Host Range of *Verticillium isaacii* and *Verticillium klebahnii* from Artichoke, Spinach, and Lettuce

Suraj Gurung and **Dylan P. G. Short**, Department of Plant Pathology, University of California, Davis, c/o U.S. Agricultural Research Station, Salinas 93905; **Xiaoping Hu**, State Key Laboratory of Crop Stress Biology for Arid Areas and College of Plant Protection, Northwest A&F University, Yangling, Shaanxi, China; **German V. Sandoya**, The Genome Center and Department of Plant Sciences, University of California, Davis; **Ryan J. Hayes**, United States Department of Agriculture–Agricultural Research Service, Salinas, CA 93905; **Steven T. Koike**, University of California Cooperative Extension, Salinas 93901; and **Krishna V. Subbarao**, Department of Plant Pathology, University of California, Davis, c/o U.S. Agricultural Research Station, Salinas

Abstract

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.

Verticillium is a genus that includes major vascular wilt pathogens. Recently, multilocus phylogenetic analyses of the genus identified five new species, including *Verticillium isaacii* and *V. klebahnii*, both of which occur in agricultural soils in coastal California and have been isolated from asymptomatic and diseased spinach and lettuce plants. Little data are available regarding their pathogenicity and virulence on a broader range of crops important to the region. Four isolates each of *V. isaacii* and *V. klebahnii* along with two reference isolates of *V. dahliae* races 1 and 2 were inoculated on eight crops (artichoke, cauliflower, eggplant, lettuce, pepper, tomato, spinach, and strawberry) in a greenhouse experiment. After 8 weeks, plants were assessed for disease severity to determine the relative host ranges of *Verticillium* isolates. Additionally, 13 lettuce lines resistant to race 1 and

partially resistant to race 2 of *V. dahliae* were screened against *V. isaacii* and *V. klebahnii* to evaluate their responses. Three of four *V. isaacii* and four of four *V. klebahnii* isolates tested were nonpathogenic on all crops tested except those indicated below. One *V. isaacii* isolate caused wilt on artichoke and ‘Salinas’ lettuce and most isolates of both species caused varying degrees of *Verticillium* wilt on strawberry. Lettuce lines resistant to *V. dahliae* race 1 and partially resistant to *V. dahliae* race 2 also exhibited resistance to all of the isolates of *V. isaacii* and *V. klebahnii*. Thus, at least some isolates in the populations of *V. isaacii* and *V. klebahnii* have the potential to become significant pathogens of coastal California crops. However, resistance developed against *V. dahliae* also offers resistance to the pathogenic isolates of both species, at least in lettuce.

Verticillium spp. are soilborne fungal pathogens that cause vascular wilts in more than 400 plant species. A comprehensive reassessment of the systematics and taxonomy of the genus *Verticillium* by Inderbitzin et al. (2011) delineated strongly supported species boundaries within the genus *Verticillium* and described five new species. The phylogenetic tree that Inderbitzin et al. (2011) constructed based on multigene phylogenies included two large clades with five species each. Species in clade Flavexudans produce a yellow pigment in culture and includes *Verticillium albo-atrum*, *V. isaacii*, *V. klebahnii*, *V. tricornis*, and *V. zaregamsianum*; in contrast, species in clade Flavonoxudans produce no pigmentation and include *V. alfalfae*, *V. dahliae*, *V. nonalfalfae*, *V. nubilum*, and the hybrid *V. longisporum*, of which *V. dahliae* is by far the most economically important, followed by *V. longisporum*. The other species from this clade cause *Verticillium* wilt in a limited number of plant species.

V. tricornis sensu stricto is morphologically cryptic with *V. isaacii* and *V. klebahnii* (Inderbitzin et al. 2011). *V. tricornis* is known to occur only in Japan, The Netherlands, and the United Kingdom. Reports of *Verticillium* wilt caused by the morphospecies “*V. tricornis*” in California and elsewhere in the United States, prior to phylogenetic circumscription, was therefore likely to be caused by either *V. isaacii* or *V. klebahnii*. Previous host range experiments based on a limited number of plant species has suggested the existence of potential differences in host ranges between the three aforementioned *Verticillium* spp. (Isaac 1953; Vallad et al. 2006).

The number of plant species known to be susceptible to *Verticillium* wilt has steadily increased as epidemics have appeared on crops

previously considered immune or not susceptible (Pegg and Brady 2002). In the late 1980s, *Verticillium* wilt caused by *V. longisporum* became severe in commercial cauliflower fields in coastal California (Koike et al. 1994). The Salinas and Pajaro Valleys are home to >85% of fresh-market spinach and 60% of lettuce production in the United States (Anonymous 2011, 2012) and, although lettuce was historically considered to be immune to *V. dahliae*, it was recognized as a host in 1995 (Subbarao et al. 1997). The disease has since expanded its geographical range to become a significant problem for lettuce production (Atallah et al. 2011; Gurung et al. 2014). As of 2013, *Verticillium* wilt on lettuce had spread to multiple fields in coastal California and caused millions of dollars in yield losses annually (Gurung et al. 2014). Although *Verticillium* wilt of lettuce caused by *V. dahliae* is currently restricted to coastal California in the United States, *Verticillium* wilt caused by *V. tricornis* was reported from Washington (Powell et al. 2013). The National Center for Biotechnology Information GenBank accessions and the phylogenetic analysis of the sequence data from Powell et al. (2013) clearly place this pathogen within *V. isaacii*. *Verticillium* wilt of spinach, although known (du Toit et al. 2005; Short et al. 2014, 2015), is not a problem in spinach production because the crop is harvested well before symptom onset, which coincides with bolting and flowering.

