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ORIGINAL PAPER

Optimized assay and storage conditions for enzyme activity profiling of ectomycorrhizae

Karin Pritsch · Pierre Emanuel Courty · Jean-Louis Churin · Benoit Cloutier-Hurteau · Muhammad Arif Ali · Coralie Damon · Myriam Duchemin · Simon Egli · Jana Ernst · Laurence Fraissinet-Tachet · Francisco Kuhar · Elvira Legname · Roland Marmeisse · Alex Müller · Petia Nikolova · Martina Peter · Claude Plassard · Franck Richard · Michael Schloter · Marc-André Selosse · Alain Franc · Jean Garbaye

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Abstract The aim of a joint effort by different research teams was to provide an improved procedure for enzyme activity profiling of field-sampled ectomycorrhizae, including recommendations on the best conditions and maximum duration for storage of ectomycorrhizal samples. A more simplified and efficient protocol compared to formerly published procedures was achieved by using manufactured 96-filter plates in combination with a vacuum manifold and by optimizing incubation times. Major improvements were achieved by performing the series of eight enzyme assays with a single series of root samples instead of two series, reducing the time needed for sample preparation, minimizing error-prone steps such as pipetting and morphotyping, and facilitating subsequent DNA analyses due to the reduced sequencing effort. The best preservation of samples proved to be storage in soil at 4–6°C in the form of undisturbed soil cores containing roots. Enzyme activities were maintained for up to 4 weeks under these conditions. Short-term storage of washed roots and ectomycorrhizal tips overnight in water did not cause substantial changes in enzyme activity profiles. No optimal means for longer-term storage by freezing at −20°C or storage in 100% ethanol were recommended.

K. Pritsch (✉) · P. Nikolova
Institute of Soil Ecology, German Research Center for Environmental Health (GmbH),
85764 Neuherberg, Germany
e-mail: pritsch@helmholtz-muenchen.de

P. E. Courty · J.-L. Churin · J. Garbaye
UMR 1136, Interactions Arbres Micro-organismes,
Centre INRA de Nancy,
54280 Champenoux, France

S. Egli · A. Müller · M. Peter
Swiss Federal Research Institute WSL,
8903 Birmensdorf, Switzerland

C. Damon · L. Fraissinet-Tachet · R. Marmeisse
UMR CNRS 5557 d'Ecologie Microbienne, Université de Lyon,
Université Lyon 1,
69622 Villeurbanne Cedex, France

B. Cloutier-Hurteau · M. A. Ali · M. Duchemin · E. Legname · C. Plassard
UMR 1222, Ecologie Fonctionelle et Biogéochimie des Sols,
INRA/IRD/SupAgro,
34060 Montpellier Cedex 01, France

F. Richard · M.-A. Selosse
UMR 5175, Centre d'Ecologie Fonctionnelle et Evolutive,
34293 Montpellier 5, France

A. Franc
UMR Biodivers Genes & Communautes, INRA Pierroton,
33612 Cestas, France

J. Ernst · M. Schloter
Institute of Soil Ecology, Terrestrial Ecogenetics,
German Research Center for Environmental Health (GmbH),
85764 Neuherberg, Germany

F. Kuhar
Institute of Soil Ecology, Helmholtz Zentrum München,
German Research Center for Environmental Health (GmbH),
85764 Neuherberg, Germany

Present Address:
F. Kuhar
Biodiversidad y Biología Experimental, CABA,
Universidad de Buenos Aires,
Buenos Aires 1428, Argentina

Present Address:
P. E. Courty
Botanical Institute, University of Basel,
4056 Basel, Switzerland

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Introduction

Ectomycorrhizae (ECM) are a major component of temperate and boreal forest ecosystems with 100–500 different species in one stand (Horton and Bruns 2001; Tedersoo et al. 2003; Richard et al. 2005). From experiments under controlled conditions, it is well-known that many ECM are capable of improving the nutritional status of their plant hosts (Abuzinadah and Read 1989; Perez-Moreno and Read 2000; Tibbett and Sanders 2002). However, considering the large number of species in ecosystems and the limited number of cultivated ectomycorrhizal fungus species that can be used in controlled experiments, there is an enormous gap in the knowledge of the functions of ECM in ecosystems. Despite the rapid progress made at the level of genome exploration of ECM (e.g., Martin and Selosse 2008; Martin et al. 2010), few methods are available to address the functional adaptation of ECM in ecosystems. One recent example is the enzymatic profiling method of field-sampled ECM (Pritsch et al. 2004; Courty et al. 2005). This method is a cultivation-independent method for studying the functional aspects of individual tips of ECM by measuring their potential extracellular enzymatic activities related to nutrient release from organic sources, such as plant litter or nutrients contained in soil organic matter (Pritsch and Garbaye 2011).

The method has been applied in a number of studies and has provided insights into the temporal and spatial dynamics of enzyme activities involved in nutrient cycling and how these activities are influenced by disturbance (Buee et al. 2007; Courty et al. 2007, 2010, 2011; Mosca et al. 2007; Rineau and Garbaye 2009). However, two major concerns have been repeatedly addressed by researchers interested in using the method of Courty et al. (2005): The assay procedure itself requires self-made materials, and two identical sets of ectomycorrhizal tips and samples need to be preserved before the tests.

To address the first problem, we aimed to simplify the assay procedure and preparation steps described by Courty et al. (2005). Specifically, we aimed to modify this method by adapting it to use purchased materials and to optimize the procedure to be performed on a single set of ectomycorrhizal tips. This would save time in preparing the samples, allow random sub-sampling of field samples instead of selecting ECM based on morphological criteria, and facilitate the identification of each assayed tip and functional analyses based on DNA sequencing. To achieve this aim, we analyzed possible methods to minimize time-consuming handling steps and to reduce incubation times without reducing the number of functional assays or the precision of the results.

The second problem was that few empirically based recommendations exist concerning sample storage and preservation for enzyme activity testing. Sampling campaigns are time-consuming, and sample processing of ECM may take weeks. Moreover, sample storage after cleaning of the roots and excision of root tips for testing has been restricted to the assay procedure itself (Pritsch et al. 2004), but during larger sampling campaigns, washed roots may need to be preserved during processing. No upper limits on storage times for fresh samples were known. Therefore, we first tested the storage of fresh root samples for up to 2 months in their original substrates versus storage as washed root samples. Second, we tested the impact of different means of storage on subsequent steps of processing for enzyme activities. For this purpose, different experiments tested storing whole fine roots compared to excised mycorrhizal tips and the possibility of stabilizing enzyme activities of excised mycorrhizal tips by adding or removing ions in aqueous solutions. Third, we tested the possibilities for long-term storage by freezing at −20°C, which is a widely applicable method for storage of biological samples (Wallenius et al. 2010), or by preservation in ethanol as an alternative, which has been used in biological samples to preserve enzymes in an active state (Kacena et al. 2004).

