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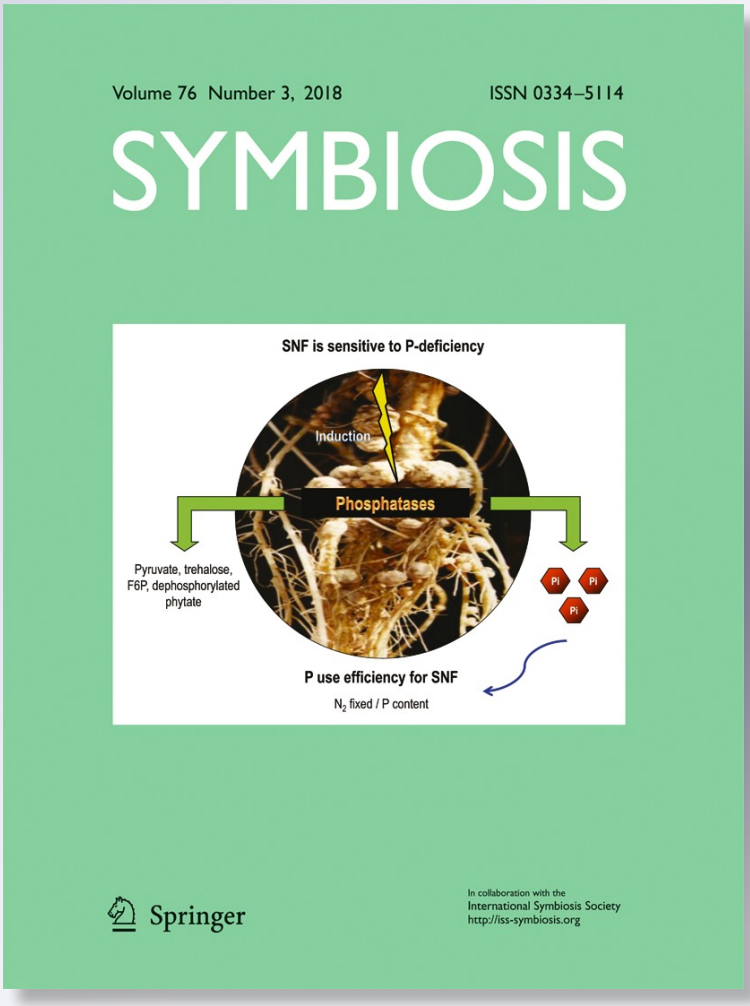

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Polyamines and flavonoids: key compounds in mycorrhizal colonization of improved and unimproved soybean genotypes

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Abstract
Modern breeding programs might have caused a reduction in plant responsiveness to arbuscular mycorrhizal fungi (AMF). Flavonoids and polyamines (PAs) are hypothesized to play a role in this symbiosis. We tested the effects of them in AMF roots of improved (I-1) and unimproved (UI-4) soybean genotypes, under the hypothesis that domestication decreased their concentration in roots, affecting AMF colonization, particularly arbuscule formation. After 20 days of treatment, AMF roots of UI-4 genotype had greater amount of total flavonoids/phenols and PAs while in I-1 genotype no differences were observed between roots of mycorrhizal (M) and non mycorrhizal (NM) plants. Exogenous application of flavonoids led to an increase in arbuscules in both genotypes. Improved-1 genotype needed higher levels of flavonoids to reach the percentage of mycorrhization achieved by UI-4 control. In regard to PAs, mycorrhizal plants of both genotypes had higher endogenous concentration than NM plants, however, the highest concentration, especially of putrescine (put) was in UI-4 M genotype. To check the participation of put in symbiosis we used RNAi silencing methodology. Down regulation of the GmADC transcript, involved in put formation, had a profound negative effect on mycorrhizal colonization and also affected the normal development of the plant. By contrast, down regulation of GmDAO, in which ADC transcript was expressed, arbuscule formation was similar to control plant. Our results suggest that mycorrhizal colonization is affected by soybean domestication particularly arbuscule formation and this effect seems to be mediated by the endogenous roots levels of flavonoids and PAs, especially put.

Keywords Arbuscular mycorrhizal fungi (AMF) · Flavonoids · Polyamines, improved and unimproved soybean genotypes

1 Introduction

The association between vascular plants and arbuscular mycorrhizal fungi (AMF) of the Phylum *Glomeromycota* are among the most ancient and widespread mutualism in terrestrial ecosystems, thus having a high ecological significance (Parniske 2008; Strack et al. 2003). The establishment of this association implies a complex exchange of signals between the two

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symbionts (Gadkar et al. 2001; Vierheilig and Piché 2002). There are several reports reviewing in detail the molecular and cellular aspects during the establishment of the AMF symbiosis, starting from early events e.g. the exudation of signaling compounds by the root (Harrison 2005; Karandashov and Bucher 2005; Balestrini and Bonfante 2005) and continuing with the formation of the appresorium, the fungal penetration into the root surface and the colonization of the intercellular space of the root cortex leading to the arbuscule formation (Harrison 1999; García-Garrido and Ocampo 2002; Pozo et al. 2002). The growth increase of the hyphae near the root of the plant is attributed to the fact that there are probably specific molecules present in the root exudates of the host indicating that in some way, a phenomenon of recognition occurs in the early stages of formation of AMF (Gianinazzi-Pearson and Gianinazzi 1989). Moreover, it has been hypothesized that at each stage different signaling events occur (Larose et al. 2002). Among the molecules involved on mycorrhizal development, flavonoids (Phillips and Tsai 1992; Hartmann et al. 2008; Hassan and Mathesius 2012; Sugiyama and Yazaki 2014) and/or polyamines (PAs) (Kusano et al. 2008; Requena et al. 2007) have been postulated by these authors.

