Title
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Author
Subbarao, Krishna V

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Characterization of *Verticillium dahliae* and *V. tricorpus* Isolates from Lettuce and Artichoke

Qing-Ming Qin, Department of Plant Pathology, University of California, Davis, c/o United States Agricultural Research Station, Salinas, CA, 93905; **Gary E. Vallad,** Department of Plant Pathology, University of Florida, Gulf Coast Research and Education Center, Wimauma 33598, and **Krishna V. Subbarao,** Department of Plant Pathology, University of California, Davis, c/o United States Agricultural Research Station, Salinas

ABSTRACT

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Verticillium isolates collected from lettuce and artichoke were characterized for morphology, growth and pathogenicity. Several isolates were identified as *Verticillium tricorpus* based on morphological and cultural characteristics, including the production of dark resting mycelia, chlamydospores, microsclerotia, and yellow to orange pigmentation in culture. Compared with isolates of *V. dahliae*, these isolates also produced microsclerotia and conidia that were significantly larger and exhibited a distinct growth pattern at varying temperatures. Using database sequence information, primers were developed from the internal transcribed spacer region to produce a diagnostic 337-bp product specific to *V. tricorpus* and used to confirm the identification of isolates. Pathogenicity tests indicated that isolates of *V. tricorpus* were weak pathogens, causing a median disease severity (DS) of <1 (0-to-5 scale) on lettuce and artichoke. In contrast, isolates of *V. dahliae* consistently caused severe wilt with a median DS of >3.5 on lettuce and 5.0 on artichoke. Although lettuce and artichoke inoculated with isolates of *V. tricorpus* exhibited reduced height and fresh foliar and root weight, the reductions were not statistically significant, unlike in plants inoculated with isolates of *V. dahliae*. Lettuce co-inoculated with isolates of *V. tricorpus* and *V. dahliae* exhibited reduced symptoms of Verticillium wilt and improved growth relative to those inoculated with *V. dahliae* alone. The early introduction of *V. tricorpus* in soil-drench inoculations appeared to provide better relief from subsequent *V. dahliae* inoculation than when the two species were co-inoculated simultaneously using the root-dip method, suggesting competitive exclusion as a plausible mechanism. A spore-polymerase chain reaction assay developed using cultured spores directly as template and primers specific to *V. tricorpus* confirmed the presence of *V. tricorpus* on inoculated roots. This work demonstrates the potential use of *V. tricorpus* to directly reduce the effect of *V. dahliae* on lettuce and artichoke and, to our knowledge, is the first reported characterization of *V. tricorpus* isolates collected from lettuce and artichoke.

Additional keywords: co-inoculation, colony morphology, cross-protection

The genus *Verticillium* consists of six phytopathogenic species: *Verticillium dahliae* Kleb., *V. albo-atrum* Reinke & Berthold, *V. nigrescens* Pethybr., *V. nubilum* Pethybr., *V. tricorpus* I. Isaac, and *V. theobromae* (Turconi) E.W. Mason and S. Hughes after the recent revision (2,23). Of the six species, *V. dahliae* and *V. albo-atrum* are well-known plant pathogens causing vascular wilt in a broad range of economically important plants, whereas the other species are considered weak pathogens or saprotrophs (2,8,23). The known host range of *Verticillium* spp. has expanded, with more than 60 additional hosts identified as susceptible to *Verticillium* spp. worldwide over the past decade (23,28).

V. tricorpus was first reported by Isaac in 1953 (11) and later was isolated from other hosts such as snapdragon, potato, *Antirrhinum* spp., mint, cantaloupe, cotton, and various weed species (10,12,14,18, 20,29). In culture, isolates of *V. tricorpus* produce erect and prostrate verticillate mycelia; three types of resting structures: microsclerotia, dark resting mycelia; and chlamydospores; and a yellow to brown pigmentation in mycelia and in the surrounding medium (13,27). In contrast, isolates of *V. dahliae* produce only conidia and microsclerotia. On modified soil extract agar (MSEA), *V. dahliae* produces globose to elongate microsclerotia that lack dark hyphae or dark mycelia and are distributed fairly uniformly around the colony, whereas *V. tricorpus* always produces dark hyphae or dark mycelia and globose to irregular microsclerotia that are much larger in size and are scattered around the colony (9). These differences easily distinguish *V. tricorpus* from other closely related species. However, morphological characteristics are affected by many factors, and loss of resting structures in vitro is common and can be rapid, so that specific isolates may not be easily identifiable (2). Therefore, additional diagnostic methods have been developed to distinguish *V. tricorpus* and *V. dahliae* from other *Verticillium* spp. (19,22).

Lettuce and artichoke are two important vegetable crops grown year-round in California, with a combined value of over $1.5 billion in Monterey County alone. The susceptibility of lettuce, once considered a nonhost of *Verticillium* species, to *V. dahliae* was first recognized in 1995 and since has become a serious threat to the lettuce industry in coastal California (28). Dufrenoy first reported *V. dahliae* on artichoke in France in 1927 (23) whereas, in the United States, infection of artichoke was not reported until 1999 (3). In addition to *V. dahliae*, *Verticillium* isolates morphologically similar to those of *V. tricorpus* also were isolated frequently from diseased lettuce and artichoke in field surveys in recent years. *V. tricorpus* has been reported to be pathogenic to tomato and antirrhinum (10–13,31), as the causal agent of dry rot of stored potato (30), and as a mild pathogen of potato (7). New pathotypes of *V. tricorpus* attacking tomato, eggplant, and potato were found in Tunisia recently (15). Although *Verticillium* spp. are widely distributed in the agricultural soils of California and affect diverse economically important crops (3,4,17), lettuce and artichoke had not been described previously as hosts of *V. tricorpus*.

The objectives of this study were to better characterize the *V. tricorpus*-like isolates recovered from diseased lettuce and artichoke for morphological and cultural characteristics, and for pathogenicity toward lettuce and artichoke individually and in co-inoculation with *V. dahliae*. A final objective was to diagnose these *V. tricorpus*-like isolates using previously developed primers specific to *V. tricorpus* by polymerase chain reaction (PCR), and to

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

Q.-M. Qin and G. E. Vallard contributed equally to this work.

Current address of Q.-M. Qin: Department of Plant Pathology and Microbiology, Texas A&M University.

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develop a rapid method to identify *V. tricorpus* on plant roots.

MATERIALS AND METHODS

Isolates of *Verticillium* spp. In all, 19 *Verticillium* isolates were prepared for these studies (Table 1). The eight isolates of *V. dahliae* and nine isolates of *V. tricorpus* were recovered from Verticillium-wilt-symptomatic lettuce and artichoke grown in coastal California, while the isolate of *V. albo-atrum* and the long-spored isolate of *V. dahliae* var. *longisporum* (also referred to as *V. longisporum*) were recovered from alfalfa and horseradish, respectively. All the isolates were single spored and maintained on potato dextrose agar (PDA) or as a spore suspension as previously described (24).

Morphology and colony growth of isolates. All cultures were grown for 4 weeks at 25°C prior to morphological characterization. The lengths and widths of 40 randomly chosen conidia, microsclerotia, and chlamydospores of the eight isolates of *V. dahliae* and nine isolates of *V. tricorpus* were measured under a microscope. During incubation, potential changes in colony morphology were recorded twice a week.

