CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

Combined Annual Reports for Spinach and Lettuce Downy Mildew projects, 2017-2018

Risk assessment, early detection, and control of downy mildew of lettuce and spinach

Steven J. Klosterman
USDA-ARS, Salinas, CA

Alexander I. Putman
University of California, Riverside, CA

Krishna V. Subbarao
University of California Davis, Salinas, CA

ABSTRACT

Downy mildew on spinach and lettuce are caused by the obligate oomycete pathogens *Peronospora effusa* (*P. effusa*), and *Bremia lactucae* (*B. lactucae*), respectively. Downy mildews are widespread and very destructive diseases on spinach and lettuce in California. Sporangia of both pathogens can be airborne, and may also initiate disease from seed or soilborne oospores. DNA-based detection assays were previously deployed to quantify the levels of airborne inoculum of *B. lactucae* from spore traps, which may be useful in disease forecasting. In this reporting period, we noted a correlation between the levels of detectable DNA of *B. lactucae* and increasing wind speeds and temperatures that occurs in late morning hours. However, because of the low number of spore trap samples placed during the period, statistical analyses revealed that the correlations observed were not significant. A major objective of the *P. effusa* research entailed examination of oospore production, survival, germination and conditions necessary for reproducible infection of spinach. During this period, we demonstrated that the sexually-produced *P. effusa* oospores germinate, from fresh leaf samples or those obtained from seeds. Germination of the oospores of *P. effusa* had not been reported in the literature for 100 years. We did not have an adequate stand of spinach in the desert to evaluate oospore production in that area. Using newly available chambers and humidity tents, we obtained reproducible infections of *P. effusa*. We previously determined that *P. effusa* could be detected in leaves in field plots, at least a week prior to leaf symptom development. We supplied material to a local biotech company to begin to explore commercializing leaf detection of *P. effusa* as a single nucleotide polymorphism assay. We tested biofungicides in an effort to reduce symptoms and sporulation of *P. effusa*. In the first experiment, none of the eleven treatments showed significant reductions of the disease relative to the untreated control. In summary, tracking the levels of windborne inoculum of the pathogen and in-field leaf detection applications may be valuable to inform efficient spray applications for disease control, and in the case of spinach, to assess whether a field is infected (for early harvest before loss) or prior to the entire field becoming infected and symptomatic. *P. effusa* is detected in nearly 21% of commercial seed lots in the form of sexually-produced oospores. Treatments that eliminate *P. effusa* on spinach seed may limit some outbreaks, especially for organic spinach.

PROJECT TITLE: Risk assessment, early detection, and control of downy mildew of lettuce and spinach.

INVESTIGATORS:

Steven J. Klosterman
USDA-ARS
1636 E. Alisal St
Salinas, CA 93905

Alexander I. Putman
Dept. of Plant Pathology
University of California, Riverside
Riverside, CA

Krishna V. Subbarao
Dept. of Plant Pathology
University of California, Davis,
c/o USDA ARS Station
1636 E. Alisal St
Salinas, CA 93905

COOPERATING PERSONNEL:

Steven T. Koike
Trical Diagnostics, Hollister, CA

Shyam Kandel, Frank Martin, Beiquan Mou
USDA-ARS, Salinas, CA

Allen Van Deynze, Mychele Batista Da Silva, Charlie Brummer, Juliana Osorio Marin, Bullo Mamo, Nikhilesh Dhar
University of California, Davis

OBJECTIVES (downy mildew on lettuce):

1) Combine the information from spore trapping at a local ranch level from lettuce and spinach fields with weather data to determine local conditions and airborne inoculum load favorable for downy mildew to time fungicide application in a forecast system

OBJECTIVES (downy mildew on spinach):

1) Examine oospore production, survival, germination and conditions necessary for reproducible infection of spinach.
2) Test biopesticides to reduce sporulation of *P. effusa* on organic spinach
3) Analyze early infection in spinach leaves (pre-symptomatic) for assessing its utility as a downy mildew disease warning for organic spinach and work with a local company to commercialize the assay.

PROCEDURES:

Data were collected for detection of *Bremia lactucae* in commercial lettuce fields near Salinas, CA in three experimental plots. Solar/battery-powered impaction spore traps (Figure 1) obtained from Dr. Walt Mahaffee (USDA ARS, Corvalis, OR) were sampled for windborne inoculum of *B. lactucae* at each location. The pairs of 1.1 mm x 40 mm stainless steel rods (Figure 1B) coated in silicone vacuum grease (Dow Corning) were collected at the 72 or 96 hour time intervals. The collected rods (with the downy mildew spores) 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. Quantitative PCR and the estimates of spore load were based on the DNA level detected was determined as described [12] and [13].

