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PII: S0981-9428(15)30049-8

DOI: 10.1016/j.plaphy.2015.07.003

Reference: PLAPHY 4221

To appear in: *Plant Physiology and Biochemistry*

Received Date: 28 March 2015

Revised Date: 24 June 2015

Accepted Date: 1 July 2015

Please cite this article as: F.B. Salazar-Badillo, D. Sánchez-Rangel, A. Becerra-Flora, M. López-Gómez, F. Nieto-Jacobo, A. Mendoza-Mendoza, J.F. Jiménez-Bremont, *Arabidopsis thaliana* polyamine content is modified by the interaction with different Trichoderma species, *Plant Physiology et Biochemistry* (2015), doi: 10.1016/j.plaphy.2015.07.003.

Arabidopsis thaliana polyamine content is modified by the interaction with different Trichoderma species

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Abstract

Plants are associated with a wide range of microorganisms throughout their life cycle, and some interactions result on plant benefits. Trichoderma species are plant beneficial fungi that enhance plant growth and development, contribute to plant nutrition and induce defense responses. Nevertheless, the molecules involved in these beneficial effects still need to be identify. Polyamines are ubiquitous molecules implicated in plant growth and development, and in the establishment of plant microbe interactions. In this study, we assessed the polyamine profile in Arabidopsis plants during the interaction with *Trichoderma virens* and *Trichoderma atroviride*, using a system that allows direct plant-fungal contact or avoids their physical interaction (split system). The plantlets that grew in the split system exhibited higher biomass than the ones in direct contact with Trichoderma species. After 3 days of interaction, a significant decrease in Arabidopsis polyamine levels was observed in both systems (direct contact and split). After 5 days of interaction polyamine levels were increased. The highest levels were observed with *Trichoderma atroviride* (split system), and with *Trichoderma virens* (direct contact). The expression levels of Arabidopsis *ADC1* and *ADC2* genes during the interaction with the fungi were also assessed. We observed a time dependent regulation of *ADC1* and *ADC2* genes, which correlates with polyamine levels. Our data show an evident change in polyamine profile during Arabidopsis – Trichoderma interaction, accompanied by evident alterations in plant root architecture. Polyamines could be involved in the changes undergone by plant during the interaction with this beneficial fungus.

Keywords: *Arabidopsis thaliana*; plant growth promotion; polyamines; split growth system; *Trichoderma atroviride*; *Trichoderma virens*.

Abbreviations:

PAs, polyamines; Put, putrescine; Spd, spermidine; Spm, spermine; Tspm, thermospermine; ADC, arginine decarboxylase; ODC, ornithine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase; SAM, S-adenosylmethionine; SAMDC, S-adenosylmethionine decarboxylase; PDA, potato dextrose agar; Tv, *Trichoderma virens*; Ta, *Trichoderma atroviride*; At, *Arabidopsis thaliana*; dpi, days post inoculation; PCA, perchloric acid; HTD, 1,7-diamino heptane; At-Ta direct contact interaction between Arabidopsis - *T. atroviride*; At-Tv direct contact interaction between Arabidopsis - *T. virens*; At/Ta without contact interaction between Arabidopsis - *T. atroviride*; At/Tv without contact interaction between Arabidopsis - *T. virens*; VOCs, volatile organic compounds.

1. Introduction

Trichoderma spp. are free-living filamentous fungi that are classified as beneficial microorganisms, and are widely distributed in soil and in a variety of ecosystems. Beneficial effects of *Trichoderma* species have been reported on several crop plants, such as tomato, cucumber, chilli pepper, corn, cactus pear, among others (Delgado-Sánchez et al. 2010; Harman et al. 2004; Mastouri et al. 2010; Yedidia et al. 2001). Among the beneficial effects of *Trichoderma* on plants are enhancement of seed germination, modulation of seed dormancy break (Delgado-Sánchez et al. 2010; 2011, 2013), plant growth promotion, increases in nutrient uptake, and improvement in biotic and abiotic stress tolerance (Contreras-Cornejo et al. 2014 a, b; Harman et al. 2004; Hermosa et al. 2012; Saenz-Mata et al. 2014;). Trichoderma species also stimulate plant defenses against pathogenic microorganisms (Brotman et al. 2013; Saenz-Mata and Jiménez-Bremont 2012, Saenz-Mata et al. 2014; Salas-Marina et al. 2015). Several *Trichoderma* species have a high impact on agriculture (Mukherjee et al. 2013), wherein *Trichoderma atroviride* and *T. virens* species are the best studied in plant interaction.

Some mechanisms by which *Trichoderma* influences plant growth and development have been proposed, which include the increment in nutrient availability (Kleifeld and Chet, 1992), the solubilization of soil nutrients (Altomare et al. 1999, Yadav et al. 2009; Yedidia et al. 2001), modification of root morphology by increasing root branching, which contributes to water and nutrient uptake (Contreras-Cornejo et al. 2014 b; Samolski et al.

2012), synthesis or stimulation of phytohormones production (Limon et al. 2004; Contreras-Cornejo et al. 2009; Gravel et al. 2007; Roco and Perez 2001; Windham et al. 1986), and volatile organic compounds production (Hung et al., 2013).

PAs are newly discovered like plant growth regulators that have been implicated in several plant growth and developmental processes, besides plant polyamines (PAs) metabolism undergoes remarkable changes during plant – microbe interactions (Jiménez-Bremont et al. 2014). These include stimulation of cell division, germination, regulation of rhizogenesis, embryogenesis, floral development, pollination, development and ripening of fruit, lignification, senescence, and abiotic and biotic stresses resistance (Alcazar et al. 2010; Gill and Tuteja, 2010; Groppa et al. 2008; Imai et al. 2004; Kakkar et al.2000; Kusano et al. 2008; Mattoo et al. 2010; Minocha et al. 2014; Ortega-Amaro et al. 2012). In plants, putrescine (Put), spermidine (Spd) and spermine (Spm) are the most abundant PAs. Put is formed by decarboxylation of arginine and ornithine, a reaction catalyzed by arginine decarboxylase (ADC; EC 4.1.1.19) or ornithine decarboxylase (ODC; EC 4.1.1.17), respectively. The addition of two successive aminopropyl groups to Put in two reactions catalyzed by spermidine synthase (SPDS; EC 2.5.1.16) and spermine synthase (SPMS; EC 2.5.1.22), leads in that order to the formation of Spd and Spm. Aminopropyl groups are formed through decarboxylation of S-adenosylmethionine (SAM) by the S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) (Kusano et al. 2008). An additional tetraamine, thermospermine (Tspm), can also be synthesized through the activity of an alternative aminopropyltransferase, named ACAULIS5 (ACL5), which is present only in plants and several prokaryotic genera (Minguet et al., 2008; Rodriguez-Kessler et al., 2010).

