Phyton-
International Journal of
Experimental Botany

Tech Science Press

DOI: 10.32604/phyton.2022.021507

ARTICLE

Participation of Auxin Transport in the Early Response of the *Arabidopsis* Root System to Inoculation with *Azospirillum brasilense*

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Received: 18 January 2022 Accepted: 09 April 2022

ABSTRACT

The potential of Plant Growth Promoting Rhizobacteria (PGPR) has been demonstrated in the case of plant inoculation with bacteria of the genus *Azospirillum* which improves yield. *A. brasilense* produces a wide variety of molecules, including the natural auxin indole-3-acetic acid (IAA), as well as other phytoregulators. However, several studies have suggested that auxin induces changes in plant development during their interaction with the bacteria. The effects of *A. brasilense* Sp245 on the development of *Arabidopsis thaliana* root were investigated to help explain the molecular basis of the interaction. The results obtained showed a decrease in primary root length from the first day and remained so throughout the exposure, accompanied by a stimulation of initiation and maturation of lateral root primordia and an increase of lateral roots. An enhanced auxin response was evident in the vascular tissue and lateral root meristems of inoculated plants. However, after five days of bacterization, the response disappeared in the primary root meristems. The role of polar auxin transport (PAT) in auxins relocation involved the PGP1, AXR4-1, and BEN2 proteins, which apparently mediated *A. brasilense*-induced root branching of *Arabidopsis* seedlings.

KEYWORDS

Auxin; *Azopirillum brasilense*; *Arabidopsis*; auxin transport; lateral roots

1 Introduction

The inoculation with bacteria of the genus *Azospirillum*, a well-known PGPR, has demonstrated the potential of these microorganisms to promote plant growth and improve crop yield in different types of soil and growth conditions. The most common phenotype in roots modulated by PGPR is the inhibition of primary root growth coupled with proliferation and growth of lateral roots and root hairs, which support vegetative, green biomass [1–3]. *Azospirillum* produces many molecules, including the natural auxin indole-3-acetic acid (IAA), and harbors lipopolysaccharides that elicit complex responses in the

plant [4]. IAA regulates virtually any aspect of the plant's development, so a large number of studies have focused on analyzing the beneficial effect of IAA produced by *Azospirillum* on cereal crops [5].

Recently, to elucidate the molecular mechanism that regulates the interaction of this bacterium with plants, the root development of *Arabidopsis thaliana* was investigated. It is crucial pointing that primary root growth is due to both root meristem cell division and cell elongation [6]. It was observed a reduction in the primary root length, an increase in the lateral root number and root hairs, as well as an elevation in the internal auxin levels [7,8]. This same phenotype was also observed when exposing the plant to high auxin concentrations [9]. In the *Azospirillum-Arabidopsis* interaction reported by Spaepen et al. [7], they evaluated the effect of the auxins produced by the bacteria when inoculating *A. thaliana* with two strains of *A. brasilense* Sp245: a wild-type strain and a mutant in auxin biosynthesis (FAJ009). The inoculation with the wild-type strain caused inhibition of primary root growth and an increase in the number of lateral roots and length of root hairs. The mutant strain did not alter the root architecture of the plant. These results allowed them to suggest that the auxins produced by the bacteria could be involved in the morphological changes of the root architecture.

Fine-tuning control of auxin homeostasis and time-space distribution is crucial to promoting growth. This is because suboptimal concentrations of auxins cannot elicit the desired physiological responses, and high concentrations can be growth repressing. Thus, auxin biosynthesis, transport, signaling, and degradation, are critical factors for sensing biotic and abiotic stimuli within cells and tissues [10]. For the plant-*Azospirillum* interaction, bacterially-produced auxins could be involved in alterations of root architecture, such as the programmed lateral root formation. Nevertheless, little information has been generated to elucidate the molecular mechanism behind this important agronomical trait. The present study aimed to uncover the genetic component of the *Arabidopsis* response pathway to auxins potentially involved in the root response to *A. brasilense*. Novel evidence was gathered that the overall bacterium increases internal auxin levels, changes the root system development, and promotes lateral root primordia initiation. We also tested how the presence of this bacterium modulated the root architecture of several *Arabidopsis* mutants by altering the auxin response pathway.

2 Materials and Methods

2.1 Disinfection, Sowing, and Incubation of Seeds

The seeds of the wild-type Ecotypes Columbia (Col-0), or *Landsberg Erecta* (Ler), transgenic lines *DR5::uidA* [11], *CycB1::uidA* [12] and *EXP7::uidA* [13], as well as the mutants in auxin synthesis *yuc2 yuc6*$^{+/-}$ [14]; auxin carriers *aux1-7*$^{+/-}$ [15], *pin1*$^{+/-}$ [16], *pin2*$^{+/-}$ [17], *pin3*$^{+/-}$ [18], *pgp1*$^{-/-}$ [19], *pgp4* $^{-/-}$ [20], *pgp19*$^{-/-}$ [21], *axr4-1*$^{+/-}$ [22], *ben2*$^{+/-}$ [23]; the auxin signaling *axr1-3*$^{-/-}$ [24], *slr1*$^{+/-}$ [25], *arf7 arf19*$^{-/-}$ [26], the mutant *tt4*$^{+/-}$ [27] and the line *35S::YUC4 DR5::uidA* [28] were all disinfected with a 5% chlorine 1% SDS solution and stratified at 4°C for 48 h [29]. Subsequently, the seeds were sown in Petri dishes containing 0.2× Murashige and Skoog (MS) culture medium, 0.5% sucrose, 0.8% agar and 200 µL/L of a vitamin solution (thiamine 1 mg/mL, pyridoxine 5 mg/mL and nicotinic acid 5 mg/mL) and incubated in a vertical position for 5 days in a plant growth chamber (Percival Scientific AR-95L, Parry, IA, USA). The photoperiod was 16 h light/8 h dark at 22°C, and light intensity was 100 µmol m^{-2} s^{-1}.

2.2 Preparation of Culture Medium

The Murashige-Skoog (MS) culture medium (Phytotechnology Laboratories) was prepared by dissolving in distilled water the amount of salts corresponding to the 0.2× concentration and 0.5% of sucrose. After adjusting the pH to 5.7, 0.8% agar was added. After that, 200 µL/L of a vitamin solution was added to the warm medium previously sterilized at 121°C for 20 min. All reagents used came from the Sigma brand unless otherwise specified.

2.3 Preparation of Culture Medium for Maintenance and Inoculation of Azospirillum brasilense Sp245

The bacteria were kept in Luria-Bartani (LB)-tetracycline culture medium (10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl, 0.186 g/L $MgSO_4$, 0.2775 g/L $CaCl_2$, 1 g/L agar and 10 µg/mL tetracycline) at 4°C, they were reseeded every 10 days in Petri dishes with LB-tetracycline medium, and incubated overnight at 37°C. Then the bacterium was incubated for 16 h at 37°C. Afterwards, an aliquot of bacteria was taken from this plate and resuspended in 1 mL of sterile distilled water to reach absorbances of 0.9, equivalent to 1×10^8 CFU/mL or 2.5×10^5 CFU. The 0.2× MS medium included 0.5% sucrose and 0.8% agar.

2.4 Transfer of Seedlings to Culture Media Supplemented with Azospirillum brasilense Sp245

Five-day-old seedlings were transferred to Petri dishes containing 0.2 × MS medium with 2.5×10^5 CFU/mL of *Azospirillum* or without the bacterium. The seeded Petri dishes were incubated for 1, 2, 3, 4, 5, 6, and 9 days in the plant growth chamber under the conditions as mentioned above.

2.5 Histochemical Activity of uidA

Transgenic seedlings expressing *DR5::uidA*, *CycB1::uidA* and *EXP7::uidA* were exposed to the bacterium for 1, 2, 3, 4, 5, 6 and 9 days. The seedlings were placed in microtiter boxes and incubated in a solution of X-Gluc (5-bromo-4-chloro-3-indolyl-β-D glucuronide) (Phytotechnology Laboratories) (1 mM in a phosphate buffer) (NaH_2PO_4 and 0.1 M Na_2HPO_4 pH 7) adding 2 mM of $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$, at 37°C for 16 h. The seedlings were then incubated in an acid solution (0.24 N HCl and 20% methanol) for 80 min at 60°C, transferred into in a basic solution (7% NaOH and 60% EtOH) for 40 min at room temperature and dehydrated with 40%, 20% and 10% ethanol solutions for 15 min [30]. Finally, 50% glycerol was added and the seedlings were incubated overnight. The seedlings were mounted on slides and observed under the microscope with a 10× objective; photographs of the primary and lateral roots were taken.

2.6 Evaluation of Root Architecture of Seedlings

To evaluate the effect of *Azospirillum* on root architecture, five-day-old Wt seedlings were transferred to culture media without and supplemented with 2.5×10^5 CFU/mL of *A. brasilense* and incubated for 1, 3 and 6 days. The parameters of root architecture were determined: Primary Root Length (PRL), Lateral Root Number (LRN) and Lateral Root Density (LRD). The PRL was measured with a ruler (cm), the LRN was obtained by counting the roots emerging from the primary root under a stereomicroscope (Iroscope ES-24 Leica) with a 4× objective, and the LRD was obtained by calculating the ratio between LRN, and PRL [31].

