**CALIFORNIA LEAFY GREENS RESEARCH PROGRAM**

Combined Annual Reports for Spinach and Lettuce Downy Mildew projects, 2016-2017

# Disease risk assessment, early detection, and disease control applications for downy mildew of lettuce and spinach.

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## ABSTRACT

Downy mildew on spinach and lettuce are caused by the obligate oomycete pathogens *Peronospora effusa* (*P. effusa*), and *Bremia lactucae* (*B. lactucae*). Downy mildews are widespread and very destructive diseases on spinach and lettuce in California. To assess the factors required for disease outbreaks on both spinach and letttuce, and also to identify potential sources of the pathogens, DNA-based assays were previously developed for *P. effusa* and *B. lactucae*. The assays were deployed to quantify the levels of airborne inoculum from spore traps. The first major objective of the *P. effusa* research for this period entailed 1) evaluation the connection between spore trap data and the levels of downy mildew in spinach; 2) analyses of early infections in spinach leaves (pre-symptomatic) for assessing its utility as a downy mildew disease warning for conventional and organic spinach; 3) further assessing the role of seedborne *P. effusa* in transmitting the pathogen; and 4) testing of biopesticides to reduce sporulation of *P. effusa* on organic spinach. Additional sexually produced oospores of *P. effusa* were detected in some commercial seed lots by seed wash-off and microscopy. The results of all of the presymptomatic leaf testing for *P. effusa* in three different field plots completed in this reporting period, indicate that *P. effusa* could be detected in the leaves at least a week prior to leaf symptom development. For the *B. lactucae* objectives, we 1) evaluated several fungicides in a replicated field study late in the season for lettuce downy mildew and 2) deployed spore traps near the fungicide trial fields to simultaneously analyze downy mildew of lettuce. These data were analyzed and used to carry out the objective to evaluate the connection between spore trap data and the levels of downy mildew disease to advise on fungicide applications. The results indicate a savings of 1.7 fungicide applications in the three field trials conducted in 2016. Tracking the levels of windborne inoculum of the downy mildew pathogens and in-field leaf detection applications can be valuable to inform efficient spray applications for disease control. Knowledge of *P. effusa* routinely detected in commercial seed lots and presence of sexually produced oospores of the pathogen indicate that treatments that eliminate *P. effusa* on spinach seed may limit some outbreaks, especially for organic spinach. We validated a DNA-based detection system for *B. lactucae*, along with in-field fungicide tests, and showed this to be a useful tool for reducing spray applications for lettuce downy mildew on a local, ranch level.

**PROJECT TITLE: Disease risk assessment, early detection, and disease control applications for downy mildew of lettuce and spinach.**

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**OBJECTIVES (downy mildew on spinach)**:

**1)** Analyze early infection in spinach leaves (pre-symptomatic) for assessing its utility as a downy mildew disease warning for organic spinach

**2)** Examine oospore survival, conditions necessary for reproducible infection of spinach, and seed infestation thresholds for reducing introduction of downy mildew

**3)** Evaluate the connection between spore trap data and the levels of downy mildew in spinach.

**4)** Testing of biopesticides for *P. effusa* on organic spinach

**OBJECTIVES (downy mildew on lettuce):**

**1)** Evaluate several fungicides that have already been identified as effective in a replicated field study late in the season for lettuce downy mildew

**2)** Deploy spore traps near the fungicide trial fields to simultaneously analyze downy mildew of lettuce

**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 or 100 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* and described previously [14] but during this reporting period, an additional 54 lot samples were examined similarly. The comprehensive manuscript describing all of these procedures for the handling of spinach seeds for detection of *P. effusa* was published in 2016 [14], including Seed viability tests (See also methods in the previous report). Oospore germination tests were done by placing oospore suspensions from seed lots on detached leaves in sterile Magenta plant tissue culture boxes in a dew chamber set for 18 C. For field detection of oospores, the oospores were washed from a symptomatic Viroflay planted in 2016 at the USDA ARS station in Salinas, and which also had small, dark irregular lesions on a leaf.

Quantitative PCR for quantification of *P. effusa* DNA was carried out using the TaqMan assay developed previously [12] that differentiates *P. effusa*, which only infects spinach [1], from the closely related pathogen of beet [3]. Detection of *B. lactucae* based on mitochondrial DNA sequences unique to the species [13].

Spore traps (Figure 1) obtained from Dr. Walt Mahaffee (USDA ARS, Corvalis, OR) were sampled (at approximately 72 hr or 96 hr intervals; biweekly for six months) for windborne inoculum of *B. lactucae* 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.

