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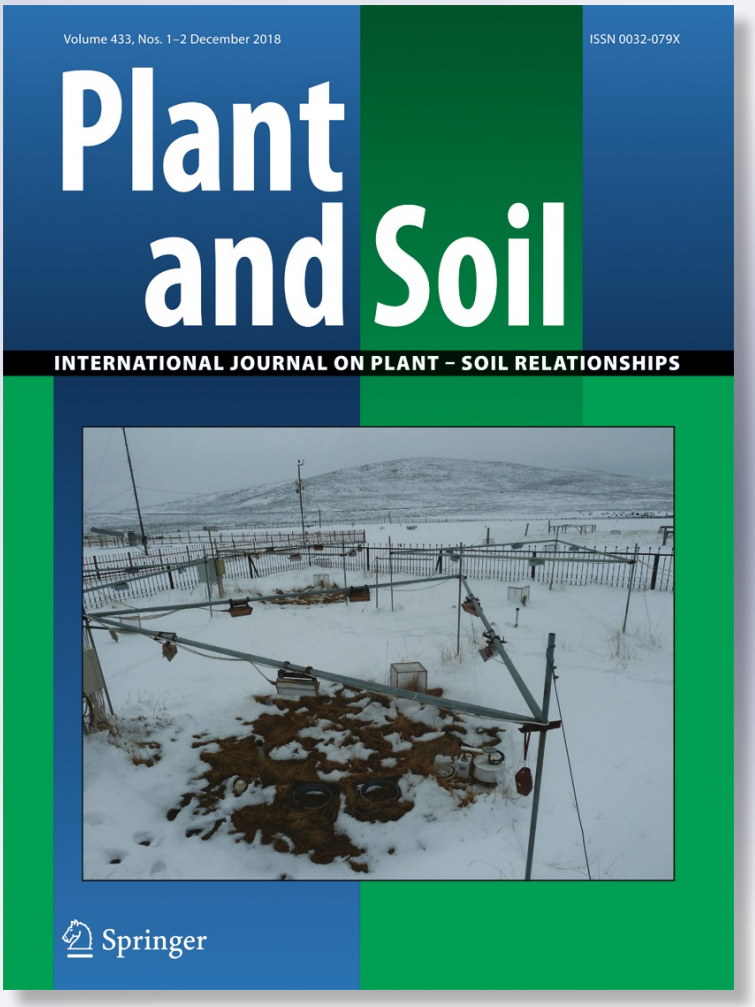


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REGULAR ARTICLE

# Simultaneous inoculation with beneficial and pathogenic microorganisms modifies peanut plant responses triggered by each microorganism

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**Abstract**
*Background and aims* Plant signaling pathways activated by single microbial species may be modified by the presence of other microbial groups. Here, link between phenotypic changes of peanut plants co- inoculated with *Bradyrhizobium* sp. SEMIA6144, *Bacillus* sp. CHEP5 and *Sclerotium rolfsii*, and molecules involved in peanut responses to each microorganism was evaluated.
*Methods* Phenolic compounds content, peroxidase activity and *AhSymRK* gene expression were evaluated in plants co-inoculated and inoculated with each microorganism.
*Results* Peroxidase activity, associated with peanut response to the pathogen, was induced earlier in plants co-inoculated than in those inoculated only with *S. rolfsii*, in coincidence with their more tolerant phenotype to this pathogen. The increase in phenolic compounds content induced by the biocontrol agent *Bacillus* sp. CHEP5 was affected by the co-inoculation with *Bradyrhizobium* sp. SEMIA6144. However, the bacterial protection against *S. rolfsii* remains unaltered. In co-inoculated plants, *AhSymRK* gene expression level was similar to plants inoculated only with the microsymbiont, in concordance with their symbiotic phenotypes.
*Conclusions* We demonstrated that responses triggered in peanut plants by single microbial species populations are modified in presence of others, highlighting the relevance to improve our understanding about plant responses to soil microbial communities.