The phase-out of the soil fumigant methyl bromide in 2015 is likely to increase the range and incidence of soilborne diseases in coastal California. Thus, it is reasonable to expect that the reservoirs of *V. dahliae* and other fungi present in the soil but currently not known to cause diseases may emerge to have a greater impact on crops in the area. In disease surveys, *V. isaacii* and *V. klebahnii* were frequently recovered from lettuce and artichoke fields in California (Qin et al. 2008). Similarly, Short et al. (2015) frequently recovered *V. isaacii*, *V. dahliae*, and *V. klebahnii* isolates during surveys of commercial spinach fields from 2010 to 2012. The relative roles of *V. isaacii* and *V. klebahnii* in the *Verticillium* wilt complex in lettuce production and other crops in coastal California are unknown. Finally, the effectiveness of *V. dahliae*-resistant lettuce accessions and cultivars, which includes race 1 resistance, mediated by the plant resistance gene *Vr1* (Hayes et al. 2011b) and several lines identified

Corresponding author: K. V. Subbarao; E-mail: kvsubbarao@ucdavis.edu

S. Gurung and D. P. G. Short contributed equally to this study.

Accepted for publication 25 January 2015.

as partially resistant to race 2 (Hayes et al. 2007), has not been tested against *Verticillium* spp. other than *V. dahliae*.

The objectives of the present study were to (i) determine the host range of multiple isolates of *V. isaacii* and *V. klebahnii* on major crops grown in coastal California relative to the two races of *V. dahliae* and (ii) test several lettuce cultivars that are partially resistant to *V. dahliae* against isolates of *V. isaacii* pathogenic to lettuce and artichoke and isolates of *V. klebahnii* pathogenic to strawberry.

Materials and Methods

Pathogenicity assays of *V. dahliae*, *V. isaacii*, and *V. klebahnii* in the greenhouse. Four isolates each of *V. isaacii* (Cello-A-Tri-P [spinach], PD 341 [lettuce], PD 661 [lettuce], and PD 752 [spinach]) and *V. klebahnii* (PD 347 [artichoke], PD 407 [lettuce], PD 458 [lettuce], and PD 659 [lettuce]), and two isolates of *V. dahliae* representing race 1 (Ls. 16) and 2 (Ls. 17) were tested on artichoke ('5473/91 black'), cauliflower ('Snowball'), eggplant ('Long Purple'), lettuce 'La Brillante' (resistant to race 1 of *V. dahliae*), lettuce 'Salinas' (susceptible to both *V. dahliae* races), pepper ('California Wonder'), spinach ('Hector'), strawberry ('Albion'), and tomato ('Beefsteak') in the greenhouse using previously described procedures (Bhat and Subbarao 1999; Hayes et al. 2011a; Vallad et al. 2006). Experiments were conducted two times. Thirty seeds of each host were sown in 200-well plug trays (Hummert International, St. Louis) filled with Sunshine Mix number 4 (Sun Gro Horticulture, Canada). Three weeks after sowing, seedlings were transferred into 0.5-liter foam-insulated cups filled with a pasteurized sand-potting soil

Table 1. Statistical analysis of variance based on the effect of individual isolates of *Verticillium dahliae*, *V. isaacii*, and *V. klebahnii* on the ranked means of Verticillium wilt severity on different hosts across two independent experiments

Effect	df _{Num}	df _{Den}	ATS	P value
Experiment	1.37	1	8.85	0.2189
Isolate	8.99	1	32.00	0.1364
Host	6.56	302	99.07	<0.0001
Isolate × host	59.10	302	4.13	<0.0001

Fig. 1. Vascular discoloration in **A**, artichoke; **B**, lettuce; and **C**, strawberry caused by the race 1 and 2 strains of *Verticillium dahliae* (Vd.Ls16 and Vd.Ls17) and an isolate of *V. isaacii* (Cello-A-Tri-P) relative to an uninoculated control plant.

mixture (2:1, vol/vol). Strawberry transplants were first established in trays containing the potting mix; after dormancy was broken and the plants had grown leaves and new roots, plants were then transplanted for inoculation. Each host-isolate treatment combination was replicated three times, with 10 plants in each replication, and was arranged in a randomized complete block design. Inoculum from all *V. dahliae*, *V. isaacii*, and *V. klebahnii* isolates were prepared as previously described by Vallad et al. (2006). Seedlings were inoculated at 4 weeks after sowing by saturating the soil in each well with a 5- to 10-ml suspension containing 1×10^7 conidia/ml in sterile distilled water. Two additional inoculations were performed on all plants with the corresponding inoculum at 1 and 2 weeks after the first inoculation. Control plants for each host were mock inoculated with sterile distilled water and were kept in a separate block. Plants were incubated on greenhouse benches for approximately 10 weeks after the first inoculations and were evaluated for Verticillium wilt severity. Each plant was uprooted gently; roots were washed free of soil, and cut longitudinally to evaluate disease severity. Verticillium wilt severity was assessed as previously described by Vallad et al. (2006). Briefly, root discoloration was rated on a 0-to-5 scale, where 0 = no vascular discoloration; 1 = 1 to 25% of the vascular tissue exhibiting discoloration; 2 = 26 to 50, 3 = 51 to 75, and 4 = 76 to 100% discoloration and no foliar symptoms; and 5 = 100% discoloration and the presence of foliar symptoms typical of Verticillium wilt.

Symptomatic root and crown tissues were washed, surface sterilized using distilled water and 5% bleach, dissected, and plated on NP-10 medium (Kabir et al. 2004). After 10 to 14 days of incubation at 22 to 24°C, plates were examined under a dissecting microscope for the presence of microsclerotia, verticillate conidiophores, and hyaline conidia, the distinguishing characteristics of *Verticillium* spp.

Evaluation of *V. dahliae* race 1-resistant and race 2-partially resistant lettuce cultivars against *V. isaacii* and *V. klebahnii*. Eight *V. dahliae* race 1-resistant cultivars ('Annapolis', 'Eruption', 'Defender', 'Pavane', 'Little Gem', 'Sentry', 'Infantry', and 'Merlot'; Hayes et al. 2007), and four *V. dahliae* race 2-partially resistant accessions (169511, 171674, 204707, and 226641; Hayes et al. 2011a) were tested against two isolates of *V. isaacii* (Cello-A-Tri-P and PD341), two isolates of *V. klebahnii* (PD458 and PD659), and a race 1 (Ls. 16) and race 2 (Ls. 17) isolate of *V. dahliae*. Isolate Cello-A-Tri-P was previously known to be pathogenic while isolates PD 341, PD 458, and PD 659 were determined to be pathogenic on strawberry from the pathogenicity assays described above.

All plant growth conditions, inoculum preparation, inoculation, and disease scoring were the same as described above. Lettuce cultivar-isolate combinations were arranged in a randomized complete block design with three replications, and each replication included 10 plants of each lettuce line. The experiment was repeated twice.

