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Growth of Mycorrhizal Fungi in Dixenic Cultures with Bacteria in Media of Different Composition

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Abstract

Studies were carried out on the effect of bacteria: *Arthrobacter globiformis*, *Bacillus subtilis* and *Pseudomonas fluorescens* on biomass production by three important ectomycorrhizal fungi: *Laccaria laccata*, *Hebeloma crustuliniforme* and *Rhizopogon vinicolor* in media of different composition. It was shown that bacteria stimulated, inhibited or did not affect significantly the biomass production by mycorrhizal fungi. 3-factor ANOVA have shown that although effect of bacteria was statistically significant ($p \leq 0.05$), composition of medium and its pH affected mycelial growth stronger than bacteria.

Introduction

It is well known that mycorrhizal phenomenon occurs in a great majority of vascular plants (woody and herbaceous ones). The plant gains manifold advantages from the association with the mycorrhizal fungus (Linderman, 1988; Harley, 1989). Different physical, chemical and biological factors affect the process of mycorrhiza formation. The effect of different soil microorganisms on the success of ectomycorrhizal inoculation seems to be of great importance (Linderman, 1988; Garbaye, 1991). It was shown that soil microorganisms, especially those originating from mycorrhizosphere, actively interact with the establishment and functioning of mycorrhizal symbiosis. They can stimulate or inhibit the mycorrhizal fungi (Bowen and Theodorou, 1979; Strzelczyk and Kampert, 1987; Friedman *et al.*, 1989; Garbaye and Bowen, 1989; Richter *et al.* 1989; Garbaye, Duponnois and Wahl, 1990; Chanway and Holl, 1991). Competition for nutrients rather than antibiotic

properties was considered by Strzelczyk (1966) to be the major factor responsible for suppression of the fungal growth in associated cultures of bacteria and fungi. From the practical point of view research on "helper" bacteria which improve the efficiency of mycorrhizal inoculation is of importance.

The aim of this work was to study the effect of bacteria found most often on the root surface of pine seedlings (coryneforms and pseudomonads) and bacilli – found only in root-free soil (Strzelczyk, Dahm and Leniarska, 1978; Różyczki, 1987) on growth of ectomycorrhizal fungi in media of various complexity and different pH values. The widespread problem of acid rains creates the necessity to study the fungus-bacteria interrelationships at different pH values.

Experimental

Material and Methods

Fungi and bacteria. Three fungi isolated from ectomycorrhizae of Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco): *Laccaria laccata* (Scop. ex Fr.) Berk and Br. (S-238), *Rhizopogon vinicolor* Smith (7534) and *Hebeloma crustuliniforme* (Bull. ex St. Am.) Quel (7650) were used in this study. These were obtained from the Pacific Northwest Research Station, Forestry Sci. Lab. in Corvallis (Oregon, USA). Bacteria: *Arthrobacter globiformis* CCM 1651 was obtained from Czechoslovak Collection of Microorganisms in Brno (Czechoslovakia), *Bacillus subtilis* T 182 – from the collection of Laboratory of Microbiology, Agricultural and Technical University in Olsztyn (Poland).

Inoculum preparation. The fungi were grown at 26°C in Petri dishes on Potato Dextrose Agar (Difco), *L. laccata* for 7 days, *R. vinicolor* and *H. crustuliniforme* for 14–21 days. Discs (1 cm in diameter) were cut from the edges of mycelium. One disc served as inoculum for 100 ml of medium in 300 ml Erlenmeyer flask. The bacteria were cultured on agar slants prepared from medium "A" according to Lochhead and Chase (1943). After 24 h of incubation at 26°C the agar slant cultures were washed off with 5 ml of sterile distilled water and 0.5 ml of bacterial suspension was used for inoculation of 100 ml of medium. The number of living cells in the suspensions was determined for each strain by plating diluted suspensions on nutrient agar and counting colonies.

