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NEW PERSPECTIVES ON THE DETERMINATION OF PHOSPHATASE ACTIVITY IN ECTOMYCORRHIZAE OF *NOTHOFAGUS OBLIQUA* FORESTS IN SOUTHERN CHILE

NUEVAS PERSPECTIVAS EN LA DETERMINACION DE LA ACTIVIDAD DE LA FOSFATASA EN ECTOMICORRIZAS DE NOTHOFAGUS OBLIQUA *EN BOSQUES DEL SUR DE CHILE*

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ABSTRACT

We present a method to localise and quantify surface-bound phosphatase activity (SBPA) in mycorrhizal fungi and mycorrhizae of *Nothofagus obliqua* (Mirb.) Oerst., using image processed confocal fluorescent microscopy. ELF-97 is a hydrophilic substrate which turns into a strongly fluorescent precipitate upon activation by phosphomonoesterases. In fungal mycelium, this technique has recently been approved by comparison with a standard method (p-nitrophenyl-phosphate). The microscopic technique based on ELF-97 revealed that *Paxillus involutus* (Batsch: Fr.) Sing. and *Austropaxillus boletinoides* (Sing.) Brsky. & Jarosch provide different adaptive strategies to changing phosphate concentrations and different pH (3-7). We also analysed SBPA in four mycorrhizal associations of *N. obliqua* and found that the organisation of the mantle played an essential role regarding the SBPA. In general, mycorrhizal roots shifted SBPA from the root to the mantle. In this context, *Pisolithus tinctorius* (Pers.) Coker & Couch proved to be the most relevant mycorrhiza partner for *N. obliqua* by increasing significantly the overall SBPA of the mycorrhiza in respect to non-mycorrhizal roots. In conclusion, ELF-97 fluorescence microscopy in combination with image processing routines determined SBPA and revealed phosphorus (P) adaptation strategies of mycorrhizal fungi and mycorrhizae on a structural-physiological level.

KEYWORDS: Confocal microscopy, ectomycorrhizae of *Nothofagus obliqua*, ELF-97, phosphatase activity, quantitative fluorescence microscopy.

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RESUMEN

Nosotros presentamos un método basado en la microscopía confocal de fluorescencia y en el análisis de imágenes, para localizar y cuantificar la actividad de la fosfatasa superficial (SBPA) en hongos ectomicorrícicos y en ectomicorrizas de *Nothofagus obliqua* (Mirb.) Oerst. El sustrato ELF-97 es hidrofílico, el cual se desarrolla en un precipitado altamente fluorescente bajo su activación a través de fosfomonoesterasas. Recientemente esta técnica ha sido aprobada mediante comparaciones con un método estándar (fosfato de p-nitrofenilo) realizadas en micelio fúngico. La técnica microscópica con ELF-97 reveló que *Paxillus involutus* (Batsch: Fr.) Sing. y *Austropaxillus boletinoides* (Sing.) Brsky. & Jarosch poseen diferentes estrategias de adaptación frente a distintas concentraciones de fosfatos y pH (3-7). Nosotros analizamos la SBPA en cuatro asociaciones micorrícicas de *N. obliqua* y encontramos que la organización del manto juega un rol esencial con respecto a la SBPA. En general, las raíces micorrizadas transfieren la SBPA de la raíz hacia el manto. En este contexto, *Pisolithus tinctorius* (Pers.) Coker & Couch demostró ser el hongo ectomicorrícico más relevante para *N. obliqua*, al incrementar significativamente el

total de la SBPA de la micorriza en relación a la raíz sin micorrizar. En conclusión, a través de la microscopía confocal de fluorescencia basada en el sustrato ELF-97 y su combinación con el procesamiento de imágenes es posible, tanto en hongos micorrícicos como en micorrizas, determinar la SBPA y revelar las estrategias de adaptación con respecto al fósforo (P) a un nivel fisiológico-estructural.

