

The biocontrol agent *Bacillus* sp. CHEP5 primes the defense response against *Cercospora sojina*

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Abstract *Glycine max* (soybean) production can be dramatically affected by frogeye leaf spot (FLS) caused by *Cercospora sojina* Hara. The inoculation of biocontrol agents may be an alternative strategy for *C. sojina* control. The native biocontrol bacterium *Bacillus* sp. CHEP5 reduced the severity of FLS in soybean by inducing systemic resistance. We suggest that the defense response was primed since the expression of the defense related gene *GmAOS* was enhanced in induced plants treated with both methyl jasmonate and *C. sojina*. Furthermore, as *GmAOS* is related to jasmonic acid biosynthesis, we assume that this phytohormone is involved in induced systemic resistance signaling defense pathway in soybean against *C. sojina*.

Keywords Soybean · *Bacillus* sp. · *Cercospora sojina* · Induced systemic resistance · Priming · Jasmonic acid

Introduction

Glycine max (soybean) is an economically very important crop throughout the world. It is susceptible to many pathogens, with great damage being caused by fungi. Frogeye leaf spot (FLS) caused by the necrotrophic fungi *Cercospora sojina* Hara can dramatically affect this crop production. FLS symptoms usually appear at V3-V4

phenological stages and led to a premature defoliation (Fehr and Caviness 1977; Mian et al. 2008).

Effective control of FLS disease can be reached by supplying a recommended fungicide, a strategy that could contribute to environmental pollution and to greater production cost, or by using resistant cultivars, which is not always practical since cultivar selection depends on the characteristics of the agricultural area. Biological control of phytopathogenic fungi is eco-friendly and a potential component of integrated disease management.

In nature, plants form beneficial associations with soil-borne microbes. Plant growth-promoting bacteria (PGPB) stimulate either directly or indirectly the plant growth. Some PGPB lessen or prevent the deleterious effect of phytopathogenic organisms by antibiosis, competition for space and nutrients, production of siderophores or induction of systemic resistance in plants against a broad-spectrum of root and foliar pathogens (Podile and Kishore 2006; Lugtenberg and Kamilova 2009).

The induced systemic resistance (ISR) is an indirect biocontrol mechanism that consists in the activation of plant defense state only upon pathogen attack (Kloepper et al. 1992). ISR involves a cascade of defense reactions that spread from the site of induction to distant parts of the plant and encompass signal transduction mediated by phytohormones, generation of phytoalexins, oxidative stress protection, synthesis of enzymes related to plant defense (phenylalanine ammonia-lyase, peroxidase, β -1,3-glucanase) and formation of structural barriers such as wall thickening, callose deposition and accumulation of phenolics (Reymond and Farmer 1998; Verhagen et al. 2004). A common feature of the resistance responses induced by beneficial microorganisms is priming. This phenomenon is characterized by a faster and/or stronger activation of cellular defense responses upon pathogen

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attack, resulting in a major resistance to its deleterious effect. Primed plants are sensitized for enhanced defense expression and thus, there is little cost in yield lost to the priming process in the absence of pathogens (Paré et al. 2005; Conrath et al. 2006; Pieterse et al. 2000; Pozo et al. 2008; Van der Ent et al. 2009; Buensanteai et al. 2009).

Although several researchers revealed that beneficial microorganisms triggered the salicylic acid-dependent defense response in the signaling pathway activated by systemic resistance inducing microorganisms (Buensanteai et al. 2009; De Mayer and Höfte 1997), the phytohormones jasmonic acid (JA) and ethylene (ET) are central players in the regulation of ISR (Van der Ent et al. 2009). Interestingly, ISR induced by PGPB is associated with an enhanced sensitivity to these hormones, instead of an augmented production of them (Pieterse et al. 2000).

JA-signaling has also been described as the main pathway in ISR against aboveground herbivores and is stimulated by root-associated microbes (Van der Ent et al. 2009).

Allene oxide synthase (AOS) is a major intermediate enzyme in the oxylipin pathway to JA biosynthesis affecting the synthesis of all JA-related compounds in plants, and therefore playing a significant role in plant defense.

It has been demonstrated that constitutive *GmAOS* overexpression conferred an enhanced tolerance to cotton worm in transgenic tobacco, by increasing activities of allene oxide synthase, peroxidase, chymotrypsin inhibitor, and the trichome. Moreover, in soybean plants highly resistant to cotton worm this gene expression was faster and greater than in susceptible cultivars.

