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Original Article

Microscopic study on colonization and antimicrobial property of endophytic bacteria associated with ethnomedicinal plants of Meghalaya

Fenella Mary War Nongkhlaw, S.R. Joshi *

Microbiology Laboratory, Department of Biotechnology Bioinformatics, North-Eastern Hill University, Shillong, India

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ABSTRACT

Microscopic visualization using transmission electron microscopy (TEM) can provide a better understanding of endophytic colonization within ethnomedicinal plants. Bacterial endophytes were found attached to the host cell wall colonizing the aerenchyma and intercellular spaces of the epidermis and outer cortex except the vascular system. Colonization was non-uniform as single cells, doublets or in the form of microcolonies. Analysis of *in vivo* antibacterial action of the methanolic extracts of the isolated endophytic bacteria against Gram-positive, *Streptococcus pyogenes* MTCC 1925 and Gram-negative, *Salmonella enterica ser. paratyphi* MTCC735 pathogens has revealed the morphological damages in the tested pathogens respectively, under scanning electron microscopy (SEM). Detached cell wall and cell burst were observed in *Streptococcus pyogenes* where as, cell blisters were shown in *Salmonella enterica ser. paratyphi.* The study on bacterial endophyte colonization process is important to better predict how endophytes interact with their host and establish themselves in the plant environment by procuring biocontrol activity.

1. Introduction

During the course of endophyte-host co-evolution, plants and bacterial endophytes have developed an intimate relationship that has probably resulted in exchange of information at the cellular and molecular levels. Colonization is believed to be essential for biocontrol [1] with endophytes protecting their host plant [2–4]. The knowledge of colonization mechanism of endophytes with their host plant at the ultrastructural level can assist in understanding their efficiency and reliability as biocontrol agents. Unfortunately, the study on endophytes associated with ethnomedicinal plants is threatened by the rapid deplenishing of rainforests together with the disappearance of traditionally used medicinal plants as a result of over exploitation. It is believed that the only direct method to provide evidence for endophytic colonization is through microscopy [5]. Therefore, this study aimed to uncover the ultrastructural colonization of naturally occurring endophytes prevalent in ethnomedicinal plants using TEM. The study included both, microscopic evidence of endophytic colonization and the isolation of endophytes from surface-disinfected tissue which satisfies the criteria to recognize "true" endophytic bacteria [6].

With the enhanced environmental awareness and increasing rate of resistance of pathogens to conventional antibiotics, the search for biological alternative to fight against prevailing persistent infections continues. This study attempted to evaluate the antimicrobial activity of

* Corresponding author at: Microbiology Laboratory, Department of Biotechnology & Bioinformatics, North-Eastern Hill University, Shillong, 93022, India.
E-mail address: srjoshi2006@yahoo.co.in (S.R. Joshi).

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the bacterial endophytes isolated from ethnomedicinal plants; *Centella asiatica*, *Houttuynia cordata* and *Potentilla fulgens* against bacterial pathogens underlining the fact that the host plants are used for similar purposes in traditional treatments. *Centella asiatica* is used as a liver tonic, antidysenteric, antiseptic and in skin healing. It is also used for increasing memory power and treatment of high blood pressure. *Houttuynia cordata* is used as an antioxidative, antimutagenic, immunologic, anti-inflammatory and in the treatment of amoebic dysentery. *Potentilla fulgens* is used in the treatment of diabetes, reduces skin inflammation, diarrhoea and as a relief for sore throat [7]. The ability of the methanolic extracts of endophytic bacteria to inhibit and cause structural deformities in pathogenic Gram-positive and Gram-negative bacteria was evaluated using SEM.

2. Materials and methods

2.1. Plant sample collection

Healthy plants (*Centella asiatica*, *Houttuynia cordata* and *Potentilla fulgens*) used by different Traditional Medicinal Practitioners (TMPs) were collected from different parts of Meghalaya [7] based on their ethnomedicinal usages. The taxonomic identity of the plants was confirmed with the help of Herbarium Curator of the parent University. All samples were collected in sterile polythene bags and brought to the laboratory and used for isolation within 24 h of collection.

