Colonization pattern of *gfp* tagged *Methylobacterium suomiens* on rice and tomato plant root and leaf surfaces

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Abstract
The localization of bacterial cells, pattern of colonization, and survival of *Methylobacterium suomiense* CBMB120 in the rhizosphere of rice and tomato plants were followed by confocal laser scanning, scanning electron microscopy, and selective plating. *M. suomiense* CBMB120 was tagged with green fluorescent protein (*gfp*), and inoculation was carried out through the seed source. The results clearly showed that the *gfp* marker is stably inherited and is expressed in planta allowing for easy visualization of *M. suomiense* CBMB120. The colonization differed in rice and tomato-intercellular colonization of surface-sterilized root sections was visible in tomato but not in rice. In both rice and tomato, the cells were visible in the substomatal chambers of leaves. Furthermore, the strain was able to compete with the indigenous microorganisms and persist in the rhizosphere of tomato and rice, assessed through dilution plating on selective media. The detailed ultrastructural study on the rhizosphere colonization by *Methylobacterium* put forth conclusively that *M. suomiense* CBMB120 colonize the roots and leaf surfaces of the plants studied and is transmitted to the aerial plant parts from the seed source.

Key Words
Methylobacterium, Green fluorescent protein, Conjugation, Colonization, Microscopy.

Introduction
Bacteria of the genus *Methylobacterium* possess one or more characteristics of plant-growth promoting bacteria (PGPB). Despite the beneficial effects, application of PGPB are often hampered in the field due to inconsistencies in the rhizosphere under different conditions. Therefore, a better understanding of the colonization pattern and the survival of introduced bacteria is a critical prerequisite (Compant *et al*. 2005). Strain CBMB120, a rhizosphere soil isolate from rice (*Oryza sativa* cv. Dong-jin) had plant growth promoting characteristics and the presence of acyl-homoserine lactone quorum sensing signal molecules for cell to cell communication has been also documented in this strain (Madhaiyan *et al*. 2006). In this study, *M. suomiense* CBMB120 was tagged with green fluorescent protein (*gfp*), and confocal laser scanning (CLSM) and scanning electron microscopy (SEM) were utilized to localize the bacterial cells in the rhizosphere in two different plant species. The persistence of the strain in the presence of indigenous microorganisms in rhizosphere was checked by selective plating.

Methods
Tagging of Methylobacterium with gfp
M. suomiense CBMB120 was tagged with *gfp* (CBMB120-*gfp*29) through triparental mating using *E. coli* S17-1 (pFAJ1820 - *Tn5gusA-gfp*). The stability of introduced marker was checked through several generations and through a starvation experiment at 4 °C. The presence of gfp was confirmed by PCR amplification using specific primers.

Plant experiments
Surface sterilized, pre-germinated rice and tomato seeds (7, 3 days for tomato and rice) after treatment with bacterial suspension (2-4 h) were transferred to phytatrays containing 200 g of sterile sand with 35-40 ml plant nutrient solution (Simons *et al*. 1996) or to multi-well trays filled with air-dried Wonjo-Mix bed soil, the vegetable raising growth medium. The phytatrays were covered with lid, sealed with parafilm. Two ml nutrient solution were added to each well in the tray at weekly intervals. The plants were grown under growth chamber conditions(25/20 °C; 70% humidity; 14/10 h).

Confocal laser Scanning and Scanning Electron Microscopy (CLSM; SEM)
Roots, leaves and hand cut transverse sections of sterile leaves and roots were mounted using Vectashield mounting medium under a coverslip. Microscopic observations were performed using Leica TCS SP2

confocal system equipped with an Ar ion laser (Gfp: excitation, 488 nm; emission filter BP 500-530). For SEM, samples were prepared according to Bozzola and Russell (1998), dried to critical point, coated with gold-palladium and visualized using a Hitachi S-2500C Scanning Electron Microscope.

Enumeration of bacterial population
The bacterial enumerations were carried out at 7, 14 and 21 days for rice and 14, 21 and 30 days for tomato. The rhizosphere soil population, the rhizoplane and endophytic colonization of roots and shoots was determined by serial dilution technique on 1/10 Tryptic soy agar (TSA) for total bacteria and AMS with antibiotics for the inoculated bacterial population.

Results
The strain CBMB120-*gfp*29 showed higher fluorescence and can be easily differentiated from the wild type and readily detected under CLSM (Figure 1). The PCR amplification with *gfp*29 specific primers as well as grown in antibiotic selective media revealed that integration of the mini-transposon into the bacterial chromosome was stable.

