies, so that diagnostic information is lost. Thus, increasing knowledge of the diversity of species that cause melon rots through accurate diagnoses of the causal pathogens is relevant, because this would allow improvement of disease management practices.

Previous studies have not been carried out in Spain on the postharvest fruit rots of melons, caused by *Fusarium* sp., which can be favoured by the climatic conditions of the Mediterranean Basin. These diseases affect melon plantations and the subsequent marketing of melon fruits.

The present study aimed to identify the *Fusarium* species causing fruit rots in cantaloupe melons (*C. melo* var. *cantalupensis*) fruits. For this purpose, the organism associated with the symptomatology was isolated, its morphological characteristics were described, and the species was identified by molecular methods. Koch's postulates were applied by means of pathogenicity tests of the percentage of infection in inoculated healthy fruit, as well as the severity of the resulting disease.

MATERIALS AND METHODS

Sampling and fungal isolation

Melon producers in Campo de Cartagena, Murcia province, Spain (37°36'18.4"N, 0°59'10.4"W), supplied cantaloupe fruits showing rot symptoms to the Phytopathology Laboratory of the Crop Protection Group of the Polytechnic University of Cartagena (UPCT) (Grupo de Protección de Cultivos de la Universidad Politécnica de Cartagena - UPCT). The fruits were harvested during July 2018, from different producing areas of Murcia province.

Fungus isolations were carried out using two different methods. Firstly, superficial mycelium developing on the skins of the fruits was transferred to Petri dishes containing Potato Dextrose Agar (PDA) (Scharlab), amended with 0.1 g L^{-1} of streptomycin sulphate. In addition, diseased fruit tissues were disinfected with 1% NaOCl for 5 min, then sprayed with 96% ethanol, and subsequently rinsed with sterilized distilled water. Fragments of approx. 0.5 × 0.5 cm of the infected tissues were aseptically taken and placed onto PDA. The culture plates were incubated in darkness at constant 26°C for 7 d. After this period, tips of hyphae from emerging colonies were transferred to new PDA plates to obtain pure cultures.

Morphology and molecular characterization of isolated fungi

The morphology of all isolates was similar, and the isolate MLFR-09 was used as a representative strain in for further study. Mycelium fragments (5 × 5 mm) were transferred to the centres of 90 mm diam. PDA plates (four replicates), which were incubated in darkness at 26°C to assess colony development. To allow the development of microscopic characteristics, the colonies were also grown on Spezieller Nährstoffarmer Agar (SNA) (Leslie and Summerell, 2006), incubated with a 12 h light/12 h darkness regime. Mycelium growth data, based on the perpendicular and horizontal diameters of colonies, were measured with a calliper. The average of both measurements was recorded after 4 d of incubation on PDA or 6 d on SNA. The colour of the colonies was evaluated after 14 d of growth. After 10 days of incubation on both media, samples were prepared for microscope observation. The criteria of Bugnicourt (1952), Nelson *et al*. (1983), and Yilmaz *et al*. (2021), taking into consideration presence of conidiophores and conidia on aerial mycelium, conidiogenous cells, and production of sporodochia and chlamydospores. The average, maximum and minimum measurements were recorded for each identified structure (n = 30). Morphological features were observed using an Olympus BX50F optical microscope, and photomicrographs were taken of each observed fungal structure using an Olympus SC20 camera.

Molecular identification and phylogenetic analysis

To confirm the identity of isolate MLFR-09, partial gene fragments of the ITS region, translation elongation factor 1-α (*TEF1-α*), and the second largest subunit of RNA polymerase (*RPB2*) were analysed. Total genomic DNA was extracted from fresh mycelium using the Animal and Fungi DNA Preparation Kit (Jena Bioscience).

The ITS region was amplified with the primer pair ITS1 and ITS5 (White *et al*., 1990), the translation elon-

gation factor 1-α (*TEF1-α*) with ef1 (O´Donnell *et al*., 2010) and ef2 (O´Donnell *et al*., 1998), and the RNA polymerase II second largest subunit (*RPB2*) with the primer pair RPB2-5F2 (Sung *et al*., 2007) and RPB2-7cR (Liu *et al*., 1999).

DNA templates were PCR-amplified on a Veriti 96-Well Thermal Cycler (Applied Biosystems). The PCR amplifications were each carried out in a total volume of 50 µL, containing 1 µL (50 pmol μL^{-1}) of each of the universal primers, 0.25 µL (5U μL^{-1}) of Taq DNA polymerase (Takata, Clontech Laboratories, Inc.), 5 µL of 10× reaction buffer, 2.5 µL of DMSO, 4 µL of dNTP mixture (10 mM), 31.25 µL of sterile filtered water, and 5 µL of DNA sample (150-200 ng genomic DNA).

PCR conditions for ITS and *RPB2* regions were as follows: an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, extension at 72°C for 1 min and a final extension at 72°C for 10 min. The PCR programme for *TEF1-α* amplification consisted of one cycle of 5 min at 94°C, 10 cycles of 45 sec at 94°C, 45 sec at 55°C, and 1.5 min at 72°C, 30 cycles of 45 sec at 94°C, 45 sec at 52°C, and 1.5 min at 72°C, and a post elongation step of 6 min at 72°C.

DNA fragments were purified using an Ultra-Clean PCR Clean-Up Kit (MoBio Laboratories Inc.). Sequencing was carried out using ABI PRISM Big-Dye Terminator Cycle Sequencing Ready reactants and an ABI3730XL sequencer (Applied Biosystems) at the Central Service of Support to Experimental Research (SCSIE) of Valencia University. Sequencing primers were the same as those used in the amplification reaction. The sequences of three repetitions of the isolate MLFR-09 were analysed.

The BLAST program was used to search homologous MLFR-09 sequences in the NCBI databases (http://www.ncbi.nlm.nih.gov/).

Pathogenicity test and evaluation of disease severity

For the pathogenicity test, healthy cantaloupe fruits at physiological maturity were used at 10 d after harvesting (≥ 10° Brix), and with diameters between 16 and 20 cm and approx. weight of 1–1.5 kg. The MLFR-09 isolate was first grown on PDA at 26°C to allow sporulation. The fruits were surface sterilized following the procedure described above. One group of fruits was wounded with a sterile needle and another group was left uninjured. A suspension of conidia (1×10^6 conidia mL^{-1}) was prepared in sterile distilled water, and 50 µL were pipetted onto the wound/surface of each healthy melon, while the experimental controls were similarly treated with sterile distilled water. Ten fruits were used per inoculation treatment, and ten were used in the control treatment. The fruits were kept at 28 ± 2°C for 7 d. Relative humidity was maintained constant by covering the fruits with plastic bags, and the test was carried out twice. Development of any symptoms was checked and recorded daily from inoculation onwards. The percentages of melons that developed disease were determined. To assess the severity of any rot in each melon, a disease severity index (DSI; Promwee *et al*., 2017) was applied, and following the scale proposed by Wonglom and Sunpapao (2020):

$$DSI\ (\%) = \frac{\Sigma\ (Score\ Amount\ of\ fruits\ )}{Maximum\ score\ \times\ Total\ number\ of\ fruits} \times 100$$

The pathogen was re-isolated and re-identified with molecular methods to verify Koch's postulates.

RESULTS

Disease description

The symptoms the disease comprised brownish areas (diam. up to 6 cm) of the melon skins, which were covered with white mycelium, on different areas of the exocarps, especially in the areas close to the stalks (Figure 1, a and b). These mycelium masses were surrounded by darkened and softened exocarps. The pathogen also colonised the inner tissues of the fruits, and internal lesions of the mesocarps reached a depth of approx. 4 cm, but which could also reach the fruit placental tissues, where sparse mycelium developed (Figure 1, c). The fruit pulp lost consistency, becoming watery as a result of cell degradation, even leading to internal browning in the areas near the skins.

Molecular and phylogenetic study of the isolated fungus

PCR amplification of ITS, *TEF1-α* and *RPB2* genes provided fragments, respectively, of approx. lengths 582, 709 and 644 bases. The PCR amplifications of the three repetitions of isolate MLFR-09 were identical, so the sequences of this isolate were deposited in GenBank under the accession number MZ355136 for ITS, OL960473 for *TEF1*-α, and OL944300 for *RPB2*. A BLAST search in GenBank (NCBI) revealed the sequence of the ITS gene region to be 100% identical to *F. annulatum* (Accession No. MH862668, reference strain CBS 738.97). Translation elongation factor 1-*α* (*TEF1-α*) showed 99.30% sequence identity with sequence

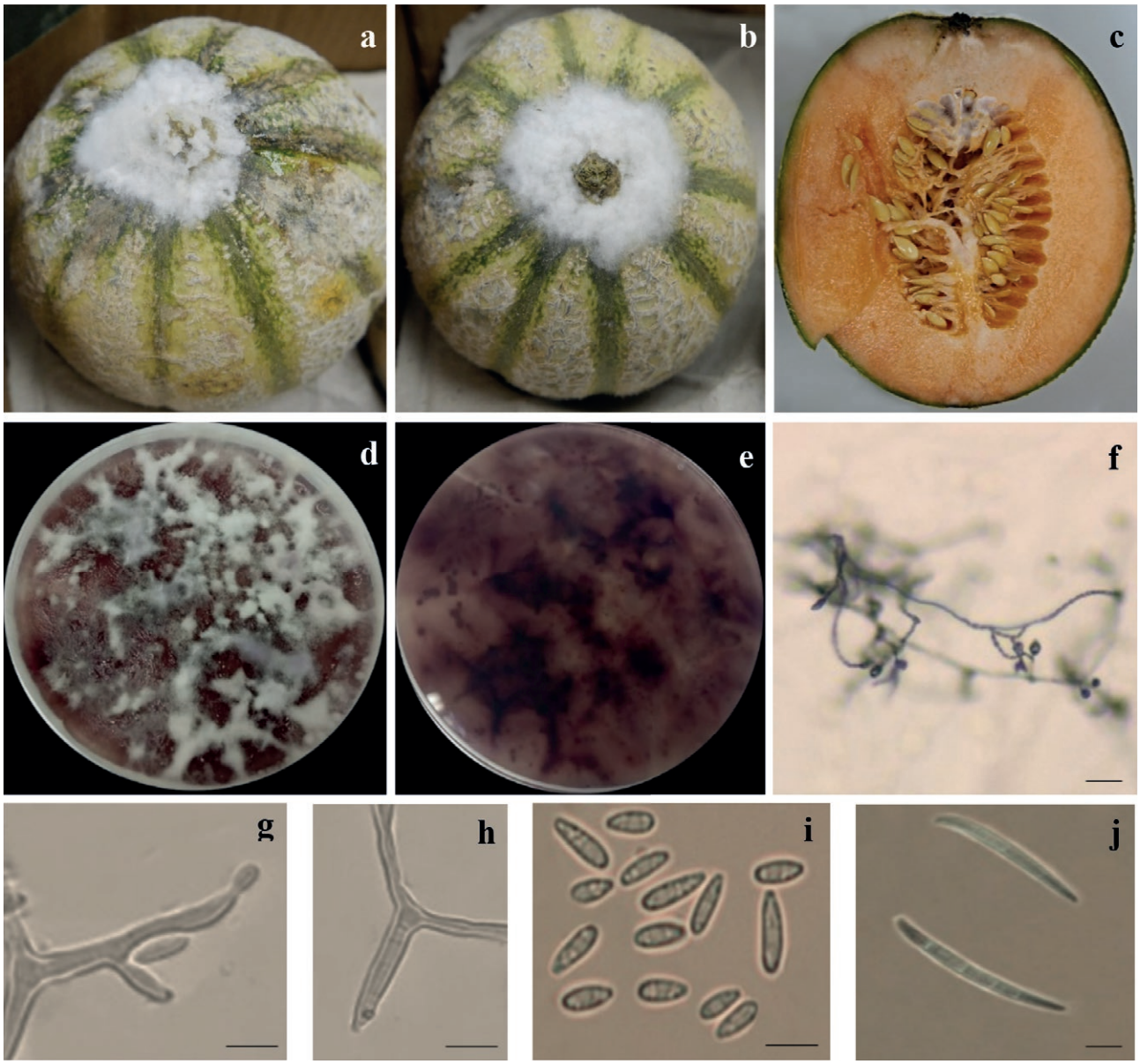

Figure 1. External disease symptoms caused by *Fusarium annulatum* in cantaloupes after natural infections (a, b), and rot of the internal tissues (c). Colony of *F. annulatum* isolate MLFR-09 on potato dextrose agar after 14 d at 26ºC, top view (d) and reverse view (e). Microconidial chains (f), polyphialide (g), monophialide (h), microconidia (i) and macroconidia (j). Scale bars: f = 20 µm, g, h, i and j = 10 µm.

MT010994, and the second largest subunit of RNA polymerase (*RPB2*) showed 99.69% sequence identity with sequence MT010983, both sequences corresponding to the CBS 258.54 type strain of *F. annulatum* (Table 1).

A BLAST search of three sequences in the *Fusarium*-ID database (Geiser *et al.*, 2004) showed that the MLFR-09 isolate belonged to the *Fusarium fujikuroi* species complex (FFSC).

Phylogenetic analysis supported the results obtained from the molecular analysis, confirming that isolate MLFR-09 was *F. annulatum*. The isolate was deposited in the culture collection (Microorganismos de la Agricultura, Poscosecha y Sostenibilidad (MAPYS), Escuela Técnica Superior de Ingeniería Agronómica (ETSIA), Universidad Politécnica de Cartagena (UPCT) (Microorganisms of Agriculture, Postharvest, and Sustainability, Higher Technical School of Agronomic Engineering, Polytechnic University of Cartagena (UPCT), Murcia, Spain).

Table 1. BLAST search for gene sequences of the *Fusarium annulatum* isolate MLFR-09, compared with the reference sequences obtained from type culture material.

GenBank accession No. (MLFR-09)	DNA target	BLAST match sequence			
		Reference accession No.	Type material	Coverage (%)	Identity (%)
MZ355136	ITS, rRNA[a]	*Fusarium annulatum* MH862668	CBS 738.97	98	100
		Fusarium concentricum MH862659	CBS 450.97	96	99.82
		Fusarium fujikuroi NR_111889	CBS 221.76	95	99.82
OL960473	*TEF1*-α[b]	*Fusarium annulatum* MT010994	CBS 258.54	100	99.30
		Fusarium globosum MT010993	CBS 428.97	100	98.31
		Fusarium fujikuroi AB725605	CBS 221.76	93	96.38
OL944300	*RPB2*[c]	*Fusarium annulatum* MT010983	CBS 258.54	100	99.69
		Fusarium concentricum MT010981	CBS 450.97	100	97.52
		Fusarium proliferatum MN534272	CBS 480.96	92	97.32

[a]ITS, internal transcribed spacer; rRNA, ribosomal gene.
[b]*TEF*-α, translation elongation factor 1-α gene.
[c]*RPB2*, second largest subunit of RNA polymerase gene.

Fungus morphology

The mean diameter of the colonies of *F. annulatum* MLFR-09 grown on PDA was 54 ± 5 mm after 4 d of incubation in darkness. Development of the isolate was also assessed on SNA after 6 d incubation, where the average diameter of the colonies was 84 ± 2 mm. The aerial mycelium developed in SNA was sparse and whitish, extending over the entire medium surface in each culture plate.

Fusarium annulatum MLFR-09 culture had characteristic violet pigmentation on PDA (Figure 1, d). On the sides, the colour was deep purple (Figure 1, e). In general, the aerial mycelium had a cottony appearance, was initially white but gradually turned violet as it aged, and becoming grey in some areas. Deep purple sporodochia were occasionally observed. The scant macroconidia had straight, thin, partitioned arrangements (3–4 septa). Macroconidium sizes ranged from 20 to 55 µm length and from 3.7 to 5 µm width; the macroconidium apical cells were blunt, and the basal cells were foot-shaped (Figure 1, j). The abundant microconidia were observed in long chains (Figure 1, f) supported by monophialides (Figure 1, h) and polyphialides (Figure 1, g). No septa were detected in the microconidia, their shapes varied from obovoid or nearly oval with a truncate bases, to fusiform, while they ranged from 5.2 to 13.5 × 2.5 to 3.2 µm (Figure 1, i). No chlamydospores were observed.

The MLFR-09 isolate was morphologically compared with the first published description of *F. annulatum* in Bugnicourt (1952), and also referenced in Nelson *et al*., (1983) and Yilmaz *et al*. (2021) (Table 2).

Pathogenicity

One week after inoculation, white mycelium covered the wounds of the fruits inoculated with *F. annulatum* MLFR-09 (Figure 2, b). No disease was detected in the uninjured melons or in the control treatment (Figure 2, a and c). Symptoms caused by *F. annulatum* MLFR-09 began to appear 3 d after inoculation. On the fruit surfaces, brown necrotic haloes initially appeared around the emerging mycelium, accompanied by the loss of structural rigidity and depression of the fruit exocarps in the same areas. The advance of the pathogen was also evident in the mesocarp of fruits, where there was a change in the colour of the pulp, which became soft with a woolly texture, reaching a depth of 3 cm after 7 d (Figure 2, d). Cross-sectioning of the fruits allowed the tissues between the mesocarp and exocarp to be analysed; the structure turned brown and oily as a result of the necrotic dehydration that had occurred.

Pathogenicity of *F. annulatum* MLFR-09 to cantaloupe fruit was confirmed, as shown in Table 3. The infection rate was 100% when the fruits were artificially wounded and 0% in the non-wounded and inoculated fruit. The mean IDS was 89.75% for wounded and 0% for non-wounded and inoculated fruit. Symptoms of fruits in which the disease developed was compared with the control treatment in which no disease appeared (Figure 2). The severity of the damage was similar to that caused to the fruits by natural infections at the time of isolation of the pathogen (Figure 1).

Microscopic analysis of the pathogen present in the wounds showed that *F. annulatum* that had been inocu-

Table 2. Morphological characteristics of *Fusarium annulatum* isolate MLFR-09, causing fruit rot in cantaloupes, compared with previous descriptions of *F. annulatum* by Bugnicourt (1952), Nelson *et al.* (1983), and Yilmaz *et al.* (2021).

Morphological characteristics	*Fusarium annulatum* grown on Corn Meal Agar (CMA)	Isolate MLFR-09 grown on Potato Dextrose Agar (PDA)
Colony top view	Aerial mycelium absent or late-developed, very light, powdery, slightly dispersed, white in colour. Blackish-purple pigments are normally formed in synthetic cultures	Aerial mycelium with cottony appearance, initially white, but gradually turning violet with age. It even had a grey colouration in some areas
Colony reverse view	*ND*	Accentuated purple colouration
Macroconidia		
Shape	Thin-walled, strongly curved and sickle-shaped, with the basal cell clearly foot-shaped	Straight and slender; the apical cell was blunt and the basal cell foot-shaped
Length (μm)	13-58	20-55
Wide (μm)	1.9-3.3	3.7-5
Number of septa	3-6	3-4
Microconidia		
Shape	Cylindrical or claviform with a truncate tip	Ovoid or nearly ovoid with a truncate tip and rarely fusiform
Length (μm)	4.7-14.4	5.2-13.5
Wide (μm)	1.7-2.3	2.5-3.2
Number of septa	0-1	0
Chlamydospores	Absent	Absent

ND = not described.

Table 3. Pathogenicity test and disease severity index (DSI) of caused by *Fusarium annulatum* isolate MLFR-09 on *Cucumis melo* var. *cantalupensis* fruits.

Inoculum	Percentage infection		Average DSI (%)	
	Wounded	Not wounded	Wounded	Not wounded
Spore suspension (MLFR-09)	100	0	89.75 ± 11	0
Sterile distilled water	0	0	0	0

lated in the pathogenicity tests was also recovered from the fruits with symptoms (data not presented). The morphological and molecular characteristics of the inoculated isolate were maintained, verifying Koch's postulates.

DISCUSSION

This research completed a morphological, molecular, and pathogenicity characterization of *F. annulatum* as a fruit rot pathogen of cantaloupe melons collected from different producing areas of Murcia province of Spain.

Fusarium annulatum is a pathogen found in tropical and temperate climates, affecting a variety of crops (Yilmaz *et al.*, 2021). According to Nelson *et al.* (1992), this fungus is considered an uncommon species, partially because *F. annulatum* has often been misidentified as *F. proliferatum* (Yilmaz *et al.*, 2021). In Spain, *F. proliferatum* has been reported as a frequent pathogen in the production of garlic, causing rots during storage (Gálvez *et al.*, 2017), while it has also been detected in strawberry crops, where it causes vascular wilt (Borrero *et al.*, 2019). In Mexico, Rivas-García *et al.* (2018) found *F. annulatum* causing rot in muskmelon, where affected fruits showed tissue darkening and thickening, with dehydrated and pinkish white exocarps. In the present case, the pink-violet colouration was not detected in the mycelium that developed in the fruit, but was detected during subsequent isolation of the pathogen on synthetic culture medium.

Fusarium annulatum was described by Bugnicourt (1952) as producing ring-shaped macroconidia. Based on this original description, Nelson *et al.* (1983) indicated that *F. annulatum* was basically a *F. proliferatum* with strongly curved macroconidia. However, Yilmaz *et al.* (2021) noted that this feature is atypical of the species because most isolates of *F. annulatum* tend to produce straight macroconidia (Figure 1, g).

Fusarium annulatum belongs to the *Fusarium fujikuroi* species complex (FFSC) (Yilmaz *et al.*, 2021), formerly designated as the *Gibberella fujikuroi* (GFC) species complex (Wigmann *et al.*, 2019). Three clades form

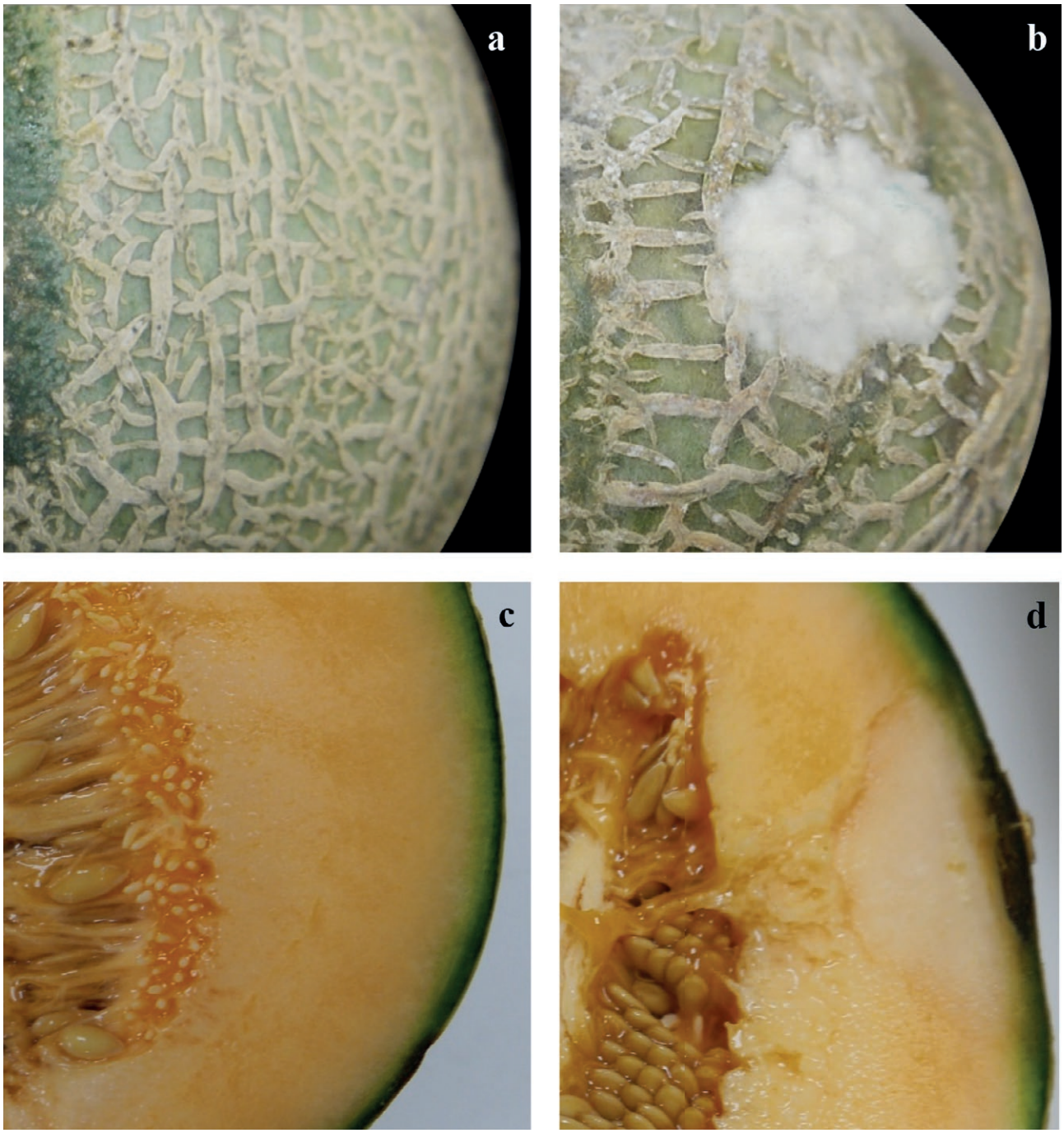

Figure 2. Morphological changes in cantaloupe (*Cucumis melo* var. *cantalupensis*) fruits, in pathogenicity tests 7 days after inoculation with *Fusarium annulatum*. a and c, Control fruit inoculated with sterile distilled water. b and d, Fruit inoculated with *F. annulatum* isolate MLFR-09.

the GFC complex, with *F. annulatum* belonging to the Asian clade (O´Donnell *et al*., 1998).

The pathogenicity test in the present study confirmed that melon fruits must be naturally wounded (mechanical injury) for the pathogen inoculations to be effective (Figure 2), as was also noted by Wonglom and Sunpapao (2020). Nuangmek *et al*. (2019) suggested that cutting the fruit peduncles during harvesting

could be the source of infections in the field. Another determining factor could be the texture of the surface tissues of some commercial melon varieties, with natural cracking facilitating pathogen infections. Fruit cracking disorders and open netting areas due to a defective synthesis of lignin and suberin are considered natural infection pathways for pathogens (Martínez *et al*., 2009). However, Champaco *et al*. (1993) commented on the possible association between the root rot caused by *Fusarium* species and fruit rot, since melons grow on the soil surface which would act as an inoculum reservoir. If pathogen penetration occurs prior to, or during, harvesting procedures, *Fusarium* survival in the soil and in the plant debris from previous crops is likely to be important.

This is the first report of *F. annulatum* causing rot in cantaloupe fruits in Spain. The results presented here provide the basis for new disease management strategies, based on the precise identification of the causal agent of this disease.

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Short Notes

Draft genome sequence of *Phyllosticta ampelicida*, the cause of grapevine black rot

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Summary. *Phyllosticta ampelicida* causes grapevine black rot, a potentially damaging disease for grape production. This paper reports the draft genome sequence of *P. ampelicida* PA1 Galicia CBS 148563, which is 30.55 Mb and encodes 10,691 predicted protein-coding genes. This is the first sequence genome assembly of *P. ampelicida*, and this information is a valuable resource to support genomic attributes for determining pathogenic behaviour and comparative genomic analyses of grapevine black rot fungi.

Keywords. Grapevine disease, microbe-plant interaction, *Vitis vinifera* L.

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Phyllosticta ampelicida (Engelm.) Aa (syn. *Guignardia bidwellii*, following the recommendation of the International Commission on the Taxonomy of Fungi, Rossman *et al*., 2015) is the causal agent of black rot of grapevine. *Phyllosticta ampelicida* (*Ascomycota*, *Dothideomycetes*, *Botryosphaeriales*, *Phyllostictaceae*) causes Black rot, which is an economically important disease, especially in grape producing regions characterised by humid growing seasons (Ramsdell and Milholland, 1988). In epidemic years, black rot can cause crop losses between 5 and 80% (Ramsdell and Milholland, 1988), and in severely affected vineyards virtually complete crop loss if not effectively managed (Rinaldi *et al.,* 2013). All *Vitis vinifera* cultivars are highly susceptible to black rot (Wilcox and Hoffman, 2019). Chemical treatments against downy and powdery mildews are sufficient to prevent black rot, although in recent years, especially because of the adoption of downy mildew *V. vinifera* resistant varieties and the increased use of active ingredients specific against Oomycetes, black rot is of increasing importance (Pertot *et al*., 2017).

All herbaceous tissues of grapevine plants are susceptible to infection by the pathogen, including leaves, shoots, tendrils, petioles and berries, with young leaves and fruit being extremely susceptible (Vezzulli *et al.,* 2022)

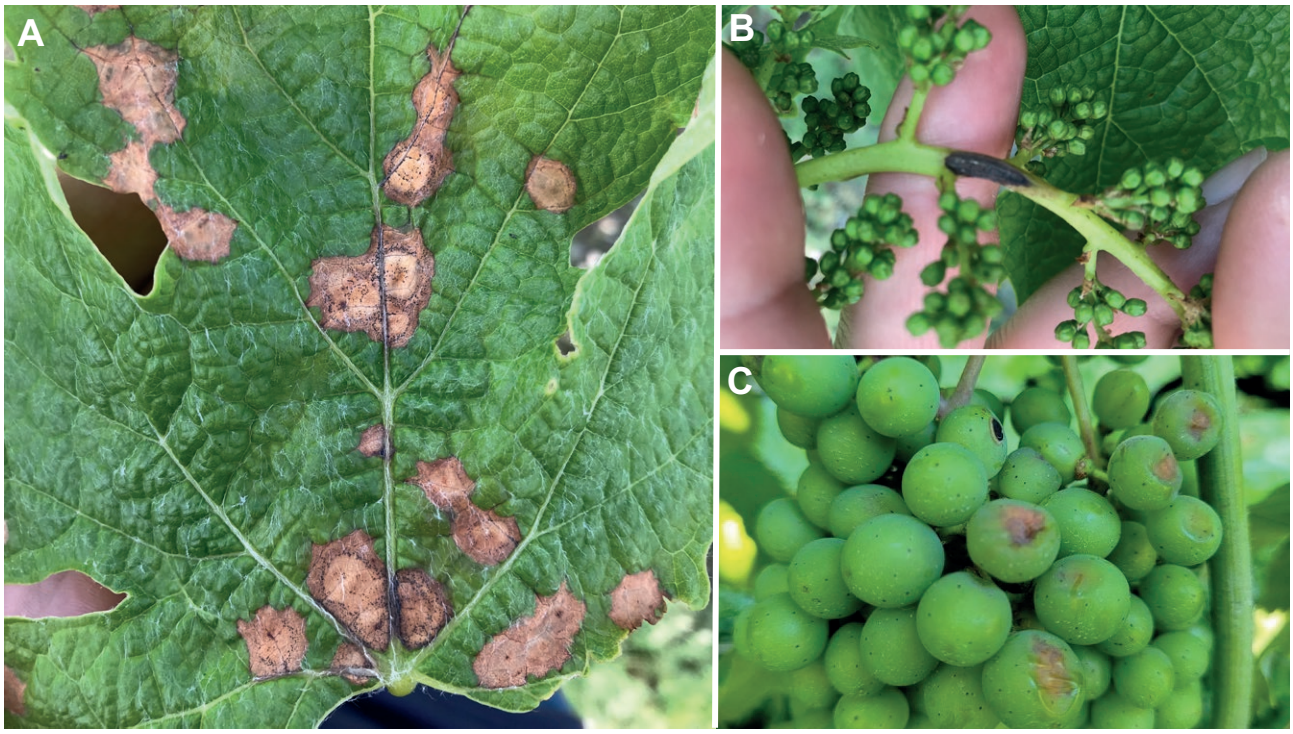

Figure 1. Symptoms on grapevine tissues caused by *Phyllosticta ampelicida*. Reddish-brown circular lesions on infected leaves, with pycnidia in the lesion centres (A), necrotic spots on the rachis (B), and brown to black spots on berries (C).

(Figure 1). Black rot is a polycyclic disease with repeated cycles of primary and secondary infections. Three *formae speciales* (f. sp.) of "*G. bidwellii*", with different host specificities, have been described (Luttrell, 1946; Luttrell, 1948). These are: "*G. bidwellii*" f. sp. *euvitis*, which is pathogenic to *V. vinifera* and to the American bunch grape species of the section *Vitis*; "*G. bidwellii*" f. sp. *muscadinii*, which is pathogenic to *V. rotundifolia* and *V. vinifera*; and "*G. bidwellii*" f. sp. *parthenocissi*, which is pathogenic to *Parthenocissus* spp. High genetic variability has been found among *P. ampelicida* isolates collected from different geographic areas (Narduzzi-Wicht *et al*., 2014; Rinaldi *et al*., 2017). Describing the *P. ampelicida* genome sequence is an important step toward enhancing understanding of the grapevine and *P. ampelicida* interaction, and will provide a basis for pathogenicity mechanism studies and development of disease management strategies.

We report the genome sequencing and assembly of the *P. ampelicida* PA1 Galicia CBS 148563, which was isolated from diseased leaves of 25-year-old *V. vinifera* 'Mencia', in Leiro-Ourense (Galicia, Spain). The strain was purified by single-spore isolation and maintained on potato dextrose agar (PDA) medium at 25°C in the darkness. DNA was extracted with NucleoSpin Tissue (Macherey-Nagel), following the manufacturer's protocol. Firstly, the ITS regions, including the 5.8S gene, were amplified with ITS1/ITS4 (White *et al*., 1990), the amplicon was then sequenced according to Eichmeier *et al*. (2010), and the sequence was submitted to GenBank (Accession No. MZ914563).

The same DNA was used for genome library construction using the Nextera XT DNA Library Preparation Kit (Illumina Inc.). The library was sequenced using MiniSeq High Output Reagent Kit (300-cycles) (Illumina Inc.) with 2 × 150PE read option. A total of 14,796,001 high-quality reads passed the filter. The sequence quality was checked using the FastQC-0.10.1 program (Andrews, 2010). A FASTX-Toolkit Clipper (http://hannonlab.cshl.edu/fastx_toolkit/), specifying the Q33 parameter, was used to remove the adaptors, and low-quality reads were discarded. Contigs of individual reads were assembled *de novo* using the SPAdes genome assembler v. 3.15.2 (Prjibelski *et al*., 2020) with default settings. *De novo* assembly of *P. ampelicida* PA1 Galicia CBS 148563 resulted in a genome size of 30,547,631 bp, G+C content of 54.49%, and 6,675 contigs, with a scaffold length at which 50% of the total assembly length is covered (N50) value of 20,626 bp and the number of contigs whose summed length is N50 (L50) of 428.

The *ab initio* gene prediction using Augustus (Keller *et al*., 2011) (-species = botrytis_cinerea -strand = both -gene model = complete) for the assembled genome of *P. ampelicida* PA1 Galicia resulted in 31,876 exons and 10,691 predicted coding sequences. Using BUSCO 5.2.2 (Manni *et al*., 2021), 745 complete single-copy proteins were identified with known functions (Supplementary Data).

Carbohydrate-active enzymes (CAZymes) that play vital roles in breakdown of host cell wall components establish successful infections were predicted, using CAT and dbCAN servers (Yin *et al*., 2012). Fifty-eight signal peptides were detected by HMMER (Zhang and Wood, 2003) using dbCAN (Supplementary data). Signal peptides act as zip codes marking the protein secretion pathway as well as the protein target location. In addition to protein targeting, a number of critical functions with or without regard to the passenger proteins have been attributed to signal peptides (Owji *et al*., 2018). A total of 43,636 translated amino acid sequences was predicted by FragGeneScan (Rho *et al*., 2010). Using Hotpep analysis (Busk *et al*., 2017), 4,914 hits of CAZyme sequences were detected. The most represented CAZymes belonged to two groups (GT41 and GT48) of glycosyl transferases. Fungal glycosyl transferases may facilitate pathogenesis of plants by enabling hyphal growth on solid surfaces, a phenomenon previously reported by King *et al*. (2017). Further classification of CAZymes based on their catalytic activity showed a high proportion of glycosyl hydrolases (39.4%), followed by glycoside transferases (31.2%), auxiliary activities (12.8%), carbohydrate-binding modules (12.8%), carbohydrate esterases (2.1%) and polysaccharide lyases (1.7%). Using MicroStation Reader BioTek ELx808BLG (Biolog Inc.) and carbon sources (CS) in FF MicroPlate (Biolog Inc. USA), consumption was detected of 72 CS by *P. ampelicida* PA1 Galicia CBS 148563 (Supplementary Data). This fungus is not included in any database of Biolog Inc.

Secondary metabolites are essential for fungal growth and development, providing protection against various environmental stresses (Calvo *et al*., 2002). The search for secondary metabolite clusters using antiSMASH fungal version (Blin *et al*., 2017) revealed the presence of 17 clusters (10 NRPS and NRPS-like, 3 T1PKS, 3 terpene and 1 betalactone).

The draft genome of *P. ampelicida* PA1 Galicia CBS 148563 reported here are of high-quality genome assemblies, which can serve as reference genomes for other species or strains within the family *Phyllostictaceae*. The genome of *P. ampelicida* PA1 Galicia CBS 148563 reported here has been deposited in GenBank under Acc. No. JAIFKG000000000.1 (BioProject No. PRJNA753299).

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Research Papers

Fig rust caused by *Phakopsora nishidana* in South Africa

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Summary. Fig rust, caused by *Cerotelium fici*, was first recorded in South Africa in 1927. Recent observations have revealed high incidence of rust and untimely defoliation of fig trees (*Ficus carica*) in residential gardens and commercial orchards. Using phylogenetic analysis, the causal organism of a fig rust isolate (PREM63073) collected in 2020 was confirmed as *Phakopsora nishidana*. Inoculation and microscope studies showed that mulberry plants were immune to *P. nishidana* isolate PREM63073. Infection of fig leaves occurred through stomata on the abaxial leaf surfaces. Very long germ tubes were observed for *P. nishidana*, often with no clear contact with the leaf surfaces and an apparent lack of directional growth towards stomata. Inoculated plants from 15 fig cultivars varied in their severity of leaf infection, whereas fruit of the cultivar Kadota developed reddish-brown blemishes without sporulation. Currently, *C. fici* and *P. nishidana* are recognised as occurring on *F. carica* in South Africa. This suggests a need to resolve the worldwide distribution and identity of the rust species involved.

Keywords. *Cerotelium fici*, *Ficus carica*, host response, infection, phylogenetic analysis.

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INTRODUCTION

Ficus belongs to the *Moraceae*, which contains 60 genera and at least 1400 species (Gerber, 2010). The edible fig, *Ficus carica* L., one of approximately 750 *Ficus* species (Lötter, 2014), is native to the Middle East and Mediterranean regions and thrives where winters are mild and summers are hot and dry (Verga and Nelson, 2014). In 2019, the top fig producing countries, with a combined harvest of just over 1.1 million tons, were Turkey, Egypt, Morocco, Iran, Algeria, Spain, Syria, United States (mostly California), Tuni-

sia, and Afghanistan (https://fao.org/faostat, accessed 16 Feb 2021). According to the South African Fig Producers Association, South Africa (SA) ranks 33rd in global production of figs, exporting 336 tons in 2018/19 and 239 tons in 2019/20, to various destinations (https://www.safigs.co.za/links/, accessed 28 April 2021).

According to Karsten (1951), Godée Molsbergen mentioned figs as one of the many cultivated plants introduced to the South African Cape region between 1652 and 1662. Initially, fig production was restricted to individual trees or single rows or avenues on farms and residential gardens. In the absence of commercial production during the 18th and 19th centuries, fresh figs were used mostly for local consumption, but also as dried products and different types of preserve (Lötter, 2014). In 1902/03, the Cape Department of Agriculture imported 140 'Smyrna' and 'Capri' trees of different cultivars (e.g., Calimyrna, Kassaba, Bardajie, and Capri No. 1, No. 2 and No. 3), providing momentum to the local industry. Once the trees were established, 'Capri' cuttings with fruit containing *Blastophaga* wasps were imported under carefully controlled conditions for the insect to serve as a pollinating vector. South African fig production, located mainly in the Western Cape Province, varied considerably over the years (Lötter, 2014). Over the past 50 years attempts to revitalize the fig industry were launched by establishing experimental blocks and importing additional cultivars from California and France.

Doidge (1927; 1950) listed fig rust specimens collected from Mozambique, Zimbabwe, and the Eastern Cape, Free State, KwaZulu-Natal, North West, and Western Cape provinces in SA as belonging to *Cerotelium fici* (Castagne) Arthur. In addition, Verwoerd (1929) reported the occurrence of *C. fici* in the Western Cape, specifically in Wynberg, Paarl and Stellenbosch, and also from drier localities such as Barrydale, Ladismith and Oudtshoorn.

Fig rust symptoms are first visible as flecks on upper leaf surfaces, followed by the appearance of sporulating pustules on the lower surfaces. Pustules can occur over entire leaf blades, but are usually clustered in areas where moisture accumulates (McKenzie, 2013). Eventually, the spots on the upper surface of each affected leaf become brown and angular and may coalesce to form larger necrotic areas, leading to defoliation under conditions of severe infection. Fruit infections are visible as blister-like rust pustules (Verga and Nelson, 2014). Mulberry (*Morus* spp.) has also been reported as a host for *C. fici*, although Laundon and Rainbow (1971) doubted the conspecificity of the rust fungi on fig and mulberry.

In 2020, a fig tree heavily infected with rust was observed in a residential garden (Figure 1). The visible impact of the disease on tree health prompted an investigation to: (i) confirm the identity of the causal organism; (ii) understand the infection process; and (iii) evaluate methods to determine the host range and response of fig cultivars to the rust isolate under controlled conditions.

MATERIALS AND METHODS

Molecular identification of the fig rust isolate

Infected fig tree leaves (cultivar 'Parisian') containing uredinia were collected on 9 February 2020 in the coastal town of Vermont, Western Cape, SA. Infected leaf tissue, accessioned as PREM63073 in the National Collection of Fungi, Pretoria, was freeze-dried. A modified cetyltrimethylammonium bromide (CTAB) method (Visser *et al*., 2009) was used to extract genomic DNA from ground leaf tissue. The 5.8S rRNA-ITS2-28S rRNA [Rust2Inv: 5'-GATGAAGAACACAGTGAAA-3' (Aime, 2006) and LR6: 5'-CGCCAGTTCTGCTTACC-3' (Vilgalys and Hester, 1990)] locus was PCR amplified, where each 10 µL reaction contained 10 ng DNA, 0.25 µM primers, a 1 × concentration of KAPA Plant PCR buffer and 0.5 U KAPA3G Plant DNA polymerase (KAPA Biosystems). The amplification regime consisted of a single 3 min denaturation step at 94°C, followed by 40 cycles of 94°C for 30 sec, 54°C for 30 sec, 72°C for 1 min, and a final 5 min elongation step at 72°C.

The amplicon was cloned into the pGem-T® Easy plasmid vector (Promega Corporation) and transferred into *Escherichia coli* JM109 competent cells. Cloned inserts of four recombinant plasmids were sequenced with the Rust2Inv, primer LR6, and internal primers LR0R (Moncalvo *et al*., 1995) and LR3 (Vilgalys and Hester, 1990), using the BigDye™ Terminator v 3.01 Sequencing kit (Thermo Fisher Scientific). Sequenced products were separated on a 3130x1 Genetic Analyzer (Applied Biosystems), using the StdSeq50_POP7 sequencing run module. Once all ambiguous nucleotides were corrected, the four overlapping sequences of each recombinant clone were assembled into a single contiguous sequence, using the online CAP3 Sequence Assembly Program (Huang and Madan, 1999). The final consensus sequence was deposited on GenBank (accession No. MZ047090).

The two allelic sequences were aligned with selected reference sequences (Table 1), using the online MAFFT web interface (Katoh *et al*., 2019). *Helicobasidium longisporum* was used as the outgroup (Maier *et al*., 2016). After trimming the 5' and 3' extending sequences, the resulting 1036 bp ITS2-28S rRNA sequences were used

Figure 1. Rust damage on leaves of a fig tree in a residential garden in Vermont, Western Cape, South Africa, in February 2020.

to determine the appropriate nucleotide substitution model with the Akaike Information Criterion (AIC) within jModelTest v 2.1.1 (Darriba *et al.*, 2012; Guindon and Gascuel, 2003). The suggested TIM2 + G + I (n = 5) nucleotide substitution model was replaced with the GTR + G + I (n = 5) model (Lecocq *et al.*, 2013) for Bayesian inference (BI) analysis in MrBayes v 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). BI analysis was started from a random tree using four Markov Chain Monte Carlo (MCMC) chains. The search was limited to 5,000,000 searches, where every 500th generation was sampled. The average standard deviation of split frequencies was examined and the analysis stopped at a value below 0.01. The first 1000 trees were discarded as burnin before analysis.

Maximum parsimony (MP) analysis was carried out in PAUP* v. 4.0b10 (Swofford, 2003), where all characters were weighted equally. The heuristic search was carried out with 1000 addition-sequence replicates, with tree bisection and reconnection (TBR) branch swopping. Ten trees per replicate were saved. Bootstrap support for proposed branches was evaluated with 1000 replicates with 100 random addition-sequence replicates and TBR branch swopping.

Spore and pustule morphology

The widths and lengths of 60 urediniospores of isolate PREM63073 were measured with an Olympus BX53 light microscope, fitted with a DP72 digital camera for image capturing with Analysis LS Research version 2.2 software (Olympus Soft Imaging System). Free-hand cross-sections of pustules (uredinia and telia) were made after soaking small pieces of dried leaves in hot water for a minimum of 30 min. Spores and pustules were examined with either a Zeiss Axioscope or a Nikon E600 microscope.

Host infection studies

During early spring 2020, young plants (< 1 m height) of 15 edible fig cultivars and a Queensland Red mulberry plant (*Morus rubra* L.) were grown in 10 L

Table 1. Taxa used in the molecular identification of the Vermont fig rust isolate PREM63073. Allelic variants of the isolate identified in the present study are indicated in bold font.

Taxon	Host	Isolate	Country	GenBank	Reference
Batistopsora crucis-filii	*Annona* sp.	BPI:863563	Guyana	DQ354539	Aime, 2006
Batistopsora crucis-filii	*Annona tomentosa*	PUR:87629	[a]-	KF528023	Beenken, 2014
Batistopsora pistila	*Annona sericea*	BPI:863563	-	KF528028	Beenken, 2014
Batistopsora pistila	*Annona spraguei*	PUR:66577	-	KF528029	Beenken, 2014
Cerotelium fici	*Ficus coronulata*	BRIP:56890	Australia	MH047209	Unpublished
Cerotelium fici	*Ficus coronulata*	BRIP:59463	Australia	MH047210	Unpublished
Cerotelium fici	*Ficus* sp.	BRIP:58068	Australia	KP753385	Maier *et al*., 2016
Cerotelium fici	*Ficus* sp.	LAH20019AM	Pakistan	MK135779	Unpublished
Cerotelium fici	*Ficus* sp.	LAH20025AM	Pakistan	MK135780	Unpublished
Coleosporium plumeriae	*Plumeria rubra*	BRIP:55387	Australia	KM249866	McTaggart *et al*., 2014
Helicobasidium longisporum	-	AFTOL-ID	-	AY885168	Unpublished
Crossopsora fici	*Ficus* sp.	BRIP:56872	Australia	MH047208	Unpublished
Crossopsora fici	*Ficus virens* var. *sublanceolata*	BRIP:58118	Australia	MH047207	Unpublished
Phakopsora annonae-sylvaticae	*Annona sylvatica*	PUR:87311	Brazil	KF528008	Beenken, 2014
Phakopsora cherimoliae	*Annona cherimola*	ZT:RB3096	-	KF528011	Beenken, 2014
Phakopsora cherimoliae	*Annona cherimola x squamosa*	PUR:89695	-	KF528012	Beenken, 2014
Phakopsora cingens	*Bridelia tomentosa*	BRIP:55628	Australia	KP729474	Maier *et al*., 2016
Phakopsora gossypii	*Gossypium* sp.	BPI:910191	USA	KY764073	Unpublished
Phakopsora jatrophicola	*Jatropha* sp.	BPI:910194	USA	KY764078	Unpublished
Phakopsora jatrophicola	*Jatropha curcas*	BPI:910195	USA	KY764079	Unpublished
Phakopsora meibomiae	*Aeschynomene* sp.	R188	Colombia	EU851164	Zuluaga *et al*., 2011
Phakopsora myrtacearum	*Eucalyptus grandis*	UP:217	Mozambique	KP729471	Maier *et al*., 2016
Phakopsora myrtacearum	*Eucalyptus* sp.	[b]PREM:61156	Kenya	KP729472	Maier *et al*., 2016
Phakopsora myrtacearum	*Eucalyptus grandis*	PREM:61155	Kenya	KP729473	Maier *et al*., 2016
Phakopsora myrtacearum	*Eucalyptus grandis*	PREM:61155	Kenya	NG060142	Maier *et al*., 2016
Phakopsora nishidana	*Ficus carica*	BPI:910197	USA	KY764080	Unpublished
Phakopsora nishidana	*Ficus carica*	BPI:842289	USA	KY764081	Unpublished
Phakopsora nishidana (as *Cerotelium fici*)	*Ficus carica*	UACH-107	Mexico	MF580676	Solano-Báez *et al*., 2017
Phakopsora pachyrhizi	*Glycine max*	BPI:871755	Zimbabwe	DQ354537	Aime, 2006
Phakopsora pachyrhizi	*Desmodium* sp.	BRIP:56941	Australia	KP729475	Maier *et al*., 2016
Phakopsora phyllanthi	*Phyllanthus acidus*	BPI:910198	USA	KY764082	Unpublished
Phakopsora phyllanthi	*Phyllanthus acidus*	ZT:RB8581	-	KF528025	Beenken, 2014
Phakopsora rolliniae	*Annona exsucca*	NY:3237	Trinidad and Tobago	KF528036	Beenken, 2014
Phakopsora tecta	*Commelina diffusa*	BPI:843896	Costa Rica	DQ354535	Aime, 2006
Phakopsora tecta	*Commelina* sp.	BRIP:56943	Australia	KP729476	Maier *et al*., 2016
Prospodium lippiae	*Aloysia polystachya*	U152	Argentina	DQ354555	Aime, 2006
Puccinia graminis	*Glyceria maxima*	BRIP:60137	Australia	KM249852	McTaggart *et al*., 2016
Puccinia junci	*Juncus tenuis*	PDD:99243	New Zealand	KX985745	Padamsee and McKenzie, 2017
UFS20_1	***Ficus carica***	**PREM:63073**	**South Africa**	**MZ047090**	**This study**
UFS20_2	***Ficus carica***	**PREM:63073**	**South Africa**	**MZ047090**	**This study**

[a] Information not available.
[b] Mycological collection at the South African National Collection of Fungi, Pretoria, South Africa.

bags filled with a mulch mixture, and acclimatized for 3 weeks pre-inoculation in a greenhouse cubicle with a temperature range of 18 to 25°C. The plants were watered every second day with reverse osmosis water, and were each fertilised once a week with 100 mL of a 0.2% (w/v) Multifeed-Classic water-soluble fertilizer [Effekto®, NPK analysis 19:8:16 (43)]. Cuttings representative of *Morus alba* L., including common or white mulberry, as well as weeping mulberry, were sampled a few hours before inoculation from confirmed sources. These cuttings were placed in 10 L containers filled with reverse osmosis water before and after inoculation.

Urediniospores of isolate PREM63073, kept at -80°C, were used in all experiments. Three weeks before inoculation, the urediniospores were increased through the inoculation of leaves of the fig cultivar Kadota. Procedures described by Boshoff *et al*. (2020) were used to heat-shock the spores and for inoculation of plants and subsequent incubation. After 3 weeks, urediniospores were collected from the Kadota leaves into size 00 gelatine capsules, by connecting an air vacuum to a cyclone spore collector (Pretorius *et al*., 2019).

The response of fig and mulberry plants, including the mulberry cuttings, to the local rust isolate, was studied in two independent trials. During the first trial, a urediniospore concentration of 98×10^4 spores mL^{-1} in 0.3 mL Soltrol® 130 isoparaffinic oil, was applied per leaf. Two leaves were inoculated per cultivar/cutting, one inoculated on the abaxial surface and the other on the adaxial surface. In the second trial, one leaf per cultivar/cutting was inoculated on the abaxial surface, using a urediniospore concentration of 87.7×10^4 spores mL^{-1} in 0.3 mL Soltrol® 130 oil, as described above. During the second trial, replicate young developing figs from the cultivar Kadota were also inoculated using the above urediniospore concentration. Inoculated leaves on plants, young figs and cuttings were regularly observed for development of first symptoms of infection. Three weeks after inoculation, the number of rust pustules per cm^2 was counted on the abaxial leaf surfaces. This was achieved by placing a rectangular template with a 1 cm^2 opening at five random positions on each assessed leaf.

Analysis of variance (ANOVA; $\alpha = 0.05$) of host response data was performed for the number of rust pustules counted per cm^2, using 'base' R functions, where the ANOVA model 'pustules ~ cultivar + trial' was applied (N = 150). Means were separated on the Minimum Significant Difference (MSD) test, using the *LSD.test* function from the 'agricolae' package, with an adjusted Bonferroni *P*-value to account for potential family-wise error rates (De Mendiburu, 2020). Data processing and analyses were performed with R version 4.0.2 (R Core Team, 2020) within R Studio version 1.2.5042 (R Studio Team, 2020). Data exploration, wrangling and visualisation were conducted using the 'Tidyverse' package (Wickham *et al*., 2019). Data scripting was conducted in rmarkdown (Allaire *et al*., 2020).

Fluorescence and scanning electron microscopy (SEM) were used to describe the host penetration process by the pathogen. Material for microscopy included newly sprouted leaves from the susceptible fig cultivars Cape White and Kadota, as well as young developing Kadota figs. Young leaves from the mulberry cultivar, Queensland Red, and freshly collected cuttings from a weeping mulberry plant, were included for fluorescence microscopy. The abaxial surfaces of three leaves per plant/cutting were each sprayed with 0.8 mL of urediniospore suspension (±1 mg mL^{-1} urediniospores in Soltrol® 130 oil), whereas dry spores were applied with a brush to young fruit. Post-inoculation treatment, dew chamber incubation and greenhouse conditions were as described above.

Fluorescence microscopy

Leaf and fig peel segments were sampled at 48 and 96 h (hpi), and 16 d post-inoculation (dpi), and were cut into 10 mm segments. Half of the samples were prepared using a modified method of Rohringer *et al*. (1977) as described by Moldenhauer *et al*. (2006), and stained leaf segments were stored in 50% (v/v) glycerol containing a trace of lactophenol to prevent deterioration and drying of fungal material.

The remaining leaf segments were processed according to the modified method (Maree *et al*., 2020) of Ayliffe *et al*. (2011), using *Triticum vulgaris* Vill. lectin (wheat germ agglutinin) fluorescein isothiocyanate conjugate (WGA-FITC) (Sigma-Aldrich). The leaf segments were stained for 16 h at room temperature with 8.3 µg mL^{-1} (w/v) of WGA-FITC probe before viewing with an Olympus AX70 microscope (Rohringer *et al*., 1977; Kuck *et al*., 1981).

For the segments stained with Uvitex 2B, the blue wavelength epifluorescence cube with an excitation filter of 330-385 nm and a barrier filter of 420 nm showed fluorescence of stained fungal tissue. Observations of WGA-FITC stained segments were made with the ultraviolet wavelength (WU) epifluorescence cube, with an excitation filter of 450-480 nm and a barrier filter of 515 nm. The microscope was fitted with a CC12 digital camera for image capturing with Analysis LS Research version 2.2 software (Olympus Soft Imaging System).

Scanning electron microscopy

Leaf and fig peel segments were sampled at 48 and 96 hpi, and 16 dpi, and were cut into 5 mm segments. Samples were fixed according to the protocol of Glauert (1974). The dried samples were directly mounted on 12.2 mm diam. metal stubs (Cambridge pin type) using double-sided carbon tape, for observations of fruit or abaxial leaf surfaces. The mounted segments were coated with gold (± 60 nm thickness) in a sputter coater (Bio-Rad), and the specimens examined with a JSM-7800F Extreme-resolution Analytical Field Emission SEM.

RESULTS

Molecular identification of the fig rust isolate

Sequencing of multiple recombinant clones identified two ITS2-28S rRNA allelic variants of isolate PREM63073, which differed with four nucleotides within the ITS2 region over the 1054 bp amplicon. A BLASTn search with the ITS2-28S rRNA locus showed that the isolate shared closest identity to *C. fici* isolate UACH-107 (MF580676; 99% identity, 994/1002 bp), *P. nishidana* voucher BPI:910197 (KY764080; 99% identity, 1019/1021 bp) and *Phakopsora myrtacearum* voucher UP:217 (KP729471; 99% identity, 1052/1059 bp). Five *C. fici* accessions (KP753385; MH047209; MH047210; MK135779; MK135780) shared between 93 and 91% identity with the fig rust isolate.

The fig rust isolate was identified using a phylogenetic approach where the topologies of the MP (results not shown) and BI (Figure 2) trees were almost identical, except for the placement of *Phakopsora gossypii*. A clade with excellent support (99% bootstrap (BS); 1.0 posterior probability (PP)) contained several isolates of *Phakopsora cherimoliae*, *P. rolliniae*, *P. annonae-sylvaticae*, *Batistopsora pistila* and *B. crucis-filii*. These were all collected from different *Annona* species hosts from the pawpaw/sugar apple family (*Annonaceae*).

Sister to this, was a clade that grouped both allelic variants of PREM63073 with one *C. fici* (likely incorrectly identified) and six other *Phakopsora* isolates. This clade

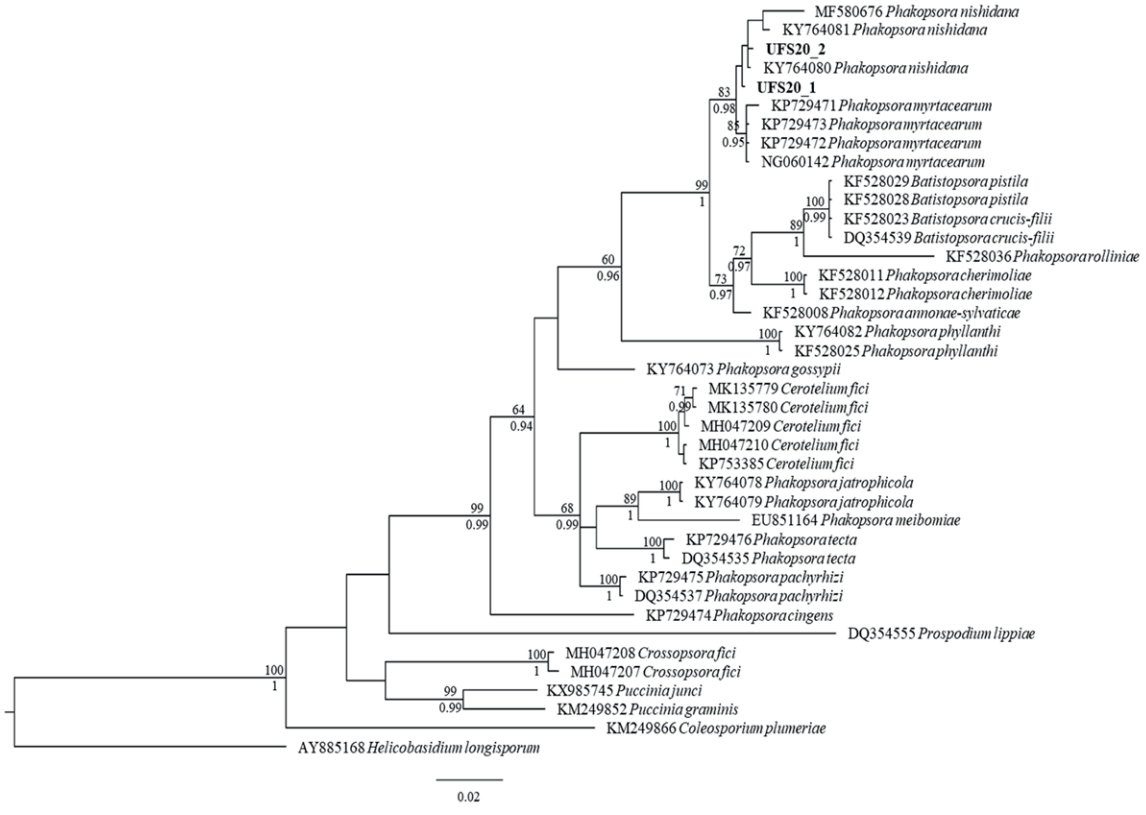

Figure 2. Identification of the Vermont fig rust (isolate PREM63073) using Bayesian inference (BI) and Maximum parsimony (MP) analyses within MrBayes and PAUP*, using the ITS2-28S rRNA locus. The two allelic variants for rust isolate PREM63073 are indicated in bold font. Bootstrap (BS) support values (>60%) are indicated above the nodes, and posterior probability (PP) values (>0.9) are indicated below the nodes.

was divided into two sub-clades (83% BS; 0.98 PP). The first sub-clade contained both PREM63073 variants, and three *P. nishidana* accessions that were all collected from *F. carica* trees from, respectively, SA, the USA and Mexico. The second sub-clade contained four *P. myrtacearum* isolates, all collected from *Eucalyptus* trees in eastern Africa.

The other five *C. fici* isolates grouped as a separate sub-clade (68% BS; 0.99 PP), within a larger clade (64% BS; 0.94 PP) containing *P. jatrophicola* (collected from *Jatropha* sp.), *P. pachyrhizi* (*Glycine max* and *Desmodium* sp.), *P. meibomiae* (*Aeschynomene* sp.) and *P. tecta* (*Commelina* sp.). The first of these five *C. fici* isolates (KP753385) formed part of a study on *P. myrtacearum* on eucalyptus trees in southern and eastern Africa, where the pathogen origin was indicated as a *Ficus* sp. in Australia (Maier *et al*., 2016). The other four were direct submissions to GenBank as unpublished studies. The first two of these accessions (MH047209 and MH047210) were part of a study of *Uredo morifolia* on mulberry in Australia, where these isolates were collected from *F. coronulata*. The other two *C. fici* accessions (MK135779 and MK135780) were collected from *Ficus* spp. in Pakistan.

Spore and pustule morphology

Uredinia were scattered over the abaxial leaf surfaces, singly but frequently becoming crowded. The surrounding leaf tissue was darkly melanised. Telia were visible as dark brown crusts surrounding many uredinia in two of the specimens examined (Figure 3, A). The uredinia were sub-epidermal, erumpent (Figure 3, B), and telia were subepidermal and non-erumpent (Figure 3, C). Paraphyses (Figure 3, D) were present at the peripheries and scattered through the uredinia. The paraphyses were hyaline, cylindrical, wall ≈0.5 µm thick, 25–48 µm long, 5–8 µm wide. The telia were typical *Phakopsora*-like, forming subepidermal, non-erumpent crusts two to five cells deep. Telial cells were light brown, not in distinct columns, 12–20(–28) × 6–10 µm, with smooth walls 1 µm thick though slightly thickened in the uppermost cells to 1.5–2 µm (Figure 3, E). Urediniospores (Figure 3, F and G) were hyaline, and globose, ellipsoid or obovoid in shape, and measured (17.4–)19–26(–32.8) × (14.9–)16–19(–20.8) µm (means = 17.5 × 23.5 µm). Their walls were ≈1 µm thick, with no readily visible germ pores.

Infection studies

No symptoms or signs developed on the inoculated leaves of mulberry cultivar Queensland Red or on the cuttings from the common or weeping mulberry plants. This apparent immunity of the tested mulberry plants was confirmed by fluorescence microscopy. Observations at either 48 or 96 hpi showed random distribution of appressoria, occasionally over stomata, on the mulberry leaves. Here, abortion of substomatal vesicles without development of haustorium mother cells (HMCs) (Figure 4, A) or non-penetrating appressoria (Figure 4, B), were observed.

Fig leaves inoculated on the adaxial surfaces did not develop meaningful infections (zero to trace infection), and the leaves remained mostly symptomless. The first signs of infection became visible as minute reddish-brown flecks for leaves inoculated on the abaxial surfaces, 7 dpi. The latent period, indicated by the first signs of sporulation, varied among cultivars, and was 10 dpi for Brunswick and Noire de Caromb. This was followed by 11 dpi for Tiger, Black Mission, Dalmatie, Ronde de Bordeaux, Col de Dame Noir, Parisian, Deanne, Adamsvy and Cape White at 11 dpi; 12 dpi for Kadota and Cape Brown; 13 dpi for Black Genoa; and 14 dpi for White Genoa. The ANOVA indicated no statistically significant differences ($F = 3.7$, $P = 0.06$) between the two experimental trial replicates for mean numbers of pustules per cm^2. However, significant differences were detected between the host cultivars ($F = 156.3$, $P < 0.05$). Cape White, Noire de Caromb (mean >20 pustules per cm^2) and Tiger (mean > 15 pustules per cm^2) were the most severely affected cultivars (Figure 5). While the Cape White and Noire de Caromb cultivars did not differ ($P > 0.05$) from one another, they were different from Tiger. The remaining cultivars, except White Genoa, produced mean numbers of pustules per cm^2 between 5.0 and 13.6, with three clusters of responses indicated by mean separation. White Genoa responded the least to inoculation, producing a mean of 3.2 pustules per cm^2. Rust severity (27 dpi) of infected leaves for cultivars Noire de Caromb (Figure 6, A), Brunswick (Figure 6, B), Adamsvy (Figure 6, C), Black Genoa (Figure 6, D), and 50 dpi for Brunswick on the adaxial (Figure 7, A) and abaxial (Figure 7, B) leaf surfaces, indicated severe disease on some cultivars.

