

## Polyamines Participate in Mycorrhizal and Root Development of Citrus (*Citrus tangerine*) Seedlings

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### Abstract

A pot experiment was conducted to study the effects of difluoromethylornithine (DFMO, an irreversible inhibitor of putrescine (Put) formation) alone or combination with Put on mycorrhizal and root development of *Citrus tangerine* seedlings inoculated with *Glomus mosseae*. DFMO significantly inhibited plant growth (plant height, stem diameter, leaf number per plant, shoot and root dry weights), mycorrhizal (root colonization and the number of entry point, vesicle and arbuscule) and root development (total length, total projected area, total surface area and total volume), chlorophyll content, photosynthesis (photosynthetic rates, transpiration rates and stomatal conductance) and soluble protein, whereas the inhibition was partly or completely reversed by Put application to DFMO treatment. DFMO applied to mycorrhizal seedlings showed the decrease of P contents of leaves and roots, the decrease of acid, neutral and alkaline phosphatase activities of rhizosphere and the increase of available P content of rhizosphere, whereas application of exogenous Put to DFMO treatment partly unchained the effects of DFMO. DFMO strongly inhibited the allocation of glucose to root but stimulated the allocation of sucrose to root and guaiacol peroxidase (G-POD) activity of root, but these effects were reversed to a certain extent when Put was included in DFMO treatment. This study thus indicates that endogenous polyamines regulate mycorrhizal development of citrus seedlings through altering the allocation of carbohydrates to root, and also participate in root development, which is negatively related to root G-POD and may be due to inducing new protein synthesis.

**Keywords:** allocation of carbohydrates to root, arbuscular mycorrhiza, citrus, difluoromethylornithine, guaiacol peroxidase, polyamines, putrescine, root morphology

### Introduction

Polyamines (PAs), mainly including diamine putrescine (Put), triamine spermidine (Spd) and tetraamine spermine (Spm), are low molecular weight aliphatic polycations that exist in all eukaryotic cells (Kusano *et al.*, 2008). Put, the precursor of Spm and Spd, is formed either by the direct decarboxylation of L-ornithine through ornithine decarboxylase (ODC) or by decarboxylation from L-arginine to agmatine by arginine decarboxylase (ADC) (El Meskaoui and Trembaly, 2009). A number of experiments have used PA inhibitors to modulate endogenous PA metabolism, in order to research the role in plants (El Ghachtouli *et al.*, 1996a; El Meskaoui and Trembaly, 2009). Difluoromethylornithine (DFMO) is an irreversible inhibitor of ODC-catalyzed steps of Put formation (Hummel *et al.*, 2002). PAs play a critical role in various plant developments, and also involve in stress responses (Takahashi and Kakehi, 2010). Additionally, PAs regulate both root development of plants and plant-microbe interactions, but their functions still remain unclear (Walters, 2000; Hummel *et al.*, 2002; Coucé *et al.*, 2004).

Arbuscular mycorrhizal (AM) symbiosis, a mutualistic association between arbuscular mycorrhizal fungi (AMF)

and 80% of terrestrial plants, provides host plant with essential nutrients and water (Paszowski, 2006; Requena *et al.*, 2007). In turn, AM symbiosis receives photosynthetic carbon of host plant for its growth. The characteristics of AM symbiosis are that fungal hyphae enter the root and grow towards the inner cortex where they penetrate the cortical cells and form arbuscules (Limpens and Bisseling, 2003). It has been shown that AMF colonization can impact root development, with consequential effects on the anatomy, physiology and morphology of roots (Berta *et al.*, 2002; Gutjahr *et al.*, 2009). However, interaction between AMF and PAs on root development of citrus is poorly known. A recent study showed that exogenous PAs (100 mg l<sup>-1</sup>) obviously stimulated mycorrhizal colonization of *Poncirus trifoliata* seedlings (Wu and Zou, 2009). Optimal concentrations (50-200 mg l<sup>-1</sup>) of exogenous PAs had significantly positive effects on spore germination and hyphal growth of *Glomus mosseae* and *Gigaspora margarita* *in vitro* culture conditions (Zhang *et al.*, 2003). Sannazzaro *et al.* (2004) reported that Put biosynthesis occurred by two alternative pathways involving ODC and ADC activities in *Gigaspora rosea*. DFMO strongly inhibited mycorrhizal infection of *Pisum sativum* but did not prevent spore germination of *Gigaspora rosea* (El Ghachtouli *et al.*,

1996a; Sannazzaro *et al.*, 2004). The inhibition of DFMO was reversed by exogenous Put. To date, information regarding the effects of a PA biosynthesis inhibitor solely or combination with PA on mycorrhizal and root development of host plants is lacking.

