CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

Annual Report, 2013-2014

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 developed to determine the amount of *P. effusa* in air and other samples. This assay may be useful in disease forecasting. The first major objective of this research entailed quantification of airborne inoculum of *P. effusa* using the DNA-based assays already developed in the 2012-13 funding period. Additional specificity tests of the DNA-based assays against other *Peronospora* species were conducted, revealing the appropriate specificity. The second major objective entailed quantification of airborne inoculum of *P. effusa* at multiple sites in the Salinas Valley using the DNA-based assay. After initial experiments revealed specific detection of *P. effusa* at spore trap sites in the Salinas Valley, an experiment was conducted in the period of late January, 2013 to June, 2013 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. A replicate experiment is currently underway at the same sites in the time frame from late January, 2014 to June, 2014. The detection and quantification of airborne *P. effusa* from these trap sights dovetails with a third objective to evaluate the connection between spore strap data and the levels of downy mildew disease and developing an information hub for growers and PCAs. Results from the initial detection period in 2013 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. 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. A final objective entailed the analyses of seedborne transmission of spinach downy mildew. Though *P. effusa* has been routinely detected by the DNA-based assays in commercial seed lots, sexually produced oospores of the pathogen have not been observed on seeds, nor was the pathogen present in seedlings that were grown from five seed lots that tested positive for *P. effusa* with the DNA-based assay. There is currently no evidence that the pathogen is viable in spinach seed lots. Oospores characteristic of *P. effusa* were detected in leaves of one spinach sample from a greenhouse study in 2013, but they have not been identified in leaves of field samples collected in the Salinas Valley. We continue to examine field samples to determine if *P. effusa* oopores are present in field samples.

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

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OBJECTIVES:

1) Quantification of airborne inoculum of *Peronospora effusa*;
2) Quantification of airborne inoculum of *P. effusa* at multiple sites in the Salinas Valley;
3) Evaluate the connection between spore strap data and the levels of downy mildew disease;
4) Analyses of potential seedborne transmission of spinach downy mildew

PROCEDURES:

We continued to monitor the available database collections of nuclear ribosomal DNA sequences from *Peronospora species* and other oomycetes by alignment and examination of sequence differences and similarities using DNasis software (Hitachi Software). Examination of several target DNA sequences, including mitochondrial and nuclear loci, have continuously revealed the highest level of DNA sequence similarity between *P. effusa* and isolates of *Peronospora schachtii* (from Swiss chard or beet). A single nucleotide polymorphism (SNP) depicted in Figure 1 was previously identified to differentiate *P. effusa* from the closely related *P. schachtii* isolates.

The reverse primer differentiates both of these pathogens from other oomycetes and actually overlaps with the probe by a few base pairs, providing additional specificity. For further verification of the specificity of the DNA-based assay, DNA samples of downy mildew-infected plants were obtained from Monterey and Santa Cruz counties in addition to those from Dr. Marco Thines (Frankfurt, Germany) and Dr. Hermann Voglmayr (Vienna, Austria), and tested against both the *P. effusa* primer set and probe, and the *P. schachtii* (from beet or Swiss Chard) primer/probe set using qPCR.

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.* The 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. To ensure that the rods were not contaminated by residual grease and dirt, the rotating arm and grommets were washed with isopropanol and wiped with a clean paper towel at each sampling. The collected rods were stored at 4°C until DNA extraction using the Nucleospin Plant II kit (Machery Nagel) following the manufacturer's protocol for isolating genomic DNA from fungi.

