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Nitrite tolerance of different ectomycorrhizal and wood- and litter-decomposing fungi

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The effect of nitrite (NO_2-) on the *in vitro* radial growth of 10 ectomycorrhizal and 11 wood- and 19 litter-decomposing fungi was studied by cultivating mycelia on malt extract (ME) agar supplemented with different amounts of $NaNO_2$. Nitrite tolerance differed very much between fungus species. *Piptoporus betulinus* and *Collybia butyracea* were the only fungi, which were not able to grow on NO_2-media, while a mycorrhizal fungus *Cenococcum geophilum* as well as a litter-decomposing fungus *Coprinus cinereus* grew even at 2500 mg l^{-1} $NaNO_2$. Generally, the litter-decomposing fungi thriving in gardens, dung and other nitrogen (N) rich places were the most NO_2-tolerant species which shows that they have adapted to continuous high supply of nitrogen. The possible mechanisms involving in NO_2-tolerance are discussed.

Key words: Nitrite (NO_2-), tolerance, ectomycorrhizal fungi, wood and litter decomposing fungi.

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Introduction

Nitrite (NO_2-) is an intermediate product in the conversion of ammonium (NH_4+) to nitrate (NO_3-), of NO_3- to nitrogen gas (N_2) in soils and of NO_3- to NH_4+ in plants and fungi. Owing to faster oxidation of NO_2- to NO_3- than of NH_4+ to NO_2-, accumulation of NO_2- is limited in soil, and its concentration does not normally exceed 1 mg g^{-1} soil (Haynes and Sherlock 1986). Concentration of NO_2- may, however, dramatically increase as a result of fertilization with urea (Löffler et al. 1986, Monaghan & Barrachloug 1992) or inorganic N (Jones & Schwab 1993, Burns et al. 1995) or of industrial N deposition as well. According to Bingham et al. (1954) concentration of NO_2- above 50 µg g^{-1} in the root zone produces toxicity symptoms. In acidic conditions, such as in Nordic forest soils, toxic level can be reached already at much lower concentration due to formation of uncharged nitrous acid (Lee 1979, Zsolbas et al. 1993).

Reduction to NH_4+ and further incorporation to organic form is a prerequisite for utilization of NO_2- in organisms. Reduction of NO_2- to NH_4+ is catalyzed by nitrite reductase enzyme (NiR) which has been found both in chlorophyllous and non-chlorophyllous parts of plants (e.g. Duncanson et al. 1992, Wray 1993, Seith et al 1994). Reduction of NO_2- in fungi has not been investigated as widely as in plants, and the studies have mainly concentrated on lower fungi, such as *Neurospora crassa* (Lafferty et al. 1974, Greenbaum et al. 1978, Exley et al. 1993) and *Candida nitratophila* (Al Kubisi et al. 1996). Reduction of NO_2- has also been observed in some wood-decomposing fungi (Gundersen 1967) as well as in an ectomycorrhizal fungus *Hebeloma*

cylindrosporum (Plassard et al. 1984). As high concentrations NO_2- is known to be toxic to fungi (Löffler et al. 1986), and therefore it has been used to inhibit infection of, for example, *Heterobasiodion annosum* in tree stumps (Gundersen 1967, Schönhar 1997).

In order to obtain more information about NO_2- tolerance among higher fungi, we investigated the ability of both ectomycorrhizal and wood- and litter-decomposing fungi to grow *in vitro* on the media with different concentrations of NO_2-.

Table 1. Fungal species used in the study.

