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Activity of different ectomycorrhizal types studied by vital fluorescence

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Abstract

Ectomycorrhizal root tips were sampled in a Norway spruce (*Picea abies* L. Karst) stand (Höglwald, Bavaria) during one growing season and analysed by fluorescence microscopy. After staining with fluorescein-diacetate, tissue fluorescence was used as an estimate of activity of six identified ectomycorrhizal types on *Picea abies (Tylospora* sp.-*Picea abies, Piceirhiza nigra, Piceirhiza gelatinosa, Xerocomus badius-P. abies, Russula ochroleuca-P. abies, Cenococcum geophilum*) and two unidentified mycorrhizas. There were differences among ectomycorrhizal types in the FDA-hydrolysing activity of the various tissue layers of the mycorrhizas: outer and inner hyphal sheath, Hartig net and stele. The differential activity in tissues was judged to be an estimate of physiological activity of the fungal symbiont and of the life span of the mycorrhiza. The differences in species activity may influence the overall activity of mycorrhizas in a forest stand.

Introduction

The ectomycorrhizal root system of forest trees is dynamic and undergoes constant ageing and regeneration. The efficiency of the system with respect to nutrient uptake versus carbohydrate demand is a function of the physiological activity of the mycorrhizas and their turnover rate. A lower production rate and long-lived mycorrhizas were shown to be more advantageous to growth of Norway spruce (*Picea abies* [L.] Karst.) than a high production of mycorrhizas with a higher turnover rate (Kottke and Agerer 1983; Kottke et al., 1993; Ritter et al., 1989). Activity and life span of individual mycorrhizas in a forest stand have been found to be influenced by soil treatments (Al Abras et al., 1988; Alexander and Fairley, 1983; Ritter et al., 1989; Ritter, 1990). In these investigations mycorrhizal types were not considered. From the results presented here we conclude that there are differences in the activity of identified mycorrhizal types independent from soil conditions, but related to the activity of the fungus involved.

Ageing of a mycorrhizal root is a process which generally starts on the surface and proceeds to the inner tissue layers (Al Abras et al., 1988; Downes et al., 1992; Duddridge and Read, 1984; Fusconi, 1983; Haug, 1987; Ritter, 1990). The progression of ageing of the mycorrhizal tissues can be studied by vital fluorescence after staining with fluorescein diacetate (Downes et al., 1992; Ritter et al., 1986). Applying this technique to investigating the mycorrhizal populations of several Norway spruce stands, Ritter (1990) observed that apart from the general rule of tissue senescence, ectomycorrhizal types differed in hydrolysing enzymatic activity. A mycorrhizal type termed "silbrig glänzend" by Ritter (1990), which was later identified as *Xerocomus badius-Picea abies* mycorrhiza, showed bright vital fluorescence in the hyphal sheath even after the Hartig net fluorescence had declined. In another type of mycorrhiza ("hellbraune"), now identified as being formed by *Tylospora* sp., low fluorescence activity was observed in the hyphal sheath, but higher activity in the Hartig net. The fluorescence activity differences may reflect the functioning of mycorrhizas (Al Abras et al., 1988) and could be ecologically important if very active or

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inactive occur as predominant types. The aim of this study was, therefore, to clarify to what extent the dominant ectomycorrhizal types differed in activity in a *P. abies* stand in the Höglwald forest near Augsburg, Bavaria. To evaluate the difference in activity among the mycorrhizal types data obtained from mycorrhizas of different soil treatments and monthly sampling were pooled. In a companion publication influence of soil treatment at the site on mycorrhizal activity was evaluated (Agerer et al., 1998; Qian et al., 1998).

