CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

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

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ABSTRACT

Downy mildew on spinach is caused by *Peronospora effusa* (*P. effusa*), an oomycete microorganism. Downy mildew is the most widespread and destructive spinach disease in California. Fungicide applications are available for the control of this disease in conventional production, but no control measures are available for organically produced spinach. To assess the factors required for disease outbreaks on spinach, and also to identify potential sources of the pathogen, a DNA-based assay was developed to determine the amount of *P. effusa* in air and other samples. This assay may be useful in disease forecasting. The first major objective of this research entailed developing of a sensitive DNA-based detection system for *P. effusa*. DNA sequences between *P. effusa* and isolates of *Peronospora farinosa* from Swiss chard (f. sp. *cicla*) or beet (f sp. *betae*) are highly similar. Because chard is grown in close association with spinach, and commonly infected with *P. farinosa cicla*, there is the potential for interference of this pathogen in assays designed for detection of *P. effusa*. Therefore the assay incorporates independent specific probes to assess the levels of both of these pathogens in the environment. Specificity of the DNA-based assay against other *Peronospora* species was verified by testing against DNA samples from different downy mildew infected host plants. A second major objective of this research entailed airborne detection and quantification of *P. effusa* using the DNA-based assay. Two experiments were conducted in 2012 to determine the feasibility of airborne detection and quantification of *P. effusa* using the DNA assay and a spore trap system. The experiments revealed detection of *P. effusa* at spore trap locations up and downwind of spinach fields. An additional on-going experiment was initiated in January, 2013 to assess the airborne inoculum level of *P. effusa* at four different sites in the Salinas Valley, including near Salinas, Gonzales, Soledad, and King City. The results suggest a continuous "blanket" of spores present throughout the valley, with times when more *P. effusa* is detectable. Ongoing work is aimed at determining how weather patterns and disease outbreaks are correlated with the fluctuations in downy mildew pathogen DNA levels detected at spore trap sites. Microscopy studies were conducted to determine if sexually produced oospores of the downy mildew pathogen are present in leaf samples and seeds. Oospores characteristic of *P. effusa* were detected in leaves of one spinach sample from a greenhouse study, although they were not identified in leaves of field samples collected in the Salinas Valley. Additional studies are underway to determine if *P. effusa* oopores are present in field samples. *P. effusa* was detectable on multiple commercial seed lots tested, as determined by the DNA-based assay. Additional work is required to determine whether *Peronopora* sp. detected on spinach seeds is viable.

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

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OBJECTIVE: **To develop a rapid assay for DNA-based detection and quantification of the downy mildew pathogen, *Peronospora effusa***.

PROCEDURES:

To select optimal primer and probe sequence for use in a DNA-based assay that employed quantitative real time PCR (qPCR), DNA sequences from *P. effusa*, *P. farinosa* and other oomycetes were aligned and examined for differences and similarities using DNasis software (Hitachi Software). Multiple primer sets were designed and tested for specific detection of *P. effusa.* Examination of several target DNA sequences, including mitochondrial and nuclear loci, revealed the highest level of DNA sequence similarity between *P. effusa* and isolates of *P. farinosa* from Swiss chard (f. sp. *cicla*) or beet (f sp. *betae*). Regions of the DNA sequences which were different were used to design primers or probes for differentiating *P. effusa* from related species. A single nucleotide polymorphism (SNP) depicted in Figure 1 was identified to differentiate *P. effusa* from the closely related *P. farinosa* f. sp. *cicla/betae* isolates. For further verification of the specificity of the DNA-based assay, DNA samples of downy mildew infected plants were obtained from Dr. Marco Thines (Frankfurt, Germany) and Dr. Hermann Voglmayr (Vienna, Austria), researchers with expertise in population analyses of *Peronospora* spp., and tested against both the *P. effusa* primer set and probe, and the *P. farinosa* (beet or Swiss Chard) primer/probe set using qPCR.

Spore traps (Figure 2) obtained from Dr. Walt Mahaffee (USDA ARS, Corvalis, OR) were sampled at approximately 48 or 72 hr intervals for windborne inoculum of *P. effusa.* The pairs of

1.1 mm x 40 mm stainless steel rods coated in silicone vacuum grease (Dow Corning) are held in place at the top of the spore trap head (Figure 2B). The metal rods spin on a solar/battery powered motor controlled arm at a high cyclic rate, enabling small particles (such as downy mildew spores) to stick to the rods. To ensure that the rods were not contaminated by residual grease and dirt, the rotating arm and grommets were washed with isopropanol and wiped with a clean paper towel at each sampling. The collected rods were stored at 4^{o}C until DNA extraction using the Nucleospin Plant II kit (Machery Nagel) following the manufacturer's protocol for isolating genomic DNA from fungi.

