INVESTIGATING BOTRYOSPHAERIA/PHOMOPSIS DISEASES OF WALNUT IN SAN JOAQUIN AND STANISLAUS COUNTIES, CALIFORNIA

Mohamed T. Nouri, Florent Trouillas, Kari Arnold, and Themis Michailides

ABSTRACT

In the last few years, Botryosphaeria and Phomopsis blight and canker diseases have been observed increasingly in walnut orchards in almost all walnut growing regions in California. Main symptoms include cankers in branches, dieback of spurs resulting from infections moving from affected fruit (fruit blight) via the peduncle or shoots through leaf and peduncle scars. Results from previous research revealed the occurrence of 10 species of Botryosphaeriaceae and two species of Diaporthe (syn. *Phomopsis*). Fungi in the Botryosphaeriaceae and Diaporthaceae spp. can be found throughout the year on diseased branches and may sometimes occur together on the same branch, which is difficult to diagnose in the field. These fungi overwinter on dead branches and shoots, and can develop both reproductive structures – pycnidia producing the water-splashed conidia and perithecia producing the airborne ascospores – with both spore types being dispersed during wet conditions. Between September 2019 through November 2020, we collected and analyzed diseased tissue samples from 43 mature walnut orchards in San Joaquin County and three orchards in Stanislaus County. Despite several management practices implemented to prevent major yield and economic losses caused by Bot/Phomopsis diseases, field survey results showed Diaporthaceae spp. to be the most prevalent fungal pathogen isolated from diseased walnut samples. Botryosphaeriaceae fungi were found occasionally. Similarly, pre-season BUDMON monitoring showed very low recovery percentage of fungi in the Botryosphaeriaceae and Diaporthaceae families. Spore trapping studies were performed to determine the abundance and seasonal spore release pattern of Botryosphaeriaceae and Diaporthaceae fungi. Based on colony counts, the population of Botryosphaeriaceae fungi was significantly lower than that of the Diaporthaceae fungi. Here we analyzed the correlation between precipitation events, irrigation and grinding of infected branches between tree rows (following maintenance/cleaning pruning) and Bot/Phomopsis spore release. Among these variables, we found a strong relationship between spore release and precipitation. The first irrigation of the season also showed a positive relationship with spore release in both orchards with sprinkler irrigation. We also detected high aerial dissemination of spores when grinding of infected branches deposited between tree rows; this information is of great importance as it helps to identify production practices responsible for the spread of these fungal pathogens within walnut orchards. Although growers are making four to five fungicide applications per season to control this disease with inconsistent results, the persistence of Diaporthaceae spp. in walnut orchards has raised the questions whether this fungus has emerged as the main blight/canker/dieback disease of walnut. Molecular identification for isolates recovered from infected spurs and branch cankers and isolates recovered from spore trapping studies revealed the presence of five new species of Diaporthe – three of them were found in both spore trapping and diseased tissue isolations. The occurrence of these new species in walnut orchard represent new reports in California. In laboratory assays using walnut fruits, we tested the efficacy of three new biocontrol products: Julietta® (plant protection product based on living Yeast); Epsilon ε-Poly-lysine (EPL) and Vintec (*Trichoderma atroviride* SC1-based Product) against two fungal isolates of *Diaporthe ambigua* and *Neofusicoccum mediterraneum*. Preliminary

results showed the biological yeast-based product Julietta® to perform well. We plan to repeat this experiment on a larger scale and evaluate the efficacy of these three biocontrol products in preventing fungal pathogen entry via pruning wounds. We are also planning to evaluate the pathogenicity of the new *Diaporthe* spp. recovered from walnut orchards.

OBJECTIVES

1) Investigate the frequency and distribution of Botryosphaeriaceae and Diaporthaceae fungi in walnut orchards from different locations in San Joaquin and Stanislaus Counties.
2) Determine the incidence of Diaporthaceae spp. and Botryosphaeriaceae spp. in asymptomatic walnut buds via the BUDMON Technique.
3) Investigate when and under what environmental conditions spores of these fungi are released in three walnut orchards using spore-trapping studies.
4) In vitro testing of three new biocontrol products for the protection of walnut fruits.

