Symbiosis, 29 (2000) 1–18
Balaban, Philadelphia/Rehovot

Co-Cultivation of N_2-Fixing Cyanobacterium *Nostoc* sp. Strain 2S9B and Wheat Callus

MIROSLAV GANTAR
Department of Biological Sciences, Florida International University, University Park, Miami, FL 33199, USA
Tel. +1-305-348-4030, Fax. +1-305-348-1986, E-mail. Gantarm@fiu.edu

Received November 23, 1999; Accepted April 23, 2000

Abstract

An association was established between the N_2-fixing cyanobacterium *Nostoc* sp. strain 2S9B and wheat callus. Cyanobacteria co-cultivated with wheat calli penetrated the callus, occupying space between the cells and colonizing the big non-embryogenic cells both inter- and intracellularlly. The callus regions composed of small embryogenic, tightly packed cells were not readily colonized. When grown in the presence of wheat callus, the cyanobacteria underwent morphological, ultrastructural, and metabolic changes, and showed an increase in the cell size (both vegetative cells and heterocysts), and an increase in biomass yield. Higher production of extracellular polymeric substance in cyanobacteria-callus suspension cultures was also observed. The nitrogen fixation rate of cyanobacteria co-cultivated with wheat callus was 2–3 times of that of free-living cultures. In cyanobacterial cells co-cultivated with wheat calli, the number of thylakoids was reduced and their morphology and orientation changed.

Keywords: Artificial symbiosis, callus, cyanobacteria, wheat

1. Introduction

The need for lesser usage of nitrogenous chemical fertilizers has prompted an extensive research in harnessing biological nitrogen fixation in agricultural

0334-5114/2000/$05.50

practice. While the legume-*Rhizobium* symbiosis (Denarie et al., 1992) has long been exploited there have also been numerous attempts to exploit N_2-fixation in non-leguminous plants. In particular, the integration of N_2-fixing microorganisms with non-leguminous plants appears to be an approach with broad application (Kennedy et al., 1997). The technique of para-nodule formation elicited by the plant hormone 2,4-D (Tchan and Kennedy, 1989) expanded the possibility of creating novel symbioses between N_2-fixing microorganisms and crop plants. However, the efficacy of these plant-bacteria associations appears to be limited by the inhibition of nitrogenase activity by O_2 (Christiansen-Weniger, 1996).

Certain nitrogen-fixing cyanobacteria enter into associations with a wide range of plants (Bergman et al., 1992). These species differentiate specialized cells called heterocysts in which nitrogenase is protected against the deleterious effect of O_2 (Wolk et al., 1994). Initiation of natural cyanobacterial-plant associations appears to require no special response by the cyanobacterium (Meeks, 1998) and is not dependent on the complex signaling mechanisms such as those found in *Rhizobium*-legume symbioses (Kijne, 1992). Therefore, cyanobacteria can be seen as prospective partners in novel associations.

There have been attempts to introduce cyanobacteria into protoplasts of plants (Burgoon and Bottino, 1976; Meeks et al., 1978) however, no stable associations were obtained. Gusev et al. (1986) reported that *Anabaena variabilis* a cyanobacterium that usually does not form natural symbiosis, was successfully introduced into tobacco callus followed by plant differentiation.

We have shown that the cyanobacterium *Nostoc* sp. strain 2S9B can colonize wheat roots (Gantar et al., 1991b) as well as hormone-induced wheat para-nodules (Gantar and Elhai, 1999). The goal of this paper was to show whether an integration of cyanobacteria into wheat callus tissue is possible and what effect does it have on cyanobacteria. To our knowledge this is the first report on novel association between one of the most important crop plants – wheat, and a strain of N_2-fixing cyanobacterium originally isolated from a soil.

2. Materials and Methods

Organisms and growth conditions

The cyanobacterium *Nostoc* sp. strain 2S9B was originally isolated from solonetz soil (soil with high concentration of NaCl) (Gantar et al., 1991a). It was made axenic by antibiotic treatment (Vaara et al., 1979) and repeated isolation of individual hormogonia. The strain was maintained in BG11 (Rippka et al., 1979), modified as previously described (Elhai and Wolk, 1990)

which either contained 17 mM $NaNO_3$, or in which $NaNO_3$ was omitted ($BG11_0$). The cultivation was carried out under a constant illumination at 20 μmol photons $m^{-2}s^{-1}$ and temperature of 24°C.

The wheat (*Triticum aestivum* L.) cultivars Penawawa, Edwall and Klassic, kindly provided by Steve Jones (Washington State University, Department of Crop and Soil Sciences, Pullman), were used in these experiments.

Initiation of callus and suspension cultures

Callus cultures of three wheat cultivars were initiated from germinating seeds. The seeds were surface sterilized by washing in 90% ethanol for 30 sec, followed by 2.5% (w/v) sodium hypochlorite for 15 min. After that, the seeds were rinsed five times in sterile BG11. Germination was carried out in a petri dish with BG11 solidified with 1% agar in the dark at 24°C. After 2 days, when the shoot reached a length of about 1 cm, both shoot and root were cut off and the seed was transferred onto Murashige and Skoog (1962) medium (MS) supplemented with 2% of sucrose, 1 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) (designated as MS/D medium) and solidified with 0.8% agar. The cutting-off procedure was repeated 2–3 times and after two months the callus, which developed from the base of the shoot, reached about 1 cm in diameter when it was used for inoculation with cyanobacteria. The calli were transferred onto fresh medium once in a week.

The wheat cell suspension culture was initiated by transferring a single callus, formed in the way described above, into a 50 ml flask containing liquid MS/D medium. The cultivation was carried out in the dark on a gyratory shaker at 24°C. The medium was replaced once in two weeks. After 2–3 months the callus proliferated giving numerous spherical cell aggregates (Wang and Nguyen, 1990).