Media for dixenic cultures, cultures conditions. Fungi with bacteria were grown at room temperature (22±2°C), in three media: according to Aschan-Norkrans (1953), to Lamb (1974) and Melin-Rama Das (1954). Composition of modified Aschan-Norkrans medium was as follows: ammonium tartrate – 5 g, KH_2PO_4 – 1 g, $MgSo_4 \times 7H_2O$ – 0.5 g, ferric citrate – 5.0 mg, $ZnSO_4 \times 7H_2O$ – 4.4 mg, $MnSO_4 \times 4H_2O$ – 5.0 mg, $CaCl_2$ – 55.5 mg, yeast extract – 250 mg, H_2O distilled – 1000 ml. Medium according to Lamb was composed of: glucose – 10 g, NH_4Cl – 0.5 g, $MgSO_4 \times 7H_2O$ – 0.5 g, KH_2PO_4 – 0.5 g, FeNaEDTA – 1 ml of 5 ppm Fe solution, thiamine – 1 mg, biotin – 0.01 mg, H_2O distilled – 1000 ml and according to Melin Rama Das of: glucose – 20 g, Malt Extract Broth (Difco) – 3 g, ammonium tartare – 0.5 g, K_2HPO_2 – 1 g, $MgSO_4 \times 7H_2O$ – 0.5 g, ferrous citrate 0.5 ml of 1% aqueous solution, zinc sulphate 0.5 ml of a 1:500 aqueous solution, thiamine – 100 µg, H_2O distilled – 1000 ml. The media were prepared in two versions of pH: 5.8 and 7.0. Five replicates were used for each experimental combination. After incubation (*L. laccata* – 14, *R. vinicolor* and *H. crustuliniforme* – 21 days) the mycelium was separated from the medium by filtration and dried to constant weight. pH of the post culture liquids were measured as well as their optical density (by use of Klett-Summerson Photoelectric Colorimeter, Model 800-3 with a blue filter No. 42) for evaluation of the bacterial growth.

biotin - 5µg
pyridoxine-100µg
nicotinic acid amide - 500µg

Data analysis. Data were processed by Student's t-test for independent samples ($p \leq 0.05$) and by 1-factor analysis of variance (ANOVA). Means were compared using Newman-Keuls multiple range

test ($p \leqslant 0.05$). 3-factor analysis of variance comparing the effects of media (1), pH (2) and bacteria (3; control without bacteria included) on the biomass yield of mycorrhizal fungus was also made. For the statistical analysis of data STATS+ and CSS: STATISTICA procedures were applied (StatSoft, Tulsa, Oklahoma, USA).

Results

The results are presented in Tables I–IV and Figs 1–3. Bacteria stimulated, inhibited or did not affect significantly the biomass production by mycorrhizal fungi. Effect of bacteria on fungal growth depended on composition of the