Flavonoids are secondary plant metabolites existing in most plants, and they also constitute a large part of root exudates and play an important role in many plant microbial interactions (Cesco 2010; Sugiyama and Yazaki 2014). They have been implicated in spore germination and hypha growth (invasion of the fungi and arbuscule formation within the roots) (Sugiyama and Yazaki 2014). They may also influence the preinfections stages of the AMF (Akiyama et al. 2002). Flavonoids as root exudates showed controversial responses, in carrot seedlings stimulated hypha growth of the AM fungus *Gigaspora margarita*, while in pea; they inhibited hypha growth of the same fungus. As flavonoids exuded by legume seedlings elicit rhizobial nod genes and also stimulate mycorrhizal colonization, it has been proposed to select and/or transform the plants to increase the exudation of these flavonoids, which can result in a double benefit for mycorrhized legumes (Gianinazzi-Pearson and Gianinazzi 1989; Rengel 2002). However, flavonoids might not be essential for the plant–fungi recognition since Becard et al. (1995) showed that maize mutant plants showing impairment in flavonoid production were able to form mycorrhizae similarly to wild type plants.

Polyamines are small polycationic compounds which are ubiquitous in all living organisms (Kusano et al. 2008). The main PAs species in plants are putrescine (put), spermidine (spd), and spermine (spm), which have been detected in different tissues so far (Kumar et al. 1997). In plants, PAs are involved in growth and development, as well as in stress responses (Groppa and Benavides 2008; Alcázar et al. 2010; Mattoo et al. 2010; Takahashi and Kakehi 2010; Alet et al. 2012). Polyamines homeostasis is governed by a dynamic balance between PAs biosynthesis and catabolism. The plant PAs biosynthetic pathway has been well documented (Bagni and Tassoni 2001; Kusano et al. 2008; Fuell et al. 2010). Putrescine biosynthesis starts with either the decarboxylation of ornithine, catalyzed by ornithine decarboxylase (ODC) or indirectly by the decarboxylation of arginine by arginine decarboxylase (ADC), via agmatine (Fuell et al. 2010). Putrescine then serves as the substrate for the biosynthesis of spd and spm via the activities of S-Adenosylmethionine decarboxylase (SAMDC) and spermidine synthases (SPDS) and spermine synthases (SPMS), Walters (2000). Polyamines catabolism is crucial in regulating PAs levels in cells. They are oxidative catabolized by amine oxidases which include copper-binding diamine oxidases (CuAO)/diamine oxidases (DAO) and FAD-binding polyamine oxidases (PAO) which are widespread in all living organisms (Cona et al. 2006). As a result of DAO activity, put can be oxidized to Δ′-pyrroline releasing ammonia and hydrogen peroxide (H_2O_2). Oxidation of spm by PAO yields 1,3aminopropylpyrroline, along with diaminopropane and H_2O_2 (Bagni and Tassoni 2001). In plants, diversified roles of DAO and PAO have been reported in cell growth (Delis et al. 2006; Tisi et al. 2011), abiotic stresses such as drought (Aziz et al. 1998) and salt stress tolerance (Cona et al. 2006; Liu et al. 2011) and defense responses (Rea et al. 2002). DAO is believed to be loosely bound to the cell wall and can be released in the apoplast (Angelini et al. 1993; Møller and McPherson 1998). There is increasing evidence that PAs are involved in mycorrhizal colonization (El Ghachtouli et al. 1996). They affect arbuscular mycorrhizal infection of pea roots by acting directly on the fungus, thus increasing contact events with host roots, or by altering host plant physiology, e.g. by interacting with pectinases, affecting adhesion and/or penetration of the plant cell wall by the AM fungus (El Ghachtouli et al. 1996). Moreover, it has been recently demonstrated that exogenous PAs applications could improve mycorrhizal development of citrus seedlings. It is likely that this occurs through transformation of root sucrose more into glucose for sustaining mycorrhizal development (Wu et al. 2012b).

On the other hand, it was demonstrated the importance of the host genotype in the establishment of this association since the degree of AMF root colonization and the positive effect of mycorrhization on plant grow can vary (Rengel 2002; Singh et al. 2012). Moreover, modern breeding programs might have caused a reduction in plant responsiveness to AMF (Pérez-Jaramillo et al. 2016). In that sense, it was reported variability in the level of AMF colonization between improved and unimproved wheat (Hetrick et al. 1993) and corn (Khalil et al. 1994) genotypes. In soybean, Heckman and Angle (1987) reported variability in root colonization by indigenous soil populations of AMF. Khalil et al. (1994, 1999) also found that both AMF colonization and plant growth change associated with AM colonization, defined as mycorrhizal dependency (MD) by Plenchette et al. (1983) increased more in unimproved soybean genotypes than in modern cultivars. More

recently, our group observed a higher arbuscules/hyphae level of AMF colonization in unimproved than improved soybean genotypes. The differences in MD responses were reflected in growth parameters and in the oxidative stress regulation, evaluated during the first days of colonization with a mixed AMF inoculum isolated from soybean roots (Salloum et al. 2016).

Since it is known that the beneficial effects of mycorrhizal symbiosis occur as a result of a complex molecular dialogue between symbiotic partners (Gadkar et al. 2001; Vierheilig and Piché 2002), the identification of the molecules involved in this process is a prerequisite for a better understanding of this interaction. Therefore, we propose to compare flavonoids and PAs concentration in roots between improved vs unimproved mycorrhizal soybean genotypes under the hypothesis that domestication of soybean genotypes decreased their concentration in soybean roots, during the first time of AMF colonization. This effect is accompanied by decreased colonization evaluated as changes in arbuscules formation and MD response. To check if flavonoids and PAs, influence mycorrhizal symbiosis, two experiments were carried out: 1) addition of flavonoids to the soil and 2) silencing the PAs pathway at ADC and DAO level, using RNA interference (RNAi).

