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Surface-bound phosphatase activity in living hyphae of ectomycorrhizal fungi of *Nothofagus obliqua*

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Abstract: We determined the location and the activity of surface-bound phosphomonoesterase (SBP) of five ectomycorrhizal (EM) fungi of *Nothofagus oblique*. EM fungal mycelium of *Paxillus involutus, Austropaxillus boletinoides, Descolea antartica, Cenococcum geophilum* and *Pisolithus tinctorius* was grown in media with varying concentrations of dissolved phosphorus. SBP activity was detected at different pH values (3–7) under each growth regimen. SBP activity was assessed using a colorimetric method based on the hydrolysis of p-nitrophenyl phosphate (pNPP) to p-nitrophenol phosphate (pNP) + P. A new technique involving confocal laser-scanning microscopy (LSM) was used to locate and quantify SBP activity on the hyphal surface. EM fungi showed two fundamentally different patterns of SBP activity in relation to varying environmental conditions (P-concentrations and pH). In the cases of *D. antartica, A. boletinoides* and *C. geophilum,* changes in SBP activity were induced primarily by changes in the number of SBP-active centers on the hyphae. In the cases of *P. tinctorius* and *P. involutus,* the number of SBP-active centers per μm hyphal length changed much less than the intensity of the SBP-active centers on the hyphae. Our findings not only contribute to the discussion about the role of SBP-active centers in EM fungi but also introduce LSM as a valuable method for studying EM fungi.

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INTRODUCTION

Phosphorus is one of the most important minerals for plant nutrition. At the same time, it is the most inaccessible soil nutrient (Holford 1997, Narang et al 2000). For this reason, plants have developed numerous morphological, physiological, biochemical and molecular adaptation strategies for its acquisition (Raghothama 1999). Among these strategies, the expression of P-cleaving enzymes (mainly phosphomonoesterases and phosphodiesterases) by mycorrhizas and EM fungi represents an important mechanism to increase P availability for plant symbionts (Jayachandran et al 1992, Smith and Read 1997, van Aarle et al 2001). Despite the numerous studies on phosphatase activity in EM fungal mycelium, little is known about adaptive changes in SBP activity in response to different soil conditions.

In this study, we mapped the distribution of SBP activity in mycelium of five EM partners of *Nothofagus obliqua* (Mirb.) Oerst: *Paxillus involutus* (Batsch : Fr.) Sing., *Austropaxillus boletinoides* (Sing.) Brsky & Jarosh, *Descolea antartica* Sing., *Cenococcum geophilum* Fr. and *Pisolithus tinctorius* (Pers.) Coker & Couch. SBP activity was determined with a colorimetric assay, based on the hydrolysis of pNPP to pNP + P, which is a well established method for quantification of phosphomonoesterase activity in EM fungi (Antibus et al 1992, Tibbett et al 1998). In addition, SBP activity was determined using a new approach; we combined confocal LSM, a simple staining procedure with the UV-sensitive fluorogenic substrate, enzyme-labeled fluorescence (ELF-97) and image processing routines. This technique enabled us to localize and quantify the SBP activity of the fungal mycelium at defined pH and phosphorus levels.

MATERIALS AND METHODS

Cultivation of EM fungi.—Cultures of the EM fungi *Paxillus involutus, Austropaxillus boletinoides* and *Descolea antartica* were obtained from fruiting bodies that were collected in temperate forests of *Nothofagus obliqua,* near Quita Calzón, 39°78′S, 73°02′W, Valdivia, X Region, Chile. Ectomycorrhi-