Inoculated figs did not show typical rust signs or symptoms. However, as the fruit matured, reddish-brown blemishes became visible on the peels (Figure 8, A). Although germinated urediniospores were visible with SEM at 48 hpi on the skin of young Kadota fruit (Figure 8, B), sometimes close to stomata, no appressoria were observed. At 96 hpi, the germ tubes were collapsed with no signs of infections. Instead, blister-like structures, usually associated with host stomata in the urediniospore treated areas, but not necessarily with presence of rust spores, were observed at 96 hpi (Figure

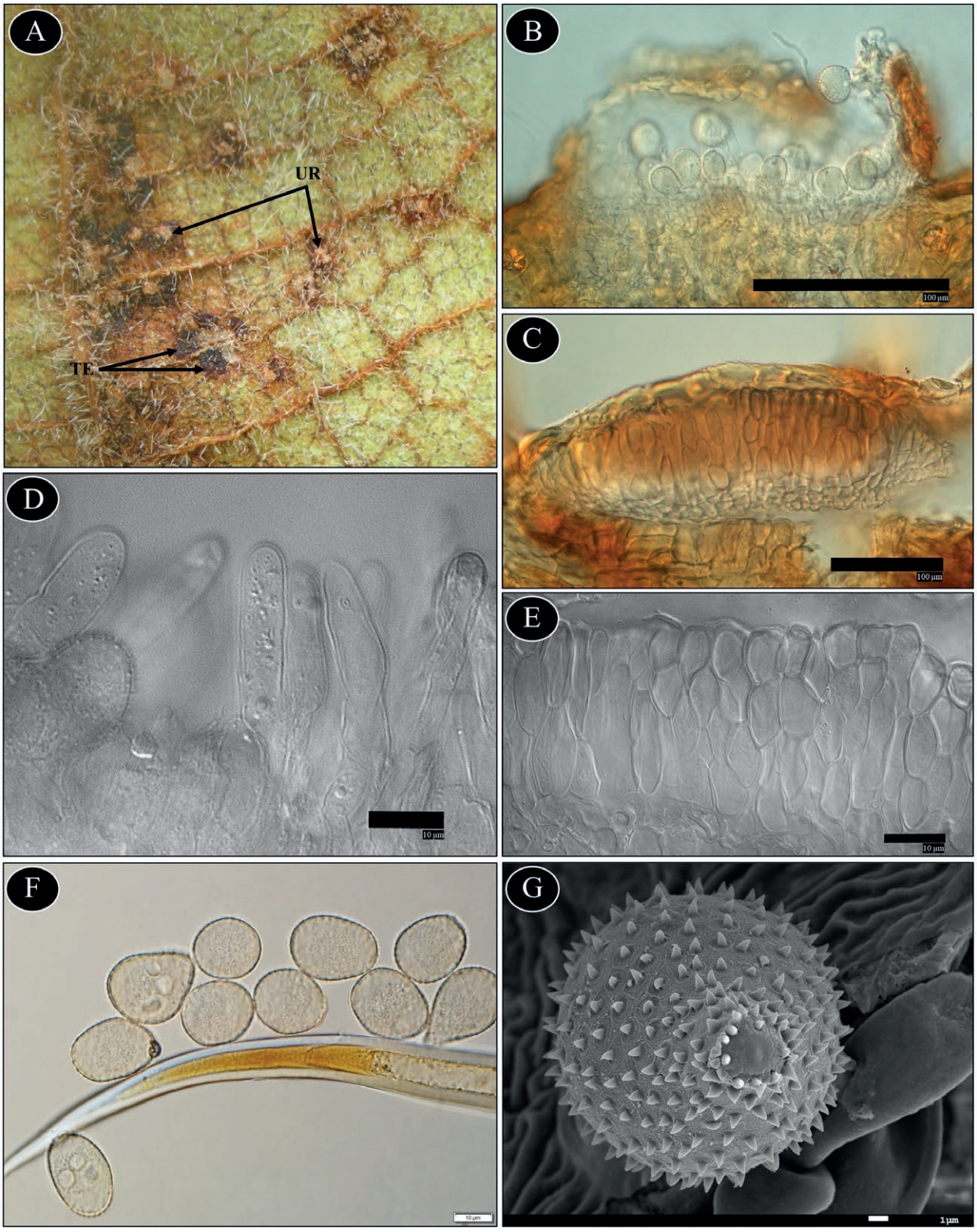

Figure 3. A, Uredinia (UR) and telia (TE) of *Phakopsora nishidana* on the abaxial surface of a leaf of *Ficus carica*. B, transverse cross section through a subepidermal erumpent uredinium, and C, a non-erumpent telium. D, thin-walled cylindrical paraphyses in a uredinium of *P. nishidana*. E, teliospores, F, thin-walled urediniospores (germ pores are obscure), and G, scanning electron micrograph showing ornamentation and hilum of a *P. nishidana* urediniospore. Scale bars in B to E represent values indicated below each bar.

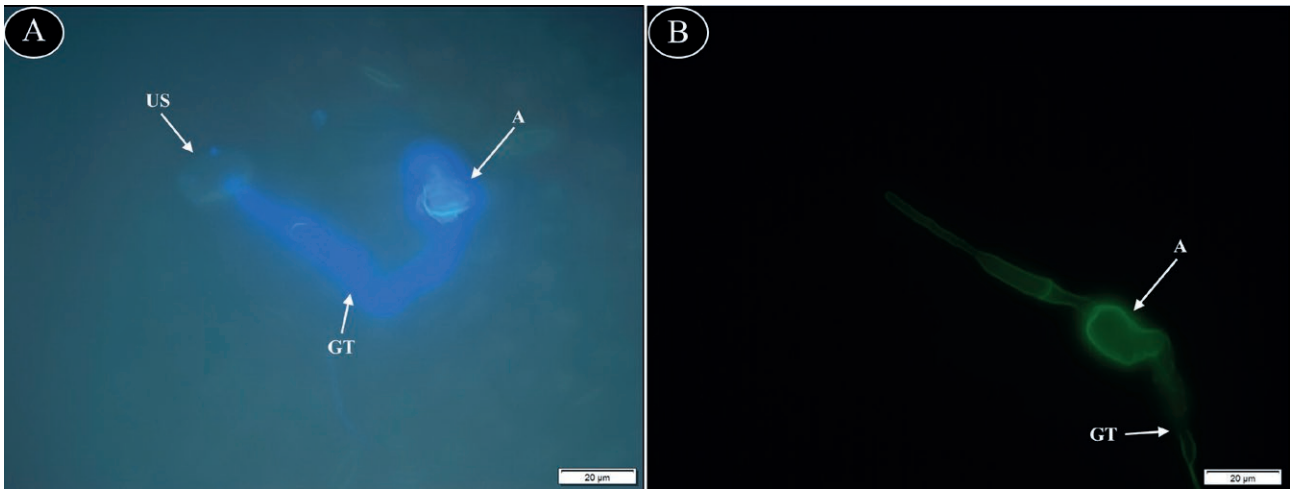

Figure 4. Microscope images of *Phakopsora nishidana* urediniospore germination, taken at 96 h post-inoculation, from A, Queensland Red mulberry leaf, indicating an early abortion, and B, mulberry, showing a non-penetrating appressorium. The urediniospore (US), germ tubes (GT) and appressoria (A) are indicated.

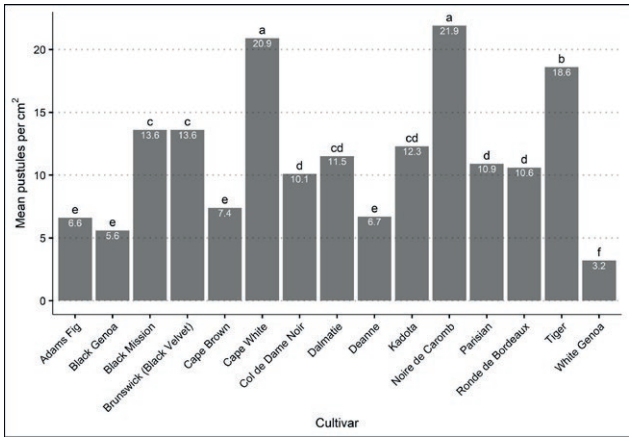

Figure 5. Mean numbers of rust pustules per cm^2 for 15 fig cultivars after inoculation with *Phakopsora nishidana*. Mean values, indicated by annotation in the bar, followed by the same letter for each cultivar did not differ ($\alpha < 0.05$), according to Fishers MSD, where MSD = 2.24 pustules per cm^2.

8, C). Inoculated areas of the fruit surfaces had sunken lesions at 14 dpi (Figure 8, D).

Light microscopy showed that leaves of Cape White and Kadota all had infection structures, typical of those formed on leaf surfaces by rust fungi during the first phases of infection cycles. At 48 hpi, these structures included germinated urediniospores, germ tubes, and appressoria forming over stomata on the abaxial leaf surfaces (Figure 9, A and B). As each appressorium matured, it was delimited from the germ tube by a septum (Figure 9, B and C) followed by collapse of the germ tube. Most of the appressoria were collapsed on top of stomata at 96 hpi (Figure 9, C). The *P. nishidana* germ tubes were very long, often extending from one trichrome to another with no apparent contact with the leaf surface (Figure 9, D). Formation of appressoria was not necessarily on the first encountered stomata (Figure 9, E), and appeared to be random. With fluorescence microscopy (not shown), only a few HMCs (mostly one or two) were visible at 48 hpi, whereas at 96 hpi, small colonies (approx. 30 HMC's) were observed. At 14 dpi, established colonies filled with urediniospores were common (Figure 9, F).

DISCUSSION

Phakopsora nishidana S. Ito is here recorded from SA, the first confirmation of this species occurring in Africa. This is the second rust fungus to be reported on cultivated fig in SA; the other species is the widely distributed fig rust, caused by *C. fici* (Doidge, 1927, 1950). The phylogenetic analysis of the present study showed that both these species belong to the *Phakopsoraceae*, as currently delimited (Aime and McTaggart, 2020). *Phakopsora nishidana* is in a distinct clade to that containing the type species, *P. pachyrhizi* Syd. & P. Syd. (Aime and McTaggart, 2020), and therefore should be assigned to a new genus. This has not been conducted in the present study, as more species need to be included in analyses before generic limits within the *Phakopsoraceae* can be accurately assessed. *Cerotelium fici* is, in the present analysis, a sister clade to *Phakopsora sensu stricto*, and

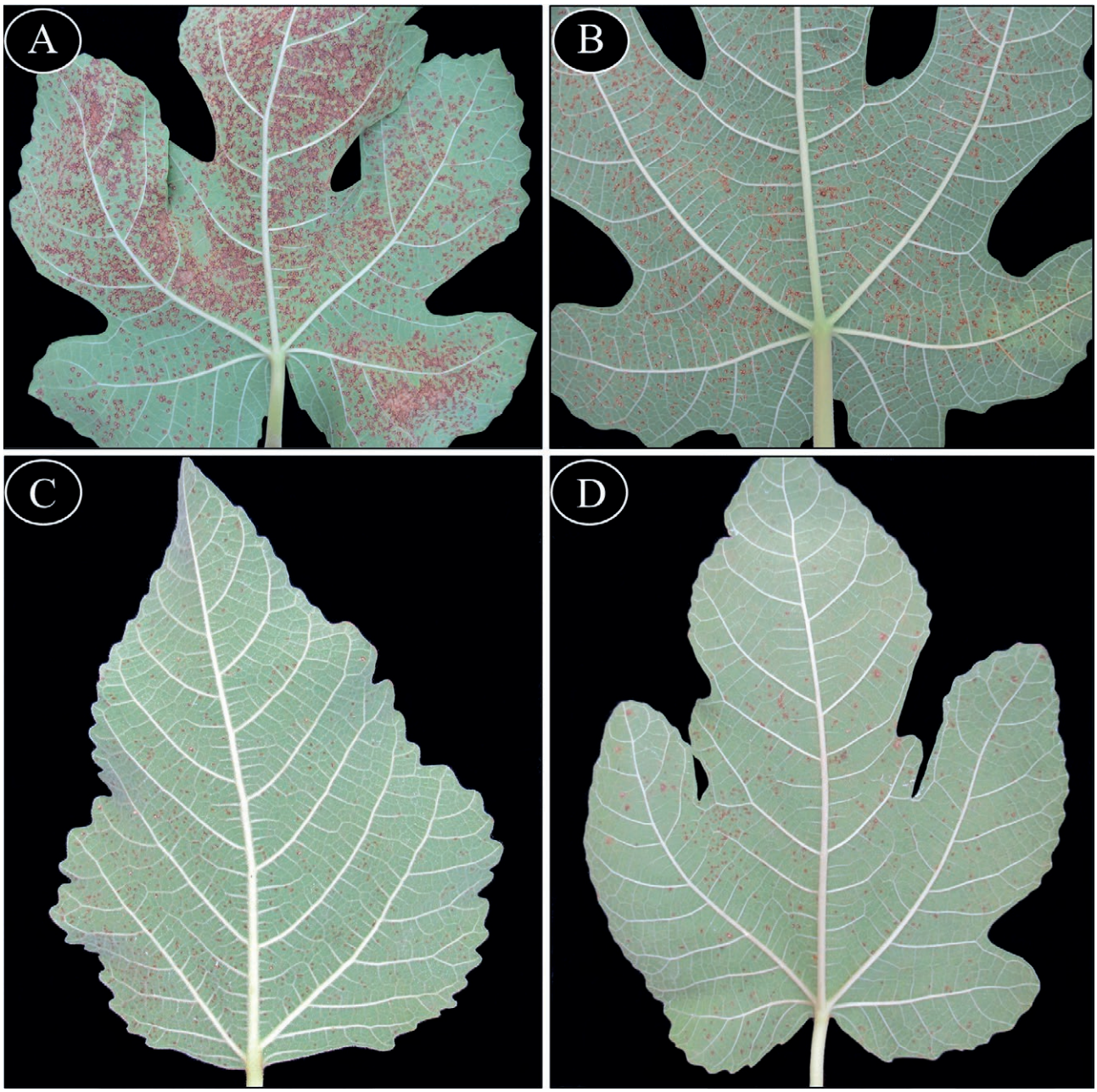

Figure 6. Fig rust symptoms on the abaxial surfaces of leaves from the fig cultivars Noire de Caromb (A), Brunswick (B), Adamsvy (C) and Black Genoa (D), 27 d post inoculation with urediniospores of *Phakopsora nishidana*.

may therefore be transferred to this genus depending on results of additional analyses.

The application of the names *Cerotelium fici* and *Phakospora nishidana* has been confused, with some workers applying the *C. fici* for the widely distributed fig rust pathogen (e.g. Laundon and Rainbow, 1971; McKenzie, 1986), whereas others have applied *P. nishidana* (e.g. Buritica, 1999; Hennen *et al.*, 2005; De Carvalho *et al.*, 2006). This resulted from differing interpretations of the species to which the first described spore stage, *Uredo fici* Castagne, belongs. Here, this is assigned to *C. fici*. The paraphyses of *P. nishidana* were described as both peripheral and intermixed with the urediniospores (Ito and Homma, 1938). In contrast, the uredinia of *U. fici* are surrounded by paraphyses (malupa-like) (Arthur, 1906), as described for *C. fici* by Butler (1914). In addi-

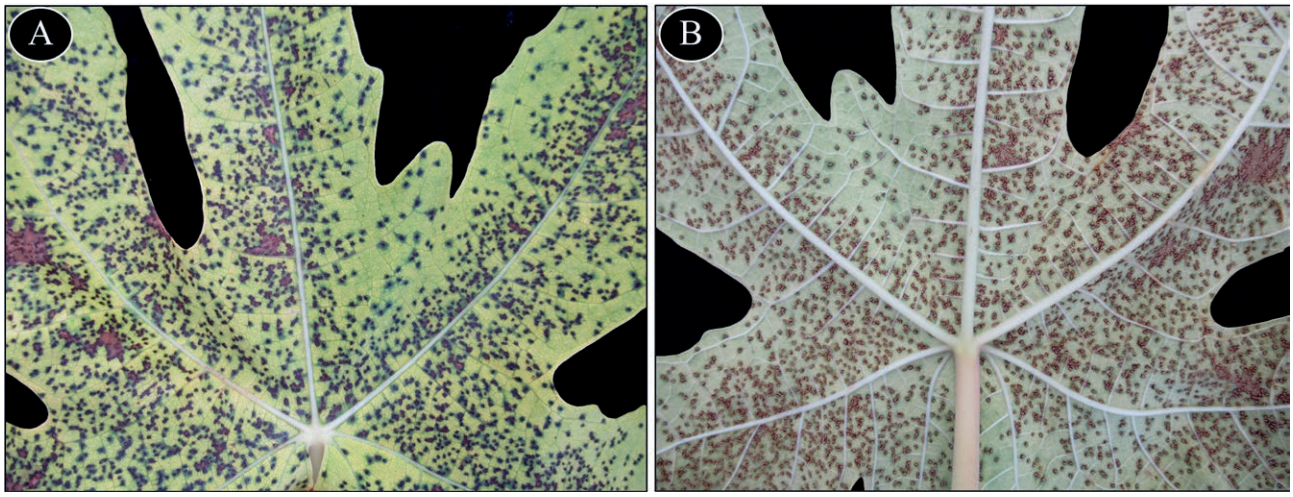

Figure 7. Fig rust symptoms on adaxial (A) and abaxial (B) leaf surfaces of the fig cultivar Brunswick, 50 d post inoculation of the abaxial surfaces with urediniospores of *Phakopsora nishidana*.

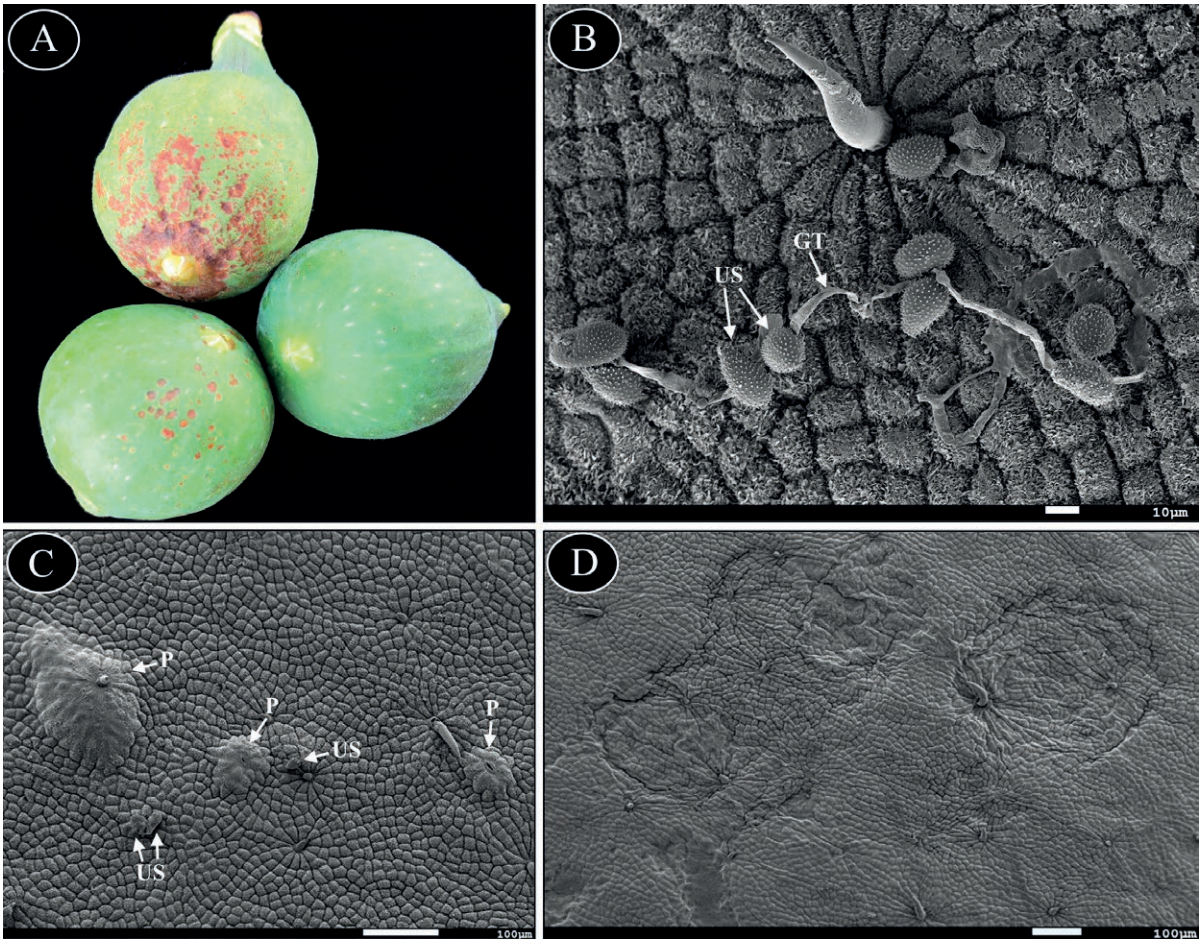

Figure 8. A, Fruit of fig cultivar Kadota after applying dry urediniospores of *Phakopsora nishidana* to young developing figs, showing blemishes (top and bottom left fruits), while the fig on the right represents an uninoculated control. B and C, scanning electron micrographs (SEMs) 96 h post inoculation, of a germ tube (GT), urediniospores (US) and blister-like pustules (P). D, SEM of sunken leaf lesions at 16 d post inoculation.

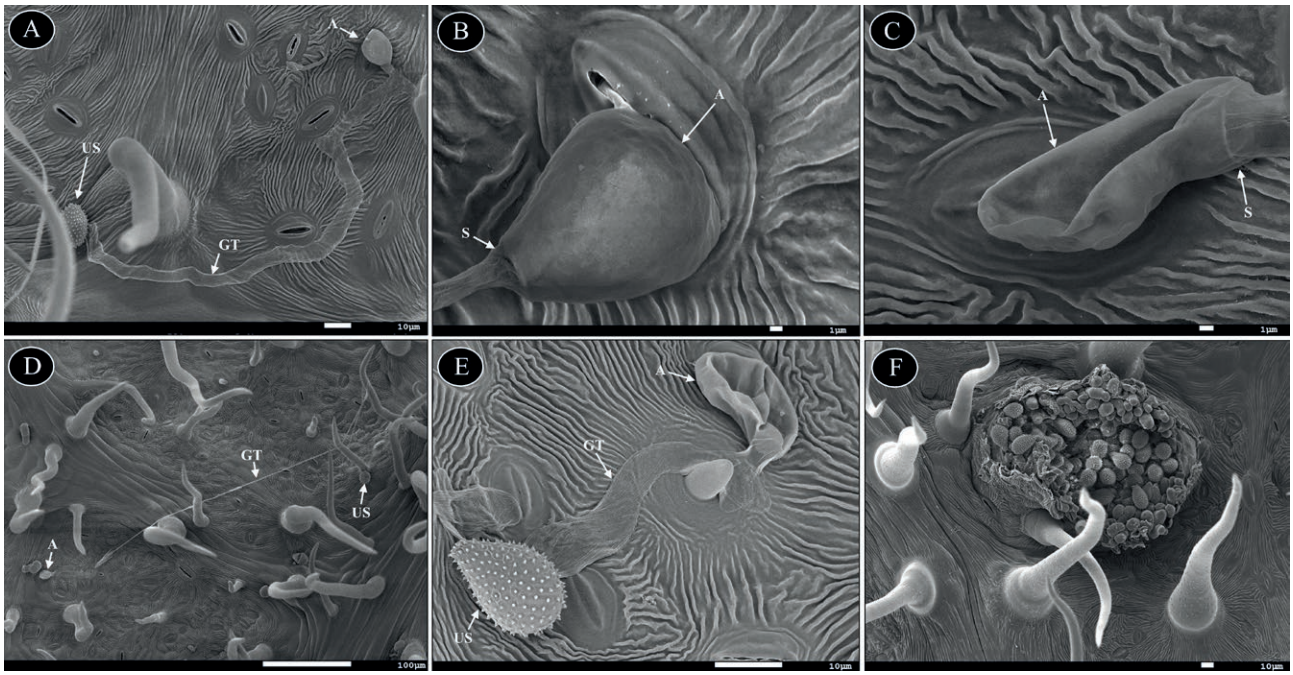

Figure 9. Scanning electron micrographs of *Phakopsora nishidana* on abaxial fig leaf surfaces, at 48 hpi (A and B), 96 hpi (C, D and E) and 16 dpi (F). Appressoria (A), germ tubes (GT), septum (S) and urediniospores (US) are indicated.

tion, Ito and Murayama (1949) assigned *U. fici* as the uredinial stage of *Phakopsora fici-erectae* S. Ito & Y. Otani ex S. Ito & Muray., not *P. nishidana*. However, *P. nishidana* was not recorded from *F. carica* (Ito and Murayama, 1949), the type host of *U. fici*. The uredinial morphology of *P. fici-erectae* should be re-examined and contrasted to that of *C. fici*, as this was beyond the scope of the present study.

The correct synonomy for the two species considered, is as follows:

***Cerotelium fici* (Castagne) Arthur, Bull. Torrey Bot. Club 44: 509 (1917)**
basionym: *Uredo fici* Castagne, Pl. Crypt. Nord France, Edn 3 34: no. 1662 (1848)
≡*Malupa fici* (Castagne) Buriticá, Revta ICNE, Instit. Cienc. Nat. Ecol. 5(2): 175 (1994)
Physopella fici (Castagne) Arthur, Résult. Sci. Congr. Bot. Wien 1905: 338 (1906)
Kuehneola fici (Castagne) E.J. Butler, Annls mycol. 12(2): 79 (1914)

Note: under the previous rules of Nomenclature, a teleomorph name could not be based on an anamorph type specimen, and therefore this species was correctly cited as *Cerotelium fici* (E.J. Butler) Arthur, as Butler's *K. fici* was then the correct basionym. However, since the principle of 'one fungus one name' has been applied, the first correctly published name becomes the basionym, even if for an anamorph.

***Phakopsora nishidana* S. Ito, Trans. Sapporo Nat. Hist. Soc. 15: 117 (1938)**
Phakopsora fici Nishida, Engei no Tome 7: 881 (1911) *nomen nudum*

Besides these species, another, *Uredo sawadae* S. Ito (Farr and Rossman, 2021) has been described from *F. carica*. Other rust species described from various *Ficus* species, other than *F. carica*, include *Phakopsora fici-elasticae* T.S. Ramakr., *Cerotelium ficicola* Buriticá & J.F. Hennen, *Crossopsora fici* Arthur & Cummins (Farr and Rossman, 2021) and various *Uredo* species that must be assigned to one of these listed telial species or as yet undescribed species.

Cerotelium fici often occurs wherever cultivated figs are produced (Laundon and Rainbow, 1971; McKenzie, 1986; Latinović *et al.*, 2015). Except for the reports of Doidge (1927; 1950), Verwoerd (1929) and Lötter (2014), no published information on fig rust in SA could be found. As the description provided by Doidge (1927) corresponds to other accounts of *C. fici* (Laundon and Rainbow, 1971; McKenzie, 1986, 2013), the historical context and urediniospore morphology suggested that

the Vermont rust isolate could be *C. fici*. Phylogenetic analysis, however, clearly differentiated between *C. fici* and *P. nishidana*. The close association of molecular data of the local fig rust examined with two *P. nishidana* accessions confirmed that isolate PREM63073 was *P. nishidana* and not *C. fici*. The placement of the Mexican *C. fici* isolate UACH-107 (GenBank accession MF580676; Solano-Báez *et al*., 2017) within this subclade indicated that it was incorrectly identified, due to a lack of adequate reference sequences. The morphology described for this isolate (Solano-Báez *et al*., 2017) is consistent with that of the uredinial state of *P. nishidana*, though the urediniospores were slightly larger (23–27 × 16–19 µm) than originally described for this species (18–24 × 16–18 µm; Ito and Homma, 1938). One of the reference sequences for *P. nishidana* (KY764080) was of a specimen that originate from Mexico (BPI 910197), confirming the presence of this species in that country. Furthermore, a general assumption that fig rust is caused by *C. fici*, as well as morphological similarities between the urediniospores of *C. fici* and *P. nishidana*, may prevent clear visual distinction between the two species.

Teliospore morphology is distinct between *C. fici* and *P. nishidana* (Ito and Homma, 1938), with the telia of *C. fici* being typical of *Cerotelium*, as erumpent and producing teliospores in short chains which are not laterally adherent. The telia of *P. nishidana* are not erumpent, with the teliospores forming crusts of laterally adherent teliospores. Telia are seldom produced by *C. fici*, having only been recorded three times (Butler, 1914; Patil and Thirumalachar, 1971; Huseyin and Selcuk, 2004), whereas telia are readily produced by *P. nishidana* (Ito and Homma, 1938). The urediniospores of these two fungi are morphologically almost identical, being hyaline, thin-walled and with inconspicuous germ pores in both species. They differ in that they are slightly smaller in *P. nishidana* (18–24 × 16–18 µm; Ito and Homma 1938: typically 19–26 × 16–19 µm, though occasionally larger or smaller in the present study), compared to *C. fici* (20–35 × 14–25 µm: Butler, 1914; McKenzie, 1986; 2013; Huseyin and Selcuk, 2004; Latinović *et al*., 2015). However, as stated above, the uredinia in *P. nishidana* are uredo-like, with paraphyses intermixed with the urediniospores, whereas those of *C. fici* are malupa-like with only peripheral paraphyses (Butler, 1914; Doidge, 1927; Ellis, 2020). As the present study collection had *Phakopsora*-like telia present, and the uredinia had paraphyses intermixed through these pustules, this confirmed the rust's identity as *P. nishidana*, despite some of the urediniospores being larger than previously described for this species.

The taxonomy of rust fungi on figs remains confused, and there is need to fully resolve the species involved and their distribution, both within and beyond fig production regions. *Phakopsora nishidana* has been recorded from eastern Asia and the Americas (Hennen *et al*., 2005; Solano-Báez *et al*., 2017; Farr and Rossman, 2021), and there is a possibility that some of the records of *C. fici* from southern Africa, as well as elsewhere in Africa, may also be *P. nishidana*. The species with *Phakopsora*-like telia described from *Ficus* hosts, including *P. nishidana*, *P. fici-erectae* and *P. fici-elasticae*, as well as *U. sawadae*, need to be compared by phylogenetic analyses. The description of the fig rust identified as *P. nishidana* from South America (Buritica, 1999; Hennen *et al*., 2005) fits the description of *P. fici-erectae* better than that for *P. nishidana*. It is possible that several species are involved, but are not recognised, as the urediniospore morphology for all is almost identical.

In SA, the Western Cape Province, specifically around the towns of Porterville, Malmesbury, Paarl and Wellington, and Napier in the Overberg, is considered as the most important fig producing region. The production conditions in this province are diverse and vary from more rust-conducive areas, with regular conditions of high humidity along the coastal regions, to semi-arid and less disease-prone areas such as Worcester, Ladysmith, Oudtshoorn and Prince Albert. Although rust regularly occurs towards the end of the production season in fig orchards in the Overberg, infection levels are generally low and without apparent economic impacts. In April 2021, however, rust incidence in orchards near Napier was high, with several trees defoliating due to rust infections. Similar to the observations of McKenzie (2013), rust pustules were typically clustered alongside leaf veins or on leaf sections exposed to moisture accumulation and retention. Seasonal variation in severity and distribution of rusts is influenced by inoculum levels, environment, and cultivar susceptibility. Wind and splashed-dispersed fig rust urediniospores require at least 14 h of wetness to germinate and infect fig leaves or fruit (McKenzie, 2013). Rogovski Czaja *et al*. (2021) showed that urediniospores of *C. fici* could germinate after 6 h, whereas successful infection of fig leaves was achieved between 18°C and 28°C.

The mulberry plants tested in the present study were all highly resistant to rust infections, indicating that the rust fungus found elsewhere on this host may be different from *P. nishidana* which infects edible fig plants. *Phakospora nishidana* is known as a pathogen only of several species of *Ficus* (Farr and Rossman, 2021), whereas *C. fici* is recorded from species of *Broussonetia*, *Ficus*, *Maclura* and *Morus* (Moraceae) (McKenzie, 1986;

Farr and Rossman, 2021). Inhibition of colony establishment at an early stage, as seen in both mulberries, was classified as abortive penetration according to Kochman and Brown (1975). In *F. carica*, infection occurs after formation of appressoria over stomata on abaxial leaf surfaces of the hypostomatic leaves. Directional germ tube growth was not observed for *P. nishidana*, but it was evident that trichomes on lower leaf surfaces interfered with germ tube extension. Infection of lower leaf surfaces, and thick upper epidermis with underlying leaf anatomical structures, may explain the predominant colonization and sporulation on the abaxial surfaces.

Selection of cultivars tested in the present study was influenced by the availability of trees at local nurseries. Although none of the cultivars was immune to rust infections, differences were observed in the severity of infections, which may impact disease development and economic losses. Parisian, the dominant purple fig cultivar grown in SA, which accounts for about 75% of fresh fig production, and Ronde de Bordeaux, were intermediate in their rust responses. Deanna is a yellow peel-coloured cultivar grown especially in the North West Province. It is early and productive, with large fruit for the local market, and was less susceptible to rust in the present study. Mean rust severity on Black Genoa, which is grown in many countries under names such as San Piero, California Brown Turkey, Roxo de Valinhos, Black Jack, and Negro Largo, was significantly less than on most other cultivars. In the present greenhouse study, cultivar responses were assessed as the number of pustules per unit leaf area. However, the severity scale developed by Da Silva *et al*. (2019) provided a useful resource should rust assessments be expanded to field trials. The present results have shown that routine methodology to study rust pathosystems of field crops is equally applicable for fig rust research, and has potential for further application in horticultural systems. Rogovski Czaja *et al*. (2021) also obtained successful *C. fici* infection using a fig leaf disc technique as well as spray-inoculation of 1-year-old potted fig plants.

Control of diseases on figs is hampered by the lack of coordinated research including data on the occurrence and economic importance of a disease such as rust. Furthermore, fresh figs produced for export must adhere to pesticide residue limits which are strictly imposed by importing countries. At present, SA is excluded from exporting to the biggest markets of the USA and China until regulatory protocols have been established, due to the phytosanitary requirements for these countries. These regulations and the relatively small area planted with commercial figs in SA have contributed to the scarcity of relevant published information on pests and pathogens on fig, as well as almost non-existence of chemicals registered to for their control. Advice on chemical control of fig rust to commercial producers and residential fig growers is currently limited due to a lack of registered products in SA. Kanguard 940™, labelled as a contact fungicide and containing plant organic acids as active ingredients, is the only disease control product registered on figs in SA (https://www.agri-intel.com; http://www.kannar.co.za; accessed 26 October 2020). Spray programmes involving this product require preventative application at 5 d intervals and good coverage of plants. However, the efficacy of this product has not been confirmed against *P. nishidana* and requires further evaluation along with other fig rust management options.

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Research Papers

Activity of essential oils from *Syzygium aromaticum* and *Rosmarinus officinalis* against growth and ochratoxin A production by *Aspergillus tubingensis* and *Aspergillus luchuensis* from Moroccan grapes

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Summary. Essential oils have been used since ancient times in traditional medicine and agri-food science to preserve food, and to combat human diseases. Essential oils (EOs) from clove and rosemary, obtained by hydro-distillation, were analyzed by GC/MS and evaluated for their antifungal activity against strains of *Aspergillus tubingensis* and *A. luchuensis* and their effect on ochratoxin A (OTA) production by these fungi. The major constituent of clove EO was eugenol (86.4%), and of rosemary EO was eucalyptol (35.9%). Mycelium growth inhibition assays showed that the EOs had dose-depend inhibition effects, which reached 36.6% for rosemary EO, and 100% for clove EO, at EO concentration of 200 µL L^{-1}. These reductions were different for different *Aspergillus* strains, the essential oil type, and the strain/oil type interaction. Analyses of OTA in the culture medium extracts of *Aspergillus* strains was carried out using HPLC-FLD, and was confirmed by LC-MS/MS for positive controls of two OTA-producing strains of *A. tubingensis*. In general, OTA was reduced (from 45 to 100% reduction), except for two strains of *A. tubingensis*, where OTA production was stimulated.

Key words. Antifungal effect, mycotoxins, biological control, clove, rosemary.

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INTRODUCTION

Aspergillus species are widely recognized as food contaminants, which may create severe economic losses, and constitute health risk for consumers due to their ability to produce mycotoxins. Ochratoxin A (OTA) is the one of the most frequent toxic secondary metabolites produced by several fungal species belonging to *Aspergillus* (e.g. *A. ochraceus, A. carbonarius, A. niger, A. alliaceus, A. tubingensis, A. luchuensis*) and *Penicillium* (e.g *P. verrucosum, P. nordicum, P. expansum*). This mycotoxin is hazardous to human health since it can be neurotoxic, carcinogenic, nephrotoxic, hepatotoxic, teratogenic and immunosuppressive (Murphy *et al*., 2006; IARC, 2009; Coronel *et al*., 2010). OTA has been classified as a possible carcinogen for humans (group 2B) by the International Agency for Research in Cancer (IARC, 1993). This mycotoxin has been frequently detected in a wide range of agricultural and food products, including cereals and derived products (Hajjaji *et al*., 2006; Gamza *et al*., 2015), cocoa (Copetti *et al*., 2010), grapes, grape and apple juices (Battilani *et al*., 2006; Leong *et al*., 2006; Selouane *et al*., 2009), and spices (Zinedine *et al*., 2007; Wan Ainiza *et al*., 2015).

To reduce potential hazards from mycotoxin-producing fungi in crops and stored agricultural products, control of mycotoxin-producing fungi using conventional fungicides is often advocated. However, the use of antifungal chemicals is progressively becoming restricted by food safety regulations, as it could lead to development of resistant strains and occurrence of residues in food, feed, crops and the environment. These fungicides may also stimulate the production of mycotoxins (Audenaert *et al*., 2010; Zouhair *et al*., 2014). Therefore, research for alternative antifungal substances is important to control the growth of spoilage fungi and risks of OTA.

Essential oils (EOs) are possible natural alternatives to synthetic fungicides. Several aromatic and medicinal plants have received attention, and have been studied for control of growth of toxigenic fungi to reduce production of mycotoxins (Lappa *et al*., 2017; Mateo *et al*., 2017; Lasram *et al*., 2019; Oliveira *et al*., 2020). EOs are complex mixtures of volatile organic compounds, mainly terpenoids, aromatic, and aliphatic components, all characterized by low molecular weights (Bassole and Juliani, 2012). The Food and Drugs Administration (FDA) classified theses oils "Generally Recognized as Safe" (GRAS) substances (Edris, 2007). Several biological activities have been attributed to EOs, including antibacterial (Risaliti *et al*., 2019) and antifungal properties (Bomfim *et al*., 2015; Santamarina *et al*., 2016). EOs contain major and minor components, and mixtures are more biologically active than major mixed constituents (Gill *et al*., 2002; Mourey and Canillac, 2002), which suggests that the minor components may have potentiating influences or synergistic effects.

Antifungal activity of clove and rosemary essential oils against mycotoxin-producing fungi has been reported (Rasooli *et al*., 2008; Bouddine *et al*., 2012; Bomfim *et al*., 2015; Boukaew *et al*., 2017). El Khoury *et al*. (2016) found that rosemary EO inhibited production of OTA by *A. carbonarius*. In addition, the antifungal activity of the major compounds (eugenol and eucalyptol) of these EOs has also been reported (Morcia *et al*., 2012; Jahanshiri *et al*., 2015; Caceres *et al*., 2016; Nazzaro *et al*., 2017).

The aim of the present work was to assess effects EOs from *Syzygium aromaticum* (clove) and *Rosmarinus officinalis* (rosemary) on growth and OTA production for eight strains of *A. tubingensis* and *A. luchuensis*.

MATERIALS AND METHODS

Fungus strains and culture conditions

Seven strains of *A. tubingensis* (MUCL54478, MUCL54481, MUCL54482, MUCL54483, MUCL54484, MUCL54485 and MUCL54486) and one strain of *A. luchuensis* (MUCL54477) were used in this study. These strains were previously isolated from grapes produced in Morocco. All strains were identified (Qjidaa *et al*., 2014), and deposited at BCCM/MUCL (Mycothèque de l'Université Catholique de Louvain, Belgium). The strains (suspensions of conidia) were maintained in 25% glycerol + 0.01% Tween 80 at -20°C. Prior to the study, OTA production of the eight strains was confirmed *in vitro* on Czapek Yeast Autolysate agar (CYA). The identification of the OTA peak in chromatograms was confirmed by methylation according to Zimmerli and Dick, (1995). Inocula for the experiments were prepared by growing each strain on CYA at 25°C for 7 d. Suspensions of conidia ($\approx 10^5$ conidia mL^{-1}) were prepared in sterile distilled water containing 0.5% Tween 80. A Thoma chamber was used to determine the final conidium concentrations.

Essential oil isolation

Dried *S. aromaticum* or dried leaves of *R. officinalis* (100 g) were hydro-distillated (3 h) using a Clevenger-type apparatus. EOs were stored at 4°C in the dark before analysis.

Antifungal tests

In vitro experiments to evaluate the efficacy of EOs against *A. tubingensis* and *A. luchuensis* were carried out using a CYA medium (0.99a_w). Conidia of *A. tubingensis* or *A. luchuensis* were each exposed to a series of increasing concentrations of the tested antifungal agents (Remmal *et al.*, 1993). The EO was emulsified with a 0.2% agar solution to disperse the constituent compounds. Volumes of this dilution were added to sterilized CYA medium and cooled to 45–50°C. The final essential oil concentrations used were 25, 50, 100 and 200 µL L^{-1}. Petri plates were prepared with the different amended media, and experimental controls containing the culture medium alone were also prepared. The plates were each single-point inoculated with 10 µL of a suspension of the appropriate conidia (≈ 10^5 conidia), and the plates were then incubated at 25°C. All the experiments were carried out in triplicate. The Petri plates were examined daily, and the diameter of resulting colonies was measured in two perpendicular directions during a period of 7 d. Linear regression of colony radius (mm) against time (d, from the day the colony was 5 mm diam.) was used to determine growth rates (mm d^{-1}). Lag phase for growth was considered as the number of days elapsed between inoculation and the time when fungal growth became evident (colony reached 5 mm diam.). Petri plates were also used for OTA extractions and determination.

Essential oils analyses

Gas chromatography-mass spectrometry (GC/MS) analyses were carried out using a Thermo Finnigan gas chromatograph directly coupled to a mass spectrometer system (model TRACE GC ULTRA S/N 20062969; Polaris Q S/N 210729). Retention Indices and the confirmation of MS identified compounds were carried out using a gas chromatograph with flame-ionization detection (GC-FID) Thermos Finnigan Trace GC 2000, under the same conditions as used for GC/MS analyses. Compounds were separated using a TR-5 capillary column (60 m × 0.32 mm, 0.25 µm film thickness). The Oven temperature was maintained at 40°C for 2 min, the programmed to 280°C at 5°C min^{-1}, and the final temperature kept for 10 min. The splitless injector was maintained at 220°C and opened after 0.85 min. The flow rate of carrier gas (helium) was 1 mL min^{-1}, and the volume of injected specimen was one µL of diluted oil in hexane. Electron impact mass spectra were recorded at 70 eV. The ion source temperature was 200°C, the interface line temperature was 300°C, and the scan mass range m/z was of 40-650. The same method was previously used by Bennouna *et al.* (2018).

Extraction and OTA analyses

Ochratoxin A was extracted using the method of Bragulat *et al.* (2001). Briefly, three agar plugs (diam. = 7 mm) were removed from the inner, middle, and outer areas of each fungus colony. The plugs were weighed and then dispensed into 3 mL vials before adding 1 mL of methanol to each vial. The vials were shaken for 5 sec with an autovortex and then incubated at 25°C for 60 min. The extracts were centrifuged three times for 10 min at 13,000 rpm. The supernatant was then filter-sterilized through a PVDF hydrophilic filter (0.22 µm) and then analyzed using HPLC technology (Agilent Technologies) with fluorescence detection (excitation 333 nm, emission 460 nm; calibration with OTA standard (Sigma Aldrich). The separation of metabolites was carried out on a C18 RP column (Zorbax SB, 4.6 × 250 mm × 5 µm particle size). The mobile phase (acetonitrile-water-acetic acid; 99:99:2, v/v/v) was pumped at 0.7 mL min^{-1}, and the injection volume reached 20 µL. Run time for samples was 30 min, with OTA being detected at about 11 min. OTA was identified by its retention time (11-11.6 min), according to the OTA standard.

Quantification of OTA was achieved by measuring peak areas according to a linear standard curve, and the limit of detection was 0.2 ppb. All analyses were repeated three times. OTA production by ochratoxigenic fungus strains was confirmed using an UPLC-MS/MS system (Waters) coupled with a triple-quadrupole (Xevo TQ-S micro) mass spectrometer (MS). The OTA was separated using an UPLC C18 column (2.1 µm particle size, 1.7 × 100 mm) with a C18 pre-column (2.1 µm particle size, 1.7 × 20 mm). Both the column and guard column were maintained at 45°C. The flow rate was 0.4 mL min^{-1} and injection volume of 2 µL. The mobile phase consisted of a gradient (Table 1) achieved with the following mobile phase: phase A) water/0.1% formic acid/5 mM ammonium formate, and phase B) methanol/0.1% formic acid/5 mM ammonium formate.

Table 1. Gradient profile of LC-MS/MS.

Time (min)	Phase A	Phase B
0	98	2
1	98	2
13	1	99
14	98	2
17	98	2

The mass spectrometry analysis ESI-MS/MS was carried out using the positive electrospray ionization mode (ESI+) in a multiple reaction monitoring mode (MRM) at capillary voltage of 3.5 kV, cone voltage of 8 V, source temperature of 150°C, and desolvation temperature of 500°C. Argon was used as desolvation with a flow rate of 1000 L h^{-1}. Full scan mode was employed in the mass range of 30-1250 Da. The transition of the most abundant product transition ion (m/z 404.2 < 239.1) and the second least abundant transition (target or confirmatory) ion (m/z 404.2 < 358.2) were selected for quantification and identification. MassLynx (V4.1) software (Waters Corporation) was used for the data acquisition.

Statistical analyses

Linear regressions of colony radius against time (d) was used to determine the growth rates (mm d^{-1}) under each set of conditions, were obtained with the program Microsoft Excel version 2013. Analysis of variance for the different growth data sets was carried out using IBM SPSS Statistics, version 20.

RESULTS

Essential oil composition

Hydro-distillation of clove and rosemary tissues yielded an average, respectively, of 3 mL and 2 mL for 100 g of the dry plant material. Based on the results of the GC/MS and GC/FID analyses, different compounds were identified in the oils (Table 2). The most abundant chemical components were eugenol (86.4%), eugenyl acetate (8.9%) and a-humulene (1.1%) for clove EO, and eucalyptol (35.9%), camphor (21.2%) and o-cymene (4.8%) for rosemary EO.

Essential oil antifungal effects on fungus lag phases and growth rates

The lag phases observed in CYA cultures supplemented with clove EO were always greater than for rosemary EO, and this under all doses tested. A concentration of 200 µL L^{-1} of clove oil completely inhibited growth of two ochratoxigenic strains of *A. tubingensis* (MUCL54482 and MUCL54484) and two non-ochratoxingenic strains *A. tubingensis* MUCL54486 and *A. luchuensis* MUCL54477. In contrast, the lag phases for the control cultures were 1 d, and for the cultures supplemented with clove EO were about 2 to 3 d for 25 to 50 µL L^{-1}, and 4 to 6 d for 100 to 200 µL L^{-1}. However, the lag phases after rosemary EO treatments were similar for all doses tested (approx. 2 d).

Statistical analyses (Table 3) revealed that factors of the EOs, EO concentrations of EOs, fungus strains, as well as their interactions, had significant effects ($P \leq 0.001$) on the antifungal activity. In control plates, mycelium growth had already reached the edges of the Petri dishes after 7 d of incubation, and the mean growth rate was 7.4 mm d^{-1}. Clove EO at different concentrations (0-200 µL L^{-1}) decreased the radial growth rate of all the tested strains (Figure 1). Clove EO applied at 25 µL L^{-1} gave mean growth rates between 6.4 and 7.3 mm d^{-1}, and mean growth rate reductions varied between 1.2

Table 2. Chemical composition of rosemary and clove essential oils.

Retention Index	Compound	Identification	Amount (%)	
			Rosemary	Clove
940	α-pinene	GC, MS	0.12	-
956	camphene	GC, MS	4.15	-
1024	γ-terpinen	GC, MS	0.35	-
1035	o-cymene	GC, MS	4.79	-
1044	eucalyptol	MS	35.88	-
1166	camphor	GC, MS	21.15	-
1186	borneol	GC, MS	4.68	-
1208	α-terpinol	GC, MS	2.90	-
1232	verbenone	MS	0.56	-
1301	bornyl acetate	MS	0.38	-
1390	eugenol	MS	-	86.41
1442	a-humulene	GC, MS	1.24	1.11
1477	germacren D	MS	0.15	0.14
1552	eugenyl acetate	MS	-	8.94
1615	caryophyllene oxide	MS	-	0.12
Total			76.35	96.72

Table 3. ANOVA for effects of essential oils and their concentrations on growth rates of strains of *Aspergillus* section *Nigri*.

Source of variation	Growth study			*P*
	DF[(a)]	MS[(b)]	F	
Strain (S)	7	2,835	14,812	0.000
Concentration (C)	4	131,808	688,548	0.000
Essential oil (EO)	1	65,125	340,206	0.000
S × C	28	0,731	3,817	0.000
S × EO	7	2,546	13,299	0.000
C × EO	4	61,150	319,441	0.000
S × EO × C	28	0,416	2,175	0.001

DF[(a)], degrees of freedom
MS[(b)], mean square

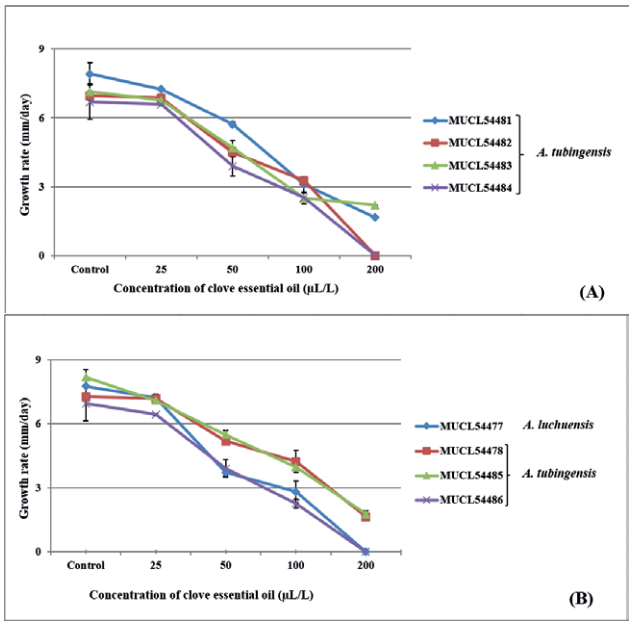

Figure 1. Mean colony growth rates (mm d^{-1}) of *Aspergillus tubingensis* and *A. luchuensis* strains on CYA medium supplemented with different concentrations of clove essential oil (A: OTA producing strains; B: non-OTA producing strains).

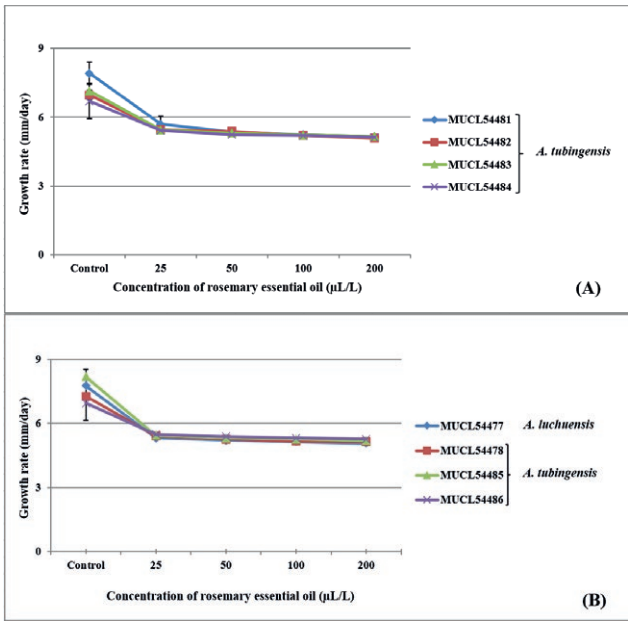

Figure 2. Mean growth rates (mm d^{-1}) of *Aspergillus tubingensis* and *A. luchuensis* on CYA medium supplemented with different concentrations of rosemary essential oil. (A: OTA producing strains; B: non-OTA producing strains).

and 13.3%. Greater concentrations of clove EO, complete (100%) inhibition of half of the tested strains. However, for the other strains, growth rate reductions compared to the control varied between 69.2 and 78.4%, with growth between 1.6 and 2.2 mm d^{-1}. Rosemary EO was less (Figures 2 and 3). At 25 µL L^{-1}, mean fungus growth rates were between 5.3 and 5.7 mm d^{-1} and resulted in reductions from 18.9 to 33.5% compared to the control. At 200 µL L^{-1} of rosemary EO, the mean growth rates were between 5.1 and 5.3 mm d^{-1}, resulting in inhibition between 23.5 to 36.6%. Clove EO therefore displayed greater antifungal potential as a mycelium growth inhibitor than rosemary EO (Figure 3).

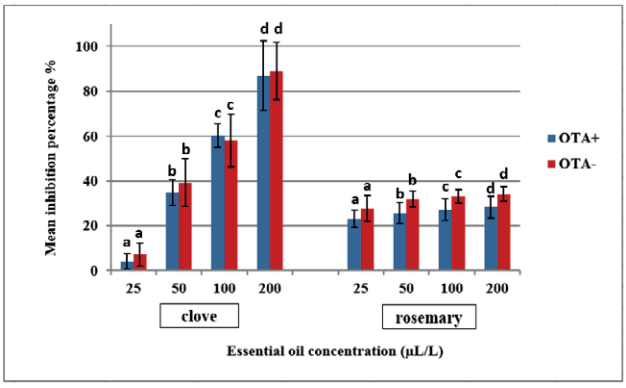

Figure 3. Mean inhibition percentages (%) of *Aspergillus tubingensis* and *A. luchuensis* on CYA medium supplemented with different concentrations of clove or rosemary essential oils. (OTA^+, OTA producing strains; OTA^- non-OTA producing strains). Means within accompanied by the same letters are not significantly different (LSD, $P = 0.05$).

Ochratoxin A production

OTA production was assessed for all treatments of cultures of *A. tubingensis* and *A. luchuensis* using HPLC-FLD (Table 4). No OTA was detected in culture extracts of non OTA producing strains, with or without clove or rosemary EOs, except for the *A. tubingensis* MUCL54478 strain grown in presence of clove EO at 25 µL L^{-1}, where 8.3 µg g^{-1} OTA was detected. For OTA producing strain controls, the OTA amounts produced were between 0.06 to 0.14 µg g^{-1}. The rosemary EO reduced accumulation of OTA for all the strains studied, except for *A. tubingensis* strains MUCL54483 and MUCL54484 at 200 µL L^{-1}, where OTA was increased (means of 0.44 µg g^{-1} for MUCL54483 and 0.22 µg g^{-1} for MUCL54484). Where clove EO was applied at 50, 100 or 200 µL L^{-1}, OTA has not been detected (depending on the strain), but exceeded the control levels of OTA, especially for strain MUCL54484 at 25 or 50 µL

Table 4. Mean OTA amounts produced by different strains of *Aspergillus tubingensis* and *A. luchuensis* on CYA medium.LOD = 0.2 µg kg^{-1}; LOQ = 0.6 µg kg^{-1}.

Essential oils	Doses (µL L^{-1})	OTA (µg g^{-1})[a] (Mean±S.D. [b])							
		OTA non-producing strains				OTA producing strains			
		A. luchuensis	*A. tubingensis*			*A. tubingensis*			
		MUCL54477	MUCL54478	MUCL54485	MUCL54486	MUCL54481	MUCL54482	MUCL54483	MUCL54484
S. aromaticum	Control	ND[c]	ND	ND	ND	0.06±0.09	0.14±0.2	0.08±0.11	0.07±0.09
	25	ND	8.29±2.69	ND	ND	<LOQ [e]	<LOQ	0.12±0.02	0.41±0.11
	50	ND	ND	ND	ND	<LOQ	<LOQ	ND	0.43±0.27
	100	ND	ND	ND	ND	<LOQ	<LOQ	ND	ND
	200	NG[d]	ND	ND	NG	ND	NG	ND	NG
R. officinalis	Control	ND	ND	ND	ND	0.06±0.09	0.14±0.2	0.08±0.11	0.07±0.09
	25	ND	ND	ND	ND	<LOQ	<LOQ	<LOQ	<LOQ
	50	ND	ND	ND	ND	<LOQ	<LOQ	<LOQ	<LOQ
	100	ND	ND	ND	ND	<LOQ	<LOQ	<LOQ	<LOQ
	200	ND	ND	ND	ND	0.1±0.04	0.06±0.01	0.44±0.16	0.22±0.16

[a] means (n=3); [b] S.D: standard deviaton; [c] ND: not detected; [d] NG: no growth; LOD: limit of detection; LOQ[e]: limit of quantification.

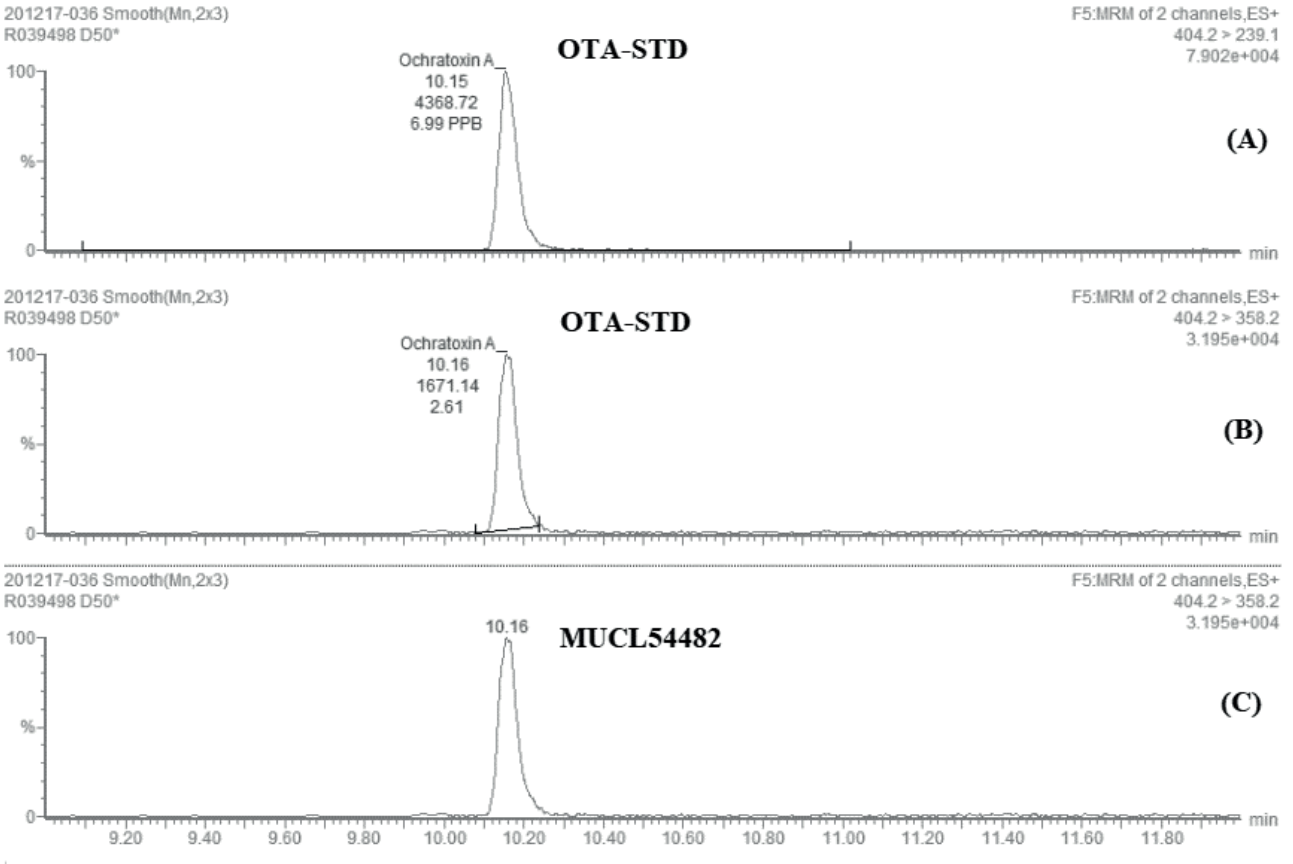

Figure 4. UPLC-MS/MS chromatograms of OTA standard (A and B) and for OTA from *Aspergillus tubingensis* strain MUCL54482 (C).

L^{-1} (respectively, 0.41 and 0.43 µg g^{-1}). Confirmation of OTA production by *A. tubingensis* strains MUCL54482 and MUCL54483 was carried out by UPLC-MS/MS, with production of 1050 µg kg^{-1} by *A. tubingensis* strain MUCL54482 (Figure 4).

DISCUSSION

GC/MS analyses of the clove and rosemary EOs from Moroccan origins showed that eugenol was the major component of Clove EO, and eucalyptol (1,8-cin-

eole) was the major compounds in rosemary EO. For clove EO, eugenol was 86.4% of the total volatile compounds. This agrees with the results of Santamarina *et al*. (2016), who showed that the proportion of eugenol in commercial clove EO was 88.6%. Bozik *et al*. (2017) also reported that eugenol was the major constituent in two commercial clove Eos, with amounts of 81.7 and 77.8%. In addition, Boukaew *et al*. (2017) and Sharma *et al*. (2017) concluded, respectively, that eugenol constituted 62.4 and 75.4% of clove EO volatiles.

Results for rosemary EO in the present study showed that eucalyptol was 35.8% of total volatiles. Risaliti *et al*. (2019) also reported similar results, with a proportion of 48.7%. Farhat *et al*. (2017) reported that the proportion of eucalyptol in Tunisian rosemary EO ranged from 20.8 to 64.7% depending according on the extraction methods used. Analysis of rosemary EO volatiles from Brazil (Takayama *et al*., 2016) and Morocco (Bouyahya *et al*., 2017) showed that the major component was eucalyptol, at 28.5% in Brazil and 23.67% in Morocco. Studies of the chemical composition of EOs from clove and rosemary revealed variability in their chemical profiles dependent on plant growing conditions, phenological stage, sampling time, and extraction and analytical methods used, and even existence of new plant chemotypes as suggested by Kokkini *et al*. (2004) and Sharma and Tripathi (2008). The chemical composition and antibacterial activity of clove and rosemary EOs has been described by several authors. However, studies on antifungal activity of these essential oils against *A. niger* aggregates and their effects on OTA synthesis are scarce.

In the present study, the lag phases, radial growth and OTA accumulation by strains of *A. tubingensis* and *A. luchuensis* were strongly influenced by clove and rosemary EOs. In general, the EO type, their concentrations, fungus isolate, and interactions between these factors had highly significant effects on radial fungus growth. For clove EO, mean inhibition rates greater than 90% were obtained at 200 µL L^{-1}, showing that this essential oil had effectiveness exceeding that for some fungicides widely used in Morocco (azoxystrobin, benomyl, hexaconazole and pyrimethanil) (Zouhair *et al*., 2014; Laaziz *et al*., 2017). Inhibition rates for clove EO were between 1.2 and 13 3% at the lowest concentration of the EO (25 µL L^{-1}), and were 69.2 to 100% for 200 µL L^{-1}. Passone *et al*. (2012) reported that the application of 500 µL L^{-1} of clove EO inhibited the growth of *Aspergillus* section *Nigri* by 48.8 to 100%, depending on EO concentration, fungus strains and water activity levels. Santamarina *et al*. (2016) reported significant antifungal activity of clove EO against *Fusarium* spp. They demonstrated that for a concentration of 300 mg mL^{-1}, there was a growth reduction of 88% for *F. graminearum*. Boukaew *et al*. (2017) also studied the antifungal effect of clove EO but on *A. flavus* responsible of the deterioration of maize in storage in Thailand. They demonstrated that low concentrations of clove essential oil (1 and 10 µL L^{-1}) induced greater growth inhibition (respectively, 85.7 and 94.0%), and complete growth inhibition (100%) was achieved at EO concentrations of 50 and 100 µL L^{-1}.

This study showed that *A. tubingensis* growth inhibition rates from treatments with rosemary EO ranged from 23.5 to 36.6%, depending on the EO concentration. Bouddine *et al*. (2012) also reported that rosemary EO showed a weak effect on *A. niger*, as assessed using the broth dilution method. Rasooli *et al*. (2008), using the disc diffusion method, showed antifungal activity of rosemary EO at 20 µL, giving 17% inhibition of *A. parasiticus*. Bomfim *et al.,* 2015) showed that the same essential oil inhibited growth of *F. verticillioides* by 29.7% at a concentration of 300 µg mL^{-1}. The weak effect (1.6 to 24.2% at 1 µL mL^{-1}) of rosemary EO on growth inhibition for fungal pathogens was also demonstrated by Matusinsky *et al*. (2015).

Investigation of synergistic or potential effects of different constituent compounds of EOs can increase insights into how EO composition affects antifungal activities. Morcia *et al*. (2012) demonstrated that eugenol, the major component of clove EO, had high activity against *A. tubingensis* and *A. carbonarius*. However, eucalyptol (the major component rosemary EO) was only active at the greatest doses tested (2%). Nazzaro *et al*. (2017) also reported that eucalyptol and eugenol exhibited important antifungal activity. Several studies demonstrated that the lipophilic properties and low molecular weights of essential oils allow them to cross cell membranes, causing irreversible cell wall and cellular organelle damage and affecting inorganic ion equilibria and pH homeostasis (Pawar *et al*., 2006; Helal *et al*., 2007).

High efficacy of EOs in disease control has been reported by several authors. The use of EOs in the packaging of fruits and other commodities was reported by Sivakumar *et al*. (2014, Guerra-Rosas *et al*. (2017), Munhuweyi *et al*. (2017), and Talebi *et al*. (2018). Campos *et al*. (2016) reported that the use of thyme and sage EOs in the headspace of strawberry packages led to decreases fruit contamination. To improve shelf-life without changing the organoleptic characteristics of jujube fruit, Nikkhah and Hashemi (2020) demonstrated that the treatments of the fruit with thyme, cinnamon or rosemary EOs (at, respectively, 0.156, 0.625, or 0.078 g L^{-1}), during 60 d of storage, did not negatively impact on

taste, texture, aroma or overall acceptance. They also suggested that using multiple essential oils at low concentrations may increase the antifungal activity because of synergistic actions. They demonstrated that, the concentration of EOs penetrating the fruit flesh remained under taste detection thresholds. According to the European Food Safety Authority (EFSA, 2012), the Acceptable Daily Intake (ADI) and Acceptable Operator Exposure Level (AOEL) for eugenol are 1.0 mg kg^{-1} body weight d^{-1}. In the present study, total inhibition for clove essential oil was obtained at 200 µL L^{-1} meaning that the use of this essential oil to conserve fruits in storage is likely to be safe for consumers. On the other hand, based on the Maximized Survey-derived Daily Intake (MSDI) approach, EFSA (2013), reported that 1,8-cineole (eucalyptol) presented no safety concern (1200 µg *capita*$^{-1}$ d^{-1})

In parallel to the fungal growth inhibitory effect by the two tested EOs, there were contradictory effects (reduction or stimulation) on OTA biosynthesis caused by the tested *Aspergillus* strains. Although several authors have reported that *A. tubingensis* produces OTA, the ability of this species to produce this toxin is controversial. Studies have shown that strains of *A. tubingensis* were able to produce a metabolite identified by HPLC-FLD as OTA (Medina *et al*., 2005; Perrone *et al*., 2006; Selouane *et al*., 2009; Chiotta *et al*., 2011). Other studies have considered that *A. tubingensis* was not an OTA producer (Abarca *et al*., 2004; Frisvad *et al*., 2011). Pantelides *et al.* (2017) also showed that none of 261 strains of *A. tubingensis* isolated from wine grapes were found to be ochratoxigenic when analyzed by UPLC-MS/MS, while some of these isolates were initially considered to be toxin producers as indicated by HPLC-FLD. In the same way, Storari *et al.* (2012) re-examined *A. tubingensis* strains previously described as ochratoxigenic using LC-MS, and could not detect the toxin, concluding that *A. tubingensis* cannot be considered as an ochratoxigenic species. This agrees with more recent publications (Gil Serna *et al*., 2019 and Tavakol *et al*., 2020). Therefore, data related to OTA production in controls in the present study was validated using an UPLC-MS/MS based method, in order to be sure that the observed peak was not an artifact (Figure 4). The present results showed that the *A. tubingensis* strains used in this study produce OTA, and this is the first report where production of OTA by *A. tubingensis* has been confirmed using the UPLC-MS/MS technique.

