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Full Length Research Paper

Investigation of endophytic and symbiotic features of *Ralstonia* sp. TSC1 isolated from cowpea nodules

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***Ralstonia* sp. TSC1 previously isolated from cowpea nodules was tagged with gfp gene by transposon insertion. The resultant *gfp*-tagged *Ralstonia* sp. TSC1 showed no difference in physiological properties such as growth rate, exopolysaccharide formation and colony morphology. When it was co- inoculated with compatible bradyrhizobial strains, the observation of fluorescence microscopy showed that TSC1 strains were colonized in subsurface of cowpea nodules and stems. In addition, TSC1 positively and negatively modulated symbiotic performance with the bradyrhizobia in terms of nodulation and symbiotic nitrogen fixation. The results suggested that *Ralstonia* sp. TSC1 is an endophyte with beneficial, neutral or detrimental effects on cowpea plants when in presence of effective bradyrhizobia.**

Key words: *Ralstonia* sp., cowpea, endophyte, green fluorescent protein, *bradyrhizobium*, nodulation.

INTRODUCTION

To accommodate species previously known as *Alcaligenes eutrophus*, *Pseudomonas solanacearum* and *Pseudomonas pickettii*, Yabuuchi et al. (1995) established the genus *Ralstonia*. Some *Ralstonia* species are environmental micro-organisms (*Ralstonia eutropha* and *Ralstonia basilensis*) living in sludge, soil and waste-water and others such as *Ralstonia solanacearum* are phytopathogenic, causing bacterial wilt on a wide range of crops (Palleroni and Doudoroff, 1971). Many species have been isolated from various clinical sources and environmental samples (*Ralstonia paucula*, *Ralstonia pickettii*, *Ralstonia mannitolytica* and *Ralstonia gilardii*). *Ralstonia taiwanensis* isolated from root nodules of *Mimosa* is the first beta-proteobacterium strain to be capable of root nodule formation and nitrogen fixation (Chen et al., 2003). In previous studies (Sarr et al., 2009), *Ralstonia* sp. TSC1 was isolated from cowpea nodules. This strain diverged by at least 5% 16S-23S ITS rRNA gene nucleotide sequence from its closest relative *R. pickettii*.

Endophytic bacteria have been defined as bacteria detected inside surface-sterilized plants or extracted from inside plants and having no visibly harmful effects on the plants (Hallmann et al., 1997). This definition includes bacteria that reside internally in plant tissues with apparently neutral behavior as well as symbionts. Numerous reports described endophytic bacteria in various plant tissues, including seeds and ovules, tubers, roots, nodules, stems and leaves, fruits (Hallmann et al., 1997; Sturz et al., 1997). Firstly described as a cowpea nodulating bacterium, repetitive re-inoculations did not prove any nodulation capability of *Ralstonia* sp. TSC1 on cowpea. Therefore, this study was mainly carried out to

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investigate the features of this strain, as if it could appear as a cowpea endophytic bacterium. Kuklinsky- Sobral et al. (2005) detected *R. pickettii* among a set of soybean bacterial endophytes. However, to date no report has been found describing cowpea endophytic bacteria. Recently, green fluorescent protein (GFP) has become a valuable tool for research regarding plant-microbe interactions in living systems. It has been used to elucidate patterns of colonization and the spread of various bacteria (Chelius and Triplett, 2000; Xi et al., 1999). The study of endophytic bacteria is important, not only for understanding their ecological role in their interaction with plants but also for their possible biotechnological applications, such as bioremediation. Some endophytes have been discovered to have beneficial effects on the host plant, such as plant growth promotion, induction of increased resistance to pathogens (Furuya et al., 1991; Trigalet et al., 1994) and environmental stress, as well as the supply of fixed nitrogen to the host plant (Mano and Morisaki, 2008). Considering these properties, the effect of co-inoculation of *Ralstonia* sp. TSC1 with compatible bradyrhizobial strains on cowpea was also investigated.

