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Azorhizobium caulinodans ORS 571–*Aspergillus spp.* biofilm in the presence of flavonoid naringenin: An extremely effective association for rice root colonization with a definite future as a nitrogen bio-fertilizer

Thilini A Perera[1], TL Shamala Tirimanne[1]*, G Seneviratne[2] & SA Kulasooriya[2]

[1]Department of Plant Sciences, Faculty of Science, University of Colombo, Colombo 03, Sri Lanka
[2]Microbial Biotechnology Unit, National Institute of Fundamental Studies, Kandy, Sri Lanka

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Use of diazotrophs to provide biologically fixed nitrogen to rice is a challenging but an important alternative to reduce the ailments of excessive use of nitrogen fertilizer on environment, health and economy of a country. The present study focuses on finding an effective strategy for induced colonization of rice roots by *Azorhizobium caulinodans* leading to significantly beneficial nitrogen providing the plant-microbe association with a very high potential to be developed as a nitrogen bio-fertilizer. *Azorhizobium caulinodans* was labelled with a green fluorescent protein (GFP) for detection *in-vivo*. Three separate rice rhizosphere fungi were tested for biofilm formation with the GFP-labelled bacterium *in-vivo* and successful formation of a biofilm was observed with *A. caulinodans-Aspergillus* spp. 1 (AAB). *A. caulinodans* in the presence of flavonoid naringenin, AAB and *A. caulinodans* in water limiting conditions were tested for induced Azorhizobial colonization of three local rice varieties. The degree of colonization and its effectiveness were screened through epifluorescent microscopy, the degree of endophytic colonization and acetylene reduction assay. Green fluorescence intensity measurements revealed that the treatments, biofilm and naringenin have induced colonization at significantly higher values. Treatment biofilm resulted in the highest nitrogenase activity. Combination of the treatments biofilm, and naringenin, reflected significantly much higher values in fluorescence intensities, acetylene reduction assay and endophytic colonizations. This study reveals that the combination of the biofilm and naringenin promote extremely efficient rice root colonization, with a definite future as a nitrogen bio-fertilizer for rice.

Keywords: Acetylene reduction assay, Endophytic colonization, Flavonoid, Fluorescence intensity, GFP, Plant – Microbe association

The utilization of nitrogen fertilizers for agriculture is neither a choice nor a virtue. Although the usage of nitrogen fertilizers are coupled with environmental and socio-economical complexities, nitrogen is the *sine qua non* of today's high yielding agriculture[1]. Sustainable and environmentally friendly method of substituting nitrogen fertilizer usage in rice has been intensely studied. A biological nitrogen-fixing (BNF) rice plant would be an ideal alternative[2].

The bacterium *Azorhizobium caulinodans* is unique over other micro-symbionts due to its ability to fix nitrogen non-symbiotically[3] even in the presence of 3% v/v oxygen[4]. This character is of extreme importance to induce BNF in non-legumes such as rice. Previous studies on the association of *A. Caulinodans* with rice roots indicated the possibility of the use of this bacterium as a bio-fertilizer. However in the previous studies nitrogen gain to the plant was not significant[5,6].

Flavonoids are known to be released by leguminous plants to attract rhizobia in root colonization and nod gene expression. Naringenin is a flavonoid which is important in rice root colonization by *A. caulinodans*. The use of flavonoid naringenin increases the *A. Caulinodans* colonization of rice roots, specifically in cortex region, lateral root cracks and in the xylem[7-11].

The present study was aimed at obtaining maximum colonization of rice roots by *A. caulinodans*, hence different strategies were employed to induce colonization (based on published literature). Biofilms are a novel mode of bio-fertilization having remarkable benefits[12]. Cell aggregations in the form of biofilms possess collective functions that are not expressed by monocultures[13,14] and they exhibit a set of emergent properties that are not expressed and differ substantially from free-living bacteria[15].

*Correspondence:
Fax: +9411585038
E-mail: shamala.tirimanne@gmail.com

Diazotrophs-containing-biofilms can be used for successful inoculation of nitrogen-fixing bacteria into the soil since the biofilm can protect the bacteria against adverse environmental conditions and competition by native soil biotic populations[16]. A biofilms formed between a fungi and a bacterium enhances growth and the survival of the bacterium in the soil (Das *et al*. 2012).

Considering the effectiveness, convenience of application and the success of biofilms achieved as biofertilizers, in this study we attempt to formulate a biofilm between *Azorhizobium caulinodans* ORS 571 and a rice rhizosphere fungus *in vitro* and to investigate the ability of the developed *Azorhizobium caulinodans* ORS 571–*Aspergillus* spp. biofilm (AAB) to colonize rice roots.

Since, the unique characters of *A. caulinodans* would foster multitude of benefits considering root colonization and nitrogen fixation, the major objective of the study was to determine the best strategy (among the treatments of flavonoids, biofilms and water stress) for induced colonization of rice roots by *A. caulinodans* that could lead to a significant beneficial nitrogen providing association. The bacterium was labelled with Green Fluorescent Protein (GFP) marker for accurate and rapid detection of the bacterium in and around the root hairs. The screening was performed by epifluorescent microscopic measurements, endophytic colonization and acetylene reduction assay measurements. This study reports the ability of rice root colonization of *A. caulinodans* by the biofilm of *A. caulinodans* – *Aspergillus* spp. and its increment in the presence of flavonoid naringenin.

