ESTABLISHMENT MECHANISM OF NITROGEN FIXING SYSTEM BY ENDOPHYTIC BACTERIA IN THE JAPANESE SUGARCANE PLANT

(内生菌による窒素固定システムがサトウキビに定着する仕組みの解明)

JOYCE PRISCA NJOLOMA

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ESTABLISHMENT MECHANISM OF NITROGEN FIXING SYSTEM BY ENDOPHYTIC BACTERIA IN THE JAPANESE SUGARCANE PLANT

A dissertation submitted to

The United Graduate School of Agricultural Sciences, Kagoshima University, Japan

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in Agriculture

JOYCE PRISCA NJOLOMA

2006

DECLARATION

I, Joyce Njoloma, 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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2006

APPROVAL OF DISSERTATION

This dissertation titled "**ESTABLISHMENT MECHANISM OF NITROGEN FIXING SYSTEM BY ENDOPHYTIC BACTERIA IN THE JAPANESE SUGARCANE PLANT**" submitted to The United Graduate School of Agricultural Sciences, Kagoshima University, Japan by Joyce Prisca Njoloma in partial fulfillment of the requirements for the degree of Doctor of Philosophy is here by approved on the recommendation of

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DEDICATION

To

VINCENT and **PATRICIA**

For their endurance.

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ABSTRACT

Sugarcane (*Saccharum* spp. L.) is one of the most important agricultural crops whose products are both for export and domestic consumption in most of the tropical and subtropical countries of the world. Several recent studies have confirmed that biological nitrogen fixation by endophytic bacteria contributes significantly to the nitrogen nutrition of sugarcane. Endophytic diazotrophic bacteria, which have been isolated in association with sugarcane plants, includes *Acetobacter diazotrophicus* and *Herbaspirillum* spp. In this research work, the main objective was to study the mechanisms involved in the establishment of nitrogen fixing system in sugarcane plant through the investigation of the mode of infection and mechanism by which the endophytic diazotrophic bacteria colonizes sugarcane after inoculation.

Herbaspirillum spp. B501*gfp*1 (B501*gfp*1), an isolate from wild rice was used as source of inoculum to investigate the interaction between nitrogen-fixing endophytic bacterium and micropropagated sugarcane plants under aseptic and non-sterile conditions. In the first place an investigation was carried out to detect if the green fluorescence protein's expression from B501*gfp*1 could be distinguished from the sugarcane tissue's background fluorescence. B501*gfp*1 bacterial cells were spot inoculated on to sugarcane tissues from 5 month-old sterile micro-propagated plants. Stem tissues and leaf sections mounted on glass slides were directly inoculated with a single touch using the tip of a syringe previously dipped into the inoculum containing 10^8 bacterial cells ml^{-1}. It was observed that GFP fluorescence could be easily distinguished in the stem than in the leaf tissues. However, the brightness level of the fluorescence varied with time as a result of

fluctuations in the bacterial cell density. The presence of chloroplasts in the leaf tissues of sugarcane requires the use of bright GFP variants when monitoring bacteria-plant interactions using *gfp* labeled bacteria.

In a study under sterile condition, two Japanese sugarcane plant cultivars (cvs) NiF8 and Ni15 were inoculated using B501*gfp*1 in 2 inoculum doses of 10^8 and 10^2 bacterial cells ml^{-1} suspension. The results obtained showed that bacterial cells colonized both the root and stem tissues, and colonization was apparent in the intercellular spaces. Higher bacterial numbers detected in plant tissues corresponded to the higher inoculum concentration treatment. Bacterial numbers also varied between the 2 cultivars with the higher values determined in cv. Ni15. Colonization ability of B501*gfp*1 in the presence of indigenous endophytes in sugarcane under non sterile condition was also carried out using the two inoculum concentrations (10^2 and 10^8 bacterial cells ml^{-1}) and in cv NiF8 only. Internal tissue colonization was observed in plants inoculated with both the 10^2 and 10^8 B501*gfp*1 bacterial cells ml^{-1} inoculum concentrations. However, extensive colonization and higher bacterial numbers were determined in the basal stem tissues of plants inoculated with the 10^8 bacterial cells ml^{-1}. Infection and establishment of the bacteria in the plants varied with respect to the inoculation technique. In the sterile plants inoculated with intact roots and basal stem, more bacteria were detected in the root tissues with the lateral root junctions as the main entry points. However, in some instances internal tissue colonization was observed to have occurred through intact root surfaces. On the other hand, plants which were inoculated with their stem bases bearing cut openings under non-sterile condition had more bacteria colonizing the stem tissues with dense establishments in the vascular bundle tissues. It can therefore be concluded

that the mode of infection and subsequent establishment mechanism in the internal tissue is facilitated by the inoculation technique and availability of entry points. This study also provides evidence for non-specificity of host plant among endophytes since *Herbaspirillum* spp. B501*gfp*1, a rice isolate could colonize sugarcane plant tissues.

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Chapter 1 GENERAL INTRODUCTION

Sugarcane (*Saccharum* spp. L.) is one of the most important agricultural crops whose products are for both export and domestic consumption in most of the tropical countries (Nickell, 1993) and in the subtropical region of Japan (DAFF, 1997). Several recent studies have confirmed that biological nitrogen fixation (BNF) by endophytic bacteria has contributed significantly to the nitrogen nutrition of sugarcane in Brazil (Boddy et al., 1991), Cuba (Dong et al., 1994), and Australia (Li and MacRae, 1991).

Various microorganisms can live in both higher and lower plants endophytically. In general, the term 'endophytic diazotrophs' includes all microorganisms that are able of colonizing the inner tissues of plants, during some periods of their life cycle, without causing any apparent damage to the host (Petrini, 1971). Thus, endophytes that are capable of invading healthy plants with intact surfaces must overcome some barriers to reach the plant interior. These bacterial endophytes can be isolated from surface-disinfected plant tissues or extracted from the internal tissues (Hallmann et al., 1997). Both gram-positive and gram-negative bacterial endophytes have been isolated from various tissues in numerous plant species. Furthermore, several different bacterial endophytes have been isolated from a single plant. Reports indicate that endophytes enter plant tissues primarily through the root zone. However, aerial portions of plants such as flowers, stems and cotyledons may also be used for entry. Thus in specific terms, the endophytes may enter internal tissues though the germinating radicles, secondary roots,

stomates or as a result of foliar damage (Kobayashi and Palumbo, 2000; Agarwal and Shende, 1987; Roos and Hattingh, 1983; Gagné et al., 1987; Leben et al., 1983). Once inside the plant, endophytes may either become localized at the point of entry or spread throughout the plant. The endophytes can reside within the cells, in the intercellular spaces or the vascular system (Hallmann et al., 1997; Bell et al., 1995; Jacobs et al.,1985; Patriquin and Döbereiner, 1978).

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. The isolation and characterization of endophytic bacteria from sugarcane and other sugar-rich crops have also been reported by several reseachers (Cavalcante and Döbereiner, 1988; Li and MacRae, 1991; Asis et al., 2000). The biological nitrogen fixing endophytic diazotrophic bacteria, which have been isolated in association with sugarcane plants includes *Acetobacter diazotrophicus, Herbaspirillum seropedicae* and *Herbaspirillum rubrisubalbicans* (Baldani et al., 1986; Cavalcante and Döbereiner, 1988; James and Olivares, 1998). However there is still some knowledge gap with respect to localization of these strains in the plant tissues (James and Olivares, 1998). Apart from the two extensively studied species of nitrogen fixing endophytes, sugarcane contains many other bacteria such as species of *Bacillus, Beijirickia, Enterobactor* and *Klebisiella* (Rennie et al., 1982; Kennedy and Tchan, 1992; Boddey et al., 1995), even though none of these bacteria are considered to be present in sufficient numbers to be of benefit to the host plant. Furthermore, it is inevitable that in addition to the beneficial diazotrophs, sugarcane contains some other endophytes which are potential pathogens. The most important of these are the xylem dwelling bacteria,

Clavibacter xyli subsp. *Xyli* and *Xanthomonas* spp. which causes ratoon stunting disease and leaf scald diseases, respectively (Purseglove, 1979).

The endophytic colonization of sugarcane by *Acetobacter* and *Herbaspirillum* spp. represents the first established case in which a monocot plant hosts a diazotrophic bacterial species which in turn supplies nutritionally significant amounts of fixed N for plant growth. In this type of association, bacteria colonize the intercellular spaces and vascular tissues of most organs of the infected plant, promote plant growth and do not cause any visible plant anatomical changes or disease symptoms (Sevilla and Kennedy, 2000). The nitrogen-fixing bacterial endophytes of sugarcane are said to provide about 4% up to 70% of the host plant's nitrogen requirement (Yoneyama et al., 1997; James, 2000) and are thought to promote plant growth through production of indole-acetic acid and other plant growth promoting substances (Dong et al., 1994; Sevilla and Kennedy, 2000). Variations in the contribution of biological nitrogen fixation to the plant nitrogen balance in different sugarcane cultivars suggest that the plant is controlling, at least in part, the efficiency of the biological nitrogen fixation process. However, there is still need to investigate the mechanisms involved in establishment of the nitrogen fixing system in this particular type of interaction. A research study was, therefore, proposed with the main goal to investigate the mode of infection and mechanism by which endophytic diazotrophic bacteria colonizes sugarcane plant tissues after inoculation. In this research study, *Herbaspirillum* spp. strain B501*gfp*1, an isolate from wild rice, *Oryza officinalis* (Elbeltagy et al., 2001) was used. *Herbaspirillum* spp. is Gram-negative bacteria capable of fixing nitrogen and promoting plant growth in different grasses (Baldani et al., 1986; James et al., 2002; Oliveira et al., 2002). In addition, studies involving light and

transmission electron microscopy have clearly demonstrated the endophytic nature of the plant-bacteria interaction (James and Olivares, 1998; Roncato-Maccari et al., 2003). At structural level, the interactions between *Herbaspirillum seropedicae* and sugarcane plants have received attention over the last decade. Studies based on chemically fixed samples of roots and shoots have demonstrated that the endophytic bacteria colonize randomly as single cells or micro colonies in the apoplast (intercellular spaces, cell wall and xylem lumen) (James and Oliviera, 1998).

Chapter 2 LITERATURE REVIEW

2.1 Sugarcane production

Sugarcane is believed to have originated from the islands of the South Pacific region and New Guinea. It is composed of six species of perennial grasses of the genus *Saccharum* L., classified in the tribe *Andropogoneae*, division *Magnoliphyta*, class *Liliopsida*, and in the order *Cyperales* of the family *Gramineae*. There are two wild species, *S. spontaneum* L., and *S. robustum* Brandes and Jeswiet ex Grassl, and 4 cultivated species, *S. officinarum* L., *S barberi* Jeswiet, *S. sinense* Roxb., and *S. edule* Hassk. (Purseglove, 1979; Wrigley, 1982). Sugarcane plant has a C-4 photosynthetic system and is one of the most efficient photosynthesizer in the plant kingdom. Sugarcane plant can achieve a photosynthetic efficiency of as high as 2%, whereas most plants on average can convert only about 0.1% of incident solar energy into biomass (Clements, 1980; Blackburn, 1984). In terms of biomass harvested, sugarcane is the world's largest crop with 691 million MT reported in 1977/78 (Irvine, 1981) and more than 1.3 billion tones in 2004 covering more than 20 million hectares throughout the tropical zone in about 107 countries (FAO, 2004). The world's leading and largest sugarcane producers are Brazil, India, China and Pakistan accounting for more than 50% of the world production (FAO 2004). The theoretical maximum yield is about 280 MT/ha/yr and seven countries (Colombia, Hawaii, Iran, Malawi, Peru, Rhodesia and Swaziland) yields an average of more than 100 MT/ha/yr. Australia, on small plots has attained more than 75% of the theoretical maximum (Irvine, 1981). In Japan, sugarcane is grown in the southern most

part on the small islands of Kyushu region, of which the leading one is the Okinawa islands. Sugar production in Japan is about 800,000 tons with sugar consumption of about 2.3 million tons and total supply of sweetener about 3.9 million tons from sugarcane and sugar beets (Figure 2-1) (Shimabukuro, 1997; Fukuda et al., 2002).

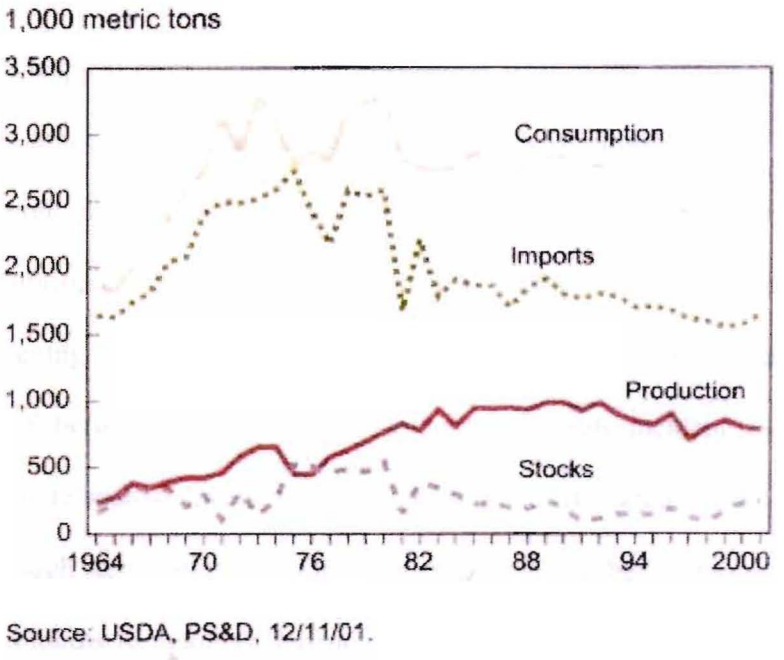

Figure 2-1. Sugar production, consumption and trade in Japan.

2.2 Sugarcane growth requirements

Sugarcane is a tropical crop, although it also grows well in the subtropical regions. The ideal weather conditions are mean day temperatures of around 30°C with adequate moisture and high incident solar radiation. It is essentially a perennial grass of the warm tropics and subtropics grown best when heavy rainfall is interspersed with bright sunshine. Ripening and harvesting require frost-free conditions with mean daily temperatures of between 10 and 20°C, dryness and high incident radiation. These conditions promote sucrose levels in the sugarcane juice (Blackburn, 1984). Sugarcane is propagated through stem cuttings and the first crop is called the "plant crop" which takes about 9 to 24 months to maturity depending on location. The subsequent crop from the cut plant crop is called "ratoon crop" and takes only 12 months to mature. Depending on agricultural practices, the length it takes before replanting varies from 4 to 10 years. Usually, each successive harvest gives a smaller yield and eventually the declining yields justify replanting. (Clements, 1980; Purseglove, 1979). Lack of multiplication procedures have been a problem in its breeding programs. Thus, it normally takes a long time to produce enough seed canes for commercial plantations. Time spent for this multiplication could also be considered of economic importance in view of higher yields that would be obtained by planting improved varieties on a larger scale. Continued contamination by systemic diseases which most often occur during multiplication stage in the open fields poses another problem. Tissue culture techniques such as meristem shoot tip culture and axillary buds methods have been used to overcome some of these sugarcane production problems (Hendre et al., 1983; Lee, 1986).

