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Production of B-group Vitamins by *Azospirillum* spp. Grown in Media of Different pH at Different Temperatures

Produktion von Vitaminen der B-Gruppe durch *Azospirillum* spp. bei unterschiedlichen Temperaturen und in Medien mit verschiedenen pH-Werten

H. Dahm[1]), H. Różycki[1]), E. Strzelczyk[1]) and C. Y. Li[2])

Key words: B-group vitamins, *Azospirillum,* pH, temperature

Summary

Studies were carried out on B-group vitamin (thiamine, biotin, nicotinic acid, riboflavin, pantothenic acid) production by 3 strains of *Azospirillum* (one derived from coniferous ectomycorrhizae and two – from sporocarps of ectomycorrhizal fungi) grown in media of different pH (5.5, 6.5, 7.5) at different temperatures (10 °C, 20 °C, 26 °C). Riboflavin was produced in largest amounts by all the strains studied; biotin was not detected in culture filtrates at all. Qualitative-quantitative composition of vitamins in post culture liquids of azospirilla depended on the temperature of growth, pH of the medium and on the strain studied. Thiamine was synthesized in largest quantities at pH 5.5 by all strains of *Azospirillum* – independently of the temperature of growth. In media of higher pH this vitamin was detected in considerably smaller amounts or was not detected at all. The smallest quantities – and the smallest numbers of vitamins produced were observed at temperature 10 °C and pH 5.5.

Zusammenfassung

Es wurde die unterschiedliche Vitaminproduktion (B-Gruppe: Thiamin, Biotin, Nicotinsäure, Riboflavin, Pantothensäure) von *Azospirillum*-Stämmen untersucht. Die Stämme wurden von Mycorrhizen (1 Stamm) an Koniferen und aus Sporokarpien von Ektomycorrhiza-Pilzen gewonnen. Die Vitaminproduktion wurde beim Wachstum unter verschiedenen Temperaturbedingungen (10 °C, 20 °C, 26 °C) und bei verschiedenen pH-Werten (5,5, 6,5, 7,5) studiert. Riboflavin wurde in größeren Mengen von allen untersuchten Stämmen produziert, Biotin dagegen wurde in keinem der Kulturfiltrate nachgewiesen. Die qualitativ-quantitative Zusammensetzung der Vitamine in den Kulturfiltraten der Azospirillen war jeweils von den Temperaturen und den pH-Werten des Mediums abhängig. Thiamin wurde am meisten bei pH 5,5 von allen Stämmen produziert, unabhängig von der Temperatur. In Medien mit höhern pH-Werten nahm die Thiaminbildung ab bzw. wurde nicht mehr nachgewiesen. Die geringste Menge an Vitaminen und die niedrigste Anzahl der Vitamine wurde bei 10 °C und bei pH 5,5 gebildet.

Introduction

In the root region (rhizosphere and rhizoplane) the microbial population is selected by compounds excreted by the roots (Rovira and Harris 1961, Katznelson 1965, Curl and Truelove 1986). The numbers and composition of these populations change constantly, depending on the type of soil and other ecological agents.

Microorganisms of the rhizosphere affect not only the growth and development of plants, but also their health (Rambelli 1973, Brown 1974). Among organic substances vitamins seem to be of special importance. They may enhance growth of mycorrhizal fungi and mycorrhizae formation (Shemakhanova 1962, Sulochana 1962, Davey 1971, Rambelli 1973, Trappe

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Microorganisms of the rhizosphere affect not only the growth and development of plants, but also their health (Rambelli 1973, Brown 1974). Among organic substances vitamins seem to be of special importance. They may enhance growth of mycorrhizal fungi and mycorrhizae formation (Shemakhanova 1962, Sulochana 1962, Davey 1971, Rambelli 1973, Trappe

1977). Some of the most promising organisms, capable of colonizing roots in large numbers and exerting beneficial effects on plants, belong to the genus *Azospirillum* (Döbereiner et al. 1976, Tarrand et al. 1978, Berg et al. 1980). *Azospirillum* is recognized as a very ubiquitous soil microorganism capable of colonizing effectively not only the roots of crop plants forest trees and weeds but also the above ground parts of the plant. It was also isolated from mycelium and from within sporocarps of mycorrhizal fungi (Li and Castellano 1987, Tilak et al. 1988). This bacterium if enough recognized could well be used as a "helper" for mycorrhizal inoculation.

