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Siderophore production by mycorrhizal sorghum roots under micronutrient deficient condition

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Abstract

It has widely been accepted that mycorrhizal symbiosis improves micronutrients uptake by most of the plants. In this study, sorghum (Sorghum bicolor L.) plants were grown in sterile perlite and were inoculated with either Glomus etunicatum (GE) or G.intraradices (GI), while the control set was left un-inoculated. Rorison's nutrient solution with three levels of 0, half and full strength (C_0, $C_{0.5}$ and C_1, respectively) of Fe, Cu, Zn and Mn was applied to the pots during 85 days of growth period. Chrome azurol-S assay was used for determination of siderophores in root leachates on 45, 65 and 85 days after sowing (DAS). Siderophore production per unit volume of root was higher in mycorrhizal than non-mycorrhizal plants. Both GE and GI were efficient fungi in this respect. Siderophore production was significantly induced at C_0 level of the micronutrients. Amount of siderophores produced on 45 and 85 DAS was more than 65 DAS. Mycorrhizal root colonization by GE or GI was not significantly affected by micronutrient levels.

Keywords: Arbuscular mycorrhizal fungi, siderophore, micronutrient deficiency, sorghum

Introduction

Arbuscular mycorrhizal fungi (AMF) enhance nutrient uptake by establishing a hyphal net inside and around the roots (Lee and George, 2005). Increment of Fe, Zn and Cu uptake by mycorrhizal plants have been reported world wide (Clark and Zeto, 1996; Caris *et al.*, 1998; Liu *et al*., 2000; Lee and George, 2005; Silvia *et al*., 2005; Schreiner, 2007). The mechanism(s) involved are not well documented, although some of these have been suggested as follows: (1) siderophore production by both fungi and roots (Caris *et al*., 1998), (2) reduction of Fe^{3+} and Mn^{4+} to Fe^{2+} and Mn^{2+} by mycorrhizal roots, which led to their higher solubility (Cairney and Ashford, 1989) and (3) organic acid and H^+ production (Cairney and Ashford, 1989; Angle *et al*., 1989). Siderophores and phytosiderophores are low molecular chelators which are produced by microbes and plant roots, respectively and have high affinity to Fe^{3+}, but they can also make complexes with other metal ions such as Zn, Cu, Mn, Cd, Cr, Ni and Al (Romheld, 1987; Buyer *et al*., 1993; Bakkaus *et al*., 2006). Siderophore is a metabolic product of a fungus (or other organism) which binds iron and facilitates its transport from the environment into the microbial cell. In fact, siderophores and phytosiderophores are secondary metabolites from inducible genes which are generally expressed by Fe^{3+} and to some extent by Zn, Cu and Mn deficiencies (Cakmak *et al*., 1994; Zhang *et al*., 1991).

Several plant species are able to produce phytosiderophore but they can also use microbial siderophores as metal ion transporters (Romheld, 1987; Leyval and Reid, 1991). Some fungal species produce more siderophore than bacteria (Milagres *et al*., 1999). Ericoid mycorrhizal fungi release ferricrocin or fusigen as the main siderophores. Ferricrocin was also shown to be produced by the ectomycorrhizal fungi, *Cenococcum geophilum* and *Hebeloma crustuliniforme*. Arbuscular mycorrhizal fungi are reported to enhance Fe-uptake rates of associated host plants which can be taken as an indication that mycorrhizal siderophores of a yet unknown structure may be involved (Lee and George, 2005; Schreiner, 2007)**.**

Mycorrhizal *Hilaria janesii* grass, which showed greater Fe uptake than non-mycorrhizal controls, tested positively when bioassayed for hydroxamate siderophores (Cress *et al*., 1986). However, it has to be stressed that these tests were carried out under non-axenic conditions; hence these results cannot be taken as proof that *Glomus* species may produce hydroxamate siderophore. In non-setile soil, it may well be that the hydroxamate siderophores detected, were produced by microorganisms other than arbuscular mycorrhizal fungi. Sound evidence for the nature of siderophore released by AMF can only be obtained when the fungi together with suitable host plants are grown under pure culture conditions.

