Annual Report to the California Leafy Greens Research Board
for the period 1 April 2009 to 31 March 2010

I. Abstract

Project Title: Evaluation of seed treatments for management of seedborne *Verticillium* in spinach, 2009-10.

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 concerns about introducing *V. dahliae* on spinach seed into fields subsequently planted to other susceptible crops. In 2008-09, 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. The trial was repeated in 2009-10 with those treatments that showed greatest potential, to assess consistency in efficacy of these treatments. A seed germination assay was used to assess whether any treatment might adversely affect seed quality (germination/vigor). No treatment improved germination, but three proprietary organic treatments significantly reduced seed germination (ACX 804 by 10%, Experimental I by 11%, and Experimental II by 37%). A freeze-blotter seed health assay was used to assess the extent to which each treatment reduced the level of *Verticillium* on spinach seed. Ten treatments reduced the incidence of *Verticillium* to <10%, the current threshold for exporting spinach seed into Mexico. The most effective fungicides (0% *Verticillium* detected) were Topsin M 70 WP, Topsin 4.5FL, Mertect 340F (alone or with Farmore D300, and Experimental I; followed by BAS 595 XG F and Seed Support II, Seedgard, Thiram 42-S, and ACX 804 (2.8 to 9.1%). Three treatments had intermediate efficacy (Captan 400C, Incotec II, and Experimental II with <30% *Verticillium*). Coronet was the least effective (34%). Some treatments prevented *Verticillium* from developing on the pericarp but the fungus was observed on the embryo through the split end of the pericarp (where the radicle emerges) or the funiculus, illustrating the systemic nature of infection of spinach seed.

The seed treatments were also evaluated for preventing seed transmission or soil infestation by *V. dahliae*. Seeds with each treatment were planted in sterilized sand and the leaves harvested after 35 days to mimic a 'baby leaf' spinach crop. The roots, crowns, and cotyledons remaining were crushed in buffer and plated on an agar medium. Seedlings that grew from non-treated seeds produced an average of 123,450 CFUs of *V. dahliae*/100 plants, demonstrating a high rate of seed transmission. Ten seed treatments reduced this to <60,000 CFUs/100 plants. The most effective were Topsin M 70WP (0 CFUs), Topsin 4.5FL (39 CFUs), Mertect 340F alone (59 CFUs), BAS 595 XGF (857 CFUs) and Mertect + Farmore D300 (2,652 CFUs). The best organic treatment was Experimental I (10,897 CFUs). Four treatments had intermediate efficacy (Seedgard with 24,797, Seed Support II with 35,937, ACX 804 with 46,406, and Thiram 42-S with 59,970 CFUs/100 seedlings). Only Thiram 42-S had a federal spinach seed treatment label in the US in 2009. However, a Special Local Needs 24(c) seed treatment registration for Topsin M 70 WP has been granted by WA State Dept. of Agric. for stock seed planted in spinach seed crops in Washington. Leaves harvested from plots with the two Mertect 340F treatments were subjected to residue analyses by Syngenta Crop Protection. The active ingredient, thiabendazole, was detected at <10 ppb in each sample. The data is being used by Syngenta to pursue a federal spinach seed treatment label for Mertect 340F.

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II. Main Body of Report

Project Title: Evaluation of seed treatments for management of seedborne *Verticillium* in spinach, 2009-10.

Project Investigator: Lindsey J. du Toit, Washington State University Mount Vernon NWREC, Mount Vernon, WA. Tel: 360-848-6140. Email: dutoit@wsu.edu

Cooperating Personnel: Mike L. Derie, Barbara J. Holmes, and Louise M. Brissey, Washington State University; Krishna Subbarao, University of California-Davis

Objectives:

Continue to assess the efficacy of conventional and organic seed treatments for eradicating *Verticillium* 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) in the original CLGRB proposal (management of *V. dahliae* associated with spinach seed and Verticillium wilt on crops that follow spinach and lettuce).