PALABAS CLAVES: Actividad fosfatasa, ectomicorrizas, ELF-97, microscopía confocal, microscopía cuantitiva de fluorescencia

INTRODUCTION

Surface-bound phosphatase activity (SBPA) of fungi, roots, and mycorrhizae is an important parameter which describes the ability of plants to catalyse phosphorous (P) mineralisation. SBPA of mycorrhizal associations is of great interest in areas of limited P supply, because it permit access to sources of phosphate which are inaccessible for plants under isolated conditions (Smith & Read 1997). This holds especially in soils of volcanic origin, which represent a natural sink for P due to their highly active soil components (Scheffer & Schachtschabel 1998). In the humid temperate forests of southern Chile, developed on volcanic soils, ectomycorrhizal *Nothofagus* species are among the most widespread overstorey dominants. However, little is known so far about the importance of mycorrhizal associations for the P-supply of *Nothofagus* under different soil conditions (Veblen *et al*. 1996, Donoso *et al*. 1999).

The present work evaluates the relevance of several ectomycorrhizae for the P-supply of *Nothofagus obliqua* (Mirb.) Oerst., introducing a new method for the determination of SBPA of fungal hyphae of *Paxillus involutus* (Batsch: Fr.) Sing. and *Austropaxillus boletinoides* (Sing.) Brsky. & Jarosch. The method developed is based on enzyme activated fluorescence of ELF-97 substrate (van Aarle *et al*. 2001) and image processed confocal Laser-Scanning-Microscopy. In addition to the determinations in fungal hyphae, we applied this technique on non-mycorrhizal root systems of *N. obliqua* and compared the results to the mycorrhizae of *P. involutus*, *Cenococcum geophilum* Fr., *Pisolithus tinctorius* (Pers.) Coker & Couch and *Descolea antartica* Sing.

MATERIALS AND METHODS

CULTIVATION OF FUNGI AND MYCORRHIZAE OF *N. OBLIQUA*

Ectomycorrhizal (EM) fungi *Paxillus involutus* and *A. boletinoides* were grown in liquid Modified Melin-Norkrans media (Molina & Palmer 1982). Fungi were cultivated in the dark at 25°C and pH = 5 with 0, 30, 60, and 100 % of dissolved phosphate (100 % = 0.5 g KH_2PO_4 l^{-1} + 0.25 g $(NH_4)_2HPO_4$ l^{-1}). Fungi were cultivated for a period of two weeks in accordance with Straker & Mitchell (1986). Roots and mycorrhizal associations of *N. obliqua* with *P. involutus*, *C. geophilum*, *P. tinctorius* and *D. antartica* were cultivated in mini-rhizotrons.

LOCALISATION AND QUANTIFICATION OF SURFACE-BOUND PHOSPHATASE ACTIVITY

EM fungi, non-mycorrhizal roots, and EM of *N. obliqua* were harvested and incubated with ELF-97 substrate solution for 15 min (Alvarez *et al*. 2002). After washing, fungal hyphae were observed directly with confocal Laser-Scanning Microscopy (LSM) (LSM 510, ZEISS, Göttingen, Germany) in specially designed microscopic object slides. Roots and mycorrhizae were embedded in agar at 40°C. After cooling, fractions were cut with a vibratom (model 1000, Lancer Sherwood Medical Company) into sections of 30 µm and observed with LSM, equipped with a 364 nm wavelength UV-argon-laser (model Enterprise II-653, Coherent, Santa Clara, CA, USA). Three-channel picture series were recorded (see Fig. 1e-h). Channel 1: bright field pictures. Channel 2: 385-470 nm band pass filter for auto fluorescence of fungal mycelium. Channel 3: 560-615 nm band pass filter for enzymatically activated ELF?-97 fluorescence intensities. SBPA was determined in EM fungi at different pH = 3-7, roots and EM of *N. obliqua* at pH = 5. Image processing routines for segmentation and quantification of SBPA and mycorrhizal morphology were written in IDL5.4 (Interactive Data Language, Research Systems, Co, USA) (Härtel *et al*. 2003).