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In this study, siderophore production by mycorrhizal sorghum roots inoculated with either *G.etunicatum* or *G.intraradices* in axenic condition, were quantitatively determined using chrome azurol-S assay (Schwyn and Neilands, 1984) by inducing micronutrients (Fe, Zn, Cu and Mn) deficiencies.

Materials and Methods

Mycorrhizal inoculum production

Two species of arbuscular mycorrhizal fungi, *Glomus etunicatum* (Becker & Gerdemann) (GE) and *G.intraradices* (Schenck & Smith) (GI) were propagated with sorghum plants in 7 liter pots containing sterile sandy loam soil (Aliasgharzad *et al.*, 2001). Rorison's nutrient solution, 20 mM $Ca(NO_3)_2.4H_2O$; 10 mM $MgSO_4.7H_2O$; 10 mM $K_2HPO_4.3H_2O$; 0.5 mM FeNaEDTA; 0.1 mM $MnSO_4.4H_2O$; 0.5 mM H_3BO_3; 0.01 mM $(NH_4)_6 Mo_7O_{24}.4H_2O$; 0.02 mM $ZnSO_4.7H_2O$ and 0.015 mM $CuSO_4.5H_2O$ in deionized water (Merryweather and Fitter, 1991) with 1/2 strength of phosphorus was added to the pots twice a week to bring the soil moisture to field capacity. Pots were kept in growth room with 28/20 ±2˚C day/night temperatures and 16h photoperiod. After four months, top plants were cut off and pot materials containing soil, mycorrhizal roots, hyphae and spores were throughly mixed and used as fungal inoculum. Root colonization percentage (Giovanetti and Mosse, 1980) and number of spores per 10 g soil (Gerdemann and Nicolson, 1963) were assessed to determine inoculum potential. Both inocula had an average of 65% root colonization and ~150 spores per 10 g soil.

Plant culture

Sorghum (*Sorghum bicolor* L.) seeds were surface sterilized with 0.5% sodium hypochlorite for 15 min, and 10 seeds were sown in pots containing 2.8 liter acid washed and sterilized perlite. Fungal inocula were rinsed three times with distilled water to minimize their micronutrients content. Each pot received 60 g mycorrhizal inoculum as a layer of 0.5 cm thickness, 5 cm below the seeds. Control pots (non-mycorrhizal) received 60 g autoclaved inoculum. Two weeks after sowing, these were thinned to 3 plants per pot. Rorison's nutrient solution with three levels of 0, half and full strength (C_0, $C_{0.5}$, C_1, respectively) of Fe, Zn, Cu and Mn was applied to the pots twice a week during total growth period of 85 days. Pots were kept in growth room with 28/20 ±2˚C day/night temperatures and 16h photoperiod.

Root leachate collection

On 45, 65 and 85 days after sowing (DAS), pot materials were eluted with two pore volume (2.5 liter) of distilled water (DW) to remove nutrients from root zone. Three hours after subjecting root to the DW, root leachates were collected by adding 500mL DW to each pot (Koa *et al.*, 2001).

Siderophores assessment

Siderophore concentrations were immediately measured in the collected leachates as described below. Chrome azurol-s (CAS) solution was prepared as described by Schwyn and Neilands (1984). Five mL of CAS solution and one mL of root leachate were mixed in a test tube and absorbance was measured at 698 nm after one hour. Siderophore concentration was calculated using a DTPA (diethylenetriaminepentaacetic acid) standard calibration curve. Standard solutions containing 0, 25, 75, 100, 125 and 150 µmol DTPA were mixed in 1/5 ratio with CAS before subjecting to spectrophotometry. The amount of siderophore was expressed in µmol per total volume of leachate in one hour (µmol pot^{-1} h^{-1}) or µmol per unit volume of root in one hour (µmol cm^{-3} h^{-1}). The latter unit was applied just for data obtained at harvest time (85 DAS). Root volume was determined by suspending total root system in water and re-measuring water height in cylinder.

