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Effect of *Chalara longipes* on Decomposition of Humic Acids from *Picea abies* Needle Litter

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ABSTRACT. The effect of the saprotrophic ascomycete *Chalara longipes* on the decomposition of humic acids was determined in a cultivation experiment. The fungus was incubated in liquid cultures in a full-strength (F system) and an organic nitrogen-free medium (F–N system), both amended with pure humic acids isolated from spruce forest litter. Fungal biomass production was highest in the F system with humic acids and lowest in the F–N system, the effect of organic nitrogen and humic acids being significant ($p < 0.05$). The presence of organic nitrogen seems to be essential for growth. The fungus utilized humic acids; molar mass distribution in media obtained by gel-permeation chromatography showed decrease in the humic acids fraction. Decolorization of the media reached 75 and 64 % in the F and F–N systems, respectively. The molar mass of humic acids probably also decreased as estimated from the increase in the absorbance A_{465}/A_{665} (A_4/A_6) ratio. It is assumed that the utilization of humic acids may be mediated by the production of organic acids (as their components) and production of some oxidative enzymes.

Abbreviations

FA fulvic acids HA humic acids HS humic substances MEA malt extract agar N_{org} organic nitrogen

HA together with FA and humins belong to HS, which represent a relatively stable organic part of soil organic matter present predominantly in soils and sediments and in lower amount in water. HS have a significant effect on the physical and chemical properties of soil. They hold ≈70–80 % of organic carbon in mineral soils. Total soil HS form ≈1.4–1.5 Eg (*i.e.* 14–15 × 10^{14} kg) of the organic carbon (Piccolo 2002). In view of their role in the turnover of organic carbon in the global ecosystem, studies dealing with HS are essential for understanding the biogeochemical processes in soil.

The genesis of HS in boreal forests begins in fallen litter needles (Schnitzer 1978). This substrate is colonized by various organisms comprising mainly fungi and bacteria, which affect the HS turnover (Gryndler *et al.* 2003). A substantial part of fungal litter colonizers is formed by specialized saprotrophic ascomycetes and basidiomycetes often limited only to the niche of this substrate. The degradation of HS by litter inhabiting basidiomycetes and bacteria has been widely investigated. Steffen *et al.* (2002) recorded a decomposition of HA from pine-spruce forest and its conversion to FA by litter inhabiting species *Collybia dryophila.* The conversion increased in the presence of Mn^{2+} ions. They concluded that Mn-peroxidase is a key enzyme in HA degradation. Using ^{13}C-NMR, Yanagi *et al.* (2002) provided valuable information on the relation between the structure of various HA and their decolorization by 3 basidiomycetes and 2 actinomycetes. Regarding wood-degrading species *Coriolus consors* they found a significant relationship between the decolorization and increasing saccharide content and the loss of aromatic carbon of HA studied; this is in agreement with Hertkorn *et al.* (2002), who compared the alteration of aquatic HS by natural microbial populations from various sites. Recording the highest alteration of riverine HS (which contained considerable saccharide content), they concluded that the structure of HS had a significant influence on their microbial alteration.

Compared to basidiomycetes, ascomycetes have as yet been largely overlooked and little information is available on the effect of individual species on HA turnover. In this paper, the effect of the saprotrophic ascomycete *Chalara longipes* on the decomposition of pure HA added to liquid media was investigated and changes in HA content and fungal growth were determined.

MATERIALS AND METHODS

Litter needles inhabiting *Chalara longipes* (PREUSS)COOKE strain CCF 3367 was isolated from litter needles collected in a well preserved spruce [*Picea abies* (L.)KARST] forest in the Bohemian Forest (*Šumava National Park*, Czechia). The uppermost litter layer was removed and needles from O_l and O_f soil horizons were collected in sterile polyethylene bags. Individual needles were washed 3× in sterile water, first washing being performed with 100 ppm Tween to remove fungal spores from the needle surface. Washed needles were incubated on Petri dishes containing 2 % MEA at room temperature. Culture was preserved on 1.5 % MEA for 6 months until use.

