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Inoculation with Pseudomonas Pseudoalcaligenes Lead to Changes in Plant Sugar Metabolism and Defense That Enhance Tolerance Against the Pathogenic Fungus Sclerotium Rolfsii

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Abstract

Certain *Pseudomonas* species promote in plants an induced systemic response (ISR), which results in pathogenic disease reduction. This is energetically expensive, implies a redistribution of sugars, and involves several enzymes such as cell-wall invertase (cwINV). The present study aimed to evaluate the role of soluble sugars and cwINV activity in the ISR of *Pseudomonas pseudoalcaligenes*-primed tomato plants challenged with *Sclerotium rolfsii*. Disease severity of infected plants was 100%, whereas that of primed plants was 43%. At 24 h after challenge, infected plants showed higher cwINV activity, increased *LIN6* and *SUS3* mRNA levels, upregulation of the defense marker gene *PR1b1*, no changes in *PR2* and *PR3* mRNA levels, and almost unchanged sugar content. Instead, primed plants displayed a lower induction of cwINV activity and gene expression, slightly increased *PR2* and *PR3* mRNA levels, and increased leaf fructose content. Cytokines also induced *LIN6* expression and cwINV activity. Altogether, these results reveal that *P. pseudoalcaligenes* triggers changes both in sugar metabolism and plant defense, leading to enhanced tolerance against *Sclerotium rolfsii*.

Keywords: invertase; PGPR; *Sclerotium rolfsii*; sugar; tomato.

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1. Introduction

Plant growth-promoting rhizobacteria (PGPR) have several positive effects on plants, acting either directly, by producing metabolites that are used as nutrients or plant growth regulators, or indirectly, by inhibiting the growth of microorganisms that are detrimental to plant development [1, 2] PGPR such as those of the genera *Pseudomonas*, *Bacillus* and *Serratia* also promote an induced systemic response (ISR), which results in priming of defense or induced resistance in the host plant. The ISR is similar to the Systemic Acquired Resistance, but the mechanism triggered primarily involves jasmonic acid (JA) and ethylene (ET) and is independent of salicylic acid (SA) [3]. In previous works, we isolated and characterized the endophytic bacterium *Pseudomonas pseudoalcaligenes* (PAC BNM0522) and showed that it is an effective biocontroller of phytopathogens such as *Sclerotium rolfsii, Fusarium solani* [4] and *Meloidogyne sp.* [5] and generates tomato plant protection by regulating the expression of genes involved in the ET pathway [6]. In warm regions, *S. rolfsii* is a major problem in tomato cultivation because it induces southern blight disease, which causes damping off, basal stem rot and wilting, and develops resistance structures (sclerotia) that allow its long-term survival in the soil [7]. Plant defenses against pathogens are costly in terms of energy and carbohydrates. Sucrose and the products of its hydrolysis, i.e. glucose and fructose, are central molecules of metabolism. Their rapid mobilization is a determining factor in plant-pathogen interactions, not only as sources of energy but also as signaling factors in the induction of defensive responses. These sugars modulate the expression not only of enzymes associated with carbohydrate metabolism, such as sucrose synthase (SuSy) and invertase (INV), but also of genes associated with defenses against pathogens [8]. Besides, in stress situations, sucrose can activate signaling enzymes such as mitogen-activated protein kinases (MAPKs) [9, 10]. SuSy (EC 2.4.1.13) catalyzes the reversible conversion of sucrose and nucleoside diphosphate (NDP) into NDP-glucose and fructose, whereas INV (EC.3.2.1.26) catalyzes the irreversible hydrolysis of sucrose into fructose and glucose. Plants have three different types of INV: cell wall INV (cwINV), vacuolar INV (vacINV) and alkaline or neutral INV, which can be distinguished by their solubility, subcellular location, optimal pH and isoelectric point. cwINV plays a crucial role in the regulation of source/sink relations because it is responsible for taking the sucrose from the phloem and allowing it to enter the cell already hydrolyzed as glucose and fructose. Besides, cwINV is induced upon pathogen infection [11, 12]. In tobacco plants infected with *Phytophthora nicotianae*, studies have shown early induction of cwINV [13]. In *Arabidopsis thaliana* infected with *Erysiphe cichoracearum*, the expressions of both cwINV and *AtSTP4*, a monosaccharide transporter, have been found increased, resulting in greater sequestration of glucose by the infected tissues [14]. In tomato plants, inoculation with beneficial microorganisms like the arbuscular mycorrhiza *Glomus intraradices* has been found to moderately induce *LIN6*, one of the isoforms of cwINV, suggesting a fine-tuning in the activation of sink metabolism in this mutualistic interaction [15]. Other authors have found that, upon infection with pathogens, soluble sugar levels decrease, probably due to increased metabolic activity of the infected tissues that would lead to the increase in ATP, necessary to reinforce the cell wall and defense enzymes. Authors in [16] observed that *A. thaliana* tissues infected with *Botrytis cinerea* drastically reduce their soluble sugar levels and that the induction of enzymes associated with their metabolism depends on the characteristics of the pathogen and its interaction with the host. The relationship between the levels of sucrose and its hydrolysis products is also an important indicator of cwINV activity after infection [16]. The leaves of tomato plants contain two isoforms of cwINV: Lin8 and Lin6 [17], being this latter

