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Mating types and physiological races of *Verticillium dahliae* in Solanaceae crops in Brazil

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Abstract *Verticillium dahliae* is a soil-borne fungal pathogen responsible for vascular wilt diseases in more than 300 dicotyledonous species, including solanaceous vegetable crops. In this study, a collection of 89 Brazilian *V. dahliae* isolates was characterized by combing molecular information for mating type and physiological race determination, as well as via virulence bioassays employing a set of differentials. Based on the virulence assays, three isolates were classified as race 1, 76 were classified as race 2, whereas ten isolates did not cause any symptom on the tested cultivars." In race-specific detection, a total of six isolates were identified as race 1, 70 as race 2, and 13 isolates displayed no amplicon with any primer set employed. Therefore, *V. dahliae* race 2 isolates are currently ubiquitous across major Solanaceae-producing areas in Brazil. Both *MAT* idiomorphs were detected, but a larger number of the *V. dahliae* isolates displayed the *MAT1–1* (82%) pattern in our molecular analyses. The simultaneous presence of both *MAT* idiomorphs opens the possibility of sporadic events of sexual reproduction among *V. dahliae* populations from Brazil, enabling the potential emergence of either new recombinant isolates with broader host ranges or novel physiological races. Our results clearly indicated the need to intensify the search for effective sources of resistance to *V. dahliae* race 2 in tomato breeding programs under Brazilian conditions.

Keywords Mating type · Pathogenicity assays · Race structure · Solanaceae vegetables · Verticillium wilt

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Introduction

Verticillium dahliae Kleb. is a soil-borne, xylem-invading fungus that can induce vascular wilt diseases in more than 300 eudicotyledonous plant species (de Jonge et al., 2012; Farr & Rossman, 2021; Klosterman et al., 2009; Reis & Boiteux, 2006a). The host range of this pathogen includes trees, ornamentals, and economically important vegetable and field crops (Farr & Rossman, 2021; Pegg & Brady, 2002; Suaste-Dzul et al., 2022). Among the most important vegetables affected by *Verticillium*-induced diseases are: tomato (*Solanum lycopersicum* L.), eggplant (*Solanum melongena* L.), and potato (*S. tuberosum* L.) as well as strawberry and lettuce (Domsch et al., 1980; Reis & Boiteux, 2006b).

The fungus is able to survive for very long periods of time in soil as melanized microsclerotia, which are resting structures produced in infected plants.

Microsclerotia can remain viable for up to 14 years even in the absence of a host plant (Carroll et al., 2018; Pegg & Brady, 2002; Vallad & Subbarao, 2008). In this scenario, the cultural control of verticillium wilt-inducing pathogens is difficult due to the long persistence of the resting structures in the field and their broad host range (Deketelaere et al., 2017). Chemical control via soil fumigants has a low efficacy (Jiménez-Díaz et al., 2012).

Management strategies aiming to reduce the inoculum in the soil include crop rotation with non-host plants, green manure, biocontrol agents, solarization, organic amendments, cover crops and living mulches (Deketelaere et al., 2017; Johnson & Dung, 2010). However, the use of resistant cultivars is one of the few economically viable alternatives of controlling verticillium wilt. Nevertheless, effective host resistance to verticillium wilt has been actively identified only in a limited number of crops, such as tomato, lettuce, potato, and cotton (Diwan et al., 1999; Hayes et al., 2007; Jiménez-Dìaz et al., 2012; Mohan et al., 1990; Schaible et al., 1951).

Tomatoes have been mainly affected by isolates of *V. dahliae,* classified as either race 1 or race 2 (Alexander, 1962; Fradin et al., 2009; Maruthachalam et al., 2010). More recently, a subgroup of isolates (initially identified as race 2) were reclassified as a novel *V. dahliae* race 3 (Ingram et al., 2020). The first source of genetic resistance to *V. dahliae* isolates was identified in accessions of the wild tomato species *Solanum pimpinellifolium* L. (Bryan, 1925). This *V. dahliae* race 1-specific resistance trait was subsequently introgressed into commercial tomato varieties (Bryan, 1925; Pegg & Brady, 2002; Schaible et al., 1951). A single dominant *Ve*-1 locus in the chromosome 9 controls the original race 1-specific resistance (Diwan et al., 1999), encoding a cell surface receptor protein (Kawchuk et al., 2001). In addition, race 1 is characterized by the presence of the effector gene *Ave1*, conferring avirulence to tomato that carry *Ve*-1 and acts as a genuine resistance gene (de Jonge et al., 2012). Over time, race 1 resistance-breaking strains, named as race 2, have become increasingly problematic in tomato crops, since its first report (Alexander, 1962; Acharya et al., 2020; Chavarro-Carrero et al., 2020; Maruthachalam et al., 2010).

In Brazil, even though *V. dahliae* race 2 isolates have been reported in tomatoes since the early 1980s (Laterrot et al., 1983), race 1 isolates predominated in this crop up to the late 1990s (Reis et al., 2007). This momentary prevalence of *V. dahliae* race 1 isolates and the widespread employment of hybrids carrying the *Ve*-1 resistance gene have resulted in the decrease of the importance of this pathogen in tomato (Miranda et al., 2010). However, sporadic reports of verticillium wilt outbreaks with significant yield losses started to be observed across major tomato-growing areas in São Paulo State (Cerezine & Kurozawa, 1992), the Federal District (Santos & Lopes, 1995), and the South and South-East regions, indicating a gradual modification in the virulence profile of the Brazilian *V. dahliae* isolates (Reis et al., 2007; Reis & Boiteux, 2006a).

The sexual stage of *V. dahliae* is not yet reported (Klosterman et al., 2009). However, genetic recombination of strains has been detected (O'Garro & Clarkson, 1992; Usami et al., 2009b). In fact, *V. dahliae* has been characterized as a heterothallic fungus with two mating type (MAT) idiomorphs, which suggests that the putative sexual stage of the pathogen might exist under natural conditions (Erincik, 2020; Milgroom et al., 2014; Usami et al., 2009b). In Ascomycete fungi, both mating-type idiomorphs are required for successful mating (Milgroom et al., 2014). In these circumstances, the presence of fungal strains with distinct MAT idiomorphs may allow the sexual exchange of genetic material. The potential occurrence of opposite mating types either in close proximity or their migration into a single field represents a risk of sexual recombination events (Baroudy et al., 2019).

Hence, the purposes of the present study were threefold: (1) assessment of the pathogenicity and virulence profiles of *V. dahliae* isolates in bioassays with race differential tomato cultivars (with and without the *Ve*-1 resistance gene) and eggplant; (2) Identification (via a PCR-based marker system) of the physiological races 1 and 2 in a collection of *V. dahliae* isolates from Brazil, mainly from Solanaceae crops; (3) assessment of the molecular diversity of MAT idiomorphs. Thus, the present work represents a considerable expansion of the knowledge about biological diversity as well as epidemiological aspects of the *V. dahliae* populations affecting Solanaceae crops in Brazil.

Material and methods

Verticillium dahliae isolates

Eighty-nine (89) isolates of *V. dahliae*, obtained mainly from Solanaceae crops in the major vegetable-

producing regions of Brazil, were used in the present study (Suppl. Table 1). All isolates were previously inoculated and re-isolated from their original hosts, fulfilling Koch's postulates, and identified at the species level via phylogenetic analysis of three genomic regions (Suaste-Dzul et al., 2022). Fungal conidia were maintained as stock in 25% glycerol at −80 °C in the collection of plant pathogenic fungi and oomycetes of Embrapa Vegetables (Embrapa Hortaliças). For routine use throughout this work, a replica of each isolate was preserved in distilled sterilized water (Castellani, 1964) at 6 °C.

