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Effect of B-Group Vitamins on Cytokinin-Like Substances Production by Ectomycorrhizal-Fungi of Pine (*Pinus sylvestris* L.)

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Abstract

The effect of thiamine, pyridoxine, nicotinic acid, pantothenic acid and biotin on the production of cytokinin-like substances (CLS) by *Rhizopogon luteolus* and *Suillus bovinus* was investigated. Pyridoxine considerably stimulated the production of CLS by *R. luteolus*. However, *S. bovinus* produced these substances only in the presence of thiamine and biotin. Results obtained by means of gas chromatography and the soybean-callus bioassay suggest, that most of the substances produced by the fungi studied were riboside 2ip and riboside zeatin. Other compounds showing cytokinin-like activity were not closer identified.

Keywords: Ectomycorrhizal fungi, vitamins, cytokinin-like substances

Abbreviations: CLS = cytokinin-like substances

1. Introduction

It is generally accepted that mycorrhizal fungi improve growth and development of associated plants through (a) increased uptake of minerals, (b) plant growth hormones production, (c) drought resistance, and (d) resistance to root pathogens (Harley, 1948; Voigt, 1971; Bowen, 1973; Slankis, 1973; Marx, 1972; France and Reid, 1983).

Ectomycorrhizal fungi are known to produce plant growth hormones such as auxins, gibberellins and cytokinins (Ulrich, 1960; Horak, 1960; Kampert and Strzelczyk, 1984, Strzelczyk and Pokojska, 1984; Strzelczyk et al., 1985). These compounds affect growth and development of the plant and are of importance in establishing and functioning of mycorrhiza (Slankis, 1973; Rambelli, 1973; Crafts and Miller, 1974).

Among the plant growth regulators, cytokinins are of special interest. They stimulate cell division, modify cell enlargement, delay senescence, inhibit root elongation and protect the plant against pathogens by increasing the synthesis of phenolic compounds (Miller, 1971; Dekhuijzen, 1976; Michniewicz, 1985). Cytokinins may modify the growth of cortical cells in such a manner as to facilitate the invasion of the root by the fungus. They also cause mobilization of nutrients to the region of mycorrhizal association (Crafts and Miller, 1974). Therefore it is not surprising that among the plant growth regulators auxins and cytokinins are being considered of utmost importance in plant-mycorrhizal fungus interrelationships.

Certain B-vitamins are either essential or stimulatory both of growth of different mycorrhizal fungi and mycorrhiza formation (Norkrans, 1950; Moser, 1959; Davey, 1971, Palmer, 1971; Rambelli, 1973; Slankis, 1973). The source of the vitamins might be contained in root exudates (West, 1939; Rovira and Harris, 1961; Sulochana, 1962) and/or excreted by microorganisms associated with the mycorrhizal roots (Lochhead, 1958; Moser, 1959; Lochhead and Burton, 1953; Strzelczyk, 1963; Rivière, 1963; Hussain and Vančura, 1970; Strzelczyk and Leniarska, 1980, 1985; Strzelczyk and Różycki, 1985). Because of the intimate contact of the root zone microorganisms with the root cells and the hyphae of mycorrhizal fungi the vitamins released by these organisms may be of great importance for the growth of mycorrhizal fungi and mycorrhiza formation.

To our knowledge there are no data on the effect of vitamins on cytokinin production by mycorrhizal fungi. The work presented here determines the effect of B-group vitamines on the production of CLS excreted from two species of mycorrhizal fungi. The effect of CLS on soybean callus yield is also determined.

2. Materials and Methods

Organisms, media and culture conditions

Two isolates of ectotrophic mycorrhizal fungi of pine — *Rhizopogon luteolus* and *Suillus bovinus* were used in this study. The fungi were cultured and stored before experiments on potato-dextrose agar (Difco). For this work they were grown in Lamb's (1974) medium — (glucose 10 g; NH_4Cl 500 mg; $MgSO_4 \cdot 7H_2O$ 50 mg; KH_2PO_4 50 mg; FeEDTA 1 mg of 5 ppm Fe solution; thiamine-HCl 1.0 mg; biotin 0.01 mg; distilled water 1 l; pH 5.5–5.7). This medium has been supplemented with B-vitamins. The following experimental combinations were applied: Lamb's medium without vitamins (control) — with biotin and thiamine 0.5 and 1.0 mg/l; with thiamine 1.0 mg/l, — pyridoxine 1.0 mg/l, — nicotinic acid — 0.5 mg/l, — pantothenic acid 0.25 mg/l, — pyridoxine 1.0 mg/l + nicotinic acid 0.5 mg/l + panthothenic acid 0.25 mg/l. Erlenmayer flasks containing 200 ml of the above liquid media were inoculated with agar discs (1 cm in diameter) cut from 10 days old agar (potato dextrose agar, Difco) cultures of the fungi. The inoculated flasks were incubated at 26°C for 21 days. Subsequently the mycelium was separated from the medium by filtration on filter paper. The mycelium was dried at 105°C to constant weight.