**Keywords** Co-inoculation · Symbiosis · Induced systemic resistance · Pathogenesis

## Introduction

The rhizosphere is inhabited by millions of (micro)organisms, including bacteria, nematodes, fungi, protozoa and arthropods (van Dam and Bouwmeester 2016). Soil microbial diversity, edaphic factors and root exudates are some of the most important determinants of root microbiota composition (Lareen et al. 2016). Plants growing in natural environments interact simultaneously with a wide diversity of beneficial and pathogenic microorganisms which may affect their growth and health. Therefore, plants must be able to restrict pathogenic interactions at the same time that they promote the association with beneficial microorganisms. Reaching this balance is complex and requires an adequate perception of invader microorganisms through molecular signals, followed by response activation that promotes colonization of beneficial microorganisms (Zipfel and Oldroyd 2017).

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It is expected that the knowledge gained on responses triggered in plants interacting simultaneously with pathogenic and beneficial microorganisms contributes to develop integrated disease management strategies, as well as to enhance the association of plants with beneficial microorganisms to mitigate stress or factors limiting crop production (Pieterse et al. 2014).

*Arachis hypogaea* L. (peanut) is one of the world's most important oilseed crops which establish a symbiotic relationship with *Bradyrhizobium* sp. SEMIA6144, a nitrogen-fixing bacterium (Fabra et al. 2010). *AhSymRK* is one of the genes involved in the peanut symbiotic signaling pathway (Sinharoy et al. 2009). Peanut productivity is affected by different biotic and abiotic stresses. Stem wilt caused by *Sclerotium rolfsii* is one of the diseases that adversely affect peanut yields all over the world's growing areas.

Previous results obtained in our laboratory demonstrated that the native isolate *Bacillus* sp. CHEP5 induces systemic resistance (ISR) in peanut, which is effective against the pathogen *S. rolfsii* (Tonelli et al. 2011). ISR is a mechanism by which some plant growth promoting bacteria (PGPB) prime plant defense system (Mauch-Mani et al. 2017). These bacteria induce latent plant defense responses that, after pathogen challenge, are expressed locally and systemically. On the other hand, we also demonstrated that the simultaneous inoculation of peanut plants with *Bradyrhizobium* sp. SEMIA 6144, *Bacillus* sp. CHEP5 and *S. rolfsii*, affects the bacterial plant growth promoting activities and the fungus pathogenic effects and consequently, the phenotype of each interaction is modified (Figueredo et al. 2017). The aim of this study was to link these phenotypic changes with variations in the activity or content of plant molecules and in the expression of genes involved in peanut responses to each microorganism.

## Materials and methods

### Bacterial strains and culture conditions

*Bacillus* sp. CHEP5 (a native biocontrol agent) (Tonelli et al. 2010) and *Bradyrhizobium* sp. SEMIA6144 (reference strain recommended as inoculant by Microbiological Resource Center, Porto Alegre, Brazil) 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 was cultured at 28 °C on Yeast Extract Mannitol broth (YEM) or YEM-agar (YEMA) (Vincent 1970).

For inocula preparation, bacterial cultures were grown until they reached an $OD_{620nm} = 1$ ($10^8$ CFU $mL^{-1}$) approximately. Then they were centrifuged at 2500 rpm for 5 min at room temperature and 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).

### Fungal culture conditions

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

For fungal inoculum preparation, sterile wet wheat seeds were 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).

### Plant material and growth conditions

*A. hypogaea* L. (var. Runner cultivar Granoleico) seeds were surface disinfected as described by Vincent (1970). Briefly, seeds were soaked in ethanol 96% for 30 s followed by 30% $H_2O_2$ for 20 min, and then washed six times with sterile distilled water. Seeds were germinated at 28 °C on sterilized Petri dishes with one layer of Whatman N°1 filter paper and moist cotton, until the radicle reached about 2–3 cm.

To avoid direct contact between the bacteria and the fungi, the methodology for plant growing conditions described by Figueredo et al. (2017) was followed. Briefly, 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 plant root reaches the bottom plastic cup through the hole connecting 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, peanut 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). Non-pathogenized and non-bacterized 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. The experiment was repeated three times with 7 replicates per treatment.

