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Effect of some herbicides on the production of lysine by *Azotobacter chroococcum*

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Summary. Production of lysine by *Azotobacter chroococcum* strain H23 was studied in chemically-defined media amended with different concentrations of alachlor, metolachlor, 2,4-D, 2,4,5-T and 2,3,6-TBA. The presence of 5, 10, and 50μg/ml of alachlor or 2,3,6-TBA significantly decreased quantitative production of lysine. However, the presence 2,4-D or 2,4,5-T at concentrations of 10 and 50μg/ml enhanced the production of lysine. Quantitative production of lysine was not affected as consequence of the addition of metolachlor to the culture medium, showing that the release lysine to the culture media by *A. chroococcum* was not affected by that herbicide.

Keywords: Amino acids – Lysine – Herbicides – *Azotobacter* – Xenobiotics

Introduction

The effect of xenobiotic compounds on the qualitative and quantitative composition of soil microflora, as well as their influence on microbial activities in soil and water, have been studied to some extentent (Pozo et al., 1994, 1995) *Azotobacter* has been widely used as indicator of the effect of agrochemical on N_2-fixation (González-López et al., 1992). Alachlor, metolachlor, 2,4,-D, 2,4,5-T and 2,3,6-TBA are herbicides widely used in agriculture to control broad-leaved annual and perennial weeds and its applied at concentrations in the range from 1.0 to 4.0Kg/ha.

Amino acids in the rhizosphere are both plant and microbial origin. The role of amino acids produced by rhizobacteria such as *Azotobacter* in their interaction with plants in practically unknown, although it is well established that some amino acids act in soil as main precursors for the synthesis of plant hormones (Arshad and Frankenberger, 1988). Plants also show responses to the exogenous application of amino acids to soils (Arshad et al., 1993).

Since 1902, bacteria of the genus *Azotobacter*, have been used as inoculant for agriculture because of their ability to fix N_2 in association with plant roots (Becking, 1992). Plant growth or yield responses to *Azotobacter* in-

oculation have been reported for wheat, corn, rye, oat and several vegetable crops (Abbas and Okon, 1993). These plant growth-promoting ability of *Azotobacter* is not only due to nitrogen fixation; suppression of pathogenic microorganisms, mobilization of soil phosphate and production of phytohormones and water soluble compound such as vitamins and amino acids by these bacteria are also involved in their synergistic interactions with plants (Jagnow, 1987). In these context, synthesis of biologically-active substances by *Azotobacter* is strongly influenced by some growth condition, such as the availability of C and N sources (Murcia et al., 1997a).

It has been previously reported that various environmental toxicants affects nitrogenase activity ATP content and synthesis of biologically-active substances by *Azotobacter* (Murcia et al., 1997a; Chung et al., 1988). In this paper we report the effect of alachlor, metolachlor, 2,4-D, 2,4,5-T and 2,3,6-TBA on lysine production by *A. chroococcum*. Chemically defined media were used to better elucidate the effect of herbicides on the synthesis of these amino acids.

Materials and methods

Microorganisms

Microorganisms used in this study were *A. chroococcum* strain H 23 (Spanish Type Culture Collection, CECT 4435), isolated from maize rhizosphere (Martinez-Toledo et al., 1985). The production of lysine was assayed using *Pediococcus acidolactici* ATCC 8042 auxotrophic according to Murcia et al. (1997b). *P. acidolactici* was maintained on Difco MRS medium and transferred into fresh medium once a week. Bacto-Lysine Assay Medium was used for detection and quantification of lysine.

Culture media

Production of lysine by *Azotobacter* strains was studied in two different culture media amended with 0.5% (w/v) glucose: 1. Chemically-defined, N-free (g per 1 of distilled water: K_2HPO_4, 0.64; KH_2PO_4, 0.16; NaCl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; $CaSO_4 \cdot 2H_2O$, 0.05; $NaMoO_4 \cdot 2H_2O$, 0.01; ferric citrate 0.02, pH 7.2); 2. Chemically-defined medium, supplemented with 0.3% (w/v) NH_4Cl.

