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M. Gryndler · J. Larsen · H. Hršelová · V. Řezáčová · H. Gryndlerová · J. Kubát

Organic and mineral fertilization, respectively, increase and decrease the development of external mycelium of arbuscular mycorrhizal fungi in a long-term field experiment

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Abstract Effects of long-term mineral fertilization and manuring on the biomass of arbuscular mycorrhizal fungi (AMF) were studied in a field experiment. Mineral fertilization reduced the growth of AMF, as estimated using both measurements of hyphal length and the signature fatty acid 16:1ω5, whereas manuring alone increased the growth of AMF. The results of AMF root colonization followed the same pattern as AMF hyphal length in soil samples, but not AMF spore densities, which increased with increasing mineral and organic fertilization. AMF spore counts and concentration of 16:1ω5 in soil did not correlate positively, suggesting that a significant portion of spores found in soil samples was dead. AMF hyphal length was not correlated with whole cell fatty acid (WCFA) 18:2ω6,9 levels, a biomarker of saprotrophic fungi, indicating that visual measurements of the AMF mycelium were not distorted by erroneous involvement of hyphae of saprotrophs. Our observations indicate that the measurement of WCFAs in soil is a useful research tool for providing information in the characterization of soil microflora.

Keywords Whole cell fatty acids (WCFAs) · Mycelium · Soil microorganisms · Fertilization · AMF

M. Gryndler (✉) · H. Hršelová · V. Řezáčová · H. Gryndlerová
Institute of Microbiology CAS, Division of Ecology,
Vídeòská 1083, CZ 142 20,
Prague 4, Czech Republic
e-mail: gryndler@biomed.cas.cz
Tel.: +420-2-41062652
Fax: +420-2-41062384

J. Larsen
Danish Institute of Agricultural Sciences,
Department of Integrated Pest Management,
Research Centre Flakkebjerg,
Flakkebjerg, 4200 Slagelse, Denmark

J. Kubát
Research Institute for Crop Production,
Drnovská 507, CZ 161 06,
Prague 6, Czech Republic

Introduction

Arable soils that have been under intensive management practices (ploughing, fertilization) for a long time substantially change their character and develop a steady state, characterized by constant values of the ratio of biomass of living organisms to total content of organic matter. This suggests a direct trophic connection between soil biota, organic matter and mineral nutrients (Anderson and Domsch 1989; Witter and Kanal 1998). Among soil organisms, arbuscular mycorrhizal fungi (AMF) are believed to be obligatory symbiotic, so that no direct relationship between them and organic matter as a source of energy is expected. In spite of this, AMF respond markedly to the presence of organic matter in their environment (St.-John et al. 1983; Joner and Jakobsen 1995) as well as to organic fertilization and substrate additives (Gryndler et al. 2001; Joner 2000; Ravnskov et al. 1999).

High-input soil management, involving mineral fertilization, can have a profound negative affect on mycorrhizal fungi, as reported by Galvez et al. (2001) who found that the abundance of spores of AMF was much lower in high-input agriculture compared to that in low-input agriculture. Some species of AMF may even be severely depressed under conventional farming where mineral fertilizers are exploited (Oehl et al. 2004). On the other hand, organic fertilization may increase sporulation of some AMF species (Douds et al. 1997) or propagule density in the soil (Harinikumar and Bagyaraj 1989). In a field experiment, fertilization (organic vs inorganic) did not affect external mycelium of AMF in sandy loam soils, but in clay soil, AMF populations were increased by manuring (Kabir et al. 1998).

The external mycelium of AMF plays a key role in the transport of nutrients from soil to host plants (Jakobsen et al. 1994; Johansen et al. 1994; Paszkowski and Boller 2002), in soil aggregation (Bethlenfalvay et al. 1999) and perhaps even in the suppression of root pathogens (St.-Arnaud et al. 1997; Larsen et al. 2003). Despite the apparent major importance of the external mycelium in the plant-beneficial features of AMF (Sanders et al. 1977; Hodge et al. 2001;

Paszkowsi and Boller 2002), quite often, only the intraradical phase of the fungus is considered when measuring AMF in both field and pot experiments (Guadarrama et al. 2004).