The aim of the present study was to evaluate whether DFMO alone or combination with Put affected mycorrhizal and root development of citrus seedlings, and sequentially to clarify the function of PA on root and mycorrhizal development.

## Materials and methods

### *Plant growth and infection*

The experiment was performed in a plastic greenhouse, lacking the light and temperature equipments. The photo flux density ranged from 600 to 850  $\mu\text{mol m}^{-2} \text{s}^{-2}$  during the entire experiment. The average day/night temperature was 26/18°C; the relative humidity was 65%-95%.

Seeds of citrus (*Citrus tangerine* Hort. ex Tanaka) were allowed to germinate on wet filter paper in Petri dishes at 25°C, after surface-sterilization with 70% ethanol for 5 min. The seven-day-old seedlings were transferred into the plastic pots (15 cm in depth and 20 cm in mouth diameter) with 3.2 kg of autoclaved (121°C, 0.11 MPa, 2 h) growth mixture of yellow soil, vermiculite and sphagnum (5:2:1, v/v/v) on March 20, 2009. The potted substrates had been inoculated with *Glomus mosseae* (Nicol. and Gerd.) Gerdemann and Trappe (BGC XZ02A) before transplant by placing 16 g of inoculum (the mixture of soil, spores, hyphae and infected roots) 5 cm below the surface of the substrate. Non-AMF pots supplied with 16 g sterilized substrate as control. These inocula were provided by the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences. The experiment was performed from March 20 to September 20, 2009.

### *Experimental design*

In a completely random designed arrangement, four treatments with five replicates each for a total of 20 pots (three seedlings/pot) were as follows: 1) non-AMF control (non-AMF), 2) *G. mosseae* only (AMF), 3) *G. mosseae* plus exogenous DFMO (AMF + DFMO), and 4) *G. mosseae* plus exogenous DFMO combination with exogenous Put (AMF + DFMO + Put).

### *Exogenous DFMO and Put treatments*

After three months of AMF inoculation, all the mycorrhizal seedlings divided into three groups. Hereinto, the first group supplied 300 ml distilled water; the second group supplied only 300 ml 5 mg l<sup>-1</sup> DFMO; the other group supplied 300 ml 5 mg l<sup>-1</sup> DFMO, and then 300 ml 100 mg l<sup>-1</sup> Put was added after 45 days of DFMO supply to reverse the inhibition of DFMO. These Put and DFMO used here were provided by Sigma, USA.

### *Growth and root morphology measurements*

The seedlings harvested after six months of AMF inoculation, and plant height, stem diameter and leaf number per plant were recorded. The shoots and roots were separated; the shoots were over-dried at 75°C for 48 h. These intact roots were rinsed with distilled water and scanned using root automatism scan apparatus, EPSON Expression/STD 4800 scanner. The acquired root images were analyzed using WinRHIZO Pro 2007b (Regent Instruments Inc., Quebec, Canada) to obtain root parameters, such as total root length, total root projected area, total root surface area, average root diameter, total root volume, etc.

After root scanned, all the roots were divided into two: one root system per pot was utilized to analyze soluble protein, guaiacol peroxidase (G-POD) and mycorrhizal development; the other two root systems per pot were used to obtain the dry weight (75°C, 48 h).

### *Mycorrhizal development*

A small quantity of one-cm length fresh root pieces was cleared with 10% KOH and stained with 0.05% trypan blue in lactophenol, according to Phillips and Hayman (1970). AM colonization and mycorrhizal structures such as entry points, vesicles and arbuscules were microscopically examined and calculated (Wu *et al.*, 2008).