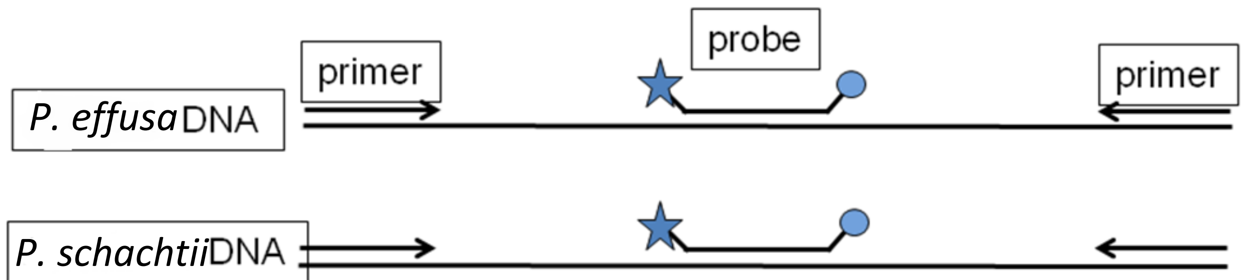

Figure 1. Illustration of the primer/probe combinations used in quantitative real-time PCR (TaqMan) assays to quantify *Peronospora effusa* and *Peronospora schachtii* (beet/chard pathogen) DNA target sequences. FAM™ and VIC® fluorescent dyes (Applied Biosystems), indicated by blue stars, were attached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

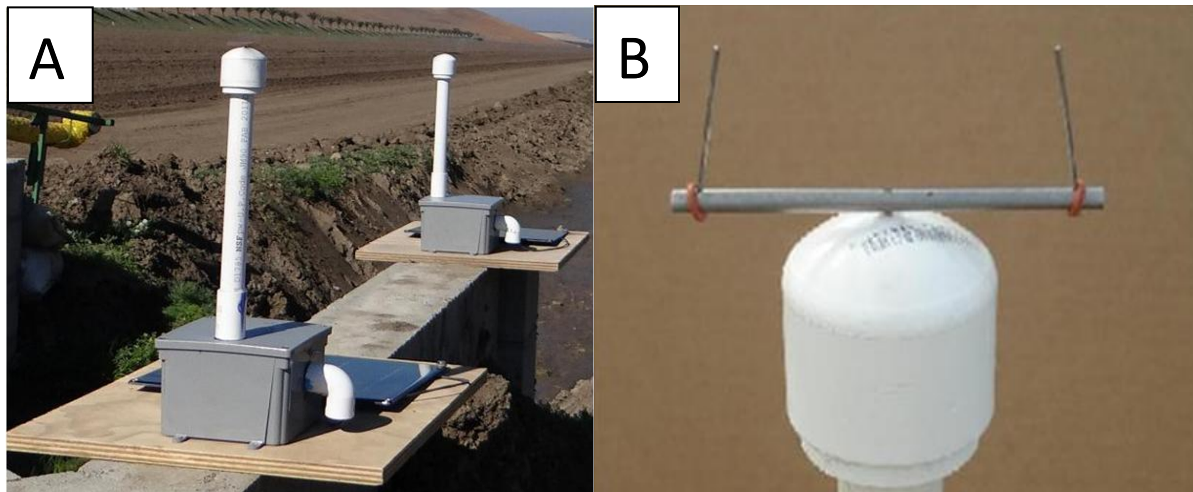

Figure 2. Spore trap system to detect *Peronospora effusa*, the causal agent of spinach downy mildew in the Salinas Valley, California. A) Two spore traps in operation south of King City, CA. B) Spore trap head with rotating arm and removable stainless steel rods.

To get an estimate of the amount of spores detected in relation to a DNA standard curve, DNA was extracted from known numbers of spores using the same procedure as for the sampling rod extraction. Spores were enumerated by dipping human hair on a spore covered leaf, counting

the spores on the hair under light microscopy and combining a clean grease-coated rod in the sampling tube for extraction. DNA target copy number was plotted relative to spore count for each sample. The trend line placed on this plot revealed values from the DNA based assay that can be correlated with spore numbers.

QPCR was carried out using dual Taqman® assays outlined in Figure 1 with a LightCycler 480 II (Roche Diagnostics). Probes labeled with FAM™ and VIC® fluorescent dyes (Applied Biosystems) were used in detection of *P. effusa* and *P. schachtii* DNA, respectively, with the appropriate filters of the LightCycler 480 II (Roche Diagnostics). The ratio of *P. effusa* to *P. schachtii* DNA amount was obtained using the method of Germer et al [7].

