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Joint Immobilization of Plant Growth-Promoting Bacteria and Green Microalgae in Alginate Beads as an Experimental Model for Studying Plant-Bacterium Interactions▽

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A simple, quantitative experimental model, offering a convenient and basic approach to studies of plant-bacterium interactions, is proposed. This involves immobilizing a unicellular, freshwater microalga, a *Chlorella* species, serving as the plant, with a plant growth-promoting bacterium, an *Azospirillum* species, in small alginate beads to allow close interaction and to avoid external interference from bacterial contaminants.

A major obstacle in the study of interactions between prokaryotes and eukaryotes is the complexity of the eukaryote partner, the latter species usually of some economic importance. Studies of basic plant-bacterium interactions of *Azospirillum* spp., plant growth-promoting bacteria (PGPB), done mainly with cereal roots, are difficult because there are multiple tissue functions and numerous possible interactions with plant roots and there is interference with the soil matrix. Consequently, there is a significantly greater quantity of information on the bacteria than on the interactions (5, 55, 56).

The *Arabidopsis thaliana* (mouse-ear cress) plant model is widespread, mainly because its 125-Mb genome is small and sequenced (1), and numerous mutants of most of its metabolic pathways are available. The rice plant has a 389-Mb genome (31) and may soon provide research opportunities with PGPB in addition to the extensive research already done with this plant (33, 34, 46, 61). However, larger plant genomes in which these PGPB commonly interact, such as maize (2.5 Gb), oat (11.4 Gb) (2), and wheat (16 Gb) (20), are unlikely to be understood in detail soon, even though some are undergoing sequencing procedures. Microalgae, on the other hand, have the smallest plant genome (~40 Mb), as yet not sequenced (V. Huss, personal communication). To our knowledge, the *Chlorella* genome is the smallest eukaryotic, photosynthetic microorganism characterized so far (28), which makes it an alternative to higher plants with large genomes interacting with PGPB.

Chlorella spp. (Chlorophyceae) are simple, nonmotile, unicellular, aquatic green microalgae. They were one of the first algae to be isolated as a pure culture in 1890 by Martinus Beijerinck (43). *Chlorella* has been used in studies of photosynthesis and respiration (30). Much of the knowledge on the synthesis of carbohydrates in microalgae has been obtained with this species (29, 49). Since the late 1940s, attention has been drawn to the potential of mass cultivation of this microalga for the production of high-value, low-volume compounds, such as pigments for food industries, including the health-food market in industrialized countries (52), and application in wastewater treatment (45). Except for symbiotic rhizobia, *Azospirillum* is the most studied agricultural PGPB (4). It is a rhizosphere-dwelling, N_2-fixing bacterium that is very versatile in its nitrogen transformations. In addition to fixing N_2 under microaerobic conditions, it denitrifies under anaerobic or microaerophyllic conditions and can assimilate NH_4^+, NO_3^-, or NO_2^- (25) and act as a general PGPB for numerous plant species (5), including *Chlorella* (21).

Alginate is the most commonly used polymer for microbial cell encapsulation, also called immobilization (54). The polymeric chain is made of mannuronic and guluronic acids in various proportions and sequential arrangements linked by calcium ions by binding consecutive blocks of guluronic acid to form gels (41).

The immobilization of microorganisms trapped in alginate beads is a widely used technique for viable microbial cells (44). It is used in several biotechnological fields: (i) as a practical way to immobilize microbes producing secondary metabolites of commercial value, (ii) as a tool in genetic manipulation to immobilize recombinant bacteria to improve plasmid stability (53), and (iii) to remove inorganic compounds and heavy metals from wastewater (58), where its greatest advantage is that it significantly facilitates harvesting of mass-produced microalgae from the water after treatment (15). Those are the three basic components of the experimental model that we are proposing.

Although several technical aspects of this model that show its use for supporting studies on wastewater treatment have previously been published (13), this is the first presentation of the entire model, as a vehicle to study plant-bacterium interactions.

Chlorella vulgaris Beijerinck (UTEX 2714; University of Texas, Austin) and *Chlorella sorokiniana* Shih. et Krauss (UTEX 1602) were used. The PGPB partners were *Azospirillum brasilense* Cd (DMS 1843; Brunswick, Germany), *A. brasilense* Sp6 (3), and *Bacillus pumilus* ES4 (formerly *Bacillus chitinolyticus* [48]). The

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TABLE 1. Studies of paired microalga-PGPB interactions using the proposed model