Soil sample was collected from the O_f horizon of Ferro-Humic Podzol in spruce forest (association *Athyrio alpestris–Piceetum* HARTMANN 1959) in the Trojmezí stand (*Bohemian Forest, Šumava National Park*, Czechia). Air-dried sample was sieved on 5-mm sieve and the sieved fraction was used for HA extraction.

HA were extracted using 0.1 mol/L NaOH after soil decalcification by 0.1 mol/L HCl, 3× reprecipitated, washed by distilled water and concentrated in *Amicon* ultrafiltration cell (filter YM 1K NMWL). Sodium humate was converted into H^+ form in a column packed with Dowex strong cation exchanger WX4, freeze-dried and stored until use. Elemental composition of HA obtained (in %): C 50.1, H 3.3, N 2.6, O 41.8, ash 2.2, P 0.2.

A nutrient broth (composition in g/L: malt extract 20, NH_4NO_3 2.0, K_2HPO_4 1.0, $MgSO_4 \cdot 7H_2O$ 0.5, KCl 0.5, $FeCl_3$ 0.01; pH 6.5; C : N ratio of 6) was used in full strength (F) or as organic nitrogen-free nutrient solution (F–N) with glucose 19.0 g/L as a source of saccharides. Freeze-dried HA were dissolved in 10 mmol/L NaOH, added to nutrient broth at a final concentration of 0.5 g/L and autoclaved for 20 min. No particulate humic material was visible in the medium. The fungal inoculum consisted of 1 mL spores and blended mycelium suspension of 3-week-old culture. CFU value for inoculation dose was $>2 \times 10^{-3}$ per mL. Cultivation was performed in triplicate in cotton-plugged 100-mL Erlenmeyer flasks with 50 mL of medium. Inoculated flasks without HA and noninoculated sterile media with and without HA served as controls. The flasks were incubated in the dark at 25 °C on a reciprocal shaker (2 Hz). Total fungal biomass was harvested by filtration (ash-free, narrow-pored filter, Filtrak no. 390) after 21 d and oven-dried for 12 h at 80 °C. Molar-mass distribution in cultivation medium was obtained by gel-permeation chromatography, using a column (V_t = 116 mL, V_0 = 34 mL) with Sephadex G-25 Fine gel. Solution of 50 mmol/L phosphate buffer (pH 6) with 50 mmol/L NaCl was used as effluent, the elution rate being ≈60 mL/h. The absorbance (A_{280}, A_{465}, A_{665}) of eluted solution was measured using UV spectrophotometers Ultrospec III and Uvicord II (*Pharmacia LKB*, Sweden). The quotient of absorbances A_{465} and A_{665} nm was used to calculate the A_4/A_6 ratio (Chen *et al.* 1977) and the decolorization at the visible spectra (665 nm).

The effects of N_{org} and HA source on fungal biomass were statistically tested using Anova (*SPSS*; 11.5 for Windows) with fixed factors (presence of N_{org} and HA). The effect of *C. longipes* on the utilization of HA was not statistically analyzed because the 3 replications did not provide sufficient number of degrees of freedom.

Spot tests of the presence of oxidative enzymes were performed according to Gramss *et al.* (1998). Three-week-old mycelia growing on 1.5 % MEA received one drop of guaiac gum, pyrogallol with 0.2 % H_2O_2 and *p*-cresol. Color change of colonies was evaluated after 3 and 24 h to establish the production of monophenol monooxygenase (EC 1.14.18.1; blue color), peroxidase (EC 1.11.1.7; brown color) and pyrocatechol oxidase (EC 1.10.3.1; yellow to red color).

RESULTS

After 21 d of incubation the mycelium of *C. longipes* formed lens-like pellets sized ≈2–4 mm in the medium; it grew also along the sides of the flasks. The pellets in samples without HA were distinctly paler than in the samples with HA.

