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Arbuscular mycorrhizal enhancement of iron concentration by *Poncirus trifoliata* L. Raf and *Citrus reticulata* Blanco grown on sand medium under different pH

Mingyuan Wang · Peter Christie · Zhiyan Xiao ·
Changping Qin · Peng Wang · Jinfa Liu · Yachao Xie ·
Renxue Xia

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Abstract The effect of the arbuscular mycorrhizal (AM) fungus (*Glomus versiforme*) on iron contents by two citrus rootstocks (trifoliate orange [*Poncirus trifoliata* L. Raf] and red tangerine [*Citrus reticulata* Blanco]) was studied in sand culture under different pH conditions. Seeds were sown in a mixed substrate (perlite/sand, 1:1 [v/v]) inoculated with or without mycorrhizal inoculum. The experiment was carried out at four pH levels by applying nutrient solution at pH 5.0, 6.0, 7.0, or 8.0 to *P. trifoliata* and pH 5.2, 6.2, 7.2, or 8.2 to *C. reticulata*. No AM colonization was found in uninoculated control (NM) and plants, and root colonization in AM plants was depressed under iron deficiency at high pH. Colonization by *G. versiforme* led to higher dry weights of shoots compared with NM treatments, suggesting that *G. versiforme* enhanced plant growth. Higher concentration of chlorophyll and active iron, lower ratios of P/Fe and

50(10P+K)/Fe were present in AM plants than NM treatments. Nevertheless, *G. versiforme* improved root Fe (III) chelate reductase activity of *P. trifoliata* and *C. reticulata*. The data indicate that plant uptake and translocation of iron were enhanced and AM fungi may be considered as a potential tool for bioremediation of citrus iron deficiency.

Keywords Chlorophyll · Fe(III) chelate reductase · P/Fe ratio · 50(10P+K)/Fe · Iron contents · Mycorrhizal colonization

Abbreviations

AM arbuscular mycorrhizal
Chl chlorophyll
FCR Fe (III) chelate reductase

M. Wang · Z. Xiao · P. Wang · J. Liu · Y. Xie · R. Xia (✉)
Key Laboratory of Horticultural Plant Biology,
Ministry of Education, Huazhong Agricultural University,
Wuhan 430070 Hubei Province, China
e-mail: renxuexia@mail.hzau.edu.cn

P. Christie
Agricultural and Environmental Science Department,
Queen's University Belfast,
Newforge Lane,
Belfast BT9 5PX, UK

C. Qin
National Key Laboratory of Crop Genetic Improvement,
National Center of Crop Molecular Breeding,
Huazhong Agricultural University,
Wuhan 430070, People's Republic of China

M. Wang
College of Chemical Engineering, Huaqiao University,
Quanzhou 362021 Fujian Province, China

Introduction

Higher plant species can be classified into either strategy I or II according to their iron uptake mechanisms under iron deficiency (Marschner et al. 1986). Strategy I species include nongraminaceous monocot and all dicots including citrus. Under iron deficiency conditions, these plants induce a redox system on cell plasma membrane followed by the H^+ and chelate production. Then ferric iron is reduced to ferrous iron by the Fe (III) chelate reductase (FCR) ($Fe^{3+} - Fe^{2+}$), which can use electrons from reduced nicotinamide adenine dinucleotide (NADH). Strategy II plants are graminaceous species characterized by the release of phytosiderophores from their roots to form stable chelates with Fe^{3+} . Ferric ions are then transported by specific carriers present in the plasma membranes of the epidermal cells (Zhang et al. 1991).

Iron chlorosis in plants is a worldwide problem, especially in calcareous and alkaline soils (Marschner 1995). Iron is very abundant in most soils, and its availability to plants is only low in higher pH soils. Iron chlorosis occurs frequently in fruit trees such as peach (Igartua et al. 2000), pear (Sanz et al. 1994), and citrus (Pestana et al. 2001). Affected leaves turn yellow while the leaf veins remain green. Symptoms may appear over the entire tree, on one side only, or be limited to individual branches. Most of the affected orchards have high soil pH, leading to low iron solubility in the soil solution. Iron chlorosis has been reported to result in decreased yield and poor quality of fruit. The total iron contents may not reflect the iron nutritional status of the plants (Pestana et al. 2003). Iron deficiency depresses the synthesis of chlorophyll, which results in the decrease of photosynthetic products, which affect plant growth. Iron nutritional status can be diagnosed by the content of active iron, biomass and chlorophyll (Chl) contents of young leaves. While the iron concentration fluctuates during the growth season, the P/Fe ratio and 50(10P+K)/Fe have consistent values. Therefore, the P/Fe ratio and 50(10P+K)/Fe are also considered as an index of iron nutrition (Köseoglu 1995; Álvarez-Fernández et al. 2005).

