Verticillium isaacii is a Pathogen and Endophyte of Potato and Sunflower in the Columbia Basin of Washington

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Abstract

The objective of this research was to test the hypothesis that *Verticillium isaacii* causes diseases of sunflower and potato plants. Two sunflower genotypes and one potato cultivar were inoculated with five *V. isaacii* isolates and three pathogenic *V. dahliae* isolates. Biomass, disease expression, and stem colonization were quantified. Overt wilt symptoms were observed on both sunflower genotypes and potato plants inoculated with a subset of the *V. isaacii* isolates. Biomass of both sunflower genotypes was not affected by *V. isaacii* infection. Tuber yields either decreased in response to

infection by one *V. isaacii* isolate or were not affected by infection. Stems of sunflower and potato plants were infected by at least four of the five *V. isaacii* isolates. A new disease of sunflower and potato is documented. Evidence that *V. isaacii* exhibits different lifestyles including pathogenicity and endophytism is presented. Finally, this research documents variation in fungal lifestyles that can exist in samples from a single field.

Keywords: fungi, field crops, etiology

The ascomycete genus *Verticillium* is comprised of 10 species (Inderbitzin et al. 2011). These species, despite their mostly clonal reproductive mode (Milgroom et al. 2016), vary both genotypically (Klosterman et al. 2009) and phenotypically in the resting structures they produce (Inderbitzin et al. 2011) and the lifestyles they exhibit (Malcolm et al. 2013). Common *Verticillium* spp. that cause wilt symptoms in plants include *V. dahliae* Kleb. and *V. longisporum* Karapapa, Bainbr. & Heale. While these species are undoubtedly important pathogens, other species in the *Verticillium* genus, although of potentially less economic importance, are important to characterize.

V. isaacii Inderb. et al. is a recently described species (Inderbitzin et al. 2011) that, like *V. dahliae*, causes disease symptoms on some plant species (Gurung et al. 2015) and asymptotically infects others (Tyvaert et al. 2014). Unlike *V. dahliae*, however, *V. isaacii* and the morphologically indistinguishable *V. tricorpus* Isaac of which it was once a cryptic species, are also associated with wilt suppressive soils and cross-protection (Davis and Sorensen 1985; Davis et al. 2000; França et al. 2013; Tyvaert et al. 2014). More specifically, *V. isaacii* was recovered from field soil associated with reduced levels of cauliflower wilt compared with other locations within the same field in Belgium (França et al. 2013). An isolate recovered from this suppressive field soil thereafter conferred cross-protection to cauliflower plants coinoculated with *V. longisporum* (Tyvaert et al. 2014). Thus, *V. isaacii* possesses a wide range of ecological lifestyles including pathogenesis (Gurung et al. 2015), endophytism, and a yet-to-be elucidated lifestyle that facilitates cross-protection (Qin et al. 2008; Tyvaert et al. 2014).

These discoveries have expanded the collective understanding of *Verticillium* biology and raised new questions. For example, do all *V. isaacii* isolates possess the ability to cause disease on some plant species, infect but incite no symptoms on others, and confer

cross-protection to other species? Evidence presented by Gurung et al. (2015) provides support for the hypothesis that different isolates possess distinct lifestyles but host responses are conditional upon the combination of isolate and host species used in inoculations. While these discoveries have contributed to our knowledge about *Verticillium* spp., it is not yet known if they can be extrapolated to other plant species in other geographical areas with other *Verticillium* isolates.

During 2016, the authors were presented with the opportunity to test this hypothesis. A grower in Washington State showed the authors a field of healthy and symptomatic sunflowers (*Helianthus annuus* L.). *V. isaacii* isolates were recovered from the stems of these sunflowers (Wheeler and Johnson 2016). Since sunflower is grown in rotation with potato, the authors recognized this as an opportunity to test the hypothesis that this fungus expresses different lifestyles with different hosts. The goal of this research was to determine if *V. isaacii* isolates recovered from sunflowers cause disease on sunflower and potato (*Solanum tuberosum* L.). While both of these plant species express wilt symptoms when inoculated with *V. dahliae* (Johnson and Dung 2010; Sadras et al. 2000), their response to *V. isaacii* has not been determined. Completion of Koch's postulates is thereby necessary to determine if interactions between *V. isaacii* and these crops constitute new diseases.

