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Original Research

Enzymatic Activity of *Thelephora terrestris* and *Hebeloma crustuliniforme* in Cultures and Mycorrhizal Association with Scots Pine Seedlings

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Abstract

The activities of enzymes such as cellulase, pectinase, proteinase, chitinase and acid phosphatase produced *in vitro* by *T. terrestris* and *H. crustuliniforme,* as well as the activity of β-glucosidase in roots of inoculated Scots pine seedlings, were studied. In pure cultures an isolate of *T. terrestris* produced nine of ten enzymes, whereas an isolate of *H. crustuliniforme* produced only five enzymes. The activity of enzymes was higher in *T. terrestris* cultures than in *H. crustuliniforme*, the only exception being neutral proteinase. It was observed that in roots of seedlings inoculated with *T. terrestris* levels of β-glucosidase was about 107 per cent higher activity than in roots of non-inoculated seedlings. The enzyme level in roots inoculated with *H. crustuliniforme* was 44 per cent lower than in those inoculated with *T. terrestris*.

Keywords: ectomycorrhizal fungi, enzymatic activity, Scots pine

Introduction

Studies of Scots pine seedlings in bare root nurseries have shown that high nitrogen content in soils can limit the development of mycorrhizae [1]. In many cases nursery soils are high in mineral and organic nutrients due to excessive fertilizer application. In such nurseries species of *Thelephora* are dominant on roots of seedlings, particularly *T. terrestris* [1-3]. This fungus thrives in soils that are rich in minerals as well as high moisture [4-6].

T. terrestris tolerates a relatively wide range of pH and different soil types [3]. These features have stimulated numerous studies aimed at widening the use of this species. This work is of both scientific and practical importance, especially in Poland, where former arable agricultural fields are to be afforested using of mycorrhiza-inoculated containerized seedlings [7]. In some cases it has been confirmed within a few years of seedling outplanting that inoculated fungi were replaced by *T. terrestris* [8], this being a fungus that can thrive on roots for many years. There is a poor understanding of the inter-relationship between this widespread and common mycorrhizal fungus and its host plants. Amongst the unknown factors, the important role played by hydrolytic and oxidative extracellular enzymes on ectomycorrhizal development was studied [9]. The essential enzymes, acid phosphatase and proteinase are particularly relevant with respect to P and N cycles [10]. In ectomycorrhizal development important roles are played by hydrolytic and oxidative extracellular enzymes [9].

Table 1. Activities of extracellular enzymes (exocellulase, endocellulase, PG, PGL, PL, acid and neutral proteinases, chitinase) and cellular extractracts (β-glucosidase and acid phosphatase) of *Thelephora terrestris* and *Hebeloma crustuliniforme* grown in pure cultures; average values ± SD (n=6) 0 - no enzymatic activity at given experiment parameters; Values in rows that are followed by a different letter indicate significant differences, t-test, P<0.05.

Enzyme-specific activity (U)		Fungi	
		Thelephora terrestris	*Hebeloma crustuliniforme*
Cellulase	Exocellulase	0	0
	Endocellulase	23.01 ± 0.52 b	0 a
	β-glucosidase	64.75 ± 2.63 b	0 a
Pectinase	PG	271.76 ± 6.15 b	42.70 ± 3.58 a
	PGL	0.77 ± 0.07 b	0 a
	PL	60.17 ± 3.74 b	0 a
Chitinase		12.91 ± 0.40 b	7.63 ± 0.18 a
Proteinase	acid	0.68 ± 0.14 b	0.40 ± 0.08 a
	neutral	0.14 ± 0.02 a	4.86 ± 1.11 b
Acid phosphatase		0.95 ± 0.08 b	0.43 ± 0.04 a

Courty et al. [11] found that some ectomycorrhizal fungi have shown very low enzymatic activity, while others were able to produce at least one specific enzyme. In Poland *H. crustuliniforme* is commonly used to inoculate Scots pine seedlings for outplanting on post-agricultural lands [12]. Ectomycorrhizas of the fungus are within a few years replaced by other fungi, among which *T. terrrestis* ectomycorrhizas are numerous [6]. The hypothesis is that persistence and abundance of some fungi on roots may reflect partly on their high enzymatic activity.