SIGNIFICANT FINDINGS

- Survey results showed Diaporthaceae spp. to be the most prevalent fungal pathogens isolated from diseased walnut samples including infected spurs and branch cankers. Botryosphaeriaceae fungi were only found occasionally.
- Molecular identification of isolates recovered from infected spurs and branch cankers and isolates recovered from the spore trapping study revealed the occurrence in walnut orchards of several new species of Diaporthe.
- Three of the new Diaporthe species were found in both spore trapping and symptomatic tissue cultures.
- We found a strong correlation between spore release and precipitation.
- The first irrigation of the season showed a strong correlation with spore release for both orchards with sprinkler irrigation.
- We also detected high aerial dissemination of spores of these pathogens when grinding infected branches between tree rows following maintenance pruning.

PROCEDURES

1) **Investigate the frequency and distribution of Botryosphaeriaceae and Diaporthaceae fungi in walnut orchards from different locations in San Joaquin and Stanislaus Counties.**

During this study, we collected samples from 46 walnut orchards. Samples including fruit and peduncle blight, twig/spurs blight, dead twigs/spurs and branch cankers, were collected and taken to the laboratory to proceed with isolation and identification works. Small wood pieces from the margins of necrotic and apparently healthy tissues were surface disinfected in 10% bleach solution for 2 min and subsequently rinsed twice with sterile water. After drying on sterile paper towels, the wood fragments were plated onto acidified potato dextrose agar (APDA; 2.6 ml of 25% [vol/vol] lactic acid per liter of medium), then incubated in the laboratory at room temperature (around 25°C) with approximately 12 h of daylight and 12 h of darkness. After ~7 days, plates

were inspected for developing fungal colonies. Pure fungal cultures were obtained by transferring single hyphal tips into new APDA Petri plates. Identification of the various isolates collected was completed using polymerase chain reaction (PCR), amplification of the internal transcribed spacer region (ITS) of the rDNA, and comparison with reference sequences from GenBank. DNA amplification of the ITS region was conducted using primers ITS1 and ITS4.

2) Determine the incidence of Diaporthaceae spp. and Botryosphaeriaceae spp. in asymptomatic walnut buds via the BUDMON technique.

From February to early March 2020 and before bud dormancy break, we tested the incidence of Diaporthaceae spp. and Botryosphaeriaceae fungi in asymptomatic walnut buds via the BUDMON technique (Michaillides et al. walnut report 2013) from 30 walnut orchards (20 orchards in San Joaquin County and 10 in Stanislaus County). Samples were collected from the cultivars Chandler, Tulare, Vina, Howard and Serr. Buds were surface disinfected as described above, then plated onto APDA and incubated in the laboratory at room temperature. Isolation results were recorded after 7 days.

3) Investigate when and under what environmental conditions spores of these fungi are released in three walnut orchards using spore-trapping studies.

Three walnut orchards were selected for this study. For both orchards, one and two, with sprinkler irrigation system – from which we recovered high incidence of Diaporthaceae spp. from diseases samples – we started this experiment by the end of February 2020, a period that coincided with late dormant season rainfalls. For orchard three – a 12-year-old Howard orchard with drip irrigation and showing very low Bot and Phomopsis disease incidence – we started the experiment the first week of April. Spores were trapped on glass microscope slides (25 by 76 mm) coated on both sides with a thin layer of white petroleum jelly and placed on walnut branches. This is a simple and rapid technique of quantifying spores from walnut orchards. Ten spore traps were placed randomly on the lower canopy of separate trees in the middle of the three orchards. Spore traps were changed weekly and individually collected in sterile 50-ml screw-cap tubes. In the laboratory, spores were removed by adding 4 ml DI water into each tube, then shaken gently by hand for 30-40 second. From the slide wash solution, two 100µl aliquots were placed on two replicate 85-mm-diameter APDA petri plates. After spreading the solution with a disposable 10 µl inoculating loop, plates were dried for 10 min inside a laminar flow hood, then incubated in the laboratory at room temperature (~ 25°C) with approximately 12 h of daylight and 12 h of darkness. Fungal colonies were counted after 7 days. Fungal colonies of Diaporthaceae and Botryosphaeriaceae fungi were identified by colony morphology and growth characteristics. Representative isolates were subcultured onto new APDA plates.

4) In vitro testing of three new biocontrol products for the protection of walnut fruits.

