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Production of amino acids by *Azotobacter vinelandii* and *Azotobacter chroococcum* with phenolic compounds as sole carbon source under diazotrophic and adiazotrophic conditions

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Summary. *Azotobacter vinelandii* strain ATCC 12837 and *Azotobacter chroococcum* strain H23 (CECT4435) were tested to grow in N-free or NH_4Cl amended chemically defined media, with protocatechuic acid or sodium p-hydroxybenzoate as sole carbon (C) sources at a concentration of 2 mmol/L. Both substrates supported grow at similar rates than bacteria grown in control media amended with 2 mmol/L sodium succinate as C source. The two strains produced aspartic acid, serine, glutamic acid, glycine, hystidine, threonine, arginine, alanine, proline, cysteine, tyrosine, valine, methionine, lysine, isoleucine, leucine and phenylalanine after 72 h of growth in chemically defined media with 2 mmol/L of phenolic compounds or sodium succinate as sole C source amended or unamended with 0.1% (w/v) NH_4Cl. Qualitative and quantitative production of all amino acids was not affected by the use of different C and N substrates.

Keywords: Amino acids – *Azotobacter* – Phenolic compounds – Soil microbiota

Introduction

Microorganisms of the genus *Azotobacter* are common inhabitants of soils of neutral pH in cold and temperate regions, where they constitute a major fraction of the free-living N_2-fixing population that keeps nitrogen cycling in the biosphere (Tchan and New, 1984). *Azotobacter* is able to fix at least 10 mgN per gram of carbohydrate (Becking, 1992). It has been shown that the soil characteristics and climate conditions affect the distribution of this microorganisms (González-López, 1992); it includes organic matter content and C/N relation. In addition, strains of this genus are often able to colonize the rhizosphere and root surfaces of many agrochemical important plants and establish, under natural conditions, non-endosymbiotic root association that lead to plant growth promotion (Revillas et al., 2000).

Agronomical exploitation of *Azotobacter*-plant interactions has been successfully tested under laboratory and field conditions (Becking, 1992), and the positive effect of inoculation of plants with these bacteria has been attributed to the cession of fixed N to the plant, enhancement of soluble P availability, antibiosis to pathogenic microorganisms and production of soluble substances such as hormones, vitamins and amino acids (Okon and Itzigsohn, 1995; González-López et al., 1999). The influence on *Azotobacter* root colonization of biologically-active substances produced and released by these microorganisms, such as vitamins (Revillas et al., 2000), on success of the establishment of root surface colonization has been proposed by not clarified.

One of the main factors that influences the biological activities of *Azotobacter* spp in soil and rhizosphere is the availability of carbon compounds. In this way, sugars and organic acids supplied in plant root exudates are readily incorporated by these microorganisms (Tchan and New, 1984). However, survival and persistence of *Azotobacter* in natural habitat is believed to be highly dependent on their ability to metabolize simple phenolic compounds, which are commonly present in soils at concentrations of 1–2 mmol/L (Wu et al., 1987). Phenolic acids in the soil come mainly from plant litter decomposition or soil contamination by agrochemical and are toxic for most microorganisms, but a few genera of bacteria and fungi are able to degrade and use them as carbon sources. In this sense, growth and N_2-fixation supported by some phenolic

compounds, such as p-hydroxybenzoic acid or protocatechuic acid, at concentrations well over the range commonly found in natural soils, have been reported for *A. vinelandii* (Moreno et al., 1999).

Production of some amino acids (methionine, lysine, arginine, tryptophane and glumatic acid) has been reported by *Azotobacter* spp during growth in culture media amended with glucose as sole carbon source under diazotrophic and adiazotrophic conditions (González-López et al., 1995). As far as in known, there are no previous data on amino acids production by *Azotobacter* spp, or any other soil bacteria when phenolic compounds are the only carbon source available at the concentrations commonly found in soils in nature.

In this paper, the production of aspartic acid, serine, glutamic acid, glycine, histidine, threonine, arginine, alanine, proline, cysteine, tyrosine, valine, methionine, lysine, isoleucine, leucine and phenylalanine by *Azotobacter vinelandii* strain ATCC 12837 and *Azotobacter chroococcum* H23 (CECT 4435) growing on chemically-defined media amended with low concentration of two phenolic compounds as sole carbon source, under diazotrophic and adiazotrophic conditions, is reported.