Two experiments were conducted to compare the response of *Verticillium* isolates to temperature. In the first experiment, the growth response of the 17 isolates from lettuce and artichoke was tested at 5, 10, 15, 20, 25, 30, and 35°C in the dark. Test cultures were initiated with a 4.5-mm-diameter mycelial plug taken from the leading edge of a colony previously grown for 2 weeks in the dark at 25°C. The diameters of colonies were measured after 2 weeks of incubation. For the second experiment, a smaller, more diverse collection of six isolates was tested (Ms.103, Ar.136, Ls.16, Ls.17, Ls.183, and Cs.225) for their growth response at 15, 20, 25, and 30°C. The diameters of colonies were then measured every 2 days. In both experiments, four plates of each isolate were incubated at each temperature and repeated once ($n = 2$).

Isolate identification by PCR. Two *V. tricorpus*-specific primers from the internal transcribed spacer (ITS) region, VtITS1: 5′-CGCCGGTACATCAGTCTC-3′ and VtITS2: 5′-ACTCCGATGCGA GCGAA-3′, developed previously (22) were used to further differentiate isolates of *V. tricorpus* from other phytopathogenic *Verticillium* spp. The primers were supplied by Sigma-Genosys (The Woodlands, TX). Amplified genomic DNA was extracted from frozen mycelia following the method described by Al-Samarrai and Schmid (1). PCR amplifications were performed with a PTC-200 thermocyler (MJ Research, Waltham, MA) in a total of 25 µl of mixture as previously described (24). Samples were overlaid with a drop of mineral oil and PCR reactions were run under an initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 50 s, and extension at 72°C for 1 min; followed by a final extension at 72°C for 5 min, and stored at 4°C until used. The PCR products were analyzed on a 1.0% agarose gel in 0.5×Tris-acetate-EDTA buffer (25).

Pathogenicity of isolates and spore-PCR. To evaluate the pathogenicity of different isolates either singly or in combination, lettuce and artichoke seed were sown into 200-well seedling trays filled with an autoclaved sand:potting soil mix (3:1, vol/vol). Seedlings were maintained on benches in a greenhouse (for experiments using root dip inoculation) as previously described (24) or in a growth chamber (for experiments using soil-drench inoculation) at 25°C and a 12-h photoperiod. For experiments using the root-dip inoculation method, 10 4-week-old seedlings with intact roots were inoculated with a conidial suspension of approximately 1.0×10^7 conidia/ml. Ten seedlings of both lettuce and artichoke were dipped in each conidial suspension treatment (50 ml) for at least 30 min. Seedlings then were transplanted individually into 0.5-liter foam-insulated cups containing a pasteurized sand:potting soil mix (3:1, vol/vol). Seedlings dipped in sterile water were treated as controls.

For soil-drench inoculation, 4-week-old seedlings were inoculated by adding 1.5 ml of conidial suspension (2.0×10^6 conidia/ml) to each seedling tray well. For mixed inoculation treatments, *V. tricorpus* isolates were applied a week prior to the addition of *V. dahliae* isolates; each isolate was applied in equal measure. A separate set of seedlings were drenched with sterile water for controls. Fifteen seedlings were inoculated with each isolate or combination of isolates. Seedlings were similarly transplanted into 0.5-liter foam-insulated cups containing a pasteurized sand:potting soil mix (3:1 vol/vol). In each experiment, plants were arranged in a randomized block design on greenhouse benches. Each treatment had three blocks, and each block contained three to five plants for each treatment. The lettuce and artichoke experiments using the root dip inoculation method were repeated once ($n = 2$) and the experiment using the soil-drench inoculation method was repeated three times ($n = 4$).

All plants were gently uprooted 7 to 10 weeks after transplanting, washed free of soil, and rated for disease severity by longitudinally cutting from the crown through the main taproot of each plant as previously described (24). Plant height and width were measured prior to uprooting. The fresh foliar and root weight were determined using a portable electric scale. For reisolation, plant roots were washed with tap water for 1 to 2 min and dried on sterile paper towel, and 1- to 2-cm sections of excised root tissue (six sections per petri dish) were placed on a modified NP-10 medium (16) and incubated in the dark at 18 to 25°C for 10 days. After 10 days of incubation, morphological characteristics such as the presence of microsclerotia, pigmentation, and melanized hyphae were examined under both stereo and compound microscopes (BX60 and SZX; Olympus, Japan), and the spores also were identified directly via the spore-PCR method using primers specific to *V. tricorpus.*

The spore-PCR assay was performed directly on conidia collected from colonies growing around root-tissue-sections previ-

Table 1. Isolates of *Verticillium* used in the study along with their host and geographic origin

Species, isolate	Originating host	Geographical location	Year isolated
Verticillium dahliae			
Cs.80	Artichoke (*Cynara scolymus* L.)	California	1991
C.312	Artichoke (*C. scolymus* L.)	California	1999
Cs.413	Artichoke (*C. scolymus* L.)	California	2000
Cs.423	Artichoke (*C. scolymus* L.)	California	2001
Ls.1	Lettuce (*Lactuca sativa* L.)	California	1995
Ls.14	Lettuce (*L. sativa* L.)	California	1996
Ls.16	Lettuce (*L. sativa* L.)	California	1996
Ls.17	Lettuce (*L. sativa* L.)	California	1996
V. tricorpus			
Cs.225	Artichoke (*C. scolymus* L.)	California	1999
Cs.234	Artichoke (*C. scolymus* L.)	California	1999
Cs.236	Artichoke (*C. scolymus* L.)	California	1999
Cs.456	Artichoke (*C. scolymus* L.)	California	1999
Ls.183	Lettuce (*L. sativa* L.)	California	1997
Ls.432	Lettuce (*L. sativa* L.)	California	2001
Ls.441	Lettuce (*L. sativa* L.)	California	2001
Ls.442	Lettuce (*L. sativa* L.)	California	2001
Ls.443	Lettuce (*L. sativa* L.)	California	2001
V. dahliae var. *longisporum*			
Ar.136[a]	Horseradish (*Armoracia rusticana*)	Illinois	1989
V. albo-atrum			
Ms.103[b]	Alfalfa (*Medicago sativus* L.)	Pennsylvania	1986

[a] Provided by D. Eastburn.
[b] Provided by B. W. Pennypacker.

ously placed on modified NP-10 medium. Conidia were collected using a rubber policeman to gently dislodge conidia around the plated root tissue in 1 ml of sterile water. The suspension was transferred to a 1.5-ml microcentrifuge tube with a pipette and brought to a 1.5-ml total volume with sterile water. The suspension was vortexed repeatedly and centrifuged at 2,300 × *g* for 1 min, discarding the supernatant in between until the final spore concentration achieved was 1.0×10^8 conidia/ml. The samples were stored at –20°C until use in PCR assays. Spore-PCR amplification was conducted in a total of 25 µl of reaction mixture containing approximately 1.5 to 2.0 µl of spore suspension (1.5 to 2.0×10^5 conidia) as template, 2.5 µl of 10× PCR buffer, 2.5 µl of 25 mM Mg^{2+}, 0.2 mM each dNTP, 0.2 µM each primer, and 1.25 units of *Taq* polymerase (Promega Corp., Madison, WI). Samples were overlaid with a drop of mineral oil and subjected to the PCR conditions described above. PCR products were analyzed on a 1.0% agarose gel.