Sugarcane plant has high demands for nutrients, especially nitrogen and potassium. Nitrogen is important in sugarcane for vigorous vegetative growth and potassium for the formation of carbohydrates through the process of photosynthesis in the leaves and the transportation of sucrose to the stems and roots. Of all the nutrient elements, nitrogen has the greatest influence on cane ripening. Multiple applications of chemical nitrogen fertilizer are often required during the growing season to sustain adequate sugarcane production on soils which lack organic nitrogen contents. Shortage of nitrogen during critical growth periods can result in stunted plants, premature ripening, reduced biomass and subsequent sugar yields (Anderson, 1990). On the other hand, if applied in excess can slow down the ripening process, especially under very wet conditions. Thus, the amount of nitrogen requirements for proper growth will depend on the soil characteristics in a given location. Sugarcane tolerates a pH range of 4.5 to 8.5 but has an optimum pH range of 6.0 to 7.5. There are many pest and diseases which may also affect sugarcane growth. The most common includes bacterial, fungal and viral diseases. The most destructive insect-pests of sugarcane are the stem borers, termites and rats. Weeds can also cause economic losses if not properly managed (Purseglove, 1979).

2.3 Composition of a mature sugarcane plant

About 15.5-24% of the total sugarcane biomass consists of soluble hexose sugars, predominantly sucrose (14.5-24.0%). Glucose and fructose are also found in varying amounts of 0.2-1.0% and 0.0- 0.5 %, respectively. Cellulose, pentoses and lignin makes up 10-16% of the total composition. Amino acids, waxes, fats and organic acids accounts for 0.8-1.8%. The inorganic constituents of about 0.2-0.7% is composed of silicon,

potassium, phosphorus, calcium, magnesium, sodium, iron, sulfur and chloride. Water constitutes the remaining percentages (about 55%) (Stuppiello, 1987).

2.4 Domestic and economic uses

Fresh cane stems are often chewed for their sweet juice. Cane sugar, syrup, molasses, wax and rum are products of sugarcane. Molasses is used as a sweetener, in industrial alcohol, cosmetic and pharmaceuticals, cleaning detergents preparations and solvents, synthetic rubber production and in combustion engines. Interest in the use of sugarcane for the production of ethanol is on the rise, in light of high oil and gasoline prices. Brazil has successfully adopted its fuel economy to replace much of its gasoline consumption with ethanol consumption. Corn is predominantly used in the United States for the production of ethanol. Sugarcane is, however, many times more efficient for the production of ethanol. A shift from corn-based ethanol to sugarcane ethanol may permit heavily oil-dependant countries such as the United States to substantially reduce its oil-dependence.

Sugar is also used as a preservative for fruits and other meat products. It is a common adjunct to unpleasant medicines (used as coating) and the juice has been used as a folk medicine remedy for colds, cough among other diseases. Refuse cane (bagasse) is used in the manufacture of paper, cardboard and fuel. A mixture of bagasse and molasses is used as cattle feed. The ground and dried cane (after juice has been expressed) makes some excellent mulch. (Watt and Breyer-Brandwijk, 1962; Lewis and Elvin-Lewis, 1977; Purseglove, 1979).

2.5 Biological nitrogen fixation in non-leguminous plants

The availability of nitrogen often limits plant growth in most agricultural ecosystems. It affects the productivity and the species composition of the plant communities and ecosystem processes at all scales. In the today's agriculture, nitrogen is one of the most widely used fertilizers and is still on the increase. Due to the ever-increasing nitrogenous fertilizer which are used for crop production and the limited supply of fossil fuel energy available to manufacture this fertilizer, it is becoming increasingly important to try to extend biological nitrogen fixation to the non-legume crop. The only biological reaction counter balancing the loss of nitrogen from ecosystems is biological nitrogen fixation. This process is unique to bacteria and archaea, and is estimated to contribute globally between 100 and 200 million tons of fixed nitrogen per year (Galloway et al., 1995; Karl et al., 2002; Hurek and Reinhold-Hurek, 2003). The best studied plant-bacteria interaction is the root nodule symbiosis between rhizobia and legumes. On the contrary, the most important crops world wide, wheat, rice, maize and sugarcane belonging to the *Gramineae* do not naturally form these specialized symbiotic structures. However, it has been shown that some of these gramineous crops can derive a substantial part of the plant nitrogen requirement from biologically fixed nitrogen.

The role of biological nitrogen fixation in non-leguminous plants began to unfold with the discovery of the semi-solid N-free media by Döbereiner's group in the 1970's (Döbereiner, 1972). Several diazotrophic species have since been isolated from different grass plants in high numbers of up to 10^7 cells per gram of fresh weight (Paula et al., 1991; Boddey et al., 1991; Baldani et al., 1997). Some of the isolated bacteria are held

responsible for the very significant nitrogen fixation observed in the field experiments using N-balance and ^{15}N isotope dilution techniques (Boddey et al., 1991; Döbereiner et al., 1993; Yoneyama et al., 1997; Asis et al., 2002). The recent evidence of significant nitrogen fixation in economically important gramineous species of sugarcane and forage grasses such as kallar (*Leptochloa fusca*) (Urquiaga et al., 1992; Malik et al., 1997), has generated tremendous interest in nitrogen fixation by non-leguminous plants. Sugarcane - endophytic bacteria interaction continues to unfold a promising model for biological nitrogen fixation in the non-leguminous crop plants.

2.6 Nitrogenase activity by endophytes in grass plants

The studies on nitrogenase activity in non-leguminous plants and associative bacteria began with the use of the acetylene reduction method to measure the capacity of gramineous plants to fix nitrogen in association with diazotrophs. The results showed high nitrogenase activity rates, however there was no direct relation to the nitrogen-fixing bacteria (Döbereiner, 1961). And the use of this technique to evaluate the biological nitrogen fixation potential of several forage grasses (*Panicum maximum, Pennisetum purpureum, Digitaria decumbens, Cynodon dactylon and Melinis minutiflora*) gave rates of 200 to 700 nmoles C_2H_4/h/g of roots, which varied with the season and stage of plant development (Day et al., 1975), soil temperature (Abrantes et al., 1976) and level of ammonium in the soil (Neves et al., 1976). In the recent decade, both ^{15}N isotope dilution and ^{15}N natural abundant studies have shown that sugarcane, rice and other grass plants can fix substantial amounts of nitrogen. However, these gramineous 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; Boddey et al., 1999).

Nitrogen fixation is a high-energy demanding process and to accomplish this process, microorganisms have developed a mechanism to turn off nitrogenase when fixed nitrogen is available to supply the organism's need. The addition of ammonium inhibits nitrogenase activity in some diazotropic bacteria, and this process is called "NH_4^+ switch-off". After a decrease in the external ammonium concentration, their enzyme activity is restored "switch on". The sensitivity to ammonium depends on the culture condition and species of the endophytes (Gordon et al., 1981; Gotto and Yoch, 1985). In the case of *Acetobacter diazotrophicus*, nitrogenase activity is partially inhibited by the addition of 20mM $(NH_4)_2SO_4$, and several amino acids have shown similar responses in the presence of 5 g glucose per litre (Stepan et al., 1991). Also, nitrogenase could be inactivated by excessive oxygen. This can occur at genetic level by acting on the nitrogenase synthesis, by causing irreversible damage to the iron protein and/ or by causing a reversible "switch-off" where the activity can return following a decrease in oxygen pressure (Goldberg at al., 1987). Thus, the oxygen levels must be high enough to maintain cell growth, but not too high to inactivate the nitrogenase enzyme.

2.7 The green fluorescent protein

2.7.1 Green fluorescence protein expression and detection in living cells

The green fluorescent protein (GFP) of *Aequorea victoria* offers a unique tool that permits the monitoring of gene expression, protein localization in living cells. GFP can be functionally expressed in different bacterial species and can be used as a reporter of gene expression, dynamic processes during bacterial development, and their behavior in complex environments. Since the introduction of GFP as a marker gene expression *in vivo* and cytological marker fused to protein-coding sequences, researchers have used GFP fusion proteins to monitor protein expression and localization within cells of all types in living tissues. It has been expressed in bacteria, yeast, slime molds, plants, drosophila, zebra fish, and in mammalian cells. As a non invasive fluorescent marker in living cells, it allows for a wide range of applications where it may function as a cell lineage tracer, reporter of gene expression, or as a measure of protein-protein interactions. (Prasher et al., 1992; Chalfe et al., 1994; Inouye and Tsuji, 1994a; Wang and Hazelrigg, 1994).

The importance of GFP as a biological marker derives from the fact that the protein's fluorescence requires no other cofactors and that the protein does not alter the normal function or localization of the fusion partner. Permeability for substrate entry and fixation are not needed to localize GFP in organelles and therefore cells marked with the GFP can be examined in living tissues. While GFP does not require any additional cofactors for fluorescence, the amount of fluorescence is not always proportional to the total pool of GFP. The GFP chromophore requires molecular oxygen and therefore fluorescence often

decreases under anaerobic or strong reducing environments (Heim et al., 1994; Inouye and Tsuji, 1994b). Temperature can also affect GFP fluorescence because the tendancy of GFP to precipitate into non-fluorescent inclusion bodies inclusion bodies increases with temperature. The fluorescence signal can be enhanced by growing bacteria aerobically at low temperatures up to 30°C (Ogawa et al., 1995; Webb et al., 1995; Cormack et al., 1996). However, when using some of the enhanced GFP mutants these requirements are less important.

GFP expression is uniquely suited to track live bacteria within complex environments and can be assayed within fractionated samples. In bacterial-plant interaction studies, the GFP labeled bacteria's localization, association and multiplication as monitored by fluorescence, can be followed temporally and spatially (Fisher and Long, 1992). Although GFP has been successfully expressed and visualized in many divergent organisms, several parameters affect the ease of detection of GFP, some of which includes the type of GFP used (mutant or wild-type forms), the concentration of the GFP and the effects of background autofluorescence (Ausubel et al., 1996). In most biological samples, there are naturally occurring fluorescent molecules which fluoresce with characteristics similar to those of GFP. The substances contribute to the background fluorescence, or autofluorescence of the specimen.

2.7.2 Fluorescent spectrum of green fluorescent protein

Wild type GFP from jellyfish has two excitation peaks, a major one at 395 nm and a minor one at 475 nm with extinction coefficient of 30,000 and 7,000 M^{-1} cm^{-1}, respectively. Its emission peak is at 509 nm in the lower green portion of the visible

spectrum. The GFP from the sea pansy exhibits a single major excitation peak at 498 nm. Crystals of GFP exhibits a nearly identical fluorescence spectrum and lifetime to that of GFP in aqueous solution.

For wild type GFP exciting the protein at 395 nm leads to rapid quenching of the fluorescence, with an increase in the 475 nm excitation band. This photoisomerization effect is prominent with irradiation of GFP by UV light. In a wide range of pH, increasing pH leads to a reduction in fluorescence by 395 nm of excitation band and an increased sensitivity to 475 nm of excitation. Some mutants of the GFP gene have been produced which have increased fluorescence. The major excitation peak has been red-shifted to 490 nm with the emission staying at 509 nm. This is better for use with standard optical filter sets, as the mutant GFPs have excitation range more compatible with the commonly available optical filters. This also is more useful for confocal laser scanning applications. The corrected excitations and emission spectra of GFP and some of its variants (termed as BlueFP, CyanFP, GreenFP, and YellowFP variants) are as shown in Figure 2-2.

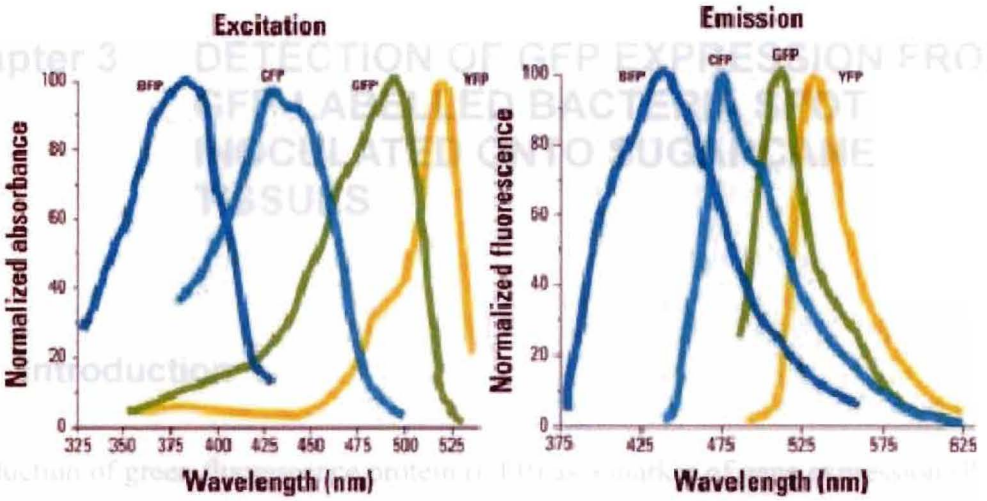

Figure 2-2. Fluorescent spectrum of green fluorescent protein.

Chapter 3 DETECTION OF GFP EXPRESSION FROM GFP-LABELLED BACTERIA SPOT INOCULATED ONTO SUGARCANE TISSUES

3.1 Introduction

Introduction of green fluorescence protein (GFP) as a marker of gene expression (Prasher et al., 1992; Chalfie et al., 1994) has facilitated research in localization and identification of the GFP tagged bacteria in the infected cells and plant tissues. Its use has become one of the powerful and valuable tools for addressing most of the biological research in plant – microbe interactions in the living sytems (Chalfie et al., 1994). The enormous advantage of using GFP over conventional microscopic probes is that it allows the observations of the protein in living cells. Bacterial cells tagged with GFP can be enumerated *in situ* and samples do not need to be fixed, hybridized or staining (Tombolini et al., 1997). GFP expression has been so unique in tracking live bacteria within complex environments. In addition, bacterial multiplication can be followed temporally and spatially (Valdivia et al., 1998; Compant et al., 2005). However, there is one major limiting factor in the detection of GFP in living organisms whose cells or tissues emit background autofluorescence such that it becomes difficult to detect the GFP's fluorescence. Some reports have indicated that the autofluorescent of chloroplasts, normally present in the upper parts of most plants can provide counter fluorescence for GFP, such that even when using the brightest GFP variant, its expression within the cells

or tissues may be unsatisfactory (Haseloff, 1998). Thus, the expression of GFP can be limited to particular cell types or tissues within a plant, as a means for visualizing GFP tagged bacterial cells. In the stem tissues, the parenchyma tissues emit most of the red autofluorescence while the vascular bundle tissues emit yellow autofluorescence. Although, research has shown that endophytes can establish inside plant tissues, multiplication of the endophytes in the tissues has not been demonstrated (Hallman et al., 1997; Compant et al., 2005). In this study *Herbaspirillum* spp. B501*gfp*1 (Elbeltagy et al., 2001), bacterial cells were spot inoculated on to sugarcane tissues and the main purpose was to detect if bacteria's green fluorescence can be distinguished from plant tissue's autofluorescence in the sugarcane plant especially in the stem and leaf tissues which contains a substantial amount of chloroplasts. The other objective was to study the behaviour of B501*gfp*1 in sugarcane plant tissues over time after inoculation.

3.2 Materials and Methods

3.2.1 A bacterial strain and inoculum preparation

Herbaspirillum spp. B501, an isolate from wild rice was kindly provided by Dr Kiwamu Minamizawa of Tohoku University, Japan. Based on its basic characteristics the bacterial strain B501 is classified as belonging to the genus, *Herbaspirillum*. However, it still differs from the earlier know three *Herbaspirillum* spp. (*H. seropedicae, H. rubrisubabicans and H. frisingense*) based on its carbon source utilization and diagnostic probe sequence (Elbeltagy et al., 2001). The green fluorescent protein (*gfp*) gene encoding GFP was introduced into B501 bacterial strain with a gfp mintransposon

pUTgfpx2 by electroporation (Unge et al., 1998) and was thereafter referred to as *Herbaspirillum* spp. B501*gfp*1 (Elbeltagy et al., 2001).

B501*gfp*1 bacteria were cultured on LB medium containing 50µg/ml kanamycin for 48 hours at 28°C. The bacterial cells were harvested from plates with a sterile loop and suspended in sterile distilled water. Inoculum density was estimated by direct cell count method using Petroff-Hauser counting chambers and adjusted by dilution to10^8 bacterial cells/ml with sterile distilled water.