MATERIALS AND METHODS

Ralstonia sp. TSC1 was tagged with a GFP-expressing plasmid (pTn*5* Km *gfp*). For the tagging, *Escherichia coli* S17-1 harboring this plasmid (Tanaka et al., 2006) was grown at 37°C in 100 ml of Luria-Bertani (LB) liquid medium supplemented with 50 ppm kanamycin for 12 h at 100 rpm, and *Ralstonia* sp. TSC1 was incubated at 30°C in 100 ml of A1E HM liquid medium (Kuykendall, 1979) for 48 h at 100 rpm. One milliliter of *E. coli* S17-1 and 5 ml of *Ralstonia* sp. TSC1 cultures were centrifuged at 6,000 rpm for 10 min at 25°C. Pellets were re-suspended in 1 ml (*E. coli* S17-1) or 3 ml (*Ralstonia* sp. TSC1) of A1E HM solution and centrifuged as above. This operation was repeated 3 times. Each strain was finally suspended in 1 mL of A1E HM solution. Mixed cell solution (50 µl *E. coli* S17- 1 plus 100 µl *Ralstonia* sp. TSC1) was applied on a sterile cellulose acetate filter (0.45 µ m, 25 mm, ADVENTEC, Tokyo, Japan) mounted on an A1E HM agar plate and incubated at 30°C for 48 h. Thereafter, the filter was suspended in 1 ml of A1E HM liquid medium in a 12 ml Falcon tube. Aliquots of the suspended solution were spread on A1E HM (50 ppm Km) agar plates incubated at 30°C to allow *Ralstonia* sp. TSC1 *gfp* colonies development. GFP fluorescence of *Ralstonia* sp. TSC1 *gfp* was confirmed under an Olympus BH2 fluorescence microscope (Olympus Corp., Tokyo) with GFP filter. *Ralstonia* sp. TSC1 *gfp* colonies were easily identified from that of *E. coli* S17-1 based on their resemblance with the wild type *Ralstonia* sp. TSC1 except the GFP pigmentation (Sarr et al., 2009). Moreover, the incubating temperature (30°C) does not help getting a good growth of *E. coli* S17-1 which grows best at 37°C.

The effect of co-inoculation of *Ralstonia* sp. TSC1 *gfp* (R) with strains of *bradyrhizobium* on the symbiotic activity of cowpea (*Vigna unguiculata* L. Walp.) cv. Melakh (Cisse et al., 1997) was assessed. Seeds of cowpea were kindly supplied by Senegal Agricultural Research Institute (Dakar, Senegal). Treatments consisted of a control (uninoculated), single-inoculations with R, *Bradyrhizobium yuanmingense* TSC10 (TSC10), *Bradyrhizobium japonicum* DTB4 (DTB4), *Bradyrhizobium elkanii* DTC9 (DTC9) (Sarr et al., 2009), *B. yuanmingense* CCBAU 10071^T (CCBAU^T) obtained from BCCM/LMG Bacteria Collection (Gent, Belgium), and co-inoculations with TSC10 + R, CCBAU^T + R, DTB4 + R, DTC9 + R. They were replicated three times; with each replicate (pot) containing 6 plants. The inoculation process was as described previously (Sarr et al., 2009) with some modifications. *Ralstonia* sp. TSC1 *gfp* and the bradyrhizobial strains used in this experiment were incubated in A1E HM liquid medium until the exponential phase and optical densities (OD_{600nm}) of cultures were measured to estimate the number of cells used in inoculations. Primarily, a curve line relationship between cells number and OD was drawn after a serial dilution of cultures at exponential phase and spreading of aliquots of known volume on A1E HM agar plates for colony count. According to their OD, cultures used for inoculation were diluted to correspond to about 1 x 10^7 cells ml^{-1}. Therefore, co-inoculation treatments contained 2 x 10^7 cells ml^{-1} (1 x 10^7 cells ml^{-1} of *Ralstonia* sp. TSC1 *gfp* cells + 1 x 10^7 cells $ml^{-1}$of *Bradyrhizobium* cells). After sowing of seeds, pots were placed into a phytotron at 25°C, 70% RH, under natural light condition and watered every 7 days with sterilized de-ionized water. ARA, nodulation phenotype (number, dry weight and mass of nodules), plant N accumulation and plant dry weight (shoot and root) were determined after 28 days (from May 13 to June 11, 2009) cultivation from 3 plant samples per replicate. Plant N accumulation was determined by the indophenol method (Ohyama et al., 1991). For ARA measurement, plant roots (per replicate) with intact nodules were severed at the cotyledonary node and placed in 100 ml conical flasks. Flask was sealed with a serum stopper and 10% air gas was replaced with acetylene (C_2H_2) gas. The nodulated roots were incubated at room temperature and 1 ml subsamples were analyzed for ethylene (C_2H_4) production at 5 and 65 min after incubation using a flame ionization gas chromatograph (Shimadzu GC-14A Kyoto, Japan). Column and injector temperatures were 35, 45°C, respectively. Carrier gas was N_2 (flow rate: 30 ml min^{-1}). ARA was calculated as µmol of ethylene produced per hour per plant. All data were subjected to ANOVA using IRRISTAT 4.0 (International Rice Research Institute, Manila, Philippines). When appropriate, Duncan's multiple range test (DMRT) at $P < 0.05$ was used to compare treatments means. Surface-sterilized cross-sections (25 - 50 µm thick) of roots, stems and nodules from the remaining 3 plants of each pot were observed under an Olympus BH2 fluorescence microscope (Olympus Corp., Tokyo) with GFP filter for observation of GFP fluorescence emitted from *Ralstonia* sp. TSC1 *gfp* cells.