Most of the molecular mechanisms by which PAs exert their biological function remain unclear. Because PAs are polycations, their biological function was associated only with their structural abilities to bind to anionic macromolecules. However, increasing evidence indicates that PAs function as signal molecules and are key players in regulatory mechanisms at the transcriptional and translational levels, also by epigenetic regulation (Fuell et al. 2010; Hanfrey et al. 2002). Moreover, studies indicate that PA signaling involves crosstalk with other pathways such as redox, nitric oxide and hormonal signaling (Takahashi and Kakehi, 2010; Tiburcio et al. 2014).

In order to elucidate whether PAs metabolism plays a role during Arabidopsis - *Trichoderma* interaction, we compared two types of interaction: one involving direct plant-fungal contact, and the other using a split growth system, which avoids the physical interaction between the plant and the fungus. For this purpose, two Trichoderma species: *T. virens* and *T. atroviride* were used, and biomass production, root architecture, and PA levels of Arabidopsis plantlets at 3 and 5 days post-inoculation (dpi) were tested. In addition, the expression levels of Arabidopsis *ADC1* and *ADC2* genes during the interaction with the fungi were analyzed. We observed that plantlets cultivated without physical contact with the fungus showed an increase in the growth and development at 3 and 5 days of interaction. Our data show changes in Arabidopsis PA contents, which are dependent on time and type of interaction, and Trichoderma species, suggesting that polyamine metabolism could be involved during this beneficial association.

2. Materials and methods

2.1 Plant material and growth conditions

Seeds of *Arabidopsis thaliana* ecotype Col-0 were surface sterilized for 10 min with 40% (v/v) chlorine solution and rinsed six times in sterile distilled water. Aseptic seeds were sown on Petri dishes (150 x 15 mm) containing 0.2x MS medium [0.2X MS salts (Phytotechnology), 1% (w/v) agar, 0.75% (w/v) sucrose (pH 7.0)] and placed at 4 °C for 2 days for vernalization. Plates were incubated at 22 ± 1 °C in a 16-h-light/8-h-dark cycle for 7 days. After, plantlets were transferred to the left side of split Petri dishes (90 x 15 mm) containing 0.2X MS medium. Thirteen days-old plantlets were inoculated with Trichoderma *spp*. spores as described below.

2.2 Fungal growth and inoculum preparation

The fungal strains used in this work were: *Trichoderma virens* (Gv29.8; Tv) and *T. atroviride* (IMI 206040; Ta). Each fungus was grown on potato dextrose agar (PDA) plates for 8 days at 28 °C. Fungal conidia were collected in 15 mL of sterile distilled water at room temperature. Total conidia were counted on Neubauer chamber in microscope at 40x magnification. Fungal spore suspension was adjusted to 1×10^6 spores per mL and used as inoculum.

2.3 Arabidopsis plants inoculation with *Trichoderma atroviride* and *Trichoderma virens*

Thirteen days-old Arabidopsis plantlets (nine plates with 3 plantlets per plate) were placed in the left side of the split Petri dish. For experiment three conditions were used: 1) control

plants without inoculum, 2) plantlets were inoculated directly at the bottom of the left side of plates (At-Ta and At-Tv), and 3) plantlets were inoculated at distance, it means in the right side of the split plate (At/Ta and At/Tv) with a suspension of 1 x 10^6 spores/mL. After 3-days and 5-days post inoculation (dpi) the plant material was collected. Estimation of primary root length (cm), emerged lateral roots [stage of development as reported by Malamy and Benfey (1997)] and lateral root density was determined by measuring 27 plantlets. The fresh weight (mg) of the seedlings was obtained on an analytical scale and the values obtained represent the means of nine groups of three seedlings of each treatment.

2.4 Free-polyamine extraction

Free polyamines were estimated as dansyl-derivatives, by reversed phase HPLC (Marcé et al. 1995), in Arabidopsis plantlets grown in several experimental conditions (Control, At-Ta, At-Tv, At/Ta and At/Tv). It was used 0.5 g of the plant material for extraction. Extraction was done with 0.8 mL 5% (v/v) perchloric acid (PCA) and then incubated overnight at 4º C. After centrifugation, 0.2 mL of the supernatants were dansylated in a mixture containing 0.1 mL of saturated Na_2CO_3, 0.2 mL dansylchloride (5 mg/mL acetone), and 1 mM 1,7-diamino heptane (HTD) as internal standard. The mixture was incubated overnight in darkness at room temperature. Reaction was stop by adding 0.1 mL proline (100mg/mL) and dansylated PAs were extracted with 0.4 mL toluene. Organic phase was vacuum-evaporated and dansylated polyamines were dissolved in 0.05 mL acetonitrile. This method does not distinguish between Spm and TSpm, so both tetraamines are identified simultaneously.

2.5 Chromatographic analysis of PAs

Polyamines were analyzed by HPLC using a 4.6×150 mm C18 reverse phase column. Column flow was 1.5 mL min^{-1}, and the elution gradient was prepared with eluent A (water) and eluent B (acetonitrile). The column was equilibrated with 70% B and 30% A before injecting 0.01 mL samples. This was followed by a linear gradient ending with 100% B at 9 min. The final step was held for 4 min before regenerating the column. Detection was done with a fluorimeter using excitation and emission wavelengths of 415 and 510 nm, respectively, according to Flores and Galston (1982). A relative calibration procedure was used to determine the polyamines in the samples, using 1,7-diaminoheptane as the internal standard and polyamine concentrations ranging from 0.3 to 1.5 nmol from Sigma-Aldrich Mexico. Results were expressed as nanomoles per gram (nmol/g) of fresh weight.