The goal of this study was to provide a protocol that requires only a single set of ectomycorrhizal tips, preserved under optimal storage conditions, handled with commercially available equipment, with a rationale for optimized handling and incubation times to facilitate the application of the enzyme activity profiling method for routine use.

Materials and methods

Origin of mycorrhizal samples

The dataset for calculating optimized incubation times was based on a sampling campaign in autumn 2007 at the field site “Kranzberger Forst” Germany (for details of the site, see Pretzsch et al. 1998). Twelve and six soil cores (4 cm diameter, 20 cm depth) were taken under beech and spruce, respectively. A total of 12 ectomycorrhizal species/morphotypes represented by at least four tips were included in this study, as shown in Table 1.

To test storage conditions, mycorrhizal samples from different field sites and from the greenhouse were used (Table 1). Field sampling was performed to collect enough material from one or a few abundant and unequivocally identifiable mycorrhizal morphotypes without following a uniform sampling scheme. In addition to field samples, ECM of *Laccaria bicolor* (Maire) P.D. Orton were used

Table 1 Fungal species, hosts, origins, and substrates of ECM tested for optimal incubation times and storage in experiments 1–5

Fungus species	Origin	Host	Substrate	Experiment	Enzymes tested
Optimization of incubation times					
Amanita rubescens Pers., *A. spissa* (Fr.) P. Kumm., *A. citrina* (Pers.), *Russula aeruginea* Fr., *Russula* sp., *Clavulina cristata* (Holmsk.) J. Schröt., *Hygrophorus olivaceoalbus* (Fr.) Fr., *Cenococcum geophilum* Fr.	Kranzberger Forst, near Freising, Germany	*F. sylvatica*	Soil		Leu Xyl, Glr, Cel, Nag, Pho, Gls, Lac
Tomentella sp. 1, *Tomentella* sp. 2, *Xerocomus* sp., *Laccaria amethystina* (Huds.) Cooke	Kranzberger Forst, near Freising, Germany	*P. abies*	Soil		
Storage experiments					
Lactarius quietus (Fr.) Fr., *Tomentella sublilacina* (Ellis & Holw.) Wakef.	Champenoux near Nancy, France	*Quercus*	Soil	1	Cel, Nag, Pho
Laccaria bicolor (Maire) P.D. Orton	Greenhouse	*P. menziesii*	Peat–vermiculite	1, 3, 5	Cel, Nag, Pho
Lactarius subdulcis (Pers.) Gray	Ramerenwald near Zurich, Switzerland	*F. sylvatica*	Non-decomposed logs of dead wood	2	Leu, Xyl, Glr, Cel, Nag, Pho, Gls
Xerocomus sp., *L. subdulcis*, *Cortinarius* sp.	Kranzberger Forst, near Freising, Germany	*F. sylvatica*	Soil	4	Cel, Nag, Pho

For abbreviations of substrates, see Table 2

1 storage in original substrate vs. tap water, *2* storage as intact clusters vs. single tips in tap water, *3* storage in tap water vs. deionized water vs. 0.2 mM $CaSO_4$, *4* storage by freezing (−20°C), *5* storage in ethanol

from controlled inoculations of Douglas fir seedlings (*Pseudotsuga menziesii* (Mirb.) Franco) following the method described by Duponnois and Garbaye (1991).

All root samples were kept in plastic bags, transported to the respective labs in Styrofoam boxes, and stored overnight at 4°C in their surrounding substrate. Details on the different mycorrhizal samples and their use in the experiments are provided in Table 1. The experiments were independent, and every experiment demonstrated one aspect of storage condition and/or storage time.

Improved protocol for enzyme activity measurements

Substrates, calibrations solutions, and buffers

Calibration solutions of 4-methylumbelliferone (MU) and aminomethylcoumarin (AMC) stock solutions were prepared as 10 mM stock solutions in 2-methoxyethanol and further diluted with sterile H_2O to concentrations of 0, 1, 2, 3, 4, and 5 μM. Calibration solutions were kept in the dark at 4–6°C and used within 1 week.

Fluorogenic substrates were prepared as 5 mM stock solutions in 2-methoxyethanol (Hoppe 1983). To provide the same buffer concentrations for all assays, stock solutions were first diluted with sterile deionized water to the desired working concentrations (Table 2), further diluted with sterile incubation buffer to the desired incubation concentrations as given in Table 2 and stored at −20°C for up to 6 months.

Diammonium 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS) was dissolved to result in a 667-μM solution in rinsing buffer, pH 4.5, and was stored up to 4 weeks in the dark at 4°C. Universal buffer was prepared as a stock solution from 12.1 g Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol), 11.6 g maleic acid, 14.0 g citric acid, 6.3 g boric acid, and 488 mL of sodium hydroxide (1 mol L^{-1}) and adjusted with deionized water to a volume of 1,000 mL.

For the incubation buffer, 100 mL of the buffer stock solution was set to the desired pH (either pH 4.5 or pH 6.5), adjusted with deionized water to a volume of 1,000 mL, and sterilized (121°C for 20 min). To prepare the rinsing buffers of pH 4.5 and 6.5, 200 mL incubation buffer at the respective pH was diluted with 100 mL of deionized water.

Tris (1 M; pH 10–11) was used to stop the fluorescence assay solutions at the end of the incubation and to increase pH for fluorescence enhancement. The sterilized buffers were stored at room temperature.

