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Long-living *Azotobacter-Chlamydomonas* association as a model system for plant-microbe interactions

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Abstract
A stable association was established between the nitrogen-fixing bacterium *Azotobacter vinelandii* and the unicellular green alga *Chlamydomonas reinhardtii* on a carbon- and nitrogen-free medium. This medium ensured a balanced ratio between the two partners, as was detected by light and electron microscopy and a molecular biological method. The respiration, photosynthetic oxygen evolution and nitrogen fixation of the associative culture were lower compared with the data obtained from pure cultures growing under optimal conditions. However, observations confirmed a cooperation that improved over time, indicating a gradual stabilization of the system.

Keywords: *Azotobacter*, *Chlamydomonas*, alga-bacterium association, microscopy, photosynthesis, nitrogen fixation, molecular detection

1. Introduction

The free-living, nitrogen-fixing *Azotobacters* are widely distributed in the soils of temperate regions. They are known as diazotrophic bacteria because they are able to fix atmospheric nitrogen. The acquisition of nitrogen from the atmosphere requires the breaking of the very stable triple covalent bond between two nitrogen atoms (N≡N) to produce ammonia. Bacteria need energy for this reduction step. The energy originates from plant photosynthesis in the form of carbohydrates and is acquired most efficiently in the case of symbiosis or association. On the other hand, the *Azotobacters* produce and excrete numerous amino acids, vitamins and plant growth regulatory substances such as auxins and cytokinins. These metabolites are important factors in their synergic interactions with plants and may promote the formation of a stable association between the partners.

This paper describes an attempt to construct a model system of such an association using a strain of the nitrogen-fixing bacterium, *Azotobacter vinelandii* and the unicellular green alga *Chlamydomonas reinhardtii*. Previous experiments showed that these partners were capable of co-existing on nitrogen-free medium, where the energy required for nitrogen fixation was provided by the effective photosynthesis of the alga (Korányi et al., 1990, 1993; Gyurján et al., 1995). An overall analysis of this long-living model system is presented here.

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2. Materials and Methods

Bacterial strains and culture conditions

The strain CCM 289 of *Azotobacter vinelandii* was used, and was maintained in nitrogen-free liquid and solid culture (Newton et al., 1953) at 26°C.

Algal culture

The wild strain DSM 375 of the unicellular green alga *Chlamydomonas reinhardtii* was used. The cells were grown at 25°C on solid TAP medium containing KNO_3 as nitrogen source (Sager and Granick, 1953).

Establishment of alga-bacterium intercellular association

The algal cells were mixed with *A. vinelandii* cells at a ratio of 1:100 in liquid medium and the suspension was spread on nitrogen-free *Azotobacter* medium solidified with 2% agar. The glucose content of the medium was initially 20 g/l, while later the associative colonies were cultured on media containing different concentrations of glucose: 20 g/l, 5 g/l, 2 g/l, 0 g/l.

Electron microscopy

For transmission electron microscopy the associative cultures were fixed in 2% (v/v) glutaraldehyde for 2 h and postfixed in 1% (w/v) OsO_4 for 2 h in 70 mM K-Na phosphate (pH 7.2). Samples were embedded in Durcupan ACM epoxy resin (Fluka Chemie AG). Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Hitachi 7100 electron microscope.

Detection of respiration and photosynthetic oxygen evolution

The respiration intensity and photosynthetic oxygen evolution of samples dissolved in 50 mM Tricin-NaOH (pH 7.8) buffer were measured using a Hansatech Oxy Lab oxygen electrode. The oxygen consumption and evolution of the samples were given in $\mu mol\ O_2\ ml^{-1}\ min^{-1}$. The chlorophyll content of the samples was determined according to Porra et al. (1989).

Molecular detection of Azotobacter vinelandii *and* Chlamydomonas reinhardtii *from cultures and co-cultures using PCR*

Genomic DNA was isolated from separate *A. vinelandii* and *C. reinhardtii* cultures and from co-cultures using the FastPrep system (Qbiogene Inc., MP Biomedicals). Cell lysis was achieved in the FastPrep instrument using the CLS-VF (for plant) lysis solution from the FastDNA Kit (Qbiogene Inc.).

