STUDIES TOWARD THE DEVELOPMENT OF MORE EFFECTIVE NITROGEN FIXING ASSOCIATION BETWEEN NON-LEGUMINOUS PLANTS AND ENDOPHYTIC BACTERIA

非マメ科植物と内生窒素固定細菌との間に成立する共生窒素固定系の強化に関する研究

A dissertation submitted to

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In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Agriculture

MUHAMMAD ZAKRIA

2008

DECLARATION

I, Muhammad Zakria, do hereby declare that this dissertation is my own piece of work except where reference is made and that it is not being concurrently submitted in candidature to this or any other University for the award of a degree.

……

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2008

APPROVAL OF DISSERTATION

This dissertation titled "**STUDIES TOWARD THE DEVELOPMENT OF MORE EFFECTIVE NITROGEN FIXING ASSOCIATION BETWEEN NON-LEGUMINOUS PLANTS AND ENDOPHYTIC BACTERIA"** submitted to The United Graduate School of Agricultural Sciences, Kagoshima University, Japan by Muhammad Zakria in partial fulfillment of the requirements for the degree of Doctor of Philosophy is hereby approved on the recommendation of

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DEDICATED

To

MY PARENTS

Who inspired me towards higher ideals of life

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Abstract

Nitrogen is one of the major limiting nutrients for crop production and its availability to the plant is critical for high yields. In nature, legumes benefit directly from biologically fixed nitrogen, provided that they are in association or symbiotic association with root-nodulating bacteria, such as Rhizobia. However, most of non-leguminous plants belonging to the Gramineae family, do not have this natural association. Among non-leguminous plants, several diazotrophic endophytes have been isolated and characterized as nitrogen fixing endophytes. Although the diazotrophic endophytes have been reported in various crops, the nitrogen supply by the indigenous endophytes is usually insufficient for the crops demand. To achieve maximum benefit from biological nitrogen fixation (BNF) by the endophytic bacteria the proper combination of host-endophyte is required. Thus, there is a possibility to introduce more effective endophyte from one host to other plants that can contribute to plant growth. A research study was therefore, proposed with the main objective to study the interaction between the diazotrophic endophytes isolated from different hosts and non-leguminous crops (non-host plants). Their colonization, nitrogen fixing and plant growth abilities were also studied. Additionally studies to find a reliable and efficient method of delivering diazotrophic endophytes were also carried out. For this purpose *Herbaspirillum* sp. strain B501*gfp*1, *Pantoea* sp. strain 18 and *Enterobacter* sp. strain 35 were used and their interaction studies were investigated in rice, sugarcane and broccoli.

The endophytic colonization, nitrogen fixation, and plant growth-promoting abilities of *Herbaspirillum* sp. strain B501 *gfp*1, which is a diazotrophic endophyte isolated from wild rice, were studied after infection (at 10^2 and 10^8 cells ml^{-1}) of seedlings of cultivated rice *Oryza sativa* cv. Nipponbare. Both doses resulted in colonization of the roots and stem (basal stem and leaf sheath). However, no colonization of leaves was observed. Higher bacterial populations were observed in the roots than stems. The bacteria colonized the intercellular spaces of the root epidermis and the spaces at the junctions of the lateral roots. They also colonized the epidermis and pericycle of the basal stem and the sub-epidermal tissues of the dermal tissue system of the leaf sheath at later stage (45 DAI). The colonizing bacteria incorporated significant amounts of $^{15}N_2$ into the infected plants. The inoculated plants also had higher dry weights and fresh weights than the control (uninoculated) plants.

To find out the influence of inoculation technique and strain specificity on the endophytic colonization of rice, two endophytic diazotrophs, *Pantoea* sp. strain 18 isolated from sweet potato and *Enterobacter* sp. strain 35 isolated from sugarcane were inoculated in cultivated rice (*Oryza sativa* cv. Nipponbare) and wild rice (*Oryza officinalis*) using two inoculation methods: rhizosphere inoculation and the root dip method. Higher bacterial densities were observed from the root dip method for both strains. The bacterial population of both strains was greater in cultivated rice than in wild rice. The use of GFP-labeled strains indicated that *Pantoea* sp. 18-2 was able to better colonize and more effectively reduce the acetylene in both hosts compared to *Enterobacter* sp. 35-1. Specifically, the combination of cultivated rice and *Pantoea* sp.

strain 18-2 gave the best association for colonization and nitrogen-fixing. The study provides evidence that endophytic bacteria can be established in rice plants other than their original hosts, suggesting a lack of host specificity, although the extent of colonization may vary depending upon the combination of host and endophyte.

The inoculation effect of nitrogen fixing endophytic bacteria isolated from monocots on a dicot plant, *Brassica oleracea* was also investigated. The bacteria used in this study were *Enterobacter* sp. strain 35 isolated from sugarcane and *Herbaspirillum* sp. strain B501 from wild rice. Under glasshouse conditions, plants inoculated with strain 35 significantly increased the fresh weight of *B. oleracea* compared to the uninoculated plants whereas, the fresh weigh of plants inoculated with strain B501 tended to be higher than that of uninoculated plants. Laboratory-scale experiment was conducted to confirm the above results and to investigate the colonization and nitrogen-fixing abilities of these bacteria. Plants inoculated with *Enterobacter* sp. strain 35-1, a GFP-labeled strain derived from strain 35, showed higher bacterial populations and acetylene reduction activity than those inoculated with *Herbaspirillum* sp. strain B501*gfp*1. Strain 35-1 colonized the intercellular spaces and the junctions of the lateral roots to the parent root. The results indicate that isolates from monocots can colonize *B. oleracea* (dicots) and promote plant growth.

Studies were also conducted to develop a suitable delivery method for introducing endophytic bacteria into new sugarcane plants at the field level. Stem pieces of cultivars NiF8 and Ni15 were inoculated with *Herbaspirillum* sp. strain B501*gfp*1 by the vacuum

infiltration method. The inoculated bacteria showed better nitrogenase activity in cultivar Ni15 than in NiF8. In further studies involving four cultivars, the population density of 10^7-10^8 CFU g FW^{-1} immediately after inoculation confirmed that strain B501*gfp*1 successfully entered the stem tissues of all the cultivars, and its presence in the stem tissues was also confirmed by fluorescence microscopic observations. Colonization was observed in the intercellular spaces and between the cell layers. Population of inoculated bacteria remained stable at 5 DAI and showed adequate amount of fixed nitrogen. The roots and aerial parts of the newly emerged plants from the inoculated stems also showed bacterial colonization when observed under fluorescence microscope. The colonization pattern differed slightly among the cultivars. The results indicate that endophytic bacteria can be introduced successfully into sugarcane stem by the vacuum infiltration method, and can colonize the new plants. Thus, this method can be used to prepare the planting material for new plants in the field, in such a way that the material carries its own inoculum source. However, the effect of indigenous bacteria on the nitrogen fixation by the inoculated bacteria needs to be investigated.

Chapter 1 GENERAL INTRODUCTION

Nitrogen is one of the major limiting nutrients for crop production and its availability to the plant is critical for high yields. According to the U.S. Agency for International Development, (1994), the total world population is expected to double in the year 2050. With this much increase in the population rate the food requirement needs to be doubled. However, the land and soil nutrients are limited. To accommodate the growing need of food more nutrient deficient land has been brought under cultivation. This increases the use of artificial fertilizers specially nitrogen fertilizers to get the maximum output. In normal farming practice, the nitrogen fertilizers are chemically synthesized by Haber-Bosch process (Burgess and Newton, 1977) that is very expensive due to the requirement of high temperature and pressure. Besides, in this process the CO_2 gas is also released to the atmosphere to cause Greenhouse Effect. Another Greenhouse gas may be N_2O which is generated by denitrifying of spilled fertilizers on land. In groundwater, there are also reports of pollution by nitrate (NO_3) dissolved from fertilizers.

A marked increase in environmental awareness and a concern for sustainable agriculture has occurred over the last decade. This awareness has highlighted the need both to further promote plant growth and yields and also to find environment friendly replacements for industrially produced nitrogen fertilizers. If the plant scientists could not be able to discover new technologies, this would lead to worldwide starvation. Hence, the Biological Nitrogen Fixation (BNF) crops would be the choice of the new generation to put the world into a sustainable living place.

In nature, legumes benefit directly from biologically fixed nitrogen, provided that they are in symbiotic association with root-nodulating bacteria, such as Rhizobia. However, non-leguminous plants, most of which belong to the Gramineae, do not have this symbiosis (Hurek and Reinhold-Hurek, 2003). Studies to plant bacteria that might make reliable contributions to the growth of non-legume cereals revealed that there are groups of bacteria which intimately associate with non-leguminous crops called diazotrophic endophytes. In general the term "endophyte" includes all the microorganisms that are able of colonizing the inner tissues of plants. The term endophyte was first introduced to the area of nitrogen fixation research associated with Graminaceous plants by Döbereiner, (1992a; 1992b). The term was defined by De Bary, (1866) cited by Stone, (1986) and refers to mycotic flora that inhabits the interior of plant tissues. The term was then applied to bacteria and was the subject of several conceptual definitions. In general, the term includes all microorganisms that are able to colonize, during some portion of their life cycle, the inner tissues of plants without causing any apparent damage to the host (Petrini, 1991).

Among non-leguminous plants, several diazotrophic endophytes have been isolated and characterized as nitrogen fixing endophytes, including *Acetobacter* (Sevilla et al., 2001), *Azoarcus* spp. (Reinhold-Hurek and Hurek, 1998a), *Serratia* spp. (Gyaneshwar et al., 2001), *Burkholderia* spp. (Baldani et al., 2000) and *Herbaspirillum* spp. (Elbeltagy et al., 2001; Gyaneshwar et al., 2002). The interaction of endophytic diazotrophic bacteria with plants has been extensively studied through the inoculation of

sugarcane and rice plants grown under sterile conditions followed by microscopic analysis. These bacteria enter the plant tissues primarily through the root zone. They colonized the spaces at the junctions of the lateral roots and the intercellular spaces of the root epidermis (Roncato-Maccari et al., 2003) and penetrate deeply to enter the internal tissues of the roots, basal stem (James et al., 2000; Zakria et al., 2007) and colonize the aerial parts by entering in the xylem tissues of the roots and stem (Hurek et al., 1994; James et al., 2000). The nitrogen-fixing bacteria are reported to provide biologically fixed nitrogen to their hosts but the amounts of nitrogen that they supply are highly variable, for example in rice it ranged from 0-36% (Malarvizhi and Ladha, 1999; Shrestha and Ladha, 1996) and in sugarcane from 4-70% of the host plant's nitrogen requirement (Yoneyama et al., 1997). The variation in amount of fixed nitrogen is believed to depend on variety, plant stage, endophyte strain, inoculation method and environmental conditions.

Objectives of the study

Although the diazotrophic endophytes have been reported in various crops, the nitrogen supply by the indigenous endophytes is inadequate for crop growth (Asis et al., 2000; Nishiguchi et al., 2005). Endophytes also show host non-specificity and can colonize different plants other than their original hosts (Dong et al., 2003; Olivares et al., 1996). Thus, there is a possibility to introduce more effective endophytes isolated from one host to other plants that can contribute to plant growth. To facilitate the use of endophytic bacteria in practical agronomic production, reliable and practical methods of inoculum

delivery must be developed. Interaction studies involving these issues have not been done in an efficient way, and these issues need to be addressed. Consequently, it may be feasible to convert the potential of this association into a standard inoculation practice in agriculture. A research study was therefore, proposed with the main objective to study the interaction between the diazotrophic endophytes isolated from different hosts and non-leguminous crops (non-host plants). Their colonization, nitrogen fixing and plant growth abilities were also studied. Additionally studies to find a reliable and efficient method of delivering diazotrophic endophytes into hosts were also carried out.

For this purpose, *Herbaspirillum* sp. strain B501*gfp*1, *Pantoea* sp. strain 18 and *Enterobacter* sp. strain 35 were selected and their interaction studies were investigated in rice, sugarcane and broccoli. *Herbaspirillum* sp. strain B501*gfp*1was originally isolated from the stems of wild rice (*Oryza officinalis*). Its colonization and nitrogen-fixing abilities have been studied in its host *Oryza officinalis* (Elbeltagy et al., 2001) and in sugarcane (Njoloma et al., 2006b). *Enterobacter* sp. strain 35 was isolated from sugarcane, which is a monocotyledon plant that belongs to the grass family, whereas *Pantoea* sp. strain 18 was isolated from the sweet potato, which is a dicotyledon plant that belongs to the Convolvulaceae family.

Chapter 2 COLONIZATION AND NITROGEN-FIXING ABILITY OF *Herbaspirillum* sp. strain B501 *gfp*1 AND ASSESSMENT OF ITS GROWTH-PROMOTING ABILITY IN CULTIVATED RICE

2.1 Introduction

Among the cereals, rice (*Oryza sativa*) is the most important crop (Boddy, 1995) and is a staple food of many people worldwide. Nitrogen is one of the major limiting nutrients for rice production and its availability to the plant is critical for high yields. In nature, several endophytic bacteria have been isolated from rice plants. Many of these bacteria have been identified as diazotrophs, such as *Pantoea agglomerans* (Feng et al., 2006; Verma et al., 2001), *Herbaspirillum seropedicae* (Baldani et al., 1986; Baldani et al., 1996; James et al., 2002), *Rhizobium* (Tan et al., 2001), *Herbaspirillum* sp. strain B501 from wild rice *Oryza officinalis* (Elbeltagy et al., 2001), *Serratia* spp. (Gyaneshwar et al., 2001; Tan et al., 2001), and *Burkholderia* spp. (Baldani et al., 2000; Gillis et al., 1995; Tran Van et al., 2000).

Studies have shown that many endophytes have a broad host range. For example, *Klebsiella pneumoniae* (Kp342), which was originally isolated from maize, can colonize the roots of rice, wheat, and *Arabidopsis* (Dong et al., 2003). *Azoarcus indigens*, which is isolated from Kallar grass, also colonizes rice and sorghum (Egener et al., 1999; Reinhold-Hurek et al., 1993; Stein et al., 1997). Similarly, *Herbaspirillum seropedicae* has been found in a variety of crops, including maize, sorghum, sugarcane, and other

Graminae plants (Baldani et al., 1986; Olivares et al., 1996). The extent of colonization and the amount of fixed nitrogen vary with environmental conditions, bacterial strains, and host plants (Boddy, 1995; Grimont and Grimont, 1991). Rice cultivars differ from each other with regard to their abilities to respond to bacterial inoculation, and these variations are partly due to the bacterial strains and partly due to the growth media used (Rolfe and Weinman, 2001). Elbeltagy et al., (2001) have emphasized the potential effects of plant stage and environmental conditions on the amount of nitrogen fixed by endophytes, and have proposed studies involving mature plants to elucidate this phenomenon. Thus, studies of the interaction between the host, the endophyte, and environmental conditions are very important for defining the nitrogen-fixing association between hosts and endophytes.

Herbaspirillum sp. strain B501 *gfp*1 (strain B501*gfp*1), which was originally isolated from wild rice (*Oryza officinalis*), is an important diazotrophic endophyte (Elbeltagy et al., 2001). Strain B501*gfp*1 colonizes the shoots and seeds of *O. officinalis* and fixes significant amounts of nitrogen, as can be observed under the fluorescence microscope after seed inoculation. This bacterium does not colonize the shoots of cultivated rice *Oryza sativa* cv. Sasanishiki, with only weak signals observed in the seeds (Elbeltagy et al., 2001). It has also been reported that strain B501*gfp*1 has the ability to colonize the roots and basal stems of sugarcane plants following root inoculation (Njoloma et al., 2006b). This indicates that under suitable growth conditions and with the appropriate inoculation technique, this bacterial strain can colonize plants other than its original host.

In a previous report (Elbeltagy et al., 2001) the colonization and nitrogen-fixing abilities of strain B501*gfp*1 for rice were studied 7 days after inoculation (DAI). However, similar studies in mature plants, especially those of cultivated rice, have not been performed. The present study aimed to address these issues and to reveal the contribution of nitrogen fixation by this bacterium to inoculated cultivated rice. Thus, the colonization and nitrogen-fixing ability of strain B501*gfp*1 inoculated at different concentrations was studied in *Oryza sativa* cv. Nipponbare. The systemic movements of the bacteria within the inoculated plants and the plant growth-promoting abilities of the bacteria were also examined.

2.2 Materials and Methods

2.2.1 Plant growth conditions

Dehulled seeds of *Oryza sativa* cv. Nipponbare were surface-sterilized with 70% ethanol for 2 min and with 5% sodium hypochlorite (NaClO) for 30 min. The seeds were then washed three times with sterile distilled water and subjected to sterility checks on nutrient agar media, to ensure sterilization efficiency.

Sterilized seeds were germinated in 4.0 × 3.5-inch plastic pots, one third of which was filled with sterilized vermiculite, and 10 ml of MS medium was added (Murashige and Skoog, 1962). Four-day-old seedlings were transferred into modified Leonard jars that were filled with sterile vermiculite containing growth medium (Fig. 2.1). The growth

medium was kept moist by a centrally positioned cotton wick that ran half the length of the jar and extended into a reservoir that contained 300 ml of MS medium with 0.5 mM KNO_3 (pH 5.6). Sterilized stones were spread on top of the vermiculite to provide a dry surface, thereby preventing the growth of any airborne bacteria or fungi on the vermiculite and reducing evaporation from the jars. The plants were maintained at 28°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. After one week, the MS medium with nitrogen was replaced with nitrogen-free MS medium.

2.2.2 Bacterial strain and inoculum preparation

Strain B501*gfp*1 was provided by Dr. K. Minamisawa, Institute of Genetic Ecology, Tohoku University, Japan. The inoculum was prepared from bacterial cultures grown in LB broth for 24 h. The bacterial suspensions were centrifuged at 6,500 rpm for 10 min at 25°C. The supernatant was discarded and the pellet was resuspended in sterile distilled water. The bacterial suspensions were adjusted to 10^2 and 10^8 cells ml^{-1} using a hemocytometer (B1587, Sun Lead Glass Company, Tokyo, Japan) under a microscope (Nikon Eclipse E600, Nikon, Tokyo, Japan).

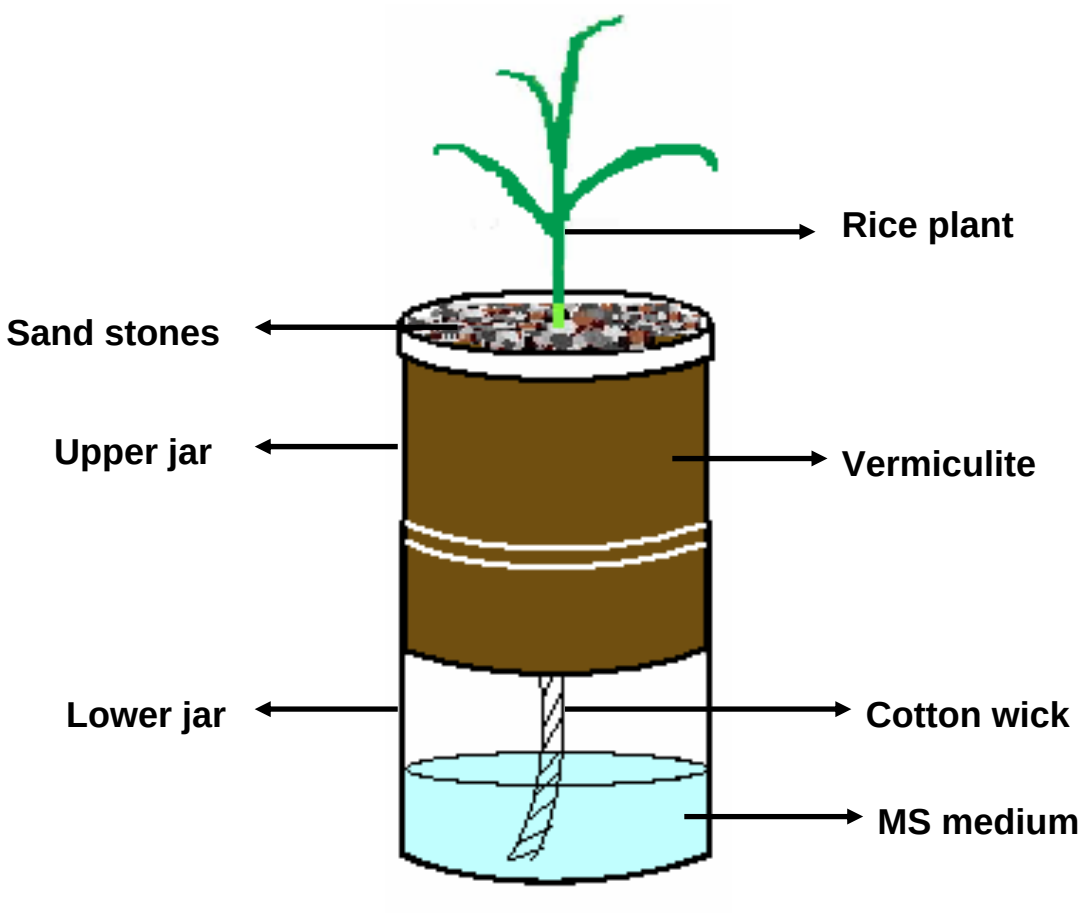


Figure 2.1. Experimental design of the modified Leonard jars.

2.2.3 Inoculation and harvesting

Roots are the main avenue of entry of diazotrophic endophytes into plants (Hurek and Reinhold-Hurek, 2003). Thus, we chose 12-day-old seedlings with well-developed root systems for inoculation of the bacteria. Plants were inoculated with 200 ml of 10^2 or 10^8 cells ml^{-1} of strain B501*gfp*1 by removing the stones that covered the vermiculite and evenly pouring the suspension onto the vermiculite in the jars, without disturbing the plant root system. For control plants, the same amount of sterile distilled water was added. After the pouring on of the inoculum, the vermiculite was covered once again with sterile stones and the plants were left to grow under the conditions described above.

The plants were harvested and sampled for analysis at 2, 8, 15, 30, and 45 DAI. Plants were divided into roots, stem (basal stem and leaf sheath), and leaves (leaf blades) for fluorescence microscopic observations and bacterial density determination.

2.2.4 Enumeration of bacteria

To estimate the population densities of strain B501*gfp*1, replicate (n=3) plants (inoculated and control) were sampled and loosely attached bacteria were removed by washing the roots and aerial parts with distilled water. The plants were cut in to small pieces, weighed, and transferred into sterile tubes. Each sample was washed three times with sterile distilled water and macerated using a mortar and pestle. The macerates were then serially diluted and plated on LB medium that contained 10 µg ml^{-1} kanamycin. The green

colonies that appeared on the plates after incubation at 28°C were counted using a Nikon SMZ 1500 fluorescence microscope.

2.2.5 Fluorescence microscopy

Plants from two separate inoculations, together with the corresponding control plants, were examined at 3, 15, 30 and 45 DAI. The inoculated plants were also examined after surface sterilization, to confirm endophytic colonization and localization of the bacteria. For surface sterilization, plant parts were treated with 70% ethanol for 1-2 min, followed by washing in sterile distilled water. For the unsterilized treatment, plant parts were washed only with sterile distilled water. To examine the localization of the inoculated bacteria in tissues of the roots and stem, transversal and longitudinal sections of 0.1-mm thickness were prepared with a Microslicer. These sections were examined for microscopic fluorescence using the Nikon Eclipse E600 (Nikon, Tokyo, Japan). Each field was observed and photographed under GFP (R)-BP, HQ (FITC)-BP filter (DM 505, BA 500-560, EX 460-500) and B-2A filter (DM 505 and EX 450-490). The images were captured using Pixera 150-CU, 5QV/2.0A (Pixera Corporation, Los Gatos, CA, USA) fitted on to the Nikon Eclipse E600 and viewed on Pixera view finder and studio 3 computer programme.

2.2.6 $^{15}N_2$ gas fixation in intact plants

At 30 DAI, plants were washed with sterile distilled water (to remove vermiculite and loosely attached bacteria) and were placed in 70-ml glass tubes that were then sealed with rubber caps. The tubes were completely filled with N-free Fahraeus liquid medium and 60 ml of this volume was then replaced with 30% (vol/vol) $^{15}N_2$ gas (99.8 atom%) and 70% (vol/vol) Ar gas, leaving 10 ml of Fahraeus liquid medium in the tubes. The tubes were incubated at 28°C under a photoperiod of 16-h light. After 72 h, the seedlings were removed from the tubes. The roots and green aerial portions were separated, dried to a constant weight at 70°C, and powdered using a mortar and pestle. The ^{15}N concentration was determined in duplicate using a mass spectrometer. The samples were analyzed using the Thermo Finnigan Delta plus Advantage system (Shoko Co. Ltd., Japan).