Root colonization

Eighty five days after sowing, plants were harvested and whole root system was washed, while removing perlite particles gently and its total volume was measured as described above. Fine feeding roots (0.5 g fresh weight) were sub-sampled, cleared in 10% KOH and stained with trypan blue. Root mycorrhizal colonization percentage was determined by gridline intersect method (Giovanetti and Mosse, 1980).

Statistical analysis

A factorial completely randomized design was used with three factors of (1) mycorrhizal fungi with three variations (*G.etunicatum*, *G.intraradices* and non-mycorrhizal), (2) nutrient solution with three concentrations of micronutrients (C_0, $C_{0.5}$ and C_1), and (3) time of leachate collection, 45, 65 and 85 DAS with three replications per treatment. Analysis of variance and mean comparison by Duncan's Multiple Range Test were carried out using MSTATC software.

Results and Discussion

Mycorrhizal roots produced considerably higher ($p<0.05$) siderophore (13.7 µmol cm^{-3} h^{-1}) than non-mycorrhizal roots (2.0 µmol cm^{-3} h^{-1}). Sorghum plants inoculated with either GE or GI were not different in siderophore production (Table 1). Obviously, non-mycorrhizal roots secrete only phytosiderophore but

mycorrhizal roots can produce both siderophore and phytosiderophore. Enhancement of siderophore and/or phytosiderophore per unit volume of root in mycorrhizal plants suggests that AM fungi may secrete siderophore by themselves and/or induce plant root to produce more phytosiderophore. Arbuscular mycorrhizal fungi are reported to enhance Fe-uptake rates of associated host plants, which can be taken as an indication that mycorrhizal siderophores of a yet unknown structure may be involved (Haselwandter, 2008). An arbuscular mycorrhizal grass species, which showed greater Fe uptake than non-mycorrhizal controls, tested positively when bioassayed for hydroxymate siderophores (Haselwandter, 1995).

Table 1. Main effect of fungal species on siderophore production and root colonization (RC)

Mycorrhiza	Siderophore		% RC
	µmol pot^{-1} h^{-1}	µmol cm^{-3} h^{-1}	
NM	39.0a*	02.0c	00b
GE	39.7a	13.7b	43a
GI	39.7a	13.7b	43a

NM, non-mycorrhizal; GE, *Glomus etunicatum;* GI, *Glomus intraradices.*
*Means in each column followed by same letter are not significantly different ($p<0.05$)

Siderophore production per total volume of leachate obtained from each pot (39-39.7 µmol pot^{-1} h^{-1}) was not significantly different in mycorrhizal and non-mycorrhizal plants (Table 1). It was due to lower root volume in mycorrhizal plants (data not shown). Higher efficiency of mycorrhizal roots in nutrients uptake (Liao *et al*., 2003; Aliasgharzad *et al*., 2009) and carbon consumption by the symbiotic fungi (Landis and Fraser, 2008) may lead to restricted root development in AMF inoculated plants. Mycorrhizal colonization percentage was not significantly different between GE and GI inoculated roots (Table 1). This means that the two fungal species have nearly the same potential in producing siderophore or inducing plant root to enhance phytosiderophore production.

Siderophore and/or phytosiderophore concentrations (11.3-25.0 µmol cm^{-3} h^{-1}) have also been adversely affected by the micronutrient levels. The highest concentration was achieved at the C_0 level. There was no significant difference between $C_{0.5}$ and C_1 in this respect (Table 2). Severe micronutrient starvation in C_0 has induced both root and AMF to secrete more siderophore. Sigel and Sigel (1998) pointed out that genes coding for siderophore in microorganisms, plants and animals are inducible and are usually expressed in response to Fe deficiency.

Table 2. Main effect of micronutrients levels on siderophore production and root colonization (RC)

Mycorrhiza	Siderophore		% RC
	µmol pot^{-1} h^{-1}	µmol cm^{-3} h^{-1}	
C_0	40.3a*	25.0a	29a
$C_{0.5}$	38.0a	15.7b	25a
C1	40.3a	11.3b	26a

NM, non-mycorrhizal; GE, *Glomus etunicatum;* GI, *Glomus intraradices.*
*Means in each column followed by same letter are not significantly different ($p<0.05$).