Previous studies in our laboratory demonstrated that the native isolate *Bacillus* sp. CHEP5 induces systemic resistance in peanut against the pathogen *Sclerotium rolfsii* (Tonelli et al. 2011). In this research work, we evaluated the ability of *Bacillus* sp. CHEP5 to induce soybean systemic defense response against *C. sojae*, and determined whether it is related to the priming of JA signaling pathway by measuring *GmAOS* gene expression in induced plants.

Materials and methods

Bacterial strain, pathogen and culture conditions

The native biocontrol bacterium *Bacillus* sp. CHEP5 was cultured at 28 °C on Luria–Bertani broth (LB) or Agar medium. The strain was kept in 20 % glycerol at –80 °C for long-term storage and in 40 % glycerol at –20 °C for short-term storage.

The fungal pathogen *C. sojae* CCC 172-09 (Carmona et al. 2009) was obtained from Centro de Referencia en Micología (CEREMIC), Universidad Nacional de Rosario,

Argentina. It was grown on potato dextrose agar (PDA) supplemented with streptomycin 100 µg ml⁻¹, at room temperature for 10 days. The phytopathogen was kept in 15 % glycerol at –20 °C for long-term storage or in PDA tubes covered with vaseline.

Plant material and growth conditions

Soybean seeds cv. DM 4676 susceptible to *C. sojae*, were surface sterilized. Briefly, the seeds were soaked in 96 % ethanol for 20 s followed by 20 % bleach for 20 min, and then washed six times with sterile distilled water (Buensanteai et al. 2009). The surface sterilized seeds were germinated at 28 °C in sterilized Petri dishes with one layer of Whatman #1 filter paper and moist cotton, until the radicle reached approximately 2 cm. Seedlings were sown in plastic cups filled with sterilized quartz sand, watered regularly and supplied once a week with Hoagland medium (Hoagland and Arnon 1950). 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 %).

Bacterial inoculum preparation

Bacillus sp. CHEP5 was cultured on LB broth for 24 h at 28 °C, until the culture reached an OD_{620nm} = 1, and the number of viable cells was determined (10⁸–10⁹ cfu ml⁻¹ approximately) (Somasegaran and Hoben 1994). The cultures were centrifuged at 4,000 g for 12 min at room temperature and the cells were re-suspended in 10 mM MgSO₄ sterile solution.

Phytopathogen inoculum preparation

Cercospora sojae spores were suspended in a sterile aqueous solution containing 0.015 % of the surfactant Tween 20 to a final concentration of 10⁶ spores ml⁻¹.

Bioassays to evaluate the induction of systemic resistance

Soybean seedlings were obtained as described above. Two days after the seedlings were sown in the cups filled with sterilized quartz sand, their roots were inoculated with 4 ml of *Bacillus* sp. CHEP5 culture to obtain a final concentration of 10⁷ cfu g⁻¹ of quartz sand. At V3–V4 phenological growth stage (10 days after bacterial inoculation), trifoliate leaves were challenged with *C. sojae* (10⁶ spores ml⁻¹) (Carmona et al. 2009). Twenty-one days after challenge inoculation, disease severity was assessed by determining the disease level and the percentage of damaged leaf area (Carmona et al. 2009). Disease rating was

expressed on the basis of symptom severity and damaged leaf area measured in challenged trifoliolate leaves: (1) healthy leaves without spots, (2) spots covering 5 % of leaves area approximately, (3) spots covering 15 % of leaves area approximately, (4) spots covering 35 % of leaves area approximately, (5) 50 % or more of their surfaces affected by the FLS. The shoot dry weight and total chlorophyll content were also determined.

Nonpathogenized and non-bacterized control plants were also included. Plants were grown under controlled environment and regularly supplied with Hoagland medium.

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

Total chlorophyll determination

The amount of total chlorophyll was determined. Briefly, 0.10–0.15 g of fresh weight soybean leaves was placed into a mortar and the tissue was ground to fine pulp after the addition of 80 % acetone. The extract was transferred to a Buchner funnel containing a pad of Whatman filter paper. While filtering the extract, the leaves pulp was grounded and the final volume of the filtrate was adjusted to 10 ml (Arnon 1949). The chlorophyll content was spectrophotometrically determined at 650 and 665 nm. The amount of total chlorophyll was calculated on the basis of μg of chlorophyll gram^{-1} of fresh leaf tissue, according to the following equation (Mc Kinney 1938):

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

Phytopathogen and methyl jasmonate treatments

Soybean seedlings were obtained and *Bacillus* sp. CHEP5 was inoculated as described before. At V3–V4 phenological growth stage, trifoliolate leaves were challenged with *C. sojina* (10^6 spores ml^{-1}) and harvested at 0 and 1 h after the phytopathogen treatment. To obtain methyl jasmonate (MeJA) treated plants, trifoliolate leaves were moistened with an aqueous solution containing 50 μM MeJA and 0.015 % of the surfactant Tween 20 (Pozo et al. 2008). Trifoliolate leaves were harvested at 0, 1 and 3 h after MeJA treatment.