expressed under conditions that require a high carbohydrate supply. In various plant species, Lin6 has also been found to be induced by cytokinins [18]. In tobacco leaves, authors in [11], showed the importance of cwINV in generating carbohydrates that are catabolized upon the activation of defense responses by RNA interference-mediated knock-down of cwINV. These authors found that lower cwINV expression resulted in compromised defense responses and a delay in the hypersensitive cell death. Several researchers have demonstrated the role of carbohydrates in activating plant defense against phytopathogens [11, 12, 13, 14, 19], but little is known about how tomato plants respond to PGPR priming regarding carbohydrate metabolism in the presence of phytopathogens. Thus, this study aimed to determine the role of sugar metabolism in the induced defense of PAC BNM0522-inoculated tomato plants challenged with *S. rolfsii*, by evaluating the role of sugar content and enzymes involved in its metabolism as well as the expression of defense genes.

2. Materials and Methods

2.1. Culture of microorganisms

Pseudomonas pseudoalcaligenes PAC BNM0522 was grown at 33ºC for 48 h in tryptic soy broth under continuous agitation (100 rpm) for 48 h. *Sclerotium rolfsii* was grown in potato dextrose agar at 25°C for 7 days as described in [6]. *S. rolfsii* was provided by Dr Mitidieri (National Institute of Farming Technology, INTA Argentina).

2.2. Plant growth conditions and inoculation

Solanum lycopersicum cv Rio Grande seeds were disinfected and sown as described in [6]. At seven days after sowing (DAS), half of the plants were inoculated with 1 ml of bacterial suspension containing 10^9 CFU and the other half were left uninoculated. The bacteria were poured at the base of the stem of each plant contained in the pot and plants were cultured for 60 days. Plants were watered every two days with 50 ml of distilled water and once a week with 50 ml of 0.25% v/v Hoagland solution [20].

2.3. Treatments

The following treatments were performed: i) no inoculation of bacteria (control), ii) inoculation with PAC BNM0522 (bacterized plants), iii) challenge with one sclerotium of *S. rolfsii* each, placed at the base of the stem, at 50 DAS (infected plants), iv) inoculation with PAC BNM0522 and challenge with one sclerotium of *S. rolfsii* each, placed at the base of the stem, at 50 DAS (primed plants) and v) application of 2 µM zeatin (ZEA) solution at the base of the stem, to induce carbohydrate metabolism, at 50 DAS [21]. Thirty plants were used of each treatment. Ten plants were withdrawn at different times (12, 24 and 48 hours after challenge, HAC) to measure cwINV and vacINV activity, sugar content, and RNA extraction. Total chlorophyll content was measured at 240 HAC. The experiment was repeated three times and determinations were performed in triplicate. The design of the experiment was based on a fully randomized statistical model. Experimental data were analyzed by ANOVA software package, and LSD was calculated using a 0.05% or 0.1% significance level. The results were analyzed using Infostat [22].

2.4. Evaluation of bacteria-induced plant growth promotion and disease evolution after challenge with S. rolfsii

Shoot and root length, fresh shoot and root weight, and plant height (measured from the soil surface to apical buds) were evaluated at 60 DAS in ten plants. Disease severity (incidence) and mortality (as percentage of seedling damping off) were evaluated daily, for 10 days, after placing the sclerotia on stems. Seedling mortality was expressed as a percentage of the total number of plants evaluated (ten plants). The experiment was repeated three times.

2.5. Soluble sugar content

The content of soluble sugars was determined according to [23] using 200-300 mg of aerial part corresponding to 10 plants. For this, 10 µl of each resuspended sample was injected in an Agilent 1100 System, with an Agilent HI-PLEX Ca (Duo) column, 300 x 6.5 mm (PL1F70-6850), refractive index detector, 85 ºC, and flow of 0.4 ml/min. The running solvent was milli-Q water. Fructose, glucose and sucrose (Sigma®) were used as standards.

2.6. Protein extracts

Thirty tomato leaves from 10 plants in each treatment were ground to a fine powder in liquid N_2 and suspended in 2 ml chilled homogenizing buffer (50 mM Na (H_2PO_4)) pH 7.5. The suspensions were centrifuged at 14,000 x g for 40 min. The pellet was resuspended in 500 µl Na (H_2PO_4) pH 7.5 and 100 µl of the resulting suspension was used to determine cwINV activity. The supernatant was used to determine vacINV activity.

2.7. INV activities

cwINV activity was determined according to the author in [24]. Briefly, the activity was assayed in 200 µl of resuspended pellet in a mixture containing 800 µl of the reaction buffer (citrate-phosphate, 100 mM, pH 5) and 200 µl of 700 mM sucrose, and incubated at 37 °C for 30 min. vacINV activity was determined in the supernatant in the same way. INV activities were stopped with Tris HCl 50 mM (pH 10) at 100 °C for 5 min. The reaction products were quantified by spectrophotometry, according to the Nelson-Somogyi method [25, 26].