Materials and Methods

Development of GFP-labelled *Azorhizobium caulinodans* ORS 571

The plasmid (pBBR5-hem-gfp5-S65T) in *E. coli* (Gm^r) was kindly provided by the University of Ghent, Belgium. *A. caulinodans* ORS 571 (Cb^r)[18] and plasmid pRK2013 in E. coli. (Km^r) were purchased from CCUG culture collection, Sweden and DZMC culture collection, Germany respectively.

The GFP plasmid was inserted in to *A. caulinodans* ORS 571 with pRK2013 as a helper plasmid[19]. The resultant mixture of plasmids was cultured on antibiotic combinations (YEB+Cb+Gm)/ (YEB+Cb)/ (YEB+Gm) and the resulted bacterial colonies were observed through an epifluorescent microscope. (Cb^r: Carbenicillin resistant, Gm^r: Gentamicin resistant, and Km^r: Kanamycin resistant).

Development of *A. caulinodans* – *Aspergillus* spp. biofilm (AAB)

Two *Aspergillus* spp. isolated from rice rhizosphere (*Aspergillus* spp. 1 and *Aspergillus* spp. 2) and one *Penicillium* spp. (provided by the culture collection of the National Institute of Fundamental Studies, Kandy, Sri Lanka) were cultured separately in Potato Dextrose Broth (PDB) for three days.

GFP-labelled *Azorhizobium caulinodans* was cultured in YEB medium for 24 h (approximately 10^8 cells/mL). Each of the fungal cultures was combined with the bacterium *A. Caulinodans* in separate flask and were allowed to grow for one week. The possibility of *A. caulinodans* to form a biofilm was investigated over one week and the progress was documented.

Different strategies/treatments for maximum colonization of *A. caulinodans* in rice roots

Seeds of three commonly cultivated Sri Lankan rice varieties, BG 366, BG 352 and BG 359 were dehusked, surface sterilized using the methods described by Rashid *et al*. 1995[20], and pre-germinated for three days in 0.8 % (V/V) agar. Pre-germinated seeds were planted in sterile pots containing 200 g of the sterilized vermiculite-perlite mixture (1:1). Twelve pre-germinated seeds were planted in each pot equidistantly. Sterilized, nitrogen-free, Fahraeus medium (50 mL/twice a week per pot) was added, and then the pots were flooded with autoclaved distilled water and were subjected to the following treatments. The pots were kept inside a greenhouse in the Department of Plant Sciences, University of Colombo, Sri Lanka.

Treatment 1: Flavonoid naringenin and A. caulinodans ORS 571

Naringenin was prepared according to the method given by Cancino *et al*. 2001[21]. Filter sterilized Naringenin at 1×10^{-4} M was added to pots containing surface sterilized, pre-germinated seeds in sterilized vermiculite and perlite medium and *A. caulinodans* ORS 571 culture (10^8 cells/mL and 5 mL per plant) was added twice a week for two weeks.

Treatment 2: A. caulinodans ORS 571–Aspergillus spp. 1 biofilm AAB

A healthy stable biofilm culture was diluted 15 fold by adding autoclaved distilled water. Five mL of the diluted biofilm culture was carefully added to each five-day old rice seedlings. Biofilm was added once a week for two weeks**.**

Treatment 3: Water limiting condition

Test plants were subjected to a water limited environment by sprinkling only $1/10^{th}$ of the volume required for flooding every other day. Five millilitres

of *A. caulinodans* ORS 571 culture was added twice a week for two weeks (for each plant). The plants were kept at reduced water levels throughout the experiment.

Control

Five millilitres of *A. caulinodans* ORS 571 was added to each plant twice a week for two weeks.

Amount of culture applied (5 mL)/ frequency of application (twice a week) and optimum time duration of measurement (15 dpi) were adopted from results of a preliminary study[22].

Each treatment was replicated three times in all three rice varieties and the experiment was arranged in a completely randomized design.

Microscopic analysis

From each plant, a single root was picked randomly. Each root was cut in to 1 cm pieces and three root pieces representing root base region, mid region and the root apex region were selected for analysis. The roots were analysed by micro-imaging, (Carl Zeiss Primostar fluorescent microscope with i-LED) fluorescent detection unit. The roots were analysed for the degree of colonization with an epifluorescent microscope. Green fluorescing regions were photographed by Axiocam ERc 5s. The Average fluorescing intensities were measured using ZEN 2012, ZEN light blue edition software[23,24].

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using SAS V 9. The differences among means were separated by Duncan's multiple range test at 0.05 probability level.

Determination of nitrogenase activity of rice plant roots

Acetylene reduction assay was conducted to determine the nitrogenase activity of plant roots that were subjected to different treatments above for two rice varieties (BG 366 and BG 352). Roots of three plants from each treatment were subjected to the assay. Plants were uprooted on the 15 dpi. Vermiculite and perlite were carefully removed and the plants were sprinkled with water. Then the roots were detached and were inserted into glass bottles (5 mL) with rubber stoppers and flushed with high purity (99%) nitrogen (N_2) gas. Five percent of the air volume was replaced with air and incubated overnight (24 h). Ten percent (10%) of the gas phase was then replaced by acetylene and was incubated for another 4 h. (Optimum incubation time was determined from a preliminary study and data not shown here). At the end of 4 h, 1 mL of gas samples from each bottle were analysed by Shimadzu GC 9 AM gas chromatograph fitted to a fused silica capillary column equipped with flame ionization detector[25].

Combined treatment of biofilm (AAB) and Naringenin

The two treatments naringenin and biofilm (treatments 1 and 2) which results in the highest rice root colonization and acetylene reduction were combined to test the combined effect on rice root colonization, nitrogenase activity and endophytic colonization by *A. caulinodans* with the rice variety BG 366.

