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Effects of Exogenous Putrescine on Mycorrhiza, Root System Architecture, and Physiological Traits of *Glomus mosseae*-Colonized Trifoliate Orange Seedlings

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Abstract

Putresince (Put) as one of the important polyamines (PAs) has been identified to regulate mycorrhizal development of citrus plants. The present study was to screen an efficient concentration of Put application at the range of 0.05-1 mM on the trifoliate orange (*Poncirus trifoliata*) seedlings colonized by *Glomus mosseae*, in terms of growth, root system architecture, and chlorophyll and carbohydrate contents. Compared to the non-Put treatment, all the Put treatments, especially 0.05 mM Put, significantly increased mycorrhizal colonization of tap root in addition to first, second, and third order lateral roots. The mycorrhizal seedlings treated by 0.05, 0.1, and 1 mM Put showed greater growth (stem diameter, height, leaf number, and fresh mass) and root morphological properties (tap root length, projected and surface areas, and volume) and higher numbers of first, second, and third order lateral roots. Bio-molecules like chlorophyll *a*, total chlorophyll, and carotenoid contents of the seedlings were significantly increased by the Put treatments at 0.05-1 mM. All exogenous Put application at the range of 0.05-1 mM significantly decreased sucrose contents but increased glucose contents of leaves and roots. This study suggests that exogenous Put can significantly improve growth performance and root system architecture, besides changes in physiological traits of AMF seedlings. The 0.05 mM concentration of Put showed the best effects.

Keywords: arbuscular mycorrhizal fungi, citrus, glucose, lateral root, polyamine, sucrose

Introduction

Polyamines (PAs) mainly including spermine (Spm), spermidine (Spd), and putrescine (Put) are a kind of organic polycations found in all compartments of the plant cell (Mapelli *et al*., 2008). PAs in plants are involved in a variety of physiological and developmental processes, such as regulation of cell proliferation, somatic embryogenesis, flower and fruit development, replication, transcription, translation, membrane and cell wall stabilization, chromatin organization, ribosome biogenesis, and programmed cell death (Hussain *et al*., 2011). PAs also act as a mysterious modulator involved in plant responses to several stresses. As a result, PAs are widely used as a new type of plant growth regulators.

Arbuscular mycorrhizal fungi (AMF), belonging to thephylum Glomeromycota, are obligate biotrophs and can form the mutualistic symbiosis with 80% of the terrestrial plants (Bainard *et al*., 2011). The arbuscular mycorrhizal (AM) symbiosis contributes the transfer of mineral nutrients and water from the soil to the host plant (Smith and Read, 2008). In return, the symbiosis obtains carbohydrates from the host plants to maintain fungal growth. A study showed that Put was the most abundant PA specie in un-germinated spores of *G. mosseae* (El Ghachtouli *et al*., 1996a). Spore germination rate and hyphal growth of *G. mosseae in vitro* were stimulated by exogenous Put at the range of 50-200 mg/L but were inhibited at 500 mg/L (Zhang *et al*., 2003). On the other hand, PAs may be an important regulatory factor in formation of AM symbiosis. In trifoliate orange (*Poncirus trifoliata*) seedlings, exogenous Spd, Spm, and Put (100 mg/L) obviously altered mycorrhizal colonization, whereas Put was the most effective (Wu and Zou, 2009). Exogenous Put (100 mg/L) also participated in regulating mycorrhizal development of *Citrus tangerine* inoculated with *Glomus mosseae* (Wu *et al*., 2010c). As stated above, Put has the best effect on mycorrhizal development of citrus plants, which is dependent on concentration of Put. Therefore, screening an efficient concentration of Put is an urgent problem in mycorrhizal research of citrus and will also provide technical reference for the regulation of citrus mycorrhiza and plant growth.

The purpose of the present study was to screen an efficient concentration of exogenous Put at the range of 0.05-1 mM in the trifoliate orange seedlings colonized by *G. mosseae*, in terms of the analysis of growth, root system architecture (RSA), chlorophyll contents, and carbohydrate contents.

Materials and methods

Experiment design

The experiment was one factorial design with completely randomized arrangement, which consisted of

0, 0.05, 0.1, 0.5, and 1.0 mmol/L concentrations of Put (namely, Put-0, Put-0.05, Put-0.1, Put-0.5, and Put-1.0) respectively applied to mycorrhizal trifoliate orange seedlings. Each treatment was repeated three replicates for a total of 15 pots.