Co-cultivation of callus and cyanobacteria

Calli of three wheat cultivars (Penawawa, Klassic, Edwall) were inoculated with the cyanobacterium *Nostoc* sp. strain 2S9B in the following way. Three days prior to inoculation with cyanobacteria, the primary calli developed and maintained on solid MS/D medium were transferred onto a MS-NH_4-free medium (Sigma) in which NH_4NO_3 was replaced with $NaNO_3$ providing total nitrogen of 40 mM (according to Sigma). This medium was designated as MS-$(NH_4)_0$, and the medium that contained 1 mgl^{-1} 2,4-D was named MS/D-$(NH_4)_0$. The omission of NH_4NO_3 was necessary since the ammonia in the MS medium suppressed the growth of cyanobacteria and eventually caused bleaching. The cyanobacterial inoculum was grown in BG11

until it entered the stationary phase and then the cells were centrifuged, washed in BG11, and resuspended to give a biomass of 100 µg chlorophyll *a* per ml. About 50 µl of cyanobacterial cell suspension was used to cover the surface of a single callus. Further cultivation was carried out in a growth chamber at 24°C under light intensity of 20 µmol photons $m^{-2}s^{-1}$ and 10/14h dark/light cycle.

Suspension culture of callus and cyanobacteria

Associative cultivation was also carried out in a liquid medium by inoculating MS/D-$(NH_4)_0$ medium with wheat calli and cyanobacteria. Wheat cell culture was obtained as described above. Five wheat cell aggregates (each 2–3 mm in diameter) were taken, washed in fresh MS-$(NH_4)_0$ medium, placed in 50 ml of MS/D-$(NH_4)_0$ and then inoculated with cyanobacterial cells giving final concentration of about 0.2 $\mu g chl^{-1} ml^{-1}$. The cultures were grown on a gyratory shaker with a rotating speed of 100 RPM under the same conditions as described above. Cyanobacterial cells from these cultures were used for electron microscopy as well as for the measurements of nitrogenase activity, cyanobacterial biomass yield, cyanobacterial cell size, and extracellular polysaccharide secretion. No attempts were made to measure the callus biomass yield.

Nitrogenase activity during the cyanobacteria-calli co-cultivation

Nitrogenase activity, determined by the production of ethylene from the reduction of acetylene (Stewart et al., 1967), was measured using a Shimadzu (GC-14a) gas chromatograph, fitted with a flame ionization detector and a 30 m GS-alumina column. A single wheat callus was placed in a 17 ml vial, which contained 2 ml solidified MS/D-$(NH_4)_0$ medium. The vial was sealed with a screw cap with a septum that enabled gas sampling. The calli were grown in the dark for seven days before they were inoculated with cyanobacteria. After inoculation, further co-cultivation of calli and cyanobacteria was carried out in the growth chamber at 24°C under 10/14h dark/light cycle. The acetylene reduction assay (ARA) was carried out by injecting the vials with 10% (v/v) of acetylene and incubating them under illumination of 30 µmol photons $m^{-2}s^{-1}$ for 1 hour. Then 1 ml of gas sample was taken and the vials were left open to ventilate for 30 min in a laminar flow cabinet. The same vials (callus-cyanobacteria cultures) were used throughout the experiment. After initial acetylene reduction determination, acetylene reduction measurements were performed every other day, 2h after the commencement of a light cycle. A cyanobacterial culture grown on the same solid medium but without callus was used as a control. The uninoculated calli did not show any ethylene production.

Nitrogenase activity in suspension culture

The cyanobacterial cells grown in a suspension culture with wheat calli were also used for nitroganase activity measurements. The control cyanobacterial cultures were grown under the same conditions but contained no wheat calli. Cultivation was carried out on a gyratory shaker at 24°C with 10/14h dark/light cycle. Acetylene reduction was measured in 1 ml of cyanobacterial cell suspension (cyanobacterial cells were not associated with wheat cell aggregates) in 17 ml vials by the procedure described above. Each ARA value is the mean of three measurements normalized against cyanobacterial chlorophyll. The chlorophyll content was used as a measure of biomass yield and was determined according to Mackinney (1941).

Differentiation of calli

Attempts were made to differentiate plants by using (i) primary calli, (ii) secondary calli obtained by cutting and subculturing primary calli, and (iii) primary calli inoculated with cyanobacteria. Two months old calli grown on MS/D-$(NH_4)_0$ medium were transferred to differentiation-inducing medium [MS-$(NH_4)_0$] which did not contain 2,4-D. Calli that were inoculated with *Nostoc* sp. strain 2S9B were cultivated for another 30 days on MS/D-$(NH_4)_0$ medium before being transferred to MS-$(NH_4)_0$ medium.

Production of extracellular polymeric substance

Nostoc sp. strain 2S9B was grown either as monoculture (control) or in suspension culture with wheat calli in MS-$(NH_4)_0$ medium for 10 days. The cells were removed by centrifugation, and to the cell-free supernatant, an equal volume of absolute alcohol was added. Extracellular polymeric substance was precipitated at –18°C for 24 h. The precipitate was removed by centrifugation at 10 K for 15 min in a high-speed centrifuge (Sorval). The pellet was dried in an oven at 60°C for three days and weighed. The obtained data are means from three separate experiments. Precipitation procedure from either non-inoculated medium [MS/D-$(NH_4)_0$] or from the medium in which only wheat calli were grown, yielded no visible precipitate.

Cell size of cyanobacteria

Cell sizes of both vegetative cells and heterocysts were determined in the monoculture (control) or in suspension cultures grown in MS/D-$(NH_4)_0$ medium for 10 days. The cell size was measured by an image processing system (Image-Pro) fitted onto a Leica light/fluorescent microscope. Sizes were determined by

measuring outlined cell images (μm^2) of at least 100 cells. The percentage of heterocysts was determined by direct observation of at least one thousand cells. The obtained data were subjected to analysis of variance.