### Determination of total peroxidase (PX) activity

Peanut stems or *S. rolfsii* mycelium (0.1 g) were homogenized with liquid nitrogen using a mortar and pestle containing appropriate buffer solution (50 mM potassium phosphate and 1 mM EDTA, pH 7.4) and 1% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant was stored at −20 °C to be used for enzymatic activity determination.

The protein concentration of the extracts was determined by the method described by Bradford (1976), using bovine albumin (1 mg $mL^{-1}$) as standard.

Total PX activity was determined by measuring increase in absorbance at 470 nm according to Sosa Alderete et al. (2009). Activity was calculated applying Lambert-Beer law using molar extinction coefficient 11.3 $mM^{-1}$ $cm^{-1}$. One unit (U) of PX was defined as the amount of enzyme that catalyses the formation of 1 μmol of product in 1 min of reaction. PX activity was expressed as U $mg^{-1}$ protein.

### Phenolic compounds determination

For extraction and quantification of phenolic compounds the methodology described by Ainsworth and Gillespie (2007) was followed. Briefly, peanut stems (0.1 g) were homogenized with liquid nitrogen using a mortar and pestle containing 1 mL of 95% cold methanol and kept in the dark at room temperature for 48 h. Each sample was then homogenized and centrifuged at 10,000 rpm for 5 min. The supernatant was removed and stored at −20 °C. Reaction mixture consisted in 100 μL of plant extract and 200 μL of 10% Folin-Ciocalteau. After 5 min, 800 μL of 700 mM $Na_2CO_3$ were added, the reaction mixture was allowed to stand for 2 h and the absorbance at 765 nm was recorded. A calibration curve was prepared for each assay using different concentrations of gallic acid (GA) in 95% methanol. Absorbance values were converted to mM gallic acid equivalent (GAE) $g^{-1}$ fresh weight (FW).

### Determination of *AhSymRK* expression levels

At 9 days post-inoculation (dpi) with *Bradyrhizobium* sp. SEMIA6144, samples from 4 plants per treatment were pooled. RNA from roots was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized from 1 μg of total RNA using the AccuScript Hi-Fi RT (Agilent, Santa Clara, CA, USA) and diluted (1:40) to perform quantitative reverse-transcriptase polymerase chain reaction (qPCR) assays using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. The sequences of primers used for qPCR amplification are provided in Supplementary Table S1. The efficiency of the PCR reactions was determined by linear regression analysis of 10-fold dilutions of cDNA and denoted with a correlation coefficient ($r^2$). Reactions were performed in a real-time thermocycler (Stratagene MX3000P; Agilent, Santa Clara, CA, USA) with settings of 95 °C for 3 min, and 40 cycles of 95 °C for 20 s and 60 °C for 20 s. At least three biological replicates were performed with technical duplicates for each sample. Results obtained from the different treatments were standardized to the Actin mRNA level. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

### Statistical analysis

Data were subjected to analysis of variance (ANOVA). Statistical significance was determined by LSD-Fisher test at $p \leq 0.05$, using Infostat software (1.0, FCA, UNC, Argentina). For comparison between two treatments we applied Student test-*t*.

## Results

### PX activity is linked to *S. rolfsii* pathogenesis

In order to determine if peanut inoculation with *S. rolfsii* induces changes in the PX activity, this was measured at

different time's post-inoculation (1, 5, 10, 24, 48 and 72 h post inoculation (hpi)).

Results showed that PX activity began to increase at 48 hpi in challenged plants compared with non-challenged healthy control plants (Fig. 1). This time coincided with an increase in *S. rolfsii* colonization of plant tissues (Supplementary Fig. 1). We confirmed by determining this enzymatic activity also in fungal mycelium that the activity measured belong only to plant tissues (data not shown). As expected, these results confirm that *S. rolfsii* induces plant defense at the site of inoculation, and indicate that the changes in peanut PX activity are associated to *S. rolfsii* pathogenesis.

