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Induced systemic resistance and symbiotic performance of peanut plants challenged with fungal pathogens and co-inoculated with the biocontrol agent *Bacillus* sp. CHEP5 and *Bradyrhizobium* sp. SEMIA6144

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ABSTRACT

Synergism between beneficial rhizobacteria and fungal pathogens is poorly understood. Therefore, evaluation of co-inoculation of bacteria that promote plant growth by different mechanisms in pathogen challenged plants would contribute to increase the knowledge about how plants manage interactions with different microorganisms. The goals of this work were a) to elucidate, in greenhouse experiments, the effect of co-inoculation of peanut with *Bradyrhizobium* sp. SEMIA6144 and the biocontrol agent *Bacillus* sp. CHEP5 on growth and symbiotic performance of *Sclerotium rolfsii* challenged plants, and b) to evaluate field performance of these bacteria in co-inoculated peanut plants. The capacity of *Bacillus* sp. CHEP5 to induce systemic resistance against *S. rolfsii* was not affected by the inoculation of *Bradyrhizobium* sp. SEMIA6144. This microsymbiont, protected peanut plants from the *S. rolfsii* detrimental effect, reducing the stem wilt incidence. However, disease incidence in plants inoculated with the isogenic mutant *Bradyrhizobium* sp. SEMIA6144 V2 (unable to produce Nod factors) was as high as in pathogen challenged plants. Therefore, *Bradyrhizobium* sp. SEMIA6144 Nod factors play a role in the systemic resistance against *S. rolfsii*. *Bacillus* sp. CHEP5 enhanced *Bradyrhizobium* sp. SEMIA6144 root surface colonization and improved its symbiotic behavior, even in *S. rolfsii* challenged plants. Results of field trials confirmed the *Bacillus* sp. CHEP5 ability to protect against fungal pathogens and to improve the yield of extra-large peanut seeds from 2.15% (in Río Cuarto) to 16.69% (in Las Vertientes), indicating that co-inoculation of beneficial rhizobacteria could be a useful strategy for the peanut production under sustainable agriculture system.

1. Introduction

Plant roots are surrounded by a variety of microorganisms that can establish different interactions among them and with roots, being beneficial and pathogenic the most extreme life styles (Lagunas et al., 2015). Microbial diversity is differentially influenced by the plants genotypes probably due to root exudates composition (Bednarek et al., 2010). Nitrogen fixing symbiosis in legumes has been extensively studied in plants inoculated only with rhizobial bacteria. However, under natural conditions rhizospheric microbes may interact with each other and with plants. These interactions may result in associative, symbiotic, neutralistic or detrimental effects depending upon the type of microorganisms involved, soil properties, environmental conditions and the plant defense response (Van der Putten et al., 2007). Synergism between different rhizospheric bacteria promote plant growth (Kannan and Sureendar 2009), but antagonistic interactions have also been reported (Maida et al., 2016). Therefore, evaluation of plant beneficial bacteria performance when co-inoculated on crop would contribute to understand how plants manage interactions with different microorganisms.

Arachis hypogaea L. (peanut), a member of the Leguminosae family, establishes nitrogen fixing symbiosis with *Bradyrhizobium* sp. Peanut is susceptible to the attack of different pathogens being *Sclerotium rolfsii* the casual agent of stem wilt disease. In previous studies we isolated the native strain *Bacillus* sp. CHEP5 able to

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protect peanut against this fungal pathogen by inducing systemic resistance (ISR) (Tonelli et al., 2011).

The main goal of this work was to elucidate, in greenhouse experiments and under field conditions, the effect of co-inoculation of peanut seedlings with *Bradyrhizobium* sp. SEMIA6144 and the biocontrol agent *Bacillus* sp. CHEP5 on symbiotic performance and induction of plant defense response against fungal pathogens.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacillus sp. CHEP5 (a native biocontrol agent, Tonelli et al., 2010; Gen Bank accession number EU723264), *Bradyrhizobium* sp. SEMIA6144 (reference strain recommended as inoculant by Microbiological Resource Center, Porto Alegre, Brasil) and *Bradyrhizobium* sp. SEMIA6144 V2 (a *Bradyrhizobium* sp. SEMIA6144 derivative *nodC* mutant, Ibáñez and Fabra, 2011) were used in this study. *Bacillus* sp. CHEP5 was cultured at 28° C on Trypticase Soya Broth (TSB) or Agar (TSA) (Britania) media. *Bradyrhizobium* sp. SEMIA6144 and *Bradyrhizobium* sp. SEMIA6144 V2 were cultured at 28° C on Yeast Extract Mannitol broth (YEM) or YEM-agar (YEMA) (Vincent, 1970). To grow *Bradyrhizobium* sp. SEMIA6144 V2, the media were supplemented with kanamicyn (100 μg mL $^{-1}$). The strains were kept in 20% glycerol at −80 °C for long-term storage and in 40% glycerol at −20 °C for short-term storage.

2.2. Fungal phytopahogen culture conditions

Sclerotium rolfsii was obtained from infected peanut plants and grew on Potato Dextrose Agar (PDA) (Kong et al., 2010) supplemented with streptomycin sulfate (100 μg mL^{-1}) at room temperature for 7 days.

The phytopathogen was kept in 15% glycerol at −20 °C and sclerotia were maintained at room temperature for long-term storage.

2.3. Bacterial inoculum preparation

Bacterial cultures (10^8 CFU mL $^{-1}$) were centrifuged at 2500 rpm for 5 min at room temperature and the cells were suspended in 0.85% NaCl sterile solution. For co-inoculation treatments, mixed cultures were prepared in a 1:1 ratio.

The number of viable cells was determined following the method described by Somasegaran and Hoben (1994).

2.4. Phytopathogen inoculum preparation

Wet wheat (*Triticum aestivum* L.) seeds were autoclaved and then infected with 5-mm diameter *S. rolfsii* mycelia plugs. They were maintained at room temperature until abundant mycelium growth was observed (7–10 days approximately) (Grupta et al., 2002).

