Annual Report 2018

Project Title: BIOLOGY AND CONTROL OF NEOFABRAEA LEAF SPOT, BRANCH CANKER AND TWIG DIEBACK OF OIL OLIVES IN CALIFORNIA

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Interpretive summary:
Surveys of table and oil olive orchards were conducted in 2017 and 2018 to determine the distribution in California of a new disease of olive, namely *Neofabraea* and *Phlyctema* leaf and shoot lesions. Surveys were conducted in Colusa, Contra Costa, Glenn, Kings, San Joaquin, San Luis Obispo, Stanislaus, and Tulare Counties and included traditional as well as high density (HD) and super high density (SHD) plantations, totaling 27 orchards. The new disease was detected mainly in the Arbosana cultivar and to a lesser extent in the Arbequina cultivar in SHD olive orchards in Glenn, San Joaquin, and Stanislaus Counties. Arbosana was highly affected by the disease, whereas it was only sporadic in Arbequina and not found in Koroneiki. Two species, namely *Neofabraea kienholzii* and *Phlyctema vagabunda*, were found to be consistently associated with the disease. Species identity was confirmed by morphology and molecular data and Koch's postulates to determine the pathogenicity of these species in olive were completed. Several field experiments were conducted over the last three fall-winter seasons to evaluate the efficacy of various fungicides to control Neofabraea and Phlyctema leaf and twig lesions. During the winter 2017-2018, we conducted additional field trials in the highly susceptible cultivar Arbosana in a commercial orchard in San Joaquin County and compared the efficacy of 8 fungicides and different application regimes (single versus multiple applications) to control this new disease. Results showed that several products were effective in reducing infection by the pathogens and limiting disease incidence. Overall, best disease control was achieved by Topsin M, Vanguard, Inspire Super, Bravo and Ziram fungicides, which provided up to 75% reduction in disease incidence. Copper fungicides did not control the disease. Comparison of different fungicide application regimes showed that one to two applications after harvest significantly reduce disease incidence. Two independent wound susceptibility trials were conducted also to determine the duration (0, 1, 2, 3, 4 or 5 weeks) when harvest wounds on leaves remain susceptible to infection, and thus determine the number and timing of fungicide applications required to control Neofabraea and Phlyctema diseases. Results showed that leaves inoculated immediately after wounding (harvest) and those inoculated one week after wounding were the most susceptible to infection. Overall, leaf wound susceptibility to infection declined substantially after 4 weeks following wounding.

This suggests that wounds had healed after 4 weeks following a wounding event at harvest and that one fungicide application after harvest followed by a second application 2 to 3 weeks later should suffice to protect olive trees from infection. Last year, both Inspire Super and Ziram fungicides were submitted for use under section 18 emergency exemption as well as for registration through the IR-4 program. The availability of these two fungicides in olive will improve control of Neofabraea and Phlyctema leaf and shoot lesions and will allow for management of fungicide resistance by rotating modes of action.

Objective 1: Determine the symptoms, geographic distribution and host range of *Neofabraea kienholzii* and *P. vagabunda* in California

Material and methods

Surveys of table and oil olive orchards were conducted in Colusa, Contra Costa, Glenn, Kings, San Joaquin, San Luis Obispo, Stanislaus, and Tulare Counties. Orchards surveyed included traditional as well as HD and SHD plantations, totaling 27 orchards. Approximately 30 to 40 olive trees per orchard were inspected for the presence of leaf and shoot lesions typical of the new diseases. Symptomatic olive tissues were taken to the laboratory to proceed with microbiological isolations to determine which pathogens may be present. Isolations were made using small pieces (3 × 3 mm) of leaves and shoots taken at the margin between necrotic and apparently healthy tissues. Plant pieces were submerged in 0.5% sodium hypochlorite for 2 min, rinsed twice in sterile water and plated onto acidified potato dextrose agar (APDA; 2.6 ml of 25% [vol/vol] lactic acid per liter of medium) for isolation of fungi. Isolation plates were incubated in the laboratory at room temperature (around 24°C) under an approximate 12 h photoperiod and plates were observed for fungal growth after 7 and 14 days. Fungal colonies consistently emerging from plated tissue pieces were transferred to potato dextrose agar (PDA) plates to be identified morphologically and molecularly.

Fruit trees as well as ornamental and native plant species surrounding olive orchards were examined to determine the host range of *Neofabraea* and *Phlyctema* fungi in California.