Procedures:

Verticillium dahliae is systemic and readily seed transmitted in spinach, raising concerns about introducing *V. dahliae* on spinach seed into fields subsequently planted to other susceptible crops. In 2008-09, 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. The trial was repeated in 2009-10 with a subset of those treatments that showed greatest potential in the 2008-09 trial, in order to assess consistency in efficacy of these treatments. The same proprietary spinach seed lot naturally infected with *Verticillium* spp. and *Stemphylium botryosum* was used to evaluate six organic and eight conventional fungicide treatments for control of these two pathogens. The treatments and rates of application are detailed in Table 1 below. For Captan 400C, Coronet, Thiram 42-S, Topsin M 70WP, Topsin 4.5 FL, 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 using four replications of 100 seeds/treatment with 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 (each blotter moistened with 12 ml sterile deionized water) in 10 cm x 10 cm clear acrylic boxes (Hoffman Manufacturing) (32 to 34 seeds/box). Seeds imbibed on the blotters in the dark for 25 h, and were then incubated at –20ºC for 25 h in the dark, followed by 21 d at 24ºC under a 12 h/12 h day/night cycle with near-UV and cool white fluorescent light by day. The seeds were examined 5, 9, 14 and 21 d after plating (8 to 100X

magnification).

The treatments were also evaluated for efficacy at preventing seed transmission and soil infestation by *V. dahliae*. For each of four replications per treatment, the seeds were planted into washed sand in a 200-cell flat (one seed/cell). In addition, non-treated seeds were also planted into each of four flats (replicates) containing semi-pasteurized (heated at 65°C for 2 h) or non-pasteurized soil (pH 5.8) sampled from a field in central Washington with no history of spinach production. The flats were set up in a randomized complete block design in a greenhouse with lights set at a 10 h daylength. After 35 d, 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 sets of 25 plants/flat. Each set of 25 seedlings was weighed and placed in a mesh-lined, BioReba ELISA extraction bag. The seedlings in each bag were then crushed using a drill press with an Agdia ball-bearing adaptor, and the crushed tissue mixed in 3 ml 0.0125M potassium buffer. A 10-fold dilution series in 0.0125M phosphate buffer was prepared, and three 0.1 ml aliquots of each dilution plated onto NP-10 agar medium (semi-selective for *Verticillium* spp.). The plates were incubated in the dark at 26°C for 27 to 28 d. Colonies typical of *V. dahliae* (radiating microsclerotia) were counted (8 to 100X magnification) to calculate the 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, leaves harvested from each replicate flat of the control, Mertect 340F, Mertect 340F + Farmore D300, Coronet, and BAS 595 XGF treatments were frozen at -80°C immediately after harvest for a month. Leaves from the two Mertect treatments and subsample of leaves from the control plots were shipped to Syngenta Crop Protection in North Carolina for thiabendazole residue analyses. Leaves from the Coronet and BAS 595 XGF treatments, subsamples of leaves from the control plots, and samples of the seeds used for these three treatments were shipped to BASF Corporation for residue analyses.

Results and Discussion:

None of the spinach seed treatments significantly improved germination compared to the control seed (78.3% germination), but three treatments reduced germination significantly (to 70.3% for ACX 804, 69.3% for Experimental II, and 49.3% for Experimental I). In the seed health assay, 10 treatments reduced the incidence of seedborne *Verticillium* from 60.3% for control seed to <10%, the current threshold for exporting spinach seed into Mexico. The most effective treatments (with 0% *Verticillium*) were Topsin M 70 WP, Topsin 4.5FL, Mertect 340F (alone and with Farmore D300), and Experimental I; followed by BAS 595 XGF and Seed Support II, Seedgard, Thiram 42-S, and ACX 804 (2.8 to 9.1%). Captan 400C, Incotec II, and Experimental II had intermediate efficacy (<30%), and Coronet was the least effective (34.3% *Verticillium* spp.). Some treatments prevented *Verticillium* spp. from developing on the pericarp, but the fungi were observed on the embryo through the split end of the pericarp (where the radicle emerges) or the funiculus, illustrating the systemic nature of infection of spinach seed from the mother plant. Control seed had 54.8% *Stemphylium botryosum* in the seed health assay. Seed treatments most effective against this leaf spot fungus (<10% infection)