Microscopy

The association of cyanobacterial cells with wheat tissue was followed by an ISI super IIIA scanning electron microscope (SEM) and light microscopy of paraffin- and resin-embedded sections. Samples for SEM were prepared by fixing the plant callus colonized with *Nostoc* sp. strain 2S9B with 2.5% glutaraldehyde, followed by washing in distilled water and freezing in liquid nitrogen. The frozen samples were fractured and dehydrated in series of alcohol and critical point dried. The dry tissue was coated with gold and observed under scanning electron microscope.

Paraffin sections were prepared by fixing the colonized wheat tissue with 2.5% (w/v) glutaraldehyde, dehydrating in series of alcohol, which was then replaced with toluene. Paraffin embedded samples were cut to fifteen μm sections and stained with toluidine blue.

Samples for transmission electron microscopy were prepared by embedding cyanobacterial cells in 1.5% (w/v) agar, fixing the agar blocks in 2.5% (v/v) glutaraldehyde, and postfixing them in 1% (w/v) OsO_4 and 2% (w/v) $KMnO_4$. Then the blocks were embedded in Spur's resin, and the sections were cut on a Reichert ultramicrotome. The thin sections were stained with uranyl acetate and lead citrate and observed on a Phillips electron microscope.

Statistical analysis

Stat-100 (Biosoft) software was used for statistical analysis.

3. Results

Co-cultivation of cyanobacteria and plant tissue

The method of inducing callus development described here (adapted from Heyser et al., 1985) permitted rapid production of plant tissue and plant cells for experiments requiring co-cultivation with cyanobacteria. Both primary and secondary calli consisted of big, tubular non-embryogenic cells and small isodiametric embryogenic cells, as previously noted (Heyser et al., 1985). The non-embryogenic cells covered the surface of the callus and could be easily detached, but they were also found inside the callus between compact clusters of small embryogenic cells.

Transfer of a two month-old primary calli onto medium without 2,4-D led to their differentiation into plants in about one month. Same effect had a prolonged cultivation of calli on MS/D without transfers to fresh medium. However, the secondary calli when transferred to 2,4-D-free medium differentiated only into roots but not shoots, therefore only primary calli were used for inoculation with cyanobacteria.

When inoculated onto wheat callus grown on a solid medium, cyanobacteria overgrew the surface of callus in about 10 days. After one month of co-cultivation, the cyanobacteria could be found inside the callus. Colonized calli remained viable for about six months, however they never differentiated into plants. Both scanning electron microscopy and light microscopy of sections confirmed the presence of cyanobacteria inside the callus tissue. Cyanobacterial filaments found in the callus periphery were embedded in their own thick mucilaginous envelopes (Fig. 1a). They were either attached to the surfaces of big non-embryogenic cells or were stretched between them (Fig. 2). In the regions where the big cells were more tightly packed, cyanobacteria filled the spaces between them (Fig. 1b). Cyanobacteria embedded in a mucilaginous matrix were also found inside the big callus cells (Fig. 1c). The mechanism by which cyanobacteria penetrate into the cells is unclear. Deeper inside the callus cyanobacteria filled spaces between clusters of small cells (Fig. 1d). However, they were never found in between small embryogenic cells. Co-cultivation in liquid culture resulted in less extensive colonization of callus than that carried out on solid medium.

Morphology of associated cyanobacteria

Nostoc sp. strain 2S9B has a complex developmental cycle, which consists of an aseriate stage (clumps of tightly packed filaments), heterocystous filaments, and short motile non-heterocystous filaments, called hormogonia. When grown in BG11 *Nostoc* sp. strain 2S9B grows almost exclusively in the form of aseriate packets. The cyanobacterium grown in medium without combined nitrogen ($BG11_0$) exhibits all three forms. During cultivation in plant tissue culture medium MS/D-$(NH_4)_0$, *Nostoc* sp. strain 2S9B appeared only in long heterocystous filaments, whether or not wheat calli were added.

Co-cultivation with the calli of all three examined wheat cultivars, led to a statistically significant increase in size of both vegetative cells and heterocysts (Table 1). The increase ranged from 10% to 60% compared to that of free-living cultures. The largest increase was noticed in the presence of Penawawa cultivar. Cyanobacteria co-cultivated with wheat calli showed an increase in heterocyst frequency, which was, however, statistically not significant (Table 1).

