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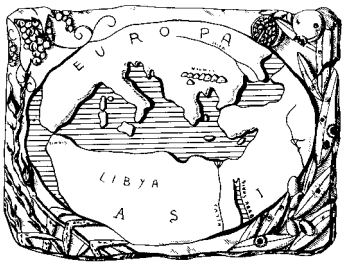

PHYTOPATHOLOGIA MEDITERRANEA

Plant health and food safety

The international journal edited by the Mediterranean Phytopathological Union
founded by A. Ciccarone and G. Goidànich

Phytopathologia Mediterranea is an international journal edited by the Mediterranean Phytopathological Union The journal's mission is the promotion of plant health for Mediterranean crops, climate and regions, safe food production, and the transfer of knowledge on diseases and their sustainable management.
The journal deals with all areas of plant pathology, including epidemiology, disease control, biochemical and physiological aspects, and utilization of molecular technologies. All types of plant pathogens are covered, including fungi, nematodes, protozoa, bacteria, phytoplasmas, viruses, and viroids. Papers on mycotoxins, biological and integrated management of plant diseases, and the use of natural substances in disease and weed control are also strongly encouraged. The journal focuses on pathology of Mediterranean crops grown throughout the world.
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Research Papers

Factors affecting *Venturia oleaginea* infections on olive and effects of the disease on floral biology

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Summary. The disease olive peacock eye was evaluated in organic olive orchards in Palestine (Asira, Burquin, Qabatia and Sir). In each orchard, six trees were randomly chosen (three pruned and three unpruned). Severity was estimated at 20 d intervals by determining the percentage of infected leaves in the upper, mid and lower parts of the canopy of each tree. Proportions were estimated of symptomatic leaves, and those with asymptomatic infections using the NaOH test. In spring, inflorescence length was determined just before flowering (white stage). The proportions of fruit set were also calculated on small, labeled branches. Among the different sites/orchards tested, there were significant differences in the infection level, which were related to climatic conditions. Pruning reduced the susceptibility of the trees to the disease. The infections were much greater in the lower parts of the canopy. Peacock eye greatly reduced inflorescence length and fruit set. *Venturia oleaginea* severity was negatively correlated with inflorescence development and fruit set, due to defoliation caused by the pathogen. Based on effects on fruit set, the disease could severely reduce olive yields. This is the first report showing quantitative relationship between olive defoliation caused by peacock eye and reproductive activity.

Key words. *Olea europaea* L., organic olive growing, pruning, peacock eye, *Spilocaea oleaginea*, Palestine.

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INTRODUCTION

Peacock eye (olive leaf spot), caused by *Venturia oleaginea* (Castagne) Rossman & Crous (= *Spilocaea oleaginea*), provokes severe damage to olive in all olive-growing countries. In recent years, the incidence and severity of this disease have increased in Palestine, likely resulting from climate change (Salman *et al.*, 2011). Peacock eye is considered the most important disease attacking olive trees in Palestine, where olive production is very important, contributing approx. 13% to the national income (Salman *et al.*, 2011; Abuamsha *et al.*, 2013; UNCTAD, 2015).

Venturia oleaginea mainly attacks olive leaves, sometimes fruit peduncles and rarely tender shoots and fruits (Agosteo and Schena, 2011). The first symptoms of the disease are small, roundish, black spots, which may be arranged in concentric circles with an olive-green to dark olivaceous centre and peripheral rings with reddish-brown to yellow or green halos (Agosteo and Schena, 2011). The infected leaves fall prematurely and defoliation negatively affects the vegetative and reproductive activities of the trees (Graniti, 1993). Tree susceptibility to the disease largely depends on environmental conditions (it is favored by high humidity of the air), plantation density, cultivar and leaf age (old leaves are less susceptible) (Graniti, 1993; Obanor *et al*., 2008, 2011). Favourable conditions for disease development are temperatures between 15 and 20 °C and persistent rain. At these temperatures, duration of leaf wetness required for infections is the least. Progress of the disease is reduced by hot, dry conditions (Obanor *et al*., 2008, 2011).

Although pruning is known to reduce humidity and shading within olive tree canopies, and consequently disease incidence and severity, no data are available on these effects. Therefore, the positive role of pruning for reducing peacock eye is hypothetical, but has never been experimentally demonstrated. Little information is available on the quantitative effects of factors that favour the disease, and on effects of the disease on the reproductive activity of the trees. Furthermore, no clear relationships are known regarding the effects of the disease on flowering, fruit set and final olive yields. It is known that the disease mainly attacks the lower parts of tree canopies, but no quantitative data are available to evaluate its distribution in the different parts of the canopies.

The aims of the present study were to: 1) acquire more knowledge on peacock eye in Palestine, and on environmental conditions which affect development and presence of the disease in different areas of the country; 2) establish and quantify the effects of pruning on the disease; 3) determine and quantify the distribution of the disease in the different parts of olive tree canopies; and 4) measure and quantify the effects of the disease on floral biology (inflorescence growth and fruit set). The acquired knowledge of factors affecting the development and severity of peacock eye will assist the definition of disease management strategies, particularly in relation to the different conditions occurring in olive producing countries of the Mediterranean area.

MATERIALS AND METHODS

This study was carried out in the West Bank, which is a landlocked territory forming the bulk of the Palestinian territories and the State of Palestine. The West Bank climate is characterized by long, hot and dry summers with no or little rainfall from the end of April-beginning of May to October. However, rainfall and temperature vary according to altitude and location (Lodolini *et al*., 2016, 2017). Four representative organic olive groves were chosen. They were located in Jenin and Nablus, which are the two provinces with the greatest concentration of cultivated olive. The groves were chosen to represent the diversity in topography, altitude and climatic conditions (temperature, relative humidity, rainfall), which characterize the study area. Two orchards were located in sub-areas (Sir and Qabatia) which are known to develop low levels of infection, and the other two were located in sub-areas with high levels of infection (Northern Asira and Burqin). The altitudes of these four areas are: Northern Asira 120 m above sea level, Sir 178 m, Burqin 233 m and Qabatia 268 m (PCBS, 2014). The olive cultivar in all four orchards was Nabali, which is the most important in Palestine. The trees were adult, spaced at 7 × 7 m and trained to the globe system. The orchards were managed with two to three tillages each year and organic fertilization. There were no treatments applied to control peacock eye.

Some climatic data (temperature, relative humidity of the air and rainfall) were recorded at meteorological stations near the olive orchards. In each orchard, six trees were randomly chosen. Three of the trees in each orchard were subjected to pruning before starting the investigation, while the other three were not pruned. This was to allow evaluation of the effects of pruning on peacock eye disease development. On each tree, nine branches were selected as follows: three in the upper part of the canopy (the upper third), three in the middle part (the middle third) and three in the lower part (the lower third). For each branch selected, all of the leaves were counted, and peacock eye symptomatic leaves were counted. These observations were repeated at approx. 20 d intervals. At the same time, the NaOH test was used to determine asymptomatic disease in leaves collected from branches close to the labelled branches. From each designated part of the canopy, three samples (one per tree) of 20 leaves were used. These leaves were immersed in a solution of 5% NaOH at 60–70°C for 2–4 min. The leaves were then examined and the numbers of infected leaves (recognizable by the appearance of dark circular spots) was recorded. The data collected from the labelled branches and the determination of the amounts of asymptomatic infection on leaves were used to calculate the proportions of fallen leaves (defoliation), symptomatic leaves and infected asymptomatic leaves for each of the three parts of the canopy. The observations

on leaves were carried out from January to the first days of May 2016, and only leaves grown in 2014 and 2015 were considered. In particular, the defoliation caused by the disease on the labeled branches was determined by counting the number of nodes on branches grown in 2014 and 2015. Because each node produces two leaves, it was possible to calculate the number of leaves present on each branch in the absence of leaf fall. Leaves survive for approx. 2.5 years, so it was hypothesized that the leaves that were no longer on the labeled branches had fallen mainly because of peacock eye. Therefore, fallen leaves caused by peacock eye were taken as the difference between the number of leaves derived from the multiplication of the number of nodes × 2 and the leaves that were present at the last sampling (effective defoliation). Because all the *V. oleaginea*-infected leaves fall, the total potential percent defoliation was calculated as the effective percent defoliation plus the percent symptomatic leaves plus the percent infected asymptomatic leaves.

The new leaves that developed from March of 2016 were not considered because of the short period from bud sprouting to the last sampling at the beginning of May.

In spring, the number of inflorescences on the labeled branches was determined, and approx. 1 month after flowering the number of fruits was also determined. This was to allow calculation of the ratio between the number of fruits and inflorescences, to give the amount of fruit set.

Immediately before flowering (when the inflorescences were white), three samples of ten inflorescences per tree were collected from each of the three parts of the canopy, and inflorescence lengths were measured.

Data were statistically analyzed by ANOVA according to a factorial experimental design (site/time of observation/pruning/part of the canopy), and the averages were compared by the Student-Newman-Keuls Test. Some data were also presented as simple linear regressions of some variables.

RESULTS

Climatic parameters of the areas studied

Some climatic parameters of the experimental sites are summarized in Table 1. Temperature (T) and relative humidity (RH) of the air were available only for the Northern Asira and Sir sites, whereas rainfall was available for all four sites. Northern Asira had lower T and higher RH than Sir. Asira and Burquin had the highest rainfall amounts.

Table 1. Climatic data of the experimental sites in Palestine examined in this study. Temperature and relative humidity values are means ± standard errors.

Area	Average air temperature (°C, Dec 2015 – May 2016)	Relative humidity of the air (%, Dec 2015 – May 2016)	Cumulated rainfall (mm, Sept 2015 – May 2016)
Northern Asira	15.1 ± 1.9	71.9 ± 4.7	560.7
Burqin			493.5
Qabatia			409.8
Sir	18.1 ± 2.1	61.7 ± 4.3	409.2

Amounts of leaf infection

Data on the effects of the factors (site/environment, time of observation, pruning, part of the canopy) on the amounts of leaf infection were statistically analyzed using a factorial design. There were statistically significant effects for all the factors, but significant interactions between factors were very few, so these are not presented.

Effects of the different sites

All the parameters used to evaluate the amounts of infection were significantly affected by the site/environment (Figure 1). The percentages of symptomatic leaves and infected asymptomatic leaves were greatest in Northern Asira, followed by much lower values in Burqin and then lower still for Qabatia and Sir. A similar trend was observed for effective and total potential defoliation. The amounts of effective and total potential defoliation were very high in Northern Asira and high in Burqin.

Effects of time

The percentage of symptomatic leaves decreased from the beginning of the experiment (January) to February, and then increased and reached a maximum in March before decreasing thereafter (Figure 2). A similar pattern was observed for the percentage of infected asymptomatic leaves.

Effects of pruning

Pruned trees had lower proportions of symptomatic and infected asymptomatic leaves than unpruned trees (Figure 3). A similar result was observed for both the

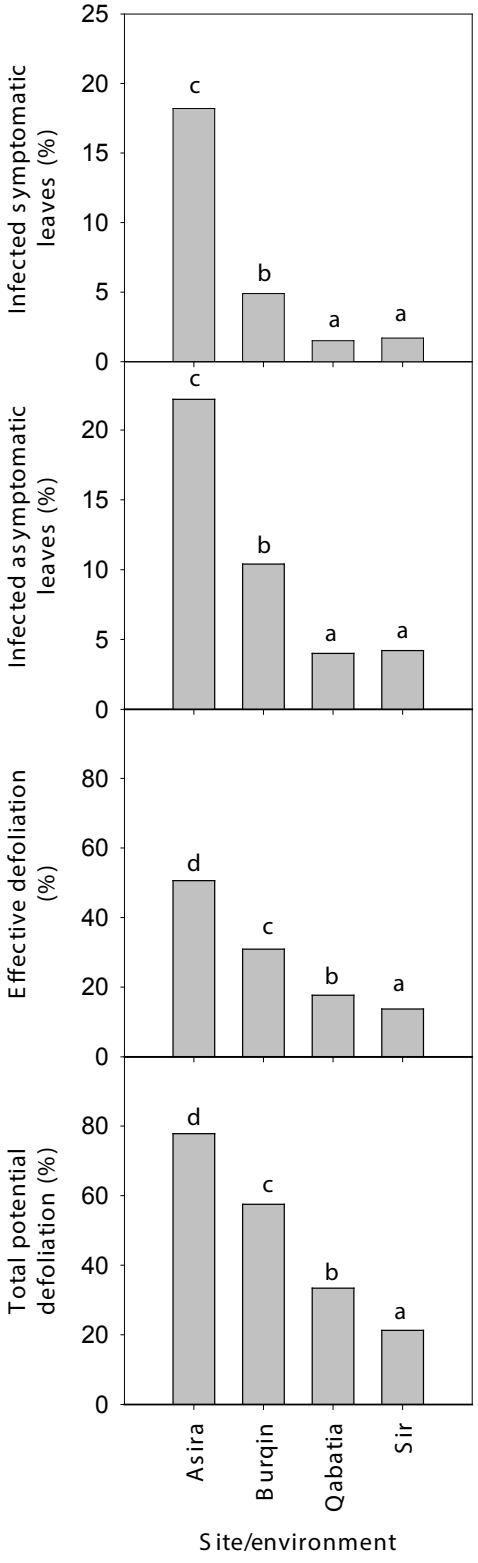

Figure 1. Means of olive tree defoliation and *Venturia oleaginea* infections as influenced by different sites/environments in Palestine. Means accompanied by the same letter are not significantly different ($P \leq 0.05$).

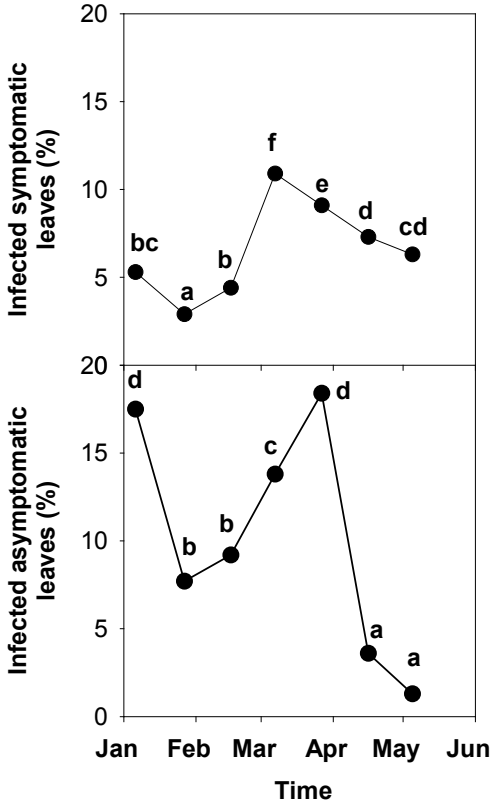

Figure 2. Mean proportions of olive leaf infections by *Venturia oleaginea* as influenced by the time of observation. Means accompanied by the same letter are not significantly different ($P \leq 0.05$).

effective and total potential defoliation proportions.

Effect of leaf position in the canopy

The proportions of symptomatic leaves and infected asymptomatic leaves were both affected by the position of the leaves in the tree canopies (Figure 4). The greatest infection percentages were always in the lower parts of the canopies and the least infection was in the upper parts. The greatest percentages of effective and total potential defoliation were in the lower parts of the canopies.

Floral biology and fruiting

All the factors considered in this study affected the mean length of the olive inflorescences (Figure 5). The longest inflorescences were found in Qabatia, whereas the shortest were in Northern Asira. Intermediate inflorescence length occurred in Burqin and Sir. The inflorescences were longer on pruned trees and in the upper parts of the canopies. Fruit set was significantly affected

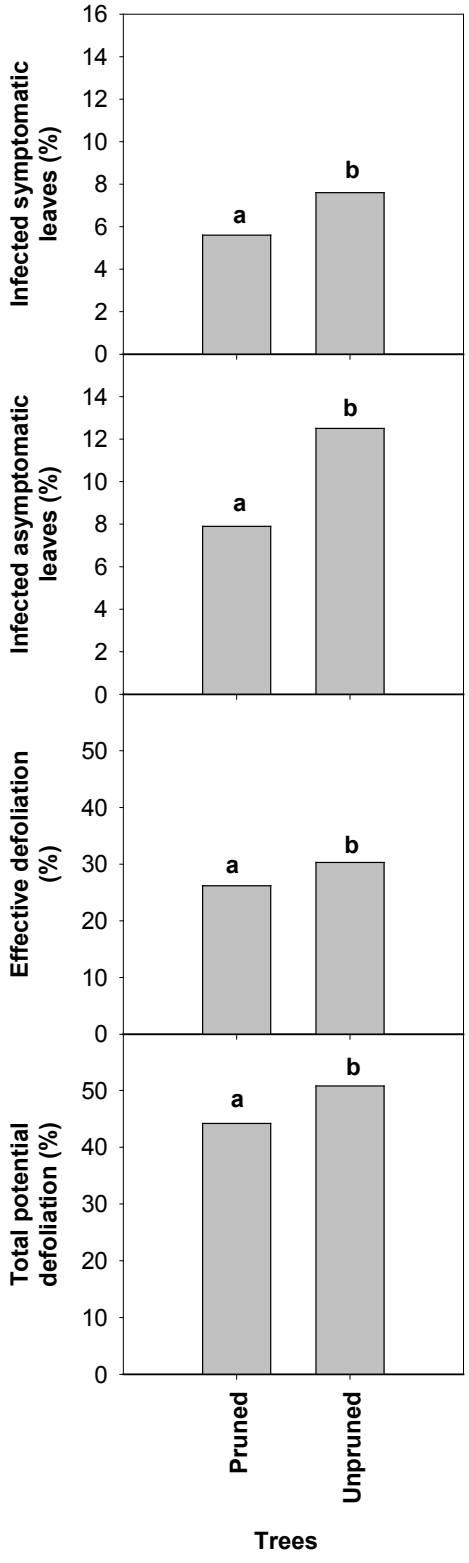

Figure 3. Means of olive tree defoliation and *Venturia oleaginea* infections as influenced by pruning. Means accompanied by the same letter are not significantly different ($P \leq 0.05$).

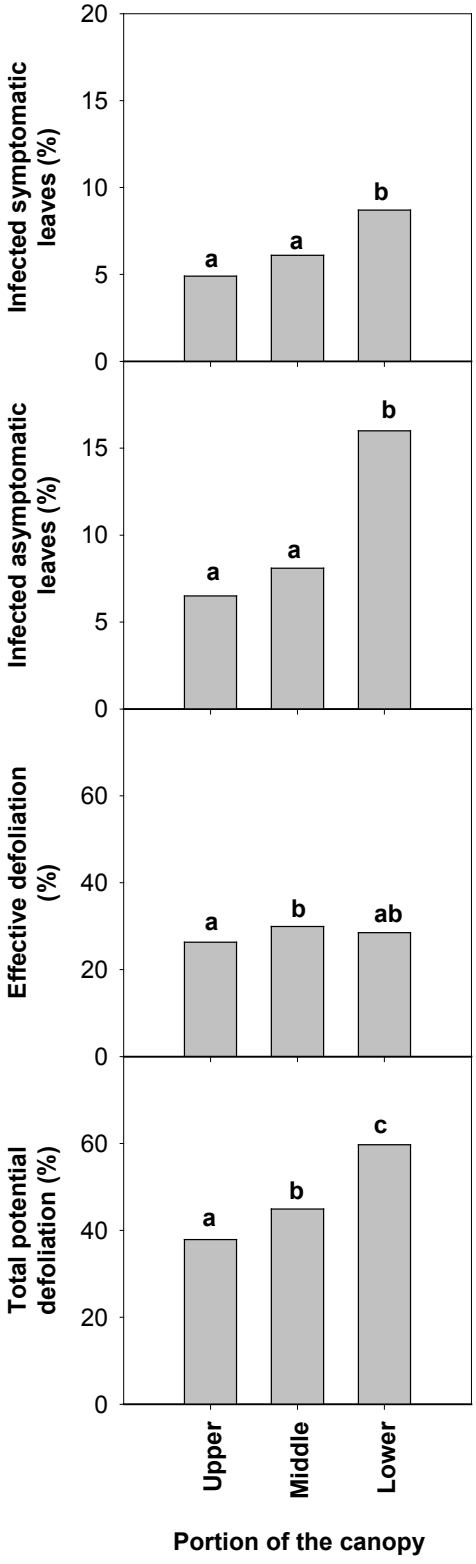

Figure 4. Means of olive tree defoliation and *Venturia oleaginea* infections as influenced by three different tree canopy positions (upper, middle and lower). Means accompanied by the same letter are not significantly different ($P \leq 0.05$).

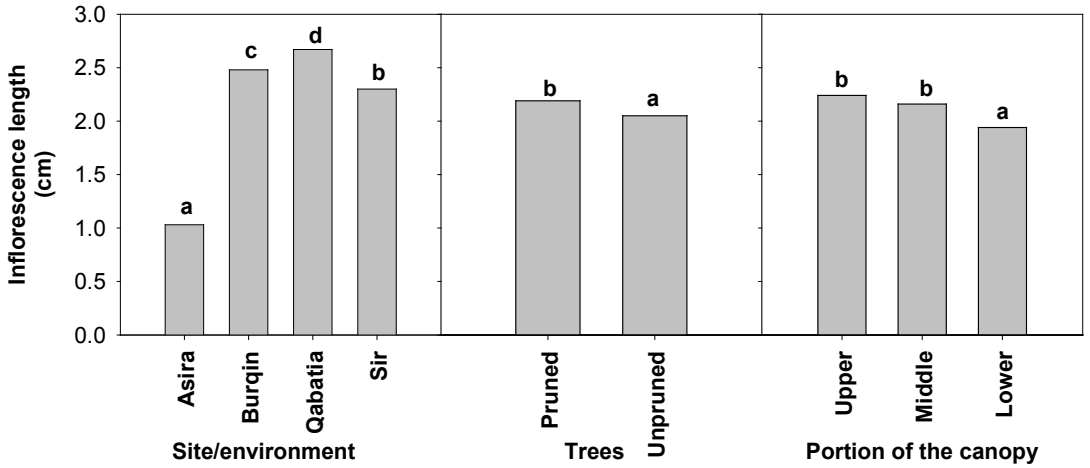

Figure 5. Mean olive inflorescence lengths as influenced by the site/environment, pruning and portion of tree canopy. Means of each factor accompanied by the same letter are not significantly different ($P \leq 0.05$).

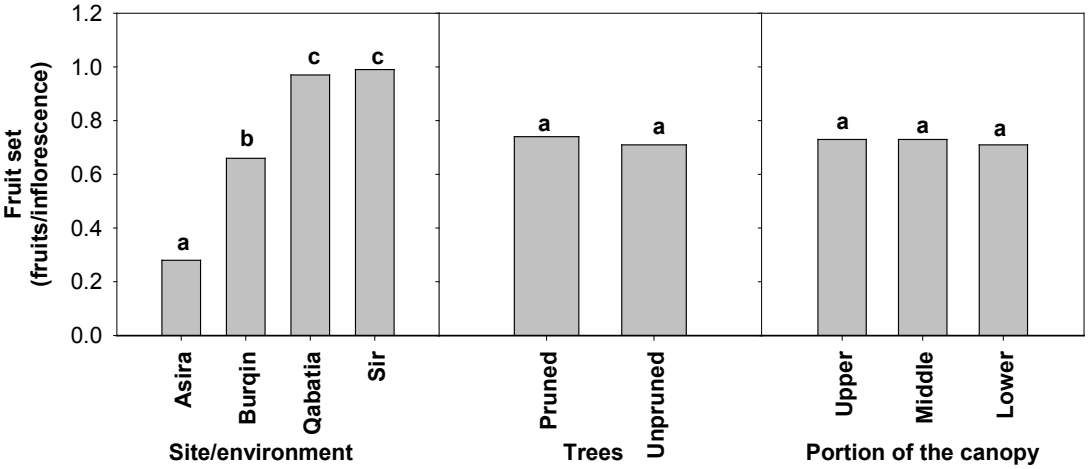

Figure 6. Mean fruit set values for olive trees as influenced by the site/environment, pruning and portion of tree canopy. Means of each factor accompanied by the same letter are not significantly different ($P \leq 0.05$).

by the site/environment, but was not affected by pruning or position in the tree canopies (Figure 6).

DISCUSSION

This investigation has shown a significant influence of all the considered factors on the severity of peacock eye occurring in olive orchards.

The effects of site/environment were most likely due to the different climatic conditions at the different experimental sites. In particular, Northern Asira, where the greatest amounts of infected leaves and defoliation were found, had the greatest rainfall and high values of RH, along with relatively cool temperatures. These conditions are particularly favourable for peacock eye development, as conidium production by *V. oleaginea* is optimal at 15°C, especially when humidity is high (Azeri, 1993; Guechi and Girre, 1994; Obanor *et al.*, 2008). In contrast, the lowest amounts of infected leaves and defoliation were observed at Sir, where the lowest rainfall and RHs, and the highest temperatures, were recorded. Using the rainfall data, which was available for all four sites, the important and positive influence of high humidity resulting from high rainfall on disease development was demonstrated. There was a significant positive linear correlation between the cumulated rainfall at the four sites and the levels of infection and defoliation that were recorded (Figure 7). For rainfall, the period

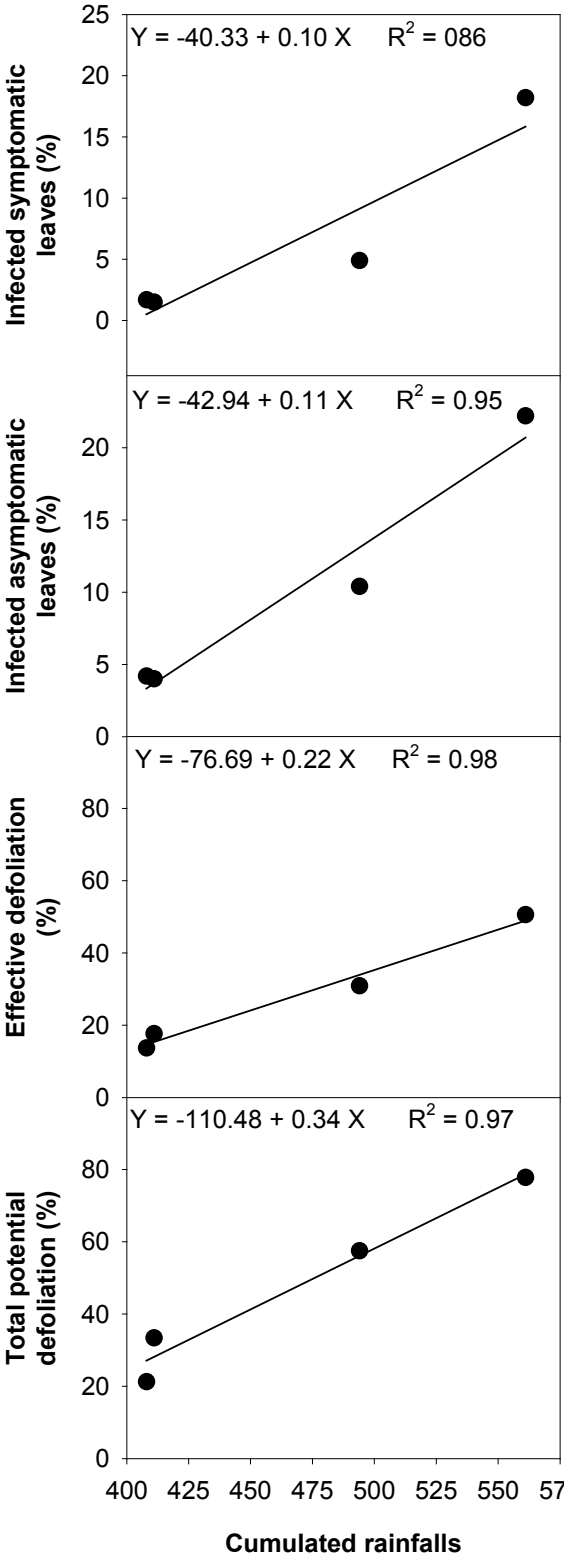

Figure 7. Relationships between cumulated rainfall in the four areas of Palestine (Asira, Burqin, Qabatia and Sir) and infections/defoliations caused by peacock eye of olive.

from September to May was considered as it is when most of the rain occurs and so it is representative of the climatic conditions of the area. Therefore, environmental factors, in particular RH and T, appear to be the major elements in determining differences in the infection and spread of peacock eye disease among the considered sites. Indeed, high humidity and relatively cooler conditions are the most suitable for the disease (Salerno, 1966; Saad and Masri, 1978; Graniti, 1993; Obanor *et al.*, 2008; 2011). These results are similar to the those of previous observations in Palestine, which showed high levels of *V. oleaginea* infection in Nablus where climatic conditions were similar to Northern Asira, and low levels of infection in Tubas where climatic conditions are similar to Sir (Salman *et al.*, 2011).

The trends of infected leaves, both symptomatic and asymptomatic, showed maximum values from the end of winter to beginning of spring. This is when very favourable temperature and humidity conditions support rapid development of peacock eye (Guechi and Girre, 1994). The subsequent decreases in the percentages of diseased leaves were probably due to losses of the infected leaves from the trees.

Lowest levels of infection were observed in pruned trees compared with unpruned ones. This can be explained by considering that thinning of the vegetation creates better conditions for lightening and aeration of the internal parts of the canopy, with a consequent decrease in RH, which is a very important factor in favoring leaf attack by *V. oleaginea* (Obanor *et al.*, 2008; 2011; Al-Jabi, 2013). However, even if the effect of pruning was supposed, the present study has given the first experimental evidence that pruning can reduce the severity of the peacock eye in olive.

Our results clearly showed gradients of peacock eye severity from the lower to upper parts of olive tree canopies. Amounts of infected leaves and defoliation decreased from the lowest to the upper parts of the canopies. This is likely to be because the lower canopy parts retain the greatest air humidities, while upper canopy parts have more light and are well-ventilated.

All the factors investigated in this study influenced the length of olive inflorescences. To determine if these differences were associated with pathogen infections, the relationship between the percentage of effective defoliation and inflorescence length was evaluated by using the average values of the combinations of the following factors: site/pruning/part of the canopy. A significant linear correlation (R^2 = 0.66) was found between these two parameters (Figure 8). This indicates that olive inflorescence length was related to the number of leaves and that the shorter length of the inflorescences in Northern

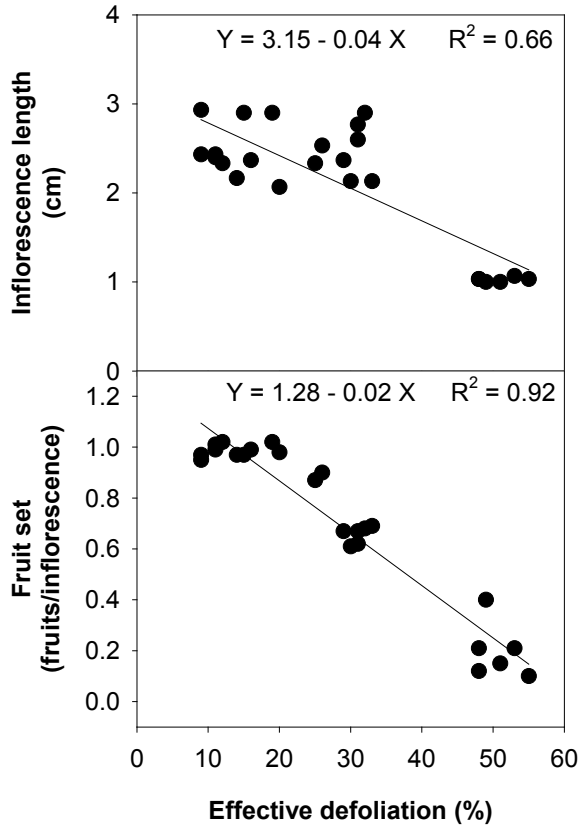

Figure 8. Relationships between effective olive tree defoliation caused by peacock eye with inflorescence lengths and fruit set. Each value represents the average of all the combinations of three factors (site, pruning, portion of canopy).

Asira, in unpruned trees and in the lower parts of the tree canopies were associated with the higher levels of defoliation caused by peacock eye.

The differences in fruit set can also be analyzed by evaluating the relationships between the proportions of effective defoliation and fruit set using the average values of the combinations of the following factors: site/pruning/part of the canopy. Again, a significant, negative, linear correlation ($R^2 = 0.92$) was found between these two parameters. This means that the differences in fruit set were also significantly associated with defoliation caused by the peacock eye disease. Examination of this relationship also indicates the effect of defoliation on the potential yield of the trees and thus the economic damage caused by the disease. Indeed, it is possible to see that depending on the level of defoliation, the number of fruits can be reduced up to one fifth (20%), when defoliation reaches values around 50%, compared to those obtainable in conditions of very low defoliation. This means that the impact of the disease on the potential production of the trees could be very high. Further investigations are required to determine the effects of peacock eye on olive fruit growth and possible fruit drop.

CONCLUSIONS

The results of this study indicate that variability of the environmental conditions in the different areas of Palestine is enough to determine differences in the susceptibility of olive to peacock eye attacks. Therefore, even though Palestinian Territories have a limited extension, there are significant environmental differences among the different areas, which determine significant changes in the susceptibility to peacock eye, which must be considered in applying site-dependent strategies for disease control.

Pruning can reduce the susceptibility of olive trees to peacock eye. Infection was generally greater in unpruned than pruned trees. To the best of our knowledge, this is the first experimental evidence showing that pruning has a positive role in reducing this disease. In Palestine the importance of olive pruning is often underestimated. Hence efforts have to be made to train farmers to apply this practice in an optimal/regular way, also taking into consideration its importance in controlling important biotic adversities, such as peacock eye disease.

There is a large gradient within olive tree canopies for susceptibility of leaves to *V. oleaginea*. Disease severity is much greater in lower than upper canopy parts. This information is important for optimization of disease control methods. Treatments must ensure uniform and complete coverage especially of the middle and lower parts of the tree canopies.

Peacock eye can reduce the lengths of olive inflorescences and fruit set. This is the first report showing direct and linear influence of defoliation caused by peacock eye on inflorescence length and fruit set, and these negative effects are associated with defoliation caused by the disease. Based on the effects of the disease on fruit set, it can be concluded that peacock eye can cause severe reductions in olive fruit yields.

The results of the present study increase knowledge of factors that affect the susceptibility of olive to infection by *V. oleaginea,* and of effects of defoliation caused by the pathogen on olive tree production. This information, besides being important for a better knowledge of the biology of peacock eye in Palestine and other Mediterranean countries, can be useful for the development of effective strategies for its control.

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Research Papers

Transmission of 16SrIII-J phytoplasmas by the leafhoppers *Paratanus exitiousus* and *Bergallia valdiviana*

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Summary. Two of the most common leafhoppers present in Chile are the Cicadellidae *Paratanus exitiosus* and *Bergallia valdiviana*. They commonly occur in vineyards of central Chile, including some vineyards infected by phytoplasmas. The present study demonstrates that *P. exitiosus* and *B. valdiviana* can transmit 16SrIII-J phytoplasmas to grapevine and periwinkle plants. This provides improved understanding of the 16SrIII-J phytoplasma epidemiology in Chilean vineyards.

Keywords. Auchenorrhyncha, transmission trials, nested-PCR, *tuf* gene, 16S rRNA gene, RFLP.

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INTRODUCTION

In Chile, grapevine yellows (GY) is associated with phytoplasmas belonging to diverse ribosomal subgroups, including 16SrI-B and 16SrI-C ('*Candidatus* Phytoplasma asteris'-related), 16SrIII-J ('*Ca*. P. pruni'-related), 16SrV-A ('*Ca*. P. ulmi'), 16SrVII-A ('*Ca*. P. fraxini'), 16SrXII-A ('*Ca*. P. solani' or "stolbur") (Gajardo *et al*., 2009; Fiore *et al*., 2015). However, the phytoplasmas in the 16SrIII-J group were prevalent in the vineyards of the central zone of the country. This phytoplasma has been reported to infect various crops and spontaneous plant species, and a draft sequence of the genome of a Chilean strain was obtained (Gonzalez *et al*., 2011; Zamorano and Fiore, 2016; Quiroga *et al*., 2017a).

Phytoplasma dissemination in the field occurs with the use of infected plant materials and by insect vector transmission. The insects generally feed on weeds and only occasionally on grapevine, allowing the transmission of phytoplasmas to this species. In order to determine which insects are involved in transmission of the 16SrIII-J group-phytoplasma in Chile, an epidemiological study was carried out in symptomatic phytoplasma-infected vineyards. During dedicated surveys, several leafhoppers species (Hemiptera, Auchenorryncha, Cicadellidae) were found to be positive for the 16SrIII-J phytoplasma (N. Fiore, unpublished data), and among them, two of the most common insect species were *Paratanus exitiousus* (Beamer) and *Bergallia valdiviana* Berg 1881. This paper reports transmission of 16SrIII-J phytoplasma to periwinkle (*Catharanthus roseus* L.) and grapevine plants by both leafhoppers.

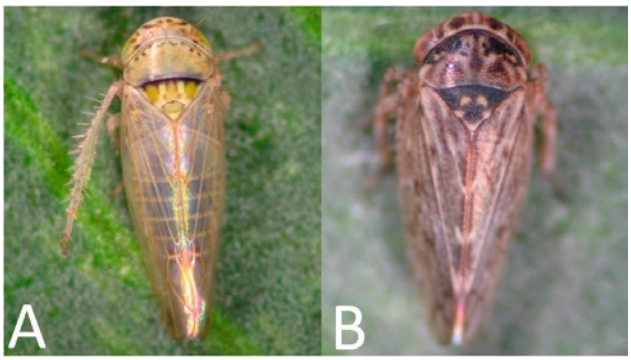

Figure 1. The two insect species used in transmission trials: A) *Paratanus exitiosus*; B) *Bergallia valdiviana.*

MATERIALS AND METHODS

In 2011 (September–December) and 2012 (January–May) surveys for *P. exitiosus* presence were carried out in three selected 16SrIII-J phytoplasma infected Chilean vineyards, two in the Metropolitana region (Buin and Pirque), and one in the Valparaiso region (Casablanca). The capture of *B. valdiviana* adults was carried out in 2012 (October–December) and 2013 (January–June) in a vineyard of cv. Pinot noir infected with 16SrIII-J phytoplasmas, in the Valparaiso region (Casablanca). *Paratanus exitiosus* and *B. valdiviana* (Figure 1) were captured using an entomological sweeping net and 150 sweepings directed to weeds present inside the vineyards. At the end of each capture, the adults of both insects were separated by species and divided in two batches. All the individuals belonging to one species were released into two entomological cages to let them feed on three grapevine plants of cv. Cabernet Sauvignon (first cage), and three periwinkle plants (second cage). The number of individuals released each month in each cage is reported in Tables 1 and 2.

Periwinkle and grapevine plants used in the transmission trials were from seeds, and tested by nested PCR (described below) to verify the absence of phytoplasmas before the transmission trials. Eighty-one periwinkle and 81 grapevine plants were used for the trials with *P. exitiosus,* while the phytoplasma transmission by *B. valdiviana* was performed to 27 periwinkle and 21 grapevine plants. All the plants were kept in a conditioned

Table 1. Numbers of *Paratanus exitiosus* individuals captured in three vineyards. Insects were released each month in cages to feed on periwinkle and grapevine plants for phytoplasma transmission trials.

Month	Number of individuals used in transmission trials to grapevine plants			Number of individuals used in transmission trials to periwinkle plants		
	Vineyard			Vineyard		
	Buin	Pirque	Casablanca	Buin	Pirque	Casablanca
September 2011	33	6**	2	32	7	2
October 2011	25**	17	20	24	18	20
November 2011	34	8	22	33**	7	22
December 2011	36	18**	9	35**	17	8**
January 2012	33	20	21	32	20	20
February 2012	31	14	30	30	14	30
March 2012	34	10	24	33	10	23
April 2012	18	5	21**	17	4	20
May 2012	33**	3	23	32	3	22

** Successful transmission trials for phytoplasma 16SrIII-J.

Table 2. Numbers of *Bergallia valdiviana* individuals captured in the Casablanca vineyard. Insects were released each month in cages to feed on periwinkle and grapevine plants for phytoplasma transmission trials.

Month	Number of individuals used in transmission trials to grapevine plants	Number of individuals used in transmission trials to periwinkle plants
October 2012	0	4
November 2012	0	16
December 2012	17	22
January 2013	20**	25
February 2013	28**	33
March 2013	24	30
April 2013	26	21**
May 2013	26	19
June 2013	0	23

** Successful transmission trials for phytoplasma 16SrIII-J.

incubator at 25ºC with 16 h of photoperiod. Each feeding period lasted until the death of all insects in the cages (four to six leafhoppers per test).

The cages were inspected on a daily basis to collect the dead insects and store them in 70% ethanol for further analyses. The cages without insects were then moved into a screen house for symptom onset. Phytoplasma detection in plants was carried out every three months. All insects from each cage were also tested in order to verify the phytoplasma presence.

Total nucleic acids were extracted from plants using a chloroform/phenol method (Prince *et al*., 1993), while insect nucleic acid was extracted with a CTAB method (Angelini *et al*., 2001). Nucleic acid was dissolved in Tris-EDTA pH 8.0 buffer and maintained at 4°C. PCR amplification was carried out using 20 ng μL^{-1} of nucleic acid. Direct and nested PCR on the *tuf* gene were performed according to the protocol of Makarova *et al*. (2012). Further direct PCR with primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) and nested PCR with R16F2n/R2 primers on the *16S rRNA* gene (Gundersen and Lee, 1996), were performed as described by Schaff *et al*. (1992). Amplicons from nested PCRs for both genes were purified using the EZNA gel extraction kit (OMEGA Bio-tek). DNA fragments were ligated into the pGEMT-Easy Cloning Kit (Promega). Putative recombinant clones were analyzed by colony PCR, and selected fragments were sequenced in both directions by Macrogen USA Corp. The sequences were then aligned with those of classified strains deposited in GenBank using BLAST engine for local alignment (version Blast N 2.2.12). The phytoplasma identification was carried out using *in silico* restriction fragment length polymorphism (RFLP) analysis with *Hha*I, *BstU*I, and *Rsa*I restriction enzymes, in the *i*PhyClassifier online tool (https://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi) (Zhao *et al*., 2009).

RESULTS

Leafhopper surveys

In the vineyard located in Buin, the *P. exitiosus* capture rate remained constant during all months, except for decreases in October 2011 and April 2012 (Table 1). In the Pirque vineyard, the greatest numbers of individuals were collected in October and December 2011, and January 2012. In the Casablanca vineyard, *P. exitiosus* was less abundant in September 2011, remained constant during the other months and increased in February 2012. These results indicated that in the surveyed areas *P. exitiosus* tended to be more abundant during summer.

Adult individuals of *B. valdiviana* were captured in both years in greatest abundance through the summer and remained constant in autumn (Table 2). This insect was of greatest abundance in the coastal valleys, where Casablanca is located. For this reason, only the insects captured in Casablanca vineyards were used for the transmission trials.

Phytoplasma identification

Phytoplasma 16SrIII-J was detected in some grapevine and periwinkle plants used in the transmission trials, and in *P. exitiosus* and *B. valdiviana* specimens that were fed on these plants. Sequences of cloned PCR fragments from both amplified genes were the same from the positive plants and insects in the *tuf* gene sequence (438 bp). On the other hand, sequence identity of the *16S rRNA* gene clones was 99.9 to 100% (1,230 bp) in the same samples. Two sequences of the *tuf* gene were deposited in GenBank under the accession numbers (Acc. No.) MH743135, MH743136, and two of the *16S rRNA* gene and were deposited under Acc. Nos MH743137, MH743138. For the *16S rRNA* gene, the nucleotide similarity percentages of the detected phytoplasmas showed a close correlation (99.8%) with those of the strain Ch10 (GenBank Acc. No, AF147706), corresponding to the chayote witches' broom phytoplasma (16SrIII-J) from Brazil. For the *tuf* gene, the greatest nucleotide identity was 100%, with the strain Hort72, belonging to a 16SrIII-J phytoplasma from sugarbeet from Chile (GenBank Acc. No. KM658259). The 16S rRNA amplicons were also subjected to *in silico* RFLP

analysis that assigned the detected phytoplasmas to the ribosomal subgroup 16SrIII-J (Figure 2).

Transmission trials with Paratanus exitiosus *to grapevine plants*

Paratanus exitiosus survived for 3 to 4 days on the grapevine plants. In the twelve months post-transmission trials, the phytoplasma presence was detected by nested PCR with primers for *tuf* and *16S rRNA* genes in five out of the 81 grapevine plants. Two plants (V47 and V78A) were from the transmission trials carried out with insects captured in Buin in two different months (respectively October 2011 and May 2012). Two plants (V43 and V61) were infected from insects captured in Pirque, during, respectively, September and December 2011. The plant V76B was infected by individuals of *P. exitiosus* captured in Casablanca, during April 2012 (Table 1). The transmission rates were 7.5% for the insects captured in Buin and Pirque, and 3.7% for those from Casablanca. At 24 months after the transmission trials were set up, two out of five grapevine plants infected with 16SrIII-J were asymptomatic (V43 and V61), while the other three plants showed short internodes, and leaves with downward curling and deformation (Figure 3).

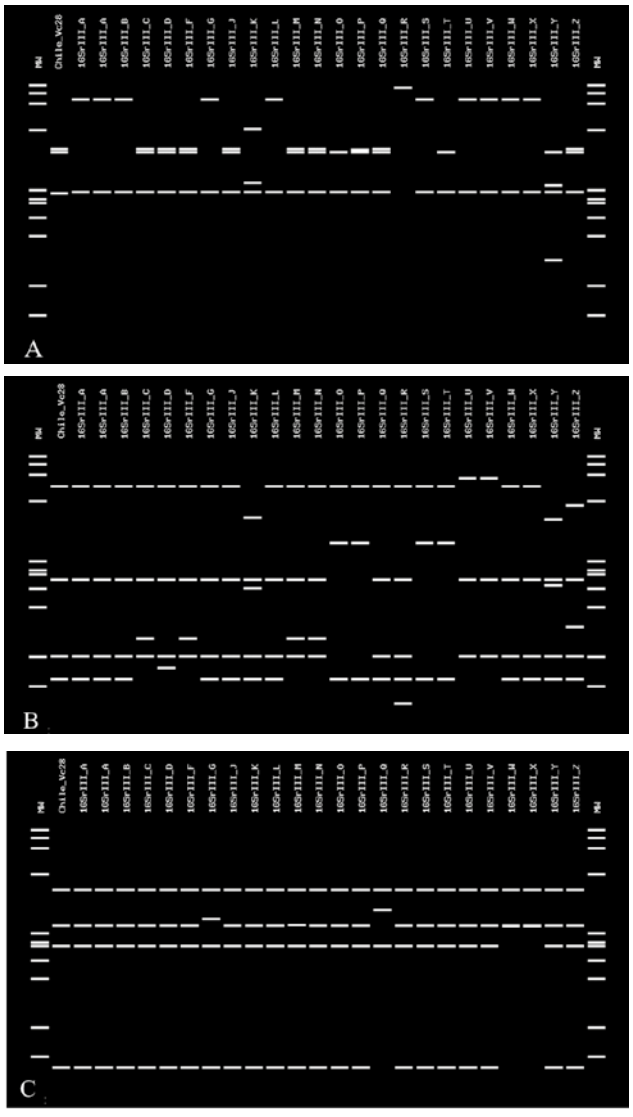

Figure 2. Virtual RFLP patterns obtained from the restriction with *Hha*I (A); *BstU*I (B) and *Rsa*I (C), of a 1,200 bp *16S rRNA* gene fragment obtained from sample VC28 (phytoplasma detected in a periwinkle plant used in transmission trials with *Paratanus exitiosus*). The phytoplasmas transmitted are classified in subgroup 16SrIII-J. The strains representative of the diverse subgroups are those enclosed in the *i*PhyClassifier (Zhao *et al*., 2009).

Transmission trials with Paratanus exitiosus *to periwinkle plants*

Paratanus exitiosus survived for 4 to 5 days on periwinkle plants. Three months after the transmission trials, in three out of the 81 periwinkle plants, the phytoplasma presence was detected by nested PCR with the primers for *tuf* and *16S rRNA* genes (Table 1). Two of the positive plants (VC28C and VC31C) were from the transmission trials carried out with insects captured in the Buin vineyard in two different months (November and December 2011, respectively). The third plant (VC33A) was infected by individuals captured in the Casablanca vineyard during December 2011. The three periwinkle plants infected with 16SrIII-J phytoplasmas showed symptoms of virescence, phyllody and witches' broom five months after the start of the transmission trials (Figure 4).

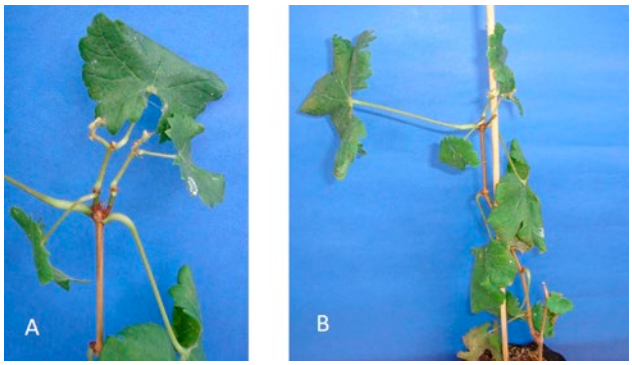

Figure 3. Symptoms associated with phytoplasma presence in grapevine after *Paratanus exitiosus* transmission. A) V43, short internodes; B) V76, leaves with downward rolling, deformations and necrosis.

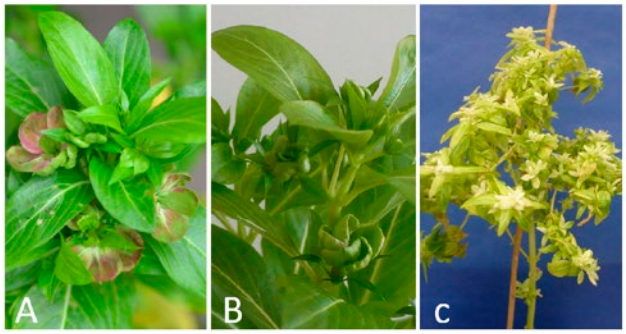

Figure 4. Symptoms associated with phytoplasma presence in periwinkle after *Paratanus exitiosus* transmission. A) VC28C, flowers with virescence; B) VC31C, flowers with virescence and phyllody; C) VC33A, plant with witches' broom, small and yellow leaves.

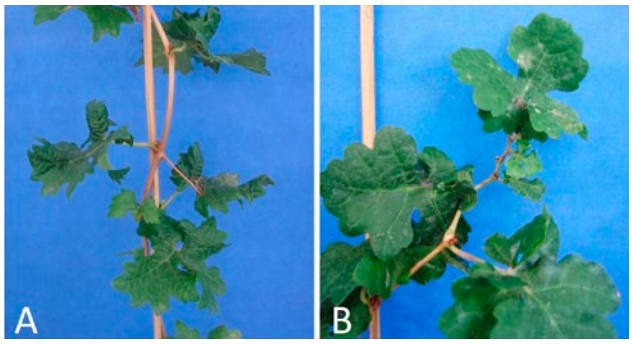

Figure 5. Symptoms associated with phytoplasma presence in grapevine after *Bergallia valdiviana* transmission. Short internodes, and leaves with downward rolling and deformation: A) VC85A; B) V86C.

Transmission trials with Bergallia valdiviana *to grapevine plants*

Bergallia valdiviana survived for 4 to 5 days on grapevine plants. At 24 months post-transmission trials, two of the 21 plants were positive for phytoplasma presence in nested PCR tests using primers for *tuf* and *16S rRNA* genes (Table 2). The positive grapevine plants (V85A and V86C) corresponded to the transmission trials carried out with insects captured in two different months (January 2013 for V85A and February 2013 for V86C). The transmission rate was 9.5%. The two grapevines infected with 16SrIII-J were symptomatic, with short internodes, and leaves with downward rolling and deformation (Figure 5).

Figure 6. Symptoms in periwinkle after *Bergallia valdiviana* transmission; A), B) VC60B, leaf deformation and severe yellowing; C) VC62B, flowers showing virescence.

Transmission trials with Bergallia valdiviana *to periwinkle plants*

Bergallia valdiviana survived for 6 to 7 days on periwinkle plants. Two out of the 27 plants were positive for phytoplasma presence, as determined with nested PCR using primers for *tuf* and *16S rRNA* genes (Table 2) 12 months after the transmission trials. These plants (VC 60B and V62B) corresponded to the transmission trials performed with insects captured in April 2013. The transmission rate was 3.7%. The 16SrIII-J infected periwinkle plants showed symptoms of virescence, leaf deformation and severe yellowing, 15 months after the transmission trials (Figure 6).

DISCUSSION

P. exitiosus and *B. valdiviana* transmitted the phytoplasma 16SrIII-J to grapevine and periwinkle plants. Both insects live on weeds and only occasionally feed on grapevine or other crops. *P. exitiosus* transmitted the phytoplasmas at a higher rate to grapevine than to periwinkle plants, while *B. valdiviana* had the same percentage of transmission to grapevine and periwinkle. Survival times of both insects on the grapevine plants was less than on periwinkle plants. The detection of 16SrIII-J phytoplasmas and the appearance of symptoms in grapevine occurred later than in the periwinkle plants. In the plants used for the transmission assays with *B. valdiviana*, phytoplasma detection occurred later in comparison with *P. exitiosus* assays and, as expected, the symptoms took longer to appear. This could indicate that the amount of inoculum of the pathogen in the *B. valdiviana* individuals used for transmission trials was less than in *P. exitiosus*, or that its transmission efficien-

cy was less. The main differences from the transmission trials were that higher transmission resulted from the *P. exitiosus* captured in the spring and summer periods, while *B. valdiviana* was only able to transmit the phytoplasma when collected at the end of summer and autumn. This vector difference could play a fundamental role in maintaining the phytoplasma population in host weeds during the periods of grapevine vegetative recess.

The phytoplasma 16SrIII-J and its newly identified insect vectors are widely distributed in Chile, infecting different weed species and crops of agronomic importance (Castro *et al*., 2000; Hepp and Vargas, 2002; González *et al*., 2010; 2011; Longone *et al*., 2011). Taking the observed *P. exitiosus* and *B. valdiviana* phytoplasma-transmission rates into account, if environmental conditions are favorable, there is high possibility that grapevine yellows outbreaks, associated with the presence of 16SrIII-J phytoplasmas, will occur in the central zone of Chile. Recent studies have indicated that climate change could modify the habitat of these species of insects, and increase their reproduction rates in the central zone of Chile (Quiroga *et al*., 2017b).

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Research Papers

Genetic and agronomic characterization of chickpea landraces for resistance to *Fusarium oxysporum* f. sp. *ciceris*

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Summary. Chickpea, with annual grain production of approx. 15 million tons, is the third largest world pulse crop, which is an important source of protein for human and animal diets. In Italy, chickpea production is mainly based on landraces cultivated on small farms. However, the attention that consumers give to local products stimulates farmers to extend the use of chickpea landraces by reintroducing them in crop rotations. Production of chickpea using landraces can be adversely affected by agronomic factors and, particularly, plant diseases such as Fusarium wilt, caused by the widespread fungal pathogen *Fusarium oxysporum* f. sp. *ciceris* (*Foc*). Therefore, studies of agronomic adaptation of landraces and on disease resistance are needed. The most important agronomic traits and levels of resistance to *Foc* were evaluated for 18 chickpea landraces collected from Central Italy. These landraces were also characterized for their genetic traits in comparison to some of the main Spanish cultivars, and to two reference cultivars with worldwide distribution. Molecular characterization showed variability in genetic and phenotypic traits among the Italian landraces. In particular, landrace 203 locally known as "Longano" was resistant to *Foc* and could be considered in chickpea breeding programmes. Comparative analyses based on molecular markers showed, with some exceptions, that the Italian landraces are genetically different compared to the main Spanish cultivars analyzed in this study.

Keywords. Fusarium wilt, chickpea biodiversity, germplasm conservation, molecular markers, disease resistance improvement.

OPEN ACCESS

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INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the only cultivated species of the genus *Cicer*, and originated in East Turkey (van der Maesen, 1987). It is a self-pollinated diploid annual plant species (2n = 2x = 16) with a small genome (740

Mbp) (Arumuganathan and Earle, 1991). Plant breeders have distinguished two chickpea types: desi and kabuli. The seeds of the desi type are normally small, angular and dark, and are produced by plants with purple flowers, while seeds of the kabuli type are large, round, white to cream, and are produced by plants with white flowers (Taylor and Ford, 2007).

Chickpea, is the third largest pulse crop in the world after dry bean and pea, with annual production of approx. 15 million tons. It is mainly cultivated on the Indian subcontinent. In Europe, the major chickpea producing country is Spain, followed by Italy, Portugal and the Balkan Countries. In Italy, chickpea is the third largest cultivated dry legume, grown on 20,025 ha, with grain production of 33,541 t (FAO, 2017). The crop is mainly grown on small farms, and landraces are particularly appreciated for local markets. The landrace varieties usually take their names from the location where they have been traditionally cultivated (Negri, 2003).

Although Italy is the second largest European producer of chickpea, cultivation is still restricted because of adverse environmental and/or agronomic factors responsible for variability in yields (Rossini, 2008). Nationally produced and imported chickpeas are mainly for human food use. The introduction of chickpea as animal feed could represent a viable alternative to soybean, enlarging its use in crop rotations, especially in arid and non-irrigated areas (Crinò and Saccardo, 2008). The use of landraces could have an important economic role, as well as social and cultural significance. Chickpea cultivation is sometimes connected to ethnic preferences of particular linguistic minorities, and also enhances plant biodiversity preservation programmes implemented by germplasm banks aimed at avoiding loss of crop plant ecotypes (Laghetti *et al.,* 2011). Cultivated landraces can be affected by a wide range of pathogens, in particular *Fusarium oxysporum* f. sp. *ciceris* (*Foc*), *Ascochyta rabiei* (Pande *et al.,* 2005; Jiménez-Díaz *et al.,* 2015), and other emerging pathogens (De Curtis *et al.,* 2014), which can cause significant yield losses.

Selection of landraces is mainly based on commercial characteristics of the grain while disease resistance is not often taken into account (Zaccardelli *et al.,* 2012).

Fusarium oxysporum f. sp. *ciceris* is the causal agent of chickpea Fusarium wilt with worldwide distribution. This Ascomycete fungus has been characterized into eight races (0, 1A, 1B/C, 2, 3, 4, 5 and 6), which are differentiated by genetic compatibility with the host and by geographical distribution. In addition to the pathogenic variability of the fungus, two distinct types, referred to as yellowing and wilting syndromes, have been distinguished on the basis of symptoms on infected plants (Trapero-Casas and Jiménez-Díaz, 1985; del Mar Jiménez-Gasco *et al.*, 2004). The pathogen is soil-borne, and can survive in the soil 6 or more years, even in the absence of the host plants, because of its durable survival structures. The infection of host plants can occur at different phenological stages with greatest incidence during the pod-forming phase, mainly when crops are subjected to water stress and sudden temperature increases. Infections occurring during vegetative and reproductive stages can result in complete yield losses. For these reasons, disease management is critical for chickpea production (Arunodhayam *et al.,* 2014).

Chickpea resistance towards the different races of *Foc* is of the "gene-for-gene" type, and different molecular markers associated with resistance are available. A gene cluster, located on Linkage Group (LG) 2 of the chickpea genetic map, confers resistance against races 0, 1, 2, 3, 4, 5 (Sharma and Muehlbauer, 2007). A second gene conferring resistance against race 0 is located on LG5 (Halila *et al.,* 2008). Molecular markers tightly linked to *Foc* resistance genes are useful tools for characterisation and genotype selection. The microsatellite TA59, located on LG2, is the marker most closely associated with resistance to *Foc* race 5 (*Foc*5) (Jendoubi *et al.,* 2017).

Flowering time is another agronomic trait defining adaptation and included in this study. Classical genetic analyses and conventional mapping studies have resulted in the identification of Quantitative Trait Loci (QTLs) on different LGs showing that genes governing this trait are distributed throughout the chickpea genome (Mallikarjuna *et al.,* 2017). Two conserved major QTLs have been identified, QTL_{DF3} on LG3 (Cobos *et al.,* 2009; Aryamanesh *et al.,* 2010) and QTL_{DF1} on LG4 (Cobos *et al.,* 2007; Varshney *et al.,* 2014).

In the present study, we focused on race *Foc*5, which is important in the Mediterranean basin (Jiménez-Díaz *et al.,* 2015). We characterised 18 landraces of chickpea from Central Italy for resistance to *Foc*5 and for some agronomic traits. Molecular markers, distributed along the chickpea genome, were used to study genetic variability among the landraces. Flowering time, an agronomic trait defining ecotype adaptation, was also investigated.

MATERIALS AND METHODS

Plant material

Eighteen Italian chickpea landraces previously collected by *Agenzia Regionale per lo Sviluppo Agricolo*

Rurale e della Pesca, Campobasso, Italy, during its institutional activity aimed at collecting and preserving traditional crops from different regional locations for a germplasm bank, were examined in the present study (Table 1). All landraces were the kabuli type, except accession number 184 which was the desi type. Forty-eight Spanish cultivars and two undomesticated *Cicer* species used in a previous study by Castro *et al.* (2010a), were also included as reference cultivars for comparative analyses, using the same molecular markers adopted for the Italian landraces.

Morphological and agronomic assessments

For each landrace, ten seeds were sown in each of three replicate plots in an experimental field at *Instituto de Investigación y Formación Agraria y Pesquera de Andalucía*, Córdoba, Spain, on January 2015. The following morphological traits were recorded: percentage of emerged plants 15 d after sowing, flowering time (assessed as 50% full opened flowers), growth habit and seed size (determined as 100-seed weights). Phenotypic evaluation of growth habit was assessed on three replicates, each of ten adult plants, for each landrace seeded in the field, based on the scale: 1 = semi erect; 2 = erect (Ali *et al.,* 2015).

Evaluation of resistance to Fusarium oxysporum *f. sp.* ciceris *Race 5*

The landraces were evaluated for wilt reaction to race 5 of *Foc* under controlled condition. The pathogenicity test was conducted in a growth chamber (daily cycle of 12 h light at 25 ± 2°C and 12 h dark at 22 ± 2°C). Lines ILC3279 and WR315 from the International Centre for Agricultural Research in the Dry Areas, which are, respectively, susceptible and resistant to *Foc*5, were included as experimental controls. Ten seeds of each landrace and control line were sown into plastic trays (60×40×10 cm - five lines per tray) that were filled with perlite. Three replicates were sown of each seedline. The trays were irrigated with nutrient solution. Conidia of *Foc*5 were obtained by growing the fungus in potato dextrose broth at 25°C and 100 rpm for at least 7 d. After incubation, fungal mycelium was removed, conidia were collected by centrifugation and their concentration adjusted to 1×10^6 conidia mL^{-1}. When plants were approx. 9 cm height they were removed from perlite and their roots were cut to approx. 5 cm lengths, and the plants were then dipped in conidial suspension for 5 min. The plants were then replanted in the same trays from which they were previously removed. Disease incidence, as percentage of dead plants, was recorded, commencing with appearance of the first symptoms on the susceptible

Table 1. Geographical origins and agronomic traits of Italian chickpea landraces assessed in this study.

Landraces	Town/Province[a]	Geographical coordinates	Type[b]	Germination (%)	Growth habit[c]	100 seeds weight (g)	Flowering time (days)
62	Cercemaggiore/CB	41° 28′ N/14° 43′ E	K	90	1	27.1	87
64	Cercemaggiore/CB	41° 28′ N/14° 43 ′E	K	80	1	36.7	87
73	Salcito/CB	41° 45′ N/14° 31′ E	K	90	1	37.3	86
76	S. Elia a Pianisi/CB	41° 37′ N/14° 53′ E	K	100	1	38.4	87
83	Casacalenda/CB	41° 44′ N/14° 51′ E	K	90	1	36.2	88
97	S. Angelo del Pesco/IS	41° 53′ N/14° 15′ E	K	100	1	35.3	86
99	Venafro/IS	41° 29′ N/14° 02′ E	K	90	1	31.7	84
111	Ripabottoni/CB	41° 41′ N/14° 49′ E	K	100	1	52.4	84
125	Morrone del Sannio/CB	41° 43′ N/14° 47′ E	K	60	1	48.6	84
147	Riccia/CB	41° 29′ N/14° 50′ E	K	80	1	42.5	84
148	Filignano/IS	41° 32′ N/14° 03′ E	K	100	1	30.5	84
160	Miranda/IS	41° 39′ N/14° 15′ E	K	90	1	35.8	87
184	Cercemaggiore/CB	41° 28′ N/14° 43′ E	D	100	1	21.9	87
203	Longano/IS	41° 31′ N/14° 15′ E	K	90	1	47.9	67
228	Riccia/CB	41° 29′ N/14° 50′ E	K	100	1	35.4	84
237	Montagano/CB	41° 39′ N/14° 40′ E	K	100	1	34.6	79
241	Riccia/CB	41° 29′ N/14° 50′ E	K	100	1	31.2	83
245	Capracotta/IS	41° 50′ N/14° 16′ E	K	90	2	27.2	87

[a] CB= Campobasso (IT), IS= Isernia (IT); [b] K=Kabuli, D=Desi; [c] 1= semi-erect, 2 = erect.

control ILC3279. Disease severity on each plant of all landraces was assessed each week for 3 weeks.

Foc symptoms and pathogen resistance were evaluated by using the following empirical disease scale: 0–10% of plants wilting = high resistance (R), 11–89% of plants wilting = intermediate resistance (I), >90% of plants wilting = high susceptibility (S) (Sharma *et al.*, 2005).

Molecular marker analyses

DNA extraction was carried out on young leaflets from five different plants of each landrace. Approximately 0.1 g of the mixed tissues was frozen in liquid nitrogen and stored at -80°C. DNA was isolated using the Plant DNAzol® Reagent (Invitrogen™). DNA was quantified by Nanodrop and used in PCR reactions to amplify the 12 microsatellite markers listed in Table 2. These molecular markers have been selected for their distribution across different linkage groups of the chickpea genome (Winter *et al.,* 2000; Millan *et al.,* 2010). Among the used markers, nine (those marked with TA prefix) were reported by Winter et al. (2000), two (CaGM14822 and CaGM07922) were reported by CAGM (http://cegresources.icrisat.org/CicArMiSatDB/, and one (H2I20) was reported by Lichtenzveig et al. (2005).

In the present study a high number of markers for LG2 (TA27, TA59, CaGM07922) was included, to target the resistance gene associated to *Foc*5 (Castro *et al.,* 2010b). The microsatellite H2I20 located on LG5 has been associated with a gene conferring resistance to *Foc*0 (Jendoubi *et al.,* 2016). The markers CaGM14822 associated with QTL_{DF1} on LG4 and TA142 linked to QTL_{DF3} on LG3 were used for their associations with flowering time (Ali, 2015).

Microsatellite alleles were visualized by electrophoresis in 2.5% (w/v) agarose, and polyacrylamide (10%, C2, 67%) gels, or with capillary electrophoresis using an automatic capillary sequencer (ABI 3130 Genetic Analyzer Applied Biosystems /HITACHI, Madrid, Spain) at the Central Research Support Service, University of Córdoba, Spain. Data from Fragment Analysis were analyzed using the GeneMapper and the Peak Studio V2.2 software packages (McCafferty *et al.,* 2012).

Statistical analyses

Assessment of Fusarium wilt resistance

In the pathogenicity tests, the number of plants showing; i) no symptoms (healthy), ii) light symptoms (yellowing and/or loss of leaf turgidity), iii) heavy symptoms (withering), or iv) dead plant, were periodically assessed. Disease symptom data were used to calculate the Area Under the Disease Progress Curve (AUDPC) with the following equation:

$$AUDPC = \sum_{i=1}^{n-1} \left(\frac{Y_i + Y_{i+1}}{2}\right)(t_{i+1} - t_i)$$

where Y_i is Fusarium wilt severity at the i^{th} observation, t_i is time (d) at the i^{th} observation, and n is the total number of observations (Campbell and Madden, 1990).

The AUDPC data from the different assessments were subjected to ANOVA using the SPSS statistics software v.25. Means were separated by Tukey's tests.

Analyses with molecular markers

Allele frequencies of data obtained in the diversity analyses were calculated and used to determine; ì) size range, ìì) number of alleles, and ììì) Polymorphism Information Content (PIC) of each marker (Shete *et al.*, 2000). The alleles were scored as present (1) or absent (0) to create a binary data matrix. This matrix was used to calculate the degree of genetic similarity between all pairwise combinations, using the Dice coefficient of similarity. Clustering of the genotypes was determined using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Statistical analyses was performed using the NTSYS-pc 2.02j software.

RESULTS

Agronomic traits

In the field assessments, seed germination was greater than 80% for all the chickpea lines except accession 125, for which 60% of seeds germinated. Only accession 245 had an erect growth habit (value 2), whereas the other landraces had semi-erect growth habits (value 1). Landraces 111, 125, 147 and 203 had large seed sizes (100-seed weights from 42.5 to 52.4 g) while landraces 62, 245 and 184 had smaller seed sizes (100-seed weights, respectively, 27.1, 27.2, and 21.9 g). Flowering began 83 d after sowing in all landraces except for landraces 237 and 203, which flowered at, respectively, 79 and 67 d (Table 1).

Evaluation of Fusarium wilt resistance

Data collected from the pathogenicity tests were used to calculate the AUDPC index, which allows disease

progression to be compared, and reveals the presence of susceptible and resistant phenotypes. Resistance in control genotype WR315 was confirmed by the absence of *Foc* wilt symptoms (AUDPC = 0). In contrast, the *Foc* susceptible control ILC3279 showed wilt symptoms at the first assessment date, and had a final average AUDPC = 14. Among all the tested landraces, accession 203 showed complete resistance to the pathogen, and disease symptoms were absent at all the assessment dates. All the other landraces developed symptoms and were susceptible to the pathogen, with AUDPC values from 10 to 15 (Figure 1).

Molecular marker analyses

The analyses were performed using twelve microsatellite markers (Table 2). All markers revealed high levels of polymorphisms, displaying a total of 100 different alleles with fragment sizes ranging from 150 to 350 bp. The number of alleles per locus varied from two to 15, with an average value of 8.33. STMS TA142 and TA78 amplified, respectively, the minimum (two) and maximum (15) number of alleles (Table 2). Eighteen out of the 100 alleles detected in the chickpea landraces were classified as 'rare' because of their low frequency (<0.03), 69 as 'common' (0.03-0.20) and 13 were considered the 'most frequent' alleles (>0.20). Only common alleles were detected at all the 12 STMS loci studied. Rare alleles per locus ranged from one to five in TA27, TA59, TA11, TA14, TA78 and TA144. The number of common alleles per locus ranged from one (CaGM07922 and TA142) to 12 (TA11 and TA78). For the most frequent alleles, zero, one or two such alleles were detected in the majority of the STMS loci, except for TA135 in which three alleles were detected (Table 2). Based on PIC values obtained, most STMS, except for CaGM07922, TA135, TA142, CaGM14822 and H2I20, were considered informative markers (PIC >0.63). The most polymorphic marker was TA78 with a PIC value of 0.88 and 15 alleles (Table 2).

All landraces were genotyped with two markers previously associated with flowering time (CaGM14822 and TA142). CaGM14822 had three alleles of 300, 320 and 350 bp, and TA142 two alleles of 150 and 160 bp. All these alleles showed clear association with early/late flowering time. The control line WR315 (early flowering) had alleles 380 and 133 for both markers, whereas the second control line ILC3279 (late flowering) had alleles 350 and 144 for both markers (Table 3).

For analyses of the *Foc*5 resistance genes located on LG2, the markers TA27, TA59 and CaGM07922 were considered. The resistant landrace 203 showed a 230/233 bp allele for TA27, a 225 bp allele for TA59 and a 350 bp

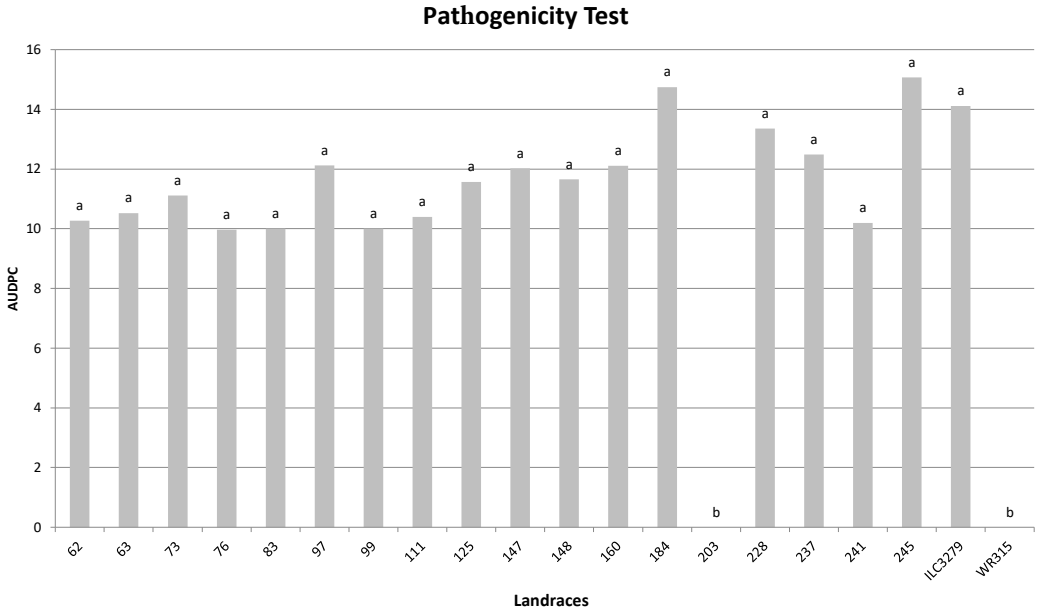

Figure 1. Average Area Under the Disease Progress Curve (AUDPC) obtained from pathogenicity tests carried out in controlled conditions on Italian chickpea landraces inoculated with *Fusarium oxysporum* f. sp. *ciceris* race 5 (*Foc*5). *Foc*5-susceptible ILC3279 and *Foc*5-resistant WR315 were included as controls. Accessions with letters in common above the columns are not significantly different ($P = 0.05$), according to Tukey's Test.

Table 2. Size ranges, numbers and frequencies of alleles and Polymorphism Information Content (PIC) observed in 18 Italian chickpea landraces, studied with 12 microsatellite markers.

Marker	Linkage Group	Size range (bp)	Nº of alleles	Rare alleles (<0.03)	Common alleles (0.03-0.2)	Most frequent alleles (> 0.2)	PIC
TA113	1	169-217	11	0	10	1	0.81
TA27	2	218-248	11	4	7	0	0.84
TA59	2	222-273	12	5	6	1	0.80
CaGM07922	2	300-350	3	0	1	2	0.34
TA142	3	150-160	2	0	1	1	0.21
TA135	3	187-199	5	0	2	3	0.59
CaGM14822	4	300-350	3	0	2	1	0.35
H2I20	5	180-230	5	0	3	2	0.39
TA11	5	220-262	14	2	12	0	0.83
TA14	6	242-278	11	3	8	0	0.82
TA78	7	191-236	15	3	12	0	0.88
TA144	8	230-254	8	1	5	2	0.73
Total			100	18	69	13	
Mean			8.33	1.5	5.75	1.08	0.63

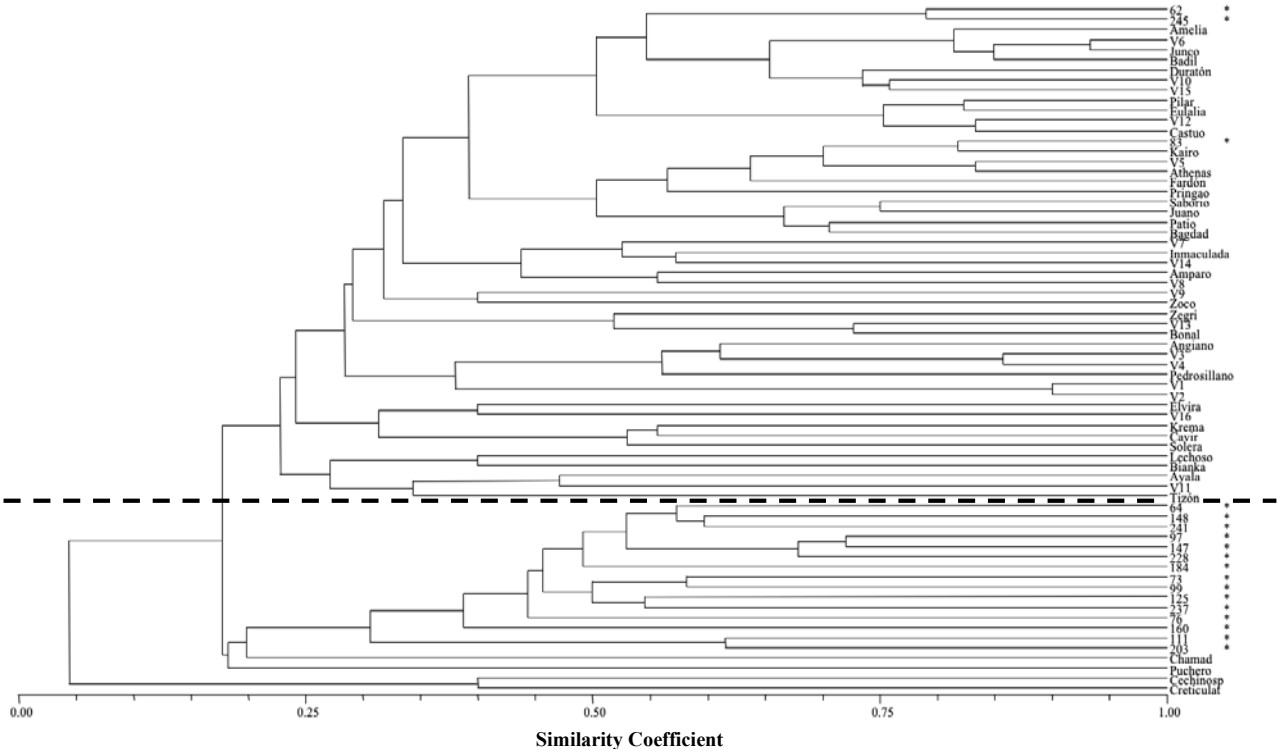

Figure 2. UPGMA dendrogram obtained from cluster analyses of 18 Italian chickpea landraces (marked with asterisks) and 48 Spanish cultivars, based on Dice coefficients of similarity, using 12 microsatellite markers selected for their Polymorphism Information Content (PIC) values. The dotted line separates two Subgroups, which mainly include Italian (Sub-group 1) and Spanish (Sub-group 2) chickpea lines. *Cicer reticulatum* and *Cicer echicnospermum* were included as outgroup controls.

Table 3. Associations between phenotypic data for flowering and resistance to *Foc*5 with microsatellite marker alleles found in the 18 Italian chickpea landraces. Microsatellite markers associated with flowering time were CaGM14822 and TA142, and with resistance to *Foc* were TA27, TA59 and CaGM07922.

Landraces	Flowering time[a]	Flowering time (days)		Resistance to *Foc*5[b]	Foc		
		CaGM14822	TA142		TA27	TA59	CaGM07922
62	T	300	150	S	236-239	246-255-273	300
64	T	300	150-160	S	221-233-242	225-234	300
73	T	300	150	S	221-227-239	231-234-237	350
76	T	300	150	S	227-230	228-234	300
83	T	350	160	S	221-236-239	243-246	350
97	T	300	150	S	221-239-242	231-234	350
99	T	300-350	150-160	S	221-227	231-234-237	300
111	T	320	150	S	221-227-230	225	300
125	T	300	150	S	221-233-236	234	350
147	T	300	150	S	239-242	222-225-234	350
148	T	300	150	S	221-227-233-236	228	300
160	T	300	150	S	221-224-230	237-240	300
184	T	300-350	150	S	221-236-239-242	246	300
203	P	320	150	R	230-233	225	350
228	T	300	150	S	221-239-242-245-248	234	320
237	T	300	150-160	S	221-230-233-236	234-237	300-350
241	T	300	150	S	218-221-227-239	225-234-237	300
245	T	300	150	S	236	252-255	300
WR315	P	380	133	R	221	225	270
ILC3279	T	350	144	S	216	234	300

[a] T = late, P = early; [b] R = high resistance, S = high susceptibility.

allele for CaGM07922. The allele displayed for TA59 in landrace 203 was also present in the resistant reference line WR315 (Table 3).

Genetic diversity analyses

The comparison between Italian landraces and Spanish cultivars revealed the presence of two genetic subgroups (Figure 2). Although with a low level of similarity of close to 0.20, subgroup 1 included 15 Italian landraces and the Spanish cultivars 'Chamad' (of unknown origin) and 'Puchero' (originating from mass selection of Spanish germplasm). Subgroup 2 included all the other Spanish cultivars and the Italians landraces 62, 83 and 245. Landraces 62 and 245, with a similarity coefficient of 0.79, were similar to the Spanish cultivars 'Amelia', 'Badil', 'Junco' and 'Duraton', with which they are grouped with a similarity coefficient of 0.55. Landrace 83 and the Spanish variety "Kairo", with a similarity coefficient of 0.83, were similar to each other, and both were grouped with the Spanish varieties "Athenas" and V5 (similarity coefficient = 0.70), that were subsequently grouped with 'Fardón', 'Pringao', 'Juano', 'Saborio', 'Bagdad' and 'Patio' (Figure 2).

DISCUSSION

Chickpea landraces could be valuable resources for improving sustainability of production on small farms of southern Italy (Negri, 2003; Crinò and Saccardo, 2008). The most important environmental and agronomic factors affecting yields are the length of cultural cycles and growth habits. Currently, the short cycle with spring sowing of chickpea crops is adopted in Italy. Although the long cultural cycle with winter sowing is potentially more productive, crop yields are often affected by adverse factors such as low seed germination rates, high disease incidence and early appearance of weeds (Rossini, 2008).

In cultivated chickpea varieties, bushy growth habit similar to that of the wild relative *Cicer reticulatum* is typical of varieties or landraces with a low amounts of selection (Cobos *et al.,* 2009). As expected, most of the tested landraces showed semi-erect growth habits (Table

1). Most of these landraces were selected in mountainous or hilly areas, where chickpeas are usually produced in marginal and minimally mechanized cropping systems. As a consequence, growers primarily selected chickpea seeds based on seed size and yield. In modern agriculture, growth habit is important for mechanical harvesting (Cobos *et al.,* 2009) and to escape weeds which are common in winter production (Rossini, 2008).

Landrace 203 ('Longano') flowered 67 d after sowing and was the earliest flowering line (Table 3). Early flowering, not affected by photoperiod and typical of plants selected and developed at low latitudes, results in a biological cycle that is 1 month shorter than for other genotypes. Early flowering genotypes escape summer drought stress at high latitudes, resulting in increased yields (Cobos *et al.,* 2009). Based on the knowledge acquired so far, there are no other Italian ecotypes with a flowering trait as early as 67 d. Flowering time is an important trait to increase profitability from chickpea crops, and early flowering allows the plants to escape biotic and abiotic stresses (Semere Mallu *et al.,* 2014). Furthermore, in the Mediterranean basin which is characterized by frequent water stress in the summer, the early flowering phenotypes can allow farmers to obtain increased yields (Siddique and Loss, 2003; Rubio *et al.,* 2004).

Morphological analyses carried out in the present study showed the potential improving chickpea landraces for traits needed to increase yields, particularly growth habit and flowering time. These improvements could be pursued by crossing different landraces to introduce required characteristics without losing the unique genetic characteristics of these ecotypes.

Most of the evaluated landraces were very susceptible to *Foc*5. The exception was landrace 203 ('Longano'), which was highly resistant to the pathogen (AUDPC = 0), and similar to the resistant ecotype WR315 used as control (Table 3 and Figure 1). Genetic resistance to *Foc*5 is conferred by a single gene located on LG2 of the chickpea map (Castro *et al.,* 2010a). High resistance to *Foc*5 in landrace 203 was observed previously in preliminary assays carried out in naturally infested experimental fields at two different locations in southern Spain (Córdoba and Escacena del Campo) (data not shown).

Differences in disease severity among the other landraces studied here are unlikely to be associated with different degrees of genetic resistance to *Foc*5, but are more likely to be from the effect of different responses to specific functions involved in the wilt stress. These may include uptake of iron and other nutrients, and response to water deficiency (Blum, 2017).

Among the tested landraces, accession 203 ('Longano') showed valuable agronomic traits, including large seed size, early flowering and, particularly, a high level of genetic resistance to *Foc*5, which is considered the most aggressive chickpea pathogen worldwide. Based on our findings, landrace 203 could satisfy the current high demand for local products by consumers, and could be a good candidate for large-scale and wide-spread use in agriculture.

Our results with STMS markers did not allow establishment of relationships between different alleles and phenotypic traits, such as flowering time with resistance to *Foc*5. The prediction of the resistance alleles with STMS markers is complex because they show extensive polymorphisms within species. Therefore, their use is not recommended for screening germplasm collections (Madrid *et al.,* 2014). Haplotypes with SNP markers characteristic of the main sources of disease resistance should also be used (Caballo *et al*., 2019). However, in the case of TA59, tightly linked to *Foc* resistance and used previously in breeding programmes (Castro *et al.,* 2010b), the alleles present in resistant and susceptible controls, respectively, were also present in the resistant landrace 203 and the susceptible line 228 (Table 3).

The study of similarity among Italian landraces and Spanish cultivars, based on molecular markers, showed that the two subgroups, in most of cases, correlated with geographic origins. Some exceptions were found in subgroup 1, where two Spanish cultivars joined with Italian landraces, and in subgroup 2, where three Italian landraces joined with the Spanish cultivars (Figure 2). This genetic similarity could be explained by historic germplasm exchanges during the Spanish domination of southern Italy during the 15th to 18th Centuries.

Future chickpea improvement programmes aimed at conservation and promotion of genetic resources should consider these landraces as good genetic resources for selection and/or breeding. In addition, our results highlight the potential of local varieties to be improved and exploited as productive and profitable crops even in marginal areas, stimulating the farmers of these areas to expand the cultivation of chickpea, and to provide alternative crops in the cropping systems.

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Research Papers

Severity of Diplodia shoot blight (caused by *Diplodia sapinea*) was greatest on *Pinus sylvestris* and *Pinus nigra* in a plantation containing five pine species

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Summary. The severity of Diplodia shoot blight in *Pinus nigra* and *P. sylvestris* seed orchards, and in *P. taeda*, *P. pinaster* and *P. radiata* industrial plantations was examined in two 20 × 20 m plots of each tree species. All 159 trees assessed in the plots showed symptoms of *Diplodia sapinea* infections. Average disease severity in the tree crowns varied from 42 to 68% (mean = 53.8%) over all tree stands. Fungus isolates obtained from affected trees were identified as *D. sapinea*, based on morphological characteristics of cultures and conidia grown on agar plates. The identifications were confirmed by sequence analysis of the ITS rDNA of a subsample of isolates. Multilocus genotyping with RAMS primers showed that 13 *D. sapinea* genets were present in the plots. *Diplodia sapinea* had serious impacts on *P. sylvestris* and *P. nigra* in the seed orchards Marmara Region of Turkey, and is likely to have been the main causal agent of shoot blight and reductions in seed production at this location.

Keywords. Disease severity, RAMS, pathogenicity, pine plantations.

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INTRODUCTION

Diplodia sapinea (Desmaz.) J. Kickx [syn. *Sphaeropsis sapinea* (*Diplodia pinea*) (Fr.: Fr.) Dyko & Sutton] causes a range of disease symptoms on conifer trees, including stunting, browning of needles, shoot blight, twig and branch dieback, crown wilt and bark cankers, root disease, and damping-off and root rot of seedlings. In addition, the fungus causes blue stain of sapwood in fallen or freshly cut timber, in which it is considered a saprotroph (Brookhouser and Peterson, 1971; Peterson, 1977). The fungus has wide distribution in temperate and tropical regions. *Pinus nigra* Arnold, *Pinus radiata* Don. and *Pinus sylvestris* L. are particularly susceptible to infec-

tion (Wingfield and Know-Davies, 1980; Palmer and Nicholls, 1985; Swart *et al*., 1987; Chou and Mackenzie, 1988; Rees and Webber, 1988; Nicholls and Ostry, 1990; Stanosz and Cummings, 1996; Stanosz *et al*., 2001). Predisposing factors such as drought stress or physical wounding through forestry practices may have important roles in increasing tree susceptibility to *D. sapinea* infections (Swart and Wingfield, 1991; Blodgett and Stanosz, 1997; Adams *et al*., 2002; Blodgett and Bonello, 2003).

Although *D. sapinea* was first reported in Turkey in 1993 on *Pinus pinea* L. and *Pinus pinaster* Ait. (Ünligil and Ertaş, 1993), little is known about the incidence and severity of Diplodia diseases, or genetic variation in the pathogen populations, in Turkish forests. Some of the most severe damage from Diplodia shoot blight has occurred in the southern part of Turkey. The pathogen was first noted on dead twigs or canker samples of *Pinus brutia* var. *eldarica* (Medw.) Silba and *Pinus brutia* Ten. in Kahramanmaraş, Turkey, in 2000 (Sümer, 2000). *Diplodia sapinea* was later shown to be the main causal agent of shoot blight of *P. brutia* in the Isparta region, in the western Taurus Mountains (Doğmuş Lehtijärvi *et al*. 2007). In 2013, similar symptoms were reported in *Pseudotsuga menziesii* (Mirb.) Franco plantations in İzmit province (Aday Kaya *et al*., 2014), and *D. sapinea* was suggested as the likely causal agent.

Genetic variation in *D. sapinea* populations has previously been determined based on morphological characteristics and virulence of the isolates (De Wet *et al*., 2002). In recent years, however, molecular genetic analyses of several populations worldwide have been performed. The results of these studies indicate high variation in the genetic diversity among *D. sapinea* populations (Burgess *et al*., 2004; Bihon *et al.,* 2012 a, b). The pathogen is predominantly asexual and wound-associated die-back appears to be caused by clones of the pathogen occurring in narrow time frames (Bihon *et al*., 2012b). However, endophytic infections show high levels of genetic diversity (Bihon *et al*., 2011, Bihon *et al.,* 2012 a, b), probably resulting from a cryptic, heterothallic sexual cycle (Bihon *et al.,* 2014).

To date, genetic differentiation and pathogenicity in Turkish *D. sapinea* isolates has not been examined. Random amplified microsatellites (RAMS) and internal simple sequence repeat (ISSR) are reliable tools for analysis of genetic variation in several important forest pathogens (Hantula *et al*., 1996). Using RAMS, Doğmuş Lehtijärvi *et al*. (2014) demonstrated that 60 isolates of *D. sapinea* in *P. nigra* and *P. sylvestris* seed orchards were genetically identical.

The objectives of the study reported here were: i) to estimate the disease severity caused by *D. sapinea* in *P. nigra* and *P. sylvestris* seed orchards, and in *P. taeda*, *P. pinaster* and *P. radiata* industrial plantations in Turkey, ii) to characterize isolates of the fungus using a combination of morphological and molecular techniques, iii) to investigate pathogenicity, host specialization and virulence of isolates, and iv) to determine genetic variation within the *Diplodia* isolates.

MATERIALS AND METHODS

Disease severity

Surveys were conducted in October 2014 in two seed orchards planted with *Pinus nigra* and *P. sylvestris*, and in industrial plantations of *P. taeda*, *P. pinaster* and *P. radiata* in the Kerpe Research Forest, İzmit province, Marmara Region of Turkey. These pine plots (sections) were located 130 to 870 m apart from each other (Table 1). Soil types of the stands were brown forest soil, light hydromorphic grey brown podzolic brown soil, and andesite brownstone soil on the main rock. For each tree species, two 20 × 20 m plots were surveyed. Diameter at breast height (DBH), tree height and disease severity caused by *D. sapinea* were recorded for all trees in the plots, and mature, open cones remaining on shoots were collected from each tree. Severity of Diplodia shoot blight was scored using a 1-5 scale (Table 2), and calculated using the Townsend-Heuberger formula (Townsend-Heuberger, 1943):

$$\text{Disease Severity (\%)} = [\Sigma(n.V)/Z.N] \times 100$$

Where
n = number of samples that are in different disease scales,
V = scale value,
Z = greatest scale value,
N = total number of samples

Estimation of inoculum densities in cones

A total of 477 cones were collected from sampled trees in the investigated plots. Three cones were taken from each tree to estimate *D. sapinea* inoculum densities. Cone samples from each tree were pooled in a 100 mL plastic beaker containing 80 mL of deionized water with two drops of Tween®80 (Sigma-Aldrich, Inc.), which was then shaken for 3 h at 24°C at 110 rpm. After shaking, the cones were removed from the beakers and the

Table 1. Locations of pine plantings assessed for Diplodia shoot dieback.

Tree species	Coordinates	Section ID	Type of stand	Size of section (ha)	Origin of tree species	Soil
Pinus nigra	41° 9' 5.83" N 30 °12'19.39" E 41° 8' 59.03" N 30°12'20.47" E	10c	Plantation	8.4	Turkey- Dursunbey	Brown forest soil
P. sylvestris	41° 9' 26.06" N 30°13'3.77" E 41° 9' 23.21" N 30°13'3.02" E	18e1, 19b	Seed orchard	34	Turkey- Sarıkamış	Brown forest soil
P. taeda	41° 9' 5.75" N 30°12'27.06" E 41° 8' 58.88" N 30°12'27.91" E	15r	Plantation	28.1	USA	Light hydromorphic gray brown podzolic brown soil
P. pinaster	41° 9' 11.73" N 30°12' 21.62" E 41° 9' 9.05" N 30°12' 25.03" E	9g	Plantation	19.1	France	Light hydromorphic gray brown podzolic brown soil
P. radiata	41° 9' 27.19" N 30°12' 29.55" E 41° 9' 23.50" N 30°12' 28.66" E	9a	Plantation	9.0	Australia	Andesite brownstone land on the main rock

Table 2. Disease severity scores used for assessments in pine stands.

Score	Damage class	Symptoms used for scoring
1	No damage	-
2	Light damage	≤five main branches with advanced defoliation
3	Moderate damage	Several (≥five) main branches or a whole crowns, groups of branches seriously damaged.
4	Severe damage	Most of the main branches seriously damaged.
5	Dead	-

volume of water in each beaker adjusted to 100 mL with deionized water. The numbers of conidia in the suspensions were enumerated with five replicate counts using a compound microscope and a haemocytometer.

Identification and assessment of genetic variation of the pathogen

Cones taken from pines were examined for the presence of pycnidia typical of *D. pinea* under a dissecting microscope (Table 3). Pycnidia were transferred to potato dextrose agar (PDA Merck 1.10130) and incubated at 24°C for 5 d. In total, 106 isolates were obtained. Morphological features of the conidia collected from pynidiaon cones were examined under a binocular microscope, and cultural characteristics of the isolates were examined using pure cultures grown on fresh PDA.

Isolates were sub-cultured to cellophane membranes covering PDA and incubated at 24°C for 7 d. The mycelium was then scraped off the membrane surfaces and ground in liquid nitrogen using a mortar and pestle. DNA was extracted using DNeasy Plant Mini Kits (Qiagen) following the manufacturer's instructions. PCR amplification of the internal transcribed spacer (ITS) region of the rDNA gene was performed using the primer set ITS1 (5'–TCCGTAGGTGAACCTGCGG–3') and ITS4 (5'–TCCTCCGCTTATTGATATGC–3') (White *et al.* 1990) in 50 µL reactions. Each reaction containing 50 ng genomic DNA, 250 nM of each primer, 200 µM of each dNTP, 25 mM $MgCl_2$, 1U *Taq* polymerase, 1 × Q solution, and 1 × PCR buffer (Promega Corporation). PCR was conducted in a Biorad MJ Mini Personal Thermal Cycler. PCR conditions were: denaturing at 95°C for 10 min, followed by 30 cycles of amplification (20 s denaturation at 94°C, 25 s annealing at 55°C, and 2 min extension at 72°C). PCR products were separated by electrophoresis in 1% (w/v) agarose.

Genetic variation among the isolates was determined using multilocus genotyping with the M13 marker (Zamponi *et al.* 2007) and RAMS-primers VDH $(TCG)_5$, DDB$(CCA)_5$ and DDB$(CGA)_5$, where B = G/T/C, D = G/A/T, H = A/T/C and V = A/C/G (Hantula *et al.* 1996).

Table 3. Diplodia shoot dieback severity assessments.

Stand No.	Tree species	No. of trees	Damage scale and disease severity (%)[1)]									
			No damage (1)		Light damage (2)		Moderate damage (3)		Severe damage (4)		Dead (5)	
			Tree No.	%	Tree No.	%	Tree No.	%	Tree No.	%	Tree No.	%
1	*Pinus sylvestris* 1	11	0	0	5	18.7	6	32.7	0	0	0	0
2	*Pinus sylvestris* 2	10	0	0	3	12	6	26.0	1	8	0	0
		21		0		15.1		34.3		4		0
3	*Pinus nigra* 1	10	0	0	0	0	3	18.0	6	48.0	1	10.0
4	*Pinus nigra* 2	9	0	0	0	0	3	20.0	6	53.3	0	0.0
		19		0		0		19.0		50.6		5.0
5	*Pinus pinaster* 1	26	0	0	0	0	12	27.7	14	43.0	0	0
6	*Pinus pinaster* 2	17	0	0	8	18.8	9	31.7	0	0.0	0	0
		43		0		9.4		29.7		21.5		0
7	*Pinus radiata* 1	21	0	0	6	11.4	8	22.8	6	22.8	1	4.8
8	*Pinus radiata* 2	12	0	0	0	0	4	20.0	8	53.3	0	0
		33		0		5.7		21.4		38.0		2.4
9	*Pinus taeda* 1	22	0	0	7	12.7	10	27.2	5	18.0	0	0
10	*Pinus taeda* 2	21	0	0	0	0	21	80.0	0	0	0	0
		43		0		6.3		53.6		9.0		0

Primers were synthesized by IonTek. A total of 50 isolates was analysed, with ten isolates from each filed plot examined. DNA was extracted from each isolate as described above and subjected to PCR. DNA was denatured at 95°C for 10 min, after which 37 cycles of amplification were carried out (30 s denaturation at 95°C, 45 s annealing at the primer dependent temperature and 2 min extension at 72°C), followed by 7 min at 72°C (Maresi *et al*. 2007).

Amplification products were separated by electrophoresis (6 V cm^{-1} for 180 min) in 1.0% agarose gels (Biobasic Inc.) and 1.0% SynerGel gels (Diversified Biotech) in TAE buffer, and lengths of the products was estimated using DNA molecular size markers with 100 bp repeats (Thermo Fisher Scientific).

The presence or absence of amplification products was scored, with only clear and reproducible markers considered for the analysis. To estimate genetic variation, analysis of molecular variance (AMOVA) was calculated based on RAMS markers. Variation was assessed among and within isolate groupings based on the host species.

Genetic variation between *D. sapinea* isolates was computed using Jaccard's coefficient of similarity in Arlequin V 6.1 of NTSYSpc 2.1 software (Exeter Software Co.) and GenAlEx 6.5 (Peakall and Smouse, 2006).

Pathogenicity, host specialization and virulence of isolates

Seedling inoculations

Pathogenicity of the *D. sapinea* isolates was assessed using potted seedlings of *P. sylvestris*, *P. nigra*, *P. radiata*, *P. taeda* and *P. pinaster*, which were obtained from local forest nurseries. Three-year-old seedlings growing in a mixture of 60% clay, 20% sand and 20% humus were placed in a growth chamber and incubated at 20°C for 28 d prior to inoculation and during incubation. Seedlings were irrigated with tap water at 48 h intervals. Twenty-five isolates of *D. pinea* grown on PDA were used for the inoculations. Seedlings were inoculated 2 cm above the root collar. The inoculation point was cleaned with 70% (v:v) ethanol. A circular 3 mm diam. wound was made in the stem of each seedling using a sterilized cork borer to remove the bark. An equal-sized agar plug colonized by *D. sapinea* was inserted into each wound and wrapped with Parafilm M®. Experimental controls were mock-inoculated with non-colonized agar plugs. Five seedlings were inoculated for each isolate-host combination, and a randomized complete block experimental design used in the trial. Inoculated seedlings were incubated in the growth chamber for 4 weeks, after which lesion lengths were measured. Random re-isolations were made onto fresh PDA to confirm *Diplodia* sp. as the causal agent of the lesions.

