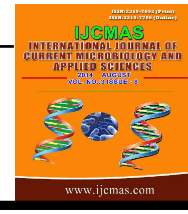

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Original Research Article

Root colonization in rice and wheat by wild type and mutant strains of *Anabaena variabilis*

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A B S T R A C T

Keywords

Cyanobacteria, Root colonization, Associative N_2-fixation, Biofertilizer

The present study was undertaken to test the potentiality of wild type and the mutant strains [multiple herbicide resistant-*Av* (MHR)^r, ammonia excretory mutant - *Av* (Eda)^r and ammonia excretory multiple herbicide resistant mutant - *Av* (MHR-Eda)^r]of *Anabaena variabilis* to colonize with the roots of rice and wheat plants. Colonization of strains was tested for their ability to adsorb to roots by chlorophyll *a* measurements and for N_2 -fixation by acetylene reduction. Structural investigations were made by light microscopy. All the strains were able to colonize the roots of rice and wheat. The chlorophyll *a* content and rates of N_2 fixation by associated cyanobacterial strains were higher compared with those in free-living condition. The potentiality of these mutationally improved biofertilizer strains to associate with the roots of rice and wheat plant keeps agricultural significance.

Introduction

The application of cyanobacteria (blue-green algae) as biofertilizer in rice cultivation has been reported to have beneficial effect on rice productivity and maintenance of soil fertility (Singh, 1961; Whitton, 2000). However, there are some drawbacks that limit the benefits of cyanobacteria biofertilizers. For example, sensitivity to herbicides and moreover they are unable to meet the total N requirements of modern, high-yielding varieties of rice. Chemical-N fertilizers are therefore used as a supplement, resulting in inhibition of any natural N_2-fixation. Furthermore, much of the fixed N is released from the cyanobacteria only after their death and decay, rather than during growth. Use of nitrogenase-derepressed, ammonia-excreting mutants would avoid inhibition of N fixation by chemical-N fertilizers, and would result in a greater release of fixed N from the cyanobacteria (Kamuru et al., 1997). However, whether such mutants can compete with natural populations in rice fields is still an open question.

To increase the benefits of cyanobacteria N_2 fixation for rice, the establishment of tighter N_2 fixing association between rice plant and cyanobacteria could potentially be an alternative to cyanobacteria biofertilizers. In such association, fixed N could be made more directly available to the rice plant, rather than only after death and decay of the cyanobacterial biomass. Under aquatic or in high-humidity habitats, N_2-fixing cyanobacteria have often been noted on rice plant surface (Toledo et al., 1995), including roots and submerged shoots (Whitton, 2000). Previous laboratory studies have shown that some and *Nostoc and Anabaena* strains are able to colonize roots of wheat, and to carry out associative N-fixation there (Gantar et al., 1991). Studies have been made that has tested the potential of rice plants to associate artificially with free-living cyanobacteria (Svircev et al., 1997), even though naturally occurring cyanobacteria in rice fields have been detected in loose association with rice root (Toledo et al., 1995; Freiberg, 1999). Investigation have also been undertaken to screen numerous *Nostoc* isolates for their ability to associate and colonize rice plant (Nilsson et al., 2002).

Keeping the above points in view earlier attempt was made to isolate a spontaneous mutant of a local rice isolate *Anabaena variabilis* exhibiting resistance to six commonly used rice field herbicides viz. Arozin, Alachlor, Butachlor, 2,4-D, Atrazine and DCMU (Singh et al., 2011; Singh et al., 2012). Further this multiple herbicide resistant strain was screened for the presence of spontaneous mutant resistant to growth inhibitory concentrations of EDA (ethylene diamine) for the isolation of ammonia excretory strain (Singh and Datta, 2013).
The goal of the present study was to explore the possibility of wild type and mutant strains of *Anabaena variabilis* for their ability to associate and colonize rice and wheat plant. Since the cyanobacterial cultures used in the present study are improved biofertilizer strains so their ability to fix nitrogen in such association is of great interest from agriculture view point.

