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Hyphal growth and mycorrhiza formation by the arbuscular mycorrhizal fungus *Glomus claroideum* BEG 23 is stimulated by humic substances

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Abstract Effects of humic substances (humic acid or fulvic soil extract) or saprophytic microorganisms (*Paecilomyces lilacinus* and an unidentified actinomycete) on growth of mycelium and mycorrhiza formation by *Glomus claroideum* BEG23 were studied in a hydroponic system. Humic substances stimulated root colonization and production of extraradical mycelium by the mycorrhizal fungus. Both humic and fulvic acids tended to decrease populations of culturable bacteria and fungi in the cultivation system, indicating a moderately antibiotic activity. The addition of saprophytic microorganisms able to use humic substances to the cultivation system further stimulated the development of the mycorrhizal fungus. However, stimulation of *G. claroideum* was also observed when the saprophytic microorganisms were heat-killed, suggesting that their effect was not linked to a specific action on humic substances. The results indicate that humic substances may represent a stimulatory component of the soil environment with respect to arbuscular mycorrhizal fungi.

Keywords Fulvic acid · Humic acid · Iron · Mycelium · Hydroponics

Introduction

Soil organic matter has been an important factor affecting the development of many soil microorganisms including arbuscular mycorrhizal fungi (AMF). Although AMF are obligate biotrophic organisms receiving nutrition from their hosts, they tend to colonize soil organic particles (St John et al. 1983) and dead plant seeds (Rydlová and Vosátka 2000). Stimulation of intraradical and extraradical hyphae has been observed in experiments in which non-sterile potting substrata or soils were amended with small amounts of organic compounds such as cellulose (Gryndler et al. 2002), chitin (Gryndler et al. 2003b), organic wastes or composts (Linderman and Davis 2001). This effect of soil organic compounds on AMF remains unexplained. It may be due to modifications in the physicochemical properties of the soil environment or to the release of trace amounts of biologically active compounds during the decomposition of organic particles by saprophytic microflora. Larger organic components (such as dead seeds) may simply provide free space for the development of AMF.

Little attention has so far been paid to the effects on AMF of relatively stable forms of soil organic matter represented by humic substances (Vallini et al. 1993), which can affect soil environment because of their capacity to bind and exchange ions present in the soil solution (Nordén and Dabek-Zlotorzynska 1996). Humic substances can affect plants in different ways (Nardi et al. 2002a). They possess a biological activity that has been compared to that of plant growth auxin regulators (O'Donnell 1973, Nardi et al. 1994) and effects may depend on their activation ("breaking out") by root exudates (Nardi et al. 2002b). Humic substances are ubiquitous soil components that must be frequently in contact with mycelium of AMF. In artificial substrata, where soil is not included, AMF may suffer from the lack of such soil organic components but little attention has been given to their effect on AMF.

The objective of the present work was to evaluate the effect of humic substances on AMF in soil-free hydroponic culture by focusing on two fractions: humic acid and the fulvic fraction of a soil extract. Since humic substances may be degraded in the soil environment by saprophytic microorganisms, the possible effect of such a process on AMF was also studied by adding saprophytic microorganisms able to degrade humic substances to the cultivation substratum.

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Materials and methods

Biological material

In all experiments, mycorrhizal treatments were inoculated with spores of *Glomus claroideum* Schenck and Smith emend. Walker and Vestberg (isolate BEG23). The saprophytic microorganisms used were the fungus *Paecilomyces lilacinus* (Thom) Samson, isolate R21, and an unidentified actinomycete, the isolate 22/1. Both microorganisms are able to grow on a fulvic fraction of soil extract and their cultures are maintained in the Laboratory of Fungal Biology, Institute of Microbiology CAS, Prague. Maize (*Zea mays* L., F1, cv. Radius) was used as a host plant in all experiments.

Extraction of humic substances from soil

A simple extraction procedure involving NaOH as the extraction agent was used to avoid eventual residual effects due to the sensitivity of AMF to traces of recently recommended extractants ($Na_4P_2O_7$, organic extractants). In order to obtain the fulvic fraction of extractable soil organic matter, a 2-kg sample of the tilled layer of an orthic luvisol collected at the Institute of Plant Production (Prague) was shaken with 4 L of 0.1 M HCl for 20 min, washed twice with 4 L of distilled water and further extracted with 2 L of 0.5 M NaOH for 20 h at 25°C. The paper-filtered extract was acidified (deionized) using Dowex 50 W up to pH 2.67. The resulting fulvic fraction of the organic matter was filtered to remove the precipitate and further neutralized by $CaCO_3$ up to pH 6.3 (measured after removing the evolved CO_2 from the solution under vacuum). This solution was designated as "Ca fulvate". The amount of dissolved oxidizable organic matter was measured according to the modified method of Sims and Haby (1971).

