

## Effect of $Cd^{2+}$ , $Zn^{2+}$ and $Cu^{2+}$ on Auxins Production by *Azospirillum* strains

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### Abstract

Studies were carried out on the effect of heavy metals on three strains of *Azospirillum*.  $Cd^{2+}$ ,  $Zn^{2+}$  and  $Cu^{2+}$  at concentrations 10 and 100 ppm inhibited the biomass yield of these bacteria. The growth of azospirilla in media containing the heavy metals at concentration 500 ppm was completely inhibited. However inhibitory effect of the heavy metals on auxin-like substances (ALS) production was not noticed. The amount of ALS produced by the *Azospirillum* strains presented as equivalents of IAA in  $\mu\text{g}/1\text{ g}$  of bacterial dry mass was, as a rule, the highest in the presence of heavy metals at concentration 100 ppm. Gas chromatography revealed the presence of IAA, IPA and IAN in the culture filtrates from *Azospirillum* strains grown with cadmium.

### Introduction

*Azospirillum* spp. constitute widespread groups of  $N_2$  fixing bacteria, beneficial to higher plants (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). Thus it can not be excluded that they may affect growth of the mycorrhizal fungi and mycorrhiza formation.

It is well known that bacteria of the genus *Azospirillum* can produce plant growth hormones (Strzelczyk *et al.* 1994; Brock and Vanderleyden, 1995; Okon and Itzigsohn, 1995). However little is known about the effects of heavy metals on azospirilla, it is realized that heavy metal contamination of agricultural and forest soils can not be appreciated without elucidating its effects on soil microorganisms. Each of bacterial species may be influenced in a different way by the heavy metals. They may affect not only the balance among the total number of bacteria but also the ratio of bacterial species (Duxbury, 1981; Duxbury and Bicknell, 1983; Ohya *et al.*, 1986).

Heavy metals are introduced to soils *via* both natural (soil and organic processes) and anthropogenic processes [fuel and coal combustion for energy generation, indus-

trial processes, vehicle use and agricultural activities (Smith, 1983)]. It is suggested that heavy metals deposited from the atmosphere to forest soils are accumulated in the upper horizons of forest floors (Miller and McFee, 1983; Friedland et al. 1984). Since this accumulation is at horizons with maximum root and microorganisms activity it was appropriate to consider the effect of some important heavy metals on *Azospirillum* isolated from mycorrhizae or from the sporocarps of mycorrhizae forming fungi.

## Experimental

### Materials and Methods

**Bacteria and culture conditions.** Three strains of *Azospirillum* were used in this study: *Azospirillum* No.1 - derived from ectomycorrhizae formed by *Rhizopogon vinicolor* Smith on the roots of Douglas fir - *Pseudotsuga menziesii*, *Azospirillum* No. 2 and No. 3 were isolated from sporocarps of ectomycorrhizal fungi *Laccaria laccata* (Scop.: Fr.) Berk. & Br. and *Hebeloma crustuliniforme* (Bull.) Quel. Bacteria were grown in liquid medium according to D ö b e r e i n e r (1980) with or without heavy metals supplementation (as acetate salts) in concentrations of 1, 10, 100 and 500 ppm. Solutions of heavy metals salts were autoclaved separately [ $\text{Zn}(\text{CH}_3\text{COO})_2 \times 2 \text{H}_2\text{O}$ ,  $\text{Cd}(\text{CH}_3\text{COO})_2 \times 2 \text{H}_2\text{O}$ ] or sterilized by filtration [ $\text{Cu}(\text{CH}_3\text{COO})_2 \times \text{H}_2\text{O}$ ] using Millipore filter 0.45  $\mu\text{m}$  pore size. 100 ml of the medium (in 300 ml Erlenmeyer flasks) were inoculated with 0.5 ml of the suspension of *Azospirillum* cells (2-days old). Filter sterilized L-tryptophan (0.2 g/l) was added to the autoclaved media prior to inoculation. Studies on the production of auxins in tryptophan-free control medium were also performed. The experiments were done in triplicate. Bacteria were grown for 5 days at 28°C. Subsequently bacteria were harvested by centrifugation at 12 000 rpm and their dry mass was estimated. In the post culture fluids the amount of auxins was determined.