Stimulation of ectomycorrhizal fungi by associated microorganisms has been noticed by Garbaye and Bowen (1989) and Duponnois and Garbaye (1990). Among such microorganisms *Azospirillum* seems to be especially promising because azospirilla are not only diazotrophic organisms but they are also plant growth regulators and vitamin producers (Tien et al. 1979, Kampert et al. 1992).

Materials and methods

Organisms and culture conditions

The organisms studied were three strains of *Azospirillum* derived from ectomycorrhizae formed by *Rhizopogon vinicolor* Smith on the roots of Douglas-fir – *Pseudotsuga menziesii* (*Azospirillum* No. 1), or from sporocarps of ectomycorrhizal fungus: *Laccaria laccata* (Scop.: Fr.) Berk. & Br. (No. 2) and from sporocarps of ectomycorrhizal fungus: *Hebeloma crustuliniforme* (Bull.) Quel. (No. 3).

The strains were obtained from the U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station, Forestry Sciences Laboratory, Corvallis, Oregon U.S.A.

The bacteria were grown in liquid medium according to Döbereiner (1980); pH of the medium was adjusted to 5.5, 6.5 or 7.5 with 1 N HCl or 1 N NaOH. Subsequently 20 g of MES buffer (Sigma) (pH 5.5 and 6.5) or the same of MOPS buffer (Sigma) (pH 7.5) was introduced to 1000 ml of the medium.

100 ml portions of the medium were transferred into 300 ml Erlenmeyer flasks and sterilized for 15 min at 117 °C and inoculated with 0.5 ml of the suspension of cells of *Azospirillum* (3-day old). Each experiment was performed in triplicate. The bacteria were grown for 7 days at 26 °C, 20 °C and 10 °C. Subsequently the bacteria were harvested by centrifugation at 14000 rpm and in the post culture fluids the amount of the vitamins were estimated.

Determination of vitamins

The methods applied were identical to those described by Rovira and Harris (1961). Vitamins in the supernatants were determined by bioassay. Lactobacilli were used as test organisms.

Dehydrated media (Difco) were applied for growth of these bacteria. Standard response curves were obtained by adding appropriate vitamin (General Biochemicals Inc., USA) at different concentrations to the Difco assay media.

Stock cultures of the Lactobacilli were kept in stabs of Micro Assay Culture Agar (Difco). The inoculum was prepared from cultures grown for 24 h at 37 °C in Micro Inoculum Broth (Difco) with the highest concentration of the vitamin used in the standard response curve. After centrifugation the bacterial mass was resuspended in saline solution and repeatedly centrifuged. 0.1 ml portions of such suspensions were used for inoculation of tubes, containing 10 ml aliquots of the Difco medium (appropriate for each vitamin), supernatant of the vitamin-producing bacteria and double distilled water. Ten replications were made in each case.

The vitamins were detected either turbidimetrically (thiamine) or acidimetrically (the remaining vitamins). The spectrophotometer S-1/K (EMCO, Plock, Poland), at a wavelength of 560 nm was applied to 18 h-old cultures in the turbidimetrical studies. Acidimetric determinations were made with 30 (biotin), 48 (pantothenic acid) and 72 h-old cultures (riboflavin and nicotinic acid). The supernatants of the cultures were titrated with 0.1 N NaOH, using 0.004% bromothymol blue as indicators.

The following test bacteria were used in the bioassay of vitamins:

Biotin, nicotinic acid, pantothenic acid – *Lactobacillus arabinosus* (WSRO 77/T44, obtained from the Culture Collection of the Technical and Agricultural University, Olsztyn).

Riboflavin – *Lactobacillus casei* (III/1 c/4), obtained from the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences, Wroclaw).

