CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

Annual Report, 2014-2015

DNA-based detection and quantification of the downy mildew pathogen, *Peronospora effusa*.

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ABSTRACT

Downy mildew on spinach is caused by *Peronospora effusa* (*P. effusa*), an oomycete microorganism. Downy mildew is the most widespread and destructive spinach disease in California. To assess the factors required for disease outbreaks on spinach, and also to identify potential sources of the pathogen, a DNA-based assay was previously developed to determine the amount of *P. effusa* in air and other samples. This assay has been deployed to quantify the levels of airborne inoculum of *P. effusa* from spore trap samples, spinach seeds, and may be useful in disease forecasting. The first major objective of this research entailed analyses of the potential role of seedborne *P. effusa* in transmitting the disease. The sexually produced oospores of *P. effusa* were detected in 13 out of 82 commercial seed lots and nearly 95% of the seed lots were positive for *P. effusa* in the DNA-based assay (quantitative PCR) testing. The second major objective entailed quantification of airborne inoculum of *P. effusa* over the winter period at a USDA (Salinas) spinach field plot in a second overwintering season. In both overwintering experiments conducted in 2013-2014 and 2014-2015 overwintering periods, the disease developed in January and the inoculum load was tracked indirectly by quantitative PCR at the onset of disease development. After initial experiments revealed specific detection of *P. effusa* at spore trap sites at the south end of the Salinas Valley, an experiment was conducted in the period of late January, 2013 to June, 2013 and in the corresponding period in 2014, to assess the airborne inoculum level of *P. effusa* at four different sites in the Salinas Valley, including near Salinas, Gonzales, Soledad, and King City. The replicate 2014 experiment for inoculum quantification was completed in this reporting period. These data were used to carry out the remaining objective to evaluate the connection between spore trap data and the levels of downy mildew disease and developing an information hub for growers and PCAs. Results from both 2013 and 2014 suggest that the increases in spore trap-detectable inoculum throughout the Salinas Valley are correlated with increasing disease incidence in the field and decreasing temperatures and higher wind speeds. Ongoing work is aimed at determining how weather patterns and disease outbreaks are correlated with the fluctuations in downy mildew pathogen DNA levels detected at spore trap sites. Work on an information hub for growers and PCAs was initiated. Tracking the levels of windborne inoculum of the pathogen is expected to yield insights on the environmental conditions that favor outbreaks of downy mildew. Knowledge of *P. effusa* routinely detected by the DNA-based assays in commercial seed lots, and the common presence of sexually produced oospores of the pathogen indicate that treatments that eliminate *P. effusa* on spinach seed may curtail the spread of the pathogen.

PROJECT TITLE: DNA-based detection and quantification of the downy mildew pathogen, *Peronospora effusa*.

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OBJECTIVES:
1) Analyses of the potential role of seedborne *P. effusa* in transmitting the disease;
2) Quantification of airborne inoculum of *P. effusa* over the winter period at a spinach field plot;
3) Quantification of airborne inoculum of *P. effusa* at multiple sites in the Salinas Valley;
4) Evaluate the connection between spore trap data and the levels of downy mildew in spinach

PROCEDURES:

To analyze seeds for the presence of *P. effusa*, 1000 seeds from most seed lots were washed with water for 5 min by vigorous vortex mixing, the debris were pelleted by gentle centrifugation for 5 min, and then the pelleted debris were analyzed under light microscopy. For some seed lots, fewer seeds were available, and 500 seeds were examined in this way. In total the sediment obtained from 82 seed lots were examined for the characteristic oospores and sporangiophores of *P. effusa* [2].

Spinach seeds were ground for qPCR as previously described [4], except that 300 seeds of each lot were ground instead of 1000 for each seed lot tested. qPCR analysis on seed was conducted using a *P. effusa* SNP-specific TaqMan assay (Fig. 1) on 59 of the seed lots.

Seed viability tests were conducted using plasmolysis tests [6]. Using the plasmolysis test, the cell membrane visibly shrinks to form a tight ball within the central oospore cavity if the oospore cellular plasma membrane is intact, and thus indicates viability.