2.2.7 Plant growth

Plant growth was determined by comparing the fresh and dry weights of the inoculated and uninoculated plants. The fresh weights of the plants were recorded at each harvest. For the dry weight calculation, plants harvested at 30 DAI were oven-dried at 70°C, and the final weight was recorded.

2.3 Results and Discussion

2.3.1 Bacterial colonization

The bacterial population density varies with the inoculum concentration and with time post-inoculation. No colonization of leaves was observed at any stage. The high inoculum dose (10^8 cell ml^{-1}) resulted in rapid colonization of roots (at 2 DAI), whereas the low inoculum dose (10^2 cell ml^{-1}) resulted in delayed (8 DAI) colonization (Table 2.1). This rapid colonization of roots is probably due to the expression of cell wall-degrading enzymes, such as cellulase and pectinase, by strain B501*gfp*1(Elbeltagy et al., 2001). Similar results (James et al., 2002; James and Olivares, 1998; Njoloma et al., 2006b; Olivares and James, 2000) have been reported for sugarcane and rice roots inoculated with *Herbaspirillum seropedicae*. The bacterial populations in the roots of high-inoculum plants were higher than those in low-inoculum plants until 15 DAI. From 30 DAI, a higher bacterial population (5.8×10^6 CFU g^{-1} FW) was observed in the roots of low-inoculum plants than in the roots of high-inoculum plants (2.4×10^6 CFU g^{-1} FW) (Table 2.1). The decreases in the bacterial numbers in the high-inoculum plants at 30 DAI and 45 DAI compared to the low-inoculum plants may be due to the difference in initial inoculum dose. In the high-inoculum plants, bacterial colonization was aggressive and rapid. Therefore, the colonized bacteria multiplied and occupied the roots quickly and the population reached a maximum between 15 and 30 DAI and started to decrease before 30 DAI. A possible reason for the decline in bacterial numbers is the non-pathogenic nature of strain B501*gfp*1, as is the case with other bacterial endophytes. The non-pathogenic nature of *Herbaspirillum* sp. has been reported previously

Table 2.1. Numbers of bacteria colonizing the roots, stems, and leaves of rice plants inoculated with low and high doses of strain B501*gfp*1.

Plant part	Inoculum concentration (cells ml^{-1})	Bacterial numbers (CFU g^{-1} FW)				
		2 DAI	8 DAI	15 DAI	30 DAI	45 DAI
Root	10^2	0	2.4×10^2	4.2×10^3	5.8×10^6	5.2×10^5
	10^8	9.77×10^3	2×10^4	6×10^4	2.4×10^6	1.2×10^5
Stem	10^2	0	0	2.0×10^2	1.0×10^3	2.4×10^4
	10^8	0	1×10^3	6.6×10^2	7.7×10^4	2.1×10^4
Leaves	10^2	0	0	0	0	0
	10^8	0	0	0	0	0

The values listed represent the means of three replicates.
FW, fresh weight; DAI, days after inoculation.

(James et al., 1997; Olivares et al., 1997; Pimentel et al., 1991). Unlike true pathogens, endophytes lack the ability to overcome the plant's defenses as the plants mature and grow in size beyond the seedling stage (Hallmann et al., 1997; James et al., 2002). Another reason may be the reduced surface area and nutrition available to the bacteria for growth and multiplication. In the present study, plants with well-developed root systems were inoculated with bacteria. Therefore, at the time of inoculation, extensive root areas were available to the bacteria for infection and colonization, which meant that they colonized and multiplied quickly within the roots. The bacterial population tended to increase until 30 DAI, and occupied most of the root area and peaked by that time-point (Table 2.1, Fig. 2.3D and Fig. 2.4C). The reduced availability of non-colonized roots, coupled with the inability to overcome the defenses of the maturing plant, probably caused the slight decrease in bacterial numbers observed at the last harvest. In the low-inoculum plants, the initial inoculum size was very small and 8 days was required for the bacteria to colonize the roots (although the fluorescent micrographs revealed some bacteria at 3 DAI, this was a rare phenomenon). After the initial colonization period, the bacteria multiplied rapidly and reached maximal density at 30 DAI, with a subsequent decline in numbers. These results are somewhat different from those presented in a previous study (Njoloma et al., 2006b), in which higher bacterial (strain B501*gfp*1) populations were associated with higher inoculum doses in sugarcane roots at 56 DAI. Strain B501*gfp*1 initially showed aggressive behavior, probably due to its ability to produce cell wall-degrading enzymes, but this behavior was suppressed once the bacteria were established within the plant (James et al., 2002). No signs of a hypersensitive reaction (host defense reaction) (Hallmann et al., 1997) were observed at the later

harvests, which is linked to the production of cell wall degrading-enzymes by phytopathogenic bacteria. The lack of defensive responses by the plants may explain why the bacteria localized primarily within the intercellular spaces.

Compared to root entry, the invasion of bacteria into the stem occurred rather late. Bacteria entered the stem at 8 DAI in the high-inoculum plants and at 15 DAI in the low-inoculum plants. Regarding this difference, it is possible that a threshold number of bacteria in the roots is required to initiate penetration of the stem. This required population size in the roots would have been achieved earlier in the high-inoculum plants than in the low-inoculum plants. The bacterial numbers in the stems of the low-inoculum plants continued to increase up to the last sampling time-point, whereas the bacterial numbers in the high-inoculum plants increased until 30 DAI but were decreased slightly at 45 DAI (Table 2.1).

The bacterial populations in the roots were always higher than in the stems for both doses of inoculum (Table 2.1). These findings are in accordance with those of Gyaneshwar et al., (2001) and James et al., (2002) and contrast with the findings of Barraquio et al., (1997) and Elbeltagy et al., (2001), who have reported that the numbers of nitrogen-fixing bacteria in rice are higher in stems than in roots.

2.3.2 Localization of Strain B501*gfp*1

Roots were found to be heavily colonized by bacteria in both the low and high inoculum treatments. Bacterial colonization of the stems was observed but there was no colonization of the leaves, as evidenced also by the population density determinations. Furthermore, the initial association of bacteria with roots was apparent at 3 DAI as small colonies in the intercellular spaces of the root surface and in the cracks of the lateral roots on the main root and on the basal stem (Fig. 2.2A and C). However, plants inoculated with the higher inoculum (10^8 cell ml^{-1}) showed greater surface colonization of the main roots, in that the bacteria colonized the intercellular spaces of the main roots and the junctions/cracks of the lateral roots on the main root (Fig. 2.2B and D). To investigate whether the bacteria penetrate the internal tissues of the roots and stem or are merely present in the rhizoplane, the inoculated plants were examined after surface sterilization. The fluorescent micrographs of surface-sterilized roots confirmed the endophytic colonization of bacteria (Fig. 2.2C and D). Similar patterns of *Herbaspirillum seropedicae* colonization of the roots of rice, wheat, maize, and sorghum have been reported previously (Roncato-Maccari et al., 2003). Examination of transversal sections of the basal stem at 3 DAI and in later stages showed the endophytic colonization of the adventitious roots on the basal stem (Fig. 2.2E). The presence of dense colonies of bacteria in intercellular spaces (Fig. 2.2C) and in cracks (Fig. 2.2B and D) of roots on the basal stem indicates that the bacteria are multiplying at theses sites. The endophytic presence of bacteria in roots (Fig. 2.2E) and the basal stem (Fig. 2.3E) suggests that bacteria reached these sites by penetrating these cracks and entered into the pericycle of

the basal stem (Fig. 2.3E-1 and Fig. 2.4F). They also colonized the veins of the outer leaf sheath of the lower stem by 15 DAI (Fig. 2.3F and F-1). Such a mode of penetration in roots and stem by endophytic bacteria has been reported previously (James et al., 2002; Roncato-Maccari et al., 2003). It was observed that the bacterial colonies on a few roots increased in number and size and formed lines along the intercellular spaces of the root surface. This phenomenon could be observed from 15 DAI (Fig. 2.3D) and at 30 DAI (Fig. 2.4C) in the high-inoculum and low-inoculum plants, respectively. Heavy colonization of the intercellular spaces of unsterilized roots (Fig. 2.3A and Fig. 2.4 A, D) and surface-sterilized roots (Fig. 2.3B and Fig. 2.4B) was apparent throughout the 45-day period. No significant upward movement of bacteria was noted in the stem, except for the rare presence of bacteria in the sub-epidermal tissues of the dermal tissue system of the leaf sheath at 45 DAI (Fig. 2.4G).

The entry of strain B501*gfp*1 into aerial tissues, colonizing the apoplast shoot tissues and coleoptiles of wild rice, has been reported previously (Elbeltagy et al., 2001). However, in the present study, colonization of aerial components was not observed, except for the leaf sheath and lower stem. Colonization of the xylem in the roots and stem by *Azoarcus* sp (Hurek et al., 1994) and *Herbaspirillum seropedicae* (James et al., 2000) has been reported previously, and this is generally believed to be the route through which endophytic bacteria move from the roots to the aerial parts. In contrast, no colonization of root xylem has been observed in studies conducted with *Serratia marcescens* (Gyaneshwar et al., 2001) and *Herbaspirillum seropedicae* (cultivar IR42) in rice, although these bacteria were observed in the stem xylem. Endophytic bacteria are mainly

located in the aerenchyma and intercellular spaces (Elbeltagy et al., 2001; Gyaneshwar et al., 2001; James et al., 2002). In the present study, heavy colonization of intercellular spaces was observed in the roots. It would have been worth conducting a microscopic study of internal root tissues (vascular vessels) as well but due to a lack of suitable laboratory facilities, this was not possible. Nevertheless, fluorescence microscopic observations of the transversal sections of the stem showed no colonization of vascular tissues. Therefore, the potential for upward movement through the transpiration stream appears to be minimal. The movement of endophytic bacteria in this situation from the roots to the stem might be due to the formation of long lines of intercellular bacteria (Fig. 2.2E-1, Fig. 2.3D and E-1, and Fig. 2.4C) along the lateral and adventitious roots, as described by Elbeltagy et al., (2001). Many endophytes (Okunishi et al., 2005) from rice, including strain B501*gfp*1, are motile in nature (Elbeltagy et al., 2001), and it is thought that motility plays an important role in entering and movement in plants (Mano et al., 2006). Therefore, it is possible that bacteria within the growth medium entered directly into the basal stem and subsequently migrated to other regions of the plant.

2.3.3 $^{15}N_2$ gas fixation in intact plants

Measuring the incorporation of ^{15}N-dinitrogen gas is a direct way to evaluate nitrogen fixation in plants. Plants inoculated with the high and low doses of bacteria contained higher amounts of fixed nitrogen than control plants. On average, 3.8 µg of nitrogen plant^{-1} (roots and green aerial portion) was fixed in 3 days, which represents 0.6% of the total nitrogen content of the plant (Table 2.2). The plants inoculated with the low-inoculum dose fixed more nitrogen than the plants inoculated with the high-inoculum

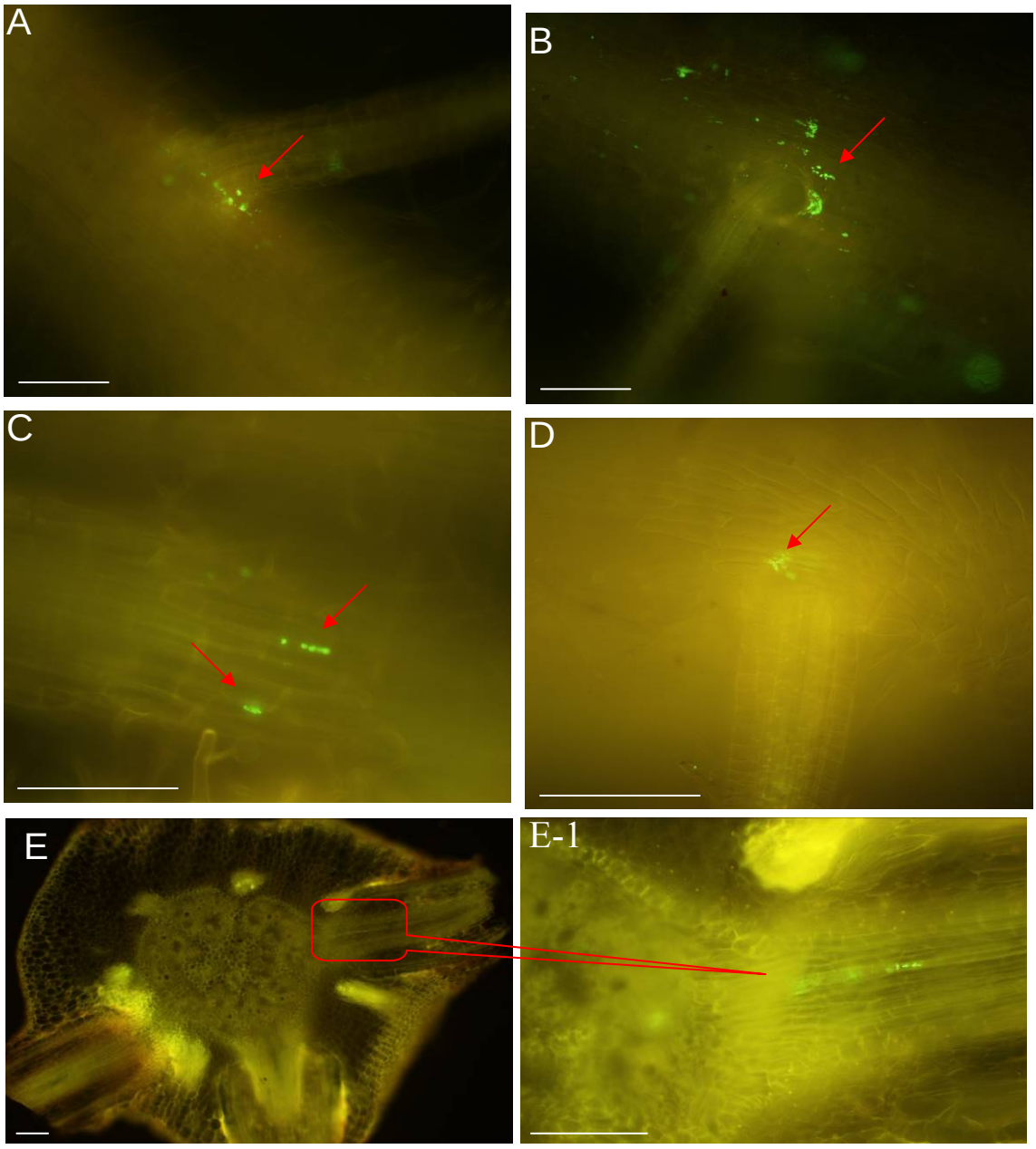
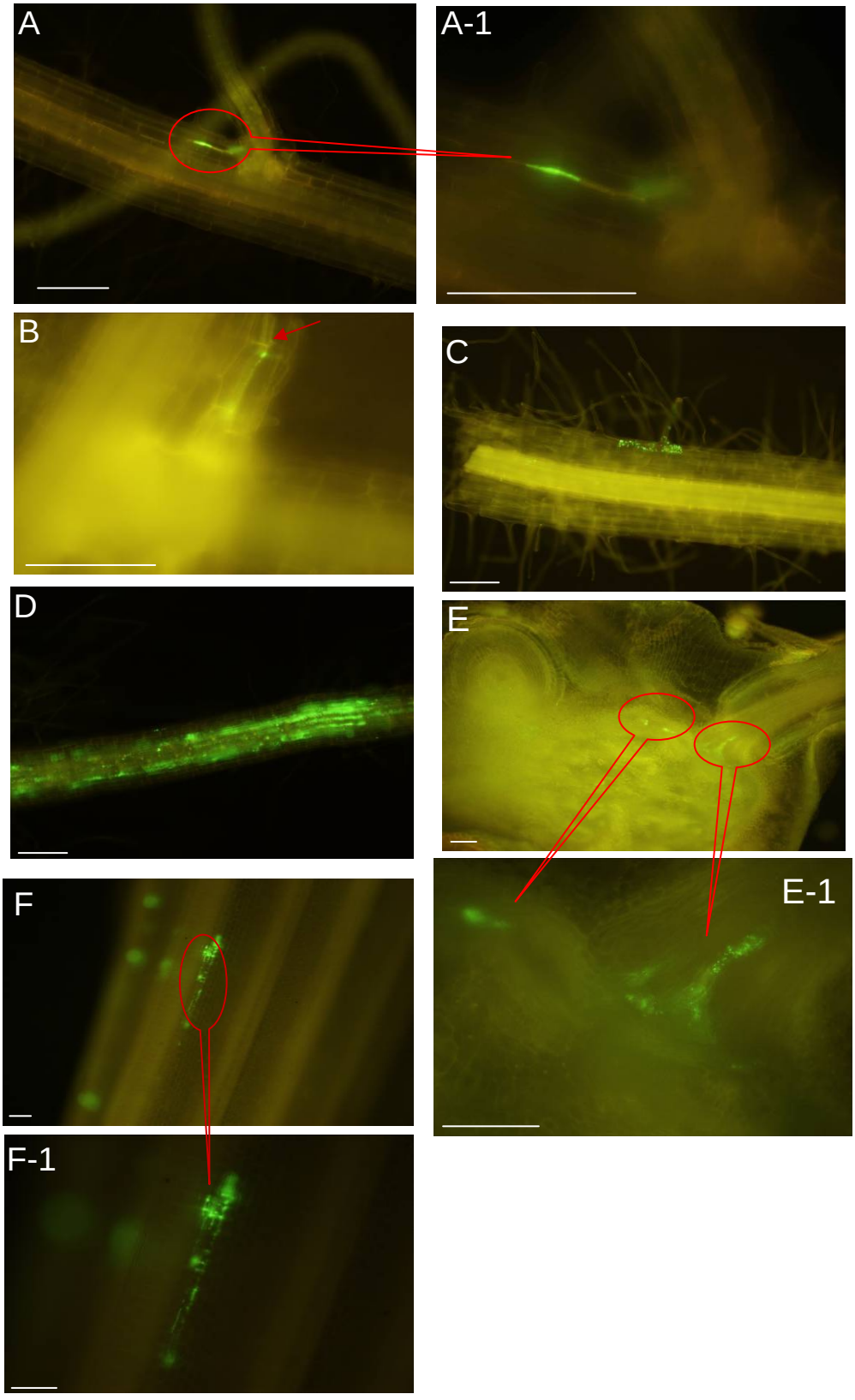

A
B
C
D
E
E-1

Figure 2.2. Fluorescent micrographs showing colonization by *Herbaspirillum* sp. strain B501*gfp*1 of roots and transversal sections of basal stem at 3 DAI. (A) Colonization of the surfaces and junctions of the lateral roots of unsterilized plants inoculated with 10^2 cell ml^{-1}. (B) Colonization of surfaces and junctions of the lateral roots on the main root of unsterilized plants inoculated with 10^8 cell ml^{-1}. (C) Bacteria are present in the intercellular spaces of surface-sterilized plants inoculated with 10^2 cell ml^{-1}. (D) Colonization of the internal tissues of a surface-sterilized root at the junction of the main and lateral roots in plants inoculated with 10^8 cell ml^{-1}. (E and E-1) Transversal section of a basal stem showing the presence of bacteria in the center of the adventitious root at 3 DAI in surface-sterilized plants inoculated with 10^2 bacteria ml^{-1}. Bar = 10 μm. The arrows show the colonization sites.


A
A-1
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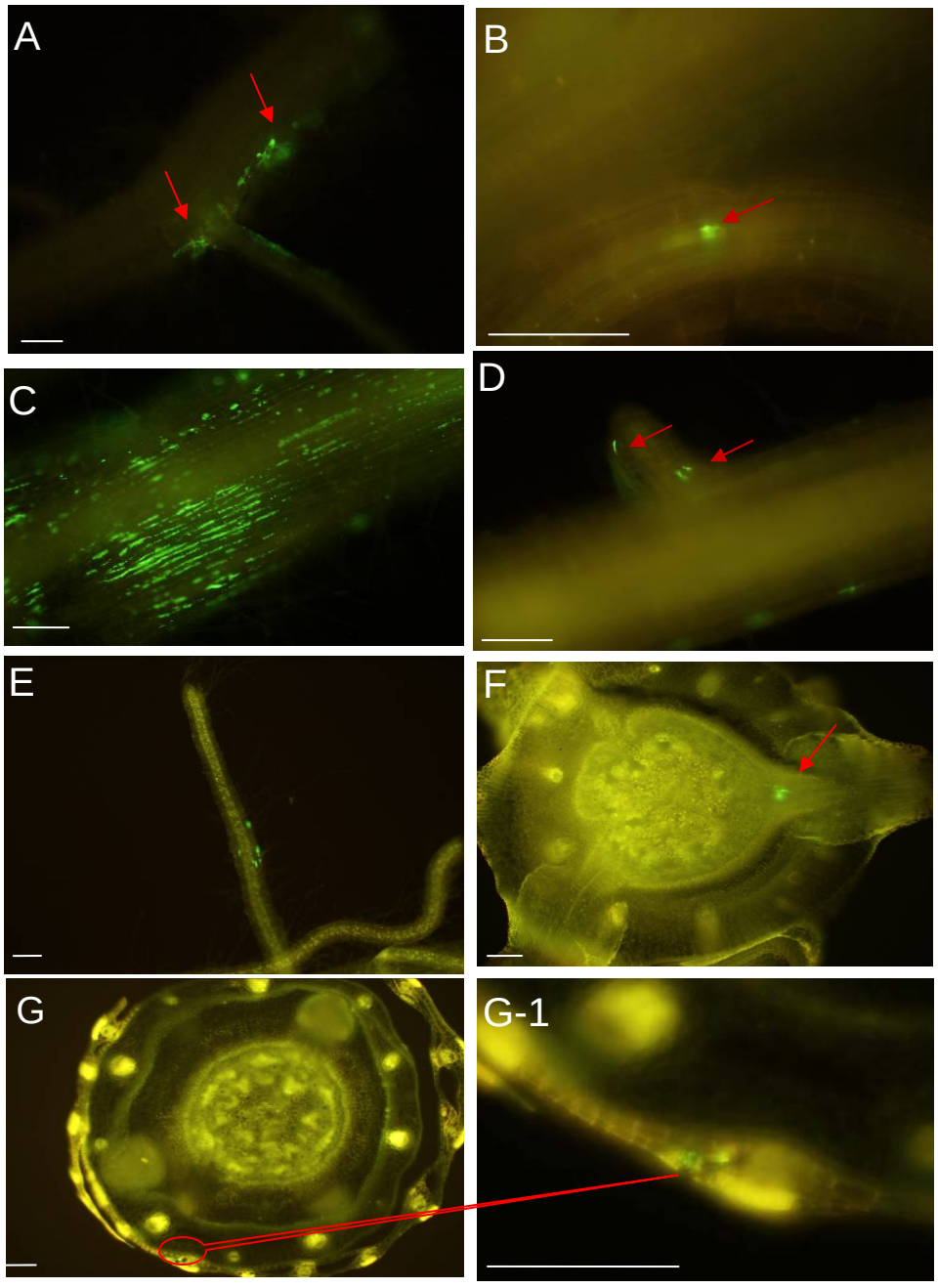
E-1
F
F-1

Figure 2.3. Fluorescent micrographs showing bacterial colonization of roots and stem at 15 DAI. (A and A-1) Intercellular spaces of unsterilized roots inoculated with 10^2 cell ml^{-1}. (B) Surface sterilized roots inoculated with 10^8 cell ml^{-1} showing endophytic colonization. (C) Bacteria inside the epidermal cells of the roots of plants inoculated with 10^2 cell ml^{-1}). (D) Roots covered with bacterial colonies (inoculated with 10^8 cell ml^{-1}). (E and E-1) Transversal sections of the basal stems of plants inoculated with 10^2 cell ml^{-1}, showing bacterial colonization of the pericycle of the basal stem and the presence of bacteria in the center of the base of an adventitious root; the bacteria seem to enter through the junction/cracks of the adventitious root on the basal stem. (F and F-1) Bacterial colonization along the veins of the leaf sheath of a plant inoculated with 10^2 bacteria ml^{-1}. Bar=10 μm. The arrows show the colonization sites.


A
B
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G
G-1

Figure 2.4. Fluorescent micrographs showing different bacterial colonization sites on the roots and a stem at 30 DAI and 45 DAI. (A) Surface colonization at 30 DAI of unsterilized root and colonization at junctions of the lateral roots on the main root of plants inoculated with 10^2 cell ml^{-1}. (B) Surface-sterilized roots of plants inoculated with 10^2 cell ml^{-1}(30 DAI). (C) Roots covered with bacterial colonies (30 DAI for plants inoculated with 10^2 cell ml^{-1}). (D) Epidermis and base of emerging lateral root at 45 DAI in plants inoculated with 10^2 cell ml^{-1}. (E) Colonization of thin hairy roots inoculated with10^2 cell ml^{-1} at 45 DAI. (F) Colonization of the pericycle of a basal stem at 45 DAI for plants that were inoculated with 10^2 cell ml^{-1}. (G and G-1) Colonization of the sub-epidermal tissues of the dermal tissue system of a leaf sheath at 45 DAI in a plant inoculated with 10^2 cell ml^{-1}. Bar = 10 μm. The arrows show the colonization sites.