Citrus is very susceptible to iron chlorosis, and iron chlorosis is the most frequent nutritional problem in this type of fruit. Different management regimes have been devised such as iron sprays, iron injection, or soil iron fertilizer application including EDDHA/Fe³⁺, EDDHMA/Fe³⁺, and the novel EDDHSA/Fe³⁺ (Álvarez-Fernández et al. 2005). Iron spraying is effective, but the frequent applications required are very time-consuming. Iron injection is a useful alternative, but pathogens and bacterial infections often occur. Iron chelate fertilizers are usually very expensive.

Arbuscular mycorrhizal (AM) fungi are widely distributed in soils and about 90% of higher plant species examined interact with AM fungi (Gadkar et al. 2001). The association with AM fungi can enhance plant growth and mineral nutrition (Toro et al. 1998; Wu and Xia 2006) and increase plant tolerance to environmental stresses such as drought conditions (Porcel and Ruiz-Lozano 2004).

AM fungi have been found to affect plant uptake of mineral nutrients, especially relatively immobile elements such as P, Zn, Cu, and Al (Smith and Read 1997; Thingstrup et al. 2000). However, few studies on the effects of AM fungi on plant iron uptake have been reported. Caris et al. (1998) found that *Glomus mosseae* significantly increased iron absorption from soils by sorghum, but no difference was found in peanut. In contrast, Pacovsky and Fuller (1988) reported that the presence of AM fungi decreased iron absorption by soybean. Different experiments in which different host plant species have been inoculated with different AM fungi under different experimental conditions have given varied results.

To study plant iron nutrition with soil is problematic because the amount of available iron is very low in soil, pH can change during the experiment, and it is difficult to separate the various abiotic and biotic mechanisms affecting iron behavior. The sand and perlite medium can hold water, avoid anaerobic conditions, and it does not contain metals after acid treatment and thus iron concentration can be easily changed for investigation purposes. Both sand and solutions have been already used in iron plant nutrition studies (González-Vallejo et al. 2000; Chouliaras et al. 2004a; Chen et al. 2005). In the present studies, two citrus rootstocks, *Poncirus trifoliata* and *Citrus reticulata*, inoculated with *Glomus versiforme* were grown in sand and perlite medium to study the effects of the *G. versiforme* on plant iron uptake treated with nutrient solutions differing in pH values because pH can affect the susceptibility of the plant to iron deficiency (more susceptible for *P. trifoliata*). The aim was to determine whether *G. versiforme* would enhance iron absorption under different pH conditions and alleviate iron chlorosis in citrus rootstocks.

Materials and methods

Sand culture and mycorrhizal inoculum

Fine sand (<2 mm diameter) and perlite [1:1 (v/v)] were mixed, treated with 1 M HCl for 48 h, then washed with tap water and rinsed in clean water for 48 h to remove excess Cl⁻ ions, which are toxic to citrus. The substrate was washed with distilled water, sterilized at 121–126°C for 2 h in an oven to eliminate all microbes, then distributed into 17-cm-diameter plastic pots with 3.0 kg per pot.

Mycorrhizal inoculum consisted of spores, hyphae, and colonized jowar root fragments from a stock culture of *G. versiforme* (Karsten) Berch provided by the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences. The inoculum added to each pot consisted of 20 g mycorrhizal inoculum per pot containing approx. 1,488 spores, which were extracted by Gerdemann and Nicolson (1963) and uninoculated controls received the same weight of autoclaved growth medium. Mycorrhizal inoculum was placed 5 cm below the seeds at sowing.

In November 2005, seeds of trifoliolate orange (*Poncirus trifoliata* L. Raf) and red tangerine (*Citrus reticulata* Blanco) were washed with tap water for 10 min, sterilized in 75% (v/v) ethanol for 10 min, then rinsed three to four times in distilled water. Seeds were germinated on wet filter paper at 26°C for 2 weeks and the seedlings sown in plastic pots, which were placed in a greenhouse with average day/night temperature of 25/15°C and relative humidity of 60%. Each pot was watered with 200 ml nutrient solution every 2 days (iron-free, Hoagland and Arnon 1938).

Experimental design

In June 2006, symptoms of iron chlorosis were observed on *P. trifoliata*. The experiment was carried out with mycorrhiza treatments (inoculation with *G. versiforme* and uninoculated control), which had been treated before sowing, and four pH levels. *P. trifoliata* seedlings were grown in substrates watered by nutrient solution with 50 μM Fe-EDTA at pH values of 5.0, 6.0 (as control), 7.0 and 8.0. *C. reticulata* seedlings were grown in nutrient solution with 20 μM Fe-ethylenediaminetetraacetic acid (EDTA) and pH values of 5.2, 6.2 (as control), 7.2, and 8.2. Iron was supplied as Fe-EDTA in the nutrient solution, 200 ml of which was applied every 2 days. Six seedlings were grown in each pot and there were six replicate pots of each treatment. Once harvested, the samples were mixed and a subsample was taken for determination of the following index.

