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Full Length Research Paper

Colonization ability of *Herbaspirillum* spp. B501gfp1 in sugarcane, a non-host plant in the presence of indigenous diazotrophic endophytes

Joyce Prisca NJOLOMA[1]*, Moriya OOTA[2], Kazuhiko TAROURA[2], Yuichi SAEKI[1] and Shoichiro AKAO[1]

[1] Department of Biochemistry and Applied Bioscience, Faculty of Agriculture, University of Miyazaki, Miyazaki, 889-2192, Japan.
[2]Miyako Branch, Okinawa Prefectural Agricultural Experiment Station, Nishizato 2071-40, Hirara, 906-0012, Japan.

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Inoculating sugarcane with a mixture of diazotrophic endophytic bacteria has shown that they can provide substantial amount of biologically fixed nitrogen to the plant. The genera of diazotrophic endophytes previously isolated from sugarcane have been reported associating with other non-leguminous plants showing a broad host range. This study examined the colonization ability of a wild rice isolate, *Herbaspirillum* spp., in sugarcane plants in the presence of indigenous endophytes using two inoculum concentrations (10^2 and 10^8 bacterial cells ml^{-1}). Internal tissue colonization was observed in plants inoculated with both the 10^2 and 10^8 B501gfp1 bacterial cells ml^{-1} inoculum concentrations. However, extensive colonization and higher bacterial numbers were determined only in the basal stem tissues of plants inoculated with the 10^8 bacterial cells ml^{-1}.

Key words: Sugarcane, wild rice isolate, indigenous endophytes, *Herbaspirillum* spp.

INTRODUCTION

Interaction studies between sugarcane and diazotrophic endophytic bacteria have shown that sugarcane can derive substantial biologically fixed nitrogen from inoculation with a mixture of diazotrophic bacteria (Oliveira et al., 2002; Muthukumarasamy et al., 1999). In these, plant-bacterial interactions, the N_2-contribution by the endophytic bacteria has been found to vary with bacterial strain and plant host. Two of the most widely studied genera among the diazotrophic endophytes are *Herbaspirillum* and *Gluconacetobacter* and both were originally isolated as endophytes of sugarcane (Baldani et al., 1986; Gillis et al., 1989). Recently, these genera have also been found associating with other non-leguminous plants in a broad host range (Chelius and Triplett, 2001; Elbeltagy et al., 2001). These diazotrophic endophytes are expected to provide a range of potential N_2-fixing bacteria to be utilized as inocula for non-leguminous plants. Asis et al. (2000) isolated putative strains of *G. diazotrophicus* and *Herbaspirillum* from sugarcane cultivar (cv) NiF8 cultivated in Miyako islands of Japan and reported that out of the 52 randomly selected isolate colonies, only 21 showed positive acetylene reduction assay (ARA). Thus, the nitrogen supply contribution from the indigenous endophytic N_2-fixing bacteria is inadequate for the crop's demand. Nishiguchi et al. (2005) also reported that the N_2-contribution by the indigenous diazotrophic endophytes existing in sugarcane cvs (Ni15, F172 and NiF8) was not enough to supply the nitrogen which the host plant demands.

One of the key features for achieving significant biological N_2-fixation between endophytes and non-leguminous plants is the possibility of adequate internal colonization by potential N_2-fixing diazotrophic endophytes. Such endophytes have to be inoculated and they successfully colonize the internal tissues. However, most of the inoculation and interaction studies of

*Corresponding Author E-mail: jnjoloma@yahoo.com Tel: +81-985-58-7207. Fax: +81-985-58-7206.

Figure 1. New sugarcane shoots. **A**. Before detaching from the sets, and **B**: detached, ready for transplanting.

