



A rapid and highly sensitive method for measuring enzyme activities in single mycorrhizal tips using 4-methylumbelliferone-labelled fluorogenic substrates in a microplate system

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Abstract

A microplate fluorimetric assay was developed for measuring potential activities of extracellular enzymes of individual ectomycorrhizal (EM) roots using methylumbelliferone (MU)-labelled fluorescent substrate analogues and microsieves to minimise damage due to manipulation of excised mycorrhizal roots. Control experiments revealed that enzyme activities remained stable over the whole time of the experiment suggesting a strong affinity of the studied enzymes to the fungal cell walls. The same mycorrhizal tips thus could be used repeatedly for enzyme detection and subsequently analysed for the projection area by automated image analysis. The developed system was evaluated on four different EM species measuring pH optimum and substrate saturation of phosphatase, chitinase and β -glucosidase. The four EM species studied were *Lactarius subdulcis*, *Russula ochroleuca*, *Cortinarius obtusus* and *Xerocomus* cf. *chrysenteron*. Depending upon the enzyme, each species exhibited different levels of enzymatic activities as well as enzyme kinetics and showed also differences in pH optima.

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1. Introduction

Ectomycorrhizae comprise an ecologically important group of around 5000–6000 species of higher

fungi associated with fine roots of the dominant temperate and boreal forest tree genera, e.g., *Pinus*, *Fagus* or *Picea*. The high structural diversity of the fungal partners is well known, and recent publications demonstrate an increasingly sophisticated knowledge on distribution and ecological preferences of ectomycorrhizal (EM) communities (Agerer et al., 2002; Tedersoo et al., 2003). In contrast to the detailed

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methods available to assess fine scale structural diversity, there is still a lack of knowledge on functions of the different species in their environment. There is some indirect and direct evidence, that ectomycorrhizae show a high functional diversity, considering their capabilities in plant protection against root-borne pathogens, plant growth promotion or increased nutrient acquisition of their host plant (Smith and Read, 1997).

Enzymatic activities of ectomycorrhizal roots may play an important role in short and direct nutrient mobilisation and transfer from forest soil and litter via ectomycorrhizal hyphae to their plant host. Their extracellular enzymes directly interact with the environment to which they are released or exposed. In forest soils, decaying plant material is the main nutrient source for all soil organisms and fungi are the most important organisms in turnover, degradation and mineralisation of carbon, nitrogen, phosphorous and others, making these nutrients again available for plants and the soil microflora (Kjoller and Struwe, 2002). Therefore, it is of great interest to measure enzymes of ectomycorrhizal roots to obtain insights in nutrient mobilisation of different species.

Studies on enzymatic activities of ectomycorrhizal roots themselves are rare (Antibus et al., 1981; Bartlett and Lewis, 1973; Buée et al., in press) and restricted to very few enzymes of high activity, e.g., phosphatase and dehydrogenase. However, detection of enzymatic activities in pure cultures as summarized by Hutchison (1991) and fruiting bodies of ectomycorrhizal fungi (Agerer et al., 2000) showed a great range of activities of oxidative and hydrolytic enzymes. One prerequisite for applying enzyme assays to nonsterile mycorrhizal samples are short incubation times, because long incubation times bear the risk of decreasing viability of the mycorrhizae or may be disturbed by the potential growth of bacteria. The requirements for enzyme assays suitable for mycorrhizal tips or mycelia are therefore high sensitivity in order to decrease incubation times, easy handling to avoid disturbance of the delicate structures and small sized assay conditions with respect to the dimension of ectomycorrhizal tips. Additionally, quantitative measurements related to the surface of mycorrhizal roots as applied by Buée et al. (in press) would allow to compare the contribution of different species to selected enzyme activities.

