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Beijerinckia derxii releases plant growth regulators and amino acids in synthetic media independent of nitrogenase activity

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ABSTRACT

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Aims: This study aims at evaluating the ability of *Beijerinckia derxii*, a free-living nitrogen (N)-fixing bacterium frequently isolated from tropical soils, to release certain plant growth regulators [indoleacetic acid (IAA), ethylene, polyamines] and amino acids into the growth medium.

Methods and Results: The production of those substances was compared using both cultures in which nitrogenase was active (N-free medium) and cultures in which nitrogenase was repressed (combined-N cultures). Those cultures were grown under agitation and in absence of agitation. Total IAA production was higher in agitated, N-free cultures but specific production was greater in combined-N cultures under agitation. Putrescine and spermidine were detected under all conditions tested. Ethylene was produced in both N-free and combined-N cultures. A greatest diversity of amino acids was released in N-free cultures.

Conclusions: There was no inhibition of the production of the analysed substances under conditions where nitrogenase was inactive.

Significance and Impact of the Study: *Beijerinckia derxii* is potentially a producer of plant-active substances; its presence in the natural environment suggests that this bacterium may contribute to the development of other living organisms.

Keywords: amino acids, *Beijerinckia derxii*, ethylene, indoleacetic acid, nitrogenase activity, polyamines.

INTRODUCTION

Dinitrogen-fixing bacteria are potential suppliers of nitrogen (N) for the environment. However, while several diazotrophic bacteria are able to synthesize plant growth regulators (PGR), the stimulation of plant growth observed when seeds and plants are inoculated with these bacteria may be either due to biological N-fixation or biologically active substances produced by the bacteria. Several micro-organisms, including soil, epiphytic and tissue-colonizing bacteria, have been found to synthesize 3-indoleacetic acid (IAA), an auxin identical to that found in plants (Patten and Glick 1996). This compound is involved in several growth and development processes in plants (Taiz and Zeiger 1998).

Polyamines (PA) such as putrescine, spermidine and spermine are known to be ubiquitous in plant, animal and microbial cells (Davies 1995). These substances can modulate the functions of nucleic acids and proteins, besides influencing membrane stability, affecting cell proliferation, and differentiation in higher organisms (Igarashi and Kashiwagi 2000; Walters 2000). Considerable interest has been shown in the regulation and in the biosynthetic steps of spermidine and spermine, as the intermediate S-adenosylmethionine (SAM) is also a source of ethylene. This plant hormone is well known as a senescence inducer and PAs have anti-senescence activity (Galston and Kaur-Sawhney 1995). The release of PA by N-fixing bacteria has not been well studied.

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Ethylene mediates many plant physiological processes, from germination of seeds to senescence of organs, and many responses to environmental stress. Ethylene production rates are regulated by other plant hormones and by ethylene itself, through different biochemical mechanisms (McKeon *et al.* 1995), including auto-inhibition. Exogenous ethylene is also effective in stimulating plant development (Taiz and Zeiger 1998). Ethylene and IAA may act together to stimulate the development of the cortical cell layer (Grichko and Glick 2001).

Some free-living, N-fixing, organisms are able to excrete by-products of N fixation such as amino acids, as detected by Pati *et al.* (1994) in *Azotobacter chroococcum*, *Beijerinckia indica* and *Corynebacterium* cultures, and by González-Lopez *et al.* (1983) in *A. vinelandii*, grown in chemically-defined media and dialysed soil media.

The genus *Beijerinckia* is frequently found in tropical soils. Analysed tropical soil (48%) samples were positive for this genus (Becking 1961a). A study of the occurrence of free-living, N-fixing bacteria in Brazilian soils revealed the presence of *Beijerinckia* in 92 of 158 soil samples, all with a pH above 6·0. *Azotobacter* was found in only 15 of the 158 soil samples examined (Döbereiner 1959). Lateritic soils, the preferential habitat of this genus, are very poor in essential nutrients such as calcium, phosphate and molybdenum, and rich in toxic ions for plants, such as iron, aluminium, titanium, etc. A correlation between the mineral requirements of *Beijerinckia* and the mineral composition of lateritic soils has been found to exist (Becking 1961b), indicating an adaptation of this micro-organism to these limiting conditions.