A comprehensive manuscript describing all of these procedures in much greater detail will be submitted for journal publication in June, 2015 (S. G. Kunjeti, A. Anchieta, K. V. Subbarao, S. T. Koike, S. J. Klosterman).

Quantitative PCR for detection of quantification of *P. effusa* DNA was carried out using the TaqMan assay developed previously [10] and shown in Figure 1. Examination of several target DNA sequences, have revealed a high level of DNA sequence similarity between *P. effusa* and isolates of *Peronospora schachtii* (from Swiss chard or beet) [3, 10]. A single nucleotide polymorphism (SNP) depicted in Figure 1 was previously identified to differentiate *P. effusa* from the closely related *P. schachtii* isolates. However, in airborne detection and quantification, qPCR using the primers and probes specific for both *P. effusa* and *P. schachtii* must be performed because of some nonspecific amplification. This doubles the time and cost of each assay. Therefore, in this reporting period, we undertook development of a new *P. effusa*-specific assay based on newly available mitochondrial sequences of *P. effusa* and analyzed by Dr. F. Martin, which can eliminate this additional cost in airborne sampling. DNA samples from various downy mildew-infected plants were tested by Drs. M. Thines and Y-J. Choi (Frankfurt, Germany) by PCR to ensure specificity. Unlike the development of *Bremia*-specific primers, there was difficulty in finding specific primer sets, but we have recently settled on a *P. effusa*-specific pair and probe.

Spore traps (Figure 2) obtained from Dr. Walt Mahaffee (USDA ARS, Corvalis, OR) were sampled three times weekly (at approximately 48 or 72 hr intervals) for windborne inoculum of *P. effusa* at each of the locations where spore traps were deployed in this reporting period. Pairs of 1.1 mm x 40 mm stainless steel rods coated in silicone vacuum grease (Dow Corning) are held in place by rubber grommets at the top of the spore trap head (Figure 2B). The metal rods spin on a solar/battery-powered motor controlled arm, enabling small particles (such as downy mildew spores) to stick to the rods. The collected rods were stored at 4^{o}C until DNA extraction using the Nucleospin Plant II kit (Machery Nagel) following the manufacturer's protocol for isolating genomic DNA from fungi. The estimate of the spore numbers based on the DNA level detected was determined as previously described [10].

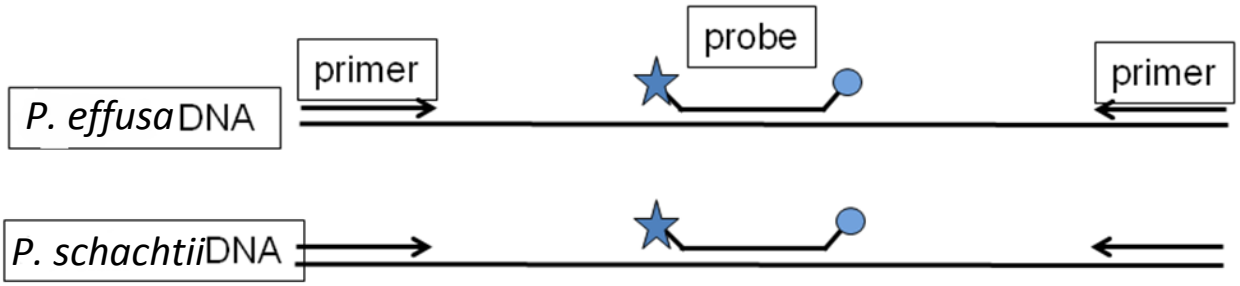

Figure 1. Illustration of the primer/probe combinations used in quantitative real-time PCR (TaqMan) assays to quantify *Peronospora effusa* and *Peronospora schachtii* (beet/chard pathogen) DNA target sequences. FAM™ and VIC® fluorescent dyes (Applied Biosystems), indicated by blue stars, were attached as labels to the probes for detection of *P. effusa* and *P. schachtii*, respectively. Probe quencher (blue circles).

