The Pathogen that Causes Verticillium Wilt of Potato, *Verticillium dahliae*, Infects and Alters Biomass of Several Rotational Crops

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ABSTRACT

Verticillium wilt, caused by the fungus *Verticillium dahliae*, reduces yields of potato in temperate production regions worldwide. Management of Verticillium wilt depends on host resistance, sanitation of infested host debris, optimization of irrigation regimes, and soil fumigation. Crop rotation also represents a potential management tactic for Verticillium wilt; however, the wide host range and survivability of *V. dahliae* may limit the effectiveness of this tactic in potato production systems. The potential of rotation crops to serve as hosts for *V. dahliae* was evaluated by inoculating mustards, grasses, and Austrian winter pea with eight strains of *V. dahliae.* The density of the primarily inoculum, survival structures called microsclerotia, was estimated from plant stems, roots and soil. Symptoms typical of wilt were not observed in any rotation crop but plant biomass of some crops infected with strains of *V. dahliae* was altered compared to non-inoculated controls. Strains were host-specific and infected a subset of the rotation crops tested but microsclerotia from at least one strain were observed on each rotation crop. Some strains were also host-adapted and differentially altered plant biomass or produced differential amounts of inoculum on rotation crops. For example, arugula and Austrian winter pea supported more inoculum of specific strains of *V. dahliae* than potato. Information about the asymptomatic and symptomatic infection and differential inoculum formation of *V. dahliae* strains on rotation crops presented here will be useful in designing rotations for management of Verticillium wilt in potato production systems.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is an economically valuable crop in the Pacific Northwest (Washington, Oregon, and Idaho) of the United States where yields account for 58% of nationwide production (cwt) (NASS 2015). Fields planted with potato may or may not be rotated with crops such as mustards, grasses and legumes. *Verticillium dahliae* Kleb. causes Verticillium wilt in potato production systems and can reduce yields up to 50% (Powelson and Rowe 1993). Disease symptoms vary among hosts but generally include stunting, wilting, and premature mortality (Horner 1954; Isaac and Harrison 1968).

Verticillium dahliae can survive in soil for at least 14 years as microsclerotia (Wilhelm 1955). Microsclerotia germinate in response to exudates from plant roots (Emmaty and Green 1969; Mol 1995a), the vegetative growth of the fungus then penetrates root tips (Perry and Evert 1983; Schreiber and Green 1963) and colonizes the root cortex and vascular system of the plant (Klosterman et al. 2009). Asexual spores of *V. dahliae*, called conidia, are generally produced during flowering, move upward through the vascular system and eventually restrict plant

transpiration (Buckley et al. 1969; Eynck et al. 2007). Microsclerotia develop during death of the infected plant in most stem and root tissues (Isaac and Harrison 1968; Mol and Scholte 1995; Perry and Evert 1984; Slattery 1981). Microsclerotia are returned to the soil by incorporation of crop residue and decomposition of infested plants.

Available practices to manage Verticillium wilt of potato include host resistance, sanitation, irrigation management, and fumigation (Johnson and Dung 2010), but fumigation is not consistently economical in potato production systems and options are increasingly limited by regulatory restrictions. Crop rotation is another practice that has been actively investigated for management of Verticillium wilt (Bhat and Subbarao 1999; Davis et al. 1996; Easton et al. 1992; Huisman and Ashworth 1976; Joaquim et al. 1988; Larkin et al. 2011; Njoroge et al. 2009; Xiao et al. 1998). *V. dahliae*, however, causes wilt in over 200 broad-leaved plants (Pegg and Brady 2002) and therefore poses significant challenges in the development of crop rotations. Additionally, *V. dahliae* asymptomatically infects a range of other plants, including both grasses and broad-leaved plants (Benson and Ashworth 1976; Evans and Gleeson 1973; Krikun and Bernier 1987, Lacy and Horner 1966, Malcolm et al. 2013; Malik and Milton 1980; Martison and Horner 1962; Mol 1995b; Mol et al. 1995; Vargas-Machuca et al. 1987; Woolliams 1966). Despite the wide host range of *V. dahliae,* variability among strains exists and some strains are host-specific and infect a subset of the host range of the species (Bhat and Subbarao 1999), and/or host-adapted and are more aggressive and produce more inoculum on a subset of the host range than other strains (Douhan and Johnson 2001). Hence, to design crop rotations that minimize the development and persistence of *V. dahliae* inoculum in potato fields, the asymptomatic host range of *V. dahliae* and the potential inoculum production on various rotation crops requires additional research.

The overall objective of this study was to determine if strains of *V. dahliae* that cause disease on potato, mint and other commercial crops can infect rotation crops of potato. Secondary objectives were to: (i) determine if microsclerotia are produced on infected rotation crops and estimate the density of inoculum in stems, roots, and soil of infected rotation crops, (ii) estimate the effects of *V. dahliae* infection on rotation crop symptom development and biomass, and (iii) estimate the incidence of infected stems of rotation crops from commercial fields with a history of Verticillium wilt of potato.

MATERIALS AND METHODS

Two experiments were conducted to estimate the inoculum density of *V. dahliae* from rotation crops of potato and mint. The experiments differed in experimental design, specifically in the number and species of rotation crop and the number and origin of *V. dahliae* strains, the number of replicates per treatment, the density of inoculum used to infest growth media, the type of growth media, the duration of the experiments, and the data collected.

Incidence of infected stems and inoculum density of two *Verticillium dahliae* strains from roots and soil of two brown mustards, white mustard, sweet corn, spring wheat and sudangrass. A greenhouse experiment was conducted to determine if rotation crops are infected

by *V. dahliae* strains from potato and peppermint. The experimental design was comprised of a two-way factorial treatment structure, with six rotation crops and three primary crops crossed with two strains and one non-infested control. Rotation crops were white mustard 'Martigena' (*Sinapis alba* L.), brown mustards 'Pacific Gold' and ISCI 99 (*Brassica juncea* L.), sweet corn 'Marvel' (*Zea mays* L.), spring wheat 'Alpowa' (*Triticum aestivum* L.), and sudangrass 'Piper' (*Sorghum drummondii* Nees ex Steud.). Primary crops were potato 'Norkotah', native mint (*Mentha spicata* L.), and peppermint 'Black Mitcham' (*M. x* piperita L.). *V. dahliae* strains used to infest soil were originally isolated from potato (653) or mint (111) (Table 1). Pots were arranged in a randomized complete block design structure with three replicates. The trial was repeated once in 2012.

