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Impact of Vitamins on Cellulolytic, Pectolytic and Proteolytic Activity of Mycorrhizal Fungi

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Abstract

The objective of the present work was to study the effect of vitamins on enzyme production in some ectomycorrhizal fungi. It was found that *Hebeloma crustuliniforme* was more cellulolytic and pectolytic than *Laccaria laccata* and *Pisolithus tinctorius*. *H. crustuliniforme* and *P. tinctorius* but not *L. laccata* produced proteolytic enzymes. The vitamins did not influence the proteolytic and pectolytic activity of the fungi. The vitamins used (especially biotin) also did not affect or repress the cellulolytic activity of the fungi studied.

Keywords: Ectomycorrhizal fungi, vitamins, cellulase, protease, polygalacturonase, pectin, pectate lyase

1. Introduction

It is known that cellulose and pectin are main components of the cell wall of plants. The ability to synthesize cellulolytic and pectolytic enzymes is widespread among plant pathogenic (Capellini and Peterson, 1966; Manka, 1981) and saprophytic microorganisms (Strzelczyk and Szpotanski, 1989).

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According to Lyr (1963) most ectomycorrhizal fungi produce cellulolytic enzymes. On the other hand, Meyer (1974) and Richards (1974) claim that ectomycorrhizal fungi do not develop outside the roots of the host plant, because of lack of enzymes hydrolyzing cellulose, pectin and other complex organic compounds and require simple organic compounds contained in root exudates for growth. Several mycorrhizal fungi have been shown to express proteolytic activity which appears to be important for growth of the mycelia and the mycorrhizal plants (Leake and Read, 1989). The breakdown of pectins in the middle lamella of the host cells during mycorrhiza formation also suggests that ectomycorrhizal fungi produce pectolytic enzymes (Duddridge and Read, 1984).

Growth of hyphae and rhizomorphs is regulated by particular interactions of the fungus with its host and the environment. For ectomycorrhizal fungi the host serves mostly as a source of energy and perhaps growth regulators such as thiamine and biotin. The host may also regulate the formation of fruiting bodies of certain ectomycorrhizal fungi (Molina et al., 1992).

Vitamins affect growth and the physiology of organisms occurring in the rhizosphere. The effect of these substances on the production of plant growth regulators by mycorrhizal fungi could be of importance in the establishment and functioning of mycorrhizae (Strzelczyk and Kampert, 1987).

Temperature and pH influence the decomposition rate of substrates such as cellulose, pectin and proteins (Hulme and Stranks, 1971). In addition, certain mineral salts, amino acids, and carbohydrates may activate cellulases, pectinases and proteases (Brumano et al., 1993; Zhu et al., 1994).

Chemical compounds especially important for the fungal symbionts are vitamins, mainly thiamine and biotin. However, thiamine is required by more species of mycorrhizal fungi than biotin. They may stimulate not only fungal growth but also modify their enzymatic activity.

Vitamins are common among organic substances produced by soil microorganisms (Riviere, 1963; Strzelczyk and Leniarska, 1985; Strzelczyk and Rozycki, 1985). The presence of these compounds in the soil is also ascribed to the release from plant and animal residues during their decomposition and to liberation from the roots of growing plants (Rovira and Harris, 1961; Oertli, 1987). Vitamins are essential or stimulatory for many saprophytic, symbiotic and pathogenic soil and root zone inhabiting microorganisms (Davey, 1971; Palmer, 1971; Slankis, 1973).

Some mycorrhizal fungi are capable of producing vitamins (Strzelczyk et al., 1991). Also numerous microorganisms isolated from the roots of forest trees synthesized these compounds (Strzelczyk and Rozycki, 1985; Strzelczyk, 1987). B-group vitamins, especially thiamine, is required by more species of ectomycorrhizal fungi than biotin (Norkrans, 1950; Slankis, 1973).

2. Materials and Methods

Organisms, media and culture conditions

Three isolates of ectomycorrhizal fungi: *Hebeloma crustuliniforme*, *Pisolithus tinctorius* and *Laccaria laccata* were used for studying the activity of cellulolytic, pectolytic and proteolytic enzymes.