Pathogenicity and virulence bioassays: Race differential cultivars, *Verticillium dahliae* inoculation, and experimental design

Two tomato cultivars, with and without the race 1-specific resistance gene *Ve*-1 (Kawchuk et al., 2001), were used as physiological race differentials: 'Ponderosa' (susceptible to races 1 and 2), and 'Floradade' (resistant to race 1 due to the presence of the *Ve*-1 gene) (Reis et al., 2007). Additionally, the eggplant hybrid 'Ciça' was used as highly susceptible indicator for verticillium wilt symptoms, due to its high susceptibility to all races of *V. dahliae*. Seeds of the tomato and eggplant cultivars were sown in polystyrene trays with 128 cells, filled with sterile substrate Plantmax®, and maintained under greenhouse conditions for two weeks. For inoculum (conidia) production, all 89 isolates were recovered on potato dextrose agar + tetracycline (PDA, tetracycline at 50 μg/mL) and maintained at 24 °C for seven days. After that, five discs of mycelium (15 mm diameter) from pure cultures were grown in an Erlenmeyer with 100 mL potato-dextrose broth for ten days at 24 °C ± 2 °C under low agitation (99 rpm) with an orbital shaker (TE-420 Tecnal). Subsequently, the spore suspension was filtered with double gauze and the inoculum concentration was estimated with a Neubauer chamber and then adjusted to 2×10^6 conidia/mL. Seedlings were inoculated when they reached two pairs of true leaves following the protocol described by Santos (1997), with modifications (Reis et al., 2007). Plants were removed from trays, and roots were gently rinsed with water to eliminate the substrate excess. The roots of seedlings were injured at the apical zone and inoculated by immersion into 50 mL of spore suspension for 3 minutes. Afterwards, the seedlings were transplanted to plastic pots (7.8 cm × 10.2 cm), containing sterilized substrate. Then 3 mL of the spore suspension were deposited in the collar region of each seedling. Seedlings in the control treatments were similarly treated with distilled water. Inoculation bioassays were conducted under greenhouse conditions (air temperature 25 ± 4 °C and 70–80% relative humidity) in a randomized block design with three experimental units (three pots with two plants each) for each differential tomato and eggplant cultivar. Assessment of symptoms was carried out 30 days after inoculation (DAI). Fungal re-isolation from the tested plants was performed for each isolate. The assays were carried out twice.

Genomic DNA extraction and race-specific detection via PCR assays

Genomic DNA of the isolates were extracted with CTAB buffer plus organic solvents according to Boiteux et al. (1999). Mycelia were harvested directly from PDA plates, blotted dry with filter paper, and frozen at −80 °C overnight. The DNA pellet was resuspended in 100 μL of TE buffer + RNAse A (20 mg/mL) (Thermo Fisher Scientific Inc., Waltham, MA, USA). Samples were then stored at −20 °C for later use. PCR determination of *V. dahliae* races was performed using the following primer pairs: VdAve1F/VdAve1R (race 1) (Usami et al., 2007), Tr1/Tr2 (race 1) (de Jonge et al., 2012), and VdR2F/VdR2R (race 2) (Short, Gurung, Maruthachalam, et al., 2014). PCR conditions to determinate pathogenic races following Short, Gurung, Maruthachalam, et al. (2014) with modifications introduced in the present study as listed in Table 1. All DNA used to specific detection of pathogenic races were tested twice to corroborate the results.

PCR assays for mating type determination

Two sets of primers were used in this study to determinate the frequency of two idiomorphs of the MAT locus in the collection of *V. dahliae* isolates: VdMAT1–1a (5'-GTC CCT GGA GGT AGG GAG TG-3′) /VdMAT1–1b (5'-TGC TTC CTC CGT CAA GAC GC-3′) (Usami et al., 2009a), and VdMAT1–2a (5'-CGA CCG CTA CTA TAT TGG CCC-3′) /VdMAT1–2b (5'-CTG CGA CAG CAG ATT CTG GGT TGC AAA GGC -3′) (Usami et al., 2009b). Multiplex PCR amplifications were performed in a final volume of 25 μL essentially as described by Usami et al. (2009a). Also, simplex PCR conditions were

standardized in a T100 PCR thermal cycler (BioRad®) with an initial denaturation step at 95 °C for 3 minutes; 32 cycles of denaturation at 95 °C for 30 seconds, annealing at 65 °C (*MAT1–1*) and 61 °C (*MAT1–2*) for 30 seconds, and extension at 72 °C for 3 minutes; and a final extension at 72 °C for 10 minutes. Expected amplicons were ~ 400 bp, and ~ 600 bp for *MAT1–1* and *MAT1–2*, respectively. All PCR reactions were repeated twice.

Results

Verticillium isolates belong to a fungal collection maintained at Embrapa Vegetables. These isolates were obtained from different hosts such as tomato, eggplant, potato, okra, strawberry, scarlet eggplant, and cacao, being collected in different regions of Brazil from 1992 to 2019. Previous molecular studies (Suaste-Dzul et al., 2022) with this set of isolates established *V. dahliae* as the only species associated with vegetable crops in Brazil.

Pathogenicity and virulence assays

Results of the inoculation assays using 89 isolates of *V. dahliae* on two tomato cultivars, and one eggplant hybrid are presented in Table 2. In our assays, typical *Verticillum* wilt-associated symptoms were observed, such as yellowing on the lower leaves, some V-shaped areas at the leaf margins progress until they turn brown, and eventually collapse. A red-to-brown discoloration inside the vascular tissue from affected plants was observed after a longitudinal cut at the basal portion of the stems (Fig. 1). The initial symptoms were detected earlier in eggplants (18–21 DAI) than in tomato (20–23 DAI). Seventy-six (76) out of 89 isolates induced severe wilt symptoms in both tomato cultivars and on 'Ciça' eggplant, which is in accordance with the virulence profile of *V. dahliae* race 2 isolates. Only, three isolates were characterized as race 1(collected before the year 2000), based on their response on tomato differentials and eggplant. Surprisingly, ten isolates were non-pathogenic on all differentials. As expected, mock-inoculated control plants displayed no symptoms. All virulent samples were re-isolated from diseased tomato or eggplant seedlings. No isolate was obtained in tentative re-isolations from the asymptomatic plants.

PCR assays for identification of *Verticillium dahliae* races 1 and 2

In the PCR assays, the 89 *V. dahliae* isolates were analyzed using previously reported race 1-specific primers VdAve1F/VdAve1R (Fig. 2). Amplification of six *V. dahliae* isolates for VdAve1 yielded a specific DNA amplicon of 1000 bp (6/89). Alternatively, PCR with the primer pair Tr1/Tr2 was used to confirm the race 1 identity of these six isolates. A DNA fragment amplification of 680 bp was obtained with Tr1/Tr2 only in five isolates from the total of samples analyzed to race 1 (5/89) (Fig. 3). In the PCR assay with the primer pair VdR2F/VdR2R, 70 out 89 *V. dahliae* isolates were classified as race 2, generating DNA amplicons of 250 bp. Samples that were positives for race 2 were negative for race 1, indicating the race-specificity of this PCR-based assay (Fig. 4). Thirteen (13) isolates were negative in both reactions either for race 1 or race 2-specific PCR, which may indicate either genetic variants of the race 1 and 2 or the presence of isolates from the recently described race 3 (Wang et al., 2021). A strong concordance was observed between the pathogenicity/virulence assays and the results of the race by PCR (Table 2). Interestingly, the ten non-pathogenic isolates displayed molecular profiles of one race or another (Table 2).