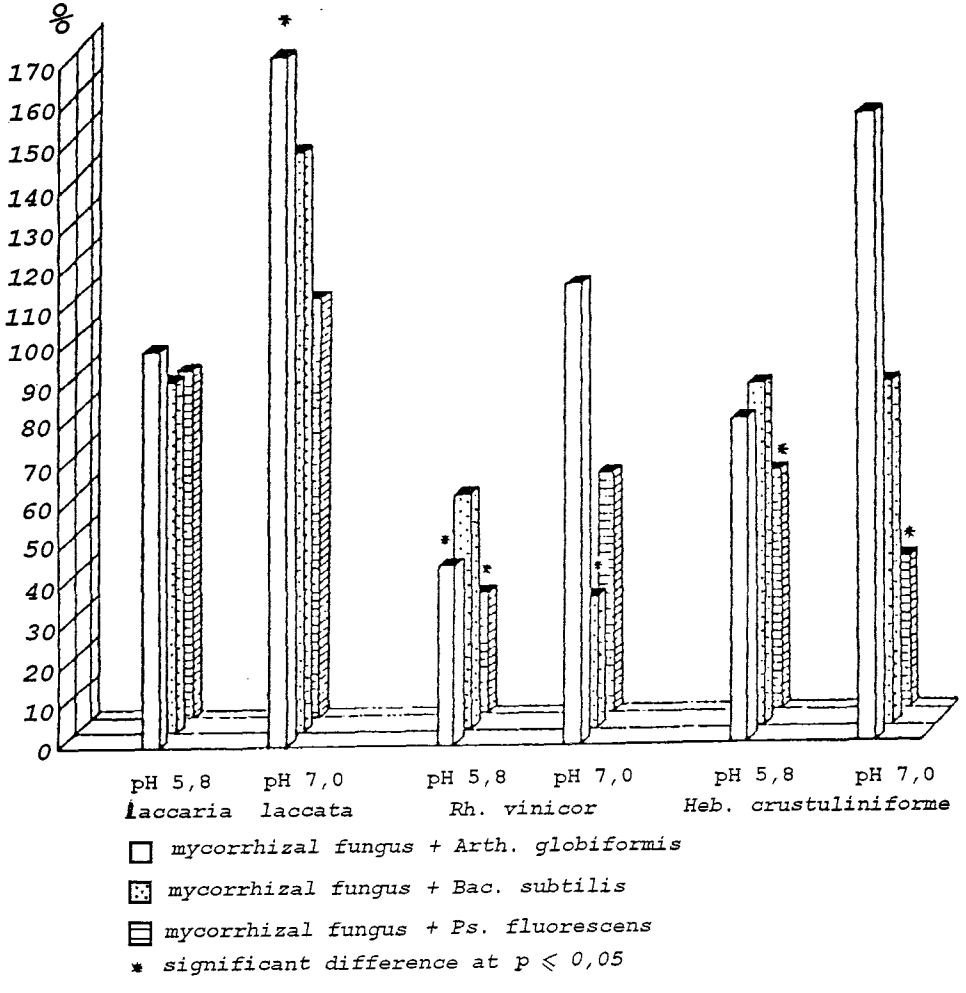

Fig. 1. Biomass production by mycorrhizal fungi in the presence of bacteria in Aschon-Norkrans medium (in % of control).