Determination of plant growth and mycorrhizal colonization

The plants were harvested after 30 days in sand culture. Shoots and roots were separated, washed, and oven-dried at 75°C for 48 h. A fraction of the fresh roots was sampled and placed in formalin/acetic acid/ethanol [FAA, 13:5:200 (v/v/v)] for 24 h for determination of AM colonization. The samples were cleared with 10% (m/v) KOH solution and stained with 0.05% (v/v) trypan blue in lactophenol (Phillips and Hayman 1970). AM colonization was observed under a compound microscope (Model XSZ-G; Chongqing, China) and the percentage of AM colonization was calculated based on the following formula:

$$\text{AM colonisation (\%)} = 100 \times \frac{\text{number of root length colonised}}{\text{total number of root length observed.}}$$

Analysis of chlorophyll content, mineral composition, and root Fe (III) chelate reductase activity

Chlorophyll was extracted from 0.2 g fresh leaves in 25-ml 95% (v/v) ethanol in the dark at room temperature (Li 2000). Chl (Chl *a* and *b*) contents were measured at 649 and 665 nm with a Shimadzu UV-2450 spectrophotometer (Tokyo, Japan). Chl *a* and *b* contents were estimated according to the equations of Winternans and Mots (1965).

Fresh leaves were washed with distilled water, dried in an oven at 105°C for 48 h. The dry weight was obtained, then subsamples were mill-ground and after dry digestion in a muffle furnace at 480°C, the ashes were used to extract Fe and K with 1 M HCl according to the method of Oserkowsky (1933). The concentrations of Fe and K were

determined with a SpectrAA 220 atomic absorption spectrophotometer (Varian, Palo Alto, CA) following standard methods (Belkhodja et al. 1998).

The remainder of the samples was dry-digested with HCl in a muffle furnace at 480°C. Phosphorus was determined by the phospho-vanado-molybdate method with a Shimadzu UV-2450 spectrophotometer (Bao 2000).

Root FCR activity was estimated by a modification of the method of Welch et al. (1993) and Albano and Miller (1996). About 50 mg fresh root tips 5 mm in length were washed for 5 min with 0.5 mM $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ and then soaked in a solution consisting of 0.5 mM $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 0.1 mM Fe (III)-EDTA (ferric ethylene diamine-tetraacetate), and 0.3 mM Na_2 -bathophenanthrolinedisulfonic acid (BPDS). The solution was adjusted to pH 5.5 with 5 mM 2-*N*-morpholino ethanesulfonic acid (MES). The samples were incubated in a water bath at 25°C for 3 h in the dark. Root FCR was estimated at 535 nm with a Shimadzu UV-2450 spectrophotometer.

Data were statistically tested by two-way analysis of variance (ANOVA) using the SAS version 8.1 software package (SAS Institute, Cary, NC). Probabilities of significance were used to test the significance among treatments and interactions, and least significant difference (LSD) ($P < 0.05$) was used to compare the means.

Results

Mycorrhizal colonization, plant growth, and contents of Chl and iron

Roots of both citrus species inoculated with AM fungi were colonized at all pH levels tested in sand culture. No AM colonization was observed in uninoculated controls (Tables 1 and 2). Percentage of root length colonized decreased from 51.3 to 28.3% in *P. trifoliata* (Table 1) and decreased significantly ($P < 0.05$) in *C. reticulata* from 55.2 to 27.2% (Table 2) with increasing pH value.

Different pH values significantly affected ($P < 0.05$) plant biomass in *P. trifoliata* seedlings (Table 1), with higher shoot dry weights in the pH controls (pH 6.0) than in the pH treatments, but AM seedlings had significantly higher shoot dry weights than did the corresponding uninoculated controls, with significant differences only at the two highest pH values (pH 7.2 and 8.2) in *C. reticulata* (Table 2).

