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NITROGEN-FIXATION BY *AZOSPIRILLUM BRASILENSE* CD IS PROMOTED WHEN CO-CULTURED WITH A MANGROVE RHIZOSPHERE BACTERIUM *(STAPHYLOCOCCUS SP.)*

GINA HOLGUIN*[1,2] and YOAV BASHAN[1]†
[1]Department of Microbiology, Division of Experimental Biology, The Center for Biological Research of the Northwest (CIB), La Paz, A.P. 128, B.C.S., 23000, Mexico and [2]Experimental Biology Laboratory, Interdisciplinary Center for Marine Sciences (CICIMAR), P.O. Box 592, La Paz, Baja California Sur, 23000, Mexico

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Summary-*Azospirillum brasilense* Cd fixed more N_2 when grown in a mixed culture with *Staphylococcus* sp., a non-N_2-fixing bacterium isolated from mangrove roots. This was not the result of an increase in the bacterial population nor of decreased O_2 concentration in the mixed culture. In mixed culture, the *Staphylococcus* population declined sharply, but not because *A. brasilense* Cd was more effective in competing for the available N. The addition of a cell-free dialyzate of *Staphylococcus* sp. culture medium to the *A. brasilense* culture significantly promoted the N_2-fixing capacity of the latter. When this dialyzate was produced by culturing Staphylococcus in N-free medium without yeast extract, the increased dialyzate activity depended on the concentration. When the dialyzate was diluted by volume to 50 and 25% of its original concentration, N_2-fixation by *A. brasilense* Cd increased significantly; when undiluted, the dialyzate failed to enhance N_2-fixation. Chemical analyses of the dialyzate by thin layer chromatography identified aspartic acid; gas chromatography revealed succinic acid to be the major organic acid component. When artificially added to the *A. brasilense* Cd culture, only aspartic acid significantly promoted N_2-fixation by *A. brasilense* Cd. The N_2-fixing ability *of A. brasilense* Cd increased significantly when grown in mixed culture with the non-N_2 fixing bacterium *Staphylococcus epidermidis*, but not with *Micrococcus lylae*, both isolated from mangrove roots. ©1997 Elsevier Science Ltd

INTRODUCTION

Azospirillum has been found to colonize, promote growth and increase the yield of numerous plant species (Bashan and Levanony, 1990; Bashan, 1993; Okon and Labandera-Gonzales, 1994; Bashan and Holguin, 1995). However, some of these effects can be enhanced when *Azospirillum* is co-inoculated with other microorganisms. A higher soybean yield was obtained when using mixed inoculants of *Azospirillum* and *Rhizobium* as compared to inoculations of *Rhizobium* alone (Singh and Subba Rao, 1979). *Azospirillum*, by enhancing the proliferation of root hairs, increased the susceptibility of forage legumes to *Rhizobium* infection (Yahalom et al., 1987). A dual inoculation of *A. brasilense* and vesicular-arbuscular mycorrhizal fungi (VAM) increased the root biomass and the absorption of phosphorus by pearl millet (Subba Rao et al., 1985a), barley yield (Subba Rao et al., 1985b) and the number of colonization sites of VAM fungi in halophytic plants growing in dunes (Will and Sylvia, 1990).

N_2-fixation may be one of the minor mechanisms involved in plant growth promotion by *Azospirillum* (Michiels *et al*., 1989; Bashan and Levanony, 1990). Despite the small importance of this mechanism to plants, the role of N_2-fixation in rhizocompetence, survival in soil and interactions of *Azospirillum* with other rhizosphere bacteria has been overlooked. The success of an *Azospirillum* inoculant in promoting plant growth will largely depend on its survival in the hostile soil environment (Bashan et al., 1995) and on its movement towards the host plant both in bulk soil and in the rhizosphere (Bashan and Levanony, 1987; Bashan and Holguin, 1994). The capacity of a bacterium to fix N_2 may improve its survival as compared to non-fixing strains.

The interaction of N_2-fixing bacteria with other bacteria can inhibit or promote their diazotrophic activity and this is quite common among microorganisms (Drozdowicz and Ferreira Santos, 1987; Isopi *et al.*, 1995). The degradation of cellulose

*Present address: Department of Biology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1
†Department of Microbiology, Division of Experimental Biology, The Center for Biological Research of the Northwest (CIB), La Paz, A.P. 128, B.C.S., 23000, Mexico, FAX: 52(112)5-47-10 or 5-3625, E-mail: bashan@cibnor.conacyt.mx

by *Cellulomonas* sp. CSI-17 provides *Azospirillum* sp. DN64 with a usable C source to obtain energy for N_2-fixation. The contribution of *Azospirillum* to *Cellulomonas* is fixed nitrogen (Halsall and Gibson, 1989). The association between diferent *Azospirillum* species and the N_2-fixer *Bacillus polymyxa,* enhanced the N_2-fixing activity of the co-cultures as compared to pure cultures of either *Azospirillum* or *Bacillus. Azospirillum* is benefited by the products which are released from the degradation of pectin by *Bacillus* (Khammas and Keiser, 1992).

The N_2-fixing activity of *A. brasilense* Cd increased significantly when grown in mixed culture with the mangrove rhizosphere bacterium *Staphylococcus* sp. (Holguin and Bashan, 1993), similarly to when *Staphylococcus* sp. was grown with the marine N_2-fixer *Listonella anguillarum* (Holguin *et al.,* 1992). This finding holds promise for the future application of mixed inoculants of *Azospirillum* in salt-affected soils.