Treatment 5: A combination of naringenin and biofilm

AAB (as in treatment 2) was added once a week for two weeks to the pots with added naringenin prepared as in section 2.3 above.

Re-isolation of endophytically colonized *A. caulinodans*

From the treatments 1, 2, 3, control and treatment 5, rice roots were first weighed and surface sterilized by the methods described by Jain & Gupta (2003)[11]. Surface sterilized roots were macerated with sterile sand and water[10]. The extracts were serially diluted and streaked on YEB agar medium with or without antibiotics Cb and Gm. The plates were incubated at 37°C for 3 days, the colonies were observed for fluorescence, counted and the number of endophytic colonies per gram fresh weight was calculated.

Results

GFP-labelling of *Azorhizobium caulinodans* ORS 571

GFP-labelling of *Azorhizobium caulinodans* ORS 571 was successfully achieved by inserting the GFP-labelled plasmid into the host *A. caulinodans* by tri-parental mating. Successful insertion was initially tested by culturing the resultant recombinants on YEB agar with Gm and Cb antibiotics which did not support the growth of the non-recombinant. GFP-labelled *A. caulinodans* was confirmed by the observation of fluorescing bacteria under blue light through an epifluorescent microscope.

A biofilm with *A. caulinodans* ORS 571

Out of the three fungal cultures isolated from the rice rhizosphere, *Aspergillus* spp. 2 and *Penicillum* spp.1 had not resulted in formation of biofilms with *A. caulinodans* ORS 571. A biofilm comprising of the fungus *Aspergillus spp* 1, and the bacterium *A. caulinodans* ORS 571 was successfully formed *in vitro.*

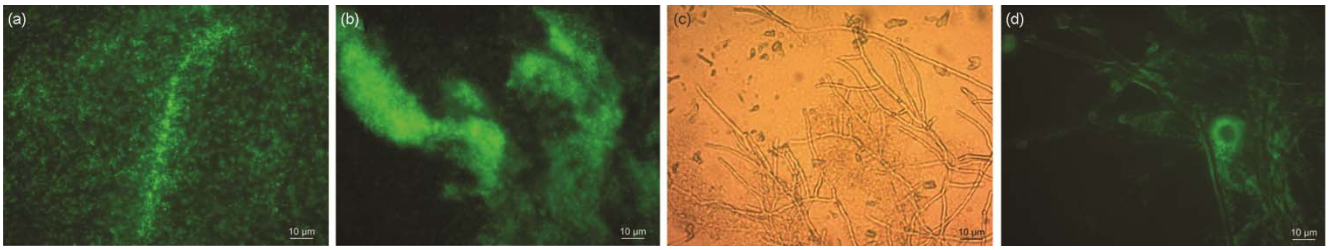

Fig. 1 — The epifluorescent micrographs showing formation of a biofilm.(A) A 2 day old biofilm: a fungal filament surrounded by bacteria *A. caulinodans* migrating towards the fungal filament (×40); (B & C) a 3-day old biofilm (fungal filaments completely surrounded by bacteria)×40 (B) under epifluorescent microscope,(C) under a light microscope. (D) 3 day old fungal filaments and fungi vesicles surrounded by fluorescing bacteria (×40).

Table 2 — Degree of colonization measured by Fluorescence intensities of the three different treatments and the control on the three rice varieties. Comparison of different methods of inoculation of *A. caulinodans* analysed by one-way ANOVA of SAS, using Duncan's Multiple range test (P =0.05). Data followed by different letters differ significantly. Numbers after ± are a Standard error of means. A Separate ANOVA was conducted for each variety for the 4 treatments.

Treatment	Mean Fluorescence intensity [a.u] Rice Varieties		
	BG 366	BG 352	BG 359
1. Naringenin + *A. caulinodans*	200.828 ± 2.09 a	225.685 ± 1.46 a	203.131 ±3.08a
2. Biofilm (AAB)	206.55 ± 2.68 a	173.083 ± 2.03 d	173.083 ± 2.03 c
3. Water limiting conditions	189.977 ± 2.1 b	187.674 c ± 2.91 c	187.601 ± 2.11 b
4. Control *(A.caulinodans* only)	177.460 ± 2.44 c	197.633 b ± 2.74 b	165.461 ± 2.73 d

On the second day after mixing of the bacterial and fungal cultures, the fluorescing bacterial cells were observed around the fungal filaments, the cells were migrating towards the fungal filaments but yet with large numbers of planktonic cells (Fig. 1A). On the third day, heavy colonization was observed on and around the fungal filaments with bacterial clouds heavily covering the fungal filament (Figs. 1B &C). In the places where the fungal mat was present in the culture, it was observed that the conidia and conidiophores were also covered with clouds of bacteria (Fig. 1D). Heavy colonization sustained for two more days after which the biofilms began to deteriorate.

Treatments for maximum colonization of *A. caulinodans* ORS 571 in rice roots

The degree of rice root colonization of GFP-labelled *A. caulinodans* directly reflected by the intensity of the green fluorescence emitted, is captured and measured using ZEN 2012, ZEN light blue edition software.