Plant culture

Seeds of trifoliate orange were disinfected in 70% of alcohol for 5 minutes, rinsed four times with distilled water and sowed in plastic pots (19 cm upper diameter × 17 cm height × 13 cm bottom diameter) filled with 3.2 kg of autoclaved (0.11 Mpa, 121°C, 2 h) yellow soil. Fifteen gram inoculum of *Glomus mosseae* (Nicolson & Gerdemann) Gerdemann & Trappe containing infected root segments of white clover (*Trifolium repens*), extraradical hyphae, and spores, was inoculated into the pots at the time of sowing. The pots were placed in a naturally ventilated plastic house from March 15 to August 24, 2011.

After 94 days of the acclimatization, the inoculated seedlings per pot were subjected to exogenous Put treatment with 250 mL of 0.05, 0.1, 0.5, and 1.0 mmol/L Put (American Sigma Company) concentrations, respectively. Non-Put pots were supplied with 250 mL distilled water as the control. All the mycorrhizal inoculated seedlings were irrigated weekly with the exogenous Put. During the entire test period, the seedlings were not supplied with any nutrients.

Variable measurements

After 10 weeks of exogenous Put treatment, shoots and roots of the mycorrhizal seedlings were separately harvested on August 24, 2011. Plant height (cm), stem diameter (cm), and leaf number per plant were determined before the harvest.

The root systems were gently cleaned with the tap water and scanned by the Epson Perfection V700 Photo Dual Lens System (J221A, Indonesia, Seiko Epson Corporation). The obtained photographs of the root systems were analyzed with the professional WinRHIZO software in 2007 version (Regent Instruments Inc., Quebec, Canada), and the traits of the RSA including total length, projected area, surface area, average diameter, and volume were recorded. The length of tap root was determined using a vernier caliper. Lateral root numbers at all levels were manually counted.

Chlorophyll content was extracted with 80% of aceton from 0.15 g of fresh leaf samples in the dark. The absorbance was measured at 663, 646 and 470 nm in a UV/VIS spectrophotometer. Several chlorophyll concentrations were calculated using the following equations (Li, 2000):

Chlorophyll $a = 12.21 \times A_{663} - 2.81 \times A_{646}$

Chlorophyll $b = 20.13 \times A_{646} - 5.03 \times A_{663}$

Carotenoid $= [1000 \times A_{470} - 3.27 \times \text{Chlorophyll } a - 104 \times \text{Chlorophyll } b]/229$

Total chlorophyll = chlorophyll *a* + chlorophyll *b*

Glucose and sucrose of leaf and root samples were extracted with 80% of ethanol and determined by the colorimetric method of Zhang and Zai (2004) at 460 and 480 nm, respectively.

Approximate 1-cm root segments from tap root and several order lateral roots were cleaned with 10% (w/v) of KOH and stained with 0.05% (w/v) of trypan blue in lactoglycerol (Phillips and Hayman, 1970). The stained root segments were examined under a biological microscope, and then the root mycorrhizal colonization was estimated using the formula of Wu and Zou (2009).

Statistical analysis

Data were analyzed using ANOVA (SAS, Version 8.1), and Duncan's multiple range test ($p<0.05$) was used to compare significance of the means.

Results and discussion

Mycorrhizal colonization of different order root systems

Previous studies have shown that PAs especially Put stimulated mycorrhizal formation and hyphal growth in *Pisum sativum* (El Ghachtouli *et al*., 1996b), *Citrus tangerine* (Wu *et al*., 2010c), and *Poncirus trifoliata* (Wu *et al*., 2010b). In the present study, compared with the non-Put treatment, all the Put treatments significantly increased the mycorrhizal colonization of the tap root, first-order later root, second-order later root, and third-order later root, except for the first-order later root treated by Put-1, the second-order and third-order later roots by Put-0.5 (Tab. 1). Meanwhile, compared to other Put treatment, the Put-0.05 treatment exhibited significantly highest effects on root mycorrhizal colonization. These results suggest that exogenous Put promoted mycorrhizal colonization at all root levels, although affected by varying Put levels.

