Annual Report for the California Leafy Greens Research Board
for the Period April 1, 2008 to March 31, 2009

I. Abstract

Project Title: Evaluation of seed treatments for management of seedborne *Verticillium* in spinach, 2008.

Project Investigator: Lindsey J. du Toit, Washington State University
Cooperator: Krishna Subbarao, University of California-Davis

Summary: *Verticillium dahliae* is systemic and readily seed transmitted in spinach, raising concern about introducing *V. dahliae* on spinach seed into fields subsequently planted to other susceptible crops. A seed lot naturally infected with *Verticillium* at 64% was used to evaluate 9 conventional and 11 organic seed treatments for control of *Verticillium* on spinach seed. A seed germination assay and a freeze-blotter seed health assay were each completed. No treatment significantly affected germination compared to non-treated seed, except Experimental II, a proprietary organic treatment that reduced germination by 12%. In the seed health assay, seven treatments reduced the incidence of seedborne *Verticillium* from 63.5% for non-treated seed to <10%, the current threshold for exporting spinach seed from the U.S. to Mexico. The most effective fungicide treatments were Topsin M 70 WP (0%), Mertect 340F applied alone (0.3%) or with Farmore D300 (0%), and BAS 595 XG F (2.0%). Three proprietary organic treatments were also highly effective: Seedgard (2.8%), Seed Support II (3.3%), and Seed Support I (7.0%). Ten treatments had intermediate efficacy. Treatments that did not significantly reduce the incidence of seed with *Verticillium* spp. included two organic treatments (ACX 801 and ACX 802), and Coronet at 200 ml/100 kg seed. In a component seed health assay, *Verticillium* was observed on 50% of the pericarps and 51% of the embryos not surface-sterilized, 46% of pericarps and 74% of embryos sterilized for 30 sec, and 34% of pericarps and 29% of embryos sterilized for 60 sec, demonstrating the internal nature of seed infection.

To assess inoculum potential, the seed lot was planted in sterilized sand and the leaves harvested after 35 days to mimic a 'baby leaf' crop. The roots, crown, and cotyledons were crushed in buffer and plated on an agar medium. *Verticillium* was detected at >9,000 CFU/100 emerged seedlings. In addition, seedlings were sampled at weekly intervals from 7 to 28 days after planting to determine the location of seedling infection by *V. dahliae*. *V. dahliae* was observed on roots as early as 7 days after planting, at a similar incidence to the percentage seed infected (~60% of seedlings). *V. dahliae* developed on the main root 10 to 20 mm behind the root tip, but not on the crown, cotyledons, or true leaves within the 4 weeks after planting. Thirteen of the seed treatments were evaluated for potential at preventing seed transmission or soil infestation by *V. dahliae*. Non-treated seeds produced an average of 174,476 CFUs of *V. dahliae*/100 plants by 35 days after planting (3,096 CFU/g fresh weight), illustrating the high rate of seed transmission. Six treatments reduced this to <50,000 CFUs/100 plants, including two organic treatments (Seed Support II and Seedgard). The most effective treatments were Topsin M 70WP and Mertect 340F (alone or with Farmore D300). Very few of the treatments evaluated in this study were registered for use on spinach seed in the U.S. in 2008. However, a 2009 Special Local Needs 24(c) seed treatment registration for Topsin M 70 WP was granted by the WA State Dept. of Agriculture for management of *Verticillium* in spinach stock seed planted in spinach seed crops in Washington State in 2009.

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II. Main Body of Report.

Project Title: Evaluation of seed treatments for management of seedborne *Verticillium* in spinach, 2008.

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Cooperating Personnel: Krishna Subbaro, University of California-Davis

Objectives:

Assess the efficacy of conventional and organic fungicides for eradicating *Verticillium dahliae* from spinach seed, preventing seed transmission of *V. dahliae*, and reducing the risk of infesting soils into which infected spinach seed is planted. This is objective 'A' of the nine objectives (A to I) listed in the original proposal (management of *V. dahliae* associated with spinach seed and Verticillium wilt on crops that follow spinach and lettuce).