Chlorophyll contents of *P. trifoliata* are shown in Table 1 and of *C. reticulata* in Table 2. The pH controls (pH 6.0 and 6.2, respectively) showed the highest Chl contents and Chl concentrations decreased with increasing pH. Chl contents decreased by up to 30.7% in AM seedlings and 26.5% in uninoculated controls (Table 1), with lower corresponding values (21.4 and 24.7%) in *C. reticulata*

Table 1 Root colonization, shoot dry weight, chlorophyll concentration, and active iron contents of leaves of *Poncirus trifoliata* L. Raf seedlings growing in sand culture in response to a factorial treatment combination of the AM fungus *Glomus versiforme* and four pH levels

Mycorrhizal status	pH value	Root colonization (%)	Shoot dry weight (g plant ⁻¹)	Chlorophyll concentration (mg g ⁻¹ FW)	Iron content (mg g ⁻¹ DW)
Mycorrhizal	5.0	51.3 a	1.88 bc	2.22 bc	13.9 bc
	6.0 (CK)	45.0 b	2.40 a	2.81 a	15.8 a
	7.0	34.3 c	1.94 b	2.57 ab	15.4 a
	8.0	28.3 d	1.65 cd	2.15 bc	15.0 ab
Non- mycorrhizal	5.0	0.0 e	1.61 d	2.05 cd	11.6 de
	6.0 (CK)	0.0 e	2.04 b	2.31 bc	12.7 cd
	7.0	0.0 e	1.65 cd	2.05 cd	12.1 d
	8.0	0.0 e	1.33 e	1.62 d	10.7 e
Significance ^a AM status		**	**	**	**
pH value		**	**	**	**
AM status×pH value		**	NS	NS	NS

Values are means of three triplicates.

Different letters within each column indicate significant differences ($P<0.05$) between treatments by DMRT.

NS not significant

*, $P<0.05$. **, $P<0.01$

(Table 2). However, no significant differences were found between high pH treatments (pH 7.0 and 8.0 for *P. trifoliata* and pH 7.2 and 8.2 for *C. reticulata*). AM *P. trifoliata* seedlings had significantly higher Chl contents than uninoculated controls (Table 1), but no inoculation effects were found in *C. reticulata* (Table 2).

The contents of iron in pH control treatments of both citrus species were highest in the respective pH treatments (Tables 1 and 2), and following the increases in pH the total contents of iron decreased. *G. versiforme* significantly affected ($P<0.05$) the contents of iron in *P. trifoliata* (Table 1). AM seedlings had significantly higher contents of iron compared to non-mycorrhizal controls. However, no

significant differences were observed between AM and uninoculated seedlings of *C. reticulata* at high pH (Table 2).

Root FCR activities and P/Fe and 50(10P+K)/Fe ratios

Root Fe (III) chelate reductase activities of *P. trifoliata* and *C. reticulata* are shown in Table 3. Root FCR activities decreased with increasing pH. AM seedlings of both citrus rootstocks had higher root FCR activities than the corresponding uninoculated controls and *P. trifoliata* showed significant differences between AM and non-mycorrhizal

Table 2 Root colonization, shoot dry weight, chlorophyll concentration, and active iron contents of leaves of *Citrus reticulata* Blanco seedlings growing in sand culture in response to a factorial treatment combination of the AM fungus *Glomus versiforme* and four pH levels

Mycorrhizal status	pH value	Root colonization (%)	Shoot dry weight (g plant ⁻¹)	Chlorophyll concentration (mg g ⁻¹ FW)	Iron content (mg g ⁻¹ DW)
Mycorrhizal	5.2	55.2 a	1.45 bcd	2.42 ab	15.3 a
	6.2 (CK)	45.1 b	1.76 a	2.55 a	15.5 a
	7.2	36.8 c	1.70 a	2.34 abc	13.0 b
	8.2	27.2 d	1.56 abc	2.23 bcd	12.1 b
Non- mycorrhizal	5.2	0.0 e	1.34 cd	2.10 cd	12.8 b
	6.2 (CK)	0.0 e	1.64 ab	2.42 ab	12.9 b
	7.2	0.0 e	1.44 bcd	2.17 acd	12.4 b
	8.2	0.0 e	1.23 d	1.94 d	11.8 b
Significance ^a AM status		**	**	**	**
pH value		**	**	*	**
AM status×pH value		**	NS	NS	NS

Values are means of three triplicates.

Different letters within each column indicate significant differences ($P<0.05$) between treatments by DMRT

NS not significant

* $P<0.05$; ** $P<0.01$

Table 3 Root ferric chelate reductase activities of *Poncirus trifoliata* L. Raf and *Citrus reticulata* Blanco seedlings growing in sand culture in response to a factorial treatment combination of the AM fungus *Glomus versiforme* and four pH levels

Mycorrhizal status	pH value	Trifoliata orange seedlings FCR ($\mu\text{mol g}^{-1} \text{h}^{-1}$)	pH value	Red tangerine seedlings FCR ($\mu\text{mol g}^{-1} \text{h}^{-1}$)
Mycorrhizal	5.0	20.2 abc	5.2	19.8 bc
	6.0 (CK)	23.8 a	6.2 (CK)	23.9 a
	7.0	22.0 ab	7.2	20.7 ab
	8.0	19.3 bc	8.2	16.1 cd
Non-mycorrhizal	5.0	18.4 bcd	5.2	17.9 bcd
	6.0 (CK)	19.5 bc	6.2 (CK)	21.2 ab
	7.0	17.6 cd	7.2	18.7 bc
	8.0	15.2 d	8.2	14.8 d
Significance ^a AM status		**		*
pH values		*		**
AM status $\times$ pH value		NS		NS

Values are means of three triplicates.

