The Three Lineages of the Diploid Hybrid *Verticillium longisporum* Differ in Virulence and Pathogenicity

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ABSTRACT

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Verticillium longisporum is an economically important vascular pathogen of Brassicaceae crops in different parts of the world. *V. longisporum* is a diploid hybrid that consists of three different lineages, each of which originated from a separate hybridization event between two different sets of parental species. We used 20 isolates representing the three *V. longisporum* lineages and the relative *V. dahliae*, and performed pathogenicity tests on 11 different hosts,

including artichoke, cabbage, cauliflower, cotton, eggplant, horseradish, lettuce, linseed, oilseed rape (canola), tomato, and watermelon. *V. longisporum* was overall more virulent on the Brassicaceae crops than *V. dahliae*, which was more virulent than *V. longisporum* across the non-Brassicaceae crops. There were differences in virulence between the three *V. longisporum* lineages. *V. longisporum* lineage A1/D1 was the most virulent lineage on oilseed rape, and *V. longisporum* lineage A1/D2 was the most virulent lineage on cabbage and horseradish. We also found that on the non-Brassicaceae hosts eggplant, tomato, lettuce, and watermelon, *V. longisporum* was more or equally virulent than *V. dahliae*. This suggests that *V. longisporum* may have a wider potential host range than currently appreciated.

Verticillium longisporum is a fungal plant pathogen that infects the vasculature (9) of Brassicaceae crops (16). One of the most severely affected hosts is oilseed rape (canola) in Europe, specifically France, Germany, Russia, and Sweden (14), where disease incidence of up to 80% (7) and yield losses of up to 50% (8) were reported. Other hosts include broccoli in Italy; cabbage in Japan and the United States (California); cauliflower in Belgium, Germany, Netherlands, and the United States (California); field mustard in Italy, Japan, and Sweden; horseradish in Japan and the United States (Illinois); and radish and turnip in Japan (14). Sugar beet in Sweden is the only non-Brassicaceae host from which *V. longisporum* has been isolated (14). *V. dahliae*, a close relative (14,15), causes Verticillium wilt (11) on a variety of different crops (16). Similar symptoms can result from infection by *V. longisporum* (27) but, in oilseed rape, *V. longisporum* induces premature ripening and early senescence (25), while wilting symptoms are absent (10).

Once established, *V. dahliae* and *V. longisporum* are difficult to eradicate (23). *Verticillium* spp. are soilborne and form microsclerotia, which are clusters of melanized cells that survive in the absence of a host for at least 14 years (30). In the proximity of a host root and in response to root exudates, microsclerotia germinate, and the hyphae penetrate and colonize the root vasculature, where they spread and proliferate into the plant vessels.

V. longisporum has an unusual evolutionary history. It is a diploid hybrid that evolved at least three different times, by hybridization between four ancestral lineages representing three different species

(15). The ancestral lineages include the unknown, hypothetical Species A1 and Species D1, the *V. dahliae* lineage D2, and the *V. dahliae* lineage D3. Species A1 hybridized with Species D1, *V. dahliae* lineage D2, and *V. dahliae* lineage D3, resulting in the *V. longisporum* lineages A1/D1, A1/D2, and A1/D3, respectively.

Hybridization is known to generate new plant pathogens (3), including *V. longisporum*, which is more virulent on oilseed rape than *V. dahliae* (9). Due to the involvement of different ancestors and potential lineage-specific genetic and epigenetic changes following hybridization (22), differences in pathogenicity and virulence may also exist between *V. longisporum* lineages. This is supported by the fact that, among *V. longisporum* lineage A1/D1 and A1/D3 isolates included in pathogenicity tests on cauliflower and oilseed rape, the *V. longisporum* lineage A1/D3 isolates were the least virulent group (15,32,33). However, only two *V. longisporum* lineage A1/D3 isolates were used, and lineage A1/D2 isolates were missing in these studies.

Therefore, the objectives of this study were to test whether the three *V. longisporum* lineages differ with respect to pathogenicity and virulence. We performed greenhouse pathogenicity assays with 20 *V. longisporum* and *V. dahliae* isolates on 11 different hosts, which included Brassicaceae hosts and other important crops.

MATERIALS AND METHODS

Pathogenicity and virulence of 20 *V. dahliae* and *V. longisporum* isolates were determined on 11 hosts. Artichoke (Asteraceae), cotton (Malvaceae), eggplant, tomato (Solanaceae), and watermelon (Cucurbitaceae) experiments were performed at the University of California (UC) Davis and cabbage, cauliflower, horseradish, oilseed rape (Brassicaceae), and linseed (Linaceae) experiments at the University of Göttingen, Germany.

Fungal strains and inoculum production. Information about the 20 fungal isolates used in the experiments including host and geographic origin is provided in Table 1. Isolates were

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previously identified based on DNA sequencing and phylogenetic analyses as *V. longisporum* lineages A1/D1, A1/D2, or A1/D3 and *V. dahliae* lineage D2 or *V. dahliae* main group by Inderbitzin et al. (15), and Tran et al. (28) for strain PD639 (=VI43).

To inoculate artichoke, cotton, eggplant, lettuce, tomato, and watermelon, all isolates were grown in 10-cm Petri plates on potato dextrose agar medium (PDA) (Sigma-Aldrich, St. Louis) at room temperature ($24 \pm 2^\circ\text{C}$) in the dark for approximately 6 weeks. Conidia stock suspensions were prepared by pouring sterile distilled water into plates and dislodging conidia with a sterile pipette tip. Stock volumes and concentrations were 50 ml and $>20 \times 10^6$ conidia ml^{-1} , respectively, as determined by using a hemocytometer. Stocks were prepared the day before inoculation and stored at 4°C overnight.

To inoculate cabbage, cauliflower, horseradish, linseed, and oilseed rape, isolates were grown on PDA (Carl Roth GmbH + Co KG, Karlsruhe) in Petri dishes for 10 days at room temperature. Two to three agar discs (5 mm in diameter) were transferred into 500-ml Erlenmeyer flasks containing 250 ml of sterile potato dextrose broth medium (Carl Roth GmbH + Co KG) and incubated for 2 weeks at 22°C under constant shaking (120 rpm) in the dark. Conidia suspensions were prepared the day of inoculation. Conidia were harvested by filtering through sterile gauze into 100-ml Erlenmeyer flasks. Spore concentrations were determined by using a hemocytometer (Thoma chamber) and adjusted to 1×10^6 conidia ml^{-1} with sterile tap water.