Preparation of filter plates and ectomycorrhizal tips before assaying

All enzyme assays on individual ectomycorrhizal tips were performed in 96-well filter plates (AcroPrepTM 96-filter plate with 30- to 40-μm mesh size, Pall, Life Sciences,

Table 2 Enzyme assay conditions for root tips according to the new optimized procedure

Substrate order in the assay, abbreviation, enzyme (EC number)		Substrate	Working solution (μM)	Incubation solution (μM)	Time (min)	Assay pH	Calibration
1 Leu	Leucine amino peptidase (3.4.11.1)	Leucine-AMC	1,200	400	70	6.5	AMC
2 Xyl	β-Xylosidase (3.2.1.37)	MU-xyloside	1,500	500	30	4.5	MU
3 Glr	β-Glucuronidase (3.2.1.31)	MU-glucuronide	1,500	500	30	4.5	MU
4 Cel	Cellobiohydrolase (3.2.1.91)	MU-cellobioydrofuran	1,200	400	30	4.5	MU
5 Nag	*N*-Acetylglucosaminidase (3.2.1.14)	MU-*N*-acetylglucosamine	1,500	500	15	4.5	MU
6 Gls	β-Glucosidase (3.2.1.21)	MU-β-glucoside	1,500	500	15	4.5	MU
7 Pho	Acid phosphatase (3.1.3.2)	MU-phosphate	2,400	800	30	4.5	MU
8Lac	Laccase (1.10.3.2)	ABTS	–	667	60	4.5	ε_{425}

Crailsheim, Germany). On each filter plate, six wells were reserved for controls for substrate background fluorescence (negative control), and another six wells were used for calibration. The wells of the filter plate corresponding to the calibration wells in the measurement plates (see below) were left empty and sealed with tape to allow the proper application of vacuum. The remaining wells were filled with 150 μL rinsing buffer at the pH corresponding to the enzyme assays. Ectomycorrhizal tips, cut to a length of 2–4 mm, were individually placed in the remaining filter wells where they stayed during the series of assays. A vacuum manifold for microplates was used to transfer liquids from the filter plates to measurement plates or to dispose of washing buffer.

Preparation of measurement plates

For each filter plate in the assay series, seven black microplates were provided for measuring fluorescence at the end of assays (one for AMC, six for MU), and one transparent flat-bottom 96-well microplate was used for the colorimetric assay (ABTS). All 96 wells of the black microplates were filled with 150 μL of Tris buffer (pH>10, 1 M) to stop the reaction and alkalinize the reagent. To those wells matching the respective positions of the sealed wells of the filter plate (see above), 100 μL calibration solutions (0, 1, 2, 3, 4, or 5 μM) were added according to the AMC or MU tests, respectively.

Assay procedure

A number of preliminary experiments were performed to test the removal of the fluorescence originating from substrates possibly remaining adsorbed to surfaces of root tips and filter plates. The applied rinsing procedure using 150 μL of rinsing buffer (Fig. 1) was found to be sufficient to remove substrates from previous incubation steps (data not shown). As previously shown (Courty et al. 2005), the order of incubation with different substrates did not change the results, and no interference between substrates (AMC and MU substrate, ABTS) was observed. As a result, we decided to first incubate ectomycorrhizal tips with the AMC substrate (Leu-AMC), then with the MU substrates (six different substrates) and finally with the ABTS substrate. Incubations took place on a microplate shaker in a climate chamber at 21°C.

To start the assay, rinsing buffer was vacuum-removed from the prepared filter plates and discarded (Fig. 1, column “buffer”). One hundred microliters of the desired incubation solution was added to all wells of the filter plate (except for the six sealed calibration wells; Fig. 1, column “substrate”); plates were placed on a microplate shaker and incubated in the dark at 22°C with the incubation times shown in Table 2. To stop the reaction, the incubated liquid of filter plates was vacuum-transferred into a prepared measurement plate (Fig. 1, column “substrate”). The measurement plate was set aside and protected from light until measurement.

The next assay cycle started by supplying the filter plate with 150 μL of the rinsing buffer with the pH appropriate for the next assay (Table 2) and the procedure was repeated as described above until all fluorescence assays were performed (Fig. 1, column “substrates 1–7”). For the laccase test with ABTS, 120 μL of incubation solution was added, and the incubated solution was finally transferred to the transparent, empty 96-well plate (Fig. 1, column “substrate 8”). The final design for multiple activity tests with eight different substrates on the seven selected ectomycorrhizal tips (incubation time, pH, type, and concentration of substrate) is summarized in Table 2.

Fluorescence and photometric measurements

All fluorescence and photometric measurements were performed on the same day as the rest of the assay. Settings

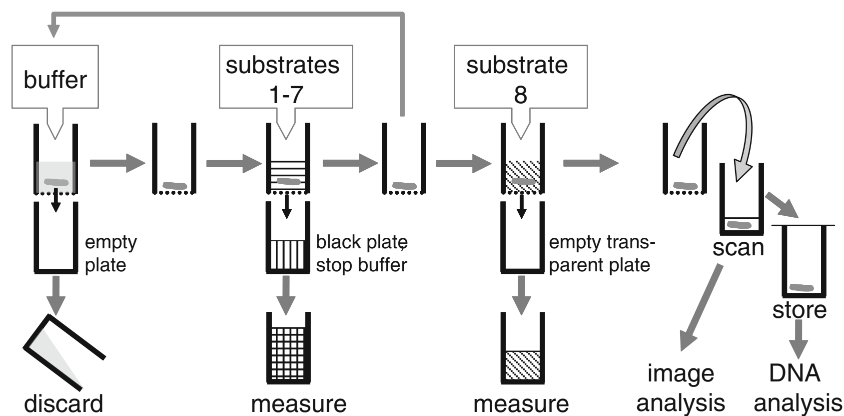

Fig. 1 Schematic of the optimized assay procedure for enzymatic profiling of ECM tips. Depicted are single wells as a representation for a whole 96-well microplate. Filter plates contain single mycorrhizal tips and the desired buffer or substrate and are used for incubation. Liquids are transferred to a receiver plate by using a vacuum chamber (not displayed here) as indicated by a *black arrow*. Contents of the receiver plate are either discarded (rinsing buffer), collected in black microplates containing stop buffer (substrates 1–7), or in a transparent microplate (substrate 8) before measuring. Following the enzyme assays, mycorrhizal tips are transferred from the filter plates to clear bottom microplates, scanned for image analyses, and finally stored at −20°C for subsequent DNA analysis

for the fluorescence measurements were 364 (±10) nm excitation and 450 (±10) nm emission in a fluorescence microplate reader after finishing the assay procedures. A slide width of 5 nm, a collection time of 0.5 s, and a sensitivity manually adjusted according to the strength of the signal was shown to be optimal on several fluorimeters used in the different labs (i.e., Varian Cary Eclipse; Victor III, Perkin Elmer; Tecan infinite M200; Xenius safas). The colorimetric ABTS assay was measured immediately in a microplate spectrophotometer at 420 (± 5) nm.