Hyphal length in external mycelium of AMF can be measured directly per unit volume of soil or cultivation substrate and combined with enzymatic activities to estimate viability (Sylvia 1988; Malcová et al. 2003), but these measurements are both extremely laborious and it can also be difficult to discriminate between the mycelium of AMF and the mycelium of other fungi. Consequently, the use of biochemical markers may be useful to provide more accurate measurements of the biomass of AMF in soil. AMF are known to contain fatty acids which are less common in other microorganisms, such as 16:1ω5, 18:1ω7, 20:1ω6 and 20:2ω6,9 (Olsson and Johansen 2000; Olsson et al. 1995; Madan et al. 2002; Balser et al. 2005).

The use of fatty acid biomarkers to quantify communities of microorganisms in environmental samples is based on the fact that they possess different kinds of fatty acids (Harwood and Russell 1984; Vestal and White 1989; Zelles 1997). In general, branched fatty acids (iso and anteiso) are specific to Gram-positive bacteria (Ratledge and Wilkinson 1988), hydroxyl and cyclic fatty acids are specific to Gram-negative bacteria (Wilkinson 1988), methylated fatty acids are common among actinomycetes (Wilkinson 1988), and soil fungi can be quantified with the fatty acid 18:2ω6,9 (Frostegård and Bååth 1996). The latter fatty acid is also found in low amounts in AMF (Larsen et al. 1998, Jansa et al. 1999). Although interpretations of fatty acid profiles from soil can be difficult, their use as biochemical markers of microbial communities has the advantage; that is, mainly the living biomass is measured because the turnover of fatty acids is rapid. A whole cell fatty acid (WCFA) method has previously been used to measure soil microbial community structure in agricultural soil systems (Cavigelli et al. 1995; Ibekwe and Kennedy 1999; Zelles 1999).

The purpose of the present work was to evaluate the effects of mineral and organic fertilization on AMF in relation to other groups of soil microorganisms. Our hypothesis that development of AMF in soil is reduced by mineral fertilization and increased by organic fertilization was tested by quantifying the growth of AMF in soil directly by measuring AMF hyphal length, root colonization and numbers of spores, and indirectly using the biomarker fatty acid 16:1ω5. The biomass of other groups of microorganisms was also estimated using fatty acid measurements. The measurements were conducted in a long-term factorial field experiment, which has been subjected to continuous fertilization treatments for decades.

Materials and methods

Field experiment and sampling

Analyses of all parameters were performed on soil samples taken from selected plots of a long-term field experiment established in 1955 at the Institute for Crop Production, Prague (altitude 340 m, average annual precipitation 464 mm, mean annual air temperature 8°C). The soil is a clay-loam Orthic Luvisol, pH 6.5, which has developed on diluvial sediments mixed with loess.

Soil samples were taken from six treatments of the experiment involving various levels of mineral (none, ordinary, elevated), combined with two levels of organic fertilization (manuring vs without manure application), in a randomized design of 12×12-m experimental plots in four replicates. The mean annual doses of mineral fertilization per hectometer squared were 63 kg N, 54 kg P_2O_5, 131 kg K_2O for the ordinary fertilization regime and 91 kg N, 71 kg P_2O_5, 176 kg K_2O for elevated fertilization. The manured plots received cattle manure (dung) twice during a 9-year crop rotation. The average annual dose of nitrogen supplied with the manure was 38.6 kg N ha^{-1}. Concentrations of oxidizable C and available P in the studied soils were reported by Gryndler et al. (2003).

Soil samples were collected from the upper soil layer (0–15 cm) in spring 2003 (June 5) and in autumn 2003 (October 23) from plots covering all the combinations of mineral and organic fertilization. Altogether, 96 samples (4 samples of approximately 100 g soil for each of four replicates per each combination of mineral and organic fertilizers) were collected twice, in the two seasons. Additionally, the same sampling design was followed in summer 2004 (July 29) to check the mycorrhizal colonization of alfalfa roots as a result of the presence of hyphae and spores present in field soil in autumn 2003.

