CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

Combined Annual Reports for Spinach and Lettuce Downy Mildew projects, 2015-2016

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

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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*), respectively and are widespread and very destructive diseases on both crops in California. To assess the factors required for disease outbreaks on both spinach and lettuce, and also to identify potential sources of the pathogens, DNA-based assays were developed for *P. effusa* and *B. lactucae*. The assays were deployed to quantify the levels of airborne inoculum from spore traps, and may be useful in disease forecasting. The first major objective of the *P. effusa* research for this period entailed analyses of the role of seedborne *P. effusa* in transmitting the pathogen. The sexually produced oospores of *P. effusa* were detected in 17 out of 88 commercial seed lots in total by seed wash-off and microscopy, and determined viable in the five of the seed lots examined. Another major objective entailed detection of early (presymptomatic) infection and colonization of spinach leaves. In all the three overwintering spinach plots established from November through February, the disease developed in January of 2013, 14 and 15. The results indicate that *P. effusa* could be detected in the leaves at least a week prior to leaf symptom development. Additional weather data were collected. Together, these data were analyzed with 2015 data and used to carry out the objective to evaluate the connection between spore trap data and the levels of downy mildew disease. Increases in detected DNA of *P. effusa* throughout the Salinas Valley were correlated with increasing disease incidence in the field and decreasing temperatures and higher wind speeds. Tracking the levels of windborne inoculum of the pathogen and in-field leaf detection applications can be valuable to time spray applications for disease control, and in the case of spinach, to assess whether a field is infected prior to visual symptoms (for early harvest before loss) or prior to the entire field becoming infected and symptomatic. 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, revealing that the assay is specific and sensitive for *B. lactucae* detection, and a useful tool for forecasting lettuce downy mildew.

PROJECT TITLES: DNA-based detection and quantification of the downy mildew pathogen, *Peronospora effusa* and Biology and epidemiology of downy mildew of lettuce.

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OBJECTIVES (downy mildew on spinach):
1) Analyses of the potential role of seedborne *P. effusa* in transmitting the disease.
2) Detection of presymptomatic infections and colonization of spinach leaves.
3) Evaluate the connection between spore trap data and the levels of downy mildew in spinach.

OBJECTIVES (downy mildew on lettuce):
1) Develop qPCR for specific detection of *Bremia lactucae* and deploy for environmental monitoring of inoculum levels.
2) Screen protectant and systemic (if available) fungicides registered to identify those efficacious against lettuce downy mildew during fall in coastal fields.

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.

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.

The comprehensive manuscript describing all of these procedures handling seeds for detection of *P. effusa* was published in 2016 [12].

Quantitative PCR for quantification of *P. effusa* DNA was carried out using the TaqMan assay developed previously [10], and a new TaqMan assay was developed and published for detection of *B. lactucae* based on mitochondrial DNA sequences unique to the species [11]. We continued 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.

Spore traps (Figure 1) were sampled three times weekly (at approximately 48 or 72 hr intervals) for windborne inoculum of *P. effusa* or *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°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] and [11].

Experimental plots at the USDA ARS station in Salinas were established in each of the overwintering periods of November until February, in 2013-2014, 2014-2015, 2015-2016 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.

For the *B. lactucae* experiments, DNA extractions from impaction spore samplers were conducted as described previously [10]. For this study, spore traps were placed at three separate

sites (King City, Castroville, and USDA-ARS Salinas). Two spore traps were set in each of the four locations near spinach and lettuce fields from April through July 2015, near King City, CA. These samples were collected on Monday and Friday of every week. Another set of two samplers was placed in Castroville, CA, next to a commercial lettuce field, and sampled from August through September 2015. Also, four samplers were set up each winter season in 2013-14 and 2014-15 at a USDA-ARS plot maintained at the Salinas, CA location, designated as USDA 2013-14 and USDA 2014-15, and spanned the lettuce-free period (December 7 to December 21). The spore samples from the Castroville and USDA 2013-14 and USDA 2014-15 locations were collected three times a week (Monday, Wednesday and Friday).

All of the qPCR experiments for *B. lactucae* were performed in 384 well plates using a LightCycler 480 II (Roche Diagnostics, Basel, Switzerland) and the *B. lactucae*-specific and sensitive TaqMan assay described in reference #11. The qPCRs were run in triplicate using one DNA extraction per spore trap. The qPCR reaction volume of 12 µl contained 200 nM probe, 200 nM each primer, 1x real master mix (5 PRIME®, Hilden, Germany), and one µl of DNA. Depending on quantities available, we used 500, 5, or 2 pg of DNA for specificity tests. The LightCycler 480 II with LightCycler 480 software (release 1.5.0) and the Absolute Quant/Fit Point analysis was used to conduct the assays with a reaction profile of 10 min at 95ºC initial denaturation followed by 55 cycles of 95ºC for 10 s and 56ºC for 30 s.