Table 2.2. Effect of inoculation with *Herbaspirillum* sp. strain B501*gfp*1 on the incorporation of $^{15}N_2$ into *Oryza sativa* cv. Nipponbare plants.

Inoculation	Treatments	Population density at 30 DAI	Occupied (%) of fixed ^{15}N in total nitrogen	^{15}N fixed (μg/plant[b])	Dry matter (mg)
None	Control	0	0	0	69±6.5
Strain B501*gfp*1	10^2	5.8×10^6	0.603±0.220	3.8±2.1	98.3±10.6
	10^8	2.47×10^6	0.194±0.050	1.24±0.12	86.6±7.5

The values listed represent the means ± SE of two replicates.
The percentage of occupied ^{15}N of the total nitrogen content of each plant was calculated using the following formula:

Occupied % of ^{15}N in total nitrogen = (sample excess % / enriched ^{15}N gas excess %) × 100

where sample excess % = sample atom % - BG atom %,
and enriched ^{15}N gas excess % = Gas atom % - BG atom %
[b]Roots and green aerial portion.
DAI, days after inoculation.

dose. The reason for this might be the higher number of bacteria (5.8×10^6 CFU g^{-1} FW) in the low-inoculum plants than in the high-inoculum plants (2.47×10^6 CFU g^{-1} FW) at 30 DAI (Table 2.2) as the $^{15}N_2$ gas concentration was also measured at 30 DAI. The percentage of fixed nitrogen in the present study is low compared to the level of 4% (Gyaneshwar et al., 2002) reported for an aluminum-tolerant rice variety infected with *H. seropedicae* Z67. However, the amount of nitrogen fixed in the present study is close to that observed in some other studies in rice. The amount of fixed nitrogen (0.6% in 3 days) in the present study is higher than the 0.135% per day observed for strain B501*gfp*1 inoculated into wild rice (*Oryza officinalis*) (Elbeltagy et al., 2001). Similarly in an another study, *H. seropedicae* Z67 has been reported to fix 0.45% nitrogen (over 3 days) in the rice variety IR42 (James et al., 2002). Gyaneshwar *et al*. observed 0.17% nitrogen fixation in rice infected with *Serratia marcescens* (Gyaneshwar et al., 2001). The variation in the amount of nitrogen fixed indicates that nitrogen fixation varies with strain, plant genotype, plant age, and environmental conditions (Boddy, 1995; Elbeltagy et al., 2001).

2.3.4 Plant growth

Comparison of the dry and fresh weights of the inoculated plants with those of the control plants suggests that strain B501*gfp*1 has growth-promoting abilities. The inoculated plants showed increased dry weights (42% and 26% for the low- and high-inoculum doses, respectively) compared to the controls (Table 2.2). Inoculation also resulted in increases in the fresh weights of plants compared to uninoculated plants (Fig. 2.5). At 45

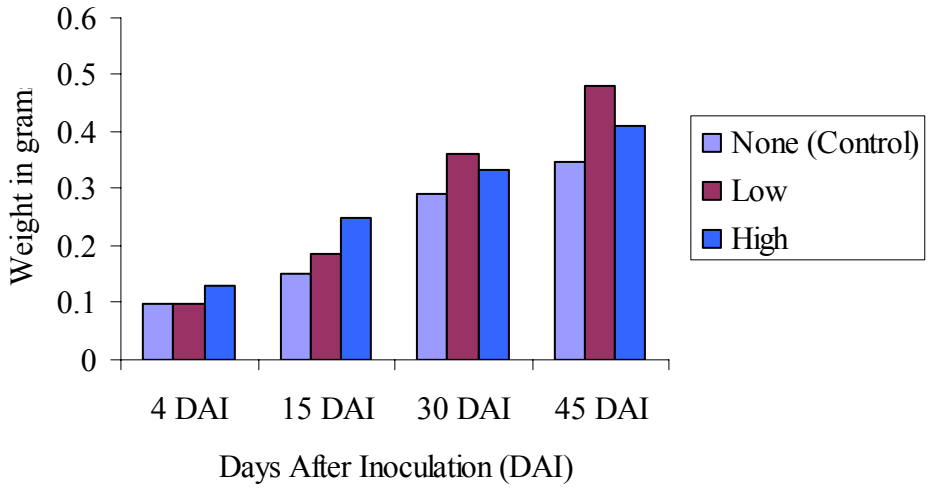

Figure 2.5. Fresh weight in grams of plants inoculated with low (10^2 cell ml^{-1}) and high (10^8 cell ml^{-1}) inoculum doses of *Herbaspirillum* sp. strain B501*gfp*1 (different sampling times). The values represent the means of two replicates.

DAI, the fresh weight of the low-inoculum plants (0.479 g plant^{-1}) was almost 17% higher than that of the high-inoculum plants (0.411 g plant^{-1}) (Fig. 2.5). It is noteworthy that increased dry weights were observed for the plants with higher nitrogen content (data not shown), which indicates that endophytic bacteria contribute to plant growth by providing fix nitrogen (James et al., 2002). However, endophytic bacteria are also reported to promote plant growth without enhancing nitrogen content, which implies that endophytic bacteria influence plant growth by means other than nitrogen fixation (Gyaneshwar et al., 2001). This enhancement of plant growth is due to the excretion of phytohormones, which lead to increases in photosynthesis and root surface and thus increase nutrient uptake (Chi et al., 2005; Dobbelaere et al., 1999; Okon and Labandera-Gonzalez, 1994).

2.4 Conclusion

Based on the findings of this study it can be concluded that strain B501*gfp*1 can colonize, fix nitrogen, and promote growth in cultivated rice (*Oryza sativa* cv. Nipponbare). It was also observed that compared to other studies (Elbeltagy et al., 2001), levels of bacterial colonization and fixed $^{15}N_2$ were higher, which may be due to differences in the experimental conditions, e.g., inoculation technique and plant growth stage at the time of inoculation. Further studies using different inoculation techniques that assess the effects of plant growth stage on endophytic colonization and amount of fixed nitrogen are necessary to shed light on the roles of these parameters in the development of rice-endophyte interactions.

Chapter 3 INFLUENCE OF INOCULATION TECHNIQUE AND STRAIN SPECIFICITY ON THE ENDOPHYTIC COLONIZATION OF RICE BY *Pantoea* sp. ISOLATED FROM SWEET POTATO AND BY *Enterobacter* sp. ISOLATED FROM SUGARCANE

3.1 Introduction

Several diazotrophic endophytes have been isolated from rice and they can provide fixed N (Gyaneshwar et al., 2001; Gyaneshwar et al., 2002; Verma et al., 2001). Many endophytes appear to have a broad host range. For example, *Herbaspirillum seropedicae* has been found in a variety of crops, including maize, sorghum, sugarcane and other Gramineae plants (Baldani et al., 1986; Olivares et al., 1996). This indicates that an endophyte isolated from one host family member can colonize other non-host members of the same family, which is suggestive of host non-specificity in Gramineae plants. Endophytes also show host non-specificity among families. *Burkholderia* sp. isolated from the onion can colonize grapes (Compant et al., 2005), potatoes and vegetables (Nowak et al., 1995). Interaction studies involving rice and endophytes isolated from other host families have not been done in an efficient way, and the issue needs to be addressed.

The nitrogen supplied by indigenous endophytic N_2-fixing bacteria is inadequate for crop growth (Nishiguchi et al., 2005). Asis et al., (2000) isolated several putative strains of *Gluconacetobacter diazotrophicus* and *Herbaspirillum* from sugarcane, and

reported that only 40% of these strains had acetylene reduction activity. Similarly, although many diazotrophic endophytes have been reported in rice, the amounts of nitrogen that they supply are highly variable, ranging from 0-36% of the total N derived from air, depending on the variety (Malarvizhi and Ladha, 1999; Shrestha and Ladha, 1996). Thus, the potential exists to introduce non-indigenous endophytes that can contribute to plant growth. Therefore, studies of the interactions among the host, endophyte and environmental conditions are very important in defining the nitrogen-fixing associations between hosts and endophytes.

Several delivery methods, such as inoculation of seeds, roots and aerial parts, have been reported for endophytic bacteria (Bressan and Borges, 2004; Elbeltagy et al., 2001; Njoloma et al., 2006b; Ruppel et al., 1992; Verma et al., 2001). None of these methods is effective to the same extent for all strains, so a specific delivery method has to be found for each endophytic bacterial isolate (Bressan and Borges, 2004). Researchers are interested in finding bacterial strains with enhanced plant-growth-promoting capabilities. As new beneficial bacterial strains are identified, the delivery of these strains to specific plant tissues becomes an issue. To facilitate the use of endophytic bacteria in practical agronomic production, reliable and practical methods of inoculum delivery must be developed.

The aims of this study were to find an appropriate inoculation method and to identify the host-specificity and differences between the hosts in terms of the quantitative assessment of endophytic colonization. For these purposes, GFP-labeled strains derived

from *Enterobacter* sp. strain 35 and *Pantoea* sp. strain 18 were used. *Enterobacter* sp. strain 35 was isolated from sugarcane, which is a monocotyledon plant that belongs to the grass family, whereas *Pantoea* sp. strain 18 was isolated from the sweet potato, which is a dicotyledon plant that belongs to the Convolvulaceae family. The strains were inoculated into O*ryza sativa* cv. Nipponbare and *Oryza officinalis* by two different methods, and their colonization and nitrogen-fixing abilities were studied.

3.2 Materials and Methods

3.2.1 Strains and inoculum preparation

Enterobacter sp. strain 35-1 (strain 35-1) and *Pantoea* sp. strain 18-2 (strain 18-2), which are the GFP-labeled strains derived from *Enterobacter* sp. strain 35 and *Pantoea* sp. strain 18, respectively, were labeled with GFP by conjugation with *Escherichia coli* strain s17-λpir containing pTn5kmgfpmut1, as described previously (Tanaka et al., 2006). The inoculum was prepared from bacterial cultures grown in LB broth for 24 h. The bacterial suspensions were centrifuged at 6,500 rpm for 10 min at 25°C. The supernatant was discarded and the pellet was resuspended in sterile distilled water. The bacterial suspensions were adjusted to 10^8 cells ml^{-1} using a hemocytometer (B1587, SLGC, Tokyo, Japan) under a microscope (Nikon Eclipse E600, Nikon, Tokyo, Japan).

3.2.2 Seed surface sterilization

Dehulled seeds of *Oryza sativa* cv. Nipponbare and *Oryza officinalis* were surface-

sterilized with 70% ethanol for 2 min and with 5% sodium hypochlorite (NaClO) for 30 min. The seeds were then washed three times with sterile distilled water and subjected to sterility checks on nutrient agar media, to ensure sterilization efficiency.

3.2.3 Plant growth conditions and inoculations

Two experiments with the two different inoculation methods, i.e. inoculation of the rice root system with bacterial suspension and inoculation by dipping the roots in bacterial suspension, were conducted to study the colonization abilities and host specificities of *Pantoea* sp. strain 18-2 and *Enterobacter sp*. strain 35-1 in cultivated rice (*Oryza sativa* cv. Nipponbare) and wild rice (*Oryza officinalis*).

3.2.3.1 Inoculation of the rice root system (rhizosphere) with bacterial suspension

Sterilized seeds were germinated in 4.0 × 3.5-inch plastic pots, one third of which were filled with water-soaked sterile vermiculite, and 10 ml of MS medium (Murashige and Skoog, 1962) were added. Three-day-old seedlings were transferred into modified Leonard jars (Fig. 2.1). The plants were maintained at 28°C under a photoperiod of 16-h light and a photon flux density of 60 μmol m^{-2} s^{-1}, which was provided by cool white fluorescent tubes. After six days, the MS medium containing nitrogen was replaced with nitrogen-free MS medium.

The plants were inoculated with 200 ml of 10^8 cells ml^{-1} of strain 35-1 or strain 18-2 by removing the sand stones that covered the vermiculite and evenly pouring the suspension onto the vermiculite in the jars, without disturbing the plant root system. For the control plants, the same amount of sterile distilled water was added. After pouring the inoculum, the vermiculite was covered once again with sterile sand stones, and the plants were left to grow under the conditions described above.

3.2.3.2 Inoculation by dipping the roots in bacterial suspension

Surface-sterilized seeds of *Oryza sativa* cv. Nipponbare were germinated in glass tubes containing 15 ml of semisolid MS medium (Murashige and Skoog, 1962), whereas *Oryza officinalis* seeds were germinated in 4.0 × 3.5-inch plastic pots containing 150 ml of semisolid MS medium. Four-day-old (in the case of the former) and nine-day-old (in the case of the latter) seedlings were uprooted and washed three times with sterile distilled water to remove the entire attached semisolid medium. The seedlings were inoculated by dipping their roots in inoculum suspension (10^8 cells ml^{-1}) of each strain for 1 min or 60 min. The inoculated roots were rinsed with sterilized distilled water three times to remove the loosely attached bacteria. For the control treatment, the roots were dipped in sterile distilled water. The seedlings were then transplanted into 4.0 × 3.5-inch plastic pots filled with water-soaked sterilized vermiculite and 50 ml of nitrogen-free MS medium. The vermiculite was covered with small sterile stones and the plants were maintained at 28°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. After one week, 100 ml of sterile distilled water was added to each pot.

3.2.4 Harvesting

The plants were harvested and sampled for analysis at 2 and 15 days after inoculation (DAI) for bacterial density determination, and at 3, 9 and 15 DAI for fluorescence microscopy observations.

3.2.5 Determination of microbial populations

To estimate the population densities, replicate inoculated and control plants (n=3) were sampled, and the loosely attached bacteria were removed by washing the roots and aerial segments with sterile distilled water. The plants were cut into small pieces, weighed, and transferred into sterile tubes. Each sample was washed three times with sterile distilled water and homogenated using a mortar and pestle. The macerates were then serially diluted and plated on LB medium containing 10 μg ml^{-1} kanamycin. The green colonies that appeared on the plates after incubation at 28°C were counted using a Nikon SMZ 1500 fluorescence microscope. For the bacterial count in plant tissues after surface sterilization, the samples were surface-sterilized with 70% ethanol for 30 sec and 1% NaClO for 1 min, and then washed with sterile distilled water. The last set of washes used to rinse the tissues was plated on LB medium to determine the efficiency of surface sterilization. The plates were observed after 2-3 days for the presence of bacterial colonies. To determine the effects of the two inoculum treatments using the root dip

method, the plants exposed to inoculum for 1 min or 60 min were subjected to population density determination at 2 DAI. To determine the rhizosphere and endophytic populations, the plants exposed to inoculum for 60 min were subjected to population density determination at 15 DAI with and without prior surface sterilization.

3.2.6 Fluorescence microscopy

The inoculated plants and the corresponding control plants were examined by fluorescence microscopy. The plant samples were washed three times with sterile distilled water. The roots were observed directly to determine the localization of the inoculated bacteria. For observation of endophytic bacteria in the stem, transversal sections of the stem were examined after surface sterilization. The samples were surface-sterilized with 70% ethanol for 1~2 min, followed by washing in sterile distilled water. Transversal sections 0.1-mm thick were prepared with a microslicer. Surface sterilization was carried out to avoid recontamination with the surface bacteria, which swim and stick to the tissues during the slicing procedure. For the leaf sheath observation, longitudinal sections of unsterilized stems were cut by hand. These sections were examined for microscopic fluorescence on a Nikon Eclipse E600 (Nikon, Tokyo, Japan).

3.2.7 Acetylene reduction assay of inoculated rice plants

The nitrogenase activity of the inoculated bacteria (colonizing the surface and the interior tissues of the plant) was determined at 15 DAI. The plants were taken out from the pots

and washed with sterile distilled water three times to remove the loosely associated bacteria. They were then placed in 70-ml glass tubes containing 10 ml of nitrogen-free Fahraeus liquid medium with sugar as the carbon source (Gyaneshwar et al., 2001), and the tubes were sealed with rubber caps. The head spaces of the tubes were filled with 10% [vol/vol] acetylene gas. The tubes were maintained at 28^{o}C under a photoperiod of 16-h light. Ethylene concentrations were measured after 24 h and 48 h using the Shimadzu GC-8A gas chromatograph. Uninoculated plants served as controls.

3.3 Results and Discussion

3.3.1 Methods of inoculum delivery into rice plants

Pantoea and *Enterobacter* belong to the family Enterobacteriaceae, the members of which are frequently associated with rice and other plants, colonizing the rhizosphere, seeds and other plant parts (Barraquio et al., 2000; Ladha et al., 1983; Verma et al., 2001). To identify a suitable method of delivering strains 18-2 and 35-1 into rice, both strains were inoculated into *Oryza sativa* cv. Nipponbare. The populations of both strains in Nipponbare following rhizosphere inoculation (with bacterial suspension) and inoculation via the root dip method were compared. To ensure the efficiency of surface sterilization, the last set of washes used to rinse the tissues was plated on LB medium. In most cases, no bacterial growth was observed. In those very few cases where colonies were observed, the number of bacteria present in the solution was subtracted from the total count of that sample, as described by James et al., (2002).

The population densities of strains 18-2 and 35-1 in *Oryza sativa* cv. Nipponbare following rhizosphere inoculation and inoculation by the root dip method are presented in Tables 3.1-3.3. The root dip method gave larger bacterial populations in the roots and aerial parts than the rhizosphere inoculation method. At 15 DAI, the population densities of *Pantoea* sp. strain 18-2 produced by the root dip method were 40-fold and 6785-fold higher in the unsterilized roots and aerial parts, respectively, than those generated by the rhizosphere inoculation method. The root dip method gave population densities of *Enterobacter* sp. strain 35-1 that were 53-fold and 245-fold higher in the roots and aerial

Table 3.1. Population densities of strain 18-2 and strain 35-1 in *Oryza sativa* cv. Nipponbare plants inoculated with bacterial suspension in the rhizosphere.

Bacteria	Plant part(s)	Population densities (CFU g^{-1} FW)	
		2 DAI	15 DAI
Strain 18-2	Root	2.6×10^5	1.0×10^6
	Aerial parts	9.5×10^4	5.6×10^4
Strain 35-1	Root	3.1×10^6	4.1×10^5
	Aerial parts	8.8×10^4	1.1×10^4

The values represent the means of three replicates.
CFU=colony-forming units, DAI=days after inoculation, FW=fresh weight

Table 3.2. Effect of inoculum exposure time on bacterial population densities of strain 18-2 and strain 35-1 in *Oryza sativa* cv. Nipponbare and *Oryza officinalis* plants inoculated by the root dip method.

Bacteria	Plant part(s)	Bacterial density (CFU g^{-1} FW)			
		Oryza sativa cv. Nipponbare		*Oryza officinalis*	
		1 min	60 min	1 min	60 min
Strain 18-2	Root	6.6×10^{6}	1.9×10^{8}	3.1×10^{5}	1.8×10^{7}
	Aerial parts	1.6×10^{5}	7.6×10^{6}	2.1×10^{4}	6.0×10^{5}
Strain 35-1	Root	1.1×10^{6}	1.4×10^{8}	7.4×10^{5}	7.6×10^{5}
	Aerial parts	5.3×10^{6}	1.5×10^{7}	1.1×10^{6}	2.6×10^{6}

The values represent the means of three replicates.
Bacterial densities were determined at 2 DAI.

Table 3.3. Bacterial numbers of strain 18-2 and strain 35-1 in *Oryza sativa* cv. Nipponbare and *Oryza officinalis* plants inoculated by the root dip method.

Bacteria	Plant part(s)	Bacterial density (CFU g^{-1} FW)				% of endophytic population to total population of associated bacteria	
		Oryza sativa cv. Nipponbare		*Oryza officinalis*		*Oryza sativa* cv. Nipponbare	*Oryza officinalis*
		(US)	(S)	(US)	(S)		
Strain 18-2	Root	4.0×10^{7}	2.5×10^{6}	2.6×10^{6}	8.6×10^{4}	6.25	3.30
	Aerial parts	3.8×10^{8}	2.3×10^{7}	1.2×10^{5}	5.4×10^{4}	6.00	45.00
Strain 35-1	Root	2.2×10^{7}	6.5×10^{6}	7.8×10^{6}	1.2×10^{3}	29.50	0.01
	Aerial parts	2.7×10^{6}	6.6×10^{5}	5.9×10^{6}	2.0×10^{3}	24.40	0.03

The values represent the means of three replicates.
US = not surface sterilized, S= sterilized
To determine the endophytic and total populations of associated bacteria, plants inoculated with 60 min inoculum treatment were subjected to population density at 15 DAI. Populations obtained without surface sterilization represents the population of total associated bacteria (surface and endophytic) whereas the populations obtained after surface sterilization represent the endophytic population.

parts, respectively, than those generated by the rhizosphere inoculation method (Tables 3.1 and 3.3). The population density in the aerial parts was more influenced by the inoculation method. One of the possible reasons might be the presence of higher bacterial populations in the roots (of the plants inoculated by the root dip method compared to the plants inoculated by the rhizosphere inoculation method), which resulted in higher colonization of the stem tissues. The other possibility might be the direct entry of the inoculated bacteria into the basal stem during the washing of the roots to remove the loosely attached bacteria after inoculation.

Seed-transmitted bacteria are already present in plants; however, endophytic bacteria enter plants through root epidermal conjunctions and cracks that occur naturally as a result of root growth (James et al., 2002; Reinhold-Hurek and Hurek, 1998b; Roncato-Maccari et al., 2003). Besides providing entry avenues, wounds create favorable conditions for the invading bacteria by allowing the leakage of plant exudates, which serve as food sources for the bacteria. Bressan and Borges, (2004) studied several inoculation methods for introducing endophytic bacteria into maize, and reported that the highest level of colonization was achieved using the pruned-root dip method. In the present study, even though the roots were not pruned, the process of removing the semisolid agar medium from the roots before root dipping may have caused injuries and wounds to the fine roots and root hairs. As a result, these wounds and other natural openings (as the roots were directly exposed to the inoculum) may have attracted the bacteria and provided additional avenues of entry, so that more bacteria may have

detected. Vermiculite is a porous material that can hold bacteria, thus limiting their contact with the roots, and this might be another possible reason for the comparatively low bacterial populations observed in plants inoculated by the rhizosphere inoculation method compared to those inoculated by the root dip method, where roots are directly exposed to the inoculum.

3.3.2 Bacteria population density determination

The population densities of strains 18-2 and 35-1 in *Oryza sativa* cv. Nipponbare and *Oryza officinalis* are presented in Tables 3.2 and 3.3. The population densities of both strains were higher in cultivated Nipponbare plants than in wild rice plants. In Nipponbare, strain 18-2 showed comparatively higher bacterial populations (1.9×10^8 CFU g^{-1} fresh weight [FW] and 4×10^7 CFU g^{-1} FW in the roots at 2 and 15 DAI, respectively), as compared to strain 35-1 (1.4×10^8 CFU g^{-1} FW and 2.2×10^7 CFU g^{-1} FW) (Table 3.2 and 3.3). The overall pattern of bacterial population growth was similar for both strains. In general, the population densities of both strains were lower in the aerial parts than in the roots. The only exception was observed for strain 18-2, in which more bacteria (3.8×10^8 CFU g^{-1} FW) were observed in the aerial parts than in the roots (4×10^7 CFU g^{-1} FW) at 15 DAI (Table 3.3).

Slightly different results were obtained for wild rice. In addition to the low bacterial populations of both strains in the wild rice plants compared with the cultivated ones, strain 35-1 showed relatively higher bacterial numbers in the roots (7.8×10^6 CFU g^{-}

^{1} FW) and aerial parts (5.9×10^{6} CFU g^{-1} FW) than strain 18-2 (2.6×10^{6} CFU g^{-1} FW and 1.2×10^{5} CFU g^{-1} FW in the roots and aerial parts, respectively) at 15 DAI. Lower bacterial numbers were observed in the aerial parts than in the roots at 15 DAI (Table 3.3).

Endophytic bacteria have been reported to colonize rice roots rapidly and in high numbers after inoculation. It has been reported that a period of 2-7 DAI is enough for the bacteria to penetrate the roots (Gyaneshwar et al., 2001; James et al., 2002). Ruppel et al., (1992) reported high numbers of *Pantoea agglomerans* in the roots and stems of wheat, ranging from 10^{6} to 10^{7} cells ml^{-1} of plant sap. Similarly, *Enterobacter cloacae* has been isolated in high numbers from rice (Mehnaz et al., 2001). For endophytes, the highest bacterial densities are usually observed in the roots, and the numbers decrease from the stem to the leaves (Lamb et al., 1996). A decline in the bacterial populations of strain 18-2 and 35-1 was observed in Nipponbare and wild rice, respectively, at 15 DAI, which may be due to the non-pathogenic nature of these strains, as is the case with other bacterial endophytes (James et al., 1997; Olivares et al., 1997; Pimentel et al., 1991). Unlike true pathogens, endophytes lack the ability to overcome the plant's defenses as the plants mature and grow in size beyond the seedling stage (Hallmann et al., 1997; James et al., 2002).