Sample processing

Soil samples were sieved (2 mm mesh) and divided into three portions: 25 g soil for WCFA measurements was frozen and freeze-dried under vacuum at −25°C for 20 h, 5 g soil was subjected to AMF hyphal length measurements, and 5 g soil was air-dried and further used for spore counting. A further 20 g soil of each spring-collected soil sample was used to estimate initial inoculum potential.

Estimation of soil inoculum potential

A biotest based on a hydroponic cultivation system was used to estimate the mycorrhiza inoculum potential of spring soil samples. Pre-germinated maize seeds were planted onto 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 after placing a 20-g soil sample at 4 cm below the level of the seeds. Planted tubes were then put into plastic tubs and supplied with a P_2N_3 mineral nutrient solution (Gryndler et al. 1992). Each tub contained 12 tubes and a volume of 2 L of the nutrient solution. In total, 8 tubs containing 96 soil samples in plastic tubes were established. The nutrient solution was renewed once a week. The hydroponic cul-

tures were kept in a growth chamber (11,000 lx, 16 h a day, 20:23°C night/day, 75% air relative humidity) for 8 weeks.

Measurement of mycorrhizal colonization of maize (biotest) and alfalfa (field)

Roots were washed from the hydroponic substratum or from soil and digested for 30 min in 10% KOH at 90°C, washed again, acidified in 4% lactic acid and stained in 0.1% trypan blue in lactoglycerol. Percentage of root length colonized by AMF was measured using the grid-line intersect method (Giovannetti and Mosse 1980).

Measurement of hyphal length

Each of the 5 g soil samples was placed in a household blender containing 500 ml of water and blended for 30 s. One millilitre 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, and total length of the mycelium was assessed using the grid-line intersect method (microscope equipped with focal plate grid 100× magnification), as described by Malcová et al. 2002.

Spore counts

Spores were extracted from soil by wet sieving (sieves 32, 250 and 500 μm mesh) and decanting (Gerdemann and Nicolson 1963) and counted microscopically at 40× magnification.

Analysis of WCFA

A 25-g sample of freeze-dried soil was powdered in a steel mill, and 1 g was subjected to lipid extraction and further analysed for the content of WCFAs, as described by Thygesen et al. (2004). Analyses of fatty acid methyl esters were performed using the Sherlock 3.1 software package (MIDI Inc., Newark, DE, USA) with an HP Chemstation (Hewlett Packard, CA, USA) and an HP5890 GC fitted with a 25-m fused silica capillary column (HP part no. 19091B-102) and hydrogen as carrier gas. The injector and detector temperatures were set to 250 and 300°C, respectively. The column temperature was programmed so that it increased from an initial 170°C to an ultimate 270°C at a rate of 5°C min^{-1}. One microlitre of sample preparation was injected. Calibration standards contained a mixture of straight chain saturated and hydroxylated fatty acid methyl esters with a length of 10–20 carbon atoms (MIDI, Part No. 1200A).

Statistics

Data were subjected to one- or three-way ANOVA and Duncan's multiple range test. To check the validity of the use of ANOVA, the homogeneity of variance was first tested using Bartlett's *F* test. Correlation coefficients were calculated to evaluate the strength of the relationship between AMF hyphal length, sporulation and abundance of distinguishable groups of soil microflora.

Results

Three-way ANOVA analysis of the effects of main experimental factors and their interactions

All parameters except WCFA 18:2ω6,9 were significantly affected by at least one experimental treatment (Table 1). Mycorrhizal parameters were mostly affected by mineral fertilization, with the exception of AMF colonization (season 2004), which responded only to organic fertilization. Similarly, the AMF inoculum potential of field soil collected in the spring 2003 showed a response to organic fertilization, and this parameter was also affected by mineral fertilization. Spore counts were affected by all three experimental factors. Marker WCFAs of actinomycetes, Gram-negative and Gram-positive bacteria were

Table 1 Statistical significance (three-way ANOVA, *P* values) of the effects of organic or mineral fertilization on soil microorganism populations in field soils