Material and methods

Microorganisms

The strains used in this study were *Azotobacter vinelandii* strain ATCC 12837 and *Azotobacter chroococcum* H23 (Spanish Type Culture Collection, CECT 4435). Both strains are amino acids produced in media amended with glucose, and have shown growth-promoting ability on plants (Rodelas et al., 1999; Pozo et al., 2000).

Growth conditions

Azotobacter spp were maintained in Burk's N-free agar slants amended with 0.5% glucose as carbon source (Pozo et al., 2003). *Azotobacter* strains were transferred from fresh agar slants to Erlenmeyer flasks with 50 ml Burk's N-free or Burk's NH^+_4 liquid medium containing 0.3% NH_4Cl amended with sodium p-hydroxybenzoate (PB), protocatechuic acid (PA) or sodium succinate (S) at concentrations of 2.0 mmol/L. Succinate was selected as control substrate as it is readily incorporated into the tricarboxilic acid cycle (TCA), as well as being the main product of both PA and PB degradation by *Azotobacter* spp (Hardison et al., 1969). All chemicals used as carbon sources were purchased from Sigma. PA, PB and S was added to culture media, after autoclaving and cooling down, from a freshly-made, filter-sterilized, 10 fold concentrated stock solution. After 72 h incubation at 28°C in a shaker (Gallemkamp, INR-200, UK) with gentle agitation (110 rpm), total cell numbers were determined using a Petroff-Hausser counting chamber (C.A. Hausser and Son, Philadelphia, PA, USA).

Amino acids productions assay

Samples from Erlenmeyer flasks containing 50 ml Burk's N-free or Burk's NH_4^+ medium amended with 2 mmol/L of the corresponding carbon source were taken after 72 h of incubation at 28°C. Aliquots were centrifuged at 5000 g for 30 min in a Sorvall RC-5B centrifuge (Dupont Instruments, Wilmington, DE, USA) at 4°C, and the supernatant fluid were passed through 0.22 μm of Millipore filter membranes. Aliquots (100 μl) of filtrate supernatants were added to Eppendorf tubes containing 200 μl of acetic acid (2 mol/L). The amino acids fraction was isolated with pre-prepared Dowex column (AG 50WX8 in the H^+ form) according to Stoll et al. (1999), and the purified amino acids were used for HPLC analysis (Water ACQO.TAG) with 6-amino quinolyl-N-hydroxysuccinimidyl carbamate derivative. The solution was analysed for amino acids by injecting 5 μl into a HPLC 2690 (Water) with a water column of 150 mm. Standard curve for amino acids was calculated from 3 concentrations (20, 50 and 100 pmol/ml of amino acids) and sensitivity of the method was in the range of 2 pmol of amino acid per ml.

Statistical analysis

Data obtained from this study were analyzed by multifactorial analysis of variance (ANOVA) with all interactions, using the software package *Statgraphics* version 5.0 (STC Inc. Rockville, MD, USA). Least significant differences (LSD) were calculated at the 95% level of significance ($p < 0.05$).

Results and discussion

Total cell numbers of *A. chroococcum* H23 and *A. vinelandii* ATCC12837 after growing for 72 h on chemically-defined media amended with 2 mmol/L of PA, PB or S as sole carbon source is shown in Table 1. No significant differences in the growth of the two *Azotobacter* strains were found on media amended with all the substrates

Table 1. Total cell numbers per ml of *Azotobacter vinelandii* ATCC 12837 and *A. chroococcum* H23 after 72 h of incubation in Burk's N-free and Burk's NH^+_4 medium amended with 2 mmol/L of protocatechuic acid (PA), sodium p-hydroxybenzoate (PB) or succinate (S) as sole carbon source

Strain	Carbon source		Log CFU/ml
ATCC 12837	PA (N-free)*		6.56
	PA (NH^+_4)		7.10
	LDS ($p < 0.05$)	0.16	
	PB (N-free)		6.89
	PB (NH^+_4)		7.03
	LDS ($p < 0.05$)	0.15	
	S (N-free)		6.60
	S (NH^+_4)		6.21
	LDS ($p < 0.05$)	0.32	
H23	PA (N-free)		7.58
	PA (NH^+_4)		7.76
	LDS ($p < 0.05$)	0.24	
	PB (N-free)		6.23
	PB (NH^+_4)		7.00
	LDS ($p < 0.05$)	0.27	
	S (N-free)		6.99
	S (NH^+_4)		7.23
	LDS ($p < 0.05$)	0.18	

* All media were inoculated with an initial cell number around 10^5 cells/ml

tested. The positive effect of the addition of 0.3% NH_4Cl to the media on growth of *Azotobacter* strains was slight.