Thiamine – *Lactobacillus fermentum* (PCM 491, obtained from the Institute of Biotechnology of Food and Agricultural Industry, Warszawa).

Results

The results of our studies are presented in Figs. 1–3.

Fig. 1 shows the production of vitamins by the strain No. 1 of *Azospirillum* grown at different temperatures and different pH values. It appears from this figure that among the vitamins studied riboflavin was synthesized in largest amounts. Biotin was not detected at all. pH affected more the production of riboflavin than temperature. More of this vitamin was detected at neutral than at acidic pH (Fig. 1). pH significantly influenced the production of thiamine by the same strain (No. 1). The largest amounts of thiamine were detected at acidic pH (5.5) – significantly different from the amounts found at pH 6.5 and 7.5. At lower temperature (10 °C) the smallest amounts of nicotinic acid were found (at pH 5.5) but pantothenic acid was not detected at all (at any pH value).

It appears from Fig. 2 that the second strain of *Azospirillum* (No. 2) was similar to strain No. 1 in respect the riboflavin production (largest amounts) despite no detection of this vitamin at temperature 10 °C, pH 5.5. Among the vitamins studied only thiamine was detected at acidic pH (5.5) and at lower temperature (10 °C). This vitamin was synthesized by the strain No. 2 (similarly as in No. 1) in higher amounts in acidic medium (pH 5.5) at all temperatures used. Along with the increase of pH and temperature the quantity of pantothenic acid produced by *Azospirillum* increased. At 10 ° and 20 °C increasing amounts of nicotinic acid produced by the strain No. 2 along with the increase of pH were observed. In case of the cultures growth at 26 °C a reverse relationship was stated. Strain No. 2 (like No. 1) did not synthesize biotin (Fig. 2).

Production of vitamins by the strain No. 3 is illustrated in Fig. 3. This strain, similarly as the two previous ones produced in largest amounts riboflavin, although this vitamin was not detected at temperature 10 °C. Pantothenic acid at this temperature was not produced either. Thiamine was detected only in acidic media (pH 5.5). Riboflavin and pantothenic acid were produced by strain No. 3 at pH 6.5 in maximum amount at 20 °C and in minimum amount at 26 °C. Biotin was not produced by the organism studied (Fig. 3).

Both temperature and pH significantly affected ($p < 0.05$) the production of vitmains by *Azospirillum* spp., as evaluated by 2-factor analysis of variance (ANOVA) (except thiamine in strain No. 1; data not shown).

Discussion

Vitamins in the rhizosphere can be of plant – as well as of microbial origin (Lochhead 1958, Rovira and Harris 1961, Katznelson 1965, Alexander 1977). Vitamins are stimulating or essential for many mycorrhizal and plant pathogenic fungi (Palmer 1971, Slankis 1973). Growth of *Basidiomycetes* is stimulated by two vitamins, of these thiamine is required by more species than biotin (Norkrans 1950, Palmer 1971). Many non-symbiotic bacteria and fungi may promote mycorrhizae formation. Such effects are ascribed to metabolites and among them also to the vitamins they produce (Slankis 1973, Rambelli 1973). According to Lochhead (1958) the main source of vitamins for the mycorrhizal fungi are likely to be rather the rhizosphere microorganisms than the plant itself.

Strzelczyk and Różycki (1985) found vitamins production to be quite common among the soil and root zone bacteria of pine. Although the amount of particular vitamins produced was different, riboflavin, thiamine and nicotinic acid were produced most frequently and in highest amounts. Biotin, on the other hand, was produced in small amounts by few isolates only. Bacteria isolated from the rhizosphere and mycorrhizosphere produced more riboflavin than those originating from the soil distant from roots.