Data analysis. Disease severity data from the greenhouse studies were analyzed using analysis of variance (ANOVA)-type statistics of ranked data using the PROC Mixed procedure in SAS (SAS Institute, Cary, NC), and the LD_CI macro was used to generate relative marginal effects for each treatment and 95% confidence intervals for detection of statistical difference between treatments (Shah and Madden 2004). Orthogonal contrasts were conducted to compare the disease levels caused by *V. isaacii* and *V. klebahnii* (at the species level with data from all species combined) with *V. dahliae* races 1 and 2 on crops in which individual isolates were pathogenic, as well as between the two races. This was done to verify whether the general response of *V. isaacii* and *V. klebahnii* aligned with race 1 or 2 of *V. dahliae*.

Results

ANOVA indicated no significant differences between the two experiments (Table 1); thus, the data from the two experiments were combined. ANOVA indicated no significant differences among isolates of *V. isaacii*, *V. klebahnii*, and *V. dahliae*. Most of the *V. klebahnii* and *V. isaacii* isolates did not cause Verticillium wilt symptoms on most hosts evaluated. Correspondingly, significant interactions were observed among hosts and isolate-host interactions (Table 1).

Pathogenicity on artichoke. Artichoke plants inoculated with *V. dahliae* isolates (Ls. 16 and Ls. 17) exhibited root discoloration (Fig. 1A). Plants were significantly stunted and had dry leaves relative

to noninoculated plants. Similarly, *V. isaacii* (Cello-A-Tri-P, PD 341, PD 661, and PD 752) and *V. klebahnii* (PD 347, PD 407, PD 458, and PD 659) also produced root discoloration. *V. isaacii* isolate Cello-A-Tri-P produced higher disease severities than the *V. dahliae* race 1 isolate Ls.16 (Table 2). Overall, mean ratings of disease severity caused by isolates of *V. dahliae* were higher than those caused by

V. isaacii and *V. klebahnii* (Table 2). *V. dahliae* and *V. isaacii* were successfully reisolated from the discolored roots of the infected artichoke plants.

Pathogenicity on cauliflower. Cauliflower plants inoculated with *V. dahliae* isolates (Ls. 16 and Ls. 17), *V. isaacii* (Cello-A-Tri-P, PD 341, PD 661, and PD 752), and *V. klebahnii* (PD 347, PD 407, PD

Table 2. Median and maximum disease severity and relative marginal effects along with 95% confidence intervals (CI) for Verticillium wilt severity ratings in relation to different hosts and isolates of *Verticillium dahliae*, *V. isaacii*, and *V. klebahnii*

Hosts, isolates ^c	Disease severity ^a		Relative marginal effect ^b	
	Median	Maximum	Estimate	95% CI
Artichoke (5473/91 black)				
Ls.16	1.43	2.50	0.69	(0.57, 0.79)
Ls.17	2.75	3.50	0.92	(0.83, 0.94)
Cello-A-Tri-P	2.00	2.43	0.71	(0.57, 0.81)
PD 341	0.92	1.29	0.46	(0.32, 0.60)
PD 661	0.47	1.14	0.33	(0.20, 0.49)
PD 752	0.31	1.40	0.36	(0.24, 0.52)
PD 347	0.69	2.17	0.45	(0.24, 0.68)
PD 407	0.32	1.67	0.37	(0.17, 0.65)
PD 458	0.21	1.86	0.34	(0.19, 0.55)
PD 659	0.34	2.29	0.37	(0.18, 0.63)
Cauliflower (Snowball)				
Ls.16	0.37	0.70	0.59	(0.39, 0.75)
Ls.17	0.57	1.00	0.62	(0.36, 0.82)
Cello-A-Tri-P	0.05	0.60	0.42	(0.24, 0.62)
PD 341	0.58	1.30	0.65	(0.42, 0.81)
PD 661	0.00	1.22	0.37	(0.19, 0.62)
PD 752	0.05	0.33	0.36	(0.23, 0.51)
PD 347	0.50	1.25	0.64	(0.40, 0.82)
PD 407	0.00	1.33	0.41	(0.20, 0.67)
PD 458	0.27	0.44	0.47	(0.31, 0.64)
PD 659	0.15	1.22	0.48	(0.26, 0.70)
Eggplant (Long purple)				
Ls.16	1.17	2.14	0.78	(0.64, 0.86)
Ls.17	4.15	5.00	0.95	(0.63, 0.99)
Cello-A-Tri-P	0.05	0.80	0.32	(0.17, 0.54)
PD 341	0.39	1.29	0.54	(0.36, 0.70)
PD 661	0.10	0.60	0.37	(0.20, 0.59)
PD 752	0.26	0.56	0.43	(0.26, 0.62)
PD 347	0.16	0.30	0.34	(0.22, 0.49)
PD 407	0.15	0.56	0.37	(0.21, 0.58)
PD 458	0.31	0.89	0.5	(0.33, 0.68)
PD 659	0.11	0.70	0.41	(0.23, 0.62)
Lettuce (Salinas)				
Ls.16	3.00	3.50	0.81	(0.67, 0.89)
Ls.17	4.50	4.67	0.85	(0.74, 0.91)
Cello-A-Tri-P	3.26	3.96	0.69	(0.54, 0.80)
PD 341	1.00	3.00	0.41	(0.23, 0.62)
PD 661	1.75	3.13	0.53	(0.32, 0.73)
PD 752	0.75	2.13	0.29	(0.14, 0.52)
PD 347	1.04	1.78	0.31	(0.17, 0.51)

(continued in next column)

^a Disease severity of Verticillium wilt was assessed visually on an ordinal 0-to-5 scale, where 0 = no vascular discoloration; 1 = 1 to 25% of the vascular tissue showing discoloration; 2 = 26 to 50, 3 = 51 to 75, and 4 = 76 to 100% discoloration with no foliar symptoms; and 5 = 100% discoloration and the presence of foliar symptoms typical of Verticillium wilt (Vallad et al. 2006). Analysis of variance indicated no significant differences between two experiments, and therefore averaged disease severity from the two experiments are shown. Median values were calculated from wilt severity values averaged over inoculated plants within replications.

^b Relative marginal effect and 95% CI are calculated from analysis of the rank values of the disease severity data.