Two isolates of *Diaporthe ambigua* (Mo-10) and *Neofusicoccum mediterraneum* (Mo-25) originally isolated from blighted walnut fruits were used in this experiment. Healthy fruits were surface disinfected for 4 min in 10% chlorine bleach solution, then rinsed twice in sterile distilled water and air-dried on sterile paper towels. A 4-mm wound was made using a sterilized cork borer on the surface of the fruit hulls and a 4-mm-diameter mycelium plug from a 7-day-old APDA culture was aseptically inserted into the wound. Non-colonized APDA agar plugs served as the

negative control. Fruits were incubated for 2 weeks in the laboratory at room temperature (around 25°C) on square plastic boxes containing wet paper towels on the bottom to keep humidity up. Each plastic box contained 10 fruits (five fruits inoculated with the isolate Mo-10 and five fruits inoculated with the isolate Mo-25). Two type of treatments were conducted. **1) Preventive:** after wounds were made, and 24h before inoculation, fruits in each box were sprayed with one of the three treatment products – Julietta, EPL and Vintec – following the recommended label rate. **2) Curative:** fruits were first inoculated with the pathogens and then 24h later were treated with the natural fermentation and biocontrol products. Fruit were evaluated for blight symptoms (necrosis) and for any potential phytotoxicity.

RESULTS AND DISCUSSION

1) **Investigate the frequency and distribution of Botryosphaeriaceae and Diaporthaceae fungi in walnut orchards from different locations in San Joaquin and Stanislaus Counties.**

Isolation work showed Diaporthaceae fungi to be the most prevalent fungal pathogens (70-80% fungal recovery) isolated from diseased walnut samples. Botryosphaeriaceae fungi were only found occasionally (5-10% fungal recovery). We also initiated preliminary taxonomic studies that revealed the occurrence in walnut orchards of five new species of Diaporthe; three of them were found in both spore trapping and symptomatic tissue cultures, and included *D. ambigua*, *D. australafricana, D. chamaeropis* and *D.* foeniculina. *Diaporthe ambigua* has been reported as a weak pathogen on grapevines in California (Lawrence et al. 2015), and moderately aggressive to pistachio wood (Nouri et al. 2019). *D. foeniculina* has recently been reported to cause black tip and necrotic spot on hazelnut kernel in Chile (Contreras et al. 2020). Additionally, *D. australafricana* has been identified to be associated with stem canker and dieback in European Hazelnut (Corylus avellana L.) and walnut branch canker in Chile (Guerrero and Pérez 2013; Jiménez Luna et al.2020). Further research to determine the pathogenicity of these new Diaporthe species will be conducted. Several studies on the effect of temperature indicated that the optimum mycelial growth and conidial sporulation for Diaporthe ranged from 20 to 30°C. Also, with the spore-trapping studies results (see below), it would be useful to further investigate whether an early spray timing would be effective to reduce the disease incidence. Diagnostic results were shared with PCAs and growers, which will allow them to adopt management decisions/approaches accordingly.

2) **Determine the incidence of Diaporthaceae spp. and Botryosphaeriaceae spp. in asymptomatic walnut buds via the BUDMON technique**

Overall, percent recovery of fungal pathogens was very low (≤ 2%) for all the orchards sampled. This result may be attributable to the dry weather conditions, which prevailed during last two seasons in the sampled orchards, especially during the sampling period (From February to early March 2020).

3) **Investigate when and under what environmental conditions spores of these fungi are released in three walnut orchards using spore-trapping studies.**

Results for January and February 2021 are not included in this report, since we are conducting a spore-trapping study for one-year cycle (Feb 28, 2020 to Feb 26, 2021). Spores were mainly captured from March to May. Overall, our spore trap study indicated that greatest spore release of Diaporthaceae and Botryosphaeriaceae spp. occurred during and following rain events. A strong correlation between spore release and precipitation was established: as precipitation increased spore release also increased (Fig. 1 to 5). For the Orchard 1 and 2 with sprinkler irrigation system, Diaporthaceae spores were also trapped during the second week of May 2020 and following the first irrigation event of the season. Here, we believe that the wetness/humidity generally in the orchard resulting from irrigation caused spores to ooze and be released from pycnidia in diseased tissues within the orchard. Also, our results showed no correlation between further in season irrigation events and the release of fungal spores of Diaporthaceae and Botryosphaeriaceae fungi. Furthermore, we noted that – across the course of the study – the amount of Botryosphaeriaceae spp. spore released was much lower than that of Diaporthaceae spp. and very few spores of Botryosphaeriaceae were trapped in the spring (ranging from 1 to 5 spores for orchard 1 and 1 to 6 spores for orchard 2) (Fig 2 and 4). The weekly average number of spores of Diaporthaceae spp. trapped ranged from 4 to 199.4 spores/trap for orchard 1 and from 2 to 175 spores/trap for orchard 2, which confirm the results of the field surveys that showed Diaporthaceae spp. to be the most prevalent fungal pathogens isolated from diseased walnut samples.

