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ORIGINAL PAPER

Arbuscular mycorrhizas alter root system architecture of *Citrus tangerine* through regulating metabolism of endogenous polyamines

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Abstract Arbusculuar mycorrhizal fungi (AMF) optimize root system architecture (RSA) of their host plants, whilst polyamines (PAs), including putrescine (Put), spermidine (Spd) and spermine (Spm), play an important role in primary, lateral and adventitious root development. However, the interaction of AMF and PAs on RSA is less studied. In a pot study, 0, 5, 10, 20 and 40 g fresh inocula of *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe were inoculated into rhizosphere of 9-day-old red tangerine (*Citrus tangerine* Hort. ex. Tanaka) seedlings. After 164 days of inoculation, AM colonization ranged from 26.6 to 54.2% and increased with the increase of *G. mosseae* inocula amount. Mycorrhization generally significantly increased RSA traits such as total length, projected area, surface area, volume, 0–1 cm classified root length, and the ratio of the 0–1 cm classified root length to the total length, but decreased root average diameter and the proportion of 1–2 cm classified root length. Mycorrhization also generally increased both leaf and root arginine and ornithine decarboxylase (ADC and ODC) activity, thus stimulating the synthesis of Put. In leaves, more Put was converted to Spd, but not to Spm, resulting in a Spd increment in leaf. By contrast, root Put was induced by AMF, which indirectly improved the root average diameter and proportion of fine roots, since the RSA traits were significantly correlated with root Put synthetases through ADC and ODC. Our results indicate that AMF might regulate endogenous PAs metabolism, thus resulting in a synergetic impact on RSA.

Keywords Arginine decarboxylase · *Glomus mosseae* · Ornithine decarboxylase · Polyamine · Putrescine · Root system architecture

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Introduction

Root system architecture (RSA), the spatial configuration of the shape and structure of root systems, influences the capacity of roots to take up soil resources (de Dorlodot et al. 2007). RSA possesses both dynamic and plastic characteristics to adapt to severe conditions, and can be altered by either abiotic (e.g., availability of soil water and nutrients) or biotic (e.g., soil microorganisms) factors (Ingram and Malamy 2010).

Arbuscular mycorrhizal fungi (AMF), a group of soil fungi symbiotically associated with roots of higher plants, enhance plant mineral nutrition and stress tolerance (Smith and Read 2008). Inoculation with AMF generally affects root longevity, architecture, and structure of host plants (Schellenbaum et al. 1991; Hodge et al. 2009; Wu et al. 2011). For example, colonization with *Glomus fasciculatum* or *G. etunicatum* increases root system branching

leading to a large proportion of high order (tertiary and above) roots in strawberry (Norman et al. 1996). Colonization with *G. intraradices* significantly enhanced the initiation of lateral roots in rice (Gutjahr et al. 2009). AM root colonization was positively correlated with fine root length and specific root length, but negatively correlated with fine root diameter in plants of grasses, secondary and mature forests in southern Brazil (Zangaro et al. 2008). The latter was also true in a pot experiment with AM trifoliate orange (*Poncirus trifoliata*) (Wu et al. 2011). Improvement of RSA including total length, total volume, total projected area and total surface area was reported in AM colonized *Citrus tangerine* and *P. trifoliata* (Wu et al. 2010a; 2011). The AMF-induced RSA alteration may be linked to an improved uptake of nutrients (Padilla and Encina 2005; Schroeder and Janos 2005) and salt tolerance (Wu et al. 2010a, 2011). In addition, Gutjahr et al. (2009) reported that AMF-induced alterations of RSA were independent of common symbiosis signaling. In contrary, infection with AMF reduced the length and the number of tap roots, and the 1st and 2nd order lateral roots of kidney bean (*Phaseolus vulgaris*) (Isobe et al. 2002), and also restricted the length, volumn and surface area of trifoliate orange (*P. trifoliata*) (Yao et al. 2009). Therefore, additional studies are required to evaluate the effects of AM on RSA.