Type of study	*Chlorella* species	PGPB strain(s)	Reference(s)
Growth promotion (dry wt, cell no., colony size, cell size)	*C. vulgaris*	*A. brasilense* Cd, Sp6, Sp245; *Azospirillum lipoferum* JA4	9, 21
	C. sorokiniana	*A. brasilense* Cd; *Bacillus pumilus*	14, 27
Hormones	*C. vulgaris*	*A. brasilense* Cd, Sp6, Sp245, FAJ0009, SpM7918; *A. lipoferum* JA4, JA4::*ngfp15*	9
Absorption of nitrogen and phosphorus	*C. vulgaris*, *C. sorokiniana*	*A. brasilense* Cd; *Phyllobacterium myrsinacearum*; *Bacillus pumilus*	11, 13, 22, 26, 27, 60
Photosynthetic pigments	*C. vulgaris*	*A. brasilense* Cd	10
Lipids	*C. vulgaris*	*A. brasilense* Cd	10
Modification of fatty acids	*C. vulgaris*	*A. brasilense* Cd	10
Cell-cell interactions	*C. vulgaris*	*A. brasilense* Cd; *Phyllobacterium myrsinacearum*	36
Delayed senescence	*C. vulgaris*	*A. brasilense* Cd; *Phyllobacterium myrsinacearum*	22, 36
Population control	*C. vulgaris*	*A. brasilense* Cd	8
Population dynamics	*C. vulgaris*	*A. brasilense* Cd	10
Mitigation of heat and intense sunlight	*C. sorokiniana*	*A. brasilense* Cd	14
Nutrient starvation	*C. sorokiniana*	*A. brasilense* Cd	26
Mitigation of pH inhibition	*C. vulgaris*	*A. brasilense* Cd	8
Mitigation of tryptophan inhibition	*C. vulgaris*	*A. brasilense* Cd	This study
Enzymes in the nitrogen cycle	*C. vulgaris*	*A. brasilense* Cd	12

procedures for culturing, immobilizing, and counting these microorganisms were described by de-Bashan et al. (8, 11). The growth promotion, ammonium assimilation, protein content, and activity of the enzyme glutamate dehydrogenase were measured as previously described (8, 12, 14, 21). All experiments were done in triplicate, where an Erlenmeyer flask served as a replicate. Each experiment was repeated two or three times in full. Results between treatments were analyzed by Student's t test at a P value of ≤ 0.05; differences between cycles of the same treatment were analyzed by one-way analysis of variance (ANOVA) at a P value of ≤ 0.05 and then by Tukey's post hoc analysis. Computations were made with STATISTICA software, version 6.0 (StatSoft, Tulsa, OK).

The following sequence of events occurs during the interaction between the two microorganisms within the polymeric sphere (Table 1). Initial immobilization of *Chlorella* spp. with a PGPB strain is described in Fig. 1A and is characterized as random immobilization of particles inside a gel matrix. Nutrients in the surrounding medium freely diffuse in the porous gel. Over time (6 to 48 h), depending on the microalga-bacterium combination, both microorganisms are found in the same cavity within the sphere, mainly just beneath the periphery of the sphere, after small parts of the internal structure of the matrix dissolve or split and separate as internal microcolonies develop and enlarge (Fig. 1B) (36). The bacteria excrete indole-3-acetic acid (IAA) and other undefined signal molecules, possibly reaching the nearby microalgal cells. At this stage, the activities of the microalgal enzymes (two were tested so far, glutamine synthetase and glutamate dehydrogenase) are not enhanced (Fig. 1B). At the next phase of interaction, beginning 48 h after joint immobilization and continuing later, the following happens: glutamate synthetase and glutamate dehydrogenase activities are enhanced, photosynthetic pigment production is enhanced, nitrogen and phosphorus uptake into microalgal organelles is enhanced, and the jointly immobilized system liberates oxygen produced by *Chlorella* spp. as a byproduct of photosynthesis (Fig. 1C). The most noticeable effect of joint immobilization is that the population of microalgae increases two- to threefold over that of immobilized microalgae (21). Experimental testing of the proposed model used several microalga-PGPB pairs. Enhanced growth promotion was demonstrated in the joint immobilization system of *C. sorokiniana* and *B. pumilus* (Fig. 1D) and that of *C. vulgaris* and *A. brasilense* Sp6 (Table 2). Enhanced ammonium absorption occurred in the same systems (Fig. 1E) at the culture and cellular levels (Table 2). The enhancing effect of several concentrations of exogenous tryptophan (precursor of the phytohormone IAA and the main mechanism by which *Azospirillum* affects the growth of *Chlorella* [9]) was tested with a combination of *C. vulgaris* and *A. brasilense* Cd (Fig. 1F to I). The increase in the production of protein was evaluated with a combination of *C. vulgaris* and *A. brasilense* Sp6 at two ammonium concentrations (Table 2), and the enhancement of the activity of glutamate dehydrogenase, a key enzyme in ammonium assimilation in plants, was evaluated with the same microalgal species but was paired with *A. brasilense* strain Sp6 (Table 2). Taken as a whole, these additional studies show that this model can serve as an appropriate experimental tool to study events related to growth and nitrogen and phytohormone cycles in eukaryotes.

