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Molecular identification of the causal agent of bacterial wilt in tomato plants in southwestern Paraná

Identificação molecular do agente causal da murcha bacteriana em plantas de tomateiro no sudoeste do Paraná

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Highlights

All 26 isolates were confirmed as *Ralstonia solanacearum* phylotype II.
The isolates are genetically similar despite belonging to different regions.
Eight groups were formed by BOX-PCR (similarity of 0.63).

Abstract

Bacterial wilt, caused by phytopathogenic species of the genus *Ralstonia,* is one of the main diseases affecting the tomato crop. The *Ralstonia solanacearum* species complex occurs as a result of variants being widely diverse in terms of adaptation to different climatic conditions, host variations, and aggressiveness, which complicate disease control recommendations. In this study, we employed molecular methods to analyze 26 *R. solanacearum* isolates collected from tomato plants cultivated in the southwest region of Paraná, Brazil. Isolates were obtained from plants exhibiting wilt symptoms in a protected cultivation system and in an open field. The specific primers 759/760 confirmed the isolates as part of the *Ralstonia solanacearum* complex, and Nmult primers were used to identify the phylotype. Variability analysis using BOX-PCR with the BOX-A1R primer on 19 isolates revealed molecular diversity. All 26 isolates were confirmed as *Ralstonia solanacearum,* belonging to phylotype II. Comparison of genomic DNA band patterns amplified by BOX-PCR indicated molecular variability, forming eight groups at a similarity level of

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0.63. These results confirm the prevalence of *R. solanacearum* phylotype II in southwestern Paraná. This information aids decision-making in disease management and contributes to breeding efforts aiming at the development of resistant cultivars.
Key words: Genetic variability. *Ralstonia solanacearum. Solanum lycopersicum.*

Resumo

A murcha bacteriana, causada por espécies fitopatogênicas do gênero *Ralstonia,* é uma das principais doenças do tomateiro. O complexo de espécies de *Ralstonia solanacearum* ocorre devido a variantes com alta diversidade em termos de adaptação a diferentes condições climáticas, variabilidade de hospedeiros e agressividade, características que interferem na recomendação para o controle da doença. Neste estudo foram analisados por métodos moleculares 26 isolados de *R. solanacearum* isolados de plantas de tomateiro cultivadas na região sudoeste do Paraná. Os isolados foram obtidos de plantas com sintomas de murcha cultivadas em sistema de cultivo protegido e campo aberto. Os iniciadores específicos 759/760 foram usados para confirmar que os isolados pertenciam ao complexo específico *Ralstonia solanacearum* e os iniciadores Nmult foram usados para identificar o filotipo. A análise de variabilidade foi realizada por BOX-PCR com o primer BOX-A1R para 19 isolados. Os vinte e seis isolados foram confirmados como *Ralstonia solanacearum* e pertencentes ao filotipo II. A comparação entre os padrões de bandas do DNA genômico amplificado por BOX-PCR demonstrou variabilidade molecular com a formação de oito grupos ao nível de 0,63 de similaridade. Esses resultados confirmam a predominância de *R. solanacearum* filotipo II no sudoeste do Paraná e auxiliam na tomada de decisões relacionadas ao manejo da doença, bem como no melhoramento genético para obtenção de cultivares resistentes.
Palavras-chave: Variabilidade genética. *Ralstonia solanacearu. Solanum lycopersicum.*

Introduction

Tomatoes rank among the most commercially important vegetable crops globally (Salim et al., 2020). Bacterial wilt, attributed to *Ralstonia solanacearum* (phylotype II) and *R. pseudosolanacearum* (phylotype I) (Lopes & Rossato, 2018; Albuquerque et al., 2021; Santiago et al., 2017; Fonseca et al., 2013; A. L. Garcia et al., 2013; Rodrigues et al., 2012), results in substantial losses in tomato production. The primary symptom is plant wilting, caused by the production of extracellular polysaccharides that obstruct xylem vessels, compromising water and nutrient transport to the plant shoots (R. O. Garcia et al., 2019).