Data analysis. Mean lengths and widths of conidia and microsclerotia of different *V. dahliae* and *V. tricorpus* isolates were computed and significant differences were determined using paired *t* tests. Initial analyses of disease severity and biomass data were consistent across repeated experiments; therefore, data were pooled and analyzed according to crop. For disease severity, the combined dataset was analyzed using a nonparametric procedure for the analysis of ordinal data in one-way (artichoke) and two-way (lettuce) factorial experiments (5,26). The overall effect of *V. dahliae* isolates, *V. tricorpus* isolates, and mixtures of the two on the severity of disease was analyzed by the analysis of variance type statistic of ranked data using the PROC Mixed procedure in SAS (version 9.1; SAS Institute Inc., Cary, NC) to generate relative marginal effects (RME), and the LD_CI macro to generate 95% confidence intervals (5,26). Replications of experiment and blocks within experiments were treated as random effects in the analysis. Linear contrasts were performed to test specific interactions within models. Data for plant height and biomass (fresh foliar and root weight) from pathogenicity experiments were similarly analyzed for a two-way analysis of variance using the PROC Mixed procedure of SAS (version 9.1; SAS Institute Inc.) with differences between least significant means tested using Tukey-Kramer pairwise comparisons.

RESULTS

Morphological characterization. During the 4-week incubation, colonies of eight isolates of *V. dahliae* (Ls.1, Ls.14, Ls.16, Ls.17, Cs.80, Cs.312, Cs.413, and Cs.423) changed from white to black as they melanized, while colonies of isolates Ls.183, Ls.442, Cs.225, Cs.234, Cs.236, and Cs.456 produced a yellow-orange pigmentation in the first 2 to 3 weeks, before turning black. However, the yellow-orange pigmentation was still apparent around the edge of the growing colony after 4 weeks of incubation. Colonies of isolates Ls.432, Ls.441, and Ls.443 changed from white to black only (Table 2).

The conidial size (length by width) of the eight isolates of *V. dahliae* ranged from 3.2 to 9.0 by 1.4 to 3.2 µm, with an average size of 5.0 by 2.2 µm. The conidial sizes of the other *Verticillium* isolates ranged from 3.5 to 11.0 by 1.8 to 3.8 µm, with an average of 6.2 by 2.6 µm (Table 2). The differences in conidial lengths and widths between these two groups of isolates were significant (Table 3). The size of microsclerotia of the eight isolates of *V. dahliae* ranged from 16.2 to 150.0 by 12.5 to 105.0 µm, with a mean size of 59.7 by 37.2 µm. Microsclerotia of the other *Verticillium* isolates ranged from 15.0 to 230.0 by 10.0 to 150.0 µm, with a mean size of 90.9 by 57.7 µm (Table 2). Again, the differences in mean microsclerotial lengths and widths between these two groups of isolates were significant (Table 3). The chlamydospores of Ls.183, Ls.432, Ls.441, Ls.442, Ls.443, Cs.225, Cs.234, Cs.236, and Cs.456 ranged from 4.5 to 13.0 by 3.5 to 9.0 µm, with a mean of 7.9 by 6.1 µm. Chlamydospores were never observed among the eight isolates of *V. dahliae* (Table 2 and *data not shown*). In addition, Ls.183, Ls.432, Ls.441, Ls.442, Ls.443,

Table 3. Comparison of the differences in lengths and widths of conidia and microsclerotia between isolates of *Verticillium dahliae* and *V. tricorpus*[a]

Comparison[b]	SD	*t* Statistic	df	Significance
Mean conidial length	0.70	0.00003	16	$P < 0.001$
Mean conidial width	0.24	0.00001	16	$P < 0.001$
Mean microsclerotial length	23.46	0.00373	16	$P < 0.01$
Mean microsclerotial width	17.02	0.01019	16	$P < 0.05$

[a] SD = standard deviation and df = degrees of freedom.
[b] *V. dahliae* versus *V. tricorpus* isolates.

Table 2. Comparison of morphological characteristics of *Verticillium dahliae* and *V. tricorpus* isolates from lettuce and artichoke[a]

	Spore		Microsclerotia		Chlamydospore		
Isolates	Length (µm)	Width (µm)	Length (µm)	Width (µm)	Length (µm)	Width (µm)	Color change[b]
V. dahliae							
Ls.1	5.3 (1.1)	2.2 (0.3)	62.1 (27.0)	35.0 (12.3)	Absent	Absent	White to black
Ls.14	5.4 (1.1)	2.2 (0.3)	57.5 (18.4)	40.5 (8.8)	Absent	Absent	White to black
Ls.16	5.5 (0.9)	2.3 (0.4)	57.6 (20.6)	41.1 (14.3)	Absent	Absent	White to black
Ls.17	5.0 (0.9)	2.4 (0.4)	53.8 (24.5)	29.8 (10.8)	Absent	Absent	White to black
Cs.80	4.3 (0.7)	2.2 (0.4)	64.9 (27.3)	40.6 (16.5)	Absent	Absent	White to black
Cs.312	4.5 (0.7)	2.1 (0.4)	57.3 (18.5)	37.0 (11.3)	Absent	Absent	White to black
Cs.413	5.0 (1.2)	2.2 (0.5)	73.7 (31.3)	41.2 (18.1)	Absent	Absent	White to black
Cs.423	5.0 (0.6)	2.1 (0.4)	52.4 (22.4)	32.1 (8.1)	Absent	Absent	White to black
Mean	5.0	2.2	59.7	37.2	…	…	…
V. tricorpus							
Ls.183	6.5 (1.8)	2.7 (0.3)	93.9 (36.7)	58.2 (23.3)	7.5 (1.5)	6.0 (1.1)	Yellow to black
Ls.432	6.3 (1.1)	2.6 (0.4)	58.6 (35.4)	37.8 (21.2)	8.2 (1.4)	6.5 (0.8)	White to black
Ls.441	6.2 (1.1)	2.8 (0.4)	85.8 (46.5)	51.9 (19.1)	8.3 (1.7)	6.2 (1.0)	White to black
Ls.442	6.3 (1.4)	2.6 (0.4)	103.6 (47.3)	71.2 (31.9)	8.5 (1.7)	6.0 (1.0)	Yellow to black
Ls.443	6.2 (1.3)	2.7 (0.4)	82.6 (36.9)	49.4 (17.3)	7.2 (0.9)	5.7 (0.8)	White to black
Cs.225	5.7 (1.1)	2.3 (0.3)	102. 4 (46.5)	71.0 (28.1)	8.1 (1.2)	6.2 (0.9)	Yellow to black
Cs.234-2	6.3 (1.0)	2.7 (0.5)	61.2 (29.0)	31.1 (10.4)	7.7 (1.5)	6.2 (1.0)	Yellow to black
Cs.236	5.8 (1.4)	2.5 (0.4)	94.2 (37.0)	56.8 (17.1)	8.1 (1.1)	6.1 (1.0)	Yellow to black
Cs.456	6.4 (1.4)	2.6 (0.4)	136.1 (43.5)	91.6 (20.1)	7.6 (1.9)	6.0 (1.3)	Yellow to black
Mean	6.2	2.6	90.9	57.7	7.9	6.1	…

[a] Average size of 40 conidia, microsclerotia, chlamydospores and standard error of different measurements in parentheses.
[b] Change of colony color.