3.2.2 Plant tissue inoculation

Five months-old sterile sugarcane plants, cv. NiF8 propagated under sterile conditions were used in this study. Plantlets were produced through meristem culture and rooted plantlets were then transferred into modified Leonard jars. Plants were supplemented with 5mg/l of KNO_3 as a nitrogen source for 1 month after which plants were maintained on N-free growth medium in the laboratory under controlled conditions (28°C and 16-h photoperiod). At 5 months of growth, selected plants were uprooted and washed under tap water and then finally rinsed with distilled water. Tissues for observations were obtained from the stem and leaf sections. Microslicer (D.S.K microslicer, DRK 1000, Dosaka EM Co. Kyoto, Japan) was used to obtain at least 0.1 mm tissue thickness of the stem section. Stem tissues and leaf sections mounted on glass slides were directly inoculated with a single touch using the tip of a syringe previously dipped into the inoculum containing 10^8 bacterial cells/ml. Tissues were kept in modified humidity chamber to prevent them from drying up quickly and also to provide moist condition for bacterial cell growth. Observations were carried out over a period of 7 days.

Chapter 3. Detection of GFP Expression

Inoculated tissues were examined for expression of GFP and autofluorescence using a Nikon Eclipse E600 (Nikon Corporation, Tokyo, Japan) equipped with 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). Filter B-2A ensures a clear distinction between the plant tissues autofluorescence from the GFP labeled bacteria. Under B-2A filter autofluorescence becomes dark brown, red or yellow. The images were captured using Pixera a digital camera system for microscopy (Pixera Corporation, Los Gatos, USA) fitted on to the Nikon Eclipse E600.

3.3 Results

3.3.1 Detection of fluorescence

Time course observations on the stem tissues showed that few hours after inoculation (Figure 3-1A and B) and when bacterial population had increased (Figure 3-1D), the expression of B501*gfp*1's green fluorescence level was distinguishable from the stem tissue's autofluorescence. Bacterial cells mortality was observed in the inoculated tissues at 2 days after inoculation (DAI) and when bacterial numbers had decreased, low levels of the bacteria's green fluorescence were observed as shown in Figures 3-1C and E. On the other hand, on the leaf surface, it was found out that the GFP expression could only be clearly distinguished under gfp filter (EX 460 -500 nm) (Figures 3-3A, C and E). Its expression was highly masked under B-2a filter by the strong red autofluorescence (Figure 3B). By the 4^{th}, the aggregated bacterial cell clusters were observed localized along the leaf veins and could only be observed emitting some yellowish fluorescence still masked by the red autofluorescence (Figures 3-3D and F).

3.3.2 Changes in the inoculated bacterial cell density with time

It was interesting to note the behavior of the inoculated bacteria in the tissues over time. Fluctuations in the bacterial cell density were observed on the tissues as bacteria settled in aggregated clusters. Soon after inoculation bacterial cells were observed scattered round the inoculated spot (Figure 3-1B) and by the second day, only few bacterial cells were observed localized mostly as clustered cells in low densities (Figure 3-1C). However, by the 4^{th} day, the surviving bacterial cells especially in the parenchyma tissues had multiplied filling up the cells in which they were localized (Figure 3-1D). On the other hand, bacterial cells localized in the xylem tissues did not show much increase in numbers in most of the samples and by the 5^{th} day, very few or no bacterial cells could be detected in the xylem tissues probably due to shortage of food resources required for growth and multiplication (Figures 3-2C and D). Some bacterial cells were also observed proliferating in the intercellular spaces (Figures 3-1D and 3-2E). The intercellular space localized bacterial cells survived for a longer period compared to bacterial cells proliferating in either the parenchyma or xylem tissues (Figure 3-1E). And on the leaf surface, bacterial proliferation was observed mostly when bacterial cells formed clusters along the leaf veins (Figure 3-3).

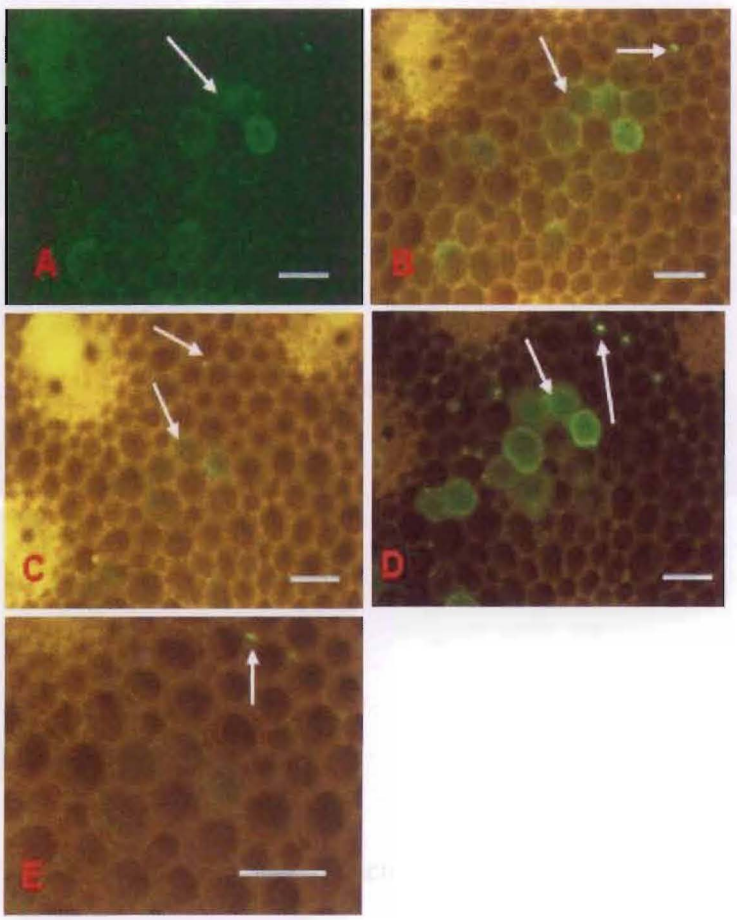

Figure 3-1. Bacteria population changes over time on the stem tissue.

An hour after inoculation (A and B), (C) at 2 DAI, Bacterial population density rose at 4 DAI (D) followed by a sharp decrease 7 days later (E). (A) as observed under gfp filter while (B, C, D and E) under B-2a filter. Scale bar = 20μm, arrows (in white colour) indicate some of the positions colonized by bacteria (in green colour).

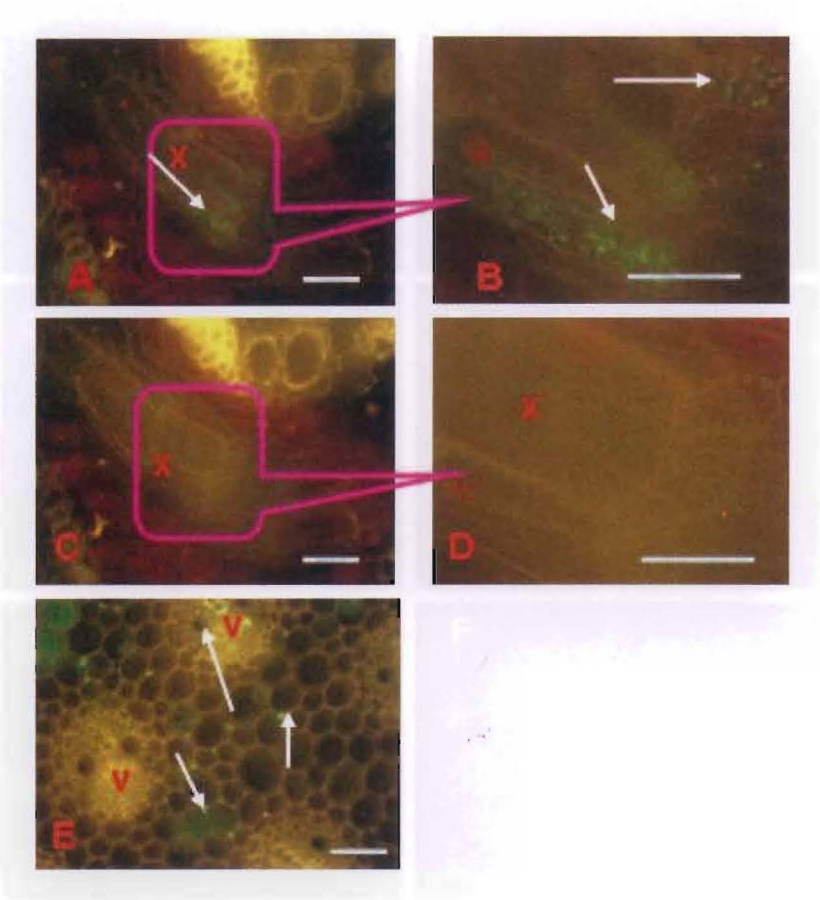

Figure 3-2. Spot inoculated bacterial cells in the xylem tissue over time.

At 2 DAI, (A and B) shows relatively low density of proliferating bacterial cells. By the fifth day, bacterial cells could no longer be visible in the xylem tissue (C and D). (E) shows less and smaller aggregates of inoculated bacteria in the vascular bundle tissues compared to numerous aggregates in the intercellular spaces in the parenchyma tissues. Scale bar = 20μm. X stands for xylem tissue and V for vascular bundle

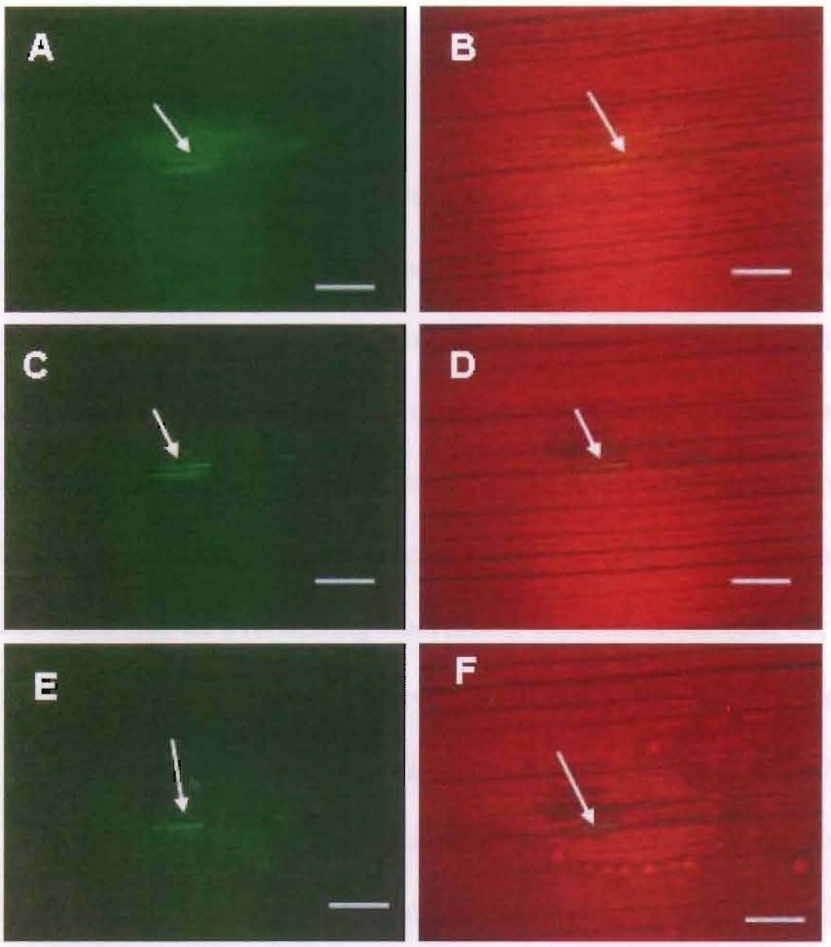

Figure 3-3. Colonization pattern of spot inoculated bacteria on the leaf surface.

(A and B) 1 hour after inoculation bacterial cells were observed spreading over the inoculated area. Bacterial cells clustered along the leaf vein, 2 DAI (B and C) and at 4 DAI (D and E). Observed under gfp filter (A, C, E) and B-2a filter (B, D, F). Arrows (in white colour) indicate observed bacteria. Scale bar = 20μm

3.4 Discussion

Low levels of the red autofluorescence were observed in the stem tissues such that expression of the green fluorescence from the inoculated strain B501*gfp*1 could be easily distinguished from the stem tissue's autofluorescence. On the leaf surfaces, higher levels of the red autofluorescence due to presence of chloroplasts made it difficult to detect the green fluorescence expression from the bacterial cells. Even after some time when the bacterial cells had aggregated, their fluorescence could only be expressed as yellowish, suggesting that there was an overlapping fluorescent expression between the leaf tissues and bacteria. Elbeltagy et al. (2001) observed B501*gfp*1's fluorescence expression in shoots of 7 day old wild rice, *O. officinalis* W0012 (its host plant) and *O. sativa* cv. Sasanishiki. He reported that yellow fluorescence mixed with red and green fluorescences was observed exclusively in shoots of the host plant, wild rice *O. officinalis*. Expression of GFP fluorescence has been demonstrated to show the ability of some GFP labelled endophytes like *Burkhoheria* sp strain PsJN and *Xylella fastidiosa* colonizing xylem vessels of *Vitis vinifera* seedlings (Compant et al., 2005; Newman et al., 2003) in which GFP fluorescence expression was clearly distinguished from the tissues autofluorescence. In this study, it has been demonstrated that the presence of chloroplasts on the leaf tissues especially, requires the use of high levels of GFP fluorescence for monitoring inoculated *gfp* labelled bacteria. There is also a possibility that in these 5 months old sugarcane plant the chloroplasts were more matured and developed resulting in emitting the strong red autofluorescence. Tombolini et al. (1997) demonstrated that GFP tagged *P. fluorescens* could be visualised in soil samples and that bacterial fluorescence was easily detected,

even after prolonged carbon starvation conditions. On the contrary, presumably due to depletion of both the energy and water resources a decrease in bacterial numbers was observed, which resulted in very low levels of GFP expression. It can be suggested that where there is some background counter fluorescence the GFP expression can be difficult to visualize when the bacteria density is low. This study demonstrates that sugarcane is one of the plants which would require the use of high level GFP expressing gene markers especially in the leaf tissues.

In plants, some of the important activities such as photosynthesis, assimilation, respiration, storage, and secretion are primarily based in parenchyma tissues. The extensive multiplication of the cells in the parenchyma tissues can be explained by the fact that these tissues are considered to be food storage tissues unlike the xylem vessels which serve mainly as water transporting tissues in the vascular bundles. This observation demonstrates that a good amount of available energy source is required to sustain higher bacterial numbers in the internal tissues. Although, bacterial cells did not extensively multiply in the xylem vessels in the present study, other studies have indicated that detected endophytes within aerial plant parts can be transported in the xylem vessels through the transpiration stream and in the intercellular spaces (James and Olivares, 1998; Gyaneshwar et al., 2001; James et al., 2002). The decrease in bacterial cell density a few days after inoculation on both the stem and leaf could have been as a result of adaptation adjustment to the new growth conditions. Cells and tissues are sensitive to their environment such that if not maintained in a proper environment they may die or behave differently. Monier and Lindow (2004) reported that amongst other factors, formation of bacteria aggregates requires conducive environment and ample

supply of carbon containing nutrient. Using whole-cell biosensors for sugars on leaves, it has been reported that the sites of abundant sugar on the leaf are few and small (Leveau and Lindow, 2001). These observations confirm that bacteria cells can multiply in the internal tissues under good environmental conditions and supply of energy source.

