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# Determination of mannitol in ectomycorrhizal fungi and ectomycorrhizas by enzymatic micro-assays

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**Abstract.** Two sensitive methods for the enzymatic determination of mannitol are described which were applied to fungal and mycorrhizal extracts. Both methods are based on the oxidation of mannitol by mannitol dehydrogenase from *Agaricus hortensis* and the fluorometric determination of the NADPH produced in this reaction. The detection limits are 125 pmol for the direct fluorometric assay and 100 fmol, when enzymatic cycling of NADPH is included. The levels of mannitol detected were 123 pmol/μg dry wt (mycelia from *Cenococcum geophilum,* cultivated on malt medium), below 0.3 or about 2.4 pmol/μg dry wt (mycelia from *Amanita muscaria,* dependent on carbon source in the cultivation medium), and between 1.9 and 5.2 pmol/μg dry wt in mycorrhizal short roots of *Picea abies/Amanita muscaria.*

**Key words:** *Amanita* – *Cenococcum* – Enzymatic cycling – Mannitol determination – Mycorrhiza

## Introduction

D-Mannitol is the most common polyol in fungi (Lewis and Smith 1967). Apart from its function as a source of metabolic carbon and energy (Lewis and Smith 1967; Blumenthal 1976; Jennings 1984), mannitol accumulation in fungi is of considerable importance for resistance to water- (Lewis and Smith 1967) and heat stress (Pons et al. 1986), and for generating osmotic potential during the growth of fruit bodies (Hammond and Nichols 1976).

For the synthesis of this sugar alcohol, Hult and Gatenbeck (1978) proposed a NADPH-regenerating "mannitol cycle" for the deuteromycete *Alternaria alternata.* This cyclic pathway has been since confirmed for ascomycetes (Martin et al. 1985, 1988; Ramstedt et al. 1987) but not for basidomycetes (Ramstedt et al. 1986, 1987). In ectomycorrhizas, the synthesis of mannitol by the fungus most probably relies on sugars delivered by the host plant. As the root of the host plant has no or only a restricted ability to metabolize mannitol, the fungal symbiont can thus deplete the root of soluble sugars (Lewis and Harley 1965; Jirjis et al. 1986).

Laborious chromatographic methods of low sensitivity have so far been applied for mannitol determination in mycorrhizas (see e.g. Niederer 1989). Spectrophotometric assays for the enzymatic determination of mannitol, as applied in medical diagnosis (see e.g. Blood et al. 1991) are also of low sensitivity and thus require relatively large amounts of sample. This severely hampers mannitol determination in mycorrhizal material where large samples are usually not available. This is even more problematic when single mycorrhizal root tips are to be assayed. We, therefore, developed micromethods for the enzymatic determination of mannitol in fungal material, and in this contribution we present an assay that allows the determination of mannitol down to about 100 fmol.

## Materials and methods

### *Materials*

Mycelium of *Amanita muscaria* (L. ex Fr.) Hooker strain MG2 (Guttenberger 1989) was isolated from a fruiting body. *Cenococcum geophilum* Fr. strain no. 117.57 was obtained from CBS Baarn, The Netherlands. Fruiting bodies of *Agaricus hortensis* (Cke.) Pilat (sensu Moser 1983) were obtained from commercial growers. The solid cultivation media contained agar 1614 (Merck, Darmstadt, Germany).

### *Culture of mycorrhizal fungi*

The cultivation media (MMN, MMNC) were identical to the media described by Guttenberger and Hampp (1992). *A. muscaria* was first grown in suspension culture (MMNC-medium) and then dispersed on cellophane sheets, which were placed in petri dishes

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filled with MMN agar (2% agar) containing different carbon sources (MMNC agar if not indicated otherwise). After incubation for 10 days (20° C, darkness), the cellophane sheets with the mycelium were quick-frozen in liquid nitrogen and stored at −80° C. *C. geophilum* was grown in liquid medium containing 2% (w/v) malt extract. The fungal material was lyophilized at −30° C, homogenized under liquid nitrogen using a "Micro-Dismembrator" (Braun, Melsungen, Germany) and stored under vacuum at −20° C.