An additional experimental plot at the USDA ARS station in Salinas was established in each of the overwintering periods of November, 2013 and 2014 and monitored until February, 2014 or 2015 respectively, to assess the presence of over wintering inoculum of *P. effusa*, and to assess the level of airborne inoculum associated with an onset of a disease outbreak. These plots in each season consisted of four 80" beds using spinach cultivar Viroflay, susceptible to all *P. effusa* races. The plot was watered twice weekly by overhead irrigation. The first observation of the disease in the USDA spinach plot was on January 21, 2014 and similarly in mid-January, 2015, and the disease progressed throughout the plot. Spore traps were placed on each of the four sides of the plot, and rods were collected at approximately 48 to 72 hr intervals and processed as described previously [10]. Disease incidence was rated using a high-density cluster sampling method, with disease incidence measured as percent of diseased leaves in a $1m^2$ plot. The plot was rated weekly for the duration of the trial beginning at full leaf stage.

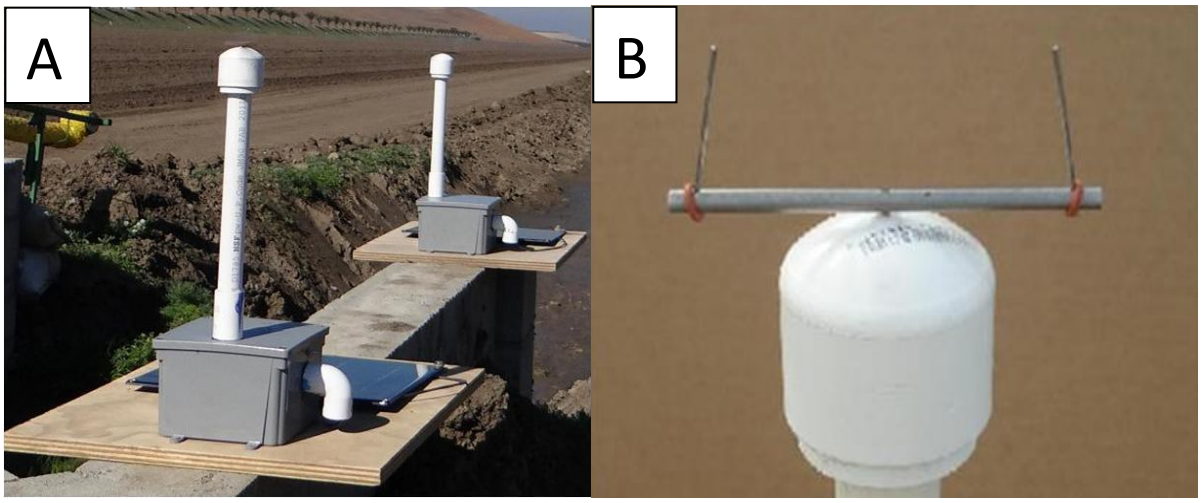

Figure 2. Spore trap system to detect *Peronospora effusa*, the causal agent of spinach downy mildew in the Salinas Valley, California. A) Two spore traps in operation south of King City, CA. B) Spore trap head with rotating arm and removable stainless steel rods.

High quality weather data from each of the trap locations was obtained from Fox Weather LLC. Logistic regression based on spore increase and decrease was used to correlate temperatures, relative humidity, and windspeed with spore load over the course of 6 time sections of the day. Summary data from weather variables such as temperature, solar radiation, windspeed, and relative humidity were directly correlated with spore load. Spinach fields nearby trap sites were monitored for disease incidence. Disease incidence was measured as percent of diseased leaves in a $1m^2$ plot. Fields were monitored using a cluster sampling method. Large fields were measured multiple times.

We conducted two biofungicide trials in the summer of 2014 in Watsonville to evaluate seven organically registered materials for managing downy mildew in organic spinach in collaboration with S.T. Koike. In both trials, spinach (cultivar Corfu) was planted on 80-inch beds in a conventional field. Materials were sprayed onto replicated plots using a backpack sprayer and delivered at 65 gallons of water per acre equivalent. The conventional fungicide Zampro was included for comparison. Disease incidence was determined as the percent of infected leaves in a 1 ft-sq area, with six measurements per replicate. Spray timing was consistent with grower practices.