Inoculum of *V. dahliae* was prepared from *V. dahliae* strains cultured in Czapek-Dox broth (MP Biomedicals, Solon, OH) on a rotary shaker at 125 rpm for 7 days at 22°C**.** Liquid cultures were mixed with 700 ml of sand (grain size between 0.6 and 2.4 mm) in 1 L flasks, and flasks were agitated to distribute propagules throughout the sand. Infested sand was distributed over the surface of a 33 x 22 x 5 cm pyrex (Pyrex, Charleroi, PA) pan lined with 2 layers of paper towels,1 layer of aluminum screen with 1.4 mm openings, 1 layer of Mira cloth (EMD Chemicals, San Diego, CA.), and dried in a laminar flow hood for 7 days. Dried sand was stored at 22°C for 4 weeks to air dry the fungal propagules (Butterfield and DeVay 1977; Galanopoulos and Tribe 1974).

Inoculum density was estimated from 10 samples of 0.1 g of sand inoculum uniformly distributed across the surface of 100 x 15 mm plastic Petri dishes (Thermo Fisher Scientific, Waltham, MA.) containing NP-10 media (Goud and Termorshuizen 2003; Kabir et al. 2004). Sand was rinsed from the surface of the media 48 h later and colonies were counted with a Nikon SMZ800 dissecting microscope at 10 to 40X (Nikon Corp., Tokyo).

Growth media was infested with approximately 30 colony-forming units (CFU)/g of each *V. dahliae* strain by mixing 3,000 g of Sunshine L2 soilless potting media (Sun Gro Horticulture, Agawam, MA) with 3 g of sterilized sand or sand infested with 30,000 CFU/g of *V. dahliae* 653 or 111. Growth medium to be planted with *Mentha* spp. were fertilized with 500 ml of 1.5 g 24-8-16 N-P-K/3.79 liters of water (Scotts Miracle Grow Company, Marysville, OH), whereas media for all other crops were fertilized with 13 g of 16-16-16 N-P-K fertilizer (Agriliance Agronomy Co., St. Paul, MN).

Plant propagules were tested for *V. dahliae* infection and infestation before planting. Basal-end sections approximately 15 mm diameter were aseptically excised from potato seed tubers, disinfested in 0.5% NaOCl for 3 min, rinsed in sterilized distilled water for 1 min, dried on sterile paper towels and placed on NP-10. Samples of 50 seed of each plant except potato and both mint species were disinfested for 1 min in 0.5% NaOCl, rinsed in sterilized distilled water for 1 min, dried on sterile paper towels, and placed on NP-10. Tuber sections and seed were incubated in the dark for 5 weeks at 22°C and then scanned for *V. dahliae* colonies with a dissecting microscope. Candidate *V. dahliae* colonies were identified by the presence of microsclerotia and fungal colony morphology (Inderbitzin et al. 2011a)**.** Only tubers and seed

sources not infected or not infested with *V. dahliae* were used in experiments. Rooted cuttings of both mint species derived from *V. dahliae*-free stock plants were used. Stock plants were tested for *V. dahliae* by incubating sterilized stem sections on NP-10 and observing cultures for the presence of *V. dahliae*. Plants were planted in 15.2 x 17.8 cm, 3 L pots (J. M. McConkey & Co., Inc., Sumner, WA).

Two 1-cm sections were sampled from stems 3 (lower) and 30 (upper) cm above the soil surface after the end of the season for the mint species or after plant senescence for all other species. Stem sections were disinfested for 3 min in 0.5% NaOCl and then rinsed for 1 min in distilled water, dried on sterile paper towels, and placed on NP-10. Stems were incubated in the dark for 5 weeks at 22°C and then scanned for *V. dahliae* colonies as described previously. Mean incidence of infected stems was calculated by averaging the incidence of infected subsamples within each replicate.

Roots were removed from growth media, rinsed with water, and dried in paper bags for 5 weeks at 22°C before further processing. Ten roots were subsampled from each root mass. Two, 3 cm long root sections were selected from each subsample, 3- and 15 cm below the plant crowns. Root sections were disinfested with 0.5% NaOCl for 3 min, rinsed in distilled water for 1 min, dried on sterilized paper towels and placed onto NP-10. Roots were incubated in the dark for 5 weeks at 22°C and then scanned for *V. dahliae* colonies as described previously. Mean root inoculum density (CFU/60 cm of root) was calculated by summing CFU/30 cm of root and averaging all subsamples within each replicate.

Five 15 x 110 mm diameter soil cores were collected from pots containing growth media. Subsamples were homogenized with a mortar and pestle and stored for 5 weeks at 22°C to air dry fungal propagules (Butterfield and DeVay 1977; Galanopoulos and Tribe 1974). Five subsamples of 0.1 g were distributed across the surface of Petri dishes containing NP-10 as described by Goud and Termorshuizen (2003). Cultures were incubated in the dark for 14 days at 22°C. Soil was rinsed from the surface of each plate and colonies of *V. dahliae* were counted. Mean soil inoculum density (CFU/g of soil) was calculated by averaging subsamples within each replicate.

Inoculum density of eight *Verticillium dahliae* strains from stems, roots and soil of arugula, Austrian winter pea, sweet corn, barley and sudangrass. A greenhouse experiment was conducted to determine if rotation crops not tested in the proceeding experiment and planted regionally are infected by and serve as inoculum reservoirs for an expanded set of *V. dahliae* strains. The experimental design was comprised of a two-way factorial treatment structure, with five rotation crops and two primary crops crossed with eight strains. Rotation crops were Austrian winter pea (*Pisum sativum* L.), arugula ‘Nemat’ (*Eruca sativa* Mill.), sweet corn, barley ‘Baroness’ (*Hordeum vulgare* L.), and sudangrass. Primary crops were potato and peppermint. *V. dahliae* strains used to infest soil are listed in Table 1 and differed in the crops from which they were isolated, vegetative compatibility groups (VCG), genotype, and mating-type (Dung et al. 2013). Pots were arranged in a randomized complete block design structure with five replicates. The trial was repeated once in 2014.

Inoculum and plant propagules were prepared as described above unless indicated otherwise. Growth media was infested as described above but inoculum density was less in this experiment. Growth media was infested with approximately 10 CFU/g of each *V. dahliae* strain by mixing 3,000 g of sterilized sand (grain size between 0.6 and 5 mm) with 30 g of sterilized sand or sand infested with 1,000 CFU/g of each *V. dahliae* strain. Plants were sown in pots with or without infested sand 24 h later. Fertilizer (100 ppm of N Peter's Professional 20-10-20 N-P-K peat-lite special (Everris International B.V., Geldermalsen, The Netherlands)) was applied daily with irrigation.

Symptom expression of rotation crops was monitored after plant emergence and weekly thereafter until harvest. Percent of whole plants exhibiting chlorosis, necrosis, and stunting were visually estimated on a weekly basis. Area under the senescence progress curve (AUSPC) was calculated for each treatment (Shaner and Finney 1977). Plant height, measured from the soil surface to the tallest plant tissue, and biomass of dried stems, seed, and roots were measured after harvest.