Seedling production and inoculation. Eleven different hosts were used, and the cultivar of each host is listed in Table 2. Artichoke, cotton, eggplant, lettuce, tomato, and watermelon experiments were performed at the UC Davis Contained Research Facility, a BSL3-P facility. For each species, one to two 128-plug trays (item CN-PLG 128; International Greenhouse Company, Danville, IL) containing autoclaved Sunshine Basic Mix number 2 potting mix (Sun Gro Horticulture, Agawam, MA) were seeded. Trays were incubated on a greenhouse bench with natural daylight at $21 \pm 5^\circ\text{C}$, covered with sterilized cheesecloth until germination, and watered daily. Artichoke, cotton, eggplant, lettuce, and watermelon seedlings were grown for 4 weeks and tomato seedlings for 3 weeks prior to inoculation. During the first run of the experiment, fertilizer was applied daily for the first 2 weeks. During the second run, fertilizer was applied upon seeding and 2 weeks thereafter. Just prior to inoculation, seedlings were gently uprooted, rinsed free of soil in tap water, and immersed in 200 ml of a conidial suspension (2×10^6 conidia ml^{-1}) in 500-ml beakers for at least 15 min in groups of four representing the four replications of each of the 114 host–fungal strain combinations (6 hosts, 19 fungal strains). Inoculations were performed separately for each host species. Conidial suspensions were replaced approximately halfway through the inoculations to ensure that all hosts were inoculated with nearly the same conidial densities. Seedlings were planted into 0.5-liter (= 16 oz.) Styrofoam cups (item 16J16; Smart and Final, Inc., Commerce, CA) containing autoclaved Sunshine Basic Mix

TABLE 1. *Verticillium* strains used in this study

Species	Lineage	Strain identifiers	Host scientific name	Host common name	Collection site	Strain origin ^a	References
<i>V. longisporum</i> ^b	A1/D1	PD348, Bob.70, 90-02	<i>Brassica oleracea</i> var. <i>botrytis</i>	Cauliflower	Salinas, CA	Subbarao Lab Collection	15
<i>V. longisporum</i>	A1/D1	PD588, CA9	<i>B. oleracea</i>	Cabbage	Gunman, Japan	Subbarao Lab Collection (Usami, Sakai)	15
<i>V. longisporum</i>	A1/D1	PD638, VI40	<i>B. napus</i>	Oilseed rape	Mecklenburg, Germany	UGoe	15,32
<i>V. longisporum</i> ^{b,c}	A1/D1	PD639, VI43	<i>B. napus</i>	Oilseed rape	Mecklenburg, Germany	UGoe	15,28,32
<i>V. longisporum</i>	A1/D1	PD640, Chalons en Champs	<i>B. napus</i>	Oilseed rape	France	UGoe	15
<i>V. longisporum</i> ^d	A1/D1	PD643, Vd02:50	<i>B. napus</i>	Oilseed rape	Kölbäck, Sweden	UGoe	15
<i>V. longisporum</i>	A1/D1	PD644, Vd4	<i>B. napus</i>	Oilseed rape	Sweden	UGoe	15,32
<i>V. longisporum</i> ^{d,e}	A1/D1	PD673, F617-9	<i>B. napus</i>	Oilseed rape	Haute-Marne, France	Subbarao Lab Collection (Typas)	15
<i>V. longisporum</i> ^b	A1/D2	PD356, Ar.139	<i>Armoracia rusticana</i>	Horseradish	Illinois	Subbarao Lab Collection	15
<i>V. longisporum</i>	A1/D2	PD402, Ar.136	<i>A. rusticana</i>	Horseradish	Illinois	Subbarao Lab Collection	15
<i>V. longisporum</i> ^b	A1/D2	PD629, 004	<i>A. rusticana</i>	Horseradish	Illinois	Subbarao Lab Collection (Barbara)	15
<i>V. longisporum</i>	A1/D2	PD730, SDV-I066-08	<i>A. rusticana</i>	Horseradish	Illinois	Subbarao Lab Collection (Babadoost)	15
<i>V. longisporum</i>	A1/D3	PD589, CA10	<i>B. oleracea</i>	Cabbage	Gunman, Japan	Subbarao Lab Collection (Usami, Sakai)	15
<i>V. longisporum</i>	A1/D3	PD614, MD73	<i>B. napus</i>	Oilseed rape	Germany	Subbarao Lab Collection (Barbara)	15
<i>V. longisporum</i> ^{b,f}	A1/D3	PD687, CBS 124.64, MUCL 9802	<i>A. rusticana</i>	Horseradish	Niedersachsen, Germany	Subbarao Lab Collection (CBS)	15
<i>V. longisporum</i> ^b	A1/D3	PD715, IPP 0119, VI32, lsp*	<i>B. napus</i>	Oilseed rape	Mecklenburg, Germany	UGoe	15,32
<i>V. dahliae</i> ^b	D2	PD327, Ca.59, B, T2	<i>Capsicum annuum</i>	Bell pepper	Gilroy, CA	Subbarao Lab Collection	15
<i>V. dahliae</i> ^b	D2	PD502, Acer.875	<i>Acer</i> sp.	Maple	Greenfield, WI	Subbarao Lab Collection (Stanosz)	15
<i>V. dahliae</i> ^b	Main group	PD322, Ls.17	<i>Lactuca sativa</i>	Lettuce	Watsonville, CA	Subbarao Lab Collection	15
<i>V. dahliae</i> ^b	Main group	PD637, Vd73	<i>Linum usitatissimum</i>	Common flax	Mecklenburg, Germany	UGoe	15,32

^a UGoe = University of Göttingen. Names of original strain providers are in parentheses when known.

^b Used in horseradish experiment.

^c Lineage determined by Tran et al. (28).