Materials and Methods

Organisms and growth conditions

The axenic clonal culture of wild type N_2-fixing cyanobacterium *Anabaena variabilis* [*Av* (P)], a rice field isolate, its spontaneous mutant [*Av* $(MHR)^r$] exhibiting resistance to the lethal dosages of herbicides: Arozin, Alachlor, Butachlor, 2,4-D, Atrazine, DCMU (Singh et al., 2011; Singh et al., 2012) its ammonia excretory mutant [*Av* $(Eda)^r$] and its multiple herbicide ammonia excretory resistant mutant [*Av* $(MHR\text{-}Eda)^r$] (Singh and Datta, 2013) was cultivated in BG_{11} medium (Rippka et al., 1979), devoid of any combined nitrogen source (N_2-medium). Cultures were incubated in an air-conditioned culture room maintained at 25° ± 1°C fitted with cool day fluorescent light. Photon flux density of light on the surface of the vessel was 45 $\mu Em^{-2} s^{-1}$ for 18 h d^{-1}.

Variety of Seeds

For Rice (*oryza sativa)* IR-36 and for Wheat (*Triticum aestivum)* Lokman variety was used.

Co-cultivation of wild type and mutant of *Anabeana Variabilis* with rice and wheat plant

Rice and wheat seeds were surface-sterilized by washing with distilled water, then in 1% sodium hypochlorite solution. The seeds were thoroughly rinsed in distilled water and the seed germination was carried out in plastic containers. Seedlings of rice after 10 days were uprooted. The roots were washed with distilled water and suspended in 15ml capacity tubes containing 10ml of BG_{11}^- medium. Exponentially grown cyanobacteria strains were harvested by centrifugation. Cyanobacterial filament were washed by repeated centrifugation and resuspended in fresh BG_{11}^{-} medium. Cyanobacterial inocula were added to a final concentration of 2µg (chl *a*) ml^{-1}. Co-cultivation was carried out at $30^{0}C$ with the plant root either exposed to light or dark periods. After co-culture for 6 days, the rice and wheat seedlings were harvested and roots excised. The roots were washed to remove loosely associated cyanobacteria, and used for assessing colonization and associative N_2 fixation.

Nitrogenase activity

Nitrogenase activity was measured using the acetylene reduction technique (Stewart et al., 1967). After 6 d of co-culture with *Anabaena* strains, the roots were excised and washed to remove loosely associated cyanoabacterial cells. The roots were then incubated with acetylene under light. The nitrogenase activity was also measured in *Anabaena* strains remaining unassociated (free-living) in the co-culture medium.

Chlorophyll *a*

Chlorophyll *a* was extracted in methanol. The absorbance at 665nm was measured and the concentration calculated according to Mackinney (1941).

Light microscopy

The surfaces and freshly cut transverse section of rice and wheat roots were prepared and examined under Olympus BX 60 light microscope.

Results and Discussion

The colonization of rice and wheat roots by wild type and mutant strains of diazotrophic cyanobacteria *Anabaena variabilis* were screened (Table 1). Colonization was defined as blue green colonies on the rice and wheat roots, visible to the naked eyes that could with stand gentle washing in distilled water (Nilsson et al., 2002) .The appearance of cyanobacterial strains on the rice and wheat root is illustrated in figure 5 &6 and light microscopy of root samples colonized is shown in figure 7 & 8. It was observed that all the four strains tested were able to colonize the roots of seedling grown in liquid medium. The level of colonization was quantified by measuring the chlorophyll *a* content tightly bound to the roots after 6 day of co-culture. Associative competence was found in all the cyanobacterial strains. *Av* $(MHR\text{-}Eda)^{r}$ displayed tight association with highest chlorophyll *a* content (5.8µgm^{-1} in rice and 5.7µgml^{-1} in wheat) followed by *Av* $(Eda)^{r}$, *Av* $(MHR)^{r}$ and wild type strain (Fig 1 &2). All the *Anabaena* strains showed enhanced nitrogenase activity when associated with rice and wheat, compared with free-living (Fig. 3 & 4). In particular *Av* $(MHR\text{-}Eda)^{r}$ exhibited highest nitrogenase activity (6.52 nmol C_2H_2 μg^{-1} Chl *a* h^{-1} with rice and 6.22 nmol C_2H_2 μg^{-1} Chl *a* h^{-1} with wheat).

Several isolates of cyanobacteria, belonging to genera *Nostoc* and *Anabaena*, are capable of forming novel associations with rice and wheat roots grown in liquid culture are reported. In this paper we investigated the ability of some mutant strains of *Anabaena variabilis* to associate with rice and wheat roots. It is clear that, under the experimental condition all the strains tested colonized with roots of rice and wheat. The associated cyanobacterial strains exhibited 25%-40% increase in chlorophyll *a* content and fixed N_2 40%-50% more efficiently than when free living. Polysaccharides of plant and cyanobacterial origin may play an important role in the attachment of cyanobacteria to roots, as has been shown for the attachment of cyanobacteria to plant cells and inert surfaces (Robins et al., 1986).