3.5 Conclusion

The results of this experiment show that GFP fluorescence of the labeled bacteria could be easily distinguished in the stem than in the leaf tissues. However, the brightness level of the fluorescence varied with time as a result of fluctuations in the bacterial cell density. The presence of chloroplasts in the leaf tissues of sugarcane requires the use of bright GFP variants when monitoring bacteria-plant interactions using *gfp* labelled bacteria. Bacterial cells are capable of multiplying in the sugarcane plant tissues especially in the parenchymatously tissues.

Chapter 4 INFECTION AND COLONIZATION OF ASEPTICALLY MICROPROPAGATED SUGARCANE SEEDLINGS BY NITROGEN-FIXING ENDOPHYTIC BACTERIUM, *HERBASPIRILLUM* SPP. B501 *GFP* 1.

4.1 Introduction

Sugarcane is one of the vegetatively propagated plants whose intercellular spaces are reported to be colonized by endophytic nitrogen-fixing bacteria (Paula et al., 1991; Reis et al., 1994; Olivares et al., 1996). The isolation and characterization of endophytic bacteria, *Acetobacter* and *Herbaspirillum* species among other species, from sugarcane and other agricultural crops have also been reported (Cavalcante and Döbereiner 1988; Li and MacRae, 1991; Dong et al., 1994; Asis et al., 2000). These bacterial endophytes may originate from seeds, soil (Mc Inroy and Kloepper, 1995b) and vegetative material (Sturz, 1995). Population dynamics of these bacterial endophytes have shown that both indigenous and introduced endophytic population seem to be higher in the roots and at the basal stem, decreasing gradually up to the stem and leaves with comparatively lower densities for the introduced ones (Poon et al., 1977; Pleban et al., 1995; Mc Inroy and Kloepper, 1995a; Lamb et al., 1996; Quadt-Hallmann and Kloepper, 1996). Studies have shown that biological nitrogen fixation by the endophytic bacteria has contributed significantly to the nitrogen nutrition of some sugarcane cultivars in Brazil (Boddey et al., 1991) and Australia (Li and MacRae, 1991). Nitrogen balance and ^{15}N aided dilution

studies have confirmed nitrogen nutrition benefits in sugarcane, however, there has been cultivar differences in amounts of fixed nitrogen ranging from 4 to over 70% Ndfa of the total nitrogen from the atmosphere (Lima et al., 1987; Urquiaga et al., 1992; Yoneyama et al., 1997; Asis et al., 2002).

The endophytic nitrogen fixing bacteria isolated from several grasses can decrease the use of nitrogenous fertilizers in the cultivation of major cereal crops. Different techniques have been used to detect and isolate endophytic bacteria mostly this has involved maceration and centrifugation of the surface sterilized plant tissues. These methods can give estimates of bacterial population and extent of colonization but fails to reveal the sites of infection of the colonization process. Another method is the use of electron microscopy which shows *in situ* colonization sites (Sevilla and Kennedy, 2000). However, preparation of samples for electron microscopy is tedious and can not be used in routine examination of multiple plant samples. During the last decade the use of reporter genes such as *gus A* gene from *E. coli* has facilitated investigations of plant-microbe interactions, including those that involve grass and endophytic diazotrophic associations (Christiansen-Weniger, 1997). *Gus A* encodes β-glucuronidase whose activity can be detected easily by a wide range of glucuronide substrates (Wilson et al., 1995). Thus, Colonization investigations of isolates in crop plants could be achieved using the *gus A* marker gene. In addition, bacterial numbers are evaluated by colony count methods.

Biomarkers have been used and proved extremely useful for reporting gene expression (Haseloff and Siemering, 1998) in localization and identification studies of the inoculated bacteria in the infected plant tissues. The gfp gene cloned from the jellyfish, *A. victoria*

(Chalfie et al., 1994), has been used as a visual marker in gene expression and protein subcellular localization in a wide range of organisms including the plant-associated bacteria *Pseudomonas* (Tombolini et al., 1997) and *Herbaspirillum* sp. (Elbeltagy et al., 2001). Unlike other markers, GFP can be visualized directly in living tissues without any prolonged and lethal histochemical staining (Haseloff and Siemering, 1998).

Interaction studies between endophytic bacteria and their host, sugarcane plants have been conducted under laboratory and greenhouse conditions by inoculating the isolated bacterial endophytes (*Acetobacter and Herbaspirillum* sp.) onto micropropagated sugarcane plants (Reis et al., 1999; James et al., 2001; Oliveira et al., 2002) and sugarcane plants developed from buds (Muthukumarasamy et al., 1999). It has been found that endophytes are capable of invading healthy plants with intact surfaces by overcoming some barriers to reach the internal plants tissues (Reinhold-Hurek and Hurek, 1998). In this study, *Herbaspirillum* spp. strain B501*gfp*1, an isolate from wild rice, *O. officinalis* (Elbeltagy et al., 2001) was used to investigate its colonization in a non-host plant, sugarcane. The effect of inoculum density on plant tissue colonization was investigated using two inoculum concentration levels and bacterial numbers in plant tissues were quantified by colony counting method.

4.2 Materials and Methods

4.2.1 Plant and culture conditions

Stalks of sugarcane cultivars (cvs) NiF8 and Ni15 obtained from Miyako Islands, Japan were used. Sets of about 3 internodes were prepared and surface sterilized for 5 minutes using 70% ethanol followed by 2% Sodium hypochlorite (NaClO) for 20 minutes. The

sterilized sets were thoroughly washed with distilled water and then planted in containers filled with sterilized vermiculite under laboratory conditions. New shoots were collected and sterilized for 2 minutes using 70% ethanol followed by 2% NaClO for 15 minutes and then washed three times with distilled water. A callus was then induced on the meristematic tips using Murashige and Skoog (MS) basal salt medium (Murashige and Skoog, 1962) (Appendix 1) supplemented with 3 mg L^{-1} 2, 4-dichlorophenoxyacetic acid (2, 4-D). At six weeks of callus growth, shoot regeneration was induced on MS medium supplemented with kinetin and 6-benzylaminopurine (BAP) each at a concentration of 0.01 and 0.2 mg L^{-1} respectively. Regenerated shoots were separated and then transferred to MS medium supplemented with 0.2 mg L^{-1} naphthaleneacetic acid (NAA) for root induction and after one week, plantlets were transferred into a hormone free MS medium for root growth (Figures 4-1 and 4-2). Subcultures were made at a monthly interval. Medium pH was adjusted to 5.8 prior to autoclaving at 121℃ for 20 minutes.

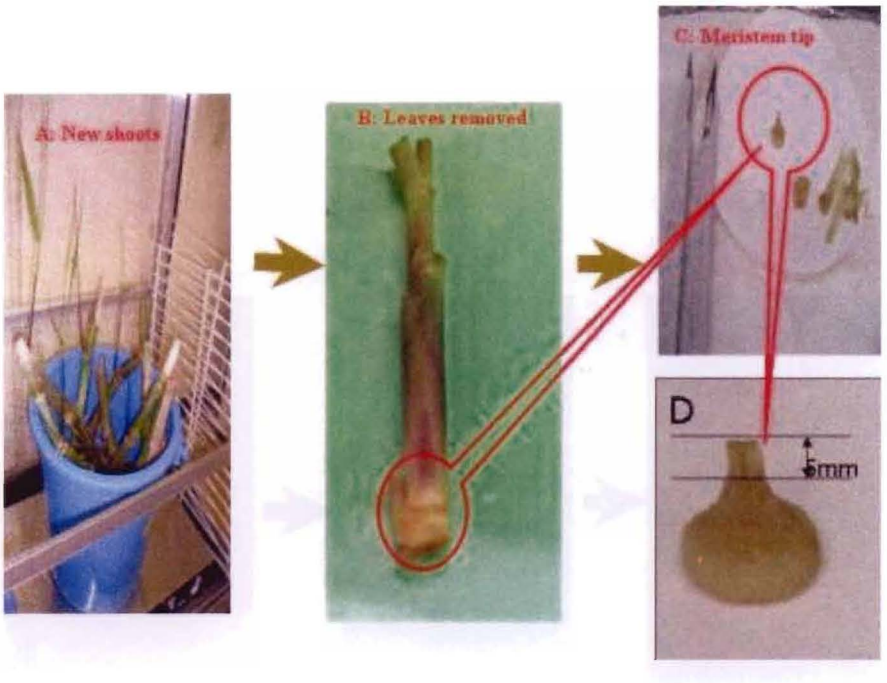

Figure 4-1. Preparation of meristem tip.

(A) New shoots of sugarcane cultivar Ni15 planted in sterilized vermiculite. (B) Shoot tips about 5 cm with the outer covering leaf shealths removed were sterilized by dipping in 70% ethanol for a minute followed by 2% NaOCl for 15 minutes with periodical shaking. Shoot tips were then washed 3 times using sterile distilled water. (C & D) Meristem tip about 5 mm for callus production was aseptically exposed by removing the surrounding primordial leaf shealths

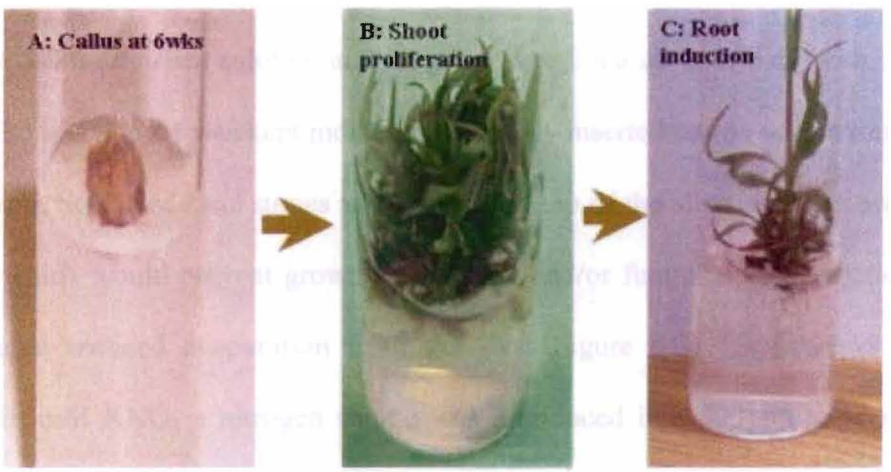

Figure 4-2. Callus induction and sterile shoot regeneration.

(A) A callus was induced on MS basal salt medium containing $2mgl^{-1}$ 2, 4-D. (B) shoot regeneration from the callus was induced on MS medium supplemented with kinetin and BAP each at a concentration of 1 mgl^{-1}. (C) regenerated shoots were then transferred to MS medium supplemented with 1 mgl^{-1} NAA for root induction and after one week plantlets were transferred into a hormone free MS medium for root growth.

Rooted plants were washed with sterile distilled water to remove the agar, separated under sterile condition and then transferred into modified Leonard jars. Two, 1 litre capacity (8.5 cm diameter and 14.5 cm length) twist pack pots (Takeya Co. Osaka, Japan) were used to assemble a single set. One pot served as a reservoir containing approximately 200 ml nutrient solution and the other was filled up to 700 ml with sterile vermiculite. The vermiculite was kept moist by a centrally inserted cotton wick extending into the reservoir. Sterilized sand stones were spread on top of the vermiculite to provide a dry surface which would prevent growth of bacteria and/or fungus on the vermiculite and also ensured reduced evaporation from the jars (Figure 4-3). Two weeks after transplanting 0.5mM KNO_3 a nitrogen source was introduced into the jars to boost up growth. Even though nitrogen is not a limiting factor for *Herbaspirillum* spp. in the interaction with its host plant (Oaks, 1992; Muthukumarasamy et al., 1999), in this study its supply was terminated after 1 month. Thereafter, N_2-free nutrient solution was used until when plants were inoculated with B501*gfp*1 (Appendix 3). All cultures were maintained at a temperature range between 25 - 28℃ 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.

4.2.2 Bacterial strain used

Herbaspirillum spp. strain B501*gfp*1 (Elbeltagy et al., 2001) was provided from the research group of Dr. K. Minamisawa, Institute of Genetic Ecology, Tohoku University, Japan. *Herbaspirillum* spp. B501*gfp*1 was isolated from stems of wild rice, *O. officinalis* W0012. The bacterium has been classified as belonging to the genus, *Herbaspirillum*. However, it differs from the known *Herbaspirillum* sp. (*H. seropedicae*, *H.*

rublisulbabicans and *H. frisingense*) isolated from the roots of rice, maize, sorghum and sugarcane in its utilization of *meso*-erythritol, *N*-acetylglucosamine and *meso*-inositol. Its endophytic colonization and in planta nitrogen fixation have been studied using both wild and rice cultivars. B501*gfp*1 colonized its host plant, wild rice, *O. officinalis*, more than the rice cultivars (Elbeltagy et al., 2001)

4.2.3 Inoculum preparation

B501*gfp*1 bacterial cells were grown on Luria Bertani (LB) medium (10 gl^{-1} tryptone, 5 gl^{-1} yeast extract, both from DIFCO Laboratories, Detroit, USA, 5 gl^{-1} NaCl, 10 % sucrose), and pH was adjusted to 7.0 before autoclaving (Appendix 2). Inoculum was prepared with bacterial suspensions from 24 hour old bacterial cultures growing on LB medium. Three milliliters of phosphate buffered saline (PBS), pH 7.2 were added to the culture plates and bacterial cells were scraped with a glass rod. The bacterial suspension was then centrifuged (3480 g, 10 min, 4 °C). The supernatant was discarded and pellet resuspended in PBS. Inoculum suspensions were adjusted to 10^2 and 10^8 bacterial cells ml^{-1} using PBS.

4.2.4 Inoculation of sugarcane plants with B501*gfp*1

Plants were inoculated with 200 ml of 10^2 and 10^8 bacterial cell suspensions. Sand stones covering the vermiculite were removed and then bacterial suspension was evenly poured onto vermiculite in the jars without disturbing the plant root system. Control plants were inoculated with sterile distilled water. After pouring all the inoculum, the vermiculite was covered again with new sterile sand stones and plants were left to grow under same conditions as described above. Sampling started 1 then 7, 28 and 56 days after

inoculation (DAI). Plants were divided into roots (main and lateral roots) and aerial (basal stem, leaf shealth and leaf blade) for microscopic fluorescence observation and bacterial density determination. Plant tissue samples to be examined were selected from both the inoculated and uninoculated (control) plants. Surfaces of intact roots, leaf sheath and leaf blades were examined for the presence of B501*gfp*1. Internal tissues of the roots, stem and leafy portion (mainly the leaf sheath) were examined by slicing the sampled portion into about 0.1 mm transversal sections. Microslicer (D.S.K microslicer, DRK 1000, Dosaka EM Co. Kyoto, Japan) was used in the first part of the experiment and later, plant sections were sliced by hand. Microscopic fluorescence was examined using a Nikon Eclipse E600 (Nikon Corporation, Tokyo, Japan) equipped with 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).

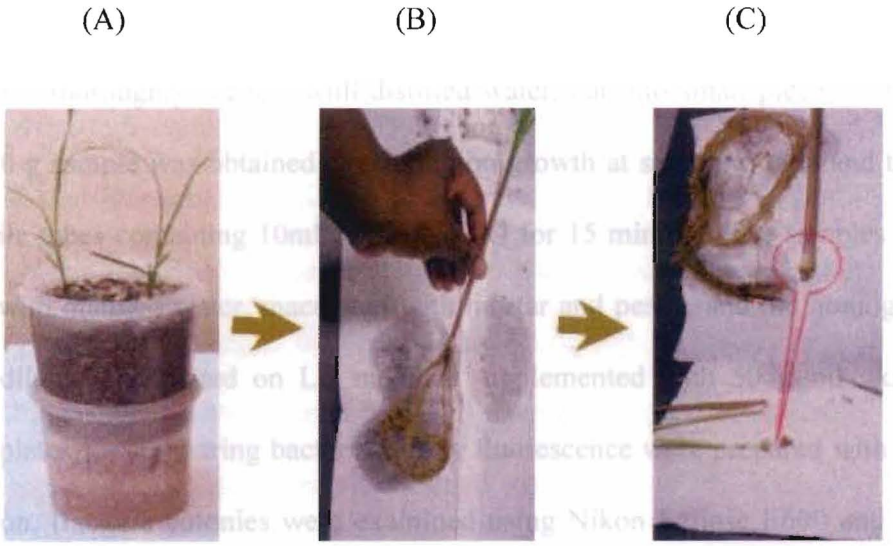

Figure 4-3. Rooted plants transferred into modified Leonard jars filled with sterile vermiculite.