Lysine production assay procedures

A. chroococcum strain H23 were grown in N-free medium amended with 0.5% glucose. The cultures were incubated with gentle agitation (100 rpm) to maintain aerobic conditions for 24 h at 28°C. After three successive transfer of the culture to fresh medium, cells were harvested by centrifugation at 10.000 × g for 10 min and resuspended in sterile phosphate buffer (150 mM, pH 7.0) at an optical density of 0.6 at 550 nm. Aliquots (1 ml) of the cell suspensions were transferred to 250 ml Erlenmeyer flasks containing 50 ml of N-free medium or NH_4 – medium amended with 0.5% glucose. Alachlor[2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl) acetamide], metolachlor [2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide], 2,4-D (2,4-dichlorophenoxy acetic acid), 2,4,5-T (2,4,5-trichlorophenoxy acetic acid) and 2,3,6-TBA (2,3,6-trichlorobenzoic acid) had been added to the media at concentrations of 0 (control), 5, 10 and 50 μg/ml. Concentrated solution of the compounds were prepared in 0.3% tween 80 (polyoxyethylene sorbitanmonooleate) to aid solubility and dispersion. Control received equal amounts of tween 80 for comparison.

Total cell number of *Azotobacter* in these cultures were determined by standard plate counts in N-free agar medium amended with 0.5% (w/v) glucose. All cultured were centrifuged at 10.000 × g for 10 min in a refrigerated (4°C) centrifuge. The supernatants were passed through sterile 0.22 μm filter membranes (Millipore) and the filtrates added (2 ml) to test tubes containing 2 ml of Difco assay medium for lysine. The standard curve for lysine was determined according to Rodelas et al. (1994). All test tubes were inoculated with 0.1 ml of standarized inoculum of the auxotrophic strain, according to Rodelas et al. (1984). The inoculated tubes were incubated for 24 h at 37°C. Total cell number of the auxotrophic strain in test tubes were determined by standard plate counts in Difco MRS.

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 *A. chroococcum* in chemically defined media amended with alachlor was inhibited, compared with the growth obtained in an unamended media (Fig. 1). Quantitative production of lysine by *A. chroococcum* in chemically-defined N-free or NH_4^+-amended in the presence of 0, 5, 10 or 50 μg/ml of alachlor, is shown in Fig. 2. Lysine production in *A. chroococcum* was decreased in the presence of alachlor. The degree of inhibition was related to the concentration of herbicide. The presence of 5, 10 and 50 μg/ml of alachlor in chemically-defined N-free medium caused 45, 85 and 95% inhibition, respectively, after 7 d; the presence of 5, 10 and 50 μg/ml of alachlor in NH_4^+-amended caused 35, 65 and 75% inhibition respectively, after 7 d. The effect or alachlor was more evident when the *Azotobacter* strains were grown in culture medium without NH_4Cl.

Growth of *A. chroococcum* strain was not significantly affected by the addition of metolachlor to culture media. When *Azotobacter* spp. strains was cultured in the presence of 5 to 50 μg/ml of metolachlor no significant effects were observed on production of lysine. Therefore the effect of metolachlor on the production of lysine by the strain H23 grown in N-free and NH_4^+-amended media shows similar pattern (Fig. 3).

Growth of *Azotobacter* sp. strain in chemically defined N-free and NH_4^+-amended in the presence of 2,4-D or 2,4,5-T was similar to the growth curves obtained in control media without herbicides. A stimulatory effect on lysine production was observed at concentration of 10 and 50 μg/ml with 2,4-D and 2,4,5-T. The production of this amino acids by *A. chroococcum* was strongly enhanced in the presence of 2,4-D, showing maximum increases versus control at the maximal dose (50 μg/ml) in both culture media (Figs. 4 and 5).