Polyamines (PAs), mainly in the form of diamine putrescine (Put), triamine spermidine (Spd) and tetraamine spermine (Spm), are organic compounds with two or more primary amino groups existing in all compartments of the plant cells (Couée et al. 2004; Fuell et al. 2010). The decarboxylation of arginine by arginine decarboxylase (ADC) or ornithine by ornithine decarboxylase (ODC) produces Put, which is then transformed to Spd and further to Spm by the Spd and Spm synthase (Kaur-Sawhney et al. 2003; Hussain et al. 2011). The oxidative degradation of Put is through diamine oxidase (DAO), and the degradation of Spd and Spm is through polyamine oxidase (PAO) (Couée et al. 2004). Like plant growth regulators, PAs play an important role in primary, lateral and adventitious root development by regulating root cell division and differentiation (Couée et al. 2004). Put, but not Spd or Spm, enhances root mycorrhizal development and plant growth of trifoliate orange (Wu et al. 2010b). However, it is unclear if the AMF-induced root alteration is related to PA metabolism.

We hypothesize that AMF may regulate the metabolism of endogenous PAs, thus resulting in a synergetic improvement of RSA. Red tangerine (*C. tangerine* Hort. ex Tanaka), a major citrus rootstock in the central and southwestern China, represents less root hair in field. The objectives of this research were to study: (1) effects of AMF inoculation on RSA and PA metabolism, and (2) relationship between RSA and PA metabolism in 6-month-old red tangerine seedlings.

Materials and methods

AMF inocula and plant growth

The AM fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe used in this study exhibits high efficiency on some RSA traits of *C. tangerine* (Wu et al. 2010a). The inocula (BGC XZ02A) of *G. mosseae* were a mixture of extraradical hyphae, infected *Sorghum vulgare* roots and growth media (soil:river sand = 1/3, v/v) and commercially supplied by the Beijing Academy of Agriculture and Forestry Science. The experiment was a factorial randomized design consisting of five inoculation dosage (0, 5, 10, 20 and 40 g fresh AMF inocula per pot, respectively described as *Gm*-0, *Gm*-5, *Gm*-10, *Gm*-20 and *Gm*-40) with five replicates for each inoculation treatment.

Seeds of red tangerine were surface-sterilized with 70% alcohol for 10 min and then germinated on sterilized moisture filter papers at 25 °C under darkness. After 9 days, six two-leaf-old seedlings (~2 cm tall) were transplanted into one plastic pot (12 cm bottom × 18 cm upper diameter × 13 cm height) containing 2.7 kg autoclaved (121 °C, 2 h) growth substrate (xanthi-udic ferralsols:vermiculite:perlite = 5:1:1, v/v/v, pH 5.8), which had 152.7, 15.1 and 22.9 mg/kg available nitrogen, phosphorus and potassium, respectively. The *G. mosseae* inocula were placed at 5 cm depth below the growth substrate. No mycorrhizal inocula were added to the non-AMF control pots, considering that only trivial amount of nutrition from the inocula could be added to the whole growth substrate. Three seedlings per pot were kept after 45 days of transplanting and continued to be grown in a plastic greenhouse at the Yangtze University campus (Jingzhou, China) from 27 March to 7 September, 2010. The light density ranged from 672 to 893 μmol/m^2/s with 24/18 °C (day/night) and 70–95% relative humidity during the entire experiment. Shoots and roots were separately harvested after 164 days of transplanting.

Analysis of root mycorrization and RSA

Root AM colonization was determined by randomly selected ten 1-cm root fragments per plant after the clearance with 10% KOH at 90 °C for 1.5 h and stain with 0.05% trypan blue for 5 min (Phillips and Hayman 1970).

After carefully washing, the intact fresh root system was rinsed with distilled water and then scanned with Epson Perfection V700 Photo Dual Lens System (J221A, Seiko Epson Corporation, Indonesia). The characteristics of RSA, including total projected area, total surface area, diameter, total volume, total length, 0–1, 1–2, 2–3, 3–4 and >4 cm classified root length (root length in diameter, namely) were automatically analyzed with a WinRHIZO Pro 2007b software (Regent Instruments Inc., Quebec, Canada).

Analysis of endogenous free PAs and PA metabolic variables

PAs were extracted according to the method of Liu and Moriguchi (2007) with minor modifications. Briefly, 0.1 g fresh leaves or 0.2 g fresh roots were ground to fine powder with liquid nitrogen and homogenized in 1 mL 5% cold perchloric acid. The homogenates were maintained at 4 °C for 30 min and then centrifuged at 4 °C and 12,235*g* for 15 min. The centrifuged residues were extracted again using the same procedure, the two supernatants were mixed together and then benzoylated according to Fu et al. (2011). Determination of the three free PAs (Put, Spm and Spd) was performed with a 1200 Agilent HPLC system (Agilent Technologies Ltd., Santa Clara, CA, USA) equipped with a C18 reversed phase column (4.6 × 150 mm, particle size 5 μm) and an UV-detector (230 nm). The program of the gradient elution was developed for ~6 h with two solvent systems: methanol (HPLC grade):ultrapure water (1:1, v/v) or methanol (100%). The retention times were 6 min, 19 min, and 53 min for Put, Spd and Spm, respectively. The mixture of Put, Spd and Spm was used as the standard.