The fluorescence measurements resulted from the treatments 1, 2, 3 and control in materials and methods (1: *A. caulinodans* ORS 571 + Naringenin, 2: AAB, 3: Water limiting condition + *A. caulinodans* ORS 571, Control (only with A. *caulinodans* ORS 571) on three rice varieties, (BG 366, BG 352, BG 359) were compared using 2-way ANOVA of SAS (Version 9.0) program. It was revealed that there is a highly significant difference between the different treatments, varieties and the combination of treatment and varieties as shown in Table 1. Since there is a significant difference, rice root colonization data of each variety were then analysed separately for each treatment (Table 2). The treatment 1 with naringenin and *A. caulinodans* resulted in significantly higher colonization than other treatments for the varieties BG 352 and BG 359. In BG 366, Treatment AAB shows a numerically slightly higher colonization (which does not differ significantly to the treatment 1). *A. caulinodans* under water limiting conditions (treatment 3) has also resulted in significantly higher colonization than the control in the rice varieties BG 366 and BG 359. In BG 352, the control (with only *A. caulinodans*) has resulted in higher colonization than the treatments 2 and 3 (Fig. 2).

Table 1 — Comparison of the 4 different treatments. Comparison of varietal and treatment effects on colonization intensity by 2 way ANOVA of SAS version 9.

Source	Mean Square	F value	PF > F
Variety	13755.6779	17.43	< 0.0001
Treatment	58805.5470	74.52	< 0.0001
Variety × Treatment	27535.9312	34.90	< 0.0001

Acetylene reduction assay of rice varieties BG 366 and BG 352

Nitrogenase activity of the plant roots colonized with *A .caulinodans* under treatments 1, 2, 3 and control [(In materials and methods (section 2))] were

measured using Acetylene Reduction Assay (ARA) to determine which treatment contributed most for nitrogenase activity inside the roots. Two rice varieties with the highest colonization intensities values (BG 366 and BG 352) were used for the experiment.

Biofilm (AAB) (treatment 2) had resulted in highest ARA values for both of the rice varieties (Table 3) of which BG 366 yielded the highest. The treatment with naringenin had also resulted in higher values than the control. However, water limiting

Table 3 — Nitrogenase activity of rice roots subjected to the four treatments (1, 2, 3 and control) in rice varieties BG 352 and BG 366 evaluated by acetylene reduction assay. Values indicate the mean of three replicates.

Treatment	ARA values (nmol C_2H_4/4 h/g fresh weight)	
	BG 366	BG 352
1. Naringenin + *A. caulinodans*	540	404
2. Biofilm (AAB)	777	600
3. Water limiting condition + *A. caulinodans*	290	294
4. Control *(A.caulinodans* only)	494	323

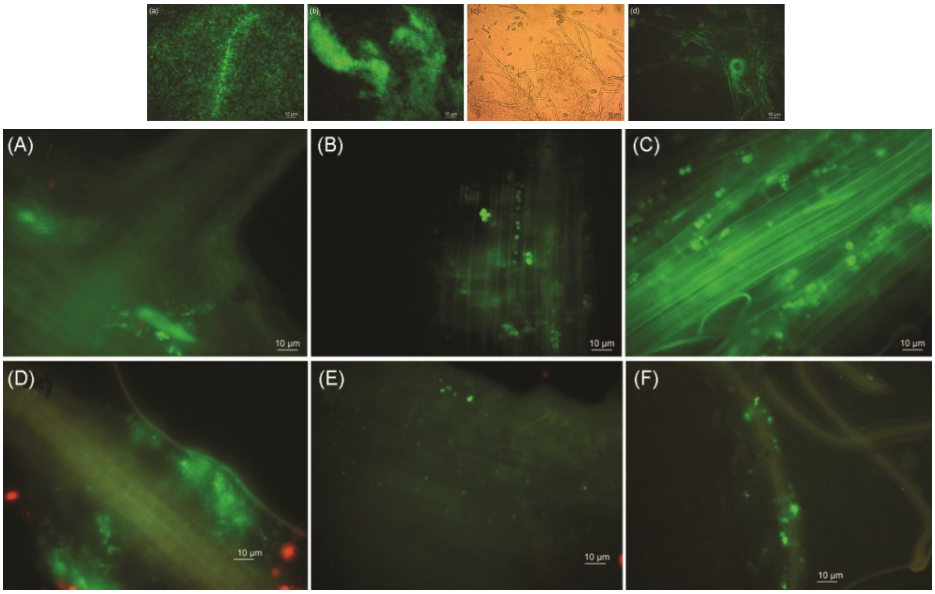

Fig. 2 — Epifluorescent micrographs of rice roots showing colonization of *A. caulinodans*: 15 days after application of treatments (× 40). (A) (B) (C) Treatment 1: plants treated with naringenin $1 \times 10^{-4}M$ and *A. caulinodans*: Colonization of the *Azorhizobium caulinodans* ORS 571 (A) lateral root cracks;(B) in the xylem region; (C) in the cortical region(D) Treatment 2: inoculation with the 3-day old biofilm (AAB only) fluorescing *A. caulinodans* appear in clusters. (E) Control: Plant inoculated only with *A. caulinodans* (F)Treatment 3: Plants subjected to limited water levels: *A. caulinodans* clusters surrounding the root.