When *Beijerinckia* was inoculated into the rhizosphere of the *Paspalum* grass, the plants had a higher N-content than the noninoculated controls, suggesting that the micro-organism may contribute to the development of this grass (Ruschel and Britto 1966). *Beijerinckia indica* (JN_1) isolated from phyllosphere (Pati and Chandra 1981) and *Beijerinckia* sp. isolated from sources other than leaf surface (Nandi and Sen 1981), when sprayed on wheat and rice plants, as a substitute for nitrogenous fertilizers, resulted in a marked improvement in yield and growth of the plants. Later, Pati *et al.* (1994) showed that the JN_1 strain was able to release amino acids into N-free broth. *Beijerinckia derxii* was shown to positively influence the growth of nondiazotrophic bacteria and to sustain their viability when grown in co-culture with them, in N-free media (Barbosa *et al.* 2000). Despite the above-described positive characteristics of this genus, very little is known about its biology and its contribution to the structure and function of its habitat.

The aim of this paper is to establish a qualitative and quantitative estimation of PGR (IAA, PAs and ethylene) and amino acids released by *B. derxii*. The production of those substances was compared using agitated and nonagitated cultures grown with N_2 (active nitrogenase) or NH_4^+ (inactive nitrogenase), as N sources. The excretion of IAA was determined during the bacterial life cycle in order to calculate the synthesis potential of single cells, as indicated by specific production, under the different experimental conditions.

MATERIALS AND METHODS

Bacterial strain

Beijerinckia derxii ICB-10 was isolated by our group from the soil, in an area covered by the so-called 'Cerrado', a type of Brazilian savannah, in the city of Pirassununga, state of São Paulo, Brazil. The bacterium was identified and catalogued as ATCC 33962. This type of soil is characterized by high acidity and low levels of N and organic material.

Culture media

LG medium (Lipman 1904) composition (mM): K_2HPO_4 (0·860); KH_2PO_4 (2·200); $CaCl_2{\cdot}2H_2O$ (0·140); $MgSO_4{\cdot}7H_2O$ (0·810); $Na_2MoO_4{\cdot}2H_2O$ (0·008); $CoCl_2{\cdot}2H_2O$ (0·006); $FeCl_3{\cdot}6H_2O$ (0·060); glucose (58·800); pH 5·7.

Modifications of LG medium are shown in Table 1. Tryptophan (Zimmer and Bothe 1988) and methionine (Arshad and Frankenberger 1992) were used as necessary precursors to the biosynthesis of IAA and ethylene, respectively. Ammonium sulphate was employed both as a combined N source and as a nitrogenase inhibitor.

Culture conditions

Beijerinckia derxii inoculum was grown in 250 ml Erlenmeyer flasks containing 50 ml LG liquid medium, for 48 h, at 30°C and 200 rev min^{-1}. The cultures used to determine IAA, PAs and amino acids were prepared as follows: 40 ml of the inoculum were transferred to each of 1 l Erlenmeyer

Table 1 Media modifications according to the analysed substance

Substance	Culture Media	Agitated/Nonagitated culture	Modification
IAA	LG	Agitated and nonagitated	–
	LG_{AS}	Agitated and nonagitated	1
	LG_{Trp}	Agitated and nonagitated	2
	LG_{TrpAS}	Agitated and nonagitated	1, 2
Ethylene	LG_{Met}	Agitated	3
	LG_{MetAS}	Agitated	1, 3
Polyamines and amino acids	LG	Agitated and nonagitated	–
	LG_{AS}	Agitated and nonagitated	1