Activity of ADC and ODC was determined according to Zhao and Liu (2000) with minor modifications. Briefly, 0.2 g fresh plant tissues was homogenized in 1.5 mL 0.1 M cold phosphate buffer (pH 6.3), which contained 5 mM EDTA, 1 mM pyridoxal phosphate, 0.01 mM polyvinyl pyrrolidone, 10 mM dithiothreitol, and 0.43 mM sodium thiosulfate, and then centrifuged at 12,000*g* at 4 °C for 40 min. The assay mixture consisted in 1 mL 0.1 M Tris–HCl buffer (pH 7.5), which contained 5 mM EDTA, 40 μM pyridoxal phosphate, and 5 mM dithiothreitol, 0.8 mL supernate, and 0.2 mL 25 mM L-Arg (for ADC) or L-Orn (for ODC), and then incubated at 37 °C for 60 min. L-Arg or L-Orn was replaced by the perchloric acid, which was the blank. 0.5 mL supernate was mixed with 1 mL 2 M NaOH and 10 μL benzoyl chloride and then stirred for 20 s. After incubation at 37 °C for 30 min, 2 mL saturated NaCl and 2 mL ether (100%) were added into the reaction mixture, which was then centrifuged at 1,500*g* at 4 °C for 5 min. After the centrifugation, 1 mL ether phase extraction was evaporated at 50 °C water bath, and the remainder was dissolved in 3 mL methanol (100%, HPLC grade). Absorbance increased 1.0 at 254 nm during 1 min was defined as one activity unit of ADC or ODC. Three parallel measures were performed.

Activity of DAO and PAO was determined according to Qin et al. (2006) with minor modifications. Briefly, 0.2 g fresh plant tissues were homogenized by 2 mL 0.1 M cold phosphate buffer (pH 7.0) and centrifuged at 10,000*g* for 20 min at 4 °C. The reaction mixture (1.8 mL) contained 1.2 mL 0.1 M phosphate buffer (pH 7.0), 0.2 mL 25 mM guaiacol, 0.2 mL 500 μg/L horseradish peroxidase, and 0.2 mL supernate, and incubated at 30 °C for 5 min. The reaction was initiated by adding 0.2 mL 10 mM Put or Spd for the determination of DAO or PAO. Put or Spd was replaced by the phosphate buffer, which was used as the blank. Absorbance increased 1.0 at 470 nm during 1 min was defined as one activity unit of DAO or PAO. Three parallel measures were performed.

Statistical analysis

Data were analyzed using the SAS statistical software, and one-factor analysis of variance (ANOVA) was used to compare the significant difference with the LSD test at $P < 0.05$. The Pearson's correlation coefficients between root AM colonization or RSA and PA metabolic variables were performed using the Proc Corr's procedure in the SAS.

Results

There was no mycorrhizal colonization in the non-AMF control seedlings. Root AM colonization with *G. mosseae* was 26.6, 38.6, 46.4 and 54.2% under the 5, 10, 20 and 40 g mycorrhizal inoculation, respectively.

Mycorrhizal inoculation significantly increased root projected area, root surface area, and root volumn but decreased averaged root diameter, compared with the non-mycorrhizal control (Table 1). The tested RSA variables were generally significantly higher under the *Gm*-20 and *Gm*-40 dosage than under the *Gm*-5 and *Gm*-10 dosage, and similar between the *Gm*-5 and *Gm*-10 or between the *Gm*-20 and *Gm*-40 dosage. The length and proportion of

Table 1 Effects of *G. mosseae* inocula on root system architecture of red tangerine (*C. tangerine*) seedlings

Data (means ± SE, $n = 5$) followed by the same letter within a column are not significantly different at $P < 0.05$

G. mosseae inocula (g)	Root projected area (cm^2)	Root surface area (cm^2)	Average root diameter (mm)	Root volume (cm^3)
Gm-0	5.9 ± 0.4c	18.4 ± 1.2c	0.64 ± 0.04a	0.30 ± 0.03c
Gm-5	7.2 ± 0.6b	22.2 ± 1.7b	0.62 ± 0.08ab	0.36 ± 0.05b
Gm-10	7.1 ± 1.2b	22.0 ± 1.7b	0.55 ± 0.02bc	0.35 ± 0.03b
Gm-20	11.7 ± 1.0a	36.8 ± 3.3a	0.54 ± 0.02c	0.51 ± 0.02a
Gm-40	11.5 ± 0.9a	36.2 ± 2.5a	0.57 ± 0.04bc	0.55 ± 0.04a

