**CALIFORNIA LEAFY GREENS RESEARCH PROGRAM**

Combined Annual Report for Spinach and Lettuce Downy Mildew projects, 2022-2023

# Downy mildew detection, epidemiology, and biopesticide evaluation

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## ABSTRACT

Downy mildew diseases on spinach and lettuce are caused by the obligate oomycete pathogens *Peronospora effusa* and *Bremia lactucae*, respectively. Both downy mildews are destructive in California. Sporangia of both pathogens can be airborne and rapidly spread between and within fields. In this reporting period, we employed a multiplex quantitative PCR assay for quantification of airborne *P. effusa* and *B. lactucae* from cyclone spore traps in the Salinas, Coachella, and Imperial Valleys of California in October-March of 2022-2023. These values were examined in relation to those collected in October-March of 2021-2022 in each of the three Valleys. *P. effusa* and *B. lactucae* were not regularly detected in the Coachella Valley. However, *P. effusa* was detectable throughout the growing and nongrowing seasons in the Salinas Valley, whereas *P. effusa* and *B. lactucae* were not detected for nearly 4 weeks during warmer periods in the Imperial Valley at the beginning of the growing season. Based upon these findings, *B. lactucae* may survive in wild lettuce or may be present in the soil as oospores. *P. effusa* is likely either soil or seed transmitted. Another objective focused on analyses of seed transmission of spinach downy mildew. In December 2022, we observed seed transmission of spinach downy mildew from seeds harboring oospores and from seeds that had been coated artificially with oospores prior to planting in isolator chambers, essentially replicating the results of the 2021-2022 experiment which also revealed seed and soil transmission of spinach downy mildew. An additional experiment conducted in a mist tent with the same seed lot samples confirmed seed transmission. We further tested whether the biopesticide AgroPro or the surfactant R-11 could reduce symptoms and sporulation of *P. effusa.* In experiments in 2022, we observed reductions in disease incidence when spinach plants were treated with the surfactant R-11 but not the biopesticide AgroPro. We previously reported that AgroPro and R-11 inhibit sporangia germination directly on water agar plates. In mist tent experiments, we observed phytotoxicity on spinach seedlings that was caused by both R-11 and AgroPro, but not on plants grown in the field. In summary, tracking the levels of windborne inoculum of *B. lactucae* and *P. effusa* in different valleys in California can be valuable to inform on the epidemiology of downy mildews for efficient spray applications for disease control. Analyses of weather parameters in relation to the levels of inoculum are ongoing. Oospores of *P. effusa* have been detected in 85 out of 448 (19%) seed lots that were examined since 2014, and oospore germination experiments in this period indicate their survival for at least six years on seed. Reductions in the amounts of infected spinach seeds entering the production stream, seed treatments, and use of surfactant-type biopesticides may help to curtail spinach downy mildew.

**PROJECT TITLE: Downy mildew detection, epidemiology, and biopesticide evaluation**


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

**1:** Compare the relative airborne detection values of *Peronospora effusa* and *Bremia lactucae* in the Salinas, Coachella, and Imperial Valleys of California.

**2:** Examine *Peronospora effusa* oospore production and seed transmission of downy mildew disease of spinach.

**3:** Complete biopesticide testing in mist tents, microplots and pre-inoculated field experiments.

**PROCEDURES:**

**Immediate objective 1:** Cyclone spore traps (Fig. 1; Root Applied Sciences) were sampled three times weekly (approximately 48 or 72 hr intervals) in the Salinas, Coachella, and Imperial Valleys of California. The traps in each location were placed at the north and south ends of each valley (one cyclone trap on each end). For each of these traps, we made the effort to keep the traps removed from the vicinity of local spinach and lettuce fields. Samples collected by cooperators A. Putman and A. Montazar were shipped to Salinas on ice packs, once per month, to prevent degradation of the samples. Following shipping, collected tubes from the cyclone spore traps were stored at 4 ºC until DNA extraction using the Nucleospin Plant II kit (Machery Nagel). We had previously been successful in devising assays for quantifying the levels of airborne spores of *P. effusa* [2,3,11] and *Bremia lactucae* [12], and we combined both mitochondrial markers in a single reaction for dual detection of both downy mildew pathogens and submitted this work for publication [4]. The spore estimates observed per sample were based on standard curve calculations prepared for both *B. lactucae* and *P. effusa*.