RESULTS AND DISCUSSION

Compared to the wild-type, cells of *Ralstonia* sp. TSC1 *gfp* showed no difference in physiological properties including the growth rate, formation of extracellular polysaccharides (EPS), colony size, pigmentation other than GFP, and colony fluidity (data not shown). For co-inoculations, photographs of two treatments (DTB4 + R, DTC9 + R) were shown to emphasize the fluorescence of *gfp*-tagged TSC1 in cowpea tissues; the other co-inoculation treatments showed similar results (data not shown) . Compared to the autofluorescence of the control (Figure 1B) and the *Bradyrhizobium* single-inoculated treatments (Figures 1D and F), GFP fluorescence (although faint) was observed in root sections of *Ralstonia* sp. TSC1 *gfp* single-inoculated (Figure 1A) and co-inoculated (Figures 1C and E) plants. This result indicated the capability for *Ralstonia* sp. TSC1 to infect cowpea root tissues. A common invasion process used

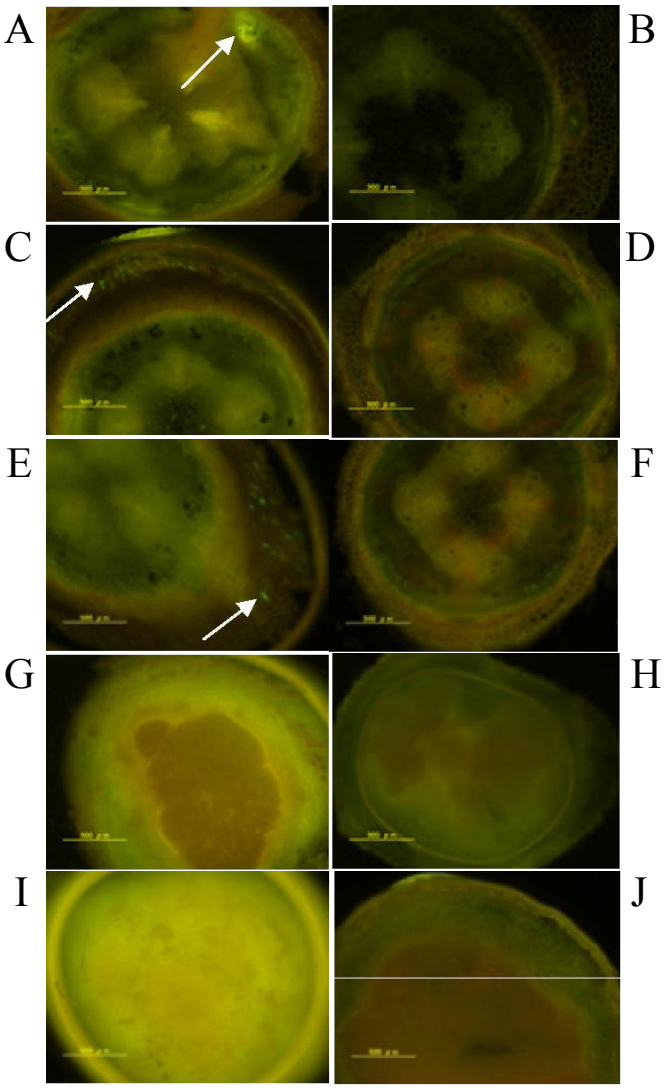

Figure 1. Observation of *Ralstonia sp*. TSC1 *gfp* (R) cells in plant roots and nodules at 4 weeks post-inoculation. Cells accumulation is shown by white arrows. (A) root of R inoculated plant, (C) root of *B. japonicum* DTB4 (DTB4) + R co-inoculated plant, (E) root of *B. elkanii* DTC9 (DTC9) + R co-inoculated plant, (G) nodule of DTB4 + R co-inoculated plant, (I) nodule of DTC9 + R co-inoculated plant. Autofluorescence observed in non-infected plant tissues are shown in right panels for comparison: (B) control (Uninoculated) plant root section, (D and F) DTB4 and DTC9 single-inoculated plant root sections, respectively, (H and J) DTB4 and DTC9 single-inoculated plant nodule sections, respectively. Root portion surrounded by the inoculated rhizosphere zone was chosen for sections. Bars represent 500 μm. Exposition time (1/4.0 s), size enlargement with Olympus BH2 fluorescence microscope = x 4.