Production of fungal biomass was enhanced in a system with organic nitrogen (F system) and in the presence of HA (Fig. 1). The positive effect of organic nitrogen and HA was statistically significant ($p < 0.05$). The shift in pH of the F system inoculated with *C. longipes* corresponded well with fungal enzymic activity and its biomass (Table I). The shift was less evident in F–N system.

Spot tests of the presence of monophenol monooxygenase and peroxidase were positive. After 1 d, mycelia and agar showed distinct color change on the spots where drops of the agent were added. Production of pyrocatechol oxidase was not detected as no color change appeared within 1 d.

Three peaks were observed on the gel-chromatographic graphs of the F samples (Fig. 2). As the A_{280} was used, they originated from compounds with carboxyphenols. The first peak with elution maximum

at 40 mL represented mainly fractions of HA and also proteins from malt extract (as seen in the control without HA). The second shoulder-like peak with elution maximum at 85 mL corresponded probably to oligopeptides. The third peak with elution maximum at 105 mL corresponded to amino acids from malt extract. An analogous peak with HA fractions was also observed in the F–N systems. Both the 2nd and 3rd peaks were nearly absent as there was no organic nitrogen originating from the medium; they being were formed by an unidentified aromatic compound. The 1st peak decreased in all variants within 21 d, as the fungus utilized HA (proteins) from media. Decolorization of the HA fraction reached 75 and 64 % in the F and F–N system, respectively. The A_4/A_6 ratio of HA significantly increased ($p < 0.05$). Both the 2nd and 3rd peaks in the F system decreased in all inoculated samples as the fungus utilized amino acids and oligopeptides. There was no difference in nitrogen uptake with or without HA, both peaks in the F–N systems being not changed.

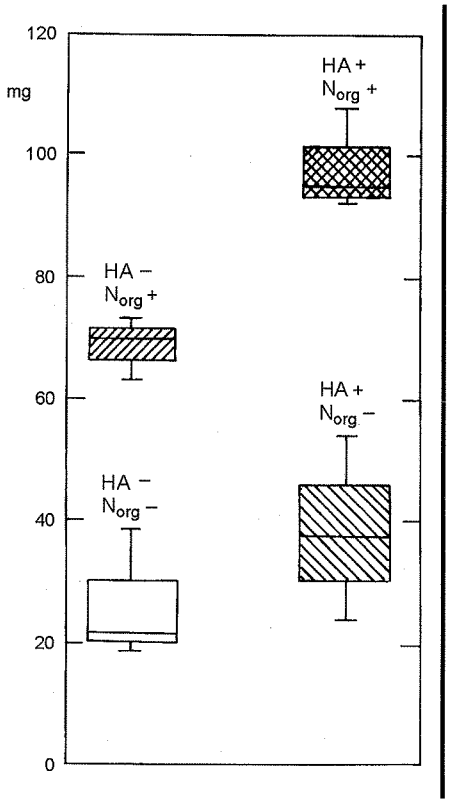

Fig. 1. The effect of N_{org} and HA on the growth of *Chalara longipes*; total fungal biomass per 50 mL media (mg) after 21 d of cultivation; *boxes* – 25–75 %, *horizontal lines* – median value.

DISCUSSION

The litter inhabiting species *C. longipes* representing the group of anamorphic ascomycetes (*Deuteromycotina*, mitosporic fungi) was found to belong to organisms capable of utilizing of spruce litter HA. This activity has so far been studied only in wood and litter decomposing basidiomycetes (Yanagi *et al.* 2002; Steffen *et al.* 2002). However, the ability of *C. longipes* seems to be higher that that previously found in basidiomycetes. The strain under study decolorized HA (present in the media) more effectively than the basidiomycetes *Coriolus consors*, *C. hirsutus* and *Lenzites betulina*; cf. Yanagi *et al.* (2002) who recorded 0–51 % decolorization of HA in visible spectra.

