

## INVOLVEMENT OF INDOLE-3-ACETIC ACID PRODUCED BY THE GROWTH-PROMOTING BACTERIUM *AZOSPIRILLUM* SPP. IN PROMOTING GROWTH OF *CHLORELLA VULGARIS*<sup>1</sup>

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**Involvement of indole-3-acetic acid (IAA), produced by the microalgae-growth-promoting bacteria *Azospirillum brasilens* and *A. lipoferum*, in promoting growth of the microalga *Chlorella vulgaris* Beij. was studied. Four wildtype strains of *Azospirillum* and their IAA-deficient mutants were co-immobilized with *C. vulgaris* in alginate beads. Cultures were grown in synthetic growth medium supplemented with tryptophan. Growth promotion of microalgae and production of exogenous IAA by *Azospirillum* spp. were monitored. All wildtype *Azospirillum* spp. produced significant but varying amounts of IAA, while their mutant forms produced significantly less. The results demonstrated a significant growth promotion in *Chlorella* cultures when immobilized with the four wildtype strains of *Azospirillum*, while very low or no enhanced growth was induced by the four IAA-deficient mutants, compared to when *C. vulgaris* is immobilized alone. A complementation experiment, where an IAA-attenuated mutant (*A. brasilense* SpM7918) was supplemented with IAA produced by its parental wildtype strain (*A. brasilense* Sp6), restored growth promotion in the microalgae-mutant culture.**

**Key index words:** *Azospirillum*; *Chlorella*; indole-3-acetic acid; microalgae; plant-growth-promoting bacteria; wastewater treatment

**Abbreviations:** IAA, indole-3-acetic acid; PGPB, plant-growth-promoting bacteria

*Azospirillum* sp. is a known plant-growth-promoting bacterium (PGPB) that enhances growth and yield of many terrestrial crop plants (Bashan and Lev-anony 1990, Okon and Labandera-Gonzalez 1994, Bashan and Holguin 1997, Bashan et al. 2004) and can promote growth of several freshwater species of the chlorophyte genus *Chlorella* (Gonzalez and Bashan 2000, Hernandez et al. 2006). As yet, the precise mode of action of *Azospirillum* on plants has not been fully defined or accepted (Bashan et al. 2004). Proposed modes of action include hormonal activity, N<sub>2</sub> fixation, undefined signal molecules enhancing proton extrusion from cells that interfere with plant metabolism, nitrite production, or the sum of activities of small magnitude mechanisms working in concert (Bashan and Holguin 1997, Bashan et al. 2004, Bottini et al. 2004).

A plausible explanation for some of the growth-promoting effects of *Azospirillum* on plants is the production of several phytohormones that alter metabolism and morphology of plants, leading to better mineral and water absorption and consequently larger and healthier plants. In unicellular microalgae, phytohormones may lead to larger cell population. The contribution of N<sub>2</sub> fixation is controversial; the other proposed mechanisms are less established. *Azospirillum* species are well known mainly for their ability to produce plant hormones in vitro, among which are indoles, mainly IAA, gibberellins (Steenhoudt and Vanderleyden 2000, Dobbelaere et al. 2003, Bashan et al. 2004, Bottini et al. 2004), and possibly other plant hormones (Somers et al. 2005). The effect of these bacterial-produced phytohormones to influence plant growth is less known.

Earlier studies on production of IAA by several strains of *Azospirillum* showed that production

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depended on the type of culture medium and availability of tryptophan as a precursor (Reynders and Vlassak 1979, Prinsen et al. 1993). However, in one study, *Azospirillum* produced IAA in the absence of tryptophan (Prinsen et al. 1993). This finding suggests that at least one tryptophan-independent pathway is present in *Azospirillum*; however, neither the precursor nor the intermediate in such a pathway was identified. Currently, three tryptophan-dependent pathways are known in *Azospirillum*: indole-3-acetonitrile (IAN), indole-3-acetamide (IAM), and indole-3-pyruvic acid (IPyA) (Zakharova et al. 1999).

The effect of IAA has been less studied in microalgae than in higher plants. IAA induces root elongation (Fallik et al. 1989, Kende and Zeevaart 1997), growth of roots and shoots in response to light and gravity (Kaufman et al. 1995), cell division in tissues (Salisbury and Ross 1994), cell differentiation, and the formation of adventitious roots (Fallik et al. 1989). The mode of action of IAA on plants is not completely established, but low levels of IAA enhance growth directly by stimulating cell division or cell elongation (Brummell and Hall 1987, Salisbury and Ross 1994).

*Chlorella vulgaris* is a commonly used microalga for various industrial applications, but mainly as an agent for tertiary wastewater treatment (de-Bashan et al. 2002, 2004, Valderrama et al. 2002, Hernandez et al. 2006). Its growth is promoted by *A. brasilense* strain Cd when co-immobilized in alginate beads (Gonzalez and Bashan 2000, de-Bashan et al. 2005).

The effect of auxins on the microalga *Chlorella* has been rarely studied. Czerpak et al. (1999) found a strong stimulation by IAA on water-soluble protein, monosaccharides-aldoheoses, and chl *a* and *b* content in *C. pyrenoidosa*. The stimulating activity of auxins was considered to be similar to responses in

vascular plants (Czerpak et al. 1999). Protein excretion by *C. pyrenoidosa* cells increased by application of auxins (indolyl-3-acetic, indolyl-3-propionic, indolyl-3-lactic, and indolyl-3-butyric acids, ~7- to 10-fold), by the auxin precursors anthranilic acid and tryptamine (250%–275%), and by chemical analogues of auxins such as 2,4-dichlorophenoxyacetic, phenylacetic, naphthyl-3-acetic, and naphthyl-3-sulphonic acids (187%–325%) (Czerpak and Bajguz 1993). In addition, application of exogenous IAA to *C. vulgaris* culture significantly increased cell multiplication (Gonzalez and Bashan 2000).

This study investigated how IAA, produced by the PGPB *Azospirillum* spp., may be involved in promoting growth of *C. vulgaris*, by cultivating three wild-type strains of *A. brasilense* and one wild strain of *A. lipoferum* and comparing the results with their comparable IAA-attenuated mutants. IAA production by the bacteria and its consumption by the microalga were measured with colorimetric and HPLC methods, and the data obtained were compared. As an additional proof for the involvement of IAA in growth of *C. vulgaris*, a complementation experiment was performed, in which the addition of IAA produced by the wildtype bacterium restored the capacity for promoting growth of its IAA-attenuated mutant.