2.7 Statistical Analysis

All the experiments were carried out at least three times independently, taking 25 seedlings per treatment as a representative sample. Statistical analysis was applied to the data obtained, with the Statistic 8.0 program, and the data were analyzed by repeated measures ANOVA. When F tests were significant, differences among means were tested using the post hoc Tukey test with a statistical significance of $P < 0.05$.

3 Results

3.1 Azospirillum Modifies Arabidopsis Root Architecture

In this study, the changes in the *Arabidopsis* root system during short times of exposure to the bacteria were analyzed. *Azospirillum* inhibited the growth of the primary root throughout all the incubation times (Fig. 1A), while the lateral root number increased markedly after three and six days of exposure to the bacteria (Fig. 1B); the lateral root density increased twenty-five times (Fig. 1C). Representative images of seedlings clearly show that *Azospirillum* induced a reprogramming of the root architecture from the third day of exposure to the bacteria (Fig. 1D).

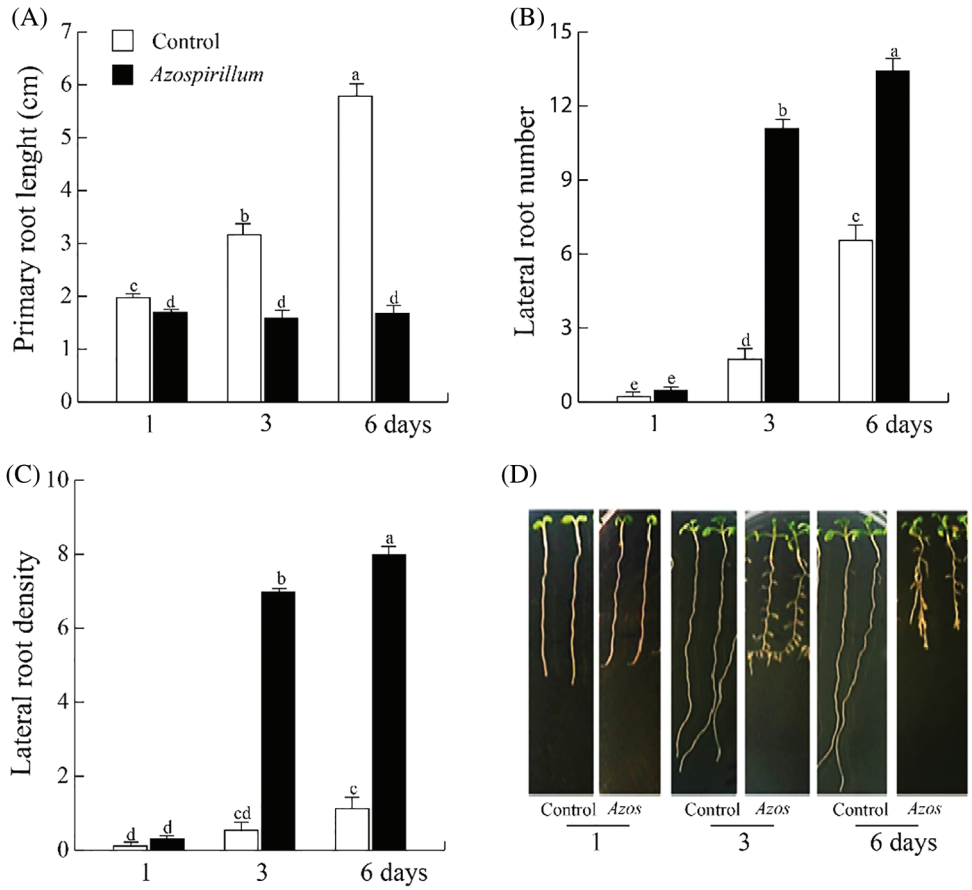

Figure 1: Effect of *Azospirillum brasilense* Sp245 on *Arabidopsis* root architecture. (A) Primary root length. (B) Lateral root number. (C) Lateral root density. (D) Representative photographs of the Wt seedlings on the control medium and inoculated with 2.5×10^5 CFU/mL of *Azospirillum*. The values shown represent the average of 25 seedlings +/− standard error (n = 3). Different letters above columns indicate significant differences between treatments on any study variable at $P < 0.05$

3.2 Azospirillum Regulates Proliferation and Elongation of Primary Root and Stimulates Lateral Root Development

To determine which process would be affected in the arrest of primary root growth in the presence of *Azospirillum* (Fig. 1A), cell division was first analyzed. For this, the line *CycB1::uidA* was used, which expresses this gene in the G_2/M phase of the cell cycle. It was observed that from the third day of exposure, the marker *CycB1::uidA* disappeared from the primary root meristem, being detected only in the lateral root meristems (Fig. 2).

On the other hand, to assess the effect of the bacteria on cell elongation, the behavior of *Exp7::uidA* on seedlings, which specifically marks the differentiation zone from any primary root was analyzed [13]. In Fig. 3, we show that *Exp7::uidA* expression extended towards the root tip and the strength in the elongation zone decreased. Together these results showed that *Azospirillum* repressed primary root growth, inhibiting both cell division and elongation, thus promoting cell differentiation at very short exposure times to the bacteria.

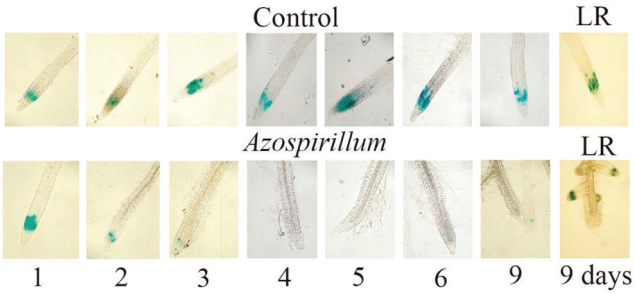

Figure 2: *Azospirillum brasilense* Sp245 modifies the *CycB1::uidA* expression in the *Arabidopsis* root. *Arabidopsis CycB1::uidA* seedlings were transferred to a control or supplemented with 2.5 × 10^5 CFU/mL of *Azospirillum* media, and incubated for 1, 2, 3, 4, 5, 6 and 9 days. At the end of the incubation time, the seedlings were treated with X-Gluc, followed by a clearing process. The seedlings were mounted on slides, and microscopic observations were made at 10× magnification. Representative photographs of the roots from the seedlings in the control medium and those inoculated with *Azospirillum* (of at least 25 seedlings from three different trials with similar results) are shown

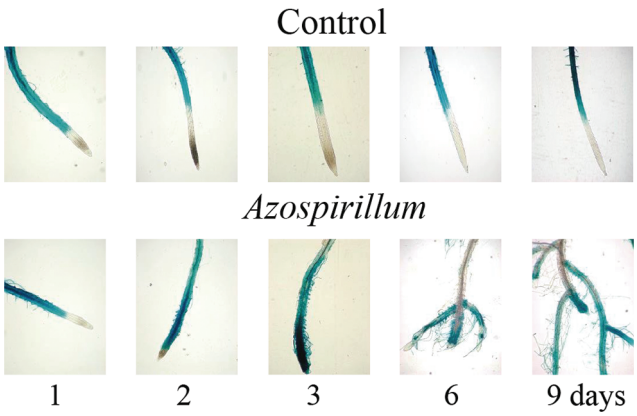

Figure 3: *EXP7::uidA* expression in *Arabidopsis* roots in the presence of *Azospirillum brasilense* Sp245. *EXP7::uidA* seedlings were transferred to a control media and those supplemented with 2.5 × 10^5 CFU/mL of *Azospirillum*, and incubated for 1, 2, 3, 6 and 9 days. At the end of the incubation time, the seedlings were treated with X-Gluc, followed by a clearing process. The seedlings were mounted on microscope slides and observations were made at 10× magnification. Representative photographs of seedling roots in control medium or incubated with *Azospirillum* media of at least 25 seedlings from three different trials with similar results are shown

Then, the effect of *A. brasilense* in stimulating the initiation and/or maturation of lateral roots was analyzed. The number of the lateral root primordia (LRP) was assessed at different developmental morphology stages, which are summarized below: I. The first evidence of LRP initiation is the appearance of closely spaced cell walls in the pericycle layer in perpendicular orientation to the root axis. II. A periclinal division occurs that divides the LRP into two layers outer layer (OL) and inner layer (IL). Hence, as the OL and IL cells expand radially the domed shape of the LRP begins to appear. III. The OL divides periclinally, generating a three layer primordium. This further emphasizes the domed shape of the LRP. IV. The IL divides periclinally, creating a total of four cell layers. At this stage the LRP has penetrated the parent endodermal layer. V. A central cell in OL1 and OL2 divides anticlinally to form four small cuboidal cells. The cells adjacent to these two cells in the OL1 and OL2 also divide, creating

an outer layer that contains 10–12 cells. The LRP at this stage is midway through the parent cortex. VI. This stage is characterized by several periclinal division. The LRP has passed through the parent cortex layer and has penetrated the epidermis. VII. It appears that many of the cells of the LRP continue to undergo anticlinal divisions. The LRP appears to be just about to emerge from the parent root [32]. *DR5::uidA* seedlings were used to facilitate the analysis of these structures. The quantification of lateral root primordia showed, that *Azospirillum* promoted both the initiation (stage I) and maturation (stages IV, V, VI, and VII) of the primordia. Later on, the number of lateral root primordia on the *Azospirillum* samples increased towards stage VII when was compared to the axenic seedlings, where lateral root primordia were at the earliest developmental stages (Fig. 4). These results showed that at very short exposure times, the bacteria promoted lateral root development.

