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Effect of Simazine on the production of lysine and methionine by *Azotobacter chroococcum* and *Azotobacter vinelandii*

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Summary. Production of lysine and methionine by *Azotobacter chroococcum* strain H23 and *A. vinelandii* strain ATCC 12837 was studied in chemically-defined medium and dialysed-soil medium, amended with different concentrations of Simazine. Responses on production due to Simazine were different for each strain and were fairly conditioned by culture media composition. Quantitative production of amino acids was significantly affected by the xenobiotic only at higher doses (50–100 μg/ml). The effect of Simazine on methionine production by strain H23 was very pronounced when bacteria were grown in dialysed-soil medium, which was specially formulated to reproduce the natural habitat of the organisms.

Keywords: Amino acids – Lysine – Methionine – *Azotobacter* – Simazine

Introduction

Azotobacter spp. are bacteria capable of nitrogen fixation, which commonly inhabit soils of neutral pH, particularly in temperate and cold climate regions of the world (Becking, 1992). These organisms can be easily isolated from the rhizosphere of a wide variety of cereals and other agricultural plants, and their potential to improve plant growth and yielding has been often reviewed (Jagnow, 1987; Becking, 1992). *Azotobacter* strains commonly synthesize biologically active substances such as plant hormones, water-soluble B-group vitamins, and several amino acids (González-López et al., 1983, 1986, 1995), although qualitative and quantitative production of these compounds is fairly influenced by some growth conditions, specially the availability and concentration of substrates used as C and N sources (González-López et al., 1995; Martínez-Toledo et al., 1996).

Amino acids in the rhizosphere are both of plant and microbial origin. The role of amino acids produced by rhizobacteria such as Azotobacter in their interaction with plants is practically unknown, although it is well established

that methionine and tryptophan act in soil as main precursors for the synthesis of plant hormones ethylene and indole-3-acetic acid, respectively. The positive influence of microbial produced L-methionine derived ethylene on pea seedlings has been reported (Arshad and Frankenberger, 1988). Plants also show responses to the exogenous application of L-methionine to soils (Arshad et al., 1993).

Xenobiotics commonly applied in agriculture affect the composition of soil microbial populations and exert some influence on enzymatic activities in soil (Pozo et al., 1994, 1995). *Azotobacter* has been widely used as indicator of the effect of agrochemicals on soil N_2-fixation (González-López et al., 1992); nevertheless, the effect of xenobiotics on the production of biologically-active substances by *Azotobacter* has received very little attention, despite of the roles attributed to these compounds in the interaction of these bacteria with plants. Simazine is an herbicide widely used in agriculture to control annual and perennial weeds, applied at concentrations in the range from 1.0 to 4.0 Kg ha^{-1}. The effect of Simazine on nitrogenase activity and ATP content of *Azotobacter* has been previously reported (Martínez-Toledo et al., 1991). In this paper we report the effect of Simazine on methionine and lysine production by *Azotobacter* spp., dealing also with the possible influence of different composition of the culture media on the effects of the xenobiotic on the synthesis of these amino acids.

Materials and methods

Microorganisms

Microorganisms used in this study were *Azotobacter chroococcum* strain H23 (Spanish Type Culture Collection, CECT 4435), isolated from maize rhizosphere by Martinez-Toledo et al. (1985), and A. vinelandii ATCC 12837, supplied by American Type Culture Collection.

The production of lysine and methionine was assayed using *Pediococcus acidilactici* ATCC 8042 auxotrophic for both amino acids. *P. acidilactici* was maintained on Difco MRS medium and transferred into fresh medium once a week. Bacto-Lysine Assay Medium and Bacto-Methionine Assay Medium were used for detection and quantification of amino acids.

Growth conditions and amino acid production assay procedures

Liberation of lysine and methionine by *Azotobacter chroococcum* and *A. vinelandii* was studied in three different culture media: 1. chemically-defined, N-free medium (g per I of distilled water: K_2HPO_4, 0.64; KH_2PO_4, 0.16; NaCl, 0.2; $MgSO_4 \cdot 7\ H_2O$, 0.2; $CaSO_4 \cdot 2\ H_2O$, 0.05; $NaMoO_4 \cdot 2\ H_2O$, 0.01; ferric citrate, 0.02; pH 7.2); 2. chemically-defined medium, supplemented with 0.3% (w/v) NH_4Cl; and 3. dialysed-soil medium, prepared according to Martinez-Toledo et al. (1996). All media were amended with 0.5% (w/v) D-glucose as carbon source. A concentrated solution of Simazine (2-chloro-4,6-bis-ethylamino-1,3,5-triazine) was added to each culture media to give final concentrations of 0 (control), 10, 50 or 100 μg/ml. 0.3% Tween 80 was incorporated into the media in order to aid solubility and dispersion. Control media received equal amounts of Tween 80 for comparison.