Ralstonia solanacearum forms a species complex, meaning it encompasses strains with distinct characteristics such as metabolic variations, geographical origins, host diversity, and optimal environmental conditions for infection. This complexity poses challenges for studying *R. solanacearum,* particularly for less experienced researchers in the field (R. O. Garcia et al., 2019). Originally classified into Phylotypes I, II, III, and IV, the *R. solanacearum* species complex was reorganized by Safni et al. (2014) into three distinct species: *R.*

pseudosolanacearum (Phylotypes I and III), *R. solanacearum* (Phylotype II), and R. syzygii, with three subspecies including Phylotype IV isolates.

Brazil, considered a putative center of origin for some *R. solanacearum* variants, harbors a diverse range of races, biovars, phylotypes, and sequevars of the pathogen. At present, races 1, 2, and 3 and biovars 1, 2A, 2T, and 3 have been identified in Brazil (Santiago et al., 2017; Lopes & Rossato, 2018). Among the reported phylotypes, besides phylotype II, native to the Americas, phylotype I has been registered only with sequevar 18, in various Brazilian states (Lopes & Rossato, 2018). Identifying *Ralstonia* variability in Brazilian regions is essential for understanding cultivar resistance, decision-making, and pathogen control strategies. Despite research efforts to develop resistant tomato cultivars, bacterial wilt remains a significant challenge in production due to the considerable pathogenic variability within the species present in Brazilian soils (Lopes & Rossato, 2018).

The molecular analysis of variability in *Ralstonia* spp. allows the identification of genetic differences between strains, contributing to a deeper understanding of diversity and facilitating the search for more effective control measures. Additionally, this approach offers a more precise and detailed assessment compared to methodologies focused on phenotypic traits (Santiago et al., 2017). In this study, molecular methods were employed to identify the causative agent of bacterial wilt in tomato plants in the southwest region of Paraná. Our objectives included identifying the species and phylotype of the pathogen and evaluating its genetic variability.

Samples were collected from tomato plants showing symptoms of bacterial wilt in production areas across the southwest region of Paraná (municipalities of Francisco Beltrão, Itapejara d'Oeste, Mariópolis, Palmas, Pato Branco, Renascença, and Verê). From 46 isolates obtained from these samples, *Ralstonia solanacearum* was confirmed through the cup test (Lopes & Rosatto, 2013). The stem portion taken from the cup test-positive plants was washed with detergent and running water to remove soil particles and contaminants. Afterward, the material was dried on paper towels and the outermost part of the stem was scraped in a laminar flow chamber using a sterilized scalpel, immersed in ethanol 70%, and flamed lightly. A 1-cm portion from one end of the stem was then placed in a 15 x 150 mm test tube containing 3 mL of autoclaved water, and bacterial cells were allowed to settle for 15 min until the water became cloudy. Subsequently, using a platinum loop, approximately 10 µL of the suspension was streaked in Petri dishes with Kelman medium without triphenyl tretrazolium chloride (Kelman, 1954) and incubated at 28 ºC for 48 h. Typical colonies were subcultured to Petri dishes containing the same medium under the same conditions.

DNA extraction from bacterial isolates followed the protocol described by Mahuku (2004). To confirm the species of the genus *Ralstonia,* oligonucleotide primers 759/760 (Opina et al., 1997) were used (759 5´GTCGCC GTCAAATCACTTTCC3´ and 760 5´GTCGCCGTCAGCAATGCGGAATCG3´). For phylotype identification, the Nmult primers (Fegan & Prior, 2005) were employed (Nmult: 21:1F5´-CGTTGATGAGGCGCGCAATTT-3´, Nmult: 21:2F5´-AAGTTATGGACGGTGGAAGTC-3', Nmult:

23:AF5´-ATTACSAGAGCAATCGAAAGATT-3', Nmult: 22:InF5´ATTGCCAAGACGAGA GAAGTA-3', Nmult: 22:RR 5´TCGCTT GACCCTATAACGAGT A-3').