2.8. Protein determination

Total protein concentration was determined by the Bradford method [27], using bovine serum albumin as standard. To determine cell wall-associated proteins, a previous alkaline hydrolysis was performed in NaOH 1 N at 100 °C for 30 min.

2.9. Total chlorophyll content

At the end of the experiment (240 HAC), ten leaves per treatment were taken for the extraction of total chlorophyll, which was determined by the method described in [28], using N,N-Dimethylformamide as a very

convenient solvent for chlorophyll extraction. The absorbance in the spectrophotometer was evaluated at 664 and 647 nm to determine the concentrations of chlorophyll a and chlorophyll b respectively, and total chlorophyll content.

2.10. RNA extraction and PCR conditions

To analyze gene expression, a pool of leaves from four plants of each treatment was ground in liquid N_2 and 100 mg of the powdered tissue was used. Extraction and purification were performed three times using an RNAeasy plant MINIKIT (Quiagen), according to the manufacturer´s instructions. First-strand cDNA was prepared using SuperScript II RNAse H-Reverse Transcriptase (Invitrogen), following the manufacturer´s specifications. Oligonucleotide primers were designed based on sequences available at the databases http://www.ncbi.nlm.nih.gov/ and http://solgenomics.net and synthesized by Operon Biotechnologies (USA). The sequences of interest were amplified by PCR using Recombinant Taq DNA Polymerase (Invitrogen). The amplification mixture contained 10 ng cDNA in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM deoxynucleotide triphosphates, 5U Taq DNA polymerase, and 2 μM of the specific primers. Target genes, primers and RT-PCR conditions are described in Table 1.

Table 1: Target genes, primers and RT-PCR conditions

Gene	Accession No.	Forward primer (5´-3´)	Reverse primer (5´-3´)	PCR conditions
Sl-LIN6	AB004558	GCTACTGAACGGTGTTTGAACGTGG	ATGCTGTGCCAAGTACTGCAAGTAA	-94ºC (1 min), 25 cycles of 94ºC (30s), 66ºC (30s) 72ºC (45s). 72ºC (4 min)
Sl-SUS3	NM_001247875.1	TGCAGGTGCAATGGCTCAACG	TCCCACACACCGGGCCTCAA	-94ºC (1 min), 25 cycles of 94ºC (30s), 65ºC (30s) 72ºC (45s). 72ºC (4 min)
Sl-UBI3	X58253.1	CGAAGCCTCTGAACCTTTCC	GGATTCCCCCAGACCAGCAG	-94ºC (1 min), 30 cycles of 94ºC (30 s), 62ºC (30 s) 72ºC (1 min). 72ºC (5 min)
Sl-ACT1	XM_004253248.1	TGGCATCATACCTTTTACA	TCCGGGCATCTGAACCCTCTC	-94ºC (1 min), 25 cycles of 94ºC (30 s), 60ºC (1 min) 72ºC (1 min). 72ºC (5 min)
Le-PR1b1	Y08804.1	TCTGGTGCTGGGGAGAATCT	TCTGGTGCTGGGGAGAATCT	-94ºC (1 min), 25 cycles of 94ºC (30 s), 55ºC (1 min) 72ºC (45s). 72ºC (5 min)
Sl-PR2(GluB)	M80608.1	GTGGCCGCAGCGCAAAGCGC	CAAGCCCTCCATTTCTGCAT	-94ºC (1 min), 25 cycles of 94ºC (30 s), 57ºC (45s) 72ºC (45s). 72ºC (5 min)
Sl-PR3 (CHI3)	Z15141.1	TGCAGGAACATTCACTGGAG	TGCAGGAACATTCACTGGAG	-94ºC (1 min), 25 cycles of 94ºC (30 s), 60ºC (30s) 72ºC (45s). 72ºC (5 min)

As the result obtained with the *Sl-ACT1* primers was the same as that obtained using the *Sl-UBI3* primers, we decided to use *Sl-UBI3* as housekeeping gene (data not shown).

Genes were amplified three times and samples (10 µl) of PCR products were separated on 1.5% agarose in TAE buffer containing 0.5 µg of ethidium bromide per ml at 65 V for 1 h. The gels were photographed using the EBOX VX2 gel documentation system (Vilmert Lourmat, France).

2.11. Endophytism evaluation

The number of bacteria inside shoots and roots was measured to ensure its presence into the plant tissue until 60 DAS and correlate it with plant metabolism changes. For this, five bacterized plants were randomly taken and the shoots and roots were disinfected with chloramine T solution (1% w/v) as described in [6]. The presence of *S. rolfii* was evaluated as the appearance of a white mycelial mass at the base of the stem, where sclerotia were placed.

