ORIGINAL ARTICLE

Colonization of Sweet Potato Roots by Rhizobacterial Isolates

FARZANA YASMIN[1], RADZIAH OTHMAN[2], NAZMUL MHM[3]

ABSTRACT

Aim: To detect the colonization of PGPR strains on roots of sweet potato plantlets. Root colonization by Plant growth-promoting rhizobacteria establishes an efficient association with a more pronounced growth enhancing effect on plants.

Methods: *In vitro* scanning electron microscopy (SEM) and transmission electron microscopy (TEM) studies were conducted to examine the colonization of PGPR strains on roots of sweet potato plantlets. Vine cuttings of Sepang Oren variety were washed several changes of sterilized distilled water and grown in nutrient solution at 7 days. The four bacterial strains *Klebisella* sp. UPMSP9, *Eriwina* sp. UPMSP10, *Azospirillum brasilense* SP7 and *Bacillus sphaericus* UPMB10 were inoculated separately at the time of planting and two different colonization areas were studied root surface and internal region of plant roots.

Results: The bacteria on the colonized roots are either single or formed small aggregates along surface of sweet potato root-hair zone. The bacteria had colonized and penetrated the intercellular space in the cortex of the root tissues.

Conclusion: The results suggest that the four bacterial strains could effectively colonize the sweet potato root surface and internal region of 7 day old plantlets by using electron microscopy.

Keywords: Sweet potato, rhizobacteria, scanning electron microscopy, colonization, biofertilizer

INTRODUCTION

Colonization of plant roots by PGPR is a very important step for establishing an effective plant–bacterial interaction. The success of inoculating plants with beneficial bacteria usually depends on the colonization potential of the introduced strains (Schippers *et al.,*1987 and Weller, 1988, Barea et al.,.2005). The bacterial growth rate may be favoured by the exudation of specific compound by the roots. The presence of flagella and polysaccharide and the ability to synthesize amino acids are important bacterial traits for effective root colonization (Vladimir *et al.,* 2001, Dutta and Podile,2010).

Plant growth-promoting rhizobacteria can colonize plant root surface and internal region. In root surface colonization, the bacteria form mainly small aggregates although many single cells may also be scattered on the root surface. In internal region colonization, PGPR cells can colonize roots by penetrating into the root intercellular spaces, and subsequently thrive as endophytes in stem, leaves, tubers and other organs (Bashan and Levanony, 1990, Compant et al., 2010). The extent of endophytic colonization of host plant roots reflects the ability of bacteria to selectively adapt to these specific ecological niches (Gray and Smith, 2005). Consequently, intimate associations between bacteria and host plants can be formed without harming the plant (Lodewyckx *et al.,* 2002). Although it is generally assumed that many bacterial endophyte communities are the product of a colonizing process initiated in the root zone, they may also originate from the rhizosphere, the phyllosphere, the anthosphere or the spermosphere (Welbaum *et al.,* 2004). Root colonization is an active process whereby bacteria survive on the roots (Kloepper, 1993 , Compant et al.,2010).

Several species of PGPR increase plant growth by producing growth regulators and increased nutrient uptake subsequently increased shoot and root dry weights. These beneficial effects are the consequence of bacterial and root interactions. Root colonization is always considered a major criterion for successful inoculation of PGPR with the host plants (Suslow, 1982 and Vladimir *et al.,* 2001, El Zemrany et al., .2006). Bacteria proliferates at the junction of the epidermal cell and regions of maximum root exudation. A much tighter binding of bacterium with surface is irreversible due to extracellular polysaccharide and fimbriae, and production of long fibrils and mucigel like substances by the bacteria. Exopolysaccharides help bacteria to anchor to root surface for better access to plant exudates and protection from being washed away. This is a secure

