Variability and geographical distribution of *Fusarium oxysporum* f. sp. *lycopersici* physiological races and field performance of resistant sources in Brazil

A.M. Gonçalves[1], H. Costa[2], M.E.N. Fonseca[3], L.S. Boiteux[3], C.A. Lopes[3] and A. Reis[3,a]

[1]Universidade de Brasília, Brasilia-DF, Brazil; [2]Incaper, Venda Nova do Imigrante-ES, Brazil; [3]National Center for Vegetable Crops Research (CNPH), Embrapa Vegetables, Brasília-DF, Brazil.

Abstract

Fusarium wilt, caused by three physiological races of *Fusarium oxysporum* f. sp. *lycopersici* (FOL), is a major tomato disease in tropical and subtropical regions of Brazil. A collection of FOL isolates from Brazil was characterized via inoculation assays using a set of *Solanum* (sect. *Lycopersicon*) accessions employed for race discrimination: 'Ponderosa' (susceptible to all races), 'IPA-5' (race 1 resistant due to the *I* locus); 'Floradade' (races 1 and 2 resistant; *I-2* gene), *Solanum pennellii* 'LA 716' (resistant to race 3; *I-3* gene) and 'BHRS 2-3' (race 3 resistant; *I-7* gene introgressed from *S. pennellii* 'PI 414773'). Race-specific molecular marker systems were also employed in these studies. Surveys indicated that races 1 and 2 are widespread in Brazil. However, because of the massive use of hybrids with both *I* and *I-2* genes, no new isolates of these two races have been incorporated into the Brazilian FOL collection since 2002. Race 3 isolates were initially restricted to commercial fields located in mild climate areas encompassing the Atlantic Forest biome in Espírito Santo and Rio de Janeiro states (southeast region). However, recent surveys indicated that FOL race 3 isolates were also present in the states of Minas Gerais (in the continental southeastern region) and Bahia (northeastern region), thus expanding the geographical distribution of this pathogen. The fast and widespread dissemination of race 3 in Brazil suggests its introduction into new producing areas via contaminated propagative material. The severe outbreaks of race 3 were the basis for the substantial replacement of susceptible hybrids by resistant ones (mainly with the *I-3* gene). 'BRS Imigrante' (released by Embrapa) was the first commercial hybrid with the *I-7* gene. 'BRS Imigrante' and the *I-3* gene-carrying hybrids show high levels of resistance in distinct production regions even when the tomato crop is established in heavily infested soils. Multi-race-resistant hybrids are now prevalent in all FOL-infested areas, increasing the selection pressure in favor of new FOL variants.

Keywords: epidemiology, *Fusarium oxysporum* f. sp. *lycopersici*, pathogen variability, resistance, soil-borne disease

INTRODUCTION

Fusarium oxysporum f. sp. *lycopersici* (Sacc.) Snyder & Hansen (FOL) is the causal agent of vascular wilt in tomatoes (*Solanum lycopersicum* L.), which is one of the major diseases affecting this vegetable crop at a global level. The virulence profile of FOL isolates affecting tomato has been grouped into three races, named 1, 2 and 3 (synonymy 0, 1 and 2; Gabe, 1975). These races are defined according to their ability to infect a distinct set of *Solanum. lycopersicum*, *Solanum pimpinellifolium*, and *Solanum pennellii* accessions carrying distinct race-specific resistance factors (Alexander and Tucker, 1945; Cirulli and Alexander, 1966; Grattidge and O'Brien, 1982).