Plant growth

Compared with the non-Put control, all four exogenous Put treatments significantly increased plant height, stem diameter, leaf number per plant, and shoot, root, and total dry weights (Tab. 2). These results confirmed the studies that PAs played important roles in plant growth

Tab. 1. Root mycorrhizal colonization of different order root systems in trifoliate orange seedlings treated by different concentrations of Put (mM)

Treatment	Mycorrhizal colonization (%)			
	Tap root	Lateral root		
		First-order	Second-order	Third-order
Put-0	13.0±1.6c	18.0±0.6c	20.0±2.2c	14.0±2.5c
Put-0.05	30.0±5.5a	34.3±3.8a	38.0±2.2a	46.3±3.8a
Put-0.1	27.0±3.0ab	27.0±4.2b	29.3±4.7b	27.8±7.8b
Put-0.5	23.7±1.1b	27.0±5.2b	18.7±1.7c	18.8±2.4c
Put-1	31.7±3.2a	15.0±1.4c	27.3±4.3b	28.3±4.9b

Note: Means ± SE (*n*=3) followed by the same letter within a column are not significant difference between various treatments at 5% level

Tab. 2. Effect of exogenous Put (mM) on growth traits of trifoliate orange seedlings colonized by *Glomus mosseae*

Treatment	Stem diameter (cm)	Height (cm)	Leaf number per plant	Dry weight (g)		
				Shoot	Root	Total
Put-0	0.238±0.004d	28.6±2.7c	25.1±0.7d	0.68±0.04c	0.23±0.01d	0.91±0.04c
Put-0.05	0.298±0.013ab	44.4±1.6a	33.3±1.5a	1.21±0.09a	0.36±0.01a	1.57±0.10a
Put-0.1	0.287±0.014b	37.4±2.5b	29.7±1.2b	1.01±0.02b	0.32±0.01b	1.33±0.01b
Put-0.5	0.269±0.007c	30.2±3.2c	25.9±1.6cd	0.71±0.05c	0.26±0.01c	0.97±0.05c
Put-1	0.308±0.010a	40.2±1.4ab	28.3±2.0bc	1.18±0.07a	0.32±0.02b	1.50±0.08a

Note: Means ± SE (*n*=3) followed by the same letter within a column are not significant difference between various treatments at 5% level

and development (Movahed *et al*., 2012; Scholten, 1998). Significant effect of Put on the growth traits of trifoliate orange ranked in the order of Put-0.05 > Put-1.0 > Put-0.1 > Put-0.5 (Tab. 2). The result is in agreement with the finding of Movahed *et al*. (2012) in strawberry applied by 0.5, 1, and 1.5 mM Put. The growth improvement might be due to the fact that Put acts as a hormonal second-messenger of cell proliferation and differentiation in many processes (Steiner *et al*., 2007).

Root system architecture

Plant roots have the roles in nutrient and water uptake, anchoring and mechanical support, storage functions, and the major interface between the plant and various biotic and abiotic factors (Smith and de Smet, 2012). Meanwhile, root system architecture (RSA) is a key determinant of nutrient- and water-use efficiency in plants. There are free and macromolecule-bound PAs presented in root systems, which play a role in root apex and during lateral and adventitious root formation (Couée *et al*., 2004). In the present study, the Put-treated seedlings generally exhibited significantly higher total length, tap root length, projected area, surface area, and volume, except root average diameter, compared to the non-Put controls (Tab. 3). The results are consistent with the result of Ben-Hayyim *et al*. (1996) in tobacco and indicate that Put also is a key factor of RSA regulation in trifoliate orange.

In the present study, the mycorrhizal trifoliate orange seedlings possessed three orders of lateral roots, and the order in number of lateral roots ranked as second-order > first-order > third-order (Tab. 4). In addition, all exogenous Put applications notably increased the numbers of the first-order lateral root and second-order lateral root, except for the second-order lateral root under Put-0.5 (Tab. 4). In contrast, only Put-0.05 and Put-1 treatments significantly increased the number of third-order lateral root in all orders. Meanwhile, 0.05 mM concentration of Put exhibited the best effects on numbers of lateral roots in all orders. This is consistent with the finding of Sun *et al*. (2010), who observed that the optimal concentration of Put to promote the number of lateral root in *Lactuca sativ*a was 0.05 mM among 0.05-1 mM. The increase in numbers of lateral roots at all orders caused by exogenous Put can, in some instances, be attributed to stimulating meristematic activity (Schwartz *et al*., 1986), inducing NO signal (Sun *et al*., 2010), and regulating levels of endogenous auxins and cytokinins (Sharma *et al*., 1997).