The inhibitory effect of rosemary EO on OTA production was also reported by El Khoury *et al.* (2016), who found that this EO inhibited production of OTA by *A. carbonarius.* Bomfim *et al.* (2015) concluded that rosemary EO reduced fumonisin B1 and B2 contamination by *F. verticillioides*. Experiments with *A. parasiticus* clearly illustrate reduction of aflatoxin production from rosemary EO (Rasooli et al., 2008). The use of eugenol also reduced aflatoxin B1 production by *A. parasiticus* and *A. flavus* (Jahanshiri *et al*., 2015; Caceres *et al*., 2016).

Several studies have shown that use of some essential oils as biocontrol agents against fungi lead to stimulation of the mycotoxin biosynthesis. Stimulation of OTA production depended on the strain, nature of the essential oil and the applied dose. Results of the present study showed that sub-lethal concentrations of EOs stimulated production of OTA by *A. tubingensis*. Lappa *et al.* (2017) demonstrated that the treatment of *A. carbonarius* with lemongrass, cinnamon or mandarin essential oils decreased growth rate, but increased OTA production. Stimulation of OTA biosynthesis by essential oils was also reported by Passone *et al.* (2012), who demonstrated that species of *A. niger* aggregate and *A. carbonarius* tended to stimulate OTA production in the presence of 1000 µL L^{-1} of poleo essential oil. In addition, Mateo *et al.* (2017) also reported stimulation of AFB1, AFB2, AFG1 and AFG2 accumulation in some cultures treated with ethylene-vinyl alcohol copolymer films containing oregano, carvacrol and cinnamon. These results are similar to those reported by Bluma and Etcheverry. (2008) for the species *Aspergillus* section *flavi*.

Mycotoxin production may be stimulated when stressful environmental conditions and sub-lethal antifungal agent doses are maintained in culture media during the growth of mycotoxin-producing fungi (Mateo *et al*., 2013; Prakash *et al*., 2015).

In conclusion, the effects of clove and rosemary EOs on fungal growth and OTA production by different *Aspergillus* isolates have shown that these oils had significant antifungal activity. Inhibition rates varied (20 to 100%) for fungus isolate and essential oil dose. In general, the statistical analyses showed that the different concentrations of clove and rosemary EOs, the *Aspergillus* strains, and their interactions, had strong effects on fungus growth. Mean inhibition rates greater than 85% were obtained at 200 µL L^{-1} of clove EO, which exceeds some fungicides (azoxystrobin, benomyl, hexaconazole and pyrimethanil) that have been tested and are widely used in Morocco. Clove EO can be used to control fungal growth and mycotoxin production as a potential alternative to chemical control. However, more research is required to evaluate the *in vivo* efficacy of these EOs, and to understand their effects on the expression of OTA biosynthesis and regulation genes.

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New or Unusual Disease Reports

First report of *Serratia marcescens* from oleander in Hungary

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Summary. Oleander (*Nerium oleander* L.) is a popular woody ornamental plant, often used for decorating public areas, terraces and gardens. Many diseases may decrease in the ornamental value of these plantings. Between 2018 and 2020, plant pathogenic bacteria of oleander were examined, and many samples of infected plants were collected from different sites in Hungary. Two non-pigmented *Serratia marcescens* isolates were identified from oleander by classical and molecular methods. The isolates caused necrotic lesions on oleander leaves. *Serratia marcescens* is known as an opportunistic mammal or plant pathogen, but non-pathogenic strains are known to be useful biological control agents or plant growth-promoting bacteria. This is the first report of the plant pathogen *S. marcescens* from oleander, and the first identification of the bacterium in Hungary.

Keywords. Bacterial disease, PCR, morphological characterization.

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INTRODUCTION

Nerium oleander (L.) is cultivated in many countries as an ornamental plant. It is native to the Mediterranean region of southern Europe and southwest Asia. In central and western Europe oleander is grown as a winter garden or patio plant. Some plant pathogenic bacteria cause serious diseases on oleander. The most widespread disease is oleander knot, caused by *Pseudomonas savastanoi* pv. *nerii* (Janse). The typical symptoms are formation of knots on stems, twigs and leaves (Bella *et al*., 2008). *Xylella fastidiosa* (Wells *et al.,* 1987), an important plant pathogen, causes a variety of diseases with severe economic impacts for agriculture, public gardens, and the environment. Symptoms of *X. fastidiosa* on oleander are chlorotic mottling and scorching of the leaves. As the disease develops, the leaves become necrotic, and severely infected plants defoliate and die (Purcell *et al*.,1999).

Serratia marcescens (*Enterobacteriaceae*) is a Gram-negative bacterium. *Serratia* genus includes 14 recognized species and two subspecies (Mahlen, 2011). *Serratia marcescens* was the first described member of the genus, and

was discovered by Bartolomeo Bizio in 1823 (Grimont *et al*., 2006). *Serratia marcescens* is ubiquitous in the environment, and is commonly found in soil, water, plants, animals, humans and foods (Cooney *et al*., 2013). It is an opportunistic mammal or plant pathogen, but non-pathogenic strains are known to be useful biological control agents or plant growth-promoting bacteria.

Serratia marcescens is also an important plant pathogenic bacterium, causing cucurbit yellow vine disease (CYVD), which was first observed in squash (*Cucurbita maxima* L.) and pumpkin (*Cucurbita pepo* L.) in the United States of America in 1988. It is now known to affect other cucurbits, including watermelon (*Citrullus lanatus* Thunb.) (Bruton *et al*., 1998), cantaloupe melon (*Cucumis melo* var. *cantalupensis* Ser.) (Bruton *et al*., 2003) and oil pumpkin (*Cucurbita pepo* var. *styriaca* L.) (Sedighian *et al*., 2018). The bacterium has also been isolated from alfalfa (*Medicago saliva*) in the United States of America (Lukezic *et al*., 1982), from onion bulbs (*Allium* sp. L.*)* (Cother and Dowling, 1986) in Australia, from bell pepper (*Capsicum annuum* L.) in Venezuela (Gillis *et al*., 2014), from sunflower (*Helianthus annuus* L.) in Russia (Ignatov *et al*., 2016), from hemp (*Cannabis sativa* L.) in the USA (Schappe *et al*., 2019), and from ginger (*Zingiber officinale* Roscoe) in China (Huang *et al*., 2020). However, plant-associated *S. marcescens* strains promote plant growth (George *et al*., 2013; Matilla *et al*., 2017; Khan *et al*., 2017; Dutta *et al*., 2020), and other strains are biological control agents (Someya *et al*., 2000; Gyaneshwar *et al*., 2001; Someya *et al*., 2005; Queiroz and Melo, 2006; Dhar Purkayastha *et al*., 2018), which can replace the use of chemical pesticides. A few strains can degrade herbicides (Silva *et al*., 2007). Pigmented *S. marcescens* has been associated symbiotically with *Rhynchophorus ferrugineus* Olivier (*Coleoptera: Curculionidae*), as the bacterium was regularly isolated from the reproductive apparatus of weevil adults and eggs and along tissues of infested palms (Scrascia *et al*., 2016). In addition, *S. marcescens* has been associated with disease and significant losses in Caribbean coral reefs (Sutherland *et al*., 2011). The bacterium is also an opportunistic human pathogen causing nosocomial infections in hospitalized patients, and may spread in epidemic proportions (Hejazi and Falkiner, 1997; Matilla *et al*., 2017; Abreo and Altier, 2019). *Serratia* species are inherently resistant to several antibiotics and can readily acquire resistance to antimicrobial agents (Mahlen, 2011).

MATERIALS AND METHODS

Eight infected plant samples were collected from Budapest's hobby gardeners between 2018 and 2020. Symptoms of browning, necrosis and deformation were observed on the seedcases and leaves of infected oleander plants. The symptomatic plant parts were delivered to the laboratory of the Department of Plant Pathology, Hungarian University of Agriculture and Life Sciences. The samples were surface-sterilized with 75% ethanol, and small tissue pieces were cut from the margins of lesions with a sterile scalpel and then macerated in sterile distilled water (SDW). Resulting suspension was streaked onto King's B agar (King *et al*., 1954) or Nutrient Agar (NA). The agar plates were incubated at room temperature (RT) for 48 to 72 h, and pure cultures of bacterial isolates were obtained by colony subculturing. Pure bacterial cultures, 24 to 48 h old, were used for further investigations.

Hypersensitive reaction (HR) was tested on tobacco leaves (*Nicotiana tabacum* L. cv. Xanthi) using pure bacterial suspension of 5×10^7 cells mL^{-1}, as determined with a spectrophotometer set at wavelength 560 nm. Leaves were assessed at 24 and 48 h post-inoculation. Gram feature was determined by the KOH test (Powers, 1995). Biochemical analysis was performed using the API 20E kit (BioMérieux).

For pathogenicity tests, oleander, sunflower and alfalfa plants, bell pepper fruit and onion bulbs were used. Stem internodes, between the first and the second leaves of oleander, sunflower and alfalfa, were injected using sterile syringes containing bacterial suspension of 5×10^7 cells mL^{-1}. Additionally, some leaves on each inoculated plant were lacerated with the inoculation syringe needle. Three inoculation replicates for each isolate were used. SDW was used for injection as a negative inoculation control. The inoculated plants were maintained at RT with a relative humidity greater than 90% for 1 week. Surface-disinfected bell pepper fruit and onion bulbs were toothpick-inoculated with bacterial suspensions (5×10^7 cell mL^{-1}), and were then incubated at RT with high relative humidity (>90%). Symptoms observations were carried out daily for 1 week post-inoculation. Negative controls were inoculated with SDW.

The amplification and sequencing of 16S rDNA was achieved using 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1389R (5'-ACGGGCGGTGTGTACAAG-3') universal primer pair (Osborn *et al*., 2000). The polymerase chain reaction (PCR) conditions were the following: initial denaturation at 94°C for 5 min, followed by 35 cycles at 95°C for 15 s, 55°C for 30 s and 72°C for 90 s, and a final extension at 72°C for 10 min. Amplification was verified on a 1% (w/v) agarose gel in 1 × TBE buffer. The PCR products were cleaned with the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH), inserted into pGEM-T Easy Vector, and trans-

formed into *Escherichia coli* DH-5 α bacterium cells. The nucleotide sequence of the PCR amplified DNA fragment from the recombinant plasmids was determined and compared with sequences from the National Center for Biotechnology Information database, using the Basic Local Alignment Search Tool (BLAST) program. Homologous sequences from other known members of the *Enterobacteriaceae*, having plant or insect associations, were included in the phylogenetic analysis for comparisons.

RESULTS

Two isolates from oleander, one each from a seedcase and a leaf, were isolated from a garden in Budapest. The colonies of both isolates were non-pigmented, shiny and round with smooth surfaces on King's B agar and NA (Figure 1). The KOH tests were positive, so both isolates were Gram-negative. The isolates induced HR on tobacco leaves 24 h post-inoculation (Figure 1). Both isolates had the same biochemical attributes. They were negative for production of sodium thiosulfate, indole, acetoin, arginine hydrolase, urease, cytochrome-oxidase, inositol, rhamnose and arabinose. They were positive for ß-galactosidase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, tryptophane deaminase, gelatinase, glucose, mannitol, sorbitol, saccharose, melibiose and amygdalin (Figure 2). The results of biochemical tests showed that these two isolates belonged to the *Enterobacteriaceae*.

Pathogenicity tests were positive for oleander, sunflower and alfalfa plants, bell pepper fruit and onion bulbs. Necrotic lesions were observed on oleander leaves around the points of injections, 3 weeks post-inoculation. The leaves of sunflower plants became yellow and wilted. Cross-sections of stems near the affected leaves showed brown discolorations in the phloem tissues. The plants wilted and completely died 1 week after inoculation. Alfalfa plants showed the same symptoms as sunflower plants, but symptoms of alfalfa plants developed more slowly than on sunflower plants. Control plants inoculated with SDW showed no symptoms. On bell pepper fruit, chlorotic lesions were observed around the inoculation sites within the 24 h post inoculation. Later, the lesions enlarged, turned brown and became mushy. There were sagging lesions around the inoculation points on onion bulbs, while the bulb scales also turned brown and became mushy. After 96 h, no symptoms were visible on control fruits (Figure 3). The pathogen of interest was the only microorganism re-isolated from lesions on the different inoculated plants, and was confirmed by PCR, fulfilling Koch's postulates.

The obtained nucleotide sequences were deposited into the GenBank database (accession numbers: MZ477518, MZ477519). The sequence identities were 98.9 to 99.8% to several *S. marcescens* strains, and the two isolates from oleander were shown to be 100% identical. Sequences that were used for the phylogenetic analysis were mainly derived from bacterium isolates from soil, food, insects or plants. The two isolates from oleander were least related to an isolate from corn

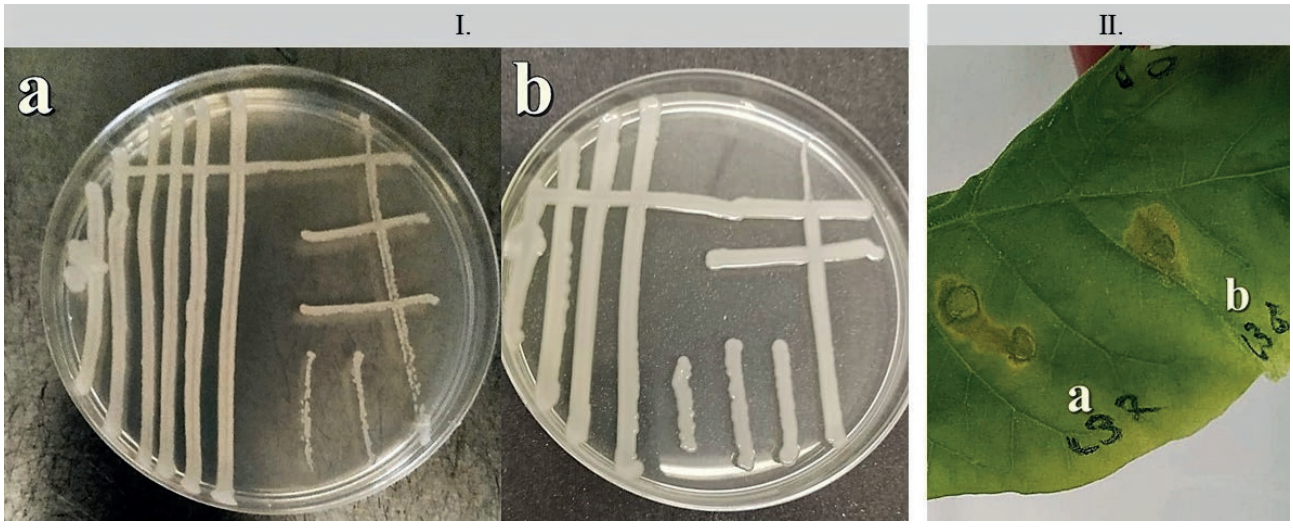

Figure 1. I. Pure cultures of bacterium colonies, after 24 to 48 h on King's B agar (a, *Serratia marcescens* isolate Bu-OleS2, and b, isolate Bu-OleS1). II. Hypersensitive reactions from infiltration of tobacco leaves 36 h post-inoculation for inoculations with, a, isolate Bu-OleS2 or b, isolate Bu-OleS1).

	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	
Bu-OleS2	+	-	+	+	+	-	-	+	-	-	
Bu-OleS1	+	-	+	+	+	-	-	+	-	-	
	GEL	**GLU**	**MAN**	**INO**	**SOR**	**RHA**	**SAC**	**MEL**	**AMY**	**ARA**	**OX**
Bu-OleS2	+	+	+	-	+	-	+	+	+	-	-
Bu-OleS1	+	+	+	-	+	-	+	+	+	-	-

Figure 2. Results from the API 20E test for two *Serratia marcescens* isolates (Bu-OleS2 and Bu-OleS1). (ONPG: β-galactosidase; ADH: arginine dihydrolase; LDC: lysine decarboxylase; ODC: ornithine decarboxylase; CIT: utilization of citrate; H2S: hydrogen sulfide; URE: urease; TDA: tryptophan deaminase, IND: indole, VP: the Voges-Proskauer test for the detection of acetoin; GEL: gelatinase, GLU: glucose, MAN: mannose, INO: inositol, SOR: sorbitol, RHA: rhamnose, SAC: sucrose, MEL: melibiose, AMY: amygdalin, ARA: arabinose).

Figure 3. Results from pathogenicity tests for different plants, from inoculation controls (upper row), or plants inoculated with two *Serratia marcescens* isolates (Bu-OleS2 or Bu-OleS1). The first column shows oleander leaves on the day of inoculation, the second and third columns show sunflower and alfalfa plants, the fourth and fifth columns show bell pepper fruit and onion bulbs at 5 to 6 d post-inoculation.

(MK461848) and most similar to isolates from sunflowers (KT741017, KT741016, KT741020, KT741019, KT741018) (Figure 4).

DISCUSSION

The two *S. marcescens* isolates obtained from oleander in this study had non-pigmented colonies that were shiny and round with smooth surfaces on King's B agar. They were Gram-negative and induced HR on tobacco leaves 24 h post-inoculation (Bruton *et al.*, 2003; Gillis *et al.*, 2014). Both isolates had the same biochemical attributes in API 20E. These results mainly correlate to previously published descriptions, but in the study of Sedighian *et al.* (2018), acetoin production was positive and tryptophane deaminase was negative, while Grimont and Grimont (2006) reported that mannitol fermentation was negative.

The pathogenicity tests were positive for oleander, alfalfa and sunflower plants, bell pepper fruit and onion bulbs. The two isolates caused necrotic lesions on oleander leaves, chlorotic and enlarged lesions on the bell pepper fruit and onion bulbs, which turned brown and mushy. In contrast, sunflower and alfalfa plants wilted and died. These reactions corresponded to those previously published for these hosts (Lukezic *et al.*, 1982; Cother and Dowling, 1986; Gillis *et al.*, 2014). The sequence of the 16S rDNA fragment was 98.9-99.8% identical to several *S. marcescens* strains. The present study isolates showed the nearest phylogenetic relationship to isolates of *S. marcescens* from sunflower.

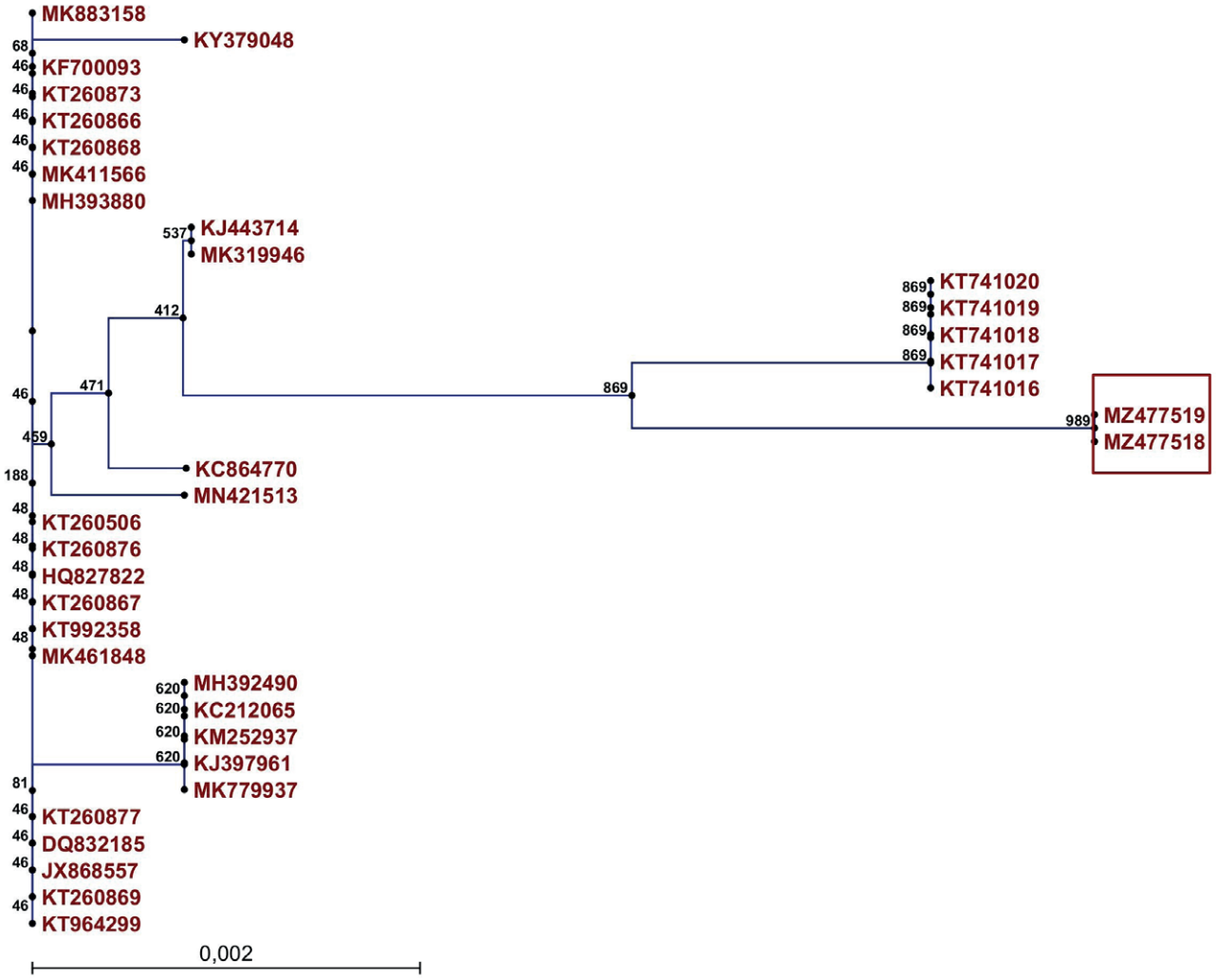

Figure 4. Phylogenetic tree of *Serratia marcescens* isolates, based on the 16S rRAS gene sequences. The tree was generated using the Neighbor-Joining method. GenBank accession numbers are shown. Hungarian *S. marcescens* isolates MZ477519 and MZ477518 are indicated with frame.

Non-pigmented strains of *S. marcescens* were previously described as the causal agent of CYVD (Bruton *et al.*, 2003; Besler and Little, 2016). However red-pigmented *S. marcescens* strains, isolated from squash in Iran (Sedighian *et al.*, 2018) or from sunflowers in Russia (Ignatov *et al.*, 2016), induced HR on tobacco leaves. The same strains were also pathogenic to zucchini (*Cucurbita pepo*). *Serratia marcescens* strains causing corn whorl rot in China produced pink or red-pigmented colonies in cultures, but HR was not reported (Wang *et al.*, 2015). In contrast, a non-pigmented *S. marcescens* strain was isolated from bell pepper in Venezuela, and it induced HR on tobacco leaves (Gillis *et al.*, 2014). The two *S. marcescens* strains from oleander were also non-pigmented and induced HR on tobacco leaves. They were also pathogenic to alfalfa and sunflower plants, to bell pepper fruit and to onion bulbs. Further studies are required to examine the effects of *S. marcescens* on oleander plants, since only one of the isolates assessed caused necrotic lesions, while no differences between the two isolates were observed on other hosts.

This is the first report of the *S. marcescens* as a pathogen of oleander, and the first identification of the bacterium in Hungary. The appearance of this new pathogen may cause serious problems on ornamentals, and possibly on other economically important hosts in Hungary.

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ABSTRACTS

Abstracts of oral and poster presentations given at the 12th International Workshop on Grapevine Trunk Diseases, Mikulov, Czech Republic, 10–14 July 2022

The 12th International Workshop on Grapevine Trunk Diseases (12th IWGTD) was held in Mikulov, Czech Republic, from 10 to 14 July, 2022. The Workshop was chaired by Dr Aleš Eichmeier and organized by Mendel University in Brno and Svaz Vinařů České Republiky/Union of Winemakers of the Czech Republic. The ICGTD Council meeting took place on 10 July, and that evening the welcome reception was opened by Rostislav Koštial (Mayor of Mikulov, Senator and viticulturist), Dr Martin Chlad (President of Svaz Vinařů České Republiky/Union of Winemakers of the Czech Republic), and Dr Svatopluk Kapounek (Vice-Rector, Mendel University, Brno).

The scientific programme was opened by Dr Alena Salašová (Dean, Faculty of Horticulture, Mendel University, Brno). The Workshop was attended by 133 researchers from 25 countries, presenting 61 oral and 49 poster papers. These presentations were in five sessions, including; Pathogen Characterization and Identification, Epidemiology, Plant-pathogen Interactions, Microbial Ecology, and Disease Management in Nurseries and Vineyards. The Disease Management session aimed to provide grape growers with an overview of practical options for trunk disease control. This session included contributions from researchers who have completed field trials on trunk disease management. David Gramaje (Instituto de Ciencias de la Vid y el Vino (ICVV), Logroño, La Rioja, Spain, outlined an evaluation of treatments for protection of grapevine pruning wounds from natural pathogen infections; Francois Halleen (University of Stellenbosch, South Africa) presented detailed results of efficiency of hot water treatments in nurseries; and Josè Úrbez-Torres (Agriculture and Agri-Food Canada SuRDC) outlined research on biological control of Botryosphaeria dieback in grapevines.

A field trip (13 July) visited vineyards in the South Moravia. Delegates were presented with an overview of grapevine production in South Moravia, followed by discussions on the main diseases (including GTDs) affecting grapevines in the Czech Republic. Vineyards affected by GTD pathogens were also visited, where the growers outlined their viewpoints. The field trip focused on presenting the current situation of GTDs in South Moravia, and the management strategies being adopted.

Student competitions for best oral and poster presentations included papers presented by 29 postgraduate students.

Delegates attending the 12th International Workshop on Grapevine Trunk Diseases, in Mikulov, Czech Republic.

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For best posters:
1st place went to Catarina Leal (University of Reims Champagne-Ardenne, France, and Polytechnic University of Valencia, Spain), "Evaluation of *Trichoderma atroviride* SC1 and *Bacillus subtilis* PTA-271 combination against grapevine trunk diseases pathogens in nursery propagation process";
2nd place went to Marcelo Bustamante (University of California, Davis, United States of America), "Investigating the role of *Fusarium* spp. in young vine decline in California";
3rd place went to María Julia Carbone (Universidad de la República, Montevideo, Uruguay), "Interactive effects of *Dactylonectria macrodidyma* inoculation on the rhizosphere and root microbiome of grapevine".

For best oral presentations:
1st place went to Catarina Leal, (University of Reims Champagne-Ardenne, France, and Polytechnic University of Valencia, Spain), "Beneficial effects of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 against the Botryosphaeria-dieback pathogen *Neofusicoccum parvum* may vary with grapevine cultivar";
2nd place went to Isidora Silva-Valderama (University of British Columbia, Vancouver, Canada), "Predicting pathogens virulence: linking host breadth and pathogenicity of the *Botryosphaeriaceae* fungal family in wine grapes (*Vitis vinifera*)";
3rd place went to Colin Todd (University of California, Riverside, United States of America), "Tracking the fungal pathobiome associated with young grapevine decline in California nurseries".

The 13th IWGTD will be held in Ensenada, Mexico, in 2025.

ORAL PRESENTATIONS

Investigating the causal agents of Sudden Vine Collapse in California. A. ESKALEN[1], E. HARDY[1], K. ELFAR[1], M. BUSTAMANTE[1], M. AL-RWAHNIH[1], C. STARR[2], S. BOLTON[3], N. MCROBERTS[1], M. BATTANY[4], L. BETTIGA[5], K. ARNOLD[6] and G. ZHUANG[7]. *[1]Department of Plant Pathology, University of California, Davis, CA 95616, USA.. [2]Pest Control Advisor. [3]Lodi Winegrape Commission. [4]UCCE Farm Advisor in San Luis Obispo/Santa Barbara counties. [5]UCCE Farm Advisor in Monterey, San Benito, and Santa Cruz Counties. [6]UCCE Farm Advisor in Stanislaus County. [7]UCCE Farm Advisor in Fresno County. E-mail: aeskalen@ucdavis.edu*

Since 2011, grape growers in the San Joaquin Delta, Central Valley, and Coastal counties of California have reported Sudden Vine Collapse (SVC), in which patches of vines within each vineyard, especially vines grafted on virus-sensitive rootstocks (e.g. Freedom, 039-16 and 101-14), quickly die with no apparent cause. In some cases, dying patches are so large that they can be seen in satellite images, with levels of loss that have caused growers to remove whole vineyards. In 2018, the disease reached an economic threshold of destruction that was affecting increasing numbers of growers, therefore gaining greater attention. In this study, four vineyards exhibiting SVC in the San Joaquin Delta were sampled during July 2019. Samples were collected from roots, rootstocks, scions, cordons, spurs, and leaves of selected vines. Samples from each tissue type were plated and cultured on PDA amended with tetracycline. Isolated fungi were identified using molecular techniques. Additionally, leaf and rootstock samples were analyzed using high-throughput sequencing to characterize the microorganisms, including viruses. Iodine tests were also carried out to evaluate the starch content and estimate the extent of girdling at the graft unions. On each vine with severe symptoms, *Grapevine leafroll-associated virus 3* and a vitivirus, *Grapevine virus A* or *Grapevine Virus F*, were present. Many Grapevine Trunk Disease (GTD) pathogens were isolated from healthy-looking and symptomatic tissues. These pathogens included *Botryosphaeriaceae*, *Fusarium* sp., and *Diaporthe* sp., among others. However, no single fungal pathogen was consistently found in affected grapevines. Graft union girdling possibly indicates rootstock reject of scions, following infections, and this prevents flow of water and nutrients throughout affected vines. Therefore, the inability of plants to transport carbohydrates leads to starch depletion in roots and subsequent lack of feeder roots, further preventing nutrient uptake. These factors all contribute to quick collapse of affected vines. We hypothesize that SVC is not caused by a single pathogen, but is a disease complex, in which vines are predisposed to root stress due to co-infections by a leafroll virus (*GLRaV-3*), vitiviruses (*GVA, GVF*) and possibly others. Infected vines rapidly die by additional infestations by fungi associated with grapevine trunk diseases.

Aspergillus vine canker: an overlooked disease of grapevine in California. M. I. BUSTAMANTE[1], K. ELFAR[1], M. ARREGUIN[1], G. ZHUANG[2] and A. ESKALEN[1]. *[1]Department of Plant Pathology, University of California, Davis, CA 95616, USA. [2]University of California, Cooperative Extension Tulare County, Tulare, CA 93274, USA. E-mail: aeskalen@ucdavis.edu*

Grapevine cankers are commonly associated with fungal pathogens of the *Botryosphaeriaceae*, *Diatrypaceae*, and *Diaporthaceae*, and these pathogens have been found and described in many cultivars and many countries. Symptoms include internal wood necroses, stunted or poor shoot development after budbreak, and dieback of cordons or entire vines. Cankered tissues may also exhibit dark fruiting bodies (pycnidia and perithecia) on the surfaces, which are responsible for releasing the spores that will lead to further vineyard infections. A different wood canker disease was first detected in the San Joaquin Valley in 1989, affecting excessively vigorous young 'Red Globe' grapevines. Since then, the disease has been observed on different cultivars, including Chardonnay, Grenache, and Crimson Seedless. The pathogen was morphologically identified *Aspergillus niger,* in the *Aspergillus* section *Nigri*, and the disease was named as Aspergillus vine canker. From 2003 to 2010, this disease was detected and monitored in Italy, affecting a number of table grape cultivars in different production regions. Recently, through a collaboration with UC Cooperative Extension farm advisors, we identified Aspergillus vine canker on Grenache and Malbec cultivars in, respectively, Fresno and Sonoma counties of California, Symptomatic vines were negative for viral infections. Affected vines are distinguishable by their prematurely senescent leaves during the fall, while healthy vines remain green. Our lab has focused on identification of the causal agent of Aspergillus vine canker in California, using molecular tools, particularly by constructing phylogenetic trees using DNA sequences of the calmodulin- gene. Preliminary data suggest that our isolates are *Aspergillus tubingensis*, which is closely related to the previously identified *A. niger.* Phylogenetic relationships between *Aspergillus* isolates from wood cankers and sour rot berries are currently being studied, to understand the etiology and epidemiology of both diseases.

Development of loop mediated isothermal amplification (LAMP) assays for rapid detection of Eutypa and Botryosphaeria dieback pathogens. R. BILLONES-BAAIJENS[1], M. LIU[1,2], M. R. SOSNOWSKI[3,4] and S. SAVOCCHIA[1,2]. *[1]Gulbali Institute, Charles Sturt University, Locked Bag 588, Wagga Wagga, NSW 2678, Australia. [2]School of Agricultural, Environmental and Veterinary Sciences, Charles Sturt University, Locked Bag 588, Wagga Wagga, NSW 2678, Australia. [3]South Australian Research and Development Institute, Adelaide SA 5001, Australia. [4]School of Agriculture, Food and Wine, Waite Research Institute, The University of Adelaide, SA 5005, Australia. E-mail: rbaaijens@csu.edu.au*

Eutypa dieback (ED) and Botryosphaeria dieback (BD) pathogens are prevalent in Australian vineyards, causing yield reductions and threatening vineyard sustainability. Current diagnostics for ED and BD rely primarily on fungal isolations and PCR-based techniques, which are time-consuming, labour intensive or require expensive equipment and skilled staff to perform analyses. Rapid and simple DNA-based methods designed for on-site diagnostics of infected grapevine plant materials allows testing for ED and BD pathogens in low resource environments. We have developed real-time LAMP assays using the Genie II instrument to detect ED and BD pathogens in infected plant material. Five species-specific LAMP assays were developed for ED pathogens, and two genus-specific assays were developed for BD pathogens. A low cost and simple DNA extraction protocol was also developed for LAMP, and was suitable for rapid DNA extraction from infected wood. All seven real-time were highly specific and suitable for detecting and discriminating different ED and BD pathogens, using gBlocks, genomic DNA and crude DNA extracted from infected plant material. All assays can detect 1-2 pg of fungal DNA, alone or in combination with plant DNA. The LAMP assays were at least two times more sensitive than conventional fungal isolation, but were less sensitive than existing qPCR assays. Development of these LAMP assays was rapid and sensitive for detecting ED and BD pathogens from infected plant material. LAMP assays are cost effective, simple, and robust diagnostic tools for assessment of infected plant material in the field or in low resource environments.

Species diversity of Diatrypaceous airborne spores in Australian vineyards. R. BILLONES-BAAIJENS[1], M. LIU[1,2], M. R. AYRES[3], M. R. SOSNOWSKI[3,4] and S. SAVOCCHIA[1,2]. *[1]Gulbali Institute, Charles Sturt University, Locked Bag 588, Wagga Wagga, NSW 2678, Australia. [2]School of Agricultural, Environmental and Veterinary Sciences, Charles Sturt University, Locked Bag 588, Wagga Wagga, NSW 2678, Australia. [3]South Australian Research and Development Institute, Adelaide SA 5001,*

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Eutypa dieback (ED) is an important grapevine trunk disease in Australia, causing significant yield reductions, and threatening vineyard sustainability. *Eutypa lata* and other Diatrypaceous fungi produce ascospores that infect through pruning wounds, resulting in cankers, dieback and eventually vine death. Understanding the prevalence and distribution of airborne spores of these fungi in vineyards will assist in elucidating their importance in disease spread, and in developing subsequent disease management strategies. The present study analysed more than 4800 DNA samples collected using Burkard spore traps from eight wine growing regions over 8 years, using multi-faceted molecular tools, to investigate diversity and abundance of these pathogens in Australian vineyards. A multi-target quantitative PCR assay using SYBR green detected Diatrypaceous spores from 21% of the samples analysed, with spore numbers and frequency of detection varying between regions. Subsequent Sanger sequencing of amplified Diatrypaceous DNA identified seven Diatrypaceous species, with *E. lata* being the most prevalent in South Australia, while *Eutypella citricola* and *Eu. microtheca* were frequently detected only in New South Wales. Two new species, *Diatrype stigma* and *Cryptosphaeria multicontinentalis* were also identified at high frequency, with *D. stigma* being detected in several regions. The data generated to date showed high species diversity of Diatrypaceous spores trapped from different wine growing regions in Australia. Data continue to be generated from spore traps deployed across Australian wine regions, so this study will further elucidate the critical times of the year when ED spores are abundant in vineyards. This will provide regionally localised data that will assist growers in making decisions for optimal timing of pruning and wound protection in their vineyards.

Implementation of droplet digital PCR to determine grapevine health status and abundance of trunk disease fungi in ready-to-plant nursery material imported into Canada. J. HRYCAN[1,2], J. THEILMANN[1], A. MAHOVLIC[1,2], J. BOULÉ[1] and J. R. ÚRBEZ-TORRES[1]. *[1]Summerland Research and Development Centre, Agriculture and Agri-Food Canada, 4200 Highway 97, Summerland, BC V0H 1Z0, Canada. [2]Department of Biology, The University of British Columbia Okanagan, 3187 University Way, Kelowna, BC V1V 1V7, Canada. E-mail: jared.hrycan@agr.gc.ca*

Fungi associated with Botryosphaeria dieback, black foot, and Petri disease are prevalent in asymptomatic and symptomatic inner tissues of nursery grapevine plants and in young vineyards. Canada is reliant on the import of ready-to-plant grapevines for industry growth, but there have been no investigations of the health status of nursery material imported for use in this country. Five nurseries, which import ready-to-plant grapevine material, were selected to identify the most prevalent grapevine trunk disease (GTD) fungi. Fifteen plants each of 'Chardonnay', 'Merlot' and 'Pinot Noir', grafted on '3309C' rootstock, were analyzed from four nurseries. The same number of self-rooted 'Chardonnay', 'Merlot' and 'Pinot Noir' plants were analyzed from one nursery. Tissue samples were collected from roots, bases, graft-unions (in grafted plants), and scions from the plants. Primers were either adapted or newly developed for use in droplet digital PCR (ddPCR), to determine the quantities of *Botryosphaeriaceae* spp., *Cadophora luteo-olivacea*, *Ilyonectria* spp., *Dactylonectria torrensensis*, *Phaeoacremonium minimum*, and *Phaeomoniella chlamydospora* from each host tissue type. Traditional re-isolations were conducted from each tissue type, and isolated fungi were identified using morphological and molecular techniques. Internal necrosis was measured at the base of each plant to further understand how levels of infection may correlate with symptom expression. All plants used in this study were infected by at least one of the above listed GTD fungi. Differences were found for pathogen concentration among plants within each host cultivar, and between cultivars from the same nursery. Differences among nurseries were also observed for the GTD fungi present. These fungi and their concentrations varied among the different parts of host plants. Overall, plant bases and graft-unions contained the highest quantities of GTD fungi.

Rapid detection of *Neofusicoccum parvum* using Loop mediated isothermal amplification (LAMP). S. ECHEVERRÍA[1,2,3], J. PÉREZ[2], R. POLANCO[1] and F. GAÍNZA-CORTÉS[3]. *[1]Universidad Andrés Bello, Centro de Biotecnología Vegetal, Laboratorio de Hongos Fitopatógenos, República 330, Santiago, Chile. [2]Universidad Andrés Bello, Centro de Bioinformática y Biología Integrativa, Laboratorio de Bionanotecnología, República 330, Santiago, Chile. [3]Centro de Investigación e Innovación, Viña Concha y Toro, k-650 km 10, Pencahue, Maule, Chile. E-mail: s.echeverraaraya@uandresbello.edu*

Neofusicoccum parvum one of the fungi associated with grapevine trunk diseases (GTDs), which are important

economic problems for viticulture in Chile. Sanitary measures, such as early detection of these pathogens, are the most efficient way to mitigate and control infections caused by *N. parvum*. qPCR is the most widely used tool for detection of this pathogen, due to its sensitivity. However, this approach is of high cost when intensifying and increasing systems for early detection of *N. parvum*. LAMP is a low-cost technique that amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions, and is a useful tool for the quantifying and intensifying detection of grapevine trunk fungi in greenhouse grown plants. we developed a set of LAMP primers for early detection of *N. parvum* based on the MAT locus. The analysis of sequencing products from the MAT 1-2-1 gene of the MAT 1-2 idiomorph of Chilean isolates allowed identification of new distinctive regions of the species. Visualization of the products obtained after LAMP amplification by colorimetry and electrophoresis showed that *N. parvum* was detected after 35 min of reaction at 65°C. This promising technique could be implemented in sanitary evaluations for the early detection of plant pathogens in high intensity production systems.

Botryosphaeria dieback is a major component of the grapevine trunk disease complex in Oregon vineyards. A. N. KC and M. HERNANDEZ. *Oregon State University, Southern Oregon Research and Extension Center, Central Point, OR, USA. E-mail: achala.kc@oregonstate.edu*

Western states of the United States of America, including California, Oregon, and Washington, are the major wine grape-producing regions of this country. While there are reports of grapevine trunk diseases (GTDs) in neighbouring states, there is limited information available on GTD prevalence in Oregon vineyards. We characterized the GTD pathogens in Oregon vineyards, and *Botryosphaeriaceae* spp. spore release in two geographically distinct regions. Northern Oregon has wet weather (1050 mm average annual rainfall), whereas, southern Oregon has dry weather (500 mm). Trunk tissue samples were collected from 29 vineyards in both regions during the fall of 2019 and 2020. Fungi were identified through culture and PCR based methods. The GTD pathogens included *Botryosphaeriaceae* spp. and *Phaeoacremonium* spp. from, respectively, 72% and 21% of the surveyed vineyards. *Phaeomoniella chlamydospora*, *Cryptovalsa ampelina*, *Truncatella angustata*, *Seimatosporium lichenicola*, *Hormonema viticola* were detected from 7% of the vineyards; and *Dactylonectria macrodidyma*, and *Pestaloptiopsis* sp. from 3 % of the vineyards. Pathogens were detected in both rainfall regions, and in young and mature vines. The presence of GTD pathogens in the Esca disease complex was affected ($P = 0.02$) by vineyard age, with pathogens being more abundant in old than in young vineyards. *Botryosphaeriaceae* species were the most commonly detected group, and their spore releases were affected by the region. In northern Oregon, the spore detection occurred between December and February. In southern Oregon, detection was between November and January. This study provides insights on common GTD pathogens in Oregon vineyards, and their spore release during the critical months of vineyard pruning, which is important knowledge for implementing disease management practices to prevent and mitigate Botryosphaeria dieback.

Carbohydrate-active enzymes and secondary metabolite production by the grapevine pathogen *Neofusicoccum parvum* Bt-67 grown on two lignocellulosic biomasses. J. D. RESTREPO-LEAL[1,2], M. BELAIR[1], J. FISCHER[3], N. RICHET[2], F. FONTAINE[2], C. RÉMOND[1], O. FERNANDEZ[2] and L. BESAURY[1]. *[1]UMR FARE 614 INRAE-URCA – Fractionnement des Agroressources et environnement, Chaire AFERE, Université de Reims Champagne-Ardenne, 51097, Reims, France. [2]RIBP USC INRAE 1488 – Résistance Induite et BioProtection des Plantes – EA 4707, Chaire MALDIVE, Université de Reims Champagne-Ardenne, 51100, Reims, France. [3]Institut für Biotechnologie und Wirkstoff-Forschung (IBWF), 55128, Mainz, Germany. E-mail: olivier.fernandez@univ-reims.fr/ludovic.besaury@univ-reims.fr*

Neofusicoccum parvum is an aggressive *Botryosphaeriaceae* pathogen associated with grapevine trunk diseases. This fungus may secrete enzymes capable of overcoming plant barriers, leading to wood colonization. In addition to roles in pathogenicity, carbohydrate-active enzymes (CAZymes) may be involved in plant cell wall degradation, and be useful for lignocellulose biorefining. *Neofusicoccum parvum* also produces toxic secondary metabolites that may contribute to its virulence. The mechanisms underlying pathogenicity and virulence of this pathogen, and its metabolism and CAZymes were examined for potential in lignocellulose biorefining. The potential of *N. parvum* Bt-67 to produce lignocellulolytic enzymes and phytotoxic secondary metabolites was examined *in vitro* on two biomasses, grapevine canes and wheat straw, in a multiphasic study combining enzymology, transcriptomic and metabolomic analyses. Enzyme assays showed greater xylanase, xylosidase, arabinosidase, and glucosidase activities when the fun-

gus was grown on wheat straw than on grapevine canes. Fourier Transform Infrared (FTIR) spectroscopy confirmed the lignocellulosic biomass degradation caused by the secreted enzymes. Transcriptomics indicated that the *N. parvum* Bt-67 gene expression in the presence of both biomass types was similar. In total, 134 genes coding CAZymes were up-regulated, and 94 of these expressed in both biomass growth conditions. Lytic polysaccharide monooxygenases, glucosidases, and endoglucanases were the most represented CAZymes, and correlated with the enzymatic activities obtained. Secondary metabolite diversity, analyzed by HPLC-UV-Vis-MS, was variable, depending on the carbon source. Preliminary results suggest that, contrary to enzymatic activities, secondary metabolite contents was greater when *N. parvum* Bt-67 was grown with canes than on wheat straw. These results provide insights into the influence of lignocellulosic biomass on virulence factor expression. This research also opens the possibility of optimizing enzyme production from *N. parvum* for lignocellulose biorefining.

Exploration of a non-enzymatic wood degradation pathway in *Fomitiporia mediterranea*, the main white rot pathogen in European Esca-affected vineyards. 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 identified as a white rot pathogen that has been associated with Esca in European vineyards. This fungus has biomolecular wood degradation mechanisms that are not yet fully understood. White rot has an enzymatic pool (laccases, Class II-peroxidases, carbohydrate-active enzymes) that can degrade lignocellulosic biomass (cellulose, hemicellulose and lignin), leaving grapevine wood as fibrous bleached residues. Comparative genomics and previously reported observations of wood cell microvoid size allowed us to formulate the hypothesis that Fmed could adopt non-enzymatic and enzymatic mechanisms to degrade grapevine wood. This hypothesis is based on the Chelator Mediated Fenton (CMF) model, proposed in the late 1990s by American researchers for brown rot fungi. According to our results, under appropriate experimental conditions as close as possible to the physiological conditions of grapevine wood, Fmed demonstrated *in vitro* ability to: *i*) produce low molecular weight (LMW) iron-chelating compounds, which can *ii*) reduce ferric iron to ferrous iron, and *iii*) initiate the redox cycle to produce hydroxyl radicals, thus satisfying all the conditions for a CMF-like non-enzymatic wood degradation mechanism. Carbohydrate oxidative experiments also showed that reducing sugars are liberated when cellulose and hemicellulose are treated with the CMF reaction induced by Fmed LMW extracts. Variations in the CMF reaction were observed among different Fmed strains. Research is ongoing to study effectiveness of the non-enzymatic pathway *in lignum* and *in planta*.

Analyzing the presence of trunk disease fungi in heritage grapevines of Baja California. C. S. DELGADO-RAMÍREZ[1], E. SEPÚLVEDA[2], C. VALENZUELA-SOLANO[3] and R. HERNÁNDEZ-MARTÍNEZ[1]. *[1]Centro de Investigación Científica y de Educación Superior de Ensenada, Baja California (CICESE), Departamento de Microbiología, Carretera Tijuana-Ensenada 3918, Zona Playitas, 22860, Ensenada, Baja California, Mexico. [2]CONACYT-CICESE. Departamento de Microbiología, Carretera TijuanaEnsenada 3918, Zona Playitas, 22860, Ensenada, Baja California, Mexico. [3]INIFAP, sitio experimental Costa de Ensenada, Mexico. E-mail: cdelgado@cicese.edu.mx*

In Baja California, Mexico, vineyards occur where plants over 50 years old are cultured without irrigation. These plants derive from "heritage vines", first acquired by Dominican missionaries in approx. 1697. The cultivar Mission stands out among them. It has vigorous foliage, high resistance to drought, and vigorous and long shoots. There are less than 38 ha of heritage vines in Baja California. These plants are established in dry conditions, with rainfall of less than 250 mm p. a. and with grown little or no pesticide and fertilizer applications. Climate change scenarios predict less rain and increased in area, so these vines are being re-evaluated by some viticulturists. It is important to determine the pathogens present that could limit their establishment. The present study analyzed trunk disease fungi in heritage vines. Wood samples were collected from eight vineyards, from plants with symptoms of dieback, dead arms, or cankers. Using PDA and water agar media, 78 fungal isolates

were recovered, including 18 with morphological characteristics similar to trunk disease pathogens. Isolate characterization was achieved using microscopy and molecular identification of ITS and EF1-α fragments. The isolates belonged to *Cytospora*, *Diaporthe*, and *Diplodia*. *Diplodia seriata* was the most abundant species, followed by *Diplodia pinea, Diaporthe ampelina* and *Cytospora parasitica*. Other identified fungi were: *Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp., *Trichoderma asperellum* and *Sordaria fimicola*. Assessment of Koch's postulates for these fungi is currently underway.

The most important fungi responsible for grapevine trunk diseases in southern Italy. A. CARLUCCI, M. L. RAIMONDO and F. LOPS. *University of Foggia, Department of Agriculture, Food, Natural resources and Engineering (DAFNE), Via Napoli 25, 71122 Foggia, Italy. E-mail: antonia.carlucci@unifg.it*

Grapevine cultivation in Italy has always been affected by biotic diseases affecting roots, trunks, cordons, leaves and berries. During the last 50 years, several studies on these diseases have highlighted the fungi that are responsible for vine and yield decline. Studies in Apulia region, of southern Italy during the last 30 years have reported the following fungi as grapevine pathogens: *Phaeoacremonium* spp., *Phaeomoniella chlamydospora*, *Botryosphaeriaceae* spp., *Pleurostoma richardsiae*, some black foot pathogens (*Dactylonetrica*, *Ilyonectria*, *Thelonectria* spp.), and other minor (less-isolated) fungi including *Seimatosporium*, *Truncatella*, *Cadophora* and *Colletotrichum* spp. Isolation frequencies of these fungi have been variable, associated with different external and internal host root, trunk, and branch symptoms, and with different vineyard cultivars and ages. Fungal pathogens were isolated from grapevine wood with different symptoms including asymptomatic samples. Forty-five to 50%, of wood tissue pieces were infected, mainly with *Botryosphaeriaceae* species; 8-15% of tissue pieces with *Phaeoacremonium* species; 3-10% with *Phaeomoniella chlamydospora*; 8–10% with black foot fungi, and the remaining 15% with other fungi. The same fungal species were also isolated from asymptomatic tissue pieces although with lower isolation frequencies, indicating that they were hemibiotrofic fungi during early years of grapevine growth. Pathogenicity tests confirmed the ability of all isolated fungi to infect grapevines. Further studies are necessary to understand which environmental, geographic, and host cultivar conditions could switch the behaviour of these fungi from hemibiotrofic to pathogenetic.

4D imaging of growing grapevines: towards non-destructive diagnosis of trunk diseases. R. FERNANDEZ[1,2,10], L. LE CUNFF[1,10], S. MERIGEAUD[3], J. L. VERDEIL[4,10], J. PERRY[5], P. LARIGNON[6], A. S. SPILMONT[7], P. CHATELET[10], M. CARDOSO[8], C. GOZE-BAC[9] and C. MOISY[1,10]. [1]IFV, French Institute of Vine and Wine, UMT *Géno-Vigne, F-34398 Montpellier, France.* [2]*CIRAD, UMR AGAP Institut, F-34398 Montpellier, France.* [3]*TRIDILOGY SARL, Groupe CRP - VIDI, Montpellier, France.* [4]*CIRAD, Phiv, Campus Lavalette, 389 Avenue Agropolis, Montferrier-sur-Lez, France.* [5]*CIVC Comité Champagne, 5 rue Henri Martin, 51200 Epernay, France.* [6]*IFV Nîmes. Pôle Rhône-Méditerranée, 7 avenue Cazeaux, 30230 Rodilhan, France.* [7]*IFV Pôle Matériel Végétal, Domaine de l'Espiguette, 30240 Le Grau du Roi, France.* [8]*BNIF University of Montpellier, Place Eugène Bataillon, Montpellier, France.* [9]*Laboratoire Charles Coulomb, University of Montpellier and CNRS, 34095 Montpellier, France.* [10]*UMR AGAP Institut, Univ Montpellier, CIRAD, INRAE, Institut Agro, Montpellier, France. E-mail: cedric.moisy@vignevin.com*

Vineyards sustainability is threatened by grapevine trunk diseases (GTDs), which irreversibly degrade internal trunk tissues and ultimately cause vine death. Foliar symptoms can appear erratically, but sanitary status of vines cannot be ascertained without injuring the plants. A novel approach was developed for GTD detection based on medical multimodal 4D imaging and Artificial Intelligence (AI)-based image processing. This allowed non-invasive GTD diagnosis. Each imaging modality contributed differently to tissue discrimination, and quantitative structural and physiological markers characterizing wood degradation were identified. Combining information on anatomical distribution of degraded tissues and vine foliar symptom history in Champagne, France, it was shown that white rot and intact tissue contents were key measurements. A model was proposed for accurate GTD diagnosis. This research opens new routes for precision agriculture, by permitting field monitoring of GTD and surveying plant health *in situ*.

Managing Esca in Bordeaux: an economic analysis of curative practices under appellation yield risk and uncertain disease severity. M. Y. E. KONAN[1,2], A. ALONSO UGAGLIA[1,3] and J. KAPLAN[4]. [1]*UMR INRAE 1065 Santé et Agroécologie du Vignoble (SAVE), F-33140 Villenave d'Ornon, France.* [2]*Bordeaux School of Economics (BSE), University of Bordeaux, Maison de l'Economie, Avenue Léon Duguit, 33608 Pessac Cedex, France.* [3]*Bordeaux Sciences Agro, 1 Cr du Général de Gaulle, 33170*

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Increased incidence of grapevine trunk diseases (GTDs) over the last 10-15 years has been reported in many grape producing countries. In France, yield losses are estimated at 4.6 hL ha^{-1} $year^{-1}$ due to Esca, the main GTD affecting grapevines in France. Despite this threat, many grape producers are reluctant to adopt field-tested preventive and curative practices. We combined an epidemiological model, developed at INRAE, for Esca infection, with an economic decision-making model, for representative vineyards in AOC Entre-Deux-Mers, AOC Pauillac, and AOC Cognac, to analyze two curative practices (curettage and complantation). Within this combined nature-human model, we analyzed how risk preferences influenced grower decisions to implement a curative practice to reduce the incidence of Esca and achieve appellation yield requirements. Different decision criteria were considered, based on risk preferences and practice efficiency, and outcomes were compared across practices, risk preferences, and efficiency to identify preferred strategies and the incremental value of high practice efficiency over low efficiency. The simulations showed that the decision of growers to implement a practice mostly favoured curettage over complantation and doing nothing, and was dependent on risk preference. In the AOC Pauillac, adopting curettage generated maximum cumulative economic loss over 40 years of 239,257 € ha^{-1}, instead of 514,615 € ha^{-1} with the decision of inaction (when practice efficiency was high). High practice efficiency also generated an additional gain of 67,147 € ha^{-1} over 40 years. These results indicate that non-economic factors may be discouraging growers from adopting curative practices, or that knowledge of the economic gains may be lacking. Providing growers with evidence of the economic benefits could lead to greater adoption of effective disease management, increased profitability, and a more sustainable wine industry in France. Investments in practice efficiency can also have substantial benefits, especially in high-valued wine grape producing regions.

Variation of mycorrhizal colonization in plants with canopy symptoms of Esca. L. LANDI, S. MUROLO and G. ROMANAZZI. *Department of Agricultural, Food and Environmental Sciences, Marche Polytechnic University, I-60131 Ancona, Italy. E-mail: g.romanazzi@univpm.it*

Esca is an important disease of grapevines, associated with several fungal pathogens, of which *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum* and *Fomitiporia mediterranea* are the most important. The onset of Esca can be affected by several factors, including host cultivar and rootstock, weather conditions, the trellising system and phytosanitary management. Effects of plant stress may also affect disease onset, which has yet to be fully clarified. Intensive investigations have been carried out on the microorganisms affecting the grapevine canopies, in particular on trunks and branches, while relatively little information is available on the colonization of root systems by pathogens and putatively beneficial microorganisms. The present study used molecular tools to identify microorganisms associated with roots of symptomatic plants, compared to apparently symptomless vines, in three vineyards of the Marche region of central-eastern Italy. Arbuscular mycorrhizal fungi (AMF) colonization intensity was greater in grapevines with symptoms of Esca (24.6 to 61.3%) than in neighbouring asymptomatic plants (17.4 to 57.6%). Specific primer pairs for native *Funneliformis mosseae* and *Rhizophagus irregularis* AMF showed more DNA copies (respectively, 67 vs 47 and 292 vs 201, in 20 µL) of both species in Esca symptomatic vines. This study indicates a possible relationship between Esca and native AMF in grapevines.

Diversity and pathogenicity of fungi causing grapevine trunk diseases in Chilean patrimonial vineyards. D. GRINBERGS, J. CHILIAN and M. REYES. *Instituto de Investigaciones Agropecuarias, INIA Quilamapu, Chillán, Chile. E-mail: dgrinbergs@inia.cl*

Trunk diseases are a major problem in Chilean commercial vineyards, reducing their productivity, longevity and grape quality. However, in ancient patrimonial vineyards, mostly containing the cultivars País, Moscatel, Carignan, País, Torontel and Moscatel, which are being rescued to make historical wines, these diseases are beginning to be studied. Preliminary identifications were performed for 235 fungal isolates from affected plants collected in Cauquenes and Itata valley, to determine the identity of the most frequently isolated species and the pathogenicity on grapevine of representative isolates. The isolates were macro- and microscopically studied after 14 d on PDA at 25°C. Isolate identity was confirmed by extracting DNA from pure PDA cultures, and genes were amplified depending on the morphological identifications. Grapevine plants were produced rooting 1-year old healthy canes ('Petit Syrah') in tap water amended with 500 ppm of indole butyric acid. Pathogenicity was assessed by inoculating fresh cuts with 0.5

cm diam. mycelium plugs of actively growing PDA colonies of *Arambarria destruens*, *Seimatosporium vitifusiforme*, *Diplodia seriata*, *D. mutila* and *Neofusicoccum parvum*. Plants (n = 7) were then incubated at 25 ± 3°C for 60 d in flowing tap water. Bark was then removed, and necrotic lesions were measured and compared (Tukey tests, $P < 0.05$). Virulence levels were detected among species, and among isolates from the same species. *Botryosphaeriaceae* species *N. parvum, D. seriata* and *D. mutila* were the most virulent, followed by *S. vitifusiforme* and *A. destruens*.

Establishment of a cell model to study molecular interactions between grapevine and Esca-associated pathogens. F. RAKOTONIAINA[1,2], C. BRETON[1], L. TESSAROTTO[2], C. CHERVIN[2], A. JACQUES[1] and O. RODRIGUES[1]. *[1]Unité de Recherche Physiologie, Pathologie et Génétique Végétales, Université Fédérale Toulouse Midi-Pyrénées, INP-PURPAN, F-31076 Toulouse, France. [2]Laboratoire de Recherche en Sciences Végétales, Université de Toulouse, CNRS, UPS, Toulouse INP, 24, chemin de Borde-Rouge, Auzeville, 31320, Castanet-Tolosan, France. E-mail: olivier.rodrigues@purpan.fr*

Esca is poorly understood trunk disease complex, which causes important economic losses in vineyards. Genetic solutions or biostimulation are the main strategies being considered for vineyard protection from these diseases. Characterization of molecular events occurring during interactions between grapevine cells and Esca-associated pathogens is a prerequisite for developing these strategies, but this is impossible to perform directly in vine trunks. Different cell strategies were developed for this purpose, including assessments of calli and leaf stomata. Guard cells, a model for the characterization of cell signalling, perceive stimuli, and respond by inducing stomatal opening/closing, which can be quantified. Assays on epidermal peels show that guard cells of *Arabidopsis thaliana* and *Vitis vinifera* can detect and respond to Esca-associated pathogens. Using pharmacological and genetic approaches, involvement of ethylene signalling in the cell responses to pathogens was assessed. Guard cell and stomatal responses are efficient and promising tools to establish a cell pathosystem model to characterize and explore molecular events that occur during grapevine reactions to Esca-associated pathogens.

Diversity of *Neofusicoccum parvum* for the production of the phytotoxic metabolites (-)-terremutin and (R)-mellein. P. TROTEL-AZIZ[1], G. ROBERT-SIEGWALD[2], O. FERNANDEZ[1], C. LEAL[1], S. VILLAUME[1], J. F. GUISE[1], E. ABOU-MANSOUR[3], M. H. LEBRUN[4] and F. FONTAINE[1]. *[1]Research Unit Résistance Induite et Bioprotection des Plantes, Université de Reims Champagne-Ardenne, RIBP EA 4707, INRAE USC 1488, SFR Condorcet FR CNRS 3417, 51100 Reims, France. [2]Independent Researcher, 49000 Angers, France. [3]Département de biologie végétale, Université de Fribourg, Chemin du Musée 10, 1700 Fribourg, Switzerland. [4]Research Group Genomics of Plant-Pathogen Interactions, Research Unit Biologie et Gestion des Risques en Agriculture, UR 1290 BIOGER, Université Paris Saclay, INRAE, Avenue Lucien Brétignières, 78850 Thiverval-Grignon, France. E-mail: patricia.trotel-aziz@univ-reims.fr; florence.fontaine@univ-reims.fr*

Plant pathogens have evolved various strategies to enter hosts and cause diseases. *Neofusicoccum parvum*, an aggressive member of the Botryosphaeria dieback consortium, can secrete the phytotoxins (-)-terremutin and (R)-mellein during grapevine colonization. Although an exogenous supply of purified phytotoxins to plantlets was shown to weaken grapevine immunity, their contributions to Botryosphaeria dieback symptoms in lignified plants remains unknown. Two *Neofusicoccum parvum* isolates and a UV induced mutant were characterized for their phytotoxin production *in vitro*, pathogenicity to grapevine, and their genome sequences. Isolate Np-Bt67 produced a high level of (-)-terremutin, but almost no (R)-mellein, and it was the most aggressive on grapevine, triggering apoplexy. Similar symptoms were not induced by purified (-)-terremutin. Isolate Bourgogne S-116 (Np-B) produced 3-fold less (-)-terremutin and large amounts of (R)-mellein, but was less aggressive on grapevine than Np-Bt67. The UV9 mutant obtained from Np-B (NpB-UV9) did not produce (-)-terremutin but overproduced (R)-mellein by 2.5-fold, and was as pathogenic as its parent. NpB-UV9 differed from its parent by simple mutations in two genes (transcription factor UCR-NP2_6692, regulatory protein UCR-NP2_9007), not located near (R)-mellein nor (-)-terremutin biosynthesis genes, but likely involved in the control of (-)-terremutin biosynthesis. Grapevine immunity was disturbed after challenge with these pathogens or purified phytotoxins, leading to upregulation of SA-dependent defences, while (-)-terremutin interfered with host JA/ET-dependent defences. These results indicate that neither (-)-terremutin nor (R)-mellein alone are essential for the pathogenicity of *N. parvum* on grapevine, since a (-)-terremutin non-producing mutant isolate which overproduced (R)-mellein *in vitro* was as pathogenic as the parent isolate. However, these phyto-

toxins could play quantitative roles in the infection processes.

Beneficial effects of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 against the Botryosphaeria-dieback pathogen *Neofusicoccum parvum* may vary with grapevine cultivar. C. LEAL[1,3], N. RICHET[1], J. GUISE[1], D. GRAMAJE[2], J. ARMENGOL[3], F. FONTAINE[1] and P. TROTEL-AZIZ[1]. *[1]University of Reims Champagne-Ardenne, Résistance Induite et Bioprotection des Plantes Research Unit, EA 4707, INRAE USC 1488, SFR Condorcet FR CNRS 3417, Reims, France.* [2]Instituto de *Ciencias de la Vid y del Vino, Consejo Superior de Investigaciones Científicas, Universidad de la Rioja, Gobierno de La Rioja, Logroño, Spain.* [3]*Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Valencia, Spain. E-mail: catarinaleal09gmail.com*

Grapevine trunk diseases (GTDs) threaten viticulture, and biocontrol strategies may be alternatives to cope with environmental concerns over pesticide use for managing these diseases. Combinations of biological control agents (BCAs) may be more effective than one BCA alone. The combination of *Bacillus subtilis* (Bs) PTA-271 and *Trichoderma atroviride* (Ta) SC1 was evaluated for the protection of *Vitis vinifera* 'Chardonnay' and 'Tempranillo' rootlings against *Neofusicoccum parvum* Bt67, an aggressive fungus that causes Botryosphaeria dieback. Indirect effects on the host of each BCA alone and in combination were assessed *in planta*, as well as their direct effects on the pathogen *in vitro*. Results indicated that: (1) the host cultivar contributes to the beneficial effects of Bs PTA-271 and Ta SC1 against *N. parvum*; and (2) the *in vitro* mutual BCA antagonism switched to the strongest fungistatic effect toward *N. parvum* in a three-way confrontation. The beneficial potential of a combination of BCAs against *N. parvum* was demonstrated, especially in 'Tempranillo'. These results highlight a common feature in both cultivars. Salicylic acid (SA)-dependent defences were low in plants protected by the BCA, in contrast to symptomatic plants. The high level of expression of SA-dependent defences in 'Tempranillo' is likely to be associated with its susceptibility to *N. parvum*, although cultivar-specific responses to the BCAs require further elucidation.