To analyze seeds for the presence of *P. effusa*, 1000 seeds from individual seed lots were washed with water, the debris was pelleted by gentle centrifugation for 5 min, and then the pelleted debris were analyzed under light microscopy. Spinach seed lots were obtained from sources previously described [4] and maintained at -20°C and some were ground as previously described [4].

Weather data, including solar radiation, was gathered from public weather stations nearby each trap. Logistic regression based on spore increase and decrease was used to correlate winds and relative humidity 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.

Four trials to test the effects of mixed seed lots were conducted in autumn, 2013 in collaboration with Holaday Seed Co (Salinas, CA). The varieties were chosen as economic varieties being used in the valley at the time. The idea was that by combining varieties that had different resistance packages, the pathogen would not be able to build up enough inoculum to cause economic damage; this strategy has been effective in other systems [6]. Mixture 1 consisted of 'Camaro', 'Cello', 'Silverwhale', and 'Tasman', while mixture 2 consisted of the same varieties as mixture 1 as well as 'Plover'. Disease incidence was measured as percent of diseased leaves in a 1m^2 plot.

RESULTS:

Additional DNA sequence analyses and assay testing in the 2013-14 reporting period indicate that the qPCR assay system developed is suitable for detection and quantification of *P. effusa* and *P. schachtii*. Single nucleotide polymorphisms (SNPs), or single nucleotide differences in the target sequence, were identified in the target DNA sequence to differentiate *P. effusa* from *P. schachtii* (Figure 1). However, even taking advantage of the SNP in the probe design, the primer/probe combination for *P. effusa* yielded a low detection signal for *P. schachtii* from *Beta vulgaris*-infected leaf tissue (Table 1). Therefore, dual qPCR analyses were subsequently performed on each sample to quantify the amounts of both the beet/chard and spinach downy mildew pathogens and the ratio of *P. effusa* to *P. schachtii* DNA amount was obtained using the method of Germer et al [7]. The TaqMan assays showed specificity when tested against a range of downy mildew DNA isolated from various host plants, including multiple related isolates from plant hosts commonly found in the U.S. (Table 1). Though the *Rumex acetosa*-infected tissue tested positive (Table 1), the host *Rumex acetosa* is not found in California or Arizona.

We had previously validated the use of the spore trap system shown in Figure 2 for detection and quantification of airborne inoculum of *P. effusa* in 2013 by the placement of spore traps near a downy mildew infected spinach field near Soledad, California. The quantity of pathogen detected was on average approximately 3400-fold higher near the infected Soledad field as

compared to the field near Salinas, where there was no nearby spinach field during the period of investigation (Please see results from previous 2012-13 report).

Table 1. Specificity of TaqMan assays for detection of *Peronospora effusa* and *P. schachtii* from downy mildew-infected plant leaves

Downy mildew host plant (common name)	Detection with *P. effusa* probe	Detection with *P. schachtii* probe
Spinacia oleracea (spinach)	+	+/-
Beta vulgaris subsp. *vulgaris* (beet/Swiss chard)	+/-	+
Chenopodium album (lambsquarters)	-	-
Atriplex patula (spear saltbush)	-	-
Spergula arvensis (corn spurry)	-	-
Dysphania ambrosioides (epazote)	-	-
Bassia scoparia (burningbush)	-	-
Chenopodium polyspermum (manyseed goosefoot)	-	-
Chenopodium bonus-henricus (good King Henry)	-	-
Rumex acetosa (garden sorrel)	-	+

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. 3). Rods were collected at approximately 48 to 72 hr intervals at all four sites from January 28, 2013 to June 7, 2013. Sampling, DNA extractions, and experimental analyses are currently ongoing for the second year data collection (2014), also in the period of January to June.