^d Lineage assessed by polymerase chain reaction only (see Table S2 in Inderbitzin et al. [15]).

^e Only used on the University of California Davis hosts.

^f Only used on the University of Göttingen hosts.

number 2. Three holes (7 mm in diameter) were punched into the bottom of each cup for drainage. An equal number of uninoculated controls were planted for each host. The experiment was conducted twice, with inoculations performed on 3 March 2012 and on 30 and 31 May 2012. Inoculated seedlings were returned to a greenhouse bench and arranged in a completely randomized block design, maintained for 2 months under daylight at $21 \pm 5^\circ\text{C}$, and watered daily for the first week and every second day thereafter. Seedlings were watered individually by hand without splashing to minimize the risk of cross contamination. Fertilizer was applied daily for the first 3 weeks during the first run of the experiment, and upon planting and every second week thereafter during the repetition of the experiment. The exact amount of fertilizer applied is unknown but the fertilizer stock consisted of CaNO_3 , Grow More (Grow More Inc., Gardena, CA), KNO_3 , MgSO_4 , and MKP and was in line with recommended rates for these crops.

Cabbage, cauliflower, horseradish, linseed, and oilseed rape seedlings were produced in a University of Göttingen climate chamber and greenhouse. Seed of cabbage, linseed, and oilseed rape were surface sterilized with 70% ethanol for 5 s and sown in sterile silica sand in seed trays (11 by 11 by 6 cm). Cauliflower seedlings were treated the same way except that a 2:1 soil-sand mixture was used. Prior to inoculation, plants were incubated for 14 days in a climate chamber at 22°C , 70% relative humidity, and illuminated 14 h/day with a light source (Osram Lumilux T8 L58W/21-840) placed approximately 100 cm above the plants (light intensity: approximately $132 \mu\text{mol m}^{-2} \text{s}^{-1}$). Horseradish seedlings were generated vegetatively from fully developed plants approximately 30 to 40 cm in height (three seedlings per mature plant) and planted in pots (9 by 9 by 9 cm) in a greenhouse using an autoclaved 3:2:1 mixture of compost soil-sand-turf. The horseradish seedlings were grown for 4 weeks in a greenhouse at 22°C with a day-and-night cycle of 14 and 10 h, respectively, and a light intensity of approximately $82 \mu\text{mol m}^{-2} \text{s}^{-1}$ achieved by a light source (Osram Lumilux 54W/840) placed at approximately 150 cm from the plants.

Cabbage, cauliflower, linseed, and oilseed rape seedlings were inoculated by a cut-root dip inoculation method (31,32). This involved careful uprooting of 14-day-old plants and removal of sand and soil particles under running tap water. Approximately one-third of the taproot was cut off and seedlings were dipped into a conidial suspension (1×10^6 conidia ml^{-1}) for 30 min. Control plants were dipped into tap water for 30 min. After inoculation, seedlings were transplanted into autoclaved 3:2:1 compost soil-sand-turf mixture in pots (7 by 7 by 7 cm), with two seedlings in each pot. Inoculations

with the 19 *Verticillium* isolates were performed separately for each host. Twenty seedlings per pathogen isolate were inoculated concurrently. Twenty seedlings per host served as control treatment. All plants were incubated for 6 weeks following inoculation.

Horseradish inoculations differed between the first and second experimental repetitions. The plants of the first repetition were first inoculated using the root dip method (31,32) without clipping of the roots. Because of a lack of foliar symptoms, the same plants were reinoculated by injecting a maximum amount of conidia suspension into the upper part of the root with a 20-gauge hypodermic needle and a 2-ml syringe (6,19). Tap water was injected into control plants. Plants of the second repetition were inoculated using root dip inoculation only. Only 10 *Verticillium* isolates and 12 horseradish seedlings were used for this experiment (Table 1). Inoculated horseradish plants were incubated for 9 weeks. Horseradish plants were fertilized weekly with a 1% NPK solution (15:11:15%).

Because 19 fungal isolates were tested on cabbage, cauliflower, linseed, and oilseed rape and 10 isolates on horseradish, there were 91 treatment combinations (isolates–plant species), including water controls. Treatments were arranged in a completely randomized block design with 20 replications for cabbage, cauliflower, linseed, and oilseed rape and 12 for horseradish. Plants were incubated under the same conditions as prior to inoculation. All experiments were performed twice.

TABLE 3. Mean disease incidence and disease score for the University of California (UC) Davis and University of Göttingen (UGoe) hosts across the two experimental replicates

Host	Mean disease incidence (%)	Mean disease score
UC Davis		
Artichoke	92	3.3
Cotton	76	1.8
Eggplant	99	4.0
Lettuce	66	1.5
Tomato	53	0.9
Watermelon	99	4.1
Mean	81	2.6
UGoe		
Cabbage	77	1.0
Cauliflower	87	1.7
Horseradish	71	1.8
Linseed	32	0.8
Oilseed rape	72	1.8
Mean	68	1.4

TABLE 2. Hosts used in this study

Host common name	Host scientific name	Cultivar	Source ^a
Artichoke	<i>Cynara scolymus</i> L.	5473/91Blk	Subbarao Lab, UC Davis
Cabbage	<i>Brassica oleracea</i> convar. <i>capitata</i> var. <i>alba</i> L. ^b	Brunswijker	NBB egesa Gartencenter GmbH, Rodenberg, Germany
Cauliflower	<i>B. oleracea</i> var. <i>botrytis</i> L.	All the Year Around	Johnsons, Kentford, Suffolk, UK
Cotton	<i>Gossypium hirsutum</i> L.	Acala Ultimate EF	Mike Davis Lab, UC Davis
Eggplant	<i>Solanum melongena</i> L.	Early Long Purple	Eden Brothers, Asheville, NC
Horseradish	<i>Armoracia rusticana</i> G. Gaertn., B. Mey. & Scherb.	... ^c	Rühlemann's Kräuter und Duftpflanzen, Horstedt, Germany
Lettuce	<i>Lactuca sativa</i> L.	Salinas	Ryan Hayes, USDA-ARS, Salinas, CA
Linseed	<i>Linum usitatissimum</i> L.	Lirina	Kiepenkerl, Bruno Nebelung GmbH, Everswinkel, Germany
Oilseed rape	<i>B. napus</i> L. var. <i>oleifera</i> D.C. ^d	Falcon	Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, Hohenlieth/Malchow, Germany
Tomato	<i>S. lycopersicum</i> L.	Earlypak 7	Subbarao Lab, UC Davis
Watermelon	<i>Citrullus lanatus</i> (Thunb.) Matsum. & Nakai	Sugar Baby	Eden Brothers, Asheville, NC

^a UC = University of California and USDA-ARS = United States Department of Agriculture–Agricultural Research Service.

