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Nitric Oxide Accelerates Mycorrhizal Effects on Plant Growth and Root Development of Trifoliate Orange

(Nitrik Oksida Mempercepatkan Kesan Mikoriza ke atas Pertumbuhan Pokok dan Perkembangan Akar Oren Trifoliat)

LI TIAN, NASRULLAH, XIAO-YUN HUANG & QIANG-SHENG WU*

ABSTRACT

*Arbuscular mycorrhizal fungi (AMF) actively colonize plant roots and thus enhance plant growth through different mechanisms. In the present study, trifoliate orange (*Poncirus trifoliata*) seedlings inoculated with* Diversispora versiformis *were subjected to 0 and 0.2 mmol/L sodium nitroprusside (SNP, a nitric oxide donor) treatments. After eight weeks, exogenous SNP considerably increased root mycorrhizal colonization by 25%, showing a positive stimulating effect of NO on mycorrhizal formation. Mycorrhizal inoculation significantly increased plant growth performance (height, stem diameter, leaf number and shoot and root dry weight) and root traits (length, projected area, surface area, volume and number of 2nd and 3rd order lateral roots) than non-mycorrhizal treatment and NO (exogenous SNP treatment) heavily strengthened the mycorrhizal effects. Moreover, NO and mycorrhization induced more fine root (0-0.5 cm) formation. There was an opposite changed trend in root sucrose and leaf and root glucose contents by SNP in AMF versus non-AMF seedlings. All these results implied that NO plays important roles in mycorrhizal formation and development and also accelerates mycorrhizal effects on plant growth and root development of trifoliate orange.*

Keywords: Arbuscular mycorrhizal fungi; carbohydrate; citrus; nitric oxide; sodium nitroprusside

ABSTRAK

*Kulat mikoriza arbuskula (AMF) mengkoloni akar tumbuhan secara aktif dan seterusnya menggalakkan pertumbuhan pokok melalui mekanisme berbeza. Dalam kajian ini, benih oren trifoliat (*Poncirus trifoliata*) yang diinokulasi dengan* Diversispora versiformis *telah diberikan rawatan 0 dan 0.2 mmol/L sodium nitropussida (SNP, penderma nitrik oksida). Selepas lapan minggu, SNP eksogenus didapati meningkatkan pengkolonian akar mikoriza sebanyak 25% dan ini menunjukkan kesan rangsangan positif NO terhadap pembentukan mikoriza. Penginokulasian mikoriza meningkatkan prestasi pertumbuhan pokok secara signifikan (tinggi, diameter batang, jumlah daun dan berat akar kering) dan ciri akar (panjang, luas unjuran, luas permukaan, isi padu, bilangan akar lateral peringkat ke-2 dan ke-3) berbanding rawatan tanpa mikoriza serta NO (rawatan SNP eksogenus) mengukuhkan lagi kesan mikoriza. Di samping itu, rawatan NO dan mikoriza mengaruh lebih banyak pembentukan akar halus (0-0.5 cm). Terdapat trend perubahan bertentangan pada kandungan sukrosa akar, daun serta glukosa akar oleh SNP dalam benih AMF berbanding tanpa AMF. Keseluruhan keputusan kajian ini menunjukkan bahawa NO memainkan peranan penting dalam pembentukan dan perkembangan mikoriza, malah mempercepatkan kesan mikoriza ke atas pertumbuhan pokok dan perkembangan akar oren trifoliat.*

Kata kunci: Cendawan mikoriza asbukula; karbohidrat; nitrik oksida; sitrus; sodium nitroprussida