One of the aims of the present study was to investigate whether endophytic bacteria from different backgrounds exhibit host specificity or preference for specific varieties of rice. Although both strains colonized both cultivated and wild rice, which

suggests the host non-specificity of these endophytic strains, they showed better colonization of cultivated rice (Nipponbare). This indicates that the extent of colonization may vary from host to host. The host non-specificity of endophytes has been reported for members of the same family and of other families. *Klebsiella pneumoniae* (Kp342), which was originally isolated from maize, can colonize the roots of rice, wheat, *Arabidopsis thaliana* and *Medicago sativa* (Dong et al., 2003). *Azoarcus indigens*, which was isolated from Kallar grass, also colonizes rice and sorghum (Egener et al., 1999; Reinhold-Hurek et al., 1993; Stein et al., 1997). Similarly, *Herbaspirillum* sp. strain B501, which was isolated from wild rice, also colonizes sugarcane (Njoloma et al., 2006b).

In order to determine the effect of the inoculum exposure time on the population density, the roots were exposed to inoculum for 1 min or 60 min and the results are presented in Table 3.2. It was observed that, although the 60-min inoculum treatment resulted in higher bacterial populations than the 1-min treatment, an exposure time of 1 min was sufficient for the bacteria to attach themselves to the roots and for subsequent colonization to occur. These results may be helpful in the development of practical techniques for the delivery of diazotrophic endophytes into rice at the field level. In rice production, seedlings are raised in a nursery and then transplanted to the field. The treatment of rice roots with diazotrophic endophytes in the nursery before transplantation may be an efficient method of delivering endophytic diazotrophic bacteria.

To investigate whether the bacteria penetrated the internal tissues of the roots and aerial parts or remained in the rhizoplane, the plants were surface-sterilized and their

population densities were determined at 15 DAI. The results confirmed the endophytic presence of both strains. In roots and aerial parts of Nipponbare, the endophytic populations of strain 18-2 made up 6.25% and 6%, respectively and of strain 35-1 made up 29% and 24.4%, respectively, of the total associated bacteria. In wild rice, the endophytic populations of strains 18-2, in roots and aerial parts respectively, made up 3.3% and 45% of the total bacterial populations. The endophytic populations of strain 35-1 in wild rice were very low (Table 3.3). The surface bacterial populations may vary at different time-points, as they are more directly exposed to adverse environmental conditions than the endophytic bacteria (Reinhold-Hurek and Hurek, 1998b). Thus, large endophytic populations are very important, in that they enable diazotrophic bacteria to fix significant amounts of nitrogen.

3.3.3 Routes of bacterial entry and spread

Fluorescence microscopic observations of the roots, stems and leaves were conducted to elucidate the routes of entry and sites of colonization of the inoculated bacteria in both host plants.

In Nipponbare, primary colonization of the roots by strain 18-2 was observed at the junctions of the lateral roots with the main root for both (1-min and 60-min) inoculum treatments (Figs. 3.1A) at 3 DAI. Relatively fewer colonization sites were observed on the roots of plants exposed to the 1-min inoculation treatment (Fig. 3.1B), compared to the 60-min treatment (Fig. 3.1A). At 9 DAI, heavy colonization of the root surfaces, with

bacteria in the intercellular spaces and in the cracks in the lateral roots on the main root (Fig. 3.1C), was visible. The intercellular spaces and cracks in the lateral roots on the main roots have been reported as entry points of endophytic bacteria in rice and other gramineous crops (Roncato-Maccari et al., 2003). Although assessment of the population density by the plate count method at 2 DAI showed colonization of the aerial parts by both strains (7.6×10^6 and 1.5×10^7 CFU g^{-1} FW for strains 18-2 and 35-1, respectively), examination of transversal sections of stem at 3 DAI showed no endophytic colonization, which indicates that the bacteria were only present on the plant surface at that time and could have been removed by surface sterilization.

The colonizing bacteria moved from the cracks in the lateral and adventitious roots and penetrated deeply to colonize the inner tissues, as evidenced by the endophytic presence of these bacteria in the bases of the adventitious roots on the basal stem (Fig. 3.1D) and in the vascular tissues of the basal stem (Fig. 3.1E) at 9 DAI. Similar modes of penetration of endophytic bacteria into the inner tissues from the sites of entry as those observed in this study have been reported for *Pantoea* sp. in rice (Verma et al., 2004) and wheat (Ruppel et al., 1992). In our previous study on *Brassica oleracea*, strain 35-1 colonized the intercellular spaces at the base of the lateral roots and at the junctions between the lateral roots and the main roots, which indicates that it may follow a similar mode of penetration in monocot and dicot roots. As observed in this study, strain 35-1 did not colonize the leaves of *Brassica oleracea* (Tanaka et al., 2006). Bacterial colonization of roots (Fig. 3.1F), vascular tissues of the stem (Fig. 3.1G), and along the veins of the outer leaf sheath (Figs. 3.1H) was apparent at 15 DAI. No colonization of the leaves was

observed at any stage. *Enterobacter* sp. strain 35-1 showed a colonization pattern similar to that of *Pantoea* sp. strain 18-2 in the roots of Nipponbare plants (Figs. 3.2A, B, C and D). However, the colonization of the leaf sheath and upper stem was not prominent, except for the colonization of cells near the emerging adventitious roots on the basal stem (Fig. 3.2E).

Both strains showed somewhat similar colonization patterns in wild rice to those seen in Nipponbare plants. Fewer colonization sites were observed on the wild rice plants as compared to the Nipponbare plants. At 3 DAI, colonization by strain 35-1 was mainly observed on the root surfaces of the wild rice plants (Fig. 3.3A). Colonization of the intercellular spaces of the root surfaces was also observed (Fig. 3.3B) at 9 DAI. Colonization of the xylem tissues of the lower stem was observed for both strains (Fig. 3.3C and 3.3D for strains 35-1 and 18-2, respectively) in the later harvests. Strain 18-2 also showed colonization of the leaf sheath (Fig. 3.3E).

Colonization of vascular tissues by endophytes has been reported in rice (James et al., 2000), and this is generally believed to be the route through which endophytic bacteria move from the roots to the aerial parts. In the present study strains 18-2 and 35-1 mainly colonized the roots, with occasional colonization of the xylem tissues of the stem. No colonization of the phloem was observed in any case. According to James and Olivares, (1998) these xylem tissues are ideal niches for endophytic bacteria, providing not only an efficient transport route throughout the plant but also an O_2-poor environment. However, Dong et al., (1997) suggested that the xylem is an unsuitable habitat for *Acetobacter diazotrophicus* in sugarcane from the anatomical and physiological point of

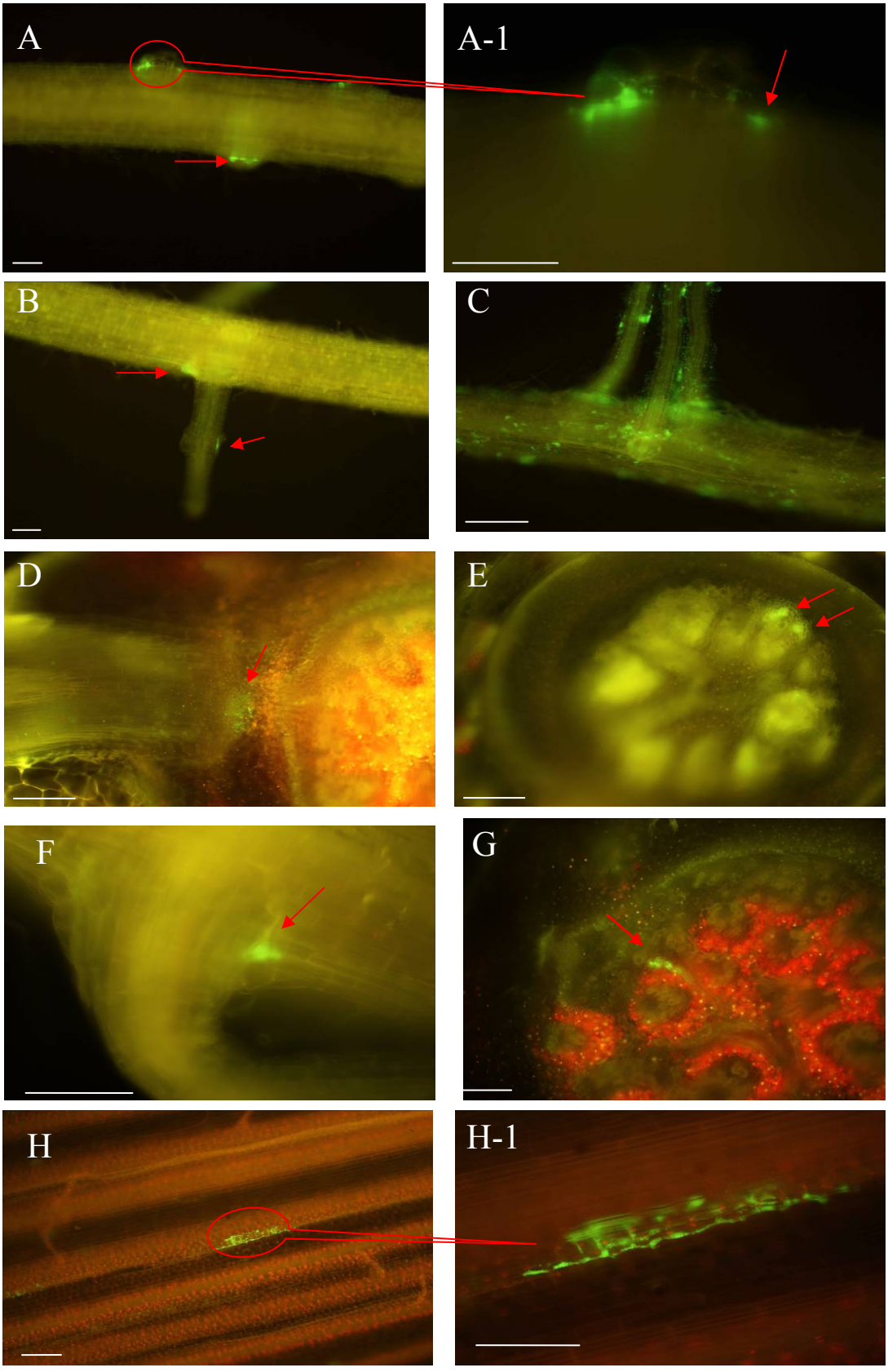
A
A-1
B
C
D
E
F
G
H
H-1

Figure 3.1. Fluorescence micrographs showing the colonization of roots and stem of Nipponbare inoculated with *Pantoea* sp. strain 18-2 by the root dip method. At 3 DAI, plants subjected to 1-min or 60-min inoculum exposure were examined; at 9 and 15 DAI, plants subjected to 60-min inoculum exposure were examined. (A and A-1) Colonization of the intercellular spaces and junctions of the lateral roots at 3 DAI (60-min inoculation treatment). (B) Colonization of the surfaces and junctions of the lateral roots on the main root at 3 DAI (1-min inoculation treatment). (C) Surface colonization of the main and lateral roots at 9 DAI. (D) Bacteria colonizing the junctions of adventitious roots on the basal stem penetrated deeply into the tissues and reached the base of an adventitious root at 9 DAI. (E) Bacteria colonizing the vascular tissues of the stem at 9 DAI. (F) Colonization of the junctions of lateral roots on the main roots at 15 DAI. (G) Bacteria reached the root initiation zone at 15 DAI and colonized the vascular cambium of the basal stem. (H) Colonization of the outer leaf sheath along the veins at 15 DAI. Bar = 10 μm. The arrows show the colonization sites.

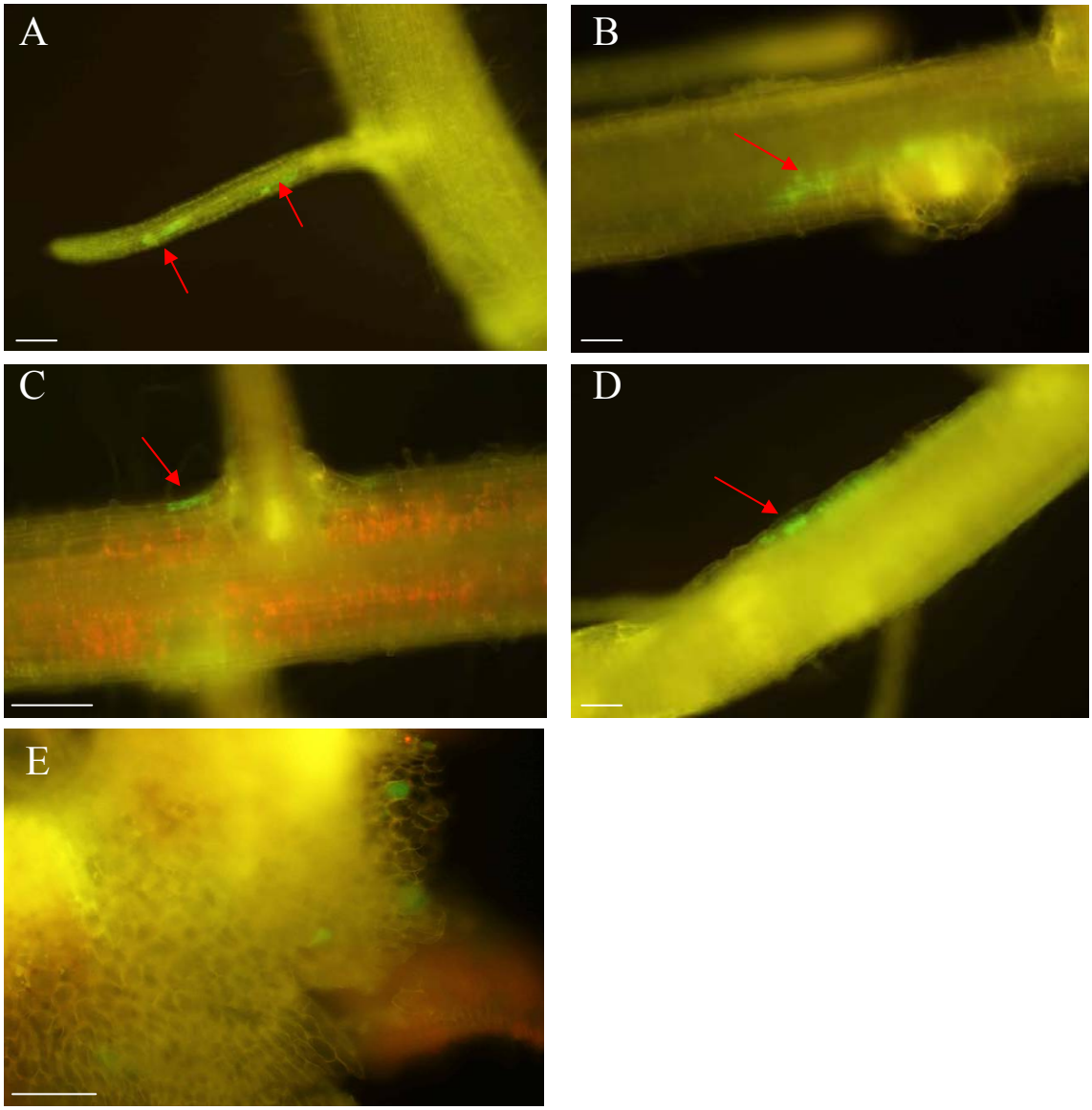

Figure 3.2. Fluorescence micrographs showing colonization of the roots and stem of Nipponbare inoculated with *Enterobacter* sp. strain 35-1 by the root dip method. At 3 DAI, plants subjected to 1-min or 60-min inoculum exposure were examined; at 9 and 15 DAI, plants subjected to 60-min inoculum exposure were examined. (A and B) Bacteria colonizing the intercellular spaces of the main roots and the cracks at the junctions of emerging roots on the main roots of plants subjected to 1-min inoculum exposure at 3 DAI. (C) Colonization of the root surface at the base of a lateral root at 3 DAI (60-min treatment). (D) Surface colonization of the root at 9 DAI. (E) Bacteria colonizing the cells near the base of an adventitious root on the basal stem at 9 DAI. Bar = 10 μm. The arrows show the colonization sites.

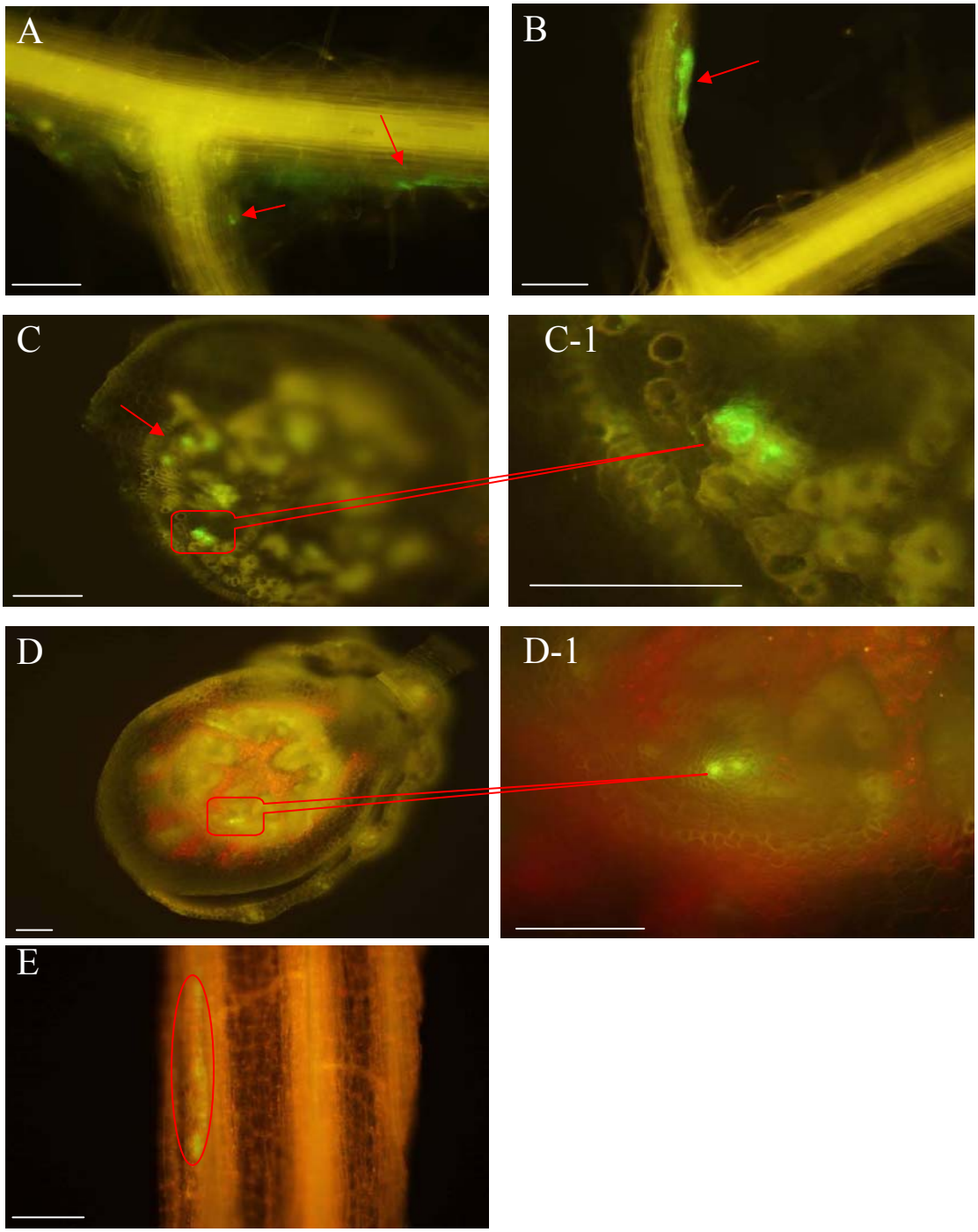
A
B
C
C-1
D
D-1
E

Figure 3.3. Fluorescence micrographs showing the colonization of the roots and stem of wild rice inoculated with *Enterobacter* sp. strain 35-1 (A-C) and *Pantoea* sp. strain 18-2 (D and E) respectively by the root dip method. At 3 DAI, plants subjected to 1-min or 60-min inoculum exposure were examined; at 9 and 15 DAI, plants subjected to 60-min inoculum exposure were examined. (A) Roots surrounded by rhizosphere bacteria, with dark-green colonies indicating the colonization of the surface and intercellular spaces at 3 DAI (60-min inoculation treatment). (B) Endophytic colonization of a thin lateral root at 9 DAI. (C and C-1) Colonization of xylem vessels of the lower stem by strain 35-1 at 9 DAI. (D and D-1) Endophytic colonization of vascular vessels by strain 18-2 at 9 DAI. (E) Bacteria colonizing the outer leaf sheath along the veins at 15 DAI. Bar = 10 μm. The arrows show the colonization sites.

view. It is not always the case that the bacteria entering the xylem conducting system reach other plant parts. According to the structural studies of xylem vessels conducted by Shane et al., (2000a; 2000b), during the maturation of the xylem elements, filters are formed within the xylem conduit system at the branch root junctions and in the nodes. These filters allow easy passage of solutes of small molecular weight, while they filter out particulate matter as small as 5 nm. Thus, any bacteria that enter the xylem of the roots and stem may be stopped at these points. This may explain the failure of these bacteria to colonize the upper stem and leaves of the rice plants in the present study.

Enterobacteriaceae species are generally considered to be rhizosphere colonizers (not endophytes) of sugarcane and other grasses, e.g., *Enterobacter* (Boddey et al., 1991; Sajjad et al., 2001), *Klebsiella* (Sajjad et al., 2001), *Enterobacter cloacae* and *Erwinia herbicola* (now *Pantoea agglomerans*), the latter found only on the exterior of the plant (Rennie et al., 1982). The results of studies in which members of the Enterobacteriaceae have been isolated endophytically from a wide variety of crops support our present findings. Asis and Adachi, (2003) have isolated the diazotrophic endophyte *Pantoea agglomerans* and the nondiazotrophic *Enterobacter asburiae* from the stem of a sweet potato species. Strains of *Pantoea agglomerans* have been isolated from the seeds of deep-water rice and have been reported to colonize these plants endophytically (Verma et al., 2001; Verma et al., 2004). *Enterobacter cloacae* (Hinton and Bacon, 1995) and *Pantoea agglomerans* (Ruppel et al., 1992), have also been described as endophytes in corn and wheat.

3.3.4 Nitrogenase activity of inoculated plants

Both strains showed significant acetylene reduction activity in LGIP medium (data not shown). The nitrogenase activity of free-living bacteria in media is quite different from their ability to fix nitrogen in plants (Egener et al., 1998). To assess the nitrogenase activity of the colonized bacteria, inoculated and uninoculated plants were subjected to acetylene reduction assay. Both types of inoculated host plants showed significantly higher acetylene reduction activity compared to uninoculated plants (Table 3.4). In Nipponbare, strain 18-2 showed higher acetylene reduction activity (2.3 $\pm$ 0.49 nmol plant^{-1} h^{-1}) than strain 35-1 (1.50 $\pm$ 0.52 nmol plant^{-1} h^{-1}). Although both strains showed relatively lower acetylene reduction activity in wild rice than in Nipponbare, they followed similar patterns, with strain 18-2 showing higher acetylene reduction activity (1.28 $\pm$ 0.59 nmol plant^{-1} h^{-1}) than strain 35-1 (0.59 $\pm$ 0.06 nmol plant^{-1} h^{-1}) (Table 3.4).

The nitrogenase activities of *Pantoea* spp. and *Enterobacter* spp. in plants, as assessed by acetylene reduction assay, have been reported previously. Verma et al., (2001) have observed high acetylene reduction activity in the aerial parts of deep-water rice colonized by *Pantoea agglomerans*. *Enterobacter cloacae* isolates from the rhizospheres of rice and maize also posses nitrogen-fixing abilities (Ladha et al., 1983; Raju et al., 1972). The acetylene reduction activity shown by both strains in Nipponbare plants was higher than that observed in some previous studies of cultivated rice. Elbeltagy et al., (2001) have reported an acetylene reduction activity of 0.18 nmol plant^{-1} h^{-1} at 7 DAI in seedlings of *Oryza sativa* cv. Sasanishiki inoculated with *Herbaspirillum*

sp. James et al., (2002) have reported acetylene reduction activity values for the wetland rice varieties IR42 (1.3 nmol plant^{-1} h^{-1}) and IR72 (0.87 nmol plant^{-1} h^{-1}) inoculated with *Herbaspirillum seropedicae*.