Material and methods

Mycorrhizas were collected from five soil treatments in an 80-years-old Norway spruce (*Picea abies*) stand. Some plots had been treated experimentally with lime or acidic water for seven years. Because the influence of soil treatments on the activity of the mycorrhizal tissues is not considered in the preseent study, no further information for the plots is given here. For details of the experimental setting and soil parameters see Kreutzer and Bittersohl (1986) and Kreutzer and Weiss (1998). Sampling took place three times, in July, September and October 1990, and was performed using a soil corer of 5 cm diameter to a depth of 15 cm. Five samples were taken within an area of about 12 m^2 each time in each plot. The soil samples were kept in plastic bags at 4 °C. Previous investigations had shown that no important change of mycorrhizal activity occurred during 10 days after sampling when the samples were kept cool (Ritter, 1990). All the mycorrhizas in the samples were identified and analysed within one week after excavation. Six types were identified as *Tylospora* sp.-*Picea abies, Piceirhiza nigra, Piceirhiza gelatinosa, Xerocomus badius-P. abies, Russula ochroleuca-P. abies* and *Cenococcum geophilum* (Agerer, 1987–1991; Gronbach, 1988; Haug, 1987; Haug and Oberwinkler, 1987; Haug et al., 1992; Taylor and Alexander, 1990). The portion of mycorrhizas that could not be identified under the dissecting microscope because of lack of characteristic hyphal sheaths was pooled. These mycorrhizas formed one quarter of the sampled mycorrhizas and belonged to two undescribed types. Table 1 gives the number and percentage of the mycorrhizas of each type analysed. In total, 1398 mycorrhizas were observed.

Table 1. Mycorrhizal types, numbers and proportion of mycorrhizas investigated by FDA-fluorescence pooled from three sampling dates in 1990

Mycorrhizal types	Numbers	Proportion of total observations
Tylospora sp.-Picea abies	420	30%
Piceirhiza nigra	243	17%
Piceirhiza gelatinosa	50	4%
Cenoccocum geophilum	61	4%
Russula ochroleuca-P.abies	143	10%
Xerocomus badius-P.abies	119	9%
Unidentified types	362	26%
Total	1398	100%

The mycorrhizas were hand-sectioned longitudinally at a thickness of about 100μm and immediately stained by fluorescein-diacetate for five min in the dark (FDA, Sigma, 0.01 mg mL^{-1} in potassium-dihydrogen-phosphate buffer, pH 7,5; Ritter et al., 1986; Ziegler et al., 1975). The sections were washed three times in phosphate buffer, protected by a microscopical cover glass and examined under an epi fluorescence microscope (Zeiss Standard; UV-filter BP 365/FT 395/LP 397). A mercury high pressure lamp HBO 100 W/2 was used as the UV-light source. The filter system allowed observation of not only the green vital fluorescence but also the blue autofluorescence of the suberized metacutin layers in order to gain information on root physiological status (Qian et al., 1998). The vital fluorescence of the different tissue layers was evaluated visually (Figure 1). A bright green colour was observed in fully active tissues (+++), fading in two steps to light green (++) and blue green (+). The latter was a mixture of the green FDA fluorescence with blue autofluorescence of suberin or lignin in the ageing tissues of the root (Haitinger, 1959) or autofluorescence from the hyphae. The dying stage (+/−) was a greenish to brownish colour and the dead tissue (−) was brown. The fluorescence of the different tissue layers, the outer and inner hyphal sheath, the Hartig net/cortical cells and the stele was recorded separately for the basal and the apical region of each mycorrhiza (Figure 1). As the fluorescence of the cortical cells was difficult to distinguish from the fluorescing hyphae in the Hartig net region it was not noted separartely. The proportion of the five stages of activity of each tissue layer was calculated based on the total number of mycorrhizas of each type observed. In all mycorrhizal types, fungal tissue of full activity was found, which indicates that there was not a problem with infiltration of the stain. No statistical analysis was carried out because of the large differences in the numbers