Results and Discussion

Neofabraea kienholzii and *P. vagabunda* leaf and twig lesions were detected mainly in the 'Arbosana' cultivar and to a lesser extent in the 'Arbequina' cultivar in SHD olive orchards in Glenn, San Joaquin, and Stanislaus Counties. Symptoms were most visible in March, which corresponds to the early spring in California. Lesions on leaves were necrotic, circular to elongated and usually occurred singly, ranging from 0.5 to 1 cm in diameter **(Fig. 1A, top row,** and **B)**. Leaf lesions occurred at sites of tree injuries caused by mechanical harvesters and included abrasion sites where leaves rub against each other. Leaf lesions caused by *N. kienholzii* and *P. vagabunda* were similar but differed clearly from peacock spot lesions, as the former were necrotic, lacked the dark green halo typically seen around peacock spot **(Fig. 1A,** bottom row**)**, and in general did not number more than one lesion per leaf **(Fig. 1A,** top row**)**. Reddish-brown lesions on shoots and twigs and occasionally cankers in branches developed at wounds caused by mechanical harvesters **(Fig. 1C, D** and **E)**. Cankers in branches appeared as sunken lesions in the bark that

elongated from the site of injury. The disease occasionally caused fruit spots in the 'Arbequina' cultivar and was visible in December on unharvested fruits remaining on trees **(Fig. 1F)**. In severely affected orchards, 'Arbosana' trees showed defoliation (Fig. 1G) and premature fruit drop. Surveys to determine the host range of *Neofabraea kienholzii* and *P. vagabunda* failed to identify additional host plants for these fungi in California. Several studies have suggested that *P. vagabunda* can survive on dead bark tissues of apple, pear, and olive trees serving as inoculum for new infections (Verkley 1999). In Michigan, this fungus has been reported to cause the coin canker disease of ash (Rossman et al. 2002). Ash trees in riparian areas as well as apple trees near olive orchards were surveyed for this study, but the olive pathogens were not detected from these host plants.

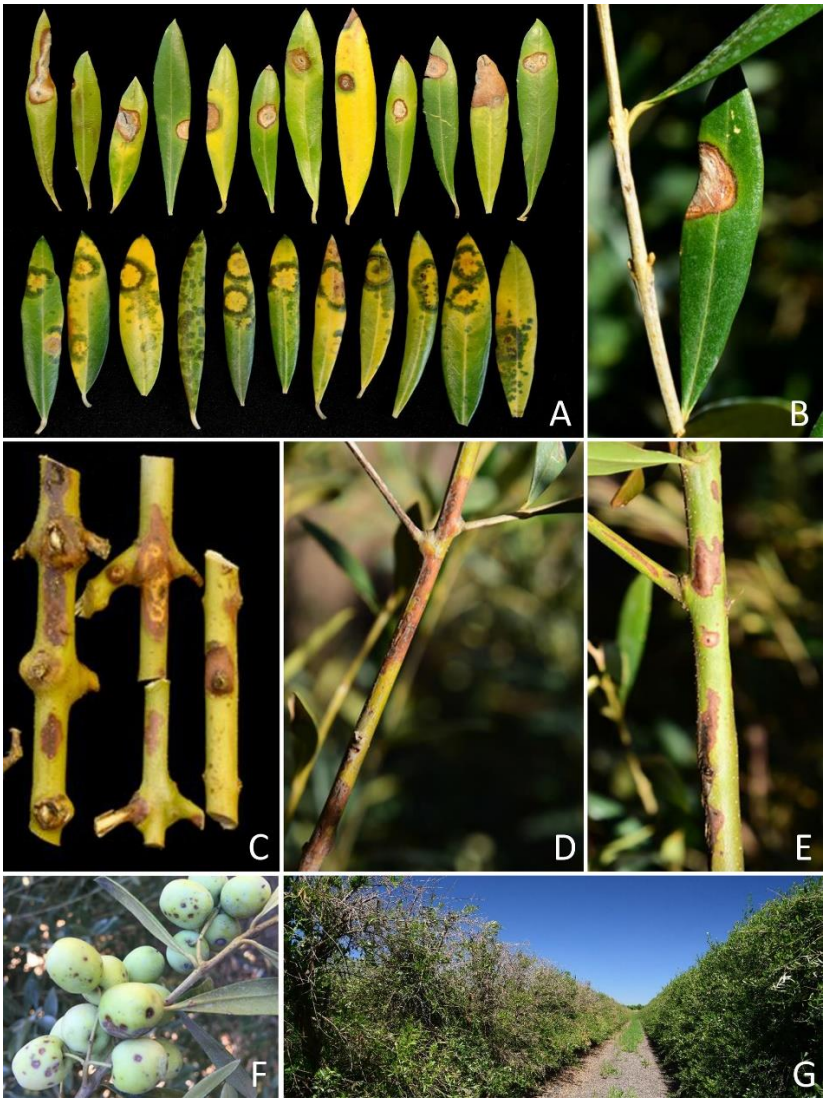

Fig. 1. Symptoms on olive caused by *Neofabraea kienholzii* and *Phlyctema vagabunda* in California. **A,** Leaf lesions of *N. kienholzii and P. vagabunda* (top row) versus peacock spot symptoms on leaves (bottom row), caused by *Fusicladium oleagineum*. **B,** Leaf lesion of *N. kienholzii.* **C, D** and **E**, Shoot lesions of *N. kienholzii* and *P. vagabunda*. F, Fruit spots in 'Arbequina' olives associated with *P. vagabunda*. **G,** Tree defoliation in a row of 'Arbosana' olives (left) heavily affected by *N. kienholzii* and *P. vagabunda* leaf and shoot lesions in comparison with a row of healthy 'Koroneiki' cultivar (right).