[1]Faculty of Science, Lincoln University, 12-18, Jalan SS6/12, Off Jalan Perbandaran, 47301 Petaling Jaya, Selangor Malaysia
[2]Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[3]Faculty of Medicine, SEGI University, Kota Damansara, 47810 Selangor, Malaysia
Correspondence to Dr Mohammad Nazmul Hasan Maziz Associate Professor in Microbiology, Faculty of Medicine, SEGi University. Kota Damansara PJU 5, 47810 Petaling Jaya, Selangor, Malaysia Email: poorpiku@yahoo.com

attachment of PGPR to the roots and long-term association with the plant (Glick *et al*., 1999 ,Shukla et al.,2011 and Drogue et al.2013). Some of the PGPR promote plant growth and suppress plant disease that might be related to the extent of colonization of the internal root tissues and establish endophytic populations. Results of the previous study clearly indicated the beneficial effect of PGPR inoculation in stimulating root and shoot growth of sweetpotato (Farzana *et al*., 2009). However, there is a need to visually confirm the colonization of bacteria on the plant roots. An in vitro study was conducted aimed to observe the ability of four rhizobacterial strains (*Klebisella* sp. UPMSP9, *Eriwina* sp. UPMSP10, *Azospirillum brasilense* SP7 and *Bacillus sphaericus* UPMB10) to colonize the sweet potato root system both on the surface and in the internal root tissues by using electron microscopy.

MATERIALS AND METHODS

Plantlet growth in vitro: The four bacterial treatments used were i) Control, ii) *Klebsiella* sp. UPMSP9, iii) *Erwinia* sp. UPMSP10, iv) *Azospirillum* sp. SP7 and v) *Bacillus* sp. UPMB10. All bacterial isolates used were local except *A. brasilense* SP7 which was originally obtained from Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA), Brazil. Vine cuttings of Sepang Oren sweet potato were grown in conical flasks (500 ml) containing 300 ml of sterilized Hoagland's nutrient solution. Preparation of bacterial inoculum was as described earlier (Farzana *et al*., 2009). The bacterial cells were harvested by centrifugation at 13,500 rpm for 10 minutes and the pellet was washed thrice with 0.85% sterilized saline solution and finally re-suspended in saline solution. The live bacterial strains (approximately 10^9 CFU mL^{-1}) were inoculated to the respective conical flask at the rate of 6 mL inoculum/flask at planting time. The conical flasks were stoppered with sponge and wrapped with aluminum foil to prevent direct illumination and were aerated with aquarium air pump connected with tubing to supply air for root respiration and bacterial growth. Air into each flask was passed through sterilized membrane filter (0.2 µm). The assembly was kept in a laminar flow maintained at $28^0/22^0$C during 12 hours light/dark cycle for seven days .

Sample Preparation for Scanning Electron Microscopy (SEM): Triplicate root samples (root–hair zones) of 7 day old plantlets were sampled for SEM study to observe the colonization of rhizobacteria on root surface. The root samples were cut into 5-10 mm pieces with a fine razor blade and were then put into separate vials and fixed in fixative solution (4% gluteraldehyde buffer) for 12-24 hours at 4^0C. The samples were then washed with 0.1M of sodium cacodylate buffer with three changes of 10 minutes each. The washed samples were then post fixed in 1% of osmium tetraoxide for 2 hours at 4^0C. They were then dehydrated serially through graded series of acetone (35, 50, 75, 95% at each step for 10 minutes) and finally with absolute acetone.

The samples were then dried in a critical point dryer (Bal-Tec™ CPD 030) and later mounted on brass stubs, sputter coated in gold (Polaron™ Equipment Ltd., SEM Coating Unit E5100) and viewed under SEM (JEOL ™6000) at an accelerating voltage of 30 KV as described by Molla *et al*., (2001).

Sample Preparation for Transmission Electron Microscopy (TEM): Triplicate root samples (lateral root zones) were sampled for TEM study to observe the colonization of rhizobacteria in the internal root region of the plantlets. The root samples cut into 1-2 mm with a fine razor blade were then put into separate vials and undergone the same preparation process as for SEM.

The specimen was then embedded into beam capsules filled up with resin. Specimen was then polymerized in the oven at 60^0C for 24-48 hours. The silver or golden sections were selected and were picked up with a grid. The grid was dried using a filter paper. Selected sections were then stained with uranyl acetate for 10 minutes and were washed with 50% filtered alcohol. Later, sections were stained with lead for 10 minutes and were rinsed with double distilled water. After air drying, they were observed under Transmission Electron Microscope (TEM) (Gyaneshwar *et al.,* 2001).

RESULTS

SEM Micrograph: The SEM micrograph showed that *Klebsiella* sp. UPMSP9, *Erwinia* sp. UPMSP10, *Azospirillum* sp. SP7 and *Bacillus* sp.UPMB10 could successfully colonize sweetpotato roots (Figure 1b-e). Uninoculated control plantlets showed no bacterial cell on roots (Figure 1a). The bacteria on the colonized roots are either single or formed small aggregates along surface of roots. Most of the cells were rod shape with variable sizes. The variable sizes of bacterial cells in the respective roots could be due to the different age. The bacterial cells of all strains studied were attached firmly on the root surface probably through production of polysaccharide substance (Skvortsov *et al*., 1995).