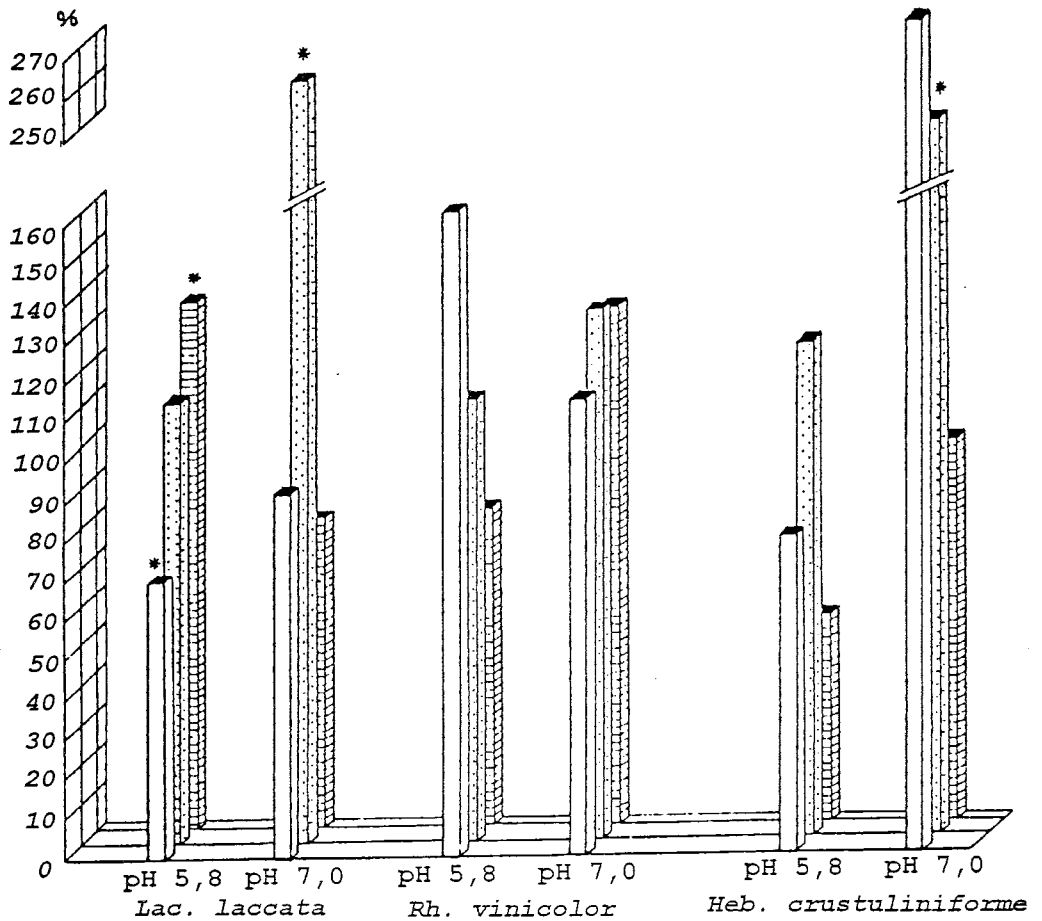

Fig. 2. Biomass production by mycorrhizal fungi in the presence of bacteria in Lamb's medium (in % of control).
Explanations: see Fig. 1

medium and its pH. *A. globiformis* significantly inhibited and *P. fluorescens* significantly stimulated the biomass yield of *L. laccata* in Lamb's medium of pH 5.8 (Table II, Fig. 1). However no effect of these bacteria on *L. laccata* in Melin-Rama Das and in Aschan-Norkrans media of pH 5.8 was observed (Tables I and III, Figs 2–3). The response of *L. laccata* on bacteria in media of pH 7.0 was different. All bacteria stimulated biomass production by *L. laccata* in Melin-Rama Das medium (Fig. 3). *A. globiformi* improved the growth of *L. laccata* also in Aschan-Norkrans medium and *B. subtilis* in Lamb's medium. Different reactions on bacteria depending on the kind of medium and its pH were noted also in *R. vinicolor* and *H. crustuliniforme*. In Aschan-Norkrans medium of

