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RESEARCH COMMUNICATION

Facilitated colonisation of the diazotroph *Azorhizobium caulinodans* in rice roots detected through GFP-labelling †

Thilini A. Perera and T.L. Shamala Tirimanne *
Department of Plant Sciences, Faculty of Science, University of Colombo, Colombo 03.

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Abstract: Rice being a non-legume, does not possess the capacity to carry out biological nitrogen fixation in symbiosis with rhizobia. *Azorhizobium caulinodans* is a micro-symbiont of *Sesbania rostrata* with several unique and beneficial characteristics that makes it a potential candidate to induce biological nitrogen fixing associations with the rice plant. Maximum colonisation of rice by the bacterium is important for high benefit. This study was aimed at finding the maximum colonisation levels of *A. caulinodans* detected by GFP-labelling of the bacterium. *A. caulinodans* was labelled with green fluorescent protein to facilitate accurate and real time observations of the bacterium in and around the root hairs. GFP-labelling was carried out by inserting the gfp-gene-containing plasmid pBBR5-hem-gfp5-S65T into *A. caulinodans* ORS 571, with a helper plasmid (pRK2013) by tri-parental-mating. The bacterium was observed to colonise rice roots by bright green fluorescence emitted by the bacterium under blue light. The fluorescence intensities reflecting the degree of colonisation of the bacteria were quantified using Zen light image analysis software and were statistically analysed. Highest colonisation was observed when 5 mL of the labelled *Azorhizobium* was applied twice a week, and the observations were made 15 days after application of the inoculum in the presence of the flavonoid naringenin.

Keywords: *Azorhizobium caulinodans*, fluorescence, GFP-labelling, rice.

INTRODUCTION

More than half of the world's population consume rice as their staple food (Mohanty, 2013). The cultivation of rice demands a high input of nitrogen fertiliser, since nitrogen is the most essential nutrient with a direct impact on vegetative growth and yield. Use of nitrogen fertiliser over a long period is known to cause serious environmental and socio-economic problems (Saikia & Jain, 2007). Biological nitrogen fixation (BNF) is the best alternative with the least negative impact. BNF is a high energy consuming reaction, which is extremely oxygen sensitive because nitrogenase, the major enzyme involved in converting dinitrogen to ammonia, is irreversibly inactivated by oxygen (Dingler & Oelze, 1985). In legumes, BNF takes place in the highly specialised nodule structure where these specific requirements are met. Induction of a non-legume crop such as rice with no nodules to fix nitrogen is therefore a challenge.

Experiments with the use of free living rice rhizosphere colonisers such as *Clostridium*, *Herbaspirillum* and *Burkholderia*, which are capable of non-symbiotic nitrogen fixation have been reported to be unsuccessful to provide significant nitrogen levels for rice due to competition by the native flora (Webster *et al*., 1997) and the usage of major portion of the fixed nitrogen for their own growth (Van Berkum & Bohlool, 1980).

Non-legume nitrogen fixation has been attempted globally in the 1990's under the directions of the International Rice Research Institute (IRRI) focusing mainly on molecular biological and genetic engineering approaches (Sofi & Wani, 2007), which have not yielded promising results (Saikia & Jain, 2007). A recent refocus on the area of study is reported again in 2011 (Beatty & Good, 2011), with the main aim of reducing the usage of nitrogen fertiliser for rice and other cereal cultivation. In this study *Azorhizobium caulinodans* was selected since

* Corresponding author (shamala.tirimanne@gmail.com)
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the bacterium possesses many unique advantageous characteristics. Although *Azorhizobium* is a micro-symbiont of the legume *Sesbania rostrata*, it can live as a free living diazotroph in soil and also has the ability to tolerate up to 3 % v/v oxygen (Kitts & Ludwig, 1994). These characteristics are remarkably advantageous for non-symbiotic nitrogen fixation.

Previous studies have shown that the flavonoid naringenin is a *nod* gene inducer (Rolfe *et al*., 1998) and a signalling molecule, which can increase azorhizobial colonisation of the non-legumes (Gough *et al*., 1997).

Concentrations of 10^{-4} M (100 $mMolm^{-3}$) / 10^{-5} M (10 $mMolm^{-3}$) naringenin has shown to increase the colonisation of *A. caulinodans* in lateral roots, cortical region (Webster *et al*., 1997; Gopalaswamy *et al.*, 2000; Jain & Gupta, 2003) and xylem (Gopalaswamy *et al.*, 2000) of rice roots. Green fluorescent protein (GFP) is a frequently used biological marker (Zimmer, 2002), which is used as a fusion tag to detect the carrier organisms, thereby allowing real time detection of the labelled organism (Tsien, 1998). Through GFP-labelling and detecting the degree of colonisation, the maximum colonisation of *A. caulinodans* can be determined under test conditions. This study focuses on labelling *A. caulinodans* with GFP, which allows obtaining accurate and real time detection of the bacterium and test conditions for maximum colonisation in and around rice roots. Inoculum volume, frequency of inoculation and the time of observation are the conditions tested for maximum colonisation.