Tab. 3. Effects of exogenous Put (mM) on root morphological characteristics of trifoliate orange seedlings colonized by *Glomus mosseae*

Treatment	Total length (cm)	Tap root length (cm)	Average Diameter (mm)	Projected area (cm^2)	Surface area (cm^2)	Volume (cm^3)
Put-0	273±35b	20±3b	0.41±0.02a	12±2c	36±5b	0.38±0.06c
Put-0.05	468±53a	30±3a	0.42±0.05a	20±4a	62±8a	0.66±0.07a
Put-0.1	452±32a	29±2a	0.44±0.01a	20±2a	63±5a	0.69±0.06a
Put-0.5	315±12b	28±2a	0.45±0.03a	14±1bc	45±4b	0.51±0.08b
Put-1	446±43a	31±2a	0.41±0.02a	18±2ab	57±5a	0.59±0.06ab

Note: Means ± SE (*n*=3) followed by the same letter within a column are not significant difference between various treatments at 5% level

Tab. 4. Effect of exogenous Put (mM) on numbers of different order lateral roots of trifoliate orange seedlings colonized by *Glomus mosseae*

Treatment	Number of lateral root		
	First-order	Second-order	Third-order
Put-0	46±2d	98±19c	3±3c
Put-0.05	63±2a	244±20a	23±4a
Put-0.1	56±2bc	193±28b	2±1c
Put-0.5	53±3c	130±10c	3±2c
Put-1	58±1b	202±22b	13±3b

Note: Means ± SE (*n*=3) followed by the same letter within a column are not significant difference between various treatments at 5% level

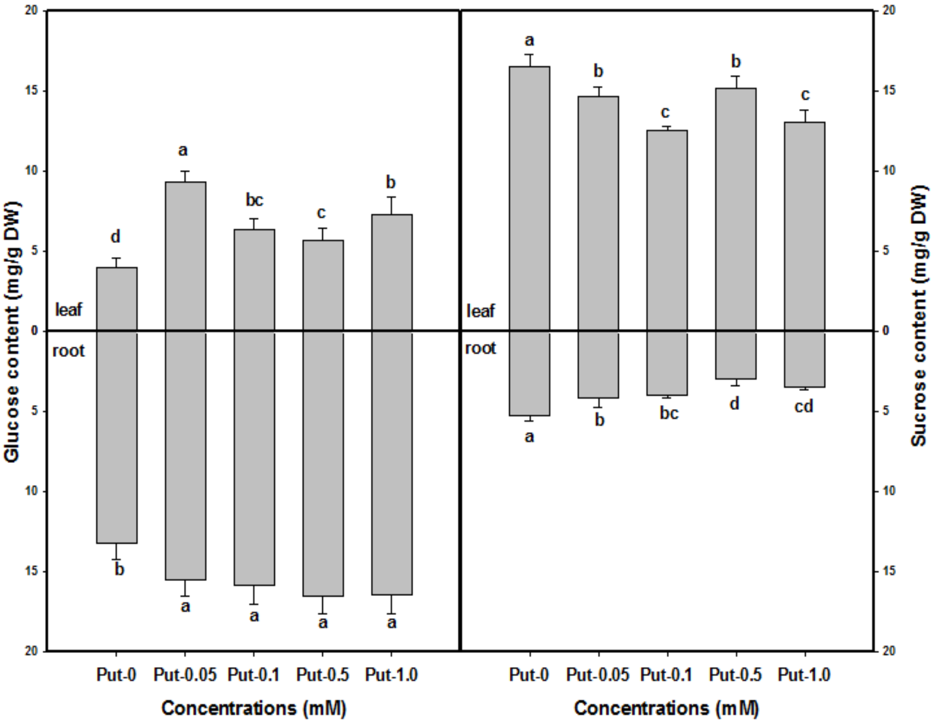

Fig. 1. Effects of exogenous Put on sucrose and glucose contents in leaf and root of trifoliate orange seedlings colonized by *Glomus mosseae*. Data (means ± SE, *n*=3) followed by the same letter above the bars are not significant difference between various treatments at 5% level