Our objective was to explore factors responsible for enhanced N_2-fixation caused by the interaction between *Azospirillum brasilense* and *Staphylococcus* sp.

MATERIALS AND METHODS

Bacteria

Azospirillum brasilense Cd ATCC 29710 and *Staphylococcus* sp. BARA9010 (Holguin *et al.,* 1992) isolated from mangrove roots were used in all experiments.

Isolation and identification of *other non N_2-fixing mangrove rhizosphere bacteria*

The isolation site was in Balandra lagoon, Baja California Sur, Mexico (Pedrin-Avilés *et al.,* 1990; Holguin *et al.,* 1992). The sampling of mangrove seedlings *[Avicennia germinans (L.)* Stern], preparation of roots, bacterial enrichment and isolation procedure, were described by Holguin *et al.* (1992) and Bashan *et al.* (1993). Rennie's medium (Rennie, 1981) was used as enrichment and growth medium. Rennie's medium was modified and consisted of three components: (i) $K_2HPO_4.3H_2O$, 2.7g, KH_2P0_4, 0.2g, NaCl, 15g, $Na_2FeEDTA$, 28.0 mg, $Na_2MoO_4.2H_2O$, 25.0 mg, yeast extract, 100.0 mg mannitol, 5.0 g, sucrose and 5.0 g, distilled water, 900 ml; (ii) 3 ml of a calcium lactate solution (3.528 dissolved in 30 ml of water); (iii) $MgS0_4.7H_20$, 3.0 g, distilled water, 100 ml. The solutions were autoclaved separately and mixed after cooling. Biotin (5 µg l^{-1}) and p-aminobenzoic acid (10 µg l^{-1}), sterilized by filtration, were added to the previous mixture. The pH was adjusted to 7.3 with NaOH.

Species identification was done by analysis through gas chromatography of cell fatty acid methyl esters (FAME) (Sasser, 1990). FAME analysis was carried out as a commercial service by Dr J.W. Kloepper's laboratory, Auburn University, Alabama, U.S.A. Strain BA9302 was identified as *Staphylococcus epidermidis* and BA9303 as *Micrococcus lylae.* The latter was isolated by a modified medium (addition of 2% NaCl and 0.03% $MgS0_4$) devised for the isolation of phosphate-solubilizing bacteria (Sundara Rao and Sinha, 1963).

Growth conditions of A. brasilense Cd *and* Staphylo*coccus sp.*

A. brasilense Cd was grown in 50 ml of Nutrient Broth (Merck) supplemented with 2% NaCl under rotary agitation (120 rev min^{-1}) for 18 h at 30°C in 250 ml Erlenmeyer flasks. The culture was washed three times at 4°C under sterile conditions at 1700 x g for 10 min with phosphate-buffer-saline (PBS) 80 mM supplemented with NaCl 50 msM, final pH 7.2. The optical density of the bacterial culture was adjusted to 1.0 at 540 nm with PBS, corresponding to 5 x 107 c.f.u. ml^{-1}. The non-N_2 fixing bacterium, *Staphylococcus* sp. was grown and washed similarly to *A. brasilense* Cd. The optical density of the culture was adjusted to 1.8 at 540 nm corresponding to 6 x 108 c.f.u. ml^{-1}. Cultures for the acetylene reduction assay were prepared as described by Holguin *et al.* (1992) and incubated without movement at 30°C for 48 h.

Acetylene (C_2H_2) reduction assay (ARA)

Acetylene reduction was assayed as described by Holguin *et al.* (1992). As a first evaluation, the C_2H_2 reduction of *A. brasilense* Cd in pure and mixed culture with *Staphylococcus* sp., was evaluated 24, 48, 72 and 96 h after inoculating *Staphylococcus* sp. into the *A. brasilense* Cd culture. A different set of bottles was used for each reading. In subsequent experiments, C_2H_2 reduction was evaluated after only 96 h. The amount of C_2H_4 produced is expressed as nanomoles C2H4 per culture or per cell 24 h^{-1} period. To calculate the total amount of C_2H_4 produced per cell, we divided the total amount of C_2H_4 produced per culture, by the total number of living bacteria present in the culture, both values expressed in log numbers. In some experiments the results are expressed as n-fold increase over the control.

Bacterial counts of A. brasilense *Cd and* Staphylo*coccus sp.*

Bacteria were counted by the conventional plate count method on solid HGB medium (Holguin *et al., 1972)* for *A. brasilense* Cd and on "Agar for staphylococci No. 110" (Bioxon, Mexico) for *Staphylococcus* sp. Counts were made in pure and mixed cultures of both bacteria after 24, 48, 72 and

96 h of inoculating *Staphylococcus* sp. into the *Azospirillum* culture. Bacterial counts of *Staphylococcus* sp. grown in HGB medium without yeast-extract were also made.

Determination of O_2 *concentration in the cultures*

The O_2 concentration was measured in pure and mixed cultures using an O_2 meter, model 54 ARC (Yellow Springs Instruments, U.S.A.) (Holguin *et al.*, 1992) and by a modification of the Winkler method (Williams and Jenkinson, 1982) 96 h after inoculating *Staphylococcus* sp. into the *Azospirillum* culture.