Materials and Methods

To determine if sunflower and potato are susceptible hosts of *V. isaacii*, Koch's postulates were conducted under greenhouse conditions during 2018. The treatment structure of the experiment involved 3 plant genotypes × 8 isolates of *Verticillium* spp. plus a noninoculated control × 5 replicates × 2 trials for a total of 270 experimental units. For the plant species, the susceptible sunflower hybrid Jupiter, the inbred line HA89, which harbors the *V_i* resistance gene (Fick and Zimmer 1974), and 'Russet Burbank' potatoes were used. For the *V. isaacii* isolates, five *V. isaacii* isolates recovered from sunflower fields sampled in Wheeler and Johnson (2016) were used. For *V. dahliae* isolates, two isolates pathogenic (isolates 1-13 and 2-13) toward sunflower (Garcia-Ruiz et al. 2014) and one pathogenic (isolate 653) toward potato (Dung et al. 2013) were used. The design structure of the experiment was a randomized complete block design.

Microsclerotial inoculum was prepared from sterilized and sieved sand, as described in Wheeler and Johnson (2016). The growth medium used was infested with 15 CFU/cm³ of *Verticillium* spp. by incorporation of 66 g of 680 CFU/g sand inoculum with approximately 3,000 cm³ of Sunshine L2 soilless potting medium (Sun Gro Horticulture, Agawam, MA) in 15.2 × 17.8 cm, 3 liter pots (J. M. McConkey & Co., Inc., Sumner, WA). Sterilized sand was used for the

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noninoculated control treatment. Thereafter, sunflower seed and potato seed tubers were planted in infested or noninfested growth medium. Seed of the sunflower genotypes were planted approximately 2 in below the surface of the potting mix. Similarly, potato seed tubers that were tested for the absence of *Verticillium* spp. (Wheeler and Johnson 2016) were planted approximately 4 in below the surface of the potting mix. Plants were grown in a greenhouse with photoperiods between 12 and 15 h/day, daytime temperatures between 13 and 29°C, and nighttime temperatures between 11 and 27°C.

Starting with the onset of the first chlorotic and/or necrotic symptoms, senescence was quantified for sunflower and potato plants every 7 days until plants expired. More specifically, the percentage of the plant that was chlorotic or necrotic was estimated visually. Estimates were subsequently converted to the area under the senescence progress curve (AUSPC) as described by Shaner and Finney (1977).

Following the senescence of all plants, aboveground biomass (dry weight) of sunflowers was quantified. Similarly, weight and number of potato daughter tubers that weighed more than 20 g were estimated. Stem infection was also quantified after plant senescence as described in Wheeler and Johnson (2016) with a slight modification. Individual stem sections were sampled from 3 and 15 cm above the soil surface for each stem of every plant. Sections were subsequently disinfested in 0.5% NaOCl for 3 min, rinsed in distilled water for 1 min, blotted dry on sterile paper towels, and plated on NP-10 medium (Kabir et al. 2004). Stem sections were then incubated in the dark for 4 weeks at 22°C and inspected for *V. isaacii* and *V. dahliae* colonies. The incidence of stem infection was calculated as the percentage of infected stems divided by the total number of stems per plant.

Estimates of disease, expressed as AUSPC values, sunflower biomass, and potato daughter tuber yields were compared with analysis of variance (ANOVA). Departures from normality and homoscedasticity were assessed with various plots and tests, including the Shapiro-Wilks and Breusch-Pagan tests. Posthoc comparisons were conducted with Tukey's honest significant difference (HSD) test if ANOVA *F*-statistics were less than alpha of 0.05. Data from both trials were combined and presented as such since no significant isolate-by-trial interactions ($P > 0.05$) were detected. All analyses were completed in R (v.3.4.3, R Foundation for Statistical Computing, Austria).

Results

Poor emergence was observed for both sunflower genotypes, HA89 and Jupiter, and despite repeated attempts to reseed and transfer pregerminated seedlings, poor emergence persisted. Specifically, 77 of 90 HA89 plants and 38 of 90 Jupiter plants emerged. Wilt symptoms were observed on HA89 sunflower plants inoculated with all five *V. isaacii* isolates (Fig. 1) with similar symptoms observed on Jupiter sunflower plants inoculated with four of the five *V. isaacii* isolates (isolates 1, 3, 4, and 5). Symptoms included stunting and asymmetrical necrosis of leaves that resulted in thin papery-like leaves that remained attached to stems. Acute wilt symptoms were observed on potato plants inoculated with two of the five *V. isaacii* isolates (isolates 2 and 3). These symptoms included mild stunting, wilting, and uniform necrosis of basal leaves that remained attached to stems, unlike the noninoculated plants (Fig. 1).