^b According to <http://plants.usda.gov/>, may be a synonym of *Brassica oleracea* L. var. *capitata*.

^c No particular cultivar.

^d According to <http://plants.usda.gov/>, may be a synonym of *Brassica napus* L. var. *napus*.

Disease assessment. *Artichoke, cotton, eggplant, lettuce, tomato, and watermelon.* Cups were removed and root systems were cleared of soil by shaking. External symptoms were noted. Roots and shoots were cut open with a scalpel parallel to the long axis to evaluate vascular discoloration, and a disease score of 0 to 5 was assigned to each plant. The disease severity rating system was based on Vallad et al. (29) but generalized to fit all hosts as follows: 0 = vascular discoloration absent; 1 = discoloration of taproot tip only; 2 = discoloration of the taproot not beyond crown; 3 = discoloration beyond crown into shoot; 4 = discoloration

throughout taproot and shoot, up to several leaves wilted, plants possibly stunted; and 5 = discoloration throughout taproot and stem, all leaves wilted or plant dead. Koch's postulates were performed on one inoculated and one control plant per host-isolate combination for the first repetition of the experiment. Side roots and remaining soil were removed and taproots were cut into five pieces that were sterilized in 10% bleach (sodium hypochlorite 0.6%) for 1 min, rinsed twice in sterilized water, dried on sterile paper towels, plated onto *Verticillium* semiselective NP-10 medium (18,26), and examined for the presence of microsclerotia

Fig. 1. Estimated relative marginal effects of *Verticillium longisporum* and *V. dahliae* disease severity scores for individual crops in the University of California Davis (top) and University of Göttingen (bottom) experiments; 95% confidence intervals are indicated by vertical lines.

TABLE 4. Analysis of variance (ANOVA) using the ANOVA-type statistic on ranked means of disease severity from the University of California (UC) Davis hosts and the University of Göttingen hosts^a

Effect	UC Davis hosts				University of Göttingen hosts			
	df _{Num}	df _{Den}	ATS	P value	df _{Num}	df _{Den}	ATS	P value
Host	4.35	571	378.83	<0.0001	2.28	245	194.76	<0.0001
Isolate	18.00	571	9.87	<0.0001	17.5	1400	50.23	<0.0001
Replication (Experiment)	6.00	593	9.31	<0.0001	37	2481	0.9	0.6454
Host × isolate	78.20	571	5.32	<0.0001	49.3	725	11.45	<0.0001
Experiment (<i>n</i> = 2)	1.00	571	1.01	0.3151	1	2423	5.27	0.0217
Host × experiment	4.35	571	17.90	<0.0001	2.97	2422	8.54	<0.0001
Experiment × isolate	18.00	571	2.18	0.0026	14.9	2420	3.26	<0.0001
Host × experiment × isolate	78.20	571	1.20	0.114	42.5	2431	5.76	<0.0001

^a UC Davis hosts are artichoke, cotton, eggplant, lettuce, tomato, and watermelon; University of Göttingen hosts are cabbage, cauliflower, horseradish, linseed, and oilseed rape.

in culture medium using a dissecting microscope 1 to 2 weeks after plating.

Cauliflower, cabbage, horseradish, linseed, and oilseed rape. Vascular discoloration was assessed as a measure of disease severity. Cauliflower, cabbage, linseed, and oilseed rape plants were removed from the pots, soil was washed off under running tap water and roots were sliced vertically with a scalpel. Discoloration was rated as follows, according to Vallad et al. (29): 0 = no vascular discoloration, 1 = 25% of vascular tissues in tap root exhibiting discoloration, 2 = 26 to 50% of vascular tissues in tap root exhibiting discoloration, 3 = 51 to 75% of vascular tissues in tap root exhibiting discoloration, 4 = 76 to 100% of vascular tissues in tap root exhibiting discoloration, and 5 = 100% of vascular tissues in tap root exhibiting discoloration extending into crown.

For horseradish, roots were cut horizontally with a sharp knife approximately 1.5 to 2 cm below the hypocotyl, and the vascular discoloration of root-cross sections was rated following the method by Khan et al. (20).

Statistical analysis. The *Verticillium* disease severity scores represented ordinal data that were analyzed using a nonparametric approach where the effects of experimental factors, including fungal strain and host, on disease severity scores were quantified through estimated relative marginal effects (RME) (5,24). Null hypotheses of treatments having no effect were tested using the analysis of variance-type statistic (ATS). Linear contrasts were constructed to compare disease severities between groups of

TABLE 5. Mean disease scores for *Verticillium dahliae* and *V. longisporum* isolates across all University of California (UC) Davis and University of Göttingen (UGoe) hosts

Lineage or species	Isolate	Mean disease scores ^a	
		UC Davis hosts	UGoe hosts
<i>V. dahliae</i>	PD322	3.7	0.7
<i>V. dahliae</i>	PD327	3.2	0.6
<i>V. dahliae</i>	PD502	3.4	0.6
<i>V. dahliae</i>	PD637	2.8	0.9
A1/D1	PD348	2.6	2.0
A1/D1	PD588	2.3	1.2
A1/D1	PD638	2.5	2.2
A1/D1	PD639	2.3	1.3
A1/D1	PD640	2.3	0.9
A1/D1	PD643	2.7	2.3
A1/D1	PD644	2.2	0.9
A1/D1	PD673	2.4	...
A1/D2	PD356	2.5	0.8
A1/D2	PD402	2.5	0.8
A1/D2	PD629	2.5	1.1
A1/D2	PD730	2.6	1.2
A1/D3	PD589	2.5	2.7
A1/D3	PD614	2.3	1.2
A1/D3	PD687	...	1.1
A1/D3	PD715	2.5	0.7

^a UC Davis hosts are artichoke, cotton, eggplant, lettuce, tomato, and watermelon; UGoe hosts are cabbage, cauliflower, horseradish, linseed, and oilseed rape. Highest and lowest scores for each location are in bold.

