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Identificação e caracterização de isolados de *Verticillium dahliae* de hortaliças e detecção de micovírus

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IDENTIFICAÇÃO E CARACTERIZAÇÃO DE ISOLADOS DE *Verticillium dahliae* DE HORTALIÇAS E DETECÇÃO DE MICOVÍRUS

Tese apresentada ao Programa de Pós-Graduação em Fitopatologia da Universidade Federal Rural de Pernambuco, como parte dos requisitos para obtenção do título de Doutora em Fitopatologia.

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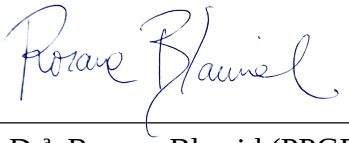

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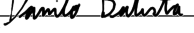

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SUMÁRIO

PÁGINA

RESUMO GERAL

A murcha de Verticillium é uma doença particularmente importante por causar grandes perdas econômicas aos produtores de hortaliças no Brasil e no mundo. Por ser um patógeno de solo e pela ausência de cultivares resistentes à raça 2, o manejo da doença tem sido pouco eficiente. Recentemente, relatos de surtos da doença vêm acontecendo em novas áreas de produção do País. Historicamente, duas espécies dentro do gênero foram associadas à murcha de Verticillium em hortaliças: *Verticillium dahliae* e *V. albo-atrum.* Mudanças na taxonomia do gênero, o reconhecimento de novas espécies patogênicas e o surgimento de três linhagens diferentes por eventos de hibridação, têm sido revelados através do novo sistema de identificação baseado numa abordagem morfológica e multigênica. No entanto, estudos de identificação de isolados de *Verticillium* no Brasil, envolvendo ambas abordagens, têm sido ausentes. Por outro lado, sobre a caracterização de isolados brasileiros quanto ao tipo de "mating" e identificação de raças fisiológicas através de ferramentas moleculares é um trabalho de pesquisa inédito. Outro aspecto extremamente inovador, é a busca de micovírus como potenciais agentes de controle biológico de *V. dahliae* que poderiam auxiliar no manejo da doença. Portanto, este trabalho teve como objetivos (1) identificar através de uma abordagem multi-locus (ITS, GAPDH e ACT) uma coleção de isolados brasileiros de *Verticillum* spp., (2) determinar a estrutura populacional de isolados de *V. dahliae* em termos da distribuição de raças fisiológicas através de bioensaios com cultivares diferenciais e via PCR, (3) determinar a distribuição e frequência dos tipos de grupos de compatibilidade sexual (*mating type*) no Brasil, e adicionalmente, (4) utilizar uma abordagem metagenômica através do sequenciamento de nova geração (NGS) para detectar micovírus que infectam *V. dahliae* e que futuramente possam ser utilizados como agentes de controle biológico. A análise multigênica revelou que existe uma única espécie, *Verticillium dahliae* Kleb., associada com sintomas de murcha em hortaliças no Brasil. Os ensaios de virulência para a determinação de raças fisiológicas foram corroborados com marcadores moleculares indicando que a maioria dos isolados que infectam hortaliças pertencem à raça 2. Foi demonstrado que *mating type* opostos estão presentes em isolados brasileiros de *V. dahliae*, sendo o *MAT-1-1* predominante. Além disso, análise de sequências de vírus obtidas por NGS, revelou a presença de um único micovírus infectando isolados de *V. dahliae*. O novo vírus foi tentativamente denominado de *Verticillium dahliae* single stranded RNA virus-strain CNPH (VdRV-strain CNPH). No presente trabalho apresentamos uma caracterização abrangente de isolados de *Verticillium* do Brasil, assim como um estudo pioneiro sobre metagenômica para a decoberta de micovírus com potencial para o controle biológico do fungo.

Palavras-chave: MATs idiomorfos, micovírus, murcha vascular de Verticillium, raças fisiológicas, sequenciamento de nova geração.

GENERAL ABSTRACT

Verticillium wilt is a particularly important disease because it causes great economic losses to vegetable producers in Brazil and worldwide. Because it is a soilborne pathogen and due to the absence of cultivars resistant to race 2, the management of the disease has been inefficient. Recently, reports of outbreaks of the disease have been occurring in new production areas of the country. Historically, two species within the genus have been associated with Verticillium wilt in vegetables: *Verticillium dahliae* and *V. albo-atrum*. Changes in the taxonomy of the genus, the recognition of new pathogenic species, and the emergence of three different lineages by hybridization events have been revealed through the new identification system based on a morphological and multigenetic approach. However, identification studies of *Verticillium* isolates in Brazil, involving both approaches, have been absent. On the other hand, on the characterization of Brazilian isolates regarding the type of "mating" and identification of physiological races through molecular tools is an unprecedented research work. Another extremely innovative aspect, is the search for mycoviruses as potential biological control agents of *V. dahliae* that could assist in the management of the disease. Therefore, this work aimed to (1) identify through a multi-locus approach (ITS, GAPDH and ACT) a collection of Brazilian isolates of *Verticillum* spp., (2) determine the population structure of *V. dahliae* isolates in terms of the distribution of physiological races through bioassays with differential cultivars and via PCR, (3) determine the distribution and frequency of sexual compatibility group types (*mating type*) in Brazil, and additionally, (4) the use a metagenomic approach through next-generation sequencing (NGS) to detect potential mycoviruses that infect *V. dahliae* isolates and that in the future could be used as biological control agents. Multigenetic analysis revealed that there is a single species, *Verticillium dahliae* Kleb. associated with wilt symptoms in vegetables in Brazil. Virulence assays to determine physiological races were corroborated with molecular markers indicating that most isolates infecting vegetables belong to race 2. It was demonstrated that opposite mating type are present in Brazilian isolates of *V. dahliae*, being *MAT-1-1* predominant. Furthermore, analysis of putative virus sequences obtained by NGS, revealed the presence of a single mycovirus infecting isolates of *V. dahliae*. The new virus was tentatively named Verticillium dahliae single stranded RNA virus-strain CNPH (VdRV-strain CNPH). In the present work we present a comprehensive characterization of Verticillium isolates from Brazil, as well as a pioneering study on metagenomics for the discovery of mycoviruses with potential for biological control of the fungus.

Keywords: MAT gene idiomorphs, mycoviruses, physiological races, next generation sequencing, Verticillium wilt.

Capítulo I

Introdução Geral

IDENTIFICAÇÃO E CARACTERIZAÇÃO DE ISOLADOS DE *Verticillium dahliae* DE HORTALIÇAS E DETECÇÃO DE MICOVÍRUS

1. INTRODUÇÃO GERAL

1.1. A Murcha de Verticillium

As murchas vasculares causadas por fungos fitopatogênicos são doenças amplamente distribuídas e são muito destrutivas, causando perdas da ordem de bilhões de dólares em culturas anuais (KLOSTERMAN et al., 2011). As murchas vasculares -que são causadas por espécies do gênero *Verticillium*- estão entre as mais devastadoras em muitas espécies de plantas. Estas doenças vasculares são causadas por um pequeno grupo de 10 espécies de fungos fitopatogênicos (INDERBITZIN; SUBBARAO, 2014), dentre as quais *Verticillium dahliae* Kleb. está entre as mais prejudiciais por tratar-se de um patógeno habitante do solo, cosmopolita e polífago que infecta mais de 200 espécies de plantas. Entre as hospedeiras de *V. dahliae* estão muitas culturas agrícolas economicamente importantes como: tomate, batata, soja, berinjela, alface, beterraba, oliveiras, algodão, cacau, entre outras (PEGG; BRADY, 2002; FRADIN; THOMMA, 2006; KLOSTERMAN et al., 2009).

Portanto, a murcha de Verticillium é uma das mais terríveis doenças vasculares das principais espécies hortícolas (JIMÉNEZ-DÍAZ et al., 2012; INDERBITZIN; SUBBARAO, 2014; ACHARYA et al., 2020). O agente causal primário, *V. dahliae* é um fitopatógeno de grande variabilidade genética, de longa sobrevivência no solo e com estilo de vida vascular (JOHNSON; DUNG, 2010; JIMÉNEZ-DÍAZ et al., 2012).

Os sintomas da doença podem incluir murcha, clorose, necrose, retardo de crescimento, bem como descoloração vascular até se tornar marrom a qual pode ser observada em seções transversais do tecido do caule das plantas infectadas (PEGG; BRADY, 2002; FRADIN; THOMMA, 2006). No entanto, os sintomas da murcha de Verticillium podem variar entre hospedeiros, sendo que não há sintomas exclusivos que pertençam a todas as plantas infectadas por este fungo (FRADIN; THOMMA, 2006). No tomateiro os sintomas externos são bem visíveis, iniciando com uma murcha moderada nas horas mais quentes do dia com recuperação da turgidez no período da noite, em seguida pode-se observar nas margens dos folíolos das folhas inferiores ou mais velhas, murcha, amarelecimento e necrose do limbo, a partir do seu bordo em forma de “V” com o vértice voltado para a nervura principal (KUROZAWA; PAVAN, 1997; INOUE-NAGATA et al., 2016).

No Brasil, desde o final dos anos 80 a doença tem sido associada com as duas espécies polífagas mais notórias dentro do gênero *Verticillium*: *V. dahliae* e *V. albo-atrum* (MENDES et al., 1998). No entanto, os trabalhos realizados por REIS; BOITEUX (2006a; 2006b) e REIS; BOITEUX; COSTA (2007), têm relevado que *V. dahliae* é aparentemente a espécie predominante, de ocorrência mais comum no País, e mais provavelmente, o único agente causal da murcha de Verticillium em tomateiro, e outras hortaliças de fruto e tubérculo como a batata (REIS; BOITEUX; 2006a; INOUE-NAGATA et al., 2016; LOPES; REIS; NARDIN, 2018).

1.2. Breve história taxonômica do gênero *Verticillium*

Verticillium é membro da Família: Plectosphaerellaceae, Ordem: Phyllachorales, Subclasse: Hypocreomycetidae, Classe: Sordariomycetes, dentro do Phylum: Ascomycota (INDERBITZIN; SUBBARAO, 2014; SUBBARAO, 2020). Atualmente, este gênero fúngico com poucas espécies é referido como *Verticillium sensu stricto (s.s.)*, cuja espécie tipo é *Verticillium dahliae*. (INDERBITZIN et al., 2011b).

Verticillium tem uma interessante e complexa história taxonômica, já que se encontra entre os gêneros mais antigos dos fungos filamentosos (INDERBITZIN; SUBBARAO, 2014). A primeira espécie do gênero foi descrita em 1816 por Nees von Esenbeck com o nome genérico de *Verticillium tenerum*, como uma única espécie saprofítica (NEES VON ESENBECK, 1816 In: INDERBITZIN et al., 2011b). Durante muito tempo, o gênero tornou-se um repositório de cerca de 190 espécies descritas, sendo vagamente delimitado formas assexuais, verticilados e de fiálides aculeiformes, caracteres pouco suficientes para uma classificação genérica precisa (ZARE; GAMS; SCHROERS, 2004).

A partir desse ponto, em 1971 o gênero foi divido em seções, *Verticillum* seção *Prostrata* (*Verticillium* section *Prostrata*) foi proposto por W. Gams para separar formas assexuais semelhantes à *Verticillium* da família Clavicipitaceae (GAMS; FISCHER; STUTTGART, 1971). Subsequentemente, Zare e Gams concentraram esforços na revisão da seção *Prostrata*, e subdividiram as espécies em diferentes gêneros (ZARE; GAMS, 2008). Com o advento da sistemática molecular, um grande número de fungos entomófagos e espécies nematófagas anteriormente classificadas como *Verticillium sensu lato* foram reclassificados nos gêneros *Lecanicillium*, *Pochonia*, *Gibellulopsis* e *Musicillium* (GAMS et al., 2005; GAMS; ZARE, 2002; INDERBITZIN et al., 2011b). Controversamente ao conhecimento atual, na literatura antiga *V. dahliae* era geralmente considerado um subgrupo dentro de *V. albo-atrum*, porém na década de 1970, foi aceito que isolados com formação de microescleródios deveriam

formar uma espécie separada chamada *V. dahliae* (ISAAC et al., 1967; SCHNATHORST, 1973; PEGG; BRADY, 2002; FRADIN; THOMMA, 2006).

Para evitar mudar os nomes dos principais patógenos de plantas, dentro do gênero *Verticillium* foi redefinido *V. dahliae* como a espécie-tipo a maneira de conservação do nome genérico, e assim evitar controvérsias taxonômicas e mudanças no gênero (GAMS et al., 2005). Para aumentar a complexidade do gênero, a primeira evidência do que formalmente se conhece como *V. longisporum*, foi apresentada na Alemanha em 1961 por Stark. Foi o primeiro pesquisador a isolar, o que ele chamou de *V. dahliae* var. *longisporum* em rábano picante (*Armorasia rusticana*) (KARAPAPA; BAINBRIDGE; HEALE, 1997). Atualmente, *V. longisporum* é um fungo fitopatogênico com própria complexidade, com uma história evolutiva incomum, por ser um híbrido diploide que consiste em três linhagens diferentes, cada uma originada de um evento de hibridação separada, e que infectam os vasos de xilema de culturas da Família *Brassicaceae*, principalmente colza (*Brassica napus* L.), couve-flor (*Brassica oleracea* var. *botrytis*), repolho (*Brassica oleracea* var. *capitata*), couve-de-bruxelas (*Brassica oleracea* var. *gemmifera*) e beterraba (*Beta vugaris* L.) (EYNCK et al., 2007; NOVAKAZI et al., 2015; DEPOTTER et al., 2016).

Até 2011, eram conhecidas e aceitas apenas cinco espécies patogênicas de *Verticillium*, sendo que as espécies *V. nigrescens* e *V. theobromae* foram reclassificadas nos gêneros *Gibellulopsis* e *Musicillium*, respectivamente, e portanto, excluídas do gênero *Verticillium s. s.* (ZARE et al. 2007; KLOSTERMAN et al. 2009). No estudo desenvolvido por Inderbitzin et al. (2011a) foram resolvidas as relações evolutivas entre as espécies de *Verticillium*. Atualmente, dentro de *Verticillium s. s.* estão reconhecidas dez espécies fitopatogênicas baseadas em análises filogenéticas multigênicas com o espaçador interno transcrito (ITS-rDNA), e sequências parciais dos genes codificadores de proteínas da actina (ACT), fator de elongação 1-alfa (EF-1α), gliceraldeído-3-fosfato desidrogenase (GAPDH) e triptofano sintase (TS) (INDERBITZIN et al., 2011a).

Verticillium dahliae sendo a espécie-tipo, é a mais amplamente distribuída e que causa mais prejuízos à agricultura no mundo (GAMS et al., 2005; ZARE et al., 2007; KLOSTERMAN et al., 2009; INDERBITZIN et al., 2011a). Entretanto, *V. longisporum* pode causar perdas de rendimento entre 10-50% em espécies de brássicas (DUNKER et al., 2008; DEPOTTER et al., 2016). As outras espécies, como *V. albo-atrum sensu stricto* tem sido relatada somente em batata com potencial para causar perdas significativas de até 50% da produção (PLATT et al., 2000; RADIŠEK; JAKŠE; JAVORNIK, 2006; USAMI et al., 2011), enquanto que *V. nonalfalfae* e *V. alfalfa* -antigamente referidas como *V. albo-atrum*- estão

associadas com perdas de até 50% no rendimento da alfafa, potato, espinafre e tomate (XU et al., 2019; INDERBITZIN et al., 2011a). No caso de *V. zaregamsianum* trata-se de um patógeno de alface relatado no Japão, enquanto que *V. tricorpus* somente é patogênico em craveiro e tomate jardim restrito a relatos feitos no Japão, Reino Unido e Holanda. As três espécies referidas como *V. nubilum, V. isaacii* e *V. klebahnii* são apenas patógenos menores em hospedeiros como batata inglesa, substrato de alface, espinafre e tomate jardím, e alface, respectivamente, além de ser considerados saprófitos do solo (KLOSTERMAN et al., 2009; INDERBITZIN et al., 2014).

Inderbitzin et al. (2011a) propõem um novo sistema taxonômico baseado numa abordagem morfológica e filogenética multigênica. O objetivo principal desse estudo foi fornecer um meio de identificação mais confiável e consistente dos principais grupos filogenéticos a nível de espécies em *Verticillium*. Além disso, os autores determinaram os nomes corretos para as espécies recuperadas por comparação com os isolados "ex-type", realizando buscas de material em herbários e revisão de literatura, assim como a descrição de novas espécies para aqueles grupos onde não havia nomes disponíveis dentro do gênero. Por tanto, atualmente para *Verticillium* estão reconhecidas com sólida estrutura taxonômica, dez espécies, cinco das quais foram novas para o conhecimento da ciência. Outro papel que continua sendo importante para diferenciar espécies é a morfologia das estruturas de repouso, porém a dependência quase completa da identificação por morfologia deve ser abandonada.

Na literatura atual, esse tipo de abordagem proposto por Inderbitzin et al. (2011a) está sendo utilizado como pré-requisito para publicação de manuscritos e assim, evitar novas confusões nas informações sobre *Verticillium*.

1.3. Raças fisiológicas em *V. dahliae*

Verticillium dahliae apresenta duas raças fisiológicas descritas (raça 1 e raça 2) em tomateiro e alface (HAYES et al., 2011; SHORT et al., 2014a). Até recentemente populações de isolados da raça 2 que infectam tomateiro foram divididos em uma raça adicional, a raça 3 (USAMI et al., 2017). Estudos sobre a raça 3 têm sido desenvolvidos em isolados coletados no Japão e nos Estados Unidos (USAMI et al., 2017; INGRAM et al., 2020).

Em tomateiro, a resistência contra *V. dahliae* para isolados raça 1 é conferida por um único gene dominante, *Ve* (DIWAN et al., 1999). O gene *Ve1* foi identificado em 1932 em acessos silvestres de *Solanum esculentum* L. denominado "Peru Wild". Consequentemente e a partir de 1950, essa resistência foi incorporada na maioria das cultivares comerciais de tomateiro (SCHAIBLE et al., 1951; GROGAN et al., 1979; DIWAN et al, 1999). Somente uma

década depois, Alexander relata em Ohio, USA, a ocorrência de uma nova raça fisiológica de *V. dahliae* causando doença em cultivares resistentes de tomateiro (ALEXANDER, 1962). A partir deste ponto, relatos similares da ocorrência da raça 2 foram feitos em diferentes países (LATERROT; MELO; BLANCARD, 1983; JONES; OVERMAN, 1986; LIGOXIGAKIS; VAKALOUNAKIS, 1992; DOBINSON; TENUTA; LAZAROVITS, 1996).

Quanto ao monitoramento e determinação das raças fisiológicas presentes no Brasil, pesquisadores da EMBRAPA hortaliças caracterizaram isolados do tomateiro e outras solanáceas relevantes, indicando que ambas estão presentes no país (REIS; BOITEUX; COSTA, 2007). Na época, a raça 1 de *V. dahliae* estava amplamente distribuída no território brasileiro, apesar de ter disso relatada somente no Estado de Pernambuco (LATERROT; MELO; BLANCARD, 1983). Com isso, estudos visando a busca de fontes de resistência para raça específica, e para ambas raças, foram mais tarde desenvolvidos (MIRANDA et al., 2010; CABRAL, 2015).

Com tudo isto, o conhecimento sobre as diferenças entre raças fisiológicas, variabilidade molecular e de resistência, ainda é limitado e complexo. Para inicialmente compreender a estrutura populacional das raças de *V. dahliae* é necessário abranger a sequência das seguintes informações: De Jonge et al. (2012) identificaram o efetor *Avr1* que codifica o fator de virulência em *V. dahliae*, o qual ativa o receptor imunológico *Ve1*. Posteriormente, no ano de 2017, foi identificada uma fonte de resistência na espécie de tomate selvagem, *Solanum neorickii* para raça 2. Esse material genético foi utilizado no desenvolvimento das cultivares de tomate "Aibu" e "Ganbarune-Karis", cuja resistência é dominada por um gene dominante único, *V2* (USAMI et al., 2017). Os autores baseados nesses resultados propuseram que a atual raça 2 de *V. dahliae*, deveria ser dividida em duas raças: raça 2, não patogênica em "Aibu", e raça 3 patogênica nesses cultivares (USAMI et al., 2017; ACHARYA et al., 2020). Recentemente, CHAVARRO-CARRERO et al., 2020 identificaram o efetor de avirulência que ativa a resistência do gene *V2*, denominado *Av2*.

1.4. Mating types em *Verticillium dahliae*

Para muitos fungos assexuados o estágio sexual é desconhecido ou raramente foi observado em condições de laboratório. Por esta razão, muitos deles estão quase sempre associados com a perda recente da sexualidade ou ocorrência de algum grau de reprodução sexual não detectada ou enigmática (BUTLER, 2007; MILGROOM et al., 2014). Tradicionalmente, considera-se que *V. dahliae* não possui estágio sexual conhecido e se reproduz assexuadamente (SHORT et al., 2014b). No entanto, evidência de heterotalismo tem

sido relatada, incluindo os genes *mating type* e específicos a meiose, embora outros estudos apontem que a estrutura populacional de *V. dahliae* seja clonal (MILGROOM et al., 2014; ERINCIK, 2020).

Nos fungos Ascomicetos, o locus de tipo acasalamento MAT controla o desenvolvimento sexual e a produção de esporos sexuais. O locus existe como duas formas alternativas, sendo um sistema de acasalamento bipolar (TURGEON, 1998). Teoricamente, a reprodução sexual em *V. dahliae* é regulada por dois idiomorfos do locus MAT referido como MAT1-1 e MAT1-2 (USAMI; ITOH; AMEMIYA, 2009a). Vários estudos indicaram que o idiomorfo MAT1-2 é altamente dominante em todo o mundo (USAMI; ITOH; AMEMIYA., 2009b; MILGROOM et al. 2014; SHORT et al., 2015), enquanto que MAT1-1 é de rara ocorrência, podendo ser esta uma das principais razões para a falta de reprodução sexual em *V. dahliae* (USAMI; ITOH; AMEMIYA, 2009b). MILGROOM et al. (2014) explica que a superabundância do MAT1-2 em *V. dahliae* pode ser parcialmente explicado pela expansão clonal de certos genótipos bem-sucedidos e altamente adaptados que não requerem reprodução sexual para completar o ciclo da doença, ao contrário de outros fitopatógenos vegetais. Outra hipótese levantada por Short et al. (2014) sobre a alta frequência MAT1-2 em alguns ou na maioria dos hospedeiros indica que poderia estar associado a uma maior virulência, onde foi observado que isolados de MAT1-2 são significativamente mais virulentos que isolados MAT1-1 (SUBBARAO, dados não publicados em SHORT et al., 2014b).