To ensure that the amplification protocol was working, standard curves using known quantities of the target DNA sequence from *P. effusa* or *P. farinosa* f. sp. *cicla* were prepared by cloning the respective target DNAs into plasmids. To get an estimate of the amount of spores detected in relation to a DNA standard curve, known numbers of spores were extracted for DNA using the same procedure as for the sampling rod extraction. Spores were enumerated by dipping human hair on a spore covered leaf, counting the spores on the hair under light microscopy and combining a clean grease-coated rod in the sampling tube for extraction. DNA target copy number was plotted relative to spore count for each sample, ranging from 6 to 112 spores. The trend line placed on this plot revealed values $\leq$ 10,000 copy number obtained from qPCR corresponded to $\leq$ 20 spores. A copy number of 150,000 corresponded to $\geq$ 100 spores.

QPCR was carried out using a Taqman® assay using the approach outlined in Figure 1 with a LightCycler 480 II (Roche Diagnostics). Probes labeled with FAM™ and VIC® fluorescent dyes (Applied Biosystems) were used in detection of *P. effusa* and *P. farinosa* f. sp. *cicla* DNA, respectively, with the appropriate filters of the LightCycler 480 II (Roche Diagnostics). The ratio of *P. effusa* to *P. farinosa* DNA amount was obtained using the method of Germer et al [6].

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

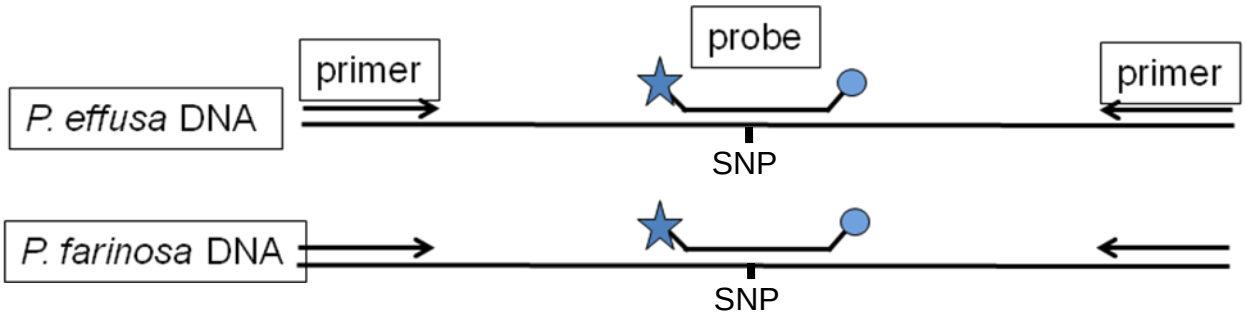

Figure 1. Illustration of the primer/probe combinations used in quantitative real time PCR assays to quantify *Peronospora effusa* and *Peronospora farinosa* DNA target sequences. FAM™ and VIC® fluorescent dyes (Applied Biosystems), indicated by blue stars, were attached as labels to the probes for detection of *P. effusa* and *P. farinosa*, respectively. Probes were quenched with MGB (blue circles).

RESULTS:

Initial tests were conducted to determine the most appropriate target DNA sequences to use in the qPCR assay. Single nucleotide polymorphisms (SNPs), or single nucleotide differences in the target sequence, were identified in the target DNA sequence to differentiate *P. effusa* from *P. farinosa* f sp. *betae* and f. sp. *cicla* (Figure 1). However, even taking advantage of the SNP in the probe design, the primer/probe combination for *P. effusa* yielded a low detection signal for *P. farinosa* from *Beta vulgaris* cultivars (Table 1). The Taqman assay did show specificity for *P. effusa* as it was tested against a range of downy mildew DNA isolated from various host plants, including multiple related isolates from plant hosts commonly found in the U.S. (Table 1). Because the primer/probe combination for *P. effusa* yielded a low detection signal for *P. farinosa* from *Beta vulgaris* cultivars (Table 1), dual qPCR analyses were subsequently performed on each sample to quantify the amounts of both the chard and spinach downy mildew pathogens.