(A) Two month after transplanting, plant were inoculated with 200ml of 10^2 and 10^8 *Herbaspirillum* strain B501*gfp*1 cell suspension by wetting the vermiculite evenly by use of pipet. (B) Sampling for fluorescent microscopic observation was done after 1, 7 and 28 days. (C) Plants were divided into upper (base of stem, leaf shealth and leaf blade) and lower (main root, adventitious, lateral and fine roots) plant parts. The upper and lower plant parts were examined for fluorescent observation. Microslicer was used for soft tissues and the thicker tissues of the root and stem were sliced by hand.

Plant samples for bacterial density determination from both inoculated and uninoculated plants were thoroughly washed with distilled water, cut into small pieces from which a 0.2 to 1.0 g sample was obtained depending on growth at sampling time and transferred into sterile tubes containing 10ml of 2% NaClO for 15 minutes. The samples were then washed with distilled water, macerated with mortar and pestle, and the homogenate was serially diluted and plated on LB medium supplemented with 50 $\mu g ml^{-1}$ kanamycin. Control plates for comparing bacteria colony fluorescence were prepared with B501*gfp*1 suspension. Bacteria colonies were examined using Nikon Eclipse E600 and those that emitted green fluorescence were counted. To evaluate the potential of B501*gfp*1 to promote growth in sugarcane, dry weight of both roots and shoots were evaluated at 7 and 56 (DAI) after drying at 60°C to constant weight. Descriptive statistic and t – test analysis to determine the mean significant differences were performed using Microsoft Excel Analysis Toolpak 2003 (Microsoft Corporation, USA).

4.3 Results

4.3.1 Bacteria density changes in tissues of sugarcane cultivars NiF8 and Ni15

Herbaspirillum spp. B501*gfp*1 bacteria population densities were detected in the root and aerial plant tissues by colony counting method. No bacteria were detected in tissues of both cvs. NiF8 and Ni15, 1 DAI. Table 4.1 shows that the population numbers in the internal tissues varied significantly between plants inoculated with 10^8 bacterial cells ml^{-1} (higher concentration) and 10^2 bacterial cells ml^{-1} (lower concentration). Differences were also observed between cultivars inoculated with the same concentration. Generally, cv. Ni15 had higher bacterial numbers with the highest population ($2.4x10^7$ CFU g^{-1} FW)

after 28 DAI in the root tissues of plants inoculated with the higher inoculum concentration. Cultivar Ni15 is said to be an early and high-sugar accumulating cultivar, a characteristic that would ensure growth of endophytic N_2 - fixing bacteria residing inside its tissues (Nishiguchi et al., 2005). The aerial parts had low bacteria counts and the lowest population density was determined in plants inoculated with lower inoculum concentration. Worth noting is that while bacteria density in tissues of sugarcane cv. NiF8 kept increasing, a decrease in bacterial numbers was observed, especially in the root tissues of sugarcane cv. Ni15 in both inoculum concentration levels

Table 4. 1. Counts of bacteria from plant tissues of sugarcane cultivars NiF8 and Ni15

Cultivar	Initial inoculum	Tissue examined	Bacterial numbers ($x10^5$ CFU g^{-1} FW) 7	28	56 (DAI)
Uninoculated		Roots	0	0	0
		Aerial parts	0	0	0
NiF8	10^8cfu ml^{-1}	Roots	0.75±0.48	2.3±0.85	5.0±0.7
		Aerial parts	0	0.38±0.25	1.2±0.32
	10^2cfu ml^{-1}	Roots	0.15±0.24	0.75±0.47	1.3±0.48
		Aerial parts	0	0	0.25±0.24
Ni15	10^8cfu ml^{-1}	Roots	18.7±2.4	237±42	33±5.1
		Aerial parts	0.50±0.28	1.5±0.64	4.0±9.1
	10^2cfu ml^{-1}	Roots	0.13±0.12	6.2±2.3	2.5±0.45
		Aerial parts	0	0.12±0.12	0.50±0.2

Values represent a mean of 4 determinations ± SE. FW stands for fresh weight.

Tables 4.2 and 4.3, show mean dry weights of cvs. NiF8 and Ni15, respectively, at 7 and 56 DAI. In each of the cultivars, there was significant increase ($P \leq 0.05$) in their mean dry weight over time. However, the means were not significantly different at each of the sampling times, but the dry weights of plants inoculated with 10^8 bacterial cells ml^{-1} in both sugarcane cultivars were relatively higher than the means of plants inoculated with the lower inoculum dose and their controls at 56 DAI. Although, the nitrogenase activity by B501*gfp*1 was not estimated in the present study, Elbeltagy et al., (2001) demonstrated its nitrogenase activity in its host plant, rice. *Herbaspirillum* spp. B501*gfp*1 showed significant acetylene reduction activity in the wild rice, *O. officinalis* W00012 compared to the cultivated *O. sativa*. In this study, B501*gfp*1 was not detected in the control plants such being the case, it can be suggested that besides other growth factors, the associated bacterial numbers in the tissues may have influenced the increase in plant's dry matter. Nishiguchi et al., (2005) estimated the contribution of N_2- fixation by host endophytic bacteria in new spouting shoots of sugarcane cvs. Ni15, F172 and NiF8 using ^{15}N dilution technique. Cultivar Ni15 yielded higher root dry weight than the other cultivars. Moreover, its estimated fixed N_2 was also higher though not enough to meet the host plant's demand.

Table 4. 2. Dry weight in sugarcane cultivar NiF8

Dry weight (g)									
	10^8			10^2			Control		
DAI	Root	Shoot	Total	Root	Shoot	Total	Root	Shoot	Total
7	0.75±0.10	1.04±0.18	1.79±0.26	0.73±0.09	1.02±0.15	1.72±0.23	0.74±0.09	1.01±0.12	1.75±0.20
56	1.83±0.23	2.11±0.19	3.94±0.41	1.75±0.25	1.89±0.11	3.64±0.37	1.67±0.20	1.71±0.21	3.38±0.41

Values represent means ± SE of 4 replicates. No significance differences between inoculated and uninoculated values. However, the inoculated plants had gained relatively higher dry weights.

Table 4. 3. Dry weight in sugarcane cultivar Ni15

Dry weight (g)									
	10^8			10^2			Control		
DAI	Root	Shoot	Total	Root	Shoot	Total	Root	Shoot	Total
7	0.97±0.15	1.10±0.15	2.10±0.30	0.93±0.07	1.11±0.09	2.04±0.16	0.96±0.10	0.91±0.07	1.75±0.20
56	2.09±0.18	2.20±0.19	4.29±0.33	1.94±0.14	2.18±0.20	4.13±0.33	1.88±0.21	2.13±0.17	4.01±0.38

Values represent means ± SE of 4 replicates. No significance differences between inoculated and uninoculated values. However, the inoculated plants had gained relatively higher dry weights.

4.3.2 Colonization of B501*gfp*1 in sugarcane cultivars NiF8 and Ni15

Colonization pattern was determined in plant tissue samples at 1, 7, 28 and 56 DAI through microscopic fluorescence observation. Bacteria presence could not be detected in the internal tissues of sugarcane from both the higher and lower dose inoculated plants of both sugarcane cvs. at 1 DAI. However, the bacteria could only be observed attached onto the root surfaces. Figures 4.4 (a; a-1) shows bacterial colony observed 1 DAI on the root surface of sugarcane plant cv. Ni15 inoculated with 10^8 bacterial cells ml^{-1}. However, the colonies were very small and only sparsely observed in the higher inoculum dose. At 7 DAI, root surfaces of both sugarcane cvs. NiF8 (Figure 4.4c) and Ni15 (Figure 4.4d) inoculated with 10^8 bacterial cells ml^{-1} showed a dense and elongated colonization pattern seemingly following intercellular spaces. As for the 10^2 bacterial cells ml^{-1} inoculated plants, colonization appeared to be spotty and in small colonies (Figure 4.4b). Moreover, the bacterial numbers were higher in the root tissues of the higher concentration inoculated plants, approximately 10^6 CFU g^{-1} FW 7 DAI (Table 1). Colonization was also observed to occur in the intercellular spaces at lateral root emergence zones (Figure 4.5a) and in the root cortical tissues (Figure 4.5b). Similarly, in the basal stem tissues, intercellular colonization was also apparent in the collenchyma (Figure 4.5c) and ground parenchyma tissues (Figures 4.5 d; e). In addition, though not frequent, colonization was seen in the xylem vessels in the vascular bundles (Figure 4.5f).

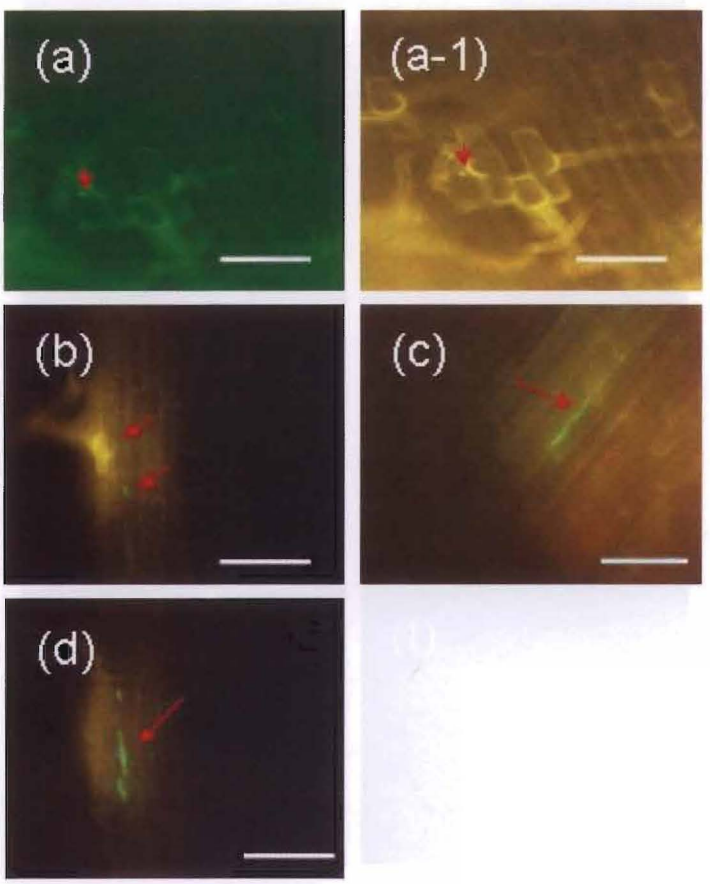

Figure 4-4. Colonization of B501*gfp*1 as observed on the root surfaces of sugarcane.

At 1 DAI bacteria colonies of B501*gfp*1 were observed on root surfaces of plants inoculated with a higher concentration inoculum suspension (10^8 bacterial cells ml^{-1}) (a) under GFP filter (a-1) B-2A filter. 7 DAI relatively large colonies were observed on roots of plants inoculated with 10^2 bacterial cells ml^{-1} (cv. Ni15) and dense colonization was observed on root surfaces of plants inoculated with 10^8 bacterial cells ml^{-1} suspension (c) cv. NiF8 (d) cv. Ni15. Arrows indicate position of bacterial colonization. Bars show 10μm.

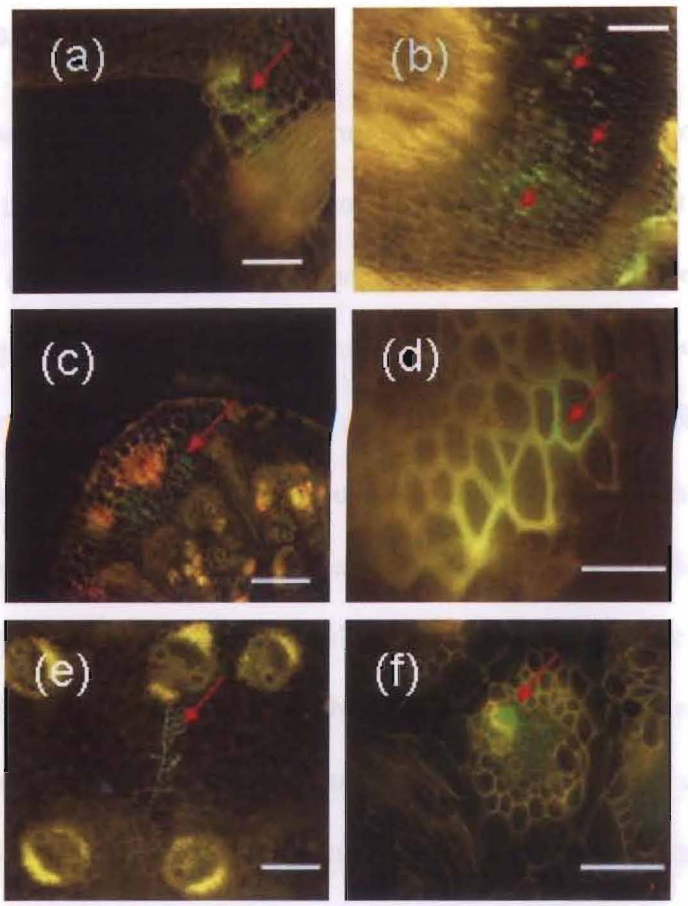

Figure 4-5. Internal colonization by B501*gfp*1 in the root and stem tissues of sugarcane.

In the root tissues, 28 DAI, intercellular colonization was observed in cv. NiF8 (a) root emergence zones and (b) root cortex tissues of plants inoculated with10^2 bacterial cells ml^{-1}. (c) 56 DAI basal stem transversal section shows B501*gfp*1 in the intercellular spaces of collenchymatous (cv. NiF8) and ground parenchyma tissues in (e) (cv. Ni15) both from plants inoculated with 10^8 bacterial cells ml^{-1}. Whereas (d) shows a weak intercellular colonization in cv. Ni15 when plants were inoculated with 10^2 bacterial cells ml^{-1}. (f) xylem vessels in the vascular bundle tissues of the stems were also colonized. Bars show 10μm.

4.4 Discussion

Densities of the inoculated B501*gfp*1 determined in the root tissues of cvs. NiF8 and Ni15showed that the bacteria were able to colonize the sugarcane plant tissues at least 7 DAI. James et al. (2001) also reported that endophytic bacteria could be re-isolated from root tissues of sugarcane 7 DAI indicating that this period is usually good enough for the bacteria to penetrate through open wounds and cracks on the roots. Reis et al. (1999) demonstrated that inoculation technique used to introduce endophytic bacteria in sugarcane micropropagated plants requires a short incubation period for the inoculated bacteria to colonize the internal tissues. Results have also shown that the higher inoculum concentration yielded more bacterial numbers in the plant tissues regardless of the inoculated cultivar within a few days compared to the lower inoculum concentration. de Weger et al. (1997) also showed that inoculum concentration had some influence on colonization of wheat roots in which germinated seeds were inoculated with bacterial suspensions varying from 10^3 to 10^8 CFU ml^{-1}. Wheat plants inoculated with higher bacteria concentrations 10^6 - 10^8 CFU ml^{-1} resulted in their roots being heavily colonized than those inoculated with lower concentrations. Baldani et al. (1992) reported that *Herbaspirillum* spp. did not survive well in soils without plants, as result higher concentrations would be necessary for a significant effect on internal tissue colonization within a short period of time. It is essential that sufficient numbers of diazotrophs should be present in the plants to account for the biological nitrogen fixation (James, 2000). Olivares et al. (1997) found out that *H. rubrisubalbicans* could survive outside the host plants and colonize their leaf surfaces, at least for a limited time. It is probably this ability that ensured increased bacteria population density with time in the sugarcane cultivars

such that as more roots emerged there was infection on the new roots and consequently a subsequent increase of the bacterial population in the plant tissues. By the time of inoculation, cv. Ni15 had more root biomass compared to cv. NiF8 and it can therefore, be suggested that their might have been an efficient initial bacteria and root contact. It is also possible that the plant genotype could have influenced the colonization of the root interior, as demonstrated by Schloter and Hartmann (1998). The author suggested that bacterial-plant interaction could be facilitated by plant cultivar as well as environmental factors.