1.5. Manejo da murcha de Verticillium

O controle da murcha de Verticillium é verdadeiramente difícil, devido às características do patógeno e a natureza da infecção (KEYKHASABER; THOMMA; HIEMSTRA, 2018). Consequentemente, o manejo da doença requer a integração de várias estratégias, pois é bem sabido que nenhuma única opção de manejo é eficaz no controle da murcha de Verticillium (WOODWARD; WHEELER, 2011; JIMÉNEZ-DÍAZ et al., 2012). Além disso, a falta de opções de controle químico que sejam economicamente viáveis para a murcha de Verticillium exige a incorporação de práticas culturais para o manejo da doença (FRADIN; THOMMA, 2006; KEYKHASABER; THOMMA; HIEMSTRA, 2018). Estratégias que reduzem o inóculo inicial incluem: a rotação de culturas, principalmente com "não-hospedeiros" do patógeno como as gramíneas e brássicas (WHEELER; BORDOVSKY; KEELING, 2019; KLOSTERMAN et al., 2009). A fumigação do solo por muito tempo foi uma estratégia indispensável para o controle da murcha de Verticillium, porém com a proibição do Brometo-de-metila, devido a questões ambientais, uma busca por alternativas para a fumigação do solo

que sejam eficazes, continua (KLOSTERMAN et al., 2009); entre outras estratégias como adubos verdes, solarização e bio-fumigantes (JOHNSON; DUNG, 2010). Por outro lado, a resistência genética restringe a infecção e colonização do patógeno, constituindo o melhor método de controle a longo prazo em todo tipo de hospedeiro (ATALLAH; HAYES; SUBBARAO, 2011; JONHSON; DUNG, 2010). Entretanto, não existem fontes de resistência à *V. dahliae* na maioria das espécies hospedeiras ou nos seus parentes selvagens. Similarmente, o biocontrole é uma estratégia de manejo vista como uma alternativa potencial ao controle químico, com a ideia de prevenir prejuízos ao meio ambiente (ACHARYA et al., 2020). De acordo com a definição de Cook e Baker (1983), "o biocontrole pode ser definido como a redução da quantidade de inóculo de um patógeno, ou sua capacidade de causar doença por meio da atividade de um ou mais (micro) organismos, exceto o ser humano". Os agentes de controle biológico (BCAs pela abreviação em inglês) são micro-organismos utilizados para controlar pragas, incluindo insetos e fitopatógenos, através da redução do inóculo do patógeno ou da sua capacidade de causar doença (RUANO-ROSA; MERCADO-BLANCO, 2015). Por conseguinte, a utilização efetiva da BCAs deve estar baseada em um conhecimento profundo dos mecanismos envolvidos no biocontrole (competição, antibiose, micoparasitismo, indução de respostas de defesa, etc.) e como os fatores bióticos interagem dinamicamente com cada patossistema. Entre os BCAs mais estudados e utilizados como biofungicida estão algumas espécies de *Trichoderma* spp., algumas espécies de bactérias, sendo as mais frequentes aquelas dos gêneros *Agrobacterium*, *Bacillus*, *Pseudomonas* e *Streptomyces*. No entanto, fungos não patogênicos e micovírus também têm sido estudados e utilizados como BCAs (MILGROOM; CORTESI, 2004; ANGELOPOULOU et al., 2014; RUANO-ROSA; MERCADO-BLANCO, 2015).

As micoviroses associadas à hipovirulência para o controle de doenças de plantas estão emergindo como uma das mais recentes estratégias de biocontrole (KUMAR; CHANDEL, 2016). O reconhecimento de que os micovírus podem induzir hipovirulência (redução da virulência) nos seus hospedeiros tem despertado grande interesse na identificação e caracterização de vírus que infectam fungos fitopatogênicos devido ao seu potencial para ser utilizados como ferramentas no controle biológico (GARCÍA-PEDRAJAS et al., 2019). Um dos casos de sucesso e bem documentados é a hipovirulência associada com a infecção por micovírus no fungo *Chryphonectria parasitica*, agente causal do cancro-da-castanheira (MILGROOM; CORTESI, 2004). Além disso, um vírus de DNA associado à hipovirulência, o SsHADV1, também mostrou a capacidade de controlar a doença de *Sclerotinia* sob condições de campo (YU et al., 2010; 2013), da mesma maneira *Rosellinia necatrix* megabirnavirus 1

(RnMBV1) mostrou um potencial significativo para o controle biológico da doença da podridão da raiz da maçã branca (CHIBA et al., 2009).

1.6. Aspectos gerais dos micovírus

Os vírus que infectam fungos (vírus fúngicos) são vírus prevalentes que se replicam dentro das células fúngicas, também denominados micovírus (KUMAR; CHANDEL, 2016). Os fungos como outros organismos vivos podem ser infectados por vírus, os quais são comumente encontrados em todos os principais grupos de fungos fitopatogênicos (GHABRIAL; SUZUKI, 2008). Assim como os vírus que infectam animais e plantas, os micovírus precisam de células vivas de outros organismos para se replicar (SON; YU; KIM, 2015). Embora compartilhem algumas características com vírus animais e vegetais, os micovírus são transmitidos intracelularmente durante a divisão celular (anastomose de hifas), esporogênese e fusão celular. Eles não possuem uma fase extracelular de infecção nos seus ciclos de vida. As partículas dos micovírus acumulam-se no citoplasma do fungo, e carecem de proteína de movimento, o que seria irrelevante dentro do fungo hospedeiro (PEARSON et al., 2009; XIE; JIANG, 2014; SON; YU; KIM, 2015; GHABRIAL; SUZUKI, 2009).

Os genomas dos micovírus são diversos: (1) RNA fita dupla com genoma segmentado (dsRNA) que atualmente estão classificados em oito famílias e um gênero sem associação à família: *Amalgaviridae*, *Chrysoviridae*, *Megabirnaviridae*, *Quadriviridae*, *Partitiviridae*, *Reoviridae*, *Totiviridae*, *Polymycoviridae* e *Botybirnavirus*; (2) RNA de fita simples (ssRNA) classificados em oito famílias com genoma senso positivo (+) ssRNA: *Alphaflexiviridae*, *Botourmiaviridae*, *Deltaflexiviridae*, *Endornaviridae*, *Gammaflexiviridae*, *Barnaviridae*, *Hypoviridae*, e *Narnaviridae*, e aqueles com genoma senso negativo (-) ssRNA: *Mymonaviridae*; e (3) genoma circular de fita simples (ssDNA) que estão atribuídos a uma única família viral, *Genomoviridae*. Até hoje, nenhum micovírus de genoma dsDNA foi relatado (GHABRIAL et al., 2015; WU et al., 2017; KOTTA-LOIZOU, 2019; MATA et al., 2020), porém existe evidência paleontológica de vírus gigantes com elementos nucleocitoplasmáticos de dsDNA (NCLDVs) infectando o genoma vários grupos de fungos (GONG; ZHANG; HAN; 2020).

1.6.1. Sintomas associados à micovírus

Geralmente, os micovírus permanecem latentes e raramente induzem sintomas em seus hospedeiros (PEARSON et al., 2009). No entanto, alguns micovírus podem alterar fenótipos que causam mudanças dramáticas nos seus hospedeiros infectados, incluindo a redução da

virulência (hipovirulência) (GHABRIAL et al., 2015; JIANG et al., 2013; PEARSON et al., 2009; NUSS, 2005).

A hipovirulência é um efeito vantajoso dos vírus que diminui a patogenicidade dos fungos fitopatogênicos contra as plantas. Num termo geral, trata-se de uma redução na capacidade de produção de doença do patógeno (KUMAR; CHANDEL, 2016). Adicionalmente, a hipovirulência pode resultar numa redução da pigmentação, redução da esporulação assexuada, perda da fertilidade e redução da taxa de crescimento do fungo hospedeiro (SON; YU; KIM, 2015; KUMAR; CHANDEL, 2016). Esse atributo (hipovirulência) geralmente é associado à presença de micovírus com genoma RNA de fita dupla (dsRNA), embora outros fatores como mutações mitocondriais, mutações nucleares e plasmídeos também já foram associados à hipovirulência (MONTEIRO-VITORELLO et al. 1995; BOLAND, 2004).

1.6.2. Formas de transmissão dos micovírus

Os micovírus podem ser transmitidos de três maneiras: (1) transmissão horizontal, (2) vertical, e (3) extracelular (MUÑOZ-ADALIA; FERNÁNDEZ; DIEZ, 2016).

A transmissão horizontal ocorre quando um micovírus coloniza um novo hospedeiro através do contato de hifas e subsequente fusão de micélios entre indivíduos (anastomose) durante a formação de heterocariontes (mediada por um sistema de auto/não-reconhecimento). No entanto, isolados da mesma espécie nem sempre são compatíveis, dentro de uma população. Neste tipo de transferência, diferentes grupos de compatibilidade vegetativa (tipos *vc* ou VCGs) desempenham um papel essencial, muitas vezes restringindo o movimento do vírus (LESLIE, 1993; MUÑOZ-ADALIA; FERNÁNDEZ; DIEZ, 2016). Portanto, esse tipo de transmissão é bastante variável uma vez que depende da compatibilidade entre as hifas, um mecanismo de reconhecimento geneticamente controlado (PEARSON et al., 2009).

Já na transmissão vertical, por divisão de células na produção de esporos sexuais ou assexuais é a mais comum e ocorre com frequência variável (COENEN; KEVEI; HOEKSTRA, 1997; PICARELLI et al., 2017) na produção de esporos sexuados e assexuados infectando novas gerações do hospedeiro (KOTTA-LOIZOU; COUTTS, 2017).

A fase extracelular no ciclo de replicação para a maioria dos micovírus conhecidos ainda não foi relatada, no entanto, há algumas exceções, como no caso de *S. sclerotium* (KOTTA-LOIZOU COUTTS, 2017). A transmissão pode ser feita, experimentalmente, pela anastomose de hifas, ou por técnicas moleculares como fusão de protoplastos, transformação, transfecção e genética reversa (PICARELLI et al., 2017). Há relatos onde partículas virais purificadas de *S.*

sclerotiorum hipovirus associado ao DNA virus 1 (SsHADV-1) infectam extracelularmente *in vitro* ou *in vivo* os protoplastos livres, hifas e fragmentos de hifas descritos em 2013 por YU et al. (2013).

1.6.3. Micovírus que infectam *Verticillium dahliae*

Até o momento, existem poucos relatos da presença de micovírus infectando micélio de *V. dahliae*. A maioria possui genomas de RNA de fita dupla (dsRNA) que mostraram senlhanças com membros do gênero *Partitivirus* (FENG et al., 2013; CAÑIZARES et al., 2015) e *Chrysovirus* (CAO et al., 2011).

Cao et al. (2011) detectaram cuatro segmentos de RNA infectando isolados de algodão coletados na província Shaanxi na China, onde cada segmento viral do genoma dsRNA continha um ORF (Open Reading Frame, pelo seu significado no inglês) codificando uma RNA Polimerase dependente de RNA (*RdRp*, pela abreviação em inglês), a proteína da cápside, uma proteína de replicação não definida e um domínio peptídico correspondente ao gênero *Chrysovirus*, o qual foi nomeado *Verticillium dahliae* chrysovirus 1 (VdCV1). Por outro lado, Feng et al. (2013) relataram um *Partitivirus* infectando também isolados de algodão provenientes da província Xinjiang na China. O genoma do novo micovírus continha dois segmentos do tipo dsRNA, o qual foi nomeado *Verticillium dahliae* partitivirus 1(VdPV1). O segmento maior (1768 pb) codifica um ORF semelhante ao domínio *RdRp*, enquanto que o segmento menor (1587 pb) contém um único ORF para a proteína putativa da cápside viral. Similarmente em 2015, em isolados de oliva na Turquia (CAÑIZARES et al., 2015) foram altamente semelhantes (94% e 91%, para o RNA1 e RNA2, respectivamente) com o *Partitivirus* previamente identificado em isolados de algodão na China (VdPV1), identificados como a mesma espécie viral com diferentes origens geográficas e hospedeiros, denominado *Verticillium dahliae* partitivirus 1 de oliva (VdPV1-ol). Além desses relatos, somente um tipo micovírus com genoma viral RNA de fita simples senso positivo (+) ssRNA tem sido recuperado de áreas de cultivo de oliva na Espanha (CAÑIZARES et al., 2017). Foi denominado como *Verticillium dahliae* RNA vírus 1 (VdRV1). Esse novo micovírus com genoma de 2631 nucleotídeos de comprimento contém dois ORFs que potencialmente codificam uma proteína hipotética com função desconhecida (ORF1) e uma proteína similar à *RdRp* de micovírus de genoma ssRNA. O mais recente relato sobre a ocorrência de dsRNA micoviral em *V. dahliae* infectando agora dois diferentes culturas: algodão e oliva, foram feitos na Turquia (HOSSEINALIZADEH; ERİNCİK; AÇIKGÖZ, 2020).

Inclusive em outros hospedeiros como no fungo *V. longisporum*, recentemente foi descoberto um novo micovírus com genoma (+) ssRNA, *Verticillium longisporum ssRNA virus* 1 (VlAV1), que junto com outros membros virais foram propostos para formar parte de uma nova família nomeada *Ambiguiviridae* (GILBERT et al., 2019).

A importância do diagnóstico precoce com sólido sistema taxonômico e a implementação de medidas que visem evitar a disseminação entre lavouras de uma mesma região, assim como a busca de variedades com resistência efectiva deve ser uma das prioridades da pesquisa contra o patógeno *V. dahliae* (REIS; BOITEUX, 2006a). Antes que isso possa ser feito, é necessária a padronização na estratégia de identificação que acompanhe as características morfológicas, assim como uma compreensão da estrutura genética dentro e entre as populações de *Verticillium* que ocorrem nas diferentes regiões produtoras do país. Por outro lado, embora muitos micovírus não tenham efeitos nos seus hospedeiros, aqueles que reduzem a virulência dos seus hospedeiros são de considerável interesse para o desenvolvimento de novas estratégias de biocontrole (GHABRIAL et al., 2015). Portanto, os estudos metagenômicos e sua aplicação abrangem um amplo escopo de pesquisa, incluindo o campo da virologia para a análise de comunidades virais. Além disso, durante a última década, o processo de detecção e descoberta de vírus a partir dos dados do sequenciamento de nova geração (NGS) vem fornecendo o avanço rápido sobre a diversidade de vírus na natureza (VILLAMOR et al., 2019). Com tudo isto, uma abordagem metagenômica resulta ser inovadora e pioneira para determinar a diversidade de micovírus em *Verticillium* spp.

Portanto, os objetivos deste estudo foram: (1) Identificar de forma precisa através de uma abordagem multi-locus (ITS, GAPDH e ACT) uma coleção de isolados brasileiros de *Verticillum* spp; (2) determinar a estrutura populacional de isolados de *Verticillium dahliae* em termos da distribuição de raças fisológicas através de bioensaios com cultivares diferenciais e a incidência relativa por PCR; (3) determinar a distribuição e frequência dos tipos de *matig type* no Brasil; (4) utilizar uma abordagem metagenômica através do sequenciamento NGS para identificar potenciais micovírus infectando isolados de *V. dahliae.* Essas informações são valiosas para o estabelecimento de estratégias de manejo da doença, assim como para os programas de melhoramento genético, visando à incorporação de resistência, e na busca de resistência ao patógeno. Além disso, uma análise abrangente do transcritoma de *V. dahliae* para examinar em detalhe a infecção por micovírus pode fornecer informações úteis em posteriores pesquisas para o desenvolvimento de agentes de controle biológico.

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Capítulo II

Molecular identification of *Verticillium* isolates causing vascular wilt in different plant species in Brazil

Molecular identification of *Verticillium* isolates causing vascular wilt in different plant species in Brazil

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Abstract

Verticillium wilt is particularly important disease because it causes great economic losses to vegetable producers in Brazil. Morphological characters and molecular identification are two tools that are necessary for an accurate diagnosis of any causal agent, including for the identification of the *Verticillium* species. This has been particularly important due the recent changes in taxonomic features, in the range of hosts, and in the geographical distribution, as well as longstanding controversies surrounding the genus. Recent outbreaks of Verticillium wilt infecting different vegetables in the country have brought back the concern to determinate the identity of the causal agent of the disease once *V. dahliae* is known as the most common species only by morphological diagnosis. We characterized a collection of 89 *Verticillium* isolates, representing a variety of vegetable hosts. A multi-locus approach (ITS, GAPDH, and ACT) was used for reliable identification of potential *Verticillium* species. Greenhouse pathogenicity tests confirmed the infection by *V. dahliae*, and the development of characteristic symptoms of the disease on their original hosts ranging from Solanaceous vegetables to other species, such as strawberry and cacao. The single and combined analysis of all DNA regions from *Verticillium* isolates correspond to a unique species, *V. dahliae*, causing vascular wilt in different horticultural crops collected in ten agricultural regions of Brazil. This information is valuable to plan strategies to manage the disease, mainly by the development of resistant cultivars.

Keywords: Multigene, phylogenetic analysis, Vegetables, *Verticillium dahliae*, Verticillium wilt.

Introduction

The genus *Verticillium* encompasses a cosmopolitan group of Ascomycota fungi, including several plant pathogenic species that cause vascular wilt diseases in commercial plant species (Klosterman et al. 2009). There are currently ten *Verticillium* species recognized (Inderbitzin et al. 2011b) and some of them are amongst the world major pathogens in agriculture (Pegg and Brady 2002).

Verticillium species are able to infect a large number of dicotyledonous plant species (Inderbitzin and Subbarao 2014), in temperate and subtropical regions (Barbara and Clewes 2003). So far, most monocotyledonous plants are considered to be non-host species of *Verticillium* spp. (Fradin and Thomma 2006). The reduced genus is now referred to as *Verticillium sensu stricto* (Inderbitzin et al. 2013), and historically, two of the most notorious and economically important species are *Verticillium albo-atrum* Reinke & Berthold and *V. dahliae* Kleb. (Klosterman et al. 2009).

For many years, *V. dahliae* was classified as a subgroup within the species *V. albo-atrum*, because it was included into the microsclerotial and dark mycelial strains. However, after much controversy it was accepted as a separate species (Fradin and Thomma 2006). Moreover, recently Inderbitzin et al. (2011a) classified and reassessed the taxonomy of the genus, and it was distinguished ten *Verticillium* species showing important changes in taxonomical features, in the range of hosts, and geographical distribution of the complex (Barbara and Clewes 2003; Inderbitzin et al. 2011b; Jing et al. 2018).

Verticillium dahliae is a soil-borne fungus and the most prominent wilt agent from this genus (Deppotter et al. 2017). The fungus colonizes the vascular system of its host plants (Reusche et al. 2014), and infection slowly progress 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 devastate a broad range of hosts and to cause serious outbreaks in new hosts or in new agricultural areas (Bhat and Subbarao 1999; López-Escudero and Mercado-Blanco 2011; Acharya et al. 2020). Control of Verticillium wilt is difficult, and also the pathogen is difficult to manage once it reaches the vascular plant tissue (Deketelaere et

al. 2017). Besides, there are currently no fungicides available to control Verticillium wilts once plants have been infected (Fradin and Thomma 2006).

In Brazil, Verticillium wilt is a particularly important disease because it causes great economic losses to vegetable producers (Reis and Boiteux 2006a). Since the late 80s, the disease has been associated with two causal agents, *V. dahliae* or *V. albo-atrum,* due to they are considered very similar in their morphological characters, especially in the presence or absence of microsclerotia (Mendes et al. 1998; Reis et al. 2007). Recent outbreaks of the disease appearing in the country have become an increasing concern in vegetable production (Reis and Boiteux 2006b; Lopes et al. 2018; Suaste-Dzul et al. 2021). However, it is important to note that *V. dahliae* has been reported as the most common occurring species in Brazil, often associated as the causal agent of wilts in several vegetables as tomato, eggplant, scarlet eggplant, strawberries, and okra (Mendes et al. 1998; Reis and Boiteux 2006a). Furthermore, in tomato, no other species than *V. dahliae* has been reported in Brazil (Reis and Boiteux 2006b). However, no study has been conducted to identify *Verticillium* species in Brazil using a multi-genic approach. For this reason, the pathogen has received considerable attention to clarify which it is the major pathogen causing Verticillium wilt recovered from vegetable growing regions through a conclusively molecular identification approach.

Thus, the objective of this study was to characterize the *Verticillium* isolates recovered from diseased tomatoes and other vegetables using molecular phylogenetic analysis, and determine if there are any *Verticillium* species within this genus in Brazil.

Material and methods

Fungal isolates

The *Verticillium* isolates used in this study were obtained from the EMBRAPA-CNPH plant pathogenic fungal collection (Empresa Brasileira de Pesquisa Agropecuária-Centro Nacional de Pesquisa de Hortaliças, Brazil) under cold storage conditions (Table 1). Fungal isolates were grown on potato dextrose agar (PDA) plates under ambient light/dark conditions at 23°C for two weeks, and their conidia were maintained as stock in 25% glycerol at -80°C in the Collection of Plant Pathogenic Fungi of Embrapa Hortaliças. A copy of each isolate was preserved at 6 °C, for been routinely used in this work.

Cultural characteristics, morphology and Sclerotia production

According to other researches, the capacity of *V. dahliae* to produce microesclerotia in PDA medium has been used as the main characteristic for discriminating between *V. albo-atrum* (Isaac 1967; Barbara and Clewes 2003; Fradin and Thomma 2006). Therefore, we conducted a phenotype classification with pure cultures of *Verticillium* isolates onto plates of PDA medium to observed their typical morphological characteristics. Three plates of each isolate were incubated at 23°C in dark conditions. Then, plates were observed daily for colony aspect and sclerotia production. During this period, it was observed the presence of fungal structures such as conidia and conidiophores and then microscopic preparations were examined with the aid of microscope (40x). Fourteen to eighteen days after the plates were completely filled by the fungus growth, they were observed for classification of sclerotia production.

DNA extraction

Genomic DNA of the isolates were extracted according to the Dellaporta et al. 1983 with modifications by Boiteux et al. (1999). Mycelia were harvested directly from PDA plates, blotted dry with filter paper, and frozen at -80°C overnight. The tissue was individually transferred into Eppendorf tubes (2 mL) containing two tungsten carbide beads (5 mm) (QIAGEN®, Germantown, MD) and 1 mL buffer lysis (50 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0, 400 mM NaCl, and 10 mM β-Mercaptoethanol). All the samples were homogenized twice at 20 Hz for 3 min with Tissue Lyser II systems (QIAGEN®, Germantown, MD). The DNA pellet was resuspended in 100 μL of TE buffer + RNAse A (20 mg/mL) (Thermo Fisher Scientific Inc., Waltham, MA). After extraction, samples were incubated at 37°C for 30 min, as part of the RNAse A treatment to remove any residual RNA present. Then, samples were stored at -20°C for later using.

PCR amplification

In order to assess the initial diversity, all isolates were first subjected to amplification of the ribosomal internal transcribed spacer region (ITS-rDNA). Different haplotypes were identified using DnaSP 4.0 (Rozas et al. 2003). A total of nine isolates representing each

haplotype was randomly chosen and subjected to a multilocus analysis, which involved: glyceraldehyde-3-phosphate dehydrogenase GAPDH and Actin (ACT). The primers and amplicon length used in the present study are listed in Table 2

PCR amplifications were performed in a Bio-Rad T100™ Thermal cycler (Bio-Rad Laboratories, Hercules, CA) in 25 µL volume reaction containing 1X buffer (10X), 2 Mm of $MgCl_2$ (50 mM), 0.2 Mm each dNTP (10 mM) (Invitrogen®), 0.4 µM of each primer (10 µM), 1 U Taq DNA polymerase recombinant (5 U/µL) (Invitrogen®), and 20 ng of genomic DNA. Conditions for PCR of ITS rDNA constituted 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 GAPDH amplification began with an initial denaturation at 95°C for 4 min; 38 cycles of 95°C for 30 s, 67°C for 30 s, and 72 °C for 45 s; and one cycle at 72 °C for 7 min. For ACT consisted of a 3 min initial denaturation 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, and followed by a final extension of 5 min at 72°C. For details about primer specific sequences and annealing temperatures, see table 2. The PCR amplification products were analyzed on 1% agarose gel in 0,5 X 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, USA). DNA purity and concentration from all samples were estimated by spectrophotometry (A260/280) using the Eppendorf Biophothometer® (Eppendorf®, AG, Germany).

The amplicons were purified with PureLink® PCR Purification kit (Invitrogen®, CA, USA) based on the selective binding of dsDNA to silica-based membrane according to the manufacturer's instructions. DNA sequencing were carried out by Macrogen Company (Seoul, Republic of South Korea).

Phylogenetic analyses

Forward and reverse sequences were assembled using the BioEdit 7.2.0 software (Hall, 1999). All obtained consensus sequences were compared to NCBI nucleotide database using the BLAST algorithm. Sequences representing ex-types and related published sequences were retrieved from GenBank (Table 1).

Multiple sequence alignments for each individual gene were estimated online using the G-INS-i strategy in MAFFT version 7 (Katoh and Toh 2013; Katoh and Yamada 2019) and manually adjusted where necessary in MEGA7 (Kumar et al. 2016).