Strains H23 and ATCC 12837 were grown for seven days at 28°C with gentle agitation (100 rpm), in 250 ml Erlenmeyer flasks with 50 ml of each culture media amended with different concentrations of Simazine. Viable counts of *Azotobacter* in these cultures were determined by dilution and plating in N-free medium solified with 1.5% agar. All cultures were centrifuged at 10,000 g for 10 min at 4°C, and the supernatants were filter-sterilized using 0.22 μm sterile Millipore membranes. The filtrates (2 ml) were added to test tubes containing 2 ml of assay medium for lysine or methionine. All test tubes were inoculated with 0.1 ml of standardized inoculum of the auxotrophic strain. The inoculated tubes were incubated for 24 h at 37°C. Total cell numbers of *P. acidilactici* in test tubes were determined by standard plate counts in Difco MRS. Amino acid production was quantified with standard curves, according to Rodelas et al. (1994).

Statistical analysis

Data obtained throughout this study were analyzed by computer-assisted one-way ANOVA, using the software package STATGRAPHICS version 5.0 (STSC Inc., Rockville, Maryland, USA, 1989). Least significant differences (LSD) were calculated at 99% level of significance ($P < 0.01$).

Results and discussion

Growth of *Azotobacter* spp. strains was not significantly affected by the addition of Simazine to culture media. Quantitative production of lysine by *Azotobacter vinelandii* strain ATCC 12837 and *A. chroococcum* strain H23, cultured in chemically-defined N-free, NH_4^+-amended or dialysed-soil media, in the presence of 0, 10, 50 or 100 $\mu g\,ml^{-1}$ of Simazine, is shown in Fig. 1. Lysine production by strain ATCC 12837 was decreased in the presence of Simazine in both ammonia-amended chemically-defined medium and dialysed-soil medium. The xenobiotic induced significant decreases of the release of lysine to the culture medium when applied at 50 μg/ml in dialysed-soil medium (46% decrease versus control) or at 100 μg/ml dose in both NH_4^+-medium and dialysed-soil medium (32% and 64% decrease, respectively). However, the production of this amino acid by *A. chroococcum* strain H23 is strongly enhanced in the presence of Simazine, showing significant increases versus control at the maximal dose (100 μg/ml) in both N-free (83% increase) and dialysed-soil media (79% increase).

The effect of Simazine on the production of methionine by strains ATCC 12837 and H23 shows a similar pattern (Fig. 2). Again, high doses of the herbicide negatively affected lysine liberation by strain ATCC 12837 in NH_4^+-medium and dialysed-soil medium (80% and 50% decrease, respectively), whereas production of methionine by strain H23 is significantly improved by all doses of Simazine assayed, both in NH_4^+-medium or dialysed-soil medium.

Data presented in this paper show that quantitative production of lysine and methionine by *Azotobacter* spp. is clearly affected by Simazine, although the observed responses on production related to the presence of different doses of Simazine were different for each strain and were fairly conditioned by culture media composition. The presence of combined N in the medium