2.6 RNA extraction and real-time RT-PCR

Total RNA was extracted from inoculated and non-inoculated whole plantlets using the Concert TM Plant RNA reagent following the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA). ADC genes expression levels were estimated by qRT-PCR as described below using the following primers: ADC1-F, ADC1-R, ADC2-F and ADC2-R (Table 1). Real-time PCR was performed in 10µL of reaction mixture made up of 5 µL of Power SYBR Green RT-PCR Mix (2x), 200 ng of each oligonucleotide, 50 ng of RNA template and 0.08µL of RT Enzyme Mix (125X) for one-step RT-PCR, using an StepOne Real-Time PCR Detection System and StepOne Software v2.1 (Applied Biosystems, Carlsbad, CA, USA). The thermal cycling conditions consisted of 30 min at

48°C (cDNA synthesis), 10 min at 95°C (activation of AmpliTaq Gold® DNA polymerase), followed by 40 PCR cycles of 15 s at 95°C (denature) and 1 min at 60°C (anneal/extend). Melting curves were performed by cycles of 15 s at 95ºC (denature), 15 at 60ºC (anneal) and 15 s at 95ºC (denature), increasing the temperature each 0.3ºC. The cycle number at threshold (Ct value) was used for calculations of relative mRNA expression levels. The Ct value of each target gene was normalized by subtraction of the Ct value from the Arabidopsis ubiquitin 5 (At3g62250) gene. The fold change in gene expression relative to control samples (Col-0) was calculated using the 2-ΔΔCt method (Livak and Schmittgen, 2001). Asterisks denote significant differences between control and plants on interaction.

2.7 Statistical analysis

The data were analyzed by using One-Way ANOVA in Graph-Pad version 5.0 software. The significance of differences between values was determined with multiple comparison Tukey´s test. A *P* value≤0.05 (five percent change) was considered on the borderline of statistical significance. The data are presented as the mean ± standard error.

3. Results

3.1 Phenotype in Arabidopsis plants during the interaction with *Trichoderma atroviride* and *Trichoderma virens*

In order to assess whether the plant-growth promoting activity of two Trichoderma species depends on the physical contact between the plant and the fungus, two different experimental conditions, direct contact (At-T) or without contact (At/T), were tested. Interaction experiments were carried out in 13-days-old *A. thaliana* Col-0 plantlets that were grown with each fungal strain, *T. atroviride* (IMI206040; Ta) or *T. virens* (GV 28.9; Tv), for 3 or 5 days.

We quantified biomass production and parameters of root architecture such as primary root length, number of emerged lateral roots, and lateral root density at 3 or 5 days post-inoculation (dpi). At 3 dpi it was clearly observed that the plantlets incubated without direct fungus contact (At/Ta; At/Tv), regardless of the species used, showed a major increase on growth and development in comparison with control plantlets and with plants grown in direct contact with the fungus, At-Ta o At-Tv, (Fig. 1). Interesting differences in the magnitude of fresh weight between the two species were observed, since *T. virens* provoked a 66%, and *T. atroviride* a 47% increase of total fresh weight, in comparison to control non inoculated plants (Fig. 2A). At 3 dpi, we also observed a significant decrease on primary root length of about 20% in the plantlets incubated in direct contact with *T. virens* or *T. atroviride*. Interestingly, no inhibition was observed in plantlets cultivated without physical fungus contact (Fig. 2B). As already described, *T. virens* induced in

Arabidopsis plantlets the major increase of fresh weight when it was inoculated on the opposite side of plates (Fig. 2A), however, no increase was observed in either the number or the lateral roots and density (Fig 2C and 2D). On the other hand, *T. atroviride* triggered a gain of about 50% in both parameters. Finally, the plantlets during physical contact with *T. virens* or *T. atroviride* showed an increase (50%) on lateral root density, but this effect is likely due the inhibition of primary root growth (Fig. 2).

In order to characterize whether the physiological changes in Arabidopsis plantlets observed at 3 dpi are potentiated at later times, we analyzed the same parameters at 5 dpi. The phenotype recorded at 5 dpi showed that growth promotion remained only in the distance condition for both species and even this effect was enhanced (Fig. 3), since an increase around 125% in the fresh weight was observed (Fig. 4A). Furthermore, the growth inhibition of the primary root length remained in plantlets during direct contact with the fungus independently of the species used. In contrast, an increase of 8% was observed in the primary root length when the plantlets were cultivated on opposite side of plates with *T. atroviride* (Fig.4B). Additionally, a 30% increase in number and density of lateral roots in comparison with data obtained at 3 dpi were observed. Intriguingly, unlike the data obtained at 3 dpi, the plantlets incubated with *T. virens* with no physical contact at 5 dpi showed an induction of 70% in both radicular parameters (Fig. 4C and 4D).

3.2 The polyamine levels of Arabidopsis plantlets change differentially during interaction with Trichoderma *spp.*

PAs have been recognized as key molecules in essential physiological processes such as plant growth and in plant-microbe interactions. For that reason, we were interested in

elucidate whether PA metabolism plays a role during *Arabidopsis - Trichoderma spp.* interaction. The free levels, of the main polyamines in plants, Put, Spd, and tetraamines (Spm + Tspm) were quantified by HPLC. The PAs profile in 16-days old Arabidopsis plantlets (control plants) showed that Spd is the most abundant PA (» 400 nm/g fresh tissue), followed by Spm + Tspm (» 300 nm/g fresh tissue) and the less abundant was Put (» 200 nm/g fresh tissue) (Fig. 5A). At 3 dpi, a significant decrease in PAs levels were recorded in all interactions regardless of the species or experimental conditions tested; however, slight differences in the magnitude of the decrease were observed between treatments. During the interaction with *T. atroviride*, the levels of Put showed the greatest reduction (50%) followed by a 20% decrease in Spm + Tspm levels in both contact (At-Ta) and not physical contact (At/Ta) in comparison with control plants (Fig. 5A). The interaction with *T. virens* provoked a clearly decrease in the Spd, and tetraamines (Spm + Tspm) levels in both experimental conditions (At-Tv, At/Tv), however a major decrease in the distance condition (At/Tv) was recorded. The Put levels reached a 50% reduction only during physical contact (Fig. 5B).

A different behavior was observed in the profile of PAs at 5dpi, with a dramatic increase in the levels of Put (200%) in the plantlets that grew in distance interaction with the *T. atroviride* strain (At/Ta), while Spd, and tetraamines (Spm + Tspm), only augmented about 30% in comparison to control plants. In contrast, during physical contact with *T. atroviride*, an increase of 50% in Put levels was detected, and the other PAs analyzed did not show significant changes (Fig. 5C). Finally, the contactless interaction with *T. virens* provoked an increase on Spd (75%) and Put (35%), while tetraamines (Spm+ Tspm) levels were unchanged. Together, these data show a differential profile of PA levels during the interaction with this fungus.