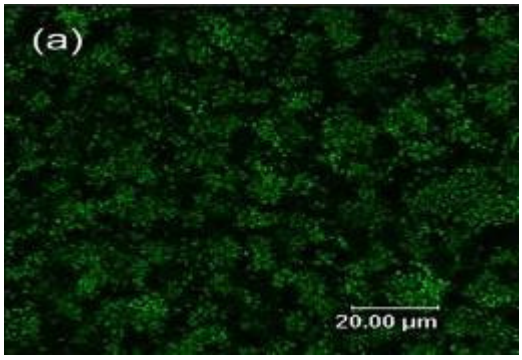

Figure 1. Confocal laser scanning microscopy of CBMB120-*gfp*29

Control plants without and with wild type CBMB120 inoculated plants observed by CLSM showed no fluorescent cells (Figure 2a). Sparsely distributed single cells of rod or circular shaped cells can be observed on the surface of rice roots (Figure 2b-c). A linear long string of closely associated cells can be observed throughout the entire length of the roots along the epidermal cell layers (Figure 2d). However, the transverse sections of rice roots obtained after surface sterilization showed no intercellular colonization. Numerous single cells were seen in the mesophyll and in the stomatal chambers of the leaves (Figure 2e-h). Similarly in tomato, fluorescent cells of CBMB120-*gfp*29 can be observed on tomato roots and leaves but the pattern of colonization remained different (Figure 3).

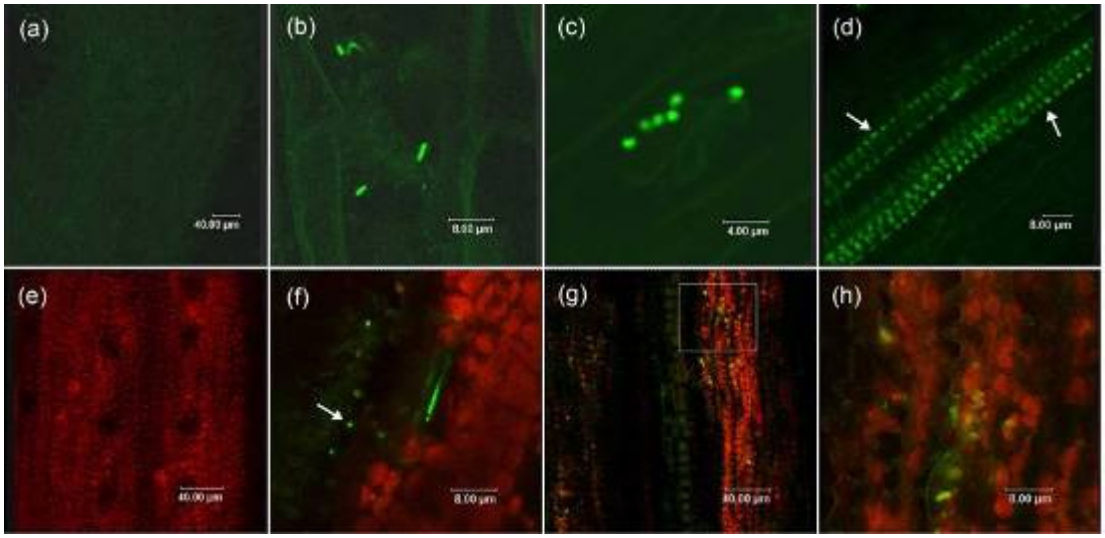

Figure 2. Colonization of rice roots and leaves by CBMB120 *gfp*29 observed through CLSM. (a) root of control plants; rod (b) and circular (c) shaped cells on the root surfaces of inoculated plants; (d) linear row of cells along the epidermal cells; (e) Leaves of control plants; (f-h) Single cells in the apoplastic region