Stems were sampled from 3 and 19 cm above the soil surface and assayed for *V. dahliae* as previously described. The 15 cm stem sections remaining after two subsamples were collected were stored in the dark for 5 weeks at 22°C to dry fungal propagules (Butterfield and DeVay 1977; Galanopoulos and Tribe 1974). Both dried stems and whole root masses were ground in a coffee grinder (KitchenAid, St. Joseph, MI) for 90 s. Five subsamples of 0.1 g were uniformly distributed across the surface of Petri dishes containing NP-10 and incubated in the dark for 14 days at 22°C. Stem and root debris were subsequently washed from media surface and colonies of *V. dahliae* were counted. Soil samples were harvested and assayed for *V. dahliae* as previously described. Mean stem, root, and soil inoculum density (CFU/g) were calculated by averaging subsamples within each replicate.

Stem samples were also visually inspected for the presence of *V. dahliae* microsclerotia with a dissecting microscope at 25X. Candidate *V. dahliae* microsclerotia were excised from stem tissue, observed and photographed with an Olympus BX53 compound microscope (Olympus America Inc., Center Valley, PA.). Microslcerotia were placed on NP-10, incubated in the dark for 14 days at 22°C and then observed weekly for *V. dahliae*.

Seed from arugula, Austrian winter pea, sweet corn, barley, and sudangrass were harvested and tested for *V. dahliae* contamination. Seed were disinfested with 95% ethanol, rinsed for 3 min in 0.5% NaOCl and then 1 min in distilled water, dried on sterile paper towels, and placed on NP-10. Seed were incubated in the dark for 5 weeks at 22°C and then scanned for *V. dahliae* colonies as described previously.

Detection of *Verticillium* spp. in commercial fields. A stratified random sample design was used to collect stems from each field (Cochran 1977). Wheel tracks in center pivot fields were used to delineate strata and samples were randomly collected within each stratum. Sampling was completed during the growing seasons in 2013 and 2014. Stems of white mustard, brown mustard 'ISCI 99', arugula, sunflower (*Helianthus annuus* L.), pea (*P. sativum* L.), timothy (*Phleum pratense* L), bean (*Phaseolus vulgaris* L.), dill (*Anethum graveolens* L.), buckwheat

(*Fagopyrum esculentum* Moench), sweet corn, wheat, barley, oats, and proso millet (*Panicum miliaceum* L.) were collected from 21 commercial fields with a history of Verticillium wilt of potato or mint in the Columbia River Basin in central Washington. In two fields brown mustard and millet or brown mustard and wheat were growing together and both rotation crops were collected. Stems were assayed for *V. dahliae* as previously described for the first greenhouse experiments. Mean incidence of infected stems was calculated within each crop from one field.

Identification of *Verticillium* spp. collected from rotation crops in fields with a history of Verticillium wilt. Strains were identified to genus and, tentatively until DNA sequencing, to species using morphological features as described in the key published by Inderbitzin et al. (2011a). *Verticillium* spp**.** were subcultured on potato dextrose agar (PDA) media overlaid with two layers of dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA.). Cultures were incubated for 10 to 15 days at 22°C and fungal propagules were collected and lyophilized before DNA extraction. Genomic DNA was extracted with a glass bead breakage method (Dobinson 1995) and DNA quality and quantity were measured with spectrophotometry and agarose gel electrophoresis of samples and known lambda DNA standards.

DNA sequences from translation elongation factor 1 alpha (*EF1-a*), glyceraldehyde-3-phosphate (*GPD*), actin (*ACT*), and tryptophan synthase (*TS*) were amplified with the primers and polymerase chain reaction (PCR) assays described by Inderbitzin et al. (2011a). Purified PCR products were obtained by adding 2 µL of ExoSAP-IT (USB Corporation, Cleveland, OH) to 5 µL of sample and incubating samples at 37°C for 15 min and 80°C for 15 min. DNA from PCR products was sequenced in forward and reverse directions by Eurofins Genomics (Eurofins MWG Operon, Louisville, KY) using cycle sequencing technology on a ABI 3730XL machine (Applied Biosystems, Foster City, CA). Two strains from every infected crop within each field and one strain from one corn field were identified with DNA sequence data. In total, 19 strains from 10 rotation crops collected from 8 of the 21 sampled fields were sequenced.

Statistical Analyses. Permutational multivariate analysis of variance (PERMANOVA) was used to test the null hypotheses that the incidence of infected stems and inoculum density from stems, roots, and soil were not different among or within crops. All variables from all experiments, except plant biomass data, were fourth-root transformed prior to analyses to scale observations to similar orders of magnitude. Bray-Curtis similarity matrices with zero-adjusted Bray-Curtis coefficients that possess desirable properties for zero-inflated data sets (Clarke et al. 2006) were generated to accommodate zero-inflated inoculum density data. PERMANOVA was used to model the incidence of infected stems and inoculum density from stems, roots, and soil as a function of crops, strains, a crop x strain interaction, blocks, trials, and a crop x strain x trial interaction. Pairwise comparisons among crops were completed within each strain. PERMANOVA was completed in PRIMER 7 (Plymouth Routine In Multivariate Ecological Research) (2015 PRIMER-E Ltd: Lutton, Ivybridge, United Kingdom).

General patterns in total inoculum density from stems, roots, and soil of crops in the experiment with eight *V. dahliae* strains were recognized with non-metric multidimensional scaling (NMDS). Inoculum density data from stems, roots, and soil was used to provide a holistic

representation of the total inoculum contributed by each crop. Interpretation of NMDS biplots does not depend on the order, scale, and direction of the axes (Legendre and Legendre 1998) but instead depends on the proximity between points, where close points are more similar to each other than distant points (Kruskal 1964). NMDS was performed with the "metaMDS" function in the "vegan" package (Oksanen et al. 2013) in R (version 3.2.1, R Foundation for Statistical Computing, Austria).

Biomass data from the experiment with eight *V. dahliae* strains were analyzed with ANOVA. Each factor was treated and tested for interactions as previously mentioned. ANOVA for dried stem and root weight was computed with PROC MIXED in SAS (version 9.2; SAS Institute, Cary, NC). Quantile-quantile plots and Shapiro-Wilks tests were used to diagnose and test for normality. Post hoc comparisons among cell means were performed and corrected for multiple comparisons with Tukey's honest significant difference using the pdmix800 macro in SAS.

