Research Abstract for the California Leafy Greens Research Board
April 2008 to March 2009

Project Title: Verticillium Wilt of Spinach: Detection, Biology and Control

Preliminary report for Objective C: Develop a sensitive, robust, standardized spinach seed health assay for *Verticillium dahliae*

Project Investigators: Steven Koike, Lindsey du Toit, and Krishna Subbarao

Summary: Verticillium wilt of lettuce has become an on-going concern since first being confirmed in the Salinas Valley in 1995. The disease has been restricted to the coastal area, and has not been seen in lettuce grown in the California desert or San Joaquin Valley. There are two races of *Verticillium dahliae* from lettuce, and both races can occur in the same lettuce field. *Verticillium dahliae* also causes Verticillium wilt of spinach. However, this disease is not seen in California because this fungus only causes visible symptoms on spinach when the plants enter the reproductive phase for flowering and seed development. Important recent research discovered that a large number of spinach seed lots were infested with *V. dahliae*. It therefore appears that infested spinach seed may have some role in development of Verticillium wilt in lettuce crops in the Salinas Valley. The overall purpose of this project is to evaluate this potential relationship between infection of spinach seed by *V. dahliae* and development of Verticillium wilt in lettuce, and to develop appropriate management guidelines based on the results.

The purpose of this specific component (Objective C) of the overall project is to evaluate various methods of assaying spinach seed for *V. dahliae* that are currently available to the seed industry, in order to develop a sensitive, robust, standardized spinach seed health assay for *V. dahliae*. Eleven spinach seed lots were obtained for this study. Both the UC Cooperative Extension lab in Salinas and the Washington State University Vegetable Seed Pathology lab in Mt. Vernon ran tests using three seed assay techniques.

Preliminary results indicate that the freeze blotter, NP-10 agar, and sorbose agar assays all allow for detection of *V. dahliae* on spinach seed. The NP-10 agar assay may be the most appropriate for accurate detection of *V. dahliae* alone. The freeze-blotter assay may more readily allow for detection of two foliar pathogens (*Stemphylium* and *Cladosporium*) in addition to *V. dahliae*. The sorbose agar assay is functional but excessive growth of secondary fungi lessens the usefulness of this medium for detecting *V. dahliae*. The overall protocol in this study entailed pre-treatment of the seed with dilute (1.2%) NaOCl. However, use of such a surface-sterilant is not suitable for all seed assay situations, e.g., when testing the efficacy of seed treatments for suppression of specific seedborne pathogens. Developing a standardized spinach seed health assay for *V. dahliae* requires consideration of the purpose of the test and the diversity of types of spinach seed samples a commercial seed testing lab might encounter, including seed samples that have been treated with fungicides or biological control agents. Such seed treatments may be intended to suppress target seedborne pathogens such as *V. dahliae*, and the particular seed assay selected may actually interfere with treatment efficacy.

Additional seed assay evaluations are in progress in both UCCE and WSU labs; final recommendations and training opportunities for commercial labs will be available once the evaluations are completed.

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Preliminary report for Objective C:

Develop a sensitive, robust, standardized spinach seed health assay for *Verticillium dahliae*

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Introduction:

Verticillium wilt of lettuce: Verticillium wilt, caused by *Verticillium dahliae*, is a major disease on a number of crops in California. For lettuce, Verticillium wilt has become a concern since first being confirmed in the Salinas Valley in 1995. The disease initially was detected in only a few fields in one area of the coast, but over the ensuing years has been found in widely separated areas. The disease has been restricted to the coastal area, and has not been seen in lettuce grown in the California desert or San Joaquin Valley growing areas. There are two races of *V. dahliae* from lettuce, and both races can occur in the same lettuce field. Researchers have recently found that lettuce seed lots can carry viable *V. dahliae* at 1 to 6% rates of contamination per seed lot.

Verticillium wilt of spinach**:** *Verticillium dahliae* also causes Verticillium wilt of spinach. However, this disease is not seen in California because this fungus only causes visible symptoms on spinach when the plants have been induced to bolt (enter reproductive phase of growth for flowering and seed development). Therefore, this problem is only observed on spinach seed crops, which are primarily grown in the US Pacific Northwest and in the northern European Union. In an important finding, du Toit et al. (2005) found that 91% of 75 tested seed lots were infested with *V. dahliae* at rates ranging from less than 1% to 85%. Koike and Subbarao (unpublished data) assayed samples of spinach seed from lots planted in the Salinas Valley from 2003 to 2005, and the results demonstrated infestation rates up to 40% per seed lot.

