SHORT REPORT

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Exogenous polyamines affect mycorrhizal development of *Glomus mosseae*-colonized citrus (*Citrus tangerine*) seedlings

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ABSTRACT: To increase mycorrhizal colonization of citrus, exogenous polyamines (PAs) including putrescine (Put), spermidine (Spd), and spermine (Spm) were applied to three-month-old *Glomus mosseae*-colonized *Citrus tangerine* seedlings. Three months after the application of PAs, all the seedlings increased mycorrhizal colonization and entry points, Put and Spm treatments significantly increased the number of arbuscules, and Spd and Put treatments notably increased the number of vesicles. Applications of exogenous Put and Spd, but not Spm, markedly increased shoot and root dry weights of the mycorrhizal seedlings. PAs significantly increased leaf glucose content of mycorrhizal seedlings, and Put and Spd treatments markedly increased leaf sucrose content of mycorrhizal seedlings. Spm treatment significantly reduced root sucrose content of mycorrhizal seedlings, and only Put treatment significantly increased root glucose content of mycorrhizal seedlings. Compared to the sole mycorrhizal inoculation, exogenous PAs significantly reduced allocation of sucrose to root, and additional Spd significantly decreased allocation of glucose to root. The results demonstrated that exogenous PAs applications could improve mycorrhizal development of citrus seedlings, possibly due to changes of leaf and root sugar content.

KEYWORDS: arbuscular mycorrhizal fungi, glucose, putrescine, spermine, spermidine, sucrose

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are obligate biotrophs which can form mutualistic symbioses, viz., arbuscular mycorrhiza (AM), with the roots of higher plants[1]. AMF can provide the hosts with water and essential nutrients. In return, photosynthetic carbon of the hosts is transported into the endosymbiotic fungi[2].

Citrus is one of the important fruit trees in the Southern China. In the natural field, citrus plants exhibit less root hairs, and thus strongly depend on AMs to substitute partly the function of root hairs[3]. Mycorrhizal colonization of citrus trees in the field of the Three-Gorge region of China was generally less than 10%, but mycorrhizal colonization of Japanese citrus trees was over 20%[4]. It is therefore important to increase mycorrhizal development of citrus in China.

Aliphatic polyamines (PAs), such as diamine putrescine (Put), triamine spermidine (Spd), and tetramine spermine (Spm), are low molecular mass polycations found in all living organisms[5]. Plant PAs have been suggested to play important roles in morphogenesis, growth, embryogenesis, organ development, leaf senescence, and abiotic- and biotic-stress responses[6]. A previous experiment showed that difluoromethylornithine treatment, with reversion by exogenous Put, strongly inhibited AM infection of *Pisum sativum*[7]. Exogenous Spm, Spd, and Put had significant effects on spore germination rate and hyphal growth of *Glomus mosseae* and *Gigaspora margarita* in vitro culture conditions at concentrations of 50–200 mg/l[8]. Thus, it seems that PAs might regulate mycorrhizal development or stimulate both mycorrhizal formation and hyphal growth. Exogenous Spm applied to *Gigaspora margarita*-colonized micropropagated grape plantlets significantly elevates AM colonization[9]. Carbon allocation patterns in citrus plants are also affected by AMF colonization[10]. Since AM symbiosis depends on the photosynthetic carbon of the host, better mycorrhizal development of hosts by exogenous PAs might depend on both plant carbon allocation to root and carbon contents of the hosts. However, the roles of PAs in plant-fungal symbiotic interactions, especially citrus-mycorrhizal interactions, are not well understood.

The objectives of the present work were to examine the effects of exogenous PAs on mycorrhizal development of citrus seedlings, and to verify if such alterations are related to the sugar content or sugar allocation.

MATERIALS AND METHODS

Spore propagation and pot culture

The AM fungus used here was *Glomus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe (Serial number: BGC XZ02A), commercially provided by the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences. It was selected because *G. mosseae* results in higher growth and better drought resistance of *Citrus tangerine* seedlings than *G. diaphanum*, *G. etunicatum*, and *G. versiforme*[11]. The AM spores were collected from the rhizosphere of *Yucca gloriosa* in Dangxiong, Sitsang Province, China, and were extracted by wet sieving and sucrose density gradient centrifugation method[12]. The classified AM spores were propagated by pot culture. A hundred isolated spores were inoculated into 2-l plastic pots containing an autoclaved mixture of soil and river sand (1:3, v/v). The propagated host was *Sorghum vulgare* Pers. After 16 weeks of spore propagation, the shoots were removed, and the infected roots and growth substrates including spores and extraradical hyphae were collected as the mycorrhizal inocula containing 33 spores/g.