2.2. Endophytic colonization study using transmission electron microscopy

The roots and leaves of *C. asiatica*, *H. cordata* and *P. fulgens* were trimmed into 1.0–1.5 mm cube size and fixed by immersion in 2–3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 at 4 °C overnight. The samples were post fixed in 2% osmium tetroxide buffered solution and were embedded in epoxy resin. Subsequently, the samples were sectioned (0.1 μm) with ultra microtome and stained with a saturated solution of uranyl acetate and lead citrate. Micrographs were produced using JEM-2100 TEM (200 kV, Jeol) at SAIF, NEHU.

2.3. Isolation and molecular characterization of endophytic bacteria

Endophytic bacteria were isolated from healthy plant samples, devoid of any apparent pathogens as per the method previously reported [7]. Total genomic DNA was extracted using HiPurA^{TM} bacterial and yeast genomic DNA Isolation Kits (Himedia, India). PCR amplification and sequencing of 16S rRNA gene were carried out in a 25 μl reaction mixture. Using general primers 27F 5′-AGAGTTTGATCCTGGCTGAG-3'and 1541R 5′-AAGGAGGTGATCCAGCCGCA-3′ with the following conditions, template DNA denaturation at 94 °C for 5 min followed by 30 cycles at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min, final step was carried out at 72 °C for 5 min and then 4 °C till infinity using PCR Gene Amp 9700 (Applied Biosystems, USA). DNA template replaced with sterile water was used as negative control. The amplified (approx. 1000 bp) 16S rRNA gene was then purified using QIAquick Gel Extraction Spin Kit (QIAGEN, Germany). The purified PCR products were bi-directionally sequenced using both forward and reverse primers in a sequencer Genetic Analyzer (ABI 3130 Applied Biosystems, USA) with Big Dye (3.1) terminator protocol. The sequences were submitted to GenBank and their accession numbers were obtained.

2.4. Bacterial methanolic extract preparation

Each isolated endophytic bacteria was grown in 100 ml Nutrient Broth and incubated at 32 °C for 3 days at 120 rpm. The bacterial culture broth was centrifuged at 10,000 rpm for 15 min and the supernatant obtained was filtered using autoclaved 0.22 μm membrane filter paper. Methanol was added to the filtrate (2:1) and stirred for 24 h for extraction. Further, it was concentrated using rotary evaporator (Stuart RE300P, UK) under reduced pressure at 45 °C to obtain the extract. The concentrated methanolic extracts of each isolate was used for performing antagonistic activity.

2.5. Antibacterial activity study using scanning electron microscopy

In this study, the morphological changes caused by bacterial endophytes towards *Streptococcus pyogenes* MTCC 1925 and *Salmonella enterica ser. paratyphi* MTCC735, as model Gram-positive and Gram-negative pathogens respectively was investigated under scanning electron microscope (JSM-6360, Jeol). The concentrated methanolic extracts (400 mg/ml) of different isolated bacterial endophytes were tested using agar well diffusion method [8]. Zones of inhibition were measured and the mean value was obtained. The region on the antibacterial plate (MHA) showing inhibition against the selected pathogen was excised by cutting the agar, fixed by immersion in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for at least 1 h. The samples were drained and placed in three consecutive 1 h washes of 0.1 M cacodylate buffer. Samples were then stored in fresh cold cacodylate buffer for transport to the electron microscopy laboratory (SAIF, NEHU). Samples were dehydrated in a series of acetone-water (20–100%) washes for 15 min each and critical-point dried with liquid $C0_2$. Finally, samples were sputter coated with a thin layer of gold-palladium and the morphological differentiation in the Gram-positive and Gram-negative pathogens was observed under scanning electron microscope.

3. Results

Using TEM, the colonization of endophytes within ethnomedicinal plants was examined and cultural method uncovered the bacterial endophytes associated with *C. asiatica*, *H. cordata* and *P. fulgens* (Table 1). Observations of transverse sectioned roots and leaves of studied ethnomedicinal plants revealed bacterial endophytes within the aerenchyma and intercellular spaces of the epidermis and the outer cortex (Fig. 1). Some bacterial endophytes appeared to be attached to the host cell wall (Fig. 2)