Despite the acute symptom expression observed in both sunflower genotypes, significant differences in disease symptoms, expressed as AUSPC, were not detected for HA89 ($F = 1.42$, $P = 0.21$) or Jupiter ($F = 0.81$, $P = 0.61$) (Fig. 2). Significant differences in disease expression were detected ($F = 4.14$, $P = 0.0005$) among potato plants inoculated with the different *Verticillium* spp. isolates (Fig. 2). However, although *V. isaacii* incited wilt symptoms on potato, these symptoms were not significantly different ($P > 0.05$) than noninoculated plants that exhibit normal chlorotic and necrotic symptoms as they senesce.

Aboveground biomass of the sunflower genotypes HA89 ($F = 0.77$, $P = 0.62$) and Jupiter ($F = 1.03$, $P = 0.42$) were not different among plants inoculated with *V. isaacii* and the noninoculated

controls (Fig. 3). Potato tuber yields differed significantly ($F = 9.60$, $P < 0.0001$) across plants inoculated with different *Verticillium* spp. and also among the isolates of *V. isaacii*. Tuber yields of plants inoculated with *V. isaacii* isolate 2 were less ($P < 0.05$) than the noninoculated controls and similar to plants inoculated with *V. dahliae* isolate 653.

Fig. 1. Symptom expression of sunflower HA89 (A) and potato 'Russet Burbank' (B) plants that were not inoculated, inoculated with *Verticillium isaacii* isolate 2, or inoculated with *V. dahliae*. Acute symptoms depicted here were especially severe and not representative of symptoms present in other treatments. Symptoms on both sunflower genotypes were characterized by stunting, asymmetrical necrosis of leaves, and dry, papery-like leaves that stay attached to plant stems. Symptoms on potato included mild stunting and wilting but mostly necrosis of basal leaves that, unlike healthy plants, stayed attached to stems.

Fig. 2. Differences in disease symptoms, expressed as the area under the disease progress curve (AUSPC) of sunflower HA89 (A), Jupiter (B), and potato 'Russet Burbank' (C) that were not inoculated (control), inoculated with *Verticillium dahliae* (653, 1-13, and 2-13), and *V. isaacii* (1, 2, 3, 4, 5). Dots represent values of each replicate and boxplots describe the distributions of the data for each treatment. Letters above boxplots were assigned with Tukey's honestly significant difference (HSD). If treatments within a plant species do not share a letter(s), they are significantly different at $\alpha = 0.05$.

Both sunflower genotypes and potato plants were infected by most isolates of *V. isaacii*, but infection was variable within and across treatments (Fig. 4). The sunflower genotype HA89 was infected by all *V. isaacii* isolates and the mean incidence of infected stems ranged from 5 to 60% (sd = 15 to 45) of stems sampled. The sunflower genotype Jupiter was infected by all but one of the *V. isaacii* isolates (isolate 4), and the mean incidence of infected stems ranged from 5 to 65% (sd = 16 to 47) of stems sampled. Similarly, all but one of the *V. isaacii* isolates (isolate 3) were recovered from potato plants over both trials, and the mean incidence of infected stems ranged from 2 to 48% (sd = 4 to 30) of stems sampled.

Discussion

The fungal genus *Verticillium* is relatively small, containing only 10 species, but members of this genus express a diversity of fungal lifestyles. For example, both *V. dahliae* and *V. isaacii* are pathogens and endophytes (Gurung et al. 2015; Malcolm et al. 2013; Qin et al. 2008; Tyvaert et al. 2014) of plants. Unlike *V. dahliae*, however, the extent to which *V. isaacii* causes disease and asymptomatically infects plants is not well understood. Evidence is presented that isolates of *V. isaacii* recovered from a single sunflower field can be pathogens and endophytes of both species.

Herein, a new disease of sunflower and potato caused by *V. isaacii* is documented. Koch's postulates were completed for both sunflower and potato. First, isolates of *V. isaacii* were isolated from sunflower plants in Washington State. Upon inoculation of two sunflower genotypes and potato with 5 *V. isaacii* isolates, wilt-like symptoms were observed in plants inoculated with one to all isolates. Finally, four of the five *V. isaacii* isolates were recovered from sunflower Jupiter and potato while all isolates were recovered from sunflower HA89. While the absence of statistically significant differences in AUSPC values between inoculated and noninoculated plants does not corroborate this claim, it also does not negate the observance of wilt-like symptoms on inoculated plants. Finally, wilt symptoms were observed across two trials and multiple sunflower and potato treatments, thereby providing evidence that these symptoms are reproducible.

