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Selection of tomato accessions resistant to Verticilium wilt[1]

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ABSTRACT

Vascular wilting in tomato plants is an important disease caused by soil-inhabiting pathogens, especially *Verticillium dahliae*, which results in significant production losses. Control measures against this disease are difficult to implement due to intrinsic pathogen characteristics, such as high adaptability to the subterranean environment, in association with the host, and development of resistance structures that remain viable in the soil for long periods. The introgression of genes that express resistance is the main control measure and requires a continuous characterization program of resistant accessions. This study aimed at identifying tomato (*Solanum lycopersicum*) accessions resistant to *V. dahlia*, by using the phenotypic and genotypic methods. The reaction of 33 tomato accessions to different *V. dahliae* isolates was reinforced by molecular analysis, through markers linked to Ve resistance genes. The combination of bioassays and specific molecular markers showed a high correlation (94.3 %), with the selection of 25 accessions resistant to *V. dahliae*.

KEYWORDS: *Verticillium dahliae*; *Solanum lycopersicum*; genetic markers; disease resistance.

RESUMO

Seleção de acessos de tomateiro resistentes à murcha-de-verticílio

Murchas vasculares em tomateiro constituem importante grupo de doenças causadas por patógenos habitantes do solo, com destaque para *Verticillium dahliae*, que ocasiona perdas significativas na produção. A dificuldade de controle dessa doença advém de características intrínsecas do patógeno, como alta adaptabilidade ao ambiente subterrâneo, em associação com o hospedeiro, e produção de estruturas de resistência que permanecem viáveis no solo por longo tempo. A introgressão de genes que expressam resistência destaca-se como principal medida de controle, o que requer um programa contínuo de caracterização de acessos resistentes. Objetivou-se avaliar e identificar acessos de tomateiro (*Solanum lycopersicum*) resistentes a *V. dahliae*, pelos métodos fenotípico e genotípico. A reação de 33 acessos de tomateiro a diferentes isolados de *V. dahliae* foi corroborada com análises empregando-se marcadores moleculares ligados aos genes de resistência Ve. A combinação de bioensaios e marcadores moleculares específicos mostrou elevada correlação (94,3 %), com a seleção de 25 acessos resistentes a *V. dahliae*.

PALAVRAS-CHAVE: *Verticillium dahliae*; *Solanum lycopersicum*; marcadores genéticos; resistência a doenças.

INTRODUCTION

Verticillium wilt, caused by variants of the *Verticillium dahliae* fungus, is one of the most destructive tomato diseases, in both salad type and industrial cultivars, especially in mild climates (Miranda et al. 2010). Due to the intrinsic characteristics of this microorganism, such as its wide range of host plants, inherent production of resistance structures (microsclerotia) and ineffectiveness of chemical control, the genetic control focusing on the use of resistant cultivars has been the most effective and feasible measure to suppress Verticillium wilt (Fradin et al. 2009).

Verticillium wilt resistance derives from the Ve gene expression, comprising highly race-specific interaction. This resistance is affected by a complex locus, consisting of two genes (Ve-1 and Ve-2) that can recognize and express resistance to *V. dahliae* and other *Verticillium* species in different hosts (Kawchuk et al. 2001, Fradin et al. 2009).

The Ve-1 gene was first identified in 1932, in *Solanum lycopersicum* var. *cerasifore* 'Peru Wild' (Schaible et al. 1951, Acciarri et al. 2007). It was mapped on chromosome 9, closely linked to the RFLP marker GP39 (Diwan et al. 1999). The introgression of this gene usually occurs through the use of resistant genotypes carrying the Ve gene (Kawchuck et al. 2001).

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The expression of Ve-1, but not Ve-2, triggers the *V. dahliae* race 1 resistance response mediated by receptor proteins with leucine-rich regions (LRR) and RLP (receptor-like kinase) (Fradin et al. 2009). These protein receptors, which belong to a protein class involved in race-specific resistance, act in basal defense and non-host resistance (Tör et al. 2009, Wu et al. 2016).

Due to the wide distribution of *V. dahliae* race 1 in countries where tomato is grown, it is important to evaluate and select accessions carrying Ve-1, since the main control method is based on introgression of dominant and specific resistance genes that can integrate the gene pyramiding process.

A more efficient pyramiding process in elite genotypes requires the combination of bioassay analysis and the use of selection assisted by molecular markers linked to effective resistance genes. Specific molecular markers for Ve-1 were already identified (Kawchuk et al. 1998).

The present study aimed at using controlled inoculations to assess the reaction of different tomato accessions to different *V. dahliae* race 1 isolates and correlate genotype responses with a series of analyses deploying molecular markers linked to the Ve-1 gene.