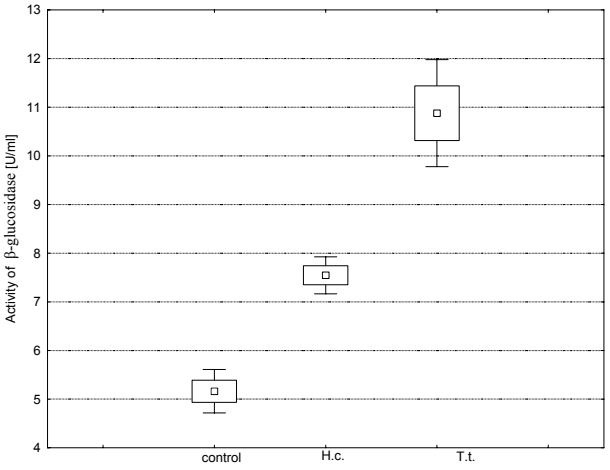

Fig. 1. β-glucosidase activity in Scots pine seedling roots: control- non-inoculated seedlings, T.t.-seedlings inoculated with mycelium of *T. terrestris*, H.c.- seedlings inoculated with mycelium of *H. crustuliniforme*, n=5, p=0.000001. Differences are significant at p< 0.05.

In order to know if this is true we investigated:

1. the activity of cellulase, pectinase, proteinase, chitinase and acid phosphatase in *in vitro* in cultures of *T. terrestris* and *Hebeloma crustuliniforme*,
2. the activity of β-glucosidase in roots of seedlings that were inoculated with mycelium of both those fungi.

The results obtained were considered likely to be helpful in inoculation process of seedlings, when using inoculum which consists of two or more fungi may have a better effect on performance of seedlings, particularly those planted on post-agricultural lands.

Material and Methods

Fungi

Thelephora terrestris (Erhr.) Fr, (isolate of IBL/ZFL/2) and *Hebeloma crustuliniforme* (Bull.) Quel., (isolate of IBL/ZFL/3) from the pure culture collection of the Polish Forest Research Institute (Department of Forest Phytopathology) were used in this study.

Culture Conditions for Enzymatic Activity

To study the cellulolytic, pectinolytic, proteolytic and chintinolytic activities, fungi were grown in MMN medium modified by Kottke et al. [13] supplemented respectively with 5g l^{-1} carboxymethylcellulose (CMC, Sigma), 5g l^{-1} citrus pectin (Sigma), 5g l^{-1} yeast extract (Difco) or 3g l^{-1} colloidal chitin (Sigma) for the induction of enzymes. To study the acid phosphatase activity, fungi were cultured in basal medium supplemented with 5g l^{-1} glucose without malt extract and reduced quantities of phosphates (down to 50 mM $(NH_4)_2HPO_4$ and 50 mM KH_2PO_4).

Aliquots (100 ml) of medium dispensed into 300 ml Erlenmeyer flasks were inoculated with 1 ml of washings prepared in 5 ml H_2Odest from 14-day-old fungal cultures grown on PDA slants at 24ºC. Three replicate flasks of each combination were used (static cultures).

Subsequently after 14 days of growth at 24ºC in the media, cultures were centrifuged at 15,000 rpm. for 10 min at 4ºC and the enzyme activities (cellulolytic, pectolytic, proteolytic and chitynolytic) were estimated in supernatant. β-glucosidase and acid phosphatase activities in mycelium extracts.

Activity of Endo-1,4-β-glucanase (EC 3.2.1.4.), Exo-1,4-β-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21)

Endo- and exogluconase activities were studied by estimation of free-reducing sugars in incubation mixture consisting of: 0.5 ml of post-culture supernatant, 1.7 ml 0.01 M acetate buffer (pH 5.5) and 0.8 ml 2% carboxymethylcellulose (Sigma) (for endo-1.4-β-glucanase) or 1% Avicel (Cellulose microcrystalline 0.1 mm, Serva for exo-1.4-β-glucanase). These samples were incubated at 50°C for 50 min. and later the reducing sugars were estimated colometrically using the Somogyi [14] and Nelson [15] method.

The endocellulase- and exocellulase-specific activity units correspond to the amounts of enzyme which released 1μmol glucose from the substrates per 1 min. per mg of protein in the post-culture liquid during the experiment.