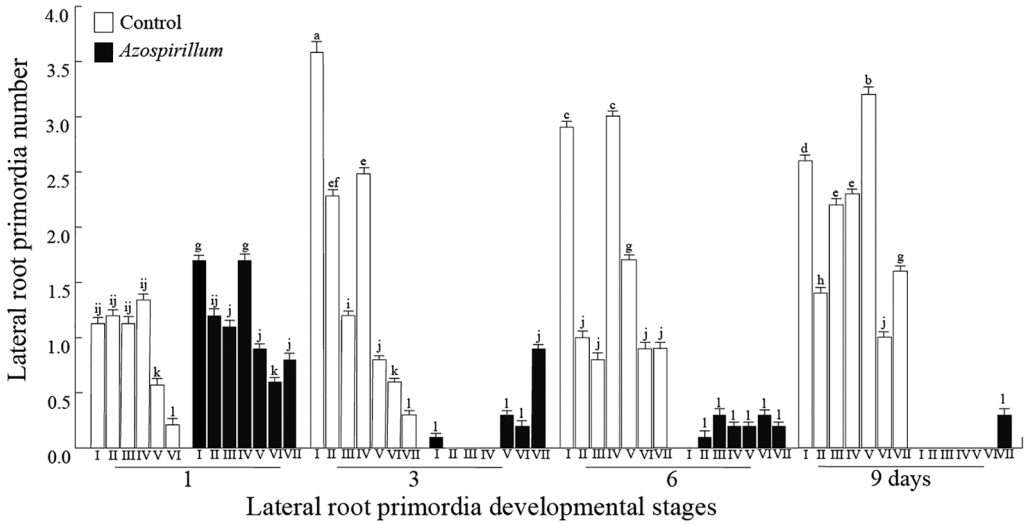

Figure 4: Lateral root primordia of *Arabidopsis* in the presence of *Azospirillum*. *DR5::uidA* seedlings were transferred to control or supplemented with 2.5×10^5 CFU/mL of *Azospirillum* media, where they were incubated for 1, 3, 6 and 9 days. On each day of exposure to the bacteria, the number of root primordia at different development stages were counted. The values represent the average of 25 seedlings +/− standard error. The experiments were repeated three times with similar results. Different letters above the histograms indicate significant differences between treatments within each day of the study

3.3 Azospirillum Stimulates Auxin Transport in Root System of Arabidopsis

The halted primary root growth in the presence of *Azospirillum* can be attributed to an accumulation of auxins at the root tip or to a decrease of auxins. To discern between these two possibilities, *Arabidopsis DR5::uidA* seedlings were used. As can be seen in Fig. 5, on day three of interaction, the level of auxins increased in primary root vascular tissue, particularly in the differentiation zone of lateral roots; this effect intensified by day fourth of exposition. The results showed that the maximum of auxins in the primary root meristem disappear from the fifth day of interaction with the bacteria, while the auxins accumulated in the vascular tissue were redistributed to lateral root meristems (Fig. 5B). Thus, the arrest of primary root growth was due, among other factors, to the disappearance of the maximum amount of auxins at the root tip.

According to the changes observed in the auxin levels in the primary root and its distribution to lateral roots in *Arabidopsis* when interacting with *Azospirillum* (Fig. 5), its polar transport (PAT) could be modulated. As N-1-naphthylthalamic acid (NPA) is a specific inhibitor of PAT [33] and has been widely

used as a valuable tool in plant development research, we evaluated the effect of NPA on changes in root architecture in presence of the bacteria. For this, Wt seedlings were transferred to culture media with 1 μM NPA to assess the interaction with *Azospirillum*. Subsequently, the material was incubated for 3, 6 and 9 days under the aforementioned conditions. The Wt seedlings grown in the presence of *Azospirillum* and *Azospirillum* plus NPA, had a decrease in root length of 70% at different times of exposure (Fig. 6A). The lateral root number in the presence of *Azospirillum* and NPA increased in a lesser proportion than on seedlings treated only with the bacterium (Fig. 6B). These results show that PAT inhibition prevents the stimulation of lateral root formation by the bacteria.

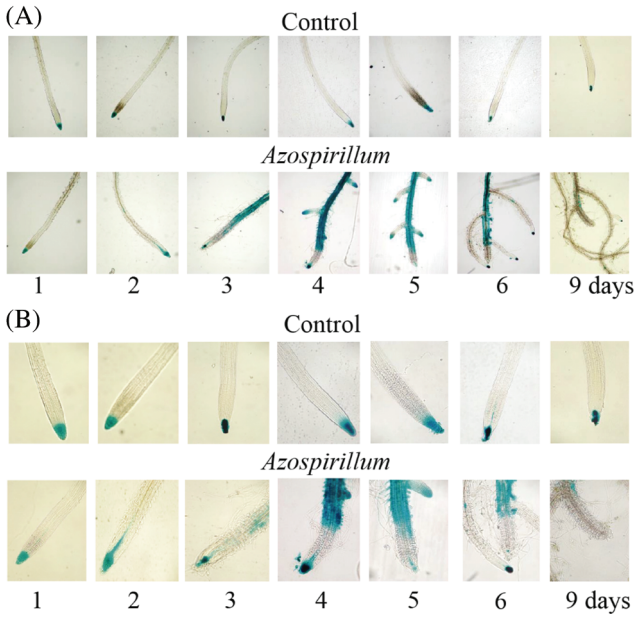

Figure 5: Effect of *Azospirillum brasilense* Sp245 on auxin levels in roots of *A. thaliana. DR5::uidA* seedlings were transferred to control or supplemented with 2.5 × 10^5 CFU/mL of *Azospirillum* media. These media were incubated for 1, 2, 3, 4, 5, 6 and 9 days. At the end of the incubation time, the seedlings were treated with X-Gluc, followed by a clearing process. The seedlings were mounted on slides and microscopic observations were made: (A) with 4× magnification and (B) 10×. Representative photographs of the roots from the seedlings in the control medium and that inoculated with *Azospirillum* of at least 25 seedlings from three different trials with similar results are shown

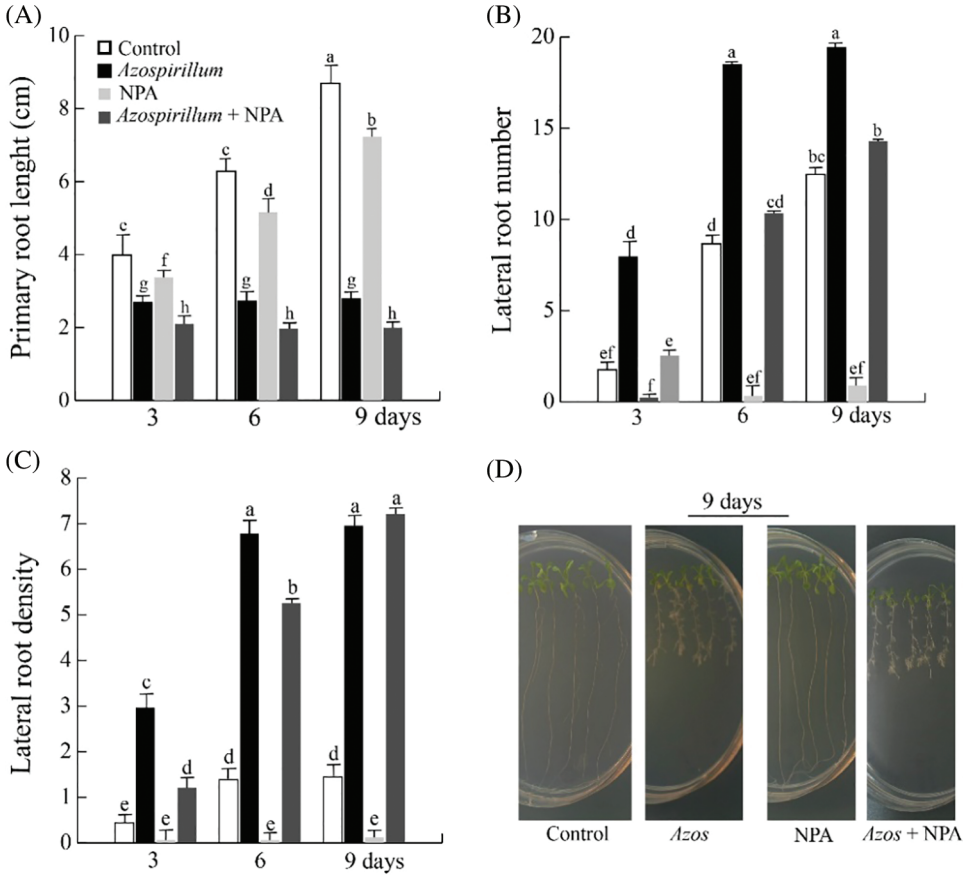

Figure 6: Effect of *Azospirillum brasilense* Sp245 on root architecture of *Arabidopsis* in the presence of NPA. *Arabidopsis* Wt seedlings were transferred to control or supplemented with 2.5×10^5 CFU/mL of *Azospirillum* media, added both media with NPA (1 µM) and incubated for 3, 6 and 9 days. (A) Primary root length. (B) Lateral root number. (C) Lateral root density. (D) Representative photographs of the seedlings are shown. The values represent the average of 25 seedlings +/− standard error. Different letters above the histograms indicate statistically significant differences between treatments using the Tukey test ($P < 0.05$). The experiments were repeated three times with similar results

Then, the effect of the bacterium in root architecture on *tt4* mutant seedling that shows an increase in PAT was analyzed. As can be seen in Fig. 7, *Arabidopsis* Ler and *tt4* seedlings, *A. brasilense* arrested the growth of the root from the first day of exposure. On the other hand, the bacteria increased lateral root formation in the *tt4* seedlings from the third day in a more significant proportion than in the Wt (Ler) line. It should be noted that the primary root of control *tt4* seedlings showed the loss of gravitropism, which was reversed in seedlings exposed to *A. brasilense*. Thus, this contrasting effect between PAT inhibition with NPA and PAT increase in *tt4* seedlings on root branching of *Arabidopsis* in the presence of *Azospirillum* suggests the participation of PAT in this plant-microbe interaction.