Susceptibility of pruning wounds to grapevine trunk disease pathogens in different Australian climates. M. R. SOSNOWSKI[1,2], M. R. AYRES[1], R. BILLONES-BAAIJENS[3] and S. SAVOCCHIA[3,4]. *[1]South Australian Research and Development Institute, Adelaide, SA 5001, Australia.* [2]*School of Agriculture, Food and Wine, Waite Research Institute, The University of Adelaide, SA 5005, Australia.* [3]*Gulbali Institute, Charles Sturt University, Wagga Wagga, NSW 2678, Australia.* [4]*School of Agricultural, Environmental and Veterinary Sciences, Wagga Wagga, NSW 2678, Australia. E-mail: mark.sosnowski@sa.gov.au*

Grapevine trunk diseases (GTDs), caused by fungi that infect pruning wounds, pose major threats to vineyard sustainability. Reports from different international regions indicate that there is variation in the duration of susceptibility of pruning wounds to infections at different times during pruning seasons. A series of wound susceptibility trials were conducted from 2013 to 2019 in three climatically different regions; McLaren Vale (MV) (warm-dry) and Adelaide Hills (AH) (warm-wet) in South Australia, and Big Rivers (BR) (hot-dry), New South Wales, using 'Shiraz' (MV and AH) and 'Cabernet Sauvignon' (BR). One-year-old canes were pruned in early (June), mid (July) and late (August) winter, followed by inoculations at weekly intervals for up to 16 weeks with the GTD pathogens *Eutypa lata* (Eutypa dieback) and *Diplodia seriata* (Botryosphaeria dieback). Wounds were very susceptible to infection by both pathogens immediately following pruning, after which susceptibility decreased rapidly over the following 14 d. From 21 d post-pruning, susceptibility was generally negligible, similar to non-inoculated controls, apart from *D. seriata* inoculations in BR, where susceptibility remained high, including in non-inoculated controls. For *E. lata*, wounds were less susceptible to infection when pruned in late winter in AH, but susceptibility was similar across the pruning times in MV. Wounds were generally more susceptible to infection by *D. seriata* in BR than in AH. These results indicate that, in AH, delaying pruning to late in the host dormant season could reduce the risk of *E. lata* infections. The greater susceptibility to *D. seriata* in BR was likely due to the pathogens prevalence in the hot-dry region, and the high inoculum doses used, compared to that of the warm-wet region of AH, where *E. lata* is prevalent. These results also highlight the importance of wound protection for the 2-week post-pruning period of greatest susceptibility.

Characterisation of the endomicrobiome of grapevine nursery plants in Australia. R. BILLONES-BAAIJENS[1,2], M. LIU[1,2], B. STODART[1,2], M. R. SOSNOWSKI[3,4] and S. SAVOCCHIA[1,2]. *[1]Gulbali Institute, Charles Sturt University, Locked Bag 588, Wagga Wagga, NSW 2678, Australia.* [2]*School of Agricultural, Environmental*

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Plant microbiomes are the communities of microorganisms that may form symbiotic, commensal, or pathogenic relationships with their hosts. Well-balanced and highly diverse microbiomes are important for increasing plant fitness by facilitating adaptation to changing environmental conditions. The endophytic microorganisms present in grapevine nursery plants were characterised, focusing on grapevine trunk disease pathogens, to understand the dynamics associated with health and fitness of planting materials and young vines in vineyards. Total DNA was extracted from inner tissues and roots of dormant grafted and own-rooted vines collected from four grapevine nurseries. All DNA samples were analysed utilising high-throughput amplicon sequencing, targeting the 16S rRNA gene and ITS sequences. Preliminary results indicated that microbial communities varied greatly between nurseries, grape varieties, and host tissue types. One nursery had greater overall microbial populations compared to the other three nurseries. 'Ramsey' had the greatest microbial population among rootstocks, while populations were similar for Shiraz and Chardonnay. Microbial populations were also greater in the roots than in above ground tissues. For the bacteria, the most predominant families were *Pseudomonadaceae* (30%) and *Enterobacteriaceae* (22%), some of which are considered harmless or symbionts, although some are also enteric microorganisms. The most abundant fungal taxa were common environmental species and beneficials, including *Trichoderma* spp. and mycorrhizal fungi. Of the grapevine trunk disease (GTD) pathogens, *Phaeoacremonium, Cadophora* and *Eutypella* were most abundant, followed by *Ilyonectria*, *Phaeomoniella*, *Fomitiporia* and *Cryptovalsa*. Further investigations are required to understand the importance of these GTD pathogens in grapevine nursery plants, and their potential impacts on vineyard establishment and longevity in Australia.

Deciphering early transcriptomic and metabolomic host responses in grapevine wood to the Esca pathogens *Phaeoacremonium minimum* and *Phaeomoniella chlamydospora*. A. ROMEO-OLIVÁN[1,*], J. CHERVIN[2,3,4,*], S. FOURNIER[2,3,4], V. PUECH-PAGES[2,3,4], C. BRETON[1], T. LAGRAVÈRE[1], J. DAYDÉ[1], B. DUMAS[2], G. MARTI[2,3,4] and A. JACQUES[1]. *[1]Unité de Recherche Physiologie, Pathologie, et Génétique Végétales (PPGV), INP PURPAN, Université de Toulouse, Toulouse, France. [2]Laboratoire de Recherche en Sciences Végétales, Université de Toulouse, CNRS, UPS, Toulouse INP, Toulouse, France. [3]Metatoul-AgromiX platform, MetaboHUB, National Infrastructure for Metabolomics and Fluxomics, LRSV, Université de Toulouse, CNRS, UPS, Toulouse, INP, France. [4]MetaboHUB-MetaToul, National Infrastructure of Metabolomics and Fluxomics, Toulouse, France. *These authors contributed equally to this work. E-mail: ana.romeo-olivan@purpan.fr*

Early host responses to the Esca pathogens *Phaeoacremonium minimum* and *Phaeomoniella chlamydospora* were compared, based on transcriptomic and metabolomic analyses, to characterise wood responses to either pathogen alone. Transcriptomic analysis identified specific sets of differentially expressed genes associated with each pathogen. Functional analysis of these genes revealed differences, mainly in "Signalling", "Hormonal signalling" and the "Biotic stress response". Metabolomic analysis highlighted a group of flavonoids and stilbenoids that were overproduced in inoculated plants, compared to non-inoculated plants. Metabolomic analysis identified specific metabolites associated with each pathogen. For *P. minimum*, inoculation was associated with the accumulation of a lipophilic flavonoid cluster. The results showed different transcriptomic and metabolomic responses, depending on the pathogen. These observations suggest that grapevine may differently 'perceive' and respond with different sets of defences to wood colonization by *P. minimum* and *P. chlamydospora*.

Comparative transcriptomic and metabolomic responses of grapevine to the biocontrol agent *Trichoderma atroviride* suggest early modifications before infections by the Esca pathogens *Phaeomoniella* and *Phaeoacremonium minimum*. A. ROMEO-OLIVÁN[1,*], J. CHERVIN[2,3,4,*], S. FOURNIER[2,3,4], V. PUECH-PAGES[2,3,4], C. BRETON[1], T. LAGRAVÈRE[1], J. DAYDÉ[1], B. DUMAS[2], G. MARTI[2,3,4] and A. JACQUES[1]. *[1]Unité de Recherche Physiologie, Pathologie, et Génétique Végétales (PPGV), INP PURPAN, Université de Toulouse, Toulouse, France. [2]Laboratoire de Recherche en Sciences Végétales, Université de Toulouse, CNRS, UPS, Toulouse INP, Toulouse, France. [3]Metatoul-AgromiX platform, MetaboHUB, National Infrastructure for Metabolomics and Fluxomics, LRSV, Université de Toulouse, CNRS, UPS, Toulouse, INP, France. [4]MetaboHUB-MetaToul, National Infrastructure of Metabolomics and Fluxomics, Toulouse,*

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The biocontrol agent (BCA) Vintec® is a commercial strain of *Trichoderma atroviride*, labelled for management of Esca in grapevines. Several *Trichoderma* species and strains have been shown to stimulate plant defence mechanisms against different phytopathogens. Early molecular responses were assessed in grapevine wood pre-inoculated with Vintec®, to later infection by the Esca pathogens *Phaeoacremonium minimum* and *Phaeomoniella chlamydospora*. Transcriptional analysis identified clusters of genes differently regulated in the presence of Vintec®, compared to non-treated plants. Phenylpropanoid metabolism and stilbene biosynthesis-related genes were represented among the genes differently expressed in the presence of Vintec®. Metabolomic analysis identified clusters of plant compounds (mainly stilbenes) synthesized in response to Vintec® and to the pathogens. Five relevant 'biomarkers' were chosen for *in vitro* evaluation of their antifungal activity against *P. chlamydospora*. These compounds may play roles in limiting *in planta* development of the pathogens. The efficacy of Vintec® may be associated with early or enhanced biochemical responses to limit host colonization by the pathogens.

Deciphering susceptibility of Greek grapevine cultivars to trunk disease pathogens through measurements of defence-related gene expression. S. TESTEMPASIS, P. NTASIOU, T. TSOUVALAS, E. MPILA and G. S. KARAOGLANIDIS. *Aristotle University of Thessaloniki, Faculty of Agriculture, Forestry and Natural Environment, Plant Pathology Laboratory, Thessaloniki, Greece. E-mail: testempa@agro.auth.gr*

Grapevine Trunk Diseases (GTDs), caused by several fungi, are major threats to viticultural production. Management of GTDs is influenced by biotic and abiotic factors including pathogen, geographic region, climate, cultivar, and cultural practices. The susceptibility was evaluated of the 13 most important Greek grape wine cultivars to three GTD pathogens (*Phaemoniella chlamydospora, Phaeoacremonium minimum* and *Diplodia seriata*). Plants of each cultivar (n = 30) were artificially inoculated and incubated in a greenhouse for 9 months. Disease severity on each cultivar was evaluated by assessing necrotic wood tissue using a 5-scale disease index. Four cultivars ('Limnio', 'Xinomavro', 'Robola', 'Kotsifali') were very tolerant to all three pathogens, and four others ('Vidiano', 'Roditis', 'Savatiano', 'Malagouzia') were very susceptible. To investigate possible mechanisms of resistance to GTDs, transcriptomic analyses were conducted on one tolerant ('Limnio') and one susceptible ('Vidiano') cultivar, by measuring expression levels of 12 defence-related genes at 12, 24, 48, 72 and 168 h post-inoculation (hpi). Early gene accumulation (12 hpi) was observed in 'Limnio' compared to 'Vidiano', in which most of the genes were overexpressed at 72 and 168 hpi. For the most tolerant 'Limnio', the pathogenesis proteins PR6, PR10, PR10.1, and chitinase were triggered. . At the same time, the biotic stress response genes Glutathione S-transferase and lipoxygenase accumulated, along with genes encoding enzymes involved in phytoalexins synthesis (stilbene synthase, phenylalanine ammonia-lyase). This is the first study deciphering susceptibility of Greek grape wine cultivars to GTDs, and enhancing understanding of the molecular basis of grapevine tolerance to the GTD complex.

Tracking the fungal pathobiome associated with young grapevine decline in California nurseries. C. TODD[1], J. F. GARCIA[2], A. JACQUES[3], D. CANTU[2] and P. ROLSHAUSEN[4]. *[1]Department of Microbiology and Plant Pathology, University of California, Riverside, United States. [2]Department of Viticulture and Enology, University of California, Davis, CA, United States. [3]Unité de Recherche Physiologie, Pathologie, et Génétique Végétales (PPGV), INP PURPAN, Université de Toulouse, Toulouse, France. [4]Department of Botany and Plant Sciences, University of California, Riverside, CA, United States. E-mail: philrols@ucr.edu*

Young grapevine decline can impact longevity and productivity of new field plantings, leading to decreased economic returns. Understanding the critical steps during plant propagation that are conducive to the spread of fungal pathogens will allow nurserymen to develop cost effective disease management practices to limit disease incidence in nursery stocks. A survey of grapevine cuttings has been conducted throughout the grapevine production process to determine how different cultural practices affect the fungal pathobiome. Identical Chardonnay and Cabernet Sauvignon clones grafted on 1103P rootstocks were collected across several nurseries at different steps in the propagation pipelines (mother field, callusing, green vines, dormant vines) for 2 consecutive years. Endophytic fungal community composition was assessed with culture-dependent and independent (high-throughput sequencing, HTS) methods, in three different trunk plant compartments (root-rootstocks, below or above graft unions). Culturable fungi were profiled using standard

isolation techniques from wood samples coupled with ITS PCR and Sanger sequencing. Fungal community composition was then profiled using metabarcoding primers designed *in silico* to target Grapevine Trunk Associated Ascomycetes (GTAA). Many known grapevine pathogens were found, including *Phaeoacremonium*, *Phaeomoniella*, *Cadophora*, *Neofusicoccum* and *Diaporthe*. HTS was more sensitive for pathogen detection than culturing. The differences in disease-associated fungal community composition were affected by nurseries, and at the different steps of the production pipeline. These results highlight several possible infection routes during plant propagation.

Esca leaf symptoms: histological alteration and relationship to physiological and nutritional status. M. V. ALARCÓN[1], L. MÉNDEZ-GRANO DE ORO[2], A. FLORES-ROCO[1], A. DE SANTIAGO[2], J. RODRÍGUEZ-PRIETO[1] and L. MARTÍN[2]. *[1]CICYTEX, Agronomy of Woody and Horticultural crops Department. Cell Biology and Microscopy Lab (LBCM). Spain. [2]CICYTEX, Plant Protection Department, Spain. E-mail: laura.martin@juntaex.es*

Manifestation of the Esca in leaves is known as "tiger stripes", and also Grapevine Leaf Stripe Diseases (GLSD). Esca is a plurennial disease, but symptoms are not regular throughout consecutive years, as a vine may show GLSD symptoms one year but be asymptomatic the next. Esca leaf manifestation was monitored from 2018 to 2021 in a vineyard of 'Tempranillo' in Extremadura (Spain). Grapevine leaves were compared under four conditions: (A) green leaves from asymptomatic vines, (S) symptomatic leaves entirely affected by the disease, (SA) green leaves from vines partially affected by Esca, and (SS) symptomatic leaves from vines partially affected by Esca. Histological, physiological, chemical, and nutritional characterizations of leaves were carried out at the time of GLSD manifestation in 2021. Morphometric measurements showed that GLSD reduced leaf areas, and damage was greatest on S and SS leaves. At the histological level, leaf cross-sections showed reduced thickness and area for S and SS than for A leaves. Similarly, decreased upper and lower epidermis tissues, and reduced thickness of palisade and spongy parenchyma tissues were observed. Using a Dualex® leaf clip sensor, decreased chlorophyll and NBI index were found on S leave, while anthocyanins indices increased. No changes were found in the flavonoid contents. Physiological activity (net photosynthesis, stomatal conductance, transpiration, water use efficiency) was low in S leaves. No significant nutrient composition deficiencies were found among the different leaf symptom conditions.

The physiology and drivers of Esca leaf symptom development in grapevine. G. BORTOLAMI[1], T. FREJAVILLE[1], J. POUZOULET[1], N. DELL'ACQUA[1], N. FERRER[1], L. GUERIN DUBRANA[1], P. LARIGNON[2], P. LECOMTE[1], G. GAMBETTA[3] and C. DELMAS[1]. *[1]INRAE, UMR 1065 Santé et Agroécologie du Vignoble, ISVV, F-33140 Villenave d'Ornon, France. [2]Institut Français de la Vigne et du Vin, Pôle Rhône-Méditerranée, 7 avenue Cazeaux, 30230 Rodilhan, France. [3]Bordeaux Science Agro, Institut des Sciences de la Vigne et du Vin, Ecophysiologie et Génomique Fonctionelle de la Vigne, INRAE, 33140 Villenave d'Ornon, France. E-mail: chloe.delmas@inrae.fr*

The development of Esca leaf symptoms is an erratic and misunderstood phenomenon, which varies among grape varieties and growing regions. Esca development is likely driven by multiple factors, such as pathogen populations, vine physiology, and climatic and viticultural conditions. Grapevine hydraulic functioning is at the core of interactions between biotic and abiotic factors during vascular pathogenesis. Host xylem tissues are colonized by many microbes, including the Esca pathogens. The ascent of sap is affected by soil water availability and climatic conditions. The present study focused on exploring (*i*) the impacts of Esca leaf symptom development on xylem hydraulic integrity, and (*ii*) the interactions between drought, climatic conditions, and leaf symptom incidence under controlled conditions and in the field. Leaf symptom development was associated with hydraulic failure caused specifically by xylem occlusion, and subsequent loss of hydraulic conductivity from trunks to leaf laminae. Xylem occlusion by tyloses was specific to Esca leaf symptoms, compared to other types of premature or autumnal leaf senescence, and varied among grape varieties. Using mature potted plants uprooted from a vineyard and naturally infected by Esca pathogens, a long drought (-1MPa of predawn water potential over 3 months) was shown to inhibit Esca leaf symptom development, suggesting that vine water status plays a key role in Esca pathogenesis. Water relations and carbon dynamics were compared during the combined effects of Esca and drought stress. Using bi-monthly leaf symptom monitoring in 50 vineyards in southern France (over several years) and statistical modelling, it was shown that soil humidity, evapotranspiration, and temperature were the key drivers of Esca incidence and phenology. These results provide new insights on the role of plant physiology and microbial communities, and their interactions with climate, as key drivers in Esca pathogenesis.

Influence of morphological traits of xylem vessels on the responses of table- and wine-grape and rootstocks to *Phaeomoniella chlamydospora*. D. GERIN, N. CHIMIENTI, A. AGNUSDEI, R. M. DE MICCOLIS ANGELINI, P. ROTONDO, F. FARETRA and S. POLLASTRO. *University of Bari "Aldo Moro", Department of Soil, Plant and food Sciences, via Amendola 165/A 70126 Bari, Italy. E-mail: stefania.pollastro@uniba.it*

Phaeomoniella chlamydospora causes severe economic losses on grapevines under field and nursery conditions. Xylem structural features could be key determinants of sensitivity or resistance of host plants. The relationships were investigated between *P. chlamydospora* infection and xylem vessel features for ten table grape, 17 wine grape varieties or clones, and three rootstocks. Observations were based on image analysis of cross sections and fungal isolation from host cuttings artificially inoculated with conidial suspension (10^6 conidia mL^{-1}) of the benomyl resistant-marked strain PCHCIA43.2 of *P. chlamydospora* at 30-90 d after inoculation (DAI). Mean vessel diameters of the table grape cultivars were from 58.8 ('Allison') to 70.5 µm ('Red Globe'). 'Allison', 'Sable' and 'Timco' had greater vessel densities than 'Italia', 'Sugar Crisp' and 'Sugraone' in the diameter classes 151–180 µm and >180 µm. Mean vessel diameters of the wine-grape varieties were from 44.8 ('Nero di Troia') to 72.3 µm ('Merlot'), and vessel densities were from 20.3 ('Nero di Troia') to 29.7 vessels mm^{-2} ('Sangiovese'). In the rootstocks, mean vessel diameters were 50.8, 54.0 and 60.9 µm for 34 E.M., 140 'Ruggieri' and 1103, and mean vessel density was always less than 25.5 mm^{-2}. The *in planta* bioassay showed that, at 90 DAI, PCHCIA43.2 was isolated from 93.3% of 'Timco' cuttings and 13.3% of 'Sugar Crisp' and 'Sugraone'. These data were positively correlated with frequencies of large vessels. With few exceptions, on wine-grape varieties, the proportions of re-isolation were related to the frequency of large vessels or vessel density. The pathogen was isolated from 33–51 % of rootstock cuttings, and infection data were more related to vessel densities than vessel size. These results highlight the relationships between susceptibility to *P. chlamydospora* and the size and density of host xylem vessels, which are candidates for further research.

Effects of tissue age on Botryosphaeria dieback caused by *Diplodia seriata* in 'Cabernet Sauvignon' plants. A. LARACH[1,2], E. SALGADO[1], P. SANHUEZA[1], A. SALINAS[1], F. CADIZ[1], M. SEEGER[2] and X. BESOAIN[1]. [1]Escuela de *Agronomía, Facultad de Ciencias Agronómicas y de los Alimentos, Pontificia Universidad Católica de Valparaíso, Casilla 4-D, Quillota 2260000, Chile.* [2]*Laboratorio de Microbiología Molecular y Biotecnología Ambiental, Chemistry Department & Centro de Biotecnología Daniel Alkalay Lowitt, Universidad Técnica Federico Santa María, Avenida España 1680, Valparaíso 2340000, Chile. E-mail: ximena.besoain@pucv.cl*

Symptoms associated with Botryosphaeria dieback (BD) can occur in young or mature vines (>7 years old). In vineyards, prevalence and severity of the disease has been observed to increase with the grapevine age. In Chile, *Diplodia seriata* is the most prevalent species in 'Cabernet Sauvignon' vines. Pathogenicity tests have been carried out in 1- to 2-year-old shoots, but no information is available on susceptibility of mature tissues to pathogen infections. Two strains of *D. seriata* (50 µL of 1.104 conidia mL^{-1}) were inoculated into wounds of tissues of 1, 2, or 10-year-old Cabernet Sauvignon plants under vineyard conditions. Lesions were compared with previous inoculations made in pieces of canes. *D. seriata* was more aggressive in 10-year-old tissues (doubling its aggressiveness) than in younger tissues, and the inoculations gave symptoms that were representative of damage observed in the field. This aspect must be considered when evaluating the importance of each pathogen causing BD, especially because aggressiveness increased when the grapevines were more than 7-years-old.

Wood degradation by *Fomitiporia mediterranea* M. Fischer: physiologic, proteomic and metabolomic approaches. M. SCHILLING[1], A. MAIA-GRONDARD[2], E. ROBERT[3], P. HUGUENEY[2], C. BERTSCH[3], S. FARINE[4] and E. GELHAYE[1]. [1]*Université de Lorraine, INRAE, IAM, Nancy, France.* [2]*INRAE, SVQV UMR-A1131, Université de Strasbourg, Colmar, France.* [3]*Université de Lorraine, INRAE, A2F, Nancy, France ISVV, F-33140 Villenave d'Ornon, France.* [4]*Laboratoire Vigne, Biotechnologies et Environnement (LVBE), Université de Haute-Alsace, Colmar, France. E-mail: marion.schilling@univ-lorraine.fr*

Fomitiporia mediterranea (Fmed) is the main fungus found in grapevine wood rot ("amadou"), one of the most typical symptoms of the grapevine trunk disease Esca. This fungus is functionally classified as a white-rot pathogen, able to degrade all wood structure polymers, including hemicelluloses, cellulose, and lignin, the most recalcitrant component. Specific carbohydrate active enzymes are secreted by fungi to degrade hemicelluloses and cellulose, which can be highly specific to the nature of polysaccharides, and peroxidases, which enable Fmed

to degrade lignin, with specificities also related to lignin composition. Besides polymers, a diverse set of metabolites often associated with antifungal activities, is found in wood, this set differing among wood species. Wood decomposers detoxify these specific compounds, and this ability may indicate adaptation of these fungi to specific environments. The present study aimed to better understand the molecular mechanisms used by Fmed to degrade wood structure, particularly its potential adaptation to grapevine wood. Fmed was cultivated on sawdust from grapevine, beech, and spruce. Measurements were made of carbon mineralization rates, mass losses, and contents of wood structure polymers, targeted metabolites (extractives) and secreted proteins. *Trametes versicolor*, a ubiquitous white-rot model organism, was used for comparison. No significant degradation was observed on spruce, but greater mass loss was measured on Fmed grapevine cultures compared to beech cultures. On both substrates, simultaneous degradation patterns were demonstrated, and proteomic analysis revealed only minor differences in secreted enzymes responsible for wood degradation. These results are a first step in the understanding the adaptation of Fmed to its ecological niche, and indicate potential for further research.

Occurrence of *Phaeomoniella chlamydospora* at different stages of grapevine propagation, and pathogen biomass fluctuation from the nursery to the field in association with the endophytic microbiome. C. TSOUKAS[1], P. ATHANASIADI[1], F. BEKRIS[2], S. VASILEIADIS[2], E. PAPADOPOULOU[2], D. KARPOUZAS[2], A. K. TZIMA[1] and E. J. PAPLOMATAS[1]. *[1]Agricultural University of Athens, Department of Crop Science, Laboratory of Plant Pathology, Iera Odos 75, 11855, Athens, Greece. [2]Laboratory of Plant and Environmental Biotechnology, Viopolis, Department of Biochemistry and Biotechnology, University of Thessaly,41500 Larissa, Greece*

Phaeomoniella chlamydospora (*Pch*), associated with young grapevine decline, has been widely reported in vine nurseries and young vines. The present study investigated the fluctuation of *Pa. chlamydospora* biomass at three stages of propagation material, in order to correlate pathogen biomass with the endophytic microbiome. Dormant cuttings (stage 1) from two Greek varieties and two rootstocks and different rootstock-scion combinations of grafted unrooted (stage 2) and rooted vines planted for 4 months in the field (stage 3), were sampled. TaqMan qPCR assays were carried out to quantify pathogen biomass within the collected samples. Mean biomass of *Pch* for all dormant cuttings (1) was 3.94 pg per 100 ng of plant DNA (3.94×10^{-5} g *Pch* DNA g^{-1} plant DNA). Mean *Pch* biomass for all rootstock-scion combinations was 1.21 pg in 100 ng plant DNA for grafted unrooted plants (2) and 62.14 pg per 100 ng of plant DNA for rooted vines (3), indicating a 50-fold increase of *Pch* biomass from the nursery to the field. To investigate correlations between *Pa. chlamydospora* biomass within the different types of propagation material and the endophytic microbiome, DNA samples with the smallest and greatest biomass values were pooled for Next Generation Sequencing analysis. Based on these results and also according to the literature, *Pa. chlamydospora* is detected in dormant cuttings derived from nursery mother vines. The large increase of pathogen biomass from the grafted unrooted vines to the grafted rooted vines confirms that the propagation process plays a crucial role in dissemination and development of Petri disease. Knowledge of the endophytic microbiome during grapevine propagation will improve understanding of the mutual interactions, and contribute to management of this important disease.

Responses of different grapevine cultivars to infections by *Lasiodiplodia theobromae* and *Lasiodiplodia mediterranea*. P. REIS[1], A. GASPAR[1], A. ALVES[2], F. FONTAINE[3] and C. REGO[1]. *[1]LEAF-Linking Landscape, Environment, Agriculture and Food, Associated Laboratory TERRA, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal. [2]CESAM—Centre for Environmental and Marine Studies, Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal. [3]Université de Reims Champagne-Ardenne, Unité Résistance Induite et Bioprotection des Plantes EA 4707 USC INRAE 1488, SFR Condorcet FR CNRS 3417, 51687 Reims, France. E-mail: pedroreis@isa.ulisboa.pt*

Botryosphaeria dieback is a grapevine trunk disease that affects all viticulture regions of the world. Species of *Lasiodiplodia* have been reported as pathogens of grapevine in several growing regions, including in Portuguese vineyards. *Lasiodiplodia* spp. particularly *Lasiodiplodia theobromae*, have been reported to be more aggressive than other *Botryosphaeriaceae* associated with Botryosphaeria dieback. The present study assessed responses of the most representative cultivars planted in Portugal, 'Touriga Nacional', 'Touriga Franca', 'Alvarinho', 'Aragonez' (= 'Tempranillo') and 'Cabernet Sauvignon', by performing artificial inoculations with *Lasiodiplodia* spp. collected from different international locations. Two experiments, each repeated twice, were performed using: (1) 2-year-old grapevines kept under controlled green-

house conditions and inoculated with six isolates of *L. theobromae* and one isolate of *L. mediterranea*; and (2) 7-year-old field-grown grapevines inoculated with two isolates of *L. theobromae*. Responses of the cultivars were assessed by evaluating the lesion length caused by the isolates, 5 months after inoculation. All the isolates were able to infect host annual shoots, since they were always re-isolated and produced internal wood discolouration. Differences were found for all isolate/cultivar combinations. For both experiments, 'Touriga Nacional' developed the largest lesions, while 'Aragonez' developed the smallest lesions following *Lasiodiplodia* spp. inoculations. Portuguese isolates were more aggressive than those from Peru which were mildly aggressive. These results are a first insight into responses of selected Portuguese cultivars to *Lasiodiplodia* species present in Portugal, but not commonly associated with Botryosphaeria dieback. This contributes to knowledge of the impacts that Botryosphaeria dieback causal agents have on important national cultivars, which will assist winegrowers to manage current cultural practices, and optimize decision making when planning establishment of new vineyards.

Development of an analytical approach using magic-angle spinning NMR for the study of molecular markers of dieback in French vineyards. C. ROBERT[1], A. GRÉLARD[1], A. SAAD[1], M. GADRAT[1], P. REY[2], C. DELMAS[2] and A. LOQUET[1]. *[1]Univ. Bordeaux, CNRS, CBMN UMR 5248, IECB, Pessac, France. [2]Univ. Pau et des Pays de l'Adour, IPREM UMR 5254, Pau, France. E-mail: c.robert@iecb.u-bordeaux.fr*

Grapevine trunk diseases (GTDs) such as Esca or Black Dead Arm have severe consequences for viticulture. The economic costs of vine replacement are high. To date, neither chemical nor biological treatments have been able to eradicate GTDs since the prohibition in Europe of sodium arsenite, and diagnostic methods for detecting GTDs are in early stages of development. Dieback of vine wood tissues is associated with necrosis processes that affect chemical structure and molecular architecture of wood. A new analytical approach is presented how, at the atomic scale, wood tissues are affected by the dieback. Magic-angle spinning nuclear magnetic resonance (MAS NMR) was used to characterize the main polymer components of vine wood extracted at different dieback stages. Apparently healthy and diseased wood tissues were compared for detection and identification of possible molecular markers of dieback. This approach allows for the identification of molecular markers such as the cellulose cristallinity, degradation of specific carbohydrate linkages and apparition of acidic degradation products, which were used to investigate wood dieback of French vineyards. This approach will open an avenue to investigate other wood tissues and vineyards impacted by GTDs.

Drought stress results in a compartment-specific restructuring of the grapevine root-associated fungal microbiome. M. J. CARBONE[1], S. ALANIZ[1], P. MONDINO[1], M. GELABERT[1], A. EICHMEIER[2], D. TEKIELSKA[2], R. BUJANDA[3] and D. GRAMAJE[3]. [1]Universidad de la *República, Facultad de Agronomía, Departamento de Protección Vegetal, Avenue Garzón 780, 12900 Montevideo, Uruguay.* [2]Mendel University in Brno, Faculty of Horticulture, *Mendeleum-Institute of Genetics, Valticka 334, 69144, Lednice, Czech Republic.* [3]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. E-mail: mjcarbone@fagro.edu.uy*

Physiological and molecular responses of grapevine to water stress have been extensively studied. However, little is known about the effects of drought on the grapevine-associated belowground fungal microbiome. Plant roots support complex microbial communities that can influence plant nutrition, growth and health. Drought-induced shifts in fungal composition in root endospheres, rhizospheres and bulk soil were assessed by ITS high-throughput amplicon sequencing (HTAS). Three irrigation regimes were imposed (100%, 50% and 25% of field capacity) to 1-year-old grapevine rootstock plants ('SO4'), when the plants had developed two to three roots. Root endosphere, rhizosphere and bulk soil samples were collected at 6 and 12 months post-plantation. Severe water deficit (25 %) modified the overall fungal composition of all three soil compartments, with root endosphere having the greatest divergence from the well-watered control (100%). Enrichment in several fungal genera was detected, including the arbuscular mycorrhizal fungus *Funneliformis* within roots at severe water deficit. The overall response of the fungal microbiota associated with black-foot (*Dactylonectria* and "*Cylindrocarpon*" genera) and the potential biocontrol agent *Trichoderma* to drought stress was consistent across compartments, with relative abundances being greater at 50–100% than at the 25% irrigation regime. These results indicate that drought stress, in addition to its well-characterized effects on plant physiology, also affect restructuring of grapevine root microbial communities.

This suggests the possibility that members of the altered grapevine microbiota could contribute to plant survival under extreme environmental conditions.

***Aureobasidium pullulans* may promote the development of foliar symptoms on Esca-diseased grapevines**. Z. KARÁCSONY[1], V. MONDELLO[2], A. SONGY[2], F. FONTAINE[2] and K. Z. VÁCZY[1]. *[1]Food and Wine Research Institute, Eszterházy Károly Catholic University, H-3300 Eger, Hungary. [2]University of Reims Champagne-Ardenne, Unité Résistance Induite et Bio-protection des Plantes (RIBP), USC INRAE 1488, Moulin de la Housse-Bat, 18-BP1039, 51687 Reims, France. E-mail: karacsony.zoltan@uni-eszterhazy.hu*

Esca is one of the most important fungal diseases infections of grapevine. Besides the main pathogens *Phaeomoniella chlamydospora* (*Pch*), *Phaeoacremonium minimum* (*Pmin*) and *Fomitiporia mediterranea*, several fungi are associated with this syndrome. While the complex nature of Esca is well known, only the pathogenic interactions between the main pathogens have been studied, and little is known about the possible roles of non-pathogenic fungi. The abundance of *Aureobasidium pullulans* (*Apu*) in the healthy wood of Esca-symptomatic 'Cabernet sauvignon' grapevines was assessed by isolation-based methods and qPCR measurements. A positive correlation was shown between the abundance of *Apu* and severity of foliar symptoms (relative area of necrosis). The *in vitro* investigation of the interactions between *Apu* and *Pch* or *Pmin* showed that *Pmin* strongly inhibited *Apu*, while there was a mutual antibiosis between *Pch* and *Apu* without toxic effects. Further studies of the *Apu-Pch* interactions suggested mutual induction of sporulation and inhibition of growth, and these effects were mediated by secreted molecules. Artificial infections of grapevine shoot sections with three isolates each of *Pch* and *Apu*, were carried out individually and in combinations. *Apu* enhanced development of foliar symptoms on *Pch*-infected plants in a strain-dependent manner. Phytotoxicity tests with water extracts from leaves severely damaged by *Apu* + *Pch* inoculations indicated that increased symptom severity was the result of the accumulation of toxic metabolites in the co-inoculated shoots. Protein and polysaccharide secretion of the examined *Pch* and *Apu* isolates was also investigated, and showed some differences. These may explain the positive effects of some *Apu-Pch* co-infections on the development of foliar Esca symptoms.

A study to characterise diversity of fungal endophytes in young nursery grapevine plants. M. AL SARRAJ[1], E. DEYETT[2], A. ROMEO OLIVAN[1], T. LAGRAVERE[1], P. ROLSHAUSEN[2] and A. JACQUES[1]. **[1]***Unité de Recherche Physiologie, Pathologie et Génétique Végétales, Université Fédérale Toulouse Midi-Pyrénées, INP-PURPAN, F-31076 Toulouse, France. [2]Department of Botany and Plant Sciences, University of California, Riverside, USA. E-mail: alban.jacques@purpan.fr*

In sharp upsurge for three decades, Esca threatens grape production from all viticulture regions. This is caused by a complex of fungi that attack perennial organs of grapevines. Disease symptoms vary according to plant age, severity of infections, and pedoclimatic contexts. Our research aims to understand the microbiome assemblage of nursery-grown plants, and its influence on physiology of the Esca pathosystem. The working hypothesis postulates that variabilities of vine microbiomes affect the early host defence responses against Esca causal agents (*P. minimum* and *P. chlamydospora*). The first step was to determine if there were differences in microbiome profiles of rooted grafts from different grapevine nurseries. In 2020, characterization of the microbiomes of grafted vines from the United States of America (California) and France (South-West). Plants were the same genetic material, Cabernet-Sauvignon grafted on Richter 110, and from two nurseries within each region. Results presented were obtained from North American plants (results from French plants are still to be analyzed). Differences between the two nurseries were apparent, in both alpha and beta diversity, thus validating the possibility of rapidly prospecting the influence of this microbiome on the responses of host wood to pathogenic agents associated with Esca. Diversity of the observed microbiomes will allow investigation of effects of these variabilities on disease outcomes.

Comparisons of grapevine-associated plant pathogenic fungal communities among different microhabitats, host cultivars and between healthy and Esca-diseased plants. A. GEIGER[1,2], C. M. LEAL[2,3], Z. KARÁCSONY[1], R. GOLEN[1], K. Z. VÁCZY[1] and J. GEML[1,2,3]. *[1]Food and Wine Research Centre, Eszterhazy Karoly Catholic University, Eger, H-3300, Hungary. [2]Doctoral School of Environmental Sciences, Hungarian University of Agriculture and Life Sciences, Gödöllő, H-2100, Hungary. [3]ELKH-EKKE Lendület Environmental Microbiome Research Group, Eszterhazy Karoly Catholic University, Eger, H-3300, Hungary. E-mail: geiger.adrienn@uni-eszterhazy.hu*

Grapevine trunk diseases (GTDs) are major threats to wine industries, causing yield losses and dieback of grapevines. While the increasing damage caused by GTDs in recent decades have spurred several studies on grapevine-associated pathogenic fungi, key questions of the emergence and severity of GTDs remain unanswered, including whether differences in richness, abundance, and composition of plant pathogenic fungi exist among below- and aboveground microhabitats, among cultivars, and among asymptomatic and symptomatic Esca-affected grapevines. DNA metabarcoding was used for soil, bark, and perennial wood samples from asymptomatic and symptomatic grapevines of two global cultivars (Chardonnay and Cabernet sauvignon) and two local cultivars (Leányka and Kékfrankos). Greater compositional differences were detected in plant pathogenic fungi within grapevine plants than among them. This was due to dominance of GTD-associated fungi in perennial wood and to a lesser extent in bark, and the dominance of non-GTD pathogens in soil, as well as the lack of differences among cultivars and among asymptomatic and Esca symptomatic grapevines. These results indicate that fungi generally associated with Esca 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 of stressed plants. Environmental factors may be particularly important for development of Esca, and studies are required to investigate effects of abiotic conditions on fungal compositional dynamics in Esca-affected plants.

Predicting pathogen virulence: linking host diversity and pathogenicity of the *Botryosphaeriaceae* fungi in wine grapes (*Vitis vinifera*). I. SILVA-VALDERRAMA[1], O. SILVA[2], J. BOULE[3], J. R. ÚRBEZ-TORRES[3] and T. J. DAVIES[1]. *[1]University of British Columbia, Botany Department, Vancouver, BC, Canada. [2]La Huarara, Santiago, Chile. [3]Summerland Research and Development Centre, Agriculture and Agri-Food Canada, Summerland, BC, Canada. E-mail: ibsilva26@gmail.com*

Plant diseases can have important negative effects on crops and human food sources. Previous research has linked host plant diversity and pathogen emergence, illustrating important connections with phylogeny of plant hosts. However, the importance of the pathogen phylogeny in shaping pathogen-host associations has been under-explored, yet this may be useful for describing pathogens likely to shift to novel hosts, and to predict potential for disease emergence following host jumps. The phylogenetic signals in host use is described for *Botryosphaeriaceae*, a widely distributed family that infects most woody perennial plants. The links between host diversity, phylogenetic relatedness, and pathogen virulence are outlined. The phylogeny of *Botryosphaeriaceae* spp. infecting grapevines (*Vitis vinifera*) was reconstructed, and whether closely related pathogens infect similar host species was examined. Virulence of the *Botryosphaeriaceae* known to infect *V. vinifera* was quantified using a high-throughput detached cane assay. A machine learning algorithm was trained to differentiate between asymptomatic and necrotic tissues, and lesion size was quantified as a proxy for virulence. The relationship between host diversity and lesion size was then modelled. Preliminary results show large differences in host diversity within the *Botryosphaeriaceae*, and a positive relationship between the phylogenetic host diversity and pathogen virulence in *V. vinifera*. This research provides a first step towards predicting virulence of a known pathogen on a novel host following a host jump. This approach could be used for coordinated global monitoring of high-risk species within *Botryosphaeriaceae*.

Exploring the microbial terroir: communities of fungi associated with grapevine trunk diseases differ among terroirs and seasons. C. M. LEAL[1,3], A. GEIGER[2,3], K. Z. VÁCZY[2] and J. GEML[1,2,3]. *[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*

In viticulture and oenology, the terroir concept is widely used to explain differences in sensory and chemical characteristics of grapes and wines. The concept is partly based on known or presumed spatial differences in edaphic and mesoclimatic factors. These environmental differences also likely affect plant-associated microbes, with possible implications for plant health and crop yield and quality. The population compositional dynamics of fungi in woody tissue of grapevines was compared, with emphasis on fungi associated with grapevine trunk diseases (GTDs). Living woody tissues were sampled from grapevine plants of one cultivar (Furmint; local white grape variety), in late winter and late summer, in three different terroirs in Tokaj, Hungary. DNA sequence data were generated by Illumina NovaSeq at

BaseClear (Leiden, the Netherlands), with fungus-specific primers targeting the rDNA internal transcribed spacer (ITS) region. Of the fifteen different GTD-associated fungus genera found, *Phaeomoniella*, *Diplodia* and *Phoma* had the greatest richness, with some variation among terroirs. Compositional differences among terroirs and sampling months were also detected, with terroir explaining 11.5% and season 6% of the variance in the community composition of GTD-associated fungi. These observed differences may be due to the inherent influence of different edaphic and mesoclimatic factors associated with different terroirs and seasonality. They affect the mycobiome composition and diversity possibly by secondary effects, since living conditions in trunk woody tissues are likely to be stable. This new information on the compositional dynamics of GTDs in wood tissues among terroirs and different seasons, has implications for plant health research.

Drought influences fungal community structure and diversity inhabiting grapevine vascular systems, and enhances *Phaeomoniella chlamydospora* abundance. M. M. MALDONADO-GONZÁLEZ[1], M. J. CARBONE[2], A. EICHMEIER[3], T. KISS[3], R. BUJANDA[1] 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]*Departmento de Protección Vegetal, Facultad de Agronomía, Universidad de la República, Avenida Garzón 780, Montevideo 12900, Uruguay.* [3]*Mendel University in Brno, Faculty of Horticulture, Mendeleum - Institute of Genetics, Valticka 334, 69144, Lednice, Czech Republic. E-mail: david.gramaje@icvv.es*

Grapevine productivity in the Mediterranean regions could be affected by global warming, resulting in increased competitiveness for water resources. Recent studies showed that water deficit can alter grapevine root-associated microbiota, particularly organisms able to alleviate effects of abiotic and biotic stress factors. Drought influences on fungal community structure and diversity inhabiting grapevine vascular systems were investigated, with special attention to the Petri and Esca disease pathogen *Phaeomoniella chlamydospora*. One-year-old grapevine rootlings grown under greenhouse conditions were exposed to three water regimes (WRs): 100%, 50% or 25% of field capacity. Wood samples were taken before planting (t0), and 1 year (t1) and two years (t2) after planting, from the base, medium and apical parts of the rootstock plants, sing a non-destructive sampling method. Fungal composition and *P. chlamydospora* abundance were assessed, respectively, using ITS high-throughput amplicon sequencing (HTAS) and droplet-digital PCR (ddPCR). Drought altered the overall fungal compositions in the vascular systems. Diversity greater at 100% WR than at the other WRs, at t1 and t2. Several fungal taxa associated with grapevine trunk diseases (GTDs) were predominant, and determined the dissimilarities among WRs. this applied for *P. chlamydospora* at 25% WR and at t1 and t2, for *Cadophora* at 50% WR at t1, and for *Cadophora* and *Ilyonectria* at 100% WR at t2. HTAS and ddPCR methods both showed increases in *P. chlamydospora* OTUs and abundance at 25% WR. Correlation analyses showed positive (*Cadophora*/*Ilyonectria*) and negative (*Cadophora*/*Phaeoacremonium*) interactions among several genera associated with GTDs. Water deficit reduced the complexity of the co-occurrence networks among taxa, resulting in the greatest interactions with dense and compact networks at 100% WR.

AMF community diversity identification and their effects on grapevine growth parameters under black foot pressure. R. MOUKARZEL[1], H. J. RIDGWAY[1,2], J. LIU[1], A. GUERIN-LAGUETTE[3] and E. E. JONES[1]. [1]*Lincoln University, Lincoln, Canterbury, New Zealand.* [2]*The New Zealand institute for Plant and Food Research Ltd, Private bag 4704, Christchurch, New Zealand.* [3]*Mycotree C/-Southern Woods Nursery, Christchurch, Canterbury, New Zealand. E-mail: romy.moukarzel@lincoln.ac.nz*

Arbuscular mycorrhizal fungi (AMF) have potential applications for sustainable agricultural ecosystems, and have been shown to reduce infections and mitigate effects of black foot on grapevine rootstocks. However, limited information is available on AMF-grapevine interactions, and especially in New Zealand. Most studies have assessed effects of one, two or a combination of only small numbers of AMF species on fungal pathogens associated with grapevines. The present study aimed to: (*i*) characterise the AMF community diversity associated with different commercial grapevine rootstocks sampled from New Zealand vineyards; (*ii*) investigate effects of AMF communities on grapevine growth parameters; and (*iii*) evaluate how young grapevine rootstocks inoculated with their 'home' and 'away' AMF communities would respond to challenges with a black foot pathogen species mixture. The AMF communities identified from these rootstocks were *Ambispora* spp., *Claroideoglomus* spp., *Funneliformis* spp. and *Glomus* spp. Community analyses demonstrated that

rootstock influenced the AMF community composition at all sites. The AMF communities had direct effects by increasing plant biomass and nutrient uptake, and indirectly by influencing chlorophyll content in grapevine leaves through the increase of specific nutrients such as K, Mn, and Zn. High disease incidence and severity did not reduce growth in vines inoculated with AMF, compared to vines inoculated with the pathogen only, and high levels of disease in rootstocks limited the effect of the AMF community, with only little evidence that AMF treatments reduced disease incidence and severity in vines. Further research is required to understand the mechanistic effects of AMF colonisation on plant growth parameters, especially under high disease pressure.

Culturome versus DNA metabarcoding: diversity of grapevine endophytic mycobiomes in old and young vines of different health status in New Zealand. N. BESSELMA[1], H. J. RIDGWAY[1,2], D. C. MUNDY[3], B. R. VANGA[2], P. PANDA[2] and E. E. JONES[1]. *[1]Faculty of Agriculture and Life Sciences, Lincoln University, PO Box 84, Lincoln 7647, New Zealand. [2]The New Zealand Institute for Plant & Food Research Limited, Private Bag 4704, Christchurch 8140, New Zealand. [3]The New Zealand Institute for Plant & Food Research Limited, Marlborough Wine Research Centre, 85 Budge Street, Blenheim 7201, New Zealand. E-mail: Noureddine.Besselma@lincolnuni.ac.nz*

Grapevines harbour diverse communities of fungi in the woody trunk tissues, the "endophytic mycota". These communities can have profound effects on host physiology, health, growth, and ability to adapt to stress. Some of these include pathogenic fungi as causal agents of grapevine trunk diseases (GTDs), with many considered latent pathogens. For GTDs, understanding the factors affecting latency is limited. This study aimed to compare the fungal endophyte community in young and old Sauvignon blanc vines, which were symptomatic or asymptomatic for GTDs, using culture-dependent and culture-independent approaches. Nine vineyards were sampled, with 60 mature vines (>10 years old) and 30 young vines (<9 years old) sampled. Each age group consisted of equal numbers of apparently healthy and symptomatic vines. Trunk cores were taken from each vine using a sterilised 4 mm drill bit, after removing the bark with a knife. Fungal communities were characterized by isolation and metabarcoding of the ITS1 region. For the culturome, a collection of 2116 endophytic fungi were recovered, representing 42 genera. Trunk microbiota were dominated by species of *Alternaria*, *Aureobasidium*, *Diplodia*, *Epicoccum*, *Phaeomoniella*, *Eutypa*, *Botrytis*, *Cladosporium*, and *Diaporthe*. Differences in the taxa recovered into culture were observed between vines of different ages and symptomology. In the metabarcoding, 1892 OTUs were obtained, and the same fungal genera were identified as the most abundant. Alpha diversity analysis revealed that greater diversity was detected in old compared to young vines, and in asymptomatic compared to symptomatic trunks. Beta diversity analysis demonstrated differentiation in the fungal communities structure for both age and health status. This study has produced new baseline information on Sauvignon blanc endophytic mycota, and further research will determine the impacts of these microbial communities on the latency of GTDs.

Can the microbiome drive suppression of grapevine trunk diseases? D. ADEJORO[1], E. E. JONES[1], H. RIDGWAY[1,2], D. C. MUNDY[3], B. VANGA[2] and S. BULMAN[2,4]. *[1]Department of Pest-management and Conservation, Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln 7647, Canterbury, New Zealand. [2]The New Zealand Institute for Plant & Food Research Limited, Lincoln 7608, Canterbury, New Zealand. [3]The New Zealand Institute for Plant and Food Research Limited, Marlborough Wine Research Centre, Blenheim, New Zealand. [4]Better Border Biosecurity (B3), Lincoln, New Zealand. E-mail: Damola.Adejoro@lincolnuni.ac.nz*

Grapevine trunk diseases (GTDs), caused by several fungi, are important diseases in New Zealand and other grape-growing countries. Control of the diseases is difficult, and there are no approved fungicides for their eradication. This has necessitated seeking alternative disease management strategies, including sustainable biological control. This study aimed to identify taxa in the grapevine microbiome that contribute to plant health. In some New Zealand vineyards, observations have revealed vines that remain healthy within backgrounds of trunk diseases. These grapevines were termed 'disease-escape' to represent their apparent health under heavy disease pressure. Recent research on grapevine microbiomes has shown that microorganisms from these 'disease-escape' plants could contribute to disease suppression. Putative disease escape vines were identified in vineyards in two grape-growing regions in New Zealand: Hawke's Bay and Canterbury. The vines were selected based on their presence in diseased areas, maturity, and absence of trunk disease symptoms. Trunk core samples were taken from the disease-escape vines and neighbouring symptomatic vines. Subsequently, the

samples' total fungal and bacterial communities were identified and compared using culture-independent DNA metabarcoding and culture-dependent approaches. After analysing the metabarcoding and culturing results, microbial taxa were identified that were more abundant in disease-escape grapevines, or that correlated negatively with GTD pathogens. The next stage of the study is to design a synthetic community using members of the taxa of interest from the disease-escape grapevines. This synthetic community (SynCom) will be introduced into young grapevines and monitored for their ability to suppress the development and severity of GTDs. The research results will provide information on the roles (if any) that the grapevine trunk microbiome plays in suppressing GTDs.

Implications of abiotic and biotic stress on *Phaeomoniella chlamydospora* colonization of young 'Merlot' grapevines. J. HRYCAN[1,2], P. BOWEN[1], T. FORGE[1], M. HART[2] and J. R. ÚRBEZ-TORRES[1]. *[1]Summerland Research and Development Centre, Agriculture and Agri-Food Canada, 4200 Highway 97, Summerland, BC V0H 1Z0, Canada. [2]Department of Biology, The University of British Columbia Okanagan, 3187 University Way, Kelowna, BC V1V 1V7, Canada. E-mail: jared.hrycan@agr.gc.ca*

Growing evidence has led to the hypothesis that some grapevine trunk disease (GTD) fungi may behave as latent pathogens, transitioning from endophytic to pathogenic lifestyles, triggered by plant stress. Two greenhouse experiments were conducted over two growing seasons, to determine the impacts of drought stress and ring nematode (*Mesocriconema xenoplax*) infestations on *Phaeomoniella chlamydospora* growth and disease development in young 'Merlot' grapevines. Research into the potential roles of arbuscular mycorrhizal (AM) fungi as stress reducers and biocontrols has also increased in recent years. The AM fungus *Rhizophagus intraradices* was inoculated into soil to investigate its potential in mitigating plant stress and reduce its impact on fungal growth. *Phaeomoniella chlamydospora* was vacuum-inoculated into the bases of dormant 'Merlot' canes at a high (25,000 spores), medium (5,000 spores), and low (1000 spores) inoculum, to investigate whether a threshold required for disease to occur would be reached more rapidly in highly than in less infected plants. Fungus quantity was determined at time of inoculation, time of planting, and upon conclusion of the experiment, using droplet digital PCR to determine the effect of stress on fungal growth. Pruning weights were measured throughout the experiment, and internal necroses were measured at the base of each grapevine at conclusion of the experiment, to monitor symptom expression. Preliminary results showed no phenotypical differences (root dry weight and pruning weights) between stressed and non-stressed plants, no matter their infection status. Percent necroses at trunk bases were greatest in water stressed grapevines, but no difference was observed in nematode infested plants. ddPCR analyses revealed increased fungal growth in water stressed plants, but not in nematode infested plants, indicating that water stress may play a role in Petri disease development in young grapevines.

Evaluating treatments for the protection of grapevine pruning wounds from 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. [3]Viticultura Viva S.L., Cabmesado 4, 31390 Olite, Spain. E-mail: david.gramaje@icvv.es*

Infection of grapevines by grapevine trunk disease (GTD) fungi primarily occurs through annual pruning wounds made during the host plant dormant seasons. This study evaluated and compared the efficacy of liquid formulation fungicide (pyraclostrobin + boscalid) and paste treatments, and biological control agents (*Trichoderma atroviride* SC1, *T. atroviride* I-1237, and *T. asperellum* ICC012 + *T. gamsii* ICC080), for their potential to prevent natural infections of grapevine pruning wounds by trunk disease fungi in two field trials over two growing seasons. Vineyards were located 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. Untreated controls were mock treated with sterile distilled water. One year after pruning, canes were harvested from vines and brought to the laboratory for assessment of *Trichoderma* spp. and fungal trunk pathogens. More than 1,000 fungal isolates associated with five GTDs (Esca, Botryosphaeria, Diaporthe and Eutypa diebacks, and Cytospora canker) were collected from the two vineyards each growing season. The efficacy of each product varied according to the

GTD fungi and the grape-growing region, although, in some cases, low incidence of some GTDs in untreated controls did not allow determining differences between treatments. In general, *T. atroviride* I-1237 was the most effective treatment, followed by pyraclostrobin + boscalid. *Trichoderma* recovery proportions ranged from 16.7 to 93.3% in Samaniego, and from 32.5 to 94.2% in Madiran. The experiment will be undertaken for a third season during 2022–2023.

Hot water treatment in grapevine nurseries: the dilemma of heat tolerant GTD pathogens. F. HALLEEN[1,2], M. WEBBER[1] and L. MOSTERT[2]. *[1]ARC Infruitec-Nietvoorbij, Private Bag X5026, Stellenbosch, 7599, South Africa. [2]Department of Plant Pathology, University of Stellenbosch, Matieland, 7602, South Africa. E-mail: halleenf@arc.agric.za*

The benefits of hot water treatments (HWT; 50°C for 30 min) in grapevine nurseries is well known, although it is also known that these do not completely eradicate all infections. A HWT protocol of 50°C for 45 min has been recommended for control of Aster Yellows in South Africa. The aim of this study was to determine effects of HWT (50°C for 45 min) on GTD pathogens in South African nurseries, firstly *in vitro*, followed by artificially inoculating rootstock cuttings of Ramsey, Richter 110, US 8-7, Paulsen 1103 and 143B Mgt. Pathogens included *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum*, *Pm. parasiticum*, *Cadophora luteo-olivacea*, *Pleurostoma richardsiae* (Petri disease), *Campylocarpon fasciculare*, *Camp. pseudofasciculare*, *Ilyonectria liriodendri*, *Dactylonectria macrodidyma* (Black foot), *Neofusicoccum australe*, *N. parvum* (Botryosphaeria canker and dieback), and *Diaporthe ampelina* (Phomopsis dieback). *In vitro* results showed that HWT completely inhibited conidium germination and mycelium growth of all pathogens associated with Black foot, Botryosphaeria canker and dieback and Phomopsis dieback. Pathogens associated with Petri disease were more heat tolerant, with *Pl. richardsiae* being the most tolerant species and *Pa. chlamydospora* the most sensitive, followed by *Ca. luteo-olivacea*. The effects of HWT temperatures greater than 50°C were also investigated. *Pleurostoma richardsiae* showed the greatest tolerance, with temperatures of up to 60°C not achieving complete control. In the *in vivo* experiments, HWT was very effective for eradicating *Pa. chlamydospora* and *Ca. luteo-olivacea*, and reduced *Pm. minimum* and *Pm. parasiticum*-associated disease incidence and severity, based on isolation studies. The effects of HWT on *Pl. richardsiae* were less consistent. Incidence of this pathogen was not reduced, but severity of the infections was reduced, although inconsistently. Although HWT may not eradicate all infections, it is recommended for use in integrated management of GTDs.

In vitro* effects of selected phenolic compounds against *Diplodia seriata, Eutypa lata, Fomitiporia mediterranea* and *Neofusicoccum parvum. K. ŠTŮSKOVÁ[1], F. FONTAINE[2], V. MONDELLO[2], E. HAKALOVÁ[1], J. WOHLMUTH[1], A. VAVŘINÍK[3], T. HELMOVÁ[3], Š. FRANKOVÁ[4] and A. EICHMEIER[1]. *[1]Mendeleum – Institute of Genetics, Mendel University in Brno, Lednice, Czech Republic. [2]Unité Résistance Induite et Bioprotection des Plantes EA4707 USC INRAE 1488, Université de Reims Champagne-Ardenne, SFR Condorcet FR CNRS 3417, Reims, France. [3]Department of viticulture and enology, Mendel University in Brno, Lednice, Czech Republic. [4]Department of Vegetable Growing and Floriculture, Mendel University in Brno, Lednice, Czech Republic. E-mail: xstusko1@mendelu.cz*

Grapevine trunk disease (GTD) pathogens cause serious damage and have a significant economic impact for cultivation of grapevines. There is no direct and effective protection against GTDs in nurseries and established vineyards. The inhibitory effects of eugenol, epigallocatechin-3-o-gallate (EGCG) and thymol against the GTD pathogens *Diplodia seriata*, *Eutypa lata*, *Fomitiporia mediterranea* and *Neofusicoccum parvum* were monitored using *in vitro* tests. Most inhibition of fungal growth was observed from eugenol, which showed the lowest EC_{50} value. For eugenol at 1.5 µL mL^{-1}, complete inhibition of growth was observed for both *F. mediterranea* and *N. parvum*, with *E. lata* and *D. seriata* also showing high inhibition values, of respectively, 99 and 98%. For eugenol, EC_{50} values of 0.94, 0.95, 0.96, and 1.00 µL mL^{-1} were obtained for, respectively, *F. mediterranea*, *E. lata*, *D. seriata*, and *N. parvum*. Thymol at the highest concentration used (45 µL mL^{-1}), inhibited all four pathogens. *D. seriata* showed 90% inhibition, *N. parvum* 88%, *F. mediterranea* 74%, and *E. lata* 67%. The thymol EC_{50} values were 25.37 µL mL^{-1} for *N. parvum*, 25.45 µL mL^{-1} for *D. seriata*, 29.02 µL mL^{-1} for *F. mediterranea*, and 34.28 µL mL^{-1} for *Eutypa lata*. To compare, EGCG did not show any statistically significant inhibitory effects on the selected GTD pathogens. The next step will be to evaluate the potential use of eugenol for control GTDs in *in planta* tests.

Minimal versus intensive: How pruning intensity affects occurrence of grapevine leaf stripe disease

and trunk wood integrity. C. KRAUS, C. RAUCH, E. M. KALVELAGE, F. H. BEHRENS and D. D´AGUIAR. *Julius Kühn-Institute, Federal Research Centre of Cultivated Plants, Plant Protection in Fruit Crops and Viticulture, 76833 Siebeldingen, Germany. E-mail: Christian.kraus@julius-kuehn.de*

Previous studies of managing grapevine trunk diseases (GTDs) have indicated that non- or minimal-pruning schemes can reduce the risk of Esca. Nevertheless, knowledge of the mechanisms behind these observations is limited. The present study investigated the effect of pruning intensity on the occurrence of grapevine leaf stripe and grapevine trunk integrity. Two German vineyards ('Dornfelder' and 'Müller-Thurgau'), partially maintained with intensive- and minimal-pruning schemes were chosen due to the accessibility of multi-annual Esca monitoring data (respectively, 5 and 6 years). Incidence of external symptoms and proportions of white rot and necrosis in trunks of Esca positive and negative vines were analysed and compared between the two pruning intensities. Only in the 'Dornfelder' vineyard, incidence of external Esca symptoms was reduced by up to 73.7% over 5 years (2017–2021) by minimal pruning compared to intensive pruning. In both vineyards, trunks of intensive-pruned vines had more pruning wounds (respectively, 86.0% and 72.9% more) than minimal-pruned vines, but also exhibited a greater trunk head circumferences (respectively 19.3% and 14.7%). Only in the 'Dornfelder' vineyard, the proportion of necrosis was greater for intensive-pruned vines (23.0%) than for minimal-pruned vines (11.5%).

Effects of pruning on desiccation cone formation of three grapevine cultivars in France. E. BRUEZ[1*], C. CHOLET[1*], T. MARTIGNON[2], M. GIUDICI[2], M. BOISSEAU[3], P. REY[4] and L. GENY[1]. *[1]UMR Oenologie, EA 4577, Université de Bordeaux, INRAE, BSA, Institut des Sciences de la Vigne et du Vin, 210 Chemin de Leysotte - CS 50008, 33882 Villenave d'Ornon, France. [2]Simonit&Sirch, Maîtres Tailleurs de Vigne, 1 Rue Porte des Benauges, 33410 Cadillac, France. [3]Hennessy Jas et Cie, 1 rue Richonne 16100 Cognac, France. [4]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 64013, Pau, France Pau et Bordeaux. *: equal contribution E-mail: emilie.bruez@u-bordeaux.fr*

Since sodium arsenite was banned, researchers have sought alternative solutions to deal with Grapevine Trunk Diseases (GTDs). Several methods, including pruning, are currently being used in attempts to prolong grapevine life. Different effects of high or short pruning in grapevine. High pruning leaves a chicot and preserves the diaphragm, unlike short pruning, which damages the diaphragm. An experiment focused on necrosis formation, and examined the relationship between spur diameter and necrosis length, and also measured the evolution of desiccation cones according to pruning type. The grapevines were pruned in February and, 4 or 8 months later, five vines per modality (short or high pruning) were sampled. There was no correlation between spur diameter and necrosis length for Cabernet sauvignon, Sauvignon blanc and Ugni blanc. However, there was a correlation between necrosis length and quality of pruning wound length for Cabernet Sauvignon and Ugni blanc. Necrosis length also varied with the vintage, particularly so for Sauvignon blanc. Overall, high pruning was effective for preventing desiccation cone development in the chicot. Keeping the diaphragm safe allowed the sap flow path to function fully.

Can spray coverage of wounds and Eutypa dieback control be improved by the addition of adjuvants to fungicides? M. R. AYRES[1] and M. R. SOSNOWSKI[1,2]. *[1]South Australian Research and Development Institute, Adelaide, South Australia 5001, Australia. [2]School of Agriculture, Food and Wine, The University of Adelaide, South Australia 5005, Australia. E-mail: matthew.ayres@sa.gov.au*

Infection of grapevine pruning wounds by *Eutypa lata*, which causes Eutypa dieback, can be efficiently and effectively controlled by the spray applications of fungicides. The level of disease control is related to wound coverage. However, as tractor-driven sprayers are designed to target leaves, they require adjustment to achieve adequate coverage of pruning wounds on dormant vines. A vineyard trial was established in winter 2017, and repeated in winter 2018, in McLaren Vale, South Australia, to evaluate whether the addition of spray adjuvants (di-1-p-menthene or trisiloxane ethoxylate) to fungicide treatments (tebuconazole or fluazinam) could improve wound coverage and protection against infection by *E. lata*. Optimal spray output to achieve adequate coverage of pruning wounds is at least 600 L ha^{-1}, but to ascertain any benefits from addition of adjuvants, a recycle sprayer applied treatments at 200 L ha^{-1}, prior to inoculation of wounds with *E. lata*. A novel technique for evaluating wound coverage was developed, using fluorescent pigment added to treatments, and was

compared with the use of water-sensitive papers (WSPs). Digital images of fluorescent pigment on wounds, captured under UV light, and from WSPs, were assessed using Image J image analysis software. These assessment showed little effect of addition of adjuvants on coverage. However, when compared directly, WSPs indicated twice the coverage than the fluorescent pigment deposited directly on pruning wounds, from the same treatment. Treated canes were removed 11 months after inoculation and assessed for pathogen recovery. Overall, there was little difference in recovery between treatments, indicating that the adjuvants did not improve efficacy of fungicides. Disease control was minimal with the application of the fungicides, reiterating the importance of applying the recommended minimum of 600 L ha^{-1} to achieve sufficient wound coverage.

Effects of biocontrol agents on *Fomitiporia mediterranea*. M. RIEDLE-BAUER[1], M. GORFER[2] and M. MADERCIC[1]. *[1]Federal College and Research Institute for Viticulture and Pomology Klosterneuburg, Wienerstraße 74, 3400 Klosterneuburg, Austria. [2]Austrian Institute of Technology, Konrad-Lorenz-Straße 24, 3430 Tulln, Austria. E-mail: monika.riedle-bauer@weinobst.at*

White rot pathogens such as *Fomitiporia mediterranea* (Fmed) and other *Fomitiporia* species are, among other fungal species, the main pathogens associated with the ESCA syndrome. Effects of the potential biocontrol agents (BCAs) *Trichoderma simmonsii* 804 and 1056, *T. citrinoviridae* 232, *Bacillus subtilis* 224, *B. subtilis* 230, *B. amyloliquefaciens/velezensis* 624, *B. amyloliquefaciens/velezensis* 2277, *B. amyloliquefaciens/velezensis* 2143, *Pseudomonas koreensis* (all isolated from grapevines) and *T. atroviridae* (Vintec, Belchim) on growth of Fmed were assessed in dual culture and wood disc assays. Mycelium disks (1 cm diam.) of Fmed cultures were placed in the centres of MEA plates. Similar disks excised from BCA cultures, were placed at the edges of the plates. The diameter of each Fmed colony was measured after 14 d. The wood disk models included either fresh or autoclaved cross sections of grapevine trunks (from dormant 10-15 y old asymptomatic vines 'Rotburger' ('Zweigelt'), approx. 4 mm thick) placed on water agar. Plates were inoculated with Fmed at either 7 d before or 7 d after treatment with the BCAs). For pathogen inoculation, mycelium disks were placed in the middle of the wood pieces, and for BCA treatment, wood pieces were briefly immersed in inoculum suspensions (bacterial isolates: OD600 0.2-0.3 in PBS; *Trichoderma* spp. 108 cfu mL^1 in tap water). Growth of Fmed on the wood disks was visually evaluated after 3-5 weeks. In the dual cultures, all BCAs reduced growth of Fmed compared to experimental controls, the *Trichoderma* species being most effective. Fmed successfully colonized the inoculated wood disks. All the *Trichoderma* species reduced pathogen growth, both on fresh and autoclaved wood, and for both inoculation time schedules. Inhibitory effects of bacterial BCAs were also recorded.

Biological control of *Phaeomoniella chlamydospora* in young grapevines with *Bacillus velezensis* K165 and *Fusarium oxysporum* F2. F. I. GKIKAS[1], A. TAKO[1], D. GKIZI[2], C. LAGOGIANNI[1], E. A. MARKAKIS[3] and S. E. TJAMOS[1]. *[1]Department of Crop Science, Agricultural University of Athens, 75 Iera Odos str., 11855 Athens, Greece. [2]Department of Wine, Vine and Beverage Sciences, University of West Attica, Ag. Spyridonos 28, 12243 Athens, Greece. [3]Department of Viticulture, Vegetable Crops, Floriculture and Plant Protection, Hellenic Agricultural Organization Demeter, Mesa Katsampas 71003, Iraklio Crete, Greece. E-mail: dgkizi@uniwa.gr*

Grapevine trunk diseases (GTDs) are major problems for viticulture, endangering sustainability of grape production. *Phaeomoniella chlamydospora* (*Pch*) is a predominant GTD-associated species in Petri disease, causing decline of young grapevines. There are no effective strategies to reduce *Pch* infections except prevention in nurseries, so there is urgent need for eco-friendly approaches to control Petri disease. The effectiveness of *Bacillus velezensis* K165 (formerly *Paenibacillus alvei* K165) and *Fusarium oxysporum* F2 against *Pch* was assessed in dual culture assays and in young grapevines. In dual culture assays, in a growth medium simulating the xylem environment, F2 decreased *Pch* growth and sporulation, whereas K165 did not have any effect on *Pch*. In rooted grapevine cuttings, K165 was applied through root drenching while F2 was applied by stem injection. K165 reduced wood discolouration, the typical symptom of *Pch* infection, whereas application of F2 by stem injection did not. Both K165 and F2 reduced the endophytic DNA amount of *Pch* compared to controls by, respectively, 90% and 82%, revealed by qPCR analysis. K165- and F2-treated grapevines harboured higher lignin levels compared to non-inoculated controls. These results indicate that F2 and K165 have potential as biocontrol agents against *Pch* in grapevines.