Fig. 2. Estimated relative marginal effects of virulence across all individual *Verticillium dahliae* and *V. longisporum* isolates grouped according to affiliation with *V. longisporum* lineages A1/D1, A1/D2, and A1/D3 and *V. dahliae*, for the University of California (UC) Davis hosts (top) and the University of Göttingen hosts (bottom); 95% confidence intervals are provided. UC Davis hosts are artichoke, cotton, eggplant, lettuce, tomato, and watermelon; University of Göttingen hosts are cabbage, cauliflower, horseradish, linseed, and oilseed rape.

isolates representing the *V. longisporum* lineages and *V. dahliae*, and to assess susceptibility to *Verticillium* infection between different hosts.

Calculations were performed using the PROC Mixed procedure in SAS (2010, version 9.3) to generate RME and the LD_CI macro to generate 95% confidence intervals (5,24). The RME values are generated by the equation $RME = (R - 0.5)/N$, where R is the mean treatment ranking and N is the total number of experimental units in the analysis. Replications nested within experiments were considered random effects in the analysis.

The UC Davis and the University of Göttingen data were analyzed separately. Differences between isolates and hosts (fixed effects) were determined for the UC Davis crops artichoke, cotton, eggplant, lettuce, tomato, and watermelon and for the University of Göttingen crops cabbage, cauliflower, horseradish, linseed, and oilseed rape. For each crop, differences between individual isolates, between *V. longisporum* lineages, and between *V. longisporum* lineages and *V. dahliae* were determined. The same differences were also assessed for the collective Brassicaceae crops and the collective non-Brassicaceae crops, except linseed, which was excluded because linseed scores were obtained at the University of Göttingen with a different rating scale.

RESULTS

Artichoke, cotton, eggplant, lettuce, tomato, and watermelon. *Verticillium* disease symptoms were observed on all hosts. Mean disease incidence, which is the percentage of plants with *Verticillium* disease symptoms, ranged from 99% in eggplant and watermelon to 53% in tomato. Mean disease scores across all isolates were highest for eggplant and watermelon, with 4.0 and 4.1, respectively, and lowest for tomato, with 1.0. The mean disease incidence across all hosts was 81% and the mean disease score was 2.6 (Table 3). RME of disease severity of hosts across all isolates of *V. dahliae* and *V. longisporum* showed significant differences (Fig. 1). The ATS of the RME showed that the effect of host was significant ($P < 0.0001$) (Table 4).

There were differences in virulence between isolates combined across all hosts tested at UC Davis. *V. dahliae* strain PD322 was the most virulent isolate, with a mean disease score of 3.7, whereas *V. longisporum* lineage A1/D1 strain PD644 was the least virulent isolate, with a mean disease score of 2.2 (Table 5). Virulence score RME of individual isolates across all hosts is shown in Figure 2, and indicates significant differences between some isolates. The ATS of the RME shows that the effect of isolate was significant ($P < 0.0001$) (Table 4).

On individual hosts, the highest disease score means observed were 5 for *V. dahliae* strains PD322 on watermelon and PD502 on artichoke. *V. longisporum* lineages A1/D1 strains PD348 and PD643 on eggplant and PD638 and on watermelon also had disease score means of 5. The lowest score was 0, by *V. longisporum* lineage A1/D1 strains PD348 and PD644 on tomato (Supplementary Table S1). The isolate RME on individual crops are shown in Figure 3. The effect of isolate was significant for all hosts ($P < 0.0001$) (Supplementary Table S2).

There were significant differences in virulence between *Verticillium* spp. and lineages across all hosts combined ($P < 0.0001$) (Table 6). *V. dahliae* was the most virulent group, with an overall disease score of 3.3, which was higher than the scores of the *V. longisporum* lineages, which varied between 2.4 and 2.5 (Table 7; Fig. 4).

On individual hosts, *V. dahliae* was significantly more virulent than *V. longisporum* on artichoke and cotton ($P < 0.0001$) but not significantly different from *V. longisporum* lineage A1/D1 on watermelon ($P = 0.57$), *V. longisporum* lineages A1/D2 and A1/D3 on lettuce ($P > 0.14$), and *V. longisporum* lineage A1/D2 on tomato ($P = 0.09$). *V. dahliae* was significantly less virulent than *V. longisporum* lineage A1/D1 on eggplant ($P < 0.0001$) (Table 8; Fig. 5).

Within species and lineages across all hosts combined, there were no significant virulence differences between isolates, except in *V. dahliae*, where strain PD322 was more virulent than strain PD637 (Fig. 2). On individual hosts within species and lineages, there were virulence differences between isolates but no single isolate was consistently most or least virulent across all hosts (Fig. 3). This

Fig. 3. Estimated relative marginal effects of virulence of individual *Verticillium dahliae* and *Verticillium longisporum* isolates on University of California (UC) Davis hosts grouped according to affiliation with *V. longisporum* lineages A1/D1, A1/D2, and A1/D3 and *V. dahliae*; 95% confidence intervals are provided. UC Davis hosts are artichoke, cotton, eggplant, lettuce, tomato, and watermelon.

agrees with the significant host–isolate interaction (Table 4), which indicates that isolate virulence is host dependent.

Cabbage, cauliflower, horseradish, linseed, and oilseed rape. *Verticillium* disease symptoms were observed on all hosts. Mean disease incidence ranged from 87% in cauliflower to 32% in linseed. Mean disease scores across all isolates were highest for horseradish and oilseed rape, with 1.8, and lowest for linseed, with 0.8. The mean disease incidence across all hosts was 68% and the mean disease score was 1.4 (Table 3). RME suggested significant differences in the response of hosts to all strains of *V. dahliae* and *V. longisporum* (Fig. 1). The ATS of the RME showed that the effect of host was significant ($P < 0.0001$) (Table 4).