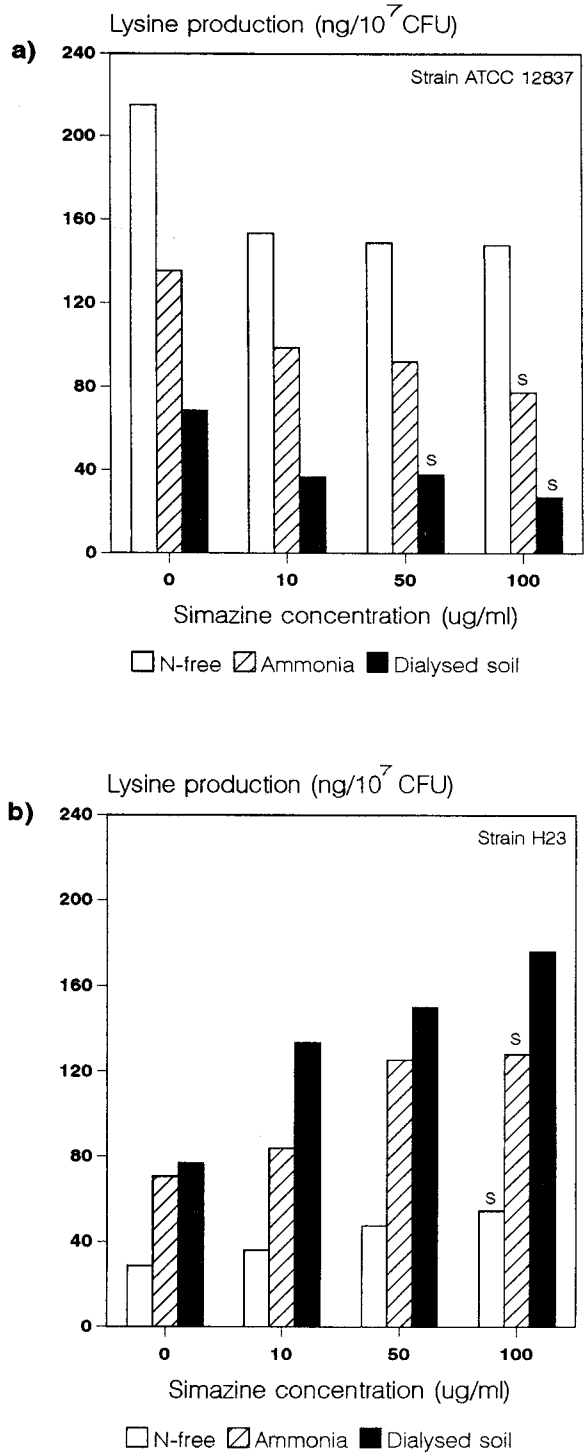

Fig. 1. Production (ng/10^7 CFU) of lysine by *A. vinelandii* strain ATCC 12837 (**a**) and *A. chroococcum* strain H23 (**b**) in chemically-defined N-free media with glucose, chemically-defined media with glucose and 0.3% NH_4Cl, and dialysed-soil media with glucose, amended with different concentrations (0, 10, 50 or 100 μg/ml) of Simazine. Values are means of five separate experiments. Bars marked with S are significant versus control ($P < 0.01$)

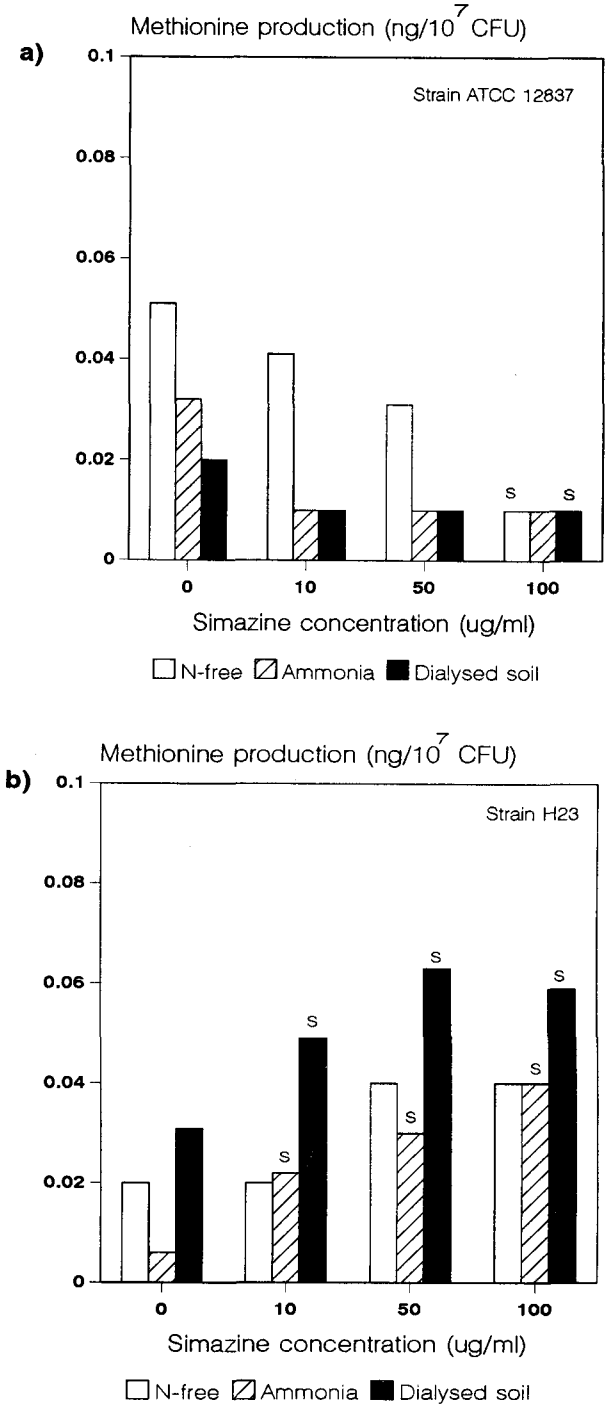

Fig. 2. Production (ng/10^7 CFU) of methionine by *A. vinelandii* strain ATCC 12837 (**a**) and *A. chroococcum* strain H23 (**b**) in chemically-defined N-free media with glucose, chemically-defined media with glucose and 0.3% NH_4Cl, and dialysed-soil media with glucose, amended with different concentrations (0, 10, 50 or 100μg/ml) of Simazine. Values are means of five separated experiments. Bars marked with S are significant versus control ($P < 0.01$)

and different C and N concentrations also affect vitamin production by *Azotobacter* spp. (González-López et al., 1983, 1991; Martinez-Toledo et al., 1996). The availability of different substrates used as carbon and energy sources also determines differences in the production of water-soluble vitamins and amino acids by diazotrophic rhizobacteria of the genus *Azospirillum* (Rodelas et al., 1993, 1994).