Objective 2: Determine the pathogenicity of *Neofabraea kienholzii* and *Phlyctema vagabunda* in the main oil olive cultivars

Material and methods

Inoculum

Pathogenicity assays were conducted on leaves, shoots and fruits. The inoculum consisted of a spore suspension (conidia) of *Neofabraea kienholzii* and *Phlyctema vagabunda.* Spores were harvested from the surface of cultures plates and were suspended in sterile water. The resulting suspension was adjusted to a final concentration of 1×10^5 spores per ml. Twenty microliters of the inoculum suspension was used to inoculate wounded olive tissues.

Leaf pathogenicity assays

Pathogenicity studies were conducted in November 2016 and 2017, respectively, to determine the ability of the newly reported fungi to cause lesions on olive leaves. Two to 3-year-old potted olive saplings of the Arbosana, Arbequina and Koroneiki cultivars were used for these assays. The experiments were conducted in a lath-house at the University of California, Davis. Leaf pathogenicity assays were conducted using 10 repetitions per treatment organized in a completely randomized design. One leaf on each of 10 trees served as one repetition. Leaves were inoculated following artificial wounding that mimicked wounds caused by mechanical harvesters. Experiments were harvested after 3 months in February 2017 and February 2018, respectively. Leaves were inspected for the development of necrotic lesions and taken to the laboratory for lesion measurement as well as attempts to recover the inoculated fungal pathogens from the symptomatic tissues.

Shoot pathogenicity assays

The ability of the various fungi to produce lesions in olive shoots was determined following pathogenicity studies in mature olive trees growing in the field. The Arbosana, Arbequina and Koroneiki cultivars were used for this experiment. Pathogenicity of various isolates was tested following inoculations of shoots in November 2016 and 2017, respectively. Inoculations of shoots were conducted following artificial wounding that mimicked wounds caused by mechanical harvesters. The bark of shoots was sliced-open using a sterile knife blade and a 20 µl propagule suspension at 1×10^5 propagules per ml was placed immediately onto the surface of the wounds. One branch on each of ten trees were inoculated with one isolate, which accounted for 10 repetitions. Additional branches were mock-inoculated with sterile water to serve as negative controls. The experiment was replicated using two representative isolates of each fungal species. Both assays consisted of two independent experiments using 1, 1- to 2-year-old shoots on each of 10, 9-year-old olive trees. Inoculated and mock-inoculated branches were collected after three months and the length of lesions was measured above and below the point of inoculation for each shoot.

Fruit pathogenicity assays

Pathogenicity studies were conducted to determine the capacity of *N. kienholzii* and *P. vagabunda* to cause disease in fruits of Arbosana, Arbequina and Koroneiki cultivars. The experiment was conducted in the laboratory using detached fruits obtained from the field. Healthy fruits were submerged for 3 min in 0.5% sodium hypochlorite solution, then rinsed twice in sterile distilled water, and air-dried on sterile paper towels. The experiment was conducted on fruits wounded with a needle and inoculated with a 10 µl drop of a conidial suspension adjusted to 1×10^5 conidia per ml. Inoculated olives (n = 6 per treatment) were

placed in moist chambers and incubated at 25°C. Inoculated fruits were assessed for fruit rot development after 14 days. The experiment was conducted twice using two different isolates for each fungal species.

Statistical analyses

All pathogenicity data were subjected to analysis of variance (ANOVA) to examine the effect of cultivars, isolates and fungal species on disease development (leaf lesion diameters, shoot lesion length and fruit lesion sizes). Data transformation were conducted when necessary to satisfy ANOVA assumptions. In case of significant effects, means were separated by Tukey's LSD test ($P < 0.05$).

Results and Discussion

In the pathogenicity assays on leaves, all cultivars tested appeared susceptible to both pathogens. In 2016, all isolates were pathogenic on leaves of the three cultivars and caused leaf lesions identical to the ones observed in the field. There were no differences in lesion sizes on the leaves of the 3 olive cultivars inoculated with two isolates of *N. kienholzii* ($P = 0.43$). In contrast, there were differences in cultivar susceptibility to isolates of *P. vagabunda*, with 'Arbosana' being more resistant than 'Arbequina' and 'Koroneiki'; the latter two showed similar susceptibility to the pathogen **(Fig. 2)**.