Table 2 Effects of *G. mosseae* inocula on the classified root length and proportion of root length classes of red tangerine (*C. tangerine*) seedlings

G. mosseae inocula (g)	Classified root length (cm)						Proportion of root length (%)				
	0–1 cm	1–2 cm	2–3 cm	3–4 cm	>4 cm	Total	0–1 cm	1–2 cm	2–3 cm	3–4 cm	>4 cm
Gm-0	79.0 ± 7.5d	10.3 ± 1.1b	1.9 ± 0.5bc	0.5 ± 0.5a	0.0 ± 0.0a	91.7 ± 7.4d	86.1 ± 1.7c	11.3 ± 1.3a	2.1 ± 0.6b	0.6 ± 0.5a	0.0 ± 0.1a
Gm-5	101.7 ± 7.0c	8.5 ± 1.4b	4.3 ± 1.8a	0.9 ± 1.0a	0.1 ± 0.1a	115.5 ± 6.6c	88.0 ± 3.3bc	7.4 ± 1.1b	3.7 ± 1.5a	0.8 ± 0.9a	0.1 ± 0.1a
Gm-10	115.3 ± 23.6c	9.7 ± 0.9b	1.3 ± 0.7c	0.1 ± 0.0a	0.0 ± 0.0a	126.4 ± 23.8c	91.0 ± 2.0ab	8.0 ± 2.2b	1.0 ± 0.5b	0.1 ± 0.0a	0.0 ± 0.0a
Gm-20	196.3 ± 10.8a	14.8 ± 2.1a	3.4 ± 0.8ab	0.2 ± 0.1a	0.0 ± 0.0a	214.7 ± 12.4a	91.4 ± 1.2a	6.9 ± 0.8b	1.6 ± 0.3b	0.1 ± 0.1a	0.0 ± 0.0a
Gm-40	175.2 ± 5.0b	19.0 ± 3.5a	3.8 ± 1.7a	0.7 ± 0.7a	0.1 ± 0.0a	193.7 ± 7.5b	90.5 ± 2.3ab	7.1 ± 1.7b	2.0 ± 0.9b	0.4 ± 0.3a	0.1 ± 0.0a

Means ± SE ($n = 5$) followed by same letter within a column are not significantly different at $P < 0.05$

