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Anatomical–physiological determination of surface bound phosphatase activity in ectomycorrhizae of *Nothofagus obliqua*

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Abstract

In roots and mycorrhizae, the activity of phosphatases is an important parameter to characterise the efficiency of plants to access non-soluble phosphate pools in soils. We have quantified surface bound phosphatase (SBP) activity in non-mycorrhizal short roots and ectomycorrhizae of *Nothofagus obliqua* with image processed confocal laser scanning microscopy (LSM) using the fluorogenic substrate ELF-97 (enzyme-labelled fluorescence). Through interactive segmentation of root cells, mantle, and SBP-active centres, this method revealed a precise anatomical–physiological description of the SBP activity in cross-sections of short roots of *N. obliqua*, and of the mycorrhizal associations of *N. obliqua* with *Pisolithus tinctorius* and with *Cenococcum geophilum* in a controlled pH range (3–7).

Our method revealed that the strategy of the examined species to vary the SBP activity was based primarily on the variation of the number and of the extension of the SBP-active centres. Fluctuations of the activities inside individual SBP-active centres were small. It was observed that non-mycorrhizal short roots of *N. obliqua* focus the distribution of SBP-active centres on the rhizodermis cells. In these cells, the SBP-active centres are distributed heterogeneously, and not preferentially in contact with the soil interface. The distribution of the SBP-active centres between the root cells and the mantle depended on the symbiont and on the pH of the buffer. The mantle hyphae of the *N. obliqua–P. tinctorius* associations promote direct contact between SBP-active centres and soil particles. In contrast, the mantle hyphae of *N. obliqua–C. geophilum* associations limit the expression of SBP-active centres to the interface between the mantle and the rhizodermis cells of *N. obliqua*. At this location, SBP-active centres are not in direct contact with any adjacent soil particles. Our observations of a pH-dependent activity, and of a mycorrhizal association-dependent activity of the SBP-active centres, together with the observed heterogeneity of the location of these centres relative to adjacent soil particles, challenge the general hypothesis that increased contact between mycorrhizae and soil results in higher efficiency of nutrient uptake.

Keywords: *Nothofagus obliqua*; *Pisolithus tinctorius*; *Cenococcum geophilum*; Ectomycorrhiza; Surface bound phosphatase activity; ELF-97; Nutrient supply; Image processed quantitative fluorescence microscopy

1. Introduction

For the uptake of phosphate from different soil components, plants have developed numerous adaptation strategies of morphological, physiological, or biochemical nature (McCully, 1999; Raghothama, 1999; Ticconi et al., 2001; Vance et al., 2003). In soils, 20–80% of P is present in the organic form, of which phytic acid (inositol hexaphosphate) comprises a major component (Richardson, 1994; Schachtman et al., 1998). One important mechanism by which organic P-compounds are transformed into plant accessible, inorganic forms is through phosphatase active enzymes that are produced by ectomycorrhizae, non-mycorrhizal short roots, extraradical mycelium and other

Abbreviations: LSM, laser scanning microscopy; SBP, surface bound phosphomonoesterase; ELF-97, enzyme-labelled fluorescence.

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biological components of the soil (Smith and Read, 1997). Among the different fractions of fungal phosphomonoesterases, wall-bound enzymes have been determined to be the most active (McElhinney and Mitchell, 1993).

In order to evaluate the importance of the surface bound phosphatase (SBP) activity for plant growth, investigators have studied different morpho-anatomical aspects of the root system, of the involved ectomycorrhizal associations, and pure cultures of mycorrhizal fungi (Lynch, 1995; Barber, 1995; Smith and Read, 1997; Narang et al., 2000). Further investigations have concentrated on the extraradical mycelium (Schachtman et al., 1998), included quantitative studies of root length (Antibus et al., 1997), or localised phosphatase active centres of fungal hyphae with microscopic techniques (Tisserant et al., 1993). van Aarle et al. (2001) visualised SBP activity in mycelium of arbuscular mycorrhizal fungi with conventional fluorescence microscopy, introducing the phosphomonoesterase-sensitive fluorogenic substrate ELF-97 into this area. Despite intense investigation, the role of SBP activity in supplying P to plants is not fully understood (see review by Joner and Johansen, 1999). One reason may be the lack of appropriate methods to study the anatomical, physiological, and biochemical properties of the root–soil interface. Kamentsky and Kamentsky (1991) demonstrated that microscope-based techniques could not only provide structural information of a fluorescent sample, but also quantitative biochemical data. From this idea, many applications have emerged (e.g. Darzynkiewicz et al., 1997; Härtel et al., 2003), and some of them have even contributed new aspects to mycorrhiza related research (Dickson and Kolesik, 1999; Cavagnaro et al., 2001; Schweiger et al., 2002).