#### MATERIALS AND METHODS

*Microorganisms and axenic growth conditions.* *C. vulgaris* (UTEX 2714) and seven strains of *Azospirillum* spp. (Table 1) were used. Prior to immobilization in beads, axenic *C. vulgaris* cultures were cultivated in sterile C30 mineral medium (Gonzalez et al. 1997) for 5–6 d under continuous agitation (150 rpm) at  $60 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  PAR and  $27^\circ\text{C}$ – $30^\circ\text{C}$ . All *Azospirillum* strains were grown in liquid nutrient broth (Sigma, St. Louis, MO, USA) at  $30 \pm 2^\circ\text{C}$  and agitation (120 rpm) for 17 h. For all experiments using the mutant strains, nutrient broth was supplemented with  $25 \mu\text{g} \cdot \text{mL}^{-1}$  kanamycin (Sigma).

TABLE 1. Production of indole-3-acetic acid (IAA) by wild and mutant bacteria strains of *Azospirillum* spp. used in this study and grown on Okon–Albrecht–Burriss (OAB) medium supplemented with tryptophan ( $200 \mu\text{g} \cdot \text{mL}^{-1}$ ) and ammonium ( $0.5 \text{ g} \cdot \text{L}^{-1} \text{NH}_4\text{Cl}$ ).

Bacteria strain	Description	Quantity of IAA produced in pure culture		Reference or source of the strain
		Salkowski ( $\mu\text{g} \cdot \text{mL}^{-1}$ )	HPLC ( $\text{ng} \cdot \mu\text{L}^{-1}$ )	
<i>A. brasilense</i> Cd	Type strain of <i>A. brasilense</i> (USA)	$91.33 \pm 0.26$	$44.01 \pm 0.17$	DSM* 1843
<i>A. brasilense</i> Sp245	Wildtype strain of <i>A. brasilense</i> (Brazil)	$52.4 \pm 0.7$	$44.53 \pm 0.78$	EMBRAPA-Rio de Janeiro, Brazil
<i>A. brasilense</i> Sp6	Wildtype strain of <i>A. brasilense</i> (Italy)	$126.6 \pm 0.33$	$51.18 \pm 0.81$	Barbieri and Galli (1993)
<i>A. lipoferum</i> JA4	Wildtype rhizosphere isolate (Brazil)	$160 \pm 0.01$	$40 \pm 0.01$	Castellanos et al. (1997)
<i>A. brasilense</i> FAJ0009	Mutant of <i>A. brasilense</i> Sp245 carrying Tn5 insertion in the <i>ipdc</i> gene (Belgium)	$23.8 \pm 0.2$	$6.96 \pm 0.01$	Vande Broek et al. (1999)
<i>A. brasilense</i> SpM7918	Derivative of <i>A. brasilense</i> Sp6, carrying another Tn5 insertion in the <i>ipdc</i> gene (Italy)	$38.8 \pm 0.6$	$15.53 \pm 0.4$	Barbieri and Galli (1993)
<i>A. lipoferum</i> JA4:: <i>ngfp</i> 15	<i>A. lipoferum</i> JA4 with a chromosomal insertion of a <i>mut2gfp</i> gene (Mexico)	$13.0 \pm 1.26$	$2.42 \pm 0.01$	Bacilio et al. (2004)

\*DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GMBH, Braunschweig, Germany.

Cultures of the seven strains of *Azospirillum* spp. used for evaluation of IAA production were grown as follows: each strain was incubated overnight in nutrient broth as described above and washed twice with saline solution (0.85% NaCl). The pellets were resuspended in Okon–Albrecht–Burriss (OAB) N-free medium (Bashan et al. 1993), and the optical density ( $OD_{540}$ ) was adjusted to 1.0 [ $10^9$  colony-forming units (CFU)  $\cdot$  mL $^{-1}$ ]. Two milliliters of the resuspended culture was inoculated in 25 mL fresh OAB medium supplemented with ammonium ( $0.5 \text{ g} \cdot \text{L}^{-1} \text{ NH}_4\text{Cl}$ ), tryptophan ( $200 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$ ), and  $25 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$  kanamycin for the mutant strains. This level of tryptophan is not deleterious to either *A. brasilense* or *C. vulgaris* (data not shown). The cultures were incubated for 48 h under the above conditions and centrifuged (Hermle model Z200A; Woodbridge, NJ, USA) for 10 min at 1,400g, the pellet was discarded, and the supernatant was transferred to clean test tubes for IAA analysis.

*Immobilization of microorganisms in alginate and cell counting.* These procedures were performed as described in de-Bashan et al. (2004). Briefly, axenic cultures (either *C. vulgaris* or *A. brasilense*) were mixed with 2% alginate solution. Initial cell concentrations among experiments was similar ( $0.5 \pm 0.2 \times 10^6$  cells  $\cdot$  mL $^{-1}$ ). The solution was dripped from a sterile syringe into 2% CaCl $_2$  solution, mixing the solution periodically. To immobilize the two microorganisms in the same bead, after washing the cultures, each of them was resuspended in 10 mL 0.85% saline solution and then mixed in the alginate. Because immobilization normally reduces the number of *Azospirillum* cells in the beads, a second overnight incubation of the beads in diluted nutrient broth was necessary.

For cell counting in each experiment, the beads were solubilized by immersing beads in 4% NaHCO $_3$  solution for 30 min. *A. brasilense* was counted by plating a series of dilutions (in 0.85% saline) on nutrient agar plates (Sigma), and *Chlorella* was counted using a light microscope (Zeiss K7C; Wetzlar, Germany) with a Neubauer hemocytometer (Hausser Scientific, Horsham, PA, USA).