ARA of A. brasilense *Cd in pure and mixed cultures with* Staphylococcus *sp. contained inside dialysis tubing*

Staphylococcus sp. suspension was centrifuged at 1700 x g for 10 min at 4°C, the supernatant was discarded and the cells were resuspended in half of its original volume in fresh HGB medium. 2 ml of this 2-fold concentrated suspension was aseptically put inside a dialysis tubing (Sigma, cellulose membrane which retains most molecules of mol. wt of 12,000 daltons or greater). The tubing containing the bacteria was tightly closed with a sterile thread and immersed into 14 ml of 48 h old cultures of *A. brasilense* Cd contained in 60 ml serum bottles. The three controls were: (i) pure culture of *A. brasilense* Cd; (ii) a mixed culture of *A. brasilense* Cd and *Staphylococcus sp.;* and (iii) empty dialysis tubing immersed in an *A. brasilense* Cd culture. ARA of the cultures was determined after 96 h.

ARA of A. brasilense Cd *after adding a cell free dialyzate* of Staphylococcus *sp*.

The C_2H_2 reduction of *A. brasilense* Cd was evaluated in pure culture compared to the addition of either a cell-free dialyzate of *Staphylococcus* sp., or dead cells (autoclaved) of *Staphylococcus sp.* to the *Azospirillum* culture. The dialyzate was obtained by introducing 2-fold-concentrated suspension of *Staphylococcus sp.* inside a dialysis tubing immersed in the same volume of HGB medium. After 96 h at 30°C under stirring conditions, the tubing was discarded, the dialyzate was recovered and 2 ml were added to the bottles containing 14 ml of *Azospirillum* culture. After an additional incubation of 24 h at 30°C, C_2H_2 reduction was determined.

Evaluation of *the activity of the dialyzate produced by growing* Staphylococcus *sp. in HGB medium without yeast-extract*

The analysis of the chemical composition of the dialyzate required the exclusion of the yeast-extract from the medium. However, it was not known if its exclusion interfered with the promotion of the C_2H_2 reduction of *Azospirillum.* Thus, we evaluated the promotional activity of the dialyzate produced by culturing *Staphylococcus* sp. in HGB medium without yeast extract. The obtained dialyzate was added to the *A. brasilense* Cd cultures at the following concentrations: 100% (non-diluted) 50 and 25% (diluted by volume in PBS).

Amino acid and organic acid analyses of *the cell-free dialyzate of* Staphylococcus sp.

Total concentration of amino acids in the dialyzate was determined according to Rosen's method (Rosen, 1957). The qualitative determination of the amino acid composition of the dialyzate was done by (i) one-dimensional; (ii) two-dimensional thin layer chromatography (TLC) on silica gel, (AI Sil G, 250 µm layer); and (iii) in automatic TLC using the Iatroscan chromatographic analyzer (MK-5 TLC-FID, Iatron Laboratories Inc., Tokyo, Japan). Separation of amino acids on one dimensional TLC was done either with Solvent I, [phenol + water (75:25, v/v)] and Solvent II [2-propanol + ammonium hydroxide (55:45, v/v)]. Since these two solvents gave good resolution, two dimensional TLC was run first with Solvent I, followed by Solvent II. The solvents used for automatic TLC were either Solvent 11 or Solvent III [2-propanol + ammonium hydroxide (70:30, v/v)]. The Pauly's reagent was used to detect tyrosine and histidine, the Sakaguchi's reagent for the detection of arginine, and the Ehrlich's reagent for the detection of tryptophan (Robyt and White, 1987). Non-inoculated medium was used as control. Automatic TLC analyzer was used for the quantitative determination of the amino acids found in the dialyzate. A conventional amino acid analyzer could not be used since the dialyzate contained high concentrations of salts which interfere in the analysis. The removal of the salts required a column not available in our laboratories. Total N in the dialyzate was quantified by the Kjeldahl method using a Kjeltec Auto-1030 analyzer (Tecator, Sweden).

The qualitative and quantitative analysis of volatile (acetic, propionic, butyric, isobutyric, valeric, isovaleric, caproic, isocaproic and heptanoic) and non-volatile organic acids (pyruvic, lactic, oxalacetic, oxalic, malonic, methylmalonic, fumaric and succinic) possibly present in the dialyzate was performed by gas chromatography (GC) using highly graded Sigma chemicals as standards. The extraction was done according to Smibert and Krieg (1981). Samples (0.5-1.0 µl) were injected in a Varian 6000 gas chromatograph (Varian Instrument Group, U.S.A.) equipped with a hydrogen flame ionization detector (FID) operated under the following conditions: a "fused silica" capillary column (Nukol, USA) (15m, 0.53 mm i.d. and 0.5 µm film thickness), an initial column temperature of 70°C for a period of 5 min, followed by an increasing rate in temperature of 8°C min^{-1} to reach a constant value of 140°C for 5 min (this program of temperature control was

run for every injection), an injector temperature of 240°C, a detector temperature of 240°C, N_2 carrier gas and H_2 for the FID were used at a flow rate of 2 and 30 ml min^{-1}, respectively, and an air flow rate of 300 ml min^{-1}.