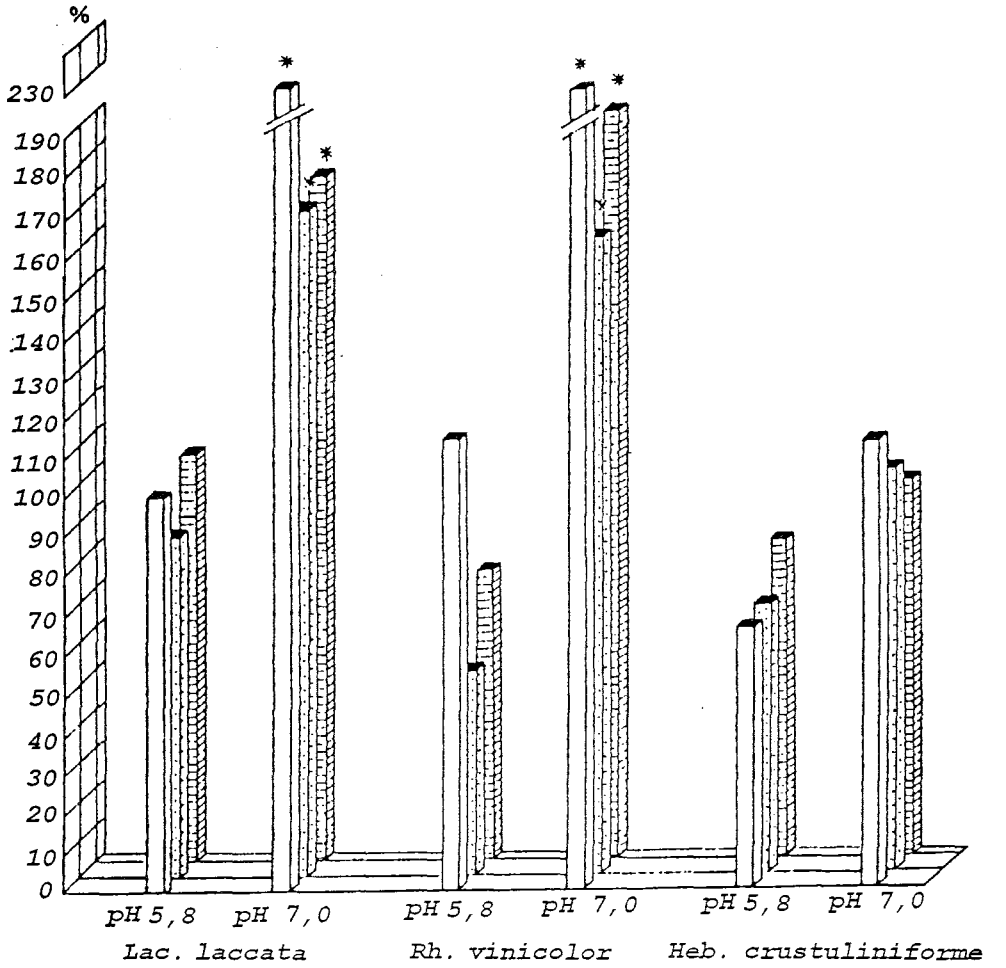

Fig. 3. Biomass production by mycorrhizal fungi in the presence of bacteria in Melin-Rama Das medium (in % of control).
Explanations: see Fig. 1.

pH 5.8 as well as of pH 7.0 mainly inhibition of *R. vinicolor* and *H. crustuliniforme* growth by bacteria was observed. In medium according to Melin-Rama Das significant stimulation of *L. laccata* and *R. vinicolor* by three bacteria took place only in media of pH 7.0 (the growth of bacteria in these conditions was the best).

Significant effects of bacteria on mycelial growth were confirmed by 3-factor ANOVA comparing the effects of medium (1), pH (2) and bacteria (3; control included). However the action of experimental factors on biomass yield of three fungi studied was in the following order: medium > pH > bacteria. This means that medium and its pH affected mycelial growth stronger than bacteria.

Table I

Biomass production by mycorrhizal fungi in the presence of bacteria in Aschan-Norkrans medium of different pH (mg dry mycelium/100 ml medium ± standard error)

Fungi	pH of medium	Control	+ *Arthrobacter globiformis*	+ *Bacillus subtilis*	+ *Pseudomonas fluorescens*
Laccaria laccata	5.8	65.1 ± 3.1 a	64.8 ± 3.9 a	56.3 ± 3.9 a	56.8 ± 5.0 a
	7.0	28.0 ± 3.7 a	48.3 ± 4.2 a	40.5 ± 4.1 a	29.5 ± 10.0 a
Rhizopogon vinicolor	5.8	11.4 ± 1.3 b	5.1 ± 1.4 a	6.6 ± 2.2 b	3.4 ± 0.7 a
	7.0	15.8 ± 3.3 a	18.3 ± 8.2 a	5.8 ± 1.1 a	9.5 ± 4.1 a
Hebeloma crustuliniforme	5.8	44.6 ± 3.0 a	36.8 ± 2.9 a	37.7 ± 6.9 a	26.6 ± 4.4 a
	7.0	14.2 ± 3.1 ab	22.4 ± 5.1 b	12.0 ± 3.0 ab	5.4 ± 1.2 a

Note: means within a given row followed by letters are signifcantly different ($p \leqslant 0.05$)

Table II

Biomass production by mycorrhizal fungi in the presence of bacteria in Lamb's medium of different pH (mg dry mycelium/100 ml medium ± standard error)

Fungi	pH of medium	Control	+ *Arthrobacter globiformis*	+ *Bacillus subtilis*	+ *Pseudomonas fluorescens*
Laccaria laccata	5.8	46.9 ± 3.3 b	33.0 ± 4.1 a	51.7 ± 5.3 bc	62.5 ± 5.1 c
	7.0	24.5 ± 9.3 a	22.5 ± 4.9 a	64.0 ± 10.7 b	19.2 ± 6.9 a
Rhizopogon vinicolor	5.8	29.1 ± 6.1 ab	47.5 ± 4.5 b	32.1 ± 4.0 ab	23.4 ± 5.6 a
	7.0	35.9 ± 2.7 a	41.4 ± 4.5 a	48.0 ± 6.1 a	47.1 ± 5.1 a
Hebeloma crustuliniforme	5.8	36.4 ± 7.1 ab	29.0 ± 6.1 ab	45.3 ± 5.4 b	19.0 ± 2.7 a
	7.0	17.9 ± 3.4 a	49.7 ± 12.6 a	44.9 ± 7.6 a	17.1 ± 5.7 a

