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Induced Systemic Resistance (ISR) in *Arabidopsis thaliana* by *Bacillus amyloliquefaciens* and *Trichoderma harzianum* Used as Seed Treatments

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Abstract: The *Trichoderma* fungal species and the bacteria *Bacillus* species were described as inducers of plant systemic resistance in relation to their antagonistic activity. The objective of this study was to evaluate the effect of selected strains of *Bacillus amyloliquefaciens* (I3) and *Trichoderma harzianum* (A) on inducing systemic resistance in *Arabidopsis thaliana* as a model for plant molecular genetics. The microorganisms were identified and were confirmed for their antagonistic potential in vitro and in vivo in previous studies. In order to explore this mechanism, two mutants of *A. thaliana* carrying a *PR1* promoter (a conventional marker of salicylic acid (SA) pathway) and *LOX2* promoter (a marker triggering jasmonic acid (JA) pathway activation) were analyzed after inoculating antagonists. Transgenic reporter line analysis demonstrated that *B. amyloliquefaciens* I3 and *T. harzianum* A induce *A. thaliana* defense pathways by activating SA and JA at a high level compared to lines treated with chemical elicitors of references (acibenzolar-*S*-methyl (Bion 50 WG (water-dispersible granule)), SA, and methyl jasmonate). The efficacy of *B. amyloliquefaciens* I3 and *T. harzianum* A in inducing the defense mechanism in *A. thaliana* was demonstrated in this study.

Keywords: seed treatment; *Bacillus amyloliquefaciens* I3; *Trichoderma harzianum* A; salicylic acid; jasmonic acid; *Arabidopsis thaliana*

1. Introduction

Inducing the plant defense mechanism is considered one of the most effective sustainable biocontrol solutions that can be implemented as a promising approach in plant protection. As a matter of fact, the plant defense mechanism can be stimulated by beneficial microorganisms that have the potential to induce a distant induced systemic resistance (ISR) in the plant. The potential to activate ISR is assigned to antagonistic microorganisms [1,2] or to their secondary metabolites [3,4]. The plant defense mechanism is typically related to ISR activation [5–8], the signaling pathways of which depend on cultivars, pathogen, and microorganism inducer [9,10]. *Bacillus amyloliquefaciens* I3 isolated from wheat leaves and *Trichoderma harzianum* A isolated from Argan tree rhizosphere in Morocco demonstrated high antagonistic potential against *Zymoseptoria tritici* in vitro and in greenhouse conditions [11]. Buensanteai et al. [12] reported that *B. amyloliquefaciens* strain KPS46 induces ISR in soybean against *Xanthomonas axonopodis* pv. *glycines*. Also, it was demonstrated that surfactine extracted from *B. amyloliquefaciens* strain S499 triggers ISR in wheat against *Z. tritici* [3]. Furthermore, Harman et al. [13] proved that *T. harzianum* strain T39 triggers ISR in many plant species against a broad range of

diseases. Likewise, *T. harzianum* Tr6 was able to induce resistance in cucumber and *A. thaliana* against *Pseudomonas* sp. Ps14 [14].

In this study, the objective was to evaluate the potential of *B. amyloliquefaciens* I3 and *T. harzianum* A to induce and activate defense pathways in *Arabidopsis* (*A. thaliana*) by seed treatment. This property was described in vitro and in vivo using mutant lines of *A. thaliana* which report the activity of gene promoters *PR1* and *LOX2*, markers of salicylic acid (SA) and jasmonic acid (JA) defense pathways, respectively. Qualitative characterization by spatiotemporal monitoring (histochemical tests) of this type of induction in planta and quantitative analysis from protein extract assays at different stages of the culture show a role of these antagonists in the induction of defense mechanisms.

2. Materials and Methods

2.1. Biological Material

Mutant lines pPR1::GUS and pLOX2::GUS (N6357 and N57953, respectively) were used as reporter lines for SA and JA activity, respectively. These lines were provided by Nottingham *Arabidopsis* Stock Center.

In-plant analysis of β-glucuronidase (GUS) activity in *Arabidopsis* lines, genetically transformed by transcriptional fusions of the *UIDA* gene with promoters of the *PR1* (PR1 line) and *LOX2* (LOX2 line) genes allows the activation of these promoters already described as markers of induction of the SA and JA pathways, respectively [15,16].

2.2. Preparation of Antagonists Inocula

Two strains with antagonistic potential were used in this study: strain I3 of *B. amyloliquefaciens* and strain A of *T. harzianum* [11]. *T. harzianum* inocula were prepared using cultures growing in PDA media (Potato Dextrose Agar) incubated seven days at 25 °C in the dark. *B. amyloliquefaciens* I3 inocula were plated in Luria Bertani (LB) Agar [17] and incubated 72 h at 27 °C in the dark.