Figure 3. Placement of spore traps at four locations in the Salinas Valley

During the period of Mar. 15 to April 3, 2013 two additional spore traps were deployed at the north and south ends of a spinach field with a known infection of downy mildew. This field was located approximately 1 mile southwest of the original Soledad spore trap site (arrow at the Soledad site in Figure 3). A window of the *P. effusa* quantification values associated with this epidemic period is shown in Figure 4. The highest readings of pathogen copy number at the trap site associated with the epidemic occurred on Mar. 20 (Fig. 4), which also corresponded to the date with lowest level of solar radiation for the month of Mar., 2013 (Fig. 4, from Statewide IPM Program, Agriculture and Natural Resources, Univ. of California, ARYOSECO.A. weather station, near Soledad, CA).

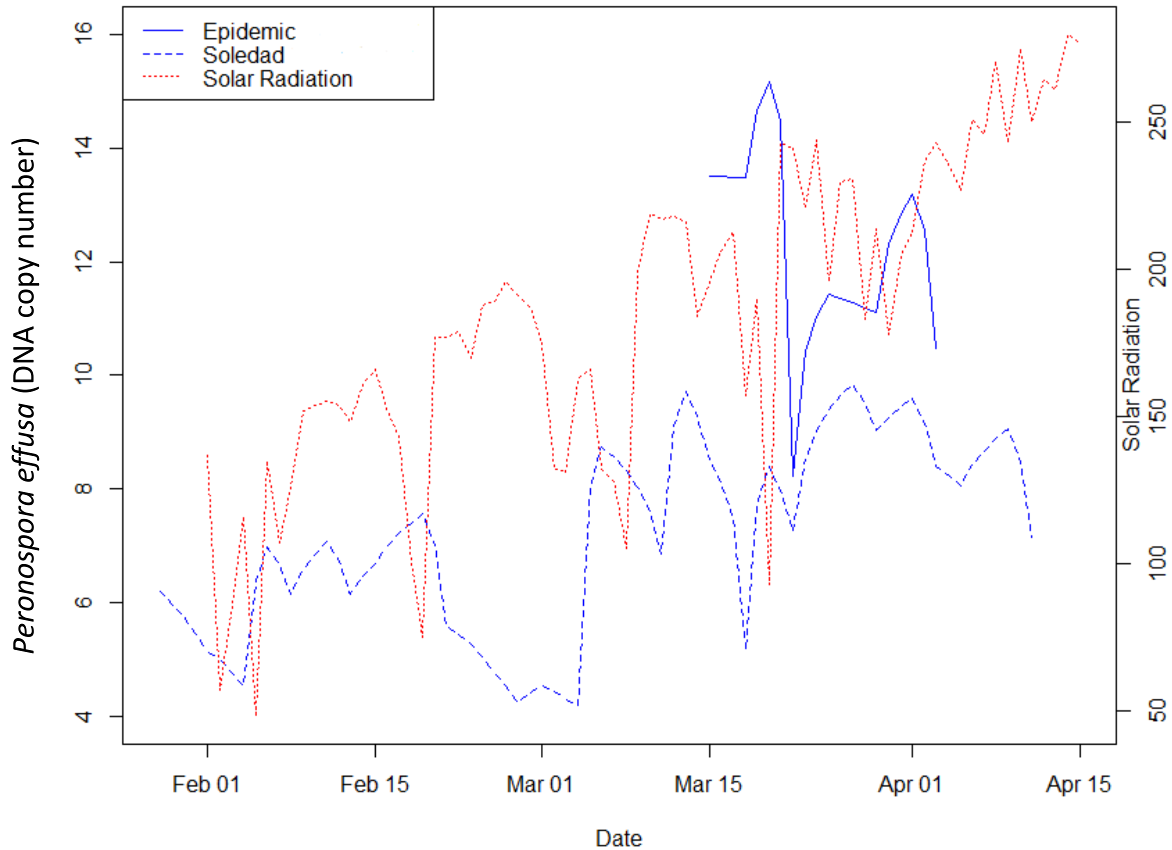

Figure 4. log copy number of *P. effusa* detected traps located next to an outbreak ("Epidemic") and away from an epidemic ("Soledad"), compared with solar radiation at the site.

