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Impact of B-group Vitamins on Growth of *Azospirillum*[*]

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Abstract

Studies on the effect of 3 vitamins (thiamine, biotin and pantothenate) at 3 different concentrations (vitamins applied separately and combination) on aerobic and microaerophilic growth of *Azospirillum* spp. were carried out. Under the aeorobic conditions, experimental factors (number of vitamins, type of vitamins and their concentrations) more strongly affected the observed (N_T) than the predicted (N_∞) growth yield. It was difficult to assess the action of vitamins on maximum aerobic growth rate (μ) and the duration of lag phase (t_L) due to high variability of these parameters within combinations. Under aerobic conditions, the growth yield of *Azospirillum* spp. was most strongly affected by the number of vitamins; under microaerophilic conditions – by their concentrations. The average microaerophilic growth yield was higher in the presence of pairs of vitamins than in case of vitamins applied separately; the reverse was true for aerobic conditions. Under aerobic conditions in most cases the vitamins did not affect significantly or slightly inhibited the growth of bacteria. Under microaerophilic conditions bacterial growth was stimulated by the vitamins.

Introduction

Azospirillum spp. constitute widespread groups of associative, N_2-fixing bacteria – beneficial to higher plants (Broek and Vanderleyden, 1995; Okon and Itzigsohn, 1995; Fallik and Okon, 1996). Azospirilla can be associated not only with the roots of plants but also with mycorrhizas and with sporocarps of mycorrhizal fungi (Li and Castellano , 1987; Tilak *et al.* 1988; Różycki *et al.*, 1992).

It is well known that bacteria of the genus *Azospirillum* can produce biologically active substances: plant growth hormones (Strzelczyk *et al.*, 1994 a; Broek and Vanderleyden, 1995; Okon and Itzigsohn, 1995), amino acids (Rodelas *et al.* 1994) and vitamins (Dahm *et al.* 1993; Rodelas *et al.* 1993). However little is known about the effects of these substances on azospirilla (Różycki *et al.*, 1992; Strzelczyk *et al.* 1994b), despite of obvious importance of studies of this kind.

* Paper dedicated to the memory of Professor W. J. H. Kunicki-Goldfinger

Experimental

Materials and Methods

Bacterial strains. The following bacteria were used in our studies:

1. *Azospirillum* sp. – strain No. 1, isolated from ectomycorrhizae of Douglas-fir formed by *Rhizopogon vinicolor* (Li and Castellano, 1987);

2. *Azospirillum* sp. – strain No. 2, isolated from within the sporocarp of *Laccaria laccata* (Li and Castellano, 1987);

3. *Azospirillum* sp. – strain No. 3, isolated from within sporocarp of *Hebeloma crustuliniforme* (Li and Castellano, 1987);

4. *Azospirillum* sp. – strain No. HCIH, isolated (1992) from vesicular-arbuscular mycorrhiza (VAM) of eucalyptus (*Eucalyptus camadulensis*), growing on southwestern coast of Taiwan;

5. *Azospirillum brasilense* Cd – type species (ATCC 29710).

Culturing. For culturing, storage and for growth experiments with *Azospirillum*, the modified Döbereiner 's (Döbereiner 1980) basal medium was used; its composition and other details were given previously (Różycki *et al.*, 1992).

Vitamin treatments. Effects of vitamins (separately and in combination) on growth of *Azospirillum spp.* were studied under aerobic and microaerophilic conditions. Vitamins with their concentrations in the medium (mg/l) were as follows: thiamine – 1, 10, 100; biotin 0.01, 0.1, 1; calcium pantothenate 1, 10, 100 (medium without vitamins served as a control). Combinations of 2 and 3 vitamins were used in concentrations given in Tables I and II (total: 22 experimental combinations).