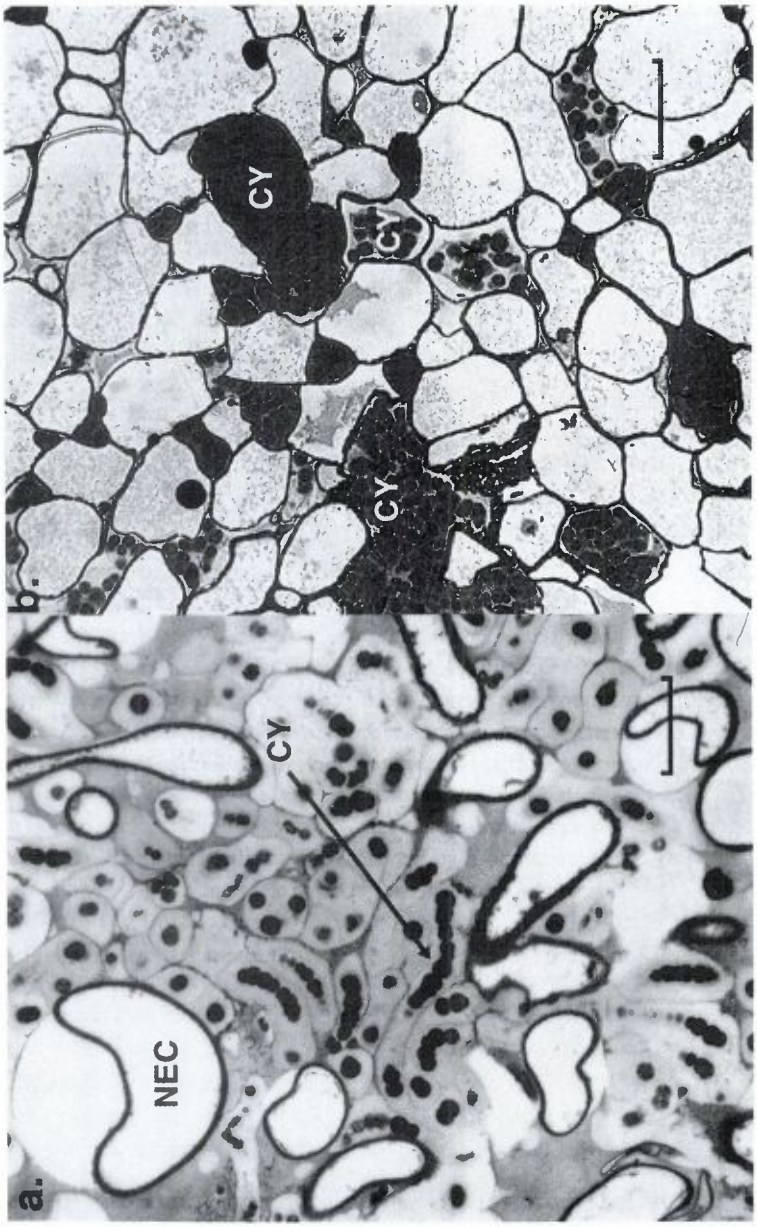

Figure 1. Cross section of colonized wheat callus embedded in resin (a,b,c,) or paraffin (d). a: Cyanobacteria colonizing the space between big non-embryogenic cells on the periphery of wheat callus. Note the thick capsules around the cyanobacterial filaments. Bar = 10 µm. b: Cyanobacteria extensively colonizing the space in-between big non-embryogenic cells inside the callus. Bar = 10 µm.

Figure 1. Continued. c: Cyanobacteria inside the big non-embryogenic cell embedded in mucilaginous matrix. Bar = 10 µm. d: The space between clusters of small embryogenic cells colonized with cyanobacteria, inside the callus. Bar = 50 µm. CY = cyanobacteria; NEC = big non-embryogenic cells; EC = small embryogenic cells.

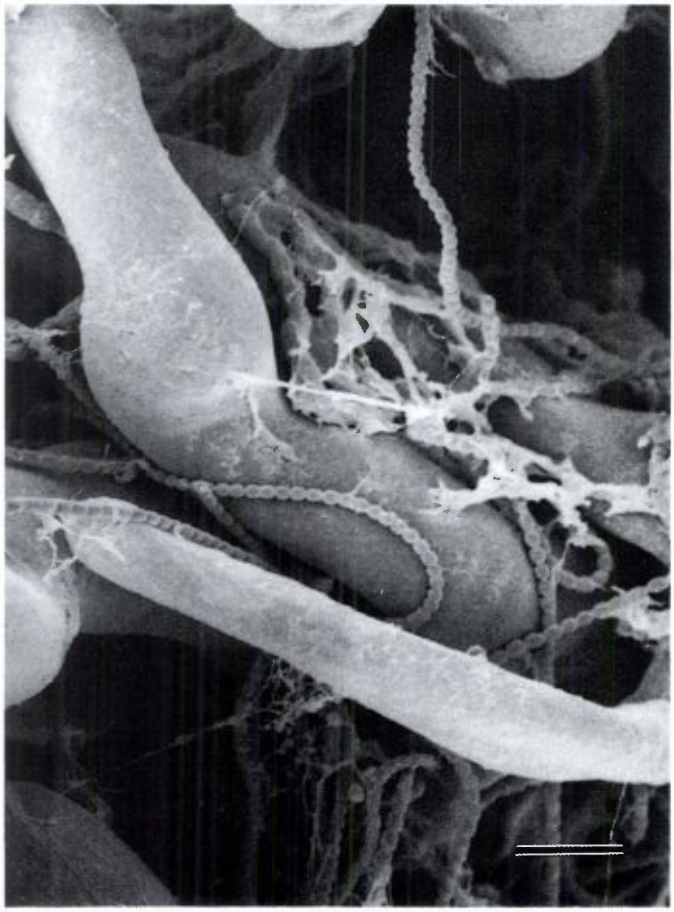

Figure 2. Scanning electron micrograph of freeze-fractured wheat (Penawawa) callus colonized internally by *Nostoc* sp. strain 2S9B. Bar = 20 µm.

In control cultures grown in MS/D-$(NH_4)_0$-vegetative cells contained up to 7 wavy thylakoids running in parallel with the cell wall (Fig. 3a). The thylakoid-free nucleoplasmic region occupied most of the cell volume. When *Nostoc* was co-cultivated with wheat calli, the number and arrangement of their thylakoids drastically changed. Thylakoids occupied most of the cell volume, became less numerous and contorted, and did not run parallel to the cell wall (Fig. 3b). The nucleoplasmic region appeared more condensed and contained numerous carboxysomes. These changes were similar to those already described in *Nostoc* in natural symbiosis with *Blasia pusilla* (Stewart et al., 1977) and *Gunnera* (Johansson and Bergman, 1992). Apart from exhibiting a

Table 1. Effect of co-cultivation of *Nostoc* sp. strain 2S9B with calli of different wheat cultivars on the cyanobacterial cell size and heterocyst frequency.

Growth condition	Size of cells (μm^2)**		% Heterocyst
	Vegetative	Heterocyst	
MS/D-$(NH_4)_0$	36.4±1.17	77.4±4.1	4.9
MS/D-$(NH_4)_0$+ Pen.*	60.2±2.40***	101.9±4.9***	5.1
MS/D-$(NH_4)_0$+ Kl.*	47.2±1.78***	95.4±2.32***	5.3
MS/D-$(NH_4)_0$+ Ed.*	54.5±2.31***	85.7±4.06***	5.7

*Wheat cultivars: Penawawa, Klassic, Edwal; ** See explanation in Materials and Methods; ***Statistically significant values, compared to the control culture grown without wheat calli [MS/D-(NH4)0], at the confidence level of 0.01.

thicker cell polysaccharide envelope, heterocysts had an ultrastructure not noticeably different from that seen in heterocysts of *Nostoc* grown in the absence of calli (not shown). The same kind of ultrastructural alteration was noticed in the presence of all three wheat cultivars.