RESULTS

Incidence of infected stems and inoculum density of two *Verticillium dahliae* strains from roots and soil of two brown mustards, white mustard, sweet corn, spring wheat and sudangrass. Both strains originally recovered from potato (653) and mint (111) were detected in stems of all broad-leaved rotation crops, brown and white mustards, and strain 111 was detected in stems of the grass, wheat (Fig. 1). Incidence of infected stems ranged from 0 to 100% and, among rotation crops, was greatest in brown mustard ISCI 199 and least in sweet corn and sudangrass. Only differences between potato and rotation crops and among rotation crops are reported for the incidence of infected stems, root, and soil inoculum density data. The grasses were less ($P = 0.02$) than potato in the incidence of infected stems. Mustards were not different from potato and rotation crops were not different ($P \geq 0.06$) from each other in the incidence of infected stems (Fig. 1).

Root infection was detected in all rotation crops (Fig. 1). Root inoculum density ranged from 0.1 to 40 CFU/60 cm of root. Soil inoculum density of *V. dahliae* strains ranged from 0.3 to 653 CFU/g of soil (Fig. 1). Rotation crops were not different ($P > 0.05$) from potato in soil inoculum density. White mustard supported more ($P \leq 0.04$) soil inoculum of strain 111 than sweet corn and wheat (Fig. 1).

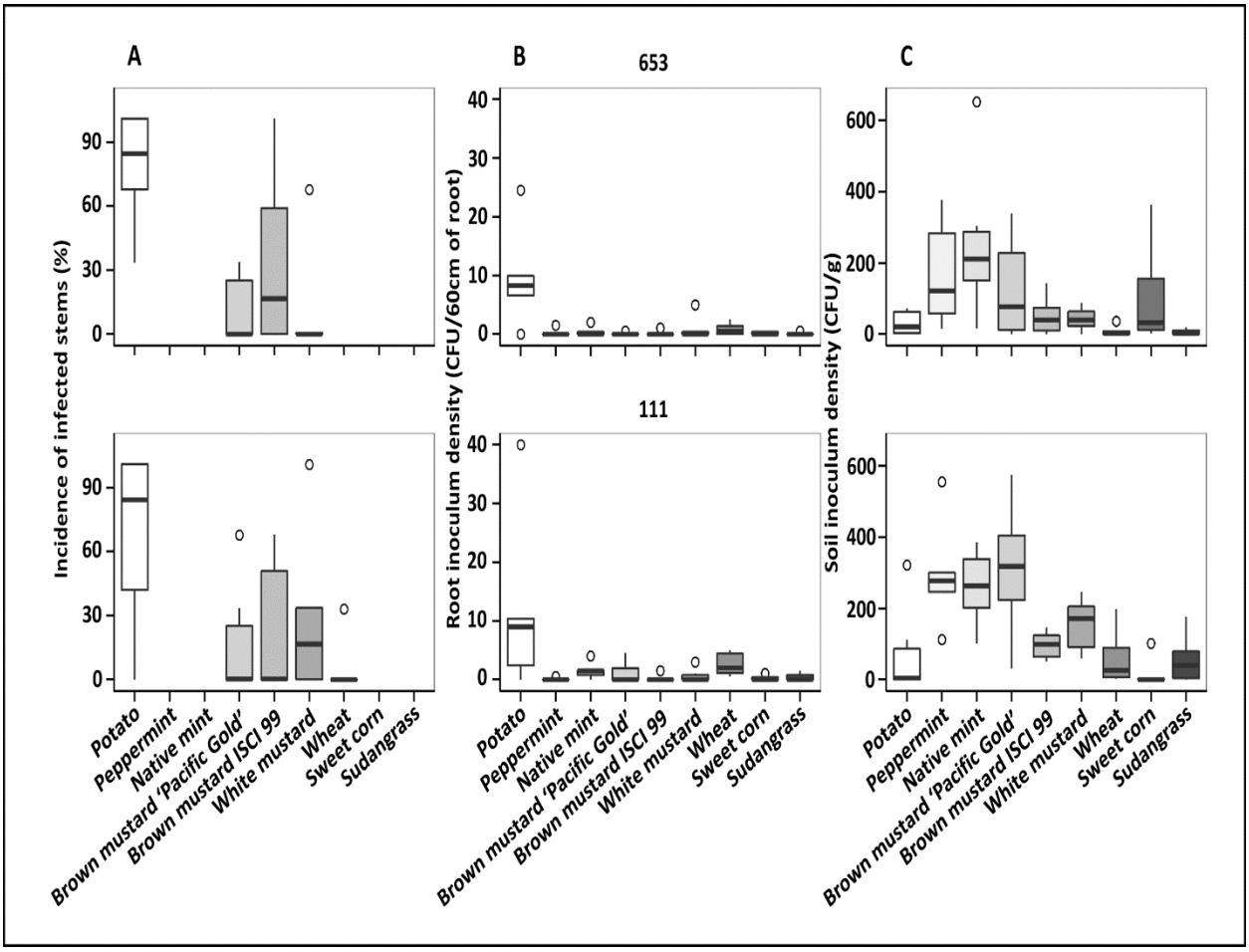

Figure 1. Inoculum density of two *Verticillium dahliae* strains from stems, roots and soil of two brown mustards, white mustard, sweet corn, spring wheat and sudangrass. **A**, Incidence of infected stems (% of infected stems), **B**, root inoculum density (CFU/60 cm of root), and, **C**, soil inoculum density (CFU/g of soil) detected from crops grown in soil infested with *V. dahliae* strains 653 and 111 are presented as boxplots. Boxplot hinges represent 1st and 3rd quartiles, whiskers extend to values within 1.5x the inter-quartile range, and circles represent outliers.

Inoculum density of eight *Verticillium dahliae* strains from stems, roots and soil of arugula, Austrian winter pea, sweet corn, barley and sudangrass. Typical Verticillium wilt symptoms were not observed in any of the rotation crops. Austrian winter pea stem weight (g) decreased by 25 to 45% and was less ($P \leq 0.04$) in plants grown in soil infested with strains 653, 111, 461, VMD-4, and VD5 VSP699 than plants from noninfested soil. Sweet corn stem weight increased by 27 to 40% and was greater ($P \leq 0.03$) in plants grown in soil infested with strains 155, 461, VMD-4, and VD5 VSP699 than plants from noninfested soils. Arugula and sweet corn root weight (g) increased and was greater ($P \leq 0.004$) in plants grown in soil infested with strain 653 and strain VMD-4 than plants from noninfested soils, respectively. Barley and sudangrass root weight decreased and was less ($P \leq 0.02$) in plants grown in soil infested with all strains and strains 653, 49.B.2010, and VD5 VSP699 than plants from noninfested soils, respectively (Fig. 2).