Bacterial growth under aerobic conditions. The vitamin stock solutions (100-fold concentrated) were filter sterilized (Milipore, pore size: 0.22 μm) and added to autoclaved basal Döbereiner 's medium, containing disodium malate (5 g/liter) and NH_4Cl (0.2 g/liter). 20 ml portions of the media in 100 ml Erlenmeyer flasks (with side arm) after autoclaving were inoculated with 0.2 ml of young (24–48 hour-old), liquid culture of a given bacterium (2 replicas per combination). The cultures were incubated at 28°C under static conditions, but with occasional shaking on Vortex mixer (immediately before optical density reading).

Optical density of the bacterial cultures was estimated on Klett-Summerson Photoelectric Colorimeter, Model 800–3 (with a blue filter No. 42) after 0, 4, 8, 12, 24, 48 and 72 hours of culturing. On the basis of resulting growth curve, the growth kinetics parameters were calculated, using Gompertz model (reparametrized Gompertz equation, according to: Zwietering *et al.* 1990) described with the following equation:

$$y = b_1 \exp\{-\exp[\mu \cdot e / b_1 \cdot (t_L - t) + 1]\}, \text{ where:}$$

y – dependent variable: transformed growth measure (optical density) at a given time point: $\ln(N/N_0)$, N – number of bacterial cells (or respective OD), N_0 – number of bacterial cells (OD) at the beginning of growth, t – independent variable (equivalent to x) time of culturing (hours), t_L – duration of lag phase (hours), b_1 – transformed value of N_∞ – expected growth yield: $\ln(N_\infty/N_0)$, where: N_∞ – growth yield at $t \rightarrow \infty$ (Klett units), μ – maximum growth rate – growth rate during the exponential phase (hour $^{-1}$; equation is given in our previous work: Różycki *et al.*, 1992), e – base of natural logarithms.

Fitting the above mathematical model to the data obtained was performed by using the computer: STATISTICA for Windows („Nonlinear Estimation" module), version 4.5, 1994 (StatSoft, Tulsa, Oklahoma, USA). Curve fitting was done using loss functional equal to: (observed values – predicted values)^2 and „quasi – Newton" estimation procedure.

Instead of absolute cell numbers we used the corresponding values of optical density (OD – expressed in Klett units) – assuming that OD is directly proportional to N.

Bacterial growth under microaerophilic conditions. Basal Döbereiner 's medium with disodium malate (5 g/liter) but without nitrogen source was used. In all experimental combina-

tions, to obtain microaerophilic conditions (in a part of volume of the medium), media were distributed in 5 ml quantities into small (140 x 14 mm) culture tubes (3 replicates per combination). After autoclaving 0.05 ml of 100-fold concentrated vitamin(s) stock solution was added to each tube (excluding the control combination) and media were inoculated with 0.05 ml of young (24–48 hour-old), liquid culture of a given bacterium. After 10 days of culturing at 28°C the final growth yield was estimated turbidimetrically. Turbidity of the culture was determined (immediately after vigorous shaking on Vortex mixer – in order to disintegrate flocks of *Azospirillum* growth) at the wavelength equal to 590 nn, using BIOLOG's turbidimeter (model 21101, BIOLOG, Hayward, California, USA).

Statistical evaluation of the results. All the results obtained from „observed" growth yield after 72 hours under aerobic – and after 10 days under microaerophilic condition, kinetic growth parameters under aerobic conditions [expected growth yield (N_∞), maximum growth rate (μ) and duration of lag phase (t_L)] were statistically evaluated using analysis of variance (ANOVA) and New-Keuls multiple range test ($p \leq 0.05$) – using STATISTICA for Windows, version 4.50, 1994 (StatSoft, Tulsa, Oklahoma, USA).

Results

Table I shows the effects of vitamins on growth kinetics parameters on four strains of *Azospirillum* spp. The „observed" growth yield (N_T) was measured after 48 hours of culturing in strains No. 1 and HCIH, and after 72 hours – in all the remaining ones. In general, the observed growth yield (N_T) was significantly higher ($p \leq 0.05$) for the control and for combinations with single vitamins („group I") than for cultures with 2 or 3 vitamins („group II"), although some exceptions of this rule could be stated (*e.g.* for thiamine 100 + pantothenate 100 having N_T as high as for group I in strain No. 1). This means that vitamins did not stimulate the growth of *Azospirillum* spp. under aerobic conditions (with few exceptions); in combinations of 2–3 vitamins (applied jointly) frequently a weak growth inhibition occurred (depending on the strain). It is noteworthy that the differences in values of expected growth yield (N_∞) in strains No. 1 and No. 3 were similar as in the case of observed growth yield (N_T). Values of N_T and N_∞ were mutually different depending on the vitamins applied, although more frequently N_T was > N_∞ (Table I).