In the present study, it has been shown that B501*gfp*1 bacteria colonized the internal tissue of sugarcane in the roots and base of the stem with apparent localization in the intercellular space and in some vascular bundle tissues. On the contrary, in the recent work by Elbeltagy et al. (2001) observations in the 7 days old rice seedlings showed that when B501*gfp*1 bacteria were inoculated onto the seeds of wild and cultivated rice plants, the endophyte best colonized the intercellular spaces of the wild rice, *O. officinalis* W0012 which is apparently its host plant compared to the cultivated rice, *O. sativa*. And no bacteria were observed in the vascular tissues of rice shoot. It can be suggested that in as much as an endophytes will be capable of infecting different plant species without showing host specificity, it localization site would differ in the associated plant species. Since B501*gfp*1 has been classified as belonging to the same genus as most of the endophytes that have been isolated from sugarcane, there is a likelihood of similar colonization pattern to the host bacteria. Bacterial colonization of the vascular system has also been reported in a number of both beneficial and pathogenic interactions between sugarcane plants and bacteria (Kao and Damann, 1980; Gagné et al., 1987; Harrison and

Davis, 1988; James et al., 1994; Bell et al., 1995; Lamb et al., 1996; Olivares et al., 1997) and in other associations between diazotrophs and grasses (Hurek et al., 1994; James et al., 1997). Furthermore, the results obtained on surface colonization shows that B501*gfp*1 could enter into the plant roots and internally colonize the tissues under conditions where minimal or no surface wounding had occurred compared to other studies in which micropropagated sugarcane plants have had to be inoculated soon after separation (Reis et al., 1999).

Besides the studies involving endophytic establishment in the Brazillian sugarcane by *Herbaspirillum* spp. using conventional microscopy (James et al., 1997; Olivares et al., 1997), cryotechnique approaches have opened a new insights for the ultrastructural aspect of the bacteria in free state and under endophytic interaction (Silva et al., 2003). Under endophytic interaction viewed by TEM after high pressure freezing followed by freezing substitution, *Herbaspirillum* spp. were observed infecting leaves as micro colonies into the lumen of the metaxylem vessel and as single cells in the apoplast. In roots, *H. seropedicae* colonized cortex apoplast, adhered to plant cell wall such as those of the xylem vessels and inside apparently dead vascular parenchyma cells. Plant roots inoculated with the bacteria showed the bacteria associated with the intercellular of the root cortex. The cryotechniques have also revealed some altered aspects of both the endophyte and the cell walls at the adhesion sites and bacteria protrusions have also been observed. Silva (2004) demonstrated with great frequency that the bacteria were associated with abundant unknown electron-dense material and some vesicular structures coming from the plant cell walls. The produced substances clearly showed that the plant cell walls were stimulated in response to the presence of the bacteria. Thus, these

bacterial endophytes could stimulate the plant cell walls not only in relation to nitrogen fixing process alone but also in some processes involving promotion of plant growth. Although, it was found out that B501*gfp*1 could in part be responsible for promoting growth in the sugarcane plants, there is need to quantify how much nitrogen can be fixed by the inoculated bacteria using the ^{15}N dilution and other technique which would give an estimation of the amount of biologically fixed nitrogen.

4.5 Conclusion

The study shows that B501*gfp*1 can colonize a non-host plant suggesting non-specificity of host plant among endophytes. It can also be concluded that higher inoculum will yield higher bacterial numbers in the internal tissues and this would probably ensure optimization for BNF in non-leguminous plants like sugarcane. However, the bacterial numbers would vary depending on the inoculated cultivars.

Chapter 5 COLONIZATION ABILITY OF *HERBASPIRILLUM* SPP. B501*GFP*1 IN SUGARCANE, A NON-HOST PLANT IN THE PRESENCE OF INDIGENOUS DIAZOTROPHIC ENDOPHYTES

5.1 Introduction

Interaction studies between sugarcane and diazotrophic endophytic bacteria have shown that sugarcane can derive substantial biologically fixed nitrogen from inoculation with a mixture of diazotrophic bacteria (Oliveira et al., 2002; Muthukumarasamy et al., 1999). In these, plant – bacterial interactions, the N_2-contribution by the endophytic bacteria has been found to vary with bacterial strain and plant host. Two of the most widely studied genera among the diazotrophic endophytes are *Herbaspirillum* and *Gluconacetobacter* and both were originally isolated as endophytes of sugarcane (Baldani et al., 1986; Gillis et al., 1989). Recently, these genera have also been found associating with other non-leguminous plants in a broad host range (Chelius and Triplett, 2001; Elbeltagy et al., 2001). These diazotrophic endophytes are expected to provide a range of potential N_2-fixing bacteria to be utilized as inocula for in non-leguminous plants. Asis et al. (2000) isolated putative strains of *Gluconacetobacter diazotrophicus* and *Herbaspirillum* from sugarcane cultivar (cv.) NiF8 cultivated in Miyako islands of Japan and reported that out of the 52 randomly selected isolate colonies, only 21 showed positive acetylene reduction assay (ARA). Thus, the nitrogen supply contribution from the indigenous endophytic N_2-

fixing bacteria is inadequate for the crop's demand. Nishiguchi et al. (2005) also reported that the N_2-contribution by the indigenous diazotrophic endophytes existing in sugarcane cvs. (Ni15, F172 and NiF8) was not enough to supply the nitrogen which the host plant demands.

Significant variations in the populations of both indigenous and introduces endophytes have been reported and that these variations could be attributed to plant source, plant age, type of tissue, sampling time and the environment (Zinniel et al., 2002). The general trend has shown that bacterial populations are larger in roots and decrease in the stems and leaves (Lamb et al., 1996). Natural endophyte concentration can vary between 2.0 and 6.0 $\log_{10}$ cfu per gram for alfalfa, sweet corn, sugar beet, squash, cotton and potato (Kobayashi and Palumbo, 2000). Similar findings have been reported for the introduced endophytic bacteria inoculated by root or seed drenching with population levels reaching between 3.0 and 5.0 $\log_{10}$ cfu per gram of plant tissues for potato and tomato (Kobayashi and Palumbo, 2000). And the levels of colonization by non pathogenic endophytes tend to be lower than the levels of colonization of pathogenic bacteria, the concentration of the later organisms range from 7.0 to 10.0 $\log_{10}$ cfu per gram of fresh plant tissues in susceptible infected plants (Grimault and Prior, 1994; Tsiantos and Stevens, 1986).

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

micropropagated seedling plants grown under sterile conditions (James et al., 1994; Reis et al., 1999; Muñoz-Rojas and Caballero-Mellado, 2003; Boddey et al., 2003). Currently, there is still little information regarding inoculation of sugarcane seedling plants propagated under non sterile conditions and on how indigenous endophytes existing in the plants would affect internal colonization by the introduced bacterial endophytes. In the earlier study (Njoloma et al., 2006 in Chapter 4) in which B501*gfp*1 bacterial cells were inoculated onto sterile sugarcane cvs. Ni15 and NiF8, it was found that the bacteria colonized the internal tissues of both the roots and basal stems of the two cultivars. *Herbaspirillum* spp. strain B501 is an isolate from wild rice, *Oryza officinalis,* known to have in planta N_2-fixation ability (Elbeltagy et al., 2001; You et al., 2005). Based on its basic characteristics, the bacterial strain B501 is classified as belonging to the genus, *Herbaspirillum.* However, it is designated as not identical to the earlier known *Herbaspirillum* species based on its carbon source utilization and diagnostic probe sequence (Elbeltagy et al., 2001). The objective of this study was to examine the internal colonization ability of B501*gfp*1 as an introduced bacterial endophyte in sugarcane (non-host plant) seedling plants propagated under non sterile conditions.

5.2 Materials and Methods

5.2.1 Determination of total bacterial population in sugarcane stems

Stalks of sugarcane cv. NiF8 obtained from Miyako islands sub-tropical agricultural experiment station, Okinawa, Japan were used. Segments of sugarcane stalks were thoroughly washed and rinsed in distilled water. Sets were cut into small stem sections and surface sterilized with 70% ethanol for 10 minutes, followed by 2% sodium

hypochlorite (NaClO) for 20 minutes. The sterilized stem pieces were thoroughly washed using sterile distilled water, peeled using a sterile knife and then cut into small pieces about 3-5 cm from which sugarcane juice was extracted.

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

5.2.2 Determination of N_2-fixing endophytic bacteria in sugarcane stems

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

5.2.3 Shoot growth and culture conditions

Sugarcane sets were prepared and planted in containers filled with non-sterilized vermiculite. The growing buds were then detached at least from 3 weeks after

germination and were transferred into modified Leonard jars (assembled using 2 plastic pots, 1L capacity, 8.5 cm diameter, 14.5 cm length (Takeya Co. Osaka, Japan)). One pot served as a reservoir containing N-free nutrient solution and the other was filled up to 700 ml with non-sterilized vermiculite covered with sand stones to prevent excess evaporation (Figure 5.1). Plants were left to grow for at least 1 month before inoculation with the B501*gfp*1 endophytic bacteria. All cultures were maintained at a temperature range between 28 - 30°C under a photoperiod of 16 h light and a photon flux density of 60 μmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes.

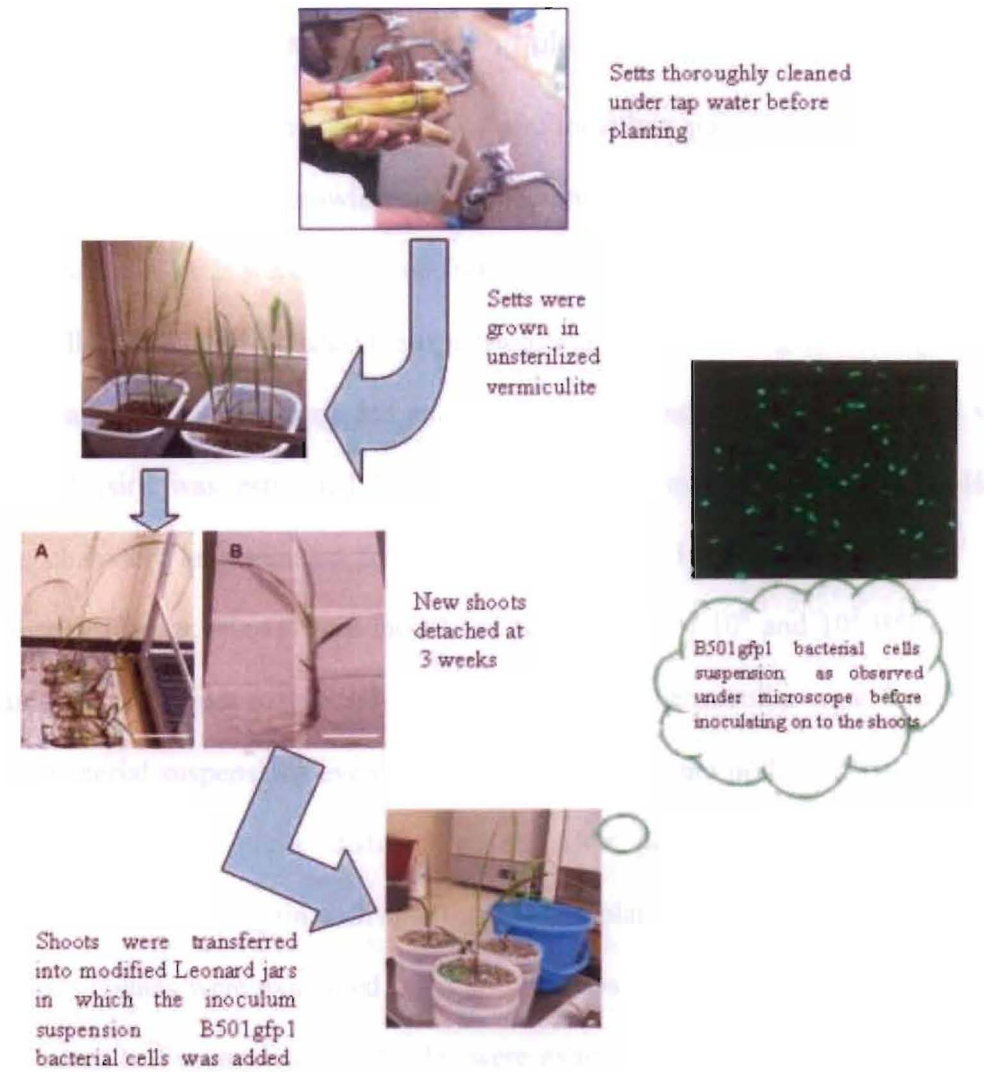

Figure 5-1. Inoculation of sugarcane seedling plants with B501*gfp*1 under non sterile condition.

5.2.4 Inoculation of sugarcane seedling plants with B501*gfp*1

B501*gfp*1 bacteria were maintained on LGIP and inoculum was prepared using 48 hours old B501*gfp*1 bacterial cells growing on LB medium containing 50 μg ml^{-1} kanamycin at 28°C. The bacterial cells were harvested from plates with a sterile loop and suspended in sterile distilled water. The bacterial suspension was then centrifuged (3,480g, 10 min, 4°C). The supernatant was discarded and pellets re-suspended in sterile distilled water. Inoculum density was estimated by direct cell count method using Petroff-Hauser counting chambers and adjusted by dilution to 10^8 and 10^2 bacterial cells ml^{-1} using sterile distilled water. Plants were inoculated with 200ml of 10^8 and 10^2 B501*gfp*1 cell ml^{-1} suspensions (Figure 5.1). Sand stones covering the vermiculite were first removed and then bacterial suspensions evenly poured onto vermiculite in the jars. Control plants were inoculated with sterile distilled water. After pouring all the inoculum, the vermiculite was covered again with small stones and plants were left to grow under same conditions. Tissues were examined at 14 and 56 days after inoculation (DAI). Surfaces of intact roots, leaf sheath and leaf blades were examined for the presence of B501*gfp*1. Internal tissues of the roots, stems and leafy sections (leaf sheath) were examined by slicing the sampled portion into about 0.1 mm transversal sections. Microslicer (D.S.K microslicer, DRK 1000, Dosaka EM Co, Kyoto, Japan) was used. Microscopic fluorescence was examined using a Nikon Eclipse E600 equipped with GFP (R)-BP, HQ (FITC)-BP filter (DM 505, BA 500-560, EX 460-500) and B-2A filter (DM 505 and EX 450-490). The images were captured using Pixera, a digital camera system for microscopy (Pixera Corporation, Los Gatos, USA) fitted on to the Nikon Eclipse.

Plant samples for bacterial density determination from both inoculated and uninoculated young sugarcane plants were thoroughly washed with distilled water, cut into small pieces from which 0.5 g sample was obtained and sterilized using 2% NaClO for 15 minutes. The samples were then washed with distilled water and grounded by hand using mortar and pestle. From each serially diluted homogenate, a 100 µl aliquot was plated on LB medium for total bacterial counts and on LGIP medium for N_2-fixing bacterial counts. Bacteria colonies on LGIP medium were examined under Nikon Eclipse E600 and those that emitted green fluorescence were counted as the inoculated B501*gfp*1 bacterial endophytes.