Cs.225, Cs.234, Cs.236, and Cs.456 produced dark resting mycelia which were absent in the isolates of *V. dahliae* (*data not shown*). The above morphological characteristics for isolates Ls.183, Ls.432, Ls.441, Ls.442, Ls.443, Cs.225, Cs.234, Cs.236, and Cs.456 were distinct from those described for *V. dahliae* or *V. albo-atrum*, but typical of *V. tricorpus* (11,13) and hereafter were referred to as such.

Response of isolates to temperature. All isolates from lettuce and artichoke grew at temperatures between 5 and 30°C (Fig. 1). The maximum growth of the eight isolates of *V. dahliae* occurred at 25°C,

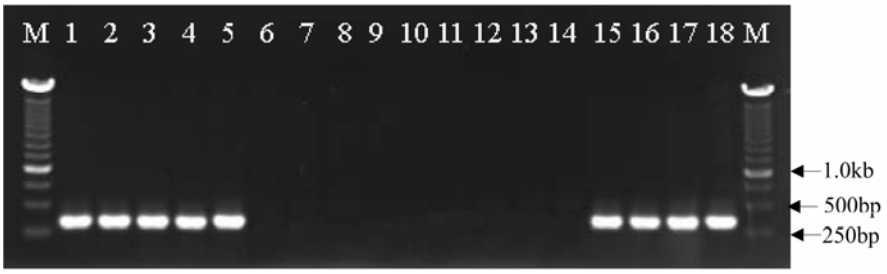

Fig. 2. Polymerase chain reaction identification of *Verticillium tricorpus* isolates with *V. tricorpus*-specific primers. Lanes 1–5 are PCR products, from *V. tricorpus* isolates Ls.183, Ls.432, Ls.441, and Ls.442 and Ls. 443 from lettuce; lanes 6–9 are from lettuce *V. dahliae* isolates Ls.1, Ls.14, Ls.16, and Ls.17; lane 10 is from control (sterile water); lanes 11–14 are from *V. dahliae* isolates Cs.80, Cs.312, CS.413, and Cs.423 from artichoke; and lanes 15–18 are from *V. tricorpus* isolates Cs.225, Cs.234, Cs.236, and Cs.456 from artichoke. Each lane was loaded with 6 µl of PCR product. M indicates 250-bp DNA ladder (Invitrogen).

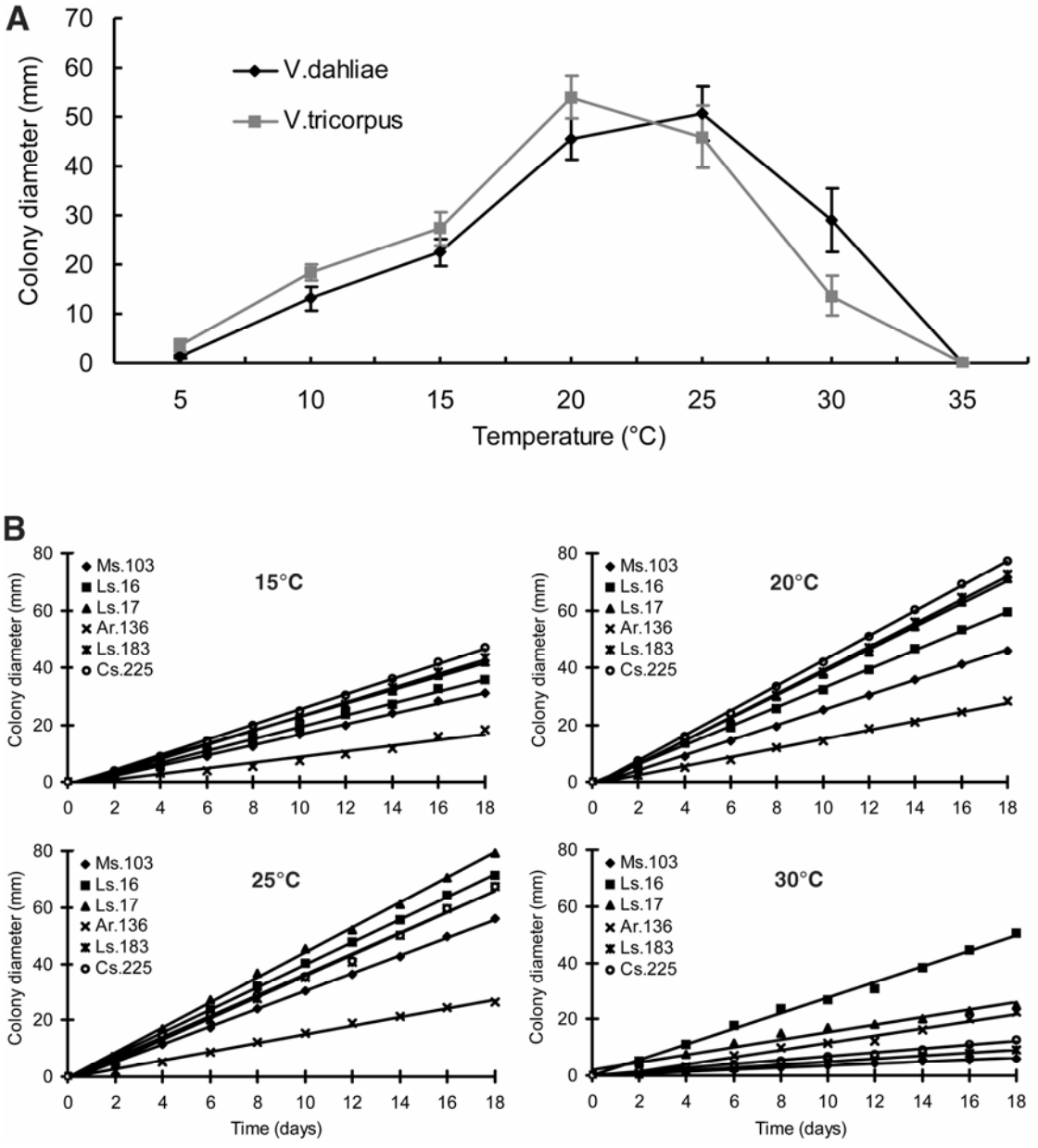

Fig. 1. Effect of temperature on the rate of growth of *Verticillium tricorpus* relative to other *Verticillium* spp. under controlled conditions. **A,** Comparison of the average rate of growth of nine isolates of *V. dahliae* (Ls.1, Ls.7, Ls.14, Ls.16, Ls.17, Cs.80, Cs.312, Cs.413, and Cs.423) versus the average of nine isolates of *V. tricorpus* (Ls.183, Ls.432, Ls.441, Ls.442, Ls.443, Cs.225, Cs.234, Cs.236, and Cs.456) following 2 weeks of incubation at various temperatures. **B,** Average growth of *V. tricorpus* isolates Ls.183 and Cs.225, *V. dahliae* isolates Ls.16 and Ls.17, *V. dahliae* var. *longisporum* isolate Ar.136, and *V. albo-atrum* isolate Ms.103 up to 18 days at 15, 20, 25, and 30°C. Growth was measured by the increased diameter (mm) of colonies on potato dextrose agar medium over time. Data represent the means of growth (± the standard deviation in B) from two independent experiments. Isolate prefixes refers to the crop of origin: "Ar" for horseradish, "Cs." for artichoke, "Ls" for lettuce, and "Ms." for alfalfa.

whereas that of the nine isolates of *V. tricorpus* occurred at 20°C (Fig. 1). The rate of growth for most *Verticillium* isolates was inhibited at 30°C, especially the nine isolates of *V. tricorpus* (Ls.183, Ls.432, Ls.441, Ls.442, Ls.443, Cs.225, Cs.234, Cs.236, and Cs.456) and the single *V. albo-atrum* isolate, Ms.103 (Fig. 1). Of all the isolates tested, isolate Ls.16 (*V. dahliae*) was the most tolerant to the elevated temperature of 30°C (Fig. 1). The growth of all isolates was inhibited at 35°C (Fig. 1). After 15 days of incubation at 35°C, colonies with older mycelia or resting structures still could be revived when transferred to 20°C, whereas younger colonies lacking older mycelia or resting structures could not be revived. None of the isolates survived after 90 days incubation at 35°C (*data not shown*).

