

# Verticillium diseases of vegetable crops in Brazil: Host range, microsclerotia production, molecular haplotype network, and pathogen species determination

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## Abstract

Recent outbreaks of *Verticillium* wilt diseases in various vegetable crops have been reported in Brazil. This fact was our initial stimulus to carry out a nationwide survey aiming to determine their causal agent(s). Thus far, *Verticillium dahliae* has been reported as the predominant species based solely on morphological traits. As other *Verticillium* species can be associated with wilt diseases, we characterized a collection of 80 isolates, collected across 10 agricultural Brazilian regions, by combining morphological, biological, and molecular traits. A multilocus approach was employed for identification of *Verticillium* species with information from three genomic regions (ribosomal internal transcribed spacer region, glyceraldehyde-3-phosphate dehydrogenase, and actin genes). Only 21 out of the 80 isolates were unable to produce microsclerotia in culture. The analyses of all genomic regions indicated *V. dahliae* as the sole species associated with vascular wilt of distinct hosts, including major solanaceous vegetables and other hosts such as strawberry, okra, and cacao. Pathogenicity tests confirmed the infection by the *V. dahliae* isolates and the development of typical disease symptoms on their original hosts. This is the first nationwide characterization of *Verticillium* isolates associated with major vegetable crops in Neotropical areas. This provides valuable information to design sound management strategies for these diseases, mainly for establishing efficient rotation systems and for the development of resistant cultivars.

## KEYWORDS

multigene, Neotropical areas, phylogenetic analysis, vegetables, *Verticillium dahliae*, *Verticillium* wilt

## 1 | INTRODUCTION

The genus *Verticillium* encompasses a cosmopolitan group of Ascomycota fungi with economic and agroecological significance (Klosterman et al., 2009). *Verticillium* species are able to infect a large number of eudicot plants in temperate and subtropical regions (Barbara & Clewes, 2003; Inderbitzin & Subbarao, 2014). So far, most monocot plants are considered as nonhosts of *Verticillium* species (Fradin & Thomma, 2006).

Currently, 10 *Verticillium* species are recognized (Inderbitzin, Davis, et al., 2011), including major pathogens in global agriculture (Pegg & Brady, 2002). After this novel taxonomic assignment, the genus is now referred to as *Verticillium sensu stricto* (Inderbitzin et al., 2013), encompassing the two most notorious and economically important species: *Verticillium albo-atrum* and *V. dahliae* (Klosterman et al., 2009). In recent decades, *V. dahliae* was classified as a member of a subgroup of strains within the *V. albo-atrum* complex, which is

characterized by the production of microsclerotia and the presence of dark mycelium. However, after many controversies, *V. dahliae* is currently accepted as a separate species (Fradin & Thomma, 2006). The reclassification and reassessment of the taxonomic status of the genus *Verticillium* was carried out using a combination of morphological and phylogenetic approaches (Inderbitzin, Bostock, et al., 2011). This classification introduced substantial changes in the range of hosts as well as in the geographical distribution of members of the genus (Barbara & Clewes, 2003; Inderbitzin, Davis, et al., 2011; Jing et al., 2018).

*V. dahliae* is a soilborne fungus and the most prominent wilt agent within this genus (Depotter et al., 2016). This fungal species colonizes the vascular system of its host plants (Reusche et al., 2014), and infection slowly progresses through the vasculature into the shoots (Carroll et al., 2018). Although *V. dahliae* is a well-studied fungus, it is still a pathogen of concern due to its ability to induce severe damage in a broad range of hosts and to cause serious outbreaks either in new hosts or in new agricultural areas (Acharya et al., 2020; Bhat & Subbarao, 1999; López-Escudero & Mercado-Blanco, 2011). Curative control of *Verticillium* wilt is difficult, especially after the pathogen reaches the vascular tissue (Deketelaere et al., 2017). For example, there are currently no efficient fungicides available to control *Verticillium* wilt once plants have been infected (Fradin & Thomma, 2006).

In Brazil, *Verticillium* wilt is a particularly important disease due to the substantial economic losses to the vegetable agribusiness (Reis & Boiteux, 2006a). Recent outbreaks of *Verticillium* wilt have increased the concern of the vegetable crop production sector in relation to this disease (Lopes et al., 2018; Reis & Boiteux, 2006b; Suaste-Dzul et al., 2021). Since the late 1980s, the disease has been associated with two causal agents, *V. dahliae* and *V. albo-atrum*, probably because of the similarity in their morphological traits, being distinguished mostly by the presence (or absence) of microsclerotia (Mendes et al., 2019; Reis et al., 2007). However, *V. dahliae* has been reported as the prevalent species affecting several vegetables such as tomato, eggplant, scarlet eggplant, strawberry, and okra (Mendes et al., 2019; Reis & Boiteux, 2006a). Furthermore, in tomato, only *V. dahliae* has been reported in Brazil (Reis & Boiteux, 2006b). Despite their importance, no comprehensive study has been conducted to identify *Verticillium* species in Brazil using more precise genetic approaches. In this context, the objective of the present study was to characterize, via molecular phylogenetic analysis, a representative collection of *Verticillium* isolates recovered from major vegetable (mainly solanaceous) crops in Brazil.

## 2 | MATERIALS AND METHODS

### 2.1 | Fungal isolates

The *Verticillium* isolates used in this study were obtained from the plant-pathogenic fungal collection of the Embrapa Hortaliças (CNPq), Distrito Federal, Brazil. This fungal collection was initiated in 1992

and is maintained under cold storage conditions. The 80 *Verticillium* isolates used in this work were originally collected across nine different Brazilian states and from the Distrito Federal. Samples from plants showing typical *Verticillium* wilt symptoms (Reis & Boiteux, 2006a, 2006b; Reis et al., 2007) were collected across the following Brazilian states: Bahia (BA,  $n = 5$ ), Ceará (CE,  $n = 2$ ), Distrito Federal (DF,  $n = 12$ ), Espírito Santo (ES,  $n = 19$ ), Minas Gerais (MG,  $n = 16$ ), Paraná (PR,  $n = 1$ ), Rio de Janeiro (RJ,  $n = 4$ ), Santa Catarina (SC,  $n = 5$ ), and São Paulo (SP,  $n = 14$ ), and two isolates from unknown locations. The sampled isolates were found in association with the following crops and in the following numbers: tomato (*Solanum lycopersicum*,  $n = 39$ ), potato (*S. tuberosum*,  $n = 13$ ), eggplant (*S. melongena*,  $n = 13$ ), scarlet eggplant (*S. aethiopicum*,  $n = 3$ ), okra (*Abelmoschus esculentus*,  $n = 1$ ), strawberry (*Fragaria × ananassa*,  $n = 9$ ), and cacao (*Theobroma cacao*,  $n = 2$ ) (Table 1). Fungal isolates were grown on potato dextrose agar (PDA) plates under light/dark conditions (12 h/12 h) at  $23 \pm 4^\circ\text{C}$  for 2 weeks. Conidia were maintained as stock in 25% glycerol at  $-80^\circ\text{C}$  in the plant-pathogenic fungal collection of CNPH. A replica of each isolate was preserved in sterile distilled water at  $6^\circ\text{C}$  (Castellani, 1963) for routine use throughout this work.

### 2.2 | Cultural characteristics, morphology, and microsclerotia production

The ability of *V. dahliae* isolates to produce microsclerotia in PDA has been used as the main morphological trait for discriminating them from *V. albo-atrum* isolates (Barbara & Clewes, 2003; Fradin & Thomma, 2006; Isaac, 1967). Therefore, we conducted an initial phenotypic classification of pure cultures of the 80 *Verticillium* isolates on PDA plates to describe their representative morphological attributes. Three plates of each isolate were incubated at  $23^\circ\text{C}$  under dark conditions and observed daily for aspects of the colonies and mycelia as well as microsclerotia production. Morphological data of the main fungal structures (conidia and conidiophores) were recorded using a Zeiss microscope (40 $\times$ ). Microsclerotia production was assessed 14–18 days after the plates were completely filled by mycelial growth.