The primers
ANF3FOR (5'-GGCCTATTTCCACGACAAGA-3' and ANF3REV (5'-ATCATCTTGGTACCGATGGC-3') were used to detect the nitrogenase 3 (*anfH*) gene of *A. vinelandii* from the culture samples. The presence of *C. reinhardtii* in the culture medium was detected using primers specific for the *p60* katanin subunit (Katan-F 5'-ACGAGGAGTGGCTCAGCGTGTTCG-3' and Katan-R 5'-GGACGCCCAAGCTTCAAATCCACG-3').

Duplex PCR was used to simultaneously detect both organisms from the samples. DNA amplification was achieved in 30 cycles, consisting of denaturation at 94°C (1 min), annealing at 55°C (1 min) and elongation at 72°C (2 min) using a Perkin Elmer Thermal Cycler. The PCR products were separated on ethidium-bromide agarose (1% w/v) gels by electrophoresis and visualised by UV transillumination.

Measurement of acetylene reduction activity

The nitrogen-fixing activity of the stable association was detected by ARA. The assay was performed as described previously (Preininger et al., 1997) except that cultures were plated on solid medium in the vial.

Detection of the amino acids secreted by A. vinelandii

The samples (bacterium-free liquid medium from 4-day pure bacterium suspension culture) were filtered through 0.45 µm and 0.22 µm membrane filters (Millex-HV Syringe Driven Filter Unit, PVDF Durapore, Non-Sterile, 0.45 µm/13 mm and 0.22 µm/13 mm, Millipore Corp., USA) consecutively.

The free amino acid contents of the filtrates were determined with an automatic amino acid analyser (BIOTRONIK LC 3000, Germany) equipped with a cation-exchange resin column (125 × 4 mm); elution with a pH gradient (4-component buffer system); flow rate, 0.20 ml min^{-1}; detection with post-chromatographic derivatization using ninhydrin reagent reaction at a temperature of 135°C; spectrophotometric measurement at λ = 570 and 440 nm (for Pro).

3. Results

Microscopy

Fig. 1 shows that the bacterium and alga partners were capable of co-existing on nitrogen-free medium containing glucose (*Azotobacter* medium). Under these conditions the algal cells are dependent on the nitrogen source originating from the diazotrophic bacterium partner. The fact that the algal cells were green (dark spots on the picture) proved that they were supplied with sufficient nitrogen to exist as vegetative cells. The multiplication of the associative culture and the ratio of the two partners in the colonies was studied on media containing various concentrations of glucose. As seen in Fig. 2A on medium containing 20 g/l glucose the bacteria overgrew the algae in the colonies, while decreasing the sugar content to 5 g/l (Fig. 2B) and 2 g/l (Fig. 2C) resulted in a reduction in bacterium multiplication. This means that the relative ratio of the algae in each colony became higher. Finally, on glucose-free medium (Fig. 2D) the growth of the two partners became balanced, with a similar rate of multiplication of algal and bacterial cells. This comparison can only be made between colonies of similar size. Fig. 3 shows part of a colony of the alga-bacterium association cultivated on

nitrogen- and carbon-free medium. Gametogenesis, a normal process in alga cultures suffering from nitrogen starvation, cannot be detected. The vegetative cells of *Chlamydomonas reinhardtii* are healthy, well developed and surrounded by numerous bacteria.

Simultaneous detection of A. vinelandii *and* C. reinhardtii *in cultures*

The molecular biological detection of the two partners verified the composition of the associated culture (Fig. 4), proving that after prolonged cultivation the associative culture consisted of *Chlamydomonas* and *Azotobacter* cells, and was not the result of contamination.