^c *V. dahliae* isolates are Ls.16 and Ls.17 (lettuce); *V. isaacii* isolates are Cello-A-Tri-P (spinach), PD 341 (lettuce), PD 661 (lettuce), and PD 752 (spinach); and *V. klebahnii* isolates are PD 347 (artichoke), PD 407 (lettuce), PD 458 (lettuce), and PD 659 (lettuce).

Table 2. (continued from preceding column)

Hosts, isolates ^c	Disease severity ^a		Relative marginal effect ^b	
	Median	Maximum	Estimate	95% CI
PD 407	1.05	2.33	0.36	(0.21, 0.55)
PD 458	1.25	2.17	0.46	(0.28, 0.66)
PD 659	0.95	1.50	0.3	(0.20, 0.43)
Lettuce (La Brillante)				
Ls.16	0.00	0.00	0.00	(0.00, 0.00)
Ls.17	2.93	4.78	0.87	(0.75, 0.92)
Cello-A-Tri-P	1.19	1.38	0.63	(0.41, 0.79)
PD 341	1.13	1.30	0.59	(0.45, 0.72)
PD 661	1.00	1.50	0.32	(0.19, 0.48)
PD 752	1.04	1.60	0.32	(0.29, 0.49)
PD 347	1.36	1.50	0.42	(0.23, 0.65)
PD 407	1.25	1.86	0.47	(0.24, 0.72)
PD 458	0.66	1.13	0.18	(0.12, 0.30)
PD 659	1.42	1.50	0.47	(0.30, 0.65)
Pepper (California Wonder)				
Ls.16	0.13	0.20	0.65	(0.46, 0.80)
Ls.17	0.00	0.00	0.35	(0.30, 0.40)
Cello-A-Tri-P	0.00	0.40	0.53	(0.33, 0.71)
PD 341	0.00	0.00	0.35	(0.30, 0.40)
PD 661	0.00	0.10	0.47	(0.34, 0.61)
PD 752	0.00	0.00	0.35	(0.30, 0.40)
PD 347	0.00	0.56	0.55	(0.33, 0.75)
PD 407	0.33	0.44	0.71	(0.46, 0.86)
PD 458	0.11	0.60	0.69	(0.45, 0.85)
PD 659	0.00	0.00	0.35	(0.30, 0.40)
Spinach (Hector)				
Ls.16	4.10	5.00	0.93	(0.87, 0.94)
Ls.17	3.70	4.20	0.86	(0.81, 0.89)
Cello-A-Tri-P	0.00	0.20	0.22	(0.15, 0.31)
PD 341	1.50	3.00	0.65	(0.48, 0.79)
PD 661	0.00	0.80	0.32	(0.36, 0.48)
PD 752	0.00	0.10	0.30	(0.32, 0.46)
PD 347	0.40	1.40	0.46	(0.47, 0.63)
PD 407	0.50	0.60	0.42	(0.31, 0.56)
PD 458	0.50	1.20	0.49	(0.32, 0.62)
PD 659	0.20	1.40	0.37	(0.42, 0.54)
Strawberry (Albion)				
Ls.16	2.20	2.60	0.39	(0.20, 0.44)
Ls.17	4.25	5.00	0.93	(0.89, 0.95)
Cello-A-Tri-P	2.13	2.40	0.40	(0.27, 0.50)
PD 341	2.00	2.40	0.31	(0.30, 0.47)
PD 661	2.38	3.40	0.50	(0.31, 0.69)
PD 752	2.22	3.60	0.46	(0.25, 0.69)
PD 347	2.85	3.40	0.71	(0.62, 0.78)
PD 407	2.55	3.00	0.57	(0.43, 0.70)
PD 458	2.10	2.80	0.35	(0.21, 0.52)
PD 659	2.20	3.40	0.47	(0.30, 0.67)
Tomato (Beefsteak)				
Ls.16	1.46	2.80	0.81	(0.58, 0.90)
Ls.17	2.81	3.50	0.92	(0.87, 0.94)
Cello-A-Tri-P	0.00	0.43	0.45	(0.33, 0.58)
PD 341	0.00	0.17	0.44	(0.33, 0.56)
PD 661	0.00	0.00	0.38	(0.34, 0.41)
PD 752	0.00	0.22	0.44	(0.33, 0.56)
PD 347	0.00	0.00	0.38	(0.34, 0.41)
PD 407	0.00	0.00	0.38	(0.34, 0.41)
PD 458	0.00	0.00	0.38	(0.34, 0.41)
PD 659	0.00	0.25	0.44	(0.33, 0.57)

458, and PD 659) exhibited no or negligible root discoloration (Table 2). Plants looked healthy and disease severity was no different from that on control plants (data not shown). Median disease severity for all isolates and species varied between 0.0 and 0.58 (Table 2).

Pathogenicity on eggplant. Eggplants inoculated with *V. dahliae* isolate Ls. 17 exhibited severe root discoloration in most plants as well as stunting and dried leaves relative to noninoculated plants. However, *V. isaacii* (Cello-A-Tri-P, PD 341, PD 661, and PD 752) and *V. klebahnii* (PD 347, PD 407, PD 458, and PD 659) produced little or no root discoloration. Overall, mean ratings of disease severity caused by isolates of *V. dahliae* were higher than those caused by *V. isaacii* and *V. klebahnii*, with the highest median disease severity of 4.15 for plants inoculated with *V. dahliae* Ls. 17 (Table 2).

Pathogenicity on Salinas and La Brillante lettuce. Plants of cultivar Salinas inoculated with *V. dahliae* isolates Ls. 16 and Ls. 17 exhibited root discoloration (Fig. 1B), as did the plants inoculated with *V. isaacii* (Cello-A-Tri-P, PD 341, PD 661, and PD 752) and *V. klebahnii* (PD 347, PD 407, PD 458, and PD 659). Of the *V. isaacii* isolates, Cello-A-Tri-P induced disease severity similar to *V. dahliae* isolate Ls. 16 (Fig. 1B). Further, *V. dahliae* and *V. isaacii* were successfully reisolated from the discolored tap roots of the infected Salinas. Overall, median ratings of disease severity produced by isolates of *V. dahliae* were greater than those caused by *V. isaacii* and *V. klebahnii*. Roots of La Brillante inoculated with *V. dahliae* isolate Ls. 17 were discolored and the plants displayed wilting symptoms; however, there was no vascular discoloration in plants inoculated with *V. dahliae* isolate Ls. 16. Even though plants of La Brillante inoculated with *V. isaacii* and *V. klebahnii* isolates displayed vascular discoloration, they had low disease severity ratings (Table 2). In general, median ratings of disease severity produced by isolates of *V. dahliae* were greater than those caused by *V. isaacii* and *V. klebahnii* (Table 2).