TEM Micrograph: The TEM micrograph showed that *Klebsiella* sp. UPMSP9, *Erwinia* sp. UPMSP10, *Azospirillum* sp. SP7 and *Bacillus* sp.UPMB10 could successfully colonize the internal sweetpotato roots (Figure 2b-e). The bacteria had colonized and penetrated the intercellular space in the cortex of the

root tissues (Fig. 2b-e). The site of emergence of lateral roots was shown to be mostly infected with bacteria, indicating the likely entry route of the endophytes. The cracks developed by the emerging lateral roots appeared to be the sites where the bacterium spread into the intercellular spaces beyond the root epidermis.

Fig. 1a: SEM micrographs of uninoculated sweet potato roots plantlets

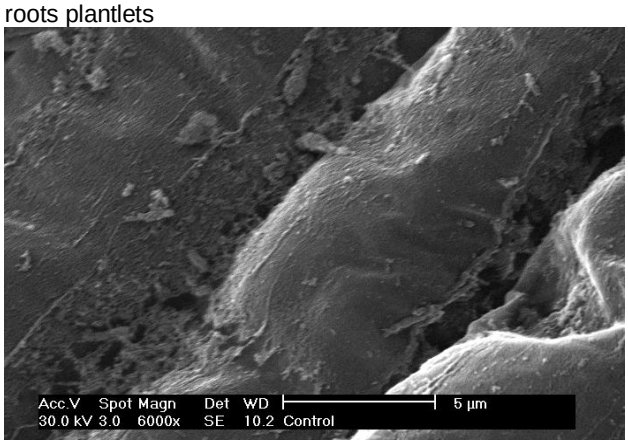


Fig. 1b: Root Surface Colonization of *Klebsiella* on sweet potato roots plantlets

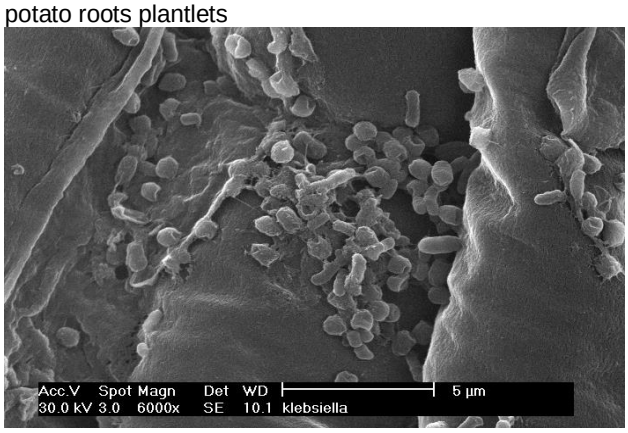


Fig. 1c: Root Surface Colonization of *Erwinia* on sweet potato roots plantlets

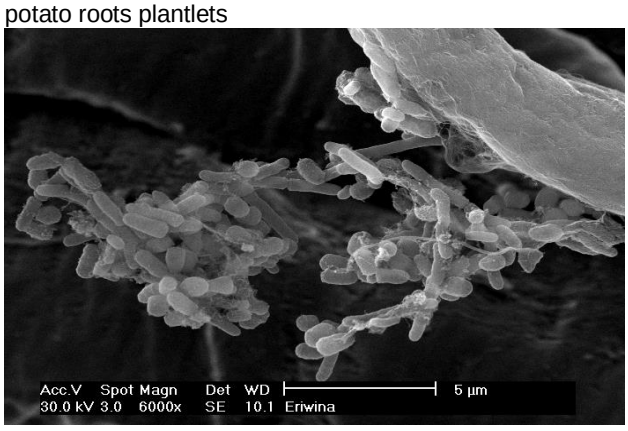


Fig. 1d: Root Surface Colonization of *Azospirillum* on sweet potato roots plantlets