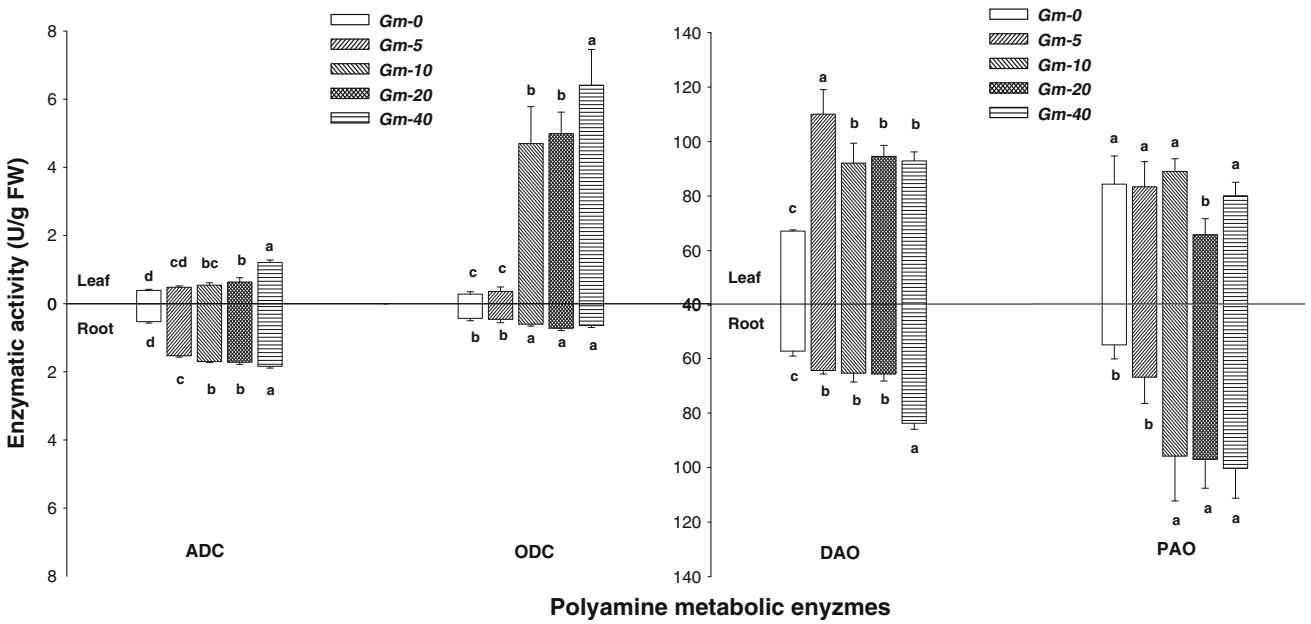

Fig. 1 Influence of mycorrhizal inocula on activity of PA metabolic enzymes in leaf and root of 6-month-old red tangerine (*C. tangerine*) seedlings. Data (means ± SE, $n = 3$) followed by the same letter above the bars are not significantly different at $P < 0.05$

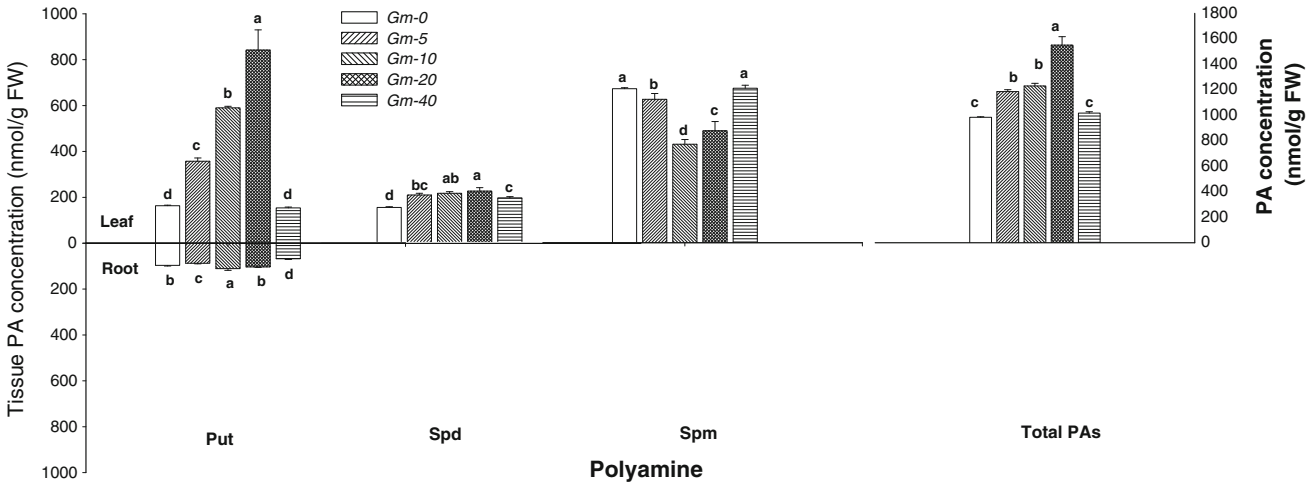

Fig. 2 Influence of mycorrhizal inocula on leaf and root Put, Spd and Spm concentrations of 6-month-old red tangerine (*C. tangerine*) seedlings. Data (means ± SE, $n = 3$) followed by the same letter above the bars are not significantly different at $P < 0.05$. Root Spd and Spm were not detectable under the determination method used in this study

the 0–1 cm classified root length and root length in total were significantly greater under the mycorrhizal than under the non-mycorrhizal inoculation, but similar in both the 3–4 and >4 cm classified root length (Table 2). Meanwhile, the length and proportion of both the 1–2 and 2–3 cm root length category varied between the non-mycorrhizal and mycorrhizal treatments.

Compared to the non-mycorrhizal control, activity of both leaf and root ADC, ODC and DAO, and root PAO was generally significantly higher under the mycorrhizal treatment and increased with the increase of mycorrhizal inocula dosage (Fig. 1). By contrast, activity of leaf PAO was similar between the non-mycorrhizal and mycorrhizal treatment, except under the *Gm*-20 dosage. In addition, ADC and PAO were generally higher in roots than in leaves whilst ODC and DAO were generally higher in leaves than in roots among the four mycorrhizal treatments. The mycorrhizal control had similar ADC and ODC activity in the leaves and roots, but higher DAO and PAO in the leaves than in the roots.