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 six time frames throughout the day. Summary data from weather variables such as temperature, windspeed, and relative humidity were directly correlated with spore load (indirectly determined by pathogen DNA amounts detected). Lettuce fields near trap sites were monitored for disease incidence. Disease incidence was measured as percent of diseased plants in each experimental plot within the field.

Correlation analysis was conducted and shown for experiment one to determine the possible relationship between weather conditions (variables such as temperature, wind speed and relative humidity) and the detectable DNA from the airborne sporangia (i.e., the Cq values from the qPCR) using statistical analysis software (R software). Experiment two was not included in correlation analysis as only a few Cq data points were available.

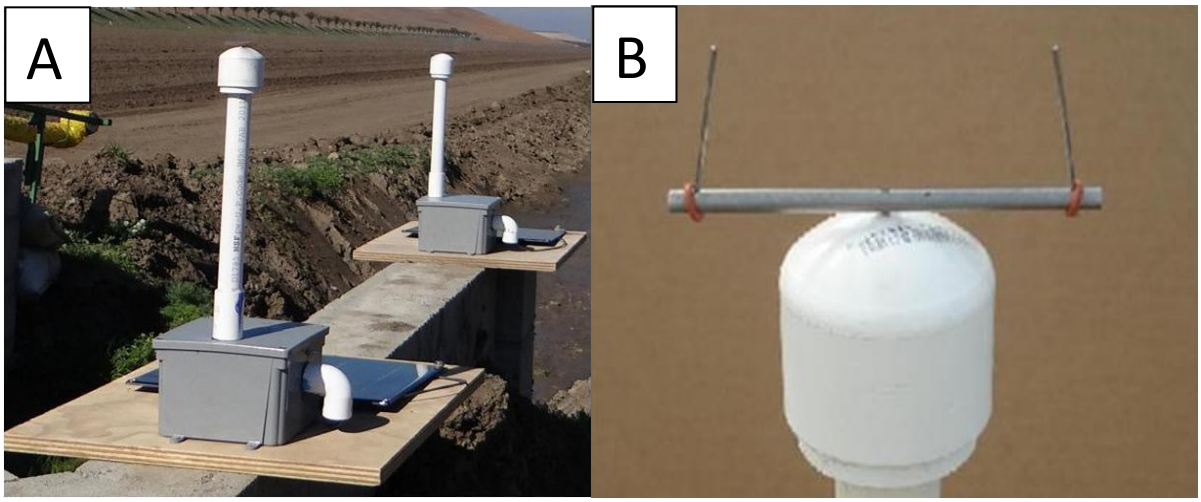

Figure 1. Spore trap system to detect *Bremia lactucae* and *Peronospora effusa*, the causal agents of lettuce and spinach downy mildew, respectively. **A)** Two spore traps in operation south of King City, CA. **B)** Spore trap head with rotating arm and removable stainless steel rods.

Biofungicide testing was conducted using experimental plots of spinach cultivar Viroflay planted at the USDA ARS station in Salinas in the summer and late fall of 2017. These plots in each season consisted of twelve 80" beds x 120 ft in length. The biofungicide treatments (Table 1) were applied by manual backpack sprays to the foliage and the spinach was monitored for disease incidence. The experiment was performed at the research field of USDA-ARS in Salinas, California. Spinach plants received brief overhead irrigation two evenings per week to maintain

the high humidity in the plant canopy. Watering was timed around biofungicide applications so as not to wash them off. In addition to the untreated control, the eleven treatments included biofungicide combinations with wetting agents, the conventional fungicide-Aliette, and an untreated control, and applied at similar or different schedules. These treatments were randomly assigned with four replications. The fungicide dose, equipment calibration, and fungicide spray schedule was maintained according to the agreement with IR-4 Project management. The application of fungicides was initiated 20 days after seed sowing and continued for six weeks. The downy mildew incidence was monitored twice; 22 and 32 days after seed sowing, respectively. Four samplings were done from each subplot containing the individual treatments, on observations of spinach plants in a 1 ft^2 area.

Table 1. Treatments and spray schedule used to test the biofungicides for the control of spinach downy mildew in the study.