Potential role of infected spinach seed in outbreaks of Verticillium wilt in lettuce: It appears that infested spinach seed may have some role in development of Verticillium wilt in lettuce crops in the Salinas Valley as the two leafy vegetables are frequently grown in rotation in this region. The overall purpose of this project is to evaluate this potential relationship between infection of spinach seed by *V. dahliae* and development of Verticillium wilt in lettuce, and to develop appropriate management guidelines based on the results.

Methods of testing spinach seed for *V. dahliae*: The ability to achieve the overall objective of this project is confounded by the current lack of an efficient, sensitive, and standardized spinach seed health assay for *V. dahliae*. Similarly, there is no standardized means of assaying lettuce seed lots for this pathogen. In 2008, du Toit (in cooperation with the spinach seed industry) completed a preliminary "blind ring test" of spinach seed health assays currently available to stakeholders by submitting samples of three commercial spinach seed lots to each of six labs (five seed testing labs and du Toit's research lab at the WSU Mount Vernon NWREC). Other than du Toit's lab, the other labs were not aware of the ring test because the objective was to see how variable the results might be among labs that currently offer spinach seed health testing. Only two labs (Naktuinbouw in The Netherlands and du Toit's lab) produced similar results, and results from the other four labs differed widely depending on the specific fungi included in their reports. The two labs with similar results were the only two that used the same protocol (a freeze-blotter seed health assay used to detect a range of necrotrophic fungi on spinach seed, including *Verticillium*). This preliminary blind ring test demonstrated clearly the need to develop a standardized spinach seed health assay for *V. dahliae* that preferably is also effective for detection of other important seedborne pathogens of spinach (e.g., *Stemphylium botryosum*, cause of Stemphylium leaf spot).

Objective:

The purpose of this specific component (Objective C) of the overall project is to evaluate various methods of assaying spinach seed for *V. dahliae* that are currently available to the seed industry, in order to develop a sensitive, robust, standardized spinach seed health assay for *V. dahliae*.

Procedures:

The Koike (UC Cooperative Extension) and du Toit (Washington State University) laboratories coordinated spinach seed testing experiments. Both laboratories received 11 commercial spinach seed lots to be tested using each of three detection methods: (1) a freeze-blotter assay; (2) NP-10 agar, a semi-selective medium for *V. dahliae*; and (3) sorbose agar. Some seed assays are in progress at the time of this report.

1) Freeze blotter assay: This method was developed by du Toit et al. (2005) modified from a protocol of Derie et al. (1988). For each seed lot tested, four replications of 100 seed were used. The seeds for each replication were placed in a stainless steel tea strainer which was then placed for 60 seconds in a 1.2% NaOCl (bleach) solution and agitated to surface-sterilize the seed.

Seeds were then triple-rinsed in sterile distilled water, placed on sterile paper towels to dry in a laminar flow hood, and placed into plastic incubation boxes. The incubation boxes are 4 inch x 4 inch clear acrylic containers (Hoffman Manufacturing, Inc.) with tight-fitting, clear lids. A sterilized steel blue germination blotter (Anchor Paper Co.) was placed in each box and moistened with 11 to 12 ml sterile distilled or deionized water. The surface-sterilized spinach seeds from each lot were then arranged on the blotters in 6 rows of 6 seed per box (maximum of 36 seed per box) using sterilized forceps, i.e., three boxes were needed for 100 seeds per replication per seed lot. The lid was then replaced on each box. The boxes were incubated in the dark for 24 hours to imbibe the seeds, placed in a freezer (-20°C) for 24 hours to freeze-kill the imbibed seed, and then placed under lights (near-UV light and cool white fluorescent light) on a 12 hour/12 hour light/dark cycle at 24°C. Seeds were examined with a dissecting stereomicroscope 5, 9, and 14 days after being placed on the blotters, to detect the presence of *V. dahliae* and other necrotrophic spinach pathogens.

Koike's UCCE lab and du Toit's WSU Vegetable Seed Pathology lab used the same protocol with slight variations. At du Toit's lab, the seeds were imbibed for 25 hours (vs. 24 hours at the UCCE lab), frozen at -20°C for 25 hours (vs. 24 hours at the UCCE lab), and then incubated in a Percival Scientific Model I30BLL incubator (vs. incubated on a lab bench).

