JAQUELINE KIYOMI YAMADA

INOCULUM DYNAMICS OF *Ralstonia* spp.: POTENTIAL SOURCES, PERSISTENCE IN A LOCAL POPULATION AND SELECTION OF PHAGES TO REDUCE BACTERIA SURVIVAL

Thesis presented to the Plant Pathology Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae.*

VIÇOSA
MINAS GERAIS – BRASIL
2018

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
Y19i
2018

Yamada, Jaqueline Kiyomi, 1989-
Inoculum dynamics of *Ralstonia* spp. : potential sources, persistence in a local population and selection of phages to reduce bacteria survival / Jaqueline Kiyomi Yamada. – Viçosa, MG, 2018.
vii, 91 f. : il. (algumas color.) ; 29 cm.

Texto em inglês.
Orientador: Eduardo Seiti Gomide Mizubuti.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Murcha-bacteriana - Controle. 2. Bacteriófagos. 3. Variação genética. 4. *Ralstonia solanacearum*. I. Universidade Federal de Viçosa. Departamento de Fitopatologia. Programa de Pós-Graduação em Fitopatologia. II. Título.

CDD 22. ed. 632.32

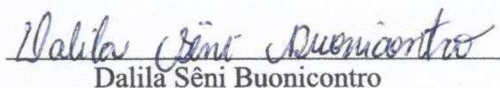
JAQUELINE KIYOMI YAMADA

INOCULUM DYNAMICS OF *Ralstonia* spp.: POTENTIAL SOURCES, PERSISTENCE IN A LOCAL POPULATION AND SELECTION OF PHAGES TO REDUCE BACTERIA SURVIVAL

Thesis presented to the Plant Pathology Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

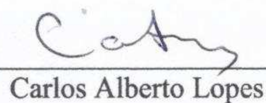
APPROVED: September 27nd, 2018.

Sérgio Oliveira de Paula



Dalila Sêni Buonicontro

José Rogério de Oliveira



Carlos Alberto Lopes

Eduardo Seiti Gomide Mizubuti
(Adviser)

AGRADECIMENTOS

Agradeço a Deus, pela vida, por estar presente em todos os momentos.

Agradeço aos meus pais, Jorge e Helena, exemplos de honestidade e humildade, todas as minhas conquistas são fruto do sacrifício deles.

À minha irmã, Michelle, por todo apoio. Obrigada Mi!

Agradeço ao Filipe Constantino Borel, pelo companheirismo e carinho, fundamental para a conclusão dessa etapa da minha vida.

Agradeço à Nina e Júlio Borel, pais de Filipe, por todo apoio.

Agradeço à Universidade Federal de Viçosa, ao Departamento de Fitopatologia e à FAPEMIG pela oportunidade e pelo financiamento desse trabalho.

Agradeço ao Professor Eduardo Seite Gomide Mizubuti pela oportunidade, paciência e conselhos.

Agradeço ao Doutor Carlos Alberto Lopes, pela contribuição para o presente trabalho e pela amizade.

Agradeço ao Professor José Rogério e ao Professor Francisco Murilo Zerbini por terem disponibilizados os laboratórios para realização desse trabalho.

Agradeço ao Professor Sérgio Oliveira de Paula, em especial ao Roberto de Sousa Dias e ao Vinicius Duarte pela colaboração e pela amizade.

Agradeço à Thaís Ribeiro Santiago e à Camila Geovanna Ferro, pelas sugestões e pela amizade.

Agradeço a todos que colaboraram com as amostras de solo e água para este trabalho: Paulo E. F. de Macedo, Amanda Guedes, Carla Santin, Leandro H. Yamada, Filipe C. Borel, Rhaphael A. Silva, Miller S. Lehner, Thaís R. Santiago.

Agradeço aos meus colegas de laboratório, em especial ao Rhaphael, Daniel, Milene, Miller, Camila, Renato e João Paulo, pela amizade.

Agradeço à Sueli, D. Neuza e Sr. Mário, pelas palavras nos momentos mais difíceis e pelo incentivo durante o doutorado.

Agradeço a todos que não foram mencionados, mas que de alguma forma contribuíram com este trabalho!

BIOGRAFIA

JAQUELINE KIYOMI YAMADA, filha de Jorge Yoshihiko Yamada e Helena Kyoko Iwai Yamada, nasceu em 18 de julho de 1989, em Londrina-Paraná. Iniciou o curso de Agronomia, na Universidade Federal de Viçosa, em março de 2007, graduando-se em janeiro de 2012. Em agosto de 2012 ingressou no curso de Mestrado em Fitopatologia, na Universidade Federal de Lavras e obteve o título de mestre em julho de 2014. No mês seguinte, ingressou no curso de doutorado em Fitopatologia pela Universidade Federal de Viçosa.

CONTENT

RESUMO.....vi

ABSTRACT.....vii

GENERAL INTRODUCTION.....1

CHAPTER 1 - ADDITIONAL ASSESSMENT OF THE SOURCES OF INOCULUM OF BACTERIAL WILT.....4

ABSTRACT.....4

INTRODUCTION.....5

MATERIAL AND METHODS.....9

RESULTS.....14

DISCUSSION.....16

ACKNOWLEDGEMENTS.....20

SUPPORTING INFORMATION.....21

REFERENCES.....27

CHAPTER 2 – GENETIC VARIABILITY OF *Ralstonia* spp. IN A MICRO-REGION.....34

ABSTRACT.....34

INTRODUCTION..... 34

MATERIAL AND METHODS 38

RESULTS.....43

DISCUSSION.....52

ACKNOWLEDGEMENTS.....57

REFERENCES.....57

CHAPTER 3 – SELECTION AND CHARACTERIZATION OF BACTERIOPHAGES POTENTIALLY USEFUL FOR THE CONTROL OF TOMATO BACTERIAL WILT (*Ralstonia* spp.).....65

ABSTRACT.....65

INTRODUCTION..... 66

MATERIAL AND METHODS 69

RESULTS.......... 74
DISCUSSION.......... 80
ACKNOWLEDGEMENTS.......... 83
REFERENCES.......... 83

GENERAL CONCLUSIONS.......... 91

RESUMO

YAMADA, Jaqueline Kiyomi, D.Sc., Universidade Federal de Viçosa, setembro de 2018. **Dinâmica de inóculo de *Ralstonia* spp.: fontes potenciais, persistência em uma população local e seleção de fagos para reduzir a sobrevivência da bactéria.** Orientador: Eduardo Seiti Gomide Mizubuti.

Ralstonia spp. são conhecidas por causar murcha bacteriana em várias plantas de interesse econômico. O patógeno possui alta variabilidade genética, ampla variedade de hospedeiros e pode sobreviver no solo mesmo na ausência de hospedeiros. A compreensão das potenciais fontes de inóculo, que contribuem para a variabilidade genética no centro de origem do patógeno é interessante para o manejo da doença. O papel dos rios, plantas daninhas e da população nativa de *Ralstonia* spp. em áreas de vegetação natural no desenvolvimento de epidemias de murcha bacteriana é pouco compreendido. A variabilidade genética entre cepas de *Ralstonia* spp. em uma região onde a doença é endêmica pode elucidar a contribuição dos meios de dispersão e fatores associados à sobrevivência. No presente estudo, a detecção de *Ralstonia* spp. em rios de diferentes biomas do Brasil revelou o potencial destes recursos naturais para dispersar o patógeno. As plantas invasoras mostraram ser importantes reservatórios de ambas as espécies de *Ralstonia* que ocorrem no Brasil e colaboram para sua sobrevivência. Métodos de detecção não foram sensíveis para confirmar a presença de *Ralstonia* spp. em amostras de solo de áreas sem ocorrência de murcha bacteriana. Quando se analisaram 204 isolados de *R. solanacearum* e 60 isolados de *R. pseudosolanacearum* obtidos do município de Coimbra, Minas Gerais, constatou-se haver baixa variabilidade genotípica e clonalidade. Nenhuma estruturação foi observada para as regiões do município, mas a composição genotípica variou entre os anos amostrados. Para o controle alternativo da murcha bacteriana, cinco fagos pertencentes à família *Siphoviridae*, ordem *Caudovirales*, foram isolados em amostras de solo. A análise molecular e a gama de hospedeiros com diferentes isolados de *Ralstonia* spp., representando o Brasil, revelaram diferenças entre os vírus. Adicionalmente, houve diferenças quanto à gama de hospedeiros quando os cinco fagos foram expostos a 24 isolados de *Ralstonia* spp. Os fagos não foram capazes de prevenir a infecção e controlar o número de células de *Ralstonia* spp. no solo. Outros métodos de aplicação são necessários para avaliar a eficiência dos fagos no controle da murcha bacteriana.

ABSTRACT

YAMADA, Jaqueline Kiyomi, D.Sc., Universidade Federal de Viçosa, September, 2018. **Inoculum dynamics of *Ralstonia* spp.: potential sources, persistence in a local population and selection of phages to reduce bacteria survival.** Adviser: Eduardo Seiti Gomide Mizubuti.

Ralstonia spp. are known to cause bacterial wilt in several plants of economic interest. The pathogen has high genetic variability, wide host range and can survive in the soil even in the absence of hosts. Understanding potential inoculum sources that contribute to genetic variability in the center of origin is interesting to the management of the disease. The importance of rivers, weeds and native population of *Ralstonia* spp. in areas of natural vegetation in the development of epidemics of bacterial wilt is poorly understood. Genetic variability among strains of *Ralstonia* spp. in a local region where the disease is endemic can elucidate the contribution of the means of dispersal and factors of survival. In the present study, the detection of *Ralstonia* spp. was attempted in water of rivers of different biomes of Brazil and revealed the potential of these natural resources to disperse the pathogen. Weeds were important reservoirs of both species of *Ralstonia* that occur in Brazil, and collaborate to their survival. Methods of detection were not sensitive to confirm the presence of *Ralstonia* spp. in soil samples from areas without the occurrence of bacterial wilt. The genetic variability of 204 strains of *R. solanacearum* and 60 strains of *R. pseudosolanacearum* from the municipality of Coimbra, Minas Gerais, was low and there was evidence of clonality in the population. The population was not genetically structured according to the geographic region in the municipality, however the genotypic composition varied in time. To assess an alternative measure to control bacterial wilt, five phages were isolated. All phages belong to the *Siphoviridae* family, *Caudovirales* order. Molecular analysis and host range with different *R. solanacearum* strains revealed differences among the viruses. There were differences in the host range when the five phages were exposed to 24 *Ralstonia* spp. strains. The phages were not able to prevent tomato infection and control the number of cells of *Ralstonia* spp. in the soil. Other methods of application are necessary to evaluate the efficiency of the phages to control of bacterial wilt.

GENERAL INTRODUCTION

Some *Ralstonia* spp. cause bacterial wilt, one of the most important plant diseases worldwide (Hayward, 1991). The control of bacterial wilt is difficult due to the ability of the pathogen to survive in the soil and due to its wide host range (Kelman, 1953; Graham et al., 1979). In Brazil, environmental conditions, as temperature and presence of natural hosts, favor the pathogen (Lopes, 2015). Brazil is a putative center of origin of *R. solanacearum* and high genetic variability is found among strains in the country (Wicker et al., 2012; Santiago et al., 2017). The high genetic variability of the pathogen population is a major challenge for the efficacy of resistant cultivars developed by breeding programs (Santiago et al., 2017).

The spread of *Ralstonia* spp. to distinct agricultural areas is supposed to be related to the ubiquity of inoculum sources and dispersal agents or routes. Nevertheless, the epidemiological relevance of both factors is poorly understood in Brazil. In a previous study, there was evidence that rivers and transportation of contaminated propagative material are important factors related to the dispersal of the pathogen. This evidence was gathered through a population genetics approach after analyzing the weak structuration of either *R. solanacearum* or *R. pseudosolanacearum* populations (Santiago et al., 2017). However, formal studies about the contribution of inoculum sources have not been properly conducted in Brazil.

The genetic structure of a local population of *Ralstonia* spp. has not been investigated in Brazil. To date, all studies about the genetics of the population of *Ralstonia* spp. were carried out using isolates collected from different areas, many of them located far from each other. From an epidemiological perspective, it would be interesting to assess how genotypes may change in a given area, across different years.

Several alternative control measures have been proposed to manage bacterial wilt. The use of bacteriophages (or phages) has been pointed as promising for the control of several bacterial disease, including wilt caused by *Ralstonia* spp. (Jones et al., 2012). Phages are specific to bacterial species, have low impact to environmental and are not toxic to eukaryotes (Buttimer et al., 2017). However, the chances of success increase when phages are obtained from the population to which they are target (Jones et al., 2012). So far, there has been no attempt to isolate and select phages effective to the control of bacterial wilt in Brazil.

The objectives of this study were to determine the contribution water of rivers, weeds, seeds and soil, associated to different biomes of Brazil as potential inoculum sources of bacterial wilt; the genetic structure of the populations of *Ralstonia* spp. in a micro-region and to select bacteriophages effective to control the disease. The specific objectives were: (1) to detect *Ralstonia* spp. in water samples of rivers, seeds of symptomatic plants, weeds and crop fields soil of different watersheds and biomes of Brazil; (2) to determine the genetic variability of local populations of *Ralstonia* spp.; (3) to select and characterize potential bacteriophages to control bacterial wilt in tomato seedlings.

References

Buttimer, C., McAuliffe, O., Ross, R. P., Hill, C., O'Mahony, J. & Coffey, A. (2017). Bacteriophages and bacterial plant diseases. *Frontiers in microbiology*, *8*, 34.

Graham, J., Jones, D. A. & Lloyd, A. B. (1979). Survival of *Pseudomonas solanacearum* race 3 in plant debris and in latently infected potato tubers. *Phytopathology*, *69*, 1100-1103.

Hayward, A. C. (1991). Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. *Annual Review of Phytopathology*, *29*, 65–87.

Horita, M., Tsuchiya, K., Suga, Y., Yano, K., Waki, T., Kurose, D. & Furuya, N. (2014). Current classification of *Ralstonia solanacearum* and genetic diversity of the strains in Japan. *Journal of general plant pathology*, *80*, 455-465.

Jones, J. B., Vallad, G. E., Iriarte, F. B., Obradović, A., Wernsing, M. H., Jackson, L. E., Balogh, B., Hong, J.C & Momol, M. T. (2012). Considerations for using bacteriophages for plant disease control. *Bacteriophage*, *2*, e23857.

Kelman, A. (1953). The bacterial wilt caused by *Pseudomonas solanacearum*. *Technical Bulletin of North Carolina Agricultural Experiment Station*, 99.

Lopes, C. (2015). A Threatening Disease of Tomato Cultivated under Warm Temperatures. *Comunicado Técnico 109*, Brasília: Embrapa Hortaliças.

Santiago, T. R., Lopes, C. A., Caetano- Anollés, G. & Mizubuti, E. S. G. (2017). Phylotype and sequevar variability of *Ralstonia solanacearum* in Brazil, an ancient centre of diversity of the pathogen. *Plant pathology*, *66*, 383-392.

Chapter 1 - Additional assessment of the sources of inoculum of bacterial wilt

Abstract

Dispersal of *Ralstonia* spp. by rivers and contaminated plant material and the importance of weeds as inoculum sources have been poorly investigated in Brazil. Water of rivers, soil of the biomes Amazônia, Cerrado and Mata Atlântica, from fields of diverse crops and areas of natural vegetation, besides soil of the rhizosphere of weeds present in tomato fields with records of bacterial wilt were sampled and analyzed to detect *Ralstonia* spp. Seeds of tomato plants artificially and naturally infected with *Ralstonia* spp. were also processed. All samples were enriched *a priori* in selective medium South Africa (SMSA) and colonies were isolated in plates containing solid SMSA. Detection of *Ralstonia* spp. was confirmed by polymerase chain reaction (PCR) with specific primers. The Co – operational PCR (CO-PCR) was also used to detect *Ralstonia* spp. Colonies of *Ralstonia* spp. were obtained from soil samples and from a commercial substrate sample. Five soil samples from eggplant fields, one from coffee field, one substrate from potato seed tuber production, two soil samples from the rhizosphere of *Amaranthus* spp., one from *Bidens pilosa* and one from *Solanum americanum* tested positive for *Ralstonia* spp. Besides these soil samples, five water samples of rivers were positive for CO-PCR detection: two samples from Amazônia, one from Cerrado and two samples from irrigation water collected from tomato fields located in the Mata Atlântica biome. *Ralstonia* spp. were not detected in tomato seeds. These results revealed potential inoculum sources, especially weeds, in areas with historical records of bacterial wilt. Additionally, rivers may be reservoirs and dispersal agents of *Ralstonia* spp.

Introduction

Bacterial wilt is a destructive disease that reduces yield of many plant species of economic importance. Plants classified in more than 50 botanical families can be affected by bacterial wilt and epidemics can develop anywhere, but severity is usually higher in the tropical regions of the world (Hayward, 1994; Elphinstone, 2005). The pathogen infects the plants through the roots, by wounds (Kelman, 1953; Hayward, 1991). The bacteria multiply in the cortex of the root to access the xylem, where it quickly reaches high numbers (Kelman, 1953). Wilt develops due to the obstruction of the vessels colonized by bacterial cells (Smith 1920; Kelman, 1953). The symptom of wilt is irreversible and infected plants usually die in a short period of time.

Bacterial wilt is caused by different species: *Ralstonia solanacearum* (formerly classified as Phylotype II) *R. pseudosolanacearum* (Phylotypes I and III), *R. syzygii* subsp. *syzygii* (Phylotype IV), *R. syzygii* subsp. *celebesensis* (Phylotype IV) and *R. syzygii* subsp. *indonesiensis* (Phylotype IV) (Safni et al., 2014). In Brazil, two species are known to cause bacterial wilt in different crops: *R. solanacearum* and *R. pseudosolanacearum* (Santiago et al., 2017).

Management of bacterial wilt is difficult because the pathogen can survive or be present in different environments/substrates (Kelman, 1953; Graham et al., 1979; Graham & Lloyd, 1979; van Elsas et al., 2005). Possible routes of transport of *Ralstonia* spp. are by water, contaminated machines and propagative materials and soil (Graham et al., 1979; Graham & Lloyd, 1979; Coutinho, 2005; Álvarez et al., 2008; Parkinson et al., 2013). The dispersal process of the pathogen in Brazil needs further studies to elucidate how it is introduced either into clean or already infested areas. For epidemics in potato fields, seed tubers are well-known sources of inoculum (Figueiró, 2008). Studies of *Ralstonia* spp. in clonal nursery of eucalyptus revealed that mini-stumps and rooting substrate are considered important inoculum sources and may be involved in

pathogen dispersal (Gonçalves Mafia et al., 2012). Additionally, water and weeds present in clonal nursery of eucalyptus can contribute to the dispersal of the pathogen (Gonçalves Mafia et al., 2012). Other potentially important dispersal routes are rivers. Along the Solimões and Amazonas rivers, in Amazonas state, Brazil, high incidence of Moko disease was observed and can be related with the dispersal of *Ralstonia* spp. (Netto et al., 2004). But, other than these studies, no other investigation was conducted and published on the role of dispersal agents and inoculum sources of *Ralstonia* spp.

Ralstonia spp. can persist in the area for a long time even in the absence of host plants (Graham & Lloyd, 1979; Stander et al., 2003). Survival of the bacterium has been associated with roots of host or non-host plants, presence of infected plant debris, in the soil, and in volunteer propagation organs, mainly tubers (Graham et al., 1979). In non-host plants, the pathogen cannot infect the roots, but it can multiply and survive associated with exudates in the rhizosphere (Álvarez et al., 2008). For this reason, weeds and invasive plants may be important to the management of bacterial wilt. In Europe, perennial bittersweet (*Solanum dulcamara*) is an aquatic plant and a problem in irrigated areas, because *Ralstonia* spp. can survive associated with its root system (Wenneker et al., 1999; Stevens & van Elsas, 2010). Thus, perennial bittersweet has been listed as an alternative host to *Ralstonia* spp. Using several methods of detection, it was possible to associate the presence of bittersweet to the incidence of bacterial wilt in surrounding areas (Elphinstone, 1996; Elphinstone et al., 1998a; Elphinstone et al., 1998b; Janse et al., 1998; Stevens & van Elsas, 2010). Other natural solanaceous weeds described as hosts of *Ralstonia* spp. are: *S. nigrum* (Hayward, 1975) and *S. cinereum* (Graham & Lloyd, 1978). Non-solanaceous weeds that allow latent infection are: *Amaranthus* spp., *Bidens pilosa*, *Galinsoga parviflora*, *Oxalis latifolia*, *Spergula arvensis*, *Tagetes minuta*, *Rumex abyssinicus*, *Physalis minima*, *Euphorbia hirta* and *Stellaria sennii* (Tusiime et al., 1998; Dittapongpitch & Surat, 2003; Wicker et al.,

2009). In Brazil, species of *Lepidium virginicum*, *Nicandra physaloides*, *S. americanum*, *Portulaca oleracea*, *Physalis angulata*, *Amaranthus* spp., *Euphorbia heterophylla*, *Crotalaria spectabilis* and *B. pilosa* are listed as potential hosts to *Ralstonia* spp. (Orozco et al., 2004). However, the tests were conducted under controlled conditions and it is not known whether there is good correspondence with epidemic development in nature. This study reinforces that weeds can be important inoculum source to the forthcoming crops in the same area.

Transmission of *Ralstonia* spp. by tomato seeds is still uncertain. Although *Ralstonia* spp. was detected in tomato fruits of infected plants (Sanchez Perez et al., 2008), the pathogen could not be detected by PCR in seeds collected from symptomatic eggplants (Ramesh et al., 2011). For this reason, more studies need to be developed to prove the capacity of *Ralstonia* spp. to infect seeds by colonization of the vascular tissue.

Ralstonia spp. have been detected in the soil up to two years after potato crops were affected by bacterial wilt (Olsson, 1976; Elphinstone, 1996). However, survival of *Ralstonia* spp. can vary as reported by Stander et al. (2003). Bacterial wilt developed in crops established in fields kept without plants (fallow) for five years (Stander et al., 2003). The ability to survive in deeper soil layers can be one explanation for the long survival period of *Ralstonia* spp. (Graham & Lloyd, 1979), once superficial soil is more exposed to desiccation than deep soil layers (Graham & Lloyd, 1979; van Elsas et al., 2000). In addition to non-host plants, crop residues can contribute to the survival of *Ralstonia* spp. Bell pepper stem and root tissues could serve as sources of inoculum of bacterial wilt (Felix et al., 2012).

In addition to the sources of inoculum associated with soil (roots, crop debris, soil particles) there are reports of water sources, mainly rivers and irrigation canals, acting as sources of inoculum or dispersal agents from where or which bacterial cells

could be introduced into fields (Hayward, 1991; Wenneker et al., 1999; Pradhanang & Momol, 2001; Netto et al., 2004; Parkinson et al., 2013). The detection of *Ralstonia* spp. in water from rivers and other sources close to solanaceous crops reinforces that these water resources are one of the main means of spreading the pathogen. In Europe, detection of the pathogen in irrigation water confirms the importance of monitoring water quality to avoid infestation of clean areas or increasing inoculum levels in already affected fields (Elphinstone et al., 1998a; Janse et al., 1998; Parkinson et al., 2013; Wenneker et al., 1999; Stevens & van Elsas, 2010). Brazil has the largest river network and is a major biodiversity resource (Brazil Ministry of the Environment). Rivers have been shown to be potential means to disperse inoculum. However, no studies were conducted to assess the contribution of water bodies as inoculum sources for bacterial wilt epidemics. Such studies need to count on precise detection methods, otherwise false negatives may occur whenever inoculum concentration is under the detection level. Studies to assess the contribution of a given potential source of inoculum for bacterial wilt epidemics depends largely on the detection methods used.