Treatment designation	**Treatment details**	**Spray schedule (applied on the days listed, beginning 20 days after sowing)**
A=T1	Untreated control	
B=T2	MBI-110	0, 7, 14, 21, 28 and 35
C=T3	Oxidate 2.0	0, 7, 14, 21, 28 and 35
	TerraGrow	0, 7, 14, 21, 28 and 35
D=T4	OS0 5%	0, 7, 14, 21, 28 and 35
	Cueva alone	4, 10, 17, 24 and 31
E=T5	Oxidate 2.0	0, 7, 14, 21, 28 and 35
F=T6	LifeGuard	0, 7, 14, 21, 28 and 35
G=T7	Zonix	0, 7, 14, 21, 28 and 35
H=T8	Oxidate 2.0	0, 7, 14, 21, 28 and 35
	Zonix	0, 7, 14, 21, 28 and 35
I=T9	Lifeguard	0, 7, 14, 21, 28 and 35
	Cueva alone	4, 10, 17, 24 and 31
J=T10	Procidic	0, 7, 14, 21, 28 and 35
K=T11	Sil-Matrix	0, 7, 14, 21, 28 and 35
L=T12	Aliette	0, 7, 14, 21, 28 and 35

We (USDA, Salinas) recently acquired a dew chamber and purchased a wall-mounted air conditioner. These items were installed in a room dedicated for this purpose to maintain cold temperatures necessary for routine *P. effusa* infection assays. Using this new equipment/facility, the conditions for infections within the growth chamber and the humidity tent were similar to those described previously [11]. The exception was that the dew chamber was maintained at cooler temperatures, in the range of 7.5 to 13.3^{o}C. Twenty-four hours following the initial inoculation, the plants were moved to a humidity tent maintained in an improvised cold room for seven days before returning overnight to the dew chamber.

To analyze seeds for the presence of *P. effusa*, 1000 seeds from seed lots were washed with water for 10 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. At least two or three replicates were performed, and an average number of oospores for all three was reported.

USDA ARS, Salinas supplied genomic DNA of downy mildew pathogens to AgBiotech, Monterey, CA to begin efforts commercialize an early detection *P. effusa* assay. The method of the assay is based on competitive allele-specific PCR (KASP) assays and takes advantage of a single nucleotide polymorphism previously identified in the rDNA sequence of *P. effusa* and *P. schachtii* [11].

RESULTS:

We had previously been successful in devising an assay for quantifying the levels of airborne spores (through DNA quantification) of *Bremia lactucae* [12]. In this period, to assess the amount of airborne inoculum of *B. lactucae* in relation to weather patterns near Salinas, spore traps (Fig. 1) were placed at either end of three different commercial lettuce fields (each planted at different times) and spore trap samples were processed twice weekly.