Table 1. Specificity of Taqman probes and primers for detection of *Peronospora effusa* and *Peronospora farinosa* f sp. *cicla*

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

Two major pilot experiments were conducted in 2012 to determine the feasibility of airborne detection and quantification of *P. effusa* using the DNA assay developed and a spore trap system as shown in Figure 2. For the first experiment, conducted May 1 to May 16, 2012, nine spore trap sites were placed near an organic spinach field south of King City, CA. For this initial experiment, three traps were placed on the northern end of an organic spinach field (northern traps), three trap sites were established in the middle of the field (middle traps), and three trap sites were established toward the southern end of the field (southern traps). The distance between the northern and southern traps was approximately one mile. The sampling rods were collected every 48 hours during the period and tested for the presence of *P. effusa* using a PCR-based DNA assay. Wind direction was recorded as north to south, and temperatures during this period reached > 90^{o}F. The second experiment, conducted in the period of July 20 to August 27, south of King City, CA, was conducted similarly, except there were a total of six traps with two traps placed north, middle, and south. Results of these experiments revealed the presence of DNA from *P. effusa*, both on the north, middle, and southern traps in both experiments.

To further assess the amount of *P. effusa* present in the Salinas Valley over time, spore traps were placed at ~10-15 mile intervals in the Salinas Valley at four different sites (Fig. 3). Rods were collected at approximately 48 to 72 hr intervals at all four sites. This experiment is ongoing and samples have been collected from January 15, 2013 to the present time (May 30). Results of this experiment in the window from Feb. 1 to Mar. 4 (Fig. 4), revealed detectable *P. effusa* DNA

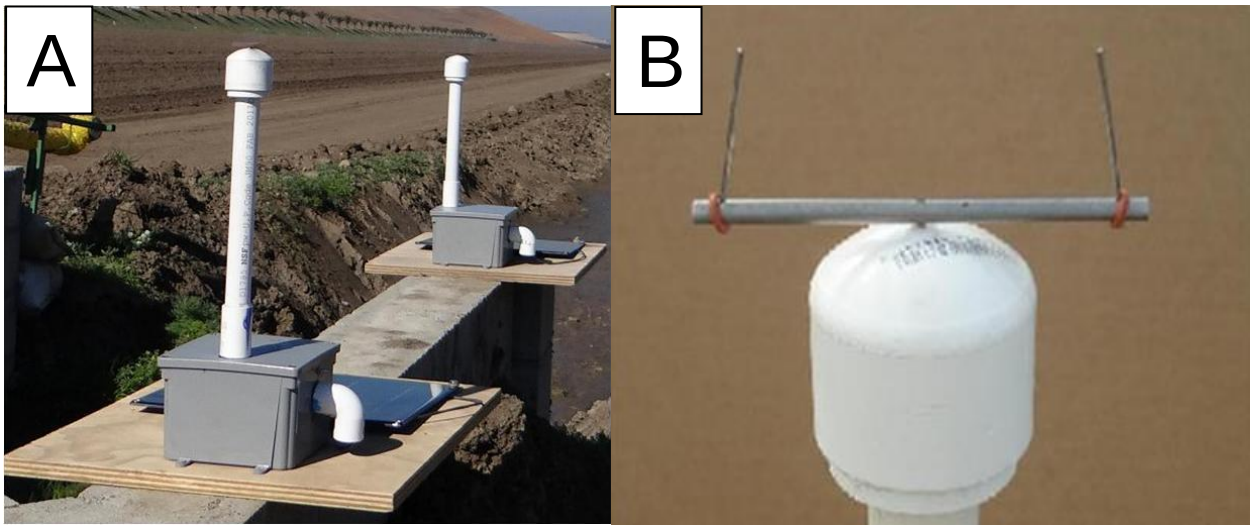

Figure 2. Spore trap system to detect *Peronospora effusa*, the causal agent of spinach downy mildew in the Salinas Valley, California. A) Two spore traps in operation south of King City, CA. B) Spore trap head with rotating arm and removable stainless steel rods.

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

with the exception of Gonzales on Mar. 1 and the Salinas, Gonzales and King City traps on Mar. 4 (Fig. 4). However, there was an increase in the amounts detected in the period of Feb. 6 to Feb. 19 at four spore trap locations (Fig. 4). The highest levels of precipitation in the period of

Feb. 1 to Mar. 4 were recorded on Feb. 8 (0.05 in.) and Feb. 19 (0.19 in.). The four dates of lowest solar radiation in the period were Feb. 2, 3, 5 and Feb. 18. (Statewide IPM Program, Agriculture and Natural Resources, Univ. of California, KINGCITY.A. weather station).