For Orchard 3 with drip irrigation system, only spores of Diaporthaceae spp. were trapped. The number of spores trapped ranged from 1 to 11 spores/trap. The spore trap study confirmed our survey results, which showed Diaporthaceae spp. to be the only fungus recovered with low percentage recovery (10%).

In Orchard 1, we also determined high aerial dissemination of spores following grinding/shredding of infected branches on the orchard floor during the 3rd week of May 2019 (Fig 1 and 2). This unique spore release event associated with shredding of dead branches also occurred shortly after maintenance pruning thus creating fresh pruning wounds, which could lead to high infection rates of pruning wounds in this orchard. This work emphasizes the importance of spore-trapping study as a valuable tool to gain knowledge of the epidemiology of the Diaporthaceae spp. and Botryosphaeriaceae spp. in California walnut orchards. Information generated from this study provides valuable information to help growers better manage canker diseases of walnut.

4) In vitro testing of three new biocontrol products for the protection of walnut fruits.

Average lesion diameters on walnut fruits for the different inoculation and biocontrol treatments are shown in Figure 6. The experiment was performed on September 10, 2020 using mycelium plug of the various pathogens. Results showed that of the three products tested, Julietta® performed well as a preventive (P) treatment against *Neofusicoccum mediterraneum* isolate (Mo-25). Lesions did not develop with the *Diaporthe ambigua* isolate (Mo-10) due to the slow growth of the fungus and unfortunately, most fruits splitted quickly after 12 days of incubation. Curative (C) treatments did not provide good disease control. Further evaluations including early season inoculation of the fruits using mycelium plug (to avoid hull split) and pruning wounds protection trials in the field will be conducted to confirm the results of our preliminary data.

Fig. 1. Total number of Diaporthaceae spores trapped per week correlated with weekly precipitation (mm) in orchard 1. CIMIS Manteca - Station 70 was used for the precipitation data collection. Note: no spores were trapped from June to December and data were combined to report monthly values due to limited space in the graph.