Recently, a technique was developed to localise and quantify the SBP activity in isolated fungal mycelium, grown in pure cultures (Alvarez et al., 2004). This technique combines confocal laser scanning microscopy (LSM), a staining procedure with the fluorogenic substrate ELF-97, and interactive image processing routines. The method allows access to the structural organisation of the phosphatase active centres on the surface of fungal hyphae and, at the same time, determines its catalytic activity through the brightness of the fluorescent spots. In the present study, the anatomical–physiological properties of SBP activity were determined in freshly extracted root material of *Nothofagus obliqua–Pisolithus tinctorius* mycorrhizae, *N. obliqua–Cenococcum geophilum* mycorrhizae, and non-mycorrhizal short roots of cultivated *N. obliqua* seedlings. Image processed fluorescent microscopy method quantified and localised the distribution of SBP-active centres in cross-sections of mycorrhizal and non-mycorrhizal short roots in a buffer controlled pH range between 3 and 7. The pH range was chosen as a physiologically relevant parameter that stimulates different responses of SBP activity in fungal and mycorrhizal samples (Antibus et al., 1986, 1992; Joner and Johansen, 1999). By combining anatomical and physiological observations, a better understanding of the symbiont-specific processes that are involved in P-uptake by *N. obliqua* would be gained.

2. Materials and methods

2.1. Cultivation of mycorrhizal and non-mycorrhizal N. obliqua seedlings

The experiments were carried out with ectomycorrhizal inocula of *P. tinctorius* (Pers.) Coker and Couch (isolate 441) and *C. geophilum* Fr. (isolate F1702), received from I. Kottke, University of Tübingen, Germany. The fungi were grown on solid Melin–Norkrans culture media (agar agar 20 g/l, malt extract 5 g/l, D-glucose 10 g/l, $(NH_4)2HPO_4$ 0.25 g/l, KH_2PO_4 0.5 g/l, $MgSO_4 \cdot 7H_2O$ 0.15 g/l, $CaCl_2$ 0.05 g/l, Fe-EDTA 0.022 g/l, NaCl 0.025 g/l, thyamine HCl 1×10^{-4} g/l) (Molina and Palmer, 1982). Fungal stock cultures were grown in the dark, at 25 °C, and at pH 5. *P. tinctorius*/*C. geophilum* showed radial growth rates of 6–7 cm/2–2.5 cm per month.

Seeds of *N. obliqua* (Mirb.) Oerst. were surface sterilized using 30% H_2O_2 solution for 3 min. They were germinated in plastic pods ($50 \times 30 \times 8$ cm), containing 5 cm of bentonite-clay substrate (3:1), previously autoclaved at 120 °C for 50 min. The further cultivation of plants took place in a phytotron (humidity: 80%; irradiation: 15.7 klux; day/night cycle: 13 h/11 h; temperature: 25/20 °C). They were fertilised every 2 weeks with Knop-solution (1:3) (Ziegler, 1998).

To generate ectomycorrhizae of *N. obliqua*, individual seedlings were removed from the bentonite-clay substrate, rinsed, and transferred to rhizotrons 3 month after germination. Rhizotrons consisted of plastic Petri dishes (diameter = 145 mm, height = 20 mm) with a 10 mm opening for the stem, filled with sterilized perlite (Kottke et al., 1987). Roots were separated from perlite by charcoal filter paper (Schleicher and Schuell, Dassel, Germany). One week after being transferred to the rhizotrons, plants were separated into three groups: (i) 12 rhizotrons with *N. obliqua* were grown without mycorrhizal inoculum, (ii) 12 rhizotrons with *N. obliqua* were inoculated with *P. tinctorius*, and (iii) 12 rhizotrons were inoculated with *C. geophilum*. For (ii) and (iii), each seedling was inoculated with 6 plugs of mycelium, placed in the Petri dishes close to young short roots according to Kottke et al. (1987). The plugs had been isolated from the leading edge of 1-month-old stock cultures with a sterile cork borer (diameter = 7 mm) and placed on fresh agar medium. After 1 week, plugs showing actively growing hyphae were used for inoculation.