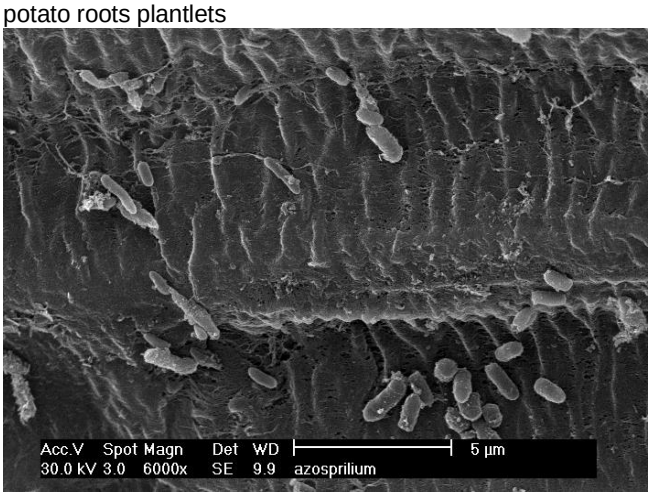


Fig.1e: Root Surface Colonization of *Bacillus* on sweet potato roots plantlets

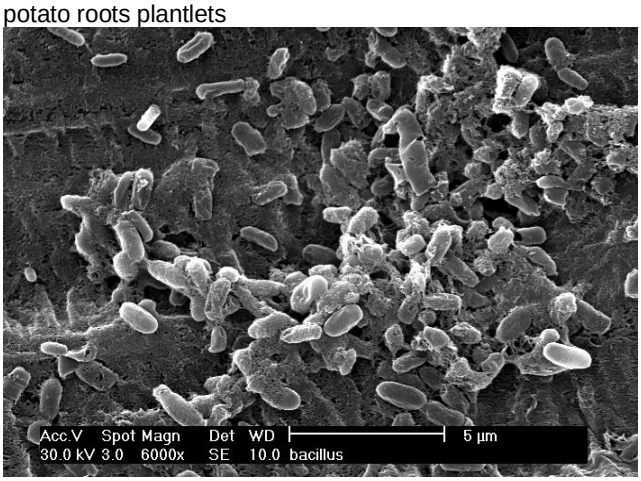


Fig. 2a: TEM micrographs of uninoculated sweet potato roots plantlets (No bacterial cells) (RC = Root Cortex, RE = Root Epidermis, ER = External Region)

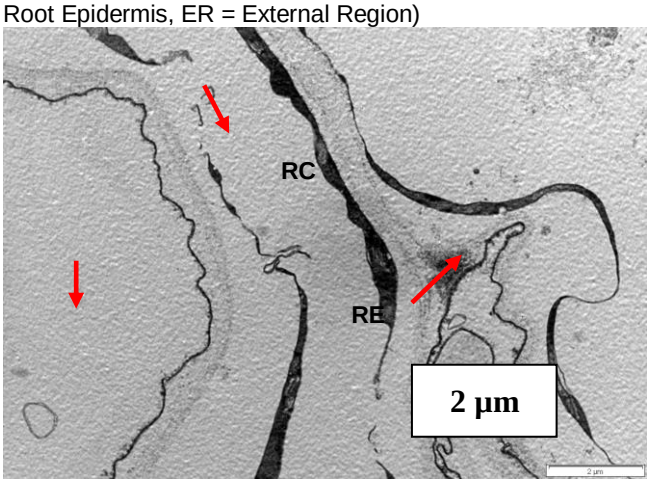
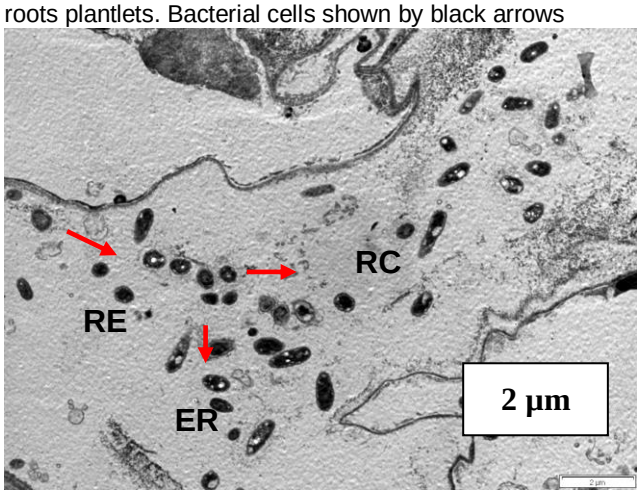
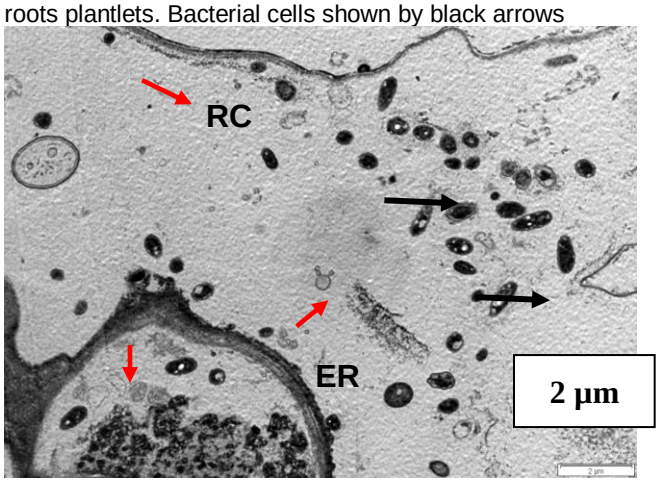