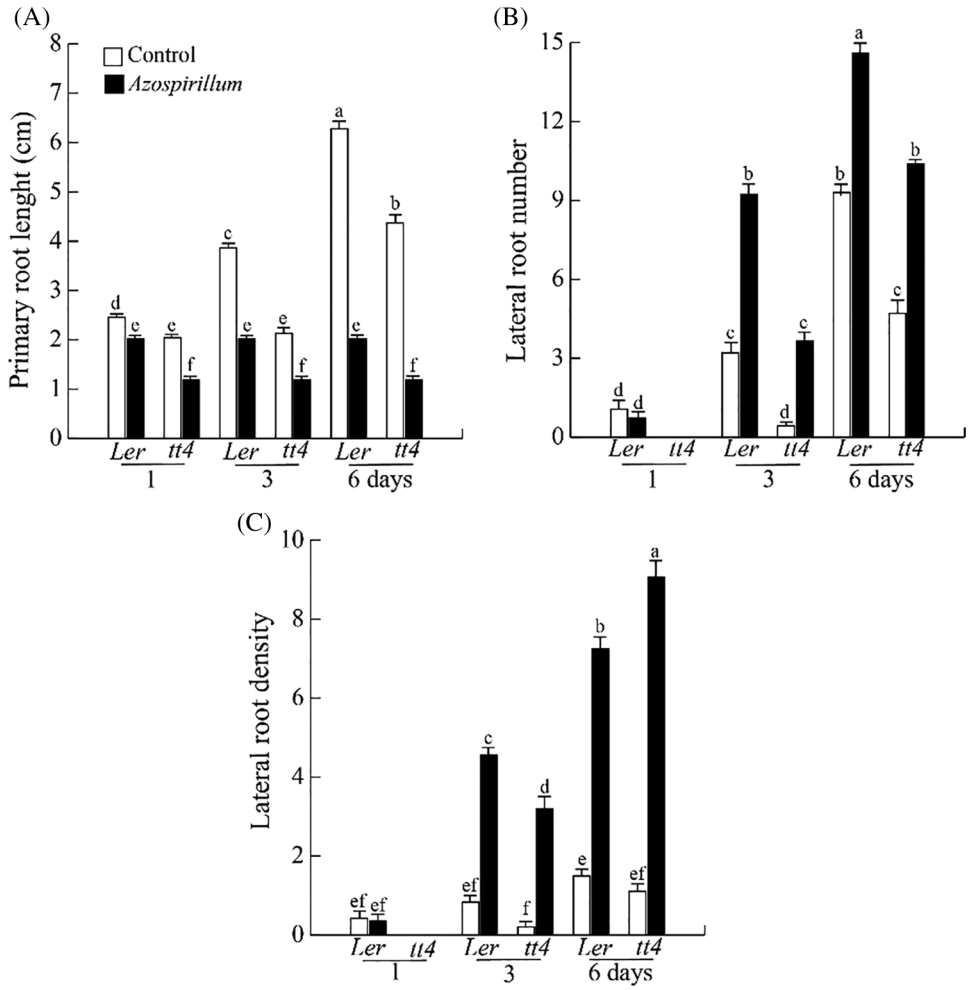

Figure 7: Effect of *Azospirillum* on root architecture of *tt4* seedlings. *tt4* seedlings were transferred to control or supplemented with 2.5 × 10^5 CFU/mL of *Azospirillum* media and incubated for 1, 3 and 6 days. (A) Primary root length. (B) Lateral root number. (C) Lateral root density. The values shown represent the average of 25 seedlings +/− standard error (n = 3). Different letters above columns indicate significant differences between treatments on any study variable at $P < 0.05$

3.4 Elements of the Auxin Response Pathway are Involved in the Effect of Azospirillum on Root Architecture of Arabidopsis

To identify genetic elements potentially involved in readjustments of the root system already influenced by *A. brasilense*, growth parameters in *Arabidopsis* Wt; and *yuc2 yuc6, aux1-7, pin1, pin2 and pin3, axr1-3, slr1*, and *arf7 arf19* mutants were evaluated. In all mutants analyzed as in Wt line, a 70% decrease in root length was observed in the presence of the bacterium (Fig. 8A). Regarding the stimulation of root branching with *Azospirillum*, in the *slr1* and *arf7 arf19* mutant seedlings, the bacterium was unable to induce the formation of these structures, while in the other mutants; there was an increase comparable to Wt line (Figs. 8B and 8C).

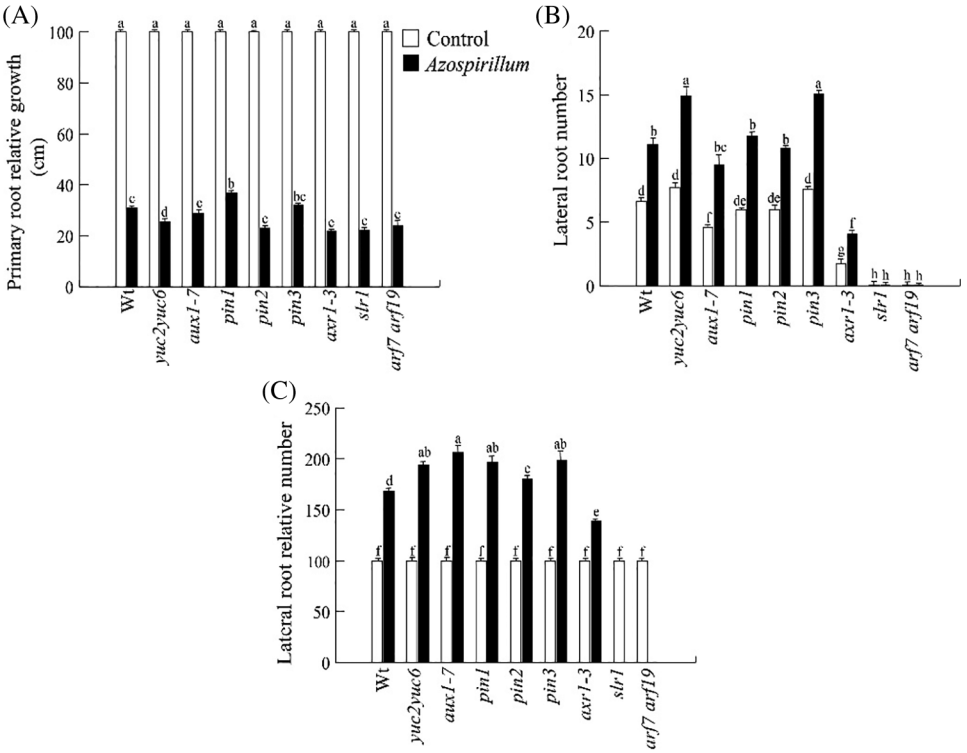

Figure 8: Effect of *Azospirillum brasilense* Sp245 on root architecture in *Arabidopsis* mutants in auxin synthesis, transport, and signaling. *Arabidopsis* mutants were transferred to control or supplemented with 2.5×10^5 CFU/mL of *Azospirillum* media and incubated for 9 days. (A) Primary root growth based on the values of the control treatment relative to 100. (B) Lateral root number. (C) Lateral root number based on the values of the control treatment relative to 100. The values shown represent the average of 25 seedlings +/− standard error (n = 3). Different letters above columns indicate significant differences between treatments on any study variable at $P < 0.05$

Next, we analyzed the effect of *Azospirillum* on mutants of other types of auxin efflux carriers such as the PGP/MDR/ABCB proteins (P-glycoprotein/multi-drug resistance/ATP-binding cassette B) [33]. For this, *pgp1*, *pgp4*, and *pgp19* mutant seedlings were supplemented with 2.5×10^5 CFU/mL of *Azospirillum* and incubated for 9 days under the conditions as mentioned above. As can be seen in Fig. 9, in the mutants *pgp1, pgp4* and *pgp19* seedlings, there was a decrease in root length of around 78% and an increase in root branching in lines *pgp4*, and *pgp19* comparable to the Wt line. Interestingly, only the *pgp1* mutant did not show an increase in root branching (Fig. 9B).

