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Early spring mycobiota of pine litter needles

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Rise in air temperature accompanied by snow-melting in early spring may affect fungi colonising litter needles. This study dealt with the diversity of communities of culturable microfungi colonising Scots pine litter at localities still covered by snow and after snowmelt. Surface-sterilised needles were cultivated on malt agar under two temperature regimes (8/9 and 15/16 °C) and outgrowing fungi were isolated into pure cultures and identified based on morphology and analysis of ITS and partial 28S rDNA regions. Thirty-one fungal species were isolated in total. Dominant colonisers with a colonisation frequency of over 10 % included *Verticicladium trifidum*, *Ceuthospora pinastri*, *Sympodiella acicola* and an unidentified member of *Helotiales*. Two basidiomycetes, *Marasmius androsaceus* and *Mycena galopus*, were also isolated relatively frequently from needles with snow cover, but under different temperature regimes. This preference for different temperatures reflects different niches that these species occupy in coniferous litter. Species richness was significantly higher on needles cultivated at lower temperatures. Widespread use of room temperature for cultivation of samples even from cold seasons may underestimate fungal diversity.

Key words: fungal diversity, fungal communities, microfungi, *Pinus sylvestris*.

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Nárůst teplot doprovázený táním sněhu na jaře může ovlivnit houby kolonizující jehlice v opadu. Tato studie se zaměřila na diverzitu společenstva kultivovatelných mikroskopických hub kolonizujících opad borovice lesní na lokalitách dosud pokrytých sněhem a na lokalitách s odtátým sněhem. Povrchově sterilizované jehlice byly kultivovány na sladinovém agaru za dvou teplotních režimů (8/9 a 15/16 °C). Vyrůstající houby byly izolovány do čistých kultur a identifikovány na základě morfologie a analýzy ITS a 28S oblastí rDNA. Celkem bylo izolováno třicet jedna druhů. Dominantními kolonizátory s kolonizační frekvencí nad 10 % byly druhy *Verticicladium trifidum*, *Ceuthospora pinastri*, *Sympodiella acicola* a neurčený zástupce řádu *Helotiales*. Dva bazidiomycety, *Marasmius androsaceus* a *Mycena galopus*, byly izolovány rovněž relativně často z jehlic pod sněhem, ale v odlišných teplotních režimech. Tato preference pro různou teplotu odráží rozdílné niky těchto dvou druhů v jehličnatém opadu. Druhová bohatost byla signifikantně vyšší na jehlicích kultivovaných při nižších teplotách. Rozšířené použití pokojové teploty pro kultivaci vzorků dokonce i ze zimních sezón může podhodnotit diverzitu hub.

INTRODUCTION

Soil in temperate coniferous forests is seasonally covered by snow for varying lengths of time. Numerous fungi benefit from the microclimatic conditions under the snow cover. So-called "snow moulds" are taxonomically diverse plant parasites. Members of this group belong to the *Xylariales* [*Monographella nivalis* (Schaffnit) E. Müll.], *Helotiales* (*Sclerotinia* spp.) and *Agaricales* [*Typhula* spp., *Coprinopsis psychromorbida* (Redhead & Traquair) Redhead, Vilgalys & Moncalvo] (Matsumoto 2009). These fungi mostly attack plants in grass-dominated ecosystems, but some species parasitise also on pine trees, e.g. *Herpotrichia juniperi* (Duby) Petrak and *Neopeckia coulteri* (Peck) Sacc. (Bazzingher 1976). Schmidt et al. (2008) mentioned among "snow moulds" also members of the *Mucorales* and *Mortierellales* that ephemerally colonised soil and rapidly disappeared after snowmelt in the subalpine pine forests of Colorado. Besides these specialised fungi, various psychrotolerant species survive under snow cover and thrive after snowmelt. Zinger et al. (2009) found that fungal diversity in arctic and alpine ecosystems was enhanced at localities with late snowmelt covered for a prolonged period compared to early snowmelt localities with patchy snow cover. Schadt et al. (2003) observed only a minor shift in fungal communities in tundra soil between winter and spring (connected with snowmelt) compared to a substantial shift from spring to summer (caused by drier and warmer conditions).