TABLE 7. Mean disease scores for *Verticillium dahliae* and *V. longisporum* lineages across all University of California (UC) Davis and University of Göttingen hosts

Lineage or species	Mean disease scores ^a	
	UC Davis hosts	University of Göttingen hosts
A1/D1	2.4	1.7
A1/D2	2.5	1.2
A1/D3	2.4	1.4
<i>V. dahliae</i>	3.3	0.8

^a UC Davis hosts are artichoke, cotton, eggplant, lettuce, tomato, and watermelon; University of Göttingen hosts are cabbage, cauliflower, horseradish, linseed, and oilseed rape. Highest and lowest scores for each location are in bold.

TABLE 6. Analysis of variance (ANOVA) using the ANOVA-type statistic on ranked means of disease severity grouped according to *Verticillium dahliae* and *V. longisporum* lineages across the University of California (UC) Davis and the University of Göttingen hosts

Effect	UC Davis hosts				University of Göttingen hosts			
	df _{Num}	df _{Den}	ATS	<i>P</i> value	df _{Num}	df _{Den}	ATS	<i>P</i> value
Host	4.84	530	257.19	<0.0001	2.28	245	194.76	<0.0001
Lineage or species	2.91	530	37.56	<0.0001	17.50	1400	50.23	<0.0001
Replication (Experiment)	6.00	662	7.46	<0.0001	37.00	2481	0.90	0.6454
Host × lineage or species	12.90	530	10.29	<0.0001	49.30	725	11.45	<0.0001
Experiment	1.00	530	1.19	0.2747	1.00	2423	5.27	0.0217
Host × experiment	4.84	530	13.53	<0.0001	2.97	2422	8.54	<0.0001
Experiment × lineage or species	2.91	530	1.72	0.1612	14.90	2420	3.26	<0.0001
Host × experiment × lineage or species	12.9	530	1.26	0.2298	42.50	2431	5.76	<0.0001

^a UC Davis hosts are artichoke, cotton, eggplant, lettuce, tomato, and watermelon; University of Göttingen hosts are cabbage, cauliflower, horseradish, linseed, and oilseed rape.

Fig. 4. Estimated relative marginal effects of virulence across all University of California (UC) Davis (top) and University of Göttingen (bottom) hosts of *Verticillium dahliae* and *V. longisporum* lineages; 95% confidence intervals are provided. UC Davis hosts are artichoke, cotton, eggplant, lettuce, tomato, and watermelon; University of Göttingen hosts are cabbage, cauliflower, horseradish, linseed, and oilseed rape.

TABLE 8. Contrasts between disease severity of *Verticillium dahliae* and *V. longisporum* lineages on individual University of California (UC) Davis hosts, University of Göttingen (UGoe) hosts, and the Brassicaceae hosts only^a

Location, host, effect	Num DF	Den DF	Value	<i>P</i> > <i>F</i>
UC Davis				
Artichoke				
A1/D1 vs. A1/D2	1	79.7	24.87	<0.0001
A1/D1 vs. A1/D3	1	42.4	14.29	0.0002
A1/D1 vs. <i>V. dahliae</i>	1	62.9	56.74	<0.0001
A1/D2 vs. A1/D3	1	35.2	0	0.9984
A1/D2 vs. <i>V. dahliae</i>	1	51.8	13.26	0.0003
A1/D3 vs. <i>V. dahliae</i>	1	46.9	8.65	0.0033
Cotton				
A1/D1 vs. A1/D2	1	62	0.06	0.8045
A1/D1 vs. A1/D3	1	36.3	1.96	0.1615
A1/D1 vs. <i>V. dahliae</i>	1	63.8	21.48	<0.0001
A1/D2 vs. A1/D3	1	40.3	2.23	0.1353
A1/D2 vs. <i>V. dahliae</i>	1	59.1	15.77	<0.0001
A1/D3 vs. <i>V. dahliae</i>	1	38.8	24.66	<0.0001
Eggplant				
A1/D1 vs. A1/D2	1	86	25.09	<0.0001
A1/D1 vs. A1/D3	1	44	1.63	0.2018
A1/D1 vs. <i>V. dahliae</i>	1	87.9	11.84	0.0006
A1/D2 vs. A1/D3	1	29.7	5.46	0.0195
A1/D2 vs. <i>V. dahliae</i>	1	54	2.56	0.1097
A1/D3 vs. <i>V. dahliae</i>	1	33.4	1.65	0.1987
Lettuce				
A1/D1 vs. A1/D2	1	70.7	17.68	<0.0001
A1/D1 vs. A1/D3	1	36.7	8.69	0.0032
A1/D1 vs. <i>V. dahliae</i>	1	50.7	22.58	<0.0001
A1/D2 vs. A1/D3	1	39.3	0.14	0.7124
A1/D2 vs. <i>V. dahliae</i>	1	51.8	1.8	0.1797
A1/D3 vs. <i>V. dahliae</i>	1	47.1	2.18	0.1402
Tomato				
A1/D1 vs. A1/D2	1	62.3	24.84	<0.0001
A1/D1 vs. A1/D3	1	49.2	14.4	0.0001
A1/D1 vs. <i>V. dahliae</i>	1	50	37.39	<0.0001
A1/D2 vs. A1/D3	1	48.6	1.79	0.1812
A1/D2 vs. <i>V. dahliae</i>	1	54.1	2.88	0.0895
A1/D3 vs. <i>V. dahliae</i>	1	46.6	8.57	0.0034
Watermelon				
A1/D1 vs. A1/D2	1	56.4	37.72	<0.0001
A1/D1 vs. A1/D3	1	63.9	109.05	<0.0001
A1/D1 vs. <i>V. dahliae</i>	1	57.9	0.33	0.5654
A1/D2 vs. A1/D3	1	46.7	3.55	0.0597
A1/D2 vs. <i>V. dahliae</i>	1	57	34.9	<0.0001
A1/D3 vs. <i>V. dahliae</i>	1	45	83.85	<0.0001
University of Göttingen hosts				
Cabbage				
A1/D1 vs. A1/D2	1	274	27.65	<0.0001
A1/D1 vs. A1/D3	1	91.6	0.93	0.3345
A1/D1 vs. <i>V. dahliae</i>	1	350	0.27	0.6042
A1/D2 vs. A1/D3	1	124	9.22	0.0024
A1/D2 vs. <i>V. dahliae</i>	1	263	31.66	<0.0001
A1/D3 vs. <i>V. dahliae</i>	1	93.9	1.71	0.1911
Cauliflower				
A1/D1 vs. A1/D2	1	313	31.5	<0.0001
A1/D1 vs. A1/D3	1	213	5.41	0.02
A1/D1 vs. <i>V. dahliae</i>	1	334	112.88	<0.0001
A1/D2 vs. A1/D3	1	192	4.79	0.0286
A1/D2 vs. <i>V. dahliae</i>	1	258	25.85	<0.0001
A1/D3 vs. <i>V. dahliae</i>	1	182	40.48	<0.0001
Horseradish				
A1/D1 vs. A1/D2	1	26.5	27.05	<0.0001
A1/D1 vs. A1/D3	1	20.4	3.06	0.0802
A1/D1 vs. <i>V. dahliae</i>	1	35.8	12.56	0.0004
A1/D2 vs. A1/D3	1	13.9	27.45	<0.0001
A1/D2 vs. <i>V. dahliae</i>	1	38.3	137.73	<0.0001
A1/D3 vs. <i>V. dahliae</i>	1	17	0.29	0.5929
Linseed				
A1/D1 vs. A1/D2	1	306	2.9	0.0885
A1/D1 vs. A1/D3	1	267	20.38	<0.0001
A1/D1 vs. <i>V. dahliae</i>	1	348	24.21	<0.0001
A1/D2 vs. A1/D3	1	256	30.98	<0.0001
A1/D2 vs. <i>V. dahliae</i>	1	284	35.13	<0.0001
A1/D3 vs. <i>V. dahliae</i>	1	218	0.07	0.7927
Oilseed rape				
A1/D1 vs. A1/D2	1	344	161.41	<0.0001
A1/D1 vs. A1/D3	1	230	61.33	<0.0001
A1/D1 vs. <i>V. dahliae</i>	1	362	283.13	<0.0001
A1/D2 vs. A1/D3	1	190	6.39	0.0115
A1/D2 vs. <i>V. dahliae</i>	1	270	20.14	<0.0001
A1/D3 vs. <i>V. dahliae</i>	1	190	36.52	<0.0001
Brassicaceae hosts				
A1/D1 vs. A1/D2	1	1070	83.49	<0.0001
A1/D1 vs. A1/D3	1	673	59.87	<0.0001
A1/D1 vs. <i>V. dahliae</i>	1	1145	411.32	<0.0001
A1/D2 vs. A1/D3	1	654	0.09	0.7593
A1/D2 vs. <i>V. dahliae</i>	1	906	105.02	<0.0001
A1/D3 vs. <i>V. dahliae</i>	1	608	86.84	<0.0001