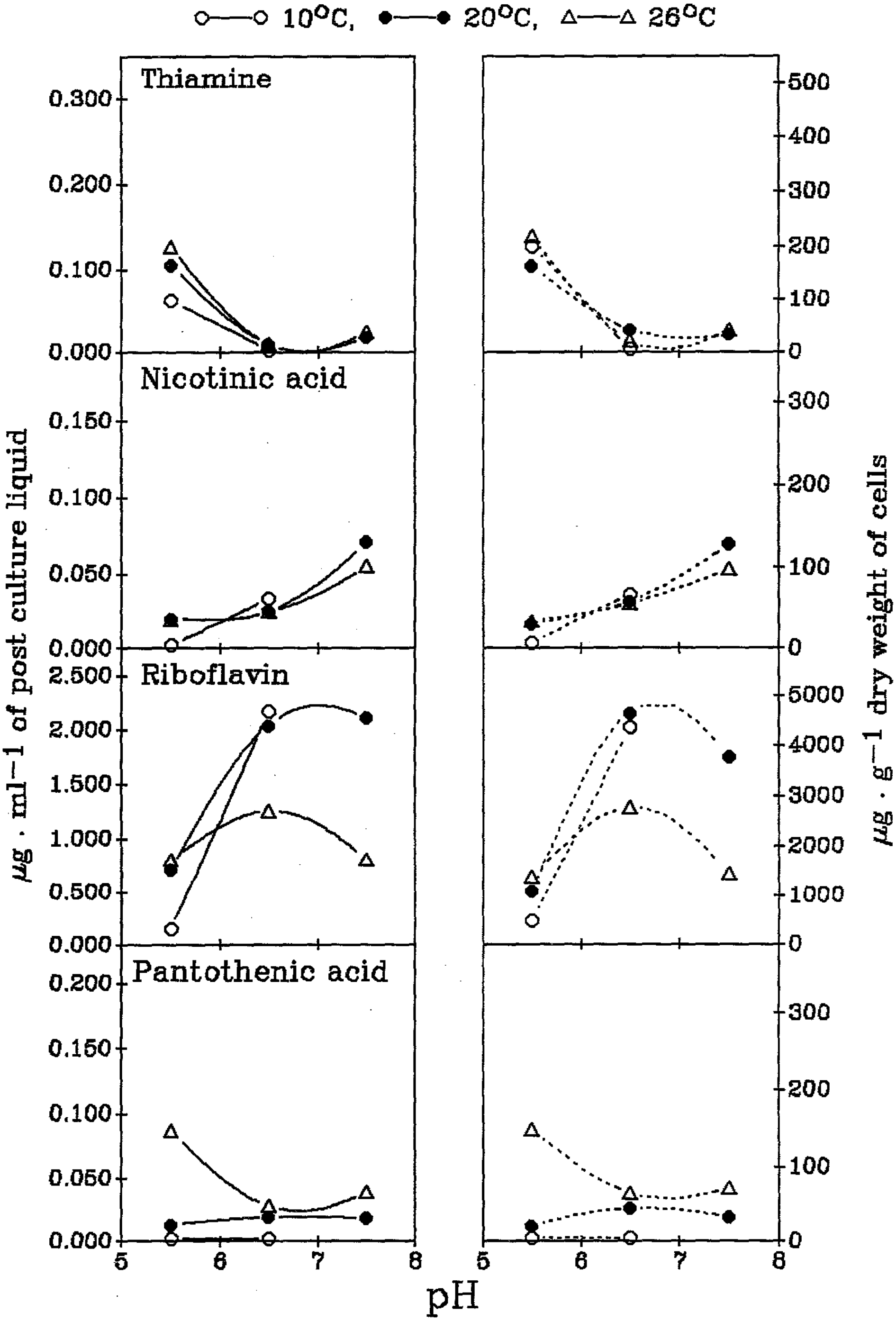

Fig. 1. Production of B-group vitamins by *Azospirillum* derived from ectomycorrhizae formed by *Rhizopogon vinicolor* (strain No. 1). Explanations: biotin was not produced (not shown); production of vitamins at 10 °C and pH 7.5 was not studied.