Humic acid was extracted from the same acid-washed soil (see above) using 0.5 M NaOH as the extractant. After a 20-h extraction at 25°C, the alkaline solution was paper-filtered and the clean, dark-coloured filtrate was cooled down to 3°C and acidified by concentrated hydrochloric acid to pH 2.0. The resulting precipitate was then washed three times by decantation, retained on filter paper, and finally resuspended in 350 mL of deionized water. The pH of the suspension was carefully adjusted to 6.3 with KOH. An aliquot (60 mL) of the suspension was evaporated and the humic acid content of the precipitate was measured gravimetrically to be 14.0 g/L.

Experiment 1: Effects of Ca fulvate on hyphal growth and mycorrhizal root colonization of maize

Pregerminated maize seeds were planted into a column of perlite in plastic tubes (length 18 cm, diameter 5 cm) covered at the bottom with canvas to ensure the inflow of nutrient solution to the perlite substratum. Two plants were planted per tube and 14 tubes were put into a plastic tub for each treatment. Each tub (50 cm length, 12 cm width, 15 cm depth) contained 2 L of nutrient solution so that the bottom of each tube was approximately 3–4 cm below the level of the nutrient solution. All inoculated treatments received 5 mL of a spore suspension containing 1,000 AMF spores per tube, applied using a pipette at a depth of 4 cm below the germinated seed. Nonmycorrhizal treatments received 5 mL per tube of a filtrate of mycorrhizal inoculum. Plants were cultivated for 12 weeks (June–August) in a greenhouse.

The experiment had a two-factorial design with two levels of mycorrhizal inoculation (control vs treatments receiving spores) and three levels of Ca fulvate (0, 100 and 600 mg oxidizable carbon per liter of nutrient solution). The concentration of Ca in treatments receiving less than the maximum amount of Ca fulvate was balanced with a corresponding amount of $CaCl_2$ to give the same amount of Ca^{2+} in all treatments (189 mg/L).

The nutrient solution in each tub initially contained (per liter) 280 mg KCl, 42 mg KH_2PO_4, 420 mg $Mg(NO_3)_2{\cdot}4H_2O$, 476 mg KNO_3, 504 mg $MgSO_4{\cdot}7H_2O$, 30 mg FeNaEDTA, 2 mg $MnCl_2{\cdot}4H_2O$, 2 mg H_3BO_3, 1 mg $ZnSO_4{\cdot}7H_2O$, 0.14 mg $CuSO_4{\cdot}5H_2O$ and 0.014 mg $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$. The pH value of the nutrient solution in all treatments was adjusted to 6.3 using KOH. The nutrient solution was not renewed and water was added so that all tubs always contained 2 L of the liquid. After 6 weeks' cultivation, the plants received a further 1200 mg KNO_3, 80 mg KH_2PO_4, 30 mg FeNaEDTA, 12 mg $MnCl_2{\cdot}4H_2O$, 5.3 mg $ZnSO_4{\cdot}7H_2O$, 3 mg H_3BO_3 and 0.26 mg $CuSO_4{\cdot}5H_2O$ per tub. The top surface of the perlite in each tube was wetted twice a week with approximately 70 mL of the solution from the tub to ensure a homogeneous distribution of mineral nutrients and Ca fulvate.

Experiment 2: Effects of Ca fulvate and saprophytic microorganisms on hyphal growth and mycorrhizal root colonization of maize

The same experimental system, mycorrhizal inoculation, cultivation period, growth conditions and nutrient solution were used in Experiment 2 as in Experiment 1 with the exception that the Ca^{2+} concentration was adjusted to 249 mg/L in all treatments. The two-factorial design had two levels of Ca fulvate (Ca fulvate at a concentration of 400 mg oxidizable carbon per liter of the nutrient solution vs controls) and four levels of inoculation: (1) non-inoculated controls receiving only the filtrate of mycorrhizal inoculum, (2) inoculated with spores of *G. claroideum*, (3) inoculated with *G. claroideum* spores plus 2 mg dry biomass equivalent of *P. lilacinus* and 4 mg dry biomass equivalent of the unidentified actinomycete, and (4) inoculated with *G. claroideum* spores and heat-killed biomass of the two saprophytic microorganisms in the same quantities. Wetting of the top of the perlite substratum (see above Experiment 1) was performed once a week.

