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Microscopic Colonization of Salt Tolerant Plant–Bacterium *Microvirga zambiensis* Strain WSM 3693 in Cotton Tissue using Auto Fluorescent Proteins GFP

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A B S T R A C T

The *Microvirga zambiensis* strain WSM 3693 is an endophytic strain isolated from saline area of kutch, Gujarat. The green fluorescent protein (GFP) was used to mark this endophyte in order to visualize and analyze the colonization processes in vivo. After inoculation for 2 to 15 d, it was visualized in cotton tissue using fluorescence microscopy. Thus, the objectives of this study were to construct gfp-marked derivatives of *Microvirga zambiensis strain* WSM 3693and to determine the endophytic patterns of colonization of cotton under gnotobiotic conditions by using genetically marked derivatives of this strain.

Introduction

Salinity is one of the major abiotic stresses that adversely affect modern agriculture and constitutes a problem everywhere in the world. More than 6% of the world's total land area is salt-affected; most of this salt-affected land has arisen from natural causes and the accumulation of salts over long periods of time in arid and semiarid zones (Rengasamy, 2002; Bui, 2013). One approach to solve the salt stress problem is the use of plant growth-promoting bacteria (PGPB).

Endophytic bacteria are bacteria that reside within living plant tissue without causing apparent harm to the host plant (Quadt-Hallmann and Kloepper, 1996), capable of establishing mutualistic associations (Hallmann *et al.*, 1997; Azevedo *et al.*, 2000), promoting plant growth and yield, suppressing pathogens, and helping to remove contaminants, among other benefits (Rosenblueth and Martínez- Romero, 2006). Investigations on the interaction of PGPB with other microbes and their effect on the physiological response of crop plants under

different soil salinity regimes are still at an incipient stage (Singh *et al.,* 2011).

During the last few years several biomarkers have become available to facilitate localization and identification of the inoculated bacteria in the infected plant tissues. Green fluorescent protein (GFP) is a valuable tool for addressing a variety of biological questions with living systems (Chalfie *et al.,* 1994). GFP is a useful biomarker for examining biological localization because the cell can be studied nondestructively and without the addition of confounding exogenous substrates or cofactors (Tombolini *et al.,* 1997).

Materials and Methods

This present study salt tolerant endophytes isolated from the rhizosphere of *Abulitonindicum* plant growing in saline area of bhachaw, kutch Gujarat. This bacterial isolates had multiple PGPR properties to promote plant growth under salt stress. This endophytes identified by bacterial DNA was partially sequencing with 16S rRNA gene using 907R (5'-CCGTCAATTC CTTTRAGTTT- 3') and 27F (5'AGAGTTT GATCCTGGCTCAG- 3') universal primer on Capillary Sequencer 3130xl genetic analyzer (Applied Biosystem). The 16S rRNA sequence of isolate was compared to NCBI data bank using BLASTn (Aravind *et al.,* 2008). Phylogenetic analysis of the 16S rRNA gene showed that the novel strains belong to the genus *Microvirga zambiensis strain* WSM 3693with 95% sequence similarity with type strains of this genus (Ardley *et al.,* 2012).