Up to the early 1990s, FOL races 1 and 2 were by far the most widely disseminated

[a]E-mail: ailton.reis@embrapa.br

pathogen variants, being present in most of the tomato-producing areas on all continents. However, the geographical distribution of FOL race 3 isolates has expanded in recent years, being already reported in Australia (Grattidge and O'Brien, 1982), Florida (Volin and Jones, 1982), California (Davis et al., 1988; Cai et al., 2003), New Zealand, United Kingdom, Venezuela, Mexico (Valenzuela-Ureta et al., 1996), Korea (Choi et al., 2013), South Africa (Jacobs et al., 2013), and Chile (Sepúlveda-Chavera et al., 2014). All three FOL races are now present in Brazil (Barboza et al., 2013). However, FOL race 3 isolates display a geographically limited distribution, being restricted to mild climate areas of the Atlantic Forest biome of the southeast region, encompassing producing areas of Espírito Santo and Rio de Janeiro states (Reis et al., 2005; Reis and Boiteux, 2007). A more recent report indicates that race 3 isolates are also present in the southern region of Bahia state (Barboza et al., 2013).

Chemical and cultural measures for controlling Fusarium wilt in tomato are expensive and not effective in most situations (Jones and Woltz, 1981). So far, the most efficient strategy for Fusarium wilt control in tomato has been the employment of cultivars with genetic resistance. Tomato breeding programs have historically worked with the introgression of genes from wild species, aiming to control *Fusarium*-induced diseases (Boiteux, 2012). Four dominant resistance factors (*I*, *I-2*, *I-3*, and *I-7*) have been genetically characterized in *Solanum* (section *Lycopersicon*) accessions and introgressed into commercial cultivars (Huang and Lindhout, 1997; Hemming et al., 2004; Catanzariti et al., 2015; Gonzalez-Cendales et al., 2016).

The *I* gene (which confers exclusive resistance to race 1) was introgressed from accessions of *S. pimpinellifolium*. The *I-2* gene (which confers resistance to race 2 and possibly also to race 1) was also introgressed from *S. pimpinellifolium* ('PI 126915'). Recent molecular analyses of a set of *S. pimpinellifolium* accessions and cultivars showed that domesticated and wild tomatoes evolved as a species complex with intense hybridization and exchange of genome segments (Zuriaga et al., 2009a; Bauchet and Causse, 2012). The interspecific variation and the absence of crossing barriers between *S. pimpinellifolium* and *S. lycopersicum* indicates a close phylogenetic relationship between the two species and shows that *S. pimpinellifolium* has been a significant source of genetic factors for many commercially important characteristics for the cultivated tomato (Zuriaga et al., 2009b). On the other hand, resistance to race 3 isolates was introgressed from accessions of the wild species *S. pennellii.* The *I-3* gene (which confers resistance to race 3) was introgressed from the accession 'LA 716'. Another gene that confers resistance to race 3 was named *I-7*, and it was introgressed from *S. pennellii* 'PI 414773' (Lim et al., 2006; Takken and Rep, 2010; Gonzalez-Cendales et al., 2016). So far, cloned genomic segments associated with resistance to FOL are the *I-2* gene (Simons et al., 1998), the *I-3* gene (Catanzariti et al., 2015) and, more recently, the *I-7* gene (Gonzalez-Cendales et al., 2016). This genomic information will allow the development of multiplex functional marker systems for monitoring the incorporation of these genetic factors into elite tomato lines.

In Brazil, work dealing with the characterization of FOL isolates is limited only to the determination of the virulence profile (i.e., physiological races) after inoculation in a set of race-differential *Solanum* (section *Lycopersicon*) accessions carrying one of the major resistance genes (Reis et al., 2005; Reis and Boiteux, 2007). In this scenario, the objective of the present work was to carry out an extensive variability analysis of Brazilian FOL isolates and to connect this information with the geographical distribution of the physiological races. A brief summary will also describe the field performance of distinct tomato resistance sources in Brazil.

MATERIAL AND METHODS

Collection of FOL isolates

The FOL isolates used in the present study belong to the collection of plant pathogenic fungi of the Plant Pathology Laboratory at the National Center for Vegetable Crops Research (CNPH) – EMBRAPA. Isolates of distinct formae speciales (*F. oxysporum* f. sp. *vasinfectum*, *F.*

oxysporum f. sp. *cubense*, *F. oxysporum* f. sp. *cepae*, and *F. oxysporum* f. sp. *lactucae*) were also included as controls in the molecular marker analyses. Isolates were stored at 10°C using the method of Castellani (1963). Recovery of the stored isolates was done by transferring mycelia to potato dextrose agar + tetracycline (50 µg mL^{-1}) under standard conditions. Morphological and cultural characteristics of the fungus were observed and compared to literature descriptions (Nelson et al., 1983; Leslie and Summerell, 2006), aiming to confirm species identity.