Experimental plot at the USDA ARS station in Salinas were 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. 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 [12, 13]. The estimate of the spore numbers based on the DNA level detected was determined as described [13] and [14]. Disease incidence was rated using a high-density cluster sampling method, with disease incidence measured as percent of diseased leaves in a 1 $m^2$ plot as described [4]

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 wind speed 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 1 $m^2$ plot.

Fields were monitored using a cluster sampling method. Large fields were measured multiple times.

A similar spinach plot with the same dimensions and maintenance was reestablished in the fall of 2016 to examine latent infections (pre-symptomatic) in spinach leaves.

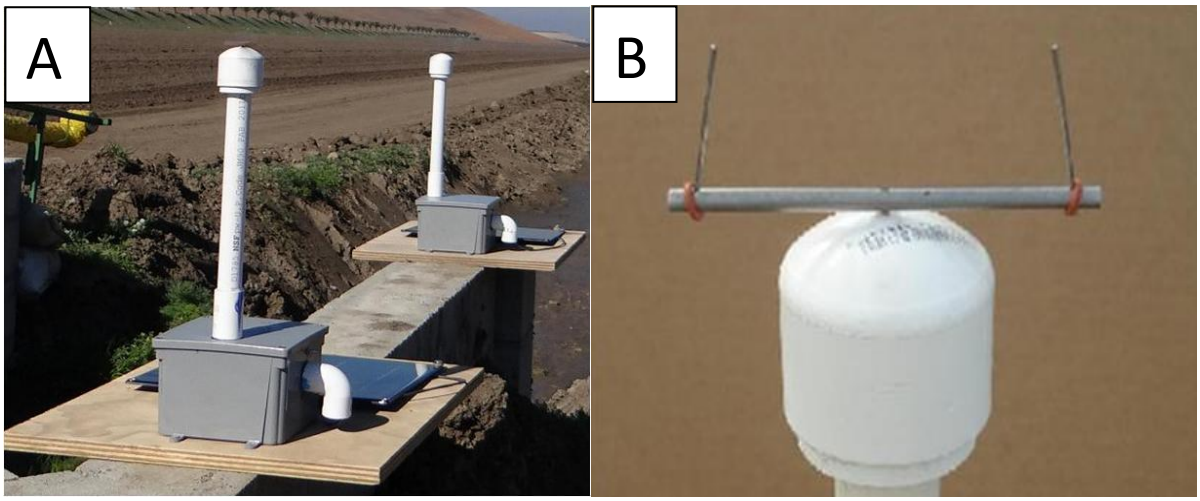


**Figure 1**. Spore trap system to detect *Bremia lactucae* and *Peronospora effusa*, the causal agents of lettuce and spinach downy mildew, respectively, in the Salinas Valley, California. A) Two spore traps in operation south of King City, CA. B) Spore trap head with rotating arm and removable stainless steel rods.

We conducted two biofungicide trials to evaluate organically registered materials for managing downy mildew in organic spinach in collaboration with S.T. Koike. Spinach 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 $ft^2$ area, with six measurements per replicate. Spray timing was consistent with grower practices.

For the conventional PCR assay for detection of *P. effusa* DNA in leaves, 5 µl of the eluate containing DNA template, extracted by NucleoSpin Plant II DNA extraction kit (Macherey-Nagel, Bethlehem, PA USA), was used in the PCRs with *P. effusa*-specific primers AS1 and PeR1 [12] on a PTC-200 thermal cycler (Bio-Rad, Hercules, CA, USA). The PCR reactions included 5 µl of extracted DNA template, 200 nM of *P. effusa*-specific primers (AS1 and PeR1), and 1x GoTaq® DNA Polymerase mix (Promega Corp., Madison, WI, USA) in a total volume of 25 µl. Reactions were performed on a PTC-200 thermocycler under the following cycling conditions: 94° C for 5 min, 35 cycles of 94° C for 30 s, 60° C for 30 s, and 72° C for 30 s. A final extension of 72° C was used for 5 min. The amplified products were loaded on 2% agarose gels in 0.5X Tris/Borate/EDTA buffer (TBE, components from Fisher Scientific, Waltham, MA USA). The gel was stained with Gel Red (Biotium Inc., Fremont, CA USA) according the manufacturer's instructions. A 100 bp ladder (Promega Corp.) was used for estimation of low molecular weight DNA products following electrophoresis and gel staining. Positive control DNA samples were derived from *P. effusa*-infected spinach leaves, or DNA extractions from sporangia washed off of leaves, while negative controls were derived from non-infected spinach tissue, or in some instances, a no template water

control was used. The specificity of primer pair AS1 and PeR1 for *P. effusa* DNA amplification in the background of spinach cultivar Viroflay DNA was previously established [12].

**RESULTS:**

**Analyses of oospores**

For the first time, during this reporting period, oospores of *P. effusa*, like the one shown in figure 2, were collected from spinach cultivar Viroflay grown in a field, in the fall of 2016, at the USDA ARS station, Salinas.