In the present paper, a microplate-based system in combination with a range of fluorescent substrates is described which was used to measure mycorrhizal enzymatic activities. To minimise damage to the mycorrhizal roots during the enzyme assays, small sieves were constructed. The mycorrhizal tips were subsequently scanned and subjected to automated image analysis to determine the projection area of the mycorrhizal roots (Buée et al., in press). With the developed system, a routine approach was established for the measurements of enzyme activities on individual mycorrhizal roots and was used for the detection of the pH optimum of each studied enzyme and all investigated species as well as the optimal substrate concentration. Among the numerous methylumbelliferone (MU)-labelled substrates, three were chosen for the establishment of the method, which should represent enzymes relevant for biological turnover processes in forest soils such as β -glucosidase (EC 3.2.1.21) involved in cellulose degradation, chitinase (EC 3.2.1.14) and an expected omnipresent highly active enzyme such as an acid phosphatase (EC 3.1.3.2) in naturally phosphorus limited acid forest litters and soils.

2. Materials and methods

2.1. Sampling

Mycorrhizae were sampled at the experimental field site Kranzberg Forest, which has been described in detail (Pretzsch et al., 1998). In brief, the sampling site is a mixed 50–70-year-old mixed spruce [*Picea abies* (L.) Karst.] and beech (*Fagus sylvatica* L.) stand located near Freising (Germany) situated in the “Tertiary hillside upper Bavaria”. The soil is gleyic cambisol and the humus form depending on the trees is mullmoder to moder under spruce and mull to mullmoder under beech. Root samples were taken by cutting blocks of at least 15×15 cm² width and 10 cm depth from the O_h and A_h horizons where over 90% of the fine roots were located using a sharp knife (Agerer, 1991). Because of the patchy occurrence of mycorrhizal species, samples were taken at trees with known mycorrhizal colonisation. The main strategy was to find enough vital tips of one species. Sampling was done at least twice for every species and every test

between July and October 2003. Roots were kept in their substrate at 4 °C until assayed within 3 days after sampling. Prior to enzyme assays, mycorrhizae were cleaned from adhering soil by gently shaking in tap water and further cleaning under a stereomicroscope. Mycorrhizal species were determined by morphological and anatomical features (Agerer, 1991). Four abundant ectomycorrhizal species were intensively studied, i.e. *Russula ochroleuca* (Pers.) Fr., *Cortinarius obtusus* (Fr.) Fr., *Xerocomus* cf. *chrysenteron* (Bull.) Quél., all on spruce (*P. abies*), and *Lactarius subdulcis* (Bull.) Gray on beech (*F. sylvatica*).

2.2. Experimental setup

2.2.1. Enzyme substrates, calibration solution and buffers

Three enzyme substrates based on 4-methylumbelliferone (MU) were studied in detail: MU-phosphate (MU-P for the detection of phosphatase), MU- β -1,4-glucopyranoside (MU-G) for β -glucosidase, and MU- β -1,4-*N*-acetylglucosaminide (MU-NAG) for chitinase activity. All chemicals were derived from Sigma-Aldrich (Germany).

Stock solutions of MU-substrates (5 mM) (Hoppe, 1983) and a 25 mM stock solution of MU for calibration were predissolved in 2-methoxyethanol (Hoppe, 1983) and further diluted with sterile distilled water to give the desired working concentrations according to the experiments as detailed below. Stock solutions, diluted substrates and calibration solutions were kept at –20 °C until use.

Incubation buffers were prepared at 75 mM concentration and sterilised. The buffers used were maleic acid/Tris for pH 2–6.5, Hepes for pH 7, which were previously tested for not interfering with the enzymes under study.

The stopping buffer used to stop enzyme reactions and to alkalize the solutions in one step contained a mixture of ethanol: Tris 2.5 M pH 10–11 (3/1 v/v).

2.2.2. Pretreatment of mycorrhizal tips

Prior to enzyme assays, single mycorrhizal tips which appeared fully vital, intact and in an active state were excised from root systems using a sharp razor blade after examination under a stereomicroscope at appropriate magnification (8–50 \times) (Agerer,

1991). Vital and intact tips could be distinguished from older roots according to their light tips, their turgescence and undamaged hyphal mantle and their vital appearance of the complete root cortex in cross-sections (Agerer, 1991). The selected tips of one species had the same diameter and were cut to the same length of 2–4 mm (depending on the species) at their basal end. This should minimise the error of assaying mycorrhizae in different physiological conditions which exist in more basal, older parts of the hyphal mantle compared to younger parts at the mycorrhizal tip. Instead of fresh and dry weights, the projection area of the mycorrhizal tips was determined as described below.