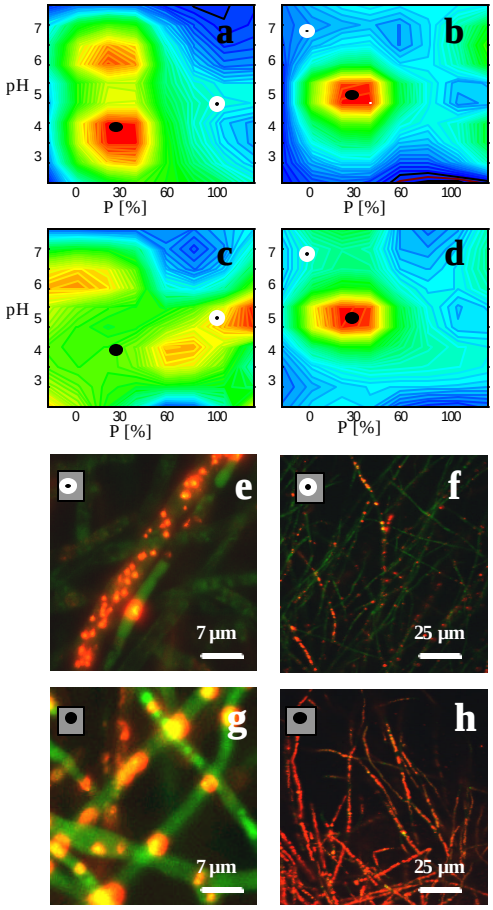

FIGURE 1. Surface bound mono-phosphatase activity (SBPA) of fungal hyphae of *P. involutus* and *A. boletinoides* as determined by image processed fluorescence microscopy based on fluorescence activation of ELF-97 substrate.
(a-b): Bivariate colour-coded presentation of SBPA per µm hyphal length of *P. involutus* (a) and of *A. boletinoides* (b).
(c-d): Bivariate colour-coded presentation of the number of fluorescent centres of precipitated ELF-97 substrate per µm hyphal length of *P. involutus* (c) and of *A. boletinoides* (d). For a-d, mean values were derived from three independent experiments.
(e-h): Representative 3-channel fluorescence images of hyphae of *P. involutus* and *A. boletinoides*. Channel 1: bright field pictures (blue). Channel 2: 385-470 nm band pass filter for auto fluorescence of fungal mycelium (green). Channel 3: 560-615 nm band pass filter for enzymatically activated ELF-97 fluorescence intensities (red). (e, g): *P. involutus* at pH = 5 and P = 100 % (e), and at pH = 4 and P = 30 % (g). (f, h): *A. boletinoides* at pH = 7 and P = 0 % (f), and at pH = 5 and P = 30 % (h). Compare to black and white circles in a-d.

RESULTS

SBPA IN MYCELIUM OF *P. INVOLUTUS* AND *A. BOLETINOIDES*

Fig. 1(a-d) shows colour-coded bivariate plots containing structural information ([number of phosphatase activated centres] / [length of the fungal hypha, µm]), and the total enzymatic activity ([Intensity of ELF-97] / [hyphal length, µm]) of SBPA. For *P. involutus*, two distinct maxima were observed for the SBPA at a P concentration of 30 %, and pH values of 3.5 and 6 (Fig. 1a). The bivariate distribution of the total enzymatic activity differed from the number of phosphatase activated centres per µm fungal hyphae (Fig. 1c). For *A. boletinoides* instead, Fig. 1(b) shows that only one optimum condition evolved for the SBPA at pH = 5 and at P concentration of 30 % during the growth period. In contrast to the results obtained with *P. involutus*, the same distribution was observed when the number of phosphatase activated centres was considered (Fig. 1d).