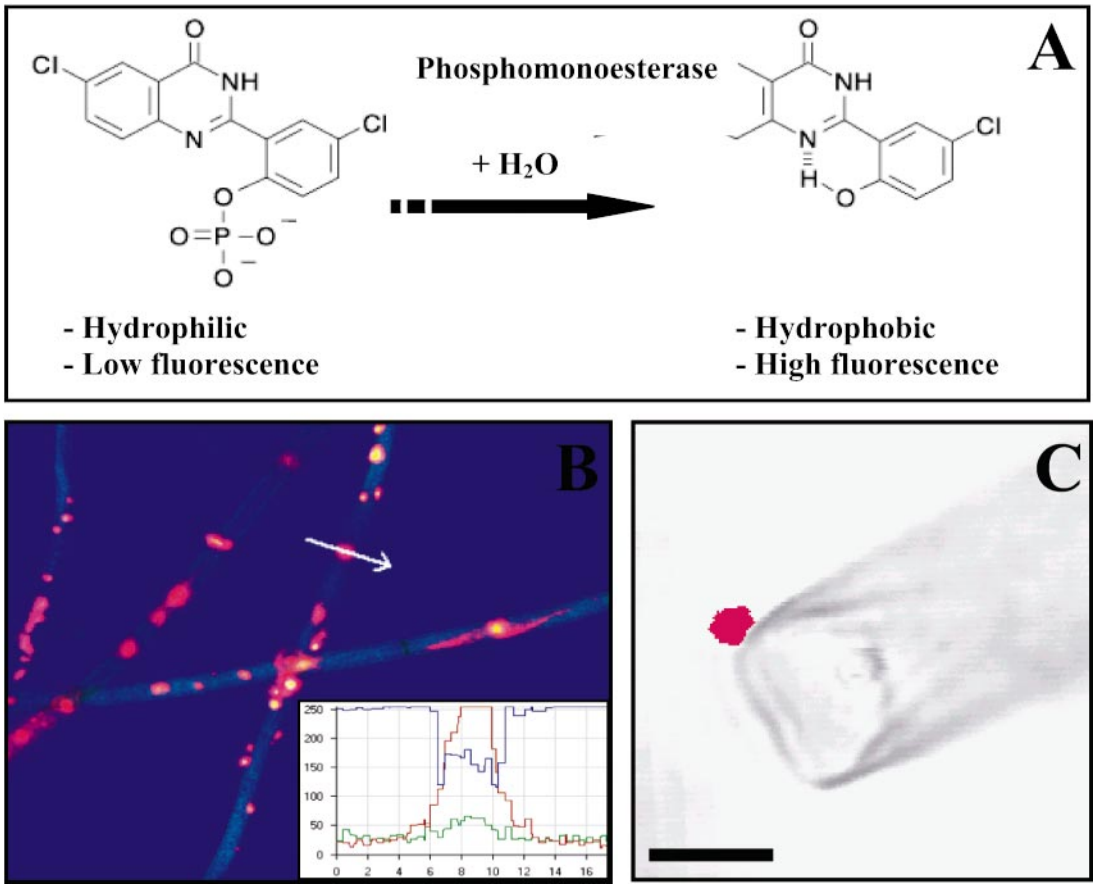

FIG. 1. Phosphomonoesterase driven activation and localization of fluorescent ELF-97 precipitate in hyphae of *C. geophilum*. A. Molecular structure of the hydrophilic, weakly fluorescent ELF-97 substrate, left, and the enzymatically activated, highly fluorescent hydrophobic ELF-97 precipitate, right. B. Three-channel confocal LSM image of in vivo stained hyphae of *C. geophilum*. Channel 1, bright field picture (blue); channel 2, 385–470 nm band pass filter for autofluorescence of fungal mycelium (green); channel 3, 560–615 nm band pass filter for enzymatically activated ELF-97 fluorescence (red); Inlet, three-channel fluorescence intensities (y-axis) on a μm scale (x-axis), following the indicated trajectory. C. Two-channel confocal LSM image of the localization of fluorescent ELF-97 precipitate on a transversal hyphal cut. Channel 1, bright field picture (black/white); channel 2, ELF-97 fluorescence (red). Black bar scales 5 μm.

zas of *Cenococcum geophilum* and *N. obliqua* also were collected at this site, and the fungi were isolated from EM mantle sections. *Pisolithus tinctorius* was obtained from cultures maintained at the Institute of Environmental Science and Technology, University of Bremen, Germany. All EM fungi were transferred from solid Melin-Norkrans (MMN) culture media (malt extract 5 g/L, d-glycose 10 g/L, $(NH_4)_2HPO_4$ 0.25 g/L, KH_2PO_4 0.5 g/L, $MgSO_4$ $7H_2O$ 0.15 g/L, $CaCl_2$ 0.05 g/L, Fe-EDTA 0.022 g/L, $NaCl_2$ 0.025 g/L, thymine HCl 1×10^{-4} g/L) (Molina and Palmer 1982) to 50 mL of liquid MMN media in culture flasks. Liquid MMN media contained 0 mM, 1.7 mM, 3.4 mM, and 5.6 mM of dissolved phosphate (5.6 mM = 0.5 KH_2PO_4 g/L + 0.25 $(NH_4)_2HPO_4$ g/L). Fungi were cultivated in the dark at 25 C and at pH 5. The cultivation period was 2 wk, as suggested by Straker and Mitchell (1986). After that period, fungi were harvested and SBP-related parameters were determined with a colorimetric method and quantitative fluorescent microscopy as described below.

Colorimetric quantification of SBP activity with pNPP.—SBP activity of all EM fungi were determined by a colorimetric

