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Influence of Mineral and Organic Fertilization on Soil Fungi, Enzyme Activities and Humic Substances in a Long-Term Field Experiment

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ABSTRACT. Changes in microfungal communities, fungal activities and humic substances (HS) in agricultural soils kept under different fertilization regimes were observed and their causal relationships were investigated in a long-term field experiment. Fertilization did not change the abundance of HS-utilizing microfungi and, except for organic amendment alone, total culturable microfungi were also unaffected by this factor. Organic fertilization increased activities of manganese peroxidase (MnP) and proteinase, but decreased endo-1,4-β-glucanase activity compared to the corresponding control without organic fertilization. In soils treated with mineral fertilizers, the activities of MnP, endo-1,4-β-glucanase and proteinase were higher than in control without any mineral treatment. Both the aromaticity of fulvic acid and the molar mass of humic acid was lower in soil with organic fertilization, which may be a result of oxidative degradation mediated by higher MnP activity observed in treatments with organic fertilization.

Abbreviations

ABTS	2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
CFU	colony forming units
FA	fulvic acid
HA	humic acid
HS	humic substances
MEA	malt extract agar
MF	mineral fertilization
MnP	manganese peroxidase
OC	oxidizable (organic) carbon
OF	organic fertilization
SGF	silica-gel supplemented with fulvic acid
SI	Sørenson index
SOM	soil organic matter
QSI	quantitative Sørenson index
RDA	redundancy analysis

Conversion of humus plays an important role in maintaining global carbon cycle and is essential for the long-term sustainability of ecosystems. HS are present in water and sediments, but the main part is deposited in soils, where they constitute an important, relatively stable source of carbon, making up to 70–80 % of the organic carbon present in the biosphere (Schlesinger 1997).

Management of plant cultures and their fertilization affect not only plants, but also soil microorganisms (Marschner *et al*. 2003) and can thus cause changes in the deposition of HS. Increased input of organic matter to the soil together with sufficient availability of mineral nutrients after MF may result in an increased rate of decomposition processes and microbial growth.

Despite the importance of HS, the response of HS in respect to various fertilization regimes has received only limited attention. It has been shown that increased resistance of HA to microbial degradation is associated with increased SOM contents (Filip and Kubát 2001). Although various microorganisms can depolymerize HS (Gramss *et al*. 1999) or change their physico-chemical features (Řezáčová and Gryndler 2006), they are considered to be very stable in soil (Hurst *et al*. 1962). In spite of the apparent significant association between SOM and microfungi (Hršelová *et al*. 1999), only few studies on the influence of various fertilization regimes on these microorganisms are available.

Here we analyzed soil samples from a field experiment run for 47 years where plots were treated by different levels of MF and OF. The objective was to contribute to an increased understanding of changes in microfungal communities, fungal activities and HS in agricultural soils caused by fertilization.

MATERIAL AND METHODS

Experimental design and locality. 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.

The field experiment had a randomized 2-factorial design with manuring as the first factor (without and with) and MF as the second factor (without, low NPK and high NPK level). Four randomly arranged replicates (12 × 12 m square plots) from each treatment were sampled.

The mean annual doses of MF per hm^2 were 63 kg N, 54 kg P_2O_5, 131 kg K_2O (low NPK) and 91 kg N, 71 kg P_2O_5, 176 kg K_2O (high NPK). The treatments with OF received 6.33 Mg of cow manure per hm^2 annually.

Soil sampling and analyses. Soil samples were collected in the spring and autumn 2003, from the upper soil layer (0–150 mm). Microfungi were assessed in the spring and slowly changing parameters characterizing HS in the autumn in the 24 samples (4 replicates per each combination of MF and OF levels). Enzyme activities were determined always in 4 subsamples per each sample (altogether in 96 subsamples and therefore in 16 replicates per each combination of MF and OF levels) in both seasons.

Isolation and characterization of HA and FA. HA and FA were prepared according to Gryndler *et al*. (2003). Ten-g sieved (2-mm mesh) soil samples were shaken with 20 mL of 0.1 mol/L HCl for 20 min, washed 2× with 20 mL distilled water and extracted with 20 mL 0.5 mol/L NaOH for 20 h at room temperature. The extract was filtered through a filter paper, acidified to pH 2.6 using HCl and filtered again to separate FA and HA. Final pH of both fractions was adjusted to 6.5. The resulting FA were characterized by the absorbance A_{280} referring to the contents of aromatic compounds, by the A_{465}/A_{665} ratio calculated from the A_{465} and A_{665} and referring to the molar mass (Chen *et al*. 1977) and by the assessment of OC in FA solution by the method of Sims and Haby (1971) with some modifications. To perform the latter, 0.5 mL of FA, 2 mL 1 % $K_2Cr_2O_7$ in concentrated H_2SO_4 and 0.5 mL of water were mixed and incubated for 1 h at 25 °C. Samples were heated for 3 h at 110 °C, left to cool and their absorbance A_{580} was measured. The content of OC was calibrated against glucose. HA were dissolved in 10 mmol/L NaOH and characterized by the A_{465}/A_{665} ratio.

