CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

Annual Report for Spinach Downy Mildew Research, 2019-2020

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ABSTRACT

Downy mildew is a very destructive disease on organic spinach in California and is caused by the obligate oomycete pathogen *Peronospora effusa*. Either airborne spores of the pathogen, infested seed or potentially soilborne oospores initiate infection. In this reporting period, DNA-based detection assays were improved and deployed to quantify the levels of airborne inoculum of *P. effusa* using spore traps. Airborne inoculum load present during a two-month cropping period in the Salinas Valley, CA was compared with that from the Coachella Valley, CA. Higher levels of airborne *P. effusa* were present in the Salinas Valley. Using plant isolators at the USDA location in Salinas, we conducted two experiments to study seed transmission. For the second straight winter season, we observed a low incidence of disease (<1 %) on spinach plants in isolators that were grown from oospore-infested seeds and a seed lot of Viroflay that was PCR-positive for *P. effusa* DNA, suggestive of seed transmission. We observed symptoms of downy mildew in our Coachella plots in the desert, but we did not detect oospores in those leaf samples that were examined. We completed testing of new *P. effusa*-specific DNA primers for leaf detection and are beginning preparations of point-of-care testing in the field. We initiated two separate field plots for biopesticide testing in the 2019-2020 period and tested the use of nitrogen dioxide (NO_2) fumigation treatment for spinach seed. The NO_2 treatment yielded highly encouraging results in 2019, as it was effective at killing two important pathogens carried on spinach seed, *Verticillium dahliae* and *P. effusa,* without significantly affecting germination. Because we learned that the use of nitrogen dioxide would not be acceptable for use for organic spinach seed without OMRI approval, we (USDA) found an EPA registration of NO_2 as a fungicide and filed a petition to obtain approval to use NO_2 as a seed treatment. In summary, tracking the levels of windborne inoculum provides valuable information on the conditions and locations conducive to downy mildew in California, and leaf detection assays provide information to assess whether a field is infected (for early harvest before loss) prior to the entire field becoming infected and symptomatic. Oospores of *P. effusa* have been detected in a total of 17% of the commercial seed lots tested since 2014, revealing that sexual reproduction of the pathogen occurs worldwide and is commonplace. This inoculum can initiate infection in a crop as demonstrated in isolators for two years. Biopesticide treatments in the field and treatments that can eliminate *P. effusa* on spinach seed may limit some outbreaks, especially for organic spinach.

PROJECT TITLE: Early detection, epidemiology, and control of spinach downy mildew.

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OBJECTIVES:

Objective 1: Examine *P. effusa* oospore production and seed transmission.

Objective 2: Determine airborne inoculum load present at two different periods of the season in the Coachella Valley, CA.

Objective 3: Test *Peronospora effusa*-specific DNA primers and develop an assay for early detection of spinach downy mildew in leaves.

Objective 4: Complete biopesticide testing and analyze nitrogen dioxide seed fumigation treatment for organic and conventional spinach.

PROCEDURES:

We obtained additional commercial seed lots for testing for the presence of *P. effusa* oospores using a wash-off method described previously [13]. Briefly, samples of 1000 or 500 seeds from seed lots were washed with water for 10 min by vigorous vortex mixing, the debris was pelleted by gentle centrifugation for 5 min, and then the pelleted debris was analyzed under light microscopy. At least three replicates were performed, and an average number of oospores for all three was obtained. In total we (USDA) have examined 299 seed lots for the presence of *P. effusa* since 2014. We examined seed transmission in both humidity tents and in plant isolators.

The leaf detection assay and the quantitative PCR (qPCR)-derived DNA from higher numbers of spores were conducted from symptomatic leaves of cultivar Viroflay grown in a dew chamber and humidity tents, both maintained in an air-conditioned room at the USDA station in Salinas. This system employs a dew chamber maintained in the range of 7.5 to 13.3^{o}C for 24 hr following initial inoculation. After this initial 24 hr period, plants were moved to a humidity tent maintained in a cold room for seven days before returning to the dew chamber for overnight incubation.