METHODOLOGY

Green fluorescent labelling of the bacterium *A. caulinodans* ORS 571 was conducted by tri-parental mating (D'Haeze *et al.,* 2004) of three bacterial strains; two *E. coli* strains as donors and *A. caulinodans* ORS 571 as the recipient. *E. coli* (strain DH5α) with the plasmid containing gfp gene (pBBR5-hem-gfp5-S65T) (D'Haeze *et al.,* 2004) was kindly provided by the University of Ghent, Belgium. *Azorhizobium caulinodans* ORS 571 (purchased from the CCUG culture collection, Sweden) and plasmid pRK2013 in *E. coli* (strain HB101) (purchased from the DZMC culture collection, Germany) were resistant to antibiotics carbenicillin (Cb) and kanamycin (Km), respectively. The DH5α strain (containing pBBR5-hem-gfp5-S65T) resistant to gentamycin (Gm) and HB101 (containing pRK2013) were grown on Luria-Bertani medium with 15 µg/mL of Gm and 50 µg/mL of Km, respectively. *A. caulinodans* ORS 571 was grown on yeast extract broth medium with 10 µg/mL carbenicillin (Cb). Gram stain, spore stain and motility tests for all three strains were carried out to confirm the viability and characteristics. The GFP-containing plasmid was inserted into *A. caulinodans* ORS 571 with pRK 2013 as a helper plasmid by the procedures given by D'Haeze *et al.* (2004). All three bacterial strains were incubated at 37 °C in an incubator shaker (125 rpm) for 24 h. Mating was performed by mixing 10^8 cells of each bacterial strain and filtering the suspensions through 0.45 µm filters. The filters were incubated at 30 °C on non-selective agar plates. The cells were then re-suspended in yeast extract broth (YEB) and the resultants were cultured on 3 antibiotic combinations (YEB+Cb)/ (YEB+Cb+Gm)/ (YEB+Cb+Gm+Km) and incubated at 37 °C overnight. The resulting bacteria were observed through an epifluorescent microscope to detect green fluorescence.

Seeds of the rice variety BG 359 were dehusked and surface sterilised according to the procedures given by Rashid *et al.* (1995) and pre-germinated in 0.8 v % agar for 3 ds. Naringenin (1×10^{-4} M) was prepared according to the procedures given by Cancino *et al.* (2001). Three-day-old seedlings were planted in pots (12 seedlings/pot) containing 200 g of sterilised vermiculite and perlite mixture (1:1 volume) with added naringenin. *A. caulinodans* culture (10^8 cells mL^{-1}) was added to pots near the roots. Two inoculum levels (2 and 5 mL) at two frequencies (once or twice a week) resulted in 4 different treatments; (2 mL - once/w), (2mL - twice/w), (5 mL - once/w) and (5 mL - twice/w).

Each treatment had 3 replicates and were arranged in a completely randomised design in the greenhouse of the University of Colombo in a contained area. Plants were allowed to grow for 15 days post inoculation (dpi), 25 dpi, and 35 dpi and uprooted for analysis. Nitrogen free Fahraeus nutrient medium (Fahraeus, 1957) was added (50 mL/twice week/pot) and adequate water levels were maintained by adding sufficient sterilised distilled water to maintain flooded conditions.

Using the best conditions that gave maximum colonisation of *A. caulinodans*, a similar experiment was carried out in the absence of naringenin to assess the effect of naringenin on colonisation.

Sampling of 3 plants from each treatment was carried out at 15 dpi, 25 dpi and 35 dpi. A single root from each plant was selected randomly and was divided into 3 sections. Each section was placed on a microscopic slide and pressed, observed and analysed by micro-imaging (Carl Zeiss primostar trinocular epifluorescent microscope with i-LED) fluorescent detection unit. The

green fluorescing regions were photographed by Axiocam ERc 5s (Carl Zeiss microscopy). The average fluorescing intensities were measured with Carl Zeiss ZEN 2012, ZEN light blue edition software (Perera & Tirimanne, 2014; Jung *et al.,* 2015; Rocha *et al.,* 2015). Statistical analysis was carried out using SAS version 9. The design of this experiment was a two factor factorial (volume and frequency) in completely randomised design (CRD) with repeated measurements over time. The analyses of the data were done accordingly.