## Phenolic compounds content increases during ISR elicited by *Bacillus* sp. CHEP5

Phenolic compounds content was determined in stems from plants inoculated with the biocontrol agent *Bacillus* sp. CHEP5 at 48 hpi with *S. rolfsii*. A significant increase in the content of these compounds was detected in these plants compared to non-inoculated plants challenged with the pathogen, and to non-challenged healthy control plants (Fig. 2). Moreover, in absence of *S. rolfsii*, phenolic compound accumulation was similar in inoculated and control plants.

Results indicated that phenolic compound accumulation at 48 hpi of *S. rolfsii* is linked to the ISR elicited by *Bacillus* sp. CHEP5.

## *AhSymRK* gene is up-regulated in early symbiotic steps of *Bradyrhizobium* sp. SEMIA6144-peanut interaction

*AhSymRK* relative gene expression was measured by qPCR in peanut roots at 9 dpi with *Bradyrhizobium* sp. SEMIA6144. A significant up-regulation was found compared with non-inoculated plants (Fig. 3), indicating that *AhSymRK* gene expression is involved in the symbiotic pathway triggered in peanut by *Bradyrhizobium* sp. SEMIA6144.

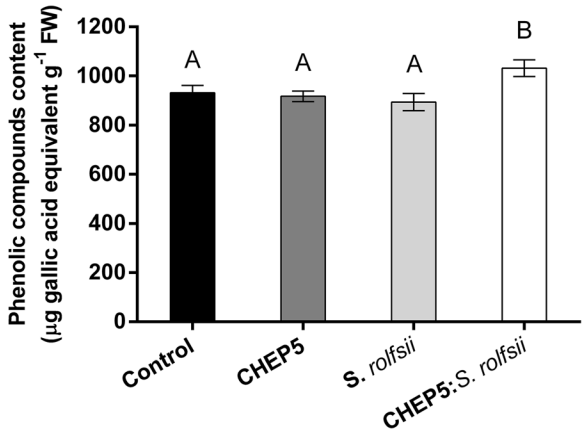


**Fig. 2** Phenolic compounds content in peanut stems. Values are the mean ± S.E. of three experiment with 7 replicates per treatment. Different letters indicate significant differences according to LSD Fisher test ($p < 0.05$)

## Co-inoculation of peanut plants with *S. rolfsii*, *Bacillus* sp. CHEP5 and *Bradyrhizobium* sp. SEMIA6144 modifies the intensity and/or timing of plant responses linked to the interaction with each microorganism

In order to determine whether responses of peanut plants to the presence of each microorganism are modified by the simultaneous interaction with all of them, we evaluated PX activity, phenolic compound content and *AhSymRK* gene expression levels, as molecular events associated to pathogenesis, ISR and symbiosis, respectively.

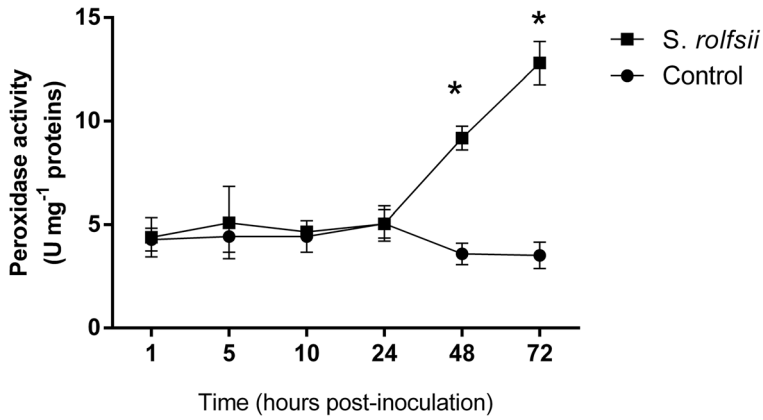


**Fig. 1** PX activity in peanut stems at different times post-inoculation with *S. rolfsii*. Values are the mean ± S.E. of three experiment with 7 replicates per treatment. Asterisks indicate significant differences according to LSD Fisher test ($p < 0.05$)