Our experiments show that *A. chroococcum* H23 and *A. vinelandii* ATCC12837 are able to degrade phenolic structures on chemically-defined media. Thus, is well know that *Azotobacter* spp can grow and biotransform a wide range of phenolic substances (Moreno et al., 1999) and complex wastes with a high phenolic compound content (Pozo et al., 2002). However, has been reported that higher concentrations (up to 5 mmol/L) of phenolic acids may be toxic for some *Azotobacter* strains (Revillas et al., 2000) or at least induce a poor utilization of these substances (Moreno et al., 1999). Obviously, our data show that the natural levels of these compounds present in soils (2 mmol/L) should not affect *Azotobacter* strains negatively, either with NH_4Cl (adiazotrophic condition) or atmospheric N_2 (diazotrophic condition) as the nitrogen source.

The production of aspartic acid, serine, glutamic acid, glycine, histidine, threonine, arginine, alanine, proline, cysteine, tyrosine, valine, methionine, lysine, isoleucine, leucine and phenylalanine by strains ATCC 12837 and H23, in both Burk's N-free and Burk's medium amended with 0.3% NH_4Cl, is shown in Tables 2 and 3. The amounts of each amino acid detected varied largely within strain; however the production of those substances was not significantly influenced by the nature of the available C and N sources. *A. vinelandii* ATCC 12837 released higher amounts (up to 30 pmol/ml) of serine, glycine, alanine and proline, whereas *A. chroococcum* H23 produced large amounts of serine, glycine, alanine, valine and leucine.

A. chrooccoccum H23 and *A. vinelandii* ATCC 12887 produced all the amino acids tested showed that those microorganisms are able to produce and liberate these biologically active substances under diazothrophic and adiazotrophic conditions. Succinate was used as control substrate as it is the main product of protocatechuic and p-hydroxybenzoic acids degradation by *Azotobacter* spp (Hardisson et al., 1969). Our results (Tables 2 and 3) show that the presence of succinate did not significantly affect amino acids production by these bacterial strains when compared with culture media amended with phenolic compounds as sole C source. Moreover, the liberation to the media of the aminoacids was independent of the phenolic compound (protocatechuic acid or p-hydroxybenzoate) available, suggested that at a concentration of 2 mmol/L (natural levels in soils) the response to the presence of different phenolic acids on amino acids production followed a similar pattern for each particular strain and amino acid.

The presence of NH_4Cl did not significantly affect amino acids production by any of the strains when the carbon source was protocatechuic acid, p-hydroxybenzoic acid or succinate. Thus, the nature of the available N source did not affected the quantitative production and

Table 2. Production (pmol/ml) of amino acids by *Azotobacter chroococcum* H23 in Burk's N-free medium and Burk's medium amended with 0.3% NH_4Cl containing 2 mmol/L of protocatechuic acid (PA), sodium p-hydroxybenzoate (PB) or succinate (S) as sole carbon source after 72 h of growth under aerobic conditions

Amino acid	PA		PB		S	
	N-free	NH^+_4Cl	N-free	NH^+_4Cl	N-free	NH^+_4Cl
Aspartic acid	4 ± 1[a]	4 ± 1	4 ± 1	5 ± 1	2 ± 1	2 ± 1
Serine	37 ± 12	46 ± 1	42 ± 7	54 ± 14	61 ± 19	45 ± 19
Glutamic acid	14 ± 6	9 ± 4	13 ± 4	13 ± 3	11 ± 4	8 ± 4
Glycine	100 ± 15	98 ± 16	76 ± 12	86 ± 16	78 ± 10	75 ± 13
Histidine	5 ± 1	6 ± 3	4 ± 2	8 ± 3	2 ± 1	9 ± 3
Threonine	16 ± 7	18 ± 5	16 ± 4	23 ± 5	23 ± 4	14 ± 4
Arginine	8 ± 3	5 ± 2	10 ± 3	20 ± 9	8 ± 3	8 ± 3
Alanine	40 ± 9	44 ± 12	37 ± 10	63 ± 13	27 ± 11	36 ± 11
Proline	12 ± 4	20 ± 4	16 ± 3	20 ± 3	23 ± 3	20 ± 2
Cysteine	11 ± 6	10 ± 7	17 ± 6	24 ± 7	15 ± 5	10 ± 6
Tyrosine	15 ± 3	15 ± 4	19 ± 2	14 ± 2	18 ± 2	19 ± 2
Valine	36 ± 8	41 ± 9	39 ± 4	51 ± 12	32 ± 6	40 ± 15
Methionine	16 ± 6	16 ± 4	12 ± 4	13 ± 4	7 ± 3	8 ± 2
Lysine	14 ± 2	18 ± 7	20 ± 5	23 ± 8	19 ± 3	16 ± 5
Isoleucine	9 ± 4	13 ± 5	10 ± 2	24 ± 9	7 ± 2	8 ± 2
Leucine	31 ± 10	37 ± 8	34 ± 7	43 ± 11	38 ± 4	48 ± 7
Phenylalanine	5 ± 1	5 ± 1	4 ± 1	8 ± 1	3 ± 1	7 ± 1