In addition to causing wilt symptoms on the sunflower and potato genotypes, one of the *V. isaacii* isolates also affected potato yields. Specifically, isolate 2 reduced yields compared with the noninoculated control, caused severe wilt on potatoes, and was consistently recovered from stems of potato and sunflower plants. Thus, isolates recovered from the same field can vary in their abilities to alter potato

yields. These observations provide additional support for the pathogenic and endophytic potential of *V. isaacii* observed by previous researchers (Gurung et al. 2015; Malcolm et al. 2013; Qin et al. 2008; Tyvaert et al. 2014). Moreover, these results motivate questions about the effects of coinfection on host plants. For example, what happens when pathogenic isolates that reduce tuber yields coinhabit the same host as less aggressive and/or endophytic isolates? Are the effects of these fungi on yields, disease expression, and stem infection additive, synergistic, or antagonistic?

As with disease symptoms and yields, stem infection was variable from *V. isaacii* isolate to isolate. Both sunflower genotypes and potato plants were infected by most of the *V. isaacii* isolates, but the incidence of infected stems varied substantially across replicates and treatments. In at least one case, an isolate (isolate 3) caused symptoms on potato but was not recovered from the plant. This discrepancy is likely an artifact of competition between *V. isaacii* and other, perhaps more competitive, saprotrophs on senescent stems. Deployment of nucleic acid-based detection and quantification methods might resolve this issue. This variability in stem infection likely reflects the overall variation in the lifestyles of these five *V. isaacii* isolates. When viewed alone, this result is not surprising; however, it does underscore the importance of including several isolates in pathogenicity assays. Indeed, the authors may have underestimated the lifestyles of *V. isaacii* herein. In short, the number of isolates used for Koch's postulates matters, at least for *V. isaacii*.

The fungus *V. isaacii* and its sister species, *V. tricorpus*, from which it was recently described (Inderbitzin et al. 2011), have a unique history within the genus *Verticillium*. Both, but especially the former, are relatively understudied. Here, the lifestyles of *V. isaacii* described in other plant species were corroborated (Gurung et al. 2015; Malcolm et al. 2013; Qin et al. 2008; Tyvaert et al. 2014), and a new disease of two sunflower genotypes and potato 'Russet Burbank' was documented. Moreover, the variation in *V. isaacii* lifestyles documented from isolates recovered from a single field emphasizes the importance of sample sizes when making inferences about the lifestyles of organisms. For example, consider the counterfactual scenario where one or several of the isolates used herein were not included. Our conclusions, knowledge about *V. isaacii* biology, and recommendations to producers might change dramatically. Moreover, a sample size of five isolates is still relatively small. Small enough, perhaps, to invoke the "law of small numbers" documented by Tversky and Kahneman (1971). What would we learn if we included 10, 20, 30, ... 100 isolates? Thus, larger sample sizes, that capture the variation in phenotypes in proportion to their frequencies in populations of interest, should be obtained when possible. Finally, the authors hope this work stimulates future projects aimed toward describing the diversity of *Verticillium* lifestyles in nature.

Fig. 3. Differences in aboveground biomass of sunflower HA89 (A), Jupiter (B), and tuber yields of potato 'Russet Burbank' (C) plants that were not inoculated (control), inoculated with *Verticillium dahliae* (653, 1-13, and 2-13), and *V. isaacii* (1, 2, 3, 4, 5). Dots represent values of each replicate and boxplots describe the distributions of the data for each treatment. Letters above boxplots were assigned with Tukey's honestly significant difference (HSD). If treatments within a plant species do not share a letter(s), they are significantly different at $\alpha = 0.05$.

Fig. 4. Stem infection of sunflower HA89 (A), Jupiter (B), and potato 'Russet Burbank' (C) plants that were not inoculated (control), inoculated with *Verticillium dahliae* (653, 1-13, and 2-13), and *V. isaacii* (1, 2, 3, 4, 5). Dots represent values of each replicate and boxplots describe the distributions of the data for each treatment.