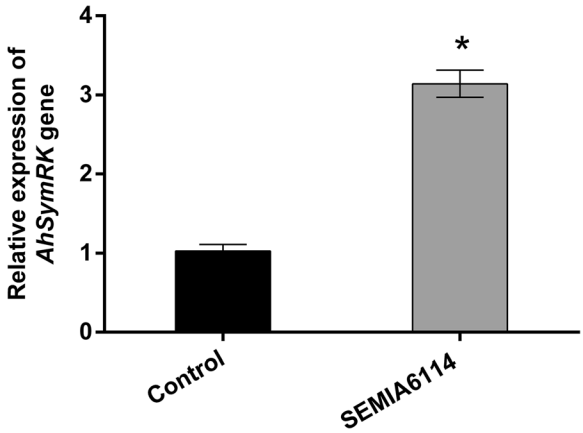


**Fig. 3** *AhSymRK* gene expression levels in peanut roots inoculated with *Bradyrhizobium* sp. SEMIA6144. Values are the mean ± S.E. of three experiment with 7 replicates per treatment. Asterisk indicates significant difference according to test t-student ($p < 0.05$)

PX activity, measured at 24 or 48 hpi with the pathogen, was not affected by the *Bradyrhizobium* sp. SEMIA6144 inoculation. However, this enzymatic activity increased in plants inoculated with *Bacillus* sp. CHEP5 and in plants co-inoculated with both bacteria at 24 hpi with *S. rolfsii*, reaching after 48 hpi similar levels to those found in plants challenged only with the pathogen (Fig. 4).

No difference in phenolic compounds content was found between non-inoculated, inoculated with *Bacillus* sp. CHEP5 or with *Bradyrhizobium* sp. SEMIA6144, and co-inoculated plants. However, at 48 hpi with *S. rolfsii*, the content of these compounds in plants singly-inoculated with *Bacillus* sp. CHEP5 or with *Bradyrhizobium* sp. SEMIA6144 was 13.5 and 12.4% higher than in plants only infected with the phytopathogen, respectively. An increase in phenolic compounds content was not observed in plants co-inoculated with both bacteria and challenged with *S. rolfsii* (Fig. 5).

*AhSymRK* gene expression reached similar levels both in plants inoculated only with the microsymbiont and in those co-inoculated with *Bacillus* sp. CHEP5. However, expression levels were reduced in the presence of *S. rolfsii*. Co-inoculation with *Bacillus* sp. CHEP5 reverted this deleterious effect caused by the pathogen (Fig. 6).

## Discussion

After plants recognize a microorganism, it is possible that they trigger a signaling pathway that culminates in a defense response and/or beneficial association. This interaction can be positive- or negatively affected by surrounding microbiota (Berg 2009). Although plant responses triggered after interaction with a single microorganism have been intensively studied (Glazebrook 2005; van Wees et al. 2008; Faulkner and Robatzek 2012), it is necessary to get deeper understanding of how plants respond when they interact simultaneously with different beneficial and/or pathogenic microorganisms.

We have previously reported phenotypic changes in peanut plants inoculated simultaneously with *S. rolfsii, Bacillus* sp. CHEP5 and *Bradyrhizobium* sp.