2.5. Peanut seeds surface disinfection and germination

Seeds of *Arachis hypogaea* L. (peanut) var. Runner cultivar Granoleico, susceptible to *S. rolfsii* root and stem wilt, were surface disinfected as described by Vincent (1970). Briefly, seeds were soaked in 96% ethanol for 30 s followed by 30% H_2O_2 for 20 min, and then washed six times with sterile distilled water. Surface disinfected seeds were germinated in dark at 28° C in sterilized Petri dishes with one layer of Whatman N°1 filter paper and moist cotton, until the radicle reached approximately 2 cm.

2.6. Evaluation of induced systemic resistance and symbiosis in co-inoculated plants

Two plastic cups filled with sterilized vermiculite were placed one above the other and connected by a hole made in the base of the upper cup. A germinated peanut seed was sown in the upper plastic cup so that the root reaches the bottom plastic cup through the hole that connected both cups.

Radicles of peanut seedlings (contained in the bottom cup) were inoculated with 4 mL (10^8 CFU mL^{-1}) of mixed (1:1) or pure bacterial culture. Seven days after bacterial inoculation, the seedlings were challenged with the pathogen by adding on the plant crown (located in the upper cup) one wheat seed infected with *S. rolfsii* mycelium (20 mg). Plants were covered with nylon bags for 72 h to favor the disease development conditions. Non-pathogenized, non-bacterized, and fertilized (KNO_3 5 mM) control plants were also included. Plants were grown under controlled environment (light intensity of 200 mmol m^{-2} s^{-1}, 16-h day/8-h night cycle, at a constant temperature of 28° C and a relative humidity of 50%), watered regularly and supplied once a week with Hoagland solution (Hoagland and Arnon, 1950). For plants inoculated with *Bradyrhizobium* sp. SEMIA6144 N-free Hoagland solution was used.

At 40 days after bacterial inoculation, assessment of disease incidence (measured as % of diseased plants) and disease severity (evaluated by total chlorophyll and nitrogen content, and shoot and root dry weights after dried at 60 °C for 10 days to a constant weight) were recorded. Nodule morphology, number and dry weight, and percentage of red nodules (red color is a consequence of nodule leghemoglobin presence) were also determined.

The experiment was repeated four times with 10 replicates per treatment.

2.7. Total chlorophyll and nitrogen contents determination

Total chlorophyll and nitrogen contents were determined at 40 days after bacterial inoculation. The method described by Arnon (1949) was used for chlorophyll determination. Briefly, 0.1 g of fresh weight peanut leaves was placed into a mortar and the tissue was grinded to fine pulp after the addition of 80% acetone. The extract was transferred to a Buchner funnel containing a pad of Whatman filter paper. The chlorophyll content was spectrophotometrically determined by measuring the absorbance at 650 nm and 665 nm. The amount of total chlorophyll was calculated according to the following equation (Mc Kinney 1939):

$$\text{Total chlorophyll} = 6.45\,(\text{Absorbance at } 665\text{ nm}) + 17.72\,(\text{Absorbance at } 650\text{ nm}).$$

Shoot nitrogen content per plant was determined using the Kjeldahl method (Kjeldahl, 1883).

2.8. Microscopic analyses of nodules

Nodules formed in peanut roots by *Bradyrhizobium* sp. SEMIA6144 were placed in FAA solution (95% ethanol: glacial acetic acid: 37–40% formaldehyde: water; 50:5:10:35, V/V). Dehydration of the samples was done following Johansen method (1940) using graduated solutions of ethanol and xylene. Tissues were embedded in Histowax (highly purified paraffin wax blended with polymer additives). Series of transversal section 6 μm thick were obtained from the sample blocks using a Minot rotary microtome. The sections were triple-stained with hematoxylin, safranin O and fast green FCF as described by Johansen (1940). A Zeiss Axiophot microscope was used to assess the histological preparations and

photomicrographs were taken with an equipment of image capture and digitalization AxioVision 4.3, with an AxioCam HRc camera.

The experiment was repeated twice with ten replicates for each treatment.

2.9. Biofilm formation assay

The bacterial ability to form biofilm "*in vitro*" was determined using the method described by O'Toole and Kolter (1998). Each well of a 96-well microtiter plate was filled with 150 μl bacterial suspension. Negative control contained sterile culture medium. Plate was incubated at 28 °C without shaking for 72 h. At this time, cell turbidity at 620 nm was measured by using a microtiter plate reader. Cultures were removed and the wells carefully washed with sterile distilled water to remove loosely associated bacteria. Then, every well was stained with 150 μl of 0.1% Crystal Violet solution for 20 min. After staining, plate was washed with sterile distilled water and 150 μl of 95% ethanol was added to each well, resuspending carefully the solution. Turbidity was determined by measurement of the absorbance of each well using a microtiter plate reader at 570 nm. The average of optical densities (OD) obtained at 620 nm and at 570 nm from the control wells was subtracted from the OD of all test wells. Biofilm formation was normalized with respect to bacterial growth to obtain the biofilm formation index (BFI), which was calculated by the following equation:

$$BFI = OD_{570}/OD_{620}.$$

The experiment was repeated three times with seven replicates for each treatment.

2.10. Isolation of bacteria from peanut root

In order to determinate the radical colonization of *Bradyrhizobium* sp. SEMIA6144 in presence or absence of *Bacillus* sp. CHEP5, the methodology described by Kuklinski-Sobral et al. (2004) with some modifications was followed. Briefly, epiphytic bacteria were isolated from roots placed in a 50 mL tube containing 10 mL of phosphate buffer saline (PBS) and they were agitated at 150 rpm for 1 h.

To isolate bacteria from inside tissues, plants roots were surface-disinfected by serial washing in 70% ethanol for 1 min, 2% sodium hypochlorite for 3 min, 70% ethanol for 30 s and six rinses in sterilized distilled water. Sterilization process was checked by plating aliquots of the wash water used in the final rinse onto YEMA and incubating the plates at 28 °C. The tissue was then pulverized in mortars with 10 mL of PBS. The samples obtained from the epiphytic and endophytic isolation procedures were diluted (10^{-1}–10^{-3}) and plated onto YEMA supplemented with cycloheximide (50 μg mL^{-1}) to control fungal growth, and chloramphenicol (40 μg mL^{-1}), ampicillin (100 μg mL^{-1}) and gentamicin (10 μg mL^{-1}) in order to select *Bradyrhizobium* sp. SEMIA6144 colonies. The plates were incubated at 28 °C for 7–10 days. The results were expressed as log colonies forming unit per gram of fresh weight (log CFU g FW^{-1}).