Both, values of maximum growth rate (μ) and duration of the lag phase (t_L) varied considerably within and between experimental combinations. Therefore almost no significant differences between the respective combinations were noted (apart from a few exceptions). The negative values of lag time for strains No. 2 and No. 3 on the one hand may point out the real lack of the lag phase in these organisms, and on the other hand – the failure in good fitting of the mathematical model used: Gompertz model (Table I).

For *Azospirillum brasilense* Cd, values of N_0 and N_∞ were almost two-fold lower than for organisms isolated from mycorrhizas or from sporocarps of mycorrhizal fungi (Table II).

Also vitamins applied in combination inhibited its growth stronger than in the case of 4 other azospirilla. However, maximum growth rate and duration of lag phase varied considerably both within – and between experimental combinations as in 4 other organism (Table II).

Table I

Effect of vitamins on the growth kinetics parameters in 4 strains of *Azospirillum* spp., isolated from mycorrhizas, grown under aerobic conditions (28°C, 72 hours).

No	Experimental combinations	Strain No. 1				Strain No. 1			
		N_T	N_∞	μ	t_L	NT	N_∞	μ	t_L
1	Control	220.0 bc	252.82 b	0.317 a	5.872 ab	218.0 b	176.52 a	0.344 a	–0.545 a
2	T1	216.00 bc	268.26 bc	0.246 a	1.617 a	215.0 b	175.07 a	0.358 a	2.810 a
3	T10	219.0 bc	251.05 b	0.235 a	6.426 ab	221.0 b	196.89 a	0.210 a	0.029 a
4	T100	218.0 bc	241.03 b	0.196 a	7.329 b	222.5 b	185.05 a	0.390 a	2.302 a
5	B0.01	212.0 bc	261.25 bc	0.274 a	3.987 ab	226.0 b	193. 90 a	0.361 a	1.534 a
6	B0.1	223.0 bc	280.21 c	0.250 a	4.366 ab	215.5 b	171.58 a	0.382 a	2.319 a
7	B1	214.0 bc	249.52 b	0.290 a	7.380 b	220.0 b	200.56 a	0.174 a	1.771 a
8	P1	207.0 b	219.55 ab	0.630 a	8.423 b	220.0 b	183.83 a	0.391 a	2.198 a
9	P10	214.0 bc	248.47 b	0.321 a	6.221 ab	222.0 b	200.62 a	0.185 a	0.853 a
10	P100	230.0 c	249.16 b	0.190 a	7.198 b	221.0 b	182.67 a	0.367 a	51.112 a
11	T1+B0.01	216.0 bc	238.88 b	0.256 a	7.638 b	224.0 b	214.22 a	0.126 a	0.908 a
12	T10+B0.1	191.0 ab	195.49 a	0.360 a	6.912 b	219.0 b	370.25 b	0.099 a	1.237 a
13	T100+B1	185.5 a	193.61 a	0.366 a	3.656 ab	195.0 a	170.16 a	0.185 a	–0.512 a
14	T1+P1	185.0 a	198.50 a	0.344 a	4.130 ab	181.0 a	154.58 a	0.260 a	–0.243 a
15	T10+P10	182.5 a	193.79 a	0.360 a	3.826 ab	182.5 a	169.11 a	0.145 a	0.101 a
16	T100+P100	204.0 b	217.24 ab	0.356 a	2.597 ab	184.0 a	146.97 a	0.307 a	–0.278 a
17	B0.01+P1	180.0 a	195.44 a	0.376 a	4.704 ab	180.0 a	156.16 a	0.174 a	0.528 a
18	B0.1+P10	182.5 a	187.86 a	0.394 a	6.712 b	179.5 a	162.54 a	0.157 a	1.442 a
19	B1+P100	190.0 ab	205.13 a	0.363 a	2.949 ab	179.5 a	154.00 a	0.211 a	–0.999 a
20	T1+B0.01+P1	183.5 a	187.15 a	0.381 a	7.459 b	180.0 a	149.28 a	0.309 a	–0.240 a
21	T10+B0.1+P10	189.0 ab	198.02 a	0.370 a	3.678 ab	179.0 a	157.56 a	0.236 a	0.292 a
22	T100+B1+P100	194.5 ab	193.52 a	0.354 a	4.804 ab	192.5 a	164.13 a	0.287 a	0.474 a