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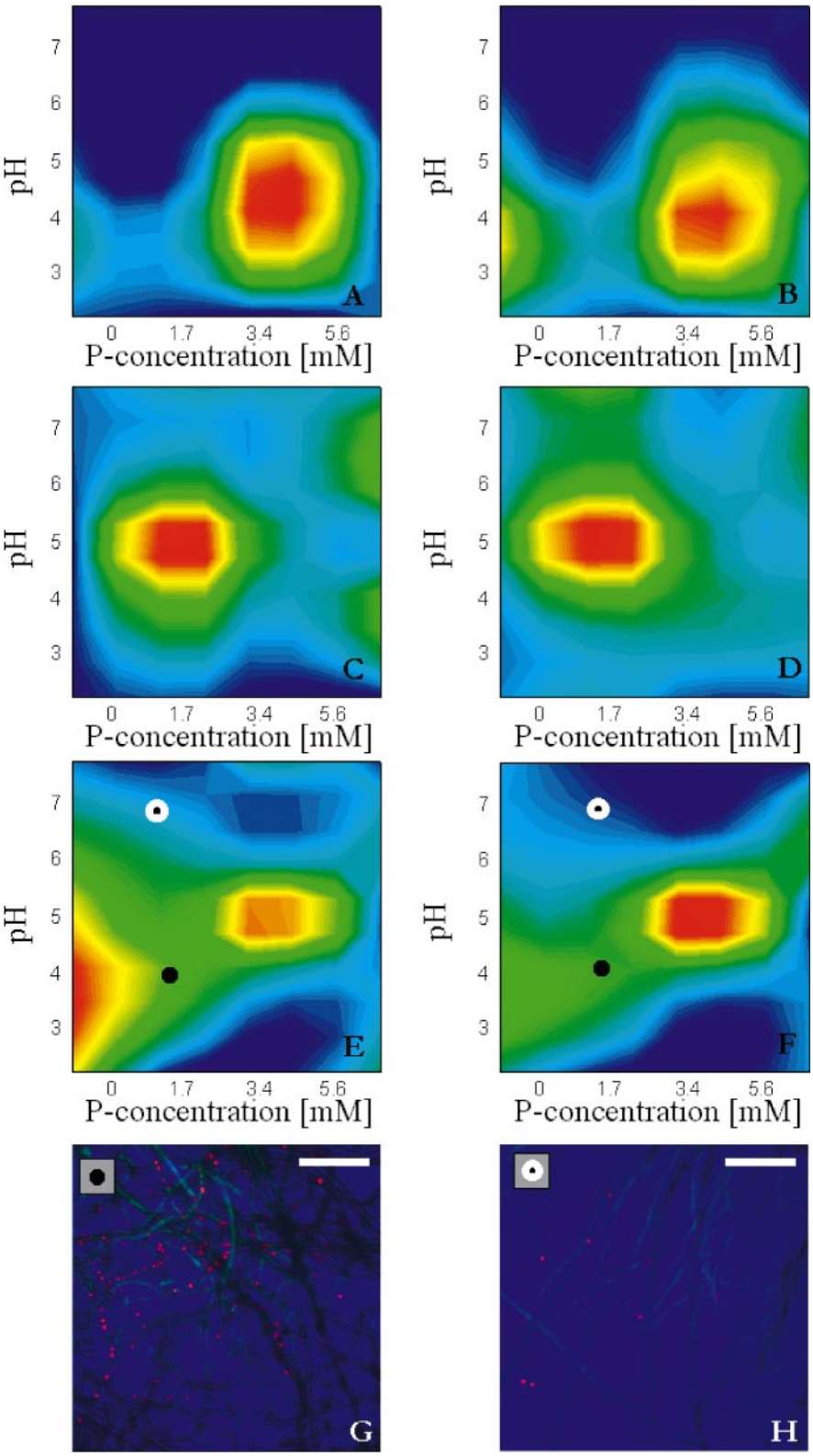
FIG. 2. Fluorescence intensity of ELF-97 precipitate and number of SBP-active centers on hyphae of *D. antartica, A. boletinoides* and *C. geophilum* as determined by image processed LSM. SBP activity per μm hyphal length is plotted in bivariate color-coded histograms for *D. antartica* (A), *A. boletinoides* (C) and *C. geophilum* (E). The number of fluorescent centers of precipitated ELF-97 substrate per μm hyphal length is plotted for *D. antartica* (B), *A. boletinoides* (D) and *C. geophilum* (F). The color code (black-blue-light blue-green-yellow-red) covers the parameter (minimum to maximum) of the respective



datasets. Three separately grown fungal samples were prepared and analyzed for each experimental condition. Black and white circles in E and F define experimental conditions for the representative three-channel fluorescence images of the hyphae of *C. geophilum* (G: pH 4/P 1.7 mM; H: pH 7/P 1.7 mM). In these images, bright field intensities (blue) were combined with autofluorescence of hyphae (green) and fluorescence intensities of the enzymatically activated ELF-97 substrate (red). White bars in G and H scale 30 μm.

standard method based on p-nitrophenyl phosphate (pNPP) (Merck 1.06850.0025, Germany) according to Tibbett et al (1998). Buffer solutions containing Tris(hydroximethyl) aminomethane (3.025 g/L), maleic acid (2.9 g/L), citric acid (3.5 g/L), boric acid (1.57 g/L), and 1 M NaOH (122 mL/L) were adjusted to pH 3–7 by 1 M HCl. SBP-active centers hydrolyse pNPP to p-nitrophenol phosphate (pNP) + P, which induces a pronounced shift in the absorption wavelength from 315 nm (pNPP) to 407 nm (pNP). Five independent experiments were conducted for each pH value and P-condition.