β-glucosidase activities were estimated in mycelium homogenates. β-glucosidase was determined by measuring the amount of p-nitrophenol released in the reaction mixtures consisting of: 0.9 ml of 0.01 M acetate buffer (pH 5.5), 50 μl of 40 mM p-nitrophenyl-β-D-glucopyranoside (Sigma) and 50 μl of the seedling root extract. The mixture was maintained at 40°C for 30 min. and after the addition of 3 ml of 1 M Na_2CO_3, the released p-nitrophenol was measured spectrophotometrically at 400 nm.

The activity unit of β-glucosidase was defined as the amount of enzyme which released 1μmol p-nitrophenol per min^{-1} under the experimental conditions.

Pectolytic Activity

Polygalacturonase (EC 3.2.1.15; PG), pectate lyase (EC 3.1.1.11; PGL) and pectin lyase (EC 4.2.2.10; PL) activity was estimated by the tyobarbituric acid (TBA) test [16]. The reaction mixture consisted of 2ml of 1% citrus pectin (Sigma) (for PL) or sodium polypectate (polygalacturonic acid, Sigma) (for PGL and PG) in 0.05M buffer pH 5.0 (PG) or 0.05M Tris-HCl buffer pH 8.0 (PL and PGL), 0.5ml of 0.01M $CaCl_2$ (PL and PGL) and 2 ml of post-culture liquid. After 3-hour incubation at 30°C the reaction was stopped by adding to each test tube 3 ml of 9% $ZnSO_4$ x $7H_2O$ as well as 0.3 ml of 0.5M NaOH. Then the samples were centrifuged for 10 min. at 14,000 rpm. Subsequently, to 1 ml of supernatant 0.6 ml 0.04 M thiobarbituric acid, 0.1 ml of distilled H_2O and 0.3 ml of 1 M HCl were added. Test tubes with the above content were placed in a boiling water bath for 30 min. After cooling down, spectrophotometric measurements were taken using Shimadzu UV VIS-1202 at 515 nm wavelength for PG and at 548 nm for PL and PGL.

The pectolytic enzyme-specific activity unit corresponds to the amount of enzyme which released 1μmol of galacturonic acid from substrate per 1 min per mg of protein in the post-culture liquid during the experiment.

Proteolytic Activity

Estimation of acid activity (EC 3.4.23.18) and neutral (EC 3.4.24.31) proteinase was performed by the method reported by Hazen et al. [17]. The reaction mixture consisted of 750 μl of post-culture liquid and 750 μl of 2% azocaseine (Sulfanilamide Azocasein, Sigma) in 0.2 M acetate buffer pH 4.5 (for acid proteinase) or 0.2 M Tris-HCl buffer pH 7.5 (for neutral proteinase). The mixture was maintained at 37°C for 4 hours. The reaction was stopped by adding 1.6 ml 7% $HClO_4$. Subsequently, the mixtures were centrifuged at 10,000 rpm and 4 ml aliquot of the supernatant was added to 450 ul of 10M NaOH. Proteolitic activity was measured colorimetrically at 440 nm wavelength.

The proteinase-specific activity unit corresponds to the amount of enzyme which released 1μmol of tyrosine from substrate per 1 min. per mg of protein during the experiment.

Chitinases

Chitinase (EC 3.2.1.14) activity was studied. The activity of these enzymes was estimated on the basis of the amount of the final product of N-acetyl-D-glucosamine (NAG) released during the reaction. The mixture consisted of: 0.6 ml of fungal filtrate and 1 ml of 1% colloidal chitin in 0.1 M acetate buffer (pH 5.5). After 3-hour incubation at 37ºC the amounts of N-acetyl-D-glucosamine released were evaluated by the Miller method [18]. The amounts of the released product were estimated spectrophotometrically at 530 nm wavelength.

The chitinase-specific activity unit corresponds to the amount of enzyme which released 1μmol of N-acethyl-D-glucosamine from substrate per 1 min per mg of protein during the experiment.