Fig. 2b: Internal Colonization of *Klebsiella* on sweet potato roots plantlets. Bacterial cells shown by black arrows



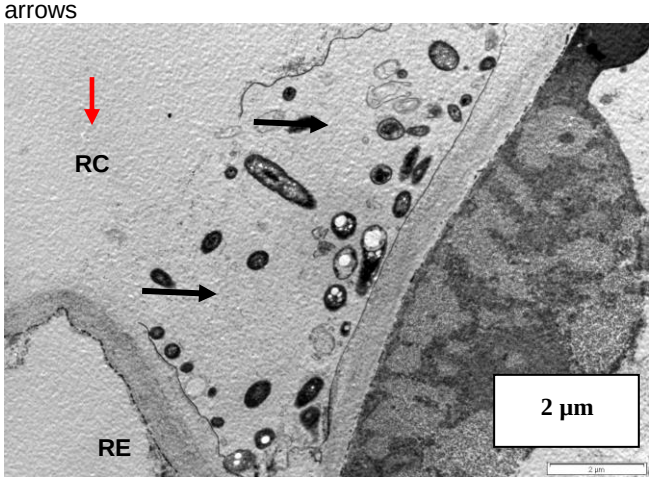
(RC = Root Cortex, RE = Root Epidermis, ER = External Region)

Fig. 2c: Internal Colonization of *Erwinia* on sweet potato roots plantlets. Bacterial cells shown by black arrows



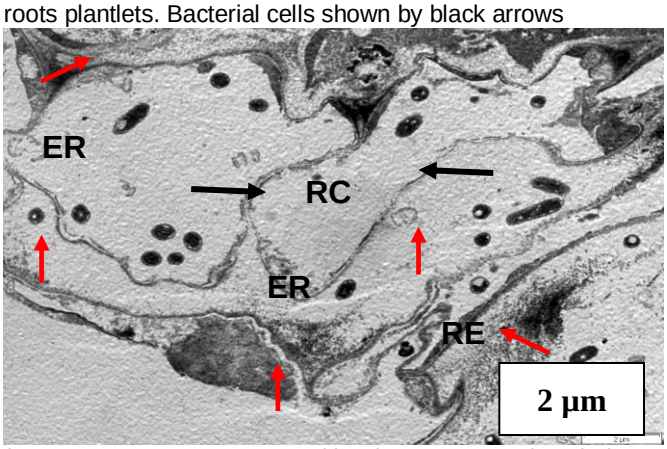
(RC = Root Cortex, RE = Root Epidermis, ER = External Region)

Fig. 2d: Internal Colonization of *Azospirillum* on sweet potato roots plantlets. Bacterial cells shown by black arrows



(RC = Root Cortex, RE = Root Epidermis, ER = External Region)

Fig. 2e: Internal Colonization of *Bacillus* on sweet potato roots plantlets. Bacterial cells shown by black arrows



(RC = Root Cortex, RE = Root Epidermis, ER = External Region)