3. Results

3.1. Bacteria-induced plant growth promotion and disease evolution after challenge with S. rolfsii

The root and aerial parts of bacterized plants were 37% and 29% heavier than those of control plants (Table 2). Also, root length and plant height in these plants were significantly increased in comparison to control plants (40% and 17% respectively). The infection with *S. rolfsii* showed an evident detrimental effect on the aerial part of seedlings (42% less biomass), but no differences in roots (Table 2). Besides, the aerial part of these plants was significantly damaged, being these plants 17% shorter than control plants.

Table 2: Effect of *Pseudomonas pseudoalcaligenes* PAC BNM0522 on tomato plant growth promotion at 60 days after sowing.

Treatment	Root FW (mg)	Aerial FW (mg)	Root length (mm)	Plant height (mm)
Control	683^{a}	1260^{b}	1575^{a}	1890^{b}
Infected	520^{a}	735^{a}	1525^{a}	1575^{a}
Bacterized	963^{b}	1627^{c}	2205^{b}	2205^{c}
Primed	846^{b}	1315^{b}	2047^{b}	2100^{bc}

Data are presented as means of ten determinations corresponding to three independent experiments. Control plants: plants none inoculated; Bacterized plants: plants inoculated with PAC BNM0522; Infected plants: plants challenged with one sclerotium of *S. rolfsii*; Primed plants: plants inoculated with PAC BNM0522 and challenged with one sclerotium of *S. rolfsii*; Different letters in each column indicate statistical differences at $P<0.05$, one-way ANOVA, using Fisher's test (FW: fresh weight). Although bacterized plants showed the highest fresh weight (shoot and root) and length, primed plants showed no evident damage on the growth parameters evaluated. Conversely, the length and fresh weight of these plants were significantly improved, showing a clear protective effect of PAC BNM0522 on *S. rolfsii*-infected plants (Table 2). All sclerotia germinated at 24 HAC and the incidence and mortality were evaluated for 10 days after pathogen challenge. Since *S. rolfsii* is not an endophyte pathogen, its presence in infected plants was evidenced by white mycelial development in the base of the stem. Infected plants showed abundant growth and high density of mycelia since

48 HAC, whereas primed plants showed delayed fungus growth and no more growth at 192 HAC. At 48 HAC, the incidence rate in infected plants was about 24%, whereas that in primed plants was 0%. Towards the end of the assay (216 HAC), the incidence rate in infected plants was 100%, whereas that in primed plants was 42.6% (57% less incidence than in the infected plants). In primed plants, the damping off caused by *S. rolfsii* was significantly suppressed as from 96 HAC in comparison with infected plants (Table 3). At this time, 17% of the infected plants and none of the primed plants had died. At the end of the experiment, maximum seedling rot in infected plants was almost 70% and less than 20% of the primed plants had died (216 HAC). After 240 HAC, all infected plants had died, whereas primed plants remained without changes.

Table 3: Influence of *Pseudomonas pseudoalcaligenes* PAC BNM0522 inoculation on the incidence of disease caused by *Sclerotium rolfsii* in tomato plants as from 50 days after sowing.

	Hours After Challenge (HAC)									
	24	48	72	96	120	144	168	192	216	240
	Incidence (%)									
Infected	6.1	24.4	27.7	30.4	67.2	79.4	91.6	96.7	100	100
Primed	0	0*	6.0*	12.21*	12.41*	18.3*	30.4*	42.6*	42.6*	44.0*
	Mortality (%)									
Infected	0	0	5.7	17.43	23.3	34.9	46.6	52.5	69.9	69.9
Primed	0	0	0	0	5.7	5.7*	11.6*	17.4*	17.6*	23.3*

The numbers in each column represent the means of ten determinations corresponding to three independent experiments. Infected plants; plants challenged with one sclerotium of *S. rolfsii*; Primed plants: plants inoculated with PAC BNM0522 and challenge with one sclerotium of *S. rolfsii*. The asterisk in each column indicates statistically significant differences at 10% level between treatment means (Fisher's test).

3.2. INV activity

As shown in the incidence and mortality assays, the differences between treatments were early detected. Thus, we next determined the activities of some enzymes involved in the sugar pathway at 48 HAC and the sucrose, glucose and fructose contents. cwINV and vacINV activities were measured to investigate the involvement of INV in sugar metabolism and its role in the defense of primed tomato plants. vacINV activity showed no differences between treatments at 24 and 48 HAC (data not shown), whereas cwINV showed no differences at 24 HAC (Figure 1A), but, at 48 HAC, increased by 100% in *S. rolfsii*-infected plants compared with control plants (1.65 µg glu/µg protein and 0.8 µg glu/µg protein, respectively) (Figure 1B). The cwINV activity of bacterized plants showed no differences with respect to that of control plants, whereas that of primed plants increased by 50%. ZEA application induced increased cwINV activity at the same level as that found in *S. rolfsii*-infected plants (Figure 1B).