For the 136 seed lots examined as of this reporting period by microscopy (including the 82 previously characterized), 29 seed lots were confirmed positive for one or more oospores (21% of the total). Seventeen 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 2B. Sporangiophores characteristic of *Peronospora* (Figure 2A) were also observed previously on some seed lot samples. The results from plasmolysis testing as described previously (14) indicated the presence of viable oospores washed from additional spinach seed lots under investigation in this reporting period.

The experiments to germinate the oospores on spinach leaves were not successful during this reporting period, but the germination of the oospores was reported previously [7].

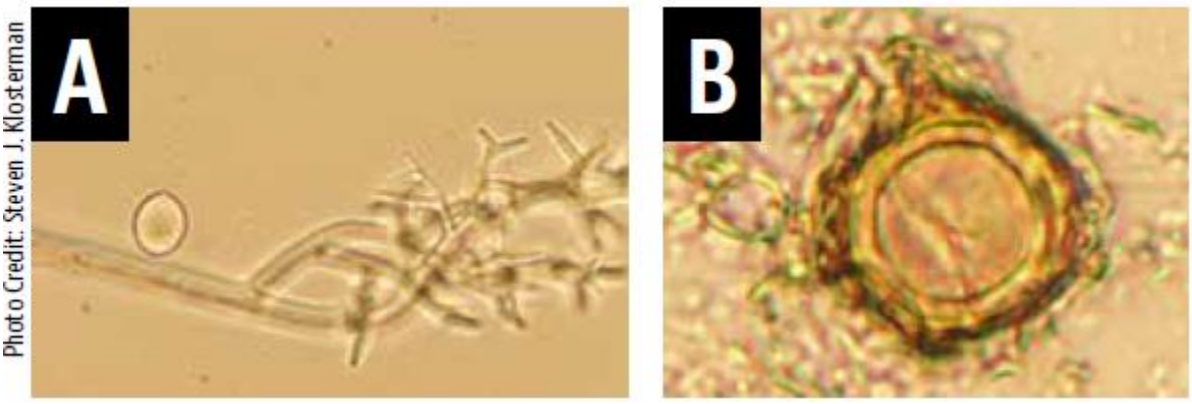


**Figure 2.** Asexual and sexual spores formed by *Peronospora effusa*, the cause of downy mildew on spinach. A) A single oval-shaped sporangium and the typical branching pattern of the sporangiophore on which the sporangia are borne; B) An oospore of the spinach downy mildew pathogen derived from a commercial spinach lot in 2014. The asexual sporangium and sexual oospore are microscopic, and each type is approximately 30 micrometers in diameter. The figure is from Klosterman, S. J. 2016. Progressive Crop Consultant, 1:12-15 [11].