Note: means within a given row followed by letters are signifcantly different ($p \leqslant 0.05$)

Table III

Biomass production by mycorrhizal fungi in the presence of bacteria in Melin-Rama Das medium of different pH (mg dry mycelium/100 ml medium ± standard error)

Fungi	pH of medium	Control	+ *Arthrobacter globiformis*	+ *Bacillus subtilis*	+ *Pseudomonas fluorescens*
Laccaria laccata	5.8	165.6 ± 12.9 a	165.6 ± 19.0 a	142.6 ± 4.8 a	170.6 ± 17.2 a
	7.0	75.9 ± 5.7 a	176.1 ± 15.5 c	128.4 ± 14.7 b	130.9 ± 6.5 b
Rhizopogon vinicolor	5.8	77.6 ± 18.1 a	88.2 ± 237 a	40.2 ± 8.2 a	56.4 ± 10.4 a
	7.0	68.6 ± 3.9 c	158.5 ± 7.2 c	110.1 ± 9.5 b	128.6 ± 7.1 b
Hebeloma crustuliniforme	5.8	81.9 ± 5.1 b	54.0 ± 6.5 a	55.8 ± 7.3 a	65.3 ± 5.4 ab
	7.0	113.1 ± 1.5 a	128.3 ± 7.7 a	116.0 ± 6.1 a	106.8 ± 10.5 a

Note: means within a given row followed by letters are signifcantly different ($p \leqslant 0.05$)

Table IV

3-factor ANOVA comparing the effects of medium (1), pH (2) and bacteria (3: control included) on biomassproduction by ectomycorrhizal fungi

Fungus	Effect of:	Variance	df	F parameter	p (significance)
Lac. laccata	Medium (M)	0.1336	2	271.570	0.0000
	pH	0.0180	1	36.521	0.0000
	Bacteria (b)	0.0016	3	3.316	0.0231
	M × pH	0.0007	2	1.508	0.2266
	M × b	0.0025	6	4.996	0.0002
	pH × b	0.0037	3	7.583	0.0001
	M × pH × b	0.0012	6	2.487	0.0280
	Error	0.0005	96	–	–
* media: Aschan-N.: 48.7 a Lamb: 40.5 a Melin-R.D.: 144.4 b pH = 5.8: 90.1 b pH = 7.0: 65.0 a control: 67.7 a Arth.: 85.0b Bac.: 80.6 ab Ps.: 78.2 ab					
Rh. vinicolor	Medium (M)	0.0689	2	174.355	0.0000
	pH	0.0145	1	36.777	0.0000
	Bacteria	0.0025	3	6.367	0.0006
	M × pH	0.0063	2	15.960	0.0000
	M × b	0.0016	6	4.160	0.0009
	pH × b	0.0016	3	4.080	0.0090
	M × pH × b	0.0014	6	3.585	0.0030
	Error	0.0004	96	–	–
* media: Aschan-N.: 9.1 a Lamb: 38.1 b Melin-R.D.: 91.0 c pH = 5.8: 35.1 a pH = 7.0: 57.1 a control: 39.7 a Arth.: 59.4b Bac.: 40.5 a Ps.: 44.7 a					
H. crustuliniforme	Medium (M)	0.0510	2	216.536	0.0000
	pH	0.0028	1	11.917	0.0008
	Bacteria	0.0011	3	4.792	0.0037
	M × pH	0.0146	2	62.009	0.0000
	M × b	0.0005	6	2.215	0.0480
	pH × b	0.0014	3	5.944	0.0009
	M × pH × b	0.0001	6	0.626	0.7088
	Error	0.0002	96	–	–
* media: Aschan-N.: 24.9 a Lamb: 32.4 b Melin-R.D.: 90.2 c pH = 5.8: 44.3 a pH = 7.0: 54.0 b control: 51.4 b Arth.: 53.2 b Bac.: 51.9 b Ps.: 40.0 a					

Explanations: Newman-Keuls multiple range test (means within a given row followed by different letters are significantly different ($p \leq 0.05$)

Newman-Keuls multiple range test points out an overall stimulatory effect of *A. globiformis* on *L. laccata* and *R. vinicolor* growth and inhibitory action of *P. fluorescens* on *H. crustuliniforme*. In the case of *Rhizopogon* all interactions (medium × pH, medium × bacteria, pH × bacteria, medium × pH × bacteria) were highly significant ($0.0001 < p < 0.009$). In *Hebeloma* the most significant interactions were: medium × pH and pH × bacteria but in *Laccaria*: medium × bacteria and pH × bacteria.