Once the species and phylotype were identified, the genetic variability of the isolates was investigated using the BOX-A1R primer (5´-CTACGGCAAGGCGACGCTGACG-3´), as described by Versalovic et al. (1994). A dendrogram with a distance matrix is constructed through clustering using the UPGMA method (Unweighted Pair Group Method with Arithmetic Mean) based on the Jaccard similarity coefficient (Jaccard, 1901).

The pair of primers (759/760) is employed for identifying the species *R. solanacearum* (senso latu). This enables the visualization of 280-bp fragments from 26 isolates in agarose gels (Figure 1 and Table S1) from Francisco Beltrão (1), Itapejara d'Oeste (8), Renascença (8), Palmas (4), Pato Branco (2), Mariópolis (1), and Verê (2). This result confirms that the isolates belong to the *R. solanacearum* species complex and align with the classification proposed by Fegan & Prior (2005). Previous investigations on the Brazilian variability of *R. solanacearum* have demonstrated the effectiveness of primers 759/760 with 53 isolates from different hosts (Santana et al., 2012), 77 isolates from the pepper crop in Northeastern Brazil (A. L. Garcia et al., 2013), 301 isolates from various Brazilian states and hosts (Santiago et al., 2017), and 108 isolates from three mesoregions in the state of Pernambuco (Albuquerque et al., 2021).

In the analysis for phylotype identification using the Nmult primer set, DNA amplification of 372-bp fragments confirms that the 26 isolates belong to phylotype II (*R. solanacearum*) (Table S1).

The taxonomic review suggested by Safni et al. (2014) associates *R. solanacearum* with what was originally described as phylotype II and links it to its geographic origin. This study confirms that isolates obtained from tomato plants in the southwest region of Paraná belong to phylotype II, consistent with previous studies highlighting its prevalence in Brazil (Lopes & Rossato, 2018; Albuquerque et al., 2021; Santiago et al., 2017; Fonseca et al., 2013; A. L. Garcia et al., 2013). While phylotype I (*R. pseudosolanacearum*) has been documented in various areas in Brazil, it is not common in the south region due to the warmer climates it demands (Santiago et al., 2017). Santana et al. (2012) reported similar findings in the analysis of 53 strains of potato, eggplant, pepper, and tomato using this molecular technique. Albuquerque et al. (2021) studied the distribution of *Ralstonia* spp. in Solanaceae in the semiarid climate and described the presence of sequevar I-17 in Brazil.