Several methods have been used for the assessment of inoculum sources of *Ralstonia* spp. (Dittapongpitch & Surat, 2003; Marco-Noales et al., 2008; Li et al., 2014; Tran et al., 2016; Perera et al., 2018). The use of semi selective medium is the most commonly adopted method to detect *Ralstonia* spp. The selective medium South Africa (SMSA) (Elphinstone et al., 1996) has been more effective in suppressing growth of other microorganisms and allowing the isolation of *Ralstonia* spp. colonies (van der Wolf et al., 1998; Pradhanang et al., 2000; Caruso et al., 2005; Pontes et al., 2017). Despite being generally considered as a useful detection method, the polymerase chain reaction (PCR) has not been successful for samples with low concentration of the target bacteria (Caruso et al., 2003). However, a combination of three primers for the 16S region has been successful for the detection of *Ralstonia* spp. in water samples

(Caruso et al., 2003). Prior enrichment with SMSA of the sample could increase the sensitivity of this technique, known as Co-operational polymerase chain reaction (CO-PCR) (Caruso et al., 2005; Marco-Noales et al., 2008). Overall, enrichment increases the likelihood of detecting *Ralstonia* spp. by different methods (Elphinstone et al., 1996; Dittapongpitch & Surat, 2003; Priou et al., 2006).

One factor that makes the detection of *Ralstonia* spp. difficult is the capacity of the pathogen to reduce its metabolism and enter a dormant-like state known as viable but non-culturable (VBNC) (Grey & Steck, 2001; Imazaki & Nakaho, 2009). When environmental conditions are favorable, *Ralstonia* spp. can be activated and multiply. Low temperature, ultrapure water or the lack of nutrients and concentration of copper ions are factors that can drive *Ralstonia* spp. into the VBNC state (van Elsas et al., 2000; van Elsas et al., 2001; Grey & Steck, 2001; van Overbeek et al., 2004). Resuscitation or recoverable cells could not be easily achieved only by increasing temperatures and providing nutrients. A long time of incubation is necessary to convert VBNC cells into active cells.

Studies about reliable methods to detect *Ralstonia* spp. in water of rivers, soil samples, seeds and in weeds are necessary to understand how the bacteria behave in tropical countries. The objective of this study was to determine the contribution of rivers, weeds, seeds and soil, associated to different biomes of Brazil as inoculum sources of *Ralstonia* spp. For this, we proposed to collect samples of possible inoculum sources and to detect *Ralstonia* spp. in these samples.

Material and Methods

Soil, water, weed and tomato seed samples

The soil samples were collected in three biodiversity-rich biomes of Brazil: Amazônia, Cerrado and Mata Atlântica. Soil samples were taken at a depth of 10 cm in

fields with and without reports of bacterial wilt epidemics and also from areas of native vegetation. One sample of 200 g was collected at each sampling site, with a hoe or a shovel. Samples were taken in the region under the influence of the rhizosphere and geographic coordinates were registered. The soil samples were placed in clean plastic bags and taken to the laboratory where they were kept at room temperature.

The water samples were collected from rivers and irrigation sources in different states of Brazil: Acre, Amazonas, Tocantins, Bahia, Distrito Federal, Goiás, Minas Gerais, São Paulo, Paraná, and Rio Grande do Sul (Figure 1). At least 500 mL of water was collected from a source using a clean plastic bottle. For each sample the geographic coordinates were registered with a portable GPS device. Water samples were stored at room temperature and taken to the laboratory.

The rhizosphere soil of weeds was collected in areas with incidence of bacterial wilt in tomato crop fields. The soil sample was stored in plastic bags and the weeds were photographed. Plant species identification was based on photographs taken *in situ*. The latitude and longitude of each sample was registered as previously described.

Fruits were collected from either artificially or naturally infected tomato plants. In laboratory, fruits were washed with detergent and rinsed in running tap water. Seeds were extracted and processed with and without fermentation. The seeds were dried under shade and room temperature conditions.

Bacterial strains and growth conditions

The Casamino acid-Peptone-Glucose agar (CPG) medium (10 g peptone, 5 g glucose, 1 g hydrolyzed casein, 12 g bacteriological agar, 1 L distilled water) was used to propagate colonies characteristic of *Ralstonia* spp. Isolation of *Ralstonia* spp. was attempted with the SMSA composed of: 10 g peptone, 5 g glucose, 1 g hydrolyzed casein, 12 g bacteriological agar, 1 L distilled water, 25 mg bacitracin, 100 mg of

polymyxin B sulfate (600,000 U), 5 mg of chloramphenicol, 0.5 mg of penicillin G, 5 mg of violet crystal and 50 mg of triphenyl tetrazolium 2,3,5-hydrochloride (Elphinstone et al., 1996). To increase the chances of recovery of *Ralstonia* spp. from water samples, 5 g of sodium pyruvate were added to each liter of SMSA medium (Imazaki & Nakaho 2010). The bacterial strains isolated from the different substrates were maintained in cryogenic storage tubes containing sterilized saline solution, 0.85 % NaCl, under room temperature.

Bacterial isolation

The adjusted methodology of Pradhanang et al. (2000) was used for the soil samples and the methodologies described by Caruso et al. (2005) and Wicker et al. (2009) were used for water samples. Briefly, for each soil sample, 1 g was suspended in 9 mL of distilled water and 1 mL of this suspension was diluted in 999 mL of water. From the last dilution, 1 mL was taken and mixed in 9 mL of SMSA broth, and kept in a rotary shaker (80 rpm) at 28 °C for 2, 4, 6 and 8 h. For each incubation period, aliquots of 100 µL was spread on three plates containing SMSA agar. Colonies were allowed to grow during 5 to 10 days at 28 °C. Colonies characteristic of *Ralstonia* spp., with a red center and whitish periphery, were cultivated in another plate with CPG medium to obtain pure cultures.

Each 500 mL of water sample was filtered through a 0.22 µm Millipore membrane filter. The filter was aseptically cut into smaller pieces and placed in 20 mL of SMSA broth and kept in a rotary shaker (80 rpm) at 28 °C for 2, 4, 6 and 8 h. As with the soil samples, 100 µL was spread onto SMSA agar. Three plates were used for each water sample. Plates were maintained at 28 °C until the development of bacterial colonies. Colonies with the appearance of *Ralstonia* spp. were streaked in another plate with solid CPG medium to obtain pure cultures.

Pathogenicity assay

The strains obtained from SMSA were transferred to plates containing CPG medium for 48 h, at 28 °C. 'Santa Clara' tomato seedlings were inoculated with a sterile toothpick laden with bacterial cells of each strain. The base of the stem of the tomato seedlings was punctured with the infested toothpick. Each strain was inoculated into three plants. The tomato plants were maintained in growth chamber at 28 °C until the development of symptoms. Strains that did not cause symptoms in inoculated plants after three weeks were considered as non-pathogenic. A positive control was comprised of a virulent strain of *R. solanacearum* (UFV 245) and the negative control was a set of plants punctured with a clean toothpick.

DNA extraction

From colonies formed after 24 h of incubation at 28 °C, a loopful of each pure colony was placed in a microtube containing 50 µL of ultrapure water. The tubes were centrifuged for 20000 *g* to form a pellet sufficient to extract DNA and the supernatant discarded. The Wizard® genomic DNA purification kit (Promega) was used according to the manufacturer's instructions for Gram-negative bacteria. The quality of DNA was analyzed by gel electrophoresis. The DNA was quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and adjusted to 50 ηg.

Species identification

Species identification was accomplished using the species-specific (*Ralstonia solanacearum*) primer pair 759/760 (Opina et al., 1997). Polymerase chain reactions were performed using the GoTaq G2 kit from Promega®. Reaction were run in the T100M Bio Rad thermocycler. PCRs were conducted at a final volume of 25 µL, containing 1 µL of DNA, 1X Colorless GoTaq Reaction Buffer, 0.2 mM each dNTP,

0.5 μM of each primer, 1.25 U of GoTaq®G2 DNA polymerase (5 U. μL^{-1}) and ultrapure water to complete the final volume. The amplified fragments were analyzed by electrophoresis in 1 % (wt/v) agarose gel, stained with GelRed (Biotium) and visualized under UV light. The comparison was done with the DNA marker of 100 base pairs (bp) and with the DNA of one strain of *R. solanacearum* (RS 279) that was submitted to the same conditions of PCR with specific primer (positive control). *R. solanacearum* or *R. pseudosolanacearum* were identified based on the presence of a single band of 282 bp.

Ralstonia spp. strains were classified in phylotypes from primers: Nmult 21:1F, Nmult 21:2F, Nmult 22:Inf and Nmult 23:AF, and a reverse primer, Nmult 22:RR (Fegan & Prior, 2005). Amplificons of 144, 372, 91 and 213 bp correspond phylotypes I, II, III and IV, respectively. The thermocycling conditions were 96 °C for 5 min for initial denaturation, 30 cycles of 94 °C for 15 s, 59 °C for 30 s, 72 °C for 30 s, and a final extension of 72 °C for 10 min. Final products of PCR were stained with GelRed ™ and subjected to 1 % agarose gel electrophoresis. Confirmation of phylotype was done by visual inspection and comparison with bands of a 1 kb plus DNA ladder (Invitrogen). Phylotypes I and III belongs to *R. pseudosolanacearum*, phylotype II corresponds *R. solanacearum* and phylotype IV, to *R. syzygii*.

Co-operational PCR (CO-PCR)

CO-PCR was performed to detect of *Ralstonia* spp. in samples that were incubated in liquid SMSA medium, under rotation (80 rpm) at 28 °C for 24 h. A volume of 5 μL was used for the CO-PCR as described by Caruso et al. (2003). The PCRs were performed in a total volume of 25 μL, in the T100M Bio Rad thermocycler. The amplified fragments were analyzed in electrophoresis in 1 % (wt/v) agarose gel, stained with GelRed (Biotium) and photographed under UV light. The comparison was done

with the DNA marker of 100 base pairs (bp) and with DNA of one strain of *R. solanacearum* (RS 279) that was submitted to the same conditions of CO-PCR (positive control). Confirmation of the detection was based on the presence of a single 408 bp amplicon.

Results

In total, 100 samples were analyzed attempting to isolate and detect *Ralstonia* spp.: 35 water samples, one substrate, four tomato seeds samples, 18 soil samples from three biomes, 33 soil samples of crops and nine soil samples associated with weeds. Localization of each water and soil sample is represented in Figure 1.

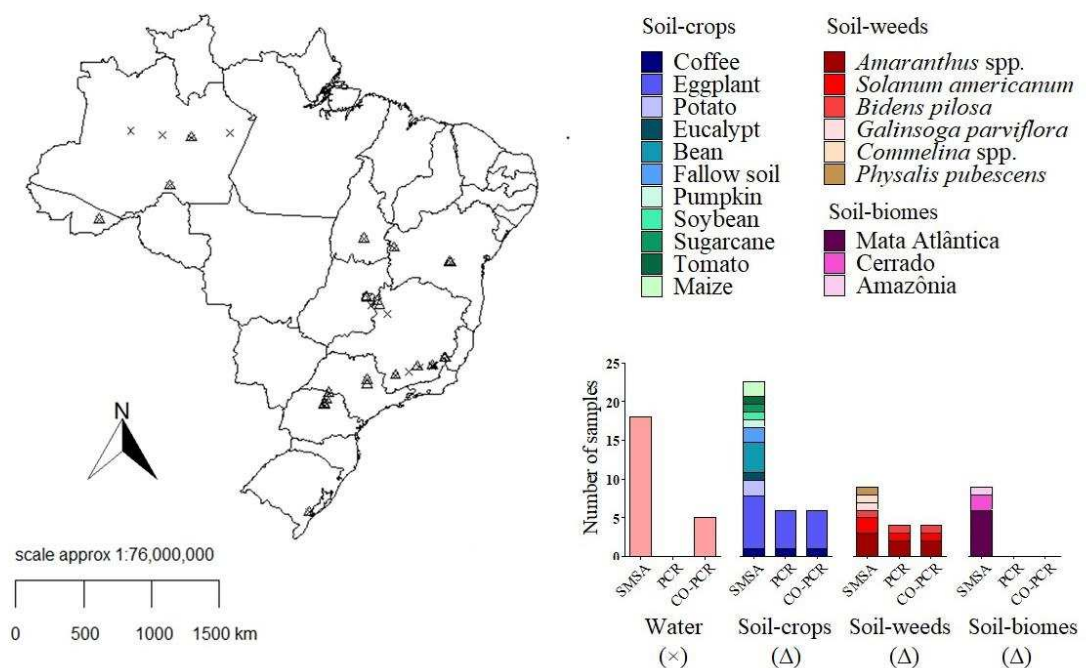


Figure 1. Map of Brazil showing the origin of water (×) and soil (Δ) samples collected in different regions. The graphic represents number of samples tested positive according to each of three methods of detection: SMSA, PCR and CO-PCR.

From 35 water samples, 18 samples resulted in colonies grown in SMSA, however PCR with specific primers were negative for *Ralstonia* spp. (Figure 1). For

five water samples, CO-PCR was positive: one sample each of the Madeira and Purus rivers, both in Amazonas state, one sample from Brasília, Distrito Federal (Federal District), and two samples from Coimbra, Minas Gerais state (Table S1).

Thirty three soil samples from agricultural areas were collected in different regions of Brazil. Five soil samples from eggplant fields located in Brasília, Distrito Federal and one soil sample from coffee field in Coimbra, Minas Gerais, were positive for *Ralstonia* spp. from CO-PCR and isolation with SMSA (Figure 1). *R. pseudosolanacearum* was detected in three samples of eggplant and *R. solanacearum* in another two samples from the same host plant. These results indicate the coexistence of both species in Brasília, Distrito Federal. Samples of crop fields in Coimbra, Minas Gerais state, were from areas with historical records of bacterial wilt. However, *Ralstonia* spp. was detected in only one sample from a coffee field. Strains obtained from this sample were classified as *R. pseudosolanacearum*. Samples from bean, maize, zucchini, cucumber, and pumpkin fields tested negative for *Ralstonia* spp. (Table S1).

One sample of commercial planting substrate from a potato seed-tuber crop was analyzed. This sample was collected from plants growing under greenhouse conditions with symptoms of bacterial wilt. Colonies of *R. solanacearum* were formed in SMSA. The CO-PCR analysis was positive for this sample (Table S1).

From 18 samples of soil from different biomes, colonies similar to *Ralstonia* spp. were recovered from nine samples: two from the Cerrado, one from the Amazônia and six from the Mata Atlântica, however none tested positive in the PCR assay. CO-PCR from enriched-samples did not detect the presence of the pathogen (Figure 1).

To assess the contribution of weeds to the survival of *Ralstonia* spp., soil of the rhizosphere was collected from weeds in areas with historical records of bacterial wilt. Nine samples were analyzed and *R. solanacearum* was detected in two samples from *Amaranthus* spp. and *R. pseudosolanacearum* was detected associated with *S.*

americanum and *B. pilosa*. CO-PCR for these samples were positive in all cases (Figure 1 and Table S1).

Four batches of tomato seeds were processed to detect and isolate *Ralstonia* spp. Three samples were from Brasília, Distrito Federal, from artificially-inoculated tomato plants. Another sample was obtained from naturally infected plant in Coimbra, Minas Gerais state. All samples of seeds tested negative for *Ralstonia* spp. regardless of the detection method (Table S1).

Discussion

We investigated samples of soil of crop fields, native plants, weeds, river waters and seeds that can be associated with the occurrence of bacterial wilt epidemics in Brazil. Several methods to detect *Ralstonia* spp. and to obtain pure cultures have been tested to analyze soil, water, weeds, and propagative parts (Nesmith & Jenkins, 1979; Chen & Echandi, 1982; Granada & Sequeira; Ito et al., 1998; Van der Wolf et al., 1998; Wenneker et al., 1999; Pradhanang et al., 2000; Dittapongpitch & Surat, 2003; Marco-Noales et al., 2008; Wicker et al., 2009; Stevens & van Elsas, 2010; Li et al., 2014; Tran et al., 2016). The use of SMSA to isolate *Ralstonia* spp. has been demonstrated to be efficient (Pradhanang et al., 2000), however, for soil samples taken from areas with no occurrence of bacterial wilt, this method was not useful. Even with prior enrichment of samples, the use of SMSA was not sensitive enough. In soil samples analyzed, SMSA isolation and CO-PCR were efficient only for samples collected from areas with previous history of bacterial wilt epidemics. Colonies similar to *Ralstonia* spp. were detected in several soil and water samples, however none was confirmed by PCR with specific primers. Similar results were reported previously, i.e. several strains obtained using selective medium were negative for PCR with specific primers (Ito et al., 1998).

Water resources can be inoculum reservoirs for bacterial wilt epidemics. *Ralstonia* spp. was confirmed only when using CO-PCR. In Thailand, The Netherlands, Martinique and Spain, the occurrence of *Ralstonia* spp. in water samples was reported after using SMSA (Wenneker et al., 1999, Stevens & van Elsas, 2010; Wicker et al., 2009, Álvarez et al., 2007). Although long-term survival of *Ralstonia* spp. in sterile water is a well-known phenomenon (van Elsas et al., 2001; Kelman, 1956), its survival in natural water conditions is apparently reduced. The presence of lytic bacteriophages and indigenous microorganisms in river water can reduce the viability of *Ralstonia* spp. populations and decline the pathogen populations (Álvarez et al., 2007). Another major factor is the VBNC state of *Ralstonia* spp. in water. Low efficiency of detection also is associated with the VBNC. This form is induced mainly by nutrient deprivation, water and soil without host, low temperature, and copper concentration (Grey & Steck, 2001; van Overbeek et al., 2004; van Elsas et al., 2001). Overall, VBNC cells are difficult to detect (Grey & Steck, 2001) and addition of sodium pyruvate in SMSA is an alternative to increase the chances of isolation (Imazaki & Nakaho, 2010). However, this modification of SMSA was not enough to obtain isolates of *Ralstonia* spp. in the present study. CO-PCR was better able to detect *Ralstonia* spp. than standard PCR assays. The technique has been shown to have high sensitivity for detection in river water samples (Caruso et al., 2003). In 35 water samples, CO-PCR confirmed the presence of the pathogen in five samples. Two of the five water samples were taken from rivers in the Amazon region, and the results corroborate the potential contribution of rivers in the Amazon to incidence of Moko disease in fields near Solimões and Amazonas rivers (Netto et al., 2004). One water sample from Brasília, in the Cerrado region, and two samples from Coimbra, Minas Gerais, collected near tomato fields with incidence of bacterial wilt also tested positive.

Detection and isolation of *Ralstonia* spp. from weeds demonstrate the important epidemiological role these plants play as inoculum source (Tusiime et al., 1998; Wenneker et al., 1999; Dittapongpitch & Surat, 2003; Wicker et al., 2009). *Ralstonia* spp. were recovered from the rhizosphere soil of the weeds, *B. pilosa*, *S. americanum* and *Amaranthus* spp. These species have been reported as plants that allow the survival of *Ralstonia* spp. (Tusiime et al., 1998; Wicker et al., 2009, Miranda et al., 2004). *Ralstonia* spp. cannot infect roots of non-hosts plants, but they can survive associated with these plants. When host plants are introduced in a field, bacteria increase in number and can cause disease. It is already known that roots of alternate hosts, debris of infected plants, volunteer tubers, and deeper soil layers can harbor *Ralstonia* spp. contributing to its survival (Graham et al., 1979; Graham & Lloyd, 1979). Detection methods are important to establish the contribution of weeds and native plants as inoculum sources. The report of the contribution of *S. dulcamara* is noteworthy (Elphinstone et al., 1998; Stevens & van Elsas, 2010; Wenneker et al., 1999). Other species of weeds can also serve as inoculum sources (Stevens & van Elsas, 2010; Wenneker et al., 1999; Dittapougpitch & Surat, 2003; Wicker et al., 2009). *Ralstonia* spp. were detected in four soil samples of the root system of weeds. Thus, these species may act as potential inoculum sources and contribute to pathogen survival. Future studies can explore more the importance of host and non-host plants in Brazil for survival of *Ralstonia* spp. In rare cases, *Ralstonia* spp. have been reported to be able to infect tomato fruits through the vascular system of the plant (Sanchez Perez et al., 2008). However, tomato seeds were negative for the presence of the pathogen even with prior enrichment, plating of the enriched samples in SMSA and CO-PCR. Failure to detect *Ralstonia* spp. associated with eggplant seeds was reported previously (Ramesh et al., 2011). As observed in the current study with tomato seeds, authors were not able to detect the pathogen in seeds of eggplant. Future studies are necessary to understand if

Ralstonia spp. are able to reach the fruit via infected vascular tissue and colonize or contaminate the fruit and seeds.

Samples collected in areas of native plants in each biome did not test positive for the presence of *Ralstonia* spp. This result does not reject the hypothesis that the pathogen is not present. Other methodologies are necessary to test these samples taken from areas without historical records of bacterial wilt. We can conclude that SMSA and CO-PCR with prior enrichment of samples are sensitive to detect the pathogen in samples collected in and nearby fields. Different sources of inoculum should be inspected oftenly so that growers can anticipate actions and prevent yield losses.

Soil samples collected from crop fields totaled 33, one sample from coffee field (Coimbra) and five samples from eggplant fields (Brasília) were positive. The presence of *R. pseudosolanacearum* in sample collected in from the rhizosphere of coffee plant is an evidence that coffee plant allow survival of this bacterial species. Lopes et al. (2015) showed, under controlled conditions, that coffee seedlings are potential hosts of *R. pseudosolanacearum*. The positive soil samples from eggplant crops were obtained from plants grown in a field with records of bacterial wilt. Other two samples from eggplant fields were negative: one from a fallow area and one from a field with resistant eggplant cultivar. Breeding programs have developed resistant cultivars and detected quantitative traits locus (QTLs) involved in the resistance, mainly to *R. pseudosolanacearum* (Salgon et al., 2017; Salgon et al., 2018). This can explain the reduction of the population of *Ralstonia* spp. in the soil at levels undetectable by our methodology.

The occurrence of *R. solanacearum* in soil, water and the rhizosphere of cultivated and non-cultivated plants in all regions in Brazil provides additional support to the hypothesis that the country is a putative center of origin of this bacterial species (Wicker et al., 2012). In addition to the practical implications of the findings of the

present study, i.e. the contribution to bacterial wilt epidemics of the potential inoculum sources, the results also allow us to infer about the role the different agents may have.

Acknowledgements

This research was financed by FAPEMIG. The authors thank Carlos Alberto Lopes for sampling soil samples and tomato seeds. We thank Bruno David Henriques, Thaís Ribeiro Santiago, Paulo Macedo, Filipe Constantino Borel, Carla Santin, Amanda Guedes, Leandro Hiroshi Yamada, Rhaphael Alves Silva, Miller da Silva Lehner and all members of our laboratory that collaborated collecting soil and water samples. We also thank José Rogério Oliveira for laboratory support.

Supporting Information

Supplementary Table S1. Samples of soil and water of rivers collected from different regions of Brazil to detect *Ralstonia* spp.