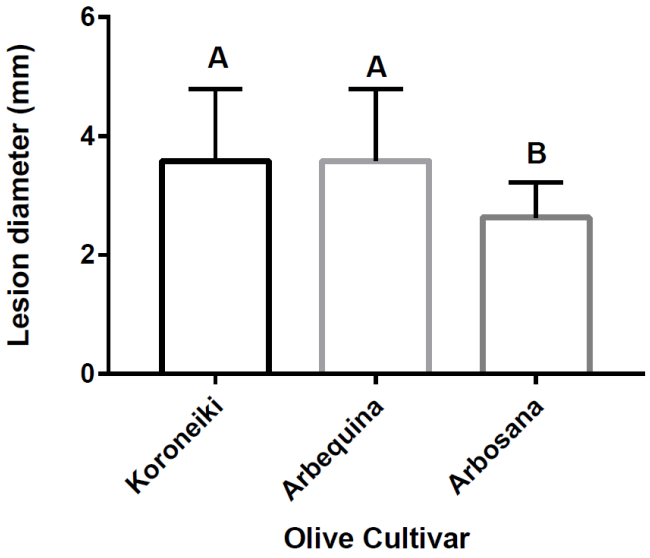

Figure 2. Lesion diameters caused by isolates of *Phlyctema vagabunda* inoculated onto leaves of three olive cultivars in 2016 in the lath-house. Data are means and standard errors (10 replicates). Data denoted by distinct letters are significantly different (Tukey's test; $P < 0.05$).

In 2017, all isolates tested caused necrotic lesions on leaves. No symptoms were produced in the mock-inoculated control plants. Lesion size produced by isolates belonging to the same species did not differ significantly between isolates ($P < 0.05$). Accordingly, lesion diameter data were combined for isolates of the same species. Average lesion size after inoculations was 6.93 mm for *P. vagabunda* isolates and 6.66 mm for *N. kienholzii* isolates, respectively. According to ANOVA, there was a significant effect of the cultivar ($P < 0.00001$) on lesion

diameter but no significant difference in aggressiveness was detected between the two fungal species (P = 0.6383). Arbosana was the most susceptible cultivar, with lesions averaging 7.2 mm in diameter, while Arbequina (4.9 mm) was the most resistant cultivar **(Fig. 3)**. Inoculation studies confirmed also that wounds were required for the pathogens to successfully infect and cause lesions in leaves of olive.

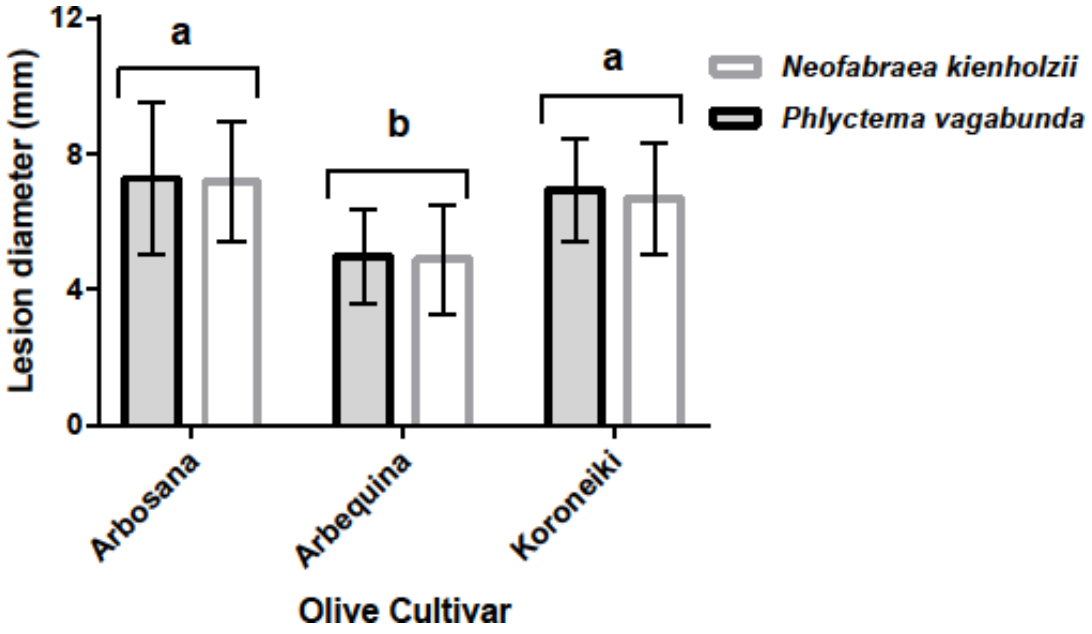

Figure 3. Lesion diameters caused by isolates of *Neofabraea kienholzii* and *Phlyctema vagabunda* inoculated on leaves of three olive cultivars in 2017 in the lath-house. Data are means and standard errors (10 replicates). Data denoted by distinct letters are significantly different (Tukey's test; P < 0.05).

In the pathogenicity assays on shoots, both 2016 and 2017 inoculations produced reddish-brown lesions in shoots that extended from the inoculation point and that were similar to those occurring in naturally infected twigs.

In 2016, average lesion size produced across all cultivars and fungal pathogens was 8.5 ± 0.9 mm. There was significant (P = 0.0203) differences in susceptibility to the pathogens among olive cultivars with Arbosana being the most susceptible cultivar (10.83 mm average lesion size) and Koroneiki the most resistant (4.66 mm average lesion size). Arbequina showed an intermediate tolerance resistance level (5.6 mm average lesion size).