Parameter	Fertilization		Season (C)	A × B	A × C	B × C	A × B × C
	Organic (A)	Mineral (B)					
AMF inoculum potential	0.0000	0.0000	–	0.1235	–	–	–
log length of AMF hyphae	0.0551	0.0000	0.0001	0.2983	0.9676	0.9666	0.0290
AMF spores	0.0000	0.0000	0.0000	0.5961	0.0000	0.0001	0.3044
log 16:1ω5 (AMF)	0.1536	0.0000	0.3496	0.1390	0.6676	0.0470	0.2830
18:2ω6,9 (saprotrophic fungi)	0.5235	0.2546	0.9213	0.9967	0.2069	0.3786	0.9330
Gram-positive bacteria/WCFA	0.0022	0.1117	0.0055	0.7304	0.0763	0.0458	0.5463
Gram-negative bacteria/WCFA	0.0219	0.0039	0.0967	0.8488	0.5851	0.7204	0.5608
Actinomycetes/WCFA	0.0007	0.8180	0.4352	0.6025	0.6371	0.0691	0.0273
Field AMF colonization (2004)	0.0000	0.1255	–	0.9768	–	–	–

AMF arbuscular mycorrhizal fungi, *WCFA* whole cell fatty acid

Table 2 Effect of fertilization on mycorrhizal parameters: inoculum potential of spring-collected field soil (as percentage of maize root colonization), length of AMF hyphae and spore counts from soil samples, and root colonization of field grown alfalfa in summer 2004

Organic fertilization	None			Manured		
Mineral fertilization	None	Ordinary	Elevated	None	Ordinary	Elevated
Spring 2003						
AMF inoculum potential (%)	31.6 b	23.8 c	18.0 d	42.5 a	28.2 bc	24.1 c
AMF hyphal length (m g^{-1} soil)	0.78 b	0.45 d	0.49 cd	0.96 a	0.55 cd	0.58 c
AMF spores (g^{-1} soil)	4.62 d	7.50 c	8.51 bc	8.79 b	10.82 a	11.34 a
Autumn 2003						
AMF hyphal length (m g^{-1} soil)	1.42 a	0.82 c	0.76 c	1.22 b	0.80 c	0.71 c
AMF spores (g^{-1} soil)	8.06 e	11.25 d	13.68 c	13.72 c	17.16 b	19.60 a
Summer 2004						
Field root colonization (%)	70.2 a	50.5 b	32.6 cd	64.1 a	44.5 bc	28.2 d

Means followed by the same letter do not differ significantly within the row by one-way ANOVA and Duncan's multiple range test at $P \leq 0.05$

influenced by organic fertilization, but an effect of mineral fertilization was only recorded for marker WCFAs of Gram-negative bacteria. Sampling season affected the length of AMF hyphae, spore numbers and marker WCFAs of Gram-positive bacteria (Table 1).

Interactions between the effects of mineral fertilization and sampling season on the concentration of 16:ω15, mycorrhizal spore counts and marker WCFAs of Gram-positive bacteria were observed (Table 1). Effects of organic fertilization and sampling season significantly interacted only for spore counts. Interactions between the effects of all three factors (mineral fertilization, organic fertilization and sampling season) on the length of AMF hyphae and marker WCFAs of actinomycetes were also significant.

AMF inoculum potential, root colonization and spore counts

Data for AMF soil inoculum potential using the root colonization biotest correlated with the length of AMF hyphae in spring soil samples (Table 2), but not with AMF spore counts. Spore counts increased with increasing mineral and organic fertilization, being most abundant in the manured treatment fertilized with an elevated dosage of mineral fertilizer (Table 2). Colonization of field grown alfalfa roots by AMF observed in 2004 gave similar trends as the biotest of infectivity the previous year: this parameter tended to follow hyphal lengths rather than spore counts. Spore counts found in spring samples were always much lower than those of soils sampled in autumn (Table 2).