Once all eight tests were performed (Fig. 1), the root tips were transferred into a clear flat-bottom 96-well microplate containing 50 µL of water per well, scanned, and the projected surface area of each root tip was evaluated using image analysis software WhinRhizo as previously described (Buée et al. 2005). After removal of the water, root tips were frozen in the plates at −20°C until used for DNA extraction and subsequent DNA-based identification methods, which are not further described here.

Calculation of enzyme activities

Enzyme activities (EA) were calculated from fluorimeter and photometer readings by the following equation:

$$\mathrm{EA} = \frac{\mathrm{sample} - \mathrm{neg}}{a \cdot \mathrm{pa} \cdot t}$$

where sample is the measured value of the sample, neg is the measured value of the negative control (substrate without sample), pa is the projection area of the mycorrhizal roots (square millimeters), and t is the incubation time (minutes). For fluorescence measurements, a is the slope of the regression line of the calibration curve (per mole).

For the ABTS test:

$$a = \frac{\varepsilon_{425} \cdot \mathrm{pl}}{\mathrm{vol}}$$

where ε_{425} is the molar coefficient of extinction for ABTS (ε_{425}=3.6×10^4 cm^2 mol^{-1}), pl is the path length (centimeters) of the well, and vol is the incubation volume (liters). EA is expressed as (moles per square millimeter per minute) of released AMC, MU, or ABTS.

Calculations for optimization of incubation times

For calculations of optimized incubation times, a data set was used based on a total of 167 ectomycorrhizal tips of 12 different ectomycorrhizal fungus species (Table 1) sampled at "Kranzberger Forst" in autumn 2007. Sample preparation and enzyme activity measurements were performed as described by Courty et al. (2005).

From the raw data of the fluorescence and photometric tests, i.e., the sample values of incubated roots (sample) and the negative control values of the uncleaved substrate (neg), possible new incubation times were calculated by the following equation:

$$\frac{\mathrm{neg}}{\mathrm{sample} - \mathrm{neg}} \cdot t_{\mathrm{old}} = t_{\mathrm{new}}$$

where t_{old} is the incubation time of Courty et al. (2005) and t_{new} is the possible new incubation time (Table 3). It was assumed that the negative control values remained constant

Table 3 Calculated new incubation times [neg/(sample−neg)×incubation time] based on enzyme activity measurements of 167 individual ECM tips of *F. sylvatica* and *P. abies* collected at Kranzberger Forst, Germany. The minimum, maximum, 25th, 50th, and 75th percentiles are shown as criteria to select appropriate incubation times for enzyme activity profiling of ECM

Substrate	Number of included values	Minimum value (min)	First quartile (25th percentile; min)	Median value (50th percentile; min)	Third quartile (75th percentile; min)	Maximum value (min)
Leu	23	5	11	66	180	250
Xyl	160	1	7	13	20	236
Glr	51	0.1	0.5	1	3	234
Cel	132	0.4	2	5	10	43
Nag	125	0.1	1	3	5	38
Gls	136	0.4	3	5	9	236
Pho	119	4	9	19	106	285
Lac	39	2	9	19	39	149

over the relevant time span of the experiment, the assumption being based on long-term experience and measurements (data not shown), and that the value of an incubated sample increases linearly with time, which has been shown for at least three enzymes by Pritsch et al. (2004). Only sample values with a ratio sample/neg>1.2 were included in the analyses because lower values were not informative as they were too close to the values of the uncleaved substrates (data not shown).

Experiments testing storage conditions

The activities of three different enzymes were measured in each of the five experiments on storage condition described below except in experiment 5, where the full set of hydrolytic enzymes was tested. The three selected enzymes (*N*-acetylglucosaminidase, acid phosphatase, and cellobiohydrolase) were chosen because they usually show high values and can be reliably measured on all ectomycorrhizal root tips, no matter the ectomycorrhizal fungal symbiont. Laccase, leucine aminopeptidase, and β-glucuronidase activities were not expressed by all ectomycorrhizal root tips (i.e., see experiment 2). According to Courty et al. (2011), cellobiohydrolase activity is strongly correlated to β-glucosidase activity, which was not included in the tests because no additional information was expected.

Storage of samples as intact soil cores, fine roots, mycorrhizal clusters, or individual ectomycorrhizal tips

Experiment 1 For comparison of sample storage in soil vs. tap water at 4°C, the samples were divided into two equal portions. One part was washed in tap water and stored at 4°C in sealed plastic bags. The other portion was kept intact; the forest soil cores containing *Lactarius quietus–Quercus* sp. and *Tomentella sublilacina–Quercus* sp. mycorrhizal roots and plastic containers with peat–vermiculite substrates containing *L. bicolor–P. menziesii* mycorrhizal roots were kept intact in their original form at 4°C in sealed plastic bags. In both cases, air was removed before sealing plastic bags. Storage times were 1, 7, 14, 28, 42, and 56 days. At each date, seven tips of each ectomycorrhizal fungus species were removed and subjected to three enzyme assays (*N*-acetylglucosaminidase, cellobiohydrolase, and phosphatase).

Experiment 2 To study whether fragmentation of mycorrhizal samples into small pieces has an influence on enzyme activities, fine roots of *Fagus sylvatica* growing under logs of dead wood colonized with *Lactarius subdulcis* (Table 1) were used. Six clusters of *L. subdulcis* mycorrhizae were gently washed in tap water and then divided into two equal portions. One portion was stored as intact clusters, and 80 single mycorrhizae from the other fraction were cut off and stored as excised roots. Both portions were stored in tap water at 4°C. Seven freshly cut root tips from each of the six fine roots and seven previously excised root tips from each of the six clusters were assayed after 0, 7, 14, 21, 28, and 35 days of storage. All seven hydrolytic enzyme activities were tested (*N*-acetylglucosaminidase, cellobiohydrolase, phosphatase, leucine aminopeptidase, xylosidase, glucuronidase, and β-glucosidase). *L. subdulcis* showed no laccase activity, which was therefore not tested.

Experiment 3 Storage in tap water, demineralized water or in a $CaSO_4$ solution (0.2 mM $CaSO_4$ in demineralized water), was tested on synthesized ECM of *P. menziesii* × *L. bicolor*. Mycorrhizal tips were picked from roots and divided into three equal portions that were stored at 4°C either in tap water, demineralized water, or a 0.2-mM $CaSO_4$ solution. $CaSO_4$ was added due to its membrane stabilizing properties (Plassard et al. 2002). After 1 or 14 days of storage in these solutions, 14 tips per treatment were subjected to three enzyme assays (*N*-acetylglucosaminidase, cellobiohydrolase, and phosphatase).