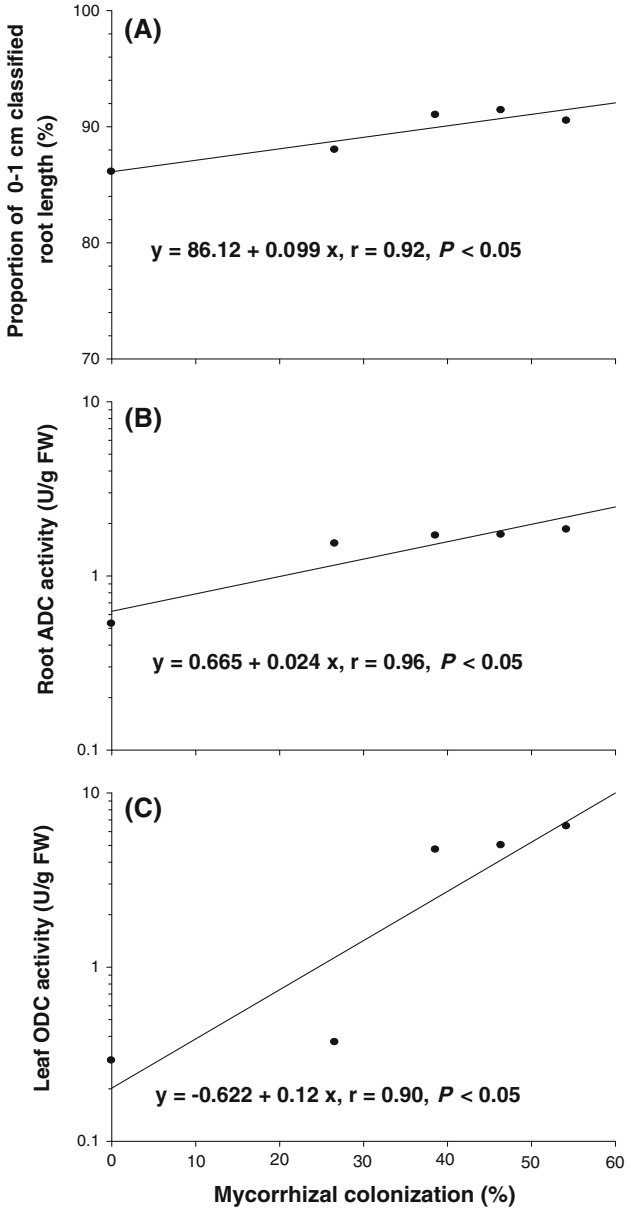

Fig. 3 Linear regression between mycorrhizal colonization and proportion of 0–1 cm classified root length in total (**a**), root ADC activity (**b**), or leaf ODC activity (**c**) in 6-month-old red tangerine (*C. tangerine*) seedlings inoculated with *G. mosseae*. Values represent the means of five replicates

In general, leaf Put and Spd concentrations were significantly higher whilst leaf Spm concentrations were lower under the mycorrhizal than under the non-mycorrhizal treatment (Fig. 2). Only root Put, but not root Spd and Spm, was detected by the method used in this study. Three effects of AMF on root Put were observed: higher in the *Gm*-10 seedlings, lower in the *Gm*-5 and *Gm*-40 seedlings, and no change in the *Gm*-20 seedlings (Fig. 2). The total three free PAs (Put + Spd + Spm) concentration of leaves was significantly higher under the mycorrhizal except for *Gm*-40 than under the non-mycorrhizal control.

Discussion

Mycorrhizal inoculation significantly improved RSA traits of red tangerine seedlings (Table 1, 2). These results are in agreement with our previous studies in citrus plants (Wu et al. 2010a, c; 2011). The present work also found an increase of the proportion of the 0–1 cm classified root length but a decrease of the proportion of 1–2 cm classified root length in the AMF colonized red tangerine, which are consistent with the results that AMF increase the length of fine roots but decrease the length of coarser roots in trifoliate orange (*P. trifoliata*) (Yao et al. 2009). A significantly positive correlation in this study was also observed between AMF colonization and proportion of 0–1 cm classified root length in the total root length (Fig. 3a; $r = 0.92$, $P < 0.05$). The result is in consistence with the results in strawberry (Norman et al. 1996) and in rice (Gutjahr et al. 2009).