3.3 Arabidopsis arginine decarboxylase genes are regulated differentially during plant-Trichoderma interaction.

The expression levels of Arabidopsis *ADC1* and *ADC2* genes during the interaction with the fungi was analyzed by qRT-PCR in 13-days-old Arabidopsis seedlings. Plantlets were grown with *T. atroviride* and *T. virens* in direct contact or separately at 3 and 5 dpi. At 3 dpi a significant repression of *ADC1* gene was observed in both experimental conditions, direct contact (At-T) or without contact (At/T). The highest reduction in expression level was observed in plants that grew separately of the fungus (Fig. 6A). Concerning *ADC2* gene, repression was also observed at 3 dpi, except for direct contact between Arabidopsis and *T. atroviride*, which showed a significant transcription induction (Fig. 6B). At later time of interaction (5 dpi), *ADC1* and *ADC2* genes were up-regulated during the interaction of Arabidopsis with both fungi (Fig. 6C, D), except for *T. atroviride* direct contact which provoked a repression of *ADC1* (Fig. 6C).

4. Discussion

A metabolic and genetic reprogramming occurs in plants as a consequence of interaction with microbes. Polyamines (PAs) are molecules implicated in plant growth and development, and in the establishment of plant microbe interactions. In this study, we have investigated PA levels of *Arabidopsis thaliana* during interaction with the beneficial fungus Trichoderma. Our interest in provide evidence of the role of the PAs during the interaction Arabidopsis - Trichoderma, were for several reasons: 1) PAs act as growth regulators (Tiburcio et al. 2014; Kim et al. 2013), 2) PAs profile undergoes remarkably changes during plant–microbe interactions (Hussain et al. 2011; Walters 2003), and 3) recent evidence places them as potential signaling molecules (Brotman et al. 2012; Jiménez-Bremont et al. 2014; Kusano et al. 2008).

Our study were performed comparing the interaction of Arabidopsis with two Trichoderma strains, and two types of interaction: one involving direct plant-fungal contact, and the other one using a split growth system, which avoids the physical interaction between both organisms. During split interaction with both Trichoderma species, we observed differences in Arabidopsis growth and development in comparison with direct interaction, such as: 1) plants reached higher development parameters such as fresh weight and primary root length, more evidently after 5 dpi; 2) the primary root growth was not inhibited compared to the Arabidopsis plants grown in direct contact with the fungi. This root growth inhibition has been previously observed during direct interaction with Trichoderma *spp.* (Contreras-Cornejo et al. 2009; 2014 a; 2014 b). A detailed study on Arabidopsis -Trichoderma direct interaction, using several Trichoderma species, showed that the inhibition of Arabidopsis primary root growth is independent of the Trichoderma species (Nieto-Jacobo et al, *in preparation*). 3) Another interesting fact was that plants grown without contact with the fungi developed the highest number of lateral roots. These physiological effects might be the consequence of different modifications that the fungus produces in the plant, such as the modulation of phytohormones and growth regulators. Interestingly, the levels of PAs determined on Arabidopsis plantlets during interaction with Trichoderma *spp.* showed a significant change in polyamine metabolism reflected as oscillations in the levels of the

PAs analyzed: Put, Spd and tetraamines (Spm + Tspm) during the time course of the interaction between the plant and the fungus. Specifically, we observed that at 3 dpi, PAs levels decreased in the plantlets with both Trichoderma species independently of the nature of the interaction (split system and contact). A modulation of PAs level has been also reported in *Lotus glaber* plants during early steps of interaction with mycorrhizal fungi (Sannazzaro et al. 2007; Hussain et al. 2011). Our data suggested that the PAs modulation observed at 3 dpi in plants during interaction with Trichoderma spp., can help the fungus in two ways: 1) to avoid its recognition as a pathogen and/or 2) to resist host defense induced at the start of the association. In contrast, at 5 dpi an increase in PAs levels with both Trichoderma species was observed. Respect to *T. atroviride* interaction, the highest increase in PAs levels was detected in the split system (without contact), whereas with *T. virens* was in contact. These differences in the profile of PAs detected in plants interacting with different fungi could be attributed to the battery of genes and the strategy of each species to achieve the interaction with the plant.

Our data at 5 dpi, showed a clear evidence for positive correlation between increased levels of PAs and increased root growth. Differences in PA concentration has been observed in plants that are interacting with fungus that improve the growth of plants. Brotman (2012) reported a metabolic profiling of 20-day-old *A. thaliana* plants growing in soil supplemented with *T. asperelloides* T-203, and they found that Put was a metabolite that increased during the interaction among other metabolites such as amino acids, sugars and citric acid cycle intermediates. This increase in Put level was related to the growth increase of Arabidopsis plants. Similar data were observed in scots pine seedlings during interaction with the ectomycorrhizal *Paxillus*, where a correlation in the increase of Put levels and the dramatically growth of seedlings was reported (Sarjala et al. 2010). These PAs variations are dependent on the stage of the symbiosis, the fungal strain and the host tissue (Kytöviita and Sarjala 1997; Sarjala et al. 2010; Fornalé et al. 1999).

Due to their important biological functions, PAs levels must be very strictly regulated at several levels: biosynthesis, conjugation, oxidation and transport. Because of that, we were interested in analyze the transcriptional levels of the arginine decarboxylase (ADC) genes.

The ADC is a rate-limiting enzyme that catalyze the first step of PA biosynthesis, in Arabidopsis there are two paralogs: *ADC1* and *ADC2* genes. The fluctuations observed in the PA levels when the plants were interacting with Trichoderma could be explained by the transcriptional profile of *ADC1* and *ADC2* genes. In most of the cases, at 3 dpi we observed that *ADC1* and *ADC2* genes were down-regulated in plant-fungal interaction; however, at 5 dpi an increase in the expression of both genes was observed. On the other hand, Arabidopsis–*T. atroviride* (At-Ta) direct interaction exhibited different ADC expression levels that those observed for the other interactions, wherein at 3- and 5-dpi, *ADC2* gene showed higher expression, while *ADC1* presented repression. This data suggests that *ADC2* gene have an important role in Arabidopsis–*T. atroviride* direct interaction. In plant pathogen interaction, it has been reported increases in *ADC* expression and Put levels. In this sense, an increase in Put levels was found in maize tumors induced by *Ustilago maydis*, where an up-regulation of the *ZmADC1* gene was also observed (Rodriguez Kessler et al. 2008). Similar results on accumulation of Put and ADC genes regulation were reported in Arabidopsis plants during interaction with *Rhodococcus fascians* (Stes et al. 2011).