Effect of the addition of aspartic and succinic acid on the C_2H_2 reduction by A. brasilense Cd

Separate solutions were prepared containing full and half concentrations of aspartic and succinic acid found in the dialyzate (1.2 and 0.6 mot for aspartic acid, and 15.3 and 7.6 mm for succinic acid), and added (2 ml) separately to 14 ml of *A. brasilense Cd* cultures and incubated for 24 h at 30°C after which the ARA was measured. Addition of a diluted dialyzate (50% by volume) to *A.* brasilense Cd culture was included as a control.

The effect of co-culture with Staphylococcus epidermidis *and* Micrococcus lylae *on the C_2H_2 reduction* of A. brasilense *Cd*

S. epidermidis and *M. lylae* were both grown and tested in co-cultures with *A. brasilense Cd,* similarly to Staphylococcus sp., to analyze their effect on the diazotrophic activity of *Azospirillum.* ARA was measured after 96 h of incubation.

Experimental design and statistical analysis

All experiments were repeated at least twice and commonly two to four times each. Each treatment included a minimum of three replicates. The ARA included at least six replicates per treatment where a replicate consisted of one culture. Data presented are the means of all replicates from all experiments showing similar trends accompanied by standard error values. Significance for two sample analyses was determined by Student's t-test at $P \leq 0.05$. For experiments which included several treatments, significance was determined by one-way analysis of variance (ANOVA) at $P \leq 0.05$. Data in percentages were transformed to arc-sin before analysis.

RESULTS

N_2-fixation and populations of A. brasilense *Cd* in *pure or mixed cultures* with Staphylococcus *sp. at different incubation* times

N_2-fixation of *A. brasilense Cd* was significantly increased in mixed culture with *Staphylococcus* sp. as compared to pure culture after 24, 48, 72 and 96 h of incubation. [Fig. 1 (a)]. N_2-fixation per cell of *A. brasilense* Cd showed a similar significant increase compared to pure culture, at all incubation times [Fig. 1 (b)]. No significant numerical differences were found in the populations of *A. brasilense* Cd in pure and mixed cultures with *Staphylococcus* sp. after 24, 48, 72 and 96 h of incubation [Fig. 2 (a)1.

The population of *Staphylococcus* sp. when grown in pure culture with yeast-extract remained stable throughout the incubation [Fig. 2 (b)]. However, when grown without yeast extract, the population after 96 h was significantly less than when grown with yeast extract. When grown in mixed culture with *A. brasilense* Cd, the number of

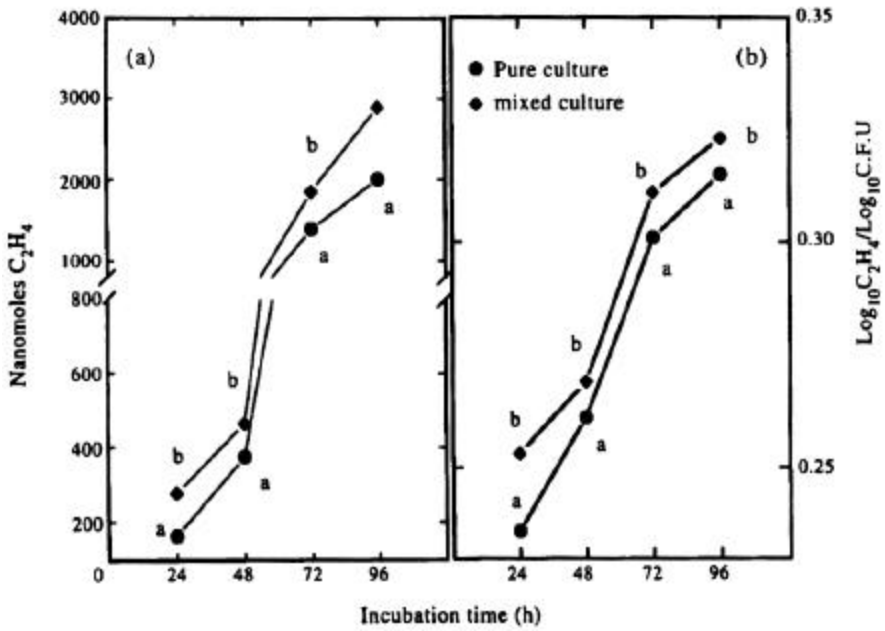

Fig. 1. C_2H_4 production (a) per culture; (b) per cell, of *A. brasilense* Cd in pure culture and in mixed culture with *Staphylococcus* sp., at different incubation times. Every pair of points at each incubation time and in each sub-figure, denoted by a different letter, differ significantly at $P \leq 0.05$ by the Student's t-test.