### 2.3 | Fungal DNA extraction

Genomic DNA of the isolates was extracted with CTAB buffer and organic solvents according to Boiteux et al. (1999). Mycelia were harvested directly from PDA plates, blotted dry with filter paper, and frozen at  $-80^\circ\text{C}$  overnight. The tissue was individually transferred into microcentrifuge tubes (2 ml) containing two tungsten carbide beads (5 mm; QIAGEN) and 1 ml buffer lysis (50 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0, 400 mM NaCl, 10 mM  $\beta$ -mercaptoethanol). All the samples were homogenized twice at 20 Hz for 3 min with Tissue Lyser II systems (QIAGEN). The DNA pellet was resuspended in 100  $\mu\text{l}$  of TE buffer + RNase A (20 mg/ml; Thermo Fisher Scientific Inc.). After extraction,

TABLE 1 Isolates of *Verticillium dahliae* used in the study

| Isolate ID | Host origin      | Geographical location <sup>a</sup> | Year of collection | ITS GenBank accession number |
|------------|------------------|------------------------------------|--------------------|------------------------------|
| Vert02     | Tomato           | SP                                 | 1992               | OM906910                     |
| Vert03     | Tomato           | SP                                 | 1992               | OM911987                     |
| Vert04     | Tomato           | SP                                 | 1992               | OK398231                     |
| Vert05     | Tomato           | DF                                 | 1994               | OM911988                     |
| Vert06     | Tomato           | DF                                 | 1995               | OM911989                     |
| Vert07     | Eggplant         | DF                                 | 1995               | OM911990                     |
| Vert08     | Eggplant         | DF                                 | 1997               | OM911991                     |
| Vert09     | Eggplant         | DF                                 | 1997               | OM907008                     |
| Vert12     | Okra             | MG                                 | 1997               | OM911992                     |
| Vert14     | Eggplant         | SP                                 | 1997               | OK398232                     |
| Vert17     | Eggplant         | SP                                 | 1997               | OK398233                     |
| Vert21     | Potato           | Unknown                            | 1997               | OM911993                     |
| Vert22     | Cacao            | BA                                 | 1997               | OK398234                     |
| Vert23     | Cacao            | BA                                 | 1997               | OM911994                     |
| Vert26     | Tomato           | Unknown                            | 1997               | OK398239                     |
| Vert32     | Tomato           | ES                                 | 2004               | OM911995                     |
| Vert34     | Tomato           | ES                                 | 2004               | OK398235                     |
| Vert35     | Tomato           | ES                                 | 2004               | OM911996                     |
| Vert36     | Tomato           | RJ                                 | 2004               | OM907132                     |
| Vert38     | Tomato           | SC                                 | 2004               | OM911997                     |
| Vert43     | Tomato           | SC                                 | 2005               | OM911998                     |
| Vert45     | Tomato           | SC                                 | 2005               | OM911999                     |
| Vert46     | Tomato           | DF                                 | 2005               | OM912000                     |
| Vert47     | Tomato           | DF                                 | 2005               | OM907133                     |
| Vert53     | Tomato           | SP                                 | 2005               | OM912001                     |
| Vert54     | Tomato           | SP                                 | 2005               | OK398238                     |
| Vert56     | Tomato           | SP                                 | 2005               | OM908366                     |
| Vert59     | Tomato           | MG                                 | 2005               | OM912002                     |
| Vert62     | Eggplant         | SP                                 | 2005               | OM912003                     |
| Vert65     | Tomato           | SC                                 | 2005               | OM912004                     |
| Vert67     | Tomato           | ES                                 | 2005               | OM912005                     |
| Vert70     | Tomato           | ES                                 | 2005               | OM908473                     |
| Vert71     | Tomato           | ES                                 | 2005               | OM912006                     |
| Vert74     | Tomato           | ES                                 | 2006               | OM912007                     |
| Vert77     | Tomato           | SP                                 | 2006               | OM912008                     |
| Vert78     | Tomato           | SP                                 | 2006               | OM912009                     |
| Vert79     | Tomato           | DF                                 | 2006               | OM912010                     |
| Vert93     | Tomato           | MG                                 | 2006               | OM912011                     |
| Vert96     | Tomato           | RJ                                 | 2007               | OM912012                     |
| Vert103    | Strawberry       | DF                                 | 2007               | OM912013                     |
| Vert106    | Tomato           | MG                                 | 2007               | OM912014                     |
| Vert110    | Scarlet eggplant | RJ                                 | 2008               | MW051677                     |
| Vert111    | Scarlet eggplant | RJ                                 | 2008               | MW051678                     |
| Vert116    | Tomato           | MG                                 | 2009               | OM912015                     |
| Vert117    | Scarlet eggplant | CE                                 | 2009               | MW051679                     |

(Continues)

TABLE 1 (Continued)

| Isolate ID | Host origin | Geographical location <sup>a</sup> | Year of collection | ITS GenBank accession number |
|------------|-------------|------------------------------------|--------------------|------------------------------|
| Vert118    | Eggplant    | CE                                 | 2009               | OM912016                     |
| Vert119    | Eggplant    | DF                                 | 2009               | OM908367                     |
| Vert121    | Tomato      | SC                                 | 2010               | OM912018                     |
| Vert125    | Tomato      | PR                                 | 2010               | OM912019                     |
| Vert129    | Eggplant    | ES                                 | 2010               | OM912020                     |
| Vert130    | Strawberry  | ES                                 | 2010               | OM912021                     |
| Vert132    | Tomato      | ES                                 | 2010               | OM908375                     |
| Vert134    | Strawberry  | ES                                 | 2010               | OM912017                     |
| Vert137    | Strawberry  | ES                                 | 2010               | OM912022                     |
| Vert144    | Strawberry  | ES                                 | 2011               | OM912023                     |
| Vert145    | Strawberry  | ES                                 | 2011               | OM908376                     |
| Vert147    | Strawberry  | ES                                 | 2011               | OM912024                     |
| Vert148    | Strawberry  | ES                                 | 2011               | OM908438                     |
| Vert150    | Strawberry  | ES                                 | 2011               | OK398236                     |
| Vert151    | Tomato      | SP                                 | 2011               | OK398237                     |
| Vert160    | Tomato      | ES                                 | 2017               | OM912025                     |
| Vert161    | Tomato      | ES                                 | 2018               | OM912026                     |
| Vert163    | Eggplant    | DF                                 | 2018               | OM912027                     |
| Vert164    | Eggplant    | DF                                 | 2018               | OM908472                     |
| Vert169    | Potato      | MG                                 | 2018               | OK398094                     |
| Vert171    | Potato      | MG                                 | 2018               | OK398095                     |
| Vert172    | Potato      | MG                                 | 2018               | OK398096                     |
| Vert173    | Potato      | MG                                 | 2018               | OK398097                     |
| Vert174    | Potato      | MG                                 | 2018               | OK398098                     |
| Vert177    | Potato      | MG                                 | 2018               | OK398099                     |
| Vert178    | Potato      | MG                                 | 2018               | OK398100                     |
| Vert179    | Potato      | MG                                 | 2018               | OK398101                     |
| Vert180    | Potato      | MG                                 | 2018               | OK398102                     |
| Vert181    | Eggplant    | SP                                 | 2019               | OM912028                     |
| Vert182    | Eggplant    | SP                                 | 2019               | OM912029                     |
| Vert184    | Tomato      | MG                                 | 2019               | OM912030                     |
| Vert185    | Tomato      | MG                                 | 2019               | OM912031                     |
| Vert186    | Potato      | BA                                 | 2019               | OK398103                     |
| Vert188    | Potato      | BA                                 | 2019               | OK398104                     |
| Vert189    | Potato      | BA                                 | 2019               | OK398105                     |

<sup>a</sup>BA, Bahia; CE, Ceará; DF, Distrito Federal; ES, Espírito Santo; MG, Minas Gerais; PR, Paraná; RJ, Rio de Janeiro; SC, Santa Catarina; SP, São Paulo; Unknown, isolates from unknown location.

samples were incubated at 37°C for 30 min as part of the RNase A treatment, in order to remove traces of residual RNA. Samples were then stored at -20°C for later use.