**Figure 1**. Cyclone spore trap (Root Applied Sciences, Berkeley, CA).

**Immediate objective 2:** We examined additional commercial seed lots for the presence of *P. effusa*, to aid in seed transmission testing. For seed testing, the standard 1000 seeds were mixed with water and briefly vortexed then centrifuged at low speed, and the sediment was examined by systematically recording counts of oospores (or lack thereof) in an area of a coverslip, under compound microscopy. The oospores are approx. 30 micrometers in diameter, are brownish in color, and have a smooth round wall [1,8,10,13].

In the winter period of 2022-2023 (November – January), two different oospore-positive seed lot samples identified were planted in glass isolators (Fig. 2) to examine disease transmission. The experimental set up was a replication of the 2021-2022 disease transmission experiment. This included planting seed coated with oospore-infested leaves and a negative control which was a Viroflay seed sample in which we did not detect oospores. Seeds were planted in the isolators at a density equivalent of 10-13 million seeds per acre. The soil was fumigated ahead of the experiments with metam sodium and leaf wetness provided by an internal overhead sprinkler system installed in each of twelve isolator compartments. Plants were evaluated after emergence through the glass of the isolators for development of disease symptoms, and ultimately opened and evaluated for symptoms at 34 days after planting and the numbers of plants with symptoms and sporulation (incidence) recorded.

**Immediate objective 3:** Due to difficulty with obtaining adequate disease pressure by relying on natural inoculum in the field and microplots, we focused on three mist tent experiments during this reporting period for biopesticide tests. However, PI Klosterman sought and obtained approval from the CDFA to conduct outdoor *P. effusa* inoculations in the future. To conduct the biopesticide testing in mist tents in the current experiments, Viroflay was grown in trays. One tray with approx. 50-60 spinach plants was used for each treatment with a total of four different treatments (water, AgroPro at 4%, R-11 at 0.125%, and AgroPro with R-11). Plants were treated with the biopesticides 24 hrs and again at 2 hrs before inoculation with downy mildew. For the mist tent experiments, disease incidence was evaluated by counting the number of infected leaves out of a subsample of 25-50 leaves at least two separate times for each treatment. This assay was repeated three times independently.

For field analysis, we established a field trial at the USDA Spence field alongside Dr. Charlie Brummer's spinach plot to facilitate downy mildew disease dispersal onto field plants. All four treatments from the mist tent experiments were used. The susceptible cultivar Viroflay, which was confirmed to be free of *P. effusa* oospores, was planted on the south side of the *P. effusa*-inoculated UC Davis spinach breeding trial in 40" beds. The plots were sprinkler irrigated. Three replicates each consisting of a 10 feet sections of spinach were treated with either AgroPro at 4%, R-11 at 0.125%, AgroPro with R-11, or water as a control. Disease incidence was evaluated by counting the number of infected leaves out of a subsample of 100-200 leaves three separate times for each treatment.

**Figure 2.** Glass isolators at the USDA ARS station in Salinas, CA.

**RESULTS:**
**Objective 1:**
***Bremia lactucae***

Replicate experiments from 2021-2022 were conducted in 2022-2023 to compare the airborne detection levels of *B. lactucae* in the Salinas, Coachella, and Imperial Valleys of California. The quantities of *B. lactucae* detected were clearly dependent on the growing season in the Salinas and Imperial Valleys, and thus the presence of the crop (Fig. 3). Unlike the 2021-2022 period, sampling values indicated reduction in the detection of *B. lactucae* in December – January 2023 in Salinas. In the Coachella and Imperial Valleys there were long spans where *B. lactucae* was not detected in October – November 2022, which could be due to the hot and dry climate in those valleys.