*Culture conditions for co-immobilized microorganisms.* After secondary incubation, the beads were washed three times with sterile saline solution. For the experiments, the beads were incubated in synthetic growth medium (SGM, Gonzalez and Bashan 2000) containing the following: NaCl,  $7 \text{ mg} \cdot \text{mL}^{-1}$ ; CaCl $_2$ ,  $4 \text{ mg} \cdot \text{mL}^{-1}$ ; MgSO $_4 \cdot 7\text{H}_2\text{O}$ ,  $2 \text{ mg} \cdot \text{mL}^{-1}$ ; K $_2$ HPO $_4$ ,  $21.7 \text{ mg} \cdot \text{mL}^{-1}$ ; KH $_2$ PO $_4$ ,  $8.5 \text{ mg} \cdot \text{mL}^{-1}$ ; NaHPO $_4$ ,  $33.4 \text{ mg} \cdot \text{mL}^{-1}$ . The concentration of nitrogen was  $25 \text{ mg} \cdot \text{L}^{-1} \text{ NH}_4\text{Cl}$ . Tryptophan ( $200 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$ ; Sigma) was filter sterilized and added to sterile SGM. For a specific experiment, synthetic IAA (Sigma) was added to the medium ( $10 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$ ). Semi-continuous cultures were incubated in cycles of 48 h, in synthetic medium (100 mL) with replacement of the solution (in each cycle) for the next cycle, but using the same bead system for immobilization. This technique was developed for wastewater treatment using this bacteria-microalgae system, and it proved to be the most efficient for removing nutrients (N and P species) from wastewater (de-Bashan et al. 2002, 2004, Hernandez et al. 2006). Experiments were performed in nonbaffled Erlenmeyer flasks at  $28 \pm 2^\circ\text{C}$  at 150 rpm and light intensity of  $60 \text{ } \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ .

*Quantification of IAA-like molecules by spectrophotometric Salkowski's assay.* IAA that was quantified in this study is the exogenous IAA liberated by the microorganisms into the growth medium. For quantitative determination of IAA-like molecules, the colorimetric Salkowski's assay was performed (Tang and Bonner 1947). Salkowski's assay, a common assay for auxin determination (Patten and Glick 1996), has been successfully used in *Azospirillum* (Hartmann et al. 1983) and, recently, for IAA-like molecule quantification in *Pseudomonas putida* (Patten and Glick 2002). Every 48 h, the culture

supernatants (1 mL) were pipetted into test tubes and 4 mL Salkowski's reagent (150 mL concentrated H $_2$ SO $_4$  dissolved in 250 mL ddH $_2$ O) on ice to cool the reaction, and then 7.5 mL 0.5 M FeCl $_3$  was added to it. The tubes containing the mixture were gently vortexed and left for 20 min for development of pink color at ambient temperature ( $26 \pm 1^\circ\text{C}$ ). The intensity of the color was spectrophotometrically determined at  $OD_{535}$  (DR/2000; Hach, Loveland, CO, USA). SGM supplemented with tryptophan and Salkowski's reagent was used for blanks. Similarly, color was also developed in a standard solution of IAA to form a standard curve. Salkowski's method can easily be performed under common laboratory conditions and is far more handy than the specific HPLC method recommended for IAA determination (described below); therefore, it can be used for multiple experiments, as in this study. The best results from each run, using the Salkowski's technique, were kept frozen for verification and quantification by HPLC.

*Verification and quantification of IAA by HPLC.* The IAA content in each of the Salkowski's assays was also verified and quantified by HPLC, using a modification of the method described by Zakharova et al. (1999): 50  $\mu$ L supernatant of the immobilized cultures was injected into an HPLC (model Agilent 1200 series; Agilent Technologies, Santa Clara, CA, USA), using the column Ultrasphere ODS, reversed-phase 4.6 mm  $\times$  15 cm, and 5 mm pore size (Beckman Instruments Inc., San Ramon, CA, USA). Separation was performed using the two-phase solvent system: Phase A, potassium phosphate 50 mM, pH 3.0; Phase B, acetonitrile (HPLC quality) at a flow rate of the eluent of 1 mL  $\cdot$  min $^{-1}$  and detection at 220 nm. IAA was quantified by integrating areas under peaks with authentic IAA (Sigma) as the standard.

*Complementation experiment: adding IAA produced by wildtype bacterium to restore effect of its IAA-attenuated mutant.* Five milliliters of preinoculum of *A. brasilense* Sp6 (wildtype) culture was inoculated into 50 mL SGM medium supplemented with tryptophan ( $200 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$ ) and incubated for 72 h as described above. Then, the culture was centrifuged at 4000g; the pellet was discarded; the supernatant was recovered, filter sterilized (0.2  $\mu$ m, Acrodisc; Pall Corporation, Ann Arbor, MI, USA), and transferred to clean glass test tubes.

In the complementation experiment, *C. vulgaris* was immobilized alone and co-immobilized with *A. brasilense* SpM7918, the IAA-attenuated mutant of *A. brasilense* Sp6, as explained previously. After the secondary multiplication phase, 4 g of beads was inoculated into 100 mL SGM medium supplemented with  $200 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$  tryptophan. In one treatment, instead of adding tryptophan, 10 mL sterile supernatant (containing IAA) obtained from culture of the parental strain *A. brasilense* Sp6 was added to 90 mL SGM. This step was performed to ensure consumption of the added IAA without interference of the small amount of IAA produced by the IAA-attenuated mutant in the presence of tryptophan. Experiments were performed in batch culture, as described above, for 96 h. Every 48 h, 1 mL of the supernatant was taken from each culture to measure IAA, and five beads were dissolved for counting the microalgae.

*Experimental design and statistical analysis.* Each experiment was performed in triplicate, where an Erlenmeyer flask served as a replicate. Each experiment was repeated, in full, two or three times. Results among treatments were analyzed by one-way analysis of variance (ANOVA) at  $P \leq 0.05$  and then by Tukey's post hoc analysis, and differences between cycles of the same treatment by Student's *t*-test at  $P \leq 0.05$ , using Statistica<sup>TM</sup> software version 6.0 (StatSoft, Tulsa, OK, USA).

## RESULTS

*Effect of exogenous IAA on growth of C. vulgaris and consumption of IAA.* Under our experimental

conditions, *A. brasilense* Cd immobilized in alginate beads produces  $4\text{--}10\ \mu\text{g}\cdot\text{mL}^{-1}$  IAA (data not shown) during a cycle of 48 h, from which *C. vulgaris* absorbed between  $0.5$  and  $1\ \mu\text{g}\cdot\text{mL}^{-1}$  during this time. The addition of  $10\ \mu\text{g}\cdot\text{mL}^{-1}$  exogenous IAA significantly increased the population of *C. vulgaris* after the third cycle of incubation (48 h, Fig. 1A). The microalgae consumed  $\sim 1.3\ \mu\text{g}\cdot\text{mL}^{-1}$  of this IAA in cycles 2 and 3 (Fig. 1B).

