Title

Biocontrol Strategies Against Grapevine Trunk Diseases Using Endophytic and Rhizospheric Bacteria and Reassessment of the Etiology of Aspergillus Vine Canker and Sour Rot of Grapes in California

Author

Bustamante, Marcelo Ignacio

Publication Date

2023

Peer reviewed|Thesis/dissertation

Biocontrol Strategies Against Grapevine Trunk Diseases Using Endophytic and Rhizospheric Bacteria and Reassessment of the Etiology of Aspergillus Vine Canker and Sour Rot of Grapes in California

By

MARCELO IGNACIO BUSTAMANTE ALVAREZ

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Plant Pathology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

Akif Eskalen, Chair

Florent P. Trouillas

Ioannis Stergiopoulos

Committee in Charge

2023

Acknowledgements

I would like to thank my major professor Dr. Akif Eskalen for the opportunity to do my Ph.D. in his laboratory and for all these years of support and mentorship. His passion for the discipline of plant pathology and his kindness in sharing his knowledge and resources has made me a better professional and scientist.

I would also like to thank my dissertation committee Drs. Florent Trouillas and Ioannis Stergiopoulos for their valuable feedback in my qualifying examination and in this dissertation.

Special acknowledgments to the different farm advisors of the University of California Cooperative Extension: Mark Battany, Larry J. Bettiga, Carmen Gispert, Glenn McGourty, Rhonda J. Smith, Gabriel A. Torres, Lynn Wunderlich, and George Zhuang for their valuable contacts with grape growers and winemakers.

Special thanks to all the members of the Eskalen Lab that helped with sample collection and processing: Karina Elfar, Karen Alarcon, Molly Arreguin, Rob Blundell, Carlos Carachure, Tyslee Chen, Teagan Haden, Erin Hardy, Masury Lynch, Edeli Reyes, Andrew Richards, Samuel Wells and Kathleen Yu.

Finally, I would like to thank my family and friends for their support and love.

Abstract

Grapevine trunk diseases (GTDs) are caused by multiple unrelated fungal pathogens, and their management is challenging for growers around the world. The current trend of reducing the use of synthetic fungicides makes biocontrol an environmentally friendly strategy to mitigate the impact of GTDs. In this dissertation, a survey that was carried out in twenty vineyards across California revealed that a subset of endophytic and rhizospheric bacterial isolates exerted inhibitory activity *in vitro* against the mycelium of *Neofusicoccum parvum* and *Diplodia seriata* (Chapter II). The bacterial isolates were identified as *Bacillus velezensis* (*n* = 154), *Pseudomonas* spp. (*n* = 12) and *Serratia plymuthica* (*n* = 2). Representative isolates (*n* = 6) of *B. velezensis*, *P. chlororaphis,* and *S. plymuthica* showed consistent levels of mycelial inhibition against eight GTD-causing pathogens (*N. parvum*, *D. seriata*, *Lasiodiplodia theobromae*, *Eutypa lata*, *Diaporthe ampelina*, *Phaeoacremonium minimum*, *Fomitiporia polymorpha* and *Ilyonectria liriodendri*). The agar-diffusible metabolites and volatile organic compounds produced by the bacterial isolates inhibited the growth of *N. parvum* and *E. lata* on a concentration and on bacterial species dependent manner. Representative isolates (*n* = 3) of each bacterial species were further evaluated for their antagonistic efficacy against fungi under field conditions (Chapter III). The isolates were grown in the laboratory and then delivered through four ways: (i) infiltrated in dormant propagation material before grafting in nursery settings; (ii) applied as a soil drench in the vineyard; (iii) injected in the trunk and cordons; and (iv) sprayed onto dormant pruning wounds of mature vines. Results showed that the isolates of *B. velezensis* (UCD10631) and *P. chlororaphis* (UCD10763) exerted a positive effect when infiltrated in propagation material and as soil drench treatments by reducing the lesion length caused by artificially inoculated GTD-causing pathogens. A better performance was observed against *E. lata* and *P. minimum* than against *N. parvum*. On separate part of this work, the etiology of Aspergillus Vine Canker (AVC) and Sour Rot (SR) of grapes was reassessed due to recent changes in the taxonomy *Aspergillus* section *Nigri* (Chapter IV). Morphological and phylogenetic analyses based on nucleotide sequences of the calmodulin (*CaM*) gene allowed the identification of the causal agents of both AVC and SR occurring in California as *A. niger*, *A. carbonarius*, and *A. tubingensis*. The most prevalent species was *A. tubingensis*, associated to both diseases, and six isolates were equally pathogenic in healthy wood and berries of 'Red Globe' grapevines. These results represent the first detection of *A. tubingensis* causing Aspergillus Vine Canker and Sour Rot of grapes in California.

Contents

Chapter I

Introduction

Chapter I: Introduction

Grapevine (*Vitis vinifera* L.) is a popular fruit crop worldwide in terms of cultivated area and volume of harvested fruit due to the high commercial value of wine, raisins, and table grapes. Production of grapes takes place on all the continents, except for Antarctica, in regions with Mediterranean and temperate climates, between latitudes 30° and 50°. In 2021, over 84.79 million tons of grapes were harvested within 7.31 million hectares distributed in 94 countries (FAO, 2023). In the United States, California produces 80% of the wine and 90% of the grapes of the country, with about 3.8 million tons crushed in 2021, valued at US$5,229 million (CDFA, 2022; Alston *et al*. 2018).

Grape cultivation is severely affected by pests and diseases, which require an intensive management program that significantly elevates production costs for growers and winemakers. The crop is susceptible to at least 29 fungal diseases, caused by a broad variety of pathogenic species, that collectively affect all the vine structures (Wilcox et al. 2015). Among them, grapevine trunk diseases (GTDs) are currently considered a major threat to the sustainability of viticulture. GTDs constitute a disease complex composed of different diseases that affect primarily the vascular system and the woody tissues of the grapevine (Gramaje et al. 2018). These diseases are characterized by chronic infections caused by different fungal pathogens that result in a rapid or slow decline of the vines and eventually leading to their death. Consequently, vineyards show progressive reduced yields and shortened lifespans. Symptoms usually include cankers and dieback in cordons and in the trunk, stunted or lack of growth throughout the season, chlorosis and shortened internodes in leaves, root necrosis, and lack of feeder roots, among others. They may appear sequentially or simultaneously, depending on factors associated with the pathogens, the plant health status and the environmental conditions. Approximately 133 fungal species within 34 genera, belonging mainly to the Ascomycota phylum, but also some Basidiomycota species have been associated with GTDs (Gramaje *et al*., 2018). The primary court of infections are wounds in woody tissue (Eskalen and Gubler, 2001; Eskalen et al. 2007; Úrbez-Torres et al. 2009, 2010; Rolshausen et al. 2010). The propagation process in nurseries and the constant pruning in vineyards involve several wounding events, thus increasing the plant susceptibility to infections (Gramaje et al. 2018). Frequently, vines are infected by more than one pathogenic species, potentially leading to more severe symptoms. In addition, some of the pathogens may be going through an endophytic phase, which is harmless to the vine until internal conditions associated

with stress and environmental factors trigger a switch in their behavior into a pathogenic one (Graniti et al. 2000; Czemmel et al. 2015; Hrycan et al. 2020).

Management of GTDs is difficult due to the multifactorial nature of each disease and their pathogens, and the constant wounding events throughout the propagation process and vineyard practices such as training and pruning (Gramaje et al. 2018). Since eradication is not possible, management requires a multidisciplinary approach including sanitation, biocontrol, prevention, and mitigation (Mondello et al. 2018). The current trend of viticulture in reducing the use of synthetic pesticides makes biocontrol an attractive and sustainable strategy to reduce the impact of GTDs (Fourie et al. 2001; Kotze et al. 2011; Yacoub et al. 2016; Pertot et al. 2017). Among the most popular biocontrol agents (BCAs), *Bacillus* and *Trichoderma* species have been extensively studied and tested in laboratory and field settings (Mondello et al. 2018). Among bacteria, different species and strains of *Pseudomonas*, *Serratia*, *Paenibacillus*, *Pantoea*, *Paraburkholderia*, and *Streptomyces* have also been evaluated in laboratory and greenhouse trials (Schmidt et al. 2001; Haidar et al. 2016a; 2016b; Andreolli et al. 2019; Martínez-Diz et al. 2020; Wu et al. 2020; Álvarez-Pérez et al. 2017). In this context, the grapevine microbiome constitutes an important source of biocontrol agents (BCAs) since they play beneficial roles in plant fitness and health (Aziz et al. 2015; Rolli et al. 2017; Deyett et al. 2017). Among these roles, grapevine endophytes have been associated with higher tolerance to disease through different mechanisms, such as competition for nutrients and space, antibiosis, interruption of pathogen signaling, detoxification of pathogen phytotoxins, and plant defense elicitation (Schmidt et al. 2001; Alfonzo et al. 2009; Compant et al. 2013; Haidar et al. 2016; Rezgui et al. 2016; Trotel-Aziz et al. 2019; Niem et al. 2020). Moreover, the application of beneficial rhizospheric bacteria into the soil can improve the health status of the soil and contribute to carbon sequestration (Dries et al. 2021). Therefore, the concept of a "balanced microbiome" has recently gained attention due to the understanding of grapevine microbial communities and their impact in disease expression (Bettenfeld et al. 2020; 2021). It has been demonstrated that vines with a higher abundance of beneficial bacteria display absence or reduced symptoms in vineyards with known history of GTDs (Bekris et al. 2021). On the other hand, the endophytic nature of latent infections caused by trunk pathogens pose an advantage for biocontrol treatments, allowing grapevines to strengthen their tolerance to biotic and abiotic stress before the switch from endophytic to pathogenic behavior (Graniti et al. 2000;

Hrycan et al. 2020). However, the use of BCAs in nurseries and vineyards is still not widely adopted (Compant et al. 2013, Cobos et al. 2022).

The main GTDs correspond to Black foot, Botryosphaeria dieback, Esca, Eutypa dieback, Petri disease and Phomopsis dieback, given their frequency, severity, and distribution worldwide (Bertsch *et al*., 2013). However, a different trunk disease known as Aspergillus Vine Canker (AVC) has sporadically been detected in North America and Europe (Michailides et al. 2002; Vitale et al. 2012; Rangel-Montoya et al. 2022). Described for the first time in the San Joaquin Valley of California in 1989, AVC symptoms include cankers in the woody tissue between the cordons and the trunk of grapevines and a premature senescence of the canopy during the fall (Michailides et al. 2002). The infections appear to begin in phloematic and cambial tissue and progress toward the surrounding healthy wood. In advanced stages, the canker may be associated with girdling of the vascular tissue, thus limiting the flow of water and nutrients between the canopy and the roots. Consequently, growers experience economic losses due to cultural practices such as retraining and replanting of affected vines. Red Globe, Crimson Seedless, Chardonnay, Grenache, and cultivars derived from these seem to be more susceptible to AVC (Michailides et al. 2002). The causal agents of AVC are black aspergilli, a group of *Aspergillus* species that produce dark-colored conidial masses (Michailides et al. 2002). These fungal pathogens are also associated with sour rot, a disease that affects grape berries, especially between veraison and harvest. Sour rot affects clusters that have been injured, leading to a rapid decay associated with different fungal pathogens, acetic acid odor and fruit flies (*Drosophila* spp.). Susceptible berries are rapidly colonized by fungi that produce black, brown, or green sporulation. These infections are followed by juice leakage that attracts fruit flies that carry acetic acid bacteria, yeasts, and other filamentous fungi. As the disease progresses, yeasts metabolize the sugars of berries into ethanol and acetic bacteria oxidize the ethanol to acetic acid, thus emitting a pungent odor, ultimately resulting in berries that turn brown and shrivel (McFadden-Smith and Gubler 2015). In California, black aspergilli have been recognized to be the dominant group in the microbial populations that initiate the disease (Rooney-Latham et al. 2008). Up to date, in California the species associated with both AVC and sour rot have been identified as *A. niger* and *A. carbonarius*, using morphological examinations and molecular analyses with ITS sequences (Michailides et al. 2002; Michailides et al. 2007; Rooney-Latham et al. 2008). Taxonomically, these species belong to the section *Nigri* (Gams et al. 1986; Houbraken et al. 2020). Numerous taxonomic rearrangements

have been implemented in section *Nigri* during the last decade due to a lack of clarity in the species delimitation. These changes have been based on morphological, physiological, and phylogenetic approaches, specifically using more informative DNA barcodes such as the calmodulin (*CaM*) and β-tubulin (*benA*) gene sequences (Hong et al. 2013; Samson et al. 2014; D'hooge et al. 2019; Houbraken et al. 2020). In Italy and Mexico, the species *A. niger*, *A. carbonarius*, *A. tubingensis*, and *A. awamori* have been identified using either *CaM* and/or *benA* (Vitale et al. 2012; Rangel-Montoya et al. 2022). In contrast, multiple *Aspergillus* species have been associated with sour rot worldwide (Hocking et al. 2007; Chiotta et al. 2011; García-Cela et al. 2014; Lim et al. 2019). Some of these species are known to produce mycotoxins, particularly ochratoxin A (OTA), fumonisins, and oxalic acid, which have been detected in wine, grape berries, vineyards, and grape juice in Europe (Bellí et al. 2002; Frisvad et al. 2018). The toxic effects of these compounds on human health are important to consider, which highlights the importance of an accurate detection of the species responsible for their production (Bui-Klimke and Wu 2015). Recently, the taxonomy of section *Nigri* has been rearranged using phylogenetic inferences from whole genome sequences, resulting in the synonymizing of multiple closely related species (Bian et al. 2022). Therefore, a reexamination of the etiology of both AVC and sour rot in California may contribute to a better understanding of the species present and their association with mycotoxin production.

The research objectives of this dissertation were divided into three chapters. The first objective contemplated the identification of endophytic and rhizospheric bacteria obtained from commercial vineyards with presence or absence of GTD symptoms and the evaluation of their potential as BCAs against frequent trunk pathogens occurring in California vineyards *in vitro* (chapter II). Second, the application and evaluation of selected isolates in nursery and vineyard settings following different biocontrol approaches (chapter III). Third, a reexamination of the identity of *Aspergillus* species associated with Aspergillus Vine Canker and Sour Rot occurring in California vineyards (chapter IV). Lastly, the main findings of these studies were discussed, and future perspectives were proposed (chapter V).

Literature cited

Alfonzo, A.; Conigliaro, G.; Torta, L.; Burruano, S.; Moschetti, G. Antagonism of Bacillus subtilis strain AG1 against vine wood fungal pathogens. Phytopathol. Mediterr. 2009, 48, 155–158.

Alston, J.M., Lapsley, J.T., Sambucci, O. 2018. Grape and wine production in California. 28p. Chapter 8. In: Martin, P.L., Goodhue, R.E., Wright, B.D. (eds.). California Agriculture: Dimensions and Issues. 2nd ed. UC Giannini Foundation of Ag. Econ. Retrieved from: https://giannini.ucop.edu/publications/cal-ag-book/

Álvarez-Pérez, J.M.; González-García, S.; Cobos, R.; Olego, M.Á.; Ibañez, A.; Díez-Galán, A.; Garzón-Jimeno, E.; Coque, J.J.R. Use of endophytic and rhizosphere actinobacteria from grapevine plants to reduce nursery fungal graft infections that lead to young grapevine decline. Appl. Environ. Microbiol. 2017, 83, e01564-17.

Andreolli, M.; Zapparoli, G.; Angelini, E.; Lucchetta, G.; Lampis, S.; Vallini, G. Pseudomonas protegens MP12: A plant growth-promoting endophytic bacterium with broad-spectrum antifungal activity against grapevine phytopathogens. Microbiol. Res. 2019, 219, 123–131.

Aziz, A.; Verhagen, B.; Magnin-Robert, M.; Couderchet, M.; Clément, C.; Jeandet, P.; Trotel-Aziz, P. Effectiveness of beneficial bacteria to promote systemic resistance of grapevine to gray mold as related to phytoalexin production in vineyards. Plant Soil 2015, 1, 141–153.

Bellí, N., Marín, S., Sanchis, V., Ramos, A.J. 2002. Ochratoxin A (OTA) in wines, musts and grape juices: occurrence, regulations and methods of analysis. Food Sci Technol Int 8, 325–335. https://doi.org/10.1106/108201302031863

Bertsch, C., Ramírez-Suero, M., Magnin-Robert, M., Larignon, P., Chong, J., Abou-Mansour, E., Spagnolo, A., Clément, C., Fontaine, F. 2013. Grapevone trunk diseases: complex and still poorly understood. Plant Pathology 62:243-265. https://doi.org/10.1111/j.1365-3059.2012.02674.x

Bettenfeld, P.; Fontaine, F.; Trouvelot, S.; Fernandez, O.; Courty, P.-E. 2020. Woody plant declines. What's wrong with the microbiome? Trends Plant Sci. 25:381–394.

Bettenfeld, P.; Cadena i Canals, J.; Jacquens, L.; Fernandez, O.; Fontaine, F.; van Schaik, E.; Courty, P.-E.; Trouvelot, S. 2021. The microbiota of the grapevine holobiont: A key component of plant health. J. Adv. Res. 40:1–15.

Bekris, F.; Vasileiadis, S.; Papadopoulou, E.; Samaras, A.; Testempasis, S.; Gkizi, D.; Tavlaki, G.; Tzima, A.; Paplomatas, E.; Markakis, E.; et al. 2021. Grapevine wood microbiome analysis identifies key fungal pathogens and potential interactions with the bacterial community implicated in grapevine trunk disease appearance. Environ. Microbiome 16:23.

Bian, C., Kusuya, Y., Sklenář, F., D'hooge, E., Yaguchi, T., Ban, S., Visagie, C.M., Houbraken, J., Takahashi, H., Hubka, V. 2022. Reducing the number of accepted species in *Aspergillus* series *Nigri*. Studies in Mycology 102: 95–132. https://doi.org/10.3114/sim.2022.102.03

Bui-Klimke, T.R., Wu, F. 2015. Ochratoxin A and human health risk: a review of the evidence. Crit Rev Food Sci Nutr.55(13):1860-9. https://doi.org/10.1080/10408398.2012.724480

CDFA. 2022. Agricultural Statistics Review 2019–2020. Available online: https://www.cdfa.ca.gov/Statistics/PDFs/2020_Ag_Stats_Review.pdf (accessed on 1 September 2022).

Chiotta, M.L., Susca, A., Stea, G., Mulè, G., Perrone, G., Logrieco, A., Chulze, S.N. 2011. Phylogenetic characterization and ochratoxin A – Fumonisin profile of black *Aspergillus* isolated from grapes in Argentina. International Journal of Food Microbiology 149:171-176. http://dx.doi.org/10.1016/j.ijfoodmicro.2011.06.002

Cobos, R.; Ibáñez, A.; Diez-Galán, A.; Calvo-Pena, C.; Ghoreshizadeh, S.; Coque, J.J.R. The grapevine microbiome to the rescue: Implications for the biocontrol of trunk diseases. Plants 2022, 11, 840.

Compant, S.; Brader, G.; Muzammil, S.; Sessitsch, A.; Lebrihi, A.; Mathieu, F. 2013. Use of beneficial bacteria and their secondary metabolites to control grapevine pathogen diseases. Biocontrol 58:435–455.

Czemmel, S., Galarneau, E.R., Travadon, R., McElrone, A.J., Cramer, G.R., Baumgartner, K. 2015. Genes expressed in grapevine leaves reveal latent wood infection by the fungal

pathogen *Neofusicoccum parvum*. PLoS One 10(3):e0121828. https://doi.org/10.1371/journal.pone.0121828

Deyett, E.; Roper, M.C.; Ruegger, P.; Yang, J.I.; Borneman, J.; Rolshausen, P.E. Microbial landscape of the grapevine endosphere in the context of Pierce's disease. Phytobiomes J. 2017, 1, 138–149.

Dries, L., Hendgen, M., Schnell, S., Löhnertz, O., Vortkamp, A. 2021. Rhizosphere engineering: leading towards a sustainable viticulture? *OENO One* *55*(2):353–363. https://doi.org/10.20870/oeno-one.2021.55.2.4534

Eskalen, A., Gubler W.D. 2001. Association of spores of *Phaeomoniella chlamydospora* and *Phaeoacremonium inflatipes*, and *Pm. aleophilum* with grapevine cordons in California. *Phytopatol. Mediterr.* 40:429-432. http://dx.doi.org/10.14601/Phytopathol_Mediterr-1613

Eskalen, A., Feliciano, A.J., Gubler, W.D. 2007. Susceptibility of grapevine pruning wounds and symptom development in response to infection by *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora*. Plant Disease 91:1100-1104. https://doi.org/10.1094/pdis-91-9-1100

FAOSTAT. Available online: http://www.fao.org/faostat/en/#data/QCL (accessed on 1 September 2022).

Fourie, P.H., Halleen, F., van der Vyver, J., Schrueder, W. 2001. Effect of *Trichoderma* treatments on the occurrence of decline pathogens on the roots and rootstocks of nursery plants. *Phytopatol. Mediterr.* 40S:473-478. http://dx.doi.org/10.14601/Phytopathol_Mediterr-1619

Frisvad, J.C., Møller, L.L.H., Larsen, T.O., Kumar, R., Arnau, J. 2018. Safety of the fungal workhorses of industrial biotechnology: update on the mycotoxin and secondary metabolite potential of *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*. Appl Microbiol Biotechnol 102:9481–9515. https://doi.org/10.1007/s00253-018-9354-1

Gams, W., Christensen, M., Onions, A.H., Pitt, J.I., Samson, R.A. 1986. Infrageneric taxa of *Aspergillus*. In: Samson, R.A., Pitt, J.I. (eds). Advances in *Penicillium* and *Aspergillus* Systematics. Springer, Boston, MA, USA: 55–62.

García-Cela, E., Crespo-Sempere, A., Ramos, A.J., Sanchis, V., Marin, S. 2014. Ecophysiological characterization of *Aspergillus carbonarius*, *Aspergillus tubingensis* and *Aspergillus niger* isolated from grapes in Spanish vineyards. International Journal of Food Microbiology 173: 89-98. https://doi.org/10.1016/j.ijfoodmicro.2013.12.012

Gramaje, D., Úrbez-Torres, J.R., Sosnowski, M.R. 2018. Managing grapevine trunk diseases with respect to etiology and epidemiology: Current strategies and future prospects. *Plant Disease* 102(1):12-39. https://doi.org/10.1094/PDIS-04-17-0512-FE

Graniti, A.; Surico, G.; Mugnai, L. Esca of grapevine: A disease complex or a complex of diseases? Phytopathol. Mediterr. 2000, 39,16–20. [CrossRef]

Haidar, R.; Roudet, J.; Bonnard, O.; Dufour, M.C.; Corio-Costet, M.F.; Fert, M.; Gautier, T.; Deschamps, A.; Fermaud, M. 2016a. Screening and modes of action of antagonistic bacteria to control the fungal pathogen Phaeomoniella chlamydospora involved in grapevine trunk diseases. Microbiol. Res. 192:172–184.

Haidar, R.; Deschamps, A.; Roudet, J.; Calvo-Garrido, C.; Bruez, E.; Rey, P.; Fermaud, M. 2016b. Multi-organ screening of efficient bacterial control agents against two major pathogens of grapevine. Biol. Control 92:55–65.

Hocking, A.D., Leong, S.L., Kazi, B.A., Emmett, R.W., Scott, E.S. 2007. Fungi and mycotoxins in vineyards and grape products. Int. J. Food Microbiol. 119:84-88. https://doi.org/10.1016/j.ijfoodmicro.2007.07.031

Hong, S.-B., Lee, M., Kim, D.H., Varga, J., Frisvad, J.C., Perrone, G., Gomi, K., Yamada, O., Machida, M., Houbraken, J., Samson, R.A. 2013. *Aspergillus luchuensis*, an industrially important black Aspergillus in east Asia. PLoS ONE 8: e63769. https://doi.org/10.1371/journal.pone.0063769

Houbraken, J., Kocsubé, S., Visagie, C.M., Yilmaz, N., Wang, X.-C., Meijer, M., Kraak, B., Hubka, V., Bensch, K., Samson, R.A., Frisvad, J.C. 2020. Classification of *Aspergillus*, *Penicillium*, *Talaromyces* and related genera (Eurotiales): an overview of families, genera, subgenera, sections, series and species. Studies in Mycology 95: 5–169. https://doi.org/10.1016/j.simyco.2020.05.002

Hrycan, J.; Hart, M.; Bowen, P.; Forge, T.; Úrbez-Torres, J.R. Grapevine trunk disease fungi: Their roles as latent pathogens and stress factors that favour disease development and symptom expression. Phytopathol. Mediterr. 2020, 59, 395–424.

Kotze, C.; van Niekerk, J.; Mostert, L.; Halleen, F.; Fourie, P. Evaluation of biocontrol agents for grapevine pruning wound protection against trunk pathogen infection. Phytopathol. Mediterr. 2011, 50, S247–S263.

Lim, Y.-S., Hassan, O., Kim, M.-K., Chang, T. 2019. First report of bunch rot caused by Aspergillus tubingensis of Shine Muscat grape in Korea. Plant Dis. 103:2953. https://doi.org/10.1094/PDIS-05-19-1104-PDN

Martínez-Diz, M.P.; Díaz-Losada, E.; Andrés-Sodupe, M.; Bujanda, R.; Maldonado-González, M.M.; Ojeda, S.; Yaocub, A.; Rey, P.; Gramaje, D. Field evaluation of biocontrol agents against black-foot and Petri diseases of grapevine. Pest Manag. Sci. 2020, 77, 697–708.

McFadden-Smith, W., Gubler, W.G. 2015. Sour rot. In: Compendium of Grape Diseases, Disorders, and Pests. 2nd ed. Wilcox, W.F., Gubler, W.D., Uyemoto, J.K. (eds.). The American Phytopathological Society, St. Paul, MN, USA.

Michailides, T.J., Peacock, W., Christensen, P., Morgan, D.P., Felts, D. 2002. First report of Aspergillus Vine Canker of table grapes caused by *Aspergillus niger*. Plant Dis. 86:75. https://doi.org/10.1094/PDIS.2002.86.1.75A

Michailides, T.J., Peacock, W., Christensen, P., Morgan, D.P. 2007. Aspergillus vine canker of table grapes caused by *Aspergillus* sect. *Nigri* in California. Phytopathol. Mediterr. 46:102-128.

Mondello, V., Songy, A., Battiston, E., Pinto, C., Coppin, C., Trotel-Aziz, P., Clement, C., Mugnai, L., Fontaine, F. 2018. Grapevine trunk diseases: a review of fifteen years of trials for their control with chemicals and biocontrol agents. *Plant Disease* 102:1189-1217. https://doi.org/10.1094/PDIS-08-17-1181-FE

Niem, J.M., Billones-Baaijens, R., Stodart, B., Savocchia, S. 2020. Diversity profiling of grapevine microbial endosphere and antagonistic potential of endophytic *Pseudomonas* against

grapevine trunk diseases. *Front. Microbiol.* 11:477. https://doi.org/10.3389/fmicb.2020.00477

Pertot, I., Caffi, T., Rossi, V., Mugnai, L., Hoffmann, C., Grando, M.S., Gary, C., Lafond, D., Duso, C., Thiery, D., Mazzoni, V., Anfora, G. 2017. A critical review of plant protection tools for reducing pesticide use on grapevine and new perspectives for the implementation of IPM in viticulture. Crop Protection 97 70-84. http://doi.org/10.1016/j.cropro.2016.11.025

Rangel-Montoya, E. A., Valenzuela-Solano, C., Hernández-Martínez, R. 2022. *Aspergillus* spp. causing Aspergillus vine canker on grapevines in Mexico. 12th International Workshop on Grapevine Trunk Diseases. Mikulov, Czech Republic.

Rezgui, A.; Ghnaya-Chakroun, A.B.; Vallance, J.; Bruez, E.; Hajlaoui, M.R.; Sadfi-Zouaoui, N.; Rey, P. Endophytic bacteria with antagonistic traits inhabit the wood tissues of grapevines from Tunisian vineyards. Biol. Control 2016, 99, 28–37.

Rolli, E.; Marasco, R.; Saderi, S.; Corretto, E.; Mapelli, F.; Cheris, A.; Borin, S.; Valenti, L.; Sorlini, C.; Daffonchio, D. Root-associated bacteria promote grapevine growth: From the laboratory to the field. Plant Soil 2017, 410, 369–382.

Rolshausen, P.E., Úrbez-Torres, J.R., Rooney-Latham, S., Eskalen, A., Smith, R.J., Gubler, W.D. 2010. Evaluation of pruning wound susceptibility and protection against fungi associated with grapevine trunk diseases. Am. J. Enol. Vitic. 61:113-119. https://doi.org/10.5344/ajev.2010.61.1.113

Rooney-Latham, S., C. N. Janousek, A. Eskalen, W. D. Gubler. 2008. First report of *Aspergillus carbonarius* causing sour rot of table grapes (*Vitis vinifera*) in California. Plant Dis. 92(4):651. https://doi.org/10.1094/PDIS-92-4-0651A

Schmidt, C.S.; Lorenz, D.; Wolf, G.A. Biological control of the grapevine dieback fungus Eutypa lata I: Screening of bacterial antagonists. J. Phytopathol. 2001, 149, 427–435.

Trotel-Aziz, P., Abou-Mansour, E., Courteaux, B., Rabenoelina, F., Clément, C., Fontaine, F., Aziz, A. 2019. *Bacillus subtilis* PTA-271 counteracts Botryosphaeria dieback in grapevine,

triggering immune responses and detoxification of fungal phytotoxins. Front. Plant Sci. 10:25. https://doi.org/10.3389/fpls.2019.00025

Úrbez-Torres, J.R., Gubler, W.D. 2009. Pathogenicity of Botryosphaeriaceae species isolated from grapevine cancers in California. Plant Disease 90(12):1490-1503. https://doi.org/10.1094/PDIS-93-6-0584

Úrbez-Torres, J.R., Gubler, W.D. 2010. Susceptibility of grapevine pruning wounds to infection by *Lasiodiplodia theobromae* and *Neofusicoccum parvum*. Plant Pathology 60(2) 261-270. https://doi.org/10.1111/j.1365-3059.2010.02381.x

Vitale, A., G. Cirvilleri, A. Panebianco, F. Epifani, G. Perreno, G. Polizzi. 2012. Molecular characterisation and pathogenicity of *Aspergillus* sect. *Nigri* causing Aspergillus vine canker of table grapes in Italy. Eur. J. Plant Pathol. 132:483–487. https://doi.org/10.1007/s10658-011-9906-z

Wilcox, W.F., Gubler, W.D., Uyemoto, J.K. 2015. Compendium of Grape Diseases, Disorders, and Pests. Second Edition. APS Press, St. Paul, MN, USA.

Wu, H.; Spagnolo, A.; Marivingt-Mounir, C.; Clément, C.; Fontaine, F.; Chollet, J.-F. Evaluating the combined effect of a systemic phenylpyrrole fungicide and the plant growth-promoting rhizobacteria *Paraburkholderia phytofirmans* (strain PsJN::gfp2x) against the grapevine trunk pathogen Neofusicoccum parvum. Pest Manag. Sci. 2020, 76, 3838–3848.

Yacoub, A., Gerbore, J., Magnin, N., Chambon, P., Dufour, M.C., Corio-Costet, M.F., Guyoneaud, R., Rey, P. 2016. Ability of *Pythium oligandrum* strains to protect *Vitis vinifera* L., by inducing plant resistance against *Phaeomoniella chlamydospora*, a pathogen involved in Esca, a grapevine trunk disease. Biol. Control 92:7-16. https://doi.org/10.1016/j.biocontrol.2015.08.005

Chapter II

Evaluation of the Antifungal Activity of Endophytic and Rhizospheric Bacteria against Grapevine Trunk Pathogens

Marcelo I. Bustamante, Karina Elfar and Akif Eskalen

Department of Plant Pathology, University of California, Davis, CA 95616, USA

This chapter has been published in the journal Microorganisms and is reproduced here. The article is available online: https://doi.org/10.3390/microorganisms10102035.

Abstract

Grapevine trunk diseases (GTDs) are caused by multiple unrelated fungal pathogens, and their management remains difficult worldwide. Biocontrol is an attractive and sustainable strategy given the current need for a cleaner viticulture. In this study, twenty commercial vineyards were sampled across California to isolate endophytic and rhizospheric bacteria from different grapevine cultivars with the presence and absence of GTD symptoms. A collection of 1344 bacterial isolates were challenged in vitro against *Neofusicoccum parvum* and *Diplodia seriata*, from which a subset of 172 isolates exerted inhibition levels of mycelial growth over 40%. Bacterial isolates were identified as *Bacillus velezensis* (*n* = 154), *Pseudomonas* spp. (*n* = 12), *Serratia plymuthica* (*n* = 2) and others that were later excluded (*n* = 4). Representative isolates of *B. velezensis*, *P. chlororaphis*, and *S. plymuthica* were challenged against six other fungal pathogens responsible for GTDs. Mycelial inhibition levels were consistent across bacterial species, being slightly higher against slow-growing fungi than against Botryosphaeriaceae. Moreover, agar-diffusible metabolites of *B. velezensis* strongly inhibited the growth of *N. parvum* and *Eutypa lata*, at 1, 15, and 30% *v/v*. The agar-diffusible metabolites of *P. chlororaphis* and *S. plymuthica*, however, caused lower inhibition levels against both pathogens, but their volatile organic compounds showed antifungal activity against both pathogens. These results suggest that *B. velezensis*, *P. chlororaphis* and *S. plymuthica* constitute potential biocontrol agents (BCAs) against GTDs and their application in field conditions should be further evaluated.