Table I cont.

No	Experimental combinations	Strain No. 3				Strain HCIH			
		N_T	N_∞	μ	t_L	NT	N_∞	μ	t_L
1	Control	199.5 b	201.44 b	0.467 a	1.048 a	229.0 b	212.69 ab	0.219 a	3.279 ab
2	T1	191.5 b	196.14 b	0.464 a	1.278 a	246.0 bc	225.51 ab	0.238 a	2.234 a
3	T10	200.5 b	202.05 b	0.444 a	1.849 a	251.0 bc	227.80 ab	0.250 a	2.932 ab
4	T100	200.5 b	202.40 b	0.473 a	0.482 a	253.0 bc	263.15 ab	0.216 a	3.371 ab
5	B0.01	192.5 b	203.69 b	0.366 a	1.213 a	250.0 bc	258.16 ab	0.210 a	3.576 ab
6	B0.1	197.5 b	204.69 b	0.338 a	1.143 a	248.0 bc	205.84 ab	0.289 a	3.209 ab
7	B1	201.0 b	206.40 b	0.274 a	2.027 a	234.0 bc	209.01 ab	0.287 a	3.848 ab
8	P1	189.0 b	194.94 b	0.356 a	1.194 a	239.0 bc	189.68 ab	0.347 a	3.268 ab
9	P10	199.5 b	201.47 b	0.278 a	2.417 a	242.0 bc	221.32 ab	0.251 a	4.161 ab
10	P100	203.0 b	200.06 b	0.379 a	1.482 a	258.0 c	274.16 b	0.170 a	2.915 ab
11	T1+B0.01	161.5 a	158.89 a	0.525 a	–0.356 a	250.0 bc	252.76 ab	0.153 a	2.856 ab
12	T10+B0.1	163.5 a	152.84 a	0.520 a	–0.660 a	207.0 ab	147.99 ab	0.340 a	3.207 ab
13	T100+B1	178.5 ab	180.16 ab	0.282 a	–0.394 a	201.0 ab	208.83 ab	0.145 a	4.062 ab
14	T1+P1	192.0 b	197.34 b	0.351 a	1.778 a	221.0 b	217.24 ab	0.200 a	4.446 ab
15	T10+P10	163.5 a	158.83 a	0.407 a	–1.117 a	213.0 ab	204.98 ab	0.221 a	2.576 ab
16	T100+P100	179.0 ab	165.70 ab	0.394 a	–0.269 a	225.0 b	260.52 ab	0.164 a	2.150 a
17	B0.01+P1	167.0 a	163.09 ab	0.382 a	–0.672 a	212.0 ab	126.89 a	0.810 b	8.921 b
18	B0.1+P10	163.5 a	153.61 a	0.528 a	–0.519 a	196.0 a	195.88 ab	0.171 a	6.150 ab
19	B1+P100	173.0 ab	174.60 ab	0.306 a	–0.048 a	195.0 a	177.67 ab	0.310 a	7.554 b
20	T1+B0.01+P1	167.0 a	165.75 ab	0.340 a	–0.497 a	201.5 ab	163.57 ab	0.372 a	6.280 ab
21	T10+B0.1+P10	169.0 a	170.39 ab	0.299 a	–0.269 a	211.0 ab	205.90 ab	0.237 a	5.308 ab
22	T100+B1+P100	181.5 ab	170.23 ab	0.392 a	0.212 a	223.0 b	182.47 ab	0.316 a	3.761