In vivo imaging of SBP activity by confocal microscopy of ELF-97 fluorescence.—SBP activity of EM fungi, cultured at different P-concentration (0 mM, 1.7 mM, 3.4 mM and 5.6 mM) were determined at different pH values (3–7) by image-processed quantitative fluorescence microscopy using the fluorogenic substrate ELF-97 (E-6588, Molecular Probes, Leiden, Netherlands; 5 mM in sterile water). Fluorescence imaging was performed by LSM (model LSM 510, ZEISS, Göttingen, Germany), equipped with a 364 nm wavelength UV-argon-ionlaser (model Enterprise II-653, Coherent, Santa Clara, California). Fungal mycelium was stained with the hydrophilic, weakly fluorescent ELF-97 substrate, which is converted into a highly fluorescent hydrophobic precipitate upon enzymatic cleavage of the phosphate residue by phosphomonoesterases (van Aarle et al 2001; FIG. 1A). After the staining procedures (see below), fungal hyphae were mounted on microscopic object slides and 3-channel picture series were recorded (63× objective, excitation at 364 nm, Intensity $I(x, y) \in [0, 255]$, $x/y \in [0, 511]$ pixels). Bright field images were detected in channel 1, whereas autofluorescence of fungal mycelium was recorded with a 385–470 nm band pass filter in channel 2. Channel 3 received light through a 560–615 nm band pass filter monitoring enzymatically activated ELF-97 fluorescence intensities (FIG. 1B, C).

Localization of SBP-active centers on fungal hyphae.—Mycelium was removed from culture medium with tweezers and rinsed with buffer solution (pH 5). Pieces of mycelium (Ø ~ 2 mm, ~0.5 mm thick) were separated with scalpels and transferred to microcentrifuge tubes (100 μL; Eppendorff). 5 μL of ELF-97 phosphatase substrate solution, previously adjusted to pH 5 by the corresponding buffer solution (2:10 v/v, buffer/ELF-97 stock solution), were added to the mycelium. After 1 h incubation, mycelium was removed from the ELF-97 staining solution, rinsed with buffer solution and embedded in 4% agar. Finally, slices of 30 μm were cut with a Vibratom (model 1000 Classic, GaLa Gabler Labor Instrumente, Bad Schwalbach, Germany), transferred to microscopic cover slides and analyzed with LSM to determine the precise localization of SBP-active centers on the hyphal structures (FIG. 1C).

Statistical quantification of SBP activity on fungal hyphae by image processed quantitative fluorescence microscopy.—Samples of mycelium were obtained as described above. Again, 5 μL of ELF-97 phosphatase substrate solution, previously adjusted to pH 3, 4, 5, 6 and 7 by the corresponding buffer solution (2:10 v/v, buffer/ELF-97 stock solution), were added to the mycelium. After 15 min incubation, samples were rinsed with buffer solution at the corresponding pH (3–7) and transferred to small notches, manually ground into the surface of microscopic object slides (radius: 1.5–2 mm; depth: ~0.5 mm). Notches were covered with buffer solution (pH 3–7) and sealed with cover slides using nail polish. Samples subsequently were scanned on the LSM as described above. Image processing routines for the separation of fungal hyphae and of individual fluorescent ELF-97 precipitate centers from their respective image background (so-called segmentation) were programmed in IDL (Interactive Data Language, Research Systems, Boulder, Colorado). Segmentation of fungal hyphae (channel 2) and segmentation of individual fluorescent ELF-97 precipitate centers (channel 3) was achieved by fixing threshold values I_T in the intensity histograms of the corresponding images. Both segmentation procedures were completed by successive treatments with opening/closing operators. From the segmented fungal hyphae and fluorescent ELF-97 centers, we calculated: (i) the total fluorescence intensity I of the ELF-97 centers per μm fungal hypha ($I \cdot \mu m^{-1}$) and (ii) the average number N of fluorescent ELF-97 centers per μm fungal hypha ($N \cdot \mu m^{-1}$).

Three separately grown fungal samples were prepared for each experimental condition (pH/P-concentration). For each experiment, 10 three-channel images were scanned and analyzed. From this data, mean values were derived and plotted in FIGS. 2, 3 and 4.

RESULTS

The basic molecular principle of the phosphomonoesterase-driven conversion of the weakly fluorescent hydrophilic ELF-97 substrate into the highly fluorescent hydrophobic ELF-97 precipitate is summarized in FIG. 1A. FIGURE 1B shows a representative 3-channel image of the distribution of the activated ELF-97 precipitate on fungal hyphae of *C. geophilum* at pH 5 (P-concentration during the growth period was 3.4 mM). The figure inlet covers the three-channel intensity distribution of the arrow across a single SBP-active center on a fungal hypha. A representative detail of the localization of a fluorescent ELF-97 precipitate on a transversal cut across the hypha of *C. geophilum* is revealed at higher magnification in FIG. 1C. The detail confirms that phosphomonoesterase-active centers are localized on the hyphal surface. All other fungi investigated in this study provided similar fluorescence staining characteristics with respect to the localization of the precipitated ELF-97 substrate.