*Effect of co-immobilization of C. vulgaris with A. brasilense Cd and Sp6 on microalgal growth and consumption of IAA.* Significant lowering of IAA concentration was detected when *C. vulgaris* was immobilized with *A. brasilense* Cd (Fig. 2C, compare  $\square$  to  $\blacksquare$ ). IAA levels were very low in cultures where the microalga was immobilized alone (Fig. 2C). Similarly, growth was promoted in the co-immobilized treatment

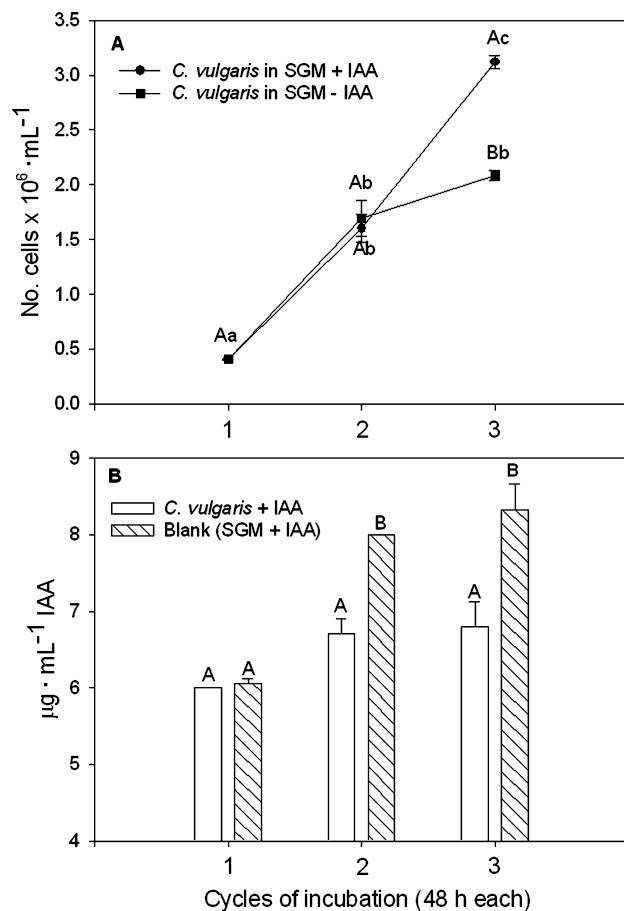


FIG. 1. Growth of *Chlorella vulgaris* in synthetic growth hormone (SGM) supplemented with  $10\ \mu\text{g}\cdot\text{mL}^{-1}$  of exogenous indole-3-acetic acid (IAA). (A) Growth of *C. vulgaris*. (B) Concentration of exogenous IAA after the growth of the microalga in the medium, measured by Salkowski's method.  $\blacksquare$  = Growth without IAA. Points on each curve denoted by a different lowercase letter differ significantly at  $P \leq 0.05$  in one-way analysis of variance (ANOVA), according to Tukey's post hoc analysis. Points denoted by different capital letters at each cycle of incubation differ significantly at  $P \leq 0.05$  in Student's *t*-test. Bars represent the SE; absence of bars indicates negligible SE.

(Fig. 2A). *A. brasilense* Sp6 produced higher amounts of IAA than *A. brasilense* Cd (Fig. 2D). When *C. vulgaris* was immobilized with *A. brasilense* Cd or *A. brasilense* Sp6, the microalga increased its growth more when co-immobilized with the PGPB than when it was grown alone. Co-immobilization with *A. brasilense* Sp6 yielded the highest population of the microalga. The difference in the amount of IAA accumulated in the microalga-free and the co-immobilized cultures (Fig. 2, C and D) was similar to the amount consumed by *C. vulgaris* cultures that were provided with exogenous IAA (Fig. 1B).

*Effect of immobilization of C. vulgaris with IAA-attenuated mutant of A. brasilense Sp6 on growth of microalgae and consumption of IAA.* The response of *C. vulgaris* to immobilization with the IAA-attenuated mutant of *A. brasilense* Sp6 (*A. brasilense* SpM7918) is shown in Figure 3. The growth of the microalga alone after two cycles is lower than when co-immobilized either with wild type *A. brasilense* Sp6 or its mutant (Fig. 3A). It is noteworthy that, after the first 48 h of incubation (Cycle 2), *C. vulgaris* grows better when co-immobilized with *A. brasilense* SpM7918, but in cycle 3, growth was similar, although there is no significant uptake of IAA from these cultures (Fig. 3, B and C). The parental strain, *A. brasilense* Sp6, significantly enhanced the growth of *C. vulgaris* during the second incubation (Fig. 3A). Consumption of IAA by the microalga in these cases is almost 50% of the amount produced by the bacteria (Fig. 3, B and C).

*Effect of co-immobilization of C. vulgaris with IAA-attenuated mutant of A. brasilense Sp245 on growth of microalga and consumption of IAA.* Similar experiments were conducted with the second IAA-attenuated mutant, *A. brasilense* FAJ0009, which was modified from the wildtype *A. brasilense* Sp245. This mutant showed no effect on growth of *C. vulgaris* when they are co-immobilized (Fig. 4A). Because there is very low IAA production by the mutant (Fig. 4, B and C), uptake by the microalga was not detected. As with other wildtypes (Cd and Sp6), the better growth response of *Chlorella* occurred with wild *A. brasilense* Sp245.

*Effect of co-immobilization of C. vulgaris with IAA-attenuated mutant of A. lipoferum JA4 on growth of microalga and consumption of IAA.* In the first 48 h of incubation, there was a significant increase in the microalga population when co-immobilized with the wild or mutant *A. lipoferum*, compared with the microalga alone (Fig. 5A). Later, the microalga growth is significantly better with wildtype *Azospirillum*. Analyzing the IAA concentration in the cultures indicates that  $>50\%$  of the IAA produced by *A. lipoferum* JA4 strain is consumed by the microalga (Fig. 5, B and C).