**Seed infestation thresholds for reducing introduction of downy mildew**

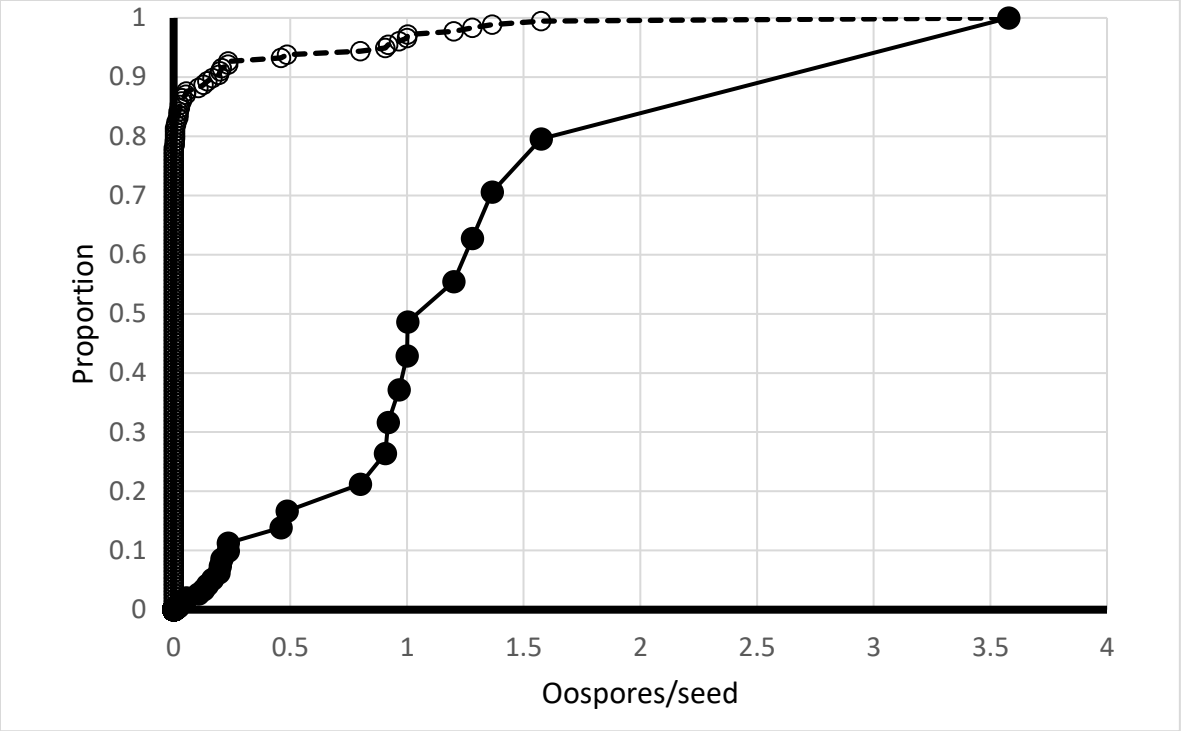


**Figure 3.** The open circles with dashed line shows the proportion of oospores removed, the solid circles with continuous line shows the proportion of seed lots. The key point from the analysis is that almost 80% of oospores occur in only 1-2% of seed lots, and these can be removed from the supply chain by setting a threshold of less than 1 oospore per 5 seeds.

One of the unresolved epidemiological issues with *P.effusa* is the extent of involvement that oospores play in the annual onset of epidemics and the rapid emergence of new virulence types when resistance genes are released in new varieties. It is known that *P.effusa* oospores do occur on spinach seeds imported to California [14], and that oospores of *P. effusa* germinate [7] and were correlated strongly with seed transmission of the disease [9]. With cooperation from stakeholders from the seed trade we were able to screen additional seed lots for the presence of oospores. Combining these new data with observations previously published by Kunjeti et al. [14] we derived a pair of cumulative distribution curves (Figure 3), one for proportion of seed lots, the other for proportion of seed-borne oospores removed, as the threshold number of oospores is varied from zero upward.