Lignan extract from knotwood of Norway spruce as a possible novel bioprotectant agent against grape-

vine trunk diseases. M. ŠPETÍK[1], J. BALÍK[2], P. HÍC[2], E. HAKALOVÁ[1], K. ŠTŮSKOVÁ[1], L. FREJLICHOVÁ[1], J. TŘÍSKA[3] and A. EICHMEIER[1]. *[1]Mendeleum—Institute of Genetics, Faculty of Horticulture, Mendel University in Brno, Valtická 334, 691 44 Lednice na Moravě, Czech Republic. [2]Department of Post-Harvest Technology of Horticultural Products, Faculty of Horticulture, Mendel University in Brno, Valtická 334, 691 44 Lednice na Moravě, Czech Republic. [3]Global Change Research Institute CAS, Bělidla 986/4a, 603 00 Brno, Czech Republic. E-mail: milan.spetik@mendelu.cz*

Grapevine trunk diseases (GTDs) are major threats to wine industries, causing significant economic loses. With no effective treatment against these diseases, there is urgent need for efficacious control disease management. An extract from the knotwood of spruce trees was assessed as an antifungal agent against GTDs. In an *in vitro* trial, the antifungal effects of the extract were assessed against strains of the most common GTD pathogens. Complete inhibition of growth was observed against *Cadophora luteo-olivacea*, *Dactylonectria torresensis*, and *Phaeoacremonium minimum*. Partial inhibition was observed against *Diaporthe ampelina* (62.5% growth reduction), *Diaporthe bohemiae* (58.4%), *Diplodia seriata* (30%) and *Eutypa lata* (79%) using 1 mg mL^{-1} of extract. A subsequent *in planta* experiment was carried out. Commercial grafts of grapevine were treated with the extract and then planted. The total genomic DNA of grapevines was extracted 10 and 180 d after the treatment. Fungal diversities of the treated or untreated plants were compared using high-throughput amplicon sequencing. Treated plants showed 76.9% lower relative abundance of *Diaporthe* and 70% lower relative abundance of *Phaeoacremonium* 10 d after treatment. A similar scenario was observed for *Cadophora* 180 d after treatment, where treated grapevines had 76% lower relative abundance of this genus compared with untreated grapevines.

Biological and chemical pruning wound protectants reduce infection of grapevine trunk disease pathogens in California. R. BLUNDELL and A. ESKALEN. *Department of Plant Pathology, University of California, Davis, California, USA. Email: aeskalen@ucdavis.edu*

Grapevine trunk diseases (GTDs) are important challenges for viticulture, curtailing vineyard longevity and productivity in most raisin, table, and wine grape production region. Vine pruning wounds are the main entry points for fungal pathogens responsible for these diseases, and pathogens enter the wounds following precipitation events. The present study evaluated the efficacy of selected chemical and experimental biological fungicides for protection of pruning wounds against GTD pathogens, *Eutypa lata* and *Neofusicoccum parvum*. The study was conducted on Chenin blanc grapevines at the UC Davis Department of Plant Pathology Field Station for 6 months. Several chemical or biological fungicides, notably fluopyram/trifloxystrobin, *Trichoderma atroviride*, *Trichoderma asperellum* + *T. gamsii*, and a blend of crab and lobster shell powder, provided protection against at least two of the canker pathogens used in this study. However, the majority of products tested did not provide simultaneous control of *E. lata* and *N. parvum*, highlighting the continuing challenge for controlling GTDs.

Impacts of a copper-hydroxyapatite formulation on the grapevine physiology, microbiome, and metabolome, for potential use against grapevine trunk diseases. V. MONDELLO[1], O. FERNANDEZ[1], J. F. GUISE[1], C. LEMAÎTRE-GUILLIER[2], R. GOUGEON[3], A. ACEDO[4], P. SCHMITT-KOPPLIN[5], M. ADRIAN[2], C. PINTO[6], P. TROTEL-AZIZ[1] and F. FONTAINE[1]. *[1]Unité Résistance Induite et Bioprotection des Plantes EA 4707 USC INRAE 1488 Université de Reims Champagne-Ardenne, SFR Condorcet FR CNRS 3417 Reims. [2]Agroécologie, Institut Agro Dijon, CNRS, Université de Bourgogne, INRAE, Université de Bourgogne Franche-Comté, F-21000 Dijon, France. [3]Institut Universitaire de la Vigne et du Vin, UMR PAM Université de Bourgogne, Institut Agro Dijon, Dijon, France. [4]Biome Makers, CA, USA. [5]Analytical BioGeoChemistry, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany. [6]Associação SFCOLAB—Laboratório Colaborativo para a Inovação Digital na Agricultura, Torres Vedras, Portugal. E-mail: florence.fontaine@univ-reims.fr; vincenzo.mondello@univ-reims.fr*

Concerns for human and environment health are driving development of innovative eco-sustainable plant protection products (PPPs). Copper (Cu) is a PPP active ingredient subjected to regulation, especially in Europe, where it is considered a "candidate for substitution". Copper is recognized as an effective fungicide, used for more than 130 years against mildew, and is the only authorized fungicide in organic viticulture. Previous studies using the new Cu-based formulation HA + Cu(II), containing 3.5% Cu, that is transported to plants by hydroxyapatite, have shown its efficiency against *Plasmopara viticola* and *Phaeoacremonium minimum* under

greenhouse conditions. These effects were related to induction of plant defence responses. The impacts of HA + Cu(II) on plant physiology were studied in greenhouse grown 'Chardonnay' and 'Cabernet sauvignon' grapevines, which were either healthy or infected by *Diplodia seriata* and *Neofusicoccum parvum*. The effects of HA + Cu(II) against GTDs were also evaluated in a vineyard ('Chardonnay', in the Champagne region, France) that was affected by Esca. Th vineyard assessment aimed to determine the impacts of HA + Cu(II) on: (*i*) incidence of Esca, (*ii*) the vine microbiome, (*iii*) vine physiology, and (*iv*) oenological parameters of grape juice. In the greenhouse assays, HA + Cu(II) application induced several defence genes without adversely affecting plant growth or photosynthetic activity, with induction levels comparable to those of a commercial product. The fungistatic effects on the two *Botryosphaeriaceae* reported *in vitro* were confirmed *in planta*. In the field, a decreasing trend was observed in the incidence of Esca cumulate over years, and no deleterious effects were detected on vine microbiota or physiology, or on the oenological properties of the grape juice. Some similarities of HA + Cu(II) treatment responses to those from sodium arsenite were observed. These results support the potential of HA + Cu(II) as a promising PPP for GTD management.

***In vitro* evaluation of endophytic and rhizospheric bacteria as potential biocontrol agents of grapevine trunk diseases**. M. I. BUSTAMANTE, K. ELFAR and A. ESKALEN. *Department of Plant Pathology, University of California, Davis, CA 95616, United States. E-mail: mibustamante@ucdavis.edu*

Management of grapevine trunk diseases (GTDs) is an ongoing challenge for viticulture, and there is a need for effective and long term sustainable disease management strategies. A total of 20 commercial vineyards throughout California were sampled over the summer of 2019 for isolating endophytic and rhizospheric bacteria from vine cordons, trunks, and roots. A collection of 1,344 bacterium isolates (1,344) were obtained and tested *in vitro* against *Neofusicoccum parvum* and *Diplodia seriata*. From these, a subset of 172 isolates exerted mycelium growth inhibition levels >40%. The majority of these isolates (154) corresponded to an undescribed *Bacillus* sp., closely related to *B. velezensis*, and the remaining were *Pseudomonas* (12 isolates), *Serratia* (two),and other genera, and were excluded from this study. Representative isolates of *Bacillus* sp., *Pseudomonas chlororaphis* and *Serratia plymuthica* were assessed in dual antagonism assays against *N. parvum, D. seriata, Lasiodiplodia theobromae, Eutypa lata*, *Diaporthe ampelina, Phaeoacremonium minimum, Fomitiporia polymorpha* and *Ilyonectria liriodendri*. Mycelium inhibition was consistent across the bacterium species, being slightly greater against slow growing fungi than against *Botryosphaeriaceae*. The volatile and agar-diffusible metabolites produced by these bacteria were also tested against mycelium growth of *N. parvum* and *E. lata, Bacillus* sp. isolates strongly inhibited the growth of both pathogens, by their diffusible metabolites at all tested concentrations (1, 15, or 30% v/v), but not by their volatile compounds. The isolates of *P. chlororaphis* and *S. plymuthica*, however, caused less inhibition of both pathogens, but a combination of volatile and diffusible metabolites was probably involved in the antifungal activity. These isolates are being assessed in field experiments for effectiveness against trunk disease pathogens.

Biological control of Botryosphaeria dieback on grapevines. J. R. ÚRBEZ-TORRES[1], J. POLLARD-FLAMAND[1,2], J. BOULÉ[1] and M. HART[2]. *[1]Summerland Research and Development Centre, Agriculture and Agri-Food Canada, 4200 Highway 97, Summerland, BC V0H 1Z0, Canada. [2]Department of Biology, The University of British Columbia Okanagan, 3187 University Way, Kelowna, BC V1V 1V7, Canada. E-mail: joseramon.urbeztorres@agr.gc.ca*

Botryosphaeria dieback (BD) is a grapevine trunk disease (GTD) that causes important grape yield losses and limits vineyard lifespans. *Botryosphaeriaceae* spp. causing BD infect grapevines through pruning wounds. Therefore, pruning wound protection is the most effective and available management strategy for this disease. Demand has increased for alternatives to chemical products and sustainable control methods to manage GTDs. With no control products currently registered in Canada against GTDs, the present research aimed to identify local *Trichoderma* biological control agents (BCAs), and evaluate their potential against the BD pathogens *Diplodia seriata* and *Neofusicoccum parvum*. A total of 29 *Trichoderma* isolates were obtained from vineyards in British Columbia. Morphological studies and phylogenetic analyses of the ITS1-5.8S-ITS2 gene and TEF-1α partial gene identified seven species (*T. asperelloides, T. atroviride, T. harzianum, T. koningii, T. tomentosum*), and two novel species, *T. canadense* and *T. viticola*. *In vitro* dual culture antagonism assays showed that several isolates inhibit pathogen mycelium growth, by up to 75%. *In planta* detached cane assays under controlled greenhouse conditions identified *T.*

asperelloides, T. atroviride and *T. canadense* isolates gave 70% to 100% pruning wound protection against *D. seriata* and *N. parvum* for up to 21 d after treatment. Field trials conducted in 'Merlot' vines in 2019 and 2020 showed that mixed-species inoculum of *T. asperelloides, T. atroviride* and *T. canadense* had high biocontrol activity against BD fungi for up to 60 d after treatment. Field results also showed that the *Trichoderma* spp. provided similar or greater pruning wound protection when compared with commercial chemical or biocontrol products. This study provides data supporting development and registration of the first control products against GTDs in Canada.

Combining a HA + Cu(II) site-targeted copper-based product with a pruning wound protection programme to prevent grapevine infection by *Lasiodiplodia* spp. P. REIS[1], A. GASPAR[1], A. ALVES[2], F. FONTAINE[3] and C. REGO[1]. *[1]LEAF - Linking Landscape, Environment, Agriculture and Food, Associated Laboratory TERRA, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal. [2]CESAM—Centre for Environmental and Marine Studies, Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal. [3]Université de Reims Champagne-Ardenne, Unité Résistance Induite et Bioprotection des Plantes EA 4707 USC INRAE 1488, SFR Condorcet FR CNRS 3417, 51687 Reims, France. E-mail: pedroreis@isa.ulisboa.pt*

Lasiodiplodia has been reported from several grape growing regions, and is one of the most rapid grapevine wood colonizers, causing Botryosphaeria dieback. These fungi can infect grapevines through wounds, and those caused by pruning are the principal sites of infection. It is therefore important to develop effective integrated disease management, which includes cultural practices, organic products, BCAs, responsible use of chemical pesticides, and appropriate management strategies, which may combine chemical and biological controls. The present study aimed to: *i*) evaluate the efficacy of Esquive®, a biocontrol agent, as grapevine pruning wound protection applied alone or in combination with a new site-targeted copper-based treatment (LC2017); and *ii*) compare their efficacy with that of the commercially available product, Tessior®. For two growing seasons, protectants were applied onto pruning wounds, while LC2017 was applied throughout the season according to the manufacturer's instructions. Pruning wounds of two different cultivars were inoculated with three isolates of *Lasiodiplodia* spp. Efficacy of the wound protectants varied between the 2 years. However, according to cultivar, the treatments controlled the pathogen to some extent. Application of LC2017 did not show clear evidence of improving control obtained by individual applications of the other products tested. However, LC2017 was fungistatic against *Lasiodiplodia* spp. *in vitro*, and has previously been shown as an elicitor against grapevine trunk diseases. This combination of two protection strategies may be a promising long-term approach to mitigate the impacts of Botryosphaeria dieback.

Relationship between *Trichoderma* recovery from pruning wounds treated with biocontrol formulations and the control of *Diplodia seriata* in Chilean vineyards. E. DONOSO, L. ROMERO, W. HETTICH, D. BASCUÑAN, C. GARCIA and J. FIGARI. *Bio Insumos Nativa SPA, Pc Antilhue Lote 4b2, Maule, Chile. E-mail: edonoso@bionativa.cl*

The use of sprayed *Trichoderma* based products has raised doubts about the relationships between recovery of *Trichoderma* from treated cuts and control efficacy against grapevine wood decay fungi. The present study aimed to assess the relationship between recovery of *Trichoderma* decreases in damage by wood fungi in vineyards. vineyards. Two experiments were established in San Felipe (Aconcagua Region) and Talca (Maule Region), using, respectively, the grapevine 'Red Globe' and 'Cabernet Sauvignon'. Twenty-four hours after pruning 100 g/hL^{-1} of the commercial product Mamull (Bio Insumos Nativa SPA) was sprayed using conventional equipment in five 0.5 ha replicates, alternated with experimental controls without treatment, under a randomized block design. At 180 d after application, samples (n = 50) were evaluated for *Trichoderma* and pathogen recovery (natural infection), and incidence and severity of lesions. At 15 d after field application, *Trichoderma* was recovered from the samples by isolating on malt extract agar. A subsample of detached canes (n = 20) was inoculated in the laboratory with *Diplodia seriata* conidium suspension and incubated in glass flasks for 30 d, for evaluation of symptoms. In the field, mean dieback incidence (mm of lesion X pathogen recovery) in the controls was 7.5% compared with 0.2 % when treated with *Trichoderma*. ($P < 0.05$ for this reduction). Average recovery of *Trichoderma* from the treated canes (52.3%), was greater ($P < 0.05$) than the control canes (11.3%). Recovery of *D. seriata* was greater ($P < 0.05$) in the control canes (16.4%) compared to those treated with *Trichoderma* (5.4%). The detached canes inoculated in the laboratory had longer ($P < 0.01$) lesions in the control (mean = 1.8 cm) compared with those from the

Trichoderma treatment (0.2 mm). Low correlation was found between the presence of *Trichoderma* and field damage (R = 0.45; $P < 0.05$). Recovery of *Trichoderma* from treated cuts was less reliable than host damage measurements for samples treated in the laboratory. The biocontrol formulation gave significant control of wood disease, and natural infection in the field, and for artificial inoculation 15 d after spray application.

Hot water treatment as a tool to produce high-quality grapevine propagation material. D. SIMON[1,2], P. WINTERHAGEN[1], R. WALTER[1], T. WETZEL[1], A. KORTEKAMP[1], A. VON TIEDEMANN[2] and J. EDER[1]. *[1]Dienstleistungszentrum Ländlicher Raum (DLR) Rheinpfalz, Breitenweg 71, D-67435 Neustadt a.d. Weinstraße, Germany. [2]Georg-August University of Göttingen, Plant Pathology and Crop Protection Section, Grisebachstraße 6, D-37077 Göttingen, Germany. E-mail: DorottyaAgnes.Simon@dlr.rlp.de*

Hot water treatment (HWT) of dormant woody plant material has been shown to reduce infections by Grapevine Trunk Disease (GTD) pathogens. In this study, HWT on individual developmental stages of three pathogens [*Phaeomoniella chlamydospora* (*Pch*), *Phaeoacremonium minimum* (*Pmi*), *Botryosphaeriaceae* (*Bot*) species] was assessed at different combinations of temperature and exposure time. Conidia and mycelium suspensions of *Pch*, *Pmi* or *Bot* were subjected to different HWTs of 30 to 45 min at 40 to 55°C, and then incubated on malt extract agar in a moist chamber to assess subsequent colony formation. In addition, field experiments were carried out to evaluate effects of HWT on the targeted fungal pathogens, and on a commercially available biological control agent (BCA) [*Trichoderma atroviride* strain SC1 (TASC1) (Vintec®, Belchim Crop Protection Deutschland GmbH). Inoculated scion cuttings grafted with healthy rootstocks following HWT (50°C, 45 min) under practical conditions, were then planted in nurseries and analysed at different times for pathogen development. The *in vitro* results indicated that *Pmi* had reduced sensitivity to HWT at the ungerminated spore stage, while *Pch* and *Bot* conidia were more sensitive to HWT. Isolations from the inoculated cuttings confirmed that infestations caused by *Pmi* and *Pch* were reduced, whereas *Bot* was completely eliminated, through HWT. No negative impacts of HWT on the commercial BCA product were detected. Therefore the antagonistic ability of the BCA against the fungal trunk pathogens should not be affected by HWTs.

Removal of trunk disease pathogens in mature grapevines using remedial surgery. E. VAN ZIJLL DE JONG[1], H. TERNENT[2], S. ST GEORGE[2], R. KALLAS[2] and M. SOSNOWSKI[3,4]. *[1]Linnaeus Limited, PO Box 1199, Gisborne 4040, New Zealand. [2]Villa Maria Estate Ltd, 118 Montgomerie Road, PO Box 43046, Mangere, Auckland, New Zealand. [3]South Australian Research and Development Institute, Adelaide, SA 5001, Australia. [4]School of Agriculture, Food and Wine, Waite Research Institute, The University of Adelaide, SA 5005, Australia. E-mail: eline@linnaeus.co.nz*

Remedial surgery has been used to improve productivity and extend life of grapevines infected by pathogens causing Botryosphaeria dieback and Eutypa dieback. Efficacy is dependent on removal of infected host wood and growth of new shoots to re-establish trunks. Remedial surgery was carried out during 3 years in mature (>18 years) commercial vineyard blocks of 'Sauvignon blanc', 'Cabernet Sauvignon' and 'Merlot'. Each year, the trunks were removed at standard distances above graft unions (200 mm for 'Sauvignon blanc', 150 mm for the other varieties). Incidence of dieback in the canopies was >90 % in 'Sauvignon blanc' and 'Cabernet Sauvignon', and increased over this time from 64 to 91% in 'Merlot'. Internal trunk staining associated with dieback was frequently detected at the top of the trunks below the heads in 'Sauvignon blanc' (98%) and 'Merlot' (84%), and at the remedial cut sites (respectively, 55 and 59%). These symptoms were not as frequent in the trunks of 'Cabernet Sauvignon' (62% of top and 10% of cut sites). Staining symptoms were also observed from spur and watershoot wounds on the trunks. Over the three growing seasons, distance of staining from remedial cut sites decreased in all three cultivars, and in 'Sauvignon blanc' and 'Merlot', incidence of staining increased at the remedial cut sites. *Botryosphaeriaceae* species were frequently detected in the trunks in advance of staining in all three cultivars. Trunks were shown to be infected with multiple species of *Botryosphaeriaceae*. When present, *Eutypa lata* was usually found together with these pathogens. *Botryosphaeriaceae* species, and occasionally *E. lata*, occurred over distances >200 mm in advance of staining. Growers are now advised to intervene early, and cut as low as practical, to improve the efficacy of remedial surgery.

Trellis systems of rootstock mother grapevines affect the wood microbiome. E. BATTISTON[1], L. BORRUSO[2], S. FALSINI[3], C. PINTO[4], T. MIMMO[2], S. CESCO[2], S. DI MARCO[5] and L. MUGNAI[1]. *[1]Department of Agri-*

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Management of rootstock mother vines is the first stage for bench-grafted grapevine production in nurseries. In source mother blocks, rootstock shoots are usually sprawled on the ground, which may favour infections by trunk disease pathogens, and compromise the quality of resulting propagation material. Trellis systems could be applied to rootstock mother vines to improve the functional leaf area and exposure to sunlight. Different trellis systems influence canopy microclimates, and the lack of contact with soil from trellising rootstock mother vines may affect epiphytic and endophytic microbial communities of resulting propagation material. DNA metabarcoding was used to investigate effects of two trellising methods (vertical trellis or transpiring fabric for protecting sprawled rootstocks from the soil), compared to traditional sprawled rootstocks, assessing on resident fungal and bacterial communities focussing on wood pathogens. The rootstock cultivars Kober 5 BB and 110 Richter were assessed. Bacteria and fungi beta-diversities, including epiphytic and endophytic communities, were affected by the temporal distribution of the rootstock. Diversity was greater when comparing a trellised rootstock ('Kober 5 BB') with the same plant material sprawled on the ground. These results demonstrated that sprawling shoots, compared to vertically-positioned shoots, were more exposed to soilborne microorganisms and pathogens, due to contact with soil inoculum, and probably due to the increased temperatures and humidity in sprawling shoots.

Biological control agents for Botryosphaeria dieback of grapevine. C. S. DELGADO-RAMÍREZ[1], E. SEPÚLVEDA[1,2], C. VALENZUELA-SOLANO[3], R. HERNÁNDEZ-MARTÍNEZ[1]. *[1]Centro de Investigación Científica y de Educación Superior de Ensenada, Baja California (CICESE), Departamento de Microbiología, Carretera Tijuana-Ensenada 3918, Zona Playitas, 22860, Ensenada, Baja California. [2]CONACYT-CICESE. Departamento de Microbiología. [3]INIFAP, sitio experimental Costa de Ensenada, Mexico. Calle del Puerto núm. 375-23, Fracc. Playa Eda.22880 Ensenada, Baja California, Mexico. E-mail: cdelgado@cicese.edu.mx*

Botryosphaeriacea cause Botryosphaeria dieback, which is responsible for damage to grapevines. In Mexico, *Lasiodiplodia brasiliensis* is a highly virulent causal agent of this disease, There is no effective control available, so integrated management includes eliminating diseased plants, and use of fungicides, wound protection, and biological control agents. The present study aimed to identify and evaluate microorganisms with biocontrol activity against Botryosphaeria dieback fungi, to provide growers with environmentally-friendly strategies for disease control. One hundred thirty-seven endophytic bacteria (137 isolates) were obtained from old grapevines, and 37 *Trichoderma* isolates were recovered from our collection. All isolates were evaluated for *in vitro* antagonistic activity against *L. brasiliensis*. Eleven *Bacillus* and four *Trichoderma* isolates were selected to assess their plant growth promotion characteristics abilities to produce volatile and non-volatile compounds with antifungal activity. Thirteen isolates were then selected for greenhouse assays. These isolates were applied directly to soil as preventive treatments, while *L. brasiliensis* was inoculated into hole made in the grapevine plants. Only the plants inoculated with *Bacillus subtilis* BEVP26 developed smaller lesions than experimental controls. Nine of the tested isolates were applied preventively in pruning wounds made on branches of grapevines established in a commercial vineyard. One hour later *L. brasiliensis* was applied at the top of each vine branch. Plants inoculated with eight of the isolates developed smaller lesions than plants inoculated only with *L. brasiliensis*. These potential biological control agents were molecularly identified as *B. subtilis*, *B. velezensis*, *T. asperellum*, or *T. longibrachiatum*.

POSTER PRESENTATIONS

Diversity of *Dactylonectria* and *Ilyonectria* species causing black foot disease in grapevine nursery stock in Uruguay. M. J. CARBONE, M. GELABERT, P. MONDINO and S. ALANIZ. *Universidad de la República, Facultad de Agronomía, Departamento de Protección Vegetal, Avenue Garzón 780, 12900 Montevideo, Uruguay. E-mail: salaniz@fagro.edu.uy*

Black foot, caused by "*Cylindrocarpon*"-like asexual morph fungi, is an important disease affecting young grapevines. Several studies have shown that grapevines are primarily infected by black foot pathogens in nurs-

eries, highlighting the role of infected nursery plants in spread of the disease. A previous study in Uruguay, recorded observation of typical black foot symptoms in grapevine planting material. These symptoms were brown to dark streaks that developed from the bases of rootstocks, wood necroses at trunk bases, sunken necrotic root lesions, and reduced root biomass. To determine the diversity of "*Cylindrocarpon*"-like fungi associated with black foot, grapevines of different cultivars grafted onto Gravesac, 1103P, SO4, 101-14 and 3309C rootstocks and with black foot symptoms, and ready to be sold to growers, were evaluated from 2017 to 2019. A total of 77 "*Cylindrocarpon*"-like strains were isolated and identified by DNA sequence analysis of the partial histone H3 gene. The BLAST search was conducted against type specimens in GenBank, and analysed phylogenetically by the Maximum Likelihood method. Five species of *Dactylonectria*, and three of *Ilyonectria*, were found. The most prevalent species was *D. macrodidyma* (32 isolates), followed by *D. novozelandica* (15), *D. torresensis* (ten), *I. liriodendri* (nine), *D. pauciseptata* (five), *D. valentina* (one), *I. robusta* (one) and *Ilyonectria* sp. (four). This study has increased knowledge of the etiology of black foot disease affecting Uruguayan nursery grapevines.

***Aspergillus* spp. causing Aspergillus vine canker on grapevines in Mexico**. E. A. RANGEL-MONTOYA[1], C. VALENZUELA-SOLANO[2] and R. HERNÁNDEZ-MARTÍNEZ[1]. *[1]Centro de Investigación Científica y de Educación Superior de Ensenada, Baja California (CICESE), Departamento de Microbiología, Carretera Tijuana-Ensenada 3918, Zona Playitas, 22860, Ensenada, B.C. Mexico. [2]INIFAP, sitio experimental Costa de Ensenada. Calle del Puerto 375-23, Playa Ensenada, 22880, Ensenada, Baja California, Mexico. E-mail: ruhernan@cicese.mx*

Aspergillus comprises a diverse group of species with high economic and social impacts. Aspergillus vine canker is rare; the fungus infects vigorous new grapevine shoots and canes when the vines are being trained, entering through wounds. Recently, grapevines with symptoms similar to Aspergillus vine canker have been observed in Mexico. Wood samples from symptomatic plants were obtained from vineyards in Sonora, Baja California, and Guanajuato. The samples were sterilized with alcohol and flame and placed onto Potato Dextrose Agar. Seventeen isolates were obtained that showed morphology similar to *Aspergillus*. colony and microscopic characteristics were observed from cultures on Czapek Yeast Extract Agar and Malt Extract Agar. Phylogenetic analysis using calmodulin (CMD) and β-tubulin (BenA) genes revealed three species of *Aspergillus*. Nine isolates were identified as *A. niger*, seven as *A. tubingensis,* and one as *A. welwitschiae*. Pathogenicity studies were carried out using 'Merlot' grapevine plants. Eleven isolates were inoculated into host through wounds and onto leaves. *Aspergillus niger* A10BCMX, *A. niger* A8SMX, and *A. tubingensis* A13SMX were the most virulent, causing lesions of up to 2 cm in length after 50 d. On the inner sides of wounds, inside host cambium, powdery black conidia were found. On the leaves, necrotic lesions, mycelia, and black conidia formed. This is the first report of *Aspergillus* species associated with Aspergillus vine canker in vineyards of Mexico.

Molecular methods for detection and quantification of *Diplodia seriata* and *Phaeomoniella chlamydospora* in grapevine plants. M. ACUÑA[1,2], R. ROA[2], P. RODRIGUEZ[2], P. ARRAÑO[2], S. VARGAS[2], F. GAÍNZA-CORTÉS[2], G. DÍAZ[1] and M. LOLAS[1]. *[1]Universidad de Talca, Facultad de Ciencias Agrarias, Laboratorio de Patología Frutal, Casilla 747, Talca, Chile. [2]Viña Concha y Toro S.A, Centro de Investigación e Innovación, Fundo Pocoa s/n, Km10 Ruta K-650, Región del Maule, Pencahue, Chile. E-mail: felipe.gainza@conchaytoro.cl*

Grapevine trunk diseases (GTDs) are important phytosanitary problems affecting vineyards. to date, 133 fungal species, in nine families, have been associated with GTDs. In Chile, *Phaeomoniella chlamydospora*, *Diplodia seriata* and *Inocutis* sp. are the fungi most frequently isolated from adult plants affected by GTDs. Diagnosis of these fungi using classical methods is laborious and time-consuming. In addition, slow growth rates of fungi in semi-selective media compared with other microorganisms can generate false negatives due to overgrowth of these fungi, resulting in underestimation of incidence levels. Methods based on molecular detection can be complementary and effective tools for detecting these wood fungi, giving rapid and precise results. Fifty-four grapevine plants (Cabernet Sauvignon clone #337, on rootstock 110-14) were inoculated with *P. chlamydospora* or *D. seriata*. Ten months after inoculation, both phytopathogenic fungi were detected and quantified by qPCR, from four zones, 5 and 15 cm above or below the inoculated zones. Both fungi were detected in the distal zones. 27 plants were infected by *P. chlamydospora*, and 24 of 27 plants were infected by *D. seriata*. These results validate the complementary use of this molecular tool along classical methods, for diagnosis and detection of *P. chlamydospora* and *D. seriata* infec-

tions, enabling their detection in asymptomatic wood in the grapevine propagation material.

Investigating the role of *Fusarium* spp. on young vine decline in California. M. I. BUSTAMANTE[1], K. ELFAR[1], M. ARREGUIN[1], R. J. SMITH[2], L. J. BETTIGA[3], T. TIAN[4], G. A. TORRES[5], G. ZHUANG[6] and A. ESKALEN[1]. *[1]Department of Plant Pathology, University of California, Davis, CA 95616, USA. [2]University of California, Cooperative Extension Sonoma County, Santa Rosa, CA 95403, USA. [3]University of California, Cooperative Extension Monterey County, Salinas, CA 93901, USA. [4]University of California, Cooperative Extension Kern County, Bakersfield, CA 93307, USA. [5]University of California, Cooperative Extension Tulare County, Tulare, CA 93274, USA. [6]University of California, Cooperative Extension Fresno County, Fresno, CA 93710, USA. E-mail: mibustamante@ucdavis.edu*

From 2018 to 2021, young wine and table grape vineyards have shown decline symptoms in several Californian counties, including Fresno, Kern, Monterey, Napa, San Joaquin, Sonoma, Tulare, Yolo, and Yuba. Symptoms were diverse and characterized by poor or no growth during the season, dieback, sap exudation, and discoloration of vascular tissues around graft unions. Absence of feeder roots and graft failure have also been observed. In ten vineyards, the estimated decline incidence ranged from 5 to 50%. Isolations were carried out at the margins of vascular discolouration of affected vines, by placing wood sections (1 × 1 mm) onto acidified potato dextrose agar, with incubation for 7 d at 25°C in the dark. Although different fungal pathogens were obtained, including *Botryosphaeriaceae*, and *Phaeoacremonium* and *Cylindrocarpon*-like species, colonies of *Fusarium* were present in isolations from all the plant samples. Pure cultures of the *Fusarium* isolates were obtained from single hyphal tips, and were further identified using a phylogenetic approach. After DNA extraction, the translation elongation factor 1-alpha (*tef1*) and the RNA polymerase II second largest subunit (*rpb2*) partial gene regions were amplified and sequenced using, respectively, the primers EF1/EF2, 5F2/7cR and 7cF/11aR. Consensus nucleotide sequences were used to search the closest species in the NCBI database using BLAST. Phylogenetic trees revealed 13 *Fusarium* species, including members of the *F. fujikuroi*, *F. oxysporum*, *F. solani*, *F. sambucinum* and *F. incarnatum-equiseti* species complexes. The most frequent species (47.4%) was *F. annulatum* (*F. fujikuroi* species complex), and pathogenicity was confirmed for this fungus and the remaining 12 species by completing Koch's postulates in one-year-old 'Chardonnay' and '1103 Paulsen' vines.

***Diaporthe* spp. associated with dieback in Baja California vineyards**. C. A. DELGADO-RAMÍREZ[1], E. A. RANGEL ONTOYA[1], J. C. LEE-CONTRERAS[2], C. VALENZUELA-SOLANO[3] and R. HERNANDEZ-MARTINEZ[1]. *[1]Centro de Investigación Científica y de Educación Superior de Ensenada, Baja California (CICESE), Departamento de Microbiología, Carretera Tijuana-Ensenada 3918, Zona Playitas, 22860, Ensenada, B.C. Mexico. [2]Universidad Autónoma de Nuevo León. Departamento de Fitopatología, Mexico. [3]INIFAP, sitio experimental Costa de Ensenada. Calle del Puerto 375-23, Playa Ensenada, 22880, Ensenada, Baja California, Mexico. E-mail: ruhernan@cicese.mx*

Diaporthe dieback, previously known as Phomopsis dieback of grapevine, is a trunk disease caused by several species of the *Diaporthe* genus. The main symptoms in affected plants are leaf spots, growth retardation, small bunches, fruit rot, and plant dieback. Some of the species associated with grapevines are *D. eres*, *D. viticola* and *Diaporthe ampelina,* with *D. ampelina* the most virulent species. In Mexico, cultivation of grapevines is of socio-economic importance, mainly in Sonora and Baja California. There are reports of species associated with Botryosphaeria dieback and Eutypa dieback, but there have been no reports of *Diaporthe* species in Mexico wine-producing regions. The present research isolated and characterized *Diaporthe* associated with grapevines. Isolates with morphological features similar to *Diaporthe* spp. were obtained from grapevine plants with dieback symptoms in different vineyards of Baja California. Identification of isolates was made using by morphological characterization, and molecular analyses using the ITS and EF1-α markers. Phylogenetic analysis allowed identification of strains of *D. ampelina, D. eres*, and *D. foeniculina*. Pathogenicity tests for these pathogens are being carried out. Although these species have been identified in vineyards in other countries, this is the first report of them on grapevines in Mexico.

The Hymenochaetaceous fungus *Arambarria destruens* associated with grapevine trunk diseases in Chilean patrimonial vineyards. J. CHILIAN, D. GRINBERGS and M. REYES. *Instituto de Investigaciones Agropecuarias, INIA Quilamapu, Chillán, Chile. E-mail: jchilian@inia.cl*

Grapevine is an important fruit crop in Chile, with 141,000 ha for wine production. Grapevine Trunk Diseases (GTDs) are well studied in commercial cultivars such as Cabernet Sauvignon, Sauvignon Blanc, Merlot and Chardonnay, but there is lack of information regarding these diseases in Patrimonial Vineyards, mostly of País, Moscatel, Cinsault and Carignan cultivars. A frequent symptom observed in Patrimonial Vineyards was trunks with yellowish, spongy wood cankers. The objective of the present study was to identify the fungi associated with these symptoms. A survey was conducted from 2019 to 2021, on Patrimonial Vineyards in the south of Chile. Fungi were isolated on quarter strength potato dextrose agar (PDA) amended with antibiotics, and then purified on PDA. Yellow cottony colonies with irregular margins and dark areas were isolated from cankers (18%), and were preliminarily identified as Basidiomycetes based on microscopic structures including basidia and basidiospores. Mycelia was collected from the edges of 20 pure PDA cultures, Genomic DNA was extracted and used to amplify the internal transcribed spacer (ITS) region and the ribosomal large subunit fragments (LSU). ITS and LSU consensus sequences were compared to reported sequences, and combined to perform a multigenic analysis. The alignments were individually edited, and then concatenated, and phylogenetic trees were created using Maximum Parsimony and Maximum Likelihood algorithms. Based on these analyses, the Basidiomycetes species associated with the described symptoms was identified as the *Hymenochaetaceae* fungus *Arambarria destruens*.

Fungal species associated with grapevine decline in China. Y. Y. ZHOU[1,2], X. H. LI[1], L. N. WU[1], H. M. ZHANG[1], W. ZHANG[1] and J. Y. YAN[1]. *[1]Beijing Key Laboratory of Environment Friendly Management on Fruit Diseases and Pests in North China, Institute of Plant Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China. [2]Center of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand. E-mail: zhwei1125@163.com*

Grapevine is an important fruit crop in China, with 765,018 ha of cultivation area, so ranking as the third in the world. To characterize fungi within the roots associated with grapevine decline, field surveys were conducted in Beijing, Hebei, Ningxia and Yunnan Provinces of China in 2021. Root samples were collected from symptomatic plants showing stunted growth, small leaves with yellow lesions, uneven sized grapes, and necrotic vascular or xylem tissues, as well as from neighbouring symptomless plants. Isolations were conducted, and isolates were identified based on morphological characteristics and multi-gene phylogenetic analyses. Ninety-three isolates were obtained from symptomatic plant samples, among which *Clonostachys* was the greatest proportion (41% of isolates), followed by *Neocosmospora* (26%), *Fusarium* (16%) and *Dactylonectria* (5%). *Rhizoctonia*, *Botrytis* and *Meyerozyma* were also isolated. From asymptomatic samples, 119 isolates were obtained. The four most frequent genera were the same as from symptomatic plants, while the proportion of *Fusarium* isolates was the greatest (28%). More genera were isolated from asymptomatic plants, including *Robillarda*, *Cylindrocladiella*, *Lasiodiplodia*, *Lophiostoma*, *Sacrocladium*, *Acrocalymma*, *Sporothrix*, *Stagonospora* and *Acremonium*. According to the phylogenetic results, the *Fusarium* isolates were clustered with three species, *F. oxysporum*, *F. commune* and *F. clavum*. *Neocosmospora* was identified as *N. solani*, *N. falciformis* and *N. pisi*. *Clonostachys* isolates were all identified as *C. rosea*. The main fungi isolated in this study were *Nectriaceae,* including *Neocosmospora* spp., *Fusarium* spp., *Dactylonectria* spp. and *Cylindrocladiella* spp., among which five species were first reported on grapevine. Pathogenicity of these fungi will be assessed, and relationships between fungal species of symptomatic and symptomless plants will be explored.

Presence and distribution of grapevine trunk diseases in the vineyards of Quebec, Canada. C. PROVOST[1], P. CONSTANT[2] and A.-A. DURAND[2]. *[1]CENTRE DE RECHERCHE AGROALIMENTAIRE DE MIRABEL, 9850 Belle-Rivière, Mirabel, Québec, Canada, J7N2X8. [2]INRS-ARMAND- FRAPPIER SANTÉ BIOTECHNOLOGIE, 531 Boul des Prairies, Laval, Québec, Canada, H7V 1B7. E-mail: cprovost@cram-mirabel.com*

Grapevine trunk diseases (GTDs) are very damaging for the sustainability of the vineyards heritage in all major wine-producing regions. GTDs can affect young plantations and aging vineyards. In both cases, risks from these diseases are increasing in Quebec vineyards, as many are more than 20 years old, and many producers plan to expand production. However, presence and distribution of GTDs in Quebec is unknown. This project characterized the distribution of GTDs in Quebec vineyards using to different criteria, including region, grapevine variety, and vineyard age. Five GTDs were assessed, including: Esca (Petri disease) (caused by *Phaeomoniella chlamydospora and Phaeoacremonium*

minimum), Eutypa dieback (*Eutypa lata*), Botryosphaeria dieback (*Botryosphaeriaceae* spp.), excoriosis (*Diaporthe ampelina*), and Black foot (*Cylindrocarpon*-like asexual morphs). Sampling was done in several vineyards, and qPCR analyses were performed to detect the GTD pathogens. Results showed the presence of all these diseases in Quebec vineyards, but mainly Botryosphaeria dieback. This information will allow identification of the scope of GTDs in Quebec vineyards, as background for study of the epidemiology of these diseases, and evaluation of cultural practices to limit their spread.

Identification and quantification of grapevine trunk disease and black-foot pathogens in soil, using real-time *PCR coupled with HRM*. S. TESTEMPASIS[1], E. STAVRIDOU[2], P. MADESIS[2] and G. S. KARAOGLANIDIS[1]. *[1]Aristotle University of Thessaloniki, Faculty of Agriculture, Forestry and Natural Environment, Plant Pathology Laboratory, Thessaloniki, Greece. [2]Institute of Applied Biosciences, Centre for Research and Technology Hellas, GR-57001 Thessaloniki, Greece. E-mail: testempa@agro.auth.gr*

Identification of plant pathogens and inoculum quantification in soil samples using conventional methods is labour-intensive and time-consuming. Therefore, development of rapid, and simple to perform PCR-based identification methods that use pathogen-specific primers, is necessary. A real-time quantitative PCR approach coupled with high-resolution melting (HRM) analysis, was developed with one primer set to identify and distinguish several fungal species associated with grapevine trunk and black-foot diseases. The developed method targeted several *Cylindrocarpon*-like asexual morphs of *Ilyonectria* or *Dactylonectria*, and *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum* and *Diplodia seriata*. The technique's reliability was first assessed on DNA extracted from pure fungal cultures. Melting curve analyses of the amplicons allowed distinction of all the target species with confidence levels > 99 %. HRM curve profiles were generated for each targeted genus/species. Identification of the target pathogens in fortified soil samples was achieved in a range confidence between 60 and 75%. The quantification of the detected pathogen DNA in the soil was assessed with quantitative PCR and sensitivity was evaluated using standard curves. These were constructed using serial dilution of plasmid calibrators containing the produced amplicons of qPCR. High PCR efficiency was reached (almost 100%), and results were reproducible throughout sample storage and calibration ranges for each specific plasmid calibrator. The reaction was linear over a large dynamic range ($R2 > 0.99$) of 5 $\log_{10}$ concentrations tested. The method was validated in soil samples from commercial grapevine nurseries, and the concentrations of detected DNA ranged from two to 10^5 copies. This study has developed a new molecular tool for detection and quantification of several GTD or Black foot pathogens in soil samples from grapevine nurseries, and may contribute to the optimization of production of multiplication material in grapevine nurseries.

Real-time PCR quantification of airborne inoculum of *Eutypa lata* and *Botryosphaeriaceae* spp. in California. J. CLERKIN, S. DUBROVSKY and A. L. FABRITIUS. *AL&L Crop Solutions, Inc., 7769 N. Meridian Rd., Vacaville, CA, U.S.A. E-mail: info@allcropsolutions.com*

Annually pruned vineyards and orchards suffer significant losses each year due to diseases caused by *Eutypa* and *Botryosphaeriaceae* fungi. Airborne spores of these fungi gain access to vines through pruning wounds, from which they grow into the wood, causing cankers, dieback and reduced growth. In California, diseases caused by *Eutypa lata* and *Botryosphaeriaceae* spp. are commonly initiated during winter, when seasonal rains trigger spore release by these fungi. The present study aimed to determine Rotorod- type spore traps could be used for capturing spores of *Eutypa lata* and *Botryosphaeriaceae* spp. from vineyard air. Real-time PCR was used to detect and quantify numbers of the spores in spore traps on weekly bases, from December till April in 2020, 2021 and 2022. Results were comparable with those obtained either by using glass slides or volumetric spore traps, and showed that *Eutypa* and *Botryosphaeriaceae* spp. spore release correlated with rain events in California. However, differences were found between release of the spores by these two groups of fungi. While *Botryosphaeriaceae* spores were present in the traps throughout each winter, *Eutypa* spores were detected sporadically. Data also suggested that at some locations, the spores were released without the presence of rain, possibly due to high humidity. Differences were also found in spore release patterns between old and newly established vineyards. In young vineyards, lower counts of *Botryosphaeriaceae* and *Eutypa* spores were detected compared to the older, established vineyards. The study also indicated that disease pressure could differ by location of the vineyard and the spore trap. Release of spores did not always correlate with the weather data, suggesting that the predictions of the spore release cannot be based only on weather forecasts.

Effect of cover crops on the dispersal of *Phaeomoniella chlamydospora* inoculum in vineyards. M. BERBEGAL[1], D. PINNA[1], E. GONZÁLEZ-DOMINGUEZ[2], G. HASANALIYEVA[3], T. CAFFI[3], V. ROSSI[3] and J. ARMENGOL[1]. *[1]Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Camino de Vera S/N, 46022-Valencia, Spain. [2]Horta srl., Via Egidio Gorra 55, 29122 Piacenza, Italy. [3]Department of Sustainable Crop Production (DIPROVES), Facoltà di Scienze Agrarie, Alimentari e Ambientali, Università Cattolica del Sacro Cuore, Via Emilia Parmense, 84, 29122 Piacenza, Italy. E-mail: jarmengo@eaf.upv.es*

Effect of different cover crops on the dispersal of *Phaeomoniella chlamydospora* inoculum was evaluated in an experimental plot in Piacenza, Italy, during two consecutive growing seasons. We hypothesized that cover crops may act as physical barriers to reduce dispersal of *P. chlamydospora* airborne and rain-splash inoculum. Spore traps (microscope slides) were placed in plots with natural grass, bare soil, or four or one cover crops grown in, respectively, the first and second seasons. Grapevine cuttings artificially inoculated with *P. chlamydospora* were placed on the soil in the middle of every two subplots per treatment; in each subplot two spore traps were located near, or 40 cm above, the inoculum source. The traps were replaced weekly from December 2018 to June 2019, and from December 2019 to May 2020. Quantitative PCR, using specific primers for *P. chlamydospora*, was used to determine DNA concentrations in the spore trap samples, and the effects of the experiment treatments on inoculum dispersal. Rainfall and average temperature data were obtained from a weather station placed in the experimental site. Soil coverage was also evaluated according to cover cropping development. Dispersal of *P. chlamydospora* was associated with rainfall events in both seasons. Results from the first season showed no reduction in *P. chlamydospora* DNA detected in grass and cover crop plots relative to the bare soil. This is most likely explained by non-uniform cover and plant development due to dry weather conditions. The second season results showed lower DNA concentrations in natural grass and cover crop plots, especially during weeks with high levels of detection, which accounted for most of the spore dispersal. Analysis for the traps placed 40 cm above the inoculum sources confirmed the reductions in spore dispersal in grass and cover crop plots, relative to the bare soil plots.

Environmental conditions influencing survival and development of reproductive structures of *Phaeoacremonium minimum* and *Phaeomoniella chlamydospora*. M. BERBEGAL[1], E. GONZÁLEZ-DOMÍNGUEZ[2] and J. ARMENGOL[1]. *[1]Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Camino de Vera S/N, 46022-Valencia, Spain. [2]Horta srl., Via Egidio Gorra 55, 29122 Piacenza, Italy. E-mail: jarmengo@eaf.upv.es*

An *in vitro* experiment was carried out to determine effects of temperature on development of perithecia of *Phaeoacremonium minimum* and pycnidia of *Phaeomoniella chlamydospora* on grapevine cuttings. Pieces of 1-year-old grapevine cuttings [(rootstock 110 Richter (110 R)] were inoculated with four isolates of *Pm. minimum* (representing complementary mating groups) or two isolates of *Pa. chlamydospora*, and were incubated at 5, 10, 15, 20, 25 or 30°C under continuous white light. After 6 weeks, the cuttings were examined under a stereoscope to evaluate formation of mature reproductive structures of the fungi. Both species produced abundant fruiting bodies at temperatures of 15 to 25°C, but *Pm. minimum* produced more perithecia at 25°C and *Pa. chlamydospora* produced more at 20°C. At 30°C, only very few reproductive structures were observed. A field experiment was conducted in two vineyards in Spain, located in Villar del Arzobispo (Valencia) and Villena (Alicante), in which 1-year-old grapevine cuttings (rootstock 110 R), inoculated with the above-mentioned isolates, were placed into perforated aluminium trays each covered with a plastic grid, and exposed to environmental conditions from December 2019 until June 2020. Cuttings were randomly collected every 15 d, and were examined under a stereoscope to determine the presence/absence of fruiting structures. Fungus isolations were also carried out to verify survival of the inoculated fungi. No fruiting bodies were observed during the experiment, but both species were recovered from the cuttings. A GLM analysis showed survival differences between species and localities with time. Differences observed between *in vitro* and field experiments indicated that development of the pathogen reproductive structures was infrequent in vineyards.

Comparing grapevine trunk disease incidence at different sites in the Tokaj wine region of Hungary. P. BALLING[1,2], T. KOVÁCS[1,2], A. KNEIP[1,2] and P. MOLNÁR[2]. *[1]Research Institute for Viticulture and Enology, Tokaj, Könyves Kálmán u. 54., H-3915 Tarcal, Hungary. [2]University of Tokaj Hegyalja, Faculty of Lorántffy, Department of Viticulture and Enology, Eötvös u.7., H-3950 Sárospatak, Hungary. E-mail: balling.peter@tarcalkutato.hu*

Grapevine Trunk Diseases (GTDs) have severe impacts in all grape-producing countries. The presence of a GTD pathogen in vines does not usually result in the immediate appearance of disease symptoms. Moreover, there is still limited information on the importance of environmental factors on disease incidence. The present study estimated occurrence of GTD in the Tokaj wine region, to gain knowledge of the biotic and abiotic factors affecting disease incidence. Four vineyards within 15 km were studied between 2016 and 2019, with different topologies, soil types, varieties, and ages. In the same period, 50 random sites across the Tokaj region were selected, to monitor GTD symptoms in different vineyards. Topology, slope characteristics, and soil type were the most important abiotic factors affecting the incidence of GTD symptoms. Vineyard age was the biotic factor with greatest effect, with disease incidence increasing with age. Neither disease incidence nor any biotic or abiotic factor was correlated with infection incidence, but there were differences among the sites. slope characteristics also influenced symptom incidence. August was the best month to detect infected vines, as symptoms were observed more frequently. Removing dead vine parts from the sites also affected disease incidence. These results indicate that infected grapevine cuttings can act as primary infection sources of healthy vines.

Spring shoot thinning wounds are susceptible to grapevine trunk disease pathogens. M. R. SOSNOWSKI[1,2] and M. R. AYRES[1]. *[1]South Australian Research and Development Institute, Adelaide SA 5001, Australia. [2]School of Agriculture, Food and Wine, Waite Research Institute, The University of Adelaide, SA 5005, Australia. E-mail: mark.sosnowski@sa.gov.au*

The grapevine trunk diseases (GTDs) Eutypa (ED) and Botryosphaeria dieback (BD) are primarily caused by infection of winter pruning wounds by spores of species of the *Diatrypaceae* (for ED) and *Botryosphaeriaceae* (for BD). Shoot thinning is undertaken during the spring to improve airflow and reduce foliar disease, increase leaf exposure to sunlight for increased photosynthesis, maintaining crop yield and quality, and reduce the number of pruning wounds in the following winter. Spore trapping in Australian vineyards has detected GTD pathogens throughout spring and summer, in association with rainfall. BD pathogens can infect green grapevine tissues, but there is no evidence of green shoot infection by ED pathogen spores. A preliminary trial was established on Shiraz vines, grown in pots in a shade house. In November 2020, green shoots were each either cut from 1 cm above the second or third nodes leaving a smooth pruning wound, or whole green shoots were each torn off at the joint between lignified cane and the shoot base, leaving a rough socket wound. Wounds were artificially inoculated with 200 spores of *Eutypa lata* (ED) or *Diplodia seriata* (BD). Spurs were removed 9 months later and assessed for presence or absence of the pathogens. *Eutypa lata* was recovered from 62% of pruning wounds and 69% of socket wounds, and isolation frequencies for *D. seriata* were 96% from pruning wounds and 91% from socket wounds. No pathogens were isolated from uninoculated controls. These results, and reports of detection of ED and BD pathogen spores throughout spring and summer, show that it is now important to determine vineyard infection risks under natural conditions following shoot thinning activities.

Investigating how *Lasiodiplodia brasiliensis* colonizes grapevine tissues. E. A. RANGEL-MONTOYA and R. HERNÁNDEZ-MARTÍNEZ. *Centro de Investigación Científica y de Educación Superior de Ensenada, Baja California (CICESE), Departamento de Microbiología, Carretera Tijuana-Ensenada 3918, Zona Playitas, 22860, Ensenada, B.C. Mexico. E-mail: ruhernan@cicese.mx*

Lasiodiplodia brasiliensis is a virulent pathogen among *Botryosphaeriaceae*, and this fungus was recently reported in Sonora and Baja California, Mexico. Little is known about how *Botryosphaeriaceae* interact with grapevines during infection processes. Grapevine colonization by *L. brasiliensis* was assessed using histological techniques. One-year-old rooted cuttings of 'Cabernet Sauvignon' were inoculated by mechanical wounding, and then maintained in greenhouse conditions for 2 months. Transverse and longitudinal sections (70 µm thick) were then obtained and stained with 0.1% Toluidine B for phenolic compounds; 5% iodine and 10% potassium iodide for starch; 0.001% Sudan black IV for suberin deposits; 0.1% phloroglucinol-HCl and Mäule stain for lignin, or 0.02% Calcofluor M2R White + 0.5% + Congo Red for cellulose and hemicellulose. Hyphae were observed using the Fontana-Masson stain. Observations were carried our using a Nikon Eclipse E200 microscope with a camera AxioCam HRc camera, and with epifluorescence microscopy using an Axio- Vert200 microscope with a HBO100 100W Mercury Lamp with ebq100 power. Cellulose and suberin were observed using a DAPI filter (excitation at 330–380 nm, emission at 420 nm), and a TEXAS RED filter (excitation at 542–595 nm, emission at 644 nm). Infected plants lacked starch in ray parenchyma cells; and cellulose, hemicellu-

lose, and lignin in the lesions. Phenolic compounds and suberin were observed in the cork and vascular cambium tissues, vascular bundles, and pith. The fungus colonized vascular cambium, vascular bundles, occlusions, and pith. Melanized hyphae were observed mainly in the pith. These observation indicate that *L. brasiliensis* overcame the defence mechanisms of host plants, and modified cell walls, mainly degrading hemicellulose and using starch as a carbon source. With time, the pathogen degraded lignin and suberin, colonizing the rays and inducing the formation of the typical Botryosphaeria canker.

Endophytic mycobiome and anthocyanins, two key components in grapevine leaves affected by 'tiger stripes'. G. DEL FRARI[1], C. INGRÀ[2], A. GOBBI[3], M. RØNNE AGGERBECK[4], T. NASCIMENTO[1], A. CABRAL[1], H. OLIVEIRA[1], A. FERRANDINO[2], L. HESTBJERG HANSEN[3] and R. BOAVIDA FERREIRA[1]. *[1]LEAF—Linking Landscape, Environment, Agriculture and Food— Research Center, Associated Laboratory TERRA, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisbon, Portugal. [2]Department of Agricultural, Forestry, Food Sciences (DISAFA), University of Turin, Largo P. Braccini, 2, Grugliasco, Torino 10095, Italy. [3]Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark. [4]Department of Environmental Science, Aarhus University, 4000 Roskilde, Denmark. E-mail: gdelfrari@isa.ulisboa.pt*

'Tiger stripe' patterns manifest in grapevine leaves affected by grapevine leaf stripe disease (GLSD) and Esca proper. In red grape varieties, affected leaves display interveinal necrotic lesions, followed by development of red lamina portions, chlorotic tissues, and green tissues, with green tissues closely associated to the main leaf veins. Two other colouring patterns are occasionally found in symptomatic leaves. One follows the sequence: necrotic-purple-green tissue, which has been correlated to the black dead arm syndrome (BDA). The other pattern follows the sequence necrotic-green tissue, and is found in pre-apoplectic shoots. Despite these differences, it is still unclear the identity of the triggering factor(s) that lead to different coloring patterns, the anthocyanins profile of symptomatic leaves, and the involvement of the endophytic mycobiome. We investigated the endophytic mycobiome composition and anthocyanins profiles of grapevine leaves with GLSD and BDA-associated symptoms. Plants of 'Cabernet Sauvignon' and 'Touriga Nacional' were used, sampling symptomatic and asymptomatic leaves in June 2020 and July 2021, in a vineyard in Lisbon. Mycobiome profiles were characterised using DNA metabarcoding (Illumina® NGS), targeting the ITS1 region, employing primer set ITS1F2-ITS2, and anthocyanins profiles were analyzed using high-performance liquid chromatography. The leaf mycobiomes analysis revealed 125 taxa with relative abundances greater than 0.1%. The most common taxa were *Plasmopara viticola* (Oomycete); *Cladosporium* spp., *Aureobasidium* spp., and *Stemphylium* spp. (Ascomycetes); and *Sporobolomyces* spp. and *Filobasidium* spp. (Basidiomycetes). Differences in alpha and beta diversity and taxa over- and under-representation were detected when examining sampling year, cultivar, and symptom type. Similarly, the anthocyanins profiles, dominated by peonidin 3-O-glucoside and cyanidin 3-O-glucoside, differed quantitatively and qualitatively for cultivar and symptom type. These data indicate strong links among symptom colouring patterns, plant responses and mycobiome profiles.

High diversity of fungal grapevine trunk pathogens isolated from young canes, and the report of *Neofabraea kienholzii* in 'Albariño' grapevines in Spain. V. REDONDO-FERNÁNDEZ[1], A. ALONSO-NÚÑEZ[1,2], N. CAMBRA-GONZÁLEZ[1], S. RODRÍGUEZ-GARCÍA[1], L. AREAL-HERMIDA[2] and C. SIEIRO[2]. *[1]ProPlantae Sanidad Vegetal SL, Rúa das Pontes Nº6 36350 Nigrán, Spain. [2]Biomedical Research Center (CINBIO), Department of Functional Biology and Health Sciences. Microbiology Unit. Universidade de Vigo, 36310 Vigo, Spain. E-mail: info@proplantae.es*

Albariño is a grapevine cultivar grown in NW Spain and northern Portugal, which produces excellent white wines with increasing export amounts. Reducing productivity has been observed in a mature Albariño vineyard near the Miño river between Galicia and Portugal. In 2021, 90 grapevine plants from three different plots were labelled and monitored. After pruning in January 2022, 1-year canes were analyzed for grapevine trunk disease (GTDs) symptoms. A total of 450 pure fungus cultures of approx. 70 morphotypes were obtained. One representative isolate for each morphotype was selected. Genomic DNA was extracted, and the Internal Transcribed Spacer (ITS) region was amplified and sequenced by Sanger dideoxy sequencing technology. Blast searches classified the isolates potentially involved in GTDs complex into at least 18 species in nine genera: *Botryosphaeria*, *Cadophora*, *Diaporthe*, *Diplodia*, *Neofusicoccum, Neopestalotiopsis, Pestalotiopsis, Phaeoacremonium* and *Phaeomoniel-*

la. An isolate of *Neofabraea* was also detected, which could also be involved in GTDs. Research is in progress to confirm the pathogen identifications. Approx. 20 morphotypes with potential activity against GTDs were also found including: *Alternaria*, *Arthrinium*, *Aureobasidium*, *Clonostachys*, *Cladosporium*, *Epicoccum* and *Trichoderma*. Next-generation sequencing provides information about microbial diversity and dynamics, while traditional culturing methods allow evaluation of potential antagonism by endophytes. This study also identified a large cultivable mycobiota associated with young grapevine shoots, that was obtained through non-destructive trunk sampling.

The BIOBESTicide project: action of *Pythium oligandrum* on grapevine trunk diseases, and its impact on microbial communities. S. LOPEZ, A. CHATAIGNER and M. C. DUFOUR. *INRAE, Bordeaux Sciences Agro, ISVV, SAVE, 33140 Villenave d'Ornon, France. E-mail: severine.lopez@inrae.fr*

Grapevine trunk diseases (GTDs) have become important in viticulture (15% yield losses). Since the 2001 ban on the use of sodium arsenate, development of alternative control strategies, including biocontrol, has become increasingly important. *Pythium oligandrum* is a promising biocontrol agent, which can improve plant health by increasing natural host defences, reduce GTDs by at least 40%. The BIOBESTicide project (BIO-Based pESTicides production for sustainable agriculture management plan) aims to commercialise production of a biopesticide solution for GTDs management. Efficiency of a *P. oligandrum*-based formulated product will be evaluated in nurseries and vineyards, and the environmental impacts of the product will be assessed to ensure its safety. An objective supported by INRAE is to increase understanding of the role of plant microbiota in plant health, and the effectiveness of biocontrol treatments. This objective is in two parts: 1) assessment of the impacts of the biopesticide on grapevine microbial communities using a microbial community diversity approach; and 2) understanding the factors contributing to success or failure of the biopesticide. An experiment on 240 grafted vines (Merlot on rootstock SO4) in semi-controlled conditions was carried out in a greenhouse. Vines were treated with a *P. oligandrum* formulation or left untreated, and were then inoculated with either *Neofusicoccum parvum* or *Phaeomoniella chlamydospora*. At different times during the following 3 months, plants were harvested and leaves, wood and rhizospheres were sampled. Potential changes in the microbial communities associated with GTD pathogen or *P. oligandrum* inoculation were assessed using Illumina high-throughput sequencing. The results will extend the approval dossier for submission in all European countries to ensure environmental safety of *P. oligandrum* as a biocontrol product.

Will it be possible to predict Esca symptom manifestation based on the grapevine wood microbiome? B. GARCÍA-GARCÍA[1], M. M. ALGUACIL[2], A. ACEDO[3] and L. MARTÍN[1]. *[1]Plant Protection Department, Extremadura Scientific and Technological Research Center (CICYTEX), Badajoz, Spain. [2]Center for Edafology and Applied Biology of Segura (CEBAS-CSIC) Murcia, Spain. [3]Biome Makers Inc., West Sacramento, California, USA. E-mail: laura.martin@juntaex.es*

Esca is an important grapevine disease, and several fungi have been described as causal agents of the syndrome because they have been isolated from degraded wood of leaf-esca symptomatic vines. The present study compared culture dependent and -independent methods for identifying and quantifying fungal communities. Twenty four wood samples from six vines (three leaf-esca symptomatic and three asymptomatic) were analysed in three ways: *i*) culture-dependent microbiological analysis; *ii*) non-culture dependent microbiome analysis using Illumina Technology; and *iii*) quantification of *Phaeoacremonium minimum* (Pm) and *Phaeomoniella chlamydospora* (Pch) by TaqMan q-PCR. Four different within vine sample points were compared to determine the most informative point for pathogen detection. These were: above the graft (A), at the strain cross (B), or arms (C and D). Fungi detected included: Pm, Pch, *Diplodia seriata*, *Diaporthe ampelina* and Basidiomycete species associated with Esca, as well as other genera including *Penicillium*, *Acremonium*, *Alternaria* and *Epicoccum*. The number of OTUs found in culture (11) represented only 0.73% of the 1512 OTUs found using Illumina. The methodology affected α-diversity indices, but sampling point did not affect the wood microbiomes. Pch was detected in 22 samples using Illumina, in nine using TaqMan q-PCR, and in four using culture dependent methods. Pm was detected in, respectively, six, five, and ten samples, indicating greater detection of this pathogen using culture dependent analysis. A non-metric multidimensional scaling (NMDS) analysis of β-diversity showed discrimination of fungal communities from Esca affected vines (with manifestation of "tiger stripe"/apoplexy in canopies). Some fungi have been selected as indicators of apoplexy. When modelling relationships between network properties and disease

phenotypes, samples without Esca symptoms tended to have lower proportions of co-exclusions.

Effects of temperature on *in vitro* biocontrol of *Diplodia seriata* by psychrotolerant *Pseudomonas* strains. A. LARACH[1,2], P. VEGA-CELEDÓN[2], P. SANHUEZA[1], N. RIQUELME[1], M. SEEGER[2] and X. BESOAIN[1]. *[1]Escuela de Agronomía, Facultad de Ciencias Agronómicas y de los Alimentos, Pontificia Universidad Católica de Valparaíso, Casilla 4-D, Quillota 2260000, Chile. [2]Laboratorio de Microbiología Molecular y Biotecnología Ambiental, Chemistry Department & Centro de Biotecnología Daniel Alkalay Lowitt, Universidad Técnica Federico Santa María, Avenida España 1680, Valparaíso 2340000, Chile. E-mail: ximena.besoain@pucv.cl*

Timely preventive control for Botryosphaeria dieback is important for disease management, and biocontrol agents have this protective effect. Conversely, from an epidemiological viewpoint, it is important to consider that the infections can occur at different temperatures. Knowledge of biocontrol capabilities of microorganisms at different temperatures will allow improvement of selection of biocontrol agents. The *in vitro* capabilities of four psychrotolerant *Pseudomonas* strains (CpR2b, GcR15a, TmR1b, TmR8) to reduce mycelium development of three *Diplodia seriata* isolates (2120, 2142 and 2183) were assessed using the agar diffusion method at low (8°C), medium (20°C), or high temperatures (35°C). At 7, 14, and 21 d after microbe confrontations, *D. seriata* colony radii were measured. The three *D. seriata* strains grew at 8°C, 20°C, and 35°C. two strains of *Pseudomonas* showed consistent inhibition of the mycelium growth of all three *D. seriata* isolates at 8°C and 20°C. However, at 35°C variation in inhibition was observed amongst the isolates.

Phylogenetic host range in the Botryosphaeriaceae. I. SILVA-VALDERRAMA[1], J. R. ÚRBEZ-TORRES[2] and T. J. DAVIES[1]. *[1]University of British Columbia, Botany Department, Vancouver, BC, Canada. [2]Summerland Research and Development Centre, Agriculture and Agri-Food Canada, Summerland, BC, Canada. E-mail: ibsilva26@gmail.com*

Botryosphaeriaceae is a diverse family composed of endophytes, pathogens, and saprobes, and is ubiquitous in terrestrial plant biomes. Anthropogenic movement of some fungi, for example, on infected host material, is a major concern, as the family includes species known to be pathogens in endophyte communities. In grapevines (*Vitis vinifera* L.), Botryosphaeria dieback is an important grapevine trunk disease, causing severe economic losses. Prevention of new infections remains the most effective disease management tool, but it is difficult to identify highly virulent pathogen isolates before they emerge. Phylogenetics are useful tools for identifying pathogens likely to shift to new hosts, and the potential for disease emergence following host jumps. However, the taxonomy of *Botryosphaeriaceae* is in flux, and there is little phylogenetic information on the species infecting grapevines. The phylogeny of *Botryosphaeriaceae* species infecting *V. vinifera* was constructed, using molecular sequence data, and the link between host breadth and pathogen phylogenetic relatedness was explored. The distribution of *Botryosphaeriaceae* hosts across the megaphylogeny of vascular plants was outlined. Variation in host breadth was large among *Botryosphaeriaceae* pathogens, revealing the complex associations linking host and fungal phylogenies. This research provides a first step towards predicting emergence of known pathogens on new hosts. This approach will be useful for coordinated global monitoring of high-risk species within *Botryosphaeriaceae*.