2.3. Arabidopsis Seedling Preparation

Ten milligrams of seeds (6000 *Arabidopsis* seeds) were stirred in 1 mL of sterilizing solution (ethanol 70%, sodium hypochlorite 10%) for 10 min, subsequently rinsed three times with ethanol 70%, and dried up using Whatman papers disinfected with ethanol.

Arabidopsis was cultivated in vitro and in vivo for studying its different early growth stages (germination, cotyledons, leaf production, and rosette production); the study focuses also on late growth stages (flowering and silique ripening). In vitro seedlings were grown in Murashige and Skoog basal salt mixture agar media in petri plates of 90 mm in diameter. In vivo seedlings were sown in pots of 7 × 7 × 8 cm, with three seeds in each pot containing 2/3 peat and 1/3 perlite.

Seeds were stratified at 4 °C in the dark before adding the antagonist suspensions. Afterward, 10 µL of the antagonist suspensions (10^9 colony-forming units (cfu)/mL) were added on the top of each seed in in vitro conditions. In the in vivo conditions, the substrate was inoculated with 8 mL of the antagonist suspensions (10^9 cfu/mL). Both plates and pots were transferred to a growth room kept at long-day conditions (19 °C/21 °C, 16/8-h photoperiod, 60% relative humidity, and 150 µE (microEinstein) of luminance).

In this study, four controls were made: a negative control in which seeds were treated with sterile milliQ water, and positive controls where seeds were treated with acibenzolar-*S*-methyl at 925 µM (Bion 50 WG, Syngenta company), SA at 140 µg/mL which induced *PR1* activity, and methyl jasmonate (MeJa) at 60 µM which induced *LOX2* activity in reporter cell lines. All plants in controls were treated using foliar spray 24 h before being stained with GUS solution.

2.4. Histochemical Staining

Firstly, 150 mL of dye solution was made from the following mixture: 25 mL of 0.2 M sodium phosphate dibasic (Na_2HPO_4), 50 mL of 0.2 M sodium phosphate monobasic (NaH_2PO_4), 1.5 mL of 50 mM potassium ferricyanide ($K_3Fe(CN)_6$), 1.5 mL of 50 mM potassium ferrocyanide ($K_4Fe(CN)_6$ 3H2O), 3 mL of 0.5 M ethylene diamine tetra acetate (EDTA), 750 µL of Triton ×100 at 20%, 2.8 mL of 40 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) dissolved in *N*,*N*-dimethylformamide, and 65.45 mL of milliQ water.

Ten plants were used to study every growth stage modality cultivated in vitro, and three plants were used to study in vivo growth modality in pots. Plants were dipped in GUS mixture for 12 h and incubated in the dark at 37 °C while stirring for 150 rotations per minute. Tissues were then washed with ethanol 70%. Stained tissues were studied with a binocular loupe, and the ratio of surface area stained over total surface area of each plant was estimated using ImageJ software. This assay was repeated three times.

2.5. β-Glucuronidase Activity Measurement

2.5.1. Protein Extraction

To extract proteins for leaves and floral stems, the SSE extraction buffer, containing 9 mL of 1M Na_2HPO_4, 680 µL of 1 M NaH_2PO_4, 4 mL of 0.2 M EDTA, 0.2 g of Triton ×100, and 0.2 g of Sarcosyl (*N*-laurylsarcosine), was completed to the final volume of 200 mL with sterile milliQ water. The following reagents were added to SSE buffer just before its use: 200 µL of 100 mM phenylmethylsulfonyl fluoride (PMSF), 800 µL of antiprotease (25-fold pastille concentrate diluted in 2 mL of milliQ water), and 200 µL of 10 mM DL-Dithiothreitol (DTT) to a final volume of 20 mL.

Regarding the roots, 5 mL of 100 mM Tris-Hcl at pH 7.6 was added to 2 mL of 10 mM $MgCl_2$, 40 µL of 0.5 M EDTA, 20 µL of 10 mM DTT, and 12.94 mL of sterile milliQ water.

