CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

Combined Annual Reports for Spinach and Lettuce Downy Mildew projects, 2021-2022

Downy mildew detection, epidemiology, and biopesticide evaluation

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ABSTRACT

Downy mildew diseases on spinach and lettuce are caused by the obligate oomycete pathogens *Peronospora effusa* (*P. effusa*), and *Bremia lactucae* (*B. lactucae*), respectively. Both downy mildews are destructive in California. Sporangia of both pathogens can be airborne and rapidly spread within and between fields. In the case of *P. effusa*, seed harbors oospores that may transmit the disease. In this reporting period, we published work on a species-specific leaf detection system for *P. effusa* and multiplexed these markers with those for *B. lactucae* for dual quantification of both pathogens after airborne sampling. Using the dual detection assay, we determined that cyclone type spore traps for airborne detection consistently outperformed the spinning rod type impaction spore traps. We deployed the cyclone spore traps in the Salinas, Coachella, and Imperial valleys in late 2021 and early 2022, for quantification of *P. effusa* and *B. lactucae.* The results of airborne sampling in each valley indicated higher amounts of both *P. effusa* and *B. lactucae* during the respective production seasons, as anticipated. However, unlike the Salinas Valley, there was a span of over one month where *P. effusa* was not detectable in the Imperial Valley, raising questions on the source of primary inoculum that may initiate disease. Another objective focused on analyses of seed transmission of spinach downy mildew. In the winter of 2021, we observed seed transmission of spinach downy mildew from seeds harboring oospores and from seeds that had been coated artificially with oospores prior to planting in plant isolators. In contrast, disease was not observed in those isolators planted with cultivar Viroflay seed which did not harbor oospores. We further tested two biopesticides in growth tent and microplot experiments to determine if each could reduce symptoms and sporulation of *P. effusa.* In experiments in 2021, we observed reductions in disease incidence when spinach plants were treated with the biopesticide AgroPro or the surfactant R-11. In addition, we have seen AgroPro and R-11 inhibit sporangia germination directly on water agar plates. In summary, tracking the levels of windborne inoculum of the pathogen in different valleys in California, and in-field leaf detection applications, may be valuable to inform efficient spray applications for disease control, and provides further insight on sources of inoculum. Oospores of *P. effusa* continue to be detected in commercial seed lots and reveal that sexual reproduction of the pathogen occurs worldwide and is commonplace. Oospores allow *P. effusa* to survive at least four years on seed. In total, we have observed oospores from seed samples of nearly 21% of seed lot samples. In addition to curtailing the amounts of infested seeds entering the production stream, the two biopesticide treatments examined may be helpful to reduce some disease incidence, especially for organic spinach.

PROJECT TITLE: Downy mildew detection, epidemiology, and biopesticide evaluation

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OBJECTIVES (downy mildew on spinach and lettuce):

1: Examine *Peronospora effusa* oospore production and seed transmission

2: Compare the effectiveness two types of spore traps for airborne detection of *P. effusa* and *Bremia lactucae*

3: Complete biopesticide testing in microplot and mist tent experiments.

PROCEDURES:

Immediate objective 1: We examined additional commercial seed lots for the presence of *P. effusa*, over the 340 lots already tested, to aid in seed transmission testing, planting for biopesticide testing, etc. For seed testing, the standard 1000 seeds were mixed with water and briefly vortexed then centrifuged at low speed, and the sediment was examined by systematically recording counts oospores (or lack thereof) in an area of a coverslip, under compound microscopy. The oospores are approx. 30 micrometers in diameter, are brownish in color and have a smooth round wall [1,2,12].

In the winter period of 2021-2022 (November – January), one of the oospore-positive seed lot samples identified was planted in glass isolators to examine disease transmission. In addition, we planted another oospore infested seed lot sample within the different sections of the isolator. We also planted seed coated with ground oospore-infested leaves. The negative control was a seed sample in which we did not detect oospores and was determined to be negative for *P. effusa* DNA

amplification in PCR tests. Seeds were planted in the isolators at a density equivalent of 10-13 million seeds per acre. The soil was fumigated ahead of the experiments with metam sodium and leaf wetness provided by an internal overhead sprinkler system installed in each of twelve isolator compartments. Plants were evaluated after emergence through the glass of the isolators for development of disease symptoms, and ultimately opened and evaluated for symptoms at 37 days after planting and the numbers of plants with symptoms and sporulation (incidence) recorded.

Immediate objective 2: Examinations of the effectiveness of two types of spore traps for airborne detection of *P. effusa and B. lactucae* was accomplished by comparing quantities of spores detected from the cyclone spore traps purchased by USDA from Root Applied Sciences (Figure 1) with the those of values obtained simultaneously from the spinning rod type spore traps (Figure 1). The traps of both types were placed at the north and south sides of a small spinach plot at the USDA Spence farm. The traps were sampled three times weekly (approximately 48 or 72 hr intervals). Collected tubes from the cyclone spore traps or rods from the impaction traps were stored at 4 ºC until DNA extraction using the Nucleospin Plant II kit (Machery Nagel). We had previously been successful in devising assays for quantifying the levels of airborne spores (through DNA quantification) of *P. effusa* [10] *Bremia lactucae* [11]. However, because in this reporting period, we completed the development publication of the *P. effusa*-specific markers [5], we combined both in a single reaction for dual detection of both downy mildew pathogens and had redone the spore curves using currently available method of spore dilution and quantification. The spore estimates observed per sample were based on these new calculations for both *B. lactucae* and *P. effusa*.