Different letters within each column indicate significant differences ($P < 0.05$) between treatments by DMRT.

NS not significant.

* $P < 0.05$; ** $P < 0.01$

seedlings at high pH (pH 6.0–8.0), but *C. reticulata* showed no such effects.

In the present investigation, the P/Fe and 50(10P+K)/Fe ratios of leaves were used to predict the chlorotic status of the two citrus rootstocks and the P/Fe and 50(10P+K)/Fe ratios determined are presented in Fig. 1 (*P. trifoliata*) and

Fig. 2 (*C. reticulata*). The ratios of P/Fe and 50(10P+K)/Fe increased with the pH from 6.0 (CK) to 8.0 in *P. trifoliata* and from pH 6.2 (CK) to 8.2 in *C. reticulata*. AM seedlings of both citrus rootstocks had lower ratios of P/Fe and 50(10P+K)/Fe than the corresponding uninoculated seedlings.

Fig. 1 Ratios of P/Fe (a) and 50(10P+K)/Fe (b) in leaves of *Poncirus trifoliata* L. Raf seedlings grown in sand culture in response to a factorial treatment combination of the AM fungus *Glomus versiforme* and four pH levels. Data ($\pm$ SE) are means of three replicates. Different letters indicate significant differences ($P < 0.05$) between treatments by DMRT

Fig. 2 Ratios of 50(10P+K)/Fe and P/Fe of leaves of *Citrus reticulata* Blanco seedlings grown in sand culture in response to a factorial treatment combination of the AM fungus *Glomus versiforme* and four pH levels. Data ($\pm$ SE) are means of three replicates. Different letters indicate significant differences ($P < 0.05$) between treatments by DMRT

Discussion

AM colonization and plant growth

AM colonization was observed in seedlings of both *P. trifoliata* and *C. reticulata* inoculated with the *G. versiforme* and no colonization was found in uninoculated plants. Increasing nutrient solution pH led to decreasing percentage of root length colonized. AM fungi have been found in soils ranging in pH from 2.7 to 9.2, but different AM fungi seem to be adapted to different pH ranges (Siqueira et al. 1984). For example, *G. mosseae* was not tolerant at pH <5.0 (Mosse 1973) and *G. versiforme* can grow well at soil pH values from 3.8 to 8.0 (Sieverding 1991), but no *Acaulospora laevis* spores were found by Porter et al. (1987) in soil of pH >6.4. Soil pH is therefore a very important factor that contributes to the distribution of AM fungi in soils. Fortin et al. (2002) obtained similar results to ours and found that high pH had detrimental effects on AM fungal spore germination and hyphal growth.

In the present study, shoot dry weights of AM plants were higher than those of non-mycorrhizal controls, and this confirms the results of Liu and Li (2000) and Colla et al. (2008). The contribution of AM fungi to host plant growth depends on the development of extraradical hyphae, which can absorb and transport water and mineral nutrients to the host plant (Faber et al. 1991).

Mineral nutrient and Chl contents and FCR activity

Six-month-old seedlings iron contents in both *P. trifoliata* and *C. reticulata* varied under different pH of nutrient solution. In this study, different pH values and iron contents of the nutrient solution were designed because *P. trifoliata* was more susceptible to iron deficiency. Here, pH affected iron absorption and translocation in both AM and non-mycorrhizal plants, and plant iron concentrations were low at low pH values (5.0 and 5.2). Similar results were reported by Clark (1997), who concluded that mineral acquisition by AM fungi was limited in acid soils. Although iron is abundant in soils worldwide, it usually exists in the Fe^{3+} form, which cannot be absorbed directly by plants.

In general, iron solubility decreases 1,000 times by an increase in soil pH of one unit (Lindsay and Schwab 1982). It is therefore difficult for dicotyledonous and non-graminaceous monocotyledonous plants (Strategy I plants, including citrus plants) to obtain soluble iron from the rhizosphere in alkaline soils (Römheld and Marschner 1983), although they may acidify rhizosphere partly. The results of the present study suggest that the *G. versiforme* had a positive effect on iron absorption by citrus rootstocks. However, the iron nutritional responses of *P. trifoliata* and

C. reticulata to high substrate pH were different. The iron content of *P. trifoliata* appeared to differ significantly at pH values of 7.0 and 8.0, but *C. reticulata* did not show this effect, which may have been due to genetic differences between the two citrus rootstocks.