When *Azotobacter* spp strains was cultured in the presence 2,3,6-TBA, a growth inhibition was detected (Fig. 6). The degree of inhibition increased as the concentration of the herbicide increased. The herbicide induced significant decreased of the release lysine to the culture media (N-free or NH_4^+-

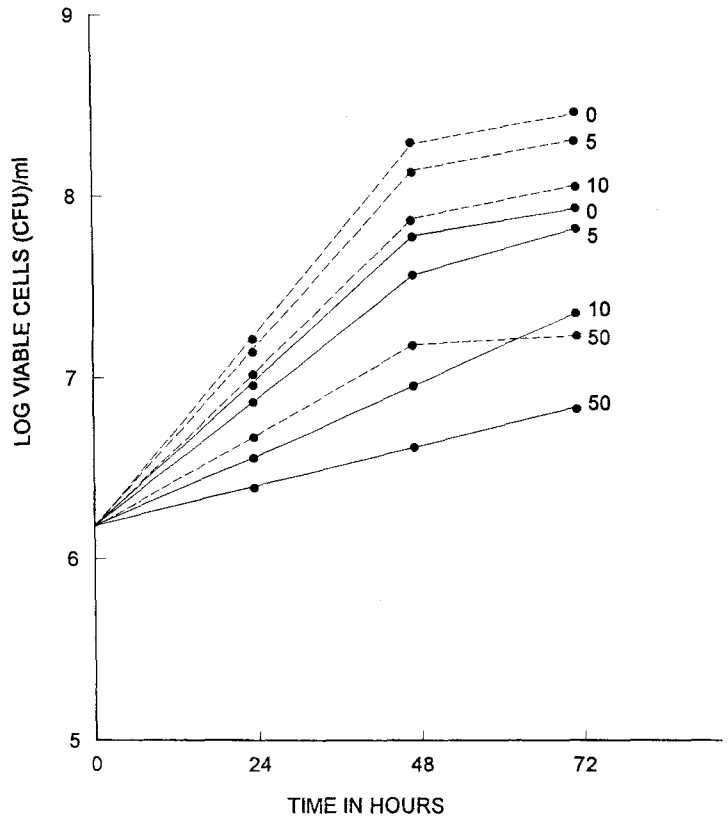

Fig. 1. Growth of *Azotobacter chroococcum* strain H23 in the presence of various concentrations of alachlor (μg/ml) N-free media (solid line) and NH_4^+-amended media (broken line). The experiment was carried out in triplicate

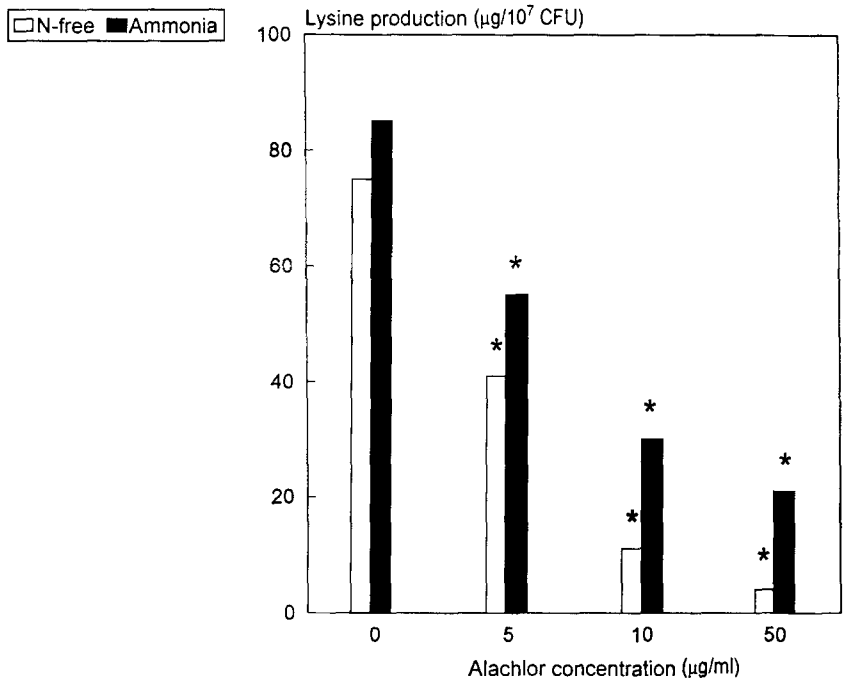

Fig. 2. Production (μg/10^7 CFU) of lysine by *Azotobacter chroococcum* strain H23 in the presence of various concentrations of alachlor (μg/ml). Values are means of five separate experiments. Bars marked with * are significant versus control ($P < 0.01$)