Storage of ECM as frozen or ethanol-fixed samples

Experiment 4 The influence of freezing at −20°C was tested for three abundant field-sampled ectomycorrhizal morphotypes (*Xerocomus* sp., *Cortinarius* sp., *L. subdulcis*) of *F. sylvatica*. After cleaning the fine roots in tap water, they were screened for vital mycorrhizal systems, and 14 tips of each of the three selected morphotypes were immediately subjected to enzyme tests (*N*-acetylglucosaminidase, cellobiohydrolase, and phosphatase). The remaining mycorrhizal systems of each morphotype were immediately frozen as separate samples and stored at −20°C for 1 or 14 days. After thawing the mycorrhizal samples at room temperature, the same enzyme assay procedure was applied as for the fresh samples. A preliminary test using whole, frozen fine roots after cleaning in tap water failed because ECM could not be longer assigned to morphotypes and vital roots be distinguished from subvital roots.

Experiment 5 Storage in ethanol (100%) was tested on synthesized mycorrhizae of *P. menziesii* × *L. bicolor*. Activities of fresh ECM were compared with activities after storage in ethanol at 4°C for 7, 14, 21, 28, and 56 days. At each date, 14 ectomycorrhizal tips were taken out of ethanol and rinsed three times with tap water by gentle shaking before subjecting them to enzyme assays (*N*-acetylglucosaminidase, cellobiohydrolase, and phosphatase).

Statistical analysis

For comparison of the average enzymatic activity among dates and between treatments in the storage treatments (Table 1) as well as for the comparison of assay results between different research groups, a non-parametric rank test (Kruskal–Wallis test) was applied. For pair-wise comparisons, Mann–Whitney *U* test was used. Analyses were performed with the statistical programs SPSS 11.5 (SPSS, USA) and R (R Development Core Team 2005).

Results and discussion

Method improvements and simplifications

Incubation times displayed in Table 3 were calculated based on enzyme activity measurements of 167 individual ectomycorrhizal tips instead of empirical testing. These results clearly show that incubation times can be reduced compared to those suggested in previous protocols (Pritsch et al. 2004; Courty et al. 2005). In our study, the median value was chosen as the minimal incubation time that should be applied (Table 3), and more stringently, the 75th percentile value was chosen as preferred incubation time whenever possible. Applying the more stringent criteria (75th percentile), incubation times for five out of eight enzyme activities could be reduced: xylosidase, cellobiohydrolase, glucuronidase, *N*-acetylglucosaminidase, and β-glucosidase. Incubation times for assaying leucine aminopeptidase and phosphatase should be prolonged, and the time necessary for laccase activity remains unchanged (Table 3).

For practical reasons, the reduction of incubation times was limited to 15 min with respect to the time needed for handling and pipetting, resulting in the selected incubation times presented in Table 2. Incubation times for glucuronidase and cellobiohydrolase could have been reduced but were kept longer than calculated to be necessary because these enzymes usually show low activity; therefore, longer incubation times may allow for an increase in the signal intensity. When these incubations are performed in one series, the resulting total net incubation time of 280 min for the new procedure was 70 min longer than the time needed for two series in the protocol of Courty et al. (2005). The longer time for incubation was compensated by optimizing the overall process, i.e., by reducing the number of samples to be assayed by half and by using a vacuum manifold in combination with a filter plate (Fig. 1). Thus, the new protocol allowed the performance of all eight enzyme assays in one series in approximately the same amount of time as the old protocol required for two separate series.

The advantages of the new assay procedure with filter plates instead of sieve strips are time savings between incubation steps, reduction in potential pipetting errors, savings of one third of the incubation chemicals as liquids are transferred without any losses, and reduction in the potential sequencing effort for DNA analyses of ECM after enzyme activity measurements by half. This facilitates identification of assayed ECM and further analysis at the gene level of the same ectomycorrhizal tips that were previously characterized for their enzyme activities.

Experiments testing storage conditions for fresh field samples

Storage of samples as intact soil cores, fine roots, mycorrhizal clusters, or individual ectomycorrhizal tips

Enzyme activities of ECM declined over 8 weeks regardless of whether the ECM were stored in soil or in aqueous solutions (experiment 1; Fig. 2). Storage of root samples in water accelerated the decline of enzyme activities compared to storage in their original substrate. Storage of undisturbed root samples for up to 28 days did not significantly change enzyme activities compared to results obtained 1 day after sampling (Fig. 2). Enzyme activities measured on ECM stored in water at 4°C significantly declined after 14 days

