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Characterization of rhizo-cyanobacteria and their associations with wheat seedlings

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Abstract

Four heterocystous cyanobacteria, belonging to the genera *Anabaena* and *Nostoc* isolated from the rhizosphere of wheat, were tested for their ability to form associations with the roots of wheat seedlings under light and dark conditions using hydroponics. The cyanobacterial strains formed close associations with wheat plants, and were able to enter through root hairs and penetrate the epidermal layer of wheat roots. Significantly higher indole acetic acid production and chlorophyll accumulation was observed in such associations, and strain N1 recorded a several-fold enhancement in acetylene reduction activity. Supplementation of the medium with glucose, yeast extract or glutamic acid also enhanced the acetylene reduction activity of the N1 strain, indicative of its photoheterotrophic ability.

Keywords: ARA; Artificial associations; IAA; Rhizosphere

Introduction

Cyanobacteria are an ancient diverse group of photosynthetic prokaryotes, which show morphological resemblances to Gram-negative bacteria but perform oxygenic photosynthesis like higher plants. Many of them also exhibit biological nitrogen fixation, and have been exploited as biofertilizers in agriculture, wherein they are known to contribute 20-25 kg N/ha/season and enhance soil fertility (Yanni 1992, Prasanna & Kaushik 2006). Cyanobacteria are almost exclusively free-living and ideally suited to an independent existence, but many of them have the capacity to form specific associations with protista, fungi and plants, ranging from unicellular algae to angiosperms (Rai *et al*. 2000, Gusev *et al*. 2002).

Scientists are now interested in creating artificial symbioses between higher plants and N_2-fixing microorganisms with a view to introducing nitrogen-fixing ability into the plants. Novel associations including those between *Rhizobium* and rice (Al-Mallah *et al*. 2002) and *Anabaena* and tobacco (Gusev *et al*. 2002) have been reported. Nilsson *et al*. (2002) tested the efficiency of numerous symbiotic cyanobacterial isolates to associate with rice, and found that under laboratory conditions a number of them were successful in forming artificial associations. Gantar *et al*. (1991a,b) observed interesting associations of selected cyanobacterial strains with wheat seedlings and characterized them on the basis of ultrastructural studies and nitrogenase activity; however, their role in plant growth promotion was not evaluated. The pre-requisite for any kind of association between cyanobacteria and plants is that the cyanobacteria must exist and proliferate in close vicinity of potential host. In our investigation, we hypothesized that the cyanobacteria isolated from the wheat rhizosphere would possess an inherent capability to establish near wheat roots, and prove better competitors in soil for developing artificial associations. Hence our aim was to isolate and characterize naturally-occurring

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cyanobacteria from the rhizosphere of wheat, and evaluate their potential for developing associations that would promote plant growth.

Materials & Methods

Cyanobacterial strains were isolated from the roots of wheat (*Triticum aestivum* variety HD2687) from experimental fields at the Indian Agricultural Research Institute by placing thoroughly washed (repeatedly with sterile water) and cut pieces of wheat roots in BG-11 medium. Standard microbiological techniques were adopted for isolation and purification of cyanobacterial strains, with incubation at 27 ± 1 ^{0}C, 3000 lux light intensity and 16L:8D cycle (Stanier *et al*. 1971). Regular monitoring for cyanobacterial growth was done by visual and microscopic examination. Identification of the strains was done using keys of Desikachary (1959).

The colonization of roots of wheat seedlings was carried out using 2-d germinated wheat seedlings. A hydroponic set was devised employing truncated plastic tips (in order to permit root growth of germinated seedlings through the tips) that were placed in test tubes containing BG-11 medium, inoculated with 5% inoculum (equivalent to 25 µg ml^{-1} chlorophyll) of 10d (log phase) cyanobacterial cultures. The setup was sterilized before placing seedlings or the cyanobacterial cultures. Colonization of wheat roots (3 seedlings per replication, i.e. 9 seedlings per treatment) was carried out in a fully factorial design, with four different strains and a non-inoculated control, in BG-11 medium with or without nitrate and in the presence and absence of light. After 12 d of co-culture, the type and degree of cyanobacterial colonization was examined visually and microscopically. The surface of fresh roots and freshly cut transverse sections were examined, before and after embedding in resin, using an Olympus (Model SC35) microscope.