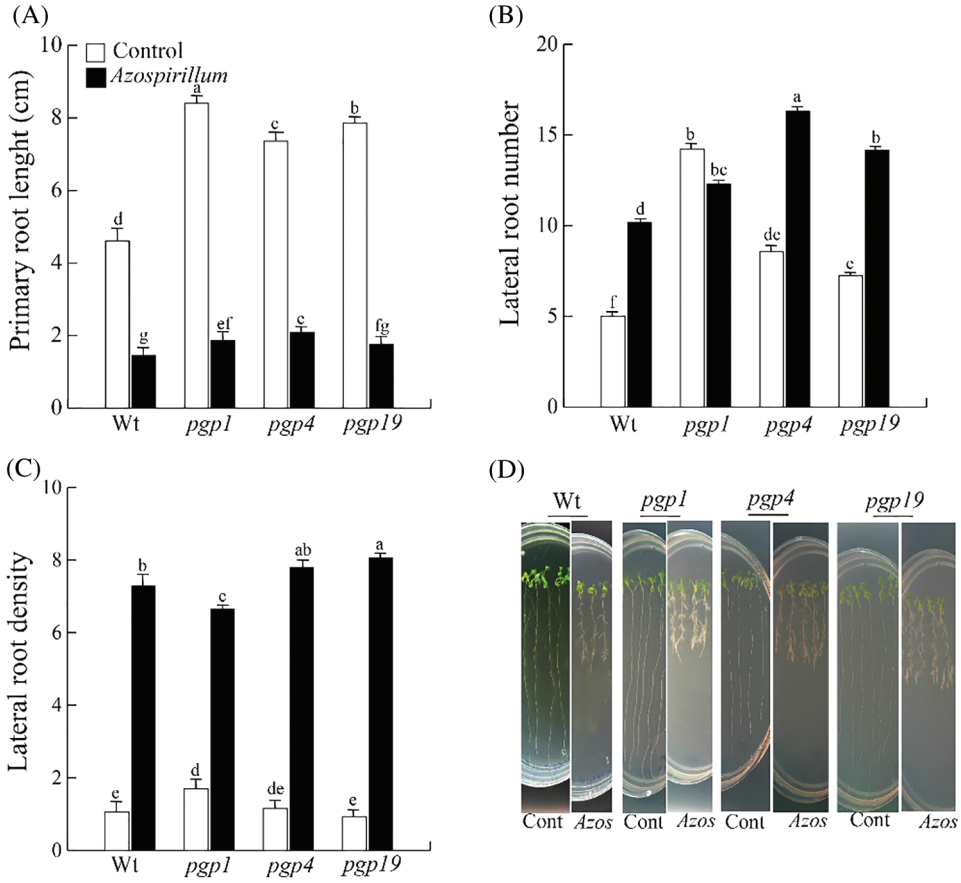

Figure 9: Effect of *Azospirillum brasilense* Sp245 on root architecture of *pgp1, pgp4*, and *pgp19* seedlings. *Arabidopsis* seedlings Wt, *pgp1*, *pgp4*, and *pgp19* were transferred to control or supplemented with 2.5×10^5 CFU/mL of *Azospirillum* media and incubated for 9 days. (A) Primary root length. (B) Lateral root number. (C) Lateral root density. (D) Representative photographs of the seedlings are shown. The values shown represent the average of 25 seedlings +/− standard error (n = 3). Different letters above columns indicate significant differences between treatments on any study variable at $P < 0.05$

Furthermore, the AXR4-1 and BEN proteins have been reported to aid in positioning of AUX/LAX and PIN carriers, respectively [22,23]. The *axr4-1* and *ben2* seedlings exposed to *A. brasilense* showed a decrease in root length equal to Wt, while the bacteria did not stimulate the root branching in *axr4-1* and *ben2* seedlings (Fig. 10). The above results suggest that PGP1, AXR4-1, and BEN2 participate in the PAT regulation, allowing *Azospirillum* to stimulate the root branching in *Arabidopsis* seedlings.

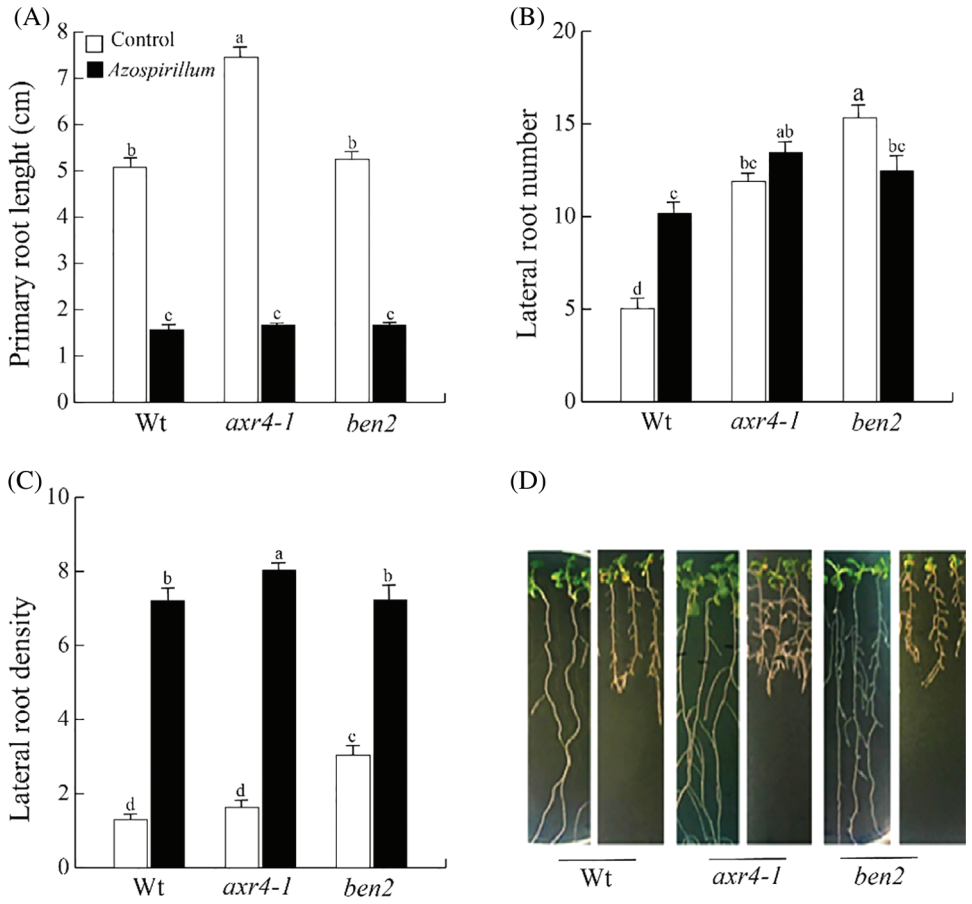

Figure 10: Effect of *Azospirillum brasilense* on root architecture of *axr4-1* and *ben2* seedlings. Mutants seedlings were transferred to control or supplemented with 2.5 × 10^5 CFU/mL of *Azospirillum* media and incubated for 9 days. (A) Primary root length. (B) Lateral root number. (C) Lateral root density. (D) Representative photographs of the seedlings in control medium and inoculated with *A. brasilense*. The values shown represent the average of 25 seedlings +/− standard error (n = 3). Different letters above columns indicate significant differences between treatments on any study variable at $P < 0.05$

3.5 Effect of Azospirillum on Root Architecture of 35S::YUC4 DR5::uidA and yuc2 yuc6 Seedlings

To assess the effect of *A. brasilense* on root architecture of *A. thaliana* seedlings with different levels of endogenous IAA, we evaluate the response of *35S::YUC4 DR5::uidA* line (which overproduces auxins) at 2.5 × 10^5 CFU/mL of *A. brasilense*. In Fig. 11A it can be seen that after three days of exposure, the bacteria arrested root growth in the same way as in Wt seedlings, while the root branching increased by 165% and 110% on the third and sixth day of the interaction (Fig. 11B). In the meantime, the double mutant *yuc2 yuc6* seedlings exposed to *A. brasilense*, showed a reduction in the root length similar to control, and an increase in the root branching of 200% and 600% on the third and sixth days, respectively (Figs. 11A and 11B).

Both lines showed an increase in the root branching; however that effect was more significant in double mutant *yuc2 yuc6* than in the *35S::YUC4* line.