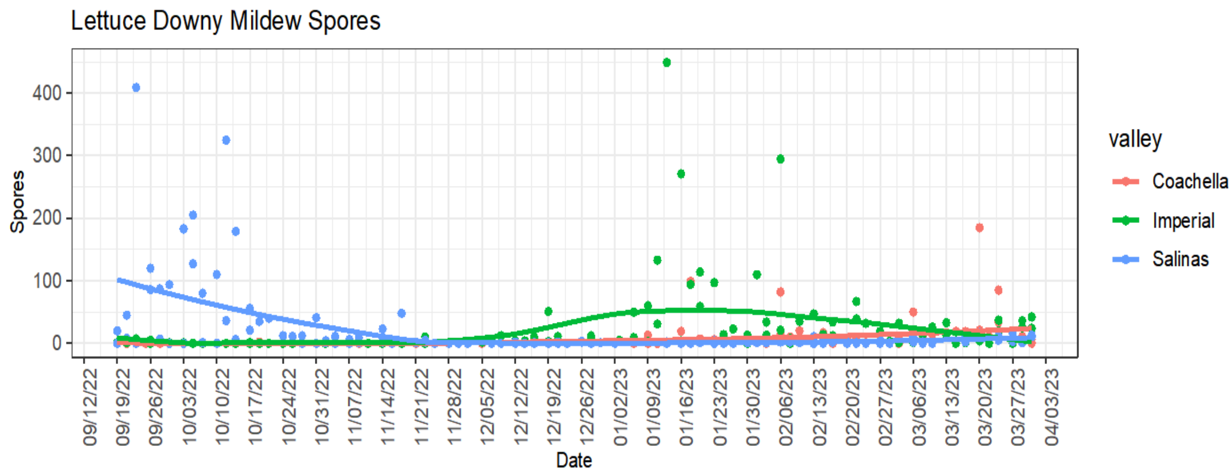


**Figure 3.** Comparison of *B. lactucae* detection in the Coachella, Imperial, and Salinas Valleys from end of September 2022 through March 2023. *B. lactucae* spore titers are the highest in the lettuce growing season of the respective valley.

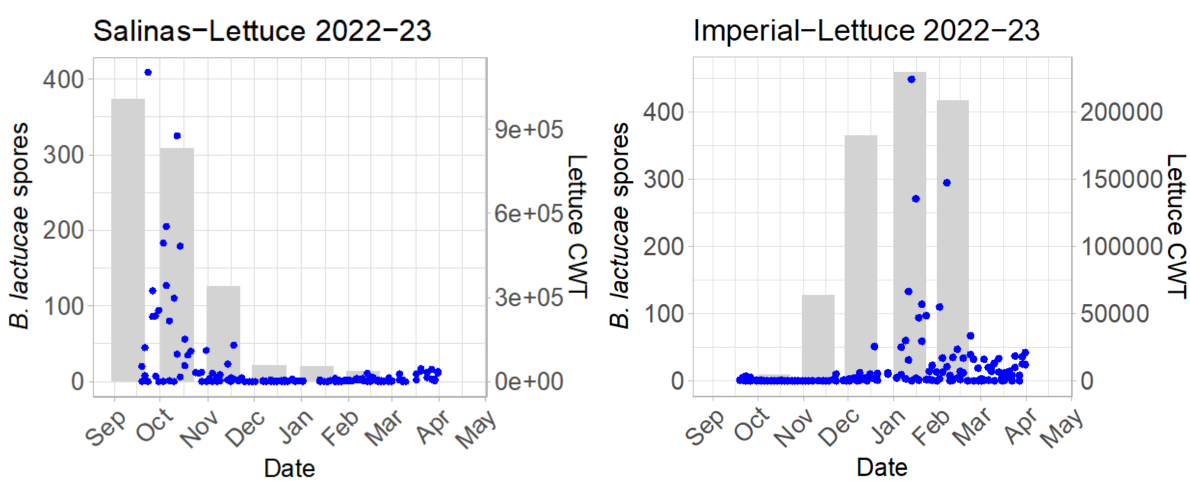


**Figure 4.** Quantification of *Bremia lactucae* spores from the end of September – March, 2022-2023 in the Salinas and Imperial Valleys overlaid with harvested crop volume. Spore counts detected are indicated by the blue dots. Note that the harvested crop volumes for Imperial lettuce represent District A (Imperial, Riverside, San Bernadino counties) and the volumes were not available for March 2023.