Mating type determination by PCR assay

Both *V. dahliae* mating types were found in Brazil. However, an overabundance of the *MAT-1-1* idiomorph was detected in the collection of isolates. The primers VdMAT1–1a and VdMAT1–1b were able to amplify a 400 bp fragment in 73 isolates (82%). Only 12 isolates (13.5%) amplified amplicons of 600 bp in size with primers VdMAT1–2a and VdMAT1–2b, and thus were classified as mating type *MAT-1-2*. Interestingly, PCR products were not obtained from four isolates corresponding either *MAT-1-1* or *MAT-1-2* idiomorph. Besides, none of the isolates had both fragments, indicating the specificity of this assay (Suppl. Table 1). The PCR reactions were performed twice and corroborated negative and positive samples, either for *MAT-1-1* or *MAT-1-2* results (Fig. 5).

Table 1 Primer information and simplex PCR conditions for physiological race determination in a collection of 89 *Verticillium dahliae* isolates

Genomic target	Primer name	Primer sequence 5′-3’	Annealing temp (°C)	Amplicon (bp)
Race 1 (*Ave*1 effector)	VdAve1F VdAve1R	AAGGGGTCTTGCTAGGATGG TGAAACACTTGTCCTCTTGCT	62	1000
Race 1 (*Ave*1 effector)	Tr1 Tr2	TGAAGTAGCCGATAGCTTTGTCTTGCC TGTCTGGATTAATCGCCGCAATAGA	64	680
Race 2 (Exonic region VDAG_05863.1)	VdR2F VdR2R	ACTTAACGAAAGCATGCGC CTTGACTTGCCGGCTCC	64	256

Discussion and conclusions

Verticillium wilt has been reported in a wide range of hosts in Brazil, being especially important in Solanaceae crops such as tomato, potato, eggplant, and scarlet eggplant (Mendes et al., 2019; Reis et al., 2007; Reis & Boiteux, 2006a; b; Suaste-Dzul et al., 2022). All 89 isolates studied here were previously identified as *V. dahliae* (Suaste-Dzul et al., 2022), according to the current classification system established by Inderbitzin et al. (2011). In the present assay, most of the isolates were collected infecting Solanaceae hosts (84.3%) as well as other crops such as strawberry, okra, and cacao (15.7%).

Conventionally, physiological race determination assays for *V. dahliae* are carried out employing a set of differential cultivars, which might be a time-consuming and cumbersome procedure (Ligoxigakis & Vakalounakis, 1994; Papaioannou et al., 2013; Reis et al., 2007; Usami et al., 2017). In addition, these assessments via inoculation should be conducted under well-controlled environmental conditions suitable for both fungus and host plant in order to avoid misleading results. However, due to some observed inconsistencies, the combination of both virulence assays and molecular markers is considered the most robust and consistent strategy for race discrimination across many vascular pathogens (Gonçalves et al., 2021). On the other hand, analyses done exclusively with molecular markers could miss important and useful information from the tomato-breeding standpoint, including the potential emergence of novel pathogen variants, as observed in Japan (Usami et al., 2017).

In the present study, we evaluated the response of the tomato cultivar ‘Floradade’ (resistant only to isolates of race 1 due to the presence of the gene *Ve*-1) and in the cultivar ‘Ponderosa’ (susceptible to races 1 and 2). Plants of the eggplant hybrid ‘Ciça’ were also used as a positive indicator of symptoms due the highly susceptible reaction of this genetic material to both races (Reis et al., 2007). The frequency of race 1 isolates (3.4%) was much smaller when compared with race 2 isolates (85.4%). It is important to highlight that in the virulence test, race 1 was found only among isolates of our fungal collection collected from 1992 to 1997. Isolates of race 2 have been more prevalent in regions of the South and South-East of the country since its first report in the North-East of Brazil (Laterrot et al., 1983). In South and South East regions, environmental conditions favor epidemic outbreaks of verticillium wilt (Reis et al., 2007; Reis & Boiteux, 2006a). We observed a dramatic dispersion of *V. dahliae* race 2 isolates to many new vegetable production areas of Brazil. Historically, race 2 was reported for the first time in Pernambuco state (Laterrot et al., 1983) then, in São Paulo (Cerezine & Kurozawa, 1992) and Distrito Federal (Santos & Lopes, 1995). Subsequently, this race was reported in the states of Rio Grande do Sul, Santa Catarina, Rio de Janeiro, Espírito Santo, and Minas Gerais (Reis et al., 2007). Hence, race 2 isolates are predominating across all major vegetable-producing areas in Brazil. The monogenic *Ve*-1-derived resistance on tomato is specific towards race 1 isolates (Fradin et al., 2009) and the *Ave1* gene was found to be a major virulence factor *V. dahliae* in tomato carrying this gene (de Jonge et al., 2012). Then, the contemporary prevalence of race 2 isolates can be explained by the selection pressure on *V. dahliae* populations due to the large-scale employment of tomato cultivars/hybrids carrying the race 1-specific *Ve*-1 resistance gene in Brazil (Reis et al., 2007). Not surprisingly, isolates collected from other vegetable crops such as eggplant, scarlet eggplant, strawberry and the potato were also classified as race 2. In the present work, we also expanded the information about the geographic

Table 2 Results of the virulence assays and results of the race 1/race 2-specific PCR test

Isolate	Cultivars			Inoculation assays	Race-determination PCR assays		
	Tomato cv.		Eggplant	Race	VdAve1F/ VdAve1R	VdR2F/ VdR2R	Race
	Floradade	Ponderosa	Ciça				
Vert02	+	+	+	**2**	–	+	**2**
Vert03	+	+	+	**2**	–	+	**2**
Vert04	–	+	+	**1**	+	–	**1**
Vert05	+	+	+	**2**	–	+	**2**
Vert06	+	+	+	**2**	–	+	**2**
Vert07	+	+	+	**2**	–	+	**2**
Vert08	+	+	+	**2**	–	+	**2**
Vert09	+	+	+	**2**	+	+	**1–2***
Vert12	–	–	–	**AVR**	–	+	**2**
Vert14	–	+	+	**1**	+	–	**1**
Vert17	+	+	+	**2**	–	+	**2**
Vert21	–	+	+	**1**	–	+	**2**
Vert22	+	+	+	**2**	–	+	**2**
Vert23	+	+	+	**2**	–	+	**2**
Vert26	+	+	+	**2**	+	–	**1**
Vert32	+	+	+	**2**	–	+	**2**
Vert34	+	+	+	**2**	–	–	**NA**
Vert35	+	+	+	**2**	–	+	**2**
Vert36	–	–	–	**AVR**	–	–	**NA**
Vert38	+	+	+	**2**	–	–	**NA**
Vert43	+	+	+	**2**	–	+	**2**
Vert45	+	+	+	**2**	–	+	**2**
Vert46	+	+	+	**2**	–	+	**2**
Vert47	+	+	+	**2**	–	+	**2**
Vert53	+	+	+	**2**	–	+	**2**
Vert54	+	+	+	**2**	–	+	**2**
Vert56	+	+	+	**2**	–	–	**NA**
Vert59	–	–	–	**AVR**	–	+	**2**
Vert62	+	+	+	**2**	–	+	**2**
Vert65	+	+	+	**2**	–	+	**2**
Vert67	+	+	+	**2**	–	+	**2**
Vert70	+	+	+	**2**	–	+	**2**
Vert71	–	–	–	**AVR**	–	+	**2**
Vert74	+	+	+	**2**	–	+	**2**
Vert77	+	+	+	**2**	–	+	**2**
Vert78	+	+	+	**2**	–	+	**2**
Vert79	+	+	+	**2**	–	+	**2**
Vert93	+	+	+	**2**	–	+	**2**
Vert96	+	+	+	**2**	–	+	**2**
Vert103	+	+	+	**2**	–	+	**2**
Vert106	+	+	+	**2**	–	+	**2**
Vert110	+	+	+	**2**	–	+	**2**
Vert111	+	+	+	**2**	–	+	**2**
Vert116	–	–	–	**AVR**	–	+	**2**
Vert117	+	+	+	**2**	–	+	**2**
Vert118	+	+	+	**2**	–	+	**2**
Vert119	+	+	+	**2**	–	+	**2**