**Determination of auxins.** Extraction, chromatography and bioassay of auxin-like substances were performed as described in detail earlier (Pokojska and Strzelczyk, 1988). The culture filtrates were acidified to pH 2 - 3 with 1 M HCl and extracted twice with peroxide-free diethyl ether. The ether fractions were evaporated at 45°C to dryness and the residue were dissolved in 1 ml of methanol. The substances contained in the methanolic solutions were separated on Whatman No. 3 filter paper in the solvent system: isopropanol: ammonia: water (10:1:1). In the eluates of the ten equal parts of paper chromatograms auxin-like substances were detected by the *Avena* coleoptile test (Nitsch and Nitsch, 1956). Gas chromatography was applied for identification of substances showing auxin activity in the biotest. Extracts of three *Azospirillum* strains grown in the control medium and in the medium containing  $\text{Cd}^{2+}$  in different concentrations were used in these studies. Shimadzu GC-14A gas chromatograph was applied with fused silica capillary column (25 m  $\times$  0.32 mm) packed with SE-54-DF-0.50. The samples were methylated with diazomethane. The column temperature was 180°C, injector and detector (FID) temperatures were 250°C. The carrier gas was  $\text{N}_2$  at a flow rate of 2  $\text{cm}^3/\text{min}$ . Indole-3-acetic acid (IAA), indole-3-propionic acid (IPA) and 3-indoleacetonitrile (IAN) were used as standards.

**Data analysis.** Data on the biomass yield and auxin-like substances production by three *Azospirillum* strains in the presence of heavy metals were processed by Student's t-test for independent samples ( $p < 0.05$ ) and by 1-factor analysis of variance (ANOVA). Means were compared using Newman-Keuls multiple range test ( $p < 0.05$ ). 3-factor of variance comparing the effects of: bacterial strains (1), heavy metals (2) and their concentrations (3); control without metals excluded) was also made. STATISTICA/win 4.50 (1994) programme was applied (Stat-Soft, Tulsa, Oklahoma, USA).

## Results

$\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Cu}^{2+}$  significantly affected the biomass yield of three *Azospirillum* strains (Table I).

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## Results

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Table I  
Effect of heavy metals on biomass production by *Azospirillum* strains

Strain No.	Experimental combinations	Dry weight (in mg/100 ml) (average $\pm$ SD)	% Control
1	Control	44.3 d $\pm$ 1.4	100
	Cd-1	41.4 d $\pm$ 7.5	92.8
	Cd-10	43.3 d $\pm$ 2.4	97.8
	Cd-100	8.6 a $\pm$ 1.5	19.4***
	Zn-1	44.2 d $\pm$ 1.5	99.9
	Zn-10	23.3 b $\pm$ 5.4	52.7**
	Zn-100	3.5 a $\pm$ 0.4	8.0***
	Cu-1	32.1 c $\pm$ 2.7	72.5**
	Cu-10	28.5 bc $\pm$ 0.8	64.5***
	Cu-100	10.2 a $\pm$ 1.5	23.0***
2	Control	35.5 c $\pm$ 4.7	100
	Cd-1	48.8 d $\pm$ 4.4	137.8**
	Cd-10	38.8 c $\pm$ 4.1	109.5
	Cd-100	7.2 a $\pm$ 0.4	20.3***
	Zn-1	47.5 d $\pm$ 1.3	134.0**
	Zn-10	14.2 b $\pm$ 0.8	40.0***
	Zn-100	1.3 a $\pm$ 2.3	3.7***
	Cu-1	39.8 c $\pm$ 6.2	110.2
	Cu-10	39.1 c $\pm$ 2.6	110.3
	Cu-100	4.9 a $\pm$ 0.5	13.9***
3	Control	49.4 g $\pm$ 0.7	100
	Cd-1	34.4 e $\pm$ 4.3	69.6**
	Cd-10	28.1 d $\pm$ 0.7	56.8 ***
	Cd-100	11.0 b $\pm$ 0.3	22.3***
	Zn-1	51.7 g $\pm$ 1.6	104.7
	Zn-10	19.0 c $\pm$ 0.6	38.5***
	Zn-100	1.0 a $\pm$ 1.7	2.0***
	Cu-1	39.0 f $\pm$ 2.7	79.0**
	Cu-10	42.1 f $\pm$ 2.5	85.3**
	Cu-100	6.1 a $\pm$ 0.9	12.4***

Explanations: 1,10,100 - concentration of heavy metal in ppm; significance of differences as compared with control (t-test): \* 0.01 < p < 0.05, \*\* 0.001 < p < 0.01, \*\*\* p < 0.001; means within a given column followed by different letters are significantly different (p < 0.05)

The growth of bacteria studied in the presence of metals at concentration of 500 ppm was completely inhibited. Strong inhibition of bacterial growth exerted metals in concentration of 100 ppm irrespective on the bacterial strain and kind of metal. In most cases the concentration of 10 ppm was also inhibitory. Metals in concentration of 1 ppm either inhibited, did not affect or stimulated the biomass production dependly on bacterial strain and metal. Generally, the concentration of metal exerted the strongest effect on biomass production.

In preliminary experiments on auxins production by three *Azospirillum* strains it was found that only trace amounts of auxin-like substances (ALS) were produced in control medium without tryptophan and quite large (300-700  $\mu\text{g}$  eq. IAA/g of dry mass) in medium supplemented with tryptophan (Table II). In further studies the impact of heavy metals on ALS production was studied in medium with tryptophan.