Protein extraction from roots, leaves, and floral stems was performed at the flowering stage. The amount of proteins in floral stems was also determined at the silique ripening stage. The test was repeated three times. Samples for each extraction were snap-frozen in liquid nitrogen and conserved at −80 °C. Floral stems and leaves were ground in the mortar using liquid nitrogen, while roots were lyophilized before grinding with small glass beads. In total, 300 mg of floral stem and leaf powder was weighed in 15-mL laboratory tubes and, subsequently, 3 mL of extraction buffer was added. All tubes were incubated in ice for 30 min, mixed every 10 min using a vortex, and then placed on a cold centrifuge (4 °C) for 10 min at 3000× *g*. The supernatant was re-centrifuged for 20 min and other 10 min at 20,000× *g*. The final volume of supernatant was conserved at 4 °C until chip analysis. For roots, 500 µL of extraction buffer was added to 100 mg of plant powder. The suspension was mixed in a vortex and placed in a cold centrifuge (4 °C) for 30 min at 14,000× *g*.

2.5.2. Preparing Protein Micro-Plate

On a 96-well micro-plate, a range of standards from 0 µg/µL to 5 µg/µL of BSA (bovine serum albumin) at 0.1 mg/mL was deposited. In each well of the micro-plate, 1 µL of plant extract, 49 µL of sterile milliQ water, and 200 µL of reactive dye Bio-Rad Protein Assay diluted at 1/4 were added. Samples were established in triplicate in the same micro-plate. The micro-plate was analyzed using a spectrophotometer at 595 nm as described in the Bradford method [18].

2.6. Statistical Analysis

SPSS 21 statistic software was applied for histochemical data analysis. Statistical analyses were established at two-factor level; the first corresponded to treatments (*B. amyloliquefaciens* I3, *T. harzianum* A, Bion 50 WG, SA, MeJa, and negative control) and the second factor indicated the growth stages (germination, cotyledons, leaf production, rosette, and flowering). ANOVA analysis was applied

for analysis of mean data variation, while the Duncan test was used for the comparison of means at $p = 0.05$.

The value of β-glucuronidase activity was transformed to square roots for data analysis. The applied factors were treatments (*B. amyloliquefaciens* I3, *T. harzianum* A, Bion 50 WG, SA, MeJa, and negative control), growth stage (flowering, silique ripening), and vegetative organs (roots, leaves, and floral stems). Variance analysis was utilized to study the variation of averages in the amount of β-glucuronidase activity using SPSS 21. Means were compared with the Duncan test at $p = 0.05$.

3. Results

3.1. β-Glucuronidase Expression by GUS Staining

The antagonist inoculation in transgenic *Arabidopsis* (*PR1* and *LOX2* reporter lines) demonstrated the induction of SA (*PR1* line) and JA (*LOX2* line) pathways. Results of β-glucuronidase (GUS) staining in both reporter lines (Figures 1 and 2) whose seeds were treated by *B. amyloliquefaciens* I3 and *T. harzianum* A revealed the effective activation of both *LOX2* and *PR1* promoters and, thus, the induction of SA and JA pathways. Similar GUS staining results were shown in lines treated with chemical elicitors (Bion 50 WG, MeJa, and SA) at different growth stages (germination, cotyledons, leaf production, rosette, and flowering).