INTRODUCTION

Nitric oxide (NO) is a crucial regulator of root growth and development (Fernández-Marcos et al. 2011). NO as an essential gas signal molecule had been intensively studied in the past, showing a pivotal role in root organogenesis (Xiong et al. 2009). In general, NO not only participates in the inhibition of primary root (PR) growth but also stimulates lateral root (LR) growth (Correa-Aragunde et al. 2006). In addition, NO also regulates the adventitious root growth of cucumber cuttings (Pagnussat et al. 2002). Root system architecture is a vital index to describe the root growth and development status and it can be modified to facilitate the capacity of nutrition-uptake under environmental stresses (nutrient limitation in particular) (Sorgona et al. 2007). Additionally, root morphology is affected by internal and external factors, including soil microorganisms. Arbuscular mycorrhizal fungi (AMF), a kind of soil inhabitant fungi, can form symbiotic associations with approximately 90% of terrestrial plants, arbuscular mycorrhizas (AMs), a kind of symbiote that can help the host plant to absorb water and nutrients (Gadkar et al. 2001). Furthermore, AMs strongly promoted root branch and increased root morphology in the host plant (Wu et al. 2016). Mycorrhizal colonization promoted the formation of LRs of high order, induced more fine roots and less coarse roots (Yao et al. 2009). Besides, AMF obtains a certain amount of plant carbohydrates from host plants to the fungal partner for its development (Bago et al. 2000).

Therefore, the relationship among AMF, NO, carbohydrate and root system architecture is complex and needs to be further investigated.

In this work, we assessed the effects of *Diversispora versiformis* and sodium nitroprusside (SNP, a NO donor) on plant growth, root morphology and carbohydrate concentration in leaf and root of trifoliate orange (a heavily mycorrhizal dependent plant) and elucidated the regulation of NO in the AMF effect on improving root morphology.

Materials and Methods

The experiment consisted of a randomized block design with two factors: two mycorrhizal treatments (*D. versiformis* and non-AMF control) and two levels of 0 and 0.2 mmol/L SNP. Each treatment was replicated three times, resulting in a total of 12 pots. Meanwhile, each pot had three seedlings.

The AM fungal strain *D. versiformis* was propagated with identified fungal spores and white clover (*Trifolium repens*) for 16 weeks in pots, thereby, containing sands, spores (20 spores/g) and infected root segments. Seeds of trifoliate orange were sterilized with 70% alcohol for 5 min and sown into plastic pots (19 cm upper diameter × 17 cm height × 13 cm bottom diameter) supplied with 4.5 kg autoclaved (0.11 MPa, 121°C, 2 h) soil and 40 g mycorrhizal inoculum. The non-AMF treatment received the same amount of autoclaved mycorrhizal inoculum. Subsequently, all the pots were placed in a plastic greenhouse of Yangtze University, where photo flux density ranged from 721 to 967 μmol/m^2/s with 25/19°C average day/night temperature and 75-95% relative air humidity. After 90 days of plant transplanting, SNP treatments were done by adding 300 mL of 0 and 0.2 mmol/L SNP solution in the interval of three days. The SNP application was lasted for 8 weeks and then the seedlings were harvested.

Plant height, stem diameter and leaf number were directly measured. The seedlings were separated into the shoot and the root, whose dry weight was measured after oven-drying at 75°C for 48 h. All the root systems were washed carefully by tap water and the number of LRs in different orders as well as the length of taproots were recorded. Then, the root system was scanned by the Epson Perfection V700 Photo Dual Lens System (J221A, Indonesia). Root morphological traits, including length, surface area, volume and average diameter were analyzed with the scanned photo by a WinRHIZO professional software in 2007 (Regent Instruments Inc, Quebec, Canada). At the same time, 1 cm long root segments were collected and stained with trypan blue, according to the protocol of Wu (2010). The root mycorrhizal colonization was counted as the percentage of infected root length against total observed root length.

Sucrose and glucose contents in leaves and roots were determined according to the method of Wu et al. (2010). Data (means ± SD, n = 3) were analyzed with ANOVA (SAS, version 8.1) and the significant differences among these treatments were compared by the Fisher's Protected Least Significant Difference (LSD) at $p<0.05$.