endophytes with sugarcane have been conducted using sterile micropropagated seedling plants grown under sterile conditions (James et al., 1994; Reis et al., 1999; Muñoz-Rojas and Caballero-Mellado, 2003; Boddey et al., 2003). Currently, there is still information gap regarding inoculation of sugarcane seedling plants propagated under unsterile conditions and on how indigenous endophytes existing in the plants would affect internal colonization by the introduced bacterial endophytes. In our earlier study (Njoloma et al., 2006) in which we inoculated *Herbaspirillum* spp. B501gfp1 (B501gfp1) onto sterile sugarcane cvs Ni15 and NiF8, it was found that the bacterial strain colonized the internal tissues of both the roots and basal stems of the two cultivars. *Herbaspirillum* spp. strain B501 is an isolate from wild rice, *Oryza officinalis,* known to have in planta N_2-fixation ability (Elbeltagy et al., 2001; You et al., 2005). Based on its basic characteristics, the bacterial strain B501 is classified as belonging to the genus, *Herbaspirillum*. However, it is designated as not identical to the earlier known *Herbaspirillum* species based on its carbon source utilization and diagnostic probe sequence (Elbeltagy et al., 2001). The objective of this study was to examine the internal colonization ability of B501gfp1 as an introduced bacterial endophyte in sugarcane (non-host plant) seedling plants propagated under unsterile conditions.

MATERIALS AND METHODS

Determination of total bacterial population in sugarcane stems

Stalks of sugarcane cv NiF8 obtained from Miyako islands sub-tropical agricultural experiment station, Okinawa, Japan were used. Segments of sugarcane stalks were thoroughly washed and rinsed in distilled water. Sets were cut into small stem sections and surface sterilized with 70% ethanol for 10 min, followed by 2% sodium hypochlorite (NaClO) for 20 min. The sterilized stem pieces were thoroughly washed using sterile distilled water, peeled using a sterile knife and then cut into small pieces about 3-5 cm from which sugarcane juice was extracted.

The bacterial population density was determined using plate count method. Bacterial colonies were counted as colony forming units (cfu) to estimate the total bacterial population in the sugarcane stalks. The extracted juice was serially diluted and from each serial dilution, 100 µl aliquots were plated on solid Luria Bertani (LB) medium containing 10 g L^{-1} Tryptone, 5 g L^{-1} Yeast extract (both from DIFCO Laboratories, Detroit, USA.) 5g L^{-1} NaCl, 100g L^{-1} sugarcane sugar, 15g L^{-1} agar and its pH adjusted to 7.2

Determination of N_2-fixing endophytic bacteria in sugarcane stems

The density of N_2-fixing endophytic bacteria was determined by plating 100 µl aliquots of the serially diluted sugarcane juice onto solid LGIP medium. The LGIP medium contained (quantities per litre); 0.2 g K_2HPO_4, 0.6 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.02 g $CaCl_2.2H_2O$, 0.002 g $NaMoO_4.2H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.02 g yeast extract, 5 ml of 0.5% bromothymol blue (BTB) in 0.2 N KOH, 100 g sugarcane sugar, 15 g agar and pH adjusted to 6.8. Single colonies of the N_2-fixing bacteria isolates were then purified on new agar plates of the same medium and some selected isolates were tested for their ARA.

Shoot growth and culture conditions

Sugarcane sets were prepared and planted in containers filled with unsterilized vermiculite. The growing buds were then detached at least from 3 weeks after germination (Figure 1) and were transferred into modified Leonard jars (assembled using 2 plastic pots, 1 L capacity, 8.5 cm diameter, 14.5 cm length (Takeya Co. Osaka, Japan)). One pot served as a reservoir containing N-free nutrient solution and the other was filled up to 700 ml with unsterilized vermiculite covered with sand stones to prevent excess evaporation. Plants were left to grow for at least 1 month before inoculation with the B501gfp1 endophytic bacteria. All cultures were maintained at a temperature range between 28 - 30˚C under a photoperiod of 16 h light and a photon flux density of 60 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes.

Inoculation of sugarcane seedling plants with B501gfp1

B501gfp1 bacteria were maintained on LGIP and inoculum was prepared using 48 h old B501gfp1 bacterial cells growing on LB medium containing 50 µg ml^{-1} kanamycin at 28˚C. The bacterial cells were harvested from plates with a sterile loop and suspended in sterile distilled water. The bacterial suspension was then centrifuged (3000 g, 10 min, 4˚C). The supernatant was discarded and pellets re-suspended in sterile distilled water. Inoculum density was estimated by direct cell count method using Petroff-Hauser counting chambers and adjusted by dilution to 10^8 and 10^2 bacterial cells ml^{-1} using sterile distilled water. Plants were inoculated with 200ml of 10^8 and 10^2 B501gfp1 cell ml^{-1} suspensions. Sand stones covering the vermiculite were first removed and then bacterial suspensions evenly poured onto vermiculite in the jars. Control plants were inoculated with sterile distilled water. After pouring all the inoculum, the vermiculite was covered again with small stones and plants were left to grow under same conditions. Tissues were examined at 14 and 56 days after inoculation (DAI). Surfaces of intact roots, leaf sheath and leaf blades were examined for the presence of B501gfp1. Internal tissues of the roots, stems and leafy sections (leaf sheath) were examined by slicing the sampled portion into about 0.1 mm transversal sections. Microslicer (D.S.K microslicer, DRK 1000, Dosaka EM Co, Kyoto, Japan) was used. Microscopic fluorescence was examined using a Nikon Eclipse E600 equipped with GFP (R)-BP, HQ (FITC)-BP filter (DM 505, BA