The summary of the 2013 spore trap study is shown in Figure 5. Overall, pathogen concentrations seem to increase exponentially over the course of the season. Disease incidence ratings taken from near the spore traps closely match the overall amounts of pathogen detected. 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. Also, days with high temperatures $\geq 75^{o}$F were correlated with the peaks of major spikes (rapid increases of detectable pathogen DNA). These high temperature correlated peaks were followed by a subsequent drop in the amount detectable pathogen DNA for six of seven of the spikes.

An additional experimental plot at the USDA ARS station in Salinas was established in November, 2013 and monitored until February, 2014 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. This plot 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 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. DNA was extracted from the rods and extracts frozen, and these samples will be analyzed for quantification purposes and comparison for the next reporting period.

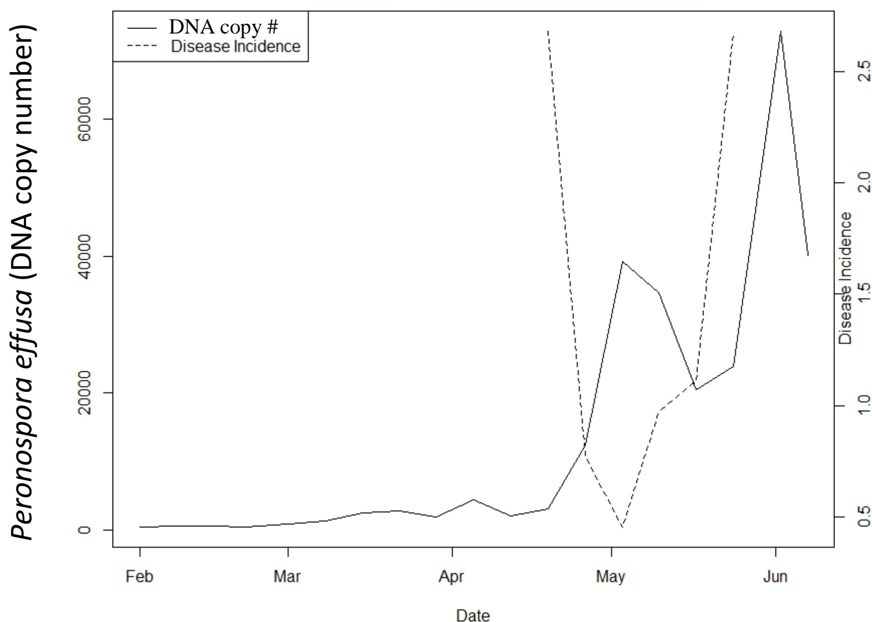

Figure 5. Weekly spore trap data of the amount of *Peronospora effusa* DNA detected and disease incidence data from the Salinas Valley in 2013.

The mixed varietal trial did not show economic value (Fig. 6). While the mixtures often performed as well as the constituent varieties, they did not reduce disease below an economic threshold. It is possible that the disease incidence results may be different at a larger scale (entire fields or regions), but without demonstrated economic control this control strategy is not likely to be adopted.

An additional objective of this research focused on determining whether *P. effusa* is present in spinach leaves as oospores and on spinach seeds. Standard PCR screening of the seed indicated that DNA from *P. effusa* was detectable on all spinach seed lots examined (18 lots total). These results were also confirmed by the analyses of DNA sequences obtained from six of the *P. effusa* PCR-positive seed lots (not shown). Furthermore, microscopy analyses of the seed surface material washed from 1000 seeds in seven seed lots revealed the presence of sporangiophores characteristic of *Peronospora* spp. in 3 of 7 seed lots examined. Oospores were not detected in the spinach seed lots examined. Oospores were detected in leaf tissue from a sample of downy mildew-infected spinach obtained from the greenhouse of Steve Koike and the downy mildew sample was originally obtained from San Benito Co. These oospores are the characteristic size (28 – 35 μm) and brownish color as those previously described elsewhere in spinach [2, 9]. Oospores have not been detected in leaf tissue collected from field samples in

California. Also, *Peronospora effusa* was not detected on cotyledons when hundreds of seedlings of each of five PCR-positive seed lots were grown out in a greenhouse test.