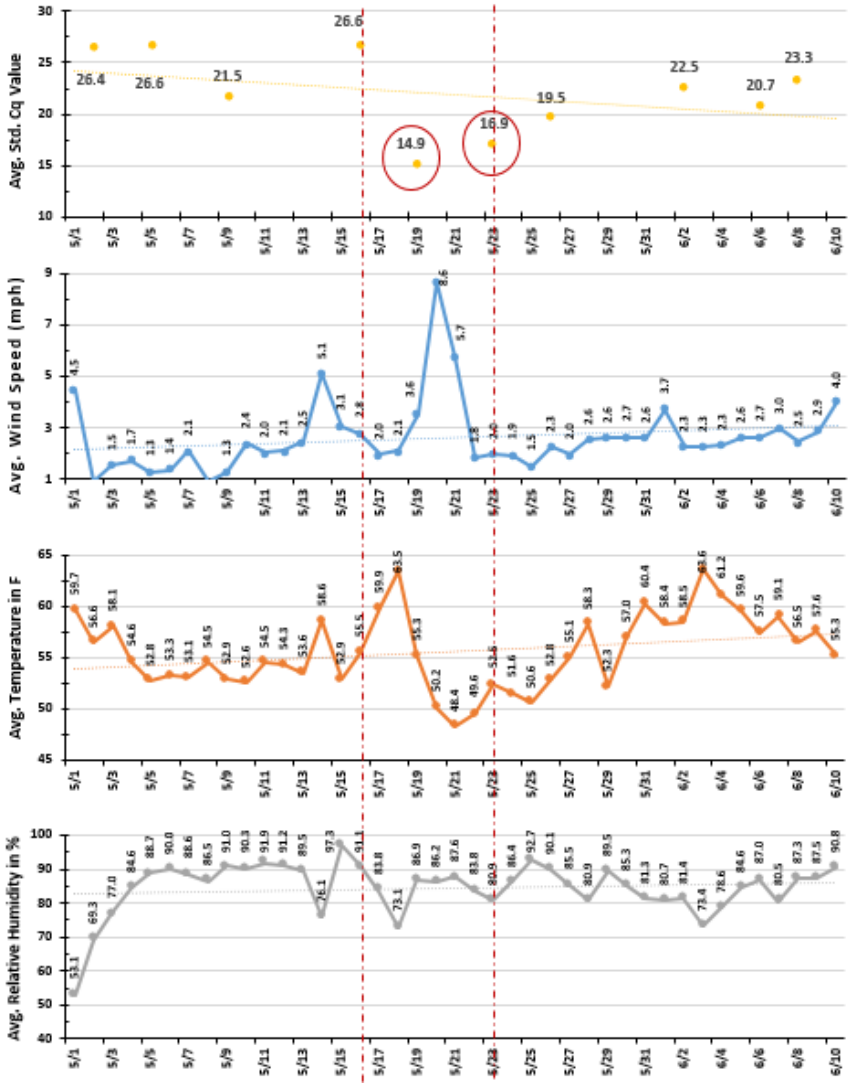

Figure 2. Correlation between weather parameters and lettuce downy mildew of spore load. Spore load was determined indirectly by monitoring *Bremia lactucae* DNA levels as previously described [13]. The circled values of 14.9 and 16.9 in the upper graph represent the dates of highest pathogen DNA detection.

Correlation between weather parameters and lettuce downy mildew of spore load is shown in Figure 2. In the upper graph of Figure 2, the Cq values that are lower correspond to the higher levels of airborne pathogen detectable. Thus in this graph circled values of 14.9 and 16.9 represent the sampling dates of highest pathogen DNA detection. Visual observations of these aligned graphs (Fig. 2) revealed that these lower values (higher pathogen) correlated with periods of highest average wind speeds and temperatures during the experiment but not relative humidity. Additional statistical analyses revealed that correlations between wind speed and temperature and inoculum load were not significant.

Peronospora effusa

Results for one experiment of the testing of multiple biofungicides for organic spinach for protection against downy mildew is shown in Figure 3. We were unable complete the second experiment despite planting because of rain toward the end of December, 2017 and beginning of Jan, 2018. In experiment 1, there were no significant differences between any of the treatments and the untreated control when disease incidence levels were compared on October 22, 2017 (Fig. 3). However, the performance was variable between different treatments, as mean comparisons indicated that biofungicides Procidic, Zonix, and the combination of OSO 5% and Cueva reduced overall reported disease incidence (Fig. 3), though again these differences were not statistically significant.