The acetylene reduction activity values represent the contribution of the total associated bacteria (endophytic and surface-colonizing bacteria). Large endophytic populations enable diazotrophic bacteria to fix significant amounts of nitrogen. However, Gyaneshwar et al., (2002) reported that most of the acetylene reduction activity comes from the bacteria on the surface, as most bacteria in their study, particularly those expressing the nitrogenase Fe-protein, were observed only on root surfaces.

In the present study, the plants were maintained in nitrogen-free Fahraeus liquid medium containing sucrose as a carbon source, as described by Gyaneshwar *et al.* (2001). It might be possible that the bacteria colonizing the surface of the roots may be liberated to the liquid medium, grow and contribute acetylene reduction activity. In this study no experiment was performed in this respect; however, Gyaneshwar et al., (2001) and James et al., (2002), in their studies on the colonization of rice by endophytic bacteria, observed nitrogenase activity (acetylene reduction activity) only after a carbon source was added to the Fahraeus liquid medium, and plants without any carbon source in the Fahraeus liquid medium failed to show any acetylene reduction activity. This indicates that endophytic bacteria need a carbon source to perform acetylene reduction. Chelius and Triplett, (2000) have also reported dinitrogen reductase activity in maize infected with *Klebsiella pneumoniae* when a carbon source was added to the medium.

Table 3.4. Acetylene reduction activities in rice plants inoculated with strain 18-2 and strain 35-1.

Plant	Bacterial strains	Ethylene evolution (nmol plant^{-1} h^{-1})
Nipponbare	18-2	2.30 $\pm$ 0.49
	35-1	1.50 $\pm$ 0.52
	Control	0.10 $\pm$ 0.02
Wild rice	18-2	1.28 $\pm$ 0.59
	35-1	0.59 $\pm$ 0.06
	Control	0.11 $\pm$ 0.02

The values represent the means $\pm$ SD of three replicates.

From these results, it is difficult to establish any relationship between the GFP expression pattern and the actual acetylene reduction activity in the plants, as the acetylene reduction activity was assessed only once, at 15 DAI. Strain 18-2 showed higher acetylene reduction activity compared to strain 35-1 in both hosts, even if its population was lower than that of strain 35-1 in wild rice. Strain 18-2 also showed higher acetylene reduction activity than strain 35-1 (data not shown) in LGIP medium under similar conditions, which may explain its higher acetylene reduction activity values in inoculated plants.

3.4 Conclusion

The results presented here show that *Pantoea* sp. strain 18 and *Enterobacter* sp. strain 35 become established in the roots and lower stems and can fix nitrogen in rice plants other than their original hosts. Although these strains do not show authentic host specificities, they appear to have preferences for specific hosts, as they show better colonization of cultivated rice than wild rice. The results presented in this study were obtained under the specific growth conditions and may be affected by the change in growth conditions.

Chapter 4 COLONIZATION AND GROWTH PROMOTION CHARACTERISTICS OF *Enterobacter* sp. AND *Herbaspirillum* sp. ON *Brassica oleracea*

4.1 Introduction

Brassica oleracea (broccoli), belonging to the Brassicaceae family, is a vegetable crop that requires great amounts of nitrogen fertilizer. To obtain a high yield, 270 to 465 kg ha^{-1} of nitrogen are needed (Feller and Fink, 2005; Herrero et al., 1999). Like other non-leguminous plants, broccoli does not have a natural association with root-nodulating bacteria such as *Rhizobia*. Several endophytic bacteria which can fix atmospheric nitrogen in their hosts have been reported in non-leguminous plants, mainly cereals. Currently, there are no reports of the isolation of and infection by diazotrophic endophytic bacteria in broccoli. Although, nitrogen-fixing *Azospirillum* strains have been isolated from the roots of *Brassica juncea* (Saha et al., 1985), *Brassica chinensis* (Chinese cabbage) and *Brassica rapa* (Chinese mustard) (Gamo and Ahn, 1991) which also belongs to the Brassicaceae family. Similarly, nitrogen-fixing pseudomonades have been reported and isolated from the roots of canola (*Brassica campestris*) (Lifshitz et al., 1986).

Many endophytic bacteria can establish themselves in plants other than their original hosts, indicating a lack of host specificity. The absence of host specificity in endophytes has been reported for members of the same family (from which they are isolated) and of other families. *Klebsiella pneumoniae* (Kp342), which was originally

isolated from maize, can colonize the roots of rice, wheat, *Arabidopsis thaliana* and *Medicago sativa* (Dong et al., 2003). *Azoarcus indigens*, which was isolated from Kallar grass, also colonizes rice and sorghum (Egener et al., 1999; Reinhold-Hurek et al., 1993; Stein et al., 1997). Similarly, *Burkholderia* sp. isolated from onions can colonize grapes (Compant et al., 2005), potatoes and vegetables (Nowak et al., 1995). Thus, the potential exists to introduce non-indigenous endophytes that can contribute to plant growth.

Bacteria belonging to the genera *Herbaspirillum* and *Enterobacter* are frequently associated with plants, colonizing the rhizosphere and other plant parts. Strains of *Herbaspirillum* have been discovered in rice, sugarcane, maize, bananas and pineapples (Baldani et al., 1986; Chelius and Triplett, 2001; Elbeltagy et al., 2001; James et al., 2002; Weber et al., 1999). *Enterobacter* strains are found in rice, maize and other grasses (Fujii et al., 1987; Raju et al., 1972)). As no endophytic bacteria have been reported in broccoli the aim of this study was to find a suitable nitrogen-fixing association between endophytic bacteria (isolated from other plants) and *B. oleracea* which can enhance plant growth. For this purpose, *Herbaspirillum* sp. strain B501 (a wild rice isolate) and *Enterobacter* sp. strain 35 (a sugarcane isolate) were used and their colonization and nitrogen-fixing abilities were studied.

4.2 Materials and Methods

4.2.1 Bacterial strains and inoculum preparation

Inoculum was prepared from bacterial cultures grown in LB broth for 24 h. The bacterial suspensions were centrifuged at 6,500 rpm for 10 min at 25°C. The supernatant was

discarded and the pellet was resuspended in sterile N-free MS medium. The bacterial suspensions were adjusted to 10^8 cells ml^{-1} using a hemocytometer under a microscope (Nikon Eclipse E600, Nikon, Japan). Two experiments, i.e. a glasshouse experiment (semi-natural conditions) and a laboratory-scale experiment (artificial conditions), were conducted. For the glasshouse experiment, the wild types (strain B501 and strain 35) of these strains were used, and for the laboratory-scale experiment, GFP-labeled strains (strain B501*gfp*1 and strain 35-1) were used.

4.2.2 Glasshouse experiment

4.2.2.1 Plant growth conditions and inoculation

Seeds of *B. oleracea* cv. Ryokurei were germinated in 250-ml plastic pots containing a commercially available potting mixture with 110 mg, 98 mg and 102 mg of nitrogen, phosphorus and potassium, respectively. One month after seeding, the plants were uprooted and the roots were washed with sterile distilled water to remove all the attached potting mixture; then the plants were transferred to modified Leonard jars (Fig. 2.1). The plants were maintained in the glasshouse under semi-natural conditions. After one week, the MS medium containing nitrogen was replaced with nitrogen-free MS medium. Next day, the plants were inoculated with 200 ml of the inoculum suspension (10^8 cells ml^{-1}) of strain B501 and 35 by removing the sand stones that covered the vermiculite and evenly pouring the suspension onto the vermiculite in the jars, without disturbing the plant root system. For the control plants, the same amount of sterile N-free MS medium was added. After pouring on the inoculum, the vermiculite was covered once again with sterile sand stones. The temperature ranged from 13 to 28°C during the experiment.

4.2.2.2 Plant growth

Plant growth was determined by comparing the fresh weights of the inoculated (with wild-type strains) and uninoculated plants. Thirty days after inoculation, replicate inoculated and control plants (n=3) were sampled and washed with tap water to remove the attached vermiculite. The roots were dried with a paper towel and weighed. The results were analyzed by the method of multiple comparison (Newman-Keuls multiple comparison of mean) using TexaSoft, WINKS SDA software, 6th edition, Cedar Hill, Texas, 2007.

4.2.3 Laboratory-scale experiment

4.2.3.1 Plant growth conditions and inoculation

Seeds of *B. oleracea* cv. Ryokurei were germinated in 250-ml plastic pots, and 30 days after seeding the plants were transplanted to modified Leonard jars (Fig. 2.1), as described previously. The plants were maintained at 13°C (night temperature) and 25°C (day temperature) 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. After six days, the MS medium containing nitrogen was replaced with N-free MS medium. The plants were inoculated with 200 ml of the inoculum suspension (10^8 cells ml^{-1}) of strains B501*gfp*1 and 35-1, as described above. Fifteen days after inoculation, 300 ml of MS medium containing K_2SO_4 (16.4 g l^{-1}) were poured onto the vermiculite in each pot.

4.2.3.2 Determination of microbial populations

To estimate the population densities, replicate inoculated and control plants (n=3) were sampled at 8 and 30 DAI, and loosely attached bacteria were removed by washing the roots and aerial segments with distilled water. The plants were divided into roots, stem and leaves, cut into small pieces, weighed, and transferred to sterile tubes. Each sample was washed three times with sterile distilled water and macerated using a mortar and pestle. The macerates were then serially diluted and plated on LB medium containing 10 μg ml^{-1} kanamycin. The green colonies that appeared on the plates after incubation at 28°C were counted using a Nikon SMZ 1500 fluorescence microscope. In order to count the bacteria in the plant tissues after surface sterilization, samples were surface-sterilized with 70% ethanol for 30 sec and 1% NaClO for 1 min, and then washed with sterile distilled water.

4.2.3.3 Fluorescence microscopy

Inoculated plants and the corresponding control plants were examined under a fluorescence microscope at 8 and 30 DAI. Plant samples were washed three times with sterile distilled water and divided into roots, stem and leaves. The roots were observed directly to determine the localization of the inoculated bacteria. In order to observe the endophytic bacteria in the stem, transversal sections of the stem were cut manually after surface sterilization. The samples were surface-sterilized with 70% ethanol for 1-2 min,

followed by washing in sterile distilled water. These sections were examined for microscopic fluorescence using a Nikon Eclipse E600 (Nikon, Tokyo, Japan).

4.2.3.4 Acetylene reduction assay (ARA) of broccoli plants

The nitrogenase activity in inoculated and control plants was determined at 8 and 30 DAI. Sampled plants were washed with sterile distilled water and placed in 400-ml plastic jars. Five ml of nitrogen-free Fahraeus liquid medium with sugar was added in each jar to supply a carbon source and to keep the roots moist, and the jars were sealed with rubber caps. The head spaces of the jars (10% [vol/vol]) were filled with acetylene gas. The jars were maintained at 25^{o}C in the dark. The ethylene gas concentration was assayed after 24 h and 48 h using a Shimadzu GC-8A gas chromatograph. The results were analyzed by the method of multiple comparison (Newman-Keuls multiple comparison of mean) using TexaSoft, WINKS SDA software, 6th edition, Cedar Hill,Texas, 2007.

4.3 Results and Discussion

4.3.1 Plant growth

The study was conducted to determine the growth-promoting and colonization abilities of two endophytes, strain B501 and strain 35, in broccoli. One of the desirable characteristics of diazotrophs is that their ability to influence plant growth through nitrogen fixation in intact plants. To evaluate the growth-promotion abilities of these endophytes, the plants were inoculated with the wild types of these strains and maintained in semi-natural conditions. A comparison of the fresh weights of the inoculated plants with those of the uninoculated plants at 30 DAI suggests that both strains have growth-promoting abilities (Fig. 4.1). Strain 35 significantly increased the fresh weight of *B. oleracea* compared to the uninoculated plants whereas fresh weigh of plants inoculated with strain B501 tended to be higher than that of uninoculated plants. Strain 35 induced 26 % higher fresh weights than strain B501 (Fig. 4.2). *Herbaspirillum* sp. has been reported to enhance plant growth in rice (James et al., 2002) and sugarcane (Njoloma et al., 2006b) by increasing their biomass. Species of *Enterobacter* are also reported to enhance plant growth in rice (Fujii et al., 1987). Endophytic bacteria increase plant growth by enhancing the nitrogen content (James et al., 2002) and by excreting phytohormones, which lead to increased photosynthesis and enhancement of the root surface and thus an increase nutrient uptake (Chi et al., 2005; Dobbelaere et al., 1999; Okon and Labandera-Gonzalez, 1994).

Figure 4.1. At 30 DAI, the plants grown in the glasshouse were harvested and their fresh weights were compared. (A) Plants inoculated with *Herbaspirillum* sp. strain B501 (1-3) and their corresponding controls (4-6). (B) Plants inoculated with *Enterobacter* sp. strain 35 (7-9) and their corresponding controls (10-12).

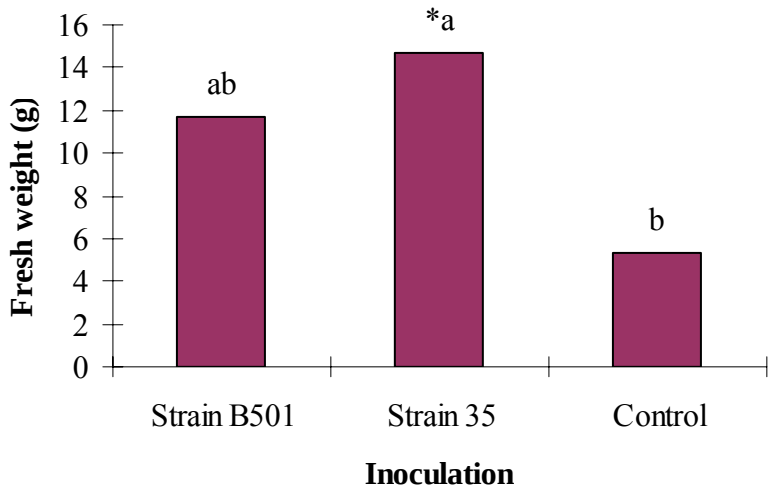

Figure 4.2. Fresh weights in grams of plants inoculated with 10^8 bacteria ml^{-1} inoculum doses of *Herbaspirillum* sp. strain B501 and *Enterobacter* sp. strain 35 at 30 DAI. The values represent the means of three replicates.

Means with a common letter suffix do not differ at a 5% level of probability (Newman-Keuls multiple comparison of mean)
*$P \leq 0.05$

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4.3.2 Population density

The population densities of strain B501*gfp*1 and 35-1 in the roots and aerial parts were determined by the colony count method. The densities of the inoculated bacteria in the roots indicated that both strains were able to colonize the broccoli plants at 8 DAI (Table 4.1). Differences were observed between the two strains. Strain 35-1 showed a more intense colonization of the roots (8.1×10^6 CFU g^{-1} FW) than strain B501*gfp*1 (1.7×10^4 CFU g^{-1} FW) at 8 DAI. Strain 35-1 also colonized the stem in high numbers (2.8×10^6 CFU g^{-1} FW), whereas no colonization of the stem by strain B501*gfp*1 was observed (Table 4.1). To investigate whether the bacteria penetrate the internal tissues of the roots and stem or are merely present epiphytically, the inoculated plants were processed after surface sterilization. Only colonization by strain 35-1 was observed in the surface-sterilized roots and stems, which indicates that the strain can colonize the internal tissues in broccoli, furthermore, strain B501*gfp*1 was probably present only epiphytically or entered the tissues in small numbers. The population count of strain 35-1 in the unsterilized roots was higher than that in the stems (Table 4.1). Higher populations of endophytic bacteria in the roots than in the stem, as we observed here, have been reported by Gyaneshwar et al., (2001) and James et al., (2002). The result of the present study is in contrast to the findings of Barraquio et al., (1997) and Elbeltagy et al., (2001), who have reported that the numbers of nitrogen-fixing bacteria in rice are higher in the stem than in the roots. Bacterial population of both strains in the roots tended to decrease at 30 DAI. Colonization of strain 35-1 was observed on the surface and in the internal tissues of root

at that stage whereas strain B501*gfp*1 only showed surface colonization of the roots. No colonization of the aerial parts by either strain was observed at that stage.

Both strains have been reported to colonize their hosts in large number in previous studies. Strain B501*gfp*1 populations of 10^7 CFU g^{-1} FW in rice shoots (Elbeltagy et al., 2001) and 10^6 CFU g^{-1} FW in sugarcane roots (Njoloma et al., 2006b) have been reported previously. Similarly, a strain 35-1 population of 10^8 CFU g^{-1} FW was observed in the Nipponbare rice variety (Table 3.2). However, in this study heavy colonization of strain 35-1 only was observed in the roots and stem at 8 DAI and population density decreases with time and no colonization of aerial part was observed at 30 DAI. The colonization by strain B501*gfp*1 was very weak at both 8 and 30 DAI. Dong *et al.* (2003) reported that the population density of grass endophytes in dicots is generally lower than the population density in grasses. They observed 100 times more cells of inoculated bacteria in grasses than in dicots. The reason may be that the apoplast volume in grasses is larger than that in dicots, and grasses secrete more sucrose into the apoplast than do dicots, thus providing a better food supply and more space for the endophytes. Another possible reason is that the host plant defense mechanism of dicots is stronger, preventing entry into the apoplast of large numbers of endophytes.

4.3.3 Localization of the inoculated bacteria in broccoli

The roots, transversal sections of the stem and leaves were observed under a fluorescence microscope to determine the route of entry and colonization sites of the inoculated

Table 4.1. Population densities of *Herbaspirillum* sp. strain B501*gfp*1 and *Enterobacter* sp. strain 35-1 in *Brassica oleracea*, at 8 and 30 DAI.

Plant parts	Bacterial numbers (CFU g^{-1} FW)			
	After 8 days		After 30 days	
	Strain 35-1	Strain B501*gfp*1	Strain 35-1	Strain B501*gfp*1
Roots (US)	8.1×10^{6}	1.7×10^{4}	4.5×10^{6}	9×10^{3}
Roots (S)	6.6×10^{4}	0	2.3×10^{4}	0
Stem (US)	2.8×10^{6}	0	0	0
Stem (S)	7.5×10^{4}	0	0	0
Leaves (US)	0	0	0	0
Leaves (S)	0	0	0	0

The values represent the means of three replicates.
US = not surface-sterilized, S = surface-sterilized, DAI= days after inoculation

bacteria in different plant tissues. The results of the fluorescence microscopic observations confirmed the results of the population density determination. At 8 DAI, strain B501*gfp*1 was apparently localized in the rhizosphere and occasionally colonized the surface and intercellular spaces of the roots (Figs. 4.3A and 4.3B). The dense rhizosphere colonization which was observed at 8 DAI was not observed at 30 DAI, and only the colonization of the intercellular spaces of the lower fibrous roots of the main and adventitious roots was observed (Figs. 4.3C and 4.3D). The entry of strain B501*gfp*1 into the stem and leaves was not detected by fluorescence-microscopic observation.

Higher colonization of the roots by strain 35-1 than strain B501*gfp*1 was observed on both sampling occasions. At 8 DAI, strain 35-1 colonized the intercellular spaces (Fig. 4.4A) of the main and adventitious roots and the junctions of the emerging roots to the parent roots (Figs. 4.4B and C). The colonization of the intercellular spaces of the lower roots was also observed at 30 DAI (Figs. 4.4D, E and F). Strain 35-1 also entered the stem and colonized the metaxylem tissues (Fig. 4.4G) to some extent, as its presence was not observed frequently under the fluorescence microscope. No colonization of the leaves by strain 35-1 was observed. The colonization of the base of the emerging lateral roots and of the intercellular spaces of the main root near the emerging lateral roots observed in this study (Fig 4.4B and C) and in our preliminary study (Tanaka et al., 2006) shows that these may be the points of entry into the roots of *B. oleracea*. A similar pattern of colonization by strain 35-1 was observed in rice (Fig. 3.2 and 3.3). Furthermore, a similar invasion by non-diazotrophic endophytes has been reported in dicotyledonous plants (Quadt-Hallmann et al., 1997; Quadt-Hallmann and Kloepper, 1996), suggesting that

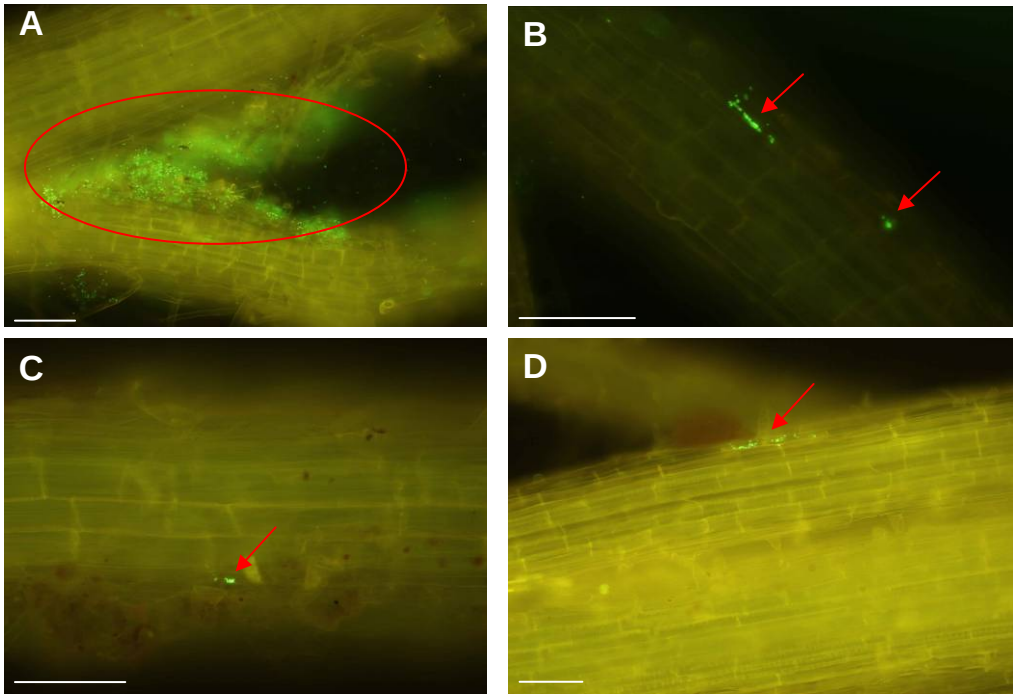


Figure 4.3. Fluorescence micrographs showing the colonization of the roots by *Herbaspirillum* sp. strain B501*gfp*1. (A) Bacteria colonizing the surface and rhizosphere of an adventitious root at 8 DAI. (B) Colonization of the intercellular spaces of the roots at 8 DAI. (C and D) Small bacterial colonies are visible along the intercellular spaces of the roots at 30 DAI. Bar = 10 µm. The arrows show the colonization sites.