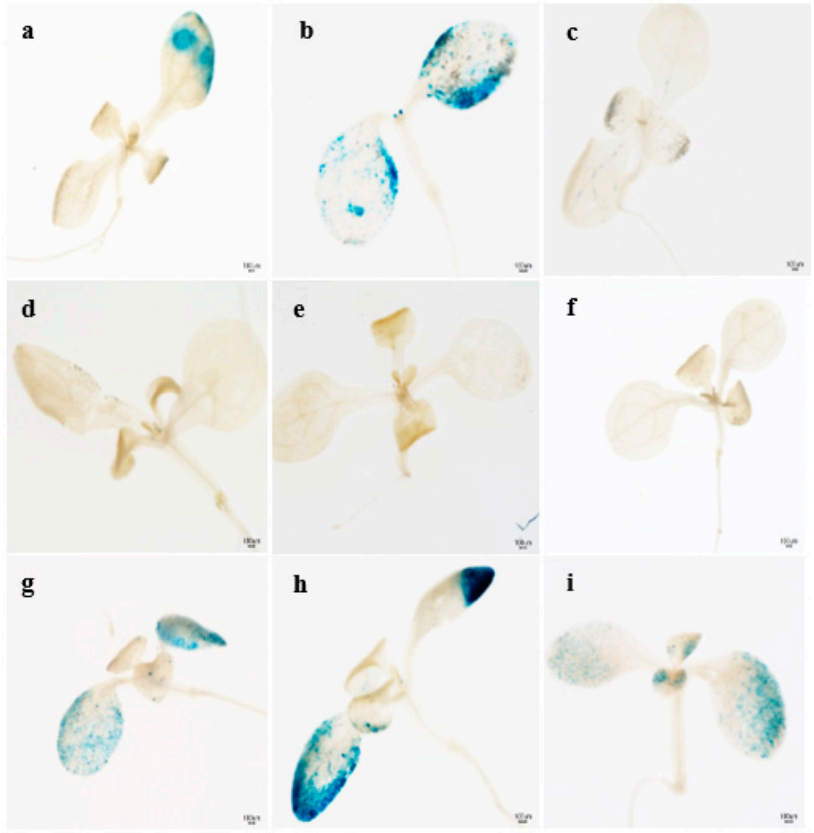

Figure 1. β-Glucuronidase (GUS) activity in pPR1::GUS (**a**–**c**,**g**,**h**) and pLOX2::GUS (**d**–**f**,**i**) lines of *Arabidopsis thaliana* at cotyledon stage (example of the plants cultivated in vitro): (**a**,**d**) seeds treated with *Trichoderma harzianum* A; (**b**,**e**) seeds treated with *Bacillus amyloliquefaciens* I3; (**g**) treatment by Bion 50 WG; (**h**) treatment by salicylic acid; (**c**,**f**) treatment by milliQ water; (**i**) treatment by methyl jasmonate.

Figure 2. β-Glucuronidase activity in pPR1::GUS (**A**–**C**,**G**,**H**) and pLOX2::GUS (**D**–**F**,**I**) lines of *Arabidopsis thaliana* at flowering stage (example of the plants cultivated in vivo): (**A**,**D**) seeds treated with *Trichoderma harzianum* A; (**B**,**E**) seeds treated with *Bacillus amyloliquefaciens* I3; (**G**) treatment by Bion 50 WG; (**H**) treatment by salicylic acid; (**C**,**F**) treatment by milliQ water; (**I**) treatment by methyl jasmonate.

GUS staining pattern in the germination stage of transgenic *Arabidopsis* lines carrying pPR1::GUS was observed in treated plants with the two positive controls, Bion 50 WG and SA. Plants inoculated with *B. amyloliquefaciens* strain I3 developed an intense histochemical staining which was dependent on the developmental stage. The former ranged from 51% in the cotyledon stage to 91% in the flowering stage. Similarly, it may be inferred that *T. harzianum* strain A could induce the *PR1* promoter, showing lower GUS staining results compared to those observed when plants were treated with *B. amyloliquefaciens*. As a matter of fact, GUS stain pattern covered 37% of the plant surface in the cotyledon stage and 80% in the flowering stage. *Arabidopsis* lines carrying pPR1::GUS and treated with *T. harzianum* A and *B. amyloliquefaciens* I3 showed a very high GUS stain pattern compared to those observed in plants treated with water at different growth stages. GUS staining covered at the most 3% of the plants in the flowering stage treated with water (Figure 3).

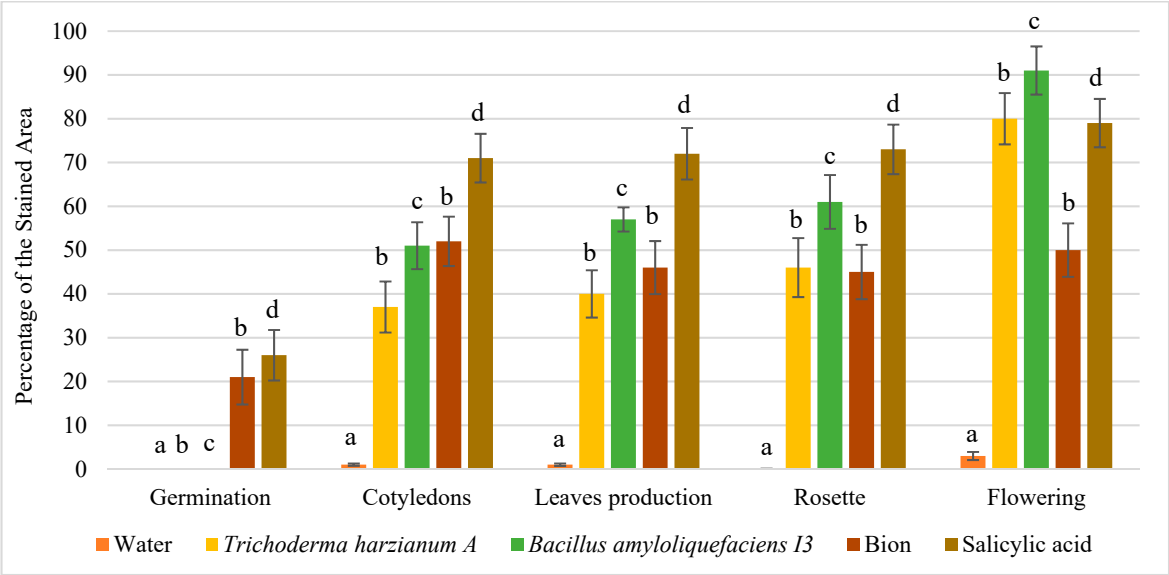

Figure 3. GUS stain pattern surface for each treatment and growth stage in the pPR1::GUS line. Bars correspond to standard errors of means. Letters denote homogeneous groups in the Duncan test at $p = 0.05$.