***Peronospora effusa***

Replicate experiments from 2021-2022 were conducted in 2022-2023 to compare the airborne detection levels of *P. effusa* in the Salinas, Coachella, and Imperial Valleys of California (Fig. 5). The quantities of *P. effusa* detected were not as dependent on the growing season in the Salinas Valley. In the Imperial Valley, there was a clear correlation between the presence of the crop and increased *P. effusa* quantities detected (Fig. 6). Unlike the 2021-2022 period, sampling values indicated a spike in increased quantities of *P. effusa* in March 2023 in the Salinas Valley. In the Imperial Valley there were long spans where *P. effusa* was not detected, as in most of October – November 2022, which could be due to the hot and dry climate in those valleys.

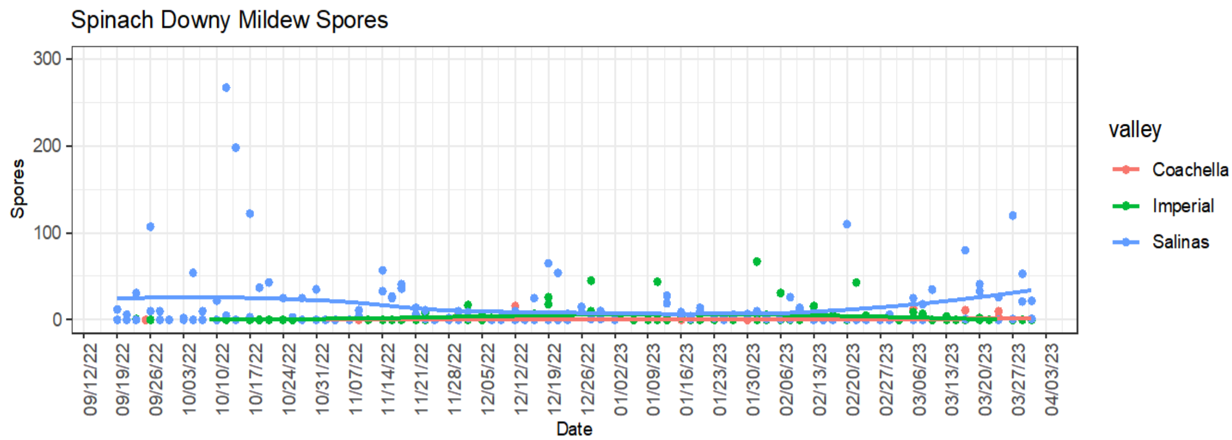


**Figure 5.** Comparison of *Peronospora effusa* detection in the Coachella, Imperial, and Salinas Valleys from end of September 2022 through March 2023.