1, Addition of 1·32 g l^{-1} ammonium sulphate (AS).
2, Addition of 0·5 g l^{-1} tryptophan (Trp) – indoleacetic acid (IAA) precursor.
3, Addition of 0·4 g l^{-1} methionine (Met) – ethylene precursor.

flasks containing 360 ml of the specific medium, according to the substance to be analysed (Table 1), and incubated at 30°C, with or without agitation (200 rev min^{-1}). To test ethylene production, 1 ml of the inoculum was transferred to a 25 ml penicillin flask containing 7 ml of LG_{Met} or LG_{MetAS} (Table 1). The flasks were fitted with rubber plugs tightened with metal cowls and then incubated for 9 days at 30°C, at 150 rev min^{-1}.

Microbiological and biochemical determinations

Samples of the LG_{Trp} and LG_{TrpAS} cultures were used to determine growth curves by colony forming unit (CFU) counts (Barbosa *et al.* 1995), nitrogenase activity, by acetylene (C_2H_2) reduction assay (ARA) (Turner and Gibson 1980) and supernatant IAA.

Prior to analysis by high-performance liquid chromatography (HPLC), sample cultures (Table 1) were centrifuged (14 000 × *g*, for 30 min, at 4°C) and filtered through Millipore membranes (0·22 μm).

The methods used to analyse IAA, PA and amino acids were described respectively in Astarita *et al.* (2003a–c). The following PA were analysed: putrescine, spermidine, spermine, diaminopentane, cadaverine, hexylamine and diaminoheptane.

Ethylene assay

In order to measure ethylene production, 1 ml of air was withdrawn from the culture flask and analysed in a gas chromatograph (Shimadzu GC-14A, Shimadzu Corporation, Kyoto, Japan) with a PORAPAK-N 80/100 – INOX column operated isothermally at 70°C with N as the gas carrier and a flame ionization detector. Pure ethylene (White Martins) was used as a standard.

Studies of all analysed substances were performed in triplicate.

RESULTS

Specific nitrogenase activity of *B. derxii* grown under agitation was highest during the exponential phase, with the activity rapidly decreasing during the stationary phase (Fig. 1). In nonagitated cultures, the enzyme activity was lower, but was maintained at comparable levels throughout the exponential and the stationary phases. In both N-free and combined-N cultures, IAA was detected after the end of the exponential growth phase (Figs 2 and 3). As no IAA was detected during the exponential phase, cultures were initiated with an elevated CFU number in order to reach the IAA production phase more rapidly.

It can be observed in Fig. 2 that, when grown in LG_{TrpAS} medium, under agitation, the bacterial population showed a

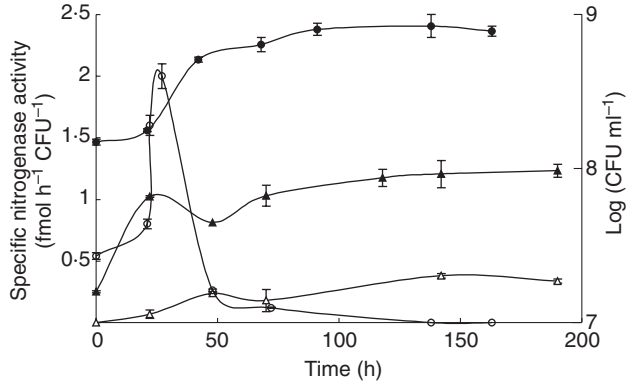

Fig. 1 Specific nitrogenase activity (△, nonagitated culture; ○ agitated culture) and growth curve (▲, nonagitated culture; ●, agitated culture) of *Beijerinckia derxii* grown in LG_{Trp}