Extraction of cytokinin-like substances (CLS)

The supernatants were adjusted to pH 2.5–3.0 with 1 M HCl and passed through a Dowex 50 WX 8 (Merck) cation exchange column H^+ form, 50–100 mesh. The column was washed with 500 ml of double distilled water and the cytokinin-like substances were eluted with 340 ml of 2 M NH_4OH followed by 680 ml of 5 M NH_4OH. The combined eluates were evaported to dryness *in vacuo* at 60°C to remove ammonia. Two ml samples from 1000 ml of the culture fluid were applied to a Sephadex LH-20 (Pharmacia, Uppsala) column (45 × 2.5 cm) and eluted with 35% ethanol. Four fractions, 10 ml each, were collected and evaporated to dryness at 50°C.

Bioassay

The following medium was used for growing soybean-callus (mg/l); KH_2PO_4 300; KNO_3 1000; NH_4NO_3 1000; $Ca(NO_3)_2 \cdot 4H_20$ 500; $MgSO_4 \cdot 7H_2O$ 71.5; KCl 65; H_3BO_3 1.6; $MnSO_4 \cdot 4H_2O$ 14; $Cu(NO_3)_2 \cdot 3H_2O$ 0.35; $ZnSO_4 \cdot 7H_2O$ 3.8; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.1; KJ 0.75; NaFe ethylenediaminetetraacetate 13.2; thiamine·HCl 0.2; nicotinic acid 0.5; pyridoxine·HCl 0.2; myo-inositol 100; sucrose 30.000; α-naphthaleneacetic acid 2; and Bacto-agar

10.000 (Miura and Miller, 1969). The pH of the medium was adjusted to 5.8 with KOH or HCl before the agar was melted. Three pieces of soybean *Glycine max* (L.) Merrill var. Acme callus ca. 45–50 mg each were placed in each flask. The cultures were grown in an illuminated chamber (continuous illumination) at 50 lux at 28°C. After 25 days of growth the increase of fresh mass of the callus was recorded. The total amount of cytokinin-like substances produced was calculated from a standard response curve prepared for pure kinetin (Serva) and expressed as equivalents of kinetin (E. kin. μg/g dry weight of mycelium).

Gas chromatography

Gas chromatography was employed for more accurate identification of the substances showing cytokinin-like activity in the *R. luteolus* culture. For this purpose "Chromatron" Gas Chromatograph (GDR) model GCHF-18 3–4 was used and the conditions were: gas flow rate (N_2)–40 cm^3/min, injectors temperature 270°C, and detectors temperature (FID) 290°C. The fractions analysed were silylated with BSTFA (N_2O-bis) trimethylsilyl (trifluoroacetamide) and kept for 1 hr at 80°C. Samples (1μl) were injected onto a glass column packed with 3% SE-30 on Gas Chrom Q (100/120 mesh) and analysed at program 4°C/min (210–260°C). Pure cytokinin substances (zeatin riboside and 6(γ,γ-dimethylallylamino purine riboside) were used as the control.

3. Results

It appears from Fig. 1 that vitamins affected the synthesizing capacity of *R. luteolus*. Especially pyridoxine added in amounts of 1 mg/l significantly influenced CLS production (1.4μg/E. kinetin/g dry weight) (Table 1 and Fig. 1), whereas 0.5 mg/l pyridoxine caused a decrease in CLS production to 0.06μg/g dry weight (Fig. 2). Thiamine added to the medium stimulated growth of the soybean callus with fractions 4–24. The substances contained in these fractions were not identified. (Fig. 1). *R. luteolus* showed good growth in media without vitamins and synthesized CLS which seem to be riboside 2iP or zeatin riboside (Fig. 2). The amount of CLS produced by *R. luteolus* in the presence of panthothenic acid or pyridoxine, nicotinic and pantothenic acids used together synthesized 0 to 1.4μg/E. kinetin/g dry weight in the presence of pyridoxine.