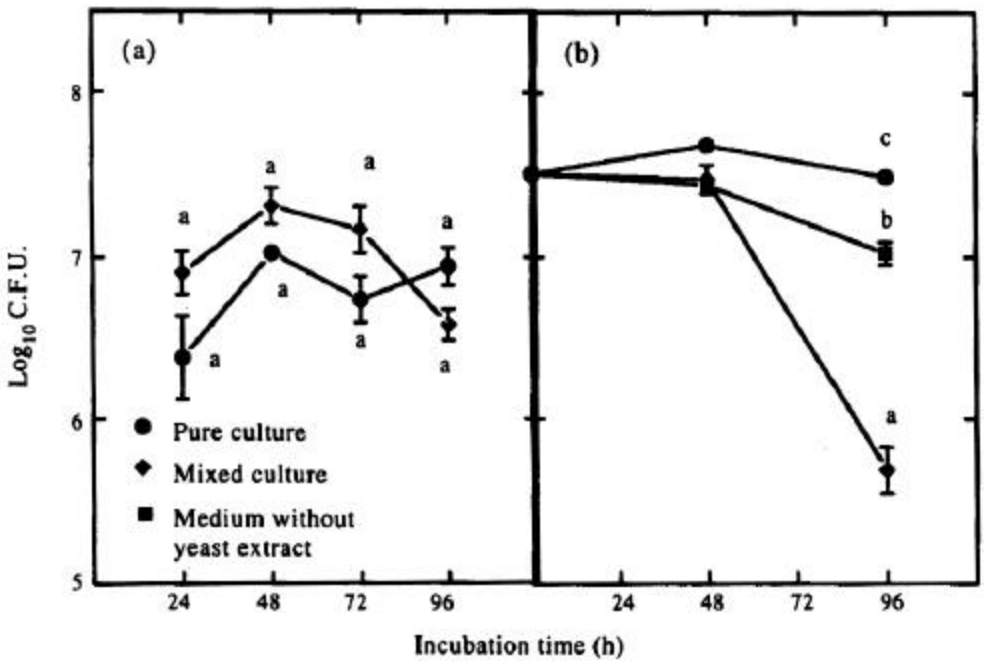

Fig. 2. Populations of (a) *A. brasilense;* and (b) *Staphylococcus* sp., in pure culture, in mixed culture and in medium without yeast extract at different incubation times. Bars represent SE. In (a) every pair of points at each incubation time denoted by a different letter, differ significantly at $P \leq 0.05$ by the Student's t-test. In (b) points denoted by a different letter differ significantly at $P \leq 0.05$ in one-way ANOVA.

Staphylococcus sp. were even lower, showing a considerable decrease in the population [Fig. 2(b)].

Dissolved O_2 concentration in pure and mixed cultures of a. brasilense Cd

The concentration of O_2 in mixed cultures of *A. brasilense* Cd was found to be significantly higher than in pure cultures after 96 h of incubation (Fig. 3) using the two different O_2 determination methods.

N_2-fixation by A. brasilense Cd *in pure and mixed cultures with* Staphylococcus *sp. cells contained inside dialysis tubing*

N_2-fixation of *A. brasilense* Cd culture was significantly higher when associated with *Staphylococcus* sp. contained inside dialysis tubing compared to the treatment which included *A. brasilense* mixed with *Staphylococcus* sp. in free association. The latter mixture also showed a significant increase in the N_2-fixation of *A. brasilense* compared to pure culture. The dialysis tubing had no effect on the N_2-fixation of *A. brasilense* Cd [Fig. 4(a)].

N_2-fixation of A. brasilense *Cd culture when mixed with a cell free dialyzate of* Staphylococcus sp. *or when mixed with a cell free dialyzate* of Staphylococcus *sp. cultured without yeast extract*

N_2-fixation of *A. brasilense* Cd was significantly higher when mixed with a cell-free dialyzate of *Staphylococcus sp.* compared to pure culture. The addition of dead cells of *Staphylococcus sp,* to the *Azospirillum* culture had no effect on the N_2-fixation of the latter [Fig. 4(b)].

A 50 and 25% diluted dialyzate of *Staphylococcus sp.* significantly and similarly promoted N_2-fixation of *A. brasilense* Cd compared to pure culture [Fig. 4(c)]. However, a non-diluted dialyzate (100%) had no effect on the N_2-fixing capacity of *A. brasilense* Cd.

Amino acid, organic acid analyses, total N and pH of the cell free dialyzate of Staphylococcus sp.

The total amino acid concentration of the dialyzate was found to be 295 ± 20 μg ml^{-1}. By separating the samples in an automatic TLC analyzer (Solvent III) and by the additional use of the Pauly, Sakaguchi and Ehrlich reagents

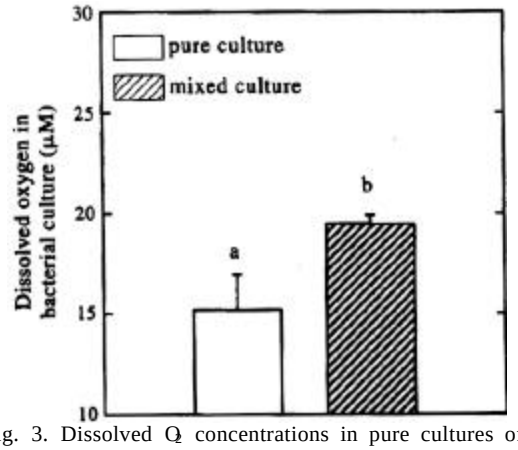

Fig. 3. Dissolved O_2 concentrations in pure cultures of *A. brasilense* and in mixed cultures with *Staphylococcus* sp. after 96 h of incubation. Bars represent SE. Columns denoted by a different letter differ significantly at $P \leq 0.05$ by the Student's t-test. Data presented was obtained by the method of Williams and Jenkinson (1982).

