Colonization of roots of rice (*Oryza sativa*) by symbiotic *Nostoc* strains

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Summary

• The lack of nitrogen in agriculture, and negative environmental effects of fertilizers, have stimulated interest in creating artificial associations between N_2-fixing cyanobacteria and rice (*Oryza sativa*).

• For the first time, numerous (57) *Nostoc* isolates from natural symbioses were screened for their ability to associate with rice. Successful colonizers were tested for N_2-fixation by acetylene reduction, and for their ability to adsorb to roots by chlorophyll *a* measurements. Paranodules were induced by 2,4-dichlorophenoxyacetic acid. And genetic fingerprints of the cyanobacteria were obtained for identification. Ultrastructural investigations were made by light and scanning electron microscopy.

• Twenty-one symbiotic *Nostoc* isolates associated with rice roots, colonizing surfaces and intercellular spaces. Adsorption was high and appeared biphasic. The rates of N_2 fixation by associated cyanobacteria were higher compared with those in free-living cyanobacteria. Paranodules were formed and colonized, but root growth was adversely affected.

• Under laboratory conditions, artificial associations were created between one-third of the screened symbiotic cyanobacteria and rice. The agricultural potential for the association appears high since the cyanobacteria adsorb tightly and fix more N_2 than when free-living.

Key words: artificial association, associative N_2-fixation, biofertilizer, cyanobacteria, *Nostoc*, rice (*Oryza sativa*).

Introduction

To support the demand for nitrogen in rice cultivation, input of chemical N fertilizers is a prerequisite. Wetland rice fields supply 86% of the global requirement for rice, and N_2-fixing cyanobacteria are common in such, often waterlogged, rice fields (Whitton, 2000). The importance of these naturally occurring prokaryotes in the nitrogen economy of rice cultivation has long been known (De, 1939; Watanabe *et al.*, 1951; Singh, 1961) and, in recent years, the use of N_2-fixing cyanobacteria as biofertilizers in rice cultivation has been popularised. Such inoculation with free-living cyanobacteria (algalization) has been shown to increase growth and crop yield of rice. It is estimated that cyanobacteria contribute 20–80 kg N ha^{-1} rice per crop on turnover of their biomass in the rice fields (Venkatraman, 1981; Albrecht *et al.*, 1991; Roger & Ladha, 1992; Ghosh & Saha, 1993, 1997; Whitton, 2000). However, there are some drawbacks that limit the benefits of cyanobacterial biofertilizers. For example, 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_2 fixation by chemical-N fertilizers, and would result in a greater release of fixed N from the cyanobacteria (Kamuru *et al.*, 1997, 1998). However, whether such mutants can compete with natural populations in rice fields is still an open question, though

immobilization of cyanobacterial inocula on substrata such as bagasse (sugarcane waste) could help by providing an unpopulated habitat (Kannaiyan *et al.*, 1997). An additional problem is that the fixed N is released in the soil where it is not only available to rice, but also to other soil organisms.

To increase the benefits of cyanobacterial N_2 fixation for rice, the establishment of tighter N_2-fixing associations between rice plants and cyanobacteria could potentially be an alternative to cyanobacterial biofertilizers, or to the transfer, by genetic engineering, of *nif* genes into rice plants. In such associations, the 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 surfaces (Toledo *et al.*, 1995; Freiberg, 1998, 1999), including roots and submerged shoots (Whitton, 2000). Previous laboratory studies have shown that some *Nostoc* and *Anabaena* strains are able to colonize roots of wheat, and to carry out associative N_2-fixation there (Gantar *et al.*, 1991a,b, 1995; Spiller *et al.*, 1993; Gantar & Elhai, 1999; Gantar, 2000). To our knowledge, only one study 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 roots (Toledo *et al.*, 1995; Freiberg, 1998, 1999; Whitton, 2000). In the former study, only three different cyanobacterial isolates were used, all lacking symbiotic competence (Svircev *et al.*, 1997).

Nostoc is one of the most versatile terrestrial N_2-fixing cyanobacteria. This genus occurs as free-living forms and in symbioses, covering a wide range of habitats, and is known to withstand a variety of modes of C and N nutrition (Potts, 2000; Rai *et al.*, 2000). In all symbioses with fungi and plants, ranging from bryophytes to angiosperms, there is biotrophic transfer of fixed N from the *Nostoc* cyanobionts to the hosts (Rai *et al.*, 2000). Furthermore, since *Nostoc* is the most common cyanobacterial genera in natural symbiotic associations (Rai *et al.*, 2000), it is likely that symbiotically competent *Nostoc* strains would be more prone than other nonsymbiotic cyanobacteria to form associations with a 'new' plant, such as rice. Until now, symbiotic cyanobacteria have not been used in the creation of artificial symbioses. The present investigation was undertaken to screen, for the first time, a large collection of symbiotic *Nostoc* strains for their ability to associate and colonize rice plants. The cyanobacteria used originated from natural symbioses with a range of host plants representing various parts of the plant kingdom. As their ability to fix N_2 in such associations is of great interest from an agricultural viewpoint, this was also investigated. Differences in colonizing efficiency between the otherwise equally symbiotically competent cyanobacterial strains will be discussed.