Representative three-channel fluorescence images of the hyphae of *P. involutus* and *A. boletinoides* (Fig. 1e-h) outline selected incubation conditions shown in Fig. 1(a-d). For *P. involutus*, a high number of individual fluorescent ELF-97 centres (with relatively small fluorescent intensities) were formed on the fungal hyphae (Fig. 1e) at P concentration of 100 % and at pH = 5. At P concentrations of 30 % and a pH value of 4 instead, a relatively small number of individual fluorescent ELF-97 centres could be observed (Fig. 1g). However, these centres yielded high fluorescence intensities. For *A. boletinoides*, only the number of phosphatase activated centres per µm fungal hyphae was changed, while the individual fluorescence intensities of the centres remained constant (Fig. 1f-h).

SBPA OF MYCORRHIZAL AND NON-MYCORRHIZAL ROOT SYSTEMS OF *N. OBLIQUA*

Three out of four mycorrhizal root systems of *N. obliqua* shifted the majority of the SBPA from the root cells to the fungal hyphae of the mantle (Fig. 2a). Only for D. *antartica*, ~80 % of the SBPA remained with the root cells and did not shift to the

mantle. As far as the relative fluorescence intensity of ELF-97 per µm² area of the mycorrhizal / non-mycorrhizal root of *N. obliqua* is concerned (Fig. 2b), only the mycorrhizae formed with *P. tinctorius* increased the total enzymatic activity in respect of the non-mycorrhizal root. *P. involutus*, *C. geophilum*, and *D. antartica* instead lowered the enzymatic activity of the root system. While Fig. 2(b) marks essential differences between the total enzymatic activities of the mycorrhizal and non-mycorrhizal roots of *N. obliqua*, Fig. 2(c) shows that the enzymatic activities of the individual phosphatase centres did not change to a great extent.

DISCUSSION

For membrane-bound PA in fungal mycelium, the microscopic method based on ELF-97 has recently been shown to reproduce results obtained with a conventional colorimetric method based on p-nitrophenyl-phosphate (Alvarez *et al*. 2002). Additionally, the microscopic method contributed valuable information about the structure and distribution of phosphatase-active spots on the fungal hyphae (Fig. 1). The different adaptive strategies by which *P. involutus* and *A. boletinoides* regulate their phosphatase activity in response to the variation of the nutrient supply with P during its respective growth periods, and in response to the pH-condition during the incubation periods, are reflected in the representative fluorescent image examples (Fig. 1e-h). While *A. boletinoides* primarily adapts to different P-supply and pH-condition by changing the number of phosphatase active centres on its hyphae (Fig. 1b, d, f, h), *P. involutus* primarily changes the activity of the individual centres (Fig. 1a, c, e, g).

P. involutus and *A. boletinoides* adapted to different growth conditions (P-concentrations) in the same way as they respond to a different pH environment (Fig. 1). As reported, a growth period of 14 days is sufficient for the adaptation of cultured fungi to different phosphorus concentrations (Straker & Mitchell 1986). In contrast to the time scale of adaptation to different growth conditions, it seems very unlikely that the fungi can actively respond to the changing pH during the short incubation period applied here (15 min. for ELF-97). A possible explication would be that the isolates re-