Before the determination of SBP activity in fungal mycelium by the ELF-97 staining technique, several control experiments were performed to justify the use of ELF-97 as a reliable substrate for the quantification of SBP activity (data not shown): (i) in the case of all fungal samples, the enzymatic reaction was not substrate limited during an incubation of 15

min—during this period, the fluorescence intensities of the centers increased linearly; (ii) in the reaction volumes, the pH remained stable during the incubation period; (iii) once activated, the quantum yield/fluorescence intensity of the ELF-97 precipitate was not affected by different buffer solutions (pH 3–7)—these results agree with earlier observations, where precipitated ELF-97 centers were not dissolved at pH below 8.5 (van Aarle et al 2001); (iv) the precipitated ELF-97 centers provided high photostability (negligible photobleaching); and (v) the two-dimensional response pattern of the SBP activity determined with ELF-97 overlaid with the response pattern of the SBP activity derived with the established method using pNPP.

In the cases of *D. antartica, A. boletinoides, C. geophilum, P. involutus* and *P. tinctorius,* the intensities and the number of SBP-active centers on fungal hyphae were derived by image-processed quantitative LSM and plotted in color-coded bivariate histograms (FIG. 2A–F; FIG. 3A, B, E, F). The colors (black-blue-light blue-green-yellow-red) cover the parameter of the data, ranging from the respective minimum to the maximum values. Representative images were chosen to show differences in the activity and in the structural organization of the SBP centers on fungal hyphae in response to different P-concentrations during the growth period of the fungi and different pH conditions during the measurements (FIG. 2G, H; FIG. 3C, D, G, H).

For all fungi, the maximum and minimum values of the respective datasets are plotted in FIG. 4. ELF-97 detects changes of the SBP activity for different experimental conditions or for different fungi. However, the unit is based on arbitrary fluorescence intensities, determined under fixed conditions with the same experimental set-up by LSM. The pNPP method instead can be calibrated to report μM $[pNP]\cdot g^{-1}\cdot h^{-1}$; it assesses enzymatic activity in a direct manner. Because the two-dimensional response pattern of the SBP activity determined with ELF-97 and pNPP were revealed to be similar (see [v] above), we used the maximum and minimum values of the pNPP protocol in FIG. 4 to report activity units that can be compared to data presented by other authors (Antibus et al 1986, 1992, Tibbett et al 1998).

In the cases of *D. antartica, A. boletinoides* and *C. geophilum* (FIG. 2A, C, E) the two-dimensional response pattern of the SBP activity overlay the pattern of the number of SBP-active centers per μm of the fungal hyphae (FIG. 2B, D, F). For these species, the changes in the number of SBP-active centers per μm of the fungal hyphae (maximum and minimum values of hatched columns in FIG. 4) exceeded the changes in the SBP activities (white columns). In the cases of *D. antartica* and *A. boletinoides,* the SBP activities (ranges between maximum and minimum as well as mean values) were similar. They outnumbered the SBP activity of *C. geophilum* (white columns, FIG. 4). In terms of the number of SBP-active centers, *A. boletinoides* provided the highest absolute mean values and flexibility (ranges between maximum and minimum values) compared to all other fungi (hatched columns in FIG. 4). In FIG. 2G–H, we show two representative fluorescence images for the hyphae of *C. geophilum,* which are similar to those of *D. antartica* and *A. boletinoides.* It is obvious from these images that the ELF-97 fluorescence intensity/phosphatase activity at a P-concentration of 1.7 mM does not change notably between pH 4 (FIG. 2G related to the black symbols in FIG. 2E, F) and pH 7 (FIG. 2H related to the white symbols in FIG. 2E, F). The number of SBP-active centers per μm hyphal length determines the changes of the SBP activity in these fungi.

In contrast to the behavior of *D. antartica, A. boletinoides* and *C. geophilum* (FIG. 2), the two-dimensional patterns of the SBP activity for *P. involutus* and *P. tinctorius* differed from the pattern based on the number of SBP-active centers per μm hyphal length (FIG. 3). In addition, *P. involutus* and *P. tinctorius* showed smaller differences between the maximum and minimum values of the number of SBP-active centers per μm versus the maximum and minimum values of the SBP activities (maximum and minimum values of hatched versus the white columns in FIG. 4). The maximum number of SBP-active centers per μm was only slightly higher for *P. tinctorius* than for *P. involutus,* while the maximum SBP activity for *P. tinctorius* outnumbered more than threefold the values determined for *P. involutus.* In addition, *P. tinctorius* provided the highest mean values of SBP activity compared to all other fungi (FIG. 4). As can be observed in the representative fluorescence images of the mycelium of *P. involutus,* the SBP activity and the number of SBP-active centers per μm hyphal length changed independently, showing no obvious correlation between these parameters (FIG. 3C, D). In the case of *P. involutus,* a small number of SBP-active centers per μm hyphal length showed high phosphatase activities at P-concentrations of 1.7 mM and pH 4 (FIG. 3C, black symbols in FIG. 3A, B), while a large number of SBP-active centers showed little phosphatase activity at a P-concentration of 5.6 mM and pH 5 (FIG. 3D, white symbols in FIG. 3A, B). In the case of *P. tinctorius* however, the number of SBP-active centers per μm hyphal length remained constant over a wide range of experimental conditions while the activity of individual SBP centers showed high variations (compare conditions for a P-concen-