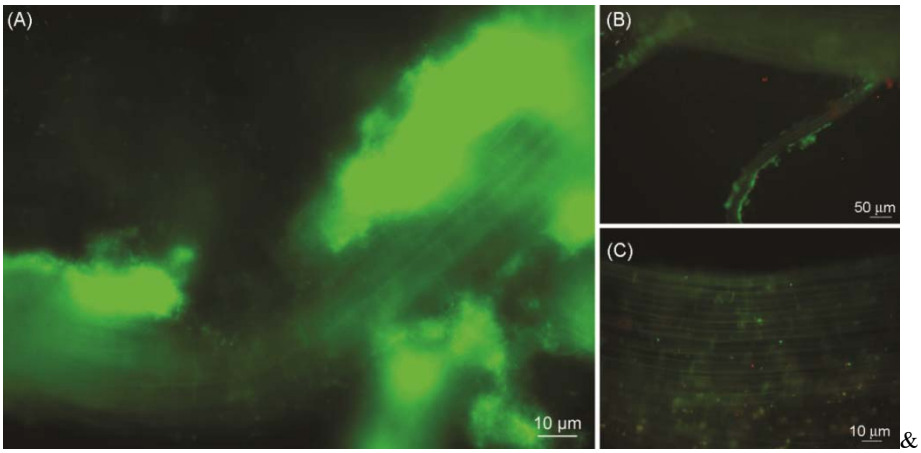

Fig. 3 — Epifluorescent micrographs of rice roots of BG 366: Combined treatments of Biofilm (AAB) and naringenin $1 \times 10^{-4}M$ and control (*Azorhizobium caulinodans* only). (A) and (B) Treatment 5: A region of root under high power (× 40) (A) and under mid power (×10) (B) of the plants after combined treatment (C) Control: A region of root treated only with *A. caulinodans* (×40)

Table 4 — Combined effect of the biofilm (AAB) and naringenin on colonization of rice roots, number of endophytes, and nitrogenase activity of rice variety BG 366. Means of fluorescence intensities followed by different letters of the alphabet differ significantly by Duncan's multiple range test (P <0.05). Number of endophytes and ARA values indicate average of three replicates

Treatment	Mean fluorescence Intensities [a.u]	No of endophytes per gram fresh weight	ARA values (nmol C_2H_4/h/g Dry weight)
1. Naringenin + *A. caulinodans*	202 b	1.46×10^5	551
2. Biofilm (AAB)	205 b	Nil	709
5. AAB+ Naringenin	221 a	9.48×10^5	1174
4. Control (only *A.caulinodans*)	178 c	4.25×10^4	393

conditions (treatment 3) had resulted in lower values for ARA than the control.

Combination of the two treatments biofilm (AAB) and Naringenin

Since the treatment 1(Naringenin + *A. caulinodans*) and treatment 2 (biofilm) has given the highest colonization intensities and ARA values, the two treatments were combined to examine the combined effect on the rice variety BG 366. The combination had resulted in statistically significantly higher *A. caulinodans* colonization (Table 4 and Fig. 3) and nitrogenase activity (Table 4).

Re-isolation of endophytically colonized *A. caulinodans*

Colonized rice roots from all the treatments (1, 2, control and 5) were surface sterilized and inoculated separately on YEB agar medium (with or without antibiotics Cb and Gm) to determine the capacity of re-isolation of the bacterium from the rice roots after treatments 1, 2 and 5. The capacity of re-isolation directly indicates the degree of endophytic colonization. Antibiotics were applied for selection of GFP-labelled endophytes. Biofilm and naringenin in combination have resulted in a 6 fold increment of the endophytic *A. caulinodans* compared to treatment 1 (Naringenin and *A. caulinodans*) and a 22 fold increment when compared to the control with only *A. caulinodans* (Table 4). Hence, the treatment biofilm and naringenin combination showed the highest capacity to re-isolate indicating the highest endophytic colonization of the bacterium. This is followed by *A. caulinodans* and naringenin (treatment 1) and then by control. This shows that the presence of naringenin enhances the endophytic colonization of *A. caulinodans*.

Discussion

Azorhizobium caulinodans is a diazotroph with unique beneficial characters that makes it a potential candidate to use as a nitrogen biofertilizer for rice through an induced nitrogen fixing association with rice plant.

GFP-labelling of *Azorhizobium caulinodans* ORS 571 was successfully achieved by insertion of GFP-labelled plasmid into the host *A. caulinodans* by tri-parental mating. The GFP-labelled *A. caulinodans* ORS 571 emitted bright fluorescent green light under blue light of an epifluorescent microscope.

Flavonoids to increase rice root colonization

In this study, the presence of naringenin had increased the rice root colonization of all three rice varieties tested (Table 2). Varieties BG 352 and BG 359 have resulted in the highest extent of colonization for the treatment with naringenin (Table 2). A highly positive correlation between rice root colonization by *A. caulinodans* and the presence of naringenin has been reported by several authors[7-11]. Flavonoids are known to be released by certain leguminous plant roots and found to induce the transcription of nod genes[26] of rhizobia and initiate the nodulation process of legumes. Goethals *et al.* (1989)[27] reported that naringenin is the most efficient nod gene inducer. Flavonoids have also been found to induce the expression of other bacterial genes with unknown functions[28,29]. Colonization of lateral root cracks (Fig. 2A), xylem region (Fig. 2B) and the cortical region (Fig. 2C) of the plant root was observed in the presence of naringenin. Several authors also have reported the possibility of induced lateral root colonization, increased number of lateral root development, crack entry invasion of the lateral roots leading to cortical and xylem colonization in the presence of 10^{-5}M or 10^{-4}M flavonoid naringenin[7-11,30].