Physiology of callus-cyanobacteria association

In the presence of wheat calli the biomass yield of cyanobacteria was significantly increased and was up to 2.5 times higher than in cultures without calli (Fig. 4a). Nitrogenase activity of *Nostoc* sp. strain 2S9B was increased 2–3 fold in the presence of wheat calli (Fig. 4b). The maximal activity in the control culture was reached 3 days after inoculation while that in the presence of wheat calli was somewhat delayed. The nitrogenase activity of free-living *Nostoc* declined gradually, in contrast to the activity of cyanobacteria co-cultivated with calli, which showed a rapid decrease after reaching the maximum. The same pattern of nitrogenase activity was recorded both when co-cultivation was carried out in liquid (Fig. 4b) or solid medium (data not shown).

Cyanobacterial co-cultivation with wheat calli brought about a viscous appearance of the cultures not seen in the wheat cell cultures grown without cyanobacteria. Production of extracellular polymeric substance in the cyanobacteria-callus suspension cultures was increased from 3 to 5 folds (Fig. 5). The largest production was recorded in the presence of Edwal cultivar, which amounted 1.60 gl^{-1} (dry weight).

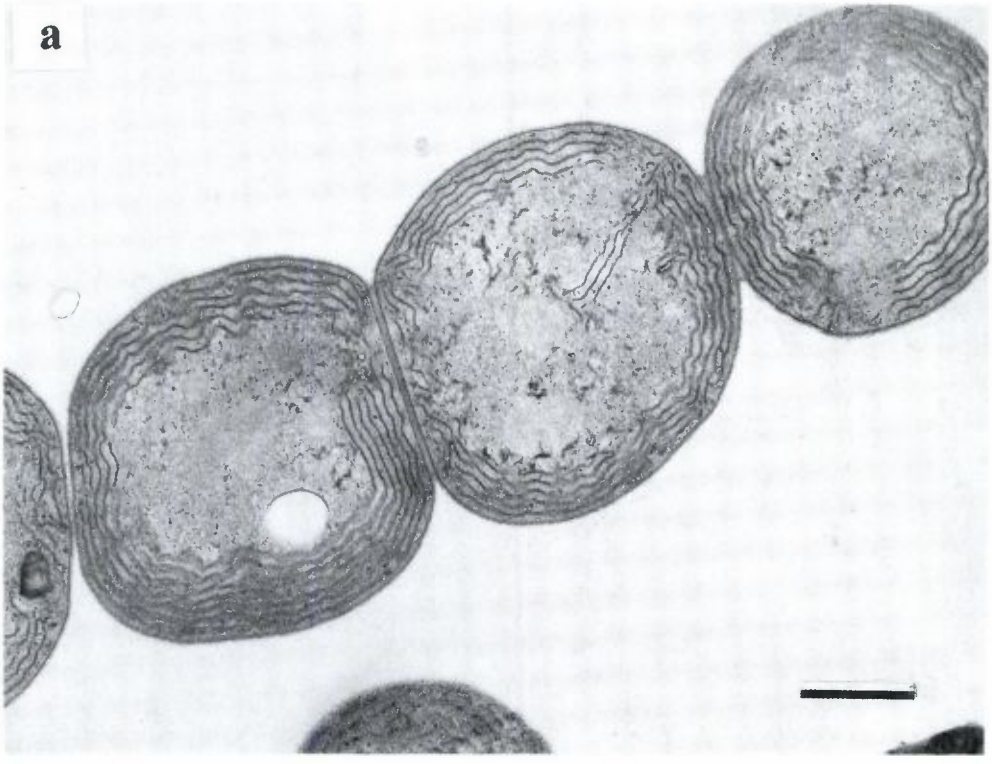

Figure 3. Electron micrograph of *Nostoc* sp. strain 2S9B in liquid MS/D-$(NH_4)_0$ medium (a) as monoculture or (b) in suspension culture with wheat callus (cultivar Klassic) grown for 10 days. Bar = 1 μm.

4. Discussion

There have been numerous attempts to co-cultivate various plants with different cyanobacteria by using isolated protoplasts, isolated cells, tissue cultures, plant regenerates, and whole plants (see Gusev and Korzhenevskaya, 1990). This report is the first attempt to co-cultivate cyanobacteria with a callus of one of the most important crop plants – wheat. We have demonstrated that the cyanobacterium *Nostoc* sp. strain 2S9B can infect and grow within the tissue of wheat callus and perform nitrogenase activity. The wheat callus infected with *Nostoc* sp. strain 2S9B was able to maintain its viability for prolonged period of time but then eventually succumbed to cyanobacteria that overgrew it. In natural symbioses, the growth rate of cyanobacteria within the