Reaction of the differential accessions to FOL isolates

The virulence profile and race identity of isolates were investigated by the root-dipping inoculation technique. The following differential tomato accessions were employed: 'Ponderosa' (susceptible to all races), 'IPA-5' (resistant to race 1/locus *I*), 'Floradade' (resistant to races 1 and 2/locus *I-2*), 'BHRS-2,3' (resistant to the three races/locus *I-7*) and *S. pennellii* 'LA 716' (resistant to the three races/locus *I-3*). Conidia were produced in potato dextrose under standard conditions (12 h light and 25±2°C) for 7 days. The suspension was filtered and adjusted to 1×10^6 spores mL^{-1}. Seeds of the differential accessions were sown in Styrofoam trays with 128 cells, filled with sterile substrate (Plantmax®). Plants with the first two pairs of true leaves fully open (about 21 days after planting) were removed from the tray cells with a gentle jet of water to preserve root integrity. The apical portion of the root system (about 2 cm) was removed and then dipped into the spore suspension (for 3 min). The plantlets were then transplanted to 1.5-kg plastic pots with sterile soil and maintained in the same greenhouse. After transplanting, 5 mL of the suspension was added to the crown area of each plantlet. The experimental plots were composed of three pots with three plants each. Disease reaction was assessed 21 days after inoculation using an ordinal scale (1 to 5): 1 = plant free of symptoms; 2 = plant without wilt symptoms but presenting conspicuous vascular browning; 3 = plants showing vascular browning symptoms and wilt symptoms but without leaf yellowing; 4 = severe wilting associated with the presence of foliar necrosis and chlorosis; 5 = dead plant (Santos, 1999). The isolates were classified as pathogenic only when they were able to induce disease symptoms. The classification of races was made according to the response observed in each differential accession. At the end of the evaluation, the fungus was reisolated from symptomatic plants and PCR was carried out to confirm the identification.

Race identification using race-specific molecular markers

Additional analyses were carried out using a molecular marker system developed for race-specific identification of FOL and *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) isolates using the set of race-specific and species-specific primers (Uni, Sp13, Sp23, and Sprl) and PCR conditions developed by Hirano and Arie (2006) using information derived from the rDNA-IGS genomic regions of *Fusarium* isolates.

RESULTS AND DISCUSSION

All isolates generated colonies with conidia and mycelium with morphological features typical of *F. oxysporum* (Nelson et al., 1983; Leslie and Summerell, 2006). All FOL isolates collected until the year 2011 had been characterized previously as to race through inoculation in accessions and differential cultivars (Urben, 1994; Reis et al., 2005, 2006; Reis and Boiteux, 2007; Barboza et al., 2013). The remaining isolates were characterized in this study. Isolates collected up to 2003 were identified as belonging exclusively to races 1 and 2. In turn, isolates collected after this period were identified as belonging exclusively to race 3, mostly from the states of Espirito Santo and Rio de Janeiro (Reis et al., 2006). Total genomic DNA extracted from this collection of isolates was used to confirm the race using the race-specific molecular marker system developed by Hirano and Arie (2006). Perfect discrimination was observed for the race 3 isolates from race 1 and race 2 isolates. Somewhat surprisingly, this molecular marker system was not efficient to discriminate any of the Brazilian race 1 and 2 isolates, with all of them displaying a race 1-specific amplicon pattern. The set of race-specific primers (Uni, Sp13, Sp23, and Sprl) was not effective in

discriminating all FOL races, being reliable only to discriminate Brazilian isolates of FOL race 3 and FORL isolates. Therefore, the development and/or validation of new, distinct race-specific markers systems is necessary in order to discriminate FOL race 1 from race 2 isolates under Brazilian conditions.