**Biopesticide trials for organic spinach**

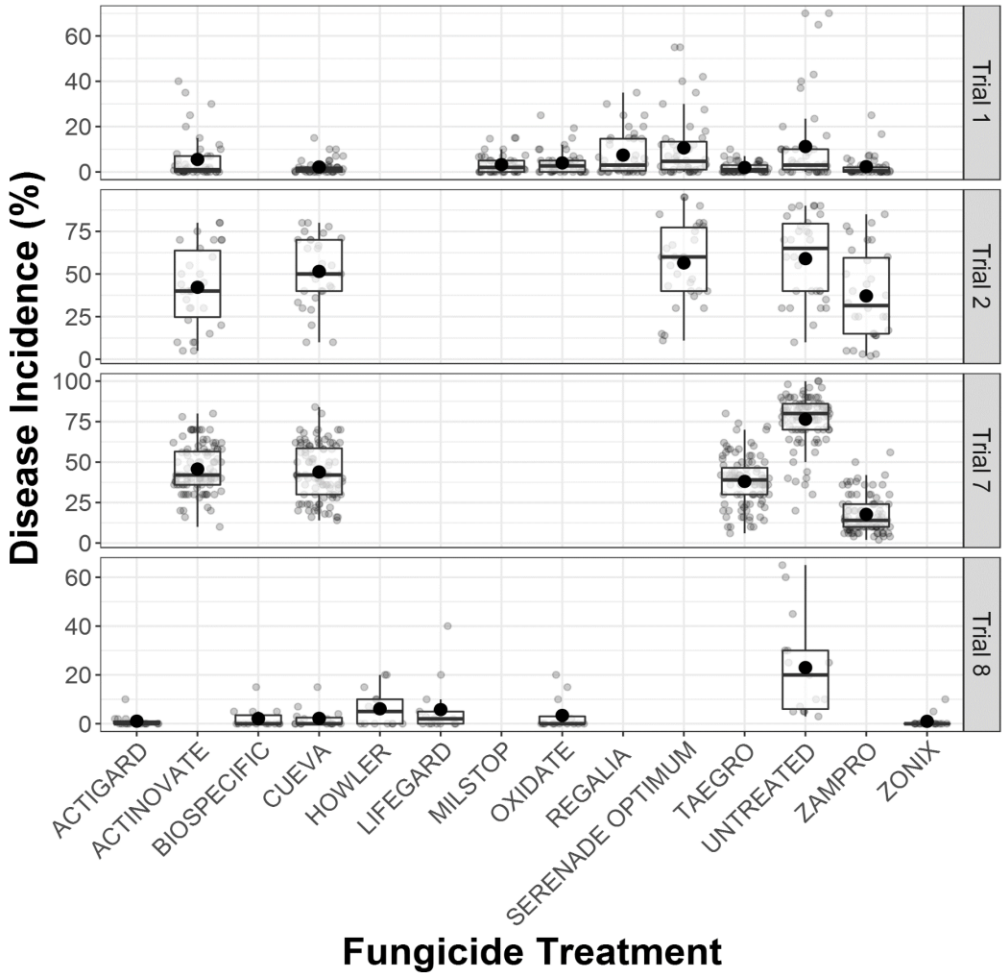


**Figure 4.** Biopesticide tests for protection against spinach downy mildew.

As in the two previous years, in 2016 we carried out efficacy trials to evaluate a number of biofungicides for their ability to control downy mildew on spinach. The trials were conducted with the cooperation of stakeholders from the seed trade and Steve Koike (UCCE), with support from USDA's IR4 program.

Broadly, the results from the 2016 trials shown in Figure 4 agree with what we saw in previous years. Some products are able to reduce the incidence of downy mildew in situations where disease pressure is low, but none of the currently available biofungicides offers reliable control under moderate to high disease pressure. Of the products tested, Cueva, Actigard, and Oxidate are somewhat successful under low disease pressure. All of the trial results to date have been published as Plant Disease Management Reports (https://www.plantmanagementnetwork.org/pub/trial/ PDMR/default.asp).