Pathogenicity on pepper. All of the *V. dahliae*, *V. isaacii*, or *V. klebahnii* isolates evaluated in this study caused negligible to no vascular discoloration on pepper (Table 2). Plants looked healthy and disease severity scores between inoculated and noninoculated control plants were not statistically different (data not shown).

Pathogenicity on spinach. Spinach plants inoculated with *V. dahliae* isolates Ls. 16 and Ls. 17 showed root discoloration. However, none of the plants inoculated with *V. isaacii* (Cello-A-Tri-P, PD 341,

PD 661, and PD 752) or *V. klebahnii* (PD 347, PD 407, PD 458, and PD 659) exhibited symptoms associated with Verticillium wilt. Overall, median disease ratings of *V. dahliae* were higher than those caused by *V. isaacii* and *V. klebahnii* (Table 2).

Pathogenicity on strawberry. All plants inoculated with *V. dahliae*, *V. isaacii*, and *V. klebahnii* showed Verticillium wilt symptoms to varying degrees (Fig. 1C). The disease severity ratings for all isolates of *V. isaacii* and *V. klebahnii* were above the minimum severity rating of 2.0 associated with isolates pathogenic on individual hosts. Overall, median disease severity ratings caused by the race 2 isolate of *V. dahliae* were higher than those produced by *V. isaacii* and *V. klebahnii*. However, all isolates of *V. isaacii* and *V. klebahnii*, including the race 1 isolate of *V. dahliae*, caused lesser disease than *V. dahliae* race 2 isolate Ls. 17 (Table 2).

Pathogenicity on tomato. On Beefsteak tomato, only *V. dahliae* isolate Ls. 17 caused vascular discoloration and wilting symptoms. Isolate Ls. 16 showed only slight discoloration and no wilting was observed (Table 2). However, none of the *V. isaacii* and *V. klebahnii* isolates caused any symptoms associated with Verticillium wilt (Table 2).

Contrasts between *V. isaacii*, *V. klebahnii*, and the two races of *V. dahliae*. Except for the contrast between race 1 of *V. dahliae* (Ls. 16 versus *V. isaacii*; Table 3) and *V. isaacii*, all other contrasts between *V. isaacii* and *V. klebahnii* and the two races of *V. dahliae* were significant, suggesting that disease severities caused by these two species were either higher or lower than those of the two races of *V. dahliae* (Table 3). Except for one isolate of *V. isaacii* on artichoke and all isolates of both *V. isaacii* and *V. klebahnii* on both cultivars of lettuce, the disease severities were lower than those caused by both races of *V. dahliae* (Tables 2 and 3). In strawberry, however, nearly all isolates of *V. isaacii* and *V. klebahnii* caused as much or higher disease severities than the race 1 isolate of *V. dahliae* (Tables 2 and 3). By far, the most virulent isolate on all hosts was race 2 (Ls. 17) of *V. dahliae* (Tables 2 and 3).

Evaluation of *V. isaacii* isolates on *V. dahliae* race 1-resistant and *V. dahliae* race 2-partially resistant lettuce cultivars. *V. isaacii* isolate PD 341 and *V. klebahnii* isolates (PD 458 and PD 659) failed to produce Verticillium wilt symptoms on susceptible Salinas. However, *V. isaacii* isolate Cello-A-Tri-P induced obvious disease symptoms on Salinas but failed to produce any disease symptoms on the other *V. dahliae*-resistant and partially resistant lettuce cultivars (Table 4).

Discussion

The current study explored the host range of two recently described *Verticillium* spp. (Inderbitzin et al. 2011) from artichoke, spinach, and lettuce fields in coastal California. Of the four isolates each of *V. isaacii* and *V. klebahnii* evaluated in this study, only one isolate of *V. isaacii* was pathogenic on the lettuce cultivar susceptible to both *V. dahliae* races 1 and 2 and artichoke. This is the first demonstration of Koch's postulates for *V. isaacii* on lettuce in California. On other hosts evaluated in this study, all isolates of these two species were nonpathogenic, except on strawberry. All eight isolates were highly pathogenic on strawberry. In addition, the effectiveness of *V. dahliae* race 1 resistance and partial race 2 resistance in lettuce cultivars and accessions was tested using two isolates each of *V. isaacii* and *V. klebahnii* identified as pathogenic on strawberry and a susceptible lettuce cultivar. All lettuce lines tested were resistant to *V. isaacii* and *V. klebahnii*. It is evident from this study that some of the *V. isaacii* isolates, including Cello-A-Tri-P, can cause disease at levels similar to *V. dahliae* race 1 isolate Ls. 16 on certain lettuce cultivars. Some of these lettuce cultivars, including PI 169511, PI 171674, PI 204707, and PI 226641, developed wilt symptoms following inoculations with *V. dahliae* race 1 isolate Ls. 16 (G. Sandoya, unpublished). Assuming that Ls. 16 and some of the *V. isaacii* isolates cause similar levels of disease, future inoculations with these isolates on some of these lettuce cultivars might result in low levels of disease symptoms in some experiments. Thus, at least some isolates in populations of *V. isaacii* and *V. klebahnii* could potentially become significant pathogens of some coastal California crops if clonal expansion of

Table 3. Orthogonal contrasts between *Verticillium isaacii* and *V. klebahnii* and type isolates for races 1 (Ls.16) and 2 (Ls.17) of *V. dahliae* on hosts in which the two former species caused Verticillium wilt