Since the 1960s, diversity and succession of culturable fungi in pine litter have been studied intensively in all seasons to determine seasonal changes in the mycobiota. In areas with substantial seasonal fluctuations in temperature and precipitation, litter needles were frequently cultivated at room temperature, which is not strictly defined but usually ranges from 20 to 25 °C (Hayes 1965, Mitchell & Millar 1978, Tokumasu et al. 1994, Tokumasu 1998). Brandsberg (1969) cultured needles also at 4 and 15 °C and found that *Beauveria bassiana* (Bals.) Vuill. and *Oidiodendron tenuissimum* (Peck) S. Hughes isolated in the winter months were able to grow and sporulate at lower temperatures. Tokumasu (1996) cultivated one set of needles sampled in winter at 4 °C. He recorded a smaller number of species, all of them also recorded from needles cultivated at room temperature. In his later study, Tokumasu (1998) supposed that the drop of temperature in late autumn negatively affects fungal colonisation of litter needles during the winter months. In his opinion, fungal colonisation of freshly fallen litter was hindered under snow cover and psychrotolerant species dominated in the short period after the snowmelt. These would-be psychrotolerant colonisers were not distinguished in his study, because needles originating from the first sampling after snowmelt (April) were cultivated at room temperature.

In this study, fungal communities colonising pine litter needles in early spring (March) were surveyed in an area occupied by Scots pine (*Pinus sylvestris* L.) forests on sandstone rocks and partly also in shaded valleys. The particular aim of this study was to compare diversity of culturable microfungi from two sites differing in snow cover and to determine the effect of cultivation temperature on the observed diversity.

MATERIALS AND METHODS

Litter needles were sampled at eight sites (at two localities) in the Bohemian Switzerland National Park (Babylon area) on March 25, 2010. Four sites without snow cover were selected on a chine of a sandstone rock, with continuous sunlight. Four sites with patches of snow (5–10 cm thick layer) were situated on shaded slopes of the hills. The localities were less than 300 m apart. The forest at these localities is dominated by *Pinus sylvestris* with interspersed spruce (*Picea abies* (L.) Karst.), and the herb layer is composed mainly of pine and spruce seedlings. The soil is sandy and very shallow (10–45 cm) and the humus form is a typical mor. In April 2007, a temperature data logger (Minikin TT, EMS Brno, Czech Republic) was buried into litter needles (2 cm deep) at a nearby locality and the daily mean temperatures were recorded for 1 year.

Needles from the uppermost litter layer were sampled into sterile plastic bags and processed in the laboratory within 24 h. Individual needle pairs were cut with scissors and a 2 cm long fragment from the middle part of the needle was surface-sterilised in 30% hydrogen peroxide for 90 s on a horizontal shaker and then placed in a Petri dish (10 fragments per dish) with 2° malt agar prepared from brewer's wort with a final sucrose content adjusted to 2% w/v and with 18 $g \cdot L^{-1}$ agar (2MA, Fassatiová 1986). Petri dishes were cultivated under two different temperature regimes: 8/9 °C and 15/16 °C (with 16/8 h cycles) in the dark. The different temperature regimes simulated the abrupt shift in winter/spring temperature conditions. Twenty fragments were cultivated for each sampling site and temperature regime, including altogether 320 needles.

Outgrowing fungi were directly identified based on morphology of sporulating colonies. If sterile colonies were formed, they were grouped into morphotypes according to physiological and morphological characteristics (e.g. growth rate, colour and macroscopic characters of colonies). A representative strain of each morphotype was transferred into a new Petri dish. Sterile cultures were kept on nutrient-poor medium potato carrot agar (Fassatiová 1986) for 6 months at a lowered temperature (18/19 °C day/night regime) combined with near-ultraviolet light (UVA) to promote sporulation.

In order to identify sterile strains, genomic DNA was isolated from 7–14 day old cultures using a Zymo Research Fungal/Bacterial kit (Zymo Research, Orange, USA). Nuclear rDNA containing internal transcribed spacers (ITS1 and ITS2), 5.8S and D1/D2 domains of the 28S region were amplified with primer sets ITS1/NL4 or ITS1/ITS4 (White et al. 1990, O'Donnell 1993). PCR products were purified using GenElute PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, USA). The same primers were used for sequencing (Macrogen Inc., Seoul, Korea).

Sequences were compared for homology in GenBank using the BLAST algorithm. Only 99–100% matches with reliable sources (ex-type sequences, taxonomic studies) were accepted as proofs of identification.