The results arising from this work evidence the communication that occurs between plants and beneficial fungus, in which plants are able to recognize fungus-derived compounds and fine tune their defense and growth responses. Our data show a differential profile of plant polyamine levels related to the time course of the interaction, the species used, and whether the interaction occurs with physical contact or not, which suggest that polyamines could be involved in this intricate signaling and reprogramming of Arabidopsis, when the plant interacts with Trichoderma *spp*.

Acknowledgements

This work was supported by the CONACYT (Investigación Ciencia Básica CB-2013-221075) funding. We are grateful to Mireya Edith Martinez and Ma. del Carmen Rocha Medina by their technical assistance in the HPLC analyses, which they were carried out at LANBAMA, IPICYT.

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Table Legends

Table 1. Primer sequences used in real-time RT-PCR analysis of Arabidopsis *ADC1* and *ADC2* genes.

Figure Legends

Figure 1. Phenotype in Arabidopsis plantlets during the interaction with *T. atroviride* and *T. virens.* Representative photographs in split Petri dishes of 13-days-old Arabidopsis (Col-0) plantlets grown on MS medium after 3 days post interaction with *T. atroviride* and *T. virens* strains. Trichoderma strains were co-cultivated in direct contact or separately from Arabidopsis plantlets. **Control** Arabidopsis plantlets grown in MS without fungus. **At-Ta and At-Tv** Arabidopsis plantlets inoculated with *T. atroviride.* And *T. virens* respectively. **At/Ta and At/Tv** Arabidopsis plantlets grown with *T. atroviride* and *T. virens* without physical contact, respectively. The experiment was performed with 9 replicates, with 3 plantlets per plate.

Figure 2. Effects of inoculation of Trichoderma species on the growth of Arabidopsis plantlets. Arabidopsis plantlets from Figure 1 were harvest to analyze: **A.** Fresh weight, **B.** Primary root length, **C.** Emerged lateral roots per plant, **D.** Lateral root density. 13 days-old Arabidopsis (Col-0) plantlets grown in split Petri dishes on MS medium after 3 days post interaction with *T. atroviride* and *T. virens* strains. Trichoderma strains were co-cultivated in direct contact or separately from Arabidopsis plantlets. **Control** Arabidopsis plantlets were grown in MS without fungus. **At-Ta and At-Tv**. Arabidopsis plantlets inoculated with *T. atroviride* and *T. virens* respectively. **At/Ta and At/Tv.** Arabidopsis plantlets grown without physical contact with *T. atroviride* and *T. virens*, respectively. Estimation of primary root length (cm), emerged lateral roots and lateral root density was determined by

measuring 27 plantlets. The fresh weight (mg) of the seedlings was obtained on an analytical scale and the values obtained represent the means of nine groups of three seedlings of each line. Different letters are used to indicate means that differ significantly ($P< 0.05$).

Figure 3. Phenotype in Arabidopsis plantlets during the interaction with *T. atroviride* and *T. virens.* Representative photographs in split Petri dishes of 13-days-old Arabidopsis (Col-0) plantlets grown on MS medium after 5 days post interaction with *T. atroviride* and *T. virens* strains. Trichoderma strains were co-cultivated in direct contact or separately from Arabidopsis plantlets. **Control** Arabidopsis plantlets grown in MS without fungus .**At-Ta and At-Tv** Arabidopsis plantlets inoculated with *T. atroviride* and *T. virens* respectively. **At/Ta and At/Tv** Arabidopsis plantlets grown with *T. atroviride* and *T. virens* without physical contact, respectively. The experiment was performed with 9 replicates, with 3 plantlets per plate.

Figure 4. Effects of inoculation of Trichoderma species on the growth of Arabidopsis plantlets. Arabidopsis plantlets from Figure 3 were harvest to analyze: **A.** Fresh weight, **B.** Primary root length, **C.** Emerged lateral roots per plant, **D.** Lateral root density. Thirteen-days-old Arabidopsis (Col-0) plantlets grown in split Petri dishes on MS medium after 5 days post interaction with *T. atroviride* and *T. virens* strains. Trichoderma strains were co-cultivated in direct contact or separately from Arabidopsis plantlets. **Control** Arabidopsis plantlets were grown in MS without fungus. **At-Ta and At-Tv**. Arabidopsis plantlets inoculated with *T. atroviride* and *T. virens* respectively. **At/Ta and At/Tv.** Arabidopsis plantlets grown without physical contact with *T. atroviride* and *T. virens*, respectively.

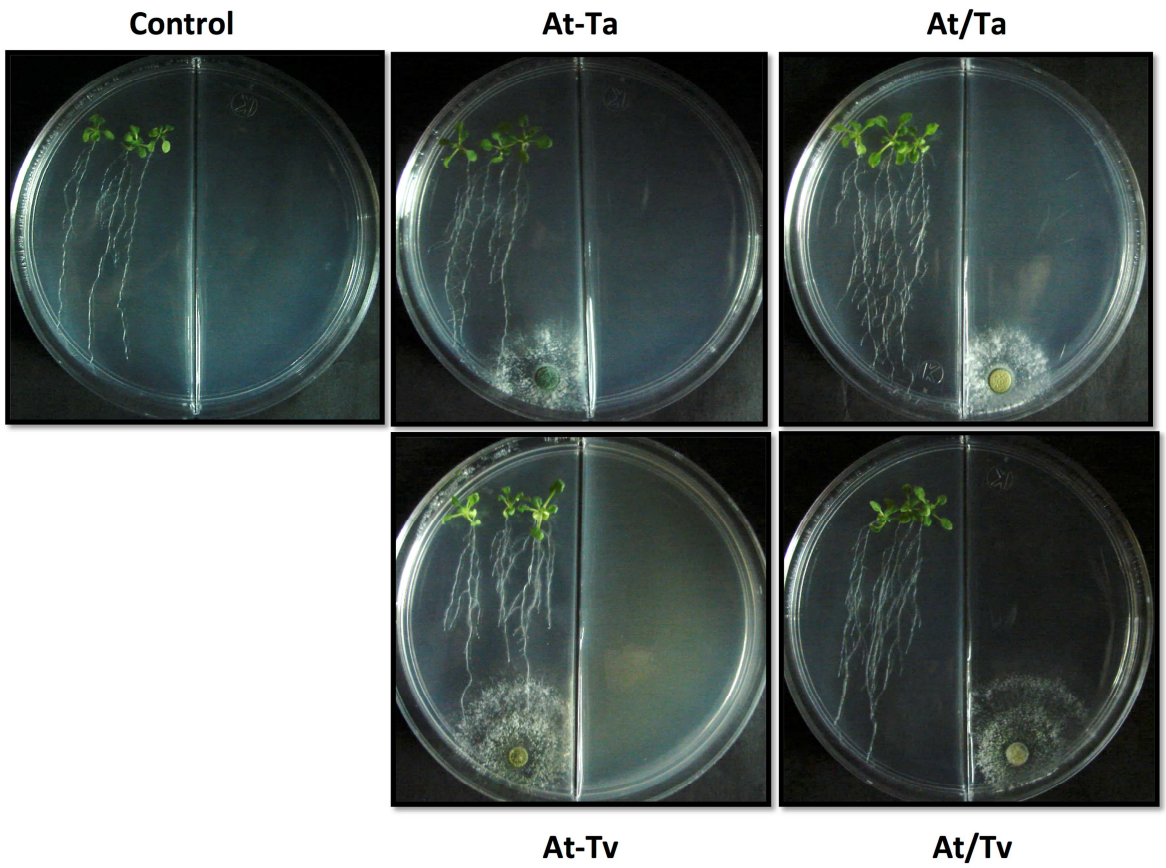
Estimation of primary root length (cm), emerged lateral roots and lateral root density was determined by measuring 27 plantlets. The fresh weight (mg) of the seedlings was obtained on an analytical scale and the values obtained represent the means of nine groups of three seedlings of each line. Different letters are used to indicate means that differ significantly ($P< 0.05$).