Table II  
Effect of heavy metals on auxin-like substances production by *Azospirillum* strains (eq. IAA -  $\mu\text{g/g}$  of dry mass)

Experimental combination	<i>Azospirillum</i> strains		
	No. 1	No. 2	No. 3
Control	298.8 a	705.3 a	411.8 a
Cd-1	200.4 a	117.1 a	1657.3 ab
Cd-10	626.2 a	628.6 a	2517.9 b
Cd-100	4782.9 b	3479.9 b	7626.2 c
Zn-1	388 a	314.9 a	968.8 ab
Zn-10	2343.2 ab	4126 bc	1902.2 ab
Zn-100	9267 c	444.4 a	222.2 a
Cu-1	3557.7 ab	596.7 a	134.5 a
Cu-10	2332.5 ab	937.6 a	260.6 a
Cu-100	5417.2 b	5230.9 c	1575.7 ab

Explanations: see Table I

After incubation of bacteria in the presence of different concentrations of  $\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Cu}^{2+}$  the amount of ALS extracted from the post-culture liquids did not change significantly or it was even larger than in the control medium. Because of biomass decreasing with increasing of heavy metals concentration the amount of ALS produced by *Azospirillum* strains given as equivalents of IAA in  $\mu\text{g/1 g}$  of bacterial dry mass was more often significantly higher in the presence of heavy metals than in control medium (Table II). Bacteria studied were the most effective producers of ALS in the

presence of heavy metals at concentrations of 100 ppm or 10 ppm for  $Zn^{2+}$  in strains No. 2 and No. 3.

Table III presents the comparison (3-factor ANOVA) of the effect of strain, heavy metal and its concentration on ALS production.

Table III

The comparison (3-factor ANOVA) of the effects of strain, heavy metal and its concentration (control excluded) on auxin-like substances production by three *Azospirillum* strains

Effect of	Variance	df	F parameter	p (significance)
Strains	17574400	2	20.49	<<0.0001
Metals	293765	2	0.34	0.7115
Concentrations	81520200	2	95.05	<<0.0001
Strains × Metals	21349000	4	24.89	<<0.0001
Strains × Conc.	9845149	4	11.48	<0.0001
Metals × Conc.	9105717	4	10.62	<0.0001
Strains × Metals × Conc.	12892000	8	15.03	<<0.0001
Error	857619			

\*

Strains: No 1 - 3213.13 b; No 2 - 1764.02 a; No 3 - 1873.95 a  
 Metals: Cd - 2404.07 a; Zn - 2219.97 a; Cu - 2227.05 a  
 Concentration: 1 ppm - 881.73 a; 10 ppm - 1741.65 b; 100 ppm - 4227.72 c

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

The influence of the experimental factors was in the following order: concentration > strain > metals. In all cases interactions between factors were significant ( $p < 0.0001$ ).

On the basis of the results obtained by the *Avena* coleoptile test and gas chromatography it is assumed that the main active substance among ALS was IAA (Fig. 1).

This auxin (located on paper chromatograms at  $R_f$  0.3-0.5) was produced in each experimental combination by three *Azospirillum* strains. Separation of auxins on chromatography paper was not good in some cases. It was probably due to large quantities of IAA produced and to simultaneous production of the other indole derivatives. Gas chromatography revealed the presence of IPA and IAN in the culture filtrates of *Azospirillum* strains obtained after the growth of bacteria in the presence of cadmium (Fig. 1). Production of other auxins can not be excluded.