Procedures:

A seed lot of a proprietary spinach cultivar naturally infected with *Verticillium* spp. was used to evaluate 11 organic and 9 conventional fungicide seed treatments for control of this pathogen. The treatments and rates of application are shown in Table 1. For Captan 400C, Coronet, Thiram 42-S, Topsin M 70WP, and BAS 595 XGF treatments, seeds were placed in a slurry of a proprietary blue colorant (7.0% by seed weight mixed 1:1 with water), to which the appropriate fungicide was added. Seeds were treated with water + colorant for the control treatment. The seeds and slurry were shaken in a flask until the slurry was adsorbed completely onto the seeds. All other treatments were applied by the registrants.

Seed germination was tested for four replications of 100 seeds/treatment using the blotter assay of the Association of Official Seed Analysts. A freeze-blotter seed health assay was also completed for four replications of 100 seeds/treatment. The seeds were placed onto damp blotters in 10 cm x 10 cm clear acrylic boxes (Hoffman Manufacturing) (34-36 seeds/box). Seeds imbibed on the blotters in the dark for 25 h, and were then incubated at –20ºC for 25 hours followed by 12 days at 24ºC under a 12 hour/12 hour day/night cycle with near-UV and cool white fluorescent light by day. The seeds were examined 5, 9, and 14 to 21 days after plating (8 to 100X magnification).

To assess inoculum potential from spinach seed, the same seed lot was planted in sterilized sand and the leaves harvested after 35 days to mimic harvest of a 'baby leaf' spinach crop. The roots, crown, and cotyledons remaining were then crushed in phosphate buffer and plated on NP-10 agar. In addition, seedlings were sampled at weekly intervals from 7 to 28 days after planting to determine the location of seedling infection by *V. dahliae*. Each week, 10 seedlings were washed, surface-sterilized with 70% ethyl alcohol, cut into 10 to 15 mm-long sections from the roots to the cotyledons/leaves (when the latter developed), and the sections plated on NP-10 agar. The sections were examined microscopically.

Thirteen of the seed treatments were also evaluated for potential efficacy at preventing seed transmission and soil infestation by *V. dahliae*. For each of four replications of each treatment and non-treated seeds, the seeds were planted into washed sand in a 200-cell flat (one seed/cell). The flats were set up in a randomized complete block design in a greenhouse with lights set at 10 hour daylength. After 35 days, the leaves of each seedling were cut using scissors to mimic harvest of a 'baby leaf' spinach crop. The roots, crown, and cotyledons remaining were washed thoroughly in running tap water. The washed seedlings were divided into four groups of 25 plants/flat. Each set of 25 seedlings was weighed and placed in a mesh-lined, BioReba ELISA bag. The seedlings in each bag were crushed using a drill press with an Agdia ball-bearing adaptor, and the crushed tissue mixed in 3 ml 0.0125M phosphate buffer. A 10-fold dilution series in 0.0125M phosphate buffer was prepared, and three 0.1 ml aliquots of each dilution plated on NP-10 agar medium (semi-selective for *Verticillium* spp.). The plates were incubated in the dark at 26^{o}C for 21 days. Colonies typical of *V. dahliae* (radiating microsclerotia) were counted (8 to 100X magnification) to calculate total number of colony forming units (CFUs) of *V. dahliae*/100 plants, and total CFUs/g fresh weight. Data were subjected to analyses of variance and means comparison using Fisher's protected least significant difference (LSD).

In addition, a component seed health assay was used to determine the location of *Verticillium* on/in spinach seed. Pericarps and embryos were manually separated from each of 100 non-treated seeds. Pericarps and embryos of each of 30 seeds were rinsed in sterile water. Pericarps and embryos of 35 seeds were surface-sterilized individually in 1.2% NaOCl for 30 sec and then triple-rinsed in sterile, deionized water. An additional 35 pericarps and embryos were surface-sterilized similarly for 60 sec and rinsed three times. The pericarps and embryos were subjected to the freeze-blotter seed health assay described above.