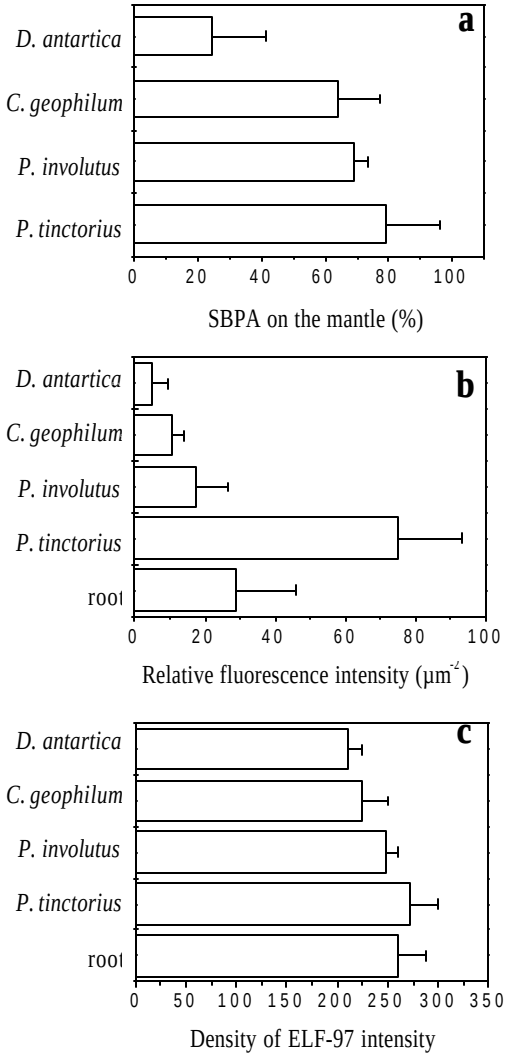

FIGURE 2. Surface bound phosphatase activity (SBPA) in root systems as determined by image processed fluorescence microscopy and ELF-97. (a): SBPA (%) on the mantle of the ectomycorrhiza of *N. obliqua* with *P. tinctorius, P. involutus, C. geophilum,* and *D. antartica* (pH=5). (b): Relative Intensity of ELF-97 fluorescence per µm² area of the mycorrhizal / non mycorrhizal roots of *N. obliqua* with *P. tinctorius, P. involutus, C. geophilum,* and *D. antartica* (pH=5). (c): Density of fluorescence of ELF-97 [I /µm²] inside of the fluorescence centres in the mycorrhizal / non-mycorrhizal root of *N. obliqua* with *P. tinctorius, P. involutus, C. geophilum,* and *D. antartica* (pH=5). For all plots, standard deviations and mean values were derived from three independent experiments.

spond to different growth conditions by varying the number (*A. boletinoides*) or the individual intensity (*P. involutus*) of phosphatase active centres, but that the exposed phosphatases are characterised by optimal activities at narrow pH ranges.

The comparison of SBPA in mycorrhizal and non-mycorrhizal roots of *N. obliqua* (Fig. 2) shows that mycorrhization does not necessarily favour the overall capacity of the root to cleave P. In fact, three out of four mycorrhizal root systems of *N. obliqua* provide less integral SBPA than non mycorrhizal roots (Fig. 2b). Out of the tested mycorrhizal associations, only *P. involutus* increases the integral SBPA significantly and concentrates nearly the entire activity on the hyphal mantle (Fig. 2b). Interestingly, *C. geophilum* and *P. involutus* still concentrate the majority of the SBPA on the hyphal mantles, although they lower the integral SBPA (Fig. 2a-b). As can be derived from Fig. 2c, overall variations of the SBPA in the tested mycorrhizal associations cannot be explained by a variation of the phosphatase activity of the existing centres (fluorescence intensity of ELF-97 per centre surface). As might be expected, the majority of the observed differences instead results from changes of the number (total surface) of the active centres.

In conclusion, the total phosphatase activity and physio-anatomical parameters derived with image processed confocal LSM in isolated fungal hyphae as well as in mycorrhizal associations were found to vary intensively between fungi. The external variation of the pH conditions and different phosphorus concentrations during the growth periods of the cultured fungi had different effects on the phosphatase activity of isolated fungal hyphae. Our newly developed method leads to a precise anatomic-physiologic description of SBPA in transversal cuts of roots or mycorrhizae *in vivo*. None of the existing methods for the determination of SBPA (Antibus *et al*. 1992, Tibbett *et al*. 1998, Tisserant *et al*. 1993) can offer comparable features. Through interactive segmentation of root, mantle, and PA, our method distinguished between SBPA expressed by the root and by the fungi. In the future, this method will be applied to root material that is directly extracted from its natural habitat in order to gain further insight into the strategies of enzyme driven P solubilisation *in situ*.

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