PCR identification. Using the *V. tricorpus*-specific primers VtITS1 and VtITS2, a diagnostic 337-bp fragment was amplified from the *V. tricorpus* isolates Ls.183, Ls.432, Ls.441, Ls.442, and Ls.443 from lettuce and isolates Cs.225, Cs.234, Cs.236, and Cs.456 from artichoke (Fig. 2). No product was amplified from any isolate of *V. dahliae*, *V. dahliae* var. *longisporum*, or *V. albo-atrum* (Fig. 2 and *data not shown*). These results corroborated the previous morphological characterization and confirmed that isolates Ls.183, Ls.432, Ls.441, Ls.442, Ls.443, Cs.225, Cs.234, Cs.236, and Cs.456 were indeed *V. tricorpus*.

Pathogenicity on lettuce. Regardless of the inoculation method, the overall effect of *V. dahliae* isolate on disease severity was highly significant (P <0.0001 for both inoculation methods; Table 4). Median disease severity scores ranged from 3.8 to 4.6 for lettuce inoculated with either *V. dahliae* isolate Ls.16 or Ls.17 alone (Table 5). Although no difference in disease severity between plants inoculated with either Ls.16 or Ls.17 alone was apparent (P = 0.4962) via the root-dip method, a significant difference (P < 0.0001) was observed with the soil-drench method, with

Table 5. Median (Med), mean ranking (R), and relative marginal effect (RME) calculated for the severity of Verticillium wilt on lettuce inoculated with isolates of *Verticillium dahliae* and *V. tricorpus*[a]

V. dahliae[b]	*V. tricorpus*[b]	Med	R[c]	RME[d]
Root-dip inoculation				
…	…	0	15.5	0.14 (0.10–0.20)
…	Ls.183	0.5	29.2	0.27 (0.20–0.35)
…	Ls.432	0	17.8	0.16 (0.10–0.27)
…	Ls.441	0.7	30.1	0.27 (0.17–0.43)
…	Ls.442	0.6	29.5	0.27 (0.18–0.38)
…	Ls.443	0.5	26.3	0.24 (0.17–0.33)
Ls.16	…	4	87.7	0.81 (0.69–0.88)
Ls.16	Ls.183	3.5	79.5	0.73 (0.60–0.83)
Ls.16	Ls.432	4.5	84	0.77 (0.55–0.90)
Ls.16	Ls.441	3.5	77.2	0.71 (0.59–0.81)
Ls.16	Ls.442	4.6	88.4	0.81 (0.66–0.90)
Ls.16	Ls.443	3.5	64.8	0.60 (0.38–0.78)
Ls.17	…	3.8	80.8	0.74 (0.57–0.86)
Ls.17	Ls.183	3.6	68.2	0.63 (0.39–0.81)
Ls.17	Ls.432	1.9	53.6	0.49 (0.30–0.68)
Ls.17	Ls.441	2	54	0.50 (0.38–0.61)
Ls.17	Ls.442	2.3	58.5	0.54 (0.34–0.72)
Ls.17	Ls.443	1	36.1	0.33 (0.18–0.53)
Soil-drench inoculation				
…	…	0	7	0.06 (0.06–0.09)
…	Ls.432	0.6	24.4	0.22 (0.19–0.26)
…	Ls.443	0.8	27.7	0.25 (0.21–0.31)
Ls.16	…	4.6	98.1	0.90 (0.86–0.93)
Ls.16	Ls.432	3.1	74	0.68 (0.57–0.77)
Ls.16	Ls.443	2.5	64.1	0.59 (0.52–0.66)
Ls.17	…	3.8	83.8	0.77 (0.71–0.82)
Ls.17	Ls.432	1.8	54.8	0.50 (0.41–0.59)
Ls.17	Ls.443	2	56.6	0.52 (0.45–0.59)

[a] Results from two and four independent experiments using the root-dip and soil-drench inoculation method, respectively. For experiments using the root-dip inoculation method, the intact roots of 4-week-old seedlings were dipped in a suspension of approximately 1.0×10^7 conidia/ml from individual isolates or mixed suspension of each *V. dahliae* and *V. tricorpus* isolate was mixed in equal measure. For soil-drench inoculation, a 1.5-ml suspension of approximately 2.0×10^6 conidia/ml was applied to the soil of individual 4-week-old seedlings grown in seedling trays. For mixed inoculation treatments, *V. tricorpus* isolates were applied a week prior to the addition of *V. dahliae* isolates in equal measure. Sterile water was used in both methods as a substitute for one or both isolates. Seedlings were transplanted individually into 0.5-liter foam-insulated cups containing a pasteurized soil mix.

[b] Seedlings were treated with a single isolate of either *V. dahliae* or *V. tricorpus*, or an isolate of each that originally was collected from field-grown lettuce (*Lactuca sativa* [Ls]); … designates control, those plants treated with sterile water as a substitute for one or both isolates.

[c] Mean rankings of Verticillium wilt severity for each isolate treatment. Disease severity was rated using an ordinal scale of 0 to 5, in which 0 = no vascular discoloration in the taproot, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76 to 100% of vascular tissues in the taproot exhibited discoloration in the absence of foliar symptoms, and 5 = 100% of vascular tissues exhibited discoloration in the taproot and the presence of foliar symptoms typical of Verticillium wilt.

[d] RME = $[(R - 0.5)/N]$; N = total experimental units in the analysis (N = 120 and 108 in root-dip and soil-drench inoculation methods, respectively). The 95% confidence intervals are in parentheses.

Table 4. Statistical analyses of variance (ANOVA) based on the effect of individual isolates of *Verticillium dahliae* and *V. tricorpus* or mixtures of isolates on the ranked means of Verticillium wilt severity and the means of lettuce growth measurements[a]

	ANOVA-type statistic (ATS), disease severity effect[b]								ANOVA F statistic (F), lettuce growth effect[c]							
	Root-dip (n = 2)				Soil-drench (n = 4)				Root weight (n = 4)				Foliar weight (n = 4)			
Isolate	df_{Num}	df_{Dem}	ATS	P value	df_{Num}	df_{Dem}	ATS	P value	df_{Num}	df_{Dem}	F	P value	df_{Num}	df_{Dem}	F	P value
V. dahliae (D)	1.77	51	68.63	<0.0001	1.82	49.3	188	<0.0001	2	88	10.64	<0.0001	2	88	138.7	<0.0001
V. tricorpus (T)	4.68	51	2.09	0.0853	1.75	49.3	10.68	0.0003	2	88	0.61	0.5457	2	88	0.54	0.583
D × T	7.87	∞	1.47	0.1629	3.07	∞	17.36	<0.0001	4	88	1.31	0.2723	4	88	3.86	0.0062

[a] Abbreviations: n = the number of independent experiments in each analysis, df_{Num} = numerator degrees of freedom, and df_{Dem} = denominator degrees of freedom.