Table 3 Effect of fertilization on WCFA markers (nmol per g dry soil) corresponding to different groups of soil microorganisms: AMF (16:1ω5), saprotrophic fungi (18:2ω6,9), Gram-positive bacteria (13:0-iso, 14:0-iso, 15:0-iso, 15:0-ante-iso, 16:0-iso, 17:0-iso, 17:0-ante-iso), Gram-negative bacteria (10:0-3OH, 12:0-2OH, 16:0-2OH, 17:0-cyclo, 19:0-cyclo ω8) and actinomycetes (16:0-10-methyl, 17:0-10-methyl, TBSA-10-Me-18:0, 19:0-10-methyl)

Organic fertilization	None			Manured		
Mineral fertilization	None	Ordinary	Elevated	None	Ordinary	Elevated
Spring						
AMF	6.65 c	3.20 e	3.93 de	9.33 b	3.90 de	3.41 de
Saprotrophic fungi	10.99 –	10.48 –	10.76 –	9.77 –	9.08 –	10.19 –
Gram-positive bacteria	16.94 de	16.05 e	19.39 cde	19.68 cd	20.36 bcd	21.28 bc
Gram-negative bacteria	4.10 c	4.24 c	5.14 abc	4.84 bc	5.30 ab	5.48 ab
Actinomycetes	6.49 e	5.80 e	7.94 a–e	8.90 abc	8.87 abc	8.56 a–d
Autumn						
AMF	9.51 b	4.40 de	3.69 de	11.52 a	5.35 cd	3.57 de
Saprotrophic fungi	7.87 –	10.38 –	9.85 –	10.42 –	13.02 –	10.35 –
Gram-positive bacteria	19.22 cde	21.50 bc	21.66 bc	23.35 ab	25.13 a	22.80 abc
Gram-negative bacteria	4.73 bc	5.42 ab	5.73 ab	5.65 ab	5.99 a	6.66 a
Actinomycetes	6.76 cde	9.03 ab	7.02 b–e	9.73 a	9.65 a	9.41 a

Means followed by the same letter do not differ significantly by one-way ANOVA and Duncan's multiple range test (over both sampling seasons) at $P=0.05$. The data on saprotrophic fungi were not evaluated by multiple range test because one-way ANOVA returned insignificant results

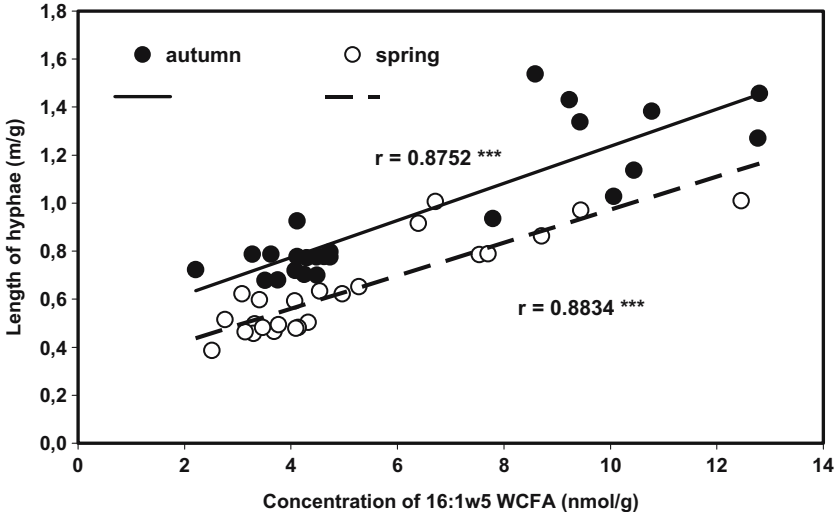

Fig. 1 Correlation between measured concentration of 16:1ω5 WCFA and length of hyphae of AMF in spring (*open circles*) and autumn (*closed circles*) soil samples. Both correlation coefficients (*r*) are significant at *P*=0.001 (*asterisk*)

Evaluation of soil mycelium of AMF

Hyphal length measurements revealed an increase in soil AMF hyphae in manured treatments with no mineral fertilization when compared with the control treatment (no fertilizer applied; Table 2). This was apparent only in the spring, and an opposite significant effect was observed in autumn. Similar effects of manuring were not observed at ordinary or elevated mineral fertilization levels. Lengths of hyphae observed in autumn were much higher than those found in spring. Any supply of mineral fertilizer significantly decreased the length of AMF hyphae, irrespective of sampling season and organic fertilization (Table 2).