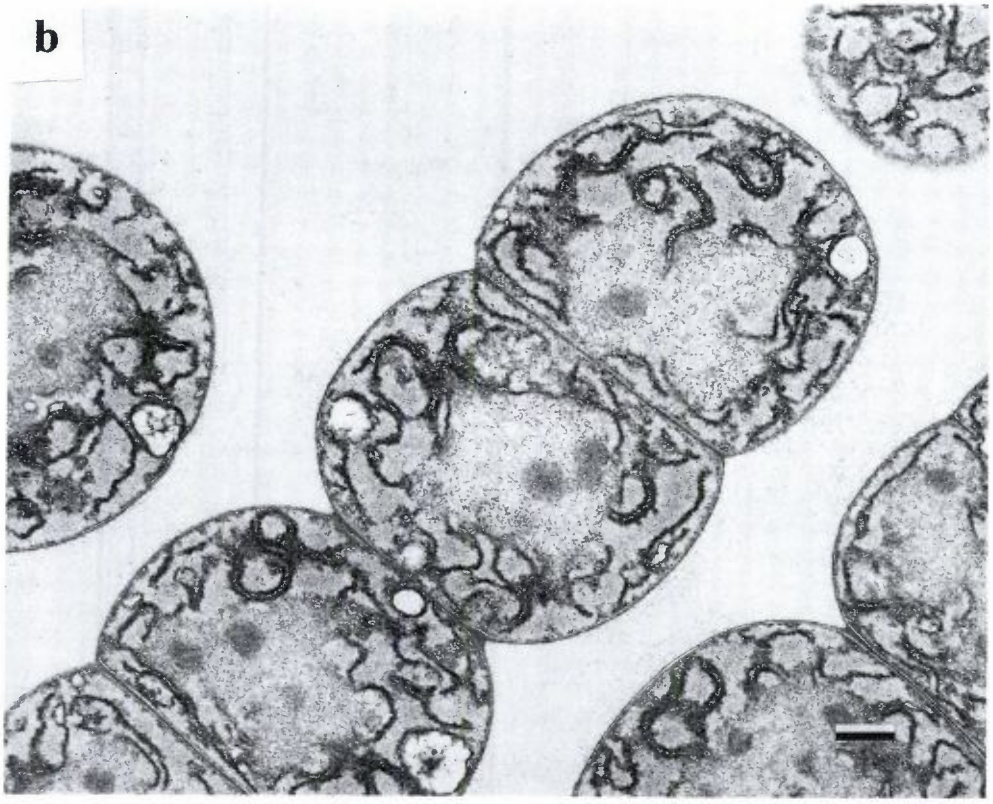

Figure 3. Continued.

symbiotic host plant appears to be regulated by an unknown mechanism (Meeks, 1998), and this regulation seems to be one of the prerequisites for the establishment of a functional symbiosis. In our experiments, the growth yield of cyanobacteria in the presence of wheat calli actually increased 2.5 times, indicating that wheat lacks the mechanism to contain the growth of associated cyanobacteria.

Unlike control calli, calli that were extensively colonized never differentiated into plants. One possible explanation for that is that the plant growth hormones produced by cyanobacteria (Metting and Payne, 1986) interfered with plant differentiation. However, whether *Nostoc* sp. strain 2S9B produces plant hormones remains yet to be proven.

On the other hand, *Nostoc* sp. strain 2S9B associated with wheat callus of all three wheat cultivars tested responded to plant signals and underwent structural and functional modifications. Some of these changes were similar to those seen in natural cyanobacterial symbiosis. For example, there is an

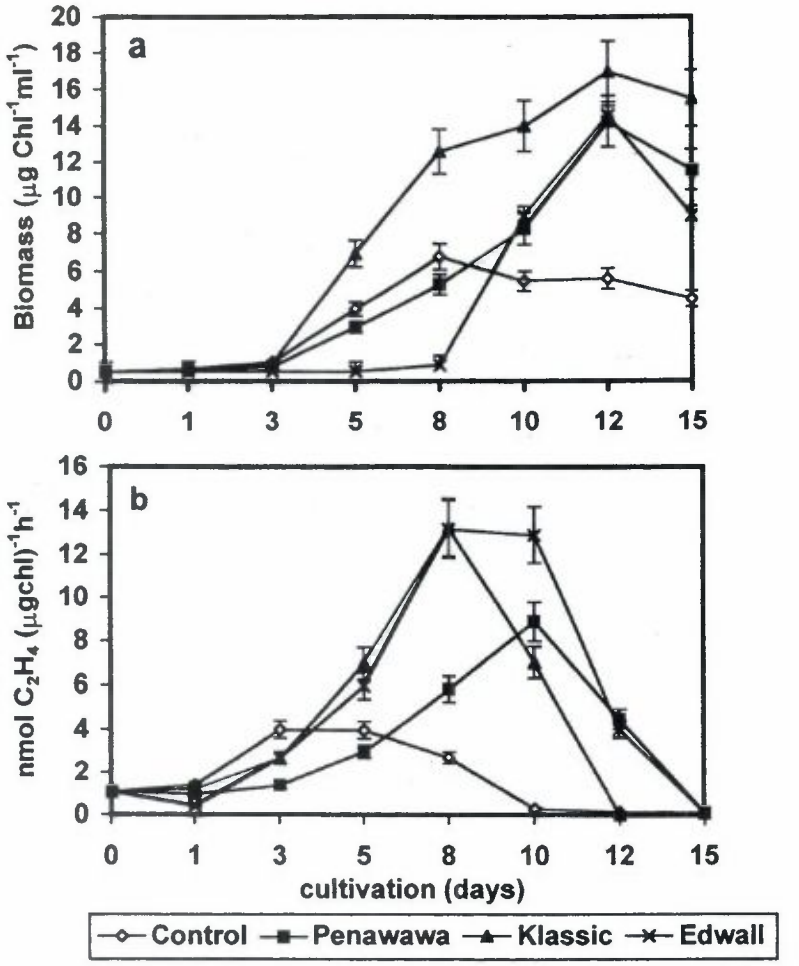

Figure 4. Biomass yield (a) and nitrogenase activity (b) of *Nostoc* sp. strain 2S9B when grown as monoculture (control) or suspension culture with wheat calli.

enhanced rate of nitrogenase activity (see Meeks, 1998) in naturally occurring symbiosis of cyanobacteria and plants. In our experiments nitrogenase activity was increased in cyanobacteria associated with wheat calli. An increase in nitrogenase activity as well as in heterocyst frequency was also described during an associative growth of alfalfa and *Anabaena variabilis* (Korzhenevskaya et al., 1993). There is a general belief that the nitrogenase enhancement can be attributed to the availability of reduced carbon supplied by the host plant, (Meeks, 1998), however, in our experiments wheat calli did not have any photosynthetic activity. This may indicate the presence of some