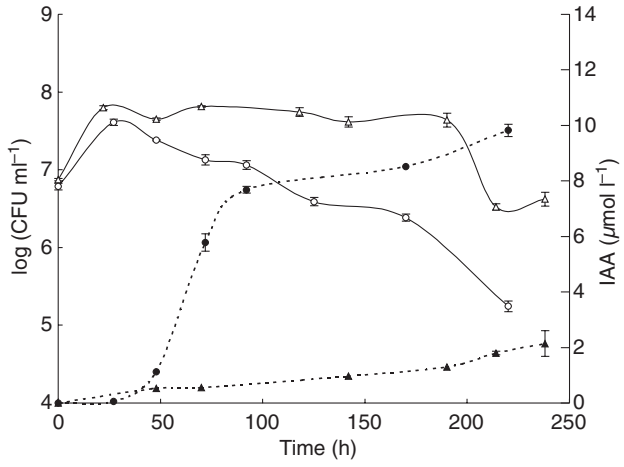

Fig. 2 Growth curves and indoleacetic acid (IAA) production by *Beijerinckia derxii* grown in LG_{TrpAS}, in agitated and nonagitated cultures (△, CFU, nonagitated culture; ○ CFU, agitated culture; ▲, IAA, nonagitated culture; ●, IAA, agitated culture)

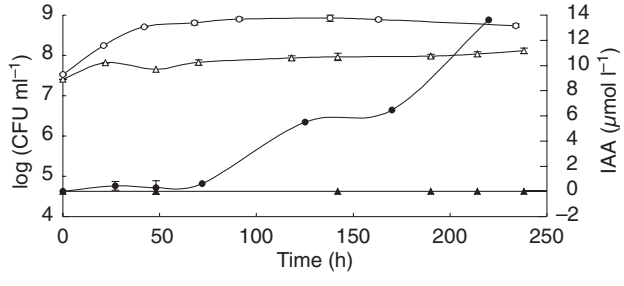

Fig. 3 Growth curves and indoleacetic acid (IAA) production of *Beijerinckia derxii* grown in LG_{Trp}, in agitated and nonagitated cultures (△, CFU, nonagitated culture; ○, CFU, agitated culture; ▲, IAA, nonagitated culture; ●, IAA, agitated culture)

decrease in the number of viable cells immediately after the exponential phase. The CFU profile curve of the still culture showed a long stationary phase followed by a sudden drop in the CFU number.

Agitation was shown to be an important factor for IAA production mainly in the case of N-free grown cells: only cultures under agitation were able to excrete this substance (Fig. 3). Cultures deprived of exogenous tryptophan did not excrete IAA.

The data from Figs 1–3 were used to calculate the specific IAA production (excreted IAA per CFU). In the agitated LG_{TrpAS} culture, the specific IAA production was about 2440 times higher than that of the LG_{Trp} culture also under agitation. The value calculated in the agitated LG_{TrpAS} culture is about 104 times larger than the highest value observed in nonagitated cultures. Under all conditions, specific IAA production values increased gradually until about 210 h and, in the case of the agitated LG_{TrpAS} culture, those values rose sharply.

Putrescine and spermidine (PAs) were detected in supernatant samples of *B. derxii* cultures grown for 240 h (Table 2). Generally, the amounts released by LG_{AS} cultures were much greater than those liberated by LG cultures. Agitation enhanced the production of putrescine and spermidine in a NH_4^+-containing medium, while in an N-free culture nonagitation conditions led to higher values for putrescine. Other PAs, such as spermine, agmatine, cadaverine and hexylamine were not detected in any of the samples.

Beijerinckia derxii was able to produce ethylene in media supplemented with methionine both in N-free or combined-N media. The latter condition was more favourable to the release of ethylene: 0·22 mM and 0·39 mM were liberated, respectively, by agitated LG_{Met} and LG_{MetAS} cultures grown for 216 h. No ethylene was detected in nonagitated cultures or in cultures growing in LG medium lacking methionine.

The results of amino acid production by *B. derxii* are shown in Fig. 4. This bacterium released most kinds of amino acids under all of the conditions assayed. Asparagine and methionine were not detected. Generally, the greatest diversity and highest concentrations of the released amino acids were detected in N-free medium under agitation. Under all tested conditions, glutamic acid was detected in high concentrations.