Contrasts	Numerator DF	Denominator DF	F	P > F
Artichoke				
Ls.16 versus <i>V. isaacii</i>	1	48	6.26	0.0158
Ls.16 versus <i>V. klebahnii</i>	1	48	12.98	0.0007
Ls.16 versus Ls.17	1	48	7.02	<0.0109
Ls.17 versus <i>V. isaacii</i>	1	48	64.08	<0.0001
Ls.17 versus <i>V. klebahnii</i>	1	48	119.11	<0.0001
Lettuce (Salinas)				
Ls.16 versus <i>V. isaacii</i>	1	48	14.18	0.0005
Ls.16 versus <i>V. klebahnii</i>	1	48	34.15	<0.0001
Ls.16 versus Ls.17	1	48	0.32	0.5717
Ls.17 versus <i>V. isaacii</i>	1	48	33.85	<0.0001
Ls.17 versus <i>V. klebahnii</i>	1	48	103.87	<0.0001
Lettuce (La Brillante)				
Ls.16 versus <i>V. isaacii</i>	1	48	6.94	0.0113
Ls.16 versus <i>V. klebahnii</i>	1	48	11.76	0.0013
Ls.16 versus Ls.17	1	48	130.63	<0.0001
Ls.17 versus <i>V. isaacii</i>	1	48	18.72	<0.0001
Ls.17 versus <i>V. klebahnii</i>	1	48	26.76	<0.0001
Strawberry				
Ls.16 versus <i>V. isaacii</i>	1	66	1.75	0.1910
Ls.16 versus <i>V. klebahnii</i>	1	66	5.80	0.0188
Ls.16 versus Ls.17	1	66	56.83	<0.0001
Ls.17 versus <i>V. isaacii</i>	1	66	144.90	<0.0001
Ls.17 versus <i>V. klebahnii</i>	1	66	56.29	<0.0001

pathogenic genotype occurs (Gurung et al. 2014). However, resistance developed against *V. dahliae* (Hayes et al. 2007, 2011a) also offers resistance against these other *Verticillium* spp.

Knowledge of the genotypic and pathogenic variation that exists within *Verticillium* spp. present in a particular agricultural region is beneficial to research programs with the goal of developing appropriate

Table 4. Median and maximum disease severity and relative marginal effects along with 95% confidence intervals (CI) for *Verticillium* wilt severity ratings in relation to different isolates of *Verticillium isaacii*, *V. klebahnii*, and *V. dahliae* on lettuce cultivars resistant to *V. dahliae* race 1 and partially resistant to *V. dahliae* race 2 isolates

Isolates, cultivars ^b	Relative marginal effects ^a		
	Median	Estimate	95% CI
Cello-A-Tri-P			
169511	0.00	0.35	(0.32, 0.37)
171674	0.00	0.47	(0.34, 0.62)
204707	0.00	0.49	(0.33, 0.64)
226641	0.00	0.41	(0.30, 0.53)
Annapolis	0.20	0.55	(0.38, 0.71)
Defender	0.70	0.72	(0.55, 0.84)
Eruption	0.00	0.35	(0.32, 0.37)
Infantry	0.00	0.35	(0.32, 0.37)
La Brillante	0.20	0.56	(0.38, 0.72)
Little Gem	0.20	0.55	(0.38, 0.71)
Merlot	0.00	0.47	(0.34, 0.62)
Pavane	0.00	0.47	(0.34, 0.62)
Salinas	2.70	0.83	(0.82, 0.84)
Sentry	0.00	0.35	(0.32, 0.37)
PD 341			
169511	0.00	0.47	(0.33, 0.63)
171674	0.00	0.35	(0.32, 0.37)
204707	0.00	0.49	(0.33, 0.65)
226641	0.00	0.41	(0.30, 0.54)
Annapolis	0.00	0.41	(0.29, 0.54)
Defender	0.00	0.41	(0.29, 0.54)
Eruption	0.00	0.35	(0.32, 0.37)
Infantry	0.00	0.49	(0.33, 0.65)
La Brillante	0.00	0.35	(0.32, 0.37)
Little Gem	0.00	0.35	(0.32, 0.37)
Merlot	0.00	0.41	(0.30, 0.53)
Pavane	0.00	0.41	(0.30, 0.53)
Salinas	0.00	0.49	(0.33, 0.65)
Sentry	0.00	0.35	(0.32, 0.37)
PD 458			
169511	0.00	0.47	(0.32, 0.63)
171674	0.00	0.41	(0.29, 0.54)
204707	0.00	0.35	(0.32, 0.37)
226641	0.00	0.35	(0.32, 0.37)
Annapolis	0.00	0.47	(0.33, 0.63)
Defender	0.00	0.35	(0.32, 0.37)
Eruption	0.00	0.42	(0.29, 0.57)
Infantry	0.00	0.35	(0.32, 0.37)
La Brillante	0.00	0.35	(0.32, 0.37)
Little Gem	0.00	0.35	(0.32, 0.37)
Merlot	0.00	0.49	(0.32, 0.65)
Pavane	0.00	0.35	(0.32, 0.37)
Salinas	0.20	0.56	(0.37, 0.73)
Sentry	0.00	0.47	(0.32, 0.63)

(continued in next column)

^a Relative marginal effect and 95% CI are calculated from analysis of the rank values of the disease severity data.

^b Isolate Cello-A-Tri-P (*V. isaacii*) was collected from a spinach plant in California. Isolates PD341 (*V. isaacii*), PD458 and PD659 (*V. klebahnii*) were collected from a lettuce field in California. *V. dahliae* isolates Ls.16 (race 1) and Ls.17 (race 2) were collected from California and were used as controls. Lettuce cultivars 169511, 171674, 204707, and 226641 are partially resistant to *V. dahliae* race 2 isolates (Hayes et al. 2011a). Similarly, Annapolis, Defender, Eruption, Infantry, La Brillante, Little Gem, Merlot, Pavane, Salinas, and Sentry are resistant to *V. dahliae* race 1 isolates (Hayes et al. 2007).

disease management strategies for that region, especially through resistance breeding. In the present study, *V. isaacii* and *V. klebahnii* collected from artichoke and lettuce fields were characterized for their pathogenicity and virulence on eight important crops. The results of this study showed that a majority of *V. klebahnii* and *V. isaacii* isolates exhibited little to no pathogenicity on most crops evaluated. However, one *V. isaacii* isolate, Cello-A-Tri-P, was pathogenic on the susceptible lettuce Salinas and artichoke. Not only did this finding demonstrate the pathogenicity of *V. isaacii* on these two crops but it also suggests that seemingly minor pathogens exhibit variation in aggressiveness under similar environmental conditions. Thus, although all of the *V. klebahnii* and three of four *V. isaacii* isolates tested were weak pathogens, the potential for substantial economic losses caused by certain isolates of these species must not be overlooked. Species of *Verticillium* are long-term soil residents and survive in field soil as resting structures. Little is known about the population structure of *V. isaacii* and *V. klebahnii* in field soils but we speculate that, under certain circumstances, severe diseases in lettuce or artichoke fields could occur if successful virulent genotypes are selected. Nevertheless, compared with *V. dahliae*, *V. isaacii*