Species
Mycorrhizal fungi
Amanita muscaria (L. : Fr.) Hook.
Amanita rubescens (Pers. : Fr.) S.F. Gray
Cenococcum geophilum (Sow.) Ferd. & Winge
Hebeloma sp.
Leccinum scabrum (Bull. : Fr.) S.F. Gray
Paxillus involutus (Batsch. : Fr.) Fr.
Suillus granulatus (L. : Fr.) Roussel
Suillus luteus (L. : Fr.) Roussel
Suillus variegatus (Sw. : Fr.) O. Kuntze
Tricholoma album (Fr.) Kumm.
Wood-decomposing fungi
Fomitopsis pinicola (Sw. : Fr.) P. Karst.
Hypholoma fasciculare (Huds. : Fr.) Kumm.
Hypholoma lateritium (Scaeff. : Fr.) Schroet.
Ischnoderma benzoinum (Wahlenb. : Fr.) P. Karst.
Kuehneromyces mutabilis (Schaeff. : Fr.) Sing. & Smith
Lentinus edodes (Berk.) Sing.
Lentinus lepideus (Fr. : Fr.) Fr.
Piptoporus betulinus (Bull. : Fr.) P. Karst.
Pleurotus pulmonarius (Fr.) Quel.
Stereum sanguinolentum (Alb. & Schw.) Fr.
Trametes versicolor (L. : Fr.) Pilát
Litter-decomposing fungi
Agrocybe molesta (Lasch) Sing.
Coprinus bisporus Lange
Coprinus cinereus (Schaeff. : Fr.) S.F. Gray
Coprinus disseminatus (Pers. : Fr.) S.F. Gray
Coprinus heptemerus M. Lange & A.H. Smith
Coprinus micaceus A (Bull. : Fr.) Fr.
Coprinus micaceus B (Bull. : Fr.) Fr.
Coprinus tuberosus Quel.
Cyathus striatus (Hudson : Pers.) Willd.
Collybia butyracea (Bull. : Fr.) Kumm.
Leucoagaricus cretaceus (Bull. : Fr.) Moser
Marasmius androsaceus (L. : Fr.) Fr
Marasmius bulliardii Quel.
Marasmius scorodonius (Fr. : Fr.) Fr.
Mycena galopus (Pers. : Fr.) Kumm.
Mycena vulgaris (Pers. : Fr.) Kumm.
Mycena vitilis (Fr.) Quel.
Micromphale perforans (Fr.) Sing.
Tephrocybe tylicolor (Fr.) Moser

Materials and methods

Fungal material

All the fungi of the present study were from the culture collection of Department of Plant Biology in University of Helsinki (Table 1). The fungi, except the genus *Coprinus*, have been isolated from surroundings of Helsinki by Dr. Veikko Hintikka. The *Coprinus* species have been isolated by Mr. Paavo Höijer, Porvoo. The fungi were cultivated on 1 % malt extract (ME) Difco or Melin Norkrans (MMN) (Marx 1969) agar slants at ca. +5 °C and were transferred to fresh media about twice a year.

Experimental design

The fungi were cultivated in Petri dishes containing 1 % ME (Difco), 1 % agar (Bacteriologique type A) and different amounts of $NaNO_2$ (Table 2).

Table 2. $NaNO_2$ concentrations and pH (H_2O) of the media at the beginning of the experiment.

$NaNO_2$ concentration (mg l^{-1})	pH
0	5.81
25	5.90
50	5.60
100	6.12
250	6.16
500	6.28
750	6.31
1000	6.32
2500	6.40

Filter-sterilized (Millipore, pore size 0.2 µm) $NaNO_2$ was added to ME agar after autoclaving. The pH (H_2O) of the media was determined before cultivation. It increased from pH 5.6 (50 mg l^{-1}) $NaNO_2$ to 6.4 (2500 mg l^{-1} $NaNO_2$, Table 2).

For the experiment, a mycelial plug, about 5 mm in diameter, was transferred to fresh medium and cultivated in the dark at 21 °C. The radial growth (mm) of the fungi was measured after the growing period of 2 (litter and wood decomposing fungi), 4 (ectomycorrhizal fungi, except *Suillus* spp.) or 7 (*Suillus* species) weeks.

Results

Responses to $NaNO_2$ differed remarkably between fungus species (Table 3). Although all ectomycorrhizal fungi were able to grow on the NO_2- media, as a group they were more sensitive to NO_2- than the wood- and litter-decomposing fungi. Within mycorrhizal fungi, *Amanita* species were the most sensitive. The growth of *A. rubescens* was almost completely inhibited even at the lowest $NaNO_2$ concentration (25 mg l^{-1} $NaNO_2$) and that of *A. muscaria* at 50 mg l^{-1} $NaNO_2$. *Cenococcum geophilum* was the exception among mycorrhizal fungi by growing at the highest concentration of $NaNO_2$ (2500 mg l^{-1}).