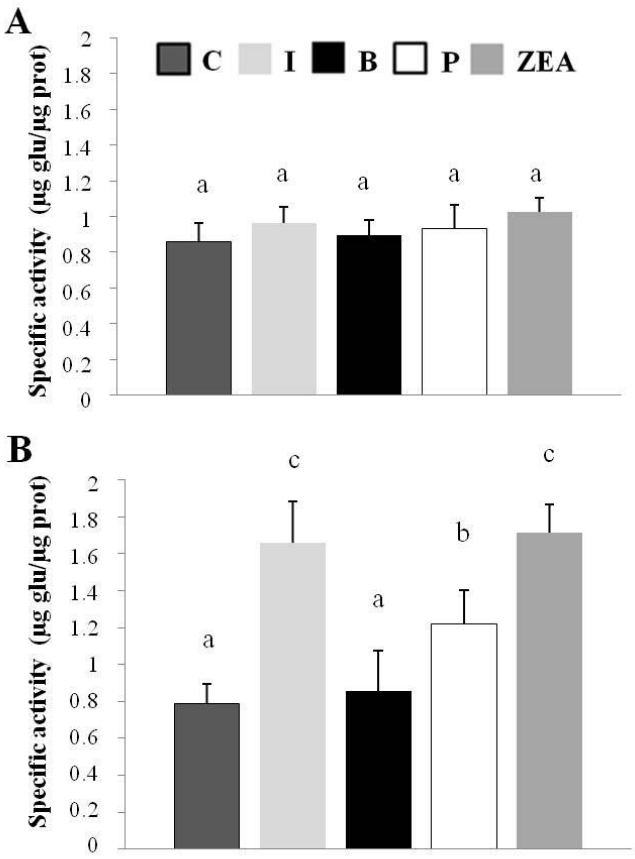

Figure 1: Specific activity of cwINV 24(A) and 48 h (B) after *Sclerotium rolfsii* challenge (HAC). cwINV activity was determined in the shoots of tomato plants at 24 and 48 h after the challenge with *S. rolfsii*. The bars represent the mean of three determinations corresponding to three independent experiments and the different letters above the bars indicate statistically significant differences at 10% level between treatment means (Fisher's test). C: Control plants none inoculated; B: Bacterized plants, inoculated with PAC BNM0522; I: Infected plants, challenged with one sclerotium of *S. rolfsii*; P: Primed plants, inoculated with PAC BNM0522 and challenged with one sclerotium of *S. rolfsii;* ZEA: Plants treated with zeatin.

3.3. Relative expression of LIN6

Since the differences in cwINV activity were recorded at 48 HAC, we also investigated *LIN6* mRNA expression earlier, at 12 and 24 HAC.At 12 HAC, *LIN6* mRNA levels of infected and bacterized plants were not different or were low as compared with those of control plants, whereas those of primed plants were overexpressed (Figure 2). At 24 HAC, *LIN6* mRNA levels of infected plants were increased (almost two-fold with respect to those of control plants), those of primed plants were reduced, and those of bacterized plants reached the lowest values, being almost undetectable and similar to those in control plants. In plants treated with ZEA, this gene was over-expressed at 12 and 24 HAC (Figure 2).

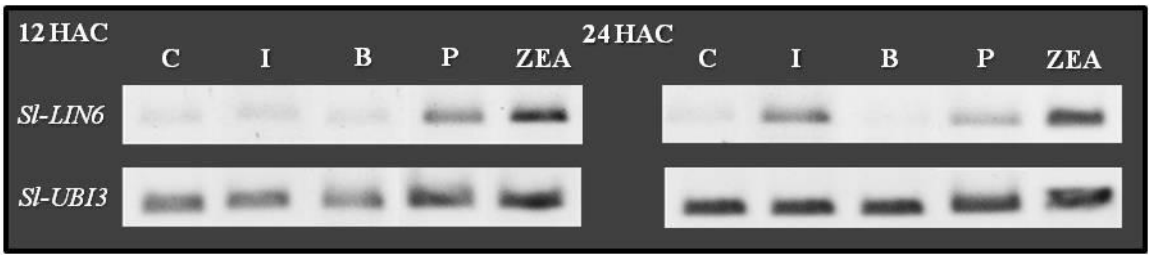

Figure 2: Expression of *Sl-LIN6* in tomato leaves at 12 and 24 h after challenge (HAC) with *Sclerotium rolfsii*. Agarose-ethidium bromide gel image of RT-PCR products amplified using specific primers for *Sl-LIN6* and *Sl-UBI3*. C: Control plants none inoculated; B: Bacterized plants inoculated with PAC BNM0522; I: Infected plants challenged with one sclerotium of *S. rolfsii*; P: Primed plants, inoculated with PAC BNM0522 and challenged with one sclerotium of *S. rolfsii*. ZEA: Plants treated with zeatin. Results corresponding to an experiment representative of three independent experiments.