MATERIAL AND METHODS

Bioassays with tomato accessions were conducted in a greenhouse, at the Universidade Federal de Goiás, Goiás State, Brazil, in 2013 and 2014, with an average temperature of 24 ºC + 6 ºC and relative humidity of 60-70 %.

The tomato collection to be evaluated for reaction against *V. dahliae* isolates was formed by three hybrids, three commercial varieties and 27 F12 lines (derived from the Vivati Plant Breeding Ltd. breeding program, Rio Verde, Goiás State). The race differentiation accessions employed were 'Ponderosa' (susceptible to race 1) (Reis et al. 2007) and 'Floradade' (resistant to race 1) (Santos 1999).

In order to identify and prove the stability of tomato accessions to *V. dahliae* race 1, two bioassays were carried out at different times, using two aggressive fungal isolates (Vet-668 collected in Campinas, São Paulo State, and Vet-152 collected in Viçosa, Minas Gerais State, both in Brazil). The first bioassay, in which the accessions were inoculated with the Vet-152 isolate, was followed by a second bioassay, where the same previously inoculated accessions were inoculated with the Vet-668 isolate.

For inoculum preparation, three mycelia discs (5 mm diameter) from each isolate, cultured on potato-dextrose-agar, were transferred to Erlenmeyer flasks containing 250 mL of a potato-dextrose autoclaved medium. After 15 days of growth in an automatic shaker at 23 ºC in the dark, the suspensions of each inoculum were homogenized and filtered, and the concentration adjusted to 1 x 10^6 microconidia mL^{-1}, using a Neubauer chamber.

Inoculation was performed using the root cutting method (Reis et al. 2007). At 20 days after emergence, tomato seedlings grown under greenhouse conditions on Plantmax® substrate were removed from polystyrene trays and submitted to root washing to remove substrate, and the apical region was cut about 2 cm above the root tip. The root cuttings were then immersed for 3 min in the spore suspension until reaching the base of the stem. After inoculation, the cuttings were transplanted into 1.0 L polypropylene pots containing sandy-loamy autoclaved soil and 5 mL of the spore suspension were added to the seedling stem.

The negative control consisted of plants with cut off roots immersed in a suspension without the presence of conidia. Disease severity was assessed at 30 days after inoculation, using the following scale from 1 to 5: 1 = no symptoms; 2 = no wilting and exhibiting slight vascular discoloration limited to the plant stem; 3 = vascular discoloration beyond the stem and/or wilting, "V" shaped necrosis or leaf yellowing; 4 = severe wilting associated with the presence of chlorosis and necrosis; 5 = death. The average disease severity in each genotype was used to classify them into two reaction classes, where accessions with average scores between 1 and 2 were considered resistant and those above 2 were considered susceptible. The experimental design was completely randomized, with 35 treatments (tomato accessions) and four replications, and each plot consisting of a pot with three plants.

For the extraction of genomic DNA, 0.2 g of tissue were collected from different accessions (20 days after emergence). DNA was purified using the 2X CTAB method with modifications (Boiteux et al. 1999) and quantified in a spectrophotometer. Genomic DNA was used as a template in detection assays based on PCR markers closely linked to the Ve-1 gene, which expresses resistance to *V. dahliae*

race 1. For PCR, 5.0 µL of Premix 2X (EmeraldAmp® GT PCR Master Mix, Takara Bio Inc., Otsu, Shiga, Japan), 1.0 µL of DNA at 100 ng μL^{-1}, 0.2 µL of each primer (2.5 pM) and Milli-Q water to 10 µL final volume were used. The primer pairs used to detect this marker were VE-B (5'-CCA-TGA-ACA-GAT-GTG-ACT-TGT-GTG-3') and VE-C (5'-AAG-TTT-CTT-ATT-TTT-CCT-TCT-CC-3') (Kawchuk et al. 1998). The PCR protocol was executed in a biocycler thermocycler (Biosystem®, Curitiba, Paraná State, Brazil) and consisted of initial denaturation at 94 ºC for 1 min, followed by 30 cycles of denaturation at 94 ºC for 30 sec, annealing of primers at 57 ºC for 1 min, extension at 72 ºC for 2 min, and a final extension step at 72 ºC for 10 min. All PCR products were separated on pre-melted 1.5 % agarose gel immersed in TBE buffer with GelRed (Invitrogen) at 80 V and viewed with a UV transilluminator. The amplification of an 800 bp fragment indicated that the tomato genotype contained the Ve-1 gene (Kawchuk et al. 1998).

RESULTS AND DISCUSSION

In the bioassays with the two *V. dahliae* race 1 isolates against 35 accessions, 28 exhibited resistance responses (including the resistant control 'Floradade'), 5 were susceptible (including the susceptible control 'Ponderosa') and 2 lines ranged from resistant to susceptible, when inoculated with different isolates (Figure 1). The predominance of tomato accessions resistant to *V. dahliae* in *S. lycopersicum* has been already reported by several authors (Acciarri et al. 2007, Miranda et al. 2010), due to the constant incorporation of the resistance Ve-1 gene in commercial lines of different breeding programs.