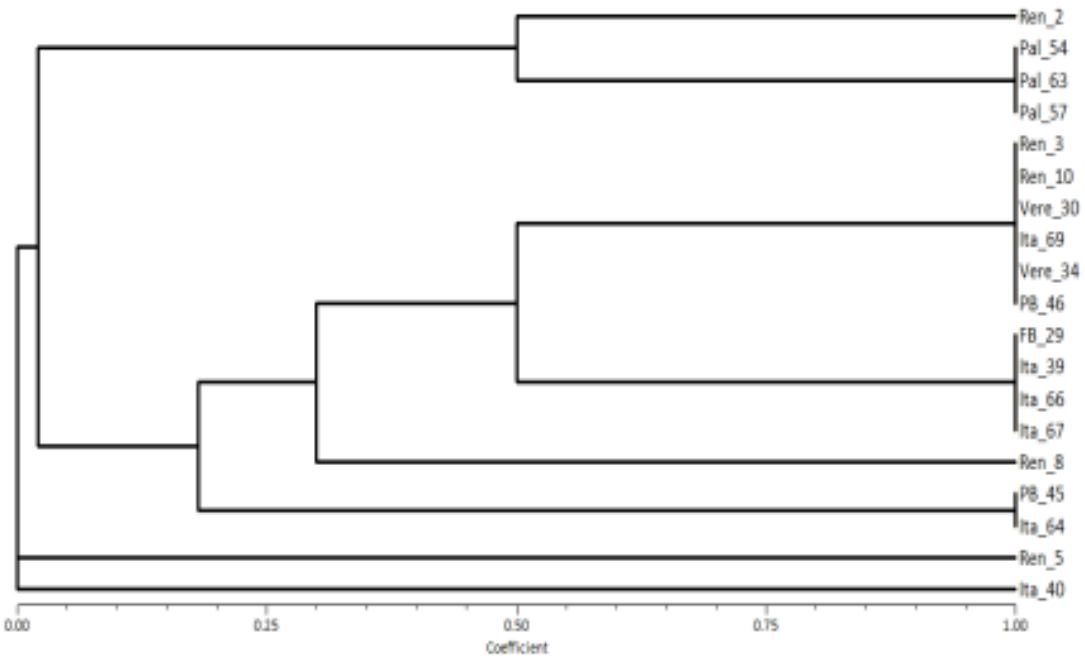

Figure 1. Dendrogram obtained from the analysis of genetic similarity of 19 Ralstonia solanacearum isolates regarding the genomic profiles obtained by BOX-PCR, using the Jaccard similarity coefficient and clustering by the UPGMA method. Average similarity of 0.63.

The BOX-PCR generated nine reproducible bands (0.5, 0.6, 0.75, 0.9, 1.0, 1.25, 1.3, 1.5, and 10 kb). Comparison of patterns from genomic DNA bands amplified by the BOX-A1R primer revealed variability in the population, resulting in the formation of eight groups with a similarity threshold of 0.63 (average similarity) (Figure 1).

Group I comprises isolates from Renascença (Ren_2); Group II includes isolates from Palmas (Palm_54, 63, and 57); Group III consists of six isolates from Renascença (Ren_3 and 10), two from Verê (Vere_30 and 34), and one each from Itapejara d'Oeste (Ita_69) and Pato Branco (PB_46). Group IV is composed of isolates from Francisco Beltrão (FB_29) and Itapejara d'Oeste (Ita_39, 66, and 67); Group V comprises a single isolate from Renascença (Ren_8); Group VI consists of one isolate from Itapejara d'Oeste (Ita_64) and one from Pato Branco (PB_45); Group VII is represented by an isolate from Renascença (Ren_5); and Group VIII contains only an isolate from Itapejara d'Oeste (Ita_40).

The BOX-PCR analysis in this study revealed genetic similarities among isolates from different production areas. Several isolates exhibited genetic identity despite being from geographically distant locations, while others from the same production site were grouped differently. This demonstrates the adaptability of *R. solanacearum* phylotype II to various soil and climate conditions, facilitating its spread as a pathogen through contaminated soil and seedlings (Denny, 2006). Similar variability among isolates from the same location was also observed in a study by Rodrigues et al. (2012). (Figure 2).

Table 1
Location and year of collection of the isolates and results of amplification (+) of 280-pb fragment from 759/760 primers (indicating *Ralstonia solanacearum*), 372-pb fragment from Nmult primers (indicating phylotype II), and BOX-A1R (+) for isolate diversity analysis