Five weeks after the inoculation, 5–6 plugs had infected the neighbouring main and lateral short roots. For anatomical verification of ectomycorrhizal formation, plastic tabs were removed from the rhizotrons and 3 root tips per plant (length = 1.5–3.5 mm) were cut at their basis

under a binocular (Stemi SV 11 Apot, ZEISS, Jena). Root tips were embedded in 4% agar and cross-sections of 100 µm were cut with a Vibratom (model 1000 Classic, GaLa Gabler Labor Instumente, Bad Schwalbach, Germany). Light microscopy revealed a paraepidermal Hartig net and a plectenchymatous mantle (results not shown).

2.2. pH-Dependent determination of SBP activity in mycorrhizal and non-mycorrhizal short roots of N. obliqua

pH-Dependent SBP activity was determined in non-mycorrhizal short roots of *N. obliqua* and in *P. tinctorius* and *C. geophilum* ectomycorrhizae, using the enzymatic activation of the fluorogenic substrate ELF-97 (Molecular Probes, Leiden, Netherlands, 5 mM). ELF-97 substrate solution was adjusted to pH 3, 4, 5, 6, and 7 by the corresponding citrate–phosphate buffer solution (2:10 v/v, buffer/ELF-97 stock solution).

For each pH condition, 3 root tips were selected from each of 3–4 replicates (rhizotrons of each respective experimental group). Mycorrhizal and non-mycorrhizal root tips of *N. obliqua* (length = 1.5–3.5 mm) were obtained as described above. Root tips were rinsed and transferred to microcentrifuge tubes (100 µl, Eppendorff). Each root tip was incubated at room temperature after the addition of 15 µl of ELF-97 substrate solution (pH 3–7). As has been reported previously for the determination of SBP activity in pure cultures of mycorrhizal fungi, the fluorescence intensity of ELF-97 increases linearly during the first 20–30 min of the incubation (Alvarez et al., 2004). This observation also applied to mycorrhizal and non-mycorrhizal root tips (results not shown). After 15 min of incubation, root tips were removed from the microcentrifuge tubes, rinsed with citrate–phosphate buffer solution in order to stop the enzymatic reaction and embedded in 4% agar.

2.3. Fluorescence imaging by LSM

For the observation of the samples with LSM (LSM 510, Zeiss, Göttingen, Germany), cross-sections of 30 µm were