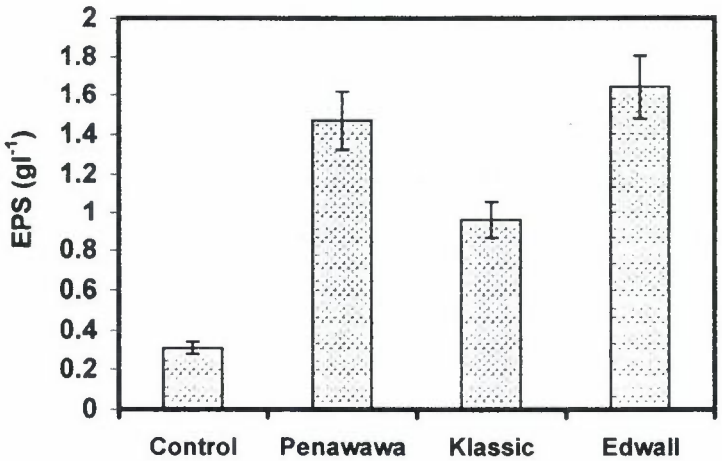

Figure 5. Production of extracellular polymeric substance (EPS) of *Nostoc* sp. strain 2S9B, when grown as monoculture (control) or suspension culture with calli of three wheat cultivars.

active factor released by the callus tissue that stimulates the nitrogenase activity. The increase in nitrogenase activity was not paralleled by a similar increase in heterocyst frequency as is usually found in natural symbioses (Bergman et al., 1996). Nevertheless, there was a significant increase in heterocyst and vegetative cell volume, a phenomenon described during cyanobacterial infection of *Gunnera* (Soederback and Bergman, 1992) and *Anthoceros punctatus* (Meeks, 1998). The significance of the change in cell size is unknown.

In natural symbioses between cyanobacteria and plants, cyanobionts are confined within structures that are filled with mucilage (see Lindblad et al., 1985; Bergman et al., 1992; Braun-Howland and Nierzwicki-Bauer, 1990). However, there is still controversy about the origin of the mucilage. Forni et al. (1998) suggested that the plant, cyanobacteria, and associated bacteria all contribute to the polysaccharide matrix of leaf cavities in *Azolla*.

We have previously shown that the extracellular polymeric substance of *Nostoc* sp. strain 2S9B, which is mostly composed of polysaccharide (Gantar et al., 1995a), is responsible for the firm attachment of cyanobacteria to the wheat root surface. Here we demonstrate an increase in synthesis of extracellular polymeric substance in the cyanobacteria-wheat callus cultures. Although the source of this polymeric substance is not clear, the presence of thick sheath around the cyanobacterial filaments, not seen in free-living cultures, as well as its absence in wheat callus suspension cultures, may point to its cyanobacterial origin. Similar thick mucilaginous envelopes were also

observed in associated growth of cyanobacteria and root cap of alfalfa (Korzhenevskaya et al., 1993). The significance and mechanism of increased polysaccharide production during co-cultivation of cyanobacteria and plants require further investigation.

In conclusion, *Nostoc* sp. strain 2S9B can co-exist within wheat callus while maintaining its N_2-fixing ability. With other characteristics such as derepressed nitrogenase, ability to grow heterotrophically (Gantar et al., 1995b), induction of hormogonia differentiation by wheat root extract (Gantar et al., 1993), *Nostoc* sp. strain 2S9B should be considered as a good candidate for artificial symbioses. However, before functional symbiosis between *Nostoc* sp. strain 2S9B and wheat can be created, further work is required to elucidate the mechanism by which the cyanobacteria interfere with plant differentiation.

Acknowledgments

This work was supported by Grant No. DE-FG02-97ER12211 awarded by the U.S. Department of Energy.

REFERENCES

Bergman, B., Rai, A.N., Johansson, C., and Soderback, E. 1992. Cyanobacterial plant symbioses. *Symbiosis* **14**: 61–81.

Bergman, B., Matveyev, A., and Rasmussen, U. 1996. Chemical signaling in cyanobacterial-plant symbioses. *Trends in Plant Sciences* **1**: 191–197.

Braun-Howland, E.B. and Nierzwicki-Bauer, S.A. 1990. *Azolla-Anabaena* symbiosis: biochemistry, physiology, ultrastructure, and molecular biology. In: *CRC Handbook of Symbiotic Cyanobacteria*. A.N. Rai, ed., CRC Press Inc., Boca Raton, pp. 65–117.

Burgoon, A.C. and Bottino, P.J. 1976. Uptake of nitrogen fixing blue-green alga *Gloeocapsa* into protoplast of tobacco and maize. *Journal of Heredity* **67**: 223–226.

Christiansen-Weniger, C. 1996. Endophytic establishment of *Azorhizobium caulinodans* through auxin-induced root tumors of rice (*Oryza sativa* L.). *Biology and Fertility of Soils* **21**: 293–302.

Denarie, J., Debelle, F., and Rosenberg, C. 1992. Signaling and host range variation in nodulation. *Annual Reviews in Microbiology* **46**: 497–531.

Elhai, J. and Wolk, C.P. 1990. Developmental regulation and spatial pattern of expression of the structural genes for nitrogenase in the cyanobacterium *Anabaena*. *EMBO Journal* **116**: 3379–3388.

Forni, C., Haegi, A., and Del Gallo, M. 1998. Polysaccharide composition of the mucilage of *Azolla* algal packets. *Symbiosis* **24**: 303–314.

Gantar, M., Obreht, Z., and Fojkar, O. 1991a. Occurrence and characterization of nitrogen-fixing cyanobacteria in different temperate soils. *Mikrobiologija* (Yu) **28**: 33–44.

