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Liberation of amino acids by heterotrophic nitrogen fixing bacteria

Minireview Article

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Summary. Large amounts of amino acids are produced by nitrogen-fixing bacteria such as *Azotobacter, Azospirillum, Rhizobium, Mesorhizobium* and *Sinorhizobium* when growing in culture media amended with different carbon and nitrogen sources. This kind of bacteria live in close association with plant roots enhanced plant growth mainly as a result of their ability to fix nitrogen, improving shoot and root development suppression of pathogenic bacteria and fungi, and increase of available P concentration. Also, it has been strongly evidenced that production of biologically substances such as amino acids by these rhizobacteria are involved in many of the processes that explain plant-grown promotion. This paper reviews literature concerning amino acids production by nitrogen-fixing bacteria. The role of amino acids in microbial interactions in the rhizosphere and establishment of plant bacterial association is also discussed.

Keywords: Amino acids – *Azotobacter* – *Azospirillum* – *Rhizobium* – *Mesorhizobium* – *Sinorhizobium*

Introduction

N_2 fixing bacteria such as *Azotobacter, Azospirillum, Rhizobium, Mesorhizobium*, and *Sinorhizobium* are well known for their ability to improve plant development and yielding when applied as inoculants for agriculture. Plant growth-promoting of these microorganisms have been reported in cereals, legumes and some others agronomical crops (Okon and Itzigsohn, 1995; Peix et al., 2001). It was first assumed that inoculation with nitrogen-fixing bacteria enhanced plant growth mainly as a result of their ability to fix N_2. Nevertheless in many cases the amount of fixed N made available for the plant would only cover a fraction of the N_2 demand; therefore, growth promotion may be attributed to other mechanisms. In fact nitrogen fixing organisms such as *Azospirillum*, directly benefit plants improving shoot and root development, increasing the rate of water and mineral uptake by roots (Baldani et al., 1997).

Azotobacter is an obligate aerobic, although it can grow under low O_2 concentration. The ecological distribution of this bacterium is a complicated subject and is related with diverse factors which determine the presence or absence of this organism in a specific soil (González-López, 1992). *Azospirillum* species belong to the facultative endophytic diazotrophs group which colonize the surface and the interior of roots, being this kind of association considered as the starting point of most ongoing *BNF* (*B*iological *N*itrogen *F*ixation) programs with non-legume plants world wide (Baldani et al., 1997).

Rhizobia are known for their ability to establish symbiotic interactions with leguminous plants by the formation and colonization of root nodules, where bacteria fix N_2 to ammonia and make it available for the plant (Peix et al., 2001). The bacteria are mostly rhizospheric microorganisms, despite its ability to live in the soil for long period of time.

Nitrogen fixing bacteria are useful organisms commercially because of their agricultural applications as inoculants (Rodelas et al., 1997; Peix et al., 2001). However, the details of the plant bacterial interactions with regards to successful bacterial root colonization is poorly known (Rodelas et al., 2002). In this way, the influence on nitrogen fixing bacterial root colonization of biologically-active substances present in plant root exudates or the liberation of substances produced by rhizobacteria, such as phytohormones (Hirsch, 1992), on success of the root colonization has been proposed by not clarified.

Many genera of soil and rhizosphere bacteria have been investigated with regard to production of biologically active substances. It has been strongly evidenced that production of phytohormones, water-soluble vitamins and amino acids by nitrogen-fixing bacteria is involved in many of the processes that explain plant growth-promotion by these bacteria. Production of amino acids by soil and rhizosphere microorganisms such as *Azotobacter, Azospirillum, Rhizobium, Mesorhizobium*, and *Sinorhizobium* has received some attention (González-López et al., 1999; Salmeron-López et al., 2004). However, practically nothing is known about the influence of growth media on the production and liberation of amino acids by nitrogen-fixing microorganisms. We will discuss the influence of growth media and their nutrient content on the liberation of amino acids by these microorganisms.

Table 1. Production (pmol/ml) of amino acids by *Rhizobium, Mesorhizobium* and *Sinorhizobium* (data from Salmerón-López et al., 2004)

Amino acid	*R. leguminasarum bv viceae*	*S. meliloti*	*M. loti*
Aspartic acid	76	36	0
Serine	258	351	57
Glutamic acid	26	32	0
Glycine	204	196	30
Histidine	66	30	0
Threonine	48	87	17
Arginine	23	24	0
Alanine	176	177	66
Proline	56	30	6
Tyrosine	39	27	16
Valine	58	51	1
Methionine	29	25	0
Lysine	30	26	0
Isoleucine	28	22	20
Leucine	30	36	2
Phenylalanine	23	24	0

0, Not detected

Liberation of amino acids by *Rhizobia*

Although some strains classified as *Rhizobium* require a large number of growth factors, including amino acids and vitamins (Jordan, 1984), most of the rhizobic strains generally do not require growth factors and are able to grow in simple mineral chemically-defined media, amended with hydrocarbon substrate (Salmerón-López et al., 2004). Production of amino acids by *Rhizobium, Mesorhizobium*, and *Sinorhizobium* has been studied in chemically-defined media, specially formulated to reproduce appropriately the natural environment of these bacteria in soil or rhizosphere (Table 1).