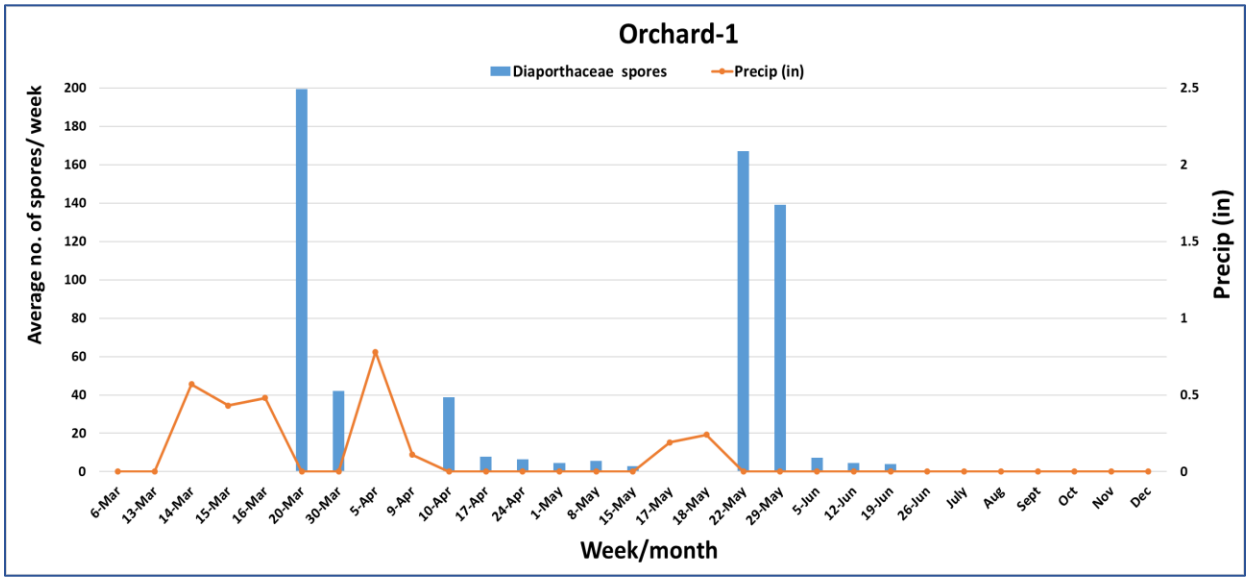


Fig. 2. Total number of Botryosphaeriaceae spores trapped per week correlated with weekly precipitation (mm) in orchard 1. CIMIS Manteca - Station 70 was used for the precipitation data collection. Note: no spores were trapped from June to December and data were combined to report monthly values due to limited space in the graph.

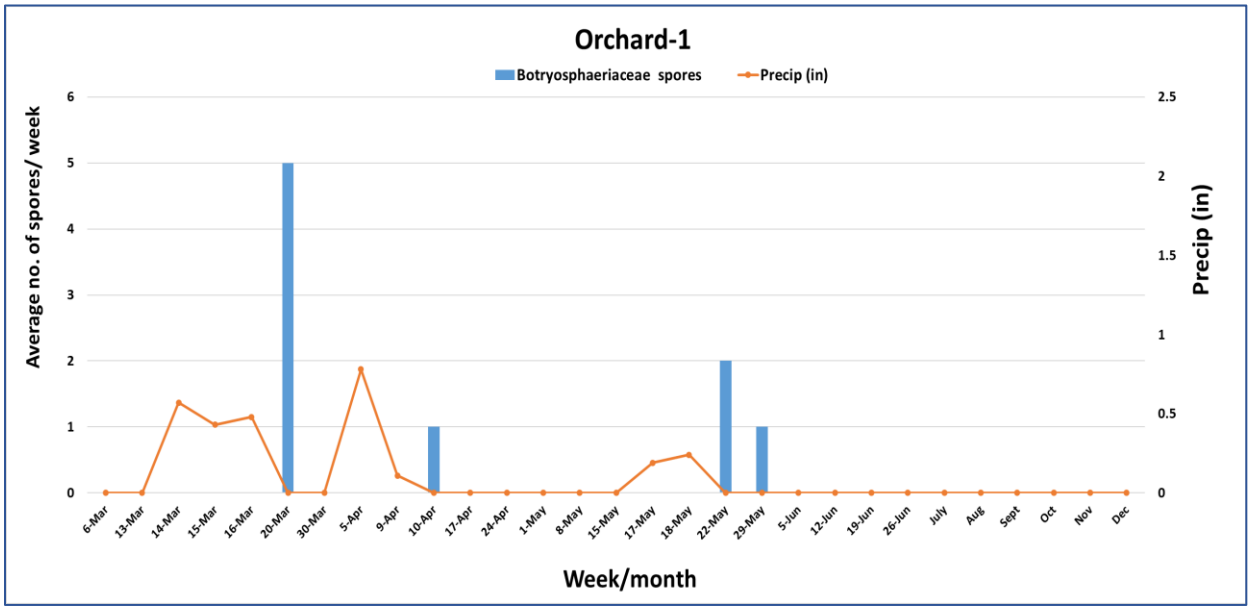


Fig. 3. Total number of Diaporthaceae spores trapped per week correlated with weekly precipitation (mm) in orchard 2. CIMIS Oakdale - Station 194 was used for the precipitation data collection. Note: no spores were trapped from June to December and data were combined to report monthly values due to limited space in the graph.