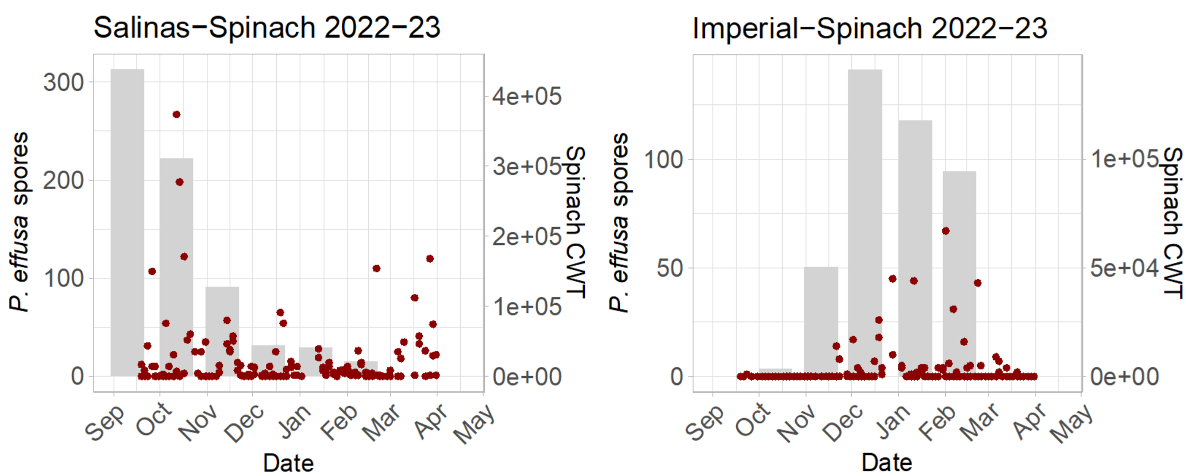


**Figure 6.** Quantification of *Peronospora effusa* spores from the end of September – March, 2022-2023 in the Salinas and Imperial Valleys overlaid with harvested crop volume. Spore counts detected are indicated by the red dots. Note the harvested crop volumes for Imperial spinach represent District A (Imperial, Riverside, San Bernadino counties) and the volumes were not available for March 2023.

**Objective 2:**

We investigated seed transmission of spinach downy mildew in the winter of 2022-2023 to confirm results from the winter of 2021-2022. In the effort to remove the possibility of windborne inoculum contaminating the experiments, we used the isolator system shown in Figure 2 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 clean non-infested cultivar Viroflay seeds, and two oospore-infested seed lots within different isolator chambers. In another treatment, the clean non-infested seed of Viroflay was coated with oospore-infested spinach leaves. Like the 2021-2022 experiment, we observed sporulation on leaves arising from oospore-infested seed (Figure 7). We also observed heavy sporulation in the isolator with plants grown from seed coated with oospore-infested leaves (Figure 7). We did not observe sporulation on the negative control seeds in the experiment (Viroflay seed with no detected oospores) as shown in Figure 7. These results confirmed seed transmission of spinach downy previously reported [7], though those earlier results were obtained using experiments without isolators and thus these experiments were necessary for confirmation of seed transmission.


**Figure 7. A)** *Peronospora effusa* sporulation on cultivar Viroflay, arising from seed coated with oospore-infested leaf debris grown in the contained isolator chamber. **B)** Healthy leaves from Viroflay seed grown in the isolators in which there were no *P. effusa* oospores. **C)** *P. effusa* sporulation on downy mildew-infected leaves from the grow out of an oospore-infested seed sample. **D)** Healthy leaves from cultivar Viroflay seed that was free of oospores.

Figure 8 from collaborator Dr. Nina Shishkoff also shows the result of a grow out from an oospore-infested spinach seed. This seedling was 1 out of 88 oospore-infested seeds to give rise to an infected seedling with visible sporangiophores of *P. effusa*. A similar result was obtained with oospore-infested seed grow outs of another cultivar, but in this instance, it was 1 out of 159 oospore-infested seeds that gave rise to an infected seedling. This result further documents the seed transmission of spinach downy mildew.