A. vinelandii was detected from the culture medium using oligonucleotide primers specific to the nitrogenase 3 (*anfH*) gene (Joerger et al., 1989). Nitrogenase 3 was chosen because it is an alternative nitrogenase encoded by the *anfHDGK* structural genes found only in *Azotobacter*

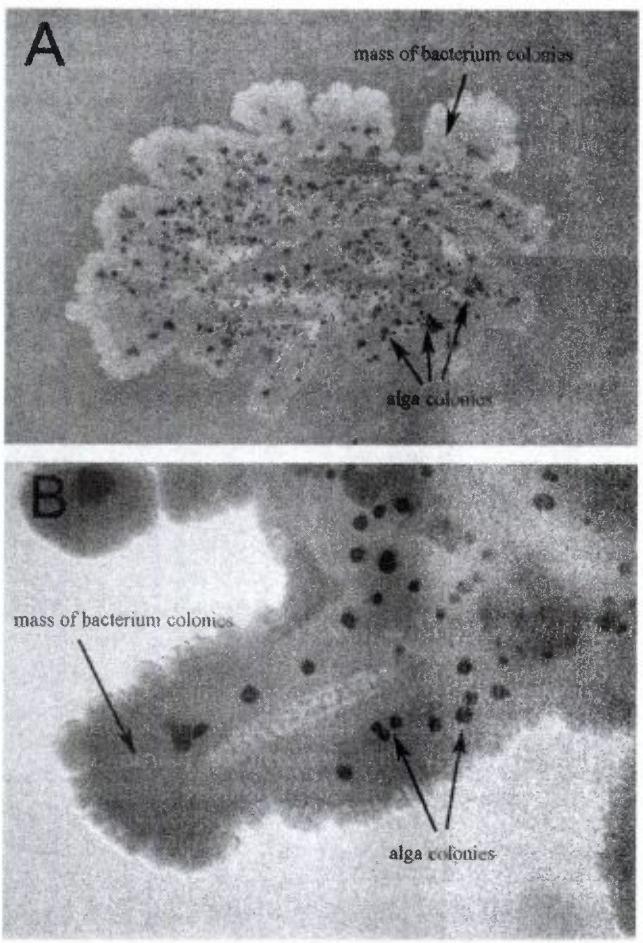

Figure 1. Co-culture of algae and bacteria growing on nitrogen-free but glucose-containing *Azotobacter* medium. Dark spots are green algal colonies in the mass of bacteria, demonstrating that algal cells are supplied with nitrogen originating from the bacteria. Nitrogen-starved algal cells would be yellow. 1B is a magnification of part of 1A.

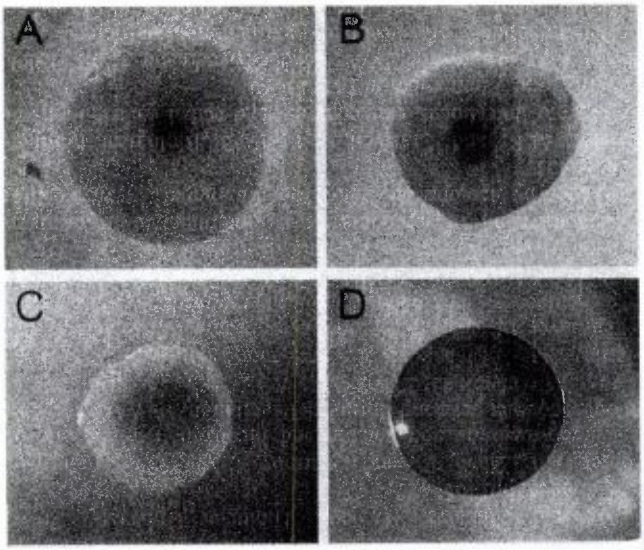

Figure 2. Comparison of associative colonies (with similar size) on *Azotobacter* media containing different amounts of glucose. A: 20 g/l glucose; B: 5 g/l glucose; C: 2 g/l glucose; D: 0 g/l glucose. Reducing the sugar content in the medium resulted in the balanced multiplication of the partners in the colonies. Algal cells are concentrated in the inner dark part of the colony