Abundances of each species (morphotype) were calculated as the number of needle fragments with colonies of a given morphotype. Abundances were joined for morphotypes later shown to be identical based on morphological or molecular data. Shannon-Wiener and Simpson indices were calculated using the Shannon-Wiener Diversity Index / Shannon Entropy Calculator (http://www.changbioscience.com/genetics/shannon.html). The statistical null hypothesis that the locality and temperature regime of cultivation are independent was tested using the Chi-square test. Differences in abundance of the most frequent species, species richness and diversity indices between localities and between temperature regimes were tested using the t-test. All analyses were performed in PAST (Hammer et al. 2001).

RESULTS

In total, the isolations from pine litter needles yielded 340 colonies belonging to 31 fungal species. Eighty-seven needles remained sterile. Twelve species sporulated in primary isolations or in pure cultures and were identified based on their morphology. No sporulation occurred after long-time cultivation of sterile strains at lowered temperature and under UV light, which were thus identified based on molecular data. Four sterile strains were identified at the genus level and the remaining 11 strains did not show similarity in their rDNA sequences higher than 95 % with any named GenBank record and were assigned to a higher taxonomic rank, such as family or order (Tab. 1). No saprotrophic fast-growing members of the *Mucorales* and *Mortierellales* mentioned by Schmidt et al. (2008) were retrieved in this study and neither any mycelial mats typical of these fungi were observed during sampling the litter surface.

The communities of culturable fungi were dominated by ascomycetes. Only three basidiomycetes were isolated, i.e. *Marasmius androsaceus* (L.) Fr., *Mycena galopus* (Pers.) P. Kumm. and an unidentified member of *Stereaceae*. The *Verticicladium trifidum* Preuss anamorph of *Desmazierella acicola* Lib. (*Pezizales*) and the hyphomycete *Sympodiella acicola* W.B. Kendr. (incertae

sedis) reached the overall highest colonisation frequency, 21.3 % and 19.1 %, respectively. The former species was more frequently isolated from the site without snow cover, but the result was not statistically significant (t-test, p=0.129). The abundance of *S. acicola* was not analysed, because this species appeared no sooner than after five months of cultivation of needles when part of Petri dishes was already discarded because of contamination by mites. Only two other species reached a frequency higher than 10 % – *Ceuthospora pinastri* (Fr.) Höhn., anamorph of *Phacidium lacerum* Fr. (*Helotiales*, 12.2 %), and another unidentified strain of the *Helotiales* labelled as *Helotiales* sp. 5 (11.6 %). Their abundance did not differ between the sites (t-test, p = 0.18 and p = 0.82, respectively). Nineteen species were recorded rarely, in less than five strains. Fungal communities retrieved under different temperature regimes were significantly associated with snow conditions at the sites (chi-square test, p = 0.025). The temperature regime was more important; species richness and Shannon-Wiener Diversity Index were significantly higher for fungal communities retrieved at lower temperature (t-test, p = 0.002 and p < 0.001, respectively).

DISCUSSION

Pine litter colonisers frequently recorded in the same area by Koukol (2007) during seasons without snow (*Verticicladium trifidum*, *Ceuthospora pinastri* and *Sympodiella acicola*) were the dominant species also in early spring collections at both localities in this study. *Verticicladium trifidum* and *Ceuthospora pinastri* showed somewhat higher colonisation frequency on needles without snow cover, but the result was not significant. For the latter species this is rather unexpected, because the teleomorph *Phacidium lacerum* parasitises on pine after snow melt and causes so-called snow blight (Hansson 2006). Tokumasu et al. (1994) recorded also *Verticicladium trifidum* as a dominant species on *Pinus sylvestris* needles in Germany throughout the year. Brandsberg (1969) found that the most commonly isolated species from litter of *Pinus monticola* Dougl. and *Pinus ponderosa* Dougl. in the USA showed limited seasonality. Fungal mycelia of interior colonisers most probably survived overwinter and began to grow immediately after the increase in temperature. Similarly, Mühlmann & Peintner (2008) observed no significant seasonal changes in the community of ectomycorrhizal fungi in roots of *Salix herbacea* L. in the Alps, where samples were taken also from the soil under snow cover.

Temperature drop may not be the only cause of lower fungal abundance. Schmidt et al. (2008) suggested that soils may become anoxic during the final stages of snowmelt. This could explain lower fungal richness at sites with snow recorded in this study. The litter layers on localities without snow were still humid enough at the time of sampling and therefore humidity was probably not a limiting factor.