DISCUSSION

The SEM micrograph showed that, locally isolated rhizobacteria (*Klebsiella* sp. UPMSP9, *Eriwina* sp. UPMSP10, and *B. sphaericus* UPMB10) and *A. brasilense* SP7 were able to colonize roots of sweet potato plants. This was important in ensuring establishment of the bacterial strains in the sweet potato rhizosphere. The bacteria were tightly bound to the root surface and irreversible, probably due to production of mucigel like substances and extracellular polysaccharide. The production of the substances have been observed in inoculated roots of other plants such as tomato, pepper, cotton and soybean, and is probably a major factor in effective root colonization (Bashan and Holguin, 1997 and Hartmann et al.,2009). Attachment of bacteria on the root surface is probably based on bacterial extracellular surface polysaccharides (Vladimir *et al.,* 2001 and Hartmann et al.,2008). The attachment of PGPR to the root is essential for the establishment of an efficient association with the plants. However, only a small percentage of root surfaces (8-20%) are actually colonized by the bacteria (Bashan *et al.,* 1991). Inability to bind firmly to the roots causes substances excreted by the bacteria to diffuse into the rhizosphere. The bacteria might be washed by water and there could be competition for attachment sites by other aggressive non-beneficial root colonizers.

There are many factors affecting plant root colonization by inoculated bacteria. Rhizobacterial motility and chemotactic movement toward roots play an important role in the colonization of roots (Scher *et al.,* 1985 and Czaban j.et al.,2007). Motility conferred by the polar flagellum of PGPR is used for swimming is important in the attachment process of bacteria to roots (Bashan and Holguin, 1997 and De weert et al., 2002).

The time taken for the bacteria to strongly bind to root surface should be short for successful colonization. This study showed that the bacteria were able to colonize roots within 7 days after inoculation. Molla *et al*., (2001) also showed that PGPR colonized root surface of soybean plant at 7 days after inoculation. Bacterial colonization could occur even at 4 days of plant growth (Mia *et al*., 1999 and Levanony *et al*., 1989) concluded that most of the bacterial cells of PGPR could associate with non-leguminous plantlets and they are located on the surface of root.

TEM micrograph showed that the four PGPR strains could colonize intercellularly in sweetpotato plantlets roots. Internal colonization by bacteria could be due to production of enzyme such as ligninase enzyme and lipopolysaccharides by the bacteria. There might be specific biochemical characteristics of strains found inside root tissues that facilitated their internal root colonization. The production of enzyme and lipopolysaccharides has been observed in internal colonization of rice, wheat, tomato and sugar/starch rich plants like sugarcane, potato and coffee (James and Olivares, 1997 and Rose et al., 2013).

The endophytic root colonization by bacteria on other plants have been shown by Ryan et al.(2008), Gyaneshwar *et al.,* (2001), Benizri *et al.,* (1997) and James *et al.,* (2001). The PGPR were able to endophytically established themselves within the root system within the vicinity of sites for lateral root emergence (Webster *et al.,* 1997 and Zachow et al.,2010). Endophytic colonization by PGPR is important as there would be less competition from other microorganisms for carbon substrates, nutrients and would be protected from high levels of O_2 present on the root surface compared to the indigenous rhizosphere bacteria (Dobereiner *et al.,* 1993; Boddey and Dobereiner, 1995; Staltzfus *et al.,* 1997, Krause et al .2011 and Malfarova et al,2013).

The non-pathogenic associations can stimulate plant growth, increase disease resistance, improve the plants ability to withstand environmental stresses, or enhance N_2 fixation, and production of other metabolites such as phytohormones and phosphate. The advantageous associations between plant and bacterial endophyte populations can lead to the development and maintenance of beneficial host-endophyte relations (Sturz and Nowark, 2000 and Ryan *et al*.,2008).

Endophytic colonization of plants by bacteria is affected by both environmental and host genetic components (Glick *et al*., 1999 and Taghavi et al.,2009). It is possible that endophytic colonization allows bacteria to establish a close communication with plants, and that the endophytic bacterial population fluctuates in number and activity depending on nutrients available to the bacteria and on the nutrient requirements of the colonized plant.

CONCLUSION

Root colonization by bacteria is an important step in the interaction of beneficial bacteria with the host plant. The SEM and TEM micrographs showed that four PGPR strains, *Klebsiella sp.* UPMSP9*, Erwinia sp.* UPMSP10*, Azospirillum sp.* SP7 and *Bacillus sp.* UPMB10 were able to colonize the surface and inside the root of sweetpotato plants. Endophytic colonization by plant growth-promoting rhizobacteria can be an effective system for the exchange of nutrients between plants and bacteria. The endophytic bacteria are promising candidates for establishing more beneficial interactions with plants and promoting plant growth.

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