Wood-decomposing fungi formed a heterogenous group in NO_2- tolerance. *Piptoporus betulinus* was the only fungus, which was not able to grow on NO_2- media (Table 3). Because it grew quite well on the control medium (no added $NaNO_2$), mycelium was viable. *Hypholoma fasciculare* and *Trametes versicolor* were the most tolerant species by growing at 750 mg l^{-1} $NaNO_2$. Host plant of the decomposing fungus had no correlation with NO_2- tolerance of the species.

Generally, the litter-decomposing fungi growing in gardens, roadsides, dung and other N rich places tolerated NO_2- best. For example, all the *Coprinus* species were able to grow at 250 mg l^{-1} or at higher concentration of $NaNO_2$. *Coprinus cinereus* would have tolerated even higher concentration than 2500 mg l^{-1}, because on this medi-

Table 3. Ability of different ectomycorrhizal and wood and litter-decomposing fungi to grow on $NaNO_2$ agar media. The highest concentration (g l^{-1}), at which growth was still recorded.

$NaNO_2$-concentration	Ectomycorrhizal fungi[1]	Wood-decomposing fungi[2]	Litter-decomposing fungi[2]
0		*Piptoborus betulinus*	*Collybia butyracea*
25	*Amanita rubescens*		*Marasmius androsaceus*
50	*Amanita muscaria*	*Lentinus lepideus*	*Micronphale perforans*
	Paxillus involutus		*Mycena vitilis*
	Suillus variegatus		*Mycena calopus*
	Suillus granulatus		
100	*Leccinum scabrum*	*Ischnoderma benzoinum*	*Mycena vulgaris*
	Suillus luteus	*Kuehneromyces mutabilis*	
	Tricholoma album	*Lentinus edodes*	
250	*Hebeloma* sp.	*Hypholoma lateritium*	*Coprinus heptemerus*
		Stereum sanguinolentum	*Coprinus micaceus* A
			Coprinus tuberosus
			Cyanthus striatus
			Marasmius bulliardii
			Marasmius scorodonius
500		*Pleurotus pulmonarius*	*Coprinus micaceus* B
		Fomitopsis pinicola	*Tephrocybe tylicolor*
750		*Hypholoma fasciculare*	*Agrocybe molesta*
		Trametes versicolor	*Coprinus bisporus*
			Coprinus disseminatus
1000			*Leucoagaricus cretaceus*
2500	*Cenococcum geophilum*		*Coprinus cinereus*

[1] 4 weeks, except *Suillus* species 7 weeks, after inoculation.
[2] 2 weeks after inoculation.

um mycelium covered half of the Petri dish. Fungi decomposing needle litter were sensitive to NO_2-; *Collybia butyracea* was not able to grow on NO_2- media at all.

Discussion

Although NO_2- plays an important role in N metabolism in both plants and fungi, as high concentrations it is a ion of possible toxicity. It can inhibit the uptake of some important cations (Zolbos et. al. 1993) and destroy both DNA and RNA by deaminating purine rings. Inhibitory effects of NO_2- are also related to its similarity to a sulphite ion (Pateman & Kinghorn 1976).

Ability to tolerate inorganic N, NO_2- included, varies greatly between fungus species. This has been observed as changes in fungal population in areas polluted by N originating from industry and agriculture (e.g. Ohenoja 1978, Brandrud 1995, Holopainen et. al 1996). In the study of Holopainen et al. (1996), ectomycorrhizal *Suillus*- and *Piloderma*-species sensitive to NO_3- and NH_4+ were replaced by more tolerant species *Cenococcum geophilum* and *Paxillus involutus* in the soil near a pulp mill in eastern Finland. In the case of *Suillus* species and *Cenococcum geophilum,* our results *in vitro* are in agreement with those obtained by Holopainen et al. (1996). In the present study, the *Paxillus involutus* strain was, however, as sensitive to NO_2- as *Suillus* species. Arnebrant (1994) observed great difference in N tolerance between two *Paxillus involutus* strains which shows that in addition to variation between different species, ability to use and tolerate N may vary remarkably within species. Therefore, it is difficult to make generalizations for species.