Cross inoculations on twigs

Host specialization and virulence of isolates were also tested on 30 cm long twigs of the five host species. All twigs were obtained from İzmit-Kerpe Research forests. The same 25 isolates as in the seedling inoculation assay were grown for 7 d on PDA and then used for twig inoculations. Twigs were under bark-inoculated 8 cm above the base of each twig using a method similar to that described above for seedling inoculations. Five twigs were used for each fungal isolate-host species combination.

In addition, five control twigs were inoculated with sterile PDA discs. Inoculated twigs were kept at 20°C in a growth chamber for 1 month before recording lesion lengths using the methods described above.

Analysis of variance (ANOVA) was performed on lesion length data using the SPSS MGLM procedure (SPSS Inc.), and differences among mean values were assessed using Duncan's multiple range test ($P < 0.05$).

RESULTS

Disease severity

All 159 trees assessed in the plots had symptoms of *Diplodia* infections. Needles on recently killed shoots were reddish brown. Needles that were dead for longer periods were greyish brown or dark grey.

The average disease severity varied from 42 to 68% (mean = 53.8%) in the stands (Table 2). The predominant disease severity class was moderate, whereas high severity was less common (Figure 1). Comparisons of the damage classes between host species indicated that light damage (score = 2) occurred more frequently in *P. sylvestris* stands, whereas moderate damage (score = 3) was greater in *P. taeda* stands. Frequencies of severely damaged and dead individuals were greatest in the *P. nigra* stands (Table 3).

Estimation of inoculum densities in cone samples

Cones from all sampled stands yielded conidia characteristic of *D. sapinea*. Pycnidia were abundant on the cones and shoots of the specimens examined. The number of conidia extracted from cones varied between tree species. Numbers of conidia were greater from cones of *P. nigra* and *P. sylvestris*, particularly when compared with those obtained from cones of *P. radiata* or *P. taeda*. There was no significant difference in the numbers of conidia (overall mean = 1.3×10^6) obtained from *P. sylvestris* and *P. pinaster* cones ($P \leq 0.05$) (Table 4).

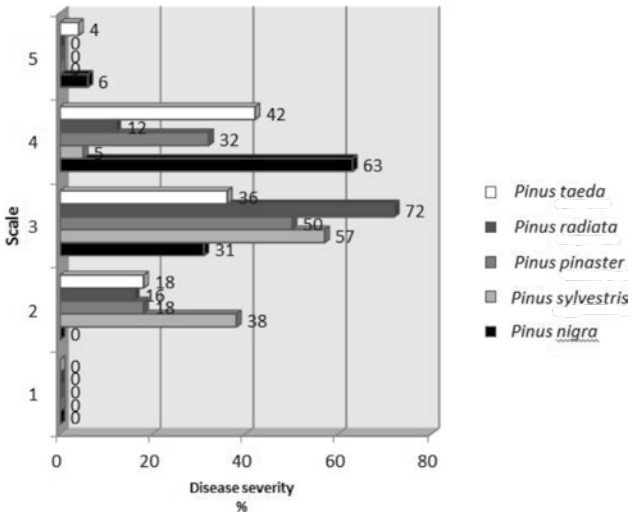

Figure 1. Percentage disease severity in each disease scale group for different *Pinus* spp.

Identification and genetic diversity in the pathogen populations

A total of 106 isolates was obtained from shoot and cone samples, and identified as *D. sapinea*. Conidia were brown to dark brown, thick-walled, with a mean width of 18.0 µm (SD ± 2.6) (range 9 to 22 µm) and mean length of 35.0 µm (SD ± 5.1) (range 20–41 µm) ($n = 100$).

Morphological identifications of the isolates were confirmed by sequence analysis of the ITS rDNA of representative isolates. The sequences showed homologies > 99% with GenBank accessions of *D. sapinea*.

Amplification of genomic DNA of 50 isolates of *D. sapinea* using the M13 and three RAMS markers produced 78 fragments of which 13 were polymorphic. All fragments were clear and reproducible, with sizes ranging from 350 to 870 base pairs (Table 5). The variation was analysed according to the presence or absence of the markers.

Table 4. Occurrence of *Diplodia sapinea* in cones of pine species and recovery of conidia in suspensions.

Host species	No. of cones examined	*Diplodia sapinea* positive (%)	Conidia per cone ($\times 10^6$)
Pinus sylvestris	54	81	1.3
P. nigra	51	88	1.1
P. pinaster	129	76	1.3
P. radiate	129	79	0.5
P. taeda	99	54	0.6
	Total = 462	Mean = 75.6	Mean = 0.96

Table 5. Presence/absence (1/0) vector of *Diplodia sapinea* isolates tested with RAMS and M13 primers.

Sample ID	Population name	CCA-870	CCA-800	CGA-750	M13-650	M13-600	CCA-570	CGA-550	CGA-510	CCA-480	TCG-440	TCG-420	CCA-400	CCA-370
D1	Pn	1	1	0	1	1	0	0	0	1	0	1	1	1
D2	Pr	0	1	0	1	0	0	0	1	1	1	1	0	1
D3	Pt	0	1	0	0	0	1	0	0	0	0	0	0	0
D4	Pp	1	1	1	1	0	1	0	0	0	1	1	0	1
D5	Ps	0	1	0	0	0	0	0	0	1	0	1	1	1
D6	Ps	0	1	0	0	0	0	0	0	1	0	1	1	1
D7	Ps	0	1	0	0	1	0	0	0	1	0	1	1	1
D8	Ps	0	1	0	0	1	0	0	0	1	0	1	1	1
D9	Ps	0	1	0	0	1	0	0	0	1	0	1	1	1
D10	Ps	0	1	0	0	1	0	0	0	1	0	1	1	1
D11	Ps	0	1	0	0	1	0	0	0	1	0	1	1	1
D12	Ps	0	1	0	0	1	0	0	0	1	0	1	1	1
D13	Ps	0	1	0	0	1	0	0	0	1	0	1	1	1
D14	Ps	0	1	0	0	1	0	0	0	1	0	1	1	1
D15	Pn	0	1	0	1	1	0	1	0	1	0	1	1	1
D16	Pn	0	1	0	0	0	0	1	0	1	1	0	1	1
D17	Pn	0	1	0	0	0	0	1	0	1	1	0	1	1
D18	Pn	0	1	0	0	0	0	1	0	1	1	0	1	1
D19	Pn	0	1	0	0	0	0	1	0	1	1	0	1	1
D20	Pn	0	1	0	1	0	0	1	0	1	1	0	1	1
D21	Pn	0	1	0	1	0	0	1	0	1	1	0	1	1
D22	Pn	0	1	0	0	0	0	1	0	1	1	0	1	1
D23	Pn	0	1	0	1	0	0	1	0	1	1	0	1	1
D24	Pp	0	1	0	1	0	0	1	0	0	0	0	0	0
D25	Pp	0	1	0	1	0	0	1	1	0	1	1	0	1
D26	Pp	0	1	0	1	0	0	1	1	0	1	1	0	1
D27	Pp	0	1	0	1	0	0	1	1	0	1	1	0	1
D28	Pp	0	1	0	1	0	0	1	1	0	1	1	0	1
D29	Pp	0	1	0	1	0	0	1	1	0	1	1	0	1
D30	Pp	0	1	0	1	0	0	1	1	0	1	1	0	1
D31	Pp	0	1	0	1	0	0	1	1	0	1	1	0	1
D32	Pp	0	1	0	1	0	0	1	1	0	1	1	0	1
D33	Pt	0	1	0	1	1	1	1	1	1	1	1	0	1
D34	Pt	0	1	0	1	1	1	1	1	1	1	1	0	1
D35	Pt	0	1	0	1	1	1	1	1	1	1	1	0	1
D36	Pt	0	1	0	1	1	1	1	1	1	1	1	0	1
D37	Pt	0	1	0	1	1	1	1	1	1	1	1	0	1
D38	Pt	0	1	0	1	1	1	1	1	1	1	1	0	1
D39	Pt	0	1	0	1	1	1	1	1	1	1	1	0	1
D40	Pt	0	1	0	1	1	1	1	1	1	1	1	0	1
D41	Pt	0	1	0	1	1	1	1	1	1	1	1	0	1
D42	Pr	0	0	0	1	0	0	0	1	1	1	1	0	1
D43	Pr	0	0	0	1	0	0	0	1	1	1	1	0	1
D44	Pr	0	0	0	1	0	0	0	1	1	1	1	0	1
D45	Pr	0	0	0	1	0	0	0	1	1	1	1	0	1
D46	Pr	0	0	0	1	0	0	0	1	1	1	1	0	1
D47	Pr	0	0	0	1	0	0	0	1	1	1	1	0	1
D48	Pr	0	0	0	1	0	0	0	1	1	1	1	0	1
D49	Pr	0	0	0	1	0	0	0	1	1	1	1	0	1
D50	Pr	0	0	0	1	0	0	0	1	1	1	1	0	1

ANOVA showed that variation among and within *D. sapinea* isolates obtained from different pine species amounted to, respectively, 44 and 56% of the total variation. Population divergence measured with F_{ST} also indicated differentiation in *D. sapinea* among *P. nigra*, *P. taeda* and *P. pinaster* (F_{ST} = 2.432) (Table 6).

Thirteen *D. sapinea* genotypes were present in all host species (Table 7). Genotypes of *D. sapinea* infecting *Pinus sylvestris* and *P. taeda* were unique to those hosts, whereas *P. nigra*, *P. pinaster* and *P. radiata* shared nine genets.

Genotype size was also estimated based on the RAMS analysis, and ranged from a single tree to approx. 115 m^2 (15 trees). Two genotypes were shared within two stand replicates. The largest genotype was located in a *P. radiata* stand.

Table 6. Genetic variation within *Diplodia sapinea* populations.

Variation source	df	SS	MS	Est. Var.	%
Between populations	4	58.640	14.660	1.359	56
Among populations	45	48.300	1.073	1.073	44
Total	49	106.940		2.432	100

Table 7. Genet size of *Diplodia sapinea* populations.

Tree species	Stand number	Genet code	Number of isolates/per genet	Estimated genet size m^2
Pinus nigra	1	1.genet	2	20
		2.genet	3	5
	2	3.genet	3	38
		4. genet	2	16
P. sylvestris	1	5.genet	2	6
		6.genet	3	18
	2	6.genet	5	100
P. pinaster	1	7.genet	1	1
		8.genet	1	1
		9.genet	3	55
	2	9.genet	5	68
P. taeda	1	10.genet	1	1
		11.genet	4	18
	2	11.genet	5	106
P. radiata	1	12.genet	1	1
		13.genet	4	114
	2	13.genet	5	93
Total		13	50	661

Pathogenicity, host specialization and virulence of isolates

Seedling inoculations

Diplodia sapinea inoculations caused dark brown to black discolouration around the inoculation points in seedlings of all pine species tested. Infection frequency in the inoculated seedlings was 100%. The pathogen was successfully re-isolated from symptomatic stem tissues, thus fulfilling Koch's postulates. Susceptibility of the tree species to *D. sapinea*, and the virulence of the *D. sapinea* isolates among those host species, differed significantly. Compared to the other *Pinus* species tested, *P. radiata* was highly susceptible to *D. sapinea*. There were also significant differences in the extents of the lesions produced by *D. sapinea* on the host species tested ($P < 0.05$) (Table 8). The longest lesions were on *P. radiata* and *P. sylvestris*. Lesion lengths caused by isolates were greater on *P. radiata*, *P. sylvestris* than *P. nigra* seedlings than on ($P < 0.05$).

Cross inoculations on twigs

Most of the isolates used in the inoculations caused lesions on the twigs of the five host species. Differences between the isolates obtained from the sampled pine species, in terms of ability to induce lesions on all tested twigs were significant ($P < 0.05$). In general, isolates from *P. taeda* caused longer lesions on *P. radiata* twigs (Table 8) than on the other host species. *Pinus radiata*, *P. sylvestris* and *P. nigra* twigs were susceptible to all isolates. MLGM analyses indicated that there was no host specificity from the cross inoculations (Table 9).

DISCUSSION

The studies reported in this paper have confirmed the presence of *D. sapinea* on *Pinus nigra*, *P. sylvestris*,

Table 8. Mean lesion lengths caused by *Diplodia sapinea* on seedlings of different *Pinus* spp. hosts.

Host	Mean lesion length (mm) (± S.D.)
Pinus radiata	18.1±0.580 A
P. sylvestris	16.5± 0.564 B
P. nigra	12.5± 0.564 B
P. taeda	5.4± 0.591 C
P. pinaster	5.4± 0.593 C

Means within a column followed by different letter are significantly different ($P < 0.05$) based on Duncan's Multiple Range test.

Table 9. Results of *Diplodia sapinea* cross inoculation of twigs.

Pinus. radiata isolates		*P. taeda* isolates		*P. pinaster* isolates		*P. sylvestris* isolates		*P. nigra* isolates	
P. sylvestris	23.8a	*P. radiata*	30.0a	*P. nigra*	13.0a	*P. nigra*	17.6a	*P. sylvestris*	15.5a
P. radiata	21.8a	*P. sylvestris*	19.3b	*P. radiata*	12.7a	*P. radiata*	14.8a	*P. nigra*	10.8b
P. nigra	10.8b	*P. nigra*	10.3bc	*P. sylvestris*	11.5ab	*P. sylvestris*	12.9ab	*P. radiata*	9.4bc
P. taeda	5.9c	*P. taeda*	5.8c	*P. pinaster*	5.3b	*P. pinaster*	6.0b	*P. pinaster*	6.3c
P. pinaster	5.3c	*P. pinaster*	4.4c	*P. taeda*	5.1b	*P. taeda*	4.5c	*P. teada*	5.7c

Means within a column followed by different letter are significantly different ($P < 0.05$) based on Duncan's Multiple Range test.

P. taeda, *P. pinaster* and *P. radiata* in the Kerpe Research Forest, in İzmit province of Turkey. On the basis of morphological characteristics of the pathogen in culture, ITS sequencing of isolates obtained from sampled trees with the symptoms and the pathogenicity tests performed on excised twigs of same host species, it was clear that *D. sapinea* was responsible for shoot blight and reductions in seed production in *Pinus* spp. trees at this location.

Typical Diplodia shoot blight symptoms (Maresi *et al*., 2007) were observed, scattered throughout the crowns of the trees. The most serious damage occurred on the middle and upper crowns. It is not clear why there was such high incidence of Diplodia shoot dieback in the Kerpe Research Forest, but there were no records to suggest that this was connected with predisposing factors such as wounding or drought. However, the trees were planted in unsuitable climatic conditions.

The design of the Kerpe research forest was not optimal for critical comparison of the tree species for disease incidence and severity, as the trees were not planted in blocks homogenous for site factors. *Pinus sylvestris* and *P. nigra* were growing on brown forest soil, *P. pinaster* and *P. taeda* on light hydromorphic grey brown podzolic soil and *P. radiata* on andesite brownstone soil on the main rock (Table 1). For tree species pairs growing on the same soil type, the disease was more severe on *P. nigra* and *P. pinaster* than on *P. sylvestris* and *P. taeda*. (Table 3).

Colony morphology of the isolates obtained here was identical to that of the *D. sapinea* described in De Wet *et al*. (2002). The presence of abundant pycnidia on branches, stems and cones of infected trees illustrated the potential of this fungus to cause epidemics when conditions are favourable for infection and disease development (Nicholls and Ostry, 1990).

In previous research, cones collected from tree canopies yielded conidia more frequently than cones collected from the ground (Doğmuş Lehtijärvi *et al*., 2014). In the present study, therefore, we sampled only from the tree canopies. It is very likely that cones in the canopies are more important for spread of the fungus than cones on the ground, and tree cones should be used for estimation of inoculum potential at particular sites (Munck and Stanosz, 2009).

Diplodia sapinea has been detected in the seeds of many *Pinus* species, including *P. radiata*, *P. nigra*, *P. sylvestris*, *P. resinosa*, as well as *P. patula*, *P. wallichiana*, *P. elliottii*, *P. taeda*, *P. oocarpa*, *P. caribaea*, *P. michoacana* and other tropical pines (Bihon *et al*., 2011). However, despite the probable critical importance of seed infection in the epidemiology of disease caused by this fungus, and implications for dissemination via plant material (e.g. from seed orchards or through international trade), seed and plant disemination is likely to be very important (Bihon *et al*., 2012a).

Diplodia sapinea causes degeneration of edible seeds on *P. pinea,* with direct economic consequences (Vagniluca *et al*., 1995; Santini *et al*., 2008). Moreover, the frequent occurrence of *Diplodia* symptoms on pine trees planted in urban landscapes and recreational areas has raised awareness of this pathogen among public administrations.

Multilocus genotyping with RAMS primers indicated that the trees in the sampled stands were colonized by thirteen *D. sapinea* genets. Genets found in *P. taeda* and *P. sylvestris* occurred only on these hosts, while the remaining genets were shared among other host species. Previous work in 2012 with *D. sapinea* in the same seed orchard showed that one genet occurred within *P. nigra* and *P. sylvestris* stands (Doğmuş Lehtijarvi *et al*., 2014). This indicates that the disease in the stand had possibly started with wound infections by one or a few clones of the pathogen (cf. Bihon *et al*., 2012b). In the present study, there were six distinguishable genets on the same hosts. The difference in number of genets could be due to spread of *Leptoglossus occidentalis* Heidemann to the study area. This alien invasive insect is a new vector of the fungus in Europe (Luchi *et al*., 2012), and could possibly have introduced more genets to the site. *Leptoglossus occidentalis* was first detected in Turkey in 2009 in Istanbul (Arslangündoğdu and Hızal, 2010), approx. 100 km from the Kerpe Research Forest. Owing to the rapid

increase in the known distribution of this insect around the Marmara Region over 2 to 3 years (Öztemiz and Doğanlar, 2015), it is possible that arrival and establishment of *L. occidentalis* in the area of the present study has affected the *D. sapinea* population, as the 13 genotypes were all closely related.

Bihon *et al.* (2012a), in examination of genetic differences between single *D. sapinea* populations from South Africa, Ethiopia, Argentina and Australia using microsatellite markers, found genetic variation between continents and within Australia.

Similarities between isolates of *D. pinea* were high when analysed with RAPD markers and isoenzyme analysis (Stanosz *et al.*, 1999). Burgess *et al.* (2004) studied six different populations of *D. pinea* isolated from *P. radiata* in New Zealand using SSR markers, and found small variations and diversity amongst populations. In the present study, the greatest numbers of polymorphic loci were in the pathogen population from *P. nigra*, with the lowest numbers of loci in the *D. sapinea* isolates from *P. radiata*.

The genetic similarities between *D. sapinea* genotypes and populations across continents suggests that the fungus has had a long asexual history of dispersal. Clones generally accumulate unique alleles in geographically isolated populations (Taylor *et al.*, 2000). In addition, plant genetic material such as seeds or seedlings for breeding programmes, has been exchanged widely between Turkey and other countries. Because *D. sapinea* is a latent pathogen, genotypes of the fungus have probably been spread with these materials (Burgess *et al.*, 2001; Flowers *et al.*, 2001).

The present study used three RAMS and M13 markers to detect genetic variation between *D. sapinea* isolates obtained from five different host pine species, although the isolate sampling was made at one site. Further analyses using SSR markers or other microsatellite markers are required to confirm these results and to determine if there is host specialization in *D. sapinea*. In the results presented here, cross inoculations suggested that there was no host specialization of this pathogen for the isolates tested.

Pathogenicity of *D. sapinea* did not relate to isolate origin or tested host in this study. This is similar to the results of Chou (1976), who found no differences in pathogenicity amongst New Zealand isolates of *D. pinea*. Doğmuş Lehtijärvi *et al.* (2009) previously reported the ability of a single isolate of *D. sapinea* to infect a range of coniferous species in Turkey, including *Cedrus libani* and *P. nigra*. In the present study, inoculations with 25 *D. sapinea* isolates resulted lesions on the tested coniferous tree species, with the greatest virulence on *P. nigra* and *P. sylvestris* and least on *P. taeda* and *P. pinaster*.

Outbreaks of disease caused by *D. sapinea* have occurred periodically wherever pines are grown. No direct and effective control measures are available for this pathogen in plantations and seed orchards. However, irrigation of seed orchards may prevent drought stress that can precede outbreaks of Diplodia shoot blight. Wounding of trees should also be minimized during forestry management to reduce infections of *D. sapinea*. Most importantly, foresters need to consider the risk of planting *P. sylvestris* and *P. nigra* in regions with unsuitable climate and location, such as low altitude, which may subject trees to stresses that predispose them to *Diplodia* infections.

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Research Papers

Phytophthora root and collar rots in fruit orchards in the Czech Republic

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Summary. A recent outbreak of *Phytophthora* diseases in fruit orchards was identified in the Czech Republic. The diseased trees showed characteristic symptoms including yellowing, wilting and sparse foliage, decreased yields, root and collar rot, and withering and dying of trees. In some orchards up to 10–15, and rarely up to 55%, of trees died. In total, 387 symptomatic trees of nine species from 44 fruit orchards, 16 samples of irrigation water from four orchards and 35 samples of nursery stock, were surveyed in 2012–2018. Oomycetes were recovered from 50.6 % of sampled trees in orchards, from 71.4 % of shipments of ex vitro-produced nursery plants, and from 93.8 % irrigation water samples. Seventeen *Phytophthora* species and 13 *Pythium* sensu lato species were recovered. The most frequent species in orchards were *Phytophthora cactorum*, *Phytophthora plurivora* and *Phytopythium vexans*. The most frequent species in nursery stock were *P. cactorum*, *Phy. vexans* and *Globisporangium intermedium*, and *Pythium helicoides*, *Phytophthora lacustris* and *Pythium litorale* were the most frequently found in irrigation water. The most frequent oomycete species recovered from nursery stock were also frequently isolated from orchards and planting material. The pathogenicity of 11 *Phytophthora* species was assessed *in vitro* in two frequently used rootstocks – ´St. Julien´ (plum) and ´M26´ (apple). This revealed that the less known and relatively rarely isolated species (*P. gregata*, *P. chlamydospora* × *amnicola*, *P. inundata*, *P. lacustris* and *P. sansomeana*) were more virulent than the more frequently occurring species *P. cactorum* and *P. plurivora*, and could pose potential threats to fruit orchards in the future. The increase of *Phytophthora* in fruit orchards is probably related to the trading and planting of infected nursery stock, to climate change and water stress, and to dismissal of the problem of *Phytophthora* diseases by stakeholders and inappropriate management. Future investigation should focus on the development of effective disease management, including assessment and selection of rootstocks for resistance.

Keywords. *Phytophthora*, *Malus*, *Prunus*, fruit orchards, rootstock.

INTRODUCTION

Phytophthora species are well known pathogens of many fruit tree species including apple, plum and pear (Mircetich and Browne, 1987; Erwin and Ribeiro, 1996; Sutton *et al*., 2014). These pathogens have been frequent-

ly reported to cause serious and increasing problems, especially in the Mediterranean and European countries (Smith *et al*., 1988; Laviola *et al*., 1990; Harris, 1991; Thomidis, 2003; Pane *et al*., 2009; Nakova, 2010a). Although research on the diversity and epidemiology of *Phytophthora* pathogens in woody plants is already a tradition in the Czech Republic (Černý *et al*., 2011; Mrázková *et al*., 2011; 2013), and a total of 26 *Phytophthora* species have been isolated from many forest and ornamental woody plants (CCPO, 2018), with many able to parasitize fruit trees (including *P. cactorum* (Lebert & Cohn) J. Schröt., *P. cambivora* (Petri) Buisman, *P. cinnamomi* Rands, *P. citrophthora (*R.E. Sm. & E.H. Sm.) Leonian, *P. cryptogea* Pethybr. & Laff., *P. plurivora* T. Jung & T.I. Burgess, *P. syringae* (Kleb.) Kleb. (Erwin and Ribeiro, 1996), investigation of these pathogens in fruit trees has not been carried out in the country.

Increases of the characteristic symptoms of *Phytophthora* diseases (root and collar rots, yellowing of foliage, withering of trees), tree death and yield reductions have been registered in many fruit orchards in different regions of the Czech Republic. The country is a regionally important producer of fruit in Central Europe. The most important fruits grown in the Czech Republic are apples (nearly 8,000 ha of orchards), plums (2,056 ha), sour cherries (1,425 ha), apricots (925 ha), cherries (901 ha), pears (745 ha) and peaches (407 ha; Buchtová, 2015). If *Phytophthora* pathogens are responsible for these diseases, they pose important and long-term risks to fruit producers. The aim of the present study was to verify whether *Phytophthora* pathogens were responsible for the recently increasing losses in Czech Republic fruit orchards, to evaluate pathogen diversity and virulence.

MATERIALS AND METHODS

Sampling and isolation

Sampling for the detection and isolation of *Phytophthora* pathogens was carried out from May to November during the fruit tree growing seasons of 2012 to 2018. Trees with sparse, yellowing or wilting foliage and declining trees were identified in the surveyed orchards. If collar rot or rot of the main roots were detected (based on the presence of of necroses, exudates, cracks in the bark, or bark discoloration), the bark of each tree was carefully removed, and the proximal transition zone between necrotised and healthy tissues was detected. Freshly colonised conductive tissues with characteristic reddish, brownish or honey colours were found, aseptically sampled (ca. 20–50 cm^2 of tissue) and stored in sterile plastic bags. In the case of root rots, samples of soil with damaged feeding roots of each tree were collected from three sites at depths of 10–20 cm in the root zone. A mixed sample of approx. 1–2 L was then created from each sampled tree. The samples were individually packaged in plastic bags and transferred to the laboratory for immediate processing.

Collected necrotised conductive tissues of collar or main roots were rinsed under tap water in the laboratory, and segments (3 × 3 mm) from the edge of each active lesion were excised. The pieces were then surface-disinfected by immersion in 95 % ethanol for 10 sec, rinsed in deionized sterile water and dried with sterile filter paper. The tissue pieces were then placed onto selective PARPNH agar medium (V8-juice 200 mL, pimaricin 10 ppm, ampicillin 200 ppm, rifampicin 10 ppm, quintozene 25 ppm, nystatin 50 ppm, hymexazole 50 ppm, agar 15 g, and $CaCO_3$ 3 g**;** Jung *et al*., 1996) in 9 cm diam. Petri dishes, and incubated at 20°C in the dark. After 2 to 5 d, the PARPNH plates were inspected for the presence of characteristic coenocytic mycelium, and hyphal tips of each isolate were transferred onto plates containing V8A medium (V8-juice 200 mL, agar 15 g, and $CaCO_3$ 3 g; Erwin and Ribeiro, 1996) for isolation of oomycetes. Separate tissue pieces were also placed in plates containing malt extract agar (Becton, Dickinson and Co.) for isolation of fungi. Pure isolates were transferred onto oatmeal agar (HiMedia Ltd) slants in tubes. Cultures of oomycetes were stored at 12°C in the Czech Collection of Phytopathogenic Oomycetes (CCPO, 2018).

Soil samples were processed using a baiting method adopted from that of Werres and Junker (2015). Approximately 150 cm^3 of soil containing damaged roots was placed in a deep plastic bowl, deionized water was added to 2–3 cm above the sample, and baits of young, healthy, previously tap water-rinsed leaves of the susceptible *Rhododendron yakushimanum* ′Silberwolke′ were added onto the water surface. These bait cultures were then incubated at room temperature in natural day/night conditions. The water was changed to prevent bacterial upsurge. When characteristic lesions on the baits appeared, the segments with necrotised tissues were excised, surface sterilized and incubated on PARPNH agar medium (described above).

Irrigation water samples were tested for the presence of oomycetes in August and September 2017 using the method of Werres and Junker (2015). Sources of irrigation water were investigated in six localities in southern Moravia and eastern Bohemia in the same fruit orchards investigated for the presence of *Phytophthora* diseases. Four washed and healthy leaves of different cultivars of susceptible *R. yakushimanum* Nakai (′Polaris′, ′Silberwolke′, ′Sonatine′) and *R.* ×*hybridum* Ker Gawl. (′Cun-

nigham White´) were placed into sterilised bags made from sparse fabric. Four bags containing leaf baits were put into the tested sources of irrigation water (small ponds, brooks and streams). In each case, the bags were left to float close under the water surface, and after 1 week, the samples were collected, transported to the laboratory and processed using leaf baits (described above).

The tests for the presence of oomycetes in nursery stocks from commercial trade were performed in the winter and spring of 2017 and 2018. In total, 33 shipments of 19 fruit tree types of *ex vitro* (produced by layering) and *in vitro* (produced from micropropagation) nursery stock of pome and stone fruits were investigated. From plants delivered from diverse national sources or from other EU countries (France, Italy, Poland, the Netherlands), at least 15 individual plants from each shipment showing damage of roots were selected and further processed. The whole root systems of plants (the material was sent as bare roots with partially cut and washed roots) were cut off and put into plastic bowls. Deionized water was added to each bowl, samples were incubated, and the isolates acquired using the baiting method (described above).

Morphological identification of isolates

Isolates were identified by comparing their colony growth patterns and morphological features with the species descriptions reported in the literature (Erwin and Ribeiro, 1996; Brasier *et al*., 2003; Gallegly and Hong, 2008; Hansen *et al*., 2009; Nechwatal *et al*., 2013; Nagel *et al*., 2013). Colony morphology was observed for 3- and 7-d-old cultures grown on corn meal agar (CMA, Sigma Aldrich), malt extract agar (MEA, Sigma Aldrich), carrot agar (CA, HiMedia Ltd), potato dextrose agar (PDA, HiMedia Ltd) and V8 juice agar (V8A), in 90 mm diam. Petri dishes at 20°C in darkness (two replications per medium). The characteristics of organism structures (oogonia, antheridia, chlamydospores and hyphal swellings) were measured after 5–10 d of incubation on V8A. Sporangia were produced after 24–48 incubation at 20°C by placement of a disk of mycelium from a 10-d-old culture grown on V8A in soil extract, prepared according to Jung *et al.* (1996). Measurements were performed with a light microscope under 400× or 1000× magnification, and 20 measurements were made for each evaluated characteristics.

Molecular characterization of isolates

The total genomic DNA was extracted from isolates using a DNeasy UltraClean Microbial Kit (QIAGEN, Germany) according to the manufacturer's instructions. For molecular identification, either the ITS region with ITS1/ITS4 primers (White *et al*., 1990) or the cytochrome *c* oxidase subunit I (COI) region was amplified with OomCoxILevup/Fm85mod primers (Robideau *et al*., 2011). All PCR reactions were performed in a Mastercycler Nexus Gradient GSX 1 thermal cycler (Eppendorf) and visualised using agarose gel electrophoresis in 1% TBE buffer along with a 100-bp DNA ladder (New England Biolabs) as a size marker. PCR products were purified and sequenced in both directions by Macrogen Inc. (KR) using the same primers applied for PCR amplification. The sequences obtained were edited and aligned in BIOEDIT v. 7.0.9.0 (Hall, 1999) and compared with the sequences present in the GenBank database using a NCBI BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) to identify the closest related sequences. Only published sequences were considered.

Pathogenicity tests

Thirteen *Phytophthora* isolates obtained during orchard and nursery stock monitoring were selected to represent the extent of orchards of pome and stone fruit and the diversity of the isolated pathogens and used in the assay. The isolates were: *P. cactorum* (isolate no. 809), *P. cambivora* (819), *P. chlamydospora* Brasier & E.M. Hansen × *P. amnicola* T.I. Burgess & T. Jung (801), *P. cryptogea* (812), *P. gonapodyides* (H.E. Petersen) Buisman (864), *P. gregata* T. Jung, Stukely & T.I. Burgess (865), *P. sansomeana* E.M. Hansen & Reeser (868) and *P. syringae* (945), which all originated from apple trees, and *P. cactorum* (795) was from cherry, *P. cambivora* (815), was from cherry, *P. inundata* Brasier, Sánch. Hern. & S.A. Kirk (924) was from wild plum, *P. lacustris* Brasier, Cacciola, Nechw., T. Jung & Bakonyi (791) from apricot, and *P. plurivora* (789) was from cherry. Fresh cultures of isolates were obtained by transferring agar plugs to Petri plates containing V8A agar and incubating at 20°C in the dark for 7 d. The isolates were each incubated in 250 mL beakers with 25 mL CMA medium amended with antibiotics (pimaricin, 10 mg; ampicillin, 250 mg; and rifampicin, 10 mg) for 7 d at 20°C. Virulence of the isolates was tested on broadly used 1-y-old apple rootstocks of ´M26´ and plum rootstocks of ´St. Julien´ using the methods of Jeffers *et al.* (1981) and Thomidis *et al.* (2008). The dormant shoots of tested rootstocks were divided into segments (length 10 cm and width 0.5–0.8 cm), using a flamed sharp knife, and were each trimmed to a slant at the base. Four segments were aseptically put into medium covered by a pathogen colony and incubated for 10 d at 20°C in the dark. After incubation, the

epidermis of each segment was carefully removed, and the length of necrosis (from the agar surface) was measured on two opposite sides. The virulence of each strain was assessed in five replicates, i.e., 40 measurements were made and evaluated for each pathogen × rootstock combination. This experiment was carried out during February 2018.

Statistical analyses

One-way analysis of variance with Welch correction (Sheskin, 2011) was used to test the hypothesis that the size of necrosis was the same for all isolates and for both rootstocks. Welch correction was used because different variances were measured. A multiple comparison procedure using Welch tests with Holm correction (Sheskin, 2011) was used to evaluate the differences between individual isolates. Statistical analyses were performed using the R statistical program (R Core Team, 2017).

RESULTS

Occurrence of Phytophthora *and other root and collar pathogens*

During this investigation thousands of trees were found with characteristic *Phytophthora* disease symptoms. These included: rot of feeding and main roots; collar rot; presence of tarry and rusty spots on bark; lesions of bark and conductive tissues; sparse, yellowing and wilting foliage; premature leaf fall; and withering and death of trees (Figures 1 and 2). Diseased trees were identified in nurseries and in young and established 10–30-y-old orchards. Symptomatic trees were individually present in orchards but were usually grouped in disease "hot spots", with each containing dying or dead trees in the middle and with less-damaged trees exhibiting yellowing foliage around the margin. Sometimes, diseased transplants were identified in highly affected parts of orchards (Figure 2). The number of diseased trees varied greatly in the investigated orchards, from a few individuals to many hundreds of trees (up to ca. 15% or more of trees in orchards).

A total of 387 symptomatic fruit trees (65.1 % were pome fruit and 34.9 % were stone fruit trees) of nine different plant species, from 44 locations, were sampled from 12 of 15 regions of the Czech Republic, during 2012 to 2018 (Figure 3). In total, Oomycete species were isolated from 196 tree samples (50.6% of those sampled), and 13 *Phytophthora* species were identified. The most frequent pathogens were *P. cactorum* (58% of *Phytophthora* isolates), which was isolated from apple (*Malus domestica* Borkh.), apricot (*Prunus armeniaca* L.), cherry (*Prunus cerasus* L.), peach (*Prunus persica* (L.) Batsch), pear (*Pyrus communis* L.), plum (*Prunus domestica* L.), quince (*Cydonia oblonga* Mill.) and wild cherry (*Prunus avium* L.), and *P. plurivora* (16%), which was isolated from apple, cherry, myrobalan (*Prunus cerasifera* Ehrh.), pear and plum (Figure 4). Other species with lesser isolation frequencies were *P. syringae*. *P. chlamydospora*×*amnicola*, *P. cambivora* and others (Table 1, Figure 4). The greatest diversity of *Phytophthora* pathogens was identified in the Hradec Králové region (eight species), the South Moravian region (7) and South Bohemian region (6; Figure 3). *Phytophthora chlamydospora* × *amnicola* (GenBank Accession No. MK012378), *P. inundata* (MK012377) and *P. sansomeana* (MK012376) were identified as new taxa for the Czech Republic. Further, many *Pythium* s. l. isolates were obtained. In total, isolates of 13 *Pythium* s. l. species were obtained from 13.5% of sampled trees of seven host species. Among the most frequent pathogenic species were *Phytopythium vexans* (de Bary) Abad, de Cock, Bala, Robideau, A.M. Lodhi & Lévesque, which was isolated from 4% of the samples from four hosts (apple, apricot, myrobalan and pear), *Globisporangium intermedium* (de Bary) Uzuhashi, Tojo & Kakish. (from apple, apricot, myrobalan, pear and plum), *G. heterothallicum* (W.A. Campb. & F.F. Hendrix) Uzuhashi, Tojo & Kakish. (from pear), *G.* cf. *mamillatum* (Meurs) Uzuhashi, Tojo & Kakish. (from pear and apricot), *G. spiculum* (B. Paul) Uzuhashi, Tojo & Kakish. (from apricot), *Phy. citrinum* (B. Paul) Abad, de Cock, Bala, Robideau, A.M. Lodhi & Lévesque (from apple and pear), *Phy. helicoides* (Drechsler) Abad, de Cock, Bala, Robideau, A.M. Lodhi & Lévesque (from apple), *Pythium* cf. *coloratum* Vaartaja (from pear), *Py.* cf. *diclinum* Tokun. (from apple) and *Py. folliculosum* B. Paul (from cherry). As well, *Phytopythium litorale* (Nechw.) Abad, de Cock, Bala, Robideau, A.M. Lodhi & Lévesque, *Phy.* cf. *ostracodes* (Drechsler) Abad, de Cock, Bala, Robideau, A.M. Lodhi & Lévesque, and *Pythium* spp. were also isolated.

Out of 16 samples of irrigation water taken from four fruit orchards, 15 (94%) contained oomycetes, and *Phytophthora* pathogens were found in nine samples (56%). The most frequently isolated were *Phytophthora lacustris* and *P. citrophthora*, followed by *P.* cf. *hydropathica* C.X. Hong & Gallegly (new for the Czech Republic), *P. plurivora* and *P. bilorbang* Aghighi, G.E. Hardy, J.K. Scott & T.I. Burgess. Three species (*P. bilorbang*, *P.* cf. *hydropathica* and *P. citrophthora*) were only isolated from irrigation water. As well, *Phytopythium helicoides*, *Phy. litorale* and *G. heterothallicum* were also isolated from irrigation water.

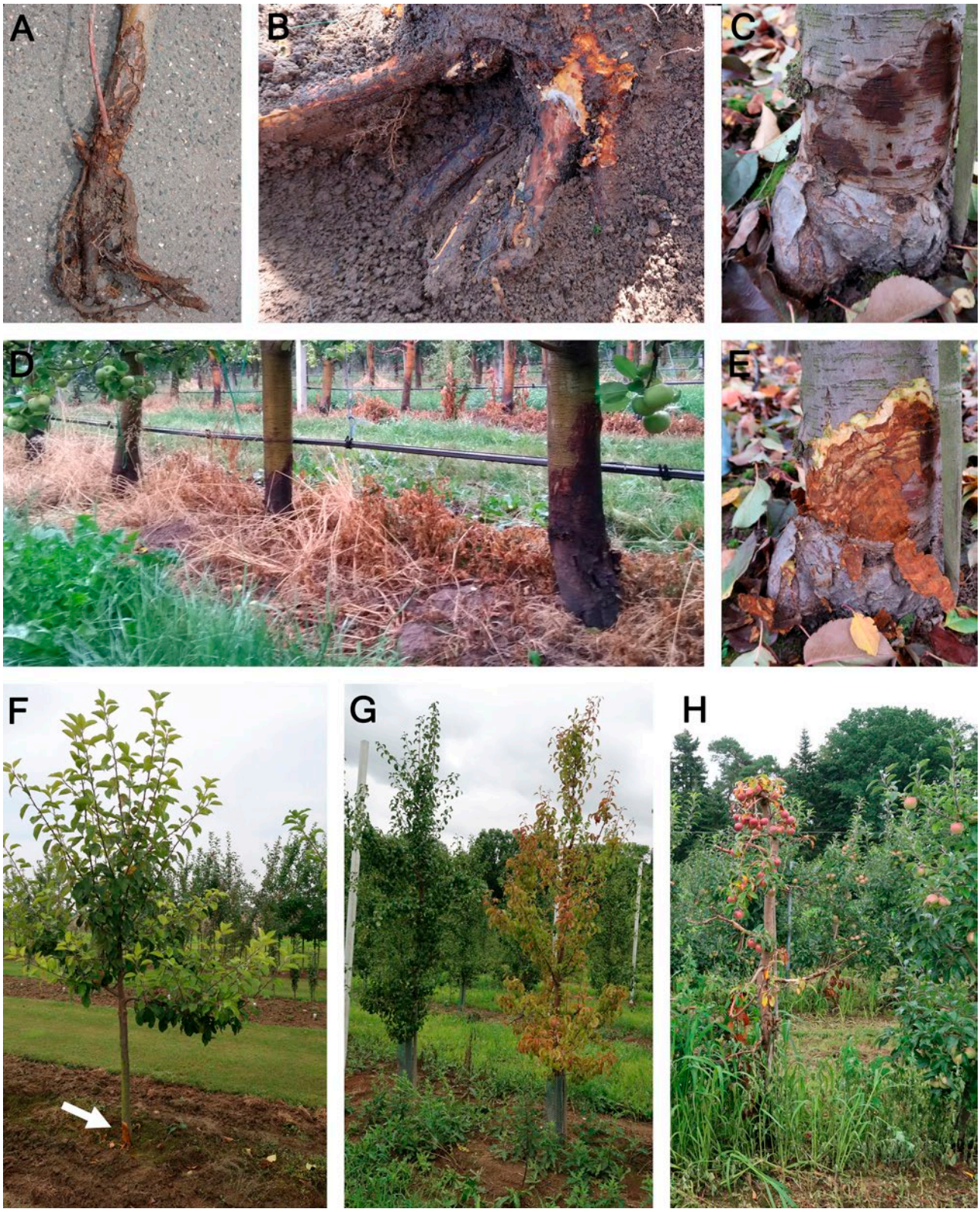

Figure 1. Characteristic symptoms of *Phytophthora* root and collar rot of *Malus domestica* (A–F, H) and *Pyrus communis* (G): A, root and collar rot of a young tree; B, root rot of a mature tree; C, characteristic tar spots on a tree collar; D, some apple orchards severely damaged by the disease; E, collar rot after the removal of bark (tree from Picture C); F, young tree with yellowed foliage resulting from collar rot (arrow); G, characteristic foliage yellowing of a pear tree; H, heavily diseased trees are characterised by production of small fruits

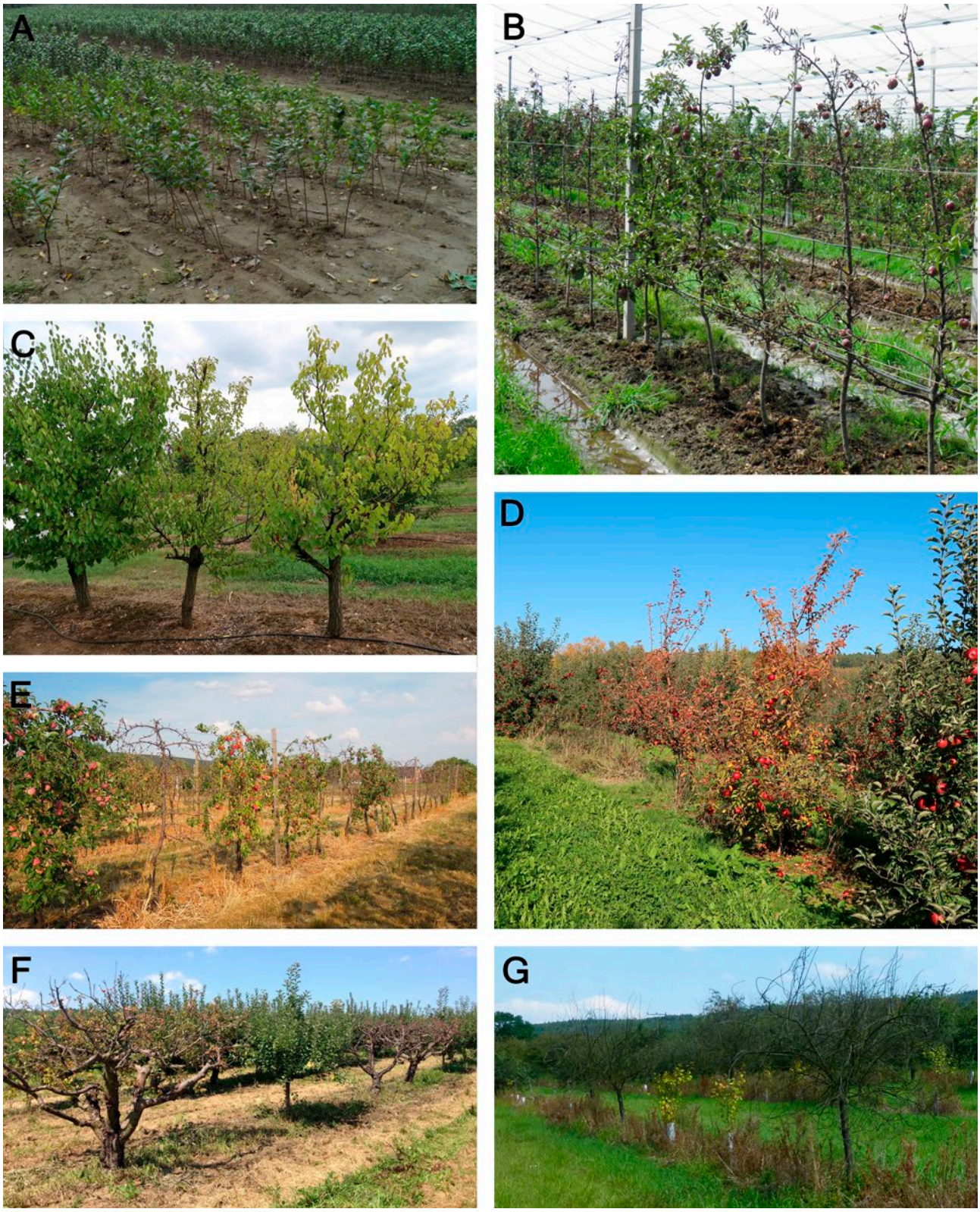

Figure 2. Characteristic damage of *Malus domestica* fruit nurseries and orchards (A, B, D–F) and *Prunus domestica* (C, G): A, yellow foliage of infected seedlings, B, an excess of water promoted the disease caused by *P. chlaymdospora* × *amnicola* in a young orchard; C, new disease outbreaks are apparent due foliage yellowing; D, premature leaf senescence and shedding of infected trees; E, characteristic disease focus in an orchard row; F, killed mature trees; G: attacked replanting in a damaged orchard.

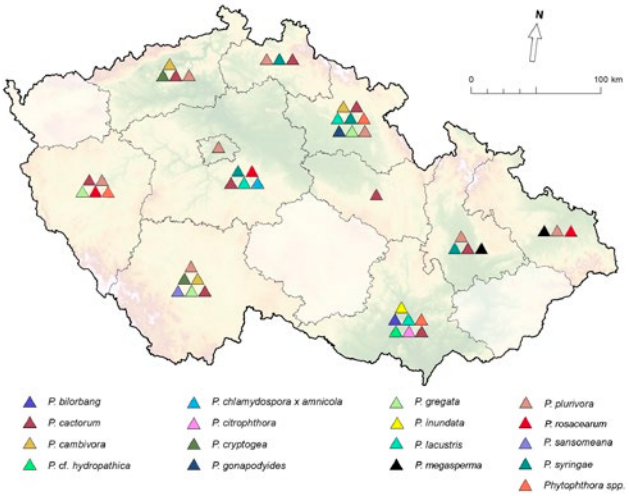

Figure 3. Regional map of the occurrence of *Phytophthora* spp. in fruit orchards (including irrigation water) in the Czech Republic. The regions without data are in dull colour.

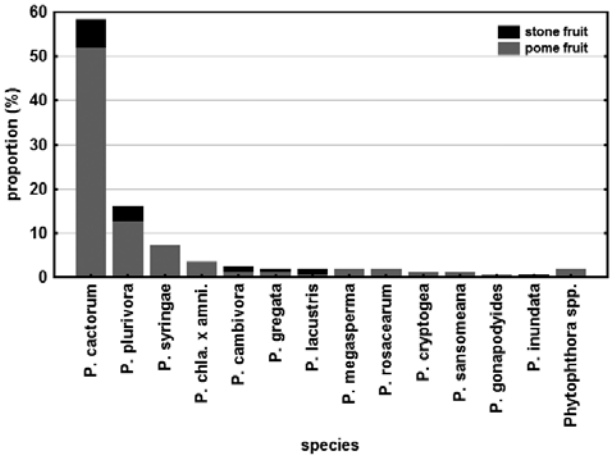

Figure 4. Frequency of occurrence of *Phytophthora* in fruit trees in the Czech Republic.

The investigation also showed that 71% of sampled lots of *ex vitro*-produced nursery stock contained plants infected by oomycetes, whereas *in vitro* plants were free of these organisms. *Phytophthora cactorum*, the only species of the genus isolated from nursery stock, was the most frequently isolated oomycete, identified in 46% of sampled groups of *ex vitro*-produced plants, and 25% of other positive samples were infested only by *Pythium* spp. (Table 2). *Phytophthora cactorum* was identified in planting material from all source countries in frequencies from 36 (the Netherlands) to 67% (Poland) of tested lots (Table 2). Some pathogenic species from *Pythium* s. l. were also obtained from nursery stock. The most frequent were *Phytopythium vexans* (29% of samples) and *Globisporangium intermedium* (14%). The other less-frequently isolated species were *Phy. citrinum*, *Py. emineosum* and *Py. litorale* (Table 2).

Some fungal pathogens were isolated from 37 (9.5 %) of samples of damaged roots and collars of fruit trees during this study. Identified pathogens causing collar and stem diseases included *Cadophora luteo-olivacea* (J.F.H. Beyma) T.C. Harr. & McNew (from collar rot of pear), *Calosphaeria pulchella* (Pers.) J. Schröt. (from canker of apricot), *Cylindrocladiella parva* (P.J. Anderson) Boesew. (from collar rot of plum), *Diaporthe eres* Nitschke, *D. rudis* (Fr.) Nitschke and *Diaporthe* sp. (from canker of apple and pear), *Valsaria insitiva* (Tode) Ces. & De Not. (from canker of apple and peach), *Leucostoma persoonii* (Nitschke) Höhn. (from canker of apple, apricot and peach), *Neonectria* sp. (from canker of plum), *Peyronellaea obtusa* (Fuckel) Aveskamp, Gruyter & Verkley (from canker of pear), *Trametes versicolor* (L.) Lloyd (silver leaf of plum). Identified root rot pathogens included *Didymella pomorum* (Thüm.) Qian Chen & L. Cai (from apricot), *Fusarium oxysporum* Schltdl. and *F. sporotrichioides* Sherb. (from apricot), *Nectria haematococca* Berk. & Broome 1873 (from apple and pear), *Roesleria subterranea* (Weinm.) Redhead (from pear), and *Valsaria insitiva* (rot of main roots of apricot). Some of these pathogens (*Cylindrocladiella parva*, *Diaporthe rudis*, *Peyronellaea obtusa* and *Roesleria subterranea*) or the associated diseases (collar rot of pear caused by *Cadophora luteo-olivacea*, silver leaf of plum caused by *Trametes versicolor,* root rot of apricot caused by *Didymella pomorum*) are the first records for the Czech Republic.

Factors potentially affecting the numbers of diseased trees were not systematically evaluated. However, it was apparent that younger orchards (up to 10 or 15 y old) were more damaged than older orchards. Diseased trees were more frequent in places with locally unsuitable soil conditions (poor drainage, high water table), and these trees apparently served as long-term reservoirs of pathogens infection in affected orchards. Unsuitable applications of water to tree collars or their direct surroundings by drip irrigation systems were also associated with collar rot development.

Influence of rootstocks and isolates on the extent of damage

The difference in the extent of damage caused by different *Phytophthora* pathogens in ´M26´ and ´St. Julien´ rootstocks was highly significant ($a = 8.7 \times 10^{-8}$). The mean extent of necrosis in ´M26´ rootstock was 15.0 mm, whereas the mean for ´St. Julien´ was 21.6 mm. The origin of the isolates (pome or stone fruits) did not have

Table 1. Overview of *Phytophthora* spp. isolated from fruit trees (host species, cultivars and rootstocks) and diseases they caused (C: collar rot, R: root rot) in the Czech Republic.

Phytophthora species	Identified host and disease			
	Species, cultivar	Disease	Rootstock	Disease
P. cactorum	*Cydonia oblonga*	R	*Cydonia oblonga*	C,R
	Malus domestica	C,R	Geneva G11	R
	Malus domestica ´Bohemia´	R	JTE	C
	Malus domestica ´Braeburn Lochbuie´	R	M9	C,R
	Malus domestica ´Champion´	R	M26	R
	Malus domestica ´Golden Delicious´	R	MA	C,R
	Malus domestica ´Heliodor´	R	MM106	C,R
	Malus domestica ´Idared´	R	*Malus domestica*	C,R
	Malus domestica ´Jonagold´	R	P14	C,R
	Malus domestica ´Luna´	C,R	Pajam	R
	Malus domestica ´Melba´	R	*Prunus avium*	R
	Malus domestica ´Minerva´	C,R	*Prunus cerasifera*	R
	Malus domestica ´Red Jonaprince´	C,R	St. Julien	R
	Malus domestica ´Red Topaz´	C		
	Malus domestica ´Remo´	R		
	Malus domestica ´Rozela´	C,R		
	Malus domestica ´Selena´	R		
	Malus domestica ´Topaz´	C,R		
	Prunus armeniaca	C,R		
	Prunus avium	R		
	Prunus avium ´Burlat´	R		
	Prunus cerasus ´Fanal´	R		
	Prunus cerasus ´Újfehértoi Fürtös´	R		
	Prunus domestica	C,R		
	Prunus persica	R		
	Prunus persica ´Favorita Morettini´	R		
	Pyrus communis	C,R		
	Pyrus communis ´Alexander Lucas´	C,R		
	Pyrus communis ´Bohemica´	C,R		
	Pyrus communis ´Conference´	C,R		
P. cambivora	*Malus domestica*	C,R	M9	R
	Prunus cerasus	C,R		
P. cryptogea	*Malus domestica* ´Topaz´	C,R	M9	R
	Malus domestica ´Topaz Red´	R	MM106	R
	Prunus domestica ´Reine Claude d'Althan´	R	*Prunus cerasifera*	R
P. gonapodyides	*Malus domestica* ´Orion´	C,R	-	
P. gregata	*Malus domestica* ´Champion´	R	M26	R
	Malus domestica ´Orion´	C,R	*Prunus mahaleb*	R
	Prunus cerasus	R		
P. chlamydospora × *amnicola*	*Malus domestica*	R	-	
	Malus domestica ´Braeburn´	R		
	Malus domestica ´Golden Delicious´	R		
P. inundata	*Prunus domestica*	R	-	
P. lacustris	*Malus domestica* ´Topaz´	R	MM106	R
	Prunus armeniaca	C,R	St. Julien	C,R
P. megasperma	*Malus domestica*	R	-	

(Continued)

statistically significant effects on the average length of necroses in either rootstock (data not presented).

The virulence of particular isolates in different rootstocks is shown in Table 3. Most of the isolates caused greater damage to ´St. Julien´ than to ´M26´. These differences were usually (with the exceptions of the strains *P. cambivora* 819 and *P. lacustris* 791) statistically supported. The greatest differences in virulence towards the

Table 1. (Continued).

Phytophthora species	Identified host and disease			
	Species, cultivar	Disease	Rootstock	Disease
P. plurivora	*Malus domestica*	R	JTE	C,R
	Malus domestica ′Bohemia′	C,R	M9	R
	Malus domestica ′Idared′	R	M26	R
	Malus domestica ′James Grieve′	C,R	*Prunus cerasifera*	R
	Malus domestica ′Red Topaz′	C,R		
	Malus domestica ′Topaz′	C,R		
	Prunus cerasifera nigra	R		
	Prunus cerasus ′Újfehértoi Fürtös′	C,R		
	Prunus domestica	R		
	Prunus domestica ′Haganta′	R		
	Prunus domestica ′Reine Claude d'Althan′	R		
	Pyrus communis	R		
	Pyrus communis ′Alexander Lucas′	C,R		
P. rosacearum	*Malus domestica*	R	M26	R
	Malus domestica ′Selena′×′Rozela′	R		
P. samsomeana	*Malus domestica* ′Topaz Red′	R	M9	R
	Pyrus communis ′Alexander Lucas′	R		
P. syringae	*Malus domestica*	R	M9	
	Malus domestica ′Melodie′	R		
	Malus domestica ′Topaz′	R		
Phytophthora spp.	*Malus domestica* ′Rozela′	R	M26	C,R
	Malus domestica ′Topaz′	R,C		
	Malus domestica ′Topaz Red′	R		

rootstocks were detected in *P. gregata* (necrosis in ′St. Julien′ was by 57% longer), *P. gonapodyides* (48%) and *P. plurivora* (46%). However, for apple ′M26′ rootstock, the three most virulent isolates were *P. cactorum* 795, *P. cactorum* 809 and *P. syringae* 945. The differences in virulence to rootstock of *P. cactorum* 795, and *P. syringae* 945 were statistically significant (Table 3).

Virulence of Phytophthora *pathogens to rootstocks*

In both infection experiments (′St. Julien′ and ′M26′), the mean necrosis lengths differed significantly among the isolates, and hypotheses of the equality of mean values of necrosis length were rejected ($P < 0.001$). The differences among the isolates were greater in ′St. Julien′ rootstock than in ′M26′.

In ′M26′ rootstock, the most pathogenic strains were *P. lacustris* (isolate no 791; mean necrosis length = 21.2 mm), *P. chlamydospora* × *amnicola* (801; mean = 20.0 mm), and *P. cryptogea* (812; mean = 17.0 mm). The isolate *P. syringae* (945) was the least pathogenic (Table 3). Differences in virulence among particular isolates were less important, and only three homogeneous groups (at $a = 0.05$), with overlaps, were established (Figure 5).

In ′St. Julien′, the most pathogenic strains were *P. gregata* (isolate 865; mean necrosis length = 35.6 mm) *P. chlamydospora* × *amnicola* (801; mean = 33.8 mm), and *P. sansomeana* (868; mean = 26.9 mm) (Table 3). The isolate *P. syringae* (945) was again the least pathogenic. Differences in virulence among particular isolates were important and five homogeneous groups ($a = 0.05$) were established (Figure 5).

DISCUSSION

The presented outcomes clearly demonstrated the importance of Oomycete pathogens in fruit orchards in the Czech Republic. It is very likely that the outcomes of this study (51% of positively tested trees, 17 *Phytophthora* and 13 *Pythium* s. l. identified species) are underestimated because of the limited number and locations of sampling, absence of sampling of asymptomatic trees, and potential displacing of *Phytophthora* pathogens by secondary fungal pathogens in lesions. Moreover, many species of *Pythium* s. l. are susceptible to hymexazol (Kato *et al*., 1990), which was used in the isolation medium utilized in this study. Documented tree losses reached 15 % or more diseased or dead trees in the most affected, usually younger, orchards (Figure 2). This was

Table 2. Overview of *Phytophthora* and *Pythium* s. l. isolations from commercial nursery material.

Host species	Rootstock	Country of origin	Year	Pathogen
Cydonia oblonga	Kwee Adams	NL	2018	*P. cactorum, Phy. vexans*
Cydonia oblonga	MA	NL	2017	*P. cactorum*
Malus domestica	A2	NL	2018	-
Malus domestica	Geneva G11	FR	2017	*P. cactorum, Phy. vexans*
Malus domestica	MM106	NL	2018	-
Malus domestica	MM106	NL	2017	*Py. litorale*
Malus domestica	M26	NL	2018	*P. cactorum, Phy. citrinum,*
Malus domestica	M9	CZ	2018	*P. cactorum*
Malus domestica	M9	CZ	2018	*G.* cf. *intermedium*
Malus domestica	M9	FR	2018	*P cactorum, Phy. vexans*
Malus domestica	M9	FR	2018	*Phy. vexans*, *Py.* cf. *litorale*
Malus domestica	M9	NL	2017	*Phy. vexans*
Malus domestica	M9	NL	2018	*P. cactorum, Phy. vexans*
Malus domestica	M9	NL	2018	-
Malus domestica	M9 Emla	FR	2018	*Py.* cf. *litorale*
Malus domestica	P14	PL	2018	*P. cactorum*, *Py. emineosum*
Prunus avium		NL	2017	-
Prunus avium		NL	2018	*Phy.* cf. *vexans*
Prunus cerasifera		CZ	2017	*P. cactorum*
Prunus cerasifera		CZ	2018	-
Prunus cerasifera		PL	2018	*P. cactorum*, *G. intermedium*
Prunus cerasifera	M29C/in vitro	IT	2017	-
Prunus domestica	St. Julien	NL	2017	*P. cactorum, Phy. vexans*
Prunus domestica	St. Julien	NL	2018	-
Prunus domestica	Wavit®/in vitro	IT	2017	-
Prunus domestica	Wavit®/in vitro	IT	2018	-
Prunus domestica × *canescens*	Gisela® 5/in vitro	IT	2017	-
Prunus domestica × *canescens*	Gisela® 6/in vitro	IT	2017	-
Prunus mahaleb		PL	2017	-
Prunus mahaleb		PL	2018	*P. cactorum*, *G. intermedium*
Prunus spinosa	WUR S766	NL	2018	-
Pyrus caucasica		PL	2017	*G.* cf. *intermedium*
Pyrus caucasica		PL	2018	*P. cactorum*

surprising, as no important problems potentially connected with oomycetous pathogens have been previously reported in the area. Moreover, *Phytophthora* pathogens have not been recently highlighted as important phytopathogenic problems in fruit production in other Central European countries, including Poland or Germany which are important fruit producers in the region. The last important *Phytophthora* outbreak in fruit trees was reported in temperate Europe more than 60 years ago (e.g., Braun, 1952; Buddenhagen, 1955; Smith, 1953). Effective control measures were developed, and more resistant rootstock began to be used, so *Phytophthora* problems became less important in modern intensive orchards (Smith *et al*., 1988). Although the most frequent *Phytophthora* pathogen (*P. cactorum*) is the same, the Czech outbreak is likely to be recent as the main problems occur in orchards planted after 2000, and is probably independent of previously described outbreaks (e.g., Braun, 1952; Buddenhagen, 1955; Smith, 1953).

The recent increase in *Phytophthora* activity could be connected to several factors. The most important is likely to be the increase of imports of infected rootstocks, as plant trade is a main pathway for spreading these pathogens (Brasier, 2008). In our investigation, the *ex vitro*-plant material from all tested countries was found to be infected, and *P. cactorum* was identified in 46.4% of tested sample lots. Another factor affecting the importance of alien *Phytophthora* spp. is likely to be changing

Table 3. Mean necrosis lengths (median and standard deviations) caused by individual isolates (isolates from pome fruit are marked by the symbol *) on ´M26´ and ´St. Julien´ rootstocks and differences in pathogenicity toward both rootstocks.

Species	Isolate No.	Mean necrosis length (mm)		Probability (*P*)
		M26	St. Julien	
P. cactorum	795	13.76 (10.34)	8.73 (2.19)	0.045
*P. cactorum**	809	16.79 (13.03)	11.38 (5.29)	0.097
P. cambivora	815	13.04 (9.30)	20.60 (7.62)	0.008
*P. cambivora**	819	11.43 (6.54)	13.89 (9.36)	0.341
*P. cryptogea**	812	16.97 (3.52)	22.61 (5.66)	< 0.001
*P. gonapodyides**	864	12.91 (6.69)	24.93 (10.82)	< 0.001
*P. gregata**	865	15.24 (4.25)	35.56 (11.38)	< 0.001
P. chlamydospora × *amnicola**	801	19.98 (7.84)	33.81 (11.76)	< 0.001
P. inundata	924	14.05 (8.96)	24.46 (11.56)	0.003
P. lacustris	791	21.16 (12.79)	26.26 (13.32)	0.225
P. plurivora	789	13.05 (6.65)	24.36 (8.19)	< 0.001
*P. sansomeana**	868	15.54 (5.73)	26.93 (6.67)	< 0.001
*P. syringae**	945	11.03 (4.71)	6.73 (7.77)	0.042

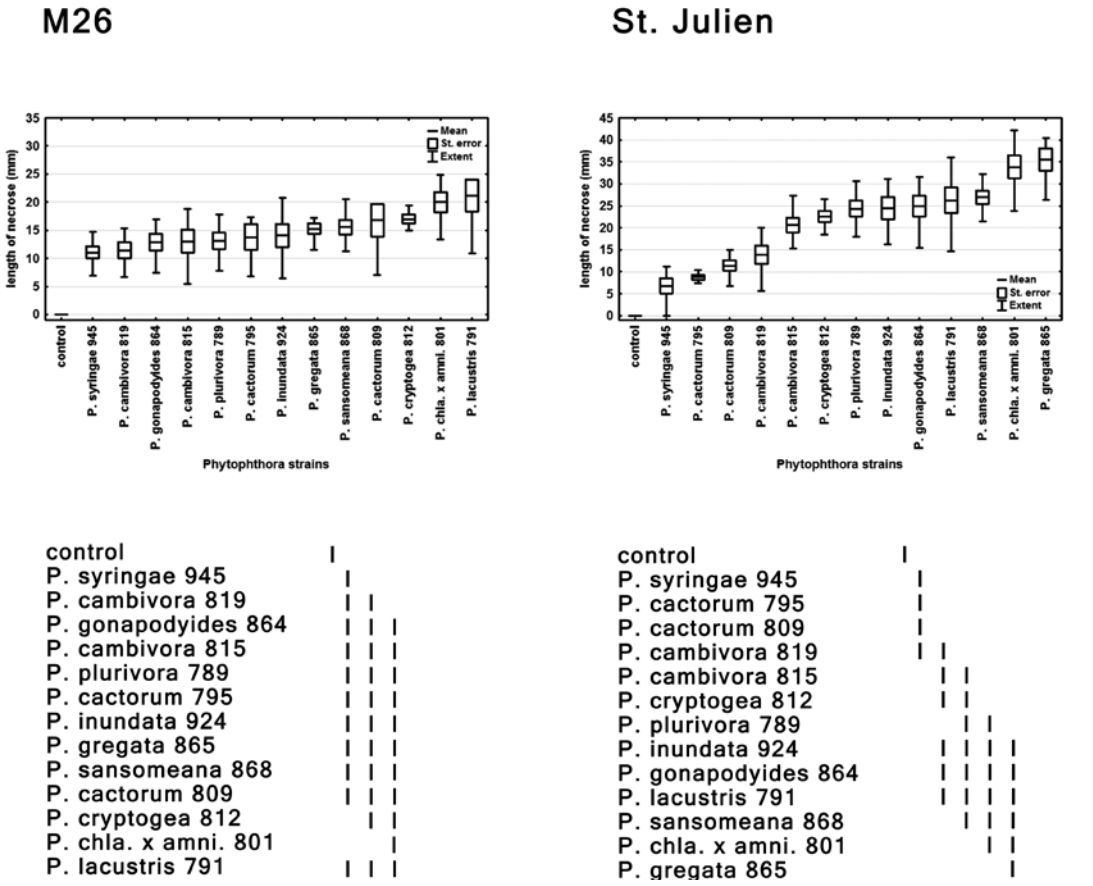

Figure 5. Virulence of *Phytophthora* isolates in ´M26´ and ´St. Julien´ rootstocks. The means, quartiles, extents and outliers are presented in the graphs and homogeneous groups in the tables.

environmental conditions (Desprez-Loustau *et al*., 2016) in Central Europe. Especially in the last 5 years, severe dry summers have occurred in the Czech Republic (Šercl *et al*., 2018), which has led to water stress on hosts and increased susceptibility to pathogens. The increased use of irrigation in orchards has provided conditions suitable to *Phytophthora* disease development. The recent Czech findings are similar to the situation in the Mediterranean region and the Balkan Peninsula, where severe root and collar rots and locally important losses caused by *Phytophthora* spp. were identified (Elena and Paplomatas, 1999; Pane *et al*., 2009; Nakova, 2010a; Kurbetli *et al*., 2017). These led to investigation of their control, and evaluation of the resistance of rootstocks, in the region (Thomidis, 2001; Boughalleb *et al*., 2006; Thomidis *et al*., 2008; Belisario *et al*., 2009).

There is poor awareness of *Phytophthora* pathogens in fruit production in the Czech Republic. These pathogens have not been systematically monitored and the causes of individual declines have apparently been misidentified, so no effective control measures have been developed. This has resulted in a limited number of fungicides effective against soil oomycetes to be registered for use in fruit orchards in this country (Anonymous, 2018). As well, the frequently used rootstocks have not been assessed for susceptibility to the most frequently occurring naturalized oomycetes. The poor knowledge and pressure for gain have led to the problems being overlooked. Implementation of unsuitable procedures in orchards and the growing of fruit trees without regard to locally unsuitable sites (high water tables, heavy or impermeable clay soils) which serve as long-term infection reservoirs in infected orchards, and repeated replanting with susceptible rootstocks, have also exacerbated disease problems.

Among the isolated pathogens, *P. cactorum* was the most frequently occurring species. This usually damaged apple trees (affecting about a half of the fruit orchards in the area; Buchtová, 2015), but it was also the most frequently occurring pathogen in stone fruit trees. This species is the most important pathogen of apple trees (Smith *et al*., 1988; Harris, 1991; Sutton *et al*., 2014), but also damages other hosts (Smith *et al*., 1988; Ogawa *et al*., 1995; Erwin and Ribeiro, 1996). Moreover, *P. cactorum* is still frequently introduced with nursery stock. The next most important and well-known *Phytophthora* pathogens of apple trees are *P. syringae* (causing losses in cool European areas; Smith *et al*., 1988; Harris, 1991), *P. plurivora*, *P. megasperma* Drechsler, *P. roseacearum* E.M. Hansen & W.F. Wilcox, *P. cryptogea*, *P. cambivora* and *P. gonapodyides* (Smith *et al*., 1988; Harris, 1991; Erwin and Ribeiro, 1996; Hansen *et al*. 2009; Sutton *et al*., 2014) were also isolated. *Phytophthora citrophthora*, another known pathogen of pome and stone fruit in Southern Europe (Elena and Paplomatas, 1999; Thomidis, 2001; Pane *et al*. 2009; Nakova, 2010b), was identified in only in irrigation, but could also damage trees in adjacent irrigated orchards.

Other isolated less recognized pathogens (*P. gregata*, *P. chlamydospora* × *amnicola*, *P. inundata*, *P. lacustris* and *P. sansomeana*) were isolated from pome or stone fruit on only a few occasions. *Phytophthora gregata* was described in 2011 from dying swampy vegetation in Australia (Jung *et al*., 2011), and is known to cause root rot in forest and ornamental plants in different stands in the Czech Republic (CCPO, 2018). This pathogen was found to cause root and collar rot in ′Orion′ apple and root rot in ′Champion′ apple and cherry. The hybrid *P. chlamydospora* × *amnicola*, first isolated in 2009 and 2010 from water in Australia and South Africa (Nagel *et al*., 2013), was isolated from only a few orchards of one company, but the affected plantations were heavily damaged, with very frequent death of trees (Figure 2B). *Phytophthora inundata*, first known in Europe as *P.* sp. O-group, was originally described in 2003 (Brasier *et al*. 2003), and is known as a pathogen of some fruit trees including almond and olive (Safaiefarahani *et al*., 2013). The pathogen was found to cause root rot of plum in the Czech Republic. *Phytophthora lacustris*, formerly named P. taxon Salixsoil, is known as a moderately pathogenic species frequently distributed in riparian stands, usually causing root rot in some European native trees. The pathogen is also a severe fine root pathogen of peach seedlings, but it was found to be non-pathogenic to cherry and myrobalan (Nechwatal *et al*., 2013). We identified *P. lacustris* as a cause of collar rot and death of mature apricot trees and root rot of apple Topaz′. *Phytophthora sansomeana* was originally described from soybean in Midwestern states of the United States of America, and was also identified from Douglas-fir, alfalfa fields, weeds, gerbera and soil (Hansen *et al*., 2009; Rahman *et al*. 2014). This pathogen was found to cause extensive root rot in apple ′Topaz red′ on M9 rootstock and pear ′Alexander Lucas′.

An investigation of oomycete contamination of commercial nursery stock revealed a high proportion of *Phytophthora* infections (up to 61%). Infected plants were identified in all tested domestic and foreign sources of planting material, with the exception of *in vitro*-produced rootstocks.

The most frequent pathogens of nursery stocks were *P. cactorum*, *Phytopythium vexans* and *Globisporangium intermedium*. Coincidentally, *P. cactorum* was the most frequent *Phytophthora* species distributed in fruit

orchards in the Czech Republic. Similarly, *Phy. vexans* and *G. intermedium* were the most frequently isolated species among *Pythium* s. l. spp. from orchards. This supports the suggestion that plant trade is the main pathway for introducing plant pathogens (e.g., Brasier, 2008; Bienapfl and Balci, 2014), and the possibility that the trade and planting of infected material is the main factor shaping oomycetous communities of orchards after their establishment. The role of later infections (from irrigation water and mechanization) could be secondary. Development and use of appropriate disease management measures should not be focused only on orchards, where, the long-term effects of permissible controls can be questionable, but should focus on fruit tree nurseries as the main sources of infection. On the other hand, although a brief investigation of the diversity of oomycetes from irrigation water did not confirm irrigation water to be the main source of pathogens, the recording of potentially important species (*P. plurivora*, *P. citrophthora*) highlights this source of inoculum of pathogenic oomycetes (e.g., Ivors and Moorman, 2014).

Both of the tested rootstocks (´M26´ and ´St. Julien´) are frequently used in the Czech Republic. The apple rootstock ´M26´ has been evaluated as less susceptible (Utkhede, 1986; Bielenin, 1995) to intermediately and highly susceptible (Browne and Mircetich, 1993; Biggs *et al*., 2012) to different *Phytophthora* species, whereas ´St. Julien´ rootstock has been evaluated as having medium susceptibility to *P. citrophthora* and as more resistant to *P. cactorum* (Elena and Tsipouridis, 2000). However, in the present study ´St. Julien´ was evaluated as being generally more susceptible than ´M26´ to *Phytophthora* spp. Nine out of eleven tested species caused more extensive damage to ´St. Julien´ than ´M26´ rootstocks. Only two species, *P. cactorum* and *P. syringae,* caused larger necroses in ´M26´ than in ´St. Julien´. These two pathogens are probably specialised to apple trees, and this result is in accordance with evaluations of these pathogens as the most important in apple trees, *P. cactorum* worldwide and *P. syringae* in northwestern Europe (Harris, 1991). This is also in accordance with fact that ´St. Julien´ is relatively resistant to *P. cactorum* (Elena and Tsipouridis, 2000). On the other hand, generalisation at the host genus level (*Malus* vs. *Prunus*) is not possible due to variation in susceptibility of different rootstocks and cultivars (see Utkhede, 1986; Browne and Mircetich, 1993; Boughalleb *et al*., 2006).

The outcomes of pathogenicity tests (Table 3, Figure 5) indicated that the lesser-known and rarely isolated species, described from other continents (*P. gregata*, *P. chlamydospora* × *amnicola*, *P. inundata*, *P. lacustris* and *P. sansomeana*), were often the most virulent in the present study. They were the most virulent species in both tests. Information on the biology and hosts of these species is scarce (see above), and they are, with exception of *P. inundata* (Safaiefarahani *et al*., 2013), not known as important fruit tree pathogens. Although the differences in susceptibility among rootstocks and virulence between *Phytophthora* species were statistically significant, these results should be verified by field inoculation experiments, which would can take months or years to provide reliable results. However, the identified damage caused by these pathogens in the field, verified by the inoculation experiments, showed that these pathogens could pose potential threats to fruit orchards in the future.

This study documented a contemporary increase in the activity of many *Phytophthora* pathogens in fruit orchards in central Europe. The involvement of some well-known species (especially *P. cactorum*, *P. plurivora*) as well as new pathogens in Europe (*P. chlamydospora×amnicola*, P. *sansomeana*), causing important losses in different regions of the Czech Republic, has been confirmed. The increase of *Phytophthora* activity is probably related to the trade and planting of infected nursery stock, to climate change and water stress, to disregard of the problems caused by *Phytophthora* diseases in fruit orchards by stakeholders, and to inappropriate disease management. This situation needs further investigation focusing on the biology of these pathogens and the development of effective disease management, including the investigation and breeding of rootstocks for resistance.

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Research Papers

Occurrence and diversity of *Grapevine leafroll-associated virus 1* in Algeria

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Summary. A survey was conducted of central and western Algerian grape cultivars and a germplasm collection to detect the presence of *Grapevine leafroll-associated virus 1* (GLRaV-1), using DAS-ELISA. The virus was found in 26 of 484 sample (5.4%). No infection was found in the germplasm collection. Analysis of the sequence of a coat protein gene region revealed that of the 17 recognised phylogenetic groups, the Algerian isolates belong to Groups I, II and XVI. This is the first study of genetic diversity of GLRaV-1 in Algeria.

Keywords. GLRaV-1, RT-PCR, genetic variability, HSP70h, CP.

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INTRODUCTION

Grapevine leafroll disease (GLD) is caused by a complex of virus species from the family *Closteroviridae*, and this disease occurs in all grape-growing regions of the world (Almeida *et al.,* 2013). It is an economically important virus disease (Alabi *et al.,* 2016). The associated viruses are referred as the grapevine leafroll-associated viruses, including GLRaV-1, -2, -3, -4; -7 and -13. GLRaV-1, GLRaV-3, GLRaV-4 and its strains and GLRaV-13 belong to the genus *Ampelovirus,* GLRaV-2 belongs to the genus *Closterovirus*, and GLRaV-7 belongs to a newly proposed genus *Velarivirus* (Martelli *et al*., 2012, Ito and Nakaune 2016).

The GLRaV-1 genome size is nearly 19.5 kb (Fazeli *et al*., 2000), from which partial sequence information was characterized (AF195822). The full-length genomic sequence of two isolates of GLRaV-1 has been determined, at ~18,730 nucleotides (nt) (isolate WA-CH) and ~18,945 nt (isolate WA-PN). Isolates WA-CH and WA-PN encode ten open reading frames, and their overall genome organization is similar to previously reported GLRaV-1 isolates (Donda *et al.,* 2017).

Grapevine leafroll-associated viruses are known to spread by grafting and by mealybug transmission. Several species of mealybugs have been shown

to transmit GLRaV-3 (Almeida *et al.,* 2013). GLRaV-1 is restricted to the phloem tissues of host plants and is frequently present with other viruses. Transmission of GLRa-1 by mealybugs follows the "semi-persistent and non circulative" mode (Tsai *et al.,* 2008), and several mealybug and soft scale species have been reported to transmit GLRaV-1 (Naidu, 2017).

Little *et al.* (2001) identified ten genes associated with GLRaV-1, several of which are hypervariable. Genetic diversity of GLRaV-1 has been less investigated compared to other leafroll disease associated viruses. Based on the HSP70h gene region, two phylogenetic groups were designed (Kominek *et al.,* 2005). Based on the heat shock 70 protein homolog (HSP70h) gene region, three phylogenetic groups were identified (Alabi *et al.,* 2011). Based on the coat protein (CP) gene, Esteves *et al.,* (2013) described eight groups. More recently, eignt groups were designated for the CP gene region and seven groups for the HSP70h gene region (Fan *et al .,* 2015).

Here we describe a survey of GLRaV-1 in grapevines in Algeria, and the chararterization of the genetic relationship of Algerian virus isolates with other isolates, in a partial fragment of the CP gene.

MATERIALS AND METHODS

In autumn 2012, 484 grapevine samples were collected from the major grapevine growing areas in the western and central regions of Algeria. Dormant canes from individual vines were randomly collected in commercial vineyards, including from eight introduced cultivars (355 samples) and two local cultivars (100 samples), as well as 18 cultivars (29 samples) from the autochthonous grapevine germplasm collection at the Institut Technique de l'Arboriculture Fruitière et de la Vigne (ITAF). All samples were tested by DAS-ELISA (Clark and Adams, 1977) for the presence of GLRaV-1, using a commercial kit (Agritest). Extracts were obtained by macerating phloem tissues of dormant canes in the grinding buffer described by the manufacturer (1 g per 10 mL). Absorbance was recorded at 405 nm using an automatic microplate reader (Labsystems Multiskan Ascent). Samples with absorbance readings equal or exceeding three times those of negative controls were considered as positive.

All positive samples were analyzed by RT-PCR using total nucleic acids extracted from 0.2 g of cortical scrapings, as described by Foissac *et al.* (2001). The negative and positive controls were provided from the virus collection of the INRAT, Tunis. Reverse transcription was performed using MMLV reverse transcriptase (200 units μL^{-1}), and PCR was carried out using the primer pairs HSP70-417F (5'- GAGCGACTTGCGACTTATCGA-3') and HSP70-737R (5'-GGTAAACGGGTGTTCTTCAATTCT-3'), designed by Osman and Rowhani (2006), and the primer GLRaV-1-CP/F (5'-CGCGCTTGCAGAGTTTAAGTGGTT-3') and GLRaV-1 CP/R (5'-TCCGTGCTGCATTGCAACTTTCTC-3') designed by Alabi *et al.* (2011), to amplify fragments of 320 bp for the HSP70h gene region and of 734 bp for the CP gene region. Some virus isolates were randomly chosen for sequencing of the CP and HSP70h gene regions (Supplementary data 1).

Mean genetic distances of the analysed 104 sequences of each group, as well as between the groups, were calculated using MEGA7 with best fit alignment (Kumar *et al.*, 2016). The Dna SP software v.5.10.01 (Librado and Rozas 2009) was used to estimate the mean values of non-synonymous to synonymous substitution ratios (dN/dS) for the different coding regions of GLRaV-1. The ratio of non-synonymous to synonymous substitutions per site (dN/dS) was also determined.The genetic diversity π and the Tajima'D value were calculated using the MEGA7 software package.

Multiple alignments of nucleotide sequences from this study and corresponding sequences available in GenBank (Supplementary data 1) were performed using ClustalW implemented in MEGA7. Using the best fit models, a phylogenetic tree was constructed for the CP gene region using the best fit alignment (585 nt) with the neighbor-joinning method with 1,000 bootstraps (Figure 1)

A search for recombination events was conducted for the CP gene region with the genetic recombination detection method (GARD) (Kosakovsky Pond *et al.*, 2006) implemented in the Datamonkey web server (https://www.datamonkey.org/).

No analyses were performed for the HSP70h gene region in this study due to the small length of the obtained sequences.

RESULTS

Results revealed that GLRaV-1 was present in 26 (5.4%) of the 484 samples tested. For the variety Dattier de Beyrouth, six of 71 samples were infected, seven of 100 for Gros noir and eight of 70 for Muscat (Table 1). No infected samples were found in the autochtonous grapevine germplasm collection at ITAF. According to previous results for the presence of GLRaV-3 in the same samples, mixed infections of GLRaV-1 and GLRaV-3 were detected in 15 samples, and infection of only GLRaV-1 was detected in 11 samples.

In order to determine the diversity of GLRaV-1 isolates from Algeria, a comparison was performed at the

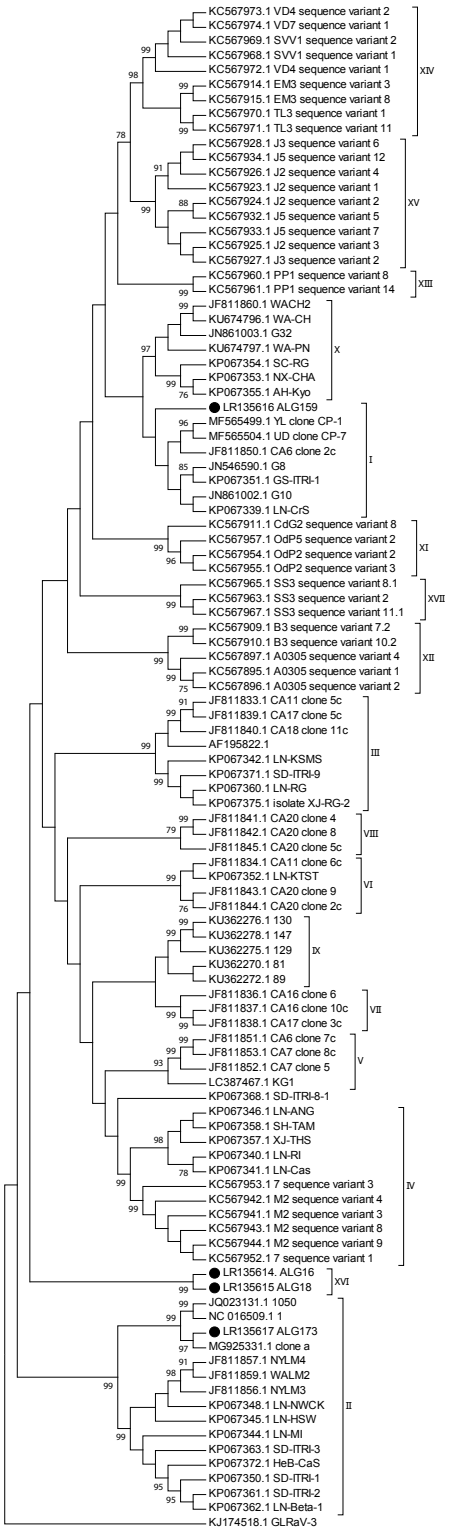

Figure 1. Phylogenetic tree of *Grapevine leafroll associated virus 1*(GLRaV-1) isolates, constructed with sequences of a 585 nt fragment of the viral coat protein gene. Percentages of bootstrap support (≥ 75%) from 1,000 replicates are indicated on relevant tree branches.

Table 1. Rates of virus infections in the surveyed grapevine varieties.

Variety	Number of samples	Number of samples infected
Dattier	71	6
Gros noir	100	7
Cardinal	52	1
Alicante	30	1
King Rubi	9	0
Muscat	70	8
Mersguira	10	0
Çinsaux	40	1
Carignon	31	1
Valensi	42	1
Autochthones	29	0
Total	484	26

nucleotide level of HSP70h and CP gene regions. For HSP70h, proportional nucleotide similarity of Algerian sequences revealed an average of 93%. Isolates ALG5, ALG128, ALG132, ALG270 and ALG420 were closely similar with 95 to 97% similarity. Isolates ALG16 and ALG18 showed 100% similarity between them. A BLASTn search showed that isolates ALG16 and ALG18 had 95% similarity to isolate WA-CH (KU674796.1), while isolates ALG420, ALG132, ALG128, ALG270 and ALG5 had 91-94% similarity between them.

For the sequence of the CP gene region, a comparison at the nucleotide level showed that ALG16 and ALG18 were identical, ALG159 showed less than 87% similarity and ALG173 less than 85% similarity compared to the three Algerian sequences. BLASTn search showed that ALG16 and ALG18 had 92-93% similarity with other sequences available in GenBank. (isolate A0305 variant 4 (KC567897) was very similar).

For the CP gene region, analyses performed with DNA sp 0.6 and MEGA7 showed that the number of non-synonymous sites was 443.91 sites out of 585, and the synonymous site was 141.09 sites out of 585. The mean dN/dS was 0.095 calculated with the Single-Likelihood Ancestor Counting (SLAC) algorithm implemented in the datamonkey server. The genetic diversity analyses gave a value of $\pi = 0.103$ and the the D Tajima's test was -0.92. The dN/dS and the Tajima's D test showed that the CP gene was under negative selection.

Tajima's D and Fu and Li's D and F statistics were calculated for groups I- and II-containing Algerian sequences. This gave a negative value for Tajima's D and positive value for Fu and Li's D and F statistics. A value of genetic divertsity π for the two groups was estimated at 0.03282 for Group I and 0.04688 for Group II (Table 2).

Table 2. Representative parameter estimates for the Algerian GLRaV-1 phylogroups.

Phylogroup	π	Tajima's D	Fu & Li's D	Fu & Li's F
I	0.03282	-1.041	1.29777	1.47497
II	0.04688	-0.056	0.26019	0.13310

No recombination event was found in the CP gene region, using the GARD algorithm implementated in the Datamonkey database (https://www.datamonkey.org).

A phylogenetic tree with four Algerian sequences and 104 other sequences downloaded from GenBank was constructed for the CP gene region using the neighbor joining method with MEGA7 software (Kumar *et al.,* 2016). Results confirmed the high diversity of the GLRaV-1 population. The tree revealed that the GLRaV-1 population clustered into 17 groups. Eight clusters were previously reported by Fan *et al.* (2015). The Algerian sequences obtained in this study clustered into three groups. The sequences of ALG16 and ALG18 clustered in a new group (Group XVI), the sequence ALG159 was in G I and ALG173 was in G II.

The within group proportional similarity ranged from 95% to 100%, indicating a close relationship between isolates from the same group, except for the Group VIII which showed 88% similarity. The between group distances ranged from 78% to 96%, showing divergence present between groups especially for some groups which had less than 80% similarity (Groups II and XVI). The tree revealed the absence of correlation between the geographical origins and phylogentic distribution for some groups. Group I was constituted by sequences from France, China and Poland, and Group II with sequences from China, the United States of America and Canada. However, some groups contained only isolates from one country. Group VII only contained sequences from the United States, Group IX contained sequences only from Turkey, and Groups XI, XII XIII, XIV and XV contained sequences only from Portugal (Supplementary data 1).

DISCUSSION

This study describes the occurrence of GLRaV-1 in different regions of Algeria, and the genetic diversity of population of this virus from other countries. There have been few studies of the diversity of this virus, which appears to be very variable.

Our results have demonstrated shown the presence of GLRaV-1 in Algeria. GLRaV-1 is less prevalent in Algeria than GLRaV-2 and GLRaV-3, which were reported in a previous study (Lehad *et al.* 2015). Mixed infections of this virus with GLRaV-3 may explain its parallel transmission by common vectors. Vector transmission of GLRaV-1 has been reported in Algeria (Bisaad *et al.,* 2017). *Parthenolecanium corni* described as vector of this virus was also observed in some vineyard in central Algeria (unpublished data). Other mealybug species, such as *Phenacoccus aceris*, were described as vectors of GLRaV-1 (Alliaume *et al.,* 2015). More research is required on the epidemiology of this virus in Algeria. It is important to determine the efficiency of mealybug transmission of GLRaV-1 compared with other viruses, in order to further understand the epidemiology of grapevine viruses in this country.

Nucleotide comparisons were carried out, and and results for HSP70 showed that the Algerian GLRaV-1 sequences had close genetic relationships ranging from 89% to 100% similarity, when CP gene region sequences were compared. However, some sequences shared less than 85% similarity. This indicates that the the HSP70h gene region was more conserved than the CP gene region. The HSP70h gene region was reported to be highly conserved in the family of *Closteroviridae* (Tian *et al*., 1996).

The genetic diversity of the Algerian sequences compared to the sequences in NCBI showed an important genetic diversity value for the CP gene region, confirmed by others (Alabi *et al.,* 2011; Esteves *et al.,* 2013). This may be explained by the natural pressure enforced on this protein. The CP gene plays an important role in plant/virus interactions. The Tajima's D test and the mean dN/dS results showed that the CP gene evolves under negative selection. Similar results were obtained for the CP and Rdrp gene of *Grapevine virus A* (Alabi *et al.,* 2014).

The Tajima's D test gave a negative value for the CP gene region. This may indicate that the gene evolved under a low frequency of polymorphism suggesting population size expansion (e.g. after a bottleneck or a selective sweep) and/or purifying selection.

Previously, two groups were reported for HSP70h gene region (Komínek *et al*., 2005). Alabi *et al.* (2011) reported three clusters, and Fan *et al.* (2015) revealed the presence of eight groups based on the CP gene region and seven based on the HSP70h region.

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Research Papers

Identification of SSR markers linked to Botrytis grey mould resistance in chickpea (*Cicer arietinum*)

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Summary. Botrytis grey mould (BGM), caused by *Botrytis cinerea*, is emerging as an important disease of chickpea in the northern and eastern parts of the Indian Subcontinent, including Nepal, Bangladesh, Pakistan, and in Australia. This fungus has a very broad host range, and sources of complete resistance to the disease have not been found in *Cicer arietinum* L. germplasm. Resistance to this pathogen has been identified in some wild *Cicer* species. A set of 371 lines, including 164 landraces and 207 interspecific derivative lines (derived from crosses of cultivated chickpea with *C. pinnatifidum, C. judaicum* or *C. reticulatum*) have been screened against Botrytis grey mould under field conditions, and using the cut twig method at the Punjab Agricultural University (PAU), Ludhiana, in 2015-16 and 2016–17. Strong correlations between the two screening methods were indicated by paired-t tests. The Bulked Sample Analysis (BSA) approach was used to screen DNA of the five most resistant and five most susceptible host lines using 300 simple sequence repeat (SSR) markers. Eighty-eight markers were polymorphic. Chi-square statistic values showed strong correlations of TA144, GA102, TA194, TA140 and TR2 with the resistant bulks, signifying their usability as putative markers linked to BGM resistance, and for development of BGM tolerant genotypes in chickpea. Future studies should rapidly ascertain marker trait associations, and identify and develop diagnostic markers that provide an accurate method of molecular tagging BGM resistant genes in chickpea.

Keywords. Chickpea, Grey mould resistance, SSR markers, Bulked sample analysis.

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INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a highly nutritious food legume, ranking as third in world importance after peas and drybeans (Bharadwaj *et al.*, 2010). Chickpea is grown on 14.56 million ha in 50 different countries, and

average yields are 1.12 t ha^{-1}(FAOSTAT, 2017). Various biotic stresses affect chickpea production globally. These include fungal, viral, and bacterial diseases, nematodes, insect pests and parasitic weeds. Transferring resistance genes to elite cultivars is one of the most efficient approaches for overcoming biotic stresses (Li *et al*., 2015). Most traits of agronomic importance are complex in nature, governed by various genes and environmental interactions (EL-Soda *et al*., 2014). The identification of linked QTLs is crucial, therefore, for understanding the molecular basis of these traits (Xu *et al*., 2010).

Botrytis grey mould (BGM), caused by *Botrytis cinerea* Pers. ex. Fr., is an economically important disease of chickpea. The occurrence of BGM has been reported in many countries, including Argentina, Australia, Canada, Columbia, Bangladesh, Nepal, Pakistan, India, Spain and the United States of America (Haware *et al*., 1998). This disease may cause yield losses upto 100% under favourable conditions (Pande *et al*., 2006a; Pande *et al*., 2002). The disease may occur at any stage of chickpea development (Hawthorne *et al*., 2006) but usually occurs at the time of flowering. Temperatures of 20-30°C and relative humidity from 70 to 100% favour BGM infections. The pathogen infects flowers most easily (Pande *et al*., 2006b, c), and infected pods may carry the infection through infected seeds to the next growing season (Nene *et al*., 2012; Matthews *et al*., 2014). Genotypes with profuse seedling growth and early flowering and canopy closure tend to develop BGM infections more often compared with other cultivars.

Botrytis cinerea has a very broad host range. Source of complete resistance to the pathogen have not been found in *C. arietinum* germplasm (Singh and Reddy, 1991), but wild *Cicer* sp. have been identified as good sources of BGM resistance. Wild *Cicer* species, including *C. judaicum, C. bijugum, C. echinospermum,* and *C. pinnnatifidum* (Singh *et al*., 1991; Haware 1998; Pande *et al*., 2002), have shown higher levels of resistance to BGM in comparison to the cultivated species. Among these wild species, *C. echinospermum* is being used for transfer of BGM resistance to the cultivated species (ICRISAT, 2007). Kaur *et al.* (2013) developed interspecific derivative lines from crosses of *C. arietinum* and *C. pinnatifidum* that were found to be highly tolerant to BGM, and this resistance can be transferred to elite lines for development of high yielding and BGM tolerant chickpea cultivars. Very few studies have been undertaken to exploit wild species and the level of diversity amongst different host plant accessions.

The present study used wild *Cicer* spp. accessions and inter-specific hybrids to identify BGM resistant genotypes and putative markers linked to BGM resistance in chickpea.

Different methods have been used for screening for BGM resistance under laboratory, greenhouse and field conditions (Gurha *et al*., 2003; Pande *et al*., 2006a, b). The cut-twig method is a non-destructive procedure for sampling, and this has been very useful in inter-specific hybridization (Kaur *et al*., 2013). In comparison to the traditional methods of breeding, time saving and cost effective approaches are now widely used in genomics and crop improvement (Sun *et al*., 2010). Marker-assisted selections and selective phenotyping can be simplified using such approaches (Xu and Crouch, 2008). Bulked BSA uses individuals with extreme phenotypes and these variants are then pooled as bulks. Bulked BSA or the Sampling-bulking method for marker development and trait mapping has been named differently as bulked segregant analysis (Michelmore *et al*., 1991) and DNA pooling (Giovannoni *et al*., 1991), and is usually achieved with molecular marker systems (Gillman *et al*., 2011; Asnaghi *et al*., 2004). Genomic regions with large differential allele frequencies between the bulks reveal association of the regions with the QTLs associated with particular trait (Deokar *et al*., 2019) (Figure 1). These QTLs are then subjected to statistical tests to verify the confidence intervals for their location (Takagi *et al*., 2013). Next Generation Sequencing-based BSA has been used for QTL mapping for different traits due to improved efficiency and affordability of NGS platforms (Deokar *et al*., 2019; Chen *et al*., 2017; Pandey *et al*., 2017; Singh *et al*., 2016; Das *et al*., 2016; Illa-Berenguer *et al*., 2015; Kaminski *et al*., 2015; Lu *et al*., 2014). BSA coupled with molecular breeding techniquesal-

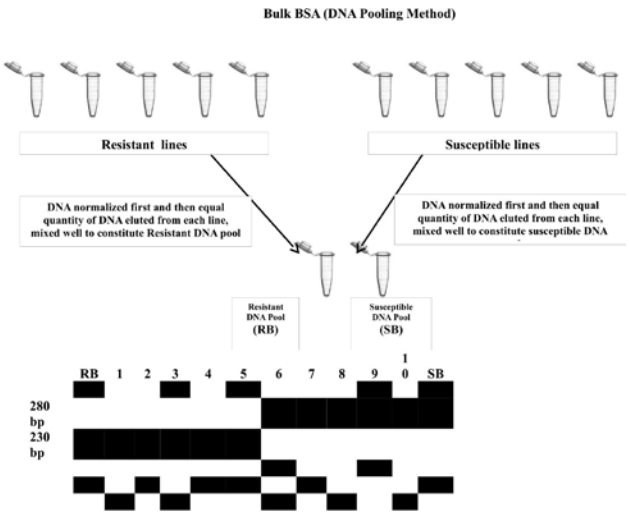

Figure 1. Figure depicting bulking sampling method where equal amount of DNA is eluted from each line after normalization and corresponding bulks generated are used for mapping genes for BGM resistance.

low rapid identification of markers, and is a promising approach for trait mapping and candidate gene discovery in plant breeding.

MATERIALS AND METHODS

Plant material

A total of 371 *Cicer* accessions, including 164 landraces and 207 F_6 interspecific derivatives lines (derived from crosses of cultivated chickpea with *C. pinnatifidum, C. judaicum* or *C. reticulatum*) were screened under field conditions and controlled conditions against *Botrytis* grey mould. The cut-twig method is non-destructive and simple, and is widely used for laboratory studies. Theaccessions were screened at the Punjab Agricultural University (PAU), Ludhiana, during 2015-16 and 2016-17. Of the 371 lines, three landraces and 23 extreme Recombinant Inbred Lines (RILs) were selected, for identification of markers linked to BGM resistance given in Table 1.

Field evaluations

The test lines were sown at plant spacings of 40 cm in 2 m length rows in fields at three replications, in a randomized complete block design (Figure 2a). The chickpea line L550 was used as a susceptible check after every eight rows in the trials. The resulting plants, at the flowering stage, were sprayed with a local isolate of *Botrytis cinerea*, in the first week of February in the evening hours. The inoculum suspension contained 10^5 conidia mL^{-1}. Isolate 24, race 510, of *B. cinerea* (Singh and Bhan, 1986) was used for screening the plants against BGM. The isolate was preserved on slants of potato dextrose agar (20g dextrose, 20g agarose, 200g potato and 1L distilled water), and were multiplied in potato dextrose broth and stored at 25°C. Following inoculation, water was applied using a sprinkler, to maintain high relative

Table 1 Plant material used in this study with, their parentage.