The usefulness of a simple experimental model for basic studies of complex interactions between plants and bacteria is obvious. This study suggests such a model. This model involves interaction between two well-studied microorganisms, *Azospirillum* and *Chlorella*, entrapped in a gel matrix.

The microorganisms *Chlorella* and *Azospirillum* were chosen for their unique characteristics. *Chlorella* possesses one of the smallest plant genomes (28) and has been one of the best-studied microalgae for almost a century. *Azospirillum* is the most studied associated PGPB (5, 25). There are numerous available mutants of these genera (3, 6, 7, 18, 24, 32, 35, 56, 59), and many species and strains are easily obtained from most culture collections. Both *Chlorella* and *Azospirillum* are easy to cultivate and maintain. Growth conditions for both organisms are straightforward and can be either autotrophic or heterotrophic. Immobilization inside a harmless and nontoxic alginate gel matrix ensures that this artificial pairing of the microorganisms limits the movement of the motile *Azospirillum*

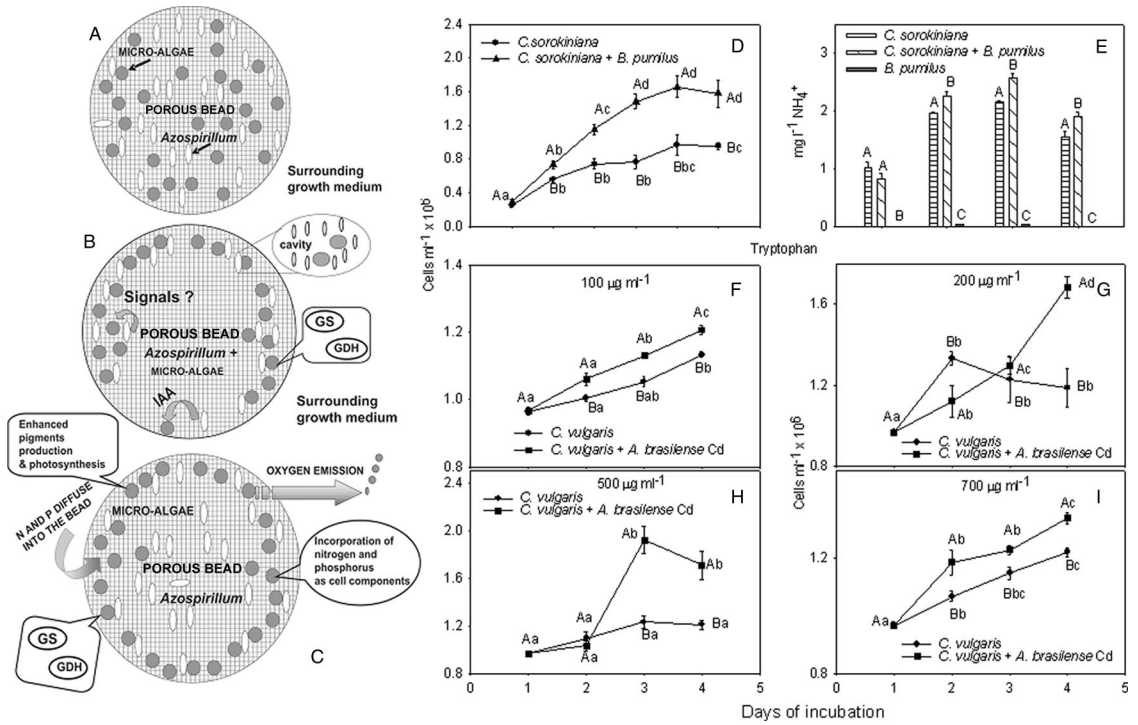

FIG. 1. Conceptual model for jointly immobilizing microalgae and PGPB in alginate spheres and testing of the model. (A) Both microorganisms are randomly immobilized in the polymeric matrix. (B) After cocultivating for a short period of time, the two microorganisms share the cavities within the sphere. Here, the PGPB excrete IAAs (and possibly other plant hormones) that enhance the growth of microalgae. (C) Nutrients from the medium diffuse into the sphere and are assimilated into cell components of the microalgae, leading to increasing population biomass and releasing oxygen to the medium. The size is not to scale. (D) Promotion of growth of *C. sorokiniana* by *B. pumilus*. (E) Enhancement of assimilation of ammonium in *C. sorokiniana* by *B. pumilus*. (F to I) Promotion of growth of *C. vulgaris* by *A. brasilense* Cd at four concentrations of tryptophan. The points on each curve denoted with different lowercase letters differ significantly at a P value of ≤ 0.05 by one-way ANOVA, according to Tukey's post hoc analysis. The points denoted by different capital letters at each cycle of incubation differ significantly at a P value of ≤ 0.05 by Student's t test. Columns denoted by different capital letters at each cycle of incubation differ significantly at a P value of ≤ 0.05 by one-way ANOVA. Whisker bars represent standard errors. The absence of a bar in panel E indicates a negligible value. GS, glutamine synthetase; GDH, glutamate dehydrogenase.