Explanations: T – thiamine, B – biotin, P – pantothenate (numbers at the abbreviations stay for concentrations of vitamins in miligrams per liter); N_T – maximum observed value of optical density [Klett units] – after 72 (or 48) hours, N_∞ – predicted (on the basis of model) growth yield (after time – ∞ in Klett units; μ – maximum growth rate [hour $^{-1}$], t_L – duration of the lag phase [hours]. Average values (n = 2) marked with the same letter do not differ significantly ($p \leq 0.05$).

Table II

Effect of vitamins on the growth kinetics parameters in *Azospirillum brasilence* Cd, grown under aerobic conditions (28°C, 72 hours).

No	Experimental combinations	N_T	N_∞	μ	t_L
1	Control	117.5 b	121.91 ab	0.112 a	5.772 a
2	T1	140.5 b	130.27 ab	0.091 a	5.439 a
3	T10	120.5 b	112.74 ab	0,171 a	8.265 a
4	T100	106.5 ab	100.89 ab	0.158 a	5.009 a
5	B0.01	117.5 b	205.16 b	0.064 a	2.833 a
6	B0.1	133.0 b	128.20 ab	0.143 a	8.840 a
7	B1	123.5 b	127.50 ab	0.062 a	9.512 a
8	P1	115.5 b	113.16 ab	0.157 a	3.127 a
9	P10	124.0 b	130.27 ab	0.075 a	8.527 a
10	P100	117.5 b	119.44 ab	0.118 a	6.861 a
11	T1+B0.01	117.0 b	123.19 ab	0.071 a	8.914 a
12	T10+B0.1	108.0 ab	97.40 ab	0.154 a	4.408 a
13	T100+B1	67.5 a	46.21 a	0.514 b	8.591 a
14	T1+P1	118.5 b	37.20 a	0.105 a	6.173 a
15	T10+P10	135.5 b	90.96 ab	0.137 a	4.630 a
16	T100+P100	84.0 ab	59.64 ab	0.462 b	6.149 a
17	B0.01+P1	104.5 ab	106.53 ab	0.098 a	5.684 a
18	B0.1+P10	102.0 ab	93.63 ab	0.153 a	2.639 a
19	B1+P100	108.5 ab	103.22 ab	0.105 a	6.295 a
20	T1+B0.01+P1	101.0 ab	82.60 ab	0.207 a	2.466 a
21	T10+B0.1+P10	103.5 ab	95.11 ab	0.184 a	1.614 a
22	T100+B1+P100	77.0 ab	70.02	0.114 a	2.413 a

Explanations: – see Table I.

Under microaerophilic conditions growth of the strain No. 1 in the presence of vitamins was in most cases significantly higher than in the culture without vitamins (Fig. 1).

The growth yield of this bacterium increased as vitamin(s) concentration increased. Thiamine, applied separately or in combination, and pantothenate were stimulatory. Some vitamins only at the highest concentrations stimulated the microaerophilic growth of strain No. 2. For the strains 3, HCIH and *Azospirillum brasilense* Cd, vitamins exerted weaker stimulatory effects than for other two strains. Thiamine did not enhance growth of strain HCIH. In general, the vitamins in highest concentrations, applied both separately and in combination, exerted a stimulatory effect on growth of the 5 strains of *Azospirillum* spp. under microaerophilic conditions (Fig. 1).