Sample	Place	Origin of the sample	Growth in SMSA [a]	PCR [b]	CO-PCR [c]	Phylotype [d]
1	Carandaí, MG	Carandaí river	+	-	-	nd
2	Paracatu, MG	Entre-Ribeiros River	+	-	-	nd
3	Cristalina, GO	Preto River	+	-	-	nd
4	Sapucaí, MG	Eleutério River	+	-	-	nd
5	Itutinga, MG	Grande river	-	nd	-	nd
6	Rio Branco, AC	River	+	unspecific	-	nd
7	Humaitá, AM	Madeira River	+	unspecific	+	nd
8	Beruri, AM	Purus River	-	nd	+	nd
9	Itacoatira, AM	Arari River	+	-	-	nd
10	Humaitá, AM	Madeira River	+	-	-	nd
11	Coari, AM	Copea Lake	+	-	-	nd
12	Juruá, AM	Juruá River	-	nd	-	nd
13	Brasília, DF	Tamanduá River	+	unspecific	+	nd
14	Coimbra, MG	São Roque River	-	nd	-	nd

15	Coimbra, MG	São Roque River	-	nd	-	nd
16	Coimbra, MG	Latão River	-	nd	-	nd
17	Coimbra, MG	Marengo River	-	nd	-	nd
18	Coimbra, MG	Marengo River	+	unspecific	+	nd
19	Coimbra, MG	São Roque River	+	unspecific	-	nd
20	Coimbra, MG	São Roque River	+	-	-	nd
21	Coimbra, MG	São Venâncio River	+	unspecific	+	nd
22	Sertaneja, PR	Paranapanema River	-	nd	-	nd
23	Águas da Prata, MG	River	-	nd	-	nd
24	Reduto, MG	Jequitibá river	-	nd	-	nd
25	Paraguaçú, MG	Sapucaí river	-	nd	-	nd
26	Tamarana, PR	River	+	-	-	nd
27	Apucarana, PR	Taquara river	-	nd	-	nd
28	Londrina, PR	Tibagi river	-	nd	-	nd
29	Luís Eduardo Magalhães, BA	River	+	unspecific	-	nd
30	Santa Rosa do Tocantins, TO	River	-	nd	-	nd
31	Pelotas, RS	River	-	nd	-	nd
32	Coimbra, MG	Marengo River	+	-	-	nd
33	Coimbra, MG	São Venâncio River	-	nd	-	nd

34	Coimbra, MG	São Venâncio River	-	nd	-	nd
35	Carandaí, MG	Marengo River	+	-	-	nd
36	Coimbra, MG	Root of *Amaranthus* sp.	+	+	+	II
37	Coimbra, MG	Root of *Solanum americanum*	+	+	+	I
38	Coimbra, MG	Root of *Bidens pilosa*	+	+	+	I
39	Coimbra, MG	Root of *Amaranthus* spp.	+	+	+	II
40	Coimbra, MG	Root of *Galinsoga parviflora*	+	-	-	nd
41	Coimbra, MG	Root of *Commelina* spp.	+	-	-	nd
42	Coimbra, MG	Root of *Physalis pubescens*	+	-	-	nd
43	Coimbra, MG	Root of *Amaranthus* spp.	+	-	-	nd
44	Coimbra, MG	Root of *Solanum americanum*	+	-	-	nd
45	Carandaí, MG	Soil of Tomato crop	+	-	-	nd
46	Unaí, MG	Soil of Bean crop	+	-	-	nd
47	Cabeceira Grande, MG	Soil of Bean crop	+	-	-	nd
48	Chapada da Diamantina, BA	Soil of potato crop	+	-	-	nd
49	Chapada da Diamantina, BA	Soil of potato crop	-	nd	-	nd
50	Chapada da Diamantina, BA	Soil of potato crop	+	-	-	nd
51	Tamarana, PR	Soil of soybean crop	-	nd	-	nd
52	Rio Branco, AC	Soil of eucalyptus crop	+	-	-	nd

53	Rio Branco, AC	Soil of Long pepper crop	-	nd	-	nd
54	Brasília, DF	Soil of eggplant crop	+	+	+	I
55	Brasília, DF	Soil of eggplant crop	+	+	+	II
56	Brasília, DF	Soil of eggplant crop	+	+	+	II
57	Brasília, DF	Soil of eggplant crop	+	+	+	I
58	Brasília, DF	Soil of eggplant crop	+	+	+	I
59	Brasília, DF	Soil of eggplant crop (Resistant clone)	+	-	-	nd
60	Brasília, DF	Fallow soil	+	-	-	nd
61	Brasília, DF	Soil of eggplant of (Resistant clone)	+	-	-	nd
62	Santa Elvira, MT	Soil of pumpkin crop	+	-	-	nd
63	Rondonópolis, MT	Soil of soybean crop	+	-	-	nd
64	Cuiabá, MT	Soil sugar cane crop	+	-	-	nd
65	Jaciara, MT	Soil of pasture crop	-	nd	-	nd
66	Santa Elvira, MT	Soil of sorghum crop	-	nd	-	nd
67	Coimbra, MG	Soil of maize crop	-	nd	-	nd
68	Coimbra, MG	Soil of zucchini crop	-	nd	-	nd
69	Coimbra, MG	Soil of cucumber crop	-	nd	-	nd
70	Coimbra, MG	Soil of coffee crop	+	+	+	I
71	Coimbra, MG	Soil of bean crop	+	-	-	nd

72	Coimbra, MG	Fallow soil	+	-	-	nd
73	Coimbra, MG	Soil of bean crop	+	-	-	nd
74	Coimbra, MG	Soil of maize crop	+	-	-	nd
75	Coimbra, MG	Soil of maize crop	+	-	-	nd
76	Luís Eduardo Magalhães, BA	Soil of soybean crop	-	nd	-	nd
77	Santa Rosa do Tocantins, TO	Soil of soybean crop	-	nd	-	nd
78	Vargem Grande do sul, SP	Potato seed (substrate)	+	+	+	II
79	Brasília, DF	Soil Cerrado biome	+	-	-	nd
80	Brasília, DF	Soil Cerrado biome	-	nd	-	nd
81	Brasília, DF	Soil Cerrado biome	-	nd	-	nd
82	Brasília, DF	Soil Cerrado biome	-	nd	-	nd
83	Reduto, MG	Soil Mata Atlântica biome	-	nd	-	nd
84	Sertaneja, PR	Soil Mata Atlântica biome	-	nd	-	nd
85	Elói Mendes, MG	Soil Mata Atlântica biome	+	-	-	nd
86	Águas da Prata, MG	Soil Mata Atlântica biome	+	-	-	nd
87	Analândia, SP	Soil Mata Atlântica biome	+	-	-	nd
88	Reduto, MG	Soil Mata Atlântica biome	+	-	-	nd
89	Londrina, PR	Soil Mata Atlântica biome	+	-	-	nd
90	Tamarana, PR	Soil Mata Atlântica biome	+	-	-	nd

91	Tamarana, PR	Soil Mata Atlântica biome	-	nd	-	nd
92	Porto, AC	Soil Amazônia biome	+	-	-	nd
93	Luís Eduardo Magalhães, BA	Soil Cerrado biome	-	nd	-	nd
94	Santa Rosa do Tocantins, TO	Soil Cerrado biome	-	nd	-	nd
95	Coimbra, MG	Soil Mata Atlântica biome	-	nd	-	nd
96	Santa Elvira, MT	Soil Cerrado biome	+	-	-	nd
97	Brasília, DF	Tomato seeds	-	nd	-	nd
98	Brasília, DF	Tomato seeds	-	nd	-	nd
99	Brasília, DF	Tomato seeds	-	nd	-	nd
100	Coimbra, MG	Tomato seeds	-	nd	-	nd

[a] (+) colonies similar of *Ralstonia* spp.; (-) no colony similar of *Ralstonia* spp.
[b] (+) amplification of sequence of 282 bp.; (-) no amplification; (unspecific) more than one amplicon of different size;
[c] (+) amplification of sequence of 408 bp.; (-) no amplification;
[d] phylotype classification: I or II
(nd) = non determined

References

Álvarez, B., López, M. M. & Biosca, E. G. (2007). Influence of native microbiota on survival of *Ralstonia solanacearum* phylotype II in river water microcosms. *Applied and environmental microbiology*, *73*, 7210-7217.

Álvarez, B., López, M. M. & Biosca, E. G. (2008). Survival strategies and pathogenicity of *Ralstonia solanacearum* phylotype II subjected to prolonged starvation in environmental water microcosms. *Microbiology*, *154*, 3590-3598.

Brazil Ministry of the Environment. Office of the National Program for Biodiversity Conservation – DCBio (2010). Fourth National Report to the Convention on Biological Diversity: Brazil. Brazilian Ministry of the Environment, Brasília, Brazil.

Caruso, P., Bertolini, E., Cambra, M. & López, M. M. (2003). A new and sensitive co-operational polymerase chain reaction for rapid detection of *Ralstonia solanacearum* in water. *Journal of microbiological methods*, *55*, 257-272.

Caruso, P., Palomo, J. L., Bertolini, E., Álvarez, B., López, M. M. & Biosca, E. G. (2005). Seasonal variation of *Ralstonia solanacearum* biovar 2 populations in a Spanish river: recovery of stressed cells at low temperatures. *Applied and environmental microbiology*, *71*, 140-148.

Chen, W. Y. & Echandi, E. (1982). Bacteriocin production and semiselective medium for detection, isolation, and quantification of *Pseudomonas solanacearum* in soil. *Phytopathology*, *72*, 310-3.

Coutinho T. A. (2005). Introduction and prospects on the survival of *Ralstonia solanacearum*. In: Allen C., Prior P., Hayward A. C. (eds). Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. *APS press*, St Paul, MN, 29–38.

Dittapongpitch, V. & Surat, S. (2003). Detection of *Ralstonia solanacearum* in soil and weeds from commercial tomato fields using immunocapture and the polymerase chain reaction. *Journal of phytopathology*, *151*, 239-246.

Elphinstone J. G. (2005). The current bacterial wilt situation: a global overview. In: Allen C., Prior P., Hayward A. C., (eds). Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. *American phytopathological society press*, St Paul, MN, USA, 9–28.

Elphinstone J. G., Stanford H. M., Stead D. E. (1998a). Detection of *Ralstonia solanacearum* in potato tubers, *Solanum dulcamara*, and associated irrigation water. In: Prior P., Allen C., Elphinstone J. (eds). Bacterial Wilt Disease: Molecular and Ecological Aspects. *Springer publishing*, Berlin, 133–139.

Elphinstone J. G., Stanford H., Stead D. E. (1998b). Survival and transmission of *Ralstonia solanacearum* in aquatic plants of *Solanum dulcamara* and associated surface water in England 1. *EPPO bulletin*, *28*, 93-94.

Elphinstone, J. G., Hennessy, J., Wilson, J. K. & Stead, D. E. (1996). Sensitivity of different methods for the detection of *Ralstonia solanacearum* in potato tuber extracts. *EPPO bulletin*, *26*, 663-678.

Enroth, H. & Engstrand, L. (1995). Immunomagnetic separation and PCR for detection of *Helicobacter pylori* in water and stool specimens. *Journal of clinical microbiology*, *33*, 2162-2165.

Felix, K. C. S., Souza, E. B., Michereff, S. J. & Mariano, R. L. (2012). Survival of *Ralstonia solanacearum* in infected tissues of *Capsicum annuum* and in soils of the state of Pernambuco, Brazil. *Phytoparasitica*, *40*, 53-62.

Figueiró, A. D. A. (2008). Detecção de *Ralstonia solanacearum* em tubérculos de batata através de PCR qualitativa e quantitativa. Dissertação (Mestrado em Fitotecnia), Universidade Federal do Rio Grande do Sul, Porto Alegre, 72 p.

Gonçalves Mafia, R., Couto Alfenas, A., Penchel Filho, R. M., Alves Ferreira, M. & Ferreira Alfenas, R. (2012). Murcha-bacteriana: disseminação do patógeno e efeitos da doença sobre a clonagem do eucalipto. *Revista árvore*, *36*, 593-602.

Graham, J. & Lloyd, A. B. (1978). An improved indicator plant method for the detection of *Pseudomonas solanacearum* race 3 in soil [Potatoes, bacterial wilt]. *Plant disease reporter*, *62*, 35-7.

Graham, J. & Lloyd, A. B. (1979). Survival of potato strain (race 3) of *Pseudomonas solanacearum* in the deeper soil layers. *Australian journal of agricultural research*, *30*, 489-496.

Graham, J., Jones, D. A. & Lloyd, A. B. (1979). Survival of *Pseudomonas solanacearum* race 3 in plant debris and in latently infected potato tubers. *Phytopathology*, *69*, 1100-1103.

Granada, G. A. & Sequeira, L. (1983). A new selective medium for *Pseudomonas solanacearum*. *Plant disease.*, *67*, 1084-1088.

Grey, B. E. & Steck, T. R. (2001). The viable but nonculturable state of *Ralstonia solanacearum* may be involved in long-term survival and plant infection. *Applied and environmental microbiology*, *67*, 3866-3872.

Hayward A. C., 1994. The hosts of *Pseudomonas solanacearum*. In: Hayward A. C., Hartman G. L. (eds). Bacterial Wilt: The Disease and its Causative Agent, *Pseudomonas solanacearum*. *CAB international*, Wallingford, UK, 9–24.

Hayward, A. C. (1975). Biotypes of *Pseudomonas solanacearum* in Australia. *Australian plant pathology society newsletter*, *4*, 9-11.

Hayward, A. C. (1991). Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. *Annual review of phytopathology*, *29*, 65–87.

Imazaki, I. & Nakaho, K. (2009). Temperature-upshift-mediated revival from the sodium-pyruvate-recoverable viable but nonculturable state induced by low temperature in *Ralstonia solanacearum*: linear regression analysis. *Journal of general plant pathology*, *75*, 213-226.

Imazaki, I. & Nakaho, K. (2010). Pyruvate-amended modified SMSA medium: improved sensitivity for detection of *Ralstonia solanacearum*. *Journal of general plant pathology*, *76*, 52-61.

Ito, S., Ushuima, Y., Fujii, T., Tanaka, S., Kameya- Iwaki, M., Yoshiwara, S. & Kishi, F. (1998). Detection of viable cells of *Ralstonia solanacearum* in soil using a semiselective medium and a PCR technique. *Journal of phytopathology*, *146*, 379-384.

Janse, J. D., Araluppan, F. A. X., Schans, J., Wenneker, M., & Westerhuis, W. (1998). Experiences with bacterial brown rot *Ralstonia solanacearum* biovar 2, race 3 in the Netherlands. In *Bacterial Wilt Disease Springer*, Heidelberg, Berlin, 146-152.

Kelman, A. (1953). The bacterial wilt caused by *Pseudomonas solanacearum*. *Technical bulletin of North Carolina agricultural experiment station*, 99.

Kelman, A. (1956). Factors influencing viability and variation in cultures of *Pseudomonas solanacearum*. *Phytopathology*, *46*, 16-17.

Li, X., Nie, J., Hammill, D. L., Smith, D., Xu, H. & De Boer, S. H. (2014). A comprehensive comparison of assays for detection and identification of *Ralstonia solanacearum* race 3 biovar 2. *Journal of applied microbiology*, *117*, 1132-1143.

Marco-Noales, E., Bertolini, E., Morente, C. & López, M. M. (2008). Integrated approach for detection of nonculturable cells of *Ralstonia solanacearum* in asymptomatic *Pelargonium* spp. cuttings. *Phytopathology*, *98*, 949-955.

Miranda, E. F. O., Takatsu, A. & Uesugi, C. H. (2004). Colonização de raízes de plantas daninhas cultivadas *in vitro* e em vasos por *Ralstonia solanacearum*, biovares 1, 2 e 3. *Fitopatologia brasileira, 29*, 121-127.

Nesmith, W. C. & Jenkins, S. F. (1979). A selective medium for the isolation and quantification of *Pseudomonas solanacearum* from soil. *Phytopathology*, *69*, 182-185.

Netto, R. A. C., Noda, H., Boher, B. & Araújo, B. G. P. (2004). Murcha bacteriana no estado do Amazonas, Brasil. *Fitopatologia brasileira*, *29*, 21-27.

Olsson, K. (1976). Experience of Brown Rot Caused by *Pseudomonas solanacearum* (Smith) Smith in Sweden 1. *EPPO bulletin*, *6*, 199-207.

Opina, N., Tavner, F., Hollway, G., Wang, J. F., Li, T. H., Maghirang, R., Fegan, M., Hayward, A. C., Krishnapillai, V., Hong, W. F., Holloway, B. W. & Timmis, J. N. (1997). A novel method for development of species and strain-specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*). *Asia-Pacific journal of molecular biology and biotechnology*, *5*, 19-30.

Parkinson, N., Bryant, R., Bew, J., Conyers, C., Stones, R., Alcock, M. & Elphinstone, J. (2013). Application of Variable Number Tandem Repeat (VNTR) Typing to Discriminate *Ralstonia solanacearum* Strains Associated with English Watercourses and Disease Outbreaks. *Applied and environmental microbiology*, *79*, 6016-6022.

Perera, A. A. U., Weerasena, O. V. D. S. J., Dasanayaka, P. N. & Wickramarachchi, D. C. (2018). Development of a rapid, sensitive and specific DNA-based method to detect *Ralstonia solanacearum* in potato for quarantine purposes. *Journal of the national science foundation of Sri Lanka*, *46*, 153-158.

Pontes, N. C., Fujinawa, M. F. & Oliveira, J. R. (2017). Selective media for detection and quantification of Brazilian *Ralstonia solanacearum* isolates in soil. *Horticultura brasileira*, *35*, 41-47.

Pradhanang, P. M. & Momol, M. T. (2001). Survival of *Ralstonia solanacearum* in soil under irrigated rice culture and aquatic weeds. *Journal of phytopathology*, *149*, 707-711.

Pradhanang, P. M., Elphinstone, J. G. & Fox, R. T. V. (2000). Sensitive detection of *Ralstonia solanacearum* in soil: a comparison of different detection techniques. *Plant pathology*, *49*, 414-422.

Priou, S., Gutarra, L. & Aley, P. (1999). Highly sensitive detection of *Ralstonia solanacearum* in latently infected potato tubers by post- enrichment enzyme- linked immunosorbent assay on nitrocellulose membrane. *EPPO bulletin*, *29*, 117-125.

Priou, S., Gutarra, L. & Aley, P. (2006). An improved enrichment broth for the sensitive detection of *Ralstonia solanacearum* (biovars 1 and 2A) in soil using DAS–ELISA. *Plant pathology*, *55*, 36-45.

Ramesh, R., Anthony, J., Jaxon, T. C. D., Gaitonde, S. & Achari, G. (2011). PCR-based sensitive detection of *Ralstonia solanacearum* from soil, eggplant, seeds and weeds. *Archives of phytopathology and plant protection*, *44*, 1908-1919.

Safni, I., Cleenwerck, I., Vos, P. D., Fegan, M., Sly, L. & Kappler,po U. (2014). Polyphasic taxonomic revision of the *Ralstonia solanacearum* species complex: proposal to emend the descriptions of the *R. solanacearum* and *R. syzygii* and reclassify current *R. syzygii* strain as *Ralstonia syzygii* subsp. *syzygii*, *R. solanacearum* phylotype IV strains as *Ralstonia syzygii* subsp. *indonesiensis* subsp. nov., banana blood disease bacterium strains as *Ralstonia syzygii* subsp. *celebesensis* subsp. nov. and *R. solanacearum* phylotype I and III strains as *Ralstonia pseudosolanacearum* sp. nov. *International journal of systemic and evolutionary microbiology*, *64*, 3087-3103.

Sanchez Perez, A., Mejia, L., Fegan, M. & Allen, C. (2008). Diversity and distribution of *Ralstonia solanacearum* strains in Guatemala and rare occurrence of tomato fruit infection. *Plant pathology*, *57*, 320-331.

Santiago, T. R., Lopes, C. A., Caetano- Anollés, G. & Mizubuti, E. S. G. (2017). Phylotype and sequevar variability of *Ralstonia solanacearum* in Brazil, an ancient centre of diversity of the pathogen. *Plant pathology*, *66*, 383-392.

Smith, E. F. (1920). The brown rot of Solanaceae. *Introduction to bacterial diseases of plants*, 177-201.

Stander, E. I. M., Hammes, P. S. & Beyers, E. A. (2003). Survival of *Ralstonia solanacearum* biovar 2 in soil under different cropping systems. *South African journal of plant and soil*, *20*, 176-179.

Stevens, P. & van Elsas, J. D. (2010). Genetic and phenotypic diversity of *Ralstonia solanacearum* biovar 2 strains obtained from Dutch waterways. *Antonie van Leeuwenhoek*, *97*, 171-188.

Tran, T. M., Jacobs, J. M., Huerta, A., Milling, A., Weibel, J. & Allen, C. (2016). Sensitive, secure detection of race 3 biovar 2 and native US strains of *Ralstonia solanacearum*. *Plant disease*, *100*, 630-639.

Tusiime, G., Adipala, E., Opio, F., Bhagsari, A. S. (1998) Weeds as latent hosts of *Ralstonia solanacearum* in highland Uganda: implications to development of an integrated control package for bacterial wilt. In: Prior, P., Allen, C. & Elphinstone, J., (eds). Bacterial Wilt Disease: Molecular and Ecological Aspects. *Springer publishing*, Berlin, 413–419.

Van der Wolf, J. M., Vriend, S. G. C., Kastelein, P., Nijhuis, E. H., Van Bekkum, P. J. & Van Vuurde, J. W. L. (2000). Immunofluorescence colony-staining (IFC) for detection and quantification of *Ralstonia* (*Pseudomonas*) *solanacearum* biovar 2 (race 3) in soil and verification of positive results by PCR and dilution plating. *European journal of plant pathology*, *106*, 123-133.

van Elsas, J. D., Kastelein, P., de Vries, P. M. & van Overbeek, L. S. (2001). Effects of ecological factors on the survival and physiology of *Ralstonia solanacearum* bv. 2 in irrigation water. *Canadian journal of microbiology*, *47*, 842-854.

van Elsas, J. D., Kastelein, P., van Bekkum, P., van der Wolf, J. M., de Vries, P. M. & van Overbeek, L. S. (2000). Survival of *Ralstonia solanacearum* biovar 2, the causative agent of potato brown rot, in field and microcosm soils in temperate climates. *Phytopathology*, *90*, 1358-1366.

van Elsas, J. D., van Overbeek, L. S., Bailey, M. J., Schönfeld, J. & Smalla, K. (2005). Fate of *Ralstonia solanacearum* biovar 2 as affected by conditions and soil treatments in temperate climate zones. In: Allen, C., Prior, P. & Hayward, A. C. (eds). Bacterial wilt disease and the *Ralstonia solanacearum* species complex. *APS press*, St. Paul, MN, USA, 39.

van Overbeek, L. S., Bergervoet, J. H., Jacobs, F. H. & van Elsas, J. D. (2004). The low-temperature-induced viable-but-nonculturable state affects the virulence of *Ralstonia solanacearum* biovar 2. *Phytopathology*, *94*, 463-469.

Wenneker, M., Verdel, M. S. W., Groeneveld, R. M. W., Kempenaar, C., Van Beuningen, A. R. & Janse, J. D. (1999). *Ralstonia* (*Pseudomonas*) *solanacearum* race 3 (biovar 2) in surface water and natural weed hosts: First report on stinging nettle (*Urtica dioica*). *European journal of plant pathology*, *105*, 307-315.

Wicker, E., Grassart, L., Coranson- Beaudu, R., Mian, D. & Prior, P. (2009). Epidemiological evidence for the emergence of a new pathogenic variant of *Ralstonia solanacearum* in Martinique (French West Indies). *Plant pathology*, *58*, 853-861.

Wicker, E., Lefeuvre, P., De Cambiaire, J. C., Lemaire, C., Poussier, S. & Prior, P. (2012). Contrasting recombination patterns and demographic histories of the plant pathogen *Ralstonia solanacearum* inferred from MLSA. *The ISME journal*, *6*, 961-974.