2) NP-10 agar assay: Originally described by Sorensen (1991), a modified NP-10 agar recipe (Table 1) developed by the Subbarao lab (Kabir et al., 2004) was used for the NP-10 agar assay. This medium has been used extensively in *Verticillium* research and is a semi-selective medium that suppresses growth of most other fungi while allowing for *Verticillium* growth and observation; this suppression is important for the detection of *V. dahliae* because *Verticillium* species are slow-growing compared with many other fungi. The medium was dispensed into 10 cm-diameter disposable plastic petri dishes. As described for the freeze-blotter assay, four replications of 100 seeds of each seed lot were surface-sterilized. The seeds were then plated directly into plates of NP-10 agar medium with 10 seeds per plate.

At the UCCE lab, the seeds were then incubated in the plates in the dark at approximately 24°C for up to 14 days. In contrast, at the WSU Vegetable Seed Pathology lab the NP-10 agar plates were placed in the same incubators as described for the freeze-blotter assay above, with near-UV light and cool white fluorescent light providing a 12 hour/12 hour light/dark cycle at 24°C. This was done to provide the seeds with the same incubation conditions as for the freeze-blotter assay. Also, the du Toit's lab had previously verified the need for this diurnal light cycle to induce sporulation by *S. botryosum*, another seedborne necrotrophic pathogen of spinach. Seeds were examined with a dissecting stereomicroscope at 5, 9, and 14 days for the presence of *V. dahliae* and other necrotrophic fungi.

3) Sorbose agar assay: Sorbose agar medium (Table 2) was also evaluated in this study because this medium is currently recommended by the US National Seed Health System (NSHS) for testing spinach seed lots for *Verticillium* (Block and Shepherd, 2008). Sorbose agar was dispensed into plastic disposable petri dishes as for the NP-10 agar assay. Four replications of 100 seeds were surface-sterilized for each of the 11 spinach seed lots, dried, and then plated onto sorbose agar as described above for the NP-10 agar assay. The plates were incubated under the

respective conditions described above for the NP-10 agar assay at the UCCE lab (in the dark) vs. the WSU lab (diurnal cycle).

Assay evaluations: At 5, 9, and 14 days the percentage of spinach seed infested with *V. dahliae* was assessed for each of the three assay methods. Initially, a spinach seed was considered positive for *V. dahliae* if black microsclerotia and verticillate conidiophores, both characteristic features of this pathogen, were observed on the seed. Counts were also made of other necrotrophic spinach pathogens and fungi observed (e.g., *Stemphylium botryosum, Cladosporium variabile, Fusarium* spp., etc.) on the seed. As the assays progressed, it became apparent that verticillate conidiophores typical of *Verticillium* spp. developed on some seed without the presence of microsclerotia on the seed and/or on the blotter or agar medium around the seed. In contrast, very rarely were microsclerotia observed on a seed without the presence of verticillate conidiophores. For this reason, both labs recorded *Verticillium* observations in three ways: i) the incidence of seed with microsclerotia typical of *Verticillium* spp., ii) the incidence of seed with verticillate conidiophores typical of *Verticillium* spp., and iii) the incidence of seed with both of these features. For future investigations, both labs collected isolates from a subset of seeds from each seed lot on which conidiophores of *Verticillium* developed in the absence of microsclerotia. Similarly, isolates were collected from seeds on which *Verticillium* conidiophores looked different than what might be considered typical for *V. dahliae*. Single-spore cultures of these isolates are being prepared for further testing to identify the species of *Verticillium*.

Table 1. Modified Sorensen's NP-10 medium

Bottle A	
Polygalacturonic acid, Na salt from orange (P-3889)	5 grams
NaOH pellets (0.025N)	1.2 grams
Distilled water	500 ml
Bottle B	
Agar (Sigma grade)	15 grams
KNO3	1 gram
KH2PO4	1 gram
KCl	0.5 gram
MgSO4-7H2O	0.5 gram
Tergitol NP-10	0.5 ml
Distilled water	500 ml
Additives	
Chloramphenicol	0.05 gram
Streptomycin sulfate	0.05 gram
Chlortetracycline	0.05 gram
Dissolve all into alcohol	1 ml

Procedure:	
Prepare separately bottles A and B.	
Autoclave bottles A and B.	
After autoclaving, let contents cool.	
Pour additives into bottle B; mix thoroughly.	
Pour contents of bottle A into B; mix thoroughly.	
Medium is sensitive to light; store poure plates in the dark.	