Table 4. (continued from preceding column)

Isolates, cultivars ^b	Relative marginal effects ^a		
	Median	Estimate	95% CI
PD 659			
169511	0.00	0.47	(0.32, 0.63)
171674	0.00	0.35	(0.32, 0.37)
204707	0.00	0.35	(0.32, 0.37)
226641	0.00	0.35	(0.32, 0.37)
Annapolis	0.20	0.54	(0.37, 0.70)
Defender	0.00	0.35	(0.32, 0.37)
Eruption	0.00	0.35	(0.32, 0.37)
Infantry	0.00	0.35	(0.32, 0.37)
La Brillante	0.00	0.35	(0.32, 0.37)
Little Gem	0.00	0.35	(0.32, 0.37)
Merlot	0.00	0.35	(0.32, 0.37)
Pavane	0.00	0.35	(0.32, 0.37)
Salinas	0.00	0.49	(0.32, 0.65)
Sentry	0.00	0.47	(0.32, 0.63)
Ls.16			
169511	0.00	0.35	(0.32, 0.37)
171674	0.00	0.35	(0.32, 0.37)
204707	0.00	0.35	(0.32, 0.37)
226641	0.00	0.50	(0.32, 0.67)
Annapolis	0.00	0.41	(0.29, 0.54)
Defender	0.00	0.41	(0.29, 0.54)
Eruption	0.00	0.35	(0.32, 0.37)
Infantry	0.00	0.35	(0.32, 0.37)
La Brillante	0.00	0.35	(0.32, 0.37)
Little Gem	0.00	0.41	(0.29, 0.54)
Merlot	0.00	0.42	(0.29, 0.57)
Pavane	0.00	0.35	(0.32, 0.37)
Salinas	4.70	0.99	(0.98, 0.99)
Sentry	0.00	0.35	(0.32, 0.37)
Ls.17			
169511	1.20	0.66	(0.45, 0.82)
171674	2.00	0.67	(0.45, 0.84)
204707	3.10	0.87	(0.85, 0.89)
226641	4.00	0.93	(0.91, 0.94)
Annapolis	3.80	0.90	(0.86, 0.93)
Defender	4.20	0.93	(0.89, 0.96)
Eruption	3.80	0.91	(0.88, 0.92)
Infantry	3.80	0.91	(0.87, 0.94)
La Brillante	4.30	0.93	(0.90, 0.95)
Little Gem	4.00	0.92	(0.90, 0.94)
Merlot	3.70	0.90	(0.87, 0.93)
Pavane	4.20	0.93	(0.89, 0.95)
Salinas	4.50	0.96	(0.92, 0.98)
Sentry	4.00	0.91	(0.88, 0.93)

and *V. klebahnii* appear to be generally less adapted to cause wilt disease in plants. The lack of susceptibility in other crops evaluated in this study suggests that *V. isaacii* may not pose a threat to these crops.

V. isaacii and *V. klebahnii* were unrecognized until Inderbitzin et al. (2011) defined these two new species from the previously described *V. tricornis* based on multigene phylogenies. *V. tricornis* had been reported as pathogenic to a number of important crops and ornamental plant species (Isaac 1953, 1967; Jabnoun-Khiareddine et al. 2006; Taylor 1968). Isaac (1953) inoculated sainfoin, potato, tomato, and snapdragon with *V. tricornis* and found *V. tricornis* to be pathogenic only on tomato. Similarly, Taylor (1968) reported *V. tricornis* to be pathogenic on eggplant, hop, and tomato but not on alfalfa. Similarly, *V. tricornis* from Tunisia had higher virulence to both tomato and eggplant (Jabnoun-Khiareddine et al. 2006). Recently, *V. tricornis* was reported to be associated with Verticillium wilt in lettuce in Washington (Powell et al. 2013). However, based on the sequences of the internal transcribed spacer regions of ribosomal DNA of these *V. tricornis* isolates (Vt.Ls.2010, Vt.Ls.2011-1, and Vt.Ls.2011-2) (Qin et al. 2008), the three isolates appear to be conspecific with *V. isaacii*. Therefore, it is quite likely that previously reported pathogenic isolates of *V. tricornis* actually belong to either *V. isaacii* or *V. klebahnii*. Except for the isolates from Washington (Powell et al. 2013), other isolates were unavailable for characterization. Thus, *V. isaacii* and *V. klebahnii* could cause disease on multiple crops consistent with the host range reported in this study.

We also attempted to delineate the potential race structure among isolates from both *V. isaacii* and *V. klebahnii* based on virulence of isolates on lettuce differentials relative to *V. dahliae* or the pattern of virulence exhibited by *V. dahliae* races on three crops on which *V. isaacii* or *V. klebahnii* isolates exhibited pathogenicity. This was done by virulence testing of multiple isolates of both species on the two differential lettuce lines routinely used to discriminate the two races in *V. dahliae* or inoculating all isolates of the three species on artichoke and strawberry. This was not a straightforward exercise, and the pattern of virulence exhibited depended on the host. Except on strawberry, isolates of *V. isaacii* and *V. klebahnii* exhibited lower virulence, collectively, than the two races of *V. dahliae*. However, one *V. isaacii* isolate (Cello-A-Tri-P) caused more severe symptoms than *V. dahliae* race 1 isolate in specific crops, including lettuce. In strawberry, however, isolates of both *V. isaacii* and *V. klebahnii* exhibited virulence intermediate to the two races of *V. dahliae*.