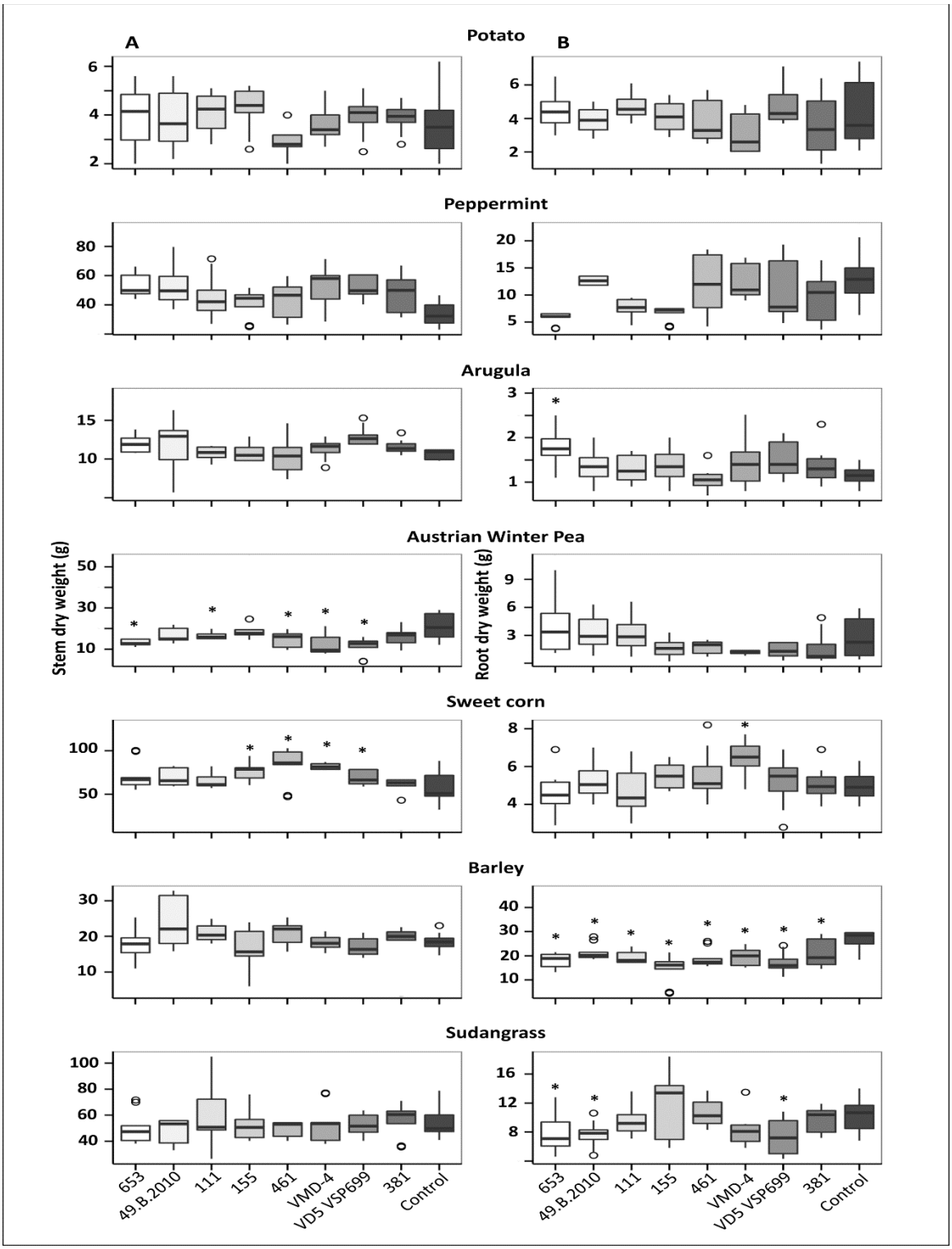

Figure 2. Biomass of potato, peppermint, arugula, Austrian winter pea, sweet corn, barley, and sudangrass grown in soil infested with eight *Verticillium dahliae* strains and a non-infested control. **A**, Stem and, **B**, root dry weight (g) data are presented as boxplots. Boxplot hinges represent 1st and 3rd quartiles, whiskers extend to values within 1.5x the inter-quartile range, and circles represent outliers. Nontransformed data are presented. Asterisks indicate significant differences ($P < 0.05$) between crops grown in infested soil and the noninfested controls.

At least one *V. dahliae* strain was detected from the stems of all rotation crops (Fig. 3). Strains 653, 49.B.2010, 111, 155, VMD-4, and VD5 VSP699 were recovered from the broad-leaved rotation crops, arugula and Austrian winter pea, and one grass, barley. Strains 461 and 381 exhibited wider host ranges than the other strains and, in addition to the aforementioned rotation crops, were recovered from sudangrass or sweet corn, respectively. Inoculum density of *V. dahliae* ranged from 0 to 1436 CFU/g of stem and, among rotation crops, was greatest in Austrian winter pea infected with strain 381. Microsclerotia were observed in stems of all infected rotation crops.

Broad-leaved rotation crops, arugula and Austrian winter pea were greater ($P \leq 0.04$) than potato in stem inoculum density of strain 155 and strains VMD-4 and 381, respectively (Fig. 3). Austrian winter pea and potato did not differ ($P = 0.07$) in stem inoculum density of strain 155. Grasses including sweet corn, barley, and sudangrass were less ($P \leq 0.05$) than potato in stem inoculum density of all strains (Fig. 3).

Rotation crops varied in the incidence of infected stems and inoculum density. The grasses were less ($P \leq 0.04$) than arugula and Austrian winter pea in stem inoculum density of strains 49.B.2010, 111, 155, 461, VMD-4, VD5 VSP699, and 381 (Fig. 3). Sweet corn and sudangrass were less ($P \leq 0.02$) than barley in stem inoculum density of strains 653, 49.B.2010, 111, 155, 461, VD5 VSP699, and 381.

Strains 49.B.2010 and 381 were detected in seed of arugula and strains 653, 49.B.2010, 461, VMD-4, and 381 were detected in seed of Austrian winter pea. Incidence of contaminated seed ranged from 0 to 0.4% in arugula and from 0 to 7% in Austrian winter pea (data not shown).

Root infection was detected on all rotation crops (Fig. 3). Root inoculum density of *V. dahliae* ranged from 0.1 to 560 CFU/g of root. Arugula was greater ($P \leq 0.03$) than potato in root inoculum density of strains 653, 49.B.2010, 111, 461, VMD-4, VD5 VSP699, and 381 (Fig. 3). Austrian winter pea was greater ($P \leq 0.02$) than potato in root inoculum density of strains 653, 461, and 381. Sweet corn and sudangrass were greater ($P \leq 0.03$) than potato in root inoculum density of strains 653 and 461 and strain 653, respectively (Fig. 3).