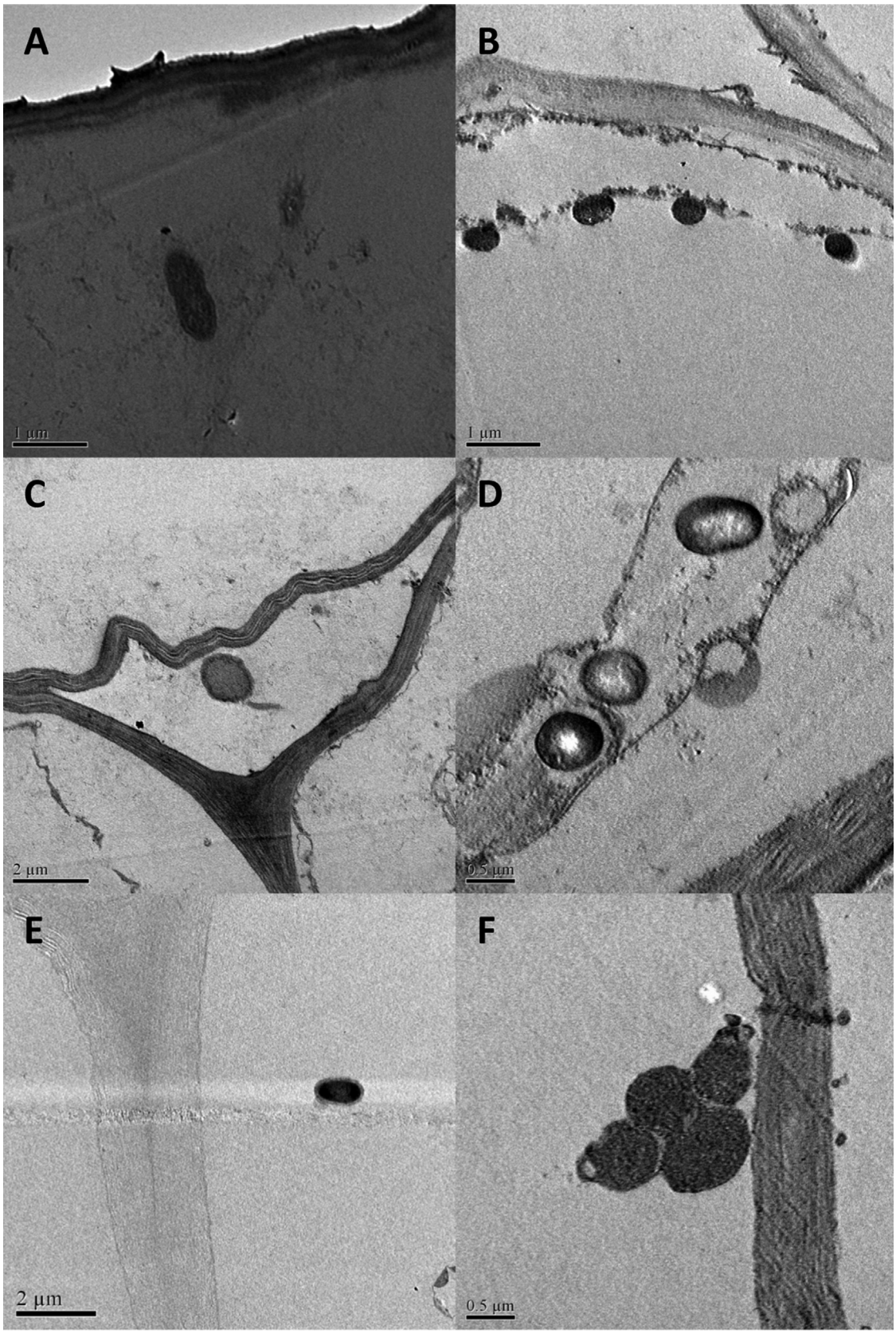

Fig. 1. Endophytic colonization within *C. asiatica* (A, B), *P. fulgens* (C, D) and *H. cordata* (E,F) as single cell and a string of bacteria in the form of microcolonies in the intercellular spaces of the epidermis and the outer cortex (A, B, E), and aerenchyma (C, D).

Table 1
Antimicrobial activity of endophytes associated with their respective ethnomedicinal plants.