included three conventional fungicides (Thiram 42-S, Coronet, and Captan 400C with 0.5, 2.0, and 9.8% seeds infected, respectively) and five organic treatments (0% for Seed Support II, 0.8% for Experimental I and Incotec II, 1.8% for ACX 804, 3.8% for Seedgard, and 6.3% for Experimental II).

In the seed transmission assay, roots, hypocotyls, and cotyledons remaining after harvest of the leaves 35 d after planting the control seed produced an average of 123,450 CFUs of *V. dahliae*/100 plants (2,026 CFUs/g fresh weight), demonstrating a high rate of seed transmission of *V. dahliae*. Ten treatments reduced this inoculum potential to <60,000 CFUs/100 plants. The most effective treatments were Topsin M 70WP (0 CFUs), Topsin 4.5FL (39 CFUs), Mertect 340F alone (59 CFUs), BAS 595 XGF (857 CFUs), and Mertect 340F + Farmore D300 (2,652 CFUs). The best organic treatment was Experimental I (10,897 CFUs). Four treatments had intermediate efficacy at reducing seed transmission (Seedgard with 24,797, Seed Support II with 35,937, ACX 804 with 46,406, and Thiram 42-S with 59,970 CFUs/100 plants).

Interestingly, planting non-treated seed into semi-pasteurized and non-pasteurized field soil vs. sand resulted in seed transmission rates of *V. dahliae* as low as that detected for the best fungicide seed treatments (42 and 89 CFUs/100 plants, respectively). This demonstrates clearly the need to evaluate the potential effects of planting media and soil properties on seed transmission rates of *V. dahliae* in spinach and in the many other crops for which this pathogen is known to be seedborne.

Comparison of results of this 2009 trial (Table 1) with results of the original, 2008-09 trial (Table 2) revealed consistency in relative efficacy of the seven conventional fungicide seed treatments evaluated in both trials. Topsin M 70WP (and the seed treatment formulation, Topsin 4.5FL, evaluated in the repeat trial) was consistently the most effective at preventing seed transmission of *Verticillium*, followed by Mertect 340F alone and BAS 595 XGF (triticonazole), with Thiram 42-S and Captan 400C showing intermediate efficacy. Seedgard (a proprietary steam treatment) and Seed Support II (unknown active ingredient) were both quite effective at reducing seed transmission rates of *V. dahliae* in the two trials. In contrast, Experimental I showed intermediate efficacy against seedborne *Verticillium* in the first trial, in which the treatment had no adverse effect on seed germination, but was highly effective at reducing seed transmission rates in the repeat trial in which the treatment reduced seed germination by 11%.

In summary, the results of this study demonstrate the strong potential efficacy of a number of organic and conventional 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. Of the treatments evaluated in this study, only Thiram 42-S had a federal spinach seed treatment label in the US in 2009. This research is expected to contribute towards registration of some organic and/or conventional treatments for management of *Verticillium* and/or *S. botryosum*, two important seedborne pathogens of spinach. The two conventional fungicides with the greatest efficacy against *Verticillium* in spinach seed were Topsin M 70WP and Mertect 340F. A 2009 review of Topsin M

70WP by the US 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. The registrant of Topsin M 70WP and Topsin 4.5FL (United Phosphorus, Inc.) is not willing to pursue a spinach seed treatment. The WA State Dept. of Agriculture has approved a Special Local Needs 24(c) seed treatment registration for Topsin M 70 WP for stock seed planted in spinach seed crops in Washington. Residue analyses by Syngenta Crop Protection detected <10 ppb thiabendazole in leaves harvested from each replicate flat planted with seed treated with Mertect 340F or Mertect 340F + Farmore D300. Thiabendazole is the active ingredient in Mertect 340F. The data is being used by Syngenta Crop Protection to pursue a federal spinach seed treatment label for Mertect 340F. Results are still pending for residue analyses of leaves harvested from the Coronet and BAS 595 XGF plots.

