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Biocontrol of *Sclerotinia sclerotiorum* (Lib.) de Bary on common bean by native lipopeptide-producer *Bacillus* strains

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ABSTRACT

Bacillus sp. B19, *Bacillus* sp. P12 and *B. amyloliquefaciens* B14 were isolated from soils of Salta province, and PGPR properties on the common bean (*Phaseolus vulgaris* L.) cv. Alubia and antagonistic activity against *Sclerotinia sclerotiorum* were studied.

It was determined that B19 and P12 increased crop germination potential (GP) from the common bean by 14.5% compared to control seeds; these strains also increased root length (10.4 and 15%, respectively) and stem length (20.2 and 30%, respectively) compared to the control; however, as for the B14 strain, no increases in growth parameters were detected. In addition, all the treatments that combined two bacilli: B14 + B19, B14 + P12 and B19 + P12, generated beneficial effects on GP and seedling growth compared to control seeds, but not compared to a single inoculant. B19 and P12 strains synthesized auxins at concentrations of 5.71 and 4.90 mg/mL, respectively, and it was qualitatively determined that they synthesize siderophores. In addition, previous studies have determined that B14 produces auxins in a concentration of 10.10 mg/mL, and qualitatively synthesizes siderophores.

The phytosanitary state of the white bean cv. Alubia control seeds revealed bacterial contamination in 87% of all the evaluated seeds and different fungi such as *Cladosporium* sp., *Fusarium* sp., and *Rhizopus* sp. Bean seeds treated with B14, B19 or P12 showed no growth of contaminating bacteria or of pathogenic fungi; in fact, bacilli inoculum development was observed in all seeds. Additionally, B19, P12 and B14 strains inhibited *in vitro* the development of 9 native *S. sclerotiorum* strains isolated from the Salta region, with FI ranging between 60 and 100%. The three *Bacillus* strains synthesized different isoforms of the lipopeptides: surfactin, iturin, and fengycin in the presence of *S. sclerotiorum*, as determined by MALDI-TOF.

In the *in vivo* trials, when common bean seeds were grown in soils contaminated with *S. sclerotiorum*, an incidence of 100% was determined when the seeds were not treated with any *Bacillus*. Seeds treated with the chemical fungicide and sown in *S. sclerotiorum*-infested soil did not produce seed emergence, while the inoculation of the seeds with B14 + P12, B14 + B19 or B19 + P12 reduced the effect of the pathogen by 46, 43 and 25%, respectively. Disease progression in B14 + P12 and B14 + B19 treatments was significantly lower than in the remaining treatments, with an AUDPC of 873.75 and 1071, respectively.

1. Introduction

Common bean (*Phaseolus vulgaris* L.) production is a key agricultural activity in northwest Argentina (NWA). Crop production is mainly concentrated (70%) in the province of Salta, with an estimated cultivated area of about 450,000 ha in the last years (De Bernardi, 2016). Sanitary status of seeds is one of the main factors influencing crop production and health and is determined by the presence or absence of

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crop associated pathogenic microorganisms. The agents causing the most devastating diseases in common bean crops can be transmitted by seeds; therefore, seeds may be a significant means of disease transmission as well as a source of primary pathogen inoculum. White mold, caused by the fungus *Sclerotinia sclerotiorum* Lib. de Bary, is a very detrimental disease affecting the common bean in the province of Salta. The fungus is favored by temperate climates, moderate temperatures and high relative humidity (Mamaní Gonzáles et al., 2015). *S. sclerotiorum* primarily spreads by spores and usually in forms of sclerotia, which may infect stems, leaves and flowers, and even spread to adjacent plants (Zhou and Boland, 1998). Sclerotia of *S. sclerotiorum* can reside in the soil for several years and, under appropriate environmental conditions, germinate to form mycelium, leading to infectious hyphae, or producinge apothecia, which release millions of airborne ascospores (Coley-Smith and Cooke, 1971; Bardin and Huang, 2001). Given its persistence in the soil or in seeds, as well as its tendency to spread, and further, the lack of resistant cultivars, this fungus can cause devastating economic losses in the crops; therefore, its management is of regional importance.

Despite the efforts made by breeding programs, several common bean cultivars used in commercial production are susceptible to white mold. While advances have been made in the development of resistant varieties, selection should also be focused on high-yield varieties (Miklas et al., 2013; Balasubramanian et al., 2014). Other recommended management strategies are crop rotation, wider seeding row spacing and treatment of seeds with chemical products (Vieira et al., 2010; Vizgarra and Mamaní Gonzáles, 2012). As for the last strategy, intensive use of chemical compounds in crop management has led to insect microbial pathogen resistance to pesticides, and has also caused serious problems for human health and the quality of the environment. Hence, over the last few years, there is a trend in Argentina to apply sustainable agricultural practices to replace, or at least supplement, the use of chemicals, and thus obtain healthy and safe food. This change requires finding non-contaminant and environmentally friendly alternatives.