S. bovinus produced CLS only in the presence of thiamine and biotin (Table 1 and Fig. 3). The greatest stimulation of CLS formation was found in fractions 8–12 and 24–36, which seem to correspond to riboside 2iP and zeatin riboside.

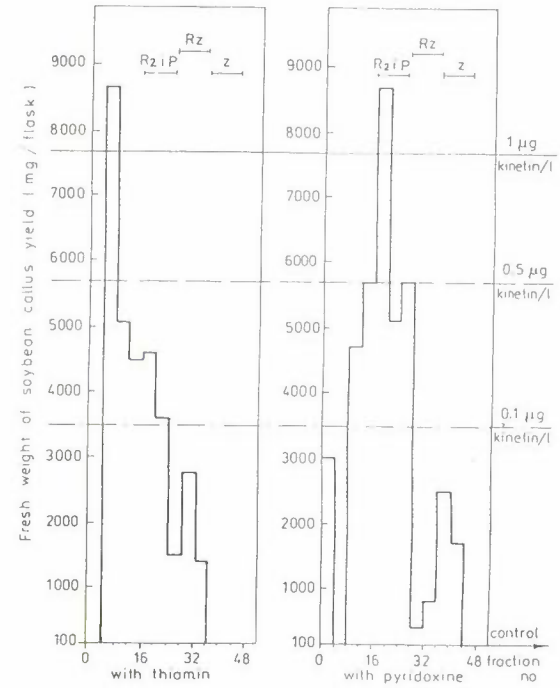

Fig. 1.
Chromatographic analysis of CLS produced by *Rhizopogon luteolus*.

Explantions to Figs. 1–4:

RZ	— Riboside zeatin
Z	— zeatin
R2iP	— riboside 6(γ, γ-dimethyl-allylamino) purine
Control	— growth of soybean callus in the medium without kinetin

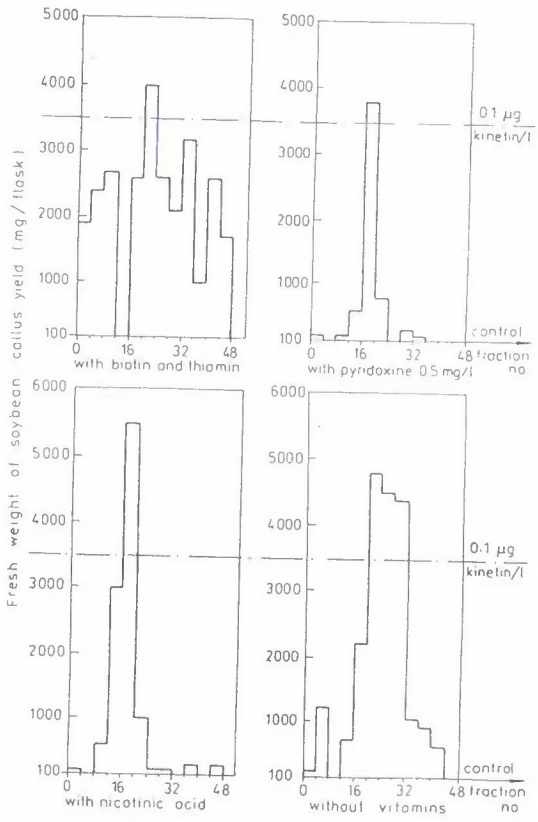

Fig. 2.
Chromatographic analysis of CLS produced by *Rhizopogon luteolus*.
The positions of R2iP, RZ and Z are the same as in Fig. 1.

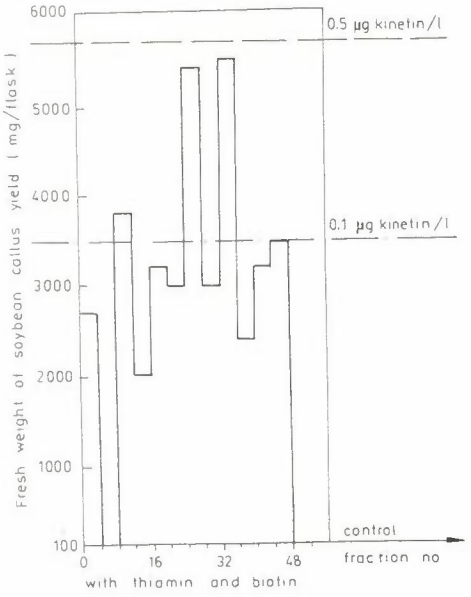

Figure 3. Chromatographic analysis of CLS produced by *Suillus bovinus*. The positions of R2iP, RZ and Z are the same as in Fig. 1.