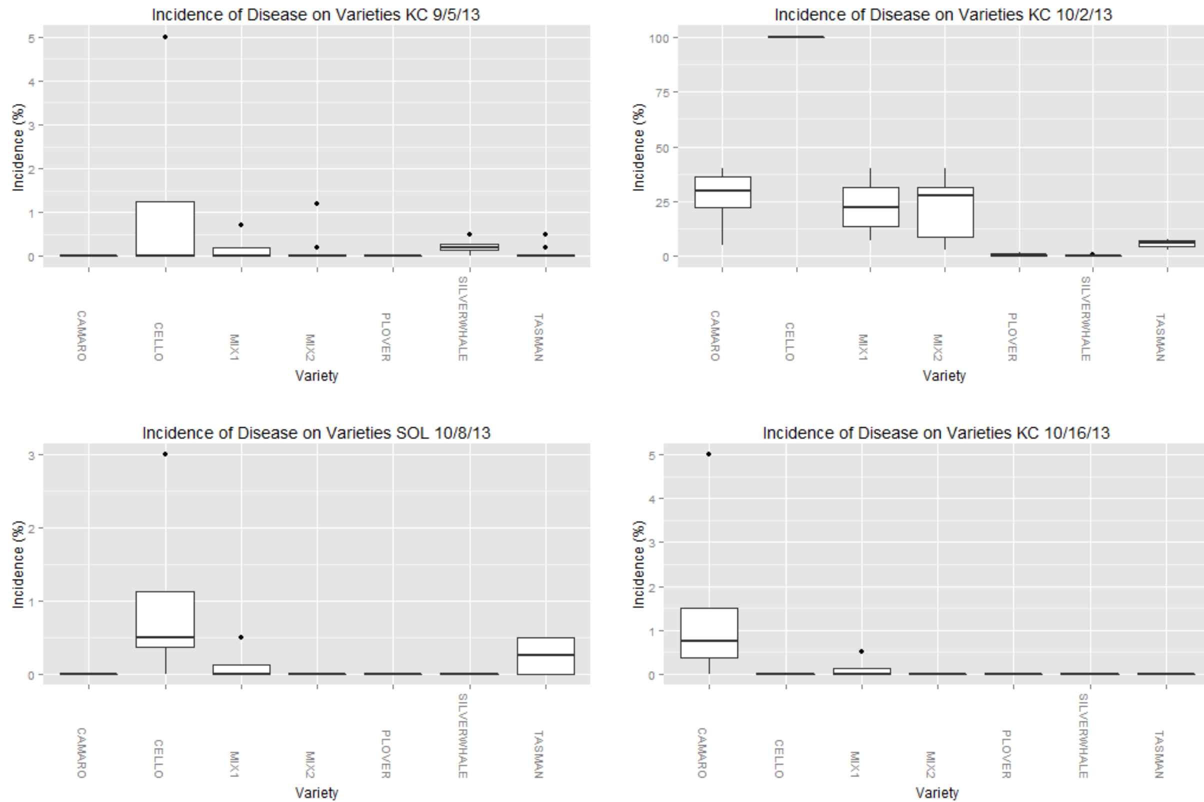

Figure 6. Disease incidence from varietal mixture trials conducted in September and October, 2013.

DISCUSSION:

The spore trap system and qPCR assay were deployed to detect both the spinach downy mildew pathogen, *P. effusa*, and the downy mildew pathogen of chard or beet, *P. schachtii* in the Salinas Valley of California. Importantly, *P. schachtii* does not infect spinach [1], 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]. Therefore, specific probes were designed for detection of each pathogen, *P. schachtii* and *P. effusa,* by taking advantage of a SNP, or single nucleotide difference, identified in the target DNA sequence. This, in combination with other DNA differences, allowed for calculation of the frequency of *P. effusa* detected in each spore trap sample.