Table.1 Visual screening of the associations of cyanobacterial strains to rice and wheat roots after co cultivation

Strains	Association with rice	Association with wheat
Av (P)	+	+
Av (MHR)[r]	+	+
Av (Eda)[r]	+	+
Av (MHR-Eda)[r]	+	+

Fig.1 Chlorophyll *a* content of cyanobacterial strains after co-culture with rice roots and when free living (bars indicating standard errors)

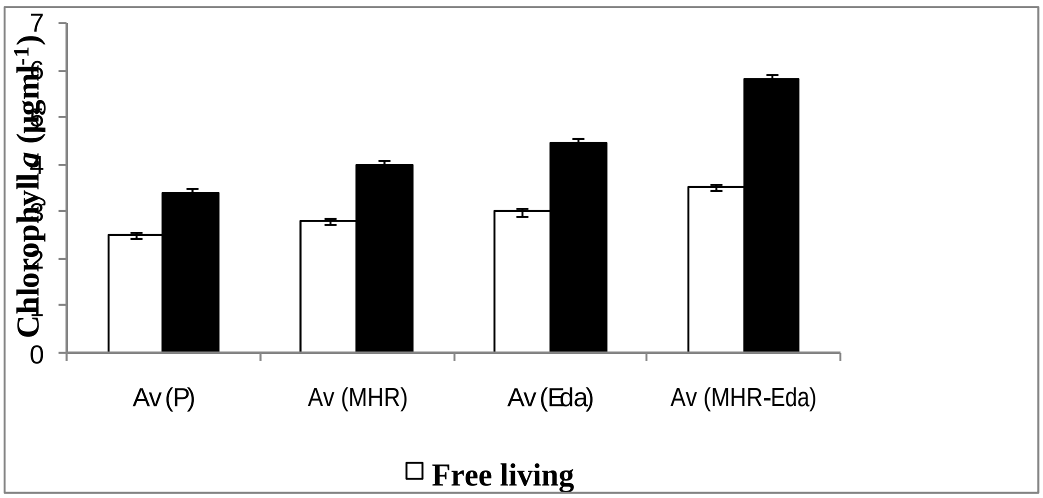


Fig.2 Chlorophyll *a* content of cyanobacterial strains after co-culture with wheat roots and when free living (bars indicating standard errors)

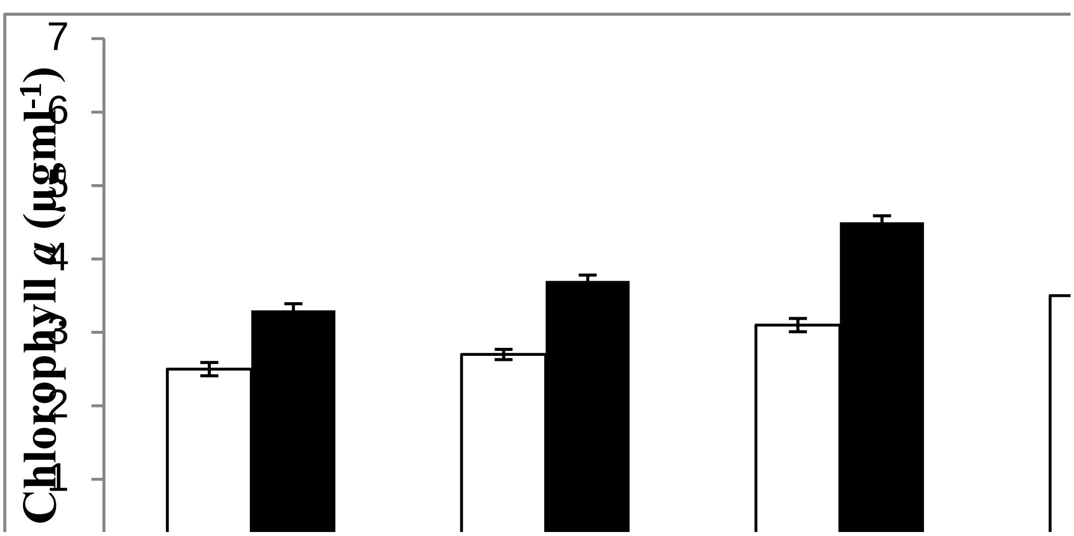


Fig.3 Nitrogenase activity in wild type and mutant strains of *Anabaena variabilis* after co-culture with rice roots and when free-living (bars indicating standard errors)