Cyclone trap samples in the Salinas, Coachella, and Imperial Valleys of California for evaluation were collected three times weekly. The traps in each location were placed at the north and south ends of each valley (one cyclone trap on each end). For each of these traps, we made the effort to keep the traps removed from the vicinity of local spinach and lettuce fields. Samples collected by cooperators A. Putman and A. Montazar were shipped to Salinas on ice packs, once per month, to prevent degradation of the samples. Following shipping, DNA extractions and quantifications were performed by a USDA technician in Salinas using the Nucleospin Plant II kit.

Immediate objective 3: To conduct the biopesticide testing in microplots, routine experimental plots of spinach cultivar Viroflay were planted at the USDA ARS station in Salinas from June to October 2021. Each plot was 4 by 8 ft in length and separated into six sections for two replications of three different treatments (water, AgroPro, Procidic with R-11) totaling three different trials. Plants were treated with the respective biopesticides and following treatment a small tray of downy mildew-sporulating plants were set next to the microplot to facilitate infection. Disease incidence was evaluated based on diseased plants out of total plants in each replicated section.

For mist tent analysis, Viroflay was grown in trays and, one tray was used for each treatment with a total of six different treatments (water, AgroPro at 4% and 0.4%, Procidic with R-11, Procidic alone, R-11 alone). Plants were treated with the biopesticides and then inoculated with downy mildew. For the mist tent experiments, disease incidence was evaluated by counting the number of infected leaves out of a subsample of 50-100 leaves at least two separate times for each treatment.

For field analysis, we established a field trial at the USDA Spence field and proceeded with the treatments that performed the best from the mist tent and microplot experiments which were AgroPro at 4% and R-11 alone (0.125%), water was used as a control. The susceptible cultivar Viroflay, which was confirmed to be free of *P. effusa* oospores, was planted on either side of the *P. effusa*-inoculated UC Davis spinach breeding trial in 40” beds. The plots were sprinkler

irrigated. Three replicates each consisting of a 5 ft section of the spinach bed was sprayed with either water, AgroPro, or R-11. Disease incidence was evaluated by counting the number of infected leaves out of a subsample of 100-200 leaves three separate times for each treatment.

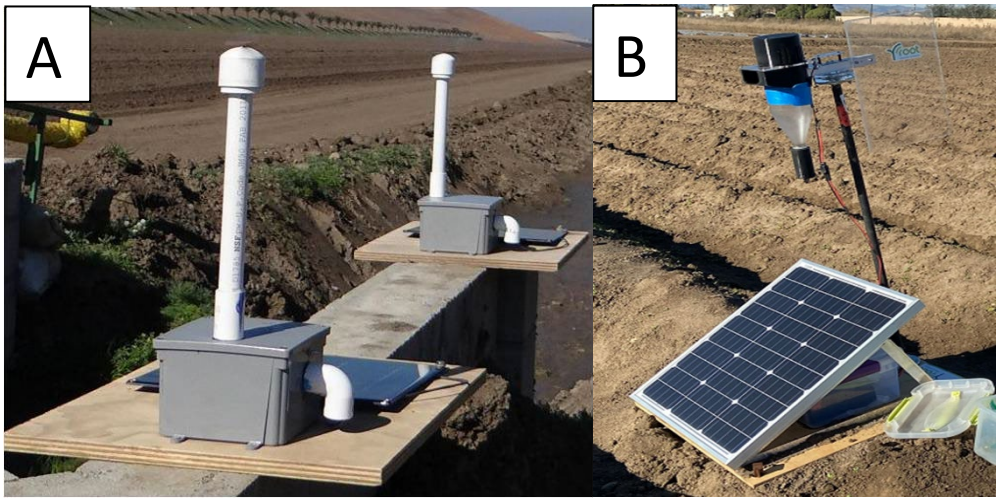

Figure 1. Two spore trap systems to detect airborne *Bremia lactucae* and *Peronospora effusa*, the causal agents of lettuce and spinach downy mildew, respectively. **A)** Spinning rod type spore trap (USDA). **B)** Cyclone spore trap (Root Applied Sciences, Berkeley, CA).

To aid in our plant inoculations, we previously acquired a dew chamber and purchased a wall-mounted air conditioner to develop a reliable system to infect spinach with *P. effusa*. These items were installed in a room on the USDA facility dedicated for this purpose, to maintain the necessary cold temperatures. Using this equipment/facility, the conditions for infections within the growth chamber and the humidity tent were as described previously [10]. The exception was that the dew chamber was maintained at cooler temperatures, in the range of 7.5 to 13.3ºC. Twenty-four hours following the initial inoculation, the plants were moved to a humidity tent maintained in the improvised cold room for seven days before returning overnight to the dew chamber.

RESULTS:

Bremia lactucae

We conducted comparisons between *B. lactucae* detection levels with the new cyclone traps and the older spinning rod type traps (Figure 1) from Nov-Dec of 2020 and repeated in May-June of 2021 with similar results. As shown in Figure 2, cyclone traps consistently detect more *B. lactucae* spores than spinning rod traps when placed at the same location. It should be noted that these comparisons were conducted next to a spinach field, not near lettuce, hence the difference in the number of spores collected by the cyclone traps is more apparent for the *P. effusa* (spinach downy mildew) comparison (Figure 4). We commonly observe some levels of airborne *B. lactucae* spores in the Salinas Valley throughout the year, even in the lettuce free period, in December [11].

