Indian Journal of Agricultural Sciences **89** (7): 1190–4, July 2019/Article
https://doi.org/10.56093/ijas.v89i7.91696

Development of propagation technique of indigenous AMF and their inoculation response in citrus

QIANG-SHENG WU[1], JIA-DONG HE[2], A K SRIVASTAVA[3], FEI ZHANG[4] and YING-NING ZOU[5]

Yangtze University, Jingzhou, Hubei 434 025, China

Received: 01 January 2019; Accepted: 22 January 2019

ABSTRACT

Arbuscular mycorrhizal fungi (AMF) have multi-pronged utility in citrus performance, while their propagation technique is a gap. In this study, indigenous AMF in rhizosphere of *Citrus unshiu* grafted on trifoliate orange were isolated from fresh root segments (Φ<2 mm), fresh rhizosphere soil (< 4 mm size), and air-dried rhizosphere soil (< 4 mm size) as AMF-source and propagated with white clover. Subsequently, indigenous AMF inocula were inoculated into potted trifoliate orange to assess the inoculated efficiency. Our results showed that AMF isolated from fresh root segments multiplied by 333.9% significantly higher than those isolated from fresh or air-dried rhizosphere soil. Similar results were obtained with regard to root mycorrhizal colonization (37.16–55.41%) and soil hyphal length (3.88–13.38 cm/g) in trifoliate orange after inoculated with AMF-source from root segments. Mycorrhizal trifoliate orange seedlings carrying AMF inoculum from fresh roots exhibited higher plant growth performance, root morphology, leaf P, K, Mg, Cu and Zn levels, and leaf superoxide dismutase, peroxidase, and catalase activities, compared to non-AMF treatment. Our study, hence, suggested that root segments would be a great choice to propagate indigenous AMF for later inoculating into the rhizosphere of target plants.

Key words: Antioxidant enzyme, Arbuscular mycorrhiza, Citrus, Root morphology, Spore

In fields, citrus plants rely strongly on soil arbuscular mycorrhizal fungi (AMF) to form arbuscular mycorrhizas (AMs) for their optimum performance through mycorrhizal extraradical hyphae in nutrient and water uptake (Srivastava *et al*. 2002, Ngullie *et al*. 2015, Zou *et al*. 2018). Citrus rhizosphere is reported to inhabit as many as 45 species of AMF, which colonize roots to enlarge the absorptive surface of roots (Wu *et al*. 2013). Earlier studies showed that AM presence could promote nutrients and water absorption, accelerate plant growth, modify root architecture and root hairs, enhance stress tolerance, increase soil fertility, and improve soil structure (Wu *et al*. 2017). Hence, AMF has been accepted as a biofertilizer and used so extensively and intensively in citrus production.

Despite these developments highlighting the utility of AMF in citrus orchards, the propagation technique of AMF has not made the desired progress over a time period. Earlier studies, though, focused on the inoculation of exotic AMF on potted citrus plants (Wu *et al*. 2013 2017) but with a limited success. In China, Shen and Wan (1994) reported that in the hotbed, excellent AMF strains could be propagated with white clover, and then the seeds of trifoliate orange were sown into the hotbed containing AMF for mycorrhization. Menge (1983) earlier proposed that isolated spores of AMF from the roots or soils in citrus inoculated into Sudan grass growing in sterilized soils in the greenhouse. After spore proliferation, the mycorrhizal inoculum was used to inoculate plant roots for containerized mycorrhizal citrus plants. In fact, distinct ecotypes of AM fungal strains behave differently in citrus rhizosphere (Wu *et al*. 2016). While application of these exotic AMF strains in citrus rhizosphere could bring some risks in form of biological invasion (Pringle *et al*. 2009). The use of native AMF is therefore, advocated for propagation and application of AMF in citriculture.