Measurements of concentrations of WCFA 16:1ω5, a biomarker for AMF, in the soil showed that any application of mineral fertilizers significantly decreased the concentration of this WCFA, irrespective of sampling season and organic fertilization (Table 3). When no mineral fertilizer was applied, manuring significantly increased the concentration of WCFA 16:1ω5 in soil. This effect was observed both in the spring and summer samples. Concentrations of this fatty acid in unfertilized soils (irrespective of organic fertilization) were significantly higher in autumn samples compared to those in spring data.

The correlation coefficient between AMF hyphal length and WCFA 16:1ω5 was positive and significant (*r*=0.607, *P*=0.01). To evaluate whether the sampling season affected this correlation and correlation of WCFA 16:1ω5 with spore counts, the data obtained in both seasons were plotted separately (Figs. 1 and 2). Data indicate a positive correlation between WCFA 16:1ω5 and visually measured hyphae. Higher amounts of this fatty acid per unit of hyphal length were observed in spring samples (Fig. 1). AMF spore counts did not positively correlate with WCFA 16:1ω5. Correlation coefficients between spore counts and WCFA 16:1ω5 (*r*=−0.44), root colonization (*r*=−0.69) or length of mycorrhizal hyphae (*r*=−0.67) were always negative, but they were significant only for autumn sampling (*P*=0.05).

Marker WCFAs of saprotrophic soil microorganisms

The WCFA 18:2ω6,9, specific to saprotrophic fungi, was unaffected by all the tested factors (Table 3). To estimate

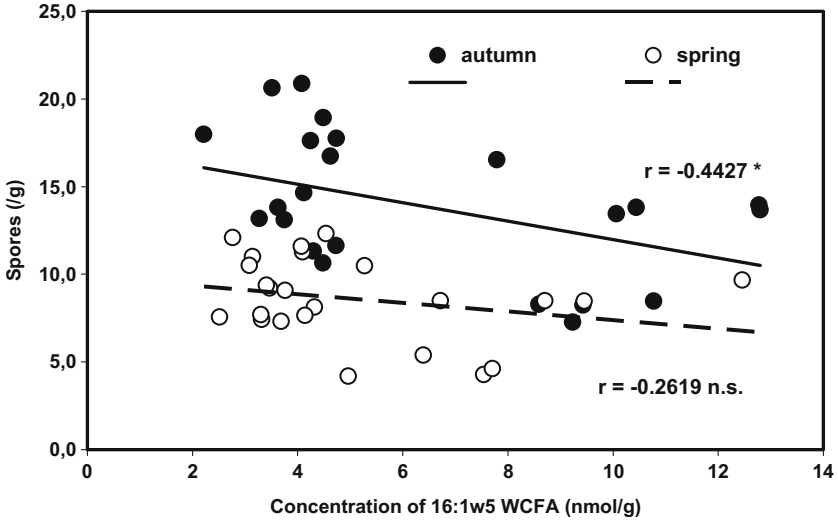

Fig. 2 Correlation between measured concentration of 16:1ω5 WCFA and counts of spores of AMF in spring (*open circles*) and autumn (*closed circles*) soil samples. Only the correlation coefficient (*r*) calculated for the autumn data is significant at *P*=0.05 (*asterisk*)

biomass of Gram-positive bacteria, Gram-negative bacteria and actinomycetes, the individual characteristic WCFA markers were added. Concentrations of summed WCFA markers of Gram-positive bacteria were significantly increased by manuring in treatments fertilized by an ordinary level of mineral fertilizer. In autumn samples, manuring further significantly increased this parameter if no mineral fertilization was applied (Table 3). WCFA markers of Gram-positive bacteria were significantly increased compared to the treatment without fertilizers in autumn samples, in manured treatments fertilized with ordinary dosages of minerals, and in manured treatments without mineral fertilization. In spring samples with an ordinary level of mineral fertilization, manuring significantly increased WCFA markers of Gram-negative bacteria, and in unmanured samples with ordinary mineral fertilization, this parameter was significantly higher in the autumn, as compared to spring sampling. Concentrations of summed WCFA markers of actinomycetes significantly increased in spring samples with zero or ordinary mineral fertilization (compared to autumn samples) if manure was applied. In the autumn samples, the lowest values were obtained in unmanured treatments with zero or elevated mineral fertilization (Table 3).