2 Materials and methods

2.1 Plant and fungal material

Two soybean genotypes, contrasting in AMF colonization efficiency (Salloum et al. 2016) were employed. The improved soybean (*Glycine max*) genotype (I-1) showed a lower AMF colonization than the unimproved soybean (*Glycine soja*) genotype (UI-4). Both genotypes belong to the germplasm collection of the Agricultural Experimental Station of the National Agricultural Technology Institute (EEA-INTA)-Marcos Juarez, Córdoba-Argentina.

The mixed AMF inoculum was isolated from soybean roots collected from a soybean monoculture system developed in EEA INTA Manfredi (Córdoba-Argentina). The inoculum was isolated and multiplied in pots containing sterile sand/soil mix (1:1 *v*/v), using soybean and *Medicago sativa* as plant-trap, under greenhouse conditions at 25 °C and watered daily with distilled water for two years. The mixed AMF inoculum has been morpho-taxonomically described in Salloum et al. (2016).

2.2 Plant-fungus bioassays

Soybean genotypes were grown in presence or absence of a mixed AMF inoculum in a chamber under controlled lighting conditions (16 h of light and 8 h of night) and temperature (average of 25 °C). The experiment had a factorial design (2 × 2), with two AMF treatments (with or without AMF inoculation), and one water regime. Soybean seeds were sterilized using hypochlorite solution at 18% for 30 s. Then, two pregerminated seeds were introduced in pots containing a substrate consisting of sand/soil mix (1:1) which was autoclaved twice for 1 h every 24 h each. The soil used in the experiment contained 4.8 ppm N-NO_3; 2.5 ppm S-SO_4; 5.9 ppm P; 3.09% organic matter; 1.79% organic carbon; 0.162% total nitrogen; 11.1 C: N ratio and pH of 6.7.

The mixed mycorrhizal inoculum consisted of 8 g of soybean root fragments, spores and mycelium isolated from trap plants. Mycorrhizal (M) plants were inoculated in the center of the pot; non mycorrhizal (NM) plants treatments received the same amount of autoclaved inoculum. Before autoclaving, the inoculum was filtered with deionized water through a 37-μm sieve (Schleicher & Schuell, Germany). The filtrate was added to the NM planting pots to provide them with the microbial populations accompanying the AMF, following Porcel and Ruiz-Lozano (2004).

Each pot received 5 ml of nutrient solution (without the presence of P) once at the start of the assay; then pots were watered with distilled water three times a week to keep moisture close to field capacity. Samples of roots were taken 20 days after treatment. The trials were repeated three times using 10 seedlings per genotype and per treatment. The AMF structures in the roots were stained according to Phillips and Hayman (1970) and colonization was measured following McGonigle et al. (1990).

2.3 Extraction and sample preparation for flavonoids and phenolics determination in roots

After a 20 days treatment M and NM soybean roots were collected dried and grounded into fine powder. These were stored in sealed plastic bag at 4 °C until ready for extraction. Powdered samples (10 mg) were extracted by soaking them in 7 ml methanol-water (70:30 *v*/v) for 24 hs in a glass container at room temperature. After cooling them at room temperature the samples were centrifuged at 4000 rpm for 10 min. The supernatant was recovered for determining flavonoids and phenolic concentration. The samples were performed in triplicate.

2.4 Determination of total phenolics concentration

Total phenolic concentration in root extracts was determined by using the Folin-Ciocalteu method (Waterman and Mole 1994; Nepote et al. 2005). The absorbance of samples was recorded at 760 nm spectrometrically (Spectrum SP-2100, Zhejiang, China). Gallic acid (GAE, Sigma-Aldrich, St. Luois, USA) was used as standard. The phenolic concentration of the roots was calculated as gallic acid equivalents (mg GAE/g Dw) sample. Dw: Dry weight.

2.5 Determination of total flavonoids concentration

To determine the total flavonoid concentration of the extracts, the aluminum chloride complex formation test was used (Luximon-Ramma et al. 2004) Quercetin (QE, Sigma Aldrich, St. Luois, USA). The concentration of flavonoids was determined as equivalents of quercetin. A calibration curve for quercetin was drawn for this purpose, and the absorbance at 367 nm was read. The flavonoid concentrations were expressed in (mg QE / g Dw).

2.6 Exogenous flavonoids application

Four treatments with ten replicates each for a total of 40 pots (one seed/pot) were as follows: (1) AMF only without flavonoids (F) (AMF + non-F), (2) 50 mg/l exogenous flavonoids plus AMF (AMF + F/50), (3) 100 mg/l exogenous flavonoids plus AMF (AMF + F/100), and (4) 150 mg/l exogenous flavonoids plus AMF (AMF + F/150). The flavonoids (Sigma, USA) were exogenously applied (100 ml) to the soil media in the start and after 10 and 20 days of AMF inoculation. The other treatments received 100 ml distilled water. The seedlings were harvested 20 days after the exogenous flavonoids treatments, and the roots were separated. The root segments were washed with 10% KOH and stained with 0.05% trypan blue in lactophenol as described in Phillips and Hayman (1970). AMF colonization and mycorrhizal structures such as hyphaes, vesicles, and arbuscules were microscopically examined and calculated (Wu et al. 2008).