Isolates of mycorrhizal fungi studied

Fungal species	No. of isolates	Year of isolation	Isolated from	Source of isolates	Mycorrhiza formation test
Hebeloma crustuliniforme (Bull. ex Fr.) Quel.	5392	1974	sporocarp	Nancy France	ECM
Pisolithus tinctorius (Pers.) Coker et Couch.	5335	1974	ectomycorrhiza of *Pinus taeda*	USA	ECM
Laccaria laccata (Scop. Fr.) Berk. et Br.)	S-238	1976	ectomycorrhiza of *Pseudotsuga menziesii*	USA	ECM

ECM = Ectomycorrhiza

The fungi were grown (without shaking) for 18 days at 23°C in 300 ml Erlenmeyer flasks with 50 ml of Lamb's (1974) medium, supplemented with 5 g/l cellulose powder (CF_{11}, Whatman) or 5 h/l carboxymethylcellulose (CMC, Koch-Light).

This medium was used for induction of cellulolytic enzymes. The same medium supplemented with 5 g/l of citrus pectin (Sigma) added aseptically after sterilization of the basal medium was prepared for induction of pectolytic enzymes. The same medium supplemented with 2 g/l of gelatin (Loba, Austria) was used for detection of proteolytic enzymes.

After sterilization (117°C for 20 min) each portion of the medium was supplemented aseptically with the following vitamins: biotin (5 µg) or

thiamine (500 µg) per 50 ml of the medium or biotin plus thiamine (5 µg and 500 µg per 50 ml of the medium respectively).

Each Erlenmeyer flask with the above media was inoculated with one disc (1 cm diameter) of the inoculum cut from the periphery of a 14 days-old culture grown on Potato Dextrose Agar (Difco). After 18 days of incubation at 23°C, the fungi were harvested by centrifugation (10,000 g) at 4°C. The activity of exo- and endoglucanase as well as of polygalacturonase (PG), pectate lyase (PGL), pectin lyase (PL) and protease was determined.

Exoglucanase activity

Two ml of the supernatant were combined with 2 ml of 0.2 M citric buffer (pH 6.0) and 2 ml of swollen cellulose (1 mg/ml) prepared according to Tansey (1971). The mixtures were kept for 2 hrs in a water bath at 37°C. After incubation, 1 ml of the mixture was combined with 1 ml of 1% solution of $ZnSO_4$ and 1 ml of 0.06 N $Ba(OH)_2$ solution and centrifuged at 9,000 g. The reducing sugars in the supernatants were detected colorimetrically using the method of Nelson (1944) and Somogyi (1952). Measurements were made in the digital spectrophotometer S-1/K (EMCO, Plock, Poland) at wavelength 625 nm.

The amounts of reducing sugars released were calculated from the equation:

$$C\ (\mu g\ ml^{-1}) = K \times E \frac{1\ ml}{625\ nm}$$

where

C = Amount of reducing sugars
K = Calibration coefficient for glucose = 87.746
E = Extinction value

An exoglucanase activity unit was assumed to represent the amount of enzyme which released 1 µg of glucose per hour under the experimental conditions used.

Endoglucanase activity

Endoglucanase activity (endo-random) was studied according to the method of Wood (1980). Six wells were cut in each Petri dish containing the test medium: 1.5% agar, 0.5% carboxymethylcellulose (CMC) and 0.2 M citric buffer of pH 6.0 (sterilized separately). Fifty µl aliquots of the fungal post-culture liquid with CMC or cellulose powder with the appropriate vitamin were pipetted into each well. The plates were incubated at 37°C for 24 hrs. Subsequently the agar medium was flooded with an aqueous solution of Congo

Red (1 mg/ml) for 15 min. Congo Red solution was then poured off and the dishes were further treated by flooding with 1 M NaCl for 15 min and observed for zones of cellulose hydrolysis. The zones of hydrolysis were stabilized by flooding the agar with 1 M HCl which changed the colour to blue and inhibited further enzyme activity.

An endoglucanase activity unit was assumed to represent the amount of enzyme of *Aspergillus niger* (Fluka) which under the experimental conditions released 1 µmol glucose (1.8×10^{-4} g) per min at 30°C and pH 4.8.

Total cellulolytic activity (exo-wise and endo-random) in the presence of Cellulose Azure

These studies were carried out using the method of Fernley (1963). Cellulose Azure (100 mg, Sigma) was dissolved in 5 ml of 0.2 M acetate buffer (pH 5.0). Aliquots (250 µl) of this solution were mixed with 250 µl of fungal post-culture liquids in Eppendorf tubes and incubated at 37°C for 24 hrs. Then, 900 µl of ethanol (95%) were pipetted into each Eppendorf tube and centrifuged at 10,000 g for 10 min. Colorimetric measurements were made using the spectrophotometer S-1/K (EMCO, Poland) at 570 nm in the presence of the blank. The reaction mixtures containing distilled water instead of post- culture liquids were used as the blank.