Analysis of below-ground grapevine microbiomes identifies *Fusarium* spp. aggravating severity of grapevine trunk diseases. Y. H. LI[1], X.H. LI[1], W. ZHANG[1], J. ZHANG[1,2], H. WANG[1], J. B. PENG[1], X. C. WANG[1] and J. Y. YAN[1]. *[1]Beijing Key Laboratory of Environment Friendly Management on Fruit Diseases and Pests in North China, Institute of Plant Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China. [2]College of Plant Protection, Hebei Agricultural University, Baoding 071000, China. E-mail: jiyeyan@vip.163.com*

Grapevine trunk diseases (GTDs) are a disease complex which is a major threat for viticulture. The microbiota colonizing the belowground parts of plants can form complex associations and promote plant productivity and health in natural environments. This present study investigated below-ground fungal communities in symptomatic or asymptomatic grapevines. Spatial dynamics of the fungal communities associated with three soil-plant compartments (bulk soils, rhizospheres, roots) were characterized using ITS high-throughput amplicon sequencing across 2 years. The diversity and composition of fungal communities were largely affected by soil-plant compartments ($P < 0.001$; 12.04% of variation explained) and sampling year ($P < 0.001$; 8.83 %), where-

as GTD symptomatology exhibited weaker, but statistically significant, association with these characteristics ($P < 0.001$; 1.29 %). Effects of GTDs symptomatology were particularly prominent in root and rhizosphere community comparisons. Some confirmed GTD-associated pathogens were detected in the communities, but their relative abundances were not related to symptomatology. Further analyses revealed that *Fusarium* spp. were enriched in symptomatic roots and rhizospheres compared to asymptomatic counterparts, suggesting that their abundances were positively correlated with symptomatic vines. Inoculation tests showed that *Fusarium* isolates, similar to *Dactylonectria macrodidyma* which is a pathogen associated with grapevine black foot. caused dark brown necrotic spots on grapevine stems and rots that blackened lateral roots. Disease indices were greater in the co-inoculation treatment than due to single inoculations with either a *Fusarium* isolate or *Dactylonectria macrodidyma*, suggesting that *Fusarium* spp. can exacerbate disease severity when inoculated with other known GTD-associated pathogens. These results demonstrate the effects of fungal root and rhizosphere microbiota on GTDs severity, and provide new insights into GTDs and potential control strategies.

Enhancing the regrowth of vines following remedial surgery. M. R. SOSNOWSKI[1,2] and M. R. AYRES[1]. *[1]South Australian Research and Development Institute, Adelaide, SA 5001, Australia. [2]School of Agriculture, Food and Wine, Waite Research Institute, The University of Adelaide, SA 5005, Australia. E-mail: mark.sosnowski@sa.gov.au*

Grapevine trunk diseases (GTDs) reduce yields, and cause vine decline and death. Remedial surgery has long been used to rejuvenate grapevines, and has more recently been demonstrated to control GTDs in Australia. Current recommendations are to cut out all visibly affected wood, and train watershoots to replace affected vines. However, as little as 42–55% of 30-year-old trunks have been reported to produce shoots following surgery of Shiraz and Cabernet Sauvignon vines. A trial was established in four rows of a 22-year-old Cabernet Sauvignon vineyard in the Clare Valley, South Australia, with 100% incidence of GTD, to evaluate methods reported anecdotally to induce watershoots on trunks following remedial surgery. Treatments included hitting with a hammer, making an X-shaped cut with a tomahawk, rubbing bark with a wire brush, application of cyanamide (Dormex) plant growth regulator, or grafting with a chip bud, and surgery was carried out in winter or spring for comparison. Over each of the following 3 years, a further four vineyard rows were subjected to remedial surgery, and stumps without watershoots were chip bud grafted. In the final year, watershoots were retained from the previous year. Natural watershoot production was 83–85%, with no difference between the seasons the vines were cut, and there was also no influence of most of the treatments applied in the first year. The exception was for grafting, which increased shoot production by 11%. In subsequent years, natural watershoot production varied from 70–78%, and grafting trunks without watershoots increased shoot growth by 6–19%. In the final year, retention of watershoots from the previous season resulted in 87–88% shoot growth, similar to that of grafting in previous years. These results indicate that regrowth of vines subjected to remedial surgery for control of trunk diseases can be enhanced by grafting or retaining shoots from the previous year.

Management of grapevine trunk diseases using remedial surgery. E. VAN ZIJLL DE JONG[1], H. TERNENT[2], S. ST GEORGE[2], R. KALLAS[2] and M. SOSNOWSKI[3,4]. *[1]Linnaeus Limited, PO Box 1199, Gisborne 4040, New Zealand. [2]Villa Maria Estate Ltd, 118 Montgomerie Road, PO Box 43046, Mangere, Auckland, New Zealand. [3]South Australian Research and Development Institute, Adelaide, SA 5001, Australia. [4]School of Agriculture, Food and Wine, Waite Research Institute, The University of Adelaide, SA 5005, Australia. E-mail: eline@linnaeus.co.nz*

Remedial surgery is increasingly used in New Zealand to improve grape yields and extend lifespans of grapevines infected with trunk diseases. Infected vines are each "renewed" by removing the diseased trunk above the graft union, and growing a new shoot to replace the old trunk. Remedial surgery was carried out in winter and spring over 3 years in mature (>18 years) commercial vineyard blocks of 'Sauvignon blanc', 'Cabernet Sauvignon' or 'Merlot' vines. This was to study disease progression and efficacy of remedial surgery, and determine the optimal time to carry out this practice. Dieback was widespread in the canopies in 'Sauvignon blanc' and 'Cabernet Sauvignon' (>90%), and only in 'Merlot' incidence of dieback increased from 64 to 91% over the three growing seasons. During this time, severity of dieback increased in 'Cabernet Sauvignon' but not in 'Sauvignon blanc' or 'Merlot'. Vine recoveries after remedial surgery were high in Merlot. In 'Sauvignon blanc' and 'Cabernet Sauvignon', some of the vines with more severe disease symptoms did not recover from remedial

surgery. For all three cultivars, there were no differences in recoveries between vines cut in winter or spring. The vines were out of production for the first growing season following remedial surgery, but by the third year there were no significant differences in yields between these vines and the untreated control vines in 'Cabernet Sauvignon' and 'Merlot'. In 'Sauvignon blanc', a change in pruning strategies affected the yield in the reworked vines. There have been no obvious signs of trunk disease in the reworked vines since the trunk surgery was carried out.

GTD prevention: A practical application of *Trichoderma* formulations under field conditions. J. J. GUERANOGALES, L. MÉNDEZ-GRANO DE ORO, M. J. DORADO-RICO and L. MARTÍN. *Plant Protection Department, Extremadura Scientific and Technological Research Centre (CICYTEX), Spain. E-mail: laura.martin@juntaex.es*

Grapevine Trunk Diseases (GTDs) are a major concern for grapevine growers all over the world, but there are no curative fungicides for these diseases. Protection of pruning wounds has been shown to be effective for avoiding wood infection by pathogens. Biological control agents (BCA) to protect pruning wounds have recently been authorized by the Spanish Ministry of Agriculture for management of GTDs. Three BCA products based on different strains of *Trichoderma* were evaluated in two commercial vineyards. In the first trial, three products were compared under uniform field conditions, in a 'Tempranillo' vineyard (head training system), 3% of Esca incidence. . In the second trial, one product were evaluated in three different years in a ' Macabeo' vineyard (transformed from double cordon to double Guyot system), with no foliar GTD symptoms. Evaluations of the disease control treatments were made by microbiological analyses of wood samples (n = 20) to isolate GTD fungal pathogens and *Trichoderma* spp. Assessment were carried out at 30 and 90 d after treatment applications. *Trichoderma* was well-established (in 50–100% of pruning wounds, and there was little difference between the three formulations. Mean Percentage of Infection (MPI: number of GTD infected samples/number of total samples × 100) showed low recovery of *Phaeomoniella chlamydospora* and *Botryosphaeriaceae* species. Efficacy of the treatments was recorded by the mean percent disease control (MPDC), calculated as 100 × (1 - (MPI treatment/MPI non-treated control). These results indicate that *Trichoderma* treatments could be effective in vineyards with head training systems, and that their efficacy is correlated with BCA implementation and also the disease pressure in vineyards.

Biological control of *Diplodia seriata* inoculum in grapevine pruning debris in Chilean orchards. E. DONOSO, L. ROMERO, W. HETTICH, D. BASCUÑAN, C. GARCIA and J. FIGARI. *Bio Insumos Nativa SPA, Pc Antilhue Lote 4b2, Maule, Chile. E-mail: edonoso@bionativa.cl*

Increased understanding of the use of biological control agents, with mechanisms other than those of chemical fungicides, has opened new opportunities for pathogen control, enabling new biocide strategies that consider periods of host tissue susceptibility and the residual efficacy times for these products. Incorporation in control strategies of biological agents active against resistant and reproductive pathogen structures (e.g. sclerotia or pycnidia) will allow to control the pathogens in new places and times, providing different tools for disease management. A field trial was carried out where a commercial formulation containing *Trichoderma* spp. and *Bionectria ochreoleca*, Mamull® (Bio Insumos Nativa SPA) was applied to Cabernet Sauvignon vine prunings, which had pycnidia of *Diplodia seriata*. Selected cane samples (n = 30), were observed under a magnifying glass, determining numbers of pycnidia cm^{-2}, and marking and coding five sectors each of 1 × 3 cm on each sample. The samples were then placed in the field, where they were sprayed with Mamull®, at 100 g hL^{-1}, using a herbicide bar and 300 L ha^{-1}. At 30 and 180 d, levels of pycnidium parasitism were assessed, and viability of released conidia was determined after addition of sterile water. Both treatments reduced viable pycnidia ($P < 0.05$; LSD tests), by 25.3% at 30 d and 72.3% at 180 days. Levels of parasitism from both treatments were 34.5% at 30 d and 59% at 180 d, but only 12.3% from the controls ($P < 0.01$; LSD tests). This trial has demonstrated a promising additional application of biocontrol agents to for control of grapevine wood pathogens.

Effects of combined treatments with LC2017 and *Trichoderma atroviride* strain I-1237 on disease development and host defence responses in grapevines infected by *Lasiodiplodia theobromae*. P. REIS[1†], V. MONDELLO[2†], I. DINIZ[1,3], A. ALVES[4], C. REGO[1] and F. FONTAINE[2]. [1]*LEAF - Linking Landscape, Environment, Agriculture and Food, Associated Laboratory TERRA, Instituto Superior de Agronomia, Universidade*

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Grapevine trunk diseases (GTDs) cause major problems for viticulture, and Botryosphaeria dieback is an important GTD. Increasing restrictions on effective chemical fungicides for mitigation of GTDs have led to expansion of research on biocontrol agents (BCAs), and new and improved fungicides with more efficient and innovative methods for pathogen control. Incorporation of these new strategies as part of integrated disease management has only recently generated interest. The present study aimed to; (*i*) evaluate effect of the combination of Esquive® (a *Trichoderma*-based product) and LC2017 (a low copper product), for control of *Lasiodiplodia theobromae*, on grapevines 'Cabernet Sauvignon' and 'Touriga Nacional' in greenhouse experiments; and (*ii*) investigate their elicitor effects on plant defence responses, through analyses of expression of a set of genes from inoculated grapevines. The pathogen was always re-isolated from the infected tissues, and able to cause wood discolouration. Touriga Nacional developed longer lesions than Cabernet Sauvignon, and applications of both products did not reduce lesion lengths when compared to LC2017 applied alone. The elicitor effect of LC2017 on grapevine defence was confirmed by gene expression analyses, and no significant differences were found between plants treated with LC2017 or with both products. A specific response related to cultivar was verified, but this apparently unique interaction between product, cultivar, and pathogen remains to be further investigated.

Will forestry waste save grapevines and help control GTDs? L. MERLEN[1,2], L. GALIMAND[1], C. TARNUS[1], C. GERARDIN[3], P. GERARDIN[3] and M. GELLON[1]. *[1]Laboratoire Vigne Biotechnologie et Environnement EA 3391, Université de Haute-Alsace, 33 rue de Herrlisheim, F-68000 Colmar, France. [2]Laboratoire de Photochimie et d'Ingénierie Macromoléculaires UR 4567, Université de Haute-Alsace, 3 bis rue Alfred Werner, F-68093 Mulhouse cedex, France. [3]Laboratoire d'Etudes et de Recherche sur le MAtériau Bois UR 4370, Université de Lorraine, Inrae, Boulevard des Aiguillettes, BP 70239, F-54506 Vandoeuvre-les-Nancy cedex. E-mail: melanie.gellon@uha.fr*

Grapevine trunk diseases are serious challenge for viticulture, causing significant economic losses on national and international scales. The three main diseases – Esca, Botryosphaeria and Eutypa diebacks – are without reliable and effective control solutions. Sodium arsenite, the only existing effective pesticide, has been banned since 2001 in France, and since 2003 in Europe. Numerous alternatives are being studied, including substances from natural origins. The wood of tree knots is an important source of extractives that meet modern pesticide acceptance criteria, and some of these compounds are being studied in human health and cancer research. Wood knots are co-products of the timber and paper industries. They can be removed from processes for which they are prejudicial, and can be valuable bio-based co-products from processing. The present study evaluated the antifungal potential of an extract from knots of Douglas fir (*Pseudotsuga menziesii*), against the GTD pathogens, *Neofusicoccum parvum* and *Fomitiporia mediterranea*. *In vitro* tests confirmed fungus growth inhibition of EC_{50}s of 0.5 to 1.0 mg mL^{-1} for both pathogens. Douglas fir knot extract therefore has potential for treatment of GTDs. Knot extracts were not toxic to callus of *V. vinifera* 'Gewurztraminer'. Protective effects of these extracts on detached canes were observed, when applied after inoculation by the pathogens. Additional greenhouse *in planta* trials, and then in vineyards, will allow evaluation of the potential of these natural substances for preventive control of GTDs.

Native isolates of *Trichoderma* spp. can protect pruning wounds against *Lasiodiplodia theobrome* in Argentinian vineyards, reducing the incidence of the disease Hoja de Malvón. V. LONGONE[1], A. PIERONI[2] and G. ESCORIAZA[1]. *[1]Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agropecuaria Mendoza, San Martin 3853 (5507), Luján de Cuyo, Mendoza, Argentina. [2]Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo, Almirante Brown 500, Luján de Cuyo, Mendoza, Argentina. E-mail: longone.maria@inta.gob.ar*

Preventing opportunistic pathogen entry through grapevine pruning woods is an important strategy for managing trunk diseases. Applications of biocontrol organisms on pruning wounds could reduce infections from

pathogens that cause "Hoja de Malvón" (HdM) a common disease in Argentina. This name recognises the symptom where host leaves resemble those of geranium. The present study aimed to assess protective effects of native *Trichoderma* isolates with *in vitro* inhibitory potential against *Lasiodiplodia theobromae* (Lt) on pruning wounds. Lt is commonly associated with HdM at different pruning times. A vineyard trial (completely randomized design) was carried out with treatments of three pruning times (early, in June; medium, July, or late, August) × five treatments (four native *Trichoderma* spp., strain ACB 3, ACB 4, ACB 20, ACB 25, and a control). After pruning, the wounds were sprayed with each biocontrol agent (BA) and the control and 24 h later, each with 50 µL (10^5 conidia mL^{-1}) of Lt inoculum. Sixty canes per treatment were collected 28 d later, and prepared for re-isolation. Effectiveness was assessed as mean percent disease control (MPDC). Pruning time × treatment interactions ($P < 0.03$) for MPDC were detected. All the BAs were re-isolated from pruning wounds at 28 d after treatment application. The most effective treatments were: for early pruning ACB 3 (81%), ACB 25 (69%), and for medium pruning ACB 3 (62%). In early and medium pruning times June and July), also ACB 4 (59%), ACB 20 (48%), ACB 25 (43%) reduced significantly pathogen infections of the pruning wounds. MPDC from all the BAs at late pruning were below 40%. Native isolates of *Trichoderma* spp. provided control against Lt mainly at early and medium pruning times, in the regional climatic conditions.

Impacts of different grapevine bench grafting methods on the xylem anatomy, hydraulic traits and wood necroses associated with young decline of grafted vines. E. BATTISTON[1], S. FALSINI[2], A. GIOVANNELLI[3], S. SCHIFF[2], C. TANI[2], R. PANAIIA[2], S. DI MARCO[4] and L. MUGNAI[1]. *[1]Department of Agricultural, Food, Environmental and Forestry Science and Technology, Plant pathology and Entomology section, University of Florence, P.le delle Cascine 28, 50144 Firenze, Italy. [2]Department of Biology, Biomorphology Laboratory, University of Florence, Via Micheli 3, 50121 Firenze, Italy. [3]Research Institute on Terrestrial Ecosystems, National Research Council, Via Madonna del Piano 10, 50019 Sesto Fiorentino, Italy. [4]Institute of BioEconomy, National Research Council, Via P. Gobetti 101, 40129 Bologna, Italy. E-mail: enrico.battiston@unifi.it*

Grapevine grafting is essential in viticulture, and bench grafting techniques have been developed to mechanize nursery processes and increase yields of viable cuttings. Bench grafting can affect the quality of propagation material and may have a role in young vine decline associated with grapevine trunk diseases. Some authors report that grafting can also influence leaf symptom development in Esca complex of diseases. This study assessed whether three bench grafting methods: omega graft, mechanical technique; whip and tongue graft, manual technique; or full cleft graft, semi-mechanical technique, affected these phenomena. The different methods were compared for their effects on anatomical development of grafting points and xylem function, considering two factors: cultivar (Cabernet Sauvignon, Glera or Teroldego) and scion/rootstock diameter (thin or large). Light microscopy observations of anatomical evolution were correlated with the grafting methods and the investigated varieties. Differences between cultivars and/or grafting methods were detected for areas of necroses on the grafted tissues. Cultivar differences in xylem parameters were also detected, while grafting type had no significant effects. However, graft type affected the intrinsic growth rate. These results confirm the potential for lesions and dysfunctionalities related to grafting method, which could induce decline of grafted vines in vineyards, due to the necrotic area detected on the grafted tissues.

Bio-products partially protect grapevine pruning wounds against infection by the trunk pathogen *Lasiodiplodia theobromae*. G. ESCORIAZA[1], V. LONGONE[1], I. FUNES PINTER[1], M. ULIARTE[1] and A. NOLI[2]. *[1]Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agropecuaria Mendoza, San Martin 3853 (5507), Luján de Cuyo, Mendoza, Argentina. [2]Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo, Almirante Brown 500, Luján de Cuyo, Mendoza, Argentina. E-mail: escoriaza.maria@inta.gob.ar*

One of the challenges related with grapevine trunk diseases ("Hoja de Malvón"; HdM) in Argentina is implementation of efficient, safe and organic control strategies. Bio-products derived from organic wastes that contain beneficial microorganisms could be alternative disease management options. Aerated (AT), non-aerated (NAT) compost teas and bio-slurry (B) were evaluated to protect pruning wounds against *Lasiodiplodia theobromae*, an important fungus associated with HdM. Sodium bicarbonate (SB), thiophanate methyl (TM) and an untreated control were also included in the field trials. The compost used to obtain AT and NAT was from exhausted grape marc, goat manure, leaves from garden

raking and alfalfa. B was brewed anaerobically from fresh material. Twelve treatment combinations [two pruning times (July or August), five products, control] were applied on 'Malbec' grapevines, in a completely randomized experimental design. After pruning, the wounds were sprayed, and 24 h later inoculated with 50 µL (10^5 conidia mL^{-1}) of conidium suspension of *L. theobromae*. Sixty canes per treatment were removed after 28 d and prepared for pathogen re-isolation. Treatment effectiveness was assessed as mean percent disease control (MPDC). TM gave more than 75% control in July and August. Although the effectiveness of AT (33%), NAT (23%) and B (24%) were less compared to TM, these treatments were more effective than SB (12%). All products showed a greater effectiveness in July than in August, where MPDC did not exceed 20%. These results indicate that beneficial microorganisms need adequate conditions, long periods, and repeated applications to become established on pruning wounds, and before challenge from the pathogen. This study is the first to assess agro-industrial residues as sustainable control alternatives against *L. theobroamae*.

Effects of hot-water treatment on grapevine viability and fungal trunk disease pathogens diversity, assessed using RNA high-throughput amplicon sequencing. M. ANDRÉS-SODUPE[1], A. EICHMEIER[2] and D. GRAMAJE[3]. *[1]Viveros Villanueva, 31251 Larraga, Spain. [2]Mendel University in Brno, Faculty of Horticulture, Mendeleum - Institute of Genetics, Valticka 334, 69144, Lednice, Czech Republic. [3]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. E-mail: laboratorio@viverosvillanueva.es*

Effects of hot-water treatment (HWT) on grapevine fungal communities have been mostly studied using culture dependent methods. Changes in potentially active fungal communities of grapevine wood were assessed after application of two HWT protocols (50°C for 45 min, or 53°C for 30 min), using RNA high throughput amplicon sequencing (HTAS) for several cultivar/rootstock combinations during two years. Fungal diversity was only reduced by both HWT protocols during the second year of study. The effects of HWT on grapevine trunk disease (GTD) pathogen abundance varied, according to the fungal genera and the HWT protocol. In some cases, HWT increased the abundance of GTD fungi (i.e. *Diaporthe*), but in other cases, GTD abundances decreased after HWT (i.e. *Dothiorella*). To evaluate the viability of planting material, treated plants were planted in a vineyard immediately after HWT, or 1, 2 or 3 months after treatments. Grapevine viability was high 1 month after treatment, but decreased as time from HWT to vineyard establishment increased. Plants established 3 months after treatments were less than 90% viable over 30% of combinations in the first year, and over more than 50% of combinations were affected in the second year.

Sensitivity of fungal grapevine trunk pathogens to treatments with electrolyzed water. A. FRINKLER[1], M. BERBEGAL[1], F. BEN ATIA[1], J. V. ROS-LIS[2], G. GAUME[3] and J. ARMENGOL[1]. *[1]Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Camino de Vera S/N, 46022-Valencia, Spain. [2]REDOLí Research Group. Universitat de València. Doctor Moliner, 50. 46100 Burjassot, Spain. [3]Aquactiva Solutions SL, Av. Blasco Ibañez 73, 46136-Valencia, Spain. E-mail: jarmengo@eaf.upv.es*

Effects were evaluated of treatments with different electrolyzed water (EW) based products on *in vitro* mycelium growth of grapevine trunk pathogens. The pathogens studied were: *Botryosphaeria dothidea* (*Bd*), *Dactylonectria torresensis* (*Dt*), *Eutypa lata* (*El*), *Ilyonectria liriodendri* (*Il*), *Lasiodiplodia theobromae* (*lt*), *Neofusicoccum parvum* (*Np*), *Phaeoacremonium minimum* (*Pmin*) and *Phaeomoniella chlamydospora* (*Pch*). Agar discs with mycelium of each fungus were treated by immersion in each of the products. The treatments lasted 30 s, or 1, 5, 15 or 30 min. Mycelium survival (%) of each fungus was determined from the different product-time combinations. Effect on conidium germination were also evaluated for *Cadophora luteo-olivacea* (*Clo*), *Dt*, *Il*, *Pm* and *Pch*. Conidium suspensions (2×10^7 mL^{-1}) of each fungus were mixed with 950 µL of different EW products for 0, 15, 30, 60 or 300 s. Exposure was ceased by adding 9 mL of neutralizing buffer (pH 7.2). Drops (20 µL) of spore suspensions were plated on water agar and incubated at 25°C for 24 h. After incubation, the drops were observed for quantification of conidium germination. All experiments were repeated once. Effects of the different products on mycelium growth and conidium germination were variable, depending on the products, treatment times and fungus species. In general, the long treatment times were more effective for reducing mycelium growth and conidium germination. *Dt*, *El* and *Np* mycelium survival was reduced after 5 min treatments with one of the products. All product-time combinations resulted in greater than 93% germination inhibition relative to the untreated controls. Electrolyzed water treatments showed promising *in vitro* results, and further

research will evaluate their effectiveness under grapevine nursery conditions.

Impacts on phenolic metabolism in 'Cabernet Sauvignon' grafts when disenfectants are used on grapevine trunk disease compromised scions. D. RUSJAN[1], D. KJUDER[1], A. ŠKVARČ[2] and M. MIKULIC-PETKOVSEK[1]. *[1]University of Ljubljana, Biotechnical faculty, Agronomy Department, Jamnikarjeva 101, 1000 Ljubljana, Slovenia. [2]Chamber of Agriculture and Forestry of Slovenia, Agriculture and Forestry Institute Nova Gorica, 5000 Nova Gorica, Slovenia. E-mail: denis.rusjan@bf.uni-lj.si*

Phenolic metabolism responses were assessed in different parts (scion, rootstock cane, roots) of grapevine grafts, after callusing and rooting to scions with different grapevine trunk disease (GTD) sanitary status. 'Cabernet sauvignon' plants were grafted, and different disinfectants were assessed for effects against GTD. Scions were from healthy mother vines: (*i*) healthy (HLT); or from GTD affected mother vines: (*ii*) infected but asymptomatic (ASYM, without any visual symptoms), or (*iii*) symptomatic (SYM, visual necroses on scions). Before grafting, the scions were treated with Beltanol, Serenade, Remedier, Bioaction, sodium bicarbonate or a combination of Beltanol and thermotherapy OR YOU MEAN HWT??? (Beltanol + TT). After callusing, the wood of SYM scions treated with Beltanol or Remedier had the greatest flavanol contents. After rooting, the graft classification showed the greatest yield (79%) of the first-quality grafts from Serenade, while the lowest (55%) from Remedier treatment. The rootstock canes of rooted grafts with HLT scions had 2.2 to 2.4-fold greater phenolic contents than the rootstock canes of grafts with SYM and ASYM scions. At rooted grafts, greatest flavanol contents were detected in SYM scions treated with Beltanol, Beltanol + TT, Bioaction or Serenade, and in ASYM scions treated with Beltanol or sodium bicarbonate. The least content of total phenolics (2.05 ± 0.08 mg g^{-1}) was measured in roots of grafts with HLT scions, but the greatest (3.99 ± 0.25 mg g^{-1}) was in roots of grafts with SYM scions. These results showed that sanitary status of scions affected phenolic metabolism of the entire graft, while the assessed biological disinfectants did not show positive impacts against GTD pathogens. This research indicates that Beltanol + TT, Remedier and Serenade treatments gave the most promising results as disinfectants against GTDs.

Evaluation of *Talaromyces pinophilus* as an antagonist of *Botryosphaeriaceae* spp. in grapevine. P. RODRÍGUEZ-HERRERA and F. GAÍNZA-CORTÉS. *Viña Concha y Toro SA, Centro de Investigación e Innovación, Fundo Pocoa s/n, Km10 Ruta K-650, Región del Maule, Pencahue, Chile. E-mail: felipe.gainza@conchaytoro.cl*

Climate change and intensive viticulture are generating early decline in grapevine plants. Grapevine trunk diseases (GTDs) have increased following the banning of fungicides (e.g. sodium arsenite) used to control these diseases, leading to decreased vineyard productivity and longevity. This has encouraged the search for GTD control alternatives, focusing on products based on beneficial endophytic microorganisms. Two isolates of *Talaromyces pinophilus* obtained from different regions of Chile were morphologically characterized and identified with molecular techniques. They were then evaluated as biocontrol agents of the main *Botryosphaeriaceae* present in Chile; *Diplodia seriata*, *Diplodia mutila* and *Neofusicoccum parvum*. Antagonistic capacity of *T. pinophilus* was evaluated in dual cultures, with each pathogen. An extract of *T. pinophilus* was also evaluated as an *in-vitro* growth inhibitor of the pathogens. A suspension of *T. pinophilus* conidia was then evaluated in unrooted vine cuttings, a reduction of damage caused by the pathogens was detected. These results showed that *T. pinophilus* good activity as a preventive biocontrol agent against the different *Botryosphaeriaceae* species, inhibiting growth of the pathogens *in-vitro* and in unrooted vine cuttings. The growth inhibition *in-vitro* ranged from 7.7 to 14.4%, while damage reduction in unrooted vine cuttings was from 68 to 92%. Additional studies should be carried out with *T. pinophilus* to investigate its potential as an alternative for GTD control.

Moss extracts affect the pathogen causing Phomopsis cane and leaf spot of grapevine. N. LATINOVIC[1], M. SABOVLJEVIC[2], M. VUJICIC[2], A. SABOVLJEVIC[2] and J. LATINOVIC[1]. *[1]University of Montenegro, Biotechnical Faculty, Mihaila Lalica 15, 81000 Podgorica, Montenegro. University of Belgrade, Institute of Botany and Botanical Garden, Faculty of Biology, Takovska 43, 11000 Belgrade, Serbia. E-mail: nlatin@ucg.ac.me*

Recent research has focused on replacing synthetic fungicides due to their potentially negative effects on human health and the environment. An option is applications of biologically active substances. Bryophytes are known to be little damaged by pathogens due to their natural chemical components, suggesting that bryophyte extracts could be assessed against fungal pathogens of crop plants. Ten ethanol extracts from mosses

were assessed for effects on colony growth of *Diaporthe ampelina*, which causes Phomopsis cane and leaf spot of grapevine. Extracts were assessed from the mosses *Abietinella abietina* (two accessions), *Dicranum polysetum, Fontinalis antipyretica, Homalothecium sericeum, Isothecium alopecuroides*, *Pseudoscleropodium purum, Racomitrium elongatum, Thuidium delicatulum* and *T. tamariscinum*. Three doses of each extract (5, 10 or 15 µL) were applied to assess *D. ampelina* colony growth inhibition on Petri dishes of potato dextrose agar. All the applied extracts inhibited the fungus compared to nil extract experimental controls. All three doses of the extracts gave inhibition, except *F. antipyretica* extract, for which only the 15 µL dose gave inhibition. There were no differences ($P > 0.05$) among the moss different extracts. However, extracts of *A. abietina* (accession II) and *T. delicatulum* gave the greatest inhibition of *D. ampelina*. These results have demonstrated the potential of mosses as environmentally-friendly treatments against fungal pathogens.

In vitro* evaluation of native *Trichoderma* strains as potential biological control agents against *Phaeoacremonium minimum. G. CARRO-HUERGA[1], S. MAYO-PRIETO[1], A. RODRÍGUEZ-GONZÁLEZ[1], O. GONZÁLEZ-LÓPEZ[2], S. GUTIERREZ[3] and P. A. CASQUERO[1]. *[1]Grupo Universitario de Investigación en Ingeniería y Agricultura Sostenible (GUIIAS), Instituto de Medio Ambiente, Recursos Naturales y Biodiversidad, Universidad de León, Avenida. Portugal 41, 24071 León, Spain. [2]Departamento de Agricultura y Alimentación, Área de Producción vegetal, Universidad de la Rioja, 26006 Logroño, Spain. [3]Grupo Universitario de Investigación en Ingeniería y Agricultura Sostenible (GUIIAS), Área de Microbiología, Escuela de Ingeniería Agraria y Forestal, Universidad de León, Campus de Ponferrada, Av. Astorga s/n, 24401 Ponferrada, Spain. E-mail: gcarh@unileon.es*

Biological control agents (BCAs) could be important options for minimizing effects of Grapevine Trunk Diseases (GTDs). These BCAs can be used in integrated pest management to increase vine health. *Trichoderma* BCAs are effective because they grow and colonize many substrates. However, recent studies have shown irregular performance of commercial products based on this fungus, so native *Trichoderma* strains isolated from vineyards could provide increased disease management efficacy. Twenty-five *Trichoderma* isolates from bark of grapevine plants in Castilla y León region (Spain) were evaluated for *in vitro* inhibition of a strain of *Phaeoacremonium minimum* (Y038-05-03a) from the same region. Dual confrontation assays of *Trichoderma* species against the pathogen were carried out, evaluating percentage of sporulation inhibition, and yellow pigment production by *Trichoderma* in an agar medium, as indications of production of diffusible antibiotic compounds (DACs). Twelve *Trichoderma* strains (T72, T74, T75, T77, T78, T105, T79, T80, T82, T84, T85 and T154) produced greatest growth inhibition. Among these, strains T75, T79, T84 and T154 sporulated over the pathogen and plates . The *Trichoderma* isolate T106 produced a yellow pigment in agar that could be of interest in the evaluation in antibiosis assays. This experiment demonstrated the importance of identifying native *Trichoderma* strains as potential biocontrol agents, and their potential for mass spore production, and for identification of other biocontrol modes of action.

Effectiveness of Mamull® (*Trichoderma* spp. and *Bionectria* spp.) for control of trunk wood diseases of table grape and blueberry. L. A. ALVAREZ[1], E. DONOSO[2] and C. TORRES[3]. *[1]Escuela Profesional de Agronomía. Universidad Nacional de Cañete, Cañete, Lima, Perù. [2]Fitonova SPA. [3]Bio Insumos Nativa SPA, Chile. E-mail: lalvarez@undc.edu.pe*

Grapevine and blueberry trunk diseases cause severe plants losses in Peru. *Botryosphaeriaceae* anamorphs are major causal agents that infect pruning cuttings. *Lasiodiplodia theobromae* dominates over other *Botryosphaereaceae* anamorphs in Peru. Chemical sprays lack prolonged protection, and use of paste formulations is expensive, has logistic complexity and is time-consuming. These factors have driven development of sprayed biological control formulations for trunk disease management. The present study evaluated effectiveness of a commercial multi-strain formulation of *Trichoderma* spp. and *Bionectria* spp. (Mamull®, Chile) in comparison with a chemical alternative. Greenhouse trials were established for each crop evaluating effectiveness of preventive and retroactive treatments. Results from preventive treatments demonstrated similar efficacy between biological and chemical products, which were better ($P < 0.01$) than the control treatment. Protection time was also extended up to 45 d. The retroactive trials showed that the biological product controlled pathogen infections with retroactive effects up to 48 h, being statistically different from the chemical and control treatments These results indicate feasibility of using biological control agents, such as Mamull®, for preventive and retroactive control of trunk diseases, with better retroactive

control than from chemical pesticides, and avoiding the logistical problems with use of paste formulations.

Efficacy of Tachigaren® SL (hymexazol 360 g L^{-1}) for suppression of grapevine branch lesions caused by *Lasiodiplodia theobromae*. L. A. ALVAREZ[1] and G. ESPINO[2]. *Escuela Profesional de Agronomía. Universidad Nacional de Cañete. Cañete, Lima, Perù. [2]Summit Agro South America SpA, Sucursal Perú. E-mail: lalvarez@undc.edu.pe*

Peru is the greatest exporter of fresh table grapes. *Lasiodiplodia theobromae* causes severe plant losses in the main grapevine production areas, and in other fruit crops. A field study assessed the efficacy of hymexazol for control of this pathogen. Two isolates of *L. theobromae* recovered from affected grapevine plants were used in field experiments. Hymexazol was applied in three doses: T1 (2.0 L ha^{-1}), T2 (2.5 L ha^{-1}), or T3 (3.0 L ha^{-1}), applied by drip irrigation with post-plant *L. theobromae* inoculation. For inoculation, a 5 mm diam. plug of the bark of each branch was removed with a cork borer, and a PDA plug (5 mm diam.) colonized by *L. theobromae* was placed onto the exposed cambium. Each inoculated area was covered to prevent wound desiccation. Non-treated plants, inoculated with each isolate, were used as experimental controls. Results were evaluated 45 d after fungicide application. Areas of developed lesions in inoculated branches were measured. Lesions from both isolates, in plants treated with hymexazol, were smaller than for non-treated controls. The factor "fungicide treatment" was statistically significant ($P < 0.05$). The average reduction in lesion size for the treatments that displayed significant differences from the nontreated controls was 64% for T1, 77% for T2, and 82% for T3. Use of hymexazol may be a worthwhile component of integrated management of *L. theobromae* in grapevine plants.

Epidemiological survey of grapevine trunk diseases in the Eger Wine Region of Hungary. A. CSÓTÓ[1,2], D. BARANYI[1], G. SZAKADÁT[3] and E. SÁNDOR[3]. *[1]Institute of Plant Protection, Faculty of Agricultural and Food Science and Environmental Management, University of Debrecen, Böszörményi út 138, H-4032 Debrecen, Hungary. [2]Kálmán Kerpely Doctoral School, University of Debrecen, Böszörményi út 138, H-4032 Debrecen, Hungary. [3]Institute of Food Science, Faculty of Agricultural and Food Science and Environmetal Management, University of Debrecen, Böszörményi út 138, H-4032 Debrecen, Hungary. E-mail: csoto.andras@agr.unideb.hu*

Grapevine trunk diseases (GTDs) cause significant losses in vineyards, and several fungi, including polyphagous pathogens of forest trees, have been recognized as GTDs pathogens. There is no completely effective disease management method for GTDs, so prevention has high importance. The present study identified favourable environmental conditions associated with disease incidence (DI) to be considered before planting. Objectives were to confirm relationships between DI and (1) cultivar susceptibility, (2) proximity of forest vegetation, and (3) effects of position on terrain slopes. Twelve vineyards older than 8 years were surveyed in two season in the Eger Vine Region, North-Eastern Hungary. Approx. 14,500 plants were monitored, including 16 cultivars. DI was surveyed separately, for (1) surveying "tiger stripe" leaf symptoms or diebacks, and for (2) surveying 100 plants randomly selected in four non-marginal rows, in each surveyed cultivar of each vineyard. Season 2020 had very high precipitation in June and October, while season 2021 was more balanced. DI ranged from 17 to 66%. Lowest DI was detected in 'Merlot, and highest DI was detected in the white grape 'Sauvignon blanc' and 'Olaszrizling', and the red grape 'Alibernet' and 'Syrah'. Ten samples were randomly collected for identification from each surveyed vineyard. Based on morphological characteristics, species of *Botryosphaeriaceae* were mainly detected from symptomatic plants. DI was greatest in vineyards close to forests or located at lower parts of slopes.

Potential GTD antagonist microfungi isolated from grapevines. A. CSÓTÓ[1,2], M. FILE[1], A. N. PITI[1], B. ELLMANN[1], K. PÁL[3], G. SZAKADÁT[3] and E. SÁNDOR[3]. *[1]Institute of Plant Protection, Faculty of Agricultural and Food Science and Environmental Management, University of Debrecen, Böszörményi út 138, H-4032 Debrecen, Hungary. [2]Kálmán Kerpely Doctoral School, University of Debrecen, Böszörményi út 138, H-4032 Debrecen, Hungary. [3]Institute of Food Science, Faculty of Agricultural and Food Science and Environmental Management, University of Debrecen, Böszörményi út 138, H-4032 Debrecen, Hungary. E-mail: csoto.andras@agr.unideb.hu*

Antagonistic microorganisms have potential as alternative controls for grapevine trunk diseases. However, there can be efficacy, safety, and production cost differences between known and newly discovered antagonists. Marketed and recently isolated Ascomycete species, with potential biocontrol activity, were studied. One *Fusarium* isolate, two of *Trichoderma* (*T. afroharzianum* and *T. simmonsii*), and two of *Clonostachys rosea*, were assessed, all of which were isolated from wood tissues

of grapevine plants from commercial Hungarian vineyards. The isolates were identified based on morphological characters and molecular sequences (ITS). *Trichoderma* species were identified based on tef1 similarities. Their growth and spore production were studied *in vitro*, and phytotoxic effects were assessed on detached leaves soaking in filtered broth, following 1 week of growth in potato dextrose (PD) broth. Biocontrol potential was assessed in PD agar plate confrontation assays against the GTD pathogens *Eutypa lata*, *Neofusicoccum parvum*, *Phaeoacremonium* sp., or *Fomitiporia* sp.). The two *Trichoderma* isolates gave 100% inhibition of each GTD pathogen in the plate confrontation tests. The *Fusarium* sp. strain had weak biocontrol potential, its metabolites were moreover phytotoxic in detached leaf tests. All of the tested strains grew well on solid media and in batch cultures, producing minimum 10^8 to 10^9 spores mL^{-1} after 4 d of growth.

Interactive effects of *Dactylonectria macrodidyma* inoculation on grapevine rhizosphere and root microbiomes. M. J. CARBONE[1], A. EICHMEIER[2], T. KISS[2], D. TEKIELSKA[2], R. BUJANDA[3], B. LÓPEZ-MANZANARES[3], S. OJEDA[3] and D. GRAMAJE[3]. *[1]Department of Plant Protection, Faculty of Agronomy, University of the Republic, Avenue Garzón 780, Montevideo 12900, Uruguay. [2]Mendel University in Brno, Faculty of Horticulture, Mendeleum - Institute of Genetics, Valticka 334, 69144, Lednice, Czech Republic. [3]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. E-mail: david.gramaje@icvv.es*

Black-foot is a soil-borne disease caused by several "*Cylindrocarpon*"-like asexual fungi, including the widely distributed *Dactylonectria macrodidyma*. Composition and interactions of rhizosphere and endosphere of fungal microbiomes of grafted grapevine plants inoculated with *D. macrodidyma* were characterized using ITS high-throughput amplicon sequencing. Root tissues and associated rhizosphere soil from plants were sampled at 0, 3, 9 or 16 months after *D. macrodidyma* inoculation. Pathogen inoculation did not affect microbiome species richness, diversity or community structure, but induced changes in the relative abundance of several microbial taxa. Inoculated plants increased the proportion of *Diaporthe*, *Cadophora* and *Glomus* in the roots, but reduced that of *Trichoderma*. In the rhizosphere, SparCC network maps showed almost equal numbers of positive (n = 122) and negative (n = 118) correlations with *D. macrodidyma*. The black foot pathogen *Ilyonectria* had positive correlation with *D. macrodidyma*, while arbuscular mycorrhizal *Glomus* was negative correlated with the pathogen. In the grapevine roots, most of the major OTUs (n = 49) were positively correlated with *D. macrodidyma* and *Ilyonectria*, but the biocontrol genus *Trichoderma* was negatively correlated. These results have important implications for further studies on grapevine/pathogen/microbiome interactions.

Evaluation of *Trichoderma atroviride* SC1 and *Bacillus subtilis* PTA-271 combination against grapevine trunk disease pathogens in nursery propagation. CATARINA LEAL[1,2], DAVID GRAMAJE[3], FLORENCE FONTAINE[2], PATRICIA TROTEL-AZIZ[2] and JOSEP ARMENGOL[1]. *[1]Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Valencia, Spain. [2]University of Reims Champagne-Ardenne, Résistance Induite et Bioprotection des Plantes Research Unit, EA 4707, INRAE USC 1488, SFR Condorcet FR CNRS 3417, Reims, France. [3]Instituto de Ciencias de la Vid y del Vino, Consejo Superior de Investigaciones Científicas, Universidad de la Rioja, Gobierno de La Rioja, Logroño, Spain. E-mail: catarinaleal09@gmail.com*

Grapevine trunk diseases (GTDs) are threats to viticulture, leading to important economic losses. In nurseries, grapevine planting material is susceptible to infection by GTD pathogens, through cuts and wounds made during the different steps in propagation processes. Without effective chemical treatments, combinations of biological control agents (BCAs) could improve propagation material protection against GTDs pathogens. Single or combined treatments with *Bacillus subtilis* (*Bs*) PTA-271 and *Trichoderma atroviride* (*Ta*) SC1 were assessed for effects on GTDs pathogen infections in grapevine material during propagation. Botryosphaeria dieback (BD) incidence and severity were reduced in propagation material treated with *Ta* SC1 and *Ta* SC1 + *Bs* PTA-271, and Black foot (BF) incidence and severity were reduced from treatments of *Ta* SC1, *Bs* PTA-271, and. The *Ta* SC1 + *Bs* PTA-271 treatment showed potential for reducing infections caused by some GTD pathogens in the nursery propagation. This combination could be an asset for integrated disease management, where multiple strategies are combined to reduce GTD infections during nursery plant production.

Incidence of newly described mycoviruses in *Diaporthe* sp. M. KOCANOVA[1], L. BOTELLA[2], M. RIEDLE-

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Diaporthe species are considered causal agents of Phomopsis dieback, one of the fungal groups causing grapevine trunk diseases (GTDs). Some types of mycoviruses reduce the virulence of fungal hosts, which provides a tool for development of biocontrol strategies. The present study focused on detection and description of mycoviruses present in *Diaporthe* isolates mainly from *Vitis vinifera* L. two RNA viruses were detected, using dsRNA purification and high-throughput sequencing (Small and Total RNA Seq). Diaporthe mitovirus 1 (DrMV1; *Mitoviridae*) has a (+)ssRNA genome (approx. 2455 nt) containing one ORF encoding the RNA-dependent RNA polymerase (RdRp). The second virus, Diaporthe negative-stranded RNA virus 1, (DNSRV1; probably *Mymonaviridae*) has (-)ssRNA genome of approx. 9372 nt, with five ORFs. The largest encodes the RdRp, and the other four have unknown function. Based on both viral RdRp sequences, specific primers were designed to screen occurrence of DrMV1 and DNSRV1 by RT-PCR in a collection of 35 *Diaporthe* isolates from different countries and hosts ((24 *Vitis* sp., seven *Junglans* sp., four Prunus sp.) Virus co-infections were detected in one *Diaporthe* isolate from Czech *V. vinifera*; one from Austrian *V. vinifera*, one from Czech *Juglans regia*, and four isolates from Slovakia *Prunus domestica* L. were positive for DNSRV1. Two isolates from Czech *V. vinifera* and *J. regia* were positive for DrMV1. Twenty-nine percent of surveyed *Diaporthe* isolates contain virus, and DNSRV1 was more abundant than DrMV1.

Biological and physical protection of grapevine propagation material from trunk disease pathogens. E. ABARQUERO[1], M. P. MARTÍNEZ-DIZ[1], A. DÍAZ-FERNÁNDEZ[1], Y. BOUZAS[1], R. BUJANDA[2], D. GRAMAJE[2] and E. DÍAZ-LOSADA[1]. *[1]Estación de Viticultura y Enología de Galicia (AGACAL-EVEGA), Ponte San Clodio s/n 32428, Leiro, Ourense. [2]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. E-mail: emilia.diaz.losada@xunta.gal*

Control of fungal trunk pathogens in grapevine nurseries is based on application of integrated disease management, which includes chemical, biological and cultural strategies. Hot water treatments (HWTs) and/or biological control agents have been recommended due to restrictions and difficulties with pesticide chemicals in many countries. The present study evaluated HWT duration and *Trichoderma atroviride* SC1 (TCH), for disease control and their effects on trunk pathogen infections. Two trials were established: (*i*) HWT, TCH or HWT + TCH applications to cuttings in hydration tanks before grafting, and (*ii*) HWT, TCH or HWT + TCH applications to grafted plants after rooting in field nurseries. In both trials, treated plants were planted in a commercial vineyard. Pathogen isolations were carried out from rootstock bases and roots at two different times: after rooting in field nurseries and after one season in a vineyard. Black foot control was low in both trials. HWT or HWT + TCH reduced Petri disease infections, mainly in grafted plants uprooted from field nurseries.

Compositions of phytopathogenic fungal communities in grapevine leaves differ for sampling months, but not between organic and conventional vineyard management. C. M. LEAL[1,3], A. GEIGER[2,3] and J. GEML[1,2,3]. *[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 health depends on plant-associated microbes, but systematic overview, and knowledge of how grapevine microbiomes are influenced by cultivation methods, are still lacking. Diversity and composition of plant pathogenic fungal communities in grapevine leaves were assessed under organic or conventional management, to assess whether differences in the fungicides used affected the leaf-associated fungal communities. DNA meta -barcoding data were generated from leaf samples ('Bianca') collected throughout a growing season at the experimental vineyard Eszterházy Károly Catholic University, Hungary. Quality-filtered sequences were

grouped into Amplicon Sequence Variants (ASVs), that were assigned to taxonomic and functional groups using In the rarefied dataset, 911 ASVs of plant pathogenic fungi represented 88 genera, of which 15 are known to be associated with grapevine trunk diseases (GTDs). *Phaemoniella*, *Diplodia*, *Stereum*, *Trametes*, and *Botryosphaeria* had the greatest ASV richness. Among non-GTD pathogens, *Cladosporium*, *Aureobasidium*, *Alternaria*, *Vishniacozyma*, and *Epicoccum* were the most diverse. The detected richness and abundance of GTD-associated fungi in leaves was noteworthy, and confirms recent results showing that many of these fungi are part of the core grapevine mycobiome. Richness and composition of plant pathogenic fungi did not differ between organic and conventionally managed grapevines, but changes were apparent among months, explaining the greatest compositional variation. This strong temporal variation in leaf-associated pathogens was probably caused by application times of fungicides, differences in sensitivity among fungal species, particularly in mid-summer, and by seasonality, i.e. leaf maturation and gradual senescence onset by September.

Bismuth subsalicylate, a fungistatic compound and plant defence stimulator with potential for management of grapevine trunk diseases. L. MERLEN[1,2], C. TARNUS[1], C. DELAITE[2] and M. GELLON[1]. [1]*Laboratoire Vigne Biotechnologie et Environnement EA 3391, Université de Haute-Alsace, 33 rue de Herrlisheim, F-68000 Colmar, France.* [2]*Laboratoire de Photochimie et d'Ingénierie Macromoléculaires UR 4567, Université de Haute-Alsace, 3 bis rue Alfred Werner, F-68093 Mulhouse cedex, France. E-mail: melanie.gellon@uha.fr*

Since use of sodium arsenite was banned in 2001, increasing numbers of grapevines affected by grapevine trunk diseases (GTDs) have been observed, and lack of highly effective control products has led research on bismuth subsalicylate (BSS). This compound has been used in pharmaceuticals (e.g. PeptoBismol®) for decades. The antifungal capacity of BSS (which contains salicylic acid) was assessed against GTD pathogens, and for ability to stimulate plant defence genes. An objective was to design an appropriate formulation for BSS which had water solubility. A suitable formulation based on a liquid polymer was developed, with small particle size which increased the bioavaibility of the compound, an extremely important feature for eventual developments. Antifungal potency of the formulated BSS against GTD pathogens was confirmed, through growth inhibition of *Neofusicoccum parvum* (isolates Bt 67 and Bourgogne), *Diplodia seriata* (98.1) and *Fomitiporia mediterranea* (PHCO36). Stimulation of defence genes was analysed by RT-qPCR on grapevine callus (VvPAL, VvEDS1, VvHSR1 overexpressed), and the non-toxicity of BSS on grapevine cells was confirmed by fluorescence microscopy. BSS was then evaluated *in planta* using vertical plant endotherapy laboratory technique, where BSS was injected directly into grapevine rotten wood where mycelium complexes are concentrated. Symptomatic grapevines (n = 100) were treated for 2 years in Alsace, Preliminary observations will be presented, taking into account the complexity of GTD symptom expression.

Trunk BioCode; a metagenomic study of grapevine trunk diseases in Portuguese vineyards, and biosensor adaptation. F. AZEVEDO-NOGUEIRA[1,2,*], A. GASPAR[3], C. REGO[3], H. M. R. GONÇALVES[4], A. M. FORTES[2], D. GRAMAJE[5] and P. MARTINS-LOPES[1,2]. [1]*University of Trás-os-Montes and Alto Douro, School of Life Science and Environment, Department of Genetics and Biotechnology, Vila Real, Portugal.* [2]*BioISI – Instituto de Biosistemas e Ciências Integrativas, Faculdade de Ciências, Universidade de Lisboa, 1749-016, Lisboa, Portugal.* [3]*LEAF - Linking Landscape, Environment, Agriculture and Food-Research Center, Associated Laboratory TERRA, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal.* [4]*REQUIMTE, Instituto Superior de Engenharia do Porto, 4200-072 Porto, Portugal.* [5]*Institute of Grapevine and Wine Sciences (ICVV), Spanish National Research Council (CSIC), University of La Rioja and Government of La Rioja, 26007 Logroño, Spain. E-mail: plopes@utad.pt; filipem.a.nogueira@gmail.com*

Increasing knowledge of grapevine trunk disease (GTD) pathogens, and their environmental interactions, may give new insights for vineyard protection. The present study investigated whether grapevine cultivars influenced the prevalence of fungi in symptomatic grapevine wood. Two grapevine cultivars ('Touriga Nacional', 'Aragonez'), were assessed in Portuguese vineyards, from different wine Appellations. Sampling was of symptomatic wood from 'Touriga Nacional' (n = 36) and 'Aragonez' (n = 61). Necrotic woody tissues were cultured onto PDA amended with chloramphenicol, and presence of GTD pathogen colonies was assessed, based on morpho- cultural characters. A distinct pattern was found among the two cultivars: *Botryosphaeriaceae* isolates were most prevalent in 'Touriga Nacional' symptomatic wood, whereas in 'Aragonez', the most abundant are fungi were related to Esca disease. These results indi-

cated that cultivars contain specific GTD fungi, related to the presence of host resistance/tolerance genes. Further studies are required to confirm this conclusion. Metagenomic analyses are being applied to the same samples to validate the data obtained by traditional means, and provide a basis for biosensor development. This will be achieved by identifying the most relevant fungus within the GTD complex affecting Portuguese vineyards. This research is within the TrunkBioCode project, which aims to develop molecular tools for early identification of GTDs in grapevine. One strategy is based on a Biosensor platform, for detection and identification of pathogenic taxa in field conditions.

***Sporocadaceae* associated with grapevine trunk diseases in Cyprus**. G. MAKRIS, M. CHRISTOFOROU and L. KANETIS. *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 biotic factors that threaten economic sustainability of grape industries. Besides well-known diseases, there is increased interest in wood-colonizing fungi that infect grapevine woody tissues, and new species are being described as GTD causal agents. More than 140 fungal species have been reported in association with GTDs, but the degree of involvement for many GTD-related species remains to be elucidated. In 2017, during a survey conducted in Cyprus, wood samples were collected from vines exhibiting typical GTD symptoms, including decline, and dead cordons and spurs. Based on morphological and multilocus phylogenetic analyses (ITS, LSU, TEF1, and TUB2), four species of *Sporocadaceae* were found in association with GTDs, including *Seimatosporium marivanicum* and *Se. vitis-viniferae*, and *Sporocadus kurdistanicus* and *Sp. rosigena*. Pathogenicity trials with seven selected isolates, representative of each species, were conducted on woody stems of 2-year-old potted grapevines ('Xynisteri'). All the isolates were pathogenic, causing dark brown to black vascular discolouration of wood tissue below the bark, extending upward and downward from the points of inoculation. The *Sporocadus* isolates (mean lesion length 6.9 to 9.2 cm) were more aggressive than *Seimatosporium* isolates (4.0 to 4.1 cm). Re-isolation percentages of the inoculated pathogens were 40% for *Se. marivanicum*, 35–67% for *Se. vitis-viniferae*, 28 - 44% for *Sp. Kurdistanicus*, and 25–32 % for *Sp. rosigena*. This is the first report of *Se. marivanicum*, *Sp. kurdistanicus* and *Sp. rosigena* causing symptoms on *Vitis vinifera* in Europe, and suggesting a potential role for *Sporocadaceae* in the GTD complex.

Grapevine trunk diseases of cold-hardy grapevine varieties in northern midwest USA coincide with wounds and winter injury. D. H. DeKREY[1], A. E. KLODD[2], M. D. CLARK[3] and R. A. BLANCHETTE[1]. *[1]Department of Plant Pathology, University of Minnesota, St. Paul, Minnesota, United States of America. [2]University of Minnesota Extension, Farmington, Minnesota, United States of America. [3]Department of Horticultural Science, University of Minnesota, St. Paul, Minnesota, United States of America. E-mail: dekre004@umn.edu*

Surveys to characterize grapevine trunk diseases have been conducted for most major grape growing regions. Many of these share mild Mediterranean-like climates, and mainly grow traditional *Vitis vinifera* cultivars. Vineyards in Northern Midwest United States of America contrast to other growing regions, with an atypical cold climate (as low as -35°C), snowy winters, wet springs, humid summers, wet autumns, and short growing seasons. A survey was carried out to identify the most prominent fungal pathogens associated with this unique climate, and the cold-hardy interspecific hybrid grapevine varieties grown in the region. From 172 samples collected, 640 isolates were obtained by culturing, and these were identified using ITS sequencing. A total of 420 sample-unique taxa were identified. Of these, opportunistic fungi of *Diaporthales, Cytospora* and *Diaporthe* spp., were most frequently identified. Species of *Phaeoacremonium*, *Paraconiothyrium*, and *Cadophora* were also prevalent. Species in *Xylariales* and *Botryosphaeriales*, which are frequently isolated in many other regions, where only isolated in small numbers. No taxa in the *Phaeomoniellales* were isolated. The compounding effects of winter injury, pathogens, and management strategies will be discussed. Difficulties for studying, understanding, and communicating knowledge on grapevine trunk disease will also be addressed.

Effects of dual inoculation (*Seimatosporium* species with/without GTD fungi) on lesion length (symptom expression) in Sauvignon blanc vines. N. BESSELMA[1], H. J. RIDGWAY[1,2] and E. E. JONES[1]. *[1]Faculty of Agriculture and Life Sciences, Lincoln University, PO Box 84, Lincoln 7647, New Zealand. [2]The New Zealand Institute for Plant & Food Research Limited, Private Bag 4704, Christchurch 8140, New Zealand. E-mail: Noureddine.Besselma@lincolnuni.ac.nz*

In a survey of the endophytic fungal diversity associated with grapevines symptomatic or asymptomatic for grapevine trunk diseases (GTDs), carried out in Marlborough, New Zealand in 2018, several pathogens were isolated. Among these, members of *Botryosphaeriaceae – Neofusicoccum parvum* (from symptomatic vines) and *Diplodia seriata* (from symptomatic and asymptomatic vines) – were recovered. These pathogens can be latent and virulent GTDs. Additionally, *Seimatosporium vitis* and *S. lichenicola* were recovered for the first time associated with GTDs New Zealand vines. Both species were isolated from symptomatic and asymptomatic tissues, but their roles as pathogens and interaction within GTD complexes is unclear. Interaction between these *Seimatosporium* spp. and *N. parvum* or *D. seriata* in the GTD complex, and effect on symptom expression, were examined. *In planta* dual inoculations with *Seimatosporium* spp. and *N. parvum* or *D. seriata* isolated from the same wood cankers were evaluated. Detached Sauvignon blanc green shoots and 2-year-old woody stems of potted plants were wounded and co-inoculated with mycelium colonised agar discs of *S. vitis* or *S. lichenicola* and *N. parvum* or *D. seriata*. Controls consisted of each fungal species inoculated alone. After 2 weeks for detached shoots and 4 months for attached shoots, lesion lengths and colonisation distances (by re-isolation) were assessed. In both assays, differences in the lesion lengths and pathogen movement were detected from co-inoculations of both *Seimatosporium* spp. with *N. parvum*. In contrast, co-inoculation of either *Seimatosporium* spp. with *D. seriata* did not develop lesions, although *D. seriata* were recovered at 5 cm upward and downward from inoculation points. No lesions developed with from *D. seriata, S. vitis,* or *S. lichenicola* inoculations. These results confirm that *Seimatosporium* spp. are involved in the GTD complex.

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Research Papers

Pseudomonas syringae pv. *syringae* causes bacterial canker on Japanese quince (*Chaenomeles japonica*)

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Summary. Japanese quince trees are grown as ornamental plants in Iran, in parks and in orchards close to stone fruit and pome fruit trees. Shoots of Japanese quince (*Chaenomeles japonica*) showing sunken brown canker symptoms were observed and collected near Sari, the center of Mazandaran province in the North of Iran, during the 2016 growing season. Gram negative bacteria isolated from symptomatic tissues were similar to *Pseudomonas syringae* pv. *syringae* (*Pss*) were pathogenic on Japanese quince and on quince (*Cydonia oblonga*) seedlings after artificial inoculation, and were re-isolated from diseased hosts. Phylogenetic tree construction using partial sequences of ITS and *rpo*D genes showed that the Japanese quince isolates were in the same clade as *Pss* strains. The isolates had ice nucleation activity, and the *Ina*K gene was amplified successfully. According to the results of phenotypic and genotypic characteristics, genomic DNA fingerprinting using REP-PCR, BOX-PCR and IS50-PCR and isolation of total cell proteins, we conclude that *Pss* is the causal agent of canker of the Japanese quince trees. Therefore, Japanese quince is a new host for *Pss* causing bacterial canker on many different host plants.

Keywords. Maule's quince, *rpo*D, BOX-PCR, REP-PCR, IS50-PCR.

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INTRODUCTION

Japanese quince (*Chaenomeles japonica* (Thumb.) Lindley ex Spach) is one of the four species of *Chaenomeles* Lindl. (*Maloidae*, *Rosaceae*). This small tree produces white to pink flowers in late winter or early spring. The flowers have five petals and can grow up to 4.5 cm in diameter. The tree is spiny and bears simple alternately arranged leaves with serrated margins. Being native to Eastern Asia and naturally grown in central and Southern Japan, it is also

cultivated in many countries including Iran (mostly together with forsythia), primarily as an ornamental plant for its showy flowers. Moreover, Japanese quince is also cultivated in Latvia, Lithuania, Russia, Poland, Belarus, Sweden, and Finland for its astringent apple-like pome fruits used in preserves and liqueurs, with potential as an alternate fruit crop (Jakobija and Bankina, 2018). Although Japanese quince is usually described as a healthy ornamental plant (Norin and Rumpunen, 2003), it can be attacked by a few pests and diseases, including aphids and scales, fungi and fire blight, reported as the main bacterial disease (Jewell, 1998).

Pseudomonas syringae is a heterogeneous group including more than 60 pathovars, belonging to the RNA homology group I of *Pseudomonas*, subclass γ from the Proteobacteria (Young, 2010). Its pathovars are host-specific on a range of plants, including beans, grasses and *Prunus* species (Little *et al*., 1998). *Pseudomonas syringae* pv. *syringae* (*Pss*) (van Hall, 1902) causes bacterial canker and blast of stone fruit trees. It is one of the most important plant pathogens, causing foliar necrosis in host plants and hypersensitive responses (HR) in non-hosts (Bender *et al*., 1999; Collmer *et al*., 2000), leading to economic losses on more than 180 plant species (Bultreys and Kaluzna, 2010; Young, 2010). *Pss* is also an ice nucleation-active (INA^+) pathogen causing frost injury to plants (Hirano and Upper, 2000).

In Iran, *Pss* strains were isolated and characterized as causal agents of bacterial canker of stone fruit hosts (cherry, peach, apricot), almond, olive, rose, mallow, pelargonium, pear, and quince, red streak of sugarcane, blast of mandarin, and leaf blight of wheat, rice, oat, barley, and cabbage, from different areas (Bahar *et al*., 1982; Banapour *et al*., 1990; Rahimian, 1995; Najafi Pour and Taghavi, 2011; Aeini and Khodakaramian, 2018; Khezri and Mohammadi, 2018; Vasebi *et al*., 2019; Basavand *et al*., 2021). Japanese quince trees showing sunken brown cankers were observed in the Sari county during the 2016 growing season. As these trees are grown in parks, often close to stone fruit and pome fruit orchards, the aim of the present study was to determine the causal agent of canker symptoms on Japanese quince using pathogenicity, phenotypic, biochemical, and molecular analyses.

MATERIALS AND METHODS

Sampling and isolation of bacteria

During the 2016 growing season, twigs of Japanese quince plants with canker symptoms and asymptomatic samples were collected from Sari in the Mazandaran province in the North of Iran. To isolate bacterial strains, plant tissues were surface sterilized, ground in 10 mL sterilized distilled water for 30 min, and a loopfull of the resulting suspension was streaked on nutrient agar with 0.5% sucrose (NAS). Resulting colonies were purified on nutrient agar, and selected according to morphology, color, and shape (Rademarker *et al.,* 2000) for further biochemical, pathogenicity, and molecular tests.

Hypersensitive response and pathogenicity tests

Hypersensitivity response tests were performed by infiltrating bacterial suspensions (1×10^8 cfu·mL^{-1}) into tobacco and geranium leaves. Inoculated leaves were examined after 24 h (Klement *et al*., 1964).

Bacteria grown on NAS for 24 h at 28°C were inoculated into liquid culture, reaching 10^7 cfu·mL^{-1}. Ten µL of each suspension were inoculated into one-year-old Japanese quince and quince seedlings by subepidermal injections, infiltrating approximately 1 cm^2 of the bark tissue of each plant (Rahimian, 1995). For each bacterial isolate, ten plants were inoculated. Seedlings were incubated at 28°C in sterilized plastic boxes (≈80% moisture) for 1 month, and then observed for canker symptoms. One-year-old seedlings inoculated with sterile water were used as negative controls (Jones, 1971). To fulfill Koch's postulates, after development of typical bacterial canker symptoms on inoculated seedlings, bacteria from each of the inoculated plants were re-isolated as above described.

Biochemical and physiological tests

All bacterial isolates were assessed for biochemical, physiological, and nutritional characteristics daily, for one month, as shown in Table 1 (Schaad *et al*., 2001). Carbohydrate utilization from glucose, fructose, sorbitol, melibiose, galactose, cellobiose, lactose, maltose or mannose (Fahy and Pearsly, 1983) was determined using the basal medium (Ayers *et al*., 1919). Nineteen isolates were selected for further analysis. The reference strain *Pss* ICMP 4916 was used in all tests as a positive control.

Isolation of total cell proteins and SDS-PAGE

Bacterial isolates were cultured on nutrient agar at 25–28°C for 24 h and single colonies were re-suspended in distilled water. Optical density of the suspensions was adjusted to 2.0 at 600 nm using a spectrophotometer. Samples were centrifuged at 2,576 g for 5 min, the pellet was re-suspended in 1 mL SDW and then dissolved in

Table 1. Phenotypic and biochemical characteristics of *Pseudomonas* strains isolated from Japanese quince in Sari.

Characteristic	Identified strain			Reference isolate	
	JQ1	JQ2	JQ3	*Pss*	*P. viridiflava*
Gram reaction	-	-	-	-	-
Potato soft rot	-	-	-	-	+
Fluorescent induction on KB	+	+	+	+	+
Oxidase production	-	-	-	-	-
Catalase production	+	+	+	+	+
Tobacco hypersensitivity	+	+	+	+	+
Geranium hypersensitivity	+	+	+	+	+
Methyl red (MR)	-	-	-	-	-
Lecithinase	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-
Utilization of citrate	+	+	+	+	+
H_2S production from cystein	-	-	-	-	-
Casein hydrolysis	+	+	+	+	+
Tween 80 hydrolysis	+	+	+	+	+
Reducing substrate from sucrose	+	+	+	+	-
Arbutin production	+	+	+	+	+
Indole production	-	-	-	-	-
Austin hydrolysis	-	-	-	-	-
Nitrate reduction	-	-	-	-	-
Fermentative metabolism	-	-	-	-	-
Oxidative metabolism	+	+	+	+	+
Ice nucleation	+	+	+	+	-
Esculin hydrolysis	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+
Syringomycine production	+	+	+	+	-
Urease production	-	-	-	-	-
α-methyl diglucoside	-	-	-	-	-
Trehalose	-	-	-	-	-
Ramnose	-	-	-	-	-
D-Fructose	+	+	+	+	+
Xylitole	-	-	-	-	-
Glucose	+	+	+	+	+
Sucrose	+	+	+	+	-
D-Manitol	+	+	+	+	+
Cellobise	-	-	-	-	-
Galactose	+	+	+	+	+
D-Mannose	+	+	+	+	+
D-Raffinose	-	-	-	-	-
D-Xylose	+	+	+	+	+
D-Ribose	+	+	+	+	+
Inositol	+	+	+	+	+
L-Arabinose	+	+	+	+	+
D-Arabitole	+	+	+	+	+
Adonitole	-	-	-	-	-
L-Serine	+	+	+	+	+
L-Asparagine	-	-	-	-	-
Melibiose	+	+	+	+	+

(Continued)

Table 1. (Continued).