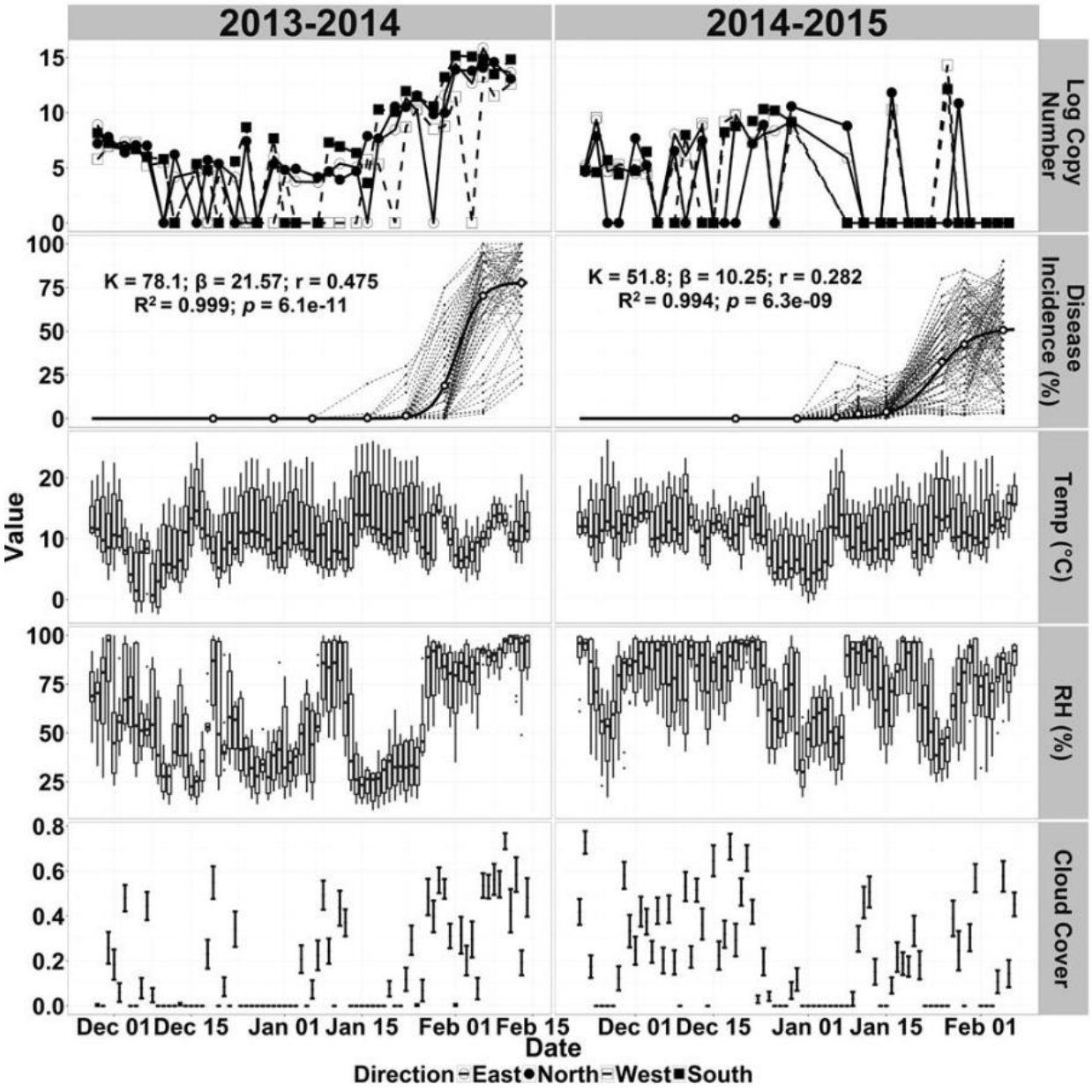


**Figure 5.** Line plot of the natural log of *Peronospora effusa* DNA copy number from four different traps, and line plots of disease incidence (%) from rated areas, temperature (C), relative humidity (%), and mean and standard error of cloud cover during the Salinas Valley epidemics in 2013–14 and 2014–15. Temperature, relative humidity, and cloud cover represent hourly data. Solid lines in disease incidence represent results of the fitted logistic growth model Y = K/[1+b(rt)] of the average spinach downy mildew disease incidence, represented by hollow white circles. Parameters for the logistic growth models are indicated within the disease incidence graphs. The figure is from Choudhury et al. [4].

**Assessment of inoculum load of airborne *P. effusa* for disease forecasting**

We had previously demonstrated that overall, *P. effusa* concentrations (measured indirectly by DNA detection and quantification) increase over the course of the season, in both the 2013 and

2014 spinach growing seasons [5]. In this reporting period, we completed the studies 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, at experimental plots established at the USDA ARS station in Salinas in each of the overwintering periods of November to February, 2013-2014, 2014-2015, 2015-2016. Disease development was observed in January of all three 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 now been fully analyzed during this reporting period, for the second season as well. 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. Analyses of weather parameters in relationship to the pathogen DNA levels detected did not reveal significant correlations that could be useful for disease prediction.

The wind pattern evident in the Salinas Valley during these experiments would have substantial impact on pathogen DNA detection, especially at more distant sites. The windroses in Figure 6 illustrate the complex wind pattern, which alternates from primarily the northwest and from southeast directions.

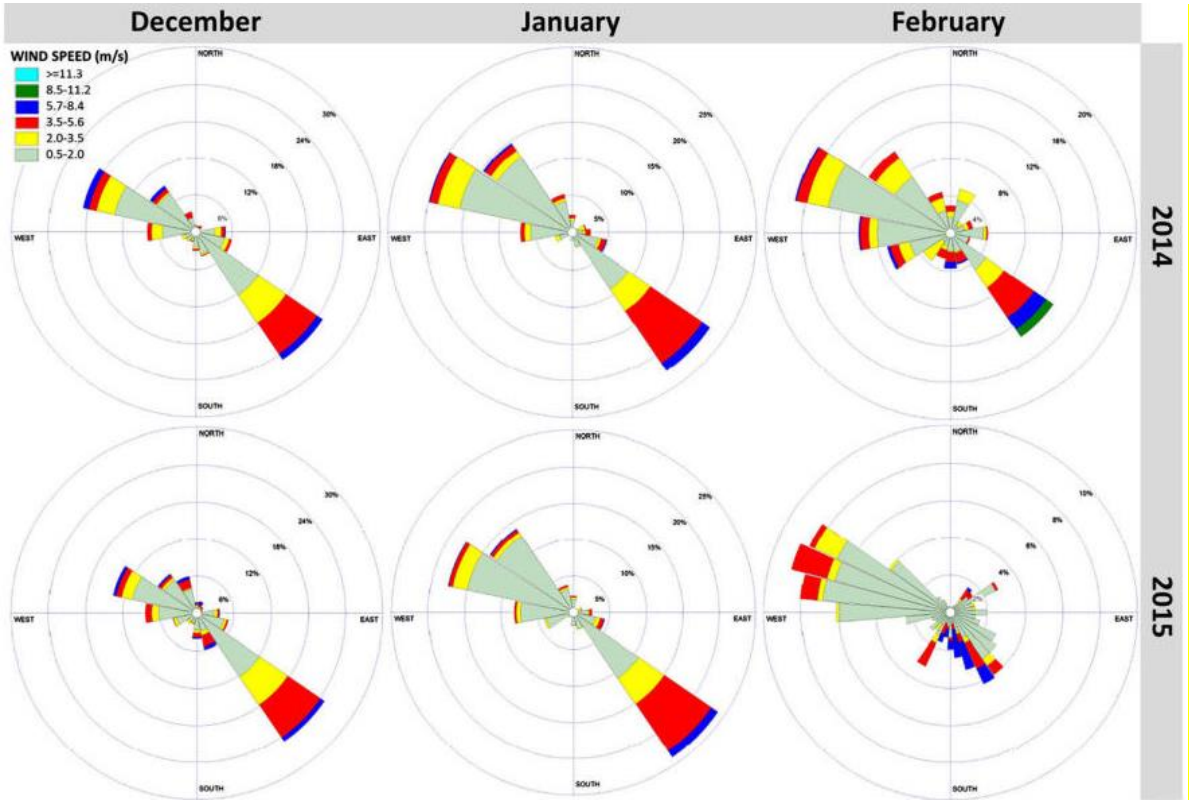


**Figure 6.** Windroses illustrating wind direction and speed at the United States Department of Agriculture–Agricultural Research Service station in Salinas, CA from the Salinas 2013–14 and Salinas 2014–15 winter seasons. The figure is from Choudhury et al. [4].