Cellulolytic activity of the fungi was compared with the standard curve for cellulose of *Aspergillus niger* (Sigma) in the presence of Cellulose Azure. The cellulolytic activity unit was defined as the amount of enzyme, which under the experimental conditions, released 1 µmol of glucose per min at 37°C and pH 5.0.

Polygalacturonase (PG), polygalacturonate lyase (PGL) and pectin lyase (PL) activities

Activity of these enzymes was estimated by the thiobarbituric acid – TBA test (Sherwood, 1966). The reaction mixtures contained 2 ml of 1% citrus pectin (PL) or sodium polypectate (PG and PGL) in 0.05 M acetate buffer, pH 5.0 (PG) or 0.05 Tris-HCl buffer, pH 8.0 (PGL or PL), 0.5 ml of 0.01 $CaCl_2$ and 2 ml of the culture filtrate. Following incubation at 30°C for 5 hrs the reaction was stopped by adding 0.3 ml of 9% $ZnSO_4$ and 0.3 ml of 0.5 N NaOH. After vigorous shaking the mixtures were centrifuged at 10,000 g for 20 min. A 0.5 ml aliquot of the supernatant was added to 0.3 ml 0.04 M thiobarbituric acid, 0.15 ml of 1 M HCl and 0.05 ml of distilled H_2O. The samples were kept for 30 min in boiling water, then cooled and scanned in the spectrophotometer S-1/K. The absorbance

at 515 nm or 548 nm was assumed as the activity of PG and PL or PGL, respectively.

The reaction mixtures containing boiled culture filtrates were used as blanks. Solution of pectolyase (mixture of different pectolytic enzymes) of *Aspergillus japonicum* (Sigma) containing 0.1–10 units/ml served as a standard of activity of polygalacturonase, pectin and pectate lyase. Pectolytic activity unit was defined as the amount of enzyme of *Aspergillus japonicum* which, under the experimental conditions, released 1 µmol (2.1×10^{-4} g) of galacturonic acid per min at 25°C and pH 5.5.

Proteolytic activity

These studies were carried out using the Hazen's (1965) method. The reaction mixtures contained 250 µl of the culture filtrate and 250 µl of 2% sulphanilamide azocasein (Sigma) in 0.2 M acetate buffer, pH 4.5 (acid protease) or in Tris-HCl buffer, pH 7.5 (alkaline protease). Following incubation at 37°C for 5 hrs the reaction was stopped by adding 800 µl of 7% $HClO_4$. Subsequently the mixtures were centrifuged at 10,000 g for 10 min at 4°C. A 1 ml aliquot of the supernatant was added to 150 µl 10 N NaOH. After vigorous shaking, the absorbance at 440 nm was assumed as the activity of proteases.

The reaction mixtures containing distilled water instead of culture filtrates were used as blanks. Solution of Fungal Protease, XIII, (Sigma) in acetate buffer, pH 4.5 served as the standard of activity of acid protease and Protease E (*Streptomyces griseus*, Sigma) in Tris buffer, pH 7.5 served as the standard of alkaline protease. As the activity of acid protease was assumed the amount of enzyme which under the experimental conditions hydrolyzed hemoglobin and released 1 µmol of tyrosine (181 µg) per min at 37°C and pH 2.8. As the activity of alkaline protease was assumed the amount of enzyme which by hydrolyzing caseine released 1 µmol of tyrosine per min at 37°C and pH 7.5.

The results of all estimations were evaluated using one and two factor ANOVA and Newman-Keul's multiple range test ($p \leq 0.05$).

3. Results

Hebelomama crustuliniforme synthesized more exoglucanase than *Pisolithus tinctorius* and *Laccaria laccata* (Table 1). In media with CMC the activity of these enzymes was more pronounced than with cellulose powder. *L. laccata* in the presence of cellulose powder did not synthesize exoglucanase at all.