Table 2 (continued)

Isolate	Cultivars			Inoculation assays	Race-determination PCR assays		
	Tomato cv.		Eggplant	Race	VdAve1F/ VdAve1R	VdR2F/ VdR2R	Race
	Floradade	Ponderosa	Ciça				
Vert120	+	+	+	**2**	–	+	**2**
Vert121	–	–	–	**AVR**	–	+	**2**
Vert125	+	+	+	**2**	–	+	**2**
Vert129	+	+	+	**2**	–	+	**2**
Vert130	+	+	+	**2**	–	+	**2**
Vert132	–	–	–	**AVR**	–	–	**NA**
Vert134	+	+	+	**2**	–	–	**NA**
Vert137	+	+	+	**2**	–	+	**2**
Vert142	+	+	+	**2**	–	+	**2**
Vert143	+	+	+	**2**	–	+	**2**
Vert144	+	+	+	**2**	–	+	**2**
Vert145	+	+	+	**2**	–	+	**2**
Vert147	+	+	+	**2**	–	+	**2**
Vert148	+	+	+	**2**	–	+	**2**
Vert149	+	+	+	**2**	–	+	**2**
Vert150	+	+	+	**2**	–	+	**2**
Vert151	–	–	–	**AVR**	+	–	**1**
Vert158	–	–	–	**AVR**	–	–	**NA**
Vert160	+	+	+	**2**	–	+	**2**
Vert161	+	+	+	**2**	+	–	**1**
Vert163	+	+	+	**2**	–	–	**NA**
Vert164	+	+	+	**2**	–	–	**NA**
Vert166	+	+	+	**2**	–	+	**2**
Vert169	+	+	+	**2**	–	+	**2**
Vert171	+	+	+	**2**	–	–	**NA**
Vert172	+	+	+	**2**	–	+	**2**
Vert173	+	+	+	**2**	–	+	**2**
Vert174	+	+	+	**2**	–	–	**NA**
Vert176	+	+	+	**2**	–	+	**2**
Vert177	+	+	+	**2**	–	+	**2**
Vert178	+	+	+	**2**	–	+	**2**
Vert179	+	+	+	**2**	–	+	**2**
Vert180	–	–	–	**AVR**	–	–	**NA**
Vert181	+	+	+	**2**	–	+	**2**
Vert182	+	+	+	**2**	–	+	**2**
Vert183	+	+	+	**2**	–	–	**NA**
Vert184	+	+	+	**2**	–	+	**2**
Vert185	+	+	+	**2**	–	+	**2**
Vert186	+	+	+	**2**	–	+	**2**
Vert187	+	+	+	**2**	–	+	**2**
Vert188	+	+	+	**2**	–	+	**2**
Vert189	+	+	+	**2**	–	+	**2**

* DNA fragments corresponding to race 1 and race 2 were detected in Vert09 isolate

AVR: Isolates considerate as non-pathogenic without evolution of symptoms when inoculated on the differential cultivars

NA: DNA from isolates that did not amplify for race 1 or race 2 with specific primers

Fig. 1 *Verticillium* wilt reaction on plant differential cultivars with 20–23 days after inoculation. Pathogenicity phenotyping of Vert04 isolate, race 1 (**a**). Pathogenicity phenotyping of Vert47, race 2 (**b**). Pathogenicity phenotyping of Vert179 isolate, race 2 (**c**)

invasion of race 2 isolates to novel locations in the states of Bahia, Ceará, Goiás, and Paraná. This relatively fast and extensive dispersion of *V. dahliae* race 2 isolates across major Solanaceae-producing areas in Brazil suggests their nationwide dissemination via contaminated propagative material (Reis et al., 2007; Reis & Boiteux, 2006a). This can be explained by the fact that most of these isolates were collected in regions where host crops are grown in succession such as, tomato and strawberry (e.g. Espírito Santo, Distrito Federal, and Santa Catarina), tomato, eggplant, and scarlet eggplant (e.g. Ceará, Espírito Santo, Minas Gerais, Rio de Janeiro, and São Paulo), and potato and tomato (e.g. Bahia).

Ten isolates previously classified as *V. dahliae* (Suaste-Dzul et al., 2022) were not able to induce symptoms in all the differential cultivars, thus they were classified as non-pathogenic (or avirulents). These results displayed some levels of discrepancy with molecular marker analyses since five of them were positive to race 2, and one isolate was classified as race 1. Notwithstanding, four isolates were found to be negative either to race 1 or race 2 markers. In addition, different levels of aggressiveness were observed (data not shown) among of the isolates, indicating the presence of either potential environmental effect (e.g. adaptation to culture

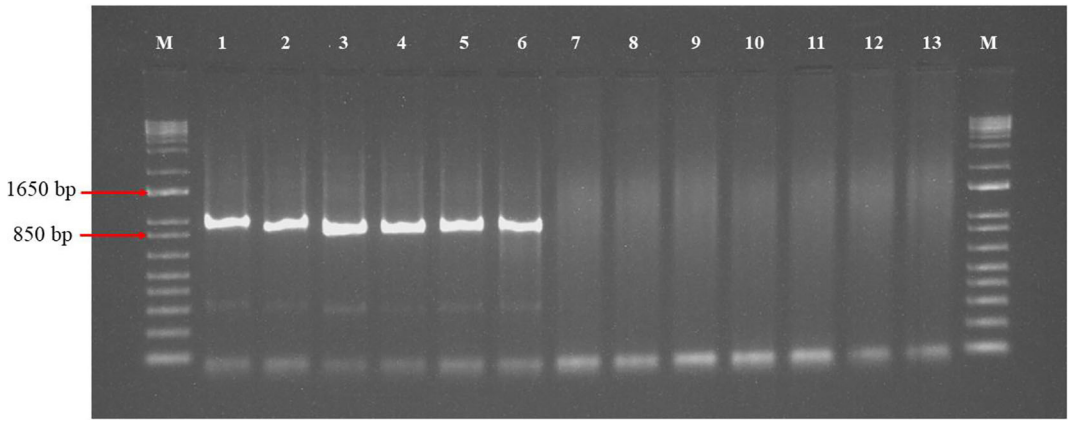

Fig. 2 Gel electrophoresis of amplicons produced by PCR assays using race 1 specific primers VdAve1F and VdAveR (1000 bp). Lanes 1–6: Vert04, Vert09, Vert14, Vert26, Vert151, Vert161 (race 1: nonpathogenic on tomato cv. Floradade, pathogenic on cv. Ponderosa, and pathogenic on eggplant cv. Ciça); Lanes 7–11: Vert17, Vert35, Vert148, Vert172, Vert182 (race 2: pathogenic on tomato cv. Floradade, pathogenic on cv. Ponderosa, pathogenic on eggplant cv. Ciça). Lane 12: No template control (NTC). Lane 13: Negative control (exogenous DNA of *Rhizoctonia solani*). Lane M: 1 Kb molecular weight marker

medium) or genetic differences among isolates for pathogenicity and virulence traits.