## 2.4 | PCR assays and Sanger DNA sequencing

In order to assess their initial diversity, the ribosomal internal transcribed spacer region (ITS-rDNA) was amplified in a subgroup of

80 isolates. Representative isolates were randomly chosen from distinct haplotypes identified through DnaSP v. 4.0 (Rozas et al., 2003). These isolates were then subjected to a multilocus analysis, which involved sequence information from the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and actin (*ACT*) genes. The PCR primer details and the expected amplicon sizes are listed in Table 2. PCRs were performed in a T100 thermal cycler (Bio-Rad) in 25 µl volume reactions containing 1× PCR buffer, 2 mM MgCl<sub>2</sub>, 0.2 mM each dNTP (Invitrogen), 0.4 µM of each primer, 1 U *Taq* DNA polymerase

**TABLE 2** Primer sequences and amplicon sizes of each gene/genomic region evaluated for identification of *Verticillium* species from different hosts in Brazil

| Locus    | Primer name | Sequence (5'-3')         | Amplicon length (bp) | Reference                         |
|----------|-------------|--------------------------|----------------------|-----------------------------------|
| ITS rDNA | Df          | CCGGTCCATCAGTCTCTCTG     | 490                  | Inderbitzin et al. (2013)         |
|          | Dr          | CTGTTGCCGCTTCACTCG       |                      |                                   |
| GAPDH    | VGPDF2      | GGCATCAACGGTTTCGGCC      | 727                  | Inderbitzin, Davis, et al. (2011) |
|          | VGPDr       | GTAGGAGTGGACGGTGGTCATGAG |                      |                                   |
| ACT      | VActF       | TAATTCACAATGGAGGGTAGG    | 588                  | Inderbitzin, Davis, et al. (2011) |
|          | VActR       | GTAAGGATACCACGCTTGG      |                      |                                   |

(Invitrogen), and 20 ng genomic DNA. PCR conditions for the ITS rDNA region were an initial denaturation step of 2 min at 94°C; followed by 32 cycles of 10 s at 94°C, 20 s at 67°C, and 60 s at 72°C; and a final extension step of 7 min at 72°C. The amplification of the *GAPDH* gene started with a denaturation step for 4 min at 95°C; 38 cycles of 30 s at 95°C, 30 s at 67°C, and 45 s at 72°C; and one cycle of 7 min at 72°C. Amplification of the *ACT* gene consisted of an initial denaturation of 3 min at 95°C; followed by 34 cycles of 30 s at 96°C, 40 s at 54.5°C, and 1 min at 72°C; followed by a final extension of 5 min at 72°C. The amplicons were analysed by 1% agarose gel electrophoresis in 0.5× Tris-borate-EDTA buffer (1.1 mM Trizma base, 900 mM boric acid, 0.5 M EDTA pH 8.0) and stained with GelRed nucleic acid gel stain (Biotium). DNA purity and concentration from all samples were estimated by spectrophotometry ( $A_{260}/A_{280}$  ratio) using a biophotometer (Eppendorf). The amplicons were purified with PureLink PCR Purification kit (Invitrogen) based on the selective binding of double-stranded DNA to a silica-based membrane according to the manufacturer's instructions. Sanger DNA sequencing was carried out at Macrogen (Seoul, South Korea).

## 2.5 | Phylogenetic analysis

Forward and reverse sequences were assembled using the BioEdit v. 7.2.0 software (Hall, 1999). Consensus sequences were compared to the NCBI nucleotide database using the BLASTn algorithm. Sequences representing ex-types and related sequences were retrieved from GenBank (Table 3). Multiple sequence alignments for each individual gene were generated online using the G-INS-i strategy in MAFFT v. 7 (Kato et al., 2019; Kato & Toh, 2013) and manually adjusted, when necessary, in MEGA 7 (Kumar et al., 2016). Phylogenetic analyses were performed using the maximum-likelihood (ML) and Bayesian inference (BI) methods for both individual and concatenated genomic information. ML and BI analyses were performed using RAXML-HCP2 v. 8.0 (Stamatakis, 2014) and MrBayes v. 3.2.1 (Ronquist et al., 2012), respectively, implemented in the CIPRES cluster (<https://www.phylo.org/portal2/home.action>). ML analyses were carried out with 1000 pseudoreplicates (-m GTRGAMMA -p 12345 -k -f a -N 1000 -x 12345) under the GTRGAMMA model. Evolution models were estimated in MrModeltest v. 2.3 (Nylander, 2004) using the Akaike information criterion for each genomic region. For the Bayesian analysis, the combined data set

was partitioned to reflect the most appropriate nucleotide substitution model for each gene/genomic region. Four Markov chain Monte Carlo chains were conducted for  $5 \times 10^7$  generations, with samplings every 1000 generations. The convergence of all the parameters was checked using Tracer v. 1.5 (Rambaut & Drummond, 2010) and the first 25% generations were discarded as burn-in. FigTree v. 1.4.3 (Rambaut, 2012) was used to visualize the phylogenetic tree.

## 2.6 | Pathogenicity tests

Isolates were employed in pathogenicity tests by inoculating their original hosts, including tomato, potato, eggplant, scarlet eggplant, okra, and strawberry. Cross-inoculation assays were also performed employing seedlings of cv. Ciça, an eggplant hybrid that is highly susceptible to *V. dahliae* isolates and used as the standard host for severe vascular wilt symptom expression. The pathogenicity of the isolates from cacao was assessed only on eggplant seedlings. The inoculation protocol was essentially as described by Santos (1997) and modified by Reis et al. (2007). The pathogenicity test was carried out twice. Bioassays were carried out in a randomized block design with three replicates (three pots with two plants each), under greenhouse conditions at  $25 \pm 4^\circ\text{C}$  and relative humidity of 70%–80%. The presence (or absence) of symptoms was assessed 30 days after inoculation. Plants were observed daily for the development of foliar symptoms, such as chlorosis, necrosis, and premature defoliation. Stems of the inoculated and control plants were taken from the pots and longitudinally sectioned to observe the presence or absence of vascular browning. Afterwards, the pathogen was reisolated from infected tissue of all original hosts and from the eggplant hybrid Ciça.