Gantar, M., Kerby, N.W., Obreht, Z., and Rowell, P. 1991b. Colonization of wheat (*Triticum vulgare* L.). I. A survey of soil cyanobacterial isolates forming associations with roots. *New Phytologist* **118**: 477–483.

Gantar, M., Kerby, N.W., and Rowell, P. 1993. Colonization of wheat (*Triticum vulgare* L.) by N_2-fixing cyanobacteria: III. The role of hormogonia promoting factor. *New Phytologist* **124**: 505–513.

Gantar, M., Rowell, P., Kerby, N.W., and Sutherland, I.W. 1995a. Role of extracellular polysaccharide in the colonization of wheat (*Triticum vulgare* L.) roots by N_2-fixing cyanobacteria. *Biology and Fertility of Soil* **19**: 41–48.

Gantar, M., Kerby, N.W., Rowell, P., and Scrimgeour, C. 1995b. Colonization of wheat (*Triticum vulgare* L.) by N_2-fixing cyanobacteria: IV. Dark nitrogenase activity and effects of cyanobacteria on natural ^{15}N abundance in the plants. *New Phytologist* **129**: 337–343.

Gantar, M. and Elhai, J. 1999. Colonization of wheat para-nodules by the N_2-fixing cyanobacterium *Nostoc* sp. strain 2S9B. *New Phytologist* **141**: 373–379.

Gusev, M.V., Korzhenevskaya, G., Pyvovarova, L.V., Baulina, O.I., and Butenko, R.G. 1986. Introduction of a nitrogen-fixing cyanobacterium into tobacco regenerates. *Planta* **167**: 1–8.

Gusev, M.V. and Korzhenevskaya, T.G. 1990. Artificial associations. In: *Handbook of Symbiotic Cyanobacteria*. A.N. Rai, ed., CRC Press, Boca Raton, FL, pp. 173–230.

Heyser, J.W., Nabors, M.W., MacKinnon, C., Dykes, T.A., Demott, K.J., Kautzman, D.C., and Mujeeb-Kazi, A. 1985. Long-term, high-frequency plant regeneration and the induction of somatic embryogenesis in callus cultures of wheat (*Triticum aestivum* L.). *Zeitschrift für Pflanzenzüchtung* **94**: 218–233.

Johansson, C. and Bergman, B. 1992. Early events during the establishment of the *Gunnera/Nostoc* symbiosis. *Planta* **188**: 403–413.

Kennedy, I.R., Pereg-Gerk, L.L., Wood, C., Deaker, R., Gilchrist, K., and Katupitiya, S. 1997. Biological nitrogen fixation in non-leguminous crops: Facilitating the evolution of an effective association between *Azospirillum* and wheat. *Plant and Soil* **194**: 65–79.

Kijne, J.W. 1992. The *Rhizobium* infection process. In: *Biological Nitrogen Fixation*. G. Stacey, R.H. Burris, and H.J. Evans, eds., Chapman & Hall, New York, pp. 349–398.

Korzhenevskaya, T.G., Baulina, O.I., Gorelova, O.A., Lobacova, E.S., Butenko, R.G., and Gusev, M.V. 1993. Artificial Syncyanoses: the potential for modeling and analysis of natural symbioses. *Symbiosis* **15**: 77–103.

Lindblad, P., Bergman, B., Hofsten, A. V., Hallbom, L., and Nylund, J.E. 1985. The cyanobacterium-*Zamia* symbiosis: an ultrastructural study. *New Phytologist* **101**: 707–716.

Mackinney, G. 1941. Absorption of light by chlorophyll solutions. *Journal of Biological Chemistry* **140**: 315–322.

Meeks, J.C., Malmberg, R.L., and Wolk, C.P. 1978. Uptake of auxotrophic cells of heterocysts-forming cyanobacterium by tobacco protoplasts, and the fate of their associations. *Plant* **139**: 55–60.

Meeks, J.C. 1998. Symbiosis between nitrogen-fixing cyanobacteria and plants. *Biosciences* **48**: 266–276.

Metting, B. and Pyne, J.W. 1986. Biologically active compounds from microalgae. *Enzyme Microbial Technology* **8**: 386–394.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue culture. *Physiologia Plantarum* **15**: 473–497.

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M., and Stanier, R.Y. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Journal of General Microbiology* **111**: 1–61.

Stewart, W.D.P., Fitzgerald, G.P., and Burris, R.H. 1967. In situ studies on N_2-fixation using acetylene reduction technique. *Proceedings of National Academy of Sciences USA* **58**: 2071–2078.

Stewart, W.D.P., Rowell, P., and Lockhart, C.M. 1977. Associations of nitrogen-fixing prokaryotes with higher and lower plants. In: *Nitrogen Assimilation of Plants*. E.J. Hewitt and C.V. Cutting, eds., Academic Press, London, pp. 45–66.

Soederback, E. and Bergman, B. 1992. The *Nostoc-Gunnera magellanica* symbiosis: phycobiliproteins, carboxysomes and Rubisco in the microsymbiont. *Physiologia Plantarum* **84**: 425–432

Tchan, Y.T. and Kennedy, I.R. 1989. Possible N_2-fixing root nodules induced in non-legumes. *Agricultural Sciences* (AIAS Melbourne New Series) **2**: 57–59.

Vaara, T., Vaara, M., and Niemela, S. 1979. Two improved methods for obtaining axenic cultures of cyanobacteria. *Applied and Environmental Microbiology* **39**: 1011–1014.

Wang, W.C. and Nguyen, H.T. 1990. A novel approach for efficient plant regeneration from long-term suspension culture of wheat. *Plant Cell Reports* **9**: 639–642.

Wolk, C.P., Ernst, A., and Elhai, J. 1994. Heterocyst metabolism and development. In: *The Molecular Biology of Cyanobacteria*, D.A. Bryant, ed. Kluwer Academic, Dordrecht, Netherlands, pp. 769–823.