Measurement of acetylene reduction activity (ARA) was measured after 12 d and used as an index of nitrogen fixation in free-living cyanobacterial cultures as well as cyanobacteria in association with roots of wheat seedlings (Jewell & Kulasooriya 1970), by gas chromatographic quantification of ethylene formed. In both the cases, 10% (v/v) acetylene was injected, after removal of equivalent amount of air, and incubated under light for 90 minutes. The triplicate sets of data were subjected to statistical analyses and are plotted as means ± standard deviations.

Cyanobacterial chlorophyll was estimated spectrophotometrically by the method of MacKinney (1941) and plant chlorophyll by the method of Jeffrey & Humphrey (1975). Chlorophyll content of wheat seedlings in association with cyanobacterial culture as depicted in the Figures represents the sum of chlorophyll content of wheat plant and cyanobacterial strains measured independently. The effect of C/N supplementation was studied using one cyanobacterial strain which exhibited enhanced ARA in association with wheat seedlings from the experiments described above; it was grown (alone or in association with wheat seedlings) in BG-11 medium (without nitrate) supplemented with glutamic acid (0.1 mg l^{-1}), yeast extract (0.1 mg l^{-1}) and glucose (0.5%) in light as well as in dark.

IAA production of the cyanobacterium-wheat association and un-inoculated wheat seedlings was measured spectrophotometrically, using extracellular filtrates, employing the method of Gordon & Weber (1951).

The triplicate sets of data for the various parameters evaluated were subjected to ANOVA (Analysis of Variance) in accordance with the experimental design (Completely Randomized Design) using the MSTAT-C statistical package to quantify and evaluate the source of variation. Standard deviations (SD) are depicted in the graphs; critical differences (CD) values were calculated for a p-level of 0.05.

Results

The experiments were undertaken with the four heterocystous strains, which included three *Nostoc* and one *Anabaena* strain. Among the three *Nostoc* strains, N2 was morphologically very different from the other two strains. It had typically blue- green coloured filaments, which were long and aggregated in clusters, while the other two *Nostoc* (N1 and N3) strains were characterized by shorter filaments. N3 exhibited very thick mucilage and cells were granulated. *Anabaena* sp. was characterized by formation of chains of akinetes.

All the heterocystous strains tested formed tight associations (which persisted even after repeated washing with running water) with the roots of wheat seedlings (Figure 1a,c) in light (both in presence and absence of nitrate). Strains N2 and N3 triggered lateral root formation in wheat seedlings, which was more extensive in N2 (Figure 1b,d). In freshly cut sections of wheat roots with cyanobacterial association (in light), a thick cyanobacterial growth was observed outside the epidermis (Figure 1e). The entry of the cyanobacterium was through the tip of root hairs, and the progressive stages of this process are depicted in Figure 1 (f, g, h). All the four strains were able to penetrate the epidermal layer of wheat root and short (2-4) celled filaments, resembling hormogonia could be located below the epidermal layer, in transverse sections (Figure 1,i). Cyanobacterial cells/filaments (1-2 celled) were also visible in the cortical region of the wheat root (Figure 1,j).

The ARA in cyanobacteria-root association grown in BG-11 medium (devoid of nitrate), involving N1 was 10 folds higher than in free-living culture (Figure 2a). A comparison of chlorophyll content of wheat seedlings with/without cyanobacteria showed that co-inoculation invariably increased the chlorophyll content of wheat seedlings both in light and dark (Figure 2b). The highest extra cellular IAA was recorded in A1 strain grown along with wheat seedlings in light. Two-way ANOVA indicated that significant differences also existed among the strains ($F_{1,4} = 18.63$; $p=0.0001$. Among the four strains tested, only A1 strain produced significantly higher amount of IAA in light as compared to dark. But in dark, N1 strain recorded highest IAA production (Figure 2c).