2.7 Polyamines determination

After a 20 day treatment 70 mg of root material was lyophilized and homogenized with 5% perchloric acid, kept 30 min on ice and centrifuged at 5000 rpm for 10 min. The supernatants were derivatized using the dansylation method described by Smith and Meeuse (1966) and 1, 6 hexanediamine was used as internal standard. Standards of put, spd and spm were dansylated simultaneously. The dansylated derivatives were extracted with 1 ml ethyl acetate. PAs were separated and identified by TLC, performed on high resolution silica gel plates (JT Baker, silica gel plates IB 2-F) using n-hexane:ethyl acetate (1:1) solvent system. Even, cad and put are difficult to separate using ethyl: acetate and hexane (1:1) as the solvent system, the TLC plates had a pore resolution enough to separate both of them. Moreover, the sheet remained in the solvent system until the front of the solvent system reach the edge of the aluminum foil, which completely separated the bands and allowed the separation and identification of cad and put. However, to confirm the identity of the separated amines, it was also used the system benzene: triethylamine (10:1). Dansylated PAs were identified by comparing the Rf values of dansylated standards. Silica plates were observed under UV light and bands corresponding to the PAs in the samples and standards were scraped off the plates and eluted with 1 ml ethylacetate. Their fluorescence was measured at 365 nm excitation and 510 emissions in a spectro fluorometer (Aminco Bowman).

2.8 Plasmid construction and generation of composite plant material

Inverted repeats were created in pRNAi by two sequential cloning steps to generate the silencing vector. Target regions corresponding to the coding sequence of *Gm*ADC (320 bp) or *Gm*DAO (337 bp) were PCR amplified and subsequently linked into the RNAi plasmid at *Asc*I-*Swa*I restriction sites in the sense orientation, or in the *Bam*HI-*Spe*I restriction sites in the antisense direction. The resulting inverted repeat construct was inserted KpnI-PacI into the pRedRoot binary vector. Target regions were amplified using the following primers:

*Gm*ADC-Up: 5'ATAT**ACTAGTGGCGCGCC**GAATGTTTTGGGTGGGGCTTAC 3′;

*Gm*ADC-Low: **5'**CGCG**GGATCCATTTAAAT**CAGCAGATAAGGCATTCTGTCAAAGG 3′.

*Gm*DAO-Up5'**ACTAGTGGCGCGCC**CAGGATGGAAARATAGAAGC3′ GGCGCGCCTCTAGAATATCATGGCTACCTCCATGGT.

*Gm*DAO-Low5'G**GATCCATTTAAAT**GCATAACWGGCCAGTCTTCC3′. ATTTAAATGGATCGCGCTGTTGATTATAACAATGGCC.

The *Spe*I-*Asc*I and BamHI-SwaI restriction sites that are included within the primer have been underlined. The resulting constructs were used for *Agrobacterium rhizogenes* K-599 mediated transformation, which allowed the generation of composite plants, mainly as described by Estrada-Navarrete et al. (2007).

Ten to twelve days after infection, plantlets exhibiting profuse hairy root formation at the site of infection were selected (Fig. 1) primary root removed by cutting approximately 1 cm below the cotyledonary node and replanted in fresh pots containing sterile sand/soil mix (1:1). Immediately after transferring to new pots, each of the *A. rhizogenes*-transformed composite plants were inoculated with AMF, and irrigated with hoagland nutrient solution (Hoagland and Arnon 1950) containing a highly reduced phosphate level. Subsequently, the plants were covered with polyethylene bags to maintain humid conditions, and returned to the plant growth chamber maintained at a 14/10 h light/dark cycle and a temperature of 25 °C. After 4–5 d of incubation, the plastic bags were perforated to facilitate the gradual acclimation of transformed plants to the environment for a few days, before they were transferred to the naturally lit greenhouse maintained at 25 °C. The plants were harvested 3–4 weeks after mycorrhyzae inoculation, and roots were analyzed for mycorrhizal formation.

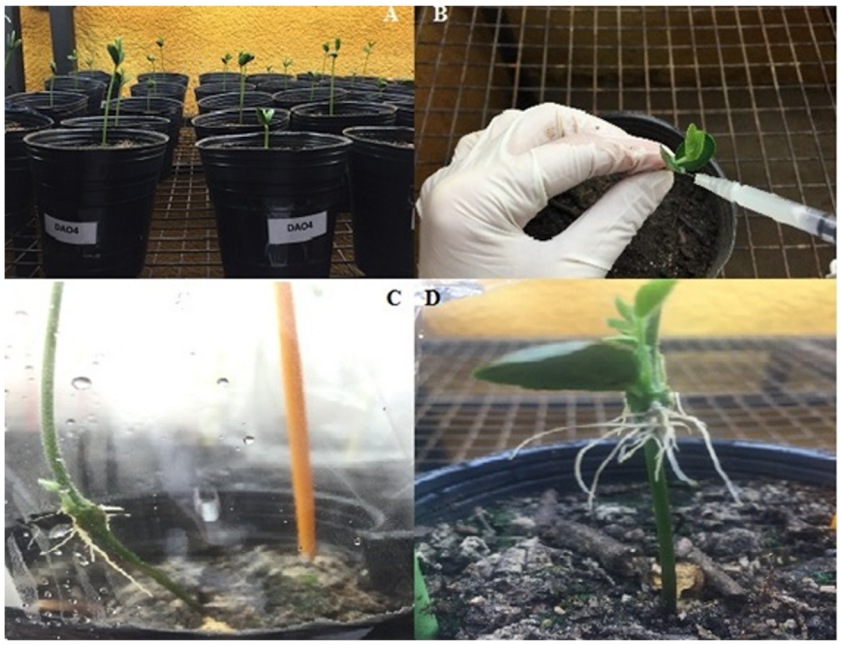

Fig. 1 Soybean hairy root transformation. A- 7 days seedlings used for infection; B- Inoculation of *A. rhizogenes* around the cotyledonary node area using a syringe needle; C- Composite plant 15 days after inoculation; D- Transformed root grown in camera for one month