Additional experiments were conducted in 2021-2022 to compare the airborne detection levels of *B. lactucae* in the Salinas, Coachella, and Imperial Valleys of California. Overall, the quantities of *B. lactucae* detected were clearly dependent on the growing season, and thus the presence of the crop (Figure 3). However, sampling values indicated the presence of *B. lactucae* in each month even at low levels in the Salinas Valley suggesting wild lettuce hosts or other

sources of airborne inoculum present in each month of sampling, even outside of the growing season when climatic conditions are less conducive to downy mildew. In the Coachella and Imperial Valleys there were week-long spans were *B. lactucae* was not detected, which could be due to the hot and dry climate in those valleys. Nevertheless, detection weekly outside the growing season suggested an alternate source of inoculum for *B. lactucae*.

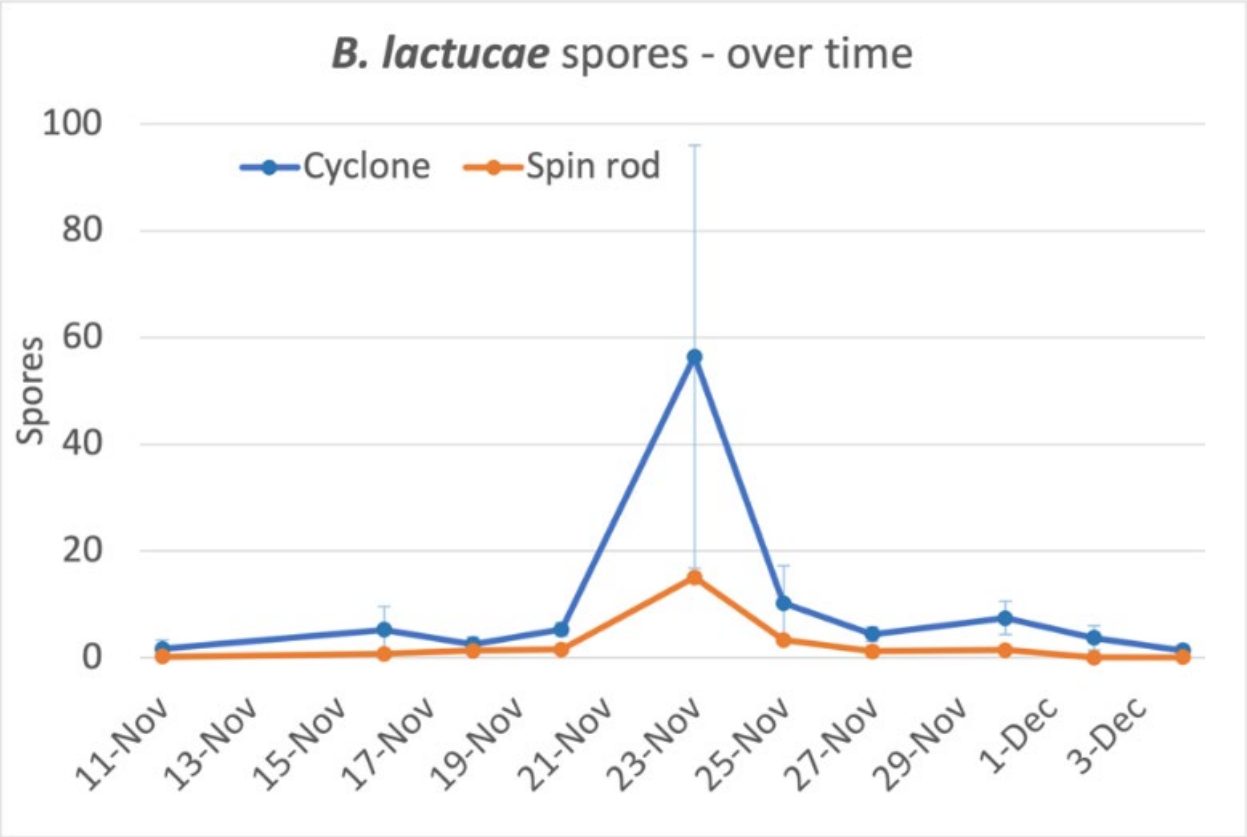

Figure 2. Spore trap type comparison Cyclone traps (blue line) collected more *Bremia lactucae* spores than spinning rod traps (orange line) at the same location. On average, the cyclone traps enabled quantification of about 7 more *B. lactucae* spores per sampling date. Samples were collected on the dates indicated on the x axis. Error bars are standard error between north and south traps. Note that this comparison took place next to a spinach field, no lettuce was nearby during the evaluation.