The transgenic *Arabidopsis* line carrying pLOX2::GUS, planted in vitro, did not show any GUS stain pattern when treated with both antagonists during all the studied developmental stages (germination, cotyledon, leaf production, rosette). On the other hand, when this line was treated with methyl-jasmonate, the GUS stain patterns ranged from 79% in germination to 81% in the rosette stage (Figure 4). Plants presented highly significant GUS stain differences in all the treatments in the flowering stage. The GUS stain patterns covered 84% of the leaf surfaces when plants were treated with *B. amyloliquefaciens* I3. The GUS stain patterns covered 82% and 78% of the leaf surfaces when treated with MeJa and *T. harzianum* A, respectively. On the other hand, the negative controls were stained on less than 2% of the total surface (Figure 4).

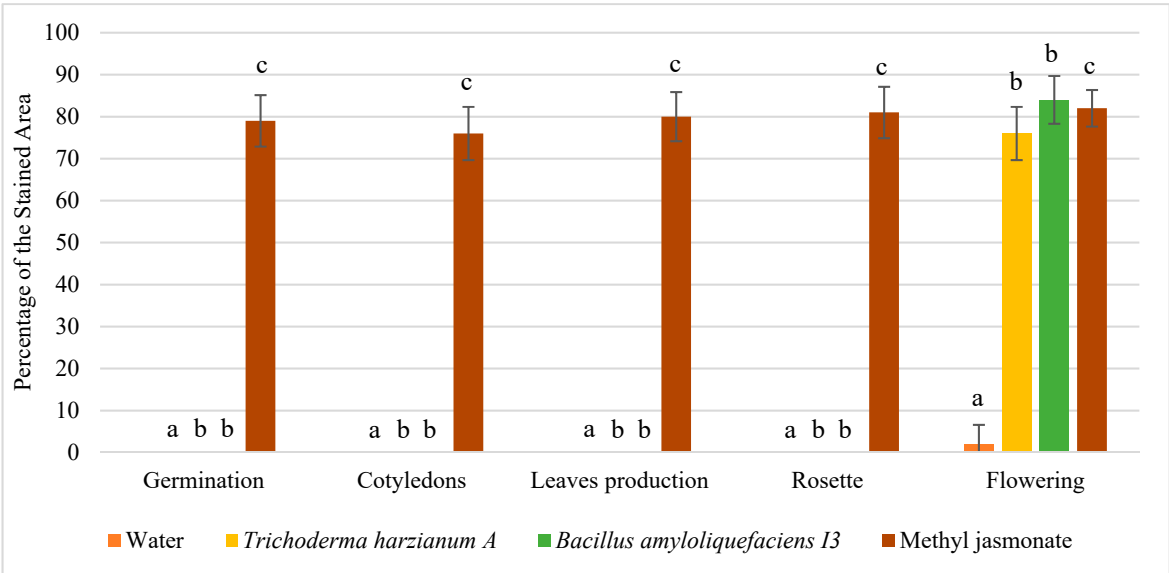

Figure 4. GUS stain pattern surface for each treatment and growth stage in the pLOX2::GUS line. Bars correspond to standard errors of means. Letters denote homogeneous groups in the Duncan test at $p = 0.05$.

3.2. Quantitative Analysis of Promoter Activation by Enzymatic Dosage of GUS in Protein Extracts

The dosage of GUS activity in the transgenic *Arabidopsis* line, carrying pPR1::GUS treated with both antagonists, demonstrated a significantly higher activity in roots and floral stems compared to that observed in leaves (Figure 5). β-Glucuronidase activity measured in the root extract was 0.78 µg/µL, 0.82 µg/µL, and 0.89 µg/µL when plants were treated with Bion 50 WG, SA, and milliQ water, respectively. However, the measured activity was 2.03 µg/µL and 1.97 µg/µL in the root extract of plants treated with *B. amyloliquefaciens* I3 and *T. harzianum* A, respectively. β-Glucuronidase activity in the root extract of plants treated with *B. amyloliquefaciens* I3 and *T. harzianum* A was comparable to that measured in floral stems. The measured activity was 2.54 µg/µL when plants were treated with *B. amyloliquefaciens* I3 and 2.07 µg/µL when treated with *T. harzianum* A. The β-glucuronidase activity in the root extract was higher than that measured in leaves.