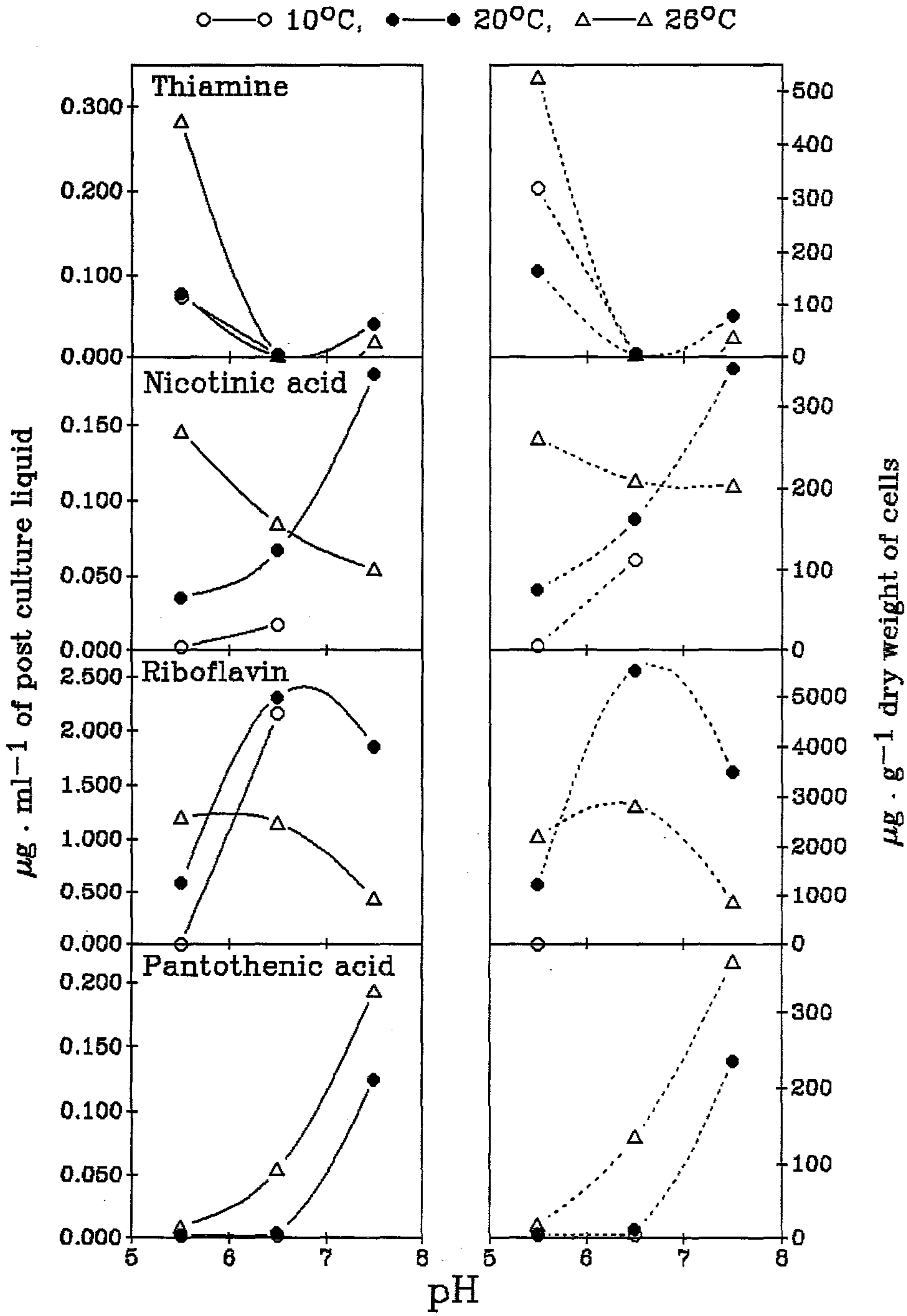

Fig. 2. Production of B-group vitamins by *Azospirillum* derived from sporocarp of Laccaria laccata (strain No. 2). Explanations – see. Fig. 1.