P. trifoliata was sensitive to iron deficiency, but *C. reticulata* was less so. The results agree with Hamzé et al. (1986) who found that *P. trifoliata* was very susceptible to iron deficiency. Pestana et al. (2005) also reported that ‘Froyer’ citrange rootstock was the most tolerant to iron deficiency, followed by ‘Taiwanica’ orange rootstock, and then ‘Swingle’ citrumelo, which was the most susceptible rootstock.

Chlorophyll concentration usually reflects the iron (including Mg) nutritional status of plants. If iron is not supplied in sufficient quantity, the synthesis of Chl precursors will be depressed, resulting in the decrease in Chl content (Mömheld and Marschner 1986). In the present study, Chl contents were higher in AM plants than in uninoculated controls, suggesting that the *G. versiforme* enhanced iron absorption from the rhizosphere and translocation to the shoots.

Iron acquisition of citrus is characterized by rhizospheric acidification and increased root ferric chelate reductase activity (Römheld and Marschner 1983). Root ferric chelate reductase activity can be used as an index to diagnose iron nutritional status and plant tolerance to iron deficiency and has been used widely in studies of herbaceous plants and woody species (Bienfait 1988; Chouliaras et al. 2004b). In the present study, root ferric chelate reductase activity decreased under high pH in both citrus rootstocks, a result similar to that of Chouliaras et al. (2004a). Moog and Bruggemann (1994) reported that decrease in root ferric chelate reductase activity at high pH values was due to its optimal pH of 5.0, and could be caused by the breakdown of root ferric chelate reductase because iron deficiency in high pH usually leads to degradation of Chl and proteins in plant leaves (Peng et al. 2001).

However, root ferric chelate reductase activity usually increased when the iron supply was insufficient. Chelate ferric iron reduction may be a long-term process (Romera et al. 1991). AM plants showed higher root ferric chelate reductase activity than non-mycorrhizal controls, suggesting that the *G. versiforme* enhanced reduction of Fe^{3+} to Fe^{2+} . Therefore, the contribution of AM fungi to iron nutritional status in plants may be due to the enhancement of root ferric chelate reductase and large amounts of extraradical hyphae that can absorb iron more efficiently from the substrate.

Ratios of 50(10P+K)/Fe and P/Fe

In general, the ratios of 50(10P+K)/Fe and P/Fe are used to analyze chlorosis status due to iron deficiency. The ratios of

P/Fe and 50(10P+K)/Fe increase when the chlorosis becomes severe (Álvarez-Fernández et al. 2005) due to the increase in P and decrease in iron. Köseoglu (1995) reported that plant roots under iron deficiency excrete H^+ at higher rates into the soil than do healthy plants. The excess H^+ contributes to the conversion of HPO_4^{2-} to $H_2PO_4^-$, which is more easily absorbed by plants, resulting in higher P concentrations in plants. Our data show that following an increase in substrate pH, both *P. trifoliata* and *C. reticulata* showed similar trends of 50(10P+K)/Fe and P/Fe. 50(10P+K)/Fe and P/Fe were lowest at pH 6.0 or 6.2, indicating that the severity of chlorosis was lowest at this pH range. Thus, pH values of 6.0 and 6.2 were the optimal levels for iron absorption by the two citrus rootstocks. AM plants had lower ratios of 50(10P+K)/Fe and P/Fe than non-mycorrhizal seedlings, suggesting a positive effect of mycorrhizal colonization on iron nutritional status.

In conclusion, the *G. versiforme* increased plant growth, contents of chlorophyll and iron, root ferric chelate reductase activity and decreased the ratios of P/Fe and 50(10P+K)/Fe. In general, there is no or less root hair of citrus in field, so the citrus growth almost depends on arbuscular mycorrhizal fungi. At present, the AM fungi inocula are commercially available (Furlan 1993; Adholeya 2003). Inoculation with AM fungi may therefore have considerable potential to remedy plant chlorosis due to iron deficiency. However, future research is required to confirm the effectiveness of different AM fungi on different fruit trees, especially under field conditions.