2.11. Isolation of Bacillus sp. CHEP5 from inside peanut nodule

To evaluate if *Bacillus* sp. CHEP5 was able to colonize peanut nodules, its presence inside this root organ was evaluated. Fifty percent of total nodules from each plant were surface-disinfected by serial washing in 96% ethanol for 2 min, 10% sodium hypochlorite for 5 min, 96% ethanol for 30 s and six rinses in sterilized distilled water. The success of the disinfection process was checked by plating aliquots of the wash water used in the final rinse onto TSA and incubating the plates at 28 °C for 24 h. Then, nodules were individually crushed in 200 μl of PBS. Appropriate dilutions (10^{-1}–10^{-3}) of these samples were plated onto TSA supplemented with cycloheximide (50 μg mL^{-1}) to control fungal growth. The plates were incubated at 28 °C for 24 h.

Table 1
Soil physical and chemical properties at the locations of the field trials.

Location	Organic matter (%)	pH (H_2O)	P (ppm)	Texture
Las Vertientes	2.42	6.78	10.1	Sandy loam
Río Cuarto	1.84	6.44	37.3	Sandy loam

2.12. BOX –PCR analysis

The fingerprinting analysis was performed in order to confirm if suspicious colonies isolated from inside peanut nodules were *Bacillus* sp. CHEP5. Approximately 10–12 colonies from TSA plates were selected to obtain DNA template. Total genomic DNA was extracted according with Walsh et al. (1991). BOX-AR1 primer sequence (5′-CTACGGCAAGGCGACGCTGACG- 3′) used in this study was described by Versalovic et al. (1994). The BOX-PCR was performed in 25 μl reaction mixture containing 10 X PCR buffer, 50 mM $MgCl_2$, 2 mM DNTPs, 50 pmol mL^{-1} BOX-AR1 primer, 1 U Taq DNA Polymerase (Promega, USA) and 6 μl DNA template solution. The temperature profile was as follows: initial denaturation at 95 °C for 7 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min, extension at 65 °C for 8 min and a final extension step at 65 °C for 16 min. PCRs were performed in a Mastercycler gradient block (Eppendorf, Germany). The BOX amplification products in 12 μl sub-samples were separated according to molecular size by horizontal electrophoresis on 1.5% (w/v) agarose gels and stained with SYBR Green.

2.13. Field assay

To evaluate field performance of *Bradyrhizobium* sp. SEMIA6144 and *Bacillus* sp. CHEP5, an experiment was carried out in two locations in the peanut-growing region of Córdoba Province, Argentina at the season 2015/2016. One of the sites (Río Cuarto) was located at 33° 9′40.38″S; 64° 8′0.45″O) and the other (Las Vertientes) at 33°18′58.14″S; 64°33′52.71″O. Chemical and physical soil properties were determined, at the beginning of the experiment, according to standard methods (Pavan et al., 1992) from samples taken from 0 to 20 cm depth (Table 1).

For inocula preparation, a *Bradyrhizobium* sp. SEMIA6144 or *Bacillus* sp. CHEP5 culture at the stationary phase of growth in YEM or TSB medium, respectively, were diluted in a 1:1 ratio with arabic gum 0.6%. The CFU mL^{-1} were determined following the method described by Somasegaran and Hoben (1994). Inoculants were stored at 4 °C until use.

Planting occurred on 22 October 2015, using cultivar "Granoleico" for all assays. Seeds were treated with the commercial fungicide Maxim®. A self-propelled pneumatic seed drill (Nova SIEMBRA) was used, with a seeding rate of 20 seeds m^{-1} and a planting depth of 3 cm. The size of each experimental plot was 1 ha with 0.7 m between rows. The following treatments were tested: a) inoculated with *Bradyrhizobium* sp. SEMIA 6144, b) inoculated with *Bacillus* sp. CHEP5, c) co-inoculated with a mixture (1:1) of both bacteria, d) uninoculated control. Treatments were laid out in a randomized complete block design. The inoculants were adjusted with water to a concentration 1.5. 10^6 CFU mL^{-1} and sprayed into the furrows immediately after seed drop but previous to furrow closure.

At time of harvest (20 April 2016), 7 samples, each of them consisting of plants from 1 m^2, were obtained, placed in an oven at 80 °C to constant weight and evaluated for pod and seed yield. The

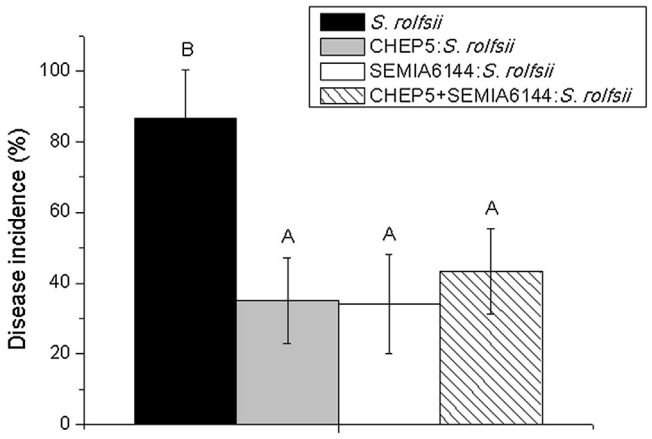

Fig. 1. Disease incidence in peanut plants inoculated with *Bacillus* sp. CHEP5 and/or *Bradyrhizobium* sp. SEMIA6144. Values are the mean ± SE. The experiment was repeated four times with 10 replicates per treatment. Different letters indicate significant differences according to LSD Fisher test ($p < 0.05$).

seeds harvested were classified as extra-large, if they did not pass through a screen with 7.5-mm openings (Bogino et al., 2006). At this time, incidence of fungal diseases was reported as percentage of affected pods, and severity was assessed using a scale of 0–4 (Marinelli and March 2005). Severity index was calculated by the following formula:

$$IS = (X_0Y_0) + (X_1Y_1) + (X_2Y_2) + (X_3Y_3) + (X_4Y_4)$$

X: severity rate, Y: proportion of infected sample.

