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Short communication

# Composition of the organic phosphorus fraction in basidiocarps of saprotrophic and mycorrhizal fungi

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ABSTRACT

In our screening, we aimed to detect phosphonates and other forms of organic phosphorus in basidiocarps and vegetative mycelia of six common basidiomycetes. Organic phosphorus-containing compounds were extracted in alkali and analysed using $^{31}$P NMR. Monoesters, diesters, pyrophosphates and polyphosphates detected in high amounts reflected the high metabolic activity in basidiocarps (growth, production of basidiospores). Phosphonates were present in all samples, in concentrations ranging from 14 mg kg$^{-1}$ of the extracted phosphorus in *Boletus badius* basidiocarp to 140 mg kg$^{-1}$ in *Amanita muscaria* vegetative mycelium. Detection of phosphonates in basidiocarps together with our previous evidence from laboratory experiments support the fungal production of natural phosphonates in forest ecosystems.



Phosphonates are remarkable organic compounds with direct covalent bonding of carbon and phosphorus atoms, and have been detected in living bacteria, protozoans, snails, stramenopiles (including oomycetes *Phytophthora infestans* and *Pythium prolatum*), invertebrates and vertebrates (including humans) (Wassef and Hendrix, 1977; Hilderbrand and Henderson, 1983; Creamer and Bostock, 1986). No phosphonates have been found in plants so far. Phosphonates are present most often in the form of phosphonolipids forming membrane components (Hilderbrand and Henderson, 1983). Other forms of phosphonates include peptide derivatives, e.g., the natural herbicide bialaphos (a tripeptide containing a C–P–C bond) produced by the actinomycete *Streptomyces hygroscopicus* (Hidaka et al., 1990).

Simple synthetic phosphonates are frequently used as fungicides (Guest and Grant, 1991). In natural ecosystems, these phosphonate-based fungicides together with herbicides and detergents may become toxic xenobiotics. Substantial attention has been paid to the screening of organisms that can degrade them; apart from bacteria, several soil anamorphic ascomycetes are able to degrade synthetic phosphonates (Kononova and Nesmeyanova, 2002). This degradation mainly occurs due to the activity of bacterial phosphonoacetate hydrolase (Ternan et al., 1998) and a very similar fungal enzyme (Klimek-Ochab et al., 2003; Forlani et al., 2006).

Natural phosphonates are frequently found in environmental samples. They were detected in grassland soils (Tate and Newman, 1982; Turner et al., 2003a,b; McDowell et al., 2007), in birch forest litter (Turner et al., 2004) and in litter under *Thuja plicata* (Cade-Menun et al., 2001). Their origin in soils has generally been attributed to "microbial activity", and direct contribution of fungi to the phosphonate fraction has not yet been considered. In our previous studies, we detected phosphonates in spruce (*Picea abies*) litter needles colonized by four saprotrophic fungal strains. No phosphonates were present in uncolonized control needles (Novák et al., 2005b; Koukol et al., 2006). To extend our knowledge of naturally occurring phosphonates in autochthonous mycoflora of temperate spruce forests, we have examined phosphonates in basidiocarps. Saprotrophic and ectomycorrhizal basidiomycetes were selected because these two ecological groups of fungi play a pivotal role in phosphorus transformation in coniferous forests (Cairney, 2005). Six fungal species were collected, representing common wood and litter decomposers and mycorrhizal fungi. Alkaline phosphorus extracts were analysed by $^{31}$P NMR spectroscopy to examine differences in the distribution of phosphorus compounds within the basidiocarps, with emphasis on the phosphonates.

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Collections were performed at well-preserved temperate coniferous forests dominated by pine (*Pinus sylvestris*) or spruce (*P. abies*) in September and October of 2005 and 2006. Collections included the following species, found at the indicated localities: *Amanita muscaria*, *Boletus badius*, and *Boletus chrysenteron* in the Blanský les Protected Landscape Area (Czech Republic); *Gymnopus acervatus* and *Thelephora terrestris* in the Bohemian Forest, Šumava National Park (Czech Republic); and *Setulipes androsaceus* near Ahvenlammi Lake (Finland). Undamaged, fresh-looking basidiocarps were collected to provide 2–20 g (dry weight) for the extraction. This amount originated from approximately 800 basidiocarps of *S. androsaceus* or 5–10 basidiocarps of the other species. Litter needles that appeared to have been colonized by the mycelium of *A. muscaria* and *B. badius* were sampled as well. Litter needles and basidiocarps were sliced on the day of collection and dried at 60 °C until constant weight, then homogenized in a laboratory grinder. Powdered samples were decalcified using 0.1 M HCl and extracted in alkali (0.1 M NaOH). The pH of the alkaline extract was reduced to 9.0 to prevent hydrolysis of phosphorus. Extracts were concentrated and washed using distilled water in an Amicon ultrafiltration cell with a YM1 membrane. The concentrated and desalted extract was freeze-dried (Novák et al., 2005a). The filtrate was concentrated and dried to measure phosphorus loss during the extraction procedure.