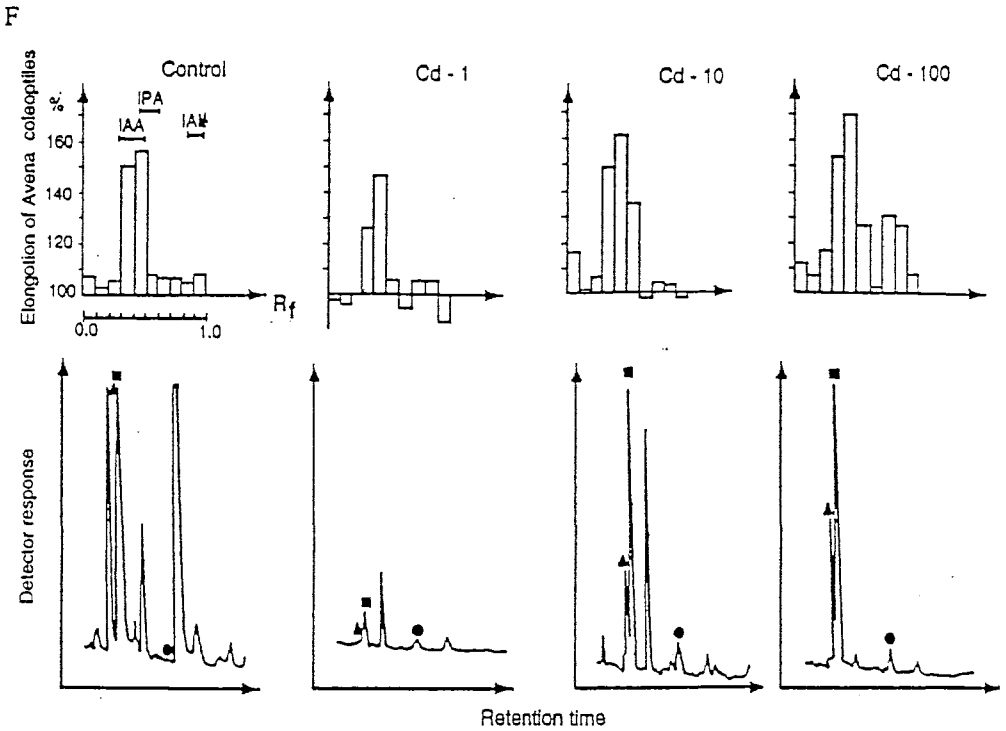


Fig.1. Paper and gas chromatography of auxins produced by *Azospirillum* No. 2 in presence of cadmium.

Explanations: ■- IAA; ▲- IAN ●- IPA

### Discussion

Heavy metals contamination of soils has become a serious problem in environmental pollution. Bacteria are generally recognized as the first organisms to be affected by these compounds (Sterritt and Lester, 1980).

During recent years, *Azospirillum* attracts the attention of many researchers because it lives in associative symbiosis with many plants, mycorrhizal fungi and sporocarps of these fungi (Li and Castellano, 1987; Li and Hung, 1987). These nitrogen fixing bacteria are also producing plant growth regulators, thus affecting growth and development of plants (Tilak *et al.* 1989; Fallik and Okon, 1996). In the interactions between plants and microorganisms the role of biological active substances is considered to be of utmost importance (Garbaye and Bowen, 1989; Gogala, 1991).

Inoculation of plants with azospirilla has been tested world-wide (Bashan and Levany, 1988). Among the mechanisms involved in this plant-bacteria interaction, hormonal effects are also suggested (Bashan *et al.* 1989). However more knowledge is required on the physiology and factors affecting the production of plant growth regulators by *Azospirillum* before this bacterium can be applied commercially (Okon and Itzigsohn, 1995, Fallik and Okon, 1996).

In field studies undoubtedly the heavy metal pollution is never due to one single metal. This makes the conclusions regarding the toxicity of heavy metals to organisms hard to draw (B ä ä t h, 1980). However to understand the relationships between the important bacteria of the genus *Azospirillum*, it is necessary to obtain basic information concerning the resistance of the bacteria to heavy metals when growing in pure culture under standard controlled conditions (Burt *et al.* 1986).

In our studies heavy metals were employed because of their importance in the natural environment and their possible involvement in the „acid rain” syndrome and because of their widespread occurrence as products of man activity.

The solubility of most metals increases with decreasing pH. Thus the natural acidic environments (*eg.* forest soils) are likely to suffer from metal toxicity (R e a d, 1986).

In our studies the strains of *Azospirillum* used were isolated from mycorrhizae or sporocarps of mycorrhizal fungi. Because organic acids and cell membranes may accumulate these compounds (B ä ä t h, 1980) *Azospirillum* may be protected from or exposed to higher concentrations of these metals. Thus these bacteria are presumably better adapted to higher concentrations of these metals than those living outside the plant.

We have found that the azospirilla revealed high sensitivity to heavy metals used in concentrations of 10 - 100 ppm. As a rule these amounts did not affect the production of ALS. Thus it seems that the production of ALS was not growth linked and inhibition of the bacterial growth must not necessarily retard the production of plant growth hormones. From our studies it also appears that  $Zn^{2+}$  was the least toxic to *Azospirillum* out of the heavy metals used. This heavy metal used at concentration of 10 ppm increased the activity of cellulolytic enzymes and was the least toxic to proteases in the ectomycorrhizal fungus *Hebeloma crustuliniforme* (Dahm and Strzelczyk, in press). According to B ä ä t h (1980) the relative toxicity of different metals is fairly constant. The following degree of toxicity appears to be most commonly found:  $Cd^{2+}$   $Cu^{2+}$   $Zn^{2+}$   $Pb^{2+}$ . We have not found the statistically significant difference between  $Cd^{2+}$ ,  $Zn^{2+}$  and  $Cu^{2+}$  in their general effect on ALS production by *Azospirillum* strains studied.

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