2.9 Isolation of RNA and semi-quantitative RT-PCR analysis

Total RNA was extracted from soybean roots using an RNA extraction Kit (Sigma). Genomic DNA was degraded with RNase-free DNaseI (Invitrogen, Carlsbad, CA, U.S.A.) by incubation in the supplied buffer from 15 min at room temperature. The purity and integrity of the RNA were determined by agarose gel electrophoresis and the A260/A230 and A260/A280 ratios. For RT-PCR experiments, 3 μg of DNase-treated total RNA was reverse transcribed into cDNA with oligo(dT) (12–18 -mer) using M-MLV[H-] Reverse Transcriptase (Promega). A fraction (about 1/20) of the first strand cDNAs was used as a template for PCR with gene specific primers in a volume of 25 μl with 1 U of Taq-polymerase (Promega), 20 μM each dNTP, and 0.25 μM of each primer. The amplification was carried out with an initial cycle of 1 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 30 s at 60 °C, and 1 min at 72 °C, and a single final cycle of 7 min at 72 °C. RT-PCR products were resolved on 1.2% (*w*/*v*) agarose gel in TRIS-acetate-EDTA buffer. As an internal control, primers EF1F (5′-TGTTGCTGTTAAGGATTTGAAGCG-3′) and EF1R (5′-AACAGTTTGACGCATGTCCCTAAC-3′) were utilized to amplify 358 bp within the soybean housekeeping gene, tefS1 (elongation factor EF-1α; GenBank accession No. X56856).

2.10 Statistical analysis

Data were statistically analyzed by analyses of variance (ANOVA). Differences among means were compared by Duncan's least significant difference tests at the significance level of $p \leq 0.05$. All statistical analyses were performed by the InfoStat Professional version 2013.

3 Results

3.1 Flavonoids and polyphenolic concentration of mycorrhizal soybean roots: Differences between improved vs unimproved soybean genotypes

After 20 days, roots of NM UI-4 showed a lower level of total flavonoids and phenols than NM I-1 one. By contrast, AMF inoculation promoted a differential increase of both specialized metabolites in soybean plants. Thus, M UI-4 genotype had greater amount of total flavonoids and phenols with respect to NM soybean plants; while in the I-1 genotype, no differences were observed between roots of M and NM plants (Fig. 2a and b).

3.2 Effect of exogenous flavonoids solution treatment on mycorrhizal development: Differences between improved vs unimproved soybean genotypes.

Exogenous application of flavonoids led to an increase in root colonization and in the number of arbuscules in both genotypes (Table 1). The positive effect was related with the concentration, being the highest flavonoids concentration applied (F/150 mg) the most effective particularly in the % of root colonization and arbuscules in both genotypes. Also, the highest concentration used in this study

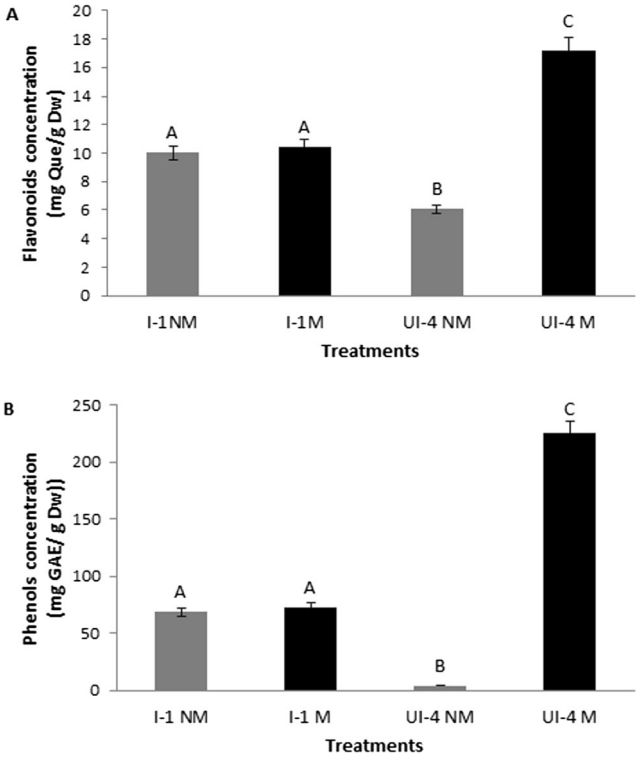

Fig. 2 Effect of arbuscular mycorrhizal fungi inoculations on total flavonoid (A) and phenolic concentrations (B) in 20 days old roots of two soybean genotypes (I-1: improved genotype and UI-4: unimproved genotype). Treatments are designed as non mycorrhizal (NM), and mycorrhizal (M) plants. Values are means based on ten biological repeats ± standard errors (SE). Means followed by the same letter does not differ ($p < 0.05$) using Duncan's multiple-range test. The flavonoids concentrations were expressed in (mg QE / g Dw) and phenols concentration as (mg/ GAE/ g Dw). Dw: Dry weight

(150 mg/l) allowed genotype I-1 to reach similar levels of mycorrhizal colonization to those acquired by the genotype UI-4 at the beginning of the experiment (Table 1).

3.3 Polyamines concentration of mycorrhizal soybean roots: Differences between improved vs unimproved soybean genotypes

The three commonly occurring PAs were detected in the roots of both genotypes, in which put was the most abundant, followed by spm and spd, respectively (Fig. 3a, b and c). The endogenous PAs concentration changed significantly between M vs NM soybean plants, especially in M UI-4 genotype, where put level showed the highest level as compared to M I-1 genotype (Fig. 3a and b). Moreover no differences were observed for the levels of spd and spm between M and NM I-1 genotype, (Fig. 3a). When we compare both M genotypes was evident that PAs endogenous concentration: put, spd and spm were higher in UI-4 than I-1 (Fig. 3c).