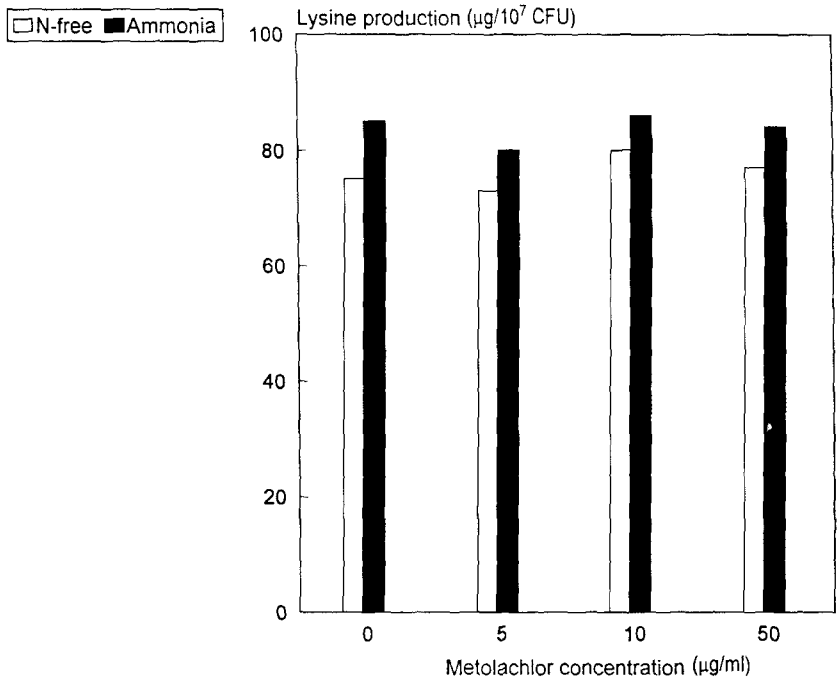

Fig. 3. Production ($\mu g/10^7$ CFU) of lysine by *Azotobacter chroococcum* strain H23 in the presence of various concentrations of metolachlor (μg/ml). Values are means of five separate experiments

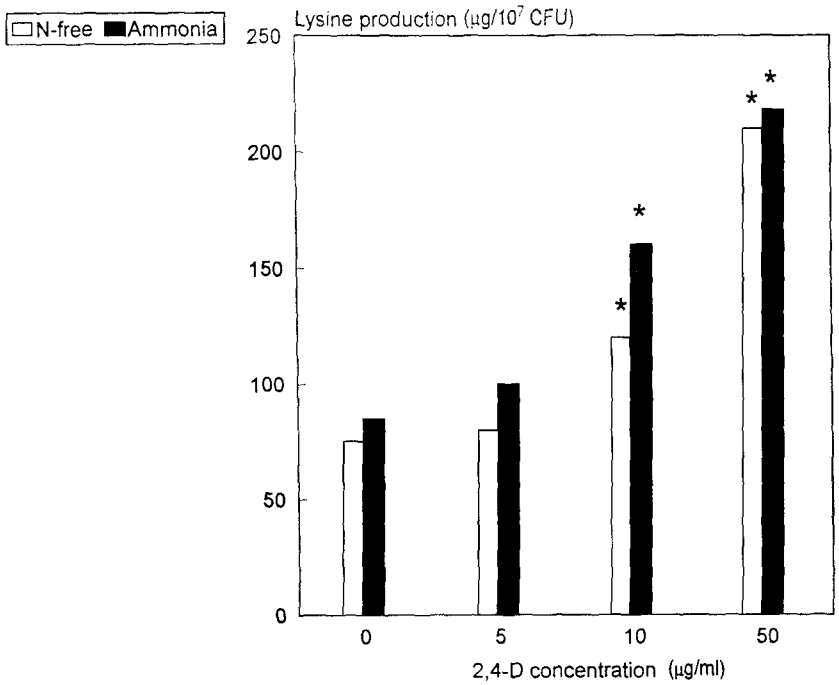

Fig. 4. Production ($\mu g/10^7$ CFU) of lysine by *Azotobacter chroococcum* strain H23 in the presence of various concentrations of 2,4-D (μg/ml). Values are means of five separate experiments. Bars marked with * are significant versus control ($P < 0.01$)