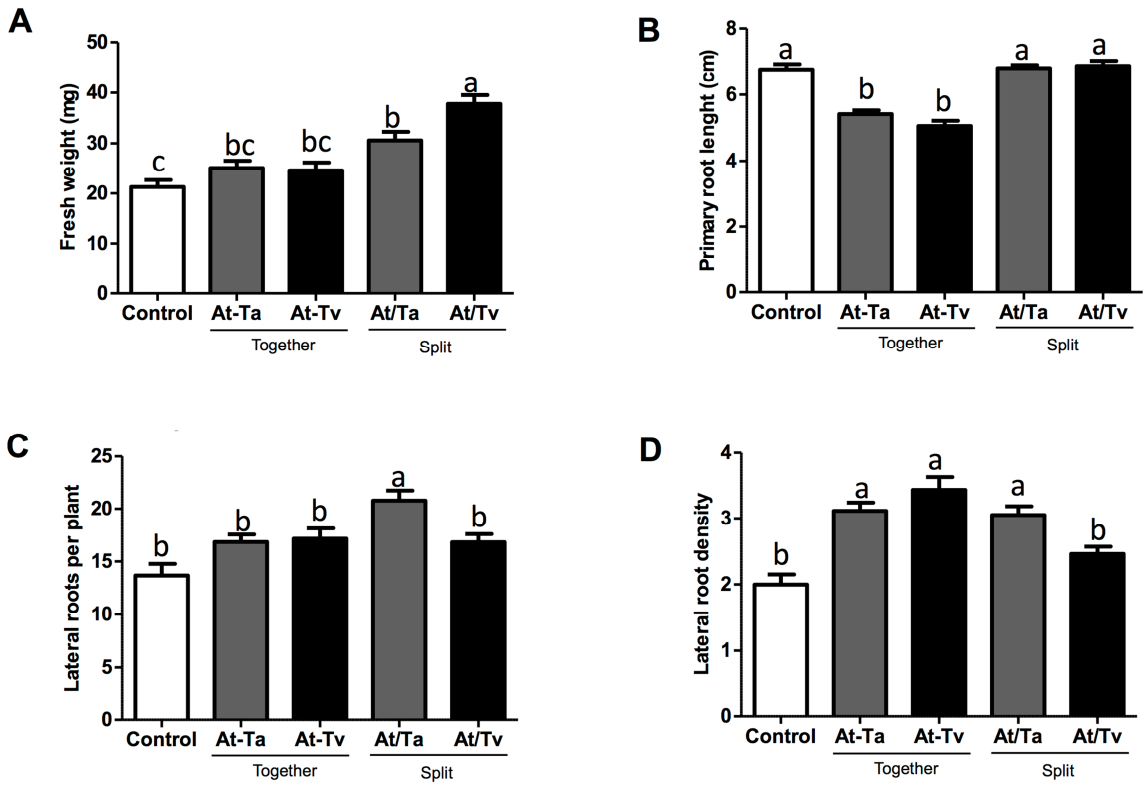
Figure 5. Polyamines levels of Arabidopsis seedlings during interaction with *Trichoderma* spp. A, C Quantification of free levels of putrescine, spermidine and tetraamines (spermine and thermospermine) in 13-days-old Arabidopsis seedlings during interaction with *T. atroviride* at 3 and 5 days post interaction respectively. **B, D** Quantification of free levels of putrescine, spermidine and spermine in Arabidopsis seedlings of 13-days-old during interaction with *T. virens* 3 and 5 days post interaction respectively. **Control** Arabidopsis plantlets grown in MS without fungus. Polyamine data from **A-D** show means + SE from three groups of 9 plantlets that were recovered from the medium. Different letters are used to indicate means that differ significantly ($P < 0.05$).