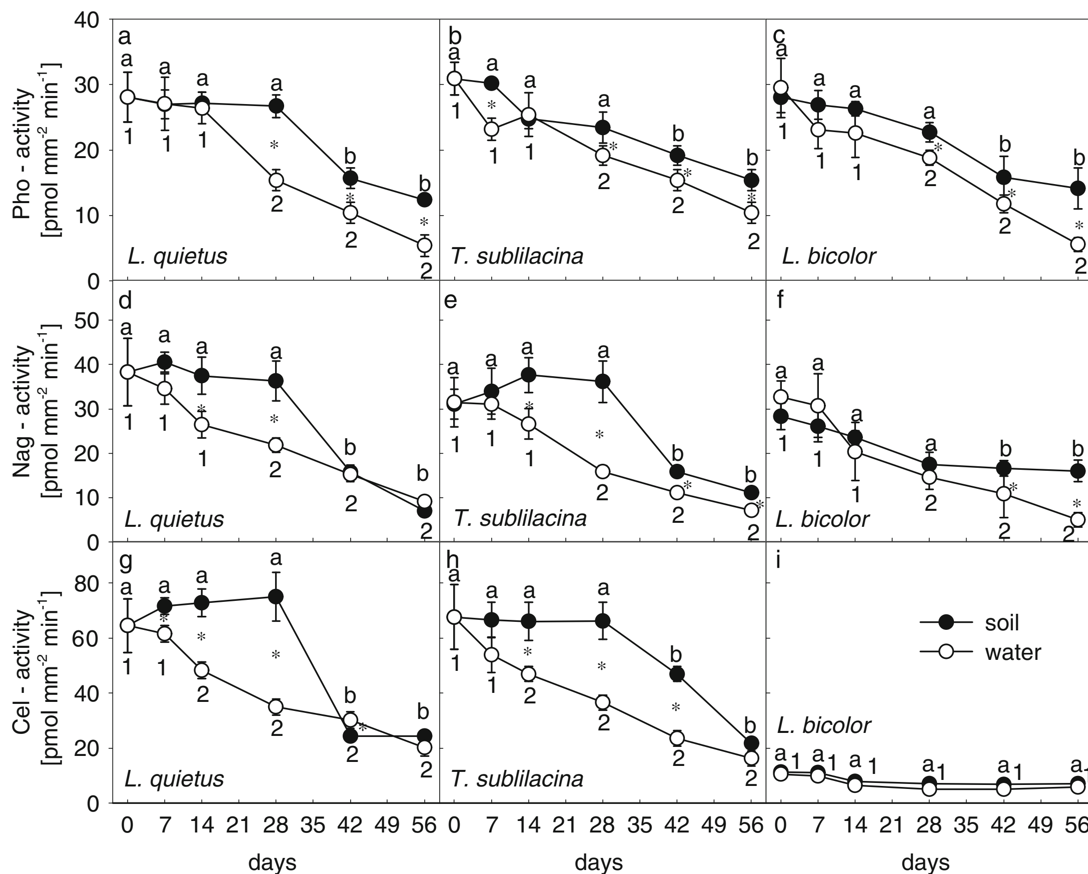

Fig. 2 Comparison of enzyme activities of ECM after storage of root samples as intact samples in their original substrate: soil (*L. quietus*, *T. sublilacina*) or peat–vermiculite (*L. bicolor*), with storage as washed root samples in water for 1, 7, 14, 28, 42, and 56 days at 4°C. **a**–**c** Phosphatase; **d**–**f** *N*-acetylglucosaminidase; **g**–**i** cellobiohydrolase. *L. quietus* (**a**, **d**, **g**) and *T. sublilacina* (**b**, **e**, **h**) ECM were collected under oak trees in Champenoux forest; *L. bicolor* (**c**, **f**, **i**) ECM were synthesized in a greenhouse in association with Douglas fir. Activities are expressed as 4-methylumbelliferone released per unit of time per ECM root tip projected area (picomoles per square millimeter per minute) for individual excised ECM root tips. *Bars* represent standard error ($n=7$). *Different lowercase letters* for soil storage and *different numbers* for water storage indicate significant differences between day 1 and the other days in the respective time series; *asterisks* indicate significant differences in enzymatic activities between water and soil storage after the same duration of storage (Kruskal–Wallis test, $p\leq0.05$)

compared to the same ECM stored in soil in all cases. Results obtained between 7 to 14 days varied with the three tested ectomycorrhizal fungus species (*L. quietus*, *T. sublilacina*, and *L. bicolor*) and the three measured enzyme activities (*N*-acetylglucosaminidase, cellobiohydrolase, and phosphatase; Fig. 2). Visual observations suggested that typical features of vital ECM, i.e., turgescent mycorrhizal tips with an intact white meristem and mantle characteristics such as color or hydrophobicity (Agerer 1991; Niederer et al. 1989), were maintained longer in samples stored in their original substrate than in water. However, the physiological vitality was not tested in our experiments. Staddon and Fitter (2001) used the nitro blue tetrazolium chloride-succinate method to compare the vitality of arbuscular mycorrhizal roots stored at 5°C in water for 1, 6, or 8 days or as intact turf sample for 7 days and then in water for 1, 7, or 14 days. They found no effect of different storage conditions. This is in accordance with our results for the time range covered. However, these authors found a strong decline in the vitality of internal hyphae and arbuscules, which they attributed to the exhaustion of carbon in these organs after removal of the shoot (Staddon and Fitter 2001), which may also explain the loss of vitality of excised roots.

Our results showing that storage in water significantly reduced enzyme activities were confirmed in experiment 2 where we tested the influence of fragmentation, i.e., excised ectomycorrhizal tips vs. intact ectomycorrhizal systems of *L. subdulcis* (Fig. 3) on seven enzyme activities after removal of the roots from their original substrate. Enzyme activities, except for leucine aminopeptidase, were the highest directly after sampling and were significantly reduced when mycorrhizae were stored in water for 7 days or longer, irrespective of whether they were stored as single tips or intact clusters (Fig. 3). Laccase activity was not

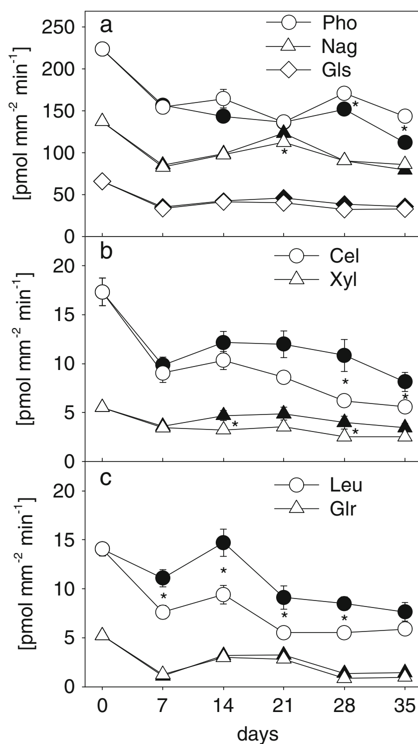

Fig. 3 Effect of storage of ECM of *L. subdulcis* with *F. sylvatica* as intact root clusters (*filled symbols*) or as excised single tips (*open symbols*) in tap water at 4°C for 0, 7, 14, 21, 28, and 35 days. Activities of **a** phosphatase (*Pho*), *N*-acetylglucosaminidase (*Nag*), and β-glucosidase (*Gls*); **b** cellobiohydrolase (*Cel*) and xylosidase (*Xyl*); and **c** leucine aminopeptidase (*Leu*) and glucuronidase (*Glr*) are expressed as the release of 4-methylumbelliferone or aminomethylcoumarin, per unit of time per ectomycorrhizal root tip projected area (picomoles per square millimeter per minute) for individual excised ECM root tips. *Bars* represent SE (n=7). Significant differences (Mann–Whitney U test) between storage conditions are indicated by an *asterisk*

detected for *L. subdulcis* (data not shown). Roots stored as intact clusters retained significantly higher leucine aminopeptidase activity compared to single clusters at days 7, 14, 21, and 28. The other enzyme activities (except phosphatase) showed a tendency toward higher activities over a longer time period when stored as clusters compared to storage as single tips, and these differences were significant only at single time points (*N*-acetylglucosaminidase, cellobiohydrolase, and xylosidase). In contrast, phosphatase activity was significantly lower after storage for 28 or 35 days as intact clusters compared to storage as excised roots. Here, it can be speculated that multiplying decomposer microorganisms (fungi, bacteria) and plant enzymes may have contributed to the increasing phosphatase activities present on ectomycorrhizal tips (Tedersoo et al. 2009; Mogge et al. 2000; Courty et al. 2011). The overall results show that storage as intact clusters retains activity slightly better than storage as single tips.