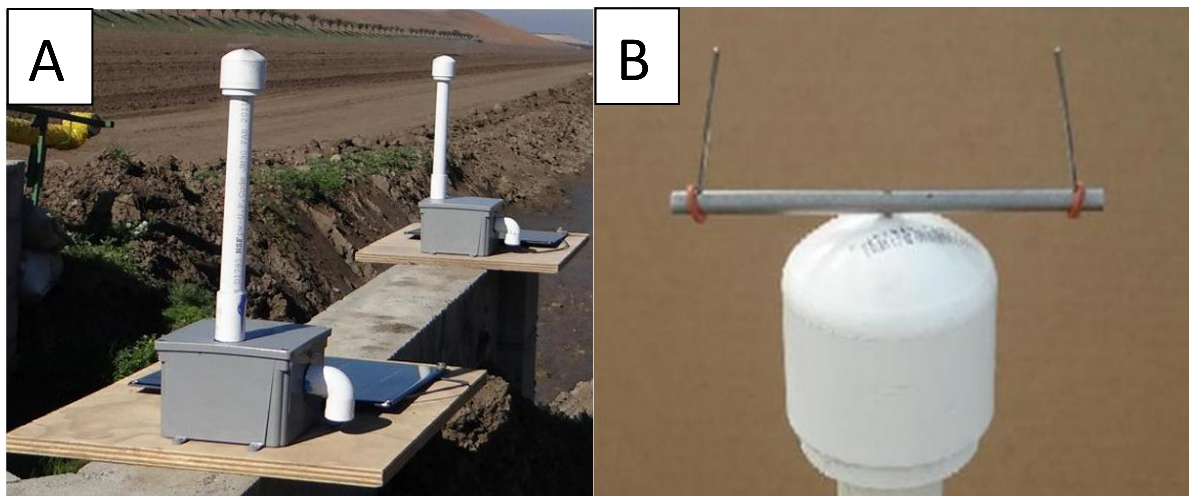

Figure 1. Spore trap system to detect *Peronospora effusa*, the causal agent of spinach downy mildew in the Salinas Valley, California. A) Two spore traps in operation south of King City, CA. B) Spore trap head with rotating arm and removable stainless steel rods.

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 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 1m^2 plot. Fields were monitored using a cluster sampling method. Large fields were measured multiple times.

We conducted two fungicide 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:

Spinach downy mildew

For the 82 seed lots were examined by microscopy, 13 seed lots were positive for one or more oospores like the one shown in Fig. 2C (16% of the total). An additional 7 seed lots were tested after the publication of Kunjeti et al. [12], and four of these were also positive for oospores. Thus 17/89 seed lots tested in total were positive for oospores. 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* [12]. Five of the seed lots contained a high abundance of oospores [12], and hence the oospores derived from them were used in additional viability experiments (Fig. 3). Figure 3 indicates an intact cell membrane which shrinks in response to 4 M sodium chloride, and the response was the same across a time course ranging from 15 min to 24 h [12]. The technique has been used to demonstrate oospore viability for other oomycetes [6]. 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 2C.

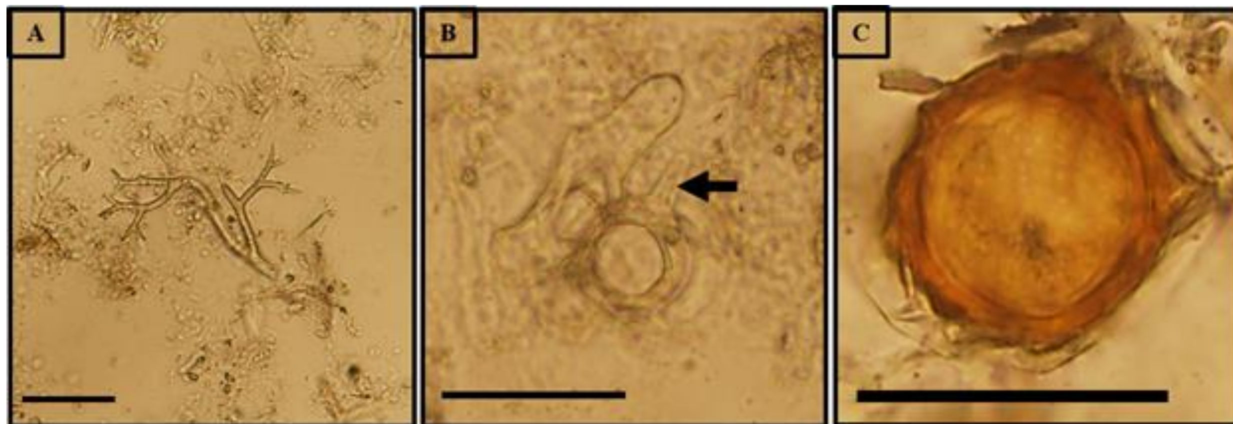

Figure 2. Different stages of *Peronospora effusa* detected in spinach seed lots. A) Sporangiophores, B) Immature oospore showing the oogonia with tapered base (arrow), and C) mature oospore. Scale bars = 50 µm. The figure is from Kunjeti et al. 2016 [12].