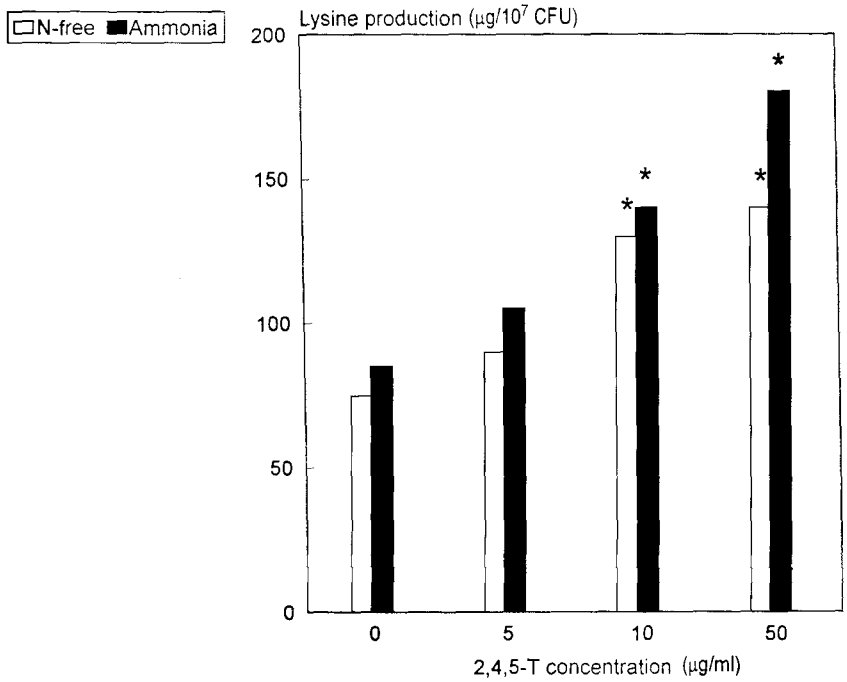

Fig. 5. Production (μg/10^7 CFU) of lysine by *Azotobacter chroococcum* strain H23 in the presence of various concentrations of 2,4,5-T (μg/ml). Values are means of five separate experiments. Bars marked with * are significant versus control ($P < 0.01$)

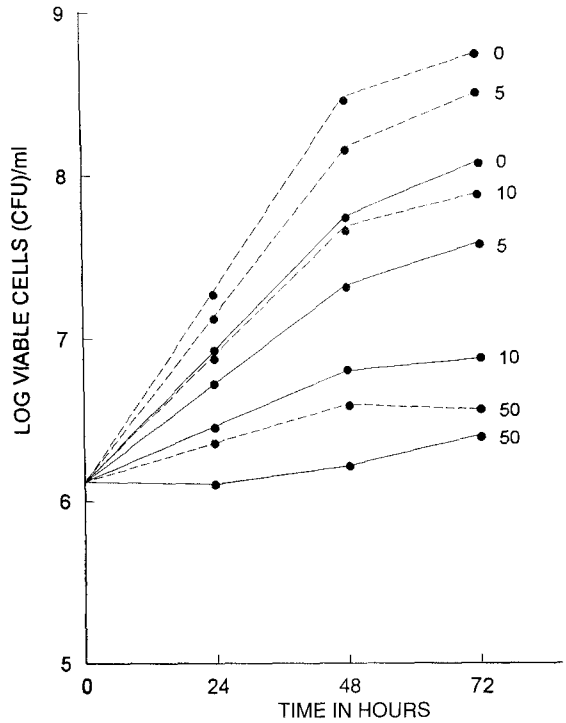

Fig. 6. Growth of *Azotobacter chroococcum* strain H23 in the presence of various concentrations of 2,3,6-TBA (μg/ml). N-free media solid line) and NH_4^+-amended media (broken line). The experiment was carried out in triplicate