Table 2 Polyamines production (μM) by *Beijerinckia derxii*, in 240 h, LG and LG_{AS}, agitated and nonagitated grown cultures

Polyamine	Medium	Agitated/Nonagitated	Production (μM)
Putrescine	LG	Agitated	7·97
		Nonagitated	64·38
	LG_{AS}	Agitated	54·39
		Nonagitated	48·75
Spermidine	LG	Agitated	47·61
		Nonagitated	10·48
	LG_{AS}	Agitated	81·88
		Nonagitated	31·08

DISCUSSION

Nitrogen-fixing bacteria are probably among the most extensively studied soil micro-organisms. Several genera of free-living, N-fixing bacteria occur in high numbers in the rhizosphere, rhizoplane and phyllosphere. *Beijerinckia* is one of the most notable among the genera occurring in the rhizosphere of a variety of tropical grasses (Berkum and Bohlool 1980). Inoculation of rice seeds with *Beijerinckia* showed that this genus is able to multiply in the soil, establishing itself in large numbers and reducing the number of other competing micro-organisms (Döbereiner and Ruschel 1961).

The results presented herein show that *B. derxii* grown in synthetic media is able to excrete different substances that have been described as capable of influencing plant development. The profiles of nitrogenase activity of this bacterium (Fig. 1) show that all the analysed substances were excreted in the presence or absence of enzyme activity, thus strongly suggesting that the excretion of the studied substances is independent of nitrogenase activity. Like other secondary metabolites (Cacciari *et al.* 1980), the excretion of IAA by *Beijerinckia* (Figs 2 and 3) occurred during the stationary growth phase. This observation has also been made in the case of other micro-organisms such as *Azospirillum* (Zimmer and Bothe 1988). Tryptophan, the precursor of IAA biosynthesis was used to stimulate its production (Zimmer and Bothe 1988), as *B. derxii* cultures deprived of exogenous tryptophan did not excrete IAA.

Assays were performed in cultures grown either with or without agitation. They showed the importance of oxygen (O_2) concentration for total and specific IAA production whereby agitation resulted in better levels of IAA excretion (Figs 2 and 3). Agitation enhances O_2 availability, a requirement for certain enzymatic transformations of tryptophan into auxins. For instance, the intermediates indole-3-acetaledhyde and indole-3-acetamide, derived from tryptophan, require oxidation for their conversion to IAA (Reinecke and Bandurski 1987). When grown in LG_{Trp}, only cultures under agitation were able to excrete IAA. *Azospirillum brasilense* Sp 13t was shown to produce IAA under microaerobic conditions (Tien *et al.* 1979) unlike *A. brasilense* Sp 7, which required O_2.

The results suggest that IAA production depends not only on O_2, but also on the availability of N. In general, the concentration of combined N derived from N fixation is lower than that observed in an artificial culture medium