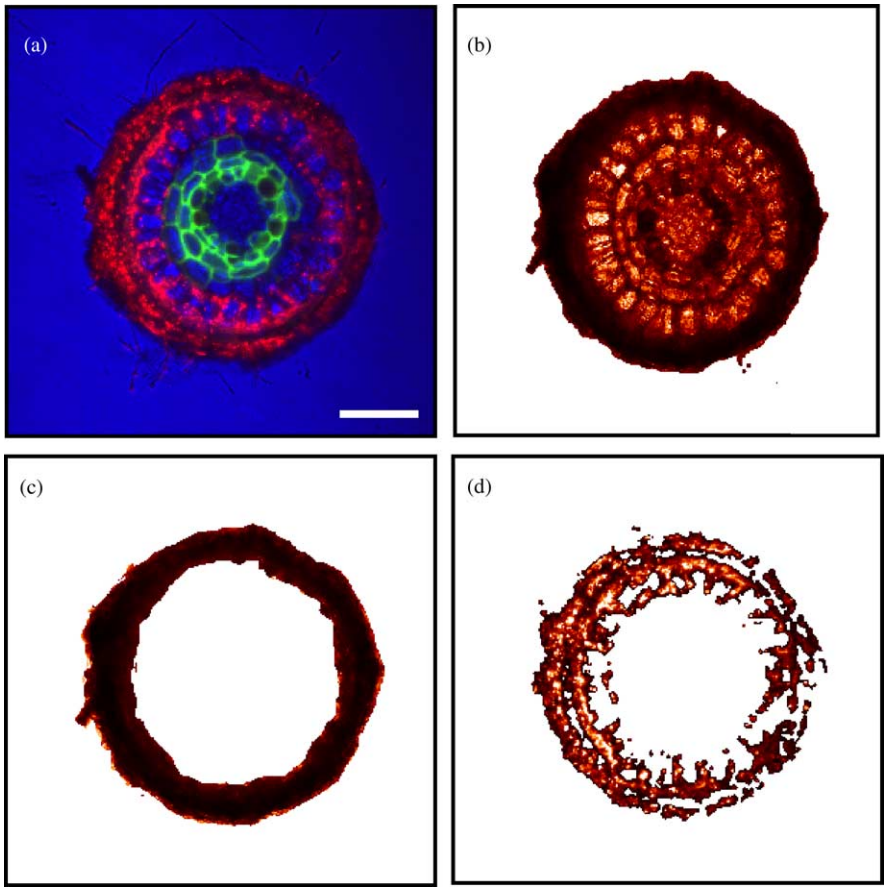

Fig. 1. Interactive segmentation of a cross-section of an *N. obliqua–P. tinctorius* ectomycorrhiza. (a) Representative 3-channel LSM image. Channel 1: bright field picture (blue). Channel 2: 385–470 nm bandpass filter for autofluorescence of fungal mycelium and root cells (green). Channel 3: 560–615 nm bandpass filter for enzymatically activated ELF-97 fluorescence intensity (red). (b) Segmentation of the complete ectomycorrhiza. (c) Segmentation of the mantle. (d) Segmentation of the enzymatically activated fluorescent ELF-97 centres on the Hartig net, the mantle, and the root cells. Bars on the lower left edge of the images represent a distance of 60 µm. (For interpretation of the reference to colour in this legend, the reader is referred to the web version of this article.)

cut with a Vibratom. The 3rd or 4th cross-section of each root tip was transferred to object slides and sealed with cover slides and nail polish. The LSM was equipped with a 364 nm wavelength UV argon ion laser (Enterprise II-653, Coherent, Santa Clara, CA, USA). Three-channel picture series were recorded (Intensity $I(x,y) \in [0,255]$, $x/y \in [0,511]$ pixels, 40× oil immersion objective). In channel 1, bright field images were recorded, whereas in channel 2, autofluorescence of mycorrhizal and non-mycorrhizal short roots of *N. obliqua* were visualised with a 385–470 nm bandpass filter. Channel 3 received light through a 560–615 nm bandpass filter, and was used to monitor enzymatically activated ELF-97 fluorescence intensities.

2.4. In vivo localisation and quantification of SBP activity by image processed quantitative fluorescence microscopy

Image processing routines to identify and separate specific regions of interest from the respective image background (i.e. object segmentation) were programmed in IDL (Interactive Data Language, Research Systems, Boulder, CO, USA). Segmentation of the mantle, of the entire ectomycorrhizae, and of the non-mycorrhizal short roots of *N. obliqua* were obtained by interactively selecting threshold values I_T in the intensity histograms of the corresponding bright field and autofluorescence channels 1 and 2. In channel 3, SBP-active centres were segmented by selecting I_T in the intensity histograms of the fluorescence