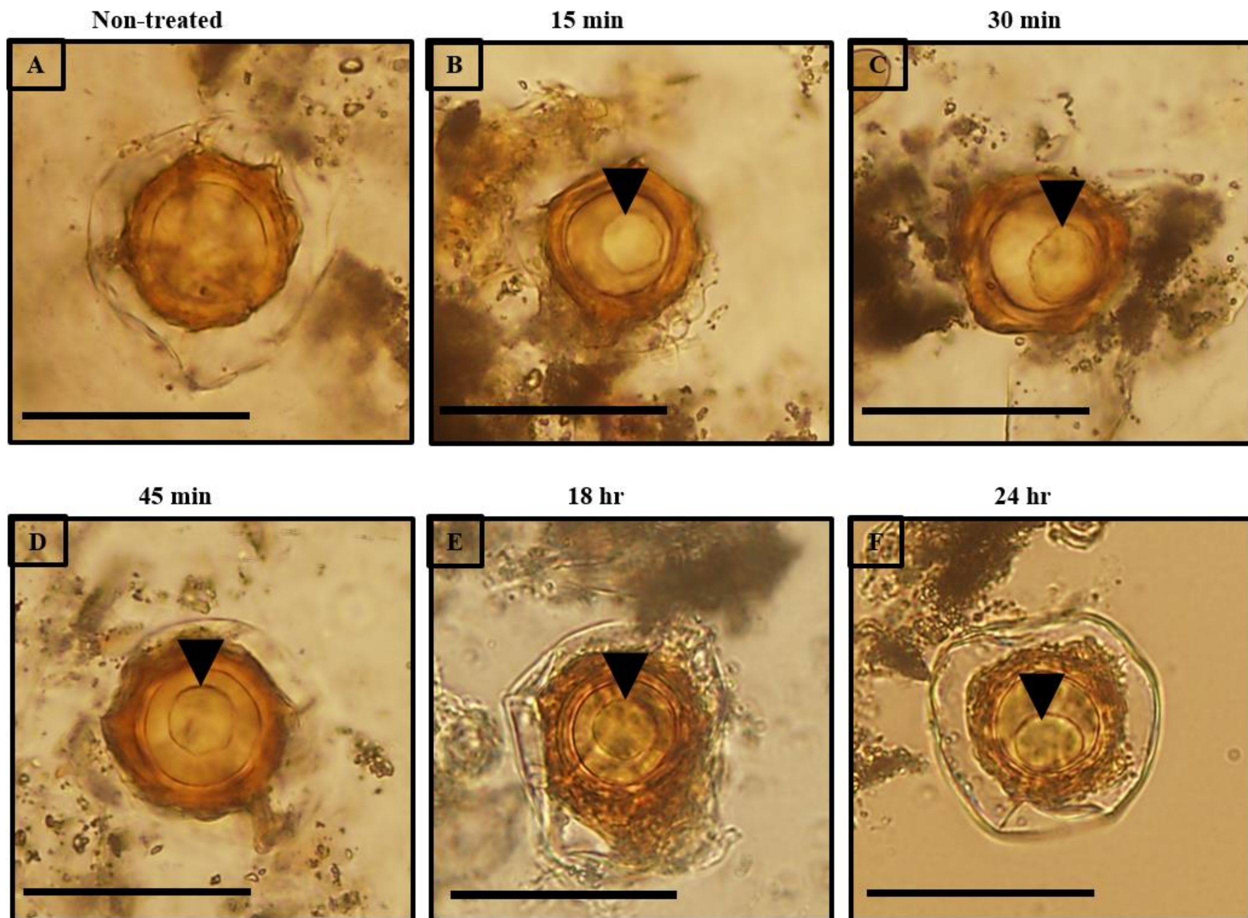

Figure 3. Effect of 4 M sodium chloride on oospores derived from spinach seed lots at different time points. A) Not treated, B) 15 min, C) 30 min, D) 45 min, E) 18 h, and F) 24 h. Scale bars = 50 µm. The figure is from Kunjeti et al. (2016), reference #12.

Sporangiophores characteristic of *Peronospora* were observed in another six different lots in total (Figure 2A).