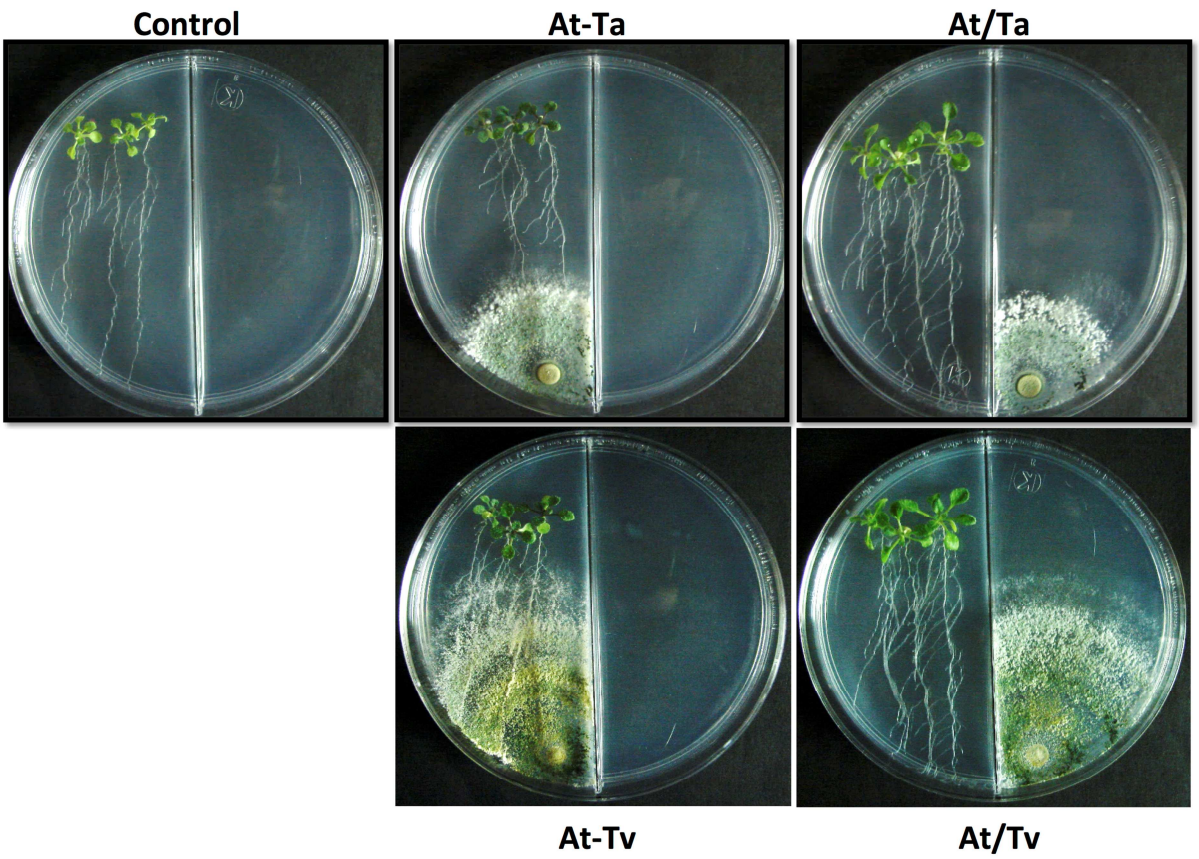
Figure 6. *ADC1* and *ADC2* genes expression from *A. thaliana* in interaction with Trichoderma spp. Thirteen days-old Arabidopsis seedlings during interaction with Trichoderma spp. at 3 and 5 days post interaction. Trichoderma strains were co-cultivated in direct contact or separately from Arabidopsis plantlets. **A, C** Expression levels of *ADC1* gene of Arabidopsis plantlets growing in contact with *T. atroviride* and T. *virens* (At-Ta and At-Tv) and growing without physical contact with *T. atroviride* and *T. virens* (At/Ta and At/Tv) at 3 and 5 dpi, respectively. **B, D** Expression levels of *ADC2* gene of Arabidopsis plantlets growing in contact with *T. atroviride* and *T. virens* (At-Ta and At-

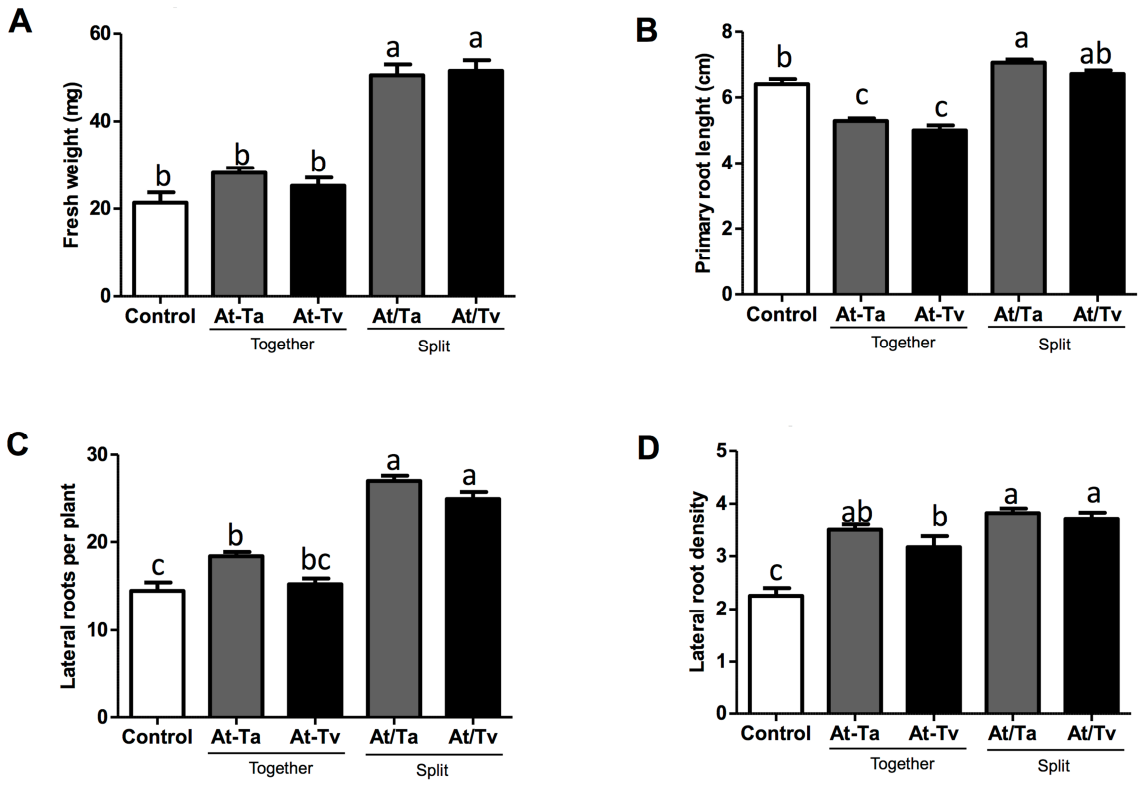
Tv), and growing without physical contact with *T. atroviride* and *T. virens* (At/Ta and At/Tv) at 3 and 5 dpi, respectively. The expression levels of *ADC1* and *ADC2* were estimated by qRT-PCR using SYBR green dye. Normalized fold change was calculated comparing the target gene expression (inoculated conditions) with a control (uninoculated Col-0), after normalization to the Arabidopsis UBQ5 gene using the (2−ΔΔCt) method. In case of ratios lower than 1, the inverse of the ratio was estimated and the sign was changed. Asterisks denote significant differences between control plants and co-cultivated plants.