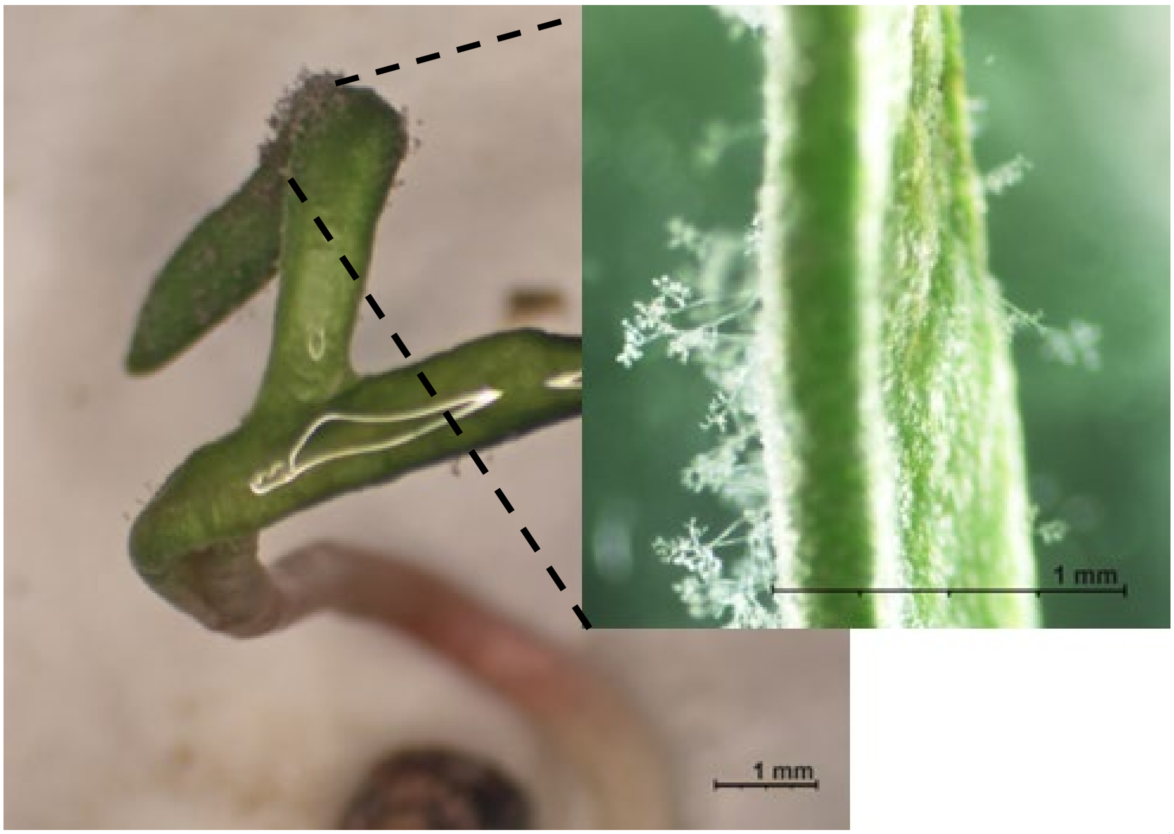


**Figure 8.** Grow out of a *P. effusa* oospore-infested seed. The enlargement shows sporangiophores emerging from a cotyledon.

**Objective 3:**

Biopesticide testing was conducted in mist tent experiments from August to October 2022. Treatments were applied to spinach 24 hrs and 2 hrs before inoculation. Table 1 is shown as a representative of one of the three replicate experiments conducted in this reporting period with AgroPro, R-11, and AgroPro plus R-11. Results from all three experiments were similar.

The results indicated a substantial reduction in average disease incidence with the use of the surfactant R-11 (Table 1). Unlike R-11, AgroPro did not substantially reduce disease incidence. When AgroPro was combined with R-11, this treatment clearly resulted in reduced disease incidence, again suggesting that the R-11 was effective in reducing downy mildew disease incidence. We also calculated disease incidence based on symptomatic true leaves in the mist tent experiments rather than whole plants, which provides a better representation of biopesticide effect as it may coat leaves more evenly that are at the top of the canopy.

**Table 1.** Biopesticide evaluations conducted in a mist tent, in October, 2022.

| Treatment | infected leaves | healthy leaves | total leaves | Disease incidence (%) | Avg. disease incidence (%) |
|---|---|---|---|---|---|
| R-11 | | | | | |
| rep 1 | 3 | 29 | 32 | 9.38 | 8.02 |
| rep 2 | 2 | 28 | 30 | 6.67 | |
| Agro Pro | | | | | |
| rep 1 | 23 | 14 | 37 | 62.16 | 55.41 |
| rep 2 | 18 | 19 | 37 | 48.65 | |
| AgroPro + R-11 | | | | | |
| rep 1 | 2 | 23 | 25 | 8.00 | 4.00 |
| rep 2 | 0 | 25 | 25 | 0.00 | |
| Water | | | | | |
| rep 1 | 21 | 9 | 30 | 70.00 | 64.17 |
| rep 2 | 21 | 15 | 36 | 58.33 | |