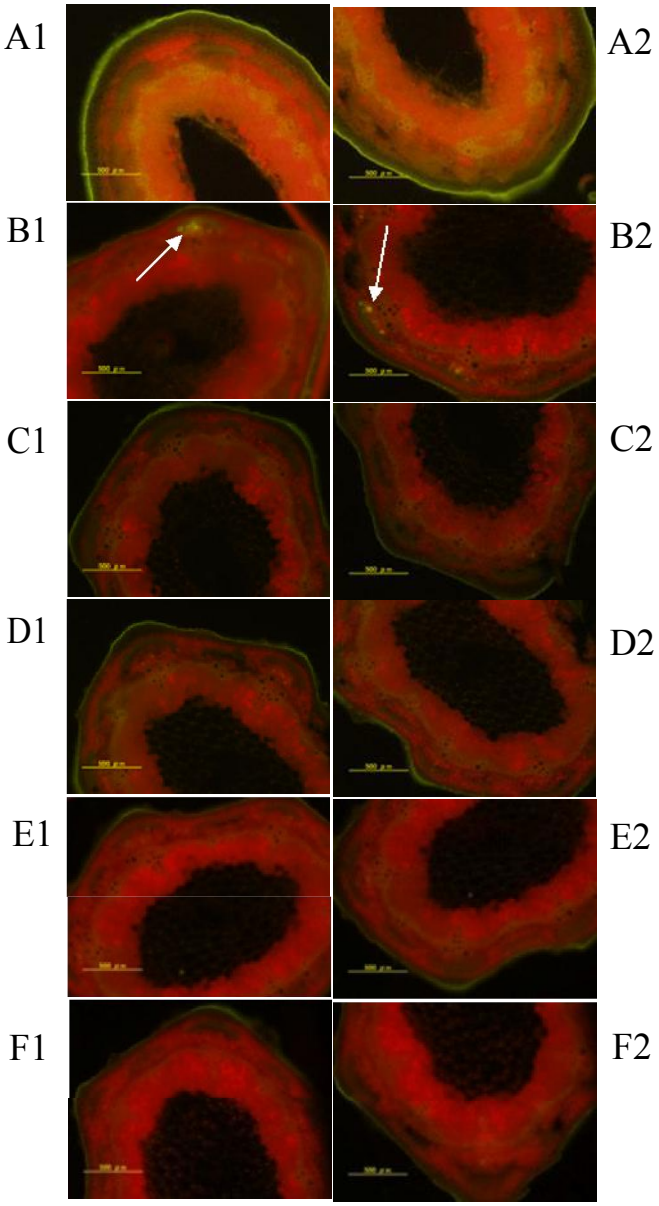

Figure 2. Observation of *Ralstonia sp*. TSC1 *gfp* (R) cells in plant stems at 4 weeks post-inoculation. Two photographs were taken for the same cross-section due to the big section size of the stems. Photographs in left panels represent the upper part and those in right panels, the lower part of the same cut. (A1-A2) control plant (uninoculated), (B1-B2) R inoculated plant, (C1-C2) DTB4 inoculated plant, (D1-D2) DTB4 + R co-inoculated plant, (E1-E2) DTC9 inoculated plant, (F1-F2) DTC9 + R co-inoculated plant. White arrows show GFP-tagged cells of *Ralstonia sp.* TSC1 *gfp*. Bars represent 500 μm. Exposition time = 1/4.0 s, size enlargement with Olympus BH2 fluorescence microscope = x 4.

by endophytic bacteria is through the wounds which occur naturally at the points of lateral root emergence and at the root tips as a result of plant growth (Huang, 1986). Fluorescence intensity emitted from nodule section of co-inoculated plants (Figures 1G and I) was stronger than that from nodules of *Bradyrhizobium* single-inoculated plants (Figures 1H and J). In Figures 1G and I, the fluorescence was detected in parenchymatic nodule tissue rather than in bacteroid filled nodule cells.

Through this result, the ability of *Ralstonia* sp. TSC1 to invade cowpea nodule tissues was also demonstrated.