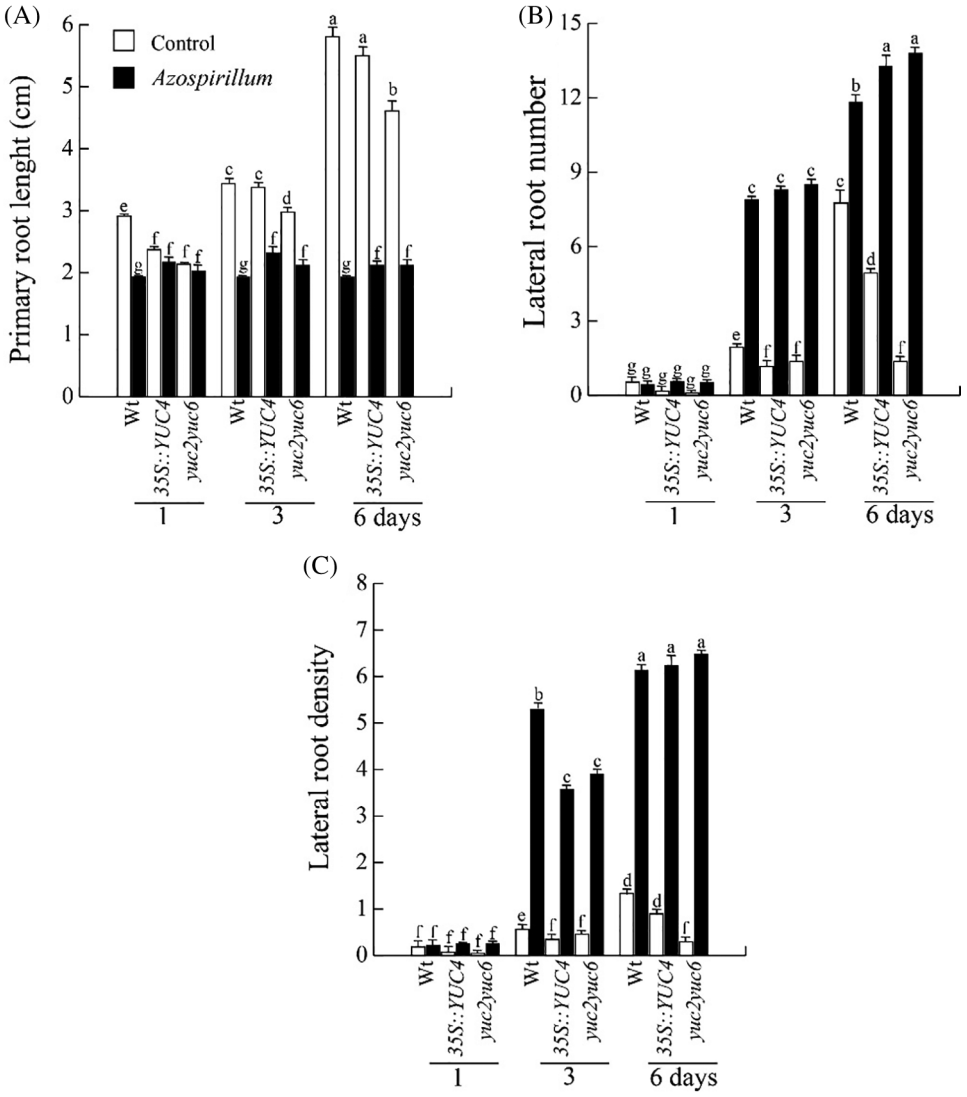

Figure 11: Effect of *Azospirillum* on root architecture of *35S::YUC4 DR5::uidA* and *yuc2 yuc6* seedlings. *35S::YUC4 DR5::uidA* and *yuc2 yuc6* seedlings were transferred to control or supplemented with 2.5×10^5 CFU/mL of *Azospirillum* media and incubated for 1, 3 and 6 days. (A) Primary root length. (B) Lateral root number. (C) Lateral root density. The values shown represent the average of 25 seedlings +/− standard error (n = 3). Different letters above columns indicate significant differences between treatments on any study variable at $P < 0.05$

3.6 Effect of Yucasin on Root Architecture of DR5::uidA Seedlings Exposed to Azospirillum

The five-day-old seedlings were transferred to culture media supplemented with 100 µM of yucasin and 2.5×10^5 CFU/mL. The plants were incubated for one, three, five, and six days of exposure to yucasin and the bacteria; at the end of these times, the radicular architecture parameters were evaluated. The results showed that *A. brasilense* and yucasin together reduce the root length and increase the root branching in the same proportion as the treatment only with the bacteria (Figs. 12A and 12B), which suggests that the effect on radicular architecture of *Arabidopsis* exposed to the bacteria is dependent of bacterial auxins and not because the stimulation of endogenous auxin synthesis. In Fig. 12D, it can be observed that seedlings in

the presence of yucasin are agravitropic, while in those exposed to the bacteria; the agravitropism is lost due to bacterial IAA.

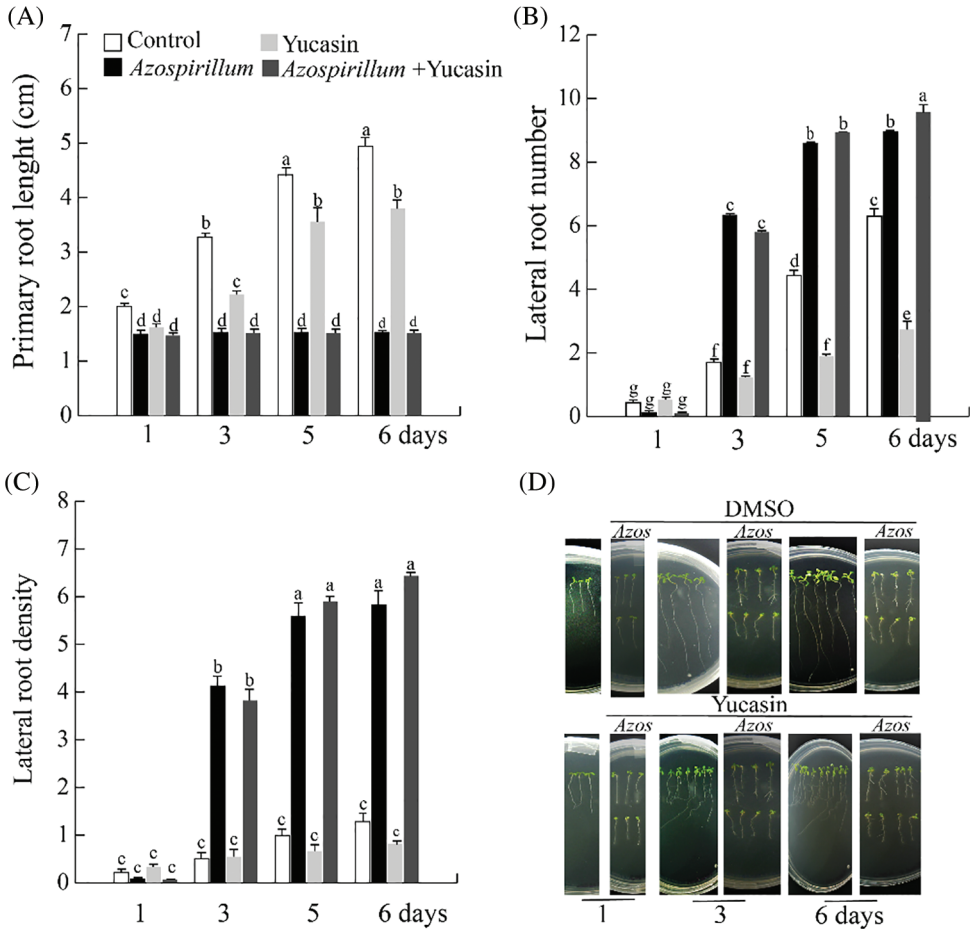

Figure 12: Effect of yucasin on root architecture of *Arabidopsis* Wt seedlings exposed to *Azospirillum*. The seedlings were transferred to control or supplemented with 100 µM yucasin and 2.5 × 10^5 CFU/mL of *Azospirillum* media and incubated for 1, 3, 5 and 6 days. (A) Primary root length. (B) Lateral root number. (C) Lateral root density. (D) Representative photographs of the seedlings exposed for 1, 3 and 6 days to *Azospirillum* and yucasin. The values shown represent the average of 25 seedlings +/− standard error (n = 3). Different letters above columns indicate significant differences between treatments on any study variable at $P < 0.05$

4 Discussion

The first reports of the effect of *Azospirillum* on wheat and *Arabidopsis* showed that the bacteria decreased the length of the root and increased the root branching when the plants interacted with *Azospirillum* for six and seven days [7,8,34–36]. It seemed to us that this time was too long and that the observed effect was the result of changes occurring at shorter times. So, we decided to evaluate what happened at shorter interaction times. Surprisingly, we found dramatic changes on the first day of exposure to the bacteria, such as the arrest of the growth of the primary root, whose length is maintained throughout the experiment (Fig. 1A), and stimulation of maturation of the lateral root primordia (Fig. 4). After three days of exposure of *Arabidopsis* to *Azospirillum*, the observed changes were very noticeable

in an increase of lateral root number (Fig. 1B), the arrest of the cell cycle in the meristem of the primary root (Fig. 2) and disappearance of the elongation zone of primary root (Fig. 3). The aforementioned data clearly indicate that the effect of bacteria on *Arabidopsis* root architecture development begins from early times of exposure.