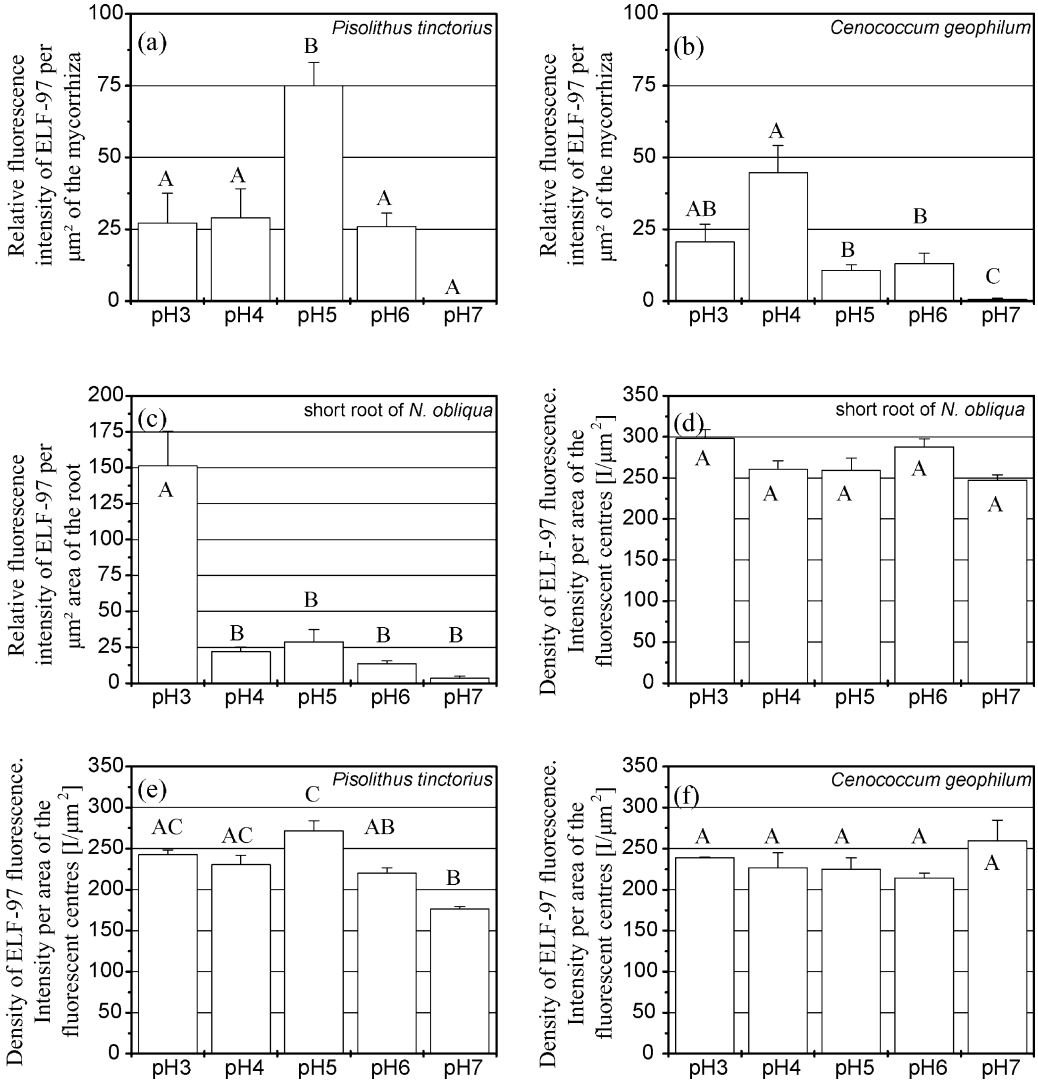

Fig. 2. The effect of the pH (3–7) on the SBP activity of mycorrhizal and non-mycorrhizal short roots. SBP activity was determined by image processed fluorescence microscopy and fluorescence activation of ELF-97. Columns represent means of 3–4 replicates ±SE. Data passed tests for normality (Kolmogorov–Smirnov) and equal variance (Levene–Median). Letters above bars indicate significant differences between treatments (Bonferroni's method, $p < 0.05$). (a,b) Relative intensity of ELF-97 fluorescence per µm² of the cross-section of the entire *N. obliqua–P. tinctorius* mycorrhizae (a) and of *N. obliqua–C. geophilum* mycorrhizae (b). (c) Relative intensity of ELF-97 fluorescence per µm² of the root surface of non-mycorrhizal short roots of *N. obliqua*. (d–f) Fluorescence density of ELF-97 [I/µm²] of the fluorescence centres for non-mycorrhizal short roots of *N. obliqua* (d), for *N. obliqua–P. tinctorius* mycorrhizae (e), and for *N. obliqua–C. geophilum* mycorrhizae (f).

activated substrate ELF-97. Segmentation of the respective regions was completed by successive treatment with opening and closing operators (Castleman, 1996). From the segmented surface areas and the calculated fluorescence intensities, the following parameters were derived: (i) the total intensity *I* of the fluorescent ELF-97 centres per area of the entire ectomycorrhiza and of the non-mycorrhizal short root [$I/\mu m^2$], (ii) the density of fluorescence of the activated ELF-97 centres per area of the fluorescent centres [$I/\mu m^2$], and (iii) the percentage of the SBP activity on the mantle in respect to the total SBP activity of ectomycorrhizae.

2.5. Data analysis

Normality of the data was assessed with Kolmogorov–Smirnov's test, equal variance with Levene–Median's method, and differences between treatments were assessed with an all pairwise multiple comparison method (Bonferroni) using SigmaStat (version 1.0, Jandel Scientific Software, San Rafael, CA, USA).