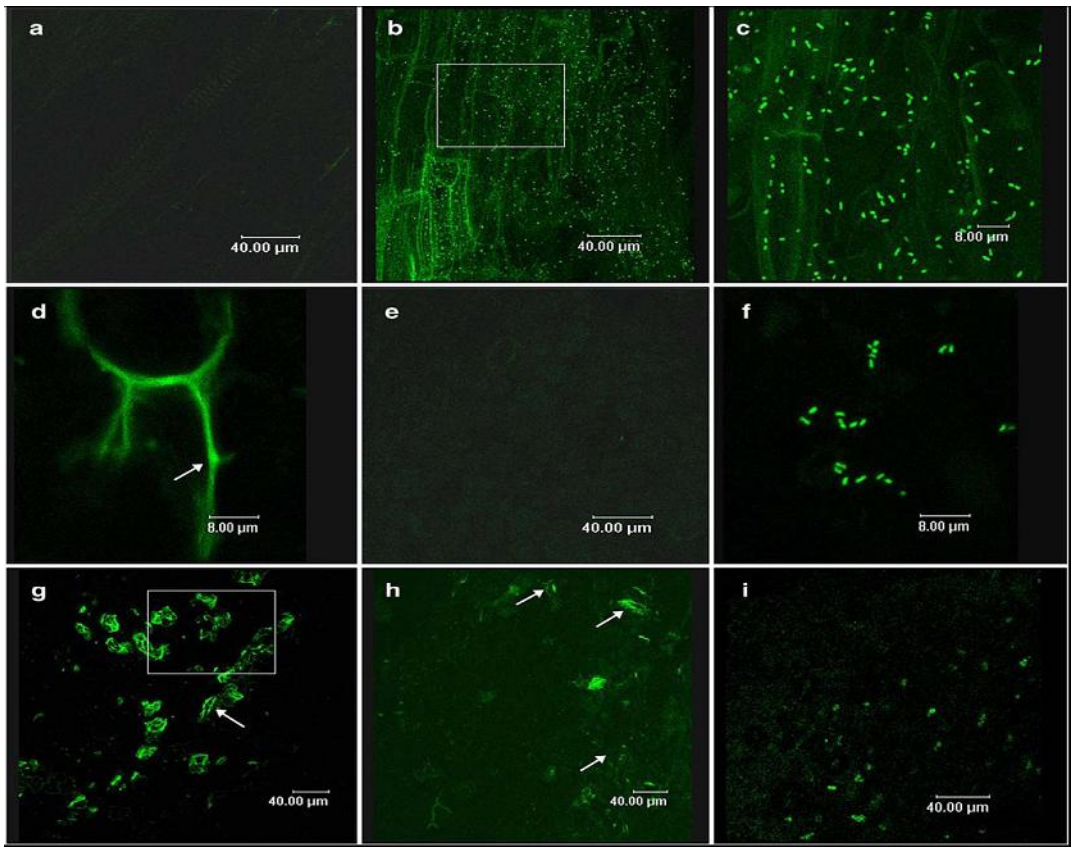

Figure 3. CLSM images showing the colonization pattern of CBMB120-*gfp*29 in tomato roots and leaves. (a) Root surface from uninoculated control. (b, c) Numerous rod-shaped cells can be observed colonizing the tomato root surface (c is an enlarged view of area pointed in b). (d) CBMB120-gfp29 penetration in to the intercellular spaces of root cortical cells can be identified by a strong fluorescence; (e–i) The colonization of leaf surfaces; (e) uninoculated control; (f) Presence of single cells on leaf surface; (g) Presence of CBMB120-gfp29 in the apoplast of leaves; (h) Cells in the substomatal chambers of leaves (i) The cells of CBMB120-gfp29 can also be observed in the surface-sterilized transverse section of tomato leaves

The results on the SEM observations for tomato is present in Figure 4. Numerous single and clusters of cells were dispersed throughout the length of the primary roots of tomato which often showed disrupted zones in roots (Figure 4a-c). Shortened, single cells were aggregated at the sites of emergence of secondary roots, which induced disruption of cortical and epidermal tissues (Figure 4d). The cells were unevenly distributed on the surface of tomato leaves with numerous shortened single cells lining the epidermal cells and clusters of single cells in the inter-cellular crevices of leaf surface and in the stomatal chambers (Figure 4e-h)