Table 1. Bacterial density determination in mature and young sugarcane tissues of cv. NiF8.

Inoculum Concentration	Time (DAI)	Tissues used	Total endophytes	Total N_2-fixing endophytes	Total InoculatedHB501gfp1
Control	At plant-ing	Stem (sugarcane juice)	1.9×10^6*	2.4×10^4 *	–
		Root	1.63×10^3	7.55×10^2	–
	14	Stem	2.62×10^3	8.95×10^2	–
		Leaf	nd	nd	–
		Root	4.11×10^3	3.33×10^3	–
	56	Stem	3.10×10^3	2.97×10^3	–
		Leaf	1.1×10^2	nd	–
10^2	14	Root	4.11×10^3	1.12×10^3	1.04×10^2 (0.1:1)
		Stem	3.34×10^3	1.70×10^3	5.01×10^2 (0.4:1)
		Leaf	nd	nd	nd
	56	Root	1.78×10^4	1.61×10^4	1.4×10^3 (0.09:1)
		Stem	3.35×10^4	6.70×10^3	2.1×10^3 (0.48:1)
		Leaf	1.20×10^2	nd	nd
10^8	14	Root	4.33×10^4	1.92×10^4	4.0×10^3 (0.27:1)
		Stem	2.81×10^4	1.47×10^4	8.1×10^3 (1.22:1)
		Leaf	1.16×10^2	nd	nd
	56	Root	4.49×10^5	5.62×10^4	4.20×10^4 (2.9:1)
		Stem	4.50×10^6	7.03×10^5	5.30×10^5 (3.1:1)
		Leaf	2.33×10^2	1.49×10^2	1.08×10^2 (2.6:1)

*: values are in cfu/ml of sugarcane juice.
– : tissues were not previously inoculated with HB501gfp1.
nd : not detected.
The value in ( ) is the ratio of the inoculated B501gfp1 to indigenous N_2-fixing bacteria detected in the tissues.
The value for the indigenous N_2-fixing bacteria was obtained by subtracting the inoculated B501gfp1 from the total N_2-fixing endophytes.
Data are means of 3 replications and 3 plants were examined per treatment.

500-560, EX 460-500) and B-2A filter (DM 505 and EX 450-490). The images were captured using Pixera, a digital camera system for microscopy (Pixera Corporation, Los Gatos, USA) fitted on to the Nikon Eclipse.

Plant samples for bacterial density determination from both inoculated and uninoculated young sugarcane plants were thoroughly washed with distilled water, cut into small pieces from which 0.5 g sample was obtained and sterilized using 2% NaClO for 15 min. The samples were then washed with distilled water and grounded by hand using mortar and pestle. From each serially diluted homogenate, a 100 µl aliquot was plated on LB medium for total bacterial counts and on LGIP medium for N_2-fixing bacterial counts. Bacteria colonies on LGIP medium were examined under Nikon Eclipse E600 and those that emitted green fluorescence were counted as the inoculated B501gfp1 bacterial endophytes.