Data were collected for airborne detection and quantification of *P. effusa* at locations in the Coachella Valley, CA, and in the Salinas Valley, CA using a pair of solar/battery-powered impaction spore traps (Figure 1) obtained from Dr. Walt Mahaffee (USDA-ARS, Corvallis, OR) or from Revolution Crop Consultants (Corvallis, OR), which manufacturers impaction spore traps for a fee. The pairs of 1.1 mm x 40 mm stainless steel rods (Figure 1B) coated with silicone vacuum grease (Dow Corning) were collected at 48- or 72-hour intervals. The collected rods (that trapped 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. Estimates of *P. effusa* spore load based on the DNA level detected were determined by quantitative PCR [11] and also using the new mitochondrial markers (unpublished).

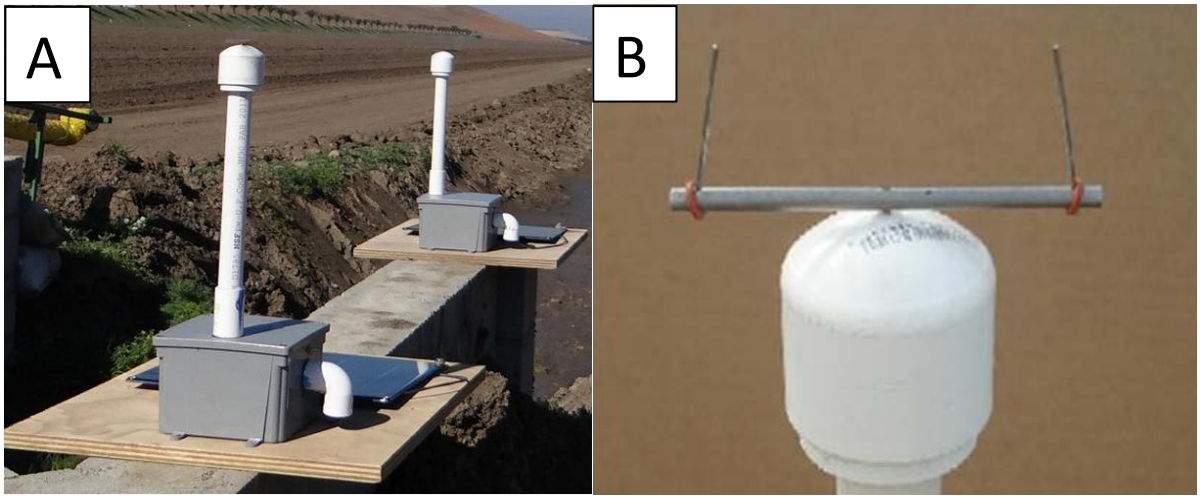

Figure 1. Spore trap system to detect *Peronospora effusa*, the causal agent of spinach downy mildew, respectively. **A)** Example of two spore traps in operation. **B)** Spore trap head with rotating arm and removable stainless-steel rods coated with grease for spore adhesion.

For the development of the recombinase-polymerase DNA amplification assay (RPA) for early detection of *P. effusa* in spinach leaves, mitochondrial DNA primers that were previously tested during 2018-2019 were used (unpublished). The assay was tested using DNA derived from *P. effusa* spores in the presence of leaf extract from spinach cultivar Viroflay. The assay was also tested using leaf samples that were sampled weekly at 12 feet intervals in small field plots.

Two small field plots were established at the USDA station in Salinas, CA in 2019-2020 for further testing of the biopesticide Procidic. Spinach cultivar Viroflay was planted in four 80" beds x 120 ft in length at the USDA-ARS station in 2019-2020 for testing. The plots were examined weekly for downy mildew disease incidence.

For nitrogen dioxide (NO_2) fumigation of spinach seeds, nitric oxide (Praxair, Inc) was released into an enclosed fumigation chamber (essentially a modified refrigerator) to react with oxygen in ambient air to form nitrogen dioxide [14, 15]. The killing of *V. dahliae* in spinach seed samples was determined by counting colonies per gram of ground seed on NP10 medium after NO_2 treatment. Seed grinding was performed as described previously [6]. Viability tests of *P. effusa* oospores was by plasmolysis as described previously [13].