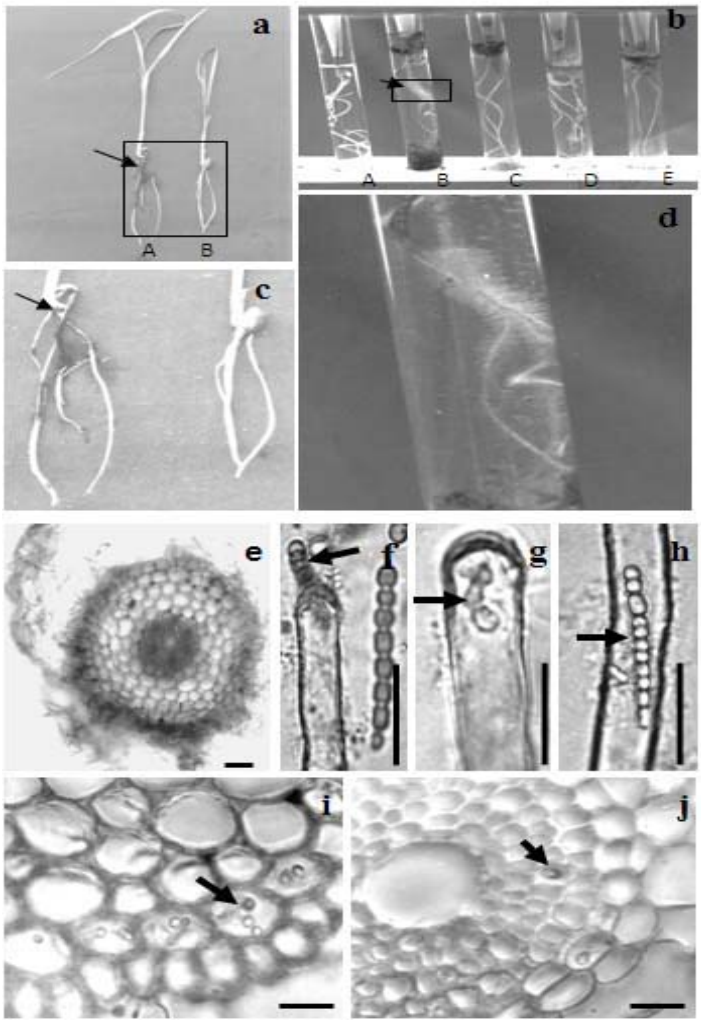

Figure 1: Cyanobacterial association with roots of wheat seedlings after 10d of co-culture in BG-11 in light. (a) Roots of wheat seedlings showing tight associations with cyanobacteria (A), wheat seedlings grown alone (B); (b) Experimental set up for evaluating association between wheat seedlings and cyanobacterial strains N1, N2, N3 and A; Control (A), N2 (B), N3 (C), N1 (D) and A (E); Arrow indicates the extensive lateral roots; (c) Magnified surface view of the roots of wheat seedlings; Arrow indicates the colonization by cyanobacteria; (d) Magnified view of the roots of wheat seedling after co-culturing with N1, showing lateral root formation; (e) Transverse sections (TS) of wheat root showing cyanobacterial packets around epidermis; (f-h) Stages in the entry of filaments into the root hairs; (i) Short filaments beneath the root epidermis and (j) cyanobacterial cells in the pericycle/endodermis region. Arrows denote the filament. The size of black bar is 20µm.

Based on the interesting behavior of N1, this strain was grown in medium supplemented with glutamic acid / yeast extract/ glucose which enhanced ARA (Figure 2d). A low level of ARA was also detected in the dark, when N1 was grown in the medium supplemented with glucose (data not shown). This cyanobacterium was observed to form close association with roots of wheat seedlings in all the treatments.