Phylogenetic analyses were performed using the Maximum Likelihood (ML) and Bayesian Inference (BI) methods for both individual and concatenated genes. 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 GTR-GAMMA model.

Evolution models were estimated in MrModeltest 2.3 (Nylander 2004) using the Akaike information criterion (AIC) for each locus. The combined data set was partitioned to reflect the most appropriate nucleotide substitution model for each of the single locus data sets for the Bayesian analysis of the combined data set. Four Markov Chain Monte Carlo (MCMC) chains were conducted for 5 $x10^7$ generations, with samplings every 1000 generations. The convergence of all the parameters was checked using Tracer v 1.5 (Rambaut and Drummond 2010) and the first 25% generations were discarded as burn-in. FigTree version 1.4.3 (Rambaut 2012) was used to visualize the phylogenetic tree.

Pathogenicity test

Pathogenicity tests were performed by inoculating the original hosts of each isolate, including plants of tomato (*Solanum lycopersicon* L.), potato (*Solanum tuberosum* L.), eggplant (*Solanum melongena* L.), scarlet eggplant (*Solanum aethiopicum* L.), okra (*Abelmoschus esculentus* L.), and strawberry (*Fragaria* x *ananassa* Duch.). Also crossed inoculations were performed in eggplant hybrid "Ciça" seedlings as this cultivar is highly susceptible to *V. dahliae*. Pathogenicity of cacao (*Theobroma cacao* L.) isolates was tested only on eggplant seedlings. Inoculation methodology was followed as described by Santos et al. (1997), with some modifications (Reis et al. 2007).

Bioassays were conducted in a greenhouse (temperature 25C ±4°C and relative humidity of 70-80%) with a randomized block design, with three replicates (three pots with two plants each). The evaluation was carried out through comparisons, taking into account

the apparition or not of symptoms 30 days after inoculation (dai). Plants were observed daily for the development of foliar symptoms, such as chlorosis, necrosis and defoliation. Afterward, the pathogen was re-isolated from infected tissue of different original hosts and, also from the eggplants which were used as indicators of severe vascular wilt symptoms.

Results

Fungal isolates

A total of 89 *Verticillium* isolates used in this work were part of a fungal collection at CNPH-EMBRAPA vegetables since 1992 originally collected from ten different states and locals in Brazil (Table 1). Samples showing the typical symptoms of Verticillium wilt (Reis and Boiteux 2006a; Reis and Boiteux 2006b; Reis et al. 2007) were collected from Bahia-BA (n=5), Ceará-CE (n=2), Distrito Federal-DF (n=12), Espírito Santo-ES (n=23), Goiás-GO (n=1), Minas Gerais-MG (n=18), Paraná-PR (n=1), Rio de Janeiro-RJ (n=4), Santa Catarina-SC (n=5), São Paulo-SP (n=13), and unknown location without further specification (n=5). Most of the isolates analyzed were sampled from tomato (n=40) and intermediate numbers were obtained from eggplant (n=16), potato (n=15), and strawberry (n=12). The smallest number of isolates was found on scarlet eggplant (n=3) and cacao (n=2), and a single isolate was obtained from okra.

Cultural characteristics and microsclerotia formation

Verticillium isolates produced white colonies with abundant fluffy mycelium on PDA medium. The colonies were creamy-white, sometimes showing orange pigmentation and gradually became densely dark at the bottom of the plate (Fig. 1). All fungal isolates presented mycelium hyaline, septate and multinucleate. The conidia were ovoid to elongated and were produced on long phialides which were positioned in a whorl shape around the conidiophores. Branching of the verticillate conidiophores and microsclerotia were consistently observed on PDA medium for the majority of isolates (Fig. 1; Fig S1). These characteristic fit with the descriptions of *V. dahliae*, therefore the isolates were identified carefully as *Verticillium dahliae*-like.

Results of the characterization of 89 isolates of *Verticillium dahliae*-like by the microsclerotia formation in culture (MS) or those that not produced microsclerotia (NoMS)

are presented in Table 3. From the total isolates analyzed, 61 (68.5%) were classified as MS and 28 isolates (31.5%) as NoMS formation in PDA (Table 3). Specifically, in tomato isolates (n=40), we obtained 57.5% MS cultures versus 42.5% NoMS grown cultures; in eggplant (n=16) resulted 62.5% MS and 37.5% NoMS cultures; 100% of potato (n=15) and okra (n=1) isolates produced MS; in scarlet eggplant (n=3) 66.6% were classified as MS versus 33.3% NoMS formation; in strawberry isolates (n=12) were 75% MS and 25% were NoMs; and isolates in cacao (n=2) resulted in 50% MS versus 50% NoMs cultures. None of the isolates formed dark mycelia.

Molecular identification

Total DNA from individual isolates were in adequate concentration and purity (260/280) ratios from 1.7 to 1.94, indicated optimal integrity and suitable for molecular analysis. Amplification of the ITS region, GAPDH, and Actin gene generated 490, 727 and 588 bp fragments, and alignments of the sequences resulted in a data set of 457, 686, 558 bp characters. BLASTn analysis of ITS sequence of 89 isolates matched 99.79-100% identity (e-value = 0) to the ITS sequences of *V. dahliae* type strain PD323. First, the ITS sequence data for 68 isolates recognized as *V. dahliae* species with 100% bootstrap support (Fig. 3) was discriminated to only nine representative isolates in the phylogenetic analysis. Then, in order to perform a preliminary exploration of the ITS data set, the haplotype network analysis was performed over the 69 isolates of *V. dahliae* (sequences obtained in this study), 10 isolates of *V. dahliae*, and eight isolates of *V. longisporum* (sequences obatained from Inderbitzin et al. 2011a). These sequences were resolved in three different haplotype groups, which resulted in a haplotype diversity of (Hd): 0.289 (Fig. S2).

For the multilocus analyses, the three single-locus datasets (ITS, GAPDH, and ACT) did not show any conflicts in tree topology with 100% support, which allowed them to be combined (Fig. 2). The most parsimonious tree based on concatenated dataset was composed of 10 *Verticillium* species that were included as reference in this analysis: *Verticillium nonalfalfae* (PD592), *V. alfalfae* (PD489), *V. isaacii* (PD660), *V. klebahnii* (PD401), *V. zaregamsianum* (PD736), *V. tricorpus* (PD690), *V. nubilum* (PD742), *V. albo-atrum* (PD747), *V. dahliae* (PD322), and *V. longisporum* (PD687) (Inderbitzin et al. 2011a). This analysis provided the most parsimonious tree showing that a single species is present in the

247 collection of the EMBRAPA vegetables, grouping the nine representative isolates into the *V.*
248 *dahliae* clade with strong bootstrap support (Fig. 2).

249 The well resolved *V. dahliae* group by multi-genic analysis included in the
250 monophyletic clade of *Verticillium* species, was compared from ITS data set of nucleotides
251 in a single analysis. All isolates from this study were included into the group of *V. dahliae*
252 isolates used as reference: PD322: lettuce, PD323: hybrid strawberry, PD327: bell pepper,
253 PD337: upland cotton, PD404: bell pepper, PD502: maple, PD617: garden tomato, PD656:
254 sunflower, PD718: oilseed rape, and PD729: horseradish. However, we observed that Vert17
255 and Vert22 isolates forming polytomic branches into the *V. dahliae* clade (Fig. 3), especially
256 clustered to the ex-type isolate (PD322), and clustered to *V. longisporum* isolates. Analyses
257 only with ITS regions apparently is not enough to separate *V. longisporum* from *V. dahliae*
258 species. However, all those isolates were identified as *V. dahliae* species based on
259 morphological characteristics, haplotype network analysis and their estimated phylogenetic
260 tree (Fig. 2).

261 On the other hand, based on GAPDH analysis of the single locus, *Verticillium* isolates
262 were located and identifying into the clade: *V. dahliae* with bootstrap support of 99% (Fig.
263 4). At the same time, analysis for the single locus ACT also showed that *V. dahliae*-like
264 isolates belongs to *V. dahliae* clade with 100% bootstrap support (Fig. 5). Analysis with these
265 partial gene sequences (GADPH and ACT) can distinguished and separate the representative
266 isolates PD348 and PD687, identified by Inderbitzin et al. (2011a) as *V. longisporum* species
267 (Fig. 4; Fig. 5).

268

269 **Pathogenicity assays**

270 The first symptoms in plant of tomato, eggplant, scarlet eggplant, potato, okra and
271 strawberry appeared in a period of 21 to 28 dai. It was observed typical symptoms of
272 Verticillium wilt, such as chlorosis on the lower leaves, typical V-shaped areas in leaf
273 margins that eventually progressed to senescence and necrosis about 1 to 2 weeks. In the
274 longitudinal cuts from the basal stems were observed a light brown discoloration produced
275 by colonization of the pathogen in the vascular tissue. Microscopical observations from
276 infected tissues showed branching conidiophores and oval free conidia or in verticillate
277 disposition (Fig. S1). All isolates used in this study were pathogenic in their original hosts,

and 88.8% were pathogenic only in eggplants when the pathogen was re-inoculated. However, we found ten isolates (11.2%) that were pathogenic in their original hosts, but not on eggplants which was used as susceptible indicator plant of Verticillium wilt symptoms in cross-inoculations. These isolates showed low levels of sporulation in PDA culture (less than 2 x 10^6 conidia/mL), therefore, less inoculum produced mild symptoms or absence of them on eggplants seedlings as follow: one isolate whose original host was okra (Vert12); seven isolates from tomato (Vert36, Vert59, Vert71, Vert116, Vert121, Vert132, Vert151), one isolate from eggplant (Vert158), and one isolate from potato (Vert180). From the 89 isolates, 100% were re-isolated from plant tissue infected or from their original host. In comparison, mock inoculated plants (control) treated with distilled water did not have noticeable symptoms.

Discussion

Although *V. dahliae* were morphologically characterized in Brazil, there is still little knowledge surrounding an accurate identification about whether there are other possible species present in the country. Therefore, for a more reliable and consistent identification of *Verticillium* isolates infecting vegetables in Brazil, it was amplified and sequenced three gene regions: the internal transcribed spacer (ITS), glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH), and actin gene (ACT) to infer a certain identification and possible relationships between those isolates.

Exist two important taxonomic controversies involving *V. dahliae*. The first was the taxonomic debate about the distinctive features separating *V. dahliae* and *V. albo-atrum* (Karapapa et al. 1997; Steventon et al. 2002; Inderbitzin et al. 2011b; Yu et al. 2016). By the mid-1970s, it was generally accepted that microsclerotial strains as two distinct species and subsequently was corroborated by phylogenetic studies clearly identified *V. albo-atrum* and *V. dahliae* as distinct taxa (Pegg and Brady 2002; Fradin and Thomma 2006; Klosterman et al. 2009). The second controversy concerned the recognition of *V. longisporum* as a separate species instead of variety of *V. dahliae* (Inderbitzin and Subbarao 2014). Most *Verticillium* isolates used in this study had been diagnosed carefully by morphological examination, including resting structure morphology, and pathogenicity (Reis and Boiteux 2006a; 2006b).

According to these examinations and microscopical observations carried out in this study, descriptions correspond to *V. dahliae.* Besides, we compared the phenotype of the colonies based on the microsclerotia formation (MS) against the absence of them in PDA culture (NoMS). Based on this characteristic, we observed two groups, the first was able to produced spherical dark microsclerotia (68.5%), which correspond to the majority of our isolates, and the second group did not form microsclerotia (NoMS) in PDA (31.5%). These results may indicate the microsclerotia viability that constitute the infective and spreadings structures of the pathogen on MS group. It is not completely clear why the capacity to produce microesclerotia in the second group was absent. Even though for many years it was thought to *V. dahliae* was considered as a microesclerotia-producing subgroup within *V. albo-atrum* (Pegg and Brady 2002; Fradin and Thomma 2006; Goud and Termorshuizen 2003), today is widely known that morphological characteristics when used for *Verticillium* species identification are unstable (Karapapa et al. 1997; Inderbitzin and Subbarao 2014). For this reason, we considerate that morphological characteristics, including resting structures can be affected by many factors under laboratory conditions, such as growth media types, low temperatures, humidity, and storage time, making them not suitable for *V. albo-atrum* and *V. dahliae* species separation.

Due to the identification of *Verticillium* based only on their morphology cannot be effective, a first alternative for phylogenetic comparisons and identification was the sequencing of the nuclear ribosomal ITS region (Pramateftaki et al. 2000; Otero et al. 2004; Qin et al. 2006; Raja et al. 2017). ITS region was chosen as the default identification tool for fungal barcodes by a consortium of mycologists (Schoch et al., 2012; Bold Systems, 2021). According to Inderbitzin et al. (2011a, 2011b) the ITS region were apparently enough to accommodate the diversity of the ten *Verticillium* species so far known. However, they also demonstrated that *V. longisporum* is an exception, because the ITS region alone cannot retrace the evolution of this species and separate it from *V. dahliae*. In figure 3, analysis of single locus ITS provided low resolution to differentiate relationships between PD348 and PD687 (*V. longisporum* isolates), which initially resulted in an unresolved consensus tree respect to *V. dahliae* group. Thus, DNA sequences comparisons based on the ITS region alone would falsely identify the *V. longisporum* lineages as *V. dahliae,* as Inderbitzin et al. (2013) have already reported. This is because ITS as a species marker is similar for some

groups of *Verticillium* species, such as *V. longisporum* lineage D2 and D3 respecto to *V. dahliae* (Inderbitzin et al. 2013). Additionally, we development haplotype network analysis on ITS representative sequences from our fungal collection, which were labeled as *V. dahliae*. Sequencing of ITS-rDNA fragment revealed the presence of three number haplotypes (H) in the overall data set with haplotype diversity value (HD=0.289) and nucleotide diversity value per site (π= 0.00176), which means low genetic variation between populations closely related of the highly conserved ITS sequences belonged to *V. dahliae* Brazilian isolates (H1, H2), and isolates used as reference of *V. dahliae* (H1, H2), and *V. longisporum* (H3).

Even though the nuclear ITS region has been utility for widely used for DNA-based identification in fungi (Schoch et al. 2012), including *Verticillium* species, except for *V. longisporum*, other variable regions have provided more information for reconstruction of phylogenetic analysis in *Verticillium* spp. The *elongation factor 1-alpha* (EF-1α), the *glyceraldehyde-3-phosphate dehydrogenase* (GADPH), the protein coding genes *actin* (ACT), and *tryptophan synthase* (TS) are among the loci highly variable and more parsimonious informative characters proposed by Inderbitzin et al. 2011a, 2011b 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 between *Verticillium* species, due to provides a higher overall support than of the single-locus phylogenies, especially when the morphological differences are minimal (Xu et al. 2019). Consequently, we sequencing GADPH and ACT locus from *V. dahliae* isolates for a more accurate identification used in this study, confirming the presence of solely one species. We did not found any conflict in single analysis between *V. dahliae* group or either *V. longisporum* lineages using these loci (Fig. 4; Fig. 5).

Our combined analyses with ITS region, GAPDH, and Actin gene resulted in the Bayesian consensus tree (see Fig. 2) were congruent with the two major clades obtained by Inderbitzin et al. (2011a). The phylogeny of our DNA sequences were very similar to the major clade named Flavnonexudans. The species that Inderbitzin et al. (2011a) analyzed were divided into: Clade Flavexudans which contained *V. albo-atrum*, *V. isaacii*, *V. klebahnii*, *V. tricorpus*, and *V. zaregamsianum*, and Clade Flavnonexudans including *V. alfalfae*, *V. dahliae*, *V. nonalfalfae*, *V. longisporum*, and the exception of *V. nubilum*, only supported by

parsimony analysis. We inferred that in the combined analysis and all single-locus datasets, the topology obtained for DNA sequences from *Verticillium* isolates collected in Brazil generate a well-supported monophyletic clade with strong support bootstrap (100%). These results were similar to the studies development by the new taxonomy system and classification of *Verticillium* proposed by Inderbitzin et al. 2011a. Therefore, we are confident that there is only a single species, *V. dahliae*, causing vascular wilt in different vegetable hosts collected in different regions of Brazil. We observed a second clade with representative isolates formed by *V. isaacii, V. Klebahnii, V. tricorpus, V. zaregamsianum*, *V. albo-atrum* e *V. nubilum* clearly distinguished from other *Verticillium* species in this study.

Verticillium dahliae is a pathogen known for lacking host specificity (Johansson et al. 2003; Gibriel et al. 2019), therefore it is considered host-adapted rather than host-specific (Douhan and Johnson 2001). As before, studies develop by Reis et al. (2006a) testing the wide circle of host plants in some isolates, also used in this study, reinforcing the knowledge about the polyphagous nature of *V. dahliae*. Our pathogenicity bioassays for a total of 89 isolates demonstrated also that the fungus is associated with symptoms of chloroses, wilting, and necrosis, until senescence of their original hosts and eggplants artificially inoculated. The fungus *V. dahliae* was consistently recovered and re-isolated from infected plant tissue, but not from the negative control plants, thus confirming Koch's postulates (Fig. 1). Many lands suitable for planting are being limited because were previously cultivated with hosts of the soil-borne fungal pathogen, which has a wide distribution thoughout the country.

Even though in Brazil there was some confusion about the causal agent of Verticillium wilt in vegetables, we corroborated by morphological characteristic and molecular markers the identity of *V. dahliae* as the only pathogen causing Verticillium wilt in those horticultural crops. Furthermore, this work confirms that the use of only morphological characteristics is not enough to certainly identify *Verticillium* species. Another important reason to avoid misidentifications using only morphological features is due to recent events of hybridization of *V. longisporum* which easily could be confused with *V. dahliae.* Besides, we believe that is necessary the use of at least two molecular markers, in addition to ITS, for the purpose of distinguish and separate *Verticillium* species.

In our research, we were able to identify by multi-locus sequencing all *Verticillium* isolates collected from vegetables in Brazil as *V. dahliae*. A more extensive research is necessary to seek the possible existence of other *Verticillium* species infecting other type of hosts in Brazil. 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 in Brazil.

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Conflicts of interest

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

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539 **Tables & figures**

540 Table 1 Fungal reference isolates and DNA sequences data from Inderbitzin et al. (2011a) that were retrieve from GenBank and used in
541 this study for phylogenetic analysis.

Species	Strain identifier	Host	Location	GenBank accessions		
				ITS	GAPDH	ACT
*Gibellulopsis nigrescens**	**PD596**	Eggplant	Japan	JN187977	JN188167	JN188103
V. albo-atrum	PD693	Irish potato	UK	JN187994	JN188186	JN188122
	PD747	Soil potato	Canada	JN188016	JN188208	JN188144
V. alfalfae	**PD489**	Alfalfa	USA	MW550073	JN188161	JN188097
	PD620	Alfalfa	Canada	HQ206851	HQ414763	HQ206965
V. dahliae	**PD322**	Lettuce	USA	HQ206718	HQ414719	HQ206921
	PD323	Hybrid strawberry	USA	HQ206719	HQ414720	HQ206922
	PD327	Bell pepper	USA	HQ206723	HQ414723	HQ206925
	PD337	Upland cotton	USA	HQ206732	HQ414727	HQ206929
	PD404	Bell pepper	USA	HQ206757	HQ414738	HQ206940
	PD502	Maple	USA	HQ206813	HQ414740	HQ206942
	PD617	Garden tomato	Brazil	HQ206850	HQ414762	HQ206964
	PD656	Sunflower	Canada	HQ206872	HQ414782	HQ206984
	PD718	Oilseed rape	France	HQ206908	HQ414803	HQ207005
	PD729	Horseradish	USA	HQ206919	HQ414811	HQ207013
	Vert04	**Tomato**	**SP, Brazil**	OK398231	-	-
	Vert14	**Eggplant**	**MG, Brazil**	OK398232	-	-
	Vert17	**Eggplant**	**SP, Brazil**	OK398233	-	-

		Vert22	**Cacao**	**BA, Brazil**	OK398234	-	-
		Vert26	**Tomato**	**Unknown**	OK398239	-	-
		Vert34	**Tomato**	**ES, Brazil**	OK398235	-	-
		Vert54	**Tomato**	**ES, Brazil**	OK398238	-	-
		Vert150	**Strawberry**	**ES, Brazil**	OK398236	-	-
		Vert151	**Tomato**	**SP, Brazil**	OK398237	-	-
	V. isaacii	PD341	Lettuce	USA	JN187963	JN188153	JN188089
		PD660	Lettuce	USA	HQ206873	HQ414783	HQ206985
	V. klebahnii	PD347	Globe artichoke	USA	JN187965	JN188155	JN188091
		PD401	Lettuce	USA	JN187967	JN188157	JN188093
	V. longisporum allele A1	**PD348**	Cauliflower	USA	HQ206738	HQ414728	HQ206930
	(species A1)	**PD687**	Horseradish	Germany	HQ206893	HQ414791	HQ206993
	V. nonalfalfae	**PD592**	Irish potato	Japan	JN187973	JN188163	JN188099
		PD808	Common hop	Slovenia	JN188020	JN188212	JN188148
	V. nubilum	PD702	Irish potato	UK	JN187995	JN188187	JN188123
		PD742	Soil	UK	JN188011	JN188203	JN188139
	V. tricorpus	PD594	Garden tomato	Japan	JN187975	JN188165	JN188101
		PD690	Garden tomato	UK	JN187993	JN188185	JN188121
	V. zaregamsianum	**PD736**	Lettuce	Japan	JN188005	JN188197	JN188133
		PD740	Tenweeks stock	Japan	JN188009	JN188201	JN188137

PD identifiers in bold represent ex-type strains.

563 *_Gibellulopsis nigrescens_* represents ex-type strain (Outgroup sequence).

564 BA: Bahia; Es: Espírito Santo; MG: Minas Gerais; SP: São Paulo; Unknown: isolates from unknown location.

565 ITS: internal transcribed spacer;

566 GADPH: glyceraldehyde-3-phosphate dehydrogenase gene;

567 ACT: Actin gene.

568 Table 2 Primer sequence and amplicon size of each gene evaluated in this study.

Locus	Primer name	Amplicon length (pb)	Sequence5'->3'	Reference
ITS rDNA	Df	490	CCGGTCCATCAGTCTCTCTG	Inderbitzin
	Dr		CTGTTGCCGCTTCACTCG	et al. 2013
GAPDH	VGPDf2	727	GGCATCAACGGTTTCGGCC	Inderbitzin
	VGPDr		GTAGGAGTGGACGGTGGTCAT GAG	et al. 2011b
ACT	VActF	588	TAATTCACAATGGAGGGTAGG	Inderbitzin
	VActR		GTAAGGATACCACGCTTGG	et al. 2011b

569

570

571 Table 3 Number of *Verticillium* isolates that produced microesclerotia (MS) or did not
572 produce microsclerotia (NoMS) associated with Verticillium wilt on different hosts in Brazil.