Ethnomedicinal plants	Endophytes with GenBank accession no.	Antimicrobial activity (mm)	
		MTCC735	MTCC1925
Centella asiatica	*Serratia marcescens* JN613282	16 ± 0.5	11 ± 0.6
	Bacillus subtilis JN613283	24 ± 0.01	–
Potentilla fulgens	*Bacillus methylotrophicus* JQ236632	13 ± 0.5	–
Houttuynia cordata	*Bacillus sp.* JX298807	–	–

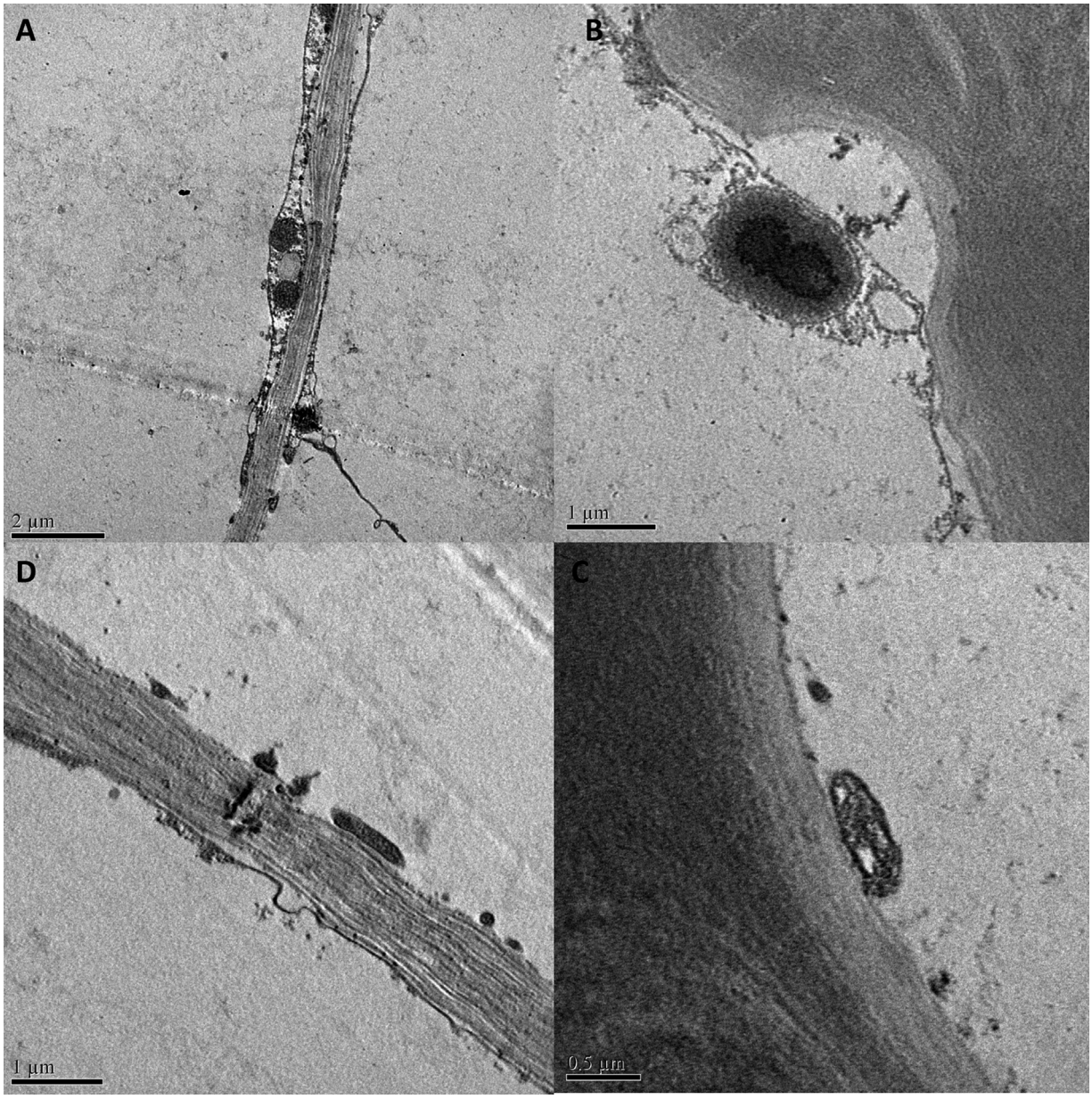

Fig. 2. Endophytes attached to the host cell wall within *P. fulgens* (A, B), *C. asiatica* (C) and *H. cordata* (D).

although there was no evidence of bacteria to penetrate the endodermis to colonize the vascular system. Endophytes colonize the tissue of ethnomedicinal plants non-uniformly as single cells (Fig. 1A, C, E), doublets (Fig. 3A) or in the form of microcolonies (Fig. 1B, D, F). Among the colonized endophytes within *P. fulgens*, some seemed to possess bacterial flagella (Fig. 3).