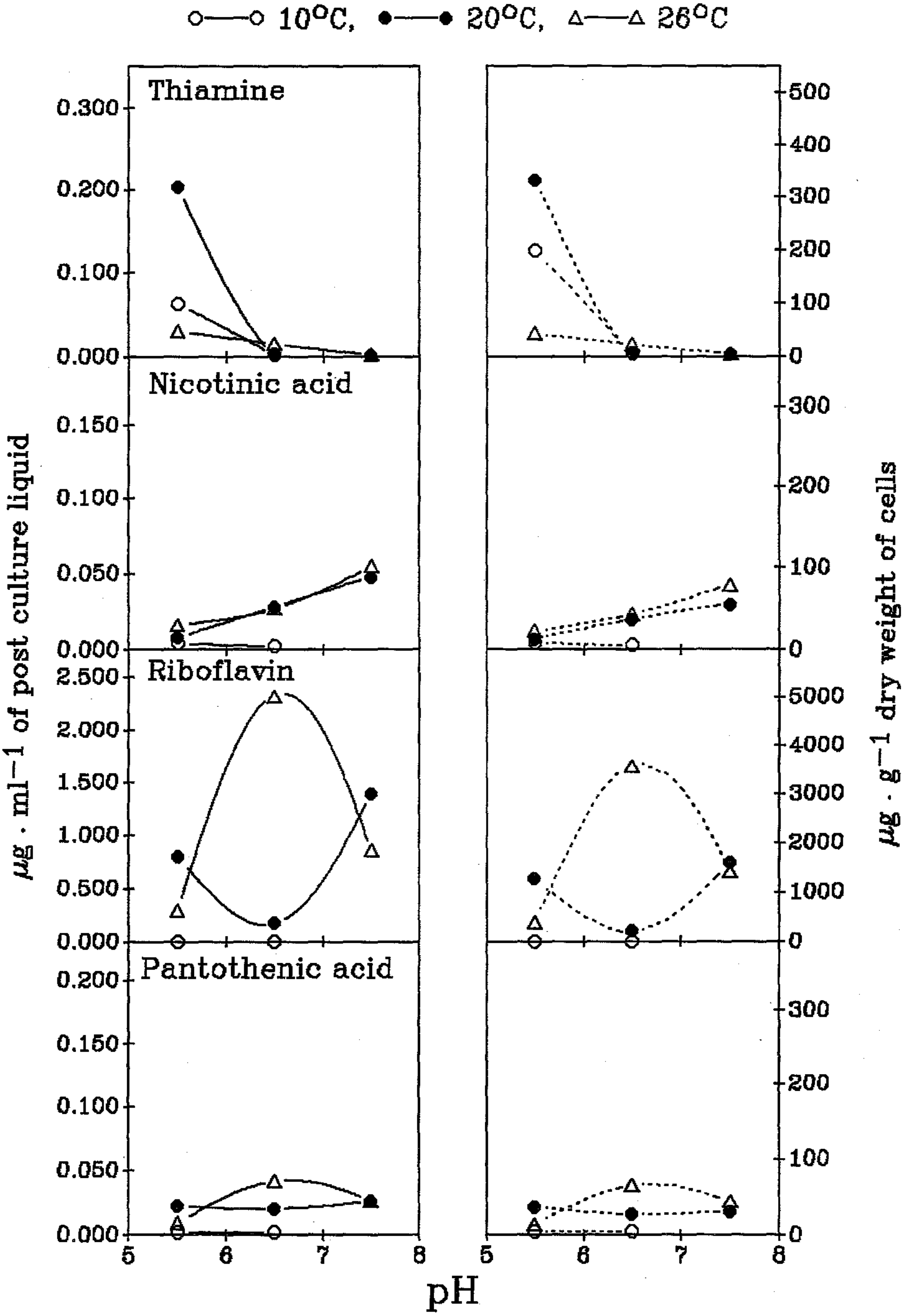

Fig. 3. Production of B-group vitamins by *Azospirillum* derived from sporocarp of *Hebeloma crustuliniforme* (strain No. 3). Explanations – see Fig. 1.

Mycorrhiza forming fungi, although requiring external B-group vitamins are also capable of producing these compounds. Production of the vitamins varied between species of fungi and was influenced by the pH of the medium. Strzelczyk et al. (1991) found, that thiamine was produced by the ectomycorrhizal fungi in minute amounts, mainly in the acidic medium. Biotin was not detected in the post culture filtrates of ectomycorrhizal fungi studies.