In this reporting period, we established a field trial in a grower field in the Coachella Valley. The susceptible cultivar Viroflay, which was confirmed to be free of *P. effusa* oospores, and three commercial cultivars, in which *P. effusa* oospores were detected, were planted on December 17, 2019. Seed was planted with a commercial precision planter with 16 lines to achieve 2.3 million seed per acre. The trial consisted of 80" to the center beds, and plots measured 30 ft. in length. There were three replicate plots per treatment, and each plot was separated by a bare bed or a bare plot on either side. The trial was sprinkler irrigated except on fungicide application days and the plot was generally maintained according to commercial standards for the Coachella Valley. Following emergence, the plots were scouted approximately weekly for symptoms of downy mildew. The trial concluded in late March.

RESULTS:

In total, the number of seed lots infested with oospores of *P. effusa* stands at 17% (52/299 seed lots) since the initial finding of oospores on modern seed lots by our lab (USDA) in 2014 [13]. We had previously verified viablity of the oospores washed from seeds since we demonstrated germination of a small number of those from seeds [8].

Seed transmission of spinach downy mildew has not been reported in the literature since 1983 [6]. We investigated seed transmission of spinach downy mildew in the winter of 2019-2020 to complement the 2018-2019 winter experiment. In the effort to remove the possibility of windborne inoculum contaminating these experiments, we used the isolator system shown in Figure 2A to prevent the introduction of windborne inoculum. We had run an experiment in early 2019 using this system in which we observed very low disease incidence (~ 1%) (unpublished) on plants grown from an oospore-positive seed lot.

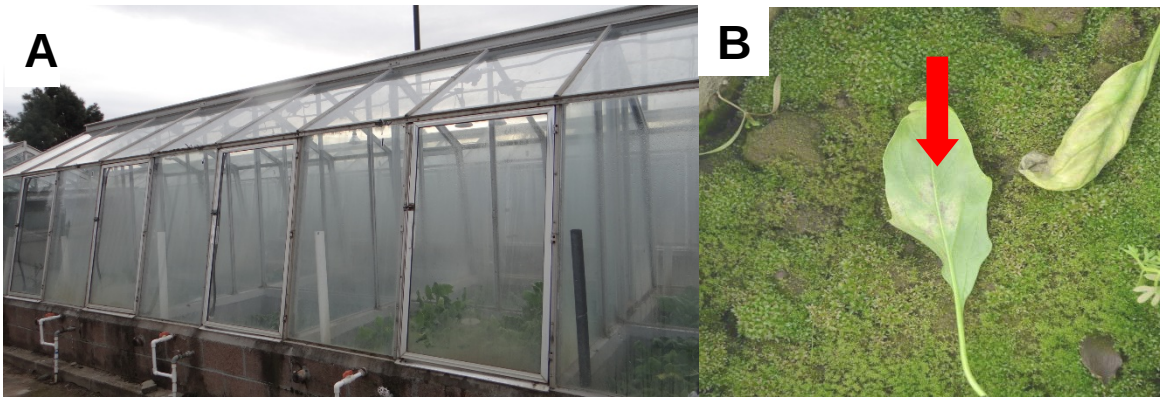

Figure 2. A) Plant isolators at the USDA-ARS station in Salinas, CA for planting *Peronospora effusa* oospore-infested seeds or those that were non-infested, and which prevent airborne spore contamination. **B)** Downy mildew sporulation on a spinach leaf (at red arrow) from a plant grown within the isolator using *Peronospora effusa* oospore-infested seeds in the 2019-2020 experiment.

In the 2019-2020 reporting period, we planted three oospore-infested seed lots within three different sections of the isolator, and a seed lot sample from which we detected no oospores. We observed sporulation on leaves (arrow; Fig. 2B) on plants grown from the same commercial seed lot that yielded downy mildew disease in early 2019. Also, unexpectedly, as in the 2018-2019 experiment, we also observed sporulation on plants that grew from the negative control seeds in the experiment which did not have detectable oospores (Viroflay). The Viroflay seeds were reexamined by the seed wash-off method using three samples each of 1000 seeds, but these retests also did not reveal the presence of oospores. However, the Viroflay seed lots tested positive by PCR for *P. effusa* at a low level of detection (data not shown).

In the plots in the Coachella Valley, symptoms and signs of downy mildew were first observed in the study area on January 24. The disease appeared to originate from a single hot spot in a replicate plot of Viroflay, and the vast majority of disease incidence was clustered in this plot and an adjacent Viroflay plot. Disease incidence was not quantified but was high in the hot spot with more than an estimated 10% of plants affected. Trace levels of disease were observed in the remaining areas of Viroflay plots. In rare instances, disease was observed in two of the commercial cultivars, but the morphology of all of these affected plants suggested they were off-types. Samples exhibiting symptoms that were suspected to be associated with oospore production were examined under the microscope, but no oospores were found. Disease progress stopped in early March.