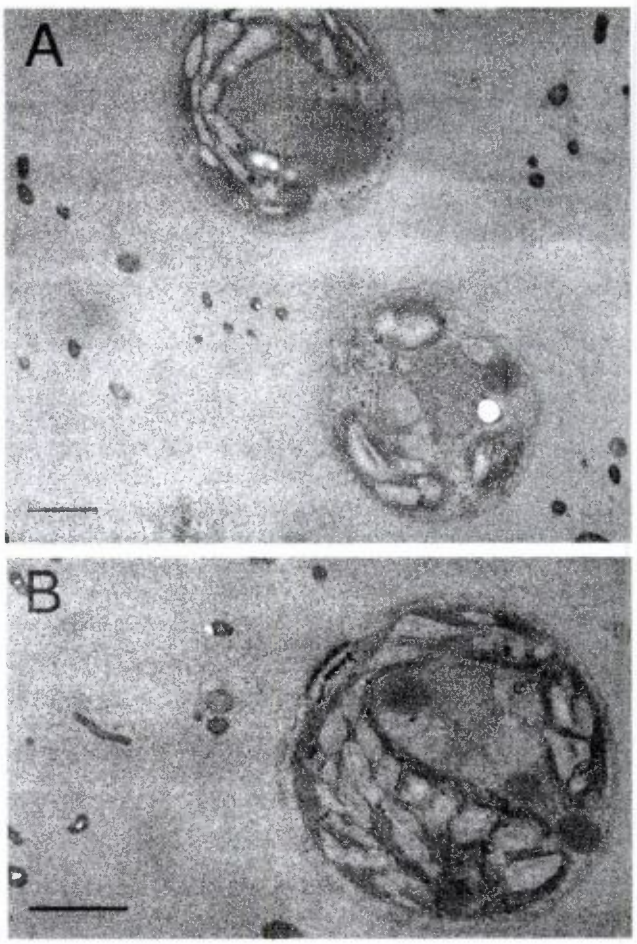

Figure 3. Electron micrograph of the associative culture showing the viability of the *Chlamydomonas* and *Azotobacter* cells.

Table 1. Respiration and photosynthetic oxygen evolution of pure and associated cultures.

	Respiration intensity (μmol O_2 ml^{-1} min^{-1} g^{-1})	Photosynthetic O_2 evolution (μmol O_2 ml^{-1} min^{-1} g^{-1})	Photosynthetic O_2 evolution (μmol O_2 ml^{-1} min^{-1} g^{-1} Chl^{-1})	Net photosynthetic O_2 evolution (μmol O_2 ml^{-1} min^{-1} g^{-1})
Azotobacter vinelandii	–1.94	–	–	–
Chlamydomonas reinhardtii	–0.75	29.10	18.70	28.35
Alga-bacterium association	–0.52	11.80	10.75	11.28

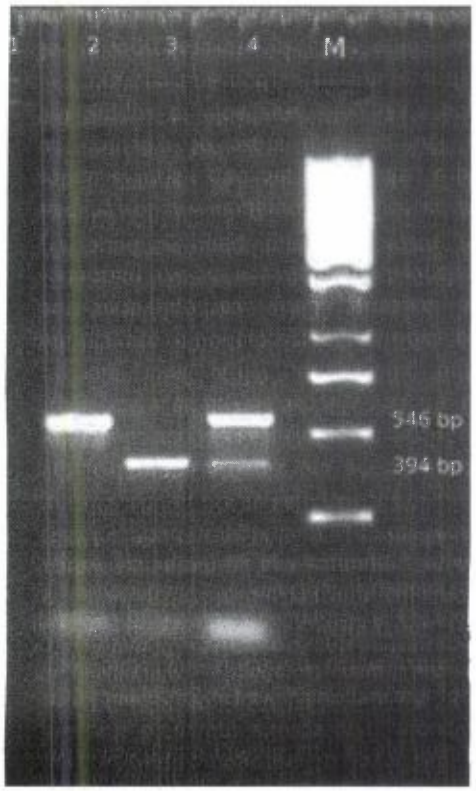

Figure 4. Detection of *Azotobacter vinelandii anfH* gene and *Chlamydomonas reinhardtii* katanin subunit of *p60* from chromosome 10. 1: No target DNA (bacterium and alga); 2: Azotobacter vinelandii culture alone; 3: Chlamydomonas reinhardtii culture alone; 4: Associative culture; 5: MW marker 500 bp ladder (250 bp, 500 bp, 1000 bp, 1500 bp ...).

vinelandii. *C. reinhardtii* was identified by detecting the katanin subunit of *p60* from chromosome 10 (Kathyr et al., 2003; Lohret et al., 1999).

