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Full Length Research Paper

Detection of gfp expression from gfp-labelled bacteria spot inoculated onto sugarcane tissues

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Green fluorescent protein (GFP) as a marker gene has facilitated biological research in plant-microbe interactions. However, there is one major limiting factor in the detection of GFP in living organisms whose cells emit background autofluorescence. In this study, *Herbaspirillum* sp. B501gfp1 bacterial cells were spot inoculated onto 5 month-old sterile micro-propagated sugarcane tissues to detect if the GFP fluorescence expression could be distinguished from the tissue's background fluorescence. 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. We 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 labelled bacteria.

Key words: GFP fluorescence, autofluorescence, bacterial cell density, sugarcane plant tissue.

INTRODUCTION

The 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. The use of GFP 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 convetional 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, hybdridized or stained (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 autofluorescence 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 this study *Herbaspirillum* sp. B501gfp1 (Elbeltagy et al., 2001), bacterial cells were spot inoculated on to sugarcane tissues with the aim to detect if bacteria's green fluorescence can be distinguished from plant tissue's autofluorescence in the sugarcane plant espe-cially in the stem and leaf tissues which contains a subs-

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tantial amount of chloroplasts. In the stem tissues, the parenchyma tissues emit most of the red autofluorescence while the vascular bundle tissues emit yellow autofluorescence. We also wanted to study the behaviour of B501gfp1 in sugarcane plant tissues over time after inoculation. 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).

MATERIALS AND METHODS

Bacteria strain and inoculum preparation

Herbaspirillum sp. B501, an isolate from wild rice was kindly provided by Dr. 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 known three *Herbaspirillum* sp. (*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* sp. B501gfp1 (B501gfp1) (Elbeltagy et al., 2001).

B501gfp1 bacteria were cultured on LB medium containing 50 µg/ml kanamycin for 48 h at 28˚C. The bacterial cells were harvested from plates with a sterile loop and suspended in sterile distilled water. Inoculum density was estimate by direct cell count method using Petroff- Hauser counting chambers and adjusted by dilution to10^8 bacterial cells/ml with sterile distilled water.

Plant tissue inoculation

Five months- old sterile sugarcane plants, cv. NiF8 propagated under sterile conditions were used. Plantlets were produced through meristem culture and rooted plantlets were then transferred into modified Leonard jars. Plants were supplemented with 5 mg/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. Observation were carried out over a period of 7 days.

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). Using filter B-2A enabled us to clearly distinguish 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.

RESULTS

Detection of fluorescence

Time course observations on the stem tissues showed that few hours after inoculation (Figures 1A and 1B) and when bacterial population had increased (Figure 1D), the expression of B501gfp1'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 1C and 1E. On the other hand, on the leaf surface, we found out that the GFP expression could only be clearly distinguished under gfp filter (EX 460 - 500 nm) (Figures 3A, C and E) . Its expression was highly masked under B-2a filter by the strong red autofluorescence (Figure 3B). By day 4, 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 3D and F).

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. We observed fluctuations in the bacterial cell density on the tissues as bacteria settled in aggregated clusters. Soon after inoculation bacterial cells were observed scattered round the inoculated spot (Figures 1B and 3A) and by the second day, only few bacterial cells were observed localized mostly as clustered cells in low densities (Figure 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 1D). On the other hand, bacterial cells localized in the xylem tissues did not show much increase in numbers in most of our samples and by the 5^{th} day, very few or no bacterial cells could be detected in the xylem tissues (Figures 2C and D). Some bacterial cells were also observed proliferating in the intercellular spaces (Figures 1D and 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 1E). And on the leaf surfaces, bacterial proliferation were observed mostly when bacterial cells formed clusters along the leaf veins (Figure 3).