Once extracted from soil and transferred to water, ectomycorrhizal tips face physical and chemical conditions that are different from soil (pH, ionic strength, air, and complete water saturation). The accelerated aging of roots excised from their carrier roots may be due to physiological processes that continue to occur at the expense of energy equivalents, which may be supplied for a longer time in larger systems compared with excised roots. This explanation is supported by results of Niederer et al. (1989), who found that fine roots still reacted to frost and desiccation exposure with an increase in trehalose content after they were excised from the carrier roots. The slow decrease in enzyme activity indicates that most exoenzymes are quite stable, with some variation between enzymes. These results confirm earlier findings that repeated rinsing over several hours did not affect four extracellular enzymes (phosphatase, cellobiohydrolase, *N*-acetylglucosaminidase, and β-glucosidase; Pritsch et al. 2004). This may be partially due to the type of association between the corresponding exoenzymes and the

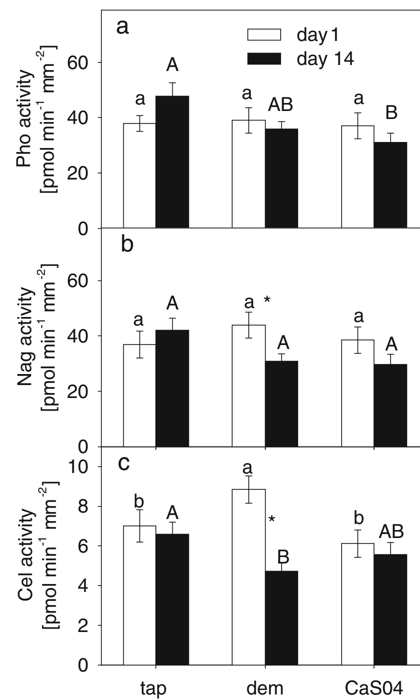

Fig. 4 Enzyme activities of ECM of *L. bicolor* × *P. menziesii* after storage in tap water (*tap*), demineralized water (*dem*), and 0.2-mM $CaSO_4$ solution (*$CaSO_4$*) for 1 or 14 days. Enzyme activities of **a** phosphatase, **b** *N*-acetylglucosaminidase, and **c** cellobiohydrolase are expressed as 4-methylumbelliferone released per unit of time per projected area (picomoles per square millimeter per minute) of individual excised ECM root tips. *Bars* represent SE (n=14). Significant differences (Kruskal–Wallis test, $p \leq 0.05$) between storage conditions (tap water, demineralized water, $CaSO_4$ solution) are indicated by *lowercase letters* after 1 day and *uppercase letters* after 14 days of storage. *Asterisks* indicate significant differences between enzyme activities after 1 and 14 days of storage in the same storage medium

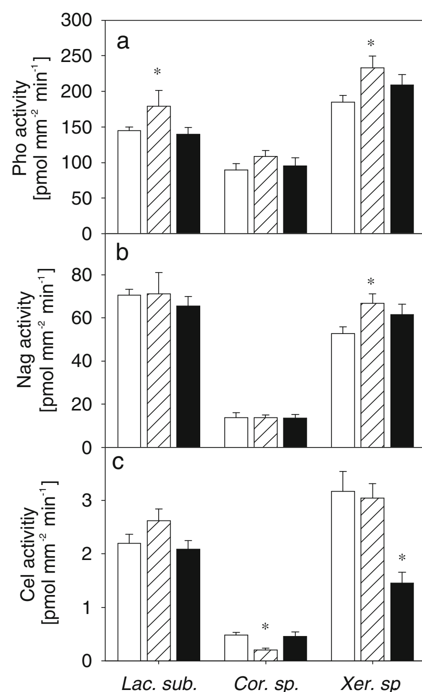

Fig. 5 Effect of storage by freezing at −20°C for 1 day (*shaded bars*) or 14 days (*black bars*) compared to field fresh samples (*empty bars*) on **a** phosphatase, **b** *N*-acetylglucosaminidase, and **c** cellobiohydrolase activities of ECM of *L. subdulcis* (*Lac. sub.*), *Xerocomus* sp. (*Xer. sp.*), and *Cortinarius* sp. (*Cor. sp.*) × *F. sylvatica* sampled at "Kranzberger Forst." Activities are expressed as 4-methylumbelliferone released per unit of time per ectomycorrhizal root tip projected area (picomoles per square millimeter per minute) for individual excised ECM root tips. *Bars* represent SE ($n=14$). *Asterisks* indicate significant differences between storage conditions within one species (Kruskal–Wallis test, $p \leq 0.05$)

fungal cell wall (Pitarch et al. 2002; Rast et al. 2003; Latgé 2007), characterized by different stable or labile bonds. In turn, decreasing enzyme activities during longer storage in water suggests either a release of enzymes from the cell wall or their degradation. A release of fungal cell wall-bound enzymes may occur through changes in pH, ionic strength of the surrounding solution, or activity of glucosidases (Rast et al. 2003). Although extracellular enzymes are relatively robust, these enzymes may eventually undergo degradation by proteolytic enzymes (Chróst and Velimirov 1991).

In experiment 3, we tested whether there is a method to preserve excised mycorrhizae during storage in water by adding or removing ions. After 1 day of storage, enzyme activities of *L. bicolor* ECM were not significantly affected by storage at 4°C in tap water, or in a 0.2-mM $CaSO_4$ solution. In contrast, cellobiohydrolase activity was significantly higher in demineralized water (Fig. 4c). After 14 days of storage, *N*-acetylglucosaminidase and cellobiohydrolase activities of *L. bicolor* ECM were not significantly affected by storage at 4°C, whereas acid phosphatase activity was significantly lower after storage in $CaSO_4$.