Some accessions, such as PX-111 and PX-112, showed partial resistance, while others, such as PX-118, PX-121 and ROME-VF, exhibited race-specific resistance. The PX-116 and PX-123 accessions displayed isolate-specific responses ranging from resistant, when inoculated with the Vet-668 isolate, to susceptible, when inoculated with Vet-157. The possibility of such phenotypic instability may be attributed, in terms of the pathogen, to the existence of a cryptic pathogenic variability within isolates of the same physiological race and an allelic variation and/or different levels of expression of the Ve gene in the host plant (O'Garro & Clarkson 1988, Cherrab et al. 2000, Tzima et al. 2011).

Although the expression of resistance to *V. dahliae* is considered dominant, incomplete or partial resistance has been reported in tomato, mainly due to germplasm allelic diversity (Miranda et al. 2010), as well as the co-evolution of race 1 with varieties that express resistance to the corresponding physiological race. The widespread use of the genetic factor (Ve gene), that prevailed for decades in the fields of tomato-producing regions, contributed to the emergence of *V. dahliae* race 1 strains that can infect cultivars containing the Ve gene. However, a limited colonization is observed in these cases. Little growth, restricted to the base of the vascular vessels in resistant accessions without symptom expression, has been observed (O'Garro & Clarkson 1988, Heinz et al. 1998). A

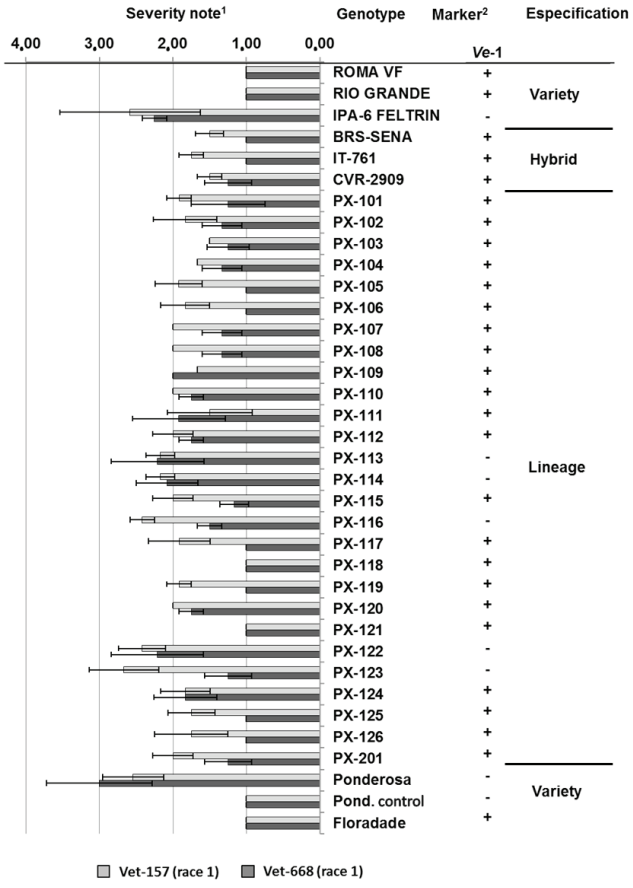

Figure 1. Phenotypic reaction of tomato accessions to *Verticillium dahliae* race 1 isolates, evaluated at 30 days after inoculation. [1] Representation of the averages and standard error of disease reaction classes, where 1.0-2.0 = resistant and 2.1-5.0 = susceptible. [2] Molecular characterization with VE markers used to detect the Ve-1 gene, which control resistance to race 1 of the pathogen.

similar evidence was found in other pathosystems (Carrer Filho et al. 2015).

Apart from possible instability of resistance, due to isolate aggressiveness and modulation of resistance gene expression, these results may be explained by the fact that a broad spectrum resistance may be associated with a set of loci that are closely linked to chromosome 9 (Kawchuk et al. 2001, Kuklev et al. 2009). Another possibility for this phenotypic instability may be the influence of genes of small effect in the host plant, responsible for modulating the expression of resistance. These small effect genes may have been lost during the introgression process through backcrossing, because of the influence of environmental components (Acciarri et al. 2007). At any rate, this instability is undesirable and may lead to potential 'resistance breaking' on the Ve-1 gene carrier.

Despite the instability observed in some accessions, the high frequency of lines showing consistent resistance against *V. dahliae* suggests that the phenotypic expression of the gene associated with Ve-1 is the predominant response.

