Effects of vitamins, temperature and pH on the biomass production by ectomycorrhizal fungi

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Studies were carried out to determine the effect of vitamins, temperature and pH on the biomass production by ectomycorrhizal fungi: *Laccaria bicolor*, *Hebeloma crustuliniforme*, *Suillus bovinus* and *Pisolithus arhizus*. Responses of particular organisms were different. Three-factor ANOVA which enables the comparison of the effect of experimental factors on biomass yield has shown that the pH of the medium had a stronger effect on *L. bicolor*, *H. crustuliniforme* and *S. bovinus* than vitamins. Temperature was the factor exerting the strongest effect on the growth of *P. tinctorius*.

INTRODUCTION

Vitamins are essential for the metabolism of animals, plants and microorganisms. They are components of coenzymes catalyzing important biochemical reactions (Oertli, 1987). Many soil bacteria and saprophytic, pathogenic as well as symbiotic fungi are capable of producing and excreting into the environment vitamins like thiamine, folic acid, nicotinic acid, biotin (Hussain, Vančura, 1970; Strzelczyk, Leniarska, 1980, 1985; Dahm, Strzelczyk, Reddy, 1989; Strzelczyk, Dahm, Pachlewski, 1991). At the same time some microorganisms are not able to produce one or several vitamins. Requirements of mycorrhizal fungi regarding vitamins were described by Norrnan (1950), Melin (1953), Slankis (1973). Among vitamins thiamine seems to be the most important one. According to Palmer (1971) thiamine must and biotin ought to be added to a satisfactory synthetic medium. Inositol, nicotinic acid and panthotenic acid also increase the growth of some fungal species (Palmer, 1971).

The ecological importance of vitamins as growth stimulators of ectomycorrhizal fungi and mycorrhizae formation is far from being elucidated. It seems that vitamins

can play an important role in interactions between plant – mycorrhizal fungus – bacteria accompanying mycorrhizae (S h e m a k h a n o v a, 1962; D a v e y, 1971). G a r b a y e (1994) suggests that the mycorrhization helper bacteria (MHB's) can produce metabolites directly used by the mycorrhiza symbionts as nutrients or enhance its metabolism (growth factor-type effect). However the author did not mention that B-vitamins could also belong to such metabolites.

The role of mycorrhizal fungi in plant growth is well established (T r a p p e, F o g e l, 1977; L i n d e r m a n n, 1988; H a r l e y, 1989). Controlled mycorrhization of tree seedlings in forest nurseries is an important cultural treatment. Practical and common use of mycorrhizae in nurseries could only be possible if sufficient quantities of viable inoculum are available (C h a l o t et al., 1988). The authors suggest that media for growing mycorrhizal fungi still need much improvement and the ability of a fungal strain to grow in pure culture should be tested when selecting mycorrhizal fungi for artificial inoculation.

The aim of this work was to determine how externally supplied vitamins (thiamine and biotin at different concentrations) together with other important environmental factors: temperature and pH affect biomass production by selected ectomycorrhizal fungi (suitable for practical use for mycorrhization of seedlings in forest nurseries).

MATERIALS AND METHODS

Origin of the fungal isolates. Four ectomycorrhizal fungi were studied:

Fungi	No. of isolate	Year of isolation	Isolated from	Source of isolation
<i>Suillus bovinus</i> (L.: Fr.) O. Kuntze	1941	1970	sporocarp	Noteć Forest, Poland
<i>Hebeloma crustuliniforme</i> (Bull.: Fr.) Quel.	5392	1974	sporocarp	Nancy, France
<i>Laccaria bicolor</i> (Maire) Pat.	S-238	1976	ectomycorrhizae of <i>Tsuga mertens.</i>	USA
<i>Pisolithus arhizus</i> (Pers.) Rausch. (= <i>tinctorius</i>)	5335	1974	ectomycorrhizae of <i>Pinus taeda</i>	USA

C u l t u r e c o n d i t i o n s. The fungi were grown in Lamb's medium supplemented with different amounts of biotin and thiamine (L a m b, 1974). The following combinations were applied: medium without vitamins (control), with thiamine at concentrations: 1, 10, 100 µg/ml, with biotin: 0.01; 0.1; 1 µg/ml, with thiamine and biotin: 1 + 0.01; 10 + 0.1; 100 + 1 µg/ml respectively. Vitamins were filter sterilized (Millipore, 0.22 µm pore size) and added aseptically to the autoclaved media.