Materials and Methods

Organisms and growth conditions

Three rice (*Oryza sativa* L.) varieties, IET 13783, IET 13459 and IPCL I-24, were obtained from India (ICAR Complex, Barapani, Shillong) and one (G-669) from China (Prof. W. W. Zheng, Fujian Academy of Agricultural Sciences, Fuzhou). The *Nostoc* strains used are presented in Table 1. All *Nostoc* strains, originating from *Gunnera* were collected by Dr E. Söderbäck (Stockholm University, Sweden), and strains from cycads were kindly donated by Dr M. Grilli Caiola (University of Rome, Italy). Cyanobacterial cultures were maintained in $BG11_0$ medium (Rippka *et al.*, 1979) at 25°C and a photon fluency rate of 50 μmol m^{-2} s^{-1}. Rice seeds were surface-sterilized by washing with distilled water, then in 1% (v : v) sodium hypochlorite solution for 5 min. The seeds were thoroughly rinsed in distilled water and the seed germination carried out on autoclaved Perlite in plastic containers. The Perlite was irrigated with a 10-fold dilution of autoclaved BG11 medium. The experiments were carried out in a growth cabinet at 30°C, at saturating relative humidity, and a 12 h light–dark cycle at a light intensity of 50 μmol m^{-2} s^{-1}.

Co-cultivation of *Nostoc* and rice plants

Seedlings of rice grown for 10 d were uprooted from the Perlite. The roots were washed with distilled water, and suspended in 15 ml capacity tubes containing 10 ml of 10-fold diluted BG11 (+N) or $BG11_0$ (–N) medium. The cyanobacteria used for inoculation to the media were grown for 4 d in batch cultures, and harvested by centrifugation. Cyanobacterial filaments were washed by repeated centrifugation and resuspending in fresh $BG11_0$ medium. *Nostoc* inocula were added to a final concentration of 2 μg chlorophyll *a* (chl *a*) ml^{-1}. Co-cultivation was carried out at 30°C with the plant roots either exposed to light (50 μmol m^{-2} s^{-1}) or in darkness, which was achieved by wrapping aluminium foil around the culture tube. The four rice varieties described previously were screened with one strain, *Nostoc* Anth, to determine which would be most suitable for co-cultivation. The variety that resulted in the highest colonization was used for the remaining experiments.

After co-culture for 4 d, the rice seedlings were harvested and the roots excised. The roots were washed to remove loosely associated cyanobacteria, and used for assessing colonization (μg chl *a* g^{-1} root dry wt) and associative N_2 fixation (nmol C_2H_2 reduced μg^{-1} chl *a* h^{-1}). Short-term experiments (30 min to 6 h) were carried out in a similar fashion in order to assess adsorption of one of the *Nostoc* strains (*Nostoc* Anth) to rice roots. Tests for root colonization were also carried out in Perlite. For this, cyanobacterial suspension was added to the Perlite in which seedlings were growing. Alternatively, seedlings of the same rice variety were uprooted, dipped in a

Table 1 Cyanobacterial strains of the genus *Nostoc*, the host and the country in which they were collected. Visual screening of the association of cyanobacteria to rice roots after co-cultivation in light and dark is presented