Discussion

The host-plant responses to mycorrhizae are the net result of the mycorrhizal fungus and its mycorrhizosphere associates (Linderman, 1988). Between ectomycorrhizal fungus and soil microflora exist interactions which may influence the survival of mycorrhizal fungus as well as mycorrhizae formation and functioning (Rambelli, 1973; Linderman, 1988; Garbaye, 1991). Microorganisms affecting mycorrhizal infection can be isolated not only from the mycorrhizal mantle or sporocarps of ectomycorrhizal fungi (Li and Castelano, 1987; Garbaye, Duponnois and Wahl, 1990) but they can be free-living soil organisms as *Agrobacterium*, *Bacillus*, *Pseudomonas*, actinomycetes and other unidentified bacteria (Bowen and Theodorou, 1979; Garbaye and Bowen, 1987; Friedman *et al.*, 1989; Leyval and Berthelin, 1989; Duponnois and Garbaye, 1990). Two rhizosphere inhabiting bacteria: *A. globiformis* and *P. fluorescens* and one typical soil microorganism – *B. subtilis* (Strzelczyk, Dahm and Leniarska, 1978; Różycki, 1987) were used in our studies. We have found that these microorganisms affected biomass production by *H. crustuliniforme*, *L. laccata* and *R. vinicolor*. The effect of bacteria depended upon composition of the medium and its pH.

The mechanisms of interactions between mycorrhizal fungi and bacteria are little understood. Duponnois and Garbaye (1990) have shown that so called "helper" bacteria can act directly by providing nutrients or growth factors to the fungus and indirectly by detoxification of the medium. Such explanation can be applied to our results. Growth of bacteria at pH 7 was better than at pH 5.8 (the best one took place in the richest medium according to Melin-Rama Das) thus more metabolites could be elaborated at pH 7. It is known that soil microorganisms produce growth factors including amino acids, vitamins, growth regulators (Hussain and Vancura, 1970; Różycki and Strzelczyk, 1986; Strzelczyk *et al.*, 1987). Garbaye (1991) as possible mechanisms of stimulating the process of mycorrhizae formation by bacteria also considers: modification of root exudates, detoxification, increase in the susceptibility of the root to penetration by the mycorrhizal fungus, interaction with the host fungus recognition mechanisms.

Inhibition of growth of mycorrhizal fungi by bacteria can result from competition for nutrients, production of detrimental metabolites including antibiotics and fungistatic substances(Friedman *et al.*, 1989; Garbaye, 1991).

We realize that our experimental model was a very simple one. The results obtained in laboratory can not be transferred without any cautions to the natural conditions. However such experiments are necessary for understanding basic physiological mechanisms involved in the fungus-bacteria interrelationships.

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Literature

Aschan K., and B. Norkrans. 1953. A study in the cellulolytic variation for wild types and mutants of *Collybia velutipes*. *Physiol. Plant.* **6:** 564–583.

Bowen G.D. and C. Theodorou. 1979. Interactions between bacteria and ectomycorrhizal fungi. *Soil Biol. Biochem.* **11:** 119–126.

Chanway C.P. and F.B. Holl. 1991. Biomass increase and associative nitrogen fixation of mycorrhizal *Pinus contorta* seedlings inoculated with a plant growth promoting *Bacillus* strain. *Can. J. Bot.* **69:** 507–511.

Duponnois R., and J. Garbaye. 1990. Some mechanisms involved in growth stimulation of ectomycorrhizal fungi by bacteria. *Can. J. Bot.* **68:** 2148–2152.

Friedman J., Hutchins A., Li C.Y., and D.A. Perry. 1989. Actinomycetes inducing phytotoxic or fungistatic activity repeated regeneration failure in southwestern Oregon. *Biol. Plant.* **31:** 487–495.