Introduction

Grapevine (*Vitis vinifera* L.) is one of the most important crops worldwide due to the high commercial value of wine, raisins, and table grapes. The cultivated area contemplates Mediterranean and temperate climate regions, between latitudes 30° and 50°, gathering approximately 7.72 million hectares (FAOSTAT, 2022). California is the largest grape producer in the United States, with 348,000 bearing hectares by 2019, of which 68.6% were destined for wine, 17.3% for raisins, and 14.0% for table grapes, altogether with a total value above USD 5.4 billion (CDFA, 2022). A wide range of pests and diseases may affect the crop; hence an intensive management program is often required, increasing production costs. Fungal diseases affecting the woody tissues, collectively known as grapevine trunk diseases (GTD), represent a major threat on

a global scale (Mondello et al. 2018). Chronic infections result in poor or no development of vegetative structures after bud break due to a malfunction of the vascular system. Symptoms are diverse and progress over time, potentially resulting in collapse and eventually in the death of the entire plant. Consequently, vineyards show significant reductions in yield and lifespan, which elevates production costs and economic losses (Mondello et al. 2018; Gramaje et al. 2018).

Botryosphaeria dieback, Eutypa dieback, Phomopsis dieback, esca and black foot are recognized as the most frequent and destructive GTDs. More than 133 unrelated fungal species have been reported to be causal agents, belonging mainly to the phylum Ascomycota and a few others to Basidiomycota (Gramaje et al. 2018). Over the last three decades, the incidence of GTDs has increased significantly worldwide. The expansion of the grape cultivated area, the transition to high-density plantations, including trellis training systems, the adoption of mechanical pruning, and the banning of effective chemical fungicides (i.e., sodium arsenite, benomyl, carbendazim, and methyl bromide) have been discussed as contributing factors (Gramaje et al. 2018). The fungal pathogens infect the grapevine primarily through pruning wounds (Eskalen and Gubler, 2001; Úrbez-Torres et al. 2010; Rolshausen et al. 2010), thus, control must include strategies to protect wounded tissues. Complete eradication is not possible; therefore, management must be focused on a multidisciplinary approach, including cultural practices and physical, biological, and chemical control strategies. In this regard, biocontrol has become increasingly attractive in viticulture, given the current trend of reducing the use of chemical pesticides due to their negative impact on the environment and workers' safety (Pertot et al. 2016; Carvalho, 2017).

The grapevine microbiome represents an important source of biocontrol agents (BCAs) since they play beneficial roles in plant fitness and health (Aziz et al. 2015; Rolli et al. 2017; Deyett et al. 2017). For instance, endophytic bacteria have the ability to enhance the grapevine tolerance to disease through different mechanisms, namely, by competition for nutrients and space, antibiosis, interrupting the pathogen signaling, or by inducing plant defenses (Rezgui et al. 2016; Compant et al. 2013). Therefore, the concept of a "balanced microbiome" has recently gained notorious attention due to recent work on grapevine microbial communities and their impact in disease expression (Bettenfeld et al. 2020; 2021). In this context, it has been shown that grapevines with a higher abundance of endophytic beneficial bacteria display less or no symptoms in vineyards with known history of GTDs (Bekris et al. 2021). On the other hand, the endophytic nature of latent infections caused by trunk pathogens pose an advantage for biocontrol treatments,

allowing grapevines to strengthen their tolerance to biotic and abiotic stress before the switch from endophytic to pathogenic behavior (Graniti et al. 2000; Hrycan et al. 2020). However, there is still a lack of variety of BCAs available for growers and nursery managers to reduce the impact of GTDs (Compant et al. 2013; Cobos et al. 2022). Hence, this study aimed to identify and evaluate in vitro the potential of endophytic and rhizospheric bacteria obtained from commercial vineyards with the absence and presence of GTD symptoms located in the main grape-growing regions in California as BCAs against GTD-causing pathogens.

Materials and Methods

Sampling and isolation of bacterial endophytes

Over the summer of 2019, twenty vineyards of wine, raisin, and table grapes were sampled across 10 counties in California (Figure 1). Eight vines were selected according to the presence (n = 3) and absence (n = 5) of externally visible GTD symptoms on each vineyard. Symptomatic vines showed cankers, dead arms, dieback, stunted shoots, or leaf tiger-stripes. Trunk, cordon, and root samples were collected from each grapevine using non-destructive methods (Hofstetter et al. 2012). Briefly, trunk and cordon samples were obtained by removing the bark (in an area of 10 cm^2) with a sterile chisel and disinfecting the surface with ethanol 70%. Once dried out, the internal wood was drilled with sterile drill bits (6.35 mm diameter), and the sawdust was collected into Whirl-Pak bags (Nasco, Fort Atkinson, WI, USA). Root samples were obtained by digging the soil with a clean shovel approximately 15 cm away from the trunk (around irrigation line) and collecting feeder roots with adhered soil in clean plastic bags (Guevara-Avendaño et al. 2018). Samples were transported to the laboratory in coolers and isolations were carried out in 90 mm Petri dishes with half-strength nutrient agar (½ NA; BD Biosciences, San Jose, CA, USA). Wood fragments were spread evenly onto the medium using a sterile tweezer. Additionally, the remaining samples were incubated in phosphate-buffered saline (PBS) for seven days, and aliquots of 100 µL were plated on ½ NA plates. Feeder root samples were shaken vigorously to remove loose soil particles, and 1 g of roots with strongly adhered soil were mixed with 99 mL of sterile distilled water in Erlenmeyer flasks. The solution was homogenized in an orbital shaker at 120 rpm for 1 min and 100 µL aliquots of 1:10 and 1:100 dilutions were plated onto the ½ NA plates. Plates were

incubated for 2 to 4 days at 26 °C in the dark and morphologically different colonies were transferred to fresh individual full-strength NA plates.

Initial screening of grapevine endophytic and rhizospheric bacteria for antifungal activity against *Neofusicoccum parvum* and *Diplodia seriata*

Bacterial isolates (n = 1344) were initially screened in vitro against the mycelium of *N. parvum* and *D. seriata* through antagonism assays described previously (Guevara-Avendaño et al. 2018). Both fungal isolates (*N. parvum* UCD7395 and *D. seriata* UCD7767) were obtained from the fungal collection of the Eskalen laboratory at the Department of Plant Pathology, University of California, Davis, that were originally isolated from GTD-symptomatic grapevine samples. A 5 mm mycelial plug of each fungal pathogen was placed at the center of 90 mm Petri dishes with potato dextrose agar (PDA) and three bacterial isolates plus a control (sterile distilled water) were inoculated at equidistant points around the mycelial plug, i.e., 3 cm from the center, using a sterile toothpick that was previously introduced in fresh bacterial culture. Plates were incubated at 25 °C for 3–7 days until the mycelium reached the border of the plate toward the controls. The radius of fungal growth was measured with a digital caliper from the center to the edge of the colony towards each treatment. The percentage of mycelial inhibition was calculated for each isolate using Equation (1):

$$\text{Percentage of inhibition (\%)} = 100\,[(R - r) / R], \quad (1)$$

where R and r corresponded to the radii of fungal growth toward the control and toward the bacterial treatment, respectively (Idris et al. 2007). The screening was initially performed using *N. parvum*, and bacterial isolates showing inhibition percentages above 40% were subsequently screened against *D. seriata* using the same methodology. Plates were prepared in triplicates and bacterial isolates that showed over 40% inhibition against the mycelium of both pathogens were selected for further analyses.

Molecular identification of bacterial isolates

Selected isolates (n = 172) were cultivated in NA plates for 24–48 h to perform DNA extraction following the protocol provided by the Quick-DNA™ Fungal/Bacterial Miniprep Kit

(Zymo Research Corp., Irvine, CA, USA). Amplifications of the 16S ribosomal DNA gene were carried out through PCR using the primers pair 27F/1492R (Heuer et al. 1997). For isolates belonging to the *Bacillus subtilis* species complex, fragments of the genes that encode for the gyrase subunit A (*gyrA*), RNA polymerase subunit B (*rpoB*), phosphoribosylaminoimidazolecarboxamide formyltransferase (*purH*), DNA polymerase III subunit alpha (*polC*), heat shock protein groEL (*groEL*) and the 16S rDNA were also amplified (Rooney et al. 2009). Given the large number of isolates associated with the *B. subtilis* species complex, seven representative isolates were sequenced for the five additional loci. PCRs were run in a T100™ thermocycler (Bio-Rad, Hercules, CA, USA). The reaction mixture consisted of 2 µL of template DNA (ca. 10 ng), 12.5 µL of GoTaq® Green MasterMix 2X (Promega, Madison, WI, USA), 9.3 µL of nuclease-free water and 0.6 µL of each primer (10 µM), completing a total volume of 25 µL. PCR conditions for the 16S rDNA gene included a hot start of 5 min followed by 35 cycles of 1 min at 94 °C for denaturation, 1 min for primer annealing at 63 °C, and 2 min at 72 °C for primer elongation, and a final step of 10 min at 72 °C. For the *gyrA*, *rpoB*, *polC*, *purH* and *groEL* amplifications, PCR conditions consisted of 35 cycles of 30 s at 94 °C, 30 s at 56 °C (*gyrA*), 52 °C (*rpoB*), 46 °C (*polC* and *groEL*) or 50 °C (*purH*) for annealing, and 1 min at 72 °C for primer extension. PCR products were submitted for Sanger sequencing to Quintara Biosciences (Hayward, CA, USA). Raw sequences were assembled using Sequencher v5.4.6 (Gene Codes, Ann Arbor, MI, USA). Consensus sequences of each isolate were compared with the NCBI nucleotide database using BLAST (http://ncbi.nlm.nih.gov/Blast.cgi (accessed on 1 September 2022)) to obtain a preliminary identification. Phylogenetic analyses were run using the 16S rDNA gene sequences of closely related species of preliminary identified isolates, and for isolates belonging to the *B. subtilis* species complex, a multi-locus approach was adopted. Alignments were carried out separately by locus using MAFFT v7 (https://mafft.cbrc.jp/alignment/server (accessed on 1 September 2022)) (Katoh et al. 2019) and included sequences of the selected bacterial isolates and closely related species (Tables S1 and S2). Alignments were depurated using Gblocks, selecting the less stringent options (Talavera and Castresana, 2007). Concatenation of the loci utilized for the *B. subtilis* species complex (16S-*gyrA*-*rpoB*-*purH*-*polC*-*purH*) was performed manually by assembling the six alignments into one, using MEGA X (Kumar et al. 2018). Phylogeny was reconstructed using the maximum parsimony method and bootstrap test with 1000 replications in MEGA X.

Dual antagonism assays of selected bacterial isolates against grapevine trunk pathogens

Six representative isolates of *B. velezensis*, *Pseudomonas chlororaphis*, and *Serratia plymuthica* (Table 1) were individually challenged against the mycelium of *N. parvum* (UCD7395), *D. seriata* (UCD7767), *Lasiodiplodia theobromae* (UCD9051), *Eutypa lata* (UCD7746), *Diaporthe ampelina* (UCD7544), *Phaeoacremonium minimum* (UCD7770), *Fomitiporia polymorpha* (UCD7757), and *Ilyonectria liriodendri* (UCD7874). All these fungal pathogens were also obtained from the fungal collection of the Eskalen laboratory mentioned above. Due to the differential growth rate among these fungi, bacterial isolates were inoculated at different times. For fast-growing fungal pathogens (*N. parvum*, *D. seriata* and *L. theobromae*), the assay was carried out in 90 mm diameter Petri dishes with full-strength PDA, where both the pathogen and the bacterial isolate were inoculated simultaneously at 22.5 mm from the center of the plate in opposite directions. The remaining pathogens with a slower growth rate (*E. lata, D. ampelina, Pm. minimum, F. polymorpha,* and *I. liriodendri)* were inoculated in 55 mm diameter PDA plates for 48 h (96 h in the case of *F. polymorpha*) prior the bacterial isolate at 10 mm from the center in opposite ways. In both cases, the pathogens were inoculated by placing a 5 mm diameter agar plug with actively growing mycelium on one side of the plate, and the bacterial isolates were streaked as a line of approximately 30 mm on the opposite side with a sterile toothpick previously inoculated with fresh bacterial culture. Incubation period ranged between three to four days for fast-growing pathogens, and fourteen days for slow-growing pathogens. Evaluations of mycelial radii were carried out when the fungal colonies of the controls reached the border of the plate in the direction of the treatment and inhibition percentages were calculated as described in Section 2.2. Each plate was prepared in triplicate, and the experiment was performed twice.

Effect of bacterial agar-diffusible metabolites on grapevine trunk pathogens

The six representative bacterial isolates were grown and fermented in LB broth for 7 days at 28 °C and 140 rpm in an orbital shaker (Incu-Shaker™ 10L, Benchmark Scientific, Sayreville, NJ, USA). Diffusible metabolites were obtained by centrifugation at 5000 rcf for 10 min and filtration of the supernatant through 0.22 µm pore size filter units (Stericup®, MilliporeSigma, Burlington, MA, USA). Cell-free filtrates were added at increasing concentrations (1, 15 and 30%

v/v) into PDA flasks when the media was approximately 50 °C after autoclaving. Control flasks did not receive bacterial filtrates. Fungal pathogens were inoculated at the center of Petri dishes containing the different treatments using 5 mm diameter plugs with actively growing mycelium. Evaluations of mycelial radii were carried out when the fungal colonies of the controls reached the border of the plate and inhibition percentage was calculated as described in Section 2.2. Each plate was prepared in triplicate, and the experiment was performed twice.

Effect of bacterial volatile organic compounds (VOCs) on grapevine trunk pathogens

The six representative bacterial isolates were used to assess the effect of their volatile organic compounds (VOCs) against the mycelial growth of *N. parvum* and *E. lata*, representing a fast and a slow growing trunk pathogen, respectively. Bacterial isolates were inoculated onto 90 mm diameter Petri dishes with Luria-Bertani agar (LB, tryptone 10 g/L, sodium chloride 10 g/L, yeast extract 5 g/L, agar 18 g/L) using a sterile toothpick, streaking the entire surface of the agar. The fungal pathogens were inoculated at the center of Petri dishes with PDA using 5 mm plugs with actively growing mycelium. Both bottoms of each Petri dish were disposed against each other and sealed with a double layer of paraffin wax (Parafilm^{TM}, Bemis Co. Inc., Neenah, WI, USA), placing the side inoculated with bacteria at the bottom. Control plates had no bacteria streaked onto the LB agar. Evaluations of mycelial radii were carried out when the fungal colonies of the controls reached the border of the plate and inhibition percentage was calculated as described in Section 2.2. Each plate was prepared in triplicate, and the experiment was performed twice.

Statistical analyses

Percentages of inhibition were subjected to analysis of variance (ANOVA) using generalized linear models with the corresponding R packages in InfoStat v2008 (Houston, TX, USA). Normality and homoscedasticity were checked and corrected when necessary and means were separated using Fisher's least significant difference test ($p < 0.05$). Data were plotted in GraphPad Prism v.5.03 (San Diego, CA, USA).

Results

Initial screening of grapevine endophytic and rhizospheric bacteria for antifungal activity against *Neofusicoccum parvum* and *Diplodia seriata*

From the field sampling carried out in 20 commercial vineyards over the summer of 2019, a collection of 1344 endophytic and rhizospheric bacterial isolates was obtained and analyzed. The antagonism assays against the mycelium of *N. parvum* and *D. seriata* revealed that 172 isolates showed mycelial growth inhibition percentages over 40% against both pathogens. Phylogenetic trees indicated that 154 isolates (89.5%) corresponded to *B. velezensis* (Figure 2), whereas the remaining belong to a range of species of *Pseudomonas* (12 isolates, Figure 3), *S. plymuthica* (2 isolates, Figure 4) and other genera (4 isolates) that were excluded from this study. The 154 isolates of *B. velezensis* were preliminary analyzed using their 16S rDNA sequences alone, which clustered them altogether in a single clade with multiple species closely related to *B. velezensis* (data not shown). However, a six-locus data set (16S rDNA-*gyrA*-*rpoB*-*purH*-*polC*-*groEL*) allowed an accurate identification. Regarding their origin, B. velezensis isolates were obtained primarily from the woody tissues of asymptomatic vines, whereas *Pseudomonas* spp. and *S. plymuthica* isolates were mainly recovered from the rhizosphere of both symptomatic and asymptomatic vines (Figure 5).

Dual antagonism assays of selected bacterial isolates against grapevine trunk pathogens

The six selected bacterial isolates inhibited the mycelial growth of almost all the pathogens over the threshold (40%), except for *L. theobromae*, in which only half of the isolates reached inhibition levels above 40% (Figure 6). Differences ($p < 0.05$) on mycelial inhibition levels were detected among the bacterial isolates on each pathogen. On Botryosphaeriaceae species, bacterial isolates of the same species did not differ on inhibition percentages, except for *P. chlororaphis* isolates against *L. theobromae*, and isolates of *S. plymuthica* against *D. seriata*. Specifically, on *N. parvum*, inhibition levels were significantly higher with UCD10763 (*P. chlororaphis*) than with UCD10756 (*S. plymuthica*). On *D. seriata*, inhibition percentages were higher with UCD10631 (*B. velezensis*) than UCD10757 (*P. chlororaphis*) and UCD10756 (*S. plymuthica*). On *L. theobromae*, both *Bacillus* isolates and UCD10763 (*P. chlororaphis*) caused higher inhibition levels than the remaining ones that did not reached the threshold of 40%. Then, on slow-growing

fungal pathogens, more differences were observed among isolates of the same species. On *E. lata*, the highest inhibition levels were observed with UCD10614 (*B. velezensis*) and UCD10763 (*P. chlororaphis*), followed by UCD10719 (*S. plymuthica*), UCD10631 (*B. velezensis*) and UCD10757 (*P. chlororaphis*), and lastly, UCD10756 (*S. plymuthica*). On *D. ampelina*, only UCD10763 (*P. chlororaphis*) was significantly higher than UCD10757 (*P. chlororaphis*). On *Pm. minimum*, inhibition levels were significantly higher with UCD10719 (*S. plymuthica*), followed by both *B. velezensis* isolates, and UCD10763 (*P. chlororaphis*) ranking third, and later UCD10756 (*S. plymuthica*) and UCD10757 (*P. chlororaphis*) ranking fourth. On *F. polymorpha*, the highest inhibitions were caused by both *P. chlororaphis* isolates, followed by *B. velezensis*, and later *S. plymuthica* ranking third. However, no differences were observed with UCD10631 (*B. velezensis*) and *S. plymuthica* isolates. Lastly, on *I. liriodendri*, UCD10719 (*S. plymuthica*) and UCD10763 (*P. chlororaphis*) caused the highest inhibition levels, followed by UCD10757 (*P. chlororaphis*), UCD10756 (*S. plymuthica*) and UCD10614 (*B. velezensis*) that ranked second, and UCD10631 (*B. velezensis*) ranking third.

Effect of bacterial agar-diffusible metabolites against grapevine trunk pathogens

The cell-free filtrates from bacterial suspensions fermented for seven days significantly ($p < 0.05$) reduced the mycelial growth of both *N. parvum* and *E. lata* (Figure 7). For both pathogens, the reduction in mycelial growth was dependent on the interaction ($p < 0.05$) between the isolate and the concentration level of metabolites in the agar. Differences in mycelial growth were detected among the bacterial isolates at all tested concentrations. The metabolites produced by *B. velezensis* isolates reached inhibition levels significantly higher against both pathogens when compared to the filtrates from *P. chlororaphis* and *S. plymuthica*. Notably, at 1% only the *B. velezensis* metabolites caused inhibition levels above 50%. Further, the metabolites of UCD10719 (*S. plymuthica*) ranked second in the inhibition of both pathogens at 15% and 30%. Specifically, at 15%, the two *P. chlororaphis* filtrates ranked third and fourth at inhibiting both pathogens, whereas the isolate UCD10756 (*S. plymuthica*) was the less toxic. At 30%, the filtrate of UCD10763 (*P. chlororaphis*) ranked third against both pathogens, whereas UCD10756 (*S. plymuthica*) ranked fourth against *N. parvum* and second against *E. lata*, and UCD10757 (*P. chlororaphis*) was the less toxic.

Effect of bacterial volatile organic compounds on grapevine trunk pathogens

The volatile organic compounds (VOCs) released by the six selected bacterial isolates caused lower inhibition levels on the mycelial growth of *N. parvum* than of *E. lata* (Figure 8). On *N. parvum*, the VOCs produced by isolate UCD10763 (*P. chlororaphis*) and both *S. plymuthica* isolates yielded a higher inhibition level than the remaining isolates, with inhibition levels from 12.3% to 15.9% in average. On the other hand, on *E. lata*, the VOCs from both *P. chlororaphis* isolates caused inhibition levels ranging from 64.3% to 70.9% in average, followed by UCD10719 (*S. plymuthica*) with an inhibition of 35.5%, significantly superior to the rest of the isolates.

Discussion

This study shows that isolates of *B. velezensis*, *P. chlororaphis*, and *S. plymuthica* obtained from GTD-symptomatic and asymptomatic grapevines have inhibitory activity against eight common fungal pathogens responsible for Botryosphaeria dieback, Eutypa dieback, Phomopsis dieback, esca, and black foot in California. Previously, other species of *Bacillus*, *Pseudomonas* and *Serratia* have been investigated for their potential as BCAs against grapevine trunk pathogens (Mondello et al. 2018). Among them, *Bacillus* spp. have been the most studied in both laboratory and field settings (Rezgui et al. 2016; Ferreira et al. 1991; Schmidt et al. 2001; Alfonzo et al. 2009; Kotze et al. 2011). Less frequently, different species of *Pseudomonas*, *Serratia*, *Paenibacillus*, *Pantoea*, *Paraburkholderia*, and *Streptomyces* have also been tested in laboratory and greenhouse trials (Schmidt et al. 2001; Haidar et al. 2016a; 2016b; Andreolli et al. 2019; Martínez-Diz et al. 2020; Wu et al. 2020; Álvarez-Pérez et al. 2017). Coincidently, this study revealed that from a subset of 172 isolates with potential biocontrol activity against GTD pathogens, the majority (89.5%) corresponded to *B. velezensis*, with a smaller proportion of *Pseudomonas* spp. (6.7%) and *S. plymuthica* (1.2%).

Selected isolates of *B. velezensis*, *P. chlororaphis* and *S. plymuthica* showed the antifungal effect when challenged directly against the pathogens and indirectly through the use of their agar-diffusible and/or volatile metabolites in vitro. Specifically, *B. velezensis* isolates showed inhibition levels above 50% against all the pathogens (except on *I. liriodendri*, with 43% of inhibition in average) by both direct confrontation and their agar-diffusible metabolites at 1, 15 and 30% *v/v*. On the other hand, *P. chlororaphis* and *S. plymuthica* isolates inhibited all the pathogens by direct

confrontation similarly to *B. velezensis*, with levels above 40% (except on *L. theobromae*, with lower levels that ranged from 31.7 to 58% of inhibition) with some differences in a few fungal pathogens. However, their agar-diffusible metabolites were not as inhibitory as the ones produced by both *B. velezensis* isolates, where concentrations above 15% *v/v* were needed to reach inhibition levels over 40% against *N. parvum* and *E. lata*. When comparing isolates of *P. chlororaphis*, the metabolites produced by isolate UCD10763 were more toxic at 15% and 30% *v/v* against *E. lata*, and at 30% against *N. parvum* when compared to isolate UCD10757. Similar observations were found between *S. plymuthica* isolates, where UCD10719 metabolites caused higher inhibition levels at 15% and 30% *v/v* against *N. parvum*, and 15% *v/v* against *E. lata*, compared to isolate UCD10756. These results highlight the importance of selecting the proper bacterial isolates that exhibit higher antifungal effects and that these could be harnessed by treating grapevines with living bacterial inoculants and/or their extracted secondary metabolites. *Bacillus*, *Pseudomonas*, and *Serratia* species secrete a diverse range of secondary metabolites that are highly inhibitory against plant pathogens. For example, *B. velezensis* secretes antibiotics such as bacillopeptines, macrolactins, bacillaene, difficidin, amylolysin, bacilysin, lantipeptides and microcins (Pan et al. 2017), cell-wall degrading enzymes such as chitinase, protease and β-1,3-glucanase (Choub et al. 2021), antimicrobial polypeptides such as iturins, fengycins, and surfactins (Liu et al. 2019), and siderophores such as bacillibactin (Chen et al. 2007). *P. chlororaphis* produce antibiotics such as phenazine, pyrrolnitrine, 2-hexyl 5-propyl resorcinol and hydrogen cyanide, and siderophores such as pyoverdine and achromobactine (Raio et al. 2021). *S. plymuthica* synthesizes antibiotics such as haterumalides, prodigiosin and pyrrolnitrin, and lytic enzymes such as chitinases and glucanases (Kalbe et al. 1996; Levenfors et al. 2004). Our results suggest a possible implication between these bacterial-derived metabolites and the antifungal activity observed against GTD-associated pathogens. Therefore, understanding the chemical diversity of these metabolites may help to understand their interactions with the physiology of the plant host and the pathogen, as well as improve processes associated with a BCA formulation such as extraction, and purification, among others.

The effect of VOCs produced by selected bacterial isolates on the mycelial growth of *N. parvum* and *E. lata* was also studied in order to elucidate other potential mechanisms of inhibition. VOCs have many functions as signaling molecules and, among them, they can have antifungal properties against different plant pathogens (Raio et al. 2021). Our results showed that only *P.*

chlororaphis and *S. plymuthica* VOCs caused a significant inhibition against *E. lata* and not against *N. parvum*. An explanation for this is that *N. parvum* has a higher growth rate and therefore did not allow any of the six bacterial isolates to produce sufficient VOCs to significantly reduce the mycelial development. Another explanation could be that *N. parvum* is not sensitive or highly tolerant to these molecules. Some of the VOCs produced by these bacterial species include 3-methyl-1-butanol and methanethiol in the case of *P. chlororaphis* (Raio et al. 2021) and sodorifen, alcohols, ketones, pyrazine, and sulfur compounds, in *S. plymuthica* (von Reuss et al. 2010; Weise et al. 2014). Interestingly, VOCs produced by *B. velezensis* isolates did not arrest the mycelial growth of neither of the pathogens, which could also be explained by a lack of sensitivity by both fungal species, or insufficient time for toxic VOCs to be produced, or even the medium composition was not suitable for VOCs production. *B. velezensis* produce diacetyl, benzaldehyde and isoamyl alcohol, which are known to be toxic VOCs to different plant pathogens such as *Botrytis cinerea*, *Penicillium italicum* and *Monilinia fructicola* (Calvo et al. 2020). Nevertheless, some of these molecules can also activate plant defense responses (Fan et al. 2018; Pršić et al. 2020), thus representing an indirect mechanism of action against GTD-associated pathogens.

We aimed to investigate the effect of selected bacterial isolates on a broad range of fungal pathogens responsible for GTDs in California. Previous studies have mainly focused on a few species, such as *E. lata* alone (Ferreira et al. 1991; Schmidt et al. 2001; Munkvold and Marois, 1993; Halleen et al. 2010) or a Botryosphaeriaceae species, usually *N. parvum* (Rezgui et al. 2016; Haidar et al. 2016b; Wu et al. 2020; Trotel-Aziz et al. 2019; Haidar et al. 2021). Several others included two or three species associated with esca, Eutypa dieback and/or Botryosphaeria dieback (Alfonzo et al. 2009; Andreolli et al. 2019; Lebrihi et al. 2009; Andreolli et al. 2016; Blundell et al. 2021; Daraignes et al. 2018). Some groups have focused on black foot pathogens (Martínez-Diz et al. 2020; Santos et al. 2016; Russi et al. 2020), but not many have contemplated multiple species representing more than three GTDs elsewhere (Kotze et al. 2011; Russi et al. 2020; Niem et al. 2020). Given the high diversity of causal agents involved with these diseases, it is critical to decipher the breadth of responses of multiple pathogens to the presence of a BCA and/or its metabolites, which will ultimately determine its effectiveness. For example, the inhibition levels on slow-growing pathogens (e.g., *E. lata, D. ampelina, Pm. minimum, F. polymorpha*) were higher than on fast growing fungi (e.g., Botryosphaeriaceae). Additionally, even between Botryosphaeriaceae, the inhibition percentages were higher on *N. parvum* and *D. seriata* than on

L. theobromae. A longer exposure to the presence of the bacterium and its metabolites during fungal growth may explain these observations. This information allows to imply that timing of the application of BCAs as a preventative strategy is critical in suppressing pathogen development.

Finally, our findings in this study revealed that the grapevine woody tissues, the rhizosphere, and the vineyard soil constitute a robust source of potential BCAs against GTDs. Our selected bacterial isolates, especially the ones identified as *B. velezensis* and *P. chlororaphis*, exhibited high levels of inhibition against eight fungal pathogens responsible for GTDs and their agar-diffusible and volatile metabolites demonstrated to be involved in the suppression mechanism. Therefore, these isolates alone or in combination could provide a broader spectrum of protection to grapevines against the development of GTD-associated symptoms. Since these isolates are natural inhabitants of grapevines, they are likely to be well adapted to their plant host (Arora et al. 2015). *B. velezensis*, *P. chlororaphis*, and *S. plymuthica* are ubiquitous inhabitants of the soil, water bodies, plant roots, and fermented foods, and have been extensively studied elsewhere for their antagonistic activity against several fungal plant pathogens and plant growth promotion capability (Alenezi et al. 2021; Arrebola et al. 2019; Kshetri et al. 2019; Soenens and Imperial, 2020). Antibiosis, lytic enzymes and siderophores are the most described mechanisms by which these bacterial species exert their beneficial effects on several plant hosts (Cleto et al. 2021; Ye et al. 2018; Anderson et al. 2020). Furthermore, *B. velezensis* and *P. chlororaphis* are known to form biofilms on plant structures, which contribute to the protection of both the plant and the bacteria from dehydration, salinity, and nutrient deficiency, especially nitrogen (Ye et al. 2018; Anderson and Kim, 2020). Currently, we are evaluating selected isolates of *B. velezensis*, *P. chlororaphis*, and *S. plymuthica* on field trials for their prevention and curative abilities against common GTDs pathogens. Result from these field studies will help to develop commercially available BCA for the management of GTDs.

Literature cited

Alenezi, F.N.; Slama, H.B.; Bouket, A.C.; Cherif-Silini, H.; Silini, A.; Luptakova, L.; Nowakowska, J.A.; Oszako, T.; Belbahri, L. 2021. *Bacillus velezensis*: A treasure house of bioactive compounds of medicinal, biocontrol and environmental importance. Forests 12:1714. https://doi.org/10.3390/f12121714

Alfonzo, A.; Conigliaro, G.; Torta, L.; Burruano, S.; Moschetti, G. 2009. Antagonism of *Bacillus subtilis* strain AG1 against vine wood fungal pathogens. Phytopathol. Mediterr. 48:155–158. https://doi.org/10.14601/Phytopathol_Mediterr-2886

Álvarez-Pérez, J.M.; González-García, S.; Cobos, R.; Olego, M.Á.; Ibañez, A.; Díez-Galán, A.; Garzón-Jimeno, E.; Coque, J.J.R. 2017. Use of endophytic and rhizosphere actinobacteria from grapevine plants to reduce nursery fungal graft infections that lead to young grapevine decline. Appl. Environ. Microbiol. 83:e01564–e17. https://doi.org/10.1128/AEM.01564-17

Anderson, A.J.; Kim, Y.C. 2020. Insights into plant-beneficial traits of probiotic *Pseudomonas chlororaphis* isolates. J. Med. Microbiol. 69:361–371. https://doi.org/10.1099/jmm.0.001157.