3.4. Total chlorophyll content

Since some pathogens promote chlorophyll loss and this leads to leaf senescence, we next investigated chlorophyll accumulation in tomato leaves at 240 HAC. Results showed that chlorophyll content was significantly higher in control plants (25.2 µg/ml ±3.4 S.E.), with no significant differences with the value found in primed plants (19.95 µg/ml ±2.6 S.E.) and being significantly decreased by 62% in infected plants (9.4 µg/ml ±1.9 S.E).

3.5. Sugar content

The relative sugar content (defined as the proportion of a specific sugar in the soluble sugar pool) was assessed to correlate with the higher activity and *LIN6* mRNA levels found. We measured the sucrose, glucose and fructose content at 48 HAC in the aerial part of the plants. Table 4 shows that pathogen infection slightly increased fructose relative content in comparison with control plants. Similar results were found in ZEA-treated plants. In contrast, bacterized plants showed higher relative sucrose content and the lowest relative glucose content. Primed plants had 38% higher relative fructose content and 33% lower relative glucose content than control plants.

Table 4: Relative content (RC) of sucrose (SUC), glucose (GLU) and fructose (FRU) in *Pseudomonas pseudoalcaligenes* PAC BNM0522-inoculated plants infected with *Sclerotium rolfsii*.

Treatment	RC SUC	RC GLU	RC FRU
Control	0.47 ^{a}	0.24 ^{c}	0.29 ^{ab}
Infected	0.48 ^{ab}	0.19 ^{bc}	0.33 ^{b}
Bacterized	0.72 ^{c}	0.04 ^{a}	0.24 ^{a}
Primed	0.45 ^{a}	0.16 ^{b}	0.40 ^{c}
ZEA	0.55 ^{b}	0.14 ^{b}	0.31 ^{ab}

The relative sugar content (defined as the proportion of mg per g of fresh weight of a specific sugar in the soluble sugar pool) was measured in the aerial tomato part at 48 hours after challenge. Data are presented as means of three determinations corresponding to ten plants each. Control plants: plants none inoculated; Bacterized plants: plants inoculated with PAC BNM0522; Infected plants: plants challenged with one sclerotium of *S. rolfsii;* Primed plants: plants inoculated with PAC BNM0522 and challenged with one sclerotium of *S. rolfsii;* ZEA: Plants treated with zeatin. Different letters in each column indicate statistical differences at P<0.10, one-way ANOVA, using Fisher's test. To explain the differences found in the sucrose content in bacterized plants, we next measured the mRNA levels of *SUS3*, the other enzyme involved in the mobilization of soluble sugars. Bacterized plants showed no *SUS3* mRNA at 12 or 24 HAC, primed plants showed appreciable *SUS3* gene over-expression at 12 HAC, and infected plants showed no differences in the *SUS3* mRNA levels with respect to control plants (Figure 3). At 24 HAC, this earlier response in *SUS3* mRNA level had almost disappeared in primed plants, while it was clearly detectable in infected plants (Figure 3).

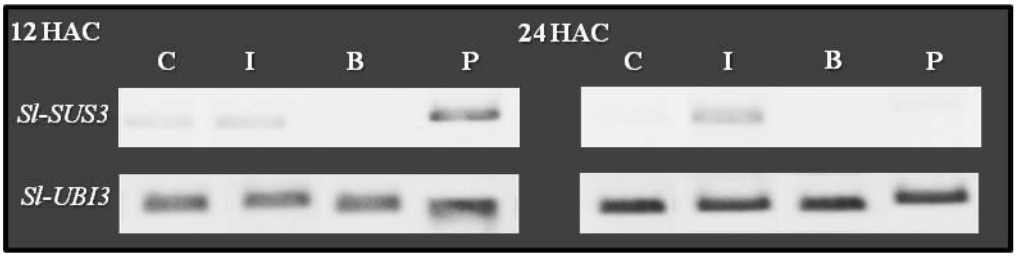

Figure 3: Expression of *Sl-SUS3* in tomato leaves at 12 and 24 h after challenge (HAC) with *Sclerotium rolfsii*. Agarose-ethidium bromide gel image of RT-PCR products amplified using specific primers for *Sl-SUS3* and *Sl-UBI3*. C: Control plants none inoculated; B: Bacterized plants inoculated with PAC BNM0522; I: Infected plants challenged with one sclerotium of *S. rolfsii*; P: Primed plants, inoculated with PAC BNM0522 and challenged with one sclerotium of *S. rolfsii*. Results corresponding to an experiment representative of three independent experiments.

3.6. Acumulation of Sl-PR transcripts

Various transcriptional studies have shown up-regulation of pathogenesis-related (PR) genes after fungal infections in response to SA, JA and ET produced by plants [29]. Thus, we next evaluated the accumulation of *PR* transcripts in tomato leaves at 12 and 24 HAC. At 12 HAC, *PR1b1* mRNA levels of infected plants were up-regulated, whereas those of bacterized plants seemed to be lower and those of the other treatments showed no differences (Figure 4). At 24 HAC, *PR1b1* mRNA levels did not change (data not shown). We also analyzed the expression of a basic β-1,3-glucanase (*Sl-PR2*, *GluB*) and a chitinase (*Sl-PR3*, CHI3). *Sl-PR2* and *Sl-PR3* transcripts were not accumulated in control or infected tomato plants, but were slightly expressed in bacterized and primed plants (Figure 4). Since PR2 is a SA signature gene product, its overexpression is expected in plants infected with biotrophic pathogens, and *PR3* mRNA is locally expressed upon pathogen attack [30].