Mycorrhizosphere and rhizosphere reactions undoubtedly are influenced by host and edaphic factors. Soil, pH, moisture, organic matter, temperature etc. can greatly influence the microbial interactions in the root zone.

Soil pH in the rhizosphere is an obvious influencing factor on microbial activity in this plant – soil region (Curl and Truelove 1986). A common assumption is that the pH of soil near the root tends to be acidic due to the release of CO_2 and organic acids by the roots (and undoubtedly also by microorganisms). In fact the pH may become either acidic or alkaline depending upon the source of nitrogen supplied as NH_4 or NO_3 (Riley and Barber 1969, Nye 1981). According to Garbaye (1991) the rhizosphere is characterized by a lower pH (due to proton extrusion linked with cation uptake and to production of organic acids) than the root-free soil. Curl and Truelove (1986) point out that the rhizosphere pH may differ from the pH of adjacent root-free soil by 1–2 units which would have a significant effect on microorganisms.

Temperature is perhaps the most biologically significant physical variable in soil. Both rhizosphere and bulk soil microorganisms are exposed to a wide range of temperatures (Curl and Truelove 1986). Therefore among other factors, pH and temperature have to be considered in studies on bacteria which could be selected as "helpers" for inoculation of plants with mycorrhizal fungi.

In our studies both pH and temperature affected the production of B-group vitamins by azospirilla. The same fact was stated in our previous studies on mycorrhizal fungi. Growth variation between and within species of ectomycorrhizal fungi in response to pH in vitro has been recorded by Hung and Trappe (1983).

Azospirilla showed maximum nitrogenase activity at pH 6.5 and above and temperature 30–35 °C. N_2-fixing *Azospirillum* was also noticed in saline and saline alkali soils (Tilak and Krishna-Murti 1981).

We have found in the present studies, that strains of *Azospirrillum* associated with coniferous mycorrhizae and with sporocarps of ectomycorrhizal fungi did not produce biotin. Also fungi from which *Azospirillum* strains were derived (*Laccaria laccata, Hebeloma crustuliniforme* and *Rhizopogon vinicolor*) did not synthesize biotin. The post culture liquids of these fungi did not contain thiamine (unpublished data). However *Azospirillum* synthesized this vitamin mainly in acidic medium (pH 5.5). Ectomycorrhizal fungus *Rhizopogon vinicolor* among the vitamins studied (biotin, thiamine, riboflavin, pantothenic – and nicotinic acid) synthesized only riboflavin. On the other hand *Azospirillum* derived from ectomycorrhizae formed by this fungus produced all vitamins studied.

Also Strzelczyk et al. (1991) observed, that production of vitamins by ectomycorrhizal fungi varied between species and different strains of the same fungus and dependend distinctly upon the pH value of the medium. Thiamine was produced by the fungi in minute amounts, mainly in the acidic medium, but largest amounts of pantothenic acid were detected at pH 7.0. Similarly one strain of *Azospirillum* (No. 2) synthesized more pantothenic acid in neutral than in acidic medium, but another strain (No. 1) produced higher amounts of this vitamin at pH 5.5 than at pH 6.5 and 7.5. Thus this production was strain depending.

Temperature also affected the production of vitamins by *Azospirillum*. The lowest quantities of these compounds were detected at 10 °C. Differences between vitamin production at 20 °C and 26 °C were smaller than those between 10 °C and the remaining two temperatures.

The results presented in this paper indicate, that vitamins production is common among the

Azospirillum strains, although amounts of particular vitamins produced were different and depended on pH and temperature. Because *Azospirillum* studied in this work was derived from within sporocarps of ectomycorrhizal fungi or from within ectomycorrhizal roots, the vitamins elaborated may affect the fungus and the plant directly.

Researches on the effect of organic substances, produced by the associated microorganisms on the plant, mycorrhizal fungus and their relations are certainly needed. They are of importance both from the scientific and practical point of view.

Acknowledgement

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