Additional tests of the assay specificity revealed that the *P. effusa* primer/probe set was capable of amplification of *P. schachtii* DNA at a low level using DNA purified directly from spores of the pathogen, and vice versa. However, in the spore trap system, the detection levels of *P. schachtii* were negligible when using the *P. effusa* primer/probe set in qPCR of spore trap samples since there is a low quantity of DNA present. Though detection of *Peronospora rumicis*, which infects garden sorrel (*Rumex acetosa*) was clearly observed using the *P. schachtii*-specific assay this host plant has not been observed in California or Arizona and thus unlikely to interfere with the dual assay developed. Other DNA samples from related pathogens were negative for

detection with the *P. effusa*-specific assay. For example, among the DNA samples obtained from downy mildew-infected plant hosts that were tested, infected lambsquarters is commonly present around spinach production areas, but yielded negative results in the sensitive qPCR analyses.

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 generally detectable per sampling site. However, there were also periods of increased pathogen detection within the background blanket level of *P. effusa* at the spore trap sites. Moreover, analyses of detection data from near a downy mildew-infected spinach field near Soledad revealed high levels of the pathogen of > 200 spores/trap. This trend continued over multiple sampling dates within 48 or 72 hr windows of detection, providing additional evidence that the spore trap/qPCR system can be useful to quantify the pathogen in the field.

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 low solar radiation (increased cloud cover) and increased *P. effusa* detection in the Feb. to Mar., 2013 windows of data analyzed. Data analyses also suggested that the sporangia/spores of *P. effusa* are present at higher temperatures than expected, leading to *P. effusa* DNA detection in sampling conditions > 90°F south of King City. However, analyses of the 2013 data revealed that days with high temperatures $\geq 75^{o}$ F were correlated with the peaks of six of seven spikes (rapid increases in spore amounts). Following six of these spikes, there were subsequent decreases in temperature and the amount of detectable pathogen, suggesting that temperatures $\geq 75^{o}$ F have an overall adverse effect on the airborne spore concentration.

The commercial spinach seed lots were not infested with downy mildew oospores, at least in the lots analyzed from Europe or the Pacific Northwest US. However, previous studies have revealed oospores of *P. effusa* when washed off of commercial spinach seed lots and in seedlings grown from seed lots that were infested with downy mildew [9]. The potential seed infestation with downy mildew requires further assessment, although currently there is no evidence that the Peronospora sp. detected on spinach seeds is viable. The seedling grow out tests from five PCR-positive seed lots did not reveal any viable pathogen in this study. Potentially, *P. effusa* may be blown onto seed crops during production, and the sensitive PCR technique is able to detect remnant DNA.

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 the soils of previously infested spinach fields have not been fully explored. We have previously observed oospores in spinach leaves that were obtained from the greenhouse of Steve Koike (UCCE, Salinas). One of the isolates of downy mildew used in this experiment was obtained from San Benito, Co., CA., and thus oospore production in California may occur frequently. Downy mildew oospores in spinach leaves have not been previously reported in California, but were reported in spinach cotyledons in the Netherlands [5] and Japan [8]. The presence of these sexual structures is indicative of mating between different strains of *P. effusa*, as reported previously [9]. 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/or soils are important primary inoculum sources, this knowledge could lead to seed or soil treatments to reduce downy mildew on spinach.

In summary, we have applied a new tool for diagnosis and quantification of the downy mildew pathogen on spinach. This tool, along with the spore trap system, has provided insight into patterns of airborne inoculum levels of the pathogen in the Salinas Valley. Year 2 spore trap data collection from all four sites is ongoing, and will provide insight over time on pathogen inoculum levels. Tracking the levels of windborne inoculum of the pathogen is expected to yield insights on the environmental conditions that favor outbreaks of downy mildew.

Additional analyses of the spore trap data are underway in conjunction with Dr. Neil McRoberts (UC Davis) to more definitively determine any correlations between weather characteristics and the amount of pathogen DNA detected. Downy mildew isolate collection is ongoing, allowing population genetics analyses as characterized in other studies for populations of *Peronospora* sp. [3].

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