The result from plasmolysis testing (Fig. 3) indicates the presence of viable oospores washed from five of the 82 spinach seed lots examined [12]. The tight ball formed in the central oospore cavity (arrow heads in figure 3) when treated with 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. All of these findings were published in detail 2016 in Plant Disease [12].

An experimental plot was 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. This plot site served dual roles for testing the spore trap system in the first two periods and for an experiment devoted to early or latent infections of leaves, prior to symptom development.

Figures 4 and 5 show one experimental set up and the results for the analysis of PCR amplification of *P. effusa* from presymptomatic and clearly infected spinach leaves. The plot shown in figure 4 was examined January 4, 2016, and pathogen and symptoms were first

observed January 8, 2016. Leaf symptoms were noted in the left-most row of the plot shown in figure 4, corresponding to leaf samples # 37 to 48. Note that most leaf samples from # 29 through 48 were positive, 16 of them, for *P. effusa* DNA amplification (Fig. 5A). These were positive for *P. effusa* at least one week prior to the observation of first symptoms.

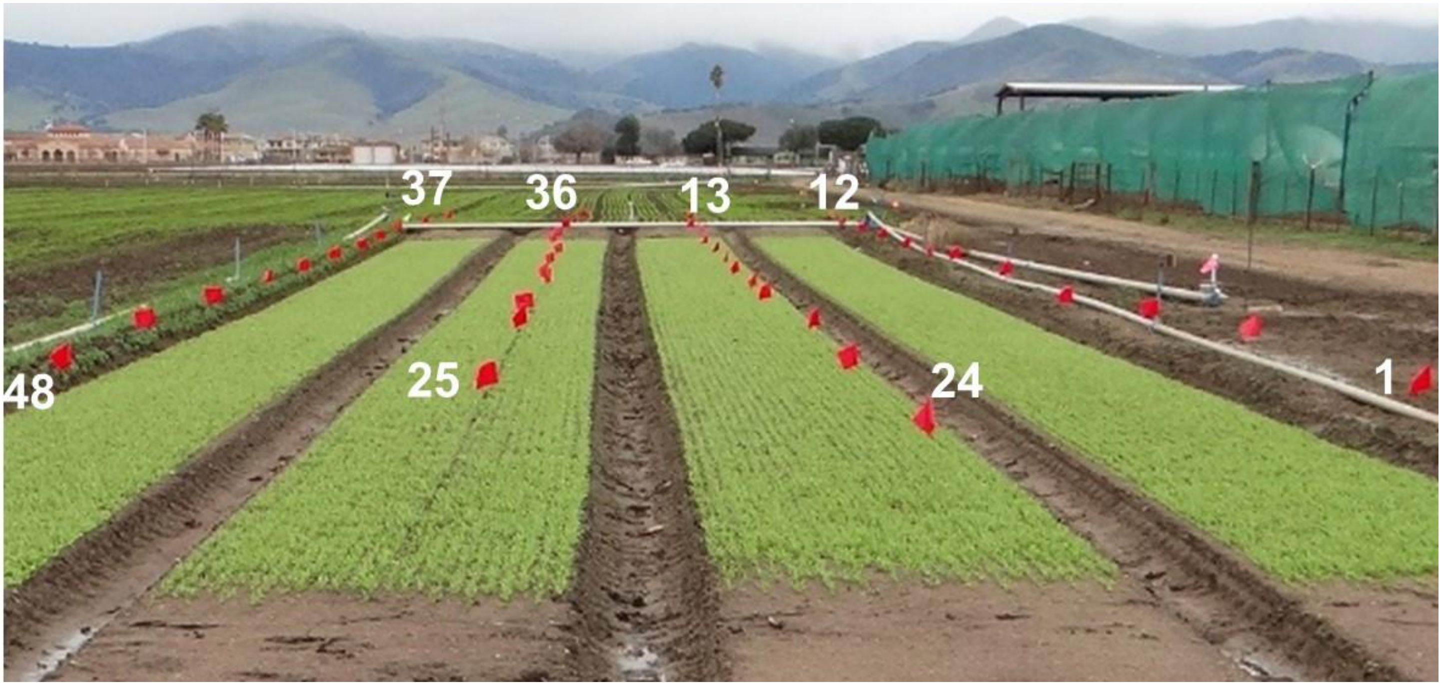

Figure 4. Small spinach plot of beds of cultivar Viroflay, maintained in November, 2015 to January, 2016 to examine early infections with *P. effusa*, prior to symptom development on leaves. The flags are spaced 10 ft apart within rows and mark the approximate location where leaves were pulled for all 48 samples each week for DNA analysis by PCR. The numbers at the end of each row bracket the all the numbered flags in a given row and hence the row 1 on the right corresponds to samples 1-12, and so on, in figure 5.