			Association with rice	
Strain	Host	Origin	Light	Dark
Nostoc can	*Peltigera canina*	Sweden	–	–
Nostoc Anth 1	*Anthoceros*	Shillong, India	+	+
Nostoc Anth 2	*Anthoceros*	Shillong, India	+	+
VRUC 103	*Macrozamia communis*	Italy	+	+
VRUC 107	*Encephalartos lehmanii*	Italy	–	–
VRUC 108	*Cycas revoluta*	Italy	–	–
VRUC 110	*Cycas circinalis*	Italy	–	–
VRUC 112	*Cycas revoluta*	Italy	–	–
VRUC 113	*Encephalartos longifolius*	Italy	+	–
VRUC 117	*Cycas revoluta*	Italy	+	+
8901:1	*Gunnera macrophylla*	New Zealand	+	–
8904	*Gunnera macrophylla*	New Zealand	–	–
8915	*Gunnera monoika*	New Zealand	–	–
8916	*Gunnera monoika*	New Zealand	+	+
8917:1	*Gunnera monoika*	New Zealand	–	–
8917:3	*Gunnera monoika*	New Zealand	+	–
8923	*Gunnera hamiltonii*	New Zealand	–	–
8924	*Gunnera hamiltonii*	New Zealand	–	+
8926	*Gunnera hamiltonii*	New Zealand	–	–
8928:1	*Gunnera hamiltonii*	New Zealand	–	–
8930	*Gunnera cordifolia*	New Zealand	–	–
8937	*Gunnera dentata*	New Zealand	–	–
8939:1	*Gunnera dentata*	New Zealand	+	–
8939:2	*Gunnera dentata*	New Zealand	–	–
8939:3	*Gunnera dentata*	New Zealand	–	–
8940	*Gunnera dentata*	New Zealand	–	–
8940:2	*Gunnera dentata*	New Zealand	–	–
8940:4	*Gunnera dentata*	New Zealand	–	–
8941:3	*Gunnera dentata*	New Zealand	+	–
8945	*Gunnera dentata*	New Zealand	–	–
8945:2	*Gunnera dentata*	New Zealand	+	+
8947	*Gunnera dentata*	New Zealand	–	–
8950:1	*Gunnera monoika*	New Zealand	+	+
8950:3	*Gunnera monoika*	New Zealand	+	+
8952	*Gunnera monoika*	New Zealand	–	–
8954:3	*Gunnera monoika*	New Zealand	–	–
8956	*Gunnera prorepens*	New Zealand	–	–
8960:3	*Gunnera prorepens*	New Zealand	–	–
8964:3	*Gunnera prorepens*	New Zealand	+	+
8978	*Gunnera* sp.	Sweden	–	–
8979	*Gunnera manicata*	Sweden	–	–
8981	*Gunnera manicata*	Sweden	+	+
8982	*Gunnera manicata*	Sweden	–	–
8983	*Gunnera tinctoria*	Sweden	–	–
8996	*Gunnera kauaiensis*	Hawaii, USA	–	–
8998:1	*Gunnera magellanica*	New Zealand	–	–
9101:1	*Gunnera magellanica*	Chile	–	–
9101:2	*Gunnera magellanica*	Chile	+	+
9102:1	*Gunnera magellanica*	Chile	+	+
9102:2	*Gunnera magellanica*	Chile	–	–
9103	*Gunnera tinctoria*	Chile	+	–
9104	*Gunnera tinctoria*	Chile	+	+
9105:2	*Gunnera magellanica*	Chile	+	–
9105:2A	*Gunnera magellanica*	Chile	–	–
9105:3A	*Gunnera magellanica*	Chile	–	–
9106:1	*Gunnera magellanica*	Chile	–	–
PCC 9229	*Gunnera monoika*	New Zealand	–	–
Nostoc sp.	Soil	Shillong, India	+	+
Nostoc sp$^{(PR)}$	Soil	Shillong, India	+	+

+, Association with rice roots; –, no association with rice roots; $^{(PR)}$, paraquat resistance mutant.

suspension of *Nostoc* Anth for 30 min and then transplanted into Perlite. During co-cultivation, the Perlite was irrigated with a 10-fold dilution of $BG11_0$ medium. The experiment was repeated three times.

Synthetic auxin treatment

In another set of experiments, synthetic auxin, 2,4-dichlorophenoxy acetic acid (2,4-D), was added into the co-culture medium at the same time as rice seedlings and *Nostoc* were combined for incubation. The experiment was performed with the same rice variety as in the screening and the cyanobacterial strain *Nostoc* Anth (used as an example of a strain that had shown association in the screening). The hormone was added to a final concentration of 1 mg l^{-1} in order to induce paranodules, after which any increase in colonization by *Nostoc* was assessed. The experiment was repeated three times.

Nitrogenase activity

Nitrogenase activity was measured using the acetylene reduction technique (Steward *et al.*, 1967). After 4 d of co-culture with *Nostoc*, rice roots were excised and washed for 1 min in an ultrasonic bath to remove loosely associated cyanobacterial cells. The roots were then incubated with acetylene under light or darkness, as appropriate. Roots from three replicates were measured together and a mean value was obtained. After estimation of nitrogenase activity, cyanobacterial chlorophyll was determined as described below. For comparative purpose, nitrogenase activity was also measured in *Nostoc* cells remaining unassociated (free-living) in the co-culture medium. Each experiment was repeated at least three times.

Chlorophyll *a* determinations and measurements of root dry weight

Chlorophyll *a* was extracted in methanol, in darkness at 4°C. The absorbance at 663 nm was measured and the concentration calculated according to McKinney, 1941). Roots from which the chl *a* of associated cyanobacteria had been extracted, were dried at 80°C for 72 h and their dry weight determined. The roots from three experimental replicates were measured together and a mean value was obtained.