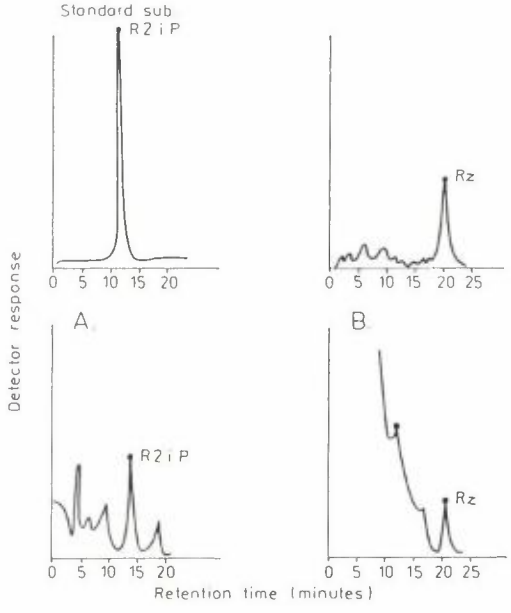

Figure 4. Chromatographic analysis of CLS in *Rhizopogon luteolus* cultures. Explanations: A — fraction No. 24-32, B—fraction No. 32-48.

The vitamins, except for thiamine and biotin, inhibited the production of CLS in *S. bovinus* despite its abundant growth.

On the basis of the fraction values as well as the data of gas chromatography, we assume that the compounds stimulating the growth of the callus are riboside 2iP and riboside zeatin (Fig. 4).

Table 1. Influence of vitamins on the amount of CLS production by *Rhizopogon luteolus* and *Suillus bovinus*.

Fungi	Vitamins added to the medium*	Dry mass of mycelium g/l	Quantity of CLS (E. kin.μg/dry mass)	Fraction number
Rhizopogon luteolus	without vitamins	0.8722	0.9745	20–32
	biotin + thiamine	1.6276	0.1220	20–24
	thiamine	3.0674	0.7174	4–24
	pyridoxine 1 mg/l	2.1191	1.3951	8–24
	"—" 0.5 mg/l	2.5891	0.0579	16–28
	nicotinic acid	0.4644	0.9689	16–20
	pantothenic acid	0.5243	0	0
	pyridoxine	0.8669		
	nicotinic acid	0.8669	0	0
	pantothenic acid	0.8669	0	0
Suillus bovinus	without vitamins	0.4052	0	0
	Biotin + thiamine	0.8217	1.1196	8–12
	thiamine	1.2453	0	0
	pyridoxine 1 mg/l	1.1954	0	0
	nicotinic acid	1.0888	0	0
	pantothenic acid	0.6338	0	0
	pyridoxine	0.6611		
	nicotinic acid	0.6611	0	0
	pantothenic acid	0.6611	0	0

* All vitamins added as indicated in methods.

4. Discussion

Extensive rhizosphere studies have shown that the root-zone microorganisms affect mycorrhizal fungi. Comprehensive reviews on this subject were published by Davey (1971), Rambelli (1973) and Slankis (1973).

Soil microorganisms were found to enhance growth of mycorrhizal fungi and mycorrhiza formation. This effect was attributed to the organic substances they release into the environment. Shemakhanova (1962) found that formation of mycorrhiza in pine roots was best supported by the presence of *Trichoderma lignorum, Azotobacter chroococum* and fluorescent bacteria.

The same observation was recorded by Malyshkin (1955). According to Vendenyapina (1955) the value if *Azotobacter* in mycorrhiza formation may be ascribed to vitamins production by the bacteria rather than nitrogen fixation or pectolytic activity.

As mentioned already ectotrophic mycorrhiza forming fungi require B-group vitamins. Thus, a supply of these compounds from external sources (root zone microorganisms) could greatly enhance growth of mycorrhizal fungi and mycorrhiza formation. Additionally the effect of these compounds on cytokinin-like substances production would affect the mycorrhiza functioning.