Enzyme measurements were carried out repeatedly on single mycorrhizal tips, by the use of sieve strips which were newly constructed to allow easy and rapid manipulation with minimal damage to the mycorrhizae. Strips consisting of eight combined tubes (8 \times 200 μ l Multi Ultra Strips[®], Roth, Karlsruhe, Germany) were modified to serve as small sieves after the bottom of every tube was cut off and replaced by fine Nylon meshes (0.25-mm mesh width) fixed by a tightly fitting plastic ring without glue to the bottom of every tube. These sieve strips exactly fit into a row of eight wells in a 96-well microtitre plate. During the enzyme assays the strips were used to incubate, shake or rinse up to eight mycorrhizal tips simultaneously and to transfer them from one row of wells in a 96-well microplate to another.

2.2.3. Microplate setup and performance of enzyme assays

EM tips were placed in seven of the eight tubes of the sieve strips, with one tube left for controlling autofluorescence of the substrate. The assays were performed at 21 °C in a climate chamber under protection from light on a microplate shaker. The procedure comprised three steps: incubation, rinsing, stopping/measurement. Incubation plates (black microplates) contained incubation wells and control wells for autofluorescence of substrates containing 100 μ l of incubation buffer and 50 μ l of substrate solution. Stopping plates (black microplates) were prefilled with 100 μ l stopping buffer and contained one row of calibration wells with 50 μ l of calibration solutions added. Rinsing plates (transparent microplates) were prefilled with 50 μ l of sterile

distilled water and 100 μ l incubation buffer of the desired pH according to the experiment. Depending on the substrate, incubation times were 5 min (MU-P), 10 min (MU-NAG), 20 min (MU-G), respectively. At the end of the respective incubation time, sieve strips were lifted from the incubation plate. From every well, 100 μ l was transferred to the corresponding row in a prepared stopping plate. The sieve strip subsequently was gently pressed on a tissue to remove all remains of liquid and then placed in the corresponding row of the prepared rinsing plate for 3 min. Before starting the next cycle of incubation, the sieve strip was removed from the rinsing plate and remaining liquid was sucked off. The next cycle started by placing the sieve strip into the next row of the incubation plate and further proceeding as described before. Depending upon the experiment, 10–12 cycles were performed with the same mycorrhizal tips. To prevent evaporation of ethanol from the stopping microplates, they were kept sealed with tape except for the pipetting steps. All enzyme assays were run at least twice independently.

Prior to measurements, the volume in stopping/measurement microplates was adjusted to 250 μ l per well by adding 50 μ l of sterile distilled water. Measurements were carried out with a Cary Eclipse Fluorescence Spectrophotometer with a microplate reader (Varian, Australia) at an excitation wavelength of 360 nm and an emission wavelength of 450 nm, slit widths of 5 nm and a voltage of 750 or 800 V dependent on the strength of the signal.

2.2.4. Controls related to fluorescence measurements

In every experimental series, controls for the auto fluorescence of the substrate were included in the respective incubation plate. Additional experiments to check autofluorescence of mycorrhizas and quenching of the fluorescence signal influenced by mycorrhizas were performed once for each mycorrhizal species. For this purpose, mycorrhizal tips were shaken for 2 h in 150 μ l of incubation buffer without substrate to control autofluorescence or in incubation buffer spiked with 300 pmol MU in 50 μ l to check quenching. Stopping and detection was carried out as described for the other assays. For the four species studied, no autofluorescence after removal of the tips from the well nor quenching of the signal was detected.