Acid Phosphatase Activity

Acid phosphatase activity was studied using the method of Kieliszewska-Rokicka et al. [19]. To obtain enzymatic samples, mycelium was centrifuged and rinsed in distilled H_2O and then in 0.01 M acetate buffer (pH 4.8), following centrifuging at 14,000 rpm for 10 min. at 4ºC. Then the mycelium was homogenized in a glass homogenizer with 0.01 M acetate buffer (pH 4.8) containing: 0.01 EDTA, 0.01% Triton X 100 and 2.5% insoluble Polyclar (PVP), in the proportion of 1 ml buffer per 100 mg fresh mycelium mass. Crushed mycelium was centrifuged at 14,000 rpm. for

10 min. at 4ºC. Supernatant was used to estimate acid phosphatase. A mixture of 3 ingredients (acetate buffer, p-nitrophenyl phosphorate and enzyme sample) was incubated at 37ºC in darkness for 1 hour. The reaction was stopped by adding to each test tube 5 cm³ 0.2 M Na_2CO_3. The amounts of p-nitrophenol were evaluated using a digital spectrophotometer (Shimadzu UV-VIS 1202) at 400 nm wavelength.

The acid phosphatase specific activity unit corresponds to the amount of enzyme which released 1 µmol of p-nitrophenol per1 min per mg of protein during the experiment.

Evaluation of β-glucosidase Activity in Seedling Roots Inoculated with Mycelium *T. terrestris* and *H. crustuliniforme*

Scots pine seedlings were grown in large test tubes of 35 (lenght) x 3.5 (∅) cm. Ingestad mineral medium (10-fold diluted) with 0.7% agar was used for culturing. For each treatment there were five replicates. Seeds of Scots pine were taken from a gene bank in Kostrzyca, Poland. To obtain aseptic seedlings pine seeds were surface-sterilized in 30% hydrogen peroxide for 20 min, and rinsed several times in sterile distilled water. The seeds were placed in Petri dishes containing 0.1% pepton and 1.5% agar medium, and were incubated at constant illumination. After 4 to 7 days, germinated sterile seeds were aseptically transferred to test tubes containing Ingestad medium. Seedlings were grown in a growth chamber with a day/night rhythm of 16/8 h and 20/18ºC. Two-month-old seedlings were inoculated with a fungal suspension of *Thelephora terrestris* or *Hebeloma crustuliniforme* grown in Melin-Norkrans liquid medium modified by Kottke, Guttenberger, Hampp, and Oberwinkler [13]. The seedlings were inoculated two months after sowing with blended mycelium of *T. terrestris* and *H. crustuliniforme*, each seedling receiving 5 ml of mycelium suspended in distilled water (0.025g of mycelium per plant). Two months after seedling inoculation with the fungi, evaluation of β-glucosidase activity in seedling roots was carried out in 5 replicates per treatment (inoculated and non-inoculated controls). Seedling roots were homogenized in a glass mortar and the macerate added to 5 ml of 0.01 M acetate buffer pH 5.5. The supernatant served as an enzymatic sample. β-glucosidase activity was estimated using the same method as in the case of pure mycorrhizal fungi cultures.

The β-glucosidase-specific activity unit corresponds to the amount of enzyme which released 1µmol of p-nitrophenol from substrate per 1 min during the experiment.

Protein Estimation in Post-Culture Liquids

Estimation of protein was performed using Lowry et al. [20] with bovine albumin as a standard.

Mycorrhizal Development

Total numbers of vital mycorrhizal root tips per seedling were counted, the tips being counted and distinguished using available identification manuals [21, 22].

Statistics

Data were analyzed using analysis of variance in the Statistica 7.1. package (StatSoft). Differences between means were evaluated with the t-test (Table 1) and LSD test (Fig. 1) at $p<0.05$.

Results

The analyses of enzymes found in post-culture liquids as well as in fungal homogenates of *T. terrestris* and *H. crustuliniforme* were qualitatively and quantitatively different (Table 1). In the case of *H. crustuliniforme* no pectate or pectin lyase activity has been found (PGL and PL). In post-culture liquids of *T. terrestris* the level of polygalacturanase (PG) was found to be several times (and significantly) higher than in post-culture liquids of *H. crustuliniforme*. In *T. terrestris* cultures significantly higher (almost twofold) levels of chitinase were observed, although the level of acid phosphatase was not significantly different. In contrast, *H. crustuliniforme* produced significantly higher amounts of neutral proteinase than *T. terrestris*. In *H. crustuliniforme* cultures no cellulase activity has been found, whereas *T. terrestris* produced endocellulase and β-glucosidase.