3. Results

3.1. Segmentation of mycorrhizal sub-structures from microscopic 3-channel images

The representative microscopic 3-channel picture in Fig. 1a contains the entire information which is needed for the subsequent image processing and data analysis. Fig. 1b shows the complete ectomycorrhiza, and Fig. 1c the mantle structure as they result from the segmentation procedures applied on the bright field and the autofluorescence images (channels 1 and 2 in Fig. 1a). In the same way, the non-mycorrhizal short roots of *N. obliqua* can be separated from its background (not shown). The area of the root cells of the ectomycorrhiza can by obtained by subtracting the surface of the mantle (Fig. 1c) from the entire ectomycorrhiza (Fig. 1b). Finally, Fig. 1d shows the area of the SBP-active centres, segmented from enzymatically activated ELF-97 fluorescence image (channel 3 in Fig. 1a). As it can be observed, ELF-97 fluorescence intensity is located on the Hartig net, the mantle, and on the root cells.

3.2. Determination of SBP activity in mycorrhizal and non-mycorrhizal short roots of N. obliqua under various pH conditions

As shown in Fig. 2a–c, the pH of the buffer influenced the SBP activity in *N. obliqua–P. tinctorius* ectomycorrhizae, in *N. obliqua–C. geophilum* ectomycorrhizae, and in non-mycorrhizal short roots of *N. obliqua*. The pH of the buffer not only affected the total fluorescence intensities of ELF-97 per μm^2 of the ectomycorrhiza surface (Fig. 2a,b), but also the fluorescence intensities per μm^2 of the root surface and the Hartig net in the same characteristic manner (data not shown). In Fig. 2a, the *N. obliqua–P. tinctorius* association showed an ELF-97 fluorescence maximum at pH 5, while the *N. obliqua–C. geophilum* association displayed a maximum at pH 4 (Fig. 2b). In Fig. 2c, the non-mycorrhizal short root of *N. obliqua* showed a high maximum at pH 3 (approx. 6 times higher than the fluorescence of the activated ELF-97 substrate at pH 5). Neither the non-mycorrhizal short root of *N. obliqua*, nor the ectomycorrhizal associations showed significant fluorescence intensities of ELF-97 at pH 7. While the data in Fig. 2a–c quantify the response of the SBP activity to pH-variations of the incubation buffer, the combination with the data in Fig. 2d–f reveals how the samples trigger this parameter. When the fluorescence intensities were based on the area of the fluorescent centres [$I/\mu m^2$] (Fig. 2d–f), there was very little (Fig. 2e) or no effect (Fig. 2d,f) of buffer pH on phosphatase activity.

As can be seen in Fig. 3, the percentage of the SBP activity on the mantle varied with the buffer pH for *P. tinctorius* and *C. geophilum* ectomycorrhizae. At pH 4–6, the dominant fraction of the SBP activity was located on the mantle of *N. obliqua–P. tinctorius* associations, while the location on the root cells was greater at pH 3 and 7 (Fig. 3a). In the *N. obliqua–C. geophilum* mycorrhizae, the peak SBP activity occurred at higher pH (5–6) and was lower than that measured in the *P. tinctorius* mycorrhizae. In response to