RESULTS:

For the 82 seed lots were examined by microscopy, 13 seed lots were positive for one or more oospores (16% of the total). Five of these seed lots contained a high abundance of oospores. The oospores obtained from the seed lots were smooth-walled, brownish in color, and similar in size (~ 30 micrometers) to oospores characterized for *P. effusa* [2] and the one shown in figure 3A.

Sporangiophores characteristic of *Peronospora* were observed in another six separate lots (data not shown). Thus, in total, approximately 23% of seed lots have detectable levels of *Peronospora*. This number is likely to be a low estimate however, since the seed wash off method used only examined windows of 1000, or in some cases, 500 seeds. Approximately 95% of the commercial seed lots tested by qPCR were positive for *P. effusa*.

The result in figure 3B from plasmolysis testing indicates the presence of a viable oospore washed from one of the spinach seed lots. The tight ball formed in the central oospore cavity (arrow head in figure 3B) when treated with 4 M sodium chloride indicates an intact membrane, and oospore viability. Additionally, because the membrane is intact, this process can be reversed, and the ball-like structure disappears upon flooding the slide with deionized water (Kunjeti et al., data not shown). A comprehensive manuscript describing all of these findings will be submitted for publication in June, 2015 (Kunjeti et al.).

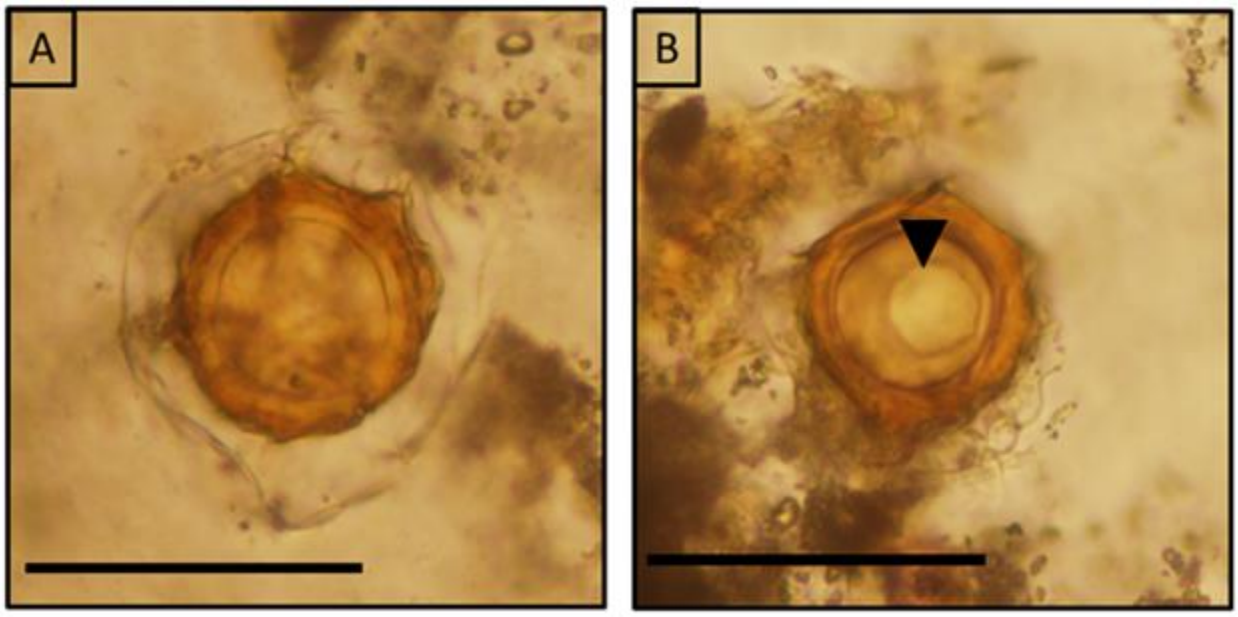

Figure 3. Plasmolysis testing on an oospore from a spinach seed lot. **A)** Not treated or **B)** treated for 15 min in 4 M NaCl.