Host	**MS**	**NoMS**	**Total isolates**
Tomato	23	17	40
Eggplant	10	6	16
Potato	15	0	15
Scarlet eggplant	2	1	3
Okra	1	0	1
Strawberry	9	3	12
Cacao	1	1	2
Total	61	28	89

573

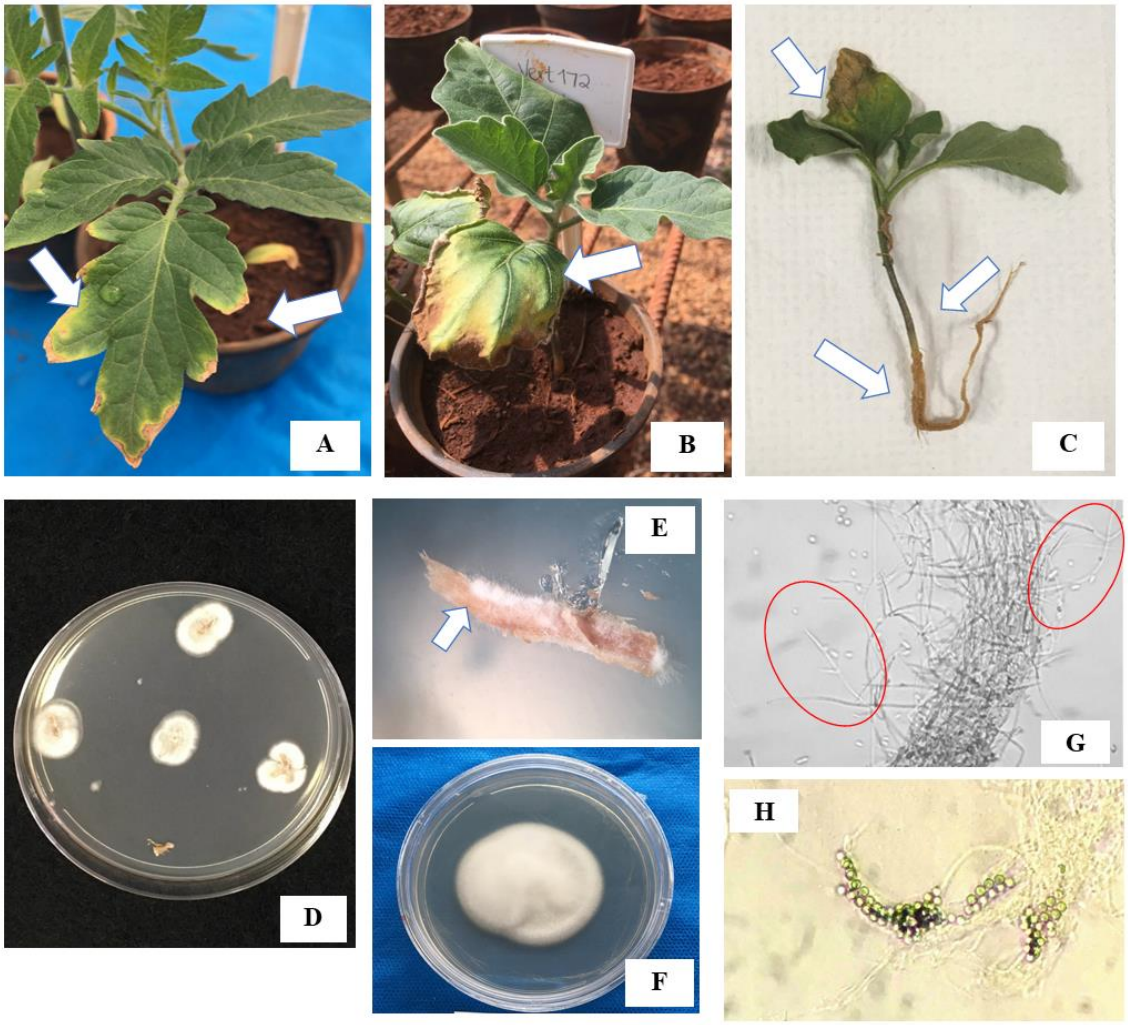
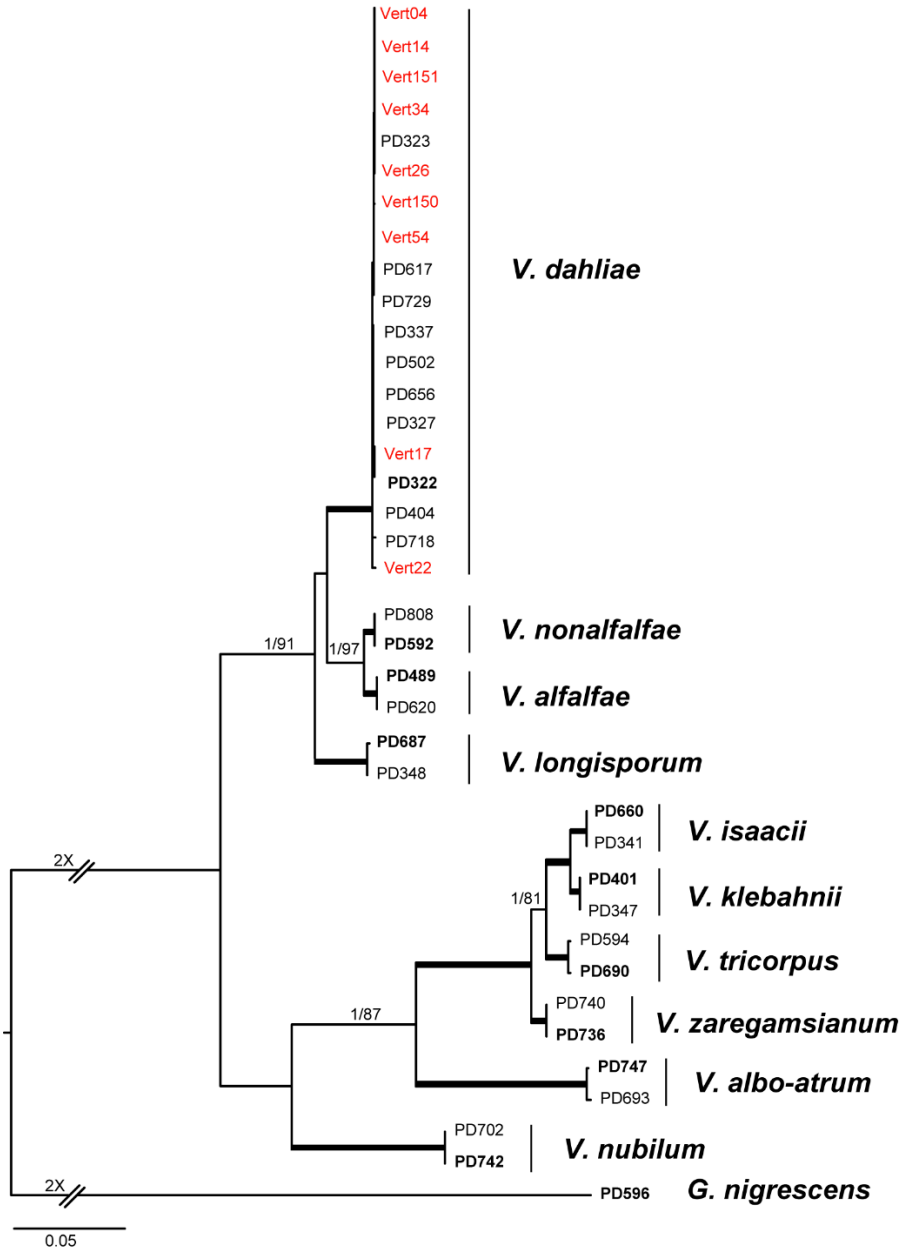
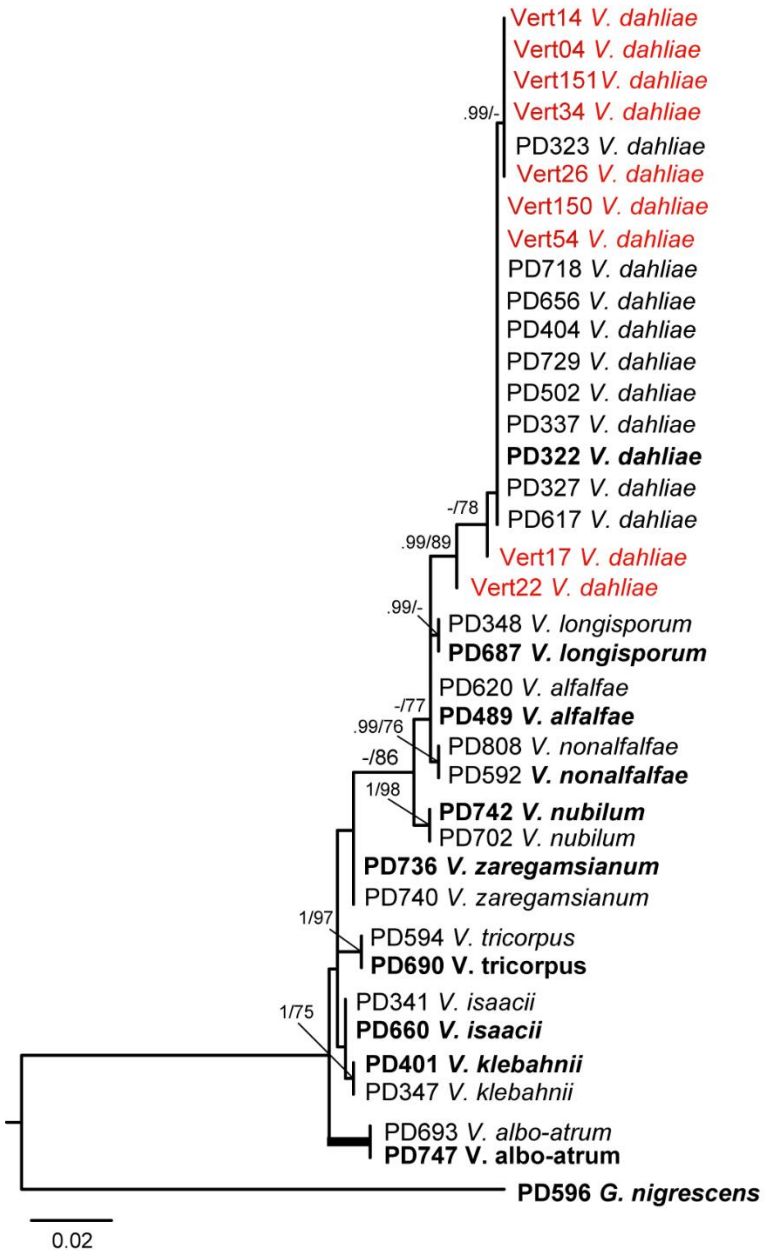


Fig. 1. Characteristics of plant pathogenic *Verticillium dahliae*. **A.** Tomato plant infected by *V. dahliae* showing initial chlorosis and necrosis. **B.** Eggplant seedling pos-inoculation with *V. dahliae* showing typical V- shaped necrosis on the leaf. **C.** Eggplant stem base with brown discoloration. **D.** *In vitro* plating of the stem of an *V. dahliae*-infected eggplant showing fungal growth in four points. **E.** Mycelial growth of *V. dahliae* on eggplant stem under optical microscope. **F.** *Verticillium* isolate re-isolation of *V. dahliae* from the stem of eggplant. **G.** *V. dahliae* conidiophores in verticilliate disposition with release of conidia. **H.** Microscopical observation of microesclerotia produced by *V. dahliae* isolates in PDA.



586

587 Fig. 2. Maximum likelihood tree of the *Verticillium* species inferred from a concatenated
588 alignment of ITS, GAPDH and ACT. Bootstrap support values (ML ≥ 70) and Bayesian
589 posterior probability values (PP ≥ 0.95) are shown at the nodes. Full supported branches
590 (ML-BI = 100 / BI-PP = 1) are indicated in bold. "-" indicates no-significant support or
591 absence of the node. "PD" identifiers in bold represent ex-type isolates of *Verticillium* species
592 obtained from the studies by Inderbitzin et al. (2011a, 2011b). "Vert" identifier is highlighted
593 in red. *Gibellulopsis nigrescens* was used as the outgroup. The scale bar indicates the
594 estimated number of substitutions per site.



595

596 Fig. 3. Maximum likelihood tree of the *Verticillium* species inferred from a single alignment
597 of ITS region. Bootstrap support values (ML ≥ 70) and Bayesian posterior probability values
598 (PP ≥ 0.95) are shown at the nodes. Full supported branches (ML-BI = 100 / BI-PP = 1) are
599 indicated in bold. "-" indicates no-significant support or absence of the node. "PD" identifiers
600 in bold represent ex-type isolates of *Verticillium* species obtained from the studies by
601 Inderbitzin et al. (2011a, 2011b). "Vert" identifier is highlighted in red. *Gibellulopsis*
602 *nigrescens* was used as the outgroup. The scale bar indicates the estimated number of
603 substitutions per site.

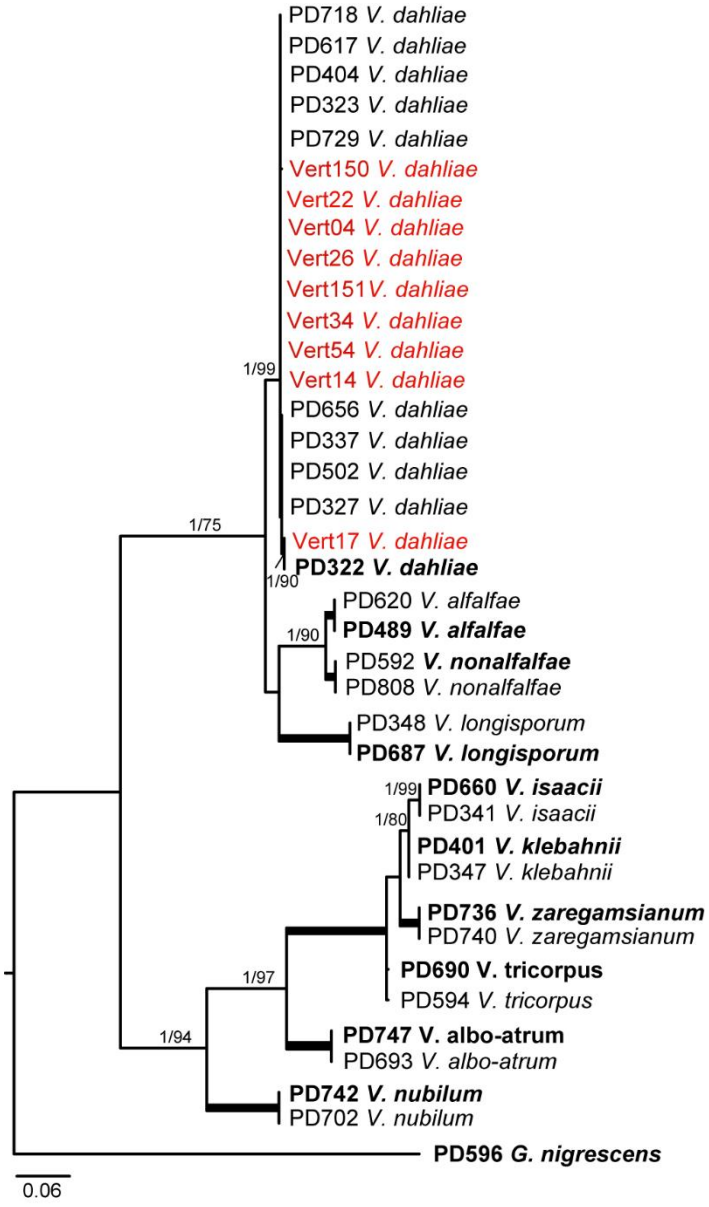
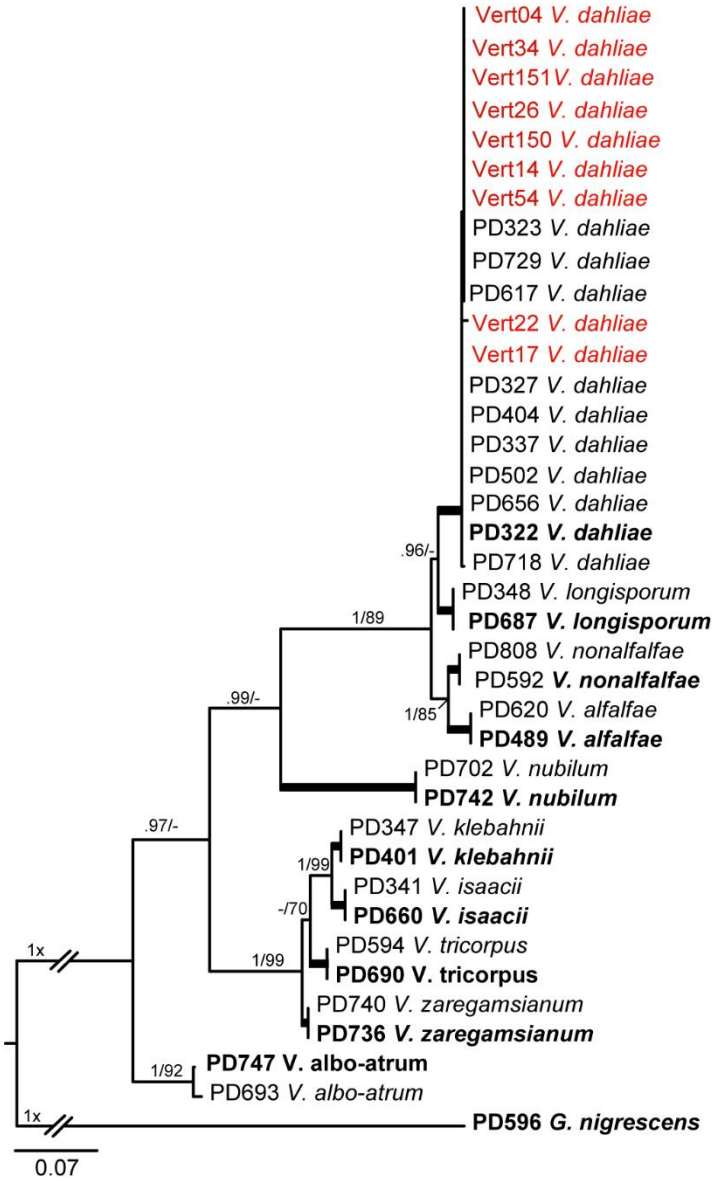


Fig. 4. Maximum likelihood tree of the *Verticillium* species inferred from a single alignment of *GAPDH* gene. Bootstrap support values (ML ≥ 70) and Bayesian posterior probability values (PP ≥ 0.95) are shown at the nodes. Full supported branches (ML-BI = 100 / BI-PP = 1) are indicated in bold. "-" indicates no-significant support or absence of the node. "PD" identifiers in bold represent ex-type isolates of *Verticillium* species obtained from the studies by Inderbitzin et al. (2011a, 2011b). "Vert" identifier is highlighted in red. *Gibellulopsis nigrescens* was used as the outgroup. The scale bar indicates the estimated number of substitutions per site.



614

615 Fig. 5. Maximum likelihood tree of the *Verticillium* species inferred from a single alignment
616 of *Actin* gene. Bootstrap support values (ML ≥ 70) and Bayesian posterior probability values
617 (PP ≥ 0.95) are shown at the nodes. Full supported branches (ML-BI = 100 / BI-PP = 1) are
618 indicated in bold. "-" indicates no-significant support or absence of the node. "PD" identifiers
619 in bold represent ex-type isolates of *Verticillium* species obtained from the studies by
620 Inderbitzin et al. (2011a, 2011b). "Vert" identifier is highlighted in red. *Gibellulopsis*
621 *nigrescens* was used as the outgroup. The scale bar indicates the estimated number of
622 substitutions per site.

623

Supplementary material

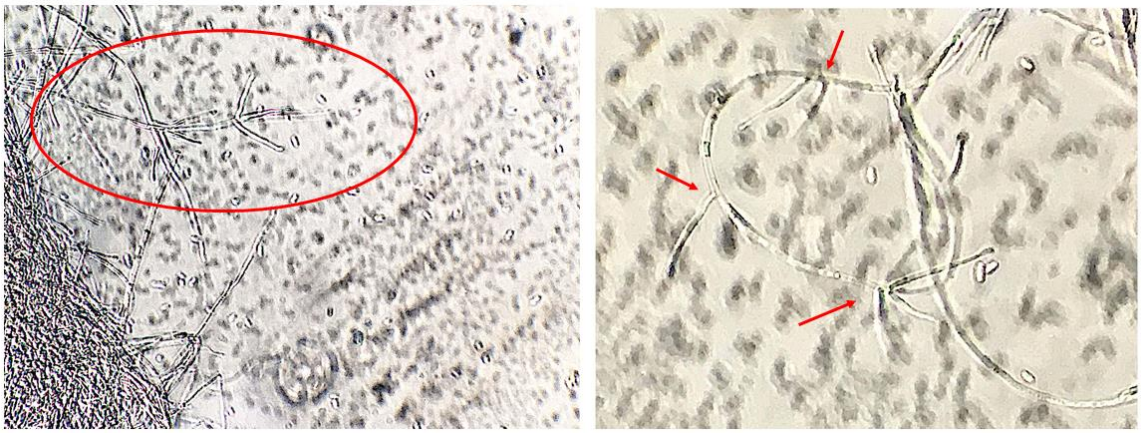
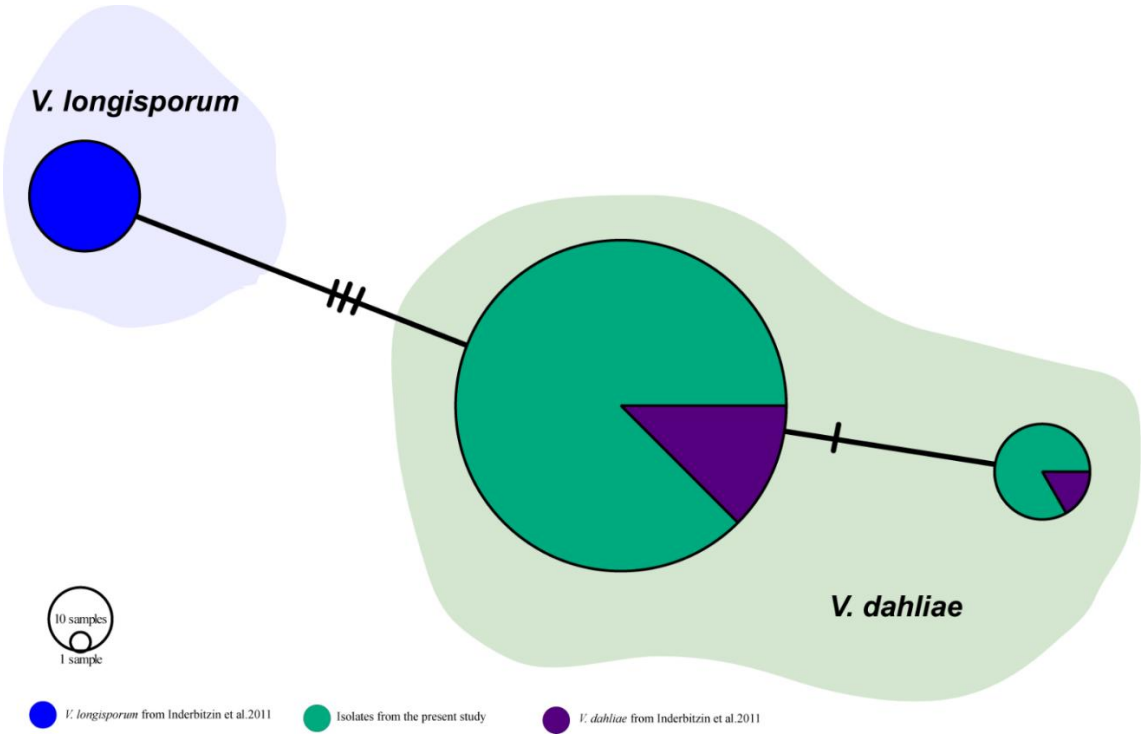


Fig. S1. Microscopical observation of *Verticillium dahliae* mycelium showing conidiophores in “verticillate” disposition and release of some conidia.



633

634 Fig. S2. Median-Joining haplotype network generated for ITS sequences alignments
635 representing *V. longisporum* and *V. dahliae* isolates obtained from Inderbitzin et al. (2011a)
636 and in this study using PopArt. Size of the circles are proportional to the number of isolates
637 with a specific haplotype and connecting lines represent the number of mutations between
638 haplotypes.