Ascomycetes have been largely overlooked in this respect but were intensively studied with regard to the melanin produced by dematiaceous mitosporic fungi, which is structurally similar to HS. This similarity was generally considered to have a direct bearing on HS formation. Several studies provided data establishing their structural relationship – similar UV/VIS and IR spectra, products of chemical degradation and also high persistence to microbial and chemical degradation (Filip *et al.* 1974, 1976; Valmaseda *et al.* 1989). However, NMR method showed also some differences between melanin and HA (Knicker *et al.* 1995). Existing results do not provide any consensus as to whether the well-humified soil organic

Table I. Decolorization by *Chalara longipes*, pH of the solution and A_4/A_6 ratio of humic acids (HA) from full strength (F) and organic nitrogen-free (F–N) system after 21 d of incubation[a]

Treatment	Decolorization, %	pH[b]	A_4/A_6 ratio
C. longipes F	0	3.9	–
control	0	6.5	–
C. longipes F + HA	75	4.0	5.5
control	0	6.4	4.6
C. longipes F–N	0	4.6	–
control	0	6.5	–
C. longipes F–N + HA	64	3.4	4.5
control	0	6.4	4.0

[a]Means of 3 replications. [b]Initial value 7.0.

matter is to a large extent of microbial origin (Russell *et al.* 1983) or if a small fraction originates from fungal melanin (Malik *et al.* 1982). Zavgorodnyaya *et al.* (2002) assumed that melanin of mitosporic fungal species must undergo a substantial transformation affecting its chemical properties if it contributes to the stable soil fraction.

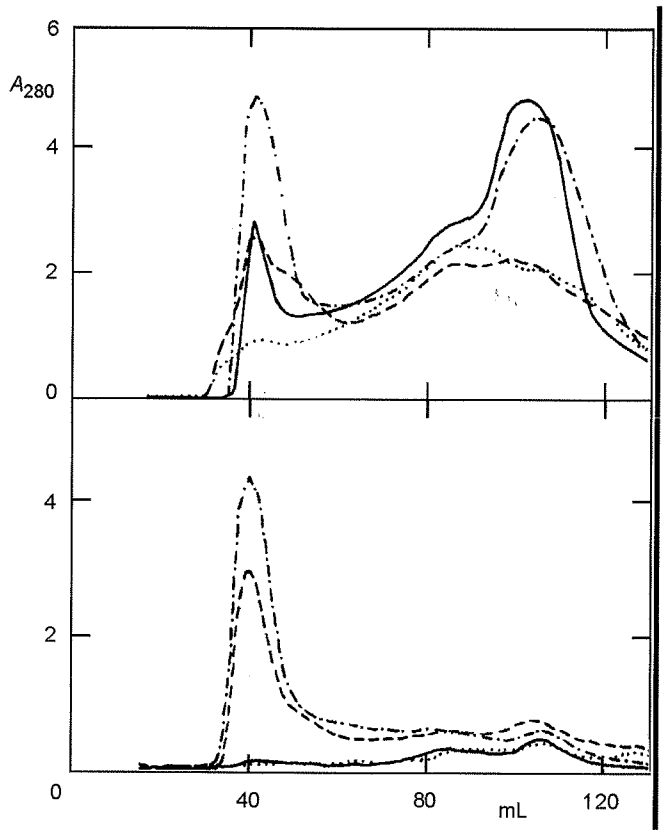

Fig. 2. Molar mass distribution (absorbance A_{280}) of humic acids (HA) after utilization by *C. longipes* (*C.l.*) in a full broth system (F) (*top*) and in a system without N_{org} (F–N) (*bottom*); gel-permeation chromatography (Sephadex G-25; elution volume, mL), mean of three replications (C – control); *top*: *dashed line* – *C.l.* F + HA, *dotted line* – *C.l.* F, *dot-and-dashed line* – C F + HA, *full line* – C F; *bottom*: *dashed line* – *C.l.* F–N + HA, *dotted line* – *C.l.* F–N, *dot-and-dashed line* – C F–N + HA, *full line* – C F–N.