Chapter 2 – Genetic variability of *Ralstonia* spp. in a micro-region

Abstract

The knowledge of the genetic variability of a pathogen that possesses good survival and dispersal capabilities is essential to understand evolutionary mechanisms and also to support effective management strategies. In the present study, the municipality of Coimbra, in Minas Gerais state, Brazil, was chosen to analyze genetic diversity. This micro-region is known for the production of fresh tomatoes and bacterial wilt is endemic. This micro-region was used as model to study the dynamics of genotypes of *Ralstonia* spp. sampled in 2015, 2017 and 2018. From 264 isolates, 60 were identified as *R. pseudosolanacearum* and 204, as *R. solanacearum*. Analyses of genetic population were based on BOX-PCR fingerprints. Both species showed low genotypic diversity. Two and 14 haplotypes were detected for *R. pseudosolanacearum* and *R. solanacearum*, respectively. Lack of population structure according to geographic region was observed for both species. The effective dispersal agents can contribute to the spread of haplotypes among regions, particularly there was evidence that water of irrigation can play a role in this process between nearby fields. But, there was evidence that haplotypes vary through time. The change in haplotypes sampled over time could have been influenced by weeds that act as potential reservoirs for both species of *Ralstonia*. From this study, management strategies can be used to avoid introduction of new haplotypes, and control of weeds, treatment of water of irrigation and sanitation of machinery, besides to be important information to assist breeding programs to understanding variability in a micro-region.

Introduction

Understanding the population genetics of a pathogen in its center of origin where the disease it causes is endemic is an interesting endeavour from both evolutionary and epidemiological perspectives. In the case of microorganisms, the pathogen population is expected to have high evolutionary potential with mixed reproductive mode (Milgroom, 2015), high genotypic variability and effective population size. Although populations of pathogenic bacteria are normally clonal, recombination events can be more frequent in some species and are important to the emergence of new genotypes (Spratt & Maiden, 1999). However, the fate of these genotypes can be determined by the environmental conditions and other factors, such as host genotype and the intensity of the processes that affect pathogen dispersal, just to mention a few examples (Spratt & Maiden, 1999). From the epidemiological perspective, the putative availability of primary inoculum must be of concern whenever and wherever fields of susceptible crops are to be installed.

Bacterial wilt has been reported in more than 250 plant species distributed in 54 botanical families and is considered one of the most devastating diseases of cultivated crops (Prior et al., 2016). A large number of hosts can be found in the Solanaceae family, some are economically important crops such as potato (*Solanum tuberosum*), tomato (*S. lycopersicum*), eggplant (*S. melongena*), sweet pepper (*Capsicum annuum*), and tobacco (*Nicotiana tabacum*). In Brazil, tomato is a high-value crop that is susceptible to *Ralstonia* spp. Approximately 4.5 million of tons of tomatoes were produced in Brazil and Minas Gerais state (MG) is the third largest producer (IBGE, 2018). In MG, the Zona da Mata region has approximately 273 ha of tomato (IBGE, 2016), it is one of the largest fresh tomato producing areas in the state. However, bacterial wilt is endemic in this region (Lopes & Rossato, 2018).

Different species of *Ralstonia* can cause bacterial wilt. This bacterium has a wide host range and is distributed in the major agricultural regions (Hayward, 1994).

Besides its capacity to survive in soil for many years, the high genetic variability of the pathogen populations make management of disease a difficult task (Kelman, 1953; Graham, 1979; van Elsas et al., 2005). The symptom of wilt is the result of the colonization of xylem vessels that triggers plant death (Kelman, 1953; Hayward, 1991; Álvarez et al., 2010). The use of host resistance is the main management strategy. Nevertheless, durable cultivar resistance is not easily achieved due to the high genetic variability of the pathogen population and the rate of recombination that contributes to generate distinct genotypes (Kelman, 1953; Graham, 1979; van Elsas et al., 2005; Wicker et al., 2012).

Using genomic and proteomic analyses, the *Ralstonia* complex was recently investigated and three species were proposed: *Ralstonia solanacearum*, *R. pseudosolanacearum*, and *R. syzygii* (Safni et al., 2014; Prior et al., 2016). In Brazil, two species were reported to occur associated with different crops, in different regions: *R. solanacearum* and *R. pseudosolanacearum* (Wicker et al., 2012; Santiago et al., 2017). *R. solanacearum* is widely distributed in the country and based on the phylogeny of the 16S-23S internal transcribed spacer (ITS) region (Fegan & Prior, 2005), two groups of individuals, phylotypes IIA and IIB, can be distinguished among the Brazilian *R. solanacearum* strains. In Brazil, phylotype IIA individuals were obtained predominantly from tomato fields distributed all over the country, while individuals of phylotype IIB were more commonly sampled from highland areas, predominantly associated with potato fields (Santiago et al., 2017). Additionally, based on the analysis of the *egl* gene sequences seven sequevars of *Ralstonia* spp. (1, 4, 18, 27, 28, 41, and 50) were recently reported in Brazil and four of them were reported for the first time (53, 54, 55, and 56) (Santiago et al., 2017).

Different molecular markers have been used to determine genetic variability of the populations of *Ralstonia* spp. Multilocus sequence typing (MLST) as repetitive

sequence-based polymerase chain reaction (BOXA1R, REP and ERIC), amplification of restriction fragments (AFLP), polymorphisms based on restriction fragment size (RFLP), simple sequence repeats (SSR) and multilocus variable number of tandem repeat analysis (MLVA) have been used (van der Wolf, 1998; Jaunet & Wang, 1999; Horita & Tsuchiya, 2000; Poussier et al., 2000; Xue et al., 2011; Siri et al., 2011). The rep-PCR fingerprints revealed clonal lineages within sequevars of *Ralstonia* spp. (Wicker et al. 2012; Lemessa et al. 2010; Norman et al. 2009). Previous studies using BOX fingerprints revealed high diversity among isolates of *R. pseudosolanacearum* in China (Xue et al., 2011), confirming the phylogenetic diversity found in 10 different sequevars described by Xu et al. (2009). Similar results were reported in Japan and Taiwan (Jaunet & Wang, 1999, Morita & Tsuchiya, 2000). On the other hand, using the same markers (BOX), the population of *R. solanacearum* in Uruguay is apparently clonal (Siri et al., 2011). The same result was found by Xue et al. (2011) for strains of *R. solanacearum* collected in China. These authors speculated that these individuals may have been introduced possibly by infected potato tubers.

In Brazil, strains of *Ralstonia* spp. obtained from ornamental plants, eucalypt, Solanaceae crops, banana and heliconia were divided into two main clusters when analyzed by rep-PCR fingerprints (Rodrigues et al., 2012). Both clusters showed high genetic diversity without any correlation with race, biovar or geographic origin. In another study, there were differences in polymorphisms among strains collected in Amazonas state, but there was no association with host or geographic location (Costa et al., 2007). In Pernambuco, bacterial wilt in bell pepper is caused by *R. solanacearum* and *R. pseudosolanacearum*, with the predominance of the latter, but, again, there was no structuring according to host species or geographic region (Garcia et al., 2013). However, also using rep-PCR fingerprints potato strains of *R. solanacearum*, in Rio Grande do Sul state, were grouped according to biovars 1 and 2 (Silveira et al., 2005).

Recently, 301 strains of *Ralstonia* spp. sampled from different regions in Brazil were analyzed using BOX fingerprints (Santiago et al., 2017). This study revealed 253 haplotypes of *R. solanacearum* and high genetic variability of the pathogen population. Movement of strains mainly from South to North in Brazil was detected and there was evidence of recombination. Overall, the results support the hypothesis that Brazil is the center of origin of *R. solanacearum*. In contrast, low genetic variability was found among *R. pseudosolanacearum* strains. Forty eight haplotypes concentrated in the North and Northeast regions were detected. These results indicate that this species was recently introduced in the country.

Knowledge of the genetic variability in a micro-region can reveal important epidemiological processes that may occur at both the spatial and temporal scales. At the spatial scale, dispersal is an important process to investigate. At the temporal scale inoculum dynamics influenced by survival (persistence in the area), and selection by hosts are of interest. Together, this knowledge is important to better support breeding programs aimed at developing resistant varieties. The objective of this study was to investigate the genetic structure of *Ralstonia* spp. at a fine scale in a tomato producing micro-region where bacterial wilt is endemic. The study was conducted in the municipality of Coimbra, Minas Gerais state, Brazil, known for its intense tomato production activity. We collected soil from the rhizosphere of both tomato and weed plants to obtain isolates of *Ralstonia* spp. and to assess the genetic variability using BOX-PCR.

Material and Methods

Soil Samples

Ralstonia solanacearum and *R. pseudosolanacearum* were obtained from the rhizosphere of symptomatic and asymptomatic tomato cultivar Aguamiel and weeds

(*Amaranthus* spp., *B. pilosa* and *S. americanum*) in commercial areas with records of bacterial wilt in the municipality of Coimbra, Minas Gerais state. The area of the municipality is 106.8 Km^2. There are two important tomato producing communities, one located to the west, São Venâncio, and the other to the east, Quartéis (Figure 2).

Soil was sampled with a hoe at a depth of 10 cm, placed in clean plastic bags, and transported to the laboratory in styrofoam boxes to be processed. The geographic coordinates of each sample were registered using a portable GPS device. To allow the identification of weeds, each plant was photographed for further examination and assessment by an expert. A total of 13 tomato fields and 160 soil samples were collected. Samples were collected in 2015 (N = 52), 2017 (N = 73), and 2018 (N = 35) in different locations (Figure 2).

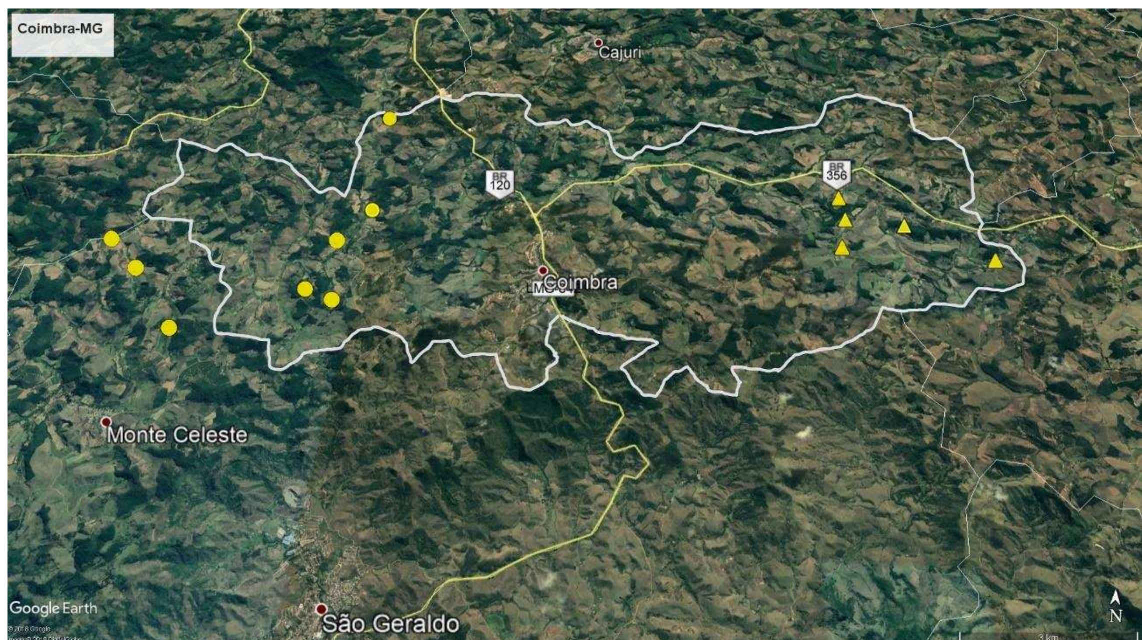


Figure 2. Map of Coimbra with points representing fields (yellow symbols) where soil samples were collected to isolate *Ralstonia* spp. Three samples (circles) were taken in areas located in a neighboring municipality (São Geraldo). Fields were located in two communities: São Venâncio (○) and Quartéis (Δ).

Isolation of *Ralstonia* spp. and growth conditions

The isolation of *Ralstonia* spp. was performed using the enrichment method adapted from Pradhanang et al. (2000). Due to the richness of microorganisms in tropical soils, a serial dilution using 1 g of each soil sample was suspended in 9 mL of distilled water. Then, subsequent dilutions in water up to 10^{-3} were conducted. One mL of the 10^{-3} dilution was added to 9 mL of selective medium South Africa (SMSA) (Elphinstone et al., 1996). The flasks were kept in a rotary shaker at 80 rpm and 28 °C for 2, 4, 6, and 8 h. After each incubation period, 100 µL was added to plates containing solid SMSA. Bacterial colonies grew for 5-10 days at 28 °C. Colonies characteristic of *Ralstonia* spp., with a red center and whitish periphery, were grown in Casamino acid-Peptone-Glucose (CPG) medium to obtain pure culture (Kelman, 1954). The plates were kept in B.O.D incubator at 28 °C for 48 h. For each soil sample, three plates with SMSA were prepared and sown. The process was repeated to soil samples for which no colonies similar to *Ralstonia* spp. could have been obtained. Pure bacterial cultures were stored in cryogenic tubes containing 0.85 % saline solution or cryogenically in 30 % glycerol at -80 °C.

DNA extraction and PCR conditions

The protocol of DNA isolation of gram-negative bacteria of the Wizard® Genomic DNA was used according to the instructions. The pellet was hydrated and resuspended with Tris-EDTA after treatment with RNAse and stored at -20 °C. The quality and concentration of the DNA were analyzed by 1 % agarose gel electrophoresis and NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The concentration of DNA was adjusted to 50 ng/µL.

Polymerase chain reactions were performed using the GoTaq G2 kit from Promega® using the T100M Bio Rad thermocycler. PCR reactions were conducted at a

final volume of 25 μL, containing 1 μL of DNA, 1X Colorless GoTaq Reaction Buffer, 0.2 mM each dNTP, 0.5 μM of each primer, 1.25 U of GoTaq®G2 DNA polymerase (5 U. μL^{-1}) and ultrapure water to complete the final volume.

Identification of species and phylotypes

The isolates were identified using species-specific primers to *Ralstonia* spp. (Opina et al., 1997). The cycling conditions were 96 °C for 3 min for initial denaturation, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s, and 72 °C for 5 min for final extension. The 282 bp amplified fragment was confirmed by 1 % agarose gel electrophoresis stained with GelRed™ and visualized under UV light. The comparison was done with the DNA marker of 100 base pairs (bp) and with DNA of one strain of *R. solanacearum* (RS 279) under the same conditions of PCR with specific primer (positive control).

Ralstonia spp. were classified in phylotypes using the primers: Nmult 21:1F, Nmult 21:2F, Nmult 22:Inf and Nmult 23:AF, and a reverse primer, Nmult 22:RR (Fegan & Prior, 2005). The phylotypes I, II, III and IV have amplicon sizes of 144, 372, 91 and 213 bp, respectively. The thermocycling conditions were 96 °C for 5 min for initial denaturation, 30 cycles of 94 °C for 15 s, 59 °C for 30 s, 72 °C for 30 s, and a final extension of 72 °C for 10 min. Final products of PCR were stained with GelRed ™ and subjected to 1 % agarose gel electrophoresis. Confirmation of phylotype was done by visual inspection and comparison with bands of a 1 kb plus DNA ladder (Invitrogen).

Genomic fingerprinting

The repetitive element PCR genomic fingerprints were generated with the BOX A1R primer (Louws et al., 1994). The thermocycling conditions were 95 °C for 7 min, 35 cycles of 1 min at 94 °C, 1 min at 53 °C, 8 min at 65 °C, and final extension of 16

min at 65 °C. Final products of PCR were stained with GelRed™ and submitted to 1.5 % agarose gel electrophoresis for 5 h at 80 V. Images were obtained from gel documentation system L-PIX EX (Loccus Biotecnologia). A binary matrix was generated after processing the gel images in PyElph 1.4 software. Presence or absence of bands as well as estimated band size were done using the 1 kb ladder as reference.

Genetic variability

The general pattern of cluster was first explored for the whole dataset, with no *a priori* groups defined. This first analysis was accomplished using principal components (PCA) using PAST 3.20 software (Hammer et al., 2001). A dendrogram using the unweighted pair group method with arithmetic mean (UPGMA) with Jaccard's coefficient of similarity from vegan package was also constructed.

As a second step, *Ralstonia* spp. strains were tentative grouped by collection year and regions in Coimbra. Levels of genetic diversity in the populations of *Ralstonia* species were evaluated by calculating potential subdivision according to geographic regions and year of bacteria isolation using the R software (R Core Team 2018). The clonal fraction was calculated for each subpopulation, based in {1 - [(N-G)/N]}*100 (N = sample size, G = number of genotypes) (Zhan et al., 2002). Genotypic diversity of each subpopulation was estimated by Stoddard & Taylor's G index (1988). Hill's number was calculated for comparisons among populations of different sample sizes (Jost 2007). Diversity indices were calculated using vegan (Oksanen et al., 2018) and vegetarian (Charney & Record, 2012) packages. Standard error of the estimates was based on 1000 bootstrap pseudoreplicates.

Genetic differentiation of subpopulations potentially structured according to geographic region or host was assessed based on G''_{ST} values calculated with GENODIVE (Meirmans and Hedrick, 2011). Analysis of molecular variance

(AMOVA), from vegan package, was used to estimate the partitioning of genetic variance among and within populations of geographic region and sampling years.

BayesAss v.1.0 program was used to estimate migration rates among regions in Coimbra. Based on Markov chain Monte Carlo (MCMC) techniques (Wilson and Rannala 2003), 1 million MCMC iterations was used with a frequency of sampling about 10000 generations and 20% of the sample was discarded as burn-in.

Results

A total of 264 isolates of *Ralstonia* spp. were obtained from soil collected in tomato fields with records of bacterial wilt. The number of isolates per field varied: 31 strains were obtained from Wilson field; 37 from Tiago; 27 from Arlindo; 46 from Amarildo; 53 from Roberto; 4 from Sebastião; 30 from Roseli; 8 from Wilson Nene; 1 each from Nilson, Assis and Everaldo; 2 from Sidney; and 23 from Leandro (Table 1). Bacterial strains were confirmed to belong to *Ralstonia* spp. with species-specific primers. Phylotype analyses from multiplex PCR determined that 60 isolates belong to phylotype I, corresponding to *R. pseudosolanacearum* and 204 strains were classified in phylotype II, corresponding to *R. solanacearum*, the predominant species in the tomato fields in Coimbra (Figure 3).

Table 1. Isolates of *Ralstonia* spp. collected in 2015, 2017 and 2018 from different regions (communities) of Coimbra, from distinct fields and host plants.

Year	Region	Field	Host	*R. solanacearum*	*R. pseudosolanacearum*
2015	São Venâncio	Wilson	Tomato	UFV 6, UFV 7, UFV 8, UFV 10, UFV 11, UFV 13, UFV 17, UFV 18, UFV 19, UFV 20, UFV 21, UFV 22, UFV 23, UFV 24, UFV 25, UFV 169, UFV 170, UFV 177, UFV 178, UFV 182, UFV 183, UFV 184, UFV 185, UFV 186, UFV 243, UFV 244, UFV 245	UFV 51, UFV 52, UFV 53, UFV 54
	São Venâncio	Arlindo	Tomato	UFV 167, UFV 168	UFV 57, UFV 62, UFV 63, UFV 64, UFV 65, UFV 79, UFV 80, UFV 81, UFV 92, UFV 94, UFV 95, UFV 96, UFV 116, UFV 117, UFV 118, UFV 119, UFV 120, UFV 121, UFV 122, UFV 123, UFV 124, UFV 125, UFV 126, UFV 127, UFV 128
	São Venâncio	Roberto	Tomato	UFV 188, UFV 189, UFV 190, UFV 191, UFV 192, UFV 193, UFV 194, UFV 195, UFV 196, UFV 197, UFV 198, UFV 199, UFV 200, UFV 201, UFV 202, UFV 203, UFV 204, UFV 205, UFV 206, UFV 208, UFV 209, UFV 210, UFV 211, UFV 212, UFV 213, UFV 214, UFV 215, UFV 216, UFV 217, UFV 218, UFV 219, UFV 220, UFV 221, UFV 222, UFV 223, UFV 224, UFV 225, UFV 227, UFV 228, UFV 257, UFV 259, UFV 260, UFV 261, UFV 262, UFV 263, UFV 264, UFV 265, UFV 266, UFV 267, UFV	

			268, UFV 269, UFV 270, UFV 271	
Quartéis	Amarildo	Pepper	UFV 29, UFV 30, UFV 31, UFV 32, UFV 33, UFV 34, UFV 35, UFV 36	
Quartéis	Amarildo	*Amaranthus* spp.	UFV 37, UFV 38, UFV 39, UFV 40, UFV 41, UFV 42, UFV 44, UFV 45, UFV 174, UFV 175, UFV 176	
Quartéis	Amarildo	Tomato	UFV 46, UFV 47, UFV 48, UFV 49, UFV 50, UFV 129, UFV 130, UFV 131, UFV 132, UFV 133, UFV 134, UFV 135, UFV 136, UFV 137, UFV 138, UFV 139, UFV 140, UFV 141, UFV 142, UFV 143, UFV 154, UFV 155, UFV 156	
Quartéis	Tiago	Tomato	UFV 71, UFV 72, UFV 73, UFV 74, UFV 75, UFV 76, UFV 171, UFV 172, UFV 173, UFV 181	UFV 59, UFV 82, UFV 83, UFV 84, UFV 86, UFV 98, UFV 99, UFV 101, UFV 107, UFV 109, UFV 111, UFV 112, UFV 113, UFV 114, UFV 115, UFV 153, UFV 157, UFV 158, UFV 159, UFV 160
Quartéis	Tiago	*Bidens pilosa*		UFV 102, UFV 103, UFV 104, UFV 106
Quartéis	Tiago	Coffee		UFV 161, UFV 162, UFV 163
Quartéis	Amarildo	*Solanum americanum*		UFV 66, UFV 67, UFV 68, UFV 70

2017	São Venâncio	Roseli	Tomato	UFV 474, UFV 475, UFV 476, UFV 477, UFV 478, UFV 480, UFV 482, UFV 483, UFV 486, UFV 487, UFV 488, UFV 489, UFV 490, UFV 491, UFV 492, UFV 493, UFV 495, UFV 497, UFV 499, UFV 576, UFV 577, UFV 578, UFV 579, UFV 580, UFV 581, UFV 582, UFV 583, UFV 584, UFV 586, UFV 587
	São Venâncio	Wilson	Tomato	UFV 501, UFV 502, UFV 505, UFV 507, UFV 525, UFV 528, UFV 530, UFV 531
	Quartéis	Sebastião	Tomato	UFV 459, UFV 460, UFV 461, UFV 464
	Quartéis	Nilson	Tomato	UFV 604
	Quartéis	Assis	Tomato	UFV 609
	São Venâncio	Everaldo	Tomato	UFV 633
2018	São Venâncio	Sidney	Tomato	UFV 832, UFV 844
	São Venâncio	Leandro	Tomato	UFV 833, UFV 834, UFV 835, UFV 836, UFV 837, UFV 838, UFV 839, UFV 840, UFV 841, UFV 842, UFV 843, UFV 845, UFV 846, UFV 847, UFV 848, UFV 849, UFV 850, UFV 851, UFV 852, UFV 855, UFV 856, UFV 857, UFV 858

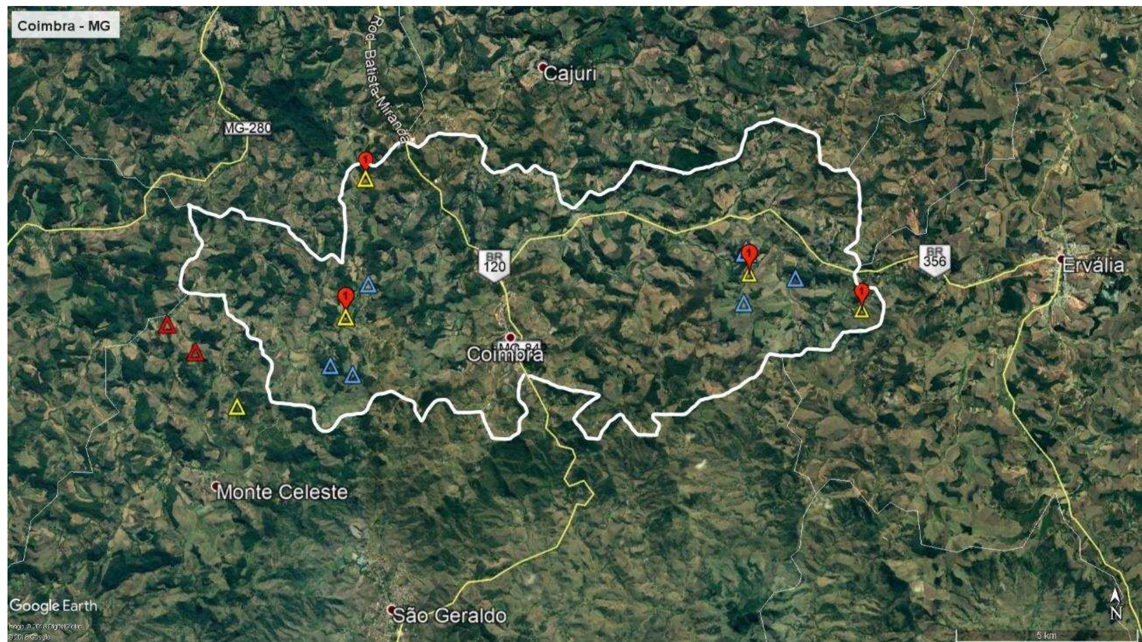


Figure 3. Map of Coimbra with points indicating fields where each species was found. *R. pseudosolanacearum* was detected in four fields (indicated with number 1). *R. solanacearum* was present in 13 tomato fields (Δ). Yellow triangles represent tomato field sampled in 2015, blue triangles are locations sampled in 2017 and red triangles are tomato field in 2018.