3.4 Effect of RNAi mediated downregulation of ADC and DAO on AMF colonization

Roots of each of three independent biological replicates of non-transformed (K599) and *GmADC*RNAi and *GmDAO*RNAi plants were analyzed by end-point PCR for the expression of *eftSI* (as a constitutive gene) and *GmADC* and *GmDAO* (as a silencing control). As expected, all replicates in K599, *GmADC*RNAi and *GmDAO*RNAi plant roots expressed the *eftSI* gene in both genotypes (Fig. 4a and b; *eftSI*). When we checked ADC transcript level of the soybean roots of the *GmADC*RNAi lines and compared to the untransformed control roots, transcripts of the ADC gene were not observed as a result of the RNAi effect in both genotypes under study (Fig. 4a, b; PrADC). Interestingly, neither was observed transcripts of the DAO gene (Fig. 4a and b; PrDAO: I1 (-ADC) and UI4 (-ADC)).

Table 1 Effect of exogenous flavonoids application on mycorrhizal development in improved and unimproved soybean genotypes. Mycorrhizal soybean plants were irrigated either with water or with different concentration of flavonoids (0, 50, 100 or 150 mg/l) in three times to the soil media: at 0, 10 and 20 days of AMF inoculation. Mycorrhizal colonization was evaluated in soybean plants of 25 days old. I-1: improved genotype; UI-4: unimproved genotype

Inoculation Treatments	Total Root Colonization (%)	Root colonization (%) by hyphae	Root colonization (%) by vesicles	Root colonization (%) by arbuscules
I-1-AMF+ F/0	20.9a	19.9a	1a	0a
I-1-AMF + F/50	34.4b	29.5b	3.3b	3.4b
I-1-AMF + F/100	34.5b	26.6c	3.3b	4.4b
I-1-AMF + F/150	39.5b	27.2c	1.7a	12.2c
UI-4-AMF+ F/0	35.5b	30.8b	2.6b	2.9b
UI-4-AMF + F/50	38.6c	30.3b	3.3b	3.6b
UI-4-AMF + F/100	43.3d	36.6d	3.3b	6.6e
UI-4-AMF + F/150	44.5d	24.7e	3.7b	16.1f

Values are means ±SD ($n = 10$ plants). The same letter within each column indicates no significant difference among treatments according to least significant difference tests at $p < 0.05$

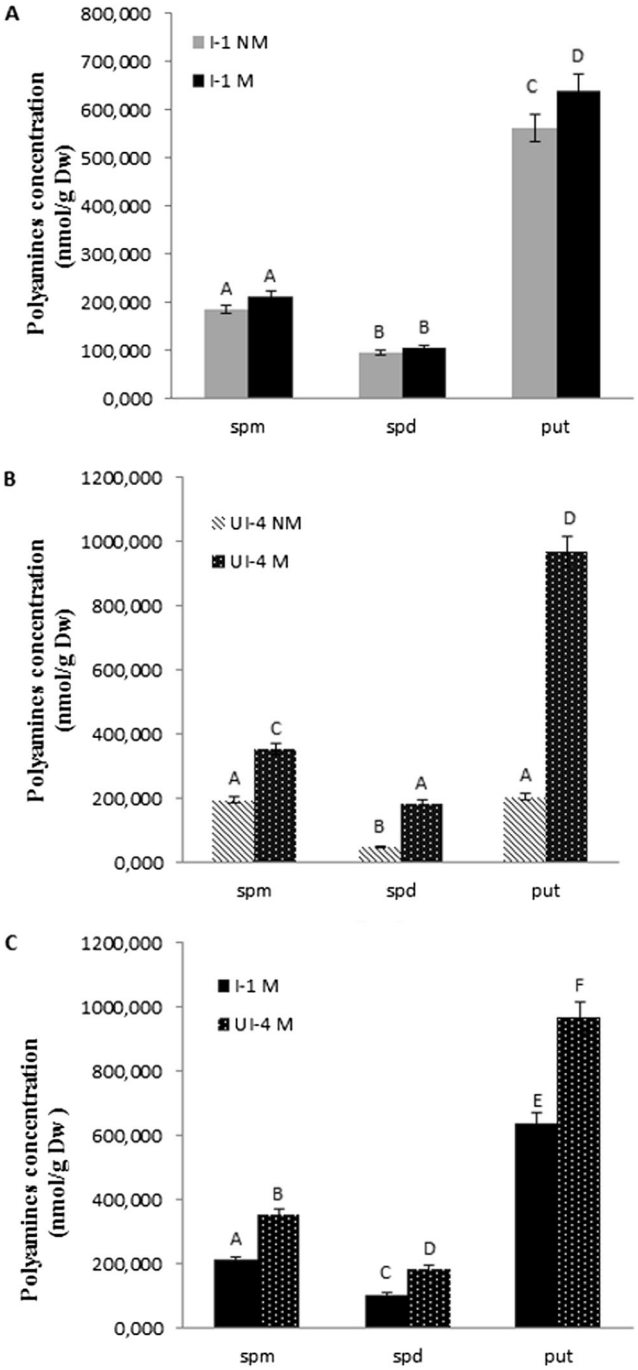

Fig. 3 Influence of mycorrhizal inocula fungi inoculation on put, spd and spm concentration in 20 days old roots of two soybean genotypes (I-1: improved genotype and UI-4: unimproved genotype). Treatments are designed as non mycorrhizal (NM), and mycorrhizal (M) plants. Put: putrescine, spd: spermidine, spm: spermine. Values are means based on ten biological repeats ± standard errors (SE). Means followed by the same letter does not differ ($p < 0.05$) using Duncan's multiple-range test. The results were expressed as (nmol /g Dw). Dw: Dry weight