To assess the presence of over wintering inoculum of *P. effusa*, and to assess the level of airborne inoculum associated with an onset of a disease outbreak, an experimental plot was established at the USDA ARS station in Salinas in each of the overwintering periods of November, 2013 and 2014 and monitored until February, 2014 or 2015 respectively. Disease development was observed in January of both seasons. Spore traps on each side of the plot enabled detection of the pathogen at the onset of disease development. The quantification of *P. effusa* DNA results have not yet been fully analyzed for the second season. Both weekly disease

incidence and pathogen inoculum levels are presented in Figure 5, which shows there was a clear correlation between the incidence of visible disease and the quantity of detected inoculum on the spore traps.

To assess the amount of airborne inoculum of *P. effusa* present throughout the Salinas Valley over time, spore traps (Fig. 2) were placed at ~10-15 mile intervals in the Salinas Valley at four different sites (Fig. 4). Rods were collected at approximately 48 to 72 hr intervals at all four sites from January 28, 2013 to June 7, 2013 and also in the similar time frame from the end January to early June in 2014.

Figure 4. Spore trap sites at four locations in the Salinas Valley

The summary of the 2014 spore trap study is shown in Figure 6. Overall, pathogen concentrations seem to increase over the course of the season. Distinct peaks in spore copy number levels were noticed, and the peaks appeared to synchronize across multiple sites. This suggests that inoculum may be spreading readily between sites. The presence of distinct peaks could be related to periods of high conducive conditions; analysis is still underway deciphering this possible connection. Disease incidence ratings taken from near the spore traps closely match the overall amounts of pathogen detected. As seen previously in 2013, there is a distinct and noticeable cycle of disease incidence increase followed by spore copy number increase. This suggests that spore traps may be able to aid in disease prediction and timing of preventative fungicides. Preliminary analyses suggest that there is weak periodicity in the detectable pathogen levels over the season, possibly due to the pathogen life cycle or the cropping period.

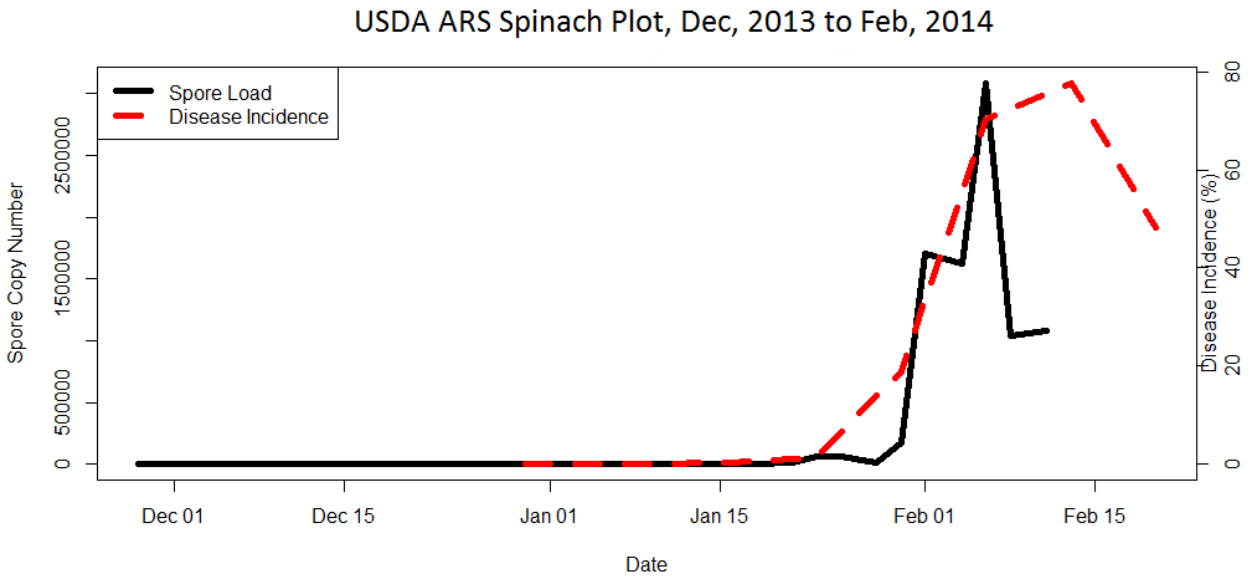

Figure 5: Weekly spore trap data of the amount of *Peronospora effusa* DNA detected and disease incidence data from a small plot study conducted at the 2013-2014 overwintering USDA ARS plot.