2.14. Statistical analysis

The data were subjected to analysis of variance (ANOVA) and analyzed by LSD-Fisher test. p values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Bradyrhizobium sp. SEMIA6144 does not affect the defense response induced by Bacillus sp. CHEP5 against S. rolfsii in peanut plants

In order to determinate if *Bradyrhizobium* sp. SEMIA6144 affects the ability of *Bacillus* sp. CHEP5 to protect peanut against *S. rolfsii* in greenhouse assays, percentage of diseased plants, total chlorophyll content and shoot and root dry weights were determined in co-inoculated and pathogen challenged plants.

As expected, considering previous results (Tonelli et al., 2011), disease incidence was reduced by 52% in peanut plants inoculated only with *Bacillus* sp. CHEP5. Values of disease incidence obtained in co-inoculated plants were similar to those from plants inoculated only with the biocontrol agent. Therefore, it is apparent that *Bradyrhizobium* sp. SEMIA6144 did not affect *Bacillus* sp. CHEP5 biocontrol capacity (Fig. 1). In concordance with this finding, plant fitness parameters (total chlorophyll content and shoot and root dry weights) from *S. rolfsii* challenged plants inoculated with each bacterium or co-inoculated with the mixed bacterial culture were significantly higher than those from uninoculated challenged plants (Tables 2 and 3). One unexpected finding is that, in plants inoculated with *Bradyrhizobium* sp. SEMIA6144 pure culture, the stem wilt incidence was reduced (Fig. 1) and the plant fitness parameters reached values similar or slightly higher (shoot dry weight) than those from *Bacillus* sp. CHEP5 inoculated plants (Tables 2 and 3), probably by the activation of plant defense response.

Table 2
Total chlorophyll content of peanut plants inoculated with *Bacillus* sp. CHEP5 and/or *Bradyrhizobium* sp. SEMIA6144, and challenged with *S. rolfsii*.

Treatments	Total chlorophyll (μg g fresh weight^{-1})
Control	111 ± 12^{B}
Fertilized (KNO_3 5 mM)	159 ± 12^{D}
CHEP5	160 ± 11^{D}
SEMIA6144	147 ± 11^{CD}
CHEP5 + SEMIA6144	157 ± 11^{D}
S. rolfsii	74 ± 10^{A}
CHEP5:*S. rolfsii*	109 ± 11^{B}
SEMIA6144:*S. rolfsii*	122 ± 12^{BC}
CHEP5 + SEMIA6144:*S. rolfsii*	135 ± 11^{BCD}

Values are the mean ± SE. The experiment was repeated four times with 10 replicates per treatment. Different letters indicate significant differences according to the LSD Fisher test ($p < 0.05$).

Table 3
Shoot and root dry weight of peanut plants inoculated with *Bacillus* sp. CHEP5 and/or *Bradyrhizobium* sp. SEMIA6144, and challenged with *S. rolfsii*.

Treatments	Shoot dry weight (mg plant^{-1})	Root dry weight (mg plant^{-1})
Control	222 ± 15^{C}	113 ± 9^{CD}
Fertilized (KNO_3 5 mM)	342 ± 18^{EF}	139 ± 11^{D}
CHEP5	268 ± 17^{D}	119 ± 10^{CD}
SEMIA6144	305 ± 17^{DE}	105 ± 9^{C}
CHEP5 + SEMIA6144	387 ± 20^{F}	106 ± 7^{C}
S. rolfsii	64 ± 13^{A}	32 ± 6^{A}
CHEP5:*S. rolfsii*	144 ± 16^{B}	64 ± 6^{B}
SEMIA6144:*S. rolfsii*	195 ± 20^{C}	52 ± 8^{B}
CHEP5 + SEMIA6144:*S. rolfsii*	183 ± 17^{BC}	73 ± 8^{B}

Values are the mean ± SE. The experiment was repeated four times with 10 replicates per treatment. Different letters indicate significant differences according to the LSD Fisher test ($p < 0.05$).

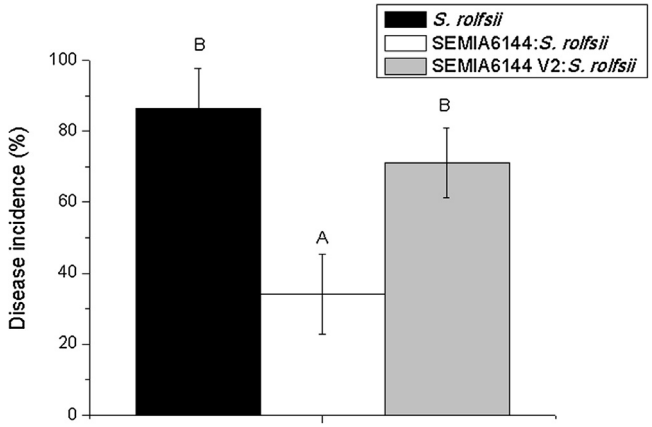

Fig. 2. Disease incidence in peanut plant inoculated with *Bradyrhizobium* sp. SEMIA6144 or its mutant *Bradyrhizobium* sp. SEMIA6144 V2. Values are the mean ± SE. The experiment was repeated four times with 10 replicates per treatment. Different letters indicate significant differences according to LSD Fisher test ($p < 0.05$).

3.2. Nod factors from Bradyrhizobium sp. SEMIA6144 contribute to the induction of peanut defense response against S. rolfsii

We evaluated whether Nod factors produced by *Bradyrhizobium* sp. SEMIA6144 are involved in the induction of peanut defense response against *S. rolfsii*. Plants inoculated with a *Bradyrhizobium* sp. SEMIA6144 derivative mutant (V2) unable to produce Nod factors showed higher disease incidence (Fig. 2) and lower shoot dry weight than plants inoculated with the wild-type strain (Table 4).