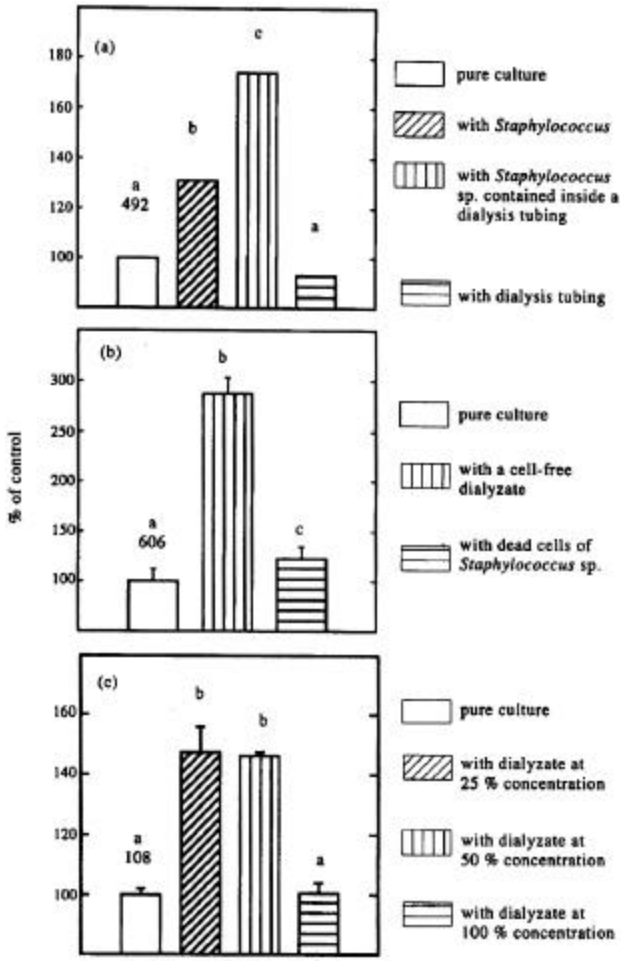

Fig. 4. Percentage of increase in the N_2-fixation production from pure culture of *A. brasilense* under different treatments involving interactions with *Staphylococcus* cells or *Staphylococcus* products. Columns in each subfigure denoted by a different letter differ significantly at $P \leq 0.05$ in one-way ANOVA. Bars represent SE. Numbers above pure culture columns indicate the actual C_2H_4 emission from that culture expressed as nanomoles of C_2H_4 produced in 24 h. These numbers serve as the base lines of the calculations presented in this figure.

, we were able to discard the presence of several amino acids in the dialyzate such as tyrosine, histidine, arginine and tryptophan. However, the automatic TLC performed in rods and the one-dimensional TLC performed on silica gel plates were not able to distinguish between aspartic acid, leucine, valine, methionine and isoleucine which gave similar R_f values. Two-dimensional TLC on silica gel plates (Solvent II followed by Solvent I) established that only aspartic acid was present in the *Staphylococcus* dialyzate [Fig. 5(A)]. This results were further confirmed by automatic TLC in solvent II [Fig. 5 (D), (E)]. The concentration of aspartic acid in the dialyzate was found to be 160 $\mu g\ ml^{-1}$ (1.2 mm).

The determination of possible organic acids in the dialyzate by GC detected mainly succinic acid in the dialyzate at a concentration of $1806 \pm 5\ \mu g\ ml^{-1}$ (15.3 mm) [Fig. 5(B), (C)]. Malonic acid was also detected at a concentration of 97 $\mu g\ ml^{-1}$. The analysis did not detect any non-volatile organic