*Complementation experiment: addition of IAA produced by wildtype bacterium to restore effect of its IAA-attenuated mutant.* Supplementing a co-immobilized culture of *C. vulgaris* and IAA-attenuated mutant *A. brasilense*

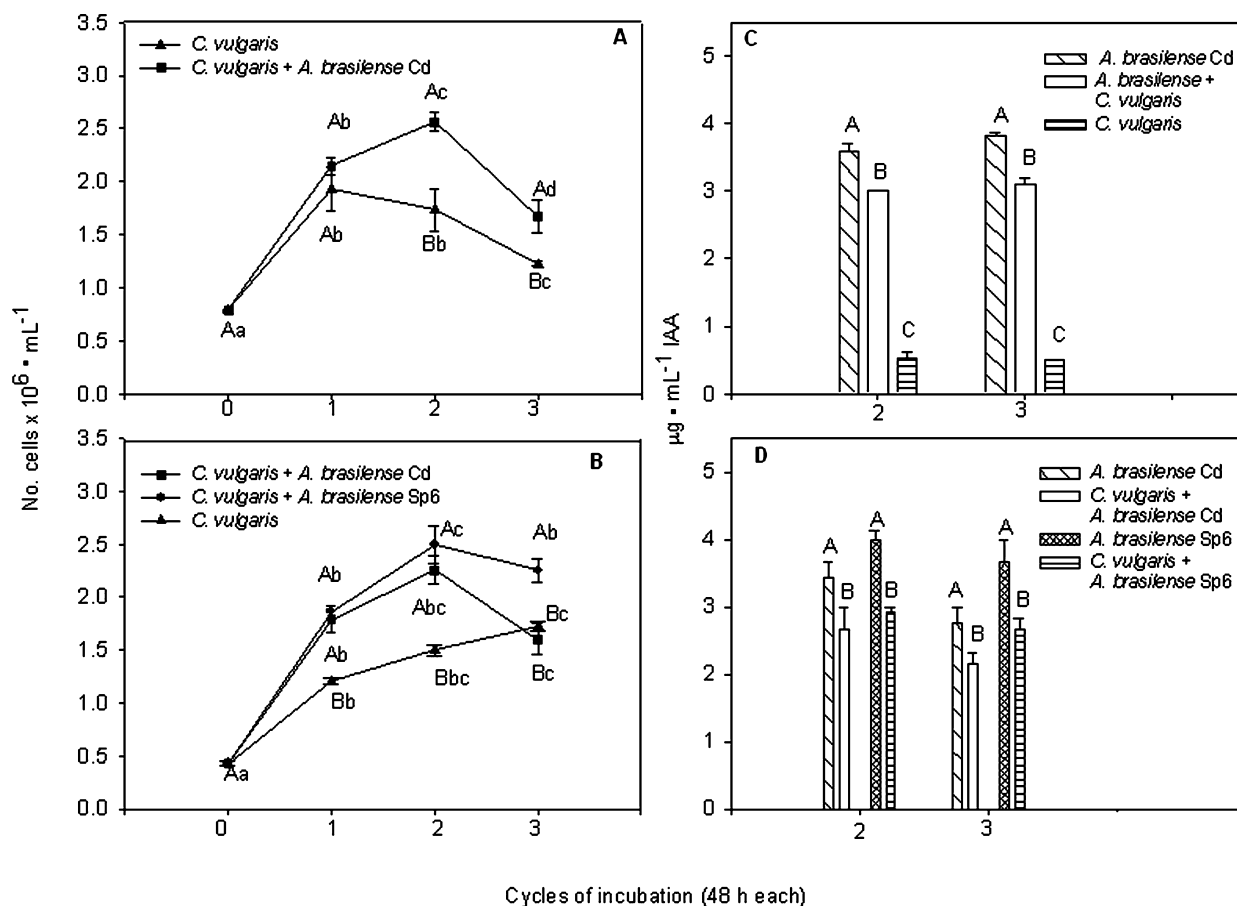


Fig. 2. *Chlorella vulgaris* co-immobilized with *Azospirillum brasilense* strains Cd and Sp6, in synthetic growth medium (SGM) supplemented with 200  $\mu\text{g} \cdot \text{mL}^{-1}$  tryptophan added in the second cycle. (A, B) Growth of the microalga. (C, D) Concentrations of exogenous indole-3-acetic acid (IAA) produced in the cultures.  $\blacktriangle$  = *C. vulgaris* alone. Points on each curve denoted by a different lowercase letter differ significantly at  $P \leq 0.05$  in one-way analysis of variance (ANOVA), according to Tukey's post hoc analysis. Points denoted by different capital letters at each cycle of incubation differ significantly at  $P \leq 0.05$  in Student's *t*-test. Bars represent the SE; absence of bars indicates negligible SE.

SpM with culture filtrate of its parental strain *A. brasilense* Sp6 that contains IAA ( $13.22 \mu\text{g} \cdot \text{mL}^{-1}$ ) restored the growth promotion effect to the culture to even larger populations of the microalga than in the presence of the parental strain (compare Fig. 6A to Fig. 3A). The IAA-attenuated mutant affected the growth of the microalga less after incubation for 96 h (Fig. 6A). At the same time, the IAA from the supernatant of the parental strains was consumed and almost completely depleted after incubation for 96 h (Fig. 6B); at that time, the population of the microalga reached its peak (Fig. 6A). The microalga itself produced negligible amounts of IAA ( $0.05\text{--}0.1 \mu\text{g} \cdot \text{mL}^{-1}$ ), while the IAA-attenuated mutant produced far smaller amounts of IAA than the wildtype, even when the microalga was present (Fig. 6B).

*Highest C. vulgaris* populations developed in association with *Azospirillum* spp. The largest populations of *C. vulgaris* developed in association with strains of *Azospirillum* spp. are summarized in Table 2. The

largest population developed occurred when *C. vulgaris* was co-immobilized with *A. brasilense* Sp6 (Table 2). *A. brasilense* Sp6 also produced the largest amount of IAA in culture (Table 2).

#### DISCUSSION

Although IAA presence in green microalgae (like *Chlorella*) is known (Dibb-Fuller and Morris 1992, Mazur et al. 2001), green algae do not produce high levels of exogenous auxin-like compounds (Stirk et al. 2002), and only scant information is available on the mechanisms of auxin transport in microalgae (Czerniak and Bajguz 1993, Czerniak et al. 1999, Gonzalez and Bashan 2000). Our results showed extremely low production of exogenous IAA with *C. vulgaris*, comparable to the known levels for *C. pyrenoidosa* (Mazur et al. 2001).