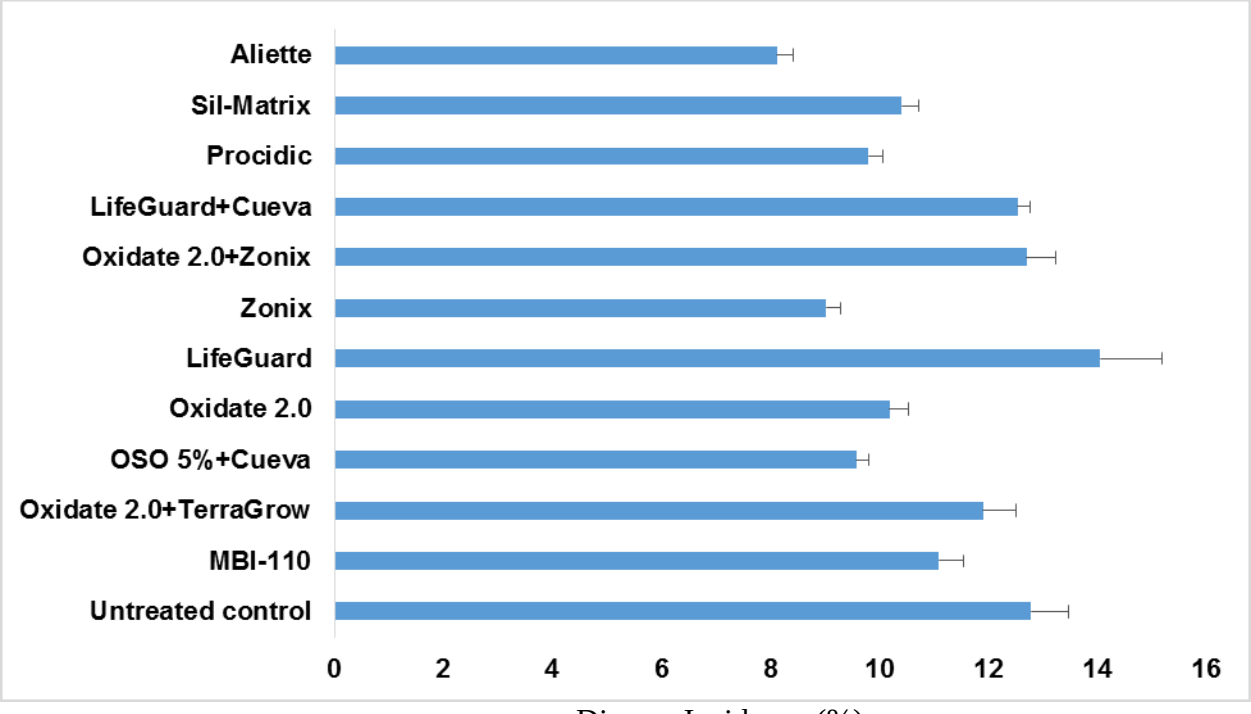

Figure 3. Downy mildew disease incidence recorded on October 22, 2017 following biofungicide treatments. Aliette is a conventional fungicide used for comparative purposes.

The results from the time point ten days earlier were similar. Though treatment with the conventional fungicide Aliette showed the lowest mean disease incidence (approx. 8%) suggestive of disease control (Fig. 3), there was overall low disease incidence recorded (13%).

Examinations of oospore germination were carried out using fresh oospores obtained from leaves of cultivar Viroflay and also from seeds of a spinach cultivar of unknown origin (which was oospore positive and harvested over 1 year ago). We observed oospore germination from leaf sample origin, initially but have more recently observed the oospore germination from those washed off of seeds and stored at 4^{o}C as shown in Figure 4. Additional pictures of higher quality will be submitted as part of a more comprehensive publication on the germination of *P. effusa* oospores.

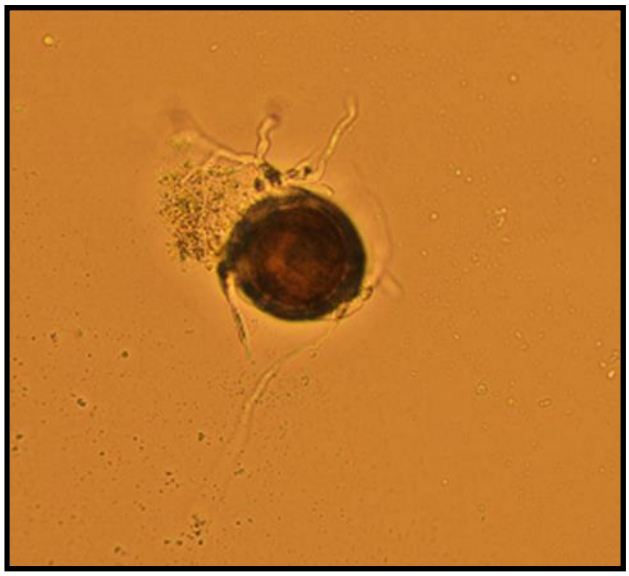

Figure 4. Oospore of *Peronospora effusa* germinating -- the first such observation since the original publication of Jakob Erikkson in 1918 [6]. The oospore is approx. 30 micrometers in diameter, the approximate value determined previously for average oospore diameter [2,13].

In this reporting period, we were not able to ascertain whether oospores were produced in a desert region of California for two reasons. First, excessive irrigation led to insufficient fertility and an outbreak of a root pathogen, and thus inadequate growth of the spinach crop through early March, 2018. Second, consistently warm and dry winter weather in Southern California led to overall low downy mildew pressure in spinach and also other crops.

We investigated seed transmission of spinach downy mildew in the winter of 2018. In the effort to remove the possibility of windborne inoculum contaminating the experiments, we used the isolator system shown in Figure 5A to prevent the introduction of windborne inoculum. The possibility of soilborne inoculum was also excluded because soil in the isolators was fumigated prior to the experiments. We planted multiple oospore infested seed lots within the different sections of the isolator, and a seed lot sample from which we detected no oospores. In one of the seed lot samples planted that contained *P. effusa* oospores, we observed sporulation on several leaves (see the red arrow; Fig. 5B). However, the door to the isolators was opened once during a nearly eight week period, prior to observation, and hence we cannot completely rule out the possibility of airborne contamination.