Table 1. Effect of co-inoculation of *Ralstonia* sp. TSC1 *gfp* with four bradyrhizobial symbiotic isolates on nodulation and nitrogen fixation.

Treatments	Nodulation			Nitrogen fixation		Total dry weight
	Nodule number	Nodule dry weight		N accumulation	ARA	
		mg plant^{-1}	mg nodule^{-1}	mg plant^{-1}	µmol plant^{-1} h^{-1}	mg plant^{-1}
Uninoculated	0 e	0.0 f	0.00 e	8.1 de	0.00 d	528 cd
TSC1 *gfp* (R)	0 e	0.0 f	0.00 e	6.9 e	0.00 d	532 cd
TSC10	48 ab	26.8 c	0.57 cd	21.5 a	0.37 d	723 a
TSC10 + R	45 bc	32.9 b	0.85 b	23.3 a	1.43 ab	707 ab
CCBAU^{I}	48 ab	19.1 de	0.40 d	9.5 cd	0.20 d	524 cd
CCBAU^{T} + R	27 d	13.5 e	0.51 cd	4.6 f	0.46 d	471 de
DTB4	32 cd	25.5 c	0.80 b	11.3 c	0.74 c	528 cd
DTB4 + R	24 d	25.1 cd	1.07 a	11.5 c	1.47 ab	607 bc
DTC9	62 a	43.5 a	0.72 bc	22.6 a	1.89 a	656 ab
DTC9 + R	30 d	23.1 cd	0.83 b	16.0 b	1.16 bc	395 e
SE	6.8	2.9	0.1	1	0.21	53
$LSD_{0.05}$	20	8.5	0.32	2.9	0.62	157

Means (from 3 replications) with the same letter in a column are not significantly different at $P < 0.05$ significance level according to the Duncan's multiple range test (DMRT). ANOVA was carried out using IRRISTAT 4.0 (International Rice Institute, Manila, Philippines). R = *Ralstonia sp.* TSC1 *gfp*, TSC10 = *B. yuanmingense* TSC10, CCBAU^{T} = *B. yuanmingense* CCBAU 10071^{T}, DTB4 = *B. japonicum* DTB4, DTC9 = *B. elkanii* DTC9, S.E = Standard Error and LSD = Less Significant Difference refer to values reported in the tow last lines, respectively.