Results and Discussion:

In the freeze-blotter seed health assay, seven treatments reduced the incidence of seedborne *Verticillium* from 63.5% for non-treated seed to <10%, the current threshold for exporting spinach seed from the U.S. to Mexico (Table 1). The most effective fungicide treatments were Topsin M 70 WP (0%), Mertect 340F applied alone (0.3%) or with Farmore D300 (0%), and BAS 595 XG F (2.0%). Three proprietary organic treatments were also highly effective: Seedgard (2.8%), Seed Support II (3.3%), and Seed Support I (7.0%). Ten treatments showed intermediate efficacy, of which three (Thiram 42-S, Captan 400C, and Experimental II) reduced the incidence of seed on which *Verticillium* spp. were observed to <30%. Treatments that did not significantly reduce the incidence of seed with *Verticillium* spp. included two organic treatments (ACX 801 and ACX 802), and Coronet at 200 ml/100 kg seed. Some treatments prevented *Verticillium* spp. from developing on the pericarp, but the fungus was then observed developed on the embryo through the split end of the pericarp (where the radicle emerges) or the funiculus (attachment to the plant), illustrating the systemic nature of infection of spinach seed from the mother plant. In the component seed health assay, *Verticillium* was observed on 50% of the pericarps and 51% of the embryos not surface-sterilized, 46% of the pericarps and 74% of the embryos sterilized for 30 sec, and 34% of the pericarps and 29% of the embryos sterilized for 60 sec, demonstrating the internal nature of spinach seed infection (*data not shown*). Non-treated seed had 37.0% *S. botryosum* in the seed health assay (Table 1). Seed treatments most effective

against this leaf spot fungus included the same three organic treatments that were highly effective against *Verticillium* (Seed Support I, Seed Support II, and Seedgard each with <1.0% *S. botryosum*) and three conventional fungicide treatments (Coronet at either rate, Farmore D300 + Mertect 340F, and Thiram 42-S all of which resulted in <5% seedborne *S. botryosum*).

None of the treatments significantly improved seed germination compared to that of non-treated seed (73.8%). Only Experimental II significantly reduced seed germination (to 61.5%). In the preliminary assessment of inoculum potential from planting spinach seed infected with *Verticillium*, colonies of *V. dahliae* were detected at >9,000 CFU/100 emerged seedlings. For seedlings sampled at weekly intervals and assayed for *V. dahliae*, the fungus was observed on the roots at each sampling period, i.e., as early as 7 days after planting, at a similar incidence to the percentage seed infected (~60%). *V. dahliae* developed on the main root approximately 10 to 20 mm behind the root tip at each sampling period, but was not observed on the crown, cotyledons, or true leaves during the 4 weeks after planting. In the greenhouse seed transmission assay of 13 seed treatments compared to non-treated seed, the non-treated seeds produced an average of 174,476 CFUs of *V. dahliae*/100 plants by 35 days after planting (3,096 CFU/g fresh weight), illustrating the high rate of seed transmission of *V. dahliae*. Six seed treatments reduced this inoculum potential to <50,000 CFUs/100 plants, including two organic treatments (Seed Support II and Seedgard with 46,349 and 38,770 CFUs/100 plants), and three conventional fungicide treatments (Topsin M 70WP at 1,487 CFUs, Mertect 340F alone at 5,378 CFUs and Mertect 340F + Farmore D300 at 1,813 CFUs/100 plants assayed).

In summary, the results of this study demonstrate the strong potential efficacy of a number of organic and conventional fungicide seed treatments for management of *Verticillium* on spinach seed. The results also highlight the need for seed treatments with multiple modes of action to address different seedborne pathogens as well as to protect developing seedlings from soilborne inoculum. Combination seed treatments (e.g., Farmore D300 + Mertect 340F, or Topsin M 70WP + Coronet + Apron XL) could play a very important role in the use of seed treatments to manage more effectively the spectrum of pathogens that can cause losses in spinach crops and/or affect crops grown in rotation with spinach. Unfortunately, most of the treatments evaluated in this study are not yet approved for use on spinach seed, although this research is expected to contribute towards registration of some of the organic and/or conventional treatments that show good potential for management of *Verticillium* and/or *S. botryosum*, two important seedborne pathogens of spinach. In March 2009 a Special Local Needs 24(c) seed treatment registration for Topsin M 70 WP was granted by the Washington State Dept. of Agriculture for management of *Verticillium* in spinach stock seed planted in spinach seed crops in Washington State. The two conventional fungicides with the greatest efficacy against *Verticillium* in spinach seed were Topsin M 70WP and Mertect 340F. A very recent review of Topsin M 70WP by the Environmental Protection Agency stated that the risk cup for this fungicide is full, i.e., in order to get approval for a seed treatment registration in spinach, some other current registration for this product will need to be dropped. Discussions are in progress with the registrant (United Phosphorus, Inc.) of Topsin M 70WP. In addition, discussions are in progress with the registrant of Mertect 340F (Syngenta Crop Protection) for obtaining federal seed treatment registrations of these products on spinach seed used to plant fresh market and processing spinach crops.