Information about the *Azospirillum* genes involved in IAA biosynthesis has been available (Costacurta et al. 1994, Steenhoudt and Vanderleyden 2000,

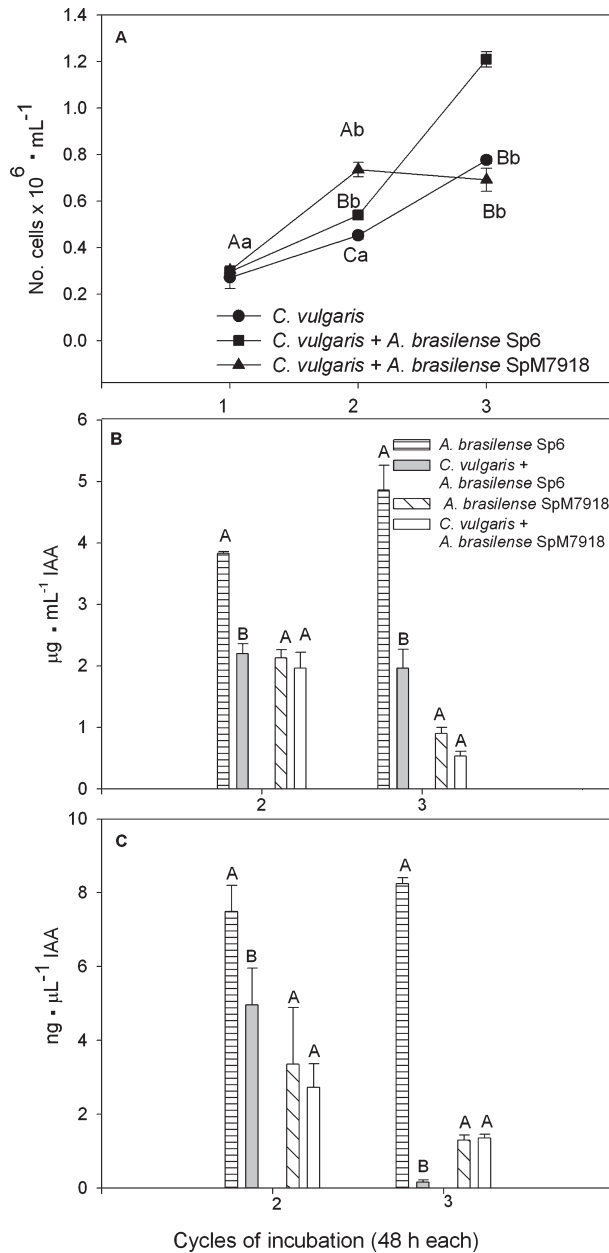


FIG. 3. *Chlorella vulgaris* co-immobilized with *Azospirillum brasilense* strain Sp6 and its IAA-attenuated mutant SpM7918 in synthetic growth medium (SGM) supplemented with  $200 \mu\text{g} \cdot \text{mL}^{-1}$  tryptophan. (A) Growth of the microalga. (B) Concentrations of exogenous IAA-like molecules by Salkowski's method. (C) Concentration of exogenous IAA produced in the medium and evaluated by HPLC method. ● = *C. vulgaris* alone. Points on each curve denoted by a different lowercase letter differ significantly at  $P \leq 0.05$  in one-way analysis of variance (ANOVA), according to Tukey's post hoc analysis. Points denoted by different capital letters at each cycle of incubation differ significantly at  $P \leq 0.05$  in Student's *t*-test. Bars represent the SE; absence of bars indicates negligible SE. IAA, indole-3-acetic acid.

Dobbelaere et al. 2003, Ona et al. 2003, 2005, Spaepen et al. 2007) ever since production of plant hormones was detected in this genus from the onset of its discovery (Tien et al. 1979). Katzy et al. (1990)

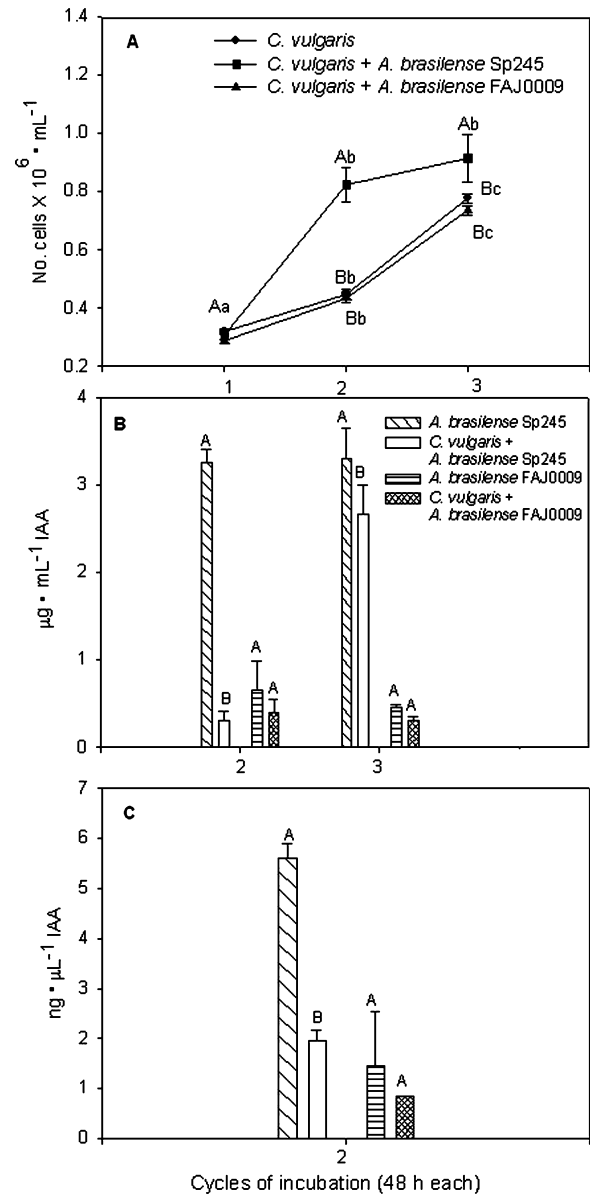


FIG. 4. *Chlorella vulgaris* co-immobilized with *Azospirillum brasilense* strain Sp245 and its IAA-attenuated mutant FAJ0009 in synthetic growth medium (SGM) with  $200 \mu\text{g} \cdot \text{mL}^{-1}$  tryptophan. (A) Growth of the microalga. (B) Concentration of exogenous IAA produced in the medium analyzed by Salkowski's method. (C) Concentration of exogenous IAA produced in the medium by HPLC method. ● = *C. vulgaris* alone. Points on each curve denoted by a different lowercase letter differ significantly at  $P \leq 0.05$  in one-way analysis of variance (ANOVA), according to Tukey's post hoc analysis. Points denoted by different capital letters at each cycle of incubation differ significantly at  $P \leq 0.05$  in Student's *t*-test. Bars represent the SE; absence of bars indicates that the SE is negligible. IAA, indole-3-acetic acid.