Table 1. Effect of vitamins on exoglucanase activity of mycorrhizal fungi grown in media containing carboxymethylcellulose (CMC) and cellulose powder (units/hour)

Mycorrhizal fungus	Substrate	Experimental combination	Average values ± standard error (n = 9)
Laccaria laccata			
	CMC		
		Control (no vitamins)	2.047406 ± 0.689397
		Biotin	4.679786 ± 4.412145
		Thiamine	0
		Biotin + Thiamine	0
	Cellulose powder		
		Control (no vitamins)	0
		Biotin	0
		Thiamine	0
		Biotin + Thiamine	0
Hebeloma crustuliniforme			
	CMC		
		Control (no vitamins)	140.9785c ± 22.20245
		Biotin	3.802326a ± 3.584867
		Thiamine	76.92399b ± 21.22404
		Biotin + Thiamine	12.86941a ± 7.229149
	Cellulose powder		
		Control (no vitamins)	102.3703b ± 6.96394
		Biotin	2.924866a ± 1.870289
		Thiamine	55.86495b ± 10.82420
		Biotin + Thiamine	0
Pisolithus tinctorius			
	CMC		
		Control (no vitamins)	11.40698a ± 10.7546
		Biotin	8.189626a ± 5.173698
		Thiamine	7.89714a ± 3.50984
		Biotin + Thiamine	22.22898a ± 10.68366
	Cellulose powder		
		Control (no vitamins)	7.604653 ± 7.169735
		Biotin	0
		Thiamine	0
		Biotin + Thiamine	0

Average values (dealing with the appropriate fungus and substrate) marked with the same letter do not differ significantly at $p \leq 0.05$.

Vitamins affected the production of exoglucanase by the three fungi differently (Table 1). Biotin and thiamine applied separately or in conjunction, in media with CMC did not influence the production of exoglucanase by *P. tinctorius*. However, with cellulose powder these vitamins repressed the production of exoglucanase in this fungus. Exoglucanase activity was not detected in culture of *L. laccata* with cellulose powder with or without vitamins (control). In media with CMC containing no vitamins and in those with biotin only a weak activity of exoglucanase was found. In cultures containing thiamine or thiamine and biotin together, these enzymes were not detected. In the post culture liquids of *H. crustuliniforme* a high activity of exoglucanase (in the presence of CMC and cellulose powder), especially in media without vitamins, was noticed. The smallest activity of exoglocanase was found in media supplemented with biotin or biotin and thiamine applied together (Table 1).

Endoglucanase activity was found only in *H. crustuliniforme*. Endoglucanase activity was detected in all experimental combinations except in the media with cellulose powder plus biotin. In media with CMC without vitamins and with cellulose powder and thiamine endoglucanase activity was high. Thus it was clear that biotin was the retarding factor (Table 2).

Table 2. Effect of vitamins on endoglucanase activity of mycorrhizal fungi grown in media containing CMC and cellulose powder (units/ml)

Mycorrhizal fungus	Substrate	Experimental combination	Average values ± standard error (n = 3)
Hebeloma crustuliniforme			
	CMC		
		Control (no vitamins)	0.117976b ± 0.024153
		Biotin	0.000654a ± 0.000276
		Thiamine	0.061688a ± 0.020318
		Biotin + Thiamine	0.000149a ± 0.00003
	Cellulose powder		
		Control (no vitamins)	0.046158a ± 0.004811
		Biotin	0
		Thiamine	0.104741b ± 0.027669
		Biotin + Thiamine	0.001989a ± 0.001191

Average values marked with the same letter do not differ significantly at $p \leq 0.05$. *Laccaria laccata* and *Pisolithus tinctorius* did not produce endoglucanases.

