

Neighboring Plants Alleviate Aluminum Toxicity on The External Hyphae of *Gigaspora margarita*

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Excessive soluble aluminum (Al^{3+}) in acidic soils is toxic to the external hyphae of arbuscular mycorrhizal fungi but it can be alleviated by other soil factors. A glasshouse experiment was conducted to study the effect of increased Al^{3+} concentration on the growth of the external hyphae of *Gigaspora margarita* in the presence of other plants near the host plants. The experiment used compartmentalized pots to facilitate the growth of mycorrhizal-inoculated host plants, external hyphae of the fungus and not mycorrhizal-inoculated neighboring plants in different compartments; and measuring the effects of Al^{3+} and of the neighboring plants on the growth of the fungal hyphae independently. Increased concentration of Al^{3+} in soil affected the growth of external hyphae of *G. margarita* negatively. However, the hyphal length density of the fungus was much higher in the pots with neighboring plants than that in the other ones, despite the Al toxicity. This indicates that the hyphae could be taken away from the toxic effect of Al^{3+} by the stimulating growth from roots of the neighboring plants.

Key words: aluminum, cowpea, external hyphae, compartmentalized pot system, *Gigaspora margarita*

Crops often suffer from adverse conditions of acid soils. Aluminum (Al) is considered the major stress factor because of its toxicity under acidic conditions. Excessive soluble-Al (Al^{3+}) in soil might create a poor plant root system by inhibiting lateral root and root hair formation, and limit the solubility and, therefore, the availability of some essential mineral nutrients, especially P (Baligar *et al.* 1995). Also, high Al^{3+} concentration can be detrimental to soil microbes that are involved in nutrient cycling and/or forming symbiotic associations with the plant roots (Wood 1995; Rohyadi 2006). As a result, the plants may have limitation in taking up nutrients and water in sufficient quantity, and consequently they grow stunted.

Arbuscular mycorrhizas (AM) are mutualistic symbioses mostly benefiting plants that have a small and/or coarse root with a lack of root hairs, and those are grown on infertile soils with particularly low available-P. Hence the symbioses can be of great importance in acid soils, where poorly developed root systems and P deficiency are the main constraints for plant growth (Miyasaka and Habte 2001). However, the AM fungi themselves are subjected to detrimental effects of mainly high Al^{3+} concentration in acidic soils. Rohyadi (2006) indicated that decreased effectiveness of an AM fungus, *Gigaspora margarita* on cowpea plant grown on acidic soils was related to the poor growth of the external fungal hyphae because of Al toxicity. It is well known that the external hyphae of AM fungi growing out from colonized plant roots play a crucial role. They develop to form a hyphal net-work in soils and function as an extension of root system to explore soil particularly beyond the depletion of the root zone, take up water and element nutrients there and then transport them to the roots of the host plants (Smith *et al.* 2000). Therefore, the inhibited growth of the hyphae may directly reduce the symbiotic effectiveness.

Only a few studies were done on the effect of Al^{3+} on the external hyphae of the AM fungi. It was reported that the growth responses of the AM fungi to Al toxicity are complex and much dependent on the fungal species (Siqueira *et al.* 1985). Recently, several studies *in vitro* showed that factors

such as CO_2 level, some chemical compounds and volatiles released by plant roots, and bacterial products, influence the growth of the fungal hyphae (Tawaraya *et al.* 1996; Nagahashi and Douds 2000, 2003; Xavier and Germida 2003; Scervino *et al.* 2005). Therefore, it is believed that the presence of other plants near their hosts will alter the response of the external fungal hyphae to toxic Al^{3+} *in situ*.

The objective of this study was to examine if the presence of neighboring plants could modify the growth response of the external hyphae of *G. margarita* to the detrimental effects of Al^{3+} .