In seedling roots inoculated by mycelium of *H. crustuliniforme,* β-glucosidase activity was 46% higher compared to non-inoculated seedlings (control) (Fig.1) and the differences were statistically significant. Seedling roots inoculated with *T. terrestris* showed significantly higher β-glucosidase activity that was about 107% in comparison with non-inoculated seedlings. At the same time the activity of enzymes was found to be (44%) lower in roots inoculated with *H. crustuliniforme* than in those inoculated with *T. terrestris*.

Discussion

Acid phosphatase found in *T. terrestris* is the enzyme responsible among others for release of non-organic phosphorus [23] or β-glucosidase. The higher content found in Scots pine seedling root inoculated with mycelium of *T. terrestris* than in seedlings inoculated with mycelium of *H. crustuliniforme* may indicate better use of nutrients where mycorrhizal *T. terrestris* fungi dominate on the roots. This hypothesis may be supported by the observation that β-glucosidase belonging to exoglucosidases is the enzyme involved in hydrolysis of phenol compounds with β-anomers of glucose [24]. The high activity of that enzyme can be essential for organic forms of phosphorus uptake as well as for utilization of nitrogen contained in proteins and chitin [11]. A difference in enzyme activity between pure cultures and mycorrhizal roots seems to be the result of different myceliums' quantities [25]. The roots were investigated a short time after inoculation.

Extracellulular chitinase activity enables the plant to obtain readily available sources of carbon and nitrogen [26]. Chitinases, 1.3- β-glucanases and proteinases [27, 28] are involved in the process of decomposition of the main

compounds of fungal cell walls. Studies of Mucha et al. [28] indicated chitinolytic activity of *A. muscaria* and *L. laccata* in the presence of chitinase inductors, such as colloidal chitine and mycelium of *Trichoderma*. However, Hodge et al. [26] studied production of those enzymes by mycorrhizal fungi *Boletinus cavipes, Paxillus involutus, Suillus variegatus, Pisolithus tinctorius* and two pathogenic fungi: *Phytophthora cinnamomi* and *Armillaria mellea*. Among mycorrhizal fungi the largest amounts of chitinase were produced by *Boletinus cavipes*, albeit the amounts were much smaller in comparison with those observed in cultures of pathogenic fungi. Significant differences in chitinolytic activity between 4 mycorrhizal fungal species in mycorrhizal association with tree roots were also shown by Pritsch et al. [25]. Our studies on chitinolytic activity have shown higher activity in *T. terrestris* than in *H. crustuliniforme* cultures, and point to the use of mycorrhizal fungi of a universal, ubiquitous type [29] like *T. terrestris* rather than the more specialized *H. crustuliniforme* for inoculation of pine seedlings. On post-agricultural lands, which are commonly rich in N, the establishment and performance of tree seedlings seems to be better when the mycorrhizas are created by fungi insensitive to higher doses of nitrogen, such as *T. terrestris* [8].

There are steadily increasing data in the literature on the ability of ectomycorrhizal fungi to synthesize cellulitic and pectolitic enzymes. These enzymes hydrolyze compounds that build cell walls of plants [10, 30, 31]. The formation of the Hartig net is possible due to the process of shifting middle lamella in the cortex of initial root bark [32], following either mechanical or enzymatic effect [32]. Studies conducted by Dahm et al. [33] on the cellulytic and pectolytic activities of ectomycorrhizal fungi *Amanita muscaria, Suillus hirtellus, Suillus luteus, Suillus bovinus, Rhizopogon luteolus*, have shown that only the latter did not produce any of the enzymes in question. Later studies by Dahm and Strzelczyk [34] indicated synthesis of cellulase and pectinase by *Hebeloma crustuliniforme*, *Laccaria laccata* and *Pisolithus tinctorius*. Among the cellulolytic enzymes, exocellulase production by these fungi was observed most often. The selected isolates of *H. crustuliniforme* did not show any cellulolytic activity, whereas those of *T. terrestris* produced endocellulase and β-glucosidase. In Scots pine seedlings inoculated with *T. terrestris,* β-glucosidase is more active than in those treated with H. crustuniliforme. Colpaert and Laere [29] described a high activity of constitutive β-glucosidase *in vitro* and its ability to amplify the defence mechanisms of the host plant. Production of proteinase by the pioneering species as *T. terrestris* has turned out to be high. Proteinases play a key role in transporting nutrients to fungi and to the symbiont plant [35].