Characteristic	Identified strain			Reference isolate	
	JQ1	JQ2	JQ3	*Pss*	*P. viridiflava*
Meso-Erithritol	+	+	+	+	+
Starch	-	-	-	-	-
Glycerol	+	+	+	+	+
Malonate	+	+	+	+	+
Citrate	+	+	+	+	+
D-Tartrat	+	+	+	+	+
L-Tartrat	-	-	-	-	-
Lactate	+	+	+	+	+
Succinate	+	+	+	+	+
Malic acid	+	+	+	+	+
Starch hydrolysis	-	-	-	-	-
Growth on 4% NaCl	+	+	+	+	+
Growth on 6% NaCl	-	-	-	-	-

+: positive, -: negative.

0.1 volume of 10% SDS (Sodium Dodecyl Sulphate (SDS) and denatured by boiling for 5 min. Cells were then fractured by sonication (UP200, Hielscher) for 1 min at low amplification in an ice-cooling bath. The resulting cell suspensions were incubated for 5 min on ice and centrifuged at 10,000 rpm for 10 min. The supernatants representing total cell protein extracts were analyzed by SDS-PAGE, following addition of an equal volume of glycerol. SDS-PAGE analysis was carried out in 10% polyacrylamide gels using the Hoefer mini-electrophoresis system (Amersham Biosciences), according to Ahmadvand and Rahimian (2005). Electrophoresis was carried out at a constant voltage of 18 mA for approx. 2 h. Gels were then stained overnight in 50% (v/v) methanol, 10% (v/v) acetic acid, 0.1% Coomassie brilliant blue and then de-stained with in 50% (v/v) methanol and 10% (v/v) acetic acid under constant shaking. Protein profiles of *Pss* ICMP 4916 and *P. viridiflava* isolated from citrus in Mazandaran were used for comparison.

Genomic DNA fingerprinting using REP-PCR, BOX-PCR and IS50-PCR

Bacterial genomic DNA was extracted according to Bertheau *et al.* (1995) and Mahmoudi *et al.* (2007). Following quality checks on 1% agarose gel, DNA samples were kept at -20°C until use. Repetitive PCR (REP-PCR) using REP1R/REP2 primers (Versalovic *et al.* 1991) and BOXAIR primers (Louws *et al.*, 1999), and Insertion sequence PCR (IS50-PCR) using 1S50 primers (Weingart and Völksch, 1997) were carried out for genomic DNA fingerprinting. Amplifications were performed in an Applied Biosystems 2720 thermal cycler (ThermoFisher) in 25 µL volumes, containing 200 mM of each dNTPs, 2 mM $MgCl_2$, 1.5 pM primers, 1 U *Taq* polymerase, and 4 µL of DNA template. Thermal cycling was carried out with an initial denaturation cycle at 95°C for 5 min, followed by 35 cycles each consisting of denaturation at 94°C for 1 min, annealing at either 40°C (for REP primers), 52°C (for BOXAIR primers), or 44°C (for IS50 primers) for 1 min, extension cycle at 72°C for 3 min, with a final extension at 72°C for 7 min. Amplified products were separated by electrophoresis at 90 V in 2% agarose gels in Tris–Borate–EDTA (TBE) electrophoresis buffer (pH 8), and bands were visualized following ethidium bromide staining.

Data analysis

Data were statistically analyzed using a numerical taxonomy [amplified fragment of each strain scored as 1 (present) or 0 (absent)] and multivariate analysis system (NTSYS, version 2.1). Forty phenotypic characters were included in the analyses. Genetic relationships within and between bacterial strains were determined by cluster analysis, using the UPGMA method on distance calculated with the Jaccard coefficient matrices (Rohlf, 1990).

Partial ITS and rpoD gene amplification

The partial internal transcribed spacer (ITS) region and the RNA polymerase sigma factor D (*rpo*D)

gene were amplified using the primer pairs ITS-F (5' GGCTGGATCACCTCCTT 3') and ITS-R (5' TGCCAAGGCATCCACC 3') and rpoD-F (5' ACCGCGCTGGACACCGAAGGT 3') and rpoD-R (5' ACCACTTCTGCTTGCTTGCGCTTGAGTG 3'). PCR amplification was carried out as described above. Thermal cycling was carried out with an initial denaturation cycle at 94°C for 5 min, 35 cycles each of denaturation at 94°C for 1 min, annealing at 58°C (ITS) and 54°C (*rpo*D) for 45 sec., extension at 72°C for 1 min, and a final extension at 72°C for 7 min (Versalovic *et al*., 1991). PCR products were purified and directly sequenced on both strands by Macrogen (South Korea). The sequences were edited using BioEdit v.7.0.5.2 and compared to the *Pss* sequences deposited in the GenBank database using BLASTn (https://www.ncbi.nlm.nih.gov). Nucleotide sequence similarity and multiple alignment and phylogenetic tree construction of partial ITS and *rpo*D gene sequences were carried out using the neighbor-joining method and bootstrap analysis replicated 1000 times, employing MEGA6 software (Tamura *et al*., 2013).

Ice nucleation activity

The ice nucleation activity of bacterial strains was conducted using the droplet-freezing method. Fifty µL droplets of bacterial suspension (each at 1×10^7 cfu·mL^{-1}) were placed on paraffin soluble in xylene-coated aluminum foil and kept at a range of temperature between -5 and -10°C. *Xanthomonas campestris* and *Pectobacterium carotovora* were used as negative controls. Ice nucleation activity was observed visually after 30 sec (Schaad *et al*., 2001). The presence of the ice nucleation *InaK* gene was tested by PCR using InaK primers (forward 5′ GGGCCACAAAAGTATCCTGA 3′ and reverse 5′ CTGTGACAAGGTCGCTGTGT 3′) specific for *Pss*. Thermal cycling was carried out with an initial denaturation cycle at 94°C for 4 min, 30 cycles of denaturation each at 94°C for 1 min, annealing at 55°C for 1 min, extension cycle at 72°C for 45 sec., followed by a final extension cycle at 72°C for 6 min (Rashidaei *et al*., 2012).

RESULTS

Isolation and biochemical tests

The main symptoms of bacterial canker of Japanese quince in the surveyed area were blossom blight, twig wilting and dieback, blights around thorns, and sunken dark brown cankers, with no symptoms detected on leaves. Typical symptoms are shown in Figure 1.

Figure 1. Canker symptoms on Japanese quince plants collected from the Sari county, Mazandaran province, Iran.

Isolation and biochemical tests

In total, 33 bacterial colonies were obtained, and 19 of them (designated JQ1 to JQ19) were chosen as representative isolates, based on the colour and morphology of the colonies and on biochemical assays. All 19 isolates were Gram negative, produced fluorescent pigment on King's Medium B, were levan-positive, oxidase-negative, potato soft rot-negative, arginine dehydrolase-negative, induced hypersensitivity-reactions on tobacco and pelargonium, with characteristics of *Pseudomonas* LOPAT group Ia. These 19 isolates were selected for further biochemical and pathogenicity tests and for ice nucleation tests; their genomic DNA was tested for REP-, BOX-, and IS50-PCR, ITS and *rpo*D gene analysis. The results of biochemical and nutritional tests are reported in Table 1. According to the morphological and biochemical assays, and to the LOPAT and GATTa identification tests, all 19 representative isolates were identified as *Pss*.

Pathogenicity tests

All 19 strains inoculated were pathogenic to Japanese quince and quince seedlings. All isolates caused water-soaked lesions on twigs within 6 d after inoculation, and developed necrotic sunken cankers after 9 d (Figure 2). No symptoms occurred on twigs inoculated with sterile water as control. The symptoms observed on the inoculated seedlings were identical to those collected from the surveyed area. Koch's postulates were confirmed following re-isolation from healthy and young Japanese quince and quince seedlings; indeed, bacterial strains with the same characteristics as those inoculated were successfully re-isolated from artificially inoculated and diseased host plants.

Analysis of total bacterial cell proteins electrophoresis

Protein profiles of ten randomly selected isolates were compared visually to the protein profiles of the *Pss*

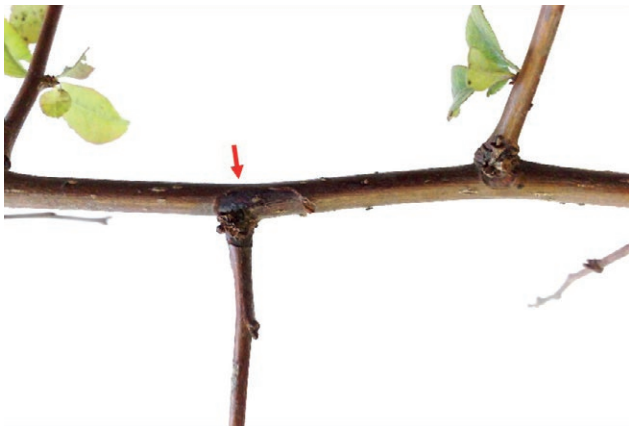

Figure 2. Canker symptoms on Japanese quince one month after artificial inoculation

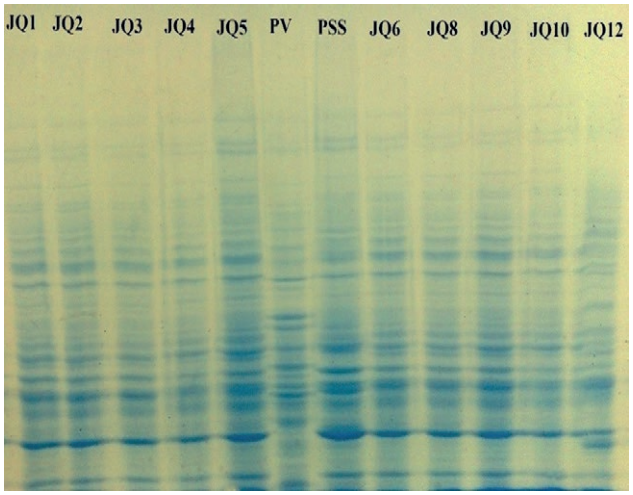

Figure 3. Electrophoretic pattern of total cell proteins of isolates JQ1, JQ2, JQ3, JQ4, JQ5, JQ6, JQ8, JQ9, JQ10, and JQ12 obtained from Japanese quince. PV, *Pseudomonas viridiflava* isolated from citrus; PSS, *Pseudomonas syringae* pv. *syringae* (reference strain ICMP 4916) isolated from citrus.

reference strain ICMP 4916 and to *P. viridiflava* isolated from citrus in the Mazandaran province. The protein profiles of the Japanese quince isolates were similar to *Pss* ICMP 4916 reference strain and differed from *P. viridiflava* (Figure 3).

Genomic DNA fingerprinting using REP-PCR, BOX-PCR and IS50-PCR

The genomic DNA fingerprints of 13 randomly selected *Pss* strains isolated from Japanese quince and of the reference isolate of *Pss* were determined using BOX-, REP- and IS50-PCR products. Overall, the results showed approximately 721 fragments, with sizes ranging between 100 and 5000 bp (Figure 4). Five main clusters were obtained, with 67% similarity considering all the three PCR methods. In REP-PCR, JQ1 and JQ3 strains clustered together in cluster 1, JQ4 and JQ9

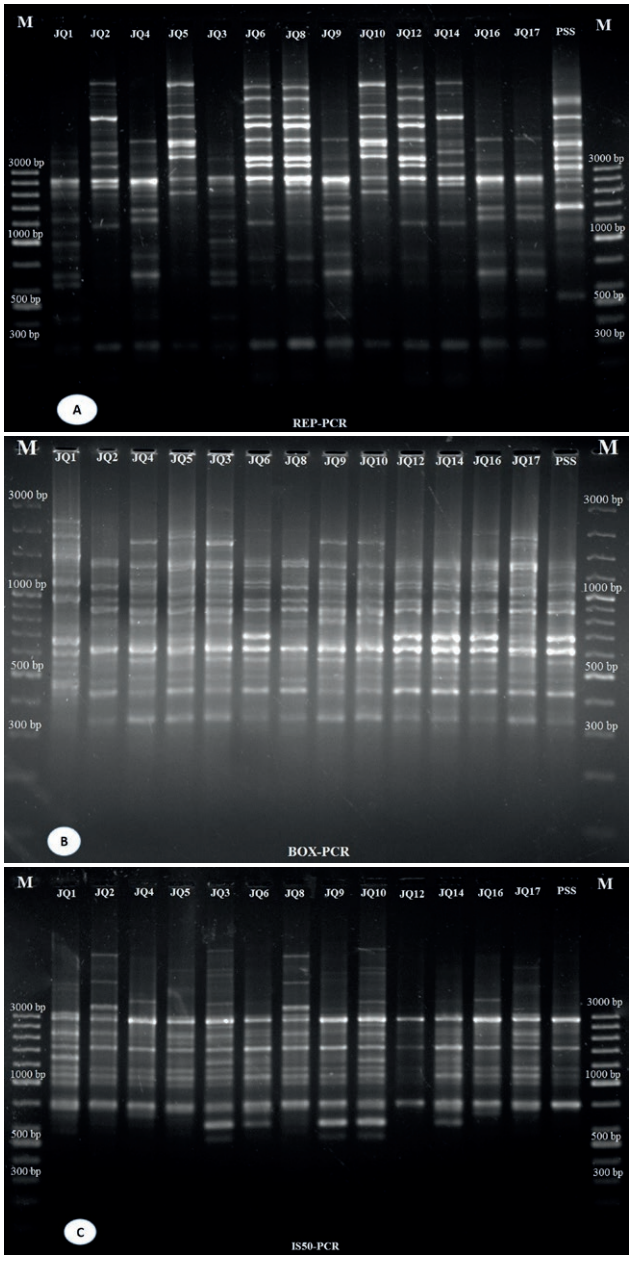

Figure 4. Fingerprints of genomic DNA from the bacterial isolates (JQ1, JQ2, JQ4, JQ5, JQ3, JQ6, JQ8, JQ9, JQ10, JQ12, JQ14, JQ16 and JQ17), obtained from Japanese quince and from *Pseudomonas syringae* pv. *syringae* (*Pss*) with REP-PCR (A), BOX-PCR (B), and IS50-PCR (C); M, 1kb DNA ladder (SMOBIOS).

strains in cluster 2, JQ6 and JQ7 strains in cluster 3, and JQ2, JQ5, JQ6, JQ8, JQ10, JQ12 and JQ14 strains in cluster 4; the reference strain *Pss* made a separate cluster (Figure 5A). According to BOX-PCR, the JQ1 strain grouped into cluster 1, JQ2 and JQ8 were grouped in cluster 2, JQ9, JQ4 and JQ10 grouped in cluster 4, and JQ6, JQ12, JQ14, JQ16 and *Pss* strains were grouped in cluster 5 (Figure 5B). The strains were differentiated into five main clusters according to IS50-PCR. JQ1, JQ5 and JQ17 strains were grouped in cluster 1, JQ4, JQ12 and JQ16 in cluster 2, *Pss* strain in cluster 3, JQ3, JQ9 and JQ10 strains in cluster 4, and JQ6 and JQ14 strains in cluster 5 (Figure 5C). The combined dendrogram from REP, BOX and IS50-PCR was also obtained for 13 strains, and obtaining seven main clusters with 67% similarity and four main groups with 56% similarity, including JQ1 and JQ16 as two separate clusters, JQ3, JQ4, JQ9 and JQ17 in cluster 3, and eight other strains in cluster 4 (Figure 6).

ITS and rpoD gene amplification

PCR amplification with specific primers for ITS and the *rop*D gene correctly amplified specific bands of 600 bp and 850 bp in all the assessed strains (Figure 7), while no amplification was obtained with negative control bacteria. The ITS sequences of two representative strains from Japanese quince, JQ4 and JQ9, had high similarity to each other (99%). The sequences of isolates JQ4 and JQ9 were deposited in the NCBI GenBank database under the respective accession numbers KJ754379 and KJ754378 (for the ITS gene) and KJ769138 and KJ769139 (for the *rpo*D gene). The results confirmed that these isolates belong to *Pss*. A Neighbor-joining phylogenetic tree with 1000 bootstrap replicates showed that isolates JQ4 and JQ9 clustered together with the *Pss* AY342179, based on the ITS gene sequence (Figure 8), and grouped together with the *Pss* isolates JX867789 and KC852112 based on the *rpo*D gene sequence (Figure 9). The isolates and strains used for phylogenetic tree construction and their GenBank accession numbers are listed in Supplementary Table 1.

Ice Nucleation activity

Ice nucleation activity was positive for all the tested strains obtained from Japanese quince, showing freezing of water droplets in less than 30 sec. In contrast, isolates of *X. campestris* and *P. carotovora* did not induce freezing of water droplets in the same timeframe (Figure 10A). Moreover, all PCR amplifications using Japanese

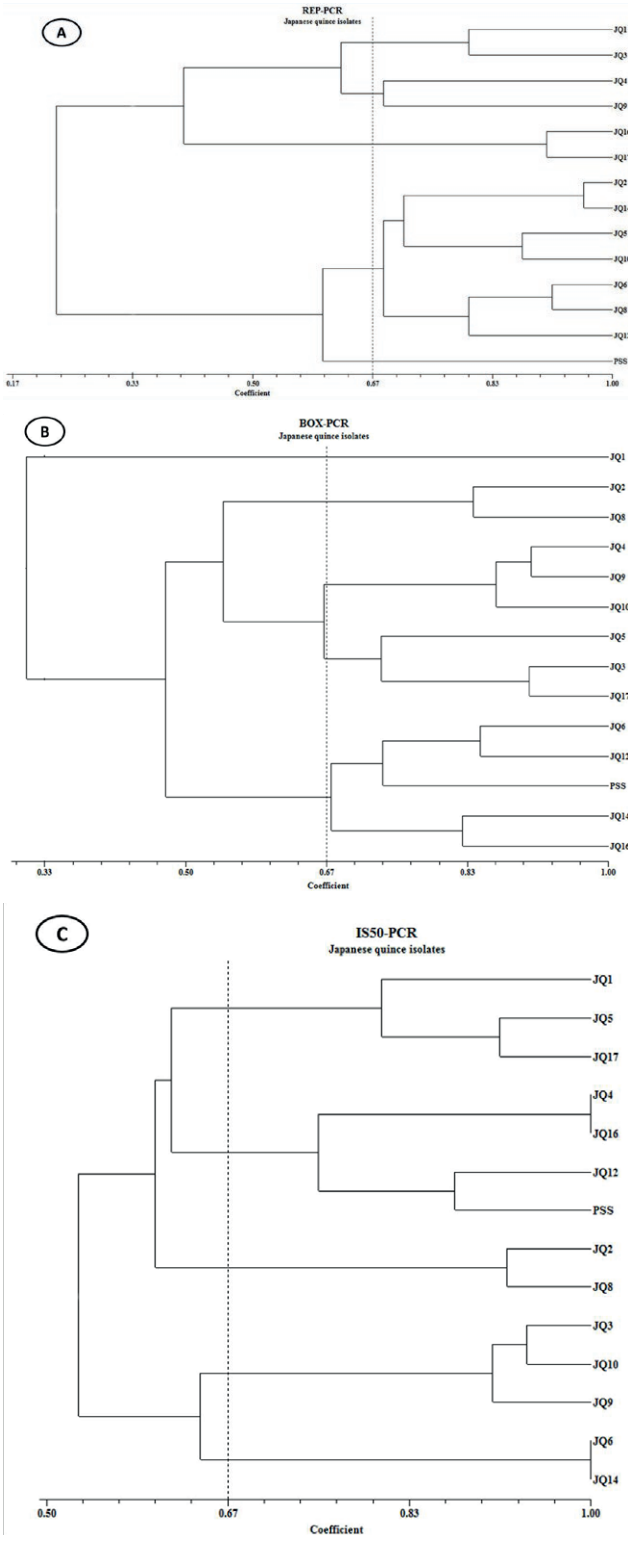

Figure 5. Dendrogram obtained by comparing bacteria isolated from Japanese quince JQ1, JQ2, JQ4, JQ5, JQ3, JQ6, JQ8, JQ9, JQ10, JQ12, JQ14, JQ16, and JQ17 and *Pseudomonas syringae* pv. *syringae* (*Pss*), with REP-PCR (A), BOX-PCR (B), and IS50-PCR (C). Five major clusters were categorized at the 67% similarity level.

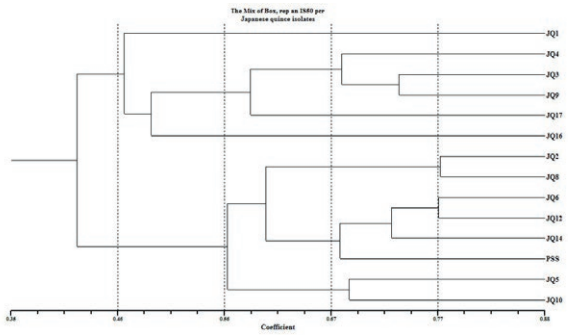

Figure 6. Dendrogram obtained by comparing the bacterial isolates derived from Japanese quince (JQ1, JQ2, JQ4, JQ5, JQ3, JQ6, JQ8, JQ9, JQ10, JQ12, JQ14, JQ16 and JQ17), and *Pseudomonas syringae* pv. *syringae* (*Pss*), by REP-PCR, BOX-PCR, and IS50-PCR assays.

quince strains resulted in products of the expected sizes (200 bp) of the *Ina*K gene (Figure 10B).

DISCUSSION

This study was carried out to identify the causal agent of canker of Japanese quince trees in the Sari County in the Mazandaran province located in the North of Iran. In total, 33 bacterial strains were isolated from canker lesions on plants located in different areas of the county. Characterization of the bacterial strains was achieved by biochemical, physiological and pathogenicity tests, indicating that the strains had phenotypic characteristics of *Pss*. Inoculations into quince and Japanese quince seedlings produced canker symptoms, consistent with previously described symptoms (Bahar *et al*., 1982; Banapour *et al*., 1990). Bacterial strains with the same features were also recovered from inoculated quince and Japanese quince seedlings, fulfilling Koch's postulates. However, as these tests do not precisely identify *Pseudomonas* strains (Peix *et al*., 2018), in order to perform a phylogenetic analysis, molecular tests based on DNA fingerprinting and partial gene sequence analysis using ITS and the housekeeping *rpo*D gene were also conducted. Thus, following BLAST analysis of the sequences of two representative bacterial strains JQ4 and JQ9 we could conclude that *Pss* was the causal agent of Japanese quince canker in the North of Iran.

All bacterial isolates belonged to *Pss*, previously reported as the causal agent of bacterial canker of many plants, including stone fruits, pear, quince, almond, olive, rose, malva and pelargonium, or causing red streak of sugarcane, blast of mandarin and leaf blight of wheat, rice, oat, barley and cabbage from different

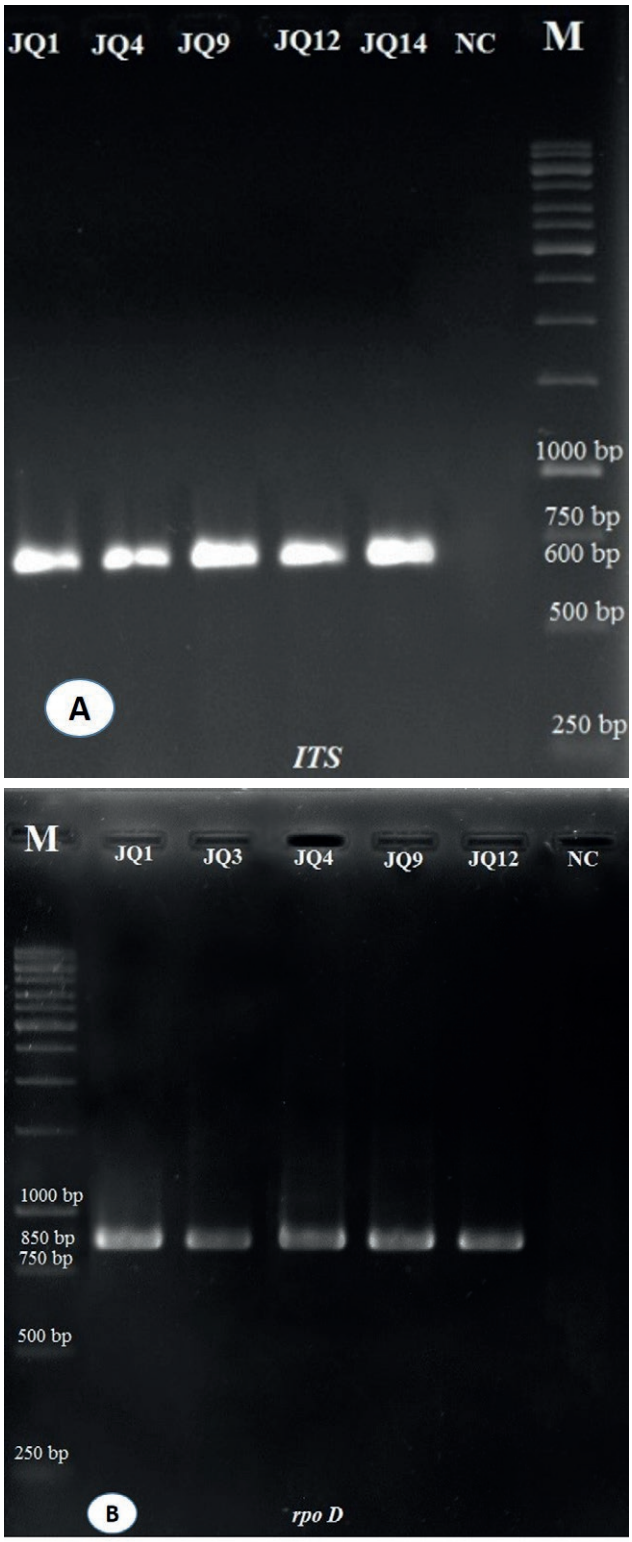

Figure 7. Agarose gel electrophoresis of the PCR products obtained with the *ITS* (A) and *rpo*D gene (B) specific primers (fragments of 600 bp and 850 bp, respectively). Japanese quince isolates (JQ1, JQ3, JQ4, JQ9, JQ12 and JQ14); NC, negative control; M, 1 kb DNA ladder.

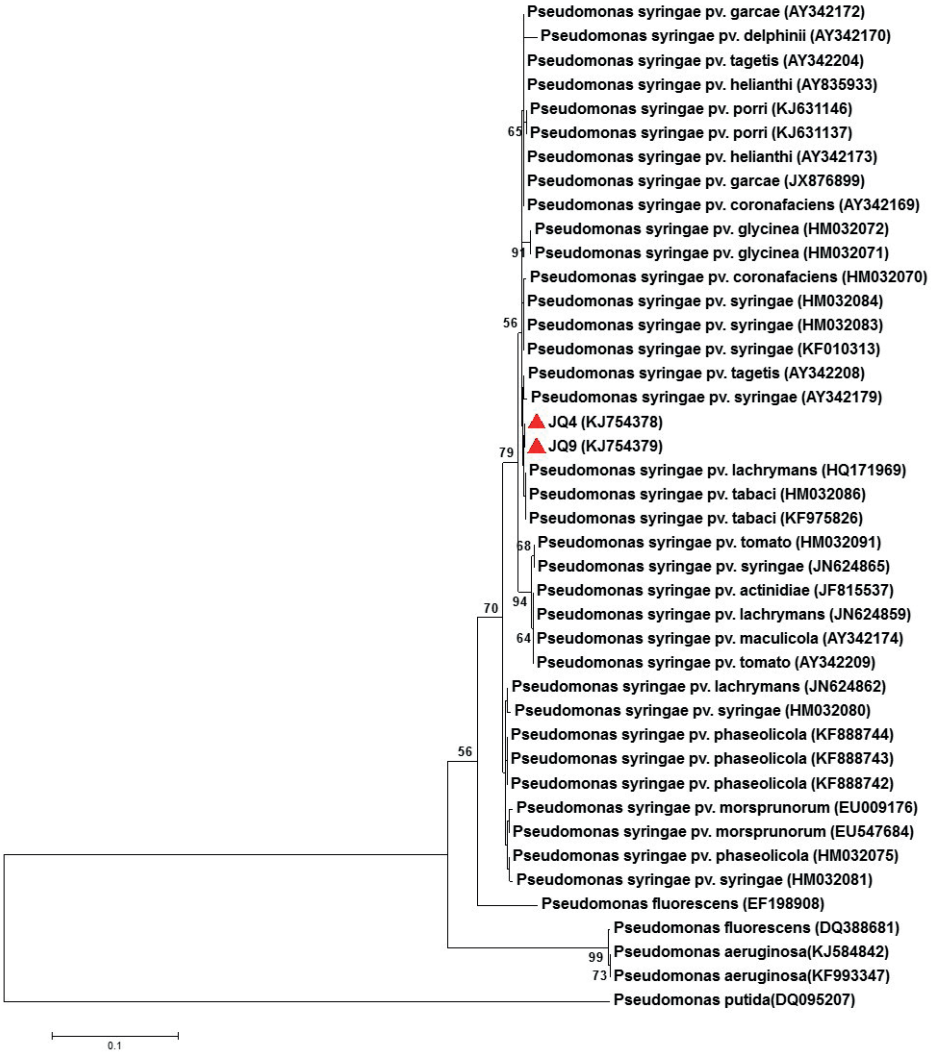

Figure 8. Phylogenetic tree obtained using the Neighbor Joining method of the ITS nucleotide sequence of Japanese quince isolates JQ4 and JQ9 (highlighted in red triangles), and of *Pseudomonas* strains available in GenBank. The GenBank accession numbers used in the analysis are shown in parentheses. Bootstrap values (expressed as percentage of 1000 replicons) are shown at the nodes.

areas of Iran (Bahar *et al*., 1982; Banapour *et al*., 1990; Rahimian, 1995; Najafi Pour and Taghavi, 2011; Aeini and Khodakaramian, 2018; Khezri and Mohammadi, 2018; Vasebi *et al*., 2019; Basavand *et al*., 2021). Genomic fingerprinting analyses, including REP-, BOX- and IS50-PCR showed some levels of variability among the *Pseudomonas* strains. The banding profiles for *Pseudomonas* isolates were also different and *Pseudomonas* isolates from the same region grouped into different clusters; therefore, genetic diversity among isolates was not related to their geographic origin. The methods here adopted are easy and rapid for distinguishing *P. syringae* at the pathovar level, with highly reproducible results. The present results agree with previous studies on genetic variation of *Pseudomonas* strains (Weingart and Völksch, 1997; Little *et al*., 1998; Louws *et al*., 1999; Najafi Pour and Taghavi, 2011; Cetinkaya Yildiz *et al*., 2016; Abdellatif *et al.,* 2020).

Noteworthy, this is the first study comparing these three genomic fingerprint analyses for the genetic characterization of *Pss* strains isolated from Japanese quince. Sequence analysis of the ITS and of the housekeeping *rpoD* gene, and the use of repetitive elements including REP-, BOX- and IS50-PCR have been described as useful methods for identification, classification and characterization of diversity of *P. syringae* isolates (Little *et al*., 1998; Najafipour and Taghavi, 2011). *P. syringae* can infect more than 200 plant species, due to a high degree

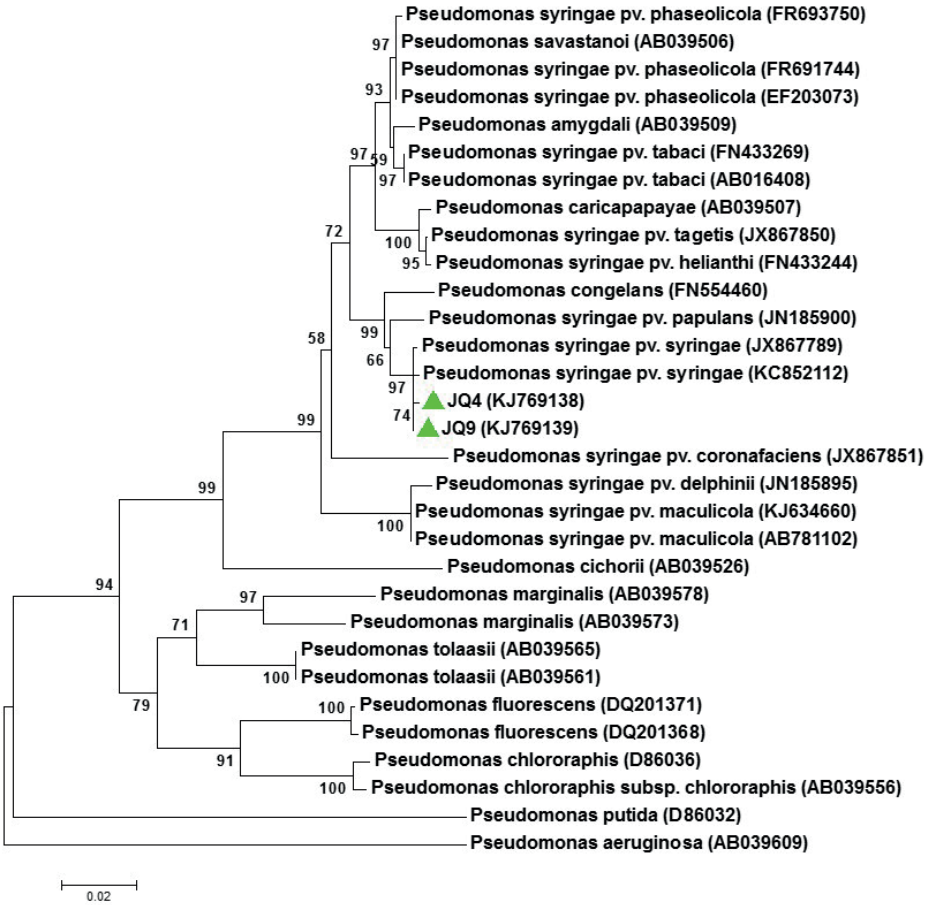

Figure 9. Phylogenic tree obtained using the Neighbor Joining method comparing the *rpoD* nucleotide sequence data of Japanese quince bacterial strains JQ4, JQ9 (highlighted in green triangles) and *Pseudomonas* strains available in GenBank. The GenBank accession numbers used in the analysis is shown in parentheses. Bootstrap values (expressed as percentage of 1000 replicons) are shown at the nodes.

of genetic variability (Ruinelli *et al*., 2019). The results of the present study show that a combination of different methods including biochemical, phenotypic, pathogenicity and molecular tests can be used for the identification of the causal agent of Japanese quince canker. Moreover, this is the first report of *P. syringae* as the causal agent of Japanese quince bacterial canker.

Bacterial canker of stone fruits, almond, olive, pear, and quince caused by *Pss* is a serious problem in all the areas where these crops are cultivated in Iran. Japanese quince, here reported as a new host for *Pss* in this country, can be grown within or near orchards and fields and can likely provide overwintering sites for *Pss,* serving as inoculum sources for disease development and outbreaks. The results of this study can facilitate further research on the ecology, epidemiology, diversity, and management of bacterial canker of Japanese quince and other host plants of *Pss*, not only in Iran but also in other countries. Further studies will allow to identify other infested areas and to identify the etiology of bacterial canker of Japanese quince in Iran.

AUTHOR CONTRIBUTIONS

TA designed and conducted experiments as a Masters student and analyzed the data. MR advised on experimental design, analyzed the data and wrote the manuscript. HR advised on experimental design, assisted with sample collection, and provided technical advice. EN and SM reviewed, critically revised and edited the manuscript

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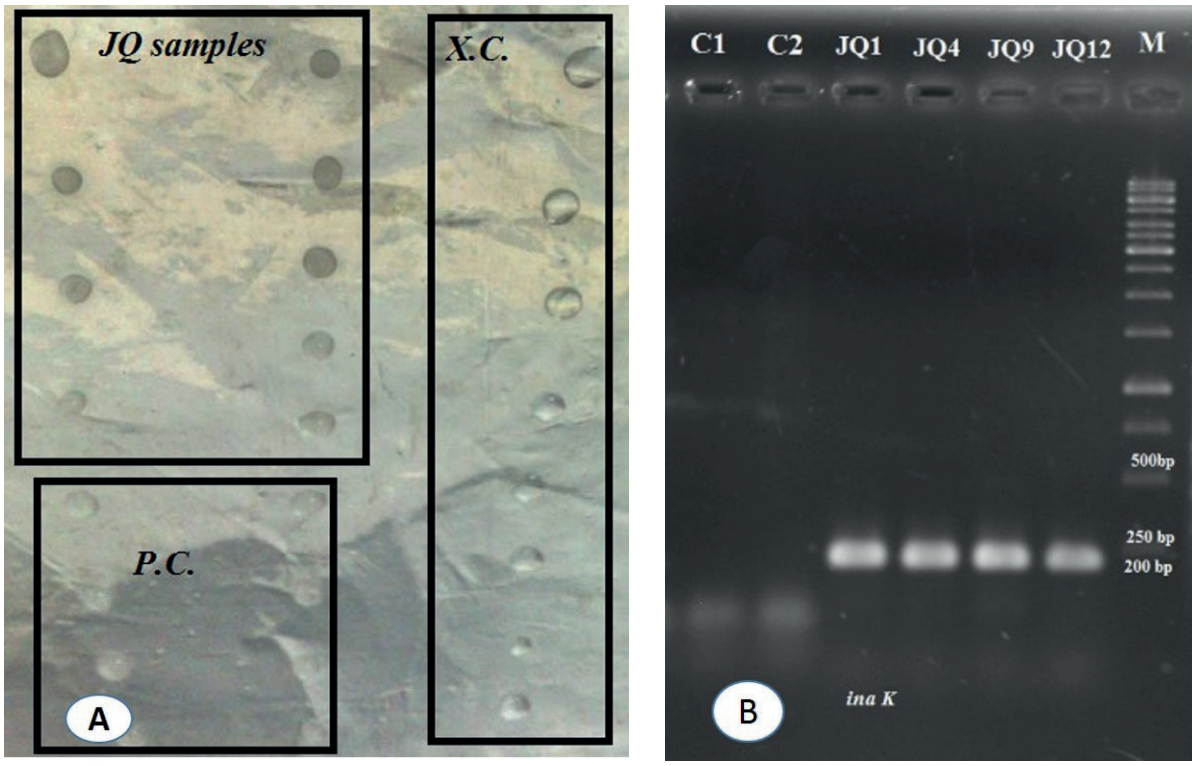

Figure 10. Ice nucleation assays of bacterial strains isolated from Japanese quince A. Ice nucleation test. Japanese quince isolates (JQ samples), *Xanthomonas campestris* (X.C.) and *Pectobacterium carotovora* (P.C.).; B. Gel electrophoresis of *Ina*K gene amplicon on 1.2% agarose gel. JQ1, JQ4, JQ9 and JQ12: isolates from Japanese quince, C1: *Xanthomonas campestris*, C2: *Pectobacterium carotovora*, M: 1 kb DNA Ladder.

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Research Papers

Alternaria species and related mycotoxin detection in Lebanese durum wheat grain

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Summary. *Alternaria* is a ubiquitous genus that may infect wheat in many countries, causing the disease black point. The present study aimed to assess contamination by fungi, of durum wheat kernels from Lebanon, and identify the main *Alternaria* species contaminants. *Alternaria* was detected in the majority (97%) of the inspected fields. Contamination by *Alternaria* differed among the samples according to their geographical origins. The greatest contamination was detected in the West Bekaa area (average 59%), followed by Akkar (55%), and lowest was observed in Baalbeck (2%). HPLC-DAD analyses performed on grain samples showed that altenuene, alternariol, alternariol monomethyl ether, and tenuazonic acid were not detected in any sample. Phylogenetic analyses, based on DNA sequences of *β-tubulin*, *glyceraldehyde-3-phosphate dehydrogenase* and *calmodulin* gene fragments, showed that *Alternaria* field strains belonged to two major sections: *Alternaria* (51%) and *Infectoriae* (40%). The remaining strains were in separate clades in sections *Ulocladioides* (3%), *Chalastospora* (3%) and *Pseudoalternaria* (3%). Although this study revealed no contamination of wheat kernels by *Alternaria* mycotoxins, the potential risk of mycotoxin accumulation remains high due to the widespread occurrence of toxigenic *Alternaria* species on kernels.

Keywords. Multi-locus gene sequencing, toxigenic fungi, *Ulocladioides, Chalastospora, Pseudoalternaria, Infectoriae*.

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INTRODUCTION

Alternaria are ubiquitous fungi, generally detected as saprophytes in soil or in plant debris, and are pathogens of several crops, including cereals, oil crops, ornamentals, vegetables, fruit trees (Logrieco *et al.*, 2009) and plants

used for their medicinal properties (Chalbi *et al*., 2020). *Alternaria* species are the main causal agent of black point in wheat, causing harvest and post-harvest damage of wheat grains. Economic damage caused by *Alternaria* infections is generally not associated with yield losses but is related to grain reduced quality of cereals (Kosiak *et al.,* 2004; Vučković *et al.,* 2012). Wheat kernels colonized by *Alternaria* species are characterized by black pigmentation in the underlying embryo regions, which compromises flour quality and grain nutritional values (Kashem *et al*., 1999).

Contamination of wheat kernels by *Alternaria* has been reported in several countries with different climates, including Italy, China, Russia, Argentina, Tunisia and Slovakia (Li *et al.,* 2000; Gannibal *et al.,* 2007; Patriarca *et al.,* 2007; Bensassi *et al.,* 2009; Mašková *et al.,* 2012; Vučković *et al.,* 2012; Ramires *et al.,* 2018; Somma *et al.,* 2019).

Several *Alternaria* species have been associated to black point symptoms, although *A. alternata* has been isolated with the greatest frequency (Somma *et al*., 2019). In addition, *A. triticina* was reported to be one of the most important causal agents of wheat leaf blight, and has been involved in black point in different geographic areas (Chaurasia *et al.,* 2000; Mercado Vergnes *et al.,* 2006; Somma *et al*., 2019). Also, *A. arborescens*, *A. infectoria* and *A. tenuissima* morpho-species have been identified in several studies (Gannibal *et al.,* 2007; Bensassi *et al.,* 2009; Scott *et al.,* 2012; Somma *et al.,* 2019; Masiello *et al*., 2020).

Some of *Alternaria* species produce secondary metabolites, including host specific toxins, which are required for fungal pathogenicity, and mycotoxins (Escrivà *et al*., 2017). The most important *Alternaria* mycotoxins are alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), and tenuazonic acid (TA), occurring with high frequency on wheat (Somma *et al.,* 2011). Based on *in vitro* and *in vivo* assays, toxicity, mutagenicity and genotoxicity of these metabolites have been proven (Lehmann *et al.,* 2006; Ostry, 2008; Zhou and Qiang, 2008), and the risks for human and animal health have been studied (Ostry, 2008; Alexander *et al.,* 2011). *Alternaria* conidia are also airborne allergens related to respiratory diseases and skin infections in humans (Cramer and Lawrence, 2003; Kilic *et al.,* 2010).

For the *Alternaria* species occurring on wheat kernels, species-specific mycotoxin profiles have not been clearly assessed. However, the morpho-species *A. alternata, A. tenuissima,* and *A. arborescens* are able to produce AOH, AME, TA, tentoxin and altertoxin (ATX)-I, II, III, whereas *A. infectoria*, a widespread species, is apparently not able to synthetize any mycotoxin (Logrieco *et al.,* 2009; Da Cruz Cabrala *et al.,* 2016).

Due to the range of demonstrated toxic effects of *Alternaria* mycotoxins, the high field occurrence of toxigenic *Alternaria* species requires correct identification to evaluate the risks they pose. *Alternaria* taxonomy is also confused. *Alternaria* species have been classified based only on morphological and physiological traits. About 280 species of *Alternaria* were described by Simmons (2007). However, due to environmental influences on the morphological traits, the close similarity between some species, and the presence of several strains with intermediary traits, many errors in *Alternaria* taxonomy and identification have occurred (Andrew *et al.,* 2009).

In consequence of molecular studies carried out using multi-locus gene sequence approaches, important taxonomic revision of *Alternaria* genus has occurred. At first, *Alternaria* morpho-species were phylogenetically analyzed and defined as species-groups (Lawrence *et al*., 2013; 2014). Further studies then elevated the species-groups to section status, defining 27 sections within *Alternaria*, also including species belonging to previous closely related genera (Woudenberg *et al*., 2015). According to this taxonomic revision, different morpho-species were synonymized, as for *A. alternata* that now includes more than 35 species (Woudenberg *et al*., 2015; Somma *et al*., 2019). The *Infectoriae* section, which mainly includes species with low or no mycotoxin capability, always forms a well-defined clade, to indicate a genus different from *Alternaria* (Somma *et al*., 2019). On the other hand, the toxigenic species are mainly placed in Section *Alternaria*, which includes the most common species on wheat, and detected in different countries.

In Lebanon, wheat grown on a total area of 41,000 ha, and producing approx. 140,000 tons of grain (FAOSTAT, 2020), is an important cereal crop used primarily for human consumption. *Alternaria* was reported on wheat kernels in Lebanon by Joubrane *et al.* (2011), but incidence was low, and data on its geographical distribution and species identification were not reported. The aims of the present study were: a) to assess contamination of durum wheat kernels by *Alternaria* species; b) to characterize, using a multi-locus sequence approach, the main *Alternaria* species occurring on wheat in Lebanon; c) to determine phylogenetic relationships between the main *Alternaria* species associated with black point; and d) to evaluate occurrence of *Alternaria* mycotoxins in wheat kernels.

MATERIALS AND METHODS

Wheat sampling and isolation of fungi

During the 2018 crop season, at harvesting time, 36 durum wheat fields were randomly selected from

the five most important Lebanese wheat production areas: Central Bekaa (ten fields), West Bekaa (12 fields), Baalbeck (five fields), Marjeyoun (five fields) and Akkar (four fields). From each field, one sample of wheat kernels was collected. Each sample consisted of approx. 1 kg of kernels, randomly gathered from five different sampling locations. In this way, 36 wheat samples were analyzed as representative of Lebanese wheat production.

For each sample, fungus isolation was carried out from 100 randomly selected kernels. The kernels were surface decontaminated with 70% ethanol (for 1 min) and 2% sodium hypochlorite (for 1 min), and then washed twice in sterile distilled water. The grains were dried on sterile filter paper under the laminar flow hood, and were then aseptically plated on potato dextrose agar (PDA) amended with 0.10 g L^{-1} streptomycin sulphate salt and 0.05 g L^{-1} neomycin. The isolation plates were then incubated at 25±1°C under a 12 h light, 12 h darkness regime for 5 d, for development of fungus colonies. The percentage of kernels contaminated by *Alternaria* species and other endophytic fungi was then determined.

Alternaria colonies originating from colonized kernels were selected to obtain mono-conidium cultures. A set of 75 representative *Alternaria* strains were selected for phylogenetic analyses, based on their geographical origins and their macro- and micro-morphological traits, according to Simmons (2007).

DNA extractions and PCR amplifications

Genomic DNA was extracted and purified from 2-d-old colonies grown on cellophane disks overlaid on PDA at 25±1°C (Habib *et al.,* 2021).

Four informative gene fragments were amplified for each fungus strain using the primer pairs gpd1/gpd2 for *glyceraldehyde-3-phosphate dehydrogenase* (*gpd*) (Berbee *et al.,* 1999), caldf1/caldr1 for *calmodulin* (Lawrence *et al.,* 2013), T1/T2 for β-*tubulin* (Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997), and alt-for/alt-rev for *allergen alt 1a* (*alt-a1*) (Hong *et al.,* 2005).

Amplification of the *gpd* partial gene was performed using GoTaq G2 Colorless Master Mix (Promega), where 25 ng of DNA template were added in mixture with a final concentration of 1× ready Master Mix, 2 mM $MgCl_2$ and 0.4 µM of each primer. *alt-a1* gene amplification was achieved using Taq DNA Polymerase (Takara) in mixture with 1× Takara PCR Buffer, 0.075 µL of Hot Master Taq DNA Polymerase (1 U μL^{-1}; 5Prime), 0.3 µL of dNTPs (10 mM), 0.45 µM of each primer and 15 ng of DNA template. *Calmodulin* and β-*tubulin* genes were amplified using Taq PCR Master Mix (Qiagen) in mixture with 1× ready Master Mix, 0.4 µM of each primer, and 50 ng of DNA template.

Amplifications were carried out in T^{100} MyCycler thermal cycler (BioRad). The PCR reactions of *gpd*, *tub* and *alt-a1* were carried out using the PCR parameters reported by Somma *et al.* (2019). PCR reactions for the *calmodulin* partial gene were: initial denaturation for 4 min at 95°C, followed by 35 cycles of 95 °C for 30 sec, 58.5°C annealing for 30 s, 72°C for 1 min, each and a final extension for 5 min at 72°C. After electrophoretic separation on agarose gel (1.5% Molecular Biology Certified Agarose, Bio-Rad Laboratories), PCR products were visualized by UV after GelRed (Biotium Inc.) staining to confirm the expected products.

Sequencing and phylogenetic analyses

Before sequencing, each PCR product was purified with the enzymatic mixture Exo/FastAP (Exonuclease I, FastAP thermosensitive alkaline phosphatase; Thermo Scientific). Sequencing of both strands of each gene was carried out using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), according to the manufacture's protocol, and the fragments were purified through Sephadex G-50 (5%) (Sigma-Aldrich), and then sequenced in an "ABI PRISM 3730 Genetic Analyzer" (Applied Biosystems). Partial sequences were assembled using the BioNumerics v. 5.1 software (Applied Maths, Inc.). A phylogenetic tree of concatenated gene sequences was generated using the maximum likelihood statistical method and bootstrap analysis (1000 replicates, removing gaps) with MEGA7 (Kumar *et al.,* 2016).

Appropriate gene sequences were downloaded from the National Center for Biotechnology Information (NCBI), and from the "*Alternaria* Genomes Database" (AGD), and were included in a phylogenetic analysis. These sequences were for the following *Alternaria* species reference strains: *A. alternata* EGS 34-016, *A. tenuissima* EGS 34-015, *A. tangelonis* EV-MIL-2s; *A. arborescens* EGS 39-128, *A. cerealis* EGS 43-072, *A. angustiovoidea* EGS 36-172, *A. consortialis* JCM 1940, *A. dumosa* EGS 45-007, *A. malorum* CBS 135.31, *A. rosae* EGS 41-130, *A. abundans* CBS 534.83, *A. triticina* EGS 17-061, *A. photistica* EGS 35-172, *A. infectoria* EGS 27-193, *A. novae-zelandiae* EGS 48-092, *A. intercepta* EGS 49-137, *A. viburni* EGS 49-147, *A. graminicola* EGS 41-139, *A. hordeicola* EGS 50-184, *A. ethzedia* EGS 37-143, *A. metachromatica* EGS 38-132, *A. oregonensis* EGS 29-194, *A. californica* EGS 52-082 and *A. conjuncta* EGS 37-139.

Mycotoxin extractions and HPLC analyses

Mycotoxin analyses were carried out for the 36 samples of wheat kernels, based on the method of Rubert *et al.* (2012), with modifications. To evaluate recovery of mycotoxins from the extraction method used, wheat samples contaminated at different amounts (0.1, 0.2 and 0.4 ppm) (n = 3) of AOH, AME, ALT and TA (Sigma-Aldrich) were assessed. Four wheat samples, G27, G25, G26 and G28 from Masiello *et al.* (2020), were also included in the analysis as positive controls for, respectively, the mycotoxins AOH, AME, ALT and TA.

Each sample of kernels (200 g) was thinly ground with an Oster Classic grinder (Madrid, Spain). Five g of each ground sample were weighed into a 50 mL capacity plastic tube containing 25 mL of methanol. For each extraction, an Ultra Ika T18 basic Ultra-turrax, Ika, (Staufen), was used for 3 min. After centrifugation, 1 mL of the supernatant was filtered on a 13 mm/0.22 µm nylon filter, and diluted before injection into high performance liquid chromatography associated with a diode array detector (LC-DAD). All the extractions were carried out in triplicate. Tenuazonic acid, AME and AOH standards were provided by SIGMA Chemical Company.

AOH, AME, ALT, and TA were determined using Merk HPLC through a diode array detector (LC-DAD) L-7455 (Merk) at 256 nm, and Hitachi Software Model D-7000 version 4.0 was used for data analyses. As the stationary phase, a Gemini C18 column (Phenomenex) 4.6 × 150 mm, 3 µm particle size was used. Themobile phase consisted of two eluents: eluent A (water with 50 µL L^{-1} trifluoroacetic acid) and eluent B (acetonitrile with 50 µL L^{-1} trifluoroacetic acid). A gradient programme with a constant flow rate of 1 mL min^{-1} was used, starting with 90% A and 10% B, reaching 50% B after 15 min and 100% B after 20 min. After that, 100% B was maintained for 1 min. The gradient was then returned to 10% B in 1 min and permitted to equilibrate for 3 min before the next analysis (Myresiotis *et al.*, 2015). The limits of detection (LOD) and quantification (LOQ) of the method used were, respectively, 0.01 and 0.1 ppm.

RESULTS

Fungus contamination of wheat kernels

The microbiological analyses carried out on the 36 wheat kernel samples showed that *Alternaria* species were the most frequent contaminants of the durum wheat kernels sampled in Lebanon. This genus was present in all the inspected fields except one, which was located in Baalbeck district. The contamination by *Alternaria* was generally high and differed among the samples according to geographic origin (Table 1). The greatest contamination was detected from West Bekaa area (average 59%; range: 36–84%), whereas the least contamination was from Baalbeck (average 2.2%; range: 0–4%).

Table 1. Fungus contamination detected in durum wheat kernels collected from different sampling areas of Lebanon.

Sampling area	Number of Fields	Fungus contamination (%)			
		Alternaria spp.		Other fungi	
		Range	Average	Range	Average
Akkar	4	46-64	55.3±5.1	0-3	1.3±0.8
Baalbeck	5	0-4	2.2±0.7	0-3	1.2±0.7
Central Bekaa	10	1-64	25.6±7.1	0-18	6.5±1.7
Marjeyoun	5	9-48	39.8±2.5	1-8	3.4±0.4
West Bekaa	12	36-84	59.0±4.1	1-25	10.2±2.4

Other fungi were also recovered from the kernels, with proportions of contamination less than that of *Alternaria*. From almost all fields, the contamination by these fungi did not exceed 10%, except in few fields located in Central and West Bekaa areas. The average values and ranges of contamination are shown in Table 1. Among these fungi, *Cladosporium* was the most frequent genus, whereas the potentially toxigenic genera *Penicillium* and *Aspergillus* were detected at low levels (1%), in, respectively, one and two fields in Central Bekaa.

Further details on the fungal contamination of kernels are provided in the Supplementary Table 1.

Phylogenetic analyses

In total, 1395 colonies of *Alternaria* were recovered from the wheat kernels assessed. From each site, pure colonies of *Alternaria* were obtained, and these were grouped based on their macro- and micro-morphological traits. Single-conidium isolates were then obtained from each group, and one to two strains per morphotype were selected from each site. In this way, 75 strains representing the population of *Alternaria* from durum wheat kernels in Lebanon (Central Bekaa, 19 strains; West Bekaa, 25 strains; Baalbeck, three strains; Marjeyoun, 12 strains; Akkar, 16 strains) underwent the phylogenetic analyses (Supplementary Table 2).

All *Alternaria* strains gave the specific PCR amplicons of the expected size for *gpd*, *calmodulin*, and *beta-tubulin* genes. Unexpectedly, 26 out of 75 field strains did not give PCR products when amplified with Alt-for/Alt-rev primer pair, so phylogenetic analyses were car-

ried out excluding *alt-a1* locus. The sequences of the three selected genes were aligned and cut at the ends to consider a common fragment for all strains.

The phylogenetic analysis of the concatenated sequences of 1451 positions resulted in a phylogenetic combining dataset comprising 90 taxa, including: 74 *Alternaria* field strains, 24 *Alternaria* reference sequences and one strain of *Stemphylium* also isolated from wheat (Altern1392) considered together with *Stemphylium vesicarium* 173-1a-13FI1M3 strain as the outgroup taxon. The phylogenetic tree, rooted to *Stemphylium* reference strain, was resolved in five well-separated clades (A-E), corresponding to *Infectoriae*, *Pseudoalternaria*, *Chalastospora*, *Ulocladioides* and *Alternaria* Sections, supported by high bootstrap values (Figure 1).

The clade A (*Infectoriae* Section) grouped 30 out of 74 field strains (41%) in three not well-supported sub-clades, except for Altern1395 which did not cluster with any strain included in this Section. The sub-clade A1 grouped 12 field strains together with the reference strains of *A. conjuncta* (EGS 37-139), *A. californica* (EGS 52-082), *A. oregonensis* (EGS 29-194), *A. hordeicola* (EGS 50-184), *A. metachromatica* (EGS 38-132), *A. graminicola* (EGS 41-139), and *A. ethzedia* (EGS 37-143). The sub-clade A2 included 13 field strains that did not cluster with any of the reference strains included in the analysis. The sub-clade A3, supported by a bootstrap value of 87, comprised four field strains showing high homology with *A. triticina* EGS 17-061 reference strain.

The two field strains Altern 1481 and Altern 1484 clustered together with *A. rosae* EGS 41-130 reference strain (clade B) belonging to *Pseudoalternaria* Section.

A well-defined clade (clade C) clustered two field strains, Altern 1381 and Altern 1382, with *A. malorum* CBS 135.31 reference strain (*Chalastospora* Section). A well-supported clade (clade D), defined as *Ulocladioides* Section, grouped *A. consortialis* JCM 1940 reference strain and two field strains, Altern 1397 and Altern1408 (Figure 1).

Of 74 *Alternaria* field strains, 38 (51%) were assigned to the *Alternaria* Section (clade E). In this section, a sub clade (Sub-Clade E1) clustered six field strains together with *A. tangelonis* (synonym of *A. gossypina*) EV-MIL-2s reference strain and *A. dumosa* EGS45-007 reference strain. A different sub-clade (Sub-Clade E2) included the reference sequences of *A. arborescens* EGS 39.128, *A. cerealis* EGS 43-072, *A. angustiovoidea* EGS 36.172 and four field strains. The strain Altern1474 showed 100% of homology with *A. arborescens* reference strain. The majority of the strains belonging to *Alternaria* section (28 of 38 strains) shared high similarity with *Alternaria* reference strains *A. alternata* EGS 34.016, and *A. tenuissima* EGS 34.015 (Sub-Clade E3). The high level of homology of these strains did not allow distinction between *A. alternata* and *A. tenuissima* morpho-species.

Mycotoxin contamination of wheat kernels

All the durum wheat samples were analyzed for the mycotoxins AOH, AME, ALT, and TA, but none of the samples were contaminated with these mycotoxins. This result was supported by inclusion of mycotoxin-positive grain samples in the same detection experiment. The grain samples G25, G26, G27, and G28 (Masiello *et al*., 2020) showed contamination with some *Alternaria* toxins. In particular, the sample G27 showed 152 ± 6 mg kg^{-1} of AOH, 231 ± 4 mg kg^{-1} of AME, 23 ± 3 mg kg^{-1} of ALT, and 189 ± 6 mg kg^{-1} of TA.

Mean recovery percentage of AOH, AME, ALT, and TA mycotoxins in the grain contaminated with 0.1 ppm was 82 ± 4%, with 0.2 ppm was 85 ± 6%, and with 0.4 ppm was 93 ± 5%.

DISCUSSION

Wheat and wheat products are among the most important staple foods consumed in Lebanon. The present study focused on characterization of *Alternaria* species isolated from durum wheat kernels during the 2018 crop season, from the five main Lebanese areas of wheat production. Almost all previous studies of mycotoxins on Lebanese wheat were focused on *Fusarium*, *Aspergillus, Penicillium*, and related mycotoxins (Joubrane *et al*., 2011; Elaridi *et al*., 2019), while occurrence of *Alternaria* has been little investigated, and has long been considered as only a quality problem for commerce.

International reports of natural occurrence of *Alternaria* species and related mycotoxins on wheat have increased. Serious toxicological risks of *Alternaria* mycotoxins for human and animals have been demonstrated (Osrty, 2008; Arcella *et al.,* 2016; Solhaug *et al.,* 2016). These risks originating from toxin levels produced by *Alternaria* species in wheat produced in Lebanon should therefore be monitored to prevent their potential harmful effects on public health.

The wheat samples analyzed in the present study were highly contaminated mainly by fungi belonging to *Alternaria*. This genus was detected from almost all sampled wheat fields, with proportions of contamination between 1 and 84%. Other reports of high levels of *Alternaria* contamination of grain (more than 90% of grains affected) have been from Serbia, Argentina, and Italy (Patriarca *et al.,* 2007; Levic *et al.,* 2012; Ramires *et*

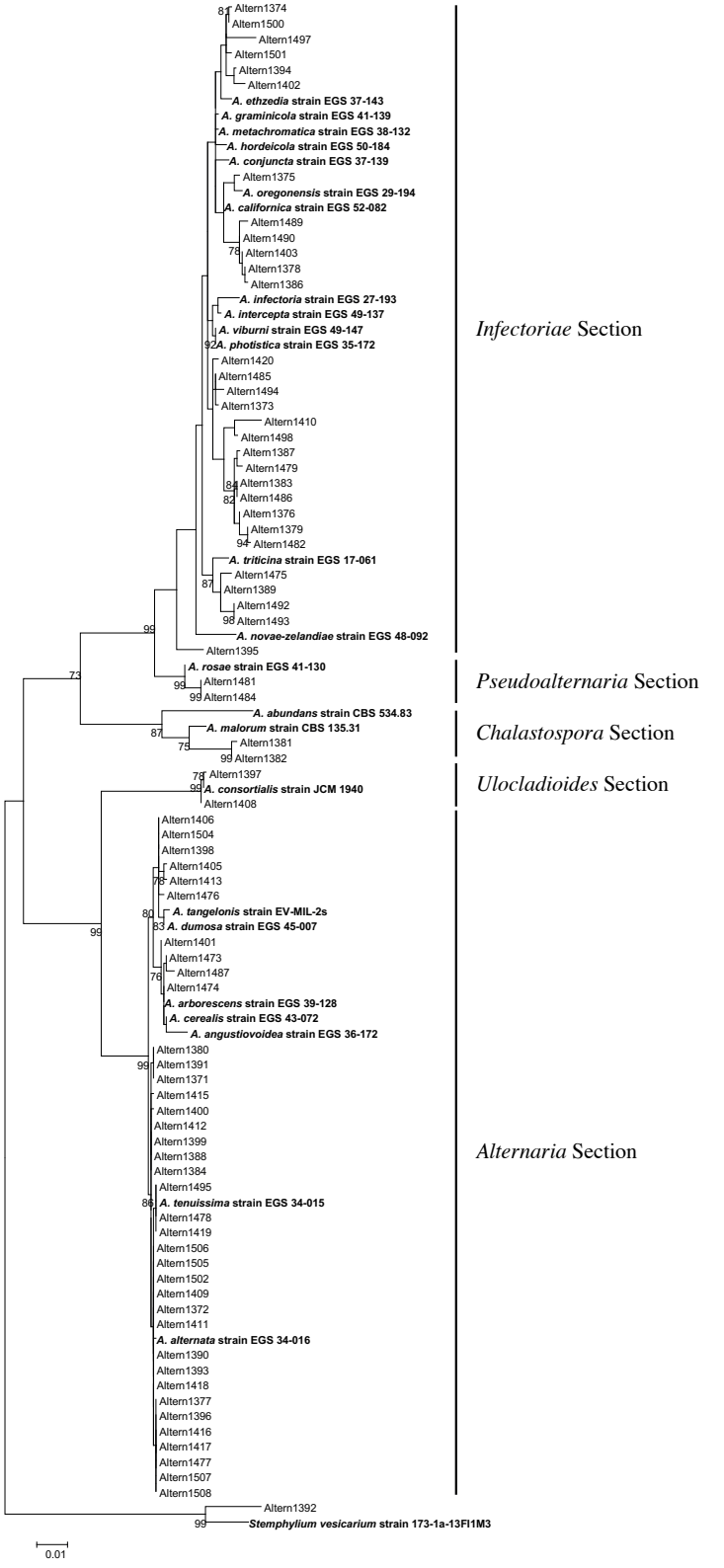

Figure 1. Phylogenetic tree generated by maximum likelihood analysis (bootstrap 1000 replicates) of combined *gpd*, *calmodulin* and β-tubulin gene sequences of 74 *Alternaria* field strains, 24 *Alternaria* reference strains, and rooted to *Stemphylium*. Bootstrap values greater than 70 are shown next to relevant branches.

al., 2018; Somma *et al.,* 2019). The results from the present study contradict those of Joubrane *et al.* (2011) for Lebanon, in which *Alternaria* species were rarely isolated (frequency of approx. 4%).

The high variability of proportions kernel fungus contamination of grains observed among the different Lebanese areas could be due to the different prevailing climatic conditions, as temperature and relative humidity are the most important physiochemical factors for *Alternaria* development (Gawai and Mangnalikar, 2018). The optimum temperature range for *Alternaria* growth is reported to be 22–28°C, and relative humidity between 60 and 100% (Gawai and Mangnalikar, 2018). This explains the lowest incidence detected in Baalbeck district, where a semi-arid climate prevails, and the average temperature and humidity were not favourable for disease development. In contrast, most of the fields with medium to high contamination percentages were mainly located in West Bekaa area, which is characterized by greater relative humidity. Nevertheless, *Alternaria* species have been isolated from other countries in a wide range of environmental conditions, because these fungi can develop in different climates at both high and low temperatures, with a wide range of relative humidity, and under multiple combinations of environmental factors (Rotem, 1994).