2.2.5. Measurements and calculations

Every series of experiments included calibrations with 0, 100, 200, 300, 400, 500 pmol MU per well and concentrations of released MU were calculated from regression curves based on these calibrations. MU release was calculated per mm² projection area of mycorrhizal tips as detailed below. Dry weights of individual ectomycorrhizal roots (data not shown) were found to be close to the detection limit (\pm 10 μ g) even when using a highly precise balance (Sartorius Micro, M25 D, Sartorius Germany). To avoid a source of error introduced by the impossibility to precisely determine the dry weight of individual tips, to improve the basis for calculations and to greatly speed up analysis, the measured enzyme activities were related to the projection area of individual mycorrhizal roots determined by automated image analysis of scanned individual tips as described by Buée et al. (in press). They found a linear and significant correlation between projection area and dry weight of mycorrhizal tips. In the present study, the image analysis software (WinRhizo 2003b, Regent Instruments, Canada) was additionally used for colour definition of mycorrhizal roots to improve the distinction of mycorrhizal roots from the background during automated analysis.

3. Results and discussion

3.1. Sensitivity, reproducibility and loss of activity during the assay procedure

The fluorescence method applied allowed a reduction in incubation times, e.g., for phosphatase from 30 to 60 min as measured with PNPP (Bartlett and Lewis, 1973; Buée et al., in press; Tibbett et al., 1998) to 5 min in the present study. Also chitinase and β -glucosidase could be easily detected after 10 and 20 min incubation, respectively. The incubation times were chosen as to allow enzymatic activities of the highest active species to be measured without diluting the reaction mixture after stopping and alkalisation.

One major concern while using single mycorrhizal tips repeatedly for measurements was the possible loss of activity during the cleaning procedure, and later, due to enzyme release into the surrounding incubation

buffer. In a preexperiment, the loss of activity during the cleaning step of mycorrhizae was assessed by taking mycorrhizal tips directly from their substrate and shaking them very briefly for a few seconds in tap water. After immediately transferring them into incubation wells with 100 μl of incubation buffer without substrate, they were shaken for 120 min. After removal of the mycorrhizae 50 μl of substrate solutions (MU-P, MU-NAG, MU-G) were added and incubated as usually. No activity was detected in these shaking solutions (data not shown). Therefore, in another experiment the maintenance of enzyme activity of individual tips during the time of the experiment was tested using an incubation buffer of pH 4.5 and substrate concentrations of 500 pmol per well.

Loss of activity at repeated measurements on single tips was shown to be not relevant within the time of the experiment (Fig. 1). Moreover, activities of all three studied enzymes were relatively constant between different measurement intervals (Fig. 1). In the total of measured intervals including the rinsing steps of at least 3 min, enzyme activities did not drop over a period of 90 min for phosphatase, 140 min for chitinase, 240 min for β -glucosidase.

Reproducibility was high, although reduced enzyme activities in single intervals were observed such as in interval 7 for phosphatase activity of *L. subdulcis* (Fig. 1). These exceptions were probably related to an unfavourable position of single tips in the sieve wells, which may have been caused by a temporarily uneven