The use of nitrogen dioxide (NO_2) was investigated for the killing of *P. effusa* and *Verticillium dahliae*, two very important pathogens that are carried on spinach seed. While oospores of *P. effusa* are thought to be primary inoculum for spinach downy mildew, *V. dahliae* causes wilt disease in subsequent lettuce crops grown in soil where the infested seeds are planted [16]. Of course, *P. effusa* affects spinach directly, but it was difficult to obtain a high enough percent of germinating oospores to properly evaluate the killing of *P. effusa* oospores by germination alone. Nevertheless, we used a plasmolysis technique to test viability of *P. effusa* oospores following NO2 treatment. All oospores from seed, examined after plasmolysis treatment, were nonviable following the 5% NO_2 treatment. In addition, the results indicated that NO_2 treatment was highly

effective in killing *V. dahliae* in seed, reducing the colonies observed in NO_2-treated seed by over 90% relative to the nontreated control (Fig. 3). The effective killing concentrations of 5% or 10% NO_2 also did not significantly affect seed germination (Fig. 4).

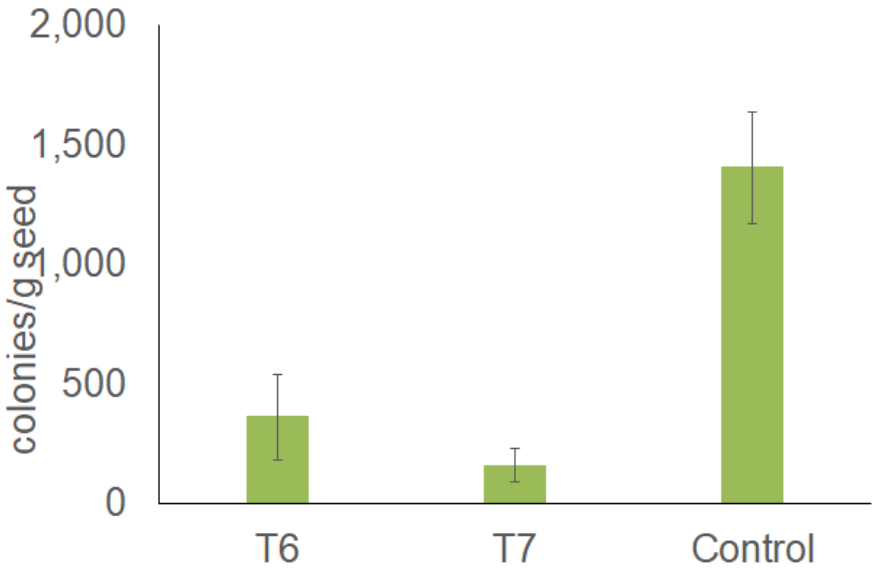

Figure 3. Test of 5% (T6) or 10% (T7) nitrogen dioxide (NO2) on the killing of *Verticillium dahliae* in ground spinach seed determined by colonies per gram of seed counted on NP10 medium. Error bars were from triplicate samples of 1 gram of seeds.