Assay to discriminate race 1 from race 2 by PCR-specific primers showed 78.6% of concordance with the results in the pathogenicity assays (Table 2). In our study, race 1 and race 2-specific primers were successfully validated to determine a rapid differentiation of these two races on a large collection of isolates from different hosts and geographic locations (Fig. 1). After screening all isolates, we found a predominance of race 2 over race 1 regardless of the host (Table 2). PCR products were not obtained from 13 isolates in the single and multiplex PCR for both races and corroborated two times (data not shown). These race-specific primers have been used and validated with high effectivity to differentiate *V. dahliae* races occurring mainly in tomato, lettuce, cotton, and olive (Maruthachalam et al., 2010; Short, Gurung, Maruthachalam, et al., 2014; Usami et al., 2007). To our knowledge, this is the first extensive study carried out for determination of pathogenic races of *V. dahliae* using molecular approaches in Solanaceae and other vegetable hosts from Brazil.

The present study represents thus far, the most comprehensive analysis of the race frequency and distribution of *V. dahliae* isolates associated with verticillium wilt of tomato and other vegetables in Brazil. Even though race identification using molecular methods is more practical than employing time-consuming inoculation tests (Usami et al., 2017), it became clear that the combination of pathogenicity tests and molecular marker assays displays some interesting advantages. As demonstrated here, analyses done exclusively with

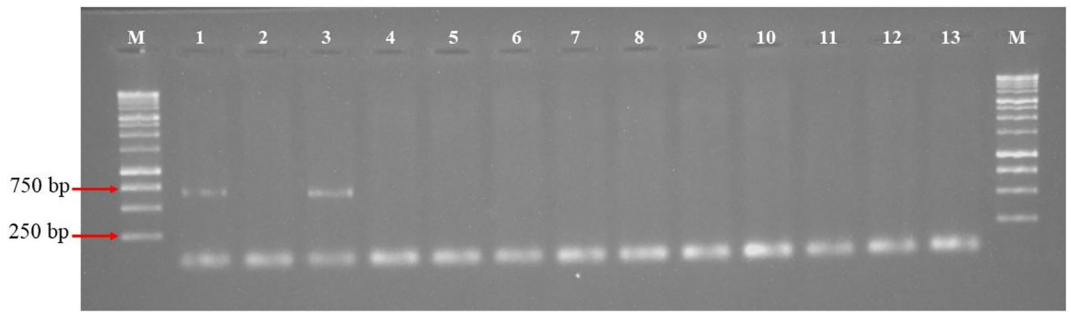

Fig. 3 Gel electrophoresis of amplicons produced by PCR assays using race 1 specific primers Tr1 and Tr2 (680 bp). Lanes **1–10:** Vert151, Vert158, Vert161, Vert163, Vert171, Vert174, Vert179, Vert180, Vert183, Vert189. Lane 11: No template control (NTC). Lane 12: Negative control (exogenous DNA from *Rhizoctonia solani*). Lane 13: No template control 2 (NTC). Lane M: 1 Kb molecular weight marker

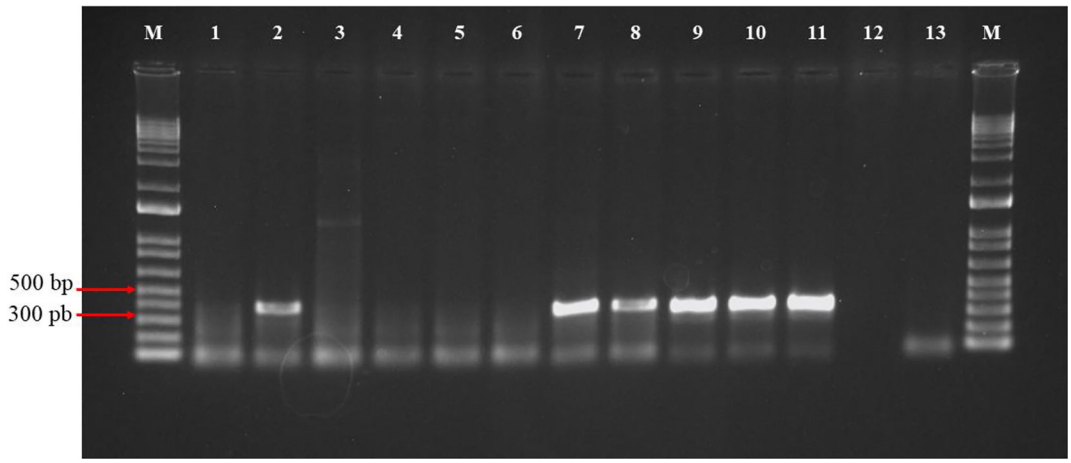

Fig. 4 Gel electrophoresis of amplicons produced by PCR assays using race 2 specific primers VdR2F and VdR2R (256 bp). Lanes 1–6: Vert04, Vert09, Vert14, Vert26, Vert151, Vert161 (race 1: non-pathogenic on tomato cv. Floradade); Lanes 7–11: Vert17, Vert35, Vert148, Vert172, Vert182 (race 2: pathogenic on tomato cv. Floradade, pathogenic on cv. Ponderosa, and pathogenic on eggplant cv. Ciça). Lane 12: No template control (NTC). Lane 13: Negative control (exogenous DNA of *Rhizoctonia solani*). Lane M: 1 Kb molecular weight marker

molecular markers could not detected important phenotypic variation across isolates, including the potential emergence of novel pathogen variants, especially new races of *V. dahliae*.