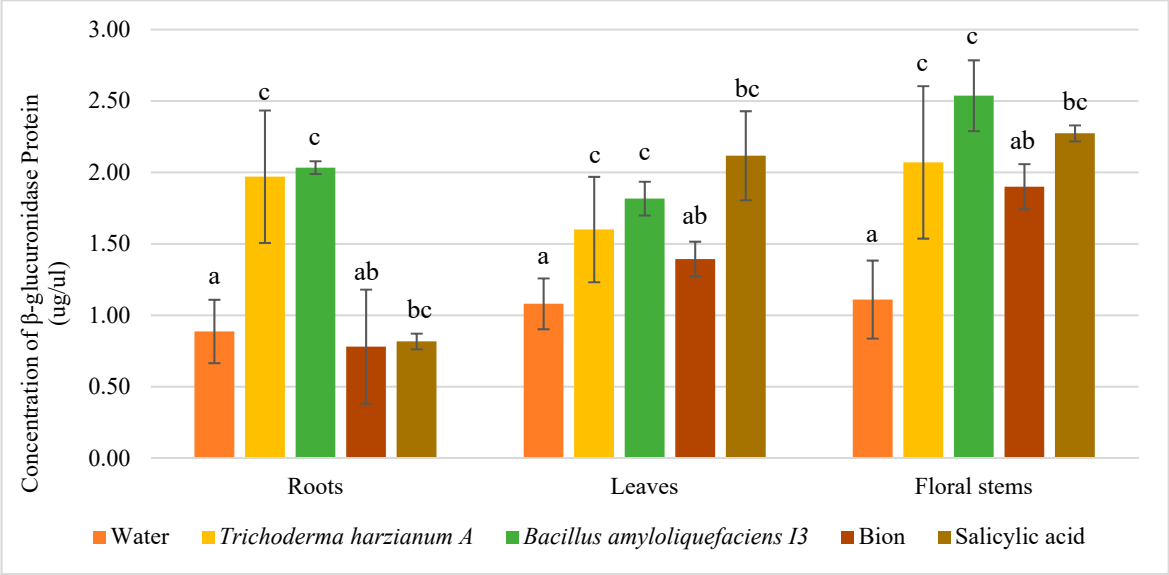

Figure 5. β-Glucuronidase activity in the extracts of roots, leaves, and floral stems in the pPR1::GUS line in the flowering stage with the following treatments: water, *Trichoderma harzianum* A, *Bacillus amyloliquefaciens* I3, and methyl jasmonate. Bars correspond to standard errors of means. Letters denote homogeneous groups in the Duncan test at $p = 0.05$.

The dosage of GUS activity in *Arabidopsis* lines carrying pLOX2::GUS showed significantly different results in all the treatments and in different tissues during plant development. The measured β-glucuronidase activity in the root extract was 1.68 µg/µL in plants treated with *T. harzianum* A and 1.55 µg/µL in plants treated with *B. amyloliquefaciens* I3. Additionally, the GUS activities were more significant in the antagonist treatments compared to samples treated with MeJa and water. The measured GUS activity in floral stems treated with *T. harzianum* A, *B. amyloliquefaciens* I3, and MeJa were 1.88 µg/µL, 2.24 µg/µL, and 2.20 µg/µL, respectively, whereas it did not exceed 1.13 µg/µL in samples treated with water. The activity in the leaf extract treated with different treatments was low compared to that of floral stems (Figure 6).