Three hundred millilitre Erlenmeyer flasks containing 100 ml of the medium were inoculated (in 5 replicates) with one disc (1 cm in diameter) of the fungi cut from the edges of mycelium grown for 7-14 days on Potato Dextrose Agar (no precultures on vitamin-free media were made).

The fungi were grown statically in media of pH 4.0 and 6.0 (this medium was buffered by use of MES-2-(N-Morpholino) ethanesulfonic acid, Sigma). Temperatures of cultivation were: 15° and 22°C. After 14 (*H. crustuliniforme*, *L. bicolor*) and 21 days of incubation (*P. arhizus*, *S. bovinus*) the mycelium was separated from the medium by filtration on filter paper and dried to constant weight at 90°C.

S t a t i s t i c a l a n a l y s i s. Data (biomass of mycelium in g/100 ml of medium) were evaluated statistically using the following methods:

— Student's t-test (for independent samples) to compare each experimental treatment with the "no vitamin" control,

— 3-factor ANOVA which enabled the comparison of the effects of: (1) temperature, (2) pH and (3) combinations with vitamins (including "no vitamin" control) on biomass yield. For statistical analysis STATISTICA/win 4.50 (1994) programme was applied (StatSoft, Tulsa, Oklahoma, USA).

RESULTS

It was found that thiamine and biotin, depending on their concentration as well as on the pH of the medium and temperature, affected the fungal biomass production. The response of particular isolates was different (Tabs. 1-2).

H. crustuliniforme reacted most strongly to vitamins. Thiamine and thiamine applied with biotin stimulated the growth of this fungus. The effect of vitamins on mycelial biomass production was considerably stronger at pH 6.0 than at pH 4.0 at both 15°C and 22°C. At pH 6.0 and temperature of 22°C the biomass of the mycelium in medium with thiamine as well as with thiamine and biotin was about three fold higher (disregarding the concentration of vitamins) as compared to the control (Tab. 1). Stimulation of biomass production by thiamine applied jointly with biotin could be ascribed mainly to thiamine. Biotin applied separately did not exert any significant effect on fungal growth.

Increase of dry mass of mycelium in the presence of vitamins was also observed in *S. bovinus*. However only at 22°C stimulatory reactions were significant (Tab. 2). At pH 4.0 the biomass of the mycelium increased with the concentration of vitamins added. At pH 6.0 the best growth of *S. bovinus* occurred in the presence of thiamine and thiamine with biotin applied at the lowest concentrations (approaching 142.3 % and 157.5 % of control growth respectively).

In *L. bicolor* vitamins, in most cases, slightly inhibited mycelial growth (Tab. 2). Statistically significant inhibition was marked at 22°C in media of pH 6.0.

In combinations with thiamine at concentrations: 1, 10 and 100 µg/ml, biotin at concentration 0.1 µg/ml as well as thiamine and biotin applied jointly (10 and 0.1 µg/ml respectively) the dry mass of mycelium reached only 50% of the control dry mass. At 15°C and pH 6.0 thiamine together with biotin applied at the lowest and at the highest concentration caused significant biomass increase (approaching 150-160 %).

The response of *P. arhizus* to vitamins applied at different temperatures and pH values was, in most cases, not statistically significant (Tab. 2). This was probably due to high internal variability of biomass data in some experimental treatments (standard error > 10 % of average). Significant increase of mycelium biomass production was observed only in the medium with thiamine at 100 µg/ml at 22°C and pH 4.0. Significant inhibition was noted in the presence of both thiamine and biotin applied separately and jointly at 15°C and pH 6.0.

Table 1

The effect of vitamins on the biomass production by *Hebeloma crustuliniforme* depending on temperature and pH

Tempera- ture	pH	Combinations	Dry weight (in mg) (Average ± stand. error) (5 replications)	% Control
15°C	4	Control	23.52 ± 1.25	100
		T-10	36.84 ± 4.14	156.63 *
		B-1	33.56 ± 2.29	142.69 **
		T-1 + B-0.01	33.06 ± 1.80	140.56 **
		T-100 + B-1	32.22 ± 1.81	136.99 **
15°C	6	Control	47.80 ± 4.73	100
		T-1	86.66 ± 13.86	181.30 *
		T-10	75.18 ± 4.20	157.28 **
		T-100	76.04 ± 3.03	159.08 **
		T-1 + B-0.01	70.50 ± 4.60	147.49 *
		T-10 + B-0.1	88.84 ± 4.22	185.86 ***
		T-100 + B-1	76.10 ± 7.46	159.20 *
22°C	4	Control	38.32 ± 3.12	100
		T-10 + B-0.1	55.94 ± 4.75	145.98 *
22°C	6	Control	33.02 ± 6.48	100
		T-1	86.38 ± 2.89	261.60 ***
		T-10	120.76 ± 6.33	365.72 ***
		T-100	106.52 ± 10.29	322.59 ***
		T-1 + B-0.01	111.80 ± 19.62	338.58 **
		T-10 + B-0.1	86.44 ± 5.84	261.78 ***
		T-100 + B-1	108.30 ± 15.58	327.98 **