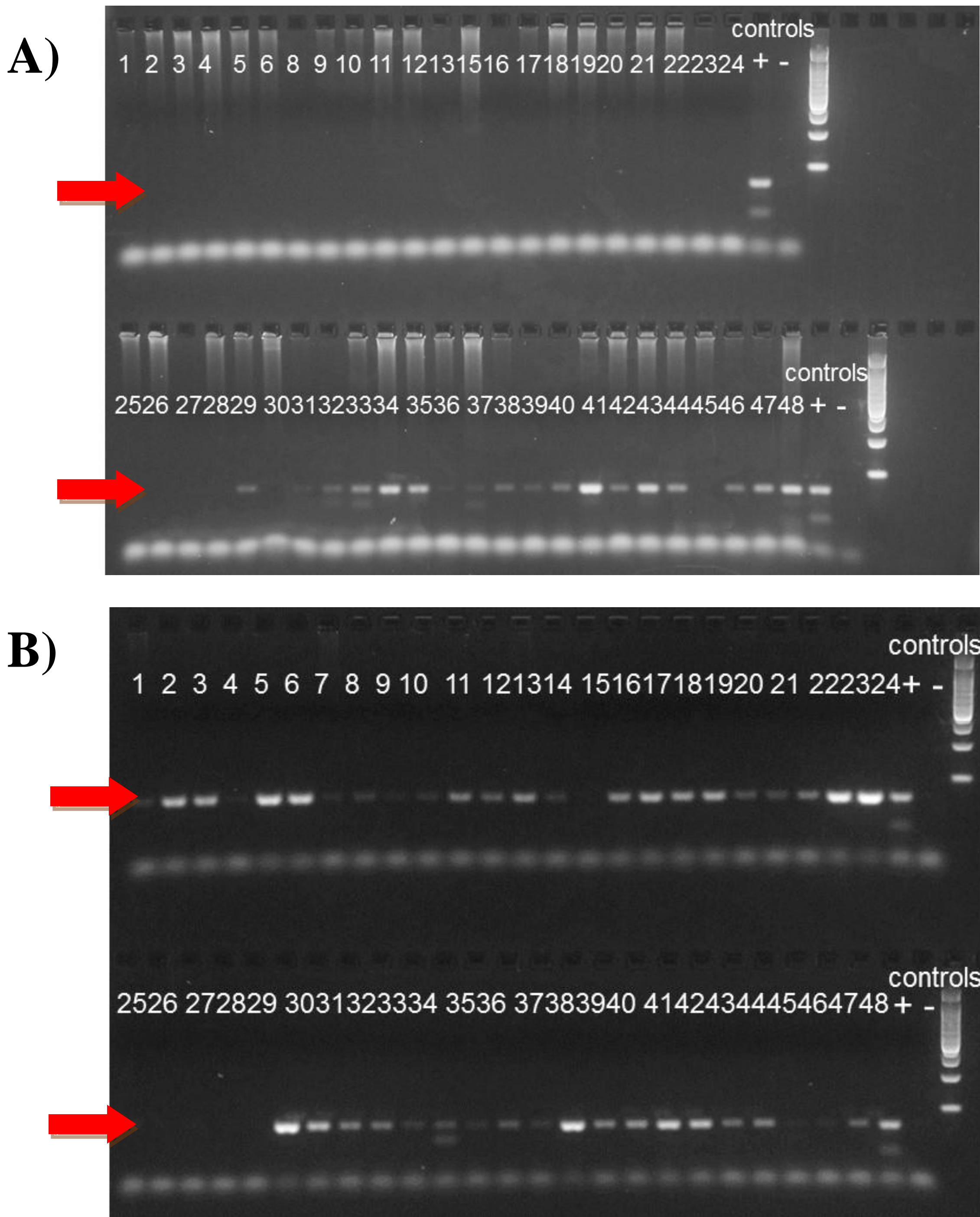

Figure 5. PCR amplification of spinach downy mildew DNA from infected leaves prior to appearance of plant symptoms. The arrow indicates the position of the PCR positive band and the positive and negative controls are PCRs conducted with infected leaves (+) or non-infected leaves (-). **A)** December 28, 2015 sampling date (five weeks after planting). DNA extractions of samples 7 and 14 yielded no usable DNA for this test and were not included. **B)** January 15, 2016 sampling date.

Analysis of the temporal dynamics of air borne inoculum and its relation to spinach downy mildew levels is now complete. We have determined that the annual pattern for the disease is an exponential increase in the level of pathogen inoculum over the growing season from February through June. The rate of increase varies from year to year depending on weather conditions, but the overall pattern is qualitatively the same, resulting in a steadily increasing risk of disease as the season progresses. Superimposed on the upward trend in inoculum in both seasons, there were periodic oscillations in the airborne inoculum level; the oscillations were approximately on cycle lengths corresponding to whole multiples of the combined length – 15 days - of the infection and latent periods of the pathogen (see Figure 6).