RESULTS

Bacterial population density in sugarcane tissues

The population densities of the diazotrophic endophytes in both the sugarcane stems prior to planting and in the young seedling plants after inoculation with B501gfp1 are presented in Table 1. In the sugarcane stems prior to planting, a total of 10^4 cfu ml^{-1} sugarcane juice was determined for the total indigenous N_2-fixing endophytes. In the young seedling, population density of the total indigenous N_2-fixing diazotrophic endophytes ranged from 10^2 to 10^3 cfu g FW^{-1} for the uninoculated plants and, from 10^3 to 10^4 and 10^4 to 10^5 cfu g FW^{-1} for the 10^2 and 10^8 B501gfp1 inoculated plants, respectively. Population density of the inoculated B501gfp1 diazotrophic endophyte was higher in the stem tissues of sugarcane plants inoculated with the 10^8 inoculum concentrations each of the sampling times. The inoculated B501gfp1 bacteria in the leafy tissues were only detected in the 10^8 B501gfp1 inoculated plants at 56 DAI. The estimated ratios of the inoculated B501gfp1 to the indigenous N_2-fixing bacteria shows that the inoculated bacteria were more than the indigenous N_2-fixing bacteria in plants inoculated with the 10^8 B501gfp1 bacterial cells ml^{-1} at 56 DAI. The results show that a higher inoculum concentration would ensure more of the inoculated bacteria infecting the tissues in the presence of the indigenous diazotrophic endophytes.

Table 2 shows the amount of ethylene each of the selected diazotrophic endophytes could produce per hour. Twenty eight isolates from the indigenous N_2-fixing diazotrophic endophytes obtained from stems of mature

Table 2. acetylene reduction assay (ARA) of sugarcane cv. NiF8 isolates and B501gfp1.

Isolates	ARA (nmol C_2H_4 tube^{-1} r^{-1})
NiF8 1	0.028
NiF8 2	0.010
NiF8 3	0.032
NiF8 4	0.032
NiF8 5	0.002
NiF8 6	0.001
NiF8 7	0.017
NiF8 8	0.005
NiF8 9	0.033
NiF8 10	0.006
NiF8 11	0.002
NiF8 12	0.002
NiF8 13	0.002
NiF8 14	0.0016
NiF8 15	0.0012
NiF8 16	0.0015
NiF8 17	0.0016
NiF8 18	0.0015
NiF8 19	0.026
NiF8 20	0.028
NiF8 21	1.178
NiF8 22	0.126
NiF8 23	0.014
NiF8 24	0.0036
NiF8 25	0.0018
NiF8 26	0.076
NiF8 27	0.0013
NiF8 28	0.0023
HB501gfp1	5.336

A 40 ml test tube containing 10 ml of semi solid (2 g L^{-1} agar) LGIP medium was inoculated with 100 µl of bacterial suspension prepared from colonies growing on the solid LGIP medium and then incubated for 7 days at 28˚C. Then 10% (v/v) of air in the headspace was replaced with acetylene gas and test tubes were further incubated for 48 h. ARA was determined using a Shimadzu GC7A gas chromatograph equipped with a flame ionization detector and a Porapack R column, Shimadzu, Kyoto, Japan).

sugarcane plants were randomly selected and tested for their ability to reduce acetylene to ethylene. Relatively higher positive ARA values were obtained in 2 (NiF8 21 and NiF8 22) of the 28 isolates, with NiF8 21 producing 1.178 nmol hr^{-1}. However, in comparison with B501gfp1 bacterial strain, all the selected bacterial endophytes showed low levels of ARA.

Colonization of the internal tissues by B501gfp1

Bacterial colonization in the young plants was also assessed at two intervals, 14 and 56 DAI. We observed internal tissues colonization by the inoculated bacteria in plants inoculated with both the 10^2 and 10^8 B501gfp1 bacterial cells ml^{-1} inoculum concentrations. In the plants inoculated with 10^2 B501gfp1 bacterial cells ml^{-1}, we observed fewer and less dense xylem vessel colonization in the stem tissues (Figure 2A). Despite the increase in the bacterial numbers to 10^3 cfu g FW^{-1} at 56 DAI (Table 1), the colonized sites remained fewer in number (Figures 2C and D) amongst the several scattered vascular bundle tissues. In addition to the vascular bundle colonization, we also observed intercellular colonization in the parenchyma tissues (Figure 2B). However, its green fluorescence expression was not very bright compared to colonies in the xylem vessels, an indication of low bacterial concentration on the site of colonization.