Even though the analysis of *V. dahliae* populations indicated a clonal structure (Milgroom et al., 2014) with no report of the sexual stage under natural conditions (Erincik, 2020; Milgroom et al., 2014; Usami et al., 2009b), the proximity of opposite mating types may represent a risk of sexual recombination (Baroudy et al., 2019). Both *MAT* idiomorphs were detected in the molecular analyses of our collection of isolates, but most *V. dahliae* isolates displayed the *MAT1–1* (82%) pattern. Inconclusive results were observed for a small subgroup of isolates (4.5%), avoiding the precise determination of their mating type via PCR. Notwithstanding, the simultaneous presence of both *MAT* idiomorphs of this pathogen opens the possibility of sporadic events of sexual reproduction among *V. dahliae*

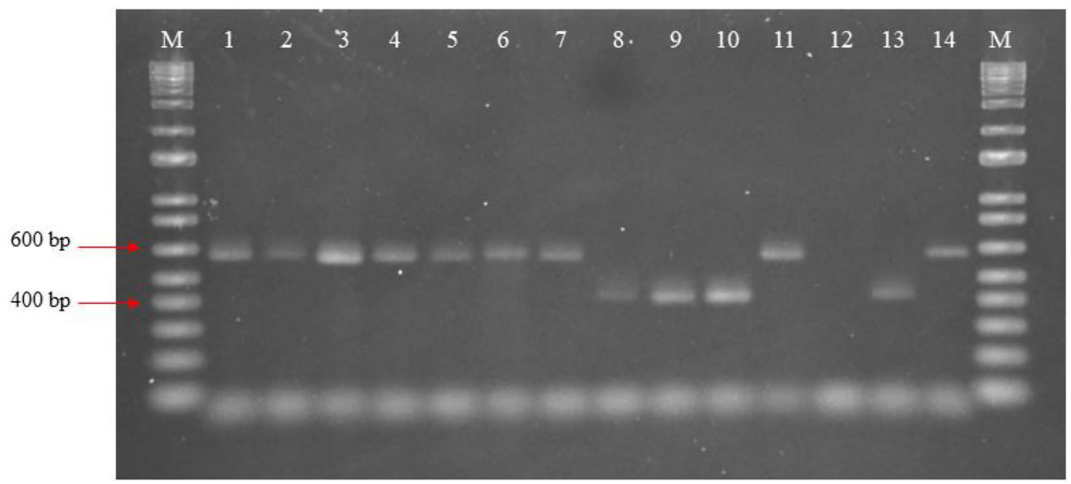

Fig. 5 Gel electrophoresis of amplicons produced by Multiplex PCR using MAT specific primers MAT1–1a/MAT1b (~400 bp), and MAT1–2a/MAT1–2b (~600 bp). Lanes 1: Vert04; 2: Vert09; 3: Vert14; 4: Vert26; 5: Vert151; 6: Vert161; 7: Vert17; 8: Vert35; 9: Vert148; 10: Vert172; 11: Vert182; Lane 12: No template control (NTC). Lane 13: Positive control to *MAT1–1* idiomorph (DNA of Vert117). Lane 14: Positive control to *MAT1–2* idiomorph (DNA of Vert166). Lane M: 1 Kb molecular weight marker

populations from Brazil, enabling the potential emergence of either new recombinant isolates with broader host ranges or novel physiological races (Table 3). Our molecular analyses showing the prevalence of *MAT1–1* isolates also revealed a peculiar scenario of mating type idiomorph distribution under Brazilian conditions. For instance, *MAT1–2* idiomorph has been found as the predominant mating type in different hosts across many areas around the world (Milgroom et al., 2014; Short, Gurung, Hu, et al., 2014; Usami et al., 2009a). Until now, *MAT1–1* isolates have been detected at the coastal areas of California (Inderbitzin and Subbarao, unpublished data cited in Atallah et al., 2010), three *MAT1–1* isolates were found among 49 Japanese isolates (Usami et al., 2009b), and only one out of five lettuce isolates in Usami et al. (2012). The reason why the *MAT1–1* isolates are prevalent in Brazil remains elusive, but one possible explanation is the occurrence of relatively few introduction events into the country territory that were followed by massive geographical distribution of a restricted number of fungal isolates via, for instance, lots of infested seeds of a leading tomato hybrid or tubers of a leading potato cultivar. Differently from other countries, *MAT1–1* predominates instead of *MAT1–2* (Table 3) in Brazil. It is an important additional information to know how *MAT1–1* was introduced in Brazil and if this mating type displays advantageous features that allow its predominance in the county.

Also, it is interestingly further research to check whether sexual recombination between Brazilian isolates of *V. dahliae* is possible since the sexual partner is apparently present. The *MAT1–1* was probably introduced in Brazil by vegetable seeds or seedlings. Brazil imports most of the tomato seeds used by growers and also most of the strawberry seedlings. After the introduction of this mating type, it was most likely dispersed in the country by contaminated seeds and seedlings.

Table 3 Summary of the results of the molecular assays to identify *MAT* type in *V. dahliae* isolates

Hosts	PCR assay results		
	MAT1–1	*MAT1–2*	No sex-related gene
Tomato	32	6	2
Eggplant	11	4	1
Potato	14	1	0
Scarlet eggplant	3	0	0
Strawberry	10	1	1
Okra	1	0	0
Cacao	2	0	0
No. of isolates	73	12	4

The former lack of robust and comprehensive information about the predominance and distribution of *V. dahliae* races and mating types was a major constraint for establishing effective crop management. In fact, the information reported here is very important to guide disease resistance breeding programs in tomato and other Solanaceae vegetables in Brazil. In the present scenario, the majority of isolates of *V. dahliae* infecting vegetables was classified as race 2. These results are of great concern, since the cultivars found in the Brazilian market do not have resistance to race 2 isolates. Another concern is the detection of both *MAT* idiomorphs, even though the majority of the *V. dahliae* isolates displayed the *MAT1–1* (82%) pattern in our molecular analyses. The simultaneous presence of both *MAT* idiomorphs of this pathogen opens the possibility of sporadic events of sexual reproduction among *V. dahliae* populations from Brazil, enabling the rare but potential emergence of either new recombinant isolates with broader host ranges or novel physiological races (Milgroom et al., 2014; Usami et al., 2009a; b).

Our results reinforce the view that verticillium wilt is currently one of the major threats to the Solanaceae vegetable agribusiness in Brazil, indicating that breeding programs should intensify the effective incorporation into adapted cultivars/inbred lines of resistance factor to *V. dahliae* race 2 already detected in tomato germplasm (Stamova, 2005; Usami et al., 2017). The verticillium wilt scenario may become more complicated in the near future due to the possible introduction into the county of isolates from the novel *V. dahliae* race 3 (Ingram et al., 2020). In fact, we detected here thirteen (13) isolates that were negative in PCR assays with both race 1 or race 2-specific markers, which may indicate either endemic genetic variants of the race 1 and 2 populations or the yet undetected presence of race 3 isolates. The availability of molecular markers for race 3-specific detection (Wang et al., 2021) will be useful in confirming the potential presence of isolates from this novel physiological race variant in Brazil.

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Declarations

Competing interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Acharya, B., Ingram, T. W., Oh, Y., Adhikari, T. B., Dean, R. A., & Louws, F. J. (2020). Opportunities and challenges in studies of host-pathogen interactions and management of *Verticillium dahliae* in tomatoes. *Plants, 9*, 1622. https://doi.org/10.3390/plants9111622

Alexander, L. J. (1962). Susceptibility of certain *Verticillium*-resistant tomato varieties to an Ohio isolate of the pathogen. *Phytopathology, 52*, 998–1000.