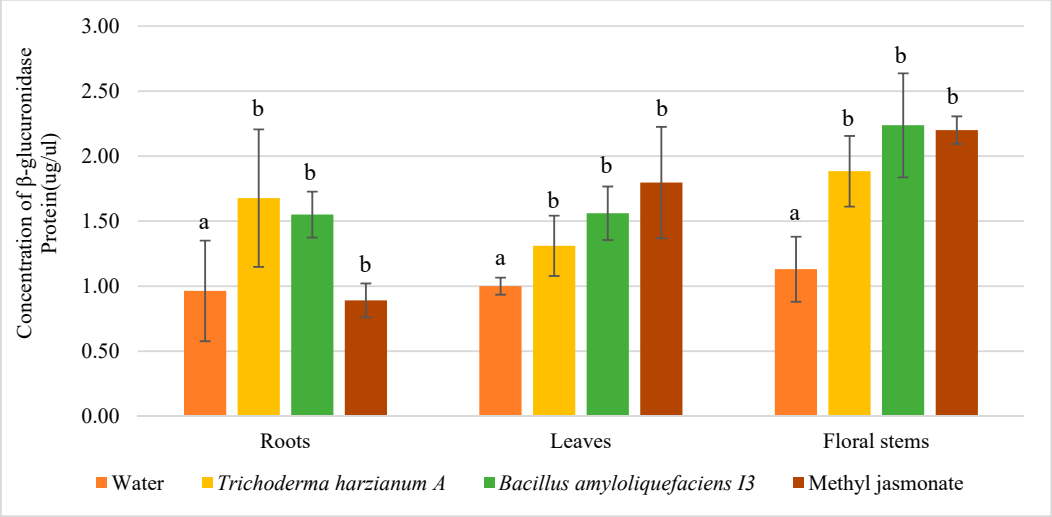

Figure 6. β-Glucuronidase activity in extracts of roots, leaves, and floral stems in the pLOX2::GUS line in the flowering stage with the following treatments: water, *Trichoderma harzianum* A, *Bacillus amyloliquefaciens* I3, and methyl jasmonate. Bars correspond to standard errors of means. Letters denote homogeneous groups in the Duncan test at $p = 0.05$.

3.3. Dosage of β-Glucuronidase Proteins in Floral Stems at Silique Ripening Stage of pPR1::GUS and pLOX2::GUS Lines

The β-glucuronidase activity in total extracts of plants treated with *T. harzianum* A and *B. amyloliquefaciens* I3 at the silique ripening stage was much higher in floral stems of transgenic lines carrying pLOX2::GUS than in plants treated with water (Figure 7). The activity was 1.43 µg/µL, 1.92 µg/µL, and 1.64 µg/µL when the pLOX2::GUS line was treated with MeJa, *B. amyloliquefaciens* I3, and *T. harzianum* A, respectively. When pPR1::GUS plants were treated with Bion 50 WG or SA, the activity of β-glucuronidase was 1.90 µg/µL compared to those treated with *B. amyloliquefaciens* I3 and *T. harzianum* A with an activity of 1.82 µg/µL and 1.45 µg/µL respectively.

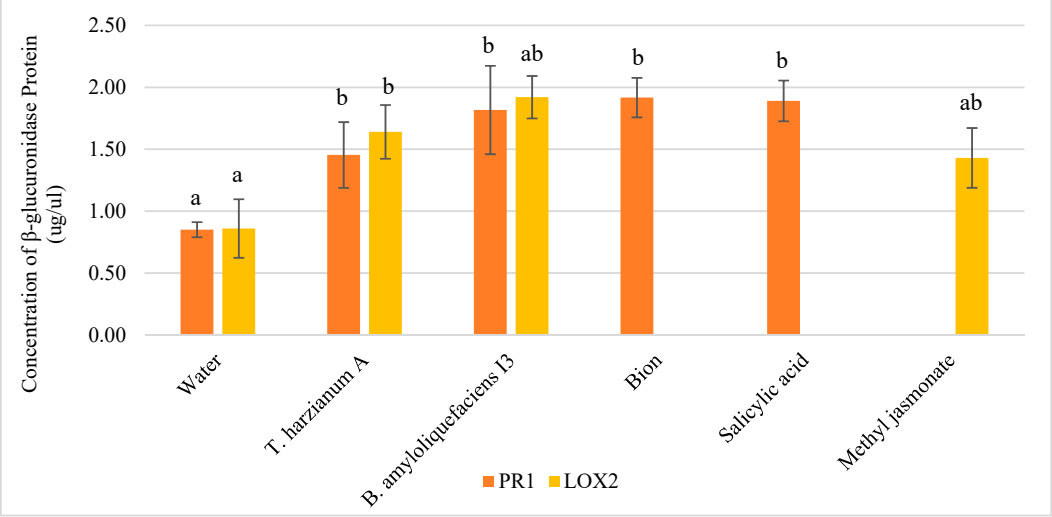

Figure 7. β-Glucuronidase activity in extracts of floral stems of the pLOX2::GUS and pPR1::GUS lines at the silique ripening stage with the following treatments: water, *Trichoderma harzianum* A, *Bacillus amyloliquefaciens* I3, methyl jasmonate, salicylic acid, and Bion 50 WG. Bars correspond to standard errors of means. Letters denote homogeneous groups in the Duncan test at $p = 0.05$.