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References

- Adholeya A (2003) Commercial production of AMF through industrial mode and its large scale application. In: Abstracts, 4th International Conference on Mycorrhizae. Montreal, Canada, pp 240
- Albano JP, Miller WB (1996) Iron deficiency stress influences physiology of iron acquisition in marigold (*Tagetes erecta* L.). J Am Soc Hortic Sci 121:438–441
- Álvarez-Fernández A, García-Marco S, Lucena JJ (2005) Evaluation of synthetic iron(III)-chelates (EDDHA/ Fe^{3+} , EDDHMA/ Fe^{3+} and the novel EDDHSA/ Fe^{3+}) to correct iron chlorosis. Eur J Agron 22:119–130
- Bao SD (2000) Soil agro-chemical analysis. China Agriculture Press, Beijing, p 495
- Belkhdja R, Morales F, Sanz M, Abadía A, Abadía J (1998) Iron deficiency in peach trees: effects on leaf chlorophyll and nutrient concentrations in flowers and leaves. Plant Soil 203:257–268
- Bienfait HF (1988) Mechanisms in Fe-efficiency reactions of higher plants. J Plant Nutr 11:605–629
- Caris C, Hördt W, Hawkins HJ, Römhelt V, George E (1998) Studies of iron transport by arbuscular mycorrhizal hyphae from soil to peanut and sorghum plants. Mycorrhiza 8:35–39
- Chen X, Wu CH, Tang JJ, Hu SJ (2005) Arbuscular mycorrhizae enhance metal lead uptake and growth of host plants under a sand culture experiment. Chemosphere 60:665–671
- Chouliaras V, Dimassi K, Therios I, Molassiotis A, Diamantidis G (2004a) Root-reducing capacity, rhizosphere acidification, peroxidase and catalase activities and nutrient levels of *Citrus taiwanica* and *C. volkameriana* seedlings, under Fe deprivation conditions. Agronomie 24:1–6
- Chouliaras V, Therios I, Molassiotis A, Diamantidis G (2004b) Iron chlorosis in grafted sweet orange (*Citrus sinensis* L.) plants: physiological and biochemical responses. Biol Plant 48:141–144
- Clark RB (1997) Arbuscular mycorrhizal adaptation, spore germination, root colonization, and host plant growth and mineral acquisition at low pH. Plant Soil 192:15–22
- Colla G, Roupheal Y, Cardarelli M, Tullio M, Rivera CM, Rea E (2008) Alleviation of salt stress by arbuscular mycorrhizal in zucchini plants grown at low and high phosphorus concentration. Biol Fertil Soils 44:501–509
- Faber BA, Zasoske RJ, Munns DN, Shackel K (1991) A method for measuring hyphal nutrition and water uptake in mycorrhizal plants. Can J Bot 69:87–94
- Fortin JA, Bécard G, Declerck S, Dalpé Y, St-Amand M, Coughlan AP, Piché Y (2002) Arbuscular mycorrhiza on root-organ cultures. Can J Bot 80:1–20
- Furlan V (1993) Large scale application of endomycorrhizal fungi and technology transfer to the farmer. In: Peterson L, Schelkle M (eds) Abstracts, 9th NACOM, Guelph, Ontario, Canada, pp 77
- Gadkar V, David-Schwartz R, Kunik T, Kapulnik Y (2001) Arbuscular mycorrhizal fungi colonization. Factors involved in host recognition. J Plant Physiol 127:1493–1499
- Gerdemann JW, Nicolson TH (1963) Spores of mycorrhizal Endogone species extracted from the soil by wet sieving and decanting. Trans Br Mycol Soc 46:235–244
- González-Vallejo EB, Morales F, Cistué L, Abadía A, Abadía J (2000) Iron deficiency decreases the Fe(III)-chelate reducing activity of leaf protoplasts. Plant Physiol 122:337–344
- Hamzé M, Ryan J, Zaabout M (1986) Screening of citrus rootstocks for lime-induced chlorosis tolerance. J Plant Nutr 9:459–469
- Hoagland DR, Arnon DI (1938) Synthetic media for hydroponic culture. California Agricultural Extension Publication 347:35–37
- Igartua E, Grasa R, Sanz M, Abadía A, Abadía J (2000) Prognosis of iron chlorosis from the mineral composition of flowers in peach. J Hortic Sci Biotech 75:111–118
- Köseoglu AT (1995) Effect of iron chlorosis on mineral composition of peach leaves. J Plant Nutr 18:765–776
- Li HS (2000) Principles and techniques of plant physiological biochemical experiment. Higher Education Press, Beijing, p 278
- Lindsay WL, Schwab AP (1982) The chemistry of iron in soils and its availability to plants. J Plant Nutr 5:821–840
- Liu RJ, Li XL (2000) Arbuscular mycorrhiza and its application. Science Press, Beijing, p 224
- Marschner H (1995) Functions of mineral nutrients: micronutrients. Iron. Mineral Nutrition of Higher Plants. Academic Press, Cambridge, p 889
- Marschner H, Römhelt V, Kissel M (1986) Different strategies in higher plants in mobilization and uptake of iron. J Plant Nutr 9:695–713
- Momheld V, Marschner H (1986) Evidence for a specific uptake systems for iron phytosiderophores in roots of grasses. Plant Physiol 80:175–180
- Moog PR, Bruggemann W (1994) Iron reductase systems on the plant plasma membrane: a review. Plant Soil 165:241–260
- Mosse B (1973) Advances in the study of vesicular-arbuscular mycorrhiza. Annu Rev Phytopathol 11:171–196