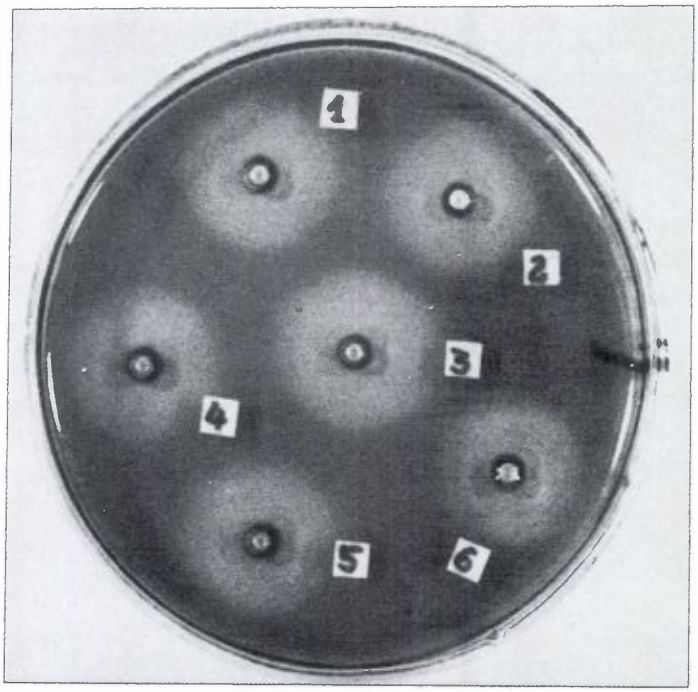

Figure 1. Endocellulase activity of *Hebeloma crustuliniforme*.
1, 2, 3 – In control media (no vitamins) with CMC.
4, 5, 6 – In control media with cellulose powder.

In the post-culture liquids of *L. laccata* trace activity of cellulase was detected only in the control media (Table 3). This would indicate that both vitamins are inhibitory for the activity of these enzymes. *H. crustuliniforme* hydrolyzed Cellulose Azure in the vitamin-free media and in the those containing thiamine. Biotin appeared to be inhibitory for the hydrolysis of cellulose in media with CMC and cellulose powder. The post culture liquids of *P. tinctorius* with Cellulose Azure exhibited traces of enzymatic activity both in the control media and in the presence of vitamins.

H. crustuliniforme exhibited a higher pectolytic activity than the other fungi (Table 4). The vitamins used either separately or together did not influence the production of pectate lyase and polygalacturonase. However, simultaneously applied biotin and thiamine significantly stimulated the activity of pectin lyase. *L. laccata* and *P. tinctorius* produced only pectin lyase in media with vitamins. The activity of these enzymes was insignificant.

H. crustuliniforme and *P. tinctorius* produced alkaline and acid proteases (Table 5). The applied vitamins did not affect the activity of these enzymes. *L. laccata* did not produce these enzymes at all.

Table 3. Effect of vitamins on total cellulolytic activity (exo-wise and endo-random) of mycorrhizal fungi grown in media with CMC and cellulose powder (units/ml) (method with Cellulose Azure)

Mycorrhizal fungus	Substrate	Experimental combination	Average values ± standard error (n = 3)
Laccaria laccata			
	CMC		
		Control (no vitamins)	0.000148 ± 0.000061
		Biotin	0
		Thiamine	0
		Biotin + Thiamine	0
	Cellulose powder		
		Control (no vitamins)	0.000398 ± 0.000132
		Biotin	0
		Thiamine	0
		Biotin + Thiamine	0
Hebeloma crustuliniforme			
	CMC		
		Control (no vitamins)	0.002981c ± 0.000091
		Biotin	0
		Thiamine	0.001880b ± 0.00006
		Biotin + Thiamine	0
	Cellulose powder		
		Control (no vitamins)	0.004126d ± 0.000016
		Biotin	0
		Thiamine	0.001753b ± 0.000058
		Biotin + Thiamine	0.001171a ± 0.000219
Pisolithus tinctorius			
	CMC		
		Control (no vitamins)	0.000477a ± 0.000167
		Biotin	0.000547a ± 0.000236
		Thiamine	0.000227a ± 0.000125
		Biotin + Thiamine	0.000083a ± 0.000008
	Cellulose powder		
		Control (no vitamins)	0.00032a ± 0.00018
		Biotin	0
		Thiamine	0.000133a ± 0.000048
		Biotin + Thiamine	0.000145a ± 0.000058

Average values (dealing with the appropriate fungus and substrate) marked with the same letter do not differ significantly at $p \leq 0.05$.