Name of genotype	Parentage/ Cross
GLW 42	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW 67	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW 69	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW107	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW108	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW115	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW174	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW185	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW501	GPF2 × [PBG 1 × (ICCV96030 × *C. pinnatifidum* 188) × ICCV96030]
GLW502	GL769 × *C. reticulatum*ILWC 129
GLW503	[PBG 1 × (ICCV96030 × *C. pinnatifidum* 188) × ICCV96030]
GLW504	[(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030] PBG 1
GLW505	GL769 × *C. reticulatum*ILWC 129
PAU7007	(GL769 × *C. reticulatum*129)× GL769
PAU7014	(GL769 × *C. reticulatum* 129)× GL769
C. judaicum ILWC-0*	-
C. judaicum ILWC-223*	-
GLW22	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW25	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW183	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
GLW186	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
C. reticulatum ILWC-292*	-
GL1001	JG62 × ICCV05530
GL1002	JG62 × ICCV05530
GLW91	(ICCV96030 × *C. pinnatifidum* 188) × ICCV96030
PBG-7	GPF2 × BG1084

*Chickpea landraces.

Figure 2. Figure showing screening of wild *Cicer* accessions under field conditions and laboratory conditions.

humidity. Symptoms of the disease became visible about 10 dafter inoculations, and disease severity was assessed for five plants per replication 7 d following inoculation. Disease severity was scored using a 1–9 scale, where 1 = no infection on any plant part; 3 = 1–2 lesions visible on leaves; 5 = burnt leaves with stem rotting; 7 = stem rotting with 50% dead leaves; and 9 = extensive stem rotting with fungal infection and 100% leaf death (Gurha *et al*., 2003). An average disease score was calculated.

Cut twig screening technique

Three twigs from each wild accession were cut and placed in a tray containing water in a completely randomized design in three replications. The twigs were then wrapped in moist cotton plugs and placed in test tubes (15 × 10 cm) freshly filled with tap water. Inoculation of the twigs was achieved by spraying conidium suspensions of *B. cinerea* (10^5 conidia mL^{-1}). Following inoculation, the test tubes were covered with wet polythene (Figure 2b). Incubation followed in a growth chamber, and BGM severity was recorded using the 1–9 severity scale (see above). Paired t-tests were performed to examine whether the BGM severity scores obtained under field and laboratory conditions were correlated (Table 2).

Genomic DNA extraction and pooled DNA analysis using SSRs

The CTAB method (Kumar *et al*., 2013) was used to extract DNA from young leaves of the twenty six extreme RILs. The concentration and purity of the DNA from the genotypes was further checked on 0.8% agarose gels and a nanodrop 1000 spectrophotometer (Thermo Scientific). Equal amounts of DNA from each line were taken after normalization and mixed well to constitute the resistant and susceptible bulks for bulked BSA. These bulks were screened for polymorphism using 300 simple sequence repeat (SSR) markers (Varshney *et al*. 2014; Gupta *et al*., 2012; Gaur *et al*., 2011); Bharadwaj *et al.,* 2010). These primers were custom synthesized from G-Biosciences, and the PCRs were carried out in the Chickpea Molecular Breeding Laboratory, Division of Genetics, ICAR-IARI. The 10 µL PCR mix consisting of 1 µL of 20 ng genomic DNA, 1.6 µL of 10× TBE buffer, 1µL of 10 mM dNTP mix, 1 µL forward and 1µL reverse primer and 0.3 µL of 3U μL^{-1}Taq polymerase (Genei), was amplified using a G-STORM thermal cycler (Labtech). The PCR reaction was set as per Yadav *et al.*(2011), with an initial denaturation at 90°C for 1 min and 30 seconds followed by 38 cycles including three different steps, including denaturation at 94°C for 20 sec, annealing at 50–58°C for 50 sec, and extension at 72°C for 50 seconds. This was then followed by a final extension step at 72°C for 7 min, before cooling to 4°C.Amplicons were resolved on 3% Agarose (Lonza) using 1.0× TBE buffer. The amplified products were separated on a horizontal gel electrophoresis system (Biorad) at 120 V for 3 h. Gel staining was by ethidium bromide (10 mg mL^{-1}), and visualized using the UVITECH Gel Documentation system (UVITECH Imaging System). Amplicons were scored as alleles for each locus. Allele sizes were determined by comparing with a standard 100 bp DNA ladder (Genei). Band patterns of the extreme bulks were compared with those of resistant and susceptible lines to confirm linkage of SSR markers with BGM resistance. Chi-square tests were performedto determine goodness of fit of the test lines for the phenotypic and SSR data, by comparing the observed frequency (O) with the expected frequency (E).

RESULTS

Identification of BGM resistance in chickpea accessions

The screening results for BGM resistance of the 26 extreme lines are presented in Table 2. The scoring results of field screening were in close agreement with those from laboratory assessments, similar scores were obtained for each accession under both conditions. Lines ILWC-0, ILWC-223, PBG-7 and nine RILs showed moderately susceptible to susceptible reactions to BGM under field and laboratory conditions. Line ILWC292 and thirteen RILs, including: GLW91, PAU7014, GLW42, GLW67, GLW 115, GLW174, GLW183, GLW186, GLW501, GLW504, GLW 506, GL1001 and GL1002, were resistant to moderately resistant to BGM,

Table 2 Mean Bortytis grey mould (BGM) severity scores for test host lines under field conditions and controlled conditions, and paired-t test analysis for correlating the respective BGM severity scores for the two screening methods (field and greenhouse).

Name of genotype	Disease reaction	Mean BGM score (1-9 scale)		Difference	D^2
		Field screening[a]	Lab screening[b]		
GLW42	Moderately resistant	5.0	5.5	-0.5	0.25
GLW67	Moderately resistant	5.0	4.5	0.5	0.25
GLW69	Moderately susceptible	8.0	8.0	0.0	0
GLW107	Susceptible	9.0	9.0	0.0	0
GLW108	Susceptible	9.0	9.0	0.0	0
GLW115	Moderately resistant	6.0	6.0	0.0	0
GLW174	Moderately resistant	5.5	4.5	1.0	1
GLW185	Susceptible	9.0	9.0	0.0	0
GLW501	Moderately resistant	5.5	5.0	0.5	0.25
GLW502	Moderately susceptible	7.0	7.5	-0.5	0.25
GLW503	Moderately susceptible	7.0	8.0	-1.0	1
GLW504	Moderately resistant	5.5	5.5	0.0	0
GLW505	Moderately susceptible	8.0	8.0	0.0	0
PAU7007	Moderately resistant	5.5	5.0	0.5	0.25
PAU7014	Resistant	4.0	4.0	0.0	0
C. judaicum ILWC-0	Susceptible	9.0	9.0	0.0	0
C. judaicum ILWC-223	Susceptible	9.0	9.0	0.0	0
GLW-22	Susceptible	9.0	9.0	0.0	0
GLW-25	Susceptible	9.0	9.0	0.0	0
GLW-183	Moderately resistant	5.0	5.5	-0.5	0.25
GLW-186	Moderately resistant	5.0	5.5	-0.5	0.25
C. reticulatum ILWC-292	Resistant	3.0	3.5	-0.5	0.25
GL1001	Moderately resistant	5.5	4.0	1.5	2.25
GL1002	Moderately resistant	5.5	5.0	0.5	0.25
GLW91	Resistant	3.0	3.0	0.0	0
PBG-7	Susceptible	9.0	9.0	0.0	0
$\bar{D}$					0.03846
$(\sum D-\bar{D})^2$					8.71153
SE of $\bar{D}$					0.34846
t					0.11037
df					25
Table value					2.06

[a]Average score from five plants per accession per replication, screened under field conditions.
[b]Average score from 12 plants (three plants per accession per replication), screened under laboratory conditions.
D = Difference between the field screening scores and laboratory screening scores.
$\bar{D}$ = Mean difference.
SE = standard error.
df = degrees of freedom.

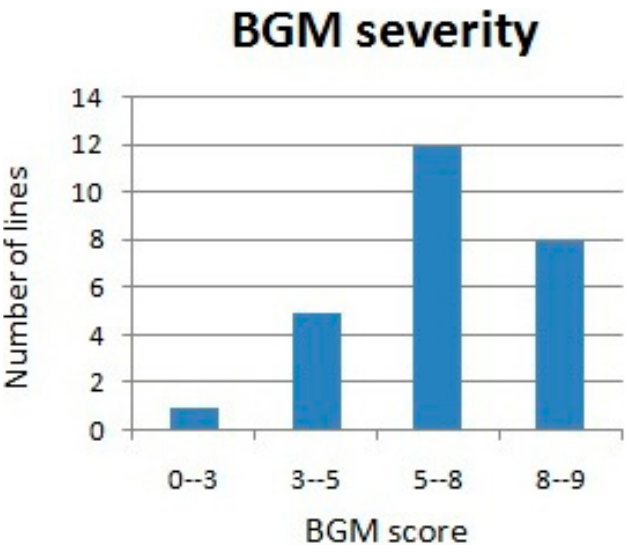

Figure 3. Frequency distribution of BGM scores of all wild *Cicer* spp. screened under field and laboratory conditions.

and could be used as resistant donors. The observed t-value of 0.1103 was much less than 2.06, (P = 0.05; d.f. = 25), showing that there was no statistically significant difference between the two screening methods (Table 2). The frequency distributions of the extreme lines for disease severity (recorded on the 1–9 scales) demonstrated a normal distribution, signifying that resistance to BGM was quantitative in nature (Figure 3).

Pooled DNA analysis using SSRs

Twenty six accession variants were selected from 371 wild chickpeas for genotyping. Of these, DNA from the five most resistant lines (ILWC 292, GLW91, GL1001, PAU7007 and PAU7014) and the five most susceptible accessions (*C. judaicum* ILWC-223, GLW22, GLW25, GLW69 and PBG7) were pooled and bulks were generated. Eighty eight markers were found to be polymorphic between the contrasting bulks, and these are list-

Table 3 List of polymorphic SSR markers used for bulked BSA in the five most Botrytis grey mould resistant and five most susceptible lines, and their corresponding bulks with their linkage groups (LGs)

Serial No.	Primer Name	LG	Serial No.	Primer Name	LG	Serial No.	Primer Name	LG	Serial No.	Primer Name	LG
1	TR43	1[a]	26	TR19	2[b]	51	NCGR209	8[d]	76	NCPGR248	*
2	TA196	3[b]	27	TS82	2[b]	52	NCPGR210	8[a]	77	NCPGR249	7[b]
3	TA144	3[c]	28	TR58	2[c]	53	NCPGR215	*	78	NCPGR250	*
4	TS5	3[b]	29	TA37	2[b]	54	NCPGR216	*	79	NCPGR252	6[d]
5	TA140	2[c]	30	GA16	2[c]	55	NCPGR218	*	80	NCPGR253	*
6	TAA104	5[b]	31	TA96	2[b]	56	NCPGR219	*	81	NCPGR254	6[a]
7	TR2	3[a]	32	TA27	2[b]	57	NCPGR220	6[a]	82	NCPGR255	6[d]
8	GA6	8[b]	33	TA21	5[b]	58	NCPGR221	3[a]	83	NCPGR267	6[b]
9	TA18	7[b]	34	CaSTMS24	2[b]	59	NCPGR224	4[a]	84	NCPGR268	7[a]
10	H3A10	5[b]	35	GA102	5[c]	60	NCPGR225	3[a]	85	NCPGR269	*
11	H2120	5[d]	36	CaSTMS22	5[b]	61	NCPGR226	6[a]	86	NCPGR272	3[b]
12	TS58	3[b]	37	NCPGR76	6[a]	62	NCPGR227	*	87	NCPGR274	6[a]
13	TAA170	4[b]	38	NCPGR78	*	63	NCPGR228	5[b]	88	NCPGR275	*
14	TA206	2[b]	39	NCPGR79	6[b]	64	NCPGR229	6[a]			
15	TA194	2[c]	40	NCPGR82	6[b]	65	NCPGR232	5[a]			
16	GAA47	4[b]	41	NCPGR84	*	66	NCPGR235	*			
17	TA3a	2[b]	42	NCPGR91	4[a]	67	NCPGR236	4[b]			
18	TR31	3[c]	43	NCPGR93	6[b]	68	NCPGR237	*			
19	TA42	7[b]	44	NCPGR95	7[b]	69	NCPGR238	6[a]			
20	TA89	4[b]	45	NCPGR96	*	70	NCPGR240	3[a]			
21	TA110	2[c]	46	NCPGR97	*	71	NCPGR241	3[a]			
22	STMS28	3[b]	47	NCPGR98	2[b]	72	NCPGR242	3[a]			
23	TR20	4[b]	48	NCPGR99	7[b]	73	NCPGR244	*			
24	CaSTMS2	4[b]	49	NCPGR100	3[a]	74	NCPGR246	*			
25	TA42	5[b]	50	NCPGR101	1[b]	75	NCPGR247	4[d]			

[a] Choudhary*et al.*, 2012; [b] Varshney *et al.*, 2014; [c] Bharadwaj *et al.*, 2010; [d] Gaur*et al.*, 2011; *Choudhary, unpublished.

Table 4 Chi-square (χ^2) test results of wild chickpeas screened using Bulked BSA for Botrytis grey mould screening.

Sno.	Marker	Observed (O)	Expected (E)	χ^2 =(Obs-Exp)^2/ Exp	χ^2 (P = 0.05)	
1	TR2	9	11	0.363636364	3.841	Strong correlation
2	TA194	11	12	0.083333333		Strong correlation
3	TA144	12	12	0.00		Strong correlation
4	GA102	11	12	0.083333333		Strong correlation
5	TA140	10	11	0.090909091		Strong correlation

Low χ^2 values indicate strong correlation with resistant allele.

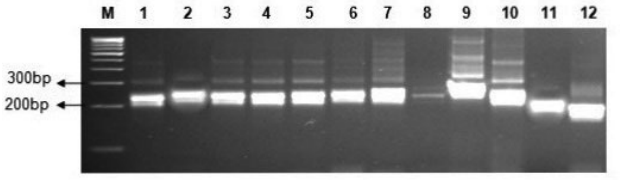

Figure 4. Gel image showing Bulked sample analysis with DNA pools generated with TA144, M: 100bp Banglore Genei ladder, 1:ILWC292,2:GL1001, 3:PAU7007, 4:PAU7014, 5:GLW91, 6:*C. judaicum* ILWC223, 7: GLW22, 8: GLW25, 9: PBG7, 10: GLW69, 11: Resistant bulk, 12: Susceptible bulk.

ed in Table 3. Most of these markers were found to be located on linkage groups (LGs) 2, 3 and 6. Anuradha *et al.* (2011) also identified three QTLs linked with BGM resistance in chickpea on LG 3 and LG 6. The BSA and chi-square statistic indicated strong correlations of TA144, GA102, TA194, TA140 and TR2 with the resistant bulks (Table 4 and Figure 4). These markers may be associated with resistance against BGM, and they could assist plant breeders in speedy development of BGM resistant cultivars.BSA combined with advanced technologies can be used to identify and develop diagnostic and constitutive markers improving the efficiency of breeding programmes and lead to animproved understanding of the molecular basis of BGM resistance.

DISCUSSION

Wild accessions serve as excellent sources of resistance to biotic and abiotic stresses, according to previous reports, and many chickpea accessions have now been identified with resistance to diseases (Madrid *et al.*, 2008). Accessions including *C. judaicum, C. pinnatifidum* and *C. echinospermum* have shown good resistance to BGM at PAU, Ludhiana, so these may be used as donors for introgressing BGM resistance in chickpea. These sources of BGM resistance were therefore used in the present study through interspecific hybridization.

Botrytis grey mould is an economically important disease of chickpea, which may cause complete yield losses under heavy rains and high humidity. It is the major production constraint limiting sustainable chickpea yields. There is an urgent need to develop varieties with resistance to BGM with greater yield stability. Screening under field and controlled conditions are time-consuming and selection of BGM-resistant genotypes may take more than 1 year or season if conventional methods are used. Screening for disease resistance may also be influenced by environmental interactions, density of pathogen inoculum, presence of some pathogens, and pathogen virulence, resulting in variable disease outcomes. Furthermore, discrepancies in scoring of disease reactions may affect the introgression of BGM resistance into elite chickpea genotypes. On the other hand, marker-assisted selection saves time in comparison to phenotypic field or greenhouse evaluations.

Bulked sample analysis and molecular markers together help discern markers associated with genes governing disease resistance in a number of plant species (Ballini *et al.*, 2008). Owing to scarcity of polymorphic markers in chickpea, BSA provides a rapid method for identifying markers linked to BGM resistance. Resistant genotypes may be identified within short periods, inferior genotypes can be excluded from the next cycle of selection, increasing the efficiency of breeding programmes.

Microsatellites combined with BSA have been used to identify molecular markers linked to genes of interest (Shoba *et al.*, 2012), and comparison of pooled DNA samples is much easier than evaluating all the individuals of different populations (Hallden *et al.*, 1997; Sweeney and Dannebeger, 1994). BSA has been successfully used in fine mapping of QTLs for *Ascochyta*blight resistance in *C. arietinum*, genes controlling powdery mildew resistance in pea (Fondevilla *et al.*, 2008), cotyledon seed colour in *Glycine max*, QTLs for rust resistance including VuUGM02, VuUGM08 and VuUGM19 in cowpea (Uma *et al.*, 2016), *Phaeoisariopsis griseola* resistance genes in *Phaseolus vulgaris* (Alzate-Marin *et al.*, 2001), 73 blast

resistance genes and 350 QTLs in rice (Ghaley *et al*., 2012),and discovery of SNPs for agronomically important traits in *Arachis hypogea* (Pandey *et al*., 2017). NGS-assisted BSA identified six candidate genes in the QTL regions on chromosomes Ca2 and Ca4 and validated for their association with *Ascochyta* blight resistance in the CPR02 population in chickpea (Deokar *et al*., 2019). A QTL-seq approach coupled with BSA identified candidate genes (Ca_04364 and Ca_04607) for 100seed weight, and one gene (Ca_04586) for total root dry weight to total plant dry weight ratio using CAPS markers in chickpea.

Anuradha *et al*. (2011) developed 126 F_{10} derived RILs) derived from a cross between a moderately BGM resistant kabuli cultivar (ICCV2) and a highly BGM susceptible desi chickpea cultivar (JG62) atthe International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. This used the single seed descent (SSD) method, and identified two QTLs for BGM resistance on LG3 and one on LG6, which after validation, can be used for marker-assisted breeding. Kaur *et al*. (2013) also developed an inter-specific population for BGM resistance using the cut twig technique. Sixty two F9 BGM resistant lines derived were evaluated for agronomically important and yield traits, and identified four lines, (GL 29029, GL29206, GL29212 and GL29081) with high degrees of resistance to BGM. These lines were crossed with the BGM-susceptible high yielding cultivar BG256 for molecular analysis, and genotyping of F_2 populations identified SSR markerspotentially linked with *Ascochyta* blight and BGM resistance genes. Of the 120 markers used, six SSRs (TA2, TA110, TA139, CaSTMS7, CaSTMS24 and TR29) were found to be polymorphic. These markers can be used for identification of markers linked to BGM resistance, and assist in marker-assisted backcrossing for resistance breeding.

In the present study, comprehensive evaluation of the test host lines and paired-t test analysis both revealed that the field and laboratory screening methods gave similar results for BGM screening of the test lines. This demonstrates that laboratory screening methods coupled with molecular marker techniques can serve as powerful tools in genetics and crop improvement. The polymorphic SSR markers identified in the present study can be used to develop chickpea cultivars with high levels of BGM resistance, that has been a challenging task due to lack of sources of high levels of resistance in cultivated chickpea. Low chi-square statistic values of five SSRs (TA144, GA102, TA194, TA140 and TR2) in comparison to the critical value at $P = 0.05$ indicated their strong correlation with the resistant bulk and BGM resistance. We therefore conclude that bulked BSA is simple and accurate method for rapidly ascertaining marker-trait associations rapidly, and may be value for molecular tagging of BGM resistant genes in chickpea.

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Research Papers

Phytoprotection potential of *Fusarium proliferatum* for control of Botryosphaeria dieback pathogens in grapevine

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Summary. The economic impact of grapevine trunk diseases (GTDs) is increasing worldwide, due to the lack of efficient and simple control protocols for these disease complexes. Possible and efficient GTD management strategies must consider the complexity of host physiological alterations affecting metabolism and defense responses determined by GTD pathogens, and linked to disease expression. In this complexity, the use of biocontrol agents could give advantages in GTD control methods. The effect of the potential biocontrol agent (BCA) *Fusarium proliferatum* was evaluated using *in vitro* tests and in dual inoculation with the Botryosphaeria dieback agent *Neofusicoccum parvum in planta*. Artificial inoculations were performed in greenhouse and vineyard experiments at three key vine growth stages, the onset of G (separated clusters), I (flowering) or M (veraison) stages. The biocontrol potential was assessed using pathogenicity tests and transcriptomic analyses. Results showed that the *F. proliferatum* has potential for phytoprotection, with disease control efficiency related to host plant growth stage. Flowering was confirmed as the growth stage when disease control was least, and efficiency of activated defense responses against pathogen infection was minimum.

Keywords. Botryosphaeria dieback, *Diplodia seriata*, *Neofusicoccum parvum*, defense responses, biological control.

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INTRODUCTION

Grapevine trunk diseases (GTDs) are recognized as one of the most destructive and important problem of grapevine worldwide (Bertsch *et al.,* 2013; Fontaine *et al.,* 2016a; Guerin-Dubrana *et al*., 2019). GTDs are caused by several unrelated fungi which are able to live and exclusively colonize the wood of grapevine perennial organs, causing wood necrosis and/or discolouration, vascular infections and decays (Mugnai *et al.,* 1999; Bertsch *et*

al., 2013). Externally, diseased vines show general and progressive dieback, often associated with specific foliar symptoms affected by different diseases and host cultivars. These diseases can initially cause loss of productivity and eventually death of affected vines. Their potential is increased by long latent asymptomatic periods, and by the capability of pathogens to infect vines through pruning wounds (Bertsch *et al.,* 2013) which may remain susceptible to GTD infections up to 4 months (Eskalen *et al.,* 2007). Among GTDs, Botryosphaeria dieback, caused by species in the Botryosphaeriaceae (Chethana *et al.,* 2016) is one of the most widespread GTDs in grape growing regions (Úrbez-Torres, 2011; Spagnolo *et al.,* 2014a; Fontaine *et al.,* 2016a). Esca disease and Eutypa dieback are also important grapevine diseases (Bertsch *et al*., 2013).

After the banning of some disease management active ingredients, such as sodium arsenite, benomyl and carbendazim-based products (Bertsch *et al.,* 2013; Fontaine *et al.,* 2016a; Gramaje *et al.,* 2018; Songy *et al*., 2019), which gave good control of some important GTDs but may have negative effects on humans and environment, a range of fungicides, natural molecules and biological control agents (BCAs) has been tested to set up efficient control strategies towards the increasing incidence of GTDs (Mondello *et al.,* 2018). To date, beyond *Trichoderma* species, other fungal and bacterial agents have been reported to be effective against GTD pathogens, although some have only been tested either *in vitro* or in nurseries (Gramaje *et al.,* 2018; Mondello *et al.,* 2018). Among these agents, several studies have shown how they could efficiently contribute in GTD management strategies. Beside other specific advantages (broad-spectrum activity and long-lasting effect as wound protectants), an interesting potential of BCAs is their effects on host/pathogen relationships, such as systemic induced resistance (SIR) responses (Handlesman and Stabb, 1996; Pal *et al.,* 2006; Berg, 2009). In this way, BCAs could reduce disease incidence by improving host plant resistance to abiotic and biotic stresses, especially for those provoked by GTDs (Sosnowski *et al.,* 2011; van Niekerk *et al.,* 2011; Spagnolo *et al.,* 2014, 2017; Pinto *et al.,* 2018). BCAs could also have positive effects on GTDs through induced changes of host plant metabolism determined by plant defense responses, and disorders caused by fungal toxins (Abou-Mansour *et al.,* 2015; Christen *et al.,* 2015; Burruano *et al.,* 2016; Fontaine *et al.,* 2016a; Trotel-Aziz *et al.,* 2019).

Previous studies focused on physiological changes occurring in GTD-affected grapevines have highlighted that: i) the primary host metabolism is involved, with carbohydrate metabolism variations also related to the activation of host defense responses (Fontaine *et al.,* 2016b); ii) defense responses are activated at least at transcriptomic levels with different induction levels for the same gene according to the host growth stage, especially for Botryosphaeria dieback (Spagnolo *et al.,* 2014, 2017); and iii) these defense responses are probably not enough to avoid the pathogen colonization and disease development, especially during flowering. At flowering, grapevines showed the greatest gene induction levels but also the greatest weakness towards the two Botryosphaeria dieback pathogens, *Diplodia seriata* and *Neofusicoccum parvum* (Spagnolo *et al.,* 2014, 2017; Fontaine *et al.,* 2016b). Furthermore, these pathogens are able to disperse their inoculum at flowering, aggravating the possibility of infections at this stage (Kuntzmann *et al*., 2009; Amponsah *et al*., 2009; Valencia *et al*., 2015).

The aim of the present study was to expand knowledge of physiological changes occurring in green stems of grapevines cv. Mourvèdre that were artificially infected with GTD pathogens at different grapevine growth stages. Furthermore, because of the close relationship between host plant physiology and GTD development, the effects were also assessed of a potential fungal BCA to limit Botryosphaeriaceae *in vitro* and *in planta* through dual inoculation tests. Assessed effects were of: i) artificial inoculation with the pathogen *N. parvum*; and ii) dual inoculation with *N. parvum* and the potential BCA *Fusarium proliferatum*, in greenhouse experiments 2 months post-inoculation and in the field at different grapevine growth stages. *In vitro* biocontrol evaluation tests, pathogenicity tests and transcriptomic analyses were carried out in this study.

MATERIALS AND METHODS

In vitro *evaluation of biocontrol ability of* Fusarium proliferatum

The *F. proliferatum* strain ("Fus") used in this study was frequently isolated epiphytically and endophytically by Larignon *et al.* (2013), from tissues of symptomless, greenhouse-trained young grapevines.

This fungus is a common pathogen of maize but can also colonize trees, vegetables and other cereals (Cendoya *et al.,* 2017). A preliminary *in vitro* evaluation of the biocontrol ability of the Fus strain towards Botryosphaeriaceae included the pathogens *Diplodia seriata* (two strains: 98-1 and 99-7, described in Reis *et al.,* 2016) and *Neofusicoccum parvum* (three strains: Bourgogne (Np Bour) (Ramirez-Suero *et al.,* 2014), Np Sainte Victoire (NpSV) (Larignon *et al.,* 2015) and Np bt67 (Trotel-Aziz *et al.,* 2019).

Two different assays were carried out. The first assessed direct antagonistic effects of the potential BCA against the pathogens, and the second was to verify any antibiotic activity of the BCA's secondary metabolites. The direct antagonistic effects of the potential BCA were evaluated by dual culture assays following the adapted protocol of Bézert *et al.* (1996). A 3 mm diam. agar culture plug of the potential BCA and a plug of the respective pathogen were placed along the same diameter in a Petri dish (9 cm diam.) containing potato dextrose agar (PDA). Controls consisted in Petri dishes each inoculated with the pathogen and with a sterile PDA plug. Each experimental treatment was replicated five times. The plates were then maintained in the dark at 24 ± 1°C. The direct biocontrol activity of *F. proliferatum* was evaluated by measuring the area of mycelial growth (expressed in cm^2), recorded each 24 h until the pathogen covered the entire Petri dish agar surface in the experimental controls. Colony area was determined by measuring the colony margins, and was calculated using the ImageJ software (http://imagej.nih.gov/ij).

The antibiotic activity of *F. proliferatum* secondary metabolites was evaluated by growing the different pathogens on PDA enriched with cultural filtrate of *F. proliferatum*. Three-d-old agar plugs of *F. proliferatum* were put into flasks (one plug per flask) containing 100 mL of sterile potato dextrose broth (PDB), and was then maintained in agitation at 100 rpm for 14 d. The cultural filtrate was collected eliminating the mycelium pellets by non-sterile filtration, and was then sterilized by filtration (0.45 and 0.22 µm pore size filters in sequence), and was then added to autoclaved liquid PDA (55°C) to obtain PDA medium with concentrations of 0 (control), 25 or 50% of culture filtrate. These plates were then each inoculated with a 3 mm plug of a pathogen in the centre of the plate. Each experimental treatment was replicated three times. The antibiotic activity of the *F. proliferatum* secondary metabolites was evaluated by daily measuring of the surface of the pathogen colony up to 9 d post-inoculation (dpi). The colony surface areas were calculated using the ImageJ free software (http://imagej.nih.gov/ij).

Greenhouse assays: plant material, fungus strains and inoculations

To evaluate the *F. proliferatum* control capability *in planta*, greenhouse co-inoculation tests with *F. proliferatum* and Botryosphaeriaceae fungi were carried out. *Neofusicoccum parvum*, due to its high aggressiveness towards *Vitis vinifera*, was preferred to *D. seriata*. Also because of the *in vitro* results, the Fus biocontrol activity was evaluated towards *N. parvum* strain NpSV, whose growth was reduced in dual cultural assay and greatly inhibited by Fus culture filtrate. Four-month-old potted plants of grapevine cv. Mourvèdre were each inoculated with *F. proliferatum* strain Fus using a PDA plug from a 7-d-old colony. One (FN+7d) and two (FN+14d) weeks post-inoculation with Fus, the plants were each co-inoculated at the same internode with the *N. parvum* strain NpSV, about 2 cm above the Fus inoculation point. Control plants were inoculated, respectively, with sterile PDA, Fus alone or NpSV alone. Five plants per experimental treatment were used. Two months after pathogen inoculation, the plants were cut, and the portion of each inoculated internode was observed and internal necrosis was measure. The presence of the potential BCA and pathogen were also verified (Figure 1). Collected data

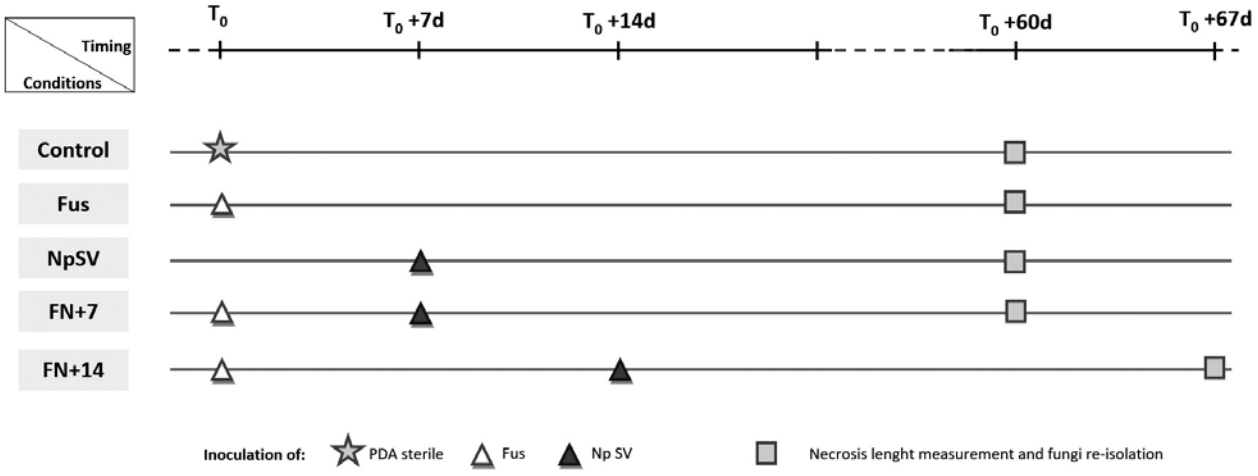

Figure 1. The co-inoculation test scheme, indicating the different conditions assayed and the timing of the inoculations BCA –pathogen, as performed in the greenhouse test.

were submitted to statistical analyses (Kruskal–Wallis and Dunn's multiple comparison tests).

Field tests: plant material, fungus strains and inoculations

To further evaluate the biocontrol activity of the Fus strain in natural conditions, and the role of host plant growth stage in disease development, a co-inoculation test was also performed in the field, as described in Reis *et al.* (2019). The experimental site was a vineyard of cv. Mourvèdre/3309, planted in 1997, and located at Rodilhan (Costières de Nîmes, France). The vineyard was owned by the Lycée agricole Marie-Durand of Rodilhan. Similar to the greenhouse essay, the Botryosphaeria dieback agent *N. parvum* strain NpSV and *F. proliferatum* strain Fus were used. These fungi were inoculated into green stems of standing vines, either alone (NpSV or Fus) or in combination (FN), using the method described by Spagnolo *et al.* (2017). In the case of the dual inoculations (FN), *N. parvum* was inoculated 7 d after Fus. The inoculations were performed at the onset of the grapevine Baggiolini phenological stages G (separated clusters), I (flowering) or M (veraison) (Baggiolini, 1952). Individual plants received the respective experimental treatments, and eight repetitions per treatment were applied. For each growth stage, non-wounded and non-inoculated green stems (C1), or stems inoculated with sterile malt agar (C2), were the experimental controls.

Observation of lesion development and re-isolation tests (for five of the 8 repetitions of each treatment) were performed at the end of vegetative season for samples from the three growth stages, as described by Larignon and Dubos (1997).

TRANSCRIPT ANALYSES

To study the effects of presence of the pathogens and the BCA on host plant physiology, inoculated stems in the vineyard were collected and submitted to transcriptomic analyses. Except for C1 stems, all the treatments were considered for transcript analysis. Samples for RNA extraction (three of 8 repetitions) were collected 20 dpi, and each consisted of the portion of the inoculated internode. For co-inoculated stems, each sample consisted of the portion of internode inoculated with both NpSV and Fus. Samples were collected, stored and processed to obtain a fine powder, according to the protocol outlined by Spagnolo *et al.* (2017).

RNA extraction

The Plant RNA Purification Reagent (Thermo Fischer Scientific Inc.) was used to extract total RNA from 2 x 50 mg of powdered green stem tissues from each assayed plant, and was DNase treated. The quality of RNA was checked by agarose gel electrophoresis, and the quantity was determined by measuring the absorbance at 260 nm.

Real-time RT-PCR analysis of gene expression

Reverse transcription was performed on 150 ng of total RNA, using the Verso cDNA synthesis kit (Thermo Fischer Scientific Inc.). Real-time PCR was performed with Absolute Blue QPCR SYBR Green (Thermo Fischer Scientific Inc.), using a CFX96 thermocycler system (Bio-Rad).

The thermal profile used was: 15s at 95°C (denaturation) and then 40 cycles each of 1 min at 60°C (annealing/extension). Melting curve assays were performed from 65–95°C at $0.5°C \cdot s^{-1}$. Melting peaks were visualized to check the specificity of each amplification. Results were expressed as the values of relative expression (ΔΔCt), corresponding to the mean of three independent experiments. The genes analyzed were considered significantly up- or down-regulated when changes in their expression were, respectively, >2-fold or <0.5-fold. The specific primers for the 16 targeted genes are listed in Table 1.

RESULTS

Evaluation of the biocontrol ability of Fusarium proliferatum

In dual culture tests, *F. proliferatum* strain Fus limited the growth of *D. seriata* and *N. parvum* strains, forming clear limits between the two touching colonies in each Petri plate after 4-7 d of co-culture, even where the differences with the controls were not statistically significant. The one exception was that of the strain Np bt67 (Figure 2, A to E). A slight decrease (7-20%) was recorded in growth of the *N. parvum* strains in dual culture with Fus, 1 d before the contact between both colonies in each plate. This effect was not recorded for the tested *D. seriata* strains (data not shown). The contact zones were often characterized by the contemporary presence of intertwining hyphae of Fus and the pathogens (Figure 2, A' and B'). Fus was also able to grow over most of the pathogens, starting at 10 dpi (Figure 2C).

Stronger Fus biocontrol activity was observed for the Fus culture filtrate (Fus CF), with differences related to

Table 1. Primers of genes analyzed by real-time reverse-transcription polymerase chain reaction.

Function	Gene	Primer Sequences	GenBank or TC TIGR* Accession Number
Housekeeping genes	*EF1* (EF1-α elongation factor)	5'-GAACTGGGTGCTTGATAGGC-3'	GU585871
		5'-AACCAAAATATCCGGAGTAAAAGA-3'	
	60SRP (60S ribosomal protein L18)	5'-ATCTACCTCAAGCTCCTAGTC-3'	XM_002270599
		5'-CAATCTTGTCCTCCTTTCCT-3'	
Phenylpropanoid metabolism	*CHI* (Chalcone isomerase)	5'-GCAGAAGCCAAAGCCATTGA-3'	NM_001281104
		5'-GCCGATGATGGACTCCAGTAC-3'	
	PAL (Phenylalanine ammonia lyase)	5'-TCCTCCCGGAAAACAGCTG-3'	X75967
		5'-TCCTCCAAATGCCTCAAATCA-3'	
	POX4 (Peroxidase-like 4)	5'-AACATCCCCCCTCCCACTT-3'	XM_002269882
		5'-TGCATCTCGCTTGGCCTATT-3'	
	STS (Stilbene synthase)	5'-AGGAAGCAGCATTGAAGGCTC-3'	FJ851185
		5'-TGCACCAGGCATTTCTACACC-3'	
Defense protein	*CHV5* (Chitinase class V)	5'-CTACAACTATGGCGCTGCTG-3'	AF532966
		5'-CCAAAACCATAATGCGGTCT-3'	
	GLUC (β-1,3 glucanase)	5'-TCAATGGCTGCAATGGTGC-3'	DQ267748
		5'-CGGTCGATGTTGCGAGATTTA-3'	
	PPO (Polyphenol oxidase)	5'-TGGTCTTGCTGATAAGCCTAGTGA-3'	XM_002727606
		5'-TCCACATCCGATCGACATTG-3'	
	PR6 (Serine-protease inhibitor 6)	5'-AGGGAACAATCGTTACCCAAG-3'	AY156047
		5'-CCGATGGTAGGGACACTGAT-3'	
	SAMS (S-adenosylmethionine synthetase)	5'-CCTGAAATCAAAGTTCTCCTTCACA-3'	XM_002266322
		5'-CCGGGCCTGAAATCAAAGTT-3'	
	TL (Thaumatin-like)	5'-CCTAACACCTTAGCCGAATTCGC-3'	AF532965
		5'-GGCCATAGGCACATTAAATCCATC-3'	
Detoxification and Stress tolerance	*epoxH2* (Epoxide hydrolase 2)	5'-TCTGGATTCCGAACTGCATTG-3'	XM_002270484
		5'-ACCCATGATTAGCAGCATTGG-3'	
	GST5 (Glutathione s-transferase 5)	5'-GCAGAAGCTGCCAGTGAAATT-3'	XM_002277883
		5'-GGCAAGCCATGAAAGTGACA-3'	
	HSP (alpha crystalline heat shock protein)	5'-TCGGTGGAGGATGACTTGCT-3'	XM_002272382
		5'-CGTGTGCTGTACGAGCTGAAG-3'	
	SOD (Superoxide dismutase)	5'-GTGGACCTAATGCAGTGATTGGA-3'	AF056622
		5'-TGCCAGTGGTAAGGCTAAGTTCA-3'	
Primary metabolism	*PglyDH* (Phosphoglycerate dehydrogenase)	5'-CGTCGAAGATGCTCAATGATGA-3'	XM_002285322
		5'-CCCCCACGAGCAACATTAATT-3'	
Water stress	*TIP1* (Tonoplast intrinsic protein)	5'-ATCACCAACCTCATTCATATGC-3'	AF271661
		5'-GTTGTTGTCTCAACCCATTTCC-3'	

* see http://www.jcvi.org/cms/research/projects/tdb/overview/

the Botryosphaeriaceae species and the strain (Figure 3, A and B). *Diplodia seriata* strains showed similar reactions to the Fus CF, with a severe growth reduction even from the lowest aliquot of filtrate. Growth of the Ds99-7 strain was inhibited until 3 dpi on 25% Fus CF and 7 dpi on 50% filtrate. This strain then had abnormal and slow growth with sparse hyphae (Figure 4). Different from *D. seriata*, *N. parvum* growth in Fus CF-amended media varied according to the strain. Fus CF showed the greatest activity against NpSV growth compared to the control, with statistically significant reductions at both 25 and 50% filtrate. In contrast, there was no statisti-

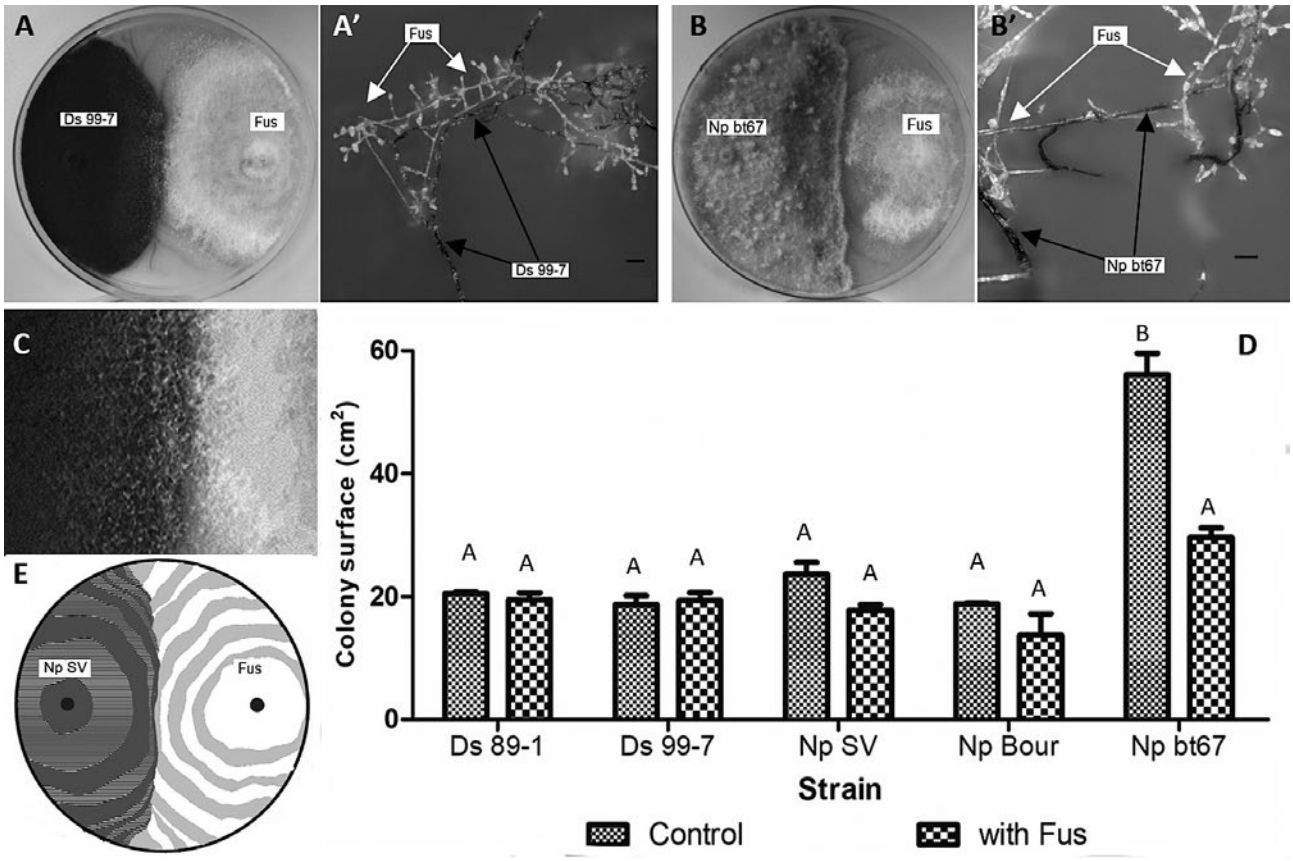

Figure 2. *In vitro* biocontrol activity of *Fusarium proliferatum* strain Fus on the tested Botryosphaeriaceae in dual culture tests. In A and B the limited growth of Ds99-7 and Np bt67 strains was noted after 4 - 7 d of dual inoculation with *F. proliferatum*. In A' and B', the respective zones of confrontation, observed with a microscope, often showed intertwining hyphae of the two strains (indicated by arrows). In C, the Fus strain are growing as white flocks over the Botryosphaeriaceae, as observed at 10 dpi. In D, the mean colony surface (in cm^2 ± SE; two-way ANOVA + Bonferroli test), of the five strains compared to the controls and recorded on the day when the colonies touched, as calculated using a graphical representation of the co-cultures (E). Scale bars = 20μm. dpi = day post inoculation. Columns accompanied by the same letter are not significant different ($P \leq 0.05$).

cally significant difference the Np Bour growth values, although Fus CF growth was stimulated growth at 25% of filtrate but inhibited growth at 50%. Intermediated response was recorded for the Np Bt67 strain, which was significantly inhibited only when growing on plates with the 50% of Fus CF.

Greenhouse pathogenicity tests

The Fus and NpSV strains were re-isolated from the edges of internal green stem lesions at 60 dpi. No fungi were isolated from vines that received sterile PDA plugs. Both the strains induced lesions starting from the inoculation points, while no lesions occurred in the control plants. The presence of *F. proliferatum* strain Fus in FN+7d co-inoculations gave a significant reduction ($P \leq 0.05$) in the lesion length caused by NpSV. In contract, NpSV gave increased of lesion lengths in co-inoculated vines when the pathogen was inoculated 14 d after inoculation with Fus (FN+14d), although the difference was statistically significant compared to control (Figure 5)

Field pathogenicity tests

The pathogen *N. parvum* and the potential BCA *F. proliferatum* were always re-isolated from the edges of the lesions associated with their inoculations, so the postulates associating disease and effects were fulfilled. No fungi were isolated from the lesions of control stems C2, indicating the lesions developed as a consequence of the wound in inoculated conditions. Mean lesion sizes associated with *F. proliferatum* in the single (Fus) and dual

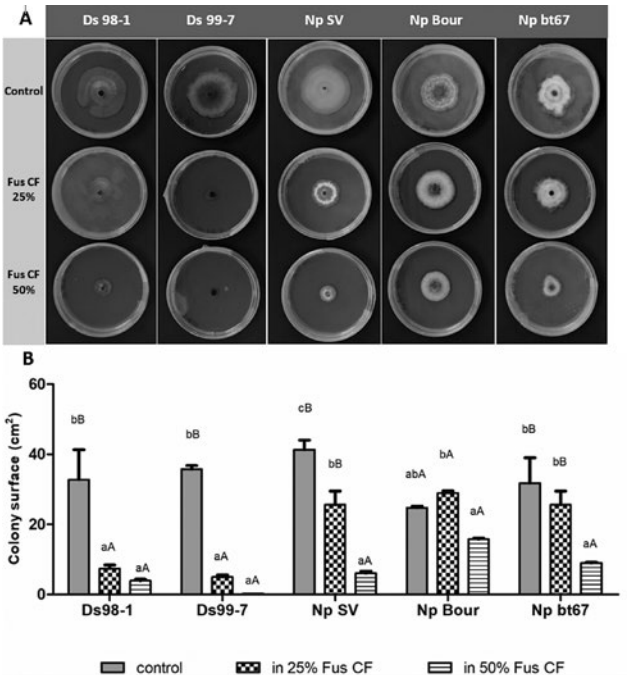

Figure 3. Biological control activity of the two different culture filtrate aliquots of *F. proliferatum* strain Fus (Fus CF) against growth of Botryosphaeriaceae fungi at 7 dpi. In B), the ANOVA results (two-way + Bonferroli test) on mean colony areas (cm^2) indicated a different Fus CF effect, for species and strain. Columns accompanied by the same letters are not significantly different (lowercase, $P \leq 0.05$, uppercase $P \leq 0.01$).

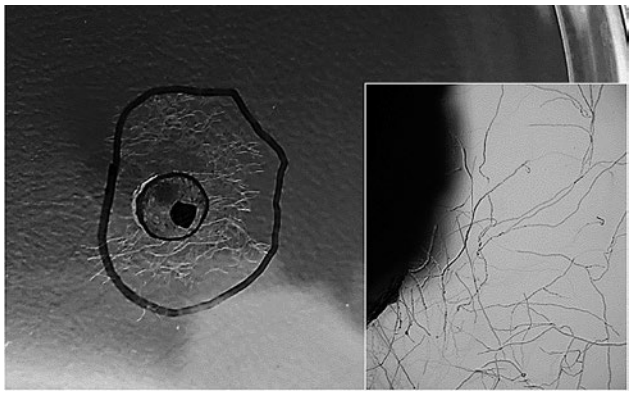

Figure 4. Detail of inhibited growth of strain Ds 99-7 on PDA plates amended with 50% of Fus cultural filtrate (Fus CF), observed with a microscope at 8 dpi. The mycelium rarely formed and had sparse hyphae.

inoculations with the pathogen (FN) were never statistically different from the controls (C2). Statistical differences were recorded for *N. parvum* in all the three vine phenological stages for single inoculation, and at the I and M stages for the in dual inoculations. At G stage, the longest lesions were recorded for Np condition (mean = 10.8 mm (± 1.3 mm)). At stages I (flowering) and M (veraison) longer lesions were associated with the pathogen from single (NpSV) and dual (FN) inoculations. At flowering, *N. parvum* produced longer lesions in the presence of Fus (mean = 24.0 mm (± 3.0 mm))

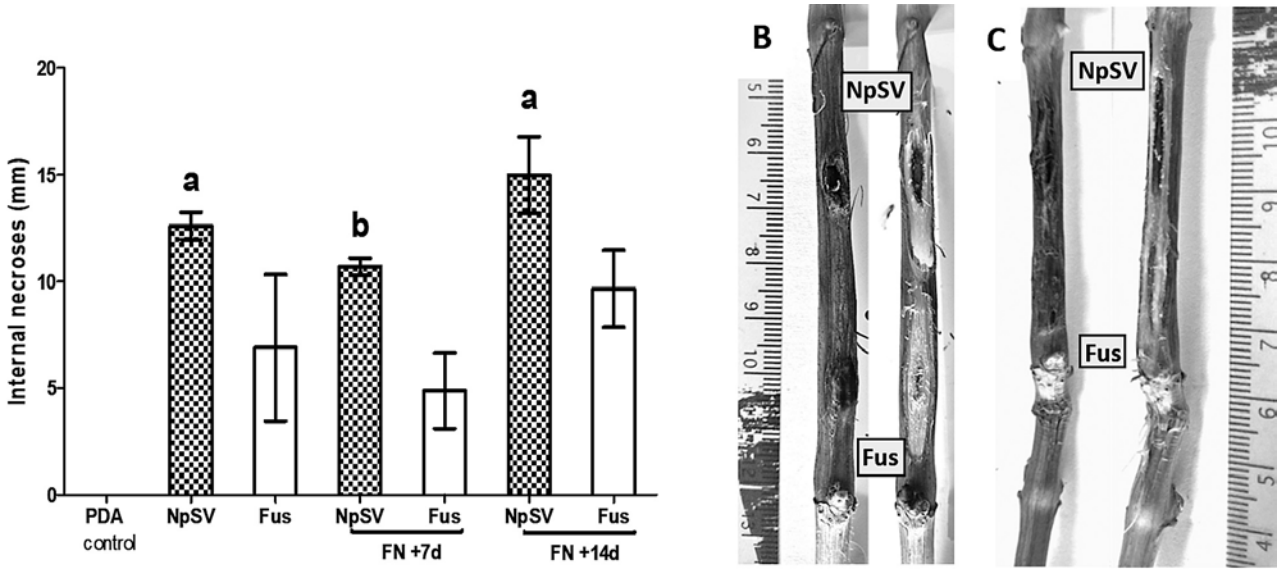

Figure 5. In A, mean lesion lengths (± SE) on rooted grapevine cv. Mourvèdre plants after inoculation with *N. parvum* strain NpSV and *F. proliferatum* strain Fus, at 60 dpi for single (NpSV, Fus) or dual inoculations (carried out in the greenhouse. Differences among the means were evaluated by the Mann-Whitney non-parametric test. Bars accompanied by the same letter are not statistically significant different ($P \leq 0.05$). In B and C, external and internal lesions produced by Fus and NpSV in FN+7d and in FN+14d co-inoculated plants.

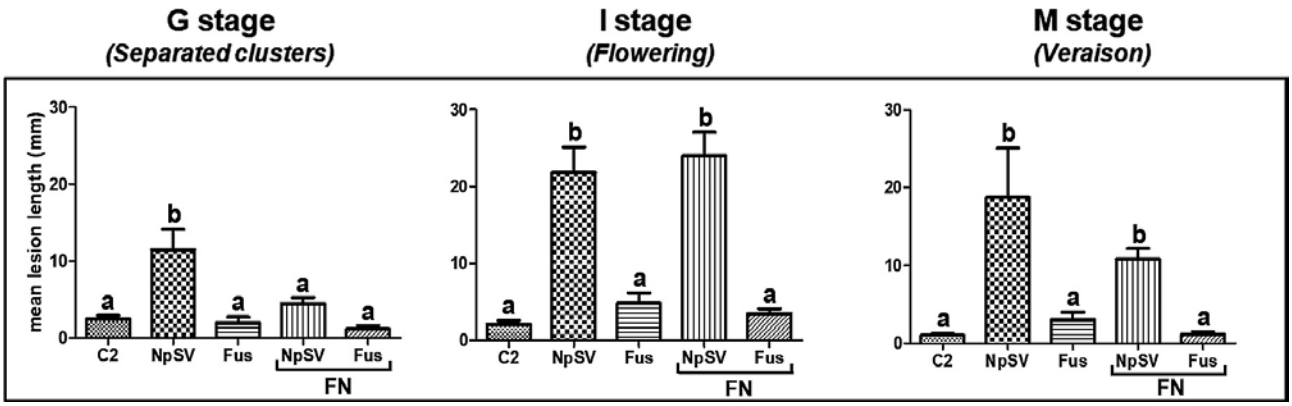

Figure 6. Mean lesion lengths (± SEs) on green grapevine stems after inoculation with *N. parvum* strain Saint Victoire and *F. proliferatum* strain Fus at the onset of the G, I and M stages, after single (NpSV, Fus) or dual (Inoculation. Control stems (C2) were wounded and inoculated with sterile malt agar. Differences among the means were evaluated by the Dunn's Multiple Comparison Test, following which the null hypothesis (equal means) was rejected in the Kruskal–Wallis test, assuming significance of $P \leq 0.05$. Columns accompanied by the same letters are not significantly different ($P \leq 0.05$).

than when inoculated alone (mean = 21.8 (± 3.3 mm)), although this difference was not statistically significant ($P > 0.05$). In contrast, at M stage (veraison), lesion lengths were greater from the *N. parvum* single inoculation (mean = 18.7 mm (± 3.4 mm)) than from the dual inoculations (mean = 10.8 mm (± 1.3 mm)) (Figure 6).

Characterization on targeted plant responses

The transcript analyses were used to evaluate the influence of the potential BCA *F. proliferatum* on host plant defense responses towards the Botryosphaeria dieback pathogen *N. parvum*. The genes were selected based on similar previous studies (Spagnolo *et al.,* 2014, 2017; Magnin-Robert *et al.,* 2011; 2014; 2016). A total of 16 genes encoding components of the phenylpropanoid pathway (genes *CHI, PAL, POX4, STS*), Pathogenesis-related (PR) and other defense proteins (*CHV5, GLUC, PPO, PR6, SAMS, TL*), proteins involved in detoxification processes (*epoxH2, GTS5, HSP, SOD*) and in primary metabolism (*PglyDH*) or water stress (*TIP1*), were chosen measure a profile of the grapevine response.

As already observed in previous studies (Spagnolo *et al.,* 2014; 2017), most of changes in the expression levels concerned the phenylpropanoid pathway and *PR*-genes, especially at the G and I vine growth stages. At these stages maximum levels of gene induction were detected in stems co-inoculated with *N. parvum* and *F. proliferatum* (FN) (Figure 7). For example, the relative expression of gene *STS* increased by up to 34-fold in FN at the G stage, and expression of *PR6* was increased almost 33-fold in FN at I stage. At flowering, a more homogenous background of upregulation was observed, determined by increased induction from both the Np and Fus inoculations, and a simultaneous decrease in some genes from the FN treatments. A general decrease in gene induction occurred at veraison (M stage), with slight upregulations occurring mostly in the co-inoculated stems (FN). Among the stress tolerance and detoxification genes, only *GST5* at the G stage and *HSP* at the I stage were slightly induced, especially in presence of the BCA either alone or in presence of the pathogen. No genes were down regulated by any of the treatments.

DISCUSSION

Antagonistic effects of Fusarium proliferatum *against Botryosphaeriaceae species*

Fusarium proliferatum strain Fus showed antagonistic effects towards *N. parvum* and *D. seriata* in the laboratory tests. The *in vitro* dual culture test highlighted the role of the potential BCA in limiting growth of the tested Botryosphaeriaceae pathogens, which were unable to overgrow the antagonist Fus when in contact with its mycelium. The slight inhibition recorded 1 d before confrontation with Fus, observed only for *N. parvum* due to the slower growth rate compared to *D. seriata*, suggested a role of antibiosis. Antibiosis was demonstrated from the results of culture filtrate test. *Fusarium proliferatum* metabolites limited the growth of the pathogens, with greater and more homogeneous inhibition of the *D. seriata* strains than of the *N. parvum* strains. The different strains of *N. parvum* showed different amounts of

Function / Gene		G stage (Separated clusters)			I stage (Flowering)			M stage (Veraison)		
		NpSV	Fus	FN	NpSV	Fus	FN	NpSV	Fus	FN
Phenyl-propanoid metabolism	CHI	1,16	0,79	1,69	2,04	2,21	2,24	1,05	0,92	1,26
	PAL	2,22	1,21	3,52	0,66	1,20	0,99	0,96	2,05	1,99
	POX4	1,81	1,07	3,45	2,68	1,48	2,02	1,11	1,37	1,53
	STS	5,71	3,08	34,07	7,13	7,50	9,27	1,06	0,95	2,19
Defense protein	CHV5	3,39	3,61	13,78	7,70	7,51	13,21	2,96	1,59	2,43
	GLU	4,69	0,96	23,30	4,70	6,80	14,29	1,93	1,16	2,71
	PPO	1,09	1,47	3,41	3,64	2,22	5,06	2,68	2,51	2,87
	PR6	3,57	2,40	7,35	12,63	25,75	32,73	0,88	0,62	1,06
	SAM	1,16	0,97	1,57	1,21	1,48	1,51	0,95	1,23	1,62
	TL	2,85	3,01	7,39	7,31	4,08	8,40	2,44	4,63	2,13
Detoxification and Stress tolerance	epoxH2	1,16	0,84	1,24	1,44	1,13	1,15	0,87	1,13	1,39
	GST5	1,93	1,18	3,60	0,85	1,47	1,26	0,80	1,01	1,24
	HSP	1,19	1,02	1,00	0,60	2,85	3,46	1,09	1,13	1,17
	SOD	1,07	0,88	1,23	1,23	1,31	1,24	0,81	0,98	1,01
Primary metabolism	PglyDH	1,56	1,05	1,36	0,62	1,13	1,47	0,82	1,05	1,40
Water stress	TIP1	0,94	0,90	1,01	3,04	1,40	1,81	0,73	0,96	0,99

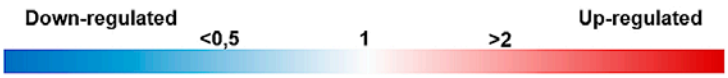

Figure 7. Expression levels of selected genes (determined with quantitative reverse-transcription polymerase chain reactions). The values (each the mean of three technical replicates) represent expression levels (ΔΔCt) of treatments relative to the controls (C2). The expression of each gene was considered up-regulated when the value was > 2-fold compared to the controls, or down-regulated when the value was < 0.5-fold compared to the controls.

growth inhibition, with NpSV as the most susceptible to Fus CF at both tested culture filtrate concentrations. *Fusarium proliferatum* was able to grow over the pathogens at 10 dpi, which also indicated direct antagonism effects, as reported previously for *F. proliferatum* and other *Fusarium* species. For instance, *F. proliferatum* was shown to be effective against the oomycete *Plasmopara viticola*, which causes grapevine downy mildew (Falk *et al.,* 1996). *Fusarium sporotrichioides* inhibited *in vitro* the growth of *Phoma negriana*, responsible of lesions and necrosis on grapevine shoots (Krol, 2008), while *F. lateritium* was shown to metabolize toxins produced by *Eutypa lata*, a cause of Eutypa dieback (Christen *et al.,* 2005). Some of the mechanisms involved in the *Fusarium* spp. biocontrol, such as mycoparasitism (Falk *et al.,*1996), extracellular β-glucosidase and endo-1,4-β-glucanase production (Bakshi *et al.,* 2001) and cyanide degradation (Christen *et al.,* 2005), were also found in other BCAs such as *Trichoderma* spp., *Ampelomyces quisqualis*, *Penicillium purpurogenum*, *Coniothyrium minitans* and *Pseudomonas fluorescens* (Haran *et al.,* 1996; Kubicek *et al.,* 1993; Rotem *et al.,* 1999; Whipps *et al.,* 2008; Winding *et al.,* 2004).

Fusarium proliferatum *reduced the necrosis produced by* N. parvum *strain NpSV* in planta

Under controlled conditions, *F. proliferatum* also showed biocontrol activity, reducing *N. parvum* strain NpSV aggressiveness when inoculated 7 d before the pathogen. In contrast, presence of *F. proliferatum* tended to increase the size of pathogen internal necroses when inoculated 14 d previously, although this effect was not statistically significant. In co-inoculations performed in the field, *F. proliferatum* showed biocontrol activity by reducing lesion sizes associated to *N. parvum* at the G (separated clusters) and, to a lesser extent, at M (veraison) grapevine stages. In contrast, the mean sizes of lesions associated with *N. parvum*, from single and dual inoculations, was greater at flowering, confirming the hypothesis that flowering is the period when the grapevine cv. Mourvèdre is more susceptible to Botryosphaeria dieback pathogens. As reported in previous studies (Spagnolo *et al.,* 2014; 2017), this susceptibility could be determined by high host plant metabolic activity focused towards inflorescence development. This metabolic re-orientation developing reproductive

organs could have also determined the inefficient biocontrol of *F. proliferatum* at I growth stage, which was unexpected considering the relative transcriptomic data. Beside biocontrol capabilities towards *N. parvum*, *F. proliferatum* was also not pathogenic to grapevine cv. Mourvèdre, although in greenhouse inoculations the strain induced longer lesions than in the field. These differences may be explained by the different host types used (cuttings *vs* standing vines), and the less stressing environmental conditions of the greenhouse compared to the field, which may have modulated the *F. proliferatum* behaviour. Greenhouse conditions could have also favored aggressiveness *F. proliferatum* and *N. parvum* in unstressed plants which were less reactive to artificial infections.

Changes induced by Fusarium proliferatum *to the grapevine*-Neofusicoccum parvum *interaction*

This also focused on the physiological changes occurring in annual stems in the host/pathogen/BCA tritrophic interaction of grapevine/*N. parvum*/*F. proliferatum,* at three host growth stages.

Fusarium proliferatum strain Fus weakly induced genes at the G stage (genes *STS*, *CHV5*, *PR6, TL*) and the M stage (*PAL*, *PPO, TL*). This indicated host plant tolerance towards *F. proliferatum* and supported the observed in field non-pathogenicity of this strain to *V. vinifera* cv Mourvèdre. In contrast, at the I stage Fus induced genes at levels equivalent to those of the pathogen *N. parvum*. This indicated a possible switch at flowering, from "neutral" to "parasitic" perception of *F. proliferatum* by the host defense system (Kogel *et al*., 2006). The greatest induction recorded for the most of genes in all FN treatments suggests the role of *F. proliferatum* strain Fus in priming the host defense system towards pathogen infections, which resulted in reduced necrosis from *N. parvum* strain NpSV observed at the G stage and to a lesser extent, at the M stage. This priming effect, elicited by several BCAs (*Trichoderma* sp., Perazzolli *et al*., 2011; *Pythium oligandrum*, Yacoub *et al*., 2016) was reported for *Aureobasidium pullulans* strain Fito_278 (Pinto *et al*., 2018) and *Bacillus subtilis* PTA-271 (Aziz *et al*., 2015; Trotel-Aziz *et al*., 2019) towards pathogens associated Botryosphaeria dieback.

Changes in the grapevine-*N. parvum* interaction determined by the potential BCA were characterized by gene expression associated either with plant defense/stress responses (secondary metabolism) or with energy metabolism (glycolysis, Krebs cycle). In agreement with Spagnolo *et al.* (2014), most of the modifications detected in the present study were more related to the host phenological stage than to a particular inoculation tested. Fasoli *et al.* (2012) also highlighted the importance of grapevine growth stage for gene expression. Gene expression in different organs is probably based on the host developmental stage rather than on organ type.

The over-expression of the genes *CHI*, *PAL*, *POX4* and especially *STS* at both the G and I stages on the infected stems from the NpSV and FN treatments could be related to activation of the phenylpropanoid pathway linked to plant defense. Phenylpropanoids are related in plant defense responses, playing preformed or inducible functions, forming physical or chemical barriers to infection, or local and/or systemic signalling for the defense gene induction (Dixon *et al.,* 2002). Similarly, stilbenes may limit development of GTD fungi in wood. *In vitro* tests have shown ability of different phenolic compounds to limit growth of several GTD-associated pathogens in the Botryosphaeriaceae and *Phaeomoniella chlamydospora* (Fontaine *et al.,* 2016b; Lambert *et al.,* 2012; Lima and Dias, 2012). Furthermore, the stilbene resveratrol could also act as a signaling molecule in the activation of defense-related responses in *Vitis* cells (Chang *et al.,* 2011).

The induction of *GLUC* expression, recorded in single or dual infected stem treatments, especially at the G (NpSV and FN) and I (NpSV, Fus and FN) growth stages, could be related to the multiple role of the related proteins. β-1,3-glucanases are abundant in plants, and are involved in cell division, movement of materials through plasmodesmata and in plant resistance towards abiotic stresses. These proteins also protect plants against fungal pathogens, alone or in association with other antifungal proteins such as chitinases or isoenzymes (Balasubramanian *et al.,* 2012). The synergistic effects between β-1,3-glucanases and chitinases towards fungal infections is frequently observed in other crops (including pea, bean, tomato, tobacco, maize, soybean, potato, and wheat), and their presence led to increased disease resistance in plants (Balasubramanian *et al.,* 2012; Saboki *et al.,* 2011; Jach *et al.,* 1995). In the present study, *GLUC* induction was always coupled with increases in chitinase *CHV5* at the G growth stage (NpSv and FN treatments), the I stage (all treatments) and the M stage (FN treatment). At the transcriptomic level, this synergism of *Gluc/CHV5* could be also have occurred in green stems cv. Mourvèdre infected by *F. proliferatum* and Botryosphaeriaceous pathogens, either alone or in combination, as observed here and in the previous study Spagnolo *et al.* (2017). For defense protein genes, the observed high induction of *PR6* genes at flowering (the I stage) from all treatments (NpSV, Fus, and FN) has been previously reported in grapevines naturally affected by

GTD pathogens (Magnin-Robert *et al.,* 2014; Falk *et al.,* 1996; Magnin-Robert *et al.,* 2014; Valtaud *et al.,* 2009; Letousey *et al.,* 2010) and in artificially inoculated plants (Camps *et al.,* 2010), and has been described in grapevine cells in the presence of extracellular compounds produced by Botryosphaeria dieback pathogens (Ramirez-Suero *et al.,* 2014). The strong induction of defense genes (*PR6, PPO, TL, GLU, CHV5*) observed in FN-treated vines at the I growth stage, was either unrelated to the reduction of lesion sizes observed at G and M stages, or not great enough to limit lesion development at flowering. The implication of inflorescences reducing responsiveness to pathogen attack in green stems (Spagnolo *et al.,* 2014; 2017), supports the observation of little biological control effect from *F. proliferatum* at flowering.

CONCLUSIONS

Results from this study agree with previous observations, and confirm the importance of flowering in the defense of grapevine cv. Mourvèdre towards the development of *N. parvum* infections, even in the presence of the potential BCA *F. proliferatum*. This fungus limited the growth of pathogens both through antibiosis and direct antagonism *in vitro*, and development of pathogen-induced necrosis *in planta*, by priming effects enhancing some plant responses to infection. Based on these results, further studies should assess the physiological mechanisms that could influence host metabolism and responses to biotic stress during flowering.

The results observed in the *in planta* dual inoculation assays suggest further analysis of possible use of *F. proliferatum* as BCA is warranted, since the priming of plant defense response is an efficient and low cost way to improve resistance to abiotic and biotic stresses (van Hulten *et al*., 2006). Toxicological studies should also be carried out when considering this fungus as a possible BCA against GTDs, as variable toxigenic potential was detected in *F. proliferatum* populations by Stepien *et al.* (2011). This would reduce the risk that toxic fungal metabolites would enter human or animal food chains. Furthermore, the role of *F. proliferatum* as opportunistic human pathogen should be also considered, since it and other *Fusarium* species are reported to be associated with infections of immunocompromised patient (Summerbell *et al*., 1988; Tulin, 2018).

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Conflicts of Interest: The authors declare no conflict of interest.

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Research Papers

Activity of Italian natural chabasite-rich zeolitites against grey mould, sour rot and grapevine moth, and effects on grape and wine composition

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Summary. The activity of Italian chabasite-rich zeolitites for control of grey mould, sour rot and grapevine moth was compared to that from synthetic fungicides and insecticides in three vineyards in the Abruzzo region of Italy. Persistence of zeolitites on grapevine canopies has enabled applications to be carried out before rainfall events, which are likely to predispose for infections by grey mould and sour rot pathogens. Applications for control of *Lobesia botrana* (grapevine moth) were carried out after the peak of the male flight, and the presence of eggs on grape berries was also assessed. Disease and pest control were very satisfactory and comparable to those obtained from synthetic fungicides and insecticides. In addition, there were no differences among treatments for yield, berry composition, or wine ethyl alcohol, pH and total acidity. Phenolic compounds increased in wine from zeolitite-treated plants where the treatment was carried out within 15 days from grape harvest. These promising results have strategic value, because zeolitites provided simultaneous control of grey mould, sour rot and *L. botrana*. However, since these compounds have been tested for the first time for the control of diseases and *L. botrana*, the results obtained in this study require further validation.

Keywords. Zeolites, vineyard, *Botrytis cinerea*, *Lobesia botrana*.

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INTRODUCTION

Botrytis cinerea (= *Botryotinia fuckeliana;* Johnston *et al.,* 2014) and *Lobesia botrana* (Denis and Schiffermüller) (Lepidoptera: Tortricidae) are among the major diseases and pests of grapevine worldwide.

Control of grey mould, caused by *B. cinerea*, is not always effective, despite the use of specific fungicides, and incurs severe economic effects for grape production (Gullino, 1992; Elad *et al*., 2007). During growing seasons characterized by rainy and wet conditions, up to 40–60% reduction in grape yields can be result from grey mould epidemics, leading to deterioration of organolectic characteristics of must and wine (Pearson and Goheen, 1988; Dubos, 2000; Elad *et al*., 2007). This pathogen is widespread in all vine-growing areas and is particularly aggressive against grapevine cultivars with tight bunches (Marois *et al*., 1986).

The pathogenic activity of *B. cinerea* is due to the ability of the fungus to synthesize lytic enzymes such as laccase and polygalacturonase (Nakajima and Akutsu, 2014), and secondary metabolites such as oxalic acid, which in turn increase the activity of polygalatturonases and laccases (Manteau *et al*., 2003), and botrydial, a phytotoxin which causes necrosis of plant cells (Colmenares *et al*., 2002).

Control of *B. cinerea* is based on at least two fungicide applications each growing season, the most important carried out at the pre-bunch closure growth stage, particularly for compact cluster cultivars. This is to protect micro-wounds that are very susceptible to the pathogen, and to reduce inoculum sources. The second application is usually during ripening, based on prevailing environmental conditions (Marois *et al*., 1986).

Leaf removal at the end of flowering improves the effectiveness of the fungicide applications. The removal of the leaves may also lead to greater coverage uniformity of the fungicide on berries and provide drying conditions that reduce bunch rot development (English *et al*., 1993; R'Houma *et al*., 1998). Removal of leaves at the 'pea-size berries' stage can also reduce infestations of *L. botrana* (grapevine moth), which in turn may favour *B. cinerea* infections from the insect feeding wounds produced on bunches (Pavan *et al*., 2016).

Effective *B. cinerea* control strategies are also based on the control of other biotic agents which facilitate occurrence of the pathogen (powdery mildew, grapevine moth), and on the correct adoption of cultural practices aimed at a reducing humidity inside grape bunches.

The use of anti-*Botrytis* fungicides can also cause residues in the harvested grapes, with different negative effects for end users, and possible onset of resistant pathogen strains (Fillinger and Elad, 2016).

In Italian vineyards, *L. botrana* has three generations per year (Pavan *et al*., 2006). After emergence, the females lay eggs on flower buttons, which can be slightly damaged by the first-generation of larvae hatched from eggs. Serious damage is caused by the second and third generations of larvae that penetrate and hollow out the berries, making the bunches more susceptible to grey mould, sour rot, and powdery mildew.

Treatments against *L. botrana* are carried out based on thresholds assessed as the presence of eggs or holes on berries in vineyards normally infested by the moth, or as 5% of bunches infested by the second and third generations in vineyards normally not infested after monitoring of the male flight activity.

Restrictions on the use of synthetic products are increasing in all wine-growing areas, for environmental protection and to limit the onset of resistant pathogen and pest strains. Application of natural compounds for management of grey mould and grapevine moth would contribute to improved production health and safety and reduce potential environmental impacts of pesticides.

Zeolites are tectosilicates and consist of a mineral family with 52 mineral species (Passaglia and Sheppard, 2001; Jha and Singh, 2016). Natural zeolites have alumino-silicate frameworks whose structures contain cavities and channels filled with water and exchangeable cations. The compensating cations may move out from the crystal structures and be replaced by other cations possessing the same positive charge (Cation Exchange Capacity; CEC). In addition, water in the channels may be removed heating from 25 to 250°C. After cooling to room temperature, water can be naturally restored (reversible dehydration). Bish and Ming (2001) and Eroglu (2014) have summarized information on zeolite crystal structure, composition, occurrence, properties and applications. For each application pure zeolites are not employed, but zeolite-rich rocks ("zeolitites") are used, and these have high contents (> 50%) of pure zeolite. The most common zeolite species in zeolitic rocks are clinoptilolite, common in many parts of Europe, and chabasite and phillipsite, which are common in different regions of Italy (Eroglu, 2014).

The effectiveness of zeolitic rocks for practical applications depends both on zeolite type and concentration. Italian chabasite-rich zeolitites are well known for their high performance for several applications (Passaglia and Sheppard, 2001). The agricultural potential of zeolites, due to their CEC (ranging from 100 to 300 meq/100g), hydration properties and adsorption capacity, has been assessed for different applications. For example, these minerals are used as soil conditioners to improve physical and chemical properties of soil, and reduce leaching of NO_3^- and $(PO_4)^{3-}$ from fertilizers, as primary sources of groundwater pollution (Malekian *et al*., 2011). Zeolite applications to soils can act as slow release fertilizers and increase water holding capacity, improving water and nutrient use efficiency (Nakhli *et al*., 2017). These minerals can also be raw materials for plant substrates,

in particular as greenhouse media for the production of vegetables or as rooting media for cuttings of fruit and ornamental plants (Pond and Mumpton, 1985).

Zeolitites have not been significantly investigated for the control of plant diseases (De Smedt *et al*., 2015), but several studies have been carried out to investigate these materials for control of harmful insects (Kljajić *et al*., 2010; De Smedt *et al*., 2016; Rumbos *et al*., 2016; Floros *et al*., 2017). To our knowledge, there is no reported research on zeolitites for control of *B. cinerea* and *L. botrana* on grapevine.

Given the peculiar water sorbent capacity of zeolitites, due to their characteristic three-dimensional structures, the aim of the present study was to verify the effectiveness of a natural Italian chabasite-rich zeolitite, sprayed onto grapevines, for effects on grey mould and grapevine sour rot. These diseases are particularly harmful in high rainfall seasons. Sour rot is difficult to control (Hall *et al*., 2018), and is characterized by a typical smell due to the formation of ethyl acetate and acetic acid on infected grapevine bunches. This disease is caused by a complex of filamentous fungi, saprophytic yeasts, and bacteria (Steel *et al*., 2013). Given the positive results for zeolitites activity against insects, we also investigated their activity against grapevine moth. The study also analysed berries and wines from cv. Montepulciano vineyards to assess variations in composition, comparing the treatments of chabasite-rich zeolitites with those of synthetic fungicides and insecticides.

MATERIALS AND METHODS

Vineyard trial

Field trials were carried out in 2015 and 2016 in a vineyard located in Ari (Chieti province), and in 2017 in a vineyard located in Città Sant'Angelo (Pescara province), in the Abruzzo region of central Italy. The vineyard in Ari was established in 2001 and with the vines trained to the bilateral Guyot system, and with vine spacings of 2.5 × 1 m. The vineyard contained Montepulciano and Cococciola cultivars, each of approx. 5000 m², and both cultivars were on rootstock 1103 Paulsen. The vineyard in Città Sant'Angelo (CSA) was approx. 10,000 m² of cv. Montepulciano grafted on rootstock SO4, was established in 1989, and was trained to the Tendone system with vine spacings of 2.5 × 2.5 m. Both vineyards were located in grape growing areas usually subjected to epidemics of grey mould and sour rot and infestations of grapevine moth.

In both vineyards and in each of the 3 years of trials, the same experimental protocol was adopted. In each of the cultivars in the Ari vineyard, and in the CSA vineyard, trials were established to compare four treatments against grey mould with putative activity against sour rot, or three treatments against grapevine moth (Tables 1 and 2). Each treatment consisted of three replicates, each consisting of a plot of 162 m² in the Ari vineyard and 405 m² in CSA. Replicates were set up in randomized block designs.

In each of the three trials, an application strategy based on Italian chabasite-rich zeolitites (ICZ), (Agrisana s.r.l.) against grey mould, sour rot and grapevine moth, was compared with a strategy based on synthetic fungicides and insecticides (SYNT). A further treatment against grey mould and sour rot (SYNT/NAT) consisted of applications of the synthetic fungicides at pre-bunch closure followed by applications with zeolitites. In all the trials, untreated treatments were used as experimental controls (Tables 1 and 2). The zeolitites mineralogical composition (percent) was: Chabasite, 68.5 ± 0.9; Phillipsite, 1.8 ± 0.4; Analcime, 0.6 ± 0.3; K-feldspar, 9.7 ± 0.7; Mica, 5.3 ± 0.6; Pyroxene, 2.9 ± 0.4; and Volcanic glass, 11.2 ± 1.0. These zeolitites, collected from quarries in Sorano (GR), central Italy, were chosen for their high content in chabasite, and had high values of CEC (2.17 meq/g) and water retention (48.5 % p/p) (Malferrari *et al*., 2013).

Applications targeting grey mould were carried out before the occurrence of conditions favorable for infection, according to weather forecasts (Tables 1, 3 and 5). Only applications at pre-bunch closure were made according to the phenological growth stage and regardless of weather conditions. The pre-infection strategy was adopted taking into account the putative adhesion, persistence and rain resistance of zeolitites. In this way the preventive activity of the synthetic products was exploited, following current control guidelines.

In the Ari vineyard, rainfall, temperature and leaf wetness duration were recorded using an agrometeorological station (DigitEco s.r.l.), which was placed at the centre of the vineyard between the two cultivars (Table 3). In the CSA vineyard, meteorological data were obtained from a control unit (Hydrographic Service - Abruzzo Region) located about 6 km from the vineyard (Table 5). The meteorological data were used to verify the amount of rainfall and leaf wetness associated with the occurrence of infections.

The applications against *L. botrana* were carried out after the insect flight peak recorded in each vineyard from four pheromone traps, and after assessment of damaged berries (Table 4).

In all vineyards, at the berry pea-size growth stage, leaf removal at the bunch zone was carried out.

Table 1. Field applications against grey mould, sour rot and grapevine moth on cv. Montepulciano and Cococciola in the Ari vineyard in 2015 and 2016.

Application date	Growth stage	Treatment	Active ingredients	Application rate (kg/L ha^{-1})
30/06/2015	73	1 - SYNT	Spinosad	0.15
		2 - ICZ	Chabasite-rich zeolitite	15
		3 - SYNT/NAT	Chabasite-rich zeolitite	15
		4 - Untreated Control	Nil	-
24/07/2015	77	1 - SYNT	Cyprodinil + Fludioxonil	0.8
		2 - ICZ	Chabasite-rich zeolitite	15
		3 - SYNT/NAT	Cyprodinil + Fludioxonil	0.8
		4 - Untreated Control	Nil	-
06/08/2015	77	1 - SYNT	Pyrimethanil	2.5
		2 - ICZ	Chabasite-rich zeolitite	15
		3 - SYNT/NAT	Chabasite-rich zeolitite	15
		4 - Untreated Control	Nil	-
14/08/2015	83	1 - SYNT	Pyrimethanil + Chlorantaniliprole	2.5 + 0.27
		2 - ICZ	Chabasite-rich zeolitite	15
		3 - SYNT/NAT	Chabasite-rich zeolitite	15
		4 - Untreated Control	Nil	-
03/09/2015	85	1 - SYNT	Cyprodinil + Fludioxonil	0.8
		2 - ICZ	Chabasite-rich zeolitite	15
		3 - SYNT/NAT	Chabasite-rich zeolitite	15
		4 - Untreated Control	Nil	-
14/07/2016	75	1 - SYNT	Pyrimethanil + Spinosad	2.5 + 0.15
		2 - ICZ	Chabasite-rich zeolitite	15
		3 - SYNT/NAT	Chabasite-rich zeolitite	15
		4 - Untreated Control	Nil	-
28/07/2016	77	1 - SYNT	Cyprodinil + Fludioxonil	0.8
		2 - ICZ	Chabasite-rich zeolitite	15
		3 - SYNT/NAT	Cyprodinil + Fludioxonil	0.8
		4 - Untreated Control	Nil	-
04/09/2016	85	1 - SYNT	Cyprodinil + Fludioxonil	0.8
		2 - ICZ	Chabasite-rich zeolitite	15
		3 - SYNT/NAT	Chabasite-rich zeolitite	15
		4 - Untreated Control	Nil	-

All pesticide applications were carried out using pneumatic sprayers, with application volume of 500 L ha^{-1}.

Disease and insect assessments

The assessments of activity against grey mould were carried out in the Ari vineyard on October 2, 2015 and October 3, 2016, close to harvesting, which was done on October 4, 2015 and October 5, 2016. In the CSA vineyard the assessments were carried out on 29 September and 11 October, 2017. For each replicate of each treatment, 60 bunches located in the central rows of the vineyard plots were considered. The percentage of infected bunches (incidence), and the percentage of infected berries (severity), were determined.

The assessments of activity against the grapevine moth were carried out on equal numbers of bunches as follows: in the Ari vineyard, on July 14, 2015 and August 4, 2016 (2nd generation infestation), and on September 10, 2015 and September 30, 2016 (3nd generation infestation); in the CSA vineyard on July 30, 2017 (2nd generation infestation) and September 29, 2017 (3nd generation infestation). The percentage of damaged bunches (incidence), and the percentage of damaged berries (severity) were determined.

The activity of the synthetic fungicides and zeolitite applications on sour rot were assessed, in Ari and CSA

Table 2. Field applications against grey mould, sour rot and grapevine moth on cv. Montepulciano in the CSA vineyard in 2017.

Application date	Growth stage	Treatment	Active ingredients	Application rate (kg/L ha^{-1})
12/07/2017	75	1 - SYNT	Pyrimethanil	2.5
		2 - ICZ	Chabasite-rich zeolitite	15
		3 - SYNT/NAT	Chabasite-rich zeolitite	15
		4 - Untreated Control	Nil	-
23/07/2017	77	1 - SYNT	Cyprodinil + Fludioxonil	0.8
		2 - ICZ	Chabasite-rich zeolitite	15
		3 - SYNT/NAT	(Cyprodinil + Fludioxonil)	0.8
		4 - Untreated Control	Nil	-
09/09/2017	83	1 - SYNT	Pyrimethanil	2.5
		2 - ICZ	Chabasite-rich zeolitite	15
		3 - SYNT/NAT	Chabasite-rich zeolitite	15
		4 - Untreated Control	Nil	-
18/09/2017	85	1 - SYNT	Cyprodinil + Fludioxonil	0.8
		2 - ICZ	Chabasite-rich zeolitite	15
		3 - SYNT/NAT	Chabasite-rich zeolitite	15
		4 - Untreated Control	Nil	-

vineyards, at the same time as the grey mould assessments, calculating the percentages of infected bunches (incidence) and infected berries (severity).

Evaluations of effects of chabasite-rich zeolitites applications on grape yields

Field applications

In the CSA vineyard in 2017, the ICZ treatment plots were first examined for the activity of the applications and then further treated with zeolitites. Each of the three plots (replicates) of the ICZ treatment was subdivided into two 202 m² sub-plots. The six sub-plots were treated with zeolitites on 29 September, 15 d before harvest. Three of the sub-plots were not subjected to further applications (ICZ-A treatment). The other three sub-plots were further treated with zeolitites on 11 October, 2 d before grape harvest, which was carried out on 13 October (ICZ-B treatment).

Evaluation of yield and vinification

At harvest, in each of the sub-plots treated on 29 September (IZS-A treatment), or treated on 29 September and 11 October (IZS-B treatment), and in each of the three plots of the SYNT treatment, 12 plants were selected from central rows of each plot or sub-plot (four plants per row), and the grape yields were determined. Berries (100) from each of the plots or sub-plots, alternately taken from the wings, tips and centres of clusters located at about the mid-point along the vine-shoots, were collected. These berry samples were analysed for soluble solids (Brix), pH and total acidity, according to the methods of the Official Gazette of the European Communities. Regulation (EEC) No. 2676/90 (Official Journal L 272, 3.10.1990) (Table 6). A 100 kg sample from 12 plants of each of the three plots (SYNT treatment) or sub-plots (IZS-A and IZS-B treatments), was then vinified (three repetitions per treatment).

Each sample was crushed with a stalk-remover grape crusher. Approximately 93 kg of crushed grapes were obtained from each 100 kg sample. A traditional vinification for red wine was performed, with the cap of skins punched down three times per day. Thirty mg L^{-1} of sulfur dioxide, 20 g hL^{-1} of commercial dry yeast (after rehydration) and 20 g hL^{-1} of fermentation activators (diammonium phosphate + thiamine hydrochloride) were added at the beginning and again at half of the fermentation process. The fermentation ended after 15 d, the samples reached maximum temperature of 30°C during this period. Pressings were carried out to separate the solids after about 15 d, and the wines were decanted. At the end of this process, and for each sample, approx. 70 L of wine was obtained.

Chemical analyses of the wines

The wine obtained was analyzed at 3 months from racking to determine concentrations of the parameters

Table 3. Rainfall, temperature and leaf wetness periods on rainy days at the ARI vineyard in 2015 and 2016.

Date	Maximum temperature (°C)	Minimum temperature (°C)	Rainfall (mm d^{-1})	Leaf wetness (h d^{-1})
20/06/2015	20.3	13.2	4.2	3
24/06/2015	22.1	14.1	30.5	16
07/08/2015	25.1	20.6	75.1	10
15/08/2015	24.4	19.4	1.1	2
16/08/2015	23.6	17.6	40.3	16
04/09/2015	21.1	16.3	13.2	20
08/09/2015	26.2	19.2	0.5	2
19/06/2016	17.4	12.5	15.2	10
20/06/2016	18.3	12.3	0.6	2
23/06/2016	24.7	18.8	1.4	2
06/07/2016	23.7	20.0	0.8	3
09/07/2016	27.8	22.1	1.6	1
15/07/2016	14.6	12.8	100.8	19
16/07/2016	14.1	12.8	47.6	21
01/08/2016	24.7	18.8	14.2	7
23/08/2016	20.5	16.4	0.4	4
31/08/2016	21.6	18.8	1.2	8
05/09/2016	23.7	16.3	2.6	6
06/09/2016	17.2	12.6	22.6	12
07/09/2016	16.2	14.0	10.6	22
08/09/2016	19.2	14.4	0.2	9
09/09/2016	21.2	17.7	1.0	3
10/09/2016	21.2	17.6	0.4	3
11/09/2016	19.5	16.9	6.0	10
12/09/2016	20.7	17.1	0.8	7
16/09/2016	21.3	17.8	7.2	4
18/09/2016	18.8	15.4	0.6	3
19/09/2016	16.3	13.2	8.4	6
21/09/2016	17.2	14.9	2.0	5

Table 4. Numbers of grapevine moth adults captured in traps in two grape cultivars in the Ari vineyard in 2015 and 2016.

Survey data	Cv. Montepulciano		Cv. Cococciola	
	Trap A Capture number	Trap B Capture number	Trap A Capture number	Trap B Capture number
15/06/2015	15	12	11	15
22/06/2015	137	139	146	29
30/06/2015	18	33	77	14
07/07/2015	7	15	5	5
14/07/2015	1	1	4	2
21/07/2015	2	3	2	2
29/07/2015	5	8	4	0
05/08/2015	18	11	6	3
12/08/2015	35	36	44	26
18/08/2015	18	11	27	8
26/08/2015	11	8	8	4
02/09/2015	6	4	3	2
10/09/2015	2	2	0	0
18/09/2015	0	2	2	0
25/09/2015	1	2	1	1
01/10/2015	4	5	0	1
14/06/2016	0	0	0	0
21/06/2016	0	0	0	0
28/06/2016	0	0	0	0
05/07/2016	8	7	8	6
12/07/2016	47	45	33	35
19/07/2016	11	12	15	18
26/07/2016	7	6	8	8
02/08/2016	0	0	0	0
09/08/2016	0	0	0	0
16/08/2016	0	0	0	0
24/08/2016	3	4	2	3
30/08/2016	0	0	0	0
07/09/2016	0	1	0	1
14/09/2016	1	0	0	0
21/09/2016	0	0	0	0
28/09/2016	0	0	0	0

reported in Table 6. Analyses were carried out in accordance with the methods outlined in the official Gazette of the European Community, Regulation (EEC) No. 2676/90 (Official Journal L 272, 3.10.1990).

HPLC Analyses

Determination of anthocyanins in red wine samples, filtered through 0.45 µm nylon filters, was carried out according to OIV-MA-AS 315-11 Method (2007). Thirty microliters of each sample was analysed by HPLC. The chromatographic system was an HPLC Waters Alliance equipped with a Waters 2695 separation module connected to a Waters 2996 photodiode array detector. Separation of analytes was carried out using a Supelcosil LC18 column (5 µm particle size, 250 × 4.6 mm I.D.), as described in the OIV Method. The system was controlled by Waters Empower personal computer software. Identification of the anthocyanins, detected at wavelength 520 nm, was based on retention times. For the calibration curves, i.e. peak area versus concentration, malvidin-3-glucoside chloride (Sigma-Aldrich) was used, resulting in the linear range of concentration between 10 and 100 mg L^{-1}. The calculated regression line was used to compute the amount of each analyte in the samples by interpolation, using the external standard method.

Table 5. Rainfall, temperature and leaf wetness periods on rainy days of the CSA vineyard in 2017.

Date	Maximum temperature (°C)	Minimum temperature (°C)	Rainfall (mm d^{-1})	Leaf wetness (h d^{-1})
17/06/2017	27.1	20.1	4.8	8
28/06/2017	30.3	20.2	1.8	3
14/07/2017	30.2	22.2	39.8	10
24/07/2017	35.4	22.2	2.2	2
25/07/2017	29.4	20.3	13.6	22
10/09/2017	26.1	17.4	18.6	8
11/09/2017	24.5	18.3	21.6	18
19/09/2017	23.4	15.2	12.4	11
20/09/2017	16.3	12.1	10.1	10
24/09/2017	23.1	14.3	11.2	9
06/10/2017	13.3	10.1	11.6	9
07/10/2017	14.2	10.2	10.4	8

Statistical analyses

Statistical analyses were performed with the Tukey's honest significant difference (HSD) test, (P = 0.05), comparing each treatment, in each vineyard and year of trial, for the data of incidence and severity of grey mould, sour rot and grapevine moth. Tukey's honest significant difference (HSD) test, (P = 0.05) was also used to compare yield and wine data from each treatment.

RESULTS

Vineyard trial

Grey mould

In 2015, in the untreated controls for cv. Montepulciano in the Ari vineyard, mean grey mould incidence was 18.3% of the bunches, and mean severity was 1.2%. In the treated plants, mean incidence was from 0 to 1.1%, and mean severity was from 0 to 0.05%. Mean incidence and severity in treated plants were statistically different compared to the untreated control plants, but not between the different treatments (Table 7).

In 2015, in the cv. Cococciola in the Ari vineyard, mean incidence of *B. cinerea* infections in bunches of the untreated controls was 27.2% and mean severity was 1.4%. Both of these values were statistically different compared with those for the treated plants, which showed mean incidence from 0 to 1.7%, and mean severity from 0 to 0.06% (Table 7). As for cv. Montepulciano, no statistically significant differences were detected for the ICZ, ICZ/SYNT and SYNT treated plants (Table 7).

In 2016, in the Ari vineyard, infections were detected in the untreated controls of both cultivars, with mean incidence of 16.7% and mean severity of 0.4% in cv. Montepulciano, and of 56.1 and 3.7% in cv. Cococciola. Also in this case, all the treated plants had significantly reduced infections compared to the untreated controls, without significant differences of activity among treatments (Table 7).

In 2017, in the CSA Vineyard, no infections by *B. cinerea* were detected.

Sour rot

In 2015 in the Ari vineyard, the untreated controls of cv. Montepulciano had mean incidence of sour rot of 37.2% and mean severity of 6.0% (Table 7). In cv. Montepulciano, no sour rot was detected from the SYNT treatment which was statistically less than for the other treated plants, and the untreated control (Table 7). The ICZ and ICZ/SYNT treatments gave similar mean sour rot incidence (respectively, 7.8 and 7.2%) and mean sour rot severity (both at 0.7%). These values were statistically

Table 6. Chemical analyses of cv. Montepulciano berries and wines obtained from vines treated with either Italian chabasite-rich zeolitites or synthetic fungicides and insecticides for control of grey mould, sour rot and grapevine moth.

Parameter	Unit of measurement	Sample	Method of analysis
Total polyphenols *	mg L^{-1}	Wines	Folin-Ciocalteau
Total anthocyanins	mg L^{-1}	Wines	Spectrophotometric
Total acidity	g L^{-1}	Berries and wines	Acid/base titration
Soluble solids	° Brix	Berries	Fheling
pH 20°C	-	Berries and wines	Potentiometric
Ethyl alcohol	% vol.	Wines	Distillation
O.D. 420-520-620 (IC_3)	-	Wines	Spectrophotometric
Anthocyanins	mg L^{-1}	Wines	HPLC

* Total polyphenols expressed as gallic acid equivalents.

Table 7. Mean incidence and severity of different control strategies for control of grey mould and sour rot based on applications of Italian chabasite-rich zeolitites (ICZ), synthetic fungicides (SYNT) or zeolitites replaced by synthetic fungicide at pre-bunch closure (SYNT/NAT), in the cv. Montepulciano and Cococciola in the Ari vineyard.

Survey	Treatment	Grey mould				Sour rot			
		Cv. Montepulciano		Cv. Cococciola		Cv. Montepulciano		Cv. Cococciola	
		Incidence[a] (%)	Severity[b] (%)	Incidence (%)	Severity (%)	Incidence (%)	Severity (%)	Incidence (%)	Severity (%)
02/10/2015	1 – SYNT	0.00 a[c]	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a
	2 - ICZ	1.11 a	0.05 a	1.67 a	0.05 a	7.78 b	0.74 b	1.11 a	0.07 a
	3 - SYNT/NAT	0.00 a	0.00 a	1.67 a	0.06 a	7.22 b	0.65 b	0.56 a	0.05 a
	4 - untreated control	18.33 b	1.19 b	27.22 b	1.37 b	37.22 c	5.99 c	8.33 b	0.71 b
03/10/2016	1 – SYNT	0.56 a	0.01 a	18.89 a	0.76 a	18.33 a	0.34 a	1.67 a	0.05 a
	2 - ICZ	1.11 a	0.01 a	20.56 a	0.90 a	18.33 a	0.41 a	2.22 a	0.04 a
	3 - SYNT/NAT	0.56 a	0.01 a	18.89 a	0.75 a	23.33 a	0.59 a	2.22 a	0.06 a
	4 - untreated control	16.67 b	0.44 b	56.11 b	3.74 b	55.56 b	2.49 b	10.00 b	0.29 b

[a] Incidence = percentage of infected bunches on the total number of bunches.
[b] Severity = percentage of infected berries on the total number of berries.
[c] Statistical analyses were performed according to Tukey's honest significant difference (HSD) test.
Different letters indicate significant differences ($P = 0.05$).

different compared to those from the untreated controls (Table 7). In cv. Cococciola, all the treatments gave similar and significantly lower values than the controls, in which mean incidence of sour rot was 8.3% and mean severity was 0.7% (Table 7).

In 2016, in the cv. Montepulciano at the Ari vineyard, mean incidence of sour rot in the untreated controls was 55.6% and mean severity was 2.5%. These were significantly greater than those from the other treatments, which did not differ significantly from each other (Table 7). In the untreated controls of cv. Cococciola at the Ari vineyard, mean sour rot incidence was 10.0% and severity was 0.3%. The other treatments gave mean incidence ranging from 1.7% to 2.2% and mean severity from 0.04 to 0.06% (Table 7).

In 2017, in the CSA vineyard, no sour rot was detected.

Grapevine moth

In 2015, after the flight peak of the second generation of *L. botrana* recorded on June 22, both cv. Montepulciano and Cococciola in the Ari vineyard were treated (Tables 1 and 4). In the assessment carried out on July 14, the untreated controls of cv. Montepulciano showed mean incidence of infestation of 33.3% and mean severity of 0.7% (Table 8). In the untreated controls of the cv. Cococciola, mean incidence of infestation was 45.0% and mean severity was 1.2% (Table 8).

Spinosad applied in the SYNT treatment at both vineyards gave complete control of the infestations in cv. Montepulciano, and low levels of infestation in cv. Cococciola at mean incidence of 2.2% and mean severity of 0.03% (Table 8).

The zeolitite applications decreased the grapevine moth infestations in both cultivars, to a lesser extent than in the SYNT treatment, but statistically different compared to the untreated controls (Table 8). In the ICZ treatment for cv. Montepulciano, mean incidence of infestation was 3.9% and mean severity was 0.05%. For cv. Cococciola mean incidence of infestation was 11.1% and mean severity was 0.17% (Table 8).

In 2015, applications with chlorantaniliprole were carried out 2 d after the grapevine moth flight peak of third generation adults recorded in both cultivars on 12 August (Tables 1 and 4). The mean incidence and severity of infestations (assessed on 10 September) in the untreated controls of cv. Montepulciano were, respectively, 8.3 and 0.19%, and for cv. Cococciola were, respectively, 27.8 and 0.7% (Table 8). The treatments with chlorantaniliprole and zeolitites, carried out in the SYNT and ICZ treatments, gave similar reductions of moth infestation, which were significantly different compared to the controls (Table 8).

In 2016, single applications of Spinosad in the SYNT treatment and zeolitites in the ICZ treatment were carried out in the Ari vineyard, on 14 July. These were against the second generation of *L. botrana*, after the peak of flights registered on 12 July (Tables 1 and 4).

Table 8. Activity of Italian chabasite-rich zeolitites (ICZ) and synthetic insecticides (SYNT) on grapevine moth bunch infestations in the cv. Montepulciano and Cococciola in the Ari vineyard in the surveys carried out at the 2nd moth generation: 14/07/2015 and 04/08/2016, and at the 3nd moth generation on 10/09/2015.

Treatment	14/07/2015		10/09/2015		04/08/2016	
	Incidence [a] (%)	Severity [b] (%)	Incidence (%)	Severity (%)	Incidence (%)	Severity (%)
Cv. Montepulciano						
1 – SYNT	0.00 a [c]	0.00 a	2.22 a	0.03 a	0.00 a	0.00 a
2 - ICZ	3.89 b	0.05 b	1.11 a	0.02 a	1.67 a	0.02 a
3 - untreated control	33.33 c	0.71 c	8.33 b	0.19 b	16.67 b	0.28 b
Cv. Cococciola						
1 – SYNT	2.22 a	0.03 a	1.67 a	0.03 a	2.22 a	0.03 a
2 - ICZ	11.11 b	0.17 b	6.11 a	0.11 a	4.44 a	0.07 a
3 - untreated control	45.00 c	1.17 c	27.78 b	0.68 b	15.00 b	0.28 b

[a], [b], [c], see Table 7.

Spinosad and zeolitite applications both significantly reduced the moth infestations compared to the controls (Table 8). In the untreated control, mean incidence of infestation was 16.7% in cv. Montepulciano and 15.0% in cv. Cococciola, while mean severity was 0.3% in both cultivars (Table 8). In the SYNT and ICZ treatments the mean incidence of infestation ranged from 0 to 4.4%, and mean severity from 0 to 0.07% (Table 8).