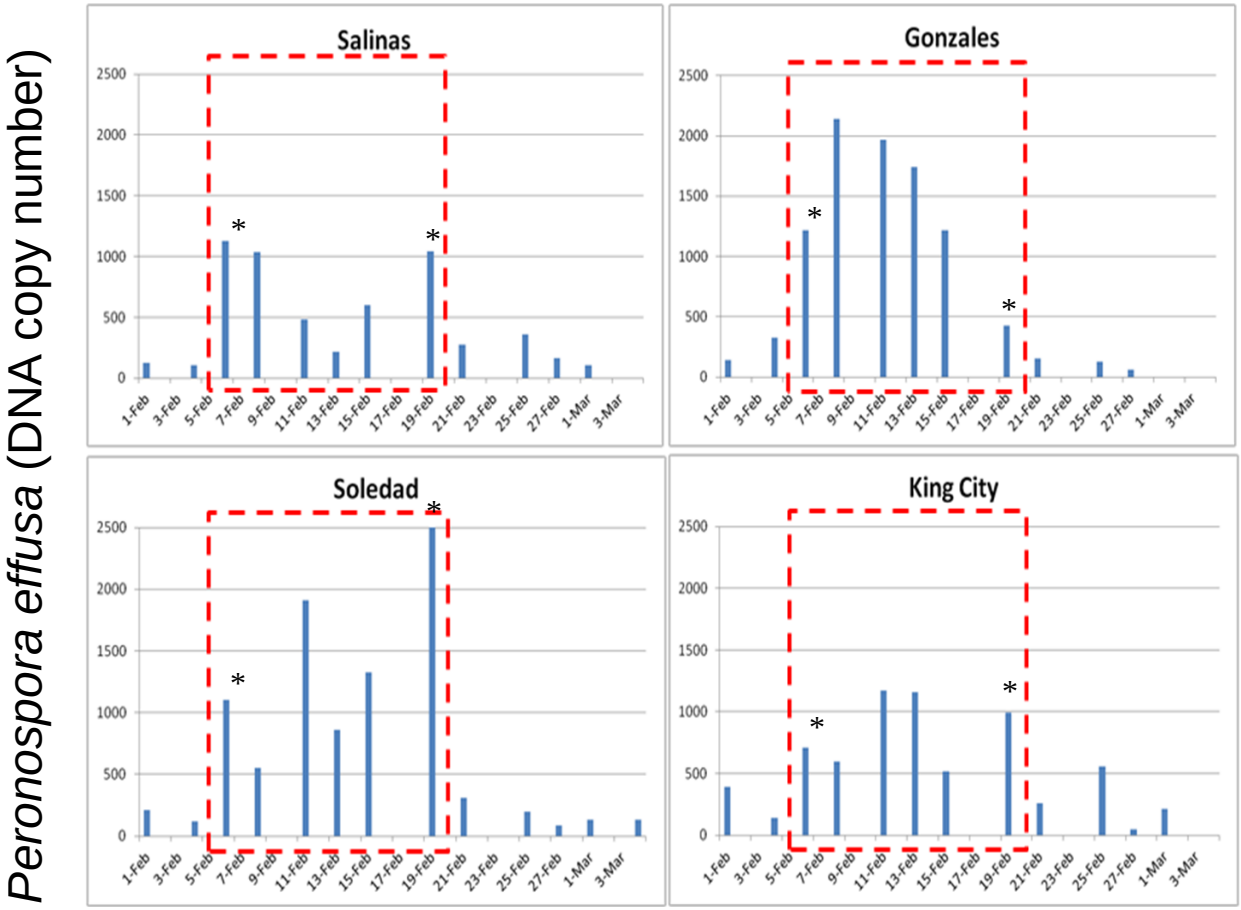

Figure 4. Quantification of *Peronospora effusa* (DNA copy number) at four spore trap sites (Feb. 1 to Mar. 4, 2013). The DNA copy number was obtained from a standard curve using a dilution of DNA target cloned from *Peronospora effusa*. *Denotes the two dates of highest precipitation.