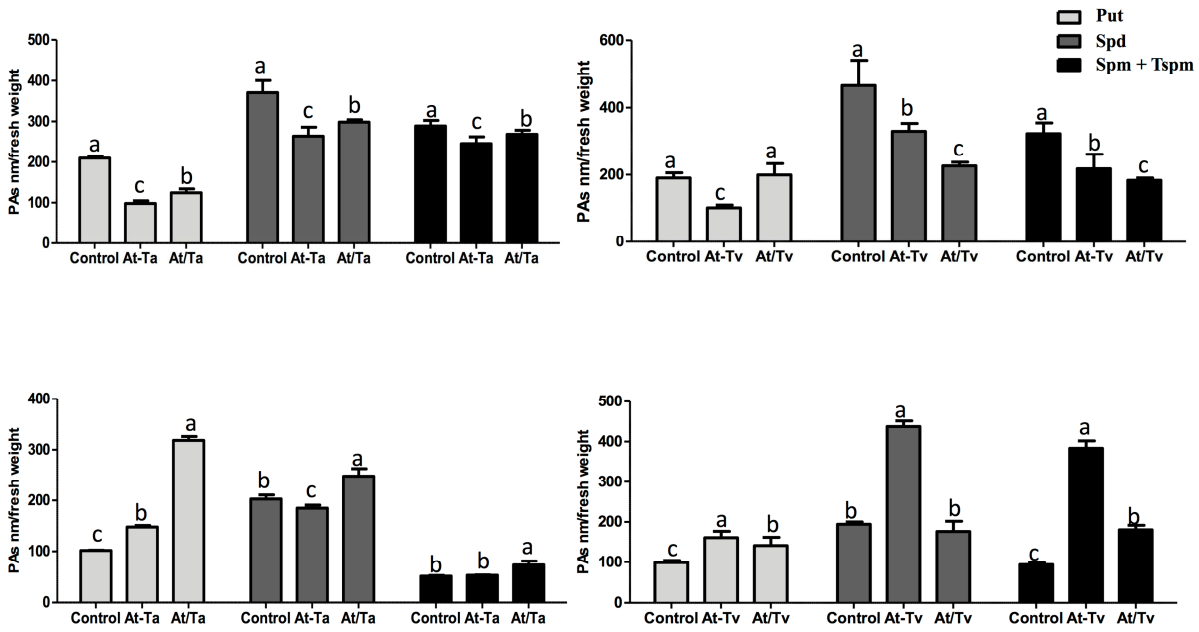
Locus	Gene	Amplicon length (bp)	Primer sequence
AT2G16500	ADC1 FW	125	5´CCTTGGCGTTTACTACTGCG
	ADC1 RV		5' CGGTGAAGATCAAAGACAGAGG
AT4G34710	ADC2 FW	145	5´GCCGTATCTTGCAACTGAGC
	ADC2 RV		5´TGCAACAACAAACCACACGA
AT3G62250	UBQ5 FW	155	5'TCGACGCTTCATCTCGTCCT
	UBQ5 RV		5'CGCTGAACCTTTCCAGATCC

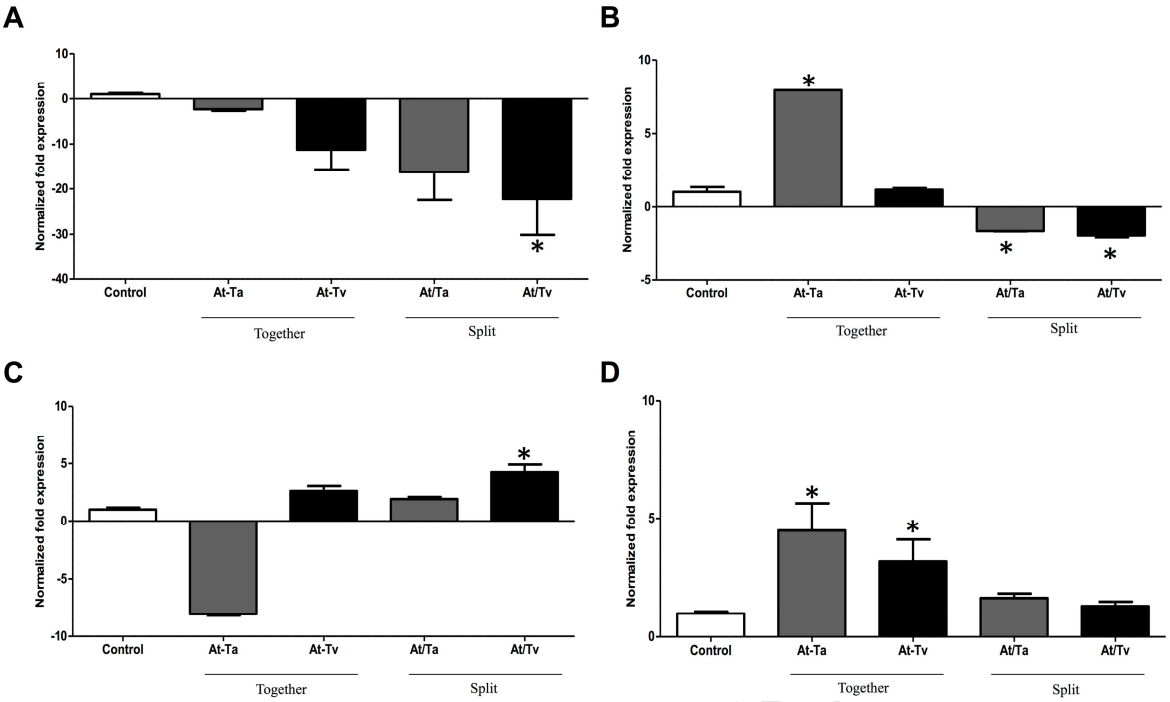












1. The largest increase in Arabidopsis biomass was observed in fungus split-interaction.
2. Changes in plant polyamine level were observed during the plant-fungus interaction.
3. Plant polyamine levels depend on Trichoderma strains, time and type of interaction

Contributions

Fatima Berenice Salazar-Badillo, Fernanda Nieto-Jacobo Diana Sánchez-Rangel, Alicia Becerra-Flora, Miguel López-Gómez designed and carried out the experiments, analyzed the results, and wrote the manuscript; Artemio Mendoza-Mendoza and Juan Francisco Jiménez-Bremont designed the research, contributed scientific advice, correction, wrote and final revision of the manuscript. All authors have read and approved the final manuscript.