Previous attempts in our laboratories (Klosterman and Mou) to routinely establish infections of *P. effusa* on spinach most likely failed because of inadequate and inconsistent temperature controls and humidity levels. For example, in a previous reporting period, we had tried Conviron growth chambers and an improvised humidity chamber within a greenhouse, but both of these approaches were not successful to achieve repeatable sporulation. During this reporting period, a dew chamber was maintained in the range of 7.5 to 13.3°C for 24 hr following the initial inoculation, and plants were moved to a humidity tent maintained in an improvised cold room (with the wall-mounted air conditioner set at the coolest temperature) for seven days before returning to the dew chamber for overnight incubation. Under these conditions, we repeated inoculations that led to infections resulting in sporulation several times.

Figure 5. A) Plant isolators at the USDA ARS station in Salinas, CA. **B)** Downy mildew sporulation on the underside of a spinach leaf (at red arrow) of a plant grown within the isolator using *Peronospora effusa* oospore-infested seed.

Results of the first attempt to produce a commercial assay for early detection of *P. effusa* are shown in Figure 6. The assay shown in Figure 6 was conducted using DNA provided by the USDA ARS, Salinas for two different *Peronospora* pathogens that differ by a single nucleotide in a region of DNA previously established [11], enabling the differentiation and successful identification of *P. effusa*. The successful assay will ultimately have to be compatible with the sampling of leaves directly. The results shown in Figure 6 are those from using purified genomic DNA of both pathogens.

DISCUSSION:

In our previous work, we have applied spore trapping and qPCR for quantification of the downy mildew pathogens of lettuce and spinach [4,5,10,11,12]. Tracking the levels of windborne inoculum of the pathogen has yielded insights on the prevalence of the downy mildew pathogens of lettuce and spinach, and increases of both pathogens have been detected in the Salinas Valley during their respective growing seasons. In this report we show the correlation between the increased wind speed and increased temperature and increased inoculum load of *B. lactucae* in the late morning hours. This is consistent with the historical research (conducted without the aid spore traps). Though a correlation between increased wind speed and increased temperature and increased inoculum was clear in two of the independent experiments, statistical analyses revealed that the results were not significant, likely owing to the limited number of spore trap samples, taken twice weekly.

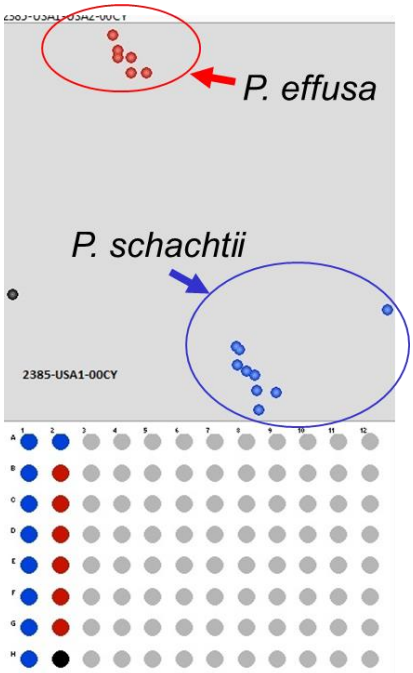

Figure 6. Differentiation of *P. effusa* (spinach pathogen) and *P. schachtii* (beet pathogen) by a single nucleotide polymorphism (SNP) assay. Image and assay courtesy of Mark Massoudi, AgBiotech, Monterey, CA.

If additional such experiments with spore traps are undertaken in the future, with the defined goal to correlate weather patterns with increased airborne pathogen detection, additional spore trap collection time points would be helpful to increase data significance. In the current experiments, we purposefully tried to use fewer traps, so to test the practicality of the trap system at a field or ranch level. Though we have determined that it would likely not be practical to install and routinely sample spore traps around lettuce ranches, we have previously shown that using the spore trap detection values directly and setting the fungicide application based on a pathogen detection threshold (Cq = 24) resulted in a savings of 1.7 fungicide sprays.

We published the work on the finding of oospores in modern spinach seed lots, and examined additional seed lots for oospores, in addition to the 82 characterized in the original publication [11]. The end result currently is that we know that about 21% of the ~ 170 commercial seed lots that have been tested contain oospores. It has been about thirty-five years since the initial report of *Peronospora effusa* on spinach seed lots. That study was conducted in Japan and a study that also provided evidence for seed transmission [8].