DISCUSSION

We observed low levels of the red autofluorescence in the stem tissues such that expression of the green

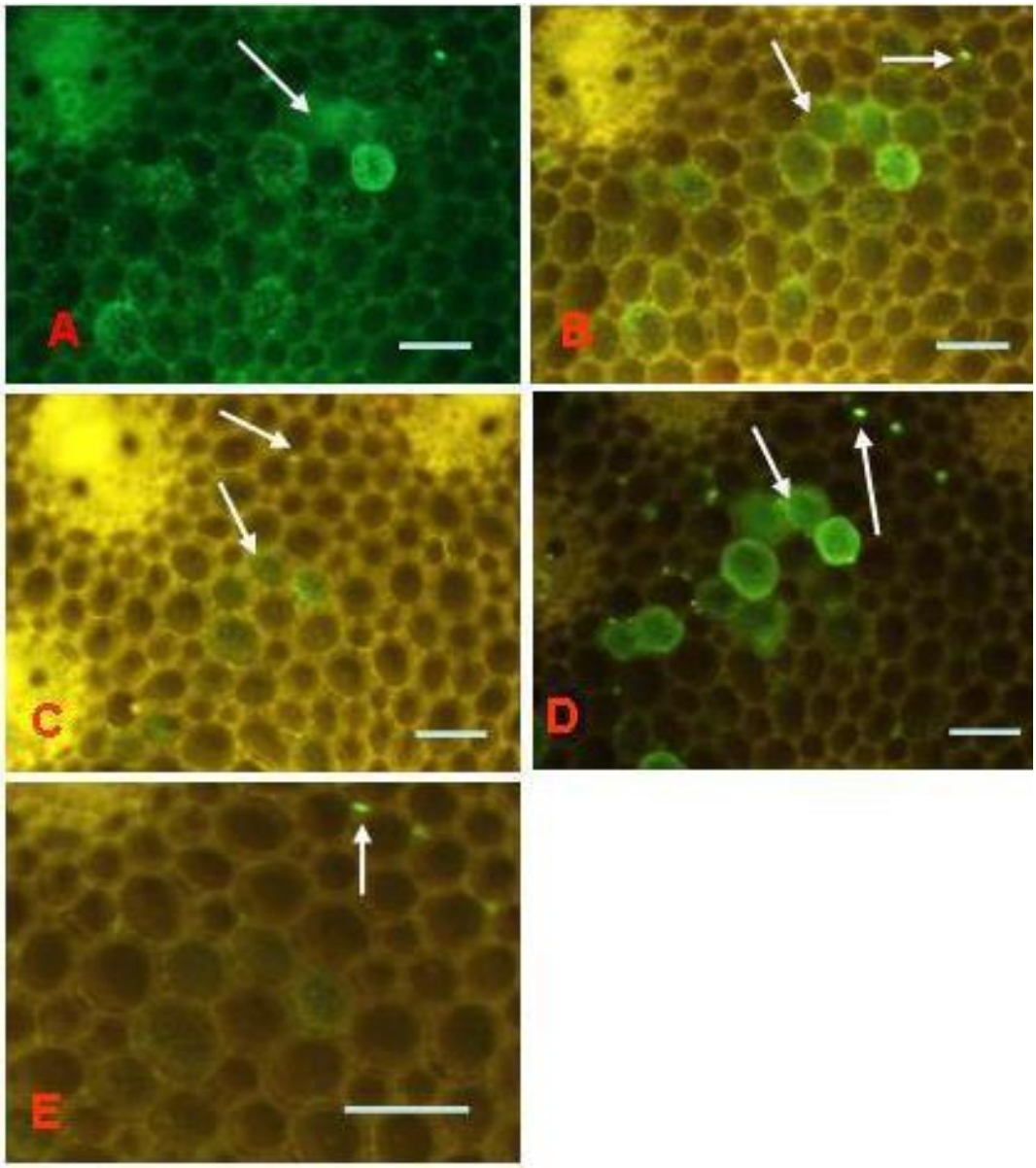

Figure 1. Bacteria population changes over time on the stem tissue (parenchyma tissues and intercellular spaces) of sugarcane plant after spot inoculation with *Herbaspirillum* sp. B501gfp1. **A** and **B**: An hour after inoculation. **C**: 2 DAI. **D**: Bacterial population density rose at 4 DAI. **E**: This was followed by a sharp decrease 7 days later. (**A**) Observed under gfp filter while (**B**, **C**, **D** and **E**) under B-2a filter. Scale bar = 20 µm, arrows (in white colour) indicate some of the positions colonized by bacteria (in green colour).