Overall, our results show that roots should not be kept in demineralized water, which may cause an osmotic disequilibrium, compromising cell integrity. For short-term storage (1 day), roots can be kept in a $CaSO_4$ solution or in tap water. Calcium addition to the external media enabled the recording of the transmembrane potential of individual hyphae of *Neurospora crassa* Shear & B.O. Dodge for up to 4 h (Slayman 1965). Bivalent cations such as Ca^{2+} and Mg^{2+} can stabilize the enzymes themselves (De Bolle et al. 1997), as well as stabilizing plasma membranes to prevent leakage of ions, changes in transmembrane potential, and changes in enzyme activities. Based on the results of experiments 2–4, in which different ECM were stored in tap water, it appears that storing ectomycorrhizal tips in tap water ranging from overnight storage to 1 week at 4°C is possible to conveniently study enzyme activities.

Storage of ECM as frozen or ethanol-fixed samples

In addition to the tests on storing fresh samples over a time span of 8 weeks, we tested two methods with the potential for longer-term sample storage: either by freezing at −20°C or by fixation in 100% ethanol (ethanol).

In experiment 4, freezing for 1 day changed enzyme activities significantly in four out of nine cases (phosphatase activity of *L. subdulcis* and *Xerocomus* sp., *N*-acetylglucosaminidase activity of *Xerocomus* sp., and cellobiohydrolase activity of *Cortinarius* sp.; Fig. 5). After 14 days at −20°C, enzyme activities of the ECM formed by three different fungal species (Fig. 5) were only significantly changed in the case of *Xerocomus* sp., with significantly lower cellobiohydrolase activity observed when comparing fresh samples and samples stored for 1 day. This result is difficult to interpret. Although some

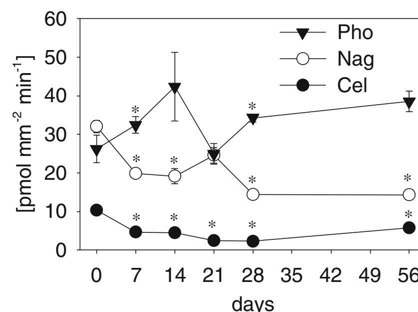

Fig. 6 Phosphatase (*Pho*), cellobiohydrolase (*Cel*), and *N*-acetylglucosaminidase (*Nag*) activities of excised ECM of *L. bicolor* × *P. menziesii* before (day 0) and after storage in ethanol (100%) for 7, 14, 21, 28, and 56 days. Activities are expressed as 4-methylumbelliferone released per unit of time per projected area (picomoles per square millimeter per minute) of individual excised ECM root tips. *Bars* represent SE ($n=14$). Significant differences (Kruskal–Wallis test, $p \leq 0.05$) between activities before (day 0) and after storage in ethanol are indicated with *asterisks*

ectomycorrhizal fungus species may be more robust against freeze-thawing than others (Lehto et al. 2008), this would not explain the result demonstrating a difference between 1 and 14 days of freezing. A likely reason for the results observed with *Xerocomus* sp. and a practical constraint in using frozen root material is the loss of typical morphological features of ectomycorrhizal tips such as color or hydrophobicity (Agerer 1991), resulting in substantial difficulties in differentiating morphotypes or distinguishing vital and older roots. As enzyme activities are dependent upon the vitality of mycorrhizae, only fully vital tips should be used for comparison of enzyme activities between ECM formed by different fungal species (Pritsch et al. 2004). In the case of *Xerocomus* sp. ECM, it is possible that a set of older tips was assayed after 14 days of storage. We did not test longer storage under frozen conditions because of the previously mentioned restrictions that we were unable to solve. However, because of the uncertainty about the reasons for the conflicting results, we conclude that freezing cannot be recommended as a long-term storage method.

In experiment 5, storage of ECM of *L. bicolor* in ethanol resulted in different effects according to the enzymes assayed (Fig. 6). Phosphatase activity was not significantly decreased by storage in ethanol compared to the values measured on fresh tips (day 0). However, the variability of the data increased, which may be explained by incomplete rehydration of enzyme proteins in some of the assays. After ethanol storage, variation coefficients for means of phosphatase activity ranged between 28% (day 0), 25% (day 7), 73% (day 14), 38% (day 21), 11% (day 28), and 25% (day 56). Recovery of phosphatase activity after ethanol fixation is in agreement with histochemical methods in which ethanol is used to fix tissues such as bones (Cosby et al. 2008) or to carry out post-fixation of preserved tissues (Kacena et al. 2004) while maintaining acid phosphatase activity. Secreted acid phosphatase released by the filamentous fungus *Aspergillus fumigatus* Fresen. into culture medium displayed hydrolytic activities after precipitation using ethanol (Bernard et al. 2002).

In contrast to phosphatase, ethanol storage significantly decreased *N*-acetylglucosaminidase activities by about 50% at days 7 and 14 and then by 70% at days 28 and 56. Day 21 was an exception with no significant reduction compared to day 0. Ethanol storage caused a strong decrease in and a high variability of cellobiohydrolase activities at all days tested. In contrast to acid phosphatase, *N*-acetylglucosaminidase and cellobiohydrolase did not withstand ethanol treatment, possibly due to irreversible changes in protein conformation, thus decreasing hydrolytic activities. Therefore, storage of ECM in ethanol may be used before measuring phosphatase activity, but this method cannot be recommended for measurements of *N*-acetylglucosaminidase or cellobiohydrolase activities.

Conclusions

Our optimization of enzyme assays on individual ECM provides reliable recommendations for sample storage and reaches a high level of standardization by replacing custom-made by purchasable materials. The main conclusion from our storage experiments is that root samples can be preserved as undisturbed root samples at 4°C in their original substrate for a maximum of 28 days without a loss of activities of *N*-acetylglucosaminidase, cellulase, or phosphatase activities. Storage in tap water should be kept to a minimum and be shorter than 1 week. Storage of single root tips over longer time periods in tap water or sampling extremely small fragments of fine roots (i.e., use of too-small coring cylinders) should be avoided when roots will be used for enzymatic activity profiling. In the future, our method may also be useful for assessing potential enzyme activities of other mycorrhizal types, i.e., ericoid and arbuscular mycorrhizae. The method has a bias for functions located at the mantle surface of ECM while excluding mycelia (Pritsch and Garbaye 2011). However, there is a potential to address mycelial parts of mid- and long-distance type ECM (Agerer 2001) as well. This type of sampling may be achieved by using mesh bags that exclude roots and favor ingrowths of ectomycorrhizal fungal hyphae and rhizomorphs (Hedh et al. 2008) in combination with ECM collected in the same vicinity. This would be challenging but could prove to be a useful extension of the protocol to cover all ectomycorrhizal exploration types, including mycelia, on a functional basis.

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