Immediately after the leaves were harvested, they were frozen in liquid nitrogen. Samples were conserved at -80°C until RNA was extracted. Plants that were not induced were used as controls.

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

RNA isolation and obtention of soybean cDNA

RNA was isolated from three replicates samples that were pooled to reduce noise arising from biological variation.

Leaf samples were ground with sterile mortars and pestles under liquid nitrogen. Total RNA was isolated using the NucleoSpin RNA Clean-Up Kit (Macherey–Nagel) according to the manufacturer's protocol. Traces of genomic DNA were eliminated by DNase digestion (Macherey–Nagel). First strand cDNA synthesis was carried out using MMLV Reverse Transcriptase 1st Strand cDNA Synthesis Kit (Epicentre) according to the manufacturer's specifications with random primers. The reaction conditions for cDNA synthesis were 2 min at 65°C , then 60 min at 37°C and finally 5 min at 85°C .

Determination of *GmAOS* transcript level

Real Time-PCR was performed in 20 μl reaction mixtures containing 2X Brilliant SYBR green QPCR master mix (Stratagene), 1:10 dilution of synthesized cDNA and 400 nM of the primers GmAOSRTF (5' GGGCCTCCC TCAATTCTTAG 3') and GmAOSRTR (5' CGCTCCGCT TCGTCTAATAC 3'). The soybean *Actin* gene was used as an endogenous control for sample normalization. *Actin* gene was amplified by using 100 nM primers GmACTRTf (5' TCGTATGAGCAAGGAAATTGG 3') and GmACTRTr (5' TAGAGCCACCAATCCAGACAC 3'). The primers were designed based on soybean *Actin* sequence (accession number V00450) and *AOS* sequence (accession number EU366252) (Wu et al. 2008), using Primer3/v4.0 software (Rozen and Skaletsky 2000).

Real-Time PCR determinations were carried out with RNA extracted from two independent biological samples and the threshold cycle (C_t) was determined in triplicate. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). Melting curves were recorded after cycle 40 by heating from 55 to 95°C .

Statistical analysis

It was performed by subjecting the data to ANOVA and analyzed by LSDs tests for statistical significance at $p < 0.05$, using Infostat software (1.0, FCA, UNC, Argentina). Student's *t* test with $\alpha = 0.05$ was performed subjecting the data to SigmaStat 3.5 software.

Results

Bacillus sp. CHEP5 systemically protects soybean plants against *C. sojina*

The ability of *Bacillus* sp. CHEP5 to systemically protect soybean plants against *C. sojina* was evaluated. Plants inoculated with *Bacillus* sp. CHEP5 previously to pathogen

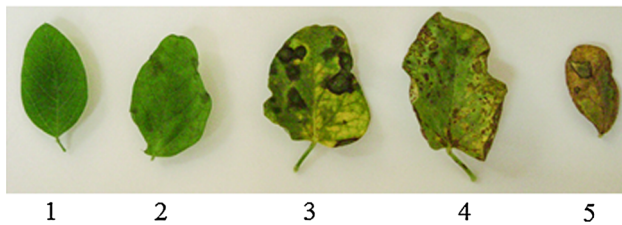
(a) Disease Level Scale from 1 to 5**(b) *C. sojae* levels 4-5****(c) *Bacillus* sp. CHEP5-*C. sojae* levels 3-4**

Fig. 1 Disease level of plants challenged with *C. sojae*. **a** Disease level scale in trifoliolate leaves. **b** Trifoliolate leaves from plants challenged with *C. sojae*. **c** Trifoliolate leaves from plants induced with *Bacillus* sp. CHEP5 and challenged with *C. sojae*

challenge, showed a reduced disease level, a decrease of approximately 40 % of the damaged leaf area and an elevated content of chlorophyll compared to non induced-challenged plants (Fig. 1 and Table 1).

Shoot dry weight increase, although not statistically significant, was also observed in induced plants compared to challenged ones (Table 1).

Bacillus sp. CHEP5 induced resistance is dependent on JA

It is known that JA has a main role in ISR signaling regulation and in plant defense response against necrotrophic fungi. Moreover, plant systemic defense induced by PGPB is usually associated with an enhanced sensitivity to this phytohormone upon pathogen attack.