Table 4
Shoot and root dry weight of peanut plants inoculated with *Bradyrhizobium* sp. SEMIA6144 or its mutant *Bradyrhizobium* sp. SEMIA6144 V2.

Treatments	Shoot dry weight (mg plant^{-1})	Root dry weight (mg plant^{-1})
Control	222 ± 15 ^{C}	113 ± 9 ^{C}
Fertilized (KNO_3 5 mM)	342 ± 18 ^{E}	139 ± 11 ^{D}
SEMIA6144	305 ± 17 ^{DE}	105 ± 9 ^{C}
SEMIA6144 V2	293 ± 12 ^{D}	108 ± 5 ^{C}
S. rolfsii	64 ± 13 ^{A}	32 ± 6 ^{A}
SEMIA6144: *S. rolfsii*	195 ± 20 ^{C}	52 ± 8 ^{B}
SEMIA6144 V2: *S. rolfsii*	122 ± 11 ^{B}	51 ± 4 ^{B}

Values are the mean ± SE. The experiment was repeated four times with 10 replicates per treatment. Different letters indicate significant differences according to the LSD Fisher test ($p < 0.05$).

These findings suggest that Nod factors from *Bradyrhizobium* sp. SEMIA6144 are contributing with the elicitation of peanut systemic resistance against *S. rolfsii.*

3.3. Bacillus *sp. CHEP5 improves the symbiotic behavior of* Bradyrhizobium *sp. SEMIA6144 in peanut plants*

To evaluate if *Bacillus* sp. CHEP5 affects the behavior of *Bradyrhizobium* sp. SEMIA6144, symbiotic parameters were determined in *S. rolfsii* challenged or unchallenged plants. Uninoculated N-free control plants were stunted, with yellow leaves while the uninoculated N-fertilized were large, with dark green leaves. These plants remained nodule free. The average shoot dry weight of the N-fertilized plants was 54% higher than that of the N-free control plants, indicating that in these conditions available nitrogen was a limiting factor for plant growth.

In co-inoculated unchallenged plants, the number of total nodules and percentage of red nodules formed were similar to those from plants inoculated only with the microsymbiont, but total nodule dry weight from each plant was increased (Table 5). Shoot dry weight and nitrogen content from these plants were increased compared with those inoculated only with *Bradyrhizobium* sp. SEMIA6144 (Tables 4 and 6). Moreover, diameters of nodule and of their nitrogen fixing zones reached in these plants the highest values (Table 7, Fig. 3)

In *Bradyrhizobium* sp. SEMIA6144 inoculated and *S. rolfsii* challenged plants, and as expected, all the symbiotic parameters were reduced drastically. However, nodule dry weight and number per plant, percentage of red nodules formed, percentage of nodulated plants as well as plant nitrogen content were improved in co-inoculated and *S. rolfsii* challenged plants (Table 5). Interestingly, by fingerprinting analysis (BOX-PCR) of colonies isolated from inside nodules formed in co-inoculated plants, it was confirmed the ability of *Bacillus* sp. CHEP5 to endophytically colonize these organs at 40 days after inoculation (data no shown).

3.4. *Bacterial root colonization and biofilm formation*

In an attempt to explain the improved *Bradyrhizobium* sp. SEMIA6144 symbiotic performance in plants inoculated with the biocontrol agent *Bacillus* sp. CHEP5, we analyzed the ability of the microsymbiont to colonize peanut root when is co-inoculated with *Bacillus* sp. CHEP5 as well as the quantity of biofilm formed by the mixed culture.

Inoculation of *Bacillus* sp. CHEP5 improved the *Bradyrhizobium* sp. SEMIA6144 root surface colonization since the number of CFU/g of root increased by a 40% (Fig. 4). Furthermore, the mixed bacterial culture formed a higher quantity of biofilm than the microsymbiont pure culture but lower compared to *Bacillus* sp. CHEP5 pure culture (Table 8).

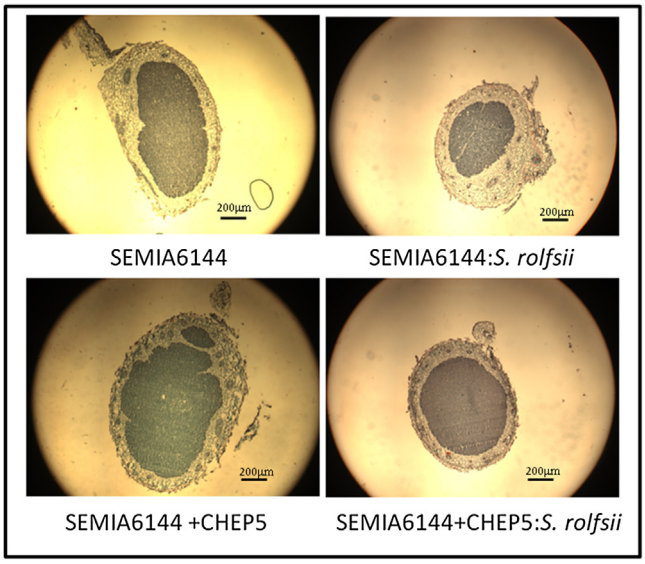

Fig. 3. Microscopic analysis of sections of peanut nodules from inoculated plants with *Bradyrhizobium* sp. SEMIA6144 alone or co-inoculated with *Bacillus* sp. CHEP5, and challenged with *S. rolfsii*. The experiment was repeated twice with 10 replicates per treatment.

Table 6
Nitrogen content in peanut plants inoculated with *Bradyrhizobium* sp. SEMIA6144 alone or co-inoculated with *Bacillus* sp. CHEP, and challenged with *S. rolfsii.*

Treatments	N (mg plant^{-1})
SEMIA6144	8.28 ± 0.22 ^{B}
CHEP5 + SEMIA6144	9.60 ± 0.21 ^{C}
SEMIA6144: *S. rolfsii*	6.10 ± 0.22 ^{A}
SEMIA6144 + CHEP5: *S. rolfsii*	7.64 ± 0.19 ^{B}

Values are the mean ± SE. The experiment was repeated twice with 5 replicates per treatment. Different letters indicate significant differences according to the LSD Fisher test ($p < 0.05$).