Rhizobium and *Sinorhizobium* produce aspartic acid, serine, glutamic acid, glycine, histidine, threonine, arginine, alanine, proline, cysteine, tyrosine, valine, methionine, lysine, isoleucine, leucine and phenylalanine when are cultured in defined media lacking growth factor using mannitol as sole carbon source. Some amino acids, such as aspartic acid or glutamic acid are detected at early stages of growth, whereas other amino acids, such as methionine are only released during the stationary phase of growth. Consequently, the data show that the liberation of amino acids by *Rhizobium* and *Sinorhizobium* is influenced by the age of the culture when these organisms are grown in chemically-defined media lacking growth factor using manitol as sole carbon source (Table 2). Thus, *Rhizobium* and *Sinorhizobium* only produce significant amounts of aspartic acid, serine, glutamic acid, glycine, histidine, threonine, arginine, alanine, proline, cysteine, tyrosine, valine, methionine, lysine, isoleucine, leucine and phenylalanine during the stationary phase of growth in mineral medium.

Mesorizhobium produced lower amounts of amino acids compared to *Rhizobium* and *Sinorhizobium* (Table 1). Thus culture supernatants of *Mesorhizobium*

Table 2. Production (ρmol/ml) of amino acids by *Rhizobium*, and *Sinorhizobium* growth in chemically-defined media lacking growth factor for 1, 2 and 3 days (data from Salmerón-López et al., 2004)

Amino acid	*R. leguminasarum bv viceae*			*S. meliloti*		
	1d	2d	3d	1d	2d	3d
Aspartic acid	0	42	80	0	0	62
Serine	0	276	240	0	0	383
Glutamic acid	0	30	30	0	48	50
Glycine	0	228	192	0	0	282
Histidine	0	36	71	0	0	49
Threonine	0	58	57	0	0	37
Arginine	0	14	21	0	0	19
Alanine	0	210	174	0	0	149
Proline	0	47	63	0	0	49
Tyrosine	0	32	43	0	2	18
Valine	0	52	55	0	7	62
Methionine	0	27	35	0	0	40
Lysine	0	21	24	0	0	28
Isoleucine	0	26	28	0	5	44
Leucine	0	34	34	0	0	43
Phenylalanine	0	16	24	0	0	31

0, Not detected

contained only 9 amino acids during the stationary phase of growth. Therefore the concentration of serine, glycine and alanine in the *Mesorhizobium* culture supernatants are significantly lower than those detected for *Rhizobium* and *Sinorhizobium*.

Liberation of amino acids by free-living N_2 fixing organisms

Free-living N_2-fixing bacteria of the genera *Azotobacter* and *Azospirillum* are well known for their ability to produce significant amounts of amino acids (González-López et al., 1995). Thus, it has been reported the liberation of amino acids such as methionine, lysine and arginine by these microorganisms during growth in culture media amended with different carbon sources under adiazotrophic and diazotrophic conditions. However, the production of amino acids by *Azotobacter* and *Azospirillum* is affected by the concentration and type of carbon source added to the culture media (Table 3). Thus, the liberation of those substances by *Azospirillum* is drastically modified when malate or gluconate are present in the culture media compared with culture media amended with fructose.

Azotobacter produce large amounts of amino acids using phenolic compounds as sole carbon and energy source at the concentrations commonly found in the soil or rhizosphere (Table 4). However and under these culture conditions, the production of these substances was not significantly influenced by the nature of the available C and N sources. Moreover, the liberation to the media of the amino acids was independent of the phenolic compound available both under diazotrophic or adiazotrophic conditions.

Discussion

The biosynthesis of phytohormones, vitamins and amino acids by nitrogen-fixing bacteria such as *Azotobacter, Azospirillum* and rhizobia is widely documental, as well as the involvement of these substances on plant responses observed after inoculation of plant with bacteria. Amino acids in the rhizosphere are both of plant and microbial origin. The role of amino acids produced by rhizobacteria in their interaction with plants in practically unknown, although it was established that some amino acids such as methionine and tryptophan act in soil as main

Table 3. Production of amino acids by *Azotobacter* and *Azospirillum* (data from González-López et al., 1995) in chemically defined media under diazotrophic and adiazotrophic conditions

Specie	Amino acid	Production (μg/ml)	Conditions of growth
AC	Glutamic acid	0.40	7 days, 0.5% glucose, N-free
		4.00	7 days, 0.5% glucose, 0.3% NH_4Cl
	Lysine	500	2 days, 0.5% glucose, N-free
		500	2 days, 0.5% glucose, 0.3% NH_4Cl
	Arginine	0.02	7 days, 0.5% glucose, N-free
		0.00	7 days, 0.5% glucose, 0.3% NH_4Cl
	Tryptophane	0.00	7 days, 0.5% glucose, N-free
		0.09	7 days, 0.5% glucose, 0.3% NH_4Cl
	Methionine	0.00	7 days, 0.5% glucose, N-free
		0.09	7 days, 0.5% glucose, 0.3% NH_4Cl
AB	Glutamic acid	0.10	3 days, 0.5% malate, N-free
		7.00	3 days, 0.5% gluconate, N-free
		14.00	3 days, 0.5% fructose, N-free
	Lysine	5.00	1 day, 0.5% malate, N-free
		20.00	1 day, 0.5% gluconate, N-free
		400	1 day, 0.5% fructose, N-free
	Arginine	12.00	3 days, 0.5% malate, N-free
		17.00	3 days, 0.5% gluconate, N-free
		25.00	3 days, 0.5% fructose, N-free
	Methionine	1.40	1 day, 0.5% malate, N-free
		2.50	1 day, 0.5% gluconate, N-free
		40.00	1 day, 0.5% fructose, N-free