Previous studies have indicated that exogenous PAs act as regulatory factors in AMF colonization (El Ghachtouli et al. 1995; Wu et al. 2010c). In the present study, mycorrhizal inoculation generally significantly affected the synthesis or degradation of endogenous PAs (Figs. 1, 2). For instance, leaf Put and Spd were significantly increased with the increase of *Gm*-5 to *Gm*-20, but decreased under the higher *Gm*-40. By contrast, leaf Spm was significantly lower below *Gm*-20 but similar at *Gm*-40 dosage (Fig. 2). Mycorrhization increased the activity of both leaf and root ADC and ODC with a significantly positive correlation (Fig. 3b, root ADC, $r = 0.96$, $P < 0.05$; Fig. 3c, leaf ODC, $r = 0.90$, $P < 0.05$). The increase of ADC and ODC indicates that AM colonization enhances Put synthesis as seen an increase of Put (Fig. 2) since the synthesis of Put is through ADC or ODC (Kaur-Sawhney et al. 2003; Hussain et al. 2011). On the other hand, under mycorrhization the decrease of Spm is due to the increase of both leaf and root DAO (e.g. less Spm precursor) and root PAO (direct Spd and Spm degradation) (Fig. 1) since the degradation of Put is through DAO while Spd and Spm are through PAO (Couée et al. 2004). Other explanations might be that mycorrhization could decrease the activity of spermine synthase, which controls the transformation of Spd into Spm, although it was not detected in the present work. Lower Spd in the non-mycorrhizal and mycorrhizal leaves than Spm and Put (Fig. 2) suggests that Spd from Put transformation might form more Spm by successive addition of aminopropyl groups in the reaction that is catalyzed by spermine synthase (Wimalasekera et al. 2011). A previous experiment identified that Put and Spd, mainly existing in the *G. mosseae* spores involved in the spore germination and hyphal growth (El Ghachtouli et al. 1996). Nevertheless, further studies are needed to analyze if the increase of PAs in plants mediated by AMF spores, and