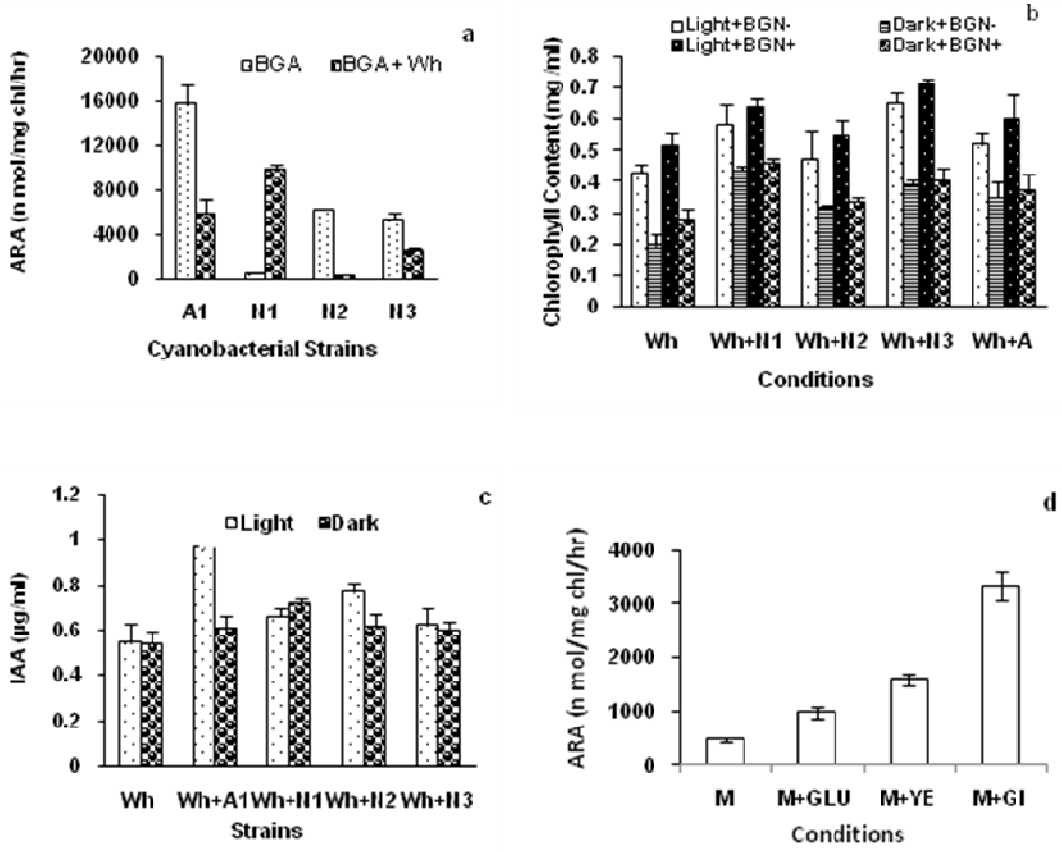

Figure 2 (a) Nitrogenase activity of rhizo-cyanobacterial isolates grown alone / in association with wheat seedlings. BGA and Wh denote cyanobacteria and wheat respectively; (b) Chlorophyll content (mg mL^{-1}) in wheat seedlings grown alone/in association with cyanobacterial strains in presence / absence of light. BGN- and BGN+ refer to BG 11 medium devoid and supplemented with nitrate respectively; (c) IAA content (µg mL^{-1}) in wheat seedlings grown in association with cyanobacterial strains in light and dark environments; (d) Nitrogenase activity of *Nostoc* sp (N1) in the presence/ absence of different C and N sources (M, Basal medium; GLU, Glutamic Acid; Gl, Glucose; YE, Yeast Extract). Vertical bars on columns represent SD (n=3).

Discussion

The rhizosphere of crop plants is a relatively unexplored frontier in terms of cyanobacterial abundance and diversity. During the current investigation, all the heterocystous cyanobacterial isolates from the rhizosphere of wheat were able to form a close association with wheat roots in hydroponic experiments. Gantar *et al*. (1991 a,b) also reported such associations with wheat roots, in which two strains of *Nostoc* were able to form tight associations with roots and penetrate the epidermis. Such isolates may be involved in close mutualistic relationships involving nutritional and biochemical

interactions with wheat roots and can be exploited as potential bioinoculants for wheat. The mechanism by which cyanobacterial cells enter the plant is not clear, but root hair exudates seem to attract the filaments and may involve recognition of some lectins (McCowen *et al*. 1987; Knight & Adams 1996) or hormogonia-promoting factor / bioactive compounds present in root exudates. Long cyanobacterial filaments were found to be closely attached to the root tip surface in all the four cases, but inside the root epidermis, 3-4 celled filaments were only observed. Two out of three *Nostoc* strains tested triggered lateral root formation, leading to increased surface area of wheat roots coming in contact with cyanobacteria, thereby aiding in better colonization.