### *Culture of mycorrhizas*

Mycorrhizas of the symbionts *A. muscaria* (L. ex Fr.) Hooker and *Picea abies* (L.) Karst. were established as described by Kottke et al. (1987) and as modified by Guttenberger (1989). For more detailed information see also Guttenberger and Hampp (1992).

### *Isolation and purification of mannitol dehydrogenase*

After removing the gills, the washed and chilled fruiting bodies of *A. hortensis* were homogenized with 250 ml isolation buffer (50 mM Tris/HCl, pH 8.05) as described by Edmundowicz and Wriston (1963). After filtration through a 200-μm nylon mesh and centrifugation (7000 × *g*, 15 min), the supernatant was dialysed against the isolation buffer. $(NH_4)_2SO_4$ was added to bring the solution to 60% saturation and the precipitated protein was removed by centrifugation (9000 × *g*, 15 min). Mannitol dehydrogenase was precipitated at 80% saturation of $(NH_4)_2SO_4$, pelleted (9000 × *g*, 15 min) and dissolved in a minimal volume of isolation buffer. This preparation was applied to a column of QAE-Sephadex (Q-25-120, Sigma, Deisenhofen, Germany) with a bed volume of 125 ml and eluted with the isolation buffer. Fractions containing active enzyme were pooled and concentrated by ultrafiltration (Centrifugal Ultrafree-20, Millipore, Eschborn, Germany). The enzyme preparation was stored at −20° C. Protein content was determined by the method of Neuhoff et al. (1979) as modified by Guttenberger (1989). Mannitol dehydrogenase activity was assayed spectrophotometrically (Uvikon 860, Kontron, Eching, Germany) by monitoring the increase in absorbance at 340 nm and 25° C. A 5-μl aliquot of the enzyme preparation was incubated in a 500-μl reaction volume containing 47 mM carbonate buffer (pH 9.8), 200 mM mannitol and 5 mM NADP.

### *Extraction of mannitol*

The lyophilized material was extracted with 65% (v/v) ethanol for 1 h at 60° C and pelleted (10000 × *g*, 5 min). The supernatant was used for mannitol determination.

### *Mannitol determination*

*Direct fluorometry.* The increase in fluorescence due to the formation of NADPH by the mannitol dehydrogenase reaction was determined in a SFM 25 fluorometer (Kontron, Eching, Germany) at wavelengths of 350 nm (excitation) and 460 nm (emission). Aliquots of extract or of mannitol standard (10 μl) were incubated in a cuvette containing 45 mM carbonate buffer (pH 9.8), 3 mM NADP and 250 mU mannitol dehydrogenase (total volume = 500 μl) for 80 min at 22° C. The change in fluorescence during the incubation was recorded and the background (fluorescence change without mannitol) was subtracted.

*Indirect fluorometry (enzymatic cycling).* The NADPH formed in the mannitol dehydrogenase reaction was amplified as described by Lowry and Passonneau (1972). Before use, the extracts were diluted to an ethanol concentration of 16.25% (v/v). Mannitol standards were also made up in 16.25% ethanol. All pipetting steps were performed on ice. Using 0.5-ml Eppendorf vials, 10-μl sample volumes were mixed with 20 μl of a solution containing 42.5 mM carbonate buffer (pH 9.8), 5 mM NADP and 750 mU $ml^{-1}$ mannitol dehydrogenase (background = without mannitol dehydrogenase; internal standard = 2 μM mannitol) in duplicate and incubated at ambient temperature for 80 min. The reaction was stopped by alkalization (10 μl 0.4 M NaOH) and the remaining NADP was destroyed by incubating the mixture for 5 min at 90° C. Aliquots of 2 μl were mixed with 50 μl of NADP(H) cycling reagent (125 mM Tris/HCl, pH 8.1, 1.25 mM glucose-6-phosphate, 12.5 mM $NH_4Cl$, 6.25 mM α-ketoglutarate, 125 μM ADP, 12 U $ml^{-1}$ glutamate dehydrogenase, 2.8 U $ml^{-1}$ glucose-6-phosphate dehydrogenase) in fluorometer tubes (Farrand-type; three replicates). After incubation for 60 min in a 37° C water bath, the cycling reaction was stopped by boiling the samples for 5 min and 1 ml indicator reagent (40 mM Tris/HCl, pH 8.1, 30 mM $NH_4Cl$, 5 mM $MgCl_2$, 110 μM EDTA, 200 μM NADP, 60 mU $ml^{-1}$ 6-phospho-gluconate dehydrogenase) was added to each fluorometer tube. Fluorescence was measured at wavelengths of 340 nm (excitation) and 455 nm (emission) in a ratio-2-fluorometer (Farrand, New York).