In 2017, average lesion size produced across all cultivars and fungal isolates was 36 ± 5.4 mm. There were significant effects of the olive cultivar (P <= 0.00108) and fungal treatments (P < 0.0001) but no effect was detected for the interaction cultivar-isolate (P = 0.3165). According to these results, Koroneiki (39.66 mm average lesion size) was significantly (P < 0.05) more susceptible than Arbosana (35.16 mm average lesion size) and Arbequina (34.62 mm average lesion size), which did not differ significantly between them (**Fig. 4**).

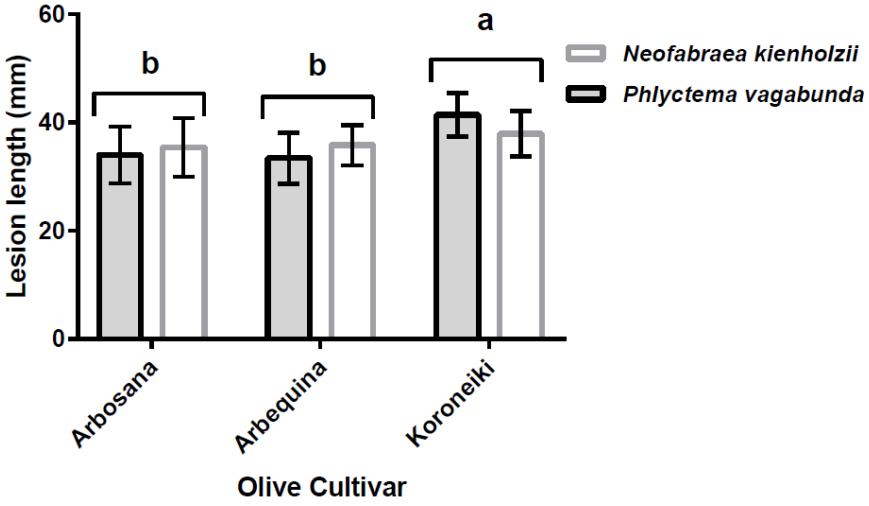

Figure 4. Lesion lengths caused by isolates of *Neofabraea kienholzii* and *Phlyctema vagabunda* inoculated on shoots of three olive cultivars in 2017 in the field. Data are means and standard errors (10 replicates). Data denoted by distinct letters are significantly different (Tukey's test; $P < 0.05$).

In the pathogenicity assays on fruits, all fungal isolates tested produced circular rot lesions extending from the inoculation points. Control fruits (mock-inoculated) did not show disease symptoms, i.e. we did not detect latent infection of the pathogen. There were significant effects on the lesion size of the fungal species ($P = 0.00103$) and olive cultivar ($P = 0.0395$) but no interaction was found between species-cultivar ($P = 0.42548$). In this assay, *N. kienholzii* was significantly more virulent than *P. vagabunda* in the three evaluated olive cultivars. Also, the cultivar Koroneiki (6.9 mm average lesion size) was significantly more susceptible than Arbequina (5.8 mm average lesion size), while Arbosana (6.3 mm average lesion size) showed an intermediate susceptibility **(Fig. 5)**.

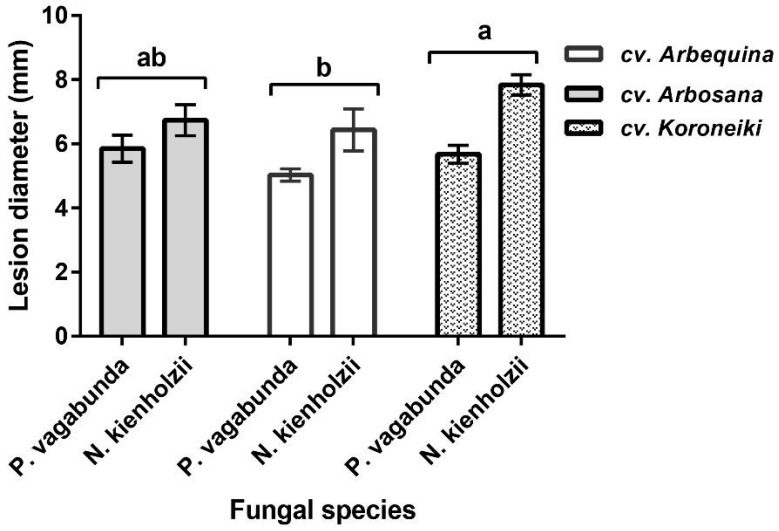

Figure 5. Lesion diameters caused by isolates of *Neofabraea kienholzii* and *Phlyctema vagabunda* inoculated on fruits of three olive cultivars in 2017. Data are means and standard errors (10 replicates). Data denoted by distinct letters are significantly different (Tukey's test; $P < 0.05$).

Objective 3: Determine the efficacy of various fungicides to control Neofabraea and Phlyctema leaf and shoot lesions.