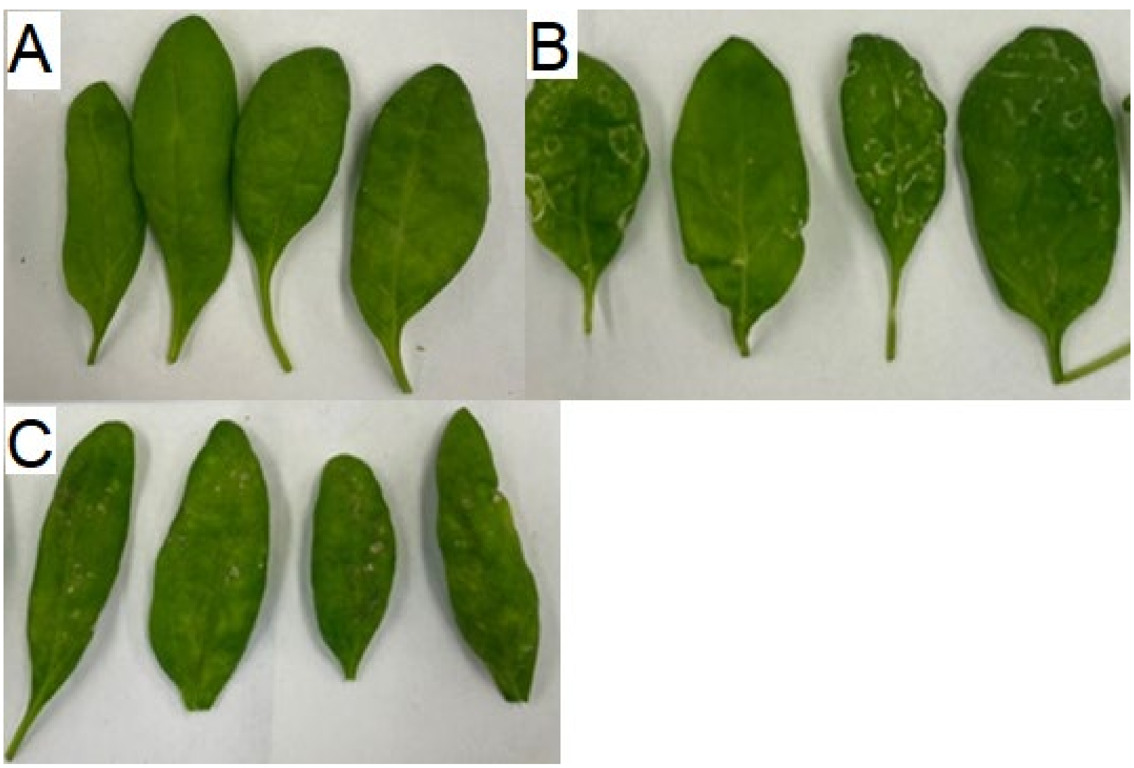


**Figure 9.** Analyses of phytotoxicity of spinach leaves from mist tent experiments following treatment with **A)** Water **B)** R-11 and **C)** AgroPro. Phytotoxicity of either R-11 or AgroPro was not observed in outdoor trials.

The AgroPro and R-11 treatments in mist tents resulted in mild phytotoxicity (Fig. 9). However, this phytotoxicity was not observed for the same concentrations of AgroPro and R-11 applied in the field or microplots, suggesting that the lighting, humidity, and other conditions within the mist tent environment may compromise spinach health.

For the field trial at Spence, the treatments included AgroPro at 4%, R-11 at 0.125%, and AgroPro with R-11 using and water as a control. Spinach plants were treated twice -- once at 24 hrs before inoculation and again at 2 hrs before inoculation. Inoculation of field plants was performed by personnel of collaborating PIs, Allen Van Deynze, Charlie Brummer. Naturally occurring downy mildew disease was also occurring due to inoculum coming from wind-blown spores in an adjacent field trial. However, the timing of initial disease appearance from naturally occurring inoculum (at about the same time as treatment application followed by subsequent inoculation) did not allow for adequate evaluation of the biopesticide products in the field during this reporting period.