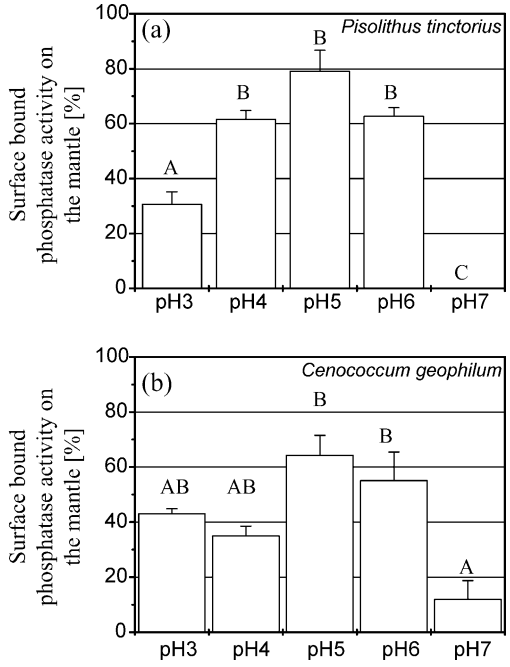

Fig. 3. The effect of the pH environment (3–7) on the percentage of the SBP activity on the mantle of *N. obliqua–P. tinctorius* mycorrhizae (a) and *N. obliqua–C. geophilum* mycorrhizae (b). SBP activity and mantle area were determined by image processed fluorescence microscopy and fluorescence activation of ELF-97. Columns represent means of 3–4 replicates ±SE. Data passed tests for normality (Kolmogorov–Smirnov) and equal variance (Levene–Median). Letters above bars indicate significant differences between treatments (Bonferroni's method, $p<0.05$).

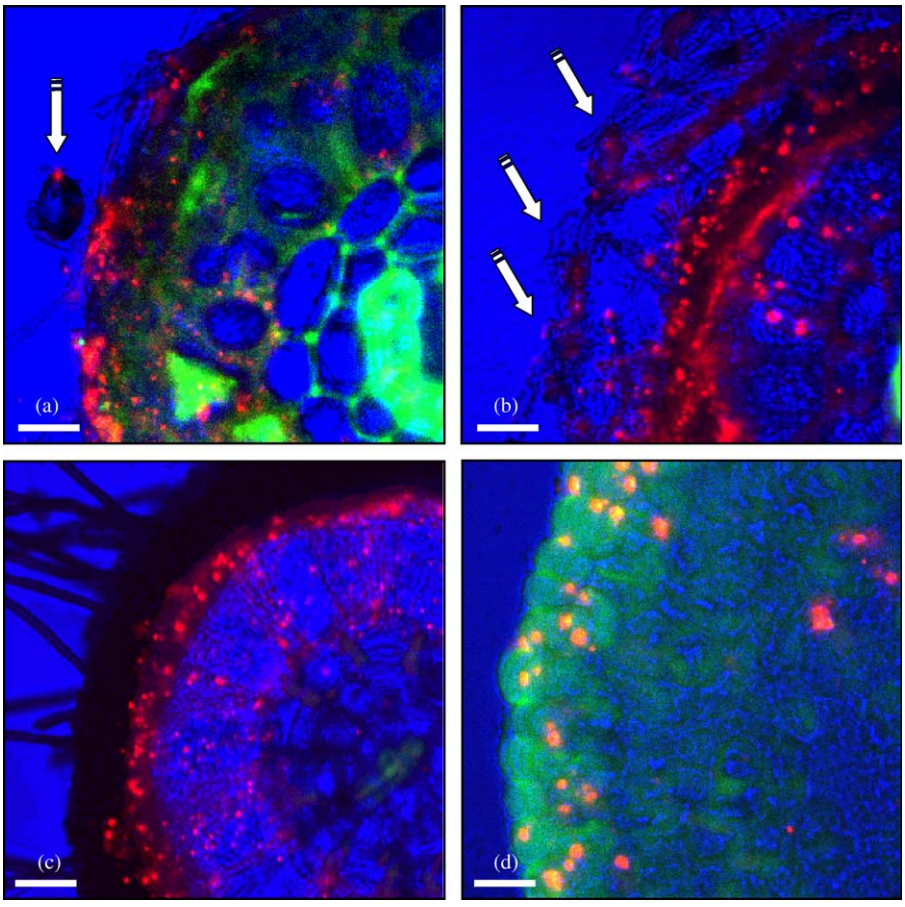

Fig. 4. Detailed images of cross-sections of *N. obliqua–P. tinctorius* mycorrhizae (a,b), of a *N. obliqua–C. geophilum* mycorrhiza (c), and of a non-mycorrhizal short root of *N. obliqua* (d). Channel 1: bright field picture (blue). Channel 2: 385–470 nm bandpass filter for autofluorescence of fungal mycelium and root cells (green). Arrows in (a) and (b) mark soil particles which are enclosed by fungal hyphae. Bars on the lower left edge of the images represent a distance of 15 μm. Fluorescence intensities were determined at pH 4 (a–c) and at pH 6 (d). (For interpretation of the reference to colour in this legend, the reader is referred to the web version of this article.)

the pH of the incubation buffer, the SBP activity on the mantle of *P. tinctorius* showed similar characteristics as the fluorescence activity per unit area of the mycorrhizae (Figs. 2a and 3a). This relationship was not observed for *N. obliqua–C. geophilum* mycorrhizae (Figs. 2b and 3b).