Rice root colonization of the *A. caulinodans* – *Aspergillus* spp. biofilm

Our attempt of developing a biofilm *in-vitro*, comprising *Azorhizobium caulinodans* and a rice rhizosphere fungus was a success. Out of the three fungal cultures tested, *Aspergillus* spp. 1 formed a biofilm while *Aspergillus* spp. 2 and *Penicillium* spp. failed. Since, the three day old biofilm (*A. caulinodans* – *Aspergillus* spp.) had fungal filaments attached with high bacterial cell densities (Fig. 1B-D) and was

observed to be stable for two more days *in vitro* prior to deterioration, the three day old biofilm was selected for rice root inoculation in this study. Observations on the 15 days after the addition of the biofilm, showed that rice roots have become colonized and the bacterial associations were observed as bright green clusters through the epifluorescent microscope (Fig. 2D). Colonization was higher in places where the number of lateral roots was high. Seneviratne *et al* (2008)[31] observed that the fungal mycelium of fungal-bacterial biofilms links the root hairs and hence provides support to maintain higher cell densities on and around root hairs whereas biofilms consisting only of bacteria cover only the root hairs, in lower densitiesof the plant root.

Water limiting conditions and colonization

Heavy colonization by *A. caulinodans* ORS 571 in the root hair zone was observed in rice plant roots subjected to a water limiting conditions. A similar observation has been reported[30] and under water limiting conditions, bacteria tend to develop into biofilms (Fig. 2F) encapsulated with extracellular polymeric substances (EPS)[32]. In all three rice varieties clumps of bacteria formed into bacterial–bacterial (Azorhizobial-Azorhizobial) biofilms were visible on the rice root surface after 15 days. Here, resource channelling to produce more EPS is suggested for *A. caulinodans*, as was evident in *Pseudomonas* sp.[33]. In response to limited availability of water, this resource channelling or sharing, leads the microbes in to a hydrated microenvironment and subsequent survival under water limiting conditions. The Extra-cellular polysaccharides that cover the biofilm are usually hygroscopic, so they create a more hydrated micro-environment in the immediate vicinity of cells leading to desiccation tolerance. Out of the three rice varieties, the varieties that are recommended for cultivation in the Dry and Intermediate zones of Sri Lanka, (BG 366, and BG 359) were observed to be colonized well (Table 2).

Acetylene reduction Assay

Acetylene Reduction Assay provides an approximation of the amount of nitrogen fixed in a particular plant[34]. In BG 366, both the highest fluorescent intensities and ARA values were observed for the treatment 2 (*A. caulinodans–Aspergillus* spp. biofilm). But in the variety BG 352, although the colonization intensities were numerically higher in the treatment 1, (Naringenin + *A. caulinodans*) the highest ARA value was gained for the treatment 2 (with the biofilm). Here we suggest that in the presence of a biofilm, even though the colonization is lower, the amount of cells that is present surrounding the roots are active, robust and act and behave efficiently fixing more nitrogen. Gonzalez and Bashan *et al* in 2000[35] have reported of alteration of metabolic activities when the micro-organisms co-exist in a community. These alterations in biofilms are mostly positive, beneficial and enhanced changes as reported by Seneviratne *et al* in 2005. These positively altered metabolic activities may have increased the nitrogen fixation in the active healthy, efficient biofilms in BG 352. In water limiting conditions (treatment 3), both BG 366 and BG 352 showed ARA values less than the control (Table 3). Alteration of metabolic processes due to water deficiency can be suggested for the lower ARA values.

Effect of biofilm and the flavonoid naringenin combination on rice root colonization

A collective effect of the treatments biofilm and naringenin in combination (that yielded significantly higher fluorescent intensity measurements and ARA values) on the BG 366 rice variety was analysed. Here, the Biofilm (AAB) was added to the pot containing naringenin instead of *A. caulinodans* (Treatment 5 above).

The combination of flavonoid naringenin and biofilm resulted in significantly high rice root colonization observed by highest fluorescent intensity mean (Table 4 and Fig. 3 A&B). Significantly higher nitrogenase activity, determined through Acetylene Reduction Assay was also evident (Table 4).

The positive effects of naringenin on rice root colonization of both *A. caulinodans* and biofilm is clearly evident as the fluorescence intensities and nitrogenase activity increase significantly whenever naringenin is used in combination with the produced, AAB (biofilm) or with the monoculture of *A. caulinodans* (Table 4). This clearly indicates that the flavonoid naringenin can increase the colonization of *A. caulinodans*.

Endophytic colonization of the rice roots by bacteria

Surface sterilized roots from the treatments 1, 2, control and 5 were crushed and streaked on YEB medium. The combination of flavonoid naringenin and biofilm resulted in the highest number of endophytic re-isolates in comparison with the other treatments (Table 4). Positive effects of naringenin on endophytic rice root colonization of both *A. caulinodans* and biofilm is apparent here as the number of

endophytic colonizers has increased whenever naringenin is used (Table 5). Epifluorescent micrographs show the endophytically colonized clumps of *A. caulinodans* in lateral root cracks (Fig. 2A), the xylem (2B) and the cortex (2C) in the presence of flavonoid naringenin. Intensity values achieved from an epifluorescent micrograph depicts the total fluorescence resulting from internal and external colonizers. The extent of bacteria re-isolated from surface-sterilized roots directly reflects the extent of endophytic colonization of *A. caulinodans.*