Capítulo III

Relative incidence and geographical distribution of physiological races and mating type determination of *Verticillium dahliae* isolates from Brazil

Relative incidence and geographical distribution of physiological races and mating type determination of *Verticillium dahliae* isolates from Brazil

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Abstract

Verticillium dahliae is an asexual soil-borne, xylem-invading, plant pathogen that is responsible for vascular wilt diseases in more than 300 dicotyledonous species, including vegetable crops such as tomato, potato, eggplant, strawberry, and scarlet eggplant. Here, 89 *V. dahliae* isolates from Brazil were characterized for their virulence on tomato and eggplant cultivars as well as for mating type and physiological race identification via molecular tools. In the virulence/race determination bioassays, the isolates were inoculated on the tomato cultivars 'Ponderosa' (susceptible to races 1 and 2) and 'Floradade' (resistant to race 1), as well as eggplant cultivar 'Ciça' (highly susceptible to both races). For molecular race determination, three specific primer pairs were used. In the mating type determination two specific primer pairs were used. In the virulence assay only three isolates were classified as race 1, whereas 76 isolates were classified as race 2. Ten isolates were avirulent in all evaluated cultivars. In the molecular race determination six isolates were identified as race 1, 70 as race 2, and 13 isolates displayed no amplicon with any primer set. The predominance of race 2 isolates can be explained by the large-scale employment of tomato cultivars/hybrids carrying the race 1-specific *Ve*-1 resistance gene in Brazil. Most Brazilian *V. dahliae* isolates reacted as *MAT1-1* (82%). However, *MAT1-2* isolates were also detected (13.5%). *Verticillium dahliae* race 2 isolates were prevalent across major vegetable crop hosts in

Brazil. This information is very important for the breeding programs aiming at the incorporation of disease resistance in tomato cultivars and in other vegetables. Another important information is the presence of both *MAT* idiomorphs of *V. dahliae* in Brazil. This pathogen variability allows, even remotely, sexual reproduction among *V. dahliae* populations, enabling the potential emergence of new pathogen races. Our results clearly indicate 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, Vegetables, Verticillium wilt.

Introduction

Verticillium dahliae Kleb., is an asexual soil-borne, xylem-invading, plant pathogen that can induce vascular wilt diseases in more than 300 dicotyledonous plant species (de Jonge et al. 2012; Klosterman et al. 2009; Farr and Rossman, 2021). The host range of this pathogen includes trees, herbaceous ornamentals, and economically important vegetable and field crops (Pegg and Brady et al. 2002, Farr and Rossman, 2021). Among the most important vegetables affected by *Verticillium*-induced diseases are tomato, lettuce, eggplant, strawberries, and potato (Domsch et al. 1980; Fradin and Thomma 2006; Reis and Boiteux 2006b).

The fungus survives 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 species (Carroll et al. 2018; Pegg and Brady 2002; Vallad et al. 2008; Wilhelm 1955). 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 is also ineffective. Different management strategies to reduce the primary inoculum in the soil include crop rotation, green manures, soil solarization, cover crops, organic amendments, and other practices (Deketelaere et al. 2017; Johnson and Dung 2010). To date, the use of resistant cultivars is one of the few viable alternatives for controlling Verticillium wilts. However, somewhat surprisingly, host resistance to Verticillium wilt has been actively

identified in a limited number of crops, such as tomato, lettuce, potato, and cotton (Diwan et al. 1999; Hayes et al. 2007; Mohan et al. 1990; Schaible et al. 1951).

The first source of genetic resistance to *V. dahliae* race 1 isolates in tomato was identified in 1925 from the wild species (*Solanum pimpinellifolium* L.). This trait was subsequently incorporated into commercial tomato varieties (Bryan 1925; Pegg and Brady 2002; Schaible et al. 1951). Hitherto, *V. dahliae* exists as two pathogenic races (named as race 1, and race 2) in tomato (Alexander 1962). A single dominant *Ve*-1 locus in the chromosome 9 controls race 1-specific resistance (Diwan et al. 1999), encoding a cell surface receptor protein (Kawchuk et al. 2001). Also, 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; Fradin and Thomma 2006). Over time, race 1 resistance-breaking strains, named as race 2, have become increasingly problematic in tomato crops, since the first report by Alexander in 1962 (Acharya et al. 2020; Chavarro-Carrero et al. 2020; Maruthachalam et al. 2010).

Even though *V. dahliae* race 2 isolates have been reported in tomatoes under Brazilian conditions since the early 1980s (Laterrot et al. 1983), race 1 isolates apparently predominated in this crop until the 1990s (Reis et al. 2007). With the prevalence of *V. dahliae* race 1 isolates, this disease was considered as being of secondary importance in tomatoes due to widespread employment of cultivars and hybrids carrying the race 1-specific *Ve*-1 resistant gene (Miranda et al. 2010). However, from 1900s to the last decade, a noteworthy emergence of novel reports of *Verticillium* wilt outbreaks with significant yield losses was observed across major tomato-growing areas in São Paulo (Cerezine et al. 1992), Distrito Federal (Santos and Lopes 1995), and the South and South-East regions of Brazil strongly indicated the chance is the virulence profile of the *V. dahliae* isolates (Reis and Boiteux 2006a; Reis et al. 2007).

The sexual stage of *V. dahliae* was not yet reported (Klosterman et al. 2009). However, genetic recombination between strains has been already reported (Usami et al. 2009b; O'Garro 1992). Recently, *V. dahliae* has been characterized as a heterothallic fungus with two MAT idiomorphs, which suggested that a putative sexual life cycle of the pathogen might exist under natural conditions (Erincik 2020; Milgroom et al. 2014; Usami et al.

2009b). Therefore, a heterothallic individual generally must encounter an individual of the opposite mating type to sexually reproduce (Baroudy et al. 2019). In Ascomycete fungi, both mating-type idiomorphs are required for successful mating (Milgroom et al. 2014). 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).

In the present study, we determinate the virulence profile of *V. dahliae* isolates in bioassays with differential eggplant and tomato cultivars and the relative incidence of physiological races by PCR in a large collection of isolates. Additionally, we investigated the diversity of MAT idiomorphs aiming to expand the knowledge about geographical distribution and frequency of distinct *V. dahliae* mating types in Brazil.

Material and methods

***Verticillium dahliae* isolates** – In this work 89 isolates of *V. dahliae*, obtained mainly from vegetables crops in the main producing regions of Brazil were used (**Table 1)**. All isolates were previously inoculated and re-isolated from their original hosts, fulfilling the Kochs's postulates.

Virulence bioassays: Race differential cultivars and plant growth conditions – In tomato the gene *Ve*-1 controls race 1-specific resistance (Kawchuk et al. 2001). Therefore, tomato cultivars used as race differentials were 'Ponderosa' (susceptible to races 1 and 2), and 'Floradade' (resistant to race 1 due to presence of *Ve*-1 gene) (Reis et al. 2007). Additionally, an eggplant (*Solanum melongena* L.) hybrid 'Ciça' was used as hypersensitive indicator for *Verticillium* wilt symptoms, due to its highly 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.

Virulence bioassays: *Verticillium dahliae* inoculum, pathogen inoculation and experimental design – For producing pathogen inoculum (conidia) all isolates of *V. dahliae* were deposited 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 Erlenmeyer with 100 mL of potato-dextrose broth (BD) for ten

days at 24°C±2°C under low agitation (99 rpm) with orbital shaker (TE-420 Tecnal). Subsequently, the spore suspension was filtered with double gauze and the inoculum concentration was estimated with the aid of a Neubauer chamber and then adjusted to 2 x 10^6 conidia/mL. Seedlings were inoculated when reached two pair of true leaves stage 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 excess of substrate. The roots of 21-day-old seedlings were injured at the apical zone and inoculated by immersion into 50 ml of spore suspension for 3 minutes. After that, the seedlings were transplant to plastic pots (7.8 cm x 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. Bioassays were conducted in a greenhouse (temperature 25C ±4°C and relative humidity of 70-80%) with a randomized block design and conducted with three experimental units per differential cultivar inoculated (three pots with two plants each). Assessment of symptoms was carried out 30 days after inoculation (DAI). The fungi were reisolated from some symptomatic tomatoes and eggplants. This assay was carried out twice.

Specific detection of pathogenic races by PCR assays – 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 et al. 2014a). PCR conditions to determinate pathogenic races were following as described by Short et al. 2014a with some modifications in this study listed in **Table 2**. All DNA used to specific detection of pathogenic races were testing twice to corroborated the results.

Mating type determination by PCR assay – To determinate the frequency of two idiomorphs of the MAT locus on isolates of *V. dahliae*, two set of primers were used in this study: VdMAT1-1a (5'-GTCCCTGGAGGTAGGGAGTG-3') /VdMAT1-1b (5'-TGCTTCCTCCGTCAAGACGC-3') (Usami et al. 2009a), and VdMAT1-2a (5'-CGACCGCTACTATATTGGCCC-3') /VdMAT1-2b (5'-CTGCGACAGCAGATTCTGG GTTGCAAAGGC -3') (Usami et al. 2009b). Multiplex PCR amplifications were performed in a volume of 25 µL as described by Usami et al. 2009a. Also, simplex PCRs conditions

were standardized in a T100 PCR thermal cycler (BioRad®) with an initial denaturation 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 the PCR reactions were repeated twice.

Results

Verticillium isolates comprise a fungal collection maintained at Embrapa Vegetables since 1992. These isolates were obtained from different host plants, such as tomato, eggplant, potato, okra, strawberry, scarlet eggplant, and cacao, being collected in different regions of Brazil. Previous molecular studies (**see chapter 2**) confirmed *V. dahliae* as the only species associated with vegetable crops in Brazil.

Virulence assays – Results of inoculation assays using 89 isolates of *V. dahliae* on two tomato cultivars, and one eggplant hybrid are presented in **Table 3.** The phenotypic reaction of tomato cultivars appeared at 20-23 days after inoculation. The first symptoms appeared a little earlier in eggplants (18-21 DAI) than in tomato seedlings. In our assays, typical *Verticillum* wilt-associated symptoms were observed, such as yellowing on the lower leaves, some V-shaped areas between the leaf margins progressing until turns brown, and eventually collapse. A red-to-brown discoloration inside the vascular tissue from affected plants was observed with a longitudinal cut at the basal portion of the stems (**Fig. 1**). Seventy-six out of 89 isolates induced severe wilt symptoms in both tomato cultivars and on 'Ciça' eggplant, indicating that those isolates belonged to race 2 of *V. dahliae*. Only, three isolates were characterized as race 1, based on their response on tomato and eggplant differentials. Surprisingly, ten isolates were non-pathogenic on all differentials. As expected, mock-inoculated control plants exhibited no disease symptoms.

Race-specific detection via PCR assays – In the PCR assays, a total of 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 primer pair Tr1/Tr2 were used to confirm the race

1 on 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 case of VdR2F/VdR2R PCR assay, seventy *V. dahliae* isolates (70/89) belonging to race 2 that produced a DNA amplicon of 250 bp were identified. Samples that previously had been positives to race 2 were negative in the PCR for race 1 (**Fig. 4**). Subsequently, thirteen isolates were negative in both reactions either for race 1 or race 2-specific PCR. A strong concordance was observed between the pathogenicity/virulence assays and the results of the race by PCR (**Table 3**).

Mating type determination by PCR assay – It was observed an overabundance of *MAT-1-1* idiomorph amplified a 400 bp fragment in 73 isolates tested (82%). Only 12 isolates (13.5%) amplified amplicons of 600 bp in size, and thus were designed as *MAT-1-2* mating type (**Table 4**). 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. The PCR reactions were performed twice and were corroborated negative and positive samples, either for *MAT-1-1* or *MAT-1-2* results.

Discussion

All 89 isolates were previously identified as *V. dahliae sensu stricto* according to the system of classification establish by Inderbitzin et al. (2011). *Verticillium* wilt has been reported in a wide range of hosts in Brazil (Reis et al. 2007; Reis and Boiteux 2006a; Reis and Boiteux 2006b; Mendes et al. 2019). However, in the present assay, most of the isolates analyzed, were sampled from Solanaceae hosts (84.3%), and some other crops such as strawberry, okra, and cacao (15.7%). Conventionally, pathogenicity assays in *V. dahliae* for the classification of the physiological races are carried out on a set of differential cultivars, which is a time-consuming and cumbersome procedure (Ligoxigakis and Yakalounakis 1994; Papaioannou et al. 2013; Reis et al. 2007; Usami et al. 2017). In addition, these tests should be conducted under strictly controlled environmental conditions suitable for both fungus and host plant in order to avoid misleading results. However, due to some observed discrepancies, the combination of both virulence assays and molecular markers is considered the most robust and consistent strategy for race discrimination. In addition, 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 a set of resistant and susceptible differential plant cultivars. Firstly, we observed consistently absence of wilt symptoms in 'Floradade' (resistant to race 1) and on the other hand, aggressive wilt symptoms on 'Ponderosa' (susceptible to races 1 and 2). Plants of the eggplant hybrid 'Ciça' were used as positive indicator of symptoms due the highly susceptible reaction of this genetic material to both races (races 1 and 2). 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 the oldest isolates of our fungal collection (1992-1997). It is known that the resistance on tomato is monogenic toward race 1 strains of *Verticillium* (Fradin et al. 2009), and it was demonstrated by de Jonge et al. (2012) that *Ave1* gene contributes to fungal virulence on tomato. Therefore, low frequency of race 1 is somewhat expected due to the large-scale employment of tomato cultivars carrying the race 1-specific resistance *Ve1* gene.

The majority of isolates (85.4%) in our study were classified as race 2. Isolates of this race have been more prevalent regions of the South and South East of the country since its first report in the North-East of Brazil. In these regions, environmental conditions favor epidemic outbreaks of *Verticillium* wilt (Laterrot et al. 1983; Reis and Boiteux 2006a; Reis et al. 2007). 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 et al. 1992) and Distrito Federal (Santos and Lopes, 1995). Subsequently, in 2007 it was reported in Rio Grande do Sul, Santa Catarina, Rio de Janeiro, Espírito Santo, and Minas Gerais (Reis et al. 2007).

In the present work, we are expanding knowledge about the geographic dispersal of this race to other states such as Bahia, Ceará, Goiás, and Paraná. This relatively fast and extensive dispersion of *V. dahliae* race 2 in Brazil suggests its introduction and nationwide dissemination via contaminated propagative material (Reis and Boiteux, 2006a; Reis et al. 2007). The current predominance of race 2 in tomato producing regions of Brazil is due to

the previous extensive use of cultivars with the *Ve1* gene that have exerted a strong selection pressure to the pathogen in favor of the pathogen race 2. Isolates collected from other vegetable crops such as eggplant, scarlet eggplant, strawberry and the potato were also classified as race 2. This can be explained by the fact that most of these isolates were collected in regions where tomato and strawberry (Espírito Santo, Distrito Federal and Santa Catarina), eggplant, and scarlet eggplant (Ceará, Espírito Santo, Minas Gerais, Rio de Janeiro and São Paulo), and potato and tomato (Bahia) are cultivated in sequence.

During the course of pathogenicity assays, we observed ten isolates not able to induce symptoms in all the differential cultivars (11.2 %), thus they were considerate as non-pathogenic (AVR). These results in disagreement with molecular analyses since only four of them were negative either to race 1 or race 2, the other five isolates were positive to race 2, and one isolate was classified as race 1. Different levels of aggressiveness among of the isolates could be associated to these unexpected results.

Race identification using molecular methods is more practical than employing time-consuming inoculation tests (Usami et al. 2017). 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 3**). In our study, race 1 and race 2-specific primers were successfully validated to determinate 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 host (Fig. S1). PCR products were not obtained from 13 isolates in the single and multiplex PCR for both races and corroborated two times (**data no shown**). These race-specific primers have been used and validated with high effectivity to differentiated *V. dahliae* races occurring mainly in tomato, lettuce, cotton, and olive (Maruthachalam et al. 2010; Short et al. 2014a; Usami et al. 2007). To our knowledge, this is the first extensive study carried out for determination of pathogenic races of *V. dahliae* using a molecular approaches in Solanaceae hosts from Brazil.

The combination of both, pathogenicity tests and molecular marker assays is considered the most robust and consistent strategy for race discrimination. Analyses done exclusively with molecular markers could miss important and useful information from a tomato breeding standpoint, including the potential emergence of novel pathogen variants,

especially new races of *V. dahliae*. In this context, the present work was carried out by combining pathogenicity tests and molecular marker assays. This research 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 (**Fig. S2; Fig. S3**).

Due the recent outbreaks of *Verticillium* wilt disease in a wide range of Solanaceae species, an important question to address is about the mating types present in the country and what is their frequency among *V. dahliae* populations in Brazil. Even though, no sexual stage has been found in any *Verticillium* species (Erincik 2020; Milgroom et al. 2014; Usami et al. 2009b), the proximity of opposite mating types represents a risk of sexual recombination (Baroudy et al. 2019), besides that *V. dahliae* has a clonal population structure with little or no evidence of recombination (Milgroom et al. 2014). Our findings revealed that 82% of isolates contained the *MAT1-1* idiomorph, whereas 13.5% belongs to *MAT1-2* idiomorph from a total of 89 *V. dahliae* isolates from different host plants, mainly Solanaceous (**Table 4**). However, some isolates (4.5%) were negative to determinate any mating type gene in the PCR. These results reveal opposites *MAT*s are present in Brazilian isolates. The majority of isolates belonged to *MAT1-1* over *MAT1-2* presence in *V. dahliae*. Controversially to our results, *MAT1-2* idiomorph is the predominant mating type and it has been obtained in high frequency in different hosts in comparison to *MAT1-1*, which is reported as rare or less distributed (Milgroom et al. 2014; Short et al. 2014b; Usami et al. 2009a). Until now, *MAT1-1* had only been found at the coastal of California comprised 30% of individual carrying *MAT1-1* (Inderbitzin and Subbarao, unpublished data founded in Atallah et al. 2010), three *MAT1-1* isolates among 49 Japanese isolates (Usami et al. 2009b), and only one of five lettuce isolates in Usami et al. (2012).

A lack of robust and comprehensively information about the predominance and distribution of *V. dahliae* races in Brazil leads to bias in the interpretation of how occurred the gene flow between the two race groups, the dispersal mechanism and transmission the one to another. Since here, differently from other countries, *MAT1-1* predominates instead of *MAT1-2* (**Fig. S4**), it is important additional information to know how *MAT1-1* was introduced in Brazil or whether spread predominantly through advantageous conditions. Also, it is interestingly further research 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.

This work showed that the majority of isolates of *V. dahliae* infecting vegetables nowadays in Brazil belong to race 2. This information is very important for the breeding programs aiming at the incorporation of disease resistance in tomato cultivars and in other vegetables. The results are of great concern, since the cultivars found in the Brazilian market do not have resistance to race 2 isolates. Another concern that this work raises is the presence of the two *MAT* idiomorphs in Brazil. This fact creates the possibility, even being unlikely (Milgroom et al. 2014; Usami et al. 2009a, 2009b), of the occurrence of sexual reproduction between populations of *V. dahliae.* This could increase the genetic variability of the fungus and enabling the emergence of new races from the pathogen. These results clearly indicate that this disease is a problem that threatens tomato and other vegetables in Brazil. In this way, tomato breeding programs should devote more efforts in the search for sources of resistance to the pathogen and the incorporation of resistance factors in new tomato cultivars.

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460 **Tables**

461

462 **Table 1** Isolates of *Verticillium dahliae* used in the study

Isolate ID	Host origin	Geographical location	Year of collection
Vert02	Tomato	SP	1992
Vert03	Tomato	SP	1992
Vert04	Tomato	SP	1992
Vert05	Tomato	DF	1994
Vert06	Tomato	DF	1995
Vert07	Eggplant	DF	1997
Vert08	Eggplant	Unknown	1997
Vert09	Eggplant	Unknown	1997
Vert12	Okra	MG	1997
Vert14	Eggplant	MG	1997
Vert17	Eggplant	SP	1997
Vert21	Potato	Unknown	1997
Vert22	Cacao	BA	1997
Vert23	Cacao	Unknown	1997
Vert26	Tomato	Unknown	1997
Vert32	Tomato	ES	2004
Vert34	Tomato	ES	2004
Vert35	Tomato	ES	2004
Vert36	Tomato	RJ	2004
Vert38	Tomato	SC	2004
Vert43	Tomato	SC	2005
Vert45	Tomato	SC	2005
Vert46	Tomato	DF	2005
Vert47	Tomato	DF	2005
Vert52	Tomato	SP	2005
Vert53	Tomato	SP	2005
Vert54	Tomato	SP	2005
Vert56	Tomato	SP	2005
Vert59	Tomato	MG	2005
Vert62	Eggplant	SP	2005
Vert65	Tomato	SC	2005
Vert67	Tomato	ES	2005
Vert70	Tomato	ES	2005
Vert71	Tomato	ES	2005
Vert74	Tomato	ES	2006
Vert77	Tomato	SP	2006
Vert78	Tomato	SP	2006
Vert79	Tomato	DF	2006
Vert93	Tomato	MG	2006
Vert96	Tomato	RJ	2007
Vert103	Strawberry	DF	2007
Vert106	Tomato	MG	2007
Vert110	Scarlet eggplant	RJ	2008
Vert111	Scarlet eggplant	RJ	2008
Vert116	Tomato	MG	2008
Vert117	Scarlet eggplant	CE	2009
Vert118	Eggplant	CE	2009
Vert119	Eggplant	DF	2009
Vert120	Eggplant	ES	2010
Vert121	Tomato	SC	2010
Vert125	Tomato	PR	2010

Vert129	Eggplant	ES	2010
Vert130	Strawberry	ES	2010
Vert132	Tomato	ES	2010
Vert134	Strawberry	ES	2010
Vert137	Strawberry	ES	2010
Vert142	Strawberry	ES	2011
Vert143	Strawberry	ES	2011
Vert144	Strawberry	ES	2011
Vert145	Strawberry	ES	2011
Vert147	Strawberry	ES	2011
Vert148	Strawberry	ES	2011
Vert149	Strawberry	ES	2011
Vert150	Strawberry	ES	2011
Vert151	Tomato	SP	2011
Vert158	Eggplant	DF	2017
Vert160	Tomato	ES	2018
Vert161	Tomato	ES	2018
Vert163	Eggplant	DF	2018
Vert164	Eggplant	DF	2018
Vert166	Potato	MG	2018
Vert169	Potato	MG	2018
Vert171	Potato	MG	2018
Vert172	Potato	MG	2018
Vert173	Potato	MG	2018
Vert174	Potato	MG	2018
Vert176	Eggplant	DF	2018
Vert177	Potato	MG	2018
Vert178	Potato	MG	2018
Vert179	Potato	MG	2018
Vert180	Potato	MG	2018
Vert181	Eggplant	SP	2019
Vert182	Eggplant	SP	2019
Vert183	Tomato	GO	2019
Vert184	Tomato	MG	2019
Vert185	Tomato	MG	2019
Vert186	Potato	BA	2019
Vert187	Potato	BA	2019
Vert188	Potato	BA	2019
Vert189	Potato	BA	2019

BA: Bahia; CE: Ceará; DF: Distrito Federal; Es: Espirito Santo; MG: Minas Gerais; PR: Paraná; RJ: Rio de Janeiro; SC: Santa Catarina; SP: Saõ Paulo; Unknown: isolates from unknown location.

Table 2 Molecular details and simplex PCR conditions to determinate pathogenic races in isolates of *V. dahliae*

Target	**Primer name**	**Sequence 5'->3'**	**Annealing temperature (°C)**	**Amplicon length (bp)**
Race 1 (*Ave*1 effector)	VdAve1F	AAGGGGTCTTGCTAGGATGG	62	1000
	VdAve1R	TGAAACACTTGTCCTCTTGCT		
Race 1 (*Ave*1 effector)	Tr1	TGAAGTAGCCGATAGCTTTGTCTTGCC	64	680
	Tr2	TGTCTGGATTAATCGCCGCAATAGA		
Race 2 (exonic region of the VDAG_05863.1)	VdR2F	ACTTAACGAAAGCATGCGC	64	256
	VdR2R	CTTGACTTGCCGGCTCC		

468 **Table 3** Results of the virulence assays and the results of the race 1/race 2-specific PCR test

Cultivars				**Inoculation assays**	**Race-PCR assays**		
Isolate	**Tomato cv.**		**Eggplant**	**Race**	**VdAve1F/ VdAve1R**	**VdR2F/ VdR2R**	**Race**
	Floradade (FD)	**Ponderosa (PD)**	**Ciça (BJ)**				
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[a]**
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**
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**

[a] 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 differential.
NA: DNA from isolates that did not amplify for race 1 or race 2 with specific primers.