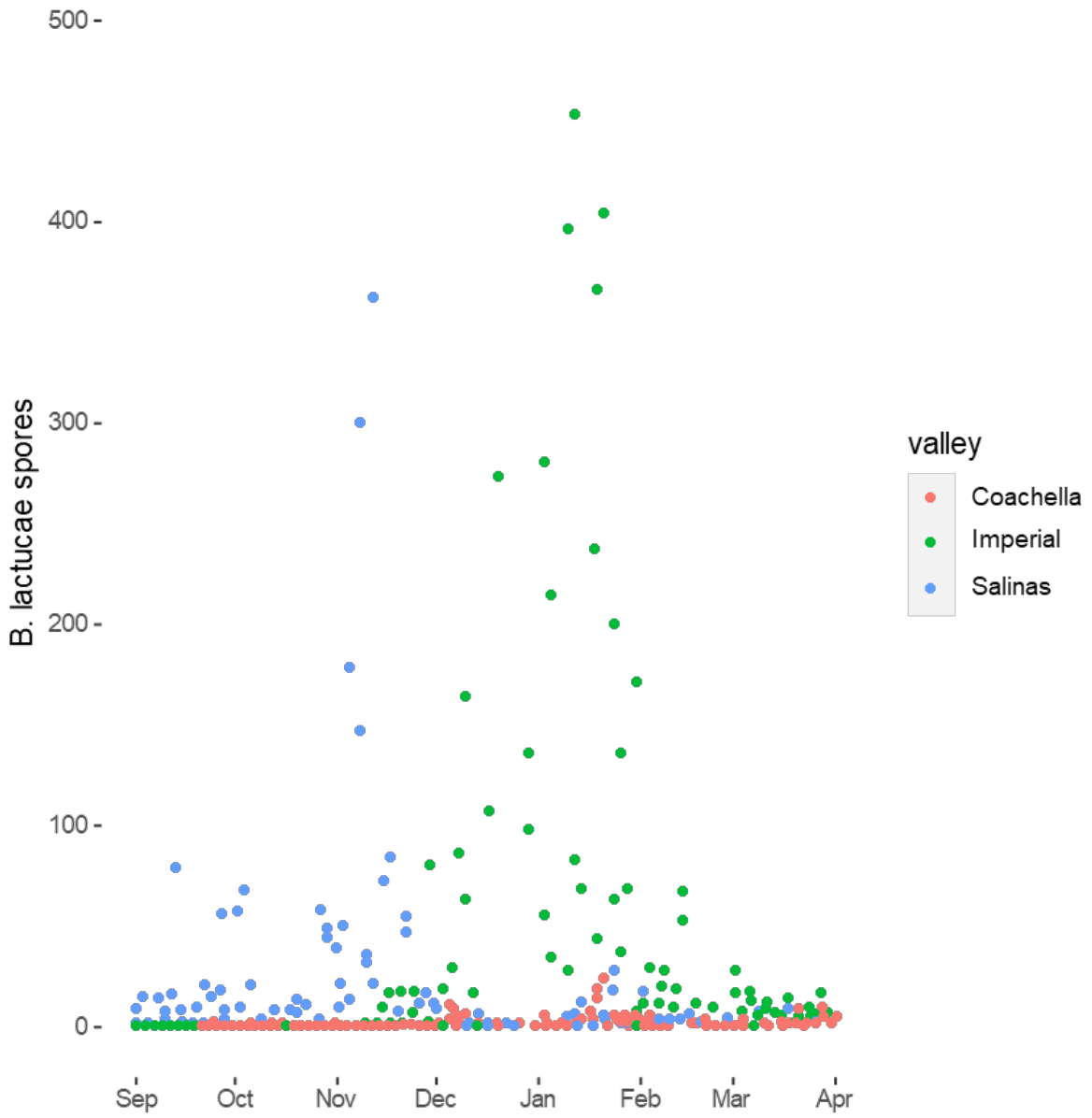

Figure 3. Comparison of *B. lactucae* detection in three valleys from September 2021 to April 2022. *B. lactucae* spore levels are the highest in the lettuce growing season of the respective valley.

Peronospora effusa

We conducted the comparison between *P. effusa* detection levels with the new cyclone traps and the older spinning rod type traps (Figure 1). As shown in Figure 4, on average the cyclone traps collected about 100 more *P. effusa* spores (with a range of 10 to 500 spores). This was repeated in May-June of 2021 with similar results.

Additional experiments were conducted in 2021-2022 to compare the airborne detection levels of *P. effusa* in the Salinas, Coachella, and Imperial Valleys of California. Like *B. lactucae*, the amounts of *P. effusa* detection were dependent on the growing season and location. But in the Coachella or Imperial Valleys there were nearly month-long spans where *P. effusa* was not detected. As shown in Figure 5, for the Salinas Valley (blue), spore counts are highest around in

the September through November period of 2021. For the Imperial Valley (green), spore counts are higher around January 2022. In the Coachella Valley (pink), spore counts remained low throughout the sample period.