Soil inoculum density of strains of *V. dahliae* ranged from 0.03 to 88 CFU/g of soil (Fig. 3). Austrian winter pea and barley were greater ($P \leq 0.04$) than potato in soil inoculum density of strain 461. Rotation crops did not differ ($P \geq 0.08$) from potato in soil inoculum density of strains 49.B.2010, VMD-4, and 381 (Fig. 3).

Axis 1 of the NMDS biplot (Fig. 4) separated crops that supported more inoculum density (left) from crops that supported less inoculum (right) independent of expression of wilt symptoms. For example, potato, which expressed wilt symptoms, and arugula and Austrian winter pea, which did not express wilt symptoms, supported more inoculum and clustered together whereas peppermint, which expressed wilt symptoms, and the grasses, which did not express wilt symptoms, supported less inoculum and clustered together.

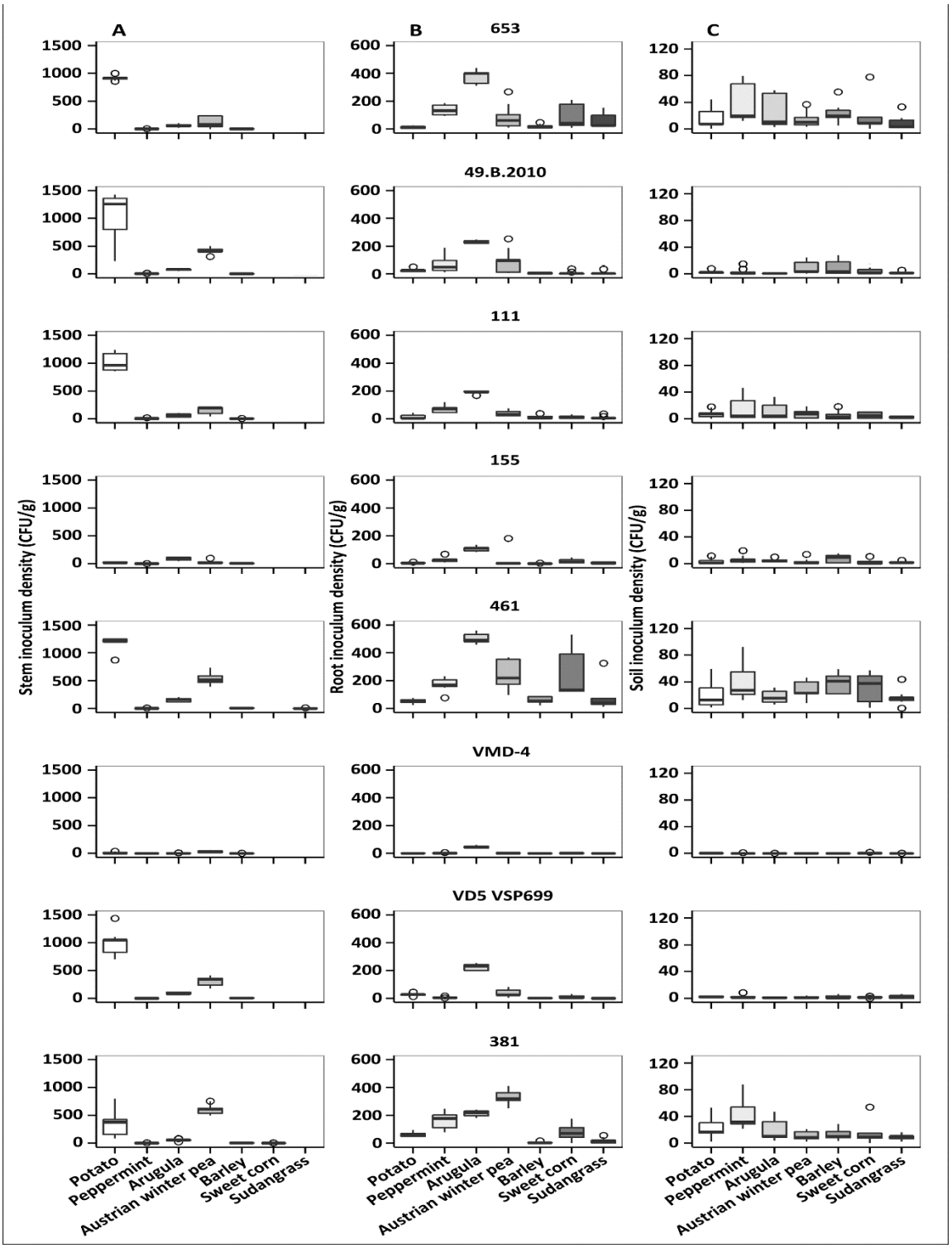

Figure 3. Inoculum density of eight *Verticillium dahliae* strains from stems, roots and soil of arugula, Austrian winter pea, sweet corn, barley and sudangrass. A, Stem inoculum density (CFU/g of stem), B, root inoculum density (CFU/g of root), and, C, soil inoculum density (CFU/g of soil) detected from crops grown in soil infested with eight *V. dahliae* strains are presented as boxplots. Boxplot hinges represent 1st and 3rd quartiles, whiskers extend to values within 1.5x the inter-quartile range, and circles represent outliers.