Table 4 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

Figures

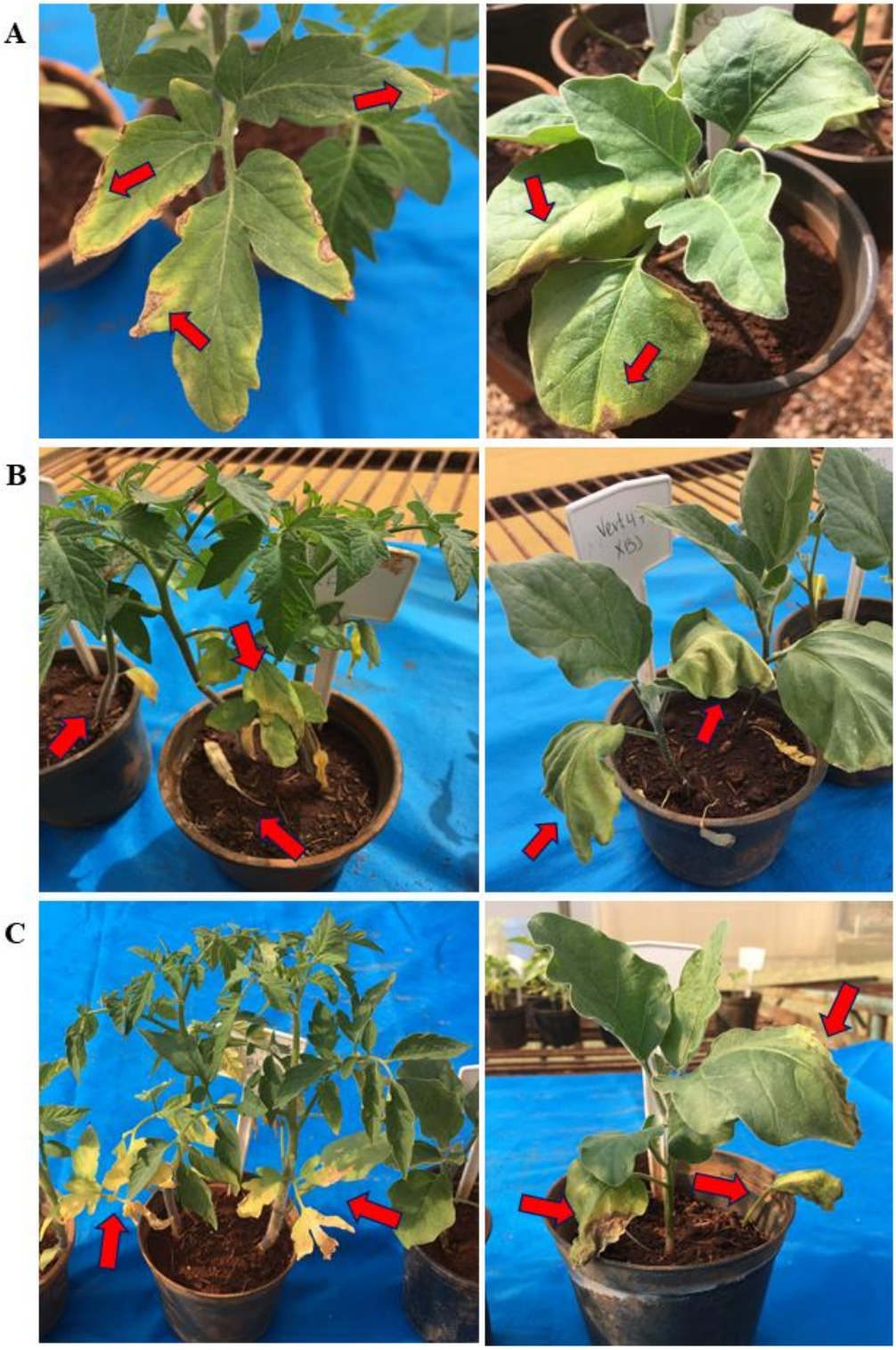


Fig. 1 *Verticillium* wilt reaction on plant differential cultivars assess with 20-23 days after inoculation. **A.** Pathogenicity phenotyping of Vert04 isolate, race 1. **B.** Pathogenicity phenotyping of Vert47, race 2. **C.** Pathogenicity phenotyping of Vert179 isolate, race 2.

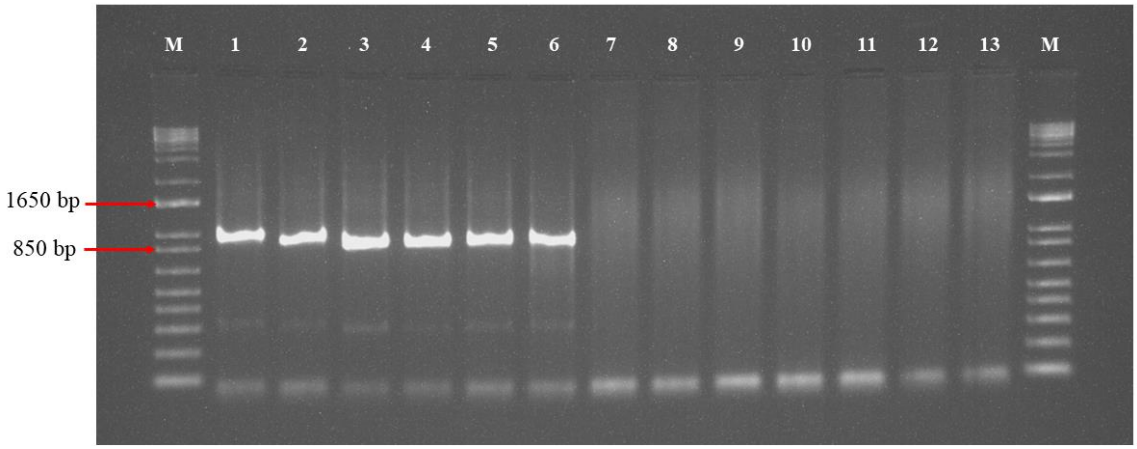


Fig. 2 Gel electrophoresis of amplicons produced by PCR assays using race 1 specific primers VdAve1F and VdAveR (1000 bp). Lanes 1-6: Vert04, Vert09, Vert14, Vert26, Vert151, Vert161 (race 1: nonpathogenic on tomato cv. Floradade, pathogenic on cv. Ponderosa, and pathogenic on eggplant cv. Ciça); Lanes 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

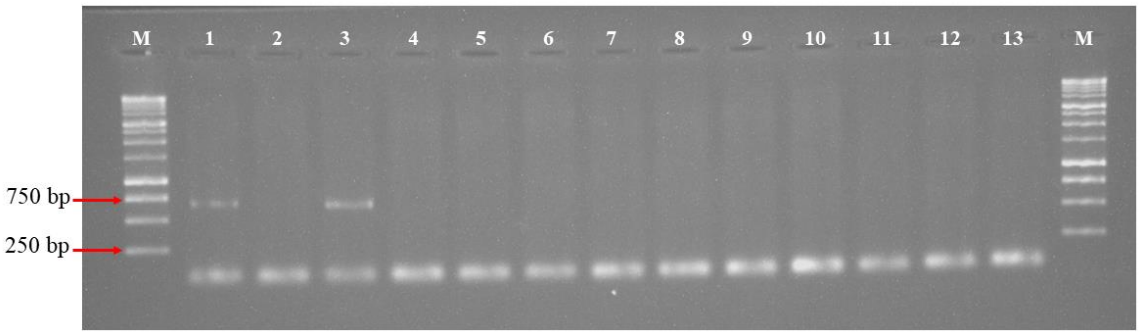


Fig. 3 Gel electrophoresis of amplicons produced by PCR assays using race 1 specific primers Tr1 and Tr2 (680 bp). Lanes **1-10:** Vert151, Vert158, Vert161, Vert163, Vert171, Vert174, Vert179, Vert180, Vert183, Vert189. Lane 11: No template control (NTC). Lane 12: Negative control (exogenous DNA from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

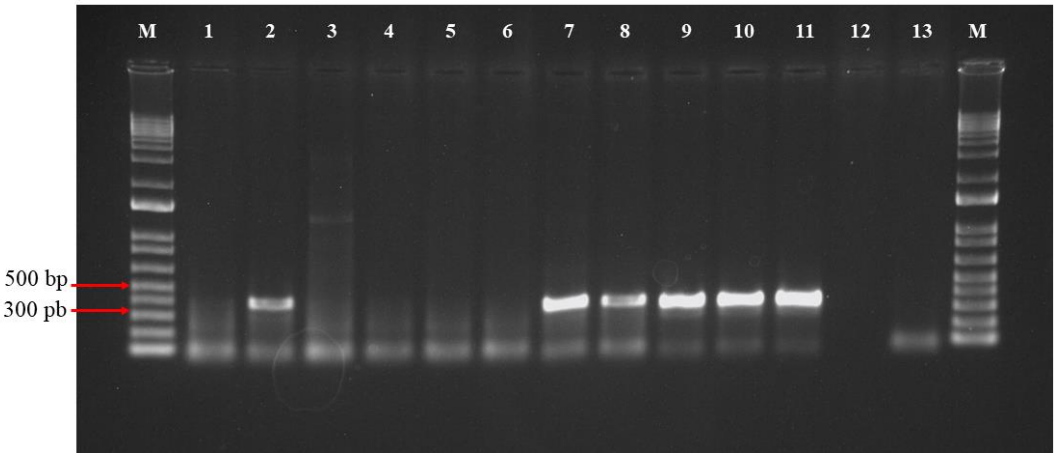


Fig. 4 Gel electrophoresis of amplicons produced by PCR assays using race 2 specific primers VdR2F and VdR2R (256 bp). Lanes 1-6: Vert04, Vert09, Vert14, Vert26, Vert151, Vert161 (race 1: non-pathogenic on tomato cv. Floradade); Lanes 7-11: Vert17, Vert35, Vert148, Vert172, Vert182 (race 2: pathogenic 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

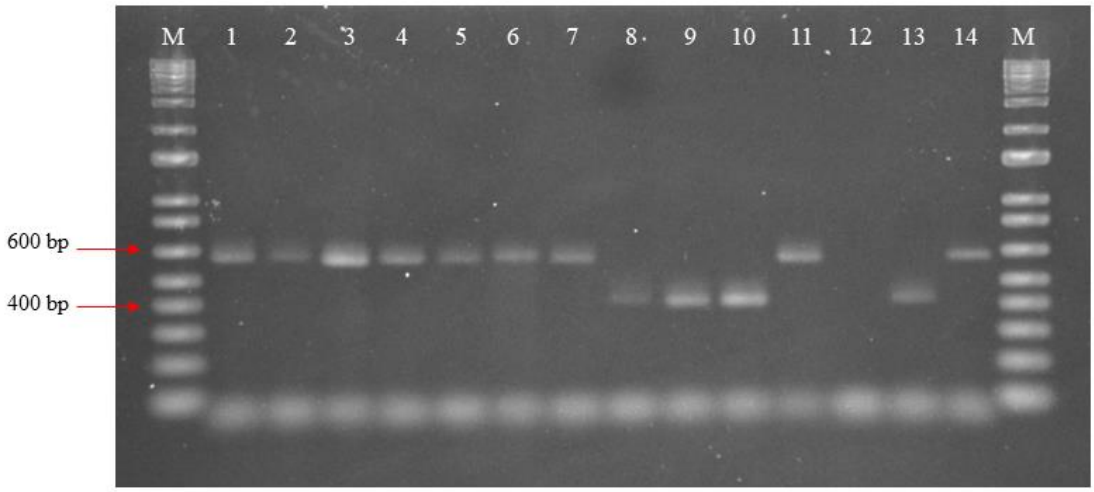


Fig. 5 Gel electrophoresis of amplicons produced by Multiplex PCR using MAT specific primers MAT1-1a/MAT1b (~400 bp), and MAT1-2a/MAT1-2b (~600 bp). Lanes 1: Vert04; 2: Vert09; 3: Vert14; 4: Vert26; 5: Vert151; 6: Vert161; 7: Vert17; 8: Vert35; 9: Vert148; 10: Vert172; 11: Vert182; Lane 12: No template 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

549 **Supplementary Material**

550

551 **Table S1** *V. dahliae* isolates used in this study along with origin, plant host, and mating types, as determined
552 by PCR assays

Isolate	Origin	Host	Mating type		
			***MAT1-1a*/ *MAT1-1b* (400 pb)**	***MAT1-2a*/ *MAT1-2b* (600 pb)**	**MAT**
Vert02	SP	Tomato	+	-	*MAT-1-1*
Vert03	SP	Tomato	+	-	*MAT-1-1*
Vert04	SP	Tomato	-	+	*MAT-1-2*
Vert05	DF	Tomato	+	-	*MAT-1-1*
Vert06	DF	Tomato	+	-	*MAT-1-1*
Vert07	DF	Eggplant	+	-	*MAT-1-1*
Vert08	Unknown	Eggplant	+	-	*MAT-1-1*
Vert09	Unknown	Eggplant	-	+	*MAT-1-2*
Vert12	MG	Okra	+	-	*MAT-1-1*
Vert14	MG	Eggplant	-	+	*MAT-1-2*
Vert17	SP	Eggplant	-	+	*MAT-1-2*
Vert21	Unknown	Potato	+	-	*MAT-1-1*
Vert22	BA	Cacao	+	-	*MAT-1-1*
Vert23	Unknown	Cacao	+	-	*MAT-1-1*
Vert26	Unknown	Tomato	-	+	*MAT-1-2*
Vert32	ES	Tomato	+	-	*MAT-1-1*
Vert34	ES	Tomato	-	-	No sex-related gene
Vert35	ES	Tomato	+	-	*MAT-1-1*
Vert36	RJ	Tomato	-	+	*MAT-1-2*
Vert38	SC	Tomato	-	-	No sex-related gene
Vert43	SC	Tomato	+	-	*MAT-1-1*
Vert45	SC	Tomato	+	-	*MAT-1-1*
Vert46	DF	Tomato	+	-	*MAT-1-1*
Vert47	DF	Tomato	+	-	*MAT-1-1*
Vert53	SP	Tomato	+	-	*MAT-1-1*
Vert54	SP	Tomato	+	-	*MAT-1-1*
Vert56	SP	Tomato	+	-	*MAT-1-1*
Vert59	MG	Tomato	+	-	*MAT-1-1*
Vert62	SP	Eggplant	+	-	*MAT-1-1*
Vert65	SC	Tomato	+	-	*MAT-1-1*
Vert67	ES	Tomato	+	-	*MAT-1-1*
Vert70	ES	Tomato	+	-	*MAT-1-1*
Vert71	ES	Tomato	+	-	*MAT-1-1*
Vert74	ES	Tomato	+	-	*MAT-1-1*
Vert77	SP	Tomato	+	-	*MAT-1-1*
Vert78	SP	Tomato	+	-	*MAT-1-1*
Vert79	DF	Tomato	+	-	*MAT-1-1*
Vert93	MG	Tomato	+	-	*MAT-1-1*

Vert96	RJ	Tomato	+	-	*MAT-1-1*
Vert103	DF	Strawberry	-	+	*MAT-1-2*
Vert106	MG	Tomato	+	-	*MAT-1-1*
Vert110	RJ	Scarlet eggplant	+	-	*MAT-1-1*
Vert111	RJ	Scarlet eggplant	+	-	*MAT-1-1*
Vert116	MG	Tomato	+	-	*MAT-1-1*
Vert117	CE	Scarlet eggplant	+	-	*MAT-1-1*
Vert118	CE	Eggplant	+	-	*MAT-1-1*
Vert119	DF	Eggplant	+	-	*MAT-1-1*
Vert120	ES	Eggplant	+	-	*MAT-1-1*
Vert121	SC	Tomato	+	-	*MAT-1-1*
Vert125	PR	Tomato	+	-	*MAT-1-1*
Vert129	ES	Eggplant	+	-	*MAT-1-1*
Vert130	ES	Strawberry	+	-	*MAT-1-1*
Vert132	ES	Tomato	-	+	*MAT-1-2*
Vert134	ES	Strawberry	+	-	*MAT-1-1*
Vert137	ES	Strawberry	+	-	*MAT-1-1*
Vert142	ES	Strawberry	+	-	*MAT-1-1*
Vert143	ES	Strawberry	-	-	No sex-related gene
Vert144	ES	Strawberry	+	-	*MAT-1-1*
Vert145	ES	Strawberry	+	-	*MAT-1-1*
Vert147	ES	Strawberry	+	-	*MAT-1-1*
Vert148	ES	Strawberry	+	-	*MAT-1-1*
Vert149	ES	Strawberry	+	-	*MAT-1-1*
Vert150	ES	Strawberry	+	-	*MAT-1-1*
Vert151	SP	Tomato	-	+	*MAT-1-2*
Vert158	DF	Eggplant	-	-	No sex-related gene
Vert160	ES	Tomato	+	-	*MAT-1-1*
Vert161	ES	Tomato	-	+	*MAT-1-2*
Vert163	DF	Eggplant	+	-	*MAT-1-1*
Vert164	DF	Eggplant	+	-	*MAT-1-1*
Vert166	MG	Potato	-	+	*MAT-1-2*
Vert169	MG	Potato	+	-	*MAT-1-1*
Vert171	MG	Potato	+	-	*MAT-1-1*
Vert172	MG	Potato	+	-	*MAT-1-1*
Vert173	MG	Potato	+	-	*MAT-1-1*
Vert174	MG	Potato	+	-	*MAT-1-1*
Vert176	DF	Eggplant	+	-	*MAT-1-1*
Vert177	MG	Potato	+	-	*MAT-1-1*
Vert178	MG	Potato	+	-	*MAT-1-1*
Vert179	MG	Potato	+	-	*MAT-1-1*
Vert180	MG	Potato	+	-	*MAT-1-1*
Vert181	SP	Eggplant	+	-	*MAT-1-1*
Vert182	SP	Eggplant	-	+	*MAT-1-2*
Vert183	GO	Tomato	+	-	*MAT-1-1*

Vert184	MG	Tomato	+	-	*MAT-1-1*
Vert185	MG	Tomato	+	-	*MAT-1-1*
Vert186	BA	Potato	+	-	*MAT-1-1*
Vert187	BA	Potato	+	-	*MAT-1-1*
Vert188	BA	Potato	+	-	*MAT-1-1*
Vert189	BA	Potato	+	-	*MAT-1-1*

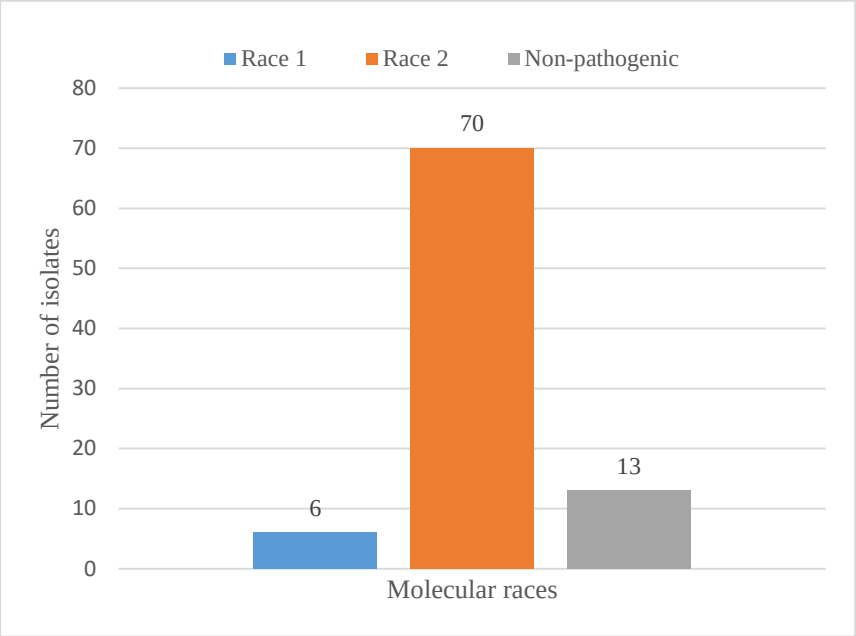


Figure S1. Molecular determination of races in a fungal collection of *V. dahliae* isolates.

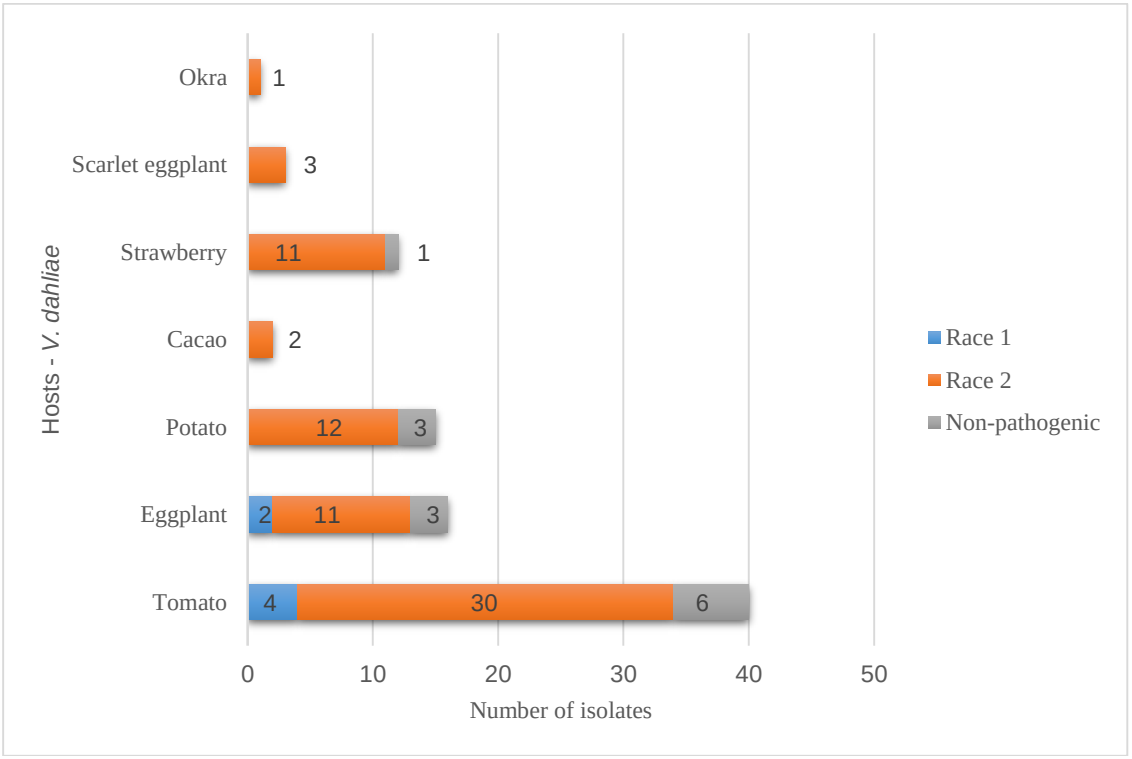
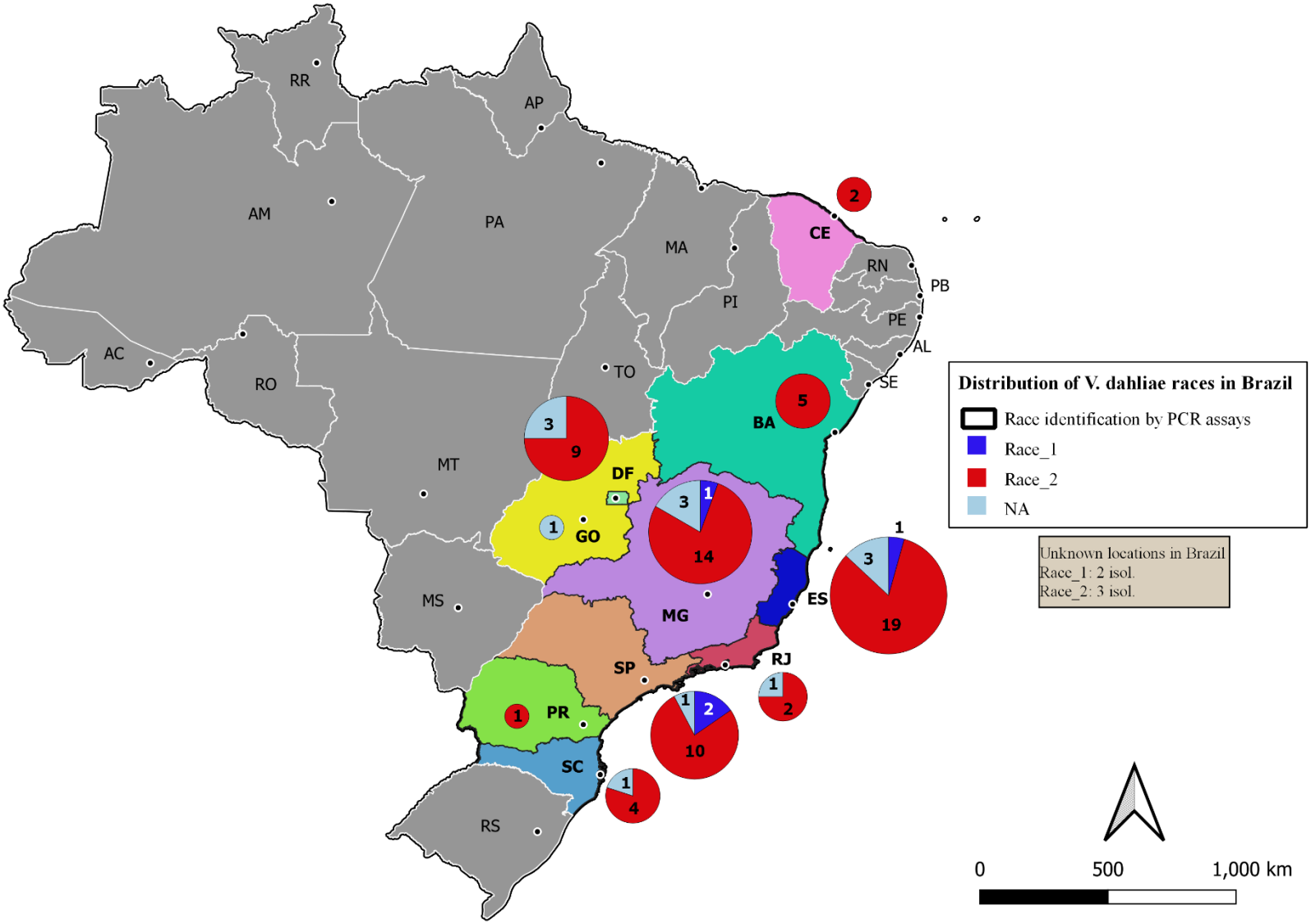


Figure S2. Race specific detection by PCR in several hosts of *V. dahliae*.