^a Brassicaceae hosts are cabbage, cauliflower, horseradish, and oilseed rape.

There were differences in virulence between isolates across all hosts combined. *V. longisporum* lineage A1/D3 strain PD589 was overall the most virulent isolate, with a mean disease score of 2.7, whereas *V. dahliae* strains PD327 and PD502 were least virulent, with a mean disease score of 0.6 (Table 5). Virulence score RME of individual isolates (Fig. 2) across all hosts indicated significant differences between some isolates. The ATS of the RME shows that the effect of isolate was significant ($P < 0.0001$) (Table 4).

On individual hosts, the highest disease score was 4.6 for *V. longisporum* lineage A1/D3 strain PD589 on oilseed rape. The lowest score was 0 by *V. dahliae* strains PD322 and PD327 and *V. longisporum* lineage A1/D1 strain PD588, all on linseed (Supplementary Table S3). The isolate RME on individual crops are shown in Figure 6. The effect of isolate was significant for all hosts ($P < 0.0001$) (Supplementary Table S4).

Fig. 5. Estimated relative marginal effects of virulence on individual University of California Davis hosts of *Verticillium dahliae* and *V. longisporum* lineages; 95% confidence intervals are provided.

There were significant differences in virulence between *Verticillium* spp. and lineages across all hosts combined (Table 7). *V. longisporum* lineage A1/D1 was the most virulent group, with an overall disease score of 1.7, followed by *V. longisporum* lineages A1/D3 and A1/D2, with scores of 1.4 and 1.2, respectively, which was significantly higher than the score of *V. dahliae* ($P < 0.0001$), which was 0.8 (Table 7; Fig. 4).

On individual hosts, *V. longisporum* lineage A1/D1 was significantly more virulent than other lineages and *V. dahliae* on cauliflower ($P = 0.02$) and oilseed rape ($P < 0.0001$), and equally virulent as *V. longisporum* lineage A1/D2 on linseed ($P = 0.0885$). *V. longisporum* lineage A1/D2 was significantly more virulent than other lineages and *V. dahliae* on cabbage ($P < 0.0001$) and horseradish ($P < 0.0001$) (Table 8; Fig. 7).

Fig. 6. Estimated relative marginal effects of virulence of individual *Verticillium dahliae* and *V. longisporum* isolates on University of Göttingen hosts grouped according to affiliation with *V. longisporum* lineages A1/D1, A1/D2, and A1/D3 and *V. dahliae*; 95% confidence intervals are provided.

Within species and lineages across all hosts combined and on individual hosts, there were virulence differences between isolates within all *V. longisporum* lineages (Fig. 6). No single isolate was most or least virulent across all individual crops, as illustrated by the significant host–isolate interaction term in Table 4.