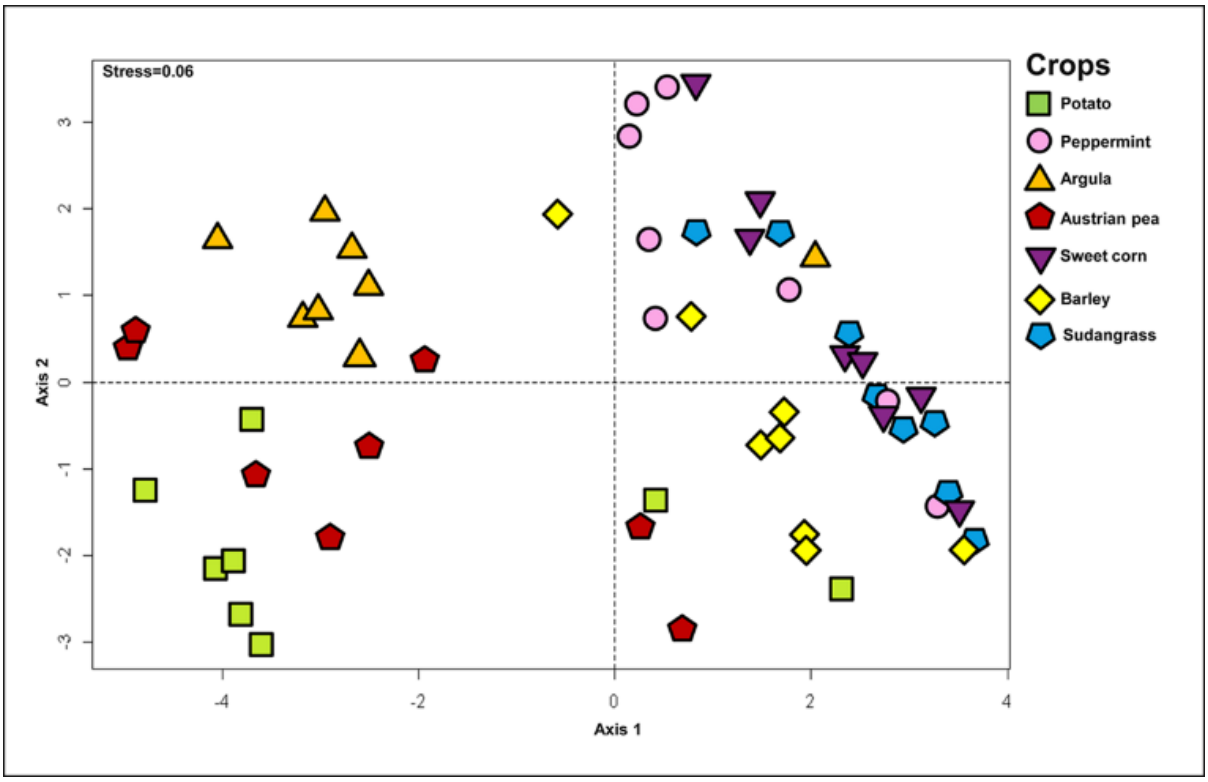

Figure 4. Similarity of inoculum density among crops infected with *Verticillium dahliae* strains plotted with non-metric multidimensional scaling. Points represent the total inoculum density detected from stems, roots and soil of crops. Crops are depicted with different shapes and colors and the eight strains are represented by the eight points within each crop.

Detection of *Verticillium* spp. in commercial fields. Incidence of fields with rotation crops infected by *Verticillium* spp. was 8 of 21 (38%) (Table 2). Incidence of stems infected with *Verticillium* spp. within each field ranged from 1 to 63%. Incidence of infected stems was greatest in brown mustard from one field. No typical wilt symptoms were observed on rotation crops collected from commercial fields. Microsclerotia of *Verticillium* spp. were observed with microscopy on stems of both brown mustard cultivars but not on other rotation crops (data not shown).

Identification of *Verticillium* spp. collected from rotation crops in fields with a history of Verticillium wilt. Fungal strains were identified as *V. dahliae* based on colony morphology. Additionally, strains were identified as *V. dahliae*, or *V. isaacii* in the case of the strains collected from sunflower, based on BLAST queries of DNA sequence data from *EF1-a, GPD, ACT* and *TS* against reference strains from Inderbitzin et al (2011a, b). Sequence data from each strain were deposited on GenBank and are presented in Table 2.

DISCUSSION

Crop rotations that minimize the development and persistence of *V. dahliae* inoculum in potato production systems are needed to supplement or replace existing management tactics. However, the impacts of crop rotation on Verticillium wilt and inoculum density of *V. dahliae* are inconsistent (Davis et al. 1994; Huisman and Ashworth 1976; Joaquim et al. 1988; Johnson and Cummings 2015; Larkin et al 2011; O'Sullivan 1978; Subbarao et al. 2007; Wheeler et al. 2012). Sources of the inconsistent results include the spatial and temporal heterogeneity of *V. dahliae* populations among and within fields (Joaquim et al. 1988; Powelson and Rowe 1993), the crops planted in rotations (Green 1969), and the lengths of rotation (Gudmestad et al. 2007; Powelson and Rowe 1993). Observed inconsistencies may also be due, in part, to differential infection by and inoculum production on crops planted in rotation with primary crops. The identification of rotation crops that minimize or reduce *V. dahliae* soil inoculum is therefore of paramount importance when designing crop rotation regimes.

This study provides evidence that the infection of numerous rotation crops by *V. dahliae* has the potential to increase inoculum levels of the organism and potentially increase losses in potato production systems in the Pacific Northwest of the United States. The expanded host range, differential inoculum production, and responses in plant biomass to infection supports a diverse life history of *V. dahliae* whereby strains can infect and incite typical symptoms in primary hosts, alter biomass, or incite no symptoms in other hosts. Before recommendations can be made additional questions need to be addressed: do microsclerotia produced on rotation crops survive and contribute to soil inoculum to the same extent as microsclerotia produced on primary crops; what crop rotation sequences minimize the potential contribution of microsclerotia to soil inoculum; are infections of rotation crops stable or will new Verticillium wilts emerge over time; are rotation crops of potato and mint infected by *V. dahliae* worldwide or regionally and; are strains of *V. dahliae* recovered from rotation crops pathogenic to potato and peppermint?

Rotation crops of potato and mint were infected by *V. dahliae* in controlled greenhouse experiments and in commercial fields in Washington State. The incidence of infected stems was relatively low in most commercial fields sampled and may be explained by the rare occurrence of asymptomatic infections under field conditions and or the use of a culture dependent detection method biased towards vigorously growing strains. The former explanation is supported by the low incidence of infected stems detected in published studies (Demirci and Genc 2009; Krikun and Bernier 1987; Malcolm et al. 2013). To understand the potential role of infected rotation crops in Verticillium wilt epidemiology the geographical distribution of infected rotation crops should continue to be investigated in regions where crops susceptible to *V. dahliae* are grown.

Detection of microsclerotia from at least one *V. dahliae* strain on each rotation crop in this study has expanded the known host range of the organism. This is the first experimental documentation of stem infection of brown mustard, white mustard, arugula, Austrian winter pea, sweet corn, and sudangrass by *V. dahliae* according to the United States Department of Agriculture Systematic Mycology and Microbiology Fungus-Host database (http://nt.ars-grin.gov/fungaldatabases/fungushost/fungushost.cfm, accessed during November, 2015).

Detection of *V. dahliae* from stems of wheat and barley corroborates previous reports (Krikun and Bernier 1987; Mathre 1986). The expansion of the known host range of *V. dahliae* is of particular importance for mustards, sweet corn, and sudangrass, which are often grown as green manures (Davis et al. 2010; Larkin and Halloran 2014) and may be efficacious in disease-suppression if plant biomass is incorporated into soils before microsclerotia form on senescing plant tissues.

The presence of different host ranges among some of the strains used in this experiment supports the existence of host specificity in *V. dahliae.* All strains colonized a subset of rotation crop stems and were therefore host-specific; however, strains 461 and 381 exhibited wider host ranges than the other strains included. These observations corroborate previous reports of host specificity (Bhat and Subbarao 1999; Krikun and Bernier 1987) and may help explain the reported variability in the effectiveness of crop rotation for managing Verticillium wilt (cited above). Hence, rotations with the grasses not included in the host range of some strains used here may be more effective in minimizing potential inoculum production and or the incidence of Verticillium wilt in primary crops than the broad-leaved crops evaluated (Davis et al. 1996, 1998; Larkin et al. 2010).