564
565 **Figure S3.** Geographical distribution of *V. dahliae* races confirmed using specific primers and collected in several hosts in Brazil.

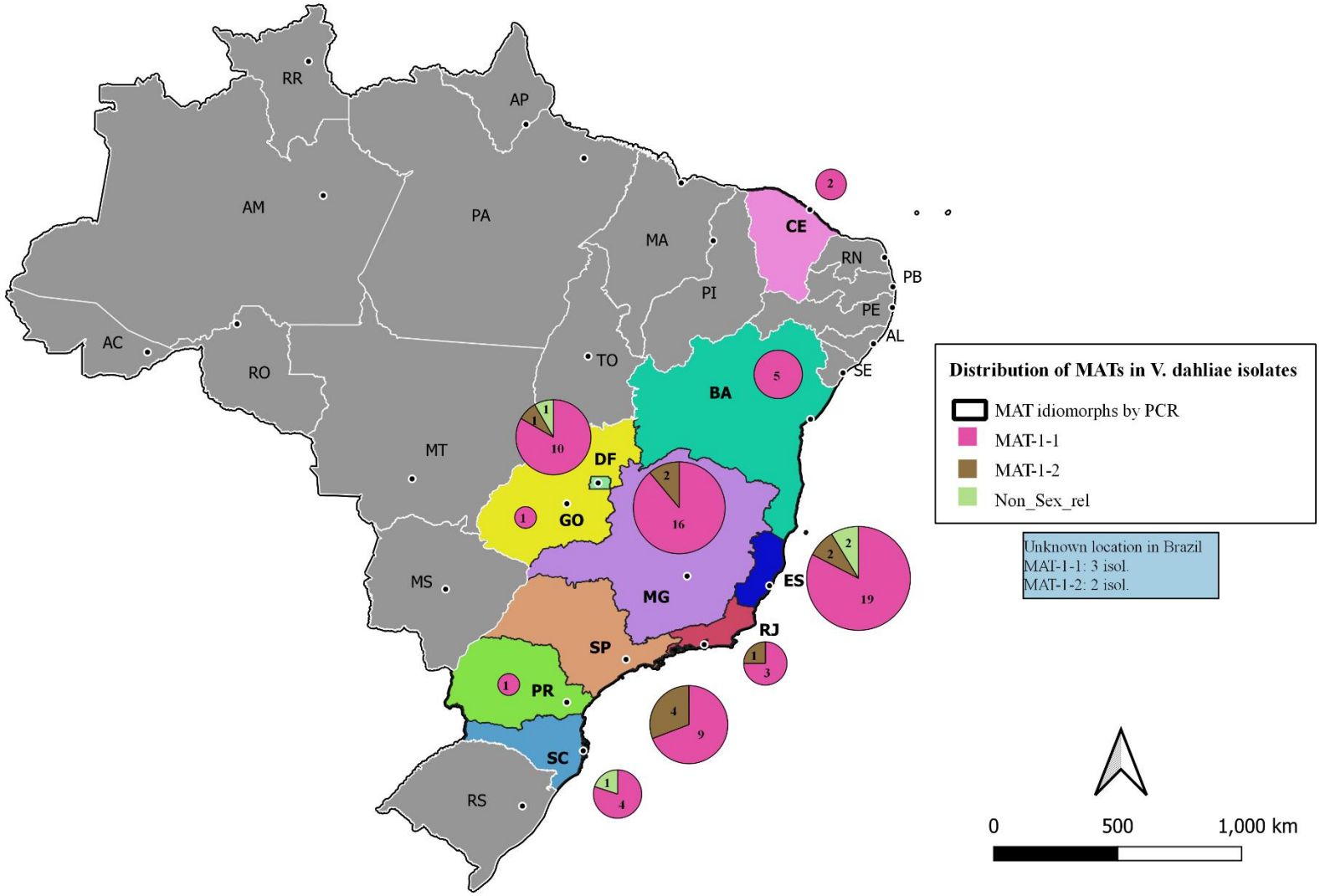


Figure S4. Geographical distribution of MAT idiomorphs of *V. dahliae* confirmed using specific primers and collected in several hosts in Brazil.

Capítulo IV

Discovery of a novel ssRNA virus infecting the phytopathogenic fungi *Verticillium dahliae* by high throughput sequencing

Discovery of a novel ssRNA virus infecting the phytopathogenic fungi *Verticillium dahliae* by high throughput sequencing

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Abstract

Mycoviruses that infect the soil-borne fungi *Verticillium dahliae,* one of the most important causal agents of vascular wilts, represent a largely unexplored group of organisms, opening an opportunity to explore them as biological control agents. In this study, we used a metagenomics approach to discover mycoviruses infecting *V. dahliae* isolates. We selected 42 isolates of *V. dahliae* obtained from different vegetable crops and localities of Brazil. Total RNA was isolated from mycelia from each sample, pooled, and the resulting cDNA libraries were sequenced by high-throughput sequencing (HTS). In the pooled RNA, we identified one single mycovirus-like sequence in a contig of 2793 nucleotides (nt). The sequence shares 75% nt identity to an unclassified (+) ssRNA virus, clustering with other unclassified members in the *Riboviria* domain. The putative ORF encodes a protein of 235 aa homologous to an RdRp-like protein. RT-PCR amplifications were performed to confirm the presence of this virus in the individual original isolates. It was positively detected in 38 (90.5%) out of 42 isolates. This study is the first to identify a mycovirus infection in Brazilian isolates of *V. dahliae*. Comparisons of this genome with those of other RNA viruses indicate that this is a new virus, tentatively named *Verticillium dahliae* single stranded RNA virus. As the control of soil-borne pathogens is complex, this virus represents a potential mycopathogenic virus for testing to induce hypovirulence in *Verticillium* isolates.

Keywords: Novel mycoviruses, ssRNA viral genome, Next Generation Sequencing, *Verticillium dahliae*.

Introduction

Vascular wilt caused by fungal pathogens are widespread and are very destructive plant diseases, responsible for enormous economic losses. *Verticillium* spp. are among the most common causal agents of these diseases (Klosterman et al. 2011). *Verticillium dahliae* Kleb. is, for excellence, the primary causal fungal agent of vascular wilts in a wide variety of crops (Pegg and Brady 2002; Bhat and Subbarao 1999). The disease is difficult to manage, and the control generally relies on the application of fungicides, though this practice may not be economically viable and environmentally friendly (Atallah et al. 2011; López-Escudero and Mercado-Blanco 2011). Consequently, a particular interest in fungal viruses, and their hypovirulent property, has been observed recently, and growingly explored because of the possibility of using them as new alternatives for the biological control of fungal diseases (Nuss, 2005). Mycoviruses, or fungal viruses, are viruses that replicate in fungal cells (Xie and Jiang 2014). Although mycoviruses are widespread in all major taxa of fungi, they surprisingly share similar properties (Pearson et al. 2009). Symptoms induced by mycoviruses can range from severe to no effects on host physiology, such that they may lead to attenuation (hypovirulence) or enhancement of fungal virulence (hypervirulence) (Ghabrial and Suzuki 2009). Dramatic changes in the infected hosts, irregular growth, abnormal pigmentation, and altered sexual reproduction have been documented as a consequence of the mycoviruses infections (Son et al. 2015). Nevertheless, the majority of studies have so far focused on mycoviruses that are associated with hypovirulence in economically important plant pathogenic fungi, because of their potential to be used as a tool for combating fungal diseases as biological control agents (Ghabrial et al. 2005; Wang et al. 2015).

The interest in studying viruses of phytopathogenic fungi has increased since the first report of mycoviruses in *Cryphonectria parasitica*, the causal agent of chestnut blight (Choi and Nuss 1992; Nuss 1992), particularly those that affect their virulence. Some examples of mycoviruses were observed in *Colletotrichum truncatum*, *Macrophomina phaseolina*,

Diaporthe longicolla, *Rhizoctonia solani* (Marzano et al. 2016; Zhong et al., 2016; Picarelli et al. 2019), *Sclerotinia sclerotiorum* (Xie et al. 2011; Liu et al. 2015; Xie et al. 2006; Mu et al. 2018), *Rosellinia necatrix* (Kanematsu et al. 2014), *Monilinia fructicola* (Tran et al. 2019), *Fusarium* species (Cho et al. 2013; Li et al. 2019), *Aspergillus* species (Banks et al. 1970; Kotta and Coutts 2017), *Botrytis cinerea* (Wu et al. 2007), *Penicillium digitatum* (Niu et al. 2016), among others.

Nowadays, metatranscriptomic and metagenomic studies have shown that mycoviruses are ubiquitous in nature (Marzano et al. 2016). Several technical advances have proven to be valuable for the discovery, detection and sequencing of previously unidentified viruses (Mokili et al. 2012; Blouin et al. 2016). High-throughput (HTS) or next generation sequencing (NGS) technologies and bioinformatics can be applied to all types of genomes, and drastically changed the research on viral pathogens, and also these technologies become one of the most used approaches to characterize fungal viruses (Mokili et al. 2012; Massart et al. 2014).

To date, there are still few reports of mycoviruses in *V. dahliae*. The majority of these mycoviruses was identified as double-stranded RNA genome viruses infecting cotton isolates in China (Cao et al. 2011; Feng et al. 2013) and from olive isolates in Turkey (Cañizares et al. 2015). So far only one positive (+) single-stranded (ss) RNA viral genome has been characterized and collected from olive growing areas in Spain (Cañizares et al. 2017). In addition, there are no other reports of mycoviruses infecting *V. dahliae*.

In Brazil, no studies were carried out aiming to detect the presence of any type of mycoviruses infecting *V. dahliae*, the causal agent of *Verticillium* wilt in several vegetable hosts. Thus for, in this work we used a deep sequencing approach to detect and identify mycoviruses that are infecting Brazilian isolates of *V. dahliae*.

Material and Methods

Fungal isolates and multiplication

A total of 42 *V. dahliae* isolates, collected from several vegetable crops in different states of Brazil, were selected for this study (Table 1). All isolates were grown on potato

dextrose agar (PDA) medium at 20-24 °C. Working cultures were handled by transferring agar plugs from stock cultures (-20°C) onto PDA in Petri dishes and incubating in the dark at 23°C for approximately two weeks. All isolates were previously identified in this study by multi-genic approach (data not shown). Besides they were tested in their ability or inability to produce microsclerotia, and any irregular mycelial sectors in PDA medium (in triplicates) at 23°C for 20 days.

RNA extraction

The mycelia of the 42 *V. dahliae* isolates were cultured on PDA and harvested directly from plates after 7 days. Total RNA was extracted individually from 100 mg of mycelia with Trizol® (Invitrogen™, CA, USA), according to the manufacturer's instructions. Immediately, fresh mycelium was frozen and ground to a fine powder in the presence of liquid nitrogen using a mortar and pestle. Subsequently, the powdered mycelium was transferred into a 1.5 mL centrifuge tube containing 1 mL Trizol® buffer. To this mixture, 300 µL phenol-chloroform (50:50) was added, agitated, and subsequently centrifuged at 12000 rpm at 4 °C for 15 min. The supernatant was taken and placed in a new sterile 1.5 mL tube containing 500 µL of cold isopropanol, incubated for 10 min (room temperature, R.T.), and then centrifuged (12000 rpm) for 2 min to form an RNA pellet. The supernatant was discarded, the pellet washed twice with 1 mL of 75% (v/v) chilled ethanol and then centrifuged at 12000 rpm for 5 min. The RNA pellet was dried with the open tubes facing up and, then, it was resuspended in 100 µL of 0.1% (v/v) Diethyl-Pyrocarbonate (DEPC)-treated water. The RNA of each isolate was resolved on 1% agarose gel (p/v) and visualized with ethidium bromide to verify integrity. Purity and RNA concentration (260/280 nm) were estimated by spectrophotometry using the Eppendorf Biophothometer® (Eppendorf®, AG, Germany).

cDNA library construction and High throughput sequencing (HTS)

A set of 42 RNA samples were combined into one pool and sequenced by HTS. Then, a total of 500 ng was separated in dried using RNAstable® (Sigma-Aldrich, Darmstadt, Germany), and sent to Macrogen, Inc. (South Korea). The rRNA was depleted with Ribo-

Zero™ rRNA H/M/R treatment (Illumina®, Inc.). cDNA libraries were constructed using TruSeq™ Stranded Total RNA sample preparation kit (Illumina®, Inc.), and sequenced on an Illumina® NovaSeq 6000 platform. The RNA-seq library generates FastQ files, and reads with lengths of 100 nucleotides (nt). The reads were trimmed with Trimmomatic v. 0.35 (Bolger et al. 2014) to removed low quality bases and eliminated adapter sequences. The trimmed reads were assembled with Velvet software (Zerbino and Birney, 2008) using standard parameters by a *de novo* approach. The resulting contigs were compared to the RefSeq viral database using MegaBLAST and imported into Geneious v. 9.1.9 software (Biomatters Ltd. Auckland, New Zealand) (http://www.geneious.com/) for mapping the reads. Amino acid sequences in contigs virus-like were analyzed by ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) to predict the potential Open Reading Frames (ORFs).

Primer design

To confirm the presence of mycoviruses infecting each fungal isolate of *V. dahliae*, we designed a pair of specific primers from the consensus sequence within a region of high coverage of reads (Table 2). Additionally, to confirm the complete genome of the mycovirus, three primers were designed: 1) Outer forward primer, 2) Outer reverse primer, and 3) inner-primer (Table 3). Primer3 plus (Untergasser et al. 2007) was used to design the primers, and the specificity was checked by primer-BLAST analysis (Ye et al. 2012) against the *V. dahliae* genome and other mycoviral genomes available in the GenBank database.

RT-PCR

The pooled RNA sample and the total RNA from each *V. dahliae* isolate were used for the detection of virus by reverse transcription – polymerase chain reaction (RT-PCR). First-strand cDNA was synthesized in two steps using Superscript™ IV reverse transcriptase (200 U/µL) (Thermo Scientific, CA, USA) according to the manufactures' protocol. In a PCR tube, 1 µL of RNA (~20 ng), 10 mM each of dNTPs mix (Invitrogen®, CA, USA), 1 µL of 10 µM random hexamer primer (5′-CGATCGATCATGATGCAATGCNNNNNN-3′), and 9 µL of DEPC treated water were combined to reach a final reaction volume of 12 µL.

The reaction was mixed and incubated at 70 °C for 10 min in a C1000 Touch PCR thermal cycler (Bio-Rad Laboratories, Inc., CA, USA), and then incubated on ice for at least 5 min. For the second step, 4 µL of 5X first strand buffer (Invitrogen®, CA, USA), 2 µL of DTT (0.1 M), 1 µL of RNaseOUT™ Recombinant Ribonuclease Inhibitor (40 U/ µL), and 1 µL of Superscript™ IV reverse transcriptase (200 U/µL) were added. The RT-mixes were incubated at 50 min at 37 °C and then, inactivated by heating at 70 °C for 15 min. cDNA from individual isolates of *V. dahliae* were used as templates for amplification of each viral fragment with specific primers, previously designed in this study, and based on the contig obtained from metagenomics analysis. PCRs were performed in a 25 µL final volume reaction containing 0.4 µM of each forward and reverse primer (10 µM each), 2 mM of $MgCl_2$, 0.4 mM of each dNTP, 1X buffer (Invitrogen®, CA, USA), 1 U *Taq* DNA polymerase, and 2 µL of cDNA as template. The amplification program was standardized as follows: 95 °C for 5 min, 32 cycles of 95 °C for 30 s, 60°C for 60 s, and 72 °C for 90 s, and a final extension of 72 °C for 5 min.

All RT-PCR products were purified using a gel extraction kit, Stratec molecular (Negev Bio Products, Israel), eluted in 20 µL of elution buffer, cloned and sequenced using the Sanger method.

cDNA cloning and sequencing

In order to validate the high quality consensus sequences from the complete genome of potential mycoviruses, the amplified PCR products were recovered from gel slices and purified with Stratec molecular kit (Negev Bio Products, Israel). PCR products were cloned into pGEM®-T Easy Vector system (Promega©, WI, USA) according to the manufacturer's instructions. Two PCR fragments corresponding to the consensus sequences obtained by HTS were amplified with Rb_383-F/Rb_2594-R to obtain a DNA insert of 2212 bp, and Rb_1143-F/Rb_2594-R to obtain a fragment of 1452 bp in length (Table 3).

Ligation reactions were incubated overnight at 4°C with the following reagents in 20 µL as final volume of reaction: 5 µL of 2X Rapid Ligation Buffer, 1 µL of T 4 DNA Ligase (3 Weiss units/ µL; Promega©, WI, USA), 1 µL of pGEM®-T Easy Vector (50 ng; Promega©, WI, USA), 12 µL of product of PCR (insert) (3:1; insert: vector molar ratio), and

1 µL of nuclease-free water. Then, 5 µL of ligation reactions were transformed into competent cells of *Escherichia coli* DH5α. Positive clones were sequenced with SP6/T7 primers by the Sanger method at Macrogen. Nucleotide and amino acid sequences obtained were analyzed with BLASTn, and compared with those in the GenBank database.

Results

Colony morphology of *V. dahliae* isolates with phenotypic alterations

All isolates used for detection of mycoviruses were previously identified as *V. dahliae* with molecular markers. From the 42 *V. dahliae* analyzed isolates, we observed that 30 isolates (71.4%) were able to produce microsclerotia, and 12 isolates (28.6%) not on the PDA medium (Table 1). Most isolates were of typical *V. dahliae* morphology, though some showed abnormal and irregular growth in mycelial sectors, fewer production of sclerotia (≥ 20 days), and production of an orange pigmentation at the bottom of the plate (Fig. 1). Comparison of somatic mycelial growth and aggressiveness for all the isolates will be further studied.

Identification of viral genomes in *Verticillium dahliae* library by HTS

Following Illumina sequencing, we obtained 54,534,666 total of reads, with total read bases of 5.5G bp and high quality scores (QC%) of Q20= 98.35%, and Q30= 95.49%. Three contig sequences associated with viruses were obtained with 1) 1743 nucleotides (nt), 2) 2793 nt, and 3) 6090 nt. Multiple alignments were performed with all contigs, suggesting that contig 1 and 2 originate from the same virus genome (99.5% Per Ident; Query cover = 95%). The two contigs share ~75% nt identity to previously described unclassified (+) ssRNA virus isolated from soil metagenomic sample and other unclassified RNA mycoviruses (Table 4). Therefore, we tentatively named this novel mycoviruses infecting *V. dahliae* isolates as *Verticillium dahliae* single stranded RNA virus-strain CNPH (VdRV-strain CNPH).

The contig 3 shares a high percentage of identity with contig 1 (98%) and contig 2 (99.5%), but only 34% and 50% of the sequence was covered by contig 1 and 2, respectively. The sequence assembled display a maximum length of 6090 nt that correspond to the nearly

full genome of mycovirus-like sequence. Using BLASTn analysis, this contig exhibited closest homology (74.9%) with *Plasmopara viticola lesion associated ambiguivirus* 2 (MN551113) identified in other fungi (Table 4). Therefore, it is necessary a further manually extended several rounds for mapping reads.

For more details, all contigs were subject to BLASTn and the results are listed in table 4.

Putative ORF prediction

A BLASTp analysis revealed a putative RNA-dependent RNA polymerase (RdRp) in the 2793 nt virus-associated contig. This new mycovirus sequence encodes a protein of 235 amino acids (aa) and 708 nt in length of an RdRp-like protein. The predicted amino acid sequence showed significant similarity (76.5%) to a reverse transcriptase (RT, RNA-dependent DNA polymerase)-like family, associated to a (+) ssRNA genome belonging to the unclassified *Riboviria* sp. (Accession: QDH88200.1; E-value = 3^{e-84}).

Presence of mycovirus in *Verticillium dahliae* isolates

At first, total RNA integrity was confirmed for each mycelium sample (Fig. S1). Then, RT-PCR was performed to detect the VdRV-strain CNPH in our sample pool with specific primers designed in this study (Table 2). We obtained an amplicon size of ~930 bp with Rib-1144-F and Rib-2056-R, corresponding to the target size of VdRV-strain CNPH (Fig. 2) in the pool. After confirming the presence of the virus in the pooled sample, each RNA sample was analyzed (Fig. 3; Table 1). We obtained a total of 38 (90.5%) positive samples for the presence of the mycovirus, while only four (9.5%) isolates were negative to VdRV-strain CNPH detection (Table 1). The negative samples were Vert93 from tomato, Vert149 from strawberry, Vert158 from eggplant, and Vert171 from potato. On the other hand, the DNA fragment corresponding to our putative mycovirus was found in the majority of Brazilian isolates that represent seven hosts (tomato, eggplant, scarlet eggplant, potato, okra, strawberry, and cacao), and in nine different states in the country (Table 1). We adopted a criterion of selection based on phenotypes with morphological alterations for *V. dahliae*

236 isolates, thus may be an indicator in the high incidence of the virus detection rate among this
237 pre-selected population.