Andreolli, M.; Lampis, S.; Zapparoli, G.; Angelini, E.; Vallini, G. 2016. Diversity of bacterial endophytes in 3 and 15 year-old grapevines of *Vitis vinifera* cv. Corvina and their potential for plant growth promotion and phytopathogen control. Microbiol. Res. 183:42–52. https://doi.org/10.1016/j.micres.2015.11.009

Andreolli, M.; Zapparoli, G.; Angelini, E.; Lucchetta, G.; Lampis, S.; Vallini, G. 2019. *Pseudomonas protegens* MP12: A plant growth-promoting endophytic bacterium with broad-spectrum antifungal activity against grapevine phytopathogens. Microbiol. Res. 219:123–131. https://doi.org/10.1016/j.micres.2018.11.003

Arora, J.; Ramawat, K.G. 2015. An introduction to endophytes. In *Endophytes: Biology and Biotechnology*, 1st ed.; Maheshwari, D.K., Ed.; Springer: Cham, Switzerland; Volume 1, pp. 1–23. https://doi.org/10.1007/978-3-319-66541-2_1

Arrebola, E.; Tienda, S.; Vida, C.; de Vicente, A.; Cazorla, F.M. 2019. Fitness features involved in the biocontrol interaction of *Pseudomonas chlororaphis* with host plants: The case study of PcPCL1606. Front. Microbiol. 10:719. https://doi.org/10.3389/fmicb.2019.00719

Aziz, A.; Verhagen, B.; Magnin-Robert, M.; Couderchet, M.; Clément, C.; Jeandet, P.; Trotel-Aziz, P. 2015. Effectiveness of beneficial bacteria to promote systemic resistance of grapevine to gray mold as related to phytoalexin production in vineyards. Plant Soil 1:141–153. https://doi.org/10.1007/s11104-015-2783-z

Bekris, F.; Vasileiadis, S.; Papadopoulou, E.; Samaras, A.; Testempasis, S.; Gkizi, D.; Tavlaki, G.; Tzima, A.; Paplomatas, E.; Markakis, E.; et al. 2021. Grapevine wood microbiome analysis identifies key fungal pathogens and potential interactions with the bacterial community implicated in grapevine trunk disease appearance. Environ. Microbiome 16:23. https://doi.org/10.1186/s40793-021-00390-1

Bettenfeld, P.; Cadena i Canals, J.; Jacquens, L.; Fernandez, O.; Fontaine, F.; van Schaik, E.; Courty, P.-E.; Trouvelot, S. 2021. The microbiota of the grapevine holobiont: A key component of plant health. J. Adv. Res. 40:1–15. https://doi.org/10.1016/j.jare.2021.12.008

Bettenfeld, P.; Fontaine, F.; Trouvelot, S.; Fernandez, O.; Courty, P.-E. 2020. Woody plant declines. What's wrong with the microbiome? Trends Plant Sci. 25:381–394. https://doi.org/10.1016/j.tplants.2019.12.024

Blundell, R.; Arreguin, M.; Eskalen, A. 2021. In vitro evaluation of grapevine endophytes, epiphytes and sap microorganisms for potential use to control grapevine trunk disease pathogens. Phytopathol. Mediterr. 60:535–548. https://doi.org/10.36253/phyto-12500

Calvo, H.; Mendiara, I.; Arias, E.; Gracia, A.P.; Blanco, D.; Venturini, M.E. 2020. Antifungal activity of the volatile organic compounds produced by *Bacillus velezensis* strains against postharvest fungal pathogens. Postharvest Biol. Technol. 166:111208. https://doi.org/10.1016/j.postharvbio.2020.111208

Carvalho, F.P. 2017. Pesticides, environment, and food safety. Food and Energy Secur. 6:48–60. https://doi.org/10.1002/fes3.108

CDFA. 2022. Agricultural Statistics Review 2019–2020. Available online: https://www.cdfa.ca.gov/Statistics/PDFs/2020_Ag_Stats_Review.pdf (accessed on 1 September 2022).

Chen, X.H.; Koumoutsi, A.; Scholz, R.; Eisenreich, A.; Schneider, K.; Heinemeyer, I.; Morgenstern, B.; Voss, B.; Hess, W.R.; Reva, O.; et al. 2007. Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42. Nat. Biotechnol. 25:1007–1014. https://doi.org/10.1038/nbt1325

Choub, V.; Ajuna, H.B.; Won, S.-J.; Moon, J.-H.; Choi, S.-I.; Maung, C.E.H.; Kim, C.-W.; Ahn, Y.S. 2021. Antifungal activity of *Bacillus velezensis* CE 100 against anthracnose disease (*Colletotrichum gloeosporioides*) and growth promotion of walnut (*Juglans regia* L.) trees. Int. J. Mol. Sci. 22:10438. https://doi.org/10.3390/ijms22191043

Cleto, S.; Haslinger, K.; Prather, K.L.J.; Lu, T.K. 2021. Natural combinatorial genetics and prolific polyamine production enable siderophore diversification in *Serratia plymuthica*. BMC Biol. 19:46. https://doi.org/10.1186/s12915-021-00971-z

Cobos, R.; Ibáñez, A.; Diez-Galán, A.; Calvo-Pena, C.; Ghoreshizadeh, S.; Coque, J.J.R. 2022. The grapevine microbiome to the rescue: Implications for the biocontrol of trunk diseases. Plants 11:840. https://doi.org/10.3390/plants11070840

Compant, S.; Brader, G.; Muzammil, S.; Sessitsch, A.; Lebrihi, A.; Mathieu, F. 2013. Use of beneficial bacteria and their secondary metabolites to control grapevine pathogen diseases. Biocontrol 58:435–455. https://doi.org/10.1007/s10526-012-9479-6

Daraignes, L.; Gerbore, J.; Yacoub, A.; Dubois, L.; Romand, C.; Zekri, O.; Roudet, J.; Chambon, P.; Fermaud, M. 2018. Efficacy of *P. oligandrum* affected by its association with bacterial BCAs and rootstock effect in controlling grapevine trunk diseases. Biol. Control 119:59–67. https://doi.org/10.1016/j.biocontrol.2018.01.008

Deyett, E.; Roper, M.C.; Ruegger, P.; Yang, J.I.; Borneman, J.; Rolshausen, P.E. 2017. Microbial landscape of the grapevine endosphere in the context of Pierce's disease. Phytobiomes J. 1:138–149. https://doi.org/10.1094/PBIOMES-08-17-0033-R

Eskalen, A.; Gubler, W.D. 2001. Association of spores of *Phaeomoniella chlamydospora*, *Phaeoacremonium inflatipes*, and *Pm. aleophilum* with grapevine cordons in California. Phytopathol. Mediterr. 40:429–432. https://doi.org/10.14601/Phytopathol_Mediterr-1613

Fan, B.; Wang, C.; Song, X.; Ding, X.; Wu, L.; Wu, H.; Gao, X.; Borriss, R. 2018. *Bacillus velezensis* FZB42 in 2018: The gram-positive model strain for plant growth promotion and biocontrol. Front. Microbiol. 9:2491. https://doi.org/10.3389/fmicb.2018.02491

FAOSTAT. Available online: http://www.fao.org/faostat/en/#data/QCL (accessed on 1 September 2022).

Ferreira, J.H.S.; Matthee, F.N.; Thomas, A.C. 1991. Biological control of *Eutypa lata* on grapevine by an antagonistic strain of *Bacillus subtilis*. Phytopathology 81:2983–287. https://doi.org/10.1094/Phyto-81-283

Gramaje, D.; Úrbez-Torres, J.R.; Sosnowski, M.R. 2018. Managing grapevine trunk diseases with respect to etiology and epidemiology: Current strategies and future prospects. Plant Dis. 102:12–39. https://doi.org/10.1094/PDIS-04-17-0512-FE

Graniti, A.; Surico, G.; Mugnai, L. 2000. Esca of grapevine: A disease complex or a complex of diseases?. Phytopathol. Mediterr. 39:16–20. https://doi.org/10.14601/Phytopathol_Mediterr-1539

Guevara-Avendaño, E.; Carrillo, J.D.; Ndinga-Muniania, C.; Moreno, K.; Méndez-Bravo, A.; Guerrero-Analco, J.A.; Eskalen, A.; Reverchon, F. 2018. Antifungal activity of avocado rhizobacteria against *Fusarium euwallaceae* and *Graphium* spp., associated with *Euwallacea* spp. nr. *fornicatus*, and *Phytophthora cinnamomi*. Antonie van Leeuwenhoek 111:563–572. https://doi.org/10.1007/s10482-017-0977-5

Haidar, R.; Amira, Y.; Roudet, J.; Fermaud, M.; Rey, P. 2021. Application methods and modes of action of *Pantoea agglomerans* and *Paenibacillus* sp. to control the grapevine trunk disease-pathogen *Neofusicoccum parvum*. OENO One 55:1–16. https://doi.org/10.20870/oeno-one.2021.55.3.4530

Haidar, R.; Deschamps, A.; Roudet, J.; Calvo-Garrido, C.; Bruez, E.; Rey, P.; Fermaud, M. 2016a. Multi-organ screening of efficient bacterial control agents against two major pathogens of grapevine. Biol. Control 92:55–65. https://doi.org/10.1016/j.biocontrol.2015.09.003

Haidar, R.; Roudet, J.; Bonnard, O.; Dufour, M.C.; Corio-Costet, M.F.; Fert, M.; Gautier, T.; Deschamps, A.; Fermaud, M. 2016b. Screening and modes of action of antagonistic bacteria to control the fungal pathogen *Phaeomoniella chlamydospora* involved in grapevine trunk diseases. Microbiol. Res. 192:172–184. https://doi.org/10.1016/j.micres.2016.07.003

Halleen, F.; Fourie, P.H.; Lombard, P.J. 2010. Protection of grapevine pruning wounds against *Eutypa lata* by biological and chemical methods. S. Afr. J. Enol. Vitic. 31:128–132. https://doi.org/10.21548/31-2-1409

Heuer, H.; Krsek, M.; Baker, P.; Smalla, K.; Wellington, E.M.H. 1997. Analysis of Actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl. Environ. Microbiol. 63:3233–3241. https://doi.org/10.1128/aem.63.8.3233-3241.1997

Hofstetter, V.; Buyck, B.; Croll, D.; Viret, O.; Couloux, A.; Gindro, K. 2012. What if esca disease of grapevine were not a fungal disease?. Fungal Divers. 54:51–67. https://doi.org/10.1007/s13225-012-0171-z

Hrycan, J.; Hart, M.; Bowen, P.; Forge, T.; Úrbez-Torres, J.R. 2020. Grapevine trunk disease fungi: Their roles as latent pathogens and stress factors that favour disease development and symptom expression. Phytopathol. Mediterr. 59:395–424. https://doi.org/10.14601/Phyto-11275

Idris, H.A.; Labuschagne, N.; Korsten, L. 2007. Screening rhizobacteria for biological control of Fusarium root and crown rot of sorghum in Ethiopia. Biol. Control 40:97–106. https://doi.org/10.1016/j.biocontrol.2006.07.017

Kalbe, C.; Marten, P.; Berg, G. 1996. Strains of the genus *Serratia* as beneficial rhizobacteria of oilseed rape with antifungal properties. Microbiol. Res. 151:433–439. https://doi.org/10.1016/S0944-5013(96)80014-0

Katoh, K.; Rozewicki, J.; Yamada, K.D. 2019. MAFFT online service: Multiple sequence alignment, interactive sequence choice and visualization. Brief. Bioinform. 20:1160–1166. https://doi.org/10.1093/bib/bbx108

Kotze, C.; van Niekerk, J.; Mostert, L.; Halleen, F.; Fourie, P. 2011. Evaluation of biocontrol agents for grapevine pruning wound protection against trunk pathogen infection. Phytopathol. Mediterr. 50:S247−S263. https://doi.org/10.14601/Phytopathol_Mediterr-8960

Kshetri, L.; Naseem, F.; Pandey, P. 2019. Role of *Serratia* sp. as Biocontrol Agent and Plant Growth Stimulator, with Prospects of Biotic Stress Management in Plant. In *Plant Growth Promoting Rhizobacteria for Sustainable Stress Management*; Sayyed, R., Ed.; Springer: Singapore; Volume 2, pp. 169–200. https://doi.org/10.1007/978-981-13-6986-5_6

Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35:1547–1549. https://doi.org/10.1093/molbev/msy096

Lebrihi, A.; Errakhi, R.; Barakate, M. 2009. New *Streptomyces barakatei* Strain, Culture Filtrate, Derived Active Compounds and Use Thereof in the Treatment of Plants. French Patent WO 2009/156687 A2. Available online: https://patentimages.storage.googleapis.com/a5/ed/da/66f8232a24aa29/WO2009156687A2.pdf (accessed on 1 September 2022).

Levenfors, J.J.; Hedman, R.; Thaning, C.; Gerhardson, B.; Welch, C.J. 2004. Broad-spectrum antifungal metabolites produced by the soil bacterium *Serratia plymuthica* A 153. Soil Biol. Biochem. 36:677–685. https://doi.org/10.1016/j.soilbio.2003.12.008

Liu, Y.; Teng, K.; Wang, T.; Dong, E.; Zhang, M.; Tao, Y.; Zhong, J. 2019. Antimicrobial *Bacillus velezensis* HC6: Production of three kinds of lipopeptides and biocontrol potential in maize. J. Appl. Microbiol. 128:242–254. https://doi.org/10.1111/jam.14459

Martínez-Diz, M.P.; Díaz-Losada, E.; Andrés-Sodupe, M.; Bujanda, R.; Maldonado-González, M.M.; Ojeda, S.; Yaocub, A.; Rey, P.; Gramaje, D. Field evaluation of biocontrol agents against black-foot and Petri diseases of grapevine. *Pest Manag. Sci.* **2020**, *77*, 697–708. https://doi.org/10.1002/ps.6064

Mondello, V.; Songy, A.; Battiston, E.; Pinto, C.; Coppin, C.; Trotel-Aziz, P.; Clement, C.; Mugnai, L.; Fontaine, F. 2018. Grapevine trunk diseases: A review of fifteen years of trials

for their control with chemicals and biocontrol agents. Plant Dis. 102:1189–1217. https://doi.org/10.1094/PDIS-08-17-1181-FE

Munkvold, G.P.; Marois, J.J. 1993. Efficacy of natural epiphytes and colonizers of grapevine pruning wounds for biological control of Eutypa dieback. Phytopathology 86:624–629. https://doi.org/10.1094/Phyto-83-624

Niem, J.M.; Billones-Baaijens, R.; Stodart, B.; Savocchia, S. 2020. Diversity profiling of grapevine microbial endosphere and antagonistic potential of endophytic *Pseudomonas* against grapevine trunk diseases. Front. Microbiol. 11:477. https://doi.org/10.3389/fmicb.2020.00477

Pan, H.-Q.; Li, Q.-L.; Hu, J.-C. 2017. The complete genome sequence of *Bacillus velezensis* 9912D reveals its biocontrol mechanism as a novel commercial biological fungicide agent. J. Biotechnol. 247:25–28. https://doi.org/10.1016/j.jbiotec.2017.02.022

Pertot, I.; Caffi, T.; Rossi, V.; Mugnai, L.; Hoffmann, C.; Grando, M.S.; Gary, C.; Lafond, D.; Duso, C.; Thiery, D.; et al. 2016. A critical review of plant protection tools for reducing pesticide use on grapevine and new perspectives for the implementation of IPM in viticulture. Crop Prot. 97:70–84. https://doi.org/10.1016/j.cropro.2016.11.025

Pršić, J.; Ongena, M. 2020. Elicitors of plant immunity triggered by beneficial bacteria. *Front.* Plant Sci. 11:594530. https://doi.org/10.3389/fpls.2020.594530

Raio, A.; Puopolo, G. 2021. *Pseudomonas chlororaphis* metabolites as biocontrol promoters of plant health and improved crop yield. World J. Microbiol. Biotechnol. 37:99. https://doi.org/10.1007/s11274-021-03063-w

Rezgui, A.; Ghnaya-Chakroun, A.B.; Vallance, J.; Bruez, E.; Hajlaoui, M.R.; Sadfi-Zouaoui, N.; Rey, P. 2016. Endophytic bacteria with antagonistic traits inhabit the wood tissues of grapevines from Tunisian vineyards. Biol. Control 99:28–37. https://doi.org/10.1016/j.biocontrol.2016.04.005

Rolli, E.; Marasco, R.; Saderi, S.; Corretto, E.; Mapelli, F.; Cheris, A.; Borin, S.; Valenti, L.; Sorlini, C.; Daffonchio, D. 2017. Root-associated bacteria promote grapevine growth: From the laboratory to the field. Plant Soil 410:369–382. https://doi.org/10.1007/s11104-016-3019-6

Rolshausen, P.E.; Úrbez-Torres, J.R.; Rooney-Latham, S.; Eskalen, A.; Smith, R.J.; Gubler, W.D. 2010. Evaluation of pruning wound susceptibility and protection against fungi associated with grapevine trunk diseases. Am. J. Enol. Vitic. 61:113–119.

Rooney, A.P.; Price, N.P.J.; Ehrhardt, C.; Swezey, J.L.; Bannan, J.D. 2009. Phylogeny and molecular taxonomy of the *Bacillus subtilis* species complex and description of *Bacillus subtilis* subsp. *inaquosorum* subsp. nov. Int. J. Syst. Evol. Microbiol. 59:2429–2436. https://doi.org/10.1099/ijs.0.009126-0

Russi, A., Almança, M.A.K., Grohs, D.S., Schwambach, J. 2020. Biocontrol of black foot disease on grapevine rootstocks using *Bacillus subtilis* strain F62. Trop. Plant Pathol. 45:103–111. https://doi.org/10.1007/s40858-019-00319-7

Santos, R.; Heckler, L.; Lazarotto, M.; Garrido, L.; Rego, C.; Blume, E. 2016. *Trichoderma* spp. and *Bacillus subtilis* for control of *Dactylonectria macrodidyma* in grapevine. Phytopathol. Mediterr. 55:293–300. https://doi.org/10.14601/Phytopathol_Mediterr-18048

Schmidt, C.S.; Lorenz, D.; Wolf, G.A. 2001. Biological control of the grapevine dieback fungus *Eutypa lata* I: Screening of bacterial antagonists. J. Phytopathol. 149:427–435. https://doi.org/10.1111/j.1439-0434.2001.tb03874.x

Soenens, A.; Imperial, J. 2020. Biocontrol capabilities of the genus *Serratia*. Phytochem Rev. 19:577–587. https://doi.org/10.1007/s11101-019-09657-5

Talavera, G.; Castresana, J. 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst. Biol. 56:564–77. https://doi.org/10.1080/10635150701472164

Trotel-Aziz, P.; Abou-Mansour, E.; Courteaux, B.; Rabenoelina, F.; Clément, C.; Fontaine, F.; Aziz, A. 2019. *Bacillus subtilis* PTA-271 counteracts Botryosphaeria dieback in grapevine, triggering immune responses and detoxification of fungal phytotoxins. Front. Plant Sci. 10:25. https://doi.org/10.3389/fpls.2019.00025

Úrbez-Torres, J.R.; Gubler, W.D. 2010. Susceptibility of grapevine pruning wounds to infection by *Lasiodiplodia theobromae* and *Neofusicoccum parvum*. Plant Pathol. 60:261–270. https://doi.org/10.1111/j.1365-3059.2010.02381.x

von Reuss, S.; Kai, M.; Piechulla, B.; Francke, W. 2010. Octamethylbicyclo[3.2.1]octadienes from *Serratia odorifera*. Angew. Chem. Int. 122:2053–2054. https://doi.org/10.1002/ange.200905680

Weise, T.; Thürmer, A.; Brady, S.; Kai, M.; Daniel, R.; Gottschalk, G.; Piechulla, B. 2014. VOC emission of various *Serratia* species and isolates and genome analysis of *Serratia plymuthica* 4Rx13. FEMS Microbiol. Lett. 352:45–53. https://doi.org/10.1111/1574-6968.12359

Wu, H.; Spagnolo, A.; Marivingt-Mounir, C.; Clément, C.; Fontaine, F.; Chollet, J.-F. 2020. Evaluating the combined effect of a systemic phenylpyrrole fungicide and the plant growth-promoting rhizobacteria *Paraburkholderia phytofirmans* (strain PsJN::gfp2x) against the grapevine trunk pathogen *Neofusicoccum parvum*. Pest Manag. Sci. 76:3838–3848. https://doi.org/10.1002/ps.5935

Ye, M.; Tang, X.; Yang, R.; Zhang, H.; Li, F.; Tao, F.; Li, F.; Wang, Z. 2018. Characteristics and application of a novel species of *Bacillus*: *Bacillus velezensis*. ACS Chem. Biol. 13:500–505. https://doi.org/10.1021/acschembio.7b00874

Tables

Table 1. Selected isolates of endophytic and rhizospheric bacteria obtained from commercial grapevines in California for *in vitro* antagonism and metabolite assays against GTD-causing pathogens.

Species	Isolate	Tissue	Vine Health Status	County	Cultivar
Bacillus velezensis	UCD10614	Cordon	Asymptomatic	Santa Barbara	Pinot Noir
	UCD10631	Trunk	Asymptomatic	San Luis Obispo	Cabernet Sauvignon
Pseudomonas chlororaphis	UCD10757	Rhizosphere	Symptomatic	Monterey	Chardonnay
	UCD10763	Rhizosphere	Asymptomatic	Riverside	Scarlet Royal
Serratia plymuthica	UCD10719	Rhizosphere	Asymptomatic	Fresno	Thompson Seedless
	UCD10756	Rhizosphere	Asymptomatic	Tulare	Thompson Seedless

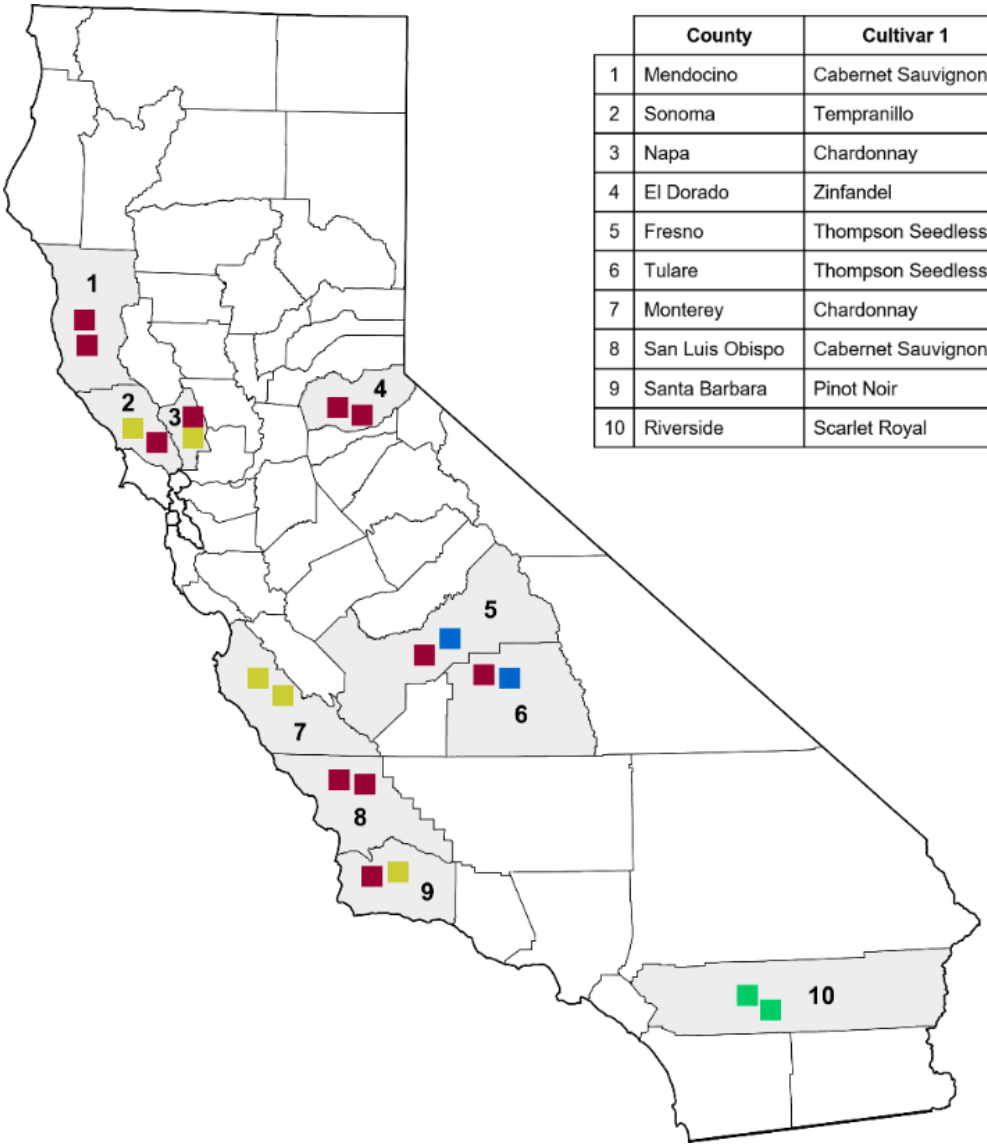
Table 2. GenBank accession numbers of strains used in the phylogenetic analysis of the *Bacillus subtilis* species complex. Sequences generated in this study are highlighted in bold.

Species	**Strain**	**16S rRNA**	***gyrA***	***rpoB***	***polC***	***purH***	***groEL***
Bacillus amyloliquefaciens	ATCC 23350^{T}	NR_118950	FN597644: 7010-9469	FN597644: 122979-126560	FN597644: 1728311-1732624	FN597644: 668235-669773	FN597644: 572972-574606
Bacillus atrophaeus	NRS-213^{T}	NR_116190	EF026731	EU138861	EU138723	EU138792	EU138585
Bacillus cereus	ATCC 14579^{T}	NR_074540	CP034551: 6195-8666	CP034551: 113629-117162	CP034551: 139550-140494	CP034551: 308734-310269	CP034551: 257545-259179
Bacillus inaquosorum	NRRL B-23052^{T}	KT989848	GQ488737	EU138812	EU138674	EU138743	EU138536
Bacillus licheniformis	ATCC 14580^{T}	X68416	AE017333: 6900-9368	AE017333: 120755-124336	AE017333: 1832492-1836808	AE017333: 707082-708620	AE017333: 626726-628360
Bacillus mojavensis	NRRL B-14698^{T}	AB021191	EU138598	EU138805	EU138667	EU138736	EU138529
Bacillus nakamurai	NRRL B-41091^{T}	LSAZ01000028	LSAZ01000005: 218-2686	LSAZ01000009: 20722-24303	LSAZ01000041: 267992-272305	LSAZ01000023: 39384-40922	LSAZ01000021: 7656-9290
Bacillus pumilus	NRRL NRS-272^{T}	NR_116191	EU138655	EU138862	EU138724	EU138793	EU138586
Bacillus siamensis	KCTC 13613^{T}	MN176482	AJVF01000039: 264-2723	KC608574	AJVF01000013: 140847-144185	AJVF01000023: 36481-38019	AJVF01000023: 98236-99870
Bacillus sonorensis	NRRL B-23154^{T}	AF302118	EU138611	EU138818	EU138680	EU138749	EU138542
Bacillus spizizenii	NRRL B-14472^{T}	CP002183: 9750-11308	EF134424	CP002183: 116233-119814	CP002183: 1683631-1687944	CP002183: 676895-678433	CP002183: 628489-630114
Bacillus subtilis	NRRL NRS-744^{T}	NR_116192	NC_000964: 6994-9459	NC_000964: 121919-125500	NC_000964: 1727133-1731446	NC_000964: 708594-710132	NC_000964: 650234-651868
Bacillus tequilensis	NRRL B-41771^{T}	NR_104919	EU138625	EU138832	EU138694	EU138763	EU138556
Bacillus vallismortis	NRRL B-14890^{T}	AB021198	EU138601	EU138808	EU138670	EU138739	EU138532
Bacillus velezensis	NRRL B-41580^{T}	AY603658	EU138622	EU138829	EU138691	EU138760	EU138553
Bacillus velezensis	NRRL BD-545	-	EU138626	EU138833	EU138695	EU138764	EU138557
Bacillus velezensis	NRRL BD-568	-	EU138631	EU138838	EU138700	EU138769	EU138562
Bacillus velezensis	NRRL BD-621	-	EU138650	EU138857	EU138719	EU138788	EU138581
Bacillus velezensis	**CE100**	**OP550064**	**OP561954**	**OP561962**	**OP561970**	**OP561978**	**OP561986**
Bacillus velezensis	**UCD10598**	**OP550065**	**OP561955**	**OP561963**	**OP561971**	**OP561979**	**OP561987**
Bacillus velezensis	**UCD10599**	**OP550066**	**OP561956**	**OP561964**	**OP561972**	**OP561980**	**OP561988**
Bacillus velezensis	**UCD10600**	**OP550067**	**OP561957**	**OP561965**	**OP561973**	**OP561981**	**OP561989**
Bacillus velezensis	**UCD10607**	**OP550068**	**OP561958**	**OP561966**	**OP561974**	**OP561982**	**OP561990**
Bacillus velezensis	**UCD10613**	**OP550069**	**OP561959**	**OP561967**	**OP561975**	**OP561983**	**OP561991**
Bacillus velezensis	**UCD10614**	**OP550070**	**OP561960**	**OP561968**	**OP561976**	**OP561984**	**OP561992**
Bacillus velezensis	**UCD10631**	**OP550071**	**OP561961**	**OP561969**	**OP561977**	**OP561985**	**OP561993**

Table 3. GenBank accession numbers of strains used for phylogenetic analyses of *Pseudomonas* spp. and *Serratia* spp. Sequences generated on this study are highlighted in bold.

Species	**Strain**	**16S rRNA**
Pseudomonas aeruginosa	LMG 1242^T	Z76651
Pseudomonas brassicacearum	DBK11^T	NR_024950
Pseudomonas chlororaphis subsp. *aurantiaca*	NCIB 10068^T	DQ682655
Pseudomonas chlororaphis subsp. *aureofaciens*	DSM 6698^T	AB680099
Pseudomonas chlororaphis subsp. *chlororaphis*	DSM 50083^T	KX186940
Pseudomonas chlororaphis subsp. *piscium*	JF3835^T	FJ168539
Pseudomonas chlororaphis	**UCD10653**	**OP550072**
Pseudomonas chlororaphis	**UCD10746**	**OP550078**
Pseudomonas chlororaphis	**UCD10748**	**OP550079**
Pseudomonas chlororaphis	**UCD10757**	**OP550080**
Pseudomonas chlororaphis	**UCD10763**	**OP550083**
Pseudomonas donghuensis	HYS^T	NR_136501
Pseudomonas donghuensis	**UCD10759**	**OP550081**
Pseudomonas granadensis	F-278,770^T	HG764746
Pseudomonas granadensis	**UCD10729**	**OP550074**
Pseudomonas koreensis	Ps 9-14^T	NR_025228
Pseudomonas koreensis	**UCD10732**	**OP550075**
Pseudomonas cf. *koreensis*	**UCD10666**	**OP550073**
Pseudomonas cf. *koreensis*	**UCD10738**	**OP550076**
Pseudomonas cf. *koreensis*	**UCD10739**	**OP550077**
Pseudomonas kribbensis	46-2^T	KT321658
Pseudomonas monteilii	CIP 104883^T	AF064458
Pseudomonas plecoglossicida	NBRC 103162^T	BBIV01000080
Pseudomonas cf. *plecoglossicida*	**UCD10762**	**OP550082**
Pseudomonas putida	NBRC 14164^T	NR_113651
Pseudomonas reinekei	MT1^T	AM293565
Pseudomonas taiwanensis	BCRC 17751^T	EU103629
Pseudomonas vranovensis	CCM 7279^T	AY970951
Serratia entomophila	DSM 12358^T	NR_025338
Serratia ficaria	DSM 4569^T	A5233428
Serratia marcescens	DSM 30121	AJ233431
Serratia odorifera	DSM 4582^T	A5233432
Serratia plymuthica	DSM 4540^T	NR_114579
Serratia plymuthica	DSM 49	AF286871
Serratia plymuthica	CKQ9	OP035846
Serratia plymuthica	CTB4	OP102591
Serratia plymuthica	KAR18	KR054980
Serratia plymuthica	PR	ON337524
Serratia plymuthica	**UCD10719**	**OP550084**
Serratia plymuthica	**UCD10756**	**OP550085**
Serratia proteamaculans	DSM 4543^T	NR_025341
Serratia quinivorans	DSM 4597^T	NR_037112

Figures



	County	Cultivar 1	Cultivar 2
1	Mendocino	Cabernet Sauvignon	Merlot
2	Sonoma	Tempranillo	Sauvignon Blanc
3	Napa	Chardonnay	Cabernet Sauvignon
4	El Dorado	Zinfandel	Syrah
5	Fresno	Thompson Seedless	Pinot Gris
6	Tulare	Thompson Seedless	Pinot Gris
7	Monterey	Chardonnay	Chardonnay
8	San Luis Obispo	Cabernet Sauvignon	Cabernet Sauvignon
9	Santa Barbara	Pinot Noir	Chardonnay
10	Riverside	Scarlet Royal	Scarlet Royal

Figure 1. Sampled vineyards across California showing counties, type of vineyard (in colored squares) and cultivars. Red squares = red wine, yellow squares = white wine, blue squares = raisin, green squares = table grape.

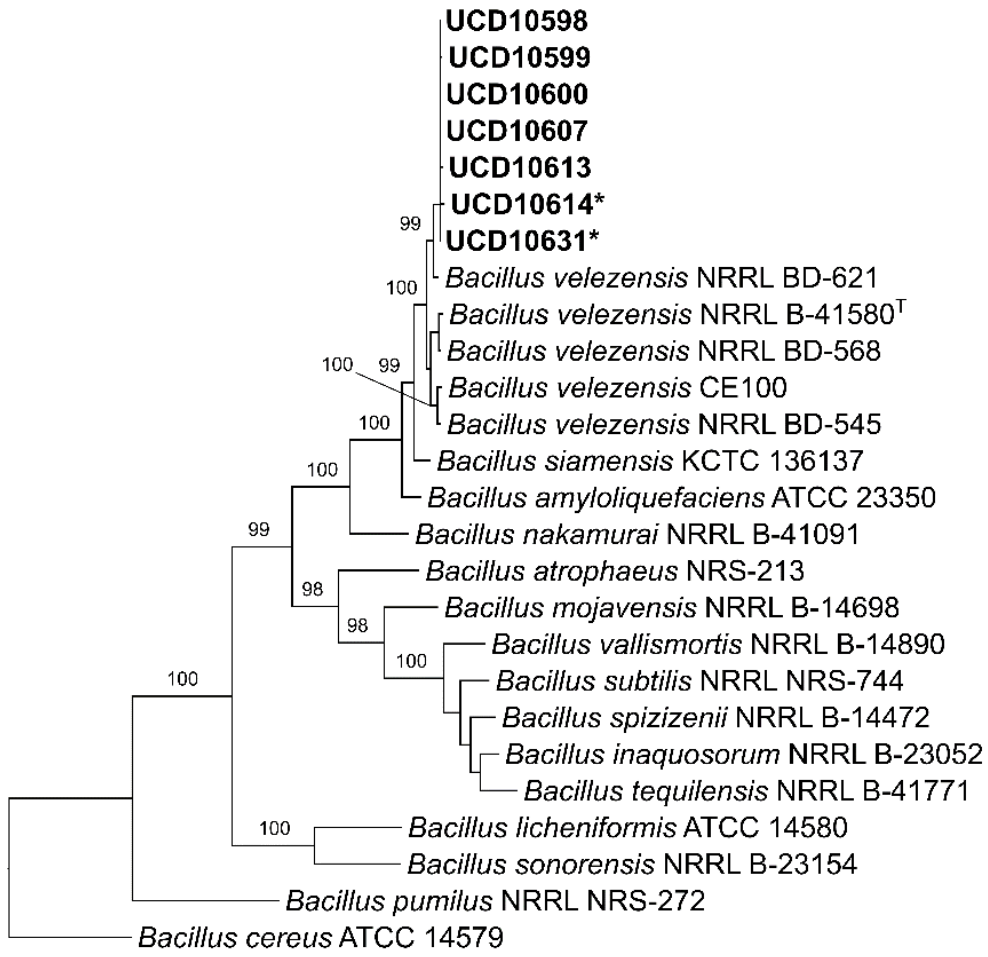


Figure 2. Most parsimonious phylogenetic analysis of seven isolates of *Bacillus velezensis* recovered from commercially grown various grapevine cultivars in California compared to closely related strains and species. The tree was inferred from a six-locus data set (16S rDNA-*gyrA*-*rpoB*-*purH*-polC-*groEL*). Numbers above branches represent non-parametric bootstrap values from 1000 replicates. *B. cereus* (ATCC 14579) was used as outgroup. ^{T} = type strain of *B. velezensis* (NRRL B-41580 = CR-502). * = isolates used in dual antagonism assays and metabolites analyses.