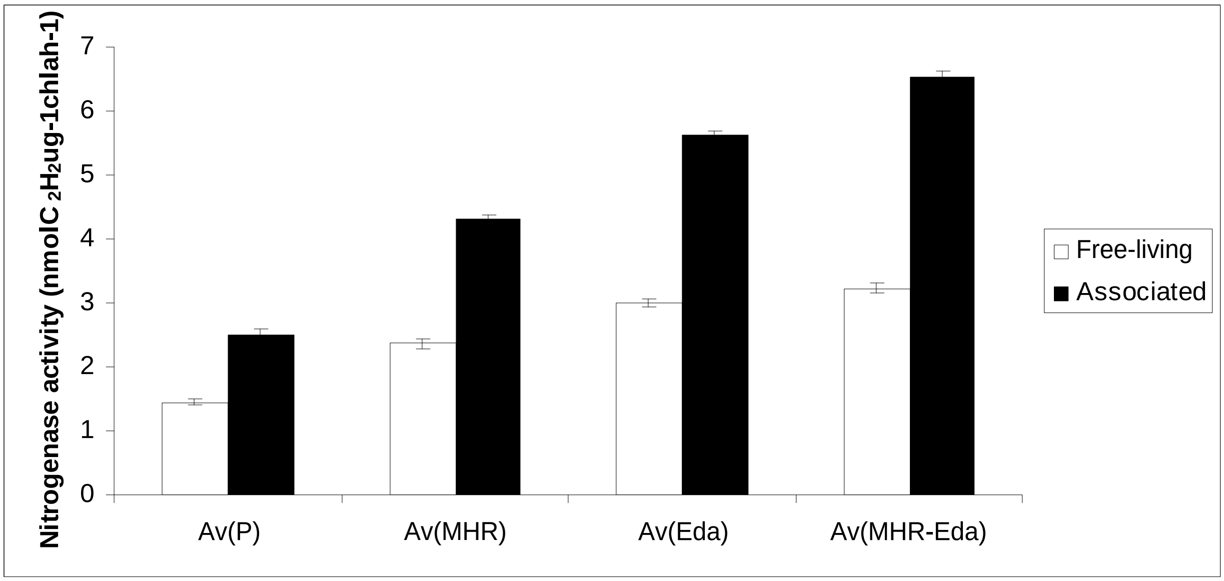


Fig.3 Nitrogenase activity in wild type and mutant strains of *Anabaena variabilis* after co-culture with rice roots and when free-living (bars indicating standard errors)