During the period of Mar. 15 to April 3, 2013 two additional spore traps (designated as Sol2 in Table 2) were deployed at the north and south ends of a spinach field with a known infection of downy mildew. This field was located approximately 1 mile southwest of the original soledad spore trap site (Sol1). Increased amounts of detectable pathogen DNA relative to the other spore trap locations were recorded on each of the Sol2 traps during the period of Mar. 15-25, with the exception of the Mar. 22 date. A window of the *P. effusa* and *P. farinosa* (chard) detection and quantificaition values from Mar. 15-25 are shown in Table 2. The highest readings of pathogen copy number occurred on Mar. 20 (Table 2), which also corresponded to the date with lowest level of solar radiation for the month of Mar., 2013 (Statewide IPM Program, Agriculture and Natural Resources, Univ. of California, ARYOSECO.A. weather station, near Soledad, CA).

The Sol2 spore traps are highlighted in green in Table 2 to indicate that these traps were the only traps placed immediately at the boundary of a known downy mildew infected spinach field.

Table 2. Taqman qPCR assays to detect *Peronospora* DNA from spore trap samples

Loc[a]	Sample	*P. effusa* %[b]	*P. farinosa* %[b]	*P. effusa* copy #[c]	Spore est[d]	Date
Sol2	611	99.98	0.02	383208.14	>100	3/15/13
Sol2	612	99.98	0.02	1080752.88	>100	3/15/13
Sal	613	82.41	17.59	149.94	<20	3/15/13
Sal	614	100.00	0.00	54.55	<20	3/15/13
Gon	615	0.00	100.00	0.00	0	3/18/13
Gon	616	6.76	93.24	104.43	<20	3/18/13
KC	617	74.92	25.08	1110.58	<20	3/18/13
KC	618	2.21	97.79	130.21	<20	3/18/13
KC	619	97.46	2.54	5123.62	<20	3/18/13
KC	620	96.15	3.85	3513.62	<20	3/18/13
Sol1	621	58.89	41.11	176.07	<20	3/18/13
Sol1	622	100.00	0.00	0.00	0	3/18/13
Sol2	623	99.41	0.59	34313.77	>20	3/18/13
Sol2	624	98.09	1.91	1395570.34	>100	3/18/13
Sal	625	0.00	100.00	0.00	0	3/18/13
Sal	626	0.00	0.00	0.00	0	3/18/13
Gon	627	0.00	100.00	0.00	0	3/20/13
Gon	628	99.51	0.49	6038.27	<20	3/20/13
KC	629	98.04	1.96	7349.48	<20	3/20/13
KC	630	78.07	21.93	2801.54	<20	3/20/13
KC	631	94.71	5.29	2056.19	<20	3/20/13
KC	632	95.20	4.80	14334.66	<20	3/20/13
Sol1	633	83.29	16.71	703.96	<20	3/20/13
Sol1	634	93.34	6.66	8082.71	<20	3/20/13
Sol2	635	99.89	0.11	4318421.62	>100	3/20/13
Sol2	636	99.81	0.19	3390286.13	>100	3/20/13
Sal	637	33.16	66.84	4349.25	<20	3/20/13
Sal	638	7.00	93.00	306.68	<20	3/20/13
Gon	639	47.70	52.30	176.28	<20	3/22/13
Gon	640	1.38	98.62	151.16	<20	3/22/13
KC	641	90.16	9.84	4697.26	<20	3/22/13
KC	642	86.93	13.07	8026.73	<20	3/22/13
KC	643	12.88	87.12	5009.78	<20	3/22/13
KC	644	67.78	32.22	4996.33	<20	3/22/13
Sol1	645	0.00	100.00	0.00	0	3/22/13
Sol1	646	97.05	2.95	2901.00	<20	3/22/13
Sol2	647	58.13	41.87	3776.42	<20	3/22/13
Sol2	648	ND[e]	ND	ND	ND	3/22/13
Sal	649	100.00	0.00	81.57	<20	3/22/13
Sal	650	59.36	40.64	191.47	<20	3/22/13
Gon	651	79.79	20.21	210.77	<20	3/25/13
Gon	652	73.54	26.46	184.37	<20	3/25/13
KC	653	0.00	100.00	0.00	0	3/25/13
KC	654	35.09	64.91	2994.70	<20	3/25/13
KC	655	84.81	15.19	3472.49	<20	3/25/13
KC	656	40.31	59.69	223.56	<20	3/25/13
Sol1	---[f]	ND	ND	ND	ND	ND
Sol1	---	ND	ND	ND	ND	ND
Sol2	657	99.67	0.33	95692.12	>20	3/25/13
Sol2	658	99.87	0.13	89015.86	>20	3/25/13
Sal	659	50.03	49.97	551.15	<20	3/25/13
Sal	660	5.38	94.62	513.52	<20	3/25/13