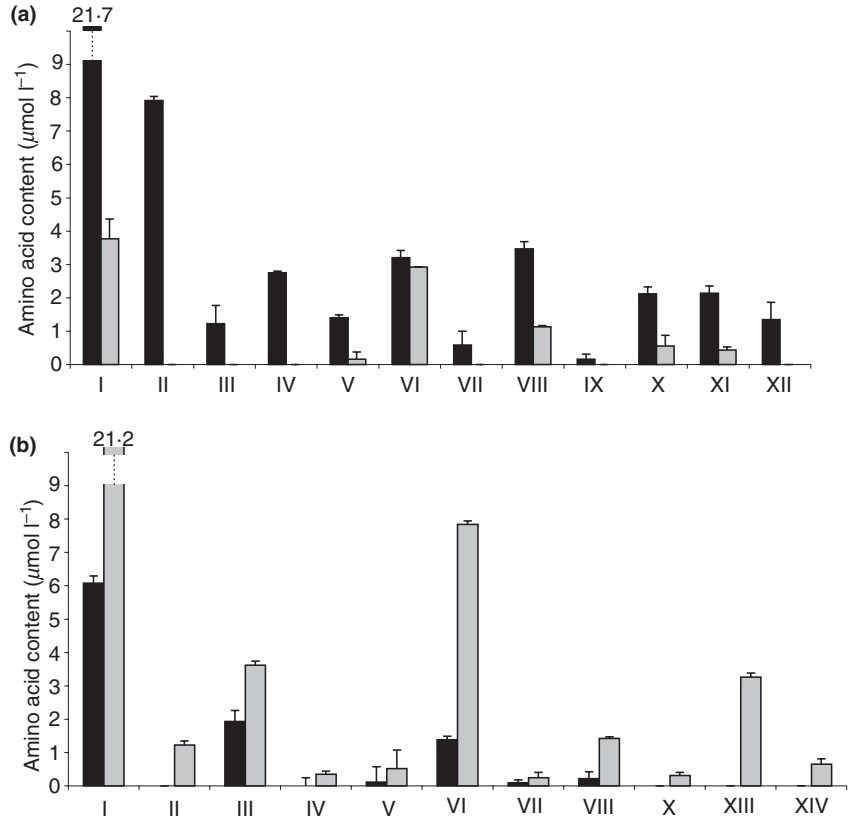

Fig. 4 Amino acids production (μM) by *Beijerinckia derxii*, in 240 h cultures, grown in LG (a) and LG_{AS} (b), under nonagitated conditions (grey) and under agitation (black). I, Glu; II, Ser; III, Gln; IV, Arg + Gly; V, Thr; VI, Ala; VII, Tyr; VIII, Val; IX, Phe; X, Ile; XI, Leu; XII, Ornithine; XIII, Lys; XIV Asp

supplemented with ammonium. Here, the best condition for IAA specific production was in LG_{TrpAS} under agitation, although lower production was observed in a LG_{Trp} culture under agitation (Fig. 2). In the nonagitated LG_{Trp} culture, the absence of IAA production (Fig. 3) suggests that, under this condition, supplies of both O_2 and N were inadequate for IAA release. These results indicate that for *B. derxii*, simultaneous, adequate concentrations of exogenous O_2 and endogenous combined N are essential for IAA synthesis. *Azospirillum lipoferum* was able to excrete similar concentrations of IAA both under N_2-fixing conditions and with ammonium (Hartmann *et al.* 1983). Thus, the response to the same environmental condition depends on the bacterial species, which indicates possible differences between biosynthetic pathways. The levels of IAA produced by *B. derxii* may be compared with that observed by Tien *et al.* (1979) in *A. brasilense* cultures. However, Garcia-Tabares *et al.* (1987) showed that *A. vinelandii*, a free-living N-fixing bacterium, was able to release about 148 μM of IAA. This value is about 10 times the highest value determined for *B. derxii*. The IAA production rate by endophytic pathogenic bacteria could be considerably greater than the IAA production rate by the plant (Bandurski *et al.* 1995), showing that the production of this substance must be finely regulated, depending on the relationship established between microorganism and plant.

The fact that IAA was released by *B. derxii* in three of the four tested conditions suggests that IAA is likely to be produced in the rhizosphere and therefore influence plant growth as observed by inoculation of *Paspalum notatum* with *A. paspali* (Barea and Brown 1974; Brown 1976). The results herein obtained are similar to the finding of Lee *et al.* (1970) in which *A. vinelandii* produced more IAA in agitated than in stationary culture and in N-free than in media supplemented with combined-N. Exogenous IAA can be taken up by the plant and, in conjunction with the endogenous plant IAA, can stimulate plant cell proliferation and/or elongation (Glick *et al.* 1998).