In the co-culturing experiment all the strains showed significantly higher amounts of IAA, which is reflective of the plant growth-promoting role of the cultures. The strain N1 also showed higher ARA in association with wheat seedlings. A similar observation, related to stimulation of ARA, was also reported in black mangrove seedlings inoculated with a filamentous cyanobacterium- *Microcoleus* sp. (Toledo *et al*. 1995). Addition of YE, Glutamic acid and glucose in nitrogen free BG-11 medium, in the present study, significantly increased ARA in N1, indicative of photoheterotrophic abilities of the strain.. The root exudates might be providing stimulatory/growth-promoting compound(s) such as those present in YE, Glutamic acid or glucose, which promote heterotrophic growth and make available energy equivalents for cyanobacteria, which in turn, exhibit enhanced ARA.

In the current study, we found that chlorophyll content of wheat seedlings was enhanced, when co-cultured with any of the four cyanobacterial strains and IAA production was shown in all the cyanobacteria-wheat associations. Interestingly, N1–wheat association showed highest IAA production in dark, among all the strains, which could be correlated to the higher chlorophyll accumulation in this association in dark. Misra & Kaushik (1989) have reported the production of growth promoting substances by cyanobacteria, which enhance germination and growth of rice seedling, however scanty published literature is available with respect to wheat. Cyanobionts in *Gunnera* are known to secrete arabinogalactan proteins that might act as signal molecules regulating plant growth and development (Bergman *et al*. 1996). Sergeeva *et al*. (2002) reported the production of indole-3-acetic acid by many free living and symbiotic cyanobacteria, which has been shown in our studies also. The significance of nitrogen fixing BGA in self maintenance of the status of tropical rice field soils and its utilization as inoculants has shown to bring about a yield improvement of 5 - 25% in rice, even in the presence of high doses of nitrogenous fertilizers (Yanni 1992).

The potential of these rhizo-cyanobacterial isolates, especially N1 strain is tremendous as a bioinoculant, because it can supplement the N-requirements and promote the growth of wheat plants through enhanced ARA and IAA production both under light and dark conditions. Such associations can also serve as useful models for a better understanding of signaling mechanisms in the rhizosphere, besides suggesting that cyanobacteria may have genetic potential to form new symbiotic systems with non-symbiotrophic plants, such as wheat.

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الملخص العربى

صفات السيانوبكتريا الجذرية وعلاقتها بنبات القمح

برنتا شاسوال١ – ريدا براسانا١ – ساسواتى نایاك١ – انشولى سوود١ – م. ر. سوسيلا٢
١. مركز الحفاظ واستخدام الطحالب الخضراء والزرقاء – معهد البحوث الزراعية الهندى – نيودلهى
٢. المعهد القومى لبحوث النبات – لوكنوا – الهند

تم إختبار قدرة اربعة انواع من السيانوبكتريا على التوافق مع جذور نبات القمح، واوضحت الدراسة ان البكتريا لها علاقة وثيقة بنبات القمح وكان لها القدرة العالية على إختراق جذور النبات من خلال الشعيرات المنتشرة على الجذور. صاحب هذا افرازات معنوية عالية فى حمض الخليك الاندولى وكمية الكلورفيل المترسب وكذلك ارتفعت كمية ن١ مما زاد من اختزال نشاط الاستيلين. وعند إضافة الجلوكوز او مستخلص الخميرة او حامض الجلتوميك فقد ادى ايضا إلى زيادة اختزال نشاط الاستيلين.