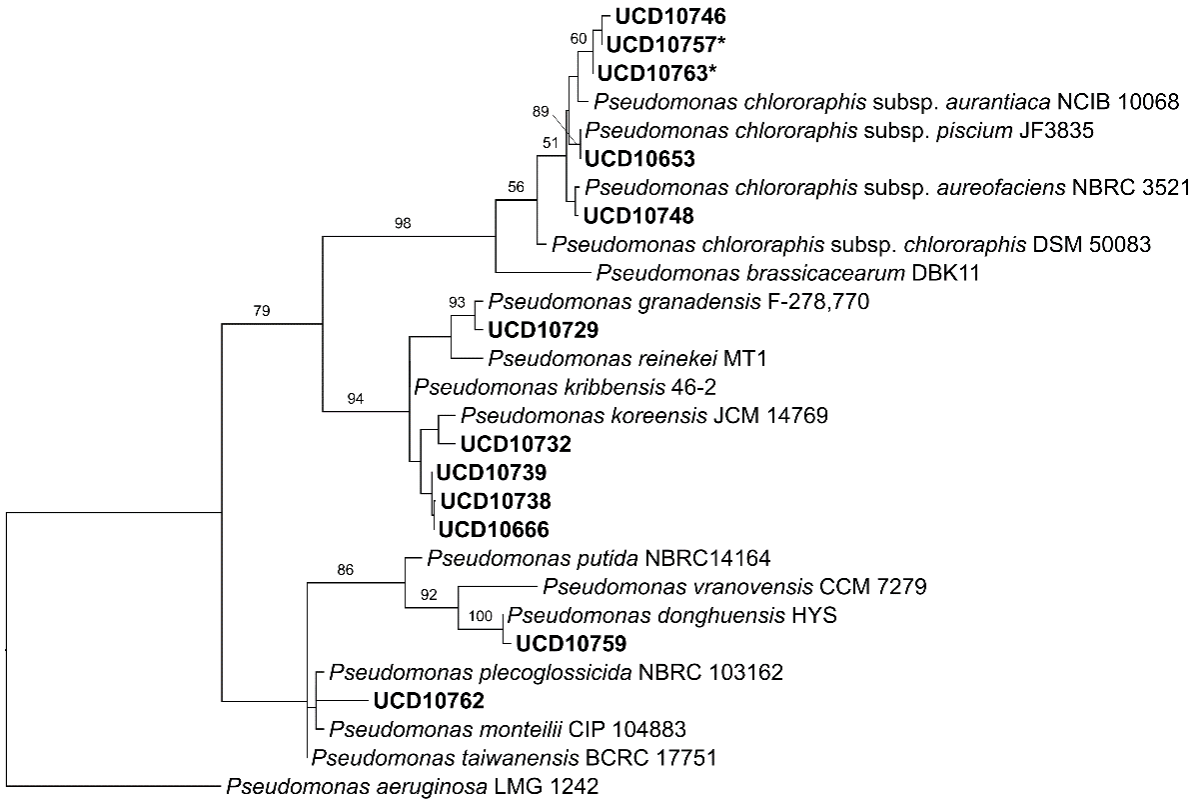


Figure 3. Most parsimonious phylogenetic analysis of 12 isolates of *Pseudomonas* spp. recovered from commercially grown various grapevine cultivars in California compared to closely related species. The tree was inferred with sequences of the 16S rDNA gene. Numbers above branches represent non-parametric bootstrap values from 1000 replicates. *P. aeruginosa* (LMG 1242) was used as outgroup. * = isolates used in dual antagonism assays and metabolites analyses.

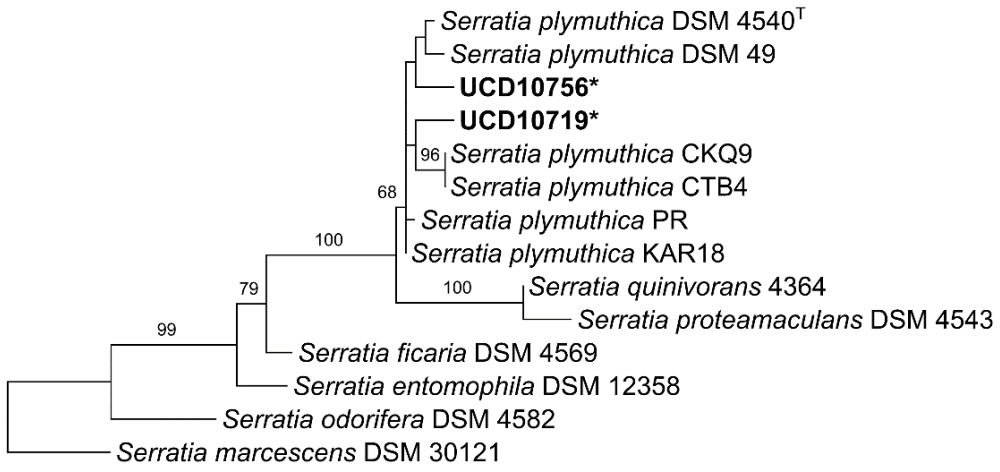


Figure 4. Most parsimonious phylogenetic analysis of two isolates of *Serratia plymuthica* obtained from commercial grapevines in California compared to closely related species. The tree was inferred with sequences of 16S rDNA gene. Numbers above branches represent non-parametric bootstrap values from 1000 replicates. *S. marcescens* (DSM 30121) was used as outgroup. ^{T} = type strain of *S. plymuthica* (DSM 4540 = K−7). * = isolates used in dual antagonism assays and metabolites analyses.

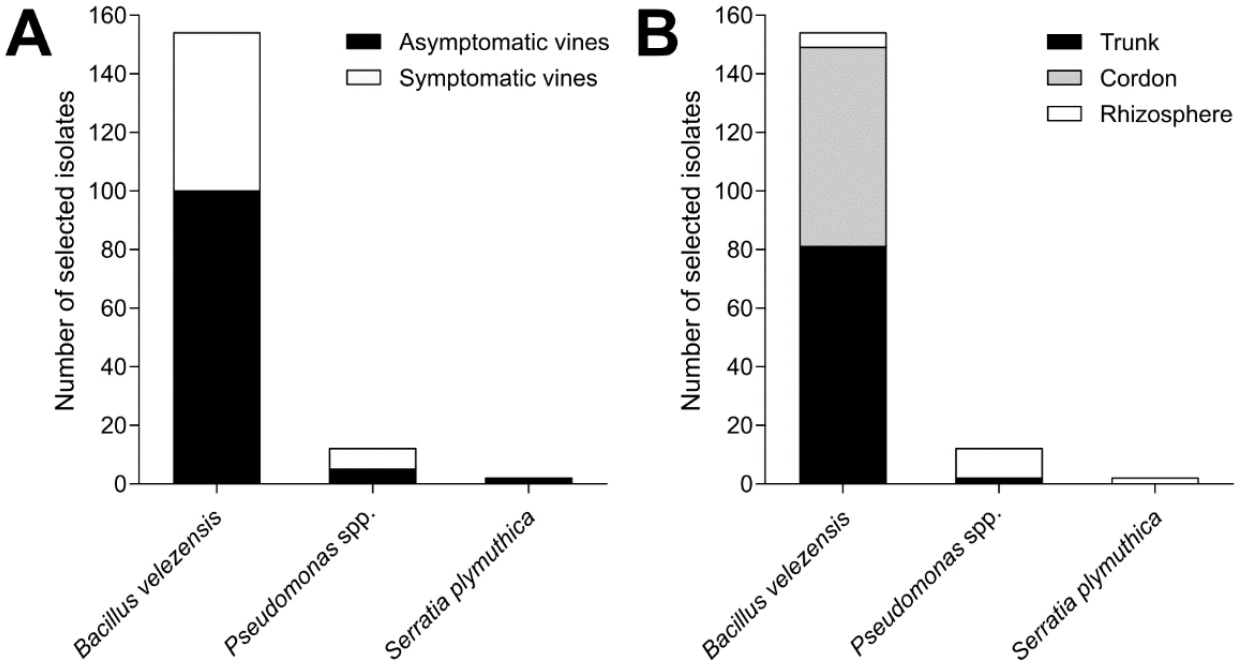


Figure 5. Distribution of selected bacterial isolates (*n* = 172) that showed inhibition levels over 40% against the mycelial growth of *Neofusicoccum parvum* and *Diplodia seriata* according to the vine health status (**A**) and the tissue they were recovered from (**B**).

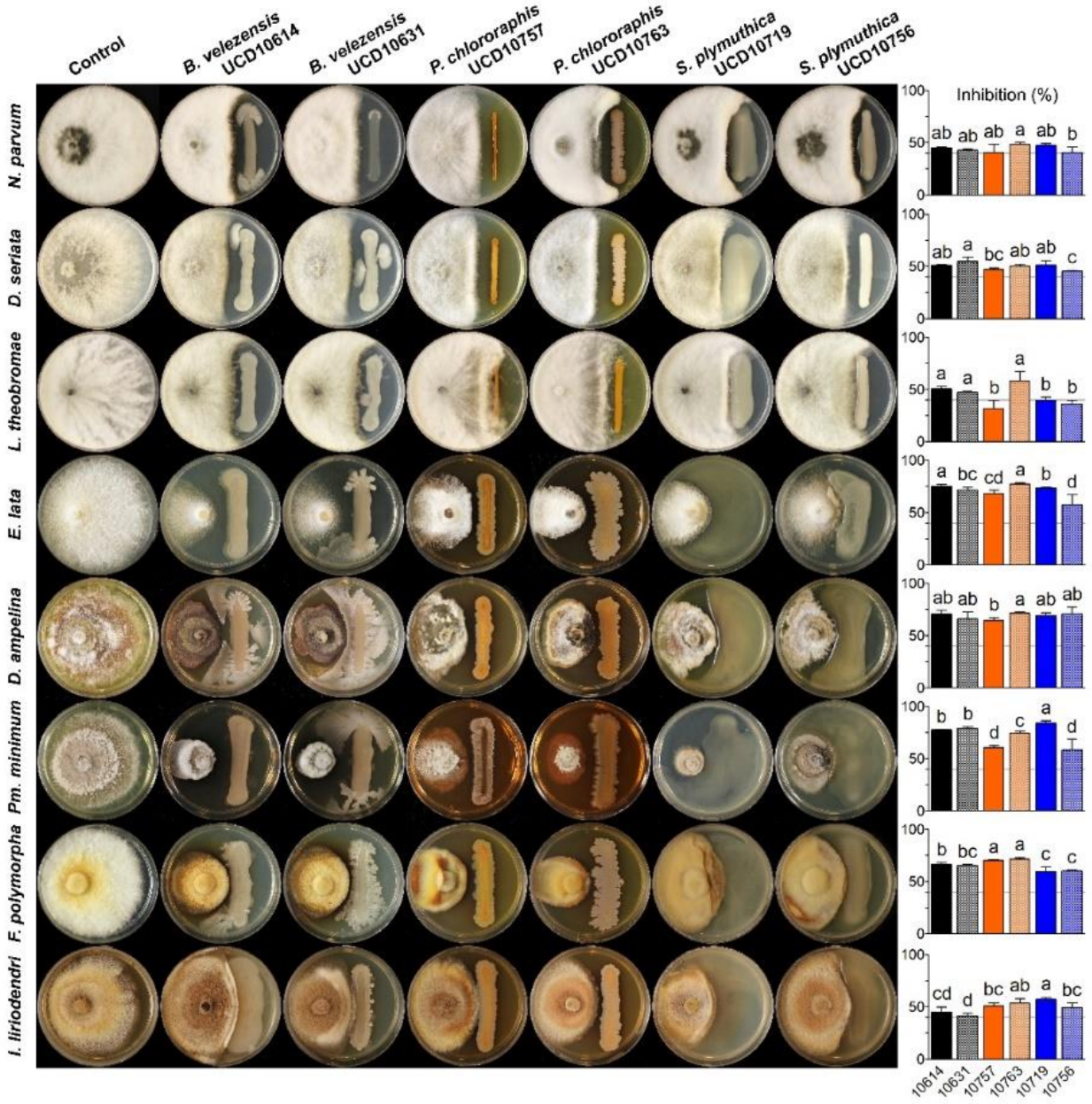


Figure 6. Inhibition levels (%) caused by selected isolates of *B. velezensis*, *P. chlororaphis* and *S. plymuthica* against *Neofusicoccum parvum*, *Diplodia seriata*, *Lasiodiplodia theobromae*, *Eutypa lata*, *Diaporthe ampelina*, *Phaeoacremonium minimum*, *Fomitiporia polymorpha*, and *Ilyonectria liriodendri*. Means with the same letter horizontally on each graph are not significantly different from each other according to the Fisher's LSD test ($p > 0.05$). Gray line represents the threshold of 40% of inhibition.

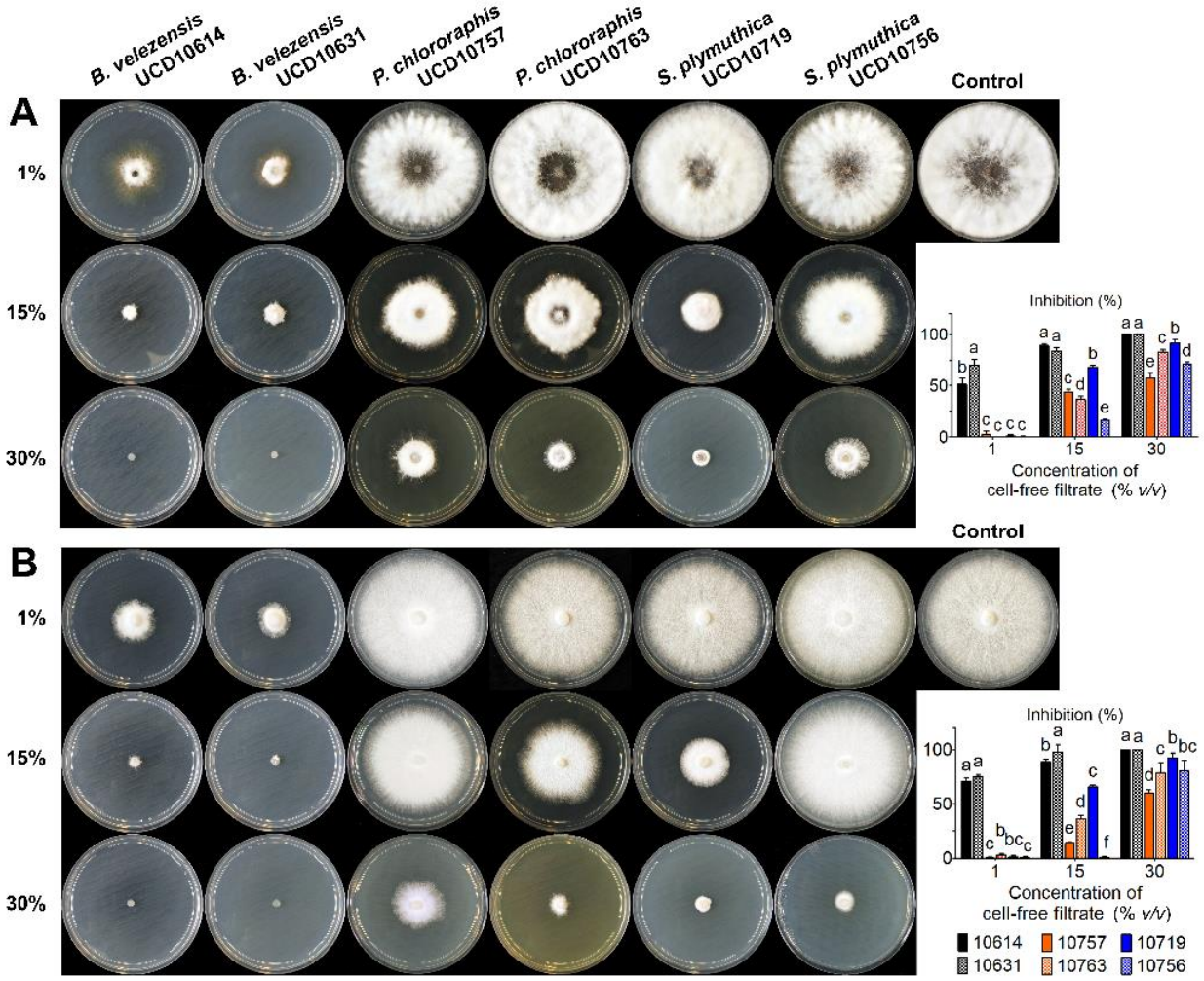


Figure 7. Inhibition levels (%) of increasing concentrations of agar-diffusible metabolites produced by selected bacterial isolates against the mycelial growth of *N. parvum* (**A**) and *E. lata* (**B**). On each graph, means with the same letter within each level of filtrate concentration are not significantly different from each other according to the Fisher's LSD test ($p > 0.05$). Legend at the bottom right shows isolate codes for both graphs.

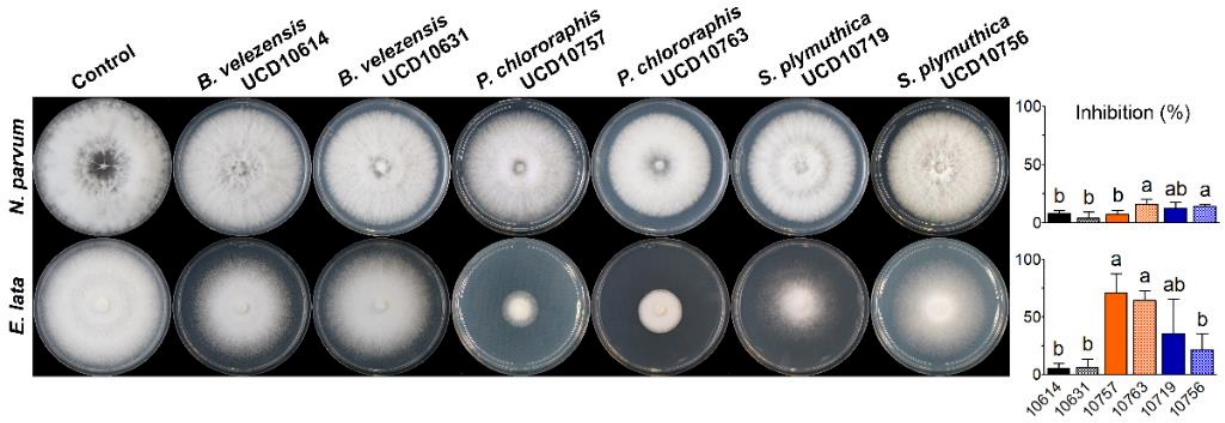


Figure 8. Inhibition levels (%) of volatile organic compounds (VOCs) produced by selected isolates of *B. velezensis*, *P. chlororaphis* and *S. plymuthica* against the mycelial growth of *N. parvum* (top) and *E. lata* (bottom). On each graph, means with the same letter horizontally are not significantly different from each other according to the Fisher's LSD test ($p > 0.05$).

Chapter III

Field Evaluation of Endophytic and Rhizospheric Bacteria as Biocontrol Agents Against Grapevine Trunk Diseases

Abstract

Grapevine trunk diseases (GTD) represent a serious threat to the sustainability of viticulture worldwide and their management remains challenging. The current trend of reducing the use of synthetic pesticides makes biocontrol an environmentally friendly strategy to mitigate the impact of GTDs. Three bacterial isolates obtained from woody tissues and the rhizosphere of grapevines were selected based on their *in vitro* antifungal activity against GTD-causing pathogens and further evaluated as biocontrol agents (BCAs) under field conditions. The BCAs were grown in liquid media and then delivered through four approaches: (i) infiltrated in dormant propagation material before grafting in nursery settings; (ii) applied as a soil drench to grapevines in the vineyard; (iii) injected into the trunk and cordons of grapevines; and (iv) sprayed onto dormant pruning wounds of mature grapevines. Results revealed that isolates of *Bacillus velezensis* (UCD10631) and *Pseudomonas chlororaphis* (UCD 10763) exerted a positive effect when infiltrated in propagation material and as soil drench treatments by reducing the size of lesions caused by artificially inoculated GTD pathogens. When comparing the levels of disease control, a better performance was observed against *Eutypa lata* and *Phaeoacremonium minimum* than against *Neofusicoccum parvum*.

Introduction

Grapevine trunk diseases (GTD) are considered a major threat to viticulture worldwide. The chronic infections caused by different fungal pathogens result in rapid and/or slow decline of the different structures of the grapevine that eventually can lead to plant death. The symptoms include cankers and dieback in spurs, cordons and trunk, chlorosis in leaves and shortened internodes, and stunted growth in shoots. Symptoms may appear sequentially or simultaneously, depending on factors associated with the fungal species involved, the plant health status and environmental conditions. These destructive diseases are caused by numerous unrelated wood-colonizing fungi that infect vines through wounds (Eskalen and Gubler, 2001; Eskalen et al. 2007; Úrbez-Torres and Gubler, 2009; 2010; Rolshausen et al. 2010). The propagation process in nurseries and the constant pruning in vineyards involve several wounding events, thus increasing the plant susceptibility to infections (Gramaje et al. 2017). A single vine can be infected by more than one fungal species, leading to more severe symptoms. Additionally, some of these pathogens

can cause latent infections, which remain unnoticed until the right conditions occur such as vine stress or favorable environmental conditions for fungal growth and colonization (Graniti et al. 2000; Czemmel et al. 2015; Hrycan et al. 2020).

Current management strategies of GTDs are limited, highly dependent on the disease and the pathogens involved, and vary among geographical regions (Gramaje et al. 2017). Since complete eradication is not possible, alternative management options require a multidisciplinary approach including sanitation, cultural practices, prevention, biocontrol, and mitigation (Mondello et al. 2018). Previous studies have demonstrated that hot water treatments (53°C for 30 min) of propagation material in nurseries can reduce the viability of these pathogens but not eliminate them (Elena et al. 2015). However, beneficial microorganisms within the plant tissues can also be eliminated, affecting the grapevine tolerance to fungal attack due to a disturbed balance between the microbial communities and the plant defense (Schulz and Boyle, 2005). This has been demonstrated in several studies, in which microbial communities from the grapevine plant (endophytes) and from the rhizosphere have a positive effect on the plant growth and also increased the tolerance to pathogens and to abiotic stress (Campisano et al. 2014; Rolli et al. 2017; Deyett et al. 2017; Guevara-Avendaño et al. 2019).

From a disease management perspective, biocontrol treatments have been demonstrated to reduce the incidence of grapevine trunk diseases (Fourie et al. 2001; Kotze et al. 2011; Yacoub et al. 2016). In California, commercially available biofungicides for grapevine are mainly either single or mixed strains of *Bacillus* or *Trichoderma* species. In general, some of the challenges for BCA performance are host colonization efficiency and/or in reduced persistence in the plant tissues under environmental conditions different from their original habitat (John et al. 2008, Aloi et al. 2015; Pertot et al. 2016). In this regard, applications of endophytic bacteria isolated from the same host can not only improve the composition of natural microbial communities but also induce plant immune responses and detoxify pathogen phytotoxins, resulting in effective protection against trunk diseases (Schmidt et al. 2001; Alfonzo et al. 2009; Haidar et al. 2016; Trotel-Aziz et al. 2019; Niem et al. 2020). Previous studies have also shown that endophytic microbes can move systemically within the plant and produce metabolites that have antifungal properties that can reduce the incidence of plant pathogens (Compant et al. 2011; Fan et al., 2018). Moreover, the application of beneficial rhizospheric bacteria into the soil can improve the health status of the soil and contribute to carbon sequestration (Dries et al. 2021).

Previously, we screened over 1,300 endophytic and rhizospheric bacterial isolates from seemingly healthy and symptomatic vineyards across California for their potential as biocontrol agents against different GTD-causing pathogens (Bustamante et al. 2022). Three bacterial species and their secondary metabolites showed promising results *in vitro* against eight pathogens responsible for Botryosphaeria dieback (*Neofusicoccum parvum*, *Diplodia seriata* and *Lasiodiplodia theobromae*), Eutypa dieback (*Eutypa lata*), Phomopsis dieback (*Diaporthe ampelina*), black foot (*Ilyonectria liriodendri*), and esca (*Phaeocremonium minimum* and *Fomitiporia polymorpha*). Selected bacterial isolates were identified as *Bacillus velezensis*, *Pseudomonas chlororaphis* and *Serratia plymuthica*. In this study, we evaluated these beneficial bacterial isolates in nursery and vineyard settings using different delivery methods. The results from this study may help in the development of effective IPM programs to control GTDs.

Materials and Methods

Bacterial and fungal isolates

Three bacterial isolates, namely *B. velezensis* (*Bv* UCD10631), *P. chlororaphis* (*Pc* UCD10763), and *S. plymuthica* (*Sp* UCD10719) were initially selected based on their antagonistic activity *in vitro* against eight common trunk pathogens (Bustamante et al. 2022). In this study, the same isolates were also evaluated as biocontrol agents against three GTD-associated pathogens in both nursery and vineyard settings. The bacterial isolates were grown in Luria-Bertani (LB) broth in an orbital shaker (150 rpm) at 28 °C for 7 days (Incu-Shaker^{TM} 10L, Benchmark Scientific, Sayreville, NJ, USA). The bacterial cultures were diluted in sterile distilled water at 1:100 for *Bv* UCD10631 and 1:10 for *Pc* UCD10763 and *Sp* UCD10719 based on the *in vitro* results associated with effective concentrations of secondary metabolites produced by these isolates against trunk pathogens (Bustamante et al. 2022). The fungal isolates used to perform artificial inoculations of treated vines were *N. parvum* (UCD7395), *E. lata* (UCD7746), and *P. minimum* (UCD7770), all obtained from the fungal collection of the Eskalen laboratory at the Department of Plant Pathology, University of California, Davis, and originally isolated from GTD-symptomatic grapevines.

Infiltration of dormant propagation material

The trial was conducted in three commercial nurseries during the winter of 2022. Cultivars included Chardonnay or Cabernet Sauvignon for scions, and 1103 Paulsen for rootstocks. Prior to grafting, bundles of dormant scion and rootstock cuttings (100 per treatment) were soaked in 50-L of suspensions each treatment in an aluminum vacuum chamber (Best Value Vacs, Naperville, IL, USA). The cuttings were harvested from the mother block 4 to 5 months before a cold storage period and later transported to a room at 25 °C to perform the experiment. Treatments included the bacterial isolates of *B. velezensis* (UCD10631, 1% v/v), *P. chlororaphis* (UCD10763, 10% v/v), and *S. plymuthica* (UCD10719, 10% v/v), commercially available biofungicides Serifel (a.i. *B. amyloliquefaciens* strain MBI600) and Vintec (a.i. *Trichoderma atroviride* strain SC1), the commercial synthetic fungicide Topsin M (a.i. thiophanate-methyl, 70%), and a water control. These treatments were diluted in non-chlorinated tap water according to label rate. The cutting bundles were submerged in the treatment solution and pressurized up to −15 inHg for 10 minutes. After the treatments, scions and rootstocks were stored in cold storage until they were grafted according to each nursery's protocol. Following grafting, plants were incubated in humid chambers to induce callus formation for about 10 days. The percentage of callus formation was evaluated to determine the potential effects of the treatment on the viability of grafted vines. The evaluation consisted of estimating the coverage area of the callus around the graft union and the basal end of the rootstock. A rating scale from 1 to 5 was used to group the percentages of callus formation, where 1 corresponded to full callused around the cutting (100%), 2 to 80-99%, 3 to 60-79%, 4 to 40-59%, and 5 to poor coverage or no development (40% or lower). Later, vines were planted in pots and incubated for 90 days in a greenhouse for rooting. Treated vines were further artificially inoculated with three fungal pathogens (*N. parvum*, *E. lata*, and *P. minimum*) using toothpicks that were previously colonized by the mycelium of each pathogen on APDA plates. For this, the vines corresponding to each treatment were divided into subgroups prior to the inoculations. The internode area of the rootstock was surface disinfected with 70% ethanol and once dry, it was drilled using a sterile 2.38-mm diameter drill bit up to approximately 3-mm of depth. A colonized toothpick (2 mm long) corresponding to each pathogen was manually inserted into the drilled wounds and wrapped with Parafilm (Bemis Co., Neenah, WI, USA) to avoid desiccation of the inoculum. Treated vines were then incubated for 3 months in the greenhouse and then transported to the laboratory for further evaluation. The bark around the inoculation point

was peeled off to expose discolored wood and lesions were measured with a digital caliper and isolations were performed from the margin of the discolored tissue onto potato dextrose agar acidified with 92% lactic acid (APDA) at 0.5 mL/L. Percentages of isolation of the pathogens and lesion length data were subjected to analysis of variance (ANOVA) using generalized linear models with the corresponding R packages in InfoStat v2008. Normality and homoscedasticity were checked and corrected when necessary and means were separated using Fisher's least significant difference test ($p < 0.05$).

Soil drench applications

The biocontrol soil drench field trials were conducted on 12-years-old Cabernet Franc vines, trained with bilateral cordon system and located at the research field station of the Plant Pathology Department of UC Davis (38.52242579764458, -121.75734549826203). Each vine was treated in the summer (July 2022) with 1 liter of solution as soil drench around the closest irrigation emitters. The trial included the above-mentioned bacterial isolates, the biopesticides Bio-Tam (a.i. *Trichoderma asperellum* strain ICC012 and *T. gamsii* strain ICC080), GCM (a.i. *B. velezensis* strain CE100), CrabLife Powder (a.i. chitin), the synthetic fungicide Rhyme (a.i. flutriafol, 22.7%), and water control. A total of nine vines were used for each treatment, and the trial was arranged in a completely randomized block design. After 30 days post-treatments, the GTD-causing pathogens *N. parvum*, *E. lata*, and *P. minimum* were inoculated using the same toothpick inoculum method described above on each cordon: 10 cm and 30 from the beginning of the cordon. Each pathogen was inoculated in one cordon of each vine with two technical replicates, completing three biological replicates per treatment. After fourteen weeks (November 2022), inoculated cordons were inspected for vascular discoloration by removing the bark with a clean knife and the length of the resulting lesions was measured. Discolored wood samples (3 x 5 mm) of the vascular streaking were plated on APDA to determine the percentage of recovery (%) of the fungal pathogens.

Trunk injections

This trial was carried out in a separate plot of the same experimental vineyard used for the soil drench trial. Vines were treated in June 2022 by injecting the three beneficial bacterial isolates in the trunk and two cordons as a preventative treatment against GTDs. A volume of approximately 300 mL of each treatment was injected using a QUIK-jet AIR® device (Arborjet, Woburn, MA). Four holes were drilled up to the depth of the pith, two at approximately 5 cm below and above the graft union and two at 15 cm from the beginning of each cordon, to ensure a proper fit of the four injectors into the woody tissue. The device was pressurized up to 30 psi and the valves of the four injectors were opened to allow the solution to circulate through before they were inserted into each hole. The treatments were pumped through the injectors for 10 min until the volume of the tank was exhausted. After 30 days following the treatments, *N. parvum* was inoculated using the same toothpick inoculum method described above on each site of the cordons: 5 cm and 15 cm from the injection point on the cordon. A total of twelve vines were used for each treatment, and the trial was arranged in a completely randomized block design. Fourteen weeks later, inoculated cordons were examined for vascular discoloration by removing the bark with sterile knives and measuring the length of the discolored lesions. Samples of the vascular streaking were taken in sterile tubes and transported to the laboratory for isolations on APDA plates. Five pieces of sampled wood were cut (3 × 5 mm) and plated on APDA to determine pathogen survival, estimated as percentage of recovery (%) out of the total samples per treatment.