238

239 **Characterization of novel mycoviruses infecting *Verticilliu dahliae* isolates**

240 To characterized the putative VdRV-strain CNPH, two primer-pairs were designed to
241 cover the complete genome of the virus. These primers target two overlapping segments of
242 the viral genome that included the RdRp-like protein. The first part consisted of a largest
243 segment located around the 5´-383 _to 3´-2594 in the genome sequence, and the second
244 middle segment was located on sense 5´- 1143 and to the end of 3´-2594 in the sequence.
245 The expected amplicon size was 2211 bp and 1452 bp (Fig. 4). They were successfully
246 amplified from the reference genome of the pooled sample, purified and cloned. A total of
247 five positives clones were obtained (Fig. S2), and sequenced. Our partial results showed two
248 fragments of 1437 bp and 1455 bp corresponding to the middle of the VdRV-strain CNPH
249 genome cloned share 56% and 55% nt identity with *Riboviria* sp. isolate H4 (MN033924).
250 Also, we retrieved two sequences of 2211 nt and 2206 nt in length from the fragments cloned,
251 which targeting the complete viral genome of the VdRV-strain CNPH. Similarities for these,
252 were found using BLASTn tool, showing 48% nt identity with *Riboviria* sp. isolate H4. The
253 analysis is still not concluded.

254

255 **Discussion**

256 *Verticillium dahliae* is the causal fungal agent of the Verticillium wilt disease
257 (Klosterman et al. 2009). Verticillium wilt outbreaks are under a high concern for the
258 Brazilian vegetable producers (Lopes et al. 2018). Moreover, the disease is difficult to
259 manage by conventional chemical application (Fradin and Thomma 2006). Many studies
260 about the potential use of mycoviruses to control plant pathogenic fungi have emerged as a
261 novel biological tool (Jiang and Ghabrial 2013; Ghabrial et al. 2015). In Brazil, the interest
262 to explore the diversity of mycoviruses infecting plant pathogenic fungi has been recently
263 expanded (Figueirêdo et al. 2012; Picarelli et al. 2019; Picarelli et al. 2020). Thus far, in this
264 study we used a metagenomic approach to unravel the virus population on a pooled RNA
265 preparation from isolates of *V. dahliae* collected in different hosts and regions of the country.

All analyzed *Verticillum* isolates from the collection of Embrapa Vegetables showed morphological features that corresponded to *V. dahliae* species, which were confirmed previously by molecular identification methods (in this study, data not shown). However, we observed in a subset of 42 isolates, phenotypes with morphological alterations such as irregular growth in sectors, irregular margins in the colony, reduction in sclerotia production, and unusual pigmentation. As previously described, the presence of viruses in some fungi is often associated with hypovirulence causing phenotypic changes in their host fungi. (Kyrychenko et al. 2018; Song et al. 2020). For this reason, the production of microsclerotia was determined, as this structure plays an important role in the disease cycle as inoculum source and long-term survival structure (Fradin and Thomma 2006). In the analysis, we observed 71.4% of biological efficiency in sclerotia production (MS) and only 28.6% loss of this attribute (NoMS). Among the MS group, in 96.6% of these isolates the VdRV strain CNPH was detected, whereas for NoMS isolates, in 75% of them the virus was detected. It suggests that the ability of producing microsclerotia was not lost for most isolates that contained the mycovirus. Besides, resting structures can be affected by many factors in laboratory conditions (Inderbitzin and Subbarao 2014), and have not been reliably attributed to the presence of virus. We believe it is necessary to perform a comparative analysis of the deleterious effects of mycoviral infection on the growth of fungal colonies of *V. dahliae*.

Mycoviruses are widely distributed in almost all fungal groups (Niu et al. 2017). However, only few reports of mycovirus infecting fungal isolates of *V. dahliae* have been generated in China (Cao et al. 2011; Feng et al. 2013), Turkey (Canizares et al. 2015), and Spain (Canizares et al. 2017). The latter reported the first (+) ssRNA mycovirus discovered in *V. dahliae* infecting olive trees and from soil samples. Here, we initiated a survey to discover mycoviruses in *V. dahliae*, which were causing vascular wilt on vegetables and other hosts. The analysis of our *V. dahliae* virome resulted in most likely in one virus sequence (Fig.2; Table 4). The virus-associated contigs were derived from a positive ssRNA virus genome. In contrast to many studies on virus detection in fungal transcriptomes (Ruiz-Padilla et al. 2021; Jo et al. 2020; Tran et al. 2019; Marzano et al. 2016), in our study we found a single viral population infecting isolates of *V. dahliae*. This result apparently is not totally uncommon since few reports have been done to characterize the viromes of *V. dahliae* in the world with low frequency of virus-associated contigs belonged to two types of genomes:

297 double-stranded RNA genomes and positive ssRNA genome (Cao et al. 2011; Canizares et
298 al. 2017).

299 The partial genome of VdRV-strain CNPH was determined to contain one deduced
300 protein. It has a high identity to genomes of mycoviruses members of unclassified (+) ssRNA
301 mycovirus and belonging to genus *Riboviria* sp. Similar to our results, the genome of
302 *Verticillium dahliae RNA virus* 1 (VdRV1) named by Canizares et al. (2017) showed
303 significant similarity (60%) with three of the unclassified (+) ssRNA mycoviruses related to
304 umbravirus. However, we were not able to find enough identity at the nucleotide or at the
305 amino acid level between VdRV-strain CNPH and VdRV reported by Cañizares et al. (2017)
306 to infer that they could be the same species. The next step of our study is to complete the
307 genome sequence, and determine the genome organization of the virus, including the
308 5´(UTR) and 3´(UTR).

309 We found a high prevalence of the mycovirus in our samples. This may indicate an
310 evidence of an efficient transmission of this mycovirus to uninfected isolates through hyphal
311 anastomosis, and perhaps by vertical transmission to the progeny, as demonstrated in the case
312 of the mycovirus in *C. parasitica* (Zhang et al. 2014; Wu et al. 2017; Yang et al. 2018). The
313 tested *V. dahliae* isolates were widely infected with one single mycovirus (VdRV-strain
314 CNPH) indicating that mycoviruses are not common in this fungi. It may also suggest that
315 the selection criteria used for this study were particularly selective for isolates infected by
316 VdRV-like viruses.

317 We believe that the detection, identification, complete characterization and biological
318 effects of mycoviruses naturally found in the plant pathogenic fungi, *V. dahliae*, could be of
319 great interest in the search for potential biological control agents. This is the first study using
320 a deep sequencing approach for mycoviral detection on isolates of *V. dahliae* performing in
321 Brazil.

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Figures and Tables with Captions

Table 1. Origin, host, production of sclerotia, and the presence/absence of (+) ssRNA in isolates of *Verticillium dahliae* collected in Brazil

Strain identifier	Host origin	Geographical location	Year of collection	Sclerotia formation	Mycovirus detection
Vert03	Tomato	SP	1992	MS (≥20 d)	+
Vert04	Tomato	SP	1992	NoMS	+
Vert05	Tomato	DF	1994	MS (≥20 d)	+
Vert06	Tomato	DF	1995	NoMS	+
Vert12	Okra	MG	1997	MS (≥20 d)	+
Vert21	Potato	Unknown	1997	MS	+
Vert22	Cacao	BA	1997	MS	+
Vert23	Cacao	Unknown	1997	NoMS	+
Vert26	Tomato	Unknown	1997	MS	+
Vert32	Tomato	ES	2004	MS	+
Vert34	Tomato	ES	2004	NoMS	+
Vert38	Tomato	SC	2004	MS	+
Vert43	Tomato	SC	2005	MS	+
Vert46	Tomato	DF	2005	MS	+
Vert47	Tomato	DF	2005	MS	+
Vert54	Tomato	SP	2005	MS (≥20 d)	+
Vert56	Tomato	SP	2005	MS	+
Vert59	Tomato	MG	2005	MS (≥20 d)	+
Vert78	Tomato	SP	2006	NoMS	+
Vert93	Tomato	MG	2006	NoMS	-
Vert110	Scarlet eggplant	RJ	2008	MS (≥20 d)	+
Vert111	Scarlet eggplant	RJ	2008	NoMS	+
Vert121	Tomato	SC	2010	MS	+
Vert125	Tomato	PR	2010	MS	+
Vert129	Eggplant	ES	2010	MS	+
Vert132	Tomato	ES	2010	NoMS	+
Vert134	Strawberry	ES	2010	MS	+
Vert137	Strawberry	ES	2010	MS	+
Vert142	Strawberry	ES	2011	No MS	+
Vert149	Strawberry	ES	2011	NoMS	-
Vert150	Strawberry	ES	2011	MS (≥20 d)	+
Vert151	Tomato	SP	2011	NoMS	+
Vert158	Eggplant	DF	2017	NoMS-Orange pigment	-
Vert160	Tomato	ES	2018	MS(≥20 d)	+

Vert163	Eggplant	DF	2018	MS	+
Vert169	Potato	MG	2018	MS	+
Vert171	Potato	MG	2018	MS	-
Vert173	Potato	MG	2018	MS	+
Vert181	Eggplant	SP	2019	MS	+
Vert182	Eggplant	SP	2019	MS	+
Vert184	Tomato	MG	2019	MS	+
Vert189	Potato	BA	2019	MS	+

BA: Bahia; DF: Distrito Federal; Es: Espirito Santo; MG: Minas Gerais; PR: Paraná; RJ: Rio de Janeiro; SC: Santa Catarina; SP: Saõ Paulo; Unknown: isolates collected from unknown location. MS: isolates that produce microsclerotia in PDA medium; NoMS: isolates that not produce microsclerotia in PDA medium.

Table 2. Oligonucleotide primers used in this study for detection of the mycovirus in *V. dahliae*

Primer name	Sequence (5' - >3')	Position in the genome	Length (nt)	Tm	GC%	Product length (bp)
Rib-1144-F	GCACGTCGAACGGTACATTG	1144-2075	20	59.91	55	932
Rib-2056-R	TCACCTTAGGGGCTTCACC		19	60.04	60	

Table 3. Oligonucleotide primers used in this study to characterize the complete genome of (+) ssRNA mycovirus

Primer name	Sequence (5' - >3')	Position in the genome	Length (nt)	Tm	GC%
Rb_383-F[a]	CATTACAGGCCGCGCTAAAC	383-402	20	59.97	55
Rb_1143-F[b]	AGCACGTCGAACGGTACATT	1143-1162	20	60.04	50
Rb_2594-R[c]	CCGTTGCCAATTACGTCGTC	2594-2575	20	59.91	55

[a] Outer forward primer

[b] Inner forward primer

[c] Outer reverse primer

344 **Table 4.** BLASTn of consensus sequences obtained from a pooled cDNA library by HTS

Contig length (nt)	BLAST-Best match	Per Ident (%)	Query cover (%)	E-value	Accession	Reference
Contig 1 (1743)	*Riboviria* sp. isolate H4 (soil metagenome)	74.19	54	6e-167	MN033924	Starr et al. 2019 UC Berkeley, USA
Contig 2 (2793)	*Riboviria* sp. isolate H4 (soil metagenome)	75.12	54	3e-153	MN033924	Starr et al. 2019 UC Berkeley, USA
Contig 3 (6090)	*Plasmopara viticola lesion associated ambiguivirus* 2	74.91	40	0.0	MN551113	Chiapello et al. 2019 Istituto per la Protezione Sostenibile Delle Piante, Itália

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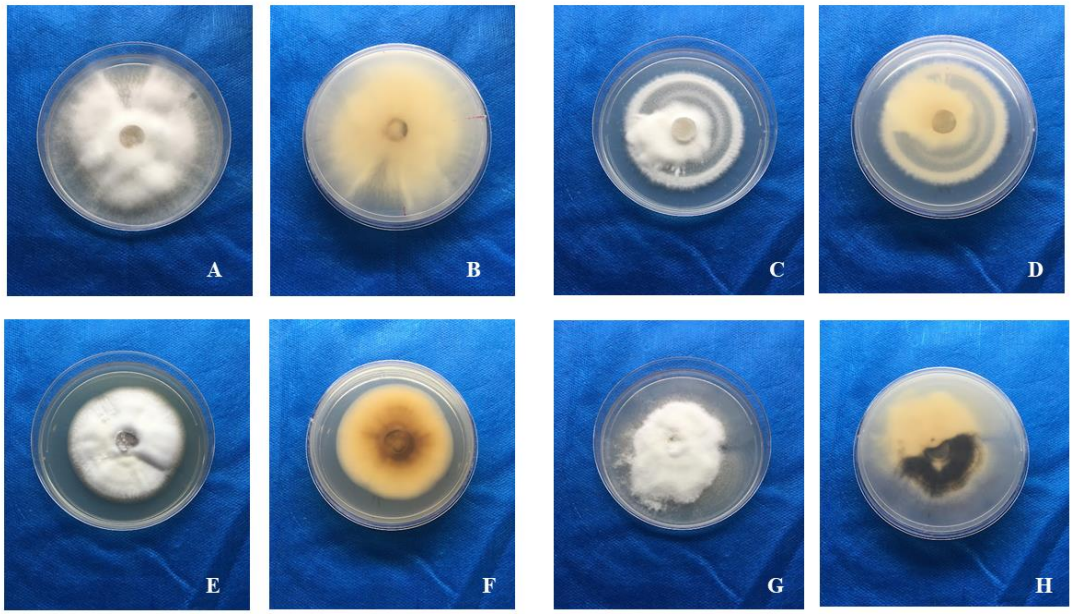

Fig. 1. *Verticillium dahliae* isolates showing typical characteristics and phenotypic alterations in their colony. **A.** Surface side from Vert26 isolate showing irregular growth in mycelial sectors. **B.** Bottom side from Vert26 isolate showing reduced microsclerotia production. **C.** Surface side from Vert04 isolate with loss of aerial hyphae formation. **D.** Bottom side from Vert04 isolate with absence of microsclerotia. **E.** Surface side from Vert158 isolate with milky-white and dense mycelium. **F.** Bottom side from Vert158 isolate showing orange pigmentation. **G.** Surface side from Vert129 isolate showing white and dense mycelium in the center and transparent margins. **H.** Bottom side from Vert129 isolate showing darkening formation of microsclerotia.

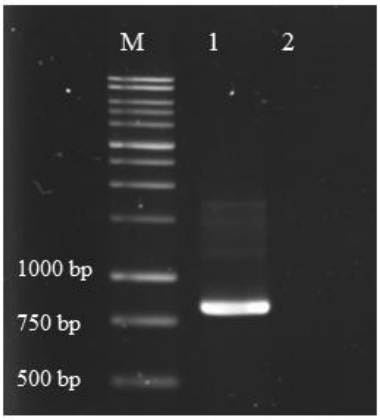

Fig. 2. RT-PCR confirmation of assembled mycovirus contigs from *Verticillium dahliae* library obtained by illumina using specific primers Rib-1144-F/Rib-2056-R (~930 bp). Lane 1: cDNA of putative VdRV-strain CNPH in sampled pool; Lane 2: No template control (NTC); Lane M: 1 Kb molecular weight marker.

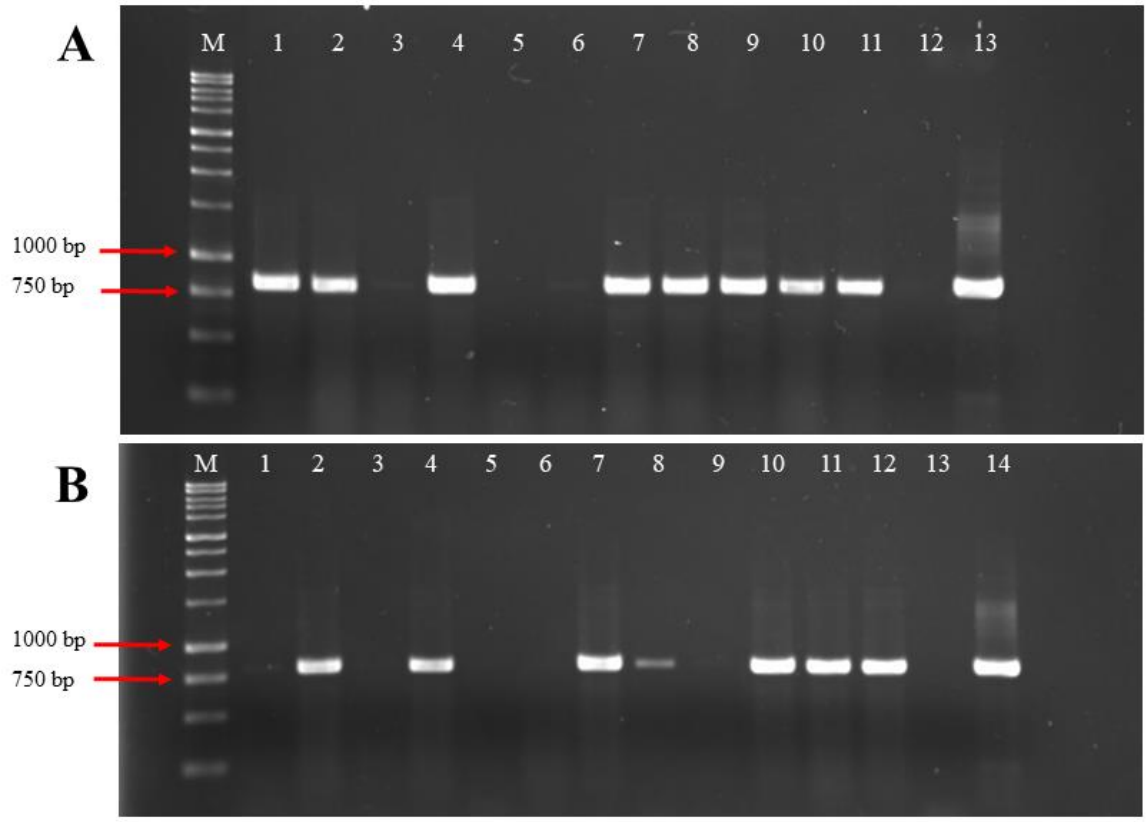

Fig. 3. Gel electrophoresis of amplicons produced by RT-PCR using specific primers Rib-1144-F/Rib-2056-R (~930 bp) for detection of VdRV-strain CNPH. **A.** Lanes 1: Vert34; 2: Vert43; 3: Vert46; 4: Vert59; 5: Vert93; 6: Vert110; 7: Vert121; 8: Vert129; 9: Vert137; 10: Vert32; 11: Vert178; 12: No template control (NTC); 13: cDNA putative VdRV-strain CNPH in sampled pool used as positive control. **B.** Lanes 1: Vert125; 2: Vert142; 3: Vert149; 4: Vert150; 5: Vert158; 6: Vert171; 7: Vert06; 8: Vert22; 9: Vert47; 10: Vert132; 11: Vert151; 12: Vert163; 13: No template control (NTC); 14: cDNA putative VdRV-strain CNPH in sampled pool used as positive control; Lane M: 1 Kb molecular weight marker.

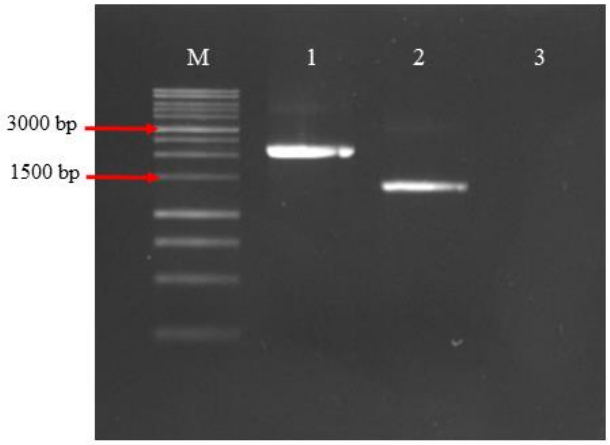

Fig. 4. Gel electrophoresis of amplicons produced by RT-PCR to characterize the putative VdRV-strain CNPH genome. Lanes 1: The largest fragment of the VdRV-strain CNPH genome sequence (2211 bp) obtained with Rib-383F/Rib-2594R; 2: The second middle fragment of the VdRV-strain CNPH genome (1452 bp) obtained with Rib-1143F/ Rib-2594; 3: No template control (NTC); Lane M: 1 Kb molecular weight marker.

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Supplementary material

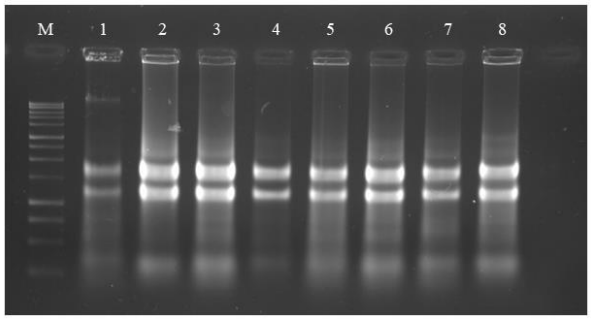

Fig. S1. RNA extraction by Trizol® reagent from a set of 42 field-collected isolates of *V. dahliae*. Lanes 1: Vert34; 2: Vert43; 3: Vert46; 4: Vert59; 5: Vert93; 6: Vert110; 7: Vert121; 8: Vert129. Lane M: 1 Kb molecular weight marker.

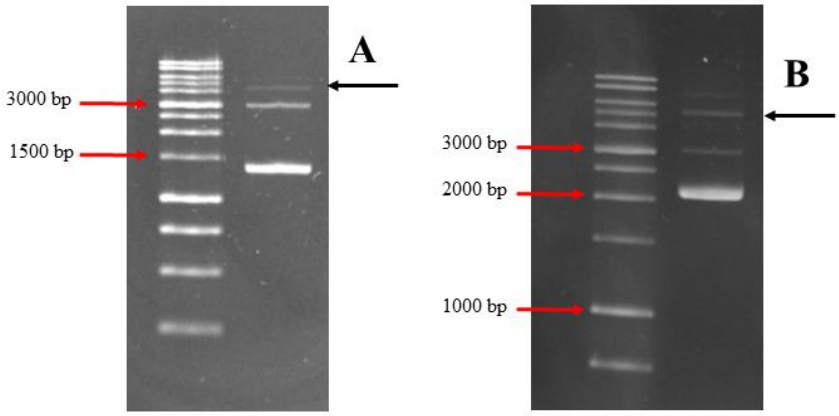

Fig. S2. Gel electrophoresis of DNA fragments corresponding to the almost full-genome of Riboviria-like inserted into pGEM®-T Easy vector. **A.** The clones constructed were digested by *Eco*RI enzyme before sequencing, where the 1452 bp band (insert) and 3.0 kb (vector) band are shown. **B**. Construction of the recombinant plasmid with DNA fragment of 2211 bp in length inserted into 3.0 kb band corresponding to the vector are shown.

Capítulo V

Conclusões gerais

CONCLUSÕES GERAIS

1. O agente causal da murcha de Verticillium em hortaliças coletadas em diferentes regiões do Brasil é *Verticillium dahliae*;

2. Nos ensaios de virulência e através de marcadores moleculares observou-se que a maioria dos isolados de *V. dahliae,* que infectam hortaliças no Brasil, pertencem à raça 2;

3. A maioria dos isolados carrega o idiomorfo *MAT1-1*, enquanto que o idiomorfo *MAT1-2* também está presente em baixa frequência entre os isolados;

4. Através do Sequenciamento de Nova Geração (NGS) foi descoberto um único micovírus infectando a maioria dos isolados avaliados de *V. dahliae,* nomedo aqui *Verticillium dahliae* single stranded RNA virus-strain CNPH (VdRV-strain CNPH);

5. Este é o primeiro estudo realizado no Brasil utilizando uma abordagem de sequenciamento nova geração para a detecção de micovírus em isolados de *V. dahliae*.