Because concentration of N in wood is low, wood-decomposing fungi would be expected to be sensitive to NO_2-. However, Gundersen (1967) found NO_2- tolerant species and strains by cultivating mycelia on sterilized pine stem disk. In his study, all the fungi tested were able to grow even at $NaNO_2$ concentration of 1000 mg l^{-1}, although the growth of, for example, *Heterobasidion annosum*, *Fomitopsis pinicola* and three *Polyporus* species was reduced significantly. The growth of *Stereum sanguinolentum* was still the same as on the control medium. Wood-decomposing fungi were, however, 10–20 times more sensitive to NO_2- than microfungi, such as some *Penicillium* and *Trichoderma* species. In the present study, *Fomitopsis pinicola* tolerated NO_2- better than *Stereum sanguinolentum*, but it was not able to grow at as high NO_2- concentration as reported by Gundersen (1967).

The concentration of N in deciduous leaves is usually higher than that in needles of conifers (Berg & Cortina 1995). Therefore, the decomposing fungi of deciduous leaves might tolerate higher N concentration than fungi decaying needles. In our *in vitro* study of NO_2- tolerance, this tendency was observed. The most tolerant fungi were, however, the species decomposing litter in gardens, dumps and roadsides rich in N. For example, the growth of most *Coprinus* species occurring in NH_4+ - and urea-rich conditions in nature, where accumulation of NO_2- is obvious, was inhibited only at very high concentrations of $NaNO_2$.

Although synthesis and regulation of NiR enzyme is important in adaptation to high concentrations of NO_2-, tolerance to NO_2- in fungi depends on several mechanisms. Some plants have been observed to oxidize NO_2- to NO_3- and in this way avoid accumulation of NO_2- (Funkhouser et al. 1981, Aslam et al. 1989). In addition, NO_3- is the ion inducing NiR enzyme (Aslam et al. 1989; Wray 1993). Fungi might have the same kind of system first to induce oxidation of NO_2- and then to increase activity of NiR with NO_3- formed. Nitrogen may be also stored and released gradually. Deposits rich in N have been found in vacuoles of ectomycorrhizal fungus *Cenococcum geophilum* after N-treatment (Kottke et al. 1995). Amino acids and proteins have been suggested to be predominant N forms in these deposits (Wallenda & Kottke 1998), but they might also contain tiny amounts of inorganic N. On the other hand, Choudary (1993) has suggested that the yeasts tolerating high concentrations of inorganic N keep the ion in a transient state until they are able to change it to a less toxic form.

Plassard et al. (1984) showed that nitrite in medium induced NiR activity in the mycelium of *Hebeloma* cylindrosporum. Induction depended, however, on pH of the media. Acidity has an important effect on both growth and N metabolism in fungi: acidic conditions increase toxicity of NO_2- (Gundersen 1967, Plassard et al. 1984, Chang & Chung 1988). The decrease of pH from 6 to 4 seems to be most critical. In our study,

$NaNO_2$ increased pH of the agar media which might compensate some toxic effects of NO_2-. On the other hand, before toxic concentrations of NO_2-, too high pH (over 6) might inhibit the growth of some ectomycorrhizal fungi originally isolated from acid soil.

Nitrous acid, formed from NO_2- in acid conditions, is known to react with phenolic compounds. This reaction has been suggested as a possible mechanism for gaseous N loss from acid soil (Christianson et al. 1979, Smith & Chalk 1980). The amount of phenolic compounds, especially tannins, have been shown to increase in plants after different stress conditions (Briggs 1991, Holopainen et al. 1996), but the importance of them during N stress is still unclear. The formation of phenols could be some kind of safety measure to prevent toxic effects of nitrous acid or NO_2-. The possibility that phenolic compounds could prevent N accumulation in cells should be investigated.

Our study shows that although *in vitro* conditions simplify responses, relationship between N concentration in the growth site of the fungus and NO_2- tolerance *in vitro* can be found. Because only one strain of a species was tested and because variation within species is known to be great, generalization of tolerance is, however, difficult. Therefore, it would be important to test more strains originating from different areas.

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