Table 2. Sorbose agar	
Bottle A	
Agar	15 grams
Sorbose	2 grams
Distilled water	1 liter
Additive	
Streptomycin	0.1 gram
(dissolve in 2 ml ethanol)	
Prepare and autoclave bottle A.	
Allow mixture to cool slightly; thoroughly mix in additive.	

Results:

This project is in progress and final results will be made available in a later report to the CLGRB. Included in this preliminary report are the following findings.

Comparison of the three spinach seed health assay methods at the UCCE lab: All three methods allowed for the observation and detection of *V. dahliae* on the commercial spinach seed lots assayed (Table 3). In most cases, for any one seed lot the recovered incidence appeared similar for the three methods. The freeze blotter method resulted in the lowest percentage recovery of *V. dahliae* for four of nine lots (lots 3, 4, 5, and 7) while the sorbose agar method had the lowest recovery for three of nine lots (lots 1, 6, and 9). The NP-10 agar method had the highest recovery

for all lots except number 2 (which was negative for *V. dahliae* with three methods). Statistical analyses remain to be conducted to see if these numeric differences are significant. Additional seed lots remain to be tested in the UCCE lab.

Table 3. Percentages of Verticillium-infested spinach seed: UCCE lab

Seed Lot	Freeze blotter	NP-10	Sorbose
1	79.0	98.5	77.5
2	0.0	0.0	0.0
3	1.3	4.5	2.5
4	51.8	75.0	64.0
5	2.5	12.3	5.8
6	41.8	82.3	21.3
7	66.8	95.0	83.3
8	0.0	0.0	0.0
9	50.0	71.5	24.5

Four replications of 100 seed each were tested.
Seed were first surface sterilized with 1.2% bleach.

The percentages of spinach seed that tested positive for *Stemphylium* species at the UCCE lab are presented in Table 4. The greatest numbers of Stemphylium-infested seed were observed on the freeze blotter assay. Few seed tested positive for this genus on NP-10 agar and sorbose agar media, but this may be the result of incubating the plates in the dark for these two methods.

Table 4. Percentages of Stemphylium-infested spinach seed: UCCE lab

Seed Lot	Freeze blotter	NP-10	Sorbose
1	1.0	0.0	2.0
2	30.0	0.0	0.0
3	0.3	3.3	0.0
4	2.5	0.8	1.5
5	2.0	0.0	0.3
6	3.3	0.0	0.0
7	1.5	0.0	0.0
8	0.0	0.0	0.0
9	8.3	1.5	0.0

Four replications of 100 seed each were tested.
Seed were first surface sterilized with 1.2% bleach.
Species of Stemphylium was not identified.

Comparisons of the spinach seed assay methods at the WSU lab:
Freeze-blotter and NP-10 agar assays have been completed at the WSU lab for 11 spinach seed lots, and the sorbose agar assay is in progress. Preliminary analyses of variance (ANOVAs) were completed for the two methods finished for all 11 lots (factorial treatment design). In summary, the main effect of seed assay method (freeze blotter vs. NP-10 agar) was significant ($P < 0.05$) for *Verticillium* spp. and for *Stemphylium* spp., i.e., averaged over the 11 lots the two methods differed significantly in the percentage of seed on which these fungi were detected (Table 5). The NP-10 agar method enabled detection of a higher mean incidence of *Verticillium* spp. (43.4 ± 4.8% averaged over 11 lots) vs. the freeze-blotter method (36.6 ± 4.8%), similar to results from the UCCE lab. Similar to the UCCE lab results, detection of *Stemphylium* spp. was highest (25.1 ± 3.0%) for the freeze-blotter assay and lower (22.3 ± 3.1%) for the NP-10 agar assay. The main effect of seed lots was also significant ($P < 0.05$) for these two genera of fungi, i.e., the 11 seed

lots differed significantly in the percentage of seed infested with *Verticillium* spp. and with *Stemphylium* spp. (Table 5). The interaction between seed health methods and seed lots was also significant, i.e., for some seed lots there was no significant difference between the two methods, but for other lots the difference was significant (Table 5).

Table 5. Mean ± standard error of the incidence (%) of seed on which *Verticillium* spp. and *Stemphylium* spp. were detected at the WSU lab for each of 11 commercial spinach seed lots.