Assays of enzymes and H_2O_2. Extraction of enzymes was done from 10 g soil samples, which were mixed with 10 mL of 50-mmol/L phosphate buffer (pH 7.0) and incubated on ice for 1 h. The suspensions were centrifuged (15000 ***g***, 15 min, 15 °C), and the resulting supernatants once more (5000 ***g***, 15 min, 15 °C). The clear supernatants were used for estimation of enzyme activities (Baldrian *et al*. 2000).

Laccase (EC 1.10.3.2) activity was measured using ABTS in citrate–phosphate buffer (100 mmol/L citrate, 200 mmol/L phosphate, pH 5.0) according to Niku-Paavola *et al*. (1990).

Activity of MnP (EC 1.11.1.13) and other peroxidases was assayed in succinate–lactate buffer (100 mmol/L, pH 4.5) according to Ngo and Lenhoff (1980).

Activity of endo-1,4-β-glucanase (CM-cellulase) was measured with azo-dyed carboxymethyl cellulose using the protocol of the supplier (*Megazyme*, Ireland).

Proteinase activity was assayed with azocasein (*Sigma*, US) by a modified method of Sethuraman *et al*. (1998). The reaction mixture containing 300 mL of 1 % azocasein in 50 mmol/L sodium acetate buffer (pH 5.0) and 750 mL sample was incubated for 30 min at 40 °C. The reaction was stopped by adding 150 mL of 40 % trichloroacetic acid and vortexing. Samples were centrifuged and the absorbance A_{440} of the supernatants was read. One unit of enzyme activity was defined as the amount of enzyme responsible for the increase of A_{440} by 1.0 per min.

The amount of H_2O_2 was measured according to Wolff (1994) using xylenol orange. All spectrophotometric measurements were done in a microplate reader (Sunrise, *Tecan*) or a UV-VIS spectrophotometer (Lambda 11, *Perkin-Elmer*).

Production of lignolytic enzymes involved in HS decomposition (Steffen *et al*. 2002; Baldrian 2006), laccase and nonspecific peroxidases, was estimated *in vitro* in the most frequently isolated microfungal species including *Alternaria alternata* (FR.) KEISSL., *Clonostachys rosea* (LINK) SCHROERS, SAMUELS, SEIFERT & W. GAMS, *Exophiala* cf. *salmonis* J.W. CARMICH, *Fusarium* sp., *Penicillium aurantiogriseum* group DIERCKX, *Paecilomyces lilacinus* (THOM) SAMSON and *Phoma* sp. to know if they can influence HS also directly by their degradation. Spot test of the presence of oxidative enzymes (Gramss *et al*. 1998) was used; their production was determined in 8-d-old fungal mycelium of 1–3 strains of each species grown on MEA (Pitt 1979) and SGF. Colonies were observed after 1, 3 and 24 h; color intensity (indicating enzyme activity) was classified using a 4-point scale varying from weak/just perceptible to very strong reaction with dark coloration.

Assessment of soil microfungi. Common soil fungi were isolated from a modified SD medium (Hršelová *et al*. 1999) and fungal utilizers of resistant forms of SOM were isolated on SGF medium (Gryndler *et al*. 2003) prepared by mixing 1 L solution of FA (0.27 g/L of OC), 52.5 mL 33 % sodium silicate and a mixture of 100 mL mineral stock solution (Gryndler *et al*. 2003), with 22.6 mL 25 % phosphoric acid. All components were autoclaved separately, cooled and mixed immediately before use. Dilution plating was used for the isolation. One g of soil of each sample was shaken for 10 min in 100 mL of sterile deionized water and 0.5 mL of the diluted suspension (final dilutions 1 : 10^3 for SGF and 1 : 10^4 for agar) was spread onto the medium in a 90-mm Petri dish, three dishes being prepared per sample and per dilution. CFU were counted after 7–10 d of incubation at 25 °C. In case of SGF, each colony was transferred to the same, fresh SGF and left to grow for another 1–3 weeks. The isolates were then obtained from the margin of resulting colonies.