In this background, the study was conducted to propagate the indigenous AMF strains of citrus field and subsequently verify the inoculation response of these indigenous AMF in citrus plants. Such results would provide a foundation for the propagation and application of indigenous AMF in citrus orchards on a commercial scale.

MATERIALS AND METHODS

Isolation of native AMF and propagation: The rhizosphere soil (< 10 cm of soil layer) and roots (Φ < 2 mm) were collected from a citrus orchard with 30-year-old *Citrus unshiu* cv. Guoqing 1 grafted on *Poncirus trifoliate* spaced

[1]Professor (wuqiangsh@163.com), [2]Postgraduate Student, [4]Doctoral Student, [5]Associate Professor, College of Horticulture and Gardening, Yangtze University, Jingzhou, Hubei, China. [3]Principal Scientist (aksrivas2007@gmail.com), ICAR-Central Citrus Research Institute, Nagpur, Maharashtra, India.

at of 3 m × 4 m in apart in campus of Yangtze University (30°362 N, 112°142 E, 36 m above sea level). From rhizosphere soil and root segments, a total of 4 treatments, replicated 5 times were formulated, which comprise T_1 as fresh roots (6 g fresh root segments having colonization of native AMF species was mixed with autoclaved (0.11 MPa, 121°C, 2 h) rhizosphere soil after removing roots/ other impurities and sieving with 4 mm size); T_2 having air-dried rhizosphere soils (Soils were dried naturally, removed root segments/other impurities and screened by 4 mm size); T_3 as rhizosphere fresh soil (Collected fresh rhizosphere soil was cleaned of roots /other impurities and sieved through 4 mm to retain native AMF species in soils; and control (CK) as sterilized soils (after removing roots and impurities and sieving through 4 mm, the rhizosphere soils were autoclaved (0.11 MPa, 121°C, 2 h) to eliminate native AMF spores). In all the four treatments, sterilized (0.11 MPa, 121°C, 2 h) river sands (3:1, v/v) were evenly mixed. Seeds of white clover (*Trifolium repens* L.) were then sown and grown in a Walk-in Artificial Plant Growth Chamber (Zhejiang Qiushi Artificial Environment Co. Ltd. China) having air temperature 28°C/18°C (12 hours in the day / 12 hours in the night), light intensity 1200 Lux, and relative air humidity 68% for spore germination. After 3 months (February 19 to May 19, 2017) of AMF propagation with host plants in pots, the indigenous AMF was collected, and the spore density of AMF was determined by wet sieving-sucrose centrifugation using the procedure as outlined by Brundrett *et al*. (1994).

Inoculation of host plants with indigenous AMF: Plastic pots having 1.5 L capacity were filled with 1.3 kg autoclaved (0.11 MPa, 121°C, 2 h) rhizosphere soils, then inoculated with 200 g inoculum of indigenous AMF (composed of infected root segments, spores and soil hyphae). Each pot had three five-leaf-age trifoliate orange seedlings raised in non-mycorrhized autoclaved sands. The trifoliate orange seedlings were harvested after three months (May 19 to August 19, 2017) of indigenous AMF-inoculation.

Variable determinations: Plant height, stem diameter, leaf number per seedling, and fresh weight of shoots and roots in trifoliate orange were measured. The mycorrhizal colonization of roots was studied by staining and examining them with the help of optical microscope (Phillips and Hayman 1970) and calculated as the percentage of mycorrhizal colonized root lengths against total observed root length. Soil spore density was determined with wet sieving-sucrose centrifugation (Brundrett *et al*. 1994). Spore propagation rate was calculated by the following formula: Spore propagation rate = (soil spore density after propagation – soil spore density before propagation)/spore density before propagation × 100%.

Leaf mineral nutrient (P, K, Ca, Mg, Fe, Cu, Mn, Zn, and B) levels were determined by the Plasma Emission Spectrometer (IRIS Advantage, American Thermoelectric Company, USA) (Black *et al*. 1982). Leaf superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities were assayed using nitrogen blue tetrazole method (Giannopolitis and Ries 1997), guaiacol method (Chance and Maehly 1955) and ultraviolet spectrophotometry (Wu 2018), respectively.