strain; consequently, the two usually affect each other, although immobilization has only a facilitating role in the interaction. The immobilization and liberation of both microorganisms from alginate beads is common microbial inoculant technology. The alginate from the giant kelp *Macrocystis pyrifera* is commonly used in food, medicine and dentistry, and pigment production and is easy to handle and very inexpensive (41).

These favorable characteristics notwithstanding, the model is not restricted to the *Chlorella vulgaris-Azospirillum brasilense* interactions that have comprised most of the studies done so far. Other PGPB, such as *B. pumilus*, and other microalgae, such as *C. sorokiniana*, were successfully tested (Table 1). These options create opportunities for endless combinations of microalgae and PGPB. Similarly, different alginates and derivatives from many macroalgae are commercially available (41, 60), even from bacteria (19), for entrapment combination schemes, as needed. Because the immobilization of microorganisms is also commonly used with other polymers (44), this model is not restricted to alginates, but each polymer has its own advantages and disadvantages.

Apart from easy technical handling of this experimental model, the logic of choosing a microalga as the plant partner is simple. The most basic definition of a green plant is that it contains chlorophylls *a* and *b*, starch as storage material inside the chloroplast, and a cell wall made of cellulose (51). Systematically, higher plants and algae are part of the same group (Chlorobionta). There is 70 to 98% genetic similarity between land plants and algae (16). The size of the organism, the number of cells, and differentiation into organs are not defining parameters of a plant. Consequently, single-celled microalgae are considered plants. The bacterial partner, *Azospirillum*, was chosen because, although its origin and main effects were studied in conjunction with agricultural plants (5), it also affects the growth of microalgae; hence, it has been designated a growth-promoting bacterium for microalgae (8, 9, 10, 11, 12, 13). It is likely that these bacteria should have a significant effect on many metabolic pathways during their interaction with microalgae.

Additionally, the use of a green microalga as the plant partner in studies of plant-bacterium interactions has practical advantages and has already been tested: (i) phytohormones that affect plants affect microalgae, including IAA, indole-3-butyric acid, jasmonic acid, gibberellins, and cytokinins (17, 40, 43, 57); (ii) enzymes associated with the nitrogen cycle, such as glutamate dehydrogenase, glutamine synthetase, and glutamate synthase, are similar in plants and green microalgae; (iii) the processes of lipid accumulation are similar; (iv) photosyn-

TABLE 2. Effects of joint immobilization of *Chlorella* and *Azospirillum* on growth rate, absorption of ammonium, protein content, and glutamate dehydrogenase activity incubation for 48 h[a]

Growth rate (K)[b]		Growth rate (K)[c]		Absorption of ammonium per cell (ng cell^{-1})[d]		Absorption of ammonium per culture (mg liter^{-1})[d]		Protein content (mg ml^{-1})[b]		Protein content (mg ml^{-1})[d]		Activity of glutamate dehydrogenase (A_{340} min^{-1} cell^{-1}, 10^{-6})[d]	
Cv	Cv + AbSp6	Cv	Cv + AbSp6	Cv	Cv + AbSp6	Cv	Cv + AbSp6	Cv	Cv + AbSp6	Cv	Cv + AbSp6	Cv	Cv + AbSp6
0.33	0.56	0.58	0.70	4.1 ± 0.0004 a	5.6 ± 0.0004 b	3.1 ± 0.2 a	4.2 ± 0.5 b	0.23 ± 0.01 a	0.55 ± 0.09 b	0.33 ± 0.08 a	0.45 ± 0.02 b	5.57 ± 0.04 a	6.51 ± 0.2 b

[a] $K = \ln (N_1/N_0)/(t_1 - t_0)$, where N_1 and N_0 are cells at day 1 and time zero, respectively. Cv, *Chlorella vulgaris*; AbSp6, *Azospirillum brasilense* Sp6. Values denoted by different lowercase letters at each parameter differ significantly at a P value of ≤0.05 by Student's t test. Values are means ± standard errors.
[b] Initial concentration of ammonium, 3 mg liter^{-1}.
[c] Initial concentration of ammonium, 10 mg liter^{-1}.
[d] Initial concentration of ammonium, 8 mg liter^{-1}.

thetic pigments and photosynthesis are similar (Table 1); and (v) many species and strains of microalgae are available for experimentation.