The BOX-PCR fingerprints of the 264 strains produced different banding pattern that ranged from 200 to 3500 bp (data not shown). For the 60 isolates of *R. pseudosolanacearum* only two haplotypes were identified and the clonal fraction of the population was 96.7%. *R. pseudosolanacearum* strains were isolated only in 2015 and from four tomato fields (Table 1). Low genotypic diversity were observed for *R. pseudosolanacearum* (Table 2), evidencing low variability in Coimbra. The PCA showed separation of strains by haplotypes (data not shown). Despite the geographical distance among fields (Figure 3), one haplotype of *R. pseudosolanacearum* was present in all four fields. In one tomato field two haplotypes of *R. pseudosolanacearum* were observed (Tiago) (data not shown).

Table 2. Genetic diversity indices of *R. solanacearum* and *R. pseudosolanacearum* strains from Coimbra, MG.

Population	N	MGL	E5	G	N_1
R. solanacearum	204	14	0.645	4.68 (4.25-5.11)*	6.70 (6.26-7.14)
R. pseudosolanacearum	60	2	0.651	1.34 (1.23-1.45)	1.53 (1.40-1.66)

N = sample size; MGL = number of haplotypes; E.5 = evenness; G = index of diversity (Stoddart & Taylor); N_1 = index of diversity genotypic (Hills number). * = 95% confidence interval of each estimate.

For *R. solanacearum*, analysis of the 204 strains showed low genotypic diversity with clonal fraction of the 93.1% and 14 haplotypes were detected. *R. solanacearum* was found in 13 tomato fields distributed in two main communities of Coimbra (São Venâncio and Quartéis) and in the three years of sampling (2015, 2017 and 2018).

Genetic structure was studied by year of sampling (2015, 2017 and 2018) and geographic region (São Venâncio and Quartéis) for *R. solanacearum*. The following fields are located in the São Venâncio region: Wilson, Arlindo, Roberto, Roseli, Wilson Nene, Everaldo, Sidney and Leandro, and region of Quartéis by Tiago, Amarildo, Sebastião, Nilson and Assis (Table 1). In 2015, 11 haplotypes were detected and the clonal fraction was 91.8%, in 2017 and 2018, six and two haplotypes were detected and the clonal fraction was 86.7% and 92.0%, respectively. Higher diversity was observed in 2015 and 2017 compared to 2018 (Table 3). When the analysis was done according to regions, 13 haplotypes were observed in São Venâncio with 91.1% of clonal fraction and six haplotypes in Quartéis with 89.7% (Table 3 and Figure 2). Higher diversity were observed in São Venâncio than in Quartéis (Table 3).

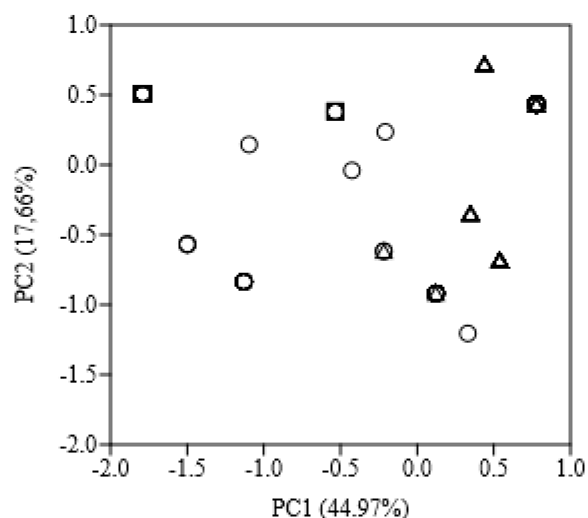


Figure 4. Principal components analysis for haplotypes of *R. solanacearum* represented by geometric forms. Each symbol indicate the year of collection of each haplotype. Circles (○) represent strains of 2015; triangles (∆), 2017; and squares (□), 2018.

According to PCA results there is evidence for a single population of *R. solanacearum* (Figures 4 and 5). The dendrogram constructed using UPGMA method for Jaccard coefficient and the results of the AMOVA confirmed PCA results (Figure 6 and Table 4). Variation of 57.21% and 51.73% was attributed among individuals within years and within regions (Table 4), respectively. Based on G''_{ST} value of the 0.11, there is no evidence of population structure according to regions, however high values of G''_{ST} indicated possible structure by years. The G''_{ST} values between 2015 and 2017 was 0.18. The G''_{ST} values between 2018 and 2015, and between 2018 and 2017 were higher than that between 2015 and 2017: 0.69 and 0.83, respectively, supporting evidence of structure by year.

Movement of *R. solanacearum* isolates between communities was observed in both ways, from São Venâncio to Quartéis (0.25) and from Quartéis to São Venâncio (0.21) (Table 5).

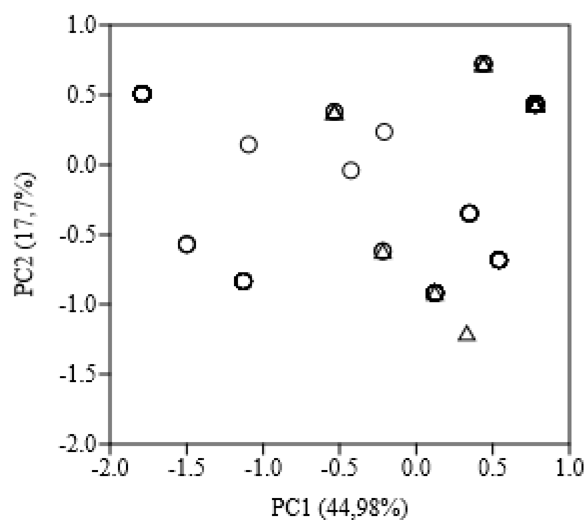


Figure 5. Result of PCA for *R. solanacearum* strains. Circles (○) represent strains of São Venâncio and triangles (Δ) are isolates of Quartéis.

Table 3. Genetic diversity indices of subpopulations of *R. solanacearum* by year or by community

Population	N	MGL	E5	G	N1
Year					
2015	134	11	0.561	3.25 (2.88-3.62)*	5.47 (4.91-6.03)
2017	45	6	0.681	3.24 (2.72-3.76)	4.38 (3.83-4.93)
2018	25	2	0.537	1.17 (1.02-1.29)	1.32 (1.15-1.49)
Total	204	14	0.645	4.68 (4.25-5.11)	6.70 (6.26-7.14)
Community					
São Venâncio	146	13	0.79	6.53 (6.06-7.00)	8.32 (7.74-8.90)
Quartéis	58	6	0.42	1.77 (1.52-2.02)	2.97 (2.50-3.42)
Total	204		0.645	4.68 (4.25-5.11)	6.70 (6.26-7.14)

N = sample size; MGL = number of haplotypes; E.5 = evenness; G = index of diversity (Stoddart & Taylor); N_1 = index of diversity genotypic (Hills number). * = 95% confidence interval of each estimate.

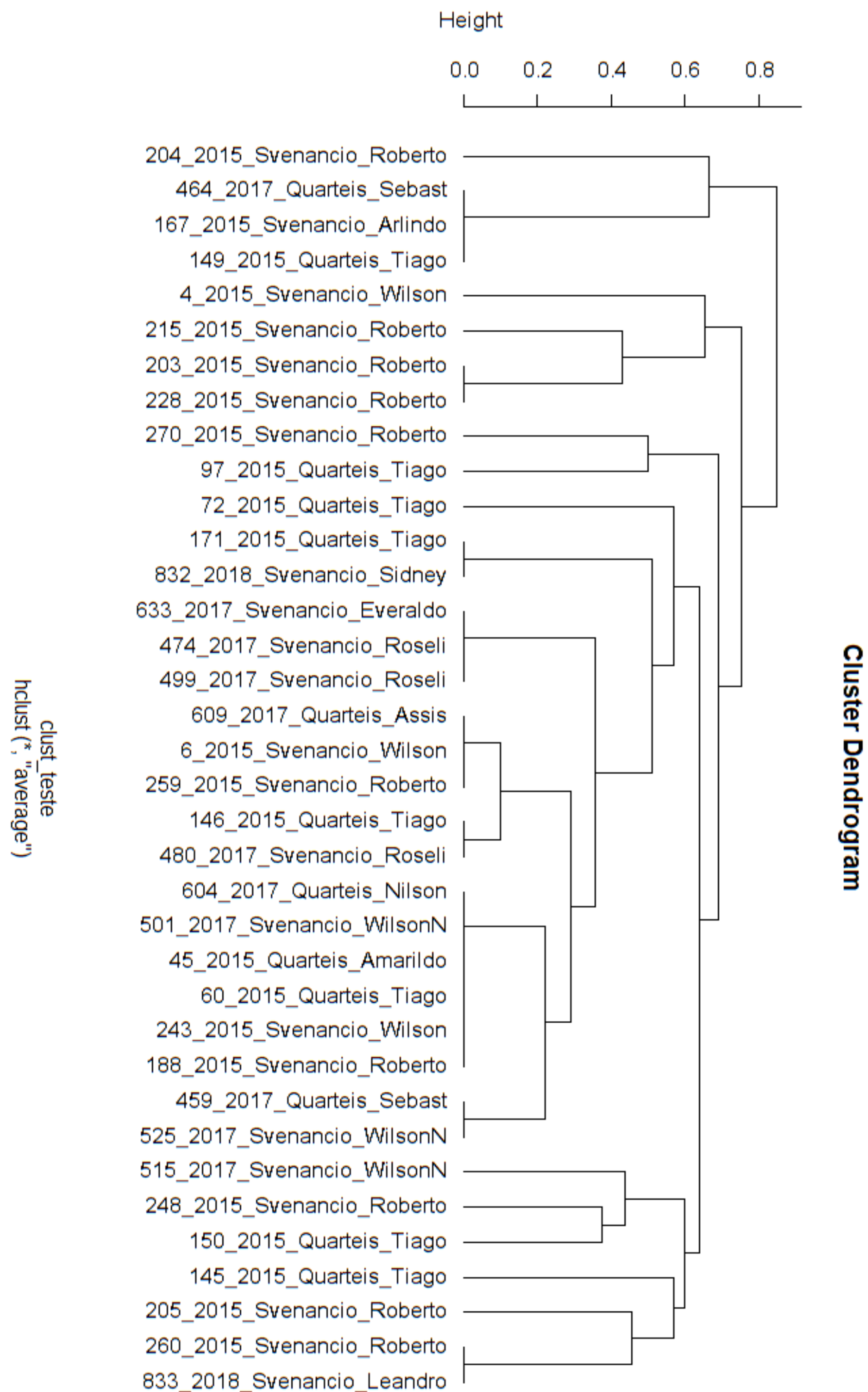


Figure 6. Dendrogram representing 36 strains of *R. solanacearum* based on BOX-PCR fingerprint, using Jaccard's coefficient of similarity. Dendrogram was reproduced by UPGMA. The strains represent haplotypes from each tomato field. Names of each branch of dendrogram are composed of (strain code)_(year of collection)_(region)_(tomato field).

Table 4. Analysis of molecular variance (AMOVA) for 204 individuals of *Ralstonia solanacearum*, using the BOX-PCR fingerprint, sampled from years 2015, 2017 and 2018, from the regions of São Venâncio and Quartéis, in Coimbra, Minas Gerais state, Brazil.

Source of Variation	d.f.	Sum of squared deviations	Proportion of variance components (%)	P-value
Among years	2	113.56	26.70	0.072
Between regions within years	2	43.60	21.57	0.001
Within regions	199	263.92	51.73	0.001
Total	203	421.09	100.00	
Between regions	1	49.05	-3.99	0.311
Among years within regions	3	108.11	46.78	0.001
Within years	199	263.92	57.21	0.001
Total	203	421.09	100.00	

Table 5. Movement rate between geographic regions with confidence intervals of 95% probability estimated by BayesAss.

	Movement from	
Movement into	São Venâncio	Quartéis
São Venâncio	-	0.21
Quartéis	0.25	-

Discussion

This is the first time the populations of *Ralstonia* spp. were intensively studied in a micro-region. Box fingerprints revealed polymorphisms among strains, as reported for *Ralstonia* spp. in other regions in the world (Ramsubhag et al., 2012; Wicker et al., 2011; Zulperi et al., 2016). When analyzed at the country-wide level, there was high genetic diversity among *R. solanacearum* strains in Brazil (Santiago et al., 2017). However, in the present study, at the micro-region level, we found relatively low genotypic variability among the strains of both species of *Ralstonia* from Coimbra.

Considering that Brazil is one putative center of origin of *R. solanacearum* we would expect higher diversity among strains of this species in our sampling. We think three factors could have affected this result: the resolution of the makers used; the size of the sampled area; and selection by host genotype.

Rep-PCR fingerprints can be considered as appropriate to detect polymorphisms to describe clonal lineages of *Ralstonia* spp. (Wicker et al. 2011; Lemessa et al. 2010; Norman et al. 2009). BOX-PCR resolution vary among strains of *Ralstonia* spp. in different regions of the world, but the marker has enough resolution to detect high genetic variability as described in Trinidad (Ramsubhag et al., 2012), in China (Xue et al., 2011), and in Taiwan (Jaunet & Wang, 1999).

The size of area sampled can also be related to low diversity found for both species. The area of the municipality of Coimbra is 106.8 km^2 and low genotypic diversity was expected. In small areas there is higher probability of detecting clonal lineages of *Ralstonia* spp. (Grover et al., 2006). Usually, in small areas number of haplotypes is restricted due to founder effects and selection by host genotype. Low genetic variability was observed among strains of *Ralstonia* spp collected in an island of India (Sakthivel et al., 2016) and in Myanmar (Kyaw et al., 2017). When analysis is based only on strains from host, as eggplant in Philippines, low genetic diversity with few haplotypes was found, suggesting clonality (Ivey et al., 2007). Coimbra is small area, with intense cultivation of a single host (tomato), low genetic variability was expected.

Even though the genotypic diversity was low, the number of haplotypes of *R. solanacearum*, 14, was higher than that of *R. pseudosolanacearum*, for which only two haplotypes were detected. The low genotypic diversity found in *R. pseudosolanacearum* supports the hypothesis of recent introduction in Brazil (Garcia et al., 2013; Santiago et al., 2017). The results reported here are similar to those reported earlier for strains

sampled from several regions of Brazil (Santiago et al., 2017). The number of haplotypes of *R*. *pseudosolanacearum* was seven times smaller than that of *R*. *solanacearum* and strains of the former were only sampled in two regions: North and Northeast (Santiago et al., 2017). In the present study, *R*. *pseudosolanacearum* was sampled only in 2015. It is not possible to ascertain the routes or processes that introduced *R*. *pseudosolanacearum* in Coimbra. We speculate that most likely this could have been associated with contaminated tomato seedlings. This could have been a single event, since no *R*. *pseudosolanacearum* strains were obtained in the 2017 and 2018 samplings. There are only two major sellers of tomato seedlings in Coimbra. Maybe introduction of *R*. *pseudosolanacearum* was through tomato seedlings coming from other providers. Unknown factors could be involved with non establishment of the species in Coimbra

There was no genetic structuring of the populations of *Ralstonia* spp. according to geographic region. In India, populations of *R. pseudosolanacearum* were not structured by host or geographic origin (Ramesh et al., 2014). The lack of structure of *R. pseudosolanacearum* according to geographic region was also reported in China and Trinidad, and the high genetic variability is probably influenced by events of recombination. In these studies, the population of *R. solanacearum* was shown to be clonal (Xue et al., 2011; Ramsubhag et al., 2012). In our study, there was evidence for structuring according to year of sampling. However, these results can be related to the disproportionate sample sizes, particularly for 2018. It was possible to obtain strains of *R. solanacearum* from only two tomato fields in 2018. For 2015 and 2017, values of G''_{ST} indicated low differentiation suggesting persistence of the population over the time period.

Movement of isolates between tomato fields could be detected using the genetic marker. Pathogen dispersal may influence genetic variability, as suggested for *Ralstonia*

spp. in Trinidad and Tobago (Ramsubhag et al., 2012). The dispersal of *R. solanacearum* in Coimbra could have been mediated by water used for irrigation between nearby tomato fields, as reported in several regions in the world (Hayward, 1991; Wenneker et al., 1999; Pradhanang & Momol, 2001; Netto et al., 2004; Parkinson et al., 2013). In England, the distribution pattern of bacterial wilt was related to the water used for irrigation (Parkinson et al., 2013). The same was suggested to explain the high incidence of Moko disease along the Solimões and Amazonas rivers, in the Amazon, Brazil (Netto et al., 2004), but no formal analysis was conducted to assess this potential route of pathogen dispersal. In chapter 1, two samples of water from Coimbra, Minas Gerais, used to irrigate tomato fields, were positive for *Ralstonia* spp. This indicate the importance of rivers for pathogen dispersal, but further studies are necessary to investigate this correlation. Shared equipments can also be related to the dispersal of *Ralstonia* spp. (Jansen, 1996; Champoiseau et al., 2009). Tomato growers in Coimbra commonly share machineries that revolve soil. Additionally, detection of *Ralstonia* spp. in rhizosphere of the weeds, *Amaranthus* spp., *Bidens pilosa* and *Solanum americanum*, reveal the importance of these plants to survival of the pathogen in infested areas. Besides that, weeds can serve as inoculum sources and can be dispersed by rivers and equipment carrying contaminated soil. After artificially inoculating the weeds *Amaranthus* spp., *B. pilosa* and *S. americanum*, they served as potential reservoir of *Ralstonia* spp. in Brazil (Orozco et al., 2004). Several studies have already reported that weeds contribute to the maintenance of the pathogen in the area (Tusiime et al., 1998; Wenneker et al., 1999; Dittapongpitch & Surat, 2003; Wicker et al., 2009).

Our results indicate low genetic variability of *R. solanacearum* in the Coimbra micro-region of intense tomato production and presence of *R. pseudosolanacearum*, both as clonal population. Several studies conducted in different regions of Brazil

demonstrated that the genetic variability and distribution of genotypes are not related to host or geographic origin of each strain. Only in Rio Grande do Sul state, strains of *R. solanacearum* sampled of potato fields are formed by two main clusters, biovar 1 and 2 (Siqueira et al., 2005). In the Amazon region, high genetic variability of isolates of *R. solanacearum* was not correlated with a geographic criterion, race or biovar. The same conclusion was reached for *R. pseudosolanacearum* in the Agreste and Zona da Mata regions of Pernambuco state (Garcia et al., 2013). Sampling issues such as repeated collection from the exact same spot and the number of strains can affect the results of genetic variability. Garcia et al. (2013) found high genetic variability of *R. pseudosolanacearum* in the Agreste and Zona da Mata regions. In contrast, Santiago et al. (2017) analyzed few isolates of this region and found low genetic variability. The 264 strains analyzed from this micro-region, representing the current situation of genetic variability of *Ralstonia* spp. in Coimbra, revealed a similar pattern found by Santiago et al. (2017).

The breeding programs aimed at developing resistant varieties, the strategic planning of management actions to reduce damage by bacterial wilt, and high-sensitivity pathogen detection methods can benefit from the studies of genetic variability of the pathogen. Because Brazil is supposedly a center of origin of *R. solanacearum* different regions may have genetically distinct local populations which may be more effectively managed by one practice or plant variety and not so effective by other(s). Besides the genetic variability among strains of *Ralstonia* spp., temperature and other environmental variables can affect cultivar resistance (French & De Lindo, 1982; Hayward, 1991; Wang et al., 2000). Control measures can be directed by the genetic variability of *Ralstonia* spp. For instance, phages are potential agents to control bacterial diseases. The genetic variability of the pathogen can affect the sensitivity of methods of detection of *Ralstonia* spp. in water of irrigation and weeds, and to avoid

importation of vegetal material with latent infection. Based on the pattern of genetic diversity strains can be analyzed to investigate recent introduction of contaminated plant material, for instance potato seed tubers, with new genotypes (Wicker et al., 2009, Marco-Noales et al., 2008; Nouri et al., 2008. Wang et al., 2017). Therefore, improvements of detection methods are necessary to inspect materials for quarantine purposes (Marco-Noales et al., 2008). This is the first report of *R. pseudosolanacearum* in tomato in the state of Minas Gerais. *R. pseudosolanacearum* have been reported associated with chickpea plants in Minas Gerais (Salcedo et al., 2017). Curiously, we were able to isolate three strains of *R. pseudosolanacearum* from a soil sample from the rhizosphere of coffee plants. Lopes et al. (2005) reported that coffee plants are potential hosts to *Ralstonia* spp. and our results reveal that coffee plant can allow the survival of the pathogen. Probably, new genotypes of *Ralstonia* spp. are introduced in the studied micro-region and that selection exerted by tomato hybrids that are regularly cultivated in the area contribute to shape the genetic variability of the pathogen. Factors that influence and contribute to the introduction of new haplotypes in the area are still unknown and need to be explored.

Acknowledgements

The research was supported by FAPEMIG. The authors thank Thaís Ribeiro Santiago and Camila Geovanna Ferro for helpful discussion and for technical support.

References

Albuquerque, G. M., Santos, L. A., Felix, K. C., Rollemberg, C. L., Silva, A. M., Souza, E. B., Cellier, G., Prior, P. & Mariano, R. L. (2014). Moko disease-causing strains of *Ralstonia solanacearum* from Brazil extend known diversity in paraphyletic phylotype II. *Phytopathology*, *104*, 1175-1182.

Álvarez, B., Biosca, E. G. & López, M. M. (2010). On the life of *Ralstonia solanacearum*, a destructive bacterial plant pathogen. *Current research, technology and education topics in applied microbiology and microbial biotechnology*, *1*, 267-279.

Charney, N. & Record, S. (2012). Vegetarian: Jost Diversity Measures for Community Data. *R package version 1.2*. https://CRAN.R-project.org/package=vegetarian

Costa, S. B., Ferreira, M. A. S. V. & Lopes, C. A. (2007). Diversidade patogênica e molecular de *Ralstonia solanacearum* da região amazônica brasileira. *Fitopatologia brasileira*, *32*, 285-294.

Dittapongpitch, V. & Surat, S. (2003). Detection of *Ralstonia solanacearum* in soil and weeds from commercial tomato fields using immunocapture and the polymerase chain reaction. *Journal of phytopathology*, *151*, 239-246.