### *Chlorophyll and photosynthesis measurements*

Photosynthetic rates (Pn), transpiration rates (E) and stomatal conductance (g<sub>s</sub>) were evaluated using a portable photosynthesis system (Li-6400, Li-Cor, Lincoln, USA) on five replications per treatment from 9:40 to 10:30 am on September 19, 2009. Measurements were recorded when the total coefficient of variation was less than 0.5%. The air pumped into the Li-6400 was passed through a buffering gallon (2.5 l) to reduce the time for measurement stabilization. Chlorophyll content was determined by the method of Lichtenthaler (1987).

### *Allocation of glucose/sucrose to root determinations*

The oven-dried plant matters were 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 2,500 g for 5 min. The centrifugal residues were extracted again using the above procedure, and the two-time supernatant was combined for glucose and sucrose assays. Glucose and sucrose contents were performed as described by Zhang and Zai (2004). Allocation of glucose/sucrose to root was quantified based on the formula:

$$\text{Allocation of glucose/sucrose to root (\%)} = \frac{S_{\text{root}}}{S_{\text{leaf}} + S_{\text{root}}} \times 100$$

Where S<sub>leaf</sub> and S<sub>root</sub> are the values of glucose/sucrose in leaf and root, respectively.

### Plant phosphorus (P), available P and phosphatase of rhizosphere measurements

Plant P content was analyzed by the vanadate-molybdate-yellow method (Chapman and Pratt, 1961). Rhizospheric soils, which were loosely adhered to the roots, were air-dried and ground through a 1 mm sieve for the analyses. Determinations of available P and phosphatase activity of rhizosphere were according to Olsen *et al.* (1954) and Zhao and Jiang (1986). Soil acid, neutral or alkaline phosphatase was extracted by sodium acetate buffer (pH 5.0), citric acid-disodium hydrogen phosphate buffer (pH 7.0) or borate buffer (pH 10.0), respectively.

### Soluble protein and G-POD measurements

Plant fresh material was homogenized in 8 ml of 0.1 mol l<sup>-1</sup> phosphate buffer (pH 7.8) in an ice bath. The homogenate was centrifuged at 4,200g for 10 min, and the supernate was used for the assays. G-POD activity was determined using the method described by Amako *et al.* (1994). Soluble protein content was assayed by a previous procedure using bovine serum albumin as the standard (Bradford, 1976).

### Statistical analysis

The experimental data were statistically analyzed by one-way variance (ANOVA) with SAS 8.1 software. The Least Significant Differences (LSD) were used to compare the means at 5% level.

## Results and discussion

Mycorrhizal colonization is a dynamic process, which can be affected by secondary metabolites (Smith and Read, 2008). Sole DFMO significantly inhibited root colonization and the number of vesicles, arbuscules and entry points in the *G. mosseae*-colonized citrus seedlings

in the experiment (Tab. 1). Put was the most abundant PA in *G. mosseae* spores (El Ghachtouli *et al.*, 1996b). Since DFMO can irrevocably inhibit ODC-catalyzed steps of Put formation (Hummel *et al.*, 2002), the mycorrhizal inhibition due to DFMO showed that endogenous Put might participate in mycorrhizal development (Tab. 1). Wu and Zou (2009) observed that Put treated *Poncirus trifolata* seedlings colonized by *G. versiforme* exhibited higher root colonization than Spm and Spd treatments. DFMO inhibited hyphal growth of *G. mosseae* and spore germination (El Ghachtouli *et al.*, 1996b) but did not affect spore germination of *Gigaspora mosseae* (Sannazzaro *et al.*, 2004), implying that the effect of PA inhibitor on mycorrhiza formation depends on AMF species. In our study, the inhibition was reversed by exogenous Put application to DFMO treatment (Tab. 1), once again suggesting that PA is an important regulated factor in plant-AMF interaction. The results are in agreement with the findings of El Ghachtouli *et al.* (1996) in the *G. mosseae*-infected *Pisum sativum*.

In the present work, inoculation with *G. mosseae* significantly increased plant height, stem diameter, leaf number per plant, shoot and root dry weights of the citrus seedlings (Tab. 2). The result is consistent with the finding of Wu *et al.* (2007) in *C. tangerine*. The growth increase due to mycorrhization was limited notably by exogenous DFMO supply, which could be broken after application of Put.