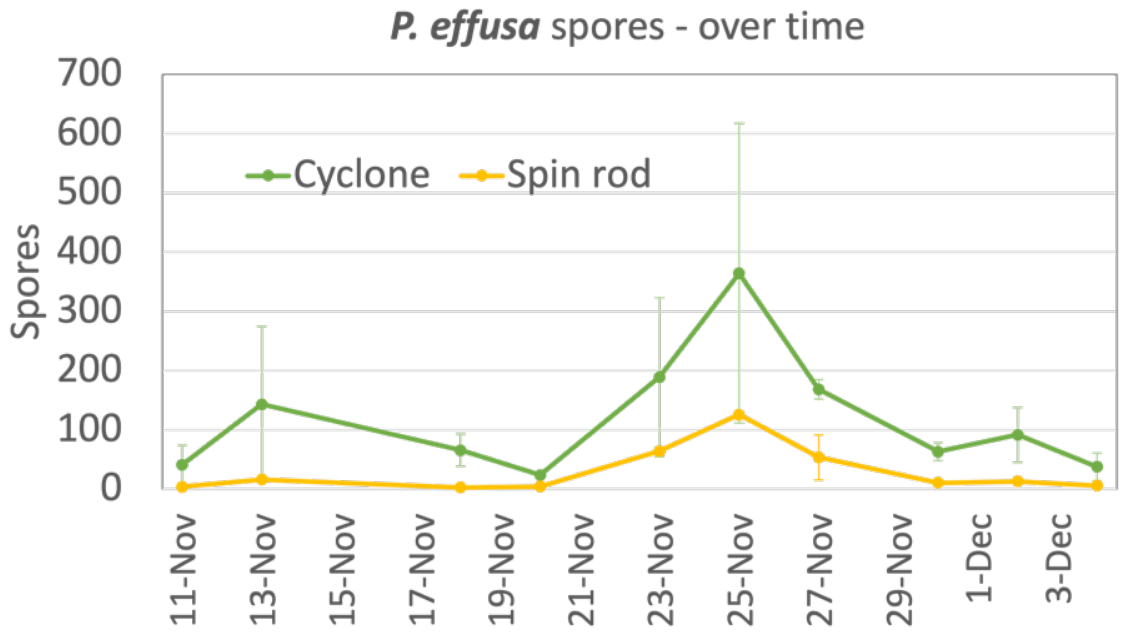

Figure 4. Spore trap type comparison. Cyclone traps (green line) collected more *P. effusa* spores than spinning rod traps (yellow line) at the same location. On average, the cyclone traps collected about 100 more *P. effusa* spores. Samples were collected on the dates indicated on the x-axis. Error bars are standard error between the north and south traps.

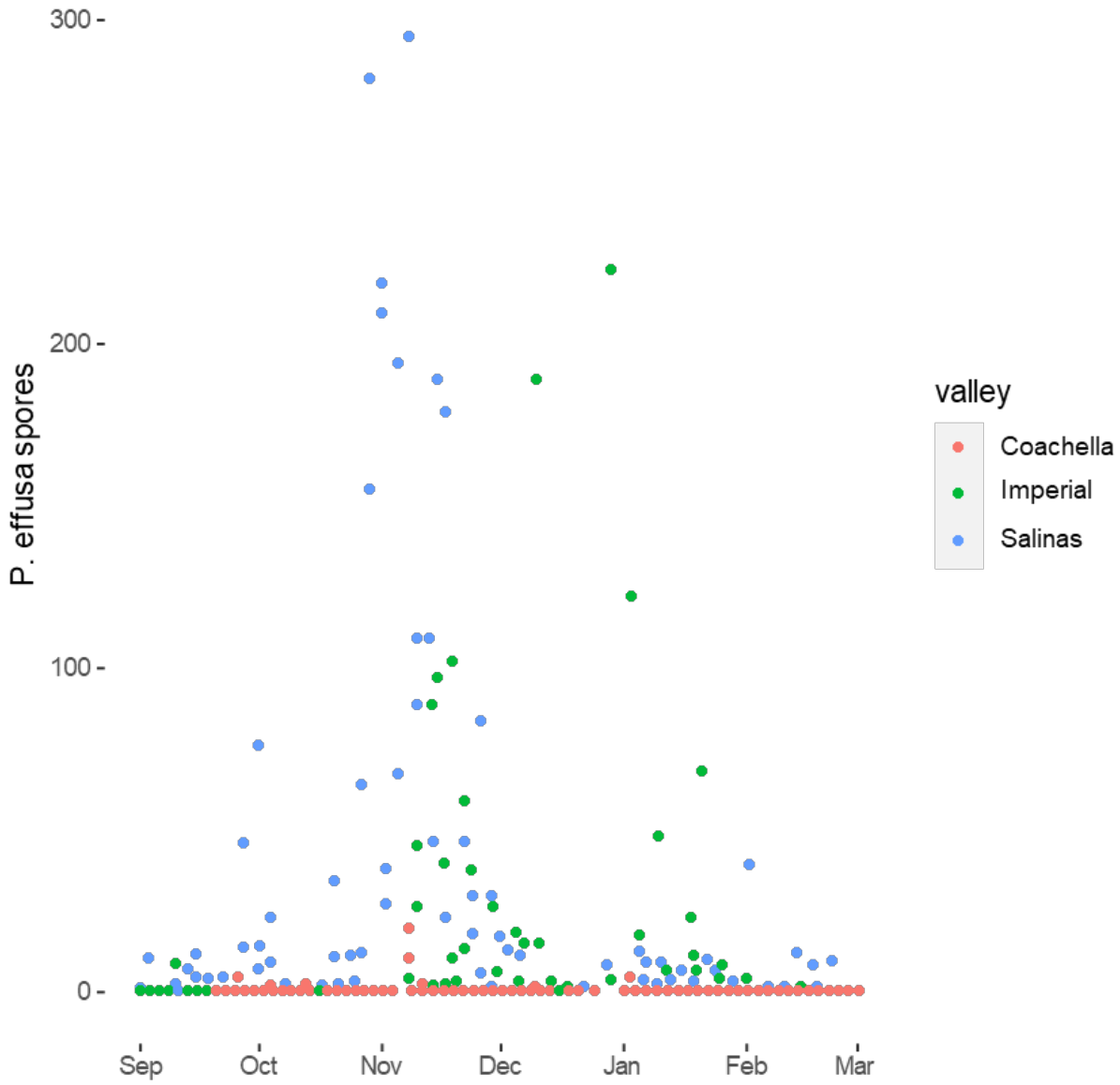

Figure 5. Comparison of *P. effusa* airborne detection in three valleys from September 2021 to April 2022. *P. effusa* spore levels are the highest in the spinach growing season of the respective valley.