Root colonization percentage was not affected ($p<0.05$) by micronutrients levels (Table 2). The maximum level of micronutrients used in this experiment, was the full strength of Rorison's nutrient solution. It has been reported that enough or higher levels of these nutrients may restrict root mycorrhizal colonization (Khaliel and Sohaibani, 1993; Liao *et al*., 2003).

Interaction effect of micronutrient levels×mycorrhizal inoculation on siderophore production indicates that in non-mycorrhizal roots the highest siderophore concentration occurred at C_0 level, but there was no difference between $C_{0.5}$ and C_1 levels. In mycorrhizal roots there was no significant difference between micronutrient levels for siderophore production (Figure 1). Mycorrhizal fungi may alleviate nutrient deficiency in plant by increasing absorbing surfaces and therefore poor induction of siderophore genes.

With an exception for GE, siderophore production was significantly reduced from 45 to 65 DAS and thereafter was markedly increased toward 85 DAS in both mycorrhizal and non-mycorrhizal plants (Figure 2). Higher demand of plant for nutrients including Fe, in flowering stage (85 DAS) may be a reason for this phenomenon, which would lead to higher siderophore production. However, additional mechanisms may be involved in GE inoculated plants for this reduction.

Conclusion

Considering µmol cm^{-3} h^{-1} as a reasonable unit for siderophore production, which was obtained at harvest time (85 DAS), it can be concluded that mycorrhizal fungi enhance siderophore production by themselves and/or by inducing plant roots. Thus, micronutrients deficiencies (especially Fe) can stimulate siderophore production by both AM fungi and plant root. Overall, it seems that inoculation of sorghum plants with either GE or GI in

micronutrient deficient condition, would help this plant to absorb enough micronutrients through chelate formation with siderophores. In case of fungi as symbionts of plants, the role of fungal capacity to synthesize siderophores, is not clear with regard to the establishment of the symbiosis. On the other hand, it is evident that such a fungal capacity may affect the Fe nutrition of associated host plants. Further study is necessary to distinguish between fungal derived siderophores and root derived phytosiderophores. A new approach involving a siderophore deficient mutant of a mycorrhizal fungus, which is already known to produce siderophore could be applied for an evaluation of the importance of siderophore biosynthesis for the establishment of the symbiosis with host plant.

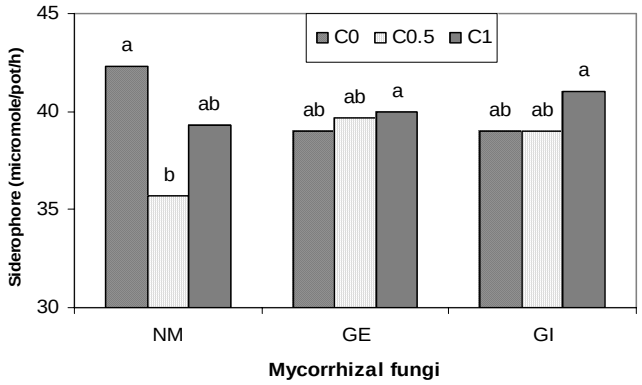

Figure 1. Effect of mycorrhizal fungi on siderophore production in variable regimes of micronutrients

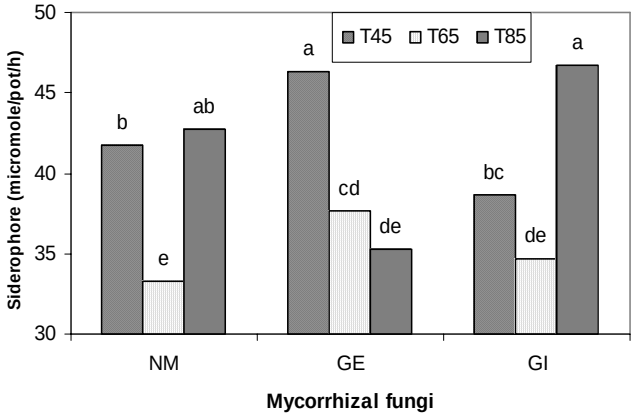

Figure 2. Effect of mycorrhizal fungi on siderophore production at different stage of plant growth

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