Upon detailed revision of the literature on the effect of PGPR in different plants, none of these interactions has reported that the arrest of primary root growth is affected by the two events that determine the development of this organ [2,7,37–39], as it happens during interaction with *Azospirillum*. In addition, the bacteria caused a depletion of root meristem of primary root after three days of exposure. Regarding the stimulation of *Azospirillum* on lateral root development, our results show that it begins on the first day when the bacteria stimulated the induction and maturation of lateral root primordia (Fig. 4). It has been widely reported that *Azospirillum brasilense* alters the root architecture of a large number of plants and this effect has been attributed to the auxins it produces [1–3,7,8,34–36]. In the *Azospirillum-Arabidopsis* interaction reported by Spaepen et al. [7], they evaluated the effect of the auxins produced by the bacteria, when inoculating *A. thaliana* with two strains of *A. brasilense* Sp245: one wild-type and the other mutant in auxin biosynthesis (FAJ009). Their results allowed them to suggest that the auxins produced by the bacteria could be involved in the morphological changes of the root architecture. The authors also analyzed gene expression by microarrays of *Arabidopsi*s roots exposed for three and seven days to *Azospirillum*. They observed that the bacterium induced changes in the expression of genes related to defense and the synthesis of cell wall components and that these were more pronounced at seven days of interaction. Regarding the expression of genes related to the auxin response pathway, they only observed an increase in the expression of a family of *GH3* genes, so this analysis did not allow them to propose a mechanism by which auxins could be involved in the changes in the root system. Our results on the auxin levels in *Arabidopsis* seedlings exposed to *Azospirillum* indicate that auxins begin to increase after three days of exposure in primary root, specifically in the differentiation zone of the lateral roots, reaching a maximum in the same zone one day later. Subsequently, auxins showed to be distributed to meristems of the lateral roots and from the fifth day on, auxins disappeared from the root meristem of the primary root (Fig. 5).

Since auxins appear to be involved in the effect of *Azospirillum* on root architecture development, we decided to investigate what elements of the auxin response pathway might be participating in the outcome. For this, we analyzed the effect of the inoculation of the bacteria on the root architecture of several mutant lines of this pathway such as: *yuc2 yuc6, aux1-7, pin1, pin2 and pin3, axr1-3, slr1*, and *arf7 arf19* mutants, affected in the genes that code for proteins that participate in the synthesis, transport, repressors and transcription factors of auxin response pathway [10]. However, we did not observe differences in the parameters of the root architecture of the mutants with respect to the control (Fig. 8). This was unexpected, especially in the auxin carrier mutants, since our results with the *DR5::uidA* line indicated that auxins increased in the vascular bundle at short exposure times and were eventually mobilized to the meristems of lateral roots (Fig. 5). To directly analyze the participation of PAT during the *Azospirillum-Arabidopsis* interaction, we used the PAT inhibitor, NPA [40]. On the other hand, flavonoids are known to affect expression, location of PIN efflux carriers [41–43] and the PIN transport cycle in endosomal vesicles [44]. Furthermore, flavonoids also modify the activity of ABCB-like auxins transporters and modulate the root gravitropism [33]. Thus, in the present study, was analyzed the effect of *A. brasilense* on root architecture of *Arabidopsis tt4* mutant seedlings with decreased levels of flavonoids [27]. The results of the experiments with NPA (Fig. 6) and with the *tt4* mutant (Fig. 7) strongly suggested that PAT was one of the factors necessary to prompt *Azospirillum*-induced changes in root architecture. So, we decided to test the *pgp1, pgp4*, and *pgp9* mutant lines of other auxin efflux carriers, and in this way, we were able to determine that the PGP1 carrier seemed to be involved in this effect (Fig. 9B).

It seems relevant to highlight that an atypical efflux carrier is involved in the mobilization of bacterial auxins, such as PGP1 (Fig. 9) and the AXR4-1 and BEN proteins (Fig. 10) that help the correct positioning of

the AUX/LAX and PIN carriers respectively, and not the classic transporters or some other element of the auxin response pathway as it occurs in most interactions with other PGPR [37–39]. We think that perhaps, the root system, faced with an excess of bacterial auxins in the root, does not respond through the usual channels and makes use of other elements that participate in PAT. Therefore, we decided to investigate what happens during the *Arabidopsis-Azospirillum* interaction, in *35S::YUC4 s*eedlings that have an excess and a double mutant *yuc2 yuc6* with a deficiency of endogenous auxins. The results in both lines showed an increase of root lateral (Fig. 11), being the effect greater in *yuc2 yuc6* than in *35S::YUC4*, which suggests that a plant with excess endogenous auxins has developed a molecular system that allows it to regulate an auxins excess.

The IAA is involved in all processes that regulate plant development. The tryptophan-dependent indolpyruvic acid (IPA) pathway is the main pathway by which *A. thaliana* synthesizes IAA [45]. In this pathway, tryptophan is converted to IPA by Trp aminotransferase, which, when it is oxidized by yucca becomes IAA. Yucasin is an inhibitor of yucca enzymes [45], so we analyzed the effect of yucasin on the root architecture of *A. thaliana* seedlings exposed to *A. brasilense*. Our results, suggests that the effect on the radicular architecture of *Arabidopsis* exposed to the bacteria dependent on bacterial auxins and not on the stimulation of endogenous auxins synthesis.

5 Conclusion

Novel evidence was gathered that bacterium *A. brasilense* increases internal auxin levels, changes the development root system and promotes the initiation and maturation of the lateral root primordia of *A. thaliana*.

Author Contributions: E. B.-P. and M. E. M.-R. conceived and designed the experiments. E. C.-F., J. A.-R., D. M. P.-S., M. F.-R. and G. F.-R. conducted experiments. E. B.-P. collected and analyzed the date. E. B.-P., M. E. M.-R. and G. F.-R. wrote the manuscript.

Funding Statement: This work was supported by the Coordinación de la Investigación Científica UMSNH. E. C.-F. and J. A.-R. were fellows of CONACYT-México.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

References

1. Dobbelaere, S., Vanderleyden, J., Okon, Y. (2003). Plant growth-promoting effects of diazotrophs in the rhizosphere. *Journal Critical Reviews in Plant Sciences, 22(2),* 107–149. DOI 10.1080/713610853.
2. Fibach-Paldi, S., Burdman, S., Okon, Y. (2012). Key physiological properties contributing to rhizosphere adaptation and plant growth promotion abilities of *Azospirillum brasilense*. *FEMS Microbiology Letters, 326(2),* 99–108. DOI 10.1111/j.1574-6968.2011.02407.x.
3. Verbon, E. H., Liberman, L. M. (2016). Beneficial microbes affect endogenous mechanisms controlling root development. *Trends in Plant Science, 21(3),* 218–229. DOI 10.1016/j.tplants.2016.01.013.
4. Méndez-Gómez, M., Castro-Mercado, E., Peña-Uribe, C. A., Reyes-de la Cruz, H., López-Bucio, J. et al. (2020). *Azospirillum brasilense* Sp245 lipopolysaccharides induce target of rapamycin signaling and growth in *Arabidopsis thaliana*. *Journal of Plant Physiology, 253(6),* 153270. DOI 10.1016/j.jplph.2020.153270.
5. Bashan, Y., de-Bashan, L. E. (2010). How the plant growth-promoting bacterium *Azospirillum* promotes plant growth-a critical assessment. In: Sparks, D. L. (Ed.), *Advances in agronomy*, pp. 2–315. USA: Elsevier Inc.
6. Barrada, A., Montané, M. H., Robaglia, C., Menand, B. (2015). Spatial regulation of root growth: Placing the plant TOR pathway in a developmental perspective. *International Journal of Molecular Science, 16(8),* 19671–19697. DOI 10.3390/ijms160819671.