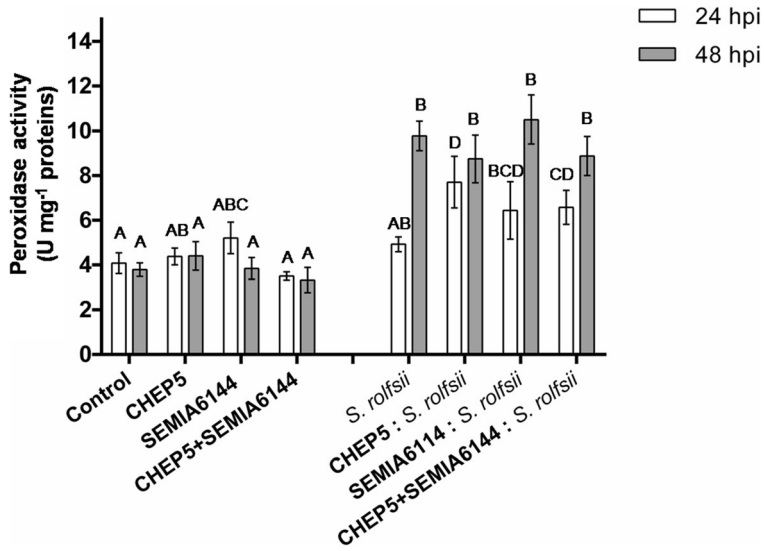


**Fig. 4** PX activity in peanut plants interacting with *Bradyrhizobium* sp. SEMIA 6144, *Bacillus* sp. CHEP5 and *S. rolfsii*. Values are the mean ± S.E. of three experiment with 7 replicates per treatment. Different letters indicate significant differences among data from the same time (24 or 48 h post-inoculation) (LSD Fisher test ($p < 0.05$))

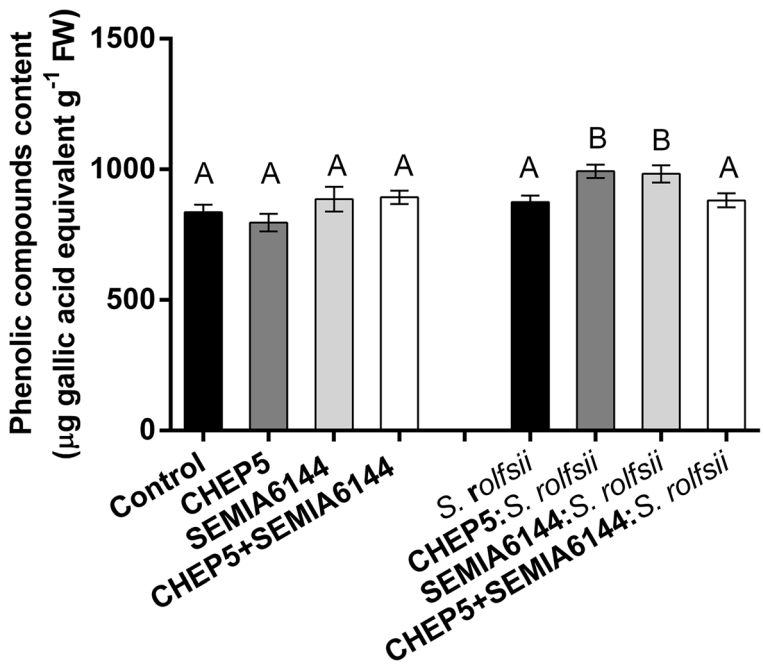


**Fig. 5** Phenolic compounds content in peanut plants interacting with *Bradyrhizobium* sp. SEMIA 6144, *Bacillus* sp. CHEP5 and *S. rolfsii*. Values are the mean ± S.E. of three experiment with 7 replicates per treatment. Different letters indicate significant differences according to LSD Fisher test ($p < 0.05$)

SEMIA6144 (Figueredo et al. 2017). Under this condition we found that a) the inoculation of plants with both PGPB (either single or mixed cultures), reduce *S. rolfsii* pathogenic effect, b) this pathogen negatively affects the symbiotic phenotype of *Bradyrhizobium* sp. SEMIA6144, and c) the presence of *Bacillus* sp. CHEP5 inactivated this harmful effect.