Table 4. Effect of vitamins on pectolytic activity (pectin lyase <PL>, pectate lyase <PGL>, polygalacturonase <PG>) in mycorrhizal fungi (units/ml)

Mycorrhizal fungus	Enzyme	Experimental combination	Average values ± standard error (n = 6)
Laccaria laccata			
	Pectin lyase (PL)		
		Control (no vitamins)	0
		Biotin	0.016164a ± 0.003801
		Thiamine	0.024489a ± 0.005304
		Biotin + Thiamine	0
Hebeloma crustuliniforme			
	Pectin lyase (PL)		
		Control (no vitamins)	0.164658a ± 0.063573
		Biotin	0.177503a ± 0.04276
		Thiamine	0.354179a ± 0.036593
		Biotin + Thiamine	0.391532b ± 0.055604
	Pectate lyase (PGL)		
		Control (no vitamins)	0.759014a ± 0.226367
		Biotin	1.171372a ± 0.249244
		Thiamine	1.406736a ± 0.300769
		Biotin + Thiamine	1.439613a ± 0.232023
	Polygalacturonase (PG)		
		Control (no vitamins)	0.746097a ± 0.513408
		Biotyn	0.349102a ± 0.113742
		Thiamine	1.09277a ± 0.280229
		Biotin + Thiamine	1.730609a ± 0.605065
Pisolithus tinctorius			
	Pectin lyase (PL)		
		Control (no vitamins)	0
		Biotin	0.019312a ± 0.002296
		Thiamine	0.016164a ± 0.003801
		Biotin + Thiamine	0.014088a ± 0.001906≥

Average values (dealing with the appropriate fungus and enzyme) marked with the same letter do not differ significantly ($p \leq 0.05$). *Laccaria laccata* and *Pisolithus tinctorius* did not produce polygalacturonase (PG) and pectate lyase (PGL).

Table 5. Effect of vitamins on proteolytic activity (acid and alkaline proteases) of mycorrhizal fungi (units/ml)

Mycorrhizal fungus	Enzyme	Experimental combination	Average values ± standard error (n = 6)
Hebeloma crustuliniforme			
	Acid proteases		
		Control (no vitamins)	0.006606a ± 0.000408
		Biotin	0.00515a ± 0.000756
		Thiamine	0.016734a ± 0.007309
		Biotin + Thiamine	0.006676a ± 0.000738
	Alkaline proteases		
		Control (no vitamins)	0.002617a ± 0.000571
		Biotin	0.003202a ± 0.000453
		Thiamine	0.002621a ± 0.000087
		Biotin + Thiamine	0.002933a ± 0.0000157
Pisolithus tinctorius			
	Acid proteases		
		Control (no vitamins)	0.000548a ± 0.000235
		Biotin	0.002814a ± 0.000586
		Thiamine	0.001789a ± 0.000391
		Biotin + Thiamine	0.007907a ± 0.002434
	Alkaline proteases		
		Control (no vitamins)	0.003634a ± 0.001113
		Biotin	0.004517a ± 0.001194
		Thiamine	0.004592a ± 0.000781
		Biotin + Thiamine	0.004158a ± 0.0009767

Average values (dealing with the appropriate fungus and enzyme) marked with the same letter do not differ significantly ($p \leq 0.05$). *Laccaria laccata* and *Pisolithus tinctorius* did not produce polygalacturonase (PG) and pectate lyase (PGL). *Laccaria laccata* did not produce acid- and alkaline proteases.

4. Discussion

The process of mycorrhiza formation begins with spore germination followed by colonization and penetration into host root tissues. A number of studies have shown that stages of this process can be either inhibited or stimulated by components of the general microflora (Linderman and Paulitz, 1990).

Infection of plants by symbiotic microorganisms may occur either by their own enzymes or by those of associated microorganisms. Duponnois (1992) hypothesized that mycorrhization helper bacteria (MHBs) could soften the cell walls and the middle lamella between the cells of the root cortex with specific enzymes thus making the fungal penetration easier. Endoglucanase, pectate lyase and xylanase were detected in pure cultures of several MHBs from the Douglas fir – *Laccaria laccata* system (Garbaye, 1994). This tends to support the hypothesis, as did the early work of Mosse (1962), which showed that some microorganisms (e.g. *Pseudomonas* sp.) producing cell wall-degrading enzymes promoted the establishment of arbuscular endomycorrhizas. Filtrates of bacterial cultures or solutions of the enzymes had the same effect.

Some mycorrhizal fungi have been shown to express proteolytic activity. The significance of these enzymes for growth of the mycelia and mycorrhizal plants was stressed by Leake and Read (1989).