Mycorrhizal symbionts depend upon their autotrophic plant hosts to supply carbon (Finlay, 2008). Mycorrhizal plants shifted an allocation of carbon to pools that are rapidly turned over roots and fungal hyphae (Rygielwicz and Andersen, 1994). In the present work, AMF inoculation notably increased chlorophyll content, Pn, *E* and *g<sub>s</sub>* of the seedlings (Tab. 3). DFMO treatment weakened the effects of AMF, but application of Put to DFMO reversed the ef-

Tab. 1. Effect of DFMO alone or combination with Put on mycorrhizal development of *Glomus mosseae*-colonized citrus (*Citrus tangerine*) seedlings

| Treatment    | Root colonization (%) | Vesicles (num./cm root) | Arbuscules (num./cm root) | Entry points (num./cm root) |
|--------------|-----------------------|-------------------------|---------------------------|-----------------------------|
| AMF          | 26.56b                | 1.4b                    | 2.1b                      | 1.6b                        |
| AMF+DFMO     | 20.33c                | 0.9c                    | 1.4c                      | 0.7c                        |
| AMF+DFMO+Put | 44.50a                | 1.7a                    | 2.2a                      | 2.1a                        |

Note: Means followed by the same letter within a column shows no significant difference among treatments (LSD<sub>0.05</sub>)

Tab. 2. Effects of DFMO alone or combination with Put on plant performance and biomass of *Glomus mosseae*-colonized citrus (*Citrus tangerine*) seedlings

| Treatment    | Plant height (cm) | Stem diameter (cm) | Leaf number per plant | Shoot dry weight (g) | Root dry weight (g) |
|--------------|-------------------|--------------------|-----------------------|----------------------|---------------------|
| Non-AMF      | 16.7d             | 0.296c             | 17.9c                 | 1.24b                | 1.11b               |
| AMF          | 21.6a             | 0.336a             | 21.4a                 | 1.71a                | 1.74a               |
| AMF+DFMO     | 18.6c             | 0.323b             | 19.4b                 | 1.30b                | 1.05b               |
| AMF+DFMO+Put | 20.0b             | 0.331ab            | 20.2ab                | 1.78a                | 1.59a               |

Note: Means followed by the same letter within a column shows no significant difference among treatments (LSD<sub>0.05</sub>)

Tab. 3 Effects of DFMO alone or combination with Put on chlorophyll and photosynthesis of *Glomus mosseae*-colonized citrus (*Citrus tangerine*) seedlings

| Treatment    | Chlorophyll<br>(mg/g) | Pn<br>( $\mu\text{mol}/\text{m}^2/\text{s}$ ) | E<br>( $\text{mmol}/\text{m}^2/\text{s}$ ) | $g_s$<br>( $\text{mmol}/\text{m}^2/\text{s}$ ) |
|--------------|-----------------------|-----------------------------------------------|--------------------------------------------|------------------------------------------------|
| Non-AMF      | 2.10c                 | 4.43b                                         | 0.55b                                      | 0.048b                                         |
| AMF          | 2.60ab                | 6.36a                                         | 1.06a                                      | 0.076a                                         |
| AMF+DFMO     | 2.45b                 | 4.15b                                         | 0.71b                                      | 0.052b                                         |
| AMF+DFMO+Put | 2.73a                 | 7.58a                                         | 1.11a                                      | 0.077a                                         |

Note: Means followed by the same letter within a column shows no significant difference among treatments (LSD<sub>0.05</sub>)

fects of DFMO. For allocation of sucrose/glucose to root, AMF decreased the allocation of sucrose but increased the allocation of glucose compared to non-AMF (Tab. 6). DFMO treatment drastically reversed the effects of sole AMF. Adding Put into DFMO treatment partly alleviated the effects of DFMO. AMF acquire hexose (glucose mainly) of hosts to maintain its development (Bago *et al.*, 2003). In better mycorrhizal roots, more sucrose contents might transform into hexose, e.g. glucose, thus resulting in the increase of allocation of glucose to root and the decrease of allocation of sucrose to root. Therefore, it concludes that PA altered the allocation of carbohydrates to mycorrhizal root through the increase of glucose and the decrease of sucrose, which would benefit mycorrhizal development.