Results and Discussion

Root mycorrhizal colonization

Root mycorrhizal colonization of trifoliate orange by *D. versiformis* varied from 42.0% to 52.7% (Table 1). Whereas, the SNP treatment induced 25% higher root mycorrhizal colonization than non-SNP treatment. Previously Calcagno et al. (2012) reported that, the exudates of *Gigaspora margarita* could induce NO accumulation, which is a novel component in the signaling pathway leading to AM symbiosis. Puppo et al. (2013) concluded that NO controlled the mycorrhizal infection process. These results suggest that, NO may play an important role in the colonization and development of root mycorrhizas.

Plant growth performance

AMF could stimulate plant growth significantly once the AM association was formed (Augin et al. 2004). The present study showed that inoculation with *D. versiformis* significantly increased plant height, stem diameter, leaf number, shoot and root dry weight by 31%, 26%, 24%, 38% and 41% under SNP conditions and by 20%, 10%, 15%, 17% and 11% under non-SNP conditions, respectively (Table 1). The AMF colonization significantly promoted plant growth and the SNP treatment further amplify the AMF effect. This could be attributed to NO, which regulates the growth of leaves and roots in certain concentrations (Cueto et al. 1996; Leshem & Wills 1998). The significant plant growth promotion of AMF+SNP treatment may be closely linked with root mycorrhizal colonization, because AMF+SNP treatment had significantly higher root colonization than AMF-SNP treatment. As previously

TABLE 1. Effects of AMF and SNP on mycorrhizal colonization and plant growth of trifoliate orange seedlings

Treatments	AMF colonization (%)	Plant height (cm)	Stem diameter (mm)	Leaf number per plant	Shoot biomass (g DW/plant)	Root biomass (g DW/plant)
Non-AMF-SNP	0 ± 0^c	17.8 ±1.4^c	2.20 ±0.13^c	18 ±2^b	0.407 ± 0.031^c	0.235 ± 0.021^b
Non-AMF+SNP	0 ± 0^c	20.6 ±1.5^c	2.49 ±0.18^a	17 ±2^b	0.494 ± 0.045^b	0.262 ± 0.020^b
AMF-SNP	42.0 ± 2.7^b	21.3 ±1.3^b	2.42 ±.0.09^b	21 ±1^a	0.478 ± 0.042^b	0.262 ± 0.023^b
AMF+SNP	52.7 ± 3.6^a	23.3 ±1.7^a	2.78 ± 0.21^a	22 ±1^a	0.561 ± 0.055^a	0.332 ± 0.030^a

Data (means ± SD, $n = 3$) followed by different letters indicate significant differences ($p<0.05$) between treatments

reported by Wu et al. (2015), root mycorrhizal colonization was positively correlated with plant growth. These findings suggest that NO improved plant growth by accelerating the colonization of AMF. However, the underlying mechanism needs to be further investigated.

ROOT MORPHOLOGY

In this study, the AMF colonization significantly increased root projected area, surface area and volume of trifoliate orange seedlings: 66%, 66% and 69% higher under SNP conditions and 43%, 43% and 56% higher under non-SNP conditions, respectively (Table 2). The result is in accordance with Wu et al. (2011). Moreover, the SNP treatment significantly promoted root projected area, surface area and volume by 19%, 19% and 18% in the non-AM seedlings, respectively. These results imply that NO (exogenous SNP treatment) strengthened the AMF effect on root morphology of trifoliate orange.

Furthermore, the AMF inoculation and SNP treatment strongly induced higher number of 2nd and 3rd order LRs (Table 2). These results are in agreement with previous works on leek (Berta et al. 1990) and tomato (Correa-Aragunde et al. 2004). In addition, the AMF and SNP treatments represented considerably higher root length, which originated from the increase of fine roots in 0-0.5 cm (Table 3). This showed that AMF mainly induce fine root formation, which would increase the chance by AMF infection (Wu et al. 2010). The AMF inoculation induced the host plant to produce auxin (Meixner et al. 2007), which is the key factor involving in root development. Furthermore, NO as the downstream product in the auxin signal transduction pathway takes part in root formation and development (Xiong et al. 2009). In a nut shell, these results suggested that AMF and NO interact synergistically to stimulate LR formation and fine root development.