Our previous studies on B-vitamins synthesis by root zone microorganisms of pine have revealed that the majority of these organisms released one to three vitamins into the medium. Some of them produced even four or five vitamins (Strzelczyk and Leniarska, 1985; Strzelczyk and Różycki, 1985; Strzelczyk, 1987).

It is obvious that many environmental factors affect the production of plant growth regulation by microorganisms, among them different organic substances of microbial origin. The affect of some B-group vitamins on cytokinin-like substances production by mycorrhizal fungi has been clearly demonstrated in the present research. It has been claimed long ago by Harley (1948) that non-mycorrhizal soil organisms should be studied in conjunction with mycorrhizal fungi. As yet data concerning such organisms or the effect of their metabolites on mycorrhizal fungi are scarce (Boven and Theodorou, 1979; Strzelczyk et al., 1985).

The present results suggest that further studies on the effect of different organic substances which are known to be elaborated by soil and root zone microorganisms on the production of plant growth regulators by mycorrhizal fungi are required. Such studies are needed for better understanding of the complex mechanisms operating between the plant-mycorrhizal fungus — accompanying soil microorganisms at the root-soil interface.

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REFERENCES

Bowen, G.D. 1973. Mineral nutrition in ectomycorrhizae. In: *Ectomycorrhizae.* G.C. Marx and T.T. Kozlowski, eds. Academic Press, New York, pp. 119–125.

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

Crafts, C.B. and Miller, C.O. 1974. Detection and identification of cytokinin produced by mycorrhizal fungi. *Plant Physiol.* **54:** 586–588.

Davey, C.B. 1971. Non-pathogenic organisms associated with mycorrhizae. *Proc. 1st North Amer. Conf. Mycor.* U.S. Dept. Agr. Forest Serv.

Dekhuijzen, H.M. 1976. Endogenous cytokinins in healthy and diseased plants. In: *Encyclopedia of Plant Physiology.* Vol. 4. R. Heitefuss and P. Williams, eds. Springer-Verlag, Berlin, pp. 526–559.

France, R.C. and Reid, C.P. 1983. Interactions of nitrogen and carbon in the physiology of ectomycorrhizae. *Can. J. Bot.* **61:** 964–984.

Harley, I.L. 1948. Mycorrhizae and soil ecology. *Biol. Rev.* **23:** 127–158.

Hussain, A. and Vančura, V., 1970. Formation of biologically active substances by rhizosphere bacteria and their effect. *Folia Microbiol.* **15:** 465–478.

Horak, E. 1960. Untersuchungen zur Wuchsstoffsynthese der Mycorrhizapilze. Inter. Mykorrhiza Symp. Weimar VEB Fischer, Jena.

Kampert, M. and Strzelczyk, E. 1984. Effect of pH on production of cytokinin-like substances by bacteria isolated from soil, rhizosphere and mycorrhizosphere of pine (*Pinus sylvestris* L.). *Acta Microbiol. Polon.* **33:** 77–85.

Lamb, R.J. 1974. Effect of D-glucose on utilization of single carbon source by ectomycorrhizal fungi. *Trans. Brit. Mycol. Soc.* **62(2):** 295–306.

Lochhead, A.G. 1958. The soil microflora the plant and the root pathogen. *Trans. Royal Soc. Can. 3rd Ser.* **LII,V:** 17–24.

Lochhead, A.G. and Burton, M.O. 1953. An essential bacterial growth factor produced by microbial synthesis. *Can. J. Bot.* **31:** 7–22.

Malyshkin, P.E. 1955. Stimulation of tree growth by microorganisms. In: *Mycotrophy of Woody Plants.* A.A. Imshenetski, ed. Acad. Sci. USSR. Trans. Israel Program for Sci. Trans., Jerusalem, 1967, pp. 211–220.

Marx, D.H. 1972. Ectomycorrhizae as biological deterens to pathogenic root infections. *Ann. Rev. Phytopath.* **10:** 429–454.

Michniewicz, M. 1985. *Fizjologia roślin.* J. Zurzycki and M. Michniewicz, M. PWRIL (Panstu. Wgd. Roln. Lesn) (in Polish), pp. 519–539.

Miller, C.O. 1971. Cytokinin production by mycorrhizal fungi. In: *Mycorrhiza.* F. Hacskaylo, ed. U.S. Dept. Agr. Forest Serv. Miscell. Publ. No. 1183, Washington, D.C., pp. 169–171.