fluorescence from the inoculated strain B501gfp1 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 B501gfp1'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

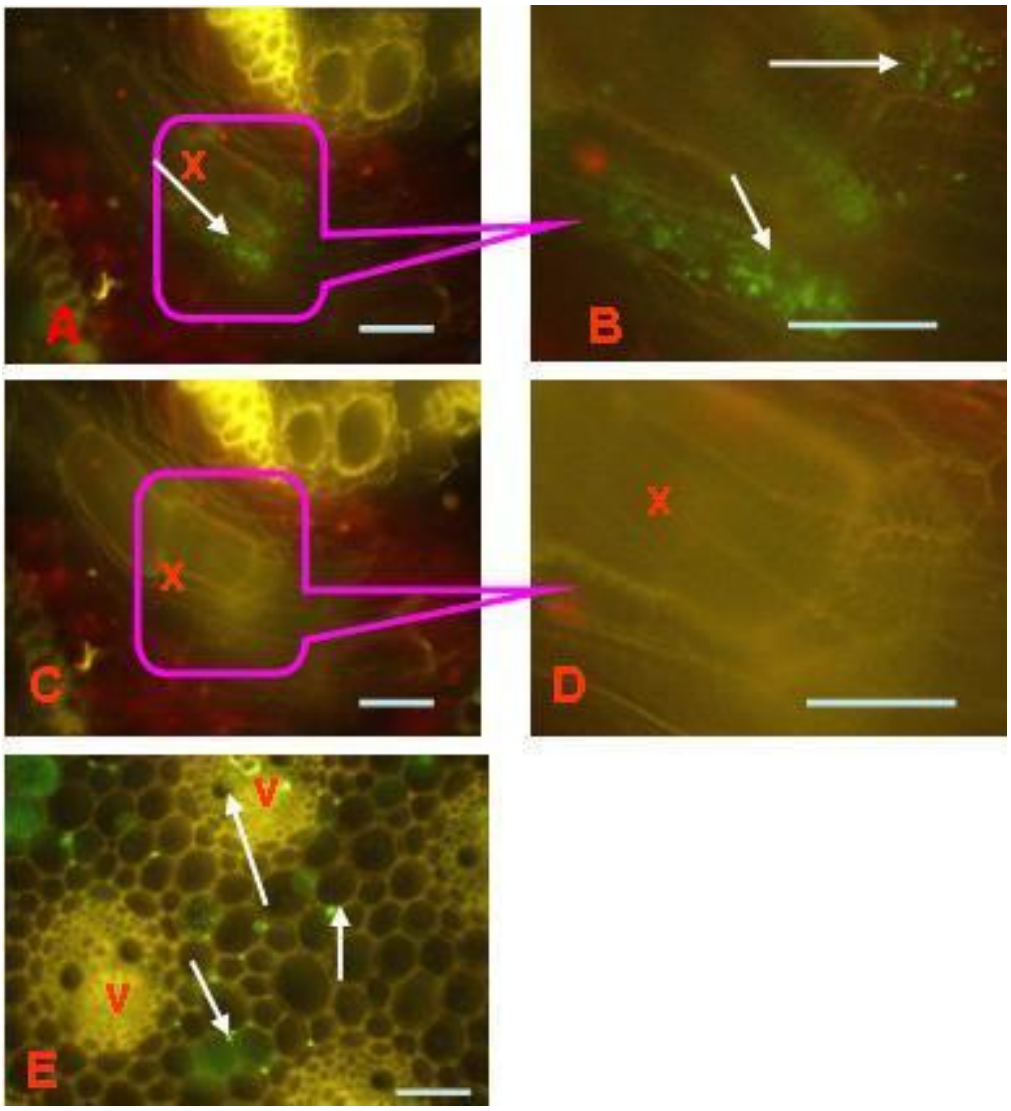

Figure 2. Spot inoculated bacterial cells in the xylem tissue over time At 2 DAI, **A** and **B**: Relatively low density of proliferating bacterial cells. **C** and **D**: By the fifth day, bacterial cells could no longer be visible in the xylem tissue. **E**: shows less and smaller aggregates of inoculated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

autofluorescence. In this study, we have demonstrated that in the presence of chloroplast especially in the leaf tissues, high levels of GFP fluorescence is required 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, we observed a decrease in bacterial numbers 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 demon