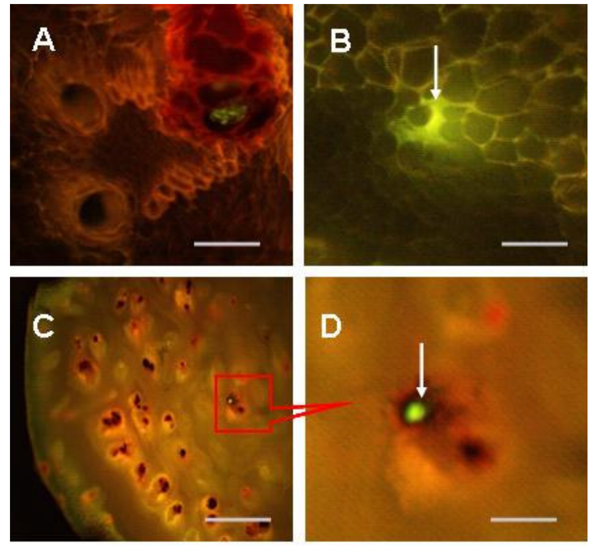

Figure 2. Internal colonization in the sugarcane stem tissues of plants inoculated with 10^2 cell ml^{-1}. Observed colonization in the xylem vessels **A**: 14 DAI. **C** and **D**: 56 DAI. **B**: Intercellular space colonization in the stems tissues at 56 DAI. White arrows show the colonized area. Scale bar: 40 µm (**A**). 10 µm (**C**), 20 µm (**B**, **D**).

Similarily, in the 10^8 B501gfp1 bacterial cells ml^{-1} inoculated plants, very few vascular bundles were densely colonized (Figure 3G) at 14 DAI with at least a minimum of 3 vascular bundles among the several bundles observed in a stem transversal section. As the bacterial concentration progressively increased in the tissues, the numbers of colonized vascular bundle tissues also increased at 56 DAI with extensive colonization. And in most of the observed stem sections, B501gfp1 could be detected in the vascular bundles and their intercellular spaces (Figure 3A). In many successive stem transversal sections, we observed vessels filled with B501gfp1 bacteria (Figure 3C) and colonization was also observed in the parenchymal cells of the xylem vessels and in their intercellular spaces (Figure 3B, C, D,). In the root tissues, dense colonization was observed in the cells at the lateral root junctions (Figure 3E, F), while in the leafy tissues,

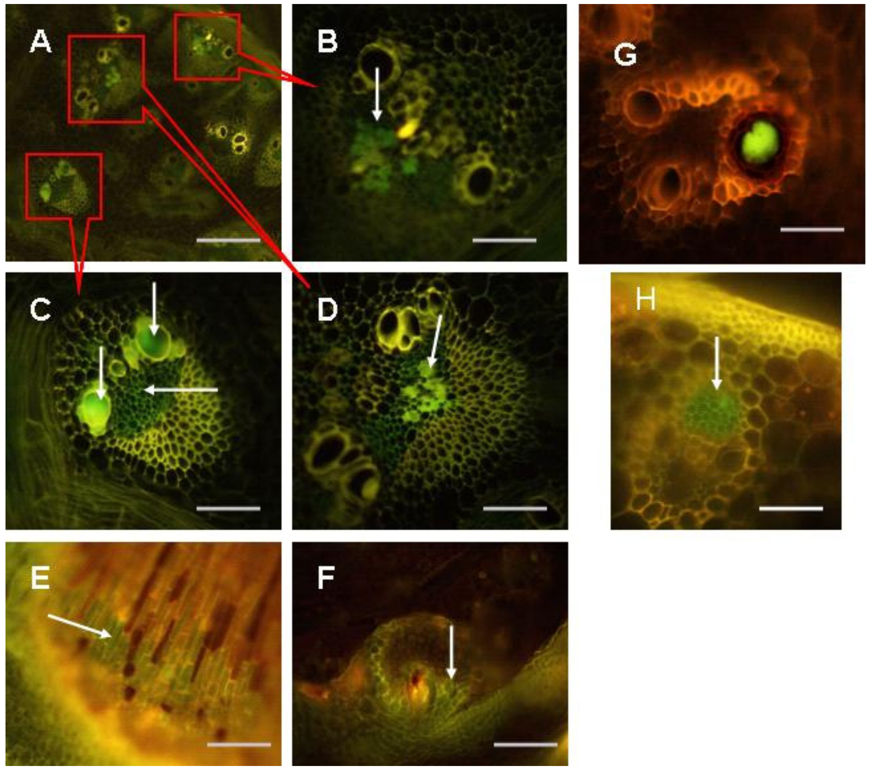

Figure 3. Internal colonization in the sugarcane root, stem and leaf sheath tissues of plants inoculated with 10^8 B501gfp1 bacterial cells ml^{-1}. Vascular bundle tissue colonization in **G**: 14 DAI and in (**A - D**): 56 DAI. Colonization in the **C**: metaxylem tissues; **B** and **D**: proto xylem tissues; **E** and **F**: lateral root junctions; and **H**: leaf sheath. Scale bar: 10 µm (**A**). 20 µm (**B, C, D, E, F**). 40 µm (**G, H**).