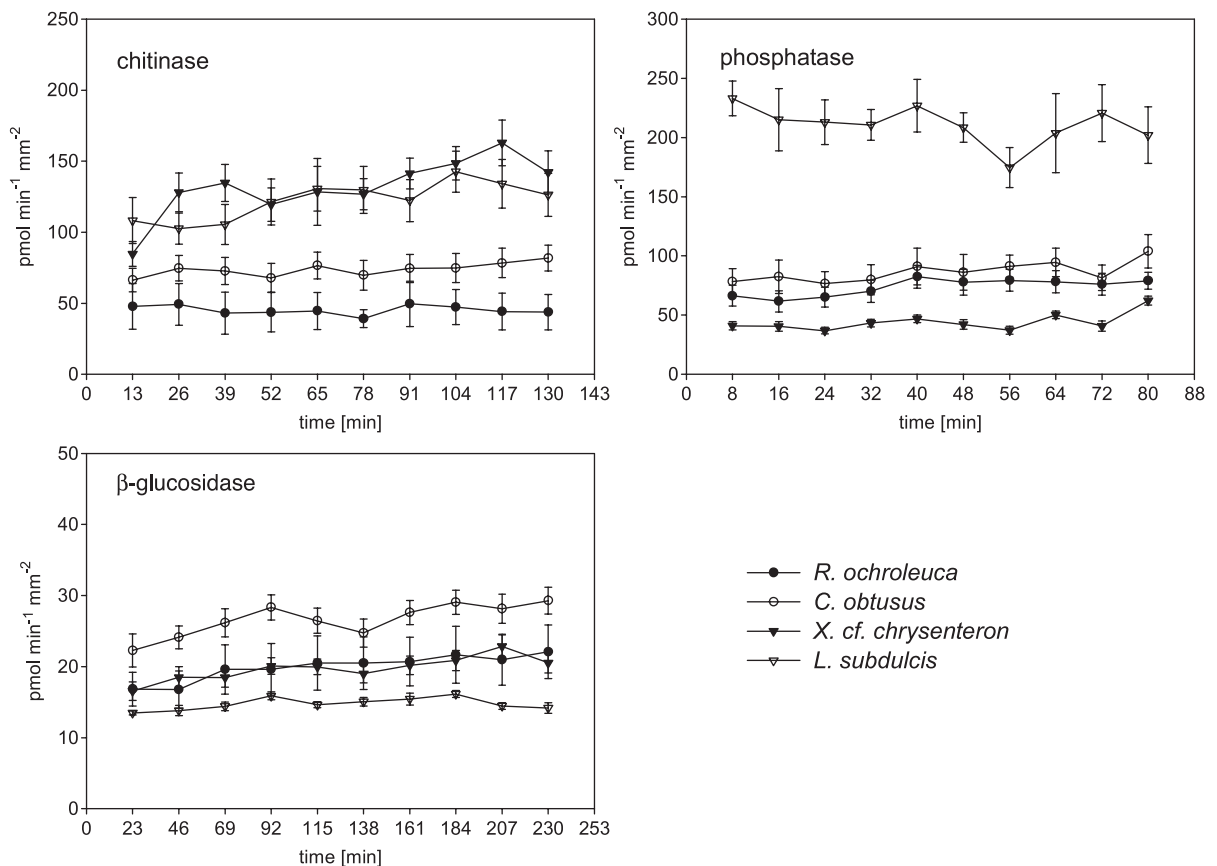


Fig. 1. Enzyme activities after repeated incubation at pH 4.5 expressed as MU release per projection area and time of individual, excised mycorrhizal tips ($n = 7$). Intervals for phosphatase were 5 min incubation + 3 min rinsing each, *N*-acetylglucosaminidase 10 min incubation + 3 min rinsing, β -glucosidase 20 min incubation + 3 min rinsing.

substrate availability resulting in a reduced rate of MU release. As enzyme activities recovered to the former level, the decrease at one time point apparently was not related to a general decrease in enzyme activity. From these results, it was concluded that repeated measurements on single tips are possible over the time range assayed with high reproducibility and without a loss of activity at the end of the measurement period. The results also show that repeated measurements on single tips should be performed to reduce the risk of false results on single tip measurements. This may be especially relevant in the case of very high enzyme activities, e.g., phosphatase and the corresponding very short incubation times.

The fact that enzyme activities did not decrease even after 240 min of assay time indicates that enzymes may be bound to cell components and were apparently not released into the surrounding in large amounts. All of the studied enzymes have been reported to occur in cell wall bound form in yeast and filamentous fungi hitherto studied (Rast et al., 2003). If this is also the case in ectomycorrhizal fungi, it would explain the long maintenance of activity and low enzyme activities after shaking mycorrhizal roots in water at least in the observed time span.

3.2. Substrate saturation

Enzyme assays were performed as described above with increasing substrate concentrations of 0, 50, 100, 200, 300, 400, 500, 600, 700, 800 pmol per well, which corresponded to 0, 0.3, 0.7, 1.3, 2.0, 2.7, 3.3, 4.0, 4.7, 5.3 μM , respectively, in the total volume of 150 μl of the incubation mixture. These experiments were carried out at pH 4.5 where all enzymes in pre experiments showed high activity.