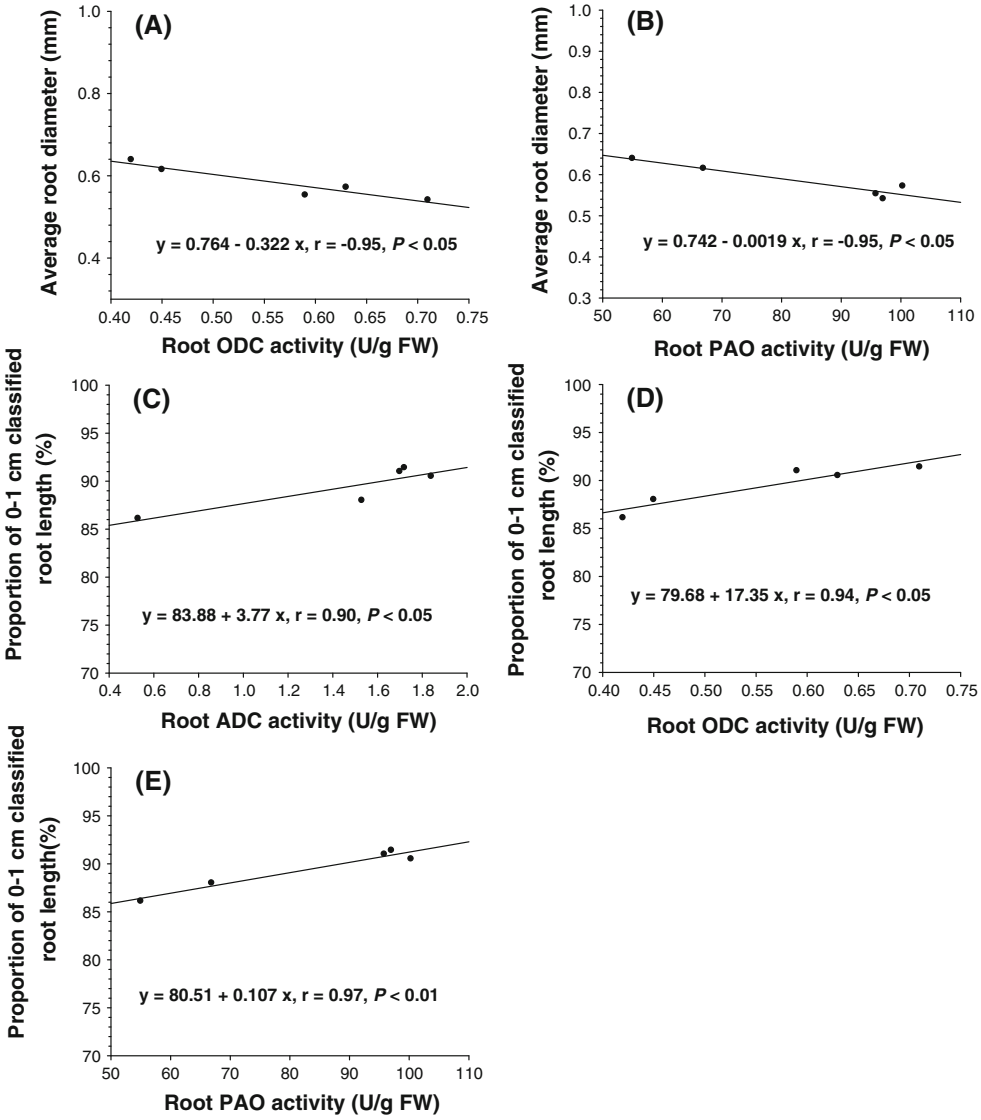

Fig. 4 Linear regression between root ADC (**a**) or ODC (**b**) activity and average root diameter and between root ADC (**c**), ODC (**d**), or PAO (**e**) and proportion of 0–1 cm classified root length in total in 6-month-old red tangerine (*C. tangerine*) seedlings inoculated with *G. mosseae*. Values represent the means of five replicates

how the increased PAs are translocated from an AMF spore to plants.

It is well documented that PAs are involved in the control of cell division and differentiation and thus play an important role in lateral and adventitious root formation (Couée et al. 2004). In plants, Put is synthesized from arginine by ADC or ornithine by ODC, and is degraded by DAO and both Spd and Spm are degraded by PAO (Couée et al. 2004). Based on the Pearson's correlation analysis, root ODC (Fig. 4a; $r = -0.95$, $P < 0.05$) or root PAO (Fig. 4b; $r = -0.95$, $P < 0.05$) had a significantly negative correlation with the average root diameter, while root ADC (Fig. 4c; $r = 0.90$, $P < 0.05$), root ODC (Fig. 4d; $r = 0.94$, $P < 0.05$), or root PAO (Fig. 4e; $r = 0.97$, $P < 0.01$) had a significantly negative correlation with the proportion of 0–1 cm classified root length of the root length in total, indicating that PAO is located in young root tissues. Gene expression analysis revealed that PAO expression occurs in cells destined to undergo lignification (Paschalidis and Roubelakis-Angelakis 2005). It seemed that in roots, Put, but not Spd and Spm, indirectly improved the average root diameter and proportion of fine roots, because the RSA traits significantly correlated with root Put synthetase (ADC and ODC). ADC is generally associated with cells undergoing cell expansion and ODC with cells undergoing cell division (Paschalidis and Roubelakis-Angelakis 2005). Furthermore, Put may reflect the sub optimal growth conditions whereas Spd and Spm may help eliminating reactive oxygen species (Hussain et al. 2011). These results from different plant and mycorrhizal fungal species confirmed our previous results that the exogenous Put, not Spd and Spm, improved RSA of *G. versiforme* colonized *P. trifoliata* (Wu et al. 2010b), a close related orange plant to the red tangerine used in this study. Meanwhile, PAO catalyzes the oxidation of Spd and Spm (but not other PAs) (Kuznetsov and Shevyakova 2007).

Higher root PAO activity under mycorrhization might result in a greater oxidative degradation of Spd and Spm with the formation of H_2O_2, a highly reactive oxygen species, although root Spd and Spm had not been detected in the present study.

Conclusion

Mycorrhizl colonization generally significantly optimized the RSA including higher proportion of fine roots, total root length, total root projected area, total root surface area, total root volume, and 0–1 cm classified root length, and thinner root diameter of the 6-month-old red tangerine seedlings. Mycorrhization also stimulated the synthesis of Put since it significantly positively correlated with ADC and ODC activity in both leaves and roots. Both Spd and Spm (the end product of their diamine precursor Put degradation) accumulated in leaves, but were not detectable in roots. These results may indicate that a rapid translocation of Spd and Spm to aboveground shoots from belowground roots. Therefore, it is also possible that AMF-induced root Put, not root Spd and Spm, indirectly improves root diameter and fine root production, because RSA traits were significantly correlated with root Put synthetases (ADC and ODC).

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