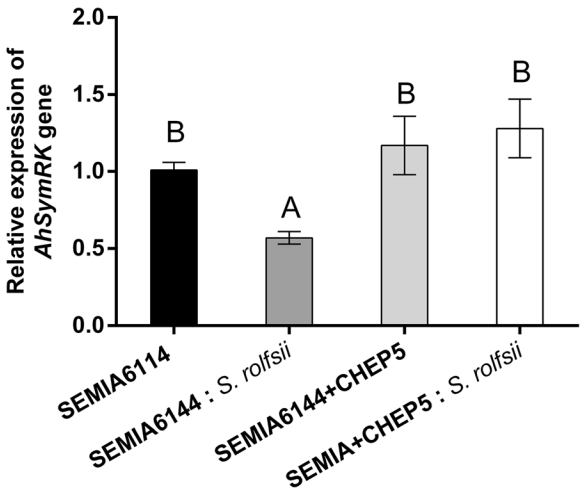


**Fig. 6** *AhSymRK* gene expression levels in peanut plants interacting with *Bradyrhizobium* sp. SEMIA6144, *Bacillus* sp. CHEP5 and *S. rolfsii*. Values are the mean ± S.E. of three experiment with 7 replicates per treatment. Different letters indicate significant differences according to LSD Fisher test ($p < 0.05$)

In this work we analyzed the possible relationship between these phenotypic changes and molecules involved in the processes that each microorganism induces in peanut: pathogenesis, ISR and symbiosis. To achieve this aim we measured PX activity, phenolic compounds content, and *AhSymRK* gene expression, respectively.

Oxidative burst, characterized by the production of reactive oxygen species (ROS), has been reported for many plant-pathogen interactions. These compounds trigger hypersensitive cell death limiting the invasion of biotrophic pathogens but facilitating the infection by necrotrophic pathogens like *S. rolfsii* (Glazebrook 2005; Lehmmann et al. 2015). Plants are able to cope with ROS harmful effects by inducing an antioxidant defense mechanism including redox metabolites and antioxidant enzymes such as PX. Plants possess many different PX that are keys for pathogen defense mechanisms and for cell wall reinforcement, since they catalyze the synthesis of suberin and lignin and their cross-linking with other cell wall polymers (Espelie et al. 1986; McDougall 1991, 1993). This is an important feature that determines plant resistance to necrotrophic pathogens, since their ability to colonize a broad spectrum of hosts mainly depends on cell wall susceptibility to lytic enzymes (Bellincampi et al. 2014). Paradoxically, PX can also generate $H_2O_2$ and subsequently OH radicals via the hydroxylic cycle (Passardi et al. 2004). However,

amounts of ROS generated by PX are low and transient, and they seem to be insufficient to trigger an oxidative burst (Almagro et al. 2008; Zhao et al. 2005).

Peanut plants challenged with *S. rolfsii* increased disease incidence and severity (Figueredo et al. 2017) which are linked to a significant increase in the PX activity from the 48 to 72 hpi. Results obtained indicated that plants inoculated simultaneously with the three microorganisms were healthier, and showed earlier and higher induction of PX activity compared with plants only challenged with *S. rolfsii*. The faster induction of PX activity confirms priming previously evidenced during ISR elicited by *Bacillus* sp. CHEP5 (Figueredo et al. 2014, 2017) and, considering that this enzymatic activity is also high, it is possible to suggest its involvement in cell wall strengthening to prevent or delay peanut tissues infection by *S. rolfsii*.