The low number of captures of third generation moth adults did not require further treatment applications in 2016 in the Ari vineyard (Table 4). During the assessment of 30 September, there was no additional damage compared to second generation moths.

In 2017, in the CSA vineyard and throughout the growing season, no insecticide applications were carried out because no moths were captured, or because there was only sporadic *L. botrana* presence (1–2 moths per trap). During the 30 July and 29 September assessments, no grapevine moth damage was found in the grapes.

Evaluations of effects of chabasite-rich zeolitite applications on yields

At harvest, no differences among treatments were detected in grape yield per plant (Table 9). Furthermore, compared to the SYNT treatment, the treatments with zeolitites (ICZ-A, ICZ-B) had no effects on accumulation of soluble solids or acidic balance of the berries.

The chemical composition of the wines obtained after 3 months of ageing is outlined in Table 10. Although the parameters of alcohol content, pH and total acidity did not vary among the treatments, the treatments gave statistically significant differences for concentrations of polyphenols and anthocyanins, and in wine colour intensity. Polyphenol content was significantly greater from the ICZ-A treatment (mean = 3213 mg L^{-1}) compared to SYNT (mean = 2922 mg L^{-1}), whereas the ICZ-B treatment gave the least amount (mean = 2475 mg L^{-1}). Concentrations of total anthocyanins in the three wines were also statistically different. The ICZ-A treatment gave greater anthocyanin content (mean = 625 mg L^{-1}) compared to the SYNT treatment (mean = 579 mg L^{-1}) and the ICZ-B treatment (mean = 486 mg L^{-1}). The concentration of anthocyanins affected the intensity of colour (IC_3), which resulted in an increased value from the ICZ-A compared to the SYNT treatment, while the ICZ-B treatment gave greater colour discharge (Table 10).

The composition of total anthocyanin fractions was determined, and the percentage of each glycosylated anthocyanin and the sum of acylated forms are outlined in Table 11. Montepulciano wines had characteristic anthocyanin profiles, with low amounts cyanidin gluco-

Table 9. Mean grape yields and grape composition parameters recorded in 2017 at harvest, for cv. Montepulciano vines in the CSA vineyard, treated with Italian chabasite-rich zeolitites (ICZ-A and ICZ-B) or synthetic products (SYNT).

Treatment	Yield (Kg $vine^{-1}$)	Soluble solids (° Brix)	pH	Total acidity (g L^{-1})
ICZ-A	10.0 a [a]	26.5 a	3.61 a	6.10 a
ICZ-B	9.8 a	26.5 a	3.61 a	6.10 a
SYNT	9.5 a	26.9 a	3.63 a	6.03 a

[a] Statistical analyses were performed according to Tukey's honest significant difference (HSD) test.
Different letters indicate significant differences ($P = 0.05$).

Table 10. Mean chemical composition values for Montepulciano d'Abruzzo wines obtained from cv. Montepulciano vines in the CSA vineyard, treated in 2017 with Italian chabasite-rich zeolitites (ICZ-A and ICZ-B) or synthetic products (SYNT), after 3 months of ageing.

Treatment	Ethyl alcohol (% vol.)	pH	Total acidity (g L^{-1})	Total polyphenols (mg L^{-1})	Total anthocyanins (mg L^{-1})	IC_3
ICZ-A	16.1 a[a]	3.65 a	5.93 a	3213 a	625 a	15.1 a
ICZ-B	16.1 a	3.67 a	5.93 a	2475 c	486 c	10.1 c
SYNT	16.3 a	3.67 a	6.03 a	2922 b	579 b	12.6 b

[a] See Table 9.

Table 11. Mean composition (%) of total anthocyanins in Montepulciano d'Abruzzo wines obtained from cv. Montepulciano vines in the CSA vineyard, treated in 2017 with Italian chabasite-rich zeolitites (ICZ-A and ICZ-B) or synthetic products (SYNT), after 3 months of ageing. Montepulciano d'Abruzzo total anthocyanins included glycosylate and acylated forms (sum of acetate and coumarate fractions).

Treatment	Delphinidin 3-G	Cyanidin 3-G	Petunidin 3-G	Peonidin 3-G	Malvidin 3-G	Acylated anthocyanins
ICZ-A	5.4 a[a]	0.5 a	9.3 a	9.6 a	58.0 a	17.6 a
ICZ-B	4.6 a	0.5 a	10.4 a	8.8 a	62.9 a	16.1 a
SYNT	4.6 a	0.5 a	8.8 a	9.0 a	60.2 a	17.4 a

[a] See Table 9.

side. The percentages of total glycosilated anthocyanins (delphinidin, cyanidin, petunidin, peonidin and malvidin), and of acylated anthocyanins (sum of acetates and coumarates) did not significantly differ among the different treatments. In particular, the glycosylated fractions were predominant, and malvidin 3-G was the major constituent in wine solution after 3 months of ageing.

DISCUSSION

Increasing awareness of the impacts of plant protection on the environment has led to restrictions on the use of synthetic pesticide products, with increasing research of predisposing epidemiological factors to diseases (Calzarano *et al*., 2018), and there has been increased research on environmentally-friendly products for disease and pest management (Calzarano and Di Marco, 2018). In the present study, Italian natural chabasite-rich zeolitites were evaluated as environmentally-friendly materials for management of grapevine grey mould, sour rot and grapevine moth.

In both years of trials carried out in the Ari vineyard, applications of zeolitites gave activity for grey mould control, which was comparable to that from synthetic fungicides. This activity was verified for applications of zeolitites against *B. cinerea* at all growth stages except the pre-bunch closure growth stage (Pearson and Goheen, 1988), and in the control strategy based only on applications of zeolitites.

The control strategies for *B. cinerea* were effective also against sour rot. The side activity of the anti-*Botrytis* fungicide mixture cyprodinil + fludioxonil against the sour rot is well known (Adaskaveg *et al*., 2011), but was greater than that from the zeolitite treatments only in 2015 in the cv. Montepulciano grapes.

However, the reductions of sour rot from the different control strategies was considerable in the presence of moderate infections, and to a lesser extent when the incidence of the disease was severe, as in 2016 in the cv. Montepulciano grapes.

The cv. Montepulciano and Cococciola have different susceptibilities to grey mould and sour rot. Severe infections of sour rot in cv. Montepulciano corresponded to minor grey mould infections. Similarly in cv. Cococciola, bunches heavily infected with *B. cinerea* were less affected by sour rot.

The activity of zeolitites against *L. botrana* was high, and was comparable to that of synthetic insecticides where mild infestations of the moth occurred. Zeolitites also gave good control of heavy infestations. This was for the second moth generation in 2015 in both cultivars in the Ari vineyard, but was less than that achieved from synthetic insecticides.

The lack of grey mould and sour rot in 2017 in the CSA vineyard could be ascribed to the dry weather conditions of that year, characterized by high temperatures

and low relative humidity. Resistance to diseases in cv. Montepulciano clone could also result from the greater thickness of berry cuticles in this cultivar compared with others, despite the occurrence of rainfall predisposing to infections (Rogiers *et al*., 2005; Mundy, 2008). The absence of grapevine moth infestations in 2017 in the CSA vineyard may have also resulted from the high temperatures that occurred (Moosavi *et al*., 2017).

The good efficacy of the applications of zeolitites towards grey mould and sour rot could attributed to the physico-chemical properties of these materials (Bish and Ming, 2001; De Smedt *et al*. 2015). Water adsorption capacity and the resulting reduction in moisture in zeolitite-treated bunches could have caused the decreases in grey mould and sour rot (Ng and Mintova, 2008; Tatlier *et al*. 2018).

Applications of zeolitites lead to the formation of microscopic layers of mineral particles (Glenn and Puterka, 2005). High hydrophilicity of these layers of chabasite-rich zeolitites results in the absorption of condensing water and elimination of free water (Tatlier *et al*., 2018). Direct contact of disease inoculum with host tissues could be reduced by a physical barrier developed on the treated plant surfaces. This barrier can also reduce spore germination and prevent or hinder microbial growth (Walters, 2006).

Studies on the activity of zeolitites against grapevine moth are lacking, but the insecticidal potential of zeolitite formulations have been evaluated for control of others insect pests. These include stored-grain insects (Kljajić *et al*., 2010; Rumbos *et al*., 2016; Kavallieratos *et al*., 2018), *Tuta absoluta* on tomato (De Smedt *et al*., 2016) and bean weevil (Floros *et al*., 2017). These studies have demonstrated that zeolitites induced high adult and progeny mortality. Investigations on mechanisms of action towards different harmful insects have also been carried out for other mineral powders similar to zeolitites, such as kaolin, a clay mineral composed of aluminosilicate.

As in the case of kaolin, the epicuticles of insect larvae may be damaged by the non-sorptive particles of zeolitites for abrasion and by adsorption of epicuticular lipids to sorptive particles. Loss of water from the insect bodies leads to the death from desiccation (Ebeling, 1971; Glenn *et al*., 1999). This specific activity has recently been observed from applying zeolitites to Pharaoh ants (Van Den Noortgate *et al*., 2018). Plants treated with kaolin clay showed a low oviposition rates by different insects. Furthermore, hatch rate of eggs and larval development significantly decreased from these treatments, causing a high insect mortality (Puterka *et al*., 2000; Knight *et al*., 2000).

The larvae on which the mineral particles adhere can also die from starvation, because they are subject to tactile deterrence leading to inability to feed (Larenzaki *et al*., 2008). Deposition of the particle films on the treated plants can also reduce the visual cues for insects, and hinder recognition of plant parts on which the adults lay their eggs (Glenn *et al*., 1999; Mazor and Erez, 2004). The possible increases in infestations of insects not directly affected by kaolin applications (Knight *et al*., 2001; Markò *et al*., 2008) were not detected in the present study.

Zeolitite activity for control of grey mould, sour rot and grapevine moth has been linked with the adhesion and persistence of the mineral deposits on the treated grapes, with increased resistance to rainfall wash-off of the mineral. This characteristic is associated with the effectiveness of preventive control strategies against grey mould, based on applications carried out just before expected rainfall.

The use of zeolitites has also proved to be effective for integrated pest management for *L. botrana*. Applications were carried out immediately after the peak of adult insect flights, assessed as presence of penetration holes on grape berries (Amo-Salas *et al*., 2011).

One of the most interesting results from the present study is the possibility of using zeolitites to simultaneously control grey mould, sour rot and grapevine moth. This could provide a clear economic advantage for viticulturists, and for the environment, in a combined disease and pest management strategy that has not been previously suggested.

Increasing of temperatures, associated with climate change, could strongly influence the production of high quality grapes, affecting the grapevine development and phenology during growing seasons. It has been established, by focusing on phenolic compounds in black berry grapevine varieties, that high temperatures negatively affect colour intensity, resulting in poor colour wines (Buttrose *et al*., 1971; Kliewer and Torres, 1972; Mori *et al*., 2007). Recent studies on cv. Sangiovese showed that exposure of grape bunches to high temperatures lead to reductions in synthesis of anthocyanin, while anthocyanin degradation was stimulated (Movahed *et al*., 2016; Pastore *et al*., 2017a; Pastore *et al*., 2017b). Thus, in hot growing seasons as in 2017 in the CSA vineyard, the contents of phenolic compounds were low in all treatments compared to the usual amounts of these compounds in cv. Montepulciano grapes (Mattivi *et al*., 2006).

The timing of zeolitite applications to grapevine canopies influenced the concentrations of phenolic compounds in grape berries and wine, without affecting the percent-

age fraction of each individual anthocyanin. No differences resulted for alcohol contents and wine total acidity. When the last treatment with zeolitites was carried out no later than 15 d from harvest (treatment ICZ-A), the polyphenol concentrations increased and the wine developed increased colour intensity. This result is similar to that from foliar applications of kaolin, which is a radiation-reflecting inert mineral able to reduce leaf surface temperatures and influence major secondary metabolism pathways leading to biosynthesis of phenolic compounds and anthocyanins (Glenn and Puterka, 2005; Song *et al*., 2012; Conde *et al*., 2016). Dinis *et al*. (2016) showed that kaolin-treated grape berries had enhanced total amounts of phenols, flavonoids, anthocyanins and vitamin C compared with grapes from untreated vines. The timing of zeolitite applications was shown to be a crucial factor. Application close to grape harvest (the ICZ-B treatment) gave a residue of inert dust that effectively decreased the wine phenol contents, affecting anthocyanin concentration and colour intensity. This was probably due to the hydrophillic properties of zeolitites, determining the adsorption of target molecules (Perego *et al*., 2013; Mercurio *et al*., 2016; Lisanti *et al*., 2017). Recent studies have shown that natural and surfactant-modified zeolites are effective sorbents for removal of organic compounds such as phenol and 4-chlorophenol from aqueous solutions (Kuleyin, 2007; Yousef *et al*., 2011).

The low content of cyanidin glucoside associated with the high content of malvidin glucoside was attributed to the specific anthocyanin pattern of cv. Montepulciano (Mattivi *et al*., 2006), and to enzymatic conversion that occurred in berries during the late growing season (Versari *et al*., 1999).

Due to lack of interference of the grape composition, particularly for phenolic compounds, the use of zeolitites could further be encouraged.

The positive results obtained in this study need to be confirmed to fully assess the potential of zeolitites as environmentally-friendly control strategies for grapevine disease and pest management. Use of these compounds could also provide clear economic advantages for grape and wine production.

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Research Papers

New records of *Penicillium* and *Aspergillus* from withered grapes in Italy, and description of *Penicillium fructuariae-cellae* sp. nov.

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Summary. *Penicillium* and *Aspergillus* are common pathogenic fungi of grapes, that occur frequently on withered berries used in the Italian passito wine production. Members of these genera isolated from withered grapes were identified using molecular and morphological approaches. The isolates were examined by amplification of internal transcribed spacer region, β-tubulin, calmodulin and RNA polymerase II second largest subunit. *Penicillium bilaiae, Aspergillus pallidofulvus* and *A. puulaauensis* are reported for the first time from *Vitis vinifera.* Two *Penicillium* isolates showed a distinct phylogenetic position and different morphological characteristics from *P. bissettii* and *P. vasconiae*, the two most closely related species. These isolates are assigned to the new species *Penicillium fructuariae-cellae*, that is here described. An *in vitro* pathogenicity assay was carried out to evaluate the infectivity to grape berries by *Penicillium* and *Aspergillus* isolates recovered in this study. All examined isolates colonized the berries when artificially inoculated, but to a lesser extent than *Botrytis cinerea*. This suggests that these fungi may contribute, with other pathogenic species, to the onset of post-harvest diseases of grapes.

Keywords. Saprophytic/pathogenic fungi, grapes, phylogeny, taxonomy, post-harvest diseases.

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INTRODUCTION

Fungal contamination of grapes causes severe and economically important losses for world food and beverage industries. Table, wine and raisin grapes can be infected by several species of fungi, on grapevines and/or during post-harvest processes. Grapes for Italian passito wine production are particularly vulnerable to fungal infections, during withering carried out in fruit drying rooms (*fruttaio*) (off-vine withering) (Mencarelli and Tonutti, 2013).

Penicillium and *Aspergillus* spp. are among the most frequent saprophytic fungal pathogens on withered grapes (Torelli *et al*., 2006; Lorenzini *et al*., 2016; Stefanini *et al*., 2017). Their presence is very important since they are

causal agents of bunch rot and can be mycotoxin producers (Torelli *et al*., 2006; Somma *et al*., 2012). In addition, grape contamination by these fungi can lower the quality of the resulting wines. The detrimental effects of withered grapes infected by *P. expansum* and *P. crustosum* on the quality of Amarone wine, a dry red passito wine, has recently been documented (Zapparoli *et al*., 2018).

During previous surveys on fungi associated with withered grapes (Lorenzini *et al*., 2016, 2018), eight species of *Penicillium* (*P. adametzoides*, *P. expansum, P. crocicola*, *P. crustosum, P. glabrum*, *P. griseofulvum*, *P. oxalicum* and *P. ubiquetum*) and five species of *Aspergillus* (*A. flavus*, *A. sydowii, A. tubingensis*, *A. uvarum* and *A. welwitschiae*) were identified by phylogenetic analyses. The placements of two isolates (*Penicillium* sp. P3 and *Aspergillus* sp. AS100) were not clear enough for reliable species delimitations.

In the present study, isolates P3 and AS100 were phylogenetically and morphologically analyzed to clarify their taxonomic positions. Three isolates of *Penicillium* and *Aspergillus*, recovered from withered grapes during the current survey, were also identified. These fungi belonged to species that are reported for the first time from *Vitis vinifera*. Two isolates were assigned to a new species of *Penicillium*. The pathogenicity of fungi isolated from grape berries was also assayed.

MATERIALS AND METHODS

On the basis of our previous studies (Lorenzini *et al*., 2016; 2018) carried out on grape berries of the Garganega and Corvina varieties, collected from fruit-drying rooms located in two Northern Italian winemaking areas (Soave and Valpolicella), five representative strains of *Aspergillus* and *Penicillium* were isolated and identified. Three isolates recovered from withered grapes in this study (designated Pdb1, Pls8 and ASls13) were isolated according to Lorenzini *et al.* (2016). The other two isolates (P3 and AS100) were obtained during a previous sampling (Lorenzini *et al.,* 2016). These isolates are deposited at the Westerdijk Fungal Biodiversity Institute (CBS, Utrecht, the Netherlands) and ITEM Agro-Food Microbial Culture Collection of the Institute of Science and Food Production (CNR-ISPA, Bari, Italy) (Table 1).

DNA was extracted from pure culture of each isolate as previously described (Lorenzini and Zapparoli, 2014). Each DNA extract was used to amplify the internal transcribed spacer (ITS) region, using primers ITS1/ITS4 (White *et al.,* 1990), partial β-tubulin gene (*benA*), using primers Bt2a/Bt2b (Glass and Donaldson, 1995), partial calmodulin gene (*CaM*), using primers cmd5/cmd6 (Hong *et al*., 2006), and parts of the second largest subunit of RNA polymerase II (*rpb2*), using primers fRPB2-5F2/fRPB2-7C (Liu *et al.,* 1999). The amplified products were purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel), and were sequenced in both directions using the same primers applied for amplification (Eurofins Genomics, Edersberg, Germany). The generated sequences were deposited at GenBank (Table 2).

Combined and individual analyses were conducted using the partial DNA sequences of five isolates recovered from withered grapes, and other reference taxa belonging to the genus *Penicillium* (sections *Lanata–divaricata* and *Sclerotiora*) and *Aspergillus* (*A. versicolor* clade and section *Circumdati*), retrieved from GenBank (Table 2). Maximum Likelihood (ML) analysis of the combined data sets was performed using MEGA7 v. 7.0.25 software. The combined data sets were analysed as three or four distinct partitions. For each individual data set, the most optimal substitution model was calculated in MEGA7 (Kumar *et al*., 2016) using the Akaike Information Criterion (AIC). Maximum Likelihood analyses of the individual data sets were also conducted using MEGA7, and robustness of the trees was evaluated by 1,000 bootstrap (BS) replicates. A second measure for statistical support was performed using Bayesian Evolutionary Analysis Sampling Trees (BEAST) Version v1.10.1, 2002-2018 (Drummond and Rambaut, 2007), and the previously obtained most optimal substitution model was used in the analyses. The Markov Chain Monte Carlo (MCMC) analysis used four chains and started from a random tree topology. Burn-in was set to 25%

Table 1. Fungus isolates recovered from withered grape berries in this study.

Specie	Isolate designation	CBS number	ISPA number	Grape variety
Penicillium fructuariae-cellae	P3	CBS 145110^{T}	ITEM 18276^{T}	Corvina
Penicillium fructuariae-cellae	Pdb1	CBS 145111	ITEM 18277	Garganega
Penicillium bilaiae	Pls8	CBS 145112	ITEM 18278	Garganega
Aspergillus pallidofulvus	ASls13	CBS 145108	ITEM 18279	Garganega
Aspergillus puulaauensis	AS100	CBS 143103	ITEM 18280	Corvina

Table 2. Isolates used in phylogenetic analyses.

Species	Strain identification	Source	Locality	GenBank numbers			
				ITS	β-tubulin	Calmodulin	*rpb2*
Aspergillus section *Circundati*							
A. affinis	ATCC MYA-4773^T	Leaf litter	Italy	GU721090	GU721092	GU721091	-
A. auricomus	NRRL 391^T	ni	ni	EF661411	EF661320	EF661379	-
A. bridgeri	NRRL 13000^T	ni	ni	EF661404	EF661335	EF661358	-
A. cretensis	NRRL 35672^T	ni	ni	FJ491572	AY819977	FJ491534	-
A. elegans	NRRL 4850^T	ni	ni	EF661414	EF661349	EF661390	-
A. fresenii	NRRL 4077^T	Soil	India	EF661409	EF661341	EF661382	-
A. insulicola	NRRL 6138^T	Soil	Venezuela	EF661430	EF661353	EF661396	-
A. melleus	NRRL 5103^T	Soil	India	EF661425	EF661326	EF661391	-
A. muricatus	NRRL 35674^T	Grassland soil	Philippines	EF661434	EF661356	EF661377	-
A. neobridgeri	NRRL 13078^T	Soil	USA	EF661410	EF661345	EF661359	-
A. occultus	CBS 137330^T	Air sample	Netherlands	KJ775443	KJ775061	KJ775239	-
A. ochraceopetaliformis	NRRL 4752^T	Scalp lesion	Brazil	EF661429	EF661350	EF661388	-
A. ochraceus	NRRL 398^T	ni	ni	EF661419	EF661322	EF661381	-
A. ostianus	NRRL 420^T	ni	ni	EF661421	EF661324	EF661385	-
A. pallidofulvus	NRRL 4789^T	ni	ni	EF661423	EF661328	EF661389	-
*A. pallidofulvus**	ITEM 18279 = CBS 145108	Vitis vinifera	Italy	MK039437	MK045335	MK045340	-
A. persii	NRRL 35669^T	Toenail	Italy	FJ491580	AY819988	FJ491559	-
A. pseudoelegans	CBS 112796^T	Soil	Costa Rica	FJ491590	AY819962	FJ491552	-
A. pseudosclerotiorum	UTHSCSA DI15-13^T	Lung biopsy	-	LT574713	LT574748	LT574783	-
A. pulvericola	CBS 137327^T	Indoor hause	Micronesia	KJ775440	KJ775055	KJ775236	-
A. robustus	NRRL 6362^T (outgroup)	Soil	Kenya	EF661176	EU014101	EF661357	-
A. roseoglobulosus	NRRL 4565^T	Decaing leaves	Bahamas	FJ491583	AY819984	FJ491555	-
A. salwaensis	DTO 297B3^T	Soil	Qatar	KJ775447	KJ775056	KJ775244	-
A. sclerotiorum	NRRL 415^T	Apple	USA	EF661400	EF661337	EF661384	-
A. sesamicola	CBS 137324^T	Sesame sead	Denmark	KJ775437	KJ775063	KJ775233	-
A. steynii	NRRL 35675^T	Grean bean coffee	India	EF661416	EF661347	EF661378	-
A. subramanianii	NRRL 6161^T	Shelled brazil nuts	Canada	EF661403	EF661339	EF661397	-
A. tanneri	NRRL 62425^T (outgroup)	Human lung	USA	JN853798	JN896582	JN896583	-
A. westerdijkiae	NRRL 3174^T = CBS 112803	*Andropogum sorghum*	South Africa	EF661427	EF661329	EF661360	-
A. westlandensis	CBS 137321^T	Air sample	Netherlands	KJ775434	KJ775066	KJ775230	-
A. versicolor clade							
A. amoenus	NRRL 4838^T	ni	ni	NR_137462	EF652304	EF652392	-
A. austroafricanus	NRRL 233^T	ni	South Africa	NR_135443	JN853963	JN854025	-
A. creber	NRRL 58592^T	Indoor air sample	USA	NR_135442	JN853980	JN854043	-

(Continued)

Table 2. (Continued).

Species	Strain identification	Source	Locality	GenBank numbers			
				ITS	β-tubulin	Calmodulin	*rpb2*
A. cvjetkovicii	NRRL 227^{T}	Soil	USA	EF652440	EF652264	EF652352	-
A. fructus	NRRL 239^{T}	Date fruit	USA	EF652449	EF652273	EF652361	-
A. griseoaurantiacus	DTO_267D8^{T}	Indoor air dust	Micronesia	KJ775553	KJ775086	KJ775357	-
A. hongkongensis	HKU49^{T}	Human nail	Hong Kong	AB987907	LC000552	LC000565	-
A. jensenii	NRRL 58600^{T}	Indoor air sample	USA	NR_135444	JN854007	JN854046	-
A. multicolor	NRLL 4775^{T} (outgroup)	ni	ni	EF652477	EF652301	EF652389	-
A. protuberus	NRRL 3505^{T}	Brined meat	UK	NR_135353	EF652284	EF652372	-
A. puulaauensis	NRRL 35641^{T}	Dead hardwood branch	USA	NR_135445	JN853979	JN854034	-
A. puulaauensis	**ITEM 18280 = CBS 145103**	**Fruit of *Vitis vinifera***	Italy	MK039438	MK045336	KU554606	-
A. sydowii	NRRL 250^{T}	ni	n	EF652450	EF652274	EF652362	-
A. tabacinus	NRRL 4791^{T}	ni	ni	NR_135361	EF652302	EF652390	-
A. tennesseensis	NRRL 13150^{T}	Ex toxic dairy feed	USA	NR_135447	JN853976	JN854017	-
A. venenatus	NRRL 13147^{T}	Toxic dairy feed	USA	NR_135448	JN854003	JN854014	-
A. versicolor	NRRL 238^{T}	ni	USA	EF652442	EF652266	EF652354	-
A.subversicolor	NRRL 58999^{T}	Coffee berry	India	NR_135446	JN853970	JN854010	-
Penicillium section *Lanata–divaricata*							
P. abidjanum	CBS 246.67^{T}	Soil	Ivory Coast	NR_111502	GU981650	KF296383	JN121469
P. amphipolaria	DAOMC 250551^{T} = KAS 2555 = CBS 140997	Leaves	Panama	KT887872	KT887833	KT887794	na
P. annulatum	CV 37 = CBS 135126^{T}	Soil	South Africa	NR_138303	JX091514	JX141545	KF296410
P. araracuarense	CBS 113149^{T}	Leaf litter	Colombia	GU981597	GU981642	KF296373	KF296414
P. austrosinenese	CGMCC 3.18797^{T} = CBS 144505	Acidic soil	China	KY495003	KY495112	KY494943	KY495061
P. bissettii	DAOMC 167011^{T} = KAS1951 = CBS 140972	Soil	Canada	KT887845	KT887806	KT887767	KY495055
P. brasilianum	CBS 253.55^{T}	Herbarium specimen	Brazil	AF178512	GU981629	AB667857	KF296420
P. brefeldianum	CBS 235.81^{T}	Human alimentary tract	ni	GU981580	GU981623	AB667857	KF296421
P. camponotum	KAS2177 = DAOMC 250557^{T} = CBS 140982	Ants	Canada	KT887855	KT887816	KT887777	na
P. caperatum	CBS 443.75^{T}	Soil	Australia	KC411761	GU981660	KF296392	KF296422
P. cataractum	CBS 140974 = KAS2145 = DAOMC 250534^{T}	Nuts of *Carya cordiformis*	Canada	KT887847	KT887808	KT887769	na
P. fructuariae-cellae	**ITEM 18276^{T} = CBS 145110^{T}**	**Fruit of *Vitis vinifera***	**Italy**	**MK039434**	KU554679	MK045337	MK520927
P. fructuariae-cellae	**ITEM 18277 = CBS 145111**	**Fruit of *Vitis vinifera***	**Italy**	**MK039435**	**MK045333**	**MK045338**	**MK520928**
P. cluniae	CBS 326.89^{T}	Soil	Spain	KF296406	KF296471	KF296402	KF296424
P. coeruleum	CBS 141.45^{T}	ni	ni	NR_138293	GU981655	KF296393	KF296425
P. cremeogriseum	CBS 223.66^{T}	Soil	Ukraine	NR_111505	GU981624	KF296403	KF296426
P. curticaule	CV2842 = CBS 135127^{T}	Soil	South Africa	FJ231021	JX091526	JX141536	KF296417
P. daleae	CBS 211.28^{T}	Soil	Poland	NR_111503	GU981649	KF296385	KF296427
P. echinulonalgiovense	CBS 328.59^{T}	Soil	Japan	GU981587	GU98163	KX961269	KX961301

(Continued)

Table 2. (Continued).

Species	Strain identification	Source	Locality	GenBank numbers			
				ITS	β-tubulin	Calmodulin	*rpb2*
P. ehrlichii	CBS 324.48^T	ni	Poland	GU981578	KF296464	KF296395	KF296428
P. elleniae	CBS 118135^T	Leaf litter	Colombia	GU981612	GU981663	KF296389	KF296429
P. excelsum	HF-2015 = CCT 7772^T	Nut shell	Brazil	KR815341	KP691061	KR815342	na
P. flaviroseum	CGMCC 3.18805^T = CBS 144479	Acidic soil	China	KY495032	KY495141	KY494972	KY495083
P. glabrum	CBS 125543^T (outgroup)			GU981567	GU981619	KM089152	JF417447
P. glaucoroseum	NRRL 908^T = CBS 138908	Soil	USA	KF296407	KF296469	KF296400	KF296430
P. globosum	CGMCC 3.18800^T = CBS 144639	Acidic soil	China	KY495014	KY495123	KY494954	KY495067
P. griseoflavum	CGMCC 3.18799^T = CBS 144525	Acidic soil	China	KY495011	KY495120	KY494951	KY495064
P. griseopurpureum	CBS 406.65^T	Soil	England	KF296408	KF296467	KF296384	KF296431
P. guangxiense	CGMCC 3.18793^T = CBS 144526	Soil	China	KY494986	KY495095	KY494926	KY495045
P. hainanense	CGMCC 3.18798^T = CBS 144527	Acidic soil	China	KY495009	KY495118	KY494949	KY495062
P. infrabuccalum	KAS2181^T= DAOMC 250537	Ants	Canada	KT887817	KT887817	KT887778	na
P. janthinellum	CBS 340.48^T	Soil	Nicaragua	NR_111504	GU981625	KF296401	JN121497
P. javanicum	CBS 341.48^T	Rut of *Cammelia sinensis*	Indonesia	NR_111511	GU981657	KF296387	JN121498
P. jianfenglingense	CGMCC 3.18802^T = CBS 144640	Soil	China	KY495016	KY495125	KY494956	KY495069
P. koreense	KACC 47721^T	Soil	Korea	KJ801939	KM000846	na	na
P. laevigatum	CGMCC 3.18801^T = CBS 144481	Acidic soil	China	KY495015	KY495124	KY494955	KY495068
P. levitum	CBS 345.48^T	Modeling clay	USA	NR_111510	GU981654	KF296394	KF296432
P. limosum	CBS 339.97^T	Marine sediment	Japan	NR_111496	GU981621	KF296398	KF296433
P. lineolatum	CBS 188.77^T	Soil	Japan	NR_111500	GU981620	KF296397	KF296434
P. ludwigii	CBS 417.68^T	Polished rice	Japan	NR_138339	KF296468	KF296404	KF296435
P. malacosphaerulum	CV2855 = CBS 135120^T	Soil	South Africa	FJ231026	JX091524	JX141542	KF296438
P. mariae-crucis	CBS 271.83^T	*Secale cereale*	Spain	NR_111506	GU981630	KF296374	KF296439
P. meloforme	CBS 445.74^T	Soil	Papua New Guinea	NR_153203	GU981656	KF296396	KF296440
P. ochrochloron	CBS 357.48^T	Copper sulphate solution	USA	NR_111509	GU981672	KF296378	KF296445
P. onobense	CBS 174.81^T	Soil	Spain	NR_111497	GU981627	KF296371	KF296447
P. ortum	CV 102 = CBS 135669^T	Soil	South Africa	NR_138304	JX091520	JX141551	KF296443
P. oxalicum	CBS 219.30^T (outgroup)	Soil	USA	MH855125	KF296462	KF296367	JN121456
P. panissanguineum	DAOMC 250562^T = KAS 2209 = CBS 140989	Termite mounds	Tanzania	KT887862	KT887823	KT887784	na
P. paraherquei	ATCC 22354 = CBS 338.59^T	Soil	Japan	AF178511	KF296465	KF296372	KF296449
P. pedernalense	CBS 140770^T	Waste compost	Ecuador	KU255398	KU255396	KY494968	KY495079
P. penarojense	CBS 113178^T	Leaf litter	Colombia	NR_138289	GU981646	KF296381	KF296450
P. piscarium	CBS 362.48^T	Cod-liver oil emulsion	Norway	NR_111507	GU981668	KF296379	KF296451
P. pulvillorum	CBS 280.39^T	Acidic soil	UK	NR_138292	GU981670	KF296377	KF296452
P. raperi	NRRL 2674 = CBS 281.58^T	Soil	UK	AF033433	GU981622	KF296399	KF296453

(Continued)

Table 2. (Continued).

Species	Strain identification	Source	Locality	GenBank numbers			
				ITS	β-tubulin	Calmodulin	*rpb2*
P. reticulisporum	NRRL 3447 = CBS 122.68^T	Soil	Japan	AF033437	GU981665	KF296391	KF296454
P. rolfsii	CBS 368.48^T	Pineapple	USA	JN617705	JU981667	KF296375	KF296455
P. rubriannulatum	CGMCC 3.18804^T = CBS 144641	Acidic soil	China	KY495029	KY495138	KY494969	KY495080
P. simplicissimum	CBS372.48^T	Flannel bag	South Africa	NR_138290	GU981632	KF296368	JN121507
P. singorense	DTO 133C6 = CBS 138214^T	House dust	Thailand	KJ775674	KJ775167	KJ775403	na
P. skrjabinii	CBS 439.75^T	Soil	Russia	NR_111498	GU981626	KF296370	EU427252
P. soliforme	CGMCC 3.18806^T = CBS 144482	Acidic soil	China	KY495038	KY495147	KY494978	KY495047
P. stolkiae	NRRL 5816 = CBS 315.67^T (outgroup)	ni	ni	NR_121233	JN617717	AF481135	JN121488
P. spiliferum	CGMCC 3.18807^T = CBS 14483	Acidic soil	China	KY495040	KY495149	KY494980	KY495090
P. subrubescens	DTO188-D6 = CBS 132785^T	Soil	Finland	KC346350	KC346327	KC346330	KC346306
P. svalbardense	CBS 122416^T	Glacial ice	Greenland	GU981603	KC346325	KC346338	KF296457
P. tanzanicum	DAOMC 250514^T = KAS 1946 = CBS 140968	ni	Tanzania	KT887841	KT887802	KT887763	KY495066
P. terrarumae	CBS 131811^T	Soil	China	KC346349	KC346326	KC346339	KC346316
P. vanderhammenii	CBS126216^T	Leaf litter	Colombia	NR_137577	GU981647	KF296382	KF296458
P. vasconiae	CBS 339.79^T	Soil	Spain	NR_138291	GU981653	KF296386	KF296459
P. viridissimum	CGMCC 3.18796^T = CBS 14484	Acidic soil	China	KY495004	KY495113	KY494944	KY495059
P. wotroi	CBS 118171^T	Leaf litter	Colombia	GU981591	GU981637	KF296369	KF296460
P. yunnanense	CGMCC 3.18794^T = CBS 14485	Acidic soil	China	KY494990	KY495099	KY494930	KY495048
P. zonatum	CBS 992.72^T	Coastal marsh soil	USA	NR_111501	GU981651	KF296380	KF296461
Penicillium section *Sclerotiora*							
P. adametzii	CBS 209.28^T	Soil	Canada	NR_103661	JN625957	KC773796	-
P. adametzioides	CBS 313.59^T	Soil	Japan	JN686433	JN799642	JN686387	-
P. alexiae	DTO 118H8^T	Soil	Tunisia	KC790400	KC773778	KC773803	-
P. amaliae	CV 1875 = CBS 134209^T	*Protea repens*	South Africa	JX091443	JX091563	JX141557	-
P. angulare	CBS 130293^T	Polypore on dead conifer	USA	KC773828	KC773779	KC773804	-
P. arianeae	DTO 20B8^T	Soil	Netherlands	KC773833	KC773784	KC773811	-
P. austrosinicum	HMAS 248734^T = CGMCC 3.18410	ni	ni	NR_153272	KX885041	KX885051	-
P. bilaiae	NRRL 3391^T	Soil	Ukraine	NR_111679	JN625966	JN626009	-
P. bilaiae	**ITEM 18278 = CBS 145112**	**Fruit of *Vitis vinifera***	**Italy**	**MK039436**	**MK045334**	**MK045339**	**-**
P. brocae	CBS 116113^T	Coffee berry	Mexico	NR_111868	KC773787	KC773814	-
P. cainii	DAOM 239914^T	Nuts of *Juglans nigra*	Canada	NR_120000	JN686366	JN686389	-
P. choerospondiatis	HMAS 248813^T = CGMCC 3.18411	ni	ni	NR_153274	KX885043	KX885053	-
P. daejeonium	CNU 100097 = KACC 46609^T	Grape fruit	South Korea	JX436489	JX436493	JX436491	-
P. exsudans	HMAS 248735^T = CGMCC 3.18412	ni	ni	NR_153273	KX885042	KX885052	-
P. guanacastense	DAOM 239912^T	Gut of caterpillar	Costa Rica	NR_111673	JN625967	JN626010	-

(Continued)

Table 2. (Continued).

Species	Strain identification	Source	Locality	GenBank numbers			
				ITS	β-tubulin	Calmodulin	*rpb2*
P. herquei	CBS 336.48^T	Leaf of *Agauria pirifolia*	France	JN626101	JN625970	JN626013	-
P. hirayamae	CBS 229.60^T	Milled rice	Thailand	JN626095	JN625955	JN626003	-
P. jacksonii	DAOM 239937^T	Forest soil	Australia	NR_111675	JN686368	JN686391	-
P. johnkrugii	DAOM 239943^T	Forest soil	Malaysia	NR_111676	JN686378	JN686401	-
P. jugoslavicum	CBS 192.87^T	Seed of *Helianthus annuus*	Ex Yugoslavia	NR_120269	KC773789	KC773815	-
P. lilacinoechinulatum	CBS 454.93^T	Soil	Japan	KC773837	KC773790	KC773816	-
P. malachiteum	CBS 647.95^T	Soil	Japan	NR_120271	KC773794	KC773820	-
P. mallochii	DAOM 239917^T	Caterpillar	Costa Rica	NR_111674	JN625973	JN626016	-
P. maximae	NRRL 2060^T	Cellophane	USA	NR_121343	KC773795	KC773821	-
P. restingae	MS-2014 = URM 7075^T	Soil	ni	KF803354	KF803349	KF803352	-
P. sanshaense	HMAS 248820^T = CGMCC 3.18413	ni	ni	NR_153276	KX885050	KX885060	-
P. sclerotiorum	NRRL 2074^T	Air	Indonesia	JN626132	JN626001	JN626044	-
P. vanoranjei	DTO 99H6^T	Soil	Tunisia	KC695696	KC695686	KC695691	-
P. verrucisporum	HMAS 248819^T = CGMCC 3.18415	ni	ni	KX885069	KX885049	KX885059	-
P. viticola	FKI-4410 = JCM 17636^T	Grape	Japan	AB606414	AB540174	AB540173	-
P. levitum	CBS 345.48^T (outgroup)	ni	ni	NR_111510	GU981654	KF296394	-

* Strains studied in this paper are in bold type;
- Sequences not used in this study;
^{T} = Ex-type strain; ni = no information about the source and locality; na = not available
ATCC, American Type Culture Collection (Manassas, VA); CBS, Westerdkjik Fungal Biodiversity Institute (Utrecht, the Netherlands); DTO, Applied and Industrial Mycology Department Collection, Westerdkjik Fungal Biodiversity Institute (Utrecht, Netherlands); NRRL, Agriculture Research Service Culture Collection (Peoria, NY); UTHSCSA, University of Texas Health Science Center (San Antonio, TX); HKU, University of Hong Kong, (Hong Kong, China). ITEM, Agro-Food Microbial Culture Collection of Institute of Science and Food Production (ISPA) (Bari, Italy); JCM, Japan Collection of Microorganism (Saitama, Japan); CGMCC. Chinese General Microbiological Culture Collection Center (Beijing, China); URM, University Recife Mycology Culture Collection (Recife, Brazil); KACC, Korean Agricultural Culture Collection (Suwon, South Korea); CCT, Coleção de Cultura Tropical (Campinas, Brazil); DAOMC, Canadian Collection of Fungal Cultures (Ottawa, Canada); DAOM, Canadian National Mycological Herbarium (Ottawa, Canada).

and Tracer v. 1.5.0 (Rambaut and Drummond, 2009) was used to confirm the convergence of chains. The phylograms obtained through the ML analyses were used for presenting the data. Phylograms were redrawn from the tree files using FigTree v1.4 2006-2012. Bootstrap values less than 70% and posterior probability (pp) values less than 0.95 were removed from the phylograms. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA7. All isolates of *Aspergillus* and *Penicillium* (Pdb1, P3, Pls8, ASls13 and AS100) were inoculated onto Czapek yeast extract agar (CYA), yeast extract sucrose agar (YES), creatine sucrose agar (CREA) (Visagie *et al.* 2014) and malt extract agar (MEA, 2% w v^{-1} malt extract, 0.1% w v^{-1} peptone, 2% w v^{-1} dextrose, 1.5% w v^{-1} agar) at 25°C in the darkness. The colony diameters (mm) were measured daily for 7 d (three replicate plates for each isolate) and the experiments were performed twice. The colony diameters were also measured on CYA at 15, 30 and 37°C. The cultural characteristics and micro-morphology of each strain was examined on CYA, YES and MEA after 7, 14 or 30 d at 25°C. Colony morphology was also examined on CREA after 7 d incubation.

Growth tests for the fungal isolates were carried out under acidic, neutral and alkaline conditions, as reported by Diao *et al.* (2018).

Preparations for microscopy were made from colonies grown on CYA, YES and MEA after 7, 14 or 30 d. Lactic acid (60% v v^{-1}) was used as mounting fluid and excess conidia were washed away with ethanol (70% v v^{-1}). ethanol. Characters were recorded and analyzed using stereomicroscopy (Leica EZ4D, Leica Microsystems). Measurements of fungal components were carried out using light microscopy (Leica DM750) equipped with camera module (Leica ICC50W). Lengths and widths were determined for 20 conidiophores, metulae and phialides, 50 conidia, 20 cleistothecia and 30 ascospores (when present) from each isolate.

The five isolates were tested for their ability to cause disease on grape berries (white fresh table and red withered wine grapes). The berries were surface sterilized by immersion for 5 min in 0.5% NaOCl solution, then rinsed twice with sterile distilled water and placed in compartmentalized square culture dishes. Suspensions of conidia were prepared, adjusted to 10^4 conidia mL^{-1} and then inoculated by berry piercing, as reported by Lorenzini and Zapparoli (2014). Mock inoculation (controls) consisted of berries wounded and inoculated with sterile water, and a positive control consisted of berries wounded and inoculated with *Botrytis cinerea* ITEM 17200. The experiment was performed twice, each with three replicates, which each consisted of 25 berries. After 7 and 14 d at 22°C, the disease index (DI) was assessed on a scale of 0 to 4, as previously described (Lorenzini and Zapparoli, 2014). Variance analysis (ANOVA) was used for the DI data to evaluate isolate differences in pathogenicity. Tukey's multiple comparison test (Tukey, HSD) was applied to determine statistically significant differences.

RESULTS

Phylogenetic analysis

Using the BLASTn tool in GenBank, the *benA*, *CaM*, ITS and *rpb2* gene sequences of Pdb1 (ITEM 18277) showed 99% similarity to *Penicillium* sp. P3 (ITEM 18276^T) (Lorenzini *et al.* 2016) for *benA*, greater than 89% similarity to *P. bissettii* and *P. annulatum* for *CaM*, greater than 98% similarity to different *Penicillium* species (e.g. *P. janthinellum, P. reticulisporum, P. ochrochloron, P. bissettii and P. javanicum*) for ITS, and 95% similarity to *Penicillium* sp. for *rpb2*. The alignment results of the *benA*, *CaM*, ITS and *rpb2* gene sequences of P3 were similar to those of Pdb1. Based on these data, the phylogenetic position of both isolates (Pdb1 and P3) was evaluated using members of *Penicillium* section *Lanata-divaricata*, according to Diao *et al.* (2018). The ML combined phylogenetic tree (*benA*+*CaM*+ITS+*rpb2*) with the greatest log likelihood (-21693.88) is shown in Figure 1a. The Pdb1 and P3 isolates were grouped together (BS/pp = 100/1), and were distantly related to *P. bissettii* DOAMC 167011 and *P. vasconiae* CBS 339.79. Data from phylogenetic analyses using *benA*, *CaM*, ITS and *rpb2* individually (data not shown) were in concordance with those based on the combine dataset. However, analyses of the combined dataset provided greater support than the individual datasets. The molecular identification of all isolates recovered in this study is based, therefore, on phylogeny from the combined dataset of gene sequences.

The comparative analysis by GenBank database of *benA*, *CaM* and ITS gene sequences of isolate Pls8 (ITEM 18278) showed 99% similarity to different strains of *Penicillium bilaiae* (section *Sclerotiora*). The phylogenetic position of Pls8 was therefore evaluated using members of *Penicillium* section *Sclerotiora*, reported by Wang *et al.* (2017). The combined phylogenetic tree (*benA*+*CaM*+ITS) with the greatest log likelihood (-11066.6111) confirmed the identification (Figure 1b), as the Pls8 (ITEM 18278) was grouped with *P. bilaiae* NRRL3391 (BS/pp = 100/1).

Comparative analysis using GenBank of *benA*, *CaM* and ITS gene sequences of ASls13 (ITEM 18279) showed 99% similarity to *A. melleus, A. pallidofulvus, A. sulphureus* and *A. petrakii* for *benA*, 99% similarity to *A.*

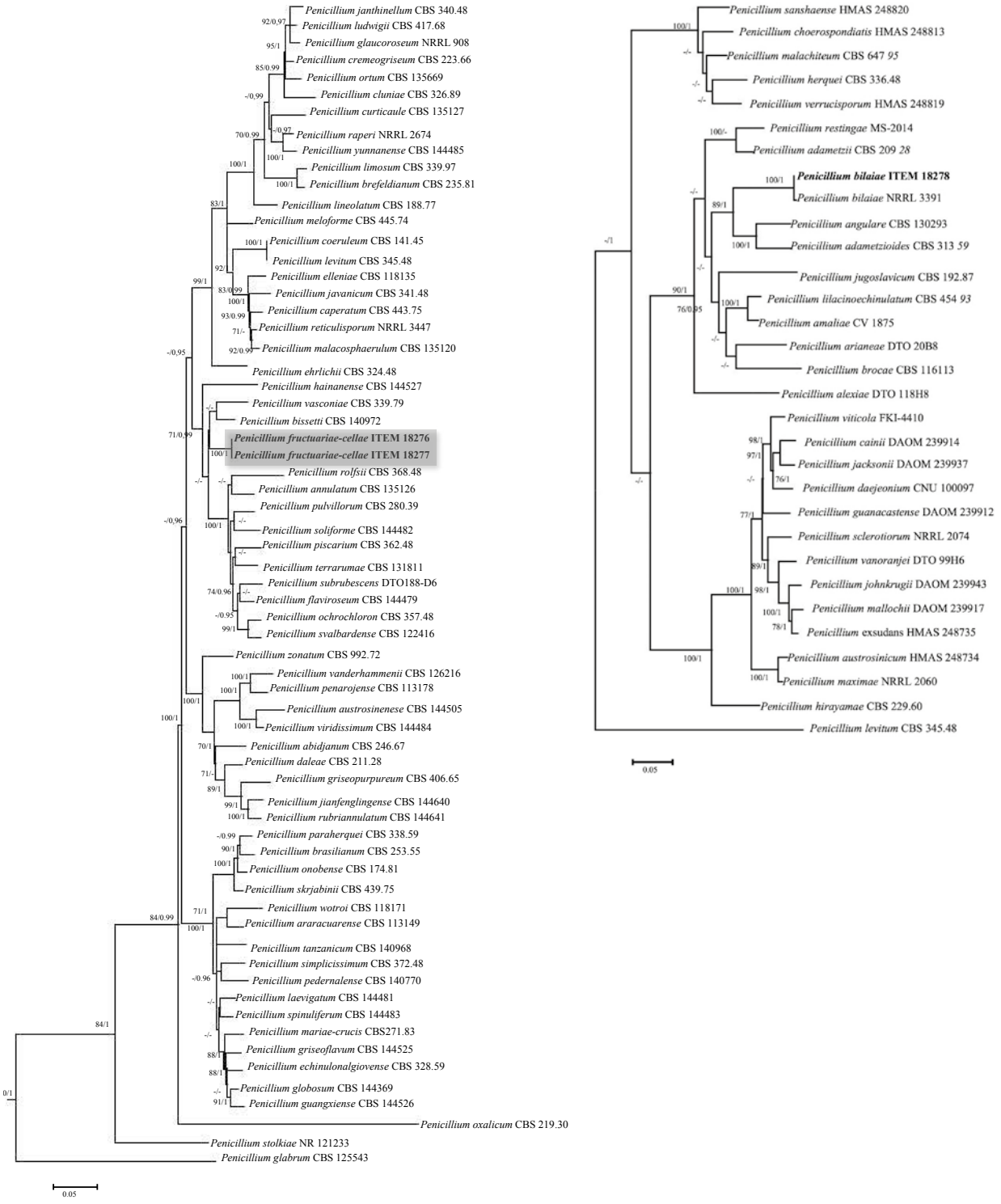
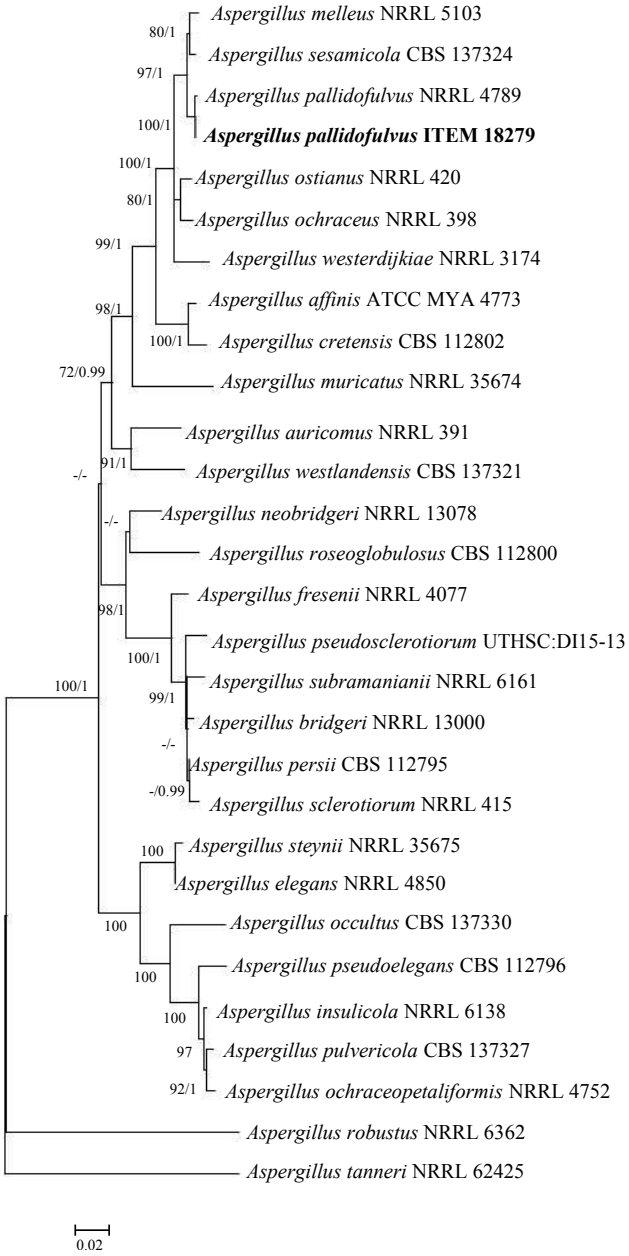


Figure 1. Phylogenetic tree of combined sequences of *Penicillium* spp. (a) Maximum Likelihood combined (*benA+CaM+ITS+rpb2*) tree of *P. fructuariae-cellae* ITEM 18277 (Pdb1) and ITEM 18276^{T} (P3) and representative taxa of *Penicillium* section *Lanata–divaricata*. *Penicillium stolkiae* CBS 315.67, *P. oxalicum* CBS 219.30 and *P. glabrum* CBS 125543 are outgroups. (b) Maximum Likelihood combined (*benA+CaM+ITS+rpb2*) tree of *P. bilaiae* ITEM 18278 (Pls8) and representative taxa of *Penicillium* section *Sclerotiora*. *Penicillium levitum* CBS 345.48 is outgroup. The BI posterior probabilities values and bootstrap percentages of the ML analysis are indicated above the nodes (BS/pp). Values less than 70% bootstrap support in the ML analysis and less than 0.95 posterior probability in the Bayesian analysis are indicated with a hyphen. Branches with high support are thickened.



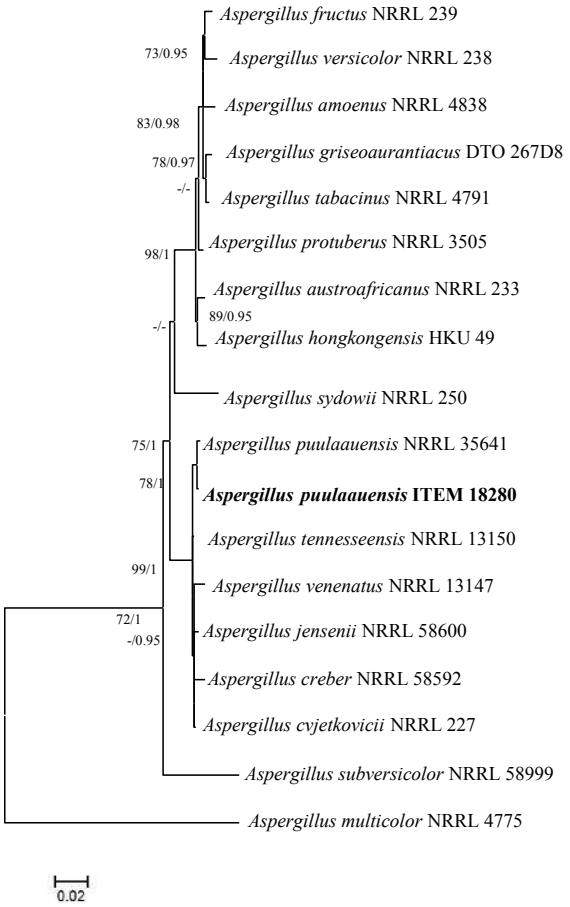


Figure 2. Phylogenetic tree of combined *benA*, *CaM* and ITS sequences of *Aspergillus* spp. (a) Maximum Likelihood tree of *Aspergillus pallidofulvus* ITEM 18279 (ASls13) and representative taxa of *Aspergillus* section *Circumdati*. *Aspergillus robustus* NRRL 6362 and *A. tanneri* NRRL 62425 are outgroups. (b) Maximum Likelihood tree of *A. puulaauensis* ITEM 18280 (AS100) and representative taxa of *Aspergillus* species belonging to *A. versicolor* clade. *Aspergillus subversicolores* NRRL 58999 and *A. multicolor* NRRL 4775 are outgroups. The BI posterior probabilities values and bootstrap percentages of the ML analysis are indicated above the nodes (BS/pp). Values less than 70% bootstrap support in the ML analysis and less than 0.95 posterior probability in the Bayesian analysis are indicated with a hyphen. Branches with high support are thickened.

pallidofulvus and different strains of *Aspergillus* sp. for *CaM*, and 99% similarity to different *Aspergillus* species (e.g. *A. ochraceus, A. melleus* and *A. pallidofulvus*) for ITS. Based on these results, the phylogenetic analysis was performed using the *Aspergillus* taxa of section *Circumdati*, according to Siqueira *et al.* (2017). The ML combined phylogenetic tree (*benA*+*CaM*+ITS) with the greatest log likelihood (-3612.040) showed that ASls13 strongly belongs to *A. pallidofulvus*, as it grouped with the relevant strain, NRRL4789 (BS/pp = 100/1) (Figure 2a).

The GenBank comparative analysis of *benA*, *CaM* and ITS sequences of AS100 (ITEM 18280) showed 99% similarity to *A. puulaauensis, A. versicolor* and *A. jensenii* for *benA*, and 99% similarity to different species of *Aspergillus* (e.g. *A. puulaauensis, A. cvjetkovicii*, *A. tennesseensis*, *A. versicolor*, *A. jensenii* and *A. creber*) for *CaM* and ITS. In the ML combined tree (*benA*+*CaM*+ITS) with the greatest log likelihood (-3938.2941), the clustering of AS100 with *A. puulauensis* NRRL35641 was highly supported (BS/pp = 78/1), as

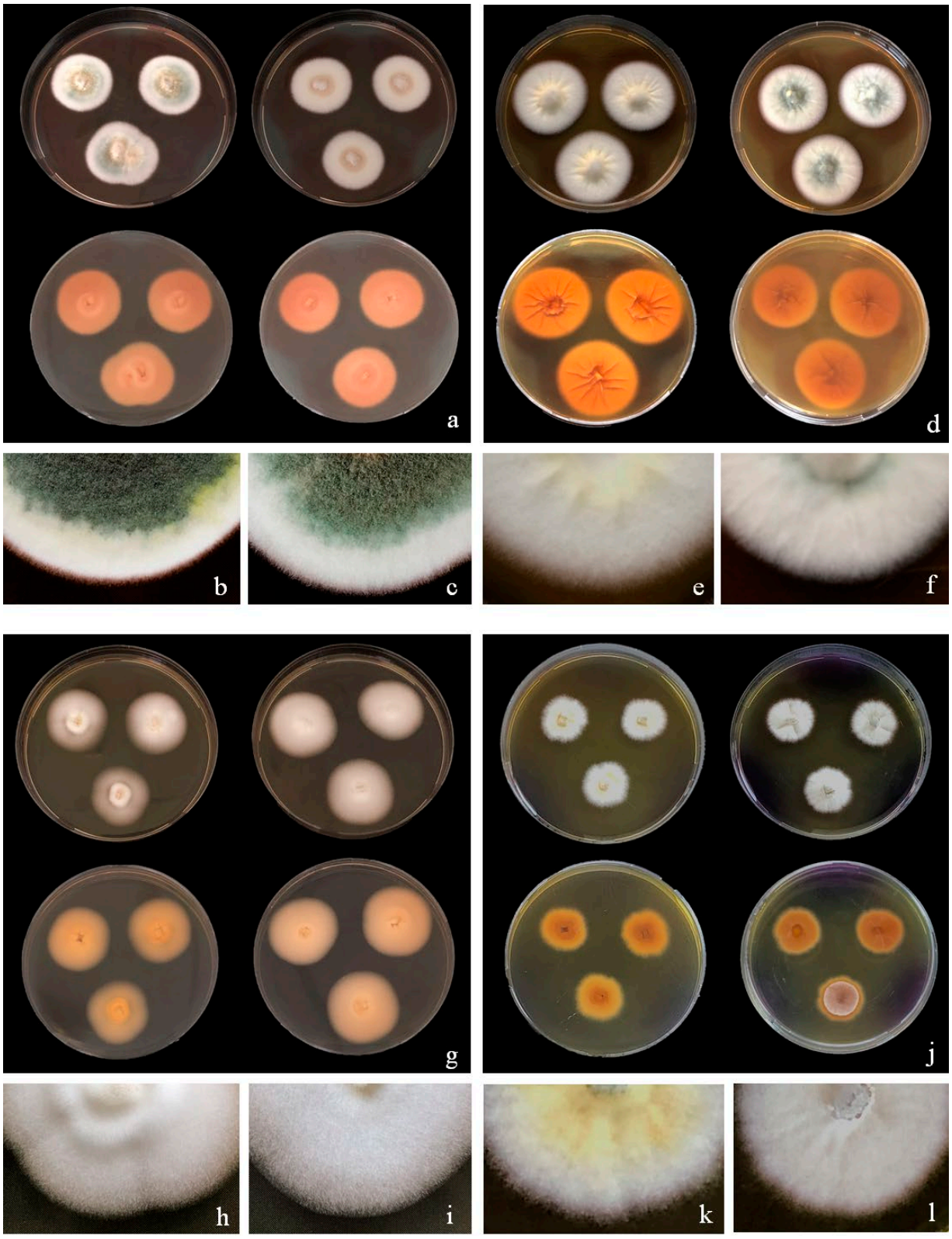


Figure 3. Macro-morphology of *Penicillium fructuariae-cellae* on culture media. Colony upper surfaces (top row) of ITEM 18277 (isolate Pdb1, left) and ITEM 18276^{T} (isolate P3, right), and reverse sides (bottom row) on CYA (a), YES (d), MEA (g) and CREA (j). Colony texture of ITEM 18277 (Pdb1) (b, e, h, k) and ITEM 18276^{T} (P3) (c, f, i, l) on, respectively, CYA, YES, MEA or CREA.

Table 3. Micromorphological characteristics of *Penicillium* and *Aspergillus* isolates after 7 d growth on different agar media. All dimensions are in µm. Numbers of metulae or phialides are indicated in parentheses..

	Media	*P. fructuariae-cellae* ITEM 18276^T (P3)	*P. fructuariae-cellae* ITEM 18277 (Pdb1)	*P. bilaiae* ITEM 18278 (Pls8)	*A. pallidofulvus* ITEM 18279 (ASls13)	*A. puulaauensis* ITEM 18280 (AS100)
Cleistothecia*	CYA	121-221 × 80-194	74-120 × 67-100	-	-	-
	YES	88-192 × 88-178	126-277 × 120-231	-	-	-
	MEA	124-191 × 77-133	35-87 × 25-56	-	-	-
Ascospore	CYA	2.5–4.5	2.5–4	-	-	-
Conidiophores/Stipes	CYA	56-200 × 2-3	55-313 × 2-3.5	34-110 × 2-3.5	155-760 × 4.5-10	115-360 × 4.5-7.5
	YES	137-413 × 2.5-4	68-227 × 2.5-5	26-97 × 2-3.5	288-630 × 4.5-10.5	168-325 × 3.5-9.5
	MEA	92-129 × 2.5-3.5§	86-120 × 2-3§	31-123 × 2-3.5	200-890 × 5-9	190-810 × 4-7
Vescicles	CYA	-	-	3.5-5	12-30	11-19.5
	YES	-	-	2.5-4.5	20-51	15-22
	MEA	-	-	3-4	13-31	9-19
Metulae/Branches	CYA	9-16 × 2-3 (2-4)	10-47 × 2-3.5 (2-4)	-	5.5-8 × 2.5-4.5	4.5-6.5 × 2-3
	YES	13-24 × 2 -4 (2-3)	8.5-18 × 2-4.5 (2-4)	-	6-12 × 3-6	3.5-7 × 2.5-4
	MEA	12-19 × 2.5-4 (2-4) **	10-26 × 2-4 (2-3) **	-	7.5-10 × 3-6	4-8 × 2-3.5
Phialides	CYA	3.5-8 × 2-3 (2-10)	4.5-10 × 2-3 (3-10)	6-9 × 2.5-3.5 (3-8)	5-8 × 2-3.5	4.5-7.5 × 2-3.5
	YES	7.5-15 × 2-4 (2-7)	5.5-11 × 2-3.5 (3-9)	5-10 × 2-3 (2-6)	6-9 × 2.5-4	4-7 × 2-3.5
	MEA	6-9.5 × 2-3 (3-5) **	5-9.5 × 2-3.5 (3-5) **	4.5-8.5 × 2-3.5 (2-4)	6.5-8 × 2.5-4	4.5-7 × 2-3.5
Conidia	CYA	2-3.5	2-3.5	2-3.5	2.5-4	2-3.5
	YES	2-4	2-3	2-3	2-3.5	2-3.5
	MEA	2-3.5 **	2-3.5 **	2-3.5	2.5-4.5	2-3.5

- = structures absent; § n < 4; * measurements based on 14 d old cultures; ** measurements based on 30 d old cultures.

shown in Figure 2b. Phylogenetic analysis based on the ITS dataset placed AS100 in a group containing most of the *Aspergillus* taxa of the *A. versicolor* clade (data not shown).

Culture and morphological characteristics

Penicillium isolates Pdb1 (ITEM 18277) and P3 (ITEM 18276^T) had similar colony morphology on the different media (Figure 3). On CYA, colonies were compact, velvety, with entire margins, and were white; initially yellowish (for Pdb1) or cream (for P3) then becoming gray-green due to abundant sporulation. Spherical or suboval cleistothecia were observed, often covered with a network of hyphae. The cleistothecia matured after three or more weeks, containing evanescent asci and hyaline ascospores, which were smooth–walled and globose to subglobose (Table 3). The colonies were surrounded by diffused soluble pigment in the agar developing as a red-brown colony halos. Hyaline exudates were also observed. Reverse sides of the colonies were light brown and pale cream shades (Figures 3a, b and c).

The growth test revealed little variability among the isolates. The colony diameters at 25°C were 36 to 41 mm for isolate Pdb1 and 43 to 48 mm for P3, at 15°C were 16 to 20 mm for Pdb1 and 17 to 20 mm for P3 mm, and at 30°C were 46 to 49 mm for Pdb1 and 47 to 49 mm for P3. The colony diameters at 37°C were 8 to 10 mm for Pdb1 and 10 to 13 mm for P3. On YES, the colonies were moderately deep and radially sulcate, with regular margins. The mycelium was white and pale yellow in the centre for Pdb1, or white and greenish to grayish in the centre for P3. Sporulation was moderate and pale yellow for Pdb1 and pale gray-green for P3. The cleistothecia were spherical or suboval. The colonies were surrounded by diffused soluble pigment in the agar, as a faint yellow zones surrounded by faint purpuric-brown halos. The colony textures were pubescent and exudates were not observed. The reverse sides of the colonies were orange to brown in colour (Figures 3b, e and f). The colony diameters were 43 to 50 mm for isolate Pdb1 and 42 to 46 mm for P3. On MEA, the colonies were compact, velvety, sometimes radially wrinkled, with entire and plane margins. The mycelia were whitish and non-sporulating mycelium. The cleistothecia were spherical or suboval, and hyaline exudates sometimes were observed. The reverse sides of colonies were yellow and white for Pdb1 and white to pale cream for P3 (Figures 3g, h and i). The colony diameters were 38 to 40 mm for isolate Pdb1 and

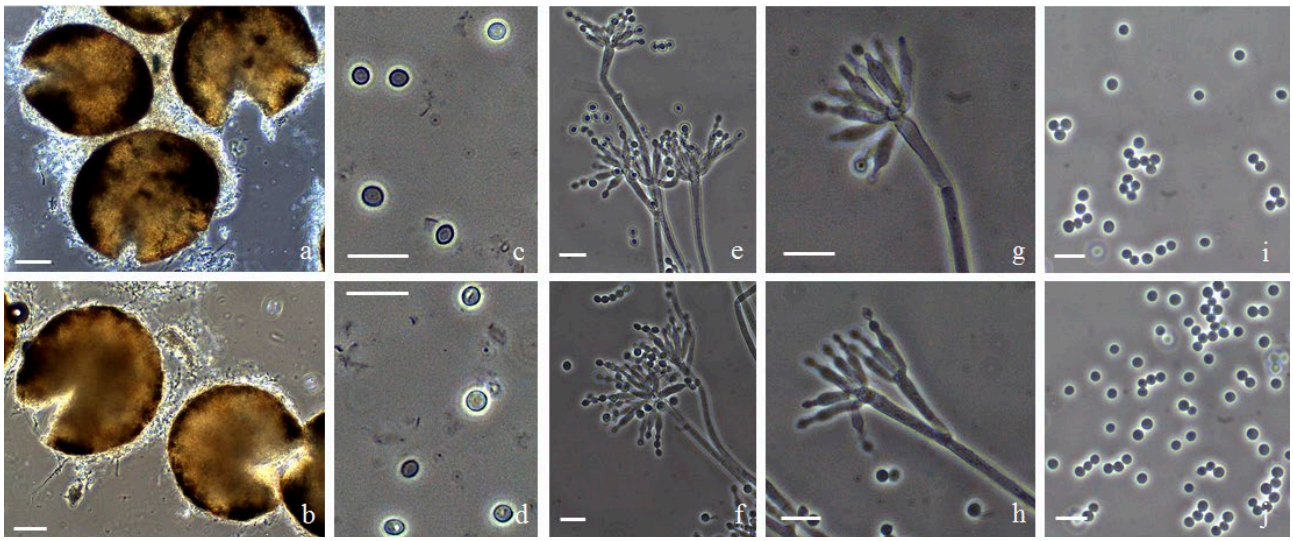


Figure 4. Micro-morphology of *Penicillium fructuariae-cellae* on CYA. Cleistothecia (a and b), ascospores (c and d), conidiophores (e to h), and conidia (i and j) of, respectively, ITEM 18277 (isolate Pdb1, top row) and ITEM 18276^{T} (isolate P3, bottom row). Bars = 50 µm (a and b), or 10 µm (c to j).

43 to 45 mm for P3. The colony diameters at 25°C after 7 d on PDA pH 4 were 37 to 40 mm for Pdb1 and 39 to 41 for P3, on ¼ strength PDA pH 7 were 35 to 38 mm for Pdb1 and 38 to 42 mm for P3, and on Horikoshi agar pH 10 were 29 to 30 mm for Pdb1 and 32 to 33 mm for P3. On CREA, the isolates grew well and produced acid (Figures 3j, k, l). On CYA and YES, conidiophores were monoverticillate or biverticillate, and a minor proportion were divaricate. Metulae were oblong and divergent, phialides were ampuliform, and conidia were smooth walled and globose to subglobose (Figure 4; Table 3). On MEA, conidiophores were rarely observed.

On CYA, colonies of *P. bilaiae* Pls8 were convex, with concentric folds, conidia were abundantly produced, and aerial mycelium was lanose and floccose and grey-green to white. The colony margins were margin entire and white and sporulation was heavy. Exudates were dark, superficial or embedded, and the colonies were surrounded by diffused soluble pigment into agar as faint purpuric-brown halos. The colony reverse sides were orange-yellow. On YES, colonies were heavily wrinkled, white and pubescent at the margins, green-gray in colour, and felty in the centres. The colonies sporulated heavily. The margins entire and exudates were not observed. The colonies were surrounded by soluble pigment diffused into the agar as faint yellow zones surrounded by faint purpuric-brown halos. The colony reverse sides were orange to brown. On MEA, the colonies were velutinous, floccose and raised in the centre, with villose white aerial mycelium and entire margins which were entire, low, plane and white. The colonies were; sporulating heavily, without exudates, and were surrounded by diffused soluble pigment into the agar as faint yellow zones. The reverse sides of the colonies were yellow to pale orange. Colony diameters were 22 to 26 mm on CYA, 20 to 22 mm on YES and 16 to 20 mm on MEA. On CREA, the fungal growth was weak with good acid production. On CYA, YES, and MEA the conidiophores were monoverticillate, the stipes were smooth walled and mostly globose vesiculate or subglobose to ellipsoidal. The phialides were ampulliforms and wide at the bases. Conidia were globose to subglobose (Table 3).

On CYA, colonies of *A. pallidofulvus* ASls13 was velutinous, the mycelium was white, and sporulation was pale yellow to cream, without exudates. Reverse sides of colonies were light brown to brown. On YES, the colonies were moderately powdery to velutinous, the mycelium was white without exudates, and sporulation was light yellow Colony reverse sides were pale brown to yellow. On MEA, the colonies were velutinous, felty and floccose and the mycelium was white with light yellow sporulation, without exudates. Colony reverse sides were cream to pale brown. Colony diameters were 56 to 60 mm on CYA, 70 to 75 mm on YES and 55 to 61 mm on MEA. On CREA, growth was weak with no acid production. On CYA, YES and MEA, the conidiophores were biseriate, stipes were hyaline to pale brown, vesicles were globose, metulae were oblong covering entire vesicles, and phialides were ampulliform. Conidia were globose, subglobose to ovoid and smooth (Table 3).

Table 4. Disease index (%) on grape berries after 14 d, for three isolates of *Penicillium* (P3, Pb1b and Pls8) and two of *Aspergillus* (ASls13 and AS100), all recovered from withered grapes, and for *B. cinerea* ITEM 17200 as positive control.

Species	Isolate designation	Disease index (%) on grape berries	
		White fresh table	Red withered wine
Penicillium fructuariae-cellae	P3	24 ± 0 a	44 ± 1 a
Penicillium fructuariae-cellae	Pdb1	23 ± 1 a[1]	44 ± 1 a
Penicillium bilaiae	Pls8	24 ± 2 a	44 ± 2 a
Aspergillus pallidofulvus	ASls13	22 ± 1 a	47 ± 2 a
Aspergillus puulaauensis	AS100	23 ± 1 a	43 ± 1 a
Botrytis cinerea	ITEM 17200	100 ± 0 b	98 ± 1 b

[1]Values (mean of three independent measurements ± standard deviation) with different letters are significantly different (ANOVA, Tukey HSD, $P < 0.05$)

On CYA, colonies of *A. puulaauensis* AS100 was sulcate and raised in the centre. Sporulation green to gray. Colony margins were regular, plane and white without exudates. Colony reverse sides were yellow to light orange. On YES, colonies were moderately powdery to felty, sulcate, raised in the centre, and white with green to grey and light pink shadows. Sporulation was green to gray. Colony margins were regular and no exudates were produced. Colony reverse sides were yellow to light brown. On MEA, colonies were sulcate, and raised in the centre. Mycelium was white and sporulation was light yellow. Colony margins were regular, plane and white, without exudates. Colony reverse sides were yellow to light orange. The colony diameters were 18 to 22 mm on CYA, 24 to 25 mm on YES and 18 to 20 mm on MEA. On CREA, growth was moderate growth without acid production. On CYA, YES and MEA, the conidiophores were biseriate, and stipes were smooth, and hyaline to light yellow. Vesicles were spatulate or subspherical, and metulae were oblong covering entire vesicles. Phialides were oblong from which globose or subglobose conidia developed (Table 3).

Pathogenicity assay

All *Penicillium* and *Aspergillus* isolates obtained in this study displayed ability to infect grape berries, although infection was much less than for *B. cinerea* (Table 4). Wounded inoculated berries initially showed mycelium around the inoculation sites and subsequently necrotic areas became visible, particularly in white fresh table berries. Red withered berries inoculated by *Penicillium* isolates Pdb1, P3 and Pls8 caused typical symptoms of *Penicillium* infection characterized by tufts of white and green mycelium erupting from the berry skins (Figure 5). Abundant sporulation was observed on berries infected by *A. pallidofulvus* isolate ASls13. The pathogens were re-isolated from inoculated berries, fulfilling Koch's postulates.

Taxonomy

The phylogenetic analysis based on four different loci and the morphological analysis showed that two isolates of *Penicillium* (Pdb1 and P3) recovered from withered grapes were distinct from any known species within the *Penicillium* section *Lanata–divaricata*. Therefore, these isolates are here described as members of a new *Penicillium* species.

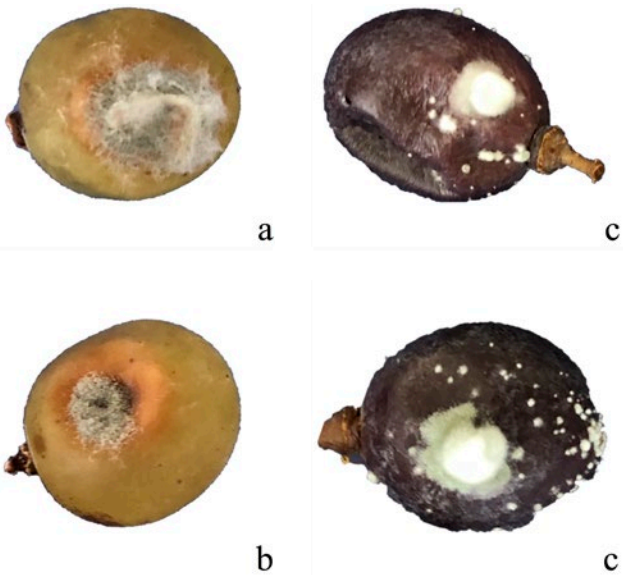


Figure 5. Details of the pathogenicity assay of ITEM 18277 (isolate Pdb1) (a and c) and ITEM 18276^T (isolate P3) (b and d) on white fresh table grapes (left) and red withered wine grapes (right).

Penicillium fructuariae-cellae Lorenzini, Zapparoli & Perrone **sp. nov.**

MycoBank: MB 831228 – Figures 3 and 4

In: subgenus *Aspergilloides*, section *Lanata–divaricata*.

ITS barcode: MK039434. Alternative markers: *benA* =KU554679; *CaM* = MK045337; *rpb2* = MK520927.

Etymology. Latin, *fructuariae-cellae*, meaning fruit-drying room for grape withering, the place where two representative strains were isolated.

Type specimen. ITALY, Verona, Marano di Valpolicella, on Corvina withered grapes stored in fruit-drying room, Dec. 2013, *coll*. M. Lorenzini and G. Zapparoli, *isol*. M. Lorenzini and G. Zapparoli, P3 (holotype CBS 145110^{T}; ex-type strain ITEM 18276^{T}).

Colony morphology. Colony diameters (mm), 7 d: CYA 43–48; YES 42–46; MEA 43–45; CREA 35–37; CYA 15°C 17–20; CYA 30°C 47–49; CYA 37°C 10–13.

Colonies on CYA after 7 d at 25°C were compact, velvety; margins entire and white; sporulation abundant, conidia *en masse* pale gray-green; cleistothecia spherical or suboval covered with networks of hyphae; asci evanescent, ascospores hyaline, smooth–walled, globose to subglobose; soluble red-brown pigments and hyaline exudates produced; colony reverse sides pale brown and pale cream. Colonies on YES after 7 d at 25°C were moderately deep, radially sulcate, with regular margins; mycelia white and green to gray; sporulation moderate, conidia *en masse* pale gray-green; cleistothecia observed; soluble yellow and faint purpuric-brown pigments produced from colonies; exudates absent; colony reverse sides orange to brown. Colonies on MEA after 7 d at 25°C were compact, sometimes radially wrinkled, with entire and plain margins with velvety texture; mycelia white; sporulation poor; cleistothecia observed; hyaline exudates sometimes observed; colony reverse sides white yellow-pale cream. Colonies grew well on CREA after 7 d at 25°C, with good acid production.

Conidiophores (on CYA) monoverticillate, biverticillate, with a minor proportion divaricate; stipes smooth, 55–313 × 2–3.5 µm, *metulae* divergent, 2–4 per stipe or branch, 9–47 × 2–3.5 µm; *phialides* ampulliform, 2–10 per metula, 3.5–10 × 2–3 µm; *conidia* smooth walled, globose to subglobose, 2–3.5 µm (mean = 2.5 µm ± 0.4 µm n = 50); *cleistothecia* covered with a network of hyphae, 74–221 × 67–194 µm (n = 20); asci evanescent; *ascospores* hyaline, smooth–walled, globose to subglobose 2.5–4.5 µm (mean = 3.0 µm ± 0.4 µm, n = 30).

Other strains examined. ITALY, Verona, Montecchia di Crosara, Garganega withered grapes stored in a fruit-drying room, Nov. 2017, *coll*. M. Lorenzini and G. Zapparoli, *isol*. M. Lorenzini and G. Zapparoli, Pdb1, ITEM 18277 = CBS 145111, ITS barcode: MK039435. Alternative markers: *benA* = MK045333; *CaM* = MK045338; *rpb2* = MK520928.

Notes. *Penicillium fructuariae-cellae* is classified in section *Lanata–divaricata*, and is distantly related to other *Penicillium* species. The multi-locus phylogeny placed it closed to *P. bissettii* KAS1951 and *P. vasconiae* CBS 339.79 (Figure 1a). *Penicillium fructuariae-cellae* produces red-brown pigments in CYA compared with *P. bissettii* and *P. vasconiae* that do not produce pigments. *Penicillium fructuariae-cellae* mainly differs from *P. vasconiae* and *P. bissettii* in conidiophore structure and size as it has longer conidiophores than *P. vasconiae*, and shorter conidiophores than *P. bissetti*. *Penicillium fructuariae-cellae* also differs from *P. bissettii* by having smooth stipes. *Penicillium fructuariae-cellae* differs from *P. vasconiae* in phialide shape (*P. vasconiae* has long tapped neck phialides) and having smooth and small conidia.

DISCUSSION

Phylogenetic analysis and morphological observations of the isolates recovered in this study from withered grapes compile the first report of *P. bilaiae*, *A. pallidofulvus* and *A. puulaauensis* from *Vitis vinifera*.

The species identification of Pls8 (ITEM 18278) as *P. bilaiae* was taxonomically clear due to its congruence with phylogenetic and morphological data from the *P. bilaiae* holotype NRRL 3391. However, isolate Pls8 showed slower growth in agar media and some micro-morphological differences (i.e. longer stipes, wider and more numerous phialides) than holotype PB-50 described by Pitt (1979) and Savard *et al.* (1994). Prior to the present study, *P. bilaiae* was detected in Portuguese grapes through morphological observations (Serra *et al*., 2005), a method that does not provide sufficient data for reliable identification at the species level. *Penicillium bilaiae* is morphologically similar to both *P. alexiae* and *P. adametzioides* (Visagie *et al*., 2013). Hence, the present study provides the first taxonomic evidence of the occurrence of *P. bilaiae* on *Vitis vinifera*.

The assignment of isolate ASls13 (ITEM 18279) to *A. pallidofulvus* was confirmed by its genealogy and macromorphology, according to the description of the holotype *A. pallidofulvus* NRRL 4749 (Visagie *et al*., 2014). However, micro-morphological observations of ASls13 showed differences in the size of its stipes and phialides (respectively smaller and shorter for ASls13 than the holotype), and with no production of sclerotia by ASls13, in contrast with the *A. pallidofulvus* holotype. This spe-

cies, recently introduced into section *Circumdati*, has also been isolated from green coffee beans in India and clinical samples (Visagie *et al*. 2014; Masih *et al*., 2016). The recovery of this species from grapes and the results of pathogenicity assays show that *A. pallidofulvus* may exhibit pathogenic behaviour in grapevine. Nevertheless, further investigation is required to determine occurrence for this fungus on withered grapes and its infectivity under post-harvest environmental conditions.

The use of multi-locus phylogenetic analysis (*CaM*, *benA* and ITS) resolved the taxonomic position of isolate AS100 (ITEM 18280), identifying it as *A. puulaauensis*. Assignment of the isolate to this species has previously proved impossible using only the *CaM* gene sequence (Lorenzini *et al*., 2016). Moreover, AS100 and the holotype *A. puulaauensis* NRRL 35641 (Jurjevic *et al*., 2012) both showed identical colony macro- and micro-morphological characters. The recovery of *A. puulaauensis* from grape berries further supports its worldwide and cosmopolitan distribution, since this species has previously been reported from disparate environments including Hawaiian plants, Atlantic sponges, Italian cheese, air samples in North America and clinical samples (Jurjevic *et al*., 2012; Siqueira *et al*., 2017; Bovio *et al*., 2018; Decontardi *et al*., 2018).

Based on multi-locus phylogenetic analyses, isolates Pdb1 (ITEM 18277) and P3 (ITEM 18276^{T}) represented a distinct species, herein named *P. fructuariae-cellae*. These two isolates form a phylogenetic cluster based on *benA*+*CaM*+ITS+*rpb2* combined gene genealogies, distinct from any currently described species in section *Lanata–divaricata,* which was recently updated with 13 new *Penicillium* species collected from Chinese acidic soils (Diao *et al*., 2018). *Penicillium fructuariae-cellae* is acid-preferential, like most of the new species described by Diao *et al.* (2018). This is a physiological characteristic congruent with the acidic habitat (grapes) from which it was isolated. Isolates of P3 and Pdb1 are distantly related to *P. vasconiae* CBS 339.79 (Ramírez and Martínez, 1980) and the recently described species *P. bissettii* (Visagie *et al*., 2016). Moreover, *P. fructuariae-cellae* displays some differences in macro-morphological characteristics (e.g. colony colour, texture and pigment production) compared with related species. The pigment production by these two isolates distinguishes them from *P. vasconiae* and *P. bissettii* that produce no pigments. *Penicillium fructuariae-cellae* produces monoverticillate, sometimes biverticillate conidiophores, whereas *P. vasconiae* is strictly monoverticillate and *P. bissettii* is biverticillate/terverticillate. The conidiophores of *P. fructuariae-cellae* are longer than those of *P. vasconiae* (< 50 μm; Ramírez and Martínez, 1980) and shorter than conidiophores of *P. bissettii* (190–670 μm; Visagie *et al.,* 2016). Its stipes are smooth, while in *P. bissettii* they are rough. Its phialides are also shorter than those of *P. vasconiae* (9–13 μm; Ramírez and Martínez, 1980). As well, of *P. fructuariae-cellae* produced smaller conidia than *P. vasconiae* (4–4.5 μm; Ramírez and Martínez, 1980). The conidial surfaces of *P. fructuariae-cellae* were smooth, while those of *P. vasconiae* are conspicuously echinulate (Ramírez and Martínez, 1980). These morphological differences together with phylogenetic information, support the uniqueness of *P. fructuariae-cellae*.

Based on the results of pathogenicity assays, the detrimental effects of these fungi on withered grapes was quite significant. Although these *Penicillium* and *Aspergillus* isolates were less pathogenic than *B. cinerea*, they could make major contributions to berry rotting. Decay of berry surfaces, like that observed on infected berries caused by each isolate due to mycelial growth and necrosis, is important for susceptibility to subsequent fungal infections by the same and other pathogens (Padgett and Morrison, 1990). Incidence and symptoms of grape diseases caused by these fungi under fruit-drying room conditions require further investigation.

In conclusion, this study describes isolates belonging to species of *Penicillium* and *Aspergillus* from withered grape berries that have not been previously reported from *V. vinifera*. A new species of *Penicillium* from this host, *P. fructuariae-cellae,* is herein described. The recovery of these species highlights the complexity of fungal species affecting withered grapes (Lorenzini *et al*., 2016; 2018). According to pathogenicity assays, *P. fructuariae-cellae*, *P. bilaiae*, *A. pallidofulvus* and *A. puulaauensis* are able to infect grapes but with much lower infectivity than *B. cinerea*, which is the most important pathogen occurring on withered grapes. It is likely that the species identified in this study are less pathogenic than other *Penicillium* and *Aspergillus* species frequently reported on withered grapes (e.g. *P. expansum, P. crustosum, A. tubingensis* and *A. uvarum*) (Lorenzini *et al*., 2016). Further investigations are necessary to ascertain the pathogenic role of *P. fructuariae-cellae, P. bilaiae, A. pallidofulvus* and *A. puulaauensis* under withering conditions, as well as their interaction with other causal fungal agents causing rots of grape berries.

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Short Notes

Introducing the potential biological control agent *Frateuria defendens* into pot- and field-grown grapevines

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Abstract. Bois Noir is a major yellows disease in grapevine with no current means of control. The endophyte *Frateuria defendens (Frd)*, isolated from the insect vector of Bois Noir, is a potential biocontrol agent for this disease. The aim of this study was to determine an efficient way to introduce *Frd* into mature vines under field conditions. Shoots of young and field-grown vines were sprayed with *Frd* suspension supplemented with either Tween 20, BB5, DX, or Triton X-100 as surfactants. The presence of *Frd* was confirmed by PCR in sprayed leaves, and in leaves below and above the sprayed site. The results showed that cells of *Frd* penetrate and move inside the vine shoots under field conditions. Highest penetration rate was achieved when leaves were sprayed with 10^8 or 10^9 colony forming units per mL (CFU mL^{-1}) of *Frd* with addition of 0.1% Tween 20. The addition of a surfactant is essential to increase the proportion of shoots with *Frd*.

Keywords. Biological control, yellows disease, phytoplasma, endophytes.

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INTRODUCTION

Bacteria from the phloem-restricted genus '*Candidatus* Phytoplasma' (Mollicutes; hereafter referred to as phytoplasma) are cell wall-less prokaryotes, formerly called mycoplasma-like organisms (Doi *et al*., 1967). Members of this genus are obligate parasites that reside only in the phloem vessels of plants and the hemolymph of their insect vectors. They are pathogens associated with yellows diseases in more than a thousand plant species worldwide, posing major threats to many agricultural crops, including stone fruit trees, vegetables and grapevine (Marcone *et al*., 2014). "Bois noir" (BN) is one of the most important grapevine yellows diseases in Europe and in the countries around the Mediterranean Sea. This disease is associated with the presence of '*Candidatus* Phytoplasma solani', (Quaglino *et al*., 2013) which is classi-

fied in the ribosomal subgroup 16SrXII-A. These phytoplasmas are mainly vectored to grapevine (*Vitis vinifera*) from weeds by the polyphagous planthopper *Hyalesthes obsoletus* Signoret (Hemiptera: Cixiidae) (Sforza *et al*., 1998). However, *H. obsoletus* is considered an occasional grapevine feeder (Foissac and Maixner, 2013). In Europe, it completes its life cycle mainly on annual herbaceous species such as bindweed and nettle (Sforza *et al*., 1998, Maixner 2009), whereas in Israel, its preferred host plant is Abraham's balm (*Vitex agnus-castus;* Sharon *et al*., 2005). Control of BN has met with major difficulties because: i) insecticide applications are ineffective due to the erratic presence of the insect on grapevines; ii) other than by injection, the target site is hard to reach with bactericides; and iii) the relatively efficient application of antibiotics is prohibited in several areas of the world due to risks to the human health and the environment. These challenges, combined with growing public pressure for the application of "green products" and the emergence of pesticide resistance, call for the development of alternative plant disease-control strategies.

One such alternative is harnessing endophytes as biocontrol agents, especially if they share the same niche as the target pathogen (Compant *et al*., 2005, 2013). Because spontaneous recoveries from phytoplasmas have been reported in grapevine, a role of endophytes in control of these diseases has been previously suggested (Compant *et al*., 2013). Bulgari *et al*. (2011) showed differences in endophyte populations monitored in infected *vs*. healthy grapevines. Moreover, yellows disease symptoms or the phytoplasma have never been recorded in Abraham's balm, the host plant of *H. obsoletus* in Israel (Sharon *et al*., 2005, 2015). Besides the obvious requirement of efficiency against the disease agent, a potential biocontrol agent must be cultured, able to reach the target sites and survive for a reasonable period inside plant tissues (Compant *et al*., 2013).

Frateuria defendens (*Frd*; formerly referred as DLB), a bacterium with endophytic characteristics, was isolated from *H. obsoletus* (Iasur-Kruh *et al*., 2016; Lidor *et al*., 2019). Its genome was fully sequenced and deposited in GenBank (accession number LFQR00000000; Lahav *et al*., 2016). The potential of *Frd* as a biocontrol agent was suggested based on the following evidence: i) it reduces the symptoms of grapevine yellows under laboratory conditions (Iasur-Kruh *et al*., 2018); ii) it can penetrate plants via leaf stomata and reside in the vascular systems including phloem vessels (Lidor *et al*., 2018); and iii) it survives inside grapevine plantlets for up to 4 weeks (Iasur-Kruh *et al*., 2018).

The interaction of *Frd* with the grapevine plants is not clear. Genome-based analysis that uses computer models for predicting metabolic interactions between bacteria showed no evidence of common nutrient uptake of the bacterium with phytoplasmas, ruling out competition over metabolites in the shared niche (Iasur-Kruh *et al*., 2018). In contrast, compounds secreted by *Frd* inhibit *in vitro* growth of *Spiroplasma melliferum*, suggesting the presence of antibiosis activity (Iasur-Kruh *et al*., 2016). Nevertheless, to test the endophyte's efficiency in mature plants and under field conditions, its penetration into field-grown grapevines must be optimized. In the case of biocontrol agents, addition of wetting agents to the cell suspensions increases their cell adhesion to leaf tissues and improves cell spread on leaf surfaces by reducing the surface tension of the suspension solution (Ongena and Jacques, 2008; Wyss *et al*., 2004).

The aim of the present study was to determine an efficient way of introducing *Frd* into mature grapevines under field conditions.

MATERIALS AND METHODS

Plants and bacteria

Semi-field experiments were conducted on 6-week-old *ex vitro* grapevine plants cv. Chardonnay in 0.3 L capacity pots, grown under controlled conditions of 25°C and a 16/8 h light/dark photoperiod (Experiment 1). Field trials were conducted on table grape grapevines cv. Early Sweet in a 5-y-old commercial vineyard, ca. 30 d after bud burst, when the shoots were 20–30 cm long (Experiments 2 and 3).

Pure cultures of *Frd*, originally isolated from *H. obsoletus* in 2011 (Iasur-Kruh *et al*., 2016), was used in all the experiments. The strain type DHoT is deposited in both the Netherlands Culture Collection of Bacteria (NCCB; 100648T) and the German Collection of Microorganisms and Cell Cultures (DSM; 106169T) (Lidor *et al*., 2019). For experimental purposes, the bacterial suspension was prepared as described by Lidor *et al*. (2018) with slight modifications. Stock cultures of *Frd*, generally kept at -80°C in a 30% glycerol, were freshly streaked onto a sugar-rich medium [6.6% sucrose, 1% sorbitol, 0.2% Luria broth, 1.5% agar, (all w/v)] and cultured at 28°C. Liquid *Frd* cultures were prepared by inoculating 3 to 5 mL starter containing nutrient broth (NB, Difco) or Luria-Bertani medium (LB, Difco) with a single *Frd* colony from the agar plates (colony morphology: smooth, circular, 1.0–1.5 mm diam., with yellow-pigment; Lidor *et al*., 2019). Starters were cultivated on a shaker for ca. 24 h at 28°C and then used to inoculate broth at a ratio of 1:100 (v/v) in an Erlenmeyer flask. Cultures of *Frd* were cultivated to a concentration of $\approx 10^9$ cfu mL^{-1} (verified by

plating). To achieve the desired concentration for spraying, the suspensions were serially diluted with tap water to 10% and 1%, which was equivalent, respectively, to 10^8 and 10^7 cfu mL^{-1}. A surfactant was added to the final suspension before spraying.

Frateuria defendens *application*

To test for ability of *Frd* to migrate within plants, the upper or lower part of each shoot was covered and sealed with a polyethylene bag to prevent wetting with the bacterial suspension (Figure 1). For these experiments, the leaves from potted plants with 3–4 nodes (ca. 15 cm long) and the leaves from the upper part of shoots (ca. 15 cm long) from field-grown grapevines were used. The uncovered parts of each plant were sprayed with a hand sprayer to run-off with *Frd* suspension supplemented with a surfactant. Each shoot of the potted plants was sprayed with 2–3 mL, while 50 mL of *Frd* suspension was applied to each field grapevine. Non-sprayed plants served in the experiments as negative controls to confirm that *Frd* was not naturally present in the grapevines. After ca. 1 h, when the suspension on the sprayed leaves had completely dried, the polyethylene cover was carefully removed from the plants.

DNA extraction and PCR analyses

To test the presence of *Frd* in the treated plants, one leaf from the covered and one from the uncovered parts of the shoots were sampled 7 d post inoculation (dpi), together with one of the uncovered leaves from the non-sprayed control shoots. Each sampled leaf was washed under running water with a commercial detergent, then externally disinfected by a 15 sec dip in 70% ethanol followed by 2 min in 0.5% sodium hypochlorite solution, followed by three consecutive washes in sterile water supplemented with 0.1% (v/v) Tween 20. The covered leaves were not washed.

DNA was extracted from 300 mg samples of leaf tissue using the cetyltrimethylammonium bromide (CTAB) method according to Lodhi *et al.* (1994). For each sample, leaf tissue was ground and mixed in 4 mL of extraction buffer (20 mM sodium EDTA, 100 mM Tris-HCl, pH 8.0, 1.4 M NaCl and 2.0% w/v CTAB). DNA was extracted with isoamylalcohol: chloroform (1:24, v/v), precipitated in cold isopropanol and 10% (v/v) 3 M sodium acetate, and stored overnight at -20°C. The pellet was then washed in 70% ethanol and dissolved in 40 µL Tris-EDTA buffer.

Specific primers based on 16S rRNA (Iasur-Kruh *et al.*, 2016) were used to determine the presence of *Frd* by PCR (DLBF: 5'-CTCTGTGGGTGGCGAGTGGC-3', DLBR: 5'-ACCGTCAGTTCCGCCGGG-3'). The PCR mix of each tube (25 µL) contained 10 µL Apex Taq DNA Polymerase Master Mix (Genorama), 5 pmol of each primer, 12.5 µL double-distilled water, and 1 µL DNA template. The PCR consisted of 35 cycles of 94°C for 0.5 min, annealing at 63°C for 0.5 min and 72°C for 0.5 min, followed by a final step of 10 min at 72°C.

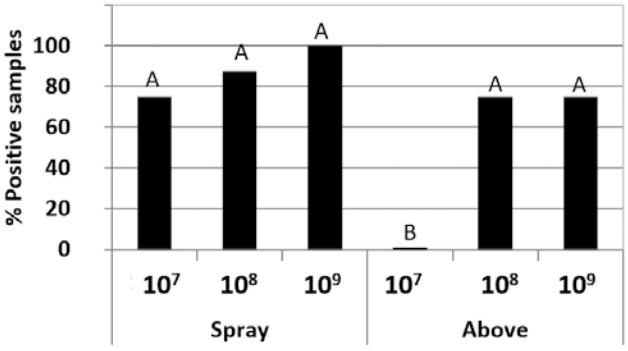

Figure 1. Mean proportions of grapevine tissue samples containing *Frateuria defendens* (*Frd*; determined by PCR) after spraying with various concentrations of bacterial suspension. *Frd* was monitored at and above spraying point of field grown grapevine plants 7 dpi (N = 8 per treatment, N = 6 per control). Since results showed statistically significant interactions between main effects (cell concentration of the sprayed suspension and point of inoculation) each variable is presented separately. Different letters indicate significant differences between categories (α = 0.05)

Experimental design and sampling

Experiment 1. To determine the most efficient surfactant, Tween 20 (TR1880-002; Tedia) and three commercially available surfactants commonly used in agricultural practice, BB5 (alkyl phenoxy polyethylene alcohol, Agrica CTS Ltd.), Triton X-100 (octyl phenyl polyether alcohol, Adama/Agan Ltd.) and DX (alkylaryl polyether alcohol, Adama/Agan Ltd.) were tested. Each surfactant was added to a 10^9 cfu mL^{-1} of the *Frd* suspension at concentrations that were chosen based on the respective manufacturers' recommendations for commercial usage (Table 1). For each treatment, the potted plants were divided into two groups: in the first group, the upper parts (three leaves) of seven plants was each covered and sealed with a plastic bag (Figure 1b); in the second group, the lower three leaves of seven plants were similarly covered (Figure 1a). The uncovered part of each plant shoot was sprayed with suspension of bacteria and surfactant. Control plants (n = 2) were not sprayed.

Experiment 2. The two most efficient surfactants determined in Experiment 1 were further tested under

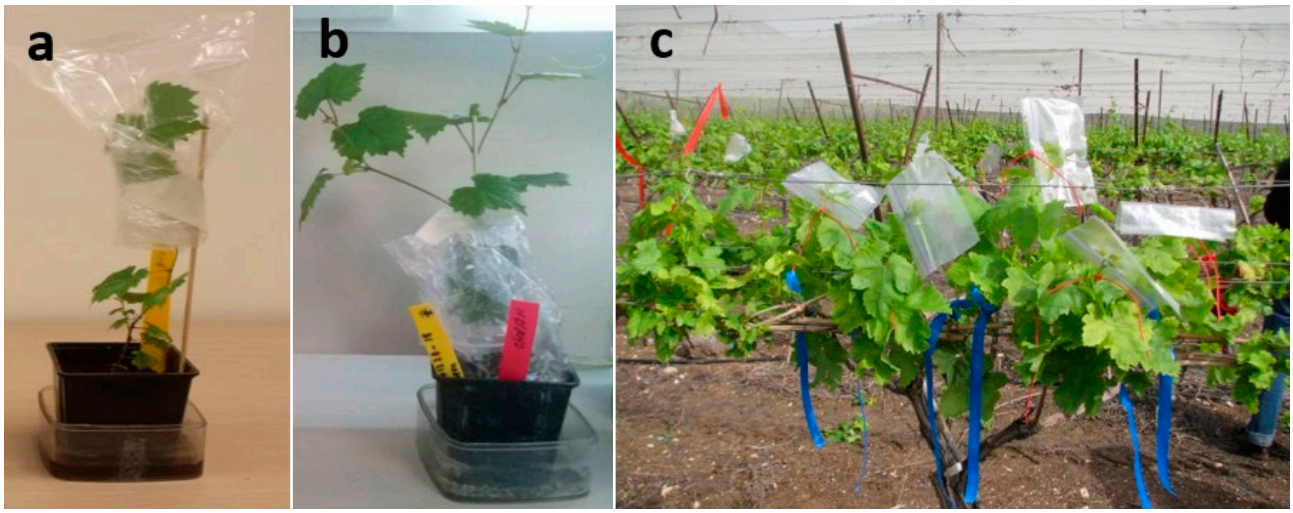

Figure 2. Spraying method. Shoots were partially covered and leaves were sampled 7 d post inoculation from uncovered and covered plant parts. Spray was applied on the shoots of: Lower (a) and upper (b) part of young plants in pots; (c) lower part of mature field grapevine (c).

field conditions. The upper part of each grapevine shoot was covered and sealed with a plastic bag (Figure 1c). Each surfactant was added to a 10^8 cfu mL^{-1} of *Frd* suspension which was sprayed on the uncovered parts of eight shoots.