Light and scanning electron microscopy

The surfaces and freshly cut transverse sections of rice roots were prepared by fixation in 2% (w : v) paraformaldehyde and 2.5% (v : v) glutaraldehyde in 0.05 M phosphate buffer, pH 7.2–7.4. The material was dehydrated in ethanol and embedded in Epon. Sections, 2-μm thick, were examined with an Olympus BX 60 light microscope. Cross-sections of roots colonized by *Nostoc* strains were also prepared for scanning electron microscopy (SEM) by sectioning with a razor blade. The sections were fixed in 2.5% (v : v) glutaraldehyde in phosphate buffer (0.05 M). The root pieces were washed in phosphate buffer and air-dried, mounted on stubs. After drying, the pieces were sputter-coated with gold and examined with a Cambridge Stereoscan 260 SEM (Cambridge Instruments Ltd; Cambridge, UK) at 10 kV.

DNA fingerprints of *Nostoc* strains

Polymerase chain reaction (PCR)-based DNA fingerprints of *Nostoc* strains were obtained using short tandemly repeated repetitive sequences (STRR-1A) as primers (Rasmussen & Svenning, 1998), and whole filaments of *Nostoc* as templates. The method used was as described by Nilsson *et al.* (2000). The experiments were repeated at least three times.

Results

Colonization of rice roots

The colonization of rice roots by 57 symbiotically competent cyanobacteria originating from a variety of natural host symbioses and two free-living cyanobacteria isolated from rice field soil (wild type and corresponding paraquat mutant) were screened (Table 1). Forty-seven of the isolated cyanobacterial strains originated from 10 species of the angiosperm family *Gunnera*, two from the liverwort *Anthoceros*, seven strains from three genera within the cycads (gymnosperm) and one strain from the lichen *Peltigera canina*. The experiments were performed on rice variety IET 13783, which proved to yield the highest root association of cyanobacteria in a screening of the four rice varieties described previously (data not shown). The appearance of cyanobacteria on the rice roots is illustrated in Fig. 1. Colonization was defined as blue-green colonies on the rice roots, visible to the naked eye, that could withstand gentle washing in distilled water. The level of colonization was quantified by measuring the amount of *Nostoc* tightly bound to the roots after 4 d of co-culture. Twenty-three (*c.* 39%) of the total 59 *Nostoc* strains tested were able to colonize the roots of seedlings grown in liquid medium (Tables 1 and 2). Associative competence was found among cyanobacterial isolates originating from all host divisions, except for the only lichen isolate tested. Twenty-one of the symbiotic cyanobacterial strains displayed a tight association with the roots, ranging from 100 to 1100 μg chl *a* g^{-1} root dry weight, while two of the strains, *Nostoc* 8924 and *Nostoc* VRUC 103, showed a lower binding capacity. The cyanobacterial biomass represented 0.6–6% of the root dry weight. The colonization of rice roots by the 21 successful, tightly binding *Nostoc* strains occurred in light as well as in darkness, and in N-free as well as in nitrate-containing media

Fig. 1 (a) The blue-green appearance of cyanobacteria on rice roots. The rice root is photographed in the tube in which the co-culturing took place. (b) Rice roots after paranodule induction by 2,4-dichlorophenoxy acetic acid (2,4-D). Cyanobacteria are seen as green spots or streaks on the root, oriented in parallel to the longitudinal axis of the root. Bar, 1 mm.

Table 2 Cyanobacterial colonization in the presence (+N) and absence (–N) of combined nitrogen, measured as chlorophyll *a* in root dry weight

	Chlorophyll *a* (μg g^{-1} root dry wt)			
	Light		Dark	
Strain	+N	–N	+N	–N
Nostoc Anth1	425	494	309	550
Nostoc Anth2	520	391	495	301
VRUC 103	73	28	32	68
VRUC 113	1093	750	270	923
VRUC 117	963	473	557	1163
8901:1	408	337	413	457
8916	270	149	159	134
8917:3	274	75	109	112
8924	12	11	–	45
8939:1	942	827	652	1138
8941:3	136	175	84	79
8945:2	141	101	73	121
8950:1	107	103	190	119
8950:3	127	182	75	54
8964:3	747	163	742	278
8981	339	361	379	147
9101:2	167	240	170	278
9102:1	347	187	368	193
9103	1136	266	353	386
9104	686	199	159	146
9105:2	600	402	372	385
Nostoc sp.	682	528	630	465
Nostoc sp$^{(PR)}$	750	571	640	642

Values are the mean of three replicates measured together; –, no colonization; $^{(PR)}$, paraquat resistant mutant.