[b] In the root-dip inoculation method, the roots of 4-week-old seedlings were dipped in a suspension of approximately 1.0×10^7 conidia/ml for 30 min. Mixed inoculation treatments used a suspension of 1.0×10^7 conidia/ml of each *V. dahliae* and *V. tricorpus* isolate in equal measure. In the soil-drench inoculation method, 4-week-old seedlings were inoculated by adding a 1.5-ml suspension of approximately 2.0×10^6 conidia/ml to each seedling tray well. For mixed inoculation treatments, an isolate of *V. tricorpus* was applied 2 week prior to the addition of the isolate of *V. dahliae*; each isolate was applied in equal measure. For both inoculation methods, seedlings were transplanted into 0.5-liter foam-insulated cups containing a pasteurized sand:potting soil mix (3:1, vol/vol), and isolates were substituted with sterile water in controls.

[c] The effect of *V. dahliae* and *V. tricorpus* isolates on lettuce growth, in terms of fresh foliar and fresh root weight, was analyzed from experiments using the soil-drench inoculation method.

Ls.17 exhibiting a 17% reduction in median disease severity (Table 5). The effect of *V. dahliae* isolate on fresh root and foliar weight also was highly significant ($P < 0.0001$ for both) for the soil-drench-inoculated lettuce plants, with plants exhibiting an average reduction of 54.7 and 21.3% for fresh foliar and root weight, respectively, relative to the noninoculated controls (Fig. 3).

The overall effect of *V. tricorpus* isolate on disease severity was highly significant ($P = 0.0003$) for the soil-drench inoculation method but less significant ($P = 0.0853$) with the root-dip inoculation method (Table 4). Regardless, the severity of disease symptoms was marginal, with median values ranging from 0.0 to 0.8 (Table 5), reflecting the ability of *V. tricorpus* isolates to cause minor vascular discoloration in lettuce. Only 2 of the 400 lettuce plants inoculated with *V. tricorpus* alone ever exhibited foliar symptoms typical of Verticillium wilt during the course of these studies. No significant effect of *V. tricorpus* isolate on lettuce fresh foliar and root weight was observed ($P = 0.5457$ and 0.5830, respectively; Table 4) in soil-drench-inoculated plants.

In general, lettuce co-inoculated with *V. dahliae* and *V. tricorpus* exhibited reduced disease severity relative to lettuce inoculated with *V. dahliae* alone. A significant effect of *V. dahliae* × *V. tricorpus* on disease severity was observed when lettuce was inoculated by the soil-drench method ($P < 0.0001$) but not by the root-dip method ($P = 0.1629$; Table 4), reflecting the significance of the inoculation method. For example, using the root-dip method, co-inoculation of *V. tricorpus* isolates Ls.432 ($P = 0.063$), Ls.441 ($P = 0.014$), and Ls.443 ($P = 0.001$) with *V. dahliae* isolate Ls.17 resulted in a significant 57% reduction in median disease severity relative to lettuce inoculated with Ls.17 alone (Table 5). However, only isolate Ls.443 effectively reduced disease severity when co-inoculated with *V. dahliae* Ls.16 by the root-dip method (Table 5), albeit with marginal significance ($P = 0.083$). For experiments using the soil-drench method, there were no statistical differences between *V. tricorpus* isolates Ls.432 and Ls.443 ($P = 0.662$) in their effective capacity to reduce disease severity ($P < 0.0001$) by nearly 50% when co-inoculated with either *V. dahliae* isolate Ls.16 or Ls.17 (Table 5). Although both isolates Ls.432 and Ls.443 reduced disease severity, neither improved fresh foliar or root weight significantly in co-inoculated treatments (Fig. 3).

Pathogenicity on artichoke. Across two independent experiments, the effect of *Verticillium* isolate on disease severity was significant ($P < 0.0001$; Table 6). Artichoke inoculated with *V. dahliae* isolates Cs.80, Cs.312, Cs.413, and Cs.423 exhibited significant stunting, reflected in fresh foliar and root weight (Fig. 4), and severe symptoms of Verticillium wilt (Table 7) relative to noninoculated controls. In contrast, *V. tricorpus* isolates Cs.225, Cs.234, Cs.236, and Cs.456 caused slight but significant ($P = 0.002$) reductions in fresh foliar and root weight (Fig. 4) and Verticillium wilt on artichoke. Mean rankings of disease severity caused by isolates of *V. dahliae* were significantly higher ($P < 0.0001$) than those caused by *V. tricorpus*, with a median disease severity for *V. dahliae* of 5.0, compared with 0.2 for *V. tricorpus*. Although some vascular discoloration was observed in the roots of some artichoke plants inoculated with isolates of *V. tricorpus*, none developed foliar symptoms.

Confirmation of the infection by *V. tricorpus* isolates. After incubation of lettuce and artichoke tissue from inoculated plants on modified NP-10 medium for 10 days in the dark at room temperature, colonies typical of *V. tricorpus* (single-celled conidia on simple conidiophores

Table 6. Statistical analyses of variance (ANOVA) based on the effect of individual isolates of *Verticillium dahliae* and *V. tricorpus* or isolate mixtures on the ranked means of Verticillium wilt severity and the means of artichoke measurements across two independent experiments

	ANOVA-type statistic (ATS) and *F* statistic (*F*)[a]				
Isolate effect[b]	**df_{Num}**	**df_{Dem}**	**ATS**	***F***	***P* value**
Disease severity	8	39.7	38.86	…	<0.0001
Foliar weight	8	40	…	12.34	<0.0001
Root weight	8	40	…	18.70	<0.0001
Plant height	8	40	…	26.80	<0.0001

[a] Abbreviations: df_{Num} = numerator degrees of freedom and df_{Dem} = denominator degrees of freedom.
[b] In the root-dip inoculation method, the roots of 4-week-old artichoke seedlings were dipped in a suspension of approximately 1.0×10^7 conidia/ml for 30 min. Mixed inoculation treatments used a suspension of 1.0×10^7 conidia/ml of each *V. dahliae* and *V. tricorpus* isolate in equal measure. Seedlings were transplanted into 0.5-liter foam-insulated cups containing a pasteurized sand:potting soil mix (3:1, vol/vol), and sterile water was used in controls.