In Brazil, the first report of a Fusarium wilt race 1 isolate was from São Paulo state (Arruda, 1941). The emergence of new races occurs in general as a result of the intensive use of cultivars with resistance to existing races of the pathogen, which may not have happened with race 2 in Brazil. Race 2 was initially described in São Paulo in production fields that had never been planted with seedlings originating from other areas, eliminating the possibility of introduction from another location and reinforcing the idea of an endemic source of isolates. Thus, the unilateral selection opportunity for this race had not occurred because the cultivar grown in the region was 'Santa Cruz' (susceptible to all races of FOL). Two hypotheses were proposed to explain the occurrence of race 2 in Brazil: (1) high frequency of mutation from race 1 to race 2; and (2) this mutation occurred in the direction of increasing the virulence profile and aggressiveness of isolates previously existing in tomato-producing regions (Tokeshi and Galli, 1966). Analysis using the race-specific molecular marker system developed by Hirano and Arie (2006) with Brazilian race 1 and race 2 isolates supports both hypotheses.

Race 3 isolates were previously reported in 1966 in São Paulo state, Brazil (Tokeshi et al., 1966). However, subsequent studies have shown that the isolates were in fact race 2 isolates, because the cultivar 'Cast-M-wd' employed is not an adequate FOL race differential (Tokeshi and Noguez, 1974). Because of this initial misclassification, there are some international reports indicating an ancient presence of race 3 in Brazil (Jones and Woltz, 1981). So, the first formal registration of FOL isolates from race 3 in Brazil was done in the state of Espírito Santo (Reis et al., 2005) and, just after, in the state of Rio de Janeiro, in race 1- and 2-resistant hybrids 'Carmen', 'Giovana', and 'Alambra' (Reis and Boiteux, 2007). After these first two records, the occurrence of race 3 remained geographically restricted to these two tomato-producing regions. All pathogenic isolates of *Fusarium* received during the timeframe of this work (2012-2015) were identified as belonging to FOL race 3. Furthermore, race 3 isolates were detected infecting tomato plants in the state of Bahia (Barboza et al., 2013), confirming the expansion of this variant of the pathogen also to the northeast region of Brazil. Subsequently, new isolates (collected between 2012 and 2015 in the region of Zona da Mata, Minas Gerais) were pathogenic to all differentials, except to accession 'BHRS2-3', also demonstrating that they belong to race 3. This was the first formal record of race 3 in the state of Minas Gerais (Gonçalves et al., 2013), highlighting the rapid progress of this variant to different producing regions in Brazil. Recent field surveys indicated that race 3 is not yet present in the processing tomato-growing regions located in central Brazil. However, samples received in early 2015 in commercial fields from the state of Goiás and the Federal District also proved to be pathogenic to all differentiating accessions except 'BHRS2-3', a characteristic of race 3 (unpublished results). These results show that, in recent years, the geographical expansion of FOL race 3 in Brazil has been very fast; in 3 years, this race has been described in four new states in the country.

In summary, inoculation tests in the set of differential accessions allowed three isolates of race 1, 24 isolates of race 2 and 77 isolates of race 3 to be cataloged, collected over the past three decades and sampling some of the major tomato-producing regions in the country. The widespread dissemination of race 3 in Brazil and around the world suggests its introduction into new tomato-producing areas via contaminated propagative material (Reis et al., 2005). Severe outbreaks of race 3 seem to be the basis for the substantial replacement of susceptible hybrids by resistant ones (mainly with the *I-3* gene). 'BRS Imigrante' (released by Embrapa) was the first commercial hybrid with the *I-7* gene. 'BRS Imigrante' as well as the *I-3*-gene-carrying hybrids display high levels of resistance in distinct production regions even when the crop is established in heavily infested soils. Multi-race-resistant hybrids are now prevalent in all FOL-infested areas, which could increase the selection pressure in favor of new FOL variants. Evaluation of commercial seed lots imported into Brazil for contamination with the pathogen may also be necessary in order to avoid

nationwide spread of this serious disease.

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