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. New pathotypes or races of the pathogen, as well as both mating types, can be dispersed on seeds. Also, the mating of different strains of *P. effusa* after bringing the mating types together in a new region has implications of quickly increasing the genetic diversity within populations, contributing to the appearance of new “races” or pathotypes. Oospores on seeds likely initiate disease outbreaks in current and new production areas, where spinach has not been planted and in areas that are not necessarily conducive to downy mildew. Given the importance of seed transmission in other downy mildew systems, treatments to reduce downy mildew on spinach may slow disease spread and pathogen

diversification. In this current report, we demonstrated that oospores of *P. effusa* derived from seed do germinate. This finding, coupled with previous evidence of seed transmission [8], further advances the importance of oospores arriving on spinach seed in initiating disease in current and new spinach production areas. In this context, it is also important to note that experiments have been performed by us (USDA) [11] and others [1] to show that other host species of plants are not infected by P. effusa, and thus the sporulation only occurs on spinach.

In this reporting period, we further investigated seed transmission of spinach downy mildew. Because a door to the isolators was opened once briefly during a nearly eight week growth period, we could not completely rule out the possibility of airborne contamination prior to the observation of sporulation. Nevertheless, this finding provides additional support to previous published findings [8] that spinach downy mildew is seed transmitted. The majority of *Peronospora* spp. causing downy mildews that have been examined for seed transmission have been confirmed as seed transmitted, and thus the findings of seed transmission of spinach downy mildew are anticipated. We also established a system using newly available resources at the USDA station in Salinas to obtain routine infections and sporulation when using fresh inoculum of *P. effusa*.

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 and soil remain to be fully explored. We routinely detect oospores in samples of cultivar Viroflay maintained at the USDA ARS station in Salinas. However, oospores have not yet been detected in leaf tissue collected from field samples (at least non-Viroflay samples) in California and at other locations in California. The appearance of oospores in non-Viroflay samples may be a rare occurrence since the appropriate mating types of *P. effusa* must occur in the same plant and some levels of resistance may preclude one or the other mating type.

The experiments initiated to assess oospore production in a California desert region were not successful due to the poor stand of spinach in the current reporting period. This did not allow evaluation. For the coming growing season, we will: 1) work with a grower for help with bedding and planting; 2) add windbreaks around the plots to increase relative humidity; 3) ensure irrigation and fertility is appropriate; and 4) ask experienced growers or PCAs to periodically confirm crop is growing adequately.

The biofungicide testing is required in one more field plot before we can fully evaluate their effectiveness for protection from downy mildew. In the current study, the level of disease incidence was not very high in the untreated control plots (~13%), and thus increased disease pressure will also be helpful in future biofungicide evaluations. There was too much rain in the winter months of 2017-18 to complete the second trial, but we have recently replanted, in part to also fulfil our obligation to testing for the IR-4 program.

We (USDA ARS) have previously shown detection of *P. effusa* DNA seven days before the appearance of symptoms in spinach leaves [14]. The previously developed approach would not allow quick turnaround time on the results to growers because it relies on conventional PCR using DNA from the smashed leaf samples [14]. The results shown herein indicate that *P. effusa* can be identified by the SNP assay (AgBiotech, Monterey, CA), and this assay takes approximately 2 hours (personal communication, Mark Massoudi). While this is fast, the successful assay that we will deploy for early detection of *P. effusa* will have to work with leaf samples directly. The samples tested thus far using the SNP assay have only been genomic DNA and therefore we must add leaf DNA for additional testing in the next round of experiments.

Additionally we will be testing new technology for in field detection with Dr. Frank Martin's group.

In this reporting period, we also continued development of a new *P. effusa*-specific assay with collaborator Dr. Allen Van Deynze based on mitochondrial sequences of *P. effusa* and analyzed by Dr. F. Martin. We have requested additional DNA samples from various downy mildew (*Peronospora spp*) strains and infected plants from Drs. M. Thines and Y-J. Choi (Frankfurt, Germany and Seoul, South Korea, respectively) to test by PCR to ensure assay specificity. The previous assay developed had a drawback in that dual assays had to be performed for both closely related pathogens *P. effusa* and *P. schachtii* (beet downy mildew) [3], and the value of *P. schachtii* had to be subtracted because low level nonspecific amplification of the *P. schachtii* DNA, especially when analyzing spore trap samples. The new assay will completely eliminate the need for dual PCR. We will use the new target mitochondrial sequence for development of leaf assays that may be used either directly by the grower or PCA in the field, or used in the development of a commercial assay.