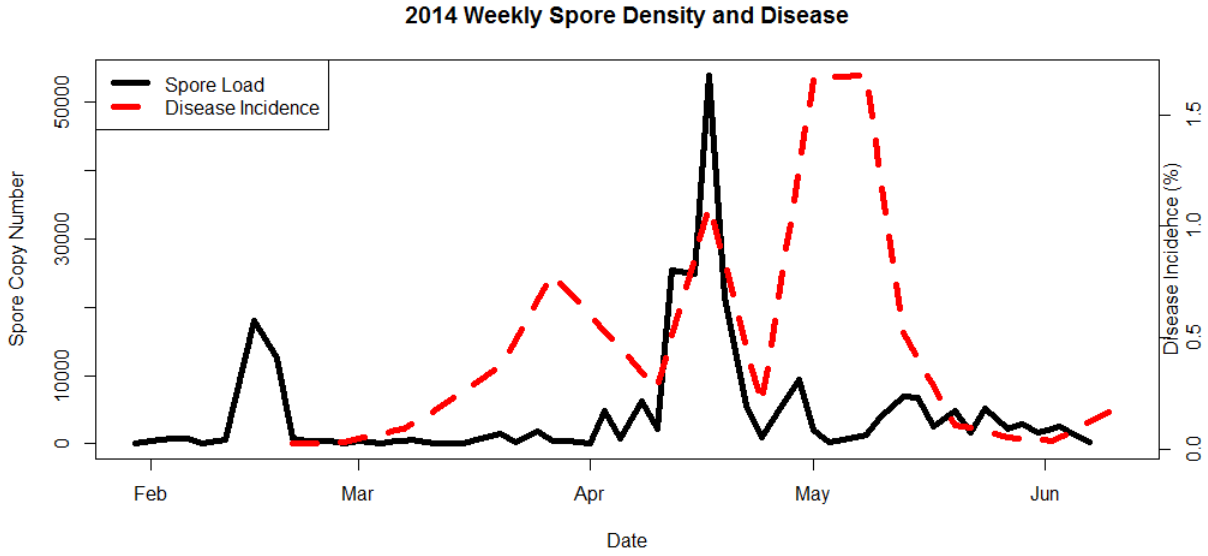

Figure 6. Weekly spore trap data of the amount of *Peronospora effusa* DNA detected and disease incidence data from the Salinas Valley in 2014.

Logistic regression-based on spore increase and decrease was used to correlate high afternoon and evening winds and low temperatures with increases in spore copy number. Direct correlation of weather variables and spore copy number suggests that stable, cool early morning temperatures promote increases in spore load. Full analysis of the role of weather variables in disease incidence and spore copy number is still underway.

Downy mildew pressure was moderate to high in the two biofungicide trials. In the first trial (figure 7A), Taegro, Cueva, and Milstop were significantly better than the untreated control, as

was the conventional Zampro treatment. In the second trial (figure 7B), only Actinovate and Zampro were better than the untreated control. No phytotoxicity was observed in any of the treatments in either of the trials.