To determine whether resistance to *C. sojae* induced by *Bacillus* sp. CHEP5 is associated with the JA signaling pathway, we analyzed *GmAOS* transcript level at 1 h post

Table 1 Severity disease indicators in soybean plants challenged with *C. sojae*

Treatments	Severity disease indicators		
	Percentage of damaged leaf area	Total chlorophyll content (μg chlorophyll/g fresh weight)	Shoot dry weight (g)
Control	ND	81.4 ± 8.9^b	0.19 ± 0.02^b
<i>C. sojae</i>	88.9 ± 5.9^a	43.6 ± 8.1^a	0.11 ± 0.02^a
<i>C. sojae</i> - <i>Bacillus</i> sp. CHEP5	48.9 ± 5.6^b	92.7 ± 8.4^b	0.13 ± 0.01^{ab}

Values are the mean \pm SE (n = 15). Different letters indicate significant differences according to LSD $p < 0.05$

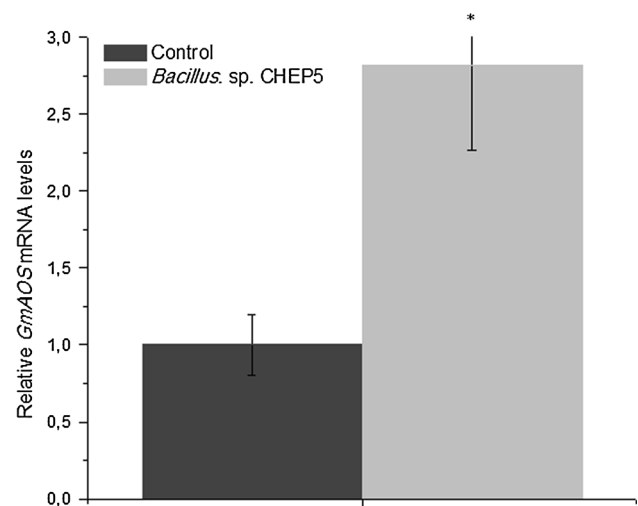


Fig. 2 Relative *GmAOS* expression in plants challenged with *C. sojae*. *GmAOS* transcript levels in noninduced control plants were set at 1. Asterisk, statistically significant differences compared with the noninduced control according to *t* test $\alpha = 0.05$

pathogen challenge. In response to *C. sojae*, an increase in the relative expression of *GmAOS* gene was observed in plants inoculated with *Bacillus* sp CHEP5 compared with the uninoculated plants (Fig. 2). Therefore, the *Bacillus* sp CHEP5-mediated resistance to *C. sojae* in soybean occurs in a JA-dependent manner.

Bacillus sp. CHEP5 primes the defense response associated to JA

Methyl jasmonate application induces the expression of all of the genes involved in JA biosynthesis, including AOS (Sasaki et al. 2001). To determine whether *GmAOS* gene is primed in the resistance response to *C. sojae* induced by *Bacillus* sp. CHEP5, we examined the *GmAOS* transcript level in *Bacillus* sp. CHEP5 inoculated or uninoculated soybean plants exposed to exogenous MeJA.

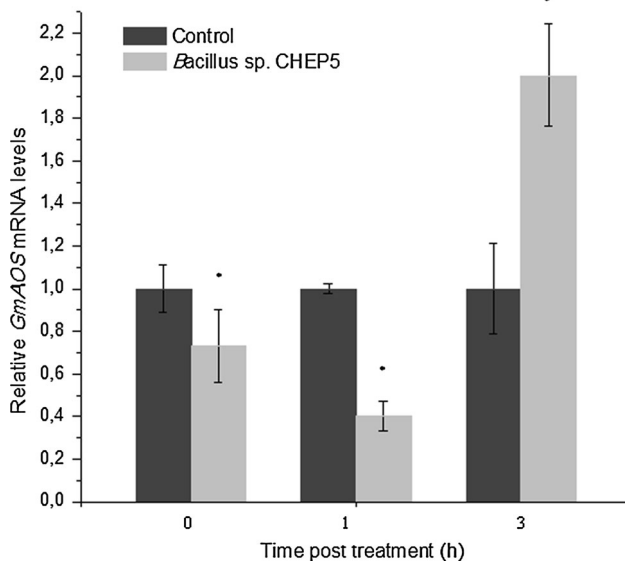


Fig. 3 Relative *GmAOS* expression in plants treated with MeJA and induced with *Bacillus* sp. CHEP5. *GmAOS* transcript levels in noninduced and MeJA treated control plants were set at 1. Asterisk, statistically significant differences compared with the noninduced control according to *t* test $\alpha = 0.05$

Inoculation of *Bacillus* sp. CHEP5 in MeJA exposed plants, led to increased *GmAOS* transcript level at 3 h after treatment (Fig. 3). These results suggest that *Bacillus* sp. CHEP5 inoculation induced in soybean plants a defense-priming mechanism that may facilitate more potent response against *C. sojina*. Noteworthy, at 0 and 1 h post MeJA treatment, *GmAOS* transcript level was lower in *Bacillus* sp. CHEP5-induced plants than in MeJA-treated plants, probably related with a decrease in the JA signaling pathway to suppress early activated defense response (Stein et al. 2008; Zamioudis and Pieterse 2012).