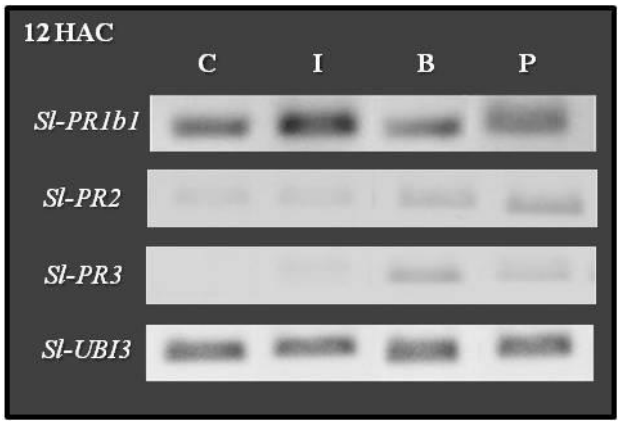

Figure 4: Expression of the *Sl-PR* genes in tomato leaves at 12 h after challenge (HAC) with *Sclerotium rolfsii*. Agarose-ethidium bromide gel image of RT-PCR products amplified using specific primers for *Sl-PR1b1*, *Sl-PR2*, *Sl-PR3* and *Sl-UBI3*. C: Control plants none inoculated; B: Bacterized plants inoculated with PAC BNM0522; I: Infected plants challenged with one sclerotium of *S. rolfsii*; P: Primed plants inoculated with PAC BNM0522 and challenged with one sclerotium of *S. rolfsii*. Results corresponding to an experiment representative of three independent experiments.

3.7. Endophytism evaluation

PAC BNM0522 was isolated from surface-disinfected tissues of inoculated plants. The number of bacteria was $3.7\ 10^5 \pm 3.25\ 10^3$ CFU g^{-1} FW and $2.3\ 10^5 \pm 1.14\ 10^3$ CFU g^{-1} FW in roots and shoots respectively (mean of four determinations ± S.E). Thereby, PAC BNM0522 was able to colonize and persist inside tissues until the end of the assay, without causing damage to tomato plants.

4. Discussion

In the present study, *S. rolfsii*-infected plants showed the lowest biomass at 60 DAS. PAC BNM0522 had positive effects on the growth of bacterized plants and on the height and biomass of primed plants, thus showing its ability to mitigate the adverse effects of this pathogenic fungus in *in vivo* assays. In primed plants, PAC BNM0522 had an inhibitory effect on mycelial growth near the beginning of the assay and reduced seedling dumping off, suggesting that it can significantly control southern blight disease. We also investigated the biochemical mechanism at play in the plant-pathogen interaction and found that cwINV activity displayed no changes in the different treatments at 24 HAC. However, at 48 HAC, cwINV activity increased strongly in response to pathogen attack both in infected and primed plants, although a bit lower in the latter. As *S. rolfsii* is a pathogenic fungus, this increase in the plant cwINV activity was expected and in agreement with results of [31,32], who found increased cwINV activity in response to infections with bacteria and fungi in tomato and barley leaves, respectively. Regarding the role of *LIN6* role in defense, we found that, at 12 HAC, *LIN6* mRNA levels of infected plants were not different from those of control plants, being almost undetectable. In contrast, primed plants showed an over-expression of this gene as from 12 HAC. In this sense, primed plants responded over-expressing this gene more quickly than infected ones, being later (at 24 HAC) strongly over-expressed in infected plants and down-regulated in primed plants. This was paralleled by a decrease in INV activity. The