3.5. *Field performance of* Bradyrhizobium *sp. SEMIA6144 and* Bacillus *sp. CHEP5 in co-inoculated peanut plants*

In order to evaluate the effect of *Bradyrhizobium* sp. SEMIA6144 and *Bacillus* sp. CHEP5 co-inoculation, pod and seed yields were evaluated. In Río Cuarto, yield of extra-large seeds increased by 2.15% in plants inoculated with *Bacillus* sp. CHEP5 compared with

Table 5
Symbiotic performance of *Bradyrhizobium* sp. SEMIA6144 in co-inoculated and *S. rolfsii* challenged plants.

Treatments	Nodule numberplant^{-1}	Nodule dry weight (mg plant^{-1})	% red nodule	% nodulated plants
SEMIA6144	21 ± 2 ^{BC}	6.5 ± 0.5 ^{B}	100 ± 0 ^{C}	100 ± 0 ^{C}
CHEP5 + SEMIA6144	25 ± 2 ^{C}	9.5 ± 0.5 ^{C}	100 ± 0 ^{C}	100 ± 0 ^{C}
SEMIA6144: *S. rolfsii*	5 ± 4 ^{A}	2.8 ± 1.2 ^{A}	13 ± 5 ^{A}	22.5 ± 2.9 ^{A}
SEMIA6144 + CHEP5: *S. rolfsii*	17 ± 3 ^{B}	7.7 ± 0.8 ^{BC}	75 ± 5 ^{B}	41.7 ± 2.4 ^{B}

Values are the mean ± SE. The experiment was repeated four times with 10 replicates per treatment. Different letters indicate significant differences according to the LSD Fisher test ($p < 0.05$).

Table 7
Diameters of nodules formed in peanut plants inoculated with *Bradyrhizobium* sp. SEMIA6144 alone or co-inoculated with *Bacillus* sp. CHEP5, and challenged with *S. rolfsii*.

Treatments	μm		NFAD/ND
	Nodule Diameter (ND)	Nitrogen Fixing Area Diameter (NFAD)	
SEMIA6144	1131.03 ± 58.03 ^{B}	823.32 ± 49.45 ^{B}	0.82 ± 0.03 ^{A}
SEMIA6144: *S. rolfsii*	866.59 ± 64.88 ^{A}	648.72 ± 47.20 ^{A}	0.80 ± 0.04 ^{A}
CHEP5 + SEMIA6144	1313.86 ± 55.23 ^{C}	1075.22 ± 42.34 ^{C}	0.78 ± 0.03 ^{A}
CHEP5 + SEMIA6144: *S. rolfsii*	1076.03 ± 58.13 ^{B}	815.00 ± 50.03 ^{B}	0.78 ± 0.02 ^{A}

Values are the mean ± SE. The experiment was repeated twice with 10 replicates per treatment. Different letters indicate significant differences according to the LSD Fisher test ($p < 0.05$).

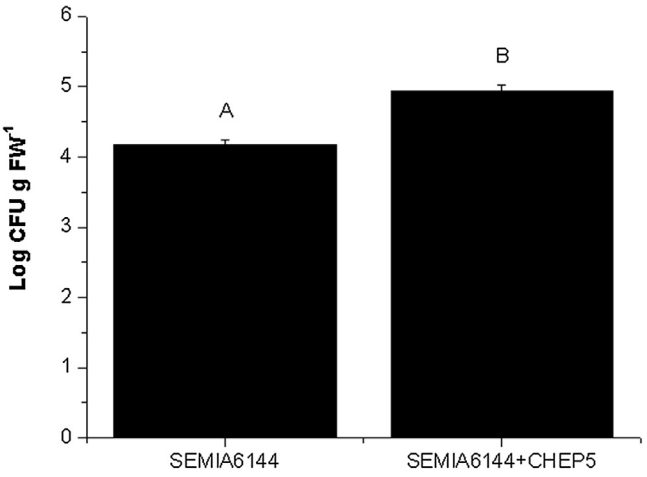

Fig. 4. Root colonization by *Bradyrhizobium* sp. SEMIA6144 in presence or absence of *Bacillus* sp. CHEP5. Values are the mean ± S.E. The experiment was repeated twice with 3 replicates per treatment. Different letters indicate significant differences according to LSD Fisher test ($p < 0.05$).

Table 8
Biofilm formation index of *Bradyrhizobium* sp. SEMIA6144, *Bacillus* sp. CHEP5 and the mixed cultures.

Treatment	BFI
Bradyrhizobium sp. SEMIA6144	0.53 ± 0.25 ^{A}
Bacillus sp. CHEP5	1.48 ± 0.20 ^{B}
Bacillus sp. CHEP5 + *Bradyrhizobium* sp. SEMIA6144	1.15 ± 0.20 ^{AB}

Values are the mean ± S.E. The experiment was repeated three times with 7 replicates per treatment. Different letters indicate significant differences according to LSD Fisher test ($p < 0.05$).

those from co-inoculated or inoculated only with *Bradyrhizobium* sp. SEMIA6144 (Table 9). In Las Vertientes, this parameter increased by 16.69% not only in plant inoculated with *Bacillus* sp. CHEP5 but also in co-inoculated ones. It is known that large seeds have greater consumer preference and receive greater prices in markets (Blengino 2014). Furthermore, inoculation with *Bradyrhizobium* sp. SEMIA6144 and/or *Bacillus* sp. CHEP5 increased both pod and seed yields (Table 9).

Unfortunately, no symptoms of wilting caused by *S. rolfsii* were found in any of the evaluated sites. However, pods with symptoms of peanut smut caused by the fungus *Thecaphora frezii* were detected in Río Cuarto. Considering that ISR is a nonspecific plant defensive response, that may protect plants against a wide spectrum of plant pathogens (Van Loon et al., 1998), peanut smut severity and incidence were analyzed.

As it was expected, inoculation of *Bacillus* sp. CHEP5 decreased both peanut smut severity and incidence. This effect was not only observed in plants inoculated only with this biocontrol agent but also in those co-inoculated (Fig. 5). Therefore, we conclude that *Bradyrhizobium* sp. SEMIA 6144 did not affected the *Bacillus* sp. CHEP5 biocontrol ability.