The results of the bioassays were supported by specific molecular markers for the Ve-1 gene (Kawchuk et al. 1998). The marker is one amplified DNA fragment with an approximate size of 800 bp, as observed in resistant controls for the corresponding gene present in tomato (Figures 1 and 2).

Analysis using molecular markers corroborated the results of the corresponding bioassays (Figures 1 and 2). Similar results were presented by Arens et al. (2010), using various markers to identify resistance to several pathogens in tomato plants, observing that, in 98 % of the time, the molecular assay showed identical results to those of its bioassay counterpart. For Ve-1, the same authors found approximately 95.5 % of compatibility between molecular and bioassay results, when other types of primers were used to detect this marker (Kawchuk et al. 2001). They argue that the genetic background, where the resistance genes were incorporated, may play an important role in the expression of resistance.

Due to the existence of genetic variability among isolates of the same phenotypic race, combined with the polyphagous nature of *V. dahliae* and the allelic diversity of Ve, the development of cultivars that exhibit a broad spectrum of resistance and dominance against race 1 of this pathogen is of utmost importance. Thus, it is necessary to emphasize the importance of combining methodological parameters to evaluate and select accessions resistant to Verticillium wilt in tomato.

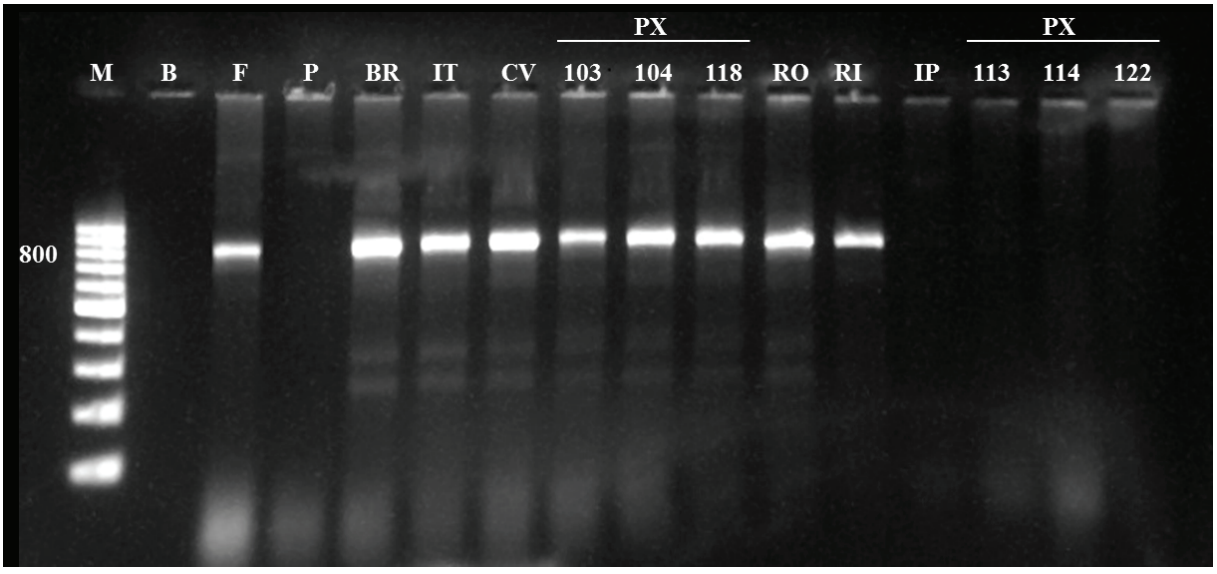

Figure 2. Amplification profile of the VE marker in tomato (*Solanum lycopersicum*) accessions, using PCR on 1.5 % agarose gel in TBE solution. Accessions resistant to *Verticillium dahliae* race 1, such as the 'Floradade' variety (F), show an 800 bp amplicon, which is absent in the 'Ponderosa' susceptible control (P). The gel illustrates the amplification patterns of BRS Sena (BR), IT-761 (IT) and CVR-2909 (CV) hybrids, from the ROMA VF (RO), RIO GRANDE (RI) and IPA-6 FELTRIN (IP) varieties and the PX lines. Marker (M) 100 bp ladder and reaction control with water (B).

Molecular evaluations, highlighted in this study, allow the detection of genes that express resistance without environmental, physiological or pathogenic aggression interference, especially when used in the first cycles of mass selection. Implementing the bioassay using two geographically distinct isolates helps to evaluate the behavior of the introduced trait inheritance in selected materials. This method is efficient for the identification of resistant materials against *V. dahliae*.

CONCLUSIONS

1. Analyses combining bioassays and specific molecular markers for the Ve-1 resistance gene showed consistent results to most of the tested accessions (94.3 %);
2. Most of the varieties, hybrids and lines evaluated carry the Ve-1 gene, which provides resistance against *V. dahliae*.

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