Statistical analysis: The data were analyzed with the ANOVA in SAS (8.1) for significance levels, and the Duncan's Multiple Range Test ($P<0.05$) was used to compare significant differences amongst treatments.

RESULTS AND DISCUSSION

Propagation of indigenous AMF: The soil spore density after propagated with white clover for three months was 81, 122, and 128 spores / 10 g dry soil under T1, T2, and T3 conditions, respectively. Treatment T_1 induced maximum spore propagation rate of 339.44% followed by 154.35% with treatment T_2 and 104.89% with treatment T_3. These observations suggested that the fresh roots possessed the maximum spore density compared with either dried rhizosphere soils or fresh rhizosphere soil. Using the Internal Transcribed Spacer Fragment sequencing, Wu *et al*. (2017) reported that AMF communities in citrus roots were much higher than citrus rhizosphere soil. Such results further indicated that AMF diversity in roots was more than those in rhizosphere soil.

Mycorrhizal status in trifoliate orange as host plant: No mycorrhiza was observed in the CK-treated trifoliate orange. Root mycorrhizal colonization with treatments T_1, T_2, and T_3 was 55.4%, 51.1% and 37.2%, soil hyphal length was 13.38, 5.93, and 3.88 cm/g soil, respectively. The response of different treatments for magnitude of root colonization and soil hyphal length could be ranked as $T_1 = T_2 > T_3$ and $T_1 > T_2 > T_3$, respectively, in the decreasing order. The observation further indicated a higher mycorrhizal colonization and soil hyphal length in mycorrized soil treated with AMF incoculum form of fresh roots. Although spore propagation rate with T_2 was marginally higher than that of T_3, the difference was non-significant. But a significantly greater inoculation effect of AMF was observed on root mycorrhizal colonization and soil hyphal length with treatment T_2 than with T_3, suggesting that moderate soil water deficit with a short time is beneficial for multiplication of AMF spores within citrus rhizosphere, even in field conditions (Jacobson 1997, Augé 2001).

Plant growth responses with indigenous AMF inoculation: After three months of indigenous AMF inoculation, no significant difference in stem diameter and root volume of trifoliate orange seedlings was observed among all the four treatments (Fig 1 and Table 1). Compared with CK treatment, T_3 treatment likewise produced no significant response on different growth parameters, viz. plant height, leaf number per plant, biomass of shoot and root, total root length, and root projected area. However, both treatments T_1 and T_2 showed a significant increase in plant height, leaf number per plant, biomass of shoot and root, over treatments CK but treatments T_1 and T_2 were at par with each other. Compared with non-AMF seedlings (CK), mycorrhizal seedlings represented by T_1 and T_2 observed a significantly higher total root length, root projected area, and

Table 1 Effects of different AMF inocula on plant growth performance and root morphology in trifoliate orange seedlings

Treatment	Growth performance					Root morphology			
	Plant height (cm)	Stem diameter (cm)	Leaf number (#/plant)	Shoot (g FW/plant)	Root (g FW/plant)	Total length (cm)	Projected area (cm^2)	Surface area (cm^2)	Volume (cm^3)
T_1	24.8±2.7a	0.25±0.02a	25±2a	1.5±0.2a	1.0±0.04a	236±19a	14.0±0.2a	19.2±0.5a	0.62±0.11a
T_2	24.3±3.5a	0.24±0.03a	23±1a	1.4±0.4a	1.0±0.06a	207±11b	13.2±0.2b	16.9±0.8b	0.74±0.13a
T_3	16.6±0.7b	0.22±0.02a	18±2b	0.8±0.1b	0.9±0.08b	198±9bc	12.7±0.7bc	15.3±0.9c	0.72±0.22a
CK	15.9±1.8b	0.23±0.04a	18±1b	0.9±0.2b	0.9±0.07b	188±6c	12.2±0.4c	13.6±0.3d	0.65±0.10a