Plant culture

Seeds of citrus (*Citrus tangerine* Hort. ex Tanaka) were surface-sterilized with 70% alcohol for 5 min, rinsed four times with distilled water and germinated on wet filter paper in Petri dishes at 25 °C. The 7-day-old seedlings were transferred into plastic pots (13 cm in depth and 18 cm in mouth diameter) containing 3.2 kg of autoclaved (121 °C, 0.11 MPa, 2 h) growing mixture of yellow soil, vermiculite, and sphagnum (5:2:1, v/v/v) on 20 March 2009. The potted substrate had been inoculated with *G. mosseae* before being transplanted by placing 16 g of inocula (growth substrates, 528 spores, hyphae, and infected roots) 5 cm below the surface of the substrate. Non-AMF pots supplied with 16 g sterilized substrate served as the control. All the pots were placed in a plastic greenhouse at the College of Horticulture and Gardening, Yangtze University, Jingzhou, China from March to September 2009. The photo flux density ranged from 600 to 850 µmol/m^2/s during the entire experiment, the average day/night temperature was 26 °C/18 °C, and the relative humidity was 65–95%.

Experimental design

In a completely random arrangement, five treatments with five replicates each for a total of 25 pots (three seedlings/pot) were as follows: (1) non-exogenous PA plus non-mycorrhizal control (non-AMF + non-PA), (2) AM *G. mosseae* only (AMF+ non-PA), (3) exogenous Put plus *G. mosseae* (AMF + Put), (4) exogenous Spd plus *G. mosseae* (AMF + Spd), and (5) exogenous Spm plus *G. mosseae* (AMF + Spm). Put, Spd, or Spm (Sigma, USA) was exogenously applied (300 ml of 100 mg/l PA) to the soil media after 3 months of AM inoculation. The other treatments received 300 ml distilled water.

Measurements of parameters

The seedlings were harvested 3 months after the exogenous PAs treatments, and the shoots and roots were separated. Pieces of 1-cm length root, taken from the middle part of the roots, were cleaned with 10% KOH and stained with 0.05% trypan blue in lactophenol[13]. AM colonization and mycorrhizal structures such as entry points, vesicles, and arbuscules were microscopically examined and calculated[14].

The shoots and the remaining roots were dried in an oven at 70 °C for 60 h and these dry weights were recorded. The oven-dried plant matter was ground and sieved through a 0.5 mm sieve. The ground material (50 mg) was placed in a 10 ml centrifugal tube containing 4 ml 80% ethanol, incubated for 40 min at 80 °C, and centrifuged at 2500g for 5 min. The centrifugal residues were extracted again using the above procedure and both supernatants were used for glucose and sucrose assays. Glucose and sucrose contents were determined as described[15]. Allocation of sugar to root, introduced to describe the effect of PAs on the allocation of sugar, was calculated as $S_{\mathrm{root}}/(S_{\mathrm{leaf}} + S_{\mathrm{root}})$ where S_{leaf} and S_{root} are the amounts of sugar in leaf and root of citrus seedlings, respectively.

Statistical analysis

The experimental data were analysed by ANOVA with SAS 8.1 software. Least significant differences (LSD) were used to compare the means at 5% level.

RESULTS AND DISCUSSION

Mycorrhizal development of citrus

Mycorrhizal structures occurred in the roots of the citrus seedlings inoculated with AMF solely or in combination with exogenous PAs (Fig. 1b, c), but not in that of non-AMF + non-PA seedlings (Fig. 1a).

Table 1 Effect of exogenous polyamines on mycorrhizal development of *Glomus mosseae*-colonized *Citrus tangerine* seedlings.