Pruning wound protection trial

Another separate plot of the same Cabernet Franc vineyard was used for pruning wounds protection trial against GTD-causing pathogens. Vines were spur pruned (3 buds) in early March of 2022 while vines were dormant, and the fresh pruning wounds were immediately sprayed with different treatments using a 1-L hand-held spray bottle until runoff. After five days, the treated canes were inoculated with a 20-µL spore suspension (10^5 conidia/mL) of *N. parvum*. The treatments included the three bacterial isolates and other experimental and commercial fungicides (Table 6). A total of four vines with 5 spurs on each vine were used for each treatment, and the trial was arranged in a completely randomized block design. Controls were treated with sterile distilled water. After a six months incubation period, treated spurs were removed and transported

to the laboratory for further evaluation. The spurs were cut in half and vascular streaking was measured with a digital caliper. Small pieces of discolored tissue (3 × 5 mm) were cultured on APDA to determine the recovery of the pathogen. The efficacy of each treatment was determined by the percent of recovery of *N. parvum*. Percentages of inhibition were subjected to analysis of variance (ANOVA) using generalized linear models with the corresponding R packages in InfoStat v2008. Normality and homoscedasticity were checked and corrected when necessary and means were separated using Fisher's least significant difference test ($p < 0.05$).

Results

Infiltration of dormant propagation material

The treatments showed different effects on the callus formation at the basal end of the rootstocks and at the graft union (Figures 1 and 2). In general, the majority of treatments did not negatively affect the rootstock callusing in the two nurseries, when compared to the controls, with similar frequencies of rootstocks showing callusing levels between 80% and 100% (Figure 1). However, in nursery 1, the infiltration with *Sp* UCD10719 caused a 100% callusing failure, which led to the discard of this treatment. At the graft union, different frequencies of callusing levels were observed among the treatments when compared to the control (Figure 2). In nursery 1, the treatments with *Sp* UCD10719 and Topsin M caused significantly lower callusing levels, with about 100% and 68% of the vines showing very poor or no callus formation, respectively. In nursery 2, the treatment with the bacterial isolates of *Sp* UCD10719 and *P. chlororaphis* caused lower quality of callusing compared to the control and the other treatments, with about 100% and 45% of the vines showing poor or no callus formation, respectively. After three months, potted vines were transported to a greenhouse located in Davis, CA, and vines with poor or no growth were discarded. Figure 3 shows the proportion of disposed vines relative to the total number of vines per treatment expressed as mortality (%). In nursery 1, the mortality was twice as the water controls after the treatments with *Sp* UCD10719, Serifel, Vintec and Topsin M, whereas in nursery 2, only *Sp* UCD10719 caused mortality levels notably higher than the water control. In both nurseries, all the vines treated with *S. plymuthica* were discarded. Treated vines were further inoculated with *N. parvum*, *E. lata* and *P. minimum* and after three months the results of infection levels and lesion lengths were analyzed (Tables 1-3). With *N. parvum* no differences were detected

in both infection percentages and lesion length, however with *E. lata* and *P. minimum* differences were found in both infection percentages and lesion length in both nurseries. Specifically, in nursery 1, lesion lengths caused by *E. lata* were significantly shorter in all the treatments. In nursery 2, all the treatments except for Vintec caused shorter lesions relative to the water control. The infection percentages were not statistically different in both nurseries. Lesions caused by *P. minimum* were significantly smaller after all the treatments in nursery 1, whereas in the second nursery they were not different than the water control. In nursery 2, *Pc* UCD10763, Vintec and Topsin M caused significantly lower infection percentages than the control. Figure 4 shows the aspect of lesions caused by the three pathogens after the treatments. The aspect of the lesions is depicted in Figure 4, where beyond the length of lesions, it is noticeable that there are reductions in area and color with the treatments, whereas the controls display darker and more profuse lesions.

Soil drench applications and trunk injections of BCAs

Percentages of infection and lesion length values from inoculations made in vines that were received treatments as soil drench are shown in Table 4. No significant reductions were observed in infection percentage and lesion length when treated vines were inoculated with *N. parvum* and *P. minimum*. However, the lesions caused by *E. lata* were significantly smaller after the treatments with *Bv* UCD10631, with a reduction of about 32% compared to the control. Similarly, Rhyme and CrabLife Powder reduced the incidence of *E. lata* by approximately 40 and 60%, respectively. Moreover, the treatments with *Bv* UCD10631, and Rhyme reduced the incidence of *P. minimum* by about 50 and 60%, respectively, compared to the control. These results suggest that applications of the beneficial bacteria as soil drench can reduce the severity of GTDs pathogens, especially of *E. lata* and *P. minimum* that are responsible for Eutypa dieback and Esca diseases, respectively.

Trunk injections

Results of the trunk injection trial are shown in Table 5. The three bacterial isolates injected into the trunk and cordons did not significantly reduce the infection percentages and the lesion length caused by *N. parvum*, compared to the control. Controls reached 45.8% of infection and

necrotic lesions of 48.5 mm in average, whereas the bacterial isolates caused infection percentages from 32.6 to 56.3% and lesions from 43.7 to 57.9 mm in average.

Pruning wound protection trial

The protection of pruning wounds showed that significant reductions in infection levels by *N. parvum* were achieved with certain treatments. The mean percentages of *N. parvum* infection (MPI) varied from 0 to 64.4% among the treatments (Table 6). Although no significant differences were detected between the positive control and the treatments, a higher control trend was observed with synthetic fungicides, sealants and fungal biofungicides, where MPI values ranged from 6.7 to 13.3%, which translates into control levels between 66.8 and 83.3%. The biofungicides based on *Trichoderma* spp. and *Aureobasidium pullulans* reached the higher levels of control (MPI between 12.2 and 25.0%), when compared to the bacterial treatments that yielded MPI between 33.3 and 64.4%. The selected isolates of *Bv* UCD10631, *Pc* UCD10763 and *Sp* UCD10719 had consistent levels of control with other bacterial biofungicides such as GCM, Theia, and Howler, that are based on other strains of *B. velezensis, B. subtilis* and *P. chlororaphis*, respectively.

Discussion

The results presented herein correspond to the field evaluation of bacterial isolates selected from previous studies, that showed strong inhibitory activity *in vitro* against eight different grapevine trunk pathogens (Bustamante et al. 2022). Experiments were conducted in nursery and field settings by implementing four different control strategies and evaluated against common canker pathogens of grapevine in California.

The infiltration trial aimed to treat dormant grapevine materials prior to grafting through infiltration of the bacterial isolates and other biological and synthetic fungicides in nursery settings. After grafting, no overall negative effects were observed on the callus formation at the basal end of the rootstocks or at the graft union (Figures 1-2). However, the treatments with Topsin M and *Pc* UCD10763 reduced the callus formation at the graft union resulting in 68% and 45% of vines with poor or no callusing, respectively. Later, when grafted vines were planted in pots, the treatments with *Sp* UCD10719, Serifel, Vintec and Topsin M caused mortality levels twice greater

of that of the water-treated controls in nursery 1, whereas only *Sp* UCD10719 resulted in mortality levels higher than the control in nursery 2. In both nurseries, all vines treated with *Sp* UCD10719 were discarded due to their lack of growth (Figure 3). This grafting failure may be explained by potential phytotoxic effects of isolate *Sp* UCD10719. This isolate was grown for 7 days and diluted to reach a concentration of 10% v/v, which could have been detrimental to the dormant material. Although, it has been vastly reviewed that *S. plymuthica* produces a broad range of secondary metabolites that are collectively considered beneficial to plants in terms of growth promotion, defense activation and antimicrobial activity (Kalbe et al. 1996; Levenfors et al. 2004; Weise et al. 2014), some strains can be harmful to plants. Such is the case, for example, of strain A153 which has been used to suppress growth of dicotyledonous weeds (Weissman and Gerhardson, 2001; Weissmann et al. 2003). Since grapes are also a dicotyledonous species, there could be a plant-suppressive effect of isolate *Sp* UCD10719 when applied at 10% v/v. Lower concentrations might help to avoid this issue. It has been shown that certain compounds such as chitosan and salicylic acid used at high concentrations may be phytotoxic to grapevines (Elmer and Reglinski, 2006; Dagostin et al. 2011). Likewise, the application of high concentrations of extracts of *Penicillium chrysogenum* and *Microcystis aeruginosa* were phytotoxic to grapevine and tomato plants, respectively (Thuerig et al. 2006; Corbel et al. 2015). Subsequently, when the fungal pathogens were inoculated, no differences were detected in both infection levels and lesion length of vines inoculated with *N. parvum* (Table 1). However, the treatments showed less necrosis between the bark and the pith, which was evident by the contrast in color and extension of the lesions between the control and the treatments (Figure 4). The lack of significant differences between treatments can be explained by the high variability of the data, which is consistent with other nursery trials that have applied BCAs to control GTDs (Leal et al. 2023). Usually, BCAs are known to reduce disease severity and incidence, but their efficacy is not always constant (Collinge et al. 2022). Therefore, it can be expected that biocontrol assays show variable levels of disease control. On the other hand, when vines were inoculated with *E. lata* and *P. minimum*, significantly shorter lesions and/or lower levels of infection were detected with some treatments (Tables 2-3). Specifically with *E. lata*, all the treatments reduced the length of lesions in both nurseries, except for Vintec in nursery 2. However, overall infection levels were not significantly decreased. Similarly, after the inoculations with *P. minimum*, all treatments, except of the *Pc* UCD10763 one, significantly reduced the length of lesions in nursery 1. In nursery 2, nonetheless, *Pc* UCD10763 significantly

reduced the infection levels, along with Vintec and Topsin M. When comparing the aspect of the lesions of *E. lata* and *P. minimum*, a reduction pattern is observed where lesions have a less profuse necrotic area and less dark color in treated vines compared to the control (Figure 4). These results could be explained by the higher virulence of *N. parvum* compared to *E. lata* and *P. minimum*, resulting in larger necrotic lesions. Therefore, these results show a positive effect of both *Bv* UCD10631 and *Pc* UCD10763 applied separately in decreasing the severity of *N. parvum*, *E. lata* and *P. minimum*. These results are consistent with other studies that have applied BCAs in nursery settings aiming to manage GTDs. Leal et al. (2022) applied a strain of *B. subtilis* alone and in combination with Vintec against Botryosphaeria dieback and black foot, prior to and after grafting, with reductions in infection rates that were variable but significant against black foot pathogens but less effective against Botryosphaeriaceae. Martínez-Diz et al. (2020) treated grafted vines with different bacterial and fungal BCAs through root dipping and observed significant reductions in incidence and severity of black foot and Petri disease pathogens. However, the vine age played a role in the effectiveness of the treatments, with different results between 2- and 3-year-old vines.

The trial involving soil drench treatments of mature vines showed that the treatments including the bacterial isolates caused no significant reductions in infection levels nor lesions length caused by *N. parvum* and *P. minimum*. However, the application of *Bv* UCD10631 significantly reduced the lesions length caused by *E. lata* by about 32%. Despite the lack of significant differences in infection percentages between treatments, there were reductions that are relevant to note. Specifically, approximately 30% of average infection reductions were observed with *Bv* CE100 (GCM) against *N. parvum*, 20% with *Pc* UCD10763, *Sp* UCD10719 and *Bv* CE100, 40% with flutriafol and 60% with chitin against *E. lata*, 50% with *Bv* UCD10631 and 60% with flutriafol against *P. minimum*. These results suggest that the incorporation of beneficial bacteria into the rhizosphere can improve the health status of grapevines by reducing the infection levels of different trunk pathogens. This phenomenon has been recently described as rhizosphere engineering, which refers to the modification of the microbial communities by inoculating the soil with beneficial microorganisms or by applying specific fertilizers, following the worldwide trend of a cleaner viticulture (Dries et al. 2021).

The injection of the three bacterial isolates into the trunk and cordon of mature vines showed no significant positive effect in reducing the infection levels and length of lesions caused by *N. parvum* (Table 5). The use of trunk injections, or endotherapy, has been studied as an

alternative management strategy against multiple pests and diseases that affect woody plants (Berger and Laurent, 2019). In the context of GTDs, only synthetic fungicides have been applied as trunk injections against Esca and Eutypa dieback in Europe (Calzarano et al. 2004; Loskill et al. 2006; Dula et al. 2007; Darrieutort and Lecomte, 2007). These investigations have shown inconsistent results of disease reduction, and therefore the implementation of trunk injections has not been extensively studied. Nevertheless, up to date, no BCAs have been applied against GTDs using this approach. Therefore, this experiment constitutes the first attempt in evaluating trunk injections of endophytic and rhizospheric bacteria against *N. parvum* in grapevines. In other pathosystems, the injection of *B. amyloliquefaciens* and *Trichoderma* spp. were proved to be effective in reducing the necrotic lesions against *Phytophthora* spp. in branches of 30-year-old beech trees (Berger et al. 2015). It has been reviewed that alternative techniques of fungicidal delivery such as trunk injections or soil application can be costly, impractical and/or percolated by irrigation or rainfall (Bertsch et al. 2013).

Lastly, as pruning wound protectants, *Bv* UCD10631, *Pc* UCD10763 and *Sp* UCD10719 were consistent in preventing infections by *N. parvum* in grapevine canes. This was the case also for the bacterial biofungicides Theia, Howler, and GCM, that are based on other strains of *B. subtilis*, *P. chlororaphis*, and *B. velezensis*, respectively. However, the infection levels of *N. parvum* after these treatments were not significantly different than the water control (MPI of 40%), with some of them showing even higher levels of infection (50.0% to 64.4% MPI). Although with minimal significant differences, a trend was observed with synthetic fungicides, sealants and fungal biofungicides, that showed lower MPI values (6.7% to 13.3%), which translates into control levels between 66.8% and 83.3%. These results may be a consequence of a low survival rate of the living bacteria in the grapevine tissue after the application on the pruning wounds. Another explanation could be a low persistence of the secondary metabolites produced by the bacterial isolates in the pruning wound tissue, which can be denatured by environmental conditions. Similarly, Halleen et al. (2010) found that the treatment of *B. subtilis* strain EE onto pruning wounds minimally reduced the incidence of *E. lata* compared to the positive control, from 48.5 to 45.5%. Different results were detected by Blundell and Eskalen (2021), who found significant levels of control of *N. parvum* and *E. lata* after treating pruning wounds with *Bacillus*-based suspensions (isolates UCD8745 and CE100). Kotze et al. (2011) observed reductions from 37.5% to 10.7% in the incidence of *E. lata* with the application of *B. subtilis* strain EE. The high variability

in GTD control levels exerted by different BCAs is not surprising, given the multifactorial dependence of these treatments to the internal conditions of the grapevine and environmental factors affecting the performance of the BCA (Mutawila et al. 2011; 2016). Nevertheless, the protection of pruning wound protection is an important preventative strategy that helps growers to decrease the incidence of GTDs and the need of replanting, as well as to significantly increase their yields (Gispert et al. 2020).

Altogether, the results presented here show the positive effects of the application of endophytic and rhizospheric bacteria using different delivery methods to control or reduce the severity of GTDs. Although the disease control levels are not comparable to synthetic fungicides, biocontrol offers a sustainable alternative that agrees with the trend of reducing the use of synthetic pesticides (Pertot et al. 2017). Furthermore, BCAs have additional beneficial effects on plants, such as plant defense activation, growth stimulation, detoxification of fungal phytotoxins because of the ability of colonizing the plant tissues, which may result in longer protection periods and increase the overall plant fitness, thus reducing the expression of symptoms (Mondello et al. 2018). From the four different delivery methods of BCAs into grapevines evaluated in this study, the infiltration of propagation material prior to grafting and the soil drench application of *Bv* UCD10631 and *Pc* UCD10763 showed the most promising results, with reductions of either the infection percentages or lesion length caused by *E. lata* and *P. minimum*. The positive outcome from these methods could be utilized to develop an effective and sustainable integrated pest management program for GTDs. In this regard, Leal et al. (2021) demonstrated that the treatment of propagation material with *B. subtilis* PTA-271 alone and in combination with Vintec had a satisfactory protective effect of Chardonnay and Tempranillo vines against *N. parvum*. The combination of a bacterial and a fungal BCA had a stronger effect in Tempranillo, suggesting that the cultivar plays a significant role in performance of BCAs against pathogens. Moreover, Pertot et al. (2016) detected that in the propagation process, hydration was the most critical stage where applications of Vintec were most effective against esca-associated pathogens, when compared to callusing and pre-planting, which could improve the timing of these applications. In mature vines, soil drench treatments of these BCAs may be included along with fertilizer applications, enriching the rhizosphere (Dries et al. 2021). The results presented in this study may help to improve the efficacy of *Bv* UCD10631 and *Pc* UCD10763 as BCAs applied to reduce the impact of GTDs in nurseries and vineyards. Possible ways to achieve this are, among others, increasing the number

of applications, combining different isolates including fungal BCAs, and improving the formulation of the BCA (concentration, medium composition, fermentation conditions).

Literature cited

Alfonzo, A., Conigliaro, G., Torta, L., Burruano, S., Moschetti, G. 2009. Antagonism of *Bacillus subtilis* strain AG1 against vine wood fungal pathogens. *Phytopathologia Mediterranea* 48:155-158. https://doi.org/10.14601/Phytopathol_Mediterr-2886

Aloi, C., Reggiori, F., Bigot, G., Montermini, A., Bortolotti, P., Nannini, R., Osti, F., Mugnai, L., Di Marco, S. 2015. REMEDIER (*Trichoderma asperellum* and *Trichoderma gamsii*): a new opportunity to control the esca disease complex. Five years of results of field trial in Italy. *Phytopatol. Mediterr.* 54:430-431.

Aziz, A., Verhagen, B., Magnin-Robert, M., Couderchet, M., Clément, C., Jeandet, P., Trotel-Aziz, P. 2016. Effectiveness of beneficial bacteria to promote systemic resistance of grapevine to gray mold as related to phytoalexin production in vineyards. *Plant and Soil* 405: 141–153. https://doi.org/10.1007/s11104-015-2783-z

Berger, C., Laurent, F. 2019. Trunk injection of plant protection products to protect trees from pests and diseases. Crop Protection 124:10483. https://doi.org/10.1016/j.cropro.2019.05.025

Berger, G., Czarnocka, K., Cochard, B., Oszako, T., Lefort, F., 2015. Biocontrol endotherapy with *Trichoderma* spp. and *Bacillus amyloliquefaciens* against *Phytophthora* spp.: A comparative study with phosphite treatment on *Quercus robur* and *Fagus sylvatica*. *J. Agric. Sci. Technol. A* 5:428–439. https://doi.org/10.17265/2161-6256/2015.06.005

Bertsch, C., Ramirez-Suero, M., Magnin-Robert, M., Larignon, P., Chong, J., Abou-Mansour, E., Spagnolo, A., Clement, C., Fontaine, F. 2013. Grapevine trunk diseases: complex and still poorly undersood. *Plant Pathology* 62:249.265. https://doi.org/10.1111/j.1365-3059.2012.02674.x

Blundell, R., Eskalen, A. 2022. Evaluation of biological and chemical pruning wound protectants to control grapevine trunk disease pathogens *Eutypa lata* and *Neofusicoccum parvum*. Plant Health Progress 23(2):197-205. https://doi.org/10.1094/PHP-08-21-0113-RS

Bustamante, M.I., Elfar, K., Eskalen, A. 2022. Evaluation of the antifungal activity of endophytic and rhizospheric bacteria against grapevine trunk pathogens. Microorganisms 10:2035. https://doi.org/10.3390/microorganisms10102035

Calzarano, F., Di Marco, S., Cesari, A. 2004. Benefit of fungicide treatment after trunk renewal of vines with different types of esca necrosis. Phytopatol. Mediterr. 43:116-124. https://doi.org/10.14601/Phytopathol_Mediterr-1725

Campisano A., Antonielli L., Pancher M., Yousaf S., Pindo M., Perlot I. 2014. Bacterial endophytic communities in the grapevine depend on pest management. PLoS One 9(11):e112763. https://doi.org/10.1371/journal.pone.0112763

Collinge, D.B., Jensen, D.F., Rabiey, M., Sarrocco, S., Shaw, M.W., Shaw, R.H. 2022. Biological control of plant diseases – what has been achieved and what is the direction? Plant Pathology 71:1024-1047. http://doi.org/10.1111/ppa.13555

Compant, S., Mitter, B., Colli-Mull, J.G., Gangl, H., Sessitsch, A. 2011. Endophytes of grapevine flowers, berries, and seeds: identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. *Microb. Ecol.* 62:188-197: https://doi.org/10.1007/s00248-011-9833-y

Compant, S., Brader, G., Muzammil, S. Sessitsch, A., Lebrihi, A., Mathieu, F. 2013. Use of beneficial bacteria and their secondary metabolites to control grapevine pathogen diseases. *BioControl* 58:435-455. https://doi.org/10.1007/s10526-012-9479-6

Corbel, S., Mougin, C., Martin-Laurent, F., Crouzet, O., Bru, D., Nélieu, S., Bouaïcha, N. 2015. Evaluation of phytotoxicity and ecotoxicity potentials of a cyanobacterial extract containing microcystins under realistic environmental concentrations and in a soil-plant system. Chemosphere 128:332-40. http://doi.org/10.1016/j.chemosphere.2015.02.008

Czemmel, S., Galarneau, E.R., Travadon, R., McElrone, A.J., Cramer, G.R., Baumgartner, K. 2015. Genes expressed in grapevine leaves reveal latent wood infection by the fungal pathogen *Neofusicoccum parvum*. PLoS One 10(3):e0121828. https://doi.org/10.1371/journal.pone.0121828

Darrieutort, G., Lecomte, P. 2007. Evaluation of a trunk injection technique to control grapevine wood diseases. Phytopathol. Mediterr. 46:50–57.

Deyett, E., Roper, M.C., Ruegger, P., Yang, J.-I., Borneman, J., Rolshausen, P.E. 2017. Microbial landscape of the grapevine endosphere in the context of Pierce's disease. *Phytobiomes* 1:138-149. https://doi.org/10.1094/PBIOMES-08-17-0033-R

Dries, L., Hendgen, M., Schnell, S., Löhnertz, O., Vortkamp, A. 2021. Rhizosphere engineering: leading towards a sustainable viticulture? *OENO One* *55*(2):353–363. https://doi.org/10.20870/oeno-one.2021.55.2.4534

Elena, G., Bella, V.D., Armengol, J., Luque, J. 2015. Viability of Botryosphaeriaceae species pathogenic to grapevine after hot water treatment. *Phytopatol. Mediterr.* 54(2):325-334. http://dx.doi.org/10.14601/Phytopathol_Mediterr-15526

Eskalen, A., Gubler W.D. 2001. Association of spores of *Phaeomoniella chlamydospora* and *Phaeoacremonium inflatipes*, and *Pm. aleophilum* with grapevine cordons in California. *Phytopatol. Mediterr.* 40:429-432. http://dx.doi.org/10.14601/Phytopathol_Mediterr-1613

Eskalen, A., Feliciano, A.J., Gubler, W.D. 2007. Susceptibility of grapevine pruning wounds and symptom development in response to infection by *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora*. Plant Disease 91:1100-1104. https://doi.org/10.1094/pdis-91-9-1100

Fan, B., Wang, C., Song, X., Ding, X., Wu, L., Wu, H., Gao, X., Borriss, R. 2018. *Bacillus velezensis* FZB42 in 2018: The gram-positive model strain for plant growth promotion and biocontrol. *Front. Microbiol*. 9:2491. https://doi.org/10.3389/fmicb.2018.02491

Fourie, P.H., Halleen, F., van der Vyver, J., Schrueder, W. 2001. Effect of *Trichoderma* treatments on the occurrence of decline pathogens on the roots and rootstocks of nursery plants. *Phytopatol. Mediterr.* 40S:473-478. http://dx.doi.org/10.14601/Phytopathol_Mediterr-1619

Graniti, A., Surico, G., Mugnai, L. 2000. Esca of grapevine: a disease complex or a complex of disease? *Phytopatol. Mediterr.* 39:16-20. https://doi.org/10.14601/Phytopathol_Mediterr-1539

Gramaje, D., Úrbez-Torres, J.R., Sosnowski, M.R. 2017. Managing grapevine trunk diseases with respect to etiology and epidemiology: Current strategies and future prospects. *Plant Disease* 102(1):12-39. https://doi.org/10.1094/PDIS-04-17-0512-FE

Granett, J., Omer, A.D., Pessereau, P., Walker, M.A. 1998. Fungal infections of grapevine roots in phylloxera-infested vineyards. *Vitis* 37(1):39-42. https://doi.org/10.5073/vitis.1998.37.39-42

Guevara-Avendaño, E., Bejarano-Bolívar, A.A., Kiel-Martínez, A.L., Ramirez-Vasquez, M., Méndez-Bravo, A., Guerrero-Analco, J., Aguirre von Wobeser, E., Sanchez-Rangel, D., Guerrero-Analco, J.A., Eskalen, A., Reverchon, F. 2019. Avocado rhizobacteria emit volatile organic compounds with antifungal activity against *Fusarium solani*, *Fusarium* sp. associated Kuroshio shot hole borer and *Colletotrichum gloeosporioides*. *Microbial Research* 219:74-83. https://doi.org/10.1016/j.micres.2018.11.009

Haidar, R., Roudet, J., Bonnard, O., Dufour, M.C., Corio-Costet, M.F., Fert, M., Gautier, T., Deschamps, A., Fermaud, M. 2016. Screening and modes of action of antagonistic bacteria to control the fungal pathogen *Phaeomoniella chlamydospora* involved in grapevine trunk diseases. Microbiological Research 192 :172–184. http://doi.org/10.1016/j.micres.2016.07.003

Hrycan, J., Hart, M., Bowen, P., Forge, T., Úrbez-Torres, J.R. 2020. Grapevine trunk disease fungi: their roles as latent pathogens and stress factors that favour disease development and symptom expression. *Phytopathologia Mediterranea* 59(3): 395-424. https://doi.org/10.14601/Phyto-11275

John, S., Wicks, T.J., Hunt, J.S., Scott, E.S. 2008. Colonisation of grapevine wood by *Trichoderma harzianum* and *Eutypa lata*. *Aust. J. Grape Wine Res.* 14:18-24. https://doi.org/10.1111/j.1755-0238.2008.00003.x

Kalbe, C.; Marten, P.; Berg, G. Strains of the genus Serratia as beneficial rhizobacteria of oilseed rape with antifungal properties. Microbiol. Res. 1996, 151, 433–439.

Kaur, S., Dhillon, G.S., Brar, S.K., Vallad, G.E., Chand, R., Chauhan, V.B. 2012 Emerging phytopathogen *Macrophomina phaseolina*: biology, economic importance and current diagnostic trends. *Critical Reviews in Microbiology* 38(2):136-151. https://doi.org/10.3109/1040841X.2011.640977

Kotze, C., Van Niekerk, J., Mostert, L., Halleen, F., Fourie, P. 2011. Evaluation of biocontrol agents for grapevine pruning wound protection against trunk pathogen infection. *Phytopathologia Mediterranea* 50:S247-S263. https://doi.org/10.14601/Phytopathol_Mediterr-8960

Leal, C., Gramaje, D., Fontaine, F., Richet, N., Trotel-Aziz, P., Armengol, J. 2023. Evaluation of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 to control Botryosphaeria dieback and black-foot pathogens in grapevine propagation material. Pest Manag Sci 79:1674-1683. https://doi.org/10.1002/ps.7339

Leal, C., Richet, N., Guise, J.-F., Gramaje, D., Armengol, J., Fontaine, F., Trotel-Aziz, P. 2021. Cultivar contributes to the beneficial effects of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 to protect grapevine against *Neofusicoccum parvum*. Front. Microbiol. 12:726132. https://doi.org/10.3389/fmicb.2021.726132

Lee, Y., Jeon, H., Kim, Y., Monkhung, S., Kim, K.-Y. 2017. Effect of gelatinase and chitinase producing microorganism on the growth of soybean and control of stink bug in field. *Korean J. Soil Sci. Fert.* 50(2):71-80. https://doi.org/10.7745/KJSSF.2017.50.2.071

Lee, J.H., Hong, A.R., Yun, J.H., Seo, S.T., Lee, J.K. 2018. Prevention of oak wilt by tree injection of culture suspension of an antifungal microorganism, *Streptomyces blastmyceticus* against oak wilt fungus, *Raffaelea quercus-mongolicae*. *J For Environ Sci* 34(5):376-381. https://doi.org/10.7747/JFES.2018.34.5.376

Levenfors, J.J., Hedman, R., Thaning, C., Gerhardson, B., Welch, C.J. 2004. Broad-spectrum antifungal metabolites produced by the soil bacterium Serratia plymuthica A 153. Soil Biol. Biochem. 36:677–685.

Loskill B., K. Rosswog, E. Kappes, B. Berkelman-Loehnertz, 2006. Investigations on the control of Esca disease by means of stem injection. IOBC/wprs Bulletin 29(11):119–122.

Martínez-Diz, M.P., Díaz-Losada, E., Andrés-Sodupe, M., Bujanda, R., Maldonado-González, M.M., Ojeda, S., Yacoub, A., Rey, P., Gramaje, D. 2021. Field evaluation of biocontrol agents against black-foot and Petri diseases of grapevine. Pest Manag Sci 77: 697–708. https://doi.org/10.1002/ps.6064.

Mayorquin, J.S., Carrillo, J.D., Twizeyimana, M., Peacock, B.B., Sugino, K.Y., Na, F., Wang, D.H., Kabashima, J.N., Eskalen, A. 2018. Chemical management of invasive shot hole borer and Fusarium dieback in California sycamore (*Platanus racemosa*) in Southern California. Plant Disease 102(7):1307-1315. https://doi.org/10.1094/PDIS-10-17-1569-RE

Michailides, T.J., Peacock, W., Christensen, P., Morgan, D.P., Felts, D. 2002. First report of Aspergillus vine canker of table grapes caused by *Aspergillus niger*. *Plant Disease* 86(1):75. https://doi.org/10.1094/PDIS.2002.86.1.75A

Mondello, V., Songy, A., Battiston, E., Pinto, C., Coppin, C., Trotel-Aziz, P., Clement, C., Mugnai, L., Fontaine, F. 2018. Grapevine trunk diseases: a review of fifteen years of trials for their control with chemicals and biocontrol agents. *Plant Disease* 102:1189-1217. https://doi.org/10.1094/PDIS-08-17-1181-FE

Mutawila, C., Fourie, P.H., Halleen, F., Mostert, L. 2011. Grapevine cultivar variation to pruning wound protection by *Trichoderma* species against trunk pathogens. Phytopathol. Mediterr. 50:S264-276.

Mutawila, C., Halleen, F., Mostert, L. 2016. Optimisation of time of application of *Trichoderma* biocontrol agents for protection of grapevine pruning wounds. Aust. J. Grape Wine Res. 22:279-287.

Niem, J.M., Billones-Baaijens, R., Stodart, B., Savocchia, S. 2020. Diversity profiling of grapevine microbial endosphere and antagonistic potential of endophytic *Pseudomonas* against grapevine trunk diseases. *Front. Microbiol.* 11:477. https://doi.org/10.3389/fmicb.2020.00477

Nouri, M.T., Zhuang, G., Culumber, C.M., Trouillas, F.P. 2019. First report of *Macrophomina phaseolina* causing trunk and cordon canker disease of grapevine in the United States. *Plant Disease* 103(3):579-579. https://doi.org/10.1094/PDIS-07-18-1189-PDN

Omer, A.D., Granett, J., Wakeman, R.K. 1999. Pathogenicity of *Fusarium oxysporum* on different *Vitis* rootstocks. *J. Phytopathology* 147:433-436. https://doi.org/10.1111/j.1439-0434.1999.tb03846.x

Pan, H.Q., Li, Q.L., Hu, J.C. 2017. The complete genome sequence of *Bacillus velezensis* 9912D reveals its biocontrol mechanism as a novel commercial biological fungicide agent. *Journal of Biotechnology* 247:25–28. https://doi.org/

Pertot, I., Caffi, T., Rossi, V., Mugnai, L., Hoffmann, C., Grando, M.S., Gary, C., Lafond, D., Duso, C., Thiery, D., Mazzoni, V., Anfora, G., 2016. A critical review of plant protection tools for reducing pesticide use on grapevine and new perspectives for the implementation of IPM in viticulture. *Crop Protection* 97:70-84. https://doi.org/10.1016/j.cropro.2016.11.025

Pertot, I., Prodorutti, D., Colombini, A. *et al. Trichoderma atroviride* SC1 prevents *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* infection of grapevine plants during the grafting process in nurseries. *BioControl* **61**, 257–267 (2016). https://doi.org/10.1007/s10526-016-9723-6

Rolshausen, P.E., Úrbez-Torres, J.R., Rooney-Latham, S., Eskalen, A., Smith, R.J., Gubler, W.D. 2010. Evaluation of pruning wound susceptibility and protection against fungi associated with grapevine trunk diseases. Am. J. Enol. Vitic. 61:113-119. https://doi.org/10.5344/ajev.2010.61.1.113

Rolli, E., Marasco, R., Saderi, S., Corretto, E., Mapelli, F., Cherif, A., Borin, S., Valenti, L., Sorlini, C., Daffonchio, D. 2017. Root-associated bacteria promote grapevine growth: from the laboratory to the field. Plant Soil 410:369–382. https://doi.org/10.1007/s11104-016-3019-6

Schmidt, C.S., Lorenz, D., Wolf, G.A. 2001. Biological control of the grapevine dieback fungus *Eutypa lata* I: Screening of bacterial antagonists. *J. Phytopathol.* 149:427-435. https://doi.org/

Schulz, B., Boyle, C. 2005. The endophytic continuum. Mycol. Res. 109(6):661-686. https://doi.org/10.1017/S095375620500273X

Simonetti, E., Hernández, A.I., Kerber, N.L., Pucheu, N.L., Carmona, M.A., García, F.A. 2012. Protection of canola (*Brassica napus*) against fungal pathogens by strains of biocontrol rhizobacteria. *Biocontrol Science and Technology* 22:111-115. https://doi.org/10.1080/09583157.2011.641519

Thuerig, B., Binder, A., Boller, T., Guyer, U., Jimenez, S., Rentsch, C., Tamm, L. 2006. An aqueous extract of the dry mycelium of *Penicillium chrysogenum* induces resistance in several crops under controlled and field conditions. European Journal of Plant Pathology 114:185–197. https://doi.org/10.1007/s10658-005-4512-6

Trotel-Aziz, P., Abou-Mansour, E., Courteaux, B., Rabenoelina, F., Clément, C., Fontaine, F., Aziz, A. 2019. *Bacillus subtilis* PTA-271 counteracts Botryosphaeria dieback in grapevine, triggering immune responses and detoxification of fungal phytotoxins. Front. Plant Sci. 10:25. https://doi.org/10.3389/fpls.2019.00025

Úrbez-Torres, J.R., Gubler, W.D. 2009. Pathogenicity of Botryosphaeriaceae species isolated from grapevine cancers in California. Plant Disease 90(12):1490-1503. https://doi.org/10.1094/PDIS-93-6-0584

Úrbez-Torres, J.R., Gubler, W.D. 2010. Susceptibility of grapevine pruning wounds to infection by *Lasiodiplodia theobromae* and *Neofusicoccum parvum*. Plant Pathology 60(2) 261-270. https://doi.org/10.1111/j.1365-3059.2010.02381.x

Weise, T.; Thürmer, A.; Brady, S.; Kai, M.; Daniel, R.; Gottschalk, G.; Piechulla, B. VOC emission of various *Serratia* species and isolates and genome analysis of *Serratia plymuthica* 4Rx13. FEMS Microbiol. Lett. 2014, 352, 45–53.