Microfungi were identified according to micro- and macromorphological characteristics (Domsch *et al*. 1980; Ellis 1971, 1976).

Data analysis. Two-way analysis of variance (ANOVA) was used in order to evaluate the effects of MF and OF on the abundance CFU, enzyme activities and parameters characterizing HA and FA at $p \leq 0.05$. Differences in community structure between various treatments were assessed using calculated SI and QSI (Mueller-Dombois and Ellenberg 1947). The lower is the value of SI or QSI the higher are differences in species composition between two compared data sets.

Effects of OF and MF on enzymic activities measured in soil, HS-characterizing parameters and relative abundance of particular microfungal species obtained on modified soil agar and on SGF were also tested by means of RDA, using Canoco, version 4.0 (TerBraak and Šmilauer 1998). The set of analyzed data contained 24 soil samples, laccase, cellulase (endo-1,4-β-glucanase), MnP activities, all measured HS-characterizing parameters and abundance of all fungal species. The data were square-root transformed and standardized through species and samples. The significance ($p \leq 0.05$) of species-environment relationship was assessed using the implemented Monte Carlo permutation test (499 permutations).

RESULTS

Effect of fertilization on saprobic microfungi. The abundance of microfungi was unaffected by both MF alone and in combination with OF, whereas OF alone caused a decrease in the abundance of microfungi (Table I). The abundance of HS-utilizing microfungi was unaffected by any type of fertilization (Table I).

Table I. Average concentration of CFU (per mg soil) and average activity of laccase (nkat/g) from soil with mineral and organic fertilization*

Fertilization		CFU/agar	CFU/SGF	Laccase
organic	mineral	spring	spring	autumn
Without	none	64.3a	57.4	1.43a
	low	52.8a	33.3	0.95b
	high	31.3ab	23.5	1.57a
With	none	11.5b	19.1	1.47a
	low	59.4a	60.5	1.85a
	high	72.6a	47.1	1.52a

**Different letters at values* – means differ significantly by 1-way ANOVA at $p = 0.05$.

A total of 46 microfungal species were identified in June 2003, *Clonostachys rosea* (LINK) SCHROERS, SAMUELS, SEIFERT & W. GAMS, *Fusarium* cf. *coeruleum* LIB. ex SACC. and *Penicillium aurantiogriseum* group DIERCKX being the most frequent among them. Twenty-eight species were obtained from the soil agar and 27 from the SGF.

SI (Table II) revealed large differences in the microfungal community structure between all the combinations of MF and OF. The differences in species composition were most pronounced between treatments with and without OF, and also between soils without MF and with lower NPK level. Quantitative differences in the abundance of particular microfungal species were also apparent in soils subjected to different fertilization, as reflected in QSI (Table II). Although the most frequently isolated microfungal species

were common for both soil agar and SGF, both SI and QSI revealed great differences between the community structure counted on agar and SGF (SI = 0.286, QSI = 0.192).

No effect of MF or OF on the abundance of particular microfungal species was found when the data were processed using RDA (Fig. 1). Here, the first two canonical axes explained 11.1 % and all axes together 37.3 % variability of the dataset.

Table II. SI (*first rows*) and QSI (*second rows*) for communities of soil culturable microfungi obtained on modified soil agar (agar) and SGF medium (SGF) from soils treated by combination of mineral and organic fertilization*

Fertilization treatment	Agar	SGF
None, low mineral	0.19	0.63
	0.29	0.47
None, high mineral	0.46	0.58
	0.29	0.36
Low, high mineral	0.53	0.58
	0.35	0.45
None, organic	0.35	0.53
	0.40	0.42

*High values indicate high similarity of fungal communities in compared soils.

Effect of fertilization on soil enzyme activities. In the autumn sampling, the effect was more pronounced than in spring (Table III). In soil harvested in the spring, a significant effect of fertilization was recorded only for MnP and nonspecific peroxidases (Table III).

The strongest effect of fertilization was found in endo-1,4-β-glucanase activity measured in the autumn. OF decreased this activity to 13 % of that in the soil without OF. In contrast, higher level of NPK nearly doubled this activity compared to the control (without any mineral treatment). MnP activity was significantly affected by fertilization in the spring as well as in the autumn. In the autumn the activity increased in organically fertilized soil up to 3× relative to that in soil without any organic treatment. Compared with control, the higher level of NPK increased the MnP activity 3–5× in the spring and only 2× in the autumn; here the proteinase activity increased under both MF and OF. Similarly, MF increased the activity of non-specific peroxidases in the spring. Combination of low MF with nil OF decreased the laccase activity (Table I).