Evidence of host-adapted *V. dahliae* strains was also presented where differences in stem inoculum density and plant biomass depended on the strain with which each crop was infected. Strains 155 and 381, for example, were host-adapted and produced more inoculum on arugula or Austrian winter pea than potato, respectively. Evidence of host-adapted *V. dahliae* strains corroborates previous reports from potato and cotton pathosystems (Douhan and Johnson 2001; Korolev et al. 2008; Rowe and Powelson 2002). The observed differences among host-specific and host-adapted strains may be explained by (i) differences in *V. dahliae* fitness, (ii) the host from which the strain was recovered (Alkher et al 2009; Fordyce and Green 1960), (iii) differences in the ability of hosts to induce germination of microsclerotia (Mol and van Riessen 1995) and resist infection or the development of microsclerotia (Krikun and Bernier 1987; Slattery 1981), and or (iv) differences in host microbiomes (Berendsen et al. 2012). The effectiveness of crop rotation for managing Verticillium wilt will therefore be contingent upon the pathogenicity of populations of *V. dahliae* present in fields, the crop planted in rotation with the primary crop, the potential contribution of inoculum formed on the rotation crops, and environmental conditions.

A range of responses in plant biomass to infection of rotation crops also supports the existence of host-adapted strains and putative nonpathogenic interactions between *V. dahliae* and some hosts. While stem weight of most rotation crops was not altered by infection, stem weight of Austrian winter pea decreased while stem weight of sweet corn increased when infected by some, but not all, *V. dahliae* strains. Mild to typical wilt symptoms were observed in potato plants grown in infested soils but these symptoms did not uniformly decrease biomass and were not reported herein. The asymptomatic infections reported here corroborate those reviewed by Malcolm et al. (2013). The symptomatic infections are similar but not identical to the alterations in the growth and development observed by Robb et al. 2007 and Veronese et al. 2003 where

some infected hosts were taller or flowered earlier or later and produced more stems than noninfected controls; however, unlike results presented here, these changes in plant growth were accompanied by mild (Robb et al. 2007) and typical (Veronese et al. 2003) symptom expression.

Characterization of the herein reported asymptomatic and symptomatic infections of rotation crops is essential to determine the sustainability of crop rotation on potato and mint cropping systems. Assignment of asymptomatic and symptomatic infections within the symbiotic continuum may be completed by a combination of serial passage experiments (Ebert 1998; Fordyce and Green 1960; Little et al. 2006) and descriptions of changes in plant growth, plant development, rates of metabolism, and tolerance to stress in response to infection (Rodriguez and Roossinck 2012). This approach may be used to test the stability of infections over time, predict the emergence of aggressive strains, and help inform growers when designing rotation crop regimes.

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TABLE 1. Eight *Verticillium dahliae* strains from various hosts, vegetative compatibility groups (VCG), genotypes, and mating-types used to inoculate rotation crops (from Dung et al. 2013).

V. dahliae strain	Host	VCG	Genotype	Mating-type	State	Year	Source
653	Potato	4A	H04	*MAT1-2*	ID	1995	R. Rowe
49.B.2010	Potato	4B	H07	*MAT1-2*	OR	2010	J. Dung
111	Peppermint	2B	H02	*MAT1-2*	WA	1996	D. Johnson
155	Peppermint	4A	H04	*MAT1-2*	ID	1996	D. Johnson
461	Tomato	2	H37	*MAT1-1*	OH	1984	R. Rowe
VMD-4	Tomato	2A/B	H38	*MAT1-2*	NY	pre-1983	M. Lacy
VD5 VSP699	Spinach	2B/4B	H07	*MAT1-2*	WA	2001	L. du Toit
381	Watermelon	2A/B	H24	*MAT1-2*	OH	1981	S. Miller

TABLE 2. Incidence of infected stems of rotation crops and Genbank accessions of *actin* (*ACT*), *elongation factor 1 alpha* (*EF1-a*), glyceraldehyde-3-phosphate (*GPD*), and *tryptophan synthase* (*TS*) sequences from *Verticillium dahliae* strains collected from 21 fields with a history of Verticillium wilt of potato or mint in the Columbia Basin, Washington.

Rotation crops sampled	n	Incidence (%)	*ACT*	*EF1-a*	*GPD*	*TS*	Species
Arugula 'Nemat'	50	0	.	.	.	.	.
Arugula 'Nemat'	50	0	.	.	.	.	.
Brown mustard[a]	150	63	KT224573, KT224574	KT224535, KT224536	KT224554, KT224555	KT224760, KT224761	*V. dahliae*
Millet	50	2	KT224575, KT224576	KT224537, KT224538	KT224556, KT224557	KT224762, KT224763	*V. dahliae*
Brown mustard[a]	50	14	KT224577, KT224578	KT224539, KT224540	KT224558, KT224559	KT224764, KT224765	*V. dahliae*
Wheat	50	2	KT224579, KT224580	KT224541, KT224542	KT224560, KT224561	KT224766, KT224767	*V. dahliae*
White mustard 'Martigena'	50	6	KT224581, KT224582	KT224543, KT224544	KT224562, KT224563	KT224768, KT224769	*V. dahliae*
Sunflower	25	24	KT224585, KT224586	KT224547, KT224548	KT224566, KT224567	KT224772, KT224773	*V. isaacii*
Pea	100	0	.	.	.	.	.
Pea	100	0	.	.	.	.	.
Bean	100	0	.	.	.	.	.

Buckwheat	100	7	KT224587, KT224588	KT224549, KT224550	KT224568, KT224569	KT224774, KT224775	*V. dahliae*
Buckwheat	100	9	KT224589, KT224590	KT224551, KT224552	KT224570, KT224571	KT224776, KT224777	*V. dahliae*
Buckwheat	100	0	.	.	.	.	.
Dill	100	0	.	.	.	.	.
Corn	100	1	KT224584	KT224546	KT224565	KT224771	*V. dahliae*
Corn	100	0	.	.	.	.	.
Corn	100	0	.	.	.	.	.
Corn	100	0	.	.	.	.	.
Oat	100	0	.	.	.	.	.
Oat	100	0	.	.	.	.	.
Barley	100	10	KT224583, KT224591	KT224545, KT224553	KT224564, KT224572	KT224770, KT224778	*V. dahliae*
Timothy	100	0	.	.	.	.	

[a] Millet was intercropped with brown mustard in one field and wheat was intercropped with brown mustard in a separate field.