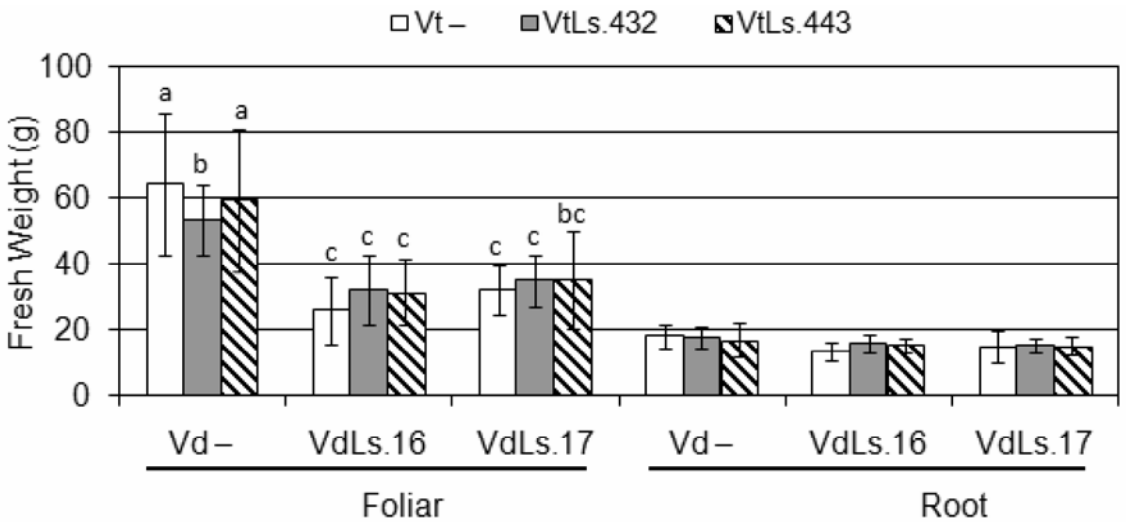

Fig. 3. Effect of individual or mixed isolates of *Verticillium dahliae* and *V. tricorpus* on the mean fresh weight of foliar and root tissues of lettuce cv. Salinas 10 to 12 weeks following inoculation. Data represent the least significant (LS) means and standard deviations (bars) from four independent experiments. Differences between foliar weight LS means with the same letters are not statistically significant according to Tukey-Kramer pairwise comparisons at the 95% confidence level. Such comparisons were not made for root weight, because the *V. dahliae*–*V. tricorpus* interaction was not significant. *V. dahliae* isolates were applied as a conidial suspension to the soil of 4-week-old seedlings prior to transplant. *V. tricorpus* isolates were applied 1 week prior to the *V. dahliae* isolate. For Vd and Vt treatments, sterile water was substituted for the *V. dahliae* or *V. tricorpus* isolate, respectively.

arranged in a verticillate manner along the axis of the hyphae microsclerotia) were observed, regardless of the presence of vascular discoloration. Yellow-orange pigment was observed in colonies formed by segments of tissue from plants inoculated with Ls.183, Ls.442, Cs.225, Cs.234, Cs.236, and Cs.456. *V. tricorpus* was recovered from 92 to 100% of inoculated plants. For both lettuce and artichoke plants, either inoculated with *V. tricorpus* alone or in combination with *V. dahliae*, spore-PCR produced the 337-bp diagnostic fragment using the *V. tricorpus*-specific primers and conidia produced by their root tissue samples as a template (Fig. 5 and *data not shown*). No PCR product was detected from root tissue samples inoculated with isolates of *V. dahliae* alone.

DISCUSSION

Nine *Verticillium* isolates (Ls.183, Ls.432, Ls.441, Ls.442, Ls.443, Cs.225, Cs.234, Cs.236, and Cs.456) from lettuce and artichoke were classed as a group distinct from other phytopathogenic *Verticillium* spp. in a recent study (24). The nine isolates produced dark mycelia, chlamydospores, and microsclerotia and exhibited a pattern of growth across varying temperatures characteristic of *V. tricorpus* isolates (11,13,27). PCR amplification of the nine isolates with primers specific to *V. tricorpus* produced a 377-bp diagnostic fragment. Based on the morphological data and the diagnostic PCR, the nine isolates were identified as *V. tricorpus*. To our knowledge, this is the first reported characterization of *V. tricorpus* isolates from lettuce and artichoke.

Some of the *V. tricorpus* isolates included in this study grew more slowly and produced smaller microsclerotia and fewer

Table 7. Median (Med), mean rankings (*R*), and relative marginal effects (RME) calculated for the severity of Verticillium wilt on artichoke caused by isolates of *Verticillium dahliae* and *V. tricorpus*

Treatment[a]	Med	*R*[b]	RME[c]
K	0.0	8.5	0.15 (0.11–0.20)
Cs.312	5.0	44.5	0.81 (0.78–0.85)
Cs.413	5.0	40.5	0.74 (0.66–0.81)
Cs.423	5.0	42.4	0.78 (0.70–0.83)
Cs.80	5.0	42.6	0.78 (0.71–0.83)
Cs.225	0.5	22.8	0.41 (0.32–0.51)
Cs.234	0.3	17.6	0.32 (0.23–0.42)
Cs.236	0.1	14.5	0.26 (0.18–0.36)
Cs.456	0.0	14.1	0.25 (0.16–0.39)

[a] Results from two independent experiments using the root-dip inoculation method.
[b] Mean rankings of Verticillium wilt severity for each isolate treatment. Disease severity was rated using an ordinal scale of 0 to 5, in which 0 = no vascular discoloration in the taproot, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76 to 100% of vascular tissues in the taproot exhibited discoloration in the absence of foliar symptoms, and 5 = 100% of vascular tissues exhibited discoloration in the taproot and the presence of foliar symptoms typical of Verticillium wilt.
[c] RME = [(R – 0.5)/N]; N = total experimental units in the analysis (N = 54). The 95% confidence intervals are in parentheses.

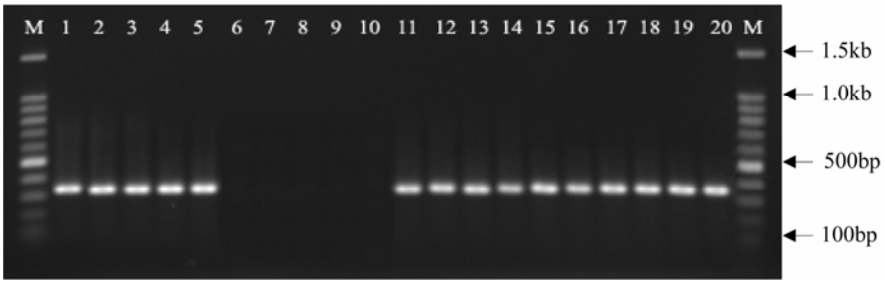

Fig. 5. Confirmation of *Verticillium tricorpus* on inoculated lettuce roots using *V. tricorpus*-specific primers in a spore polymerase chain reaction assay. Lanes 1–5 are from lettuce plants inoculated with *V. tricorpus* isolates Ls.183, Ls.432, Ls.441, Ls.442, and Ls.443, respectively; lanes 6–9 are from lettuce plants inoculated with *V. dahliae* isolates Ls.1, Ls.14, Ls.16, and Ls.17, respectively; lane 10 is from noninoculated plants; lanes 11–15 are from plants co-inoculated with *V. dahliae* isolate Ls.16 and *V. tricorpus* isolates Ls.183, Ls.432, Ls.441, Ls.442, and Ls.443, respectively; and lanes 16–20 are from plants co-inoculated with *V. dahlia* isolate Ls.17 and *V. tricorpus* isolates Ls.183, Ls.432, Ls.441, Ls.442, and Ls.443, respectively. PCR products were loaded as follows: 6 µl on lanes 1–5 and 10 µl on lanes 6–20. M indicates 100-bp DNA ladder (Promega Corp.).