The four different mycorrhizal species showed clear differences of enzyme activities depending on the substrate concentration (Fig. 2). Highest activities of all enzymes were measured for phosphatase, followed by chitinase and β -glucosidase. Phosphatase activity was extraordinarily high in *L. subdulcis* and did not reach saturation even at the highest concentration applied with 800 pmol per well (Fig. 2). In contrast, *C. obtusus*, *X. cf. chrysenteron* and *R. ochroleuca* had similar phosphatase activities and apparently reached saturation between 700 and 800

pmol per well. Chitinase activity greatly differed between species as also observed by Hodge et al. (1995) in pure culture assays with different ectomycorrhizal and pathogenic fungi. In the present study, again, *L. subdulcis* showed highest activities with saturation reached at 700–800 pmol per well. *C. obtusus* had very little chitinase activities. Chitinase activities of *X. cf. chrysenteron* and *R. ochroleuca* were similar at concentrations below 300 pmol per well. Above 300 pmol per well, chitinase activity of *X. cf. chrysenteron* reached saturation and did not further increase, while in *R. ochroleuca* higher concentrations of MU-NAG decreased activity. In the case of *R. ochroleuca*, inhibition of enzyme activity at higher substrate concentrations is likely to have occurred.

β -Glucosidase activity was lowest in *R. ochroleuca*, while *L. subdulcis*, *C. obtusus* and *X. cf. chrysenteron* had very similar endpoint activities, but very different kinetics (Fig. 2). Among the latter three fungi, *C. obtusus* very rapidly reached substrate saturation between 100 and 200 pmol per well, while the others reacted much slower.

3.3. Optimal pH

For determination of pH range and pH optimum of enzymes, microplates were prepared with increasing pH between 2 and 7.0 at 0.5 pH steps and 500 pmol substrate per well. The highest phosphatase activity was measured in all species between pH 4.5 and 5.5 (Fig. 3). Although all four species had some phosphatase activity at very acidic pH < 3.5, a strong increase in activity was observed above pH 4.0 and a very dramatic decrease between pH 5.5 and 6.0 with an almost complete loss of activity at pH 7. Very similar pH optima for acid phosphatase activity were observed on beech mycorrhizae which have not been closer specified (Bartlett and Lewis, 1973).

β -Glucosidase activity was highest between pH 4.0 and 4.5 with *X. cf. chrysenteron* showing the highest activity of all fungi. *C. obtusus* had a slightly more acidic optimum at pH 4.0 than *R. ochroleuca* and *L. subdulcis*, both with an optimum close to pH 4.5.

pH optima for chitinase activity were pH 6.0 for *L. subdulcis*. *X. cf. chrysenteron* had one optimum at pH 5.0–5.5 and a smaller peak was observed at pH 2.5,