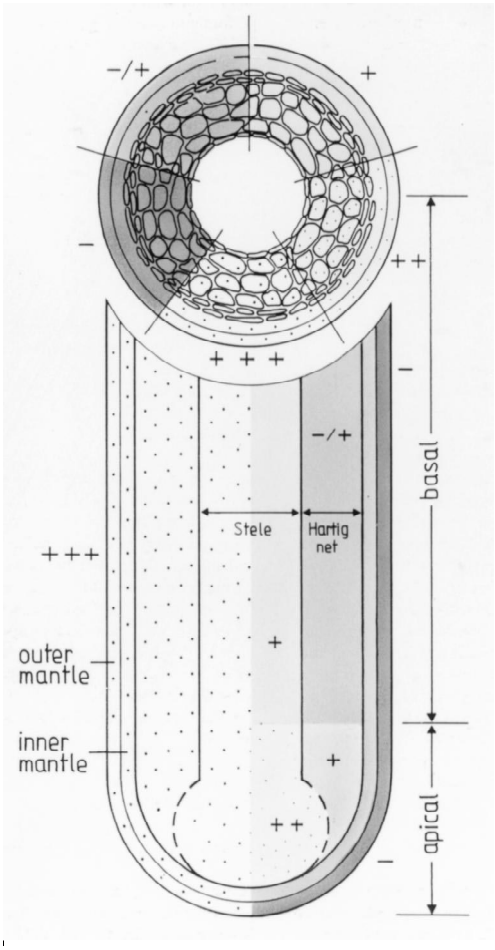

Figure 1. Scheme illustrating the fluorescence intensities after FDA-staining in different ages of root and ectomycorrhizal tissues. The radial section indicates level of fluorescence activity: +++ bright green = full activity, ++ light green = intermediate activity, + blue green = low activity, −/+ brownish = very low activity, − brown = inactive tissue. Longitudinal section: left side mycorrhiza of full activity; right side sequence of ageing in the tissue layers and the basal and apical regions occurring in ectomycorrhizas and corresponding to FDA fluorescence.

of each mycorrhizal type observed. The mycorrhizal types were not found in same numbers at the different sampling dates. Therefore, no discrimination of seasonal influences was performed.

Results

Fluorescence activity in the hyphal sheath layers

The outer sheath layer varied in fluorescence activity in the mycorrhizal types (Table 2). The greatest proportion of highly active (+++) mycorrhizas was 25% for *Xerocomus badius-P. abies* at the basal region and 19% at the apical region. In *Piceirhiza gelatinosa*, only 4.7% and 2.3% were active in the basal and apical region, respectively. The mycorrhizas, *Russula ochroleuca-P. abies, Piceirhiza nigra, Tylospora* sp.-*P. abies* and the two unidentified types, only rarely displayed full activity in the outer sheath layer. The inner hyphal sheath layer displayed higher fluorescence activity than the outer layer in most of the mycorrhizas (Table 2). In *Xerocomus badius-P. abies*, 23% of the sectioned mycorrhizas were highly fluorescent at the apex and 27% at the base. Only 3.5% of the inner sheaths of *Russula ochroleuca-P abies* mycorrhizas were fully active in the apical region and 0.7% in the basal region. A high proportion of active mycorrhizas of *Piceirhiza gelatinosa* (apically 7%, basally 2.3%) was observed, but a comparatively low proportion of *Tylospora* sp.-*P.abies* (2.4% apically, 0% basally), and the unidentified types (3% apically, 1.1% basally were active. In more than 90% of the latter mycorrhizas, the hyphal sheaths lacked activity while 10–20% of the inner hyphal sheaths of *Piceirhiza nigra, Russula ochroleuca-P. abies*, and *Piceirhiza gelatinosa* showed weak activity (+/−).

Fluorescence activity in the Hartig net

Differences among mycorrhizal types were also detected in fluorescence activity of the Hartig net layer (Table 2). The two mycorrhizal types, *Piceirhiza nigra* (basal region 11% apical region 14.5%) and *Russula ochroleuca-P. abies* (basal region 9.8%, apical region 13.3%), yielded the highest proportion of fully vital Hartig net. *Piceirhiza gelatinosa* had the lowest proportion of inactive Hartig net and the highest in the intermediate stages. *Xerocomus badius-P-abies* mycorrhizas displayed low activity in the Hartig net layer. The proportion with full activity in the Hartig net was only 5.3% in the apex as well as in the base. The proportion of inactive tissue was 62.1%. Low activity of the Hartig net region was also displaygd by *Tylospora* sp.-*P. abies, Cenococcum geophilum* and the unidentified types. Generally, in the Hartig net the intermediate levels activity of (++ and +) appeared more frequently and the inactive state less frequently than in the hyphal sheath.