	Verticillium spp.		*Stemphylium* spp.	
Seed lot	Freeze-blotter	NP-10 agar	Freeze-blotter	NP-10 agar
1	89.5 ± 1.3	93.3 ± 1.9	22.5 ± 3.1	8.0 ± 1.5
2	0	0	62.0 ± 1.1	70.5 ± 2.0
3	3.8 ± 1.3	13.5 ± 1.0	7.8 ± 1.7	8.8 ± 2.0
4	63.3 ± 3.6	65.0 ± 1.1	7.8 ± 2.0	7.3 ± 1.0
5	6.8 ± 2.0	6.8 ± 0.8	50.3 ± 2.0	47.8 ± 3.8
6	57.5 ± 2.1	70.0 ± 2.8	36.0 ± 1.9	33.3 ± 2.7
7	81.5 ± 2.2	88.0 ± 1.1	10.0 ± 2.0	6.8 ± 1.0
8	1.8 ± 0.9	12.0 ± 2.7	1.3 ± 0.8	0.8 ± 0.3
9	35.8 ± 1.7	51.8 ± 1.7	44.3 ± 5.2	27.5 ± 2.2
10	36.5 ± 3.1	44.0 ± 1.8	17.5 ± 1.2	14.8 ± 2.6
11	26.3 ± 0.8	33.5 ± 1.8	17.3 ± 2.1	20.5 ± 2.1
Mean	36.6 ± 4.8	43.4 ± 4.8	25.1 ± 3.0	22.3 ± 3.1
LSD for method of assay[*]	1.69		1.62	
Prob > F for method of assay[#]	0.0008		0.0111	

[*] LSD = Fisher's protected least significant difference based on the analysis of variance (ANOVA).

[#] Probability the F value is not significant for the method of assay (in the ANOVA).

Overall, results for *Verticillium* detected on the 11 spinach seed lots were very similar between the two labs, i.e., lots on which low incidences of *Verticillium* spp. were observed on the seed at the WSU lab (Table 5) also had very low incidences of seed with *V. dahliae* at the UCCE lab (Table 3); this similarity also applied for lots with intermediate and higher levels of infestation. Some of the minor differences in incidences between the two labs probably reflect the fact that results for the UCCE lab only included seeds on which the fungus had characteristic features of *V. dahliae* (verticillate conidiophores as well as microsclerotia), whereas results for the WSU lab included all *Verticillium* spp. observed. The latter will be further defined once species identification of single-spore isolates from the seeds is completed. Isolates collected from the UCCE tests will also be characterized to species. Refer also to some of the comments in the Discussion below.

In contrast to the *Verticillium* results, a much lower incidence of seed infested with *Stemphylium* spp. was detected at the UCCE lab (Table 4) compared to the WSU lab (Table 5), both with the NP-10 agar assay and the freeze-blotter assay. The UCCE lab detected *Stemphylium* at a range of

0-30% on NP-10 agar and 0-2.0% for the freeze-blotter assay, compared with the WSU results of 0.8-70.5% on NP-10 agar and 1.3-62.0% with the freeze-blotter assay. A low incidence of *Stemphylium* spp. detected with the NP-10 agar assay at the UCCE lab is not unexpected given the plates were incubated in the dark (a diurnal cycle is required to induce this genus to sporulate), although this does not account for the low incidence with the freeze-blotter assay. Identification of *Stemphylium* spp. at the WSU lab is based on observing typical conidia and conidiophores of the genus and/or observation of pseudothecia (which can be distinguished microscopically from similar fungal fruiting bodies such as pycnidia and perithecia). Therefore, to assess whether the discrepancy in results for *Stemphylium* spp. between the two labs might be associated with recognition of these two characteristic features, results for *Stemphylium* spp. at the WSU lab will be analyzed separately based on the incidence of seed with and without pseudothecia.

Discussion:

This project is in progress and final conclusions are not yet available. Included in this preliminary report are some observations on results for the three seed assay methods from the two participating labs.

Freeze blotter observations:
By day 14, many of the seeds were covered by extensive fungal growth that can obscure *V. dahliae* colonies. Therefore, this final evaluation date may not be needed. Similar observations have been made previously (du Toit and Derie, 2008). However, du Toit also noted that this longer duration for the freeze-blotter assay may be necessary when testing seed lots that have been treated (e.g., with fungicides, biological agents, or disinfectants), because such seed treatments can significantly delay development of fungi on the seed, including *V. dahliae*. Samples of treated seed lots may have to be assayed for as long as 21 days. This aspect should be examined in additional studies.