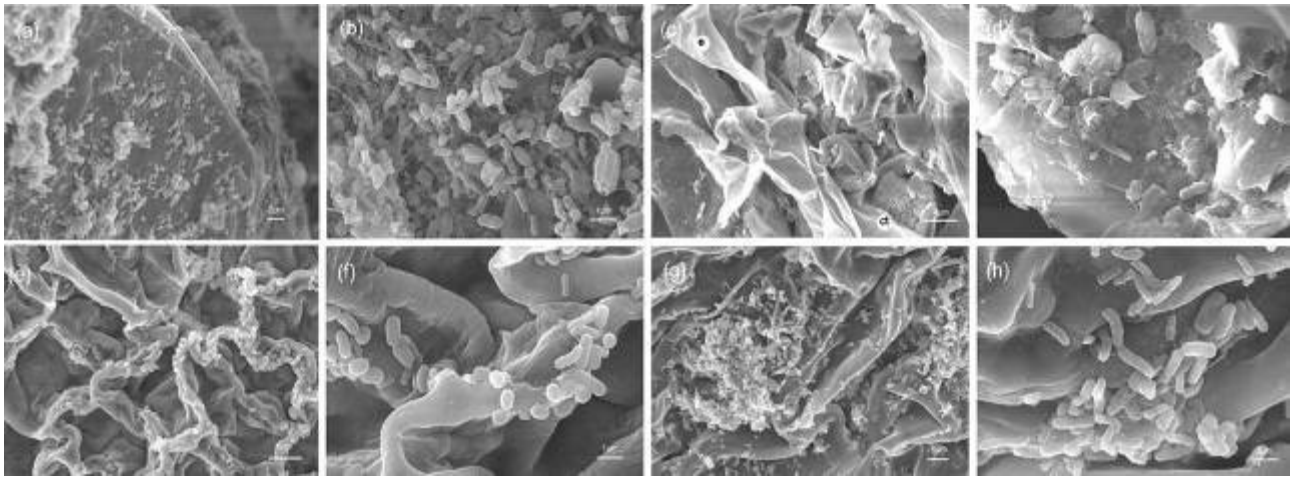

Figure 4. Distribution of *M. suomiense* CBMB120 on tomato roots and leaves of inoculated plants. e - Epidermis of primary root; ct - cortical tissues. The cells in the root surface showed adhesion filaments with in them and the rhizoplane.

Quantitative data on the survival of CBMB120-*gfp*29 in tomato and rice was obtained by selective plating on AMS with methanol supplemented with kanamycin (Table 1). No background growth was observed from non-inoculated control plants and the inoculated strain densities were higher in the rhizoplane than in the phylloplane. A considerable population was present in the rhizosphere soil also.

Table 1. Population of CBMB120-*gfp*29 in different parts of rice and tomato at three sampling periods

DAI	Population (log cfu g^{-1} sample)				
	Rhizosphere	Rhizoplane	Phylloplane	Root interior	Shoot interior
14*	3.95±0.14b	4.52±0.30a	3.24±0.14a	3.53±0.19a	3.26±0.15ba
21	4.81±0.12a	4.34±0.20a	3.25±0.14a	2.97±0.10b	3.30±0.12a
30	2.76±0.06c	3.16±0.09b	2.31±0.12b	1.12±0.07c	3.18±0.10b
LSD	0.18	0.41	0.05	0.25	0.09
7	3.98±0.16b	5.64±0.14a	3.25±0.14c	3.50±0.06c	2.87±0.16b
14	3.63±0.13c	5.57±0.21a	4.15±0.09a	4.59±0.11a	3.06±0.15a
21	4.06±0.15a	5.25±0.14b	3.76±0.21b	4.05±0.14b	2.47±0.21c
LSD	0.06	0.16	0.24	0.17	0.14

DAI- days after bacterial inoculation; * the days of bacterial enumeration. Within each column, values followed by the same letter are not statistically different at $P \leq 0.05$

Conclusion

M. suomiense CBMB120, a rhizosphere soil isolate colonize the roots and leaf surfaces of plants without host speciation. The strain is transmitted to the aerial plant parts from the seed source rather than from environmental sources and able to persist in the rhizosphere in the presence of indigenous microorganisms.

References

Bozzola JJ, Russell LD (1998) Electron microscopy, 2nd edn. Jones & Bartlett, Sudbury, MS

Compant S, Reiter B, Sessitsch A, Nowak J, Clement C, Barka EA (2005) Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. *Appl. Environ Microbiol.* **71,** 1685–1693

Madhaiyan M, Poonguzhali S, Ryu JH, Sa TM (2006) Regulation of ethylene levels in canola (*Brassica campestris*) by 1-aminocyclopropane-1-carboxylate deaminase-containing. *Methylobacterium fujisawaense*. *Planta*. **224,** 268–278

Simons M, van der Bij AJ, Brand I, de Weger LA, Wijffelman CA, Lugtenberg BJJ (1996) Gnotobiotic system for studying rhizosphere colonization by plant growth promoting *Pseudomonas* bacteria. *Mol. Plant Microbe Interact* **9,** 600–607.