During the field experiments, climatic conditions (rainfall, temperature and relative humidity) were also monitored. Data recorded were in the normal range for the geographic area where the experiments were carried out (data not shown).

4. Discussion

The rhizosphere contains a wide diversity of microorganisms that interact with each other in a negative or positive manner (Bais et al., 2006). Although soil microorganisms are associated with plant diseases, many bacteria are beneficial for plant productivity. For instance, rhizobial bacteria establish a symbiotic interaction with legumes which introduces 50 to 70×10^6 ton nitrogen per year into the soil (Herridge et al., 2008), reducing the use of fertilizers. Other soil bacteria inhibit plant pathogens proliferation and/or reduce disease incidence by different mechanisms, helping plants to growth (Copping and Menn, 2000). ISR emerged as an important mechanism by which some plant growth promoting bacteria (PGPB) prime the whole plant body for enhanced defense against a broad range of pathogens. This state of resistance is characterize by the activation of latent defense responses that are expressed upon pathogen challenge not only at the site of induction but also systemically in plant parts spatially separated from the inducer (Pieterse et al., 2014).

Table 9
Effect of peanut co-inoculation on seed yield, pod yield and percentage of extra-large seeds.

Location	Treatment	Pod yield (Kg ha^{-1})	Seed yield (Kg ha^{-1})	% extra-large seeds
Las Vertientes	Control	5124.02 ± 224.79 ^{A}	3313.68 ± 272.76 ^{A}	49.84 ± 2.79 ^{A}
	SEMIA6144	5818.21 ± 189.98 ^{B}	4312.02 ± 263.76 ^{BC}	51.12 ± 3.05 ^{AB}
	CHEP5	6595.63 ± 205.20 ^{C}	5021.46 ± 251.21 ^{C}	58.16 ± 2.58 ^{BC}
	SEMIA 6144 + CHEP5	5837.10 ± 205.34 ^{B}	4162.61 ± 252.53 ^{B}	59.94 ± 2.59 ^{C}
Río Cuarto	Control	6527.45 ± 266.18 ^{AB}	5145.16 ± 278.80 ^{A}	93.73 ± 0.55 ^{A}
	SEMIA6144	5844.88 ± 291.59 ^{AB}	4760.81 ± 273.50 ^{A}	93.20 ± 0.51 ^{A}
	CHEP5	6713.58 ± 295.51 ^{B}	5441.76 ± 278.70 ^{A}	95.75 ± 0.60 ^{B}
	SEMIA 6144 + CHEP5	5759.20 ± 259.90 ^{A}	4616.74 ± 329.89 ^{A}	93.35 ± 0.51 ^{A}

Data represent the mean ± SE of seven replicates. Within a column for a given location, means followed by the same letters are not significantly different according to LSD Fisher test ($p < 0.05$).

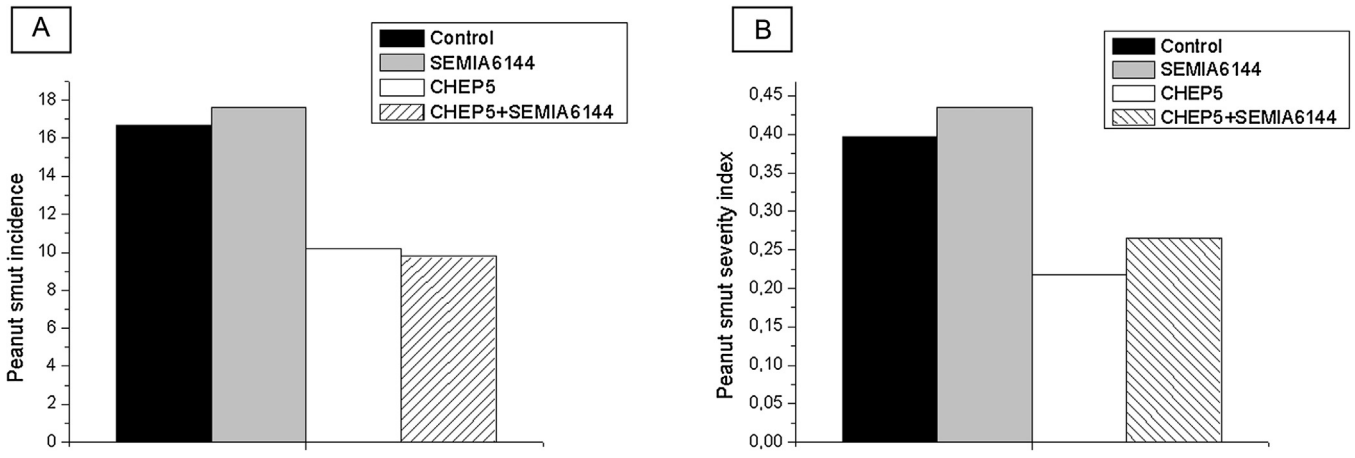

Fig. 5. A Peanut smut incidence expressed as percentage of affected pods, B. Peanut smut severity index. Data represent the mean ± SE of seven replicates.

Much of what we know about the interactions among plants and soil microorganisms is based upon studies that have been performed in associations between a single microorganism and a plant root. However, our understanding of the interactions among multiple soil microorganisms is lacking, and has hindered the possibility to predict the success of beneficial bacterial inoculation to promote plant growth at field. Therefore, it is necessary to redirect research focus to examine how multiple signaling pathways between root plants and different microorganisms impact on plant growth.

We have previously isolated from peanut rhizosphere the strain *Bacillus* CHEP5, identified as a biological control agent that protect peanut against *S. rolfsii* by ISR when plants are growing in nitrogen supplemented medium (Tonelli et al., 2010, 2011). In this work we demonstrated that the efficiency of *Bacillus* sp. CHEP5 to protect peanut plants both against *S. rolfsii* (in greenhouse assays) and *Thecaphora frezii* (in field trials) was not altered by the presence of the nitrogen fixer strain *Bradyrhizobium* sp. SEMIA6144. The unexpected finding (obtained from greenhouse assay) that the stem wilt incidence was also reduced in plants inoculated only with the microsymbiont suggests that this bacterium is also able to activate plant defense response against *S. rolfsii*. Considering that the experimental conditions used in this assay guarantees temporal and physical separation between the phytopathogen and this bacterium, we suggest that ISR is the mechanism involved in the defense response induced by *Bradyrhizobium* sp. SEMIA6144.