The usefulness of molecular and biochemical studies can be illustrated with a study using various IAA-attenuated mutants of several *Azospirillum* species. These mutants failed to promote the growth of the microalgal cells compared to wild types (9). Similarly, slight changes in the enzymatic activities of glutamate dehydrogenase and glutamine synthetase could be accurately measured following joint immobilization of the two microorganisms (12). There are additional hypothesized possibilities for this model, including the study of bacterial interactions with cell walls of plants. The cell walls of green microalgae are related and have many structural features in common with those of land plants. Both types of cell walls contain cellulose, hemicellulose, pectin, and glycoproteins, although the chemical composition of green microalgal cell walls is more diverse (38, 39, 42, 47). The general similarity raises the potential usefulness of this model to study the invasive entry of PGPB, such as *Azospirillum* and *Azorhizobium*, known for harmless invasive entry through cracks in the roots (23, 37, 50) that do not harm the host plant or its defense responses. Thus, the *Chlorella* cell wall remains a valid representation of cell walls of higher plants that are addressed by this model. Because thousands of cells were needed to measure minute changes in the growing population, their enzymatic activities, and cell-cell interactions, a model that allows for the growth, harvesting, and counting of populations of single cells is advantageous. Furthermore, a potential assessment of this model as a preliminary screening tool of candidates for agricultural and forestry inoculants was only initiated but can be expanded. For example, two PGPB strains, *Bacillus pumilus* ES4 and RIZO1, first screened as potential PGPB by this model (Table 1), were later used as inoculants for sorghum (M. E. Puente, E. Ortiz, and Y. Bashan, unpublished data) and eroded land reforestation by legume-family trees (Y. Bashan, B. Salazar, M. E. Puente, and R. Linderman, unpublished data). With this line of reasoning, the proposed model, involving the immobilization of two species, might significantly facilitate molecular and physiological studies on cell-cell recognition and attachment, cell wall receptors, chemotaxis, proton extrusion, metabolic pathways of eukaryotes affected by prokaryotes, and photosynthesis that have not yet been pursued.

The practical and analytical aspects of this model are considerable. All ingredients are inexpensive, and the microorganisms are easy to cultivate and test in standard microbiology facilities. The results are available on a microbial time scale (days to weeks). Reproducibility is very high, and the replicate is merely an Erlenmeyer flask, allowing as many replicates as needed in a small space and in a soil-free system. By analyzing hundreds of published results using this system, it appears that the standard error is very low and allows detection of minute effects between the interacting organisms. So far, we have not observed any disadvantages in experiments conducted over the past 10 years.

In summary, *Chlorella* responds to interaction with *Azospirillum* in ways that are very similar to the interaction responses of higher plants by enhancing its growth and changing its metabolism. The system of immobilization is easy to handle, is inexpensive, and produces rapid results. For basic studies of

the physiology and molecular biology of plant-bacterium interactions, we recommend a practical experimental model involving an immobilization system containing two microorganisms.