Cross-sections also indicated stem colonization of *Ralstonia* sp. TSC1 *gfp* when it was inoculated alone (Figure 2: B1 and B2) as compared to the autofluorescence in stem sections of the control (Figure 2: A1 and A2) and *Bradyrhizobium* single-inoculated (Figure 2: C1-C2 and E1-E2) plants. Unexpectedly, it was difficult to observe the GFP fluorescence in stem cross-sections of co-inoculated plants (Figure 2: D1-D2 and F1-F2). Mano and Morisaki (2008) stated that because of the breaks in the endodermis, the bacteria colonizing the cortex traverse the endodermis into the vascular tissue and can infect plant stems through the vascular system. Therefore, the presence of GFP fluorescence in root and nodule sections of co-inoculated plants and its absence in stems of co-inoculated plants could be a sampling-related problem or a competition between *Ralstonia* sp. TSC1 *gfp* and the associated bradyrhizobia, repressing *Ralstonia* in the lower parts of plants. Such competition is often observed in soil-born microbes (Denison and Kiers, 2004). The overall observation on root, nodule and stem infection clearly indicated the endophytic properties of *Ralstonia* sp. TSC1 on cowpea. Hallmann et al. (1997) indicated that the most common taxa among the endophytic bacteria isolated from tissues of various plants are the former Pseudomonas group (*Pseudomonas*, *Burkholderia*, *Phyllobacterium*) and *Enterobacteriaceae* (*Enterobacter*, *Erwinia*, *Klebsiella*). The present result supports that of Kuklinsky-Sobral et al. (2005) who also found *R. pickettii* among a set of endophytic bacterial species obtained from soybean, extending the endophytic bacterial community to *Ralstonia* genus. However, firstly describes the endophytic properties of *Ralstonia* sp. TSC1 on cowpea.

As for the symbiotic activity, no nodule formation was recorded in plants inoculated with *Ralstonia* sp. TSC1 *gfp* alone, which logically led to no ethylene production during ARA measurement (Table 1). Differences were observed between single inoculation of compatible bradyrhizobial isolates and co-inoculation with *Ralstonia* sp. TSC1. With TSC10 and DTB4, *Ralstonia* sp. TSC1 significantly enhanced nodule dry weight. With DTC9, *Ralstonia* sp. TSC1 severely reduced nodulation and nitrogen fixation. This detrimental effect indicates that *Ralstonia* sp. TSC1 may be antagonistic of strain DTC9. Recent investigations show that certain types of bacteria thrive best when associated with each other. Again there are numerous instances in which bacteria exert antagonistic action toward each other (Pengnoo et al., 2006). This does not provide the opportunity to apply *Ralstonia* sp. TSC1 and *B. elkanii* DTC9 simultaneously to cowpea for increasing crop production. Although, total nitrogen did not significantly increase (Table 1), ARA was significantly enhanced when *Ralstonia* sp. TSC1 was combined with TSC10 and DTB4. Similarly, Peterson et al. (1996) and Srinivasan et al. (1997) reported that co-inoculation with *Bacillus* spp. and *Bradyrhizobium* sp. was more effective in nitrogen fixation and nodule formation that the *Bradyrhizobium* sp. alone. With CCBAU^{T}, the presence of *Ralstonia* sp. TSC1 reduced N accumulation and nodule number. The results suggested *Ralstonia* sp. TSC1 modulates symbiotic performance of bradyrhizobia with cowpea plants. Some *Ralstonia* sp.

are known as a plant pathogens (Palleroni and Doudoroff, 1971). Bacterial pathogenesis leads to decreased plant yield. However, dry weight and N accumulation of *Ralstonia* sp. TSC1 *gfp* single-inoculated plants were not significantly different from that of the control. Furthermore, signs of wilting were not observed on *Ralstonia* sp. TSC1 inoculated plants (data not shown), indicating that this strain is not pathogenic on cowpea.

This work has clarified that *Ralstonia* sp. TSC1 was a cowpea endophyte without nodulation capability when inoculated alone. Its positive nodulation previously reported (Sarr et al., 2009) was probably due to a contamination from strains of *Bradyrhizobium* in the course of the inoculation or during the first steps of bacterial isolation from cowpea nodules. The present study has demonstrated the beneficial, detrimental or neutral effects of *Ralstonia* sp. TSC1 on the different *Bradyrhizobium* strains. This finding is in accordance with Sturz et al. (2000) who stated that endophytic bacteria embracing a wide variety of species and genera from nonpathogenic relationships with their hosts: some beneficial, some neutral, and some detrimental. Beyond the understanding of the endophytic features of *Ralstonia* sp. TSC1 and its ecology, this work has important applications on crop production. At specific combinations with some bradyrhizobia, the use of this endophyte can contribute to increase the BNF on cowpea, reducing the use of nitrogen fertilizers.

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