Enzyme production by the most frequently isolated fungi. In-vitro production of extracellular lignolytic enzymes potentially involved in HS degradation was recorded for all species. Laccase activity was, however, detected in only one organism, *Phoma* sp., and only when tested on MEA. On the other hand, except for *P. aurantiogriseum* group, all the microfungi produced peroxidases. Stronger intensity of color reactions indicating enzyme activities was observed on SGF than on MEA.

Table III. Average soil enzyme activities (pkat/g), H_2O_2 content (μmol/g) and characteristics of HA and FA from soil under combinations of mineral and organic fertilization*

Enzyme	Season**	Mineral fertilization			Organic fertilization	
		none	low	high	none	applied
Endo-1,4-β-glucanase	S	3.50	3.83	4.33	3.67	4.00
	A	0.13b	0.29a	0.25a	0.39a	0.06b
Proteinase	A	0.31b	0.39ab	0.54a	0.34b	0.49a
Laccase	S	1.67	2.33	2.50	2.17	2.17
Mn-peroxidase	S	3.67b	9.67a	11.7a	8.33a	8.18a
	A	6.33b	12.2a	12.8a	5.17b	15.7a
Other peroxidases	S	4.33b	18.5a	22.7a	12.0a	18.3a
	A	6.50b	18.7a	22.7a	13.2a	18.7a
H_2O_2	S	0.46	0.33	0.48	0.43	0.42
	A	0.34a	0.27ab	0.23b	0.27a	0.29a

**Different letters at values* – means differ significantly by 2-way ANOVA at $p = 0.05$. **A – autumn, S – spring.

Effect of fertilization on HA and FA. According to the results of the two-way ANOVA, the treatment with OF significantly affected resistant fractions of SOM, both A_{280} of FA and A_{465}/A_{665} ratio of HA being influenced (Table IV). Effect of MF on other characteristics of resistant forms of SOM was not detected.

For average values of all mentioned characteristics *see* Table IV. A_{280} of FA was substantially higher in soil with OF compared to treatments without OF. A_{465}/A_{665} ratio of HA was slightly but significantly increased by OF. A significant correlation ($p = 0.0215$) between A_{280} and OC of FA was noticed.

DISCUSSION

Effect of fertilization on microfungi. The decrease in microfungal CFU abundance caused by OF is in contrast with the data of Marschner *et al*. (2003), who found fungal biomass in soil to be unaffected by OF and MF. Our results also disagree with the data of Fliessbach and Mäder (2000) and Schjønning *et al*. (2002), who recorded higher microbial biomass in organically than in conventionally managed plots and with the results of Gryndler *et al*. (2003), who observed higher CFU counts in organically treated soils compared to soil without any treatment. A possible explanation of this may consist in severe drying of the soil during the summer and autumn preceding the sampling. This drying most likely affected the whole community of soil organisms including saprotrophic fungi and its consequences might persist for the next vegetation period.