B501gfp1 bacteria were observed only in the outer sheath in few vascular bundle tissues (Figure 3H). Moreover, bacterial density in the leafy tissues was detected in very low numbers (Table 1).

DISCUSSION

In this study, we detected high B501gfp1 bacterial numbers and also observed extensive colonization in the stem tissues. The higher numbers of B501gfp1 in the stem tissues may have been due to the availability of the organic carbon sources which supports bacteria growth (Dong et al., 1994; James et al., 1994). We also previously demonstrated that B501gfp1 bacterial cells could multiply in the stem tissues especially in the parenchymatous cells which are considered to be food storage organs in plants (Njoloma et al., 2005). Bacterial colonization of the vascular system and the intercellular spaces in the apoplast has been widely reported in association with most gramineae plants (Dong et al., 1994; Sprent and James 1995; Lamb et al., 1996, James 2000; McCully, 2001). Bressan and Borges (2004) reported that the highest internal colonization in the maize root and stem tissues was obtained by pruned-root and dip method. This method might ensure existence of entry points such that infection and its subsequent internal colonization became easier. In this study, even though the roots were not pruned, the process of detaching new shoots from the sets created an opening at the base of the stem. As a result, this may have provided for entry of the introduced endophytic bacterial cells and consequently more bacteria were detected in the stem tissues than the root tissues. However, application of these methods for practical use may be difficult since the large openings could also allow entry by pathogenic bacteria microbes into the host plant. On the other hand, in our previous study (Njoloma et al., 2006) in which sterile sugarcane seedling plants cv NiF8 were also inoculated with the 2 inocula levels (10^2 and 10^8 B501gfp1 cells ml^{-1}), we observed that bacterial numbers were higher in the root than the stem tissues. In addition, even with the lower inoculum concentration, high numbers were detected in the root tissues at 56 DAI. Unlike in the current study, we could only detect such numbers (10^5 cfu g FW^{-1}) in plants inoculated with the higher inoculum concentration. It can therefore be suggested that the inoculation technique used has some significant impact on the bacterial numbers, their localization and the subsequent internal tissue colonization. And in addition, in the presence of the indigenous endophytic bacteria, the inoculated bacteria may have encountered some competition over growth resources resulting in their slow multiplication. On the other hand, with a high initial inoculum concentration, more of the B501gfp1 bacteria could be detected in the tissues compared to indigenous N_2-fixing bacteria. It is therefore suffice to indicate that in order to achieve adequate internal colonization by an introduced bacterial strain in the presence of the indigenous bacterial population, a higher initial inoculum concentration must be used.

In the colonization of the internal tissues, we observed that few sites were infected by the B501gfp1 in both the 10^2 and 10^8 B501gfp1 cells ml^{-1} inoculated plants at 14 DAI. In the leafy tissues colonization was observed only in the outer leaf sheath in some of the 10^8 B501gfp1 cells ml^{-1} inoculated plants. We earlier reported the effect of sugarcane tissues' autofluorescence (red fluorescence by chloroplasts) on the expression of B501gfp1 spot inoculated on to the tissues (Njoloma et al., 2005). We found that when concentration of B501gfp1 bacterial cells in the tissues decreased, its gfp expression could not be observed. Thus, in sugarcane tissues the chloroplasts provides a counter fluorescence which masks the expression of the bacteria's green fluorescence. In gfp labeled bacteria, the green fluorescence gives an indication for the presence of the gfp-labelled bacteria (Njoloma et al. (2005). This could be an explanation for the few observed infection sites; there could have been more of the infected sites with very low bacteria numbers to be detected in a strong tissue autofluorescence background.

In this study, we have presented the potential of *Herbaspirillum* spp. B501gfp1 to extensively colonize the sugarcane plant tissues in the presence of naturally inhabiting endophytes under unsterile growth condition. However, the biological N_2-fixing ability of B501gfp1 in sugarcane plants will have to be rigorously evaluated using methods which would quantify the biologically fixed N by B501gfp1 besides its colonization ability in the sugarcane plant.

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