Brassicaceae versus non-Brassicaceae hosts. To illustrate susceptibility of Brassicaceae and non-Brassicaceae hosts against

V. dahliae and *V. longisporum*, we generated a Brassicaceae dataset that comprised the data from cabbage, cauliflower, horseradish, and oilseed rape. The effect of lineage and species was significant in the Brassicaceae dataset (Supplementary Table S5). Across all Brassicaceae hosts combined, *V. longisporum* lineage A1/D1 was the most virulent group and *V. dahliae* was least virulent (Table 8). This was contrasted with the non-Brassicaceae experiment involving

Fig. 7. Estimated relative marginal effects of virulence on individual University of Göttingen hosts of *Verticillium dahliae* and *V. longisporum* lineages; 95% confidence intervals are provided.

artichoke, cotton, eggplant, lettuce, tomato, and watermelon, where *V. dahliae* overall was the most virulent group, and the three *V. longisporum* lineages were equally virulent (Fig. 8).

DISCUSSION

We demonstrated that there are significant differences in virulence and pathogenicity among the *V. longisporum* lineages and between *V. longisporum* and *V. dahliae*. *V. longisporum* was more virulent than *V. dahliae* on all Brassicaceae hosts tested, including cabbage, cauliflower, horseradish, and oilseed rape, but virulence of *V. longisporum* depended on the host and lineage. *V. longisporum* lineage A1/D1 was the most virulent group across all Brassicaceae crops and, among individual crops, *V. longisporum* lineage A1/D1 was the most virulent group on oilseed rape and marginally more virulent on cauliflower than *V. longisporum* lineage A1/D3. *V. longisporum* lineage A1/D2 was the most virulent group on cabbage and horseradish, whereas *V. longisporum* lineage A1/D3 was never the most virulent lineage on any of the hosts tested. However, *V. longisporum* lineage A1/D3 strain PD589 was among the most virulent isolates on oilseed rape.

Our finding that *V. longisporum* lineage A1/D1 overall is more virulent on cauliflower and oilseed rape than *V. longisporum* lineage A1/D3 agrees with Zeise and von Tiedemann (32,33), whose *V. longisporum* groups *lsp* and *lsp** correspond to *V. longisporum* lineages A1/D1 and A1/D3, respectively (15). Babadoost et al. (1) showed that *V. longisporum* lineage A1/D2 isolates were virulent on horseradish. Our assays confirmed these results, and also showed that *V. longisporum* lineage A1/D2 was most virulent on horseradish among the *V. longisporum* lineages and *V. dahliae*. It is interesting that all *V. longisporum* lineage A1/D2 isolates used in our study were originally isolated from horseradish. However, the horseradish isolates were not host specific, because *V. longisporum* lineage A1/D2 was also the most virulent group on cabbage.

The fact that *V. longisporum* was more virulent on the Brassicaceae crops than *V. dahliae* was expected, because *V. longisporum* is a pathogen of Brassicaceae, whereas *V. dahliae* generally affects other hosts (9). The only non-Brassicaceae host from which *V. longisporum* was isolated was sugar beet (16). However, in our assays, at least one of the *V. longisporum* lineages was more or equally virulent than *V. dahliae* on eggplant, tomato, lettuce, and watermelon, suggesting that, under field conditions, *V. longisporum* may have a wider potential host range than is currently appreciated.

Our studies led to the following practical considerations. *V. longisporum* lineage A1/D1 was previously introduced into the Salinas Valley of California, where it caused Verticillium wilt on cauliflower (21,27) but has since disappeared. Our results show that *V. longisporum* lineage A1/D1 is also among the most virulent groups on other crops grown in California, including eggplant and watermelon, which are potential hosts should *V. longisporum* lineage A1/D1 be reintroduced. Another issue is Verticillium infection of oilseed rape, which is a potentially yield-limiting disease in Europe but is absent from North America (12). The disease is caused by *V. longisporum* lineage A1/D1, which is known from Europe, California, and Japan but has not been documented from Canada or the oilseed-rape-growing regions of the United States (15). However, *V. longisporum* lineage A1/D2 is known from horseradish in Illinois, which is in relative proximity to some of the major oilseed-rape-growing areas in the United States, including Minnesota, North Dakota, and Oklahoma (<http://www.uscanola.com/>). We were unable to include North American varieties of oilseed rape in our assays but, on the European oilseed rape varieties, *V. longisporum* lineage A1/D2 was the least virulent lineage of *V. longisporum* and, thus, *V. longisporum* lineage A1/D2 may not pose a risk to the North American oilseed-rape-growing regions.

Our results lay the framework for investigations into the genetic bases of the virulence differences between the *V. longisporum* lineages and between *V. longisporum* and *V. dahliae*. These differences may be due to genetic changes associated with the hybridization process, as has been documented in plants (13), and also to the unique genetic contributions made to the hybrid by the individual parental lineages. Brasier (3,4) speculated that previously geographically separated nonpathogens could hybridize to form new pathogens. This appears to be the case for *V. longisporum*, particularly for *V. longisporum* lineage A1/D1, which, overall, was the most virulent lineage of *V. longisporum* in Brassicaceae crops. None of the ancestral species of *V. longisporum* lineage A1/D1, Species A1 and Species D1, have ever been found (15), suggesting that Species A1 and Species D1 are not pathogenic on any of the agricultural crops and are more likely to be associated with nonagricultural hosts, or they are nonpathogenic saprotrophs, similarly to *V. nubilum* (14,17). Very little is known about the natural hosts of *Verticillium* spp. (16). Such knowledge, particularly pertaining to Species A1 and Species D1, would be crucial to understanding the genetics of the origin of *V. longisporum* lineage A1/D1, one of the most serious oilseed rape pathogens today.

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Fig. 8. Comparison of estimated relative marginal effects (RME) of virulence between Brassicaceae and non-Brassicaceae hosts for *Verticillium dahliae* and *V. longisporum* lineages; 95% confidence intervals are provided. The Brassicaceae and non-Brassicaceae data were generated in different experiments analyzed separately, so that the confidence intervals cannot be used to compare Brassicaceae and a non-Brassicaceae RME. Noncrucifer RME are from Figure 4 (top). Brassicaceae hosts are cabbage, cauliflower, horseradish and oilseed rape; non-Brassicaceae hosts are artichoke, cotton, eggplant, lettuce, tomato, and watermelon.

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