Microsclerotia typically were observed by day 9, but sometimes only by day 14. The number of *Verticillium* microsclerotia present on seed can vary significantly. On one seed the entire surface may be covered with these small black structures, while on an adjacent seed only a few microsclerotia may be visible. The evaluator will therefore need to be careful not to miss the seed having these low numbers. This is particularly true for seed on which fast-growing fungi such as *Alternaria* spp. are abundant and may readily obscure *V. dahliae*.

A number of seeds were infested with fungi that, by day 9, had formed only verticillate conidiophores. Because microsclerotia were not present, the UCCE lab did not count these as *V. dahliae*. When these isolates were transferred to general mycological media, the isolates often did not look like *V. dahliae*. However, in some cases the resulting growth did appear consistent with *V. dahliae*. Further analysis will be done by the Subbarao lab to identify these ambiguous isolates. At the WSU lab, when microsclerotia were not observed on the seed in the freeze-blotter assay, the seed was picked up off the blotter at the final reading to inspect the surface of the blotter immediately beneath the seed. Often microsclerotia had started to form on the blotter immediately beneath the seed by the final (14 day) reading. This may account for the higher

incidence of *Verticillium* spp. detected with the freeze-blotter assay at the WSU lab vs. the UCCE lab (Tables 5 and 3, respectively).

A number of seeds were infested with a fungus that formed very distinct verticillate conidiophores that appeared to be significantly larger and more robust than the conidiophores of *V. dahliae*. Again, the Subbarao lab will examine these and determine if these may be *V. dahliae*. Similarly, the WSU lab observed and subcultured isolates with this characteristic. Tentative identification of these isolates at the WSU lab with known isolates of different *Verticillium* spp. suggests some of these may be *V. tricorpus*.

At the WSU lab, some seeds of certain seed lots had very sparse verticillate conidiophores that grew close to the surface of the seed without microsclerotia developing on the seed. Subcultures of isolates of these fungi onto general agar media, on which they formed abundant chlamydospores but no microsclerotia, and comparison of the isolates with known *Verticillium* spp., suggests they may be *V. nigrescens* (which has been re-named *Gibellulopsis nigrescens*; Zare et al., 2007).

As previously established (Hernandez-Perez and du Toit, 2006; du Toit et al., 2007) the freeze blotter method also allows for detection of *Stemphylium botryosum* and *Cladosporium variabile,* two other seedborne pathogens of spinach. This technique, therefore, has multiple uses if the intent is to screen for additional necrotrophic fungi and not only *V. dahliae*. Seed industry personnel have expressed preference for a single assay to detect multiple spinach pathogens, given the cost charged for such assays by commercial seed testing labs and the number of seed lots that need to be tested by seed companies.

NP-10 agar observations:
V. dahliae colonies grow well on this medium and are very easy to identify. Radiating fans of microsclerotia extend well beyond the seed and are easy to see in the agar medium, in most cases.

An occasional *V. tricorpus* (a species that may not be pathogenic to spinach or lettuce, or may be weakly pathogenic) was seen on the NP-10 agar plates; on this medium, the species forms a distinct yellow pigment, the microsclerotia tend not to form in a radiating pattern, and the species is readily separated from *V. dahliae* (unlike the freeze-blotter assay).

On this medium, *Stemphylium* was occasionally confirmed by the UCCE lab, and *Cladosporium variabile* was not identified by either lab. NP-10 agar may be less suitable for detection of these spinach pathogens, although the incidence of seed on which *Stemphylium* was detected on NP-10 agar by the WSU lab was much more similar to the freeze-blotter assay compared with the UCCE lab results. On NP-10 agar, the WSU lab noted that pseudothecia of *Stemphylium* were occasionally found on the seed, but mostly observed sunken in the agar medium in close proximity to the seed, frequently without observation of conidia and conidiophores. Immature pseudothecia in the agar medium were pale brown, but by day 9 and 14 the pseudothecia had typically matured to a dark brown or black color. Pseudothecia were distinguished from pycnidia of other fungi by the smaller size of the latter, which develop an exit pore (ostiole) that allows spores to be dispersed, sometimes accompanied by the presence of a curled mass of spores (spore

tendril or cirrhus). In addition, some of these other fungi formed pycnidia or perithecia with distinct setae that are never observed on pseudothecia of *Stemphylium*.