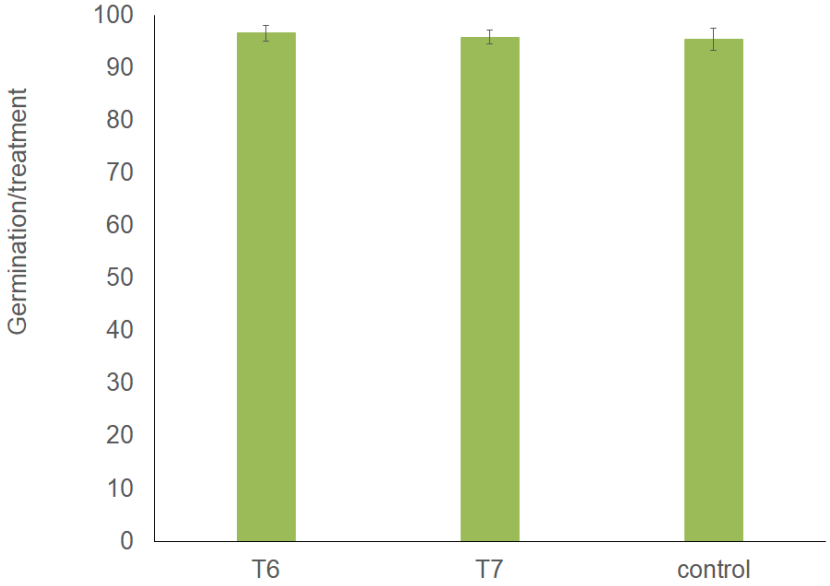

Figure 4. Test of seed germination follow seed treatments of 5% (T6) or 10% (T7) nitrogen dioxide (NO_2) treatments. The control seeds were untreated. The mean and standard deviation (error bars) were calculated from the analyses of seed germination of five replicate samples of 100 seeds each.

The results from Table 1 are from the use of the RPA assay to detect *P. effusa* in spinach leaf extract. The limit of detection of the RPA assay is nearly 900 femtograms of DNA, an amount that is approximately 11 spores of *P. effusa* in the background of spinach leaf extract, and takes ~20 minutes to obtain detection.

Table 1. Detection (RPA assay) of *P. effusa* DNA with and without spinach leaf extract

***P. effusa* DNA concentration (nanograms)**	**Average** minutes to detection (no spinach leaf extract)**	**Average minutes to detection (with spinach leaf extract)**
1	**7.62**	**8.42**
0.2	**8.32**	**9.13**
0.04	**9.58**	**9.7**
0.008	**10.12**	**12.63**
0.0016	**11.35**	**14.67**
0.0009	**13.12**	**20.32**

**Three reactions.

Results from the spore trapping and quantification of *P. effusa* indicated increased spore load of *P. effusa* in the Salinas versus the Coachella Valley during September 17 to November 11, 2019 period (Fig. 5). Spore quantities derived from qPCR analysis reached a maximum of 632 spores on the October 11 sampling date in the Salinas Valley sampling. There was clearly a spike quantities of *P. effusa* spores detected between October 7 and October 17 in the Salinas Valley (Fig. 5). Many sampling points for both the Salinas and Coachella Valleys showed no detection during this time frame (Fig. 5).

We also tested a multiplex qPCR assay for spore trap application that combined the new *P. effusa*-specific DNA target of the RPA assay with the previously developed *Bremia lactucae* qPCR assay [12] for simultaneous quantification of the spinach and lettuce downy mildew pathogens (data not shown).

DISCUSSION:

We previously published the work on the finding of oospores in modern spinach seed lots and examined additional seed lots for oospores [13]. In aggregate, about 17% of the 299 commercial seed lots contained oospores. The presence of oospores in spinach seed lots suggests long term survival of the pathogen on seed, which can disperse the pathogen worldwide [8,10].

Though evidence of spinach downy mildew seed transmission was reported in the early 1980s [6], we undertook experiments in this reporting period that further advances the importance of oospores on spinach seed initiating disease in the current and new spinach production areas.