MATERIALS AND METHODS

The fungus used in this study was *G. margarita* Becker & Hall (BEG collection), which is tolerant to and working effectively under acidic soil conditions (Rohyadi *et al.* 2004). The fungus was propagated in pot cultures of *Trifolium subterranean* L. in a sand and soil (90:10, w/w) mixture for 4 months. Another pot without the fungus to provide mycorrhiza-free inoculums was included. The cultivar of cowpea used was Red Caloona supplied by CSIRO Tropical Agriculture, Brisbane, Australia. The growth medium was a mixture of sand and acidic podsol soil of grey sandy loam (90:10, w/w). The mixture was firstly fertilized with (*in mg kg⁻¹ medium*) 59.4 NH_4-N , 178.2 NO_3-N , 36 P, 54 S, 214.2 K, 18.9 Mg, 114.3 Ca, 13.5 Na, 8.1 Cl, 2.7 Fe, 0.45 B, 0.45 Mn, 0.225 Zn, 0.036 Cu and 0.009 Mo. Then it was treated with 0, 75, 150 or 300 $Al_2(SO_4)_3$. The concentrations of Al^{3+} (measured based on the method of Close and Powell 1989) in the final established media (A_0 , A_1 , A_2 or A_3) were 0.4, 1.1, 4.1 or 7.3 $mg kg^{-1}$ soil respectively.

This experiment was conducted under glasshouse conditions. It was a 2 x 3 x 2 factorial experiment consisting of two levels of mycorrhiza with (M_1) and without (M_0) inoculation. Three concentrations of Al^{3+} established in soil were 1.1, 4.1 and 7.3 $mg kg^{-1}$ soil (denoted as A_1 , A_2 and A_3), and the presence and absence of neighboring plants were denoted as N_1 and N_0 respectively. There were six replicates per treatment combination.

The pot system used was made of a PVC pipe (Fig 1), divided into three compartments by two vertical 30 μm screen meshes that allow hyphae to pass but exclude plant roots. The first compartment, the host plant compartment (HPC), was for plants inoculated or not inoculated with mycorrhizal fungal structures, designated as the *host plants*. They supplied the external fungal hyphae being tested. The second compartment in the middle of the pot, the external hyphal compartment (EHC), was a root free compartment provided for the external hyphae extending from mycorrhizal-colonized roots of the host plants in the HPC. The third compartment, the neighboring plant compartment (NPC), was for non-inoculated plants, denoted as *neighboring plants*, which acted as a growth promoter for the fungal hyphae to traverse the EHC. In order to provide favorable conditions for plant roots, the HPC and NPC were filled with growth medium not treated with Al, A_0 (pH 5.3, containing 0.4 mg Al^{3+} kg^{-1} soil and 26 ppm Bray-1 P), whereas the medium in EHC was treated with Al at different concentrations. Therefore, the basic purpose of using the compartmentalized pot system is to allow the external fungal hyphae from mycorrhizal-colonized roots of the host plants in the HPC to extend into the EHC, interact with Al and possibly traverse to the NPC, and finally colonize roots of neighboring plants there. The effects of the Al^{3+} concentrations in the EHC and the presence of neighboring plants in the NPC on the growth of the external hyphae was determined by the extending of the external hyphae in the EHC and the colonization of neighboring plants in the NPC by the AM fungus.

At the beginning of the experiment, HPC and NPC were filled with 320 g of A_0 soil, but only the HPC was inoculated with 10% (w/w) of pot culture inoculums of *G. margarita* or was left not-inoculated to provide mycorrhizal and non-mycorrhizal colonized host plants. Two pre-germinated seeds of cowpea (cv. Red Caloona) were transplanted into all of the HPC and a half of the NPC, and then left grown for two weeks. The EHC was then filled with 260 g of A_1 , A_2 or A_3 soils in the

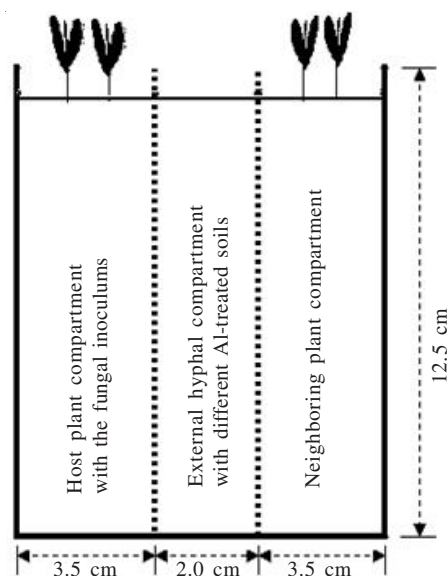


Fig 1 Schematic representation of the compartmentalized pot system. The compartments are separated by two vertical 30 μm nylon screen meshes (■ ■ ■) that can be crossed by fungal hyphae but not by plant roots.

same way. Reverse Osmotic (RO) water, adjusted to pH 4.6 with H_2SO_4 , was used on a weight loss basis to maintain the moisture content of the growth media at the field capacity (about 0.1 g g^{-1} soil) and the pH of soil in the EHC, at about 4.7 throughout the experiment.