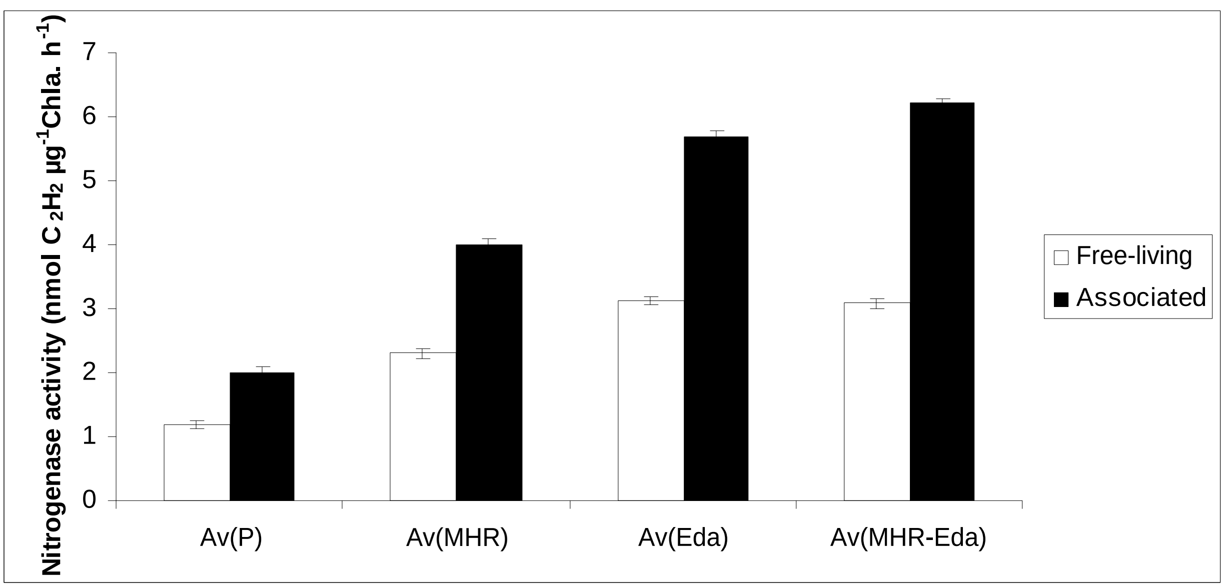
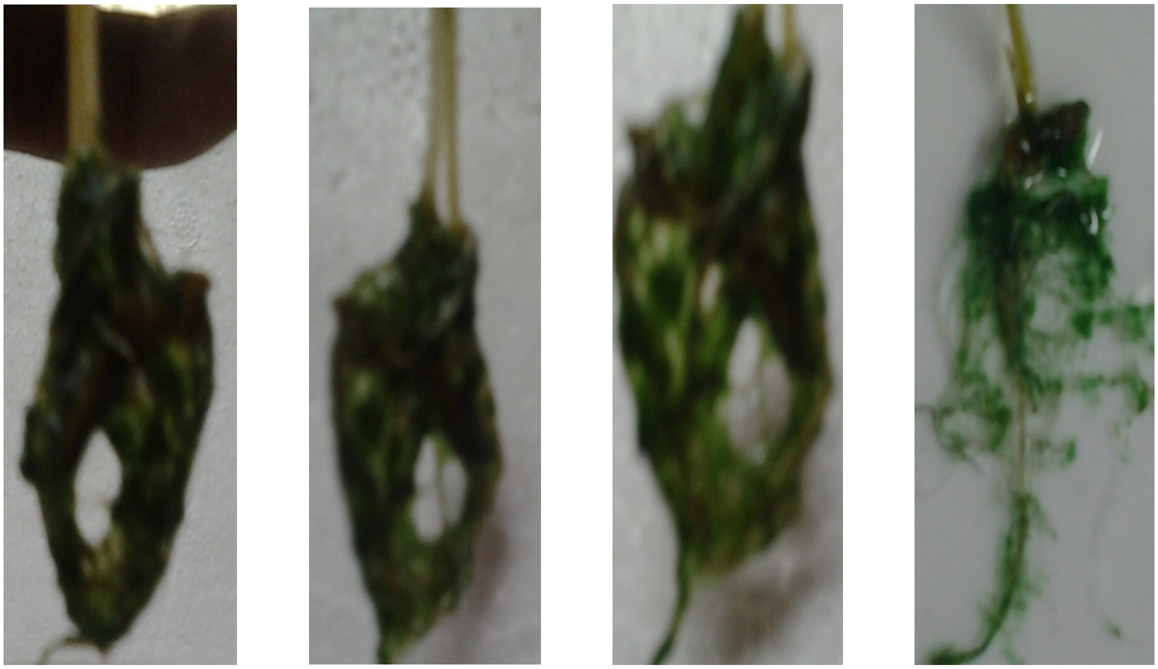
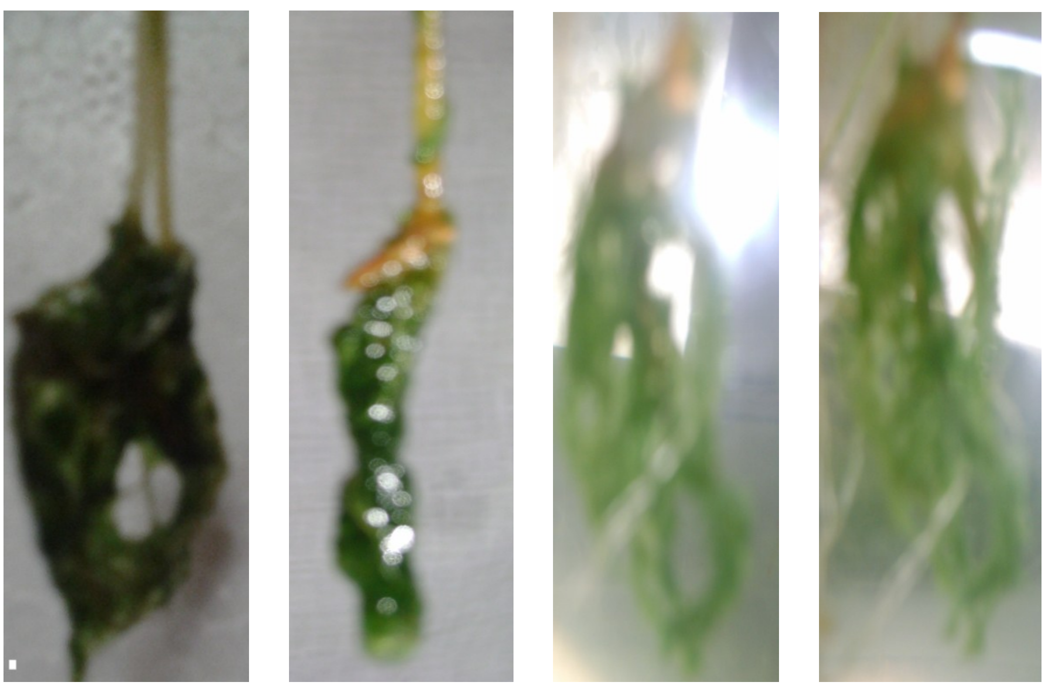