In the bacterial re-isolation experiment, while the combination of naringenin and biofilm resulting 9.48×10^5 cells per gram fresh weight root, the biofilm resulted in no endophytic re-isolates (Table 4). Several plausible arguments could be drawn into attention for the above observation, keeping in mind that the experiments were conducted in axenic environments. By past literature, it has been increasingly realised that the BFs are effective than mono or mixed cultures of microbial biofertilizers[36]. BF increases interaction among microbes and release compounds such as low molecular weight sugars and amino acids which induce to break dormancy of cysts, spores, akinetes, etc., in the soil microbial seed bank[37,38]. This could lead to the emergence of diverse microflora. Addition of a biofilm could create these changes in an actual soil environment hence more nitrogen fixers and more biofilms surrounding the root other than the inoculated microbes could be formed. In the biofilm only treatment, no internally colonized bacteria had resulted in any of the replicates. *Aspergillus* spp. which was used for the biofilm formation, is a rice rhizosphere colonizer and the addition of the only biofilm may be limiting it to reside in the rice rhizosphere zone outside the roots whereas the free-living *A. caulinodans*, coupled with the signalling molecule naringenin (treatment 1) leads the bacteria to endophytic colonization. It can be speculated that when the *A. caulinodans* is in a biofilm, due to the attachments they may hinder the endophytic colonization. When the biofilm is combined with naringenin, the huge number of bacteria residing on the roots as a biofilm or in the planktonic form in the close proximity (Fig. 1A), may also be channelled towards the inside of the root. This leads the combined effect of biofilm and naringenin to be the most effective and efficient method of colonization of the rice roots internally and externally.

Developing a nitrogen bio-fertilizer for non-legume rice has several challenges. Easy, sustainable and agronomically significant method should be formulated to make the maximum benefit of unique characters of *A. caulinodans*. Here we have tested practical strategies (given as treatments) that could be easily adopted to enhance rice root colonization of *A. caulinodans* and the potentials of these treatments to be used outside the greenhouse and laboratory conditions as a true nitrogen biofertilizer for rice. It can be concluded that the developed *A. caulinodans-Aspergillus* spp. biofilm in the presence of naringenin, is a highly effective rice root colonizer with a definite future for development as a nitrogen-fixing biofertilizer for rice which will be an eco-friendly agronomically significant alternative for nitrogen fertilizer usage.

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References

1 Ladha JK, Tirol-Padre A, Reddy CK, Cassman KG, Verma S, Powlson DS, Kessel CV, Richter DB, Chakraborty D & Pathak H, Global nitrogen budgets in cereals: A 50-year assessment for maize, rice, and wheat production systems. *Sci Rep*, 6 (2016) 1.

2 Sofi P & Wani S, Prospect of Nitrogen Fixation in Rice. *Asian J Plant Sci*, 6 (2007) 203.

3 De Brujin FJ, *The unusual symbiosis between the diazotrophic stem-nodulating bacterium Azorhizobium caulinodans ORS 571 and its host, the tropical legume Sesbania rostrate*. In: Plant-Microbe interactions, (Ed, NET N McGraw-Hill, New York) 1989, 457.

4 Kitts C & Ludwig RA, *Azorhizobium caulinodans* respires with at least four terminal oxidases. *J Bacteriol*, 176 (1994) 886.

5 Ladha JK, Garcia M, Miyan S, Padre AT & Watanabe I, Survival of *Azorhizobium caulinodans* in the soil and rhizosphere of wetland rice under Sesbania rostrate rice rotation. *Appl Environ Microbiol*, 55 (1989) 454.

6 Van Niewanhove C, Merckx R, Holm LV & Vlassak K, Dinitrogen fixation activity of *Azorhizobium caulinodans* in the rice (*Oryza sativa* L.) rhizosphere assessed by nitrogen balance and nitrogen-15 dilution methods. *Biol Fertil Soils*, 33 (2001) 25.

7 Webster G, Gough C, Vasse J, Batcheolar CA, O'Callaghan KJ, Kothari SL, Davey MR, Denarie J & Cocking EC, Interaction of rhizobia with rice and wheat. *Plant Soil*, 194 (1997) 115.

8 Boddy RM, Biological Nitrogen fixation in sugar cane: A key energetically viable biofuel production. *Crit Rev Plant Sci*, 14 (1995) 263.

9 Reddy PM, Ladha JK, So RB, Hernandez RJ, Ramos MC, Angeles OR, Dazzo FB & De Brujin FJ, Rhizobial communication with rice roots: Induction of phenotypic changes, mode of invasion and extent of colonization. *Plant Soil*, 194 (1997) 81.

10 Gopalaswamy G, Kannaiyan S, O'Callaghan KJ, Davey MR & Cocking EC, The xylem of rice (*Oryza sativa*) is colonized by *Azorhizobium caulinodans*. *Proc Royal society*, 267 (1999) 103.

11 Jain V & Gupta K, The flavonoid naringenin enhances intercellular colonization of rice roots by *Azorhizobium caulinodans. Biol Fertil Soils*, 38 (2003) 119.

12 Ricci EC, Investigating the role of Pseudomonas sp. and Bacillus sp. biofilms as plant growth promoting inoculants, Doctoral dissertation, McGill University, Montreal Quebec, Canada, December 2015.

13 Davey ME & O'Toole GA, Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol Res*, 64 (2000) 847.

14 Shapiro JA, Thinking about bacterial populations as multicellular organisms. *Annu Rev Microbiol*, 52 (1998) 81.

15 Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA & Kjelleberg S, Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol*, 14 (2016) 563.

16 Jayasinghearachchi HS & Seneviratne G, A bradyrhizobial–Penicillium spp. Biofilm with nitrogenase activity improves N_2 fixing symbiosis of soyabean. *Biol Fertil Soils*, 40 (2004) 432.

17 Das N, Basak LVG & Salam JA, Application of biofilms on remediation of pollutants–an overview. *J Microbiol Biotechnol Res*, 2 (2012)783.