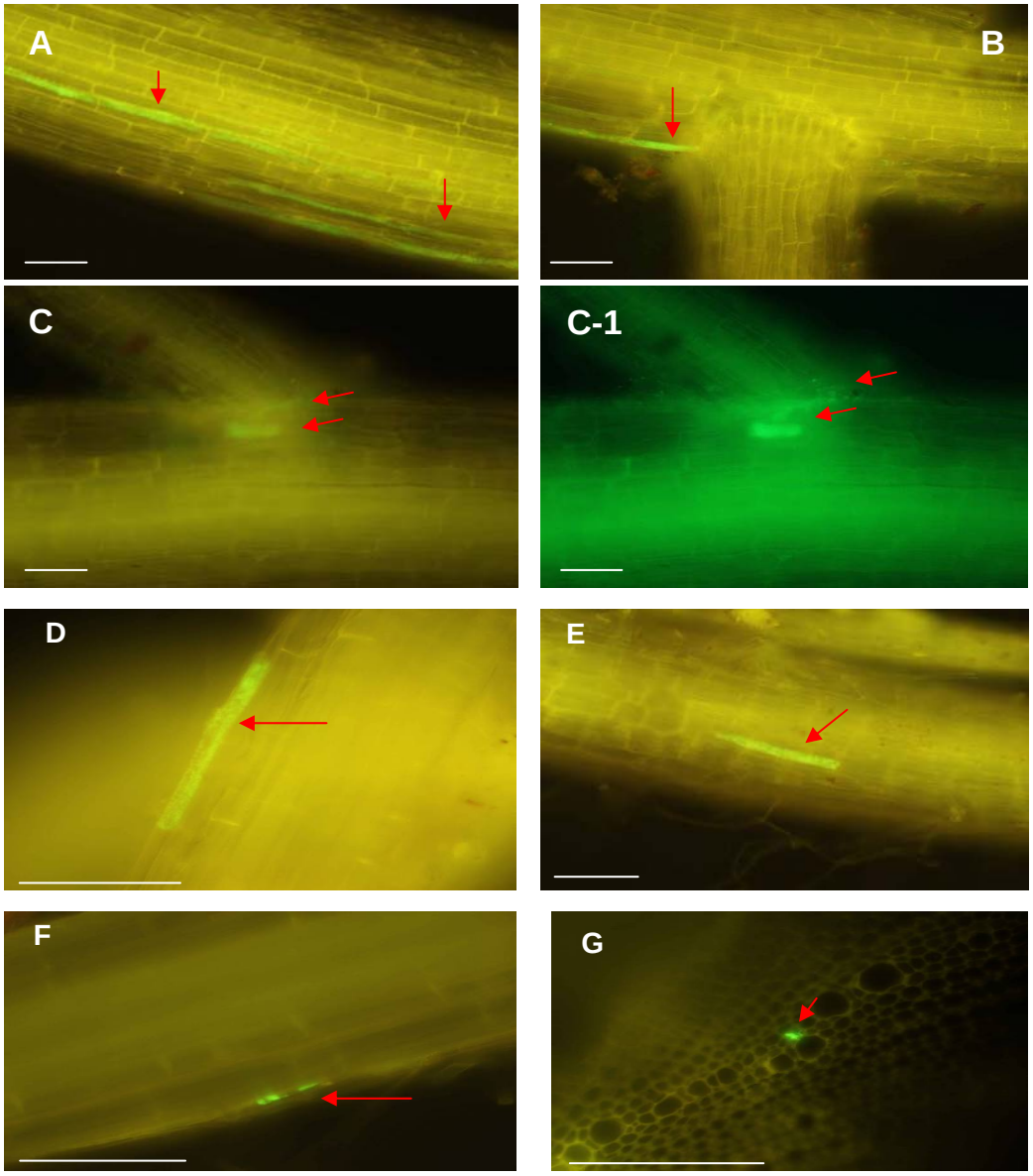


Figure 4.4. Fluorescence micrographs showing colonization of the roots and stem by *Enterobacter* sp. strain 35-1. (A) Endophytic colonization of the roots forming long lines of bacteria along the intercellular spaces at 8 DAI. (B) Bacteria colonizing the junction of the lateral root to the main root and intercellular spaces at 8 DAI. (C and C-1) Colonization at the base of the lateral root emerging from the main root at 8 DAI. (D, E and F) Bacterial colonization of the roots at 30 DAI. Bacteria colonizing the intercellular spaces under the root surface (D and E) and the surface (F). (G) Transversal sections of the stem showing the presence of bacteria in the metaxylem tissues at 30 DAI. Bar = 10 µm. The arrows show the colonization sites.

endophytic bacteria might have similar mechanisms of interaction with both monocotyledonous and dicotyledonous plants.

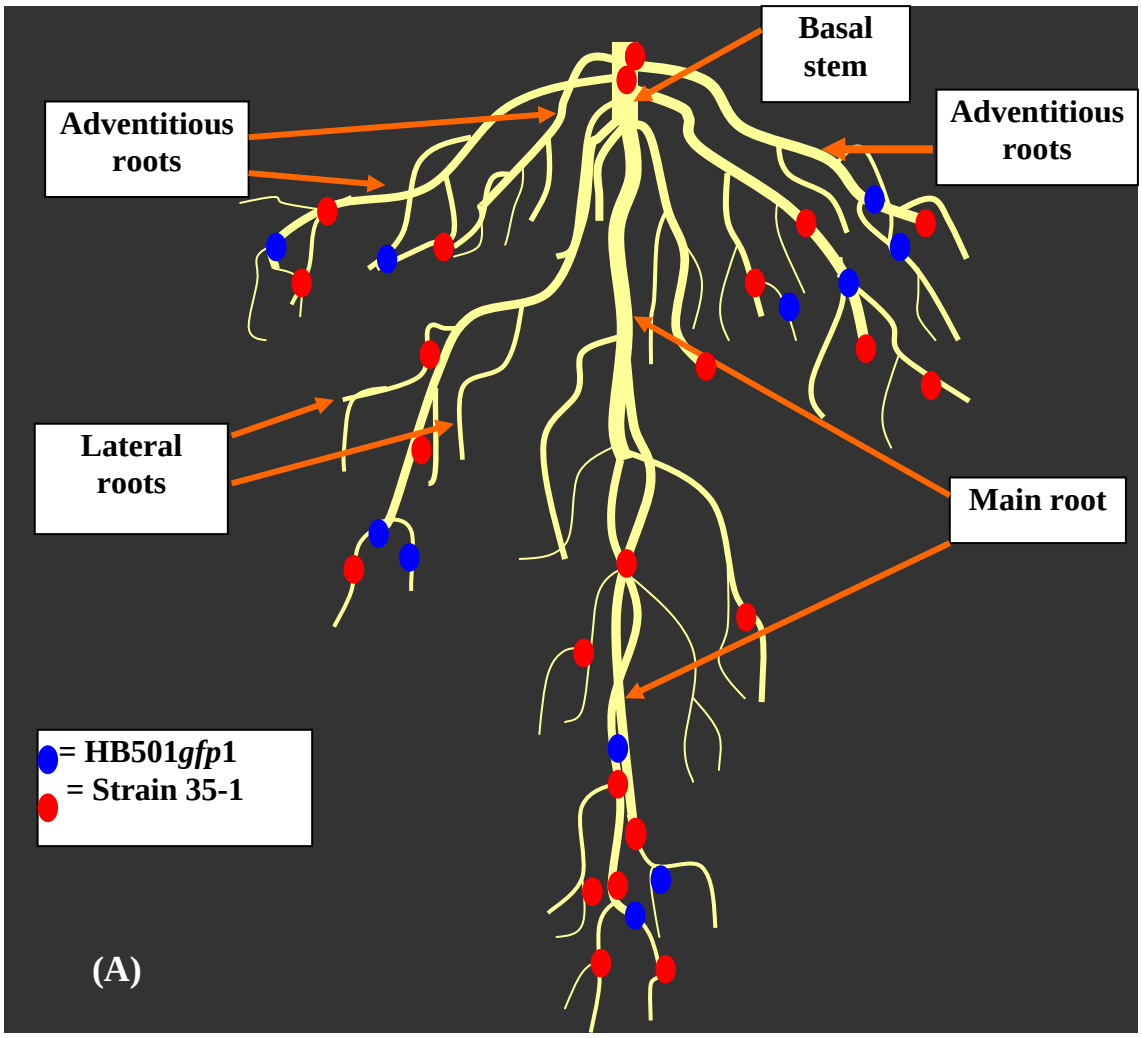
Mostly the colonization of the lower parts (young emerging roots) of the main and adventitious roots by both strains was observed in this study on both sampling occasions (Figs. 4.5A and B). The exact reason for this was not understood, but one possibility might be the contribution and composition of the root exudates from young and old roots, which need to be investigated. Myers et al., (2001) have reported that the microbial population size and diversity in soil were controlled by the amount and types of available organic compounds entering the soil through different pathways, of which the contribution from root exudates was regarded as the most important. The possible roles of the host defense mechanism, the age of the plant at the time of inoculation and the structure of the roots might be the other reasons of the low bacterial population observed in the aerial parts which gradually decreased with time in this study.

Fluorescence-microscopic observations of the transversal sections of the stem showed sparse colonization of vascular tissues. Therefore, the potential for upward movement through the transpiration stream appears to be minimal, which may explain the reason for their low numbers in the aerial parts. In this situation, the movement of endophytic bacteria from the roots to the stem might be due to the formation of long lines of intercellular bacteria along the lateral and adventitious roots (Figs. 4.4A and E), as described by Elbeltagy et al., (2001) for strain B501*gfp*1 in rice.

4.3.4 Nitrogenase activity in broccoli plants

The nitrogenase activity of inoculated bacteria in intact plants was assessed by acetylene reduction activity. Plants inoculated with strain 35-1 showed higher acetylene reduction activity compared to uninoculated plants on both sampling occasions, whereas acetylene reduction activity of plants inoculated with strain B501*gfp*1 tended to be higher than that of uninoculated plants. At 8 DAI plants inoculated with strain 35-1 showed significantly higher acetylene reduction activity (4.37 nmol hr^{-1} $plant^{-1}$) compared to uninoculated plants, whereas non-significant results were observed between the two strains at that time. At 30 DAI plants inoculated with strain 35-1 showed significantly higher acetylene reduction activity (8.79 nmol hr^{-1} $plant^{-1}$) compared to strain B501*gfp*1 and uninoculated plants. Strain 35-1 also showed significantly high acetylene reduction activity at 30 DAI compared to 8 DAI (Table 4.2).

The reason for the high acetylene reduction activity of strain 35-1 might be due to its presence in the internal tissues and on the root surface. In this study strain B501*gfp*1 only colonizes the surface of the roots and that might be the reason of the relatively lower acetylene reduction activity of plants inoculated with strain B501*gfp*1 compared to that with strain 35-1. Gyaneshwar et al., (2002) have reported the acetylene reduction activity for the surface colonizing bacteria, as most bacteria in their study, particularly those expressing the nitrogenase Fe-protein, were observed only on root surfaces. The transfer of nitrogen from the root surface to plants in cases where large bacterial populations are present on the root surface or in the rhizosphere presumably occurs through bacterial


Basal stem
Adventitious roots
Adventitious roots
Lateral roots
Main root
= HB501*gfp*1
= Strain 35-1
(A)

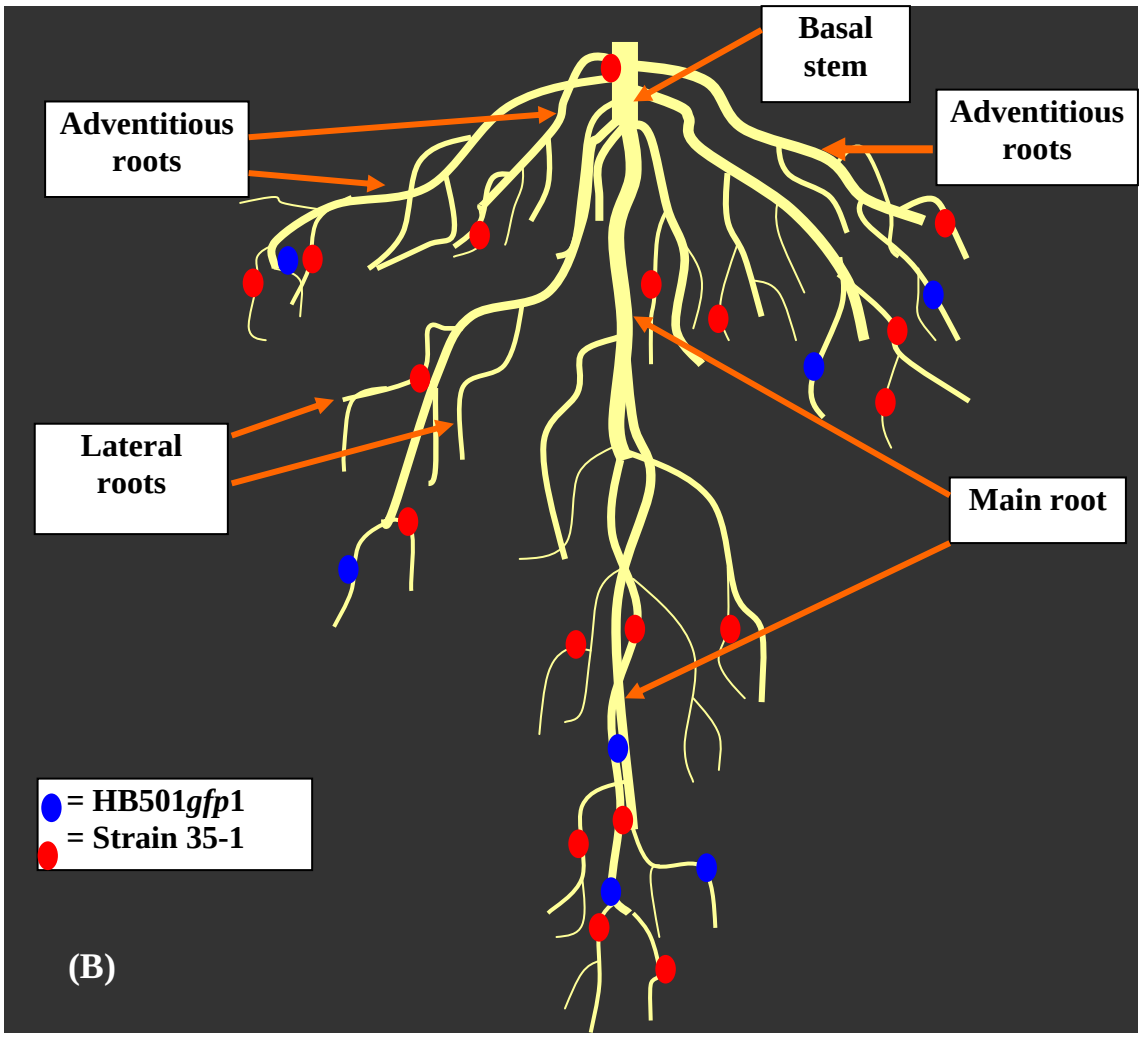


Figure 4.5. Colonization sites of *Herbaspirillum* sp. strain B501*gfp*1 and *Enterobacter* sp. strain 35-1 on the roots of *Brassica oleracea* at 8 DAI (A) and 30 DAI (B).

Table 4.2. Acetylene reduction activity of broccoli plants inoculated with *Herbaspirillum* sp. strain B501*gfp*1 and *Enterobacter* sp. strain 35-1.

Inoculation	Acetylene reduction activity (nmol plant^{-1}h^{-1})		
	8 DAI	30 DAI	Significance
Uninoculated	0.22[a]	1.00[a]	
Strain 35-1	4.37[b]	8.79[b]	*
Strain B501*gfp*1	2.45[ab]	2.05[a]	
Significance	*	*	

Means with a common letter suffix in a column do not differ at a 5% level of probability (Newman-Keuls multiple comparison of mean)
*$P \leq 0.01$

decomposition (Okon et al., 1983). A significant increase in plant growth, in parallel with significant quantities of fixed nitrogen, observed by Gyaneshwar et al., (2002) and James et al., (2002), in rice inoculated with *Herbaspirillum seropedicae* suggests that the endophytic bacteria contribute to plant growth by providing fix nitrogen. However, endophytic bacteria are also reported to promote plant growth without enhancing nitrogen content, which implies that endophytic bacteria influence plant growth by means other than nitrogen fixation (Gyaneshwar et al., 2001) such as the excretion of phytohormones (Chi et al., 2005; Dobbelaere et al., 1999; Okon and Labandera-Gonzalez, 1994).

Higher acetylene reduction activity of inoculated plants compared to the uninoculated plants in this study illustrate that higher fresh weight of the inoculated plants may be due to the nitrogenase activity of these bacteria. The plants were kept in the dark for determination of acetylene reduction activity in this study as reported by James et al., (2002). The acetylene reduction activity could have been different if the plants were exposed to the light as reported by (You et al., 2005). In their study on rice by strain B501*gfp*1 they found that the amount of *nif*H transcription increases when plants were exposed to the light. Thus it would be worth to estimate the actual amount of nitrogen fixed by these bacteria under different conditions in future studies to develop a suitable interaction between these bacteria and broccoli plants.

4.4 Conclusion

The study showed that strains 35 and B501 could colonize broccoli plants, more so strain 35. The results of the population density, fluorescence microscopy, and acetylene reduction activity analyses support the findings of the glasshouse experiment. Strain 35-1 produced both epiphytic and endophytic populations, whereas strain B501*gfp*1 produced mainly epiphytic populations. This may be one of the reasons for the better performance of strain 35 in broccoli, as plant growth promotion is often greater when induced by endophytes than by epiphytic or rhizospheric bacteria (Chanway et al., 2000; Conn et al., 1997). To our knowledge, apart from our preliminary work (Tanaka et al., 2006), this is the first detailed work on the possible role of diazotrophic endophytes in growth promotion of broccoli. The results indicate that, to some extent, epiphytic populations (as observed in the case of strain B501*gfp*1) may also be involved in increased plant growth.

Chapter 5 A NEW TRIAL ON INOCULUM DELIVERY INTO THE SUGARCANE SETTS.

5.1 Introduction

Nitrogen supply from indigenous endophytic bacteria is often insufficient for the crop's demand (Nishiguchi et al., 2005); the answer might be to introduce non-indigenous endophytes that can contribute to plant growth. To obtain a significant BNF contribution in non-leguminous plants, the presence of an adequate endophytic population and colonization by potential nitrogen-fixing endophytes (indigenous or introduced endophytes) is highly desirable.

Several delivery methods, such as inoculation of seeds, roots and aerial parts, have been reported for endophytic bacteria (Bressan and Borges, 2004; Elbeltagy et al., 2001; Njoloma et al., 2006b; Ruppel et al., 1992; Verma et al., 2001). In the field, sugarcane is propagated by sugarcane setts. The indigenous endophytes present in these mother stems (setts) act as a source of inoculum and colonize the roots and stem of the new plants (Asis et al., 2000; Njoloma et al., 2006a). In laboratory and greenhouse studies on the sugarcane-endophyte interaction, introduced endophytes are generally delivered by the inoculation of roots (Njoloma et al., 2006b; Sevilla et al., 2001) and stem (Olivares et al., 1997; Pimentel et al., 1991). The inoculated bacteria colonize the roots, intercellular spaces and xylem vessels (James et al., 1994), and move to the aerial parts through these vessels. The endophytic population in sugarcane consists of both diazotrophic and non-diazotrophic bacteria, and they compete each other and with the

introduced endophytes for space and nutrition; therefore, in order to achieve adequate internal colonization by the desired bacteria, a higher initial inoculum concentration is required (Njoloma et al., 2006a). Inoculation of plants by these methods is convenient and easy to handle at the laboratory and greenhouse level, but practical techniques for the field level must be developed. The field inoculation method should take account of the actual aspects of sugarcane cultivation out in the field, because the application of the methods used in laboratory or greenhouse in the field might not be feasible or easy.

The aim of this study was to find a reliable and efficient method of delivering diazotrophic endophytes in adequate populations into sugarcane setts. For this purpose, GFP (Green Fluorescence Protein) labeled *Herbaspirillum* sp. strain B501*gfp*1 (strain B501*gfp*1) was introduce into the stems of four sugarcane cultivars, NiF8, Ni15, F172 and Rk86-129 by the vacuum infiltration method and its colonization ability, survival and nitrogenase activity inside the stems was also studied.

5.2 Materials and Methods

Two experiments were conducted. In the preliminary experiment, sugarcane cultivars (NiF8 and Ni15) were inoculated and their acetylene reduction activity was measured, and on the basis of the results of the first experiment, a follow-up experiment was conducted with cultivars NiF8, Ni15, F172 and RK86-129.

5.2.1 Introduction of endophytic bacteria into sugarcane stems by the vacuum infiltration method

5.2.1.1 Plant material

Saccharum officinarum L. (cultivars NiF8 and Ni15) aged 13-15 months old (ratoon sugarcane), grown in the field at the University of Miyazaki, Japan were sampled in January 2007. The cultivars were planted in the summer of 2004 and were fertilized with 30kg N $ha^{-1}year^{-1}$. Mature, stalks were randomly selected from separate plants. Stalks were cut and cleaned of their leaves and leaf sheaths, washed with detergent, and rinsed with tap water. About 10-cm-long internodes of each cultivar were selected from the lower part of the sugarcane stalk. From one stalk two internodes (stem pieces) were collected (Fig. 5.1A and B).

5.2.1.2 Measurement of brix (%)

Sugarcane juice from cultivars NiF8 and Ni15 was extracted manually using a juice extractor. The Brix value (%) was measured in triplicate by a refractometer.

5.2.1.3 Inoculum preparation

Inoculum of strain B501*gfp*1 was prepared from a culture grown in LB broth for 24 h. The bacterial suspensions were centrifuged at 6,500 rpm for 10 min at 25°C. The supernatant was discarded and the pellet was resuspended in sterile distilled water. The

Figure 5.1. Sugarcane cultivars at various stages during the study. (A) Sugarcane stalks. (B) Two sugarcane stem pieces (internodes) obtained form one sugarcane stalk. (C) Stem pieces with nodes and buds before inoculation. (D & E) Young sugarcane plants grown from inoculated stems at 40 DAI. (F) Basal and lower stem of sugarcane plant after removing the necrotic leaf sheaths used for the population density determination and fluorescence microscopic observations.

bacterial suspensions were adjusted to 10^8 cells ml^{-1} using a hemocytometer under a microscope (Nikon Eclipse E600, Nikon, Japan).

5.2.1.4 Inoculation of sugarcane stems

One of the two stem pieces from a stalk of either cultivar was subjected to inoculation and the other stem piece served as a corresponding control. The sugarcane stem pieces were labeled and placed in 800-ml plastic jars. The bacterial suspension was poured into the jars until the stem pieces were submerged completely, leaving some empty headspace. The jars were closed with a lid having an outlet in the center. The rubber plug for this outlet was prepared separately, having two glass tubes inserted in it. The plug was then fixed in the lid outlet in such a way that the ends of the inserted tubes remained above the inoculum suspension. The outside end of glass tube (A) was attached to a vacuum pump by means of a rubber tube. A stopper was placed on the outer end of the glass tube (B). All the joints were sealed and airtight (Fig. 5.2).

The vacuum pump was turned on to remove the air present in the jars and inside the sugarcane stems. The evacuation continued for 10 minutes. This gave a low pressure in the jars. Air was then allowed to enter the jars gently by opening the stopper of the glass tube (B) so that the inoculum could infiltrate into the low pressure spaces inside the sugarcane stems created by the vacuum. The stems were immersed in the inoculum for 5 minutes (Fig.5.2). For the control, the same procedure was carried out with sterilized distilled water. The treated stems were subjected to $^{15}N_2$ gas replacement.

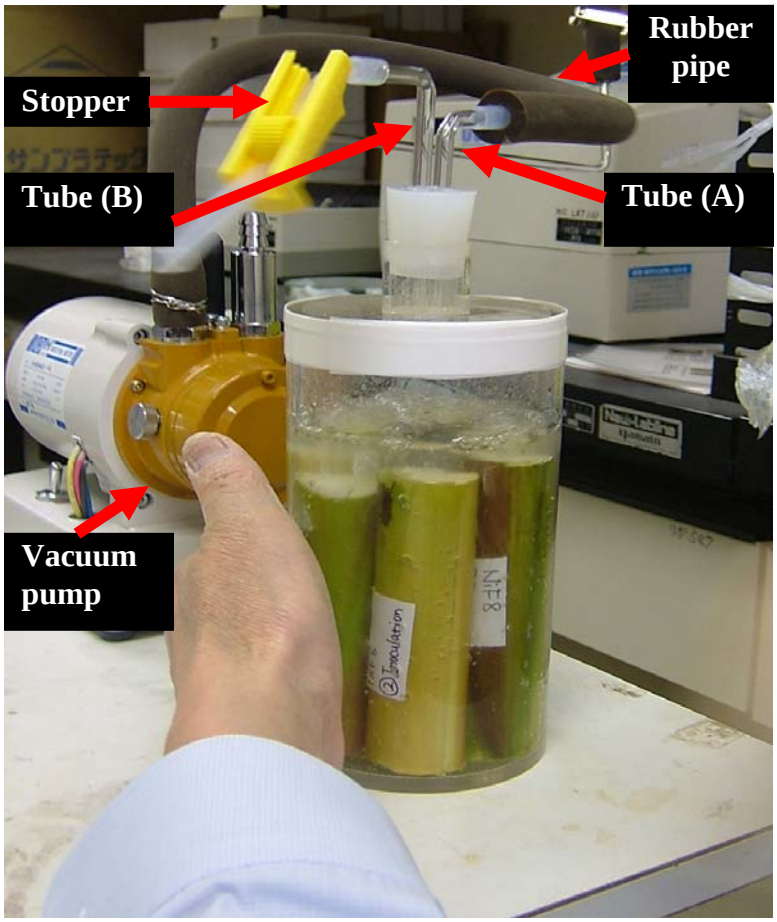


Figure 5.2. Inoculation of sugarcane stem pieces with *Herbaspirillum* sp. strain B501*gfp*1 by the vacuum infiltration method.

5.2.1.5 Replacement of $^{15}N_2$ gas

The stems (treated with inoculum or water) were placed in 400-ml jars separately. The jars were filled with water and closed with a lid having a rubber plug. Two glass tubes were inserted into the rubber plug of the outlet. To the inner end of the glass tube (A) a rubber pipe was attached which ran to the bottom of the jar. To the outer end of the glass tube (A) another rubber pipe was attached for water discharge. The outer end of the glass tube (B) was attached to a gas cylinder containing $^{15}N_2$ gas (Fig. 5.3). All the joints were sealed and airtight. The water was then replaced with a mixture of 30% (vol/vol) $^{15}N_2$ gas (99.8 atom%) and 70% (vol/vol) Ar gas. The jars were incubated at 28°C in the dark. After 7 days, the stems were removed from the jars. Each stem was cut into two halves longitudinally. One half of each stem was dried to a constant weight at 80°C and powdered using a chemist's mortar and pestle. The $^{15}N_2$ concentration was determined in triplicate using a mass spectrometer (Thermo Finnigan DeltaPlus Advantage System, Shoko Co. Ltd., Japan).