Data (means±SD, n=5) followed by different letters among treatments indicate significant differences at the 5% level between treatments. T1, the inoculums from fresh roots as indigenous AMF propagated source; T2, the inoculums from air-dried soils as indigenous AMF propagated source; T3, the inoculums from rhizosphere fresh soils as indigenous AMF propagated source; CK, the inoculums from sterilized soils as indigenous AMF propagated source.

root surface area. Amongst different treatments, treatment T_1 re-showed the response of highest magnitude on plant growth performance and root morphology. Hence, treatments T_1 and T_2, especially T_1, heavily significantly promoted the plant growth and root morphology, whereas treatment T_3 remained ineffective with respect to these attributes. These results could be effectively linked to spore propagation rate, subsequently root mycorrhizal colonization and mycorrhizal length in mycorrhizosphere (Zou *et al*. 2017, Liu *et al*. 2018). The effect of treatment T_1 on total root length, root projected area and root surface area of trifoliate orange as host plant was significantly higher than treatment T_2. It was further confirmed that the spore density and mycorrhizal development were higher in position of treatment T_1 than treatment T_2. Mycorrhizal plants develop better root architecture, conducive to plant colonization, growth and stress tolerance (Wu and Zou 2017).

Leaf nutrient composition responses with indigenous AMF inoculation: In the present study, significant difference in leaf Ca content was observed while comparing the effectiveness of all the four treatments (Table 2). Compared with CK, treatment T_1 and T_2 were associated with a significant increase in leaf P, K, Mg, Cu and Zn levels, but leaf Mn, Fe and B concentrations substantially decreased. The stimulatory effect of AMF inoculation was of higher magnitude with treatment T_1 than either T_2 or T_3. The treatment T_3 also significantly increased leaf P, K, Mg and Zn concentrations, but decreased leaf Mn, Fe and B concentrations, as compared with CK. On the whole, leaf P, K, Mg, Cu and Zn levels were significant higher in T_1 and T_2 treatments than in T_3 treatment (Table 2). Better mineral levels in mycorrhizal trifoliate orange seedlings could be attributed to effective intervention of mycorrhizal extra radical hyphae in mineral absorption from the mycorrhized soil (Leake *et al*. 2004), as a function of improved root morphology in AMF seedlings (Wu *et al*. 2017), which in turn aided in accumulating mineral nutrients, thereby, promoting the development of AMF-mycelium to add the better nutrient foraging ability of host plants (Bonfante and Genre 2010).

Fig. 1. Plant growth responses of trifoliate orange seedlings colonized by different AMF inoculums. T1, the inoculums from fresh roots as indigenous AMF propagated source; T2, the inoculums from air-dried soils as indigenous AMF propagated source; T3, the inoculums from rhizosphere fresh soils as indigenous AMF propagated source; CK, the inoculums from sterilized soils as indigenous AMF propagated

Leaf antioxidant enzyme profile responses with indigenous AMF inoculation: AMF inoculation is reported to impart better biochemical preparedness to the host plant (Liu *et al*. 2018). The activities of SOD, POD and CAT in leaves with treatment T_1 were, respectively observed 307.71%, 74.48% and 75.38% higher than with CK (data not shown). The activities of SOD, POD and CAT observed a significant increase by 120.96%, 18.29% and 70.07%, respectively, with treatment T_2 over CK. Compared with CK, SOD activity in leaves under treatment T_3 increased by 94.07%, but without any significant response on activities of POD and CAT. Compared with treatments T_3, the activities of SOD, POD and CAT in leaves were significantly increased with treatment T_1. Mycorrhizal plants, therefore, showed higher activity of antioxidant enzymes in the host plants to develop a greater resilience against biotic/abiotic stresses. Our observations are consistent with the studies of Huang *et al*. (2014) highlighting the response of inoculating AMF on trifoliate orange under drought stress. The changes in antioxidant enzymes were also reported to be closely related with AMF colonization, number of arbuscules, and amount of mycorrhiza-induced calmodulin (Huang *et al*. 2014).