The primers amplified a 546 bp and a 394 bp fragment from *A. vinelandii* and *C. reinhardtii*, respectively, enabling the simultaneous visualisation of both fragments from one PCR reaction (duplex PCR).

Respiration and photosynthetic oxygen evolution

The data in Table 1 represent the means of three independent measurements. They clearly demonstrate that the respiration and photosynthetic oxygen evolution of the association was significantly lower on nitrogen- and carbon-free medium than in free cultures growing under optimal conditions. However, the difference in the nitrogen content between the nitrogen-containing and nitrogen-free media was much more pronounced than the decline in respiration and photosynthetic oxygen evolution under starvation conditions where the partners were interdependent. This proves that the algal cells were physiologically active even under nitrogen-free conditions as a result of the associative partnership. Therefore, although these activities were lower than optimal, the forced association is nevertheless very promising.

Table 2. Amino acids secreted by *Azotobacter vinelandii*.

Amino acid	%
Pro	13.5
Glu	10.1
Phe	8.4
Asp	7.5
Leu	7.1
Gly	6.8
Ala	6.6
Thr	6.2
Val	6.1
Ser	5.6
His	4.7
Tyr	4.3
Ile	4.0
Lys	3.6
Arg	3.0
Cys	1.3
Met	1.1
Total	100.0

Measurement of acetylene reduction activity (ARA)

Compared to the control (pure alga culture) the associative cultures containing bacteria were able to fix atmospheric nitrogen. This ability was detected by the formation of ethylene from acetylene (Fig. 5). Ethylene formation was analysed qualitatively, not quantitatively, as proposed by Urquiaga et al. (1989) and Peoples and Craswell (1992).

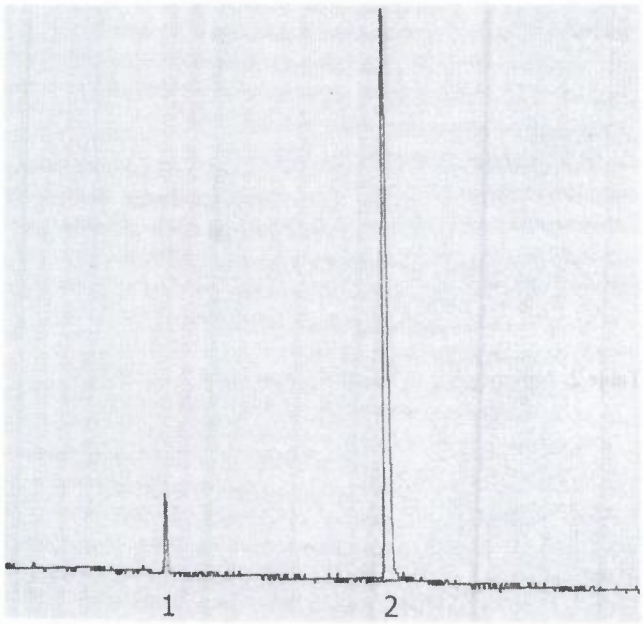

Figure 5. Acetylene reduction assay from the associative culture for the detection of nitrogen fixation. Peaks: 1: ethylene, 2: acetylene.

Detection of the amino acids secreted by A. vinelandii

Since the associative culture of algae and bacteria was cultivated in nitrogen-free medium, the algal cells must have taken up the nitrogen compounds fixed and secreted by the bacteria. Measurements on filtered, bacterium-free liquid medium in an amino acid analyser showed that 17 different amino acids were produced (Table 2). The most abundant free amino acids were proline (14%), glutamate (10%) and phenylalanine (8%), which made up 32% of the total amino acid content. The relative amounts of the other amino acids were similar: 7% (Asp, Leu, Gly, Ala), 6% (Thr, Val, Ser), 5% (His), 4% (Tyr, Ile, Lys), 3% (Arg) and 1% (Cys, Met).