Experiment 3: Effect of humic acid on hyphal growth and mycorrhizal root colonization of maize

The experimental system and conditions were the same as those used in Experiments 1 and 2 except that six planted tubes constituted one treatment. Each planted tube was inoculated with 400 *G. claroideum* spores at a depth of 4 cm below the seed. Ca concentration was adjusted to 249 mg/L (as in Experiment 2). The growth period was 60 days in a growth chamber (11,000 lx, 16 h day, 20/23° night/day, 75% relative humidity). Experiment 3 had two treatments: a control treatment and a treatment receiving 20 mL of a suspension containing 0.28 g of humic acid (dry weight) per tube. The suspension was applied at the same depth as the fungal spores.

Experiment 4: Effects of Ca fulvate and humic acid on the carbon dioxide concentration in the growth substratum, mycorrhizal root colonization and abundance of saprophytic bacteria and fungi

Three maize-planted tubes (replicates) containing perlite were placed into three separate plastic vessels (13 cm in diameter, 15 cm in height) per treatment. Each vessel contained 500 mL of the nutrient solution used in the other experiments (Ca^{2+} was balanced to 249 mg/L). The cultivation period and growth chamber conditions were the same as in Experiment 3.

Experiment 4 comprised three treatments: controls receiving a 5-mL suspension of 400 *G. claroideum* spores without Ca fulvate or humic acid, and inoculated plants treated with either Ca fulvate (400 mg oxidizable carbon per liter) or humic acid (0.28 g per tube). Each tube was perforated at a depth of 3 and 8 cm. The perforations were sealed with rubber to enable sampling of the internal air using a syringe. A 0.5-mL sample of air was taken at 3 and 8 cm in each tube on days 1, 30 and 60 of cultivation. Concentrations of CO_2 and oxygen were measured in the air samples using gas chromatography. Total counts of culturable bacteria and saprophytic fungi were estimated in the homogenized substratum using the dilution plate technique with the medium T3 and the modified Smith and Dawson procedure, respectively (Gryndler et al. 2003a).

Plant harvest and analyses

At harvest, root systems were washed with tap water; root aliquots were cleared in 10% KOH and stained with trypan blue to measure the percentage mycorrhizal root length using the grid-line intersect method (Giovannetti and Mosse 1980). The biomass of roots and shoots was estimated after drying at 105°C. Iron concentration in roots and shoots was measured in the control and mycorrhizal treatments of Experiments 2 and 3 using atomic absorption spectrophotometry after mineralization in a mixture of concentrated sulfuric acid/hydrogen peroxide (1:2) at 360°C.

The perlite substratum was collected in the first three experiments and the total length of extraradical hyphae of *G. claroideum* measured as described by Malcová et al. (2002). Each substratum sample was homogenized and an approximately 5-g aliquot blended for 30 s in a household blender containing 500 mL water. One milliliter of the resulting suspension was pipetted onto a membrane filter (24 mm diameter, 0.4-μm pore size) and vacuum filtered. The mycelium retained on the membrane filter was stained with a drop of 0.05% trypan blue in lactoglycerol. The total length of mycelium was then assessed using the grid-line intersect method (microscope equipped with focal plate grid; 100× magnification). The results were expressed in meters of hyphae per gram of air-dried substratum.

Statistical analysis of the data

Data were evaluated using one- or two-way ANOVA and by Duncan's multiple range test or by Student's *t* test. *F* tests were performed to check the homogeneity of variance in all data sets. Data describing the percentage of root length colonized were arcsin transformed ($y=\arcsin\sqrt{x}$) before analysis.

