Final Research Report to the California Leafy Greens Research Board
April 2009 to March 2010

I. Abstract

Project Title: Verticillium Wilt of Spinach: Detection, Biology and Control. 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* associated with lettuce. *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*. Therefore, infested spinach seed may have some role in development of Verticillium wilt in lettuce crops in the Salinas Valley because spinach and lettuce are commonly 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.

The purpose of this specific component (Objective C) of the project is to evaluate various methods of assaying spinach seed for *V. dahliae* 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 Mount Vernon, WA ran tests using each of three seed assay methods – an NP-10 agar assay, a freeze-blotter assay, and a sorbose agar assay.

All three methods enabled detection of *Verticillium* on the commercial spinach seed lots assayed (Figure 1), but the incidence of seeds with *Verticillium* was greatest for the NP-10 agar assay (46.2% averaged across all 11 seed lots), followed by the freeze-blotter assay (35.5%), and then the sorbose agar assay (30.2%). The NP-10 agar assay appears to be the most sensitive. The sorbose agar assay is functional but excessive growth of secondary fungi reduces the utility of this medium and makes the assay far more tedious and time-consuming than the other two methods. The overall protocol entailed pre-treatment of the seed with dilute (1.2%) NaOCl. However, use of a surface-sterilant is not suitable for all seed assays, e.g., when testing the efficacy of seed treatments. 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.

A lab workshop, taught by du Toit and Koike, was held in Davis, CA in March 2010 to train participants on how to assay spinach seed for *Verticillium* species using the three seed health assays evaluated in this study, the advantages and disadvantages of each assay, and identification of various pathogenic and non-pathogenic fungi found on spinach seed.

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II. Main Report

Project Title: Verticillium Wilt of Spinach: Detection, Biology and Control. 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. Two races of *V. dahliae* have been identified in association with lettuce, and both races can occur in the same lettuce field. Researchers have found that lettuce seed lots can carry viable *V. dahliae* at incidences ranging from 1 to 6% per seed lot for most lots.

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. du Toit et al. (2005) found that 91% of 75 commercial seed lots produced in different countries were infested with *V. dahliae* at rates ranging from <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: In addition to several other crops susceptible to Verticillium wilt that are grown in rotation with lettuce in the Salinas Valley, it appears 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 assess potential variation in results among labs that currently offer spinach seed health testing. Only two labs (Naktuinbouw in The Netherlands and du Toit's lab at WSU) 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).

Objectives:

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. Testing of all 11 lots was completed by both labs in 2009.

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 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 to 25 hours to imbibe the seeds, placed in a freezer (-20°C) for 24 to 25 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 at the UCCE lab.

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 promoting microsclerotium formation; 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 onto 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 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 seeds with the same incubation conditions as the freeze-blotter assay. Also, 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).

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.	

Assay evaluations: At 5, 9, and 14 days after placing seed on the blotters or agar media, 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 seeds with microsclerotia typical of *Verticillium* spp., ii) the incidence of seeds with verticillate conidiophores typical of *Verticillium* spp., and iii) the incidence of seeds 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 tested further to identify the species of *Verticillium*.

Statistical analyses: Results were subjected to analyses of variance (ANOVAs) using SAS Version 9.2, with labs and seed health assays as fixed effects, and seed lots and replications as random effects in the model statement.

Results and Discussion:

Verticillium: The ANOVA revealed no significant difference between the UCCE and WSU labs with respect to the incidence of *Verticillium* detected on 11 seed lots. Averaged across all three methods and 11 seed lots, the incidence of seeds with *Verticillium* was 37.5% for the UCCE lab vs. 37.3% for the WSU lab. However, the incidence of seeds on which *Verticillium* was observed differed significantly ($P < 0.01$) among the three seed health assays (method) and the 11 seed lots. There was also a significant interaction between methods and seed lots, i.e., for some seed lots there was no significant difference among the methods, but for other lots the differences were significant. All three methods allowed for detection of *Verticillium* on the commercial spinach seed lots assayed (Figure 1), but the incidence of seeds with *Verticillium* was greatest for the NP-10 agar assay (46.2% averaged across all 11 seed lots), followed by the freeze-blotter assay (35.5%), and least for the sorbose agar assay (30.2%) with the three methods each significantly different.