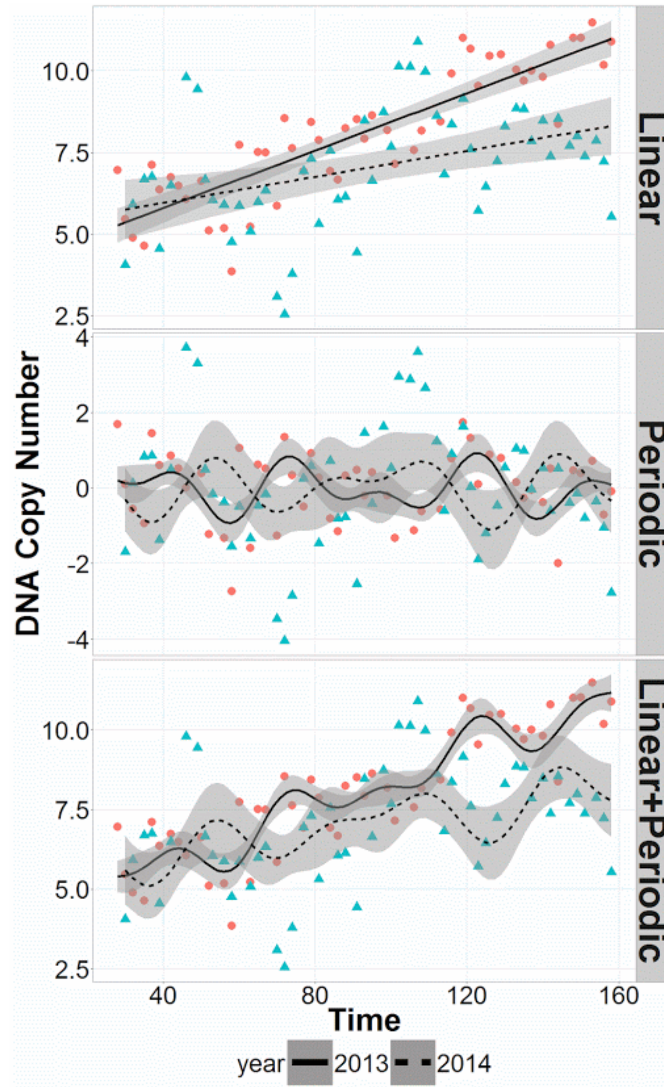

Figure 6. Airborne inoculum levels for *Peronospora effusa* in two seasons.

Two papers based on the spore trapping work for *P. effusa* were submitted to Phytopathology in this current reporting period. To date one has been accepted and revised, and the second is awaiting editorial decision. We are currently working to combine information from the spore trapping work with data from crop scouting to produce a web-based interface for displaying downy mildew risk.

***Peronospora effusa* race succession**

Using data collected by Dr Steve Koike and Dr Jim Correll on downy mildew off-types we have been able to develop an analysis of the rate of virulence race turnover in the spinach downy mildew pathogen. We used the record sheets produced when off-types are tested against the differential series of spinach varieties in Salinas by Dr Koike to look at the diversity of the samples submitted for analysis. The records available covered the period from 2004 to 2014. Over the same period we can track the total number of known mildew races and the number of races observed in the Salinas region each year. These two numbers allow us to calculate two different upper limits on the level of possible diversity against which we can benchmark the observed diversity.