Root is a key component of the soil-plant-atmosphere continuum and is influenced by endogenous factors (e.g. hormone) and exogenous factors (e.g. numerous biotic and abiotic factors, soil microorganisms) (Osmont *et al.*, 2007). It is well documented that AMF can modify root morphology of hosts (Atkinson *et al.*, 2003; Gutjahr *et al.*, 2009; Orfanoudakis *et al.*, 2010). For the root morphological traits, sole AMF inoculation notably increased total length, total projected area, total surface area and total

volume but decreased average diameter when compared to non-AMF control (Tab. 4), which is consistent with the previous studies on grapevine and beach plum (Augín *et al.*, 2004; Zai *et al.*, 2007). Compared to alone AMF treatment, dual AMF+DFMO treatment significantly affected the root morphological traits, showing the decrease of total length, total projected area, total surface area and total volume and the increase of average diameter. However, Put application to DFMO treatment partly reversed the effects of DFMO on the root morphological traits. These results demonstrate that endogenous Put participates in root development of citrus seedlings. The results are in agreement with the findings of Hummel *et al.* (2002) in *Pringlea antiscorbutica*.

Soil P affects root morphological and physiological characteristics that are important for P uptake (Hajabbasi and Schumacher, 1994). Mycorrhizal symbioses are known to improve immobile nutrient uptake from soils, especially P (Smith and Read, 2008). In the present experiment, sole AMF colonization significantly increased P contents of leaves and roots, acid, neutral and alkaline phosphatase activities of rhizosphere but decreased available P content of rhizosphere, compared with non-AMF treatment (Tab. 5). Our results are in agreement with the previous

Tab. 4 Effect of DFMO alone or combination with Put on root morphological traits of *Glomus mosseae*-colonized citrus (*Citrus tangerine*) seedlings

| Treatment    | Average diameter<br>(mm) | Total length<br>(cm) | Total projected area<br>( $\text{cm}^2$ ) | Total surface area<br>( $\text{cm}^2$ ) | Total volume<br>( $\text{cm}^3$ ) |
|--------------|--------------------------|----------------------|-------------------------------------------|-----------------------------------------|-----------------------------------|
| Non-AMF      | 1.17c                    | 215.6c               | 26.4c                                     | 80.9d                                   | 2.28d                             |
| AMF          | 1.13d                    | 276.9a               | 31.7a                                     | 100.3a                                  | 2.93a                             |
| AMF+DFMO     | 1.26a                    | 210.6c               | 28.6b                                     | 86.9c                                   | 2.52c                             |
| AMF+DFMO+Put | 1.21b                    | 244.6b               | 29.1b                                     | 90.5b                                   | 2.71b                             |

Note: Means followed by the same letter within a column shows no significant difference among treatments (LSD<sub>0.05</sub>)

Tab. 5 Effect of DFMO alone or combination with Put on leaf and root P, rhizosphere available P and rhizosphere phosphatase of *Glomus mosseae*-colonized citrus (*Citrus tangerine*) seedlings

| Treatment    | P content<br>(mg/g) |       | Phosphatase activity of rhizosphere<br>(mg hydroxybenzene/g) |         |          | Available P content of rhizosphere<br>(mg/kg) |
|--------------|---------------------|-------|--------------------------------------------------------------|---------|----------|-----------------------------------------------|
|              | Leaf                | Root  | Acid                                                         | Neutral | Alkaline |                                               |
| Non-AMF      | 5.52c               | 1.07c | 0.75b                                                        | 0.07c   | 0.014b   | 5.57a                                         |
| AMF          | 9.03a               | 3.08a | 0.94a                                                        | 0.30a   | 0.052a   | 3.44b                                         |
| AMF+DFMO     | 7.51b               | 2.05b | 0.74b                                                        | 0.24b   | 0.021b   | 5.03a                                         |
| AMF+DFMO+Put | 9.14a               | 3.36a | 0.80b                                                        | 0.30a   | 0.045a   | 3.11b                                         |

Note: Means followed by the same letter within a column shows no significant difference among treatments (LSD<sub>0.05</sub>)