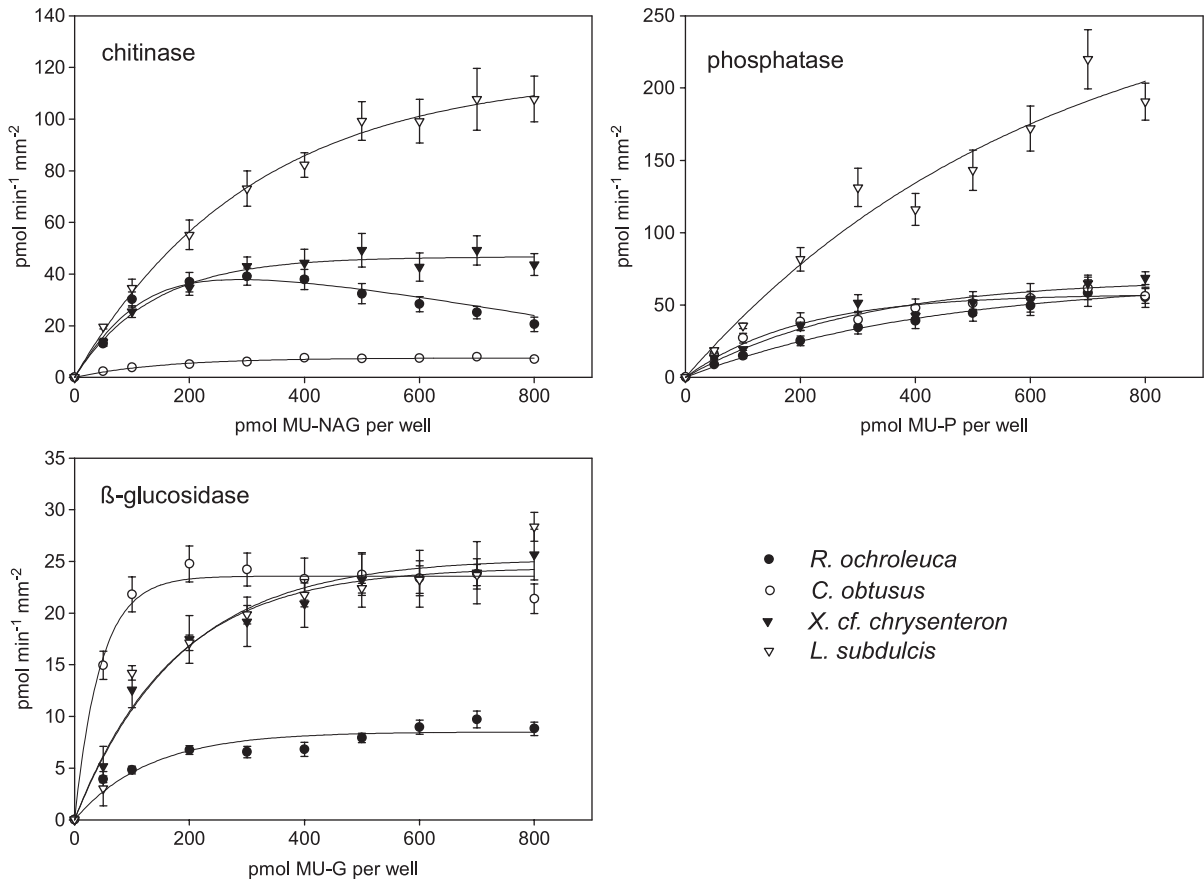


Fig. 2. Enzyme activities at increasing substrate concentrations (pmol MU per well) expressed as MU release per projection area and time of four mycorrhizal species using repeated measurements on the same tips ($n=7$).

while *C. obtusus* showed optimum activity at pH 3.5 and another smaller peak at pH 5.5. *R. ochroleuca* showed highest activity around pH 3.5. Remarkably, all fungi showed comparatively high activity over the total pH range between pH 2.5 and 6.5. Only at the extreme ends of the investigated pH range (pH 2 and 7) a clearly reduced activity was measured. The broad range of relatively high chitinase activities with seemingly more than one pH optimum and without a distinct pH optimum as compared to the other enzymes suggests that more than one chitinase may have contributed to the total enzyme activity. Studies on purified chitinases from filamentous fungi studied up to now show the occurrence of different chitinase classes and also a range of pH optima from as acidic as 3.5 of a *Trichoderma harzianum* (Rifai) exochitinase (Deane et al., 1998), up to pH 7 of an extracel-

lular chitinase in *Monascus purpureus* (Went) (Wang et al., 2002).

In the ericoid mycorrhizal fungus *Hymenoscyphus ericae* [(D.J. Read) Korf and Kernan], low pH optima for phosphatase and other enzymes have been interpreted as an adaptation to acidic mor humus soil (Cairney and Burke, 1998). This could also be applied to the studied EM species inhabiting a similarly acidic environment with pH 3–4 in the A_H horizon and an even lower pH in the litter layer (O_H) at the experimental plot (Pretzsch et al., 1998). Species-specific differences in enzyme activities directly measured on mycorrhizal tips have been restricted to measurements of acid phosphatase but have been observed by Buée et al. (in press) as well as by Antibus et al. (1981). The findings of the present study which showed differences in pH optima, level of activities, and affinity to