The results from aerobic (N_T and N_∞) and the microaerophilic conditions were additionally analysed with 2- and 3-factor ANOVA with the following experimental factors considered (considering no vitamin control excluded):

I. For 2-factor ANOVA: 1. Vitamins [a) applied separately, b) applied in combination]. 2. Vitamin concentrations.

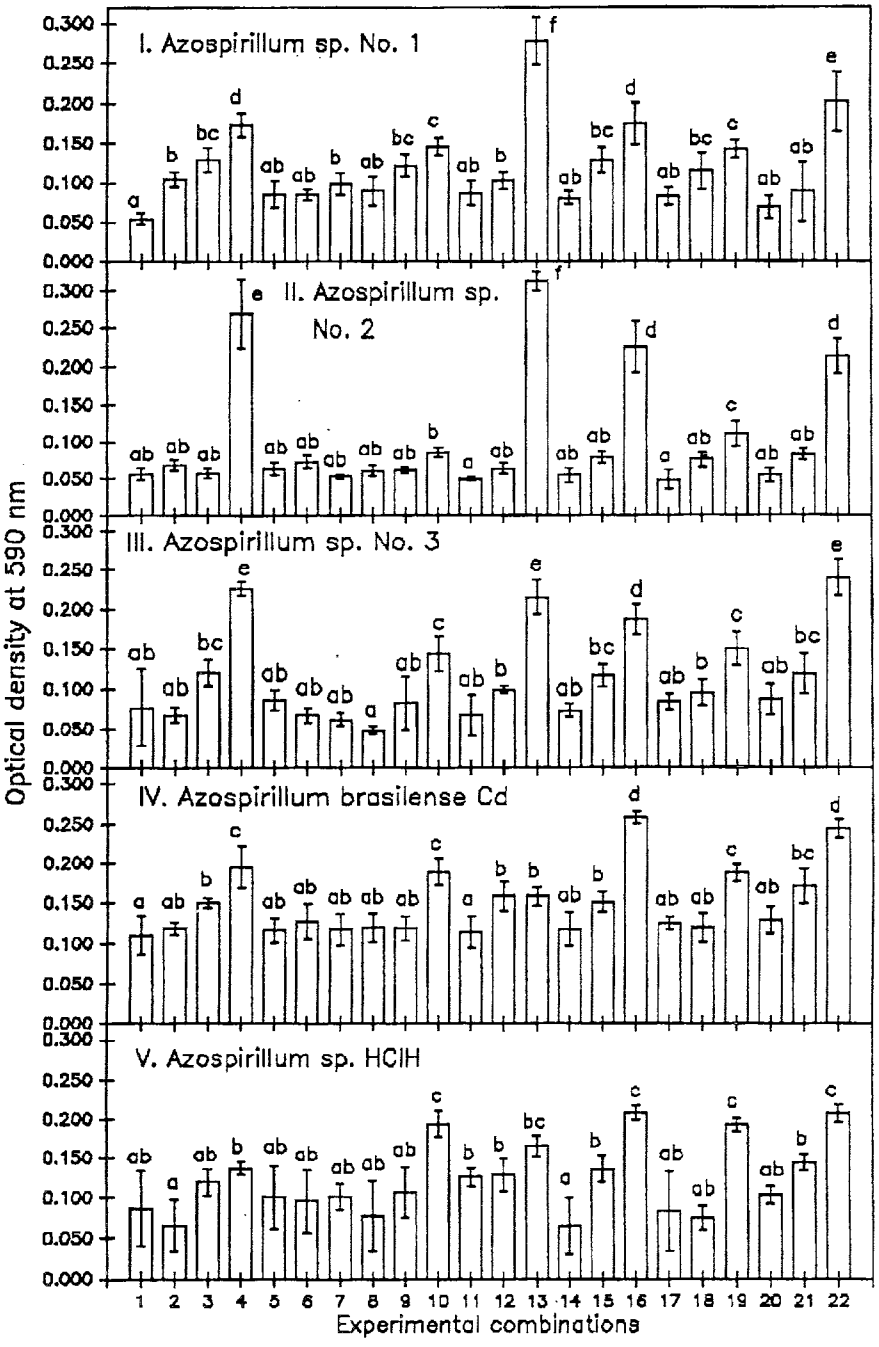

Fig. 1. Effect of vitamins on growth of *Azospirillum* spp. under microaerophilic conditions (28°C, 10 days).
Average values (± standard deviations, n = 5) marked with the same letter do not differ significantly ($p \leq 0.05$). Experimental combinations are the same as in Tables I and II.