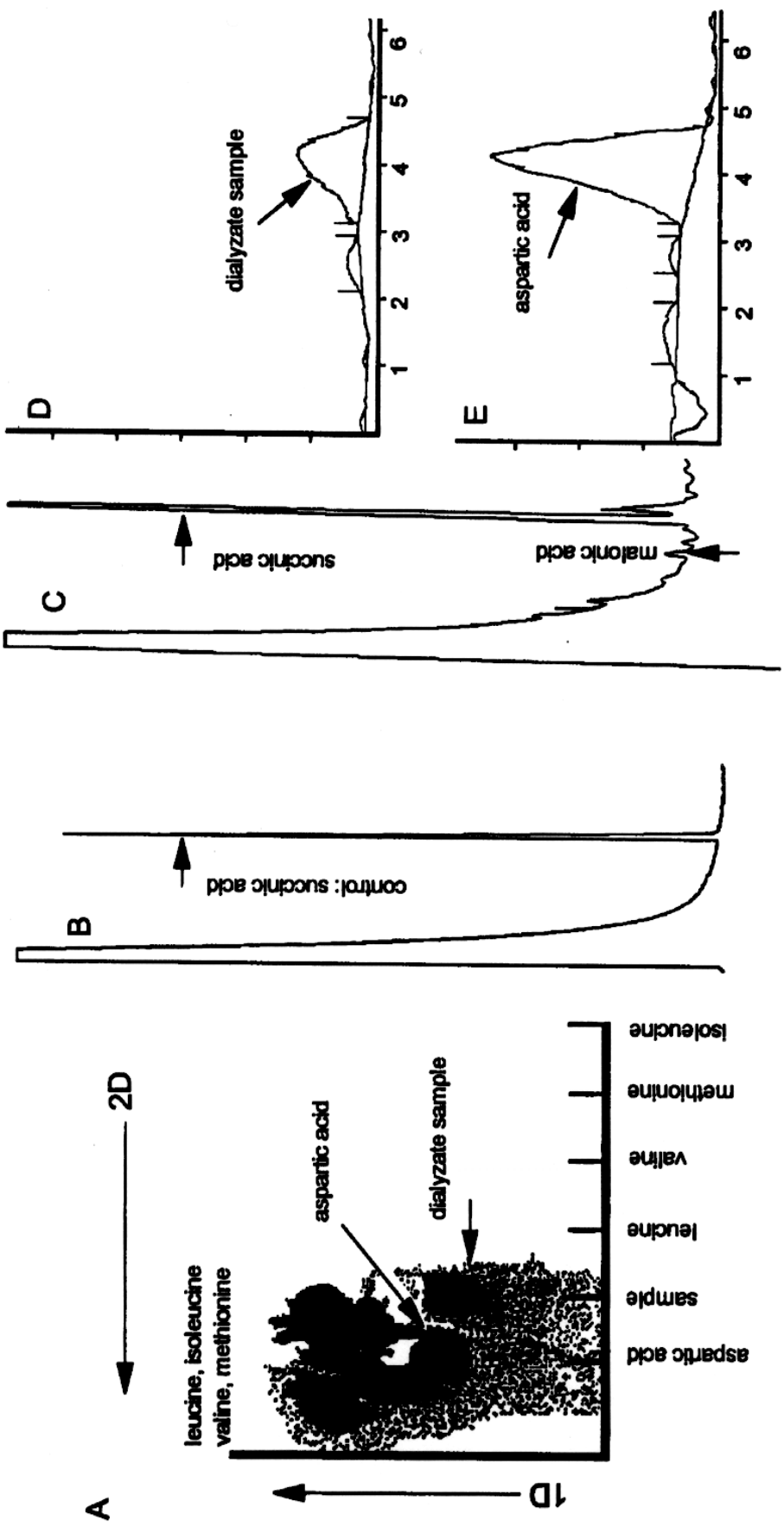

Fig. 5. Computer enhanced image of two dimensional TLC of amino acid standards and a dialyzate sample run on silica gel plate (A). Computer enhanced image of organic acids separation obtained by GC, control (B), and dialyzate sample (C). Computer enhanced image of amino acids separation obtained by automatic TLC, dialyzate sample (D) and standard (E).

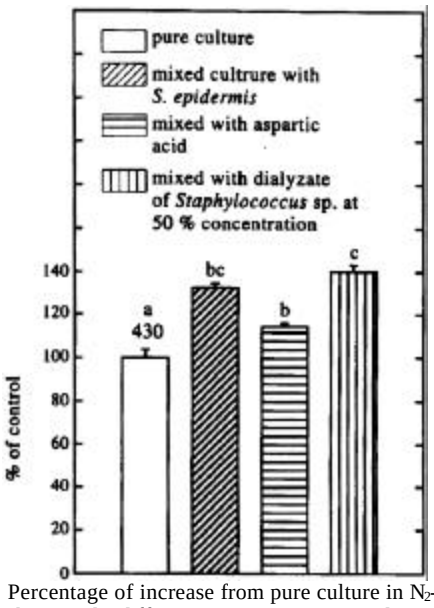

Fig. 6. Percentage of increase from pure culture in N_2-fixation of *A. brasilense* under different treatments: in pure culture, in mixed culture with *S. epidermidis*, with aspartic acid, with dialyzate of *Staphylococcus* at 50% concentration. Columns denoted by different letters differ significantly at $P \leq 0.05$ in one-way ANOVA. Bars represent SE. Numbers above pure culture columns indicate the actual C_2H_4 emission from that culture expressed as nano moles of C_2H_4 produced in 24 h. These numbers serve as the base lines of the calculations presented in this figure.

acids in the dialyzate.

The concentration of total N in the dialyzate was 33 ± 5 $\mu g\ ml^{-1}$. The pH of the dialyzate showed a slight, but significant, increase averaging 0.11 units above the pH in non-inoculated medium. No amino acids or organic acids were found in the non-inoculated medium.

Effect of aspartic, succinic acid and the mangrove rhizosphere bacterium Staphylococcus *epidermidis or Micrococcus lylae on the N_2- fixation of* A. brasilense *Cd*

The addition of aspartic acid (0.6 mm) to *the A. brasilense* Cd cultures significantly increased the N_2-fixation of *Azospirillum* compared to pure culture (Fig. 6). The addition of 0.2 mm did not affect the N_2-fixing activity of *Azospirillum*. However, the N_2-fixation of *A. brasilense* Cd mixed with the diluted dialyzate was significantly higher than the treatment which included only the addition of aspartic acid. The addition of succinic acid in both concentrations had no effect on the N_2-fixation of *Azospirillum* (data not shown).

N_2-fixation of *A. brasilense* Cd was significantly increased when grown in mixed culture with *S. epidermidis* (Fig. 6), but was unaffected when co-cultured with *M. lylae* (data not shown).