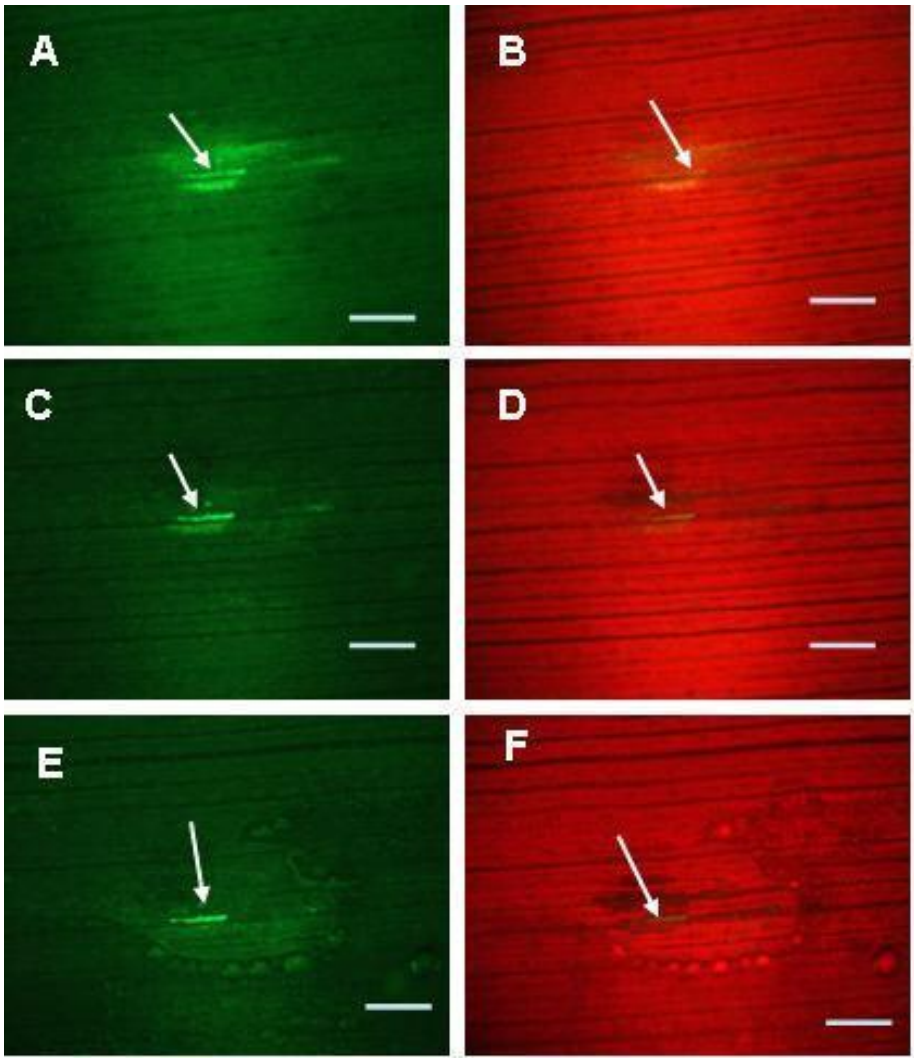

Figure 3. Colonization pattern of spot inoculated bacteria on the leaf surface. **A** and **B**: 1 h after inoculation bacterial cells were observed spreading over the inoculated area. Bacterial cells clustered along the leaf vein, 2 DAI (**C** and **D**) and at 4 DAI (**E** and **F**). Observed under gfp filter (**A**, **C**, **E**) and B-2a filter (**B**, **D**, **F**). Arrows (in white colour) indicate observed bacteria. Scale bar = 20 µm.

strates 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 than they would in a more suitable environment. 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.

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