Plants were harvested 6 and 10 weeks (H_1 and H_2) after transplanting. The dry biomass and root length of the host plants, the dry biomass of the neighboring plants, the mycorrhizal colonization on the roots of these plants, and the length density of the external fungal hyphae in the EHC were measured. Plant biomass was weighed after it was dried at 70 °C for 48 hours. To measure root length and percentage of mycorrhizal colonization, root samples were cleared with 10% KOH, and stained with trypan blue in lacto-glycerol and then assessed under a dissecting microscope using a gridline intersect method (Giovannetti and Mosse 1980). To measure length density of the external fungal hyphae in the HPC, representative samples of the growth media were taken from the compartment. The hyphae in the soil samples were then extracted following the aqueous membrane-filtration method of Jakobsen *et al.* (1992), and assessed under microscopic observation (*see detail in Rohyadi 2006*).

The data were statistically analyzed using ANOVA after grouping them into plant compartments and harvest times. The LSD tests at $p \leq 0.05$ were then applied to determine the significant differences among ways of treatment.

RESULTS

Growth of Host Plant. Dry biomass of the host plants, measured at any harvest was not affected by the Al^{3+} concentration in the EHC or by the presence (N_1) and the absence (N_0) of neighboring plants in the NPC, but was significantly affected by mycorrhizal inoculation. The dry biomass of mycorrhizal (M_1)-plants was significantly higher than that of non-mycorrhiza (M_0) (Table 1). Similarly, mycorrhizal inoculation significantly increased the root length of the host plants in the HPC regardless of the presence or absence of neighboring plants. Aluminum concentration and the presence of neighboring plants had no significant effect on the growth of roots irrespective of the mycorrhizal status of plants.

Mycorrhizal Colonization on Host Plants. Plants not inoculated with AM fungus exhibited no evidence of AM fungal colonization, while the extent of roots colonized by AM fungus and the root length of host plants were stimulated by inoculation (Table 1). In general, mycorrhizal colonization increased from the first to the second harvest. However, the increases were not related to the Al^{3+} concentration in the EHC. The presence of neighboring plants reduced mycorrhizal colonization observed in the HPC at the second but not at the first harvest.

The effect of Al^{3+} concentration and neighboring plants on the length of the mycorrhizal colonized roots in the HPC was nil at the first harvest. At the second harvest, the adverse effect of Al^{3+} on the mycorrhizal-colonized root length was dependent on the presence or absence of neighboring plants. It was found that the presence of neighboring plants was more detrimental than that the adverse effect of Al^{3+} on the root colonization by the fungus.

Table 1 Growth and AM fungal colonization on roots of host plants in the HPC in response to mycorrhizal inoculation, Al³⁺ concentration in the EHC and the presence of neighboring plants in the NPC

Treatments			Plant dry biomass (mg)		Root length(cm)		Root colonization (%)		Mycorrhizal root length (cm)	
Mycorrhizal inoculation in the HPC	mg Al ³⁺ kg ⁻¹ soil in the EHC	Neighboring plants in the NPC	H ₁	H ₂	H ₁	H ₂	H ₁	H ₂	H ₁	H ₂
Not inoculated	1.1	N ₀	245 ^a	441 ^b	627 ^b	1202 ^b	0	0	0	0
		N ₁	272 ^a	460 ^b	706 ^{ab}	1107 ^b	0	0	0	0
	4.1	N ₀	260 ^a	411 ^b	682 ^b	1072 ^b	0	0	0	0
		N ₁	248 ^a	419 ^b	647 ^b	1049 ^b	0	0	0	0
	7.3	N ₀	249 ^a	361 ^b	657 ^b	1026 ^b	0	0	0	0
		N ₁	235 ^a	392 ^b	639 ^b	1035 ^b	0	0	0	0
Inoculated	1.1	N ₀	311 ^a	829 ^a	876 ^a	1420 ^a	42 ^a	77 ^a	368 ^{ab}	1093 ^a
		N ₁	327 ^a	815 ^a	849 ^a	1488 ^a	46 ^a	57 ^b	391 ^{ab}	848 ^b
	4.1	N ₀	341 ^a	792 ^a	872 ^a	1535 ^a	50 ^a	76 ^a	436 ^a	1167 ^a
		N ₁	345 ^a	814 ^a	829 ^a	1483 ^a	42 ^a	56 ^b	349 ^b	830 ^b
	7.3	N ₀	361 ^a	797 ^a	882 ^a	1496 ^a	48 ^a	73 ^a	423 ^a	1092 ^a
		N ₁	325 ^a	745 ^a	791 ^a	1458 ^a	42 ^a	51 ^b	332 ^b	744 ^c