Results

Experiment 1: Effects of Ca fulvate on hyphal growth and mycorrhizal root colonization of maize

Ca fulvate and mycorrhizal inoculation had significant effects on root dry weight, mycorrhizal colonization and

Table 1 Effect of Ca fulvate and mycorrhizal inoculation on length of extraradical mycorrhizal mycelium in the substratum, on root mycorrhizal colonization and on maize growth (Experiment 1)

Parameter	NM FA0	NM FA100	NM FA600	M FA0	M FA100	M FA600
Shoot dry weight (g)	4.38 a	4.44 a	4.22 a	4.22 a	5.07 a	4.28 a
Root dry weight (g)	1.02 ab	0.89 c	0.91 abc	1.04 ab	1.09 a	1.04 ab
Root colonization (%)	0	0	0	22.4 c	27.3 b	33.5 a
Length of mycelium (m/g)	0.07	0.03	0.15	5.99 b	7.32 ab	9.83 a

Means followed by the same letter do not differ significantly (within the row) by one-way ANOVA and Duncan's multiple range test at $P=0.05$. Percentage of colonization and hyphal length data obtained in the uninoculated controls were not included in the statistical analysis because their variance differed significantly from that in the mycorrhizal treatments

NM uninoculated control, *M* mycorrhizal inoculation, *FA0*, *FA100* and *FA600* treatments receiving 0, 100 and 600 mg of oxidizable organic carbon (as Ca fulvate) per liter, respectively

length of mycelium in the substratum (Table 1). Root dry weight was significantly greater in mycorrhizal than non-mycorrhizal treatments at 100 mg Ca fulvate per liter. Mycorrhizal colonization as well as the length of mycelium of the mycorrhizal fungus in the substratum increased with the increasing concentrations of Ca fulvate.

Experiment 2: Effects of Ca fulvate and saprophytic microorganisms on hyphal growth and mycorrhizal root colonization of maize

When the substratum amended with Ca fulvate was inoculated not only with *G. claroideum* spores but also with the two microorganisms able to use fulvic acid (Experiment 2, Table 2), significant responses were observed in all the parameters measured, except for shoot growth and iron concentration (two-way ANOVA).

Mycorrhizal colonization significantly increased when Ca fulvate was present in the nutrient solution compared with the mycorrhizal treatment with no fulvate amendment. Mycorrhizal colonization further increased when the two saprophytic microorganisms were added into the substratum. Heat inactivation of the two saprophytic microorganisms together with Ca fulvate elicited the highest levels of mycorrhizal colonization. Similar results were obtained also for the extraradical mycorrhizal mycelium. The presence of Ca fulvate substantially increased the amount of hyphae in the substratum. The highest value was found in a treatment receiving the heat-inactivated saprophytic microorganisms together with fulvate. The concentration of iron in plant roots was substantially decreased in the treatments receiving fulvate compared with those with no fulvate added.

Table 3 Effect of humic acid on plant growth and mycorrhizal root colonization on mycorrhizal mycelium in the substratum (Experiment 3)

Parameter	Without humic acid	Humic acid added
Shoot dry weight (g)	1.79 a	1.65 a
Root dry weight (g)	0.52 a	0.53 a
Root colonization (%)	37.6 b	49.4 a
Length of mycelium (m/g)	6.88 b	17.78 a
Concentration of iron in shoots (μg/g)	7.3 a	15.3 a
Concentration of iron in roots (μg/g)	63 a	71 a

Means followed by the same letter are not significantly different (within the row) by the Student's *t* test at $P=0.05$

Experiment 3: Effect of humic acid on hyphal growth and mycorrhizal root colonization of maize

Amendment of the substratum with humic acid (Experiment 3, Table 3) resulted in a slight but significant increase in mycorrhizal colonization. The concentration of iron in the shoots and roots did not differ between treatments. The length of extraradical mycelium in the substratum significantly increased in the treatment receiving humic acid. No significant effect of the humic acid on plant growth was observed.

Table 2 Effect of Ca fulvate, mycorrhizal inoculation and amendment of substratum with microbial biomass on plant growth, root colonization, length of extraradical mycorrhizal mycelium and concentration of iron in plant tissues (Experiment 2)