## Results

### *Activity of the purified mannitol dehydrogenase*

The specific activity obtained by the purification procedure was 54 U/mg protein. As no reduction of NADP occurred in the presence of L-arabitol, erythritol, galactitol, glucose, glucose-6-phosphate, glutamate, mannitol-1-phosphate and sorbitol, the enzyme preparation was considered to be pure enough for mannitol determination.

### *Mannitol determination by direct fluorometry*

In order to check the linearity of the assay, mannitol standards down to 65 pmol per fluorometer tube were measured. As shown in Fig. 1, the increase in fluorescence was linear with respect to the mannitol concentration and differed significantly from the background down to 125 pmol ($P < 0.05$, using Student's *t*-test). Linearity was maintained up to 10 nmol mannitol.

For samples of *C. geophilum,* the linearity of the assay was determined using different amounts of mycelium (Fig. 2).

### *Mannitol determination by enzymatic cycling*

Due to the enzymatic cycling of NADPH, the limit of detection could be lowered to 100 fmol mannitol contained in 2 μl. At this concentration (50 nM), the resulting fluorescence was still significantly higher than the background ($P < 0.001$, using Student's *t*-test; Fig. 3). Probably due to constituents of the sample material that inhibited enzyme activity in the cycling reaction, the detection limits for mannitol standards in the pres-

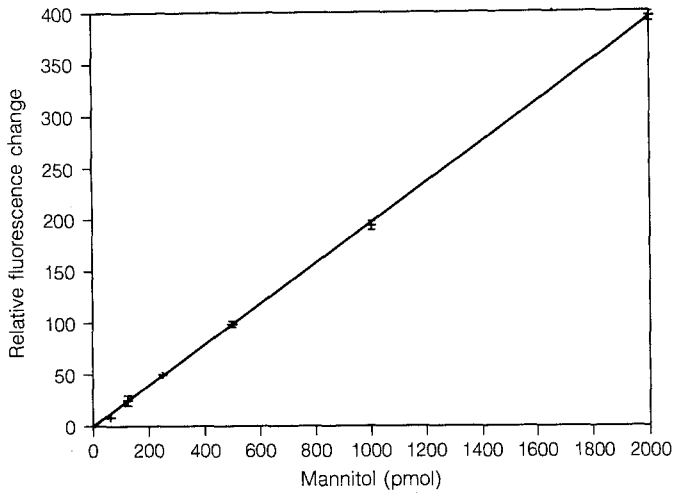


**Fig. 1.** Mannitol determination by direct fluorometry. The change in relative fluorescence during the incubation was recorded at wavelengths of 350 nm (excitation) and 460 nm (emission) and the background (without mannitol) was subtracted. Data are the means of three replicates ±SD