Weissmann, R., Uggla, C., Gerhardson, B. 2003. Field performance of a weed-suppressing *Serratia plymuthica* strain applied with conventional spraying equipment. BioControl 48:725–742. https://doi.org/10.1023/A:1026355930252

Weissmann, R., Gerhardson, B. 2001. Selective plant growth suppression by shoot application of soil bacteria. *Plant Soil* 234(2): 159–170. https://doi.org/10.1023/A:1017916717160

Yacoub, A., Gerbore, J., Magnin, N., Chambon, P., Dufour, M.C., Corio-Costet, M.F., Guyoneaud, R., Rey, P. 2016. Ability of *Pythium oligandrum* strains to protect *Vitis vinifera* L., by inducing plant resistance against *Phaeomoniella chlamydospora*, a pathogen involved in Esca, a grapevine trunk disease. Biol. Control 92:7-16. https://doi.org/10.1016/j.biocontrol.2015.08.005

Tables

Table 1. Mean percentages of infection (MPI) and lesion length caused by artificial inoculations with *Neofusicoccum parvum* on previously treated vines in two nurseries during the summer of 2022.

Treatment	Nursery 1		Nursery 2	
	***N. parvum* MPI (%)**	**Lesion length (mm)**	***N. parvum* MPI (%)**	**Lesion length (mm)**
Control	95.00 a	48.14 a	88.75 a	36.95 a
B. velezensis UCD10631	90.00 a	49.09 a	100.00 a	35.71 a
P. chlororaphis UCD10763	100.00 a	47.28 a	93.75 a	32.64 a
Serifel®	70.00 a	40.62 a	93.75 a	41.67 a
Vintec®	100.00 a	46.64 a	93.75 a	36.52 a
Topsin M®	86.67 a	42.42 a	75.00 a	36.04 a

Table 2. Mean percentages of infection (MPI) and lesion length caused by artificial inoculations with *Eutypa lata* on previously treated vines in two nurseries during the summer of 2022.

Treatment	Nursery 1		Nursery 2	
	***E. lata* MPI (%)**	**Lesion length (mm)**	***E. lata* MPI (%)**	**Lesion length (mm)**
Control	45.00 a	23.65 a	17.50 b	38.64 a
B. velezensis UCD10631	65.00 a	14.34 b c	38.75 a b	16.72 b
P. chlororaphis UCD10763	55.00 a	12.01 c	50.00 a	19.81 b
Serifel®	47.88 a	14.58 b c	5.00 b	20.70 b
Vintec®	69.40 a	12.21 b c	22.50 a b	35.34 a
Topsin M®	53.33 a	17.69 b	33.75 a b	21.92 b

Table 3. Mean percentages of infection (MPI) and lesion length caused by artificial inoculations with *Phaeoacremonium minimum* on previously treated vines in two nurseries during the summer of 2022.

Treatment	Nursery 1		Nursery 2	
	***P. minimum* MPI (%)**	**Lesion length (mm)**	***P. minimum* MPI (%)**	**Lesion length (mm)**
Control	70.00 a b	28.01 a	91.65 a	12.42 a b
B. velezensis UCD10631	40.00 b	9.09 c	67.50 a b	9.16 b
P. chlororaphis UCD10763	45.00 a b	11.08 a b c	43.75 b c	7.48 b
Serifel®	50.00 a b	11.06 c	81.25 a	11.98 a b
Vintec®	80.00 a	8.58 c	30.00 c	11.67 a b
Topsin M®	75.53 a	18.72 b	50.00 b c	12.80 a

Table 4. Mean percentages of recovery of the pathogen and lesion length caused by artificial inoculations with *N. parvum*, *E. lata* and *P. minimum* on cordons of 'Cabernet Franc' vines that received soil drench treatments during the summer of 2022.

Treatment	***N. parvum***		***E. lata***		***P. minimum***	
	MPI (%)	**Lesion length (mm)**	**MPI (%)**	**Lesion length (mm)**	**MPI (%)**	**Lesion length (mm)**
Control (water)	58.33 a	64.10 a	41.67 a	40.71 b	83.33 a	26.78 c
B. velezensis UCD10631	66.67 a	82.33 a	66.67 a	27.57 c	41.67 a	30.99 b c
P. chlororaphis UCD10763	83.33 a	70.73 a	33.33 a	38.61 b c	58.33 a	26.02 c
S. plymuthica UCD10719	58.33 a	84.51 a	33.33 a	61.20 a	75.00 a	42.53 a
Rhyme®	75.00 a	90.98 a	25.00 a	46.73 b	33.33 a	30.93 b c
Bio-Tam®	50.00 a	82.04 a	66.67 a	47.34 a b	75.00 a	30.02 b c
GCM	41.67 a	78.50 a	33.33 a	41.00 b	83.33 a	36.56 a b
Crab-Life®	66.67 a	62.06 a	16.67 a	50.61 a b	58.33 a	37.66 a b

Table 5. Mean percentages of recovery and lesion length caused by artificial inoculations with *N. parvum* on cordons of 'Cabernet Franc' vines that were treated with injections on both trunk and cordons during the summer of 2022.

Treatment	MPI (%)	Lesion length (mm)
Control	45.83 a	48.46 b
B. velezensis UCD10631	42.36 a	57.96 a
P. chlororaphis UCD10763	56.25 a	51.36 a b
S. plymuthica UCD10719	32.63 a	43.70 b

Table 6. Mean percentages of infection (MPI) caused by *N. parvum* after treating pruning wounds of 'Cabernet Franc' canes with experimental and commercial fungicides at the UC Davis Plant Pathology research field during the 2022 season.

Treatment	Active ingredient	MPI (%)†	
Non-inoculated control	N/A	0.0	f
Esendo®	Azoxystrobin + *Pseudomonas chlororaphis* AFS009	6.7	ef
Luna® Sensation	Fluopyram + Tebuconazole	8.3	ef
VitiSeal™ 1 L	Acrylic co-polymer	11.1	ef
UCD8189/8344	*Aureobasidium pullulans* UCD8189/8344	12.2	def
Topsin M®	Thiophanate-methyl	13.3	cdef
Guarda®	Thyme oil	13.3	cdef
Bio-Tam®	*Trichoderma asperellum* ICC012 + *T. gamsii* ICC080	13.3	cdef
Vintec®	*Trichoderma atroviride* strain SC1	24.4	bcdef
Botector®	*Aureobasidium pullulans* DSM14940/149411	25.0	bcdef
CrabLife® Powder	Chitin	26.7	bcdef
Rhyme®	Flutriafol	33.3	abcdef
TrichosSym Bio	*Trichoderma harzianum* T78	33.3	abcdef
Bv UCD10631	*Bacillus velezensis* UCD10631	33.3	abcdef
UCD8717	*Trichoderma hamatum* UCD8717	35.6	abcdef
Inoculated control	N/A	40.0	abcde
GCM (gelatinase and chitinase microorganism)	*Bacillus velezensis* CE100	50.0	abc
Theia®	*Bacillus subtilis* AFS032321	51.1	ab
Sp UCD10719	*Serratia plymuthica* UCD10719	52.2	ab
Howler®	*Pseudomonas chlororaphis* AFS009	56.7	ab
Pc UCD10763	*Pseudomonas chlororaphis* UCD10763	64.4	a

† Means followed by the same letter are not significantly different according to Fisher's LSD test (α=0.05).

Figures

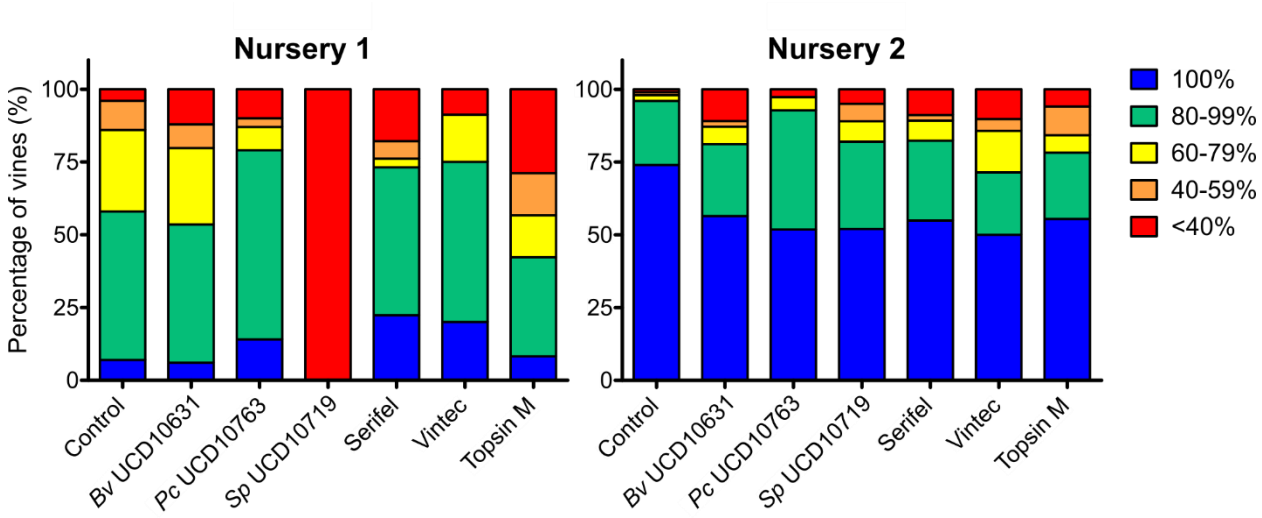


Figure 1. Distribution of the callus formation rating (%) at the basal end of the rootstock of vines treated with experimental and commercial fungicides in two nurseries.

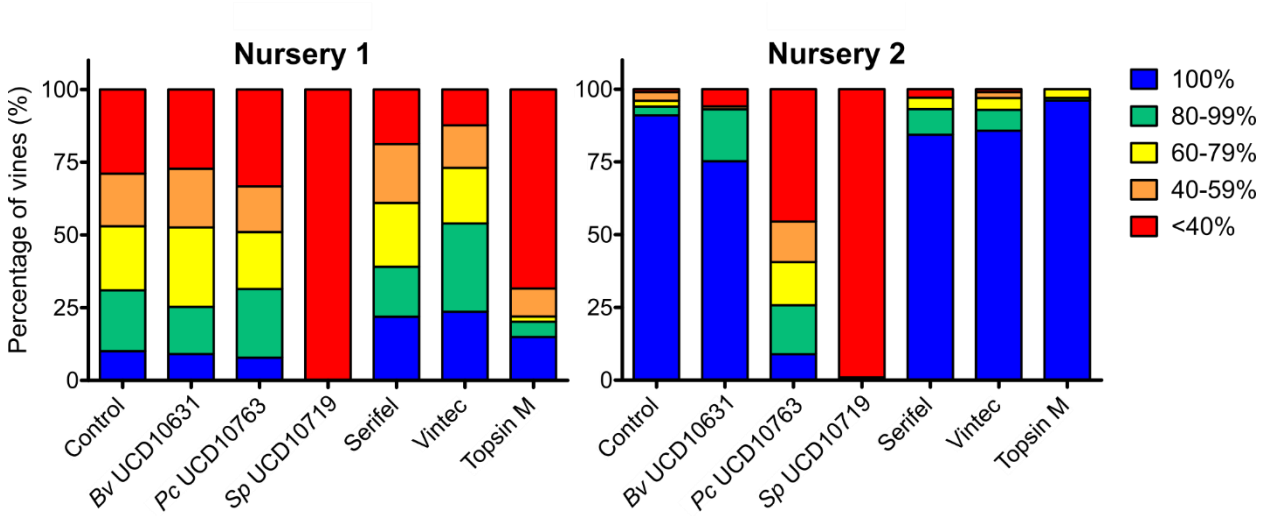


Figure 2. Distribution of the callus formation rating (%) at the graft union of vines treated with experimental and commercial fungicides in two nurseries.

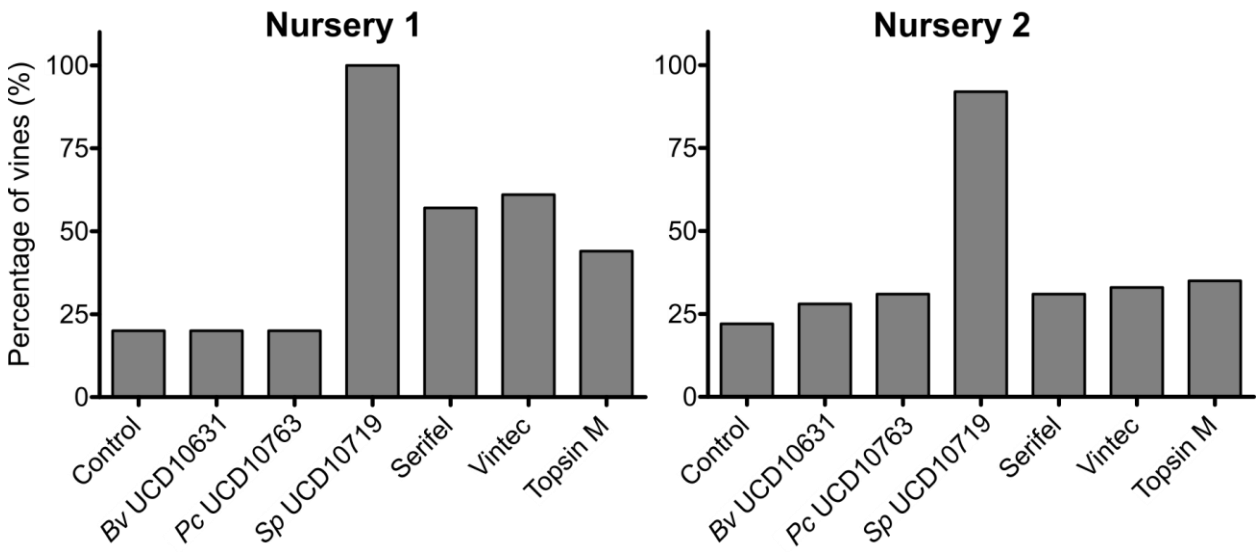


Figure 3. Mortality of treated vines expressed as the proportion of discarded vines (%) in two nurseries.

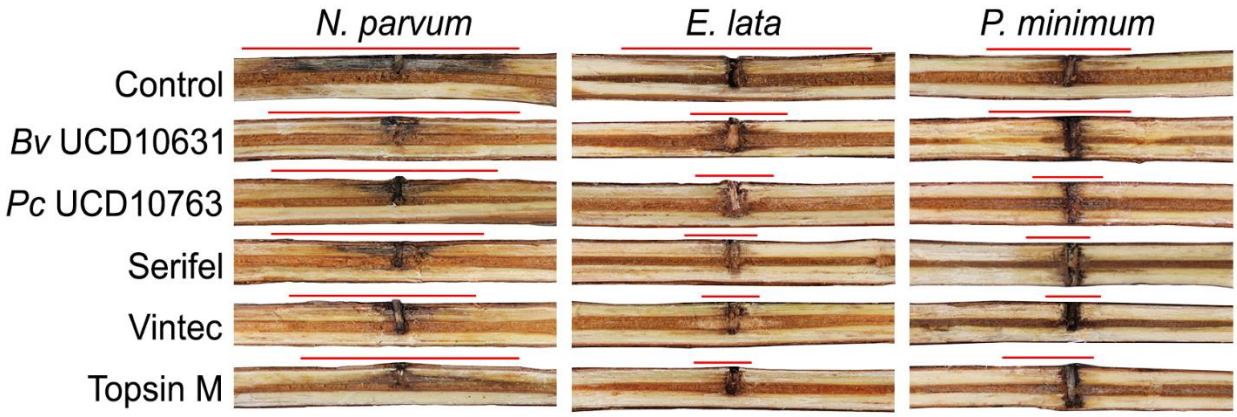


Figure 4. Resulting lesions caused by artificial inoculations with *N. parvum*, *E. lata* and *P. minimum* on grapevine rootstocks previously infiltrated with bacterial isolates and other fungicides in the nursery prior grafting. Red lines indicate the lesion length measured.

Chapter IV

Reassessing the Etiology of Aspergillus Vine Canker and Sour Rot of Table Grapes in California

Abstract

Fungal taxonomy is in constant flux and the advent of reliable DNA barcodes has allowed to improve the accuracy in the detection and identification of plant pathogens. In California, Aspergillus Vine Canker (AVC) and Sour Rot (SR) are economically important diseases that affect the wood and fruit of grapevines, respectively, and their causal agents are primarily species of *Aspergillus* known as black aspergilli (*Aspergillus* section *Nigri*). During the last decade, the taxonomy of these fungi has been rearranged several times using morphological, physiological and genetic analyses, resulting in the incorporation of multiple cryptic species that are difficult to distinguish. Therefore, in this study, we aimed to reassess the etiology of AVC and SR using a combination of morphological observations with phylogenetic reconstructions based on nucleotide sequences of the calmodulin (*CaM*) gene. Results revealed that the isolates causing AVC from recent isolations formed a strongly supported clade with strains of *A. tubingensis*, whereas the isolates obtained from initial surveys when the disease was discovered were confirmed as *A. niger* and *A. carbonarius*. Similarly, the isolates obtained from table grapes displaying sour rot symptoms and from spore traps placed in those vineyards were identified primarily as *A. tubingensis*, followed by *A. niger* and *A. carbonarius*. Notably, the isolates herein identified as *A. niger* formed a subclade with strains previously known as *A. welwitschiae*, a species that was recently synonymized with *A. niger*. Overall, the most prevalent species was *A. tubingensis*, associated to both AVC and SR, and representative isolates recovered from AVC-symptomatic wood, berries with sour rot symptoms, and spore traps were equally pathogenic in healthy wood and berries of 'Red Globe' grapevines. This study constitutes the first detection of *A. tubingensis* causing both Aspergillus Vine Canker and Sour Rot of grapes in California.

Introduction

Grapevine (*Vitis vinifera* L.) is one of the most popular fruit crops worldwide considering the size of the cultivated area and volume of harvested fruit. Approximately 84.79 million tons of grapes were harvested in 2021 within 7.31 million hectares across 94 countries (FAO 2023). In the United States, California produces 80% of the wine and 90% of the grapes of the country, with a total of 3,880,141 tons crushed in 2021, valued at about US$5,229 million (CDFA, 2022; Alston *et al*. 2018). The crop is seriously affected by pests and diseases, requiring an intensive

management program to sustain production that leads to significant increases in costs for growers. Specifically, grapevines are susceptible to 29 fungal diseases, caused by a broad variety of pathogens (Wilcox et al. 2015). Aspergillus Vine Canker and Sour Rot are known diseases affecting both wine and table grape cultivars in California.

Aspergillus Vine Canker was detected for the first time in the San Joaquin Valley in 1989. The symptoms include cankers in the woody tissue between the cordons and the trunk of grapevines and a premature senescence of the canopy during the fall (Michailides et al. 2002). The infections seem to start in the phloem and cambium tissues and then continue into the healthy wood of the trunk, cordons, and spurs. In severe cases, the canker can cause girdling of the vascular tissue, restricting the water and nutrient flow between the foliage and the roots. The pathogen eventually produces black conidiospores on the surface and underneath the bark of cankered tissues which is crucial to distinguish it from other trunk pathogens that usually form fruiting bodies such as pycnidia or perithecia on the surface of cankered wood. In contrast, the black spore masses (conidial heads) are produced and easily detached from short conidiophores and not within any known fruiting bodies. Red Globe, Crimson Seedless, Chardonnay, Grenache, and related cultivars seem to be more susceptible than others (Michailides et al. 2002). In 2021, the disease was detected again occurring in Grenache and Malbec vineyards located in Fresno and Sonoma counties, respectively. As a consequence, growers experienced economic losses due to cultural practices such as retraining and replanting affected and/or killed vines.

Sour Rot, on the other hand, affects grape berries that have been injured, leading to a rapid decay associated with different fungal pathogens, acetic acid odor and fruit flies (*Drosophila* spp.). Wounds usually occur due to wind, birds, or insects after veraison when berries begin to ripen. Other common grapevine diseases such as powdery mildew and esca may predispose the berry skin to cracking (Smith et al. 2019). Berries of cultivars that develop tight clusters and/or have rapid berry growth rates are also more prone to crack (Crandall et al. 2022). Under California conditions, susceptible berries are rapidly colonized by fungi that produce black, brown, or green sporulation. These infections lead to juice leakage that attracts fruit flies along with acetic acid bacteria, yeasts, and other filamentous fungi. As the disease progresses, yeasts convert sugars into ethanol and acetic bacteria oxidize the ethanol to acetic acid in the grape, thus emitting a pungent odor. This process seems to be highly favored by fruit flies, which act as vectors (Hall et al. 2018).

In late stages, decayed berries display a brown color and a shriveled appearance (McFadden-Smith and Gubler 2015).

The causal agents of both diseases are black aspergilli, a group of *Aspergillus* species that produce dark-colored conidial masses (Michailides et al. 2002; Rooney-Latham et al. 2008). Particularly, during the development of sour rot, these fungi dominate the populations of pathogens that initiate the disease (Rooney-Latham et al. 2008), however, other microorganisms such as acetic acid bacteria, yeasts, and other ascomycetes contribute with subsequent infections associated with acetic acid smell emanation and complete decay of berries. So far, in California the species associated with Aspergillus Vine Canker and Sour Rot have been identified as *A. niger* and *A. carbonarius*, primarily by morphological examinations and molecular tools using ITS sequences (Michailides et al. 2002; Michailides et al. 2007; Rooney-Latham et al. 2008). Taxonomically, these species belong to the section *Nigri*, under the subgenus *Circumdati* (Gams et al. 1986; Houbraken et al. 2020). However, the delimitation of species within section *Nigri* has been controversial, resulting in numerous rearrangements during the last decade based on morphological, physiological, and phylogenetic approaches, specifically using more informative DNA barcodes, such as the calmodulin (*CaM*) and β-tubulin (*benA*) genes (Hong et al. 2013; Samson et al. 2014; D'hooge et al. 2019; Houbraken et al. 2020; Bian et al. 2022). In this regard, the species causing Aspergillus Vine Canker in Italy and recently in Mexico have been identified as *A. niger*, *A. carbonarius*, *A. tubingensis*, and *A. awamori*, using either *CaM* or *benA* (Vitale et al. 2012; Rangel-Montoya et al. 2022). In contrast, multiple *Aspergillus* species have been associated with sour rot worldwide (Hocking et al. 2007; Chiotta et al. 2011; García-Cela et al. 2014; Lim et al. 2019). Some of these species are known to produce mycotoxins, particularly ochratoxin A (OTA), fumonisins, and oxalic acid, which have been detected in wine, grape berries, vineyards, and grape juice in Europe (Bellí et al. 2002; Frisvad et al. 2018). The evidence of the potential toxic effects of these compounds to human health has been reviewed elsewhere, highlighting the importance of detecting black aspergilli and their mycotoxins (Bui-Klimke and Wu 2015). The main objective of this study was to reexamine the identity of *Aspergillus* species associated with Aspergillus Vine Canker and Sour Rot occurring in California vineyards.

Materials and Methods

Sampling of symptomatic grapevine material

During the 2021 growing season, two wine grape vineyards (cvs. Malbec and Grenache) exhibiting Aspergillus Vine Canker symptoms were reported by farm advisors of the University of California Cooperative Extension in Fresno and Sonoma counties. Affected vines were inspected in the vineyards and samples of symptomatic wood were collected by removing a portion of the vine, including healthy and cankered tissue. Similarly, two table grape vineyards (cvs. Allison and Autumn King) located in Kern County were sampled for grape clusters and berries exhibiting sour rot symptoms during the harvest and after cold storage (one month post-harvest). Additionally, spore traps made of petroleum jelly-coated microscope slides (Eskalen and Gubler 2001) were used to monitor the dispersal of conidia during the 2021-22 growing season in those two vineyards in Kern County. Temperature and relative humidity data were downloaded from weather station (#182) managed by the California Irrigation Management Information System within the California Department of Water Resources database (https://cimis.water.ca.gov).

Fungal isolations and identification

Samples of grapevines with AVC symptoms were debarked and surface disinfected in the laboratory by spraying 70% ethanol. Once the disinfectant dried out, isolations were performed by cutting small wood pieces (3 × 5 mm) from the margin of necrotic lesions and plating them on potato dextrose agar acidified with 92% lactic acid (APDA) at 0.5 mL/L. Similarly, berry samples with sour rot symptoms were also surface disinfected with 70% ethanol in the laboratory, and small pieces (4 x 4 mm) of the margin of the rotting tissue were plated on APDA. The culture plates were incubated at 25 °C for seven days.

To recover fungal spores from the traps, slides were rinsed with 5 mL of 0.05% Tween in 50 mL falcon tubes and shaken by hand for 1 min to dislodge spores from the petroleum jelly coating. The resulting suspension was diluted 1:10 in sterile distilled water and aliquots of 50 µL were plated on PDA amended with a non-ionic surfactant (Igepal® CA-630, Solvay S.A., Brussels, Belgium) using an L-shaped sterile glass rod to spread the fungal spores evenly. Plates were incubated at 25 °C for seven days and colony forming units (CFU) of fungi were counted.

Following their morphological features, *Aspergillus*, *Botrytis*, *Cladosporium*, and *Penicillium* colonies were tentatively identified using the taxonomic keys of Crous et al. (2019), and Barnett and Hunter (1999).

Fungal identification

Single spore fungal cultures were grown on PDA for seven days for morphological examinations and DNA extractions. Conidial size and shape were measured in a compound microscope (Leica DM500B, Wetzlar, Germany) at 400x and 1000x magnifications. For molecular identification, the genomic DNA was extracted from freshly grown mycelium using the NucleoSpin® Plant II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Given the high phylogenetic resolution of the calmodulin (*CaM*) gene alone in separating species within section *Nigri*, a portion of approximately 720 bp was amplified via polymerase chain reaction (PCR) using the primer pair CL1/CL2A (O'Donnell et al. 2000; Samson et al. 2014). The reactions were run in a T100™ thermocycler (Bio-Rad, Hercules, CA, USA) and had a volume of 25 µL that consisted of 12.5 µL of DNA polymerase master mix (GoTaq® Green MasterMix 2X, Promega, Madison, WI, USA), 9.3 µL of nuclease-free water, 0.6 µL of each primer (10 µM), and 2 µL of DNA template (ca. 10 ng). PCR parameters consisted of a 3 min denaturation step at 95 °C followed by 35 cycles of 30 s at 95 °C, 45 s at 49 °C for annealing, and 60 s at 72 °C, with a final extension of 7 min at 72 °C. The PCR products were Sanger sequenced by Quintara Biosciences (Hayward, CA, USA) and contig sequences were assembled using Sequencher v5.4.6 (Gene Codes, Ann Arbor, MI, USA). Consensus sequences were compared with the NCBI nucleotide database using BLAST (http://ncbi.nlm.nih.gov/Blast.cgi) and type-material strains of closely related species with biseriate phialides (D'hooge et al. 2019) were considered to construct a sequence database (Table 2). Maximum parsimony (MP) and maximum likelihood (ML) phylogenetic analyses were used for identification. Sequences were aligned with MAFFT 7 (Katoh et al. 2019) and depurated using Gblocks with the less stringent settings (Talavera and Castresana 2007). Phylogenies were inferred in MEGA11 (Tamura et al. 2021) and bootstrapped 1,000 times for each method. Phylograms were compared and support values were combined with TreeGraph2 (Stöver and Müller 2010). The final phylogram was edited in Inkscape 0.92 (http://inkscape.org).

Pathogenicity tests

Koch's postulates were conducted in healthy-looking lignified canes and berries of 'Red Globe' grapevines (10 years old) located in the Armstrong Research Field at UC Davis. A total of six representative isolates of *A. tubingensis* were used in both experiments: two isolates originally obtained from wood canker (UCD10422 and UCD10494), two from sour rot symptomatic berries (UCD9799 and UCD10591) and two from spore trap samples (UCD10589 and UCD10590), plus a control (no inoculum). For the pathogenicity tests on wood, inoculations were made on 1-year-old lignified canes after surface disinfecting the internode area with 70% ethanol. A 5-mm segment of toothpicks completely colonized with each fungal isolate was inserted into a 1-mm diameter hole made with a sterile drill bit of 3/32 inch diameter, following the methods of Sparapano et al. (2000). Controls consisted of 5 mm sterile non-colonized toothpick segments. Inoculated points were wrapped with Parafilm (Bemis Co., Neenah, WI, USA) to avoid secondary contaminations and dehydration. After 12 weeks of inoculations, canes were cut and transported to the laboratory for evaluations. The bark was examined and peeled off to measure the length of necrotic lesions along the wood around the inoculation point. Isolations were performed by culturing small pieces of the margin of the lesions (3 × 5 mm) on APDA plates.

The pathogenicity test on berries was performed on mature berries (mean total soluble solids 19%) that were harvested freshly from the same vineyard mentioned previously. Clusters were first rinsed with soapy water (1 drop of dish soap in 0.5 L of water) for 3 min and then rinsed with sterile distilled water to wash off the excess soap. Individual berries from each cluster were excised from the rachis with their pedicels attached and then sprayed with ethanol 70% for surface sterilization. Once the surface of the berries was completely dry, an aliquot of 20 µL of a conidial suspension (10^5 conidia/mL) of each isolate was placed on the cheek of separate berries. Each isolate was inoculated on a set of 10 berries and replicated four times. The sets also included wounded and nonwounded berries. Wounds were made with a syringe needle (31G) by prickling the cheek of each berry at three equidistant points. The inoculated berries were incubated for 1 week at room temperature (20 ±2 °C) in closed plastic containers for 7 days until symptoms developed. Each set of inoculation experiments was evaluated by counting the number of berries that developed a soft rot with black aspergilli sporulation on top and performing isolations from the margin of the rotting pulp.

All experiments were conducted twice. The percentage of recovery of each isolate was recorded as the number of plates showing the corresponding isolate by the total of canes and berries inoculated. Lesion length values were subjected to analysis of variance (ANOVA), using generalized linear models with the corresponding R packages in InfoStat v2008. Normality and homoscedasticity were checked and corrected when necessary and means were separated using Fisher's least significant difference test ($\alpha = 5\%$). Data were plotted in GraphPad Prism v.5.03.

Results

Fungal isolations from symptomatic grapevines and spore traps

From the Grenache and Malbec wine vineyards exhibiting AVC symptoms (Figure 1A-D), 10 isolates of black aspergilli were obtained. From the Allison and Autumn King table grape vineyards, 255 isolates of black aspergilli were recovered from berries exhibiting sour rot symptoms from separate clusters (Figure 1E-G). The incidence of sour rot ranged from 30.0 to 89.3% at harvest and 0.3 to 3.4% after cold storage. From spore traps, a high prevalence of *Aspergillus* spp. was recovered in both 'Alison' and 'Autumn King' vineyards in Kern County, with over 10 colony forming units on average per trap (14 days) recovered in late May, September, and October, when vines were in pre-bloom and post-veraison, respectively (Figure 1). No correlation ($P > 0.05$) was found between the temperature, relative humidity and the frequency of *Aspergillus* spores in these vineyards (Figure 2).