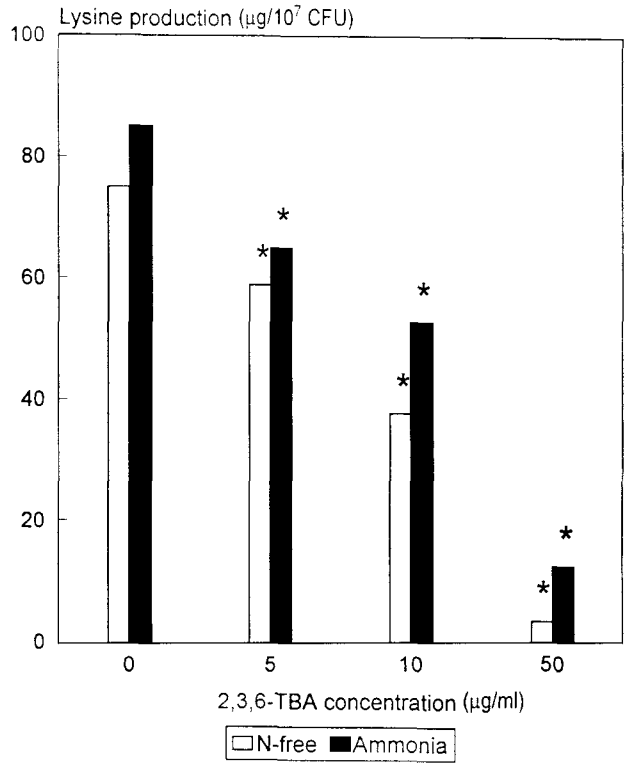

Fig. 7. Production (μg/10^7 CFU) of lysine by *Azotobacter chroococcum* strain H23 in the presence of various concentrations of 2,3,6-TBA (μg/ml). Values are means of five separate experiments. Bars marked with * are significant versus control ($P < 0.01$)

amended) when applied as 5, 10 and 50μg/ml (Fig. 7). The presence of 5, 10 and 50μg/ml of 2,3,6-TBA in N-free medium caused 22, 48 and 95% inhibition respectively (after 7 d incubation); the presence of 5, 10 and 50μg/ml in NH_4^+-amended medium caused 23, 37 and 85% inhibition respectively.

Data presented in this paper show that quantitative production of lysine by *A. chroococcum* strain H23 is clearly affected by alachlor, 2,4-D, 2,4,5-T and 2,3,6-TBA, although the observed responses on production is fairly influenced by the concentration and agrochemical in particular. The presence of combined N in the medium no significant affect lysine production by *A. chroococcum* strain H23. However Martinez-Toledo et al. (1996) reported that different C and N concentration affect pantothenic acid and thiamine production by *Azotobacter vinelandii.*

The toxic effect of alachlor and 2,3,6-TBA to *A. chroococcum* could be associated the inhibition of biological activity in the presence of this herbicides. Numerous studies have indicated both in pure culture and mixed population the negatives effects of herbicides on the biological activity of *Azotobacter* (Gadkavi, 1987; Mackenzie and MacRae, 1972; Tam and Trevors, 1981). In this sense, *Azotobacter spp.* are often used for testing the effect of environmental toxicants (Chung et al., 1997).

Lysine is the amino acid that *Azotobacter* and other diazotrophs microorganisms produces in larger amounts (Gonzalez-Lopez et al., 1995). In this context has been suggested that these organisms could be useful in developing new methods for the industrial production of lysine. Our results clearly show that production of this amino acids by *A. chroococcum* can be significantly increased by the addition of small amount of 2,4-D and 2,4,5-T to the culture media. Further study is needed to understand the mechanism of such effect and their industrial application.

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