Elphinstone, J. G., Hennessy, J., Wilson, J. K. & Stead, D. E. (1996). Sensitivity of different methods for the detection of *Ralstonia solanacearum* in potato tuber extracts. *EPPO bulletin*, *26*, 663-678.

Fegan, M. & Prior, P. (2005). How complex is the "*Ralstonia solanacearum* species complex?". In: C. Allen, P. Prior, and A. C. Hayward, eds. Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. *American phytopathological society*, St. Paul, MN., 449-461.

French, E. R., & De Lindo, L. (1982). Resistance to *Pseudomonas solanacearum* in potato: specificity and temperature sensitivity. *Phytopathology, 72*, 1408–1412.

Graham, J., Jones, D. A. & Lloyd, A. B. (1979). Survival of *Pseudomonas solanacearum* race 3 in plant debris and in latently infected potato tubers. *Phytopathology*, *69*, 1100-1103.

Guinard, J., Latreille, A., Guérin, F., Poussier, S. & Wicker, E. (2017). New multilocus variable-number tandem-repeat analysis (MLVA) scheme for fine-scale monitoring and microevolution - related study of *Ralstonia pseudosolanacearum* phylotype I populations. *Applied and environmental microbiology*, *83*, e03095–e03016.

Hammer, Ø., Harper, D.A.T., Ryan, P.D. (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia electronica*, *4*, 9pp. http://palaeo-electronica.org/2001_1/past/issue1_01.htm

Hayward, A. C. (2000). *Ralstonia solanacearum*. *Encyclopedia of microbiology*, *4*, 32-42.

Hayward, A. C. (1991). Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. *Annual review phytopathology*, *29*, 65-87.

Hayward, A.C. (1994). The hosts of *Pseudomonas solanacearum*. In: Hayward, A. C. & Hartman, G. L. (eds). Bacterial Wilt: The disease and its causative agent, *Pseudomonas solanacearum*. *CAB international*, Wallingford, UK, 9-24.

Horita, M., & Tsuchiya, K. (2001). Genetic diversity of Japanese strains of *Ralstonia solanacearum*. *Phytopathology*, *91*, 399-407.

Ivey, M. L. L., Gardener, B. B. M., Opina, N. & Miller, S. A. (2007). Diversity of *Ralstonia solanacearum* infecting eggplant in the Philippines. *Phytopathology*, *97*, 1467-1475.

Jaunet, T. X., & Wang, J.-F. (1999). Variation in genotype and aggressiveness diversity of *Ralstonia solanacearum* race 1 isolated from tomato in Taiwan. *Phytopathology*, *89*, 320-327.

Jost, L. (2007). Partitioning diversity into independent alpha and beta components. *Ecology*, *88*, 2427-2439.

Kamvar, Z. N., Brooks, J. C. & Grünwald, N. J. (2015). Novel R tools for analysis of genome-wide population genetic data with emphasis on clonality. *Frontiers in genetics*, *6*, 208.

Kamvar, Z. N., Tabima, J. F. & Grünwald, N. J. (2014). Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ*, *2*, e281.

Kelman, A. (1953). The bacterial wilt caused by *Pseudomonas solanacearum*. *Technical bulletin of North Carolina agricultural experiment station*, 99.

Lemessa, F., Zeller, W. & Negeri, D. (2010). Genetic diversity among strains of *Ralstonia solanacearum* from Ethiopia assessed by repetitive sequence-based polymerase chain reaction (rep-PCR). *EJAST*, *1*, 17-26.

Louws, F. J., Fulbright, D. W., Stephens, C. T. & De Bruijn, F. J. (1994). Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars

and strains generated with repetitive sequences and PCR. *Applied and environmental microbiology*, *60*, 2286-2295.

Mahbou Somo Toukam, G., Cellier, G., Wicker, E., Guilbaud, C., Kahane, R., Allen, C. & Prior, P. (2009). Broad diversity of *Ralstonia solanacearum* strains in Cameroon. *Plant disease*, *93*, 1123-1130.

McDonald, B. A. & Linde, C. (2002). Pathogen population genetics, evolutionary potential, and durable resistance. *Annual review of phytopathology*, *40*, 349-379.

Milgroom, M. G. & Fry, W. E. (1997). Contributions of population genetics to plant disease epidemiology and management. *Advances in botanical research academic press*, *24*, 1-30.

Netto, R. A. C., Noda, H., Boher, B. & Araújo, B. G. P. (2004). Murcha bacteriana no estado do Amazonas, Brasil. *Fitopatologia brasileira*, *29*, 21-27.

Nouri, S., Bahar, M. & Fegan, M. (2009). Diversity of *Ralstonia solanacearum* causing potato bacterial wilt in Iran and the first record of phylotype II/biovar 2T strains outside South America. *Plant pathology*, *58*, 243-249.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Henry, M., Stevens, H., Szoecs, E. & Wagner, H. (2018). Package vegan: Community Ecology Package. *R package version 2.5-2*. https://CRAN.R-project.org/package=vegan

Opina, N., Tavner, F., Hollway, G., Wang, J. F., Li, T. H., Maghirang, R., Fegan, M., Hayward, A. C., Krishnapillai, V., Hong, W. F., Holloway, B. W. & Timmis, J. N. (1997). A novel method for development of species and strain-specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*). *Asia-pacific journal of molecular biology and biotechnology*, *5*, 19-30.

Parkinson, N., Bryant, R., Bew, J., Conyers, C., Stones, R., Alcock, M. & Elphinstone, J. (2013). Application of Variable Number Tandem Repeat (VNTR) Typing to Discriminate *Ralstonia solanacearum* Strains Associated with English Watercourses and Disease Outbreaks. *Applied and environmental microbiology*, *79*, 6016-6022.

Poussier, S., Prior, P., Luisetti, J., Hayward, C. & Fegan, M. (2000). Partial sequencing of the *hrpB* and endoglucanase genes confirms and expands the known diversity within

the *Ralstonia solanacearum* species complex. *Systematic and applied microbiology*, 23, 479-486.

Pradhanang, P. M. & Momol, M. T. (2001). Survival of *Ralstonia solanacearum* in soil under irrigated rice culture and aquatic weeds. *Journal of phytopathology*, *149*, 707-711.

Pradhanang, P. M., Elphinstone, J. G. & Fox, R. T. V. (2000). Sensitive detection of *Ralstonia solanacearum* in soil: a comparison of different detection techniques. *Plant pathology*, *49*, 414-422.

Prior, P., Ailloud, F., Dalsing, B. L., Remenant, B., Sanchez, B. & Allen, C. (2016). Genomic and proteomic evidence supporting the division of the plant pathogen *Ralstonia solanacearum* into three species. *BMC genomics*, *17*, 90.

R Core Team (2018). R: A language and environment for statistical computing. *R Foundation for Statistical Computing*, Vienna, Austria. URL https://www.R-project.org/.

Ramesh, R., Achari, G. A. & Gaitonde, S. (2014). Genetic diversity of *Ralstonia solanacearum* infecting solanaceous vegetables from India reveals the existence of unknown or newer sequevars of Phylotype I strains. *European journal of plant pathology*, *140*, 543-562.

Ramsubhag, A., Lawrence, D., Cassie, D., Fraser, R., Umaharan, P., Prior, P. & Wicker, E. (2012). Wide genetic diversity of *Ralstonia solanacearum* strains affecting tomato in Trinidad, West Indies. *Plant pathology*, *61*, 844-857.

Ravelomanantsoa, S., Vernière, C., Rieux, A., Costet, L., Chiroleu, F., Arribat, S., Cellier, G., Pruvost, O., Poussier, S., Robène, I. & Guérin, F. (2018). Molecular Epidemiology of Bacterial Wilt in the Madagascar Highlands Caused by Andean (Phylotype IIB-1) and African (Phylotype III) Brown Rot Strains of the *Ralstonia solanacearum* Species Complex. *Frontiers in plant science*, *8*, 2258.

Rodrigues, L. M. R., Destefano, S. A. L., Da Silva, M. J., Costa, G. G. L. & Maringoni, A. C. (2012). Characterization of *Ralstonia solanacearum* strains from Brazil using molecular methods and pathogenicity tests. *Journal of plant pathology*, *94*, 505-516.

Safni, I., Cleenwerck, I., Vos, P. D., Fegan, M., Sly, L. & Kappler, U. (2014). Polyphasic taxonomic revision of the *Ralstonia solanacearum* species complex:

proposal to emend the descriptions of the *R. solanacearum* and *R. syzygii* and reclassify current *R. syzygii* strain as *Ralstonia syzygii* subsp. *syzygii*, *R. solanacearum* phylotype IV strains as *Ralstonia syzygii* subsp. *indonesiensis* subsp. nov., banana blood disease bacterium strains as *Ralstonia syzygii* subsp. *celebesensis* subsp. nov. and *R. solanacearum* phylotype I and III strains as *Ralstonia pseudosolanacearum* sp. nov. *International journal of systemic and evolutionary microbiology*, *64*, 3087-3103.

Santana, B. G., Lopes, C. A., Alvarez, E., Barreto, C. C., Allen, C. & Quirino, B. F. (2012). Diversity of Brazilian biovar 2 strains of *Ralstonia solanacearum*. *Journal of general plant pathology*, *78*, 190-200.

Santiago, T. R., Lopes, C. A., Caetano- Anollés, G. & Mizubuti, E. S. G. (2017). Phylotype and sequevar variability of *Ralstonia solanacearum* in Brazil, an ancient centre of diversity of the pathogen. *Plant pathology*, *66*, 383-392.

Silveira, J. R. P., Duarte, V., Moraes, M. G. D., Oliveira, A. M. R. D., Barni, V. & Maciel, J. L. N. (2005). Caracterização de estirpes de *Ralstonia solanacearum* isoladas de plantas de batata com murcha bacteriana, por PCR-Rep e RAPD. *Fitopatologia brasileira*, *30*, 615-622.

Siri, M. I., Sanabria, A. & Pianzzola, M. J. (2011). Genetic diversity and aggressiveness of *Ralstonia solanacearum* strains causing bacterial wilt of potato in Uruguay. *Plant disease*, *95*, 1292-1301.

Smith, E.F. 1896. A bacterial disease of the tomato, eggplant and Irish potato. *U.S. Department of agriculture bulletin*, *12*, 1-26.

Spratt, B. G. & Maiden, M. C. (1999). Bacterial population genetics, evolution and epidemiology. *Philosophical Transactions of the Royal Society of London B: Biological sciences*, *354*, 701-710.

Stoddart, J. A. & Taylor, J. F. (1988). Genotypic diversity: estimation and prediction in samples. *Genetics*, *118*, 705-711.

Stukenbrock, E. H. & McDonald, B. A. (2008). The origins of plant pathogens in agro-ecosystems. *Annual review of phytopathology*, *46*, 75-100.

Tusiime, G., Adipala, E., Opio, F., Bhagsari, A. S. (1998) Weeds as latent hosts of *Ralstonia solanacearum* in highland Uganda: implications to development of an integrated control package for bacterial wilt. In: Prior, P., Allen, C. & Elphinstone, J.,

(eds). Bacterial Wilt Disease: Molecular and Ecological Aspects. *Springer publishing*, Berlin, 413–419.

Van der Wolf, J. M., Vriend, S. G. C., Kastelein, P., Nijhuis, E. H., Van Bekkum, P. J. & Van Vuurde, J. W. L. (2000). Immunofluorescence colony-staining (IFC) for detection and quantification of *Ralstonia* (*Pseudomonas*) *solanacearum* biovar 2 (race 3) in soil and verification of positive results by PCR and dilution plating. *European journal of plant pathology*, *106*, 123-133.

van Elsas, J. D., van Overbeek, L. S., Bailey, M. J., Schönfeld, J. & Smalla, K. (2005). Fate of *Ralstonia solanacearum* biovar 2 as affected by conditions and soil treatments in temperate climate zones. In: Allen, C., Prior, P. & Hayward, A. C. (eds). Bacterial wilt disease and the *Ralstonia solanacearum* species complex. *APS press*, St. Paul, MN, USA, 39.

Wang, J. F., Olivier, J., Thoquet, P., Mangin, B., Sauviac, L. & Grimsley, N. H. (2000). Resistance of tomato line Hawaii 7996 to *Ralstonia solanacearum* Pss4 in Taiwan is controlled mainly by a major strain-specific locus. *Molecular plant-microbe interactions*, *13*, 6-13.

Wang, L., Wang, B., Zhao, G., Cai, X., Jabaji, S., Seguin, P. & Chen, H. (2017). Genetic and pathogenic diversity of *Ralstonia solanacearum* causing potato brown rot in China. *American journal of potato research*, *94*, 403-416.

Wenneker, M., Verdel, M. S. W., Groeneveld, R. M. W., Kempenaar, C., Van Beuningen, A. R. & Janse, J. D. (1999). *Ralstonia* (*Pseudomonas*) *solanacearum* race 3 (biovar 2) in surface water and natural weed hosts: First report on stinging nettle (*Urtica dioica*). *European journal of plant pathology*, *105*, 307-315.

Wicker, E., Grassart, L., Coranson- Beaudu, R., Mian, D. & Prior, P. (2009). Epidemiological evidence for the emergence of a new pathogenic variant of *Ralstonia solanacearum* in Martinique (French West Indies). *Plant pathology*, *58*, 853-861.

Wicker, E., Lefeuvre, P., De Cambiaire, J. C., Lemaire, C., Poussier, S. & Prior, P. (2012). Contrasting recombination patterns and demographic histories of the plant pathogen *Ralstonia solanacearum* inferred from MLSA. *The ISME journal*, *6*, 961.

Xu, J., Pan, Z. C., Prior, P., Xu, J. S., Zhang, Z., Zhang, H., Zhang, L.Q., He, L.Y. & Feng, J. (2009). Genetic diversity of *Ralstonia solanacearum* strains from China. *European journal of plant pathology*, *125*, 641-653.

Xue, Q. Y., Yin, Y. N., Yang, W., Heuer, H., Prior, P., Guo, J. H., & Smalla, K. (2011). Genetic diversity of *Ralstonia solanacearum* strains from China assessed by PCR-based fingerprints to unravel host plant-and site-dependent distribution patterns. *FEMS Microbiology ecology*, *75*, 507-519.

Yabuuchi E., Yoshimasa K., Yano I., Hotta H. & Nishiuchi Y. (1895). Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia solanacearum* gen. nov.: Proposal of *Ralstonia pickettii* (Ralston, Palleroni e Doudoroff, 1973) comb. nov., *Ralstonia solanacearum* (Smith, 1986) comb. nov., and *Ralstonia eutropho* (Davis, 1969) comb. nov. *Microbiology and immunology*, *39*, 897-904.

Yamada, T., Satoh, S., Ishikawa, H., Fujiwara, A., Kawasaki, T., Fujie, M. & Ogata, H. (2010). A jumbo phage infecting the phytopathogen *Ralstonia solanacearum* defines a new lineage of the *Myoviridae* family. *Virology*, *398*, 135-147.

Zulperi, D., Sijam, K., Ahmad, Z. A. M., Awang, Y., Ismail, S. I., Asib, N. & Hata, E. M. (2016). Genetic diversity of *Ralstonia solanacearum* phylotype II sequevar 4 strains associated with Moko disease of banana (*Musa* spp.) in Peninsular Malaysia. *European journal of plant pathology*, *144*, 257-270.

Chapter 3 – Selection and characterization of bacteriophages potentially useful for the control of tomato bacterial wilt (*Ralstonia* spp.)

Abstract

Bacterial wilt (BW) is caused by *Ralstonia* spp. and is one of the most destructive disease of plants in the world. The control of BW is difficult due to the capacity of the pathogen to survive in soil, its high genetic variability and wide host range. The use of bacteriophages seems to be an interesting alternative to control BW. Due to the specificity of these viruses to bacterial species or even strains it is necessary to isolate phages from populations and places where they are expected to be used. In this study, we proposed to select and characterize phages to control bacterial wilt. Five phages were isolated from soil samples collected in the municipality of Coimbra, Minas Gerais state, Brazil. *In vitro*, the phages were challenged with 24 strains of *R. solanacearum* from different plant hosts and regions of Brazil. The five phages had different capacities to infect bacterial strains. φRS 576 infected 16 bacterial strains; φRS633, 14; φRS475, 12; φRS519, 7; and φRS618, 6. Nucleic acids of each phage strain were extracted and analyzed as well as the nature and size of the genome. All strains had DNA as nucleic acid, φRS519 and φRS618 have genome size of 80 kb and the other phages, 56 kb. Based on the analysis of electron micrographs, all phages are from the *Siphoviridae* family, *Caudovirales* order. Efficiency of phages to control BW was evaluated in tomato seedlings in growth chamber. The phages were tested either individually or in a mixture (5 phages). The roots were treated with phages and transplanted in soil infested with five *Ralstonia* spp. strains. After one week, all phage-treated tomato seedlings wilted. When the number of colony-forming unit per gram of soil (CFU.g^{-1}) was evaluated, no significant differences were observed between the treatments of phages

and the positive control. Phage are potential agents to control BW, however, additional experiments are needed to adjust application mode and frequency.

Introduction

Bacteriophages or phages are viruses that infect prokaryotes. These viruses are known for a long time and can act as agents capable of regulating bacterial population in different environments (Abedon, 2009; Koskella & Brockhurst, 2014). Most phages are species-specific infecting agents, but higher specificity can be observed even at the strain level, i.e. some phages can infect only some individuals of a given species (O'Flaherty et al., 2009; Álvarez & Biosca, 2017). The high host-specificity allows the use of phages to control bacteria of interest in the food industry (Schnabel & Jones, 2001; Goode et al., 2003; Bai et al., 2016), that are zoonotic pathogens (Atterbury et al., 2003; Atterbury, 2009), as well as those pathogenic to humans (Wright et al., 2009), or plants (O'Flaherty et al., 2009; Yamada, 2012; Frampton et al., 2012; Buttimer et al., 2017; Álvarez & Biosca, 2017). Usually, phages prevent bacterial development due to the lysis effect as a result of either the lysogenic or lytic cycle. When the phage genome is integrated into the bacterial genome and multiply together, the bacterial cycle is known as lysogenic cycle (Weinbauer, 2004). The lytic cycle is characterized by the capacity of the phage genome to alter the cell machinery to replicate itself and form new particles. As a consequence, rapid cell death and lysis can take place and phages are released (Weinbauer, 2004).

Several studies demonstrated the efficiency of phages to control bacterial diseases of plants. Phages isolated from the soil and apples with fire blight (*Erwinia amylovora*) had a wide host range, ideal to formulate a cocktail of different strains (Schnabel & Jones, 2001). Another example is the management of soft rot caused by several pectinolytic bacteria. Soft rot is a difficult disease to control, but phages seem

promising to be used in potato crops (Adriaenssens et al., 2012; Czajkowski et al., 2014; Czajkowski et al., 2015). For tomato bacterial spot, caused by *Xanthomonas campestris* pv. *vesicatoria*, the spray of phages to control the disease has been shown to be effective (Balogh et al., 2003; Obradovic et al., 2004).

Bacterial wilt is one of the most important plant diseases worldwide. This disease is considered by many as the second most devastating bacterial plant disease (Mansfield et al., 2012). Bacterial wilt is caused by different *Ralstonia* species: *R. solanacearum*, *R. pseudosolanacearum*, *R. syzygii* subsp. *indonesiensis*, *R. syzygii* subsp. *celebesensis*, and *R. syzygii* subsp. *syzygii* (Safni et al., 2014). These pathogens infect more than 250 plant species distributed in 54 botanical families (Prior et al., 2016). Besides the wide host range these pathogens have the ability to survive associated with asymptomatic plant materials or in soil for many years (Kelman, 1953; Graham et al., 1979; van Elsas et al., 2005 Graham & Lloyd, 1979; Elphinstone et al., 1998; Janse et al., 1998; Parkinson et al., 2013). Once infection has occurred, plant wilt is practically irreversible. Thus, management of bacterial wilt is a challenge. Currently, very few options are available to try to prevent wilt epidemics and most practices are preventative, such as choosing planting areas without historical records of the disease. Crop rotation is used to reduce inoculum in the field, but in many cases, the frequency and extension of the rotations are not effective enough to prevent epidemics (Stander et al., 2003). Chemical control with antibiotics is not effective and has serious environmental issues (Hartman & Elphinstone, 1994). Therefore, one potentially useful alternative method is biological control using phages (Monk et al., 2010).

The use of phage is safe because these viruses are specific to certain bacterial species, phages do not affect eukaryotes and there is reduced environmental impacts (Buttimer et al., 2017; O'Flaherty et al., 2009). However, phage particles can have reduced efficiency when exposed to environmental factors. Temperature, desiccation,

pH, salt concentration, UV light, among others can degrade or inactivate phage particles (Erskine, 1973; Iriarte et al., 2007; Kimura et al., 2008). Formulations of phages have been developed to increase durability and improve adherence to the foliar surface (Balogh et al., 2003; Iriarte et al., 2007). Another potential pitfall to phage usage is resistance to phage infection (Weinbauer, 2004). Although bacteria can become resistant to phages, management of phage-resistant strains is easier to handle compared to strains resistant to agrochemicals (Blunchoth et al., 2015; Buttimer et al., 2017). Selection of new phages to target bacterial population can solve the problem. Nowadays, interest in phages has risen because of the lack of alternatives to control bacterial disease and the increase in bacterial populations resistant to antibiotics. Phages are particularly important for agriculture in Brazil, because epidemics of destructive bacterial diseases can affect many crops and antibiotics cannot be used under field conditions.

Phage-based commercial products are available and can be used in the field. Agriphage™ is a formulated product recommended to control *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* in the United States. Different phages are selected every year to ensure disease control. For *Ralstonia* spp., different phages have been characterized, but there is no commercial product yet (Fujiwara et al., 2008; Yamada et al., 2007; Kawasaki et al., 2009; Askora et al., 2009; Yamada et al., 2010; Fujiwara et al., 2011; Young et al., 2012; Addy et al., 2012; López-Soto et al., 2014; Kalpage & de Costa, 2015; Blunchoth et al., 2015; Brunchoth et al., 2016; Wei et al., 2017; Ahmad et al., 2017). The major families of phages that can be used in agriculture are *Myoviridae*, *Siphoviridae* and *Podoviridae*. These families are the most abundant and they are classified in the *Caudovirales* order (Ackermann, 2007).

The phages of *Ralstonia* spp. were efficient to control bacterial wilt (Fujiwara et al., 2011; Bae et al., 2012; López-Soto et al., 2014; Wei et al., 2017). However, because

of the high genetic variability in populations of *Ralstonia* spp., the use of a mix with different phages may be required to increase the effectiveness of control. A diversity of phages have been found in Japan (Fujiwara et al., 2011), Thailand (Bhunchoth et al., 2015), Korea (Murugaiyan et al., 2011), Kenya, and China (Wei et al., 2017). Most likely, for each region a specific selection of phages may be necessary. For this reason, we propose to isolate, select and characterize phages of *R. solanacearum*, from a region in Brazil where tomato crops are established year-round and bacterial wilt is endemic. The long-term goal is to develop a biological control agent effective against bacterial wilt caused by Brazilian strains of *R. solanacearum* and *R. pseudosolanacearum*.

Material and methods

Soil samples

Soil samples were collected in fields planted to tomatoes in areas with incidence of bacterial wilt, in Coimbra, Minas Gerais state, Brazil. Samples of soil, at least 200 g, were taken with a hoe at a depth of 10 cm. For each field, samples were collected near to asymptomatic plants. Geographic coordinates were registered using a portable GPS device. Strains of *R. solanacearum* were obtained from these soil samples and used to isolate bacteriophages

Bacterial cultures and growth conditions

Ralstonia spp. was cultured on CPG medium (10 $g.L^{-1}$ of peptone, 5 $g.L^{-1}$ of glucose and 1 $g.L^{-1}$ of casein) in a rotary shaker, 80 rpm, for 12 h at 28 °C. The bacterial cultures were maintained in cryogenic storage tubes with saline solution 0.85%, at room temperature. Five isolates of *R. solanacearum* were used to try to isolate the bacteriophages.