Investigated isolates of fungi were taken from mycorrhizas of seedlings growing on post agricultural lands and the DNA sequences show that the isolates represent the same strains [8]. However, given the variability of mycorrhizal isolates [10, 28, 29], there is need to conduct further studies on the enzymatic activity of *T. terrestris,* as well as on several other mycorrhizal symbionts and strains recommended for inoculation of Scots pine. Since in Poland the aim of the afforestation plan is reforesing about 1 million ha of soils not cultivated by farmers [7], it is of great importance to select isolates or strains of mycorrhizal fungi that can provide nutritional benefits on non-forest soils at the initial phase of seedling growth.

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References

1. RUDAWSKA M. The study of agents regulating mycorrhizal of Scots pine seedlings (summary of postdoctoral lecturing qualification). Institute of Dendrology, Kórnik, **1998**
2. URSIC M., PETERSON R.L., HUSBAND B. Relative abundance of mycorrhizal fungi and frequency of root rot on Pinus strobus seedlings in a southern Ontario nursery. Can. J. For. Res. **27**, 54, **1997**
3. COLPAERT J.V. Thelephora. In: Ectomycorrhizal Fungi. Key Genera in Profil. (Eds) J. W.G Cairney, SM Chambers, Springer, **1999**.
4. VILLENEUVE N., LE TACON F., BOUCHARD D. Survival of inoculated Laccaria bicolor in competition with native ectomycorrhizal fungi and effects on the growth of outplanted Douglas-fir seedlings. Plant Soil **135**, 95, **1991**.
5. GARBAYE J., CHURIN J.L. Growth stimulation of young oak plantations inoculated with the ectomycorrhizal fungus Paxillus involutus with special reference to summer drought. Forest Ecol. and Manag. **98**, 221, **1997**.
6. HILSZCZAŃSKA D., SIEROTA Z. Persistence of ectomycorrhizas of *Thelephora terrestris* on outplanted Scot pine seedlings. Acta Mycol. **41,** (2), 313, **2006**.
7. FONDER W. Management and economic aspects of afforestation in Poland. Pro. Agr.Sci. **49/54**, (3), **2002** [In Polish].
8. HILSZCZAŃSKA D., MAŁECKA M., SIEROTA Z. Changes in nitrogen level and mycorrhizal structure of Scots pine seedling inoculated with *Thelephora terrestris*. Ann. For. Sci. **65,** 409, **2008**.
9. ABUZINADAH R.A., READ D.J. The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. I. Utilization of peptides and proteins by ectomycorrhizal fungi. New Phytol. **103**, 481, **1986**.
10. CAIRNEY J.W.G., BURKE R.M. Physiological heterogenity within fungal mycelia: an important concept for a functional understanding of the ectomycorrhizal symbiosis. New Phytol. **134**, 685, **1996**.
11. COURTY P.E., PRITSCH K., SCHLOTER M., HARTMANN A., GARBAYE J. Activity profiling of ectomycorrhiza communities in two forest soils using multiple enzymatic tests. New Phytol. **167**, 309, **2005**.
12. KOWALSKI S. Mycorrhizae significance for forest trees as well as issues concerning the induction project of Polish complex mycorrhization technology. Tech. Improve. For. **76**, 16, **2000** [In Polish].