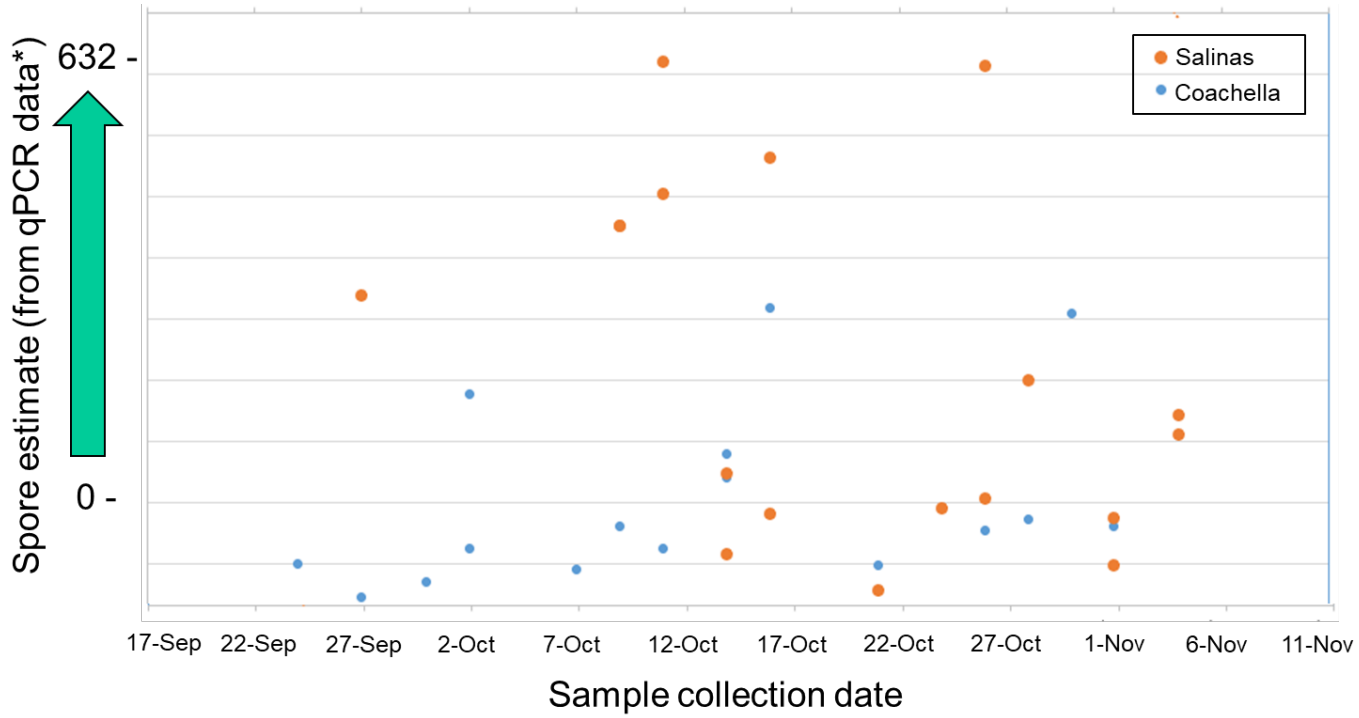

Figure 5. Spore trap-based detection and quantification of *Peronospora effusa* DNA by quantitative PCR (qPCR) in samples from the Coachella and Salinas Valleys of California in the period of September 17 – November 11, 2019. *Based on the qPCR values and the value of an estimated 50 ribosomal DNA copies per spore. Detection values below zero represent positive detection of P. effusa DNA, but not at the level equivalent to > 1 spore.

For the second consecutive winter season, we found seed transmission of downy mildew in plant isolators at the USDA station. The isolator system filters out microorganisms the size of bacteria (5-10 µm) and thus downy mildew observed within this system must have come from infested seeds. Since downy mildew spores are approximately 30 µm [1,8], they are unlikely to have passed through the isolator filtering system. The puzzling finding is that the negative control of Viroflay seeds (without **detectable** oospores) yielded spinach plants that were positive for downy mildew development in a few of these isolator experiments. The most likely explanation is that the Viroflay seed lot contained oospores at a low level undetectable in seed wash-offs. Previously, we have observed DNA amplification of *P. effusa* by PCR-based tests in about 90% of seed lots that were negative in wash-off tests [13]. The Viroflay seed lot used in this study for the isolators was also positive by qPCR analysis at a low level of detection and therefore, we are cloning and sequencing of the PCR fragment for final verification. Of the many *Peronospora* spp. causing downy mildews that have been examined for seed transmission, the majority have been confirmed to be seed transmissible. Findings of likely seed transmission in this study spinach downy mildew continue to agree with this trend. 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 unanswered at this time. Some of these studies and seed transmission studies are still ongoing in collaboration with Dr. J. Correll.

In our previous work, we have applied spore trapping and qPCR for quantification of the downy mildew pathogens of lettuce and spinach [2,3,11,12]. 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 [2,3,12]. In this reporting period, we applied spore trapping and quantification of *P. effusa* DNA to examine the relative spore load at both the Salinas and Coachella Valleys in California. As anticipated, there were higher levels of *P. effusa* detected in the Salinas Valley compared to the Coachella Valley during mid-September to mid-November 2019. But interestingly, there was an obvious spike in *P. effusa* detected during October 9-17 in the Salinas Valley. It will be interesting going forward to examine why these spikes in quantities occur in relation to cropping and weather events, which may be useful to exploit for disease forecasting purposes. We were not able to analyze the second detection period because of difficulties in maintaining the spore traps. Nevertheless, we reinitiated experiments and we have collected additional spore trap samples for quantification in this reporting period that we will analyze for the next reporting period.