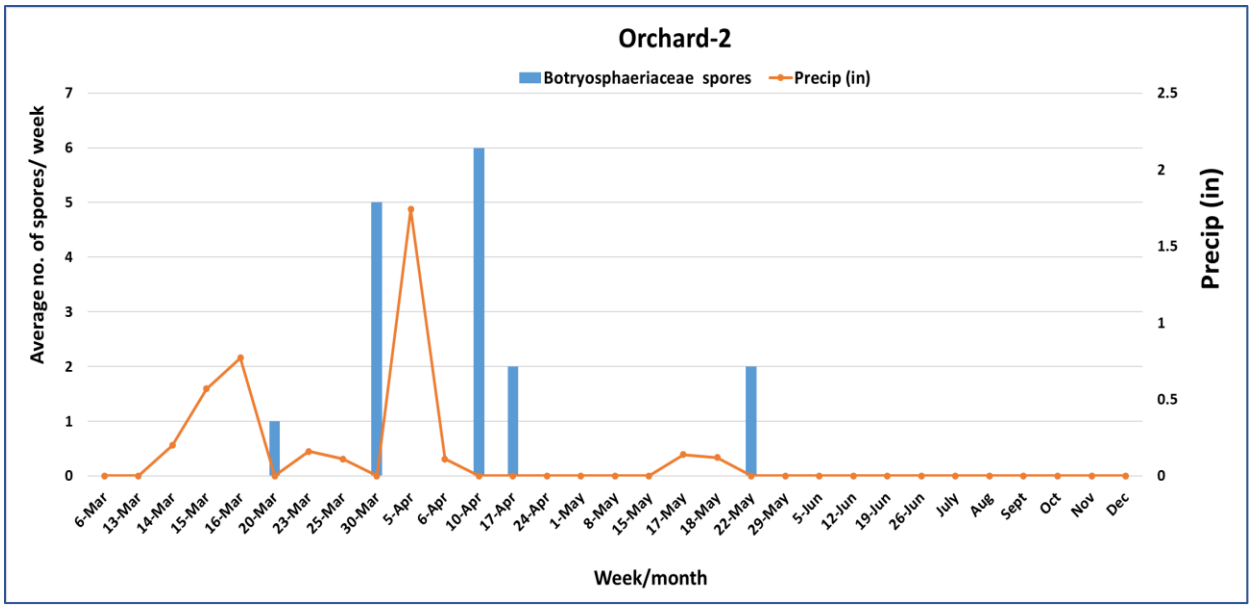


Fig. 4. Total number of Botryosphaeriaceae spores trapped per week correlated with weekly precipitation (mm) in orchard 2. CIMIS Oakdale - Station 194 was used for the precipitation data collection. Note: no spores were trapped from June to December and data were combined to report monthly values due to limited space in the graph.