5.2.1.6 Acetylene reduction assay (ARA) of stems

The other half of each stem was cut longitudinally and placed in 45-ml glass tubes. The tubes were closed with a rubber cap and the headspaces of the tubes were replaced with 10% (vol/vol) acetylene gas. The tubes were kept at 28°C in the dark. The ethylene gas concentration was assayed after 18 h and 30 h using a Shimadzu GC-8A gas chromatograph. The results were analyzed by the method of multiple comparison (Newman-Keuls multiple comparison of mean) using TexaSoft, WINKS SDA software,

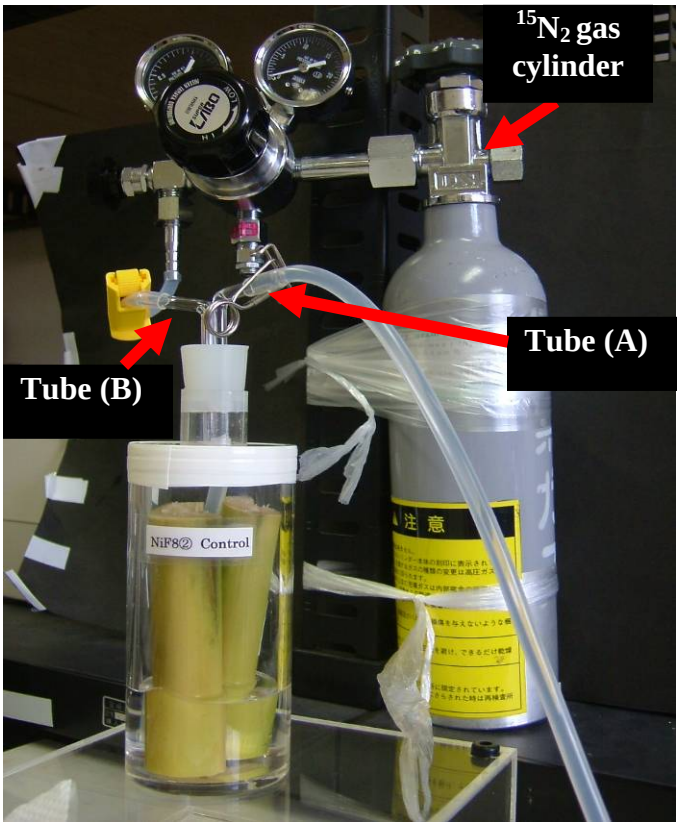


Figure 5.3. Replacement of $^{15}N_2$ gas (30% (vol/vol) $^{15}N_2$ gas (99.8 atom%) and 70% (vol/vol) Ar gas) in the jars containing treated stem pieces.

6th edition, Cedar Hill, Texas, 2007.

5.2.2 Infection, colonization and evaluation of the nitrogen fixing ability of strain B501*gfp*1 in the stems and young sprouts of sugarcane cultivar Ni15, NiF8, F172 and RK86-129

5.2.2.1 Plant material preparation and inoculation

On the basis of the results of the first experiment, a follow up experiment was conducted with cultivars NiF8, Ni15, F172 and RK86-129. Three stalks of each cultivar, aged 5-6 months old (ratoon sugarcane) grown in the above mentioned field were collected in August 2007. About 10-cm-long stem piece (internode) from each stalk of each cultivar was selected from the lower part of the sugarcane stalk. Each stem piece was cut longitudinally into two halves. One half of each stem piece was treated with inoculum as described above and the other half was treated with water and served as a corresponding control. A portion from the treated stem pieces (inoculated or control) was used for fluorescence microscopic observation and bacterial population density determination immediately after inoculation. The remaining portion of all the stems was subjected to $^{15}N_2$ gas incorporation.

In addition, stem pieces of about 10 cm-length with nodes and buds (Fig. 5.1C) were also selected and treated with inoculum or water (control) as mentioned above. The treated stems were buried in wet vermiculite bed and maintained at 28°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. The vermiculite was watered regularly and 40 DAI the plants were harvested and processed for fluorescence microscopic observations and bacterial density determination.

5.2.2.2 Replacement of $^{15}N_2$ gas

The inoculated and control stems were placed in 400-ml jars separately and the air was replaced with a mixture of 30% (vol/vol) $^{15}N_2$ gas (99.8 atom%) and 70% (vol/vol) Ar gas as described above. The 5% (vol/vol) of gas mixture ($^{15}N_2$ and Ar gas) was then replaced with O_2 gas leaving a final concentration of 95%:5% (mixture of $^{15}N_2$ and Ar:O_2) in each jar. The jars were incubated at 28°C in the dark. After 5 days, the stem pieces were removed from the jars. A portion of the stem pieces was used to determine the ^{15}N concentration and the data was analyzed by the method of multiple comparison as described above. The remaining portion of each stem was subjected to fluorescence microscopic observations and bacterial population density determination.

5.2.2.3 Fluorescence microscopy

The inoculated stems and the corresponding control stems were examined under a fluorescence microscope immediately and 5 DAI. A stem cutting (longitudinally) was obtained from each inoculated and control stem piece, peeled to remove the epidermis and cut lengthwise into fragments of about 3-5 cm. Longitudinal sections of the stem

were cut manually. These sections were examined for microscopic fluorescence using a Nikon Eclipse E600 (Nikon, Tokyo, Japan). The roots and aerial parts of the newly emerged plants from the inoculated and control stems were also observed under the fluorescence microscope at 40 DAI. The roots and leaves were observed directly and for observations of endophytic bacteria in the stem, transversal sections of 0.1mm were prepared with a microslicer after removing the necrotic leaf sheaths near the basal stem (Fig. 5.1F) and examined.

5.2.2.4 Bacterial density determination

The population densities of the replicate (n=3) inoculated and control stems were determined immediately and 5 DAI. The peeled internodes were washed three times with sterile distilled water. Sugarcane juice was extracted manually using a juice extractor. The juice was then serially diluted and plated on LGIP medium containing 10µg ml^{-1} kanamycin. The green colonies that appeared on the plates after incubation at 28°C were counted using a Nikon SMZ 1500 fluorescence microscope. The results were analyzed by the method of multiple comparison (Newman-Keuls multiple comparison of mean) using TexaSoft, WINKS SDA software, 6th edition, Cedar Hill, Texas, 2007.

The bacterial population densities in roots and aerial parts of the plants emerged from the treated stems was determined at 40 DAI. The roots of the uprooted plants were thoroughly washed with distilled water and the plants were detached from the mother stems (Fig. 5.1D and E). The plants were divided into roots and aerial parts. The plant

samples were cut into small pieces, washed vigorously with sterilized distilled water three times and grounded by hand using mortar and pestle. The extract was plated on kanamycin coated plates after serial dilutions and observed under fluorescence microscope.

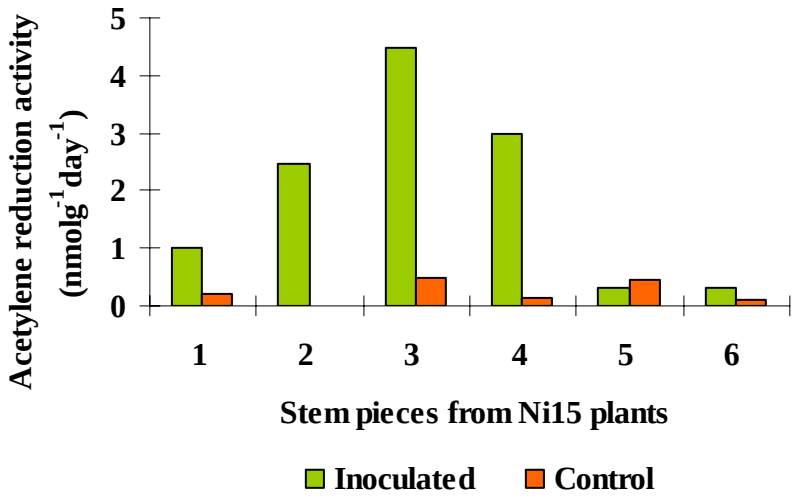
5.3 Results and Discussion

The aim of this study was to establish an efficient method of inoculum delivery into sugarcane setts which can ensure a high initial inoculum quantity for subsequent colonization of newly sprouted shoots at the field level. In the first experiment, the cultivars were inoculated by the vacuum infiltration method and the nitrogenase activity of the inoculated bacteria inside the stems was determined based on the $^{15}N_2$ gas incorporation and acetylene reduction at 7 DAI. The inoculated bacteria showed very little incorporation of nitrogen in both cultivars (Table 5.1); however, strain B501*gfp*1 showed significantly higher acetylene reduction activity (1.9 nmol g FW^{-1} day^{-1}) in Ni15 than in NiF8 (0.082 nmol g FW^{-1} day^{-1}) (Table 5.2). The inoculated stems of Ni15 also showed significantly high acetylene reduction activity compared to their corresponding control stems (Table 5.2 and Fig. 5.4A), whereas non-significant results were observed in cultivar NiF8 (Table 5.2 and Fig. 5.4B). The result suggests that higher populations of strain B501*gfp*1 may have entered and colonized the Ni15 stems than the NiF8 stems. De Bellone and Bellone, (2006) reported that the distribution of endophytic bacteria in the sugarcane plant depends on the content of different organic acids and the sugar concentration, and that these contents vary according to the age, variety of the plant and the part of the stem. Both Ni15 and NiF8 are high-sugar-accumulating cultivars (Table 5.3), both have almost the same level of sugar contents, a characteristic that would ensure the growth of endophytic N_2-fixing bacteria residing inside the tissues (Nishiguchi et al., 2005). So, the difference in the acetylene reduction values between the two cultivars might be due to factors other than the sugar concentration.

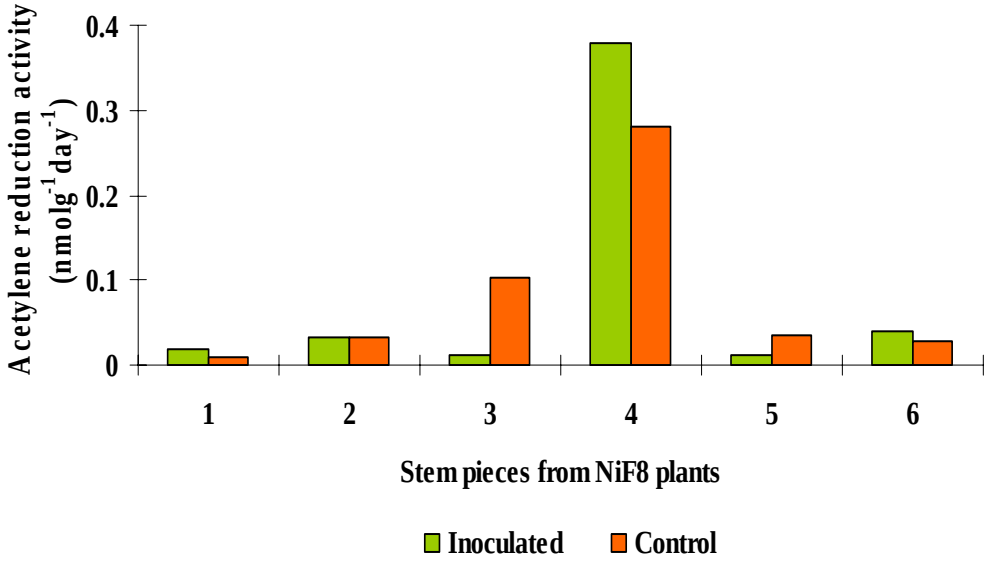
Table 5.1. Percentage (%) of fixed ^{15}N in the total nitrogen inside the stems of sugarcane cultivars Ni15 and NiF8 inoculated with *Herbaspirillum* sp. strain B501*gfp*1.

Sugarcane cultivar	% of fixed ^{15}N in total nitrogen
Ni15 (inoculated)	0.0017 ± 0.0005
Ni15 (control)	0
NiF8 (inoculated)	0.0014 ± 0.0005
NiF8 (control)	0

The values of the occupied (%) of fixed ^{15}N represent means ± SD of three replicates.
The percentage of occupied ^{15}N of the total nitrogen content of each stem was calculated using the following formula:
Occupied % of ^{15}N in total nitrogen = (sample excess % / enriched ^{15}N gas excess %) × 100,
where sample excess % = sample atom % - background atom %,
and enriched ^{15}N gas excess % = Gas atom % - background atom %.



(A)



(B)

Figure 5.4. Acetylene reduction activity of cultivar Ni15 (A) and NiF8 (B) inoculated with *Herbaspirillum* sp. strain B501*gfp*1at 7 DAI.

Table 5.2. Acetylene reduction activity of sugarcane cultivars Ni15 and NiF8 at 7 DAI.

Treatment	Acetylene reduction activity ($nmol\ g^{-1}day^{-1}$)		Significance
	Ni15	NiF8	
Inoculated	1.93 [a]	0.08 [a]	*
Control	0.23 [b]	0.08 [a]	
Significance	*		

Means with a common letter suffix in a column do not differ at a 5% level of probability (Newman-Keuls multiple comparison of mean)
*$P \leq 0.001$

Table 5.3. Brix values (%) in cultivars NiF8 and Ni15.

Cultivar	Brix (%)
NiF8	19.46 ± 1.18
Ni15	19.86 ± 0.23

The values represent the means ± SD of three replicates.

On the basis of these preliminary results, a follow up experiment with cultivars NiF8, Ni15, F172 and RK86-129 was conducted. The population density of strain B501*gfp*1 in stems of these cultivars immediately after inoculation ranged from 10^7 to 10^8 CFU g FW^{-1}) which showed that the bacteria successfully entered the tissues of these cultivars (Table 5.4). No significant difference in bacterial population density was observed between the four cultivars at that stage. A comparatively high population density (10^7 CFU g FW^{-1}) of strain B501*gfp*1 was observed in cultivars Ni15 and RK86-129 compared to other cultivars at 5 DAI. However, no significant increase or decrease in the population density was observed in the cultivars at 5 DAI compared to the population density immediately after the inoculation except for the cultivar NiF8 where significantly low populations were observed at 5 DAI (Table 5.4).

The presence of the inoculated bacteria in the stem tissues was also confirmed by the fluorescence microscopic observations immediately and 5 DAI (Fig. 5.5 and 5.6). The bacteria immediately after inoculation seemed to be present largely in the spaces between cell layers (Figs. 5.5A, C, D and G). The colonization of the intercellular spaces was also observed (Figs. 5.5 B, E, F and H). A somewhat similar colonization pattern of sugarcane stem tissues was observed in all the four cultivars at 5 DAI (Fig 5.6). Colonization was generally observed in the intercellular spaces and between the cell layers (Fig. 5.6A, C, and G-K) in all the cultivars. Colonization of the sub-epidermal tissues was also observed in cultivars NiF8 and Ni15 (Fig. 5.6 B and D). Dong et al., (1994) have suggested that the sucrose storage tissues of the stems of mature sugarcane stalks are the most likely location for symbiosis with endophytic bacteria, as the intercellular spaces are a rich

Table 5.4. Population density of *Herbaspirillum* sp. strain B501*gfp*1 in stems of different sugarcane cultivars immediately and 5 DAI.

Sugarcane cultivar	Mean log CFU ml[juice]$^{-1}$		Significance
	Just after inoculation	5 DAI	
NiF8	8.27[a]	6.29[a]	*
Ni15	7.34[a]	7.44[a]	
F172	7.67[a]	6.56[a]	
RK86-129	7.22[a]	7.24[a]	
Significance			

Means of population density with a common letter suffix in a column do not differ at a 5% level of probability (Newman-Keuls multiple comparison of mean).

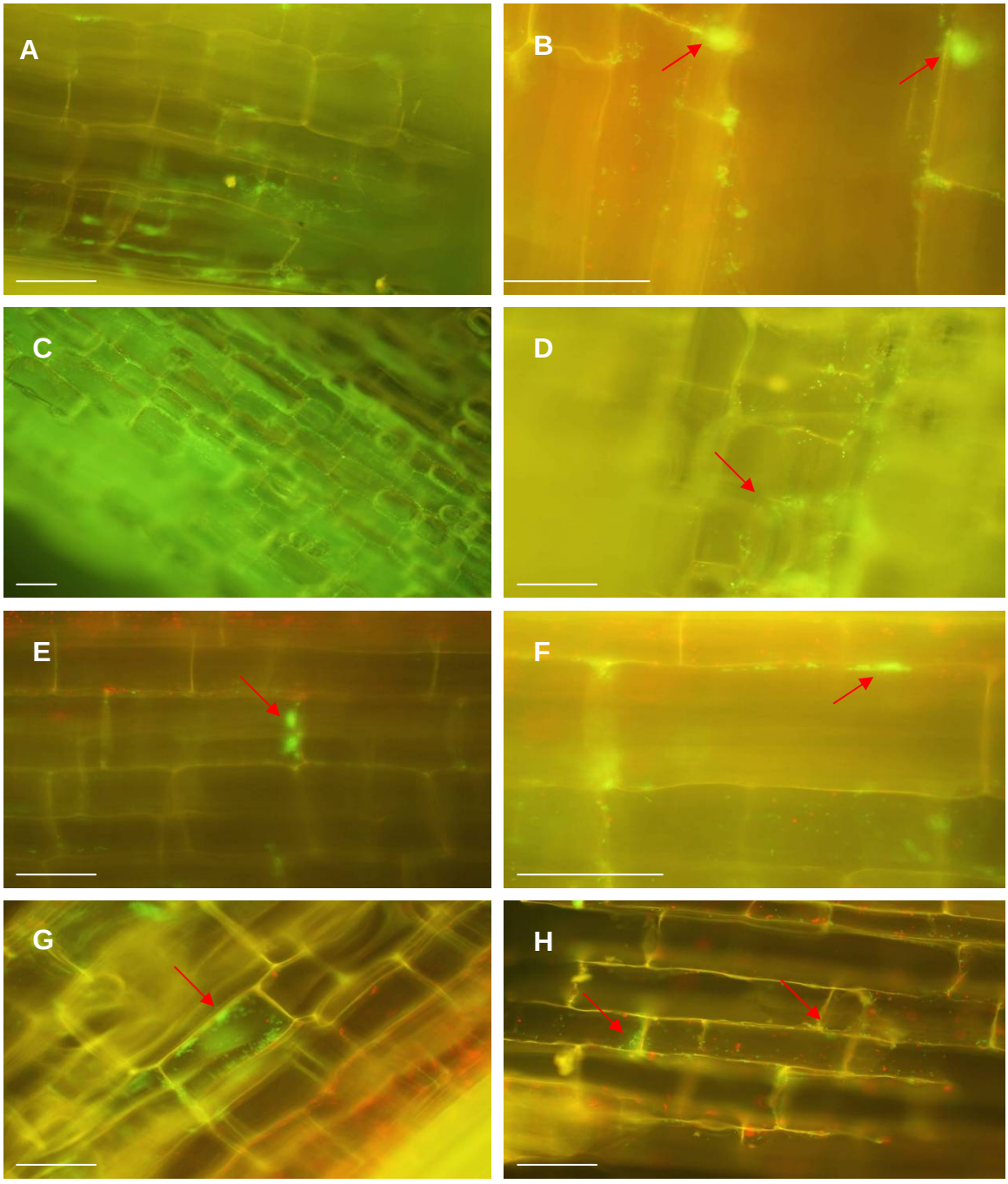


Figure 5.5. Fluorescence micrographs showing colonization of stem by strain HB501*gfp*1 immediately after inoculation. (A and B) NiF8, (C and D) Ni15, (E and F) F172, (G and H) RK86-129. Bar= 10 µm. The arrows show the colonization sites.

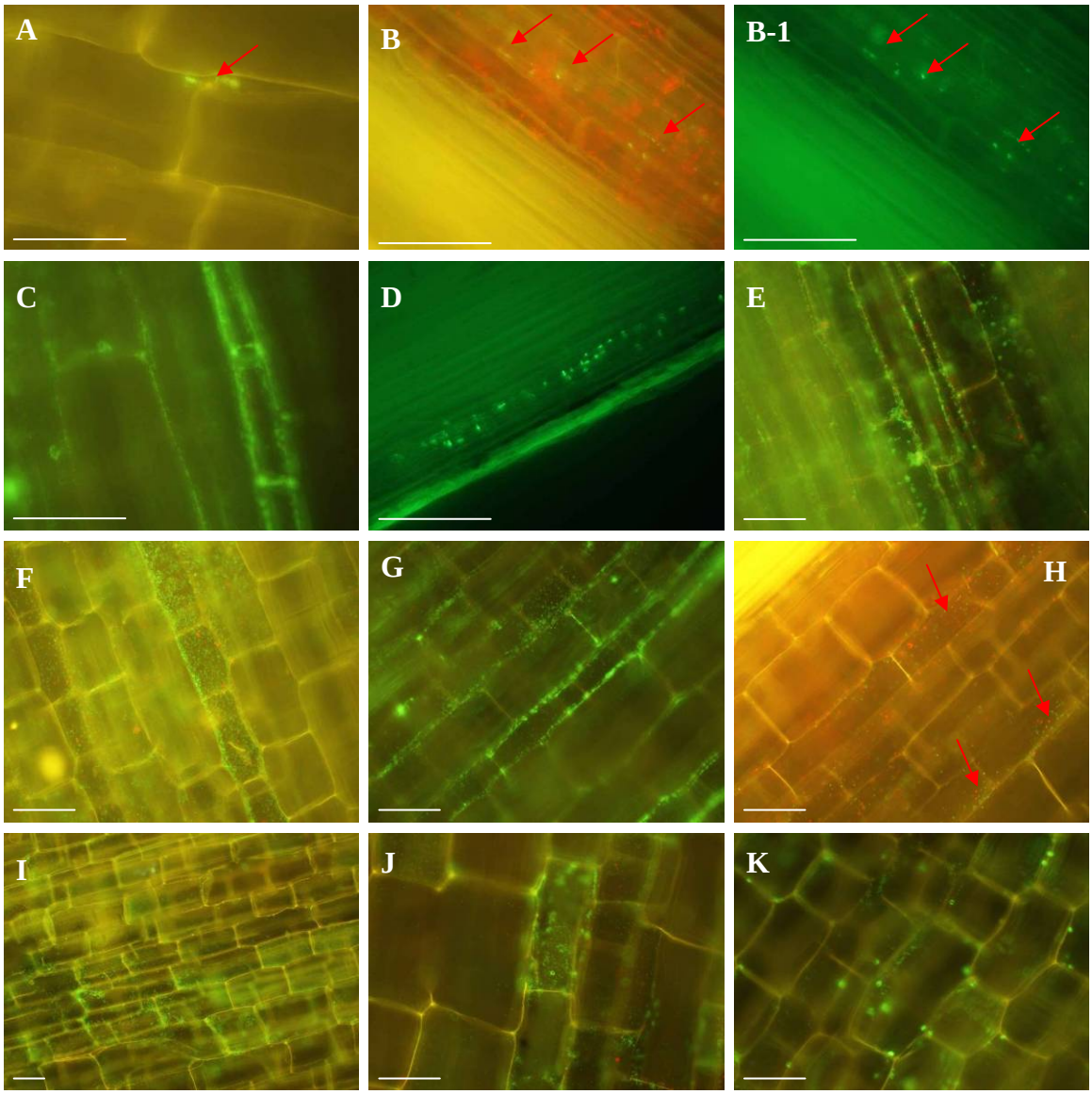


Figure 5.6. Fluorescence micrographs showing colonization of stem by strain HB501*gfp*1 at 5 DAI. (A and B) NiF8, (C-E) Ni15, (F-H) F172, (I-K) RK86-129. Bar= 10 µm. The arrows show the colonization sites.

source of sucrose. In the present study, strain B501*gfp*1 also entered the intercellular spaces of the stem tissues, which may increase its chances of staying for a longer period of time inside the stem.