(Tables 1 and 2). In general, colonization was higher in light and in nitrate-containing media. However, many strains showed consistently high levels of root colonization under all conditions. These included: *Nostoc* sp., *Nostoc* Anth and *Nostoc* strains 8901:1, 8939:1, 8964:3, 8981, 9103, 9105:2, VRUC 113 and VRUC 117. Highly associative strains were found originating from all the host plant types (liverworts, cycads, and *Gunnera*), but the free-living *Nostoc* strain (wild-type) isolated from rice field also displayed high colonization. Colonization of rice roots in Perlite was investigated using one cyanobacterial strain, *Nostoc* Anth (chosen as an example of a cyanobacterial strain that had formed an association in the liquid cultures). However, the rate of colonization was slower and the extent of binding lower than that in liquid co-cultures (data not shown). In an attempt to enhance the creation of a more durable colonization, 2,4-D was added in the culture media to induce paranodule formation on the rice roots. After co-culture, paranodules appeared over the entire root surface (Fig. 1). However, because this did not lead to enhanced colonization and the growth of roots was decreased, this experimental approach was abandoned. Examination of the colonized root surfaces and transverse sections of the roots after 4 d of co-culture, using light and scanning microscopy (Fig. 2), showed that *Nostoc* filaments were intimately associated with the root epidermis. The *Nostoc* filaments occurred in streaks or patches that followed the contours of the outer surface layer of the root epidermis. Association with root hairs was also observed. Occasionally, the *Nostoc* cells occurred intercellularly in the epidermal layer of the root. These observations could represent early events in the process of colonization and, with time, the association may proceed further. During the initial stages (1–3 d) of co-culture, mobile *Nostoc* hormogonia were common in the growth medium. As in other symbiotic interactions (Rai *et al.*, 2000), these are probably important for reaching the site of colonization on the rice roots. After association with the rice roots, the hormogonial stage was followed by re-differentiation into mature vegetative filaments with heterocysts.

The process of adsorption of cyanobacteria to the rice roots was studied in short-term experiments using *Nostoc* Anth (Fig. 3) and the same rice variety as was used for the screening. The result indicated a biphasic pattern of adherence of *Nostoc* to the rice roots. A rapid first phase lasted less than 1 h. This was followed by a second phase after a lag period. Based on these experiments, rice seedlings were dipped into cyanobacterial suspensions (*Nostoc* Anth) for 30 min and thereafter planted in Perlite to examine whether this method of cyanobacterial inoculation would lead to successful colonization.