SUCROSE AND GLUCOSE CONTENTS IN LEAF AND ROOT

In comparison with non-AMF-SNP treatment, AMF-SNP treatment and non-AMF+SNP treatment significantly increased glucose contents by 50% and 48% in leaves and 16% and 44% in roots, respectively (Figure 1). Meanwhile, the glucose contents under AMF+SNP treatment were significantly less than AMF-SNP and non-AMF+SNP treatments both in leaves and roots. Greater root AMF colonization under AMF+SNP conditions may utilize more root glucose, thereby resulting in lower glucose contents under AMF+SNP treatment than AMF-SNP and non-AMF+SNP treatments. These results suggested that there has a suppressive effect between NO and AMF in promoting glucose content. Furthermore, compared with non-AMF-SNP treatment, the AMF inoculation significantly increased leaf sucrose content by 222% under SNP treatment and by 205% under non-SNP treatment (Figure 2). These results indicate that there has a synergistic effect between NO and AMF in accelerating the formation of sucrose in leaves. Additionally, compared with non-AMF-SNP treatment, root sucrose content significantly increased by 48% and 37% under AMF-SNP and non-AMF+SNP treatments, respectively, while decreased by 5% under AMF+SNP treatment. It suggests that there has an inconsistent effect between NO and AMF in facilitating sucrose accumulation in roots. We concluded an opposite trend in root sucrose and leaf and root glucose contents by SNP in AMF versus non-AMF seedlings. However, the elucidation of the biochemical mechanisms by which NO participated in this signaling pathway is still in its infancy and needs to be further investigated. In general,

TABLE 2. Effects of AMF and SNP on root morphology and lateral root number of trifoliate orange seedlings

Treatments	Average diameter (mm)	Projected area (cm^2)	Surface area (cm^2)	Volume (cm^3)	Number of lateral roots		
					1st order	2nd order	3rd order
Non-AMF-SNP	0.45 ± 0.04^b	12.4 ± 1.1^d	39.1 ± 3.1^d	0.445 ± 0.039^d	47.3 ±2.6^a	115.0 ±8.0c	10.6 ±0.9c
Non-AMF+SNP	0.45 ± 0.03^b	14.8 ± 0.9^c	46.6 ± 3.9^c	0.525 ± 0.049^c	42.9 ±3.7^a	134.4 ±11.2b	12.8 ±1.1c
AMF-SNP	0.50 ± 0.03^a	17.7 ± 1.1^c	55.8 ± 2.6^b	0.694 ± 0.054^b	44.4 ±3.3^a	146.4 ±13.3b	17.7 ±1.6b
AMF+SNP	0.46 ± 0.03^b	20.6 ± 1.8^a	64.8 ± 5.2^a	0.751 ± 0.066^a	47.6 ±3.9^a	168.2 ±10.5a	27.6 ±1.1a

Data (means ± SD, n = 3) followed by different letters indicate significant differences (p<0.05) between treatments

TABLE 3. Effects of AMF and SNP on root length of trifoliate orange seedlings

Treatments	Different length root (cm)						Total root length (cm)
	0.0<L≤0.5	0.5<L≤1.0	1.0<L≤1.5	1.5<L≤2.0	2.0<L≤2.5	2.5<L≤3.0	
Non-AMF-SNP	223.5 ± 18.2^c	38.9 ± 3.4^c	6.0 ± 0.5^b	3.1 ± 0.2^b	2.4 ± 0.1^b	2.0 ± 0.1^b	276.6 ± 24.1^d
Non-AMF+SNP	270.7 ± 20.1^b	45.0 ± 3.5^b	5.7 ± 0.4^b	2.3 ± 0.1^c	2.7 ± 0.1^a	2.4 ± 0.2^a	330.2 ± 32.4^c
AMF-SNP	277.5 ± 26.3^b	65.0 ± 3.5^a	8.4 ± 0.4^a	3.6 ± 0.3^a	1.9 ± 0.1^d	2.4 ± 0.2^a	361.8 ± 26.4^b
AMF+SNP	362.5 ± 24.5^a	66.8 ± 5.3^a	8.0 ± 0.7^a	3.4 ± 0.2^a	2.1 ± 0.1^c	2.0 ± 0.1^b	448.6 ± 32.0^a