Phenolic compounds are secondary metabolites usually related to defense responses in plants. Some derivates of phenolic compounds have structural functions (lignin, suberin) while others are antimicrobial agents, antioxidants, etc. (flavonoids, stilbenes, phytoalexins) (Balasundram et al. 2006). Many studies reported an increase in phenolic compounds content during ISR (Doley et al. 2017; Jain et al. 2012; Singh et al. 2013, among others). Previously, we demonstrated that in the ISR mediated by *Bacillus* sp. CHEP5 against *S. rolfsii*, the activity of the enzyme phenylalanine ammonia-lyase (PAL), first enzyme in the phenylpropanoid pathway, increases at 24 hpi and 30 dpi with the phytopathogen (Tonelli et al. 2011; Figueredo et al. 2014). In the present study we found an increase in phenolic compounds content in plants inoculated with *Bacillus* sp. CHEP5 and challenged with *S. rolfsii* at 48 hpi. The same result was found in plants inoculated with *Bradyrhizobium* sp. SEMIA6144 and challenged with *S. rolfsii*. Thus, it is possible to suggest that the increase in phenolic compounds content in peanut plants inoculated with *Bacillus* sp. CHEP5 or *Bradyrhizobium* sp. SEMIA6144 contributes to cell wall strengthening. Similarly to the increase in PX activity, phenolic compounds accumulation could delay *S. rolfsii* infection while the plants activate other defensive responses. On the other hand, phenolic compounds content was lower in plants co-inoculated with *Bacillus* sp. CHEP5 and *Bradyrhizobium* sp. SEMIA6144 and challenged with *S. rolfsii* than in plants inoculated with each bacterium and challenged with the pathogen. However, there were no differences in plant protection against *S. rolfsii* between plants treated with one or both bacteria (Figueredo et al. 2017). It is possible to suggest that in these plants some groups of phenolic compounds, like phytoalexins, could be increased conferring resistance to peanut plants against *S. rolfsii*, without significant changes in the total content of phenolic compounds.

One of the most studied interactions between microorganisms and plants is that established by legumes and rhizobia. At the beginning, a specific molecular dialogue between legumes and their cognate is necessary for the recognition of both symbiotic partners. After that, a symbiotic signaling pathway is activated in which, amongst others, the symbiotic gene *SymRK* participate. Sinharoy et al. (2009) demonstrated that *SymRK* is involved in the early stages of the symbiotic peanut-*Bradyrhizobium* interaction. In this work, we found that *AhSymRK* gene expression was up-regulated in roots at 9 dpi with *Bradyrhizobium* sp. SEMIA6144, and that co-inoculation with *Bacillus* sp. CHEP5 did not affect the expression of this gene. Instead of that, we also determined that in plants inoculated with *Bradyrhizobium* sp. SEMIA6144 and challenged with *S. rolfsii*, *AhSymRK* gene expression level was significantly decreased, as it was expected considering the altered symbiotic phenotype of these plants (lower nodule number compared to control plants) (Figueredo et al. 2017). These findings are indicating that, under this condition, the pathogenic pathway prevailed over the symbiotic one, probably affecting nodule development. Similar results have been reported in the model legumes *Lotus japonicus* (Lopez-Gomez et al. 2012) and *Medicago truncatula* (Chen et al. 2017). The effect of *S. rolfsii* on the *AhSymRK* gene expression was reverted by the co-inoculation with *Bacillus* sp. CHEP5, in concordance with the unaltered symbiotic phenotype of these plants (Figueredo et al. 2017).

## Conclusions

Previous studies of plant-microorganism interactions have been mainly performed considering a single microorganism. Only limited studies of plants interacting simultaneously with multiple organisms have been conducted. Our effort to identify the effect of peanut co-inoculation with two beneficial bacteria (*Bradyrhizobium* sp. SEMIA 6144 and *Bacillus* sp. CHEP5) and a fungal phytopathogen (*S. rolfsii*) started with the analysis of phenotypic changes in these plants

(Figueredo et al. 2017). Here, to identify possible links between plant phenotypes and changes in molecules involved in the biological effect caused by each microorganism (nitrogen fixing symbiosis, biological control by ISR, and pathogenesis), *AhSymRK* gene expression, phenolic compounds content and PX activity, were determined in these plants. Almost all results obtained are consistent with the plant's phenotype observed, and demonstrate that the pathways triggered by each bacterium are affected by the pathogen. However, harmful effects induced by the fungus are attenuated in plants co-inoculated compared with single inoculated plants. Data from this work highlight the relevance of a more detailed understanding on how plant responds to associated microbial communities and not only to single microbial species populations, rarely encountered in the environment. Moreover, this knowledge should be taken into consideration for selection of bacterial inoculants to improve plant performance. In this sense, results from this work provided new insights in the benefits of *Bradyrhizobium* sp. SEMIA 6144 and *Bacillus* sp. CHEP5 co-inoculation to overcome fungal pathogenic effects.

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