4. Discussion

Various studies were conducted on the induction of systemic resistance by beneficial microbes leading to a decrease in incidence or severity of diseases in different plant pathosystems [19,20]. It was reported that different species of *Trichoderma* spp. and *Bacillus* spp. colonize the rhizosphere of different plants, protecting them against a wide range of aerial and soilborne pathogens [21–23]. In recent years, increasing attention was accorded to the study of direct interactions between *Trichoderma* spp./*Bacillus* spp. and plants, including molecular studies on plant defense mechanisms that allowed a better understanding of how antagonists interact with plants [12,24–26]. In the present study, the induction of SA and JA pathways by *B. amyloliquefaciens* I3 and *T. harzianum* A from the cotyledons to the flowering stage was demonstrated using GUS staining patterns (Figures 1 and 2). The GUS staining pattern in the plant surface was significantly higher ($p = 0.05$) when plants were treated with both antagonists at the flowering stage compared to the percentage of GUS dyes in plants treated with the commonly tested chemical references, Bion 50 WG and SA (Figure 3). There was no visible GUS staining in pLOX2::GUS lines observed in in vitro cultivated plants; however, in the flowering stage, the GUS staining was observed. On the other hand, the GUS staining pattern was very high in plant leaves treated with *B. amyloliquefaciens* I3 and *T. harzianum* A, which demonstrates the potential of strains A and I3 in inducing the JA pathway (Figure 4). It is important to note that both transgenic plants exhibited a positive GUS staining and were also induced in the flowering and the silique ripening stages, while the antagonist inoculation was established in the substrate during the early developmental stages (Figures 2 and 7). In addition to GUS staining, the activity of β-glucuronidase (GUS) in different plant tissues in the flowering stage revealed SA and JA pathway induction in roots, leaves, and floral stems in both pPR1::GUS and pLOX2::GUS lines, with a higher activity in floral stems (Figures 5 and 6). Furthermore, the β-glucuronidase activity was significantly higher at $p = 0.05$ in floral stems of pLOX2::GUS than that of the pPR1::GUS line in the silique ripening stage.

Our results are in agreement with the study conducted by Niu et al. [27], who demonstrated that *B. cereus* AR156 induces ISR in *A. thaliana*, by simultaneously activating SA and JA pathways. Similarly, van Wees et al. [10] proved that strain WCS417r of *Pseudomonas fluorescens* induces ISR in tomato and *A. thaliana* against *Pseudomonas syringae* pv. *tomato* and *Fusarium oxysporum* f. sp. *raphanin*, respectively. Ryu et al. [28] also demonstrated that different plant growth-promoting rhizobacteria (PGPR) (*Serratia marcescens* 90–166, *Bacillus pumilus* SE34, *P. fluorescens* 89B61, *P. fluorescens* 89B27, *B. amyloliquefaciens* IN937, and *B. subtilis* IN937) induce SA and JA pathways in cucumber. However, it is important to note that surfactines and fengycines, which are lipo-peptides produced by the genus of *Bacillus*, may interact with plant membrane cells and disturb them (provoking stress), leading to a similar defense reaction in the plant that may be confused with ISR induction [4].

In a previous study, Yoshioka et al. [29] revealed that *Trichoderma asperellum* colonizing *A. thaliana* roots induced ISR through the SA signaling cascade. Additionally, plants treated with *Trichoderma* filtrates induced SA and jasmonate/ethylene (JA/ET) pathways. Alizadeh et al. [14] confirmed that *T. harzianum* Tr6 activated SA and JA pathways in transgenic *A. thaliana* myb72 and sid2 against *Botrytis cinerea*. A similar result was obtained by Elsharkawy et al. [6], who demonstrated that *T. asperellum* SKT-1 strain induced ISR (SA and JA/ET) in *A. thaliana* against the Y strain of the cucumber mosaic virus (CMV-Y).

Our results demonstrated the potential of the two antagonists to systemically induce SA and JA pathways in *A. thaliana*. According to the biocide effect, the antagonists may offer more sustainable plant protection in field conditions using the treated seeds with *B. amyloliquefaciens* I3 and *T. harzianum* A. This was confirmed by Barakat et al. [30], who showed the antagonistic potential of I3 and A strains against *Z. tritici* by seed coating in greenhouse and in field conditions (unpublished data).

Author Contributions: B.I. conceived, designed, and performed experiments and wrote the paper; C.N. supervised, contributed in data analysis and translation, and reviewed the paper; G.P. was responsible experiment validation, supervision, and review; E.B. and E.G.M. were responsible for supervision and review; A.S. contributed in protein extraction; N.M. was responsible for protein extraction and dosage; M.M. supervised the study.

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Conflicts of Interest: The authors declare no conflicts of interest.

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