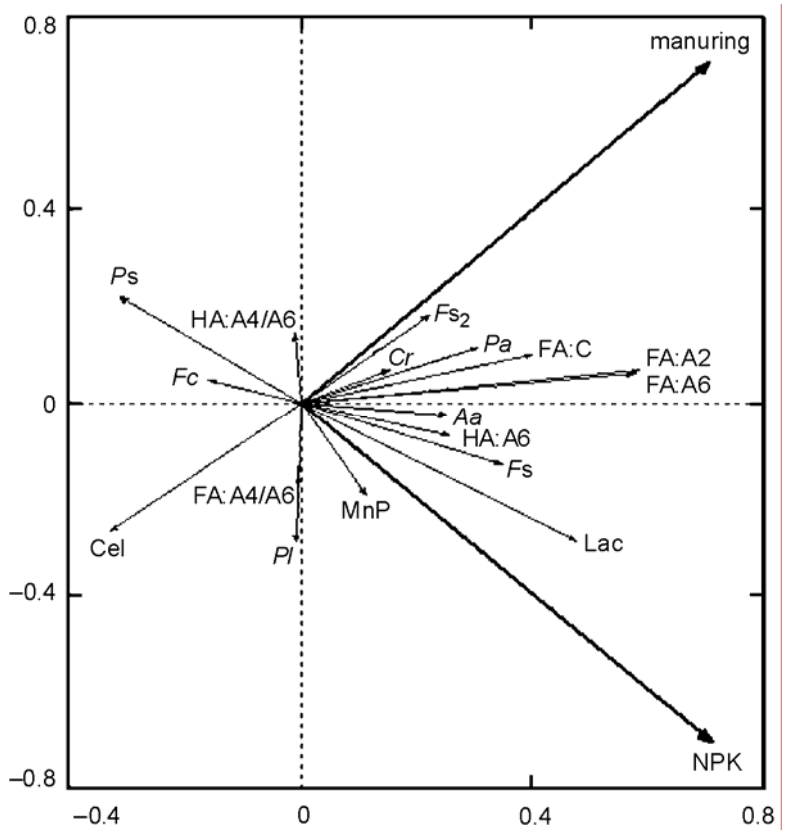

Fig. 1. Ordination biplot from redundancy analysis presenting the effect of mineral and organic fertilization on HS-characterizing parameters, enzyme activities and microfungi isolated on SGF (p-value = 0.04, F-ratio = 1.31 in Monte Carlo permutation test). The *direction* of the *arrows* indicates the tendency of the fungus to be detected under specific type of fertilization or together with specific enzyme activity or parameters characterizing HS. The *arrow length* indicates the strength of the tendency observed expressed as the correlation coefficient. For further details *see* the text.

Parameters: *Aa* *Alternaria alternata*; *Cr* *Clonostachys rosea*; *Fc* *Fusarium* cf. *coeruleum*; *Fs*, Fs_2, *Fusarium* spp.; *Pa* *Penicillium aurantiogriseum* group; *Pl* *Paecilomyces lilacinus*; *Ps* *Phoma* sp. (*all* CFU counts)

FA:A2	A_{280} of FA	FA:A4/A6	A_{465}/A_{665} ratio of FA	HA:A6	A_{665} of HA
FA:A6	A_{665} of FA	FA:C	total OC in FA	HA:A4/A6	A_{465}/A_{665} ratio of HA
Cel	cellulase activity	Lac	laccase activity	MnP	manganese peroxidase activity

Effect of fertilization on enzyme activities in soil. Both mineral and organic treatment affected most of the activities. Similarly to Marschner *et al*. (2003), enzyme activities, proteinase activity in particular, tended to increase in soils with OF. These activities could release nutrients from the soil and also from HS. Increased activity of lignolytic enzymes (MnP under OF and both MnP and other peroxidases under MF) is important because it can degrade HS (Hofrichter *et al*. 1998; Steffen *et al*. 2002).

The limited response of enzyme activities to fertilization in the spring 2003 compared to the autumn 2003 was probably also caused by the extremely dry period preceding this harvest, as soil humidity is supposed to be an important limiting factor of microbial growth and activity.

Table IV. Average values of parameters characterizing HA and FA from soil under combinations of mineral and organic fertilization in autumn*

Parameter**	Mineral fertilization			Organic fertilization	
	none	low	high	none	applied
FA to A_{465}/A_{665}	16.0	15.8	15.9	16.1	15.7
FA to A_{280}	2.43a	2.87a	3.45a	2.40a	0.992b
FA to C	0.361	0.366	0.356	0.355	0.367
HA to A_{465}/A_{665}	4.61a	4.56a	4.68a	4.38b	4.85a

**Different letters at values* – means differ significantly by 2-way ANOVA at $p = 0.05$.
**C – total oxidizable carbon (g/L).

Ability of frequently isolated fungi to utilize FA. Direct identification on the basis of morphological characteristics allowed us to detect particular microfungal species. The most frequently occurring species (*A. alternata*, *C. rosea*, *Fusarium* sp., *P. lilacinus, P. aurantiogriseum*, *Phoma* sp.) grew on the medium where FA were the only C source, and produced lignolytic enzymes. Therefore, these species are most probably involved in decomposition of FA. Claus and Filip (1998) and Gramss *et al.* (1999) also document that at least some soil microfungi are involved in HS decomposition.

Effect of fertilization on HS. Our results indicate that OF causes changes in resistant parts of SOM. Molar mass of HA was slightly lower and the amount of aromatic compounds of FA was higher in soil with OF. This does not correspond with the data of Filip and Kubát (2001), who indicated increasing resistance of HA to microbial degradation with an increase in SOM contents; however, they used different methods. In analogy to Steffen *et al.* (2002), our finding of decreasing molecular size and molar mass as well as decreasing FA-aromaticity could be caused by higher MnP activity in soil with OF.

The discrepancy between poor response of CFU of culturable fungi to fertilization and recorded effects of the same factor to some soil enzymic activities and HS-describing parameters indicate that different methods of the analysis of soil mycoflora have to be developed for the detection of biomass of specific fungi able to modify humic substances. Nevertheless, our results indicate that the activity of soil microflora degrading HS is strongly affected by fertilization regime.

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