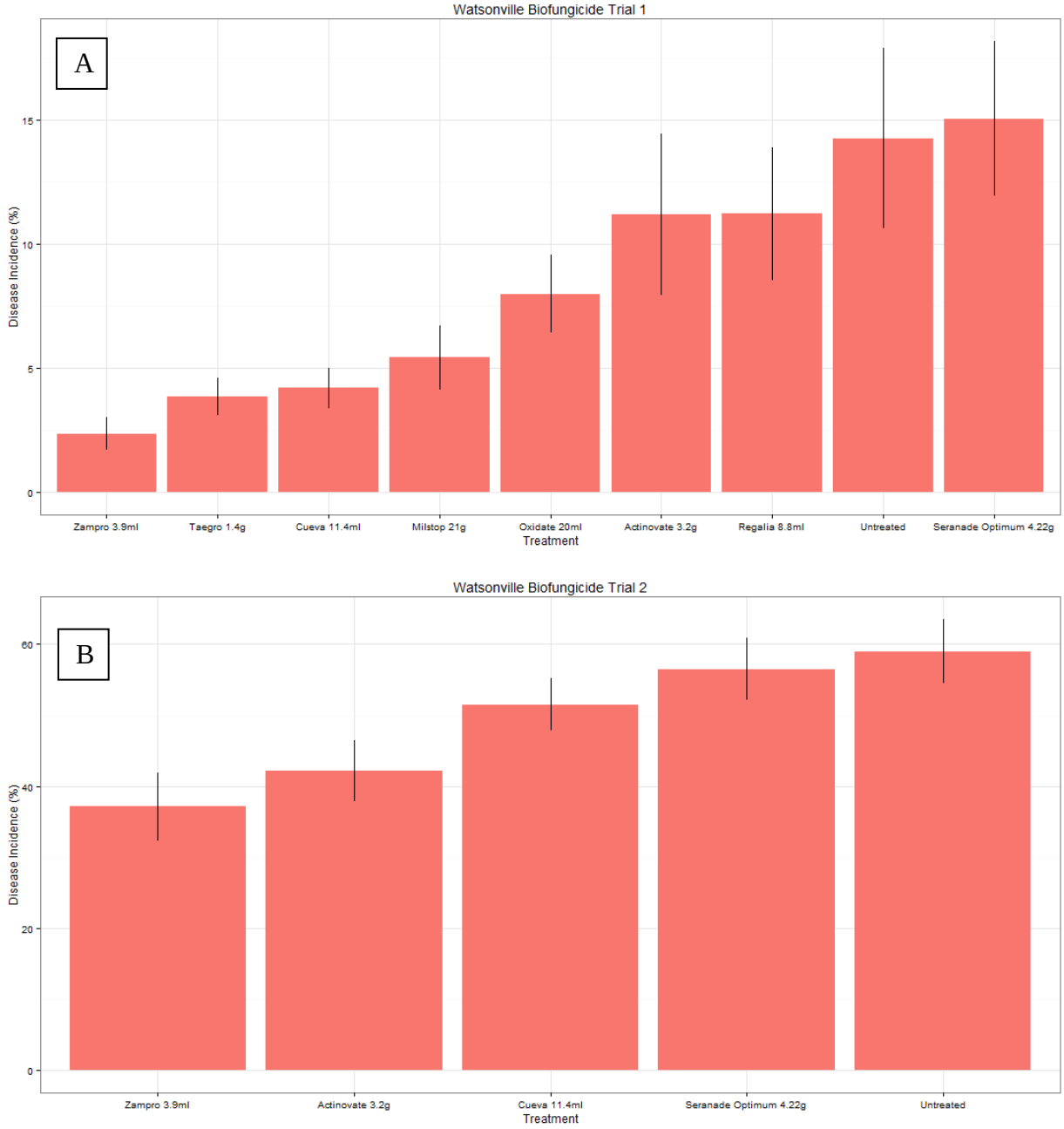

Figure 7: Disease incidence ratings from two biofungicide trials conducted in Watsonville

DISCUSSION:

It has been over thirty years since the initial report of *Peronospora effusa* on spinach seed lots in a study conducted in Japan [8]. This previous study also provided evidence for transmission of *P. effusa* on spinach seed [8]. This current report documents the finding of oospores characteristic of *P. effusa* in 16% of modern spinach seed lots. The analysis suggests

that additional seed lots are also infested with oospores, as seed wash off method only examined windows of 1000, or in some cases, 500 seeds, and nearly 95% of the lots tested were qPCR-positive. This report further documents the viability of some of the oospores detected on spinach seed lots. A comprehensive manuscript on *P. effusa* detection and the viability tests of oospores will be submitted for journal publication June, 2015.

Additional questions concerning the levels of production of oospores in spinach fields in California, and length of time that the pathogen can survive as oospores in seed remain to be fully explored. We had previously observed oospores in leaf tissue from a sample of downy mildew-infected spinach obtained from the greenhouse of Steve Koike and the downy mildew sample used in inoculations was originally obtained from San Benito Co. However, oospores have not yet been detected in leaf tissue collected from field samples in California and this may be due to a seldom occurrence of the appropriate mating types of *P. effusa*, occurring in the same location.

