Biological Control 80 (2015) 14–22

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Biological control of eucalyptus bacterial wilt with rhizobacteria

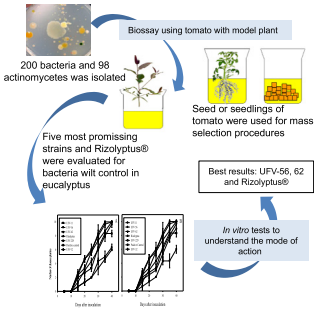
Thais R. Santiago [a], Cristhian Grabowski [b], Mauricio Rossato [a], Reginaldo S. Romeiro [a], Eduardo S.G. Mizubuti [a,*]

[a] Universidade Federal de Viçosa (UFV), Departamento de Fitopatologia, 36570-000 Viçosa, MG, Brazil
[b] Universidad Nacional de Asunción, Departamento de Protección Vegetal s/n, San Lorenzo, Central, Paraguay

HIGHLIGHTS

- UFV-56 (*Bacillus thuringiensis*) and UFV-62 (*B. cereus*) suppressed eucalyptus wilt.
- Selection of antagonists using tomato as a model system was a successful approach.
- UFV-56 apparently reduced bacterial wilt by producing HCN and volatile compounds.
- UFV-62 apparently reduced bacterial wilt of eucalyptus by producing siderophores.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 19 February 2014
Accepted 9 September 2014
Available online 22 September 2014

Keywords:
Ralstonia solanacearum
Biocontrol
Tomato
PGPR

ABSTRACT

The antagonistic potential of 298 rhizobacteria obtained from the rhizosphere and rhizoplane of tomato and eucalyptus plants was assessed for the control of bacterial wilt of eucalyptus caused by *Ralstonia solanacearum*. Several tests were performed using tomato plants as a screening system to select efficient rhizobacteria. Different methods for antagonist delivery and pathogen inoculation were evaluated: (1) seeds were microbiolized (soaked for 12 h in a suspension of the antagonist propagules) and germinated seedlings had their roots immersed in the pathogen inoculum suspension; (2) seedlings originated from microbiolized seeds were transplanted to soil infested with *R. solanacearum* and (3) roots of seedlings were immersed in a suspension of propagules of the antagonist and subsequently in a suspension of *R. solanacearum*. Nine isolates (UFV-11, 32, 40, 56, 62, 101, 170, 229, and 270) were selected as potential antagonists to *R. solanacearum* as they suppressed bacterial wilt in at least one of the methods assessed. The selected antagonists were evaluated against two isolates of *R. solanacearum* using *in vitro* and *in vivo* (inoculated eucalyptus) tests. Isolates UFV-56 (*Bacillus thuringiensis*), UFV-62 (*Bacillus cereus*) and a commercial formulation of several rhizobacteria (Rizolyptus®) suppressed bacterial wilt in eucalyptus protecting the plants during the early stages of development.

1. Introduction

Eucalyptus has become an important forest species for wood, pulp, biomass and other uses worldwide. In tropical areas, bacterial wilt caused by *Ralstonia solanacearum* (Yabuuchi et al., 1995) has caused extensive damage in plantations and is a limiting biotic factor to eucalyptus cultivation in some regions (Alfenas et al., 2006). The disease was already reported in the main producing regions of Australia (Akiew and Trevorrow, 1994), Uganda (Roux et al., 2001), South Africa (Coutinho et al., 2008), China (Wu and Liang, 1988), Taiwan (Wang, 1992), Venezuela (Ciesla et al., 1996) and Brazil (Dianese et al., 1990). Besides direct crop losses that may reach 70% (Ran et al., 2005b), there is an increase in production costs,

* Corresponding author.
E-mail address: mizubuti@ufv.br (E.S.G. Mizubuti).

http://dx.doi.org/10.1016/j.biocontrol.2014.09.007
1049-9644/

mainly of those related to pathogen eradication and greenhouse structural adaptations aimed at minimizing the risks of contamination of seedlings, mini-stumps and other vegetative propagules (Alfenas et al., 2006).

Genetic and ecological characteristics of the pathogen impose difficulties to the control of bacterial wilt of eucalyptus. *R. solanacearum* is known to have high genetic variability (Hayward, 1991) and there is variation in host range, geographic distribution, pathogenicity and physiological properties. Before the genomics era, strains of *R. solanacearum* used to be classified based on the differential pathogenicity to distinct hosts (Buddenhagen et al., 1962) and also on biovars, defined as physiologic groups that vary regarding the capacity to use carbohydrates (Hayward, 1994). Recently, new groups of the pathogen have been proposed by Fegan and Prior (2005). Individuals can be grouped based on multiplex PCR, and several sequevars, based on the partial sequence of the endoglucanase gene (*egl*) are defined (Poussier et al., 2000). Currently, these properties form the basis of the most accepted classification system.

R. solanacearum race 1 is the most widespread variant of the pathogen associated with eucalyptus plants, however biovars vary according to region. In South America biovar 1 is the most commonly found, while in Asia and Australia biovar 3 is the most prevalent (Coutinho et al., 2008; Wu and Liang, 1988). Recently, Marques et al. (2012) described isolates of *R. solanacearum* race 3 biovar 2 pathogenic to eucalyptus plants in Brazil. Additionally, isolates of sequevars 37, 41 and a new sequevar of the phylotype II group were recently described in eucalyptus plants (Fonseca et al., 2013).