Explanations: T – thiamine; B – biotin; 0.01, 0.1, 1 etc. – concentration of vitamin in µg/ml; significance of differences as compared with control (t-test): * 0.01 < p < 0.05, ** 0.001< p < 0.01, *** p < 0.001

Table 2

The effect of vitamins on the biomass production by ectomycorrhizal fungi
depending on temperature and pH

Fungus	Temperature	pH	Combinations	Dry weight (in mg) (Average $\pm$ stand. error) (5 replications)	% Control
<i>Suillus bovinus</i>	15°C	4	Control	91.88 $\pm$ 7.5 non significant differences	100
	15°C	6	Control	49.76 $\pm$ 6.07 non significant differences	100
	22°C	4	Control	88.44 $\pm$ 6.11	100
			T-100	121.38 $\pm$ 7.51	137.25 *
			B-1	134.08 $\pm$ 11.72	151.61 *
			T-100 $\pm$ B-1	117.42 $\pm$ 5.09	132.77 *
	22°C	6	Control	54.48 $\pm$ 4.32	100
			T-1	77.54 $\pm$ 2.64	142.33 **
			T-10	66.90 $\pm$ 1.17	122.80 *
			T-1 $\pm$ B-0.01	85.82 $\pm$ 6.47	157.53 **
			T-10 $\pm$ B-0.1	76.50 $\pm$ 4.16	140.42 *
			T-100 $\pm$ B-1	78.20 $\pm$ 5.04	133.63 *
<i>Laccaria laccata</i>	15°C	4	Control	98.18 $\pm$ 26.4 non significant differences	100
	15°C	6	Control	118.96 $\pm$ 18.53	100
			T-1 $\pm$ B-0.01	190.44 $\pm$ 15.57	160.09 *
			T-100 $\pm$ B-1	179.98 $\pm$ 15.58	151.29 *
	22°C	4	Control	55.84 $\pm$ 6.33 non significant differences	100
	22°C	6	Control	127.36 $\pm$ 9.31	100
			T-1	62.76 $\pm$ 4.45	49.28 ***
			T-10	70.34 $\pm$ 2.84	55.23 ***
			T-100	66.26 $\pm$ 6.05	52.03 ***
			B-0.1	63.30 $\pm$ 2.27	49.70 ***
<i>Pisolithus arhizus</i>	15°C	4	Control	35.66 $\pm$ 6.00 non significant differences	100
	15°C	6	Control	93.90 $\pm$ 5.47	100
			T-100	55.24 $\pm$ 5.95	58.83 **
			B-0.01	63.78 $\pm$ 7.90	67.92 *
			T-1 $\pm$ B-0.01	52.26 $\pm$ 4.10	55.65 ***
			T-10 $\pm$ B-0.1	68.94 $\pm$ 5.36	73.42 *
	22°C	4	Control	113.12 $\pm$ 8.72	100
			T-100	148.52 $\pm$ 6.56	131.29 *
	22°C	6	Control	123.46 $\pm$ 15.57	100
			B-0.1	57.86 $\pm$ 4.31	46.87 **

Explanations: see Table 1

Table 3

The comparison (3-factor ANOVA) of the effects of temperature, pH and vitamins (control included) on biomass production by four ectomycorrhizal fungi