Similarly, when the level of transcription of DAO was checked in *GmDAO*RNAi lines no transcripts of the DAO were observed as a result of the RNAi effect in both genotypes under study, however the transcription of the ADC gene was

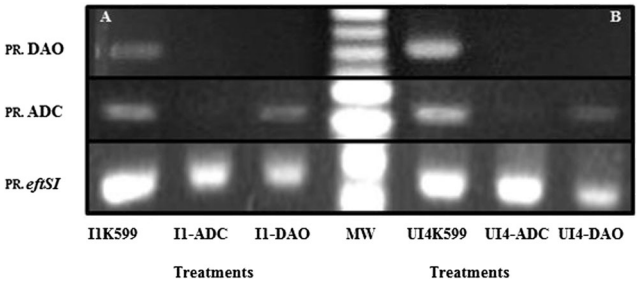

Fig. 4 RT-PCR analysis of the accumulation of GmADC and GmDAO transcript in two soybean genotypes. (I-1: improved genotype and UI-4: unimproved genotype) *eft*SI (constitutive gene), K599 not transformed plant, MW. Molecular marker; (-ADC): silenced roots in arginine decarboxylase. (-DAO): silenced roots in diamine oxidases

not affected (Fig. 4a and b PrADC: I1(-DAO) and UI4 (-DAO). *GmDAO*RNAi plants appeared normal and healthy as the untransformed control plants. By contrast, it was evident a clear phenotypic effect of RNAi down regulation of ADC, where significant difference was observed in the plant height and in the number and size of the leaves related to control plants (k599 1 and 4) *GmDAO*RNAi plants (Fig. 5a and b). Both genotypes respond similar to the down regulation of DAO and ADC genes.

We also inoculated both genotypes to determine if AMF colonization is affected by RNAi mediated disruption of GmDAO and GmADC in roots. Root colonization ranged from 26 to 30% in control and *GmDAO*RNAi plants and both have similar % of hyphae and arbuscule (Table 2). However, down regulation of GmADC decreased the % of mycorrhization from 71 in I-1 to 67% in UI-4 genotype with similar reduction in the % of hyphae and no arbuscules formation (Table 2).

4 Discussion

The knowledge of the dialogue between plants and AMF is crucial to understand the different capacity of AMF colonization between different genotypes. In this paper we deal with the importance and participation of two metabolites, flavonoids and PAs, implicated in the mycorrhizal colonization. Since domestication of plant species may have affected the ability of plants to maintain beneficial associations with rhizosphere microbes (Rengel 2002; Singh et al. 2012; Pérez-Jaramillo et al. 2016), our study makes a novel contribution by analyzing the participation of PAs and flavonoids in the regulation of AMF colonization. To our knowledge, the comparison of the amount of these metabolites between roots of AMF unimproved vs AMF improved soybean genotypes, is original and will provide some clues to improve the understanding in mycorrhizal colonization of soybean and the metabolites involved.

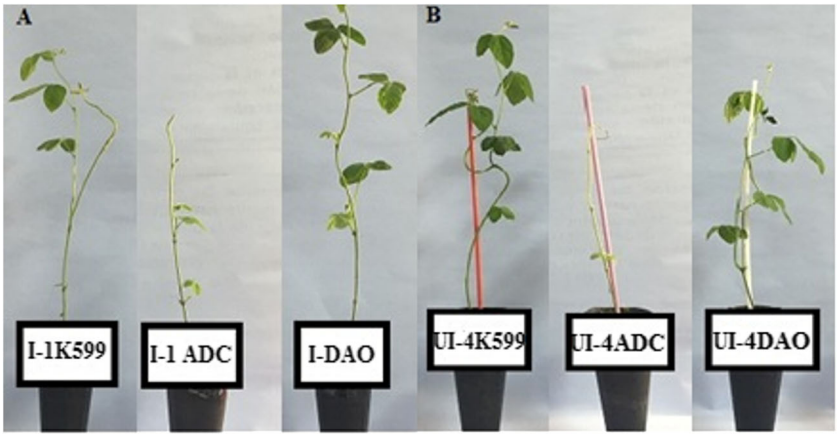

Fig. 5 Effect of RNA interference mediated silencing of ADC and DAO on soybean plants development. **a**) I-1: improved soybean genotype; **b**) UI-4: unimproved soybean genotype; K599: not transformed plant; (-ADC): silenced roots in arginine decarboxylase. (-DAO): silenced roots in diamine oxidases

Plant flavonoids are metabolites implicated in the presymbiotic development of the AMF in soil and in the recognition process of plant-AMF interaction (Sugiyama and Yazaki 2014). However there are no reports at the plant level, comparing their effect on AMF colonization of improved and unimproved soybean genotypes. Thus, we observed a higher endogenous concentration of flavonoids and total phenols in roots of M plants compared to NM plants and it was especially higher in unimproved soybean genotype than improved ones. Interestingly, we also found a relation between the percentage of mycorrhization and fungal structures, as arbuscules formation, according to the concentration of flavonoids and total phenolics, thus being greater in UI-4 than I-1.

On the other hand, in our studies it was observed that the application of exogenous flavonoids improved mycorrhizal colonization, especially the formation of arbuscules in I-1 soybean genotype, allowing it to reach a level similar to UI-4 soybean genotype at the beginning of the experiment. Although UI-4 genotype showed higher percentages of mycorrhization than those observed in genotype I-1 at all concentrations studied. The differences observed between the concentrations were more evident in I-1 genotype, which could indicate that the endogenous levels of flavonoids in UI-4 genotype is very near to the optimum required to reach good levels of mycorrhization.

The interaction of plants with beneficial microorganisms also induces changes in PAs metabolism and the AMF colonization appears to depend on PAs levels (Yao et al. 2010). El Ghachtouli et al. (1995) have suggested that PAs play the important role in the initial stages of the mycorrhizal infection, moreover various PAs were found in the spores of AMF (Sannazzaro et al. 2004).