determined that IAA production in *A. brasilense* strain Sp245 is controlled by the 85-Mda plasmid that occurs naturally in this bacterium. So far, it has not been possible to isolate *Azospirillum* mutants that were completely unable to synthesize IAA, resulting from chemical or Tn5 mutagenesis. Only a few

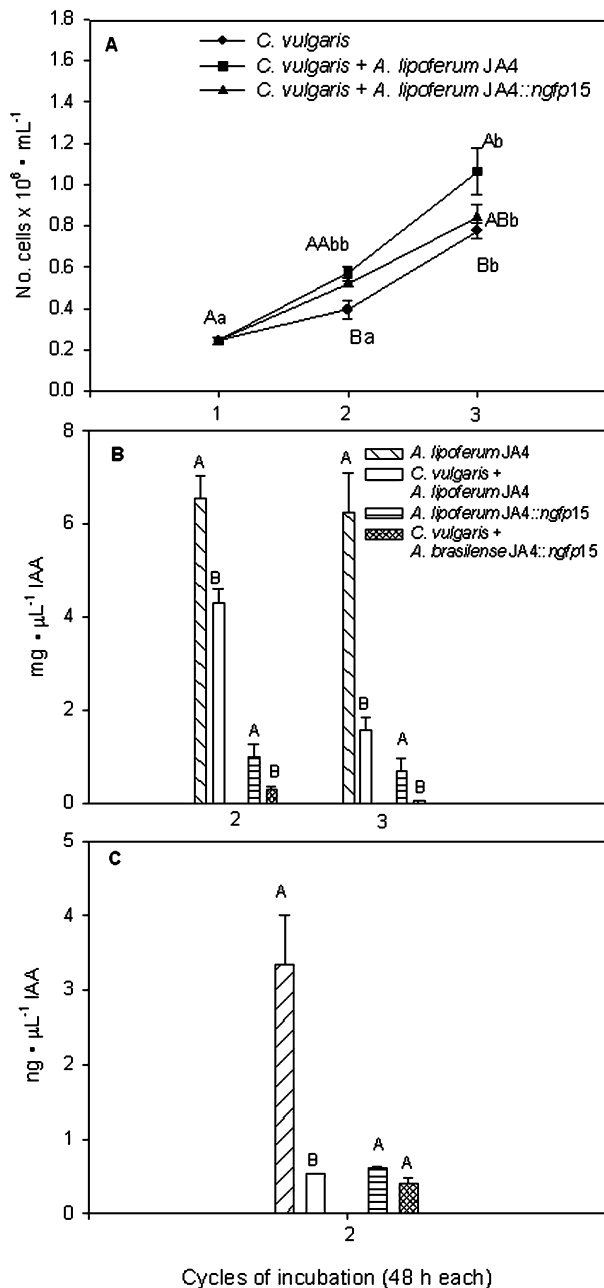


FIG. 5. *Chlorella vulgaris* co-immobilized with *Azospirillum lipoferum* strain JA4 and its IAA-attenuated mutant JA4::ngfp15 in synthetic growth medium (SGM) with  $200 \mu\text{g} \cdot \text{mL}^{-1}$  tryptophan. (A) Growth of the microalga. (B) Concentration of exogenous IAA produced in the medium analyzed by Salkowski's assay. (C) Concentration of exogenous IAA produced in the medium analyzed by HPLC method. ● = *C. vulgaris* alone. Points on each curve denoted by a different lowercase letter differ significantly at  $P \leq 0.05$  in one-way analysis of variance (ANOVA), according to Tukey's post hoc analysis. Points denoted by different capital letters at each cycle of incubation differ significantly at  $P \leq 0.05$  in Student's *t*-test. Bars represent the SE; absence of bars indicates that the SE is negligible. IAA, indole-3-acetic acid.

IAA-attenuated strains were produced. This occurs because *Azospirillum* spp. produces IAA via an unstable indole pyruvic acid intermediate. The

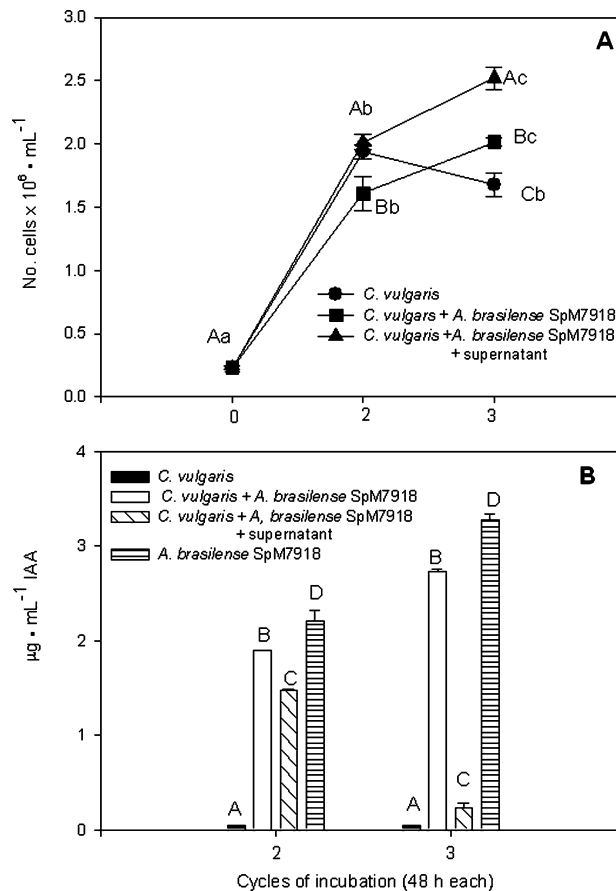


FIG. 6. *Chlorella vulgaris* immobilized with IAA-attenuated mutant SpM7918 in synthetic growth medium (SGM) supplemented with 10 mL supernatant of *Azospirillum brasilense* Sp6 containing  $13.22 \mu\text{g} \cdot \text{mL}^{-1}$  IAA. (A) Growth of the microalga. (B) Concentrations of exogenous IAA-like molecules using Salkowski's method. ● = *C. vulgaris* alone. Points on each curve denoted by a different lowercase letter differ significantly at  $P \leq 0.05$  in one-way ANOVA, according to Tukey's post hoc analysis. Points denoted by different capital letters at each time of incubation differ significantly at  $P \leq 0.05$  in one-way analysis of variance (ANOVA). Columns denoted by different capital letters at each time of incubation differ significantly at  $P \leq 0.05$  in one-way ANOVA. Bars represent SE; absence of bars indicates negligible SE. IAA, indole-3-acetic acid.