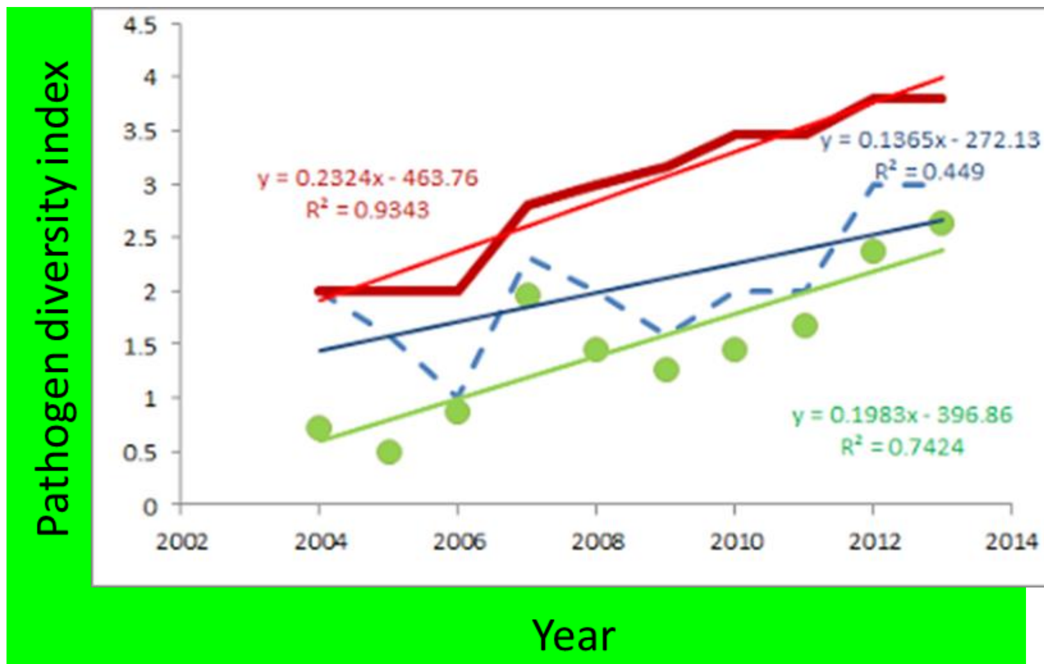

Figure 7: Diversity in *Peronospora effusa* virulence type diversity over 10 years. The green points are the observed levels of diversity. The blue dashed line is the maximum possible diversity in each year given the observed number of virulence types observed. The thick red line is the maximum possible diversity given the known number of virulence types, globally, up to the year in question. The thin lines in each case are the linear trend for the data.

Lettuce downy mildew

Detection and quantification of airborne *B. lactucae* near lettuce crops provides an estimation of the inoculum load. We developed a qPCR-based assay using a target sequence in mitochondrial DNA for specific detection of *B. lactucae* [11]. Validation using amplicon sequencing of DNA from 83 geographically diverse isolates, representing 14 *Bremia* spp., confirmed that the primers developed for the TaqMan assays are species-specific and only amplify template from *B. lactucae* [11]. DNA from a single sporangium could be detected at a Cq value of 32, and Cq values >35 were considered as nonspecific. Some of these values, depicted as mean Cq are shown in figure 6, and derived from reference #11. For this study, the

assay was deployed using spore traps in the Salinas Valley, CA. The deployment of this sensitive *B. lactucae*-specific assay resulted in the detection of the pathogen during the two-week lettuce-free period as well as during the cropping season (Fig. 6), although the levels were higher (lower Cq) in 2014-2015, presumably because of rainfall in this period, and not in the same period in 2013-14. Based on our standard of detection limits, most of the samples were considered negative for detection in the 2013-2014 period. Additional experiments were conducted near lettuce fields near King City and Castroville, revealing much higher levels of pathogen detection (lower Cq values) (Fig. 8). These results demonstrate that this assay will be useful for quantifying inoculum load in and around the lettuce fields and facilitating the timing of fungicide applications. Since no disease developed in the fungicide trial conducted in fall 2015, timing of fungicides based on the spore trap data could not be validated nor the efficacy of the different fungicide treatment combinations be tested.