II. For 3-factor ANOVA (both control and 3-vitamin combinations had to be excluded): 1. Number of vitamins (one or two). 2. Kind of vitamins. 3. Vitamin concentrations. (Data not shown in the documentation).

The results of these analyses can be briefly summarized as follows: 1. Experimental factors affected N_T more strongly than N_∞. 2. Under aerobic conditions the growth yield of *Azospirillum* spp. was most strongly affected by the number of vitamins and by their concentrations under microaerophilic conditions. 3. Under microaerophilic conditions the average growth yield was higher in the presence of pairs of vitamins than in case of vitamins applied separately; the reverse was true for aerobic conditions. 4. Under aerobic conditions in most cases the vitamins did not affect significantly or slightly inhibited the growth of bacteria, but under microaerophilic conditions the growth was stimulated by these compounds.

Discussion

The results of our study were quite similar to those obtained in the previous study on the utilization of various carbon and energy sources by the same organisms (Różycki *et al.*, 1992). Variability in growth kinetics could be caused by uncontrolled differences in the physiological state and in the viability of inoculum. It is known that the physiological state of inoculum strongly affects the growth kinetics of the subsequent culture (Vandenhove *et al.* 1993). Variability in bacterial growth kinetics parameters has been studied by Zwietering *et al.* (1994).

It is difficult to explain the weak inhibitory effects of vitamins on the aerobic growth of 5 strains of *Azospirillum* spp. due to the lack of appropriate data in the literature. We can assume that inhibition of the growth of *Azospirillum* spp. by vitamins, noted during later phases of growth could be caused by synergistic action of these compounds with some toxic metabolites – causing transition of the culture to its stationary growth phase. This hypothesis seems to be confirmed by stimulatory action of vitamins on growth of the strain No. 1 during its early stages of growth and its inhibition during later stages. Azospirilla have the ability to produce vitamins (except biotin) which has also been considered (Dahm *et al.*, 1993; Rodelas *et al.*, 1993); in that respect these bacteria do not differ from other soil microorganisms: saprophytic, pathogenic and symbiotic to plants (Dahm *et al.*, 1989; Strzelczyk *et al.* 1991; Dahm and Strzelczyk, 1995). Therefore it can be assumed that the weak inhibitory action of egzogenous vitamins to aerobic growth of *Azospirillum* spp. observed in the present work caused by excessive accumulation of these compounds in the medium.

The inhibitory action of vitamins on aerobic growth of azospirilla (in many cases nonsignificant) observed in our work – does not preclude the practical possibilities of use of vitamins as constituents of biostimulators enhancing the growth of these beneficial bacteria. It is known that vitamins can act synergistically with other biologically active substances in stimulation of growth and development of plants and microorganisms (Oertli , 1987). Therefore B-group vitamins have been used yet as a constituent of the biostimulator – ROOTS, enhancing the growth and regeneration of plant roots and stimulating the symbiotic nitrogen fixation in nodules of leguminous plants (Berlyn and Russo, 1990 a, b).

Stimulatory effects of vitamins (except of biotin) on microaerophilic growth of azospirilla in nitrogen-free medium is in contradiction with the results of our earlier studies (Różycki *et al.*, 1992), where no significant impact of vitamins on oxygen-limited growth of the same bacteria was noted. However, vitamins in the cited work were applied in only one concentration (0.1 mg/liter) – lower than concentration used in this study (1–100 mg/ml; thiamine and pantothenate only). Therefore we can suppose that thiamine and pantothenate (applied separately and in combination) did not act as growth factors to the bacteria studied, but rather as a nitrogen source.

Biotin used in this study with concentrations 100-fold lower than other vitamins could not act as the starter nitrogen source. It also did not seem to be a growth factor (due to lack of stimulatory action of its medium concentration: 0.1 mg/liter, recommended by Krieg and Döbereiner, 1984).

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