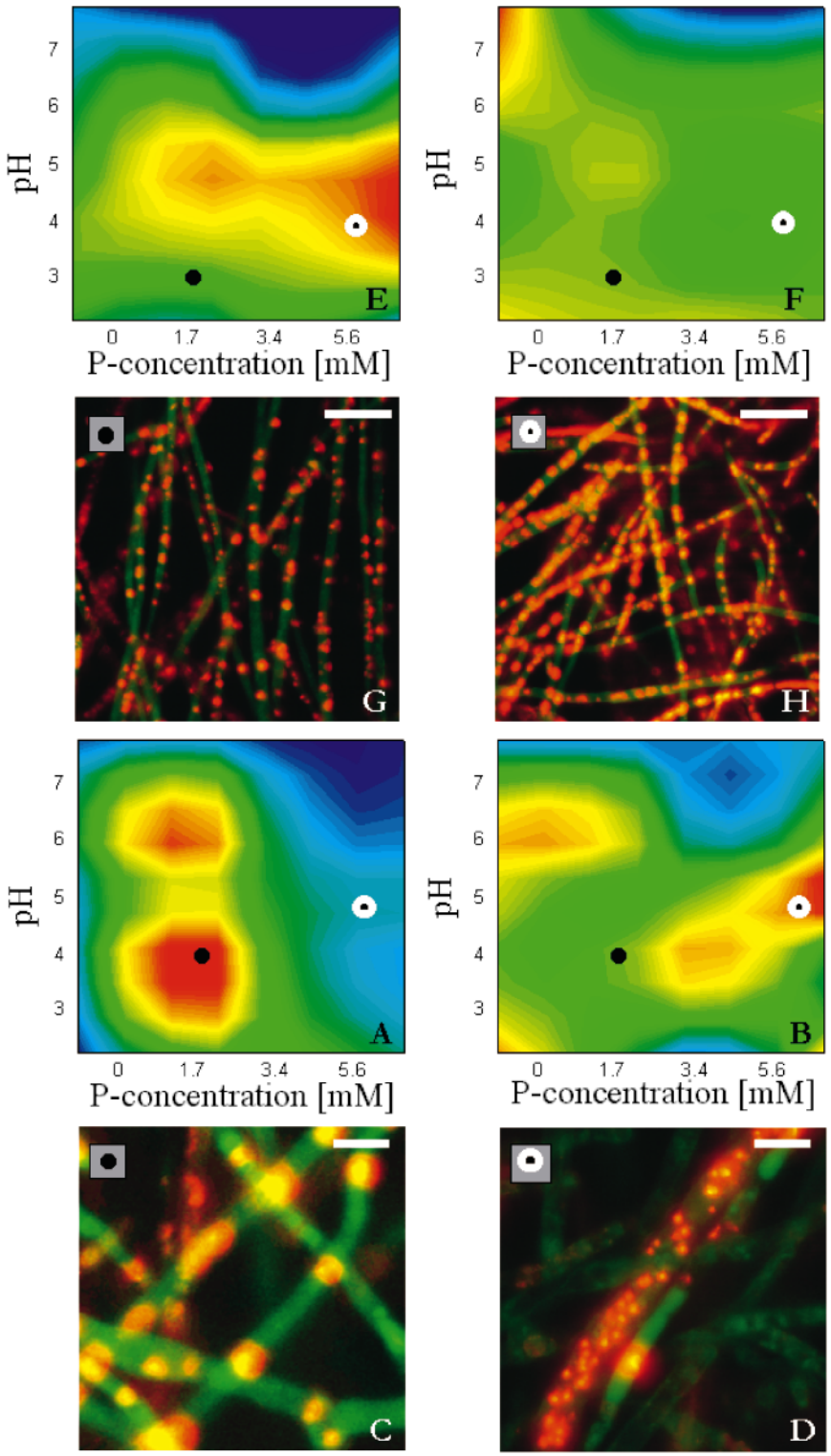


FIG. 3. Fluorescence intensity of ELF-97 precipitate and number of SBP-active centers on hyphae of *P. involutus* and *P. tinctorius* as determined by image processed LSM. SBP activity per μm hyphal length is plotted in bivariate color-coded histograms for *P. involutus* (A) and *P. tinctorius* (E). The number of fluorescent centers of precipitated ELF-97 substrate per μm hyphal length is plotted for *P. involutus* (B) and *P. tinctorius* (F). The color code (black-blue-light blue-green-yellow-red) covers the parameter (minimum to maximum) of the respective datasets. Three separately grown fungal samples were prepared and analyzed for each experimental condition. Black and white circles in the bivariate histograms define experimental conditions for the representative three-channel fluorescence images of the hyphae of *P. involutus* (C: pH 4/P 1.7

tration of 1.7 mM and pH 3 with 5.6 mM and pH 4 in FIG. 3G, H, black and white symbols in FIG. 3E, F).