Treatment	RC (%)	Ves	Arb	EP
AMF+Put	47.8^{a}	17.8^{a}	4.4^{a}	2.0^{a}
AMF+Spm	44.6^{b}	6.4^{b}	3.6^{b}	1.3^{b}
AMF+Spd	39.0^{c}	16.5^{a}	0.5^{c}	0.9^{c}
AMF+non-PA	35.8^{d}	6.0^{b}	0.5^{c}	0.7^{d}

Same letter within each column indicates no significant difference among treatments (LSD, $p < 0.05$).
RC = root colonization; Ves = vesicles (no./cm root); Arb = arbuscules (no./cm root); EP = entry points (no./cm root)

Exogenous PAs did affect the mycorrhizal development of *G. mosseae*-colonized seedlings (Table 1). All the PAs markedly increased AM colonization and entry points. The ranking of three PAs for both AM colonization and entry points was as follows: Put > Spm > Spd. The results agree with previous findings that exogenous Put, Spm, and Spd treatments significantly increased the mycorrhizal infection in the myc^{+} pea (*Pisum sativum*) and the number of appressoria formed in the myc^{-} pea[16]. The present study also observed that Put and Spm treatments significantly increased the number of arbuscules, and Spd and Put treatments notably increased the number of vesicles, suggesting that vesicles and arbuscules may be stimulated by different PA species or be dependent on PA species. In the three PAs species, the stimulated effects were highest in Put-applied seedlings, which may be explained in two ways: that Put is a precursor in the Spd and Spm biosynthesis[17], or that Put is the most abundant PA in un-germinated spores of *G. mosseae*[18]. Recent experiments indicate that the PA stimulation to mycorrhiza could be reversed when the exogenous PA biosynthesis inhibitor, methylglyoxal bis-guanylhydrazone, is included in the PA treatment[9]. These results imply that exogenous PA, especially Put, exhibits a significantly stimulating effect on mycorrhizal development in ex vitro conditions and is an important regulatory factor in plant-AM fungus interactions.

Citrus growth

A sole AMF inoculation significantly increased the shoot and the root dry weights of the citrus seedlings (Table 2) in agreement with a previous report using *C. tangerine* inoculated with *G. mosseae*[11]. Exogenous Put and Spd applications but not Spm markedly increased the shoot and the root dry weights of the mycorrhizal seedlings, suggesting that the growth promotion of mycorrhizal plants by application of PAs depends on exogenous PA species. In addition, exogenous Spm treatment significantly increases the biomass production of *Gigaspora margarita*-colonized grape (*Vitis vinifera*)[9]. Improved growth of mycorrhizal plants because of PAs application may be attributed to mycorrhizal improvement, increase of endogenous IAA levels[19], or the improvement of root system architecture[20].

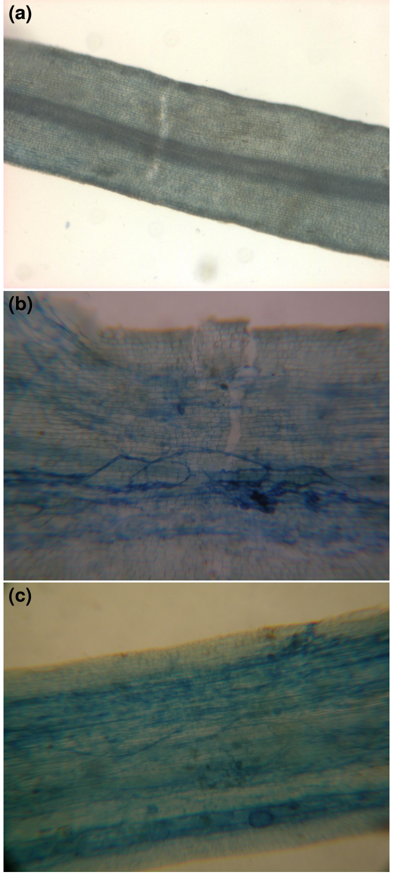

Fig. 1 Mycorrhizal infection of citrus (*Citrus tangerine*) seedlings (a) non-mycorrhizal infection of non-AMF seedlings; (b) mycorrhizal infection of alone AMF seedlings; (c) mycorrhizal infection of AMF + Put seedlings.

Sucrose and glucose content of citrus

As obligate symbionts, AMF must obtain most of their carbon from the host roots[21]. The amounts of carbon allocated to the fungus vary from 4% to 20% of the plant's total carbon budget[22]. The present experiment showed that the sole AMF inoculation markedly increased leaf sucrose content and leaf and

Table 2 Effects of exogenous polyamines and *Glomus mosseae* on shoot dry weight, root dry weight, sucrose and glucose contents, and allocation of sugar to root of *Citrus tangerine* seedlings.