7. Spaepen, S., Bossuyt, S., Engelen, K., Marchal, K., Vanderleyden, J. et al. (2014). Phenotypical and molecular responses of *Arabidopsis thaliana* roots as a result of inoculation with the auxin-producing bacterium *Azospirillum brasilense*. *New Phytologist, 201(3),* 850–861. DOI 10.1111/nph.12590.
8. Méndez-Gómez, M., Castro-Mercado, E., Peña-Uribe, C. A., Reyes-de la Cruz, H., López-Bucio, J. et al. (2020). TARGET OF RAPAMYCIN signaling plays a role in *Arabidopsis* growth by *Azospirillum brasilense* Sp245. *Plant Science, 293,* 110416. DOI 10.1016/j.plantsci.2020.110416.
9. Yoon, E. K., Kim, I. W., Yang, J. H., Kim, S. H., Lim, J. et al. (2014). A molecular framework for the differential response of primary and lateral roots to auxin in *Arabidopsis thaliana*. *Journal of Plant Biology, 57(5),* 274–281. DOI 10.1007/s12374-013-0239-7.
10. Vanneste, S., Friml, J. (2009). Auxin: A trigger for change in plant development. *Plant Cell, 136(6),* 1005–1016. DOI 10.1016/j.cell.2009.03.001.
11. Ulmasov, T., Murfett, J., Hagen, G., Guilfoyle, T. J. (1997). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell, 9(11),* 1963–1971. DOI 10.1105/tpc.9.11.1963.
12. Colón-Carmona, A., You, R., Haimovitch-Gal, T., Doerner, P. (1999). Technical advance: Spatio-temporal analysis of mitotic activity with a labile cyclin-GUS fusion protein. *The Plant Journal, 20(4),* 503–508. DOI 10.1046/j.1365-313x.19999.00620.x.
13. Cho, H. T., Cosgrove, D. J. (2002). Regulation of root hair initiation and expansin gene expression in *Arabidopsis*. *Plant Cell, 14(12),* 3237–3253. DOI 10.1105/tpc.006437.
14. Cheng, Y., Dai, X., Zhao, Y. (2006). Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissue in *Arabidopsis*. *Genes and Development, 20(13),* 1790–1799. DOI 10.1101/gad.1415106.
15. Pickett, F. B., Wilson, A. K., Estelle, M. (1990). The *aux1* mutation of *Arabidopsis* confers both auxin and ethylene resistance. *Plant Physiology, 94(3),* 1462–1466. DOI 10.1104/pp.94.3.1462.
16. Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D. et al. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell, 115(5),* 591–602. DOI 10.1016/s0092-8674(03)00924-3.
17. Xu, W., Jia, L., Baluška, F., Ding, G., Shi, W. et al. (2005). PIN2 is required for the adaptation of *Arabidopsis* roots to alkaline stress by modulating proton secretion. *Journal of Experimental Botany, 63(17),* 6105–6114. DOI 10.1093/jxb/ers259.
18. Friml, J., Wiśniewska, J., Benková, E., Mendgen, K., Palme, K. (2002). Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. *Nature, 415(6873),* 806–809. DOI 10.1038/415806a.
19. Geisler, M., Blakeslee, J. J., Bouchard, R., Lee, O. R., Vincenzetti, V. et al. (2005). Cellular efflux of auxin catalyzed by the *Arabidopsis* MDR/PGP transporter AtPGP1. *The Plant Journal, 44(2),* 179–194. DOI 10.1111/j.1365-313X.2005.02519.x.
20. Terasaka, K., Blakeslee, J. J., Titapiwatanakun, B., Peer, W. A., Bandyopadhyay, B. et al. (2005). PGP4, an ATP binding cassette P-glycoprotein, catalyzes auxin transport in *Arabidopsis thaliana* roots. *Plant Cell, 17(11),* 2922–2939. DOI 10.1105/tpc.105.035816.
21. Noh, B., Bandyopadhyay, A., Peer, W. A., Spalding, E. P., Murphy, A. S. (2003). Enhanced gravi- and phototropism in plant *mdr* mutants mislocalizing the auxin efflux protein PIN1. *Nature, 423(6943),* 999–1002. DOI 10.1038/nature01716.
22. Hobbie, L., Estelle, M. (1995). The *axr4* auxin-resistant mutants of *Arabidopsis thaliana* define a gene important for root gravitropism and lateral root initiation. *The Plant Journal, 7(2),* 211–220. DOI 10.1046/j.1365-313x-1995.7020211.x.
23. Tanaka, H., Kitakura, S., Rakosová, H., Uemura, T., Feraru, M. I. et al. (2013). Cell polarity and pattering by PIN trafficking through early endosomal compartments in *Arabidopsis thaliana*. *PLoS Genetics, 9(5),* e1003540. DOI 10.1371/journal.pgen.1003540.
24. Lincoln, C., Britton, J. H., Estelle, M. (1990). Growth and development of the *axr1* mutants of *Arabidopsis*. *Plant Cell, 2(11),* 1071–1080. DOI 10.1105/tpc.2.11.1071.

25. Fukaki, H., Tameda, S., Masuda, H., Tasaka, M. (2009). Lateral root formation is blocked by a gain-of-function mutation in the *SOLITARY-ROOT/IAA14* gene of *Arabidopsis*. *The Plant Journal, 29(2),* 153–168. DOI 10.1046/j.0960-7412.2001.01201.x.
26. Okushima, Y., Fukaki, H., Onoda, M., Theologis, A., Tasaka, M. (2007). ARF7 and ARF19 regulate lateral root formation via direct activation of *LBD/ASL* genes in *Arabidopsis*. *Plant Cell, 19(1),* 118–130. DOI 10.1105/tpc.106.047761.
27. Burbulis, I. E., Iacobucci, M., Shirley, B. W. (1996). A null mutation in the first enzyme of flavonoid biosynthesis does not affect male fertility in *Arabidopsis*. *Plant Cell, 8(6),* 1013–1025. DOI 10.1105/tpc.8.6.1013.
28. Munguía-Rodríguez, A. G., López-Bucio, J., Ruiz-Herrera, L. F., Ortiz-Castro, R., Guevara-García, A. A. et al. (2020). *YUCCA4* overexpression modulates auxin biosynthesis and transport and influence plant growth and development via cross with abscisic acid in in *Arabidopsis thaliana*. *Genetics and Molecular Biology, 43(1),* e20190221. DOI 10.1590/1678-4685-gmb-2019-0221.
29. Kellermeier, F., Amtmann, A. (2013). Phenotyping jasmonate regulation of root growth. In: Goossen, A., Pauwels, L. (Eds.), *Jasmonate signaling, methods in molecular biology*, pp. 25–32. New York, USA: Springer Science Business Media.
30. Jefferson, R. A., Kavanagh, T. A., Bevan, M. W. (1987). GUS fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal, 6(13),* 3901–3907. DOI 10.1002/j.1460-2075.1987.tb02730.x.
31. López-Bucio, J., Hernández-Abreu, E., Sánchez-Calderon, L., Nieto-Jacobo, M. F., Simpson, J. et al. (2002). Phosphate availability alters architecture and cause changes in hormone sensitivity in *Arabidopsis* root system. *Plant Physiology, 19(1),* 244–256. DOI 10.1104/pp.010934.
32. Malamy, J. E., Benfey, P. N. (1997). Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. *Development, 124(1),* 33–36. DOI 10.1146/annurev.cellbio.13.1.779.
33. Bailly, A., Sovero, V., Vincenzetti, V., Santelia, D., Bartnik, D. et al. (2008). Modulation of P-glycoproteins by auxin transport inhibitors is mediated by interaction with immunophilins. *Journal of Biological Chemistry, 283(31),* 21817–21826. DOI 10.1074/jbc.M709655200.
34. Spaepen, S., Vanderleyden, J., Okon, Y. (2009). Plant growth-promoting actions of rhizobacteria. *Advances in Botanical Research, 51,* 283–320. DOI 10.1016/s0065-2296(09)51007-5.
35. Méndez-Gómez, M., Barrera-Ortiz, S., Castro-Mercado, E., López-Bucio, J., García-Pineda, E. et al. (2021). The nature of the interaction *Azospirillum-Arabidopsis* determines the molecular and morphological change in root and plant growth promotion. *Protoplasma, 258(1),* 179–189. DOI 10.1007/s00709-020-01552-7.
36. Dobbelaere, S., Croonenborghs, A., Thys, A., Broek, A. V., Vanderleyden, J. (1999). Phytostimulatory effect of *Azospirillum brasilense* wild type and mutant strains altered in IAA production on wheat. *Plant and Soil, 212(2),* 153–162. DOI 10.1023/A:1004658000815.
37. Contesto, C., Milesi, S., Mantelin, S., Zancarini, A., Desbrosses, G. et al. (2010). The auxin-signaling pathway is required for the lateral root response of *Arabidopsis* to the rhizobacterium *Phyllobacterium brassicacearum*. *Planta, 232(6),* 1455–1470. DOI 10.1007/s00425-010-1264-0.
38. Chamam, A., Sanguin, H., Bellvert, F., Meiffren, G., Comte, G. et al. (2013). Plant secondary metabolite profiling evidences strain-dependent effect in the *Azospirillum-Oryza sativa* association. *Phytochemistry, 87(12),* 65–77. DOI 10.1016/j.phytochem.2012.11.009.
39. Sukumar, P., Legue, V., Vayssieres, A., Martin, F., Tuskan, G. A. et al. (2013). Involvement of auxin pathways in modulating root architecture during beneficial plant-microorganism interactions. *Plant Cell Environment, 36(5),* 909–919. DOI 10.1111/pce.12036.
40. Nagashima, A., Uehara, Y., Sakai, T. (2008). The ABC subfamily B auxin transporter AtABCB19 is involved in the inhibitory effects of N-1-naphthyphthalamic acid on the phototropic and gravitropic responses of *Arabidopsis* hypocotyls. *Plant and Cell Physiology, 49(8),* 1250–1255. DOI 10.1093/pcp/pcn092.
41. Brown, D. E., Rashotte, A. M., Murphy, A. S., Normanly, J., Taque, B. W. et al. (2001). Flavonoids act as negative regulators of auxin transport *in vivo* in *Arabidopsis*. *Plant Physiology, 126(2),* 524–535. DOI 10.1104/pp.126.2.524.

42. Peer, W. A., Bandyopadhyay, A., Blakeslee, J. J., Makam, S. N., Chen, R. J. et al. (2004). Variation in expression and protein localization of the PIN family of auxin efflux facilitator proteins in flavonoid mutants with altered auxin transport in *Arabidopsis thaliana*. *Plant Cell, 16(7),* 1898–1911. DOI 10.1105/tpc.021501.
43. Santelia, D., Henrichs, S., Vincenzetti, V., Sauer, M., Bigler, L. et al. (2008). Flavonoids redirect PIN-mediated polar auxin fluxes during root gravitropic responses. *Journal of Biological Chemistry, 283(45),* 31218–31226. DOI 10.1074/jbc.M710122200.
44. Geldner, N., Friml, J., Stierhof, Y. D., Jürgens, G., Palme, K. (2001). Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature, 413(6854),* 425–428. DOI 10.1038/35096571.
45. Nishimura, T., Hayashi, K., Suzuki, H., Gyohda, A., Takaoka, C. et al. (2013). Yucasin is a potent inhibitor of YUCCA, a key enzyme in auxin biosynthesis. *The Plant Journal, 77(3),* 352–366. DOI 10.1111/tpj.12399.