Miura, G.A. and Miller, C.O. 1969. Cytokinins from a variant strain of cultured soybean cells. *Plant Physiol.* **44:** 1035–1038.

Moser, M. 1959. Pilz und Baum. *Schweiz. Zschr. Pilzkunde.* **37:** 37–53.

Norkrans, B. 1950. Studies on growth and cellulolytic enzymes of *Tricholoma* with special reference to mycorrhiza formation. *Symp. Bot. Uppsal.* **11:** 1–26.

Palmer, J.G. 1971. Techniques and procedures for culturing ectomycorrhizal fungi. In: *Mycorrhizae.* F. Hacskaylo, ed. U.S. Dept. Agr. Forest Serv. Miscell. Publ. No. 1189, Washington, D.C., pp. 132–144.

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

Rivière, J. 1963. Action des microorganismes de la rhizosphère sur la croissance du blè. II. Isolement et caracterisation des bactèries produisant des phytochormones. *Ann. Inst. Pasteur* **105:** 303–310.

Rovira, A.D. and Harris, J.R. 1961. Plant root excretions in the rhizosphere effect. V. The exudation of B-group vitamins. *Plant and Soil* **14:** 199–214.

Shemakhanova, N.M. 1962. In: *Mycotrophy of Woody Plants.* A.A. Imshenetski, ed. Acad. Sci. USSR. Trans. Israel Progr. for Sci. Transl., Jerusalem, 1967, pp. 329–333. (cf. Davey, C.B. 1971)

Slankis, V. 1973. Hormonal relationships in mycorrhizal developments. In: *Ectomycorrhizae — Their Ecology and Physiology.* G.C. Marks and T.T. Kozlowski, eds. Academic Press, New York, pp. 231–298.

Strzelczyk, E. 1963. Studies on the rhizosphere microflora of plants resistant and susceptible to soil-borne diseases. *Acta Microbiol. Polon.* **12:** 211–223.

Strzelczyk, E. and Leniarska, U. 1980. Synthesis of vitamins by bacteria isolated from the root surface of pine seedlings (*Pinus sylvestris* L.). *Polish J. of Soil Sci.* **1:** 32–39.

Strzelczyk, E. and Pokojska, A. 1984. Production of auxins and gibberellin-like substances by mycorrhizal fungi, bacteria and actinomycetes isolated from soil and the mycorrhizosphere of pine (*Pinus sylvestris* L.). *Plant and Soil* **81:** 185–194.

Strzelczyk, E., Kampert, M., and Michalski, L. 1985. Production of cytokinin-like substances by mycorrhizal fungi of pine (*Pinus sylvestris* L.) in culture with and without metabolites of actinomycetes. *Acta Microbiol. Polon.* **34:** 177–186.

Strzelczyk, E. and Leniarska, U. 1985. Production of B-group vitamins by mycorrhizal fungi and actinomycetes isolated from the root zone of pine (*Pinus sylvestris* L.). *Plant and Soil* **86:** 387–394.

Strzelczyk, E. and Różycki, H. 1985. Production of B-group vitamins by bacteria, isolated from soil, rhizosphere and mycorrhizosphere of pine (*Pinus sylvestris* L.). *Zbl. Mikrobiol.* **140:** 293–301.

Strzelczyk, E. 1987. Production of B-group vitamins by non-mycorrhizal fungi associated with the roots of forest trees. *Acta Microbio. Polon.* (in press).

Sulochana, C.B. 1962. B-vitamins in root exudates of cotton. *Plant and Soil* **16:** 335–346.

Ulrich, J.M. 1960. Auxin production by mycorhizal fungi. *Physiol. Plant.* **13:** 429–443.

Vendenyapina, N.S. 1955. Effect of *Azotobacter* on growth of oak seedlings. *Izd. Akad. Nauk SSSR.* Trans. Israel Program Sci., Jerusalem, 1967, pp. 253–259.

Voigt, G.K. 1971. Mycorrhizae and nutrient metabolization. In: *Mycorrhizae.* F. Hacskaylo. USDA, Forest Serv. Miscell. Publ. No. 1189, Washington, pp. 122–131.

West, P.M. 1939. Excretion of biotin and thiamine by roots of higher plants. *Nature* **144:** 1050–1051.