## 3 | RESULTS

### 3.1 | Cultural and morphological characteristics and microsclerotia formation

All *Verticillium* isolates produced white colonies with abundant cottony mycelium on PDA. The colonies were creamy-white, sometimes showing orange pigmentation, and gradually became densely dark at the bottom of the plate (Figure 1a). All fungal isolates displayed hyaline, septate, and multinucleate mycelium. The conidia were ovoid

TABLE 3 Reference isolates of *Verticillium* species and DNA sequence data retrieved from GenBank (Inderbitzin, Bostock, et al., 2011) and used in this study for phylogenetic analysis

| Species                                      | Strain <sup>a</sup> | Host              | Location          | GenBank accession number |                    |                  |
|--|---------------------|-------------------|-------------------|--------------------------|--------------------|------------------|
|  |                     |                   |                   | ITS <sup>b</sup>         | GAPDH <sup>c</sup> | ACT <sup>d</sup> |
| <i>Gibellulopsis nigrescens</i> <sup>e</sup> | <b>PD596</b>        | Eggplant          | Japan             | JN187977                 | JN188167           | JN188103         |
| <i>V. albo-atrum</i>                         | PD693               | Potato            | UK                | JN187994                 | JN188186           | JN188122         |
|  | <b>PD747</b>        | Potato            | Canada            | JN188016                 | JN188208           | JN188144         |
| <i>V. alfalfae</i>                           | <b>PD489</b>        | Alfalfa           | USA               | MW550073                 | JN188161           | JN188097         |
|  | PD620               | Alfalfa           | Canada            | HQ206851                 | HQ414763           | HQ206965         |
| <i>V. dahliae</i>                            | <b>PD322</b>        | Lettuce           | USA               | HQ206718                 | HQ414719           | HQ206921         |
|  | PD323               | Strawberry        | USA               | HQ206719                 | HQ414720           | HQ206922         |
|  | PD327               | Bell pepper       | USA               | HQ206723                 | HQ414723           | HQ206925         |
|  | PD337               | Cotton            | USA               | HQ206732                 | HQ414727           | HQ206929         |
|  | PD404               | Bell pepper       | USA               | HQ206757                 | HQ414738           | HQ206940         |
|  | PD502               | Maple             | USA               | HQ206813                 | HQ414740           | HQ206942         |
|  | PD617               | Tomato            | Brazil            | HQ206850                 | HQ414762           | HQ206964         |
|  | PD656               | Sunflower         | Canada            | HQ206872                 | HQ414782           | HQ206984         |
|  | PD718               | Oilseed rape      | France            | HQ206908                 | HQ414803           | HQ207005         |
|  | PD729               | Horseradish       | USA               | HQ206919                 | HQ414811           | HQ207013         |
|  | <b>Vert04</b>       | <b>Tomato</b>     | <b>SP, Brazil</b> | OK398231                 | OK513235           | OK513223         |
|  | <b>Vert14</b>       | <b>Eggplant</b>   | <b>MG, Brazil</b> | OK398232                 | OK513233           | OK513224         |
|  | <b>Vert17</b>       | <b>Eggplant</b>   | <b>SP, Brazil</b> | OK398233                 | OK513231           | OK513225         |
|  | <b>Vert22</b>       | <b>Cacao</b>      | <b>BA, Brazil</b> | OK398234                 | OK513232           | OK513226         |
|  | <b>Vert26</b>       | <b>Tomato</b>     | <b>Unknown</b>    | OK398239                 | OK513236           | OK513227         |
|  | <b>Vert34</b>       | <b>Tomato</b>     | <b>ES, Brazil</b> | OK398235                 | OK513237           | OK513222         |
|  | <b>Vert54</b>       | <b>Tomato</b>     | <b>ES, Brazil</b> | OK398238                 | OK513238           | OK513228         |
| <b>Vert150</b>                               | <b>Strawberry</b>   | <b>ES, Brazil</b> | OK398236          | OK513234                 | OK513229           |                  |
| <b>Vert151</b>                               | <b>Tomato</b>       | <b>SP, Brazil</b> | OK398237          | OK513239                 | OK513230           |                  |
| <i>V. isaacii</i>                            | PD341               | Lettuce           | USA               | JN187963                 | JN188153           | JN188089         |
|  | <b>PD660</b>        | Lettuce           | USA               | HQ206873                 | HQ414783           | HQ206985         |
| <i>V. klebahnii</i>                          | PD347               | Globe artichoke   | USA               | JN187965                 | JN188155           | JN188091         |
|  | <b>PD401</b>        | Lettuce           | USA               | JN187967                 | JN188157           | JN188093         |
| <i>V. longisporum</i> allele A1 (species A1) | <b>PD348</b>        | Cauliflower       | USA               | HQ206738                 | HQ414728           | HQ206930         |
|  | <b>PD687</b>        | Horseradish       | Germany           | HQ206893                 | HQ414791           | HQ206993         |
| <i>V. nonalfalfae</i>                        | <b>PD592</b>        | Potato            | Japan             | JN187973                 | JN188163           | JN188099         |
|  | PD808               | Hop               | Slovenia          | JN188020                 | JN188212           | JN188148         |
| <i>V. nubilum</i>                            | PD702               | Potato            | UK                | JN187995                 | JN188187           | JN188123         |
|  | <b>PD742</b>        | Soil              | UK                | JN188011                 | JN188203           | JN188139         |
| <i>V. tricorpus</i>                          | PD594               | Tomato            | Japan             | JN187975                 | JN188165           | JN188101         |
|  | <b>PD690</b>        | Tomato            | UK                | JN187993                 | JN188185           | JN188121         |
| <i>V. zaregamsianum</i>                      | <b>PD736</b>        | Lettuce           | Japan             | JN188005                 | JN188197           | JN188133         |
|  | PD740               | Ten weeks stock   | Japan             | JN188009                 | JN188201           | JN188137         |

Abbreviations: BA, Bahia; ES, Espírito Santo; MG, Minas Gerais; SP, São Paulo; Unknown, isolates from unknown location in Brazil.

<sup>a</sup>PD identifiers in bold represent ex-type strains.

<sup>b</sup>ITS, internal transcribed spacer.

<sup>c</sup>GAPDH, glyceraldehyde-3-phosphate dehydrogenase gene.

<sup>d</sup>ACT, Actin gene.

<sup>e</sup>*Gibellulopsis nigrescens* represents ex-type strain (outgroup sequence).





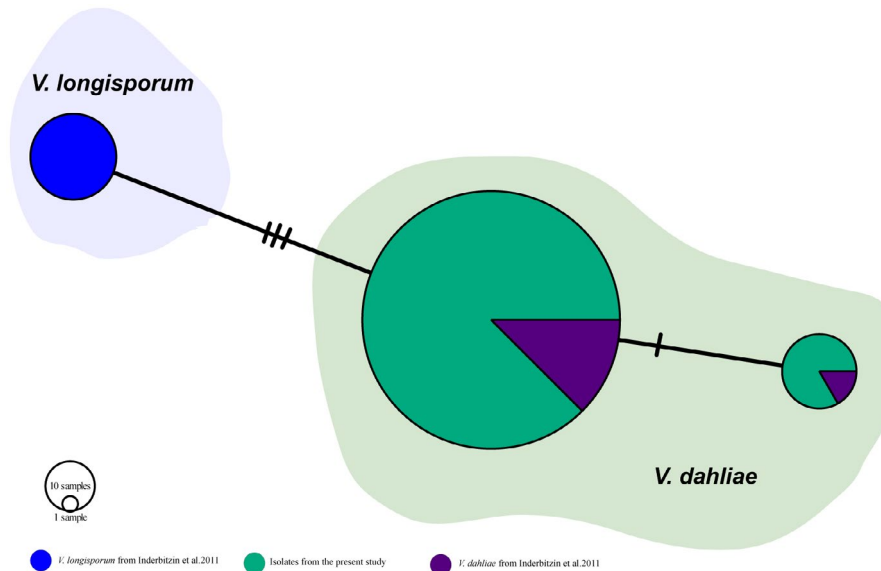
**FIGURE 1** Characteristics of plant-pathogenic *Verticillium dahliae*. (a) Typical growth of *V. dahliae* isolated from the eggplant stem. (b) Mycelial growth of *V. dahliae* on eggplant stem under optical microscope. (c) Tomato plant infected by a *V. dahliae* isolate showing chlorotic leaf border and initial necrosis. (d) Eggplant seedling showing typical V-shaped necrosis on the leaf. (e) In vitro plating of the stem of an *V. dahliae*-infected eggplant showing fungal growth in four points