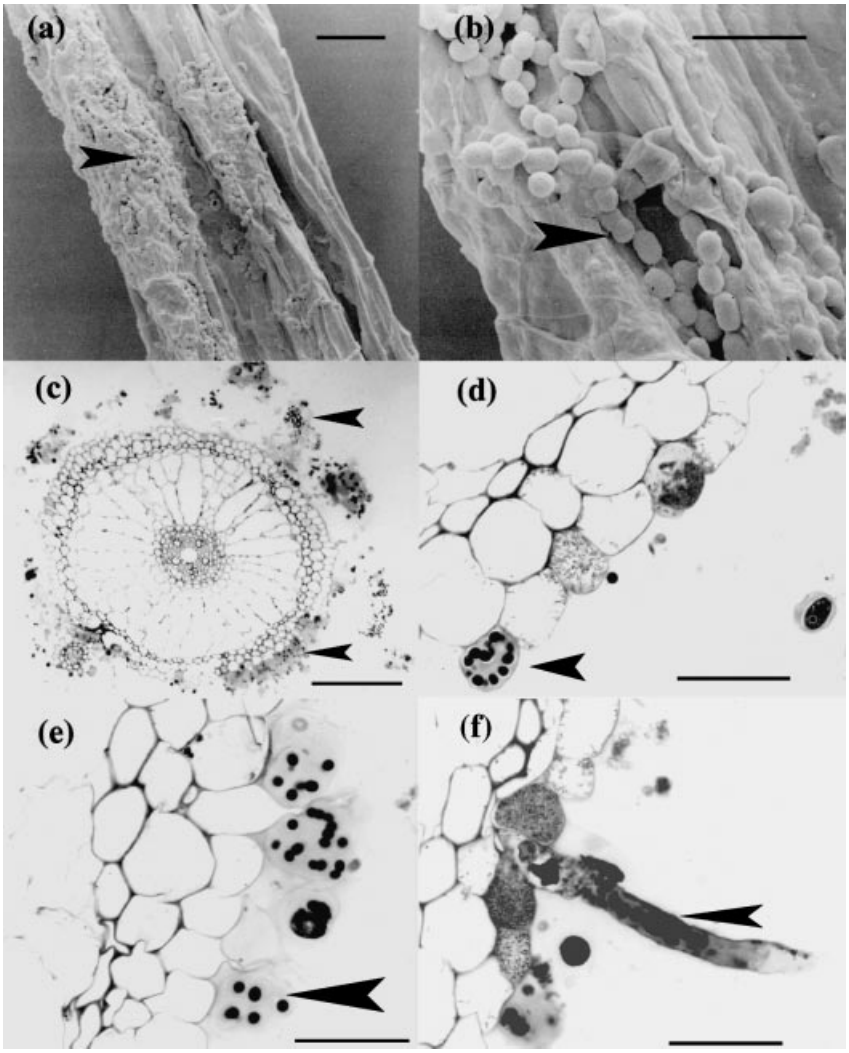

Fig. 2 (a) Scanning electron micrograph of a rice root infected with cyanobacteria. Cyanobacteria (arrow) are visible as a sheath of filaments covering the root surface. Bar, 50 μm. (b) Enlargement of the rice root with cyanobacteria (arrow) primarily in cracks in the root surface. Bar, 20 μm. (c) A transverse section of the infected rice root visualized through light microscopy. Cyanobacteria are seen as dark, mucilage-embedded packages (arrows) surrounding the epidermal layer. Bar, 0.5 mm. (d) An enlargement of the micrograph in (c) displaying a cyanobacterial package (arrow) found intercellularly in the epidermal layer of the root. Bar, 0.02 mm. (e) Mucilaginous cyanobacteria present in close proximity to the epidermal layer of the root. Bar, 0.02 mm (f) A root hair possibly containing cyanobacteria (arrow) inside. The darker cells at the base of the root hair possibly contain bacteria. Bar, 0.02 mm.

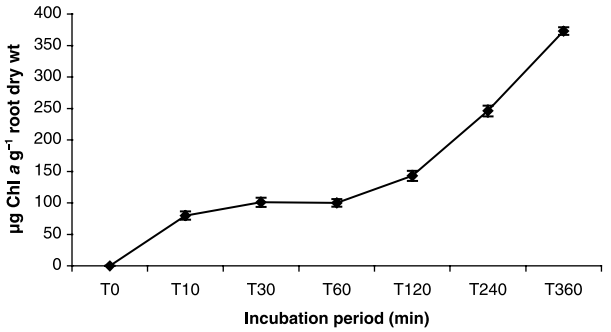

Fig. 3 Cyanobacterial adsorption to rice root, visualized as μg chlorophyll *a* (chl *a*) g^{-1} root dry weight during an incubation period of 360 min. The values are the means ± SE of three experiments.

After 1 wk of growth on Perlite, successful association was confirmed. Thus, when rice seedlings are uprooted for transplantation into rice paddies, cyanobacterial inocula could be administrated to the roots simply by keeping rice seedlings in a pool of cyanobacterial suspension for a few hours.

Associative N_2-fixation

Most *Nostoc* strains showed enhanced nitrogenase activity when associated with rice roots under light and nitrogen-free conditions, compared with when free-living (Table 3). In particular, strains 8945:2, 8950:3, 9101:2 and 9104 exhibited very high activities in association. However, there was no correlation between the tightness of root colonization by the cyanobacteria and their nitrogenase activity (Table 3). Nitrate had a more pronounced negative effect on nitrogenase activity in unassociated cyanobacteria compared to those attached to the rice roots. As expected, the presence of nitrate was inhibitory, but significant nitrogenase activity still occurred in strains 8916, 8941:3 and 8964:3. In darkness, nitrogenase activity was lower, both in the presence and absence of nitrate, compared with that in the light. However, substantial nitrogenase activities were detected in the dark under nitrogen-free conditions in strains VRUC 103 (153%), 8901:1 (20%), 8941:3 (44%), 8945:2 (15%) and 8964:3

Table 3 Acetylene reduction in the 23 successfully colonizing cyanobacterial strains after co-culture with rice roots and when free-living. The rates were obtained in light and dark and in the presence (+N) or absence (–N) of nitrogen