impact of INV inhibition due to bacterial inoculation supports a role of cwINV in plant defense. We think that bacteria manipulate the expression of defense genes to ensure their establishment but warrant the alert state that allows a rapid and efficient response to face the pathogen (primed state) without exhausting the reserves [6]. In mycorrhizal tomato roots, Authors in [15] found a moderate induction of *LIN6* expression compared with high stress-stimulated induction and suggested that the activation of sink metabolism in the mutualistic interaction is finely tuned to avoid stress-induced defense reactions. By using acarbose (a competitive chemical INV inhibitor), previous studies have clearly demostrated the role of cwINV in defense, since it turns tomato plants more sensitive to the pathogenic bacterium *Pseudomonas syringae* [33, 34]. *LIN6* expression is suppressed by SA, supporting the fact that *LIN6* is an inducible compound of the defense/stress response pathway that is antagonistically regulated by JAs [35]. *S. rolfsii* induce the JA/ET pathway in affected plants. Therefore, we think that the *LIN6* over-expression found in pathogen-affected plants responds to the high endogenous ET level triggered by *S. rolfsii* in tomato seedlings [6]. This induction indicates that cwINV is important in the heterotrophic metabolism in response to stress-related stimuli. The resulting increased supply of carbohydrates provides metabolic energy for the activation of a cascade of defense reactions. *LIN6* expression in tomato seedlings is also induced by cytokinin and auxin [34]. In the present study, ZEA, induced the expression of this gene at both 12 and 24 HAC, showing a role of INV during growth processes. SuSy plays several different roles supplying hexoses for cell respiration and precursors for callose and cellulose for defense [36]. In primed plants, *SUS3* was expressed since 12 HAC but its expression decreased over time. In infected plants, *SUS3* was much less strongly expressed and became more consistently increased at 24 HAC. Bacterized plants showed no *SUS3* expression in all assays. The pattern of *SUS3* expression was similar to that found for *LIN6*, demonstrating the importance of both enzymes in carbohydrate metabolism during pathogen attack. The photosynthetic capacity of infected plants was seriously compromised developing chlorosis before the dumping off. Instead, primed plants had almost the same chlorophyll content as control plants and no symptom was evident during the experiment. This result found in primed plants might partly support the improved plant growth found in these plants after pathogen stress and reinforces the priming status of this plant. Bacterized plants recorded the highest total chlorophyll content. Similar results were previously shown by authors in [31]. The high cwINV activity and *LIN6* mRNA level found in infected plants was paralleled by a low chlorophyll content. Authors in [31] pointed out that a decreased rate of photosynthesis in tomato plants leads to cwINV stimulation to mobilize hexoses at the infection site during *Xanthomonas* pathogen attack. In *P. fluorescens* ISR-induced *Arabidopsis* plants, Authors in [37] found that 50 metabolites were differentially regulated. Among these, amino acids and sugars were the main differentiated primary metabolites and, specifically, glucose and fructose act as signaling molecules. Here, we found that *S. rolfsii* slightly induced a differential fructose accumulation in tomato plants at 48 HAC, accompanied by a small decrease in the amount of glucose. However, primed plants reached significantly higher fructose and lower glucose content than control plants. Fructose has been described in relation with a specific pathway involving the ABA and ET-signaling pathway [38] and its specific role in the response to the attack of fungus *B. cinerea*. After pathogen infection, host glucose and fructose are dictinctly used, strongly suggesting an adjustment of the relative fructose content, required for enhanced plant defense in tomato plants. The induction of *PR1b1* in response to *S. rolfsii* infection was an early event in both infected and primed plants, accompanied with *LIN6* and *SUS3* enhanced expression since 12 HAC and being the latter associated with significant higher fructose content. Our results are in agreement with those showed by author in

[39], who found that sugar content and PR1b expression levels increase concurrently in situations of high susceptibility and concluded that the two phenomena could show a dependency link. In addition, *PR1b* is positively affected by the presence of ET, an essential component for *S. rolfsii* pathogenicity [40, 41]. Previously, we found that ET levels in *S. rolfsii* infected plants were significantly higher [6]. So, this result is in agreement with that reported by [41] in tomato plants. No accumulation of *Sl-PR2* or *Sl-PR3* transcripts was found in infected plants. The biotrophic pathogens stimulate SA pathways, which induce the activation of SA signature genes (PR2 among others), and that necrotrophic pathogens stimulate JA pathways, which induce the activation of JA signature genes (PR3 among others). Hence, the lack of *PR2* messenger in infected tomato plants was expected because *S. rolfsii* is a necrotrophic pathogen and, in the ISR pathway, the accumulation of PR3 product is only local [30]. In this sense, since we measured the systemic response to *S. rolfsii* infection in tomato leaves, the PR3 mRNA levels were as expected, and no changes were found with respect to control plants. Bacterized plants showed the highest sucrose accumulation, meaningful absence of glucose, and lowest amount of fructose, at 48 HAC. The down-regulation of *LIN6* and *SUS3* expression since 12 HAC was in parallel with the low expression of *PR1b1* genes, preventing stress-induced defensive reactions in this symbiotic interaction. Thus, our results reinforce the idea of the down-regulation mechanism used by this bacterium, as previously described [6]. The low PR2 and PR3 messenger levels detected in bacterized plants are in response to MAMPs from bacteria that induce MAMP-triggered immunity in plants [42, 43] .

5. Conclusion

Taken together, the results here presented show that that *P. pseudoalcaligenes* persists in a high number until 60 DAS, benefiting plant growth, and that its use as a biocontrol agent represents an improved strategy to ameliorate biotic stress at least towards *S. rolfsii*. Enhanced tolerance against this pathogen is the result of chages in sugar content and in the expression of genes involved in sugar metabolism. To our knowledge, this is the first time that a dependency between sugar metabolism and defenses are described in Pseudomonas-primed plants. Because this work is a first approach to the study of these mechanisms, it is necessary to continue investigating more thoroughly, and through other methodologies (quantitative PCR, for example) to achieve more precise evaluations.

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