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Evaluation of biological control and rhizosphere competence of plant growth promoting bacteria

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ABSTRACT

Bacillus mycoides B38V, *Burkholderia cepacia* 89, and *Paenibacillus riograndensis* SBR5 have previously shown plant growth promoting (PGP) characteristics. This work aims to evaluate the biological control and rhizosphere competence of these PGP bacteria. Several bacterial properties involved in bacteria-plant or bacteria-fungi relationships were evaluated, including hydrolytic enzyme production, emulsification index, and biofilm formation. Antagonistic tests were performed against filamentous fungi, yeast and bacteria. Greenhouse assays were carried out to test rhizosphere competence and the ability of these bacteria to promote the growth of four wheat cultivars. Wheat seeds were inoculated with *Bipolaris cynodontis, Drechslera tritici-repentis* and *Fusarium graminearum* and the biocontrol ability of *B. cepacia* 89 was tested. An initial characterization of the antifungal metabolite of strain 89 was conducted. All bacteria showed competitive characteristics that could enhance rhizosphere competence either through hydrolytic enzyme production or through antagonistic activities. *B. cepacia* 89 showed a wide range of *in vitro* activity against filamentous fungi. The initial characterization of its active metabolite indicates that it could constitute a new antifungal compound. Both *B. mycoides* B38 V and *B. cepacia* 89 showed remarkable antifungal activity in addition to other PGP characteristics, representing potential inoculants and biocontrol agents.

1. Introduction

Plant growth promoting bacteria (PGPB) are capable of promoting plant growth either directly or indirectly. For instance, these bacteria can act as biofertilizers, phytostimulators, or stress controllers (Lugtenberg and Kamilova, 2009). Fungal diseases are an important source of plant stress and are responsible for a large amount of annual crop loss. Wheat is one of the most important crops produced worldwide and is commonly damaged by fungal diseases. Symptoms in these cases include root rot, leaf spot, seedling and head blight, and seedling black point caused by *Bipolaris sorokiniana* (Kumar et al., 2002; Poloni et al., 2009); tan spot caused by *Drechslera tritici-repentis* (Perelló et al., 2003; Santos et al., 2002); and damping-off, crown rot, and seedling blight caused by *Fusarium graminearum* (Huang and Wong, 1998). Common strategies for fungal disease management comprise pesticide use and agricultural practices such as crop rotation, tillage, organic amendments, and cultivar breeding for resistance (Raaijmakers et al., 2009). Besides the low efficiency of these approaches, there are increasing public environmental and health concerns regarding fungicide residues. These factors, in addition to the development of pathogen resistance to some pesticides, highlight the need for reliable and eco-friendly alternatives (Lorentz et al., 2006).

The use of PGPB inoculants for the promotion of plant growth and biological control of plant diseases has gained focus as an alternative to the use of chemical fertilizers and pesticides. To be effective, PGPB must be able to colonize plant roots and survive in the rhizosphere at least for the period in which they exert their positive effects on plants. Therefore, inoculants should express characteristics that enable them to compete spatially and nutritionally with well-established indigenous microorganisms. This collection of traits is called rhizosphere competence (Bevivino et al., 1998; Raaijmakers et al., 2009).

Burkholderia spp. are common inhabitants of soil (Costa et al., 2014) and are especially present in the rhizosphere of Gramineae

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species such as wheat (Balandreau et al., 2001; Parke and Gurian-Sherman, 2001) and maize (Arruda et al., 2013; Bevivino et al., 1998). They can also be found as endophytes of grapevine (Compant et al., 2005) and sugarcane (Mendes et al., 2007) and as symbionts of legumes (Bontemps et al., 2010; Howieson et al., 2013). Similarly, *Bacillus* and *Paenibacillus* spp. are ubiquitous in soil and have been isolated from the rhizosphere of many different plants (Costa et al., 2014; Govindasamy et al., 2011). There are many studies reporting the beneficial effects of these three genera on plants and their biocontrol of soil-borne diseases (Bevivino et al., 2000; Chen et al., 2013; Haggag and Timmusk, 2008; Kumar et al., 2012; Lorentz et al., 2006; Shaharoona et al., 2007). These microorganisms can promote plant growth directly through nitrogen fixation, phosphate solubilization, and the production of phytohormones and ACC deaminase; and indirectly by the production of antagonistic compounds like hydrolytic enzymes, siderophores and a range of antibiotics (Costa et al., 2014; Govindasamy et al., 2011; Parke and Gurian-Sherman, 2001; Suárez-Moreno et al., 2012). *Bacillus mycoides* B38 V, *Burkholderia cepacia* 89, and *Paenibacillus riograndensis* SBR5 were isolated in Southern Brazil and have previously shown PGP characteristics (Ambrosini et al., 2015; Beneduzi et al., 2010; dos Passos et al., 2014). In this work, we performed further characterization of these three bacteria and evaluated their biocontrol and rhizosphere competence properties.