	Acetylene reduction (nmol C_2H_2 reduced h^{-1} μg^{-1} chlorophyll *a*)							
	Associated				Free-living			
	Light		Dark		Light		Dark	
Strain	+N	–N	+N	–N	+N	–N	+N	–N
VRUC 103	–	1.58	–	2.42	–	–	–	6.41
VRUC 113	–	1.53	–	–	–	0.05	0.93	–
VRUC 117	–	0.45	–	0.32	–	0.72	–	–
8901:1	0.79	7.31	0.18	1.43	0.33	2.71	0.08	0.09
8916	1.25	4.52	–	0.84	0.42	2.76	–	0.62
8917:3	0.31	2.57	0.83	0.59	0.41	0.19	0.14	0.12
8924	–	–	–	–	–	2.17	–	–
8939:1	–	1.15	–	0.07	–	1.35	0.03	–
8941:3	2.28	4.12	2.03	1.82	0.28	0.88	0.05	1.65
8945:2	0.10	31.35	0.86	4.85	0.09	0.73	0.12	0.16
8950:1	0.25	9.71	–	0.49	0.05	3.23	0.06	0.06
8950:3	1.75	49.43	0.89	0.74	0.87	2.86	0.17	0.68
8964:3	3.13	3.89	1.46	1.71	2.01	1.64	0.12	0.55
8981	0.59	4.72	0.18	0.43	0.08	2.78	0.05	0.14
9101:2	0.91	13.53	0.43	1.14	0.24	3.41	0.08	0.14
9102:1	0.55	2.63	0.24	0.40	0.08	2.74	0.06	0.10
9103	0.06	11.44	0.21	0.03	0.09	11.54	0.06	0.21
9104	0.88	15.32	0.30	0.66	0.28	3.49	0.08	0.52
9105:2	0.85	9.03	0.16	0.26	0.87	1.34	0.14	0.18
Nostoc Anth 1	1.86	8.50	1.41	2.45	0.15	3.38	0.20	0.25
Nostoc Anth 2	1.89	6.91	0.80	1.65	0.77	4.85	0.18	1.13
Nostoc sp.	1.46	9.95	1.02	1.47	0.16	4.85	0.20	0.40
Nostoc sp.$^{(PR)}$	1.60	10.10	1.27	1.50	0.15	4.80	0.18	0.45

The values are the mean of three experimental replicates measured together; –, no acetylene reduction; $^{(PR)}$, paraquat resistance mutant.

(44%). As in the light, nitrate inhibited nitrogenase activity in the dark, except in strains 8941:3 and 8964:3, where nitrate had little or no effect. The nitrogenase activities of unassociated (free-living) *Nostoc* appeared very low compared with those reported for laboratory cultures of heterocystous cyanobacteria. However, it should be noted that our assays were performed on young cyanobacterial inocula (4 d old) in co-cultures and the nutrient medium used ($BG11_0$) was 10-fold diluted. Furthermore, the temperature during co-cultivation was optimized for rice growth and was therefore higher than that normally used for cyanobacterial cultivation.

PCR fingerprints of *Nostoc* strains

To facilitate identification, STRR-PCR fingerprints were obtained from all cyanobacterial strains tested. Most strains have previously been identified using the same method (Rasmussen & Svenning, 1998; Nilsson *et al.*, 2000), while PCR fingerprints of the remaining strains are presented in Fig. 4. All isolates in the present study displayed individual fingerprints, with the exception of the two strains collected from symbiosis with *Anthoceros*, which had identical fingerprints. These strains may therefore be closely related, or even the same strain, while the remaining cyanobacterial strains were not related. An interesting observation is that the wild-type *Nostoc* sp. showed a different fingerprint from its two mutants, *Nostoc* sp. (PR) and *Nostoc* sp. (AR; this strain was not used in the screening but included in the PCR identification assay to evaluate the differences in fingerprints seen in mutants from the same strain). This indicates that the mutation causing herbicide resistance also caused rearrangements of the repetitive sequences, STRR. *Nostoc* PCC 9229 was included as a positive control. The fingerprints obtained may be used for identification of successful colonizers and tracking of individual strains in the field. Combined with denaturing gradient gel electrophoresis (DGGE) patterns of the cyanobacterial strains, individual cyanobacterial strains that associate with rice roots in a mixture of strains may be revealed.

Discussion

In this paper, we present data from the first screening of a large collection of naturally symbiotic cyanobacterial strains for