Table 2 Effects of different AMF inocula on leaf mineral levels in trifoliate orange seedlings

Treatment	P/g/kg	K/g/kg	Ca/g/kg	Mg/g/kg	Cu/mg/kg	Zn/mg/kg	Mn/mg/kg	Fe/mg/kg	B/mg/kg
T_1	2.81±0.09a	8.95±0.54a	26.97±1.78a	2.51±0.08a	12.36±0.96a	27.96±1.69a	34.07±2.21b	238±10b	69.5±3.6b
T_2	2.31±0.13b	8.75±0.31b	26.18±0.94a	2.60±0.17a	10.80±0.61b	28.89±1.20a	29.25±1.51c	159±13c	47.9±2.9c
T_3	2.17±0.08c	7.64±0.65b	25.34±0.96a	2.21±0.06b	8.55±0.56c	23.48±1.09b	35.28±1.85b	240±12b	67.8±3.0b
CK	1.38±0.10d	6.75±0.26c	26.91±1.28a	1.95±0.05c	7.90±0.48c	17.18±1.20c	43.08±1.49a	292±14a	92.9±2.3a

Data (means±SD, n = 5) followed by different letters among treatments indicate significant differences at the 5% level between treatments. T_1, the inoculums from fresh roots as indigenous AMF propagated source; T_2, the inoculums from air-dried soils as indigenous AMF propagated source; T_3, the inoculums from rhizosphere fresh soils as indigenous AMF propagated source; CK, the inoculums from sterilized soils as indigenous AMF propagated source.