The wide host range and the ability of the bacteria to survive in the rhizosphere of alternative hosts, cultivated or not, or even in bare soil, favor the maintenance of epidemiologically relevant population in the soil. Additionally, *R. solanacearum* can be easily dispersed by infected seedlings, irrigation and rain water and by tools used for cultural practices (Coutinho et al., 2008; Hayward, 1994). In addition to the genetic and ecological characteristics that challenge bacterial wilt control, no chemical compounds are effective against the pathogen and crop resistance as well as cultural control measures are practically ineffective (Javier, 1994; Lopes and Takatsu, 1997).

Biological control of eucalyptus bacterial wilt mediated by rhizobacteria can be an alternative to disease management. Rhizobacteria are natural soil inhabitants capable of colonizing the root system of plants and several characteristics enable them to be used as antagonists to plant pathogens (Antoun and Kloepper, 2001). Rhizobacteria have demonstrated good colonization and survival in the rhizosphere. Furthermore, most of the bioactive products produced by the antagonists have long shelf life (Schisler et al., 2004).

Most successful attempts to control eucalyptus bacterial wilt were achieved with strains of *Pseudomonas fluorescens* characterized as a plant growth promoting rhizobacteria (PGPR). The incidence of bacterial wilt in eucalyptus seedlings treated with this biocontrol agent was reduced by 45% (Ran et al., 2005a,b). Nevertheless, despite the promising characteristics of *Pseudomonas* spp., to date, the most commonly reported group of rhizobacteria used as biocontrol agents is comprised of gram-positive bacteria, mainly by the species of *Streptomyces* and *Bacillus* (Emmert and Handelsman, 1999; Koberl et al., 2013). These bacteria can colonize the rhizosphere of plants in different habitats and they can form resistant spores which can play an important role for the development of stable formulated products.

Many studies have been conducted to explore the biocontrol capacity of these organisms, and their capacity to produce antibiotics makes them a target for the biological control of plant diseases (Raaijmakers and Mazzola, 2012; Yanes et al., 2012; Raaijmakers et al., 2002). Few studies in the world have been conducted to assess the use of rhizobacteria as biological control agent of eucalyptus bacterial wilt, none in Brazil. The main objective of this study was to obtain rhizobacteria isolates to be used as a biocontrol agent of bacterial wilt of eucalyptus seedlings. To achieve this goal we isolated rhizobacteria (actinomycetes and bacteria) from the rhizosphere and rhizoplane of tomato and eucalyptus plants and screened them regarding their capacity of promoting biocontrol of bacterial wilt using tomato plants as a model system. Finally, we assessed the efficiency of the selected rhizobacteria in reducing wilt intensity in eucalyptus seedlings and attempted to elucidate the most likely mode of antagonism involved.

2. Material and methods

2.1. Bacterial strains and growth conditions

The strain of *R. solanacearum*, phylotype II, biovar I, coded as RS 295 belongs to the *R. solanacearum* culture collection of Embrapa Hortaliças and was used in the experiments. This strain was isolated from a eucalyptus plant collected in the municipality of Carbonita, Minas Gerais state, in southeast Brazil.

To obtain the inoculum suspension of *R. solanacearum*, the strain was streaked on 523 medium (Kado and Heskett, 1970) and incubated for 48 h at 28 ± 1 °C. After incubation, saline solution (0.85% NaCl) was used to wash the colonies and the suspension was collected in a becker. The concentration of the bacterial cell suspension was adjusted to $OD_{540} = 0.2$, which corresponded to approximately 5×10^7 CFU/mL. Rhizobacteria and actinomycetes were isolated as previously described (Romeiro, 2007). Ten grams of root or soil from the eucalyptus rhizosphere were mixed with saline solution (0.85% NaCl) and kept under agitation for 24 h at 28 °C in an Erlenmeyer flask. Diluted soil samples (10^{-7} and 10^{-8}) were taken and streaked onto 523 medium in Petri plates which were kept at 28 ± 1 °C for 24 h. Individualized colonies of different color, size and shape, were transferred to test tubes containing 523 medium. The isolates were maintained in tubes containing sterilized water.

For isolation of actinomycetes, soil samples from eucalyptus and tomato rhizosphere were processed as described before, but soil suspension in saline solution were kept at 70 °C for three days (Pramer and Schmidt, 1964) before being subjected to serial dilutions and streaked onto 523 medium. The colonies that developed on 523 medium were transferred to test tubes containing soil-agar extract medium.

2.2. Mass selection

Seeds or seedlings of tomato cv. Santa Clara were used for mass selection procedures. Eucalyptus plants were not used at this stage. Tomato is highly susceptible to bacterial wilt and allows for fast plant and disease development, thus we used this plant species as a model. The experiments were conducted from January to May 2010, under greenhouse conditions, with favorable conditions for the development of the disease (maximum 30 °C and minimum 22 °C). After 2 days, the plate was flooded with saline solution (0.85% NaCl) and the suspension was adjusted to $OD_{540} = 0.2$. A mixture of 80% dystrophic red-yellow Latosol soil and 20% sand was used as non-sterilized substrate, henceforth referred to as soil mixture, in which tomato plants were grown. To investigate the potential of rhizobacteria as biocontrol agent, each antagonist candidate strain was assessed under three combinations of delivery-inoculation procedures as described below.