**DISCUSSION:**

In the current reporting period, we used the recently developed multiplex qPCR assay prepared by Clark et al. [4] for the quantification of airborne *P. effusa* and *B. lactucae* simultaneously to compare the airborne detection levels of *P. effusa* in the Salinas, Coachella, and Imperial Valleys of California. In the 2022-2023 period there was little downy mildew detected from either spinach or lettuce in the Coachella Valley, possibly due to the lower volume of spinach and lettuce crops grown in Coachella relative to Imperial and Salinas. In the Imperial and Salinas Valleys, the amounts of *B. lactucae* and *P. effusa* detected were dependent on the growing season and location. There were generally more of both pathogens present during the respective growing seasons in each location. But remarkably in the Imperial Valley there were nearly month-long spans around October in both 2021 and 2022 where *P. effusa* and *B. lactucae* were not detected. This period represents a hot and dry period (average daily high temperature was 91$^{o}$ F in October 2022), but it is also beginning of the growing season for spinach and lettuce in the Imperial Valley and thus crops are present. This suggests that, because we know windborne sporangia of *P. effusa* are short-lived, especially under hot conditions [5], both airborne pathogens are eliminated and there is an alternative source of inoculum (other than airborne) that results in disease. Based upon our more conclusive findings of seed transmission of spinach downy mildew in this reporting period, and those from the past [7], the most likely explanation is that the primary inoculum source of *P. effusa* that initiates disease must reside in the spinach seed. However, our current work also confirmed that oospore-infested leaf material in the soil can transmit spinach downy mildew and thus we cannot rule out that oospores present in soil may act as primary inoculum. Additional work for the next period will focus on comparing weather parameters in correlation with spore quantities detected in each valley.

Since *P. effusa* is heterothallic, two strains of different mating type are required to form the sexual oospores [6]. Examination of the germination of oospores from older seed lot samples in this period revealed that the oospores can survive long term, at least for ~ 6 years on seed at room temperature (K.J. Clark and S.J. Klosterman et al. unpublished), and further confirms viability of oospores from seeds [8,13]. The presence of oospores on 19% of spinach seed lots (85/448) indicates that survival of the pathogen on seed is commonplace. As a result, new pathotypes or races of the pathogen, as well as both mating types, can be dispersed from different locations and then arrive on seeds in the Salinas Valley. 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; it is established that sexual reproduction contributes to the evolution of resistance-breaking isolates of *P. effusa* [14]. Thus, new pathotypes arriving on seed and those that may arise from gene shuffling that occurs during sexual reproduction once the different mating types are brought in proximity may initiate disease outbreaks. Given previous evidence of seed transmission [7], this further advances the importance of oospores arriving on spinach seed in initiating disease in current and new spinach production areas.

Importantly, the current work in collaboration with Dr. Nina Shishkoff revealed grow outs of oospore infested seeds resulted in downy mildew infested seedlings. This was not demonstrated previously. The finding of downy mildew growing from oospore-positive seed was observed at a low percentage on two different cultivars. In one cultivar the occurrence was 1 out of 88 seeds examined, and in the second, it was 1 out 159. Nevertheless, in a baby leaf spinach field planted with 3 million or more seeds per acre, there would be ample chances for infections under the appropriate conditions.

Due to difficulty with obtaining consistent disease pressure by relying on natural inoculum in the field and microplots, we primarily focused on three mist tent experiments during the 2022-2023 reporting period. However, PI Klosterman sought and obtained approval from the CDFA during this reporting period to conduct outdoor *P. effusa* inoculations with an endemic California isolate in future field trials. Thus, in this reporting period, testing was conducted in mist tents to evaluate the effectiveness of the biopesticide AgroPro and the surfactant R-11 for protection from spinach downy mildew. During previous biopesticide evaluations, we observed that among all biopesticides evaluated, only the biopesticide Procidic provided protection against downy mildew [9] – however, from recent experiments in the mist tent looking at the chemicals separately it became apparent that the non-ionic surfactant mixed with Procidic for application (R-11) was providing most of the protection. This is encouraging since surfactant products like R-11 have been approved for use in organic production. In these current studies, we found that the R-11 can inhibit downy mildew disease incidence on spinach. However, AgroPro did not inhibit downy mildew disease incidence on spinach in the mist tent experiments. In the next reporting period, field experiments at the USDA Spence farm will be conducted to evaluate R-11 as a preventative treatment prior to inoculation of plants with a spinach downy mildew isolate endemic of California.

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