Since *P. effusa* is heterothallic [9], two strains of different mating type are required to form the long-lived (1-2 years) sexual oospores. The presence of oospores on spinach seed indicates long term survival of the pathogen on seed, which may be transmitted to new areas. In support of this, one of the oospore-positive seed lots, seed lot 82, was from a grower in Arizona that suspected that the downy mildew pathogen was carried on the seed and an oospore was detected in this lot. Mating of different strains of *P. effusa* has implications of quickly increasing the genetic diversity within populations, potentially contributing to the appearance of new races of the pathogen. If seed and soils are important primary inoculum sources, as indicated for seed in this study, this knowledge could lead to treatments to reduce downy mildew on spinach.

The qPCR assay was deployed as described previously [10] to detect both the spinach downy mildew pathogen, *P. effusa*, and the downy mildew pathogen of chard or beet, *P. schachtii* from the airborne sampling devices (spore traps) at multiple locations in the Salinas Valley of California. Importantly, *P. schachtii* does not infect spinach [1, 10], although beet and Swiss chard plants are commonly infected with this pathogen in California. The presence of the pathogens could potentially interfere with an assay designed for specific detection of *P. effusa*, which only causes downy mildew on spinach [1, 10]. Therefore, specific probes were previously designed for detection of each pathogen, *P. schachtii* and *P. effusa*, by taking advantage of a SNP, or a single nucleotide difference, identified in the target DNA sequence [10]. This, in combination with other DNA differences, allowed for calculation of the frequency of *P. effusa* detected in each spore trap sample. However, due to the complications that arise using this approach (increased time and cost) and cross reactivity with *Peronospora* spp. found outside the U.S., we undertook development of a new *P. effusa*-specific qPCR assay based on mitochondrial DNA target sequences, which shows promise in reducing the time and cost of the assay by one-half, and increasing the specificity of the assay for worldwide usage.

Data analyses from the initial spore trap experiments in 2012 and the larger ongoing two year Valley experiment initiated in 2013 suggest that there is a low level "blanket" of airborne *P. effusa* spores generally present throughout the Salinas valley. Based upon correlations between actual spore numbers and DNA copy number detection by qPCR, < 20 spores are typically detectable per sampling site. However, there were also periods of clearly increased pathogen detection within the background blanket level of *P. effusa* at the spore trap sites.

More analyses are required to assess correlations between weather variables and increases and decreases of *P. effusa* DNA detectable by spore trap sampling. Preliminary findings revealed a correlation between high afternoon windspeed and low afternoon and evening temperature and

relative humidity and increased spore copy number. Additional analyses of the spore trap data are underway to more definitively determine any correlations between weather characteristics and the amount of pathogen DNA detected.

In summary, the data indicate that the sexual oospores of *P. effusa* appear rather common in commercial spinach seed lots. Additional experiments to determine the longevity of the oospores derived from seed will be undertaken in the future. In addition, we have applied spore trapping and qPCR for quantification of the downy mildew pathogen on spinach. Further tracking the levels of windborne inoculum of the pathogen is expected to yield insights on the environmental conditions that favor outbreaks of downy mildew.

Acknowledgements: We are thankful for funding from the California Leafy Greens Research Program and provision of spinach seed lot samples from Alf Christensen Seed Co. (Sakata), Germains Seed Technology, and Rijk Zwaan. We would like to thank Taylor Farms and Dobler and Sons for their help in organizing fungicide trials. We are also thankful for the assistance of Amy Anchieta (USDA ARS, Salinas) and Kat Kammeijer (UC Cooperative Extension, Salinas), in conducting qPCR experiments and providing downy mildew samples, respectively, and Lorena Ochoa (USDA ARS) for collecting spore trap samples. Sergio Jiménez (Hartnell College student intern, Salinas) and Ruben Pena (USDA ARS) assisted with spore trap maintenance and sample collection. Robin Choudhury (UCD) conducted spore trap data analysis. Dr. Sridhara Kunjeti (UCD) conducted analysis of the new *P. effusa*-specific assay. The work is also supported by CDFA grant SCB14043.

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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