Garbaye J. 1991. Biological interactions in the mycorrhizosphere. *Experientia* **47:** 941–943.

Garbaye J., and G.D. Bowen. 1989. Stimulation of ectomycorrhizal infection of *Pinus radiata* by some microorganisms associated with the mantle of ectomycorrhizas. *New Phytol.* **112:** 383–388.

Garbaye J., Duponnois R., and J.L. Wahl. 1990. The bacteria associated with *Laccaria laccata* ectomycorrhizas or sporocarps: effect on symbiosis establishment on Douglas fir. *Symbiosis* **9:** 267–273.

Harley J.L. 1989. The significance of mycorrhiza. *Mycol. Res.* **92:** 129–139.

Hussain A., and V. Vancura. 1970. Formation of biologically active substances by rhizosphere bacteria and their effect on plant growth. *Folia Microbiol.* **15:** 468–478.

Lamb R.J. 1974. Effect of D-glucose on utilization of single carbon sources by ectomycorrhizal fungi. *Trans. Brit. Mycol. Soc.* **63:** 295–306.

Leyval C., and J. Berthelin. 1989. Interactions between *Laccaria laccata*, *Agrobacterium radiobacter* and beech roots: influence on P, K, Mg, and Fe mobilization from minerals and plant growth. *Plant and Soil* **117:** 103–110.

Li C.Y., and M.A. Castellano. 1987. *Azospirillum* isolated from within sporocarps of the mycorrhizal fungi *Hebeloma crustuliniforme*, *Laccaria laccata* and *Rhizopogon vinicolor*. *Trans. Brit. Mycol. Soc.* **88:** 563–565.

Linderman R.G. 1988. Mycorrhizal interactions with the rhizosphere microflora. The mycorrhizosphere effect. *Phytopathology.* **78:** 366–370.

Lochhead A.G., and F.E. Chase. 1943. Qualitative studies of soil microorganisms. V. Nutritional requirements of the predominant bacterial flora. *Soil Sci.* **55:** 185–195.

Melin E., and V.S. Roma Das. 1954. Influence of root metabolites on the growth of tree mycorrhizal fungi. *Physiol. Plant.* **7:** 851–858.

Rambelli A. 1973. The rhizosphere of mycorrhizae, p. 299–349. In: Ectomycorrhizae – Their Ecology and Physiology, G.C. Marks and T.T. Kozlowski, eds. Academic Press, New York.

Richter D.L., Zuellig T.R., Bagley S.T., and J.N. Bruhn. 1989. Effects of red pine (*Pinus resinosa* Ait.) mycorrhizoplane-associated actinomycetes on *in vitro* growth of ectomycorrhizal fungi. *Plant and Soil* **115:** 109–116.

Różycki H. 1987. Bacteria and actinomycetes in soil, rhizosphere and mycorrhizosphere of pine (*Pinus sylvestris* L.). *Pol. J. Soil. Sci.* **20:** 53–59.

Różycki H., and E. Strzelczyk. 1986. Free amino acids production by actinomycetes isolated from soil, rhizosphere and mycorrhizosphere of pine (*Pinus sylvestris* L.). *Zbl. Mikrobiol.* **141:** 423–429.

Strzelczyk E. 1966. Effect of associated growth of bacteria from rhizosphere and non-rhizosphere soil on growth of fungi. *Ann. Inst. Pasteur., Suppl. 3,* **111:** 312–317.

Strzelczyk E., Dahm H., Kampert M., Pokojska A., and H. Różycki. 1987. Activity of bacteria and actinomycetes associated with mycorrhiza of pine (*Pinus sylvestris* L.). *Angew. Bot.* **61:** 53–64.

Strzelczyk E., Dahm H., and U. Leniarska. 1978. Influence of microorganisms contained in productive and non-productive pine forest soil on growth of pine seedlings (*Pinus silvestris* L.). *Stud. Soc. Sci. Torunensis,* Sectio D (Botanica), X (5): 1–19.

Strzelczyk E., and M. Kampert. 1987. Growth of mycorrhizal fungi cultured with bacteria. *Angew. Bot.* **61:** 157–162.