The fluorescent images in Fig. 4 reveal more details about the distribution of the SBP activity across the mantle. For *N. obliqua–P. tinctorius* ectomycorrhizae, SBP-active centres are distributed homogeneously across the mantle (Fig. 4a,b). Extramatrical hyphae can be identified which envelope individual clay particles and thereby establish a close contact surface between the substrate and the SBP-active centres (see arrows). These clay particles adhered tightly to the root by ectomycorrhizal mycelium. The mantle structure of *N. obliqua–C. geophilum* ectomycorrhizae is organised in a different form (Fig. 4c). All SBP-active centres of the mantle hyphae are located in close vicinity to the interface with the radical cells, and the emanating hyphae of *C. geophilum* did not exhibit SBP-active centres. In non-mycorrhizal roots, the SBP-active centres are located in proximity to the rhizodermis cells (Fig. 4d).

4. Discussion

The results presented here show that image processed quantitative fluorescence microscopy can be adopted for the simultaneous determination of qualitative and quantitative parameters of SBP activity in cross-sections of *N. obliqua–P. tinctorius* mycorrhizae, *N. obliqua–C. geophilum* mycorrhizae, and non-mycorrhizal short roots of *N. obliqua*. The dominant factor for the changes of the SBP activity resulted to be the number or the expansion of the segmented phosphatase active centres, because the fluorescence densities of the individual fluorescent centres show none or only small variations. In this context, the non-mycorrhizal short roots of *N. obliqua* and both ectomycorrhizae provide similar responses in respect to pH-variations of the incubation buffer. In isolated ectomycorrhizal fungi, varying environmental conditions (P-concentrations and buffer pH) induced two fundamentally different response patterns of the SBP activity (Alvarez et al., 2004). For *C. geophilum*, SBP activity was regulated primarily by the number of SBP-active centres on the hyphae. For *P. tinctorius* instead, the number of SBP-active centres per μm hyphal length

changed much less than the intensity of the SBP-active centres on the hyphae. The direct comparison to the data presented here shows that pH-induced changes of the SBP activity in isolated fungal mycelium of *C. geophilum* are similar to those observed in ectomycorrhizal associations with *N. obliqua*. For *P. tinctorius* instead, pH-induced changes of the SBP activity showed different characteristics in both systems. Evidently, the formation of ectomycorrhizae can change the pH-selective responses of the SBP activity in selected fungi. Finally, the formation of ectomycorrhizae not only affects the SBP activity of the fungal partner, but also those of the root cells of *N. obliqua*, because the maximum SBP activity of the unmycorrhized short root at pH 3 was not reflected by the mycorrhizal associations.

In conclusion, the observed heterogeneity in the localisation of the SBP-active centres challenges the assumption that an increased contact surface between mycorrhizae and soil particles necessarily implies higher absorption of nutrients (Häussling and Marschner,1989; Rousseau et al., 1994; Lynch, 1995). The absence of SBP-active centres on the emanating hyphae and on the periphery of the mantle of *C. geophilum* suggests that its physical contact surface is irrelevant in this context. Therefore, the relevance of the physical contact surface of the SBP-active centres to the soil particles must be controlled for each specific ectomycorrhizal association.

The application of the presented method is not limited to short roots of cultivated seedling, it could also be applied in combination with freshly extracted samples from experimental areas. The controlled variation of the pH under laboratory conditions can only be a first step to a better characterisation of the morpho-anatomical, physiological, and biochemical properties of SBP-activity in the rhizosphere. Further external factors with strong impact on phosphatases are P and N composition of the soil or temperature (Antibus et al., 1992; Kieliszewska-Rokicka, 1992; Joner and Johansen, 1999). Additionally, the spectrum of active phosphatases can vary within the same mycorrhizal fungus species according to the involved plant symbionts (Ho, 1989). Finally, the age of mycorrhizae or of plant symbionts can also influence the SBP activity (Antibus et al., 1986). Since no data have been published so far for the species presented here, our data stand on its own. The advantage of the introduced method to quantify and localise the catalytic activity of the SBP should stimulate further investigations in different associations. It should also be possible to extend its application to other important soil components such as rhizomorphs, further emanating elements or extraradical mycelium.

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