Figure.5 Association of wild type and mutant strains of *Anabaena variabilis* in rice plant



Root+ *Av* (P) Root+ *Av* (MHR)^r Root+ *Av* (Eda)^r Root + *Av* (MHR-Eda)^r

Figure.6 Association of wild type and mutant strains of *Anabaena variabilis* in wheat plant



Root + *Av* (P) Root + *Av* (MHR)^r Root + *Av* (Eda)^r Root +*Av* (MHR-Eda)^r

Fig.7 Light microscopy of rice root sample colonized by cyanobacterial strain *Av* (MHR-Eda)[r]

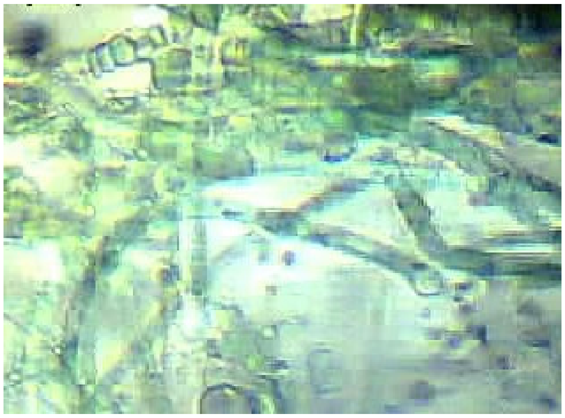
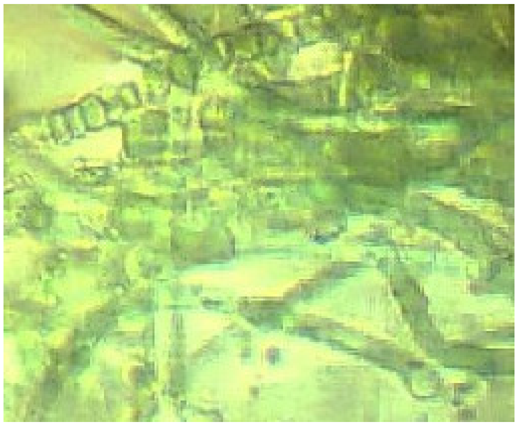


Fig.8 Light microscopy of wheat root sample colonized by cyanobacterial strain *Av* (MHR-Eda)[r]



Furthermore, plant roots secrete chemical signals such as flavonoids, that facilitate the nitrogenase activity (Theunis et al., 2004; Mathesius, 2008). These results indicate that the plant and/or products released positively influence the nitrogenase activity of associated cyanobacteria, as has previously been shown in cyanobacteria–wheat associations (Gantar et al., 1991a,b, 1995; Nilsson et al., 2002). Similarly colonization and nitrogen fixation of significant higher growth and filamentous cyanobacteria *Microcoleus* species has been observed in presence of roots of black mangrove (Toledo et al., 1995). The successful colonization of seedling following exposure to such improved cyanobacterial biofertilizer strains exhibiting multiple herbicide resistance and ammonia excretory properties is an important finding as it will simplify the inoculation procedure when used in field. In accordance with agricultural practice, the biofertilizer strains can be adsorbed to rice seedling in between uprooting of seedling and their transplantation into the rice fields. The potentiality of root colonization by such developed mutants of *Anabaena variabilis* proved them as ideal biofertilizer strains. Further work is in progress to assess the detail qualitative approach of the type of association exhibited by the mutant strains.

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