Fluorescence activity in the stele

The tissues of the stele, including the meristem, displayed intermediate levels of activity in all mycorrhizal

Table 2. Percentage of mycorrhizas of each mycorrhizal type displaying one of the five levels of fluorescence activity in the outer hyphal sheath, the inner hyphal sheath, the Hartig net and the stele of basal (Bas.) and apical (Api.) region, respectively. For explanation of the levels of fluorescence activity see Figure 1

	+++	++	+	+/–	–
Outer sheath activity mycorrhizal type					
Bas. *Tylospora* sp.	0	0	0.5	0.2	99.3
Api	0.2	0.2	1.9	1.2	96.4
Bas. *Piceirhiza nigra*	0	1.3	1.3	0	97
Api.	0.4	1.7	1.7	0	96.6
Bas. *Xerocomus badius*	25.3	4.2	4.2	0	67.4
Api.	19.9	0	6.3	0	69.5
Bas. *Russula ochroleuca*	0.3	0	0.7	1.4	97.2
Api.	0.7	4.7	4.9	4.9	89.5
Bas. *Piceirhiza gelatinosa*	2.3	4.7	16.3	0	76.7
Api.	4.7	0	4.7	0	86
Bas. *Cenococcum geophilum*	0	0	0	0	100
Api.	0	0	0	0	100
Bas. unidentified types	0.8	0	1.1	0.8	97.2
Api.	1.7	0.8	3	1.9	92.5
Inner sheath activity mycorrhizal type					
Bas. *Tylospora* sp.	0	1.2	2.9	4.3	91.4
Api	2.4	4.8	3.8	1	88.1
Bas. *Piceirhiza nigra*	9.4	6.8	10.3	0.9	72.6
Api.	8.5	9.4	12	0.9	69.2
Bas. *Xerocomus badius*	27.4	2.1	3.2	0	67.4
Api.	23.2	5.3	5.3	0	66.3
Bas. *Russula ochroleuca*	0.7	3.5	16.1	7	72.7
Api	3.5	6.3	21	4.2	65
Bas. *Piceirhiza gelatinosa*	2.3	4.7	14	2.3	76.7
Api.	7	2.3	7	0	83.7
Bas. *Cenococcum geophilum*	0	0	2.9	0	97.1
Api.	0	0	2.9	0	97.1
Bas. unidentified types	1.1	0.3	2.5	1.9	94.2
Api.	3	1.7	4.1	0.6	90.6
Hartig net activity mycorrhizal type					
Bas. *Tylospora* sp.	2.1	4.8	11.2	18.8	63.1
Api.	1.7	7.9	13.1	17.1	60.2

Table 2. continued

Bas. *Piceirhiza nigra*	11.1	17.5	14.5	7.7	49.1
Api.	14.5	18.4	15	8.5	43.6
Bas. *Xerocomus badius*	5.3	2.1	21.1	9.5	62.1
Api.	5.3	5.3	18.9	10.5	60
Bas. *Russula ochroleuca*	9.8	10.5	18.9	21.7	39.2
Api.	13.3	12.6	15.4	20.3	38.5
Bas. *Piceirhiza gelatinosa*	4.7	25.6	27.9	20.9	20.9
Api.	2.3	27.9	30.2	16.3	23.3
Bas. *Cenococcum geophilum*	0	5.9	23.5	0	70.6
Api.	2.9	8.8	26.5	2.9	58.8
Bas. unidentfied types	0.8	6.9	18.5	25.1	48.6
Api.	1.1	9.1	18.8	24	47
Stele activity mycorrhizal type					
Bas. *Tylospora* sp.	2.9	12.1	26	27.9	31.2
Api.	3.8	15	25	28.3	27.6
Bas. *Piceirhiza nigra*	6	20.9	31.6	17.9	23.5
Api.	8.5	23.5	29.1	18.1	20.1
Bas. *Xerocomus badius*	14.7	13.7	17.9	31.6	21.1
Api.	17.9	10.5	20	30.5	6.3
Bas. *Russula ochroleuca*	7	18.9	36.4	31.5	7
Api.	11.2	19.6	31.5	30.8	4.7
Bas. *Piceirhiza qelatinosa*	9.3	27.9	44.2	14	4.7
Api.	9.3	27.9	41.9	14	7
Bas. *Cenococcum geophilum*	5.9	17.6	41.2	2.9	32.4
Api.	5.9	29.4	32.4	0	32.4
Bas. unidentified types	3.3	14.6	37	29.6	15.5
Api.	5.5	16.3	34	24.9	19.3