Table 1. Evaluation of conventional fungicide and organic seed treatments for management of *Verticillium* in spinach seed.

Seed treatment and rate of product/100 kg seed	Seed health assay: % Seed infected		Seed germination assay: % Seed germinated (21 days)	Greenhouse *V. dahliae* seed transmission assay (35 days)	
	Verticillium spp.	*Stemphylium botryosum*		CFUs/ 100 plants	CFUs/ g fresh weight
Control	63.5 a[d]	37.0 ab	73.8 abcd	174,476 ab	3,096 a
ACX 802[a] ……..	66.0 a	27.0 bc	73.5 bcd	-	-
ACX 801[a] ……..	59.0 ab	27.5 bc	74.0 abcd	-	-
Coronet 200 ml ……..	54.5 abc	1.0 ijkl	77.8 abc	-	-
Vortex 6.1 g …….....	51.0 bc	27.0 bc	77.5 abcd	-	-
Coronet 400 ml ……..	41.5 cd	0.8 jklm	78.0 abc	69,628 abcd	1,447 ab
ACX 804[a] ……..	37.0 d	5.5 gh	76.5 abcd	123,606 a	2,000 a
Experimental I[a] ……..	36.0 de	13.8 de	70.8 d	-	-
Incotec I[a] …….	32.5 ef	4.3 hi	75.8 abcd	-	-
ACX 803[a] ……..	30.8 ef	21.8 cd	78.5 ab	-	-
Incotec II[a] ……..	30.3 ef	5.0 hi	72.3 bcd	121,843 bcd	2,086 ab
Experimental II[a] ……..	25.3 g	10.8 efg	61.5 e	62,944 abcd	1,186 ab
Captan 400C 391 ml ...	25.0 fg	9.0 fg	80.5 a	119,302 abc	2,211 ab
Thiram 42-S 521 ml ...	17.8 gh	2.8 hij	72.8 bcd	104,043 abc	1,815 ab
Seed Support I[a] ……..	7.0 hi	0.0 m	74.0 abcd	232,588 abc	3,677 ab
Seed Support II[a] ……..	3.3 ij	0.3 lm	77.8 abc	46,349 cde	767 b
Seedgard[a] ……	2.8 kl	0.5 klm	71.5 cd	38,770 def	667 ab
BAS 595 XGF[b] ……..	2.0 ij	13.5 def	72.0 bcd	29,055 efg	530 b
Mertect 340F 122 ml ..	0.3 kl	35.5 ab	72.8 bcd	5,378 fg	100 c
Mertect 340F 122 ml + Farmore D300[c] ……	0.0 l	1.8 ijk	72.5 bcd	1,813 g	33 c
Topsin M 70WP 500 g	0.0 l	50.3 a	74.8 abcd	1,487 g	29 c
LSD (Pr < 0.05) ……..	Rank	Rank	6.78	Rank	Log

[a] ACX 801, ACX 802, ACX 803, ACX 804, Experimental I, Experimental II, Incotec I, Incotec II, Seed Support I, Seed Support II, and Seedgard are all proprietary products developed by companies for use in certified organic vegetable crops. Seedgard is a proprietary steam seed treatment of Lantmännen and Incotec (Uppsala, Sweden). None of these products was registered for use in organic spinach production in the U.S. in 2008.

[b] BAS 595 XGF = seed treatment formulation of triticonazole (BASF Corporation) at 100 g active ingredient (a.i.)/100 kg seed.

[c] Farmore D300 = seed treatment with azoxystrobin + fludioxonil + mefenoxam applied by Syngenta Crop Protection at 2.5 + 2.5 + 7.5 g a.i./100 kg seed, respectively.

[d] Means with the same letter in a column are not significantly different based on Fisher's protected least significant difference (LSD). 'NS' = means not significantly different at $P = 0.05$. 'Log' and 'Rank' indicate original means are shown but means separation is based on log transformation and Friedman's non-parametric rank test, respectively, because of heterogeneous variances and/or non-normal variances in the analyses of variance.