The breakdown of pectins in the host during mycorrhiza formation also suggests that ectomycorrhizal fungi produce pectolytic enzymes (Duddridge and Read, 1984). When pectin was used as the sole carbon source , some fungi grew (Giltrap and Lewis, 1982) while others did not (Lindeberg and Lindeberg, 1977), indicating that certain ectomycorrhizal fungi were capable of producing pectinases. Also our studies show that ectomycorrhizal fungi are capable of synthesizing pectolytic enzymes in media with pectin as the sole source of carbon. *H. crustuliniforme* produced more of these enzymes and they were of greater diversity than those formed by *L. laccata* and *P. tinctorius*. Experiments in which lignin and cellulose were used as the carbon sources have shown that certain mycorrhizal fungi can break down these compounds (Trojanowski et al., 1984; Dahm et al., 1987; Maijala et al., 1990). In our work all ectomycorrhizal fungi studied produced exoglucanases but endoglucanases were found only in *H. crustuliniforme*.

Root exudates and chemical processes occurring in the root region of plants were studied by many workers. The main interest however has focused on aspects other than vitamins. Baya et al. (1981) found the ability of synthesizing vitamins to be common in soil bacteria. Thiamine, niacin and pantothenic acid are produced in large amounts by bacteria and fungi isolated from the rhizosphere of different plant species (Strzelczyk et al., 1991; Dahm et al., 1993; Rodelas et al., 1993). Soil microorganisms may enhance mycorrhiza formation – among other factors possibly also by supplying vitamins to the rhizosphere (Strzelczyk and Leniarska, 1985).

It is known that ectomycorrhiza-forming fungi require B-group vitamins, especially thiamine (Slankis, 1973). Mycorrhiza-forming fungi although requiring external supply of B-group vitamins are also capable of producing these compounds (Strzelczyk et al., 1991). Vitamins affect not only growth but

also the physiology of organisms occurring in the rhizosphere (Davey, 1971; Oertli, 1987). The possible effect of these substances on production of plant growth regulators by mycorrhizal fungi could be of importance in the establishment and functioning of mycorrhizae (Strzelczyk and Kampert, 1987).

Our studies on the influence of vitamins on enzymatic activity have shown that vitamins vary in their effects on the production of exoglucanases. Biotin and thiamine with CMC did not influence the production of these enzymes by *P. tinctorius*. However, in the media with cellulose powder these vitamins repressed the production of exoglucanases in this fungus. In the post culture liquids of *L. laccata* the activity of exoglucanase was weak or was not detected. We found in the post culture liquids of *H. crustuliniforme* a high activity of exoglucanase. This appeared in media without vitamins and indicated that vitamins are not required for the production of the above enzymes by this fungus.

Endoglucanase activity was detected only in the cultures of *H. crustuliniforme*. Biotin was a retarding factor in the production of this enzyme.

Extracellular activities of pectolytic enzymes are influenced by many factors. Extracts of plants inhibit pectic enzymes (Bateman and Miller, 1966). Tannic acid and some phenolic compounds showed a marked inhibitory effect on pectolytic enzyme activity (Elegado and Fujio, 1994). We found *H. crustuliniforme* to exhibit a higher pectolytic activity than *L. laccata* and *P. tinctorius*. The vitamins used did not affect the production of pectate lyase and polygalacturonase. However, biotin and thiamine stimulated the activity of pectin lyase.

In the studies on proteolytic activity we found that *H. crustuliniforme* and *P. tinctorius* but not *L. laccata* produced alkaline and acid proteases. The applied vitamins did not affect the activity of these enzymes. Zhu (1991) stated that acid protease is a major component of the proteolytic activity of *H. crustuliniforme* and may play an important role in protein nitrogen utilization by this fungus. Although proteinases have been studied in a number of fungi, still little is known about these enzymes produced by mycorrhizal fungi.

Different environmental factors and among them vitamins affect biomass production by mycorrhizal fungi (Pokojska et al., unpublished data). They also affect enzyme production by these fungi.

The liquid culture system, in vitro, used here for screening extracellular activity may be modified to investigate enzymes secretion of ectomycorrhizal fungi grown in association with the host plant root. Such experiments should reveal how enzyme secretion is influenced by root exudates and growth stimulating factors and whether it plays a role in the establishment of the symbiosis.

Acid phosphatase activity in the rhizoplane and rhizosphere of wheat and onion was increased by colonization with the mycorrhizal fungus *Glomus mosseae* (Dodd et al., 1987). It is unknown whether the enzyme production was by the mycorrhizal fungus or by associated microorganisms.

Nothing is known about production of cellulolytic and pectolytic enzymes by mycorrhizal fungi in association with plants.

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