Acknowledgements: We are thankful for funding from the California Leafy Greens Research Program, the California Department of Food and Agriculture, grant SCB14043, and the IR-4 program for biofungicide testing. We are thankful for the assistance of Amy Anchieta (USDA ARS, Salinas) in conducting qPCR experiments and Lorena Ochoa and Daniel Machado (USDA ARS) for collecting spore trap samples. We appreciate provision of spinach seed samples from the spinach seed production and distribution companies and access to lettuce fields for the spore trap studies.

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

References:

1) Byford, W. J. 1967. Host specialization of *Peronospora farinosa* on *Beta*, *Spinaciae*, and *Chenopodium*. Trans. Br. Mycol. Soc. 50:603-607.

2) Choi, Y.-J., Hong, S.-B., Shin, H.-D. 2007. Re-consideration of *Peronospora farinosa* infecting *Spinacia oleracea* as distinct species, *Peronospora effusa.* Mycological Research 111: 381-391.

3) Choi, Y.-J., Klosterman, S.J., Kummer, V., Voglmayr, H., Shin, H.-D., Thines, M. 2015. Multi-locus tree and species tree approaches toward resolving a complex clade of downy mildews (Straminipila, Oomycota), including pathogens of beet and spinach. Molecular Phylogenetics and Evolution. 86:24–34.

4) Choudhury, R. A., Koike, S. T., Fox, A., Anchieta, A., Subbarao, K. V., Klosterman, S. J., and McRoberts, N. 2017. Spatiotemporal patterns in the airborne dispersal of spinach downy mildew. Phytopathology 107:50-58.

5) Choudhury, R. A., Koike, S. T., Fox, A. D., Anchieta, A., Subbarao, K. V., Klosterman, S. J., and McRoberts, N. 2016. Season-long dynamics of spinach downy mildew determined by spore trapping and disease incidence. Phytopathology 106:1311-1318.

6) Eriksson, J. 1918. Zur Entwickelungsgeschichte des Spinatschimmels (*Peronospora spinaciae* (Grew.) Laub.). Arkiv Botanik 15:1-25.

7) Etxeberria A, Mendarte S, Larregla S, 2011. Determination of viability of *Phytophthora capsici* oospores with the tetrazolium bromide staining test versus a plasmolysis method. Rev Iberoam Micol 28, 43-9.
8) Inaba, T., Takahashi, K., Morinaka, T. 1983. Seed transmission of spinach downy mildew. Plant Disease. 67:1139-1141.
9) Inaba, T. Morinaka, T. 1984. Heterothallism in *Peronospora effusa*. Phytopathology. 74:214-216.
10) Klosterman, S. J., 2016. Spinach downy mildew – Threat, prevention and control. Progressive Crop Consultant 1:12-15.
11) Klosterman, S.J., Anchieta, A., McRoberts, N., Koike, S.T., Subbarao, K.V., Voglmayr, H., Choi, Y.-J., Thines, M., Martin, F.N. 2014. Coupling spore traps and quantitative PCR Assays for detection of the downy mildew pathogens of spinach (*Peronospora effusa*) and beet (*Peronospora schachtii*). Phytopathology, 104:1349-1359.
12) Kunjeti, S. G., Anchieta, A.,Martin, F. N., Choi, Y.-J., Thines,M., Michelmore, R. W., Koike, S. T., Tsuchida, C., Mahaffee, W., Subbarao, K. V., and Klosterman, S. J. 2016. Detection and quantification of *Bremia lactucae* by spore trapping and quantitative PCR. Phytopathology 106:1426-1437.
13) Kunjeti, S.G., Anchieta, A.G., Subbarao, K.V., Koike, S.T., Klosterman, S.J. 2016. Plasmolysis and vital staining reveal viable oospores of *Peronospora effusa* in spinach seed lots. Plant Disease. 100:59-65.
14) Subbarao, C.S., Anchieta, A.G., Ochoa, L., Dhar, N., Kunjeti, S.G., Subbarao, K.V., Klosterman, S.J. Detection of latent infections of *Peronospora effusa* in spinach. Plant Disease. https://doi.org/10.1094/PDIS-12-17-1956-RE