As far as the location of the maximum activity of SBP activity was concerned, *D. antartica, C. geophilum, A. boletinoides* and *P. tinctorius* showed a single maximum, varying between pH 4 and 5. *P involutus* instead showed two maxima: one at pH 4 and another at pH 6. In terms of the P-concentration, *D. antartica* and *C. geophilum* showed a maximum at P = 3.4 mM, *A. boletinoides* and *P. involutus* at P = 1.7 mM and *P. tinctorius* showed a plateau-like maximum between P = 1.7–5.6 mM (FIG. 2A, C, E; FIG. 3A, E).

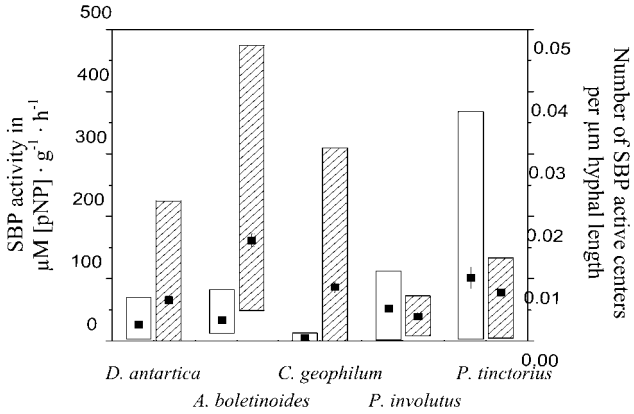


FIG. 4. Mean values (black squares), maximum values and minimum values of SBP activity as determined with pNPP (upper and lower values in white columns) and of the number of fluorescent SBP-active centers per µm hyphal length (upper and lower values in hatched columns) for *D. antartica, A. boletinoides, C. geophilum, P. involutus* and *P. tinctorius*. Maximum, minimum, mean values and corresponding standard errors were derived from datasets in FIGS. 2 and 3 for the number of fluorescent SBP-active centers. The SBP activities based on pNPP were determined from datasets equivalent to FIGS. 2 and 3, on the basis of five independent experiments.

DISCUSSION

Considering the important role of SBP activity in plant growth, it is surprising that many aspects of its function are unknown or only poorly understood. The established methodologies for the investigation of the SBP activity in fungal mycelium and in mycorrhizas can be divided roughly into quantitative and qualitative methods. A frequently used quantitative method for the determination of different phosphatase-active fractions is based on a simple colorimetric assay. While the colorimetric substrate pNPP determines the specific activity of phosphomonoesterases (Antibus et al 1992, Tibbett et al 1998), the cleavage of bis-pNPP is determined by the activity of phosphodiesterases (Antibus et al 1997). On the other hand, previous studies based on microscopic techniques have been focused solely on the localization of phosphatases. While Tisserant et al (1993) visualized the location of alkaline phosphatases using Fast Blue RR salt in combination with conventional light microscopy, van Aarle et al (2001) introduced the fluorogenic substrate ELF-97 for the reliable visualization of enzymatically active phosphomonoesterase centers by fluorescence microscopy in the UV range (see FIG. 1A). The first observations that microscope-based laser-scanning cytometry yielded quantitative data comparable to data yielded by flow cytometry were reported more than a decade ago (Kamentsky and Kamentsky 1991). Since then, many different applications taking advantage of structural and quantitative fluorescence microscopy have emerged in science (Fanani et al 2002, Härtel et al 2003).

In this study, we have presented a combination of structural and quantitative data for the SBP activity in the mycelium of five EM fungi and evaluated changes in enzyme activity in relation to P and pH. The combination of confocal fluorescence microscopy and image processing routines provides information on both the activity of the SBP ([fluorescence intensity of activated ELF-97]/[hyphal length in µm]), and the number of SBP-active centers per unit of hyphal length. It must be considered that the SBP-active centers covered by fungal hyphae could not be counted with our method. But, because ELF-97 centers distribute isotropically on the surface of the fungal hyphae, the frequency of the accessible centers on the hyphae represents a valid approximation of the overall changes of the number SBP-active centers on the hyphae. McElhinney and Mitchell (1993) quantified different fractions of fungal phosphomonoesterase activities in four EM isolates, among them *P. involutus*. Apart from the extracellular enzyme activities, their results showed that less than 4% of the total nonsoluble enzyme activity corresponded to the cytoplasmatic fraction. The vast majority of the phosphomonoesterase activity however was detected in the surface-bound fraction, located at the outer cell wall and membrane (see FIG. 1B, C).