We investigated seed transmission of spinach downy mildew further in the winter of 2021-2022. In the effort to remove the possibility of windborne inoculum contaminating the experiments, we used the plant isolator system shown in Figure 6 to prevent the introduction of windborne inoculum. The possibility of soilborne inoculum was also excluded because soil in the isolators was fumigated prior to the experiments. We planted two oospore infested seed lots within the different sections of the isolator, seed coated with oospore-infested leaves, and a seed lot sample from which we detected no oospores. In the commercial seed lot sample planted that contained on average 687 *P. effusa* oospores per 1,000 seed, we observed heavy sporulation on leaves (Figure 6). We also observed heavy sporulation in the isolator with plants grown from seed coated with oospore-infested leaves. Also, unlike some past experiments we did not observe

sporulation on the negative control seeds in the experiment (Viroflay seed with no detected oospores).

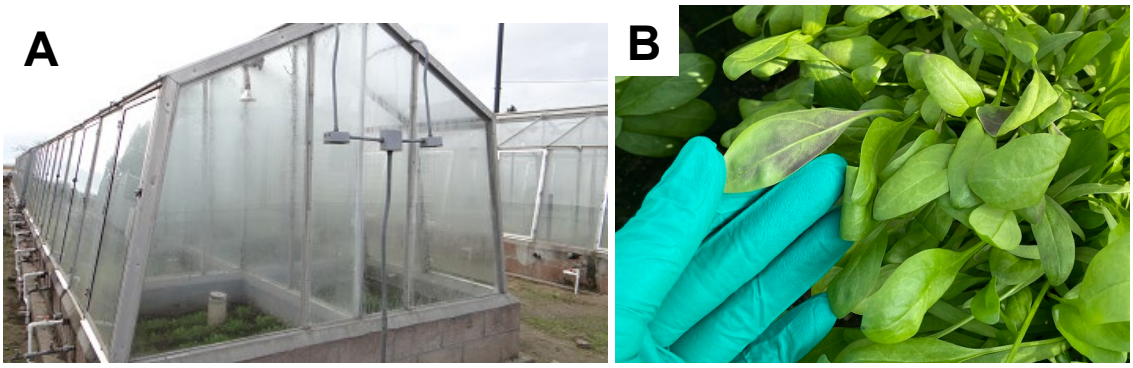

Figure 6. A) Plant isolators at the USDA ARS station in Salinas, CA for planting *Peronospora effusa* oospore-infested seeds or those that were non-infested. **B)** Downy mildew sporulation and chlorotic lesions on spinach leaves of spinach grown within the isolator using *P. effusa* oospore-infested seeds.

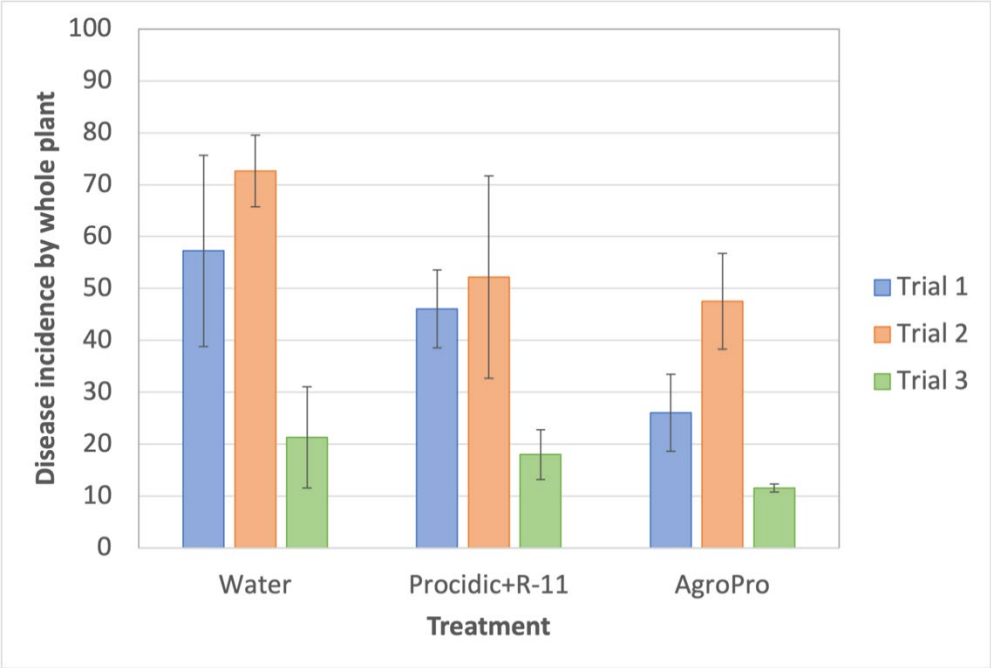

Figure 7. Summary of three separate biopesticide trials performed on spinach plants in microplots at the USDA-ARS Salinas. Disease incidence is an average of two biological replicates. Error bars are standard deviation from the two biological replicates for disease incidence scoring of 50-100 spinach plants. Each trial is represented by a different color. AgroPro was used at 4%, Procidic was used at 0.2% and R-11 at 0.125% according to the manufacturer's instructions.