Ralstonia species was determined using PCR with species-specific primers (Opina et al., 1997). *R. solanacearum* (former phylotype II) was further characterized by the amplification of the 372 bp-fragment of the 16S-23S intergenic spacer region, as described by Fegan & Prior (2005).

Isolation of phages

Four mL of ultrapure water were added to 2.0 g of soil samples and vortexed for 5 min. The soil suspension was centrifuged at 8000 *g* for 5 min. The supernatant was then filtered through a membrane (0.22 µm pore size; Millipore). A plaque forming assay was set using five *R. solanacearum* strains: UFV 475, UFV 519, UFV 576, UFV 618, and UFV 633. Each bacterial strain was used to isolate phage from the corresponding soil sample. Aliquots of 100 µL of the soil supernatant filtered were added to 250 µL of bacterial suspension of *R. solanacearum* (OD_{600} = 0.4) and incubated for 2 h at 28 °C. One hundred µL of the soil filtrate and 250 µL of the bacterial suspension were mixed in 5 mL of 0.8 % CPG soft agar and placed on CPG plates containing 1.5 % agar. After 24 h of incubation at 28 °C, the formation of lysis plates was visually assessed. Bacteriophages were propagated for three generations. A lysis plate was cut and placed in 10 mL of bacterial suspension in an Erlenmeyer flask in a rotary shaker at 80 rpm for 2 h. The suspension was used for plaque forming assay, as described (Adams, 1959). This process was repeated two more times to obtain pure cultures of the phages.

Propagation of phages

A lysis plate formed in the third generation of propagation was cut and transferred to 1 L of *R. solanacearum* cell suspension (10^8 CFU/mL) and incubated under shaking at 28 °C. After 24 h, the suspension was centrifuged at 8000 *g* at 4 °C for

removal of bacterial debris. The supernatant was filtered through a 0.22 μm membrane filter. The filtrate suspension of phages was mixed with 0.5 M NaCl and 5 % PEG (wt/v) and stored at 4 °C overnight to precipitate the virus. The mixture was centrifuged at 15000 *g* at 4 °C and the pellet was suspended with 1 mL of SM buffer containing 100 mM NaCl, 8 mM $MgSO_4.7H_2O$, 50 mM Tris-HCl pH 7.5, 0.01 % (w/v) gelatin and stored at 4 °C. A serial dilution of the bacteriophage stock solution was made to estimate the number of bacteriophages, using the plaque assay.

Morphological characterization

Each phage suspension was placed under a layer of 20 % sucrose solution and subjected to ultracentrifugation at 35000 *g* for 40 min. The pellet was resuspended in ultrapure water and stored at 4 °C. Twenty µL of the purified particles were placed on formvar-coated copper grids negatively stained with 0.5 % uranyl acetate and examined under a Zeiss EM 109 TEM electron microscope operating at 80 kV. The average virion dimensions were estimated based on the means of measurements of at least three particles of each isolate. Phage images of transmission electron microscopy were analyzed with the software ImageJ (National Institutes of Health, https://imagej.nih.gov/ij/).

Nucleic acid extraction from bacteriophages

The nucleic acid of each phage was extracted by the phenol extraction method (Sambrook & Russell, 2001). A volume of 947.5 µL of the bacteriophage suspension was gently mixed with 2.5 µL of proteinase K (20 $mg.mL^{-1}$) and 50 µL of 10 % (w/v) SDS and incubated for 1 h at 56 °C. After cooling to room temperature, the solution was divided into two microtubes, each containing 500 µL of the solution, and an equal volume of phenol was added. The tubes were gently shaken to form an emulsion and

centrifuged at 4000 *g* for 5 min. The supernatant was transferred to a new microtube containing equal volumes (1:1) of chloroform and phenol solution. The solution was gently mixed and centrifuged at 4000 *g* for 5 min. A volume of 300 to 400 µL of the supernatant was transferred to a new microtube containing the same volume of chloroform. The tube was gently shaken and centrifuged at 8000 *g* for 5 min. The supernatant was removed and mixed with the double volume of absolute ethanol in a new microtube and centrifuged at 11000 *g* for 7 min. The supernatant was discarded and the pellet washed with 70 % ethanol and centrifuged at 11000 *g* for 7 min. The supernatant was discarded and the pellet dried at room temperature. The nucleic acid was resuspended with 20 µL of TE (pH 7.6). The nucleic acid concentration was determined using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific) and the integrity of nucleic acid was checked by agarose gel 1 % electrophoresis.

Nature of the nucleic acid

The extracted nucleic acids were adjusted to obtain a concentration of 50 ng.µL^{-1} and aliquots of each phage were treated with DNAse I (Qiagen) and RNAse A (Promega) according to the manufacturer's instructions. To confirm the nature of the nucleic acids, the final product was visualized on agarose gel electrophoresis. Degradation or not of a nucleic acid by the enzymes was used to infer its nature.

Pulsed field gel electrophoresis

The size of the genome was estimated by pulsed field gel electrophoresis (PFGE). A volume of 20 µL of the extracted DNA was added to 20 µL of 2 % TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3). The samples were subjected to a 2 % agarose gel and ran at 6 V/cm, under a 120 degree angle with a

switching time of 1 to 6 seconds at 14 °C for 12 h. The genome size was estimated with Pulse Marker 0.1-200 kb by Quantum-capt® software.

Host range

The host specificity of the isolated phages was evaluated against 24 isolates of *Ralstonia* spp. from the collection of EMBRAPA Hortaliças (Brasília, Brazil) and from isolates sampled in Coimbra, Minas Gerais.

The plaque assay and the spot test were used. Bacterial suspension was adjusted to 10^8 CFU. mL^{-1} for each bacterial strain. An aliquot of 250 µL of bacterial suspension was mixed in 5 mL of CPG soft agar and spread in plates containing CPG agar. For each *R. solanacearum* strain, three plates were used. Each plate was divided in six spaces and in each space one drop of 10 µL of a suspension at 10^{10}, 10^9, 10^8, 10^7, 10^6, 10^5 plaque forming unit (PFU).mL^{-1} was deposited. After incubation at 28 °C for 24 h, the presence of a lysis zone on the spot inoculations, for each bacterial strain, was interpreted as sensitivity and the absence of lysis zone as resistance to phage infection.

Efficiency of bacteriophages to control bacterial wilt in tomato plants

This experiment was conducted to evaluate the efficacy of each phage when applied singly or in a mixture of all phages to control bacterial wilt in tomato plants. The root system of tomato seedlings with first pair of leaves expanded was cut with disinfested scissors and immersed in phage suspensions (10^8 PFU.mL^{-1}) for 15 min. Tomato seedlings were then transplanted to pots containing 2 kg of soil infested with 10 % mix (v/wt) of five strains of *Ralstonia* spp. at 10^8 CFU.mL^{-1} (UFV 633, GMI 1000, RS 376, RS 378, and RS 322). Bacterial strains were chosen based on the *in vitro* host range assay. Seedlings were watered during the experiment using chlorinated water. The experiment was composed of five phage isolates, a mixture of all five phages, negative

and positive controls. Negative control consisted of pots with clean soil (not infested with *Ralstonia* spp.) irrigated with chlorinated water. The positive control pots contained soil infested with *Ralstonia* spp. strains, but no phage was added and pots were irrigated with chlorinated water. Each treatment was applied to five experimental units (e.u.) (one e.u. = one pot with 10 seedlings). All pots were kept in a walk-in growth chamber set at 28 °C with artificial photoperiod of 12 h.

The assessment of disease intensity was accomplished when tomato plants of the positive control appeared wilted. The percentage of wilted plants in each treatment was calculated and 2 Kg of soil was collected to determine the number of colony forming units per gram of soil. Soil was homogenized and an aliquot of 1 g was suspended with 9 mL of distilled water. A serial dilution was done and 100 µL of the 10^{-4} dilution was spread in plates containing SMSA. After incubation at 28 °C for 48 h, the number of CFU.g^{-1} of soil was estimated from the average of three plates for each experimental units.

Results

Isolation of phages and determination of their host specificity

Five strains of *R. solanacearum* (UFV 475. UFV 519, UFV 576, UFV 618 and UFV 633) were isolated from the 40 soil samples collected from tomato fields, and five phages were obtained.

Phage φRS576 had the widest host range and infected 16 *Ralstonia* spp. strains. Phage φRS633 infected 14 strains of *Ralstonia* spp. and φRS475 infected 12 strains of *Ralstonia* spp. Phages φRS618 and φRS519 infected six and seven of 24 isolates of *Ralstonia* spp. isolates, respectively. Phage φRS618 could not infect *R. solanacearum* UFV 519 from which φRS519 was obtained. Both phages infected *R. solanacearum* UFV 475, UFV 576, UFV 633 and UFV 618. These bacterial isolates were collected in

Coimbra, Minas Gerais, the same location from where the phages were obtained. In addition to these isolates of *R. solanacearum*, phages φRS618 and φRS519 also infected isolates GMI1000, which was obtained from a tomato plant, and RS 224 from a passion fruit plant (Table 6). The following isolates of *R. solanacearum* were resistant to phages: RS 188 (*Talinum fruticosum*), RS 479 (*Pelargonium* spp.), RS 251 (*Solanum tuberosum*), RS 129 (*Cucumis sativus*), RS 56 (*S. melongena*) and K 60 (*S. lycopersicum*) (Table 6).

Table 6. Host range of phages with different *Ralstonia* spp. strains.

			Phage				
Strain	Local	Host	576	475	633	618	519
RS 210	AM	*Musa* spp.	+	+	+	-	-
RS 359	TO	*Solanum melongena*	+	-	+	-	-
RS 177	PA	*Piper hispidinervum*	+	+	-	-	-
RS 188	PA	*Talinum triangulare*	-	-	-	-	-
RS 479	SP	*Pelargonium* spp.	-	-	-	-	-
RS 376	MT	*Solanum lycopersicum*	+	-	+	-	-
RS 322	TO	*Solanum lycopersicum*	+	-	+	-	-
RS 387	MG	*Solanum tuberosum*	+	-	+	-	-
RS 380	CE	*Capsicum* spp.	+	-	-	-	-
UFV 576	MG	*Solanum lycopersicum*	+	+	+	+	+
UFV 475	MG	*Solanum lycopersicum*	+	+	+	+	+
UFV 633	MG	*Solanum lycopersicum*	+	+	+	+	+
UFV 618	MG	*Solanum lycopersicum*	-	+	-	+	+
UFV 519	MG	*Solanum lycopersicum*	-	+	-	-	+
RS 292	TO	*Solanum aethiopicum*	+	+	+	-	-
RS 378	AM	*Solanum lycopersicum*	+	-	+	-	-
RS 251	PR	*Solanum tuberosum*	-	-	-	-	-
RS 129	DF	*Cucumis sativus*	-	-	-	-	-
GMI 1000	French Guiana	*Solanum lycopersicum*	+	+	+	+	+
RS 224	PA	*Passiflora edulis*	+	+	+	+	+
RS 56	PE	*Solanum melongena*	-	-	-	-	-

K 60	USA	*Solanum lycopersicum*	-	-	-	-	-
RS 267	BA	*Eucalyptus globulus*	+	+	+	-	-
RS 411-1	AC	*Piper hispidinervum*	+	+	+	-	-
24			16	12	14	6	7

Morphological analysis of *R. solanacearum* phages

All phages have an icosahedral head and a tail (Figure 7). The head of φRS519 measured 53 nm in diameter, the contractile tail measured 213 nm in length and 7 nm in diameter (Figure 7A). The head of φRS618 measured 79 nm in diameter, the flexible tail 261 nm in length and 10 nm in diameter (Figure 7B). The head of φRS576 measured 55 nm in diameter, the non-contractile tail 88 nm in length and 15 nm in diameter (Figure 7C). The head of φRS633 measured 50 nm in diameter, the non-contractile tail 96 nm in length and 12 nm in diameter (Figure 7D). The head of φRS475 measured 52 nm in diameter, the non-contractile tail 103 nm in length and 15 nm of diameter (Figure 7E). All measures were approximated, since it is not possible to obtain exact dimensions. Based on these dimensions and morphology, all phages belong to the *Siphoviridae* family, order *Caudovirales*.

Molecular characterization of *R. solanacearum* phages

The nucleic acid of all five phages was DNA (Figure 8). The nucleic acid samples were degraded by Dnase I. The genome sizes estimated by PFGE were 80 kb for φRS519 and φRS618, and 56 kb for φRS576, φRS475 and φRS633 (Figure 9). There was a similar RAPD banding pattern for primers OLP5 and P2 for φRS576, φRS475, and φRS633 (Figure 10). Although φRS519 and φRS618 have similar genome sizes, the RAPD pattern was different. Phage φRS519 had a distinct banding pattern to that of φRS576, φRS475, and φRS633. However, only OLP5 primer annealed to the genome of φRS519. For φRS618, primer OLP5 did not anneal to the genome.

Amplifications were possible only for primer P2, and the polymorphisms differed from those of φRS576, φRS475 and φRS633 (Figure 10).

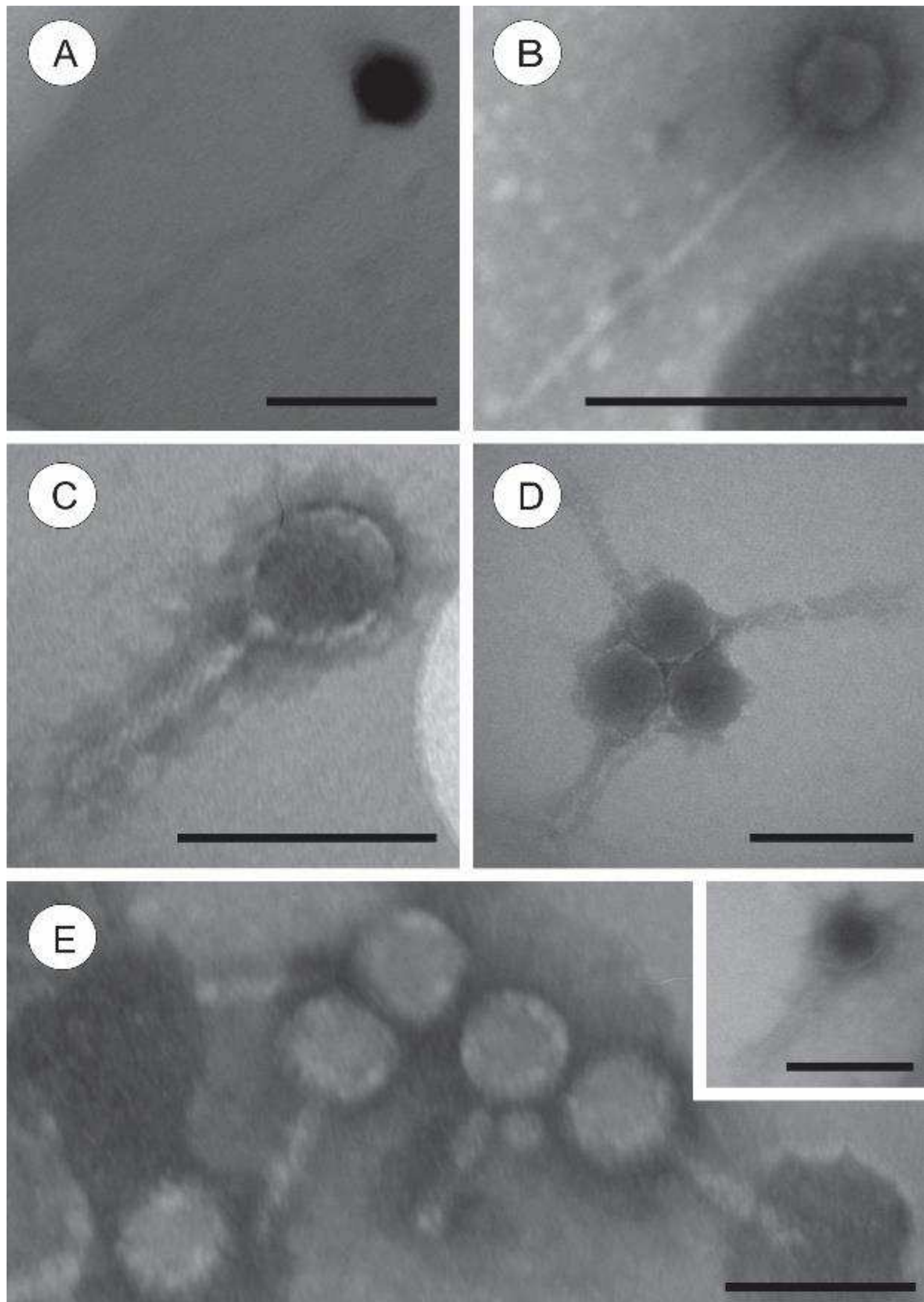


Figure 7. Electron micrographs of *Ralstonia* spp. phages φRS519 (A), φRS618 (B), φRS576 (C), φRS633 (D), and φRS475 represented by empty (ghost particles) and the inset highlights the full particle (E). Scale bars represent 100 nm (A), 200 nm (B), 100 nm (C), 100 nm (D), and 100 nm (E).

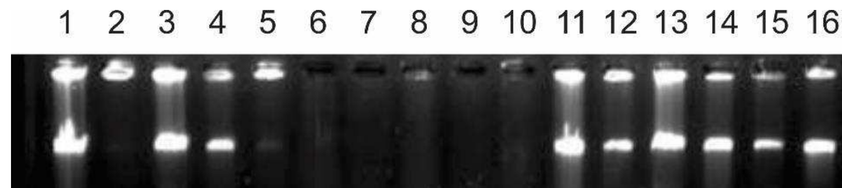


Figure 8. Nature of nucleic acid of phages that induce lysis on *Ralstonia* spp. cells. For each phage, genome was extracted and treated with RNase and DNase. In the sequence of agarose gel: nucleic acid of phages treated with RNase (lanes 1 - 5): lane 1, φRS475; lane 2, φRS576; lane 3, φRS519; lane 4, φRS618; and lane 5, φRS633. Nucleic acid treated with DNase (lanes 6 - 10): lane 6, φRS475; lane 7, φRS576; lane 8, φRS519; lane 9 φRS618; and lane 10, φRS633. Nucleic acid not subjected to enzyme treatment: lane 11, φRS475; lane 12, φRS576; lane 13, φRS519; lane 14, φRS618; lane 15, φRS633; and lane 16, DNA of the lambda phage as control.

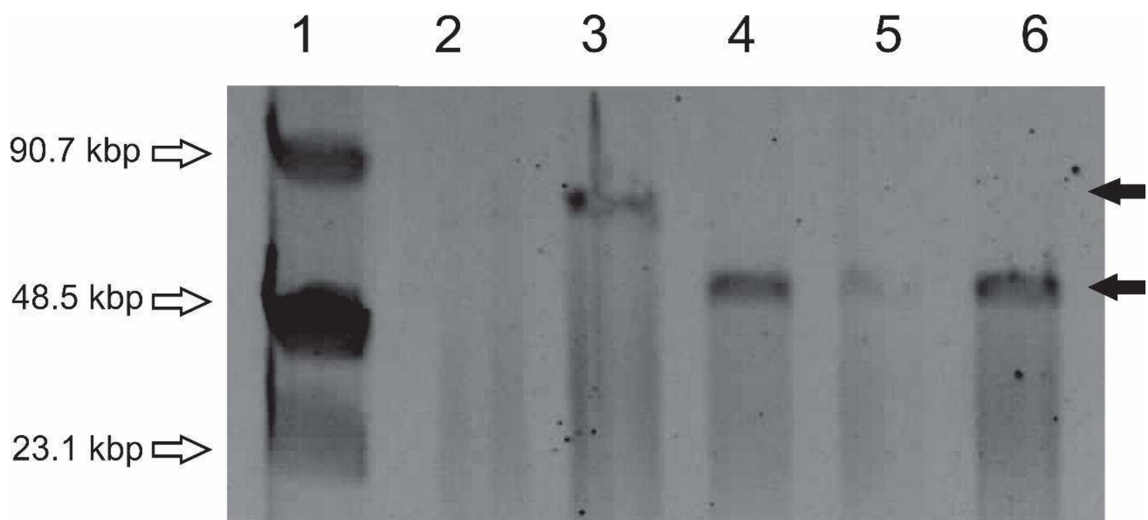


Figure 9. Genome size of bacteriophages, that induce lysis on *Ralstonia* spp. cells, determined by pulsed field gel electrophoresis. Lane 1 represents Pulse Marker 0.1-200 kb. White arrows indicate marker fragment size. Black arrows indicate phage genome sizes: 80 Kb for φRS519 (lane 2) and φRS618 (lane 3); and 56 kb for φRS576 (lane 4), φRS633 (lane 5) and φRS475 (lane 6).

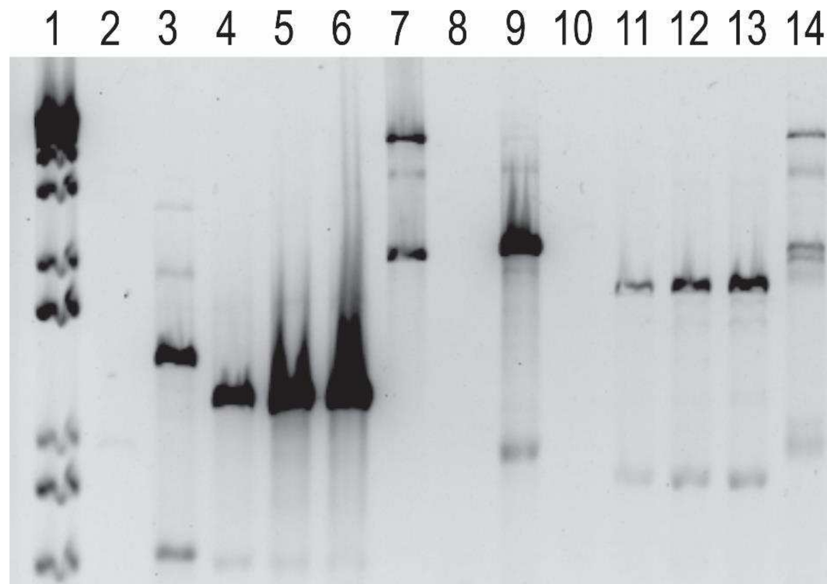


Figure 10. Banding pattern of bacteriophages, that induce lysis on *Ralstonia* spp. cells, generated with two RAPD primers (P2 and OPL5). Lane 1 = 1kb Marker plus. Polymorphisms with primer P2 are represented φRS519 in lane 2, φRS618 in lane 3, φRS475 in lane 4, φRS576 in lane 5, φRS633 in lane 6, and the λ phage in lane 7. The negative control is in lane 8, amplifications with primer OPL5 are represented φRS519 in lane 9, φRS618 in lane 10, φRS475 in lane 11, φRS576 in lane 12, φRS633 in lane 13 and the λ phage is in lane 14.

Efficacy of bacteriophages to control bacterial wilt in tomato plants in the greenhouse

One week after transplanting, tomato plants in all treatments and the positive control were wilted (data not shown). Only tomato plants treated with water did not show symptoms of bacterial wilt. When CFU.g^{-1} of soil was evaluated, there was no difference between phage treatments and the positive control (Figure 11).