Parameter	FA	Control	Mycorrhizal	Mycorrhizal + saprophytes	Mycorrhizal + inactivated saprophytes	Effect of FA by two-way ANOVA
Shoot dry weight (g)	−	4.85	4.41	4.29	4.46	n.s.
	+	5.04	4.73	5.05	4.54	
	Effect of inoculation by two-way ANOVA: n.s.					
Root dry weight (g)	−	0.71 a	0.68 a	0.78 a	0.67 a	$P=0.0119$
	+	0.61 a	0.59 a	0.66 a	0.63 a	
	Effect of inoculation by two-way ANOVA: n.s.					
Root colonization (%)	−	0	17.4 e	28.7 d	35.5 c	$P=0.0000$
	+	0	35.5 c	42.8 b	50.1 a	
	Effect of inoculation by 2−way ANOVA: $P=0.0000$					
Length of mycelium (m/g)	−	0.06	1.24 d	2.46 c	3.81 b	$P=0.0000$
	+	0.05	4.29 b	4.18 b	6.73 a	
	Effect of inoculation by two-way ANOVA: $P=0.0000$					
Concentration of iron in shoots (μg/g)	−	12.4	17.4	n.d.	n.d.	n.s.
	+	10.2	14.0	n.d.	n.d.	
	Effect of inoculation by two-way ANOVA: n.s.					
Concentration of iron in roots (μg/g)	−	81.2 a	69.2 a	n.d.	n.d.	$P=0.0004$
	+	26.1 b	24.1 b	n.d.	n.d.	
	Effect of inoculation by two-way ANOVA: n.s.					

Biomass of saprophytes capable to grow on fulvate as carbon source was used either intact or inactivated by heat. Means followed by the same letter do not differ significantly by one-way ANOVA and Duncan's multiple range test at $P=0.05$. Percentage of colonization and hyphal length data obtained in the uninoculated controls were not included in the statistical analysis because their variance differed significantly from that in the mycorrhizal treatments
FA Ca fulvate, *n.s.* not significant, *n.d.* not determined

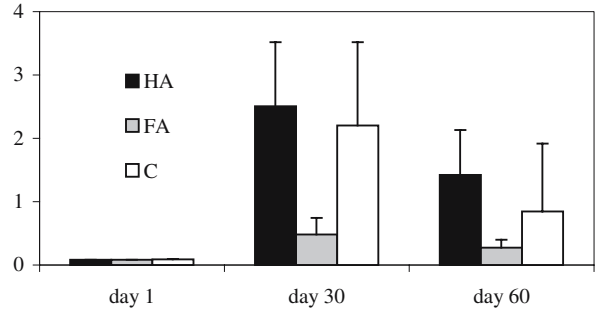

Fig. 1 Effect of amendment of cultivation substratum by humic acid (HA, *black columns*) and Ca fulvate (FA, *gray columns*) on concentration of carbon dioxide (%) in the gaseous phase of the substratum as compared with unamended control (C, *open columns*). The concentration of carbon dioxide was measured at a depth of 8 cm (Experiment 4). *Vertical lines* represent the standard deviation of the mean (n=3)

Experiment 4: Effects of Ca fulvate and humic acid on carbon dioxide concentration in the growth substratum, mycorrhizal root colonization and abundance of saprophytic bacteria and fungi

At day 30, a decrease in CO_2 concentration was found at a depth of 8 cm in a substratum receiving Ca fulvate (Fig. 1) as compared with the control treatment and the substratum amended with humic acid. Amendment with humic acid did not significantly change the concentration of CO_2 in the substratum. The concentrations of CO_2 at the depth of 3 cm were much lower than those presented in Fig. 1 (not shown). The presence of Ca fulvate as well as of humic acid in the substratum caused a decrease in the bacterial populations measured in the substratum at day 60 (Fig. 2). Populations of saprophytic fungi were apparently decreased only in the treatment in which humic acid was supplied. Mycorrhizal colonization was not significantly changed in the presence of either organic substance.

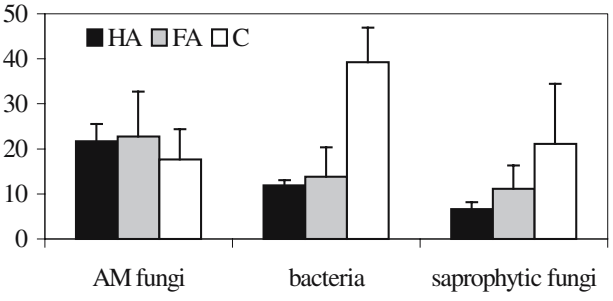

Fig. 2 Effect of amendment of cultivation substratum by humic acid (HA, *black columns*) and Ca fulvate (FA, *gray columns*) on root mycorrhizal colonization (%) and on concentration of colony-forming units of bacteria ($\times 10^{-6}$ g^{-1}) and saprophytic fungi ($\times 10^{-2}$ g^{-1}) in the substratum. Results are compared with control treatment (C, *open columns*) with no application of humic substances. The data were obtained at day 60 of Experiment 4. For other explanations see Fig. 1