4. Discussion

The artificial association established by us, represented a model system in which the nitrogen supply could be clearly detected phenotypically. The basis for this model system was the fact that nitrogen starvation combined with illumination causes gamete formation from vegetative green cells of *Chlamydomonas* (Sager and Granick, 1954). During gametogenesis both chloroplast and cytoplasmic ribosomes are degraded and cell division is stopped (Sears et al., 1980; Siersma and Chiang, 1971; Martin et al., 1976) and the gametes turn yellow. If a nitrogen source is provided in the medium, the gametes revert to metabolically active green vegetative cells. This phenomenon makes the level of nitrogen supply in the association clearly visible. The results indicated that the bacterial partner *Azotobacter vinelandii* was capable of providing sufficient nitrogen to maintain the conditions required by vegetative green algal cells. As was mentioned above, bacteria not only fix nitrogen but also produce and secrete growth regulators, vitamins and amino acids, which influence the metabolism of the algae (Scott, 1984; Gonzalez-Lopez et al., 1983; Revillas et al., 2000). During the 2-year experimental period the glucose content of the medium (basic *Azotobacter* medium) was decreased step by step from 20 g/l to 0 g/l. The glucose served as a starter for the initiation of the partnership, while later glucose deprivation helped to stabilize the association to prevent the overgrowth of the bacteria. Only carbonhydrate-free medium ensured a balanced ratio between the two partners.

The intensity of respiration and photosynthetic O_2 evolution in the alga-bacterium association was lower than that recorded for pure cultures of the two partners under optimal conditions. The question arose of whether the photosynthesis of the algal cells was able to supply the bacterial partner with a source of carbon and energy. The excretion of various compounds by several species of *Chlamydomonas* has been reported. An early study by Allen (1956) documented the excretion of glycolate, oxalate and pyruvate by *C. reinhardtii.* Collins and Kalnins (1967) reported excretion of several a-keto acids by *C. reinhardtii.* Dissolved amino acids and sugars were also found in the culture medium after the growth of *C. reinhardtii* by Vogel et al. (1978). However the ability of *Azotobacters* to uptake and utilize these excreted compounds is not well documented. In addition there are no data about incidental components which might be produced by the partners and have a negative effect on the cooperation. However, the present observations confirm that cooperation improves over time, indicating the stabilization of the partnership.

REFERENCES

Allen, M.B. 1956. Excretion of organic compounds by *Chlamydomonas. Archives of Microbiology* **24**: 163–168.

Collins, R.P. and Kalnins, K. 1976. Keto acids produced by *Chlamydomonas reinhardtii. Canadian Journal of Microbiology* **13**: 995–999.

Gonzalez-Lopez, J., Salmerin, V., Moreno, J., and Ramos-Cormenzana, A. 1983. Amino acids and vitamins produced by *Azotobacter vinelandii* ATCC 12837 in chemically-defined media and dialysed soil media. *Soil Biology and Biochemistry* **15**: 711–713.

Gyurján, I., Korányi, P., Preininger, E., Varga, Sz.S., and Paless, Gy. 1995. Artificial plant-*Azotobacter* symbiosis for atmospheric nitrogen fixation. In: Azospirillum *VI and Related Microorganisms.* NATO ASI Series, Series G. "Ecological Sciences", vol. 37. Fendrik, I., del Gallo, M., Vanderleyden, J., and Zamaroczy, M., eds. Springer-Verlag, Berlin, Heidelberg, New York. pp. 401–413.

Joerger, R.D., Jacobson, M.R., Premakumar, R., Wolfinger, E.D., and Bishop, P.E. 1989. Nucleotide seqence and mutational analysis of the structural genes (*anfHDGK*) for the second alternative nitrogenase from *Azotobacter vinelandii*. *Journal of Bacteriology* **171**: 1075–1086.

Kathyr, P., LaVoie, M., Brazelton, W.J., Haas, N.A., Lefebvre, P.A., and Silflow, C.D. 2003. Molecular map of the *Chlamydomonas reinhardtii* nuclear genome. *Eukaryotic Cell* **2**: 362–379.