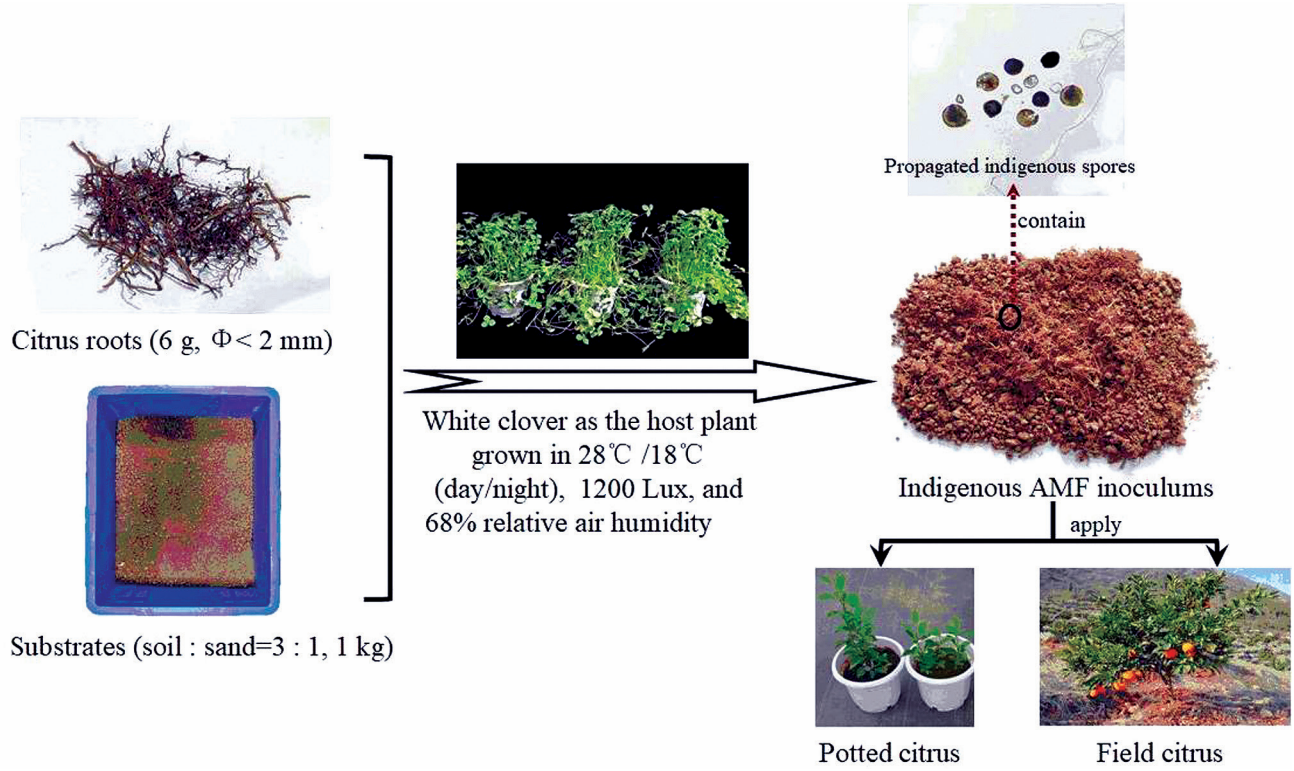

Fig 2 Schematic representation of protocol for propagation of indigenous AMF from citrus orchard and later inoculation in potted and field citrus.

In short, the combination of fresh root segment and white clover served as an effective indigenous spore propagation method of AMF, the role of which was duly verified by the magnitude of response observed in mycorrhized trifoliate orange. Such an attempt could be effectively developed in citriculture. As a result, the present study clearly confers a feasible protocol for rapid proliferation of indigenous AMF to citrus orchard (Fig 2). The full potential of AMF as a biofertilizer is to be exploited in future.

ACKNOWLEDGEMENTS

This study was supported by the Hubei Agricultural Science and Technology Innovation Action Project, the Hubei Agricultural Major Technical Cooperation Project, and the Plan in Scientific and Technological Innovation Team of Outstanding Young Scientists, Hubei Provincial Department of Education (T201604).

REFERENCES

Allen M F. 2009. Water relations in the mycorrhizosphere. *Progress in Botany.* Vol 70 pp 257–76. Lüttge U, Beyschlag W, Büdel B and Francis D (Eds). Springer-Verlag, Berlin, Heidelberg.

Amiri R, Nikbakht A and Etemadi N. 2015. Alleviation of drought stress on rose geranium [*Pelargonium graveolens* (L.) Herit.] in terms of antioxidant activity and secondary metabolites by mycorrhizal inoculation. *Scientia Horticulturae* **197**: 373–80.

Augé R M. 2001. Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. *Mycorrhiza* **11**: 3–42.

Bethlenfalvay G J and Ames R N. 1987. Comparison of two methods for quantifying extraradical mycelium of vesicular-arbuscular mycorrhizal fungi. *Soil Science Society of America Journal* **51**: 834–37.

Black C A, Evans D D, White J L, Ensminger L E and Clark F E. 1982. *Methods of Soil Analysis*, pp 228–34. American Society of Agronomy, Wisconsin, USA.

Bonfante P and Genre A. 2010. Mechanisms underlying beneficial

plant-fungus interactions in mycorrhizal symbiosis. *Nature Communications* **1**: 48.

Brundrett M, Melville L and Peterson L. 1994. *Practical Methods in Mycorrhiza Research*. Mycologue Publications, University of Guelph, Guelph, Ontario, Canada.

Chance B and Maehly A C. 1955. Assay of catalases and peroxidases. *Methods in Enzymology* **2**: 764–75.

Giannopolitis C N and Ries S K. 1977. Superoxide dismutase. I. Occurrence in higher plants. *Plant Physiology* **59**: 309–14.