Fun- gus	Effect of:	Variance	df [®]	F parameter	p (significance)
<i>Laccaria laccata</i>	Temp. (+)	32770	1	41.839	< 0.0001
	pH (+)	68480	1	87.422	< 0.0001
	Vitamins (+)	7870	9	10.051	< 0.0001
	Temp. x pH	1400	1	1.785	0.1834
	Temp. x Vit.	1420	9	1.816	0.0690
	pH x Vit. (+)	1620	9	2.072	0.0350
	T. x pH x Vit. (+)	1610	9	2.055	0.0366
	Error	780	160	—	—
<i>Hebeloma crustuliniforme</i>	Temp. (+)	7520	1	27.801	< 0.0001
	pH (+)	64800	1	239.608	< 0.0001
	Vitamins (+)	4000	9	14.749	< 0.0001
	Temp. x pH	610	1	2.266	0.1342
	Temp. x Vit. (+)	1060	9	3.936	0.0002
	pH x Vit. (+)	1770	9	6.529	< 0.0001
	T. x pH x Vit. (+)	770	9	2.851	0.0038
	Error	270	160	—	—
<i>Pisolithus arhizus</i>	Temp. (+)	170070	1	222.377	< 0.0001
	pH (+)	4330	1	5.657	0.0186
	Vitamins (+)	2610	9	3.417	0.0007
	Temp. x pH (+)	40920	1	53.506	< 0.0001
	Temp. x Vit. (+)	1580	9	2.065	0.0356
	pH x Vit. (+)	1670	9	2.189	0.0254
	T. x pH x Vit.	1160	9	1.521	0.1447
	Error	760	160	—	—
<i>Suillus bovinus</i>	Temp. (+)	7990	1	33.170	< 0.0001
	pH (+)	81010	1	336.162	< 0.0001
	Vitamins (+)	860	9	3.583	0.0004
	Temp. x pH (+)	1770	1	7.355	0.0074
	Temp. x Vit. (+)	530	9	2.217	0.0235
	pH x Vit. (+)	1090	9	4.540	< 0.0001
	T. x pH x Vit.	310	9	1.304	0.2386
	Error	240	160	—	—

Explanations: [®] – degrees of freedom; (+) – factor significantly affecting the biomass of fungi (p < 0.05)

Table 3 presents the results of the comparison (3-factor ANOVA) of the effect of temperature, pH and vitamins (with control treatment) on biomass production by four ectomycorrhizal fungi. The influence of the experimental factors on mycelial growth of *L. bicolor* was in the following order: pH > vitamins > temperature ($p < 0.001$ for each factor). In two cases interactions were significant (pH x vitamins; T x pH x vitamins) but the significance level was not strong ($0.01 < p < 0.05$). It is clear from Fig. 1 that the growth of *L. bicolor* was inhibited by vitamins (except for T-1 + B-0.01 where stimulation occurred) and the effect of vitamins on biomass production by this fungus was similar at both values of pH, but stronger at pH 6.0 than at pH 4.0. A diminishing order of experimental variables (pH > vitamins > temperature) was also found for *H. crustuliniforme*. However in the case of this fungus, the effects of pH and vitamins were even stronger than in *Laccaria*. Very highly significant ($p < 0.0001$) interaction : pH x vitamins was observed too. Fig. 1 shows that only at pH 6.0 thiamine (applied both separately and in combination with biotin) highly stimulated the growth of *Hebeloma*. In *P. arhizus* the effect of temperature was greater than that of any other factor (Tab. 3). In addition temperature interacted very strongly with pH (Fig. 1 a). Effects of the experimental variables on biomass yield in *S. bovinus* were as follows: pH > temperature > vitamins. In case of *Suillus* and *Hebeloma* highly significant ($p < 0.0001$) interactions between pH and combinations (various vitamin treatments) were observed. Fig. 1 b shows that every vitamin (applied both separately and in combination) at its highest concentration, significantly stimulated biomass production by *Suillus bovinus* only at pH 4.0.

Fig. 1 a. Two-way interaction plots of experimental factors affecting the biomass in ectomycorrhizal fungi:
 pH x temperature

Pisolithus arhizus
 $F_{(9, 160)} = 2.19; p < .0254$

Fig. 1 b. Two-way interaction plots of experimental factors affecting the biomass in ectomycorrhizal fungi: pH x vitamin treatments

Ctrl – control; T – thiamine; B – biotin; 0.01, 1, 100 etc. – concentration of vitamin in $\mu\text{g/ml}$