Biopesticide testing was conducted in both microplot and mist tent experiments from June to October 2021. The preliminary microplot trials indicated that AgroPro at 4% may inhibit downy mildew disease (Figure 7). Further testing in the mist tent also indicated inhibition of downy mildew disease development with treatments of AgroPro at 4% and 0.4%, and R-11 (Figure 8). We also calculated disease incidence based on symptomatic leaves in the mist tent experiments rather than whole plants, which provides a better representation of biopesticide effect as it may coat leaves more evenly that are at the top of the canopy.

For the field trial at Spence in November of 2021 we proceeded with the best treatments from the microplot and mist tent experiments which were AgroPro at 4% and R-11 (at 0.125%), while using water only as a control. Spinach plants were treated twice (November 10th and 17th) before evaluation of disease incidence by leaf (Figure 9). Downy mildew disease was naturally occurring with inoculum coming from wind-blown spores in an adjacent field trial.

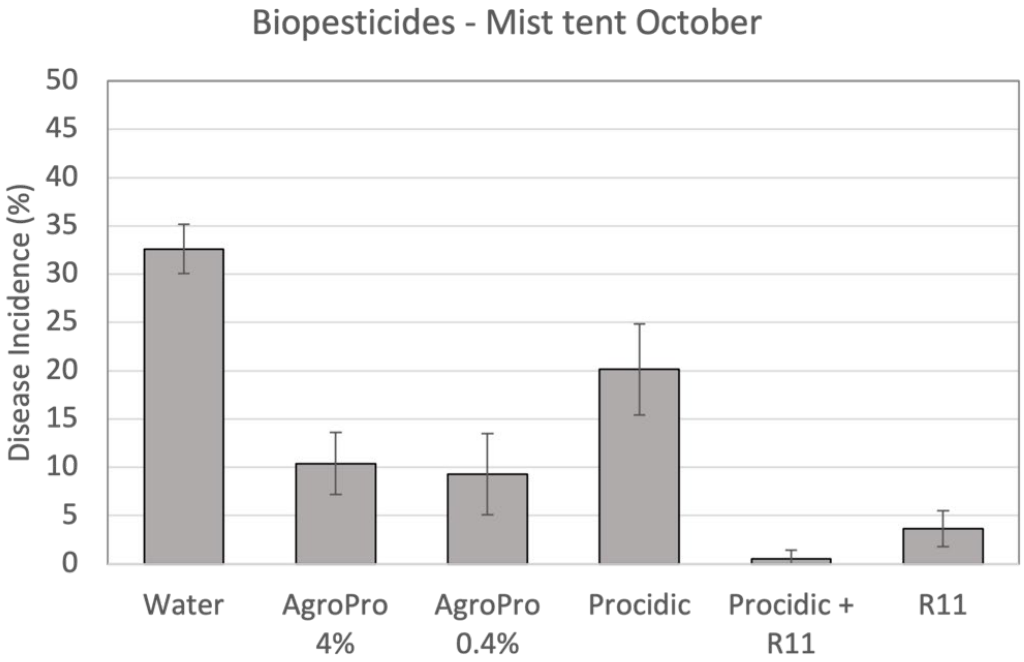

Figure 8. Results from the biopesticide trials performed on spinach plants in the mist tent. Plants were inoculated with downy mildew post treatment. Disease incidence was evaluated 7 days after inoculation. Error bars are standard deviation from three biological replicates for disease incidence scoring of 50-100 spinach leaves. Disease incidence represents an average of the three biological replicates. Procidic was used at 0.2% and R-11 at 0.125% according to the manufacturer's instructions.

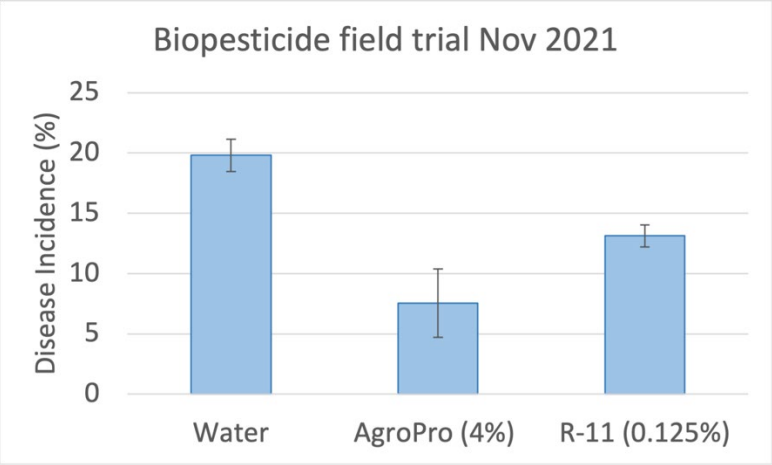

Figure 9. Results from the biopesticide trials performed on spinach plants in a field trial at Spence in November 2021. Error bars are standard deviation from three biological replicates for disease incidence scoring of 100-200 spinach leaves. Disease incidence represents an average of the three biological replicates.