- Oserkowsky J (1933) Quantitative relation between chlorophyll and iron in green and chlorotic pear leaves. *Plant Physiol* 8:449–468
- Pacovsky RS, Fuller G (1988) Mineral and lipid composition of *Glycine-Glomus- Bradyrhizobium* symbioses. *Physiol Plant* 72:733–746
- Peng XX, Zhang JP, Li MQ (2001) Reduction of ferric oxalate by cucumber leaves. *J Plant Physiol Mol Biol* 27:505–508
- Pestana M, Correia PJ, Varennes AD, Abadía J, Faria EA (2001) The use of floral analysis to diagnose the nutritional status of orange trees. *J Plant Nutr* 24:1913–1923
- Pestana M, Varennes AD, Faria EA (2003) Diagnosis and correction of iron chlorosis in fruit trees: A review. *J Food Agric Environ* 1:46–51
- Pestana M, Varennes AD, Abadía J, Faria EA (2005) Differential tolerance to iron deficiency of citrus rootstocks grown in nutrient solution. *Sci Hortic—Amsterdam* 104:25–36
- Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans Br Mycol Soc* 55:158–161
- Porcel R, Ruiz-Lozano JM (2004) Arbuscular mycorrhizal influence on leaf water potential, solute accumulation, and oxidative stress in soybean plants subjected to drought stress. *J Exp Bot* 55:1743–1750
- Porter WM, Robson AD, Abbott LK (1987) Field survey of the distribution of vesicular- arbuscular mycorrhizal fungi in relation to soil pH. *J Appl Ecol* 24:659–662
- Romera FJ, Alcantara E, Dela Guardia MD (1991) Characterization of the tolerance to iron chlorosis in different peach rootstocks grown in nutrient solution. 2. Iron-stress response mechanisms. *Plant Soil* 130:121–125
- Römheld V, Marschner H (1983) Mechanism of iron uptake by peanut plants. I. Fe^{III} reduction, chelate splitting, and release of phenolics. *Plant Physiol* 71:949–954
- Sanz M, Montañés L, Carrera M (1994) The possibility of using analysis to diagnose the nutritional status of pear trees. *Acta Horticulturae* 367:290–295
- Sieverding E (1991) Vesicular–arbuscular mycorrhiza management in tropical agrosystems. *Deutsche Gesellschaft Technische Zusammenarbeit (GTZ) GmbH, Eschborn*, p 371
- Siqueira JO, Hubbell DH, Mahmud AW (1984) Effect of liming on spore germination, germ tube growth and root colonization by vesicular–arbuscular mycorrhizal fungi. *Plant Soil* 76:115–124
- Smith SE, Read DJ (1997) *Mycorrhizal symbiosis*, 2nd ed. Academic Press, San Diego, CA, p 605
- Thingstrup I, Kahiluoto H, Jakoben I (2000) Phosphate transport by hyphae of field communities of arbuscular mycorrhizal fungi at two levels of P fertilization. *Plant Soil* 221:181–187
- Toro M, Azcón R, Barea JM (1998) The use of isotopic dilution techniques to evaluate the interactive effects of *Rhizobium* genotype, mycorrhizal fungi, phosphate-solubilizing rhizobacteria and rock phosphate on nitrogen and phosphorus acquisition by *Medicago sativa*. *New Phytol* 138:265–273
- Welch RM, Norvell WA, Schaefer SC, Shaff JE, Kochian LV (1993) Induction of iron(III) and copper(II) reduction in pea (*Pisum sativum* L.) roots by Fe and Cu status: does the root-cell plasmalemma Fe(III)-chelate reductase perform a general role in regulating cation uptake. *Planta* 190:555–561
- Wintermans JF, Mots A (1965) Spectrophotometric characteristics of chlorophylls *a* and *b* and their pheophytins in ethanol. *Biochem Biophys Acta* 109:448–453
- Wu QS, Xia RX (2006) Arbuscular mycorrhizal fungi influence growth, osmotic adjustment and photosynthesis of citrus under well-watered and water stress conditions. *J Plant Physiol* 163:417–425
- Zhang FS, Römheld V, Marschner H (1991) Role of the root apoplasm for iron acquisition by wheat plants. *J Plant Physiol* 97:1302–1305