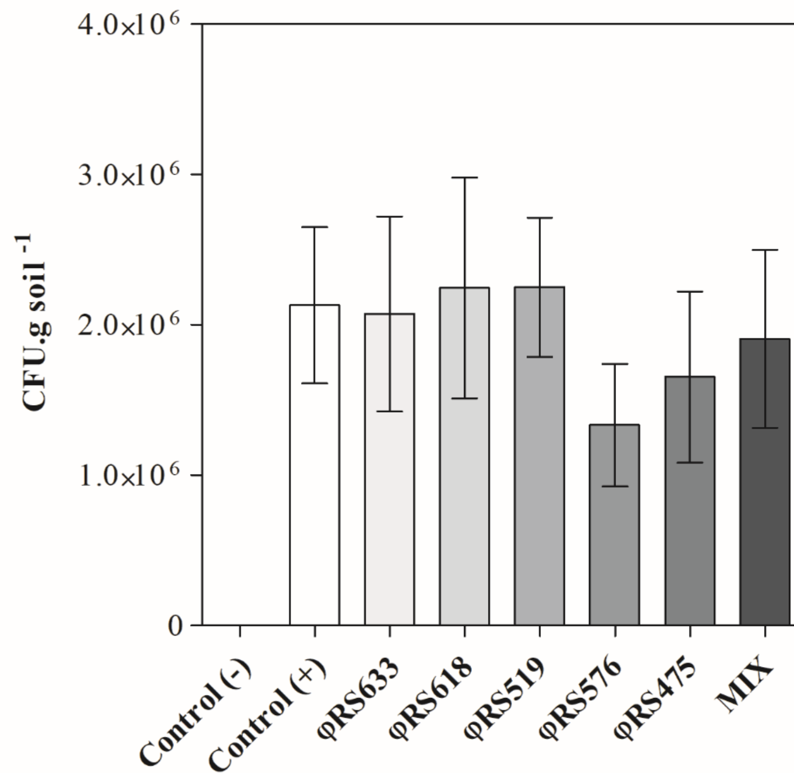


Figure 11. Efficiency of phages to control bacterial wilt in tomato seedlings. Average number of CFU.g^{-1} soil based on 5 replicates of each treatment: negative control (without both phages and *Ralstonia* spp.), positive control (water and *Ralstonia* spp.), φRS633, φRS618, φRS519, φRS576, φRS475 and the mixture of five phages (MIX). Error bars represent one standard deviation.

Discussion

Five bacteriophages were isolated from soil samples collected from tomato fields located in Coimbra, Minas Gerais state, Brazil. Based on their morphology, these phages belong to the family *Siphoviridae*, order *Caudovirales*. Microscopy images revealed that φRS475, φRS576, and φRS633 are morphologically similar. Similarities among φRS475, φRS576 and φRS633 were demonstrated in genome size, 56 kb, and RAPD banding pattern with both primers. Phages φRS519 and φRS618 differ in sizes and in RAPD banding pattern, but have the same genome size, 80 kb. Genome size may not be associated with diversity among phage isolates. The analysis of phages capable

of infecting *Campylobacter jejuni* revealed that the diversity of receptors led to different conclusions compared to the analysis of genome size (Sørensen et al., 2015). Additionally, the method used to detect differences in polymorphisms was not able to show genomic differences. Phages φRS475, φRS576 and φRS633 have the same patterns of RAPD bands. RAPD markers do not have enough resolution to investigate possible fine differences at the genome level. Thus, sequencing of phage genome is necessary to investigate the genetic differences among the isolates obtained in this study.

The bacteriophages characterized in this study were isolated from the soil of the same region, but they had marked differences in virulence against different isolates of *Ralstonia* spp. The differences among φRS475, φRS576 and φRS633 can be related to the composition of receptors that are key to the phages to infect bacteria. Receptors are localized in the surface of the bacterial membrane and phage interaction with a bacterial cell depends on the biochemical composition of the constituents (Rakhuba et al., 2010). Therefore, the molecular analyses conducted in this study were not enough to reveal difference among φRS475, φRS576 and φRS633, for which there is no difference regarding host range.

Phages were not able to protect tomato seedlings from pathogen infection. Seven days after inoculation, all tomato plants wilted, except those that were transplanted in substrate without inoculum in the soil (negative control). One potential aspect that may have compromised the expected protection by phages was the concentration of virus used. The dose of virus may not have been satisfactory to promote lysis without causing infection. Caudate phages are known to have enzymes that degrade the membrane of bacteria when applied in high concentration (Ackermann, 1999) and to be used as biological control agents, it is recommended to apply phages in high concentration for better results (Goode et al., 2003).

Preventative application of phage have shown better results to control bacterial wilt in tomato plants (Fujiwara et al., 2011; Elhalag et al., 2018; Bhunchoth et al., 2015). However, even though the root system of the tomato seedlings used in the current study were treated previously to transplantation to an infested soil, all seedlings treated with phage and the positive control wilted. These results can be related to the use of a mixture of five *Ralstonia* spp. isolates for the inoculation. The patterns of phage infection when determining the host range were used to select the five isolates of *Ralstonia* spp. It may be possible that some isolates of *Ralstonia* spp. escaped infection by the phages and caused wilt in the tomato seedlings. Elhalag et al. (2018) reported on high efficiency of a phage to control bacterial wilt in soil infested with three isolates. However, all three isolates of *Ralstonia* spp. were hosts of the phage that was used. This can explain the phage efficiency to control bacterial wilt. Several studies have shown phage efficiency only when one isolate of *Ralstonia* spp. was used, and when this isolate is a host of the phage (Fujiwara et al., 2011; Blunchoth et al., 2015). The results reported in the present study need to be carefully explored further to avoid failures of wilt control when using the phages. It is also possible that a new set of phages will be required to manage bacterial wilt.

The OmniLytics phage mix showed better results when applied immediately after inoculation of *Ralstonia* spp. (Iriarte et al., 2012). Treatment with a mix of five phages was expected to be more effective in reducing the number of *Ralstonia* spp. cells than other treatments. However, the concentration of a given phage in the mixture could have been lower than its concentration when applied singly. *Ralstonia* spp. strains that were not infected by phages could multiply and cause disease in tomato plants. Phage application prior or immediately at transplantation have shown better results to bacterial wilt control (Addy et al., 2012; Fujiwara et al., 2011; Iriarte et al., 2012). Application of phages was not efficient to control bacterial wilt when conducted three days before or

after inoculation with *Ralstonia* spp. (Iriarte et al., 2012). But, in our study, no success was achieved even with phage treatment prior to transplantation.

Failure to control the disease using phage could also be related to the mode of application. Roots immersed in phage suspension could not have enough number of phage to control *Ralstonia* spp. For better results, it may be required to combine the two modes of application: immerse roots in phage suspension and soil drenching with phage suspension at the base of the tomato seedlings after transplant. Additionally, it may be worth considering the increase in frequency of phage application after transplant. Future studies will be conducted to select new phages from other regions of Brazil and formulate different mixtures with more diverse phages.

Acknowledgments

The research was supported by FAPEMIG. The authors thank Sérgio Oliveira de Paula, Roberto Sousa Dias for assistance in TEM analysis and Vinicíus Duarte da Silva for size genome analysis with PFGE. We thank Francisco Murilo Zerbini for laboratory use. We thank Camila Geovanna Ferro for helpful discussions. We also thank Carlos Alberto Lopes for providing the bacterial isolates used in this work.

References

Abedon, S. T. (2009). Phage evolution and ecology. *Advances in applied microbiology*, *67*, 1-45.

Ackermann, H. W. (1999) Tailed bacteriophages: the order *Caudovirales*. *Advances in virus research*, *51*, 135–201.

Ackermann, H. W. (2007). 5500 Phages examined in the electron microscope. *Archives of virology*, *152*, 227-243.

Adams, M. H. (1959). Bacteriophages. *Interscience publishers*, New York.

Addy, H. S., Askora, A., Kawasaki, T., Fujie, M. & Yamada, T. (2012). Utilization of filamentous phage φRSM3 to control bacterial wilt caused by *Ralstonia solanacearum*. *Plant disease*, *96*, 1204-1209.

Adriaenssens, E. M., Mattheus, W., Cornelissen, A., Shaburova, O., Krylov, V. N., Kropinski, A. M. & Lavigne, R. (2012). Complete genome sequence of the giant *Pseudomonas* phage Lu11. *Journal of virology*, *86*, 6369-6370.

Ahmad, A. A., Kawabe, M., Askora, A., Kawasaki, T., Fujie, M. & Yamada, T. (2017). Dynamic integration and excision of filamentous phage XacF1 in *Xanthomonas citri* pv. *citri*, the causative agent of citrus canker disease. *FEBS open bio*, *7*, 1715-1721.

Álvarez, B. & Biosca, E. G. (2017). Bacteriophage-based bacterial wilt biocontrol for an environmentally sustainable agriculture. *Frontiers in plant science*, *8*, 1218.

Askora, A., Kawasaki, T., Usami, S., Fujie, M. & Yamada, T. (2009). Host recognition and integration of filamentous phage ϕRSM in the phytopathogen, *Ralstonia solanacearum*. *Virology*, *384*, 69-76.

Atterbury, R. J. (2009). Bacteriophage biocontrol in animals and meat products. *Microbial biotechnology*, *2*, 601-612.

Atterbury, R. J., Connerton, P. L., Dodd, C. E., Rees, C. E. & Connerton, I. F. (2003). Application of host-specific bacteriophages to the surface of chicken skin leads to a reduction in recovery of *Campylobacter jejuni*. *Applied and environmental microbiology*, *69*, 6302-6306.

Bae, J. Y., Wu, J., Lee, H. J., Jo, E. J., Murugaiyan, S., Chung, E. & Lee, S. W. (2012). Biocontrol potential of a lytic bacteriophage PE204 against bacterial wilt of tomato. *Journal of microbiology and biotechnology*, *22*, 1613-1620.

Bai, J., Kim, Y. T., Ryu, S. & Lee, J. H. (2016). Biocontrol and rapid detection of food-borne pathogens using bacteriophages and endolysins. *Frontiers in microbiology*, *7*, 474.

Balogh, B., Jones, J. B., Momol, M. T., Olson, S. M., Obradovic, A., King, P. & Jackson, L. E. (2003). Improved efficacy of newly formulated bacteriophages for management of bacterial spot on tomato. *Plant disease*, *87*, 949-954.

Bhunchoth, A., Blanc-Mathieu, R., Mihara, T., Nishimura, Y., Askora, A., Phironrit, N., Leksomboon, C., Chatchawankanphanich, O., Kawasaki, T., Nakano, M. & Fujie, M.

(2016). Two Asian jumbo phages, ϕRSL2 and ϕRSF1, infect *Ralstonia solanacearum* and show common features of ϕKZ-related phages. *Virology*, *494*, 56-66.

Bhunchoth, A., Phironrit, N., Leksomboon, C., Chatchawankanphanich, O., Kotera, S., Narulita, E., Kawasaki, T., Fujie, M. & Yamada, T. (2015). Isolation of *Ralstonia solanacearum*- infecting bacteriophages from tomato fields in Chiang Mai, Thailand, and their experimental use as biocontrol agents. *Journal of applied microbiology*, *118*, 1023-1033.

Buttimer, C., McAuliffe, O., Ross, R. P., Hill, C., O'Mahony, J. & Coffey, A. (2017). Bacteriophages and bacterial plant diseases. *Frontiers in microbiology*, *8*, 34.

Czajkowski, R., Ozymko, Z. & Lojkowska, E. (2014). Isolation and characterization of novel soilborne lytic bacteriophages infecting *Dickeya* spp. biovar 3 ('D. solani'). *Plant pathology*, *63*, 758-772.

Czajkowski, R., Ozymko, Z., de Jager, V., Siwinska, J., Smolarska, A., Ossowicki, A., Narajczyk, M. & Lojkowska, E. (2015). Genomic, proteomic and morphological characterization of two novel broad host lytic bacteriophages ΦPD10. 3 and ΦPD23. 1 infecting pectinolytic *Pectobacterium* spp. and *Dickeya* spp. *PLoS one*, *10*, e0119812.

Elhalag, K., Nasr- Eldin, M., Hussien, A., & Ahmad, A. (2018). Potential use of soilborne lytic *Podoviridae* phage as a biocontrol agent against *Ralstonia solanacearum*. *Journal of basic microbiology*, *58*, 658-669.

Elphinstone J. G., Stanford H. M., Stead D. E. (1998). Detection of *Ralstonia solanacearum* in potato tubers, *Solanum dulcamara*, and associated irrigation water. In: Prior P., Allen C., Elphinstone J. (eds). Bacterial Wilt Disease: Molecular and Ecological Aspects. *Springer publishing*, Berlin, 133–139.

Erskine, J. M. (1973). Association of virulence characteristics of *Erwinia amylovora* with toxigenicity of its phage lysates to rabbit. *Canadian journal of microbiology*, *19*, 875-877.

Fegan, M., & Prior, P. (2005). How complex is the *Ralstonia solanacearum* species complex. *APS press*, 449-461.

Frampton, R. A., Pitman, A. R. & Fineran, P. C. (2012). Advances in bacteriophage-mediated control of plant pathogens. *International journal of microbiology*, *2012*, 1-10.

Fujiwara, A., Fujisawa, M., Hamasaki, R., Kawasaki, T., Fujie, M. & Yamada, T. (2011). Biocontrol of *Ralstonia solanacearum* by treatment with lytic bacteriophages. *Applied and environmental microbiology*, *77*, 4155-4162.

Fujiwara, A., Kawasaki, T., Usami, S., Fujie, M. & Yamada, T. (2008). Genomic characterization of *Ralstonia solanacearum* phage φRSA1 and its related prophage (φRSX) in strain GMI1000. *Journal of bacteriology*, *190*, 143-156.

Goode, D., Allen, V. M. & Barrow, P. A. (2003). Reduction of experimental *Salmonella* and *Campylobacter* contamination of chicken skin by application of lytic bacteriophages. *Applied and environmental microbiology*, *69*, 5032-5036.

Graham, J. & Lloyd, A. B. (1979). Survival of potato strain (race 3) of *Pseudomonas solanacearum* in the deeper soil layers. *Australian journal of agricultural research*, *30*, 489-496.

Graham, J., Jones, D. A. & Lloyd, A. B. (1979). Survival of *Pseudomonas solanacearum* race 3 in plant debris and in latently infected potato tubers. *Phytopathology*, *69*, 1100-1103.

Hartman, G. L., & J. G. Elphinstone. (1994). Advances in the control of *Pseudomonas solanacearum*. In: Hayward, A. C. & Hartman, G. L. (ed.). Bacterial wilt: the disease and its causative agent, *Pseudomonas solanacearum*. *CAB international press*, Wallingford, United Kingdom, 157–177.

Iriarte, F. B., Balogh, B., Momol, M. T., Smith, L. M., Wilson, M. & Jones, J. B. (2007). Factors affecting survival of bacteriophage on tomato leaf surfaces. *Applied and environmental microbiology*, *73*, 1704-1711.

Iriarte, F. B., Obradović, A., Wernsing, M. H., Jackson, L. E., Balogh, B., Hong, J. A., Momol T., Jones J. B. & Vallad, G. E. (2012). Soil-based systemic delivery and phyllosphere in vivo propagation of bacteriophages: two possible strategies for improving bacteriophage persistence for plant disease control. *Bacteriophage*, *2*, 215-224.

Janse, J. D., Araluppan, F. A. X., Schans, J., Wenneker, M., & Westerhuis, W. (1998). Experiences with bacterial brown rot *Ralstonia solanacearum* biovar 2, race 3 in the Netherlands. In *Bacterial Wilt Disease Springer*, Heidelberg, Berlin, 146-152.

Kalpage, M. D. & De Costa, D. M. (2015). Isolation of bacteriophages and determination of their efficiency in controlling *Ralstonia solanacearum* causing bacterial wilt of tomato. *Tropical agricultural research*, *26*, 140-151.

Kawasaki, T., Shimizu, M., Satsuma, H., Fujiwara, A., Fujie, M., Usami, S. & Yamada, T. (2009). Genomic characterization of *Ralstonia solanacearum* phage φRSB1, a T7-like wide-host-range phage. *Journal of bacteriology*, *191*, 422-427.

Kelman, A. (1953). The bacterial wilt caused by *Pseudomonas solanacearum*. *Technical bulletin of North Carolina agricultural experiment station*, 99.

Kimura, M., Jia, Z. J., Nakayama, N. & Asakawa, S. (2008). Ecology of viruses in soils: past, present and future perspectives. *Soil science and plant nutrition*, *54*, 1-32.

Koskella, B. & Brockhurst, M.A. (2014). Bacteria–phage coevolution as a driver of ecological and evolutionary processes in microbial communities. *Fems microbiology reviews*, *38*, 916–931.

López-Soto, F. S. M., Allende-Molar, R., Márquez-Zequera, I., Heredia, J. B., León-Félix, J., Valdez-Torres, J. B., Bojórquez-Ramos, C. & García-Estrada, R. S. (2014). Agricultural soil supports the survival of *Ralstonia solanacearum* bacteriophage φRsS6 with biocontrol potential. *Journal of experimental biology and agricultural sciences*, *2*, 90-97.

Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., Dow, M., Verdier, V. Beer, S. V., Machado, M. A., Toth, I., Salmond, G. & Foster G. D. (2012). Top 10 plant pathogenic bacteria in molecular plant pathology. *Molecular plant pathology 13*, 614–629.

Monk, A. B., Rees, C. D., Barrow, P., Hagens, S. & Harper, D. R. (2010). Bacteriophage applications: where are we now?. *Letters in applied microbiology*, *51*, 363-369.

Murugaiyan, S., Bae, J. Y., Wu, J., Lee, S. D., Um, H. Y., Choi, H. K., Chung, E., Lee, J.H. & Lee, S. W. (2011). Characterization of filamentous bacteriophage PE226 infecting *Ralstonia solanacearum* strains. *Journal of applied microbiology*, *110*, 296-303.

Obradovic, A., Jones, J. B., Momol, M. T., Balogh, B. & Olson, S. M. (2004). Management of tomato bacterial spot in the field by foliar applications of bacteriophages and SAR inducers. *Plant disease*, *88*, 736-740.

O'Flaherty, S., Ross, R. P. & Coffey, A. (2009). Bacteriophage and their lysins for elimination of infectious bacteria. *FEMS microbiology reviews*, *33*, 801-819.

Opina, N., Tavner, F., Hollway, G., Wang, J. F., Li, T. H., Maghirang, R., Fegan, M., Hayward, A. C., Krishnapillai, V., Hong, W. F., Holloway, B. W. & Timmis, J. N. (1997). A novel method for development of species and strain-specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*). *Asia-Pacific journal of molecular biology and biotechnology*, *5*, 19-30.

Parkinson, N., Bryant, R., Bew, J., Conyers, C., Stones, R., Alcock, M. & Elphinstone, J. (2013). Application of Variable Number Tandem Repeat (VNTR) Typing to Discriminate *Ralstonia solanacearum* Strains Associated with English Watercourses and Disease Outbreaks. *Applied and environmental microbiology*, *79*, 6016-6022.

Prior, P., Ailloud, F., Dalsing, B. L., Remenant, B., Sanchez, B. & Allen, C. (2016). Genomic and proteomic evidence supporting the division of the plant pathogen *Ralstonia solanacearum* into three species. *BMC genomics*, *17*, 90.

Rakhuba, D. V., Kolomiets, E. I., Dey, E. S. & Novik, G. I. (2010). Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell. *Polish journal of microbiology*, *59*, 145-155.

Safni, I., Cleenwerck, I., Vos, P. D., Fegan, M., Sly, L. & Kappler, U. (2014). Polyphasic taxonomic revision of the *Ralstonia solanacearum* species complex: proposal to emend the descriptions of the *R. solanacearum* and *R. syzygii* and reclassify current *R. syzygii* strain as *Ralstonia syzygii* subsp. *syzygii*, *R. solanacearum* phylotype IV strains as *Ralstonia syzygii* subsp. *indonesiensis* subsp. nov., banana blood disease bacterium strains as *Ralstonia syzygii* subsp. *celebesensis* subsp. nov. and *R. solanacearum* phylotype I and III strains as *Ralstonia pseudosolanacearum* sp. nov. *International journal of systemic and evolutionary microbiology*, *64*, 3087-3103.

Schnabel, E. L. & Jones, A. L. (2001). Isolation and Characterization of Five *Erwinia amylovora* Bacteriophages and Assessment of Phage Resistance in Strains of *Erwinia amylovora*. *Applied and environmental Microbiology*, *67*, 59-64.

Sørensen, M. C. H., Gencay, Y. E., Birk, T., Baldvinsson, S. B., Jäckel, C., Hammerl, J. A., Vegge, C.S., Neve, H. & Brøndsted, L. (2015). Primary isolation strain determines both phage type and receptors recognised by *Campylobacter jejuni* bacteriophages. *PLoS one*, *10*, e0116287.

Stander, E. I. M., Hammes, P. S. & Beyers, E. A. (2003). Survival of *Ralstonia solanacearum* biovar 2 in soil under different cropping systems. *South African journal of plant and soil*, *20*, 176-179.

van Elsas, J. D., van Overbeek, L. S., Bailey, M. J., Schönfeld, J. & Smalla, K. (2005). Fate of *Ralstonia solanacearum* biovar 2 as affected by conditions and soil treatments in temperate climate zones. In: Allen, C., Prior, P. & Hayward, A. C. (eds). Bacterial wilt disease and the *Ralstonia solanacearum* species complex. *APS press*, St. Paul, MN, USA, 39.

Wei, C., Liu, J., Maina, A. N., Mwaura, F. B., Yu, J., Yan, C., Zhang, R. & Wei, H. (2017). Developing a bacteriophage cocktail for biocontrol of potato bacterial wilt. *Virologica sinica*, *32*, 476-484.

Weinbauer, M. G. (2004). Ecology of prokaryotic viruses. *FEMS microbiology reviews*, *28*, 127-181.

Wright, A., Hawkins, C. H., Änggård, E. E. & Harper, D. R. (2009). A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic- resistant *Pseudomonas aeruginosa*; a preliminary report of efficacy. *Clinical otolaryngology*, *34*, 349-357.

Yamada, T. (2012). Bacteriophages of *Ralstonia solanacearum*: their diversity and utilization as biocontrol agents in agriculture. In: Kurtboke, I. (ed). Bacteriophages. *InTech*-Open Acess Publisher, Rijeka, Croatia, 113-139.

Yamada, T., Kawasaki, T., Nagata, S., Fujiwara, A., Usami, S. & Fujie, M. (2007). New bacteriophages that infect the phytopathogen *Ralstonia solanacearum*. *Microbiology*, *153*, 2630-2639.

Yamada, T., Satoh, S., Ishikawa, H., Fujiwara, A., Kawasaki, T., Fujie, M. & Ogata, H. (2010). A jumbo phage infecting the phytopathogen *Ralstonia solanacearum* defines a new lineage of the *Myoviridae* family. *Virology*, *398*, 135-147.

Young, J. C., Dill, B. D., Pan, C., Hettich, R. L., Banfield, J. F., Shah, M., Fremaux, C., Horvath, P., Barrangou, R. & VerBerkmoes, N. C. (2012). Phage-induced expression of CRISPR-associated proteins is revealed by shotgun proteomics in *Streptococcus thermophilus*. *PloS one*, *7*, e38077.

GENERAL CONCLUSIONS

Our study is the first analysis of inoculum sources of *Ralstonia* spp. in Brazil, and showed that rivers and weeds can also be inoculum sources. Removal of weeds in areas with historic records of bacterial wilt should be considered as a cultural practice to be adopted. . Better detection methods of bacterial detection need to be developed for different purposes, including the study of sources of inoculum. Effective dispersal agents are responsible for the lack of structuring of *R. solanacearum* and *R. pseudosolanacearum* in Coimbra, MG. Water of irrigation has been implicated in pathogen dispersal. Strategies to avoid introduction of new haplotypes in this region is fundamental to increase the effectiveness of resistance of the cultivars. Phages showed potential to infect several *Ralstonia* spp. strains from different regions and plant hosts. The phages isolated in this study belong to the same family, but there are differences among them. Selection of phages in different regions of Brazil is desirable to formulate mixture with high genetic variability of phages. Despite phages were not able to avoid infection in assay with tomato seedlings, new experiments need to be developed to evaluate new forms of application of phages.