Different species of the genus *Bacillus* have been widely used both as potential plant growth-promoting rhizobacteria (PGPR) in agriculture, due to their capacity to promote plant growth and as biocontrol agents (Schenck zu Schweinsberg-Mickan and Müller, 2009; Jakab et al., 2011; Pérez-García et al., 2011; Laditi et al., 2012; Stefan et al., 2013). These bacteria have an antagonistic effect against different plant pathogens, which is conferred by their potential to synthesize a wide array of metabolites with antagonistic activity, such as lipopeptides of surfactins, iturins, fengycins, polimixins, kurstakins, and bacitracins (Hathout et al., 2000; Stein, 2005; Price et al., 2007; Ongena and Jacques, 2008; Banat et al., 2010; Yánez-Mendizábal et al., 2011; Béchet et al., 2012; Cawoy et al., 2014; Thais et al., 2015; Chandler et al., 2015; Torres et al., 2016; Zouari et al., 2016; Torres et al., 2017; Sabaté et al., 2017).

The aim of this work was to evaluate the effect of different native strains of the genus *Bacillus* isolated from soils of Salta province as potential PGPR and biocontrol agents, especially in the incidence of the fungus *S. sclerotiorum* (seed and seedling), on the common bean crop.

2. Materials and methods

2.1. Bacterial isolation from soil

Rhizosphere soil samples (10 cm depth) were taken from the central-eastern region of the province of Salta, which is the area of the province where most bean crops are cultivated under different production systems (24°52′23.72″S 64°14′54.46″W). Serial dilutions were performed, inoculated in BHI (Brain Heart Infusion, Britania, Argentina) broth and incubated at 37 °C for 24–48 h. Strains exhibiting visible morphological characteristics of the *Bacillus* strain were preselected and their structure was confirmed via optical microscopic observation. The selected strains were preserved in BHI broth with 20% v/v glycerol at −20 °C.

2.2. In vitro plant growth-promoting attributes of isolates

Plant growth-promoting bacteria (PGPB) activities of isolates were determined following standard procedures. The solubilization of inorganic phosphate was measured using the methods described by Goldstein (1986). Auxin and cyanide production were detected using the method described by de Brito Alvarez et al. (1995). Siderophore production was tested on TSA (Tripteina Soya Agar, Britania) medium supplemented with 8-hydroxyquinoline (de Brito Alvarez et al., 1995).

2.3. Phylogenetic characterization

DNA was extracted from *Bacillus* spp. B19 and P12 with an active culture after incubation in 5 mL of Brain Heart Infusion broth (BHI, Britania, Argentina) at 37 °C for 24 h, according to the method of Miller (1972). For the characterization, the strains were genetically characterized by analyzing the 16S rRNA subunit, and sequencing was performed on both strands by the commercial sequencing services of Macrogen Inc. (Seoul, Korea). 16S was carried out using nucleotide single universal strand primers S-D-Bact-0008-a-*S*-20 (AGAGTTTGATC CTGGCTCAG) and S-D-Bact-1495-a-A-20 (CTACGGCTACCTTGTTA CGA) (Daffonchio et al., 1998). The extracted genomic DNA was amplified in a 25 µL reaction mixture containing: 0.2 µL Taq polymerase, 2.5 µL buffer STR, 0.1 µL primer, 17.5 µL PCR water and 5 µL DNA sample. Amplification consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 50 °C for 2 min and 72 °C for 2 min, and a final extension at 72 °C for 7 min. Control reaction mixtures lacking template DNA were also included in each experiment. The PCR products were separated in 0.8% agarose gel electrophoresis running at 65 V for 50 min. Gel patterns were visualized by ethidium bromide staining, and photographs taken under UV light. Online search for similarity was carried out at GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov).

2.4. Effect of Bacillus cell culture on common bean seed health

For the following tests, a strain previously used for other studies, *B. amyloliquefaciens* B14 (Sabaté et al., 2017), was incorporated due to the fact that it has beneficial properties on the growth of common black bean cv. Nag 12, and as a biocontrol agent against other pathogens of this crop.

Seeds of the white common bean cv. Alubia were initially sterilized in 70% alcohol for 30 s and then in 1% sodium hypochlorite solution for 1 min. After this treatment, the seeds were inoculated, submerged for 30 min with the 48-h-old cell culture of B19, P12 and B14, at a concentration of 1×10^8 cells per mL. Non-inoculated seeds were used as control. Seeds were placed in Petri dishes (9 cm in diameter) containing Potato Dextrose Agar (PDA, Britania); five bean seeds per Petri dish per treatment were placed equidistant from one another and the dishes were then incubated in a heater at 26 °C for 10 days. After the incubation period, the presence or absence of seed-borne pathogenic microorganisms and other microorganism contaminants in the seeds was assessed. The assays were performed in triplicate.

2.5. Effect of Bacillus cell culture on common bean growth

White common bean cv. Alubia seeds were initially sterilized and inoculated with the 48-h-old cell culture of B14, B19 or P12, as mentioned above. The effect of the combination of two of these strains, grown in monoculture, was also tested, as follows: B14 + B19, B14 + P12, and B19 + P12, in 1:1 proportion. Furthermore, the commercial chemical fungicide, Maxim®Evolution Rizobacter (tiabendazol 15 g/L, fludioxonil 2.5 g/L, metalaxil-M 2 g/L), commonly used in the region for this crop, was tested; it was applied to seeds following the