Discussion

Within the concentration range used, humic acid showed a moderate stimulatory effect on root colonization by *G. claroideum* BEG23 whilst development of the extraradical mycelium was substantially increased. This is in contradiction with the observations of Vallini et al. (1993), who observed an inhibition of hyphal growth of *G. mosseae* when the soil was amended with sodium humate. We cannot explain this discrepancy by a difference in the concentration of the humic acid/humate used since the 280 mg humic acid per tube used in the present study is equivalent to 800 mg humic acid per liter of the perlite substratum, similar to the amount of humic acid applied as soluble sodium humate at a rate of 800 mg/kg soil by Vallini et al. and which decreased hyphal growth of *G. mosseae* by more than 60%. The inhibitory effects of soluble humate may be related to changes in pH after partial biodegradation or precipitation in the soil environment. Since we applied humic acid in its undissolved form, in which the cations neutralizing the acidity of humic acid are not present, pH changes and release of sodium during microbial transformation of the humic acid could not take place. The potential acidity of humic acid itself did not result in a negative effect on *G. claroideum*, probably because it was applied undissolved. As such, it could act as an ion exchanger that partially stabilized cation concentrations in the nutrient solution. The soluble fraction of soil organic matter containing fulvic acid (here designated as Ca fulvate) also stimulated development of *G. claroideum*. Unfortunately, there are no data on AMF by other authors that can be compared with our results. The only work on mycorrhizal fungi was published by Tan and Nopamornbodi (1979), who observed a significant stimulation of the growth of the ectomycorrhizal fungus *Pisolithus arhizus* (*tinctorius*) at concentrations of 640–1,600 mg/L fulvic acid.

The nature of the stimulatory effects of the two types of humic substances may differ since they differently affected the concentration of carbon dioxide in the perlite substratum, with Ca fulvate decreasing the CO_2 concentration and humic acid not. The chemical nature of humic substances changes during the extraction procedure. For example, some extracted forms of humic organic matter are solubilized by alkali, and their biological activity may thus substantially increase. This is probably the case here for fulvic acid, which remains soluble after acidification of alkaline soil extracts and may possess moderately antimicrobial activity at higher concentrations (Visser 1984). This would explain the decrease in bacterial numbers in the treatment receiving Ca fulvate in the present study. Humic substances are taken up by biota (Steinberg et al. 2003) and cause toxic effects. However, a more indirect effect through the influence of humic substances on root physiology (including respiration) and on the production of root exudates cannot be excluded.

In order to determine whether the effect of fulvic compounds on *G. claroideum* could be affected by microorganisms potentially able to degrade fulvic acid, maize plants were also inoculated with living or heat-inactivated *P.*

lilacinus and an actinomycete able to use soil fulvic acid as the sole carbon source. Both of these microorganisms stimulated the development of the mycorrhizal fungus even when they had been killed by heat, so that their effects could not be attributed to their modifying the humic substances added to the cultivation substratum. The observed effects of the two microorganisms may be linked to their biomass representing a further enrichment of the rooting environment.

One explanation for the observed effects of the humic substances on *G. claroideum* may consist in a modification of the nutrient solution by the addition of humic substances. Soil humic substances are known to interact with polyvalent inorganic cations (e.g., Nordén and Dabek-Zlotorzynska 1996), which electrostatically bind to negatively charged functional groups of fulvic acid and may thus substantially change their physicochemical character. At the same time, this reaction removes free cations from the solution. Iron can be taken as an example of a cation that is firmly bound to humic substances. This may explain the decreased concentration of iron in maize plants grown in the substratum supplied with fulvic acid, and the conditions in which iron is in low availability may favour physiological functions of mycorrhizal mycelium (absorption and transport). Although mycorrhizal mycelium can improve the iron nutrition of plants (Caris et al. 1998), no increase was observed in the concentration of iron in the mycorrhizal maize treatments. Iron concentration in the maize plants was not significantly affected by humic acid; the humic substances thus behaved differently and their effects on *G. claroideum* were related to other factors than a change of iron uptake by the organism.

In conclusion, application of humic substances as the additives to a non-soil perlite substratum substantially improved the intraradical and extraradical development of *G. claroideum* BEG23. AMF may suffer from the absence of some soil components when cultured hydroponically and the soil organic matter including humic substances may be necessary for extensive growth of their mycelium. Further research will be focused on the effects of humic substances of different origin on several species of AMF to verify whether our results are of a general character.

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