Treatment	Dry weight (g/plant)		Sucrose content (mg/g)		Glucose content (mg/g)		Allocation of sugar to root (%)	
	Shoot	Root	Leaf	Root	Leaf	Root	Sucrose	Glucose
AMF+Put	1.18^{a}	0.71^{a}	7.87^{a}	4.47^{ab}	27.0^{b}	24.0^{a}	34.6^{c}	47.1^{a}
AMF+Spm	1.11^{ab}	0.58^{bc}	6.50^{b}	3.79^{b}	25.6^{b}	22.4^{ab}	36.9^{c}	46.8^{a}
AMF+Spd	1.18^{a}	0.62^{b}	7.45^{a}	4.34^{a}	32.0^{a}	20.4^{c}	36.8^{c}	38.9^{c}
AMF+non-PA	1.02^{b}	0.55^{c}	6.36^{b}	4.27^{a}	23.9^{c}	21.0^{bc}	40.2^{b}	46.8^{a}
Non-AMF+non-PA	0.80^{c}	0.43^{d}	4.47^{c}	4.42^{a}	21.1^{d}	17.0^{d}	49.7^{a}	44.6^{b}

Same letter within each column indicates no significant difference among treatments (LSD, $p < 0.05$).

root glucose content, compared to the non-AMF+non-PA treatment (Table 2) in agreement with the previous studies on rose and citrus[11,23]. AMF acquire hexose and transform it into trehalose and glycogen[21]. In the present experiment, a sole AMF inoculation significantly increased root glucose content but not sucrose content, implying that more sucrose may be transformed into glucose to sustain the mycorrhizal development. Compared to the sole AMF treatment, additional PAs obviously affected the sucrose and glucose contents of leaf and root (Table 2); leaf sucrose content increased by 23.7% and 17.1% in the Put and Spd applied seedlings, respectively. Spm treatment significantly reduced the mycorrhizal root sucrose content by 11.2%. Also, Spm, Spd, and Put significantly increased the leaf glucose content of mycorrhizal seedlings by 7.0%, 34.0%, and 13.0%, respectively, and only Put treatment in the three PAs significantly increased the root glucose content of mycorrhizal seedlings by 14.5%. Similarly, exogenous Spd has been shown to increase soluble sugar (sucrose represents about 50% of the total soluble sugar) content in both leaves and inflorescences of grape[24]. Certain PA changes are correlated with changes in the structure and function of the photosynthetic apparatus[25]. Therefore, these results showed that exogenous PAs altered the sucrose and glucose contents in mycorrhizal plants to regulate mycorrhizal development.

Allocation of sucrose and glucose to root

Exogenous PAs and AMF significantly affected the allocation of sucrose and glucose to roots. Compared with the non-AMF+non-PA, the sole AMF treatment significantly reduced the allocation of sucrose to root but increased the allocation of glucose to root, suggesting that mycorrhizal presence might result in more root sucrose transformed into hexose (Table 2). Therefore, the increased allocation of glucose to root due to mycorrhization might result from sucrose translation. AMs take up glucose from hosts mainly to maintain its growth and development[21,26,27]. Therefore, the increased allocation of glucose to root would be propitious to AM establishment. Compared to the sole AMF inoculation, exogenous PAs treatments significantly reduced the allocation of sucrose to root, suggesting that more sucrose contents translate into hexose (Table 2). Additional Put and Spm did not affect the allocation of glucose to roots, but Spd significantly decreased the allocation of glucose (Table 2). However, the fact that PAs did not affect or significantly reduced the allocation of glucose/sucrose to root cannot be explained, because to date, no information is available about the regulatory effect of PAs on the allocation of sugar to root in higher plants. It seems that AMs may participate in the effect because PAs regulate mycorrhizal development. Further studies will be conducted to clarify the effect of dual AMF and PAs on the allocation of sugar to root.

CONCLUSIONS

In general, exogenous PAs obviously increased mycorrhizal development of *G. mosseae*-colonized citrus seedlings, which may be related to the leaf and root sugar content of but not to the allocation of sugar to the root. As a result, PAs especially Put can be regarded as one important regulator of mycorrhiza and growth in citrus trees in the field. However, the optimal concentration of PAs in mycorrhizal citrus needs to be selected because too high a concentration (of 500 mg/l) of PAs notably inhibited hyphal growth of AMF[8].

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