Although several studies have focused on *Alternaria* and closely related genera, *Alternaria* taxonomy remains controversial. Since morphological characterization can bring misidentification in taxonomy, genetic characterization is recommended for the identification at species level, or rather at Section level (Andersen *et al.,* 2009; Lawrence *et al.,* 2013; Woudenberg *et al*., 2015; Somma *et al.,* 2019). The identification of *Alternaria* species in the present study was based on sequence analyses of the fragments of *gpd* and *β-tubulin*, previously used for phylogenetic studies (Woudenberg *et al*., 2015; Somma *et al*., 2019). Based on Lawrence *et al.* (2013), the *calmodulin* gene was also included since it strongly supported the differentiation of *Alternaria* species, mostly those belonging to the *Infectoriae* Section (Lawrence *et al*., 2014). The *alt-a1* fragment, previously and widely used to characterize *Alternaria* species from wheat (Somma *et al*., 2019; Masiello *et al*., 2020), was also included in the present study. Several field strains did not amplify with *alt-a1* primers. The phylogenetic tree obtained with the remaining three gene fragments (*gpd, tub, calmodulin*) showed that all the strains that failed with *alt-a1* gene amplification belonged to *Infectoriae* Section, and were the majority of this section, i.e. 26 out of 30 strains (87%). Yet, the *alt-a1* gene was excluded from our phylogenetic analysis. A possible explanation to its failure in this study could be that a large proportion of strains of the *Infectoriae* Section from Lebanon had nucleotide polymorphisms in the sequence fragment within the annealing site of the primers. This hypothesis should be verified with further investigation.

The strains phylogenetically identified in the present study belonged mostly to *Alternaria* (51%) and *Infectoriae* (40%) sections. The *Alternaria* section clade showed that the sequences of *A. alternata* and *A. tenuissima* species are indistinguishable, and thus the two species can be merged in the same species *A. alternata*, as was proposed by Woudenberg *et al.* (2015) after whole genome and trancriptome analyses, and then confirmed by Somma *et al.* (2019). Furthermore, *A. tangelonis* synonymized under *A. gossypina* formed a supported clade from *A. alternata* in the present study. This species can be easily distinguished from *A. alternata*, in agreement with Woudenberg *et al.* (2015), who defined it as a new species. It is likely that *A. arborescens* and *A. cerealis*, defined as a species complex (Woudenberg *et al*., 2015), formed a well-separated clade from *A. alternata* in the present analysis.

On the other hand, *A. consortialis*, belonging to section *Ulocladioides*, also formed a separate clade, as in Woudenberg *et al.* (2013). Simmons *et al.* (1967) firstly established *Ulocladium* section, based upon morphological characteristics, and concluded that several atypical *Alternaria* and *Stemphylium* species should be classified as *Ulocladium*.

In addition, two field strains from wheat were assigned to a separate clade for *Chalastospora gossypii*, formerly *A. malorum* or *Cladosporium malorum* (Dugan *et al.* 1995), belonging to *Chalastospora* Section (Andersen *et al.* 2009, Lawrence *et al.* 2013). Only a few studies have reported the presence of *A. malorum* on wheat (Goetz and Dugan, 2006). This species was isolated from soil in Lebanon (Braun *et al.,* 2003), and has been reported on several host plants, including grape, chickpea, apple, and *Prunus*, and proven to be pathogenic (Zhang *et al.,* 2000; Dugan *et al.* 2002; 2005; Goetz and Dugan, 2006; Andersen *et al.,* 2009).

Based on the data obtained with the multi-locus phylogenetic tree, differentiation between *Alternaria* and *Infectoriae* sections was clearly defined, in accordance with Lawrence *et al.* (2014) and Somma *et al.* (2019). These authors deeply investigated and clustered the species of *Infectoriae* section in a phylogenetically well-defined clade very distant from the *Alternaria* clade. Field strains belonging to *Infectoriae* section represented 40% of the total number of the *Alternaria* strains, although *Infectoriae* section was shown to be the most prevalent in cereals in other studies (Dugan and Peever, 2002; Perelló and

Sisterna, 2006; Perelló *et al*., 2008). Detection of *A. triticina* in wheat kernels from Lebanon, generally considered a weak pathogen of wheat (CPC, 2018) and reported to cause leaf spot on wheat plants (Perello and Larran, 2013), agrees with recent studies carried out in Italy (Ramires *et al.,* 2018; Somma *et al*., 2019).

A clade strongly supported as the sister group of section *Infectoriae* was defined in the present study as *Pseudoalternaria*. This group was introduced as a new asexual section by Lawrence *et al.* (2016), and was typified by *A. arrhenatheri, A. parvicaespitosa* and *A. rosae* (Gannibal and Lawrence, 2016). The presence of strains belonging to *Pseudoalternaria* section was therefore demonstrated in Lebanon. These strains were previously detected in wheat in Argentina (Perello *et al*., 2008), Iran (Poursafar *et al*., 2018) and Italy (Ramires *et al*., 2018; Masiello *et al*., 2020).

Therefore, data from the present study, on the clades identified by phylogenetic analyses, agree with previous taxonomic and phylogenetic studies, showing no correlation between geographic origins and species identifications.

Species belonging to *Alternaria* section have been widely shown to produce the mycotoxins TA, AME, AOH, ALT and ATX (Logrieco *et al.,* 2009). On the other hand, studies on species of section *Infectoriae* have never shown their capability to produce mycotoxins (Andersen *et al*., 2009), although some metabolite production (e.g. of infectopyrones and novae-zelandins, which are not produced by other *Alternaria* species) has been demonstrated (Andersen *et al*., 2009, Christensen *et al*., 2005). The present results disagree with many other reports on *Alternaria* from wheat (Patriarca *et al.,* 2007; Bensassi *et al.,* 2009; Kahl *et al.,* 2015; Ramires *et al*., 2018; Somma *et al*., 2019, Masiello *et al*., 2020), because all the samples of wheat kernels from Lebanon were free of *Alternaria* toxins. One explanation could be the *Alternaria* species composition of the whole Lebanese population. Species belonging to *Alternaria* section, known to be mycotoxin producers, were detected only at 51%. On the other hand, despite the high contamination of kernels by *Alternaria*, symptoms of black point were not as frequent. This could indicate that the infections by the pathogen at harvest were at early stages, so the contamination by mycotoxins could be still undetectable.

The absence of mycotoxin contamination in Lebanese wheat could also be related to environmental factors affecting *Alternaria* mycotoxin production, as reported in Amatulli *et al.* (2013). Mycotoxins can also be produced during either pre- or post-harvest stages of transportation and storage, or during processing procedures. In general, the main factors affecting mycotoxin production are nitrogen and carbon sources, temperature, water activity (a_w) and pH (Yu *et al*., 2005; Ogelgsang *et al*., 2008). As previously reported (Oviedo *et al*., 2009, 2010; Pose *et al*., 2010), the greatest toxin production occurs with 0.98 a_w and at 25°C. Lower a_w and temperature conditions probably characterize Lebanon regions, and this could have inhibited mycotoxin production. Also, Brzonkalik *et al.* (2011, 2012) showed that the production of AOH and AME was increased by phenylalanine, whereas some nitrogen sources can inhibit production. Carbon and nitrogen sources influenced the polyketide mycotoxin, and TA biosynthesis was mainly influenced by carbon source. Thus, further investigations are strongly encouraged to determine the environmental factors involved in the inhibition of mycotoxin production by these *Alternaria* strains in Lebanese wheat.

CONCLUSIONS

This study has highlighted the widespread occurrence of contamination of wheat grain by *Alternaria* species, with contamination rates ranging from 2 to 59% depending on location. Most of the isolated strains belonged to *Alternaria* and *Infectoriae* sections. Some strains (9%) belonging to sections *Ulocladioides*, *Chalatospora* and *Pseudoalternaria* were identified for the first time in Lebanon. All samples were assayed for the presence of the most important *Alternaria* mycotoxins (AOH, AME, ALT, and TA), but none of these mycotoxins were detected. Contamination by *Alternaria* spp. can reduce the quality of wheat grain. Further research is required to verify the mycotoxin production capability of *Alternaria* strains from Lebanese wheat.

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Research Papers

Occurrence of cherry viruses in South Tyrol (Italy) by comparing growth periods in two consecutive years

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Summary. Sweet cherries (*Prunus avium* L.) are important as fruit crops, and can be affected by numerous viruses. An investigation on the occurrence of the three most common viruses of sweet cherry was carried out in commercially managed orchards in South Tyrol (Italy). The incidence of apple chlorotic leaf spot virus (ACLSV), Prunus necrotic ringspot virus (PNRSV) and prune dwarf virus (PDV) was investigated using enzyme-linked immunosorbent assays (ELISA) and the reverse transcriptase-polymerase chain reaction technique (RT-PCR) in spring 2018 and 2020, and during the summer and autumn of 2020. All three viruses were detected in the surveyed orchards. Comparative analyses showed that detection was more effective with RT-PCR than with ELISA, especially for detecting PNRSV and PDV. Mixed infections were detected in all the surveyed orchards. The results also showed clear differences between and during host growth periods, likely due to a variable virus concentration in the host trees.

Keywords. Apple chlorotic leaf spot virus (ACLSV), Prunus necrotic ringspot virus (PNRSV), Prune dwarf virus (PDV), sweet cherry, *Prunus avium*.

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INTRODUCTION

Italy has among the worlds' greatest growing areas of sweet cherries (*Prunus avium* L.). In 2018, this country ranked fifth, with a growing area of 29,010 ha, after Syria Arab Republic (30,317 ha), United States of America (34,400 ha), Chile (39,645 ha), and Turkey (82,729 ha) (FAO, 2022). Almost 90% of Italian sweet cherries are cultivated in the regions of Puglia, Campania, Emilia Romagna, and Veneto. However, sweet cherry production does not have particular temperature requirements for fruit ripening, so cultivation in altitudes up to 1,300 m a.s.l. is possible (Zago, 2003; Gamper, 2010). Hence, cherry cultivation can be an economically important supplementary crop for small farmsteads in Alpine regions.

New cherry plantings require substantial investments for protective nets against hail, birds, and insects, as well as for rain covers (Gamper, 2013). Nevertheless, good returns can be achieved for premium quality cherries

produced in South Tyrol. These result from favourable market situations in late summer, when cherries grown in warmer cultivation areas are no longer being offered, and growers from the alpine areas can supply markets at profitable prices (Zago and Ropelato, 2009; Gamper, 2010). Therefore, cherry production has increased in the last decade in South Tyrol, the northernmost province of Italy (Pirazzoli and Palmieri, 2019), and is currently producing sweet cherries on approx. 100 ha (Catalano, 2013; Martini, 2021).

Plant viruses can cause many plant diseases characterised by severe symptoms but can also remain latent in host plants (Anderson *et al*., 2004; Strange and Scott, 2005; Hull, 2013). Cherry plants can be infected by at least 29 viruses (Myrta and Savino, 2005; Kamenova *et al*., 2019). Three economically important viruses of commercial cherry cultivars are apple chlorotic leaf spot virus (ACLSV), Prunus necrotic ringspot virus (PNRSV) and prune dwarf virus (PDV) (Menzel *et al*., 2003; Yu *et al*., 2013; Rubio *et al*., 2017).

ACLSV is a member of *Trichovirus* (*Betaflexiviridae*, Martelli *et al*., 1994; Adams *et al*., 2004; Watpade *et al*., 2013), and is known to infect pome and stone fruits. The concentration of ACLSV in infected trees is low and irregular, hence reliable detection is difficult (Polák and Svoboda, 2006; Katsiani *et al*., 2014). Generally, ACLSV-infected trees are symptomless, but can be responsible for cherry necrosis (Liu *et al*., 2014), fruit deformation and discoloration, as well as graft incompatibilities in nurseries (Rana *et al*., 2011; Sánchez *et al*., 2015). However, symptoms and severity depend largely on the infected *Prunus* species and/or the infecting virus strain (Osman *et al*., 2016; Rubio *et al*., 2017). PNRSV belongs to *Ilarvirus* (*Bromoviridae*), and may cause infections of all *Prunus* species (Mekuria *et al*., 2003; Fiore *et al*., 2008). Cherry trees infected with PNRSV can exhibit a wide range of symptoms, which may include foliar mosaics, ring-shaped or spotted chlorotic areas, but symptoms may also be latent (Oliver *et al*., 2009; Sánchez *et al*., 2015). Furthermore, infections with PNRSV may lead to reduced numbers of flower buds, which leads to yield losses of 20 to 56% (Wang *et al*., 2018), and culminates in death of infected trees (Song *et al*., 2013). Like PNRSV, PDV belongs to *Ilarvirus* (*Bromoviridae*) (*Öztürk* and *Çevik*, 2015), and can infect different *Prunus* spp. (Kozieł *et al*., 2020). Depending on environmental conditions, virus strain, and plant host, PDV-infected trees often remain symptomless. This virus may cause chlorosis, yellowing, mosaic, ringspot, necrosis, and malformations on leaves, as well as reduction numbers of fruits (Predajňa *et al*., 2017). Viruses such as ACLSV, PNRSV, and PDV can occur as mixed infections in cherry orchards, and may result in decline, low yields, and unusual fruit disorders, with crop losses of up to 57% (Mandic *et al*., 2007; Yardimci and Culal-Klllc, 2011). ACLSV is only known to be transmitted by grafting and other vegetative propagation techniques. In contrast, PNRSV and PDV can also be transmitted horizontally in pollen and vertically in seeds (Rubio *et al*., 2017).

Phytopathological alterations observed in many Italian cherry cultivation areas in the last two decades have led to detailed studies of the presence of fruit tree viruses (Aparicio *et al*., 1999; Myrta *et al*., 2003; Matic *et al*., 2007; Babini *et al*., 2014). None of these studies included cherry plantings in the South Tyrol province, there is no information on the virus phytosanitary status of this small cultivation area. However, due to the expansion of cherry cultivation and the associated high investment costs in this mountainous area, it is important to obtain information on virus occurrence, to avoid yield losses caused by viruses. Reliable diagnoses of these viruses are also important for maintaining sustainable plant production. This will assist in selection of pathogen-free propagative plant material in nurseries and in orchards used for commercial production.

Routine virus diagnoses in cherry trees are based on enzyme-linked immunosorbent assays (ELISA) and the reverse transcriptase-polymerase chain reaction (RT-PCR) (Barba *et al*., 2015). There are issues with both of these methods that have been outlined elsewhere (Mekuria *et al*., 2003; Noorani *et al*., 2013; Hu *et al*., 2014; Rubio *et al*., 2017). Furthermore, the seasonal dynamics of virus concentrations throughout growing seasons may affect assay reliability (Tsai *et al*., 2012).

The aims of the present study were: (a) to obtain an overview of virus incidence in commercially managed cherry orchards in South Tyrol; (b) to compare ELISA and RT-PCR for routine diagnoses in cherry virus detection; and (c) to assess annual and seasonal fluctuations of concentrations of ACLSV, PNRSV, and PDV in South Tyrolean sweet cherry orchards.

MATERIAL AND METHODS

Sampling procedure

Eight commercially managed orchards were selected in the Val Venosta/Vinschgau valley (South Tyrol, Italy) (Table 1). The first sampling was carried out on each site in spring 2018, during the full bloom host stage (end of April until early May). Flowers were manually sampled from 30 randomly selected trees at each site (n = 240). All samples were tested immediately after sampling for the presence of three viruses (ACLSV, PNRSV, and

Table 1. Main sampling site parameters and sampling periods at each site, for virus assessments in South Tyrol cherry orchards. Site altitudes indicated (meters above sea level; m a.s.l).

Site	Location	Altitude (m a.s.l.)	Planting year	Sampling periods
A	N 46°60'92" E 10°75'47"	730	2017	Spring 2018, spring 2020
B	N 46°60'99" E 10°75'47"	730	2010	Spring 2018, spring 2020
C	N 46°63'28" E 10°64'95"	891	2015	Spring 2018, spring 2020
D	N 44°66'95" E 10°54'78"	918	2008	Spring 2018, spring 2020, summer 2020, autumn 2020
F	N 46°68'48" E 10°53'30"	1,037	2017	Spring 2018, spring 2020, summer 2020, autumn 2020
G	N 46°68'77" E 10°53'71"	1,037	2017	Spring 2018, spring 2020
H	N 46°57'40" E 10°80'19"	1,901	2016	Spring 2018, spring 2020
I	N 46°55'82" E 10°78'14"	1,901	2008	Spring 2018, spring 2020

PDV) using ELISA (Table S2). Nine trees per orchard, including those which were positive in the ELISA results 2018 (except for four trees tested positive for one of the viruses had to be replaced by negative trees, due to agronomical reasons: one tree tested positive for PDV in each of orchards A and D, and one tree tested positive for ACLSV in each of orchards D and F), were used for RNA extractions and were again sampled in spring of 2020 during full bloom at the end of April until early May, depending on the sampling site. To determine seasonal dynamics of the virus distribution in commercially managed orchards, the same nine trees of the field sites D and F, which were already tested in spring (2018 and 2020), were sampled again in summer (August) and autumn (October) 2020.

All samples (flowers, buds, and leaves) were taken randomly from different branches at different heights all around each assessed tree. Each individual sample consisted of either ten flowers in spring 2018/2020 or ten buds and ten leaf discs in summer and autumn 2020. Each sample was pooled, placed in a plastic bag, put into a cooling box, and brought to the laboratory for further processing.

Laboratory analyses

Enzyme-linked immunosorbent assays (ELISA). 0.5 g of single petals per flower were homogenised together with 4.75 mL of extraction buffer in a universal bag (Bioreba AG,) using an automatic homogenizer (Bioreba AG). The ELISA tests were carried out according to the manufacturer's instructions, with the commercially available Double Antibody Sandwich Assay (DAS-ELISA) (LOEWE® Biochemica GmbH). Absorbance values were detected at 405 nm with FLUOstar OPTIMA microplate reader (BMG Labtech). Data analyses were carried out according to the Bioreba AG technical information (BIOREBA, 2011). The cut-off was set-up individually for each plate. Absorbance values were sorted in ascending order and a histogram for each plate was created. In the resulting histograms, negative and background values could be easily distinguished from potential positive values, which were characterized by an abrupt increase in the OD value. The mean value and the standard deviation calculated from values before this abrupt increase were used to calculate the cut-off as:

$$\textit{mean value} + 3 \times \textit{standard deviation} + 10\%$$

Total RNA isolations

Immediately after sampling, petals (spring 2018 and 2020) or buds together with leaf discs (August 2020 and October 2020) were cut into pieces with a scalpel and mixed homogeneously. Approx. 0.2 g of the blended tissues was homogenized using a TissueLyser II (Qiagen), for which adaptors were pre-cooled in liquid nitrogen. RNA was extracted with RNeasy Plant Mini Kits (Qiagen) according to manufacturer's instruction. Extraction vials were cooled on ice between the extraction steps.

RT-PCR assays

One-step multiplex RT-PCR was carried out using the SuperScript™ III One-Step RT-PCR System with the Platinum™ Taq DNA Polymerase kit (Invitrogen by Life Technologies), and the primer pairs ACLSV_s/ACLSV_a, PNRSV_s/PNRSV_a, and PDV_s/PDV_a (Sanchez-Navarro *et al.,* 2005), at final concentration of 0.25 pmol μL^{-1} for each primer. A region of the chloroplast gene *rbcL*, which encodes the large subunit of ribulose bisphosphate carboxylase, was used as internal control. For the *rbcL* gene, primers Rbcl_s/Rbcl_a (Sanchez-Navarro *et al.,* 2005) were used, at final concentrations of 0.05 pmol μL^{-1}. Thermal cycling (Sanchez-Navarro *et al*., 2005) was: an initial cycle of 50°C for 30 min for cDNA synthesis, followed by a denaturation step of the RT enzyme at 94°C for 2 min, 40 cycles each at 94°C for 15 s, 50°C for 30 s, 68°C for 1 min, and a final incubation at 68°C for 7 min. The amplified PCR products were separated on 2%

agarose gels stained with GelRed® Nucleic Acid Gel Stain (Biotium Inc.), and were visually checked using Chemi-Doc™ MP with Image Lab™ v.4.0.1 (Bio-Rad Laboratories Inc.). On each gel, an artificial ladder was included, made from a mixture of amplicons of each target. This mix was prepared by cDNA amplification of reference gene fragments for ACLSV, PDV, PNRSV, and *rbcL*, with the same primers used for the RT-PCR from formerly tested positive cherry petals (Figure S1). Amplicons were cloned into the pJET1.2/blunt Cloning Vector (Thermo Fisher Scientific s.p.a.) according to the manufacturer's instructions. Inserts were amplified and sequenced with vector-specific forward and reverse primers to confirm amplification of the gene fragments by LGC Biosearch Technologies. The quality of the sequencing data was controlled with the software Geneious v.11.1.5 (Biomatters Ltd.), and sequence identity was confirmed by BLASTn search. The sequences of the three viruses were deposited in the NCBI GenBank under the accession numbers OM585596 for ACLSV, OM585598 for PNRSV, and OM585597 for PDV. Appropriate amplicons were purified with the QiaQuick PCR Purification Kit according to the manufacturer's instructions (Qiagen), and were mixed at approximately equal concentrations for the internal controls.

RESULTS

ELISA detection of viruses in 30 randomly selected cherry plants at each orchard site

All three assessed viruses were detected in the sampled cherry orchards in spring 2018 (Table 2). ACLSV was detected in four orchards, and PDV in five orchards, while PNRSV was detected in two orchards. Only orchard H showed no virus infection. The greatest number of ACLSV-positive trees were detected in orchard D in 11 samples. However, no obvious symptoms were observed on cherry trees during the orchard monitoring, except for samples I29 and I30 which were manifesting scattered chlorotic areas on the leaves. Those trees were tested positive for ACLSV (Table S1). In general, 15 (6.3 %) out of the 240 trees tested positive by ELISA for ACLSV, two (0.8 %) for PNRSV, and 11 (4.6 %) tested positive for PDV.

The following results presented focus on selected samples (n = 9 per orchard), as described in the Material and Methods (above).

Comparison of ELISA and RT-PCR detection methods in 2018

When the results of ELISA and RT-PCR were compared, not all samples positively detected by ELISA were positive by RT-PCR, and *vice versa* (Tables 3 and 4). In general, ELISA was less sensitive for detection of the viruses than RT-PCR, regardless of the virus type. However, the largest difference between the results from both techniques was for PDV, followed by PNRSV and ACLSV.

Table 2. Summary of ELISA results for ACLSV, PNRSV, and PDV on 30 randomly selected samples collected in 2018 from commercial cherry orchards in South Tyrol.

Site	No. of trees tested	ACLSV	PNRSV	PDV
A	30	0	0	4
B	30	0	0	1
C	30	0	0	2
D	30	11	0	1
F	30	1	1	0
G	30	1	0	0
H	30	0	0	0
I	30	2	1	3
Positive trees		15	2	11
Overall proportion infected		6.3%	0.8%	4.6%

Table 3. Comparison of results (based on 72 samples) obtained by ELISA and RT-PCR techniques for cherry tree samples collected during spring 2018, presented per orchard and virus. For both ELISA and RT-PCR, petals were used to determine apple chlorotic leaf spot virus (ACLSV), Prunus necrotic ringspot virus (PNRSV), and prune dwarf virus (PDV).

Site	No. samples tested	ELISA/RT-PCR		
		ACLSV	PNRSV	PDV
A	9	0/0	0/3	3/3
B	9	0/0	0/2	1/2
C	9	0/4	0/2	2/2
D	9	8/7	0/1	0/3
F	9	0/0	1/2	0/2
G	9	1/1	0/4	0/3
H	9	0/1	0/6	0/6
I	9	2/2	1/1	3/3
Positive trees		11/15	2/21	9/24
Proportions infected (%)		15.3/20.8	2.8/29.2	12.5/33.3

Table 4. Comparison of diagnostic results obtained from RT-PCR and ELISA for the detection of apple chlorotic leaf spot virus (ACLSV), Prunus necrotic ringspot virus (PNRSV), and prune dwarf virus (PDV). A total of 72 trees were tested by each method in 2018, and again in 2020 (144 test results). Petals were used as starting material. Neg. = negative; Pos. = positive.

Virus	Test	RT-PCR Neg.	RT-PCR Pos.
ACLSV	ELISA Neg.	113	11
	ELISA Pos.	3	17
PNRSV	ELISA Neg.	113	25
	ELISA Pos.	1	5
PDV	ELISA Neg.	104	27
	ELISA Pos.	4	9

Occurrence of mixed virus infections in 2018 and 2020 or both years based on RT-PCR results

Single infections with either ACLSV, PNRSV, or PDV were more predominant than mixed infections by the three viruses. (Table 5). Based on RT-PCR, the most common co-infection was by PNRSV and PDV (16.7%). Mixed infections by ACLSV and PDV were found in two samples (C18 and D08). Mixed infection by ACLSV and PNRSV was detected in only one sample (D13). Mixed infections by ACLSV, PNRSV and PDV were detected twice (A13 and H15).

Annual and seasonal fluctuations of viruses in cherry plants based on RT-PCR

Not all infections determined in 2018 were confirmed in 2020, and *vice versa*. ACLSV was detected in 11 identical samples of both years, PNRSV in four, and PDV in seven (Table S2). Generally, the number of positive samples during full bloom was greater in 2018 than in 2020 in all orchards (Table S2).

Detailed results of the infection rates of orchard D and F for all four sampling times (two years, three different seasons) are shown in Figure 1. The greatest numbers of virus positives were detected in samples taken during the full flowering stage, regardless of year and virus. ACLSV was detected in seven out of nine samples from block D in spring samples of 2018 and 2020. In summer 2020 only five and in autumn 2020 only three samples tested positive for this virus. The same applied for PNRSV and PDV. In orchards D and F, infected samples were detected when sampling was carried out during full bloom (in 2018 and 2020). Three samples tested positive in both years, spring 2018 and 2020 (PDV: D02 and F16; PNRSV: F16), while for the other samples (PDV: D06, D08, F10 and F15 for PDV; PNRSV: D13 and F20), positive infections were detected only during spring sampling in 2018 or 2020. The sample F10 was an exception, as it tested consistently positive for PNRSV throughout the vegetation periods.

Table 5. Number of trees tested positive by RT-PCR for apple chlorotic leaf spot virus (ACLSV), Prunus necrotic ringspot virus (PNRSV), and prune dwarf virus (PDV), either in 2018 and 2020 or both years. Nine trees were tested in each of eight orchards (based on 72 samples).

Site	Single infections			Mixed infections			
	ACLSV	PNRSV	PDV	ACLSV + PNRSV	ACLSV + PDV	PDV + PNRSV	ACLSV + PNRSV + PDV
A	0	2	2	0	0	1	1
B	0	0	0	0	0	2	0
C	4	1	1	0	1	1	0
D	5	0	2	1	1	0	0
F	0	2	1	0	0	2	0
G	1	3	2	0	0	1	0
H	0	1	2	0	0	4	1
I	2	1	3	0	0	1	0
Positive trees	14	10	13	1	2	12	2
Proportion infected	19.4%	13.9%	18.1%	1.4%	2.8%	16.7%	2.8%

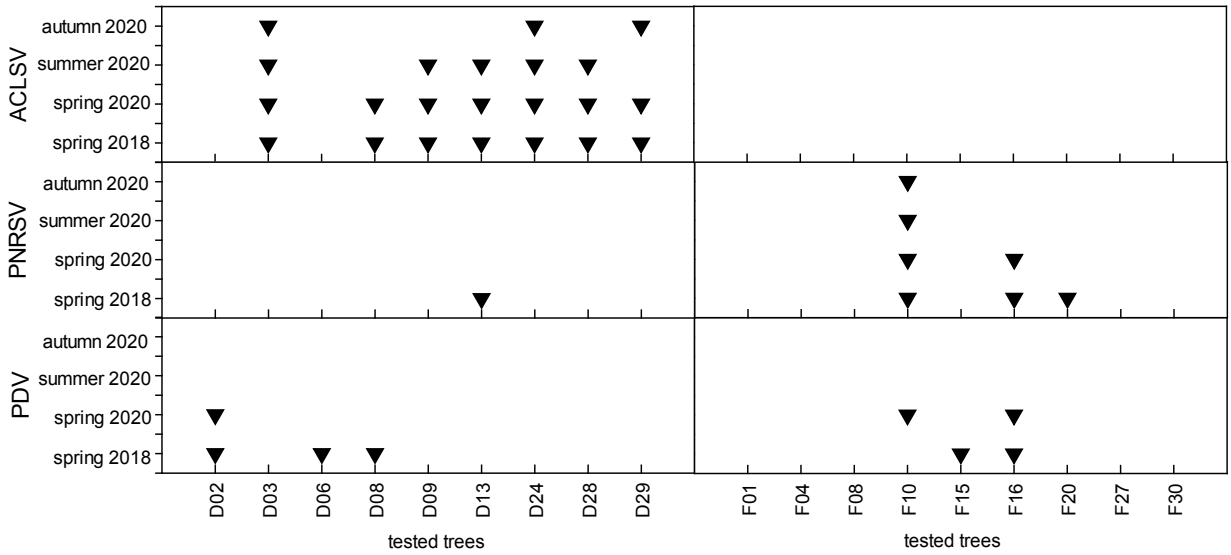

Figure 1. Seasonal fluctuations in detection of virus positive trees using RT-PCR during autumn 2020, summer 2020, spring 2020 and spring 2018 (y-axes for apple chlorotic leaf spot virus (ACLSV), Prunus necrotic ringspot virus (PNRSV) and prune dwarf virus (PDV). Each triangle indicates positively tested trees (n = 9) for orchards D (left) and F (right).

DISCUSSION

This study, using both ELISA and RT-PCR, confirmed the presence of ACLSV, PNRSV, and PDV in commercial sweet cherry orchards in South Tyrol, the northernmost province of Italy (Tables 2 and 3). Previous studies have reported of several *Prunus* species infected with these viruses in Europe and in Italy. These viruses have also been detected in plums, apricots, almonds, peaches, and sweet cherries (Barba *et al*., 1985; Savino *et al*., 1991; Myrta *et al*., 2003; Myrta and Savino, 2005; Paduch-Cichal *et al*., 2007). Recent investigations showed that over 40% of cherries in the Emilia Romagna region of Italy tested positive for ACLSV, PNRSV, and PDV (Babini *et al*., 2014).

Targeted detection of plant viruses relies on diagnosis method and choosing the correct host growth stage for sampling. Routine diagnosis for virus detection in cherry fruit trees is generally performed by ELISA and RT-PCR (Barba *et al*., 2015). In the present study, comparison of ELISA and RT-PCR as diagnostic methods for the detection of cherry viruses resulted in considerable differences, especially for detection of PNRSV and PDV, but less for ACLSV (Table 3). These results were in line with previous studies, which have reported greater infection rates for PNRSV and PDV using RT-PCR than using ELISA (Sánchez-Navarro *et al*., 1998; Yardimci and Culal-Klllc, 2011; Gospodaryk *et al*., 2013). For example, Gospodaryk *et al.* (2013) reported a nearly two-fold greater infection rate for PNRSV and PDV obtained by RT-PCR than by ELISA. These results suggested greater sensitivity for RT-PCR than ELISA, especially for the detection of PNRSV and PDV. Furthermore, Hadidi *et al.* (2011) suggested that *Ilarvirus* particles are unstable in ELISA buffers. However, a few samples detected by ELISA tests were not detected by RT-PCR (Table 4). Nevertheless, as extensively discussed in previous reports, ELISA remains the most widely used approach for detection of viruses in large numbers of samples (Mekuria *et al*., 2003; Noorani *et al*., 2013; Hu *et al*., 2014; Rubio *et al*., 2017). This is probably because the development of a multiplex RT-PCR system can be laborious and time consuming, but is a rapid, reliable, and cost-effective method (Wei *et al*., 2008). RT-PCR has been successfully used for simultaneous detection of several stone fruit viruses, even with low virus titre and in the presence of inhibitors (Bariana *et al*., 1994; Kummert *et al*., 2001). Therefore, focus in the present study was placed on the results obtained with RT-PCR.

Based on RT-PCR results of selected trees (n = 9 per orchard), PNRSV and PDV were both detected in all eight of the surveyed South Tyrolean commercial sweet cherry orchards, while ACLSV was not detected

in two of the orchards (B and F, Table 3). Considering the relatively high number of trees infected with ACLSV, especially at the sampling sites C and D, it is likely that virus-infected plant material was used when establishing these orchards (Figure 1). However, the certified status of the propagation material at purchase is known only for orchards A, F, and H. These orchards were Conformitas Agraria Communitatis (CAC) certified at the time of planting. Unlike ACLSV, PNRSV and PDV can be transmitted by grafting or vegetative propagation techniques and in pollen (Barba *et al*., 2015). Hence, pollen and seed transmission of PNRSV and PDV may have contributed to the occurrence of these in all the surveyed orchards. Viruses with these transmission modes have high distribution potential.

Most surveyed trees (n = 9 per orchard) were infected either with ACLSV, PNRSV, PDV, or with combinations of these viruses (Table 5). Mixed infections of these viruses were reported for cherry orchards in previous studies (Myrta *et al*., 2003; Yardimci and Culal-Klllc, 2011; Gospodaryk *et al*., 2013). In the present study, the combination of PNRSV and PDV occurred most frequently. As suggested by Gospodaryk *et al.* (2013), mixed infections may be the result of the different transmission modes, geographical origins, and grafting, which may contribute to wide distribution of these viruses.

In the present study, virus detection using both ELISA and RT-PCR was found to be variable, with clear differences between and during host growth periods. Detections were maximum during full flowering in spring of both years (2018 and 2020; Table S2 and Figure 1). Uneven distribution of virus in individual trees is a likely explanation for the detection discrepancies between and during growth periods. (Knapp *et al*., 1995; Spiegel *et al*., 1997; Marbot *et al*., 2003). The same phenomenon could also explain differences between sampling years, and influences of weather conditions are also likely to have contributed to the seasonal and annual discrepancies experienced in the present study.

Honjo *et al*. (2020) suggested that in natural systems, the concentration of viruses within hosts may change greatly during growth periods, depending on virus replication and host growth. In the present study, the uneven virus distribution of PNRSV and PDV within trees varied particularly between the two years. For example, only three samples tested positive in spring 2018 and 2020, while for six trees a positive infection was detected at least once, either in spring 2018 or 2020. However, samples F10 (for PNRSV), and D03 and D24 (for ACLSV) showed constant infection status throughout the growth periods. It is likely that the virus concentration was high in these trees, and/or the viruses were uniformly spread throughout the host trees. To obtain reliable results and to prevent false negatives, samples consisted of pooled homogenized tissues, taken from around each tree at different heights. However, even pooling the collected samples from different parts of each same tree, may not sufficiently overcome the effect of uneven virus distribution. Further studies are required to take account of the discrepancies observed. Greater numbers of samples per tree should be examined. Although care was taken to avoid errors during processing samples, human error can never be excluded. Furthermore, no differences in virus prevalence were found for different altitudes or ages of trees, although more studies are required to confirm these results.

CONCLUSIONS

Virus infections can seriously compromise the phytosanitary status of fruit tree nurseries, and commercially managed orchards. In the present study, all three assessed viruses (ACLSV, PNRSV and PDV) were detected in cherry orchards, either by ELISA or RT-PCR. However, RT-PCR was more sensitive than ELISA for detection of these viruses, and especially for PNRSV and PDV. Virus detection was variable and was different during different host growth stages. Future research should include host sampling from different altitudes, orchard ages, host cultivars, and propagation material at time of acquisition, to better understand the observed phenomena.

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A SYBR Green qPCR assay for specific detection of *Colletotrichum ocimi*, which causes black spot of basil

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Summary. *Colletotrichum ocimi* causes black spot of basil (*Ocimum basilicum*) and is a serious threat to basil cultivation as it compromises leaf production. The pathogen also infects seeds, which could become primary sources of inoculum for spread of black spot. A SYBR Green real-time PCR assay was developed to detect *Colletotrichum ocimi* in basil leaves and seeds, based on the partial *β-tubulin* (*tub2*) gene sequence. Two primer sets were designed and tested. The selected primer pairs produced amplicons of 130 bp. The real-time PCR assay was validated for analytical specificity, sensitivity, selectivity, repeatability and reproducibility. The assay was specific for *C. ocimi* with respect to ten *Colletotrichum* spp. and to another 12 pathogens of basil plants. Sensitivity was 1 pg μL^{-1} of genomic fungal DNA and amplification analyses were not influenced by basil genomic DNA. The assay detected and quantified *C. ocimi* in artificially inoculated basil leaves. This is the first specific primer set for *C. ocimi*, which allows rapid detection and quantification of the pathogen is a useful tool for diagnostics in plants. Detection in seeds would also be possible, but will require an optimized extraction method. The qPCR detection of *C. ocimi in planta* can contribute to adoption of effective preventive disease management strategies.

Keywords. Anthracnose, molecular diagnostics, *Ocimum basilicum*, seeds.

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INTRODUCTION

Colletotrichum includes important species pathogenic to a range of plant hosts (Dean *et al.*, 2012). *Colletotrichum ocimi* causes black spot of basil (*Ocimum basilicum*), an economically important crop cultivated in many countries, and, particularly, in the Mediterranean area (Damm *et al.*, 2014). Black spot is the common name for this anthracnose disease, which can affect basil leaves and seeds (Gullino *et al.*, 1995), and is a serious threat to seed-pro-

duction companies and basil producers (Cacciola *et al.*, 2020). Gullino *et al.* (1995) reported isolation of a *Colletotrichum* sp. from basil, which was initially identified as *C. gloeosporioides*. (Damm *et al.* (2014) later allocated these isolates to the *C. destructivum* species complex (SC), describing it as *C. ocimi*. Several other *Colletotrichum* species have been reported on basil: *C. capsici* in India (Alam *et al.*, 1981), *C. destructivum* in Italy (Cacciola *et al.*, 2020), *C. siamense* in Malaysia (Ismail *et al.*, 2021) and *Colletotrichum* sp. in Florida, United States of America (Alfieri *et al.*, 1984).

Before the 1990's, species of *Colletotrichum* were identified using a combination of morphological and cultural characters. The most investigated features were shape and size of conidia and appressoria, presence or absence of setae, sclerotia, acervuli, sexual morphs, and cultural characteristics (Cannon *et al.*, 2000). However, these characters alone were considered inadequate for species identification, due to their variability depending on environmental and culture conditions (Cai *et al.*, 2009). Molecular tools have been considered more reliable and were subsequently used along with morphological observations. Particularly, multi-locus phylogenetics is used for *Colletotrichum* species identification (Hyde *et al.*, 2009). Up to 13 different loci are available for delineation of species within a SC (Liu *et al.*, 2016; Talhinhas and Baroncelli, 2021). Bhunjun *et al.* (2021) considered the ITS region as useful for reaching a SC-level identifications, with *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) and *β-tubulin* (*tub2*) as the most informative loci for species-level identification. However, the polyphasic approach suggested for *Colletotrichum* species identification is laborious and time-consuming (Cai *et al.*, 2009; Du *et al.*, 2021).

The quantitative real-time PCR technique has been used as a rapid and sensitive diagnostic method for the identification of *Colletotrichum* spp. (Mirmajlessi *et al.*, 2015). Schena *et al.* (2017) developed a duplex qPCR TaqMan method to detect and quantify *C. godetiae* and *C. acutatum sensu stricto* in olive tissues. An assay for the simultaneous detection of *C. acutatum* and *C. gloeosporioides* from infected strawberry leaves by real-time PCR was developed by Rahman *et al.* (2019). Du *et al.* (2021) elaborated a real-time PCR method to detect, quantify and monitor *C. siamense* infecting rubber trees, and Kamber *et al.* (2021) developed a specific quantitative real-time TaqMan PCR assay that allowed rapid and reliable detection and quantification of *C. lupini* in infected seeds and plant material. These specific molecular techniques provide effective and rapid tools for *Colletotrichum* species identification and quantification in symptomatic and asymptomatic plant tissues. These methods could be implemented by phytosanitary services and companies to assess the health status of seeds and propagation material (Kumar and Gupta, 2020). However, no methods have yet been developed to identify *C. ocimi* or other species of the *C. destructivum* SC.

Considering the important losses that black spot disease can produce on basil seedlings and plants, the present study aimed to develop a SYBR Green qPCR assay to facilitate the detection and quantification of *C. ocimi* in basil leaves and seeds.

MATERIALS AND METHODS

Fungal isolates and DNA extraction

Isolates of *C. ocimi* (Table 1) were taken from stock cultures maintained at -80°C in the culture collection of the AGROINNOVA Centre of Competence (University of Torino), and grown on potato dextrose agar (PDA, VWR Chemicals) amended with 25 μg μL^{-1} of streptomycin sulphate (PDA-S, Sigma-Aldrich). Genomic DNA was extracted from 0.1 g of mycelium, using the E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-Tek), following the manufacturer's instructions, and was stored at -20°C. The DNA concentration of each sample was measured using NanoDrop 2000 (Thermo Fisher), and was then adjusted to 1–50 ng μL^{-1}.

Species-specific primer design

Damm *et al.* (2014) reported that the partial *tub2* gene sequence of *C. ocimi* was less than 97% similar to sequences of this locus of other *Colletotrichum* spp., so this region was selected for primer design. Sequences of the *tub2* region retrieved from the GenBank database were: *Colletotrichum ocimi* (CBS 298.94, KM105502; CVG189, MN535124; CVG190, MN535125; CVG193, MN535126; CVG200, MN535128; CVG 202, MN535129; CVG 203, MN535127; CVG 204, MN535130 and CVG 205, MN535131), and *Colletotrichum* spp. within the *C. destructivum* SC (*C. americae-borealis* CBS 136232, KM105504; *C. bryoniicola* CBS 109849, KM105461; *C. destructivum* CBS 136228, KM105487; *C. higginsianum* CPC 19379, KM105464; *C. lentis* CBS 127604, JQ005850; *C. lini* CBS 172.51, JQ005849, and *C. utrechtense* CBS 130243, KM105481). Selection of species within the *C. destructivum* SC was based on phylogenetic distance within the complex, with closely related species selected. All sequences were aligned with the software Mega v. 7 (Kumar *et al.,* 2016), and a region with high polymorphism with respect to the non-target species was selected for primer design. Two primer pairs were designed and

Table 1. List of isolates used in this study to assess the specificity of the primer pairs designed for the detection of *Colletotrichum ocimi* (TubOc_23fw - TubOc_190rev and TubOc68fw - TubOc_197rev).

Sample number	Species/Sample	ID Code	Host
1	*Colletotrichum ocimi*	CVG189	*Ocimum basilicum*
2	*Colletotrichum ocimi*	CVG190	*Ocimum basilicum*
3	*Colletotrichum ocimi*	CVG193	*Ocimum basilicum*
4	*Colletotrichum ocimi*	CVG200	*Ocimum basilicum*
5	*Colletotrichum ocimi*	CVG202	*Ocimum basilicum*
6	*Colletotrichum ocimi*	CVG204	*Ocimum basilicum*
7	*Colletotrichum ocimi*	CVG205	*Ocimum basilicum*
8	*Colletotrichum fioriniae*	CVG175	*Salvia leucantha*
9	*Colletotrichum bryoniicola*	CVG257	*Salvia nemorosa*
10	*Colletotrichum fructicola*	CVG170	*Salvia greggii*
11	*Colletotrichum utrechtense*	CBS 130243	*Trifolium pratense*
12	*Colletotrichum americae-borealis*	CBS 136232	*Medicago sativa*
13	*Colletotrichum lentis*	CBS 127604	*Lens culinaris*
14	*Colletotrichum higginsianum*	CPC 19379	*Brassica chinensis*
15	*Colletotrichum lini*	CBS 172.51	*Linum usitatissimum*
16	*Colletotrichum destructivum*	CBS 136228	*Crupina vulgaris*
17	*Stagonosporopsis vannaccii*	PHB1	*Ocimum basilicum*
18	*Stagonosporopsis vannaccii*	PHB8	*Ocimum basilicum*
19	*Alternaria arborescens*	PHB29	*Ocimum basilicum*
20	*Alternaria alternata*	BASALT 5/10	*Ocimum basilicum*
21	*Alternaria tenuissima*	BASALT 2/10	*Ocimum basilicum*
22	*Plectosphaerella cucumerina*	CVG886	*Ocimum basilicum*
23	*Rhizoctonia solani*	22reis	*Ocimum basilicum*
24	*F. oxysporum* f. sp. *basilici*	FOB001	*Ocimum basilicum*
25	*Myrothecium verrucaria*	BAS 5-18	*Ocimum basilicum*
26	*Myrothecium follicola*	BAS 4-18	*Ocimum basilicum*
27	*Myrothecium roridum*	BAS cv Eleonora	*Ocimum basilicum*
28	*Sclerotinia* sp.	36bas	*Ocimum basilicum*
29	*Peronospora belbahrii*	-	*Ocimum basilicum*
30	*Colletotrichum nigrum*	CVG171	*Salvia greggii*

validated *in silico* using the reference strains listed above, using NCBI Primer-Blast (Ye *et al.*, 2012): TubOc_23fw - TubOc_190rev amplifying 168 bp and TubOc_68fw - TubOc_197rev amplifying 130 bp (Table 2).

Table 2. Sequences of the primers designed for the specific detection of *Colletotrichum ocimi*.

Primer name	Primer sequence 5'->3'	Amplicon (bp)
TubOc_23fw	GCCTTTTGGTGCGTAGTCA	168 bp
TubOc_190rev	GGTGAATACGTGGTCAGGGC	
TubOc_68fw	CGACCTGGAAAGGAATAACTCGT	130 bp
TubOc_197rev	GGTTAGCGGTGAATACGTGGT	

End-point PCR amplification, analytical specificity and sensitivity

Preliminary tests were carried out to define the optimal final concentrations of $MgCl_2$ (0.5, 1 and 1.5 mM),

primers (0.5 and 1 µM), DMSO (0.5, 0.7 and 1.2 µL) and template (20, 50 and 100 ng μL^{-1}). The end-point PCR reactions were carried out using a Taq DNA polymerase kit (Qiagen), in a total volumes of 25 µL each. The optimized mixture composition was as follow: 2.5 µL of Qiagen Buffer 10×, 0.5µL $MgCl_2$ (25mM), 0.5 µL dNTPs (10mM), 0.5 µL of each primer (10µM), 0.2 µL of Qiagen Taq DNA polymerase (5U) and 1 µL of DNA as template (20-50 ng μL^{-1}) The thermal cycler conditions were: 94°C for 3 min, followed by 30 cycles each of 94°C for 45 s, 64°C for 45 s, and 72 °C for 1 min, and a final extension cycle at 72 °C for 5 min. The PCR products were examined by electrophoresis on 1% agarose gels (VWR Life Science AMRESCO^R biochemicals), stained with GelRed^{TM} in Tris-acetate buffer. The primers were tested to evaluate their specificity using strains of species within the *C. destructivum* SC (Damm *et al*., 2014) and of species reported as pathogens on basil plants and seeds (Garibaldi *et al*., 1997; Gilardi *et al*, 2018). All strains used are listed in Table 1. Analytical sensitivity tests were carried out by conducting PCR reactions using *C. ocimi* DNA of strain CVG190 (Guarnaccia *et al*., 2019), which was 10-fold serially diluted (10 ng μL^{-1} to 100 fg μL^{-1}). The Limit of Detection (LOD) of these tests was assessed.

SYBR green real-time PCR (qPCR) assay development

The primers which provided the best results in conventional PCR analyses were selected and used for qPCR with SYBR Green. The real-time PCR assays were carried out using a StepOne Plus™ Real-Time PCR System thermal cycler (Applied Biosystems). Preliminary tests were conducted to define the optimal final concentrations of the primers (3, 1 or 0.3 µM) and the optimal annealing temperature (60 or 64°C). Reactions were carried out with the optimised mixture composition in a total volume of 10 µL, using 5 µL of 10× Power SYBR Green Mastermix, 0.3 µL of each primer (100 µM) and 1 µL of DNA as template (20–50 ng μL^{-1}). The optimal amplification conditions were: 95°C for 3 min, followed by 40 cycles each at 95°C for 15 s and at 64°C for 35 s. The melting curves were acquired after each run by ramping the temperature from 60°C to 95°C. Each reaction was performed in triplicate, and the results were displayed using the StepOne software.

SYBR green real-time PCR (qPCR) assay validation

The protocol was verified by evaluation of analytical specificity, sensitivity, selectivity, repeatability and reproducibility. For specificity, qPCR was performed in triplicate on all the strains listed in Table 1, using strains of *C. ocimi* as positive controls. Analytical sensitivity of the qPCR assay was evaluated by a standard curve obtained using *C. ocimi* DNA of strain CVG190 10-fold serially diluted, ranging from 10 ng μL^{-1} to 100 fg μL^{-1}. The LOD of the method was assessed, along with the correlation coefficient (R^2) between the cycle threshold (Ct) and the initial concentration of genomic DNA and the mean relative efficiency. Another test was conducted on *C. ocimi* genomic DNA mixed with host plant DNA, to simulate interference from host plant DNA and to establish selectivity. Plant DNA was extracted after grinding in liquid nitrogen, using an E.Z.N.A. Plant DNA kit according to the manufacturer's instructions. *Colletotrichum ocimi* DNA of strain CVG190 was ten-fold serially diluted (from 10 ng μL^{-1} to 100 fg μL^{-1}) with basil genomic DNA (10 ng μL^{-1}) extracted from healthy leaves. Each reaction was performed in triplicate and the results were displayed through the StepOne software. The standard curve was used as internal control to quantify *C. ocimi* DNA in different samples. Three independent assays were conducted to determine the repeatability of the method. The reproducibility was assessed by two different operators on different days.

Detection of Colletotrichum ocimi *in artificially inoculated basil leaves*

Nine artificially inoculated leaf samples of *O. basilicum* were collected from basil plants of the 'Genovese' type cultivar 'Italiano classico' (Royal Seeds). Conidial suspensions (10^6 conidia mL^{-1}) were sprayed onto pathogen-free 2-month-old plants, which were considered pathogen-free because no visual symptoms were found on leaves examined under a stereo-microscope (Leica EZ4). Control plants were sprayed with sterile water. The plants were then covered with transparent plastic film to maintain high relative humidity, and were transferred to a growth chamber maintained at 25°C with a 12 h light 12 h dark regime. The plastic film was removed at 3 d post-inoculation (Guarnaccia *et al*., 2019). DNA was extracted from 0.1 g fresh weight of symptomatic or non-inoculated leaves. DNA was measured using NanoDrop 2000 (Thermo Fisher Scientific) and diluted to reach a concentration of 10 ng μL^{-1}. The obtained DNA was analysed with the SYBR green Real-time PCR (qPCR) assay described above.

Detection of Colletotrichum ocimi *in artificially inoculated and non-inoculated commercial basil seeds*

Conidial suspensions in water could not be used to inoculate seeds, due to gum production by basil seeds

after their immersion. Glycerol has been reported as plasticiser of this gum (Amini *et al.*, 2015). Preliminary tests were therefore conducted by soaking seeds in glycerol solutions to find the best concentration to avoid gum production (data not shown). To inoculate basil seeds, a conidial suspension of *C. ocimi* was prepared in 70% glycerol (VWR Chemicals), with a final concentration of 10^6 conidia mL^{-1}. Seed samples were exposed to thermal shock for 30 s in liquid nitrogen and then soaked for 1 h in the suspension. Control seed samples were soaked in 70% glycerol. During soaking, samples were gently shaken at 95 rpm. Each seed sample was then recovered on plastic plates with sterile absorbent paper and incubated at 25°C for 72 h in the dark. Two inoculation trials were conducted, each using six batches of seeds (1 g of seeds for each batch) of the 'Genovese' type cultivar 'Edwina' (Enza Zaden), with three replicates per batch for a total of 18 samples. After the first trial, DNA of each inoculated sample was extracted directly after the inoculation. After the second trial, each inoculated sample was first washed with 5 mL of sterile distilled water to remove glycerol residuals, and DNA extraction was then carried out. Ten commercial batches of seven basil cultivars of the 'Genovese' type were each sampled in triplicate (30 samples) of commercial basil seeds. (Table 3). The seeds had no visible symptoms under a stereo-microscope (Leica EZ4). The cultivars were selected among those known to be susceptible to black spot (Guarnaccia *et al.*, 2019; Cacciola *et al.*, 2020). After grinding in liquid nitrogen, DNA of 0.1 g of each seed sample was extracted using an E.Z.N.A. Plant DNA kit, according to the manufacturer's instructions. DNA concentrations were measured using NanoDrop 2000. The obtained DNA was analysed with the SYBR green Real-time PCR (qPCR) assay described above.

Table 3. List of commercial basil seed cultivars of the 'Genovese' type reported as susceptible to black spot, and used in the present study to detect and quantify the presence of *Colletotrichum ocimi*.

Batch number	Cultivar
1	Aromatico
2	Genovese Gecom FT
3	Genovese ISI 602 F1
4	Italiano classico
5	Italiano classico
6	Italiano classico
7	Italiano classico
8	Italiko
9	Profumo
10	Superbo

RESULTS

Species-specific primer design, analytical specificity and sensitivity

The two designed primer sets were tested to assess their analytical specificity and sensitivity in end-point PCR. The primer pair TubOc_23fw - TubOc_190rev amplified DNA of *C. ocimi* strains, but also gave non-specific amplification with DNA of *C. bryoniicola* (CVG257), *C. americae-borealis* (CBS 136232), *C. lini* (CBS 172.51), *C. destructivum* (CBS 136228) and *Sclerotinia* sp. The primer pair TubOc_68fw - TubOc_197rev gave best results, by amplifying only DNA of *C. ocimi* strains (Figure 1, A and B). In analytical sensitivity tests, the LOD of the end-point PCR was 1 ng μL^{-1} for the primer set TubOc_23fw - TubOc_190rev, and 0.1 ng μL^{-1} for the primer set TubOc68fw - TubOc_197rev (Figure 2). The analytical specificity and sensitivity assays showed that the primer pair TubOc68fw - TubOc_197rev was appropriate for subsequent assays (Supplementary Figure 1).

SYBR green real-time PCR (qPCR) assay validation

The SYBR green real-time PCR with the selected primer pair was able to amplify the 130 bp fragment of the *tub2* partial gene of the *C. ocimi* strains, but not of the other species listed in Table 1. The specificity of the primers was also confirmed by presence of a single dissociation peak in the melting curve at 81.84 ± 0.22°C (Figure 3). The DNA of *C. ocimi*, serially diluted from 10 ng μL^{-1} to 100 fg μL^{-1} in sterile distilled water, was used to build a standard curve to evaluate the analytical sensitivity of the detection method (Figure 4, Supplementary Table 1). The LOD of the test was at 1 pg μL^{-1} (Ct = 36.45 ± 0.44). The correlation coefficient (R^2) between the cycle threshold (Ct) and the initial concentration of genomic DNA was >0.99. The mean value of the regression slope was –3.22, and the mean relative efficiency was 104%, which showed good qPCR efficiency (Adams, 2006). The presence of plant DNA together with fungal DNA had no influence on the selectivity of the primers in the SYBR green assay (Supplementary Table 2). A similar PCR efficiency and a reliable correlation between the Ct values and the amount of DNA of *C. ocimi* was

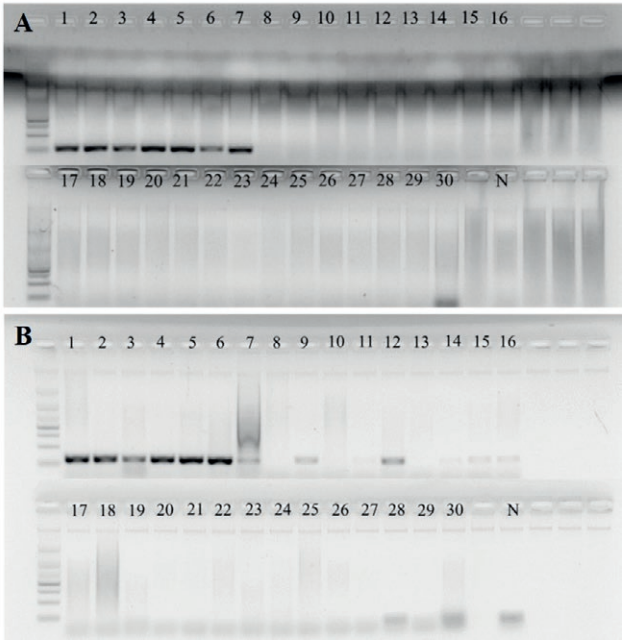

Figure 1. A) End-point polymerase chain reaction (PCR) specificity of the primer set TubOc_68fw TubOc_197rev. The numbers of tested *Colletotrichum ocimi* strains correspond to those reported in Table 1. N = negative control. **B)** End-point polymerase chain reaction (PCR) specificity of the primer set TubOc_23fw TubOc_190rev. The numbers of the tested strains correspond to those reported in Table 1. N = negative control.

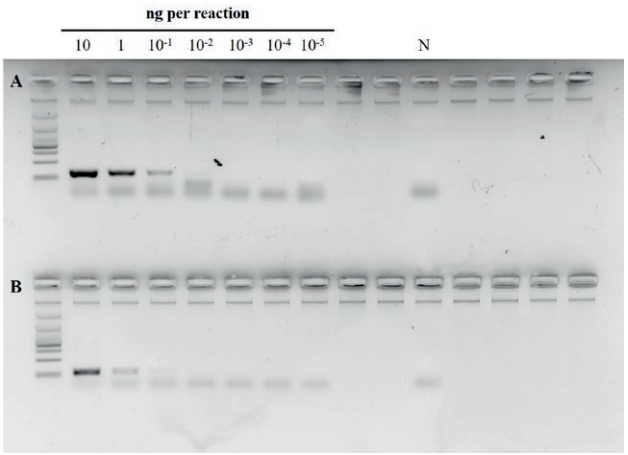

Figure 2. End-point polymerase chain reaction (PCR) analytical sensitivity of the two primer sets with a 10-fold serial dilution at the indicated concentration of genomic DNA of *Colletotrichum ocimi* strain CVG190. A = primer set TubOc_68fw TubOc_197rev; B = primer set TubOc_23fw TubOc_190rev. N = negative control.

found from the amplification of *C. ocimi* DNA of strain CVG190 diluted in basil genomic DNA, compared to amplification of the samples diluted in water. The assay

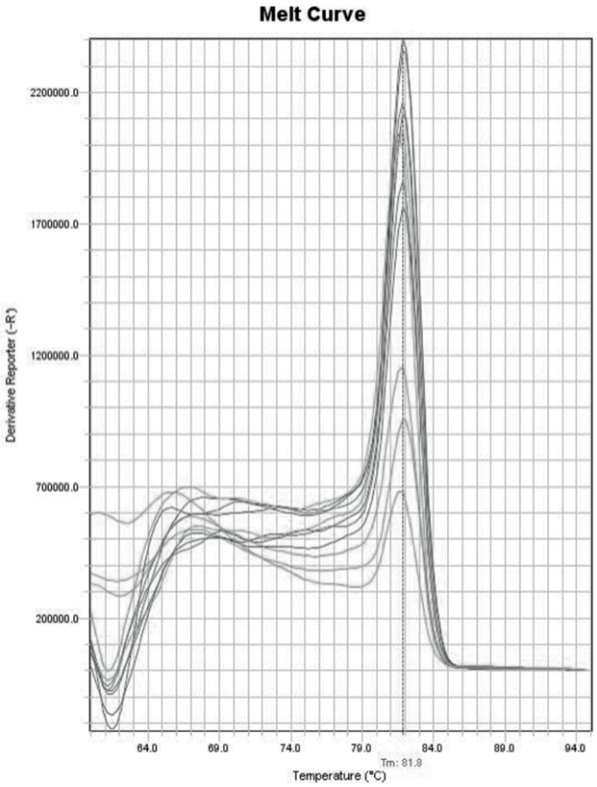

Figure 3. Single dissociation peaks of melting curves at 81.84 ± 0.22°C, which confirmed the specificity of the primer set TubOc_68fw TubOc_197rev. The peaks were obtained from the amplification of the dilutions (from 10 ng μL^{-1} to 100 fg μL^{-1}) in water of the *Colletotrichum ocimi* genomic DNA of strain CVG190.

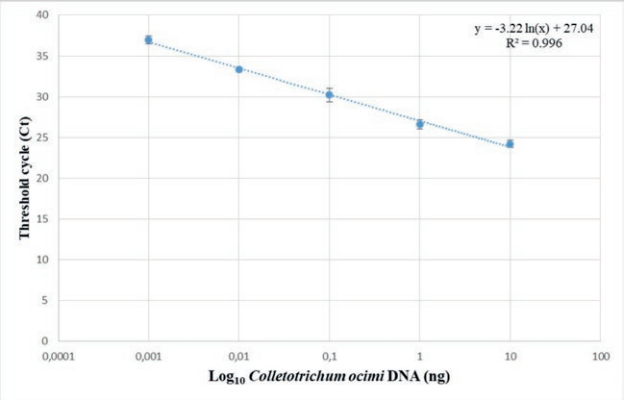

Figure 4. Standard curve obtained with *Colletotrichum ocimi* genomic DNA (strain CVG190). The Ct values were plotted against the *C. ocimi* DNA concentrations (from 10 ng μL^{-1} to 1 pg μL^{-1}). Standard deviation bars, the linear regression equation and the R^2 value are displayed on the graph. The dilutions were run in triplicate.

was performed on different days by two different operators and its reproducibility was confirmed.

Detection of Colletotrichum ocimi *in artificially inoculated basil leaves*

The SYBR green assay was used to detect and quantify *C. ocimi* in symptomatic basil leaves. The assay detected presence of *C. ocimi* in eight of the nine samples tested. In seven samples, the pathogen was detected at 0.247 to 0.001 ng μL^{-1}. One sample was below the LOD of the test (Ct = 38.26 ± 0.72). DNA samples obtained from non-inoculated basil leaves were negative for the presence of *C. ocimi*.

Detection of Colletotrichum ocimi *in artificially inoculated and commercial basil seeds*

The SYBR green assay was used to detect and quantify *C. ocimi* in artificially inoculated and in commercial basil seeds. For the artificially inoculated seeds, the first assay was conducted with DNA of basil seeds directly extracted after inoculation. The Ct values determined from four samples (one replicate each) were below the LOD of the assays (Ct = 37.13; 38.20; 39.03 or 38.15). For all other replicates, the assay was negative for the presence of *C. ocimi*. A second assay was therefore conducted on DNA of seeds extracted after inoculated seed washings. This second assay was detected presence of *C. ocimi* in four out of 18 tested samples. In two samples, the pathogen was found in a range of 0.004 and 0.001 ng μL^{-1}. The other two samples were below the LOD (Ct = 37.73 ± 0.09 and Ct = 38.93 ± 0.26). DNA samples obtained from non-inoculated basil seeds were confirmed as negative for the presence of *C. ocimi*. The assay was negative for the presence of *C. ocimi* in the commercial basil seeds sampled.

DISCUSSION

Molecular techniques are reliable and effective for the specific detection and quantification of plant pathogens (Mirmajlessi *et al.*, 2015). The present study describes the development of end-point PCR and SYBR Green real-time qPCR assays for the specific detection of *C. ocimi* in basil leaves and seeds. Both techniques were used to assess their analytical specificity and sensitivity. Selectivity, repeatability and reproducibility of the SYBR Green real-time qPCR assay were also determined. The amplification reactions were performed by increasing the annealing temperature to 64°C. With the obtained increased stringency, one primer set (TubOc_23fw -TubOc_190rev) still gave non-specific amplification. However, the amplification reaction with the other primer set (TubOc68fw - TubOc_197rev) allowed the specific detection of *C. ocimi*, avoiding the problem of cross-amplification. The LOD of the test was 1 pg μL^{-1} of *C. ocimi* DNA. This detection threshold was within the range of different assays that target other *Colletotrichum* spp. such as *C. lupini* (10 pg μL^{-1}), *C. godetiae* and *C. acutatum s.s.* (10 pg μL^{-1}) and *C. theobromicola* (1.4 pg μL^{-1}) (Schena *et al.*, 2017; Kamber *et al.*, 2021; Kaur *et al.*, 2021). Both analytical sensitivity and selectivity tests showed good qPCR efficiency within the established range of acceptability for developing a new detection method, from 90 to 110% (Adams, 2006).

This new assay can be applied to DNA directly extracted from fresh plant material and was not affected by co-extracted plant DNA. The assay allowed detection and quantification of *C. ocimi* in symptomatic artificially inoculated basil leaves. The pathogen was also detected from 0.1 g fresh weight of symptomatic leaves, in a range of 0.247 and 0.001 ng μL^{-1}. Future studies are planned to test the method also on naturally symptomatic leaves.

For seeds, the assay conducted with DNA directly extracted from seeds inoculated with conidial suspension of *C. ocimi* in glycerol permitted the detection of the pathogen on one replicate of four samples. The obtained values were positive, but below the LOD of the method, and since they were observed only in one replicate, the repeatability parameter of the assay could be affected (Cardwell *et al.*, 2018). A second assay was therefore conducted washing inoculated seeds before DNA extraction to reduce glycerol residuals. This second trial gave detection the pathogen on four inoculated samples out of 18, and quantification was possible on two of these samples. The second extraction method was used for quantification of *C. ocimi* on a subset of commercial seeds selected for testing, where the pathogen was not detected since no amplification curves and no peaks in the melting curves were observed during the qPCR analyses.

Globalization of markets and the centralization of companies in some regions have contributed to long distance exchanges of plant materials and seeds. Due to this international trade, different pathogens can be transmitted *via* uncertified material, causing disease outbreaks in new areas (Gullino *et al.*, 2014; Munkvold and Gullino, 2020). Specific pathogen detection methods are therefore required. The assay developed in the present study is the first molecular diagnostic tool developed to detect *C. ocimi*, and is a useful tool for the rapid identification and quantification of the pathogen on symptomatic basil leaves. For seeds, the method had a low detection success rate. Further research is therefore required to improve DNA extraction. This could be the most

important step, due to the production of gum and presence of possible inhibitors within the basil seed coats or endosperm.

Traditional approaches to identify *Colletotrichum* spp. based on morphological and molecular data require isolation, DNA extraction and multi-locus sequencing, are time-consuming, and are applicable only to a restricted range of samples due to the high cost of the analyses (Bhunjun *et al.*, 2021). Although a precise cost analysis should be carried out, SYBR Green real-time PCR with melting curve analysis is reported to be cost-effective, easy to use, and have optimal efficiency for small amplicons (Capote *et al.*, 2012). For this reason, this technique was selected to develop the protocol described in the present study. The technique produced reliable and accurate diagnoses, and can reduce the necessary analysis time to a few days instead of weeks.

Colletotrichum ocimi can be transmitted *via* basil seeds, as reported in previous studies (Guarnaccia *et al.*, 2019; Cacciola *et al.*, 2020). Thus, detection of the pathogen on inoculated seeds, demonstrated in the present study, even at a low rate, confirms that this diagnostic tool could be useful for further investigations of commercial seeds, with an improved DNA extraction method. Furthermore, the end-point PCR assay could be used as a screening method to detect the presence or absence of *C. ocimi*, and to estimate its relative quantity through agarose-gel visualization.

No specific primers have been previously designed to detect *C. ocimi*. The developed and validated assay described here was shown to be specific for its target organism. Further studies are planned, aiming to improve detection of the pathogen on seeds or seedlings and to develop effective and preventive disease management strategies for basil production.

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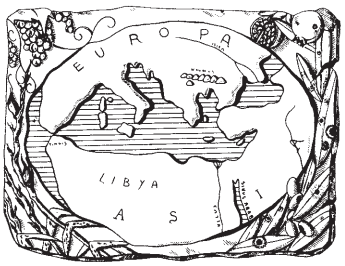

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