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Literature Cited

- Davis, J. R., Everson, D. O., Sorensen, L. H., and Schneider, A. T. 2000. Associations of *Verticillium tricorpus* with soil suppressiveness of *Verticillium* wilt of potato. Pages 347-351 in: Advances in *Verticillium* Research and Disease Management. E. C. Tjamos, R. C. Rowe, J. B. Heale, and D. R. Fravel, eds. American Phytopathological Society, St. Paul, MN.
- Davis, J. R., and Sorensen, L. H. 1985. Association of *Verticillium tricorpus* with *Verticillium dahliae* and potato early dying. *Phytopathology* 75:1279.
- Dung, J. K. S., Peever, T. L., and Johnson, D. A. 2013. *Verticillium dahliae* populations from mint and potato are genetically divergent with predominant haplotypes. *Phytopathology* 103:445-459.
- Fick, G. N., and Zimmer, D. E. 1974. Monogenic resistance to *Verticillium* wilt in sunflowers. *Crop Sci.* 14:895-896.
- França, S. C., Spiessens, K., Pollet, S., Debode, J., Rooster, L. D., Callens, D., and Höfte, M. 2013. Population dynamics of *Verticillium* species in cauliflower fields: Influence of crop rotation, debris removal and ryegrass incorporation. *Crop Prot.* 54:134-141.
- García-Ruiz, R., García-Careros, A. B., and Molinero-Ruiz, L. 2014. A new race of *Verticillium dahliae* causing leaf mottle of sunflower in Europe. *Plant Dis.* 98:1435.
- Gurung, S., Short, D. P. G., Hu, X., Sandoya, G. V., Hayes, R. J., Koike, S. T., and Subbarao, K. V. 2015. Host range of *Verticillium isaacii* and *Verticillium klebahnii* from artichoke, spinach, and lettuce. *Plant Dis.* 99:933-938.
- Inderbitzin, P., Bostock, R. M., Davis, R. M., Usami, T., Platt, H. W., and Subbarao, K. V. 2011. Phylogenetics and taxonomy of the fungal vascular wilt pathogen *Verticillium*, with the descriptions of five new species. *PLoS One* 6:e28341.
- Johnson, D. A., and Dung, J. K. S. 2010. *Verticillium* wilt of potato-the pathogen, disease and management. *Can. J. Plant Pathol.* 32:58-67.
- Kabir, Z., Bhat, R. G., and Subbarao, K. V. 2004. Comparison of media for recovery of *Verticillium dahliae* from soil. *Plant Dis.* 88:49-55.
- Klosterman, S. J., Atallah, Z. K., Vallad, G. E., and Subbarao, K. V. 2009. Diversity, pathogenicity, and management of *Verticillium* species. *Annu. Rev. Phytopathol.* 47:39-62.
- Malcolm, G. M., Kuldau, G. A., Gugino, B. K., and Jiménez-Gasco, Mdel. M. 2013. Hidden host plant associations of soilborne fungal pathogens: An ecological perspective. *Phytopathology* 103:538-544.
- Milgroom, M. G., Del Mar Jiménez-Gasco, M., Olivares-García, C., and Jiménez-Díaz, R. M. 2016. Clonal expansion and migration of a highly virulent, defoliating lineage of *Verticillium dahliae*. *Phytopathology* 106:1038-1046.
- Qin, Q.-M., Vallad, G. E., and Subbarao, K. V. 2008. Characterization of *Verticillium dahliae* and *V. tricorpus* isolates from lettuce and artichoke. *Plant Dis.* 92:69-77.
- Sadras, V. O., Quiroz, F., Echarte, L., Escande, A., and Pereyra, V. R. 2000. Effect of *Verticillium dahliae* on photosynthesis, leaf expansion and senescence of field-grown sunflower. *Ann. Bot. (Lond.)* 86:1007-1015.
- Shaner, G., and Finney, R. E. 1977. The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. *Phytopathology* 67:1051-1056.
- Tversky, A., and Kahneman, D. 1971. Belief in the law of small numbers. *Psychol. Bull.* 76:105-110.
- Tyvaert, L., França, S. C., Debode, J., and Höfte, M. 2014. The endophyte *Verticillium* Vt305 protects cauliflower against *Verticillium* wilt. *J. Appl. Microbiol.* 116:1563-1571.
- Wheeler, D. L., and Johnson, D. A. 2016. *Verticillium dahliae* infects, alters plant biomass, and produces inoculum on rotation crops. *Phytopathology* 106:602-613.