Experiment 3. To determine the minimum bacterial concentration to give detectable *Frd* establishment in plant tissues in the field, the upper parts of the shoots of field-grown grapevines were covered (Figure 1c) and the uncovered parts were sprayed with *Frd* suspensions (treatments) at concentrations of 10^9, 10^8 or 10^7 cfu mL^{-1}. Tween 20 (0.1%) was added to each suspension as a surfactant. Each treatment was applied to eight shoots, and six marked non-sprayed shoots served as experimental controls.

Table 1. Mean proportions of grapevine tissue samples containing *Frateuria defendens* (*Frd*; determined by PCR) after spray application of bacterial suspensions on different leaf positions on potted grapevine plants 7 dpi (N = 7 per treatment). Main effects: surfactant type, and leaf position relative to sprayed site. Statistical significance was calculated separately for each main effect.

Treatment (%)	% Samples positive for *Frd*			
	On sprayed leaf	Above sprayed leaf	Below sprayed leaf	Main effect: surfactant ($P = 0.51$)
Tween 20 (0.1)	100	57	86	81 A[a]
BB5 (0.2)	100	29	86	71 A
DX (0.5)	100	57	57	71 A
Triton X-100 (0.03)	100	43	43	62 A
Main effect: leaf position ($P < 0.0001$)	100 a	46 b	68 a	

[a] Different letters indicate significant differences between categories ($\alpha = 0.05$): uppercase letters indicate differences between surfactants; lowercase letters indicate differences between leaf positions on the shoots.

Statistical analyses

Descriptive statistics, analysis of variance and Fischer LSD tests were performed with JMP software. Because the non-sprayed shoots in all experiments served as negative controls to confirm that no spray had drifted (*Frd* not present), they were not included in the statistical analyses. The statistical analysis of Experiment 1 showed no significant interactions between the main effects (data not shown). However, in Experiment 3 the statistical analysis showed a significant interaction between cell concentration of sprayed suspension and leaf position, (Figure 2).

RESULTS AND DISCUSSION

Frateuria defendens has been reported as a potential biocontrol agent against phytoplasmas and other phloem-restricted pathogens (Lidor *et al.*, 2018). Because *in planta Frd* occupies the same niche as the target organism and penetrates via plant leaves (Iasur-Kruh *et al.*, 2018; Lidor *et al.*, 2018), it could serve as control against phloem residing pathogens. The presence of *Frd* in leaves of field-grown grapevines 7 dpi was confirmed by PCR (Table 1, Figure 1). In contrast, *Frd* was not detected in

control plants in Experiments 1 or 2, confirming that the bacterium did not naturally inhabit the grapevines. This also indicated that the bacterium was present in the leaf samples solely from its penetration following applications on the leaves.

To promote the potential of *Frd* for application to grapevines, three types of surfactant that are commercially available and commonly used in agricultural practice were compared to Tween 20. When young plants were sprayed, there was no significant difference among the different surfactants, although Tween 20 gave the best results (Table 1). Adding Tween 20 or BB5 to the solution resulted in a better penetration of *Frd* into leaves, as was shown by the greater percentage of positive of leaves below the spraying point scompared to DX and Triton X-100 (Table 1). Therefore, Tween 20 and BB5 were further tested under field conditions. In this experiment, *Frd* was detected in 63% of the samples from leaves above the spraying points, 7 d post-Tween 20 application, whereas all the samples from the BB5 treatment were negative. Therefore, Tween 20 was used in the experiment to optimize bacterial concentration in the spraying solutions (Experiment 3).

Frd has been shown to penetrate perennial and annual plant species of various plant families (Lidor *et al*., 2018), where GFP-labeled bacteria were observed in the vascular systems. The results from the present study broadened these observations by showing that *Frd* can move inside grapevines both upward and downward from original application points in young plants (Table 1). The ability of *Frd* to penetrate the leaves of mature grapevines and move one node upward in the plants was also demonstrated under field conditions (Figure 2).

Spraying is the best method for introducing *Frd* in the plant tissues (Iasur-Kruh *et al*., 2018, Lidor *et al*., 2018). Since spraying intervals in agricultural practice range from several days to weeks, the mobility of the biocontrol agent inside treated plants is critical for its colonization of the non-sprayed plant parts that grow between spray applications. The fact that *Frd* cells penetrate and move along grapevine shoots increases the bacterium's potential as a biocontrol agent (Table 1, Figure 2).

The cell concentration in the applied suspensions possibly affected *Frd*'s ability to penetrate leaves under field conditions. In non-sprayed leaves of treated plants (above the spraying point), *Frd* was detected 7 dpi only when its concentration in suspension was 10^8 cfu mL^{-1} or greater. This indicates that penetration rate and survival are both concentration-dependent (Figure 2). Since laboratory assessment of growth rate showed that *Frd* accumulates in the growth medium to 10^9–10^{10} cfu mL^{-1} within 3–4 d (Lidor *et al*., 2018), it is possible to dilute the growth culture 10- and 100-fold without affecting the rate of penetration into the plant tissues.

The fact that *Frd* reduced yellows symptoms in grapevine plantlets (Iasur-Kruh *et al*., 2018) combined with the results of the present study lead to the hypothesis that the bacterium is a potential biocontrol agent. The results indicate that in order to verify the efficacy of *Frd* to reduce symptoms of yellows disease in grapevine under field conditions, foliar spray should be applied at 7 d intervals with bacterial suspensions of 10^8 cfu mL^{-1} supplemented with 0.1% Tween 20.

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New or Unusual Disease Reports

Morphological and molecular characterization of *Neoscytalidium novaehollandiae*, the cause of *Quercus brantii* dieback in Iran

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Summary. During the period from 2013 to 2015 a tree health survey was conducted in Persian oak (*Quercus brantii* Lindl.) forests throughout Kermanshah province in Western Iran. Oak trees showing dieback of branches and stems were sampled, and fungal colonies resembling those of *Neoscytalidium* sp. were obtained from diseased tissues. Based on morphology and phylogeny of DNA sequence data for the internal transcribed spacer (ITS) rDNA, nuclear ribosomal large subunit (LSU) and translation elongation factor 1-alpha (TEF 1α) gene regions, all isolates were identified as *Neoscytalidium novaehollandiae*. Pathogenicity tests were carried out on 2-year-old, potted *Q. brantii* plants and on detached branches under partially controlled conditions. Pathogenicity tests showed that all the isolates were able to infect, and cause disease symptoms on, inoculated branches and plants. This is the first report of *N. novaehollandiae* as the causal agent of *Q. brantii* dieback.

Keywords. Brant's oak, Persian oak, emerging fungal pathogens, Kermanshah forests.

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INTRODUCTION

Zagros oak forests are the largest forest type in Iran, covering ca. 40% of the country's forest area (Sagheb-Talebi *et al*., 2004). The genus *Quercus* L. (*Fagaceae*) is one of the most diversified groups of temperate trees, with approx. 500 species distributed worldwide (Mehrnia *et al*., 2012). Kermanshah oak forests in West Iran are currently affected by serious oak dieback symptoms, including extensive leaf yellowing and dieback of branches and stems. Recent studies showed that numerous pathogenic fungi and oomycetes are involved in the dieback, including *Biscogniauxia mediterranea*, *Fusicoccum quercus*, *Ophiostoma querci*, *Diplodia mutila, Apiognomonia quercina, Stereum rugosum*, *Pezicula cinnamomea*, *Ceratocystis fagacearum*, *Dothiorella iberica*, and *Phytophthora ramorum* (Simonin *et al*., 1994; Grünwald *et al*., 2012; Mirabolfathy, 2013; Lynch *et al*., 2013).

The genus *Neoscytalidium* (Botryosphaeriaceae) was recently established by Crous *et al*. (2006). *Neoscytalidium dimidiatum* (Penz.) Crous and Slip-

pers, has a broad host range and wide distribution (Ray *et al*., 2010), and has often been reported to be associated with trees showing symptoms of decline, such as wilting and dieback. The fungus has been isolated from deciduous fruit trees and shade trees in Egypt, South Tunisia, California, Jamaica, Iraq, Niger, West Africa, India, China and Europe (Nattrass, 1933; Calavan and Wallace, 1954; Sommer, 1955; Meredith, 1963; Natour and El-Haider, 1967; Giha, 1975; Reckhans and Adamou, 1987; Cao and Wang, 1989; Granata and Sidoti, 1991; Harsh and Tiwari, 1992; Matheron and Sigler, 1993; Elliott *et al*., 1997; Msikita *et al*., 1997; Tsahouridou and Thanassoulopoulos, 2000; Elshafie and Ba-Omar, 2001; Namsi *et al*., 2010). Pavlic *et al*. (2008) described a new species isolated from asymptomatic and dying branches of *Acacia synchronica*, *Adansonia gibbosa*, *Crotalaria medicaginea* and *Grevillea agrifolia*, and named it as *Neoscytalidium novaehollandiae*. This fungus has since been reported as a pathogen of *Mangifera indica* in Australia (Ray *et al*., 2010), and was isolated from bark beetle galleries in *Ulmus densa* in China (Zhu and Liu, 2012). In Iran, *N. dimidiatum* has been isolated from *Ficus religiosa*, *Psidium guajava*, *Pistachia vera* and *Punica granatum* (Aminaee and Ershad, 1993; Ghelichi *et al*., 2012).

Despite the importance attributed to *Neoscytalidium* species as disease agents of fruit and forest trees, no study has been carried out to investigate a possible association between *N. novaehollandiae* infections and the extensive oak dieback that has been recently observed in Kermanshah province in Western Iran. The aim of the present study was to verify this putative association by isolating and identifying *Neoscytalidium* species from *Quercus brantii* individuals showing dieback symtoms in Iran, and to clarify the possible involvement of these fungi in the disease by means of artificial inoculations in controlled conditions.

MATERIALS AND METHODS

Sampling and morphological characterization of fungi

During the period from 2013 to 2015, a tree health survey was conducted in *Q. brantii* forests throughout Kermanshah province in Western Iran. Samples were collected from oak trees exhibiting symptoms of yellowing of the leaves and dieback of branches, and these were brought to the plant pathology laboratory in the Department of Plant Protection of Razi University, Kermanshah for further examination. Wood pieces were surface sterilized by 0.5% sodium hypochlorite for 1–3 min, rinsed with sterile distilled water, blotted dry with sterile paper towels and plated onto potato dextrose agar (PDA) and malt extract agar (MEA) amended with chloramphenicole (25 µg mL^{-1}). Micromorphological features of the fungal mycelium were studied using a light microscope (Olympus model BX-51), and images were captured with a camera (Canon Powershot model SX10). Fifty measurements of the observed fungal structures, including arthrospores, muriform conidia and pycnidiospores, were made using the BioloMICS software (Robert *et al*. 2011).

Molecular phylogenetic identification of fungi

The DNA was extracted from 5-d-old mycelium from seven isolates cultivated on PDA, using the method described by Gardes *et al*. (1991). The ITS-1 and ITS-4 primer pairs (White *et al*., 1990) were used to amplify the ITS1+5.8S+ITS2 of the ribosomal RNA. The EF1F and EF2R primer pair (Burgess *et al*., 2005) was used to amplify the portion of the translation elongation factor 1-alpha (EF1) gene. The LROR and LR5 primer pair (Slippers *et al.,* 2013) was used to amplify the nuclear ribosomal large subunit (LSU) gene region. All PCR reactions were conducted in 25 µL containing 20 ng genomic DNA, 1 µM of each primer, 100 µM of each dNTP, 0.5 U Taq DNA polymerase (CinnaGen, Iran), 1.5 mM of MgCl2, 2.5 µL of 10 × PCR buffer (CinnaGen, Iran), and 14.5 µL H_2O. The PCR reactions were performed in a T-Personal thermocycler (Biometra). PCR conditions were as follows: an initial denaturation step of 5 min at 95°C, 35 cycles each at 95°C for 60 s, annealing at 55°C for 80 s, elongation at 72°C for 90 s, followed by a final elongation step of 10 min at 72°C. The amplification products were visualized by electrophoresis in a 1% TBE-agarose gel. After sequencing (Tech Dragon,), the nucleotide sequences were edited using BioEdit Sequence Alignment Editor v. 7.2.5 software (Hall, 1999), and a similarity search in the GenBank sequence database was performed using BLAST service in NCBI (http://blast.ncbi.nlm.nih.gov). All sequences were deposited in GenBank. For phylogenetic analysis, ITS, LSU and EF1 data sets from this study were combined with sequences of *N. novaehollandiae* downloaded from GenBank (Table 1). *Lecanosticta acicola* and *Spencermartinsia viticola* were used as an outgroup (Slippers *et al*., 2013). DNA sequences were aligned with ClustalW (http://www.clustal.org/download, Thompson *et al.* 1994), and manually edited with BioEdit v. 7.2.5. Phylogenetic analyses were performed with MEGA5 software (http://megasoftware.net/) using neighbor-joining and maximum likelihood (ML) methods (Tamura *et al*., 2011). The best fit nucleotide substitution model (Tamura 3-parameter) was based on the

Table 1. Isolates of fungi used in this study, their locations and their NCBI GenBank. Sequence accession numbers for the Large Subunit of the nuclear ribosomal RNA (LSU), Internal Transcribed Spacer (ITS) and Translation elongation factor 1-alpha (EF-1) gene portions.

Species	Isolate ID	Location	GenBank Accession No.		
			LSU	ITS	EF1
Neoscytalidium novaehollandiae	CMW 26170	South Africa	KF766374	-	-
N. novaehollandiae	CBS 122071	Australia	-	KF766207	EF585580
N. novaehollandiae	NeNo1	Iran	MH899579	KY499712	MF662595
N. novaehollandiae	NeNo2	Iran	MH899580	KY499713	MF662596
N. novaehollandiae	NeNo3	Iran	MH899581	MH883623	MH885094
N. novaehollandiae	NeNo4	Iran	MH899582	MH883624	MH885095
N. novaehollandiae	NeNo5	Iran	MH899583	MH883625	MH885096
N. novaehollandiae	NeNo6	Iran	-	MH883626	MH885097
N. novaehollandiae	NeNo7	Iran	-	MH883627	MH885098
N. novaehollandiae	WAC12688	Australia	NG059496	EF585543	EF585575
N. dimidiatum	IP127881	Netherlands	DQ377925	AY819727	EU144063
N. dimidiatum	NDFB003	Mexico	MF508740	EF585537	EF585577
N. dimidiatum	CBS:125695	Netherlands	KX464535	GU172385	GU172417
N. dimidiatum	CBS:125616	Netherlands	KX464534	GU172389	GU172421
N. dimidiatum	CBS 312.90	Netherlands	DQ377924	GU172383	GU172415
Barriopsis fusca	CBS 174.26	Netherlands	KF766317	KF766149	KF766395
B. iraniana	IRAN 1448C	Iran	KF766318	KF766150	FJ919652
Botryosphaeria agaves	MFLUCC 10-0051	Thailand	JX646807	JX646790	JX646855
B. corticis	CBS 119047	Netherlands	EU673244	DQ299245	EU017539
B. dothidea	CMW 8000	South Africa	KF766319	KF766151	AY236898
Cophinforma atrovirens	MFLUCC 11-0425	Thailand	JX646817	JX646800	JX646865
C. atrovirens	MFLUCC 0655-11	Thailand	JX646818	JX646801	JX646866
Dichomera saubinetii	CBS 990-70	Netherlands	DQ377888	KF766153	KF766396
Diplodia africana	CBS 120835	Netherlands	KF766322	KF766155	KF766397
D. allocellula	CMW:36468	South Africa	JQ239410	JQ239397	JQ239384
D. allocellula	CMW:36469	South Africa	JQ239411	JQ239398	JQ239385
Dothiorella brevicollis	CMW:36463	South Africa	JQ239416	JQ239403	JQ239390
D. brevicollis	CMW:36464	South Africa	JQ239417	JQ239404	JQ239391
Endomelanconiopsis endophytica	CBS 120397	Netherlands	EU683629	KF766164	EU683637
E. microspora	CBS 353.97	Netherlands	KF766330	KF766165	EU683636
Kellermania anomala	CBS 132218	Netherlands	KF766343	KF766173	KF766404
K. confusa	CBS 131723	Netherlands	KF766344	KF766174	KF766405
Lasiodiplodia crassispora	CBS 118741	Netherlands	DQ377901	DQ103550	EU673303
L. gonubiensis	CMW 14077	South Africa	KF766361	KF766191	DQ458877
L. parva	CBS 456.78	Netherlands	KF766362	KF766192	EF622063
L. pseudotheobromae	CBS 116459	Netherlands	EU673256	KF766193	EF622057
Macrophomina phaseolina	CBS 22733	Netherlands	KF766364	KF766195	KF766422
Neofusicoccum eucalypticola	CMW 6539	South Africa	KF766368	KF766201	AY615133
N. luteum	CMW 10309	South Africa	KF766369	KF766202	KF766424
N. parvum	CMW 9081	South Africa	KF766371	KF766204	KF766426
Phaeobotryosphaeria eucalypti	MFLUCC 11-0579	Thailand	JX646819	JX646802	JX646867
P. porosa	CBS 110496	Netherlands	KF766375	KF766210	EU673130
Pseudofusicoccum adansoniae	CMW 26147	South Africa	KF766386	KF766220	EF585571
P. ardesiacum	CMW 26159	South Africa	KF766387	KF766221	EU144075
Spencermartinsia viticola	CBS 117006	Netherlands	KF766392	KF766228	AY905559

bayesian information criterion (BIC) and was implemented in MEGA 5. The confidence values of phylogenetic trees were assessed by calculating 1,000 bootstrap re-samplings.

Growth studies

All fungus isolates obtained from oak trees from throughout Kermanshah province were transferred to PDA plates and incubated at different temperatures at 5°C intervals from 10 to 45°C. Three plates were used for each isolate and temperature combination. Diameters of individual colonies were measured after 1 week of incubation.

Laboratory pathogenicity test

All isolates (n = 14) were used for laboratory pathogenicity testing on detached branches, under partially controlled conditions. The middle parts of healthy oak branches (each 20 cm long) were surface sterilized with 75% ethyl alcohol. A superficial wound was made in each branch by plunging a 10 mm diam. cork borer into the bark to a depth of 0.5 cm, and a mycelium plug obtained from the margin of a growing fungal colony was placed in the wound and wrapped with Parafilm. Inoculated branches were stored in sterilized glass jars each containing a moist sponge, and were then incubated on a 12 h : 12 h light:dark cycle at 25°C for 25 d until the appearance of the disease symptoms (Banihashemi and Javadi, 2009).

Greenhouse pathogenicity test

Pathogenicity of the isolates was also tested in a greenhouse under partially controlled conditions (natural day/night length, 25–27°C, 60–70% RH). Stems (approx. 15 mm diam., and 25-30 cm length) from 2-year-old seedlings (three plants per fungal isolate) were used. The stems were each inoculated ten cm above soil line using a 5 mm diam. cork borer to expose the cambium. A plug of mycelium (5 mm diam.) was placed into the wound, with the mycelium facing the cambium, and the inoculation point was sealed with Parafilm to reduce desiccation. The length of lesions that formed under the stem bark and on the cambium were measured after 6 weeks. Surface sterilized stem pieces were then taken from necrotic tissues and were plated on PDA, and if *N. novaehollandiae* was re-isolated, Koch's postulates were fulfilled.

Host range study

Detached branches of seven woody plant species sampled from the studied oak forest, including *Ficus carica*, *Acer monspessulanum*, *Crataegus aronia*, *Amygdalus scoparia*, *Cornus mas*, *Pistacia atlantica* and *Pinus eldarica,* were inoculated with three representative fungal isolates under laboratory conditions, using the method described above. Symptoms developing on the branches were assessed 25 d after inoculation.

RESULTS AND DISCUSSION

Fourteen isolates resembling *Neoscytalidium* were recovered from diseased oak trees displaying dieback symptoms, collected in oak forests throughout Kermanshah province in Western Iran. Affected oak trees showed various symptoms including; yellowing of the leaves, dieback of branches and stems, reddish-brown cankers on branches, and internal dark brown vascular necrosis in cross sections of the branches and stems (Figure 1). *Neoscytalidium*-like colonies were consistently isolated from symptomatic tissues on PDA and MEA. Fungal colonies in these cultures were dark blackish, and the hyphae were medium to dark brown and smooth. Arthroconidia were medium to dark brown, smooth, mostly non-septate but occasionally with one dark transverse septum, cylindrical, spherical to subspherical and measured 4–10 × 3–6 µm (Figure 2D). Dictyospores were spherical to subspherical, measuring 6.7–10.3 × 4.8–9.9 µm (Figure 2E).

All the *Neoscytalidium*-like isolates that were sequenced showed 100% sequence homology with *N. novaehollandiae* (GenBank KF766207, Slippers *et al*., 2013) at all three sequenced regions, i.e. ITS (Genbank accession nos KY499712, KY499713, MH883623, MH883624, MH883625, MH883626, MH883627), EF1 (accession nos MF662595, MF662596, MH885094, MH885095, MH885096, MH885097, MH885098) and LSU (accession nos MH899579 to MH899583). In phylogeny trees based on ITS, LSU and EF1 sequences, isolates from Iran clustered to a distinct monophyletic clade together with *N. novaehollandiae* isolates described by other authors (Figures 3 and 4).

Based on morphology and phylogenetic analyses, all isolates were identified as *N. novaehollandiae*. Pavlic *et al*. (2008) showed that *N. novaehollandiae* produced muriform, Dichomera-like conidia that distinguish this species from known *Neoscytalidium* species. The ITS regions of the genomic ribosomal RNA gene and part of the tranlation elongation factor 1-alpha gene were previ-

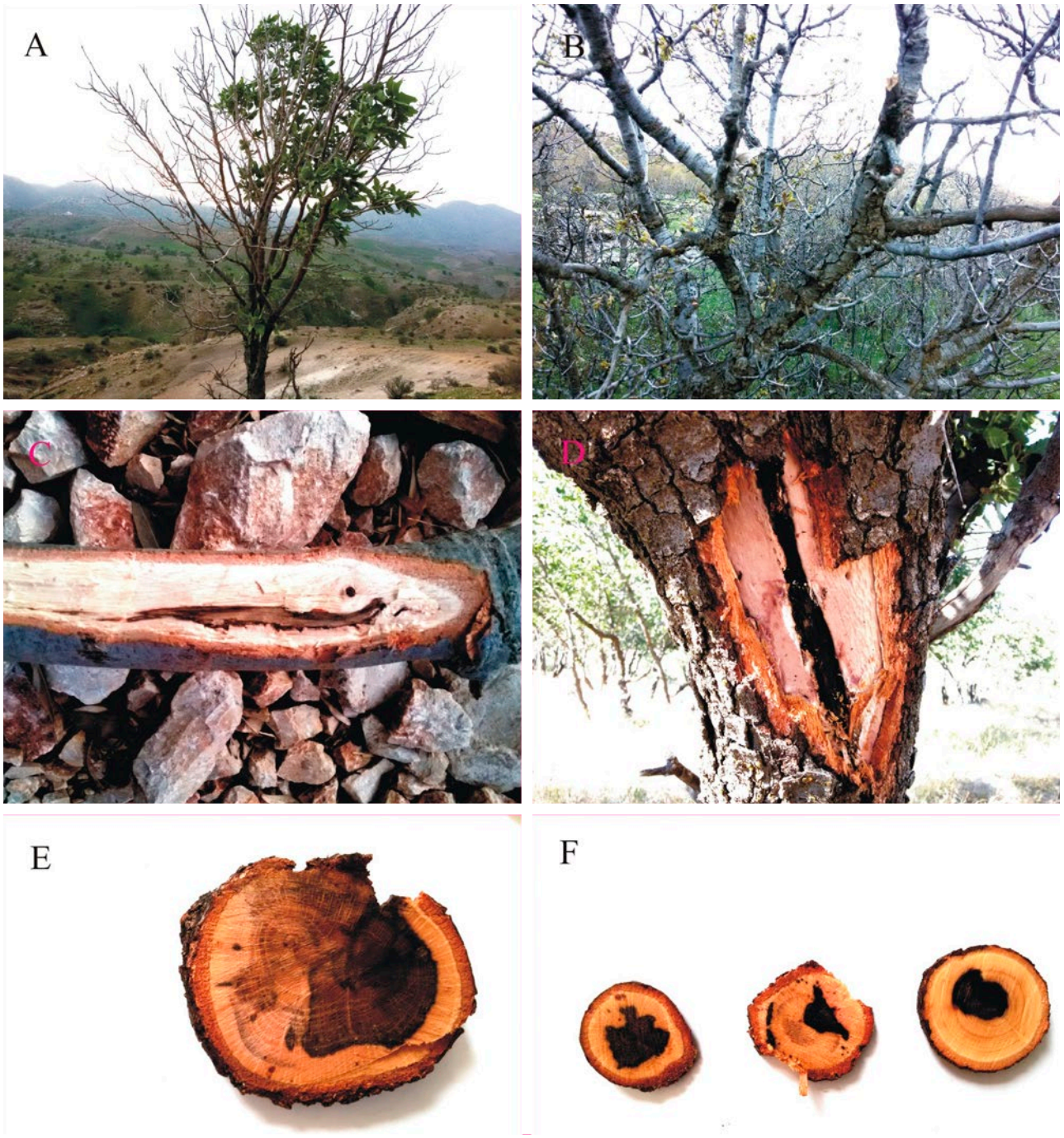

Figure 1. Symptoms of dieback and decline (A and B), canker (C and D, longitudinal sections), and watery necrosis and back staining (E and F cross sections), caused by *Neoscytalidium novaehollandiae* on Persian oak trees in Iran.

ously successfully applied for discriminating *Neoscytalidium* species (Burgess *et al*. 2005; Ray *et al*. 2010). An inferred phylogeny based on ITS, LSU and EF1 sequences clustered the isolates in a distinct monophyletic group consisting of sequences of *N. novaehollandiae* generated in previous studies (Figures 3 and 4). The phylogenetic trees inferred by both neighbor-joining (not shown) and maximum likelihood methods showed very similar topology, although there were minor differences in the bootstrapping percentages.

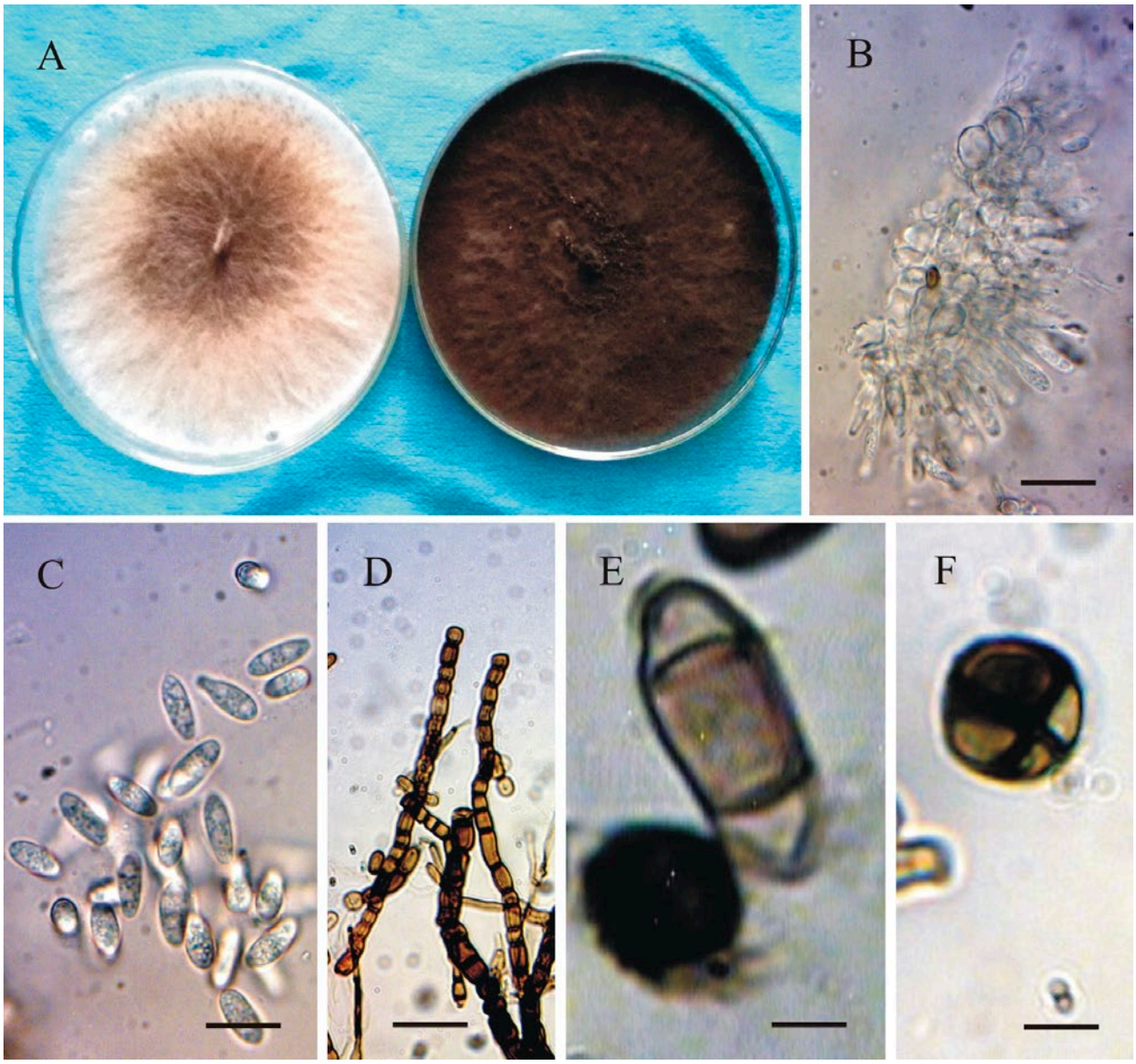

Figure 2. Morphological features of *Neoscytalidium novaehollandiae* isolates from diseased Persian oak trees: A,1-week-old colony grown on PDA; B, conidiogenous cells with developing conidia; C, immature conidia; D, arthrospores; E, mature conidium; and F, muriform conidia. (Bars = 5 µm in and 17 µm in B. C, D and F).

Pathogenicity tests revealed that all 14 isolates of *Neoscytalidium* from this study were pathogenic to *Q. brantii* seedlings. Fourty five days after inoculation, cankers extending both upward and downward from the points of inoculation were evident on the stems of all the inoculated seedlings (Figure 5). Inoculated plants also showed yellowing, wilting and internal necrosis. *Neoscytalidium novaehollandiae* was re-isolated (100%) from the inoculated seedlings, thus fulfilling Koch's postulates. No symptoms were observed on the mock-inoculated controls.

Neoscytalidium novaehollandiae was described from Australia as a pathogen of *Mangifera indica* (Pavlic *et al*., 2008; Ray *et al*., 2010). This fungus was also associated with dying branches of *Adansonia gibbosa*, *Crotalaria medicaginea, Acacia synchronica*, and *Grevillea agrifolia* in Western Australia (Pavlic *et al*., 2008). Results from the present study showed that *N. novaehollandiae* isolates produce cankers on *Q. brantii* seedlings. This report is the first describing occurrence, molecular characterization and pathogenicity confir-

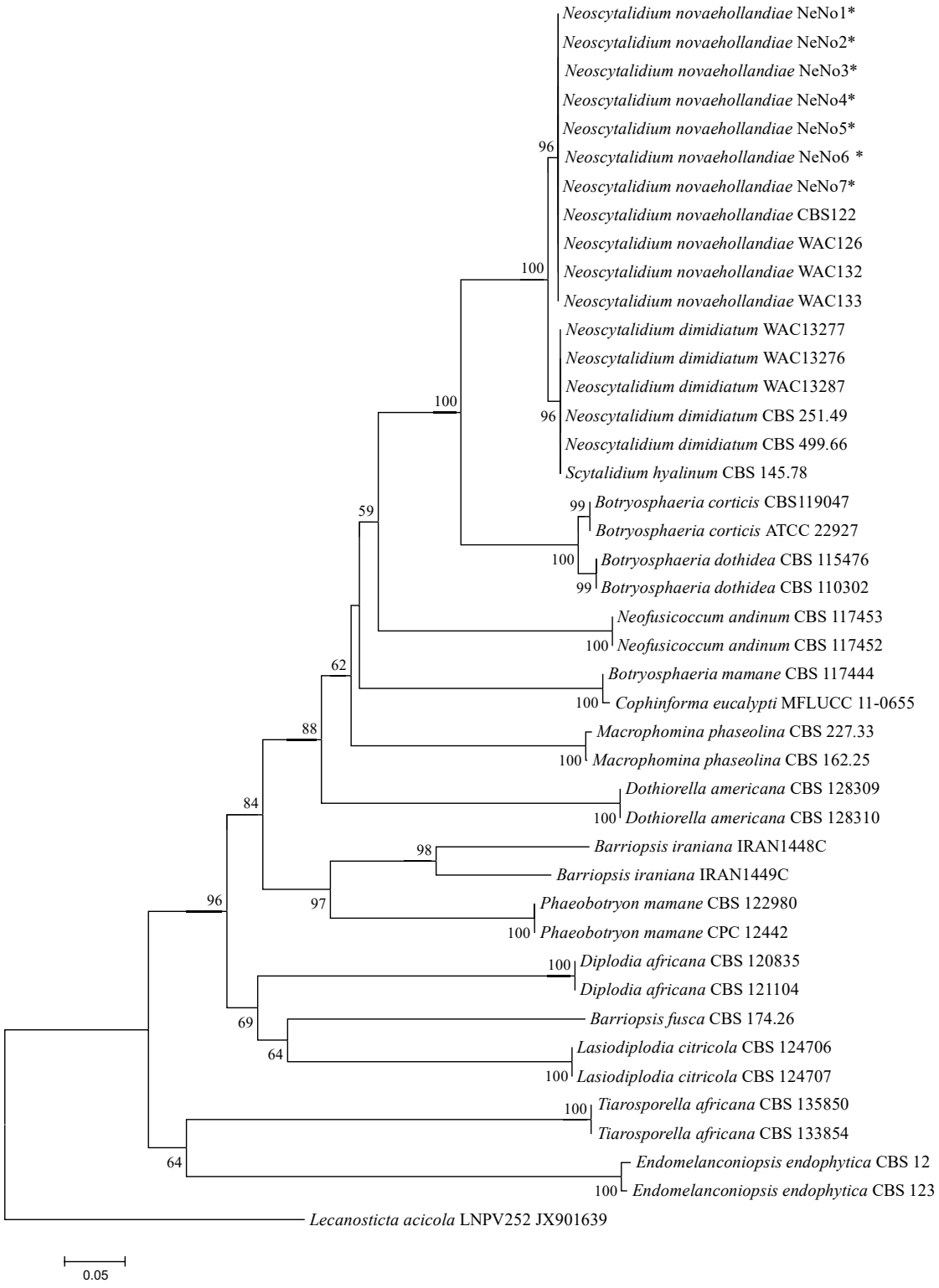

Figure 3. Maximum likelihood phylogram generated in Mega 5 from the alignment of 42 combined internal transcribed spacer (ITS) and translation elongation factor 1-alpha (EF1) gene portion datasets, using the Tamura 3-parameter model with complete deletion gap handling and 1,000-replication bootstrapping. The values associated with each horizontal the line denote bootstrap support for the node. Isolates of *Neoscytalidium novaehollandiae* cluster to a highly supported terminal clade separate from isolates of *N. dimidiatum*. The isolates from Iran are each marked with a star.

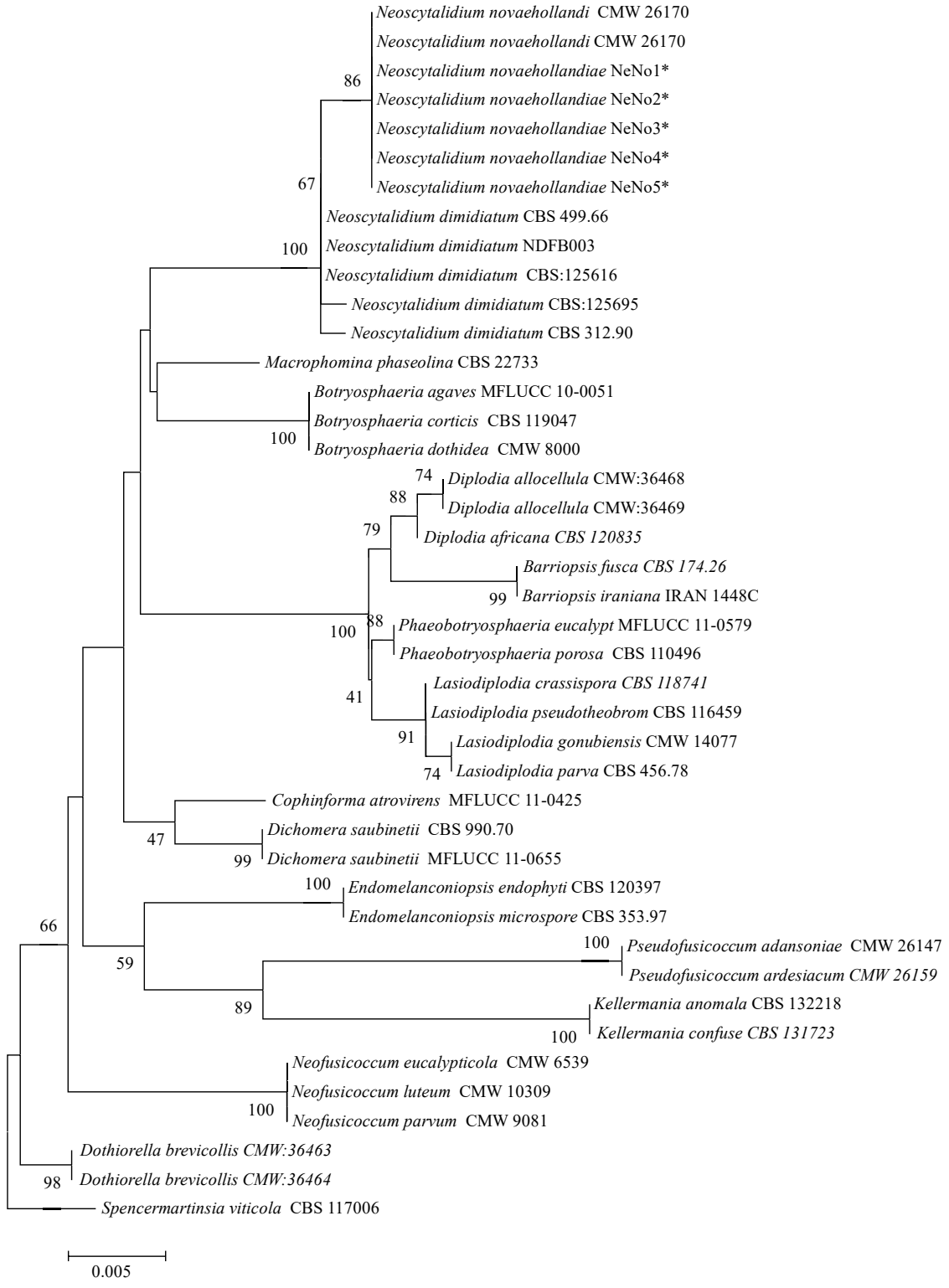

Figure 4. Maximum likelihood phylogram generated in Mega 5 from the alignment of 42 combined internal transcribed spacer (ITS), translation elongation factor 1-alpha (EF1) and nuclear large subunit (LSU) gene portion dataset using Tamura 3-parameter model with complete deletion gap handling and 1000-replication bootstrapping. The values above the line denote bootstrap support for the node. Isolates of *Neoscytalidium novaehollandiae* reside in a highly supported terminal clade separate from isolates of *N. dimidiatum*. The isolates from Iran are marked with an asterisk.

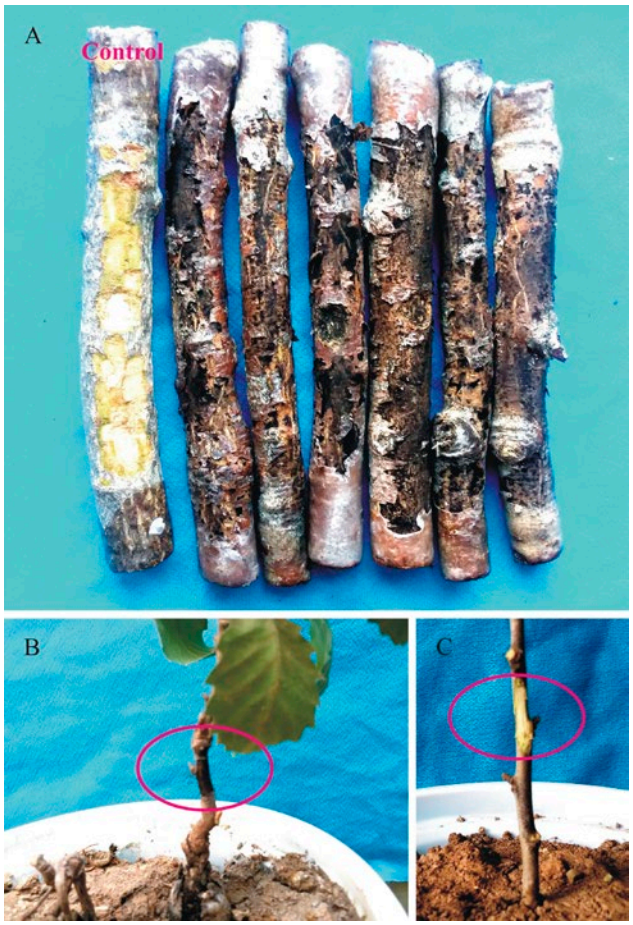

Fig.5. Results of pathogenecity tests performed by inoculating *Neoscytalidium novaehollandiae* isolates from declining Persian oak trees in Persian oak seedlings under laboratory and greenhouse conditions: A. Canker on detached stems, B. Vascular extent of necrotic tissue in an oak seedling 45 days post inoculation with *Neoscytalidium novaehollandiae*, C. Control.

mation for *N. novaehollandiae* causing Persian oak dieback in Iran.

The host range study showed that *N. novaehollandiae* isolates from Iran produced cankers on branches of all other inoculated tree species except for *Pinus*. This information may be useful in the management of this pathogen especially in urban areas.

The minimum, optimum and maximum temperatures for growth of the *N. novaehollandiae* isolates were, respectively, 10, 35 and 40°C, and no growth occurred at 45°C. Similarly, Jamali and Banihashemi (2010) reported that maximum temperature for growth of *N. dimidiatum* was 37°C. *Neoscytalidium* has been reported to be aggressive on drought stressed hosts (Calavan and Wallace, 1954). In spite of the tolerance of Persian oak species to non-optimum temperatures, the emergence of *N. novahollandiae* in *Q. brantii* forests may be related to climate change. Recent temperature increases in the region may have prolonged the host growing seasons, while shortage of rainfall has probably increased the intensity of dehydration in this forest. Desiccation of woody tissues due to drought stress leads to reduced mechanical strength of the bark-wood bonds, and may result in bark cracks that can be invaded by opportunistic fungal pathogens (Bettucci *et al*., 1999).

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New or Unusual Disease Reports

Leveillula lactucae-serriolae on *Lactuca serriola* in Jordan

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Summary. Jordan contributes significantly to the Near East plant biodiversity with numerous primitive forms and species of crops and their wild relatives. Prickly lettuce (*Lactuca serriola*) is a common species in Jordan, where it grows in various habitats. During a survey of wild *Lactuca* distribution in Jordan in August 2007, plants of *L. serriola* with natural infections of powdery mildew were observed at a site near Shobak (Ma'an Governorate). *Lactuca serriola* leaf samples with powdery mildew infections were collected from two plants and the pathogen was analyzed morphologically. Characteristics of the asexual and sexual forms were obtained. Sequence analyses of the rDNA ITS region and D1/D2 domains of the 28S rDNA were used to obtain phylogenetic data, and to reach taxonomic conclusions about these specimens. Molecular determination, performed by sequencing of the ITS region, proved its identity with the type material of *Leveillula lactucae-serriolae*. Sequencing of the 28S rDNA region provided the first verified GenBank record of *Leveillula lactucae-serriolae* deposited in this public nucleotide repository. This is the first taxonomically verified record of *L. lactucae-serriolae* on *L. serriola* growing wild in Jordan, and one of the first records of the fungus in the Near East.

Keywords. Lettuce powdery mildew, morphology, Near East, prickly lettuce, ITS and and 28S rDNA region.

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INTRODUCTION

Powdery mildews are biotrophic pathogenic fungi from the order Erysiphales, which mostly exhibit exoparasitic life strategies (Braun and Cook, 2012). On the genus *Lactuca*, at least three biologically and ecologically different genera of powdery mildews (*Golovinomyces*, *Podosphaera*, *Leveillula*) are known (Lebeda and Mieslerova, 2011), and a *Pseudoidium* type of powdery mildew (without exact determination) was detected on *L. viminea* in the south France (Lebeda *et al*., 2002). However, from this survey, it is also evident that the genus *Leveillula* is rare on *Lactuca* species. *Leveillula* has 40 species, and is an endoparasitic genus (Braun and Cook, 2012). It is considered to be adapted to xerophytic conditions, and is mainly distributed in arid and

warm areas of Africa, Asia, southern Europe, and southern North America to South America (Braun and Cook, 2012). The most known and common pathogen is *Leveillula taurica*, which has a broad host range. *Leveillula* spp. have been recorded on host 221 species in 78 genera of the Asteraceae. As a result, there is high genetic diversity in *Leveillula* spp. affecting Asteraceae (Palti, 1988).

Two different species concepts of *Leveillula* have been previously applied (Braun and Cook, 2012). These pathogens were regarded as highly specialized strains, as well as races which were able to infect a wide range of hosts belonging to various plant families. This ability was recently confirmed by the phylogenetic examinations of Khodaparast *et al*. (2001). In the past, Salmon (1900) recognized a single species, *Erysiphe taurica sensu latissimo*. He reduced all related taxa to synonymy with this species. Jaczewski (1927) divided *L. taurica* into numerous "formae" – i.e., one forma for each host genus. Golovin (1956) represented the other extreme in the treatment of *Leveillula*. He tried to split *Leveillula* into numerous species based on conidium shape and size as well as host range. He proposed one species for each host family, and his classification was, therefore, schematic and did not solve the phylo-taxonomic problems.

The detailed studies (including morphological and molecular analyses) of *Leveillula* were conducted mainly by Khodaparast and colleagues (Khodaparast *et al*., 2001; Khodaparast *et al*., 2007; Khodaparast *et al*., 2010; Khodaparast *et al*., 2012). In the most recent work, Khodaparast *et al*. (2012) performed phylogenetic analyses of ITS rDNA of 76 *Leveillula* specimens from different host families, and these suggested the maintenance of high phylogenetic variability in *Leveillula* on Asteraceae. The phylogenetic tree showed that powdery mildew on: a) *Carthamus, Crepis, Gundelia* and *Helianthus* established clade No. 1; b) *Cirsium, Lactuca serriola, Echinops* formed clade No. 2; c) *Centaurea, Launaea, Picris, Thevenotia* formed clade No. 5, and d) *Chondrilla, Acroptilon, Artemisia* and *Lactuca orientalis* were each in a separate clade. The phylogenetic variability confirmed that *Leveillula* spp. have probably colonized the Asteraceae several times during evolution, because isolates from this host family include genetically divergent taxa comprising several independent lineages (Khodaparast *et al*., 2001).

The study of *Leveillula* species solely on Asteraceae was done mainly by Khodaparast *et al*. (2010). They evaluated *Leveillula* collections on Asteraceae from Iran and recognized six species, namely *L. guilanensis*, *L. lactucae-serriolae*, *L. lactucarum*, *L. picridis*, *L. thevenotie* and *L. taurica* s. lat., which could be separated into three morphological groups. Braun and Cook (2012) described nine *Leveillula* spp. on the family Asteraceae, namely *L. asterisci*, *L. guilanensis*, *L. helichrysi*, *L. lactucae-serriolae*, *L. lactucarum*, *L. lappae*, *L. osteospermi*, *L. picridis* and *L. thevenotiae*. The occurrence of *L. taurica* on Asteraceae was left as questionable. Among others, it was established that *Lactuca* spp., not only in Iran, are infected by at least two different *Leveillula* spp. (*L. lactucae-serriolae* and *L. lactucarum*) (Khodaparast *et al*., 2010; Braun and Cook, 2012).

The *Leveillula* species known currently (Braun and Cook, 2012) are distinguished from each other mainly by features of the conidia, especially by the shape and length/width ratio of primary and secondary conidia and by the form of conidium surfaces determined using scanning electron microscopy (SEM). The importance of conidial features was also proposed by Braun (1995), and before him by Golovin (1956), Rostam (1983), Durrieu and Rostam (1984), Heluta and Simonyan (1987, 1988), Simonyan and Heluta (1987, 1989). Voytyuk *et al*. (2009) performed detailed SEM examinations of *Leveillula* conidia and confirmed that variation in their surface structures provided taxonomically relevant traits allowing differentiation between allied taxa. Later, Khodaparast *et al*. (2012) acknowledged that many collections of *Leveillula* strains on different hosts showed conidial morphology which was usually consistent for a strain on a single host species. Besides the analyses completed by Khodaparast *et al*. (2001, 2012) of the ITS region, Voytyuk *et al*. (2009) also used *tub2* gene. However, resolutions in both ITS and *tub2* gene trees were not fully sufficient because taxa that are morphologically distinguishable are not well resolved genetically.

There is little known of the geographical distribution of *L. lactucae-serriolae*. Voytyuk *et al*. (2009) reported this pathogen on *L. serriola* from Armenia, Iran and Israel. However, during field investigations in 2004–2007, this species was not recorded in Israel (Voytyuk *et al*., 2009). In recent publications (Braun and Cook, 2012), *L. lactucae-serriolae* is reported on *Lactuca azerbaijanica*, *L. scarioloides* and *L. serriola* from Asia (Iran, Israel, Lebanon, Turkmenistan) and Caucasus (Armenia), but not from Jordan. Currently, only Qasem and Abu-Blan, (1986) have reported a survey and identification of powdery mildews on economic and wild hosts in Jordan. Only *L. taurica* was confirmed in this survey, but this pathogen was not found on *Lactuca* spp. In the 1980s the taxonomy of powdery mildews and the possibilities of accurate species identification was generally on a low scientific level. From this time the attention of researchers in Jordan has been focused mainly on powdery mildews on economic crops (e.g., barley, tomato, cucurbits, grapes) (e.g., Abu-Blan and Khalil, 2001; Abdel-Ghani *et al*., 2008; Mansour *et al*., 2014).

The aim of the present study was to provide an accurate description and taxonomic position of *Leveillula* spp. found on wild *L. serriola* in Jordan.

MATERIALS AND METHODS

Lactuca *spp. distribution*

The character of the populations of wild *Lactuca* species in Jordan and the presence of powdery mildew on plants in their natural habitats was monitored during a field trip from 25–27 August 2007. Plants were studied at nine sites along a North (32°39'16,47''N) to South (30°19'48,59''N) transect, oscillating around the latitude of 35°40'E (Table 1). All sites were in the Mediterranean bioclimatic region. However, Shobak is located on its marginal part, near the Trans-Turanian region as defined by Al-Jaloudy (2006). While sites 1 to 7 were in northern areas with annual average rainfall of 200 to 400 mm, the annual average rainfall at site 9 (Petra) is 100 to 200 mm, and at site 8 (Shobak) is 50 to 100 mm (Fanack Water Editorial Team, 2017).

Site 1 was in the Jordan Valley, sites 2 to 7 were in the North of the Irbid Plateau, and site 9 (Petra) was in the South. Sites 8 (Shobak) and 9 were both on the marginal part of Steppe, and were strongly influenced by the Eastern Desert (Badiah).

Plants with morphological traits typical of *L. serriola* were observed at all of the sites except site 8. Plants with traits of *L. aculeata*, i.e., with dense and sharp spines on stems and cauline leaves, were observed at sites 1, 2, 3, 6 and 7. Plants with traits of *L. saligna*, i.e., with acute leaf apices and narrow lobes on cauline leaves, were observed at sites 1, 4, 7 and 8 (Table 1).

Plants naturally infected by powdery mildew were observed at site 8 (Shobak). Leaf samples with powdery mildew infections were collected from two different plants at this site.

Morphological examination of powdery mildew

Two powdery mildew samples collected on individual plants of *L. serriola* were used. Pieces of severely infected leaves were used for evaluation by light microscopy. As only dry leaf samples were analyzed, the modified method of Shin (2000) was used, i.e., heating of 'herbariumized' plant tissues in fuchsine in lactic acid. For statistical analyses (means, standard deviations and ranges), 30 measurements of each characteristic were used where possible (MS Excel, 2010).

Molecular examination

Genomic DNA was extracted from fungal mycelium scraped from two herbarium specimens of *L. serriola* (OL35561, OL35562) using the SDS extraction method (Edwards *et al*., 1991). The ITS region (ITS1-5.8S rDNA-ITS2) and the 5′ end of the 28S rDNA region (including D1 and D2 domains) were amplified separately by two polymerase chain reactions (PCRs) with nested primer sets. For amplification of the ITS region, the powdery mildew specific PMITS1/PMITS2 primers (Cunnington *et al.,* 2003) and ITS1-F/ITS4 primers (White *et al.,* 1990; Gardes and Bruns, 1993) were used. Amplification of D1/D2 domains of the 28S rDNA was performed according to Takamatsu *et al*. (2013), using primer sets PM3/TW14 and NL1/TW14 for the two nested PCR

Table 1. List of monitoring sites with wild *Lactuca* species in Jordan in 2007.

Site number	Name of location	Latitude	Longitude	Altitude (m a.s.l.)	Character of habitat	*Lactuca* [a] forma	Number of samples
1	Umm Qais, Cadara	32°39′16,47′′N	35°40′45,72′′E	353	stony slope	*serriola*	1
2	West from Jerash, Olive Branch hotel	32°17′46,30′′N	35°51′17,01′′E	932	stony slope, south exposition	segregating *serriola/ integrifolia*	2
3	Old Jerash, entrance to Roman City	32°16′45,01′′N	35°53′13.32′′E	590	sandy soil	*Lser, Lint* [as above]	3
4	Mount Nebo, monastery, monument	31°46′02,08′′N	35°43′33,21′′E	680	near road	*Lser*	4
5	Mount Nebo	31°46′00,30′′N	35°43′42,60′′E	671	near road	*Lser*	1
6	Madaba	31°43′12,22′′N	35°47′40,25′′E	787	ruderal place	*Lser*	5
7	Madaba, archeological park	31°42′57,47′′N	35°47′44,71′′E	780	stony background	*Lser*	1
8	Shobak	30°31′30,45′′N	35°35′21,33′′E	1304	along the road	*Lser*	2
9	Petra, Canyon	30°19′48,59′′N	35°26′26,77′′E	877	stony background	*Lser*	1

[a] Taxonomic status confirmed: *Lser – L. serriola* f. *serriola*; *Lint – L. serriola* f. *integrifolia*

runs. All PCR reactions were conducted in 15 µL reaction volume, containing 1.2 µL of DNA (50 ng μL^{-1}), 0.3 µL of each primer (10 µM), 3 µL of 10× Reaction Buffer, 0.24 µL of 10 mM dNTP´s, 0.08 µL of GoTaq G2 DNA Polymerase (Promega) and 9.88 µL of PCR grade water, and were carried out in an Eppendorf Mastercycler ProS (Eppendorf). The following conditions were used for the PCRs: 5 min at 95°C; 35 cycles of 45 sec at 95°C, 45 sec at 60°C for the first PCR or 55°C for the second PCR, 1 min at 72°C, and a final extension (7 min at 72°C). PCR products were purified using the GenElute PCR Clean-Up Kit (Sigma-Aldrich) and sequenced (Macrogene Europe) using the following primers: ITS1-F/ITS4 for the ITS region and NL1/NL2/NLP2/TW14 for 28S rDNA (Takamatsu *et al*., 2013). Geneious 7.1.8 (Biomatters Ltd) was used for contig assembly from partial reads, the editing of base calls and concatenation of partial genomic regions. The resulting nucleotide sequences were deposited in the NCBI database (accession numbers MG881818, MG881819, MG878434, MG878435) and used to search against the NCBI database using BLAST. All sequences having the similarity values equal or greater than 99% for ITS and 97% for 28S rDNA were compared using MEGA 7 software (Kumar *et al*., 2016). Subsequently, Maximum Likelihood (ML) and Maximum Parsimony (MP) phylogenetic trees were constructed to trace the relationships among selected GenBank records and sequences obtained in this study. Moreover, for the ITS tree we used additional sequences representing the main *Leveillula* groups outlined by Khodaparast *et al*. (2012). The best-fit evolution model (*Tamura-3 parameter* with *gamma distribution*) was selected with Find Best DNA/Protein Models option implemented in MEGA 7. Alignment gaps were treated as missing information.

RESULTS

Symptoms of infection

Symptoms of powdery mildew infection consisted of extensive growth of white, superficial coatings on upper and lower leaf surfaces. Newly infected leaves had sparse coverings of powdery mildew. As the disease progressed, white mycelia completely covered both leaf surfaces (Figure 1).

Morphology of the fungus

The morphological features of both herbarium samples of powdery mildew on *Lactuca* spp. are summarized as follows. Mycelium was external and (probably) internal, which was hard to confirm because of the age of the herbarium specimens. White, dense and persistent, mostly superficial mycelium occurred on leaves. Two types of conidia were produced separately on conidiophores (Figure 2, A-J). Primary conidia were lanceolate, long with pointed apices and rounded bases, and measured 39–56 × 11–13 µm, with length to width ratios of 3.2–4.9. Secondary conidia were mostly clavate, 39–66 × 11–16 µm, with length/width ratios of 2.9–4.9. Germtubes were recorded on primary and secondary conidia; arising mostly from an end of each germinating conidium, rarely from the side. Germinating conidia usually had singly long germtubes, the apices of which were mostly simple, but sometimes curved (Figure 2, K-N). Conidiophores with primary and secondary conidia were observed (Figure 2, O and P), and these were 117–244 µm long, with foot cells measuring 46–175 µm long, 6–8.5 µm wide and mostly with 2–4 distal cells. Chasmothecia were also observed, but these were probably not mature. They were 92–219 µm in diameter, with very short and few appendages, and without asci (Figure 2, Q).

Figure 1. Symptoms of powdery mildew infections on lower sides of leaves of A, *Lactuca serriola* f. *integrifolia*, and B, *L. serriola* f. *serriola*.

The shape of primary and secondary conidia of the *Leveillula* found on the *Lactuca* spp. accessions in Jordan were very similar to micrographs of primary and secondary conidia of *L. lactucae-serriolae* from Iran, published by Khodaparast *et al.* (2012).

Molecular identification of powdery mildew

The nucleotide sequences of the 28S rRNA gene and ITS regions were determined for the two analysed speci-

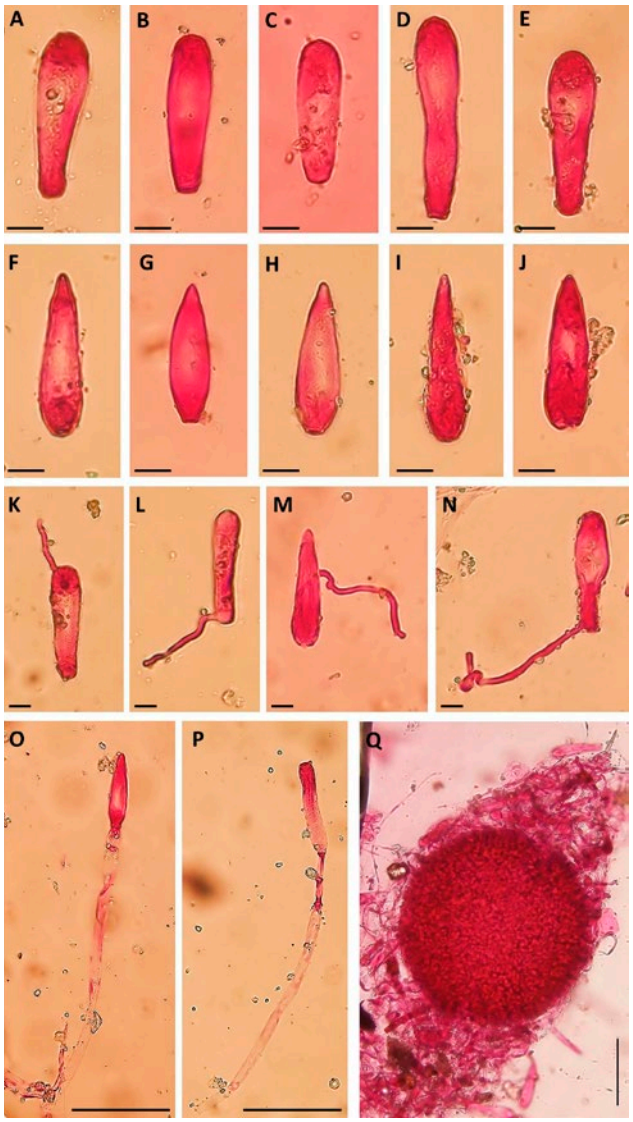

Figure 2. Micrographs of *Leveillula lactucae-serriolae*. A-E, shape variability of primary conidia; F-J, secondary conidia; K-N, germ development from primary and secondary conidia; O, conidiophore with primary conidia; P, conidiophore with secondary conidia; Q, chasmothecium. Bars = 10 µm in A-N) and 50 µm in O-Q).

mens. Identical nucleotide sequences for both specimens were obtained, with the total lengths of 673 bp for ITS and 875 bp long contig for 28S rRNA.

Comparison of the ITS from this study with sequences available in the GenBank database revealed 100% similarity with the "type" record of *L. lactucae-serriolae* (Accession no. AB044375) infecting *Lactuca serriola* from Iran (Khodaparast *et al.*, 2001). There is another ITS record in the GenBank database (Accession no. HQ821500) described as *L. lactucae-serriolae*, which was extracted from *Hexinia polydichotoma* by Xu *et al.* (2011). To inspect these records in more detail, we aligned the above ITS sequences with selected sequences representing the main *Leveillula* groups outlined by Khodaparast *et al.* (2012), and performed phylogenetic analyses. The resulting ML tree proved the identity of all *Leveillula* samples originating from *L. serriola*, which formed a separate sub-group within clade No. 2, consisting of "type" specimens of *L. lactucae-serriolae* AB044375 and the two specimens sequenced in this study (Figure 3). On the other hand, the sequence HQ821500 extracted from *Hexinia polydichotoma* by Xu *et al.* (2011) fell within clade No. 1, together with *Leveillula taurica* samples originating from Korea and China.

The BLASTn search of part of the sequenced 28S rDNA region revealed 99% similarity to 28S rDNA sequences of different *Leveillula* species: *L. lactucae-serriolae* (HQ821501 ex *Hexinia polydichotoma*) reported by Xu *et al.* (2011); *L. duriaei* (AB080475 ex *Salvia nemorosa*) and *Leveillula* sp. (AB080478 ex *Chondrilla juncea*), reported by Takamatsu *et al.* (2008) (Figure 3).

DISCUSSION

As mentioned above, the only study of powdery mildews occurring in Jordan was that of Qasem and Abu-Blan (1986), where only *Leveillula taurica* was confirmed. However, occurrence of powdery mildew species is rarely limited by state borders. In surrounding Near East countries, powdery mildews have been surveyed, but not with the same intensity in various countries. In the reviews of El-Kazzaz *et al.* (1989), Voytyuk *et al.* (2009), Severoglu and Ozyigit (2012) and Kabaktepe *et al.* (2015), the most common powdery mildew on Asteraceae was *Leveillula taurica*. However, in Turkey *L. lactucae-serriolae, L. lactucarum* and *L. picridis* were also recorded (Kabaktepe *et al.*, 2015). Voytyuk *et al.* (2009) reported *Leveillula lactucae-serriolae, L. picridis* and *Leveillula* spp. on Asteraceae in Israel. *Leveillula* spp. was separated into three species, and *L. osteospermi* and *L. wasseri* were described as new species (Voytyuk et al., 2009; Braun and Cook, 2012). Detailed studies of powdery mildews in Iran were completed by Khodaparast *et al.* (2001, 2007, 2010, 2012, 2016), and the pathogens were mainly in *Leveillula*. These authors introduced some new species of *Leveillula*, including *L. guilanensis* and *L. lactucae-serriolae,* on Asteraceae (Khodaparast *et al.*, 2002).

According to our observations, the shapes of primary and secondary conidia of *Leveillula* found on *Lactuca* spp. accessions in Jordan are very similar to those in micrographs of *L. lactucae-serriolae* published by

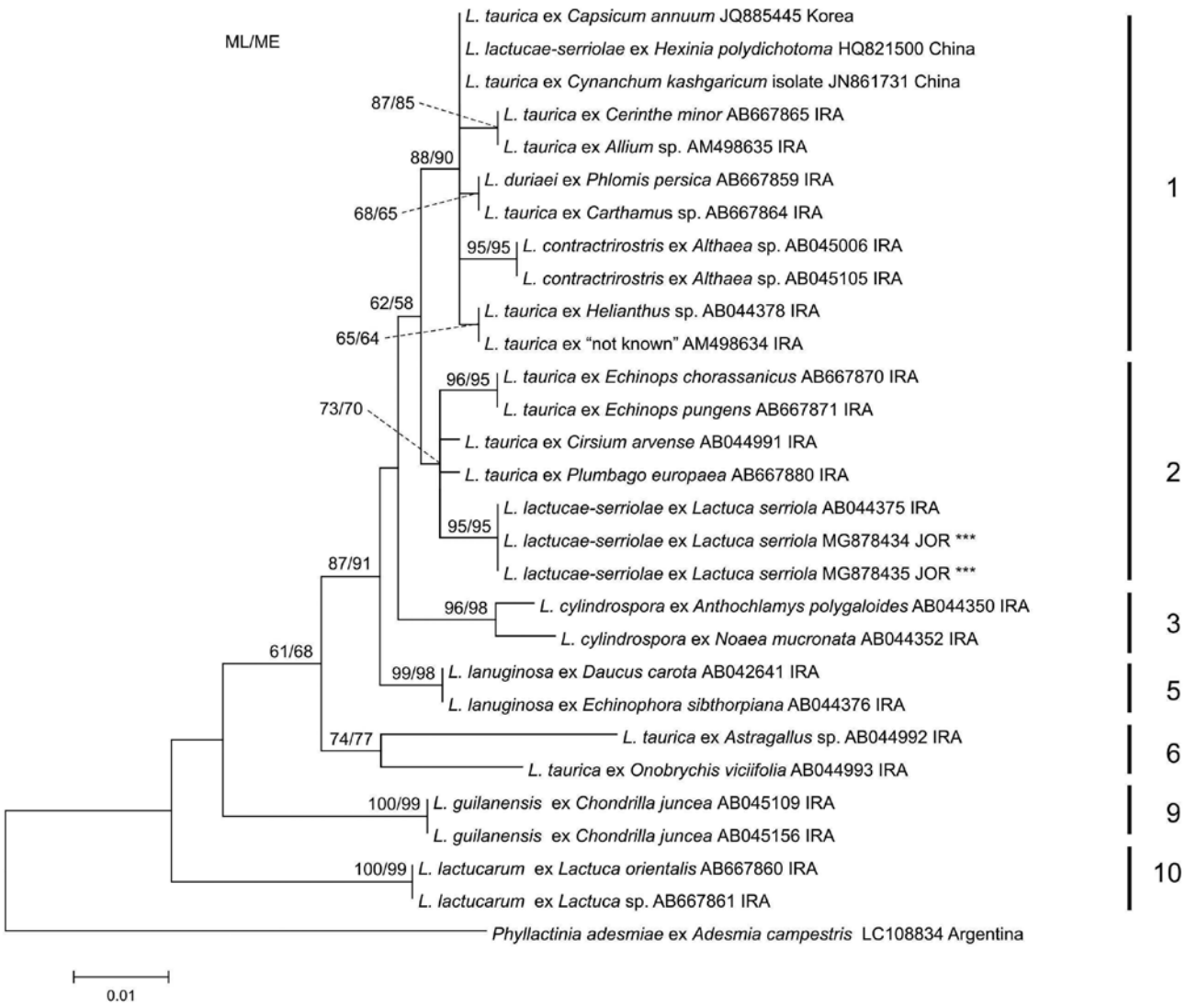

Figure 3. Maximum-Likelihood tree based on ITS nucleotide sequences of 28 taxa of *Leveillula* and single outgroup taxa. Numbers above branches indicate bootstrap values based on 1000 replications of ML and ME phylogeny (Bootstrap values less than 50% are not shown). Numbers to the right indicate the clades designated by Khodaparast *et al.* (2012). *** indicates specimens analysed in the present study.

Khodaparast *et al.* (2012). Differences were found in size, but not in shape, since in our observations (Table 2) the sizes of both types of conidia were smaller than those described by Khodaparast *et al.* (2012). Khodaparast *et al.* (2002), who first introduced *L. lactucae-serriolae,* described this species as morphologically very close to *L. taurica*, since the primary conidia of the two species are similar, but *L. lactucae-serriolae* differed in having more distinctly clavate (widest in the upper half) secondary conidia. However, this species is genetically clearly distinct, forming a separate clade (Khodaparast *et al.*, 2001), so it cannot be conspecific with *Leveillula taurica*. According to Braun and Cook (2012), *L. lactucae-serriolae* occurs on *Lactuca* (*L. azerbaijanica, L. scarioloides, L. serriola*) in Asia (Iran, Israel, Lebanon, Turkmenistan, and Armenia).

Another newly described powdery mildew species on *Lactuca* spp. in Jordan is *Leveillula lactucarum*, known for 30 years, and described by Braun and Cook (2012) on *Chondrilla, Hexinia,* and *Lactuca* (*L. orientalis, L. tatarica, L. viminea*) in Asia (Afghanistan, China, Iran, Kazakhstan, Kyrgyzstan, Turkey, Turkmenistan, Armenia, and Azerbaijan), and Europe. These authors suspected that other collections belonged to this group. *Leveillula lactucarum* is well characterized as having subcylindrical primary conidia which are narrowed toward pointed apices. This contrasts with the long, lanceolate primary conidia and more or less subclavate secondary

Table 2. The main morphological characteristics of *Leveillula lactucae-serriolae* samples originated from Jordan (collected by A. Lebeda, in 2007) and Iran (Khodaparast *et al.*, 2002).

Origin (host plant species and country)	Conidia[a]						Conidiophores[a]		Chasmothecia[a]
	Primary			Secondary					
	Mean length (µm) ± SD (min – max)	Mean width (µm) ± SD (min – max)	Length/ Width Ratio	Mean length (µm) ± SD (min – max)	Mean width (µm) ± SD (min – max)	Length/ width Ratio	Mean length (µm) ± SD (min – max)	Mean basal cell (µm) ± SD (min – max)	Mean number of distal cells ± SD (range)	Mean diameter (µm) ± SD (min – max)
Lactuca serriola f. *integrifolia* (Jordan)	50.8 ± 3.3 (43.9 – 53.7)	12.4 ± 0.5 (12.2 – 13.4)	4 ± 0.2 (3.6 – 4.2)	52.8 ± 5.5 (39 – 61)	13.9 ± 1.1 (10.9 – 15.9)	3.8 ± 0.5 (2.9 – 4.9)	146 ± 38 (117.1 – 200)	70.7 ± 23.5 (46.3 – 102.5)	2.7± 0.4 (2 – 4)	154.1 ± 43.9 (92.7 – 219.6)
Lactuca serriola f. *serriola* (Jordan)	50.4 ± 4.6 (39 – 56)	12.1 ± 0.8 (11 – 13.4)	4.1 ± 0.4 (3.2 – 4.9)	52.4 ± 5.5 (39 – 65.9)	13.2 ± 1.1 (11 – 15.9)	3.9 ± 0.5 (2.9 – 4.8)	190 ± 40.5 (126.9 – 244)	108.4 ± 32.6 (53.7 – 175.7)	2.8 ± 0.8 (2 – 4)	154.5 ± 13.8 (134.2 – 170.8)
Lactuca serriola (Iran)	53 – 75	13 – 18	3.1 – 5	49 – 75	13 – 18	n.a.[b]	n.a.	n.a.	n.a.	187 – 225

[a] Means ± SDs and ranges, where available.
[b] Information not available.

conidia of *L. lactucae-serriolae*. Furthermore, the micrographs of primary and secondary conidia of *L. lactucarum* on *Lactuca* (e.g. *L. orientalis*) presented by Khodaparast *et al.* (2012) show conidia that are very different from our samples.

Leveillula lactucae-serriolae and *L. lactucarum* are well supported molecularly and morphologically. The taxonomic positions of several species were generally well supported by morphology especially that of primary conidia (Khodaparast *et al.*, 2012). *Leveillula lactucae-serriolae* was placed in clade No. 2, while *L. lactucarum* was placed in clade No. 10, different from *Leveillula taurica* (clade No. 1).

Nucleotide sequences of two genomic regions were determined in the present study. ITS proved that all *Leveillula* samples originating from *L. serriola* (including "type" material from Iran), formed a well resolved *L. lactucae-serriolae* sub-clade on the ML phylogenetic tree (Figure 3). In contrast, GenBank record HQ821500 deposited as *L. lactucae-serriolae* extracted from *Hexinia polydichotoma* fell within the *L. taurica* samples. Although Xu *et al.* (2011) stated that the BLASTn search of HQ821500 returned 99% similarity with "type" *L. lactucae-serriolae* record AB044375, these authors ignored 100% identity of HQ821500 to 11 *Leveillula taurica* ITS records (JN861731, JQ885445). It is evident that the taxonomic denomination of HQ821500 is doubtful and should be corrected to *L. taurica* or *Leveillula* sp.

We cannot perform direct comparison of the 28S rDNA sequence to the "type" material of *L. lactucae-serriolae* since it is not deposited in GenBank. Nevertheless, the inspection of 28S rDNA nucleotide alignment of *Leveillula* sequences obtained in the present study with the most similar GenBank records (i.e. HQ821501, AB080475, AB080478 having 99% identity; alignment not shown) proved that none of the analysed sequences is identical with the two *L. lactucae-serriolae* specimens from Jordan. These differ from others in a single SNP with record HQ821501 (*L. lactucae-serriolae* ex *Hexinia polydichotoma*) and another SNP with *L. duriaei* (AB080475 ex *Salvia nemorosa*). The two deposited sequences (HQ821501 and AB080475) are also not identical, and there is high probability that each of these sequences represents a different taxon. Moreover, the record of HQ821501 published by Xu *et al.* (2011) is linked to the problematic ITS record of HQ821500 discussed above, and most likely does not represent 28S rDNA of *L. lactucae-serriolae*. Therefore, it should be corrected to *Leveillula* sp.

In conclusion, the sequencing of the ITS region of powdery mildew obtained from two *Lactuca serriola* plants in Jordan proved their identity with "type" mate-

rial of *Leveillula lactucae-serriolae* described by Khodaparast *et al*. (2012). Sequencing of 28S rDNA region provided the first verified GenBank record of *Leveillula lactucae-serriolae* deposited in a public nucleotide repository. The sequencing of both genomic regions clearly throws doubt on the *L. lactucae-serriolae* record on *Hexinia polydichotoma*. This first record of *L. lactucae-serriolae* on wild *Lactuca* species in Jordan is, however, not surprising because the environmental conditions in this area favoured the occurrence of powdery mildew of this genus.

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New or Unusual Disease Reports

Xylella fastidiosa causes leaf scorch of pistachio (*Pistacia vera*) in Iran

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Summary. Since the early 2010s, pistachio (*Pistacia vera* L.) leaf scorch symptoms have been observed in orchards in several provinces in Iran. Seventeen of 83 symptomatic leaf samples from pistachio plants from 21 orchards were positive for the presence of *Xylella fastidiosa*, as detected by DAS-ELISA with *X. fastidiosa*-specific antibodies and by PCR assays with *X. fastidiosa*-specific primers. A Gram-negative bacterium similar to *X. fastidiosa* was isolated into solid media. DAS-ELISA and PCR confirmed the identity of the isolated bacteria as *X. fastidiosa*. Koch's postulates were fulfilled by artificially inoculating isolates obtained from pistachio showing leaf scorch to healthy pistachio and *Nicotiana tabacum* (cv. White Burley). Selected isolates induced leaf scorch symptoms when inoculated on tobacco and pistachio seedlings grown under greenhouse conditions. Early leaf scorch symptoms appeared approx. 2 months after inoculation of tobacco and approx. 3 months for pistachio. Reisolation of *X. fastidiosa* from inoculated and symptomatic plants and DAS-ELISA and PCR tests confirmed the identity of the re-isolated bacteria to be *X. fastidiosa*. On the basis of disease symptoms, pathogen isolation, pathogenicity tests and positive diagnosis by DAS-ELISA and PCR, *X. fastidiosa* is concluded to be the causal agent of pistachio leaf scorch in Iran. This is the first report of isolation and pathogenicity of *X. fastidiosa* in pistachio worldwide.

Keywords. Iran, isolation, DAS-ELISA, pathogenicity test, PCR.

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INTRODUCTION

Xylella fastidiosa Wells is a xylem-limited bacterium that can cause plant diseases in a wide range of plant hosts throughout the world. This fastidious bacterium is classified as a single species, although genetic studies support multiple subspecies (Schaad *et al.,* 2004). The pathogen is currently subdivided into four subspecies: *X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *multiplex*, *X. fastidiosa* subsp. *sandyi* and *X. fastidiosa* subsp. *pauca*, with a proposed fifth subspecies ('*X. fastidiosa* subsp. *morus*') still under scrutiny. The agent of leaf scorch affecting pear trees in Taiwan has recently been

found to differ genetically from *X. fastidiosa* to warrant classification as a novel species, denoted *Xylella taiwanensis* (Su *et al*., 2016). Since the first report in grape, *X. fastidiosa* has been isolated and identified from an increasingly large number of plant hosts, with or without symptoms, and is recognized to be the causal agent of various diseases (Baldi and La Porta, 2017). Diseases caused by this pathogen include Pierce's disease (PD) of grape, almond leaf scorch (ALS), alfalfa dwarf, citrus variegated chlorosis, leaf scorch of oak, pear leaf scorch and oleander leaf scorch (OLS) (Purcell, 2013), and the olive quick decline symdrom (Luvisi et al., 2017; Saponari *et al*., 2018).

According to the latest report from EFSA (EFSA, 2018), 563 plant species are reported to be infected by *X. fastidiosa*. These host include hundreds of plant genera in 82 botanical families. Although confirmed records of presence of the pathogen come from some European countries, field epidemic outbreaks occur only in southern Italy and in insular (Corsica) and continental (French Riviera) France and in the Balearic Islands and Alicante in mainland Spain (Saponari *et al*., 2018; Ramazzotti et al., 2018). In the European Union, several cultivated plants of high economic value (e.g. olive trees, almonds, cherries) or wide-spread ornamental plants (e.g. myrtle-leaf milkwort, oleander) have been identified as hosts of *X. fastidiosa*. Many other widespread plant species remain potential hosts in the European Union territory (EFSA, 2018).

Xylella fastidiosa was previously reported from symptomatic almond and grape plants in Iran, with identification based on graft transmission, isolation on culture media, pathogenicity tests, and positive reactions in DAS-ELISA and PCR assays specific for the bacterium. (Amanifar *et al.,* 2014; Amanifar *et al.,* 2016). In the last decade symptoms of leaf scorch and dieback have been observed in many pistachio orchards in regions of Iran. However, based on the available information, there are no reports of isolation and pathogenicity studies of *X. fastidiosa* in pistachio.

Pistachio is the fifth most important commercial nut crop in the world and has been cultivated in different countries including Iran, Turkey, other Mediterranean countries, and the USA. According to the Food and Agriculture Organization (FAO, 2012), approx. 85% of the world pistachio production currently comes from these countries. Iran is the biggest world exporter, with 40% production of world pistachio and having 61% of the world market (Amiri Aghdaie, 2009).

Although *X. fastidiosa* has already been identified by ELISA from pistachio samples from California, culture on media and PCR results did not confirm the presence of this bacterium (Costa *et al*., 2004). In the present study, we report the isolation of *X. fastidiosa* strains from symptomatic pistachio plants in Iran, and confirm its pathogenicity on this host for the first time.

MATERIALS AND METHODS

Sampling

Four pistachio growing provinces in Iran, Kerman, Khorasan Razavi, Qazvin, and Yazd, were visited during each summer and autumn from 2012 to 2017, and plants exhibiting leaf scorch symptoms were sampled. Eighty-three samples from 21 pistachio orchards were collected. Each sample was from one tree. The samples consisted of stems and leaves. The distance between two sampled orchards was at least 500 m. Two samples were collected from each hectare of each pistachio orchard where leaf scorch symptoms were found. The stem and leaf samples were placed in individual plastic bags and transferred to the laboratory. Each sample was divided into three subsamples for pathogen isolation and testing for *X. fastidiosa* using DAS-ELISA and PCR (Schaad *et al*., 2001; Bextine and Miller, 2004; Amanifar *et al.,* 2014).

Bacterial cultures

Tissue pieces (each of length 3–5 cm) of pistachio petioles and midribs from plants exhibiting leaf scorch symptoms were transferred to sterile plastic bags, surface sterilized by soaking for 3 min in 5% sodium hypochlorite solution and 3 min in 70% ethanol, and then rinsed three times for 5 min in sterile distilled water. The tissues were then transferred to new plastic bags containing 3 mL of sterile succinate-citrate-phosphate buffer (1 g L^{-1} disodium succinate, 1 g L^{-1} trisodium citrate, 1.5 g L^{-1} K_2HPO_4, 1 g L^{-1} KH_2PO_4, pH 7). After a 20 min incubation period at room temperature, 100 µL of the liquid were streaked onto each of the agar media; PD2 (4 g L^{-1} tryptone, 2 g L^{-1} soytone, 1 g L^{-1} trisodium citrate, 1 g L^{-1} disodium succinate, 0.5 g L^{-1} $MgSO_4$-$7H_20$, 1.5 g L^{-1} K_2HPO_4, 1 g L^{-1} KH_2PO_4, 0.01 g L^{-1} hemin chloride (0.1% in 0.05 N NaOH), 2 g L^{-1} bovine serum albumin, 15 g L^{-1} agar) (Davis *et al*., 1980); PW (4 g L^{-1} phytone peptone, 1.2 g L^{-1} trypticase, 0.5 g L^{-1} $MgSO_4$-$7H_20$, 1.5 g L^{-1} K_2HPO_4, 1 g L^{-1} KH_2PO_4, 0.01 g L^{-1} hemin chloride (0.1% in 0.05 N NaOH), 4 g L^{-1} L-glutamine, 6 g L^{-1} bovine serum albumin, 15 g L^{-1} agar) (Schaad *et al*., 2001); Xf-D1 (3 g L^{-1} L-glutamine, 1 g L^{-1} L-asparagine, 0.5 g L^{-1} L-cysteine, 1.5 g L^{-1} Trisodium citrate, 1.5 g L^{-1} Disodium succinate, 10 mL Hemin chloride (0.1% in 0.05%

NaOH), 3 g L^{-1} Potato starch, 15 g L^{-1} Agar) (Almeida et al., 2004); or nutrient agar (28 g L^{-1}) (Schaad *et al*., 2001). The petioles and midribs were also crushed in the plastic bags using a pair of sterile pliers, and 50 µL of extract from each sample were streaked onto culture media. The plates were incubated at 28°C for 4 weeks and examined with a stereomicroscope at weekly intervals for the presence of *Xylella*-like colonies (Wells *et al*., 1987; Chen *et al*., 2007). Seven to 20 d after plating on PD2 and PW media, small white colonies, if found, were re-streaked for three passages onto PD2 and PW media to ensure the purity of the strains. Isolates were stored in PD2 broth plus glycerol at -20°C. For Gram tests, bacterial cells were Gram stained and observed at 1000× magnification using phase contrast microscopy (Schaad *et al*., 2001). Colonies on two media cultures were tested by DAS-ELISA and PCR.

DAS-ELISA tests

Serological detection of *X. fastidiosa* by *PathoScreenR XF* kit was performed according to the manufacturer's instructions (Agdia, Inc.). DAS-ELISA was used to detect *X. fastidiosa* in symptomatic samples of pistachio. One gram of petiole or midrib tissue from each sample was surface sterilized by soaking for 5 min in 70% ethanol, rinsed twice for 3 min in sterile distilled water, transferred to a plastic bag with 3 mL of extraction buffer and crushed with a pestle at room temperature. The sap was loaded into wells coated with *X. fastidiosa*–specific antibodies. The cultures were tested as plant samples. For this purpose bacterial isolates cultured on PD2 and bacterial cells suspended in extraction buffer were also used as antigen. The optical absorbance of the samples was measured using a plate reader (KC4, v.3.1, Bio-Tek), at 630 nm. All samples were tested in duplicate wells, and the plates each included positive and negative controls. A suspension of an antigen of *X. fastidiosa* included in the ELISA kit was also used as the positive control, and a non-infected pistachio plant was used as a negative sample control. Samples with absorbance values above the average absorbance values of the known negative samples plus three times the standard deviation were considered positive for *X. fastidiosa* (Sutula *et al*., 1986).

DNA extractions

Total DNA was extracted from fresh pistachio samples using the Qiagen Plant DNAeasy mini kit (Qiagen, Inc.), according to the manufacturer's instructions. The cetyltrimethylammonium bromide (CTAB) method (Ausubel *et al*., 1992, Amanifar *et al*., 2014) was used to extract DNA from bacterial cells, which had been scraped from PD2 agar plates after 6 d incubation at 28°C. Five microliters of the DNA extracted from each culture was subjected to electrophoresis in 1% agarose gels. The gels were stained with ethidium bromide, and DNA bands observed with ultraviolet light were used to estimate DNA concentrations. The DNA solutions were each diluted to 5 ng μL^{-1} and stored at -20°C.

PCR assays

Three primer sets, RST31/RST33 (Minsavage *et al*., 1994) targeting the *RNA-Pol sigma-70 factor* gene, 272-1-int/272-2-int (Pooler and Hartung, 1995) targeting an *hypothetical protein HL*gene, and Dixon454fa/Dixon-1261rg (Chen *et al*., 1995) targeting the *16S rDNA* gene, were utilized for amplification of parts of the *X. fastidiosa* genome, and the resulting products were sequenced. PCR reactions were carried out using the TaKaRa TaqTM (Hot Start Version, Takara Bio Inc.) in 25 µL volumes. The components for the each PCR reaction included 12.5 µL of Master Mix (2 × Premix), 0.1 µM of each primer (1 µL) and 8 µL of water. Templates consisted of 300 ng of extracted DNA from each plant sample, extracted bacterial DNA, or a small portion of the bacterial colony suspended in distilled water for whole-cell PCR. The amplification programme consisted of an initial denaturation step at 95 °C for 5 min; 35 cycles of 95 °C for 45 s, annealing temperatures or 57°C for the *16S rDNA* gene, 56°C for the *hypothetical protein HL*gene for 30 s, or 55°C for the *RNA-Pol sigma-70 factor* gene for 30 s, and 72°C for 30 s; and a final elongation step of 72°C for 8 min. PCR products were separated by electrophoresis on 1% agarose gels run at 5 V cm^{-1} for 1 h, stained with ethidium bromide (10 µg L^{-1}), and visualized with UV light.

All collected plant samples and bacterial isolates were tested with PCR for detection of *X. fastidiosa*. Five plant samples and four bacterial isolates (for three primer sets) were sequenced and some sequences were submitted to GenBank. PCR products were purified and sent for sequencing by Macrogen. Sequences obtained from the *RNA-Pol sigma-70 factor*, *hypothetical protein HL*gene and *16S rDNA* genes were analyzed and some sequences were deposited in the GenBank database (International Nucleotide Sequence Database Collaboration) under accession numbers MG601055 (isolate), MG517438.1 (plant sample), MG458715 (strain raf8), MG732899 (isolate Pistachio-Raf83), MG732900 (isolate Pistachio-Raf83), MG732901 (isolate Pistachio-Raf5) for isolates and plant samples from pistachio.

Plant bioassays and pathogenicity tests

Bioassays were conducted on seedlings of *Nicotiana tabacum* (cv. White Burley) maintained in a greenhouse. After seeds germinated, the seedlings were kept at 25°C in an incubator until the two-leaf stage. The plants were then transferred to pots each containing a nutrient/soil growth medium and were then kept in a greenhouse at 27±6°C. Plants at the 4-leaf stage were inoculated with bacterial suspensions. Two isolates (Pistachio-Raf83 and Pistachio-8) of *X. fastidiosa* from pistachios (from two different regions) were used for inoculations. PD2 medium plus glycerol (100 µL each) containing bacteria that had been stored at -20°C was propagated on solid PD2 medium, and the cultures were incubated for 7 d at 28°C. Bacterial suspensions in sterile distilled water were precipitated at a velocity of 5,000 rpm (4,192 × g). Resulting pellets were resuspended in distilled water and a spectrophotometer was used to adjust bacterial concentrations to approx. 2×10^6 CFU mL^{-1} (absorbance of 0.4 at 600 nm). Two hundred microliters of bacterial suspension were injected into the main veins and petioles of two leaves of each plant using an insulin syringe (Francis *et al*., 2008). Inoculated plants were covered with plastic bags for 24 h. Control plants were inoculated with distilled water. For each isolate, three tobacco plants were inoculated. All inoculated plants were maintained in the greenhouse and fertilized with irrigation water. Plants were monitored for symptoms on a weekly basis for 3 months. Leaves of inoculated and control plants were assayed for *X. fastidiosa* infection 2 months after inoculation, using DAS-ELISA. Leaves were also used for re-isolation of *X. fastidiosa*. Re-isolated bacterial colonies were tested by PCR using RST31/RST33 primers, to confirm identification of the isolated bacterium as *X. fastidiosa* (Ledbetter and Rogers, 2009).

Pathogenicity tests were also conducted on seedlings of pistachio (cv. Badami) grown and maintained in a greenhouse. Based on the results of the bioassay tests on *N. tabacum*, Pistachio-Raf83 strain of *X. fastidiosa* caused more severe symptoms than Pistachio-8 strain in the tobacco leaves, so Pistachio-Raf83 was selected to for the pathogenicity test on pistachio seedlings. The strain was grown in liquid PD2 medium at 28°C with shaking (70 rpm) for 8 d. The resulting bacteria were centrifuged to obtain a pellet, which was resuspended in 5 mL of distilled water. A spectrophotometer was used to adjust bacterial concentrations to approx. 10^5 CFU mL^{-1} (absorbance of 0.3 at 600 nm). For each inoculation, a cell suspension drop (approx. 20 µL) was placed on the young stem of each non-infected pistachio plant (as confirmed by PCR), near a petiole. Each inoculated stem was pricked five times through the cell suspension using a syringe needle. Control plants were treated in the same way except that distilled water was used instead of bacterial suspension. Nine pistachio plants were inoculated with bacterial suspensions, and three were inoculated with distilled water. All plants were kept in a greenhouse (at 27±6°C) and fertilized with irrigation water. Plants were monitored for symptom development and recorded on a weekly basis for 1 year (Chang and Donaldson, 2009, Amanifar *et al*., 2014). Leaves of inoculated and control plants were assayed for *X. fastidiosa* infections 8 months after inoculation, using the commercial DAS-ELISA *PathoScreen* kit. Leaves were also used for re-isolation of *X. fastidiosa*. Re-isolated bacterial colonies were tested by PCR using RST31/RST33 and 272-1-int/272-2-int primers to confirm identification of the isolated bacteria (Ledbetter and Rogers, 2009).

RESULTS

Orchard survey

In pistachio orchards in Kerman, Qazvin, Yazd and Khorasan Razavi provinces, the first symptoms of pistachio leaf scorch appeared in August each year, as leaf discolourations. The tips and margins of leaves initially turned light gray-green, and this progressed towards the midribs during the summer (Figure 1). Marginal scorching of leaves began as early as July and continued to increase during the summer. Symptoms sometimes appeared first on one branch of a tree or a portion of one scaffold. Over the years, symptoms spread to larger areas of the trees and sometimes the entire canopies

Figure 1. Pistachio tree with leaf scorch symptoms on a branch, caused by *Xylella fastidiosa*.

were affected. Diseased trees bloomed and leafed out later than trees without symptoms, and they were less productive and had reduced terminal growth. Diseased trees usually survived for at least 3 years.

Bacterial cultures

Nineteen isolates of *X. fastidiosa* were obtained from 83 pistachio samples (Table 1). Bacterial colonies were observed on media 10 to 17 d after plating. The PD2 medium was more suitable than PW for bacteria isolations, though PW medium was a good growing medium. Isolates did not grow on nutrient agar. The size of isolated bacterial colonies on PD2 medium varied from 0.2 mm to less than 1 mm in diameter (Figure 2). Isolated colonies on PD2 medium were circular and white-opal in colour using reflected light from above. Bacterial cells from all isolates were determined to be Gram-negative. DAS-ELISA and PCR tests confirmed that the colonies recovered on both media were from *X. fastidiosa*.

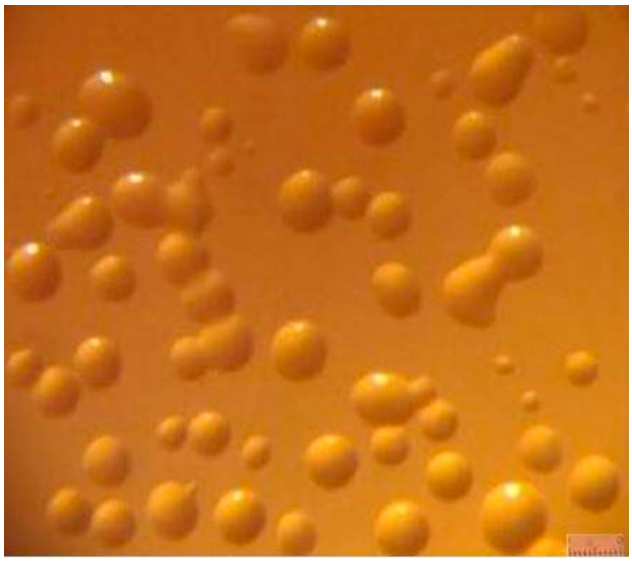

Figure 2. Colony morphology of a *Xylella fastidiosa* strain isolated from pistachio on PD2 agar medium, after incubation at 28°C after 12 days (Scale bar = 1 mm).

DAS-ELISA

Fifty-two of 83 pistachio plants tested positive for *X. fastidiosa* by DAS-ELISA (Table 1). Despite the presence of leaf scorch symptoms, not all samples tested positive for infection with *X. fastidiosa*. In Kerman and Yazd provinces 100% of the samples tested positive for the bacterium. Petiole and midrib extracts of pistachio trees that tested positive for *X. fastidiosa* had average (two replicates) absorbance values at 630 nm of 0.64 ± 0.33, compared with that of 0.124 ± 0.06 for the known negative control.

PCR assays

All three primers used effectively amplified their respective genes in the genome of *X. fastidiosa* from pistachio and culture colonies (Figure 3). Results of sequence analyses of the *RNA-Pol sigma-70 factor, 16S*

Table 1. Results of orchard survey for *Xylella fastidiosa* in symptomatic pistachio plant samples from Iran.

No. of plant samples[a]	No. of orchards[b]	Sampling month and year	Sampling province	Assay result		
				Culture (positive/tested)	DAS-ELISA (positive/tested)	PCR[c] (positive/tested)
10	2	September 2012	Kerman	2/10	4/10	4/6
4	1	August 2012	Qazvin	0/4	0/4	0/2
5	1	September 2012	Khorasan Razavi	1/5	3/5	1/3
11	3	September 2013	Kerman	3/11	11/11	2/5
6	2	September 2013	Khorasan Razavi	1/6	4/6	1/2
7	2	September 2014	Qazvin	0/7	0/7	0/3
24	5	September 2015	Kerman	5/24	17/24	4/7
4	1	October 2015	Khorasan Razavi	1/4	3/4	1/2
10	3	August 2016	Kerman	4/10	8/10	4/6
2	1	October 2017	Yazd	2/2	2/2	2/2

[a] Each sample was representative of an individual tree.
[b] The distance between two sampled orchards was at least 500 m.
[c] Tested by PCR using RST31/RST33, Dixon454fa/Dixon1261rg and 272-1-int/272-2-int primers.

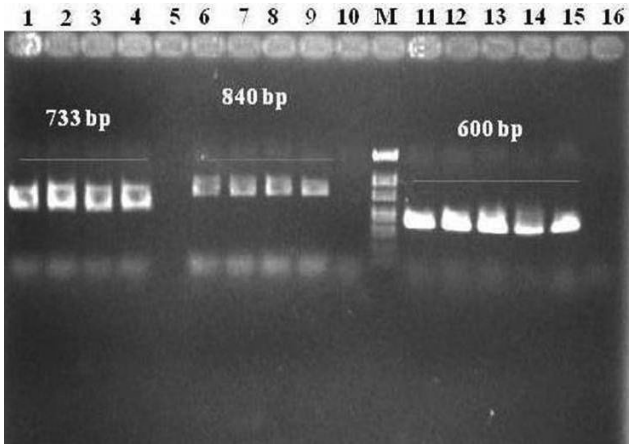

Figure 3. Gel electrophoresis pattern of PCR amplification products using *Xylella fastidiosa* specific primers. PCR products with primers RST31/RST33 from bacterial colonies isolated from pistachio (lane 1), from pistachio petioles (lane 2), from bacterial colonies isolated from grape (positive control, lane 3), from bacterial colonies isolated from almond (positive control, lane 4), from healthy pistachio petioles (negative control, lane 5). PCR products with primers Dixon454fa/Dixon1261rg from bacterial colony isolated from pistachio (lane 6), from pistachio petioles (lanes 7 and 8), from bacterial colonies isolated from almond (positive control, lane 9), from bacterial colonies isolated from grape (negative control, lane 10). PCR products with primers 272-1-int/272-2-int from bacterial colonies isolated from pistachio (lane 11), from pistachio petioles (lanes 12 and 13), from bacterial colonies isolated from almond (positive control, lane 14), from bacterial colonies isolated from grape (positive control, lane 15), from healthy pistachio petioles (negative control, lane 16). 100 bp DNA ladder (lane M).

rDNA, and *hypothetical protein HL* genes indicated that the isolates from pistachio were distinguishable from all the reference strains of *X. fastidiosa* subspecies in Genbank. In particular, nucleotide Blast analyses of *RNA-Pol sigma-70 factor, 16S rDNA* and *hypothetical protein HL* genes of pistachio isolates showed the greatest identity (96–100%) with those present in the Genbank (Table 2).

Plant bioassays and pathogenicity tests

Five days after *X. fastidiosa* inoculation, chlorosis symptoms appeared in tobacco leaf lamina. These symptoms gradually disappeared, and symptoms appeared in margins and tips of leaves. Early symptoms were leaf chlorosis and immediate mild necrosis, which gradually progressed in marginal leaf tissues to necrosis after 2 months (Figure 4, middle). Symptomatic plants were also each stunted, with dieback of branches (Figure 4, right). Control plants did not show any symptoms.

Six pistachio seedlings showed symptoms of leaf scorch under greenhouse conditions about 8 months after inoculation with bacterial cells (Figure 5). Early symptoms of the disease appeared about 3 months after inoculation (Figure 5). Control plants did not show any symptoms. Koch's postulates were proven using pistachio isolates inoculated onto pistachio seedlings.