types (Table 2). Fully active or inactive tissues were uncommon. The differences between the mycorrhizal types were therefore much less pronounced in the stele than in the fungal layers. *Tylospora* sp.-*P. abies* and *Cenococcum geophilum* showed the lowest proportion of activity. *X. badius-P. abies* displayed a comparatively high proportion of fully active stele tissue, 14.7% in the basal and 17.9% in the apical region. Inactive tissues were found in 21% of the samples in the basal region and 6.3% in the apical region of the mycorrhizas of this type. In *R. ochroleuca-P.abies,* an even lower proportion of inactive tissue was detected (7% basally, 4.7% apically). *Piceirhiza gelatinosa* was intermediate in the proportion of inactive tips.

Ranking of the mycorrhizal types

The mycorrhizal types were ranked according to the proportion of the fully active and of the inactive states in the different tissue layers (Figure 2). The intermedi-

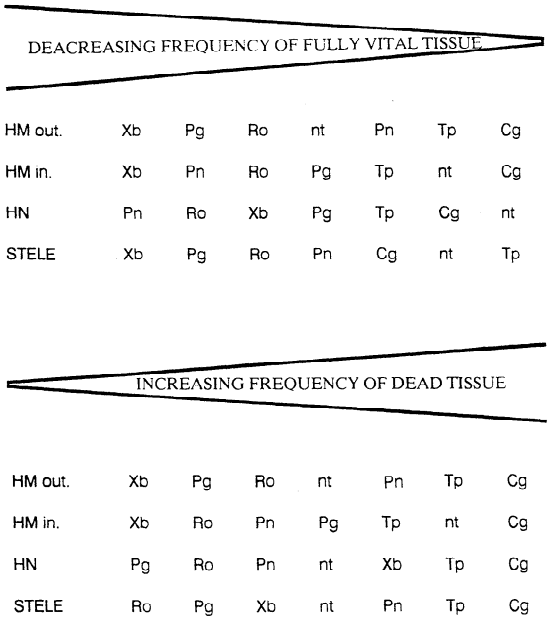

Figure 2. Ranking of the diverse types according to the proportion of the fully active and of the inactive states in the different tissue layers. Tp = *Tylospora* sp. - *Picea abies*, Pn = *Piceirhiza nigra*, Xb = *Xerocomus badius-Picea abies*, Ro = *Russula ochroleuca-Picea abies*, Pg = *Piceirhiza gelatinosa*, Cg = *Cenococcum geophilum*, nt = unidentified types. HM hyphal sheath, out. outer, in. inner hyphal sheath, HN Hartig net.

ate states were not regarded in the scheme. The diagram shows that there is not a strong connection between these two activity states of the tissues indicating differences in the ageing process among the fungi. The diagram summarizes the most important differences among the mycorrhizal types as described above.