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REFERENCES

1. **Arabidopsis Genome Initiative.** 2000. Analysis of the genome of the flowering plant *Arabidopsis thaliana*. Nature **408**:796–815.
2. **Arumuganathan, K., and E. D. Earle.** 1991. Nuclear DNA content of some important plant species. Plant Mol. Biol. Rep. **9:**208–218.
3. **Barbieri, P., and E. Galli.** 1993. Effect on wheat root development of inoculation with an *Azospirillum brasilense* mutant with altered indole-3-acetic acid production. Res. Microbiol. **144:**69–75.
4. **Bashan, Y., and L. E. de-Bashan.** 2005. Bacteria/plant growth-promotion, p. 103–115. *In* D. Hillel (ed.), Encyclopedia of soils in the environment, vol. 1. Elsevier, Oxford, United Kingdom.
5. **Bashan, Y., G. Holguin, and L. E. de-Bashan.** 2004. *Azospirillum*-plant relationships: physiological, molecular, agricultural, and environmental advances (1997-2003). Can. J. Microbiol. **50**:521–577.
6. **Bottini, R., F. Cassán, and P. Piccoli.** 2004. Gibberellin production by bacteria and its involvement in plant growth promotion and yield increase. Appl. Microbiol. Biotechnol. **65:**497–503.
7. **Burhenne, N., and R. Tischner.** 2000. Isolation and characterization of nitrite-reductase-deficient mutants of *Chlorella sorokiniana* (strain 211-8k). Planta **211:**440–445.
8. **de-Bashan, L. E., H. Antoun, and Y. Bashan.** 2005. Cultivation factors and population size control uptake of nitrogen by the microalgae *Chlorella vulgaris* when interacting with the microalgae growth-promoting bacterium *Azospirillum brasilense*. FEMS Microbiol. Ecol. **54:**197–203.
9. **de-Bashan, L. E., H. Antoun, and Y. Bashan.** 2008. Involvement of indole-3-acetic-acid produced by the growth-promoting bacterium *Azospirillum* spp. in promoting growth of *Chlorella vulgaris*. J. Phycol. **44**:938–947.
10. **de-Bashan, L. E., Y. Bashan, M. Moreno, V. K. Lebsky, and J. J. Bustillos.** 2002. Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae *Chlorella* spp. when co-immobilized in alginate beads with the microalgae-growth-promoting bacterium *Azospirillum brasilense*. Can. J. Microbiol. **48:**514–521.
11. **de-Bashan, L. E., J. P. Hernandez, T. Morey, and Y. Bashan.** 2004. Microalgae growth-promoting bacteria as "helpers" for microalgae: a novel approach for removing ammonium and phosphorus from municipal wastewater. Water Res. **38:**466–474.
12. **de-Bashan, L. E., P. Magallon, H. Antoun, and Y. Bashan.** Participation of glutamate dehydrogenase and glutamine synthetase in ammonium assimilation by *Chlorella vulgaris* jointly immobilized with the microalgae growth-promoting bacterium *Azospirillum brasilense*. J. Phycol., in press.
13. **de-Bashan, L. E., M. Moreno, J. P. Hernandez, and Y. Bashan.** 2002. Removal of ammonium and phosphorus ions from synthetic wastewater by the microalgae *Chlorella vulgaris* co-immobilized in alginate beads with the microalgae growth-promoting bacterium *Azospirillum brasilense*. Water Res. **36:**2941–2948.
14. **de-Bashan, L. E., A. Trejo, V. A. R. Huss, J. P. Hernandez, and Y. Bashan.** 2008. *Chlorella sorokiniana* UTEX 2805, a heat and intense sunlight tolerant microalgae with potential for removing ammonium from wastewater. Bioresour. Technol. **99:**4980–4989.
15. **de la Noüe, J., and N. de Pauw.** 1988. The potential of microalgal biotechnology: a review of production and uses of microalgae. Biotechnol. Adv. **6:**725–770.
16. **Devereux, R., A. R. Loeblich III, and G. E. Fox.** 1990. Higher plant origins and the phylogeny of green algae. J. Mol. Evol. **31:**18–24.
17. **Dibb-Fuller, J., and D. A. Morris.** 1992. Studies on the evolution of auxin carriers and phytotropin receptors: transmembrane auxin transport in unicellular and multicellular Chlorophyta. Planta **186:**219–226.
18. **Dobbelaere, S., A. Croonenborghs, A. Thys, A. Vande Broek, and J. Vanderleyden.** 1999. Phytostimulatory effect of *Azospirillum brasilense* wild type and mutant strains altered in IAA production on wheat. Plant Soil **212:**155–164.
19. **Gacesa, P.** 1998. Bacterial alginate biosynthesis—recent progress and future prospects. Microbiology **144:**1133–1143.
20. **Gill, B. S., R. Appels, A. M. Botha-Oberholster, C. R. Buell, J. L. Bennetzen, B. Chalhoub, F. Chumley, J. Dvorák, M. Iwanaga, B. Keller, W. Li, W. R. McCombie, Y. Ogihara, Q. Francis, and S. Takuji.** 2004. A workshop report on wheat genome sequencing. Genetics **168:**1087–1096.
21. **Gonzalez, L. E., and Y. Bashan.** 2000. Growth promotion of the microalga *Chlorella vulgaris* when coimmobilized and cocultured in alginate beads with the plant-growth-promoting bacterium *Azospirillum brasilense*. Appl. Environ. Microbiol. **66:**1527–1531.
22. **Gonzalez-Bashan, L. E., V. Lebsky, J. P. Hernandez, J. J. Bustillos, and Y. Bashan.** 2000. Changes in the metabolism of the microalgae *Chlorella vulgaris* when co-immobilized in alginate with the nitrogen-fixing *Phyllobacterium myrsinacearum*. Can. J. Microbiol. **46:**653–659.