There was absence of morphological alterations in the host cell wall and the bacterial cells seemed healthy in appearance. Although, structural plant defence reactions

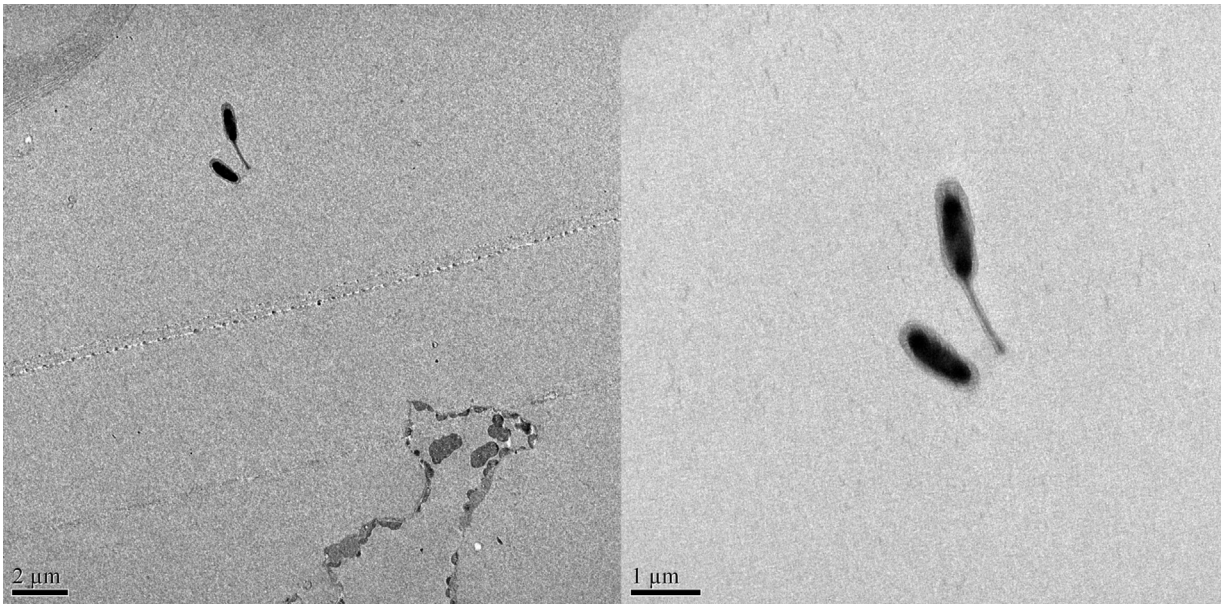

Fig. 3. Endophytic colonization within *P. fulgens* that possess bacterial flagella.