[a] Values are mean of three replicates (±standard error)

Table 3. Production (pmol/ml) of amino acids by *Azotobacter vinelandii* ATCC 12837 in Burk's N-free medium and Burk's medium amended with 0.3% NH_4Cl containing 2 mmol/L of protocatechuic acid (PA), sodium p-hydroxybenzoate (PB) or succinate (S) as sole carbon source after 72 h of growth under aerobic conditions

Amino acid	PA		PB		S	
	N-free	NH^+_4Cl	N-free	NH^+_4Cl	N-free	NH^+_4Cl
Aspartic acid	2 ± 1[a]	5 ± 1	3 ± 1	5 ± 1	5 ± 1	3 ± 1
Serine	44 ± 5	67 ± 13	28 ± 10	61 ± 16	51 ± 13	47 ± 14
Glutamic acid	2 ± 1	8 ± 3	5 ± 2	10 ± 4	12 ± 3	9 ± 3
Glycine	60 ± 6	64 ± 8	77 ± 5	57 ± 10	69 ± 8	67 ± 12
Histidine	4 ± 2	4 ± 2	4 ± 1	2 ± 1	9 ± 3	5 ± 3
Threonine	12 ± 4	20 ± 9	17 ± 2	27 ± 7	14 ± 7	16 ± 7
Arginine	19 ± 3	13 ± 3	16 ± 2	13 ± 3	17 ± 3	19 ± 4
Alanine	33 ± 10	44 ± 4	42 ± 10	51 ± 16	32 ± 11	36 ± 11
Proline	37 ± 10	46 ± 8	33 ± 14	31 ± 8	20 ± 5	23 ± 5
Cysteine	18 ± 3	13 ± 5	16 ± 5	18 ± 6	13 ± 2	14 ± 6
Tyrosine	20 ± 7	26 ± 7	13 ± 6	19 ± 4	19 ± 7	13 ± 6
Valine	13 ± 3	16 ± 6	21 ± 6	22 ± 4	22 ± 4	16 ± 7
Methionine	10 ± 3	15 ± 6	14 ± 6	11 ± 4	7 ± 3	6 ± 3
Lysine	18 ± 6	25 ± 8	15 ± 4	16 ± 6	21 ± 4	24 ± 7
Isoleucine	10 ± 4	13 ± 5	10 ± 3	14 ± 3	7 ± 2	8 ± 2
Leucine	22 ± 4	24 ± 6	28 ± 9	25 ± 7	19 ± 3	26 ± 7
Phenylalanine	6 ± 2	6 ± 1	9 ± 3	10 ± 3	6 ± 2	4 ± 2

[a] Values are mean of three replicates (±standard error)

liberation of amino acids in chemically-defined media amended with the phenolic compounds.

González-López et al. (1995) reported that the availability of NH_4Cl affected the production of some amino acids by *Azotobacter* spp when the microorganisms were grown on glucose as sole carbon source suggested that the presence of combined N on amino acids production by *Azotobacter* spp seems to be related to the special pathways for the production of such compounds. However the results of our study show that the production of amino acids by *Azotobacter* spp grown in media amended with 2 mmol/L of organic compounds such as phenolic acids and succinate was not affected by the nature of the available N source. In this sense, aromatic compounds often support high rates of nitrogenase activity (D'Mello et al., 1997) in several free-living N-fixing bacteria including *Azotobacter.*

Azotobacter spp obtain the energy for N_2 fixation from soil and plant root exudates. The chemical composition and processes in the rizhosphere have recently gained much attention from student of soil-plant interactions but the main interest has focused on aspects other than amino acids (Broughton et al., 2003). However the exogenous application of biologically-active substances such as vitamins and amino acids, can have a direct effect on plant cells functions as well as indirect effects on plant growth (Oertli, 1987; Streit et al., 1996). Consequently, the liberation of amino acids by *Azotobacter* spp could be one of the mechanisms that explain the positive effects of these plant growth promoting bacteria on plants and also their positive interactions with other microorganisms in the soil and rizhosphere.