The higher specific IAA production levels in a N-containing culture under agitation cannot be attributed to only IAA release by dead cells, as the rise in IAA concentration was proportionally higher than the rate of decrease in the number of viable cells. Different results were found by Sarwar *et al.* (1992), who observed that the addition of NH_4NO_3 had a strong inhibitory, concentration-dependent, effect on tryptophan-derived auxin formation in the soil. The authors attributed this decrease in auxin production to the utilization of the tryptophan for microbial

growth once the cells were not limited in N (as NH_4NO_3) and carbon sources.

The PAs, putrescine and spermidine were detected in all conditions tested (Table 2). Species of *Azotobacter* and other free-living N-fixating bacteria were able to produce putrescine, spermidine and also cadaverine (Goris *et al.* 1998). In the present paper, the highest levels of released putrescine were observed in nonagitated N-free cultures in which no IAA was detected (Fig. 3). The level of PA excretion shown by *B. derxii* under all conditions tested indicates that there was enough N to maintain the necessary intracellular levels of that substance while still excreting low concentrations of it into the surrounding medium. The PA content of cells is regulated in an elaborate fashion (Igarashi and Kashiwagi 1999). When PA contents increase, an induction of the degradation and excretion systems is accompanied by inhibition of PA uptake and biosynthesis (Igarashi and Kashiwagi 2000). In *Escherichia coli*, putrescine is normally synthesised in excess, and the excess is excreted from cells in order to maintain an adequate concentration for a better cell performance (Kashiwagi and Igarashi 1988). A similar mechanism of regulation for both putrescine and spermidine may be ascribed to *B. derxii*.

The higher ethylene concentration in NH_4^+ grown cultures suggests that the presence of combined N favoured cellular ethylene biosynthesis. Similarly, *Azospirillum* sp. can produce ethylene with either N_2 or NH_4^+ as a N source and, under the latter condition, excretion of ethylene was three times higher (Thuler *et al.* 2003).

Ethylene is a common constituent of the soil gas phase under aerobic and anaerobic conditions and its concentrations are usually high enough to influence plant growth (Smith 1976). Soil ethylene levels depend on soil composition and bacterial activity (Grichko and Glick 2001). Bacterial species, such as *Pseudomonas syringae* have been found to produce ethylene both *in planta* and *in vitro* (Weingart and Völksch 1997). Jackson and Campbell (1975) were the first to reveal the likely significance of soil ethylene to plant growth. They showed that the addition of a nutrient solution containing $^{14}C_2H_4$ to the root led to a fast movement from the roots to the shoots. Despite the fact that the effects of exogenous ethylene on plant growth support the idea that microbial ethylene may play a crucial role in plant physiology, little direct evidence is as yet available (Arshad and Frankenberger 1992).

The diversity of amino acids released by *B. derxii* (Fig. 4) was higher than that observed for *B. indica* (Pati *et al.* 1994), but in lower concentrations. The high concentration of glutamic acid detected under all conditions tested, suggests that *B. derxii* actively excretes amino acids as the CFU number remained stable in N-free medium under agitation. The excretion of amino acids may be seen as a way for the bacteria to maintain a low intracellular N level, necessary for active N_2 fixation. However, this phenomenon may be particularly advantageous to the naturally surrounding environment where these N-containing substances may be directly assimilated and incorporated into the proteins of plants and a number of other living organisms.

As the environmental conditions vary, it is to be expected that the availability of N sources to living organisms fluctuates. Therefore, if the substances reported in this study are relevant to the interactions of *B. derxii* with other organisms, the fact that their production is not inhibited under either N-free or combined N conditions may be interpreted as being favourable to the sustainability of those interactions.

The release of certain plant-active substances was observed under laboratory conditions, in the presence of either N_2 or NH_4^+ as N sources. This observation suggests that bacteria of the genus *Beijerinckia*, when living on the roots or in the rhizosphere, or in the phyllosphere of plants, may also release these substances, making them available to the plants. These findings should encourage further development of these studies in order to provide a better understanding of the possible contributions of this microorganism to its habitat.

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