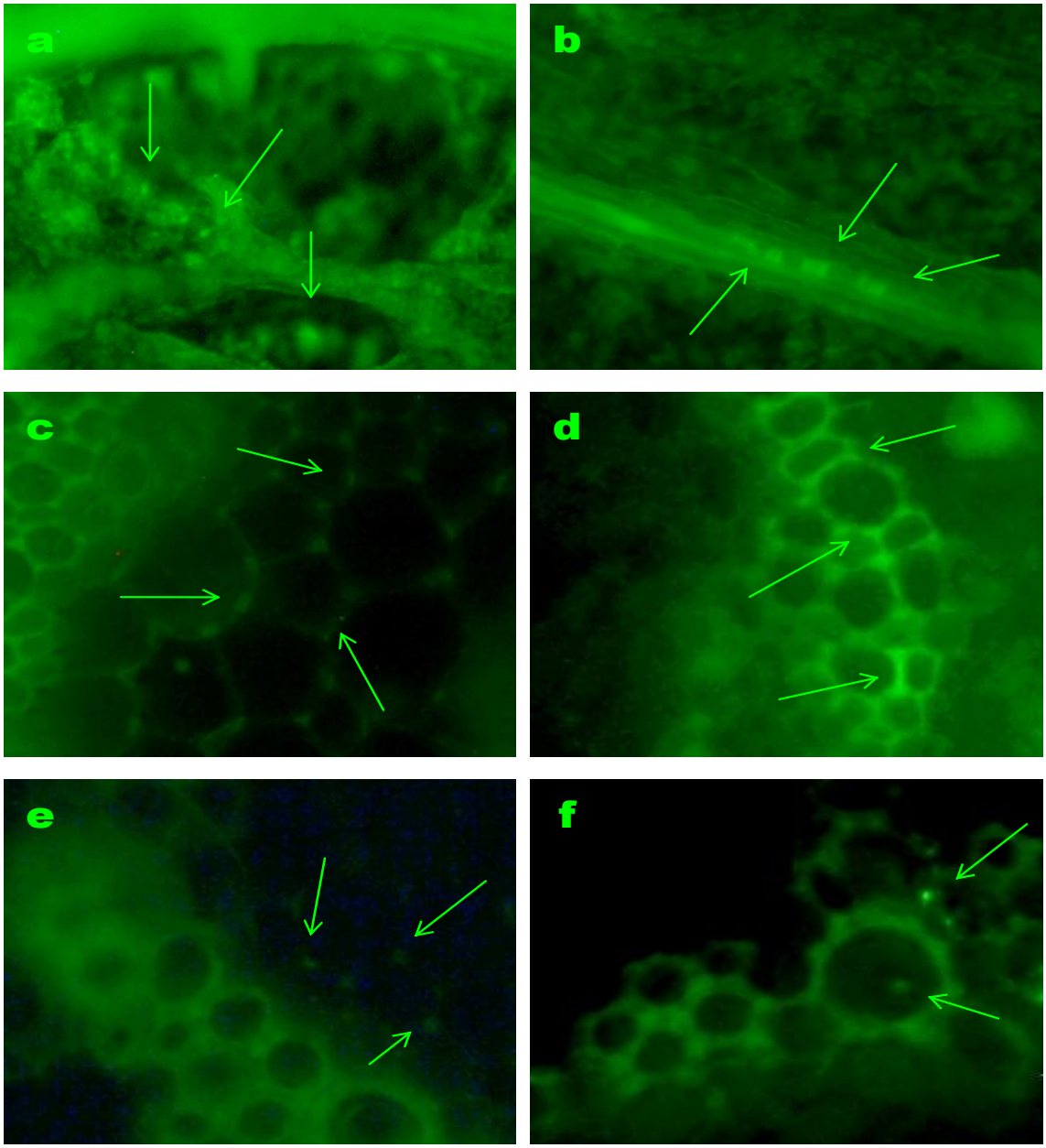
To study colonization of cotton tissue, endophytic bacterium was tagged with pGLO plasmid containing GFP gene expressed under control of ampicillin and arabinose and it was introduced by transformation - up taking GFP gene. The gfp-tagging protocol was used as perpGLO™ Bacterial Transformation Kit (CatLog Number 166-0003EDU BIO-RAD). The surface sterilized seed of cotton variety Raxak BGII were used for confirmation of endophyte. The cotton seeds were occupied from cotton Research Station, Junagadh Agriculture University, Junagadh and surface sterilized with 0.1% $HgCl_2$ for 2 min then again washed with sterile distilled water for 5 min. The seed were soaked in 25ml of GFP-tagged culture and then colonization of endophytic bacteria was checked on cotton seedlings by providing the natural system of soil under the controlled conditions in an environmental chamber (22-24°C, 10 h day/light) for 15 days. Soil used in process was autoclaved three times after intervals of 24 hours (Torres *et al.,* 2013, Tanaka *et al* 2006). After 15 days of growth in an environmental chamber (22-24°C, 10 h day/light), cotton seedlings were removed from the tubes and washed in running tap water, placed separately on blotting paper for absorbing access water then sections were cut. Hand cut section of live leaves, stem and roots were examined using AxioImager-Z fluorescence Microscope (ZEISS) and images were captured with a camera, using the software Zen. The filter set in Zeiss with a 450-490 nm band-pass excitation and 550 nm emissions was used for the GFP examination.

Results and Discussion

Hand cut transversal section of plant seen in Fluorescence microscopy showed Salt tolerant endophytic strain *Microvirga zambiensis* strain WSM 3693 colonized the stele of the lateral roots and vascular bundle of cotton root (Fig. 1e&f). GFP- tagged cells were also observed to the cortex area of the basal stem and in the stem xylem (Fig. 1c&d) and it also localized intracellular and vein of leaf (Fig. 1a&b). In summary, the gfp-tagged endophyte strain *Microvirga zambiensis* used in our study proved to be stable for at least 15

day generations as well as after colonization of the tissues under non-selective conditions. Moreover, no damages were observed in inoculated plants, making these gfp-tagged halotolerant bacterium especially *Microvirga zambiensis* strain WSM 3693 inhabiting salty and arid ecosystems has a great potential to contribute to promoting plant growth under the harsh salinity conditions and also useful to deliver enzymes or other proteins in planta.

Fig.1 Colonization of cotton seedlings were inoculated with endophytes isolates *Microvirga zambiensis* strain WSM 3693 and microscopically analyzed for GFP fluorescence. GFP-expressing cells colonizing (arrows) a,b-leaf, c,d-stem, e,f-root



Similar this work recently, the strain *P. agglomerans* 33.1: pNK GFP was able to colonize sugarcane plants, promoting their growth. The growth promotion observed in colonized plants may be related to the ability of *P. agglomerans* 33.1– 36 synthesize IAA and solubilize phosphate (Quecine *et al.*, 2012). Lacava *et al.*, (2007) reported *Klebsiella pneumoniae* 342, isolated from corn and subsequently labeled with the green fluorescent protein (GFP) was able to colonize and *Catharanthus roseus* and *Citrus sinensis*. Reporter gene like green florescent protein (GFP) has been wildly used for these studies. Endophytes either become localized at the point of entry or are spread throughout the plant (Sharma *et al.*, 2005)

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