to elongated in shape and were produced on long phialides, which were positioned in a spiral distribution around the conidiophores. Branching of the verticillate conidiophores and microsclerotia were consistently observed on PDA for most isolates (Figure 1b). All these morphological features are in accordance with descriptions of *V. dahliae*-type strains. Therefore, all these Brazilian isolates were preliminarily identified as *V. dahliae*-like. An additional characterization of these 80 *V. dahliae*-like isolates was carried out taking into account their ability to either produce (code =MS) or not (code =NonMS) microsclerotia in culture. From the total of analysed isolates, 59 (73.8%) were classified as MS and 21 isolates (26.2%) as NonMS formation in PDA. Specifically, in tomato isolates ( $n = 39$ ), 59% were MS versus 41% NonMS; in eggplant ( $n = 13$ ) 76.9% were MS and 23.1% NonMS; 100% of potato ( $n = 13$ ), strawberry ( $n = 9$ ), and okra ( $n = 1$ ) isolates were MS; in scarlet eggplant ( $n = 3$ ) 66.7%

were MS versus 33.3% NonMS; and in cacao ( $n = 2$ ) 50% of the isolates were MS versus 50% NonMS. None of the isolates displayed dark mycelium.

### 3.2 | Molecular screening of 80 *V. dahliae*-like isolates

A preliminary analysis based on ITS sequence variation among the 80 isolates revealed two distinct haplotypes (H1 and H2). BLASTn searches using ITS sequences showed 99.8%–100% similarity ( $e$ -value = 0) of the isolates to *V. dahliae* type (PD323). In addition, a single ITS tree clustered the tested isolates with *V. dahliae* with high bootstrap support and Bayesian posterior probability values (BI-PP) (Figure S1). All ITS sequences generated in the present study



**FIGURE 2** Median-joining haplotype network generated for the ribosomal internal transcribed spacer region (ITS) sequence alignments representing *Verticillium longisporum* and *V. dahliae* isolates obtained from Inderbitzin, Bostock, et al. (2011) and in this study using PopArt. Circle size is proportional to the number of isolates with a specific haplotype and connecting lines represent the number of mutations between haplotypes

were deposited in GenBank (Table 1). To ensure the identity of our isolates, they were also included in a network analysis involving *V. dahliae* and *V. longisporum* isolates (Inderbitzin, Bostock, et al., 2011), which resulted in three different haplotype groups (Figure 2). Based on these results, we selected nine representative isolates to perform the multilocus analyses (Figure 3).

### 3.3 | Multilocus analyses of *V. dahliae*-like isolates

The isolates from different hosts were grouped with the *V. dahliae* clade with strong support by both ML and BI analysis in the concatenated tree (Figure 3) and the individual gene trees (Figures 4, 5, and 6). Brazilian *V. dahliae*-like isolates were included in the group of reference *V. dahliae* isolates, including PD322 (lettuce), PD323 (strawberry), PD327 (bell pepper), PD337 (cotton), PD404 (bell pepper), PD502 (maple), PD617 (tomato), PD656 (sunflower), PD718 (oilseed rape), and PD729 (horseradish) (Figure 3). Our *Verticillium* isolates were recovered as monophyletic with high support level in the *ACT* and *GAPDH* trees (Figures 5 and 6). In the ITS tree, the isolates Vert17 and Vert22 formed polytomic branches in the *V. dahliae* clade (Figure 4).

### 3.4 | Pathogenicity assays

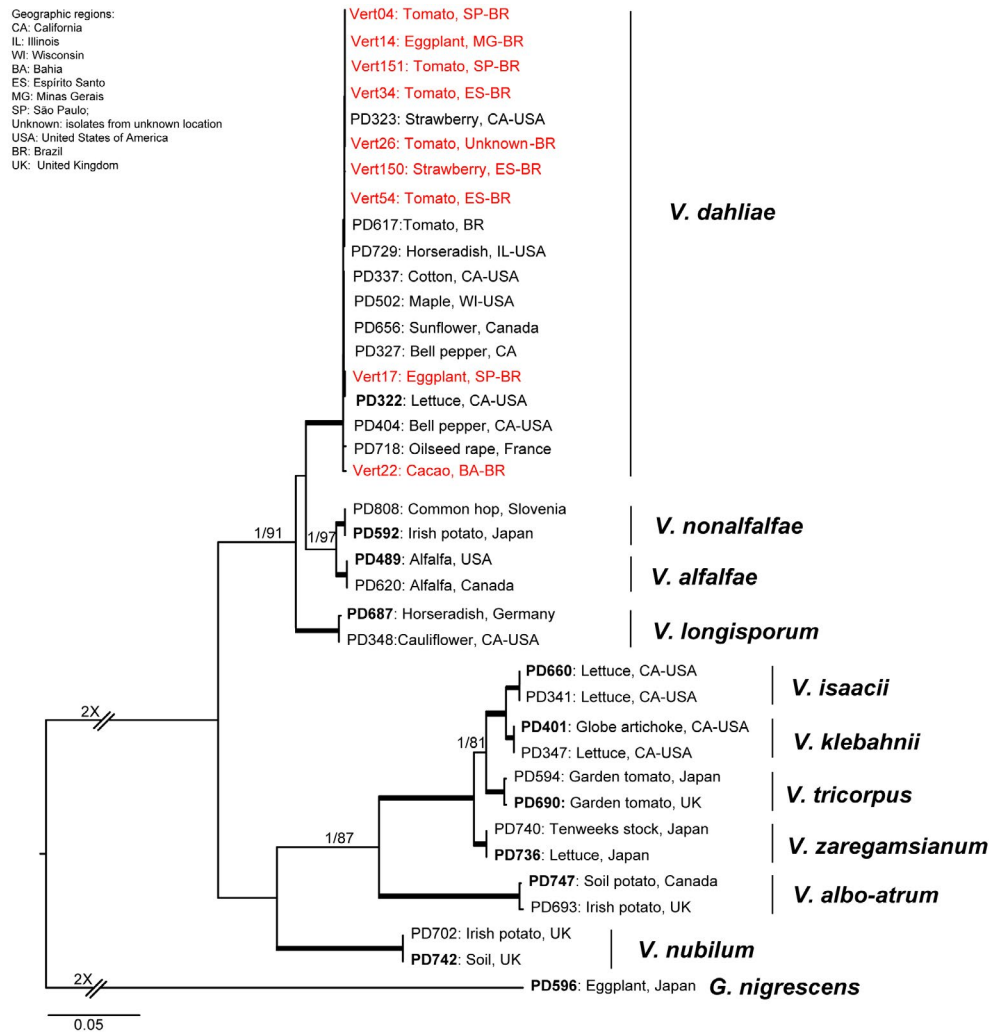
The first symptoms in the inoculated tomato, eggplant, scarlet eggplant, potato, okra, and strawberry plants appeared from 21 to 28 days after inoculation. Typical symptoms of *Verticillium* wilt were observed, such as chlorosis on the lower leaves and typical V-shaped areas in leaf margins that eventually progressed to senescence and

necrosis after about 1–2 weeks (Figure 1c,d). Longitudinal sections of the basal stems displayed light brown discolouration, indicating the colonization of the vascular tissue by the pathogen. Microscopical observations from infected tissues, maintained in a wet chamber for 2 days, showed branching conidiophores and oval conidia (either free or in verticillate arrangement; Figure 1b). All Brazilian isolates were pathogenic to their original hosts, and 88.8% were pathogenic only to eggplants upon reinoculation. However, nine isolates (11.2%) were pathogenic to their original hosts but not on eggplants (employed as our standard susceptible host) in cross-inoculation assays. Interestingly, these isolates displayed low levels of sporulation in PDA culture ( $<2 \times 10^6$  conidia/ml), suggesting that lower inoculum production might be associated with either mild or absence of symptoms in eggplant seedlings. The least aggressive isolates were Vert12 from okra; seven isolates from tomato (Vert36, Vert59, Vert71, Vert116, Vert121, Vert132, and Vert151), and one from potato (Vert180). All 80 isolates were reisolated from infected plant tissue or from their original hosts (Figure 1e). In comparison, mock-inoculated plants (controls) inoculated only with distilled water did not display conspicuous symptoms and we were unable to reisolate the pathogen from these samples.