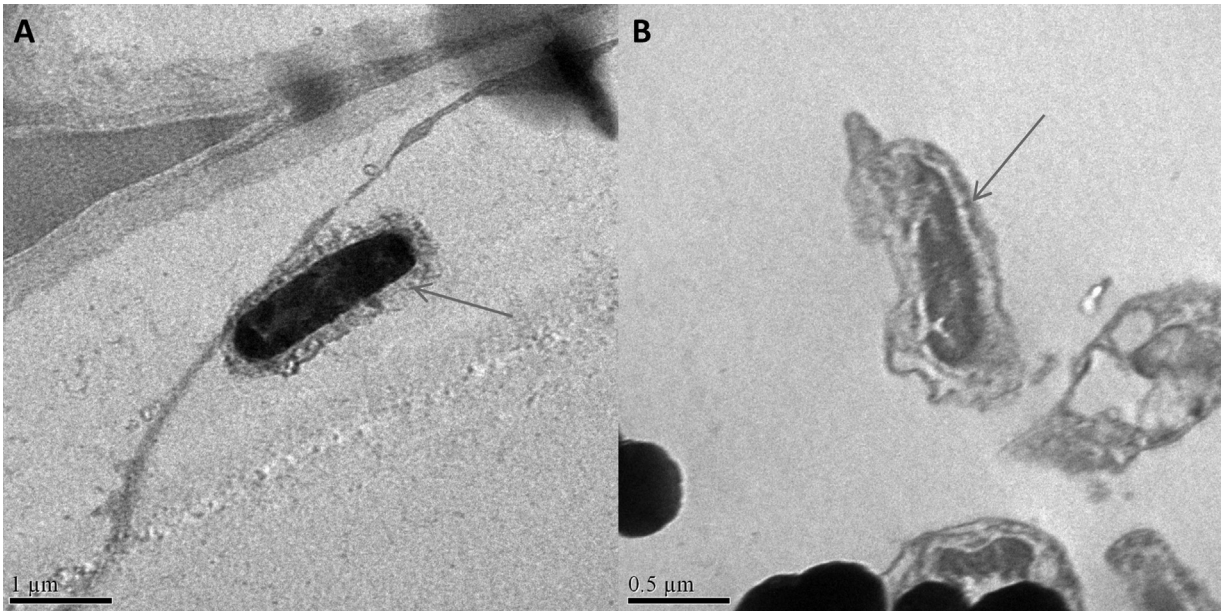

Fig. 4. Gum like material surrounding bacterial colonization within *H. cordata*. Arrows indicate the gum like material.

appeared in some bacteria within *H. cordata*, indicated by gum like surrounding material (Fig. 4). This may have occurred in response to plant defence mechanism in controlling endophytic colonization. In the leaves, although the bacteria were fewer in number, some had readily colonized the aerenchyma and the intercellular spaces.

The antimicrobial activity of the tested methanolic extracts of the isolated bacterial endophytes was evident in the agar well diffusion assay (Table 1, Fig. 5A, B). SEM revealed gradual morphological changes in the growth of both Gram-positive and Gram-negative pathogenic bacteria (Fig. 5C), indicated by the zone of inhibition showing the affected (Fig. 5Ca) and unaffected region (Fig. 5Cb). Scanning electron microscopy imaging analysis of the *in vivo* action of endophytic methanolic extracts against the tested pathogens revealed the morphological damages of the cell membrane and cell wall structure at 6000×. Distinct signs of damages in the Gram-positive pathogen (*Streptococ-*