accumulated intermediate, in most mutants, spontaneously breaks down and produces some IAA (Steenhoudt and Vanderleyden 2000). These known mutants produce 0.2%–5% of the level of IAA produced by the wildtype. One strain of *A. irakense* released about one-tenth the amount of IAA into the medium than *A. brasilense* Sp7 (Zimmer et al. 1991). Two mutants of *A. brasilense* produced only 2%–5% of the IAA produced by the parental strains (Prinsen et al. 1993, Vande Broek et al. 1999). A mutant of *A. brasilense* that was modified to harbor the *gfp* gene produced <0.25% IAA relative to its parental strain (Rodriguez et al. 2006). Zimmer et al. (1991) proposed that *Azospirillum* possesses either more than one copy of the genes involved in IAA biosynthesis or has more than one pathway for synthesizing IAA.

TABLE 2. Maximum production of indole-3-acetic acid (IAA) by wild and mutant bacteria strains of *Azospirillum* spp. in immobilized systems and the largest population of *Chlorella vulgaris* developed when immobilized with these strains.

Bacterial strain	Maximum production of IAA ( $\mu\text{g} \cdot \text{mL}^{-1}$ )*	Maximum population of <i>C. vulgaris</i> (cells $\cdot \text{mL}^{-1}$ )
<i>A. brasilense</i> Sp 6	7.76 $\pm$ 0.14 a	2.49 $\pm$ 0.17 a
<i>A. brasilense</i> Cd	6.16 $\pm$ 0.16 b	2.25 $\pm$ 0.13 a
<i>A. brasilense</i> Sp245	3.3 $\pm$ 0.35 c	1.52 $\pm$ 0.08 b
<i>A. lipoferum</i> JA4	6.10 $\pm$ 0.4 b	1.06 $\pm$ 0.11 c
<i>A. brasilense</i> FAJ0009	2.1 $\pm$ 0.35 d	1.08 $\pm$ 0.02 cd
<i>A. brasilense</i> SpM7918	2.13 $\pm$ 0.13 d	1.31 $\pm$ 0.11 bcd
<i>A. lipoferum</i> JA4::ngfp15	1.0 $\pm$ 0.26 d	0.77 $\pm$ 0.05 cd
None	ND	1.38 $\pm$ 0.12 bc

Numbers denoted by different letters in each column differ significantly at  $P \leq 0.05$  by one-way analysis of variance (ANOVA).

ND, not detected.

\*Colorimetric method.

To demonstrate involvement of IAA produced by *A. brasilense* on population growth of the microalga *C. vulgaris*, it would be preferable to use IAA-deficient mutants, but such mutants do not exist. Two attenuated mutants (Barbieri and Galli 1993, Vande Broek et al. 1999) and an IAA-attenuated mutant of *A. lipoferum* were employed in this study. This study showed that wildtype strains of *A. brasilense* (Sp6 and Sp245) and *A. lipoferum* (JA4) enhanced the growth of *C. vulgaris* in a fashion similar to the known control strain *A. brasilense* Cd, when co-immobilized with the microalga (Gonzalez and Bashan 2000); all four wildtypes produced significant amounts of IAA. However, our data show that there is an upper limit of IAA, its concentration unknown as yet, that the microalga can respond to; although *A. brasilense* Sp6 produced more IAA than *A. brasilense* Cd, its capacity to enhance microalgal growth was not greater. In contrast, when *C. vulgaris* was co-immobilized with IAA-attenuated mutants, the concentration of IAA in the medium was low and had an insignificant effect on population growth of the microalga; concomitantly, consumption of IAA from the medium by the co-immobilized microalga was lower ( $0.8 \mu\text{g} \cdot \text{mL}^{-1}$ , the maximum amount consumed when growing with the mutants) than the level when the microalga was growing alone ( $1.2$ – $1.5 \mu\text{g} \cdot \text{mL}^{-1}$ ). In a complementation experiment, the culture supernatant, containing IAA from a parental strain, was added to cultures of the microalga and an IAA-attenuated mutant of this strain that has no growth-promotion effect. This supernatant restored growth promotion to the microalgal culture. Fluctuation in growth of the microalga when the mutants are used can be explained by the fact that these strains produce 2%–5% IAA of

the parental strain; variable amounts of IAA affect the growth of the microalgal population. Cultivation factors, such as nitrogen species, pH, and presence of a carbon source, were recently demonstrated to control the population of *C. vulgaris* (de-Bashan et al. 2005). Release of large amounts of IAA by *Azospirillum* spp. cultures is probably controlled by the stationary phase of the bacteria cells after depletion of the carbon source in the medium used in batch culture, which causes arrest of growth (Ona et al. 2003, 2005). This phenomenon may explain why major IAA-enhanced growth of *C. vulgaris* occurred only after three cycles of growth and one of the benefits it draws from its association with *Azospirillum* spp. is a supply of IAA.

Since no measurable, liberated IAA production occurred without tryptophan (data not shown), our results corroborate previous observations that in vitro IAA production in *Azospirillum* is induced by the presence of tryptophan (Fallik et al. 1989, Katzy et al. 1990, Zimmer et al. 1991). *A. brasilense* Cd can promote the growth of *C. vulgaris* even without the addition of exogenous tryptophan (Gonzalez and Bashan 2000). In such cases, no measurable exogenous IAA can be detected in the growth medium. It is assumed that all bacteria-produced IAA was consumed by the microalga. By adding tryptophan to the culture medium, as demonstrated in this study, detection of exogenous IAA in the culture medium was possible. Similarly, the phytostimulatory auxin effect of *Azospirillum* sp. on wheat was further enhanced by adding tryptophan and could be mimicked by replacing *Azospirillum* sp. cells by IAA (Dobbelaere et al. 1999). The usefulness of co-immobilization technology to treat domestic wastewater has been demonstrated (de-Bashan et al. 2004, Hernandez et al. 2006), although scaling up is still under development (L. E. de-Bashan, unpublished data). The basic microalga-bacteria interaction investigated here demonstrated one possible mechanism responsible for the successful operation of this microalgal technology.

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