## 4 | DISCUSSION

Although *V. dahliae* isolates have been morphologically characterized, there is still a lack of studies employing accurate identification methods for the *Verticillium* species associated with wilt diseases of vegetable crops in Brazil. Therefore, for a more reliable and consistent identification, we amplified and sequenced three genetic regions: the internal transcribed spacer (ITS), glyceraldehyde-3-phosphate



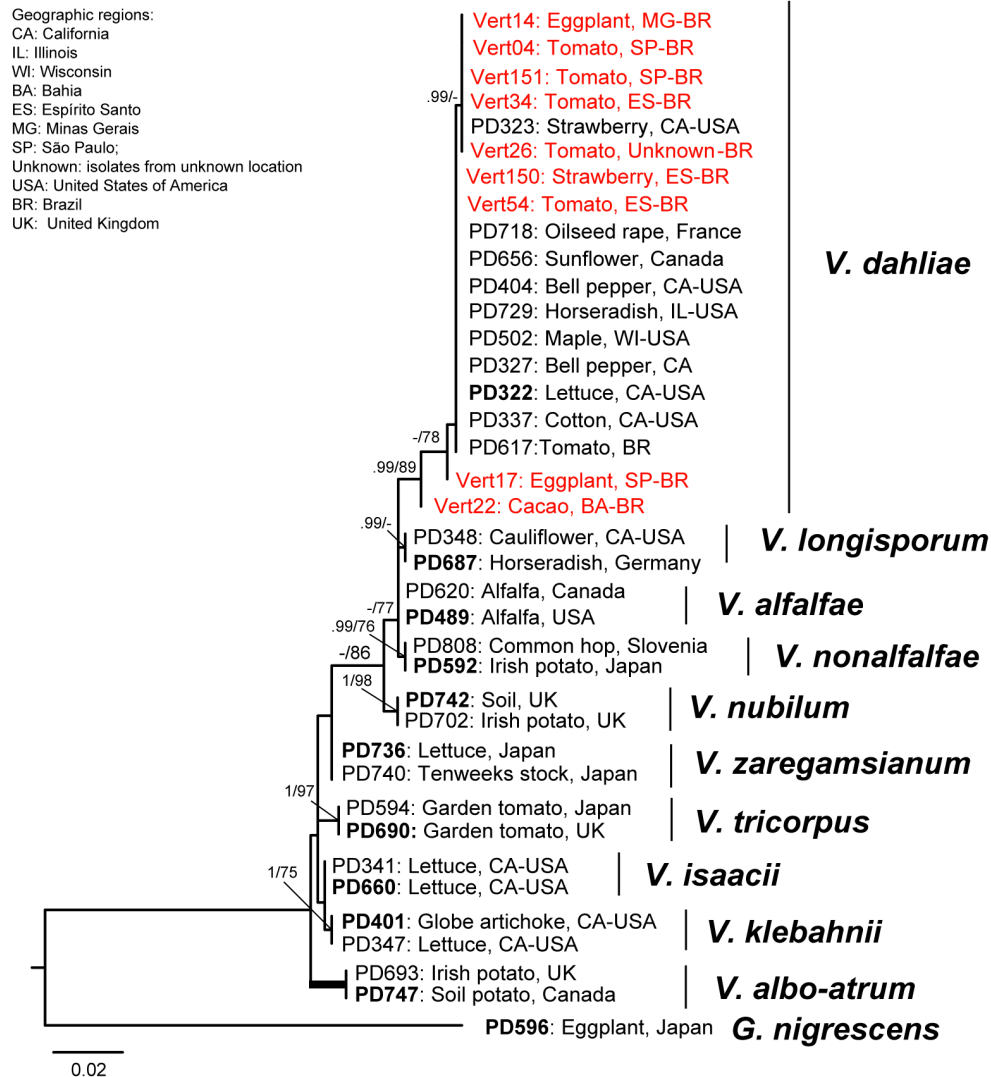


**FIGURE 3** Maximum-likelihood tree of the *Verticillium* species inferred from a concatenated alignment of the ribosomal internal transcribed spacer region (ITS), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and actin (*ACT*) genes. Bootstrap support values (ML  $\geq 70$ ) and Bayesian posterior probability values (PP  $\geq 0.95$ ) are shown at the nodes. Full supported branches (ML-BI = 100/BI-PP = 1) are indicated in bold. “–” indicates no significant support or absence of the node. ‘PD’ isolates in bold represent ex-type isolates of *Verticillium* species obtained from the studies by Inderbitzin, Bostock, et al. (2011), Inderbitzin, Davis, et al. (2011). ‘Vert’ isolates analysed in this study are highlighted in red. Hosts and geographic origins are given. *Gibellulopsis nigrescens* was used as the outgroup. The scale bar indicates the estimated number of substitutions per site

dehydrogenase gene (*GAPDH*), and actin gene (*ACT*), to infer a more certain identification and possible relationships between the isolates.

There are two major important taxonomic controversies involving *V. dahliae*. The first is the definition of the distinctive features that discriminate between *V. dahliae* and *V. albo-atrum* isolates (Inderbitzin, Davis, et al., 2011; Karapapa et al., 1997; Steventon et al., 2002; Yu et al., 2016). Historically, it was generally accepted that microsclerotial (MS) and non-microsclerotial (NonMS) strains corresponded to two distinct species. Subsequently, phylogenetic studies reinforced the view that *V. albo-atrum* and *V. dahliae* are, in fact, two distinct taxa (Fradin & Thomma, 2006; Klosterman et al., 2009; Pegg & Brady, 2002). The second controversy is the recognition of *V. longisporum* as a separate species and not as a variation within the *V. dahliae* species (Inderbitzin & Subbarao, 2014). Most

*Verticillium* isolates used in the present study were identified as *V. dahliae* according to morphological examination, including resting structure morphology, as well pathogenicity and virulence profiling (Reis & Boiteux, 2006a, 2006b). In addition, in the present study we observed two phenotypical groups in relation to the fungal colonies. The first group was composed of isolates able to produce spherical, dark microsclerotia, which correspond to the majority (73.8%) of our isolates. The second group (26.2%) was composed of isolates unable to produce microsclerotia in PDA. Even though for many years it was thought that *V. dahliae* was a microsclerotia-producing subgroup within *V. albo-atrum* (Fradin & Thomma, 2006; Goud & Termorshuizen, 2003; Pegg & Brady, 2002), today it is widely known that this characteristic is unstable and should be used with caution as a reliable tool for *Verticillium* species identification (Inderbitzin & Subbarao, 2014; Karapapa et al., 1997). For this