Morphological and molecular fungal identification

The 287 isolates of black aspergilli were cultured on malt extract agar for morphological examination of the colonies following the recommended methods for *Aspergillus* (Samson et al., 2014). The isolates grew as white mycelium with abundant black sporulation at the center. Ten isolates from AVC, 26 isolates from SR, and 4 isolates from spore traps were selected by their morphological features, with colonies showing minimal differences in color, sporulation abundance, density, and distribution across the mycelium. Under the microscope, the sporulation was composed by conidial masses composed by unbranched conidiophores with conidial heads at their ends that produced globose dark brown conidia. The conidial heads were biseriate, composed

of a vesicle (conidial head) bearing numerous metulae (supporting cells) attached to phialides (conidiogenous cells) that form conidia at the tip. Conidia were dark brown, globose to subglobose, with fine to rough ornamentation. The majority of the isolates showed conidial diameters that ranged from 2.8 to 4.8 (3.7) µm, whereas a small subset had larger conidia, ranging from 5.5 to 9.9 (7.1) µm. After DNA extraction, amplifications, and sequencing of the *CaM* gene, the length of resulting sequences ranged from 722 to 740 bp. Search queries in the NCBI database using BLAST revealed matches with the type strains of *Aspergillus tubingensis* (CBS 134.48), *A. niger* (as *A. welwitschiae*, CBS 139.54) and *A. carbonarius* (CBS 111.26), with 99.44%, 100%, and 99.21% of identity, respectively. For the phylogenetic analyses, the best substitution model for the maximum likelihood inference was Kimura 2-parameter with gamma-distributed rate of evolution (K2G) based on the Akaike information criterion (AIC) value obtained in MEGA 11. Both maximum parsimony and maximum likelihood inferences showed an almost identical topology, with high supported clusters (97-100% bootstrap values) that included reference strains of the above-mentioned species and the respective isolates analyzed here. The two analyses were combined into one phylogram, showing both bootstrap values (Figure 3).

Pathogenicity tests

The pathogenicity tests were done with *A. tubingensis* isolates obtained from symptomatic woody grape samples with AVC, berries with sour rot, and from spore traps placed in vineyards with sour rot history were all pathogenic on both healthy-looking grapevine canes and mature berries of the cultivar Red Globe (Figure 4A).

The inoculations performed on canes developed dark brown to black lesions in the cambium, ranging from 11.5 to 92.1 mm long (average 40.1 mm), with no significant differences between isolates (Table 1). In three separate replicates of the inoculations with isolates UCD10422, UCD9799, and UCD10591, sporulation was detected on the surface of the bark, emerging from and around the inoculation point (Figure 4B). From canes that were inoculated without wounds, no necrotic lesions were observed under the bark, however, a small amount of *Aspergillus* colonies were recovered (20 to 30%). Control canes remained healthy. All of the isolates used in this trial were successfully recovered (100%) from the resulting lesions, thus fulfilling Koch's postulates.

Similarly, only wounded berries developed the characteristic symptoms of sour rot with black sporulation in the berry experiments. As the lesions progressed from the point of inoculation, the berry skin started to crack, allowing the fungus to colonize further and sporulate, resulting in a star-shaped black rot (Figure 4C). After seven days of incubation at room temperature (20 ±2 °C), the diameter of the resulting rot ranged from 11.8 to 26.4 (19.9) mm. Isolations from the margins of the rotting lesions yielded a 100% recovery of the six isolates from inoculated berries, whereas controls and non-wounded berries remained symptomless, thus completing Koch's postulates. Isolates obtained from sour rot samples and from spore traps showed bigger lesions (Table 1), compared to the isolates obtained from cankers.

Discussion

Black aspergilli are known to cause Aspergillus Vine Canker (AVC) and Sour Rot (SR) of grapes in California. Both diseases have been previously associated with *Aspergillus niger* and *A. carbonarius,* and the identification was based on morphological features along with molecular analyses using ITS sequences (Michailides et al. 2002, 2007; Rooney-Latham et al. 2008). The occurrence of AVC has also been reported in Italy and Mexico, caused by the species *A. awamori*, *A. carbonarius*, *A. niger* and *A. tubingensis*, all members of the section *Nigri* with biseriate conidiophores (Vitale et al. 2008, Vitale et al. 2012; Rangel-Montoya et al. 2022). These studies utilized the taxonomy that recognized cryptic species within the aggregate clade of *A. niger*, such as *A. awamori*, *A. foetidus*, *A. lacticoffeatus* and *A. welwitschiae* (Perrone et al. 2011; Hong et al. 2013). Various other species have subsequently been introduced since then, such as *A. vinaceus*, *A. chiangmaiensis*, *A. pseudopiperis* and *A. pseudotubingensis*, using multi-locus phylogenetic analyses coupled with secondary metabolite profiles and morphological characterizations (da Silva et al. 2020, Khuna et al. 2021). On the other hand, numerous species belonging to the sections *Nigri*, *Flavi*, *Terrei*, and *Versicolores*, among others, have been identified worldwide associated with sour rot of grapes in the world (Tjamos et al. 2004; Nally et al. 2013; Gao et al. 2020; Cosseboom and Hu, 2021). However, in Mediterranean climate regions, members of section *Nigri* are the most frequently found associated with this disease (McFadden-Smith and Gubler, 2015).

Recently, taxonomic revisions in the section *Nigri* separated the group in series (Houbraken et al. 2020), and several cryptic species were further synonymized with *A. niger* and *A. tubingensis*

given their genetic proximity using multiple phylogenetic analyses with three barcodes (*CaM*, *BenA* and *rpb2*), complemented with whole genome sequences (Bian et al. 2022). Based on these taxonomic arrangements, the results of this study revealed that *A. niger*, *A. carbonarius* and *A. tubingensis* are the causal agents of both AVC and SR in California, with a higher incidence of the latter species in populations obtained from different vineyards exhibiting symptoms of AVC or SR (Figure 2). Since *A. niger* and *A. carbonarius* were previously known pathogens causing both diseases, Koch's postulates were evaluated using representative isolates of *A. tubingensis* obtained from grapevine samples exhibiting AVC and SR symptoms, and from spore traps. The pathogenicity of the six *A. tubingensis* isolates was subsequently confirmed on healthy lignified canes and ripen berries of 'Red Globe' grapevines (Figure 3). Consequently, this study constitutes the first detection of *A. tubingensis* associated with AVC and SR diseases in California. In addition, an isolate obtained in 2002 from grapevine cankers occurring in Australia (UCD-E066) was identified as *A. niger*. This punctual detection suggests that AVC might have a broader distribution than what is currently reported until now and that more studies are required to understand the role of AVC as part of the GTDs complex.

So far, typical cankers associated with GTDs are known to be caused by several unrelated Ascomycete fungi in the Botryosphaeriaceae, Diatrypaceae and Diaporthaceae families, which occur in most of the grapevine cultivars worldwide (Gramaje et al. 2018). Although the cankers caused by these pathogens have an undistinguishable appearance in the affected woody tissue, the overwintering structures they form are different. For example, pycnidia are the most frequent fruiting body that indicate the presence of Botryosphaeriaceae and/or Diaporthaceae (e.g., *Diaporthe ampelina*), whereas perithecia embedded in a black stroma are rather indicators of Diatrypaceae (e.g., *Eutypa lata*) (Gramaje et al. 2018). In contrast, the species *A. niger* and *A. carbonarius* form an overwintering structure denominated as everted polymorphic stroma that bears conidia and has only been observed inside of grape berries affected by sour rot (Pisani et al. 2015). This stroma was not observed associated with vines affected by AVC. Rather, the pathogens were found sporulating from the symptomatic tissue without forming fruiting bodies, which is evident by their powdery loose aspect instead of a compact structure that contains the spores inside. This may favor airborne dissemination of conidia around the infected vine even under drought conditions. Morphologically, *A. tubingensis* and *A. niger* are almost identical and are difficult to distinguish, nonetheless, *A. carbonarius* produces significantly larger conidia, facilitating its

identification (Samson et al., 2007). In recent years, DNA sequencing has allowed to clearly separate species of section *Nigri*, particularly the partial regions of the calmodulin (*CaM*) and β-tubulin (*benA*) genes have shown higher resolution than ITS (Varga et al., 2011; Perrone et al., 2011). When comparing the two, *CaM* alone has shown to be sufficiently informative and it has been suggested to be the secondary identification barcode for *Aspergillus* spp., after ITS (Hubka and Kolarik, 2012; Samson et al. 2014; Palumbo and O'Keeffe, 2014; Massi et al. 2017). Therefore, a combination of morphological examinations and phylogenetic reconstructions using *CaM* sequences are sufficient for a proper identification of these fungi. The three species *A. niger*, *A. carbonarius* and *A. tubingensis* herein identified have been associated with the production of ochratoxin A and fumonisins (Medina et al. 2005; Chiotta et al. 2011). These mycotoxins have multiple toxic effects on humans and are naturally found in agricultural products derived from the grape production, such as wine, grape juice, and table grapes worldwide (Battilani and Pietri, 2002; Leong et al. 2006; Perrone et al. 2006, Díaz et al. 2009). Therefore, management of *Aspergillus* spp. in the vineyard is critical to reduce the potential contamination risk with these compounds in the food chain.

It is widely accepted that *Aspergillus* spp. require wounds to infect grape berries (Jarvis and Traquair, 1984). Likewise, infections of trunk diseases are considered to take place when spores land on wounds of grapevines and other woody crops (Gramaje et al. 2017; Guarnaccia et al. 2022). This was true in our pathogenicity tests on berries; however, it was possible to recover *Aspergillus* colonies from healthy looking canes that were inoculated without wounds. Similarly, another study showed that *A. carbonarius* was able to cause infections in unwounded berries, although in lower levels compared to wounded fruit (Lappa et al. 2018). These findings are relevant for management strategies, since the ability of a pathogen to cause infections without wounds increases the risks for infection events when sufficient inoculum is present in the field and environmental conditions favor its dispersal to susceptible plants. Therefore, more attention should be paid to deploying management methods to minimize the risk of infections. Moreover, it has been discussed that the infections associated with grapevine trunk diseases (GTDs) may remain latent without causing symptoms until the plant and the environmental conditions are conducive for the pathogens to a transition from an endophytic into a pathogenic behavior (Graniti et al. 2000; Hrycan et al. 2020). Therefore, management of *Aspergillus*-associated diseases must consider prevention of the main factors that lead to infections, which are injuries of berries and of the woody

structures (growth cracks in vigorously growing cultivars). For sour rot control, the application insecticides and fungicides during berry development and cultural practices associated with increasing ventilation of canopies and reducing the compactness in clusters (Duncan et al. 1995; Visconti et al. 2007). For AVC, it must be taken into consideration that the adoption of mechanical pruning and harvesting may increase the injury risk in the woody tissues of vines, therefore opening courts for canker development. Moreover, cracks on the trunk caused by natural growth and injuries caused by wires in the cordons should also be considered, given the fact that the disease was first observed associated to these factors (Michailides et al. 2002).

Predictions on climate change suggest hotter and drier conditions which would favor *A. tubingensis* and *A. niger* over *A. carbonarius* (García-Cela et al. 2013), which is consistent to present findings, indicating a higher prevalence of the former two species (Perrone et al. 2007; Lasram et al. 2012). This future scenario is positive since ochratoxin A production is higher and more frequent in *A. carbonarius* populations than in *A. niger* and *A. tubingensis* (Bau et al. 2005; Medina et al. 2005; Battilani et al. 2006; Guzev et al. 2006). Lastly, this study demonstrates that the use of both morphological and phylogenetic analyses using *CaM* nucleotide sequences were sufficient and accurate in identifying the black aspergilli species causing Aspergillus Vine Canker and Sour Rot of grapes in California under the current taxonomy of the section *Nigri*.

Literature cited

Abarca, M.L., Bragulat, M.R., Castella, G., Cabañes, F.J. 1994, Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. Appl. Environ. Microbiol. 60:2650-2652. https://doi.org/10.1128/aem.60.7.2650-2652.1994

Alanio, A., Beretti, J.-L., Dauphin, B., Mellado, E., Quesne, G., Lacroix, C., Amara, A., Berche, P., Nassif, X., Bougnoux, M.-E. 2011. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry for fast and accurate identification of clinically relevant *Aspergillus* species. Clinical Microbiology and Infection 17(5):750-755. https://doi.org/10.1111/j.1469-0691.2010.03323.x

Alston, J.M., Lapsley, J.T., Sambucci, O. 2018. Grape and wine production in California. 28p. Chapter 8. In: Martin, P.L., Goodhue, R.E., Wright, B.D. (eds.). California Agriculture:

Dimensions and Issues. 2nd ed. UC Giannini Foundation of Ag. Econ. Retrieved from: https://giannini.ucop.edu/publications/cal-ag-book/

Barnett, H.L., Hunter, B.B. 1998. Illustrated genera of imperfect fungi. 4th ed. American Phytopathological Society Press. St. Paul, MN, USA. 218p.

Battilani, P., Pietri, A. 2002. Ochratoxin A in grapes and wine. European Journal of Plant Pathology 108: 639-643. https://doi.org/10.1007/978-94-010-0001-7_5

Battilani, P., Barbano, C., Marin, S., Sanchis, V., Kozakiewicz, Z., Magan, N., 2006. Mapping of *Aspergillus* section *Nigri* in Southern Europe and Israel based on geostatistical analysis. Int. J. Food Microbiol. 111:S72–S82. https://doi.org/10.1016/j.ijfoodmicro.2006.03.014

Bau, M., Bragulat, M.R., Abarca, M.L., Minguez, S., Cabañes, F.J. 2005. Ochratoxin A producing fungi from Spanish vineyards. In: Hocking, A.D., Pitt, J.I., Samson, R.A., Thrane, U. (eds) Advances in Food Mycology. Advances in Experimental Medicine and Biology 571. Springer, Boston, MA. https://doi.org/10.1007/0-387-28391-9_10

Bellí, N., Marín, S., Sanchis, V., Ramos, A.J. 2002. Ochratoxin A (OTA) in wines, musts and grape juices: occurrence, regulations and methods of analysis. Food Sci Technol Int 8, 325–335. https://doi.org/10.1106/108201302031863

Bian, C., Kusuya, Y., Sklenář, F., D'hooge, E., Yaguchi, T., Ban, S., Visagie, C.M., Houbraken, J., Takahashi, H., Hubka, V. 2022. Reducing the number of accepted species in *Aspergillus* series *Nigri*. Studies in Mycology 102: 95–132. https://doi.org/10.3114/sim.2022.102.03

Bui-Klimke, T.R., Wu, F. 2015. Ochratoxin A and human health risk: a review of the evidence. Crit Rev Food Sci Nutr.55(13):1860-9. https://doi.org/10.1080/10408398.2012.724480

CDFA (California Department of Food and Agriculture). 2022. California Agricultural Statistics Review 2021-2022. Available online: https://www.cdfa.ca.gov/Statistics/PDFs/2022_Ag_Stats_Review.pdf

Chiotta, M.L., Susca, A., Stea, G., Mulè, G., Perrone, G., Logrieco, A., Chulze, S.N. 2011. Phylogenetic characterization and ochratoxin A – Fumonisin profile of black *Aspergillus* isolated from grapes in Argentina. International Journal of Food Microbiology 149:171-176. http://dx.doi.org/10.1016/j.ijfoodmicro.2011.06.002

Crandall, S.G., Spychalla, J., Crouch, U.T., Acevedo, F.E., Naegele, R.P., Miles, T.D. 2022. Rotting grapes don't improve with age: Cluster rot disease complexes, management, and future prospects. Plant Dis. 106:2013-2025. https://doi.org/10.1094/PDIS-04-21-0695-FE

Creppy, E.E. 2002. Update of survey, regulation and toxic effects of mycotoxins in Europe. Toxicology Letters 127:19-28. https://doi.org/10.1016/S0378-4274(01)00479-9

Crous, P.W., Verkley, G.J.M., Groenewald, J.Z., Houbraken, J. 2019. Fungal Biodiversity. Westerdijk Fungal Biodiversity Institute. Utrecht, Netherlands. 425p.

Da Silva, J. J., Iamanaka, B. T., Ferranti, L. S., Massi, F. P., Taniwaki, M. H., Puel, O., Lorber, S., Frisvad, J. C., Fungaro, M. H. P. 2020. Diversity within *Aspergillus niger* clade and description of a new species: *Aspergillus vinaceus* sp. nov. J. Fungi 6:371. https://doi.org/10.3390/jof6040371

Díaz, G.A., Torres, R., Vega, M., Latorre, B.A. 2009. Ochratoxigenic *Aspergillus* species on grapes from Chilean vineyards and *Aspergillus* threshold levels on grapes. International Journal of Food Microbiology 133:195–199. https://doi.org/10.1016/j.ijfoodmicro.2009.04.018

Duncan, R. A., Stapleton, J. J., Leavitt, G. M. 1995. Population dynamics of epiphytic mycoflora and occurrence of bunch rots of wine grapes as influenced by leaf removal. Plant Pathol. 44:956-965. https://doi.org/10.1111/j.1365-3059.1995.tb02653.x

D'hooge, E., Becker, P., Stubbe, D., Normand, A.-C., Piarroux, R., Hendrickx, M. 2019. Black aspergilli: A remaining challenge in fungal taxonomy? Medical Mycology 57:773–780. https://doi.org/10.1093/mmy/myy124

Eskalen, A., Gubler, W.G. 2001. Association of spores of *Phaeomoniella chlamydospora*, *Phaeoacremonium inflatipes*, and *Pm. aleophilum* with grapevine cordons in California. Phytopatol. Mediterr. 40:429-432. https://doi.org/10.14601/Phytopathol_Mediterr-1613

FAO. 2023. FAOSTAT, FAO Statistics Division. Food and Agriculture Organization of the United Nations. Retrieved on April 20th, 2022, from http://www.fao.org/faostat/en/#data/QC

Frisvad, J.C., Møller, L.L.H., Larsen, T.O., Kumar, R., Arnau, J. 2018. Safety of the fungal workhorses of industrial biotechnology: update on the mycotoxin and secondary metabolite

potential of *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*. Appl Microbiol Biotechnol 102:9481–9515. https://doi.org/10.1007/s00253-018-9354-1

Frisvad, J.C., Thrane, U., Samson, R.A., Pitt, J.I. 2006. Important mycotoxins and the fungi which produce them. In: Hocking, A.D., Pitt, J.I., Samson, R.A., Thrane, U. (eds) Advances in Food Mycology. Advances in Experimental Medicine and Biology, vol 571. Springer, Boston, MA. https://doi.org/10.1007/0-387-28391-9_1

Gams, W., Christensen, M., Onions, A.H., Pitt, J.I., Samson, R.A. 1986. Infrageneric taxa of *Aspergillus*. In: Samson, R.A., Pitt, J.I. (eds). Advances in *Penicillium* and *Aspergillus* Systematics. Springer, Boston, MA, USA: 55–62.

Gao, H., Yin, X,, Jiang, X., Shi, H., Yang, Y., Wang, C., Dai, X., Chen, Y., Wu, X. 2020. Diversity and spoilage potential of microbial communities associated with grape sour rot in eastern coastal areas of China. PeerJ 8:e9376 http://doi.org/10.7717/peerj.9376

García-Cela, E., Crespo-Sempere, A., Ramos, A.J., Sanchis, V., Marin, S. 2014. Ecophysiological characterization of *Aspergillus carbonarius*, *Aspergillus tubingensis* and *Aspergillus niger* isolated from grapes in Spanish vineyards. International Journal of Food Microbiology 173: 89-98. https://doi.org/10.1016/j.ijfoodmicro.2013.12.012

Gramaje, D., Úrbez-Torres, J.R., Sosnowski, M.R. 2017. Managing grapevine trunk diseases with respect to etiology and epidemiology: Current strategies and future prospects. Plant Dis. 102(1):12-39. https://doi.org/10.1094/PDIS-04-17-0512-FE

Guarnaccia, V., Kraus, C., Markakis, E., Alves, A., Armengol, J., Eichmeier, A., Compant, S., Gramaje, D. 2022. Fungal trunk diseases of fruit trees in Europe: pathogens, spread and future directions. Phytopathologia Mediterranea 61(3): 563-599. https://doi.org/10.36253/phyto-14167

Guzev, K., Danshin, A., Ziv, S., Lichter, A. 2006. Occurrence of ochratoxin A producing fungi in wine and table grapes in Israel. International Journal of Food Microbiology 111:S67-S71. https://doi.org/10.1016/j.ijfoodmicro.2006.03.003

Hall, M.E., Loeb, G.M., Cadle-Davidson, L., Evans, K.J., Wilcox, W.F. 2018. Grape sour rot: A four-way interaction involving the host, yeast, acetic acid bacteria, and insects. Phytopathology 108:1429-1442. https://doi.org/10.1094/PHYTO-03-18-0098-R

Hashimoto, A., Hagiwara, D., Watanabe, A., Yahiro, M., Yikelamu, A., Yaguchi, T., Kamei, K. 2017. Drug sensitivity and resistance mechanism in *Aspergillus* section *Nigri* strains from Japan. Antimicrob Agents Chemother. 61: e02583–16. https://doi.org/10.1128/AAC.02583-16

Hendrickx, M., Beguin, H., Detandt, M. 2012. Genetic re-identification and antifungal susceptibility testing of *Aspergillus* section *Nigri* strains of the BCCM/IHEM collection. Mycoses 55: 148–155. https://doi.org/10.1111/j.1439-0507.2011.02049.x

Hocking, A.D., Leong, S.L., Kazi, B.A., Emmett, R.W., Scott, E.S. 2007. Fungi and mycotoxins in vineyards and grape products. Int. J. Food Microbiol. 119:84-88. https://doi.org/10.1016/j.ijfoodmicro.2007.07.031

Hong, S.-B., Lee, M., Kim, D.H., Varga, J., Frisvad, J.C., Perrone, G., Gomi, K., Yamada, O., Machida, M., Houbraken, J., Samson, R.A. 2013. *Aspergillus luchuensis*, an industrially important black Aspergillus in east Asia. PLoS ONE 8: e63769. https://doi.org/10.1371/journal.pone.0063769

Houbraken, J., Kocsubé, S., Visagie, C.M., Yilmaz, N., Wang, X.-C., Meijer, M., Kraak, B., Hubka, V., Bensch, K., Samson, R.A., Frisvad, J.C. 2020. Classification of *Aspergillus*, *Penicillium*, *Talaromyces* and related genera (Eurotiales): an overview of families, genera, subgenera, sections, series and species. Studies in Mycology 95: 5–169. https://doi.org/10.1016/j.simyco.2020.05.002

Hubka, V., Kolarik, M. 2012. β-tubulin paralogue *tubC* is frequently misidentified as the *benA* gene in *Aspergillus* section *Nigri* taxonomy: primer specificity testing and taxonomic consequences. Persoonia 29:1-10. https://doi.org/10.3767/003158512X658123

Jarvis, W.R., Traquair, J.A. 1984. Bunch rot of grapes caused by *Aspergillus aculeatus*. Plant Dis. 68:718-719. https://doi.org/10.1094/PD-68-718

JECFA (Joint FAO/WHO Expert Committee on Food Additives). 2001. Evaluation of certain mycotoxins in food: fifty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives. World Health Organization, Geneva, Switzerland.

Katoh, K.; Rozewicki, J.; Yamada, K.D. 2019. MAFFT online service: Multiple sequence alignment, interactive sequence choice and visualization. Brief. Bioinform. 20:1160–1166.

Khuna, S., Suwannarach, N., Kumla J, Frisvad, J.C., Matsui, K., Nuangmek, W., Lumyong, S. 2021. Growth enhancement of Arabidopsis (*Arabidopsis thaliana*) and onion (*Allium cepa*) with inoculation of three newly identified mineral-solubilizing fungi in the genus *Aspergillus* section *Nigri*. Frontiers in Microbiology 12: 705896. https://doi.org/10.3389/fmicb.2021.705896

Lappa, I.K., Mparampouti, S., Lanza, B., Panagou, E.Z. 2018. Control of *Aspergillus carbonarius* in grape berries by *Lactobacillus plantarum*: A phenotypic and gene transcription study. Int J Food Microbiol 275:56-65. https://doi.org/10.1016/j.ijfoodmicro.2018.04.001

Lasram, S., Oueslati, S., Mliki, A., Ghorbel, A., Silar, P., Chebil, S. 2012. Ochratoxin A and ochratoxigenic black *Aspergillus* species in Tunisian grapes cultivated in different geographic areas. Food Control 25:75-80. https://doi.org/10.1016/j.foodcont.2011.10.006

Lim, Y.-S., Hassan, O., Kim, M.-K., Chang, T. 2019. First report of bunch rot caused by *Aspergillus tubingensis* of Shine Muscat grape in Korea. Plant Dis. 103:2953. https://doi.org/10.1094/PDIS-05-19-1104-PDN

Massi, F.P., Sartori, D., Ferranti, L.S., Iamanaka, B.T., Taniwaki, M.H., Vieira, M.L.C., Fungaro, M.H.P. 2016. Prospecting for the incidence of genes involved in ochratoxin and fumonisin biosynthesis in Brazilian strains of *Aspergillus niger* and *Aspergillus welwitschiae*. International Journal of Food Microbiology 221:19–28. http://dx.doi.org/10.1016/j.ijfoodmicro.2016.01.010

McFadden-Smith, W., Gubler, W.G. 2015. Sour rot. In: Compendium of Grape Diseases, Disorders, and Pests. 2nd ed. Wilcox, W.F., Gubler, W.D., Uyemoto, J.K. (eds.). The American Phytopathological Society, St. Paul, MN, USA.

Medina, A., Mateo, R., López-Ocaña, L., Valle-Algarra, F.M., Jiménez, M. 2005. Study of Spanish grape mycobiota and ochratoxin A production by isolates of *Aspergillus tubingensis* and other members of *Aspergillus* section *Nigri*. Applied and Environmental Microbiology 71(8):4696-4702. https://doi.org/10.1128/AEM.71.8.4696–4702.2005

Melki Ben Fredj, S., Chebil, S., Lebrihi, A., Lasram, S., Ghorbel, A., Mliki, A. 2007. Occurrence of pathogenic fungal species in Tunisian vineyards. International Journal of Food Microbiology 113:245-250. https://doi.org/10.1016/j.ijfoodmicro.2006.07.022

Michailides, T.J., Peacock, W., Christensen, P., Morgan, D.P., Felts, D. 2002. First report of Aspergillus Vine Canker of table grapes caused by *Aspergillus niger*. Plant Dis. 86:75. https://doi.org/10.1094/PDIS.2002.86.1.75A

Michailides, T.J., Peacock, W., Christensen, P., Morgan, D.P. 2007. Aspergillus vine canker of table grapes caused by *Aspergillus* sect. *Nigri* in California. Phytopathol. Mediterr. 46:102-128.

Nally, M.C., Pesce, V.M., Maturano, Y.P., Toro, M.E., Combina, M., Castellanos de Figueroa, L.I., Vazquez, F. 2013. Biocontrol of fungi isolated from sour rot infected table grapes by *Saccharomyces* and other yeast species. Postharvest Biology and Technology 86:456-462. https://doi.org/10.1016/j.postharvbio.2013.07.022

O'Donnell, K., Nirenberg, H. I., Aoki, T., Cigelnik, E. 2000. A multigene phylogeny of the *Gibberella fujikuroi* species complex: detection of additional phylogenetically distinct species. Mycoscience, 41, 61–78.

Palumbo, J.D., O'Keeffe, T.L. 2014. Detection and discrimination of four *Aspergillus* section *Nigri* species by PCR. Letters in Applied Microbiology 60, 188-195. https://doi.org/10.1111/lam.12358

Perrone, G., Stea, G., Epifani, F., Varga, J., Frisvad, J.C., Samson, R.A. 2011. *Aspergillus niger* contains the cryptic phylogenetic species *A. awamori*. Fungal Biology 115: 1138–1150. https://doi.org/10.1016/j.funbio.2011.07.008

Perrone, G., Susca, A., Cozzi, G., Ehrlich, K., Varga, J., Frisvad, J.C., Meijer, M., Noonim, P., Mahakamchanakul, W., Samson, R.A. 2007. Biodiversity of *Aspergillus* species in some

important agricultural products. Stud. Mycol. 59:53–66. https://doi.org/10.3114/sim.2007.59.07

Pisani, C., Nguyen, T. T., Gubler, W. D. 2015. A novel fungal fruiting structure formed by *Aspergillus niger* and *Aspergillus carbonarius* in grape berries. Fungal Biol. 119:784-790. https://doi.org/10.1016/j.funbio.2015.05.002

Rangel-Montoya, E. A., Valenzuela-Solano, C., Hernández-Martínez, R. 2022. *Aspergillus* spp. causing Aspergillus vine canker on grapevines in Mexico. 12th International Workshop on Grapevine Trunk Diseases. Mikulov, Czech Republic.

Rooney-Latham, S., C. N. Janousek, A. Eskalen, W. D. Gubler. 2008. First report of *Aspergillus carbonarius* causing sour rot of table grapes (*Vitis vinifera*) in California. Plant Dis. 92(4):651. https://doi.org/10.1094/PDIS-92-4-0651A

Samson, R.A., Noonim, P., Meijer, M., Houbraken, J., Frisvad, J.C., Varga, J. 2007. Diagnostic tools to identify black aspergilli. Studies in Mycology 59: 129–145. https://doi.org/10.3114/sim.2007.59.13

Samson, R.A., Visagie, C.M., Houbraken, J., Hong, S.-B., Hubka, V., Klaassen, C.H.W., Perrone, G., Seifert, K.A., Susca, A., Tanney, J.B., Varga, J., Kocsubé, S., Szigeti, G., Yaguchi, T., Frisvad, J.C. 2014. Phylogeny, identification and nomenclature of the genus *Aspergillus*. Studies in Mycology 78: 141–173. https://doi.org/10.1016/j.simyco.2014.07.004

Smith, R.J., Bettiga, L.J., Gubler, W.G. 2019. Summer bunch rot (sour rot). In: UC IPM Pest Management Guidelines: Grape. Publication 3448. University of California Agriculture and Natural Resources. Davis, CA. pp.121-122.

Sparapano, L., Bruno, G., Ciccarone, C., Graniti, A. 2000. Infection of grapevines by some fungi associated with esca. II. Interaction among *Phaeoacremonium chlamydosporum*, *P. aleophilum* and *Fomitiporia punctata*. Phytopathol. Mediterr. 39:53-58. https://doi.org/10.14601/Phytopathol_Mediterr-1543

StoverStöver, B.C.; Müller, K.F. 2010. TreeGraph 2: Combining and visualizing evidence from different phylogenetic analyses. BMC Bioinform. 11:7.

Talavera G., Castresana, J. 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst Biol. 56(4):564-77. https://doi.org/10.1080/10635150701472164

Tamura, K., Stecher, G., Kumar, S. 2021. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Molecular Biology and Evolution 38(7):3022–3027. https://doi.org/10.1093/molbev/msab120

Tjamos, S.E., Antoniou, P.P., Kazantzidou, A., Antonopoulos, D.F., Papageorgiou, I., Tjamos, E.C. 2004. *Aspergillus niger* and *Aspergillus carbonarius* in Corinth raisin and wine-producing vineyards in Greece: population composition, ochratoxin A production and chemical control. J. Phytopathology 152:250–255. https://doi.org/10.1111/j.1439-0434.2004.00838.x

Varga, J., Frisvad, J.C., Kocsubé, S., Brankovics, B., Tóth, B., Szigeti, G., Samson, R.A. 2011. New and revisited species in *Aspergillus* section *Nigri*. Stud Mycol. 69: 1-17. https://doi.org/10.1016/10.3114/sim.2011.69.01

Visconti, A., Perrone, G., Cozzi, G., Solfrizzo, M. 2007. Managing ochratoxin A risk in the grape-wine food chain. Food Additives and Contaminants 25(2):193-202. https://doi.org/10.1080/02652030701744546

Vitale, A., Castello, I., Polizzi, G. 2008. First report of Aspergillus Vine Canker on table grapes caused by *Aspergillus niger* in Europe. Plant Dis. 92:1471. https://doi.org/10.1094/PDIS-92-10-1471B

Vitale, A., G. Cirvilleri, A. Panebianco, F. Epifani, G. Perreno, G. Polizzi. 2012. Molecular characterisation and pathogenicity of *Aspergillus* Sect. *Nigri* causing Aspergillus vine canker of table grapes in Italy. Eur J Plant Pathol 132:483–487. https://doi.org/10.1007/s10658-011-9906-z

Wilcox, W.F., Gubler, W.D., Uyemoto, J.K. 2015. Compendium of Grape Diseases, Disorders, and Pests. Second Edition. APS Press, St. Paul, MN, USA.

Tables

Table 1. Lesion length and diameter (mm) values obtained in wood and ripen berries of 'Red Globe' grapevines inoculated with *Aspergillus tubingensis* isolates (pathogenicity tests).

Isolate	Origin	Wood lesion length (mm)	Berry lesion diameter (mm)
UCD10422	AVC	51.5 a	19.2 bc
UCD10494	AVC	32.3 a	18.5 c
UCD9799	Sour rot	37.1 a	20.6 a
UCD10591	Sour rot	33.2 a	20.6 a
UCD10589	Spore trap	48.3 a	20.2 ab
UCD10590	Spore trap	37.8 a	19.9 ab

Table 2. Strains used in the phylogenetic analysis of the *Aspergillus* species associated with Aspergillus Vine Canker and Sour Rot of grapes in California. Sequences generated in this study are highlighted in bold.