Table 2. (footnotes)
a = Locations of Gonzales (Gon) King City (KC), Soledad field site 1 (Sol1), Soledad field 2 (Sol2), Sal (Spence road farm near Salinas). The green highlighting indicates location and results obtained from placing spore traps immediately adjacent to a downy mildew-infected spinach field. **b** = Percentage of DNA target detection attributed to *P. effusa* or *P. farinosa* f. sp. *cicla/betae* in qPCR reactions. **c** = Copy number derived from a standard curve with *P. effusa* plasmid DNA dilution. **d** = Estimate of spore number calculated from qPCR copy number as described in the procedures. **e** = Sampling rod not present. **f** =Samples not taken because spore traps were locked within a fenced facility at location Sol1 on this date.

Based upon the calculations of downy mildew spores per pathogen DNA copy number, as described in the procedures, there were fewer than 20 spores detected on most samples at the four locations (Fig. 4, Table 2, and in time periods not shown). However, the results of the Sol2 traps indicated > 100 spores on many of these samples, such as sample numbers 611, 612, 624, 635 and 636 (Table 2).

An additional objective of this research focused on determining whether *P. effusa* is present in spinach leaves as oospores and on spinach seeds. Standard PCR screening of the seed indicated that DNA from *P. effusa* was detectable on all spinach seed lots examined (16 lots total). These results were also confirmed by the analyses of DNA sequences obtained from six of the *P. effusa* PCR-positive seed lots (not shown). Furthermore, microscopy analyses of the seed surface material washed from 1000 seeds in seven seed lots revealed the presence of sporangiophores characteristic of *Peronospora* spp. in 3 of 7 seed lots examined (Figure 5A). Oospores were not detected in the spinach seed lots examined. Oospores were detected in leaf tissue from a sample of downy mildew-infected spinach obtained from the greenhouse of Steve Koike (Figure 5B) and the downy mildew sample was originally obtained from San Benito Co. These oospores are the characteristic size (28 – 35 µm) and brownish color as those previously described elsewhere in spinach [2, 8].

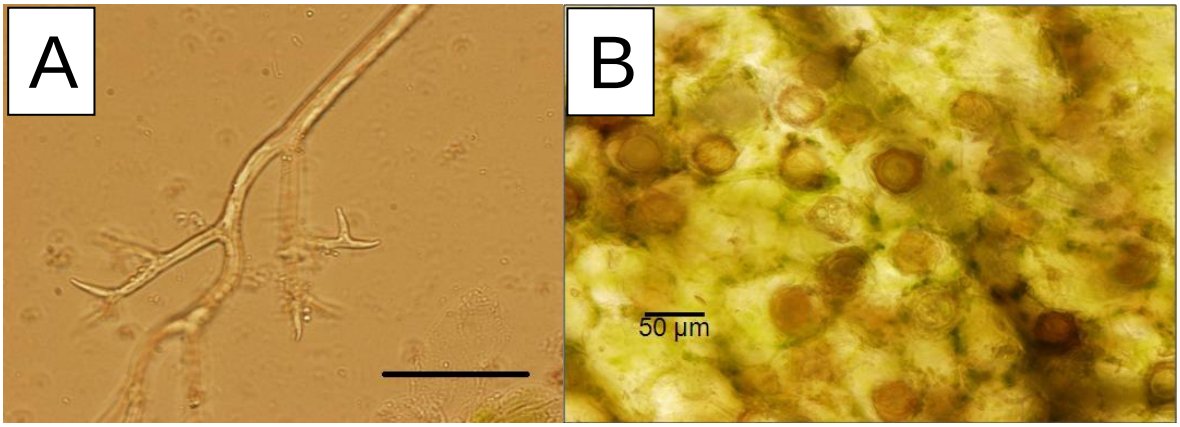

Figure 5. Detection of *Peronospora* spp. A) washed from spinach seed and B) as oospores embedded in leaves. Scale bars are 50 micrometers.