Table 1. 2009 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		Germination assay: % Seed germinated (21 d)	*V. dahliae* seed transmission assay (35 d after planting)	
	Verticillium spp.	*Stemphylium botryosum*		CFUs/100 plants	CFUs/g fresh weight
Control, sand[z]	60.3 a[v]	54.8 a	78.3 ab	123,450 ab	2,026.0 a
Coronet 400 ml	34.3 ab	2.0 ef	77.5 ab	131,273 a	2,119.3 a
Experimental II[y]	21.1 b	6.3 cd	69.3 d	137,650 ab	2,207.5 a
Incotec II[y]	18.5 b	0.8 fg	77.5 ab	91,500 bc	1,680.1 abc
Captan 400C 391 ml	14.3 c	9.8 c	81.0 ab	106,025 ab	1,755.4 ab
ACX 804[y]	9.1 c	1.8 ef	70.3 cd	46,406 cd	760.4 cd
Thiram 42-S 521 ml	6.5 c	0.5 fg	80.3 ab	59,970 cd	975.6 bcd
Seedgard[y]	5.5 c	3.8 de	82.3 a	24,797 d	344.9 de
Seed Support II[y]	2.8 d	0.0 g	77.3 ab	35,937 cd	599.0 cd
BAS 595 XGF 200 ml[x]	2.8 d	22.1 b	75.3 bcd	857 ef	13.4 fg
Experimental I[y]	0.0 e	0.8 fg	49.3 e	10,897 e	187.2 ef
Mertect 340F 122 ml + Farmore D300[w]	0.0 e	11.3 c	83.5 a	2,652 e	40.1 f
Mertect 340F 122 ml	0.0 e	53.0 a	80.5 ab	59 fgh	0.9 ghi
Topsin 4.5FL 649 ml	0.0 e	61.5 a	75.8 bc	39 gh	0.6 hi
Topsin M 70WP 500 g	0.0 e	56.3 a	79.0 ab	0 h	0.0 i
Control, non-pasteurized soil[z]	NA	NA	NA	42 efg	0.8 fgh
Control, semi-pasteurized soil[z]	NA	NA	NA	89 efg	1.5 fgh
LSD (Pr < 0.05)	Rank	Arcsin	6.28	Rank	Rank

[z] Spinach seed was planted into washed sand in a 200-cell flat for each replication of each seed treatment. In addition, control seeds were planted into field soil from central Washington (soil pH 5.8) in four replicate 200-cell flats. The field soil was either non-pasteurized or partially-pasteurized to assess the potential impact of soil vs. sand as well as soil microbial activity on seed transmission of *V. dahliae*.

[y] ACX 804, Experimental I, Experimental II, Incotec II, Seedgard and Seed Support II are proprietary products developed by companies for certified organic crops. Seedgard is a proprietary steam seed treatment of Lantmännen and Incotec (Uppsala, Sweden). These products were not registered for use in organic spinach production in the U.S. in 2009.

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

[w] 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.

[v] Means followed by the same letter within a column are not significantly different at $P = 0.05$. 'NA' = not applicable to that assay. 'Arcsin' and 'Rank' indicate original means are shown but means separation is based on arcsin square root tranformation and Friedman's rank test, respectively, because of heterogeneous variances and/or non-normal residuals in the analyses of variance.

Table 2. 2008 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)	*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 proprietary products developed by companies for certified organic vegetable crops. Seedgard is a proprietary steam seed treatment of Lantmännen (Uppsala, Sweden) and Incotec. None of these products was registered for use in organic spinach crops in the US 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 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 residuals in the analyses of variance.