Tab. 1. Fungal species isolated from Scots pine needles in March 2011, their putative identification and colonisation frequency from needles sampled at sites with/without snow cover and cultivated under a lower (8/9 °C) and a higher temperature regime (15/16 °C).
[1] only sterile morphotypes were sequenced and representative sequences were included in GenBank.
[2] colonisation frequency of each species (morphotype) was calculated as the number of needle fragments with colonies of a given morphotype divided by the total number of needles (320).
ND – identification was not carried out due to fungal growth cessation.
* Petri dishes with primary isolates were discarded because of contamination by mites prior to determination of the colonisation frequency of *S. acicola*.

Fungal identification	**Order**	**GenBank Acc. Nr.** [1]	**Colonisation frequency**[2]			
			without snow		**with snow**	
			9 °C	**15 °C**	**9 °C**	**15 °C**
Verticicladium trifidum	*Pezizales*	–	7.5	7.2	4.4	2.2
Sympodiella acicola	*Venturiales*	–	13.8	0.3	5.0	*
Ceuthospora pinastri	*Helotiales*	–	3.8	4.1	3.1	1.3
Helotiales sp. 5	*Helotiales*	FR846473	5.9	0.3	0.9	4.4
Scleroconidioma sphagnicola	Dothideomycetes inc. sed.	–	1.6	4.4	1.9	1.3
Marasmius androsaceus	*Agaricales*	–	0.0	0.0	0.0	7.8
NK255	ND	–	2.2	0.0	0.9	0.0
Mycena galopus	*Agaricales*	FR846482	0.0	0.0	3.1	0.0
Helotiales sp. 6	*Helotiales*	FR846477	1.6	0.0	1.3	0.0
Hormonema dematioides	*Dothideales*	–	0.0	0.6	0.0	1.6
Helotiales sp. 8	*Helotiales*	FR846475	0.6	0.0	1.3	0.0
Aureobasidium pullulans	*Dothideales*	–	0.6	0.3	0.0	0.6
Cladosporium herbarum	*Capnodiales*	–	0.3	0.6	0.0	0.0
Chalara sp.	*Helotiales*	–	0.0	0.0	0.9	0.0
Leptostroma pinastri	*Rhytismatales*	–	0.6	0.0	0.0	0.0
NK260	ND	–	0.6	0.0	0.0	0.0
Hyaloscyphaceae sp.	*Helotiales*	–	0.6	0.0	0.0	0.0
Phoma macrostoma	*Pleosporales*	FR846476	0.6	0.0	0.0	0.0
NK278	ND	–	0.6	0.0	0.0	0.0
Cylindrocarpon magnusianum	*Hypocreales*	–	0.3	0.0	0.3	0.0
Selenosporella curvispora	Sordariomycetes inc. sed.	–	0.0	0.6	0.0	0.0
Stereaceae sp.	*Stereales*	FR846481	0.0	0.0	0.6	0.0
Helotiaceae sp.	*Helotiales*	FR846483	0.0	0.0	0.6	0.0
Helotiales sp. 1	*Helotiales*	FR846484	0.0	0.0	0.6	0.0
Heyderia abietis	*Helotiales*	FR846485	0.0	0.0	0.6	0.0
Helotiales sp. 2	*Helotiales*	FR846474	0.3	0.0	0.0	0.0
Helotiales sp. 3	*Helotiales*	FR846478	0.3	0.0	0.0	0.0
Helotiales sp. 4	*Helotiales*	FR846479	0.3	0.0	0.0	0.0
Epicoccum nigrum	*Pleosporales*	FR846480	0.3	0.0	0.0	0.0
Helotiales sp. 7	*Helotiales*	FR846472	0.0	0.3	0.0	0.0
Trichoderma sp.	*Hypocreales*	–	0.0	0.0	0.3	0.0

The effect of cultivation temperature on the fungal diversity was much more profound. The lower temperature regime (8/9 °C) was more convenient for fungal growth and a significantly more species-rich fungal community was retrieved. The average temperature in the litter layer on a sun-exposed rock ranged from 0 to 10 °C in March. In April, temperature rose to 16 °C during sunny days (Fig. 1). Some of the rare fungi obtained from the March sampling and cultivated at 8/9 °C may represent species (or strains) that have an optimum for growth at lower temperatures and are replaced by other fungi after the increase in temperature in April. These obviously do not belong to psychrophilic specialists because they were able to grow in pure cultures kept at room temperature. In previous studies, some rare fungi may have been overlooked when needles sampled in various seasons were cultivated at room temperature only (Hayes 1965, Mitchell & Millar 1978, Tokumasu et al. 1994, Tokumasu 1998). On the other hand, Tokumasu (1996) did not isolate different fungi from needles sampled in winter months and kept at 4 °C when compared to those cultivated at laboratory temperature.