13. KOTTKE I., GUTTENBERGER M., HAMPP R., OBERWINKLER F. An in vitro method for establishing on coniferous tree seedlings. Trees **1**, 191, **1987**.
14. SOMOGYI M. Notes on sugar determination. J. Biol. Chem., **195**, 19, **1952**.
15. NELSON N. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. **153**, 375, **1944**.
16. JOHANSSON M. Pectic enzyme activity of spruce and pine strains of Heterobasidion annosum (Fr.). Bref. Plant. Pathol **33**, 333, **1988**.
17. HAZEN G.G., HAUSE J.A., HUBICKI J.A. An automated system for the quantitative determination of proteolytic enzymes using azocasein. Ann N. Y. Acad. Sci **130**, 761, **1965**.
18. MILLER G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. **31**, 426, **1959**.
19. KIELISZEWSKA-ROKICKA B., RUDAWSKA M., LESKI T. Influence of aluminium on mycelium growth and acid phosphatase activity of Scot pine ectomycorrhizal symbionts. In: R. Siwecki (ed.) III National Symposium, Kórnik, Publ. Sorus: pp. 471-479, **1994**.
20. LOWRY O.H., ROSENBROUGH N.J., FARR R.J., RANDALL R.J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265, **1952**.
21. AGERER R. Colour Atlas of Ectomycorrhizae, Einhorn Verlag, Schwabisch-Gmünd., **1987-2006**.
22. AGERER R., RAMBOLD G. DEEMY-An information system for characterization and determination of ectomycorrhizae, http://www.deemy.de, Munich, Ludwig Maximilians University, **2004-2007**.
23. TIBBET M., CHAMBERS S.M., CAIRNEY J.W.G. Methods for determinating extracellular and surface-bound phosphate activities in ectomycorrhizal fungi. In: Varma A. (Ed.), Mycorrhiza manual, Springer, New York, pp. 217-226, **1998**.
24. PIŚLEWSKA M. Role of β-glucosidase from Lupinus albus L. cell walls in metabolism of secondary phenol metabolites. A doctorate thesis. Institute of Bioorganic Chemistry Polish Academy of Sciences, Poznań, **2000**.
25. PRITSCH K., RAIDL S., MARKSTEINER E., BLASCHKE H., AGERER R., SCHLOTER M., HARTMANN A. A rapid and highly sensitive method for measuring enzyme activities in single mycorrhizal tips using 4-methylumbelliferone-labelled fluorogenic substrates in a microplate system. J. Microbiol. Meth. **58**, 233, **2004**.
26. HODGE A., ALEXANDER I.J., GOODAY G.W. Chitinolytic enzymes of pathogenic and ectomycorrhizal fungi. Mycol. Res. **99**, 935, **1995**.
27. SCHICKLER H., CHET I. Heterologous chitinase gene expression to improve plant defense against phytopathogenic fungi. J. Industr. Microbiol. Biotechnol. **19**, 196, **1997**.
28. MUCHA J., DAHM H., STRZELCZYK E., WERNER A. Synthesis of enzymes connected with mycoparasitism by ectomycorrhizal fungi. Arch. Microbiol. **185**, 69, **2006**.
29. COLPAERT J.V., VAN LAERE A. A comparison of the extracellular enzyme activities of two ectomycorrhizal and a leaf-saproptrophic basidiomycete colonizing beech leaf litter. New Phytol. **133**, 133, **1996**.
30. DURALL D.M., TODD A.W., TRAPPE J.M. Decomposition of 14C-labelled substrates by ectomycorrhizal fungi in association with Douglas fir. New Phytol. **127**, 725, **1994**.
31. LEAKE J.R., DONNELLY D.P., BODDY L. Interactions between ecto-mycorrhizal and saprotrophic fungi. In: van der Heijden M.G.A., Sanders I.R. (Eds.), Mycorrhizal ecology, Springer, Berlin Heidelberg: pp. 345-372, **2002**.
32. RUDAWSKA M., LESKI T., GORNOWICZ R. Mycorrhizal status of Pinus sylvestris nursery stock in Poland as influenced by nitrogen fertilization. Dendrobiology, **46**, 49, **2001**.
33. DAHM H., STRZELCZYK E., MAJEWSKA L. Cellulolytic and pectolytic activity of mycorrhizal fungi, bacteria and actinomycetes associated with the roots of Pinus sylvestris. Pedobiologia **30**, 73, **1987**.
34. DAHM H., STRZELCZYK E. Impact of vitamins on cellulolytic, pectolytic and proteolytic activity of mycorrhizal fungi. Symbiosis **18**, 233, **1995**.
35. DIGHTON J. Phosphatase production by mycorrhizal fungi. Plant Soil **71**, 455, **1983**.