DISCUSSION:

The spore trap system and qPCR assay were successfully deployed to detect both the spinach downy mildew pathogen, *P. effusa*, and the downy mildew pathogen of chard or beet, *P. farinosa* f sp. *cicla/betae* in the Salinas Valley of California. Importantly, the chard or beet pathogens do

not infect spinach [1], although their host plants are commonly infected with closely related *P. farinosa* f sp. *cicla/betae* in California. The presence of the pathogens could potentially interfere with an assay designed for specific detection of *P. effusa*, that only causes downy mildew on spinach [1]. Therefore, specific probes were designed for detection of each pathogen, *P. farinosa* f sp. *cicla*/*betae* and *P. effusa* by taking advantage of a SNP, or single nucleotide difference, identified in the target DNA sequence. This, in combination with other DNA differences, also allowed for the differentiation of other downy mildew strains from other plant hosts.

Tests of the assay specificity revealed that the *P. effusa* primer/probe set was capable of amplification of *P. farinosa* f sp. *cicla/betae* DNA at a low level using DNA purified directly from spores of the pathogen and vice versa. However, in the spore trap system, the detection levels of *P. farinosa* f sp. *cicla/betae* would be negligible when using the *P. effusa* primer/probe set in qPCR of spore trap samples since there is a low quantity of DNA present. Other DNA samples from related pathogens were negative for detection with the *P. effusa* assay. For example, among the DNA samples obtained from downy mildew-infected plant hosts that were tested, infected Lamb's Quarters is commonly present around spinach production areas, but yielded negative results in the sensitive qPCR analyses.

Data analyses from the initial spore trap experiments in 2012 and the larger ongoing experiment initiated in 2013 suggest that there is a "blanket" of airborne *P. effusa* spores generally present throughout the Salinas valley. Based upon correlations between actual spore numbers and DNA copy number detection by qPCR, < 20 spores are generally detectable per sampling site. However, there were also periods of increased pathogen detection within the background blanket level of *P. effusa* at the spore trap sites. Moreover, analyses of detection data from near a downy mildew-infected spinach field near Soledad revealed high levels of the pathogen of > 100 spores/trap. This trend continued over multiple sampling dates within 48 or 72 hr windows of detection, providing additional evidence that the spore trap/qPCR system can be useful to quantify the pathogen in the field.

More analyses are required to assess correlations between weather variables and increases and decreases of *P. effusa* DNA detectable by spore trap sampling. Preliminary findings revealed a correlation between low solar radiation (increased cloud cover) and increased *P. effusa* detection in the Feb. to Mar., 2013 windows of data analyzed. Data analyses also suggested that the sporangia/spores of *P. effusa* are present at higher temperatures than expected, leading to *P. effusa* DNA detection in sampling conditions > 90^{o}F south of King City, in May, 2012.

The commercial spinach seed lots were not infested with downy mildew oospores, at least in the seven lots analyzed from Europe or the Pacific Northwest US. However, previous studies have revealed oospores of *P. effusa* when washed off of commercial spinach seed lots and in seedlings grown from seed lots that were infested with downy mildew [8]. The potential seed infestation with downy mildew requires further assessment.

Additional questions concerning the levels of production of oospores in spinach fields in California, and length of time that the pathogen can survive as oospores in the soils of previously infested spinach fields have not been fully explored. In this work, oospores were observed in spinach leaves that were obtained from the diagnostic greenhouse of Steve Koike (UCCE, Salinas). One of the isolates of downy mildew used in this experiment was obtained from San Benito, Co., CA., and thus oospore production in California may occur frequently. Downy mildew oospores in spinach leaves have not been previously reported in California, but were reported in spinach cotyledons in the Netherlands [5] and Japan [7]. The presence of these sexual structures is indicative of mating between different strains of *P. effusa*, as reported previously

[8]. Mating of different strains of *P. effusa* has implications of quickly increasing the genetic diversity within populations, potentially contributing to the appearance of new races of the pathogen. If seed and/or soils are important primary inoculum sources, this knowledge could lead to seed or soil treatments to reduce downy mildew on spinach.

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

Additional analyses of the spore trap data are underway in conjunction with Dr. Neil McRoberts (UC Davis) to more definitively determine any correlations between weather characteristics and the amount of pathogen DNA detected. Analyses are also underway to determine if *P. effusa* is viable on spinach seeds, and if oospores are present on seeds and form in spinach leaf tissue in fields. Downy mildew isolate collection is ongoing, allowing eventual population genetics analyses as characterized in other studies for populations of *Peronospora* sp. [3].

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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. USDA is an equal opportunity provider and employer.

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