DISCUSSION

The concept of multi-organism inoculants as an agricultural practice superior to single-organism inoculation is decades old. Yet, as far as we know, no inoculant composed of two bacterial species (apart from a mixture of strains from the same species) passed the experimental stage. (Lindermann and Paulitz, 1990; Li *et al*., 1992; Garbaye, 1994; Polonenko, 1994; Rice *et al*., 1994)

Although the inoculation of plants with plant growth-promoting Rhizobacteria may occur naturally, it is mainly an artificial agricultural procedure. As such, *Azospirillum* species originating from Brazil, Pakistan and Iraq have been inoculated onto plants such as the giant cardon cactus in Mexico (Puente and Bashan, 1993) or weeds in Israel (Bashan and Holguin, 1995) that were probably never exposed to these species before. Thus, novel combinations of *Azospirillum* with other bacteria from diverse sources should be evaluated since they may have a potential as future inoculants. As an example for such an approach, Staphylococcus sp., a marine non-N_2 fixing mangrove rhizosphere bacterium, was found to increase the N_2-fixation of the mangrove rhizosphere N_2-fixer *Listonella anguillarum* (Holguin *et al*., 1992). Preliminary experiments showed that it had a similar effect on the terrestrial *Azospirillum* as well. Such a combination was chosen for the development of a mixed inoculant, especially for use under the salt-stressed conditions found in many semi-arid soils.

N_2-fixation is one of the main characteristics of *Azospirillum* cells, although it is probably a minor mechanism in plant growth enhancement (Bashan, 1993). We found that in mixed culture, Staphylococcus sp, is capable of promoting the N_2-fixation of *A. brasilense* Cd. This promotion was not accompanied by a parallel growth of *Azospirillum* populations. Likewise, Drozdowicz and Ferreira Santos (1987) found that out of 50 Gram-negative and 29 Gram-positive bacteria, only six affected the growth and N_2-fixation of either *A. brasilense* Cd *or A. lipoferum* Br17. They concluded that the stimulation or inhibition of nitrogenase activity resulted from the direct action of the factors on the enzyme's activity or synthesis, but not from its effect on the growth of *Azospirillum* cells.

Our attempt to relate the increased nitrogen fixation to reduced O_2 concentration in the mixed culture (which would be favorable to the microaerophilic *Azospirillum*), revealed that the oz concentration in mixed cultures was higher than the optimal concentration for N_2-fixation of *Azospirillum* (0.6 - 8 μM) (Okon *et al*., 1983; Hurek *et al*., 1987, 1988). Therefore, we concluded that oxygen was not a major factor responsible for the promotion of N_2-fixation. However, this does not exclude the possibility that the mixed culture provided culture conditions which indirectly increased the tolerance of nitrogenase to higher

concentrations of O_2, e.g. mixed cultures of *A. brasilense* and *Arthrobacter giacomelloi* showed high nitrogen. ase activities under otherwise inhibiting O_2 concentrations (Cacciari *et al.,* 1989).

Separating *A. brasilense* Cd from *Staphylococcus* sp. by dialysis tubing in the mixed culture and using cell free dialyzates of *Staphylococcus sp.* helped us to conclude that low molecular weight substances leaking out of the dialysis tubing were responsible for the promotion in the N_2-fixation of *Azospirillum.* The activity of the *Staphylococcus sp.* dialyzate on N_2-fixation was found to depend on its concentration. However, the concentration of total N found in the dialyzate (33 mg 1^{-1}) is lower than the 50 mg N 1^{-1} require to repress nitrogenase in *A. brasilense* (Das and Mishra, 1982a). Similarly to our results, diluted cell-free filtrates of *Arthrobacter giacomelloi* significantly stimulated *A. brasilense* nitrogenase activity and cell growth, whereas the whole filtrate inhibited both the activity and growth *(Lippi et al.,* 1992).

In other diazotrophic microorganisms, amino acids influence N_2-fixation activity (Hartmann *et al.,* 1988). Das and Mishra (1982b) found aspartic acid to stimulate nitrogenase activity of *A. brasilense* Sp7 at 2 mm concentration, while higher levels of aspartic acid (10 mm) did not promote N_2-fixation of *A. brasilense* Sp7 (Hartmann *et al.,* 1988), a strain closely related to strain Cd used in our study. The incorporation of aspartic acid, which was the sole amino acid found in the *Staphylococcus* sp. dialyzate, into the *A. brasilense* Cd cultures promoted its N_2-fixation similarly to histidine, which promoted N_2-fixation in several strains of *A. brasilense.* The enhancement in N_2-fixation of *A. brasilense* Cd by the addition of aspartic acid alone was lower than by adding the diluted dialyzate. This suggests that aspartic acid is probably only one of the metabolites in the dialyzate which have the ability to promote nitrogenase activity.

When Drozdowicz and Ferreira Santos (1987) tested the efect of 79 bacterial species on the nitrogenase activity of *Azospirillum* in co-cultures, they found no correlation between bacterial taxonomy and its positive or negative efect on the diazotrophic activity of *Azospirillum.* However, the fact that another staphylococcus isolated from the mangrove rhizosphere, *Staphylococcus epidermidis,* was also capable of promoting the diazotrophic activity of *A. brasilense* Cd points out the potential of positive interactions between the genera *Azospirillum* and *Staphylococcus,* which, to the best of our knowledge, was not evaluated before.

In conclusion, mixed cultures of A. *brasilense* Cd with either the non- N_2-fixing bacteria *Staphylococcus* sp. or *S. epidermidis* significantly enhanced its N_2-fixation. The enhancement was probably due in part to the release of aspartic acid from *Staphylococcus* sp. cells.

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