Species[a]	**Isolate / strain**[b]	**Host / substrate**[c]	**Location**	**Isolation date**	***CaM* GenBank**
Aspergillus brasiliensis	CBS 101740^T	Soil	São Paulo, Brazil	unknown	AM295175
A. carbonarius	CBS 111.26^T	Paper	unknown	unknown	AJ964873
A. carbonarius	IHEM 661	Indoor air, bakery	France	1981	MH645014
A. carbonarius	IHEM 25902	Human sputum	France	2012	MH645015
A. carbonarius	**UCD11285**	*Vitis vinifera* cv. Autumn King (Sour Rot)	Kern County, CA, USA	2021	**OQ861011**
A. carbonarius	**UCD-E099**	*Vitis vinifera* cv. Red Globe (AVC)	Parlier, CA, USA	2004	**OQ861012**
A. carbonarius	**UCD-E100**	*Vitis vinifera* cv. Red Globe (AVC)	Parlier, CA, USA	2004	**OQ861013**
A. ellipticus	CBS 707.79^T	Soil	Costa Rica	1962	AM117809
A. eucalypticola	CBS 122712^T	*Eucalyptus* sp.	Australia	2007	EU482433
A. heteromorphus	CBS 117.55^T	Culture contaminant	Brazil	unknown	AM421461
A. ibericus	CBS 121593^T	*Vitis vinifera*, berries	Portugal	2001	AJ971805
A. japonicus	CBS 114.51^T	unknown	unknown	1984	AJ964875
A. luchuensis	CBS 205.80^T	Awamori-koji	Okinawa, Japan	unknown	JX500071
A. luchuensis (=*A. acidus*)	CBS 564.65^T	Awamori-koji	Okinawa, Japan	unknown	JX500074
A. niger	CBS 554.65^T	Tannic acid fermentation	Connecticut, USA	unknown	AJ964872
A. niger (=*A. welwitschiae*)	CBS 139.54^T	*Welwitschia mirabilis*	Namibia	2013	KC480196
A. niger (=*A. welwitschiae*)	IHEM 2969	Soil	India	1985	MH644941
A. niger	IHEM 17902	Human, chronic sinusitis	Belgium	2000	MH644965
A. niger	IHEM 24454	Human, otitis	India	1974	MH644956
A. niger	ITEM 7090	Grapes	Italy	unknown	FN394672
A. niger	**UCD11277**	*Vitis vinifera* cv. Autumn King (Sour Rot)	Kern County, CA, USA	2021	**OQ861014**
A. niger	**UCD11281**	*Vitis vinifera* cv. Autumn King (Sour Rot)	Kern County, CA, USA	2021	**OQ861015**
A. niger	**UCD11298**	*Vitis vinifera* cv. Allison (Sour Rot)	Kern County, CA, USA	2021	**OQ861016**
A. niger	**UCD-E048**	*Vitis vinifera* cv. Red Globe (AVC)	Tulare, CA	1997	**OQ861017**
A. niger	**UCD-E066**	*Vitis vinifera* cv. Red Globe (AVC)	Parlier, CA	2005	**OQ861018**
A. niger	**UCD-E098**	*Vitis vinifera* (AVC)	Australia	2002	**OQ861019**
A. sclerotiicarbonarius	CBS 121057^T	Robusta coffee bean	Thailand	2006	EU159235
A. sclerotioniger	CBS 115572^T	Coffee bean	Karnataka, India	1996	EU163271
A. tubingensis	CBS 134.48^T	unknown	France	unknown	AJ964876
A. tubingensis	IHEM 10349	Grains	China	1997	MH644918
A. tubingensis	Strbr	*Fragaria* × *ananassa* cv. Monterey	California, USA	2019	MK636653
A. tubingensis	**UCD9799**	*Vitis vinifera* cv. Autumn King (Sour Rot)	Kern County, CA, USA	2021	**OQ446426**
A. tubingensis	**UCD10419**	*Vitis vinifera* cv. Grenache (AVC)	Fresno, CA, USA	2021	**OQ861020**
A. tubingensis	**UCD10422**	*Vitis vinifera* cv. Grenache (AVC)	Fresno, CA, USA	2021	**OQ861021**

A. tubingensis	**UCD10488**	*Vitis vinifera* cv. Grenache (AVC)	Fresno, CA, USA	2021	**OQ861022**
A. tubingensis	**UCD10490**	*Vitis vinifera* cv. Grenache (AVC)	Fresno, CA, USA	2021	**OQ861023**
A. tubingensis	**UCD10491**	*Vitis vinifera* cv. Grenache (AVC)	Fresno, CA, USA	2021	**OQ861024**
A. tubingensis	**UCD10492**	*Vitis vinifera* cv. Grenache (AVC)	Fresno, CA, USA	2021	**OQ861025**
A. tubingensis	**UCD10493**	*Vitis vinifera* cv. Grenache (AVC)	Fresno, CA, USA	2021	**OQ861026**
A. tubingensis	**UCD10494**	*Vitis vinifera* cv. Malbec (AVC)	Sonoma, CA, USA	2021	**OQ861027**
A. tubingensis	**UCD10589**	Spore trap	Kern County, CA, USA	2021	**OQ861028**
A. tubingensis	**UCD10590**	Spore trap	Kern County, CA, USA	2021	**OQ861029**
A. tubingensis	**UCD10591**	*Vitis vinifera* cv. Autumn King (Sour Rot)	Kern County, CA, USA	2021	**OQ861030**
A. tubingensis	**UCD10832**	Spore trap	Kern County, CA, USA	2021	**OQ861031**
A. tubingensis	**UCD10835**	Spore trap	Kern County, CA, USA	2021	**OQ861032**
A. tubingensis	**UCD11276**	*Vitis vinifera* cv. Autumn King (Sour Rot)	Kern County, CA, USA	2021	**OQ861033**
A. tubingensis	**UCD11278**	*Vitis vinifera* cv. Autumn King (Sour Rot)	Kern County, CA, USA	2021	**OQ861034**
A. tubingensis	**UCD11279**	*Vitis vinifera* cv. Autumn King (Sour Rot)	Kern County, CA, USA	2021	**OQ861035**
A. tubingensis	**UCD11280**	*Vitis vinifera* cv. Autumn King (Sour Rot)	Kern County, CA, USA	2021	**OQ861036**
A. tubingensis	**UCD11282**	*Vitis vinifera* cv. Autumn King (Sour Rot)	Kern County, CA, USA	2021	**OQ861037**
A. tubingensis	**UCD11283**	*Vitis vinifera* cv. Autumn King (Sour Rot)	Kern County, CA, USA	2021	**OQ861038**
A. tubingensis	**UCD11284**	*Vitis vinifera* cv. Autumn King (Sour Rot)	Kern County, CA, USA	2021	**OQ861039**
A. tubingensis	**UCD11286**	*Vitis vinifera* cv. Autumn King (Sour Rot)	Kern County, CA, USA	2021	**OQ861040**
A. tubingensis	**UCD11287**	*Vitis vinifera* cv. Autumn King (Sour Rot)	Kern County, CA, USA	2021	**OQ861041**
A. tubingensis	**UCD11288**	*Vitis vinifera* cv. Allison (Sour Rot)	Kern County, CA, USA	2021	**OQ861042**
A. tubingensis	**UCD11289**	*Vitis vinifera* cv. Allison (Sour Rot)	Kern County, CA, USA	2021	**OQ861043**
A. tubingensis	**UCD11290**	*Vitis vinifera* cv. Allison (Sour Rot)	Kern County, CA, USA	2021	**OQ861044**
A. tubingensis	**UCD11291**	*Vitis vinifera* cv. Allison (Sour Rot)	Kern County, CA, USA	2021	**OQ861045**
A. tubingensis	**UCD11292**	*Vitis vinifera* cv. Allison (Sour Rot)	Kern County, CA, USA	2021	**OQ861046**
A. tubingensis	**UCD11293**	*Vitis vinifera* cv. Allison (Sour Rot)	Kern County, CA, USA	2021	**OQ861047**
A. tubingensis	**UCD11294**	*Vitis vinifera* cv. Allison (Sour Rot)	Kern County, CA, USA	2021	**OQ861048**
A. tubingensis	**UCD11295**	*Vitis vinifera* cv. Allison (Sour Rot)	Kern County, CA, USA	2021	**OQ861049**
A. tubingensis	**UCD11296**	*Vitis vinifera* cv. Allison (Sour Rot)	Kern County, CA, USA	2021	**OQ861050**
A. tubingensis	**UCD11297**	*Vitis vinifera* cv. Allison (Sour Rot)	Kern County, CA, USA	2021	**OQ861051**
A. tubingensis	**UCD11299**	*Vitis vinifera* cv. Allison (Sour Rot)	Kern County, CA, USA	2021	**OQ861052**
A. vadensis	CBS 113365^{T}	Air	Egypt	2001	EU163269
A. vadensis	IHEM 26351	Human, sputum	France	2011	MH644878

[a] Names in parenthesis represent species that are now synonyms.

[b] Type-material strains are accompanied with ^{T}. Isolates obtained from this study are highlighted in bold.

[c] In parenthesis is indicated the disease where the isolate was recovered from. AVC=Aspergillus Vine Canker.

Figures

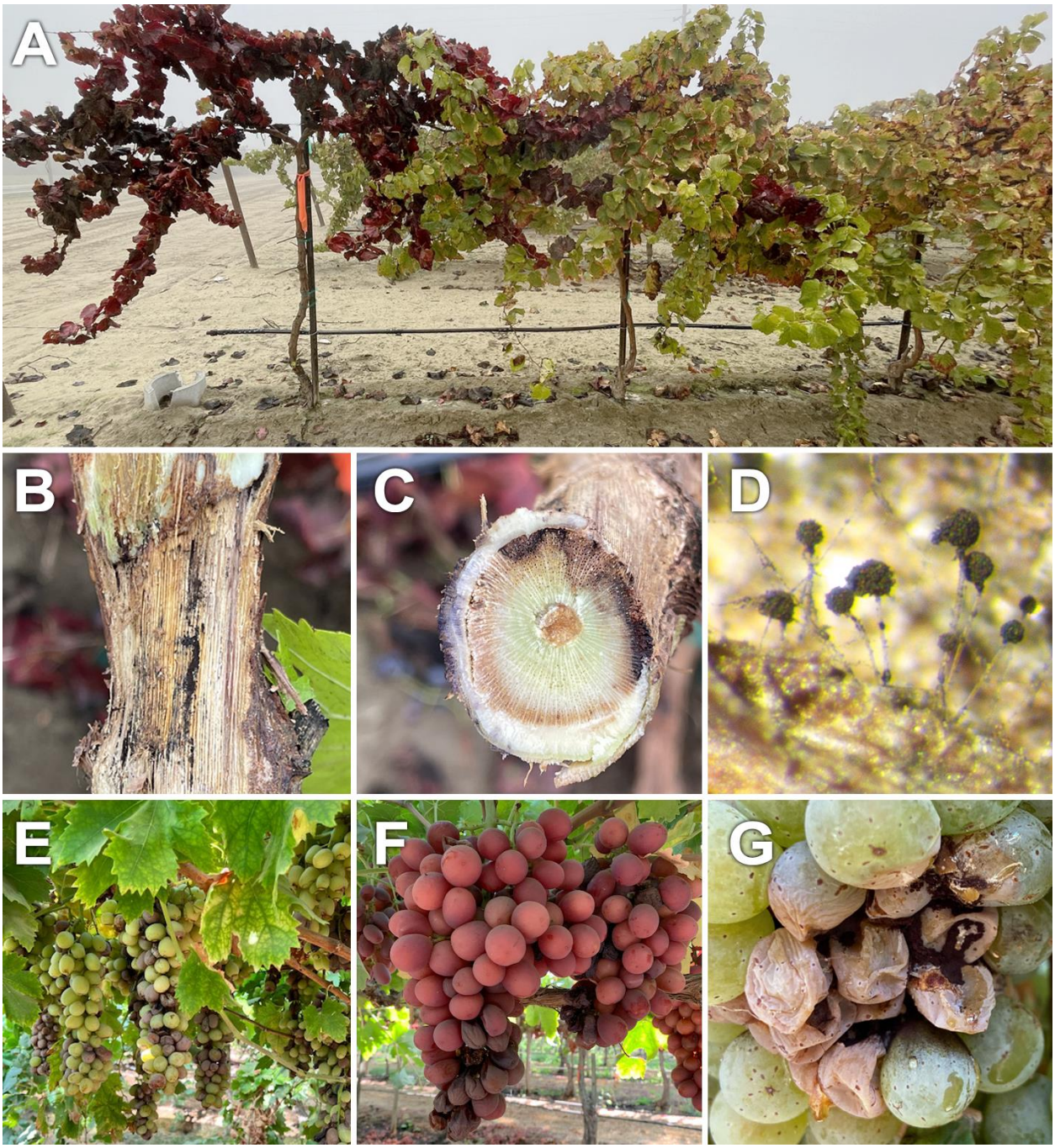


Figure 1. Symptoms of Aspergillus Vine Canker (A-D) and Sour Rot of grapes (E-G) caused by species of *Aspergillus* section *Nigri*. (A) Premature senescence of the canopy during the Fall. (B) Sporulation on cankered tissue. (C) Cross-section of a trunk showing cankers. (D) Conidial heads emerging from the bark. (E-F) Sour rot symptoms on Autumn King and Allison grape clusters. (G) Sporulation of black aspergilli on decayed berries.

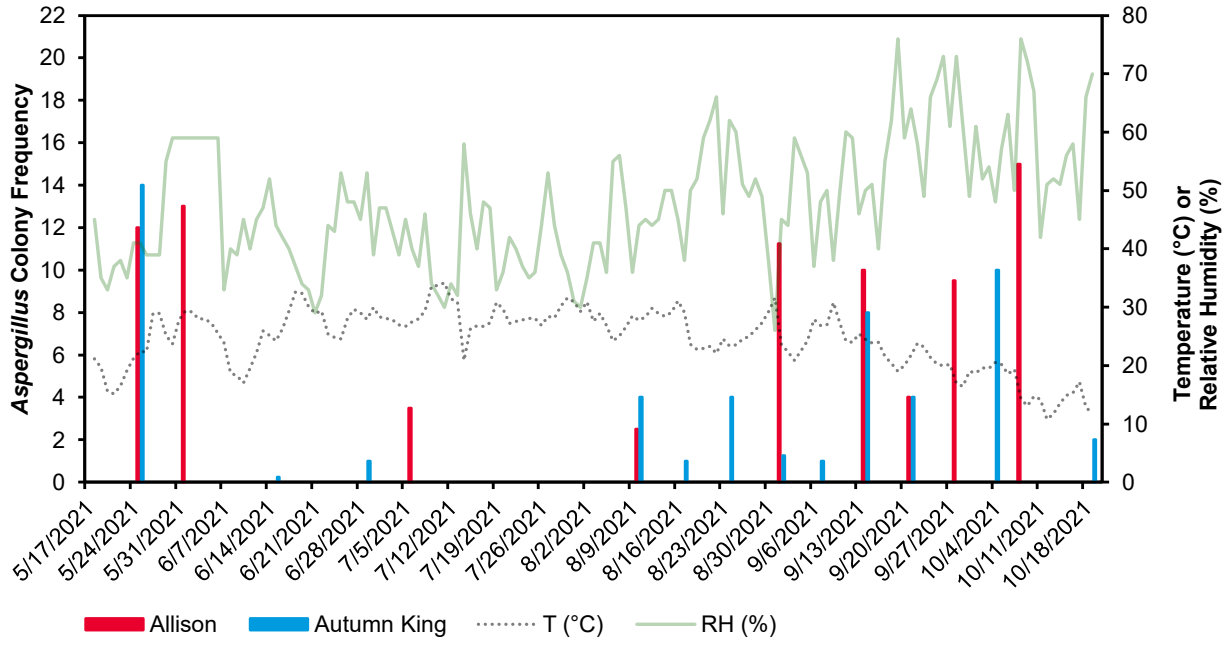


Figure 2. Frequency of *Aspergillus* colonies obtained from spore traps, average temperatures (°C) and relative humidity (%) for the Delano CIMIS weather station in Kern County. Bars indicate mean colony forming units collected every two weeks per vineyard (cultivar Allison in red and Autumn King in blue). Mean temperatures and relative humidity values are shown as lines, respectively.

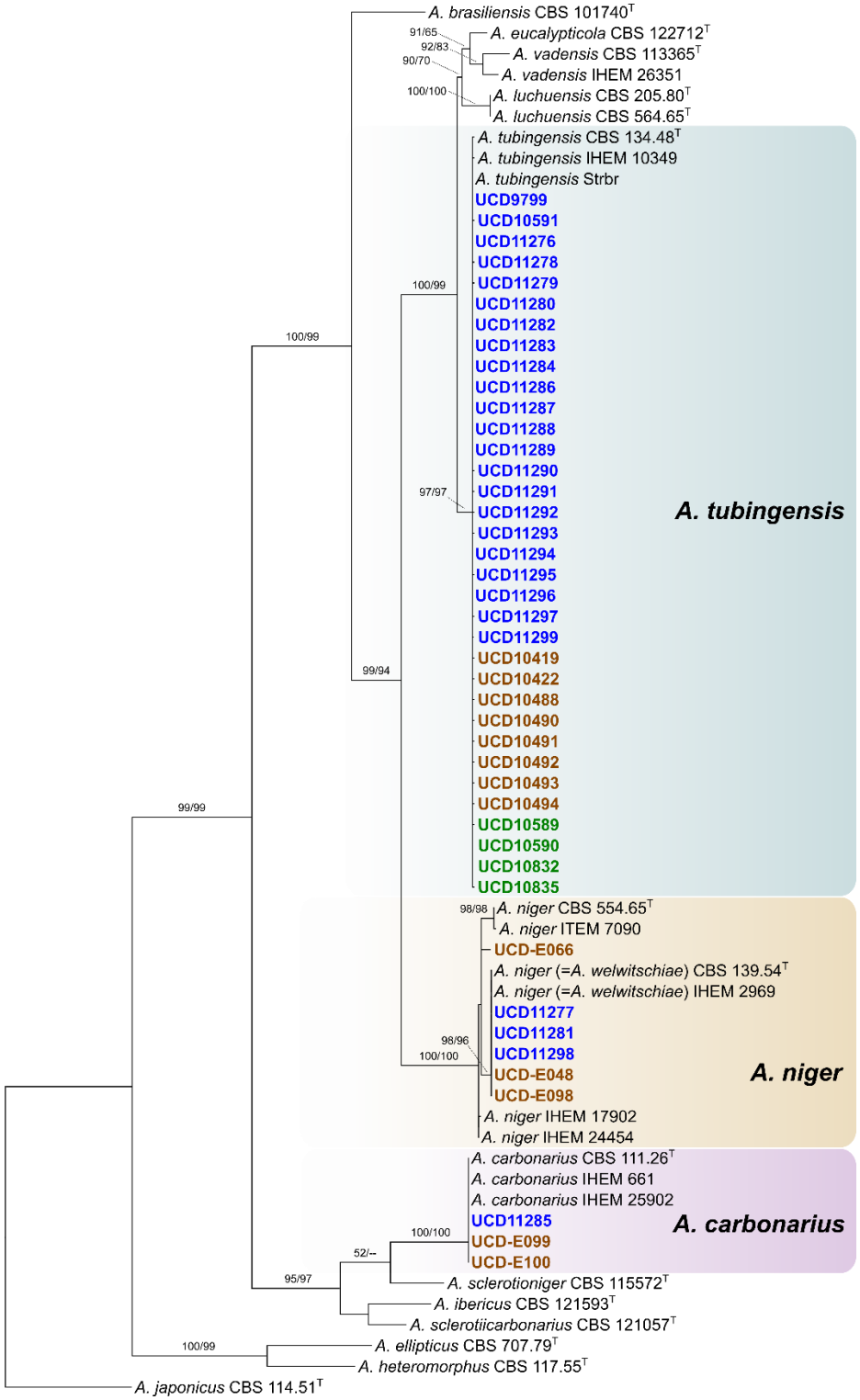


Figure 3. Phylogenetic reconstruction of isolates of *Aspergillus* spp. associated with Aspergillus Vine Canker (AVC) and Sour Rot (SR) of grapevines in California compared to closely related species of section *Nigri*. The tree was inferred using partial sequences of the calmodulin (*CaM*)

gene and rooted with *A. japonicus* (CBS 114.51). Numbers above branches represent bootstrap values from maximum parsimony and maximum likelihood analyses, both with 1,000 replicates. In bold: Isolates obtained in this study. In blue: SR isolates; in brown: AVC isolates; in green: Spore trapping isolates; and ^{T}=Reference strains.

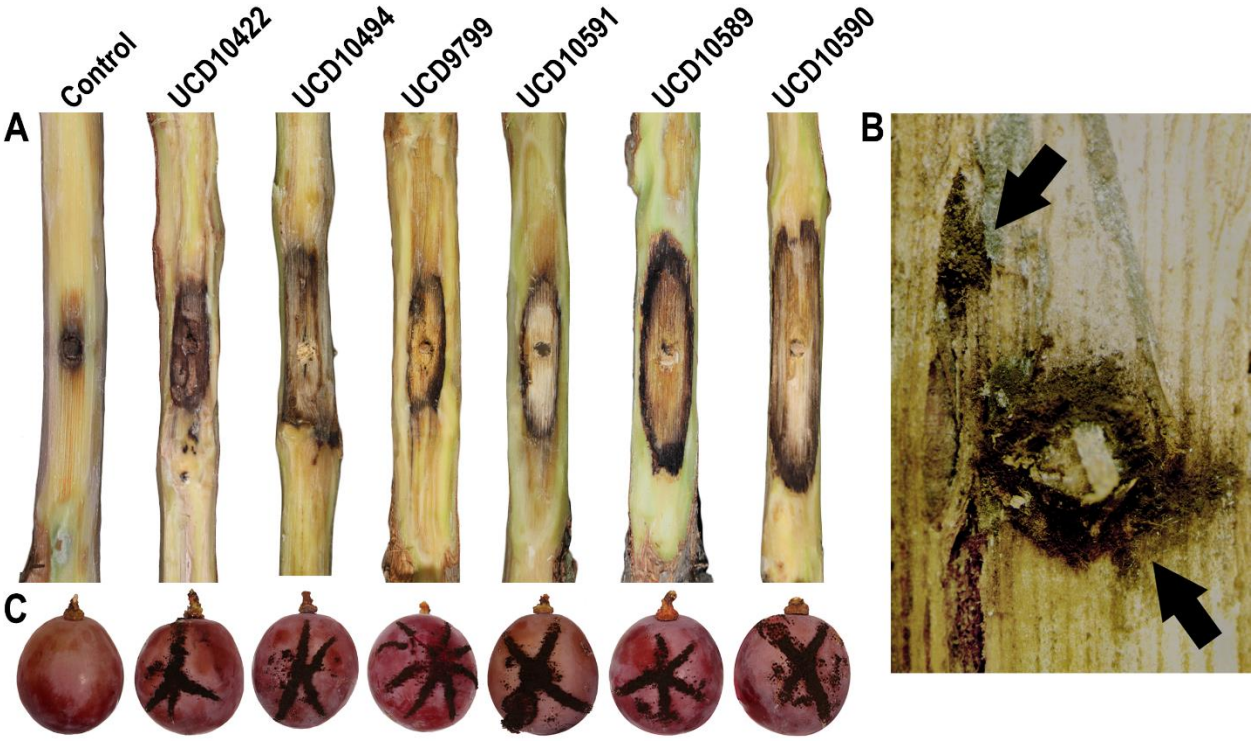


Figure 4. Pathogenicity test of *Aspergillus tubingensis* isolates on debarked wood (A-B) and berries © of 'Red Globe' grapevines. Isolates were originally obtained from symptomatic vines with Aspergillus Vine Canker (UCD10422 and UCD10494), from berries infected by sour rot (UCD9799 and UCD10591), and from those collected in the spore traps (UCD10589 and UCD10590). (A) Necrotic lesions in the wood caused by the different isolates and the control. (B) Sporulation of black aspergilli protruding the bark of an inoculated 'Red Globe' cane. Top arrow shows sporulation emerging from the lesion underneath the bark and bottom arrow indicates sporulation from the inoculation point. (C) Symptoms of sour rot caused by the different isolates and the control.

Chapter V

Discussion

Chapter V: Discussion

The results presented in this dissertation show that representative isolates of *Bacillus velezensis*, *Pseudomonas chlororaphis*, and *Serratia plymuthica*, originally obtained from grapevines with presence and absence of grapevine trunk disease (GTD) symptoms, have inhibitory activity *in vitro* against eight common fungal pathogens responsible for Botryosphaeria dieback, Eutypa dieback, Phomopsis dieback, esca, and black foot in California vineyards (Chapter II). These isolates were further applied in field settings and evaluated against artificial inoculations of different trunk pathogens (Chapter III).

A collection of 1,344 bacterial isolates were recovered from trunk, cordons, and rhizosphere of sampled vines and 12.8% showed inhibitory effects against *Neofusicoccum parvum* and *Diplodia seriata* (n = 172). Of this subset, 89.5% were identified as *B. velezensis*, whereas 6.7 corresponded to *Pseudomonas* spp. and 1.2% to *S. plymuthica*. Representative isolates of *B. velezensis* (UCD10614, UCD10631, *P. chlororaphis* (UCD10757, UCD10763) and *S. plymuthica* (UCD10719, UCD10756) exhibited antifungal activity by two mechanisms, directly by simultaneous confrontations in potato dextrose agar, and indirectly through their agar-diffusible metabolites and/or volatile organic compounds (VOCs). Specifically, all the bacterial isolates showed inhibition levels above the threshold (40%) against most of the pathogens when challenged directly, except for *L. theobromae*, where three isolates showed lower inhibition levels. However, the effect of their agar-diffusible metabolites was different across bacterial species. The diffusible metabolites of *B. velezensis* were the most toxic, inhibiting the mycelial growth of both *N. parvum* and *E. lata* at 1, 15 and 30% *v/v*, whereas the metabolites produced by *P. chlororaphis* and *S. plymuthica* only inhibited the pathogens at concentrations above 15% *v/v*, with significant differences between isolates. These results highlight the importance of selecting bacterial isolates for biocontrol purposes according to the potency of their antifungal activity. Later, it was observed that only the volatile organic compounds produced by *P. chlororaphis* and *S. plymuthica* caused a significant inhibition of the mycelial growth of *E. lata*. No inhibitions were detected from the *B. velezensis* isolates against *N. parvum* and *E. lata*, or from the other bacterial species against *N. parvum*. These compounds are also known to act as signaling molecules associated with activation of plant defense responses (Fan et al. 2018; Pršić et al. 2020), thus offering an indirect mechanism of protection.

Later, the isolates were applied in nursery and field settings implementing four different strategies and evaluated against common pathogens occurring in California. The first trial was performed in two nurseries where dormant propagation material was treated with the bacterial isolates and other biological and chemical fungicides prior grafting using a pressurized vacuum chamber. The treatments with *S. plymuthica* UCD10719 caused mortality in 100% of the vines on both nurseries. Later, when the trunk pathogens were inoculated, no differences were detected in both infection levels and lesion length of vines inoculated with *N. parvum*, however, both *B. velezensis* UCD10631 and *P. chlororaphis* UCD10763 significantly reduced the lesions length caused by *E. lata*. Later, *B. velezensis* UCD10631 only reduced the lesion length caused by *P. minimum* in nursery 1, whereas *P. chlororaphis* UCD10763 significantly suppressed the infection levels in nursery 2. Therefore, these results show a positive effect of both *B. velezensis* and *P. chlororaphis* applied separately in decreasing the impact of disease development of common trunk pathogens. These results are consistent with other studies that have applied BCAs in nursery settings aiming to manage GTDs (Leal et al. 2023; Martínez-Diz et al., 2020).

The trial involving soil drench treatments of mature vines showed that the treatments including the bacterial isolates caused no significant reductions in infection levels nor lesions length caused by *N. parvum* and *P. minimum*. However, the application of *B. velezensis* UCD10631 significantly reduced the lesions length caused by *E. lata* by about 32%. These results suggest that the incorporation of beneficial bacteria into the rhizosphere can improve the health status of grapevines by reducing the infection levels of different trunk pathogens. The injection of the three bacterial isolates into the trunk and cordon of mature vines showed no significant positive effect in reducing the infection levels and lesions lengths caused by *N. parvum*. In fact, the injections of *B. velezensis* UCD10631 caused about 20% of larger lesions compared to control. Altogether, the field evaluations show the positive effects of applying endophytic and rhizospheric bacteria using different strategies of biocontrol to prevent and reduce the impact of GTD-causing pathogens. From the four strategies, the infiltration of propagation material prior grafting and the soil drench application of the bacterial isolates *B. velezensis* UCD10631 and *P. chlororaphis* UCD10763 show the most promising results, with reductions of the infection levels of pathogens responsible for Botryosphaeria dieback, Eutypa dieback and esca. These methods could be included in an integrated pest management program targeting GTDs.

On a separate subject, the etiology of Aspergillus Vine Canker (AVC) and sour rot (SR) of grapes occurring in California was reexamined in Chapter IV given the recent rearrangement of the taxonomy of *Aspergillus* section *Nigri* (Bian et al. 2022), and the fact that the identification of the species associated to both diseases has primarily been based on morphological features and ITS sequences (Michailides et al. 2002, 2007; Rooney-Latham et al. 2008). Isolates recovered from symptomatic grapevine samples showing AVC and SR were analyzed using their calmodulin (*CaM*) gene sequences and representative isolates were tested for pathogenicity in healthy lignified canes and berries. The results revealed that *A. niger*, *A. carbonarius* and *A. tubingensis* are the causal agents of both AVC and SR in California, with a higher incidence of *A. tubingensis* among isolates obtained from different vineyards exhibiting symptoms of AVC or SR. Of these, only *A. niger* and *A. carbonarius* were previously reported associated to both diseases. Therefore, six isolates of *A. tubingensis* were tested and confirmed to be pathogenic on canes and berries of 'Red Globe' grapevines. All the isolates caused sour rot symptoms only in wounded berries and canes, however it was possible to recover *Aspergillus* colonies from unwounded canes. This may suggest that *Aspergillus* is able to colonize the wood of grapevines as an endophyte without causing disease. Previously, it has been discussed that some of the infections associated with GTDs have a latent period, where the pathogen can colonize the plant without causing symptoms until the environmental conditions are conducive, usually associated with stress (Graniti et al. 2000; Hrycan et al. 2020). From an epidemiology perspective, these findings indicate that the risks for infection events by *Aspergillus* spp. are higher when sufficient inoculum is present in the field and environmental conditions favor its dispersal to susceptible vines. Therefore, management of *Aspergillus*-associated diseases must consider prevention of the main factors that lead to infections, which are injuries of berries and of the woody structures, ventilation of the canopy and compactness of clusters, among others (Duncan et al. 1995; Visconti et al. 2007). The results of this chapter constitute the first detection of *A. tubingensis* associated with AVC and SR diseases in California. Morphologically, *A. tubingensis* and *A. niger* are almost identical and are difficult to distinguish, nonetheless, *A. carbonarius* produces significantly larger conidia, facilitating its identification (Samson et al., 2007). In conclusion, the use of both morphological and phylogenetic analyses using *CaM* nucleotide sequences were sufficient and accurate in identifying the black aspergilli species causing Aspergillus Vine Canker and Sour Rot of grapes in California under the current taxonomy of the section *Nigri*.

Literature cited

Bian, C., Kusuya, Y., Sklenář, F., D'hooge, E., Yaguchi, T., Ban, S., Visagie, C.M., Houbraken, J., Takahashi, H., Hubka, V. 2022. Reducing the number of accepted species in *Aspergillus* series *Nigri*. Studies in Mycology 102: 95–132. https://doi.org/10.3114/sim.2022.102.03

Duncan, R. A., Stapleton, J. J., Leavitt, G. M. 1995. Population dynamics of epiphytic mycoflora and occurrence of bunch rots of wine grapes as influenced by leaf removal. Plant Pathol. 44:956-965. https://doi.org/10.1111/j.1365-3059.1995.tb02653.x

Fan, B., Wang, C., Song, X., Ding, X., Wu, L., Wu, H., Gao, X., Borriss, R. 2018. *Bacillus velezensis* FZB42 in 2018: The gram-positive model strain for plant growth promotion and biocontrol. *Front. Microbiol*. 9:2491. https://doi.org/10.3389/fmicb.2018.02491

Leal, C., Gramaje, D., Fontaine, F., Richet, N., Trotel-Aziz, P., Armengol, J. 2023. Evaluation of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 to control Botryosphaeria dieback and black-foot pathogens in grapevine propagation material. Pest Manag Sci 79:1674-1683. https://doi.org/10.1002/ps.7339

Martínez-Diz, M.P., Díaz-Losada, E., Andrés-Sodupe, M., Bujanda, R., Maldonado-González, M.M., Ojeda, S., Yacoub, A., Rey, P., Gramaje, D. 2021. Field evaluation of biocontrol agents against black-foot and Petri diseases of grapevine. Pest Manag Sci 77: 697–708. https://doi.org/10.1002/ps.6064.

Michailides, T.J., Peacock, W., Christensen, P., Morgan, D.P., Felts, D. 2002. First report of Aspergillus Vine Canker of table grapes caused by *Aspergillus niger*. Plant Dis. 86:75. https://doi.org/10.1094/PDIS.2002.86.1.75A

Michailides, T.J., Peacock, W., Christensen, P., Morgan, D.P. 2007. Aspergillus vine canker of table grapes caused by *Aspergillus* sect. *Nigri* in California. Phytopathol. Mediterr. 46:102-128.

Pršić, J.; Ongena, M. 2020. Elicitors of plant immunity triggered by beneficial bacteria. Front. Plant Sci. 11: 594530. https://doi.org/10.3389/fpls.2020.594530

Rooney-Latham, S., C. N. Janousek, A. Eskalen, W. D. Gubler. 2008. First report of *Aspergillus carbonarius* causing sour rot of table grapes (*Vitis vinifera*) in California. Plant Dis. 92(4):651. https://doi.org/10.1094/PDIS-92-4-0651A

Samson, R.A., Noonim, P., Meijer, M., Houbraken, J., Frisvad, J.C., Varga, J. 2007. Diagnostic tools to identify black aspergilli. Studies in Mycology 59: 129–145. https://doi.org/10.3114/sim.2007.59.13

Visconti, A., Perrone, G., Cozzi, G., Solfrizzo, M. 2007. Managing ochratoxin A risk in the grape-wine food chain. Food Additives and Contaminants 25(2):193-202. https://doi.org/10.1080/02652030701744546