	Isolates	Local	Year	Location	759/760 (280 pb)	Nmult (372 pb)	BOX-A1R
1	FB_29	Francisco Beltrão, PR	2017	25°58'45.92"S 52°51'26.54"W	+	+	+
2	ITA_39	Itapejara d'Oeste, PR	2017	25°58'45.92"S 52°51'26.54"W	+	+	+
3	ITA_40	Itapejara d'Oeste, PR	2017	25°58'45.92"S 52°51'26.54"W	+	+	+
4	ITA_64	Itapejara d'Oeste, PR	2017	25°58'45.92"S 52°51'26.54"W	+	+	+
5	ITA_ 66	Itapejara d'Oeste, PR	2017	25°58'45.92"S 52°51'26.54"W	+	+	+
6	ITA_67	Itapejara d'Oeste, PR	2017	25°58'45.92"S 52°51'26.54"W	+	+	+
7	ITA_69	Itapejara d'Oeste, PR	2017	25°58'45.92"S 52°51'26.54"W	+	+	+
8	ITA_72	Itapejara d'Oeste, PR	2017	25°58'45.92"S 52°51'26.54"W	+	+	-
9	ITA_76	Itapejara d'Oeste, PR	2017	25°58'45.92"S 52°51'26.54"W	+	+	-
10	MAR_14	Mariópolis, PR	2017	26°18'47.52"S 52°34'17.78"W	+	+	-
11	PA_51	Palmas, PR	2018	26°31'27.44"S 51°58'35.99"W	+	+	-
12	PA_54	Palmas, PR	2018	26°31'27.44"S 51°58'35.99"W	+	+	+
13	PA_57	Palmas, PR	2018	26°31'27.44"S 51°58'35.99"W	+	+	+
14	PA_63	Palmas, PR	2018	26°31'27.44"S 51°58'35.99"W	+	+	+
15	PB_45	Pato Branco, PR	2018	26°13'56.04"S 52°36'45.16"W	+	+	+
16	PB_46	Pato Branco, PR	2018	26°13'56.04"S 52°36'45.16"W	+	+	+
17	REN_2	Renascença, PR	2018	26°12'15.87"S 52°59'46.85"W	+	+	+
18	REN_3	Renascença, PR	2018	26°12'15.87"S 52°59'46.85"W	+	+	+
19	REN_4	Renascença, PR	2018	26°12'15.87"S 52°59'46.85"W	+	+	-
20	REN_5	Renascença, PR	2018	26°12'15.87"S 52°59'46.85"W	+	+	+
21	REN_8	Renascença, PR	2018	26°12'15.87"S 52°59'46.85"W	+	+	+
22	REN_9	Renascença, PR	2018	26°12'15.87"S 52°59'46.85"W	+	+	-
23	REN_10	Renascença, PR	2018	26°12'15.87"S 52°59'46.85"W	+	+	+
24	REN_12	Renascença, PR	2018	26°12'15.87"S 52°59'46.85"W	+	+	-
25	V_30	Verê, PR	207	25°53'15.31"S 53°00'14.02"W	+	+	+
26	V_34	Verê, PR	2017	25°53'15.31"S 53°00'14.02"W	+	+	+

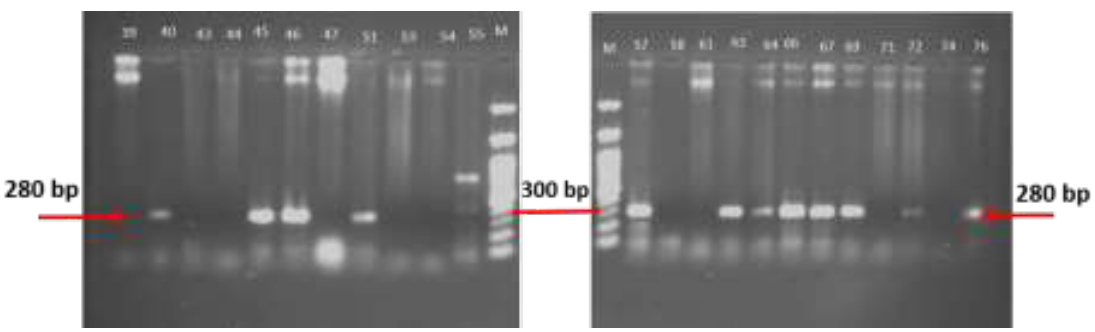

Figure 2. Agarose gel electrophoresis (1%) with the result of PCR with primers 759/760 for identification of phytopathogenic species of the genus Ralstonia. M: 1 Kbp DNA Ladder.

The limited variability observed in the analyzed isolates of *R. solanacearum* from tomato plants in the southwest region of Paraná may provide valuable guidance for selecting resistant genotypes, with a focus on this variant. Additionally, it highlights the importance of implementing meticulous crop rotation practices to reduce the risk of selecting hosts susceptible to potential losses.

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