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 at the WSU lab also had low incidences at the UCCE lab, e.g., lots 2 (0% for both labs), 3, 5, and 8 (Figure 1); this was true, too, for seed lots with intermediate (lots 9, 10, and 11) and higher levels of infestation (lots 1, 4, 6, and 7). Minor differences detected between the two labs may 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.

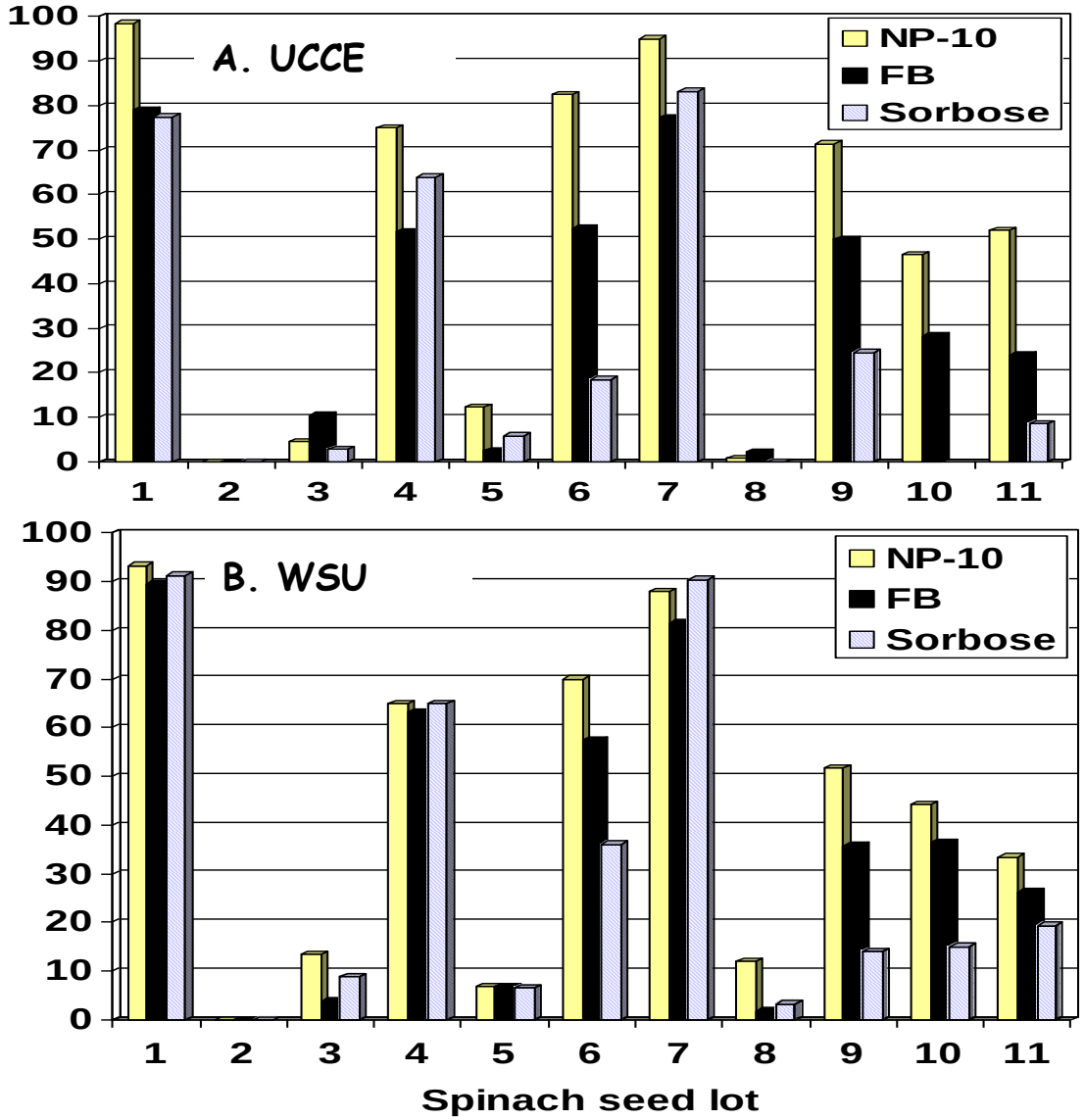

Figure 1. Incidence of spinach seeds on which *Verticillium* was observed for each of 11 seed lots tested using each of three seed health assays, an NP-10 agar assay (NP-10), a freeze-blotter assay (FB), and a sorbose agar assay (Sorbose), at both the University of California Cooperative Extension (UCCE) lab and the Washington State University (WSU) lab.

At the UCCE lab, the NP-10 agar method had the highest recovery for all lots except lots 2 (on which *V. dahliae* was not detected for any of the methods), 3, and 8; the freeze blotter method resulted in the lowest recovery of *V. dahliae* for 3 of the 11 lots (lots 4, 5, and 7); the sorbose agar method had the lowest recovery for 7 of the 11 lots (lots 1, 3, 6, 8, 9, 10, and 11). At the WSU lab, the NP-10 agar method had the highest recovery for all lots except lot 2 (on which *V. dahliae* was not detected for any of the methods), 5 (similar for all three methods), and 7; the

freeze blotter method resulted in the lowest recovery of *V. dahliae* for 4 of the 11 lots (lots 1, 3, 4, 7, and 8); the sorbose agar method had the lowest recovery for 5 of the 11 lots (lots 5, 6, 9, 10, and 11).

Stemphylium: The ANOVA revealed a highly significant difference between the UCCE and WSU labs with respect to the incidence of seeds on which *Stemphylium* was detected for the 11 seed lots (Figure 2). Averaged across all three methods and 11 seed lots, the incidence of seeds with *Stemphylium* was 1.8% for the UCCE lab vs. 23.9% for the WSU lab. This discrepancy between labs occurred because the seeds were incubated in the dark at the UCCE lab for the two agar methods. A day/night cycle is necessary to induce sporulation by *Stemphylium* to aid in the detection and identification of the fungus.