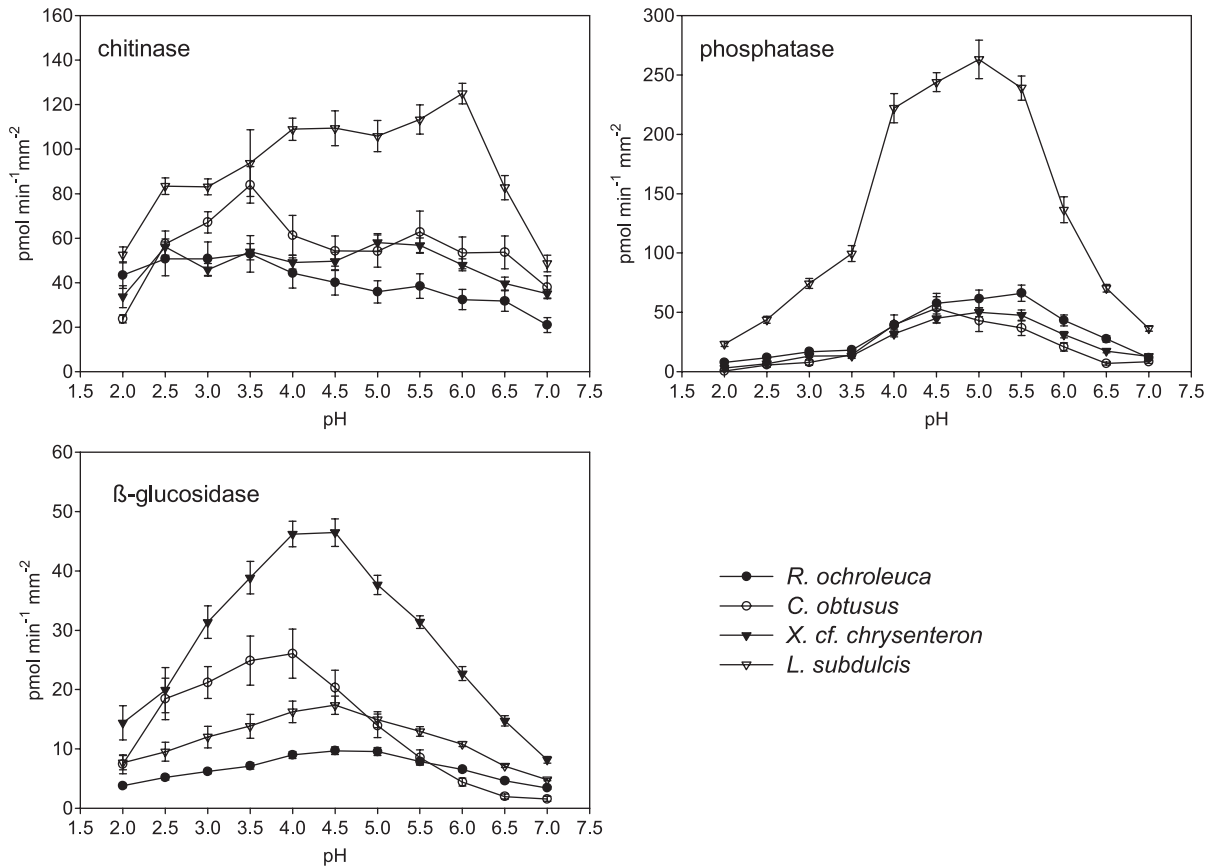


Fig. 3. Enzyme activities at increasing pH expressed as MU release per projection area and time of individual excised mycorrhizal roots ($n=7$).

substrates additionally for chitinase and β -glucosidase are indicating also species specific activities for these enzymes.

4. Conclusions

The microplate system combined with MU-labelled substrates including the microsieves has the advantage of allowing rapid processing of large numbers of samples combined with a high sensitivity for enzyme detection in small sample volumes. The possibility to make multiple measurements on single tips reduces considerably the number of tips necessary for enzymatic studies. The easy alteration of the experimental setup according to the probe of interest and the number of additional fluorescence-labelled enzyme substrates available allows a more general

application of the here presented method. First results from measurements of different enzymes on the same mycorrhizal tips were promising and would enable the characterisation of enzyme activity patterns of a larger range of different mycorrhizal species. Thus, the developed method may help to increase knowledge on functional differentiation of EM roots and possibly also their extramatrical mycelium collected in a naturally heterogeneous environment such as forest soil and litters.

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