DISCUSSION:

In our previous work, we have applied qPCR for quantification of the downy mildew pathogens of lettuce and spinach in airborne samples and in leaves for early detection systems [3,4,5,9,10,11,14]. Tracking the levels of windborne inoculum of the pathogen has yielded insights on the prevalence of the downy mildew pathogens of lettuce and spinach and increases of both pathogens have been detected in the Salinas Valley during their respective growing seasons [4,11]. By placing spore traps near grower lettuce fields that exhibited downy mildew, we had shown that using the spore trap detection values directly with a set threshold for spray application resulted in a savings of 1.7 fungicide sprays versus calendar sprays. Though it would likely not be practical to install and routinely sample spore traps around lettuce ranches because of labor costs in addition to trap maintenance costs, the insights obtained from these experiments improve our understanding of optimal conditions and locations for disease development.

In the current reporting period, we have shown that the newly available cyclone type traps clearly outperformed the spinning rod type traps that were previously used for airborne pathogen detection in the Salinas Valley [3,4,11]. In this reporting period, we gained additional insights from spore trapping comparisons from multiple valleys in California. Experiments were conducted in 2021-2022 to compare the airborne detection levels of *P. effusa* in the Salinas, Coachella, and Imperial Valleys of California. Like the findings on the levels of *B. lactucae*, the amounts of *P. effusa* detection were dependent on the growing season and location. But remarkably in the Coachella or Imperial Valleys there were nearly month-long spans where *P. effusa* was not detected. This finding suggests that, because we know windborne sporangia of *P. effusa* are short-lived, especially under hot conditions, there is an alternative source of inoculum (other than airborne) that results in disease. Based upon our findings of seed transmission of spinach downy mildew in this reporting period, the most likely explanation is that the primary inoculum source of *P. effusa* that initiates disease must reside in the spinach seed. Additional work will focus on also comparing weather parameters in correlation with spore quantities detected in each valley.

Two strains of different mating type are required to form the sexual oospores in *P. effusa*, and the oospores can survive at least for ~ 4 years on seed (Clark et al. unpublished). New pathotypes

or races of the pathogen, as well as both mating types, can be introduced to current and new production areas on seeds. The mating of different strains of *P. effusa* after bringing the mating types together in a new region has implications of increasing the genetic diversity within populations; it is clearly established that sexual reproduction contributes to the evolution of resistance-breaking isolates of *P. effusa* [13]. Given previous evidence of seed transmission [7], this work further advances the importance of oospores arriving on spinach seed in initiating disease. We (USDA) also demonstrated germination of the oospores [8] after this had not been reported in the literature for nearly 100 years [6].

It has been about forty years since the initial report of seed transmission of spinach downy mildew [7]. In this current reporting period, seed transmission studies conducted in the winter of 2021-2022 yielded convincing evidence of seed transmission of spinach downy mildew in plant isolators. The disease incidence was very high in those isolators planted with infested seed or those planted with seeds coated with oospores obtained from leaves. In these experiments, the negative controls, planted with Viroflay seed alone, did not yield downy mildew symptoms or signs.

In some previous experiments conducted in the isolators in 2018-2020, the observed disease incidence was low. Also, importantly, the negative control of Viroflay seed in the earlier experiments, without detectable oospores, was positive (also at low incidence) for *P. effusa* symptoms and sporulation. The finding of infection and sporulation in the Viroflay used in the experiment was explained by one of several possibilities: 1) the Viroflay seeds were positive for oospores but were not detectable using the typical seed wash-off tests; 2) hyphae of *P. effusa* were present on seed but not observed, and these could have transmitted the disease; and 3) there was soil contamination of *P. effusa* that was not adequately removed during fumigation one year earlier. Of these possibilities, the first seems most reasonable since the wash-offs from seed are usually negative but DNA detection is positive, meaning that we likely missed oospores in counting only the wash-off of two or three thousand seeds. The latter two are not plausible since hyphae of the pathogen are short-lived and fumigation would be anticipated to kill whatever small number of oospores that could have been present in the soil from planting spinach one year earlier. Though the data from the current seed transmission experiment strongly supports the previous finding of seed transmission tests that to our knowledge were not conducted in isolators [7], we will try to replicate the experiment in isolators prior to publication. Determining the conditions most conducive to oospore survival and germination would also provide useful information for the elimination or reduction of viable oospores on seeds.

Biopesticide testing was conducted to evaluate the effectiveness of two biopesticides for protection from spinach downy mildew. In these studies, we found that the biopesticide AgroPro and surfactant R-11 can inhibit downy mildew disease progression on spinach. Previously we found the biopesticide Procidic to provide protection against downy mildew, however from recent experiments in the mist tent looking at the chemicals separately it has become apparent that the surfactant commonly mixed with Procidic for application (R-11) is providing most of the exhibited protection. We intend to replicate the field trial at Spence field, and experiments are ongoing to examine further the direct inhibition of spore germination in response to these biopesticides on water agar plates.

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ARS) for helping set up the field trials for biopesticide testing. We appreciate provision of spinach seed samples from the spinach seed production and distribution companies and access to lettuce fields for the spore trap studies.

Mention of trade names or commercial products in this research report is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture or the University of California Davis. USDA is an equal opportunity provider and employer.

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