So far only several studies have tested the effect of N_{org} deficiency on the growth of saprotrophic fungi. The essential importance of N_{org} for ectomycorrhizal fungi is widely accepted (Chalot and Brun 1998). Our study suggests that the presence of N_{org} is essential for the growth of *C. longipes* which was demonstrated in conditions with NH_4NO_3, as the only source of N where the fungal growth was strongly inhibited. This result is also in agreement with a study of *Pleurotus ostreatus* liquid fermentation where the lack of N_{org} had a significant negative effect on the fungal biomass and production of xylan endo-1,3-β-xylosidase (xylanase; 3.2.1.32) (Qinnghe *et al.* 2003), and with the study of *Agaricus bisporus*, where ammonium as the N source inhibited the production of glutamate–ammonia ligase (glutamine synthetase; EC 6.3.1.2) (Kersten *et al.* 1999). However, the production of fungal biomass in the broth amended with HA was significantly higher than in pure nutrition broth; this suggests that HA may have a positive effect on the growth and biomass production of *C. longipes*. Enhancement of fungal growth but not complete substitution of N_{org} source by HA utilization can be therefore anticipated as the process of HA utilization was more effective in full broth medium. The lack of N_{org} may probably also prevent the germination of spores. The growth of *C. longipes* in F–N system was limited to the growth of mycelial particles from inoculum.

The Sephadex dextran gel is widely used for separation based on molecular size. Sephadex G-25 Fine was selected because it effectively separated compounds from the media to 2 distinct fractions (Figs 1 and 2). The former contained high-molar-mass compounds (HA and proteins) and the latter low-molar-mass compounds (amino acids). This explained the parallel changes of HA and media content, nevertheless further separation within these fractions was not achieved. On the other hand, the molar-mass changes within the first fraction may be estimated using the A_4/A_6 ratio. Chen *et al.* (1977) showed that this ratio best correlated with the molar mass and also other structural characteristics of HA. If this relation is accepted, then the increase in the ratio during incubation in this study suggests decreasing the molar mass of HA (Table I). According to Chen *et al.* (1977) the A_4/A_6 ratio is not dependent on the content of HA and the decrease of HA content during the incubation period therefore did not affect the ratio.

The decrease of the high-molar-mass fraction after 21 d is in contrast with the observation of Zavgorodnyaya *et al.* (2002) who studied degradation of HA and fungal melanins by chernozem microbial population. They recorded a distinct increase of high-molar-mass compound fraction after 16 d, as some products of fungal metabolism had been incorporated in the suspension, followed by a decrease slightly below the initial level as these products were utilized by the fungi. In our study the high-molar-mass fraction content distinctly decreased as the HA and proteins from the media were almost fully utilized and no compounds were probably present in the media.

A simple model of fungal degradation of HA may be proposed to account for our results. When we accept the supramolecular view of HA assumed by Picollo (2002), their degradation by *C. longipes* may be promoted by the production of organic acids by the fungus into the suspension. This decreases considerably the pH. At low pH the supramolecular structure of HA is disrupted by the amphiphilic effect of organic acids and small units of HA are released. These units are utilized by the fungus – either accumulated in mycelium or degraded by oxidative enzymes such as monophenol monooxygenase or peroxidase.

The following questions remain:

(*1*) Is the ability to utilize the HA common within the group of saprotrophic ascomycetes?
(*2*) What is the fate of HA utilized by the fungus and what nutritional benefits does the consumption of HA bring to *C. longipes*?

Valuable information on the direct effect of fungal enzymes on the structure of HA units can be obtained using methods such as ^{13}C-NMR or HPLC.

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