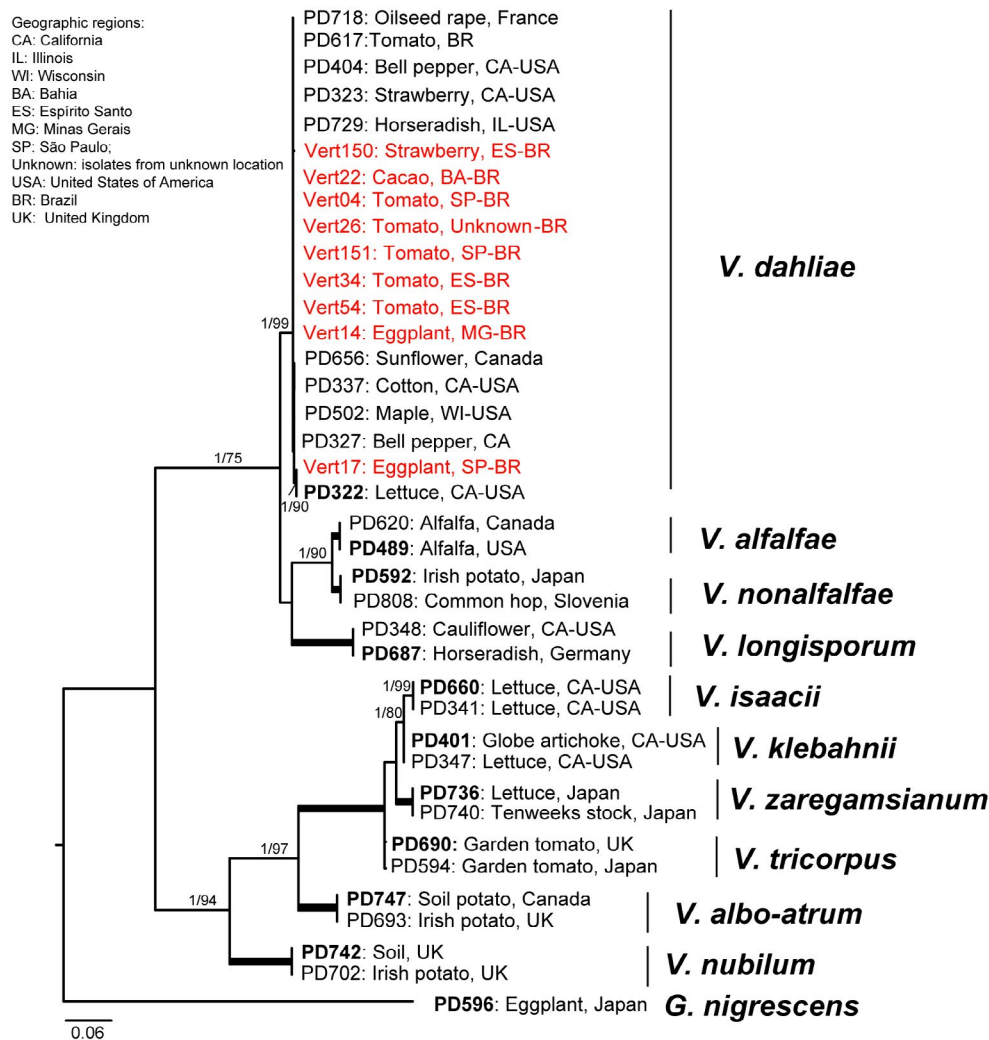


**FIGURE 4** Maximum-likelihood tree of the *Verticillium* species inferred from a single alignment of the ribosomal internal transcribed spacer region (ITS) region. Bootstrap support values (ML  $\geq$  70) and Bayesian posterior probability values (PP  $\geq$  0.95) are shown at the nodes. Full supported branches (ML-BI = 100/BI-PP = 1) are indicated in bold. “-” indicates no significant support or absence of the node. ‘PD’ isolates in bold represent ex-type isolates of *Verticillium* species obtained from the studies by Inderbitzin, Bostock, et al. (2011), Inderbitzin, Davis, et al. (2011). ‘Vert’ isolates analysed in this study are highlighted in red. Hosts and geographic origins are given. *Gibellulopsis nigrescens* was used as the outgroup. The scale bar indicates the estimated number of substitutions per site

reason, we considered that morphological characteristics, including resting structures, can be affected by many factors under laboratory conditions, such as growth media type, low temperature, humidity, and storage time, making them not suitable for *V. albo-atrum* and *V. dahliae* species separation. It is not yet known why the capacity to produce microsclerotia was lost in a subgroup of isolates and this observation deserves further investigation.

It is currently a consensus that the identification of *Verticillium* based only upon morphological attributes is neither effective nor reliable. In this scenario, analysis of phylogenetically informative genomic regions such as the ITS has been used as a more reliable diagnostic tool (Otero et al., 2004; Pramateftaki et al., 2000; Qin et al., 2006; Raja et al., 2017). The ITS region was chosen as the default identification tool for fungal barcodes

by a consortium of mycologists (Bold Systems, 2021; Schoch et al., 2012). According to Inderbitzin, Bostock, et al. (2011) and Inderbitzin, Davis, et al. (2011), the genetic information derived from the ITS region is able to accommodate the diversity of the 10 *Verticillium* species known so far. However, *V. longisporum* is a major exception, because the ITS region alone could not retrace the evolution of this species and separate it from *V. dahliae*. Our analysis employing only ITS information provided low resolution to differentiate PD348 and PD687 (two reference *V. longisporum* isolates), resulting in an unresolved consensus tree in relation to the *V. dahliae* group (Figure 4). Thus, DNA sequence comparisons based on the ITS region alone would incorrectly identify *V. longisporum* as *V. dahliae* lineages, as previously reported by Inderbitzin et al. (2013).