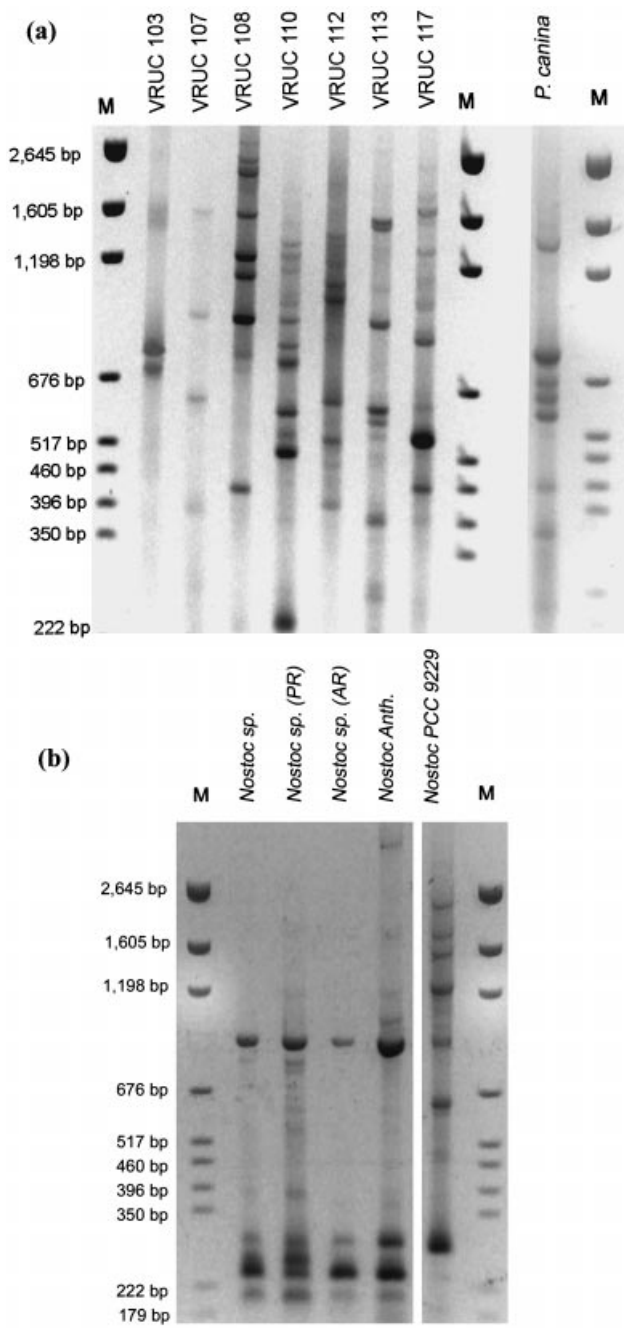

Fig. 4 (a) Polymerase chain reaction (PCR) fingerprints using short tandemly repeated repetitive (STRR) sequences as primers, obtained from cyanobacterial isolates originating from symbioses with cycads (VRUC) and from the lichen *Peltigera canina*. M, molecular markers in base pairs. (b) The STRR-PCR fingerprints of cyanobacterial isolates originating from soil (*Nostoc* sp., *Nostoc* sp. (PR), and *Nostoc* sp. (AR)) and from the symbiotic host *Anthoceros* sp. (*Nostoc* Anth). The strain *Nostoc* PCC 9229 was included as a control. M, molecular markers in base pairs.

their ability to associate with rice roots. It is clear that, under the experimental conditions used, a large number (21 out of 57 symbiotic strains) of the strains tested associated tightly with roots of rice. There was, however, no correlation between the country of origin of the cyanobacterial strains tested and their associative success on rice roots. Division, genus or species of the host from which the cyanobacteria were collected did not have any clear effect on the outcome of the association. Cyanobacteria isolated from the host *Gunnera* were over-represented in our experiments, since *Gunnera*, like rice, is an angiosperm (in contrast to the other symbiotic hosts, Table 1), making representatives from this natural association potentially more effective in any association with rice. The associated cyanobacteria fixed N_2 up to two times more efficiently than when free-living. 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) Several of the strains that colonized the rice root and fixed N_2 were also capable of N_2 fixation in the presence of nitrate and in the dark. These attributes are of importance when such strains are to be used in rice fields.

Ultrastructural investigations revealed a tight and sometimes intercellular association between cyanobacteria and the root epidermis. However, cyanobacteria were never found inside tissues or cells of rice. These data may suggest that cyanobacteria associated with rice do not form intracellular association of the type previously reported for wheat (Gantar *et al.*, 1991a,b, 1995; Spiller *et al.*, 1993; Gantar & Elhai, 1999; Gantar, 2000).

The capacity for N_2 fixation and transfer of N to the host is crucial in natural symbioses and may also be so in artificial association with rice. However, since most of the rice-associated cyanobacteria were able to fix N_2, and since there was no correlation between the capacity for colonization and N_2 fixation activity, this competence does not seem to determine success by a particular cyanobacterium. Furthermore, since not all otherwise symbiotically competent strains colonized rice roots and since there exists a large variation in the degree of colonization among the strains that did associate, we suggest that some specificity mechanism(s) may be operating, even if the new host is not involved in natural symbioses. Signals may be released by the roots and perceived by the cyanobacteria, and vice versa, and these signals now need to be identified.

The successful colonization of seedlings following exposure for 30 min to cyanobacteria and transplantation in Perlite, is an important finding as it will simplify the inoculation procedure when used in field. It shows that, in accordance with agricultural practice, cyanobacterial strains can be adsorbed to rice seedlings in-between uprooting of seedlings and their transplantation into the rice fields. Further work is in progress to assess the transfer of N from associated cyanobacteria to the rice plant, and to create herbicide-resistant mutants of the selected *Nostoc* strains for use in rice fields. In addition, a superior colonizer among the tested cyanobacterial strains will be sought through competition experiments.

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