RESULTS AND DISCUSSION

Azorhizobium caulinodans and the two *E. coli* strains showed growth in the respective media in the presence of relevant antibiotics to which they are resistant. Figure 1(a) shows that *A. caulinodans*-purchased culture is resistant only to the antibiotic carbenicillin. All three bacterial strains were observed to be Gram negative, non-spore formers and *A. caulinodans* was highly motile as expected. Twenty four hours after tri-parental mating, bacterial cells were observed on non-selective YEB agar plates containing the filters. Successful insertion of the plasmid was initially tested by culturing the resultant recombinant on YEB agar with Gm and Cb antibiotics, which do not support the growth of non-recombinant. Plasmid pRK2013 (Km resistant) in HB101 acts only as a helper in facilitating the tri-parental mating and do not get incorporated into *A. caulinodans*. Hence *A. caulinodans* does not grow when YEB plates are added with Km [Figure 1(b)]. The GFP-labelled live *A. caulinodans* ORS 571 emitted bright green fluorescence under the blue light of an epifluorescent microscope [Figure 1(c)]. Movement of the labelled bacteria was observed in and around the rice roots. The degree of rice root colonisation of the GFP-labelled *A. caulinodans* is reflected by the intensity of the green fluorescence emitted. The emitting fluorescence is captured and measured by ZEN 2012, ZEN light blue edition software, which was linked to an epifluorescent microscope. The bacteria were observed as individual cells and/or clusters inside and on the rice roots.

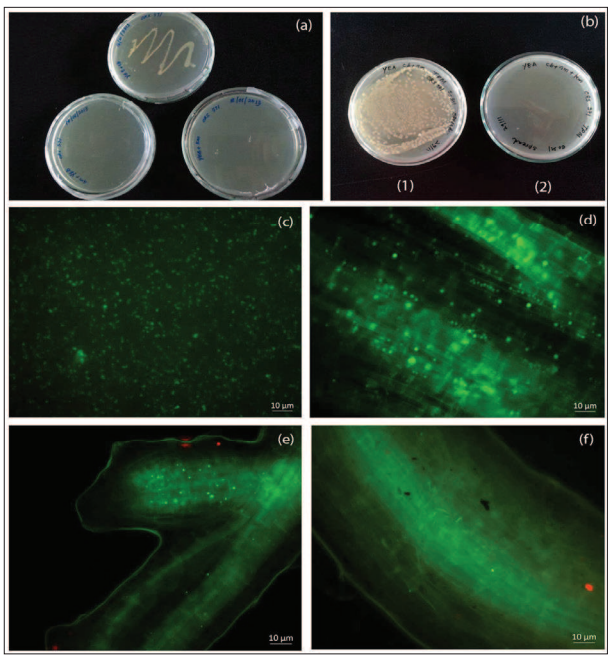

Figure 1: (a) and (b) *Azorhizobium caulinodans* grown on yeast extract agar; (a) purchased culture showing resistance only to antibiotic carbenicillin; (b) *A. caulinodans* after tri-parental mating showing resistance to the antibiotic Cb + Gm (1), but not showing growth when all three antibiotics Cb + Gm + Km are combined (2); (c) epifluorescent micrograph of fluorescing *A. caulinodans*, after introduction of the gfp plasmid by tri-parental mating; (d), (e), (f) epifluorescent micrograph of the rice roots showing colonisation of *A. caulinodans*, 15 days after inoculation (× 40); (d) in the cortical region; (e) in the lateral roots; (f) epifluorescent micrograph of the cortical region of a rice root with the same treatment as in (d), but without the addition of naringenin.

Intense bacterial colonisation was observed clearly in the root cortical region [Figure 1(d)] and in the lateral roots [Figure 1(e)]. Significantly higher level of colonisation was observed in the cortical region in the presence of naringenin [Figure 1(d)] compared to Figure 1(f), where only naringenin was absent. This confirms the enhancement of colonisation of *A. caulinodans* by naringenin in rice roots. Similar results have been observed by Jain and Gupta in 2003, where the use of 10^{-4} M and 10^{-5} M naringenin had significantly increased the colonisation by *A. caulinodans* in the rice root surface, lateral root cracks and root tips. Gopalsawamy *et al.* (2000) has reported the increment of colonisation of the lateral root cracks, cortex, as well as the xylem of the rice root in the presence of naringenin.

In the statistical analysis the significance of the main effect of time; the two way interaction effect of time and volume; the two way interaction effect of time and frequency; and the three way interaction effect of time, volume and frequency were tested with error b (Table 1). The adjusted F indicated that the only significant effects were two way interactions of Time × Volume and Time × Frequency (Table 1). The two way interaction Volume × Frequency was not significant, indicating that the effect of volume is independent from the effect of frequency (Table 1). Therefore, least significant difference (LSD) was used to compare the two levels of volume (2 mL and 5 mL) and the two levels of frequencies (once a week and twice a week) by keeping the three levels of time factor (15 dpi, 25 dpi and 35 dpi) constant (mean comparisons are given in Table 2).