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mM; D: pH 5/ P 5.6 mM) and *P. tinctorius* (G: pH 3/P 1.7 mM, H: pH 4/P 5.6 mM). In these images, bright field intensities (blue) were combined with autofluorescence of hyphae (green) and fluorescence intensities of the enzymatically activated ELF-97 substrate (red). In C and D, white bars scale 7 µm. In G and H, white bars scale 15 µm.

As becomes obvious from the bivariate histograms of FIGS. 2 and 3, the examined mycorrhizal partner of *N. obliqua* can be characterized by two fundamentally different response patterns with respect to P and pH: (i) in the cases of *D. antartica, A. boletinoides* and *C. geophilum,* the color-coded histograms showing SBP activity/hyphal length and the number of fluorescent centers are almost identical (cf. FIG. 2A, B, FIG. 2C, D, FIG. 2E, F); (ii) in the cases of *P. involutus* and *P. tinctorius,* the histograms are completely different (cf. FIG. 3A, B, FIG. 3E, F).

In group (i), the changes in SBP activity of the fungal hyphae parallel the number of SBP-active centers per μm hyphal length, suggesting that changes in enzyme activity are induced primarily by changes in the number of SBP-active centers on the hyphae. In all experiments, EM fungi were grown at different P-concentrations for 2 wk before the measurements. A growth period of 2 wk is sufficient for the adaptation of cultured fungi to different P-concentrations, according to Straker and Mitchell (1986). On the other hand, variation of the pH condition was controlled only during the 15 min of the experimental incubation with the fluorogenic substrate ELF-97 and during measurements. Considering the different time scales, it is important to say that there were no fundamental differences in the response of the SBP activity in combination with the frequency of SBP-active centers. With respect to pH variations at constant P, or in relation to varying P-concentrations at constant pH, the changes of the SBP activity are basically the same. This observation supports the conclusion that *D. antartica, A. boletinoides* and *C. geophilum* adapt to different levels of P during the growth period by changing the number of SPBA centers on their hyphae. These centers are pH selective and develop optimum activity at specific pH (*D. antartica* at pH 4–5, *A. boletinoides* and *C. geophilum* at pH 5). In other words, the three fungal species selectively express pH-sensitive enzymes in response to different levels of P-nutrition.

In contrast to group (i), fungi in group (ii) exhibited no obvious correlation between the number of SBP-active centers per μm hyphal length and the SBP activity of the fungal hyphae for *P. involutus* and *P. tinctorius.* Instead, two different phenomena can be observed:

P. involutus. It adapts to varying P/pH conditions not only by changes in the number of SBP-active centers but also by changes of the SBP activity. Both parameters vary in a noncorrelated manner. At a P-concentration of 1.7 mM, SBP-active centers strongly change the SBP activity (pH maxima at pH 4 and 6), while the number of SBP-active centers at pH 6 is higher than at pH 4 (FIG. 3A, B). In contrast, while the number of SBP-active centers at pH 4 is lower at P-concentrations of 1.7 than 3.4 mM, the SBP activity of the fungal hyphae is higher.

P. tinctorius. The number of SBP-active centers per μm hyphal length remained constant over an expanded range of varying P-/pH conditions (FIG. 3F). The adaptation of *P. tinctorius* to varying P/pH conditions occurred mainly through extensive changes in phosphatase activity within a constant number of SBP-active centers (FIG. 3G, H). These results could be explained by the assumption that the SBP-active centers of *P. tinctorius* contain pH-sensitive enzymes, which reach their maximum activity between pH 4 and 5.

The differences in the response pattern to varying P/pH conditions between groups (i) and (ii) also are supported by the results shown in FIG. 4. In group (i), the absolute variations in the number of SBP-active centers clearly dominate the changes in the SBP activity. In group (ii), this condition is reversed. Small changes in the number of SBP-active centers are dominated significantly by pronounced fluctuations in SBP activity.

In conclusion, the adaptations of EM fungi in response to P-concentration and pH are species specific. The sensitivity and the flexibility of the responses are surprising, regarding the small variations in P and pH and the brief periods of exposure to different pH values. With respect to the variations in pH, the fluctuations of the SBP activity and the location of its maximum values are comparable to previously published results (Antibus et al 1986, 1992, McElhinney and Mitchell 1993). As far as the responses to changing P-concentrations during the growth period are concerned, inverse correlation with the SBP activity were reported (Alexander and Hardy 1981, Pacheco et al 1992) but other authors contradict this hypothesis (Straker and Mitchell 1986). In this aspect, our data add additional information to a nonuniform background and have to stand on their own.

Apart from the results discussed above, it should be said that the staining procedure for the SBP-active centers with the fluorogenic substrate ELF-97 can be performed easily and reliably in living sample material. Quantitative and qualitative characterization of the SBP activity in fungal samples, extracted directly from the natural habitat, and the possible extension of the method to roots, mycorrhizal and bacterial soil components will provide more information about this poorly understood system in the future.

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