Tab. 6 Effects of DFMO alone or combination with Put on soluble protein, G-POD and allocation of sugar to root of *Glomus mosseae*-colonized citrus (*Citrus tangerine*) seedlings

| Treatment    | Soluble protein<br>(mg/kg) |        | G-POD<br>(U/g) |         | Allocation of sugar to root<br>(%) |         |
|--------------|----------------------------|--------|----------------|---------|------------------------------------|---------|
|              | Leaf                       | Root   | Leaf           | Root    | Sucrose                            | Glucose |
| Non-AMF      | 20.63b                     | 22.84b | 17.70a         | 61.94a  | 49.45a                             | 44.76c  |
| AMF          | 25.85a                     | 25.38a | 8.83a          | 8.84c   | 42.64b                             | 48.88a  |
| AMF+DFMO     | 15.86d                     | 7.64d  | 26.54a         | 44.31ab | 47.16a                             | 48.04b  |
| AMF+DFMO+Put | 18.55c                     | 10.90c | 35.39a         | 26.36bc | 48.61a                             | 48.15ab |

Note: Means followed by the same letter within a column shows no significant difference among treatments (LSD<sub>0.05</sub>)

observations in *Sclerocarya birrea* (Muok and Ishii, 2006). AMF can stimulate the production of soil phosphatase, especially acid phosphatase, which mediated the release of inorganic P (Wang *et al.*, 2006). So, better mycorrhizal development implied the higher phosphatase activity of rhizosphere, increasing the uptake of soil available P. As a result, leaf and root P contents would be increased by mycorrhization. The effects of AMF were drastically reversed when DFMO was included in AMF treatment (Tab. 5), showing the decrease of P contents of leaves and roots, acid, neutral and alkaline phosphatase activities of rhizosphere and the increase of available P content of rhizosphere, compared to the sole AMF. However, application of exogenous Put to DFMO treatment partly unchained the effects of DFMO. These influences of DFMO alone or combination with Put might be related to the effects on mycorrhizal development.

In addition to P, plant G-POD has shown the fundamental role in controlling root development (Hatzilazarou *et al.*, 2006). Inoculation with AMF significantly limited G-POD activity of root (Tab. 6). DFMO+AMF treatment completely reversed the effects of sole AMF treatment, exhibiting the increase of G-POD activity of root. However, adding Put into DFMO treatment partly mitigated the effects of DFMO. Changes in the levels of G-POD activity are related to lignin, and thus G-POD has been regarded as a candidate for lignin-forming enzymes (Chen *et al.*, 2002). DFMO treatment increased root G-POD activity in the experiment. This implied the inducement of lignin formation, which results in premature cessation of root growth (Lucca Zanardo *et al.*, 2009). Moreover, AMF colonization seems to be stronger in juvenile than in adult plants (Padilla and Encina, 2005). These results suggested that G-POD activity of root was negatively correlated with root development, and PAs might regulate G-POD activity of mycorrhizal roots but not leaves, which controlled root development.

In our experiment, AMF colonization increased soluble protein contents of leaves and roots, showing that AMF induce new protein synthesis (Tab. 6). The result is in harmony with the findings of Manoharan *et al.* (2008), who observed that AMF inoculation stimulated soluble protein contents of five selected tree seedlings. DFMO+AMF treatment completely reversed the effect of sole AMF treatment, exhibiting the decrease of solu-

ble protein contents of leaves and roots. However, adding Put into DFMO treatment mitigated the effects of DFMO. These changes in soluble protein may be due to the result which PAs regulate protein synthesis (Algranati and Goldemberg, 1977). It is reasonable to guess that improved mycorrhizal root morphology due to PA might be related to new protein synthesis by PA.

### Conclusions

As stated above, the changes of mycorrhizal development due to DFMO alone or combination with Put showed that PAs are an important regulated factor participating in mycorrhizal development. Better mycorrhizal development might stimulate phosphatase activity of rhizosphere and increase the uptake of available P from rhizosphere, resulting in the increase of P in plant tissue. Additionally, PAs altered the allocation of carbohydrates to mycorrhizal root through the increase of glucose and the decrease of sucrose, which would benefit mycorrhizal development. Endogenous Put participates in root development of mycorrhizal citrus seedlings, which is negatively related to root G-POD and may be due to inducing new protein synthesis.

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