Two basidiomycetes representing typical pine litter colonisers (*Marasmius androsaceus* and *Mycena galopus*) were both isolated from needles under snow cover, but not under the same temperature regime. These two species share the same substrate, but do not compete for resources, because they are differently affected by biotic and abiotic factors. Newell (1984) found that the two fungi were horizontally restricted in distribution due to selective grazing by litter collembolans. *Marasmius androsaceus*, which was preferred by *Onychiurus latus* Gisin, was restricted to the uppermost litter layer to avoid contact with the *Onychiurus latus*, which was more frequent in deeper horizons. Without grazing, *Marasmius androsaceus* was in laboratory conditions more competitive in the colonisation of litter needles compared to *Mycena galopus*, but this ability was reverted when being grazed. In this study, the temperature seemed to have also effect on their coexistence. *Marasmius androsaceus* was isolated at 15 °C, suggesting that it is less psychrotolerant than *Mycena galopus*, but may be more tolerant to increase of temperatures in the uppermost litter layer.

Ten strains of *Helotiales* remained unidentified even at the genus level. One of the most frequent species (*Helotiales* sp. 5) was most closely related to a sequence obtained from an uncultured fungal clone from pine B-horizon soil under *Pinus taeda* L. in Duke Forest, Durham, N.C., USA (Acc. Nr. AY969711, similarity 396/401 bp) isolated in the study of O'Brien et al. (2005). The other species of *Helotiales* had similarity in their ITS or 28S rDNA regions ranging from 94 to 99 % with soil and litter colonisers and also with fungi isolated from ectomycorrhizal root tips. Helotialean species are common saprotrophic or ectomycorrhizal colonisers of litter needles. Tedersoo et al. (2010) discussed the diversity of Helotialean species forming various associations with plant roots. The study of these species is hampered by their limited sporulation in pure cultures and un-

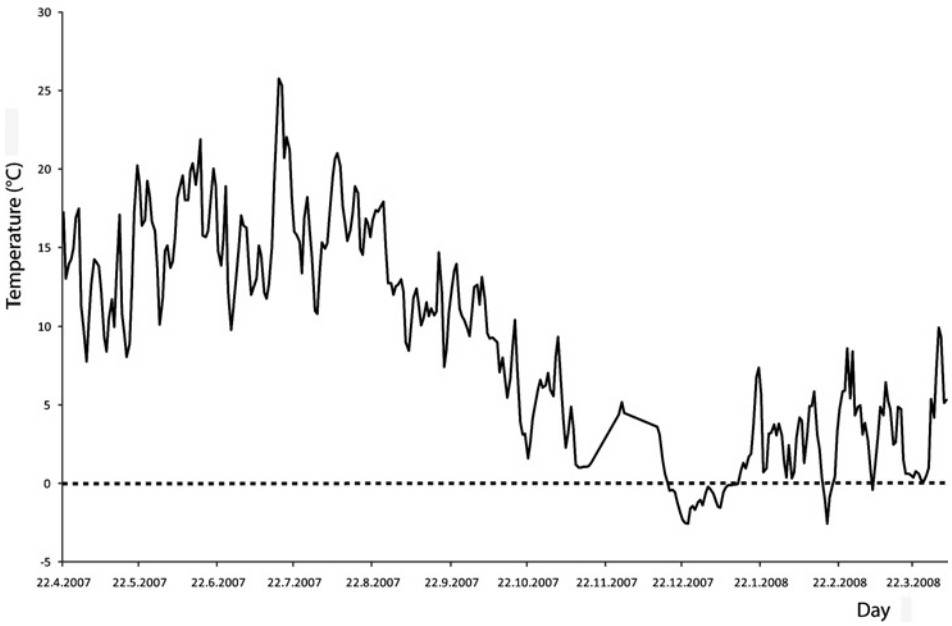

Fig. 1. Mean daily temperatures in pine litter measured from April 2007 to March 2008. The dashed line indicates 0 °C.

known teleomorph affinity, which disables assignment to known species or description of new taxa. Some of these strains might be identical to those recorded in this area in spring and autumn sampling (Koukol 2007), but others may represent mycobiota active only during the snow-melting period. This cannot be verified, because most fungal species in the study of Koukol (2007) were identified based on morphology and are not represented by any sequence in GenBank.

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