**Assessment of inoculum load of airborne *B. lactucae* disease forecasting**

The previously developed qPCR assay specific to *B. lactucae* coupled with a solar-powered spore trap system (Figure 1) for detection of *B. lactucae* was deployed in the current work to measure *B. lactucae* spore load at three commercial fields that each contained experimental plots.

Based on the inoculum thresholds detected, fungicides applications were scheduled. Following spray advisories conserved approximately 1.7 sprays on average, relative to the calendar-based sprays. This suggests that deployment of this approach in commercial fields can be useful to reduce grower costs and fungicide use in lettuce production while also decreasing the development of fungicide resistance in *B. lactucae*. A manuscript is currently in preparation with more details on the three field trials, and effectiveness of the fungicides used.

**Detection of latent infections of *Peronospora effusa* in spinach leaves**

Figure 7 illustrates the detection of *P. effusa* DNA, at least seven days prior to symptom development in the field. A full research manuscript describing the research and results is in the final stages of revision for submission for publication.

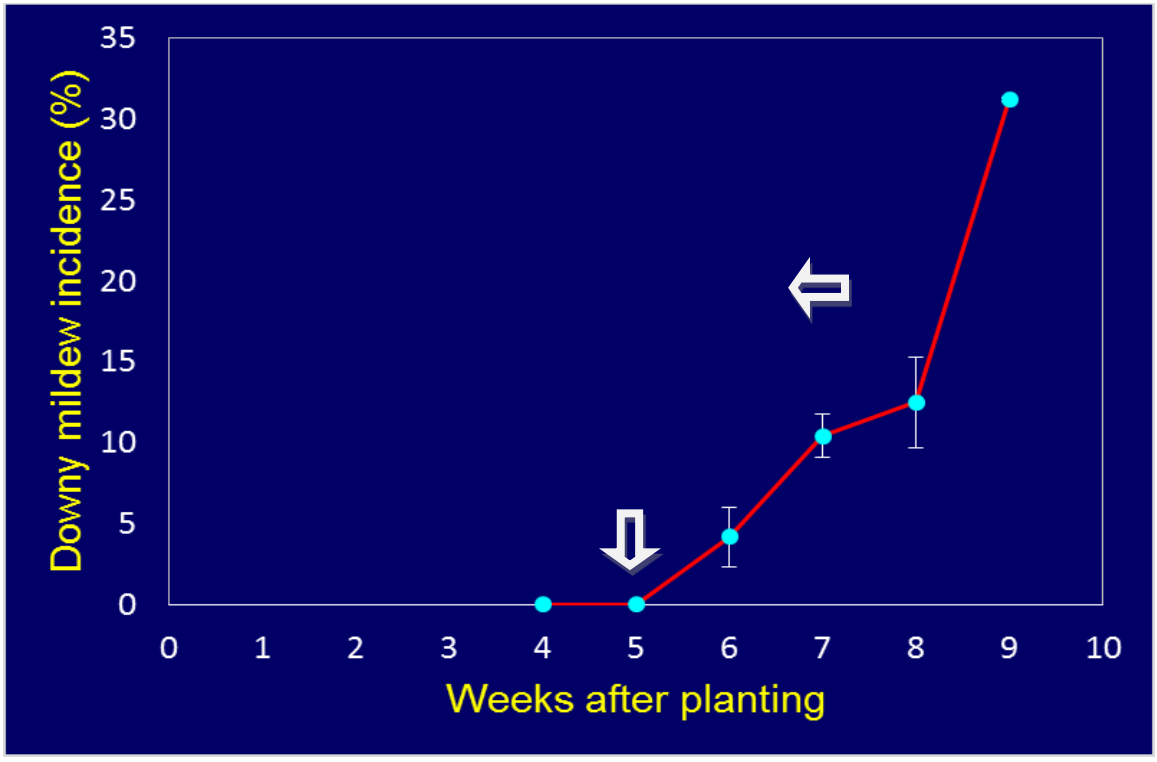


**Figure 7.** Conventional PCR test of leaf samples positive for *P. effusa* DNA detection. The arrow marks DNA detection relative to downy mildew disease incidence.