^aMeans within a selected column followed by different superscripts are significantly different based on the LSD-test at p ≤ 0.05. HPC: host plant compartment; EHC: external hyphal compartment; NPC: neighboring plant compartment. H₁ and H₂: harvest at 6 and 10 weeks after transplanting.

Growth of External Hyphae. The growth of external fungal hyphae in the EHC increased with harvest times; the extent was dependent on the Al³⁺ concentration and the presence of neighboring plants in the NPC. However, there was no a significant interaction between the two factors. The hyphal length density (HLD) decreased with increased Al³⁺ concentration, but the adverse effect of Al³⁺ was nullified by the presence of neighboring plants (Fig 2).

Mycorrhizal Colonization and Growth of Neighboring Plant. Table 2 clearly shows the fungal hyphae, which traverse the EHC, were able to definitely colonize roots of plants in the NPC. At H₁, the colonization was less than 20%, but increased to more than 40% at H₂. It also shows that different Al³⁺ concentrations in the EHC affect the colonization rates differently. At H₁, compared to the root colonization in pots with A₁, a significant reduction in the percentage of colonized roots only occurred in pots with A₃; meanwhile, at H₂ such reduction was already evident in pot with A₂. In general, the growth of the neighboring plants increased, but was not related to soluble-Al concentration in the EHC. Differences in plant growth occurred between plants in pots inoculated with (M₁) and without mycorrhiza (M₀). The dry biomass of the neighboring M₁-plants was higher than that of the

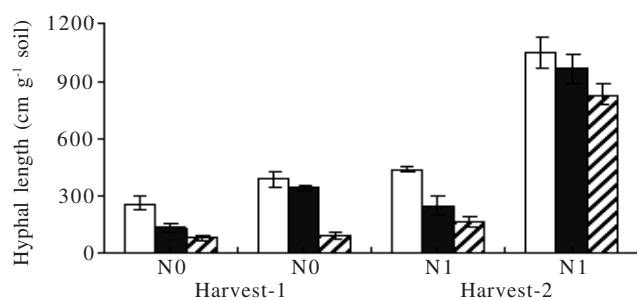


Fig 2 The influence of Al³⁺ concentrations and neighboring plants on hyphal length density of *Gigaspora margarita* in the external hyphal compartment (Bars represent means ± sem, n=3); □, A₁; ■, A₂; ▨, A₃ are growth media with 1.1, 4.1 and 7.3 mg Al³⁺ kg⁻¹ soil; N₁ and N₀ are pots with and with no neighboring plants.

Table 2 Dry biomass and root colonization of the neighboring plants relating to mycorrhizal inoculation in the HPC and Al³⁺ concentrations in the EHC observed at two harvest times

Treatments	mg Al ³⁺ kg ⁻¹ soil in the EHC	Plant dry biomass (mg)		Root Colonization (%)		
		H ₁	H ₂	H ₁	H ₂	
Mycorrhizal inoculation in the HPC	Not inoculated	1.1	210 ^a	382 ^b	0	0
		4.1	210 ^a	399 ^b	0	0
		7.3	195 ^a	397 ^b	0	0
Inoculated	1.1	255 ^a	509 ^a	18 ^a	59 ^a	
	4.1	247 ^a	532 ^a	17 ^a	48 ^b	
	7.3	242 ^a	589 ^a	8 ^b	39 ^{bc}	

^aMeans within a selected column followed by different superscripts are significantly different based on the LSD-test at p ≤ 0.05; HPC: host plant compartment; EHC: external hyphal compartment; H₁ and H₂: harvest at 6 and 10 weeks after transplanting.

neighboring M₀-plants regardless of the Al³⁺ concentration in the EHC, even though it was only significant at H₂ (Table 2).