23. **Gopalaswamy, G., S. Kannaiyan, K. J. O'Callaghan, M. R. Davey, and E. C. Cocking.** 2000. The xylem of rice (*Oryza sativa*) is colonized by *Azorhizobium caulinodans*. Proc. R. Soc. Lond. B **267:**103–107.
24. **Hartmann, A., M. Singh, and W. Klingmüller.** 1983. Isolation and characterization of *Azospirillum* mutants excreting high amounts of indoleacetic acid. Can. J. Microbiol. **29:**916–923.
25. **Hartmann, A., and W. Zimmer.** 1994. Physiology of *Azospirillum*, p. 15–39. *In* Y. Okon (ed.), *Azospirillum*/plant associations. CRC Press, Boca Raton, FL.
26. **Hernandez, J. P., L. E. de-Bashan, and Y. Bashan.** 2006. Starvation enhances phosphorus removal from wastewater by the microalga *Chlorella* spp. co-immobilized with *Azospirillum brasilense*. Enzyme Microb. Technol. **38**:190–198.
27. **Hernandez, J.-P., L. E. de-Bashan, D. J. Rodriguez, Y. Rodriguez, and Y. Bashan.** Growth promotion of the freshwater microalga *Chlorella vulgaris* by the nitrogen-fixing, plant growth-promoting bacterium *Bacillus pumilus* from arid zone soils. Eur. J. Soil Biol., in press.
28. **Higashiyama, T., and T. Yamada.** 1991. Electrophoretic karyotyping and chromosomal gene mapping of *Chlorella*. Nucleic Acids Res. **19:**6191–6195.
29. **Hosono, H., I. Uemura, T. Takumi, T. Nagamune, T. Yasuda, M. Kishimoto, H. Nagashima, N. Shimomura, M. Natori, and I. Endo.** 1994. Effect of culture temperature shift on the cellular sugar accumulation of *Chlorella vulgaris* SO-26. J. Ferment. Bioeng. **78:**235–240.
30. **Ilangovan, K., R. O. Cañizares-Villanueva, S. González Moreno, and D. Voltolina.** 1998. Effect of cadmium and zinc on respiration and photosynthesis in suspended and immobilized cultures of *Chlorella vulgaris* and *Scenedesmus acutus*. Bull. Environ. Contam. Toxicol. **60:**936–943.
31. **International Rice Genome Sequencing Project.** 2005. The map-based sequence of the rice genome. Nature **436**:793–800.
32. **Ishikawa, E., H. Sansawa, and H. Abe.** 2004. Isolation and characterization of a *Chlorella* mutant producing high amounts of chlorophyll and carotenoids. J. Appl. Phycol. **16:**385–393.
33. **Kennedy, I. R., A. T. M. A. Choudhury, and M. L. Kecskes.** 2004. Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biol. Biochem. **36:**1229–1244.
34. **Ladha, J. K., and P. M. Reddy (ed.).** 2000. The quest for nitrogen fixation in rice. International Rice Research Institute, Los Baños, Laguna, Philippines.
35. **Landstein, D., S. Arad, Z. Barak, and D. M. Chipman.** 1993. Relationships among the herbicide and functional sites of acetohydroxy acid synthase from *Chlorella emersonii*. Planta **191:**1–6.
36. **Lebsky, V. K., L. E. Gonzalez-Bashan, and Y. Bashan.** 2001. Ultrastructure of co-immobilization of the microalga *Chlorella vulgaris* with the plant growth-promoting bacterium *Azospirillum brasilense* and with its natural associative bacterium *Phyllobacterium myrsinacearum* in alginate beads. Can. J. Microbiol. **47:**1–8.
37. **Levanony, H., Y. Bashan, B. Romano, and E. Klein.** 1989. Ultrastructural localization and identification of *Azospirillum brasilense* Cd on and within wheat root by immuno-gold labeling. Plant Soil **117:**207–218.
38. **Loos, E., and D. Meindl.** 1982. Composition of the cell wall of *Chlorella fusca*. Planta **156**:270–273.
39. **Malis-Arad, S., M. Friedlander, R. Ben-Arie, and A. E. Richmond.** 1980. Alkalinity-induced aggregation in *Chlorella vulgaris*. I. Changes in cell volume and cell-wall structure. Plant Cell Physiol. **21:**27–35.
40. **Mazur, A., A. Konop, and R. Synak.** 2001. Indole-3-acetic acid in the culture medium of two axenic green microalgae. J. Appl. Phycol. **13:**35–42.
41. **McHugh, D. J.** 2003. A guide to the seaweed industry. FAO Fisheries technical paper no. 441. FAO, Rome, Italy.
42. **Northcote, D. H., K. J. Goulding, and R. W. Horne.** 1958. The chemical composition and structure of the cell wall of *Chlorella pyrenoidosa*. Biochem. J. **70:**391–397.
43. **Oh-Hama, T., and S. Miyachi.** 1992. *Chlorella*, p. 3–26. *In* M. A. Borowitzka and L. J. Borowitzka (ed.), Microalgal biotechnology. Cambridge University Press, Cambridge, United Kingdom.
44. **O'Reilly, A. M., and J. A. Scott.** 1995. Defined coimmobilization of mixed microorganism cultures. Enzyme Microb. Technol. **17:**636–646.
45. **Oswald, W. J.** 1992. Microalgae and wastewater treatment, p. 305–328. *In* M. A. Borowitzka and L. J. Borowitzka (ed.), Microalgal biotechnology. Cambridge University Press, Cambridge, United Kingdom.
46. **Perrine, F. M., J. Prayitno, J. J. Weinman, F. B. Dazzo, and B. G. Rolfe.** 2001. *Rhizobium* plasmids are involved in the inhibition or stimulation of rice growth and development. Aust. J. Plant Physiol. **28:**923–937.
47. **Piro, G., M. Lenucci, G. Dalessandro, N. La Rocca, N. Rascio, I. Moro, and C. Andreoli.** 2000. Ultrastructure, chemical composition and biosynthesis of the cell wall in *Koliella antarctica* (Klebsormidiales, Chlorophyta). Eur. J. Phycol. **35:**331–337.