Lysine is the amino acid that *Azotobacter* produces in larger amounts (Gonzalez-López et al., 1995); thus, it has been reported that these organisms could be applied in a future for the production of this amino acid in batch cultures. In this context, our data clearly show that liberation of lysine by *A. chroococcum* strain H23 can be significantly increased by the addition of Simazine to the culture media. These studies probably deserve more attention in the future.

References

Arshad M, Frankenberger WT (1988) Influence of ethylene produced by soil microorganisms on ethyolated pea seedlings. Appl Environ Microbiol 54: 2728–2732

Arshad M, Hussain A, Javed M, William T (1993) Effect of soil applied L-methionine on growth, nodulation and chemical composition of *Albicia lebbeck*, L. Plant Soil 148: 129–135

Becking JH (1992) The family *Azotobacteraceae*. In: Balows A, Trüper GH, Dworkin M, Harder W, Schleifer KH (eds) The prokaryotes, 2nd edn. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications, vol 4. Springer, Berlin Heidelberg New York Tokyo, pp 3144–3170

González-López J, Salmerón V, Moreno J, Ramos-Cormenzana A (1983) Amino acids and vitamins produced by *Azotobacter vinelandii* ATCC 12837 in chemically-defined media and dialysed soil media. Soil Biol Biochem 15: 711–713

González-López J, Salmerón V, Martínez-Toledo MV, Ballesteros F, Ramos-Cormenzana A (1986) Production of auxins, gibberellins and cytokinins by *Azotobacter vinelandii* ATCC 12837 in chemically-defined media and dialysed-soil media. Soil Biol Biochem 18: 119–120

González-López J, Martínez-Toledo MV, Reina S, Salmerón V (1991) Roots exudates of maize and production of auxins, gibberellins, cytokinins, amino acids and vitamins by *Azotobacter chroococcum* in chemically-defined media and dialysed soil media. Tox Environ Chem 33: 69–78

González-López J, Martínez-Toledo MV, Salmerón V (1992) Effect of the insecticide methidathion on agricultural soil microflora. Biol Fertil Soils 13: 173–175

González-López J, Martínez-Toledo MV, Rodelas B, Pozo C, Salmerón V (1995) Production of amino acids by free-living heterotrophic nitrogen-fixing bacteria. Amino Acids 8: 15–21

Jagnow G (1987) Inoculation of cereal crops and forage grasses with nitrogen-fixing rhizosphere bacteria: possible causes of success and failure with regard to yield response – a review. Z Pflanzen Bodenk 150: 361–368

Martínez-Toledo MV, González-López J, de la Rubia T, Ramos-Cormenzana A (1985) Isolation and characterization of *Azotobacter chroococcum* from the roots of Zea mays. FEMS Microbiol Ecol 31: 197–203

Martínez-Toledo MV, González-López J, de la Rubia T, Moreno J, Ramos-Cormenzana A (1988) Grain yield response of *Zea mays* (hybrid AE 703) to *Azotobacter chroococcum* H23. Biol Fertil Soils 6: 170–176

Martínez-Toledo MV, Salmerón V, González-López J (1991) Effect of Simazine on the biological activity of *Azotobacter chroococcum*. Soil Sci 151: 459–467

Martínez-Toledo MV, Rodelas B, Salmerón V, Pozo C, González-López J (1996) Production of pantothenic acid and thiamine by *Azotobacter vinelandii* in a chemically-defined medium and a dialysed soil medium. Biol Fertil Soils 22: 131–135

Pozo C, Salmerón V, Rodelas B, Martínez-Toledo MV, González-López J (1994) Effects of the herbicide alachlor on soil microbial activities. Ecotoxicol 3: 4–10

Pozo C, Martínez-Toledo MV, Salmerón V, Rodelas B, González-López J (1995) Effect of chlorpyrifos on soil microbial activity. Environ Toxicol Chem 14: 187–192

Rodelas B, Salmerón V, Martínez-Toledo MV, González-López J (1993) Production of vitamins by *Azospirillum brasilense* in chemically-defined media. Plant Soil 153: 97–101

Rodelas B, Salmerón V, Martínez-Toledo MV, González-López J (1994) Production of amino acids by *Azospirillum brasilense* in chemically-defined medium amended with malate, gluconate or fructose. Soil Biol Biochem 26: 301–303

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