DISCUSSION

It was shown that the mycorrhizal fungi studied were able to grow in Lamb's medium without any externally supplied vitamins. Fungi of the genera: *Laccaria*, *Hebeloma*, *Suillus* and *Pisolithus* apart from *Amanita* and *Rhizopogon* seem to be the best growing mycorrhizal symbionts (Molina, Trappe, 1982). However vitamins affected the fungal biomass production. Thiamine – especially at pH 6.0 – strongly stimulated the growth of *H. crustuliniforme*. A significant increase of the dry mass of mycelium in the presence of thiamine and thiamine together with biotin was observed in *S. bovinus* cultured at 22°C. In the case of *L. bicolor* and *P. arhizus* the inhibition of fungal growth by vitamins was observed more often than stimulation. Melin (1953) indicated that complexes of vitamins may either stimulate or inhibit the growth of mycorrhizal fungi. A comparison of the effect of vitamins, temperatures and pH on the biomass production showed that the pH of the medium had a stronger effect on the growth of *L. bicolor* and *S. bovinus* than vitamins. Temperature was the factor exerting the strongest effect on the growth of *P. arhizus*. Strzelczyk, Dahm, Pachlewski (1991) have shown that the production of vitamins by mycorrhizal fungi depended distinctly upon the pH of the medium. The authors suggested that mycorrhizal fungi may produce or require vitamins depending on the pH of the environment. However no experimental data confirming or rejecting this hypothesis have so far been published.

It seems that none of the fungi studied in our work had obligatory thiamine and/or biotin requirements. Such requirements are known to occur only in some genera of ectomycorrhizal symbionts, e.g. *Lactarius* and *Russula* (Melin, 1953; Melin, Rama Das, 1954; Slankis, 1973; Li – personal information). The “no vitamin” control used in the present work did not contain vitamins. However this did not rule out the presence of traces of vitamins in controls introduced with the inoculum, since no precultures in vitamin-free media were made. Our aim was to study the effect of external vitamins on mycelial growth at concentrations higher than the physiological ones, and not the absolute vitamin requirements. In one case (*Hebeloma crustuliniforme*) we have shown that thiamine was the essential growth stimulating factor which can be used to improve biomass yield in this fungus.

It is well known that pH exerts strong effect both on mycelial growth and some physiological processes in mycorrhizal fungi as well as on mycorrhizae formation (Hung, Trappe, 1983; Gay, 1986; Metzler, Oberwinkler, 1987; Strzelczyk, Dahm, Pachlewski, 1991; Strzelczyk, Pokojka, Kampert, 1992; Strzelczyk, Kampert, Pachlewski, 1994; Lehto, 1994; Ek et al., 1994). In many cases great variations (both inter- and intraspecific) in growth reactions to pH were observed (Hung, Trappe, 1983). The results of the present study confirm these findings. Considering the fact that many fungi had their pH optimum between the values 4 to 6 and that they grew best *in vitro* at higher pH than the pH of soils which fungi originated from (Hung, Trappe, 1983) we decided to use pH 4 and 6 in our studies.

In studies regarding the effect of pH on growth of ectomycorrhizal fungi one has to be aware of its possible interactions with other environmental factors, e.g. nitrogen level. It is known that nitrogen in excess inhibits the growth of mycelium but the possible interaction of nitrogen with pH still remains unknown (Ek et al. 1994; Arnebrant, 1994).

Temperature exerted the weakest effect on the growth of mycorrhizal fungi (except for *Pisolithus arhizus*). Cline, France, Reid (1987) indicated quite high intra- and interspecific growth variation in ectomycorrhizal fungi depending on temperature. They also indicated optimum temperature for fungal growth ranging from 16°C to 32°C depending on the genus, species or fungal strain. Differences in growth reactions to temperature (compare *Pisolithus arhizus* and *Suillus bovinus*) were also noted in the present study. In our work temperatures of 15° and 22°C were applied assuming that they are quite close to those occurring in cool temperate climatic zone (where some of the fungi originated from and where they could be potentially applied for artificial mycorrhizal inoculation). The growth of *Pisolithus arhizus* was much more dependent on temperature than in the case of the other fungi and temperature very strongly interacted with pH. This fungus seemed to require higher temperature for its optimal growth than the other species. These observations confirm the results of other authors (Cline, France, Reid, 1987; Bowen, 1994). In the present work high interactions between experimental factors were observed – especially between pH and vitamin treatments. There are no data available in literature on interactions of this kind. They are not easy for biological interpretation and it is difficult to predict their practical implications. We can assume that every mycorrhizal fungus intended to be used for mycorrhizal inoculation has very high growth rate over wide range of pH and temperature and independently of the presence/absence of any growth stimulation factor(s). Thus one could expect rather low interactions between the factors affecting the biomass production by such an organism. Among the fungi used in our work *Laccaria bicolor* seemed to satisfy the above requirements.

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