The inoculated bacteria showed an adequate amount of fixed nitrogen in the second experiment compared to the first experiment (Table 5.5). Although the amount of fixed ^{15}N (0.57 µg g[DW]$^{-1}$) by the strain B501*gfp*1 tended to be higher in cultivar Ni15 than other cultivars, statistically non-significant results were observed among the four cultivars. The relatively high amount of fixed nitrogen observed in the second experiment may be due to the reason that we used mature sugarcane stalks in the first experiment and young stalks in the second experiment, as higher amounts of fixed nitrogen have been reported in young sugarcane plants than in the mature sugarcane plants (personnel communication). The other possible reason might be the time of harvest of the sugarcane stalks as in the first experiment the stalks were harvested in the early spring and in autumn in the second experiment. $^{15}N_2$ gas incorporation is a direct method of measuring the biologically fixed nitrogen and several researchers have reported the biological fixed nitrogen by the endophytes by this method. Ruschel et al., (1975) used $^{15}N_2$ gas incorporation in sugarcane to quantify the BNF and observed significantly high incorporation of $^{15}N_2$ gas in one cultivar. However, Hoefsloot et al., (2005) observed no significant ^{15}N incorporation in South African sugarcane cultivars by this method. The ^{15}N-aided and ^{15}N isotope dilution N balance studies by Lima et al., (1987) and Urquiaga et al., (1992) revealed that some of the sugarcane cultivars obtained more than 60-70% (150 kg N ha^{-1} year^{-1}) of the total nitrogen from BNF. Asis et al., (2002) reported that the

Table 5.5. Effect of inoculation with *Herbaspirillum* sp. strain B501*gfp*1 on the incorporation of $^{15}N_2$ into sugarcane stems.

Sugarcane cultivar	Occupied (%) of fixed ^{15}N in total nitrogen			Total N (%)	^{15}N fixed by strain B501*gfp*1 (μg g[DW]$^{-1}$)
	Strain B501*gfp*1 + Indigenous bacteria (Inoculated)	Indigenous bacteria (Control)	Strain B501*gfp*1		
NiF8	0.115 ± 0.050	0.097 ± 0.074	0.020 ± 0.040	0.199 ± 0.087	0.42[a]
Ni15	0.060 ± 0.027	0.044 ± 0.030	0.016 ± 0.005	0.337 ± 0.140	0.57 [a]
F172	0.144 ± 0.030	0.101 ± 0.036	0.040 ± 0.060	0.256 ± 0.065	0.33 [a]
RK86-129	0.171 ± 0.033	0.112 ± 0.013	0.060 ± 0.040	0.096 ± 0.035	0.51 [a]

The values listed represent the means ± SD of three replicates.
Means of ^{15}N fixed by strain B501*gfp*1 (μg g[DW]$^{-1}$) with a common letter suffix in a column do not differ at a 5% level of probability (Newman-Keuls multiple comparison of mean).

The percentage of occupied ^{15}N of the total nitrogen content of each stem was calculated using the following formula:
Occupied % of ^{15}N in total nitrogen = (sample excess % / enriched ^{15}N gas excess %) × 100
where sample excess % = sample atom % - background atom %,
and enriched ^{15}N gas excess % = Gas atom % - background atom %

sugarcane cultivar NiF8 can obtain up to 38% of the total nitrogen from BNF by the ^{15}N dilution and natural ^{15}N abundance technique. Similarly BNF contribution ranging from 0-70% in different sugarcane cultivars have been reported using various measurement studies (Boddey et al., 2001; Oliveira et al., 2003). From these studies it is clear that sugarcane plants differ in their capacity to support nitrogen fixation and these may be variety-specific depending on the plant genome, growth and environmental conditions (Yoneyama et al., 1997).

The acetylene reduction activity shown by the control stems in first experiment (Fig. 5.4) and ^{15}N fixation in the second experiment (Table 5.5) indicate that the few stalks of these cultivars may have nitrogen fixing indigenous endophytes which may compete with the inoculated bacteria for resources. In studies by Njoloma et al., (2006a) it was observed that in the presence of the indigenous endophytic bacteria, strain B501*gfp*1 when inoculated in low concentration have encountered some competition over growth resources resulting in their slow multiplication but with high initial inoculum concentration higher numbers of strain B501*gfp*1 compared to the indigenous bacteria were detected. The effect of indigenous bacteria on colonization of the inoculated bacteria needs to be investigated in detail.

In order to find whether the inoculated bacteria colonized the newly emerged plants or not, the plants emerged from the inoculated and control stems were examined for population density determination and fluorescence microscopic observations at 40 DAI. The results of population density showed that the strain B501*gfp*1 successfully

entered in the roots of all the cultivars. Highest population density was observed in the roots of cultivar Ni15 (1.9×10^5 CFU g FW^{-1}), however, non-significant results were observed among the cultivars. The bacteria also colonized the aerial parts of cultivar F172 and RK86-129 (Table 5.6). The colonization of the new roots and shoots of these cultivars was also confirmed by the fluorescence microscopic observations (Fig. 5.7). Higher colonization of the roots was observed in cultivar Ni15 followed by cultivar Rk86-129. Colonization of the intercellular spaces on the root surface and internal tissues was generally observed in all the cultivars; NiF8 (Fig. 5.7A and B), Ni15 (Fig. 5.7D, E and F), F172 (Fig. 5.7H), RK86-129 (Fig. 5.7I and J). Occasionally bacteria also entered inside the cells in roots of cultivar F172 (Fig. 5.7G) and RK86-129 (Fig. 5.7K). In cultivars NiF8, the scattered presence of bacteria was observed near the metaxylem tissues (Fig. 5.7C) though the bacterial presence in aerial parts of NiF8 was not detected by plate count method. Colonization of the sub-epidermis of basal stem was also observed in cultivar RK86-129 (Fig. 5.7L). No leaves colonization was observed in any cultivar by fluorescence microscopy. Colonization of the sugarcane roots and stem by the strain B501*gfp*1 has been reported in our previous study (Njoloma et al., 2006b), where higher populations were observed in cultivar Ni15 than in NiF8. Higher populations of strain B501*gfp*1 in roots compared to the aerial parts as observed in this study were also observed in sugarcane (Njoloma et al., 2006b) and rice (Zakria et al., 2007) in our previous studies though inoculated with different method. In contrast, higher population densities of strain B501*gfp*1 in stem than roots have been reported in its original host (wild rice) (Elbeltagy et al., 2001).

Table 5.6. Population density of *Herbaspirillum* sp. strain B501*gfp*1 in different plant parts of sugarcane cultivars at 40 DAI.

Sugarcane cultivar	Bacterial numbers CFU g[fresh weight]$^{-1}$		Significance
	Root	Aerial parts	
NiF8	4.7×10^{4a}	0	
Ni15	1.9×10^{5a}	0	
F172	7.2×10^{4a}	6.6×10^{4a}	
RK86-129	2.7×10^{3a}	6.0×10^{2a}	
Significance			

Means of population density with a common letter suffix in a column do not differ at a 5% level of probability (Newman-Keuls multiple comparison of mean).

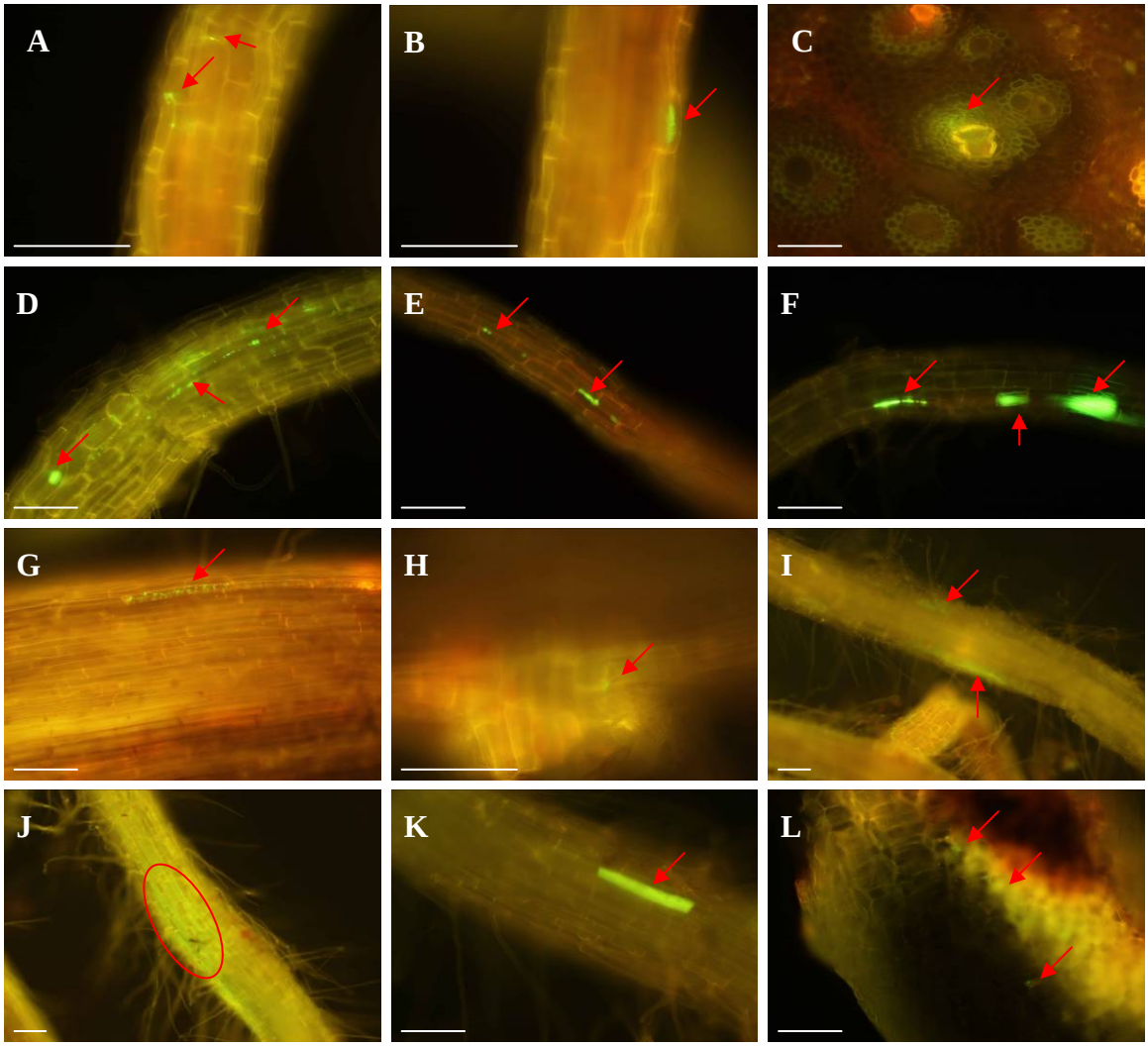


Figure 5.7. Fluorescence micrographs showing colonization by strain B501*gfp*1 of roots and stems of newly grown plants from the inoculated stems of four sugarcane cultivars. (A-C) NiF8, (D-F) Ni15, (G and H) F172, (I- L) RK86-129. Bar= 10 µm. The arrows show the colonization sites.

5.4 Conclusion

From the results of this study, it can be concluded that endophytic bacteria can be introduced successfully into sugarcane stems by vacuum infiltration method. They can survive in the stem tissues, since a stable population was observed five days after inoculation and can colonize the newly emerged plants. Thus, this method can be used to prepare the planting material so that it carries its own inoculum source for the new shoots in the field. The population of the colonized bacteria may vary in roots and aerial parts depending on the cultivar. This is a preliminary study, and further studies involving different endophytic strains and cultivars should be carried out to get the best combination of host and endophyte, which can provide maximum BNF at the field level.

Chapter 6 GENERAL DISCUSSION AND CONCLUSION

The inoculation of endophytic bacteria may increase the plant growth, but the response depends both on the endophyte and host genotype. To achieve maximum benefit from BNF by the endophytic bacteria the proper combination of host-endophyte is required, which also needs a delivery method suitable to the actual aspects of the crop out in the field. Furthermore, the inoculated endophyte must be a good colonizer and should express nitrogenase activity in association with plants.

Several inoculation methods have been reported for the endophytic bacteria depending on the host type. Among various inoculation methods used in rice, seed inoculation by the endophytic bacteria is reported by several resarchers (Elbeltagy et al., 2001; Feng et al., 2006). Additionally, in rice, inoculation of roots in rhizosphere (Gyaneshwar et al., 2001) and by immersing the roots in inoculum suspension (Fujii et al., 1987; Malik et al., 1997) have also been reported. Similarly, in sugarcane the endophytes were studied by the methods of roots rhizosphere inoculation (Njoloma et al., 2006b; Sevilla et al., 2001), immersion of roots in inoculum suspension (Rojas and Mellado, 2003) and stem treatment (Olivares et al., 1997; Pimentel et al., 1991). The potential of plants to respond to inoculation with *Herbaspirillum* sp. and other endophytes have been evaluated by many experiments. The initial results showed no positive response for sorghum (Pereira et al., 1988) and maize (Pereira and Baldani, 1995). In the case of rice, inoculation promoted a yield increase equivalent to the treatment with 40 kg of N/ha (Pereira and Baldani, 1995). However, large advances have been made in the last few

years through a highly detailed study based on selection of *Herbaspirillum* strains in association with rice plants grown under gnotobiotic conditions (Baldani et al., 2000; Elbeltagy et al., 2001). It was observed that the response of the rice variety to inoculation was dependent on the strain used. In some cases, highly significant increases in phytomass accumulation were observed with the inoculation of some strains, whilst the opposite was observed for others (Baldani et al., 2000). Evaluation of these strains under greenhouse and field conditions also showed significant increases in yield, however this increase is depended on the rice cultivar tested (Guimarães et al., 2000). The inoculation of *Pantoea agglomerans* in rice is also reported to increases the plant growth and affects the allocation of host photosynthates (Feng et al., 2006). Inoculation experiments with *Pantoea agglomerans* (formerly *Enterobacter agglomerans)* on wheat and barley in temperate regions have demonstrated the possibility of increasing yield up to 500kgha^{-1} (Ruppel et al., 1989).

Seed-transmitted bacteria are already present in the plant; however, diazotrophic endophytic bacteria must colonize the root surface prior to entering the plant (Gyaneshwar et al., 2001; James et al., 2002; Roncato-Maccari et al., 2003). The mode of infection and colonization pattern observed in rice, broccoli and sugarcane by the endophytes in this study is somewhat similar to other studies reported for the endophytes. The initial step consists of the attachment of the bacteria to the root surface. At this stage, the localization of colonizing cells at the intercellular junctions of root's epidermis is consistent with the suggestion that micro-colonies develop where bacteria find an increased concentration of carbon sources, thus explaining the preference of bacteria for this region of the root tissue (Bacilio-Jimenez et al., 2001; Bennett and Lynch, 1981). The

entry of bacteria into the root is most likely an active process, which might be mediated by enzymes degrading plant cell wall polymers (Reinhold-Hurek et al., 1993).

Root surface colonization was followed by a second stage, characterized by cortical infection, and a third stage, which consisted of stele and xylem invasion. The *Herbaspirillum* invasion seems to occur primarily through the lateral root emergence sites. Previously, Gyaneshwar et al., (2002), James et al., (2002) and Roncato-Maccari et al., (2003) documented *Herbaspirillum* invasion of rice, wheat, maize and sorghum at lateral root cracks. In wheat colonization of intercellular spaces of the roots and on the root surface was observed by *Pantoea agglomerans* (Ruppel et al., 1992). The present study of rice, together with that of Gough and associates (1997) with *Arabidopsis* and those of James and Olivares, (1998) and Olivares and James, (2000) with sugar cane, have shown that endophytes has a preference for infecting plants via such an aggressive means of entry. A similar pattern of invasion has also been described for sugarcane infection by *Gluconacetobacter diazotrophicus* (James et al., 1994) and also for non-diazotrophic endophytic bacterial infections, such as *Pseudomonas solanacearum* of dicotyledonous plants (Vasse et al., 1995 and Quadt-Hallmann et al., 1997). Gyaneshwar et al., (2001) observed large numbers of bacteria within the aerenchyma, intercellular spaces, in the gaps between the leaf sheaths and within some of the xylem vessels in stem. Inside the plant, bacteria were mainly localized within the aerenchyma and intercellular spaces. These apoplastic locations seem to be the preferred sites of the few endophytic diazotrophs so far examined in rice. For example, Hurek et al., (1994) and Egener et al., (1999) have shown dense colonies of *Azoarcus* in the aerenchyma of rice (and Kallar

grass) roots, and James et al., (2000) have presented micrographs showing *H. seropedicae* extensively colonizing the intercellular spaces and aerenchyma of roots, stems, and leaves. According to Hurek et al., (1991; 1994) another route of entry is the root tip at the zone of elongation and differentiation. The bacteria can invade inter- and intracellularly and may penetrate into the central tissues. Quadt-Hallmann and Kloepper, (1996) observed a systemic colonization of *Enterobacter asburiae* in cotton plants specifically in the roots surface, within epidermal cells, and in side intercellular spaces of the root cortex close to the conducting elements indicating that endophytes may follow similar mode of colonization in monocots and dicots.

Bacterial colonization of the vascular system and the intercellular spaces in the apoplast has been widely reported in association with most gramineae plants (James, 2000; Lamb et al., 1996; McCully, 2001; Sprent and James, 1995). James et al., (2000; 1997; 1994) have reported that the xylem elements could provide the required low pO_2 level and a site for exchange of metabolites that are necessary for nitrogen fixation. However, Dong et al., (1994; 1997) suggested that xylem is an unsuitable habitat for *A. diazotrophicus* in sugarcane from the anatomical and physiological points of view. On the other hand, N_2 fixation is an energetically demanding process, and carbon sources tend to be low in xylem, suggesting that conditions in the xylem are not conducive for N_2 fixation (Gyaneshwar et al., 2001). In this work, *Herbaspirillum* sp. strain B501 never entered vascular tissues, apparently preferring the intercellular spaces whereas, *Enterobacter* sp. strain 35-1 and *Pantoea* sp. strain 18-2 could colonize the vascular tissues of rice to some extent.

Both epiphytic and endophytic populations were observed in rice and broccoli in this study and the results of previous studies also showed that the bacterial population on the root surface may be as important as, if not more important than, the bacterial population within the plant (Gyaneshwar et al., 2002).

Nitrogen fixation by endophytic bacteria has contributed significantly to the nitrogen requirement of sugarcane in Brazil (Boddey et al., 1991; Reis et al., 1994), Cuba (Dong et al., 1994), Australia (Li and MacRae, 1991) and Japan (Yoneyama et al., 1997). $^{15}N_2$ gas incorporation is a direct method of measuring the biologically fixed nitrogen whereas; acetylene reduction assay is an indirect method of measuring nitrogen fixation in plants. However, indications of the presence of N_2-fixation can be reliably obtained with this sensitive technique (Vessey, 1994). In the seventies, a significant advance in the area of BNF in grasses was the introduction of the acetylene reduction method. The use of this technique to evaluate the BNF potential of several forage grasses (*Panicum maximum, Pennisetum purpureum, Brachiaria mutica, Digitaria decumbens, Cynodon dactylon* and *Melinis minutiflora*) gave rates of 239 to 750 nmols C_2H_4/h/g of roots, which varied with the season and stage of plant development (Day et al., 1975). Due to the potential of BNF in grasses, the studies were directed towards plants of higher agricultural and economical importance such as cereals. The association of sugarcane roots with bacteria was first examined by Döbereiner et al., (1972) using the ARA. Values of ARA around 10,000 nmols C_2H_4/h/g dry roots were detected in maize plants grown in pots containing very wet soils and under high light intensity (Dommergues et al., 1973). Similarly, Staphorst and Strijdom, (1978) showed acetylene-reduction activity in

roots from South African sugarcane plants. On the other hand Hoefsloot et al., (2005) observed no significant acetylene reduction activity and ^{15}N incorporation in South African sugarcane cultivars. Ruschel et al., (1975) obtained significant incorporation of ^{15}N gas into a sugarcane cultivar. De-Polli et al., (1980) also confirmed the BNF in sugarcane cultivar batatais by the incorporation of ^{15}N into the plant tissues. Later, Boddey et al., (1983) used the ^{15}N dilution technique and demonstrated a BNF contribution of 20 kg N/ha/year in plants grown under field conditions. The N_2 fixation in laboratory and field grown plants is generally reported by the methods of ^{15}N dilution and natural ^{15}N abundance technique (Asis et al., 2002; Boddey et al., 1991). Lima et al., (1987) and Urquiaga et al., (1992) showed large contribution of biologically fixed nitrogen in sugarcane, which they estimated to be in excess of 60 to 80% of the total plant N, equivalent to over 200 kg N $ha^{-1}year^{-1}$. ^{15}N isotope dilution techniques have also been used for quantifying associativeN_2- fixation in wheat (Lethbridge and Davidson, 1983; Rennie et al., 1983), maize (Rennie, 1980), rice (Shrestha and Ladha, 1996; Ventura and Watanabe, 1983), and other grasses (Boddey et al., 1983; Boddey and Victoria, 1986; Malik et al., 1988; Malik and Zafar, 1985; Malik et al., 1987).

Despite their nitrogen fixing abilities the endophytic bacteria are also reported to promote plant growth by excretion of phytohormones, which lead to an increased root surface and thus to an increased nutrient uptake (Okon and Labandera-Gonzalez, 1994). Phytohormonal activities of the strain *Pantoea agglomerans* and its growth-promoting effect on wheat plants has been reported by Scholz and Ruppel, (1992) and recently in rice by Feng et al., (2006). *Gluconacetobacter diazotrophicus* has shown to produce plant

growth-pomoting substances (Bastian et al., 1998). Mutant strains with different levels in IAA production have shown altered effects on root morphology (Dobbelaere et al., 1999).

In this study we have shown that the extent of colonization and nitrogen fixation by an endophyte in its host may vary with the inoculation technique and age of plant at the time of inoculation. As strain B501*gfp*1 showed significant colonization of cultivated rice when inoculated in rhizosphere at seedling stage in this study, while, its failuer to colonize the cultivated rice considerably when seed inoculated was done (Elbeltagy et al., 2001). It also showed higher amount of fixed nitrogen in this study compared to its nitrogen fixation in wild rice (the orignal host) (Elbeltagy et al., 2001). Simillarly, in comparison study of two inoculation methods i.e. rhizosphere inoculation and root dip method for *Pantoea* sp. strain 18-2 and *Enterobacter* sp. strain 35-1, dipping method resulted in better colonization of cultivated rice which also showed the importance of inoculation technique in colonization by the endophytic bacteria. Additionally, it was confirmed that the strains 18-2 and 35-1 also bear non-host specific characetristics, being isolated from different hosts they were able to colonize non-host plants like rice and broccoli. The inoculation of sugarcane stems by vacuum infiltration and the subsequent colonization of the new plants showed that this method has the potential of delivering the inoculum at the field level for the new plants.

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APPENDIX

Appendix 1. Medium for bacteria growth.

Components	LGIP medium[1] (g/L)	Luria Bertani medium (LB)[2] (g/L)
K_2HPO_4	0.2	
KH_2PO_4	0.6	
$MgSO_4.7H_2O$	0.2	
$Na_2MoO_4.2H_2O$	0.02	
$CaCl_2.2H_2O$	0.02	
$FeSO_4.7H_2O$	0.01	
NaCl		5
Yeast extract		5
Trypton		10
Bromothymol blue (BTB)	5ml (0.5% in 0.2N KOH)	
Sucrose	100	100
Agar－semisolid(Liquid)	2	
Agar solid	20	20
	pH 6.8	pH 7.2

[1]Cavalcante, V.A and J. Döbereiner. 1998. A new acid tolerant nitrogen fixing bacterium associated with sugarcane. Plant Soil. 108: 23-31.

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Appendix 2. Composition of MS-Medium.

Components	Concentration of medium (mg/L)	Concentration of stock (g/L)	Amount required ml/L
MS Stock 1		**(10x)**	**100**
$CaCl_2.2H_2O$	440	4.400	
$MgSO_4.7H_2O$	370	3.700	
KH_2PO_4	170	1.700	
K_2 SO_4 (in case of deficiency)		16.40	
MS Stock 2		**(100x)**	**10**
$MnSO_4.4H_2O$	22.3	2.230	
H_3BO_3	6.20	0.620	
$ZnSO_4.7H_2O$	8.60	0.860	
KI	0.83	0.083	
$Na_2MoO_4.2H_2O$	0.25	0.025	
$CoCl_2.6H_2O$	0.025	0.0025	
$CuSO_4.5H_2O$	0.025	0.0025	
MS Stock 3		**(100x)**	**10**
$FeSO_4.7H_2O$	27.8	2.78	
Na_2-EDTA	37.3	3.73	
Or Ferric citrate	24.4	2.44	
KNO_3	0.5 mmol	10.1g/100ml **(100x)**	**1**
pH 6.8			

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Appendix 3. Composition of Fahraeus liquid Medium.

Components	Concentration per liter
Sucrose	2.00 g
K_2HPO_4	0.17 g
KH_2PO_4	1.00 g
$MgSO_4.7H_2O$	0.25 g
Nacl	0.16 g
Fe citrate	1.20g
$CaCl_2.2H_2O$	1.20g
KNO_3	
Biotin	10µg (0.00001g)
Gibson trace elements	1 ml
	pH 6.5-6.8

Composition of Gibson trace elements

Components	Concentration per liter
H_3BO_3	2.8og
$MnSO_4.4H_2O$	2.03g
$ZnSO_4.7H_2O$	220.0g
$CuSO_4.5H_2O$	80mg
$Na_2MoO_4.2H_2O$	90mg

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