2. Material and methods

2.1. Bacterial strains

P. riograndensis SBR5 is a nitrogen-fixing bacteria isolated from the rhizosphere of wheat (*Triticum aestivum* L., cv. BRS Louro), and was able to promote plant growth of this cultivar in greenhouse assays (Beneduzi et al., 2010). *B. mycoides* B38V was isolated from the rhizosphere of sunflower (*Helianthus annuus* L.), for which the bacteria was shown to promote growth (Ambrosini et al., 2015). *B. cepacia* 89 is a soil bacterium isolated from Brazilian grasslands and was shown to protect apple trees from *Colletotrichum gloeosporioides* infection *in vivo* (dos Passos et al., 2014). This bacterium was fully identified by the amplification of the 16S rRNA gene, as previously described (Bach et al., 2011). The approximately 1500-bp sequence was assembled and analyzed with the Staden Pregap4/Phred software of the Staden Package (http://staden.sourceforge.net/) and is deposited in GenBank under accession number KR013050. The bacteria were subcultured on King B medium (KB; Glickmann and Dessaux, 1995), which contains in g L^{-1}: peptone 20, K_2HPO_4 1.15, $MgSO_4$ 1.5, glycerol 15 and agar 17.

1.1. Enzyme activity assays

Bacteria were tested for hydrolytic enzyme production spot inoculating agar plates and measuring halo formation upon solubilization of the following tested substrates: starch, carboxymethyl cellulose, pectin, skim milk, tributyrin, xanthan gum and xylan for amylase, cellulase, pectinase, protease, esterase, xanthanase and xylanase production, respectively (Bach et al., 2011; Blanco and Pastor, 1993; Cadmus et al., 1982; Cao et al., 1992; MacFaddin, 2000). When necessary, additional reagents were added for halo verification. Results were expressed by calculating the ratio of the clearance zone (CZ) and colony size (CS) diameters after 48 h at 28 °C (CZ/CS; Bach et al., 2011). Rhodamine B agar plates were used to determine lipolytic activity through the production of an orange pigment visible under UV light. Catalase production was determined by reacting bacterial cultures with 3% hydrogen peroxide. Urease production was verified observing the color change of urea broth due to alcalinization of medium (MacFaddin, 2000).

Chitinase activity was evaluated by cultivating bacteria for 7 days at 30 °C, 125 rpm, in crab chitin broth, which contained in g L^{-1}: crab chitin 10, NaCl 0.5, K_2HPO_4 0.3, KH_2PO_4 0.4, followed by an enzymatic assay with chitin azure (Pedraza-Reyes and Lopez-Romero, 1991). Briefly, 0.5 mL of the supernatant was added to 0.5 mL of 1 mg mL^{-1} chitin azure (Sigma Chemical, England) dissolved in PBS buffer (0.1 mM, pH 7.0). Microtubes were incubated for 3 h at 37 °C, 200 rpm, centrifuged (13,000 × *g* for 15 min), and the absorbance was read at 575 nm. All activity measurements were carried out in triplicate. Positive controls were used for confidence of assays.

1.2. Additional characterization

The production of biosurfactants was evaluated indirectly by measuring the emulsification index (E24; Velho et al., 2011). Supernatants were obtained by centrifugation (13,000 × *g*, 15 min) of bacterial cultures grown in KB broth for 24 h at 28 °C, 125 rpm, followed by filtration through a 0.22-μm membrane (Millipore, USA). Two milliliters of this supernatant was added to olive oil, mixed with a vortex for 2 min, and allowed to stand for 24 h at room temperature. Evaluations of the emulsification layer were done after 24 and 120 h.

To evaluate biofilm formation, bacteria were statically grown at 28 °C in regular KB or Tryptic Soy Broth (TSB; Sigma) or adding 1% glucose to these media until the cultures reached an optical density (OD) higher than 0.8. Then, bacteria were diluted 1:100 with the same culture medium, were inoculated in a microtiter plate, and incubated at 28 °C for 72 h. Determination of biofilm formation was carried out according to Stepanovic et al. (2007), where an OD_{570} that was three standard deviations (SD) above the mean OD_{570} of the negative control was considered a positive result.

Soft-rotting of potato was verified by growing the bacteria on fresh tubers that had been previously disinfected and observing the change of color to dark brown (Wastie et al., 1988).

1.3. Antagonistic activity

Antifungal activities were evaluated by seeding 10^4 spores of filamentous fungi onto KB or Potato Dextrose Agar plates (PDA, Oxoid), followed by inoculation of 20 μL of PGP bacteria in the exponential growth phase (approximately 10^8 CFU mL^{-1}). Likewise, bacteria and yeast were grown for 24 h and spread on KB plates, followed by inoculation of PGP bacteria. Inhibition zones between fungi/bacteria/yeast and PGP bacterial strains were measured after 24 h for bacteria/yeast or after fungi developed spores.

1.4. In vivo *plant growth promotion assay*

The plant growth promotion assay was carried out according to Beneduzi et al. (2008) with slight modifications. The seeds of four cultivars of wheat, CD104, Guamirim, Quartzo and Marfim, were surface-disinfected, germinated in a humid environment in Petri dishes for 5 days (24 °C) and seeded in 300-mL plastic pots (8 × 10 cm) filled with sterile vermiculite. Then, 1 mL of 10^8 CFU mL^{-1} of each bacterium was inoculated by directly irrigating the substrate. Control plants received only saline solution (0.85 % NaCl). Pots received 100 mL of 25% Hoagland's nutrient solution (Ambrosini et al., 2012) on the first day and sterile distilled water afterward. Plants were kept in a growth chamber with a photoperiod of 16 h light and 8 h dark at 24 °C for 30 days. The chlorophyll content (Lichtenthaler, 1987), root and shoot length, and dry weight of at least 12 plants were evaluated for each treatment. Additionally, in order to estimate the rhizosphere density of inoculated bacteria, vermiculite that was loosely