Discussion

Some root colonizing nonpathogenic bacteria may trigger disease resistance in the host plant, a phenomenon that has been termed ISR and that is effective against different types of plant pathogens. The ISR is generally regarded as a widespread and conserved trait, since the phenomenon is known for many plant species (Van Loon et al. 1998, 2008; Verhagen et al. 2010). Thus, one of the objectives of this work was to determine if *Bacillus* sp. CHEP5, previously reported as ISR-inducing bacteria in peanut (Tonelli et al. 2011), protects soybean from FLS foliar disease by activating systemic responses.

Plants inoculated with *Bacillus* sp. CHEP5 previously to pathogen challenge showed a reduce disease level with healthier, greener leaves compared to non inoculated-challenged plants.

Bacterial induction of resistance in the plant is systemic, as *Bacillus* sp. CHEP5 is applied onto the roots and the response is detected in the shoot system, where the pathogen *C. sojina* is applied. Hence, it may be attributed to ISR.

Induction of systemic resistance in soybean plants by gram positive strains has been previously described. Buensanteai et al. (2009) reported that the inoculation of soybean seeds with the strain *Bacillus amyloliquefaciens* KPS46 induced systemic protection against the leaf disease caused by *Xanthomonas axonopodis* pv. *Glycines*, reducing the percentage of infected leaf area. This protection encompassed primed and incremented activity of defense related proteins and phenolic content. It was also determined that both SA- and JA-dependent signaling pathways are systemically triggered by KPS46 seed treatment.

It is known that the oxylipin JA and its derivatives play an important role in the regulation of plant-beneficial microbe interactions being central players in the regulation of ISR (Van der Ent et al. 2009). Moreover, JA is the major signaling molecule in plant defense response against herbivores and necrotrophic pathogens (Froehlich et al. 2001; Pieterse et al. 2012; Wasternack and Hause 2013; Ye et al. 2013).

Allene oxide synthase is one of the first enzymes of the oxylipin pathway to JA biosynthesis. The second objective of this work was to determine if the mechanism to induce systemic resistance of *Bacillus* sp. CHEP5 involves the priming of JA-dependent pathway. The increased *GmAOS* transcript level in *Bacillus* sp. CHEP5 and pathogen challenged plants strongly suggest that the enhanced soybean resistance to *C. sojina* attack induced by this bacterium occurs in a JA-dependent manner.

Although induced defense responses allow plants to conserve energy in the absence of pathogen challenge, plants may suffer damage during the lag phase required to mount defenses once an attack occurs (Frost et al. 2008). To compensate for this vulnerability, some plants have evolved defense-priming mechanisms that facilitate more rapid and effective responses to pathogen challenge, with low metabolic requirements. Defense priming can lead to an enhanced state of readiness for the induction of defense responses, allowing plants to be physiologically prepared to induce more potent defense responses upon subsequent pathogen attack (Ye et al. 2013).

Taking into account that the resistance to *C. sojina* enhanced by *Bacillus* sp. CHEP5 involves the JA-signaling pathway, we further examined whether this bacterium induces defense-priming mechanisms by measuring *GmAOS* expression in inoculated or uninoculated plants exposed to exogenous MeJA application. The fact that *Bacillus* sp. CHEP5 inoculation induced a more effective defense response than MeJA alone after 3 h treatment

indicates that it is serving as an apparent priming agent for JA response.

Moreover, the transcript level of *GmAOS* was higher in plants induced with *Bacillus* sp. CHEP5 after 1 h *C. sojina* challenge than in noninduced plants, suggesting that the defense response upon phytopathogen attack is primed faster than with MeJA treatment.

In conclusion, results from this study demonstrated that *Bacillus* sp. CHEP5 induced the systemic resistance in soybean and reduced the severity of FLS. In addition, it is shown that JA-dependent pathway is involved in the response triggers by *Bacillus* sp. CHEP5. Irrespective of the pathway involved, this strain is a putative biocontrol agent to be used in intensive soybean production to decrease chemical inputs, although this ability needs to be proven in field assays.

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