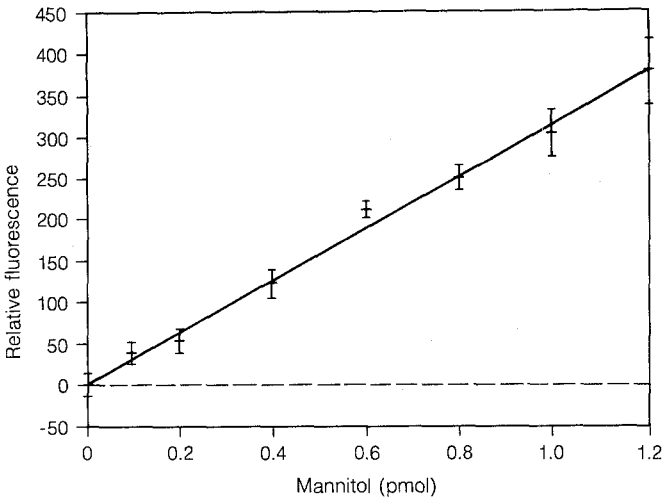


**Fig. 3.** Mannitol determination by enzymatic cycling. Relative fluorescence was measured at wavelengths of 340 nm (excitation) and 455 nm (emission) and the background (without mannitol) was subtracted. Data are the means of six replicates ±SD

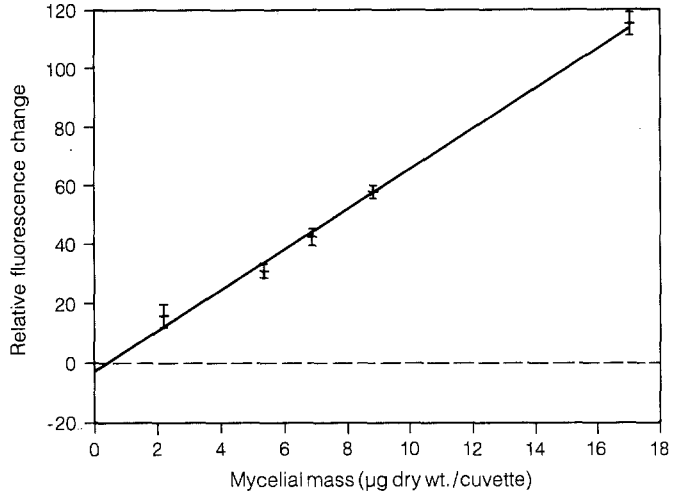


**Fig. 2.** Mannitol determination in *Cenococcum geophilum* mycelium by direct fluorometry. The change in relative fluorescence during the incubation was recorded at wavelengths of 350 nm (excitation) and 460 nm (emission) and the background (without mannitol) was subtracted. Data are the means of three replicates ±SD

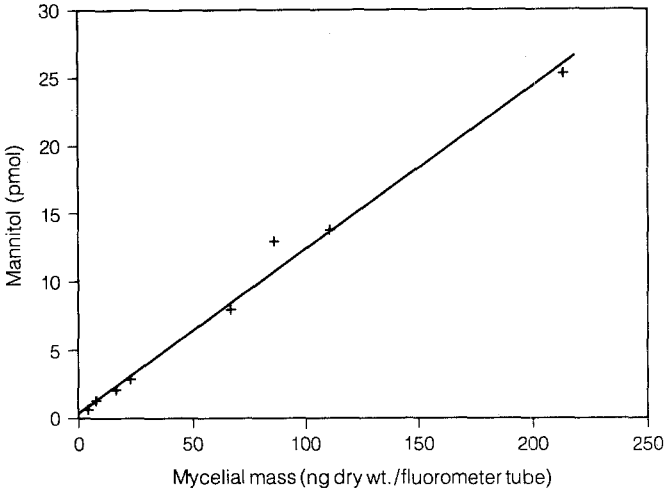


**Fig. 4.** Mannitol determination in *C. geophilum* mycelium by enzymatic cycling. Data are the means of six replicates

ence of mycelial extracs of *A. muscaria* (2 µg material per fluorometer tube) and root extracts of *P. abies* (0.5 µg material per fluorometer tube) were higher than in the absence of extract (Table 1).