DISCUSSION

None of the previous field surveys for *X. fastidiosa* in host plants has shown that pistachio is a natural host of this fastidious pathogen (Baldi and La Porta, 2017; EFSA, 2018; Saponari *et al*., 2018). We report the presence of *X. fastidiosa* in some regions of Iran in pistachio trees with leaf scorch symptoms, using three detection methods of culturing, DAS-ELISA and PCR. Scorching of leaf margins is the most common symptom caused

Table 2. Maximum nucleotide identity (%) of selected gene sequences between *Xylella fastidiosa* strains from pistachio and *X. fastidiosa* strains from other hosts present in Genbank. Respective accession numbers for isolates from pistachio are the following: *RNA-Pol sigma-70 factor* (MG601055), *16S rDNA* (MG517438.1, MG458715) and *Hypothetical protein* (MG732899, MG732900, MG732901). Accession numbers relative to strains are reported in order.

Gene	Pistachio isolate	Identity (%)	GenBank Accession No.	Host
RNA-Pol sigma-70 factor	Pistachio-Raf8	99	KT764083, KT764082	*Coffea arabica, Coffea arabica*
		98	CP000941, EU334069	*Prunus dulcis, Quercus palustris*
16S rDNA	Pistachio8	100	KF463301	*Prunus dulcis*
		99	DQ991190, CP020870.1, CP010051.1	*Carya illinoinensis*, *Olea europaea*, *Citrus aurantium*
	Pistachio-Raf3	100	KF463301	*Prunus dulcis*
		99	DQ991190.1	*Carya illinoinensis*
Hypothetical protein HL	Pistachio-Raf3	99	CP006740, EU021997, AE009442	*Morus* sp., *Persea americana, Vitis vinifera*
	Pistachio-Raf83	96	CP000941	*Prunus dulcis*

Figure 4. Healthy plant of tobacco (control, left), progressive development of tobacco leaf symptoms after inoculation with *Xylella fastidiosa*. Necrosis of leaves 40 days after inoculation (middle). Stunting of tobacco plant four months after inoculation (right).

by *X. fastidiosa*, but this symptom is not normally present until late in the growing season when temperatures are high, and plants are water-stressed (Hopkins, 1989). We observed severe symptoms of pistachio leaf scorch with increasing temperature and probably the maximum water demand by pistachio plants in August each year. The scorching symptoms, commonly induced by *X. fastidiosa,* result from blocking the host vascular systems. These symptoms can be confused with other factors such as drought, salt toxicity, or herbicide injury (Mircetich *et al*., 1976). Although *X. fastidiosa* was previously isolated and reported on the grape in Qazvin province of Iran (Amanifar *et al*., 2014), *X. fastidiosa* has not been detected in this province, although severe symptoms of leaf scorch have been observed in pistachio trees. The soil in these regions is salty, and these symptoms in Qazvin province are probably related to salt toxicity.

Disease progression in pistachio orchards during this study showed that severity increased from year to year and included the following stages (Figure 1): slight leaf scorch on individual host branches (first year), some branches with leaf scorch symptoms (second year), severe leaf scorch and leaf stunting (third year) and severe leaf stunting, dieback and death of affected trees (fourth year). Symptoms were fully developed by early September each year. Symptoms appeared first in single branches or limbs and spread throughout the entire canopie in the subsequent 3-4 years. Trees infected by *X. fastidiosa* eventually died.

These symptoms are similar to several leaf scorch diseases caused by *X. fastidiosa* on almond in Iran (Amanifar *et al*., 2016) and some other hosts (Hearon *et al*., 1980; Montero-Astua *et al*., 2008). Distribution of the disease in each orchard was irregular, often with single trees affected. This situation has also been observed in Iran in plantings of almond and grapevine with, respectively, leaf scorch and Pierce's disease (Amanifar *et al*., 2014; Amanifar *et al*., 2016), but mortality of pistachio trees was greater than in almond and grape.

Comparison of growth characteristics of *X. fastidiosa* isolates from pistachio with almond and grape isolates (Amanifar *et al.,* 2014) showed that colonies of *X. fastidiosa* from grape appeared on PD2 and PW culture media after approx. 7 d at about 28°C, but almond and pistachio isolates were slower growing appearing at least 10 d after culturing. All isolates from grape grew on the Xf-D1 and PD2 media. Almond and pistachio isolates did not grow on Xf-D1, but were able to grow on PD2 after 10 d. Maximum survival on PD2 media was 2 weeks after the appearance of bacterial colonies. We found differences in the colony morphology of grape, almond and pistachio isolates from Iran, specifically concerning colony margins which deserve further studies. Chen *et al*. (2007) showed that colony morphology has the potential to be used as a diagnostic tool for *X. fastidiosa* strain characterization.

Grape isolates of *X. fastidiosa* (Amanifar *et al*., 2014) did not amplify fragments in PCR using the Dixon454fa/Dixon1261rg primer set, but almond isolates (Amanifar *et al*., 2014) were amplified with this primer pair (Figure 3), Therefore, the pistachio isolates belong to a a subspecies multiplex, since these primers are specific for this subespecies (Figure 3).

Based on differences in *RNA-Pol sigma-70 factor, 16S rDNA* and *Hypothetical protein HL* genes sequencing and differences in biological and morphological traits of media cultures for Iranian isolates of *X. fastidiosa* it can be assumed we have two subspecies of this bacterium in Iran. Group I subsp. *fastidiosa* (grape isolates) and Group II subsp. *multiplex* (almond and pistachio isolates). However, further investigations are progressing for sequencing of more genes and specific phylogenetic analysis.

Figure 5. Pathogenicity tests: healthy plant of pistachio (uninoculated control, top left); initiation of pistachio leaf scorch symptoms 3 months after inoculation (top right); disease progress 1 year after inoculation under greenhouse condition (bottom).

DAS-ELISA was used as a conventional method for detection of *X. fastidiosa* in plants (Henneberger *et al*., 2004). Although PCR has been reported to be 100 times more sensitive than DAS-ELISA (Minsavage *et al*., 1994), in our tests with field plants it was not more sensitive. Other molecular techniques for *X. fastidiosa* identification/detection include PCR and PCR derivatives, including RFLP and RAPD analysis, as well are real-time PCR and loop-mediated isothermal amplification (LAMP). Extraction of *X. fastidiosa* DNA from culture and host species for PCR and related molecular analyses has been achieved from tissue by both standard commercial column kits and by basic CTAB or, in the case of cultures, Tris-EDTA-Sarkoysl techniques (EPPO, 2019).

About 62% of samples with typical leaf scorch symptoms were infected with *X. fastidiosa* as determined by DAS-ELISA and about 22% detected by culturing. From about 47% of the tissue samples tested, DNA of *X. fastidiosa* was extracted based on PCR results. Both DAS-ELISA and PCR have a potential for false positive and false negative reactions, which is always unclear, but one of the main causes is plant infection by other bacteria in addition to infection with *X. fastidiosa* (Sherald and Lei, 1991; Minsavage *et al*., 1994; Pooler and Hartung, 1995; Chen *et al*., 2007). In this study, we isolated other bacteria species in addition to *X. fastidiosa*, some of which were very slow to grow. These bacteria may interact with *X. fastidiosa*, which requires additional research.

The distribution range of *X. fastidiosa* is usually limited to tropical and subtropical areas, although leaf scorch diseases caused by *X. fastidiosa* also occur in colder climates, e.g. oak leaf scorch occurs as far north as Canada (Goodwin and Zhang, 1997) and almond leaf scorch occurs in regions with very cold winters in Iran (Amanifar *et al*., 2016). In this study, *X. fastidiosa* was detected from pistachio samples collected from arid-warm regions with cold winters of Iran.

Xylella fastidiosa is no longer a plant pathogen limited to a few countries in the Western Hemisphere, where its geographical distribution has expanded dramatically (Almeida and Nunney, 2015). One of the most relevant pending questions is what drives host specificity in this pathogen; in other words, why do pathogen genotypes cause disease in one plant species and not another, while still being able to colonize various plant species with different degrees of success but without inducing symptom expression (Almeida and Nunney, 2015). Our knowledge in Iran is still very limited about various aspects of this fastidious pathogen. Complementary studies are necessary to determine the molecular and biological characteristics of Iranian isolates of *X. fastidiosa* from different hosts and their host range and its insect vectors. Given the world-wide relevance of Iranian pistachio production and quality and the importance of the diseases caused by *X. fastidiosa* in fruit trees, it is necessary to deepen the knowledge on this pathogen in pistachio trees to better manage the disease.

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New or Unusual Disease Reports

Black rot of squash (*Cucurbita moschata*) caused by *Stagonosporopsis cucurbitacearum* reported in Italy

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Summary. *Stagonosporopsis cucurbitacearum* (syn. *Didymella bryoniae*) can affect cucurbits through induction of black rot. This pathogen produces irregular white spots covered with pycnidia on infected cucurbit fruit. Twenty squash fruit (cv. Butternut) with black rot symptoms were collected in Italy from two locations: Osimo (AN) and Montacuto (AN), in the Marche region. Several fungal colonies were isolated from these fruit, the morphological features of which corresponded to *S. cucurbitacearum*. This identification was confirmed using multiplexing of three microsatellite markers and by sequence analysis using internal transcribed spacers. The sequence identity for the internal transcribed spacer regions was greater than 98% compared with sequences of *S. cucurbitacearum* in the NCBI database. This is the first report of *S. cucurbitacearum* on *Cucurbita moschata* fruit with black rot symptoms in Italy.

Keywords. Butternut squash, black rot, ITS sequencing, microsatellite markers.

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INTRODUCTION

Black rot (BR) of cucurbits is caused by the fungal pathogen *Stagonosporopsis cucurbitacearum* (Fr.) Aveskamp, Gruyter & Verkley (Aveskamp *et al.*, 2010) (anamorph *Phoma cucurbitacearum* (Fr.) Sacc.), synonym *Didymella bryoniae* (Fuckel) Rehm, which is one of the most important pathogens on cucurbits worldwide (Li *et al.*, 2015; Yao *et al.*, 2016). *Stagonosporopsis cucurbitacearum* and *S. citrulli* can infect several species of Cucurbitaceae, including watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) (Rennberger and Keinath, 2018; Babu *et al.*, 2015; Huang and Lai, 2019), muskmelon (*Cucumis melo* L.) (Nuangmek *et al.*, 2018), squash (*Cucurbita maxima* Duch, *Cucurbita moschata* Duch) (Keinath, 2014), and pumpkin (*Cucurbita pepo* L.) (Grube *et al.*, 2011). These fungi can cause infection of the stems, leaves,

roots, seeds, and fruit of these host plants (Keinath, 2011).

Infected fruit manifest large irregular-shaped spots and black rot (Choi *et al*., 2010). Fruiting bodies are found in the oldest parts of lesions, because *S. cucurbitacearum* is a necrotrophic fungus (Keinath, 2014). Keinath (2011) reported that *S. cucurbitacearum* produces black mycelia inside melon and giant pumpkin (*C. maxima*) fruit. The ideal conditions for disease development include humidity greater than 90%, leaf wetness and temperatures from 16 to 24°C (Park *et al*., 2006; Seebold, 2011). BR can reduce preharvest and postharvest yields (de Neergaard, 1989), and cause up to 15% fruit loss (Keinath, 2000). *Stagonosporopsis cucurbitacearum* has only been reported once in Italy, in 1885 on *C. melo* L., and it was described as *Didymella melonis* Pass. by Giovanni Passerini (Corlett, 1981). Our investigations aimed to identify the causal agent of black rot symptoms on squash fruit.

MATERIALS AND METHODS

Fungal isolation

Twenty fruit of squash (*C. moschata*; cv. Butternut) with symptoms of black rot were collected from Osimo (AN) and Montacuto (AN), in the Marche region of Italy in September and October 2018. Small infected pieces of squash peel (≈2 mm) were cut from the fruit, sterilized with 1% sodium hypochlorite solution for 2 min, washed three times with sterilized distilled water, placed into 90 mm diam. Petri dishes containing water agar (Bacteriological agar; Liofilchem), and incubated at 24°C. After 7 d, pycnidia were excised from developing fungus colonies, placed into Petri dishes containing potato dextrose agar (Liofilchem), and incubated at 24°C. Identification of the fungus was carried out according to the colour and shape of the colonies, and to the size of the conidia produced from pycnidia (50 conidia measured).

DNA amplification and phylogenetic studies

The fungal genomic DNA was extracted from 100 mg of mycelia of isolates grown on potato dextrose agar as pure cultures, following the protocol proposed by Varanda *et al*. (2016). The DNA was amplified in a rapid PCR based assay for distinguishing the three morphologically similar species (*S. cucurbitacearum*, *S. citrulli*, and *S. caricae*) by multiplexing of the three microsatellite markers *Db01*, *Db05* and *Db06* (Brewer *et al*., 2015). The primer pairs ITS_1 and ITS_4 (White *et al*., 1990) were then used to amplify the internal transcribed spacers (ITS). The PCR products were separated on 1.5% agarose electrophoresis gels, stained with Red Gel (Biotium), and visualized, with images captured using an imagining system (Gel Doc XR; BioRad). Selected PCR amplicons were purified and sequenced by Genewiz, and the sequences have been deposited with Genbank (accession numbers: isolates ID1, MK330934; ID3, MK330935; ID9, MK330936; for ITS regions). The nucleotide sequences were subjected to Blast analysis to determine the relative similarities with other sequences available in Genbank.

RESULTS AND DISCUSSION

In the two surveyed locations in Italy, black rot symptoms occurred on butternut squash fruit. The initial symptoms were brown circular spots with exudates on the fruit surfaces (Figure 1A). Over time, the spots became white and were covered with black fruiting bodies (Figure 1B, C). After 8 d incubation on water agar, some pycnidia were seen (using a stereomicroscope) to be developing in rows on the fruit peel (Figure 2A). On crushing the pycnidia, the conidia inside were cylindrical, mostly non-septate and a few one-septate, and measuring 4 to 11 µm × 2 to 5 µm (Figure 2B, C). The isolates on potato dextrose agar showed white mycelia on the colony upper surfaces top and black mycelia on the undersides. These morphological characteristics coincided with those known for *S. cucurbitacearum* (Keinath *et al*., 1995; Koike, 1997; Choi *et al*., 2010; Keinath, 2013).

Morphological identification was supported by the multiplex amplification using the primers *Db01*f/r, *Db05* f/r and *Db06* f/r, which yielded two amplicons (220 bp and 280 bp), characteristic for *S. cucurbitacearum*. The presence of an amplicon of 280 bp and the lack of an amplicon of about 360 bp indicated that three isolates (ID1, ID3, and ID9) were *S. caricae* or *S. citrulli*, as reported by Brewer *et al*. (2015) (Figure 3). Blast analysis showed 98% to 99% similarity for the ITS regions compared to other sequences of *S. cucurbitacearum* already in the NCBI database, as shown in Table 1. Therefore, the isolates ID1, ID3, and ID9 from butternut squash are confirmed as *Stagonosporopsis cucurbitacearum*.

Stagonosporopsis spp. is a major pathogen of cucurbits worldwide, and it occurs everywhere these crops are grown (Stewart *et al*., 2015; Mancini *et al*. 2016; Nuangmek *et al*., 2018). Gummy stem blight and BR can affect all part of cucurbit plants, including stems, leaves, roots, seeds, and fruit. This pathogen is seed- and soilborne, and it can remain for long periods in the seeds and in the soil. Infected seed has continued to spread

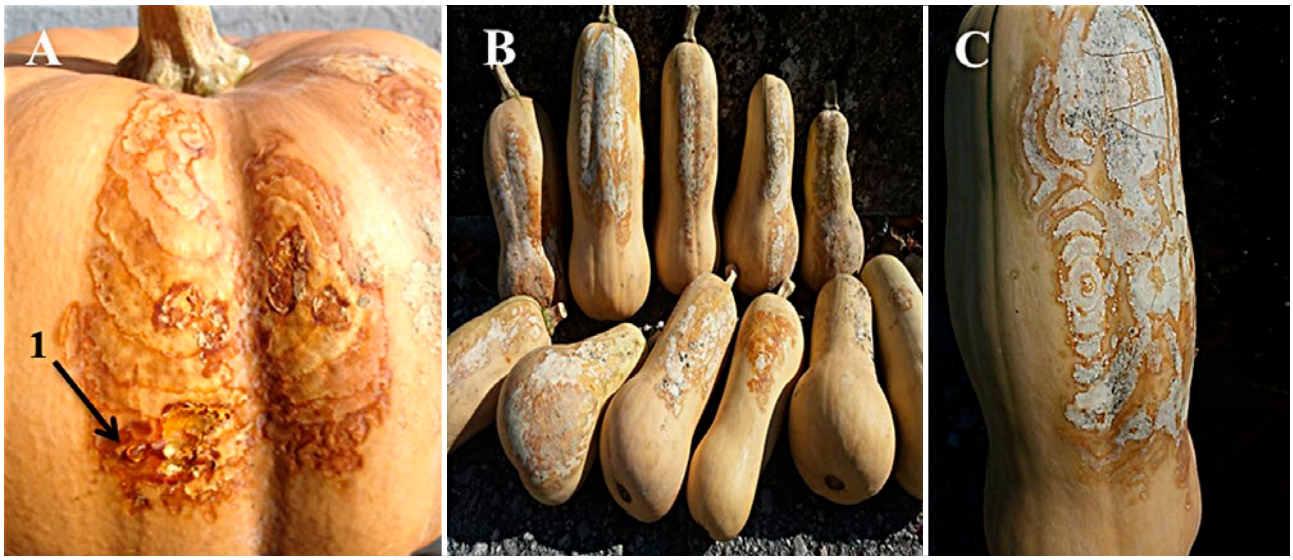

Figure 1. Typical symptoms of black rot caused by *Stagonosporopsis cucurbitacearum* on butternut squash fruit. **A,** Infected fruit showing exudate (arrow 1). B and C, Irregularly circular and white spots covered by pycnidia.

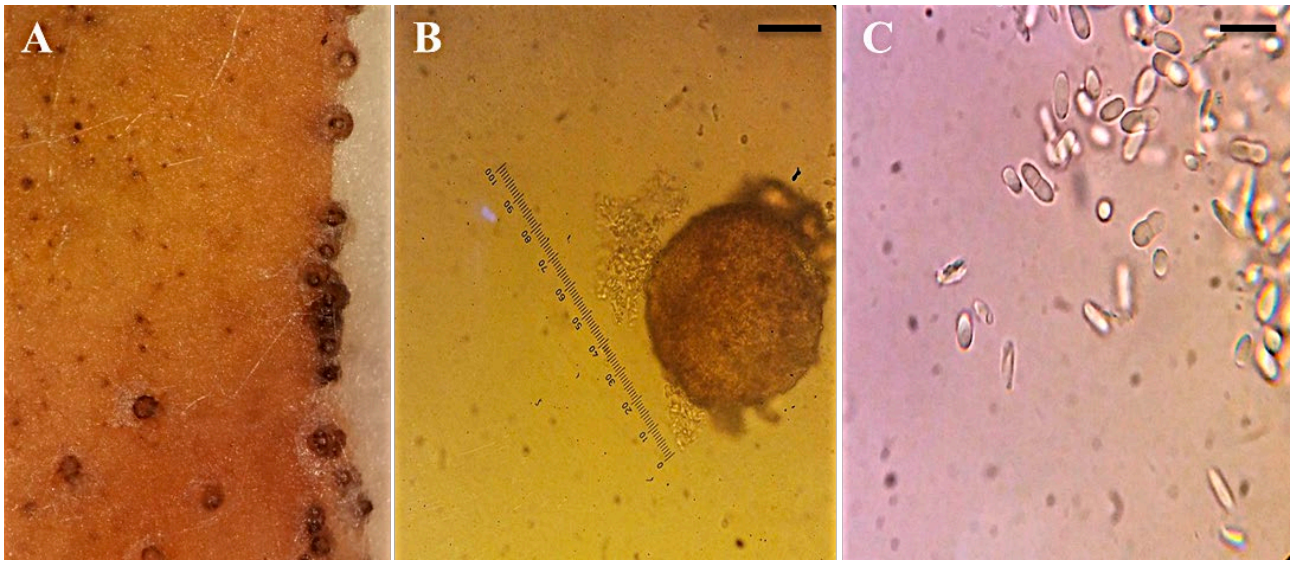

Figure 2. Morphological characteristics of *Stagonosporopsis cucurbitacearum*. A, Row of pycnidia on peel from a squash fruit under the stereomicroscope. B, Pycnidia under the microscope. Scale bar = 50 µm. C, Aseptate and one-septate pycnidiospores. Scale bar = 10 µm.

the pathogen around the world (Keinath, 2011). Seedborne pathogens can reduce of the quantity and quality harvested fruits and/or seeds, and their management is crucial for profitable production (Mancini *et al.*, 2014). On cantaloupe, field losses due to *S. cucurbitacearum* can reach 100% under conditions conducive to infection (Nuangmek *et al.*, 2018), and on watermelon, Gummy stem blight and BR can cause significant production losses, both in the field and postharvest (Maynard and Hopkins, 1999). No commercial cultivar of any cucurbit species has resistance to Gummy stem blight (Keinath, 2017).

Somai *et al.* (2002) have already reported *S. cucurbitacearum* for butternut squash in the United States of America. In Italy, this pathogen has been reported on *C. melo* (Corlett, 1981). To our knowledge, this is the first report of *Stagonosporopsis cucurbitacearum* on squash in Italy.

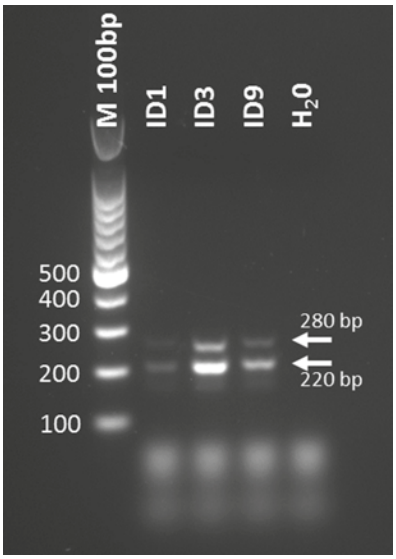

Figure 3. Polymerase chain reaction (PCR)-based marker for distinguishing *Stagonosporopsis cucurbitacearum*. Lane M is a 100-bp ladder with sizes of visible fragments indicated. Three fungal isolates (ID1, ID3 and ID9) from butternut squash fruit were analyzed with PCR-based markers using three sets of primers (Db01, Db06 and Db06) in a single reaction. Two amplicons of 220 and 280 bp were produced and no fragment of 360 bp was visible, despite the presence of the microsatellite locus Db01.

Table 1. Comparison of sequence similarities of *Stagonosporopsis cucurbitacearum* isolates with sequences already in the NCBI database.

Fungal species	Isolate number	NCBI accession no.	Nucleotide similarity (%)	Query cover
S. cucurbitacearum	ID1	EU167573	99%	100%
S. cucurbitacearum	ID3	MG009202	98%	100%
S. cucurbitacearum	ID9	KF990402	99%	99%

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New or Unusual Disease Reports

Syringa vulgaris is a new host for cucumber mosaic virus

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Summary. Virus-like symptoms consisting of light mosaic, chlorotic spots and oak chlorotic line patterns were observed on lilac plants (*Syringa vulgaris* L.) growing in a public garden in Imola (Emilia Romagna region, Italy). The causal agent was identified as cucumber mosaic virus (CMV) on the basis of biological, serological and nucleotide sequence properties of partial coat protein and movement protein genes, and the isolate was designated SYV. The CMV-SYV isolate caused mosaic symptoms on indicator plants of *Nicotiana tabacum* cv. Xanthi-nc, *N. rustica* and *Cucumis sativus*, while symptoms of local necrotic spots or pin points were observed on inoculated leaves of *Vigna unguiculata* and *Vicia faba*. To assess genetic differences between CMV-SYV and other known CMV isolates, phylogenetic analyses were carried out using 16 nucleotide sequences of coat protein and movement protein genes, including for SYV. The CMV-SYV isolate was most related to CMV subgroup IA isolates, and had 85.1-100% nucleotide sequence similarity to subgroup I isolates. This is the first report of CMV infecting *S. vulgaris*.

Keywords. Lilac, CMV, RT-PCR, subgroup identification.

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INTRODUCTION

Syringa vulgaris L. (lilac or common lilac; Oleaceae) is a popular spring-blooming ornamental shrub widely used in public and private gardens, for its large, showy and often fragrant inflorescences. Numerous lilac hybrids and cultivars have been developed for horticultural use. These selections exhibit variation in flower colour, period of blooming, and growth habit, and they are generally vegetatively propagated by rooting softwood cuttings to maintain genetic constancy (Hartmann *et al*., 1990). Risks of transmitting infectious diseases through vegetative propagation, particularly those caused by viruses, can be important. To combat virus diseases carried through vegetative propagules, adoption of preventive phytosanitary measures is essential, including correct pathogen diagnoses. Tomato mosaic virus (ToMV), arabis mosaic virus (ArMV), lilac leaf chlorosis virus (LLVC), toma-

to black ring virus (TBRV), tomato bushy stunt virus (TBSV), cherry leaf roll virus (CLRV), elm mottle virus (EMoV), strawberry latent ringspot virus (SLRSV), lilac ring mottle virus (LiRMoV) and tobacco rattle virus (TRV) have been previously described from lilac (Novák and Lanzová, 1975; Novák and Lanzová, 1977; van der Meer *et al*., 1976; van der Meer, 1976; Brunt, 1978; Canova and Betti, 1987; Castello *et al*., 1992; Cooper, 1993; Sharma-Poudyal *et al*., 2016).

Most of these viruses elicit similar symptoms in lilac, consisting mainly of chlorotic spots and line patterns on the leaves (van der Meer, 1976). This symptomatology is not indicative on the particular virus involved, and to get correct diagnoses, virus-specific detection methods are required. In this paper, we report the identification and characterization of a cucumber mosaic virus (CMV) isolate belonging to subgroup IA, from symptomatic lilac plants showing chlorotic ringspots and line patterns. This is the first documented serological and molecular evidence for the occurrence of CMV in lilac, causing similar symptoms to other viruses associated with that plant host.

MATERIALS AND METHODS

Since 2016, lilac plants showing virus-like leaf symptoms, including light mosaic, chlorotic ringspots and oak chlorotic line patterns (Figure 1), were observed in a public garden in Imola (Emilia Romagna region, Italy). Symptoms progressively and completely regressed after blooming, during the summer. In spring 2018, symptomatic leaves were collected from two plants, and were used in bioassay, serological and molecular tests.

In bioassays, three plants each of *Chenopodium quinoa*, *Nicotiana rustica*, *N. tabacum* cv. Xanthi and *Vigna unguiculata* cv. Black eye were mechanically inoculated with sap obtained by macerating symptomatic lilac leaves in 0.03 M phosphate buffer (pH 7). The same leaves were tested for the presence of CMV, alfalfa mosaic virus (AMV) and ToMV, using double-antibody sandwich (DAS)-ELISA commercial diagnostic kits (Bioreba AG; respectively, Art. Nr. 160675, 140575 and 152675 for the three viruses), including negative and positive controls. Samples from symptomatic and healthy leaves were also assessed for the presence of ilarviruses and nepovirus, using reverse transcription (RT)-PCR tests on total RNA extracted with the E.Z.N.A.® plant RNA kit (Omega Bio-tek, USA), and genus-specific primer pairs (Wei and Clover, 2008; Untiveros and Perez-Egusquiza, 2010). For the (RT)-PCR tests, the procedure of Parrella and Greco (2016) was used. Total RNA extracted from leaves of a healthy lilac plant was used as negative control, while total RNA extracted from tobacco plants infected with isolates of tomato ring spot virus (ToRSV) was used as positive control for nepovirus and parietaria mottle virus (PMoV) was used as positive control for ilarvirus (Parrella, 2002). Positive controls were derived from the collection of virus isolates at the Istituto per la Protezione Sostenibile delle Piante del CNR (IPSP-CNR), maintained as dehydrated leaves and stored over calcium chloride at 4°C.

In addition, and to confirm preliminary positive results obtained using CMV-specific ImmunoStrip® (Agdia Inc.) on symptomatic leaves from the two lilac plants, RT-PCR tests were also conducted using two pairs of CMV specific primers on total RNA isolated from infected and healthy lilac leaf tissues. Primer pair

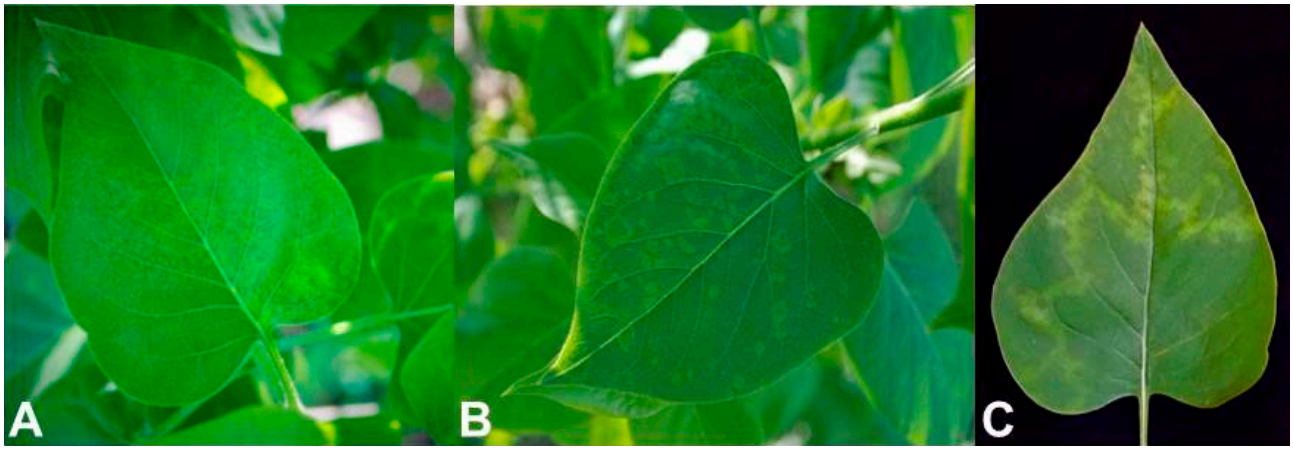

Figure 1. Natural symptoms on the leaves of *Syringa vulgaris* (lilac) plants infected by cucumber mosaic virus (CMV) isolate SYV, growing in a public garden (Northeast of Italy). Light mosaic (A), chlorotic spots (B) and oak chlorotic line patterns (C) were observed.

CMV1/CMV2 was used to amplify the partial coat protein gene (CP), and part of the 3'-noncoding region of CMV RNA3 (Singh *et al.* 1995; Parrella and Sorrentino 2009), and primer pair CMVMP1 (5'-ATGGCTTTC-CAAGGTACCA-3') and CMVMP2 (5'-CTAAAGAC-CGTTAACCACC-3') was used to amplify the entire Open Reading Frame (ORF) of the movement protein gene (MP). PCR conditions were as follows: initial denaturation cycle for 3 min at 92°C, followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 60°C, extension for 1.5 min at 72°C. In the final cycle, the elongation time at 72°C was 7 min. PCR products of the expected length (approx. 500 bp for CMV1/CMV2 and 840 bp for CMVMP1/CMVMP2) were separated by electrophoresis in agarose gels and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). DNA sequencing was carried out at Eurofins Genomics. Nucleotide sequence data were assembled using BioEdit Version 5.0.9 (Hall, 1999), and then compared with corresponding regions of the other CMV isolates retrieved from the GenBank database. Multiple nucleotide sequence alignments were performed by using the Clustal W program, while phylogenetic and molecular evolutionary analyses were inferred using the Maximum Likelihood method (Felsenstein, 1981), both packaged in the MEGA X software (Kumar *et al*., 2018). Peanut stunt virus (PSV; NC002040) was used as the outgroup to root the phylogenetic tree.

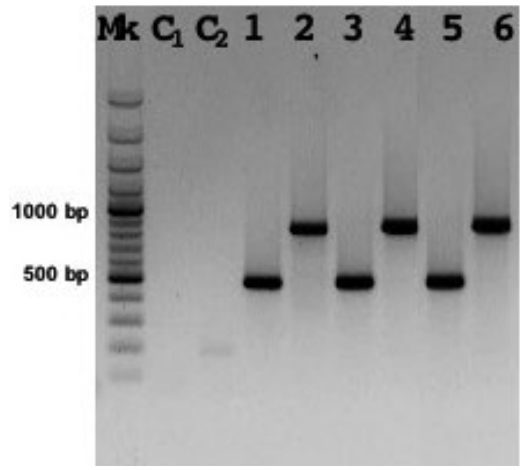

Figure 2. Results obtained with CMV1/CMV2 (lanes C1, 1, 3 and 5) and CMVMP1/CMVMP2 (lanes C2, 2, 4, and 6) primer pairs in reverse transcription (RT)-PCR tests, using total RNA extracted from healthy and symptomatic leaves of *Syringa vulgaris* (lilac): lane Mk, DNA ladder; lanes C1 and C2, negative controls (healthy lilac plant); lanes 1 and 2, amplicons obtained with RNA extracted from the lilac plant n. 1; lanes 3 and 4, amplicons obtained with RNA extracted from the lilac plant n. 2; lanes 5 and 6, positive controls (RNA of the CMV-PAE isolate; Parrella and Sorrentino, 2009).

RESULTS AND DISCUSSION

Mechanical inoculation from symptomatic lilac leaf samples induced local chlorotic lesions on *C. quinoa* and *V. unguiculata* observed 5 days post-inoculation, and vein clearing and light mosaic in *Nicotiana* spp. observed 14 days post-inoculation.

In double-antibody sandwich (DAS)-ELISA tests, only CMV was identified from both lilac plants. These results were confirmed by RT-PCR with CMV-specific primer pairs, and amplification products were obtained from the naturally infected lilac plants and mechanically inoculated plants, as well as from the PAE1 isolate of CMV (Parrella and Sorrentino, 2009) used as positive control. No amplification products were obtained from healthy plants (Figure 2). The sequences obtained from the two plants with the CMV1/CMV2 primers were identical, as well as those obtained with the CMVMP1/CMVMP2 primers, and these were deposited at GenBank (Acc. No. MH433673 for the partial CP gene and Acc. No. MH433674 for MP gene). The partial CP sequence of CMV-SYV was 96.5–98.6% similar to that of subgroup IA, 92.8–94.4% similar to subgroup IB and 77.0–76.5% similar to subgroup II, while the MP sequence was 96.4–99.0% similar to that of subgroup IA, 91.9–94.0% similar to subgroup IB and 79.2–79.6% similar to subgroup II. These results agree with those reported elsewhere concerning the nucleotide similarities within and among CMV isolate subgroups (Roossinck, 2002; Moury, 2004). Both of the CMV-SYV sequences obtained showed greatest sequence similarity at the nucleotide level with the CMV-Fny isolate (98.6% for the partial CP gene and 99.0% for the MP gene).

CMV1/CMV2 primers have been designed to produce amplicons of different sizes to distinguish CMV isolates belonging to subgroups I or II (Singh *et al.*, 1995; Parrella and Sorrentino, 2009). Based on the length of the amplicons obtained with these primers (487 bp), the CMV isolate from lilac (named SYV, from *Syringa vulgaris*) belonged to subgroup IA (Singh *et al*., 1995). This was further confirmed by the detection of one *Hpa*II restriction site in the 487 bp sequence, as previously proposed for the CMV isolates belonging to subgroup IA (Parrella and Sorrentino, 2009).

The bootstrap maximum-likelihood trees for nucleotide sequences of the CMV-SYV isolate and the two corresponding genomic regions of CMV isolates retrieved from the GenBank database and identified by the CMV1/CMV2 (partial CP gene) and CMVMP1/CMVMP2 (MP ORF) primer pairs, are presented

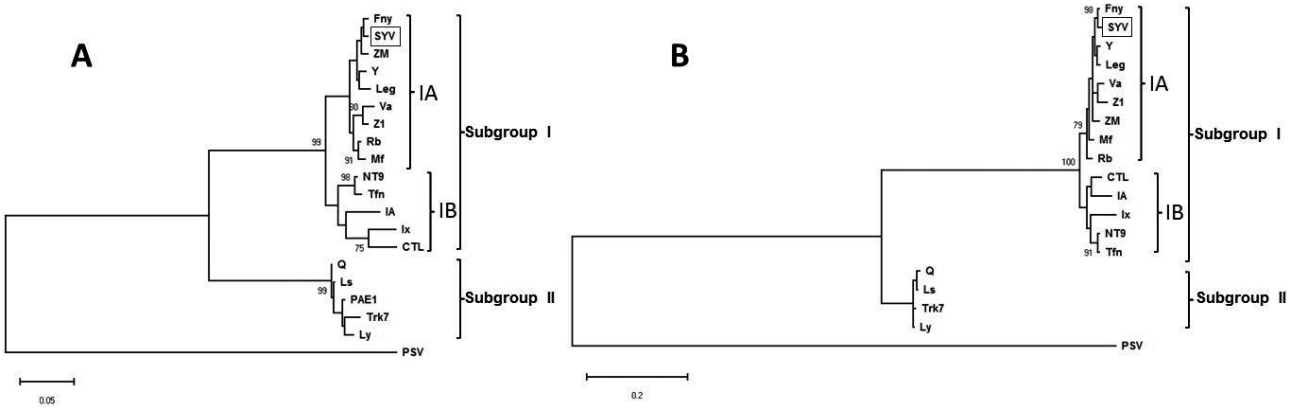

Figure 3. Phylogenetic trees derived from nucleotide sequences of the CMV RNA fragments obtained with the primer pairs CMV1/CMV2 and CMVMP1/CMVMP2 of SYV with the corresponding nucleotide regions of previously reported CMV isolates retrieved from GenBank (accession numbers and, when available, natural host plants and geographic origins are reported in parentheses for each CMV isolate): ZM (JN180311; *Zea mais*; South Korea); Rb (GU327365; *Rudbeckia hirta* var. pulcherrima; South Korea); Z1 (GU327368; *Cucurbita pepo*, South Korea); Fny (D10538; *Solanum lycopersicum*; United States of America); Y (D12499; Japan); Leg (D16405; *Vigna unguiculata*, Japan); Va (JX014248; *Vigna angularis* var. nipponensis, South Korea); Mf (AJ276481; South Korea); NT9 (D28780; *Solanum lycopersicum*; Taiwan); Tfn (Y19626; *Solanum lycopersicum*; Italy); Ix (U20219; *Ixora* sp.; Philippines); CTL (EF213025; *Brassica chinensis*; China); IA (AB042294; Indonesia); Q (M21464; Australia); Trk7 (L15336; *Trifolium repens*; Hungary); Ly (AF198103; *Lupinus angustifolius*; Australia); Ls (AF127976; USA); PAE1 (FN257306; *Passiflora edulis*; Italy); PSV (NC002040). The position of the SYV isolate is shown in the box. Peanut stunt virus (PSV) was used as an outgroup. Phylogenetic analyses were performed employing the maximum likelihood method packaged in the MEGA X software. Bootstrap values ≥ 70% are indicated at each node.

in Figures 1A and 1B. Analyses for both fragments showed that CMV isolates could be divided into two subgroups, as subgroup I and II. As expected, subgroup I was further divided into subgroups IA and IB. For CMV-SYV, this analysis showed that it belongs to subgroup IA and is closely related to the CMV-Fny, in both the genetic regions studied. Therefore, these results further confirmed that CMV-SYV belongs to subgroup IA of CMV.

Several viruses have been described in lilac (Brunt, 1978; Canova and Betti, 1987; Cooper 1993; Sharma-Poudyal *et al*., 2016). However, until now the presence of CMV infections in this host have not been reported. Results of the present study indicate that the CMV-SYV isolate systemically infected lilac plants causing symptoms including light mosaic, chlorotic ringspots and oak chlorotic line patterns on the host leaves (Figure 1). CMV is efficiently transmitted by more than 75 species of aphids (Hemiptera: *Aphididae*), in a non-persistent, stylet-borne manner, and these insects are the main vectors disseminating CMV in nature (Jacquemond, 2012). Nevertheless, since lilac is generally vegetatively propagated by basal shoots or root sprouts, it is important that mother-plants are carefully checked for virus infections to avoid spreading virus-infected material. To our knowledge, this is the first report of natural CMV infections in lilac.

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New or Unusual Disease Reports

First report of *Rhodococcus* spp. isolates causing stunting and lateral stem proliferation of *Iresine herbstii* 'Aureo-Reticulata' in Tunisia

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Phytopathogenic isolates of *Rhodococcus fascians,* a Gram-positive bacterium, cause disease on numerous plants by inducing fasciation and leafy galls (Goethals *et al*., 2001). The altered plant morphology resulting from these infections cause serious economic losses to ornamental industries (Putnam and Miller 2007). In April 2017, over 80% of micropropagated *Iresine herbstii* 'Aureo-Reticulata' plants in a greenhouse in northern Tunisia exhibited growth abnormalities similar to those caused by *Rhodococcus* spp. (Stamler *et al*., 2015). These symptoms included stunting and shoot proliferation (Figure 1). To isolate epiphytic populations of *Rhodococcus* spp., affected tissues were placed onto D2 agar (Kado and Heskett, 1970) amended with cycloheximide (2%). To detect endophytic bacterial populations, plant tissues were surfacetreated with 0.5% sodium hypochlorite for 30 s, then for 1 min with 70% ethanol, followed by rinsing three times in sterile distilled water. The tissues were then ground in phosphate buffered saline (pH 7.4) prior to dilution plating. Isolation plates were incubated in the dark at 27°C for 4 d, for detection of epiphytic populations, or 15 d for detection of endophytic populations. Bacterial colonies with colours ranging from cadmium yellow and deep chrome to deep orange were selected for purification and identification. Genomic DNA was extracted from resulting isolates, and diagnostic PCR was used for isolate identification, through 16S rDNA amplification and sequencing (Stamler *et al*., 2015). Representative 16S rRNA sequences from six isolates were deposited in GenBank (MH685556 to MH685561).

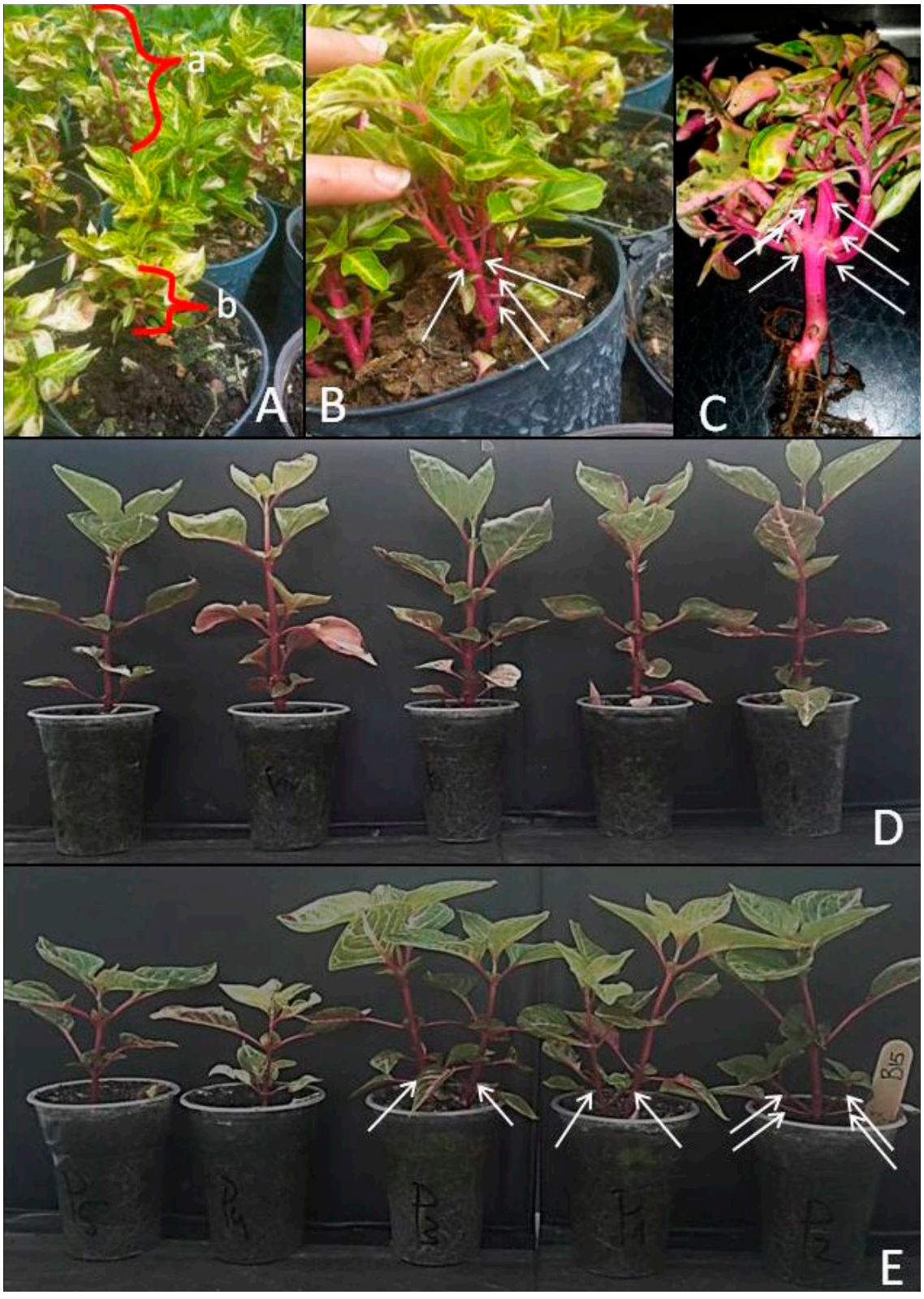

Figure 1. Symptomatic nursery-grown *Iresine herbstii* 'Aureo-Reticulata' plants were stunted (b), as compared to asymptomatic plants (a) (A), and exhibited stem proliferation (arrows) (B and C). Uninoculated control plants (D) developed single stems; plants inoculated with isolate 6 (E) developed multiple stems (arrows).

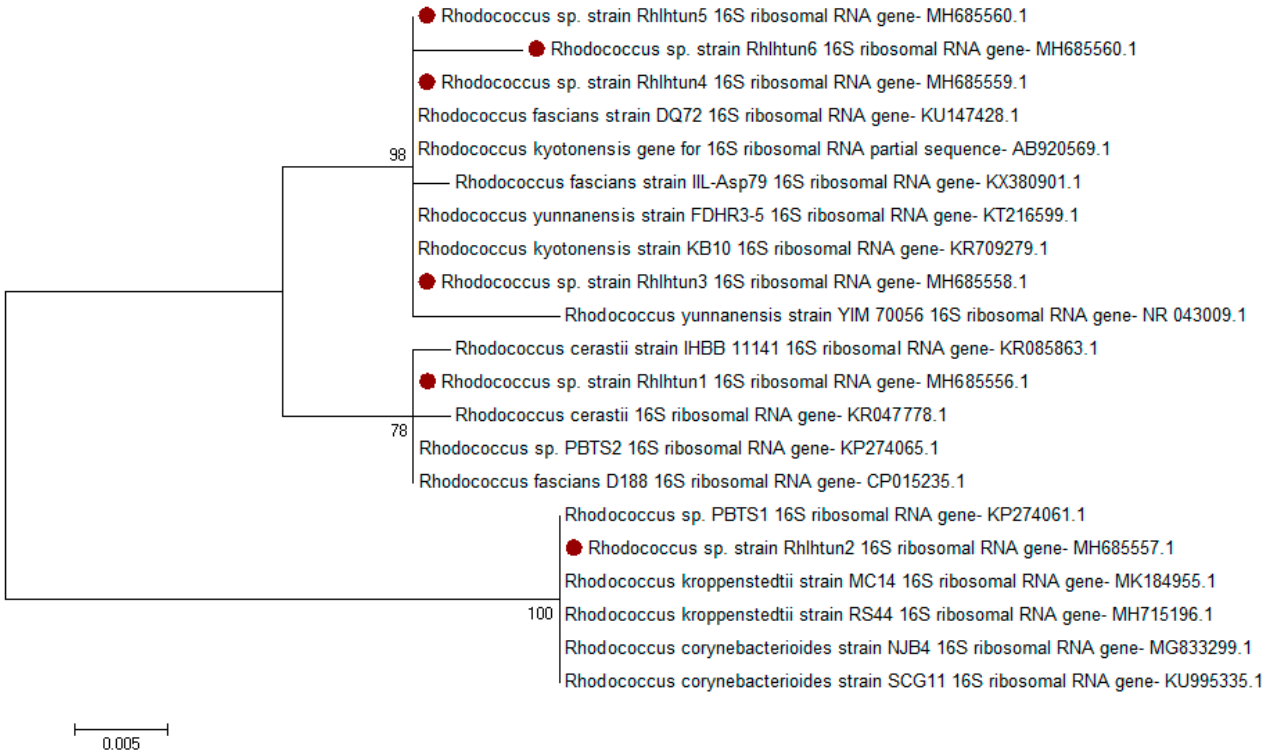

Figure 2. Phylogenetic tree based on the 16S rDNA genes of *Rhodococcus* spp. (labelled in red points), that were isolated from *Iresine herbstii* plants in northern Tunisia. The neighbour joining tree based on 16S rRNA was constructed using the Mega software version 6.06, bootstrapped 1,000× with a support threshold of 70%, corresponding to reference sequences with GenBank accession numbers.

Table 1. Symptom development on *Iresine herbstii* 'Aureo-Reticulata' in response to inoculation with six isolates of *Rhodococcus* spp.

Treatment[S]	Mean plant height (cm)[T]	Incidence of plants with lateral branches (%)[U]	Mean No. of stems per plant[V]	Mean No. of nodes per cm[W]	Mean shoot biomass (g)[X]	Mean root biomass (g)[X]	Mean total plant biomass (g)[Y]
Uninoculated	17.4 a[Z]	0	1.0 b[Z]	0.48 a[Z]	0.5 a[Z]	0.7 a[Z]	1.2 a[Z]
Isolate 1	14.1 ab	0	1.0 b	0.97 b	0.6 a	0.5 a	1.1 a
Isolate 2	9.5 b	0	1.0 b	0.70 b	0.4 a	0.6 a	1.0 a
Isolate 3	15.2 ab	0	1.0 b	0.72 b	0.7 a	0.6 a	1.3 a
Isolate 4	10.2 b	40	1.8 a	1.64 b	0.4 a	0.5 a	0.9 a
Isolate 5	10.1 b	0	1.0 b	1.17 a	0.4 a	0.3 b	0.4 a
Isolate 6	12.5 ab	60	2.6 a	0.97 ab	0.8 a	0.4 b	1.2 a

[S]Micropropagated *I. herbstii* were inoculated by dipping plantlets into bacterial suspensions of each of 6 *Rhodococcus* spp. isolates originating from symptomatic plants; uninoculated control plants were dipped in sterile buffer.
[T]Plant height was determined from the soil surface to the apical meristem 100 d post-inoculation.
[U]The percent of plants (n = 5) producing lateral stems was calculated.
[V]The mean number of stems per plant was determined by adding the main stem and lateral stems for each plant. One stem per plant indicates no lateral stem growth.
[W]Node density was calculated as the number of nodes per unit length of stem. Plants with high node density exhibited compact structure.
[X]Roots were cut from shoots and tissues were dried at 55°C for 48h prior to weighing.
[Y]Total plant biomass was calculated by summation of the root and shoot biomass.
[Z]Means associated with different letters indicate statistically significant differences ($P \leq 0.05$).

The isolates were closely related (>99% similarity) to *Rhodococcus* spp. accessions in GenBank. Isolate 1 was 99% identical to *R. fascians* D188 and *R. cerastii*, isolate 2 was 100% identical to *R. corynebacterioides* and *R. kroppenstedtii*. Isolates 3, 4, 5 and 6 were 99% identical and clustered with the group of *R. fascians*, *R. kyotonensis* and *R. yunnanensis* isolates (Figure 2).

Bacterial suspensions (0.7 OD600) of each isolate were prepared in buffer (10mM $MgCl_2$/MES), and micropropagated *I. herbstii* plants (n = 5) were inoculated by briefly dipping whole plants into the bacterial suspension of each isolate. Uninoculated control plants (n = 5) were dipped in sterile buffer. The plants were then transplanted into potting mix and incubated in a greenhouse maintained at 26°C with a 16 h photoperiod and 70% relative humidity. After 100 d, plant height and degree of lateral branching, and pathogen recovery, were assessed.

The degree of stunting and lateral shoot development varied between isolates. Plants inoculated with isolates 2, 4, and 5 were 41-46% shorter ($P \leq 0.05$) than uninoculated plants. Plants inoculated with isolates 4 and 6 produced more lateral stems ($P \leq 0.05$) than uninoculated plants (Table 1), and plants inoculated with isolate 6 exhibited more stems per plant than for all the other isolates (Table 1; Figure 1D). Epi- and endophytic populations of each isolate were recovered from inoculated plants but not from uninoculated plants, and identities of recovered isolates were confirmed through amplification of the specific virulence *vicA* gene. This completed Koch's postulates for the isolates.

To our knowledge, this is the first report of *Rhodococcus* spp. causing disease on *I. herbstii*, and the first report of pathogenic *Rhodococcus* spp. in Tunisia. These isolates are closely related (>98% similarity) to *Rhodococcus* isolates causing the pistachio bushy top syndrome, a recently-identified disease also associated with micropropagated plants (Stamler *et al.*, 2015). Further studies are necessary to determine genetic factors underlying specific host-isolate interactions.

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ABSTRACTS

Abstracts of oral and poster presentations given at the 11th International Workshop on Grapevine Trunk Diseases, Penticton, British Columbia, Canada, July 7–12, 2019

The 11th International Workshop on Grapevine Trunk Diseases (IWGTD) was held in Penticton, British Columbia, Canada, on July 7–12, 2019. This workshop was chaired by Dr. Úrbez-Torres and organized by the Agriculture and Agri-Food Canada Plant Pathology Laboratory at the Summerland Research and Development Centre (SuRDC). The 11th IWGTD also marked the 20th Anniversary of the workshop, which has been organized every two years since 1999. Delegates were presented the first day with an overview of the Okanagan Valley wine grape growing region of British Columbia by Dr. Bowen, Research Scientist and Plant Physiologist at the SuRDC.

The workshop was attended by 151 participants from 21 countries. In total, 64 oral and 48 poster papers were presented in four different sessions, including Pathogen Detection and Identification, Epidemiology, Host-Pathogen Interactions, and Disease Management. In addition, a special session on Grapevine Trunk Diseases (GTD) control was organized by the organizing committee the morning of Friday July the 12th with the goal to provide the industry and grapegrowers with a practical summary and overview of the current management options against GTD as well as the economic impact they cause. The invited speakers for this session were Dr. Gramaje from the "Instituto de Ciencias de la Vid y el Vino (ICVV)" in Logroño, La Rioja, Spain, who presented an overview on GTD management in nurseries and newly established vineyards; Dr. Sosnowski from the South Australian Research and Development Institute (SARDI), who presented an overview on GTD management in mature vineyards; Dr. Kaplan from the California State University in Sacramento, who presented an overview on the economic consequences of GTD and the potential gains from adopting preventative pruning practices and vine surgery; and Dr. Úrbez-Torres from Agriculture and Agri-Food Canada SuRDC, who presented an overview of the current status of GTD in Canada and their control.

Two field trips were organized during the workshop. The first field trip took place on the afternoon of July 9th with visit to vineyards in the North Okanagan. Delegates were presented with an overview of grapevine production in the Okanagan Valley followed by discussions on the main diseases affecting grapevines in the Valley, including GTD and visit to experimental trials where the SuRDC Plant Pathology team were conducting GTD spore trapping studies within the epidemiological research being conducted in British Columbia for GTD. The second field trip took place all day of July 11th and delegates visited two vineyards located in the South Okanagan Valley. The field trip was focused on presenting the current situation of GTD in The Okanagan Valley and management strategies being put in place.

It is worth to highlight that a student competition with awards to best oral and poster presentations was organized at the 11th IWGTD. This is the first time that such competition has been organized for the IWGTD. A total of 20 graduate students (MSC or PhD) participated in the competition. For best poster presentations, 1st place went to Pierluigi Reveglia, a joint PhD student between Charles Sturt University, Wagga Wagga, New South Wales, Australia and the University of Naples, Italy, for his paper "Isolation and characterisation of phytotoxins produced by the Botryosphaeriaceae and their role in grapevine trunk diseases"; 2nd place went to Edelweiss Rangel Montoya from the "Centro de Investigación Científica y de Educación Superior de Ensenada", Baja California, Mexico for her paper "Characterization of species of Lasiodiplodia *associated with grapevines in Mexico"; and 3rd place went to Daina Grinbergs from the "Instituto Nacional de Investigaciones Agropecuarias", Chillán, Chile for her paper "Fungal pathogens associated to grapevine trunk diseases from patrimonial vineyards in Chile". For best oral presentations, 1st place went to Jinxz Flamand from Agriculture and Agri-Food Canada Summerland RDC for his paper "Identification and characterization of potential biocontrol agents for the management of grapevine trunk diseases in British Columbia"; 2nd place went to Clément Labois from "Université de Haute-Alsace" Colmar, France for his presentation "Metabolomic studies of two* V.

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vinifera *subspecies during infection by* Neofusicoccum parvum*, a Botryosphaeria dieback pathogen – On the road to identify infection and resistance biomarkers"; and 3rd place went to María del Pilar Martínez-Diz from the "Estación de Viticultura e Enoloxía de Galicia" Ourense, Spain for her paper "Natural infections of pruning wounds by fungal trunk pathogens in mature vineyards in Galicia (northwest Spain)".*

The next 12th IWGTD will be held in Czech Republic in 2022 as the Council Members of the ICGTD decided that from now on the IWGTD will be organized every three years.

Invited lecture to celebrate the 20th anniversary of the International Council on Grapevine Trunk Diseases and Workshops

The mystery was young vine decline - the clue was black goo. LUCIE MORTON. *Morton Viticulture, PO Box 5607, Charlottesville, VA 22905, USA. E-mail: mortonviticulture@gmail.com*

A detective story unfolded in the world of viticulture that resulted in us being here today in beautiful British Columbia. Black goo was the term I coined to describe a dark, gummy ooze in xylem vessels that looks black to the naked eye. Without knowing the cause, my observation was that vines with the black goo symptom could appear normal for many years, decline prematurely, show intermittent dieback, or die suddenly. In 1990, I was asked to explain what would cause a very young vine to grow very vigorously up the stake, suddenly collapse in August, and grow very poorly, if at all the next year. Latent viruses and graft incompatibility, all supposedly linked to the susceptibility of the rootstock variety were common diagnoses. Although no specialist in grape pathology, I was deep into research on grapevine rootstocks and did not find these explanations satisfactory. I thought that fungi should be given consideration along with viruses and viroids. Little did I know that opening the mind to fungi can be like opening Pandora's Box! Had the black goo symptom simply remained one of many in the complex syndrome of esca in old vines, it might never have caught my attention. But when it appeared in the rootstocks of young grafted vines, it was impossible to ignore. Wherever my literature searches and travel took me around the world, I sought out plant pathologists who might provide insight into the symptom of black goo. A stroke of good fortune came one day in 1995 after complaining to a friend at UC Davis about the poor-quality images in a photocopy Luigi Chiarappa's "Wood decay of grapevine and Black Measles (1959)." There was one grainy photo of sectioned arms inoculated with *Cephalosporium* sp. that looked as if it might show black goo streaking. She suggested that I contact Luigi directly as he should be had just retired to Davis. It was at Luigi's California home where the

International Council on Grapevine Trunk Diseases was founded on July 21, 1998. And it was his coordination with Laura Mugnai and Giuseppe Surico that led to the next ICGTD meeting coinciding with the International Workshop on Grapevine Trunk Diseases Esca and Grapevine Declines hosted by the *Mediterranean Phytopathological Union* in Tuscany, Italy, October 1-2, 1999. This 20th anniversary meeting is a testament to our founding vision that a voluntary and well-coordinated research effort is the best way to bring the swiftest resolution to any problem.

ORAL PRESENTATIONS

Pathogen Detection and Identification

Ten years of investigation on the fungi associated with GTDs in west Sicily. VINCENZO MONDELLO[1], SELENE GIAMBRA[2], GAETANO CONIGLIARO[2], L. SANTOS[3], A. ALVES[3] and SANTELLA BURRUANO[2]. *[1]SFR Condorcet FR CNRS 3417, Université de Reims Champagne-Ardenne, Résistance Induite et Bioprotection des Plantes, RIBP EA 4707, BP 1039, Reims, Cedex 2 51687, France. [2]Università degli Studi di Palermo, Dipartimento di Scienze Agrarie, Alimentari e Forestali, viale delle Scienze, 4, 90128 Palermo, Italy. [3]Departamento de Biologia & CESAM, Universidade de Aveiro 3810-193 Aveiro, Portugal. E-mail : vincenzo.mondello@univ.reims.fr; santella.burruano@unipa.it*

After the first report of *Lasiodiplodia mediterranea* in 2008, as causal agent of a grapevine decline, epidemiological surveys on western-Sicilian vineyards with symptoms of decline highlighted the widespread presence of several GTD-associated pathogens. To date, these diseases represent a serious menace for the grape-growing regions worldwide, since no efficient and simple method is available for their control. We report the results of a 10-year study (2008-2018) on the taxonomy, pathogenicity and phylogeny of fungi associated with GTDs in West Sicily. In detail, observations were carried out in seven vineyards (cv. Alicante Bouchet, Grecanico, Grillo, Inzolia, Merlot, Muller Thurgau) located in three different provinces (2 in Agrigento, 1 in Caltanissetta and 5 in Trapani). Vines were visually inspected for GTDs symptoms, all the diseased vines were uprooted and submitted to laboratory for analyses. Fungal isolates were collected and identified at species level both traditionally and by molecular methods, using ITS and/or EF1-α regions. The latter were also used in phylogenetic analyses of Botryosphaeriaceae, the most abundant group isolated during this study. The pathogenicity of the collected strains, including different isolates of the same species were studied through *in planta* artificial inoculations. The results of our study, confirming the presence of various GTDs also in western Sicilian vineyards, showed a great abundance of Botryosphaeriaceae such as *Diplodia seriata, Neofusicoccum parvum, N. vitifusiforme* and *L. mediterranea* associated to wood necrosis in mature vineyards. As expected, the four Botryosphaeriaceae species showed different virulence producing significantly different vascular discoloration length. In young vines *Phaeomoniella chlamydospora, Phaeoacremonium* sp., *N. parvum, Neonectria* sp., *Ilyonectria* sp. and *Cadophora luteo-olivacea* were found associated to wood necrosis/discoloration. Interestingly, these fungi were selectively associated to specific symptoms and/or trunk parts, especially in the areas at root level and around the grafting point. Pathogenicity tests of some of the other fungi collected are still in progress. To our knowledge, this is the first report of *C. luteo-olivacea* on grapevine in Italy.

Identification of new pathogens associated with grapevine trunk diseases in Hungary: a review. KALMAN ZOLTAN VACZY. *Food and Wine Research Institute, Eszterházy Károly University, 3300 Eger, Eszterházy tér 1., Hungary. E-mail: vaczy.kalman@uni-eszterhazy.hu*

During the past years, more and more fungal pathogens have been described as associated with grapevine trunk diseases (GTDs). Similarly, new results on GTD have been recently published in Hungary. During an extensive study of fungal species associated with GTDs, roots and trunks of grapevine plants exhibiting typical GTD symptoms were collected at random from diverse cultivars in different Hungarian vineyards. Several hundreds of fungal strains were isolated from these samples and their identification was carried out based on colony morphology, conidiogenesis and conidial morphology for sporulating cultures and routine DNA sequencing and analysis for non-sporulating cultures. The objectives of this work were to identify the isolates obtained either by morphological characterization or based on DNA barcode sequences, and to assess or confirm the pathogenicity of selected strains on grapevine. New results obtained in this five-year study included (a) first report of pathogenic strains of *Seimatosporium vitis* associated with GTD symptoms in Hungary and worldwide; (b) first European record of *Dothiorella omnivora* and *Cytospora viticola* from grapevine, and associated with GTDs; (c) description of *Diaporthe hungariae* as a new *Diaporthe* species associated with several major diseases

of grapevines; and (d) the first report of *Neofabrea kienholzii* as GTD related pathogen. The results obtained revealed a great diversity of fungal pathogens that are linked to GTDs, and the complex background of this disease complex.

Wood-rotting Basidiomycetes associated with Esca in North America. KENDRA BAUMGARTNER[1], ALBRE BROWN[1], RENAUD TRAVADON[2] and DANIEL P. LAWRENCE[2]. *[1]United States Department of Agriculture - Agricultural Research Service, Davis, California, USA. [2]Department of Plant Pathology, University of California, Davis, California, USA. E-mail: Kendra.Baumgartner@ARS.USDA.GOV*

Phaeomoniella chlamydospora and wood-rotting Basidiomycetes, namely *Fomitiporia* spp., are known Esca pathogens. The importance of their sequence of infection in disease development, however, is not clear. To determine the impact of dual inoculations on wood symptoms, we inoculated potted *Vitis vinifera* 'Crimson Seedless' with *P. chlamydospora*, either alone or in combination with one of the following Basidiomycetes: *Coprinellus radians*, *Fomitiporia langloisii, F. polymorpha*, and newly described *Tropicoporus texanus*. All isolates originated from vines with leaf symptoms of Esca in California or Texas. In plants inoculated only with a Basidiomycete, wood lesions were characterized by orange discoloration. Such lesion in plants inoculated with *C. radians, F. langloisii,* or *T. texanus*, species not previously reported from grape, were not significantly different in length from that of known Esca pathogen *F. polymorpha* (ranging from 45 to 100% of the length of the woody stem, after 1 year), and all were larger than that of the wounded, non-inoculated controls. In dual inoculations, we tested the effects of sequential infection (*P. chlamydospora* first, Basidiomycete 6 months later, and vice versa), compared to simultaneous infection. After 1 year, plants inoculated with *P. chlamydospora* either alone or in combination with a Basidiomycete did not differ significantly in the length of lesions typically associated with *P. chlamydospora*, specifically dark black lines in the wood. Further, there was no effect of sequence of inoculation on the length of such *P. chlamydospora* lesions or Basidiomycete lesions, both of which were present in dual-inoculated plants. Because leaf symptoms were rare, it is difficult to determine how sequence of infection by *P. chlamydospora* and Basidiomycetes affects them. Nonetheless, our finding of no differences between sequential or simultaneous dual-inoculations, compared to single inoculations, in development of wood symptoms suggests that infection by Basidiomycetes may not require initial infection by *P. chlamydospora*.

Early season symptoms on grapevine stem and inflorescences associated with Botryosphaeriaceae species. PEDRO REIS[1], ARTUR ALVES[2], FLORENCE FONTAINE[3], INÊS LOURENÇO[4], JOSÉ SARAMAGO[4] and CECILIA REGO[1]. *[1]LEAF - Linking Landscape, Environment, Agriculture and Food, School of Agriculture, University of Lisbon, Lisboa, Portugal. [2]Department of Biology, CESAM – Centre for Environmental and Marine Studies, University of Aveiro, Aveiro, Portugal. [3]SFR Condorcet FR CNRS 3417, Université de Reims Champagne-Ardenne, RIBP EA 4707, BP 1039, Reims, Cedex 2 51687, France. [4]BASF Portuguesa, S.A. Rua 25 de Abril, 1, 2689-538 Prior Velho, Portugal. E-mail: pedroreis@isa.ulisboa.pt*

Botryosphaeria dieback is one of the most important grapevine trunk diseases affecting vineyards worldwide. Several species of the Botryosphaeriaceae family in the genera *Neofusicoccum*, *Diplodia* and *Lasiodiplodia*, have been associated with Botryosphaeria dieback symptoms. These fungi are xylem inhabiting and attack the framework of grapevines, causing typical wedge-shaped perennial cankers in the wood that can lead to death of vines. These pathogens were also reported to cause early season symptoms including leaf and berry symptoms. However, these symptoms may be mistaken with symptoms from other diseases such as downy mildew. Currently, studies on what Botryosphaeriaceae species are commonly associated with early season symptoms in grapevines are limited. Therefore, during the 2018 season, when grapevines were on the E-L 23 - 25 phenological stage, samples showing necrosis on green shoots and dried inflorescences were collected in several vineyards spread throughout the central regions of Portugal. Fungal isolations were performed by plating symptomatic tissues onto potato dextrose agar (PDA) amended with chloramphenicol. DNA were extracted from the 23 Botryosphaeriaceae isolates recovered from the samples using a Qiagen® extraction kit. DNA sequence analyses of ITS region and part of the elongation factor 1-αgenes revealed that the two major species frequently associated with the symptoms were *Diplodia seriata* and *Neofusicoccum parvum*. Representative isolates of each species that were selected for pathogenicity tests were able to reproduce the early season symptoms in 1-year old grapevine plants confirming these species were the primary pathogens of the disease. Since the appearance of these early season symptoms have not yet

been described, thus, further studies is needed to further understand the importance of these pathogens the early seasons symptoms observed in the vineyards.

Grapevine trunk diseases symptoms present in rootstocks and scions cuttings in the main grapevine nursery in Uruguay. MARÍA JULIA CARBONE, MATÍAS GELABERT, PEDRO MONDINO and SANDRA ALANIZ. *Department of Plant Protection, Faculty of Agronomy, University of the Republic, Av. Garzón 780, CP 12900, Montevideo, Uruguay. E-mail: mariajulia.93@hotmail.com*

Grapevine trunk diseases are one of the most important problems for grapevine nurseries. Rootstock and scion mother fields are usually infected by the fungi included in this complex. The symptoms present and their incidence on cuttings just after being cut from rootstocks and scions mother vines was investigated for the first time in the main grapevine nursery of Uruguay. The rootstocks evaluated were SO4, Gravesac, 1103P, 3309C and RR101-14 and the scions were Tannat, Marselan, Albariño, Merlot and Lácrima Christi. Fifty cuttings 110 cm long of each rootstock and scion were carefully observed by cross-sectional and longitudinal cuts. Isolations from symptomatic and asymptomatic samples were performed and identified by morphological and DNA analysis. The incidence of symptoms observed in the basal and distal parts of each rootstock cuttings were S04 16% and 15%, Gravesac 18% and 4%, 1103 P 6% and 4%, 3309 C 38% and 26% and RR 101-14 4% and 8%, respectively. In the scions were Tannat 48% and 2%, Marselan 18% and 6%, Albariño 30% and 18%, Merlot 14% and 4% and Lácrima Christi 20% and 8%, respectively. Presence of dark xylem vessels and necrotic streaks was the main symptom observed and secondly it was internal necrotic wedge-shaped staining in the cross section. Several Botryosphaeriaceae species, *Diaporthe* species and *Phaeomoniella chlamydospora* were the fungi identified. This work confirms the presence of grapevine trunk diseases in cuttings used in the main grapevine nursery in Uruguay, emphasizing the importance of developing management strategies to minimize the incidence of these diseases.

Characterization and pathogenicity of black-foot disease pathogens isolated from symptomless grapevine nursery stock in Spain. MARCOS ANDRÉS-SODUPE[1], CARMEN BERLANAS[1], REBECA BUJANDA[1], EMILIA DÍAZ-LOSADA[2], BEATRIZ LÓPEZ-MANZANARES[1], MARÍA DEL PILAR MARTÍNEZ-DIZ[2], SONIA OJEDA[1] and DAVID 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]Estación de Viticultura e Enoloxía de Galicia (AGACAL-EVEGA), Ponte San Clodio s/n 32428-Leiro-Ourense, Spain. E-mail: marcos.andres@icvv.es*

In this study, 3,426 grafted plants ready to be sold to producers were surveyed from 15 grapevine nursery fields in northern Spain from 2016 to 2018. In all, 1,427 black-foot pathogen isolates were collected from the non-necrotic inner tissues of surface sterilized symptomless secondary roots and characterized based on morphological features and comparison of DNA sequence data of the nuclear ribosomal DNA-internal transcribed spacer region, histone H3, translation elongation factor 1-α and β-tubulin genes. Eleven species belonging to the genera *Dactylonectria*, *Ilyonectria*, *Neonectria* and *Thelonectria* were identified, namely *D. alcacerensis*, *D. macrodidyma*, *D. novozelandica*, *D. pauciseptata*, *D. torresensis*, *I. liriodendri*, *I. pseudodestructans*, *I. robusta*, *N. quercicola*, *N. sp.* 1 and *T. olida*. In addition, two species are newly described, namely *D. riojana* and *I. vivaria*. Twenty-four isolates representing the 13 black-foot species were inoculated in grapevine seedlings cultivar Tempranillo. The pathogenicity tests detected virulence diversity among fungal species and between isolates within each species. The most virulent species was *D. novozelandica* isolate BV-760, followed by *D. alcacerensis* isolate BV-1240 and *I. vivaria* sp. nov. isolate BV- 2305. The present study improves our knowledge on the etiology and virulence of black-foot disease pathogens, and opens up new perspectives in the study of the endophytic role of these pathogens on grapevines.

Diversity of *Cylindrocarpon*-like species associated with roots of grapevine and other fruit and nut crops in California. DANIEL P. LAWRENCE[1], MOHAMED T. NOURI[2] and FLORENT P. TROUILLAS[2]. *[1]Department of Plant Pathology, University of California, Davis, CA, 95616, USA. [2]Department of Plant Pathology, University of California, Kearney Agricultural Research and Extension Center, Parlier, CA, 93648, USA. E-mail: dlawrence@ucdavis.edu*

Black foot disease is a common and destructive root disease of grapevine caused by taxonomically diverse cylindrocarpon-like fungi in many viticultural areas of the world. This study identified 12 cylindrocarpon-like fungal species from five genera associated with black

foot disease of grapevine and other root diseases of fruit and nut crops in the Central Valley Region of California. Morphological assessments paired with multi-locus sequence typing of four loci, internal transcribed spacer region of nuclear rDNA ITS1–5.8S–ITS2 (ITS), beta-tubulin (*TUB2*), translation elongation factor 1-alpha (*TEF1*), and histone (*HIS*), revealed ten previously described species; *Campylocarpon fasciculare*, *Dactylonectria alcacerensis*, *D. ecuadoriensis*, *D. macrodidyma*, *D. novozelandica*, *D. torresensis*, *D. valentina*, *Ilyonectria capensis*, *I. liriodendri*, *I. robusta*, and two new species, *Neonectria californica* sp. nov., and *Thelonectria aurea* sp. nov. Phylogenetic analyses of the ITS+*TUB2*+*TEF1* combined dataset, a commonly employed dataset used to identify filamentous ascomycete plant pathogens, was unable to assign some species, with significant support, in the genus *Dactylonectria*, while all other species in other genera were confidently identified. The *HIS* marker was essential either singly or in conjunction with the aforementioned genes for accurate identification of most *Dactylonectria* species. Results from isolations of diseased plant tissues revealed potential new host associations for almost all fungi recovered in this study. This work is the basis for future studies on the epidemiology and biology of these important and destructive plant pathogens.

Genetic analysis of *Dactylonectria torresensis* populations from grapevine using microsatellite markers. CARMEN BERLANAS[1], JOSEP ARMENGOL[2], MÓNICA BERBEGAL[2], AKILA BERRAF[3], ANTONIA CARLUCCI[4], ALES EICHMEIER[3], FRANCOIS HALLEEN[5], MARÍA DEL PILAR MARTÍNEZ-DIZ[6,7], LIZEL MOSTERT[8], PEDRO REIS[9] and DAVID 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]Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain. [3]Mendel University in Brno, Faculty of Horticulture, Mendeleum - Institute of Genetics, Valtick_a 334, 69144, Lednice, Czech Republic. [4]Department of Science of Agriculture, Food and Environment, University of Foggia, Via Napoli, 25, 71122 Foggia, Italy. [5]ARC Infruitec-Nietvoorbij (The Fruit, Vine and Wine Institute of the Agricultural Research Council), Private Bag X5026, Stellenbosch 7599, South Africa. [6]Estación de Viticultura e Enoloxía de Galicia (AGACAL-EVEGA), Ponte San Clodio s/n 32428-Leiro-Ourense, Spain. [7]Universidade da Coruña, Facultade de Ciencias, Zapateira, 15071 A Coruña, Spain. [8]Department of Plant Pathology, University of Stellenbosch, P/Bag X1, Matieland 7602, South Africa. [9]LEAF - Linking Landscape, Environment, Agriculture and Food, School of Agriculture, University of Lisbon, Lisboa, Portugal. E-mail: carmen.berlanas@icvv.es*

The soil-borne fungus *Dactylonectria torresensis* is the most common causal agent of black-foot disease of grapevine in Europe. Despite the importance and wide distribution of *D. torresensis*, no information is still available on the pathogen´s population structure. In this study, the genetic diversity and subdivision among and within populations of *D. torresensis* was investigated in Spain and other Mediterranean countries. In total, 190 isolates from two regions in northern Spain (La Rioja and Navarra), Algeria, Italy, and Portugal were genotyped. Eleven newly developed polymorphic simple-sequence repeat markers revealed 144 multilocus genotypes (MLG) in the Spanish population, and 112 MLGs globally. Discriminant analysis of principal components (DAPC) showed that populations from northern Spain are structured into six groups. Globally, five distinct genetic clusters were observed. Two of these clusters included only Spanish MLG, other two clusters consisted on Spanish and Portuguese MLG and the last cluster included Spanish, Algerian and Italian MLG. In general, pairwise G´st values showed very low genetic differentiation, especially among populations from Spain and Portugal and within Spanish populations. High genotypic diversity indicated that sexual reproduction might occur among *D. torresensis* isolates. However, linkage disequilibrium analyses did not support the hypothesis of random mating. The population genetic structure of *D. torresensis* in Europe is likely associated with the spread of isolates by infected propagation material.

Global patterns of genetic diversity and population subdivision in *Phaeomoniella chlamydospora*. RENAUD TRAVADON[1], PHILIPPE E. ROLSHAUSEN[2], DAVID GRAMAJE[3], FRANCOIS HALLEEN[4], JOSÉ RAMÓN ÚRBEZ-TORRES[5], SAMI J. MICHEREFF[6], MARCUS A. K. ALMANÇA[7], WALTER D. GUBLER[1], JOSEP ARMENGOL[8] and KENDRA BAUMGARTNER[9]. *Department of Plant Pathology, University of California, Davis, USA. [2]Department of Botany and Plant Sciences, University of California, Riverside, USA. [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. [4]ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa. [5]Agriculture and Agri-Food Canada, Science and Tech-*

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Phaeomoniella chlamydospora is a Petri disease and Esca pathogen reported from all major grape-growing regions of the world. This ascomycete fungus colonizes xylem vessels, impairing the translocation of water and nutrients. Sexual fruiting bodies of the fungus have not been found in vineyards. Asexual spores are thought to be the cause of infections in the vineyard and nursery. As such, the pathogen population presumably displays low levels of genetic diversity. Using 18 published microsatellite markers, we genotyped approximately 250 isolates collected from distant regions of the world: Brazil, British Columbia in Canada, South Africa, Spain, and the northeastern and western United States. Preliminary findings from analyses of all North American isolates revealed a high haplotypic diversity, suggesting that sexual reproduction generates new allelic combinations. Clustering analyses revealed three genetic groups of isolates. Two genetic groups spanned the California and British Columbia collections, whereas the third group was comprised mainly of northeastern US isolates. High genetic subdivision (Fst = 0.28; $P < 0.001$) suggests that genetic and/or ecological factors may maintain genetic differentiation among these three groups. Significant differences in levels of genetic diversity were found among the groups, with the highest gene diversity (H = 0.83) and allelic richness (A = 9.4) in the northeastern US. A high diversity of *Vitis* species in this region, and possibly differences in pathogen life history, may explain these findings. With the current, additional genotyping of isolates from Brazil, South Africa, and Spain, we will determine a more accurate picture of the distribution of genetic diversity of this pathogen at a multi-continental scale, in order to pinpoint centers of genetic diversity in the pathogen population and potential introduction routes among grape-growing regions.

Sensing grapevine trunk disease using metabolic markers. DION MUNDY[1], BHANUPRATAP VANGA[2], NIGEL JOYCE[2], PETER JAKSONS[2], CATHERINE SANSOM[3], NIGEL PERRY[3] and MONIKA WALTER[4]. *[1]The New Zealand Institute for Plant and Food Research Ltd (Plant & Food Research), Marlborough Wine Research Centre, PO Box 845, Blenheim 7240, New Zealand. [2]Plant & Food Research, Christchurch 8140, New Zealand. [3]Plant & Food Research, Department of Chemistry, University of Otago, Dunedin 9054, New Zealand. [4]Plant & Food Research, Motueka 7198, New Zealand. E-mail: Dion.mundy@plantandfood.co.nz*

Grapevine trunk disease (GTD), caused by a variety of different pathogens, including *Eutypa lata* and *Phaeomoniella chlamydospora*, is a major problem for commercial vineyards. Non-destructive detection using technologies such as field sensors that detect metabolic markers associated fungal infections could allow for targeted disease management. Understanding the chemical profiles during infection is the first step for developing these sensors. Mature Sauvignon blanc plants with presence or absence of one or more GTD-causing pathogens (confirmed by next generation sequencing) and grapevine cuttings inoculated with *E. lata* were analysed for changes in metabolite compounds. Non-volatiles were measured by solvent extraction and ultra-high performance liquid chromatography – high resolution mass spectrometry (LC-MS), and volatiles by headspace - solid phase microextraction - gas chromatography - mass spectrometry. A range of potential non-volatile markers were identified. Some were likely to be produced from the fungal pathogens causing disease, and others would be plant metabolites associated with disease response or vine stress. *E. lata* infection correlated with the unique eutypines and metabolites detected, whereas *P. chlamydospora* infection correlated with coumaryl derivatives and benzoic acid markers. Stilbene isomers and polymeric forms upregulated in response to all fungal pathogens. Vine stress was additionally shown by high ratios of sucrose to fructose or glucose with fungi present. No volatile marker compounds associated with fungal infection were found. The changes in leaf metabolite profiles identified in this study could be used to develop sensing technologies for detection of GTD in vineyards. These tools would allow non-destructive detection of infections, aiding vineyard and greenhouse decision-making for disease management.

Into the wood – monitoring fungal pathogen's development and impact on wood tissues through non-destructive imaging. CÉDRIC MOISY[1], ROMAIN FERNANDEZ[1], MAÏDA CARDOSO[2], SAMUEL

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Imaging approaches and image processing have considerably improved plant phenotyping and are nowadays increasingly used for phenotypic trait measurement. Magnetic Resonance micro-Imaging (MRI) and X-ray computed micro-tomography are non-destructive imaging techniques based on the magnetic properties of atomic nuclei, and the relative radio-density, respectively. They are widely used for medical diagnosis although these techniques remain rare in plant studies. However, they could enable the study of complex plant-pathogen interactions, as observed in grapevine trunk disease (GTD). Our research aims to estimate the benefits of imaging approaches for detection of GTD, and to evaluate the impact of fungal pathogens on host tissues through non-destructive monitoring directly into the wood. Using µ-MRI and X-ray µ-CT, we performed a dynamic monitoring of wood colonization by fungi artificially inoculated under controlled conditions. Interesting results were thereby collected on both the progression of each fungus in the different tissues and their impact on the live plant. In parallel, we were also able to detect, localize and quantify different types of degraded tissues in old grapevine plants collected in vineyards. Altogether, these results demonstrated that non-destructive imaging provides informative techniques to monitor pathogenic fungi progression in the wood. They could lead to the development of new markers for monitoring trunk diseases, and for phenotyping genetic resources for their level of tolerance. Imaging approaches open new perspectives for increasing our knowledge on GTD. These news tools could also be developed for evaluating varietal tolerance in breeding programs, and for measuring the real effect and efficacy of new molecules or biocontrol agents onto the pathogen propagation in the wood. Non-destructive imaging approaches would then benefit both the wine growing industry and the researchers.

Molecular detection of Grapevine Trunk Diseases fungi in Chilean vineyards. FELIPE GAINZA-CORTÉS, PAULINA ARRAÑO-SALINAS, ROSA ROA-ROCO, PABLO RODRIGUEZ-HERRERA, IVO AGNIC, DANIELA TORRES, PAMELA ESPINOZA, CARLOS VALDIVIA and ALVARO GONZALEZ. *Viña Concha y Toro S.A, Center for Research and Innovation, Fundo Pocoa s/n, Km10 Ruta K-650, Pencahue, Región del Maule, Chile. E-mail: felipe.gainza@conchaytoro.cl*

Fungi associated with Grapevine Trunk Diseases (GTDs) have received increased attention in vineyards worldwide during recent years. It has been widely stablished that mother-plant block sanitary status and nursery practices are both implicated in the spread of GTDs, affecting vineyard productivity, longevity and grapes quality. In this context, the implementation of an early diagnosis system would complement and positively impact the management and mitigation of this problem. Since 2016, The Center for Research and Innovation of Viña Concha y Toro (VCT) have implemented PCR based methods (quantitative and end-point) for the detection of the 5 main worldwide GTDs-associated fungi (*Diplodia seriata*, *Neofusicoccum parvum*, *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum* and *Eutypa lata*) and the 12 main viruses associated to grapevines. In this work, we present the results of the analysis of more than 1600 plants (five rootstocks and 24 clonal selections belonging to five cultivars) in VCT vineyards. From the total plants analyzed for GTDs, it was observed a higher incidence of Diplodia complex and *Eutypa lata* showed the highest incidence of detection. Additionally, a 73% of Diplodia complex and 40% of *Eutypa lata* incidence was observed for Cabernet sauvignon; 39% and 19% for Sauvignon blanc, respectively; 13% and 21% for Chardonnay, respectively; and 37% and 25% for the evaluated rootstock, respectively. As a result of this survey, it was possible to identify 907 plants free of four of the most detrimental viruses, among these, 313 plants were free of all 12 viruses. Within the 313 high standard virus-free plants, we identify 184 GTD-free plants. Those virus/GTD-free plants have been multiplied and used in the plantation of new, healthier mother-plant blocks that will allow VCT to increase the phytosanitary status and longevity of its vineyards.

The potential of LAMP assays for the detection of grapevine trunk disease pathogens. JAMES WOODHALL[1], ALEXANDRA OROPEZA[1], GUIDO MARCHI[2] and LAURA MUGNAI[2]. *[1]University of Idaho, Research and Extension Center, Parma, Idaho, USA. [2]Department of Agricultural, Food, Environmental and Forestry Science and Technology (DAGRI), Plant pathology and Entomology section, University of Florence, P.le delle Cascine, 28, 50144 Firenze, Italy. -mail: jwoodhall@uidaho.edu*

Early, accurate diagnosis is essential for successful treatment of a plant disease. Although diagnosis can sometimes be made from symptoms alone, laboratory testing is often required. Molecular tests such as real-time PCR are sensitive and accurate but are frequently confined to the laboratory and take several hours to complete. Loop mediated isothermal amplification (LAMP) offers a rapid and portable alternative for molecular testing. In this study, separate LAMP assays were developed for *Phaeomoniella chlamydospora* and *Eutypa lata*, causal agents of a vascular disease within Esca complex including Petri disease in young vineyards, and of Eutypa dieback respectively. For *P. chlamydospora*, primers were designed from the rDNA ITS region, whilst for *E. lata*, primers were designed from sequences derived from unique RAPD fragments. Assays were specific to the target pathogen and tested against DNA originating from 30 isolates representing closely related species and other grapevine trunk disease pathogens. The assays were sensitive, with a time to positive with DNA from pure culture within ten minutes on a Genie III (Optigene, UK), although the equivalent TaqMan real-time PCR assays were ten times more sensitive. Detection in grapevine wood was possible using an on-site DNA extraction method. Wood was sampled using a cordless drill and transferred to a 5 mL tube containing 1 mL of PEG/sodium hydroxide buffer and a ball bearing. After vigorous shaking by hand, the lysate was diluted tenfold and used in the LAMP reaction. Wood samples using the in-field extraction method were positive within 10-30 minutes. In conclusion, LAMP offers a rapid and portable diagnostic and detection tool. Although, real-time PCR is suited to high throughput testing where sensitivity is paramount within a fully equipped laboratory, LAMP is a viable alternative for use in low resource labs and when on-site testing is necessary.

Loop mediated isothermal amplification (LAMP) assays to detect *Diplodia seriata* and *Neofusicoccum parvum* isolated from Chilean grapevine wood samples. FELIPE SÁEZ CORTEZ[1,2], ÁLVARO CASTRO OYARZÚN[2] and RUBÉN POLANCO OTEIZA[1]. *[1]Laboratory of Phytopathogenic fungi, Center of Vegetal Biotechnology, Faculty of Life Sciences, University Andrés Bello, Avenida República 330, Santiago, Chile. [2]UC Davis Chile Life Science Innovation Center. Avenida Andrés Bello 2299, piso 11, Providencia. E-mail: f.sezcortez@uandresbello.edu*

Members of Botryosphaeriaceae family of phytopathogenic fungi have been reported to cause grapevine trunk diseases (GTD) worldwide, causing a devastating impact in fruit yield and the lifespan of the plants. In Chile, *Neofusicoccum parvum* and *Diplodia seriata* are two members of the Botryosphaeriaceae that are commonly isolated and identified as trunk disease causing agents. Early detection of these pathogens, particularly in the stages of clonal propagation of the grapevines could help screen out infected but asymptomatic material. Loop mediated isothermal amplification (LAMP) is a molecular technique performed at a constant temperature of 60 to 65°C using either two or three sets of primers and a polymerase with high strand displacement activity. This technique has the potential to be fast, sensitive and highly specific. It can also be used in low resource labs or on-site as DNA amplification can be observed visually, through either the increase in the turbidity when amplification occurs, or with the aid of colorimetric indicators such as hydroxynaphtol blue (HNB). In this study, we successfully developed LAMP assays to detect *Neofusicoccum parvum* and *Diplodia seriata*. Amplification was determined through turbidity evaluation and HNB colorimetric indicator. The assays were highly specific and did not detect DNA extracted from a range of other GTD fungi. This study shows the potential of LAMP for the rapid detection of two GTD fungi in grapevine material.

Droplet digital PCR technology for detection and quantification of black-foot disease pathogens and *Cadophora luteo-olivacea* from grapevine nursery stock and vineyard soil. MARÍA MERCEDES MALDONADO-GONZÁLEZ[1], MARCOS ANDRÉS-SODUPE[1], MÓNICA BERBEGAL[2], REBECA BUJANDA[1], EMILIA DÍAZ-LOSADA[3], MARÍA DEL PILAR MARTÍNEZ-DIZ[3,4] and DAVID GRAMAJE[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]Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain. [3]Estación de Viti-*

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Cylindrocarpon-like asexual morphs and *Cadophora luteo-olivacea* are fungal pathogens frequently isolated from planting material in grapevine nurseries and young vineyards, causing black-foot and Petri disease, respectively. Accurate, early and specific detection and quantification of these fungi are essential to alert growers and nurseries about their presence in soil, and to prevent their spread in grapevine using certified pathogen-free planting material. We comparatively assessed the accuracy, efficiency, and specificity of droplet digital PCR (ddPCR) and real-time PCR (qPCR) techniques for the specific detection and quantification of *Cylindrocarpon*-like asexual morphs in grapevine root, rhizosphere and vineyard soil, and *Cadophora luteo-olivacea* in vineyard soil and grapevine wood. In both cases, ddPCR was more sensitive to lower target concentrations than qPCR. Plant-soil fraction did not affect the abundance of black-foot associated pathogens in vineyards. *Cadophora luteo-olivacea* was detected in 31.7% of analyzed plants with variable abundances. Few samples tested positive for the target fungus in soil. Roots of asymptomatic vines were found to be a microbial niche that is inhabited by black-foot disease fungi, which opens up new perspectives in the study of the endophytic role of these pathogens on grapevines. Further research is needed to determine minimum infection thresholds needed for trunk disease fungi to start infection in the field and to understand which planting conditions will enhance disease proliferation and benefit pathogen colonization.

Characterization and detection of grapevine fungal pathogens using Fatty Acid Methyl Ester analyses (FAME). CHRISTOPHER M. WALLIS. *U.S. Department of Agriculture, Agricultural Research Service, San Joaquin Valley Agricultural Sciences Center, Crop Diseases, Pests and Genetics Research Unit, Parlier, CA 93648, USA. E-mail: christopher.wallis@usda.gov*

Grapevines can become infected by a variety trunk disease-causing fungi including those from genera such as *Diplodia, Neofusicoccum, Lasiodiplodia*, and others. These diverse pathogens can vary greatly in disease severity and symptom expression. Therefore, proper identification is necessary to determine the best management strategy. Although sequence-based genotyping can distinguish these fungal canker pathogens, there is a need for faster, cheaper ways to distinguish casual agents of trunk diseases. To address this, fingerprinting of fatty acids that comprise cell membranes of each pathogen was performed using gas chromatography on methyl esters (FAME). This was done to create profiles for over 20 different fungal species, with additional analyses that followed to verify accuracy of identification. With FAME, a total of 20 samples from culture can be analyzed in as little as two hours, and costs far lower than that of DNA-based techniques at less than 10 cents a sample. Ongoing work is examining ways to extract and analyze fungal FAMEs directly from plant tissues to avoid the need for culturing. FAME allows an alternative to DNA-based identification and could be employed in cases where nucleic acid degradation is a concern, or if an alternative methodology is required for regulatory actions.

Epidemiology

Incidence and severity of Botryosphaeria dieback in Chilean vineyards cv. Cabernet Sauvignon assessed in 2010 and 2018. XIMENA BESOAIN[1], CAROLINA TORRES[1], MICHAEL SEEGER[2] and ALEJANDRA LARACH[1]. *[1]Escuela de Agronomía, Facultad de Ciencias Agronómicas y de los Alimentos, Pontificia Universidad Católica de Valparaíso, San Francisco s/n La Palma, 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*

In Chile, one of the main crops is the grape (*Vitis vinifera*), for the production of both table grapes and wine. For wine production there are around 140,000 hectares and Cabernet Sauvignon is the main cultivar. Grapevine trunk diseases (GTDs), have become relevant in recent years, both in the world and in Chile, due to the greater appearance of symptoms associated with these type of disease. The main consequence of the plant dieback is the loss of productivity of the vineyards, due to the death of a part and/or the entire plant. Therefore, the objectives of this study were to estimate the incidence and severity of the GTDs and the impact on vineyard production during two years, 2010 and 2018. For this reason, eight blocks from five vineyards in O´Higgins Region and six blocks from five vineyards in Maule Region were sampled, each block consisting of 100 plants. In the O´Higgins Region, there was an increase in both incidence and severity from 2010 to 2018, with

an 82.4% and 43.6%, respectively in 2018. On the contrary, in the Maule Region, a decrease in the incidence and severity between 2010 and 2018 was observed, with an incidence of 87.2% and severity of 51.6% in 2018. In relation to the yield, in 2010-11 an average loss of almost 5 tons/ha was estimated for the O'Higgins Region and 6 tons/ha for the Maule Region. Average loss is currently being measured for the 2018-2019 harvest in both vineyards. Our results clearly demonstrated the importance and impact of GTDs in central Chile.

The diversity and epidemiology of Botryosphaeriaceous grapevine trunk disease pathogens occurring on other woody hosts in the winelands of South Africa. FRANCOISE HALLEEN[1,2], IHAN L. DU PLESSIS[1,2], LIZEL MOSTERT[2], BERNARD SLIPPERS[3] and JAN H. NAGEL[3]. *[1]Plant Protection Division, ARC Infruitec-Nietvoorbij, Private Bag X5026, Stellenbosch 7599, South Africa; [2]Department of Plant Pathology, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa; [3]Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0001, South Africa. E-mail: halleenf@arc.agric.za*

Species from the Botryosphaeriales are the most common and widespread pathogens of woody hosts globally and often affect fruit industries. These pathogens affect grape industries by causing yield losses and shortening the productive lifespan of infected grapevines. Farmers are limited to using good management practices to manage these fungi. Such management strategies are primarily aimed towards preventing pathogens from spreading between infected and healthy grapevines, but disregard the possible movement of pathogens from other host species. The broad host ranges of many Botryosphaeriaceae species, however, mean that alternative hosts of these grapevine pathogens can serve as sources of inoculum. To investigate this, a Botryosphaeriaceae species diversity survey was performed on 37 different host species that commonly occur throughout the winelands in Western Cape Province of South Africa. In total 950 Botryosphaeriaceae strains were isolated from visibly diseased hosts. These represented twenty-six different Botryosphaeriaceae species, six of which could not be identified to species level and represent new species. The grapevine pathogen, *Neofusicoccum stellenboschiana*, was amongst the most common and was isolated from 24 different host species. Populations of *N. stellenboschiana* from different hosts at three different locations were subsequently characterized using seven microsatellite markers. The results showed shared genotypes of *N. stellenboschiana* on the grapevines and other hosts at all of the locations, indicating that this species can spread between these hosts. These findings suggest that alternative hosts of grapevine trunk disease pathogens pose a risk to vineyards, and that these are not addressed by current management practices.

Standardizing Botryosphaeriaceae infection levels in experimental grapevine plant materials. REGINA BILLONES-BAAIJENS[1], MARK SOSNOWSKI[2,3], MATTHEW AYRES[2] and SANDRA SAVOCCHIA[1]. *[1]National Wine and Grape Industry Centre, Faculty of Science, Charles Sturt University, Wagga Wagga, NSW, Australia. [2]South Australian Research and Development Institute, Adelaide, SA, Australia. [3]School of Agriculture, Food and Wine, The University of Adelaide, Waite Campus, Glen Osmond, SA, Australia. E-mail: rbaaijens@csu.edu.au*

Grapevine nursery plants have been reported with latent infections of grapevine trunk disease (GTD) pathogens. However, the link between these latent infections and GTD symptoms observed in vineyards is still unclear. Studies have also shown that water-stress can increase the susceptibility of young vines to GTDs. Accurate quantification of infection levels from naturally-infected young vines is difficult since latent infections have no internal or external symptoms and may be randomly distributed within a vine. Thus, investigations on the effects of water stress on young vines artificially inoculated with three different conidial concentrations of GTD pathogen may provide insight on the infection thresholds that result in disease expression in vines. The objective of this study was to develop an inoculation method to standardize GTD infection levels in young vines. A published vacuum-inoculation method was evaluated for inoculating Shiraz cuttings with 300 (low), 3,000 (moderate) and 30,000 (high) conidia of *Neofusicoccum luteum* while Ringer's solution was used to inoculate control vines. The vacuum-inoculation was a reliable method as evidenced by qPCR analyses, resulting in the conidia infecting the basal, middle and apical part of the inoculated canes. The qPCR analyses further differentiated the low, moderate and high infections with the highest amount of pathogen detected from canes inoculated with 30,000 conidia and lowest from those inoculated with 300 conidia. No pathogen was detected in any of the control canes. This study showed the vacuum-inoculation method was suitable for introducing different concentrations of *N. luteum* spores into dormant canes without significant impact on plant viability.

The method was used to standardize infection levels of *N. luteum* in ~400 Shiraz rootlings in newly established glasshouse and shade house experiments. Observations on the effect of water stress on Botryosphaeria dieback symptom expression is on-going.

Saprophytic colonization of the bark by *Neofusicoccum* species mediates subsequent infection of grapevines through wounds. AMNA SHAFI[1,2], HAYLEY RIDGWAY[1,3], MARLENE JASPERS[1] and EIRIAN JONES[1]. *[1]Department of Pest-management and Conservation, Faculty of Agriculture and Life Sciences, Lincoln University, PO Box 85084, Lincoln, Canterbury, New Zealand. [2]Directorate General of Agriculture Research and Planning, Khyber Pakhtunkhwa, Agriculture University Peshawar, Pakistan. [3]The New Zealand Institute for Plant & Food Research Ltd, Lincoln 7608, Canterbury, New Zealand. E-mail: Eirian.Jones@lincoln.ac.nz*

Botryosphaeriaceae species infect grapevines via wounds. A previous study isolated Botryosphaeriaceae at higher frequencies from the bark than the underlying wood of asymptomatic grapevines canes from vineyards, suggesting they were latent on surface tissues. This study investigated the colonization of the bark as a saprophytic link to infection of the underlying wood. The bark of trunks of Sauvignon blanc and Pinot noir potted vines were inoculated by spraying an area of 3 cm length with ~1 mL of a *Neofusicoccum luteum* or *N. parvum* conidial suspensions (10^4/mL). Control vines were inoculated with sterile water. After 1 hour (T1), 2 days (T2) or 7 days (T3) a cut was made in the bark and through to the wood 1 cm above the inoculation area using a sterile scalpel. After 24 h, isolations were carried out from surface sterilized bark and wood. Infection incidence did not differ significantly between species or grapevine cultivar. Infection incidence of the bark was 100% and associated wood of the central inoculated section was 76.3%, 83.3% and 90.2% for T1, T2 and T3, respectively indicating bark infection progressed rapidly into the adjacent wood. Infection of bark and wood 1 cm above the inoculation point increased with incubation time, being 25% for bark and wood at T1 and 71% and 67% for bark and wood, respectively at T3. Infection of the bark and wood 1 cm below the inoculated area was 0%. This study showed that the pathogens remained latent in the bark and, when the cane was wounded, that the pathogen progressed towards the wound. Fluorescent microscopic observations of bark and underlying wood sections of shoots inoculated onto the bark, but without wounding, showed germinating conidia and mycelium in the bark near lenticels, and mycelia in the underlying wood, indicating that the pathogens had entered through lenticels.