A weak but significantly positive correlation was found between 16:1ω5 and 18:2ω6,9 (r=0.298, P=0.01), the latter being a marker of saprotrophic fungi. No correlation between WCFA 18:2ω6,9 and hyphae of AMF was observed, indicating almost total independence of the two parameters.

Discussion

The main hypothesis that mineral fertilization would reduce the development of the soil compartment of AMF was confirmed under field conditions. To our knowledge, this is the first report of the influence of mineral fertilization on external mycelium of AMF under field conditions. Positive correlations between hyphal length measurements and the AMF biomarker WCFA 16:1ω5 confirmed the mycorrhizal origin of the measured hyphae. The reduction in mycelial biomass of AMF in soils most likely resulted from reduced colonization of host plant roots. This was observed not only at the beginning of our study in the biotest with maize, but also in the following year later, in terms of alfalfa root colonization in the experimental field plots. These observations were consistent with previous ones and tend to confirm that inhibition of AMF development by mineral fertilization is a general phenomenon in the arbuscular mycorrhizal symbiosis (Hayman 1982; Thomson et al. 1992; Olsson et al. 1997).

Our observations further indicate that not only AMF but also other groups of soil microorganisms are affected by mineral and organic fertilization, whilst saprotrophic fungi appear to be almost unaffected by such treatments. This finding agrees with that of Marschner et al. (2003) who also observed that biomass of soil fungi measured as 18:2ω6,9 does not respond to different fertilization regimes.

Our findings that organic fertilization alone increased development of AMF mycelium, measured in terms of hyphal length and concentrations of WCFA 16:1ω5, is in agreement with previous studies from pot experiments (Joner 2000, Joner and Jakobsen 1995, Ravnskov et al. 1999, Gryndler et al. 2002). Such increased development of AMF in soil added with organic matter could be related to a general increase in soil biological activity, where AMF may benefit from the release of growth-stimulating substances. Whilst carbon in AMF mycelium proliferating in organic matter most likely originates from plant photosynthates (Gavito and Olsson 2003), mycelial growth of AMF may benefit from the release of other nutrients such as N from the organic matter, as suggested by Ravnskov et al. (1999).

Higher amounts of WCFA 16:1ω5 per unit hyphal length were observed in spring samples. Consequently, it is not possible to precisely calibrate hyphal length against the concentration of WCFA 16:1ω5, because the content of this fatty acid varies between seasons, which may be caused by a different degree of depletion of fungal lipid reserves over seasons. Also, correlation analysis of spore density and WCFA 16:1ω5 revealed that the concentration of this signature fatty acid does not positively correlate with the amount of AMF spores in the soil sample. Thus, at least in the present experiment, the concentration of WCFA 16:1ω5 in the soil may probably be considered as a measure of overall living AMF biomass, as opposed to hyphal length and spore counts which include both living and dead biomass (Olsson 1999). This may result from some of the counted spores being dead, and their lipids were degraded or exploited by the saprotrophic microflora.

The lack of correlation between AMF hyphal length and WCFA 18:2ω6, 9, the biomarker of saprotrophic fungi, is an important finding from a methodological point of view. This finding indicates that measurements of AMF hyphal lengths are not distorted by erroneous involvement of hyphae of other soil fungi. Hence, the method we have used to estimate AMF biomass is probably sufficiently selective to distinguish between AMF and other soil fungi. In conclusion, our results indicate that both organic and inorganic fertilizations influence AMF development in soil, and that the biomarker WCFA 16:1ω5 may represent a reliable measure of AMF biomass in soils.

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