We also completed the development of a new *P. effusa*-specific recombinase-polymerase DNA amplification (RPA) assay with collaborator Dr. Allen Van Deynze based on the mitochondrial sequences of *P. effusa* identified by Dr. F. Martin (unpublished). As indicated, the assay could detect 11 spore equivalents of *P. effusa* within 20 minutes using a simple isothermal amplification device. This new assay eliminates the need for dual PCR. We will begin testing this assay in the field for point-of-care usage. The RPA assay could be turned over for potential PCA usage in the field by hooking up a mobile isothermal device to a 12-volt power supply connection commonly found in pick-up trucks. We (USDA-ARS) have previously shown detection of *P. effusa* DNA seven days before the appearance of symptoms in spinach leaves [17]. 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 ground leaf samples [17]. These approaches were more expensive and took longer to obtain an answer (about 3 to 4 hours with set up). Thus, the new RPA assay is more promising.

Since *P. effusa* is heterothallic [7], two strains each of a different mating type are required to form the long-lived oospores. We routinely detect oospores in leaves of cultivar Viroflay grown in the field at the USDA-ARS station in Salinas, suggesting the presence of both mating types in the Salinas Valley. In a joint study with Dr. J. Correll, oospores also were detected in leaf tissue collected from numerous commercial cultivars grown in field trials in the Salinas Valley of California [4], further supporting that mating types are commonplace in the Salinas Valley. Dissemination of different pathotypes and mating types on seed is a concern [10], and therefore reductions of oospores on seeds are advisable to limit the spread of the pathogen.

NO_2, produced by the combination of nitric oxide and oxygen, is highly effective at killing the fungus *Aspergillus flavus* [14], which produces aflatoxins harmful to humans commonly found in contaminated peanuts and corn seeds. Our data in this report revealed that NO_2 is effective at killing two problematic plant pathogens *V. dahliae* and *P. effusa*, present in/on spinach seeds. We observed this by counting the colonies present on nutrient selective media for *V. dahliae* and

by plasmolysis tests for *P. effusa* oospores. It is difficult to examine the viability of *P. effusa* oospores directly by germination of oospores because the optimal conditions for oospore germination have not been worked out and only a small percentage germinates under conditions that we currently use. Since spinach and lettuce and other leafy greens are grown in a rotation in Salinas Valley, eliminating seed borne pathogens from spinach seeds benefits many crops including lettuce and spinach. Unlike many other seed treatments that have been tested, such as steam and hot water, 5 or 10% NO_2 did not significantly affect seed germination. Because no one has put forward an Organic Materials Review Institute (OMRI) petition to get approval to use NO_2 as a seed treatment, we (USDA) conducted the necessary literature research on NO_2 and put forth a petition for its usage in March 2020 for the purpose of treating spinach seed to kill plant pathogens in enclosed chambers that are properly exhausted. In the course of our literature searches, we found an Environmental Protection Agency registration of NO_2 under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), which may support its usage as a seed treatment (Please see Report Appendix on Page 13). We are still waiting to learn whether its usage will be approved for treating organic spinach seeds.

The most likely source for the downy mildew epidemic observed in susceptible cultivar Viroflay in the Coachella Valley plots is externally produced aerial spores. Downy mildew was observed in a commercial field in the Coachella Valley in the last week of December, therefore aerial spores were likely present and able to cause disease in our plots. However, if aerial spores were present that were associated with disease in a commercial field, it is unclear why disease did not develop in the commercial cultivars in our trial. It is possible that these cultivars possessed resistance sources that the cultivar in the commercial field did not, but the identity of the cultivars are unknown. An alternative explanation is that the outbreak was caused by seedborne inoculum that was not detectable by the wash-off method. For the commercial cultivars in the trial, disease did not develop despite being planted with seed lots infested with *P. effusa.* Significant rain fell Dec. 23 to 27. After this, conditions of cool temperatures and moderate humidity were generally favorable for downy mildew disease development, and leaf wetness was observed on most visits to the plots in the few weeks after emergence. However, it is unknown if these conditions are similar to conditions required for oospore germination and seedborne transmission. An additional possibility is that the size of our plots reduced the chances of observing a transmission event. Given the size of the plots, the detection limit in this trial is a plant infection rate of 0.01%. If the rate of actual disease transmission is small, the number of seeds in each plot in our trial may be too small of a sample size for the rare event to occur.