Early detection project - detection and quantification of *Phaeomoniella chlamydospora* and *Botryosphaeria* spp. in *Vitis vinifera* wood samples. SZABINA LENGYEL[1], RANDALL E. GOLD[2], JOCHEN FISCHER[1], ALEXANDER YEMELIN[1], ECKHARD THINES[1] and ANNETT KÜHN[2]. [1]*Institut für Biotechnologie und Wirkstoff-Forschung gGmbH, Erwin-Schrödinger-Straße 56, D-67663 Kaiserslautern, Germany.* [2]*BASF SE, Agricultural Center, Speyerer Straße 2, D-67117 Limburgerhof, Germany. E-mail*: *lengyel@ibwf.de*

Phaeomoniella chlamydospora (PCH), associated with esca, and Botryosphaeriaceae spp. (BOT), associated with dieback, are among the most important pathogens causing grapevine trunk diseases (GTDs) and therefore severe economic losses in wine-growing regions all over the world. The control and management of these diseases are challenging since the related visible symptoms occur mostly after a long latency period, when the wood is already compromised. The aims of this project were to detect and quantify fungi via their DNA in wood samples before the symptoms appear and to evaluate the efficacy of the BASF wound protectant Tessior® under field conditions. The method was developed using samples collected from grapevine plants showing typical esca foliar symptoms ("tiger-stripes") of different levels. Wood chips were received from the trunks using a 5 mm diameter drill bit. After evaluating various DNA extraction protocols, the most successful one was optimized for grapevine wood tissue. Quantitative Real-Time PCR (qPCR) method to detect and quantify PCH and BOT in wood samples. Compering the severity of symptoms with the results obtained by qPCR, a clear correlation was observable: the more symptomatic the plant appeared, the higher the amount of PCH DNA was determined in the samples. BOT DNA was also found in some samples, the highest amount in the dead plant. Long-term field trials were established between 2014 and 2015 in several countries in Europe, where each year a treatment was performed with Tessior® after pruning. In order to increase the infection pressure, the wounds were artificially inoculated with spores of PCH and BOT in some vineyards. In 2018, samples were collected from the spurs below pruning wounds of the previous year and the DNA amounts of PCH and BOT were determined by qPCR. The above described method has

been proved to be fast and accurate to detect and quantify DNA amounts of PCH and BOT in grapevine wood. Furthermore, the efficacy of Tessior® wound protectant has been also verified.

Minimal vs. intensive pruning: does the training system influence occurrence of Esca (GLSD) foliar symptoms? CHRISTIAN KRAUS[1,2], RALF VOEGELE[2] and MICHAEL FISCHER[1]. *[1]Julius Kühn-Institute, Institute for Plant Protection in Fruit Crops and Viticulture, 76833 Siebeldingen, Germany. [2]University of Hohenheim, Department of Phytopathology, 70599 Hohenheim, Germany. Email: christian.kraus@julius-kuehn.de*

The Esca complex is one of the most destructive grapevine trunk diseases (GTDs) worldwide. Several factors, such as plant age, grapevine cultivar, or pattern of precipitation have been identified as possible driving forces of the disease. In the present study, a four years monitoring of Esca foliar symptoms (namely grapevine leaf stripe disease [GLSD]) in vineyards subdivided into minimally (Semi minimal pruned hedge) and intensively (Vertical shoot positioned) pruned sections was conducted to investigate a possible impact of the training system on the incidence of GLSD. In addition, the occurrence of GLSD over the season and a possible influence of cultivar, plant age and precipitation on symptom development were studied. All investigated parameters, i.e. cultivar, plant age and precipitation, were shown to have at least some influence on symptoms incidence, even though some of the results were inconsistent over the period of monitoring. Concerning the influence of the training system, in 2015 no differences between minimally pruned (1.8%) and intensively pruned (1.9%) vines were found. However, in the following year minimally pruned vines (6.9%) expressed significantly more symptoms than intensively pruned vines (4.9%). In the years 2017 and 2018 the opposite was the case: 2.6% and 2.4%, respectively, of the minimally pruned vines showed GLSD symptoms, while for the intensively pruned vines the mean values were 4.5% and 3.6%, respectively. The seasonal patterns of symptom appearance were identical for the years 2015, 2016 and 2017. In 2018, however, the maximum peak of newly symptomatic vines was reached four weeks earlier, which correlated with the phenological development of the vines. The training system did not affect the seasonal symptom appearance. Our data should help to better understand the relation between incidence of GLSD in the course of the year and possibly influencing parameters.

Natural infections of pruning wounds by fungal trunk pathogens in mature vineyards in Galicia (northwest Spain). MARÍA DEL PILAR MARTÍNEZ-DIZ[1,2], ALES EICHMEIER[3], MARCOS ANDRÉS-SODUPE[4], REBECA BUJANDA[4], ANGELA DÍAZ-FERNÁNDEZ[1], EMILIA DÍAZ-LOSADA[1], MARÍA MERCEDES MALDONADO-GONZÁLEZ[4], SONIA OJEDA[4] and DAVID GRAMAJE[4]. *[1]Estación de Viticultura e Enoloxía de Galicia (AGACAL-EVEGA), Ponte San Clodio s/n 32428-Leiro-Ourense, Spain. [2]Universidade da Coruña, Facultade de Ciencias, Zapateira, 15071 A Coruña, Spain. [3]Mendel University in Brno, Faculty of Horticulture, Mendeleum - Institute of Genetics, Valticka 334, 69144, Lednice, Czech Republic. [4]Instituto de Ciencias de la Vid y del Vino (ICVV), Consejo Superior de Investigaciones Científicas - Universidad de la Rioja - Gobierno de La Rioja, Ctra. LO-20 Salida 13, Finca La Grajera, 26071 Logroño, Spain. E-mail: pilar.martinez.diz@xunta.gal*

Information on the natural infection rates of pruned canes caused by fungal trunk pathogens is scarce. This study aimed to determine the pathogenic fungal microbiome infecting the pruning wounds in six vineyards in Galicia, Spain, belonging to three Denomination of Origin (D.O. Valdeorras, D.O. Ribeiro and D.O. Rías Baixas) in 2017–2018. Vines were pruned in each vineyard in mid-autumn (November) leaving six buds. Fungal microbiome was identified by ITS high-throughput amplicon sequencing (HTAS) from this pruning wood in 150 canes chosen at random in each vineyard. Three months later (late winter, February), two-node canes were cut leaving four buds and fungal microbiome was identified again by HTAS from this pruning wood. Vines were then definitively pruned to two buds in mid-spring (May), and identification of the fungal microbiome by HTAS was repeated from this pruning wood. The main fungal pathogens identified in this study belonged to the genera *Cadophora*, *Cytospora*, *Diaporthe*, *Phaeoacremonium*, *Phaeomoniella* and the families Botryosphaeriaceae and Diatrypaceae. A strong seasonal effect on pathogen infections was observed for most species, with a higher fungal abundance detected after the late pruning (February-May) as compared with that of the early pruning (November-February). Under the environmental conditions and the geographical locations of this study, our results showed that the rate of natural infection of pruning wounds was lower following early pruning (mid-autumn) than late pruning (late winter). Thus, early pruning could be used in combination with other control measures to reduce the infections caused by the grapevine trunk pathogens during the pruning season in Galicia, Spain.

Temporal dispersal pattern of *Phaeomoniella chlamydospora* in Spanish vineyards. ELISA GONZÁLEZ-DOMÍNGUEZ[1], CARMEN BERLANAS[2], DAVID GRAMAJE[2], JOSEP ARMENGOL[3], VITTORIO ROSSI[4] and MÓNICA BERBEGAL[3]. [1]*Horta srl., Via Egidio Gorra 55, 29122 Piacenza, Italy.* [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.* [3]*Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain.* [4]*Department of Sustainable Crop Production - DI.PRO.VE.S., Facoltà di Scienze Agrarie, Alimentari e Ambientali, Università Cattolica del Sacro Cuore, Via Emilia Parmense, 84, 29122 Piacenza, Italy. E-mail: mobermar@etsia.upv.es*

Phaeomoniella chlamydospora is one of the most important pathogens associated with esca disease of grapevine. This fungus reproduces asexually by conidiophores produced in the mycelia or by pycnidia. In this work, the dispersal of aerial inoculum was studied to improve the knowledge about *P. chlamydospora* epidemiology. The main objectives were to monitor the dispersal pattern of this pathogen in two wine producing regions in Spain and to associate this pattern with the environmental conditions. Sampling was performed from October to May in two vineyards affected by grapevine trunk diseases, located in Valencia and Logroño provinces. Five spore traps (microscope slides coated with silicone) were replaced weekly during two seasons in Valencia (2015-2017) and three seasons in Logroño (2015-2018). The DNA concentration of *P. chlamydospora* was determined using a previously validated real time PCR-based protocol. Inoculum of *P. chlamydospora* was detected consistently throughout the investigated months at both locations in all the seasons, except in Logroño 2017/2018. DNA of *P. chlamydospora* was detected in 71 of the 139 sampling periods with rain (96.6% of the total DNA captured) and in 20 of the 31 periods without rain (3.4% of the total DNA). The dynamic of *P. chlamydospora* inoculum release during the season was best described by a Gompertz equation when time was expressed as hydrotermal time (i.e., a combination of accumulated temperature and relative humidity), with R^2>0.7 and CCC=0.87. The information obtained and the equation developed will help to identify the periods with high risk of inoculum dispersal, and to adopt efficient management strategies based on this information.

Spore dispersal patterns of Diatrypaceae and Botryosphaeriaceae species in Australian vineyards. REGINA BILLONES-BAAIJENS[1], SANDRA SAVOCCHIA[1], MEIFANG LIU[1], MATTHEW AYRES[2] and MARK R. SOSNOWSKI[2,3]. [1]*National Wine and Grape Industry Centre, School of Agricultural and Wine Sciences, Charles Sturt University, Locked Bag 588, Wagga Wagga NSW 2678, Australia.* [2]*South Australian Research and Development Institute, GPO Box 397, Adelaide SA 5001,* [3]*School of Agriculture, Food and Wine, The University of Adelaide, Waite Campus, Glen Osmond SA 5064, Australia. E-mail:* rbaaijens@csu.edu.au

Eutypa dieback (ED) and Botryosphaeria dieback (BD) are considered the two most important grapevine trunk diseases in Australia, causing significant yield reduction and threatening the sustainability of Australian vineyards. Spores of the causal pathogens are generally dispersed by rain splash and wind and infect pruning wounds resulting in cankers, dieback and eventually death of vines. Thus, understanding the spore dispersal patterns of these pathogens will help determine the critical times of the year when spores are abundant in Australian vineyards. This will assist growers in making decisions on optimal timing of pruning and wound treatment. This study investigated the spore dispersal patterns of ED (Diatrypaceae) and BD (Botryosphaeriaceae) pathogens in four wine growing regions in Australia. Burkard spore traps were deployed in South Australia (Barossa Valley and Coonawarra) and in New South Wales (Hunter Valley and Griffith) between 2014 and 2016. Spore trap tapes were collected and replaced monthly at each site and analysed for Diatrypaceae and Botryosphaeriaceae spores using qPCR assays. The 3-year study showed spore dispersal of ED and BD pathogens was sporadic and varied between regions, season and year. In South Australia, ED and BD spores were primarily detected in late winter and early spring while in New South Wales, a high number of spores were trapped over summer. Spores were generally recorded during or immediately after rain but not all rain events resulted in spore detection. The spore numbers and frequency of detection varied between years with the highest number of spores being recorded in 2016, particularly for Diatrypaceae species. Additional spore traps were deployed in 2017 across Australian wine regions and will be monitored for a further 3 years. Computer modelling of data will also investigate the role of other weather factors (temperature, relative humidity, dew point, wind direction) on the detection of these spores.

Development and implementation of a droplet digital™ PCR assay for epidemiological studies of Botryosphaeria dieback of grapevines in British Columbia. DANIEL T. O'GORMAN, MELANIE WALKER, JANE THEILMANN, JULIE BOULÉ and JOSÉ RAMÓN ÚRBEZ-TORRES. *Summerland Research and Development Centre, Agriculture and Agri-Food Canada, 4200 Highway 97, Box 5000. Summerland, BC V0H 1Z0, Canada. E-mail: daniel.ogorman@canada.ca*

Botryosphaeria dieback (BD) is one of the most predominant grapevine trunk diseases in British Columbia (BC). The fungal species associated with BD infect grapevines primarily through pruning wounds. Pycnidia, found on previously infected vines, produce spores which are released under favourable environmental conditions. Identifying the conditions that favour spore release and the corresponding infection periods is key to developing control measures. The two main objectives in this project were: firstly, to adapt a qPCR assay originally developed to detect BD species, for use with droplet digital™ PCR (ddPCR™, Bio-Rad Laboratories, Inc.); and secondly, to quantify BD spores in commercial vineyards using the newly adapted ddPCR method. Burkard spore traps (Burkard Manufacturing Co. Ltd.) were placed in five different vineyards in the Okanagan Valley of BC and were set to collect either daily (2 sites) or weekly (3 sites). Total genomic DNA was extracted using a modified protocol of the DNeasy PowerSoil Kit (MO BIO Laboratories). BD spore counts were quantified using ddPCR with either *Botryosphaeria*-specific primers used alone or, *Botryosphaeria*-specific primers and probe. Data generated using the assay with *Botryosphaeria*-specific primers only were compared with data using *Botryosphaeria*-specific primers and probe. Although trends in the resulting data were similar, the ddPCR assay using primers and probe was shown to be more specific and had a higher level of sensitivity. Resulting trends in both datasets showed BD spore release events occurring from late March to October and were absent or greatly reduced through the winter. Generally, the first spore release was detected at the end of winter when average daily temperatures climbed above zero to around 5°C or higher and often correlated, though not always, with rainfall. These results will assist future research projects aimed to develop BD control strategies including timing for both cultural practices (i.e. pruning) and biological and chemical product applications.

Host-Pathogen Interactions

Metabolic markers of grape infection with Esca disease. DIANA PIMENTEL[1], ALEXANDER ERBAN[2], PEDRO REIS[3], FLÁVIO SOARES[1], CECÍLIA REGO[3], JOACHIM KOPKA[2], and ANA MARGARIDA FORTES[1]. *[1]University of Lisbon, Faculty of Sciences, BIOISI, 1749-016 Lisboa, Portugal. [2]Max-Planck-Institut für Molekulare Pflanzenphysiologie, 14476 Potsdam-Golm, Germany. [3]LEAF - Linking Landscape, Environment, Agriculture and Food, School of Agriculture, University of Lisbon, Lisboa, Portugal. E-mail: amfortes@fc.ul.pt*

Grapevine trunk diseases are amongst the major challenges for viticulture. Among these, Botryosphaeria dieback, Esca complex, Eutypa dieback and Phomopsis dieback are the most common. The main causal agents of the Esca Proper are *Phaeoacremonium* spp., *Phaeomoniella chlamydospora* and *Fomitiporia* spp. Because the pathogens cannot be detected in leaves and berries of infected plants, the discovery of molecular and metabolic markers of the disease may lead to early detection of infected plants and elucidate on the factors responsible for the progression of the disease. Berries and leaves from control and symptomatic grapevines were collected in 2016 at harvest stage (five biological replicates) from a 17 years old vineyard of Portuguese cultivar Aragonez (= Tempranillo) that had been monitored for three years. The samples were used for metabolomics and targeted qPCR analysis of genes involved in hormonal and phenylpropanoids metabolism and biotic stress response. Principal component analysis of GC-MS data revealed that infected samples were clearly discriminated from controls. However, metabolic reprogramming due to infection was more evident in berries than in leaves. Several volatiles, fatty acids, triterpenoids and phenylpropanoids, putatively involved in defense, were present in higher amounts in infected berries. On the other hand, undergoing qPCR analysis suggested the involvement of salicylic acid signaling in the stress response. Molecular data is being integrated with metabolomics data in order to provide insights on the mechanisms involved in Esca disease affecting grapevines worldwide. Additionally, these results will contribute to predict the impact of infection on wine quality.

A comparison between constitutive and inducible chemical defenses against the esca disease in grapevine. PIEBIEP GOUFO and ISABEL CORTEZ. *Centre for the Research and Technology of Agro-Environment and Biological Sciences, Departamento de Agronomia,*

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Plants are able to counteract microorganisms by establishing physical and chemical barriers, which can be preformed/constitutive or induced. Phenolic compounds are among the most recognized chemical defenses in plants. This study was undertaken to test the hypothesis that grapevine responds to wood infection by esca fungi by systemically inducing the biosynthesis of phenolics in asymptomatic leaves. In a naturally infected vineyard, the occurrence of esca syndromes was annually evaluated on *Vitis vinifera* L. cv. Malvasia, and brown streak, petri disease, leaf stripe, apoplexy and black measles vines identified. Plants reacted to esca through the production of phenolics in a diversified way, depending on the presence or absence of foliar symptoms. In asymptomatic leaves of brown streak vines, total phenolics decreased relative to healthy vines, independent of the class assayed i.e., phenolic acids, anthocyanins, flavonoids, and proanthocyanidins. These decreases were concomitant with those of several phenolics identified by liquid chromatography e.g., caftaric acid, kaempferol-3-*O*-glucoside, *trans*-resveratrol. A declining trend was also observed in asymptomatic leaves of leaf stripe and black measles vines, which did not support the hypothesis of a systemic induction of phenolic defenses. Plants responded to foliar symptom development by increasing levels of phenolics and inducing compounds undetected in healthy leaves, and these changes correlated with the extent of lesions on the leaf surface. For example, an increase of 75% between healthy and apoplexy leaves was observed for kaempferol-3-*O*-glucuronide. It appeared that the sequence of events in the defense response of grapevine include first the recruitment of preformed phenolics before visible browning of leaves, and second the synthesis of specific phenolics with the onset of symptoms. Decreased phenolic levels in asymptomatic leaves suggest that high amounts of constitutive compounds might be associated with the long latency time and lack of symptoms between continuous years, which are characteristic traits of esca disease.

Metabolomic studies of two *V. vinifera* subspecies during infection by *Neofusicoccum parvum*, a Botryosphaeria dieback pathogen – on the road to identify infection and resistance biomarkers. CLEMENT LABOIS[1,2], MARY-LORENE GODDARD[1,2], HELENE LALOUE[1], PETER NICK[3], CELINE TARNUS[1], CHRISTOPHE BERTSCH[1] and JULIE CHONG[1]. [1]*Laboratoire Vigne, Biotechnologie et Environnement EA3991, Université de Haute-Alsace, 33 rue de Herrlisheim, 68008 Colmar, France.* [2]*Laboratoire d'Innovation Moléculaire & Applications UMR CNRS 7042-LIMA, Université de Haute-Alsace/Université de Strasbourg, 3bis rue Alfred Werner, 68093 Mulhouse, France.* [3]*Botanical Institute, Molecular Cell Biology, Karlsruhe Institute of Technology, Karlsruhe, Germany. E-mail: Julie.chong@uha.fr*

In the last decades, vineyards worldwide have been facing the resurgence of wood diseases with dramatic impact on viticulture sustainability and wine production. Botryosphaeria dieback is one of the main grapevine trunk diseases. Unfortunately, to date, no efficient treatment is convenient to prevent, protect or limit the progression of these diseases. We have previously shown that some wild grape *Vitis vinifera* subsp. *sylvestris* accession were less susceptible to the infection of Botryosphaeriaceae. This result is of great interest to study Botryosphaeria dieback resistances. In this context, grape canes from different accessions of *Vitis vinifera* subsp. *sylvestris* and different cv. of *Vitis vinifera* subsp. *vinifera* were infected with *Neofusicoccum parvum*. Necrosis area were measured seven days after infection. Targeted metabolomic studies were further performed to quantify the primary metabolites by GC-MS (sugars, organic acids and amino acids) at different times after infection. In the same way, untargeted metabolomics studies of secondary metabolites were achieved by LC-MS/MS. This approach identified biomarkers of the fungal infection. In addition, several secondary metabolites showed a specific accumulation pattern between the two-grapevine subspecies (*vinifera* and *sylvestris*). Specific accumulation pattern of secondary metabolites could be linked to their differences in trunk disease pathogen resistance.

Clone-dependent expression of grapevine esca disease. FLORIAN MORET[1], CHRISTELLE LEMAÎTRE-GUILLIER[1], CLAIRE GROSJEAN[2], GILLES CLEMENT[3], CHRISTIAN COELHO[4], LUCILE JACQUENS[1], JONHATAN NEGREL[1], REGIS GOUGEON[4], GUILLAUME MORVAN[5], GREGORY MOUILLE[3], SOPHIE TROUVELOT[1], FLORENCE FONTAINE[6] and MARIELLE ADRIAN[1]. [1]*Agroécologie, AgroSup Dijon, CNRS, INRA, Univ. Bourgogne, Univ. Bourgogne Franche-Comté, F-21000 Dijon, France.* [2]*Chambre Régionale d'Agriculture de Bourgogne Franche-Comté, 21110 Bretenière, France.* [3]*Institut Jean-Pierre Bourgin, INRA, AgroParisTech, CNRS, Université Paris-Saclay, 78000 Versailles, France.* [4]*UMR PAM Université de Bour-*

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The occurrence of grapevine trunk diseases (GTDs) increases gradually within vineyards, leading to important economic losses. Several factors could affect grapevine susceptibility to GTDs, especially climate, vine age, soil fertilization, and also rootstock, cultivar and clone. For a cultivar, the level of disease expression can vary with region and from year to year. In this context, our objective was to assess if the expression of esca disease, one of the most widespread GTDs, was also clone-dependent. Two clones (76 and 95) of the Chardonnay cultivar, grown in the same plot, were compared according to their developmental and physiological traits, metabolome, and esca foliar symptom expression. Agronomical data and symptom expression were recorded during summer 2015, and metabolome analyses were performed in leaf samples collected from visually healthy vines as control (C), and from both symptomatic (D+) and asymptomatic (D-) shoots of esca-affected vines. The bud burst percentage and the fertility ratio were significantly lower for clone 76 than for clone 95, whereas the plant vigor was similar. The percentage of vines expressing the apoplectic and chronic forms of esca disease was low but slightly higher for clone 95. Global GC-MS analysis highlighted a clone-dependent metabolic fingerprint of disease expression. Additional targeted HPLC analyses showed opposite variations in the accumulation of *trans*-caffeoyltartaric acid, quercetin-O-glucoside, quercetin-O-galactoside, and kaempferol-O-glucoside (higher levels in control leaves of clone 76 than in diseased ones, and the opposite for clone 95). An original approach, 3D fluorescent analysis, was also used to compare samples and also pointed out significant differences in disease expression between clones. Altogether, this study highlights a clone-dependent metabolic response related to esca-disease expression, and the potential of the 3D fluorescence analysis as a new method to detect it. It would be interesting to extend the analysis to other clones and varieties.

Two infections, one grapevine: How infection by one pathogen may impact that of a second pathogen via induced changes in host physiology. CHRISTOPHER M. WALLIS[1], KENDRA BAUMGARTNER[2] and ERIN GALARNEAU[3]. *[1]U.S. Department of Agriculture, Agricultural Research Service, San Joaquin Valley Agricultural Sciences Center, Crop Diseases, Pests and Genetics Research Unit, Parlier, CA 93648. [2]U.S. Department of Agriculture, Agricultural Research Service, Davis, CA 95616. [3]Department of Plant Pathology, University of California, Davis, CA 95616. E-mail: christopher.wallis@usda.gov*

Grapevine trunk diseases are often characterized by mixed infections resulting from colonization of different pruning wounds by spores of a variety of trunk pathogens. Despite being located on different branches, a first infection may induce systemic changes in host physiology that could alter progress of an independent infection elsewhere on the vine. However, studies examining multiple fungal infections on the same grapevine host are lacking. To investigate the interactions of two separate infections in a single vine, a trunk pathogen was inoculated two months after initially infecting the vine with a trunk pathogen on a different branch. The lesion growth of the second infection was assessed and compared with the growth of infections on plants not previously infected. Furthermore, we analyzed amino acid, sugar, phenolic compound, and terpenoid levels in grapevine stem tissues just prior to the second infection to observe if the initial infections shifted host physiology to disfavor pathogen establishment and growth. Initial inoculation with Botryosphaeria-dieback pathogen *Diplodia seriata* was associated with reduced lesion lengths of subsequent inoculation with *D. seriata,* Botryosphaeria-dieback pathogen *Neofusiccocum parvum* and Esca pathogen *Phaeomoniella chlamydospora*. Preliminary results suggest these reductions were associated with changes in host physiology including shifts in amino acid, sugar, phenolic compound, and terpenoid levels. Follow-up studies are underway to confirm this phenomenon and examine other pathogen combinations. Results should provide information on how grapevine physiology could be manipulated to better increase disease resistance and allow fine-tuning of integrated pest management programs based how presence of one pathogen may affect another.

Genetic basis of xylem morphology in grapevine: impact on hydraulic conductivity and resistance to *P. chlamydospora*. JEROME POUZOULET[1], SABINE GUILLAUMIE[1], REMI CHARBOIS[1], LAURENT LAMARQUE[3], GREGORY A. GAMBETTA[1], SYLVAIN DELZON[3], CHLOE E. L. DELMAS[2] and NATHALIE OLLAT[1]. *[1]UMR 1287 EGFV, ISVV, INRA, 33882 Villenave d'Ornon, France. [2]UMR 1065, SAVE, INRA, 33140*

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Vascular diseases of cultivated grapevine, *Vitis vinifera* L. *sativa*, are factors that can considerably limit vineyards productivity and longevity. Among these diseases, Esca disease is one of the most prevalent and destructive worldwide. While complete resistance toward Esca causal agents does not exist within the *Vitis* genus, differences in the ability of cultivars to limit the movement of pathogens have been reported. Previous studies provided clues about the role of xylem vessel diameter in the ability of some commercial cultivars to respond to the infection of an Esca causal agent, *Phaeomoniella chlamydospora*. Here, we tested this concept within a grapevine rootstock experimental progeny (F2, *V. riparia* cv. Gloire de Montpellier x *V. vinifera* cv. Cabernet Sauvignon). Two hundred and sixty-one genotypes from the progeny were characterized for various xylem morphological traits and a QTL analysis was performed over 2 years of observation. Our results showed that strong and stable QTLs associated with various xylem morphological traits can be found in this progeny, including for the diameter of vessels. Based on this analysis, subsets of genotypes were further characterized for functional traits (hydraulic conductivity, resistance to *P. chlamydospora*). A QTL found for vessel diameter was seen to impact xylem hydraulic conductivity significantly. In addition, our results confirmed that within this progeny as well, the density of vessels of wide diameter was positively correlated with the level of susceptibility to the pathogens. This study provides useful insights about genetic basis responsible for xylem morphological traits in perennial plants, and their potential impact on vascular diseases resistance.

Correlating xylem vessel size of grapevine cultivars and esca disease incidence in the field. ENZO FOGLIA, LUCIA LANDI and GIANFRANCO ROMANAZZI. *Department of Agricultural, Food and Environmental Sciences, Marche Polytechnic University, Via Brecce Bianche, I-60131 Ancona, Italy. E-mail: g.romanazzi@univpm.it*

Esca is one of the most important grapevine trunk diseases. This disease seriously affects vineyard productivity and longevity, being the main cause of the death of vines in most viticultural areas. Previous studies suggest that wide xylem vessel diameter favor the development of *Phaeomoniella chlamydospora*, one of the fungi involved in esca. The aim of this work was to determine the anatomical measurements (diameter and frequency) of xylem vessels of grapevine cultivars with different susceptibilities to esca. In this study 27 white-berried and 24 red-berried grapevine cultivars, grown in the same experimental vineyard were analyzed and the vessel dimension characteristics were correlated with esca incidence previously detected. The cultivars showed significant differences in vessel parameters. However, no linear relationship was detected among the vessel size and esca incidence in the field. Overall, white-berried cultivars showed wider vessel diameter than red-berried cultivars. From our investigations there is no clear relationship between vessel size and esca incidence. Vessel anatomy profiles can be useful for further investigations on sensititvity of grapevine genotypes to grapevine trunk diseases.

Exploring the hydraulic failure hypothesis on esca leaf symptom formation. GIOVANNI BORTOLAMI[1], GREGORY A. GAMBETTA[2], SYLVAIN DELZON[3], LAURENT J. LAMARQUE[3], PASCAL LECOMTE[1], JEROME POUZOULET[2] and CHLOE E. L. DELMAS[1]. *[1]Santé et Agroécologie du Vignoble, INRA, 33140 Villenave d'Ornon, France. [2]Bordeaux Science Agro, Institut des Sciences de la Vigne et du Vin, Ecophysiologie et Génomique Fonctionelle de la Vigne, INRA, 33140 Villenave d'Ornon, France. [3]BIOGECO, INRA, Univ. Bordeaux, 33615 Pessac, France. E-mail: chloe.delmas@inra.fr*

Plant vascular disorders are sometimes identified by conspicuous leaf scorch symptoms but the exact mechanisms driving leaf symptoms remains unknown. Two of the main hypotheses, presence of air embolism or tyloses/gels, rely on the disruption of xylem integrity but they still need to be investigated. In this study we explore the xylem integrity in esca symptomatic leaves of naturally field-infected grapevines (*V. vinifera* cv. Sauvignon) using traditional light microscopy and non-invasive, *in vivo* imaging via x-ray microtomography (microCT). This method allows for the visualization and quantification of embolism and vessel functionality in esca symptomatic leaf petioles and midribs. We survey annual stems and leaves using qPCR to determine if two of the main pathogen species associated with esca, *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum*, were present in these annual organs. Our results demonstrated that symptomatic leaves are not associated with air embolized xylem conduits. In symptomatic leaves, high percentages of xylem vessels were not functional due to nongaseous embolisms formed by gels and/or tyloses. However, the severity of esca leaf symptoms was not correlated to the proportion of non-functional ves-

sels. *P. chlamydospora* and *P. minimum* were undetected in the vine's distal organs confirming that the symptoms and vascular occlusions likely occur at a distance from the trunk where fungal infections occur. Studying xylem water transport and vessel integrity during esca pathogenesis is fundamental and critically important if we are to understand esca etiology. Our observations inform new perspectives on esca symptom expression where two of the underlying hypotheses (elicitor/toxin and hydraulic failure) are not necessarily mutually exclusive

Effect of water and heat stresses on the physiology of Ugni blanc infected by *Neofusicoccum parvum*. AURÉLIE SONGY[1], PHILIPPE LARIGNON[2], CHRISTELLE LEMAÎTRE-GUILLER[3], JEAN-FRANÇOIS GUISE[1], OLIVIER YOBREGAT[4], CÁTIA PINTO[1], VINCENZO MONDELLO[1], CINDY COPPIN[1], GUILLAUME ROBERT-SIEGWALD[5], MARC-HENRI LEBRUN[5], PHILIPPE SCHMITT-KOPPLIN[6,7], CHRISTOPHE CLÉMENT[1], MARIELLE ADRIAN[3] and FLORENCE FONTAINE[1]. *[1]Université de Reims Champagne-Ardenne, Unité Résistance Induite et Bioprotection des Plantes EA 4707, Bât.18, BP 1039, 51687 Reims cedex 2, France. [2]Institut Français de la Vigne et du Vin, Pôle Rhône-Méditerranée, 7 avenue Cazeaux, 30230 Rodilhan, France. [3]Agroécologie, AgroSup Dijon, CNRS, INRA, Univ. Bourgogne, Univ. Bourgogne Franche-Comté, F-21000 Dijon, France. [4]Institut Français de la Vigne et du Vin, Pôle Sud Ouest, Vinnopôle – Brames Aigues, BP 22, 81310 Lisle sur Tarn, France. [5]UMR Bioger, INRA AgroParisTech, Avenue Lucien Brétinières, 78850 Thiverval-Grignon, France. [6]Helmholtz Zentrum München, Analytical Biogeochemistry, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany. [7]TU München, Lehrstuhl für Analytische Lebensmittelchemie, Wissenschaftszentrum Weihenstephan, Alte Akademie 10, 85354 Freising, Germany. E-mail: aurelie.songy@univ-reims.fr*

Botryosphaeria dieback is one of the most widespread and prevalent grapevine trunk diseases (GTDs) in Europe. Symptoms include necrosis of perennial organs of grapevine while external symptoms include bud necrosis, leaf discoloration and dead arms although incidence and severity vary from year to year. The causal agents of this disease are Botryosphaeriaceae species with *Neofusicoccum parvum* being one of the most prevalent. Their aggressiveness were reported to be influenced by environmental factors particularly temperature increasing disease severity. Thus, our study investigated the influence of heat and water stress on the grapevine / *N. parvum* interaction. Cuttings of Ugni Blanc, a cultivar known to be susceptible to GTDs, were artificially infected by *N. parvum* and subjected to two soil water status and two heat conditions (three days at 35°C or no heat stress). Plant growth, photosynthetic activity and the necrosis induced by *N. parvum* were monitored. The whole plant responses against these biotic and abiotic stresses, alone or in combination were investigated using transcriptomic and metabolomics analyses. Our results showed the two abiotic stresses altered the photosynthetic activity of the vines. The response to infection differed depending on the abiotic stresses applied as revealed by specific transcriptomic and metabolomic profiles. The impact of heat and water stresses seems greater when they occurred simultaneously due to reduced plant growth and increased aggressiveness of *N. parvum*. The impact of infection on the metabolism was greater after a heat stress, compared to water stress, but highest severity was observed r when these two stresses were present. The lipids and secondary metabolites were the two plant compounds greatly impacted by the infection. Globally, these results may partly explain the seasonal variability of GTD symptom expression in the vineyards that may be influenced by abiotic stresses that can occur separately or simultaneously.

Investigating the production and translocation of phytotoxic metabolites by Australian Botryosphaeriaceae spp. in artificially-inoculated and naturally-infected vines. PIERLUIGI REVEGLIA[1], REGINA BILLONES-BAAIJENS[1], ALESSIO CIMMINO[2], ANTONIO EVIDENTE[2] and SANDRA SAVOCCHIA[1]. *[1]National Wine and Grape Industry Centre, School of Agricultural and Wine Sciences, Charles Sturt University, Locked Bag 588, Wagga Wagga NSW 2678, Australia. [2]University of Naples Federico II, Department of Chemical Sciences Complesso Universitario Montesant'Angelo, Via Cintia 4, 80126, Naples, Italy. E-mail: preveglia@csu.edu.au*

Grapevine trunk diseases (GTDs) caused by pathogenic fungi are considered a serious problem to worldwide. Some GTD pathogens produce phytotoxic metabolites (PMs) that play an important role in their virulence. It is hypothesized that these PMs are translocated into the leaves causing foliar symptoms. However, foliar symptoms in vines affected with Botryosphaeria dieback have never been reported in Australian vineyards. A previous investigation demonstrated that different PMs, including R-(-)-mellein, were produced *in vitro* by Australian Botryosphaeriaceae isolates. To gain insight into the role of PMs in the virulence and symptomatology of the pathogens, production of PMs *in planta* were investigated by

molecular and analytical chemistry techniques. Wood samples from vines naturally-infected with Botryosphaeria dieback were collected from three vineyards in New South Wales, Australia. For artificially-inoculated vines, one-year-old rootlings (cv. Chardonnay and Cabernet Sauvignon) were inoculated with *Diplodia seriata*, *Spencermartinsia viticola* and *Dothiorella vidmadera*. All symptomatic and asymptomatic wood samples were analysed by cultural isolations and qPCR to detect and quantify the pathogens. An LC-MS/MS protocol was optimized for the detection of spencertoxin, protocatechuic alcohol and *R*-(-)-mellein from infected wood. *R*-(-)-mellein was detected by LC-MS/MS but was only present in symptomatic wood and its amount correlated with the amount of pathogen DNA detected by qPCR. These results suggest that this PM was not translocated as previously hypothesized. The inability to detect the other two PMs could be explained by the following: they could be under the detection limit, they could be produced during the later stages of infection, they could be broken down into other compounds or detoxified by the plant. Our findings suggest that *R*-(-)-mellein may not be directly involved in the development of foliar symptoms and these symptoms may be caused by a combination of diverse factors which require more in-depth studies.

Mechanistic overview of the chelator-mediated Fenton (CMF) system in brown rot fungi and its potential role in fungal grapevine trunk diseases. BARRY GOODELL[1], JODY JELLISON[2] and E. PETIT[1]. *[1]Department of Microbiology, 102 Morrill Science Center IV-N, 639 N. Pleasant St., University of Massachusetts, Amherst, MA 01003 USA, [2]Center for Agriculture, Food and the Environment, 319 Stockbridge Hall, University of Massachusetts, Amherst, MA 01003 USA. E-mail: bgoodell@umass.edu*

Brown rot fungi comprise only 6% of all known Basidiomycota species, yet they degrade approximately 80% of wood, by mass, in terrestrial environments. Unlike white rot fungi, brown rots do not possess lignin degrading enzymes and have abandoned both peroxidases and many carbohydrate-active enzymes (CAZymes) as they evolved from white rot progenitors. Concurrently, many brown rot fungi evolved a unique low molecular weight mechanism for initiating decay in wood known as the chelator-mediated Fenton (CMF) system to depolymerize both cellulose and lignin components of wood. In this system, low molecular weight iron-binding fungal metabolites are produced, and at the correct pH these metabolites also redox-cycle to generate a stream of hydroxyl radicals which have been demonstrated to be responsible for much of the depolymerization of the plant/wood cell wall components in brown rot. These metabolites can diffuse through cell walls rapidly to depolymerize both lignin and holocellulose components. It is this depolymerization, particularly of the cellulose backbone of the elementary wood fibrils, that dramatically reduces the mechanical properties of wood undergoing brown rot attack. It is unknown if the stem necrosis observed in some grapevine trunk diseases (GTD) is associated with low molecular weight fungal metabolites or similar types of CMF reactions. However prior research has demonstrated that *Phaeomoniella* and *Phaeoacremonium* genera produce iron-binding redox-cycling compounds that are capable of generating hydroxyl radicals associated with cellulose degradation. Further, *Fomitiporia mediterranea* is an intermediate species between white rots and brown rots and may possess CMF capability. We propose, with French and Italian collaborators, that examination of the role of low molecular weight iron-binding fungal metabolites may permit a better understanding of the mechanisms behind some types of fungal GTDs, and that this understanding may lead to new targets for therapeutic treatments and IPM to combat GTDs.

Non-Enzymatic *in lignum* degradation mechanism: a way to control Grapevine Trunk Disease? SAMUELE MORETTI[2], ROMAIN PIERRON[1], ANDREA PACETTI[2], MELANIE GELLON[1], CELINE TARNUS[1], STEFANO DI MARCO[3], LAURA MUGNAI[2], BARRY GOODELL[4], ERIC GELHAYE[5], CHRISTOPHE BERTSCH[1] and SIBYLLE FARINE[1]. *[1]Laboratoire Vigne Biotechnologies et Environnement UR-3991, Université de Haute Alsace, 33 rue de Herrlisheim, 68000 Colmar, France. [2]Department of Agricultural, Food, Environmental and Forestry Science and Technology (DAGRI), Plant pathology and Entomology section, University of Florence, P.le delle Cascine, 28, 50144 Firenze, ITALY. [3]CNR IBIMET, Via Gobetti 101, 40129 Bologna, Italy. [4]Microbiology Department, University of Massachusetts. 102 Morrill, Science Center IV-N, 639 North Pleasant St. Amherst, Massachusetts 01003, USA. [5]Unité Mixte de Recherches 1136 Université de Lorraine/INRA, Interactions Arbres / Micro-organismes, Faculté des sciences, Bd des aiguillettes, 54500 VANDOEUVRE-Lès-NANCY France. E-mail: sibylle.farine@uha.fr*

For many years wood decay by fungi was assumed to be caused exclusively by extracellular cellulases and lignin-degrading peroxidase enzymes produced by certain

Ascomycota and Basidiomycota species. It is now recognized that enzymatic action alone does not explain how "brown rot" wood decay occurs. A non-enzymatic pathway generating hydroxyl radicals deep within wood cell walls has now been demonstrated to be responsible for the depolymerization of both cellulose and lignin during brown rot wood decay, and extracellular cellulases are then produced secondarily to permit further deconstruction of the plant cell walls. Because iron-binding compounds mediate this degradation of wood, the mechanism observed has been termed the "chelator-mediated Fenton" (CMF) reaction. Decay species such as *Fomitiporia mediterranea* (*Fmed*) are intermediate between brown and white rot fungi and this species is known to be involved in Esca disease in *Vitis vinifera*. However, it is unknown whether *Fmed* or other grapevine trunk disease fungal species produce low molecular weight (LMW) iron-binding compounds to promote CMF reactions and wood decay. The aim of our work is to better understand the role of CMF reactions *in lignum* during Esca infection in order to develop control strategies. Indeed, assuming that the iron-binding and the redox capacity of the LMW metabolites from the pathogen may be important in disease development, any Biological Control Agent (BCA) that could play a role in interfering with the fungal CMF mechanism may help to reduce the pathogenic effects and spread of the causal fungi. For this purpose, we are investigating the ability of the fungi associated with Esca to produce iron-binding metabolites and oxalic acid; both playing an essential role in the CMF reaction. We are also considering the degradation capacity of oxalic acid by different *Trichoderma* species to explore if oxalate oxidase increases the activity of these biocontrol agents.

Ecological role of phytopathogens in vineyard microbial ecosystem: pathogenic synergies and antagonisms from global mycobiome studies. RÜDIGER ORTIZ-ÁLVAREZ[1], CÁTIA PINTO[1], CRISTINA PEÑAS[1], AVA MEHRPOUR[1], LUIS LOPEZ[1], CHARLES RAVARANI[1], IGNACIO BELDA[1,2] and ALBERTO ACEDO[1]. *[1]Biome Makers, 890 Embarcadero Drive West Sacramento, CA 95605, USA. [2]Unit of Biodiversity and Conservation, Rey Juan Carlos University, 28933 Móstoles, Spain. E-mail: catiapinto@biomemakers.com*

Soil and vine are naturally colonized by a complex microbial ecosystem, and its balance is critical for plant health and productivity. However, understanding how such microbial networks interact with the plant is a primary challenge not only to develop a model to predict plant disease scenarios but also to identify microorganisms with beneficial potential. In this context, the aim of this study was to explore the fungal communities from 404 vineyard soil samples by using ITS metabarcoding DNA sequencing. For this, samples from vineyards under different management regimes (conventional, organic, and biodynamic) and from different geographic and climate regions of Spain and USA were compared, and a deep analysis of the fungal pathogen species related with grapevine trunk diseases (GTDs) was performed. Results from the probabilistic method to infer co-occurrences and co-exclusions in two soil metacommunities (Spain and USA) showed both positive and negative associations between microorganisms and grapevine. Thus, the list of all the significant pairs included potential positive associations between groups of pathogens, acting as potential co-infection complexes (such as *Ilyonectria liriodendri* and *Ilyonectria robusta*); and, also negative associations of multiple pathogens and other microorganisms present in the soil, which may act as broad biocontrol agents (i.e., 21 species linked to both *Cadophora luteo-olivacea* and *Campylocarpon fasciculare*). Quantifying per sample the number of species negatively associated to plant pathogens, may help to assess: (1) the risk of a plant to harbor a disease, (2) the biocontrol potential of the soil, and (3) if there are links between the soil community structure and the presence of plant pathogens. Altogether, this model contributes to the understanding of the role of the microbial structure and functioning in the vulnerability of a vineyard soil ecosystem. We are convinced that by understanding these ecological roles this would foster the innovation on vine sector.

Synergistic interactions between *Fomitiporia mediterranea* and wood-inhabiting bacteria promote grapevine-wood degradations. RANA HAIDAR[1,2,3], AMIRA YACOUB[1,2], ANTOINE PINARD[1,2], JESSICA VALLANCE[2,1], STEPHANE COMPANT[4], ANTOINE LOQUET[5] and PATRICE REY[1,2]. *[1]INRA, ISVV, UMR1065 Santé et Agroécologie du Vignoble (SAVE), F-33140 Villenave d'Ornon, France. [2]Université de Bordeaux, ISVV, UMR1065 SAVE, Bordeaux Sciences Agro, F-33140 Villenave d'Ornon, France. [3]Tichreen University, Faculty of Science, Biology Department, PO Box 2231, Latakia, Syrian Arab Republic. [4]AIT Austrian Institute of Technology GmbH,Bioresources Unit, Konrad-Lorenz-Straße 24, Tulln 3430, Austria. [5]Institut de Chimie et Biologie des Membranes et des Nanoobjets, CNRS, Université de Bordeaux, 33077 Bordeaux, France. E-mail: amira.yacoub@inra.fr*

Nowadays, viticulture worldwide is confronted with heavy economic losses caused by Grapevine Trunk Diseases (GTDs). Fungi have been described as the main cause of grapevine trunk diseases. Among these fungi, *Fomitiporia mediterranea,* is considered as the main white wood rotting Basidiomycota associated with GTDs. Bacteria diversity is very high in the wood of grapevine but little information is available about their functions and their interactions with fungi inhabiting the wood. We therefore isolated bacterial strains from different anatomical parts (i.e. trunk and cordon) and different type of tissues (i.e. necrotic or not) of GTD-symptomatic grapevines. Two hundred thirty seven bacterial strains were isolated from grapevine-wood samples and, based on their 16S rRNA genes, assigned to bacterial species, OTUs belonging to *Xanthomonadaceae* were the dominant taxonomic groups, at the family level. Fifty nine bacterial strains representing the various OTUs were screened for their ability to degrade the three main components of wood, i.e. cellulose, hemicellulose and lignin, by using different selective media. Their ability to inhibit *F. mediterranea* was also studied. Based on a microcosm experiment, the hypothesis that some bacterial strains inhabiting wood interact with *F. mediterranea* to promote grapevine wood degradation was tested. *Results demonstrated,* for the first time, a synergetic interaction between *F. mediterranea* and the two bacterial strains: *Chryseobacterium* sp. and *Paenibacillus* sp., to degrade the grapevine-wood structures. The whole-genomes of the 2 bacterial strains were sequenced because of their interest in wood degradation. *After wood samples inoculations with F. mediterranea* alone, the bacteria alone, and *F. mediterranea* plus *Paenibacillus* sp., analysis of the wood-degradations that occurred in the cellulose, hemicelluloses, and lignin, were investigated by Solid-State Nuclear magnetic resonance (NMR) method. The contribution *of* bacteria *an*d fungi alone, or in synergy, in wood degradations will be discussed with regards to GTDs.

The control and control basis of grapevine trunk diseases in China. JIYE YAN, WEI ZHANG, QIKAI XING, JUNBO PENG, MEI LIU, YING ZHOU and XINGHONG LI. *Beijing Key Laboratory for Environmental Friendly Management on Pests of North China Fruits, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China. E-mail: jiyeyan@vip.163.com*

Grapevine trunk diseases (GTDs) are widely spread in almost all grapevine cultivating countries, causing serious economic losses. At present, six species of *Botryosphaeriaceae*, including *Botryosphaeria dothidea*, *Lasiodiplodia theobromae*, *Neofusicoccum parvum*, *Diplodia seriata*, *L. pseudotheobromae* and *N. mangiferae*, were proved to be associated with GTDs in China. Recent studies have established a correlation between environmental factors and GTDs occurrence; however, less is known about the factors that can trigger these diseases. Therefore, de novo sequencing of *L. theobromae*, one of the most prevalent species in China, was conducted and resequencing was done for *B. dothidea* and *N. parvum* in this study. Our data showed that gene families associated with cell wall degradation, nutrient uptake, secondary metabolism and membrane transport, which contribute to adaptations for wood degradation were expanded in botryosphaeriaceous genomes. Further, transcriptome analysis was performed and the results revealed that genes involved in carbohydrate catabolism, pectin, starch and sucrose metabolism, and pentose and glucuronate inter-conversion were induced during the infection process of *L. theobromae*. Furthermore, genes involved in carbohydrate-binding modules and the lysine motif domain and glycoxyl hydrolase gene families were found that can be induced by high temperature. Among these genes, over-expression of two selected putative lignocellulase genes led to increased virulence in the transformants. These results demonstrate the importance of high temperatures in the opportunistic infections of *Botryosphaeriaceae* species. The current study also presents a set of *Botryosphaeriaceae* specific effectors related to pathogenicity. In conclusion, these findings significantly improve our understanding of the determinants of pathogenicity or virulence in *Botryosphaeriaceae* species and provide new insights for developing new strategies to control them.

***Lasiodiplodia gilanensis* used a model for understanding the pathogenicity of Botryosphaeriaceae.** EDELWEISS AIRAM RANGEL-MONTOYA[1], MARCOS PAOLINELLI[2,3] and RUFINA HERNANDEZ-MARTINEZ [1]. *[1]Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE), Carretera Ensenada-Tijuana, 3918, Zona Playitas, 22860, Ensenada, B.C. México. [2]Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agropecuaria de Mendoza, San Martin 3853 (5507), Luján de Cuyo, Mendoza, Argentina. [3]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Godoy Cruz 2290 (C1425FQB) CABA, Buenos Aires, Argentina. E-mail: erangel@cicese.edu.mx*

Members of the Botryosphaeriaceae are one of the primary fungal pathogens causing trunk diseases on grapevines. Among them, *Lasiodiplodia* spp. are reported as

the most aggressive, causing degenerative diseases, dieback, and plant death. To understand the pathogenicity of the Botryosphaeriaceae we are using *Lasiodiplodia gilanensis* as a model. A transcriptional study suggested that this fungus is capable of using the phenylpropanoid precursors and salicylic acid to avoid the host defense response of the plant. Several genes encoding enzymes involved in different melanin synthesis pathways, 3,4-dihydroxyphenylalanine (DOPA)-melanin, 1,8-dihydroxynaphthalene (DHN)-melanin, and pyomelanin were identified, and their production evaluated, concluding that the fungus use different types of melanin to overcome environmental stress. An *in silico* analysis shows the presence of those genes in all the available genomes of Botryosphaeriaceae in GeneBank, evidencing the importance of the melanin in this family. *Lasiodiplodia gilanensis* also produces siderophores of catechol of hydroxamate-type, as well as naturally esterified fatty acids; those compound might have a role in plant growth regulation. A plethora of hydrolytic enzymes is also produced, including xylanases, ligninases, cellulases, pectinases, cutinases, and hemicelluloses. An organic compound also produced is oxalic acid, reported as a pathogenicity factor in other fungi; its role in *L. gilanensis* is under evaluation. Microscopical observations showed that the fungus uses the starch deposited in the ray cells as carbon source, induces the production of suberin and phenolic compounds, and colonizes the vascular cambium, ray parenchyma, and the vascular bundles. In summary, these studies extend our understanding of the pathogenicity of a widely distributed pathogenic fungus.

Screening of biocontrol agents against black-foot and Petri diseases under field conditions. MARÍA DEL PILAR MARTÍNEZ-DIZ[1,2], MARCOS ANDRÉS-SODUPE[3], REBECA BUJANDA[3], EMILIA DÍAZ-LOSADA[1], MARÍA MERCEDES MALDONADO-GONZÁLEZ[3], SONIA OJEDA[3], PATRICE REY[4], AMIRA YACOUB[4] and DAVID GRAMAJE[3]. *[1]Estación de Viticultura e Enoloxía de Galicia (AGACAL-EVEGA), Ponte San Clodio s/n 32428-Leiro-Ourense, Spain. [2]Universidade da Coruña, Facultade de Ciencias, Zapateira, 15071 A Coruña, Spain. [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. [4]INRA, ISVV, UMR1065 SAVE, F-33140, Villenave d'Ornon, France. E-mail: pilar.martinez.diz@xunta.gal*

Experimentally, most of the studies on biocontrol agents (BCAs) have been applied so far in vines under controlled conditions and little information is still available about the effectiveness of this strategy under field conditions. In this study, two field experiments were established to evaluate the effect of five BCAs (*Streptomyces* sp. E1+R4, *Pythium oligandrum* Po37, and commercial products containing *Trichoderma atroviride* SC1, *T. koningii* and *Pseudomonas fluorescens+Bacillus atrophaeus*) root treatments on black-foot and Petri disease fungal infection in one-year-old dormant grafted plants prior to dispatch. In April 2017, vines were hot-water treated at 53°C for 30 min and roots were immediately soaked in BCAs suspensions for 24 h. Two additional applications of BCAs were applied by drip irrigation in May 2017 and 2018. In each field, 50% of the vines were evaluated in February 2018 and the remaining 50% in February 2019. The fungal incidence and severity in roots and at the base of the rootstock in all vines and the total root mass and shoot weight in 3-year-old vines were determined. The effectiveness of some BCAs in reducing the incidence and severity of both diseases was dependent on the plant zone analysed and the plant age. *Streptomyces* sp. E1+R4, *Pythium oligandrum* Po37 and *Trichoderma atroviride* SC1 were able to reduce significantly fungal incidence and severity in specific scenarios. BCA treatments had no effect on the shoot weight, and root weight was significantly lower in all BCA treatments with respect to the control. This study represents the first approach to evaluate the effectiveness of different antagonistic microorganisms (bacteria, fungi and oomycete) to control black-foot and Petri disease under field conditions. Investigation of BCA able to prevent or at least reduce the development of GTDs should be considered a research priority based on the restriction and difficulties that chemicals are facing in most countries around the world.

Identification and characterization of potential biological control agents for the management of grapevine trunk diseases in British Columbia. JINXZ POLLARD-FLAMAND, JULIE BOULÉ, MELANIE WALKER, DIANA BALCAEN, PORTIA McGONIGAL, DANIEL T. O'GORMAN and JOSÉ RAMÓN ÚRBEZ-TORRES. *Agriculture and Agri-Food Canada, Summerland Research and Development Centre, Summerland, British Columbia V0H1Z0, Canada. E-mail: joseramon.urbeztorres@canada.ca*

Species within the *Trichoderma* genus are widely used biocontrol agents (BCA) capable of protecting plants by actively antagonizing plant pathogenic fungi, including grapevine trunk disease (GTD) pathogens, through a wide array of mechanisms. Despite this, no BCA prod-

ucts are registered in Canada for the control of GTD. Accordingly, the main objectives of this research were i) to characterize *Trichoderma* spp. from the Okanagan Valley in British Columbia (BC) by means of morphological, biological, and molecular studies and ii) to screen for isolates that can be used as BCA against Botryosphaeria dieback fungi in BC. A total of 29 *Trichoderma* isolates were obtained from grapevines in BC and phylogenetic analyses of the ITS1-5.8S-ITS4 and TEF-1α genes allowed us to identify eight species in BC, including *T. asperellum, T. atroviride, T. harzianum, T. koningii, T. koningiopsis, T. viride* and two *Trichoderma* sp. novel. Characterization studies included experiments to determine optimum temperature for mycelial growth and spore germination. The antagonistic capabilities of *Trichoderma* isolates against *Diplodia seriata* and *Neofusicoccum parvum* were screened in vitro via dual culture assay and results revealed 26 isolates to provide more than 50% mycelium growth inhibition against both fungi. The best performing isolate from each *Trichoderma* sp. was then selected for screening their potential as BCA *in plantae* via a detached cane assay (DCA) under greenhouse conditions. Canes were pruned and treated with a total of 50,000 spores from each individual *Trichoderma* sp. Pruning wounds were then challenged with a total of 5,000 conidia of *D. seriata* or *N. parvum* 24h, 7d and 21d after treatment. The three best performing isolates were re-evaluated as double and triple species combination suspensions. Fungal re-isolation results show 100% reduction of infection from 1d to 21d post treatment with combined BCAs compared to inoculated controls. This research represents the first steps towards developing BCA for management of GTD in Canada.

Disease Management

Ozone dissolved into water: an innovative tool in grapevine nursery for young plants production? ANA ROMEO-OLIVAN[1,‡], MARIELLE PAGÈS-HOMS[1,‡], HUBERT CROS[2], OLIVIER YOBREGAT[3], CORALIE BRETON[1], ROMAIN PIERRON[1,*], FRÉDÉRIC VIOLLEAU[4] and ALBAN JACQUES[1]. *[1]Unité Pathologie, Physiologie et Génétique Végétales (PPGV), INP PURPAN, Université de Toulouse, France. [2]Pépinière Daydé, Montans, France. [3]Institut Français de la Vigne et du Vin, pôle Sud-Ouest, Lisle sur Tarn, France. [4]Laboratoire de Chimie Agro-Industrielle, INP-PURPAN, Université de Toulouse, France. [*] Present Address: LVBE, Université de Haute Alsace, Colmar, France. [‡] These authors contributed equally to this work. E-mail: ana.romeo-olivan@purpan.fr*

Grapevine trunk diseases (GTDs) can cause severe symptoms and eventual death. GTDs can infect nursery plants at different propagation stages and symptoms may appear one year later. Grapevine cultivation is in need of a high volume of phytosanitary products. However, a reduction on pesticide use is a requirement for a sustainable viticulture. Ozone is a promising alternative for controlling microbial infections in plant production processes. The goal of this study was to evaluate the efficiency of ozonated water against different fungal species associated with GTDs *in vitro*, *in planta* and in nursery conditions. The effect of ozone on the plant growth was also studied. *In vitro*: Spore solutions of *Phaeoacremonium minimum* (*P.min*) and *Phaeomoniella chlamydospora* (*P.ch*) were treated with ozonated water or water (control). Spore germination was evaluated after 5 days. *In planta*: Cuttings of *Vitis vinifera* L. Cabernet-Sauvignon clone 15 were injured and infected by *P.min* and *P.ch*. Immediately after infection, injuries were treated with ozonated water or water (control). Fungal development was assessed by q-PCR. Nursery-like assays: Plants were treated with ozonated water or antifungal products within the different steps of the plant propagation process. Viability and contamination with *P.min* and *P.ch* were evaluated. Irrigation tests: Cuttings were irrigated with ozonated water or demineralized water (control) for 6 weeks. We assessed scion bud sprouting and rootstock root growth. The results obtained were: *In vitro*: While spores on control samples germinated normally, no spore germination was observed in the ozone treated samples, showing a powerful sporicidal effect. *In planta*: Results showed a lower number of copies of both fungi in treated plants in an early evaluation after infection compared to control plants, suggesting that ozone would retard the fungal colonization in cutting-wounding conditions. Nursery-like assays: The viability and sanitary quality of plants was comparable for both treatments. Irrigation test: Ozone treatments accelerated bud sprouting and the stimulation root growth, suggesting that ozone might favor plant development.

Hybrid trunk disease evaluation: a serendipitous opportunity. PAUL E. READ, BENJAMIN A. LOSEKE and STEPHEN J. GAMET. *Department of Agronomy and Horticulture, University of Nebraska Viticulture Program, University of Nebraska, Lincoln, NE 68583 USA. E-mail: pread@unl.edu*

Declining yields in Midwest and Nebraska vineyards have been variously attributed to aging of the vines, winter injury or other environmental factors. Only recently have grapevine trunk diseases (GTD) become suspect.

A serendipitous opportunity arose for the University of Nebraska Viticulture Program (UNVP) when a 20-year-old research planting was required to be terminated in August, 2018. Because of the projected termination, UNVP personnel "harvested" and evaluated for GTD multiple complete vines of over 25 cultivars of hybrid grapevines, most of which were at or approaching 20 years of age. All were trained to a high-wire double cordon system. Evaluations were based upon observations of the visible symptoms illustrated by cross-sections at five locations: 15 cm from the distal end of the cordon, mid-cordon, crown, 90 cm above the ground, and 10 cm above-ground. All of the evaluated vines exhibited recognizable symptoms of one or more GTD, with some showing text-book symptoms of Eutypa and/or Botryosphaeria. In general, the severity of symptoms progressed from a low rating at Position 1 (distal part of cordon) to greater severity at Position 5 (10 cm above the ground) for 18 of the cultivars examined. However, two cultivars showed a discernible decrease in severity from position 5 to Position 1 ('Norton' and 'Brianna'). In addition to these two cultivars, 'Petite Pearl', 'Frontenac Gris', 'Frontenac Blanc' and 'Sabrevois' ratings were considerably better than the 18 cultivars that exhibited the progression of severity from position 1 to Position 5. It is tempting to speculate that the apparent tolerance of 'Norton', which also had significantly higher yields than any of the other cultivars, may have been conferred by its purported *Vitis aestivalis* ancestry. Furthermore, 'Sabrevois' had far less GTD than its sibling 'Saint Croix', while 'Brianna' exhibits differing morphological traits than otherwise similar cultivars. We postulate that genetic relationships may play a part in understanding the tolerance of some cultivars to GTD and should be studied further.

Special Session on Grapevine Trunk Diseases control

Disease Management 101: Grapevine trunk diseases control in nurseries and young vineyards. DAVID GRAMAJE. *Instituto de Ciencias de la Vid y del Vino, Consejo Superior de Investigaciones Científicas - Universidad de la Rioja - Gobierno de la Rioja, Logroño 26071, Spain. E-mail : david.gramaje@icvv.es*

A sizeable but unknown percentage of existing grapevine nursery stock produced under conventional nursery practices is likely to be infected with a broad range of taxonomically unrelated pathogens associated with several grapevine trunk diseases (GTDs), namely black-foot and Petri diseases, and Botryosphaeria dieback. These infections can eventually debilitate and kill infected plants after they are planted in the vineyard. Production practices in nurseries provide many opportunities for infection, through poor sanitation practices or introducing infected asymptomatic cuttings from mother vines. Infected plants may initially have no visible symptoms, but they may become apparent after a certain period of time when exposed to field stresses and depending on the level of infection. Detection and identification of these pathogens is challenging, as it requires destructive sampling from different plant parts due to their uneven distribution within the host. In this talk, I evaluate the currently known management strategies applied in nurseries and young vineyards. I will also give an overview of how to minimize the economic impact of these pathogens and to improve the quality of planting material.

Disease Management 101: Grapevine trunk diseases, control in mature vineyards. MARK R. SOSNOWSKI. *South Australian Research & Development Institute, GPO Box 397, Adelaide SA 5001, Australia, 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) have become a major concern worldwide, causing significant economic impact by reduced production and vineyard longevity. Eutypa, Botryosphaeria and Phomopsis dieback, and esca affect mature vineyards. These diseases are caused by a wide range of fungal pathogens producing a range of symptoms including: leaf and shoot distortion and discolouration, wood cankers and dieback, internal wood necrosis and staining, poor growth, and eventually vine death. The prevalence of GTDs has significantly increased with changes in production practices, loss of effective chemicals, predominance of susceptible cultivars and ageing of vineyards. Pruning wounds are the main infection portal for these pathogens and inoculum sources include a wide range of alternative hosts such as fruit crops and many introduced and/or native tree species. A good understanding of the etiology, biology and epidemiology of GTD fungi has led to the development of effective management strategies in the vineyard. Cultural practices, such as the timing of pruning, can influence the likelihood of infection and disease control can be achieved through early adoption of preventative strategies to protect pruning wounds. Paints and pastes are used for protection of large reworking wounds. Fungicide and biocontrol products, used for annual pruning wounds, can be applied most efficiently with spray machinery. Removing infected wood by remedial surgery and retraining grapevines can provide curative control.

The economic consequences of grapevine trunk diseases and the potential gains from adopting preventative pruning practices and vine surgery. JONATHAN D KAPLAN[1], RENAUD TRAVADON[2], MAX NORTON[3] and KENDRA BUAMGARTNER[4]. *[1]Department of Economics, California State University, Sacramento, 6000 J St. Sacramento, CA 95819, United States of America. [2]Department of Plant Pathology, University of California, Davis, One Shields Avenue, Davis, CA 95616, United States of America. [3]Vancouver School of Economics, University of British Columbia, 6000 Iona Drive, Vancouver, BC, Canada. [4]United States Department of Agriculture, Agricultural Research Service, Davis CA 95616, United States of America. E-mail: kaplanj@csus.edu*

Grapevine trunk diseases threaten the economic viability of vineyards worldwide. In California, the causal fungi are thought to infect pruning wounds with rain, mainly during the dormant season. Infections can reduce yield by over 90% during what would otherwise be a vineyard's most productive years. Revenue falls precipitously as well, leading to significantly shortened profitable lifespans. Preventative practices (pruning-wound protectants, delayed pruning) adopted before symptoms appear (<10 years old) can potentially reduce yield losses and extend a vineyard's profitable lifespan. After symptoms are apparent (~10 years old), vine surgery (or trunk renewal), involving the retraining of a vine from a shoot just above the graft union, can be performed as well to mitigate further losses. To estimate the economic benefits from adopting preventative practices and vine surgery alone or in tandem, we simulated alternative scenarios depicting their adoption on symptomatic vines in a representative California 'Cabernet-Sauvignon' vineyard, which faces time-varying yield effects from infection. We found that preventative practices adopted early (≤5 years old) and vine surgery adopted in mature vineyards (>10 years old) significantly reduced yield losses, raised revenue, and extended vineyard profitable lifespans by over 100%. Adoption of preventative practices in year 10, however, showed limited economic value. The analysis also showed that vine surgery alone outperformed early-adopted preventative practices, but in tandem they performed best, with greater gains the earlier preventative practices began. Further, the greatest gains from vine surgery occurred when performed on all symptomatic vines beginning in year 11 (when no preventative practices are adopted earlier) and up to year 14 (when adopting preventative practices in year 3). As such, we recommend growers pursue preventative practices in young vineyards and then vine surgery after symptoms appear in approximately 20% of vines or before year 15, to maximize vineyard profitability and longevity.

From nursery to vineyard: working towards the development and implementation of management strategies against grapevine trunk diseases in Canada. JOSE RAMON ÚRBEZ-TORRES, DANIEL T. O'GORMAN, JULIE BOULÉ, MELANIE WALKER and JINXZ POLLARD-FLAMAND. *[1]Agriculture and Agri-Food Canada, Summerland Research and Development Centre, Summerland, British Columbia V0H1Z0, Canada. E-mail: joseramon.urbeztorres@canada.ca*

The long term economic viability of the grapevine industry relies on healthy planting material and practical disease management strategies in vineyards. Nowadays, grapevine trunk diseases (GTD) are considered one of the most important biotic factor limiting both grapevine production and lifespan of vineyards worldwide. Etiological, biological and epidemiological studies conducted by the Plant Pathology laboratory at the Summerland Research and Development Centre (SuRDC) since 2010 have significantly contributed to better understand the current status of GTD diseases in British Columbia (BC), laying the foundation for the development of effective disease control strategies. However, contrary to most grape-growing countries around the world, neither cultural practices nor registered products are currently available to mitigate the impact of these diseases in Canada. This presentation will give an overview of the research conducted to date with regard the status of GTD in BC and how research findings are being put into practice to develop and implement the first management strategies against GTD in both young and mature vineyards in Canada.

Trial with an innovative product based on a biocompatible drug delivery system on GTDs incidence, disease development and vine physiology. VINCENZO MONDELLO, OLIVIER FERNANDEZ, PATRICIA TROTEL-AZIZ, CHRISTOPHE CLÉMENT and FLORENCE FONTAINE. *SFR Condorcet FR CNRS 3417, Université de Reims Champagne-Ardenne, Résistance Induite et Bioprotection des Plantes, RIBP EA 4707, BP 1039, Reims, Cedex 2 51687, France. E-mail: vincenzo.mondello@univ-reims.fr; florence.fontaine@univ-reims.fr*

CA3356 is a control product based on plant extracts and oligo elements (Zn and Cu) under registration in Italy, France and Spain. Its specificity lays in the low Cu con-

tent (35g/L) and in the presence of the hydroxyapatite, a "biomimetic" drug carrier able to be absorbed, to move and delivers active ingredients inside the plant. Previous greenhouse tests showed its efficiency in controlling *Plasmopara viticola* and *Phaeomoniella chlamydospora* infections on grapevine. The new regulation of Cu treatments in vineyards (4kg/Ha/year) imposed by the European Union in 2019, encouraged us in testing this product also towards control of GTDs. We analysed its effects (5 treatments/year) on Esca incidence in the field (cv Chardonnay) and on the *D. seriata* and *N. parvum* disease development in greenhouse (cv Chardonnay and Cabernet sauvignon, 2 post-infection treatments). For the latter trials, we studied the effect of CA3356 on plant physiology through measures of the main biometric and photosynthetic parameters, as well as the impact on primary metabolism and plant defense response, by the transcriptomic analyses of thirteen-targeted genes. Results showed a certain reduction trend in cumulate Esca incidences that need to be confirmed by additional experimental replicates. In the greenhouse trial, CA3356 was able to reduce the presence of the target pathogens, but without reducing necrosis length. CA3356 showed a positive role on vine physiology by increasing the photosynthetic activity for both cultivars and of the fresh weight on cv. Chardonnay. At transcriptomic level, CA3356 induced the most of the targeted genes, especially those related to plant defense response such as chitinase, glucanase and other *PR*-related genes. These inductions were higher than those determined by ASM (S-methyl benzo [1,2,3] thiadiazole-7-carbothioate), a molecule able to activate systemic resistance (SAR) in plant, present in commercial products and here utilized as positive control.

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Evaluation of the fungicide Tessior (boscalid and pyraclostrobin) for control of grapevine trunk diseases in Greece. A. SAMARAS[1], P. NTASIOY[1], S. TESTEMPASIS[1], S. THEOCHARIS[2], S. KOUNDOURAS[2] and G. KARAOGLANIDIS[1]. *[1]Plant Pathology Laboratory, Aristotelian University of Thessaloniki, Thessaloniki, 54124, Greece. [2]Laboratory of Viticulture, Aristotelian University of Thessaloniki, Thessaloniki, 54124, Greece. E-mail: gkarao@agro.auth.gr*

Grapevine trunk diseases (GTDs) are among the most important grape diseases affecting yield, wine quality and vineyard longevity. Due to the lack of availability of chemical products with high efficacy, the control of GTDs in Europe relies mainly on cultural measures and some biofungicides. Recently, a new chemical product was released into the European market for the control of GTDs, under the commercial name Tessior®, containing the fungicides boscalid and pyraclostrobin. A series of field experiments were established in Greece aiming to investigate the effectiveness of this new product in protecting pruning wounds from infections caused by *Phaeomoniella chlamydospora* and *Diplodia seriata*. Applications were conducted before (protective) or after (curative) wound inoculation with the pathogens following pruning. Results showed that the applications of Tessior contributed to a significant reduction of disease incidence and severity when applied either on the day of pruning or at least 6 days after pruning. In contrast, delayed applications of 13 or 29 days after pruning and inoculation did not contribute to pathogen control, leading to a disease incidence similar to that observed in the untreated control plants. To further investigate the effectiveness of the protective activity of Tessoir in controlling GTDs, two long-term experiments were established in newly planted vineyards, in 2015. Applications of Tessior were initiated from the 1st year of planting, while artificial inoculations with either *P. chlamydospora* or *D. seriata* were initiated 2 years after establishment. Preliminary results of these long-term experiments confirm the high efficacy of the product. This study highlights the effectiveness of Tessior and the appropriate application time to achieve the highest efficacy.

Factors affecting progress of grapevine trunk disease in New Zealand vineyards. MARK R. SOSNOWSKI[1,2] and DION C. MUNDY[3]. *[1]South Australian Research and Development Institute, GPO Box 397, Adelaide SA 5001, Australia. [2]School of Agriculture, Food and Wine, The University of Adelaide, Waite Campus, Glen Osmond SA 5064, Australia. [3]The New Zealand Institute for Plant and Food Research Limited, Marlborough Wine Research Centre, PO Box 845, Blenheim 7240, New Zealand. E-mail: mark.sosnowski@sa.gov.au*

Eutypa and Botryosphaeria dieback are major grapevine trunk diseases (GTDs) in New Zealand, causing significant economic losses, as they do worldwide. This has led to concerted efforts, with support from the newly established Bragato Research Institute, to better understand the progress of GTDs over time and some factors affecting disease incidence. In December 2013, 697 vineyard blocks were selected to represent a cross-section of ages (4 years and older) and varieties planted in the major growing regions of Marlborough and Hawke's Bay. In a

randomly selected section of each block, 200 vines were visually assessed and incidence of vines with dieback and foliar symptoms recorded. Information on variety, clone, and rootstock, along with pruning and disease management strategies, was collected. In November 2018, the same blocks were revisited and assessed as previously, although 95 blocks (14%) had been removed and 14 blocks (2%) reworked. The overall mean incidence of dieback increased from 8 to 20% and foliar symptoms 0.1 to 0.3% over the 5 years across all of the blocks assessed, which corresponded with an increase in average vine age from 12 to 17 years. Overall trends in 2018 revealed variation in dieback incidence between vine ages, varieties, clones, rootstocks and pruning styles, with signs of short-term success using remedial surgery to control GTDs. Details of this study will be presented and implications of the results discussed, with particular reference to management of GTDs and future research requirements.

Factors involved in the failure of grapevine nursery stock in Israel. DAVID EZRA, MENACHEM BORENSTEIN, RAN SHULHANI and DANI SHTIENBERG. *Department of Plant Pathology & Weed Research, ARO, The Volcani Center. P.O.Box 15159, Rishon LeZion, 7528809, Israel. E-mail: dezra@volcani.agri.gov.il*

Grapevine nurseries across Israel experience difficulties producing healthy, robust table grapes and wine grapes during the last year. The causes for this failure were unknown. Due to the importance of these industries in Israel, a national project was initiated aiming at identifying the factors involve and developing a practice that would enable the production of healthy nursery stock. Pathogens were not found to be a factor. Instead, one of the main causes of the failure was the timing of collection of rootstock cuttings. Cuttings were collected too early, before canes on the mother vine became fully dormant, or too late, after the mother vine had terminated their dormancy period, reduced the rooting capabilities of the cuttings significantly. Lack or inappropriate rooting of the rootstocks caused poor development of the rootings and subsequently resulted in their degeneration and decay. Factors related to the handling of the propagation material in the nurseries were of significance as well. In Israel, some of the nurseries store cuttings at 4ºC before grafting and callusing. It was found that keeping the rootstock propagation material at 4ºc for an appropriate time increases their rooting potential, whereas keeping them too long decreased their rooting potential. Further, the optimum time of cold storage varied based on when during the dormant season cuttings were collected from the mother vines. This presentation will report the procedures used to identify the factors involved in failure to produce healthy table grape and grapevine planting material in Israel and describe the practices for producing healthy plants.

Physiological and qualitative consequences of curettage on grapevine grape berry and young wine of Sauvignon variety from the Bordeaux region. CELINE CHOLET[1], EMILIE BRUEZ[1], CECILE THIBON[1], PASCALINE REDON[1], PASCAL LECOMTE[2], PATRICE REY[2], TOMMASO MARTIGNON[3], MASSIMO GIUDICI[3], PHILIPPE DARRIET[1] and LAURENCE GÉNY[1]. *[1]EA 4577, Unité de recherche œnologie, INRA, USC 1366 Œnologie, Université de Bordeaux / Institut des Sciences de la Vigne et du Vin, 210 Chemin de Leysotte - CS 50008, F-33882 Villenave d'Ornon, France. [2]UMR 1065 Santé et Agroécologie du Vignoble, Institut National de Recherche Agronomique/ Bordeaux Sciences Agro / Institut des Sciences de la Vigne et du Vin, 71, Avenue Edouard Bourleaux, INRA Domaine de la Grande Ferrade - BP81, 33883 Villenave d'Ornon Cedex, France. [3]Simonit&Sirch, maitres tailleurs de vigne, 1 Rue Porte des Benauges, 33410 Cadillac – Bordeaux, France. E-mail: celine.cholet@u-bordeaux.fr*

Esca, one of the most devastating grapevine trunk diseases, has increased in incidence worldwide over the past decade. Currently, there are no chemical products registered for Esca, as sodium arsenite was banned in 2001 in France and in 2003 in Europe. As a non-chemical therapeutic treatment, curettage has been adopted by growers, but published studies evaluating its efficacy are rare. Curettage consists of physical removal of the white-rotted wood (i.e., amadou), caused by Basidiomycete wood-rotting fungi associated with Esca. It is thought to preserve the healthy part of the vine from being contaminated by these fungi. The aim of this study was thus to examine the influence of curettage on vine growth, nutrient content, and fruit and wine quality. This work presents a synthesis of 3-year experiment of Sauvignon vines curetted in 2014 in a plot in Bordeaux. There were three treatments: (i) untreated, asymptomatic vines, (ii) untreated vines with leaf symptoms (control), and (iii) treated vines (in 2014) with no leaf symptoms following treatment. For each treatment, fertility and growth, chemical composition of the fruit and wine, and sensory quality of the wine were evaluated. Our results showed that in comparison with control vines, treated vines had lower vigor and fertility, but there was no effect of treat-

ment on fruit quality. Wine made with fruit from treated vines was not different from that of control vines in terms of aromatic markers. Wine made with fruit from control vines was less appreciated by a sensory panel, and was characterized by a decrease in aromatic markers and an increase in oxidative markers. Our findings suggest that curettage may minimize the impact of Esca on wine quality, albeit after 3 years. As such, curettage can be considered effective for management of Esca in the short term.

A new architecture of the vine: an original wrestling track. CHRISTOPHE BERTSCH. *Laboratoire Vigne Biotechnologies et Environnement UR-3991, Université de Haute Alsace, 33 rue de Herrlisheim, 68000 Colmar, France. E-mail : christophe.bertsch@uha.fr*

The different varieties of *Vitis vinifera* have varying sensitivities; none of them appear to be resistant or tolerant to grapevine trunk diseases. However, *Vitis vinifera* Subsp. sylvestris, the ancestor of our vineyard seems to have the capacity to contain the fungi implied in the disease. From a conceptual point of view, it seems difficult today, with the environmental constraints, to eradicate all the fungi involved in trunk diseases. We must therefore also exploit the resistance processes of the plant. In this context, the history of phylloxera tells us certainly the most beautiful and most effective biological approach: the use of American rootstocks resistant to the devastating insect. The weak point of our current vine about pathogens involved in trunk diseases is not the roots, as for phylloxera, but the trunk, a true bio-reactor for the fungi involved. We propose to rethink the architecture of our vine like the use of rootstocks to fight against phylloxera. It is perfectly possible to insert between the graft and the rootstock a "trunk" tolerant to trunk diseases (sylvestris). This innovative approach may produce vines tolerant to the diseases. The first grafting trials done are very promising. A strong point of this method is that it would contain this scourge, without using chemicals, in the same way that the use of rootstocks ended the phylloxera crisis.

An overview of lessons learnt in the application of *Trichoderma* products in grapevine nurseries. WYNAND VAN JAARSVELD[1], ELODIE STEMPIEN[1], ROMAIN PIERRON[1], FRANCOIS HALLEEN[1,2] and LIZEL MOSTERT[1]. *[1]Department of Plant Pathology, University of Stellenbosch, Private Bag X1, Matieland, 7602, South Africa. [2]Plant Protection Division, ARC Infruitec-Nietvoorbij, Private Bag X5026, Stellenbosch, 7599, South Africa.Email: lmost@sun.ac.za*

Trichoderma spp. hold potential to be used for the management of black foot disease (BFD). Apart from hot water treatment of dormant nursery vines, no treatment is available to prevent nursery vines from becoming infected with BFD pathogens. In the past variable success of colonization of *Trichoderma* spp. were found when applied to nursery vines. Therefore, different methods of application of *Trichoderma atroviride* were tested on commercially planted nursery vines. One hundred graftlings were used per treatment, replicated five times and repeated over two seasons. To assess the efficacy of different *Trichoderma* products one hundred graftlings were used per treatment, replicated four times and repeated over two seasons. For both seasons the trials were uprooted after 7 months and isolations made from the base and roots of the vines. *Trichoderma* and BFD pathogens were recorded. For both trials the number of certifiable vines of the *Trichoderma* treatments were not significantly more than the untreated controls. *Trichoderma* treatments did not cause additional root growth, which might be due to the vigorous rootstock cultivar Ramsey that was used. When comparing tissue parts, the base of the vine and top part of roots had significantly higher *Trichoderma* colonization than the middle and bottom parts of the roots. Only in one season (2016/17) of the trial evaluating different products did all of the *Trichoderma* treatments significantly lower the black foot infections in the bases of the untreated controls from 6.5% to as low as 1.0%. The different application methods clearly showed that dipping of basal ends in the dry formulation consistently gave the highest colonization. The 1 hour soak of bases of vines was not effective. The field drenching was significantly less effective than the dry formulation application. These results provide valuable insights into the application of *Trichoderma* products in grapevine nurseries.

Evaluation of different strains of *Trichoderma* spp. against Grapevine Trunk Diseases *in vitro* and colonization of Arbuscular Mycorrhizal Fungi in roots of nursery grapevine plants. CATARINA LEAL[1,2], ROSA ROA-ROCO[1], PAULINA ARRAÑO-SALINAS[1], PABLO RODRIGUEZ-HERRERA[1], IVO AGNIC[1], DANIELA TORRES[1], CARLOS VALDIVIA[1], MAURICIO LOLAS[3], ALVARO GONZALEZ[1] and FELIPE GAINZA-CORTÉS[1]. *[1]Viña Concha y Toro S.A, Center for Research and Innovation, Fundo Pocoa s/n, Km10 Ruta K-650, Pencahue, Región del Maule, Chile. [2]Universidade de Lisboa,*

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Grapevine Trunk Diseases (GTDs) impact economic sustainability of the wine industry affecting vineyards worldwide without efficient control methods. As consequence of this emerging problem, several studies aim to find eco-friendly solutions to control these diseases. *Trichoderma* spp. and Arbuscular Mycorrhizal Fungi (AMF) show high potential in decreasing the incidence of GTDs in vineyards as well as increasing the ability of vines to survive in stress conditions. During season 2016–2017, the Center for Research and Innovation of Viña Concha y Toro (VCT) have tested different *Trichoderma* strains (T1: based in *T. atroviride*; T2: *T. gamsii*; T3: *T. virens*; T4 and T5: *T.* spp.) against *Diplodia seriata*, *Neofusicoccum parvum*, *Phaeomoniella chlamydospora*, *Inocutis* spp. and *Eutypa lata* under *in vitro* assays. Moreover, grapevine nursery plants were inoculated in field nursery with commercial product based on AMF (M1: *Rhizophagus irregularis*, *Glomus. mosseae*; and M2: *G. brasillanum, G. clarum, G. deserticota, R. irregularis, G. mosseae, G. monosporum, Gigaspora margarita)* in order to evaluate the colonization capacity. By determining the occupied area over time for each strain of *Trichoderma* and its ability to inhibit the growth of each GTD, it was possible to determine that T1 was the most effective strain against all the GTDs tested, followed by T3 and T2 (respectively) that show some potential inhibition of the pathogens growth. T5 shows low capacity to cope with the GTDs evaluated. In the case of AMF evaluation, both M1 and M2 showed significant differences in frequency (F%), colonization intensity (M%) and arbuscles abundance (A%) compared with non-inoculated plants. M2 showed significant higher nitrogen and arginine contents in roots compared with M1-inoculated and control plants. Together, these results shows the potential use of this beneficial microorganisms in order to strengthen and improve the performance of VCT grapevines against GTDs.

Influence of different application times of *Trichoderma* in the nursery for the reduction of wood pathogens and quality improvement of grafted cuttings. GIUSEPPE CARELLA[1], ALESSANDRA BENIGNO[1], , ELISA METRUCCIO[2], SAMUELE MORETTI[1], FABIO OSTI[2], ANDREA PACETTI[1], LAURA MUGNAI[1] and STEFANO DI MARCO[2]. *Istituto di Biometeorologia, CNR, Via Gobetti 101, 40129 Bologna, Italy. [2]Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente, Sezione Patologia vegetale e Entomologia, Università degli Studi di Firenze, P.le Cascine 28, 50144 Firenze, Italy. E-mail: s.dimarco@ibimet.cnr.it*

Wood pathogens of grapevine are recognized to be already present in propagation material. These infections together with field infections through pruning wounds, which represent the main source of infection cause decline symptoms in the young vineyards. More recently, the availability of biological control products to be applied in the control of GTDs had a large increase. Application timing of a product based on *Trichoderma atroviride* SC1 (Vintec®) was tested for two years in a commercial nursery to establish the influence of application timing on the quality of the grafted vine and level of natural infections in the nursery. The formulation was applied at three stages: rehydration, callusing, basal callus formation followed by soil drenching. In addition, the effect of the combined applications was also tested. All treatments were compared with controls (another Trichoderma-based product, plants treated with synthetic fungicide and untreated plants). Surveys included assessment of plant quality and viability first, after callusing and later after a growth season in the nursery soil. At uprooting root development was analysed by video image analysis and quality assessed as percentage of certifiable plants. The final grafted vines, ready for sale, were used for isolating the mycoflora, thus analyzing the influence of application at different stages of the grafted cuttings production on the fungal mycoflora and in particular fungal colonization by wood pathogens. The rehydration stage showed to be the better performing application in reducing fungal infections, whereas the applications at the basal callus followed by soil drenching produced plants with a better root quality, and a different increase of the certifiable plants number depending on the rootstock tested. The use of biological control products requires a lot of care in detecting the most useful application timing, avoiding overlapping of needless treatments.

Effects of *Trichoderma asperellum* and *Trichoderma gamsii* and hot water treatment on *Phaeomoniella chlamydospora* in grapevine plant propagation material. STEFANIA POLLASTRO[1,2], DONATO GERIN[1], CRESCENZA DONGIOVANI[3], GIULIO FUMAROLA[3], MICHLE DICAROLO[3], RITA MILIVIA DE MICCOLIS ANGELINI[1,2], CATERINA ROTOLO[1] and FRANCESCO FARETRA[1,2]. *[1]Department of Soil, Plant and Food Sciences and [2]Selge Network, University of Bari Aldo*

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Phaeomoniella chlamydospora, associated with Petri disease and Esca in young and mature plants, respepectively affects plant propagation material frequently as latent infections. Curative methods are few and scarcely effective and hence proactive prevention measures are indispensable to limit the presence of the pathogen especially in young vineyards. In this study, the biofungicide Remedier™ (ISAGRO S.p.A, Milan, Italy) composed by *Trichoderma asperellum* icc012 and *T. gamsii* icc080 and hotwater treatment (HWT) were applied individually and in combination for controlling *P. chlamydospora* during the propagation process in nursery and at planting in pots. Cuttings of 1103 Paulsen (P) rootstock or grafted plants cv. Italia/1103P were used in experiments carried out in the nursery (trials 1 and 2), and grafted plants Italia/1103P in the pot experiment (trial 3). The biofungicide was applied by soaking cuttings during the hydration stage, at planting and 15, 30 and 60 days afterwards. Propagation material artificially inoculated with a conidal suspension (10^6 spores mL^{-1}) of *P. chlamydospora* strain CIA43.2, marked with benomyl resistance, was used after the first BCAs application. In trial 3, HWT plants were also compared. In all trials, the appropriate untreated and non-inoculated controls were also used. At uprooting, quality status of each plant was assessed at three different points and the presence of *P. chlamydospora* was evaluated by isolation on appropriate media. BCA-treated vines showed a significant ($P \leq 0.05$) increase in stems and roots development even as compared to untreated ones subjected to HWT. *Phaeomoniella chlamydospora* and Botryosphaeriaceae sp., the fungi isolated with prevalence higher than 10%, were significantly reduced (up to 100%) by HWT. BCAs reduced *P. chlamydospora* CIA43.2 up to 70%. These results contribute to an integrate management of esca and Petri disease in grapevine.

Evaluation of biocontrol agents against fungal pathogens associated with grapevine trunk diseases. ISIDORA SILVA-VALDERRAMA[1], DIANA TOAPANTA[1], GONZALO DIAZ[2], MAURICIO LOLAS[2] and ALVARO CASTRO[1]. *[1]UC Davis Chile Life Science Innovation Center. Avenida Andrés Bello 2299, piso 11, 7511303 Providencia, Chile.[2] Laboratorio de Patología Frutal, Facultad de Ciencias Agrarias, Universidad de Talca. Avenida Lircay s/n, 3462227 Talca, Chile. E-mail: alvcastro@ucdavis.edu*

Grapevine trunk diseases (GTDs) are a major problem for the wine industry worldwide, leading to reductions in vineyard productivity and longevity. In Chile, several fungal phytopathogens have been identified and isolated from internal GTDs symptomatic plant tissue, most of them from the Botryosphaeriaceae family. The aim of this study was to evaluate *in vitro* and grapevine canes potential biocontrol agents (BCAs) against *Diplodia seriata* and *Neofussicoccum parvum*. 387 endophyte and epiphyte fungal isolates were obtained from vines located in organic and integrated-pest management vineyards. Identification was performed by ITS sequencing and the isolates were classified into 56 different genera. A small group of fungi previously reported as BCAs and with antagonistic activity were selected. Isolates from *Trichoderma* sp., *Clonostachys* sp., *Chaetomium* sp., *Purpureocillium* sp., *Epicoccum* sp. and *Cladosporium* sp., in comparison with registered products Mamull and Tifi (*Trichoderma atroviridae* based), showed pathogen growth inhibition from 30% to 100% against *D. seriata* and *N. parvum* in dual culture agar plate evaluations. Strains of *Trichoderma* sp., *Clonostachys* sp. and *Epicoccum* sp. were evaluated in qualitative bioassays using grapevine canes. Four strains exhibited 100% growth inhibition against the two pathogens. Differences were found depending on the matrix used for the test, meaning PDA (potato dextrose agar), PA (plant agar) or grapevine pruning material. In addition, scale-up tests were made in order to allow field-level strain-efficacy evaluations. Therefore, the establishment of a novel pipe-line for biocontrol of trunk diseases, according to the internal localization of the pathogens in the plant, allowed the identification of promising endophytic fungal biocontrol agents isolated from grapevines. These experiments demonstrated potential for the development of a commercial biocontrol product which enables the establishment of prophylactic steps against GTDs fungi as an essential part of an integrated disease management program.

The use of three biocontrol agents alone or in combination to control *Neofusicoccum parvum*, a Grapevine Trunk Disease pathogenic fungus. AMIRA YACOUB[1,2], RANA HAIDAR[1,2], JONATHAN GERBORE[3], MARIE-CECILE DUFOUR[1,2], REMY GUYONEAUD[4] and PATRICE REY[2,1]. *[1]INRA, UMR 1065 Santé et Agroécologie du Vignoble, ISVV, F-33140 Villenave d'Ornon, France. [2]Université de Bordeaux, Bordeaux Sciences Agro, UMR 1065 Santé et Agroécologie du Vignoble, F-33175 Gradignan, France. [3]Université de Bordeaux, ISVV, Unité de recherche Œnologie EA 4577, USC*

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Neofusicoccum parvum is one of the most virulent fungal pathogens involved in Grapevine Trunk Diseases (GTDs). Using biocontrol agents (BCAs) may be a promising method to limit the extension of this pathogen, and one of the strategies for enhancing plant protection is to combine BCAs. Previous studies showed that some microorganisms such as the oomycete, *Pythium oligandrum*, and bacteria, *Pantoea agglomerans* and *Brevibacillus reuszeri*, applied individually on young vines, reduced necrosis caused by GTD pathogens. In the present experiment, *P. oligandrum*, *P. agglomerans* and *B. reuszeri* (inoculated at trunk or root levels) were applied individually or in combination to evaluate their effectiveness against *N. parvum* (inoculated at the trunk level). Results showed that 5 months after *N. parvum* inoculation, necrosis size was reduced by about 60% in most treatments. Efficacy was very similar when BCA were applied individually or in combination. However, for two modes of BCA combinations, the efficacy had decreased. These results suggest that there was no synergistic effect between these BCAs to control *N. parvum* attacks. In order to develop an adapted control strategy, the molecular events occurring during the tripartite interaction: grapevine/BCAs/*N. parvum* have been investigated. Wood samples have been collected 0 and 14 days post-inoculation and the expression of a set of 96 genes ("NeoViGen96"chip) implicated in *Vitis vinifera* defense mechanisms were analyzed. Gene expressions were quantified by real-time-PCR. Studied genes include 26 genes encoding PR proteins; 18 and 3 genes involved, respectively, in secondary metabolites and indole biosyntheses; 14 genes involved in wall thickness enhancement; 15 and 4 genes involved, respectively, in signaling and oxylipine pathways. Preliminary results will be presented.

Vertical vegetal endotherapy: a new mode of treatment to cure grapevine trunk diseases? ANDREA PACETTI[2], ROMAIN PIERRON[1], SIBYLLE FARINE[1], LAURA MUGNAI[2], CELINE TARNUS[1], CHRISTOPHE BERTSCH[1] and MELANIE GELLON[1]. *[1]Laboratoire Vigne Biotechnologies et Environnement UR-3991, Université de Haute Alsace, 33 rue de Herrlisheim, 68000 Colmar, FRANCE. [2]Department of Agricultural, Food, Environmental and Forestry Science and Technology (DAGRI), Plant pathology and Entomology section, University of Florence, P.le delle Cascine, 28, 50144 Firenze, Italy. E-mail: melanie.gellon@uha.fr*

Esca is usually described as one of the most devastating grapevine trunk diseases. This disease has been increasing in the last 30 years, and since sodium arsenite was banned in 2001, there is no solution available to effectively cure the disease. One potential technique could be plant endotherapy, which is used in fruit tree cultivation and in urban landscape trees. It has many advantages, particularly in terms of the environment as, compared to spraying, it allows a low input as for treatment doses and limits product drift in soil, air and water. This technique of vegetal endotherapy consists of drilling and injecting chemicals compounds or beneficial microorganisms directly into the plant. It has many advantages, particularly for the environment. Indeed, compared to a conventional spray, this technique allows a reduction in treatment doses and limits product drift into the soil, air and water, thus reducing the impact on non-target organisms. Recently, it has been reported that the impacts of Esca could be minimized by mechanical curettage of the white rot. The recent study of the mechanisms of sodium arsenite showed that the molecule is concentrated in white rot after spray application to the wood. All of these results suggest that some fungal activity in the white rot may have a role in Esca symptoms, specifically leaf symptoms. We adapted plant endotherapy to grapevine by drilling a hole vertically in the trunk to reach and treat directly the white rot, with chemical compounds at different concentrations, and therefore the pathogens associated to it. We apply a "chemical curettage" to vines with leaf and wood symptoms of Esca. Experiments initiated in an Alsace vineyard in 2018, showed a fairly promising trend with a reduction in the expression of leaf symptoms by up to 50% in some varieties (*V. vinifera* cv Gewurztraminer and Riesling) treated by this method. The experiments need to be repeated, monitored and extended on other regions, over several years, to see if this trend is confirmed and if this method could be used to reduce the impact of Esca.

Pruning time can reduce grapevine trunk diseases infection under British Columbia environmental conditions. JOSE RAMON ÚRBEZ-TORRES, DANIEL T. O'GORMAN, JULIE BOULÉ, MELANIE WALKER and JINXZ POLLARD-FLAMAND. *[1]Agriculture and Agri-Food Canada, Summerland Research and Development*

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Grapevine trunk disease pathogens within the Botryosphaeriaceae and Diatrypaceae families infect grapevines primarily through pruning wounds. Airborne spores are discharged from fruiting bodies (pycnidia or perithecia) under optimum environmental conditions, land on susceptible pruning wounds and start infection after germinating. These infections develop into wood necrosis and perennial cankers, which lead to grapevine dieback and eventual death of the plant. Understanding the spore seasonal abundance of these fungi is critical to determine low risk infection periods in which pruning can be conducted. It has been shown in some grape-growing regions that either early or late pruning can reduce GTD infections in vineyards. Accordingly, the main objective of this study was to determine best pruning time to minimize GTD infection caused by the botryosphaeriaceous fungi *Diplodia seriata* and *Neofusicoccum parvum* under British Columbia environmental conditions. Chardonnay and Merlot grapevines (6 vines/treatment) were pruned the 15th of each month, including December, January, February, March, and April. Then, 50 uL of 10^5 conidia/mL suspensions of *D. seriata* and *N. parvum* were used to artificially inoculate the pruned vines on March 16th, April 16th, May 15th, and June 15th, which correlates with the highest presence of Botryosphaeriaceae spores in the Okanagan Valley. The trial was conducted in two consecutive years (2016–2017 and 2017–2018). Results showed that pruning wounds made in mid-December and mid-January were significantly less susceptible to *D. seriata* and *N. parvum* infection compared to pruning wounds made in February, March and April. Pruning wounds made in mid-February were highly susceptible (>60% infection) to infections happening in March. However, pruning wound susceptibility decreased over time from 50% to 10% when infection happened in April, May and June. This study suggests that early pruning in winter can significantly minimize the infection cause by *D. seriata* and *N. parvum* in the Okanagan Valley.

POSTER PRESENTATIONS

Incidence and symptoms of grapevine trunk diseases on nursery finished plants in Uruguay. MARÍA JULIA CARBONE, MATÍAS GELABERT, PEDRO MONDINO and SANDRA ALANIZ. *Department of Plant Protection, Faculty of Agronomy, University of the Republic, Av. Garzón 780, CP 12900, Montevideo, Uruguay. E-mail: salaniz@fagro.edu.uy*

The occurrence of grapevine trunk diseases is one of the most important problems in young vineyards and nurseries worldwide. The large number of cuts and wounds made during the propagation process make the planting material vulnerable to infection by fungal trunk pathogens. The symptoms present and its incidence on nursery finished plants were investigated by first time in the main grapevine nursery of Uruguay. The plants evaluated were Tannat/RR101-14, Merlot/Fercal, Prosecco/SO4, Moscatel de Hamburgo/Gravesac, Tannat/3309C and Tannat/1103P. Twenty grafted plants of each combination scion/rootstock were carefully observed by cross-sectional and longitudinal cuts. Isolations from symptomatic samples were performed and identified. The incidence of symptoms was 100% in all nursery plants evaluated. The symptoms observed were black discoloration and necrosis of wood tissue developed from the base of the rootstock, necrotic streaks and internal necrotic wedge-shaped staining in the cross section. Species associated to Petri disease, Black foot, Phomopsis dieback and Botryosphaeria dieback diseases were isolated. This work confirms the presence of grapevine trunk diseases in plants produced in the main grapevine nursery in Uruguay. This emphasizes the importance of developing management strategies to minimize the incidence of grapevine trunk diseases in nurseries.

Effect of water stress and plant inoculation with wood-inhabiting bacteria on the symptoms caused by the grapevine trunk pathogen *Neofusicoccum parvum*. RANA HAIDAR[1,2,3], AMIRA YACOUB[1,2], ANTOINE PINARD[1,2] and PATRICE REY[1,2]. *[1]INRA, ISVV, UMR1065 Santé et Agroécologie du Vignoble (SAVE), F-33140 Villenave d'Ornon, France. [2]Université de Bordeaux, ISVV, UMR1065 SAVE, Bordeaux Sciences Agro, F-33140 Villenave d'Ornon, France. [3]Tichreen University, Faculty of Science, Biology Department, PO Box 2231, Latakia, Syrian Arab Republic. E-mail: amira.yacoub@inra.fr*

Esca and Botryosphaeria dieback are considered to be major factors limiting grapevine productivity. In the context of these diseases, the influence of combined biotic and abiotic factors, however, is still not well understood. Thus, in the present study, the combined effect of a biotic (bacteria) and an abiotic (water stress) factors on the symptoms caused by the Botryosphaeria dieback pathogen *Neofusicoccum parvum* were evaluated. Cabernet Sauvignon cuttings were co-inoculated individually with *N. parvum* or in combination with two wood-inhabiting bacteria, *Bacillus pumilus* and *Xanthomonas* sp., in normal or reduced watering condi-

tions. Nine months after inoculation, the effect of water stress, as well as the effect of bacteria / *N. parvum* interaction on the external and internal lesions caused by *N. parvum,* were assessed. The presence of *N. parvum* in the various wood samples was confirmed by qPCR. Results demonstrated that the combination of the abiotic and biotic factors have greater influence on the cankers lesions than on the necrosis lengths. The detection and the quantification of *N. parvum* DNA by qPCR revealed that this pathogen was found in higher concentrations in water-stressed vines than in well-watered vines. Interestingly, *N. parvum* concentrations were higher in the plants co-inoculated with *B. pumilus*, compared to those inoculated only with *N. parvum*. The relationship between factor-inducing stress and growth of *N. parvum* in inoculated vines and the special interaction between *N. parvum* and *B. pumilus* will be discussed.