Atallah, Z. K., Maruthachalam, K., du Toit, L., Koike, S. T., Davis, R. M., Klosterman, S. J., Hayes, R. J., & Subbarao, K. V. (2010). Population analyses of the vascular plant pathogen *Verticillium dahliae* detect recombination and transcontinental gene flow. *Fungal Genetics and Biology, 47*, 416–422. https://doi.org/10.1016/j.fgb.2010.02.003

Baroudy, F., Putman, A. I., Habib, W., Puri, K. D., Subbarao, K. V., & Nigro, F. (2019). Genetic diversity of *Verticillium dahliae* populations from olive and potato in Lebanon. *Plant Disease, 103*(4), 656–667. https://doi.org/10.1094/PDIS-03-18-0420-RE

Boiteux, L. S., Fonseca, M. E. N., & Simon, P. W. (1999). Effects of plant tissue and DNA purification method on RAPD-based genetic fingerprinting analysis in carrot. *Journal of American Society for Horticultural Science, 124*(1), 32–38. https://doi.org/10.21273/JASHS.124.1.32

Bryan, M. K. (1925). Phytopathological notes. *Verticillium wilt of tomato. Phytopathology, 15*, 187–188.

Carroll, C. L., Carter, C. A., Goodhue, R. E., Lawell, C. Y. C. L., & Subbarao, K. V. (2018). A review of control options and externalities for *Verticillium* wilts. *Phytopathology, 108*(2), 160–171. https://doi.org/10.1094/PHYTO-03-17-0083-RVW

Castellani, A. (1964). The water cultivation of pathogenic fungi. *Annales des Societés Belges de Médicine Tropicale. Parasitol. Mycol., 44*, 217–219.

Cerezine, P. C., & Kurozawa, C. (1992). Variabilidade patogênica de *Verticillium albo-atrum* em tomateiro. *Pesquisa Agropecuária Brasileira, 27*, 1153–1160.

Chavarro-Carrero, E. A., Vermeulen, J. P., Torres, D. E., Usami, T., Schouten, H. J., Bai, Y., Seidl, M. F., & Thomma, B. P. H. J. (2020). Comparative genomics reveals the in planta-secreted *Verticillium dahliae* Av2 effector protein recognized in tomato plants that carry the V2 resistance locus. *Environmental Microbiology, 23*(4), 1941–1958. https://doi.org/10.1111/1462-2920.15288

de Jonge, R., Van Esse, H. P., Maruthachalam, K. M. D., Bolton, P., Santhanam, M., Keykha, S., Zhang, Z., Usami, T., Bart, L., Subbarao, K. V., & Thomma, B. P. H. J. (2012). Tomato immune receptor Ve1 recognizes effector of multiple fungal pathogens uncovered by genome and RNA sequencing. *PNAS, 109*, 5110–5115. https://doi.org/10.1073/pnas.1119623109

Deketelaere, S., Tyvaert, L., França, S. C., & Höfte, M. (2017). Desirable traits of a good biocontrol agent against *Verticillium* wilt. *Frontiers in Microbiology, 8*, 1186. https://doi.org/10.3389/fmicb.2017.01186

Diwan, N., Fluhr, R., Eshed, Y., Zamir, D., & Tanksley, S. D. (1999). Mapping of Ve in tomato: A gene conferring resistance to the broad-spectrum pathogen, *Verticillium dahliae* race 1. *Theoretical and Applied Genetica, 98*, 315–319. https://doi.org/10.1007/s001220051075

Domsch, K. H., Gams, W., & Traute-Heidi, A. (1980). *Compendium of soil Fungi* (pp. 829–845). Academic.

Erincik, B. G. (2020). Mating types of *Verticillium dahliae* isolates from cotton in Aydın Province/Turkey. *Journal of Plant Disease and Protection, 127*(11), 677–681. https://doi.org/10.1007/s41348-020-00321-1

Farr, D. F., & Rossman A. Y. (2021). Fungal Databases, U.S. National Fungus Collections, ARS, USDA. https://nt.ars-grin.gov/fungaldatabases/. (Accessed April 13, 2022).

Fradin, E. F., Zhang, Z., Juarez-Ayala, J. C., Castroverde, C. D. M., Nazar, R. N., Robb, J., Liu, C.-M., & Thomma, B. P. H. J. (2009). Genetic dissection of *Verticillium* wilt resistance mediated by tomato Ve1. *Plant Physiology, 150*(1), 320–332. https://doi.org/10.1104/pp.109.136762

Gonçalves, A. M., Cabral, C. S., Reis, A., Fonseca, M. E. N., Costa, H., Ribeiro, F .H. S., & Boiteux, L. S. (2021). A three-decade survey of Brazilian *fusarium oxysporum* f. sp. *lycopersici* races assessed by pathogenicity tests on differential tomato accessions and by molecular markers. Journal of Applied Microbiology, 131, 873–884. https://doi.org/10.1111/jam.14966.

Hayes, R. J., Vallad, G. E., Qin, Q. M., Grube, R. C., & Subbarao, K. V. (2007). Variation for resistance to *Verticillium* wilt in lettuce (*Lactuca sativa* L.). *Plant Disease, 91*(4), 439–445. https://doi.org/10.1094/PDIS-91-4-0439

Inderbitzin, P., Bostock, R. M., Davis, R. M., Usami, T., Platt, H. W., & Subbarao, K. V. (2011). Phylogenetics and taxonomy of the fungal vascular wilt pathogen *Verticillium*, with the descriptions of five new species. *PLoS One, 6*, e28341. https://doi.org/10.1371/journal.pone.0028341

Ingram, T. W., Oh, Y., Adhikari, T. B., Louws, F. J., & Dean, R. A. (2020). Comparative genome analyses of 18 *Verticillium dahliae* tomato isolates reveals phylogenetic and race specific signatures. *Frontiers in Microbiology, 11*, 573755. https://doi.org/10.3389/fmicb.2020.573755

Jiménez-Díaz, R. M., Cirulli, M., Bubici, G., Jimenez-Gasco, M. M., Antoniou, P. P., & Tjamos, E. C. (2012). Verticillium wilt, a major threat to olive production: Current status and future prospects for its management. *Plant disease*, St Paul, *96*, 304–329.

Johnson, D. A., & Dung, J. K. S. (2010). *Verticillium* wilt of potato – The pathogen, disease, and management. *Canadian*

Journal of Plant Pathology, 32(1), 58–67. https://doi.org/10.1080/07060661003621134

Kawchuk, L. M., Hachey, J., Lynch, D. R., Kulcsar, F., van Rooijen, G., Waterer, D. R., Robertson, A., Kokko, E., Byers, R., Howard, R. J., Fischer, R., & Prüfer, D. (2001). Tomato Ve disease resistance genes encode cell surface-like receptors. *PNAS, 98*, 6511–6515. https://doi.org/10.1073/pnas.091114198

Klosterman, S. J., Atallah, Z. K., Vallad, G. E., & Subbarao, K. V. (2009). Diversity, pathogenicity, and management of *Verticillium* species. *Annual Review of Phytopathology, 47*, 39–62. https://doi.org/10.1146/annurev-phyto-080508-081748

Laterrot, H., Melo, P. C. T., & Blancard, D. (1983). Ocorrência da raça 2 de *Verticillium* em tomate (*Lycopersicon esculentum* Mill.) no Estado de Pernambuco, Brasil. *Horticultura Brasileira, 1*, 22–25.

Ligoxigakis, E. K., & Vakalounakis, D. J. (1994). The incidence and distribution of races of *Verticillium dahliae* in Crete. *Plant Pathology, 43*, 755–758. https://doi.org/10.1111/j.1365-3059.1994.tb01616.x

Maruthachalam, K., Atallah, Z. K., Vallad, G. E., Klosterman, S. J., Hayes, R. J., Davis, R. M., & Subbarao, K. V. (2010). Molecular variation among isolates of *Verticillium dahliae* and polymerase chain reaction-based differentiation of races. *Phytopathology, 100*(11), 1222–1230. https://doi.org/10.1094/PHYTO-04-10-0122

Mendes, M. A. S., Urben, A. F., & Dianese, J. C. (2019). *Fungi on plants in Brazil, second ed*. Escola Nacional de Gestão Agropecuária.