DISCUSSION

The results of this study showed that the toxicity of high Al³⁺ concentrations in soil solution to the external hyphae of *G. margarita* was evident. The growth of the hyphae was depressed particularly when neighboring plants were absent. This supports and extends previous results of work *in vitro* indicating the inhibitory effects of Al³⁺ on spore germination, germ tube elongation (Siqueira *et al.* 1985), and root colonization of the fungus (Rohyadi 2005). On the other hand, in pots with neighboring plants the growth of the external fungal hyphae considerably increased (Fig 2), which corresponds with the decrease in percentage of the mycorrhizal colonization on roots of their host plants (Table 1). This suggests the importance of the presence of neighboring plants for the growth of external hyphae of the fungus. Previously, Mummey *et al.* (2005) showed the

significance of neighboring plants on the diversity and community composition of the AM fungi in the field.

Most studies on the interaction of AM fungi and plant roots have been carried out using *in vitro* systems. The significant effects of the presence of plant roots on hyphal growth have been demonstrated. Sbrana and Giovannetti (2005) showed a chemotropism in elongation of germ tube of *Glomus mossea* in response to the presence of plant roots. It seems that the plant roots produced influential stimuli for the growth of the AM fungal hyphae. Root exudates, in this case, play a key role during the pre-symbiotic phase. For instance, it was reported that hyphal growth of *G. margarita* was stimulated by root exudates of onion (Tawarayama *et al.* 1996) and tomato (Scervino *et al.* 2005). Also, a component of root exudates of *Lotus japonicum*, identified as strigolactone, induced an extensive branching of the fungal hyphae at very low concentration (Akiyama *et al.* 2005). In general, some phenolic and flavonoid compounds, and/or volatile metabolite exuded by roots of mycotrophic plants have been recognized as chemical stimuli for hyphal elongation and branching (Nagahashi and Douds 2003; Akiyama *et al.* 2005), or as a chemical signal leading the hyphae to grow forward (Vierheilig *et al.* 1998; Steinkellner *et al.* 2007). Therefore, in the present study *in situ* it was possible that some eliciting factors produced by roots of the neighboring plants have triggered most of the external hyphae of the fungus to grow out of the HPC, and so it left a small number of the fungal inoculums to initiate new root colonization in the plant compartment.

Moreover, in this study it was found that colonization of the roots of neighboring plants by the fungal hyphae traversing the EHC reached about 40% (Table 2), suggesting that exposure to increasing Al³⁺ concentrations did not really affect the function of the external hyphae of *G. margarita* as inoculums. The growing hyphae remained viable to initiate new colonization. This is very important for field practice since spores begin most colonization by this fungus. Other results on the increased growth of neighboring plants (Table 2) also indicate that the fungal hyphae did not suffer from the loss of their ability to improve the growth of cowpea plant after being exposed to high concentrations of Al³⁺, even though there was a delay in seeing a significant contribution to the plants.

To summarize, the results of this study have broadened our understanding on eco-physiological aspects of AM symbiosis in acid soils particularly with problems of Al toxicity. It is clear that increasing concentrations of soluble-Al might directly reduce the growth rate of the external hyphae of *G. margarita* in soil, but functions of the hyphae as inoculums and as an extension of the plant root system were not really affected. Meanwhile, the presence of the roots of neighboring plants stimulated the external hyphae to grow further, irrespective of the toxic Al³⁺ in soil. These results, in general, suggest that plants need more AM symbioses to grow better particularly under adverse soil conditions, which is comparable to the requirement of the AM fungi themselves to get carbon from the plants. Therefore, from this point of view, developing a suitable cropping system is essential for empowering AM fungi to be persistent and be able to continually develop their external hyphae. Further studies

however are needed to investigate deep mechanisms and other soil factors involved in triggering the growth of the fungal hyphae under regimes of different mycotrophic crop species, and of adverse soil conditions.

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