18 Dreyfus B, Garcia J & Gills M, Characterization of *Azorhizobium caulinodans* gen nov sp Nov a stem nodulating nitrogen fixing bacterium isolated form *Sesbania rostrate*. *Int J Syst Bacteriol*, 38 (1988) 89.

19 D'Haeze W, Gao M & Holsters M, A gfp reporter plasmid to visualize *Azorhizobium caulinodans* during nodulation of Sesbania rostrate. *Plasmid*, 51 (2003) 185.

20 Rashid H, Yokoi S, Toriyama K & Hinata K, Transgenic plant production mediated by Agrobacterium in Indica rice. *Plant Cell Rep*, 15 (1995) 727.

21 Cancino GO, Gill MIS, Davey MR, Power JB & Lowe KC, Naringenin enhanced efficiency of gus activity in *Passiflora mollissima* (H B.K) Bailey. *Univ Sci*, 9 (2001) 47.

22 Perera TA & Tirimanne TLS, Facilitated colonization of the diazotroph *Azorhizobium caulinodans* in rice roots detected through GFP-labelling. *J Natl Sci Found*, 45 (2017) 193.

23 Rocha MC, Grady JP, Grünewald A, Vincent A, Dobson PF, Taylor RW & Rygiel KA, A novel immune-fluorescent assay to investigate oxidative phosphorylation deficiency in mitochondrial myopathy: understanding mechanisms and improving diagnosis. *Sci Rep*, 5 (2015) 15037.

24 Jung AR, Kim RY, Kim HW, Shrestha KR, Jeon SH, Cha KJ, Kim DS & Lee JY, Nano-engineered polystyrene surfaces with Nano pore array pattern alters cytoskeleton organization and enhances induction of neural differentiation of human adipose-derived stem cells. *Tissue Eng Part A*, 21 (2015) 2115.

25 Seneviratne G & Jayasinghearachchi HS, A rhizobial biofilm with nitrogenase activity alters nutrient availability in a soil. *Soil Biol Biochem*, 371 (2005) 975.

26 Rolfe BG, Verma DPS, Potrykus I, Dixon R, McCully M, Sautter C, Denarie J, Sprent J, Reinhold-Hurek B, Vanderleyden J, Ladha JK, Dazzo FB, Kennedy I & Cocking EC, *Round table Agriculture 2020: 8 billion people*. In: Current plant science and biotechnology in agriculture: biological nitrogen fixation for the 21st century, (Ed Elmerich C, Kondorosi A, Newton WE, Dordrecht, The Netherlands: Kluwer) 1998, 685.

27 Goethals K, Gao M, Tomekpe V, Van-Montagu M & Holster M, Common nod ABC genes in Nod locus I of *Azorhizobium caulinodans*: nucleotide sequence and plant inducible expression. *Mol Gen Genet*, 219 (1989) 289.

28 Krishnan HB & Pueppke SG, Flavonoids inducers of nodulation genes stimulate *Rhizobium fredii* USDA257 to export proteins in to the environment. *Mol Plant-Microbe Interact*, 6 (1993) 107.

29 Perret X, Fellay R, Bjourson AL, Cooper JE, Brenner S & Broughton WJ, Substraction hybridization and shotgun sequencing, A novel approach to identify symbiotic loci. *Nucleic Acids Res*, 22 (1994) 1335.

30 Webster G, Jain V, Davey MR, Gough C, Vasse J, Denarie J & Cocking EC, The flavonoid naringenin stimulates the intercellular colonization of wheat roots by *Azorhizobium caulinodans*. Plant cell environ, 21 (1998) 373.

31 Seneviratne G, Keckskes ML & Kennedy IR, *Biofilmed-biofertilisers: Novel inoculants for efficient nutrient use in plants*. In: Efficient Nutrient Use in Rice Production in Vietnam Achieved using Inoculant biofertilizers, (Ed Kennedy IR, Choudhury ATMA, Kecskes ML & Rose MT Proceedings of a project (SMCN /2002/073) (Workshop held in Hanoi Vietnam 12-13 October 2007. ACIAR Proceedings No 130, Canberra) 2008, 126.

32 Chang WS & Halverson LJ, Reduced water availability influences the dynamics, development, and ultrastructural properties of *Pseudomonas putida* biofilms. *J Bacteriol*, 185 (2003) 6199.

33 Roberson E & Firestone M, Relationship between desiccation and exopolysaccharide production in a soil *Pseudomonas* sp. *Appl Environ Microbiol*, 58 (1992) 1284.

34 Boddy RM, Methods of quantification of nitrogen fixation associated with Gramineae. *Crit Rev Plant Sci*, 6 (1987)209.

35 Gonzalez-Bashan LE, Lebsky VK, Hernandez JP, Bustillos JJ & Bashan Y, Changes in the metabolism of the microalga Chlorella vulgaris when immobilized in alginate with the fixing *Phyllobacterium myrsinacearum*. Can J Microbiol, 46 (2000) 653.

36 Seneviratne G & Kulasooriya SA, Reinstating soil microbial diversity in agro ecosystems: The need of the hour for sustainability and health. *Agric Ecosyst Environ*, 164 (2013) 181.

37 Saini HS, Bassi PK, Consolaction ED & Spencer MS, Interactions among plant hormones, carbon dioxide, and light in the relief of thermo inhibition of lettuce seed germination: studies in a flow through gaseous system. *Can J Bot*, 64 (1986) 2322.

38 De Bore W, Folman LB & Summerball RC, Living in a fungal world impact of fungi on soil bacterial niche development. *FEMS Microbiol Rev*, 29 (2005) 795.