Milgroom, M. G., Jiménez-Gasco, M., del, M., Olivares-García, C., Drott, M. T., & Jiménez-Díaz, R. M. (2014). Recombination between clonal lineages of the asexual fungus *Verticillium dahliae* detected by genotyping by sequencing. *PLoS One, 2*, 106740. https://doi.org/10.1371/journal.pone.0106740

Miranda, B. E. C., Boiteux, L. S., Cruz, E. M., & Reis, A. (2010). Fontes de resistência em acessos de *Solanum* (secção *Lycopersicon*) a *Verticillium dahliae* raças 1 e 2. *Horticultura Brasileira, 28*(4), 458–465. https://doi.org/10.1590/S0102-05362010000400014

Mohan, S. K., Davis, J. R., Corsini, D. L., Sorensen, L. H., & Pavek, J. J. (1990). Reaction of potato clones and accessions of *Solanum* spp. to *Verticillium dahliae* Kleb. And its toxin. *Potato Research, 33*(4), 449–458. https://doi.org/10.1007/BF02358022

O'Garro, L. W., & Clarkson, J. M. (1992). Variation for pathogenicity on tomato among parasexual recombinants of *Verticillium dahliae*. *Plant Pathology, 41*(2), 141–147. https://doi.org/10.1111/j.1365-3059.1992.tb02331.x

Papaioannou, I. A., Ligoxigakis, E. K., Vakalounakis, D. J., Markakis, E. A., & Typas, M. A. (2013). Phytopathogenic, morphological, genetic and molecular characterization of a *Verticillium dahliae* population from Crete, Greece. *European Journal of Plant Pathology, 136*(3), 577–596. https://doi.org/10.1007/s10658-013-0189-4

Pegg, G. F., & Brady, B. L. (2002). *Verticillium wilts*. CABI International.

Reis, A., & Boiteux, L. S. (2006a). Murcha-de-*Verticillium*: um sério problema para o cultivo de hortaliças no Brasil. *Brasília: Embrapa Hortaliças. Circular Técnica, 40*, 11.

Reis, A., & Boiteux, L. S. (2006b). Círculo de hospedeiras de isolados de *Verticillium dahliae* obtidos de tomateiro, quiabeiro e morangueiro. *Boletim de Pesquisa e Desenvolvimento, 21*, 16.

Reis, A., Boiteux, L. S., & Costa, H. (2007). Determinação de espécies e de raças de isolados de *Verticillium* oriundos de diferentes Estados do Brasil. *Boletim de Pesquisa e Desenvolvimento, 31*, 13.

Santos, J. R. M. (1997). Methodology for screening tomato for fusarium wilt, Verticillium wilt, gray leaf spot, early blight and *Septoria* leaf blight. In *In: International conference on the processing tomato. First international symposium on tropical tomato diseases, proceedings Alexandria* (pp. 164–166). ASHS IPA.

Santos, J. R. M., & Lopes, C. A. (1995). Ocorrência de *Verticillium dahliae* raça 2 em tomateiro no Distrito Federal. *Fitopatologia Brasileira, 20*, 355.

Schaible, L., Cannon, O. S., & Waddoups, V. (1951). Inheritance of resistance to *Verticillium* wilt in a tomato cross. *Phytopathology, 41*, 986–990.

Short, D. P. G., Gurung, S., Hu, X., Inderbitzin, P., & Subbarao, K. V. (2014). Maintenance of sex-related genes and the co-occurrence of both mating types in *Verticillium dahliae*. *PLoS One, 9*, e112145. https://doi.org/10.1371/journal.pone.0112145

Short, D. P. G., Gurung, S., Maruthachalam, K., Atallah, Z. K., & Subbarao, K. V. (2014). *Verticillium dahliae* race 2-specific PCR reveals a high frequency of race 2 strains in commercial spinach seed lots and delineates race structure. *Phytopathology, 104*, 779–785. https://doi.org/10.1094/PHYTO-09-13-0253-R

Stamova, L. (2005). Resistance to *Verticillium dahliae* race 2 and its introgression into processing tomato cultivars. *Acta Horticulturae, 695*, 257–262. https://doi.org/10.17660/ActaHortic.2005.695.28

Suaste-Dzul, A. P., Veloso, J. S., Costa, H., Boiteux, L. S., Lourenço, V., Jr., Lopes, C. A., & Reis, A. (2022). Verticillium diseases of vegetable crops in Brazil: Host range, microsclerotia production, molecular haplotype network, and pathogen species determination. *Plant Pathology, 71*, 1417–1430. https://doi.org/10.1111/ppa.13562

Usami, T., Ishigaki, S., Takashina, H., Matsubara, Y., & Amemiya, Y. (2007). Cloning of DNA fragments specific to the pathotype and race of *Verticillium dahliae*. *Gen. Plant pathology, 73*, 89–95. https://doi.org/10.1007/s10327-006-0334-4

Usami, T., Itoh, M., & Amemiya, Y. (2009a). Mating type gene *MAT1-2-1* is common among Japanese isolates of *Verticillium dahliae*. *Physiological and Molecular Plant Pathology, 73*, 133–137. https://doi.org/10.1016/j.pmpp.2009.04.002

Usami, T., Itoh, M., & Amemiya, Y. (2009b). Asexual fungus *Verticillium dahliae* is potentially heterothallic. *Journal of General Plant Pathology, 75*(6), 422–427. https://doi.org/10.1007/s10327-009-0197-6

Usami, T., Itoh, M., Morii, S., Miyamoto, T., Kaneda, M., Ogawara, T., & Amemiya, Y. (2012). Involvement of two different types of *Verticillium dahliae* in lettuce wilt in Ibaraki prefecture, Japan. *Journal of General Plant Pathology, 78*(5), 348–352. https://doi.org/10.1007/s10327-012-0394-6

Usami, T., Momma, N., Kikuchi, S., Watanabe, H., Hayashi, A., Mizukawa, M., Yoshino, K., & Ohmori, Y. (2017). Race 2 of *Verticillium dahliae* infecting tomato in Japan can be split into two races with differential pathogenicity on resistant rootstocks. *Plant Pathology, 66*(2), 230–238. https://doi.org/10.1111/ppa.12576

Vallad, G. E., & Subbarao, K. V. (2008). Colonization of resistant and susceptible lettuce cultivars by a green fluorescent protein-tagged isolate of *Verticillium dahliae*. *Phytopathology, 98*(8), 871–885. https://doi.org/10.1094/PHYTO-98-8-0871

Wang, D., Zhang, D. D., Usami, T., Liu, L., Yang, L., Huang, J.-Q., Song, J., Li, R., Kong, Z.-Q., Li, J.-J., Wang, J., Klosterman, S. J., Subbarao, K. V., Dai, X.-F., & Chen, J.-Y. (2021). Functional genomics and comparative lineage-specific region analyses reveals novel insights into race divergence in *Verticillium dahliae*. *Microbiology Spectrum, 9*(3), e01118–e01121. https://doi.org/10.1128/Spectrum.01118-21