Discussion

In previous investigations, the activity of mycorrhizas in forest plots was estimated from vital fluorescence after staining with FDA on the level of hyphal sheath, Hartig net, apical meristem and stele activity (Kottke et al., 1993). It was, however, observed that the mycorrhizal types may repeatedly differ in activity. More sophisticated analyses on the activity of the tissues were, therefore, carried out here differentiating five steps of activity in each tissue layer. The visual estimation of fluorescence activity is crucial because of subjective influence and most critical for the estimation of the intermediate steps. All people involved in this investigations were, therefore, trained on the same material to cope with this problem. The high number of sectioned mycorrhizas additionally compensates for the personal errors. From earlier investigations of Norway spruce in Western Germany over several seasons, we found that at least 30 mycorrhizal rootlets were required for accurate estimates of fluorescence of mycorrhizae (Kottke et al., 1993; Ritter, 1990). At the moment, no other method exists to evaluate the activity of mycorrhizas on the type level in forest stands in satisfactorily large samples. In the future, confocal fluorescence microscopy may be applied for quantification of fluorescence intensities.

The results obtained here are in good agreement with the common knowledge of ectomycorrhizal development. The fungus colonizes the rootlets sequentially from the basal to the apical region and establishes the Hartig net continuously from the root surface to the endodermis (Kottke and Oberwinkler, 1986). The growth of the hyphal sheath was shown to occur at the root surface, in the innermost part of the hyphal sheath (Fusconi, 1983). The youngest hyphae are therefore found near to the root apex and in the inner layer of the hyphal sheath and Hartig net. The ageing process starts at the bases of the mycorrhiza and in the outer hyphal sheath. Fluorescence intensities revealed the same general pattern, which confirms the reliability of the method (Figure 1).

Only *X. badius-P. abies* seems to be an exception to this rule, since its sheath displaed activity even when the Hartig net no longer showed activity. The activity was higher in the basal than in the apical part of the hyphal sheath. Ultrastructural investigations are currently carried out for better understanding of this situation.

The data yielded information about differences in activity among mycorrhizal fungal species. This is concluded from the fact that the largest differences were found in the activities of the hyphal sheaths. There are mycorrhizal types which display high FDA-hydrolizing activity in the hyphal sheath more frequently than others, e.g. *X. badius-P. abies*, *Piceirhiza gelatinosa* and *R. ochroluca-P. abies* compared to *Tylospora* sp.-*P. abies* or *Cenococcum geophilum*. The 2.5% of actively fluorescing sheaths of *Tylospora* sp. mycorrhizas reveal that the stain was able to penetrate into the hyphae and the low activity was not due to low infiltration. Downes et al. (1992) using the same method also found low hydrolysing activity in *Tylospora fibrillosa-Picea sitchensis* mycorrhizas grown in Perspex root observation chambers. Only the activity of the sheaths of *Cenococcum geophilum* may

be underestimated as the melanins in the cell walls of the dark fungus absorb the UV light in part reducing fluorescence emission. But the high percentage of inactive stele tissue suggests that most of the *Cenococcum geophilium* mycorrhizas were no longer alive.

The main role of the hyphal sheath is storage of nutritive elements like phosphorus and nitrogen (Kottke et al., 1995a). As storage products were only found in living fungal cells (Kottke et al., 1995a), high activity of the fungal sheath may be an important advantage for ectomycorrhizas on nutrient depleted soils. Storage of elements will also be necessary for survival during drought or frost periods. The worldwide distribution of ectomycorrhizal trees correlates well with such climatic situations (Read, 1991). On the basis of the results presented here it is likely that *Tylospora* sp.-P. abies has a low storage efficiency, whereas *X. badius-P. abies* may have a higher uptake rate and storage capacity. Cairney and Alexander (1992) found lower phosphate absorption rate in older compared with young mycorrhizas of *T. fibrillosa.* Phosphorus concentration was exceptionally high in mycorrhizas formed by *X. badius* when compared to other mycorrhizal types (Haug et al., 1992).

The activity of the Hartig net was generally higher than that of the hyphal sheaths and differences between species were more modest, therefore. The bidirectional transport between fungus and root cortex may still be active after the death of the sheath. Solute flow was shown to be retained after death of the sheath of mycorrhizas formed by *Tuber* spp. (Kottke et al., 1995b).

Comparing activity in the hyphal sheath and Hartig net of the six mycorrhizal types it becomes clear that the fungal species is controlling the activity of the mycorrhiza. It is concluded that the species composition of the mycorrhizal population will influence the activity of mycorrhizas in a forest stand.

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