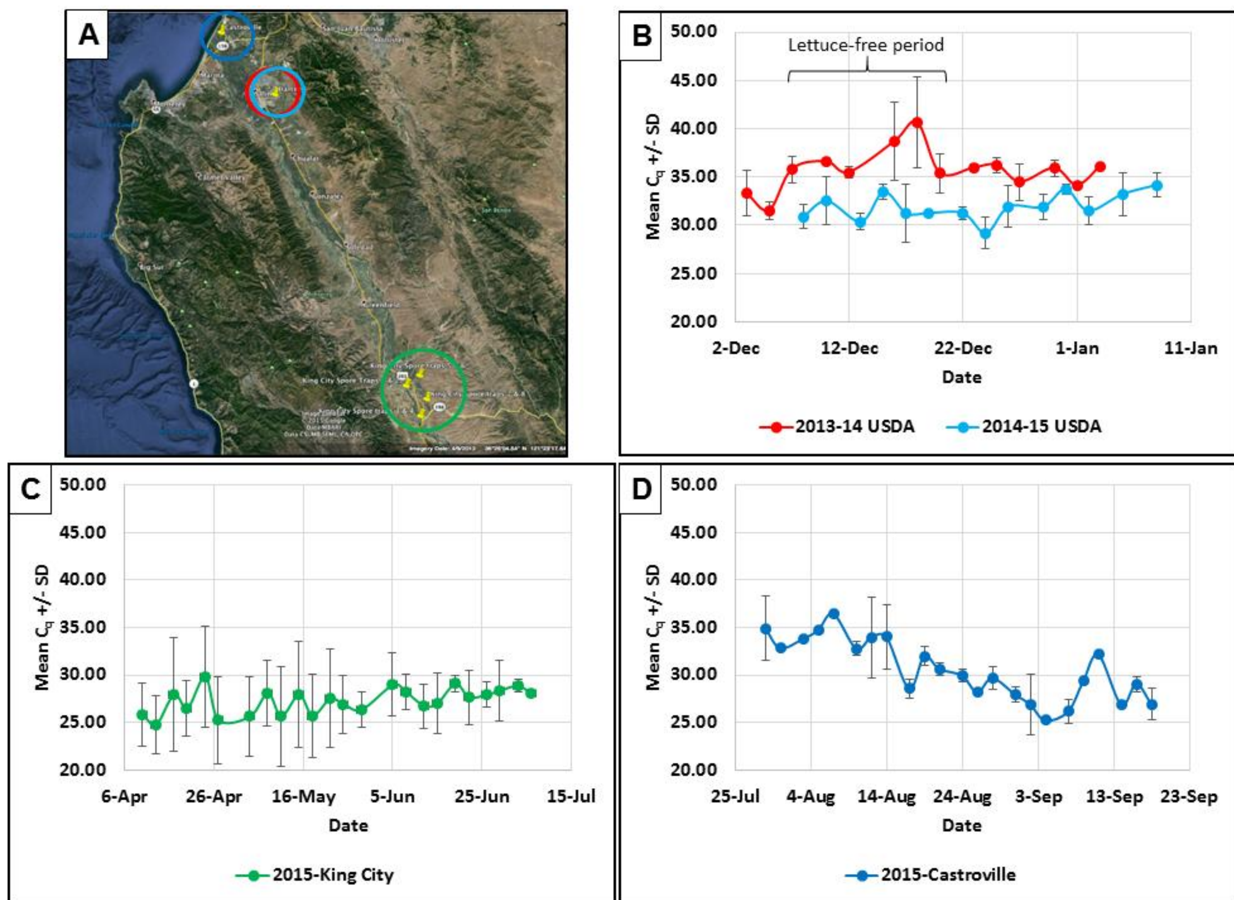

Figure 8. Relative frequency of target amplification of *Bremia lactucae* from impaction spore traps at three locations in the Salinas Valley, CA. A) Map showing spore trap locations; green, 2015-King City; dark blue, 2015-Castroville; red (2013-14 USDA) and light blue (2014-15 USDA) circles. B) The dots indicate the relative frequency of B. lactucae amplification from spore traps at USDA (red and light blue dots) locations; C) at King City (green dots); D) at Castroville (dark blue dots). Error bars represent standard deviation from eight (2015-King City), and four (2015-Castroville and 2013-14, 2014-15 USDA) spore traps.

DISCUSSION:

We published the work on the finding of oospores in modern spinach seed lots, and examined seven additional seed lots for oospores in addition to the 82 characterized in the publication [12]. 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 in 5/6 cultivars tested [8]. This current report further documents the finding of oospores characteristic of *P. effusa* in about 19% total (17/89) of the modern spinach seed lot samples examined within the past couple years in our laboratory (Klosterman). 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 [12]. 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 was published, 2016 [12].

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.

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.

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]. 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. We have the new materials in place to begin testing the new TaqMan assay for *P. effusa* detection.

Lettuce downy mildew has been managed with a combination of host resistance and fungicide applications with success over the years. Fungicide applications are routinely made under the assumption that inoculum is always present during favorable environmental conditions.

This approach often leads to fungicide resistance in *B. lactucae* populations. We have developed a highly specific and sensitive mitochondrial DNA-based assay for *B. lactucae*, and published this field-validated technique recently [11]. This assay is proving valuable currently to assess the amount of airborne inoculum available, and using this knowledge to try to time fungicide applications with Steve Koike in commercial field plots (unpublished). We currently have spore traps in the field, and will be conducting two field trials in succession to try to time fungicide applications based upon the amount of *B. lactucae* inoculum detected.

We have also gained some valuable epidemiological insight on the distribution of *P. effusa* in the Salinas Valley, CA. Previous analyses of the connection between different environmental conditions, inoculum levels and subsequent disease incidence have largely confirmed what was suspected for the spinach downy mildew pathogen. Given an overall conducive environment for disease development, slightly increased risk of disease is predicted by warmer temperatures, higher humidity and low wind speed. Spore trap *P. effusa* DNA copy numbers higher than e^8 indicate an increased risk of disease 10-12 days later.

We are also currently reaching out to industry like Trace Genomics, to turn the technologies over to a private industry that will be able to run spore trap samples and quick leaf detection assays for *P. effusa*. We will make a more of an effort to do this in the current funding period, as indicated in 2016-2017 downy mildew proposal.

In summary, the data indicate that the sexual oospores of *P. effusa* appear rather common in commercial spinach seed lots (17/89 total), and that they are viable. Additional experiments to determine the survival and germination of the oospores derived from seed will be undertaken in the future, as well as examination of seed treatments to kill them. 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, and we anticipate turning over this technology to the private industry and helping to deploy it for downy mildew detection for both lettuce and spinach.

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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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