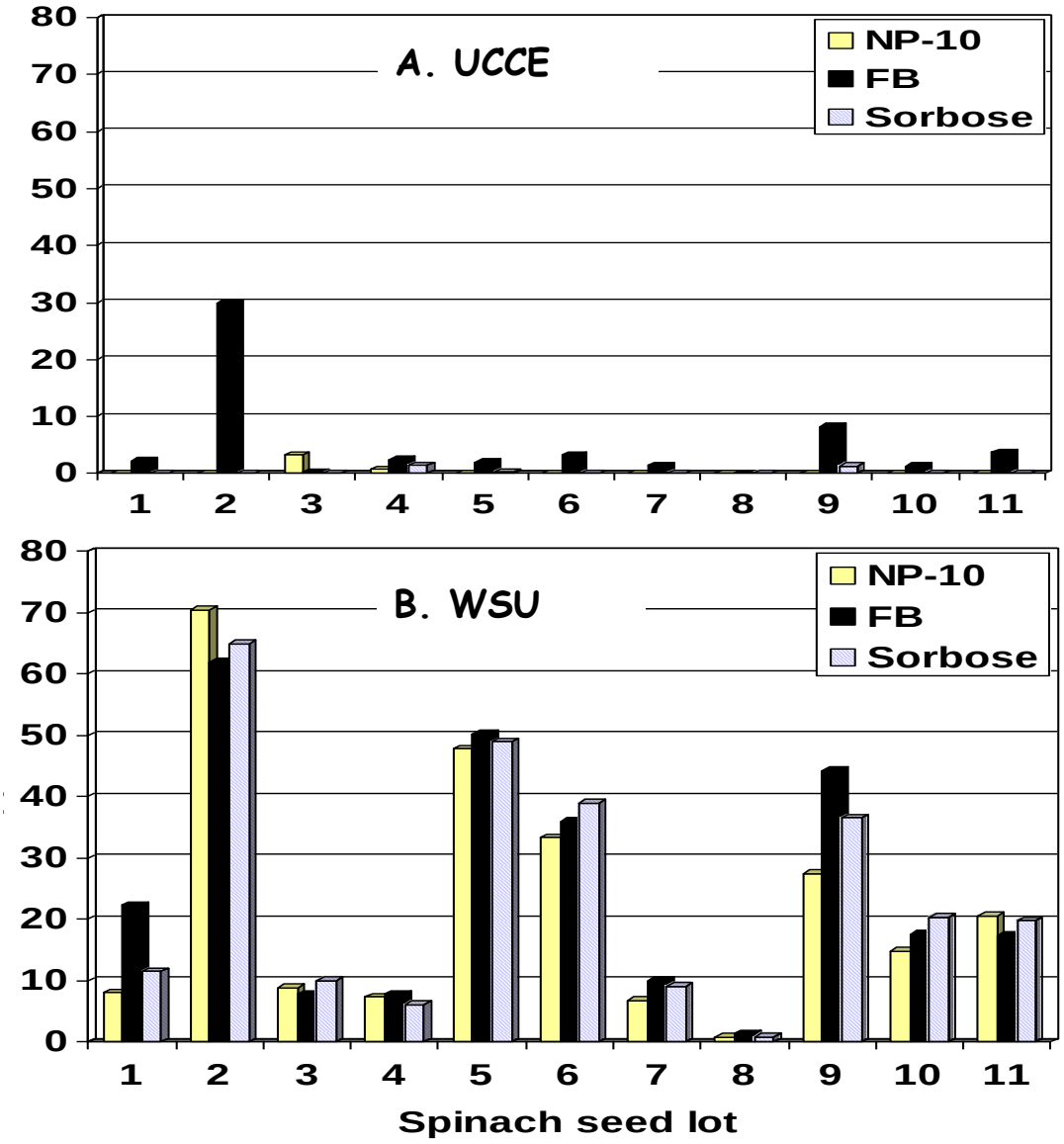

Figure 2. Incidence of spinach seeds on which *Stemphylium* was observed for each of 11 seed lots tested using each of three seed health assays, an NP-10 agar assay (NP-10), a freeze-blotter assay (FB), and a sorbose agar assay (Sorbose), at both the University of California Cooperative Extension (UCCE) lab and the Washington State University (WSU) lab.

The incidence of seeds with *Stemphylium* differed significantly ($P < 0.01$) among the three seed health assays and 11 seed lots, and there was a significant interaction between methods and seed lots (Figure 2). At the WSU lab (discounting results for the UCCE lab because of the absence of light for the agar methods), all three methods enabled detection of *Stemphylium* on the seed lots. The incidence of seeds on which this fungus was detected was greatest for the freeze-blotter (25.1% across the 11 lots) and sorbose agar (24.3%) assays, and least for the NP-10 agar assay (22.3%, which was not significantly different from that of the sorbose agar assay).

Conclusions:

Freeze blotter assay:
By day 14, many of the seeds were covered by extensive fungal growth that can obscure *V. dahliae* colonies. This final evaluation date may not be needed. However, this longer duration may be necessary when testing seed lots that have been treated (e.g., with fungicides, biological agents, or disinfectants), because seed treatments can delay development of fungi on the seed, including *V. dahliae*. Samples of treated seed lots may need to be assayed as long as 21 days.