Material and methods

Fungicides in different FRAC groups (different modes of action) were screened in the field to determine their efficacy against *Neofabraea* and *Phlyctema* pathogens. In 2017-2018, product testing was organized in two field trials (Trial 1 and Trial 2) established in a super-high-density olive orchard near Walnut Grove, CA Experimental units consisted of two adjacent trees arranged in each of four blocks using a randomized complete block design. Trial 1 tested a single spray application at harvest. Trial 2 tested two spray applications, at harvest on 11/22/2017 and a second application approximately 6 weeks later, on 01/05/2018.

Eight products were tested in the field during the fall and winter **2017-2018**:

- Topsin M (thiophanate-methyl – group 1)
- Inspire Super (difenoconazole/cyprodinil – group 3+9)
- Kocide 3000 (copper hydroxide)
- Tebucon (tebuconazole – group 3)
- Rhyme (flutriafol – group M3)
- Vanguard WG (cyprodinil – group 9)
- Ziram (ziram – group M3)
- Bravo (chlorothalonil – group M5)

For all these experiments, foliage infection relied on natural inoculum. All fungicide treatments were evaluated for their ability to protect olive trees against Neofabraea and Phlyctema twig and leaf lesions and were compared with a water treated control. Trials were rated 4 months after harvest. The amount of leaf infections was evaluated for each fungicide tested and treatments that resulted in the lowest amount of leaf spots were considered as most effective against Neofabraea and Phlyctema diseases.

Results and Discussion

Results of 2017-2018 field experiments are presented in **Figs. 6** and **7.** Trial 1 (single application at harvest) confirmed the efficacy of the products tested in reducing leaf lesion development, except for Kocide 3000. The 7 other products provided satisfactory disease control, with Topsin M and Vanguard as the most efficient products providing up to 70% disease reduction compared to the water-only treated control **(Fig. 6)**. Results of Trial 2 (two spray applications, 6 weeks apart) were very similar to those of Trial 1, confirming Topsin M and Vanguard as the most efficient products **(Fig. 7)**.

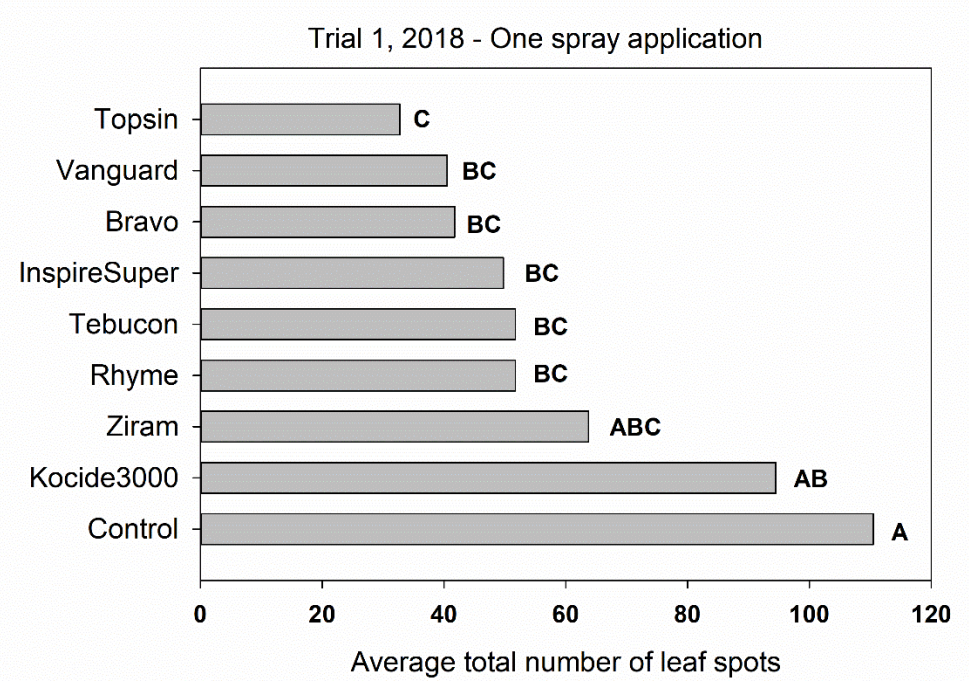

Figure 6. 2017/2018 - Trial 1, single spray application: Average number of leaf lesions per olive tree according to various fungicide treatments and compared to the water treatment a copper treatment (Kocide 3000).