48. **Puente, M. E., Y. Bashan, C. Y. Li, and V. K. Lebsky.** 2004. Microbial populations and activities in the rhizoplane of rock-weathering desert plants. I. Root colonization and weathering of igneous rocks. Plant Biol. **6:**629–642.
49. **Ramazanov, A., and Z. Ramazanov.** 2006. Isolation and characterization of a starchless mutant of *Chlorella pyrenoidosa* STL-PI with a high growth rate, and high protein and polyunsaturated fatty acid content. Phycol. Res. **54:** 255–259.
50. **Ramos, H. J. O., L. D. B. Roncato-Maccari, E. M. Souza, J. R. L. Soares-Ramos, M. Hungria, and F. O. Pedrosa.** 2002. Monitoring *Azospirillum*-wheat interactions using the *gfp* and *gusA* genes constitutively expressed from a new broad-host range vector. J. Biotechnol. **97:**243–252.
51. **Raven, P. H., R. H. Evert, and S. E. Eichhorn.** 1992. Biology of plants, 5th ed. Worth Publishers, New York, NY.
52. **Richmond, A.** 1990. Handbook of microalgal mass culture. CRC Press, Boca Raton, FL.
53. **Romo, S., and C. Perez-Martinez.** 1997. The use of immobilization in alginate beads for long-term storage of *Pseudoanabaena galeata* (Cyanobacteria) in the laboratory. J. Phycol. **33:**1073–1076.
54. **Smidsrød, O., and G. Skjak-Braek.** 1990. Alginate as immobilization matrix for cells. Trends Biotechnol. **8:**71–78.
55. **Spaepen, S., J. Vanderleyden, and R. Remans.** 2007. Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiol. Rev. **31:** 425–448.
56. **Steenhoudt, O., and J. Vanderleyden.** 2000. *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol. Rev. **24:**487–506.
57. **Stirk, W. A., V. Ordog, J. Van Staden, and K. Jager.** 2002. Cytokinin-and auxin-like activity in cyanophyta and microalgae. J. Appl. Phycol. **14**:215–221.
58. **Tam, N. F. Y., and Y. S. Wong.** 2000. Effect of immobilized microalgal bead concentration on wastewater nutrient removal. Environ. Pollut. **107:**145–151.
59. **Vande Broek, A., M. Lambrecht, K. Eggermont, and J. Vanderleyden.** 1999. Auxins upregulate expression of the indole-3-pyruvate decarboxylase gene in *Azospirillum brasilense*. J. Bacteriol. **181:**1338–1342.
60. **Yabur, R., Y. Bashan, and G. Hernández-Carmona.** 2007. Alginate from the macroalgae *Sargassum sinicola* as a novel source for microbial immobilization material in wastewater treatment and plant growth promotion. J. Appl. Phycol. **19:**43–53.
61. **Yanni, Y. G., R. Y. Rizk, F. K. Abd El-Fattah, A. Squartini, V. Corich, A. Giacomini, F. deBruijn, J. Rademaker, J. Maya-Flores, P. Ostrom, M. Vega-Hernandez, R. I. Hollingsworth, E. Martinez-Molina, P. Mateos, E. Velazquez, J. Wopereis, E. Triplett, M. Umali-Garcia, J. A. Anarna, B. G. Rolfe, J. K. Ladha, J. Hill, R. Mujoo, P. K. Ng, and F. B. Dazzo.** 2001. The beneficial plant growth-promoting association of *Rhizobium leguminosarum* bv. *trifolii* with rice roots. Aust. J. Plant Physiol. **28:**845–870.