Table 1: Repeated measures analysis of variance (ANOVA) for two factor factorial (volume and frequency) experiment in completely randomised design (CRD) with repeated measurements over time

Source	DF	Type III SS	Mean square	F value	Pr > F	Adj Pr > F	
						G - G	H - F
Volume	1	39924.47	39924.47	17.83	< .0001		
Frequency	1	95858.99	95858.99	42.81	< .0001		
Volume × Frequency	1	777.19	777.19	0.35	0.5565		
Error a	176	394101.84	2239.22				
Time	2	50045.79	25022.89	20.91	< .0001	< .0001	< .0001
Time × Volume	2	7184.07	3592.03	3.00	0.0510	0.0546	0.0531
Time × Frequency	2	58694.62	29347.31	24.52	< .0001	< .0001	< .0001
Time × Volume × Frequency	2	2693.07	1346.54	1.13	0.3231	0.3231	0.3242
Error b	352	421250.96	1196.74				

Greenhouse - Geisser (G - G) Epsilon = 0.9352
Huynh-Feldt (H-F) Epsilon = 0.9610

Table 2: Mean values (± SE) of fluorescence intensities and frequency measures between two levels of volume and two levels of frequency for each of three time periods

Time period	Volume	Fluorescence intensity	Frequency	Fluorescence intensity
15 dpi	2 mL	155 ± 5.59 [b]	Once a week	141 ± 4.52 [b]
	5 mL	182 ± 5.56 [a]	Twice a week	196 ± 5.35 [a]
25 dpi	2 mL	169 ± 4.31 [b]	Once a week	174 ± 4.32 [a]
	5 mL	184 ± 3.60 [a]	Twice a week	180 ± 3.72 [a]
35 dpi	2 mL	149 ± 4.01 [a]	Once a week	144 ± 3.65 [b]
	5 mL	158 ± 3.43 [a]	Twice a week	163 ± 3.63 [a]

Note: Means with the same letter are not significantly different

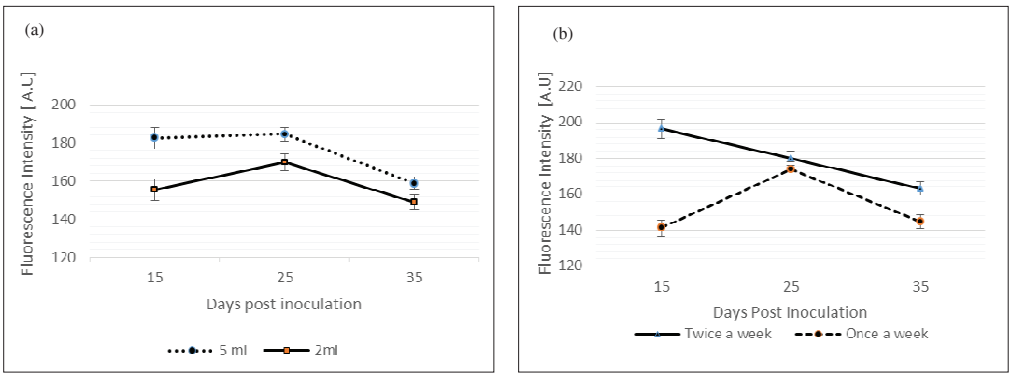

Figure 2: Variation of fluorescence intensity in response to the volume (a) and frequency of application (b) during 15, 25 and 35 days post inoculation

The application of 5 mL of the inoculum has yielded significantly higher intensities of fluorescence than 2 mL application. On the 35th dpi also the 5 mL application has resulted in a higher bacterial colonisation numerically (Figure 2).

When considering the frequency of application of the inoculum, twice a week application has yielded significantly higher values than once a week application on the 15th and the 35th dpi. On the 25th day, although a numerically higher value was obtained for twice a week application, it is not statistically significant (Table 2).

In Figures 2 (a) and (b) the trends of bacterial colonisation over time is plotted. Both graphs clearly show that the highest colonisation is obtained on the 15th day.

High colonisation of rice roots by *Azorhizobium* was observed by Gopalaswamy *et al.* (2000) on the 21st dpi, Jain and Gupta (2003) after 14 dpi and Reddy *et al.* (1997) up to 30 dpi. In the present study we have tested 15th, 25th and 35th dpi to capture the range of time duration. The highest colonisation in our study was observed on the 15th dpi when 5 mL of inoculum volume was applied twice a week.

CONCLUSION

Azorhizobium caulinodans ORS 571 was labelled successfully with green fluorescence protein through tri-parental mating in the presence of pRK 2013 as the helper plasmid. The labelled bacterium was subjected to real time detection of the degree of colonisation under different conditions, and the study showed that colonisation of *A. caulinodans* can be induced by the flavonoid naringenin. Five milliliters of the inoculum, added twice a week and observed for 15 days post inoculation resulted in the highest intensity of fluorescence demonstrating the highest level of bacterial colonisation.

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