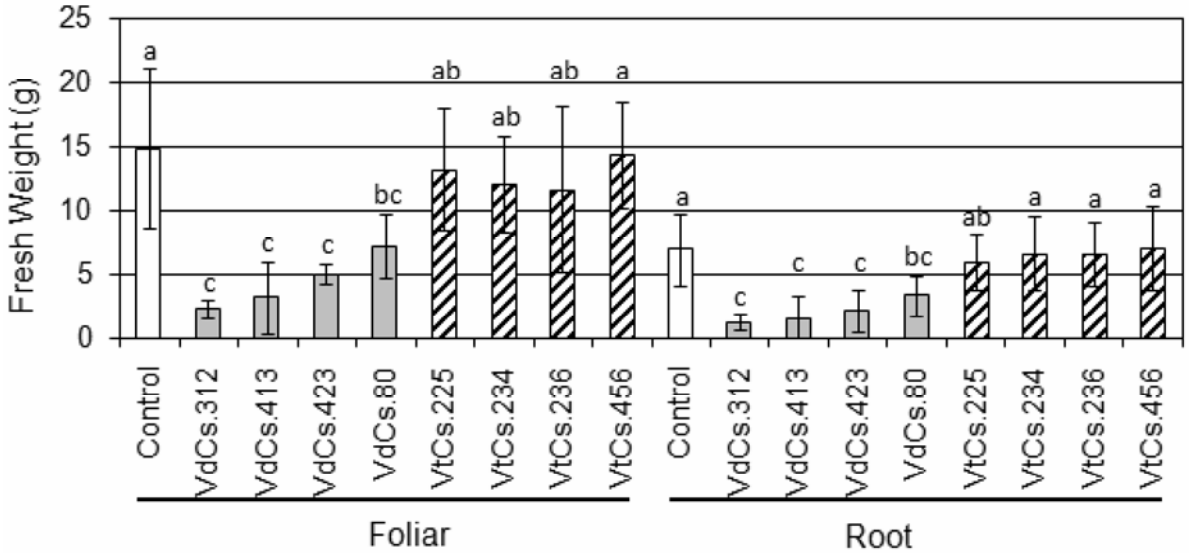

Fig. 4. Effect of individual isolates of *Verticillium dahliae* and *V. tricorpus* on the mean fresh weight of foliar and root tissues of artichoke 10 to 12 weeks following inoculation. Data represents the least significant (LS) means and standard deviations (bars) from four independent experiments. Differences among foliar weight LS means and among root weight LS means with the same letters are not statistically significant according to Tukey-Kramer pairwise comparisons at the 95% confidence level. Isolates were applied as a conidial suspension to the soil of 4-week-old seedlings prior to transplant. Sterile water was used as a control.

dark resting mycelia and chlamydospores than is typical of this species. These same atypical *V. tricorpus* isolates also failed to produce the characteristic yellow-orange pigment when grown in culture on PDA. These characteristics made it difficult to identify these isolates quickly. The development of PCR primers specific to *V. tricorpus* should facilitate rapid identification of atypical *V. tricorpus* isolates in future studies.

Using the spore-PCR assay in conjunction with *V. tricorpus*-specific primers aided in the rapid detection of *V. tricorpus* on collected root samples compared with traditional culturing methods or typical PCR methods relying on prior DNA isolation. However, because PCR is performed directly on the spore suspension without any purification, the density of spores in the suspension can have a significant effect on the assay. Having too many or too few spores can result in a failed PCR reaction. For *V. tricorpus*, a concentration of 1.0×10^5 to 1.0×10^6 conidia per 25-µl reaction volume provided adequate amplification.

Regardless of the severity of symptoms, the recovery of *V. tricorpus* from inoculated lettuce and artichoke was very high. The spore-PCR assay consistently produced the 337-bp diagnostic band from all samples from plants inoculated with *V. tricorpus* isolates. These results are consistent with previous reports of high recovery of *V. tricorpus* from hosts such as eggplant, snapdragon, tobacco, lupin, cotton, and potato (7,10,11,18,29). MacGarvie and Hide (20) estimated that *V. tricorpus* occurred in 72.5% of the seed-potato stocks after examining 225 stocks. In field experiments, recovery of *V. tricorpus* from lettuce seedlings was nearly 100% during the first few weeks following emergence (G. E. Vallad, *unpublished data*). These results suggest that *V. tricorpus* can readily colonize plant roots. However, significant differences were observed in the pathogenicity of *V. tricorpus* isolates toward lettuce and artichoke. The majority of the *V. tricorpus* isolates used in this study could be considered weakly pathogenic, causing only mild (medium disease severity <1) but statistically significant symptoms relative to noninoculated lettuce and artichoke plants. This is in agreement with others (2,13,23) who regard *V. tricorpus* as mildly or weakly pathogenic to hosts.

Verticillium spp. cause specific responses such as early flowering and dying on susceptible hosts (32). Although early flowering of lettuce and premature death of artichoke was quite common among plants inoculated by *V. dahliae,* none of these symptoms occurred on plants inoculated with *V. tricorpus* isolates used in these studies. Of the 400 lettuce plants inoculated with *V. tricorpus* over the course of these studies, only 2 (inoculated with isolates Ls.432 and Ls.441) exhibited foliar symptoms of Verticillium wilt. In Tunisia (15), it was reported that *V. tricorpus* isolates were highly virulent on eggplant, tomato, and potato, with inoculated plants exhibiting typical foliar wilt symptoms and stunting. A reduction in soybean yield linked to *V. nigrescens* without apparent disease symptoms (33) also was reported. Under greenhouse conditions, the fresh foliar and root weights of plants inoculated with *V. tricorpus* were consistently less than the uninoculated controls, although this trend was statistically negligible. Whether any *V. tricorpus* isolates exist in California with an increased virulence toward lettuce and artichoke, such as those reported in Tunisia (15), is unclear and should be investigated further, as well as the impact of *V. tricorpus* populations on lettuce and artichoke production.

Lettuce co-inoculated with certain isolate combinations of *V. dahliae* and *V. tricorpus* exhibited significantly reduced symptoms of Verticillium wilt, suggesting that specific *V. tricorpus* isolates may protect lettuce against *V. dahliae* through competitive exclusion or cross-protection. Overall, soil-drench inoculations, where the isolate of *V. tricorpus* was applied a week prior to the isolate of *V. dahliae*, appeared to be more robust at reducing Verticillium wilt symptoms relative to the root-dip inoculation method. This suggests that competition plays a major role, because the earlier introduction period would give *V. tricorpus* more time to colonize the root surface, including potential infection sites. However, there was evidence of specificity between certain isolates of *V. dahliae* and *V. tricorpus*. In the root-dip inoculation experiments, *V. tricorpus* isolates Ls.443, Ls.441, and Ls.432 effectively reduced disease severity against *V. dahliae* isolate Ls.17 but not Ls.16. Of the *V. tricorpus* isolates characterized here, Ls.443 consistently reduced symptoms of Verticillium wilt when co-inoculated with isolates of *V. dahliae* independent of the inoculation method.

Melouk and Horner (21) described cross-protection of peppermint and spearmint against *V. dahliae* when previously inoculated with a related isolate of *V. nigrescens*. More recent reports found *V. tricorpus* to be associated with Verticillium wilt suppression in potato fields (6). Thus, some *V. tricorpus* isolates may act as potential suppressors of Verticillium wilt. So far, however, little is known of the mechanism by which *V. tricorpus* interferes with the more virulent *V. dahliae*. More work is needed to further understand how isolates of *V. tricorpus* and *V. dahliae* colonize and interact on a common host over time. Future co-inoculation studies using transformed isolates of *V. dahliae* and *V. tricorpus* expressing different fluorescent protein color variants may shed light on this topic.

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