The WSU lab noted that *Fusarium* spp. developed faster and did not seem to spread as quickly on NP-10 agar plates compared with the freeze-blotter assay. In addition, the presence of bacteria on the spinach seed also seemed to be more prevalent (or, at least, more readily observed) with the NP-10 agar assay compared to the freeze-blotter assay.

The NP-10 agar recipe includes three antibiotics that are effective against a spectrum of microorganisms. As are result, this medium may have adverse effects on some biological control seed treatments, e.g., actinomycetes, bacteria, etc. Therefore, NP-10 agar is not an appropriate medium on which to test spinach seed that has been treated with biological control agents that could be affected adversely by these antibiotics. This is an important consideration for commercial seed testing labs, i.e., to ascertain clearly with clientele whether any of the seed lots to be tested may have been treated with biological agents.

Sorbose agar observations:
V. dahliae does not grow well on this medium and colonies + microsclerotia do not extend very far away from the seed into the medium. Infested seed are mostly identified when microsclerotia and conidiophores are present on the seed surface.

Because *V. dahliae* is mostly present on the seed, the growth of secondary, contaminating fungi significantly obscures observation of *V. dahliae* on the seed. In some cases, even by the first reading (day 5) such growth can prevent observation for *V. dahliae*. This is a severe limitation for this medium, particularly for seed lots that have high incidences of fast-growing saprophytic fungi such as *Alternaria* spp.

As noted above for NP-10 agar, sorbose agar contains the antibiotic streptomycin, so this medium may not be appropriate for testing spinach seed lots that have been treated with biological agents that could be affected adversely by this antibiotic.

General comments:
For both the freeze-blotter and NP-10 agar assays, the last reading (14 days) is mostly to confirm identification of some fungi on some seed. This last reading may not be necessary except when evaluating seed treatments that delay development of fungi, or if waiting for specific morphological characteristics to develop for identification of specific fungi, e.g., microsclerotia, chlamydospores, and/or conidiophores of *Verticillium* spp., or pseudothecia and conidia of *Stemphylium* spp.

For *Verticillium* spp., the presence of microsclerotia and conidiophores were recorded separately for both the NP-10 agar and the freeze-blotter assays at the WSU lab, because this may potentially be useful for differentiating species (results will be presented in a later report, so this remains to be confirmed). In the UCCE lab, *Verticillium* observations were divided into "confirmed *V. dahliae*" with both microsclerotia and conidiophores, and *Verticillium* species in which only conidiophores were seen.

Stemphylium was identified at the WSU lab by the presence of characteristic conidia and conidiophores; however, if conidia were not observed, then pseudothecia alone were taken as confirmation of *Stemphylium*. For the freeze-blotter assay, pseudothecia were most commonly observed on the seed but sometimes also developed on the blotter in the vicinity of the seed. The ability to detect conidia and pseudothecia of *Stemphylium* spp. was sometimes compromised by the presence of fast-growing fungi (e.g., *Alternaria* spp.). Although this lab does not normally tally the two morphological structures separately, this was done for the NP-10 agar and sorbose agar assay in this study to assess whether the media might affect how *Stemphylium* develops on spinach seed compared to the freeze blotter assay (this was the first time the WSU lab had tested spinach seed using the two agar media).

Summary:

Preliminary results indicate that the freeze blotter, NP-10 agar, and sorbose agar assays all allow detection of *V. dahliae* on spinach seed. The NP-10 agar assay may be the most appropriate for accurate and sensitive detection of *V. dahliae* alone. The freeze-blotter assay also more readily allows for detection of two foliar pathogens (*Stemphylium* and *Cladosporium*) in addition to *V. dahliae*. The sorbose agar assay is functional but excessive growth of secondary fungi lessens the usefulness of this medium for detecting *V. dahliae* and makes the assay much more time-consuming than the other two methods. The overall protocol in this study entailed pre-treatment of the seed with dilute (1.2%) NaOCl. However, use of such a surface-sterilant is not suitable for all seed assay situations, e.g., when testing the efficacy of seed treatments for suppression of specific seedborne pathogens. Developing a standardized spinach seed health assay for *V. dahliae* requires consideration of the purpose of the test and the diversity of types of spinach seed samples a commercial seed testing lab might encounter, including seed samples that have been treated with fungicides or biological control agents. Such seed treatments may be intended to suppress target seedborne pathogens such as *V. dahliae*, and the particular seed assay method selected may actually interfere with treatment efficacy.

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