***Botryosphaeriaceae* species: the causal agents of grapevine dieback in Algeria**. FAIZA AMMAD[1], MESSAOUD BENCHABANE[1], PASCAL LECOMTE[2] and AMEUR CHERIF[3]. *[1]Département de Biotechnologie, Faculte SNV , Université de Blida 1, Blida. [2]INRA, UMR 1065, Santé Végétale, ISVV Bordeaux-Aquitaine, France. [3]Laboratoire microorganismes and biomolécules actives, Département de biologie, Faculté des sciences de Tunis, Campus Universitaire, 2092 Tunis, Tunisia. E-mail : sahraoui_a_f@yahoo.fr*

Grapevine (*Vitis vinifera*) dieback is an increasing problem in Algeria. Field surveys conducted during spring season between 2006 and 2012 showed that the average incidence of diseased grapevines was from 1.43% to 25.11% and the severity index (to estimate the mean value of the dieback class in vine) was 0.65 to 2.25. Cross sections of symptomatic wood of Cabernet Sauvignon, Cardinal, Carignan, and Syrah were collected in four vineyards in Algeria showing different symptoms such as sectorial brown colored necrosis, central and sectorial gray necrosis, and central light-brown necrosis. Three Botryosphaericeae species, including *Botryosphaeria dothidea*, *Neofusicoccum parvum* and *Diplodia seriata* were isolated from the infected wood and identified based on morphological characters and analyses of the internal transcribed spacer region (ITS) and β-tubline nucleotide sequences. DNA sequences revealed 99 % homology with 10 isolates of *B. dothidea*, 18 isolates of *Diplodia seriata* and 7 isolates of *Neofusicoccum parvum*. The field surveys revealed the presence of both anamorph and teleomorph stages just for *Botryosphaeria dothidea* and *Diplodia seriata* produced on dead and infected wood. Inoculation of grapevine plantlets (Hmar Bouamr) with all three Botryosphaeriaceae species produced necrosis and vascular lesions in the wood after a 6-week incubation; *Neofusicoccum parvum* isolates were the most virulent, followed by *D. seriata* and *B. dothidea*. All species tested were re-isolated from lesions on infected plantlets. Our results represent the first report of a canker disease of grapevine associated with Botryosphaeriaceae species in Algeria.

Differences between small RNA profiles of *Vitis vinifera* cv. Chardonnay infected by *Diaporthe eres* and *Diaporthe bohemiae* as non-pathogenic on grapevines. ALES EICHMEIER[1], ELISKA PENAZOVA[1], JAKUB PECENKA[1], EVA ONDRUSIKOVA[1], JANA MOUDRA[1], ROBERT POKLUDA[2], AKILA BERRAF-TEBBAL[1], DARIUSZ GRZEBELUS[1,3], JANA CECHOVA[1] AND MIROSLAV BARANEK[1]. *[1]Mendel University in Brno, Faculty of Horticulture, Mendeleum - Institute of Genetics, Valticka 334, 69144, Lednice, Czech Republic, [2]Mendel University in Brno, Faculty of Horticulture, Department of Vegetable Science and Floriculture, Valticka 334, 69144, Lednice, Czech Republic, [3]Institute of Plant Biology and Biotechnology, Faculty of Biotechnology and Horticulture, University of Agriculture in Krakow, Krakow, 31425, Poland. E-mail: ales.eichmeier@mendelu.cz*

Diaporthe spp. are important pathogens, also saprobes and endophytes on grapevines. Several species are known to cause cane bleaching, swelling arm and trunk cankers. In this study, we explore the differentially expressed small RNAs in response to *Diaporthe eres* (CPC 28220) as pathogenic and *Diaporthe bohemiae* (CPC 28223) as non-pathogenic in *Vitis vinifera* cv. Chardonnay cultivated *in vitro*. The group of small RNAs was investigated because of increasing evidence that a class of small non-coding endogenous RNAs, known as microRNAs (miRNAs), play an important role in post-transcriptional gene regulation during plant development and response to biotic and abiotic stresses. We cultivated cv. Chardonnay on DKW/Juglans medium, when the plants were in size of 5 cm, we inoculated them through the second upper leaf by a sterile needle inoculated with i) *Diaporthe eres*; ii) *Diaporthe bohemiae*; and iii) empty prick. Each treatment was sustained through five samples. After ten days post-inoculation, we harvested the plants and RNA was extracted with PureLink™ Plant RNA Reagent (Thermo Fisher Scientific). Small RNA libraries were constructed using TruSeq Small RNA Library Preparation Kit (Illumina). All the steps were carried out two times to evaluate two

variants important for statistics. Three treatments were sequenced twice using a MiniSeq (Illumina) and MiniSeq High Output Reagent Kit (75-cycles). Group of miRNAs appeared differentially within analyzed treatments. Subsequently, we analyzed the sequencing data to identify putative miRNA targets and explore their involvement in possible pathogen response pathways. Our results may contribute to the understanding of the role that miRNA pathways play during plant pathogenesis, and may be crucial in understanding disease symptom development in grapevines infected by pathogenic *Diaporthe eres* and non-pathogenic *Diaporthe bohemiae*.

A LysM domain-containing protein LtLYSM1 functions as a major virulence factor during *Lasiodiplodia theobromae* infection. JUNBO PENG, XINGHONG LI, WEI ZHANG, QIKAI XING, MEI LIU and JIYE YAN. *Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China. E-mail: pjb169961@163.com*

Lasiodiplodia theobromae is a plant pathogen, which has the ability to infect and cause serious damage to a diverse range of fruit crops including apple, peach, pear, blackberry, and grape in China. However, the pathogenic mechanisms of this fungus have not yet been completely explored. In this study, a LysM domain-containing protein LtLysM1 was identified in *L. theobromae*. Overexpression of *LtLYSM1* in *L. theobromae* resulted in increased pathogenicity on *Vitis vinifera*, indicating that LtLysM1 functions as an important virulence factor. Moreover, the expression profile of gene *LtLYSM1* during infectious stages was detected, and results indicated that the transcriptional level of the *LtLYSM1* gene was significantly increased at 48 hours post inoculation. Additionally, the signal peptide of LtLysM1 could bring about the secretion of yeast invertase. Furthermore, interacting proteins of LtLysM1 were obtained by yeast two hybridization library screening, and we are going to lighten the regulatory mechanisms among LtLysM1 with its interacting targets.

Ecophysiological shifts induced by Esca disease on *Vitis vinifera* L. LORIS OUADI[1], EMILIE BRUEZ[3], CHLOÉ DELMAS[1], SYLVIE BASTIEN[1,2], PASCAL LECOMTE[1], CINDY COPPIN[4], FLORENCE FONTAINE[4], JEAN-CHRISTOPHE DOMEC[2] and PACTRICE REY[1,2]. *[1]INRA, ISVV, UMR1065 Santé et Agroécologie du Vignoble (SAVE), F-33140, Villenave d'Ornon,France. [2]Bordeaux Sciences Agro, INRA UMR1391 Interactions Sol Plante Atmosphère (ISPA), F-33140, Villenave d'Ornon, France. [3]Université de Bordeaux, ISVV, UR Œnologie, F-33140 Villenave d'Ornon, France. [4]SFR Condorcet FR CNRS 3417, Université de Reims Champagne-Ardenne, Résistance Induite et Bioprotection des Plantes, RIBP EA 4707, BP 1039, Reims, Cedex 2 51687, France. E-mail: chloe.delmas@inra.fr*

Esca is a widely reported Grapevine Trunk Diseases (GTDs), caused by a broad range of taxonomically unrelated fungal pathogens that colonize and damage the conductive vascular tissues, thus interfering with the vine physiology and potentially leading to plant death. However, the quantification of the effect of Esca on whole-plant water transport disruption remains unknown. Here a detailed analysis of xylem-related physiological parameters in grapevines that expressed Esca-foliar symptoms was carried out. The experiments were conducted in a vineyard from the Bordeaux region (France) on 16-year-old vines of the cultivar Cabernet-Sauvignon (*Vitis vinifera*), which have been monitored for Esca-foliar symptoms since 2005. For two consecutive years (2017, 2018), a set of healthy and Esca-infected grapevines has undergone detailed physiological measurements. Foliar transpiration, stomatal conductance, chlorophyll activity, and sap flow were regularly recorded to compare the physiological response of plant that remained healthy or developed Esca-foliar symptoms during the timespan of the experiment. To complete the two-year monitoring, healthy and Esca-symptomatic leaves were sampled throughout the season to measure the expression levels of genes involved in primary metabolic functions, using qPCR analysis. This study reveals that sap flow density and whole-plant transpiration of Esca-infected vines is significantly lower, several weeks before the first foliar symptoms appear.

Emerging patterns in the dispersal of Botryosphaeria and Eutypa dieback pathogen spores in New Zealand vineyards. ELINE VAN ZIJLL DE JONG[1], YVONNE WRUCK[1] and MARK R. SOSNOWSKI[2,3]. *[1]Linnaeus Limited, PO Box 1199, Gisborne 4040, New Zealand. [2]South Australian Research and Development Institute, GPO BOX 397, Adelaide SA 5001, Australia. [3]School of Agriculture, Food and Wine, The University of Adelaide, Waite Campus, Glen Osmond SA 5064, Australia. E-mail: mark.sosnowski@sa.gov.au*

Botryosphaeria dieback (BD) and Eutypa dieback (ED) are becoming an increasing problem in New Zealand. The incidence of dieback is rising rapidly according to

recent surveys, with *Eutypa lata* and at least six different Botryosphaeriaceae species present in New Zealand vineyards. Spores of these fungi are spread by water splash and wind and infect vines through pruning wounds leading to canker, dieback and eventual vine death. As part of a major research effort supported by the newly established Bragato Research Institute, to optimise pruning wound protection strategies, two Burkard spore traps have been placed in vineyards located in the major wine producing regions of Marlborough and Hawke's Bay to monitor the dispersal of BD and ED pathogen spores over different seasons and years. Spore tape samples collected daily from the two sites have been analysed by quantitative real-time PCR (qPCR) for the target spores, and compared against daily temperature and rainfall data. Spores of BD and ED pathogens were detected throughout the year in both regions and tended to be detected during and after rain events. Different patterns are emerging between the two regions in the frequency and quantity of spores detected for each of the pathogens during different seasons, which will be presented. Spore monitoring continues and will be expanded to other regions with differing climates, and the data will be used to inform disease management strategies for the New Zealand wine industry.

Characterization of *FmMCA* gene, a unique *metacaspase* from grapevine trunk disease phytopathogen *Fomitiporia mediterranea*. PEIJIE GONG[1], JULIE CHONG[1], SIBYLLE FARINE[1] and CHRISTOPHE BERTSCH[1]. *[1]Laboratoire Vigne Biotechnologies et Environnement, Universite de Haute-Alsace, 33 rue de Herrlisheim, Colmar, 68000, France. E-mail: peijie.gong@uha.fr*

In both plants and pathogens, programmed cell death (PCD) is an indispensable process that removes redundant cells during development, or genetically damaged cells during the development of multicellular organisms, or as ultimate mean to block the spread of pathogens in innate immunity and defense (so called hypersensitive response). Grapevine trunk disease (GTD) symptoms includes bud necrosis, shoot dieback, leaf spots, fruit rots. All these processes are relative with regulated cell death. However, the cause of these symptoms and their relationship with wood necrosis are far away from understood. Therefore, it is worth to consider how to conquer infection of GTD pathogens, for instance *Fomitiporia mediterranea (Fmed)*, using the way of suppressing cell death or necrosis in grapes. In our study, a unique metacapase (MCA) called *Fmca1,* was identified in pathogen *Fmed*. As the unique MCA member in *Fmed*, we deduced *Fmca1* predominantly regulate the execution of cell death in most case of PCD. Multi-sequence Alignment analysis with Fmca1 protein-sequence and other putative MCA genes (from fungi, bacteria, and plant) have been completed via MEGA software. The results showed that the fungi-associated MCA can be classified into same class. Furthermore, we revealed that Fmca1 shares closest relationship with MCA *Eutypa lata* and *Neofusicoccum parvum*. Last but not least, we found putative 3D protein structure of Fmca1 belongs to type I metacaspase, which contains a classical structure: beta strands in centre, surrounded by alpha helixes as outer ring.

Comparative genomics of *Dactylonectria torresensis* strains from grapevine, soil and weeds highlight potential mechanisms in pathogenicity and lifestyle. DAVID GRAMAJE[1], CARMEN BERLANAS[1], EMILIA DÍAZ-LOSADA[2], MARÍA DEL PILAR MARTÍNEZ-DIZ[2,3], LIVIO ANTONIELLI[4], MARKUS GORFER[4] and STÉPHANE COMPANT[4]. *[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]Estación de Viticultura e Enoloxía de Galicia (AGACAL-EVEGA), Ponte San Clodio s/n 32428-Leiro-Ourense, Spain. [3]Universidade da Coruña, Facultade de Ciencias, Zapateira, 15071 A Coruña, Spain. [4]AIT Austrian Institute of Technology GmbH, Center for Health & Bioresources, Bioresources Unit, Konrad-Lorenz Strabe 24, 3430 Tulln, Austria. E-mail: david.gramaje@icvv.es*

The soil-borne fungus *Dactylonectria torresensis* is the most common causal agent of black-foot disease in Europe. However, there is a lack of understanding on how this fungus induce symptoms on plants. In this study, we sequenced and analysed the genomes of three *D. torresensis* isolates collected from asymptomatic vine, weed, and soil. The main objectives of this study were to (i) identify the genomic characteristics of these fungi, (ii) understand the genetic variation among the sequenced species, (iii) identify genes potentially involved in niche specialization within species, (iv) identify fungal adaptations to the endophytic lifestyle, and (v) identify unique and shared genes and pathways related to fungal virulence. Genomes sequenced were further compared to those of 30 fungal species including root and aerial pathogens, white rot degraders, saprotrophs, dark septate endophytes or mycorrhiza. *Dactylo-*

nectria torresensis strains present genomes comprised between 64 and 65 Mbp with up to 18,548 predicted genes for each strain. Average nucleotide identity shows that strains are different according to genome contents. Clusters of orthologous groups were compared, and clusters of genes related to necrosis were detected in all strains (necrosis and ethylene inducing peptides, necrosis inducing proteins) as well as genes related to heavy metal resistance. Interestingly, an expanded high number of genes related to carbohydrate active enzymes were detected in each *Dactylonectria* strain, especially those related to glycoside hydrolases that could be involved in plant tissues penetration or pathogenicity. Genes of *D. torresensis* corresponding to secretome and small secreted proteins were further characterized. Antismash analysis demonstrated the presence of gene clusters such as fujikurin-like genes that have been linked to fungal pathogenicity. These results highlight several gene clusters present in *D. torresensis* strains that could be linked to pathogenicity, plant tissues maceration and degradation, adaptation to heavy metal contaminated soils, and endophytic lifestyle.

Increased levels of C18 fatty acids prior to visible leaf stripe disease symptoms in grapevine colonized by *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora*. ISABEL CORTEZ and PIEBIEP GOUFO. [1]*Centre for the Research and Technology of Agro-Environment and Biological Sciences, Departamento de Agronomia, Universidade de Trás-os-Montes e Alto Douro, Quinta de Prados, 5000-801 Vila Real, Portugal. E-mail: pgoufo@utad.pt*

Lipids play signaling roles in plant resistance against pathogens, modulating basal, effector-triggered, and systemic immunity. Two major mediators of defense signaling pathways are jasmonic and salicylic acids. Jasmonic acid is derived from C18:3n3, while increased levels of 18:1n9, C18:2n6, and 18:3n6 have been associated with enhanced synthesis of salicylic acid. In this study, fatty acids levels were determined in asymptomatic leaves of grapevine plants showing esca symptoms in the woods, leaves or berries, in order to characterize events preceding symptom appearance, and the duration of chemical signals with disease progression. As compared to healthy control leaves, fatty acids accumulated to varying levels in asymptomatic leaves in response to esca. The presence of brown wood-streaking in the trunk was associated with the accumulation of C18:0 and C18:3n6 in asymptomatic leaves, indicating an active communication of signals between woods and leaves. A sharp increase in C17:1n7, C18:1n9t, C18:2n6c, C18:2n6t, C20:4n6, and C22:6n3 levels was observed in asymptomatic leaves of vines already exhibiting necrotic leaf stripes. Asymptomatic leaves of vines with black measles berries also showed esca-induced increases in C18:2n6c and C18:2n6t, which strongly support the involvement of C18 fatty acids in hormonal-mediated signaling in grapevine response to esca. Vines likely utilize alterations in fatty acids levels as a signal for inducing defense gene activation. However, the intensity of the signal is low when fungi are still in the trunk, involving only C18:0 and C18:3n6. With early appearance of foliar symptoms, the signal is amplified with more unsaturated fatty acids acting as transduction mediators. The additional knowledge on defense mechanisms in grapevine–esca interaction gained from this study could lead to ways to exploit host resistance responses to control the disease, in light of the finding that increasing C18 levels via genetic mutations upregulates the expression of resistance genes in several plants.

Population genetic analysis of *Dactylonectria torresensis* from grapevine, soil and weeds in northern Spain. CARMEN BERLANAS[1], LUIS ALBERTO CEDEÑO-MACIAS[2], MARCOS ANDRÉS-SODUPE[1], BEATRIZ LÓPEZ-MANZANARES[1] and DAVID 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]*Universidad de La Rioja, Avda. de la Paz, 93, 26006 Logroño, La Rioja. E-mail: carmen.berlanas@icvv.es*

Dactylonectria torresensis is the most common fungal species associated with black-foot disease of grapevine in Europe. The aim of this study was to analyze the genetic diversity among *D. torresensis* isolates obtained from asymptomatic grapevines, soil and weeds in two field nurseries located in Navarra (Spain) and in a young vineyard located in La Rioja (Spain). A total of 213 isolates collected from grapevine (n=199), weeds (n=4) and soil (n=10) were genotyped for single nucleotide polymorphisms (SNPs) detected by sequencing a partial region of the histone H3 (*his*3) gene. Based on the phylogenetic analysis, 12 haplotypes were found in the *D. torresensis* population. The most abundant haplotype included 141 isolates. The phylogenetic tree formed two clades, one of them containing 8 out of the 12 haplotypes. All isolates obtained from plants, soil and weeds were closely related. In addition, there were no significant differences in the haplotype distribution of

plots within Navarra region. Movement of haplotypes between both regions may have occurred repeatedly, given the low level of geographic genetic structure and the frequent retrieval of the same haplotype in distant locations. Sexual reproduction events and recombination should not be excluded due to the high genotypic diversity found within *D. torresensis* population. Our results also confirm the hypothesis that *D. torresensis* has an endophytic lifestyle on grapevine and on alternative hosts such as weeds.

The holobiont, a biological lever to manage some declines of grapevine. SOPHIE TROUVELOT[1], FLORENCE FONTAINE[2], HELOISE MAHE[3], PAULINE BETTENFELD[1,2], EVERT VAN SCHAIK[1], MARIELLE ADRIAN[1], FRANCOIS DAL[4], OLIVIER FERNANDEZ[2], CLAIRE GROSJEAN[5], PHILIPPE LARIGNON[6], GREGORY MOUILLE[7], FABRICE MARTIN-LAURENT[1], OLIVIER ZEKRI[8], SAMUEL MONDY[1], DANIEL WIPF[1] and PIERRE-EMMANUEL COURTY[1]. *[1]Agroécologie, AgroSup Dijon, CNRS, Univ. Bourgogne, INRA, Univ. Bourgogne Franche-Comté, Dijon, France. [2] SFR Condorcet CNRS 3417, Université de Reims Champagne-Ardenne, Unité Résistance Induite et Bioprotection des Plantes, Reims, France. [3] CNIV, 12 rue Saint-Anne, Paris, France. [4] SICAVAC Laboratoire oenologique, Sancerre, France. [5] Chambre Régionale d'Agriculture de Bourgogne Franche-Comté, Bretenière, France. [6] Institut Français de la Vigne et du Vin, Pôle Rhône-Méditerranée, Rodilhan, France. [7] Institut Jean-Pierre Bourgin, INRA, AgroParisTech, Centre National de la Recherche Scientifique, Université Paris-Saclay, Versailles, France. [8] Pépinières Mercier-Novatech, Le Gué De Velluire, France. E-mail: sophie.trouvelot@u-bourgogne.fr, Pierre-Emmanuel.Courty@inra.fr*

The concept of a multicellular organism to describe complex organisms composed of groups of analogous cells called tissues and organs with specific functions, shows currently some limitations. An organism such as a plant does not live alone, but closely associated with different microbial communities making up its microbiomes. The microbiomes, specific to a tissue, interact with the plant and modulate some of its functions, including physiology and immunity. The host and the associated microbiomes define a holobiont, whose function is influenced by the spatio-temporal dynamics of their interactions. Thus, a dysfunction of the grapevine holobiont (linked for example to inappropriate technical itineraries or to climate change) could lead to the development of diseases or physiological disorders, similarly to the alteration of gut microbiota observed in numerous human diseases as type 2 diabetes, obesity and irritable bowel syndrome. The analysis of microbial communities associated with a plant such as grapevine has until recently been limited by the fact that most microorganisms cannot be cultured *in vitro*. New methods (named -omics) based on the analysis of genome, metabolome and proteome allow to define the taxonomic and functional characteristics of the different microbiomes associated with a plant, in particular organisms (*i.e.* bacteria, fungi) that are not cultivable. In the Holoviti project, we aim to characterize the role of microbiomes in grapevine homeostasis by describing and comparing the holobiont of i) healthy grapevines and ii) grapevines affected by three declines (Esca / BDA, fanleaf and linked to 161-49C rootstock). One of our objectives is to identify taxonomic and functional bio-indicators (linked to either microbes or the plant) of the host sanitary status. One prospect could be handle the microbial component of the holobiont to improve some ecosystemic services, such as the control of pathogens or the fight against abiotic stresses.

Characterization of species of *Lasiodiplodia* associated with grapevines in Mexico. EDELWEISS AIRAM RANGEL-MONTOYA[1], MARCOS PAOLINELLI[2,3], CESAR VALENZUELA-SOLANO[4] and RUFINA HERNANDEZ-MARTINEZ[1]. [1]*Centro de Investigación Científica y de Educación Superior de Ensenada, Baja California (CICESE), Carretera Tijuana-Ensenada 3918, Zona Playitas, 22860, Ensenada, B.C. Mexico.* [2]*Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agropecuaria de Mendoza, San Martin 3853 (5507), Luján de Cuyo, Mendoza, Argentina.* [3]*Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Godoy Cruz 2290 (C1425FQB) CABA, Buenos Aires, Argentina.* [4]*Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Campo Experimental Costa de Ensenada, Av. del puerto 375-23 fraccionamiento playa ensenada, Ensenada B. C. Mexico. E-mail: erangel@cicese.edu.mx*

Botryosphaeria dieback is one of the most aggressive diseases of grapevine caused by fungi in the Botryosphaeriaceae family. The fungi invade the vascular tissue mainly through pruning wounds, which can lead to perennial cankers, necrotic lesions, decline and eventually death of the vine. Among these fungi, members of the genus *Lasiodiplodia* have been reported as the most virulent. Recent studies have revised the taxonomy and phylogeny of *Lasiodiplodia* and as a result, eight-

een new species have been described worldwide. This study aimed to identify the *Lasiodiplodia* species associated with grapevine in Mexico. Canker samples were obtained from ten vineyards established on the states of Sonora and Baja California and isolations were made on Potato Dextrose Agar (PDA). From colonies showing smoke-gray abundant aerial mycelia, twenty-two isolates with hyaline and pigmented longitudinally striate, dark brown conidia were selected. The phylogenetic tree generated using *EF1-α* and ITS sequences revealed the presence of six different *Lasiodiplodia* species in these vineyards. Eight isolates were identified as *L. exigua,* eight *L. theobromae*; two *L. gilanensis*, two *L. crassispora*, and one isolate for each species of *L. parva* and *L. brasiliensis*. Pathogenicity studies showed *L. parva* VSM16a and *L. gilanensis* MXCS01 isolates to be the most virulent and caused lesions of up to 7 cm in length. *Lasiodiplodia crassispora* Vid5 and VSM1b isolates as well as *L. theobromae* VSM6a isolate caused lesion below 3 cm. Except for *L. theobromae*, all the identified species are new reports for Mexico.

Grapevine cultivar variability in colonization by *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum*. MARÍA DEL PILAR MARTÍNEZ-DIZ[1,2], MATTHEW AYRES[3] and MARK R. SOSNOWSKI[3]. *[1]Estación de Viticultura e Enoloxía de Galicia (AGACAL-EVEGA), Ponte San Clodio s/n 32428-Leiro-Ourense, Spain. [2]Universidade da Coruña, Facultade de Ciencias, Zapateira, 15071 A Coruña, Spain. [3]South Australian Research and Development Institute, GPO Box 397, Adelaide SA 5001. E-mail: pilar.martinez.diz@xunta.gal*

The use of tolerant cultivars is a key element in an Integrated Pest Management program and would be one of the most effective means of controlling grapevine trunk diseases, not only reducing disease losses, but also decreasing the need for chemical control strategies. The aim of this study was to evaluate the extent of colonization by fungi associated with esca disease in a detached cane assay with twenty grapevine cultivars from the South Australian Research and Development Centre germplasm collection. In June 2018, one-year-old canes were harvested and stored at 4°C. Two weeks later, dormant grapevine canes were cut into 10 cm single-node sections and placed into holes in polystyrene boards which were floated on water in plastic tubs in a greenhouse. Wounds on top of twelve canes per cultivar were inoculated with spore solutions (800 spores per wound) of either *Phaeomoniella chlamydospora* or *Phaeoacremonium minimum*. Non-inoculated controls were also included. Canes were harvested seven weeks after establishment and the extent of colonization by the fungi was assessed by isolation onto potato dextrose agar. Both pathogens colonized the xylem vessels of all cultivars but the extent of fungal growth within woody tissue varied significantly. Canes of all cultivars were colonized between 3 and 34 mm (*P. chlamydospora*) and between 9 and 48 mm (*P. minimum*) from the wound site. This variation could suggest different disease progression depending on the cultivar and pathogen tested, indicating possible disease tolerance in some cultivars. Further investigation is required to confirm the results and better understand the way that fungi colonize the tissues and how some cultivars are able to restrict this colonization.

The most informative loci for identifying grapevine trunk disease pathogens. DAVID GRAMAJE[1], ANA CABRAL[2], LIZEL MOSTERT[3], DANIEL P. LAWRENCE[4], FLORENT P. TROUILLLAS[4], JOSÉ RAMÓN ÚRBEZ-TORRES[5] and ARTUR ALVES[6]. *[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]LEAF, Linking Landscape, Environment, Agriculture and Food, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal. [3]Department of Plant Pathology, Stellenbosch University, Private Bag X1, Matieland, 7602. [4]Department of Plant Pathology, University of California, Kearney Agricultural Research and Extension Center, Parlier, CA, 93648, USA. [5]Summerland Research and Development Centre, Agriculture and Agri-Food Canada, 4200 Highway 97, Box 5000. Summerland, BC V0H 1Z0, Canada. [6]Departamento de Biologia, CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal. E-mail: david.gramaje@icvv.es*

Grapevine trunk disease (GTD) fungi are highly diverse and species recognition is largely dependent on multi-locus DNA phylogenetic analyses. The DNA sequences frequently used for this multi-locus approach, include the internal transcribed spacer region (ITS1-5.8S-ITS2) of the ribosomal DNA and fragments of the translation elongation factor 1-α (*TEF1*), β-tubulin (*TUB2*), histone (*HIS3*), calmodulin (*CAL*), and actin (*ACT1*) genes, depending on the taxonomic group under consideration. In this study, we provide information concerning the best loci combinations to better estimate species boundaries in GTD fungi in the families Botryosphaeriaceae, Diaporthace-

ae, Diatrypaceae, Phaeomoniellaceae, Togniniaceae, and Nectriaceae belonging to Ascomycota, and the Hymenochaetales in the Basidiomycota. Accurate identification of these pathogens is an essential first step towards meaningful studies on epidemiology and disease management of GTDs.

Effect of pruning wounds on cones of desiccation on Ugni blanc grapevines in Charentes, France. EMILIE BRUEZ[1], CELINE CHOLET[1], TOMMASO MARTIGNON[3], MASSIMO GIUDICI[3], FLORIAN PRULEAU[4], MATHILDE BOISSEAU[4], PASCAL LECOMTE[2], PATRICE REY[2] and LAURENCE GENY[1]. *[1]EA 4577, Unité de recherche œnologie, INRA, USC 1366 Œnologie, Université de Bordeaux / Institut des Sciences de la Vigne et du Vin, 210 Chemin de Leysotte - CS 50008, F-33882 Villenave d'Ornon, France. [2]UMR 1065 Santé et Agroécologie du Vignoble, Institut National de Recherche Agronomique/ Bordeaux Sciences Agro / Institut des Sciences de la Vigne et du Vin, 71, Avenue Edouard Bourleaux, INRA Domaine de la Grande Ferrade - BP81, 33883 Villenave d'Ornon Cedex, France. [3]Simonit&Sirch, maitres tailleurs de vigne, 1 Rue Porte des Benauges, 33410 Cadillac – Bordeaux, France. [4]Hennessy Jas et Cie, 1 rue Richonne 16100 Cognac, France. E-mail: emilie.bruez@u-bordeaux.fr*

Practical methods such as pruning are used in vineyards to sustain grapevines. The aim of this study was to analyze the effect of different sized pruning wounds to limit the percentage of Esca-diseased vines in Charentes (France) that was 12.7% in 2017. The experiment focused on the closing over of the pruning wound and the appearance of the cone of desiccation in the wood. Two Ugni blanc vineyards of same age showing a low vigor and a high vigor were studied. Two different pruning wounds were made, that were respectful or not of sap flow and leave or not a knot. Four (spring) and 8 (autumn) months after pruning, the form, length and size of the cones of desiccation of five plants were studied. The cones were cut longitudinally and photographed. The images were analyzed using ImageJ software. The results showed different forms and sizes of necrosis in the shoot pruning area, depending on the type of pruning but also depending on the plant vigor. The environmental conditions, such as wind direction, landscape slope, between both vineyards could explain the differences. In conclusion, the type of pruning wound has an effect on the creation of the cone of desiccation and may have an influence the pathogen entry and particularly for Esca pathogens.

Economic impact of grapevine trunk diseases in a mature vineyard of the Protected Designation of Origin "Manchuela", Spain. RAMONA MARÍA MUÑOZ[1], ANDREA CAMPOS[1], MARÍA LUISA LERMA[1], PURIFICACIÓN CASTILLO[1], JOSÉ ANTONIO MONREAL[2] and DAVID GRAMAJE[3]. *[1]Servicio de Diagnóstico y Asistencia Fitosanitaria (SEDAF), Instituto Técnico Agronómico Provincial de Albacete (ITAP), Parque Empresarial Campollano, 2ª Avenida, 61, 02007 Albacete, Spain. [2]Departamento de Producción Vegetal y Tecnología Agraria, Universidad de Castilla La Mancha, Campus Universitario s/n, 02071 Albacete, Spain. [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, 26071 Logroño, Spain. E-mail: david.gramaje@icvv.es*

Grapevine trunk diseases (GTDs) impact grapevine health in all major grape-growing regions of the world, limiting vineyard productivity and longevity. These diseases represent one of the major threats to future economic sustainability of viticulture. In order to evaluate the impact of GTDs, approximately 1,000 vines in a mature vineyard (33 years old) of cv. Bobal, located in the Manchuela wine region, were monitored for external symptom expression including foliar discoloration and dead arms and spurs, at least once a year from 2003 to 2018. Yield and crop losses were estimated according to the number of dead vines in 2017 and 2018. Annual vine mortality rate ranged between 0 (2004) and 0.73% (2010), and the cumulative mortality at the end of the study (2018) reached 5.13%. A slight increase in dead plants was recorded in 2010, coinciding with the conversion of the vineyard from gobelet to bilateral cordons. In 2017 and 2018, the disease was estimated to cause yield losses of at least 470 and 675 kg/ha, respectively. The direct crop loss to grape grower was estimated at 429 and 266 euros/ha for the 2017 and 2018 crop year, respectively. Estimations of replacement cost of dead vines accounted for more than 630 euros/ha. Investigation on the fungal communities inhabiting the necrotic wood tissues led to the identification of *Diplodia seriata* as the most frequent pathogen associated with internal wood symptoms, followed by *Phaeomoniella chlamydospora*. Considering the large investments in vineyard establishment and maintenance, a long lifespan of vines is essential for economic success in vineyards.

***In vitro* screening of *Trichoderma* species isolates for potential bio-control of black foot disease causing pathogens in grapevine nurseries.** WYNAND VAN

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Black foot disease (BFD) of grapevines is a decline and dieback disease caused by a soil-borne pathogen complex including *Ilyonectria*, *Dactylonectria* and *Campylocarpon* species. Apart from hot water treatment of dormant nursery plants, no treatment is available to prevent nursery plants from becoming infected with BFD pathogens after planting. *Trichoderma* species are well known biocontrol agents and have shown good efficacy to prevent grapevine trunk disease infections of pruning wounds. However, the efficacy of *Trichoderma* species against BFD pathogens have not been determined *in vitro*. The following *Trichoderma* species *Trichoderma atroviride*, *T. fertile*, *T. harzianum* and *T. virens* were evaluated *in vitro* against four of the most commonly occurring BFD pathogens, *Ilyonectria liriodendri*, *Dactylonectria macrodidyma*, *Campylocarpon fasciculare* and *Campylocarpon pseudofasciculare*. The effects of volatile organic compounds (VOCs) and diffusible antibiotic compounds (DACs) produced as well as the direct antagonistic effects were determined in Petri dish assays. The percentage growth inhibition was determined for both the volatile and diffusible antibiotic assays. Microscopic observations were made of the interaction zone for the dual plate assay. Higher growth inhibition of the black foot pathogens were obtained with the DACs than the VOCs. For both classes of compounds *D. macrodidyma* were found to be more sensitive (maximum of 100% inhibition by DACs and 66.94% by VOCs). In some cases growth stimulation was observed for isolates of *I. liriodendri*, C. *fasciculare* and C. *pseudofasciculare,* depending on the *Trichoderma* spp. isolate. Several interactions were macroscopically observed between *Trichoderma* spp. and the pathogen isolates, which included overgrowth or partial overgrowth, often associated with sporulation of the *Trichoderma* spp., and to a lesser extent arrested growth or the formation of inhibition zones between *Trichoderma* and the pathogen isolates. In general, *in vitro* efficacy was dependant on *Trichoderma* spp. isolates as well as the black foot species isolates.

Outbreak of young grapevine diseases in new table grape varieties in Chile. JAIME AUGER [1], CLAUDIO OSORIO-NAVARRO[1], LAURA POZO[1] and MARCELA ESTERIO[1]. *[1]Depto. de Sanidad vegetal, Fac. de Cs. Agronómicas, Universidad de Chile. Codigo postal 8820808, Santiago-Chile. E-mail: jauger@uchile.cl*

Chile is the world's largest table grape exporter, with North America accounting for 47% of total exports in 2016–2017. Peruvian table grape expansion, which has some strategic advantages to the early growing Chilean regions, and development of late harvested varieties in California, have impacted the table grape Chilean market, causing a decline in demand. Growers are now renewing their vineyards with new varieties which hold good shipping conditions plus better size, color and flavor profiles. New vineyards were established with effective and good farming practices that avoid biological and mechanical stress. However, in the last two growing seasons grapevine decline has caused serious economic losses to the renewed vineyards with novel varieties. The symptoms include reduced vigor, xylem vessels plugging, significant delay in bud break and trunk dieback. Decline symptoms were particularly significant in some varieties with almost 30% of plants lost. In symptomatic plants *Phaeoacremonium* sp. was detected using specific primers Pm1 and Pm2. In order to ensure detection, a restriction-based strategy was developped using EcoR1 enzyme, which present a conserved and unique restriction site in the DNA fragment Pm1/Pm2 in all pathogenic *Phaeoacremonium* strains reported to date. *Phaeoacremonium minimum, Phaeoacremonium mortoniae* and *Phaeoacremonium parasiticum* were identified. Contrasting symptoms severity suggest different defense responses, therefore we focus in studying expression of central genes involved in pathogen response (*PR* genes, flavonoid biosynthesis and hormone response). Understanding the etiology and epidemiology of the grapevines trunk diseases is a big concern for growers because of the high susceptibility of the currently planted new table grapes varieties. This problem became worse considering the absence of adequate sanitary tests, management of the propagation plant material in the Chilean nursery and the inefficiency of the vineyard control treatments.

Root colonization and host defence activation of grapevine rootstock by the biological control agent *Trichoderma atroviride*. ELODIE STEMPIEN[1], ROMAIN PIERRON[1], ILKA ADDENDORF[1], FRANCOIS HALLEEN[1,2] and LIZEL MOSTERT[1]. *[1]Department of Plant Pathology, University of Stellenbosch, Private Bag X1, Matieland, 7602, South Africa; [2]Plant Protection Division, ARC Infruitec-Nietvoorbij, Private Bag X5026, Stellenbosch, 7599, South Africa. E-mail: lmost@sun.ac.za*

Biocontrol agents (BCA) such as *Trichoderma atroviride* hold the potential to control soil borne diseases and promote plant growth. Very little is known about the colonization pattern of *T. atroviride* of grapevine roots and the activation of Induced Systemic Resistance *in planta*. Therefore, a laboratory model was developed to measure the colonization of grapevine roots and its impact on host resistance activation. Cuttings of the rootstock Richter 110 (*Vitis berlandieri* x *Vitis rupestris*), US 8-7 [Jacquez (*V. aestivalis* × *V. cinerea* × *V. vinifera)* × Richter 99] Ramsey (*V. champinii*), and Paulsen 1103 (V. berlandieri × *V. rupestris*) were made from one-year-old dormant canes and rooted in a mist bed. When sufficient roots and shoots developed, the plants were potted in perlite. One week after potting, plants with six fully developed leaves were drenched with a spore suspension (10^8 spores per plant) of *T. atroviride* strain T-77. Three weeks post inoculation, *T. atroviride* were re-isolated from root and crown sections. *Trichoderma atroviride* was successfully re-isolated from of all the treated rootstock plants. The colonization is significantly higher in the roots (60 to 80%) than in the bases (15 to 40%), however, not significantly different between the four cultivars. The induction of defence genes were assessed with RT-qPCR in Richter 110 and US 8-7 plants three weeks post *T. atroviride* strain T-77 inoculation. Defence gene induction were normalized to the expression of two reference genes, *VvEF1y* and *VvActin*. *VvSTS* and *VvCHIT4C* genes were significantly induced by *T. atroviride* in US 8-7, but not in Richter 110. Furthermore, *T. atroviride* T-77 was transformed with a fluorescent tdTomato gene to allow visualization of infection structures on roots by confocal laser scanning microscopy using the same model. These results give new insights in the mechanisms of grapevine-Trichoderma interactions to ensure that the potential BCA is well established inside its host plant and that it can potentially be used to prevent pathogen establishment.

Metatranscriptomic approach to characterize the microbiome and identify molecular markers for early detection of "Hoja de malvón" disease in *Vitis vinifera* cv. Malbec. MARCOS PAOLINELLI[1,2], GEORGINA ESCORIAZA[1], CECILIA ALICIA CESARI[1], RUFINA HERNÁNDEZ-MARTÍNEZ[3], SEBASTIÁN GÓMEZ-TALQUENCA[1] and SANDRA GARCÍA-LAMPASONA[1]. [1]*Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agropecuaria de Mendoza, San Martin 3853 (5507), Luján de Cuyo, Mendoza, Argentina.* [2]*Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Godoy Cruz 2290 (C1425FQB) CABA, Buenos Aires, Argentina.* [3]*Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE), Carretera Ensenada - Tijuana No. 3918, Zona Playitas, 22860, Ensenada, B.C., México. E-mail: paolinelli.marcos@inta.gob.ar*

Argentinian viticulture is markedly affected by the grapevine trunk disease "Hoja de malvón." Similar to Esca disease, *Phaeoacremonium* spp., *Phaeomoniella chlamydospora* and Botryosphaeriaceae spp. are the pioneer fungi, but in a secondary stage of the disease, the Basidiomycota *Arambarria* sp. (formerly, *Inocutis jamaicensis*) was included. Currently, detection of the pathogen is done through culture-dependent techniques since molecular culture-independent methods are poorly developed. The objective of this work was to design a metatranscriptomic approach for the simultaneous exploration of the microbiome and the host gene expression, to identify molecular markers that help with the early detection and the monitoring of the disease progression in vineyards. Twenty-three year-old own-rooted *Vitis vinifera* cv. Malbec plants were selected to obtain a pool of wood chips from the trunk and arms from either symptomatic or asymptomatic plants. Total RNA was extracted and processed for RNAseq using Illumina HiSeq4000. The metatranscriptome renders an average of 37 million 100 bp PE reads per sample. Around 81% of reads were mapped on the genome of *V. vinifera* cv. *Pinot Noir* and used for gene expression analysis. The remaining unmapped reads were used for microbiome characterization through two bioinformatics strategies: 1) rRNA reads assembly and taxonomic assignment using SILVASSU and RDP_ITS database 2) Taxonomic assignment and relative quantification based on uniquely kmer mapping of reads on microbial genome database. Culture-based and culture-independent methods coincide in the detection of the most abundant species (i.e., *Alternaria* sp.), but culture-independent and kmer-based technique detect a vast diversity of microorganisms never identified before using culture-based strategies. Based on the differential abundance of disease-associated pathogens, host functional enrichment on differential expressed genes was evaluated. Results indicate that polyamines genes are candidates for molecular markers of the disease.

Effect of a formulation containing biocontrol agents against *Diplodia seriata*, on the development of Botryosphaeria dieback in vine canes. JAVIERA MOLINA[1], TAMARA NÚÑEZ[1], KARINA VALDÉS[2], KATHERINA AGUIRRE[1], LUZ MARÍA PÉREZ[1] and JAIME R. MON-

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Diplodia seriata is one of the fungal pathogens associated with Botryosphaeria dieback in *Vitis vinifera*. Chemical fungicides have been used for its control, which can impact the environment along with human and animal health. Thus, it is important to find alternatives to these chemicals such as the use of biocontrol agents. The mixture of two Ascomycota fungi named Trizian1 and Closea1 (MBTC®), has proved its ability to control *D. seriata* under laboratory, greenhouse and field conditions. MBTC is the active ingredient of a new bioformulation (Formulation-MBTC), which must be tested for the biocontrol of *D. seriata*, both *in vitro* and *in vivo*, and compared to the biocontrol effect of MBTC. In vitro assays used dual cultures experiments, while *in vivo* assays used wounded vine canes (simulating pruning wounds) inoculated with *D. seriata*, in the presence and absence of Formulation-MBTC. The biocontrol effect of Formulation-MBTC was compared with that of MBTC. Controls were set up using the excipients of the formulation, methylthiofanate (MTF) and a commercial biofungicide containing *Trichoderma* sp. Results showed there were no differences between Formulation-MBTC and MBTC on the antagonism against *D. seriata* in *in vitro* assays, and that Formulation-MBTC maintained the biocontrol activity of MBTC on *D. seriata*, in *in vivo* assays, being identical to that of MTF. It may be concluded that Formulation-MBTC may be used for the control of *D. seriata*.

Shifts in fungal community composition during the propagation process in grapevine nurseries inferred by high-throughput amplicon sequencing. DAVID GRAMAJE[1], REBECA BUJANDA[1], ALES EICHMEIER[2], MILAN SPETIK[2], JESSICA VALLANCE[3] and PATRICE REY[3]. [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, 26071 Logroño, Spain.* [2]*Mendel University in Brno, Faculty of Horticulture, Mendeleum - Institute of Genetics, Valticka 334, 69144, Lednice, Czech Republic.* [3]*INRA, ISVV, UMR1065 SAVE, F-33140, Villenave d'Ornon, France INRA, ISVV, UMR1065 SAVE, F-33140, Villenave d'Ornon, France. E-mail: david.gramaje@icvv.es*

The objective of this study was to investigate the changes in the composition of fungal communities at different stages of the grapevine propagation process by high-throughput amplicon sequencing (HTAS). A non-destructive method was used to collect grapevine wood from the xylem vessels of rootstocks 110 Richter and 41 Berlandieri at four stages of the propagation process: cuttings before cold storage (1), after hydration (2), grafted plants after callusing (3) and prior to dispatch (4). Grapevine wood was collected from the base, middle and apical sections of the rootstocks. DNA from the three plant parts was pooled and fungal communities from these samples were analysed by HTAS of the DNA-ITS2 region. Alpha diversity of fungal communities in the xylem vessels did not differ significantly between genotypes. Temporal shifts in fungal microbiome composition were observed when studying the genotypes separately, with fungal communities identified after hydration more diverse than in the other nursery stages. Several fungal genera associated with grapevine trunk diseases (GTDs) were identified (i.e, *Cadophora*, *Phaeoacremonium*, *Phaeomoniella*), with their populations generally increasing towards the last stages of the propagation process. This study provides new insights into the ecology of fungal communities in grapevine nurseries, and opens up new perspectives in the study of grapevine-GTDs interactions.

Pruning wound colonization of *Trichoderma* native strains from Castilla y León (Spain) in semi-field conditions in winter. GUZMÁN CARRO-HUERGA[1], SARA MAYO-PRIETO[1], ÁLVARO RODRÍGUEZ-GONZÁLEZ[1], ÓSCAR GONZÁLEZ LÓPEZ[1], JAVIER SAIZ-GADEA[1], SAMUEL ÁLVAREZ-GARCÍA[1], SANTIAGO GUTIÉRREZ[2] and PEDRO ANTONIO 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, España.* [2]*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, España. E-mail: gcarh@unileon.es*

Trichoderma spp. are a well-known Biological Control Agents (BCA) against Grapevine Trunk Diseases (GTDs). However, nowadays we are far from controlling efficiently these pathogens, so it is necessary to evaluate the influence of weather conditions on *Trichoderma* colonization. The aim of this experiment was to assess if *Trichoderma*

can persist during winter in order to protect the plants in the most critical period of infection, twelve weeks after prunning. So that, we want to avoid the primary point of entry, annual pruning wounds, after having sprayed vines with *Trichoderma* native strains. Two isolates from vineyards of cv. Tempranillo in Castilla y León were tested; T071 isolated from soil and T154 isolated from plant. One-year-old dormant grapevine grafted plants of Tempranillo/110 Ritcher combination were used. A solution of $2x10^6$ *Trichoderma* spores of T071 and T154 were applied within 24 hours after pruning in December. Vine plants were harvested (eight replicates per treatment) twelve weeks after *Trichoderma* native strains application and cut into 3-cm-long pieces up to 15 cm. Bark was removed and different segments were surface sterilized to isolate *Trichoderma* isolates, 7 chips per plate and three plates per each segment were cultured on Rose Bengal Chloramphenicol Agar (RBCA) and incubated during 7 days at 23°C. After twelve weeks T071 showed 70.31% and T154 showed 89.58% of recovery at 0 cm (surface). Significant differences were shown between treatments (P=0,01). A low percentage of recovery was obtained at 3 cm and none at 6 cm, 9 cm, 12 cm and 15 cm. In control treatments no *Trichoderma* strains were re-isolated. This study shows that good results can be obtained using native *Trichoderma* strains isolated from the same location and type of tissue in order to improve colonization and demonstrates that both strains has colonized pruning wounds.

Development of rapid molecular assays for the detection of grapevine canker pathogens from woody plant tissue. XUEWEN FENG , SVETLANA DUBROVSKY and ANNA LIISA FABRITIUS. *AL&L Crop Solutions Inc., Vacaville, CA, 95688, Untied States. E-mail: info@allcropsolutions.com*

Grapevine trunk diseases caused by Botryosphaeria spp., *Eutypa* and other closely related species, Petri disease (young vine decline or young esca) caused by *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum,* and black foot disease caused by *Cylindrocarpon* spp. are the four leading disease problems in grape production regions worldwide. Symptoms include cankers, dieback, slow decline, and vine death, all of which increase production costs and yield losses. While several groups of fungi cause trunk diseases in older vineyards (>10 years), Petri disease, black foot disease-associated pathogens and Botryosphaeriaceae spp. also affect newly planted vineyards. Early and accurate diagnosis of the grapevine trunk diseases plays an important role in facilitating the growers to take proactive cultural and chemical measures for disease management. Microbiological plating is one of the major approaches for plant disease diagnosis, and usually requires the pieces of infected plant tissue to be placed on various nutrient media. This study compared the sensitivity of SYBR- Green Real-time PCR (qPCR) tests in various combinations of DNA extraction and microbiological plating methods. Woody tissue chips were sampled from the graft union of seven symptomatic grapevines and were subject to DNA extraction right after sampling, 5 days after microbiological plating on microbiological media, and 10 days after microbiological plating on microbiological media respectively. SYBR- Green qPCR tests were then conducted for the detection of Botryosphaeriaceae spp., *Eutypa* spp., *P. chlamydospora*, *P. minimum,* and *Cylindrocarpon* spp. The results indicate that microbiological plating before DNA extraction significantly increases qPCR sensitivity for the detection of trunk disease and Petri disease pathogens.

Evaluating techniques to induce water-shoot production following remedial surgery to control grapevine trunk diseases. MARK R. SOSNOWSKI[1,2] and MATTHEW R. AYRES[1]. *[1]South Australian Research and Development Institute, GPO Box 397, Adelaide SA 5001, Australia. [2]The University of Adelaide, Waite Campus, Glen Osmond, SA, Australia. E-mail: mark.sosnowski@sa.gov.au*

Remedial surgery (also known as vine surgery or trunk renewal) is an effective method of controlling the grapevine trunk diseases Eutypa and Botryosphaeria dieback. It involves cutting visibly affected wood from vine cordons and trunks, and retraining a basal shoot (aka watershoot) into a new canopy. However, it has been reported that, for some own-rooted cultivars, shoot production may be limited to as few as 60% of vines, and even fewer in grafted vines. In 2017, a trial was established to evaluate techniques anecdotally reported to improve water-shoot production in an own-rooted 'Cabernet Sauvignon' vineyard planted in 1995 in the Clare Valley, South Australia. Vines were assessed with 100% incidence of external symptoms of Eutypa dieback and Botryosphaeria dieback. All vines underwent remedial surgery by cutting trunks with a chainsaw to just above the irrigation wire. Beforehand, cross-sectional cuts starting at the top of the trunk revealed wood symptoms of Eutypa dieback and Botryosphaeria dieback in 87% of vines, with far fewer vines, 28%, with wood symptoms at the base. Treatments for inducing water-shoots were

undertaken in an area on the lower trunk predesignated by painting a white circle. They included hitting with a hammer, making an X-shaped cut with a tomahawk, rubbing bark with a wire brush and application of a hormone used to break dormancy (Dormex®). In September 2017, a spring remedial surgery was undertaken, and treatments applied, as described above. Grafting treatments were conducted in October. Assessment in February 2018 revealed water-shoots were readily produced on 80-92% of treated and untreated control trunks, but rarely (<18%) originated within the painted white circle, suggesting little influence of most treatments. No significant differences were observed between winter and spring remedial cuts, or treatments, other than grafting, which increased reworking success from 85 to 98%. This research shows that, of the treatments tested, only grafting significantly improved water-shoot production following remedial surgery. Trials continue to investigate seasonal variability of water-shoot production and effectiveness of grafting.

High prevalence of *Diplodia seriata* in vineyards cv. Cabernet Sauvignon located in the central valley of Chile. ALEJANDRA LARACH[1], CAROLINA TORRES[1], NATALIA RIQUELME[1], MICHAEL SEEGER[2] and XIMENA BESOAIN[1]. *[1]Escuela de Agronomía, Facultad de Ciencias Agronómicas y de los Alimentos, Pontificia Universidad Católica de Valparaíso, San Francisco s/n La Palma, 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: alejandra.larach.v@mail.pucv.cl*

Chile is currently the fourth largest exporter of wine in the world, with a planted area of more than 140,000 hectares, concentrated mainly in the regions of O'Higgins and Maule. Cabernet Sauvignon is the main variety planted with more than 30% of the total area. Botryosphaeria canker is one of the main grapevine trunk disease (GTD), with *Diplodia seriata* being the most frequently isolated species. Therefore, in Cabernet Sauvignon it is important to know the prevalence of the Botryosphaeriaceae species over the years. The aim of this work is to identify and quantify the pathogens associated with the death of vines in cv. Cabernet Sauvignon vineyards located in the regions of O'Higgins and Maule, and compare this data with records on fungal infections of 8 years earlier. During the fall and spring of 2018, samples were taken from 14 vineyards (the same ones sampled in 2010), obtaining pieces of wood from rotten wood, cankers or necrotic stripes, which were placed on plates with acidified potato dextrose agar media and malt extract agar. Each isolate obtained was identified morphologically and molecularly (ITS and β-tubulin). In 2010 the main species detected over 39 isolates were: *D. seriata* (69.2%), *D. mutila* (12.8%), *Neofusicoccum parvum* (10.3%), *N. australe* (5.1%) and *Spencermartinsia viticola* (2.6%). In 2018, out of 44 isolates (corresponding to 98% recovery), the main species detected were: *D. seriata* (72.7%), *D. mutila* (4.5%), *Diaporthe* sp. (4.5%), *N. parvum* (2.3%) and *Neopestalotiopsis* sp. (2.3%). Thus, *D. seriata* is consistently the main isolated pathogen of cv. Cabernet Sauvignon vineyards with symptoms of GDTs in Chile.

Evaluating spray coverage and fungicide efficacy when using adjuvants for pruning wound protection. MATTHEW AYRES[1] and MARK SOSNOWSKI[1,2]. *[1]South Australian Research and Development Institute, GPO Box 397, Adelaide SA 5001, Australia. [2]The University of Adelaide, Waite Campus, Glen Osmond, SA, Australia. E-mail: mark.sosnowski@sa.gov.au*

Eutypa dieback, which causes significant impacts to vineyards worldwide, can be controlled by applying fungicides to pruning wounds using tractor-driven sprayers, with efficacy correlated to wound coverage. In winter 2017, a vineyard trial was established in McLaren Valley, South Australia to evaluate the effect of using spray adjuvants with fungicide on wound coverage, using fluorescent dye and fungicide efficacy as assessment criteria. A recycle sprayer was used to apply two fungicide treatments; fluazinam (Emblem®) and tebuconazole (Gelseal™) with or without a wetter (trisiloxane ethoxylate; DuWett®) or a sticker (di-1-p-menthene; Flextend®). In order to determine if adjuvants can increase coverage, spray treatments were applied at a low water rate of 200 L/ha to simulate poor coverage. Following treatment, wounds were inoculated with *Eutypa lata* ascospores. To assess wound coverage, fluorescent dye was added to the treatments and water sensitive papers were attached to four positions on a post and six randomly selected positions on cordons and spurs. A selection of treated canes was immediately removed from vines and returned to the laboratory for assessment under UV light. Digital images were captured and analysed using Image J software, revealing little difference between treatments for coverage. Image analysis of water sensitive paper indicated relatively higher wound coverage compared with fluorescent dye. After 12 months, the inoculated canes

were removed from vines and returned to the laboratory for isolation on agar media. There was no difference between treatments for recovery of *E. lata*. This preliminary data suggests there may be no advantage in using adjuvants when applying fungicides to pruning wounds with tractor-driven sprayers for protection against infection by *E. lata*. The use of fluorescent dye in treatments may be a more accurate tool than water sensitive paper for evaluating spray coverage on the pruning wound surface. The trial was repeated in winter 2018 to confirm the results.

Fungal pathogens associated with Grapevine Trunk Diseases from patrimonial vineyards in Chile. DAINA GRINBERGS, JAVIER CHILIAN, MARISOL REYES, JEAN FRANCO CASTRO, MACARENA DEL RÍO and ANDRÉS FRANCE. *Instituto de Investigaciones Agropecuarias. Av. Vicente Méndez 515, Chillán, Chile. E-mail: dgrinbergs@inia.cl*

Grapevine is one of the most important fruit crops in Chile, either for wine or fresh fruit production. There are 141,000 ha for wine production with Cabernet Sauvignon as the main cultivar. Trunk diseases are a major problem in grapevine, reducing the productivity, quality and longevity of vineyards. In Chile, the problem has been studied in commercial cultivars. In recent years, there has been an effort to make historical wines, rescuing old winemaking techniques and vines, like the patrimonial cultivars Carignan, País, Torontel and Moscatel. The objective of this work was to characterize the fungal species causing cankers in these cultivars, which has never been investigated. A survey was conducted from different ancestral vineyards in Cauquenes and Itata valleys. Diseased trunks and cordons showed yellowish-spongy-wood decay sectors with a dark brown margins and cankers, i.e. dark-brown-hard V-shaped-lesions. Wood samples were collected from symptomatic plants of 14 different patrimonial cultivars. Fungi were isolated plating wood sections that were previously disinfected using sodium hypochlorite-0.1%, ethanol-70%, rinsed with sterile-distilled-water and flamed. Wood pieces were plated on 25%-acidified-PDA or water-agar, then incubated at 25°C and darkness until mycelial development, and transferred to PDA to obtain pure cultures. The isolates were identified by their cultural characteristics on PDA and MEA, morphometry of the fungal structures, and by the analyses of nucleotide sequences of ITS1-ITS4 and Ef728-Tef1R. A total of 115 isolates were obtained and Botryosphaeriaceae spp. were the most common fungi isolated (37%), with *Diplodia* (78%) as the most frequent genus in this group, followed by *Neofusicoccum*, *Dothiorella* and *Botryosphaeria*. Among basidiomycete isolates (21%) *Fomitiporia* was the most common genus. The third group in importance was *Phaeoacremonium* spp. (15-isolates, 13%), and *Seimatosporium* spp. (13-isolates, 11.3%). There were less frequent genera like *Phaeomoniella* and *Diaporthe*. Future studies will be developed to assess the pathogenicity and virulence of these fungi.

Evaluating natural alternative products for the control of eutypa dieback in grapevine. MATTHEW AYRES[1] and MARK SOSNOWSKI[1,2]. *[1]South Australian Research and Development Institute, GPO Box 397, Adelaide SA 5001, Australia. [2]School of Agriculture, Food and Wine, The University of Adelaide, Waite Campus, Glen Osmond, SA, Australia. E-mail: mark.sosnowski@sa.gov.au*

Eutypa dieback is a major grapevine trunk disease throughout the world, and the most effective means of control is to prevent infection of pruning wounds. Research has shown that synthetic fungicides are effective, and a number of products are now registered in Australia and New Zealand for this purpose. However, there are limited natural alternative products registered for pruning wound protection against Eutypa dieback. Detached cane assays using single node Shiraz canes were used to evaluate vanillin (at 5 and 10 mg/ml), chitosan (at 12.5 and 25 mg/mL), previously reported with activity against trunk disease pathogens, and the yeast-like fungus *Aureobasidium pullulans* (Botector®), registered for control of botrytis bunch rot. The fungicide tebuconazole (Gelseal™), registered for control of Eutypa dieback, was included as a reference, as well as negative (no treatment, no inoculum) and positive (no treatment, inoculated) controls. All treatments (except negative control) were inoculated with 100 ascospores of *Eutypa lata*, the primary causal agent of eutypa dieback, and the canes grown in tubs of water for 5 weeks prior to their removal to the laboratory for isolation on agar medium. Vanillin had no effect on recovery of *E. lata* compared with the positive control, while chitosan, particularly at the higher concentration, significantly reduced recovery, to a similar level to canes treated with Gelseal. No *E. lata* was recovered from the canes treated with Botector, and all agar plates contained *A. pullulans*. The results of this experiment indicate that chitosan and Botector may have potential as pruning wound protectants against infection by trunk disease pathogens. Ongoing research is evaluating these products against *N. luteum* (Botryosphaeria die-

back) and Botector will be further evaluated in vineyard trials.

Characterization of *Trichoderma* isolates from southern Italy and their potential use as biocontrol agents against grapevine trunk diseases. EUGENIA TOMASELLI[1], JULIE BOULÉ[2], MELANIE WALKER[2], JINXZ POLLARD-FLAMAND[2], STEFANIA POLLASTRO[1] and JOSÉ RAMÓN ÚRBEZ-TORRES[2]. [1]*Universitá degli studi di Bari "Aldo Moro", facoltá di Agraria,* via G. Amendola, 165/A 70126 Bari (BA), Italy. [2] *Agriculture and Agri-Food Canada, Summerland Research and Development Centre, Summerland, British Columbia V0H1Z0, Canada. E-mail: e.tomaselli1@studenti.uniba.it*

Grapevine trunk diseases (GTD) are one of the most economic important diseases on grapevines causing decline, loss of productivity and eventual death of the vine. In the last decade, the interest by the grape and wine industry in developing and implementing sustainable and biological control practices against diseases, including GTD is raising. Accordingly, the objectives of this study were to i) identify and characterize *Trichoderma* isolates from southern Italy by morphological and molecular studies and to ii) determine their potential as biocontrol agents against the GTD canker-causing fungi *Diplodia seriata*, *Neofusicoccum parvum* and *Eutypa lata*. A total of 16 *Trichoderma* isolates from Italy were studied at the Agriculture and Agri-food Canada research centre located in Summerland, British Columbia. Morphological characterization included studies to determine the optimum temperature for micelial growth, which showed to be between 25 and 30°C for all 16 isolates. Multi-locus phylogenetic analyses of the ITS1-5.8S-ITS2, TEF-1α and RPB2 genes revealed six species, including *T. atroviride, T. harzianum, T. koningiopsis, T. longibrachiatum, T. paraviridescens,* and *T. spirale*. The antagonistic capabilities of all *Trichoderma* isolates were screened against *D. seriata*, *N. parvum*, and *E. lata* in vitro via dual culture assay. Results revealed all 16 isolates to provide more than 50% mycelium growth inhibition for *D. seriata*, 14 isolates for *N. parvum* and 6 isolates for *E. lata*. The best performing isolates acroos all three species were selected to further screen in plant their potential as BCA via a detached cane assay (DCA) under controlled growth chamber conditions. Chardonnay dormant canes were pruned and inoculated with 50 µL of a 10^6 spores/mL of each *Trichoderma* sp. Then, a 50 µL of a 10^5 conidia/mL of *D. seriata* and *N. parvum* and an approximate 10^5 mycelial pieces/mL of *E. lata* were used to challenge the treated pruning wounds 24h, 7d and 21d after treatment. DCA results will be presented and discussed. This study aims to provide alternative sustainable control solutions against GTD.

Effect of native and exotic ground cover on grapevine pathogens and disease severity. ANDREW RICHARDS[1], MIRANDA HART[1], JOSÉ RAMÓN ÚRBEZ-TORRES[2], TOM LOWERY[2] and PAT BOWEN[2]. [1]*University of British Columbia Okanagan, 3333 University Way, Kelowna, BC V1V 1V7, Canada.* [2]*Summerland Research and Development Centre, Agriculture and Agri-Food Canada, 4200 Highway 97, Box 5000. Summerland, BC VOH 1Z0, Canada. E-mail: andrew.richards@ubc.ca*

Young vine decline (YVD) occurs when grapevines experience stunted growth, reduced yield, delayed fruiting, and root necrosis, often leading to dieback in vineyards worldwide. These symptoms are largely due to soil-borne fungal pathogens including *Ilyonectria* species which may be present in the soil or brought to the area by contaminated nursery plants. Recent restrictions in fungicide use and high density planting regimes have exacerbated the incidence of YVD in vineyards. Public demand for low-impact sustainable agriculture has urged researchers to test alternative approaches to combat soil-borne diseases. Cover crops are plants that are grown alongside main crops or during non-production seasons in order to maintain and enhance soil composition. These plants provide several well-known benefits and may serve as a line of defense against soil-borne fungal pathogens by harbouring suppressive microbes and producing volatile root exudates. We tested whether cover crop diversity and provenance affects the persistence and infectivity of YVD pathogens in agricultural soil. In a greenhouse experiment, we inoculated orchard soil with a 10^6 conidia dilution of *Ilyonectria liriodendri* isolates (PARC60, PARC340, PARC393) prior to seeding with native and commercial cover crop mixtures. We trained soil for 3 and 4 months then replaced cover crops with *Vitis vinifera* cuttings, which were grown for 4 and 5 months. The concentration of *I. liriodendri* decreased significantly in soil samples over 3 months. We isolated the introduced pathogen from 99% of root samples, excluding fallow treatments which did not contain the inoculant. Cover crop diversity and provenance resulted in similar necrotic damage in grapevine cross sections and grapevine physiology traits. Future diversity studies should include incorporation of cover crop residues, span multiple growing seasons and assess whether cover crops are harbouring grapevine trunk disease pathogens in their roots.

Spatio-temporal distribution of Esca disease in commercial vineyards cv. Verdicchio and Chardonnay. JONATHAN CONCAS, SERGIO MUROLO, VALERIA MANCINI, RENZO FOGLIA and GIANFRANCO ROMANAZZI. *Department of Agricultural, Food and Environmental Sciences, Marche Polytechnic University, Via Brecce Bianche, 60131 Ancona, Italy. E-mail: g.romanazzi@univpm.it*

Esca is a multiple fungal syndrome which can cause serious economic damages for grapevine in different viticultural areas. The aim of this study was to monitor the spatial and temporal epidemiology of esca disease incidence in three commercial vineyards (OS1, OS2, CAST) located in Marche region (Italy). During two years (2017–2018) of surveys, a consistent percentage of plants showing "leaf tiger-stripes" (ranging from 10% to 50%) and apoplectic stroke (ranging from 5% to 20%) was recorded. The data collected during the surveys were statistically elaborated by SYSTAT v.13 software, which generated maps of esca disease spread and severity. The presence of inter-row and intra-row disease gradient was determined by a regression curve, which represents the percentage of symptomatic plants in relationship with the distance from the edge of the vineyard. The data processing allowed to verify in 2017 and 2018 that i) none of the vineyards showed a gradient of esca disease among the rows; ii) there was a west-east gradient of disease in the vineyard OS1, and in the vineyard OS2 a greater concentration of disease in the center than the edges was observed. Particularly significant was the correlation recorded between the esca disease incidence and different combination of rootstocks (420A, SO4, 41B, 110R) in vineyard OS1. The obtained result represents a further set of information that can allow a better understanding of the epidemiology of esca.

***Vitis vinifera* resistance induced by *Pythium oligandrum* to control *Neofusicoccum parvum*, a pathogenic fungus involved in grapevine trunk diseases.** AMIRA YACOUB[1,2], RANA HAIDAR[1,2] JONATHAN GERBORE[3], CLEMENTINE MASSON[1,2] MARIE- CECILE DUFOUR [1,2] and PATRICE REY[1,2]. *[1]INRA, UMR 1065 Santé et Agroécologie du Vignoble, ISVV, F-33140 Villenave d'Ornon, France. [2]Université de Bordeaux, Bordeaux Sciences Agro, UMR 1065 Santé et Agroécologie du Vignoble, F-33175 Gradignan, France. [3]Université de Bordeaux, ISVV, Unité de recherche Œnologie EA 4577, USC 1366 INRA, Bordeaux INP, F-33140 Villenave d'Ornon, France. [4]BIOVITIS, 15400 Saint Etienne de Chomeil, France. E-mail: amira.yacoub@inra.fr*

Grapevine trunk diseases (GTDs) are on the rise in vineyards all over the world. Many pathogens are involved in these diseases, including *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum*, *Fomitiporia mediterranea* and *Neofusiccoccum parvum*. Nowadays, few treatments are available to control these diseases. Accordingly, biocontrol of pathogens implicated in GTDs was developed using microorganisms as the oomycete *Pythium oligandrum*, which naturally colonized grapevine roots in the vineyards of several countries worldwide. Recent studies showed that this oomycete induced grapevine resistance and reduced significantly *P. chlamydospora* necrosis). In this study, the ability of *P. oligandrum* to induce grapevine resistance against *N. parvum* was evaluated. Two greenhouse assays showed that the necrosis of Cabernet Sauvignon cuttings caused by *N. parvum* was reduced by 65% when *P. oligandrum* colonized the root system of young vines. The expression levels of a set of 96 genes ("NeoViGen96"chip) involved in various grapevine defense pathways were studied by real-time PCR, at the trunk level. This analysis aims to determine plant responses after inoculation by *P. oligandrum* and/or *N. parvum*, at three different sampling time points (0, 14 and 150 days after *N. parvum* inoculation). Overall, our results showed that the sampling time point has an important effect on studied gene expression levels, whatever the treatment applied. Moreover, at each sampling time point, specific grapevine responses to the different treatments (control, *P. oligandrum*, *N. parvum*, *P. oligandrum* + *N. parvum* treatments) were also differentiated. When *P. oligandrum* colonizes grapevine root systems, infection with the pathogen is associated with a more intense up-regulation of certain genes: PR protein, stilbene synthase and signaling pathway genes were overexpressed. A priming effect of plant defense system is probably induced in presence of *P. oligandrum*.

Isolation and characterisation of phytotoxins produced by the Botryosphaeriaceae and their role in grapevine trunk diseases. PIERLUIGI REVEGLIA[1], REGINA BILLONES-BAAIJENS[1], ALESSIO CIMMINO[2], ANTONIO EVIDENTE[2] and SANDRA SAVOCCHIA[1]. *[1]National Wine and Grape Industry Centre, School of Agricultural and Wine Sciences, Charles Sturt University, Locked Bag 588, Wagga Wagga NSW 2678, Australia. [2]University of Naples Federico II, Department of Chemical Sciences Complesso Universitario Monte Sant'Angelo, Via Cintia 4, 80126, Naples, Italy. E-mail: preveglia@csu.edu.au*

Botryosphaeria dieback (BD) is considered a serious

problem worldwide causing cankers, dieback and eventually death of vines. Foliar symptoms due to BD have been reported in some vineyards overseas. The foliar symptoms caused by grapevine trunk diseases are usually associated with the production of phytotoxic metabolites (PMs) produced by the pathogen that are translocated into the leaves. However, foliar symptoms due to BD have not been reported in Australian vineyards to date. This study investigated the production of PMs by the most virulent and widespread Botryosphaeriaceae species associated with BD in Australia. Isolates of *Diplodia mutila*, *D. seriata*, *Dothiorella vidmadera*, *Neofusicoccum australe*, *N. luteum* and *Spencermartinsia viticola* were grown in liquid media and PMs were extracted from the culture filtrates. The PMs were purified using a chromatographic method while their chemical characterization was performed by spectroscopic method, essentially 1D and 2D NMR. The purified PMs from all species showed different degrees of toxicity when tested on grapevine leaves at different concentrations. *R*-(-)-mellein and other well-known PMs were isolated from *D. mutila*, *D. seriata, N. australe* and *N. luteum* culture filtrates. Two novel toxic quinones (diploquinone A and B) were isolated from the culture filtrates of *D. mutila* while two novel compounds, spencertoxin and spencer acid, were isolated from the culture filtrates of *S. viticola*. Furthermore, six polyphenols were isolated from the culture filtrate of *D. vidmadera*, one being characterized as 5-hydroxymethyl-2-isopropoxyphenol, a novel compound. The isolation and characterization of the PMs secreted by these fungi are fundamental to understanding the potential role of these PMs in the virulence and symptomatology of Botryosphaeria dieback pathogens in grapevines.

Effect of volatile compounds produced by *Trichoderma* spp. against *Phaeoacremonium minimum*. SAMUEL ÁLVAREZ-GARCÍA[1], GUZMÁN CARRO-HUERGA[1], SARA MAYO-PRIETO[1], ÁLVARO RODRÍGUEZ-GONZÁLEZ[1], SANTIAGO GUTIÉRREZ[2] and PEDRO ANTONIO 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, España. [2] 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, Avenida Astorga s/n, 24401 Ponferrada, España. E-mail: salvg@unileon.es*

During the last decades, grapevine trunk diseases (GTDs) have become a major issue for viticulture causing significant reduction on grape production and damage to the plants. Among the fungi associated with GTDs, *Phaeoacremonium minimum* plays a key role in the development of the disease. One approach to face this threat is the use of biological control agents (BCAs) against these pathogens. *Trichoderma* spp. is a widespread genera of fungi used as BCAs in agriculture. One of the control mechanism shown by *Trichoderma* is the release of active volatile compounds. The aim of this study was to evaluate *in vitro* the effect of volatile metabolites produced by three *Trichoderma* strains (T71: *T. gamsii*; T130: *T. gamsii*; T154: *T. harzianum*) against *Phaeoacremonium minimum*. The pathogen was grown for 7 days in the centre of 90mm Petri dishes with PDA prior to the addition of the BCA. A 6mm plug of *Trichoderma* was placed in the centre of PDA Petri dishes and the lids of both pathogen and BCA dishes were removed and placed together along their edge, facing each other, downwards and upwards, respectively. This was considered as day 0, and diameters of the pathogen colonies were measured in day 7, 14 and 21. The results showed a high volatile-mediated antifungal activity of all *Trichoderma* strains evaluated, with inhibition percentages of; T71: 96.87%; T130: 98.44% and T154: 100%, leading an abrupt halt in *P. minimum* growth from day 0 onwards. While controls without *Trichoderma* showed a steady growth from day 0 to day 21. The results demonstrate that *Trichoderma* volatile metabolites can play a significant role in its antagonistic activity against *P. minimum*. More assays are needed to elucidate these volatile-mediated fungal interactions.

Time-course transcriptional analysis of grapevine inoculated with the fungal pathogen *Lasiodiplodia theobromae*. WEI ZHANG[1], JIYE YAN[1], XINGHONG LI[1], QIKAI XING[1], JUNBO PENG[1] and WENSHENG ZHAO[2]. *[1]Beijing Key Laboratory for Environmental Friendly Management on Pests of North China Fruits, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China. [2]State Key Laboratory of Agrobiotechnology and MOA Key Lab of Pest Monitoring and Green Management, China Agricultural University, Beijing 100193, China. E- mail: zhwei1125@163.com*

Grapevine Botryosphaeria dieback, which is caused by Botryosphaeriaceae fungi, threatens the yield and quality of grapes in the past few decades. Chemical control strategies are often observed to be ineffective in controlling the disease worldwide. Increasing our understanding of the molecular resistant mechanisms of grapevine

to pathogens would facilitate the development of pathogen-tolerant grape varieties. Therefore, our study aims are to uncover the defense response of grapevine against to *Lasiodiplodia theobromae*, which is the most aggressive pathogen of grapevine Botryosphaeria dieback in China, and to obtain the potential resistance associated genes in grapevine. Here, RNA sequencing analysis was used to profile the transcriptome of grapevine green shoots infected with *L. theobromae* over a time course of 4, 8 and 12 hours post inoculation. A total of 5181 genes were identified as differentially expressed genes (DEGs), and DEGs were more abundant over time. Further analysis revealed that many of these DEGs are involved in plant-pathogen interactions, hormone signal transduction, and phenylpropanoid biosynthesis pathway, suggesting that innate immunity, phytohormone signaling, and many phenylpropanoid compounds, which constitute a complex defense network in plants, are involved in the response of grapevine against *L. theobromae* infection. This study provides novel insights into the molecular mechanisms of plant–pathogen interactions that will be valuable for the genetic improvement of grapevines.

Antifungal effects of *Bacillus subtilis* and *Pantoea agglomerans* on grapevines cv. Cabernet Sauvignon and Italia against two GTD pathogens, *Neofusicoccum parvum* and *Phaeomoniella chlamydospora*. AWATEF REZGUI[1,2], ASMA BEN GHNAYA CHAKROUN[1], EMILIE BRUEZ[2,3], MAYSSA ARFAOUI[1,2], AMIRA YACOUB[2], MOKHTAR DRIDI[1], NAJLA SADFI ZOUAOUI[1], PATRICE REY[2,3] and JESSICA VALLANCE[2,3]. *[1]Laboratoire Microorganismes et Biomolécules Actives, Faculté des Sciences de Tunis, Université de Tunis El Manar, 2092, Tunisia. [2]INRA, UMR1065 Santé et Agro-écologie du Vignoble (SAVE), ISVV, 33140 Villenave d'Ornon, France. [3]Université de Bordeaux, Bordeaux Sciences Agro, UMR1065 SAVE, 33140 Villenave d'Ornon, France. E-mail: amira.yacoub@inra.fr*

In Tunisia, knowledge about Grapevine trunk diseases (GTDs) symptoms and the associated microflora, is still incomplete despite their ability to cause considerable damage to vineyards. In that context, the fungal microflora inhabiting the wood tissues of Tunisian GTD-foliar symptomatic vines was studied in order to identify the pathogens responsible for wood decay. Microbiological isolations and sequencing of the ITS region allowed us to identify for the first time in Tunisia three fungi previously described in the literature to be associated with GTDs, including *Lasidiodiplodia pseudotheobromae*, *Neofusicoccum parvum* and *Schizophyllum commune*. Their pathogenicity was confirmed *in planta*, and *in vivo* and *in planta* competitions between strains were noticed. The bacterial microflora colonizing the non-necrotic and necrotic wood tissues of Tunisian mature grapevines was also investigated in order to find a suitable Biological Control Agent (BCA) that can be applied under Tunisia conditions. The 19 most abundant cultivable strains, selected based on their morphology, were assigned to *Pantoea*, *Pseudomonas*, *Curtobacterium* and *Bacillus* species based on the 16S rRNA and *rpo*B genes. Microbiological and molecular screenings revealed that those 19 strains had an *in vitro* antagonistic effect against the 3 fungal pathogens previously isolated, with *B. subtilis* B6 being the most promising BCA. Finally, the efficacy of *Bacillus subtilis* B6, isolated from a Tunisian vine (cv. Italia); *Pantoea agglomerans* S5, originated from a French vine (cv. Cabernet Sauvignon); and the combination B6+S5, was investigated in controlling *N. parvum* (Tunisian strain) and *Phaeomoniella chlamydospora* (French strain) in young vines cvs. Italia and Cabernet Sauvignon. Depending on the pathogen and the cv. used, the severity of internal symptoms (necrosis length) varied. In terms of plant protection, the most efficient BCA to reduce *in planta* necrosis caused by the pathogens in both cvs was the combination of the two B6+S5 bacteria.

Construction of a grapevine yeast two-hybrid cDNA library to identify host targets of the *Lasiodiplodia theobromae* effector LtCRE1. QIKAI XING, WEI ZHANG, JUNBO PENG, YANG CAO, LINGXIAN LI, XINGHONG LI and JIYE YAN. *Beijing Key Laboratory of Environment Friendly Management on Fruits Pests in North China, Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China. E-mail: qikaixing@163.com*

Botryosphaeria dieback is a destructive woody plant disease caused by pathogens of the fungal family Botryosphaeriaceae. Effectors are molecules secreted by fungal pathogens that manipulate host physiological and biochemical events, thereby facilitating infection or triggering defense responses. One of the cysteine rich effectors (named CRE1) was identified from *Lasiodiplodia theobromae* based on its strong cell death-suppression activities and was proved to play critical roles in pathogen pathogenicity and host immune response. However, the biological mechanism of LtCRE1 during interaction remains to be determined. In this study, a grapevine yeast two-hybrid cDNA library was constructed to screen putative host proteins interacting with LtCRE1.

Mate & Plate™ yeast two-hybrid library method was used for constructing the grapevine cDNA library from pooled mRNA, which were extracted from plants challenged with *L. theobromae* CSS-01s isolate, and several hormones associated with environmental stress responses. The constructed library consisted of 8.8×10^6 independent clones with an average insert size of 1 kb. *LtCRE1* gene was ligated into the pGBKT7 vector (pGBKT7-LtCRE1) and was considered as the bait after evaluating the expression, auto-activation and toxicity tests in the yeast strain Y2HGold. The yeast two-hybrid screening was performed via co-transforming the bait strain and the cDNA library. Moreover, a total of 57 candidate-interacting proteins were identified. These data suggested that the cDNA library is in high quality and suitable for identifying the interacting proteins of *L. theobromae* effectors.

Esca and training systems in the viticulture region of Charentes, France. CELINE BENETREAU[1], PASCAL LECOMTE[1*], BARKA DIARRA[1], JESSICA VALLANCE[1], MARIE-CECILE DUFOUR[1], CAROLE COUTURE[1], LORIS OUADI[1], EMILIE BRUEZ[2], CHLOE DELMAS[1], MATHILDE BOISSEAU[3], SANDRINE WEINGARTNER[3] and PATRICE REY[1]. *[1]UMR 1065 Santé et Agroécologie du Vignoble, Institut National de Recherche Agronomique / Bordeaux Sciences Agro / Institut des Sciences de la Vigne et du Vin, 71 Ave. Edouard Bourleaux, INRA Domaine de la Grande Ferrade,* CS 20032*, 33882 -Villenave d'Ornon Cedex, France. [2]EA 4577, Unité de recherche œnologie, INRA, USC 1366 Œnologie, Université de Bordeaux / Institut des Sciences de la Vigne et du Vin, 210 Chemin de Leysotte - CS 50008 - 33882 Villenave d'Ornon cedex, France. [3]Hennessy Jas et Cie, 1 rue Richonne, 16100 - Cognac, France. E-mail: pascal.lecomte@inra.fr, chloe.delmas@inra.fr*

The relationship between cultural practices and the development of the Grapevine Trunk Diseases (GTDs), namely Esca, has been the topic of many studies in recent years. To further examine the role of some specific training systems as aggravating factors, a study was carried out in the Charentes region (France). In 2016, 4 pairs of vineyards (cv. Ugni blanc), in which Esca was the predominant GTD, were selected and surveyed. Each pair consisted of vineyards located in similar environments and comparable age, but they varied in their training systems. For each vineyard pair one vineyard was 'espalier cordon', manually or mechanically pruned, and the other was 'espaliers Guyot-arcure', manually pruned. The prevalence of Esca was assessed in winter and summer, based on trunk and leaf visual symptoms. Two pairs of vineyards were selected to collect leaf-asymptomatic grapevines. Vines were cut longitudinally and photographed to assess the inner necrosis surface. Half of vines were used to collect wood samples for identification and quantification of the main pathogens associated with Esca, by microbiology and/or quantitative PCR. Results were in line with those obtained in other previous experiments: vines with a low aerial volume and short cordons and trunks, such as those with Guyot-arcure (or arch-Guyot) training, were generally more affected by Esca (high incidence and necrotic surface) than those with long arms or trunks (espalier cordons). However, Esca pathogens, mostly located in the arms, were equivalent in number in both training systems. On the whole, this study confirmed the aggravating role of training systems that confine grapevines to a small aerial volume. This may encourage growers or "appellations" to change the training systems and distances between each vines.

This study was funded by the industrial chair "Gtdfree" jointly supported by the French National Agency for Research (ANR) and the Jas Hennessy Company.

Spatial dynamics of bacterial community in different organs and wood tissues of grapevines. AWATEF REZGUI[1], GUILHERME MARTINS[2,3], SYLVIE BASTIEN[1,2], AMIRA YACOUB[1], PATRICE REY[1,2], ISABELLE MASNEUF[2,3] and JESSICA VALLANCE[1,2]. *[1]INRA, UMR 1065 Santé et Agroécologie du Vignoble, ISVV, F-33140 Villenave d'Ornon, France. [2]Université de Bordeaux, Bordeaux Sciences Agro, UMR 1065 Santé et Agroécologie du Vignoble, F-33175 Gradignan, France. [3]Université de Bordeaux, ISVV, Unité de recherche Œnologie EA 4577, USC 1366 INRA, Bordeaux INP, F-33140 Villenave d'Ornon, France. E-mail: amira.yacoub@inra.fr*

The spatial dynamics of bacterial communities inhabiting different organs (i.e. leaves, berries, cordons, trunks, barks and roots) and wood tissues (i.e. non necrotic, necrotic and white rot in cordons and trunks) of 15-year-old grapevines cv. Cabernet Sauvignon having expressed or not esca foliar symptoms, were investigated through metagenomic approaches. A quantitative analysis of the different wood tissues found in cordons and trunks using image processing software did not highlight any differences between necrotic wood and white rot in symptomatic and asymptomatic vines. Bacterial cell counts revealed that each sampled organ and tissue was heavily colonized ($1{,}8 \times 10^9$ CFU.g^{-1} on average).

Bacterial diversity per type of vine and organ/tissue (66 samples in total) was then analysed based on tag-encoded 454 pyrosequencing of the 16S rRNA V5-V6 region. No fewer than 80974 quality sequences were obtained, corresponding to 1203 Operational Taxonomic Units (OTUs). OTUs mostly belonged to the Proteobacteria phylum (56%) followed by Actinobacteria (18%) and Bacteroidetes (12%). Alpha diversity analysis revealed that greater diversity was detected in the woody compartments, i.e. cordons, trunk, bark and roots, when compared to the aerial parts, i.e. berries and leaves. Beta diversity analysis demonstrated that differentiation of the structure of the bacterial communities was organ- and tissue-dependent (healthy vs. necrotic), rather than between esca-symptomatic and asymptomatic plants. Three main compartments were defined and differed significantly: (i) aerial parts, i.e. leaves and berries; (ii) wood parts, i.e. cordons, trunks and barks; and (iii) roots.

Bacterial screening for the biological control of grapevine trunk diseases-associated fungi. CARMEN SANJUANA DELGADO-RAMÍREZ[1], EDELWEISS AIRAM RANGEL-MONTOYA[1], EDGARDO SEPULVEDA[1] and RUFINA HERNÁNDEZ-MARTÍNEZ[1]. [1]*Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE), Carretera Ensenada Tijuana No. 3918, Zona Playitas, 22860, Ensenada, B.C., México. E-mail: ruhernan@cicese.mx*

Grapevine trunk diseases (GTD) are presently considered one of the most important menaces for viticulture worldwide. They are caused by different fungal species that mostly infect the vascular system of the vine, affecting vineyard longevity and causing yield losses. Fungi associated with trunk diseases identified in Mexico include *Diplodia corticola, D. seriata, Lasiodiplodia theobromae, Neofusiccocum australe,* and *N. vitifusiforme.* Among them, *L. theobromae* is found to be the most aggressive. This work aimed to invesitage for potential biological control agents suitable for the dry environment prevalent in Baja California. Soil samples were obtained from the rhizosphere of the shrub *Solanum hidsianum*, a plant distributed along the peninsula that is highly resistant to soil desiccation. Bacterial isolates were obtained using different culture media (Luria-Bertani, Yeast-Mannitol, ISP4, Yeast-Peptone, Potato-Dextrose Agar, and Kings B) and then evaluated, using dual plate assays against *L. theobromae*. From a total of 346 bacteria isolates, 49 showed a percentage of inhibition higher than 30%, but only nine inhibit above 60% of the growth of *L. theobromae*. Identification, by 16S rRNA sequencing, indicates that seven isolates belong to the genus *Bacillus* and two to *Streptomyces*, among them *Bacillus* spp., *B. siamensis*, *B. subtilis, B. tequilensis*, *Streptomyces luteoverticillatus,* and *S. youssoufiensis*. Further characterization showed that these strains can solubilize potassium and phosphorus and to produce indoleacetic acid or siderophores, activities that have been previously determined to be involved in plant growth promotion. To conclude, potential biological control agents were identified which might help with the management of GTD in dry environments.

Characterization of rhizosphere microbial communities associated with grapevine rootstocks and their interactions with black-foot disease. CARMEN BERLANAS[1], MÓNICA BERBEGAL[2], GEORGINA ELENA[2], MERIEM LAIDANI[2], JOSÉ FÉLIX CIBRIAIN[3], ANA SAGÜES-SARASA[3] and DAVID 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]*Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain.* [3]*EVENA/INTIA, Departamento de Desarrollo Rural, Medio Ambiente y Administración Local, Gobierno de Navarra C/ Valle de Orba 34, 31390 Olite, Spain. E-mail: carmen.berlanas@icvv.es*

The soil microbiome has a great interest for its potential in improving plant nutrient utilization and suppressing soil-borne diseases. This study aimed to unravel the effects of rootstock genotype on microbial communities in the rhizosphere of grapevine. For this purpose, the rhizosphere of five grapevine rootstocks (110R, 1103P, 140Ru, 41B and 16149C) was sampled at two sampling moments (June and November) in two Spanish vineyards located in Olite, Navarra (7-year-old) and Aldeanueva del Ebro, La Rioja (25-year-old) over two years. The composition of bacterial and fungal communities was examined using high-throughput amplicon sequencing of 16S rDNA gene and ITS region. Quantitative PCR approach was used to measure the relative abundance and dynamic changes of fungal pathogens associated with black-foot disease. Host genetic control of the microbiome was evident in the rhizosphere of the mature vineyard. Microbiome composition also shifted as year of sampling, and fungal diversity varied with sampling moments. Linear discriminant analysis identified specific bacterial (i.e., *Bacillus*) and fungal

(i.e., *Glomus*) taxa associated with grapevine rootstocks. Host genotype did not predict any summary metrics of rhizosphere a and b diversities in the young vineyard. Regarding black-foot associated pathogens, a significant correlation between sequencing and quantitative PCR was observed. Results proved to be significantly influenced by rootstock genotype, year of sampling and vineyard location. The findings of this study provide evidence that rootstock genotype affects the process of microbial recruitment in the rhizosphere of grapevine, and this could be related to the plant age or the habitat (soil type and climate).

***Trichoderma atroviride* SC1 provides long-term protection from nursery to vineyard against fungal grapevine trunk pathogens.** MÓNICA BERBEGAL, ANTONIO RAMÓN-ALBALAT, MAELA LEÓN and JOSEP ARMENGOL. *Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Camino de Vera S/N, 46022 - Valencia, Spain. E-mail: mobermar@etsia.upv.es*

The most important objectives to be covered by a fungal grapevine trunk diseases (GTDs) integrated management program are: i) the improvement of the phytosanitary quality of the vines produced in the nursery and ii) the prevention of infections of pruning wounds in the vineyard from the moment of planting. In this research, nursery and vineyard experiments were set up to evaluate the ability of the biological control agent *Trichoderma atroviride* SC1 to reduce infections of GTDs pathogens in grapevine planting material during the propagation process. Additionally, SC1 was assessed for the long-term protection on grapevine plants in young vineyards during two growing seasons. In the 2015 growing season, cuttings of 110 R rootstock subsequently grafted with Garnacha Tintorera cultivar were treated with a suspension of formulated *T. atroviride* SC1, which was applied during the nursery propagation process. Later, in 2016 and 2017, treatments with *T. atroviride* SC1 were applied to the same plants at planting moment in two different commercial vineyards (2016) and at the first pruning (2017). Plants, treated with water at each of the previously described moments were used as controls. The effect of the treatments was compared by fungal isolation followed by molecular identification of the colonies (2015, 2016 and 2017) and by measuring undried shoot weight (2016 and 2017). Results of this study confirmed that nursery application of *T. atroviride* SC1 reduced GTDs pathogens incidence and severity on grapevine propagation material after the grafting process. Moreover, additional *T. atroviride* SC1 treatments performed during two growing seasons in vineyards planted with these grafted plants also contributed to maintain reduced levels of GTDs infections when compared with untreated plants. *Trichoderma atroviride* SC1 can be used to manage GTDs infections in nurseries, and also to maintain an improved phytosanitary status of the plants when establishing new vineyards.

Bud necrosis associated with *Diplodia seriata* in California vineyards. JOSÉ RAMÓN ÚRBEZ-TORRES[1] and LUIS SÁNCHEZ[2]. *[1]Agriculture and Agri-Food Canada, Summerland Research and Development Centre, Summerland, British Columbia V0H1Z0, Canada. [2]E. & J. Gallo Winery, 600 Yosemite Blvd, Modesto, California 95354, USA. E-mail: joseramon.urbeztorres@canada.ca*

A wide range of different foliar and vascular symptoms are known to be caused by Grapevine trunk diseases (GTD) fungi, including short internodes, cupped and chlorotic leaves, poor vigor, "tiger-leaf stripes", black measles, spur and cordon dieback, wood necrosis and streaking, and perennial cankers. In the past, few reports have also associated bud necrosis with species in the Botryosphaeriaceae family; though not much information is available on this. Primary bud necrosis (PBN) in grapevines is primarily associated with physiological causes, including excess of vigor due to high levels of irrigation and fertilization, shading, winter damage and/or spring frost. However, a different necrosis named inflorescence primordium necrosis (IPN) was observed affecting numerous vineyards in California causing significant production losses. The objective of this study was to determine if causes other than physiological were associated with IPN. In total, 135 buds were collected from 9 different vineyards affected with IPN, including Chardonnay, Pinot noir, Syrah, Cabernet sauvignon, and Merlot. Buds from basal nodes 1 to 3 were collected during mid-dormancy, dissected and the percentage of buds showing IPN recorded. Isolations were conducted from both symptomatic and asymptomatic buds on potato dextrose agar in an attempt to identify a potential biotic cause for IPN. Results showed 68% of the collected buds to have IPN. Isolations and morphological identifications revealed *Alternaria* sp. to be the most prevalent fungus isolated from IPN symptoms (60%), followed by *Diplodia seriata* (13%). Both *Alternaria* sp. and *Diplodia seriata* were not isolated from healthy buds. Identification of *D. seriata* was confirmed by sequencing the ITS1-5.8S-ITS2 gene and the TEF-1α partial gene. This study suggests *Alternaria* sp. and the GTD fungus *D. seriata* to play a role on IPN in California vineyards. Further under-

standing on the cause of IPN will assist to develop and implement effective management strategies against the fungi involved.

Exploit biodiversity in viticultural systems to reduce pest damage and pesticide use, and increase ecosystems services provision: the BIOVINE Project. VITTORIO ROSSI[1], MÓNICA BERBEGAL[2], JOSEP ARMENGOL[2], PATRIK KEHRLI[3], AURORA RANCA[4], SASA SIRCA[5] and DANIEL WIPF[6]. *[1]Department of Sustainable Crop Production, Università Cattolica del Sacro Cuore, via Emilia Parmense 84, 29122 Piacenza, Italy. [2]Instituto Agroforestal Mediterráneo, Universitat Politécnica de Valéncia, Camino de Vera S/N, 46022 Valencia, Spain. [3]Département fédéral de l'économie, de la formation et de la recherche, Agroscope, Domaine stratégique de recherche Protection des végétaux, Route de Duillier 50, CP 1012, 1260 Nyon1, Suisse. [4]Research Station for Viticulture and Enology Murfatlar, Calea Bucuresti, nr. 2, Murfatlar, Romania. [5]Plant Protection Department, Agricultural Institute of Slovenia, Hacquetova ulica 17, 1000 Ljubljana, Slovenia. [6]Agroécologie, AgroSup Dijon, CNRS, INRA, Univ. Bourgogne Franche-Comté, 21000 Dijon, France. Email: mobermar@etsia.upv.es*

Organic vineyards still rely on large external inputs to control harmful organisms (i.e., pests). The BIOVINE project aims to develop natural solutions based on plant diversity to control pests and reduce pesticide dependence. The capability of plants of increasing the ecosystem resistance to pests and invasive species is a well-known ecosystem service. However, monocultures (including vineyards) do not exploit the potential of plant diversity. BIOVINE aims to develop new viticultural systems based on increased plant diversity within (e.g., cover crops) and/or around (e.g., hedges, vegetation spots) vineyards by planting selected plant species for the control of arthropods, soil-borne pests (oomycetes, fungi, nematodes), and foliar pathogens. The Project will pay special attention to fungal grapevine trunk pathogens. Candidate plants will be identified by a literature review, and the selected ones will be tested in controlled environment or small-scale experiments. The ability of the selected plants to: i) attract or repel target arthropod pests; ii) conserve/promote beneficials; iii) control soil-borne pests by means of biofumigation; iv) carry mycorrhizal fungi to the vine root system to increase plant health (growth and resistance); and v) control foliar pathogens by reducing the inoculum spread from soil, will be investigated. New viticultural systems able to exploit plant diversity will then be designed based on results of BIOVINE activities, following a design-assessment-adjustment cycle, which will then be tested by in-vineyard experiments in France, Italy, Romania, Slovenia, Spain and Switzerland for a 2-year period. Innovative viticultural systems should represent an improved way for pest control in organic viticulture, meanwhile they should positively affect functional biodiversity and ecosystem services. New control strategies may provide financial opportunities to vine growers and lower their reliance on pesticides.

Financial support was provided by transnational funding bodies, being partners of the H2020 ERA-net project, CORE Organic Cofund, and the cofund from the European Commission.

Grapevine belowground compartments affect fungal trunk pathogens composition. MARÍA DEL PILAR MARTÍNEZ-DIZ[1,2], MARCOS ANDRÉS-SODUPE[3], REBECA BUJANDA[3], EMILIA DÍAZ-LOSADA[1] and DAVID GRAMAJE[3]. *[1]Estación de Viticultura e Enoloxía de Galicia (AGACAL-EVEGA), Ponte San Clodio s/n 32428-Leiro-Ourense, Spain. [2]Universidade da Coruña, Facultade de Ciencias, Zapateira, 15071 A Coruña, Spain. [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: pilar.martinez.diz@xunta.gal*

Plant compartments of grapevine provide unique niches that drive specific microbiome associations. The microbiota colonizing the rhizosphere and the endophytic compartment (endorhizosphere) contribute to plant growth, productivity, carbon sequestration and phytoremediation. The main objective of this study was to investigate how fungal communities are enriched in different habitats inside and outside of grapevine roots, with special attention to pathogens associated with grapevine trunk diseases (GTDs). For this purpose, the spatial dynamics of the fungal communities associated with three soil-plant compartments (bulk soil, rhizosphere and endorhizosphere) in 5 young vineyards in La Rioja (Spain) were characterized by ITS high-throughput amplicon sequencing (HTAS). The fungal diversity was similar in both bulk soil and rhizosphere compartments at all taxonomic levels. A significant reduction in species richness was found in the endorhizosphere relative to bulk soil and rhizosphere, with distinct community structure especially at lower-order taxonomic analysis.

HTAS analysis showed a significantly higher abundance of fungi belonging to the genera *Cadophora* and *Ilyonectria* at rhizosphere and endorhizosphere compartments as compared with bulk soil. *Phaeoacremonium* spp. were significantly more abundant in the rhizosphere than in the other compartments, with very low fungal abundance in the endorhizosphere. Roots of asymptomatic vines were found to be a microbial niche that is inhabited by GTD fungi, which opens up new perspectives in the study of the endophytic role of these pathogens on grapevines. Results obtained in this study provide helpful information to better understand how grapevine shapes its fungal microbiome and the implications for vineyard management and productivity.

Epidemiology of Diatrypaceae spp. in California vineyards. JOSÉ RAMÓN ÚRBEZ-TORRES[1], CARMEN GISPERT[2] and FLORENT P. TROUILLAS[3]. *[1]Agriculture and Agri-Food Canada, Summerland Research and Development Centre, Summerland, British Columbia V0H1Z0, Canada. [2]University of California Cooperative Extension, Riverside County, Indio, California 92201, USA. [3]Department of Plant Pathology, University of California, Davis and Kearney Agricultural Research and Extension Centre, Parlier, California 93648, USA. E-mail: joseramon.urbeztorres@canada.ca*

The seasonal abundance of Diatrypaceae spp. spores was studied in California vineyards by using Burkard volumetric spore traps and glass microscope slides covered with petroleum jelly placed on grapevine cordons. Burkard spore traps were located from October to April during the 1999-2000 and 2000-2001 seasons in vineyards at Santa Helena (Napa Co.), Healdsburg (Sonoma Co.), and Lodi (San Joaquin Co.). Petroleum jelly slides were placed from September 2006 to May 2009 in the arid grape-growing region of Coachella Valley. Results from these studies showed Diatrypaceae spores to be released after a rain event from October to April at Santa Helena, Healdsburg and Lodi vineyards. However, the largest amount of spores trapped after a rain event were primarily recorded during the months of November and January. Results from Burkard volumetric spore traps showed releasing of Diatrypaceae spores to continue at least for a period of 20 hours after the end of the rain event. Petroleum jelly spore traps showed Diatrypaceae spores to be released from October to March in the vineyard located in Coachella Valley. Interestingly, spore released was detected the first two season with no rain event. Further analyses showed overhead sprinkling irrigation used in this vineyard to correlated with spore released during that period. Epidemiological studies are critical to understand the seasonal spore release of GTD spores, which can be used to target pruning wound protection and thus, improve contrl of these diseases. This work suggests that overhead sprinkling irrigation is sufficient to initiate spore release of Diatrypaceae spp. in vineyards located in arid grape-growing regions where rain event are limited.

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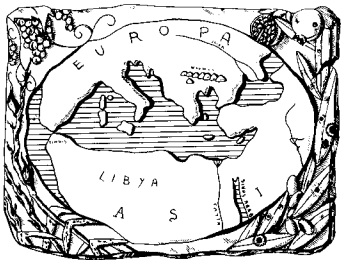

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