The $^{31}$P NMR spectra of extracts diluted in 0.1 M NaOD were recorded on a Bruker Avance DRX 500 spectrometer operating at a 202.45 MHz frequency using the following parameters: recycle delay of 2 s, number of scans 10 000, broad-band decoupling and temperature 298 K. The FID was processed with line broadening of 10 Hz, chemical shifts were measured relatively to an external standard (85% $H_3PO_4$). Quantitative analysis was achieved using instrumental integration of NMR peaks; the content of individual phosphorus forms was expressed relatively to the extracted phosphorus. The phosphorus content in extracts from basidiocarps was of the same order among all fungal species (Table 1).

The extraction method used was primarily developed for soil organic phosphorus, which is well preserved against hydrolysis and filtration loss by humified organic matter, namely humic acids (Novák et al., 2005a). The treatment of basidiocarps and alkaline extracts was accompanied by a 50% loss of phosphorus during concentration on the nanofilter. Using $^{31}$P NMR spectroscopy, we determined that the filtrate contained no other phosphorus forms than the retentate. Phosphate monoesters and orthophosphates dominated in the filtrates, with some fraction of these forms likely originating from hydrolysed phosphorus-containing compounds (Turner et al., 2003b). Phosphonates were present at similar concentrations in both the filtrates and the retentate. Nevertheless, the advantage of this extraction method is the high quality $^{31}$P NMR spectrum with good resolution and clear minor signals (Fig. 1).

Phosphate monoesters include various chemical substances playing specific roles in cell growth and development (e.g., cAMP, inositol phosphates, phosphorylated sugars). Their presence in basidiocarps was expected due to intensive growth and production of basidiospores.

Phosphate diesters, represented namely by DNA with its signal around 0 ppm, were the second most abundant phosphorus form. Less stabile RNA hydrolysed during the extraction (Turner et al., 2003a). Polyphosphates representing typical fungal phosphorus storage compounds were present in all basidiocarps. Several differences, such as high concentrations of phosphate diesters detected in the basidiocarp of *B. chrysenteron* or low content of polyphosphates in the basidiocarp of *A. muscaria* can be explained by the different age of basidiocarps and different humidity at the time of sampling rather than by fungal species.

Phosphonates were detected in all basidiocarp samples and the substrates colonized with their respective mycelia (Table 1). The highest phosphonate content was recorded in the extract from the vegetative mycelium of *A. muscaria* (140 mg kg$^{-1}$ of extracted phosphorus).

In particular, the presence of phosphonates in the basidiocarps of *S. androsaceus* confirms the findings in our previous laboratory experiments, where spruce litter needles colonized separately with two strains of *S. androsaceus* contained phosphonates, but uncolonized control needles did not (Novák et al., 2005b; Koukol et al., 2006).

In our previous study, phosphonates were detected in alkaline extracts from spruce litter needles colonized by the ascomycetes *Ceuthospora pinastri*, *Mollisia minutella* and *Scleroconidioma sphagnicola* (Koukol et al., 2006). No phosphonates, however, were detected in the extract from the needles colonized by *Penicillium* cf.

**Table 1**
Extracted phosphorus (P) content and P forms in alkaline extracts from basidiocarps and vegetative mycelia of six mycorrhizal and saprotrophic fungal species analysed by $^{31}$P NMR

| Fungal species – sample description | Extracted P (mg kg$^{-1}$ dry mass) | P forms, concentration of extracted P (mg kg$^{-1}$) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Phosphonates | Orthophosphate | P monoesters | Phospholipids | P diesters | Pyro- and polyphosphates |
| | | 15–20 ppm | 5.5–7 ppm | 3–5.5 ppm | 1.5–3 ppm | −2 to 1.5 ppm | −23 to −3.5 ppm |
| *Mycorrhizal fungi* | | | | | | | |
| *B. badius*[a] | | | | | | | |
| Basidiocarp | 10 700 | 14 | 384 | 6478 | 45 | 2122 | 1670 |
| Litter + mycelium | 1700 | 39 | 105 | 459 | 103 | 659 | 311 |
| *A. muscaria* | | | | | | | |
| Basidiocarp | 5900 | 26 | 3046 | 544 | 196 | 1904 | 148 |
| Litter + mycelium | 3200 | 140 | 679 | 846 | 130 | 1014 | 367 |
| *B. chrysenteron* | | | | | | | |
| Basidiocarp | 8300 | 123 | 2940 | 1715 | 722 | 2042 | 708 |
| *T. terrestris* | | | | | | | |
| Basidiocarp | 7200 | 31 | 1179 | 2216 | 51 | 2175 | 1535 |
| *Saprotrophic fungi* | | | | | | | |
| *S. androsaceus* | | | | | | | |
| Basidiocarp | 6500 | 23 | 2974 | 1045 | 47 | 1824 | 628 |
| *G. acervatus* | | | | | | | |
| Basidiocarp | 8900 | 79 | 1634 | 2428 | 34 | 3403 | 1362 |

[a] Data partially from Novák et al. (2006).