In conclusion, the results of this study show that large amounts of amino acids are produced by two strains of *Azotobacter* with phenolic compounds as sole carbon sources supplied at the concentrations commonly found in the soil and rizhosphere. However, the amino acids production under these conditions followed pattern different from those reported when *Azotobacter* was grown in media amended with sugars both under adiazotrophic or N_2-fixing conditions.

References

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

Broughton WJ, Zhang F, Perret X, Staehelin C (2003) Signals exchanges between legumes and *Rhizobium*: agricultural uses and perspectives. Plant Soil 252: 129–137

D'Mello R, Purchase D, Poole R, Hill S (1997) Expression and content of terminal oxidases in *Azotobacter vinelandii* grown with excess NH_4^+ and modulated by oxygen supply. Microbiology 143: 231–237

González-López J (1992) Microorganisms diazotrofos asociados a raíces de plantas no leguminosas. González-López, Lluch C (eds) Interacción

planta-microorganismo: Biología del nitrógeno. Editorial Rueda, Madrid, pp 71–96

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

González-López J, Martínez-Toledo MV, Rodelas B, Salmeron V (1999) Effect of some herbicides on the production of lisine by *Azotobacter chroococcum*. Amino Acids 17: 165–173

Hardisson C, Sala-Trepat JM, Stainer RV (1969) Pathways for the oxidation of aromatic compounds by *Azotobacter.* Gen Microbiol 59: 1–11

Moreno J, Vargas-García C, López MJ, Sánchez-Serrano G (1999) Growth and exopolysaccharide production by *Azotobacter vinelandii* on soil phenolic compounds. J Appl Microbiol 86: 439–445

Oertli JJ (1987) Exogenous application and vitamins as regulators for growth and development of plants – a review. Z Pflanz Bodenkunde 150: 375–391

Okon Y, Itzigsohn R (1995) The development of *Azospirillum* as a commercial inoculate for improving crop yields. Biotechnol Adv 13: 415–424

Pozo C, Martínez-Toledo MV, Salmeron V, Rodelas B, González-López J (2000) Effects of benzidine and benzidine analogous on the growth and nitrogenase activity of *Azotobacter.* Appl Soil Ecol 14: 183–190

Pozo C, Martínez-Toledo MV, Rodelas B, González-López J (2002) Effects of culture conditions on the production of polyhydroxyalkanoates by *Azotobacter chroococcum* H23 in media containing a high concentration of alpechín (wastewater from olive oil mills) as primary carbon sources. J Biotechnol 97: 125–131

Pozo C, Martínez-Toledo MV, Rodelas B, González-López J (2003) Response of soil microbiota to the addition of 3-3′diaminobenzidine. Appl Soil Ecol 23: 119–126

Revillas JJ, Rodelas B, Pozo C, Martínez-Toledo MV, González-López J (2000) Production of B-group vitamins by two *Azotobacter* strains with phenolic compounds as sole carbon source under diazotrophic and adiazotrophic conditions. J Appl Microbiol 89: 486–493

Rodelas B, González-López J, Pozo C, Salmeron V, Martínez-Toledo MV (1999) Response of faba bean (*Vicia faba* L.) to combined inoculation with *Azotobacter* and *Rhizobium leguminosarum* bv. *viceae*. Appl Soil Ecol 12: 51–59

Stoll B, Burrin DG, Henry JF, Jahoor F, Reeds PJ (1999) Dietary and systemic phenylalanine utilization for mucosal and hepatic constitutive protein synthesis in pigs. Am J Physiol 276: 49–57

Streit WR, Joseph CM, Philips D (1996) Biotin and other water-soluble vitamins as key growth factors for alfalfa root colonization. Mol Plant Microb Inter 9: 330–338

Tchan YT, New PB (1984) Genus I *Azotobacter* beijerinck 1901. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 1. Williams & Wilkins, Baltimore, pp 220–229

Wu FJ, Moreno J, Vela GR (1987) Growth of *Azotobacter vinelandii* on soil nutrients. Appl Environ Microbiol 53: 489–494

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