Microsclerotia of *Verticillium* typically were observed by day 9, but sometimes only by day 14. The number of *Verticillium* microsclerotia present on seed can vary significantly, particularly for seeds on which fast-growing fungi such as *Alternaria* spp. are abundant and may 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 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 formed on the blotter beneath the seed by the final 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.

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*. Tentative identification of these isolates by comparison with known isolates of different *Verticillium* spp. suggests these may be *V. tricorpus*, a species believed to be non-pathogenic to spinach and lettuce.

At the WSU lab, some seeds of some seed lots developed sparse, verticillate conidiophores that grew close to the surface of the seed without microsclerotia developing. Subcultures of isolates were made onto agar media, on which they formed abundant chlamydospores but no microsclerotia. Comparison of these isolates with known *Verticillium* spp., suggests they may be *V. nigrescens* (recently 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:
V. dahliae colonies grow well on this medium and are usually easily identified. Radiating fans of microsclerotia extend beyond the seed in the agar medium, in most cases. An occasional *V. tricorpus* was seen on NP-10 agar plates, on which this species forms a distinct yellow pigment, the microsclerotia tend not to form in a radiating pattern. The species is readily separated from *V. dahliae* on this agar medium, unlike the freeze-blotter assay.

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. On NP-10 agar, the WSU lab noted that pseudothecia of *Stemphylium* were occasionally found on the seed, but mostly observed 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 had matured to a dark brown or black color. Pseudothecia were distinguished from pycnidia of other fungi by size (pycnidia are typically smaller), and the lack of an ostiole and cirrhi. In addition, some of these other fungi formed pycnidia or perithecia with distinct setae, which are never observed on pseudothecia of *Stemphylium*.

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 by antibiotics. It is important for commercial seed testing labs to ascertain clearly with clientele whether any seed lots to be tested have been treated with biological agents.

Sorbose agar:
V. dahliae does not grow well on this medium as colony growth and 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 of *V. dahliae*. This is a major limitation of sorbose agar medium, particularly for seed lots that have high incidences of fast-growing saprophytes such as *Alternaria* spp.

Sorbose agar contains streptomycin, so this medium may not be appropriate for testing seed lots that have been treated with biological agents that could be affected by this antibiotic.

Summary:

Although the freeze blotter, NP-10 agar, and sorbose agar assays all enabled detection of *V. dahliae* on spinach seed, the NP-10 agar assay may be the most sensitive for detection of *V. dahliae*, followed by the freeze-blotter assay. The sorbose agar assay is functional but excessive growth of secondary fungi reduced the utility of this medium for detecting *V. dahliae* and made the assay very time-consuming. The protocol in this study entailed pre-treatment of the seed with dilute (1.2%) NaOCl. However, use of a surface-sterilant is not suitable for all seed lots, e.g., when testing the efficacy of biological seed treatments. 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.

Following completion of this project, Koike and du Toit organized a *Verticillium* seed health assay lab workshop in Davis, CA on 31 March 2010. The workshop was attended by about 30 people from private and public seed testing labs, seed company pathologists, regulatory agencies, and consultants. A diversity of spinach seed lots was set up with all three seed health assay methods (sorbose, NP-10, and freeze-blotter) at various stages of development on the day of the workshop, along with cultures of various necrotrophic pathogens of spinach were provided for participants to examine microscopically. Spinach plants were also inoculated with the two leaf spot pathogens and the anthracnose pathogen, for participants to observe symptoms of these diseases.

As a follow-up to this project, with funding from members of the International Seed Health Initiative Vegetable Technical Group (ISHI VTG), du Toit is working with members of the ISHI VTG to complete a 'ring test' of spinach seed lots tested with the freeze-blotter and NP-10 agar assays. Fungicide-treated and non-treated samples of each of three spinach seed lots are being assayed with both methods by nine participating labs in the USA and EU, in an effort to finalize an internationally-accepted, standardized assay for *Verticillium* on spinach seed. The ring test is expected to be completed by the end of 2010.

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