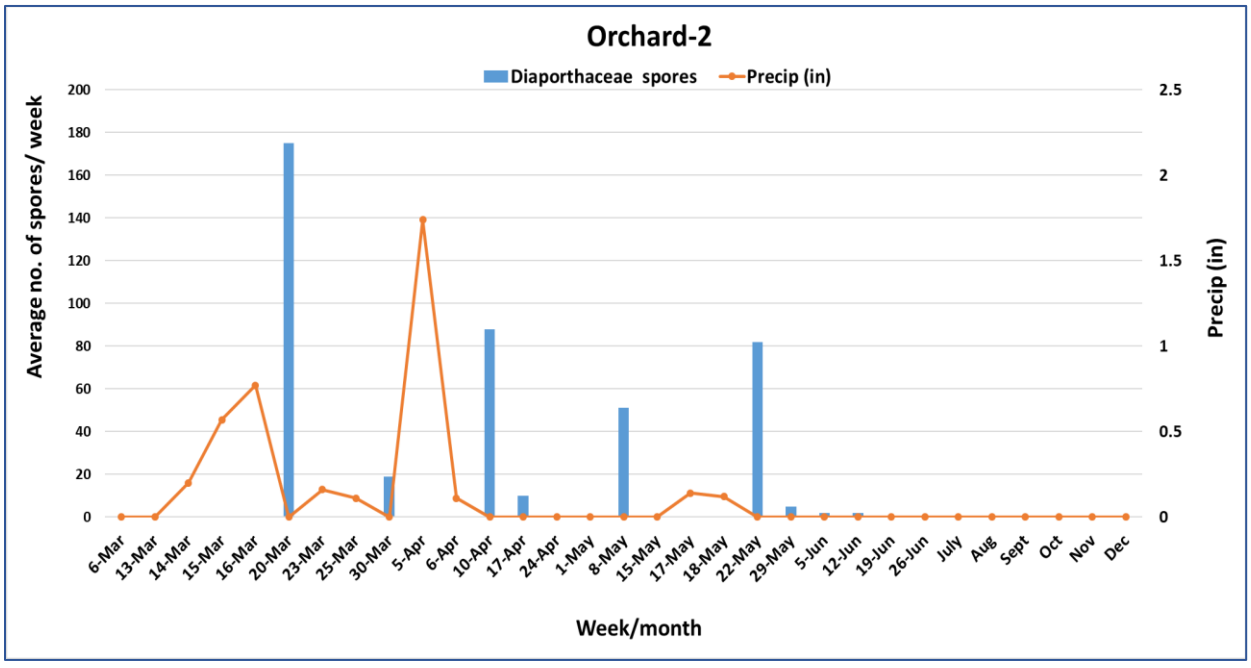


Fig. 5. Total number of Diaporthaceae spores trapped per week correlated with weekly precipitation (mm) in orchard 3. CIMIS Manteca - Station 70 was used for the precipitation data collection. Note: no spores were trapped from June to December and data were combined to report monthly values due to limited space in the graph.

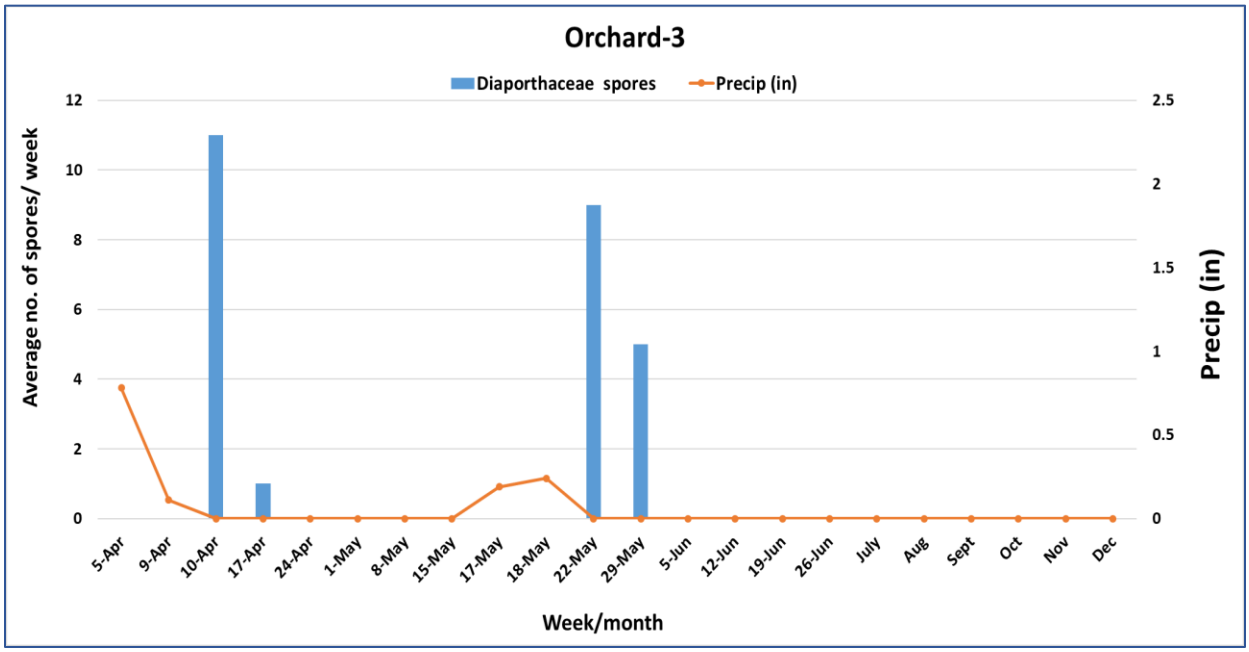
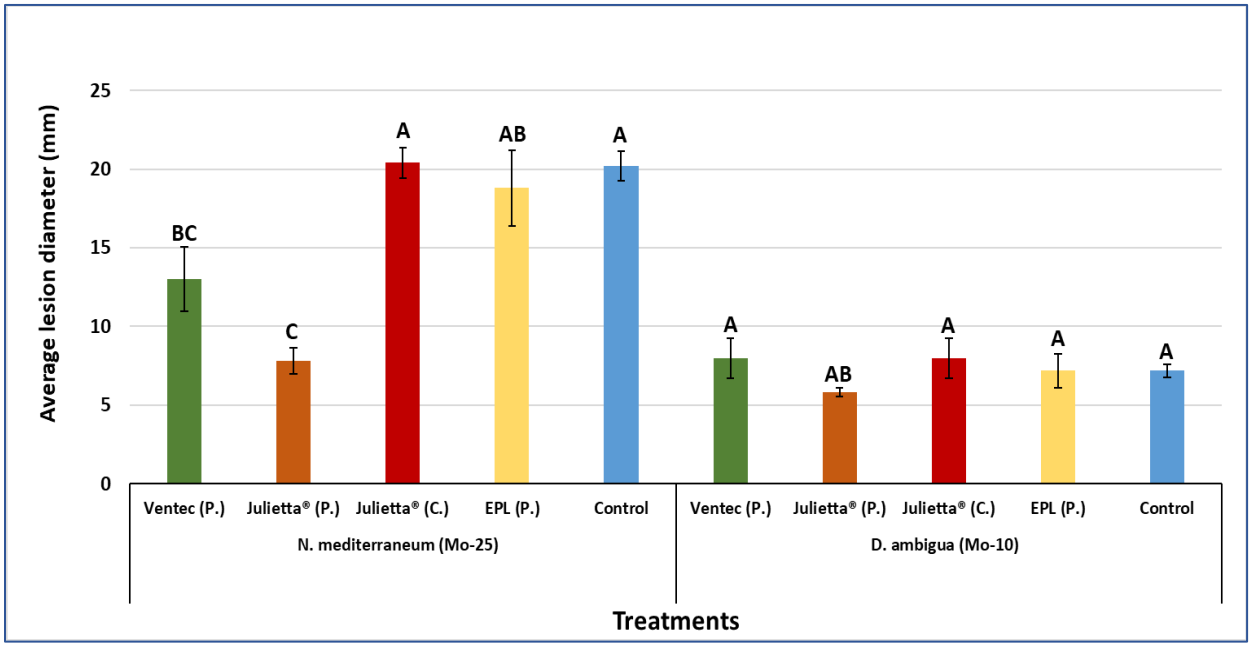