It has been reported that the initial reaction of legumes to rhizobial colonization resembles the response to pathogens, but the plant immune system is suppressed when symbiotic signaling is initiated (Santos et al., 2001; Jamet et al., 2007; Rubio et al., 2009). In peanut, we have previously demonstrated that at the early stage of the interaction with *Bradyrhizobium* sp. SEMIA6144 (10 min after inoculation), takes place an oxidative burst characterized by the root accumulation of H_2O_2, reaching the basal levels at 240 min after inoculation (Muñoz et al., 2015). On the other hand, It is widely accepted that legume plants exert a significant degree of control over this symbiotic interaction, limiting the number of nodules formed (Gage 2004). This suppression of nodulation appears to be dependent of signaling molecules from the shoot such as salicylic acid and jasmonic acid, which are also involved in induced plant response against pathogens. Therefore, interactions between signaling pathways involved in plant defense response and those induced by rhizobia might be expected to impact on plant-pathogens interactions. We hypothesize that nitrogen fixing symbiosis sensitizes aboveground peanut parts for a more effective activation of defense response against *S. rolfsii*, phenomenon known as priming (Conrath et al., 2006). In this sense, Azpilicueta et al. (2004) reported that the systemic induction of phytoalexins synthesis in peanut plants inoculated with *Bradyrhizobium* sp. SEMIA6144 is the mechanism involved in the protection against *Aspergillus niger* and *Cladosporium cucumerinum*.

Nod factors are lipo-chito-oligosaccharides synthesized by rhizobia and there are evidences suggesting that the plant perception of these molecules has evolved from recognition of more general elicitors of plant defense such as chitin fragments (Cullimore et al., 2001). Moreover, plants can detect phytopathogens using receptors that are evolutionarily related to those employed by legumes to detect symbiotic rhizobia (Samac and Graham 2007).

The diminution of the defense response against *S. rolfsii* in plants inoculated with the isogenic strain *Bradyrhizobium* sp. SEMIA6144V2 (unable to synthesize Nod factors) compared with the wild-type strain indicates a role for these molecules in the induction of ISR. These findings are in agreement with results from Rey et al. (2013). These authors reported that *Medicago truncatula* mutants, unable to perceive Nod factors, were more susceptible to the pathogens *Aphanomyces auteiches* and *Colletotrichum trifolii* than wild-type plants.

Various studies have shown that co-inoculation of rhizobia with *Bacillus* remarkably enhanced nodulation and growth of legume crops (Halvenson and Handelsman, 1991; Rajendran et al., 2008; Elkoca et al., 2008; Egamberdieva et al., 2010). However, in this work it was found that not the number but the nodule dry weight, diameters of their fixing zones and shoot nitrogen content were higher in co-inoculated unchallenged plants compared with that from plants inoculated with *Bradyrhizobium* sp SEMIA6144. As nitrogen fixation in peanut is closely related with nodule dry weight and this parameter is more important than nodule number for nitrogen fixation under stress conditions (Pimratch et al., 2008), the improved symbiotic parameters of these plants (Table 5) could be related to a higher bacteroid number and/or nitrogenase activity.

Interestingly, fingerprinting analysis (BOX-PCR) of colonies isolated from inside nodules formed in co-inoculated plants revealed that *Bacillus* sp. CHEP5 is able to endophytically colonize these organs, in accordance with our previous report demonstrating the presence of *Gammaproteobacteria* inside peanut nodule (Ibáñez et al., 2009). Considering that it has been informed that the nitrogen fixation activity of non-rhizobial PGPB have small or non-detectable effect on the plant growth (Vessey, 2003), the improved symbiotic behavior of peanut microsymbiont in co-inoculated plants should be probably related to a direct effect on either the bradyrhizobia or the plant. Siderophore production has been related with the ability of non-rhizobial strains to increase symbiotic performances in legumes since these iron-

chelating compounds are known to have growth stimulating effects on other target species in the same niche (Joshi et al., 2008). However, as we have previously demonstrated, *Bacillus* CHEP5 is unable to produces siderophores (Tonelli et al., 2010).

Co-inoculation not only improved the symbiotic performance in unchallenged plants but also in *S. rolfsii* challenged plants. Moreover, in addition to nodule dry weight and shoot nitrogen content, nodule number, percentage of nodulated plants and of red nodules formed were also increased in these plants. It has been reported that the production of volatile compounds by PGPB could contribute to the enhanced symbiotic performance of pathogen challenged legumes (Ballhorn et al., 2013). However, as we have previously demonstrated that *Bacillus* sp. CHEP5 is unable to produce volatile compounds against *S. rolfsii* (Tonelli et al., 2010), this effect cannot be attributed to these molecules. Considering that successful biofilm formation and root colonization are relevant to obtain effective legume nodulation (Bloemberg and Lugtenberg, 2001; Lagendijk et al., 2010), an explanation for the beneficial effect of *Bacillus* sp. CHEP5 on the *Bradyrhizobium* sp. SEMIA6144 symbiotic performance in *S. rolfsii* challenged plants could be that, at the time of pathogen inoculation, root colonization by the microsymbiont was increased in co-inoculated plants.

Results from this study clearly demonstrate that the native biocontrol agent *Bacillus* sp. CHEP5 acts synergistically with *Bradyrhizobium* sp. SEMIA6144 improving the symbiotic interaction with peanut (possibly by rhizobial colonization enhancement) even in plants challenged with *S. rolfsii*, and that this rhizobial strain does not interfere with the ISR triggered by *Bacillus* sp. CHEP5. Moreover, in this study we demonstrated that the use at field of this mixed inoculant provides natural protection against phytopathogens and increases the yield of extra-large peanut seeds.

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