Korányi, P., Gyurján, I., Stefanovits, P., Bóka, K., and Szigeti, Z. 1990. Characterisation of an artificial nitrogen-fixing alga-bacterium ectocytobiosis. *Symbiosis* **8**: 175–187.

Korányi, P., Varga, Sz.S., Preiniger, E., and Gyurján, I. 1993. Symbiotic integration of diazotrophic bacteria with plant cells in artificial cytobioses. In: *Endocytobiology V*. Sato, S., Ishida, M., and Ishikawa, H., eds. Tübingen University Press, Tübingen, pp. 465–472.

Lohret, T.A., Zhao, L., and Quarmby, L.M. 1999. Cloning of *Chlamydomonas p60* katanin and localization to the site of outer doublet severing during deflagellation. *Cell Motility and the Cytoskeleton* **43**: 221–231.

Martin, N.C., Chiang, K.S., and Goodenough, U.W. 1976. Turnover of chloroplast and cytoplasmic ribosomes during gametogenesis in *Chlamydomonas reinhardtii*. *Developmental Biology* **51**: 190–201.

Newton, J.W., Wilson, P.W., and Burris, R.H. 1953. Direct demonstration of ammonia as an intermediate in nitrogen fixation by *Azotobacter*. *Journal of Biological Chemistry* **204**: 445–451.

Peoples, M.B. and Craswell, E.T. 1992. Biological nitrogen fixation: Investment, expectations and actual contributions to agriculture. *Plant and Soil* **141**: 13–39.

Porra, R.J., Thomson, W.A., and Kriedemann, P.E. 1989. Determination of accurate extinction coefficient and simultaneous equations for essaying chlorophyll a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta* **975**: 384–394.

Preininger, E., Zatykó, J., Szücs, P., Korányi, P., and Gyurján, I. 1997. *In vitro* establishment of nitrogen-fixing strawberry (*Fragaria* x *ananassa*) via artificial symbiosis with *Azomonas insignis*. *In Vitro Cell and Developmental Biology – Plant* **33**: 190–194.

Revillas, J.J., Rodealas, B., Pozo, C., Martnez-Toledo, M.V., and Gonzalez-Lopez, J. 2000. Production of B-group vitamins by two *Azotobacter* strains with phenolic compounds as sole carbon source under diazotrophic and adiazotrophic conditions. *Journal of Applied Microbiology* **89**: 483–493.

Sager, R. and Granick, S. 1953. Nutritional studies with *Chlamydomonas reinhardtii*. *Annals of the New York Academy of Sciences* **56**: 831–838.

Sager, R. and Granick, S. 1954. Nutritional control of sexuality in *Chlamydomonas reinhardtii* *The Journal of General Physiology* **37**: 729–742.

Scott, T.K. 1984. Hormon regulation of development II. In: *Encyclopedia of Plant Physiology*, Vol. 10, Scott, T.K., ed. Springer, Berlin.

Sears, B.B., Boyton, J.E., and Gillham, N.W. 1980. The effect of gametogenesis regimes on the chloroplast genetic system of *Chlamydomonas reinhardtii*. *Genetics* **96**: 95–114.

Siersma, P.W. and Chiang, K.S. 1971. Conservation and degradation of chloroplast and cytoplasmic ribosomes in *Chlamydomonas reinhardtii*. *Journal of Molecular Biology* **58**: 167–185.

Urquiaga, S., Botteon, P.B.L., and Boddey, R.M. 1989. Selection of sugar cane cultivars for associated biological nitrogen fixation using ^{15}N-labelled soil. In: *Nitrogen Fixation with Non-Legumes*. Skinner, F.A., Boddey, R.M., and Fendrik, I., eds. Kluwer Academic Publishers, Dordrecht, pp. 311–319.

Vogel, S.L., Frisch, H.L., and Gotham, I.J. 1978. Qualitative assay of dissolved amino acids and sugars excreted by *Chlamydomonas reinhardtii* (Chlorophyceae) and *Euglena gracilis* (Euglenophyceae). *Journal of Phycology* **14**: 403–406.