In order to detect possible losses of mannitol during the extraction procedure we used internal standardization; mannitol (2 pmol per fluorometer tube) added to the extraction step was determined in the presence of different amounts of freeze-dried homogenates of *A. muscaria* mycelium and compared to an external standard (not heated in ethanol). Small amounts (0.5 µg) of fungal material did not influence recovery of the mannitol standard, whereas higher amounts reduced the recovery by about 13%.

**Table 1.** Detection limits for mannitol in the cycling assay ($P < 0.001$, Student's *t*-test)

| Assay | Detection limit (pmol) |
|---|---|
| Standard without extract | 0.1 |
| Standard with *Amanita muscaria* extract (2 µg/fluorometer tube) | 0.6 |
| Standard with *Picea abies* root extract (0.5 µg/fluorometer tube) | 0.4 |

As with the direct fluorometric assay, different amounts of *C. geophilum* mycelium were used to check the linearity of the mannitol determination in fungal material (Fig. 4). The calculated mannitol content was 123 pmol/µg dry wt. Because mannitol was not detect-

able (<0.3 pmol/μg dry wt.) in *A. muscaria* mycelium grown on MMNC agar, the mycelium was cultivated on MMN agar containing different carbon sources (100 mM glucose, 100 mM fructose, 50 mM glucose plus 50 mM fructose, 50 mM trehalose, 50 mM sucrose). Mannitol was only detectable (>0.3 pmol/μg dry wt.) in mycelium grown on MMN medium with 100 mM fructose; the mannitol content was 2.4±0.6 pmol/μg dry wt. (mean of three series of assays, in which different amounts of mycelium were employed, ±standard deviation). Mannitol was also detectable in mycorrhizal root tips of the system *P. abies/A. muscaria*, with amounts between 1.9 and 5.2 pmol/μg dry wt.

## Discussion

### *Mannitol determination*

By applying fluorometry, the detection limit for mannitol was lowered from the nmol (Berezenko and Sturgeon 1991; Blood et al. 1991) to the pmol range (direct fluorometry). Enzymatic cycling of NADPH further enabled us to extend the lower detection limit down to 100 fmol. This is a prerequisite for the analysis of the small sample sizes typical of symbiotic interactions such as mycorrhizal root tips.

Until now, mannitol was mainly determined by chromatography. This approach is hampered by the restricted solubility of mannitol during extraction with pyridine, the need for derivatization, and the low sensitivity (Niederer 1989). The enzymatic method described here circumvents these problems. In particular, the high sensitivity allows histochemical analysis of mannitol distribution in subsections of ectomycorrhizas as described for sugars (Rieger et al. 1992). In view of the fungus-specific location of mannitol, this method may also be a valuable tool to determine the degree of mycorrhization of root tips, substituting for the HPLC-dependent determination of ergosterol, another fungus-specific compound (Martin et al. 1990; Nylund and Wallander 1992).

### *Mannitol content of ectomycorrhizal fungi and ectomycorrhizas*

As expected, the mycelium mannitol content of the ascomycete *C. geophilum*, which has an operating mannitol cycle (Martin et al. 1985), is much higher than that of the basidomycete *A. muscaria*. The fact that mannitol accumulation in *A. muscaria* mycelium is highest on a fructose medium indicates that mannitol is directly synthesized by mannitol dehydrogenase and not by mannitol-1-phosphate dehydrogenase, which was not detectable, (data not shown; see also Ramstedt et al. 1987) and mannitol-1-phosphatase. In this context, it is interesting that mannitol also accumulates in mycorrhizas formed between *P. abies* and *A. muscaria*. This implies that in this symbiotic association the fungus takes up fructose, possibly derived from sucrose supplied by the host. The mannitol content (between 1.9 and 5.2 pmol/μg dry wt. is lower than that found in the mycorrhizas investigated by Niederer (1989), who found that different types of mycorrhizas from the forest contained mannitol at levels of approximately, 5–66 pmol/μg dry wt. The uptake of host-derived sugars together with the spacial distribution and metabolism of mannitol in the *Picea/Amanita* system will be the focus of our future research.

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