In our study, PAs concentration specially put, was increased in soybean roots by AMF inoculation. Interestingly, PAs concentration, especially put, were higher in the UI-4 genotype than I-1 one, suggesting that soybean domestication may have affected the ability of plants to maintain a higher level of PAs, particularly put, in roots. In this context, Wu et al. (2010b) observed that put but not spd or spm, enhances root mycorrhizal development and plant growth of trifoliate orange. Similarly, put increased mycorrhizal development and colonization in *Citrus tangerine* (Wu et al. 2010a), *Citrus limonia* (Yao et al. 2010), *Poncirus trifoliate* (Wu et al. 2012b) *and Freesia hybrida* (Rezvanypour et al. 2015). Moreover the possible participation of put in mycorrhizal development has been investigated using exogenous applications of DL-α-difluoromethylornithine (DFMO) a competitive inhibitor of ODC catalyzed steps of put formation (Hummel et al. 2002). DFMO strongly inhibited mycorrhizal infection of *Pisum sativum* and the inhibition was reversed

Table 2 Effect of RNA interference mediated silencing of ADC and DAO on root mycorrhizal development of two soybean genotype seedlings: improved (I-1) and unimproved (UI-4) genotypes colonized by mixed AMF inoculum. K599: non-silenced roots (control). -ADC: silenced roots in arginine decarboxylase. –DAO: silenced roots in diamine oxidases

Treatments	Total Root Colonization (%)	Root colonization (%) by hyphae	Root colonization (%) by vesicles	Root colonization (%) by arbuscules
I-1-K599	28a	22.5c	–	
I-1 –DAO	26a	26d	–	5.5f
I-1 –ADC	8b	8e	–	–
UI-4-K599	30a	23.3c	–	6.6f
UI-4-DAO	26.6a	20c	–	6.6f
UI-4-ADC	10b	10e	–	–

Values are means ±SD ($n = 10$ plants). The same letter within each column indicates no significant difference among treatments according to least significant difference tests at $p < 0.05$

when exogenous put was included in the DFMO treatment supporting the importance of put in arbuscular mycorrhizal infection (El Ghachtouli et al. 1996).

However, evidence to support the direct role of put in these processes remains elusive. Development of molecular techniques has facilitated PAs modulation by overexpression or down regulation of the genes for PAs biosynthetic enzymes in transgenic or mutant plants, thus revealing the precise roles of PAs. In this paper we report the use of an RNAi silencing approach to reduce ADC activity in soybean roots with the aim of assessing the capacity of transgenic plants to promote mycorrhizal formation. We reasoned that if the ADC mediated-pathway is indeed the preferential route to put formation that have been implicated in the stimulation of mycorrhizal formation and hypha growth in *Citrus tangerine* (Wu et al. 2010c) and *Poncirus trifoliate* (Wu et al. 2010b; Wu et al. 2012a), we might have expected that the down regulation of the ADC transcript would have had a markedly depressive effect upon mycorrhizal formation. The results show that although AMF colonization decreases drastically, it does not disappear. El Ghachtouli et al. (1996) mentioned that higher plants and bacteria use both, the ADC and ODC pathways for synthesizing polyamines, while fungi possess only ODC for polyamine biosynthesis. In our study we have used ARNi silencing associated to ADC pathway in roots, consequently ODC pathway could be active in soybean roots synthesizing put.

In order to gain further insight in the participation of put in the process of mycorrhization we decide to down regulate the expression of DAO. The degradation of put is done by means of DAO, because it oxidizes the primary amino group of put and cadaverine (Smith and Barker 1988). In GmDAO silencing plants where the ADC transcript is expressed the mycorrhizal colonization is similar to control plant, despite DAO transcript is not expressed (Fig. 4, Table 2).

Our results showed that down regulation of the GmADC transcript in roots had a profound negative effect on mycorrhiza colonization (Table 2) and also affected the normal development of the plant (Fig. 5). The observed phenotypes included stunted growth; no stem branching and small leaves in both I-1 and UI-4 genotypes (Fig. 5). By contrast, down regulation of the GmDAO transcripts in roots were similar to the control K-599, in both I-1 and UI-4 genotypes. Similar results were observed in an amiR: ADC line of NM - *A. thaliana* by Sánchez-Rangel et al. (2016), suggesting that ADC play a crucial role in plant growth and development during life span. To our knowledge, this is the first time that using this methodology we can observe some phenotypic evidence of the silencing at the shoot level in AM soybean.

Flavonoids and PAs compounds have been mentioned as signaling factors exuded by host roots and allowing colonization by the AM fungi (El Ghachtouli et al. 1996; Rengel 2002; Sugiyama and Yazaki 2014; Rezvanypour et al. 2015). In our study we observed that endogenous concentrations of flavonoids and PAs in roots were differentially regulated between NM improved and unimproved soybean plants. Thus a lower flavonoids and PAs metabolites level were found in UI-4 than I-1. Interestingly with AMF colonization a significantly higher increase in these metabolites was evident in UI-4 than I-1, suggesting that soybean domestication influenced the ability of response of flavonoids and PAs in M soybean roots.

5 Conclusion

Our results suggest that domestication in soybean affected mycorrhizal colonization and arbuscule formation. This effect seems to be mediated by the endogenous roots levels of flavonoids and PAs especially put, since the improved genotype needs more amount of them to reach the same percentage of colonization than unimproved soybean genotypes. In order to confirm this hypothesis we are conducting controlled studies that include a higher number of M and NM improved soybean genotypes to test flavonoids and PAs metabolites in roots, as selection criteria of soybean genotypes with increased AMF colonization capacity.

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