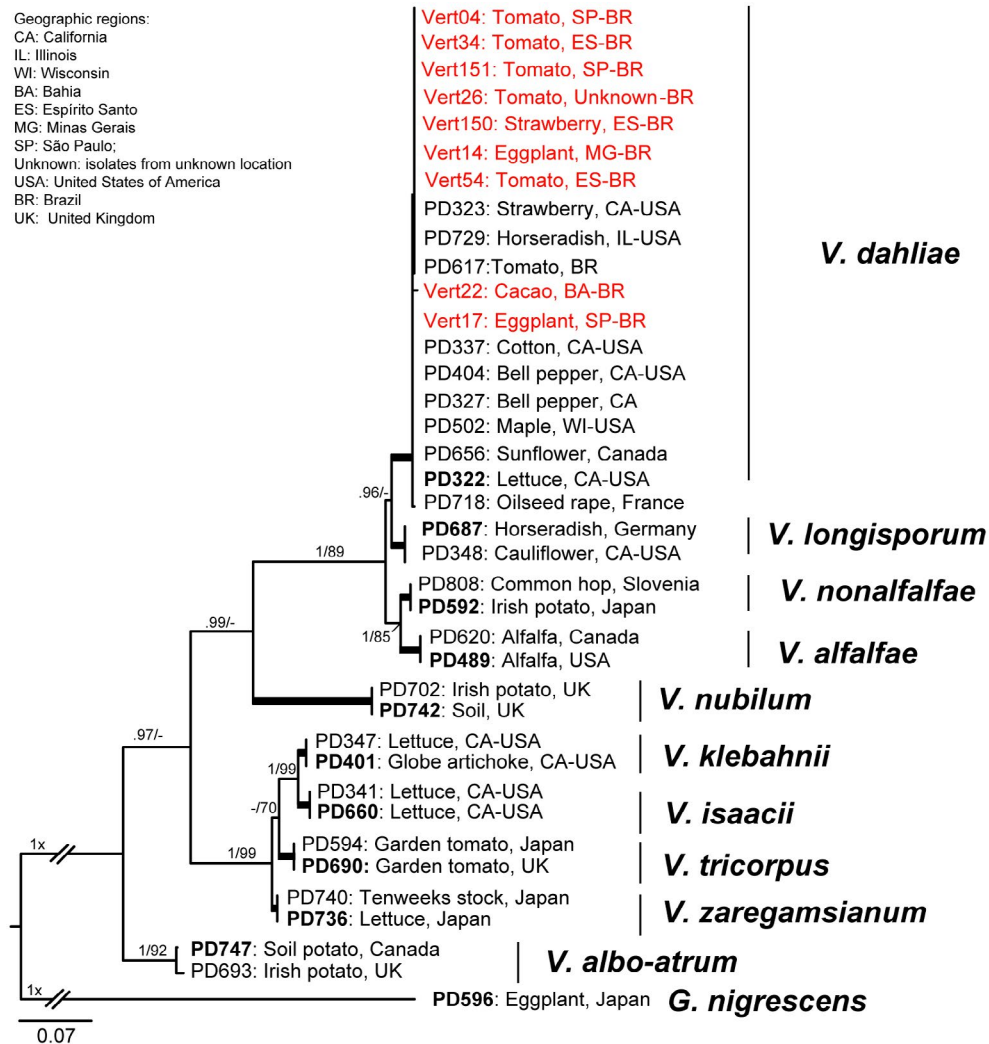


**FIGURE 5** Maximum-likelihood tree of the *Verticillium* species inferred from a single alignment of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene. Bootstrap support values (ML  $\geq$  70) and Bayesian posterior probability values (PP  $\geq$  0.95) are shown at the nodes. Full supported branches (ML-BI = 100/BP-PP = 1) are indicated in bold. “-” indicates no significant support or absence of the node. ‘PD’ isolates in bold represent ex-type isolates of *Verticillium* species obtained from the studies by Inderbitzin, Bostock, et al. (2011), Inderbitzin, Davis, et al. (2011). ‘Vert’ isolates analysed in this study are highlighted in red. Hosts and geographic origins are given. *Gibellulopsis nigrescens* was used as the outgroup. The scale bar indicates the estimated number of substitutions per site

Here, we also carried out haplotype network analysis employing ITS information with 80 isolates from our fungal collection that were identified as *V. dahliae*. Sequencing of the ITS fragment revealed the presence of three haplotypes (H) in the overall data set, with haplotype diversity (HD) value of 0.289 and nucleotide diversity value per site of  $\pi = 0.00176$ , indicating low genetic variation across the Brazilian populations. However, for the highly conserved ITS sequences, the Brazilian isolates of *V. dahliae* and the reference *V. dahliae* isolates grouped in two distinct haplotypes (H1 and H2), while the isolates of *V. longisporum* grouped in another separate haplotype (H3).

Even though the nuclear ITS region is useful for DNA-based identification in fungi (Schoch et al., 2012), other informative genomic regions have provided more information for the phylogenetic analysis of *Verticillium* species, especially in relation to the uncertain

relationship between *V. longisporum* and *V. dahliae*. In this scenario, Inderbitzin, Bostock, et al. (2011) and Inderbitzin, Davis, et al. (2011) proposed the employment of another set of parsimony-informative loci such as the elongation factor 1- $\alpha$  (*EF-1 $\alpha$* ), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), actin (*ACT*), and tryptophan synthase (*TS*) to infer relationships between members of *Verticillium*. An increasing number of studies are using this new molecular taxonomic system to identify, separate, and infer phylogenetic relationships among *Verticillium* species, because it provides a higher overall support than single-locus phylogenies, especially when the morphological differences are minimal (Xu et al., 2019). Thus, we sequenced the *GAPDH* and *ACT* loci from our isolates for a more accurate identification, confirming the presence of *V. dahliae* as the sole wilt-inducing fungal species associated with major solanaceous vegetables in Brazil. Analysis of the partial *GAPDH* and *ACT* could



**FIGURE 6** Maximum-likelihood tree of the *Verticillium* species inferred from a single alignment of the actin (ACT) gene. Bootstrap support values (ML  $\geq$  70) and Bayesian posterior probability values (PP  $\geq$  0.95) are shown at the nodes. Full supported branches (ML-BI = 100/BI-PP = 1) are indicated in bold. “-” indicates no significant support or absence of the node. ‘PD’ isolates in bold represent ex-type isolates of *Verticillium* species obtained from the studies by Inderbitzin, Bostock, et al. (2011), Inderbitzin, Davis, et al. (2011). ‘Vert’ isolates analysed in this study are highlighted in red. Hosts and geographic origins are given. *Gibellulopsis nigrescens* was used as the outgroup. The scale bar indicates the estimated number of substitutions per site

distinguish and separate the representative isolates PD348 and PD687, identified by Inderbitzin, Bostock, et al. (2011) as *V. longisporum* species. No conflict was observed across the single-locus analyses using these sets of genomic information (Figures 5 and 6).

Our combined analyses encompassing the ITS, *GAPDH*, and *ACT* regions (Figure 3) agreed with the two major clades obtained by Inderbitzin, Bostock, et al. (2011). The *Verticillium* species analysed by Inderbitzin, Bostock, et al. (2011) were divided into clade Flavexudans (containing *V. albo-atrum*, *V. isaacii*, *V. klebahnii*, *V. tricorpus*, and *V. zaregamsianum*) and clade Flavnonexudans (including *V. alfalfae*, *V. dahliae*, *V. nonalfalfae*, *V. longisporum*), with the exception of *V. nubilum*, only supported by parsimony analysis. The phylogeny of our DNA sequences was in agreement with the major clade Flavnonexudans. We inferred that in the combined analysis and all single-locus data sets, the topology obtained for DNA

sequences from *Verticillium* isolates collected in Brazil generated a well-supported monophyletic clade with strong bootstrap support and BI-PP. These results were in full agreement with the new taxonomy system of *Verticillium* classification proposed by Inderbitzin, Bostock, et al. (2011).

Therefore, our results strongly indicate that *V. dahliae* is the sole vascular wilt-inducing species in vegetable crops across 10 geographical regions of Brazil. However, *V. dahliae* is a pathogen well-known to lack host specificity (Johansson et al., 2003), therefore it has been considered host-adapted rather than host-specific (Douhan & Johnson, 2001). A previous host range study carried out by Reis and Boiteux (2006a), testing a subset of isolates used in the present study, reinforced the polyphagous nature of *V. dahliae*.

The causal agent(s) of *Verticillium* wilt in vegetable crops in Brazil was uncertain because it was based solely upon



morphological traits. The present study, using information from three genomic regions, indicates *V. dahliae* as the major pathogen causing Verticillium wilt, especially in the solanaceous crops such as tomato, potato, eggplant, and scarlet eggplant. Therefore, the previous reports of *V. dahliae* on tomato, eggplant, scarlet eggplant, okra, strawberry, and cacao (Farr & Rossman, 2021; Mendes et al., 2019) were confirmed here by the use of molecular tools. The present work is the first report of *V. dahliae* causing vascular wilt on potato crops in Brazil. Previously, Verticillium wilt of potato was reported in the southern region of Brazil and, based solely upon morphological traits, the causal agent was described as *V. albo-atrum*. Here, we were able to identify via multilocus sequencing all *Verticillium* isolates mainly collected from solanaceous vegetables in Brazil as *V. dahliae*. This is the first nationwide characterization of *Verticillium* isolates associated with major vegetable crops in Neotropical areas. This is valuable information for the design of sound management strategies for these diseases, mainly for the establishment of efficient rotation systems and for the development of resistant cultivars. More extensive studies are also necessary to characterize the *Verticillium* species associated with other groups of vegetable crops in the country. All this information is crucial for understanding the biology of the pathogen, the epidemiology of the disease on each host plant, and for planning management strategies for Verticillium wilt control on vegetables.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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