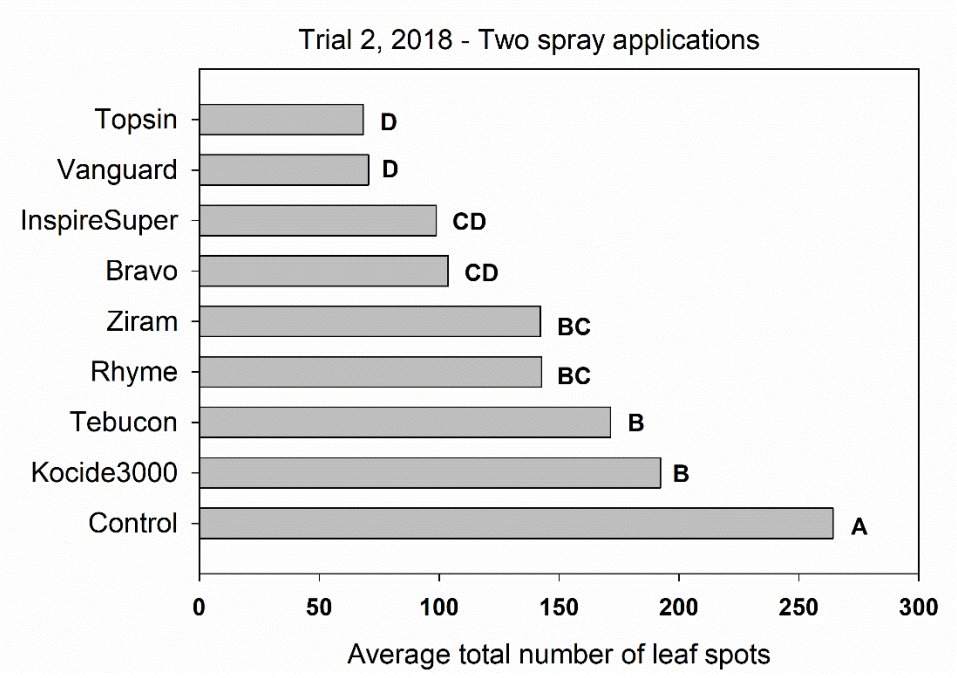

Figure 7. 2017/2018 - Trial 2, two spray applications: Average number of leaf lesions per olive tree according to various fungicide treatments and compared to the water treatment a copper treatment (Kocide 3000).

Objective 4: Submission of fungicides to the IR-4 program and for emergency exemption (section 18).

Material and methods

Submission of fungicides to the IR-4 program were initiated in 2018 in coordination with Dr. Jim Adaskaveg's work on the management of peacock spot (cf. Peacock Spot Research Annual Report 2018). Two fungicides were identified that are potentially registerable. These have different modes of action, have low resistance potential, and are efficacious against Neofabraea and Phlyctema twig and leaf lesions as well as peacock spot. Additionally, registrants of each fungicide were contacted for approval for the proposed usage on olive and proposed labels were prepared. Subsequently, an IR-4 nomination was made based on the proposed usage (rates, timing, etc.) and IPM compatibility. Dr. Jim Adaskaveg traveled to the IR-4 Food Use Workshop in St. Louis, MO, in September 2018 to defend theses nominations. An emergency registration was also requested, and application was coordinated by the OOCC. As part of the section 18 application process, olive fruits from our fungicide trials were submitted for pesticide residue analysis to Environmental Micro Analysis, Inc., Woodland, California in July 2018.

Results and Discussion

Three fungicides were nominated to the IR-4 program in 2018: ziram (Ziram 76WDG), difenoconazole/cyprodinil (Inspire Super), and thiophanate-methyl (Topsin-M). Ziram and Inspire Super were approved for residue trials at the National Food Use Workshop in Sept. for registration on olives. Strong support was provided based on the after-harvest and winter season usage with expected zero to limit-of-detection residues on the crop in the following harvest season. Ziram is a FRAC Code M3 whereas Inspire Super is a FRAC Code 3/9. Thus, integration of multi-site modes of action for both products was also established as an effective anti-resistance strategy. Topsin-M was not accepted due to a low probability of registration because of the EPA Re-registration Eligibility Decision concerning its human safety and the potential for selection of resistance in the *F. oleaginum*, *N. vagabunda* and *P. kienlozii* pathogen populations (cf. Jim Adaskaveg: Peacock Spot Research Annual Report 2018). Ziram and Inspire Super were also submitted for section 18 emergency exemption and the present fungicide trials were used for pesticide residue analysis. Results from analyses of the Environmental Micro Analysis laboratory revealed that ziram, difenoconazole/cyprodinil and thiophanate-methyl residues were non-detectable from olive fruits collected in July 2018 from trees previously treated in November 2017 (after harvest) with one and two application of the various fungicides (Trial 1 and 2).

Objective 5: Determine the duration of harvest wound susceptibility to infection in olive leaves and timing of fungicide applications

Material and methods

Additional trials were set up in November 2016-17 and 2017-18 to assess the duration of wound susceptibility in leaves mechanically wounded during harvest. Trials included

one assay conducted in a lath house in Davis, CA (2016-2017) as well as a field assay conducted in an orchard near Walnut Grove, CA (2017-2018). Knowledge of the duration, following harvest, of leaf wound susceptibility will help determine the number of fungicide applications required to protect wounds from infection by *Neofabraea* and *Phlyctema* pathogens. For this study, olive leaves were manually wounded in November with scissors to mimic wounds caused by mechanical harvesters. Wounded leaf subsets were then inoculated either directly after wounding or after 1, 2, 3, 4, or 5 weeks following wounding. Individual wounds on leaves were inoculated with 20 µL of a 1×10^5 spores mL-1 spore suspension of *Neofabraea kienholzii*, the most common pathogen of this complex found in olive. Each treatment was replicated 10 times. At the end of the experiment, leaves were collected and brought to the laboratory to proceed with fungal isolations, assess the percent fungal recovery and determine the susceptibility of wounded leaves according to the timing of infection following wounding in November (harvest). Data were analyzed in the statistical software R.