5.3 Results

5.3.1 Bacterial population density in sugarcane tissues

The population densities of the diazotrophic endophytes in both the sugarcane stems prior to planting and in the young seedling plants after inoculation with B501*gfp*1 are presented in Table 5.1. In the sugarcane stems prior to planting, a total of 10^4 cfu ml^{-1} sugarcane juice was determined for the total indigenous N_2-fixing endophytes. In the young seedling, population density of the total indigenous N_2-fixing diazotrophic endophytes ranged from 10^2 to 10^3 cfu gFW^{-1} for the uninoculated plants and, from 10^3 to 10^4, and 10^4 to 10^5 for the 10^2 and 10^8 B501*gfp*1 inoculated plants, respectively. Population density of the inoculated B501*gfp*1 diazotrophic endophyte was higher in the stem tissues of sugarcane plants inoculated with the 10^8 inoculum concentrations each of the sampling times. The inoculated B501gfp1 bacteria in the leafy tissues were only detected in the 10^8 B501*gfp*1 inoculated plants at 56 DAI. The estimated ratios of the

inoculated B501*gfp*1 to the indigenous N_2-fixing bacteria shows that the inoculated bacteria were more than the indigenous N_2-fixing bacteria in plants inoculated with the 10^8 B501*gfp*1 bacterial cells ml^{-1} at 56 DAI. The results show that a higher inoculum concentration would ensure more of the inoculated bacteria infecting the tissues in the presence of the indigenous diazotrophic endophytes.

Table 5. 1. Bacterial density in mature and young tissues of cultivar NiF8

Inoculum Concentration	Time (DAI)	Tissues used	Total endophytes	Total N_2-fixing endophytes	Total Inoculated B501*gfp*1
Control	At planting	Stem (juice)	1.9×10^6 *	2.4×10^4 *	--
	14	Root	1.63×10^3	7.55×10^2	--
		Stem	2.62×10^3	8.95×10^2	--
		Leaf	nd	nd	--
	56	Root	4.11×10^3	3.33×10^3	--
		Stem	3.10×10^3	2.97×10^3	--
		Leaf	1.1×10^2	Nd	--
10^2	14	Root	4.11×10^3	1.12×10^3	1.04×10^2 (0.1:1)
		Stem	3.34×10^3	1.70×10^3	5.01×10^2 (0.4:1)
		Leaf	nd	nd	nd
	56	Root	1.78×10^4	1.61×10^4	1.4×10^3 (0.09:1)
		Stem	3.35×10^4	6.70×10^3	2.1×10^3 (0.48:1)
		Leaf	1.20×10^2	Nd	Nd
10^8	14	Root	4.33×10^4	1.92×10^4	4.0×10^3 (0.27:1)
		Stem	2.81×10^4	1.47×10^4	8.1×10^3 (1.22:1)
		Leaf	1.16×10^2	nd	nd
	56	Root	4.49×10^5	5.62×10^4	4.20×10^4 (2.9:1)
		Stem	4.50×10^6	7.03×10^5	5.30×10^5 (3.1:1)
		Leaf	2.33×10^2	1.49×10^2	1.08×10^2 (2.6:1)

Data are means of 3 replications and 3 plants per treatment were examined.

*: values are in cfu/ml of sugarcane juice.

– : tissues were not previously inoculated with HB501gfp1.

nd : not detected.

The value in ( ) is the ratio of the inoculated B501*gfp*1 to indigenous N_2-fixing bacteria detected in the tissues. The value for the indigenous N_2-fixing bacteria was obtained by subtracting the inoculated B501*gfp*1 from the total N_2-fixing endophytes.

Table 5.2 shows the amount of ethylene each of the selected diazotrophic endophytes could produce per hour. Twenty eight isolates from the indigenous N_2-fixing diazotrophic endophytes obtained from sugarcane juice collected from stems of mature sugarcane plants were randomly selected and tested for their ability to reduce acetylene to ethylene. Relatively higher positive ARA values were obtained in 2 (NiF8 21 and NiF8 22) of the 28 isolates, with NiF8 21 producing 1.178 nmol hr^{-1}. However, in comparison with B501*gfp*1 bacterial strain, all the selected bacterial endophytes showed low levels of ARA.

-Table 5. 2. ARA of sugarcane cultivar NiF8 isolates and B501 *gfp*1

Isolates	**ARA (nmol C_2H_4 $tube^{-1}$ hr^{-1})**
NiF8 1	0.028
NiF8 2	0.010
NiF8 3	0.032
NiF8 4	0.032
NiF8 5	0.002
NiF8 6	0.001
NiF8 7	0.017
NiF8 8	0.005
NiF8 9	0.033
NiF8 10	0.006
NiF8 11	0.002
NiF8 12	0.002
NiF8 13	0.002
NiF8 14	0.0016
NiF8 15	0.0012
NiF8 16	0.0015
NiF8 17	0.0016
NiF8 18	0.0015
NiF8 19	0.026
NiF8 20	0.028
NiF8 21	1.178
NiF8 22	0.126
NiF8 23	0.014
NiF8 24	0.0036
NiF8 25	0.0018
NiF8 26	0.076
NiF8 27	0.0013
NiF8 28	0.0023
HB501*gfp*1	5.336

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

Table 5. 2. ARA of sugarcane cv. NiF8 isolates and B501 *gfp*1

Isolates	**ARA (nmol C_2H_4 $tube^{-1}$ hr^{-1})**
NiF8 1	0.028
NiF8 2	0.010
NiF8 3	0.032
NiF8 4	0.032
NiF8 5	0.002
NiF8 6	0.001
NiF8 7	0.017
NiF8 8	0.005
NiF8 9	0.033
NiF8 10	0.006
NiF8 11	0.002
NiF8 12	0.002
NiF8 13	0.002
NiF8 14	0.0016
NiF8 15	0.0012
NiF8 16	0.0015
NiF8 17	0.0016
NiF8 18	0.0015
NiF8 19	0.026
NiF8 20	0.028
NiF8 21	1.178
NiF8 22	0.126
NiF8 23	0.014
NiF8 24	0.0036
NiF8 25	0.0018
NiF8 26	0.076
NiF8 27	0.0013
NiF8 28	0.0023
HB501*gfp*1	5.336

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

5.3.2 Colonization of the internal tissues by B501*gfp*1

Bacterial colonization in the young plants was also assessed at two intervals, 14 and 56 DAI. Internal tissues colonization by the inoculated bacteria in plants inoculated with both the 10^2 and 10^8 B501*gfp*1 bacterial cells ml^{-1} inoculum concentrations could be observed. Fewer and less dense xylem vessel colonization in the stem tissues were observed in the plants inoculated with 10^2 B501*gfp*1 bacterial cells ml^{-1} (Figure 5.2A). Despite the increase in the bacterial numbers to 10^3 cfu gFW^{-1} at 56 DAI (Table 1), the colonized sites remained fewer in number (Figures 5.2C and D) amongst the several scattered vascular bundle tissues. In addition to the vascular bundle colonization, intercellular colonization in the parenchyma tissues was also observed (Figure 5.2B). However, its green fluorescence expression was not very bright compared to colonies in the xylem vessels, an indication of low bacterial concentration on the site of colonization.

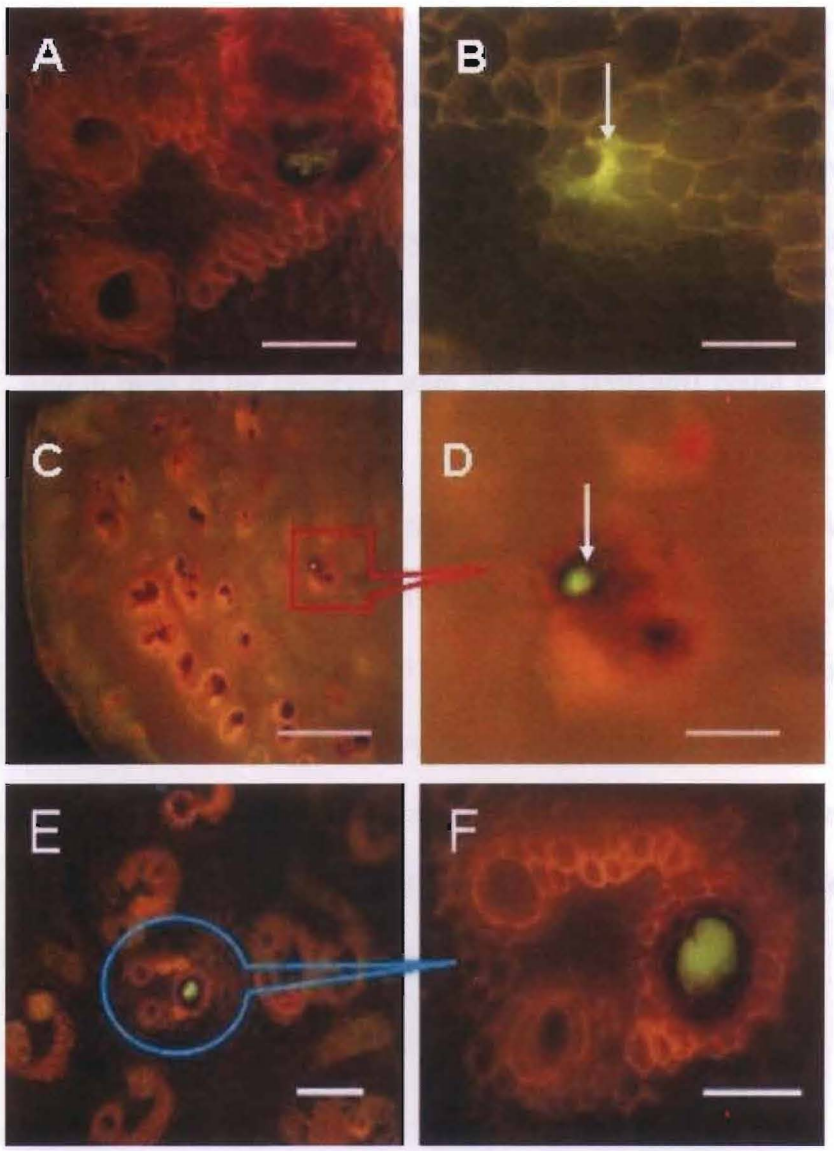

Figure 5-2. Internal colonization in the sugarcane stem tissues inoculated under non sterile condition.

In the xylem vessels of the 10^2 bacterial cells ml^{-1} inoculated plants, A: 14 DAI., (C and D): 56 DAI. B: shows intercellular space colonization 56 DAI. And in the 10^8 B501*gfp*1 bacterial cells ml^{-1} inoculated plants, E and F: 14 DAI. White arrows show the colonized area. Scale bar: 40 μm (A, F). 10 μm (C), 20 μm (B, D, E).

Similarily, in the 10^8 B501*gfp*1 bacterial cells ml^{-1} inoculated plants, very few vascular bundles were densely colonized (Figures 5.2E and F) at 14 DAI with at least a minimum of 3 vascular bundles among the several bundles observed in a stem transversal section. As the bacterial concentration progressively increased in the tissues, the numbers of colonized vascular bundle tissues had also increased at 56 DAI with extensive colonization. And in most of the observed stem sections B501*gfp*1 could be detected in the vascular bundles and their intercellular spaces (Figure 5.3A). In many successive stem transversal sections, vessels filled with B501*gfp*1 bacteria were observed (Figure 5.3C) and colonization was also observed in the parenchymal cells of the xylem vessels and in their intercellular spaces (Figures 5.3B, C and D). In the root tissues, dense colonization was observed in the cells at the lateral root junctions (Figures 3E and F), while in the leafy tissues, B501*gfp*1 bacteria were observed only in the outer sheath in few vascular bundle tissues (Figure 5.3G). Moreover, bacterial density in the leafy tissues was detected in very low numbers (Table 5.1).

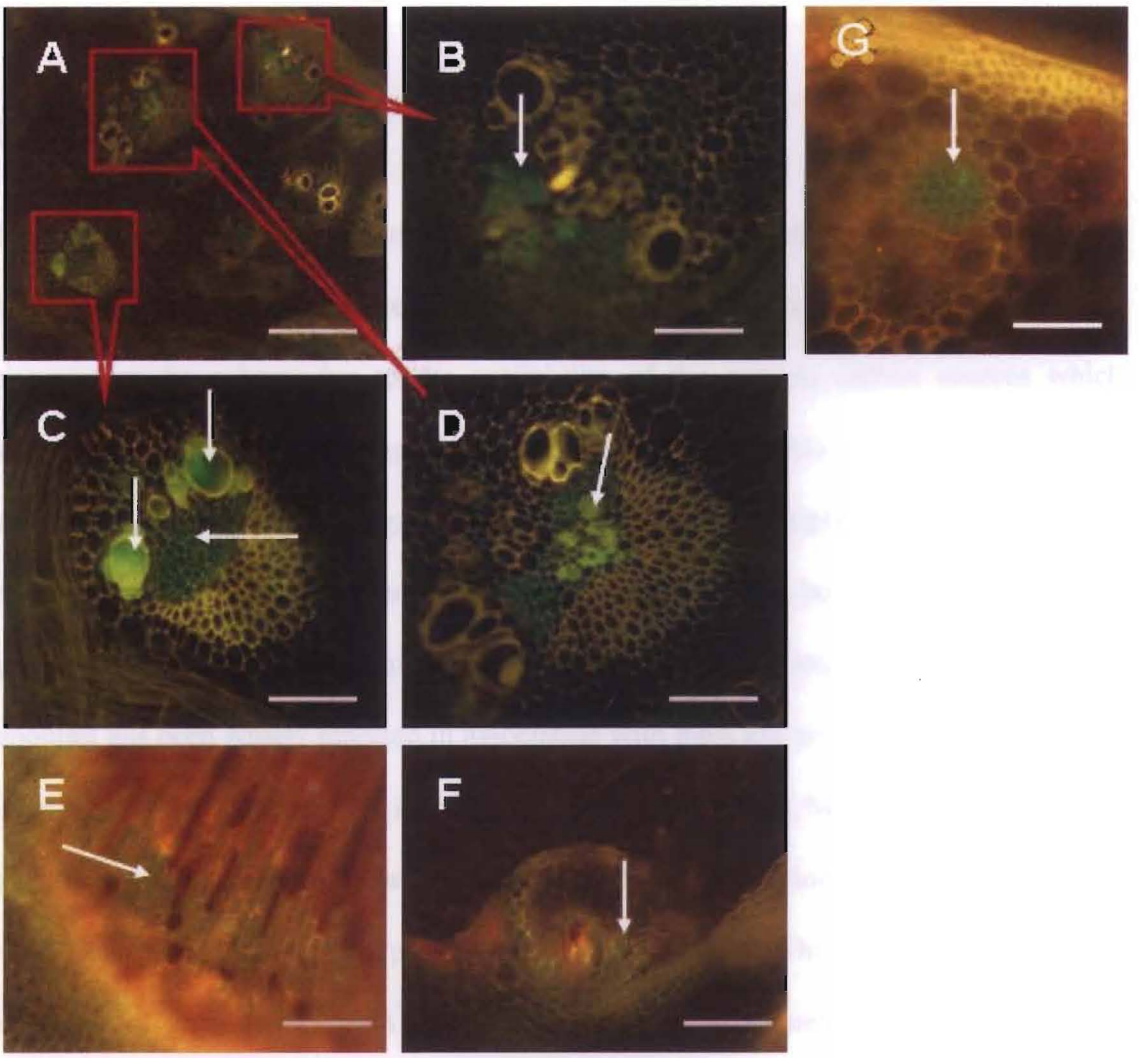

Figure 5-3. Internal colonization in the sugarcane root, stem and leaf sheath tissues of plants inoculated with 10^8 B501*gfp*1 bacterial cells ml^{-1}.