**DISCUSSION:**

We published the work on the finding of oospores of *P. effusa* in modern spinach seed lots, and examined 56 additional seed lots in this reporting period for oospores -- in addition to the 82 characterized in the publication [14]. It had been over thirty years since the initial report of *Peronospora effusa* on spinach seed lots in a study conducted in Japan [9]. The previous study also provided evidence for transmission of *P. effusa* on spinach seed in 5/6 cultivars tested [9]. This current report further documents the finding of oospores characteristic of *P. effusa* in about 21% total (29/136) of the modern spinach seed lot samples examined within the past few years in our laboratory (Klosterman). The previous work suggested that additional seed lots are also infested with oospores, as seed wash off method only examined windows of 1000 seeds (and sometimes less), and nearly 95% of the lots tested were qPCR-positive [14]. A comprehensive manuscript on *P. effusa* detection and the viability tests of oospores was published, 2016 [14].

Since *P. effusa* is heterothallic [10], two strains of different mating type are required to form the long-lived (2-3 years) sexual oospores. The presence of oospores on spinach seed coupled with our survival assays [8, 14] indicates long term survival of the pathogen on seed, which may be transmitted to new areas. Mating of different strains of *P. effusa* has implications of quickly increasing the genetic diversity within populations, contributing to the appearance of new "races" or pathotypes. With the increased movement of different mating types and pathotypes on spinach seed, as well as the rapid airborne spread of these genotypes, it is probable that the race structure no longer holds true for *P. effusa*, and thus, rather than 15 or 16 "races" of the pathogen, there are likely dozens if not hundreds more different pathotypes. It will be of interest to monitor overall changes in the genetics of the pathogen populations over time, as applied for some other *Peronospora* species [6, 15].

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, prior to this current reporting period (fall, 2016), 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. 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 have not yet been able to observe oospore germination, but this has been described elsewhere [7].

The qPCR assay was deployed as described previously [12] to detect the spinach downy mildew pathogen, *P. effusa*, and quantify the amounts of airborne inoculum (indirectly by examination of the amount of DNA detected). The levels of DNA from airborne spores of *P. effusa* were assessed near a field of susceptible plants in Salinas, CA during the winter months of 2013/14 and 2014/15 using rotating-arm impaction spore-trap samplers. Low levels of *P. effusa* DNA were detectable from December through February in both winters but increased during January in both years, in correlation with observed disease incidence; sharp peaks in *P. effusa* DNA detection were associated with the onset of disease incidence. The incidence of downy mildew in the susceptible field displayed logistic-like dynamics but with considerable interseason variation. Analysis of the area under the disease progress curves suggested that the 2013–14 epidemic was significantly more severe than the 2014–15 epidemic. Spatial analyses indicated that disease incidence was dependent within an average range of 5.6 m, approximately equivalent to the width of three planted beds in a typical production field. The spatial distribution of spores captured during an active epidemic most closely fit a power-law distribution but could also be fit with an exponential distribution. These studies revealed two important results in the epidemiology of spinach downy mildew in California.

First, they demonstrated the potential of impaction spore-trap samplers linked with a qPCR assay for indicating periods of high disease risk, as well as the detection of long-distance dispersal of *P. effusa* spores. Second, at the scale of individual crops, a high degree of spatial aggregation in disease incidence was revealed.

Regarding the biofungicide studies, it is difficult to make definitive statements about the cost-effectiveness of biofungicide use when the trial results swing between some beneficial effect and no effective control.

In summary, we have applied spore trapping and qPCR for quantification of the downy mildew pathogens of lettuce and spinach. 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 have been detected in the Salinas Valley during the growing season. Ideally, we would want to place a single spore trap on a tower or building and monitor inoculum levels and provide disease risk advisories for the entire Salinas Valley. However, our data clearly indicate there "blanket" of airborne *P. effusa* and *B. lactucae* spores generally present throughout the Salinas Valley, and complex wind patterns that preclude such large area predictions. However, there are also periods of clearly increased pathogen detection within the background blanket level of *P. effusa* at the spore trap sites. It is these pathogen detection levels within the background, coupled with disease conducive conditions that could be exploited on a more local ranch level to reduce the number of sprays required for downy mildew disease control, for both *P. effusa* and *B. lactucae*. A manuscript is currently in preparation describing a savings of 1.7 fungicide sprays for control of *B. lactucae*, over three field experiments.

However, an even more effective, accurate, and less costly approach for early pathogen detection may be the deployment of the DNA assay to detect the pathogen in the field, as latent infections in leaves. The use of this assay was demonstrated to detect *P. effusa* seven days in advance of disease symptoms in the spinach leaves. A manuscript is currently in preparation fully describing the methods and the results from three independent field trials to assess the application of this approach for early detection of infected leaves.

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