Graham J H and Syvertsen J P. 1984. Influence of vesicular–arbuscular mycorrhiza on the hydraulic conductivity of roots of two citrus rootstocks. *New Phytologist* **97**: 277–84.

Huang Y M, Srivastava A K, Zou Y N, Ni Q D and Wu Q S. 2014. Mycorrhizal-induced calmodulin mediated changes in antioxidant enzymes and growth response of drought-stressed trifoliate orange. *Frontiers in Microbiology* **5**: 682.

Jacobson K M. 1997. Moisture and substrate stability determine VA-mycorrhizal fungal community distribution and structure in an arid grassland. *Journal of Arid Environments* **35**: 59–-75.

Leake J, Johnson D, Donnelly D, Muckle G, Boddy L and Read D. 2004. Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. *Canadian Journal of Botany* **82**: 1016–45.

Liu C Y, Zhang F, Zhang D J, Srivastava A K, Wu Q S and Zou Y N. 2018. Mycorrhiza stimulates root-hair growth and IAA synthesis and transport in trifoliate orange under drought stress. *Scientific Reports* **8**: 1978.

Menge J A. 1983. Utilization of vesicular-arbuscular mycorrhizal fungi in agriculture. *Canadian Journal of Botany* **61**: 1015–24.

Ngullie E, Singh A K, Sema A and Srivastava A K 2015. Citrus growth and rhizosphere properties. *Communications in Soil Science and Plant Analysis*. **46**: 1540–50.

Phillips J M and Hayman D S. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society* **55**: 158–61.

Pringle A, Bever J D, Gardes M, Parrent J L, Rillig M C and Klironomos J N. 2009. Mycorrhizal symbioses and plant invasions. *Annual Review of Ecology Evolution & Systematics* **40**: 699–715.

Shen T H and Wan S L. 1994. A practical study on the propagation and inoculation technique of VA mycorrhizal fungi in citrus. *Acta Agriculturae Jiangxi* **6**: 25-30 (in Chinese with English abstract).

Srivastava A K, Shyam Singh and Marathe R M 2002. Organic citrus: Soil fertility and plant nutrition. *Journal of Sustainable Agriculture* **19**: 5–29.

Wu Q S and Zou Y N. 2017. Arbuscular mycorrhizal fungi and tolerance of drought stress in plants. *Arbuscular Mycorrhizas and Stress Tolerance of Plants*, pp 25-42. Wu Q S (Ed). Springer Nature Singapore Pvt Ltd, Singapore.

Wu Q S, Liu C Y, Zhang D J, Zou Y N, He X H and Wu Q H. 2016. Mycorrhiza alters the profile of root hairs in trifoliate orange. *Mycorrhiza* **26**: 237–47.

Wu Q S, Srivastava A K and Zou Y N. 2013. AMF-induced tolerance to drought stress in citrus: A review. *Scientia Horticulturae* **164**:77–-87.

Wu Q S, Srivastava A K, Zou Y N and Malhotra S K. 2017. Mycorrhizas in citrus: Beyond soil fertility and plant nutrition. *Indian Journal of Agricultural Sciences* **87**: 427–43.

Wu Q S, Sun P and Srivastava A K. 2017. AMF diversity in citrus rhizosphere. *Indian Journal of Agricultural Sciences* **87**: 653–66.

Wu Q S. 2018. *Experimental Guidelines in Plant Physiology*. Chinese Agricultural Press, Beijing, China.

Zou Y N, Srivastava A K and Wu Q S. 2018. Water redistribution in mycorrhizosphere of trifoliate orange. *Indian Journal of Agriculture Sciences* **88**: 1198–201.

Zou Y N, Wang P, Liu C Y, Ni Q D, Zhang D J and Wu Q S. 2017. Mycorrhizal trifoliate orange has greater root adaptation of morphology and phytohormones in response to drought stress. *Scientific Reports* **7**: 41134.