Vascular bundle tissue colonization in the 10^8 bacterial cells ml^{-1} inoculated plants in (A - D): 56 DAI;. C: Colonization in the metaxylem tissues. B and D: Proto xylem tissues. E and F: Lateral root junctions. G: Leaf sheath. Scale bar: 10 μm (A). 20 μm (B, C, D, E, F). 40 μm (G).

5.4 Discussion

In this study, high numbers of B501*gfp*1 bacterial were detected and also observed extensive colonization in the stem tissues. The higher numbers of B501*gfp*1 in the stem tissues may have been due to the availability of the organic carbon sources which supports bacterial growth (Dong et al., 1994; James et al., 1994). Njoloma et al. (2005) also demonstrated that B501*gfp*1 bacterial cells could multiply in the stem tissues especially in the parenchymatous cells which are considered to be food storage organs in plants. Bacterial colonization of the vascular system and the intercellular spaces in the apoplast has been widely reported in association with most gramineae plants (Dong et al., 1994; Sprent and James, 1995; Lamb et al., 1996; James, 2000; McCully, 2001). Bressan and Borges (2004) reported that the highest internal colonization in the maize root and stem tissues was obtained by pruned-root and dip method. This method might ensure existence of entry points such that infection and its subsequent internal colonization became easier. In this study, even though the roots were not pruned, the process of detaching new shoots from the sets created an opening at the base of the stem. As a result, this may have provided for entry of the introduced endophytic bacterial cells and consequently more bacteria were detected in the stem tissues than the root tissues. However, application of these methods for practical use may be difficult since the large openings could also allow entry by pathogenic bacteria microbes for the host plant. On the other hand, in the previous study (Njoloma et al., 2006) in which sterile sugarcane seedling plants cv. NiF8 were also inoculated with the 2 inocula levels (10^2 and 10^8 B501*gfp*1 cells ml^{-1}), higher bacterial numbers were observed in the root than the stem

tissues. In addition, even with the lower inoculum concentration, high numbers were detected in the root tissues at 56 DAI. Unlike in the current study, bacterial numbers in the range of 10^5 cfu gFW^{-1} could only detect in plants inoculated with the higher inoculum concentration. It can therefore be suggested that the inoculation technique used has some significant impact on the bacterial numbers, their localization and the subsequent internal tissue colonization. In addition, in the presence of the indigenous endophytic bacteria, the inoculated bacteria may have encountered some competition over growth resources resulting in their slow multiplication. On the other hand, with a high initial inoculum concentration, more of the B501*gfp*1 bacteria could be detected in the tissues compared to indigenous N_2-fixing bacteria. It is therefore suffice to say that in order to achieve adequate internal colonization by an introduced bacterial strain in the presence of the indigenous bacterial population, a higher initial inoculum concentration must be used.

In the colonization of the internal tissues, few sites were infected by the B501*gfp*1 in both the 10^2 and 10^8 B501*gfp*1 cells ml^{-1} inoculated plants at 14 DAI. In the leafy tissues colonization was observed only in the outer leaf sheath in some of the 10^8 B501*gfp*1 cells ml^{-1} inoculated plants. Njoloma et al. (2005) reported the effect of sugarcane tissues' autofluorescence (red fluorescence by chloroplasts) on the expression of B501*gfp*1 spot inoculated on to the tissues. It was reported that when concentration of B501*gfp*1 bacterial cells in the tissues decreased, its *gfp* expression could not be observed. Thus, in sugarcane tissues the chloroplasts provides a counter fluorescence which masks the expression of the bacteria's green fluorescence. In *gfp* labeled bacteria, the green fluorescence gives an indication for the presence of the *gfp*-labelled bacteria (Njoloma et

al. (2005). This could be an explanation for the few observed infection sites, there could have been more of the infected sites with very low bacteria numbers to be detected in a strong tissue autofluorescence background.

5.5 Conclusion

From the results it can be concluded that B501*gfp*1 has the potential to extensively colonize the sugarcane plant tissues in the presence of naturally inhabiting endophytes under non sterile growth condition. Dense and extensive colonization can be achieved using high bacterial inoculum concentration. Therefore, it is suffice to say that in order to achieve adequate internal colonization by an introduced bacterial strain in the presence of the indigenous bacterial population, a higher initial inoculum concentration must be used.

Chapter 6 GENERAL DISCUSSION AND CONCLUSION

Bacterial endophytes have been reported to colonize one or more of all the major organs and tissues of plants (James and Olivares, 1997; Reinhold-Hurek, 1998). In general these microbes are thought to be confined to the regions of the apoplast, however, most recently substantiated reports reveal the possibility of the intercellular colonization (James, 2000). At present, great focus is in the introduction and manipulation of the endophytic bacteria to provide a consistent and effective increase in the productivity of crops, not only by endophyte production of fixed nitrogen, but also by the production of plant growth-regulating compounds, allelopathic or antibiotic compounds (Kobayashi and Palumbo, 2000; Sturz et al., 2000).

Establishment of these endophytes in plants and formation of an effective association with the crops depends on a number of factors some of which are as follows. The bacterial endophyte must be a good colonizer by gaining a rapid and widespread entry into the plants under field conditions. The bacteria must colonize an environment that supports it for proper functioning within the plant. Thus, there must be a suitable supply of fixed carbon, optimal pH, oxygen tension, moisture among other appropriate substrates. In addition, the localization of the endophyte must not significantly impair normal plant performance (McCully, 2001; Stone et al., 2001).

Chapter 6. General discussion and conclusion

The results of this research study in which sugarcane seedlings growing in both sterile and non sterile vermiculite were inoculated under laboratory condition, using two inoculum concentrations (10^2 and 10^8 bacterial cells per ml) have shown that the bacteria endophyte B501*gfp*1 was able to colonize the sugarcane plants tissues grown in both the sterilized and non sterilized vermiculite. Slight differences were observed with respect bacterial numbers in the tissues of the two sugarcane cultivars (NiF8 and Ni15). And higher bacterial numbers were obtained in plants inoculated with a higher 10^8 bacterial cells per ml inoculum concentration. Worthy mentioning here, is that the localization and establishment of the bacteria in the plants varied with respect to the inoculated plant sample. In the sterile plants inoculated with intact roots and basal stem, B501*gfp*1 bacteria were found to be localized in the root tissues with the lateral root junctions as the main entry points. However, in some instances internal tissue colonization was observed to have occurred through intact root surfaces. On the other hand, plants which were inoculated with their stem bases bearing cut openings had more bacteria colonizing the stem tissues with dense establishments in the vascular bundle and parenchyma intercellular spaces. Thus, without causing visible disease symptoms the infection followed by colonization of the sugarcane plants by B501*gfp*1 was successful in both sterile and no sterile plants.

Kluepfel (1993) summarized the way in which bacteria generally get entry into plants. He pointed out that the invasion is usually through natural openings and wounds. Natural openings include points of emergence of lateral roots, and stomata or hydathodes. Where as wounds include abrasion of roots by soil and damage of any part by pests and pathogens and harvesting methods of perennial crops (Gagné et al., 1987). In the case of

pathogenic bacteria further movement of the endophytes is accompanied by plant cell damage. The success in the case of rhizobia in forming symbiotic associations has been associated with the refinement of such pathogenic situation (Djordjevic et al., 1987). One feature of this refinement is the localized entry into the root hairs or in some cases between epidermal cells. Evidence from endophytic nitrogen fixing bacteria suggests that most of the endophytes take entry into the plants through natural openings or wounds. Cases of entry through intact surfaces have been demonstrated by *Azoarcus* spp. *Acetobacter diazotrophicus*, *Alcaligenes faecalis* and *Pantoea agglomerans* all of which were associated with grasses and other crop plants (Hurek et al., 1994; James et al., 1994; You et al., 1990; Ruppel et al., 1992). In all these cases, bacterial cells were labeled with molecular markers in order to be certain of their identity and bacteria were observed penetrating to the root xylem. James et al. (1994) clearly showed entry into sugarcane by *Acetobacter* both at the lateral root emergence points and also in the root cap or the apical meristem region. The latter have only the primary wall layers, a condition suggested to be necessary by Sprent and Faria (1988) based on their work with rhizobial systems.

Thus the evidence that endophytic bacteria can enter intact surfaces as well as wounded tissues is now being strongly established. However, there is still some knowledge gap for convincing evidence that entry progresses beyond the cell walls without causing severe cell damage and /or probably death. Hurek et al. (1994) reported that plant cells were apparently destroyed by the introduced bacterial endophyte *Azoarcus* after the bacteria had penetrated the cell wall. However, they found no signs of phytopathogenicity such as visible necrosis. On the contrary, observations on light and transmission electron micrographs leads to the conclusion that plants cells containing bacteria are not healthy.

On the other hand, in the case of scanning electron microscope studies it is not very apparent to say whether the plant cells are healthy or not (Christiansen-weniger and Vanderleyden, 1994). Once the endophytes enter the plant tissues, some reports have indicated that movement across the root to the xylem occurs between the cells and that bacteria can be transmitted by vegetative propagation of the host as in the normal practice in cultivation of sugarcane and sweet potato. Indeed *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* are apparently obligate endophytes not found living freely in soils. However, this does not preclude their occurrences on damaged or senescence roots and stems from where new infection might originate. *Herbaspirillum seropedicae* can also be transmitted by seed in the case of rice (Baldani et al., 1993; Döbereiner et al., 1993).

The xylem tissue vessel provides discrete apoplastic pathways that are well adapted for rapid and reliable delivery of water and solutes from roots to the leaves. However, in some studies it has been observed that when xylem vessels are wounded or invaded by microbes, the xylem parenchyma cells respond by secreting mucilaginous and phenolic compounds into the vessel lumen (Dong et al., 1997; James et al., 1997; Crews, 1998). Therefore, it seems unlikely that vessels containing the defensive mucilage would continue to function normally in water conduction (McCully, 2001).

The major location of bacteria that establish themselves within living tissues is the intercellular space which is part of the apoplast within the parenchyma tissues. The intercellular space extends throughout the plant, except in the vascular bundle tissues. Until recently, this space was thought to be gas filled. Michael et al. (1999) demonstrated

that the small intercellular spaces in the tips of grass roots have hydrophobic lining and appears gas filled, but once wetted this lining disappears. And this would probably allow other fluids like liquids to flow in and out of the spaces. The only intercellular space environment that has been partially characterized is that of the stem parenchyma of sugarcane. The spaces are filled with a solution containing on average about 12% sucrose, with a pH of about 5.5. This composition is very close to that of the preferred culture medium for diazotrophic bacterium A*cetobacter diazotrophicus* isolated from the sugarcane plant tissues. The volume of intercellular space available for colonization varies greatly, for example in the case of Kallar grass which grows in waterlogged soils has extensive aerenchyma which may contain large numbers of cells of *Azoarcus* spp. (Reinhold et al., 1987). It is known that the glycoprotein found in intercellular spaces of the cortex of some legume nodules which sometimes surrounds the rhizobia is synthesized inside the plant cells and transported across the intercellular space system (de Lorenzo et al., 1993). Therefore, if the material surrounding the endophytic bacteria in the intercellular is of a similar nature, it could act as a major source of C and a minor source for N. On the other hand, it could be possible that the bacteria have adapted to use products of host defence reaction for their own metabolism. In addition, a more likely role of the matrix is that it acts to reduce pO_2, thereby facilitating induction of nitrogenase synthesis by the bacteria. The importance of optimal pO_2 can not be overemphasized, since it does not only affect nitrogenase induction (Alexander and Zuberer, 1989), but also efficiency of nitrogen fixation. In the case of *Azoarcus* spp. when incubated under low pO_2 can adapt its respiratory pathway and fix nitrogen at very high efficiencies, possibly in magnitudes higher than that of bacteriods of

Bradyrhizobium spp. (Hurek et al., 1994a). If this observation holds true for cells inside the host grasses and for most endophytic bacteria, then the case for significant nitrogen fixation by these systems will be greatly strengthened.

Based on the findings of this research study, it can be concluded that the mode of infection and subsequent establishment mechanism in the internal tissue is facilitated by the inoculation technique and availability of entry points. Although the growing condition of the laboratory plants could partially represent the undisturbed field-grown plants, it is still necessary to have the identified efficient endophytes under laboratory conditions rigorously evaluated for their efficient colonization in the actual field conditions. In addition, potential endophytes can be selected for their susceptible plant cultivars and that cultural practices can be developed for the practical utilization of the efficient nitrogen fixing endophytic bacteria in the non-leguminous plants.

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APPENDIX

Appendix 1. Murashige and Skoog salts stock solution and media composition

Components	Stock solution Amount (g/L)	Callus Induction Amount per Litre	Shoot proliferation Amount per Litre	Root Induction Amount per Litre
KNO_3 NH_4NO_3 $CaCl_2.2H_2O$ $MgSO_4.7H_2O$ KH_2PO_4	19.0 16.5 4.4 3.7 1.7	100 ml	100 ml	100 ml
Mn $SO_4.4H_2O$ H_3BO_4 $ZnSO_4.7H_2O$ $Na_2MoO_4.2H_2O$ $CuSO_4.5H_2O$ $CoCl_2.6H_2O$ KI	2.23 0.62 0.86 0.025 0.025 0.025 0.083	10 ml	10 ml	10 ml
$FeSO_4.7H_2O$ Na_2-EDTA	2.78 3.73	10 ml	10 ml	10 ml
Glycine Nicotinic acid Thiamine.HCl Pyrodoxine.HCl	0.2 0.05 0.01 0.05	10 ml	10 ml	10 ml
Myo-inositol	10.0	10 ml	10 ml	10 ml
2, 4-D				
Ki				
BAP				
NAA				
Sucrose				
Gellan Gum				
	pH 5.8	pH 5.8	pH 5.8	pH 5.8

Murashige and Skoog, 1962

Appendix 2. Medium for bacteria growth

Components	LGIP medium [1] Amount (g/L)	Luria Bertani[2] Amount (g/L)
K_2HPO_4	0.20	
KH_2PO_4	0.60	
$MgSO_4.7H_2O$	0.20	
$Na_2MoO_4.2H_2O$	0.02	
$CaCl_2.2H_2O$	0.02	
$FeSO_4.7H_2O$	0.01	
NaCl		5.0
Yeast extract	0.02	5.0
Biotin	-	
Pantothenic	-	
Nicotinic acid	-	
Sodium Lactate	-	
Mannitol	-	
Sodium Malate		
Na_2Fe EDTA		
Para amino benzioc acid		
Tryptone		10.0
Sucrose	100.0	100.0
Bromothymol blue	5.0ml (0.5% in 0.2N KOH)	
Agar (semi-solid)	2.0	
Agar (solid medium)	20.0	15
	pH 6.8	pH 7.2

[1] Cavalcante, V.A and Dobereiner, J. 1998.
[2] Lennox, E.S 1955

Appendix 3. Composition of the nitrogen supplemented culture solution

Components	Concentration of stock Solution	Amount needed per Litre
K_2HPO_4 K_2SO_4	8.0 g/L 2.48 g/L	20ml
$CaCl_2.2H_2O$	44 g/200 ml	2 ml
$MgSO_4.7H_2O$	37 g/100 ml	1 ml
$MnSO_4.4H_2O$ H_3BO_4 $ZnSO_4.7H2O$ $NA_2MoO_4.2H_2O$ $CuSO_4.5H_2O$ $CoCl_2.6H_2O$	102 mg/100 ml 143 mg/100 ml 110 mg/100 ml 10 mg/100 ml 3.9 mg/100 ml 3.4 mg/100 ml	1 ml
$FeSO_4.7H_2O$ Na_2-EDTA	2.78 g/ 200 ml 3.73 g/200 ml	5 ml
KNO_3		0.505g
Ph 6.8		