We were unable to complete the biopesticide testing of Procidic (active ingredient citric acid) a third time in the 2019-2020 period because of rain in the first field plot trial, and lack of disease until it was too late for the other trial. However, we did publish the results of the 2017 and 2018 field tests of biopesticides [9]. In both studies, the level of disease incidence was not high in the untreated control plots (~13%), and thus the rationale for repeating the trial for Procidic in this current reporting period. The spray application of Procidic resulted in statistically significant lower disease incidence with 90% statistical confidence in 2017 and 2018 but none of the other products gave statistically significant downy mildew control on spinach [9].

In summary, two experiments have supported seed transmission of spinach downy mildew in plant isolators though we would like to conduct a third seed transmission experiment and clone the *P. effusa*-specific DNA fragment from the Viroflay negative control lot before finalizing our

conclusions. We are hopeful the petition to use NO_2 will be OMRI-approved for use on spinach seeds to kill oospores. Spore trapping experiments examining airborne spore load of *P. effusa* in the Salinas and Coachella Valleys over the same two-month period revealed marked differences and additional data and their analyses in the future could be helpful in forecasting spinach downy mildew. We completed the development of an early detection RPA assay that must be field tested for point-of-care use. Although the level of disease pressure in the two earlier trials was low, the use of the biopesticide Procidic provided significant disease control, and hence Procidic is under further investigation to limit downy mildew symptoms on spinach.

Acknowledgments: We are thankful for funding from the California Leafy Greens Research Program, and the California Department of Food and Agriculture, grants SCB14043 and SCB37719. We are thankful for the assistance of Amy Anchieta (USDA-ARS, Salinas) in conducting all qPCR experiments and Lorena Ochoa and Daniel Machado (USDA-ARS) for collecting spore trap samples, Adrian Zendejas (Desert Mist Farms) for hosting the Coachella trial. We appreciate the 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**.**

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Report Appendix

U.S. ENVIRONMENTAL PROTECTION AGENCY
Office of Pesticide Programs
Antimicrobials Division (7510P)
1200 Pennsylvania Ave., N.W.
Washington, D.C. 20460

NOTICE OF PESTICIDE:
_X_ Registration
___ Reregistration
(under FIFRA, as amended)

EPA Reg. Number:	Date of Issuance:
89265-1	4/15/16

Term of Issuance:
Unconditional

Name of Pesticide Product:
NOXILIZER NO_2 STERILANT

Name and Address of Registrant (include ZIP Code):
Noxilizer, Inc.
1450 South Rolling Road
Baltimore, MD 21227

Note: Changes in labeling differing in substance from that accepted in connection with this registration must be submitted to and accepted by the Antimicrobials Division prior to use of the label in commerce. In any correspondence on this product always refer to the above EPA registration number.

On the basis of information furnished by the registrant, the above named pesticide is hereby registered under the Federal Insecticide, Fungicide and Rodenticide Act.

Registration is in no way to be construed as an endorsement or recommendation of this product by the Agency. In order to protect health and the environment, the Administrator, on his motion, may at any time suspend or cancel the registration of a pesticide in accordance with the Act. The acceptance of any name in connection with the registration of a product under this Act is not to be construed as giving the registrant a right to exclusive use of the name or to its use if it has been covered by others.

This product is unconditionally registered in accordance with FIFRA section 3(c)(5) provided that you:

1. Submit and/or cite all data required for registration/reregistration/registration review of your product when the Agency requires all registrants of similar products to submit such data.

2. The data requirements for storage stability and corrosion characteristics (Guidelines 830.6317 and 830.6320) are not satisfied. A one year study is required to satisfy these data requirements. You have 18 months from the date of registration to provide these data.

Signature of Approving Official:	Date:
Jacqueline Hardy, Product Manager 34 Regulatory Management Branch II, Antimicrobials Division (7510P)	4/15/16

EPA Form 8570-6