AC, *Azotobacter chroococcum*
AB, *Azospirillum brasilense*

Table 4. Production (ρmol/ml) of amino acids by *Azotobacter* in chemically-defined media amended with 2 mmol/l of different phenolic acids under diazotrophic and adiazotrophic conditions for 72 h (data from Revillas et al., 2005)

	PA				PB				S			
	N-free		0.3% NH_4Cl		N-free		0.3% NH_4Cl		N-free		0.3% NH_4Cl	
	AC	AV	AC	AV	AC	AV	AC	AV	AC	AV	AC	AV
Aspartic acid	4	2	4	5	4	3	5	5	2	5	2	3
Serine	37	44	46	67	42	28	54	61	61	51	45	47
Glutamic acid	14	2	9	8	13	5	13	10	11	12	8	9
Glycine	100	60	98	64	76	77	86	57	78	69	75	67
Histidine	5	4	6	4	4	4	8	2	2	9	9	5
Threonine	16	12	18	20	16	17	23	27	23	14	14	16
Arginine	8	19	5	13	10	16	20	13	8	17	8	19
Alanine	40	33	44	44	37	42	63	51	27	32	36	36
Proline	12	37	20	46	16	33	20	31	23	20	20	23
Cysteine	11	18	10	13	17	16	24	18	15	13	10	14
Tyrosine	15	20	15	26	19	13	14	19	18	19	19	13
Valine	36	13	41	16	39	21	51	22	32	22	40	16
Methionine	16	10	16	15	12	14	13	11	7	7	8	6
Lysine	14	18	18	25	20	15	23	16	19	21	16	24
Isoleucine	9	10	13	13	10	10	24	14	7	7	8	8
Leucine	31	22	37	24	34	28	43	25	38	19	48	26
Phenylalanine	5	6	5	6	4	9	8	10	3	6	7	4

PA, Protocatechuic acid; *PB*, P-hydroxybenzoate; *S*, Succinate; *AC*, *Azotobacter chroococcum*; *AV*, *Azotobacter vinelandii*

precursors for the synthesis of plant hormones ethylene and indole-3-acetic acid, respectively (Murcia et al., 1997). Plants also show significant response to the exogenous application of L-methionine to soils (Arshad et al., 1993). In this context, the production of large amounts of amino acids by rhizobacteria such as *Azotobacter, Azospirillum, Rhizobium, Mesorhizobium*, and *Sinorhizobium* could be of importance on plant-grown promotion and consequently useful in agricultural technologies.

Production of amino acids by nitrogen-fixing bacteria has been studied in chemically-defined media and also when these bacteria grow in more complex media, specially formulated to reproduce appropriately the natural environment of these bacteria in soil or rhizosphere. Thus, concentration of carbon source is a determining parameter for qualitative and quantitative production of amino acids by these microorganisms. Concentration of available carbon is also the factor that most dramatically affects the growth and biological activities of these bacteria in nature (Moreno et al., 1990). The persistence of some nitrogen-fixing organisms in natural environments have been found strongly related to the ability for using aromatic compounds as carbon substrates (Juárez et al., 2005). Several strains of *Azotobacter* can support growth and N_2-fixation in chemically-defined media amended with phenolic acids such as p-hydroxybenzoate and protocatechuic acid, even when only limited amounts of these substances, similar to those commonly found in soil and rhizosphere (1–2 mM), are present as sole carbon sources. We have also found evidence of the production of amino acids by *Azotobacter* spp in mineral media amended with low concentrations of some phenolic compounds as sole carbon source (Revillas et al., 2005).

In conclusion, the data suggest that the production of amino acids by nitrogen-fixing bacteria in nature is in fact influenced by many parameters, which are difficult to reproduce under laboratory conditions. However the results reviewed here show that the production and liberation of amino acids is a common feature among strains of diazotrophic rhizobacteria of the genera *Azotobacter, Azospirillum, Rhizobium, Mesorhizobium*, and *Sinorhizobium*, although the qualitative and quantitative production of these substances is clearly affected by the availability of different substrates used as carbon and nitrogen sources, C and N concentrations and ratios, temperature and pH, and can be also influenced by more complex factors such as composition of plant root exudates or interactions with xenobiotics present in soils. Since these growth regulators can potentially affect the development of plant as well as growth of both beneficial and pathogenic microorganisms, the study of the role of amino acids in plant-bacterial interactions may deserve more attention in the future.

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