Results and Discussion

Results of the leaf wound susceptibility study revealed that freshly wounded leaves were most susceptible to infection by *Neofabraea* pathogen during the first two weeks following wounding **(Figs. 8** and **9)**. This suggests that fungicide application to protect leaves should be applied immediately after harvest. Leaf wound susceptibility decrease significantly as early as after two weeks following wounding. A substantial decline in wound susceptibility in leaves occurred at four weeks following wounding, suggesting wound had healed after four weeks **(Figs. 8** and **9)**. These findings suggest that infection risk after four weeks following harvest (wounding) are low and that fungicide treatments are no longer required after this time period.

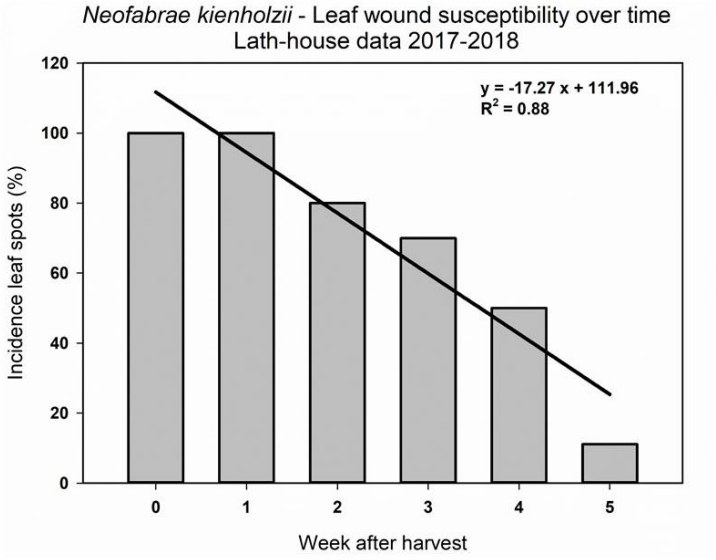

Figure 8. 2017/2018 – Lath house experiment, Davis, CA. Leaf wound susceptibility over time following a unique wounding event during harvest in November. Leaf wound susceptibility decreases with wound age, with 4 and 5-week-old wounds being less susceptible to infection by *Neofabraea* pathogen.

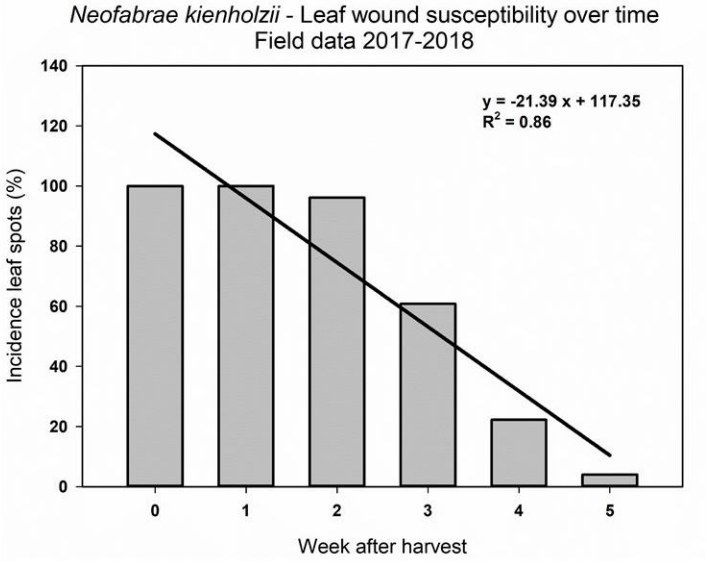

Figure 9. 2017/2018 – Field experiment, Walnut Grove, CA. Leaf wound susceptibility over time following a unique wounding event during harvest in November. Leaf wound susceptibility decreases with wound age, with 4 and 5-week-old wounds being less susceptible to infection by *Neofabraea* pathogen.

Objective 6: Outreach and Education:

Information from this research project was presented at the Sacramento Valley Olive Day in July 2018 organized by farm advisor Dani Lightle. Information obtained from this work was shared among all farm advisors working with olives and was used to train various growers and orchard managers in San Joaquin, Stanislaus, Colusa and Glenn Counties. Information about this new disease was also distributed to growers through various UCCE and OOCC networks. This research was also presented at the 2017 annual meeting of the American Phytopathological Society, Pacific Division in Riverside, CA. A publication entitled "Identification and characterization of Neofabraea kienholzii and Phlyctema vagabunda causing leaf and shoot lesions of olive in California" was recently submitted to Plant Disease journal of the American Phytopathological Society. This work was also published in October 2018 edition of CAPCA ADVISER magazine (VOL. XXI, No. 5, pp:86-88).

Acknowledgments

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