Chlorophyll

PAs are involved in photosynthetic functions (Margosiak *et al*., 1990). In the present work, supplied exogenous Put significantly increased contents of chlorophyll *a*, carotenoid, and total chlorophyll, compared with the non-Put treatment (Tab. 5). Meanwhile, 0.05 mM concentration of Put represented the best effects. Content of chlorophyll *b* was not affected by the exogenous Put treatments, except that Put-0.5 application significantly decreased content of chlorophyll *b*. This is in agreement with the result of Unal *et al.* (2008) in *Phtyscia semipinnate* by additional PAs under UV-A radiation. In fact, chloroplasts themselves enrich a certain amount of PAs including Put, which can benefit formation of chlorophyll, especially chlorophyll *a* but not chlorophyll *b* (Shu *et al*., 2012). However, high concentrations of PAs may also destroy the structure of chloroplasts, which is dependent on light conditions. In the present study, 1 mM concentration of Put still protects the structure of chloroplast, and a 0.05 mM concentration of Put would have a better ability to increase concentrations of chlorophyll in mycorrhizal trifoliate orange seedlings.

Carbohydrates

Not only chloroplasts but also photosynthetic subcomplexes possess certain PAs involved in stabilizing structure and function of the photosynthetic apparatuse (Demetriou *et al*., 2007), resulting in an increased photosynthetic rate. The present study showed that exogenous Put significantly decreased leaf and root sucrose content but increased leaf and root glucose content, compared with the non-Put control (Fig. 1). Chen *et al.* (2011) found the decrease of leaf carbohydrates and root total sugar and sucrose in *Cucumis sativus* supplied with exogenous Spd. These results are in agreement with the finding of Wu *et al.* (2010a) in *Citrus tangerine* colonized by *G. mosseae*. On the other hand, root mycorrhizal symbiosis could change the allocation of plant carbohydrates (Pang and Paul, 1980). Typically, AMF need to consume hexose

Tab. 5. Effects of exogenous Put (mM) on chlorophyll and carotenoid contents of trifoliate orange seedlings colonized by *Glomus mosseae*

Treatment	Chlorophyll *a* (mg/g FW)	Chlorophyll *b* (mg/g FW)	Total chlorophyll (mg/g FW)	Carotenoid (mg/g FW)
Put-0	1.28±0.10c	0.63±0.06a	1.91±0.11c	0.21±0.03b
Put-0.05	1.87±0.03a	0.58±0.01a	2.45±0.04a	0.37±0.04a
Put-0.1	1.75±0.09ab	0.56±0.14a	2.31±0.15a	0.35±0.09a
Put-0.5	1.68±0.09b	0.41±0.11b	2.09±0.10b	0.33±0.03a
Put-1	1.71±0.07b	0.63±0.03a	2.34±0.04a	0.32±0.04a

Note: Means ± SE (*n*=3) followed by the same letter within a column are not significant difference between various treatments at 5% level

to sustain its growth and development (Bago *et al*., 2003). Through the phloem shoot sucrose is transported down to reach the roots, where the sucrose is rapidly hydrolysed to glucose and fructose (Hammond and White, 2008). In addition, root mycorrhizal symbiosis mainly utilizes glucose, which is transformed into trehalose and glycogen for both mycorrhizal development and sink storage. Better root mycorrhizal colonization in the Put-treated seedlings suggests that the mycorrhizas might consume more glucose and thus form a mycorrhizal carbon sink. In such processes, mycorrhizal roots may require an increased sucrose via the phloem transport to the root. As a reult, exogenous Put would interact with arbuscular mycorrhizas to alter carbohydrate transport and decomposition. Further experiments on carbon consumption are required to confirm this hypothesis.

Conclusions

The present study showed that exogenous Put application at the range of 0.05-1 mM could obviously increase the infection of roots at all levels by *G. mosseae*. In addition, exogenous Put has the enhanced effects on plant growth, RSA, and contents of chlorophyll *a*, total chlorophyll, and carotenoid in the mycorrhizal seedlings. Exogenous Put may thus interact with AMF, thereby increasing glucose contents but decreasing sucrose contents in leaves and roots. A 0.05 mM Put concentration showed the best effects on regulating mycorrhizal colonization and growth of mycorrhizal plants.

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