V. isaacii isolates associated with disease symptoms were reisolated from symptomatic lettuce plants of cultivar Salinas and artichoke on NP-10 medium (Kabir et al. 2004). More interestingly, isolates of *V. isaacii* and *V. klebahnii* were also reisolated from inoculated plants that remained asymptomatic throughout the experiments, including strawberry. It appears that, although the *V. klebahnii* isolates tested were nonpathogenic, they still colonized the root tissue like other pathogenic species such as *V. dahliae* and *V. albo-atrum*. Reisolation of nonpathogenic *V. isaacii* and *V. klebahnii* species from asymptomatic plants indicates that these fungi have the potential to reproduce on nonhosts and augment soil populations similar to the pathogenic isolates of *V. dahliae*.

In conclusion, of the *V. klebahnii* and *V. isaacii* isolates characterized in this study, only one isolate of *V. isaacii* was pathogenic on susceptible lettuce cultivar Salinas and artichoke. Thus, these species may represent potential threats to agriculture over the long term if field soils become densely infested with virulent isolates. However, resistance developed against races 1 and 2 of *V. dahliae*, at least in lettuce, should restrict the establishment and multiplication of these species in production systems.

Acknowledgments

Funding for this study was provided by USDA-NIFA-SCRI grant number 2010-51181-21069 and the California Leafy Greens Research Board. We thank R. Marchebout for her excellent technical assistance and K. Maruthachalam for assistance with strawberry experiments.

Literature Cited

- Anonymous. 2011. 2011 Agricultural Statistics Annual. United States Department of Agriculture–National Agricultural Statistics Service. Online publication http://www.nass.usda.gov/Publications/Ag_Statistics/2011/index.asp
- Anonymous. 2012. Agricultural Commissioner's Office Monterey County Crop Report 2011. <http://www.co.monterey.ca.us/Home/ShowDocument?id=1481>
- Atallah, Z. K., Hayes, R. J., and Subbarao, K. V. 2011. Fifteen years of Verticillium wilt of lettuce in America's salad bowl: A tale of immigration, subjugation, and abatement. *Plant Dis.* 95:784-792.
- Bhat, R., and Subbarao, K. 1999. Host range specificity in *Verticillium dahliae*. *Phytopathology* 89:1218-1225.
- du Toit, L. J., Derie, M. L., and Hernandez-Perez, P. 2005. Verticillium wilt in spinach seed production. *Plant Dis.* 89:4-11.
- Gurung, S., Short, D., Atallah, Z. K., and Subbarao, K. 2014. Clonal expansion of *Verticillium dahliae* in lettuce. *Phytopathology* 104:641-649.
- Hayes, R. J., Maruthachalam, K., Vallad, G. E., Klosterman, S. J., and Subbarao, K. V. 2011a. Selection for resistance to Verticillium wilt caused by race 2 isolates of *Verticillium dahliae* in accessions of lettuce (*Lactuca sativa* L.). *HortScience* 46:201-206.
- Hayes, R. J., McHale, L. K., Vallad, G. E., Truco, M. J., Michelmore, R. W., Klosterman, S. J., Maruthachalam, K., and Subbarao, K. V. 2011b. The inheritance of resistance to Verticillium wilt caused by race 1 isolates of *Verticillium dahliae* in the lettuce cultivar La Brillante. *Theor. Appl. Genet.* 123:509-517.
- Hayes, R. J., Vallad, G. E., Qin, Q.-M., Grube, R. C., and Subbarao, K. V. 2007. Variation for resistance to Verticillium wilt in lettuce (*Lactuca sativa* L.). *Plant Dis.* 91:439-445.
- 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.
- Isaac, I. 1953. A further comparative study of pathogenic isolates of *Verticillium*: *V. nubilum* Pethybr. and *V. tricornis* sp. nov. *Trans. Br. Mycol. Soc.* 36:180-182.
- Isaac, I. 1967. Speciation in *Verticillium*. *Annu. Rev. Phytopathol.* 5:201-222.
- Jabnoun-Khiareddine, H., Daami-Remadi, M., Hibar, K., Ayed, F., and El Mahjoub, M. 2006. Pathogenicity of Tunisian isolates of three *Verticillium* species on tomato and eggplant. *Plant Pathol. J.* 5:199-207.
- 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.
- Koike, S. T., Subbarao, K. V., Davis, R. M., Gordon, T. R., and Hubbard, J. C. 1994. Verticillium wilt of cauliflower in California. *Plant Dis.* 78:1116-1121.
- Pegg, G. F., and Brady, B. L. 2002. *Verticillium* Wilts. CABI Publishing, New York.
- Powell, M., Gundersen, B., Miles, C., Coats, K., and Inglis, D. A. 2013. First report of Verticillium wilt on lettuce (*Lactuca sativa*) in Washington caused by *Verticillium tricornis*. *Plant Dis.* 97:996.
- Qin, Q.-M., Vallad, G. E., and Subbarao, K. V. 2008. Characterization of *Verticillium dahliae* and *V. tricornis* isolates from lettuce and artichoke. *Plant Dis.* 92:69-77.
- Shah, D., and Madden, L. 2004. Nonparametric analysis of ordinal data in designed factorial experiments. *Phytopathology* 94:33-43.
- Short, D. P. G., Gurung, S., Koike, S. T., Klosterman, S. J., and Subbarao, K. 2015. Frequency of *Verticillium* species in commercial spinach fields and transmission of *V. dahliae* from spinach to subsequent lettuce crops. *Phytopathology* 105:80-90.
- Short, D. P. G., Gurung, S., Maruthachalam, K., Atallah, Z. K., and Subbarao, K. 2014. *Verticillium dahliae* race 2-specific PCR reveals a high frequency of race 2 strains in commercial spinach seed lots and delineates race structure. *Phytopathology* 104:779-785.
- Subbarao, K. V., Hubbard, J. C., Greathead, A. S., and Spencer, G. A. 1997. Verticillium wilt. Pages 26-27 in: *Compendium of Lettuce Diseases*. R. M. Davis, K. V. Subbarao, R. N. Raid, and E. A. Kurtz, eds. American Phytopathological Society, St. Paul, MN.
- Taylor, J. 1968. Host range of *Verticillium tricornis* (Isaac). *N. Z. J. Agric. Res.* 11:521-523.
- Vallad, G. E., Qin, Q.-M., Grube, R., Hayes, R. J., and Subbarao, K. V. 2006. Characterization of race-specific interactions among isolates of *Verticillium dahliae* pathogenic on lettuce. *Phytopathology* 96:1380-1387.