Note: L: Length

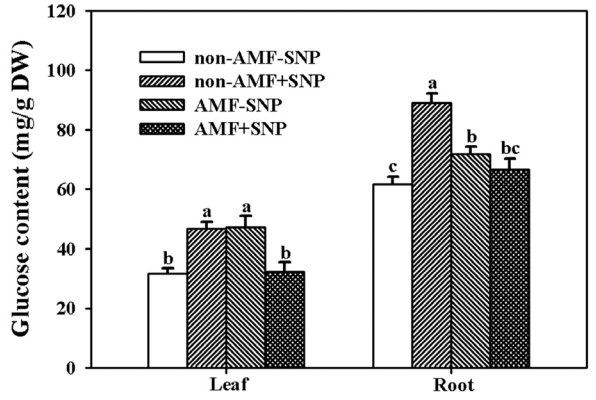

FIGURE 1. Effects of AMF and SNP on leaf and root glucose contents of trifoliate orange seedlings. Data (means ± SD, $n = 3$) are significantly different ($p<0.05$) if followed by different letters above the bars

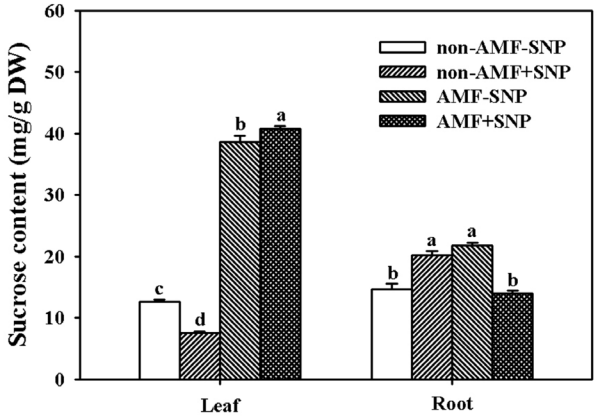

FIGURE 2. Effects of AMF and SNP on leaf and root sucrose content of trifoliate orange seedlings. Data (means ± SD, $n = 3$) are significantly different ($p<0.05$) if followed by different letters above the bars

AMF colonization and SNP treatment induced higher glucose contents and lower sucrose contents in roots of trifoliate orange. As previously reported by Arenas-Huertero et al. (2000), higher sucrose inhibits root growth because of the accumulation of abscisic acid, while glucose facilitates root growth because it plays important roles in the signaling pathway of gibberellins and cytokinins (Hu et al. 2009).

CONCLUSION

The present results confirmed that NO participated in mycorrhizal development. Such positive effect of NO on mycorrhizas would accelerate AM roles in plant growth and root morphology (especially fine roots) of trifoliate orange. X.Y.H. and Q.S.W. designed the experiment; X.Y.H. conducted the experiment; L.T. and X.Y.H. did the data analysis; L.T. and N. drafted the manuscript; Q.S.W. and N. reviewed the manuscript. The authors state that there is no conflict of interest.

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Li Tian, Xiao-Yun Huang & Qiang-Sheng Wu*
College of Horticulture and Gardening
Yangtze University, Jingzhou
Hubei 434025
China

Li Tian & Qiang-Sheng Wu*
Institute of Root Biology
Yangtze University, Jingzhou
Hubei 434025
China

Nasrullah
Department of Plant Sciences
Quaid-I-Azam University, Islamabad
Pakistan

Nasrullah
Department of Biology and Ecology
Nankai University, Tianjin
China

Qiang-Sheng Wu*
Department of Chemistry, Faculty of Science
University of Hradec Kralove, Hradec Kralove
Czech Republic

*Corresponding author; email: wuqiangsh@163.com

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