Fig. 6. Average lesion diameter on walnut fruits: In vitro testing of one natural fermentation product (Epsilon ε-Poly-lysine (EPL)) and two new biocontrol products (Julietta® – Yeast based product – and Vintec – Trichoderma atroviride SC1-based Product) for the protection of walnut fruits. Columns with different letters indicate treatment means that are significantly different ($P < 0.05$).



ACKNOWLEDGEMENTS

We would like to thank the California Walnut Board for funding. I am very grateful to Shirley Velia Alvarez, our field & lab technician in San Joaquin County, for all the field and laboratory work she provided on this project.

REFERENCES:

Contreras, J. G., Gutierrez, R. G., Contrera, K. O., and Fuentealba, S. P. 2020. First Report of *Diaporthe foeniculina* Causing Black Tip and Necrotic Spot on Hazelnut Kernel in Chile. Plant Dis. 104(3):975.

Jiménez Luna, I., Cadiz, F., Aravena, R., Larach, A., Besoain, X., Ezcurra, E., and Rolshausen, P. E. 2020. First Report of *Diaporthe cynaroidis* and *D. australafricana* Associated with Walnut Branch Canker in Chile. Plant Dis. 104(10):2732.

Lawrence, D. P., Travadon, R., and Baumgartner, K. 2015. Diversity of Diaporthe species associated with wood cankers of fruit and nut crops in northern California. Mycologia 107:926-940.

Nouri, M. T., Lawrence, D. P., Holland, L. A., Doll, D. A., Kallsen, C. E., Culumber, C. A., and Trouillas, F. P. 2019. Identification and pathogenicity of fungal species associates with canker diseases of pistachio in California. Plant Dis. 103:2397-2411.

Guerrero, J., Pérez, S. 2013. First Report of *Diaporthe australafricana*-Caused Stem Canker and Dieback in European Hazelnut (Corylus avellana L.) in Chile. Plant Dis. 97(12):1657.