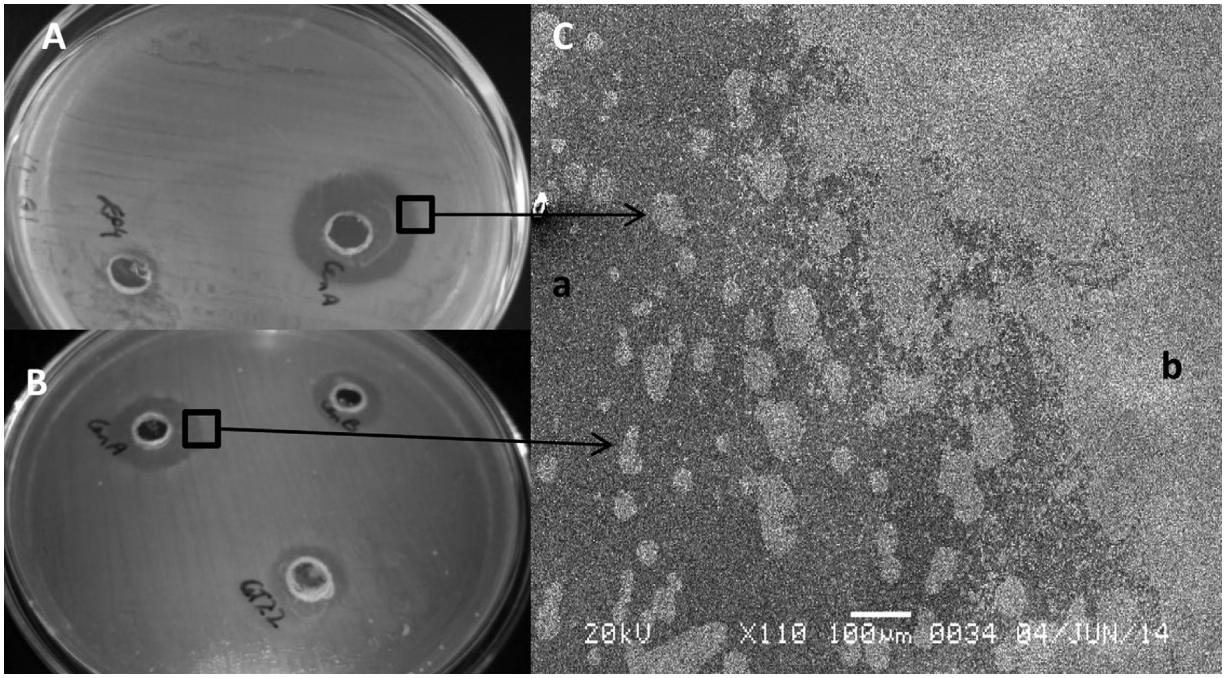

Fig. 5. Antimicrobial activity using agar well diffusion method against bacterial pathogens MTCC1925 (A) and MTCC735 (B) caused by endophytic methanolic extract. Scanning electron micrograph showing gradual morphological changes in the growth of pathogens (C) at the zone of inhibition, with the region of inhibition indicating maximum changes in pathogen growth (Ca) and the unaffected region (control) (Cb). MTCC = Microbial Type Culture Collection and Gene Bank.

cus pyogenes) were visualized, such as; detached cell wall and cell burst (Fig. 6A) caused by endophytic methanolic extract of *Serratia marcescens,* associated with *C. asiatica* (Table 1). However, such morphological changes were not observed in the cells (Fig. 6B) present in the unaffected region (Fig. 5Cb). Similarly, the effect of antibacterial activity of the endophytic methanolic extracts of *S. marcescens*, *Bacillus subtilis*, and *B. methylotrophicus* associated with ethnomedicinal plants, *C. asiatica* and *P. fulgens* (Table 1) against Gram-negative pathogen (*Salmonella enterica ser. paratyphi*) was shown by cell blisters on the surface, mostly at the polar and septal regions of the cell (Fig. 6C–E) compared to unaffected cells (Fig. 6F).

4. Discussion

Endophytes have been visualized as single cells, doublets or forming a string of bacteria [9,10]. However, biofilm formation was not observed in endophytes colonizing ethnomedicinal plants, as visualized in previous reports [11]. In the present study, endophytes were not found to colonize the vascular system which corroborates earlier reports [12]. Non-uniform bacterial colonization in the roots and leaves of ethnomedicinal plants can be explained by the presence of varying root/leaf exudation pattern. Similar observations were made among epiphytes colonizing plant external surfaces [13]. The presence of bacterial flagella may possibly result from quorum sensing and presence of different attractive or repulsive compounds that affect bacterial colonization [14,15]. Bacterial flagella allow endophytes to get into contact with exudates [16] and chemotaxis driven by flagella play an important role in colonization. However, only a few endophytes were visualized with flagella, this reveals that flagella alone is not responsible in endophytic colonization, as shown by fluorescent *Pseudomonas* and *Serratia* strains in wheat [17]. Endophytes secrete secondary metabolites as a mechanism that enables them to colonize internal host tissue [18].

Some bacteria may be neutral or deleterious in regard to plant growth, whereas other microbes support their hosts [19,20]. Consequently, plants commence different defense mechanisms in controlling endophytic colonization. The different defense mechanisms in controlling endophytic colonization includes strengthening of cell wall, establishment of gum formation as surrounding material have been observed [21–23].

Endophytes are of interest for application in agriculture either as biofertilisers or for phytoremediation applications [24,25]. Several studies have suggested the treatment of host plants with selected endophytic bacteria could sensitize plants to reduce disease incidence and severity [26].

SEM has uncovered the ultrastructural damages on both cell wall and cytoplamatic membrane of Gram-positive and Gram-negative pathogens caused by endophytic methanolic extracts [27]. Similar topography of cell lesions at the polar and septal regions has been reported previously in Gram-negative, *Escherichia coli* [28]. These observations confirm endophytic bacteria as natural inhabitants of plants and influence the plant physiology in such a way that increases their host resistance upon pathogen attack [3].

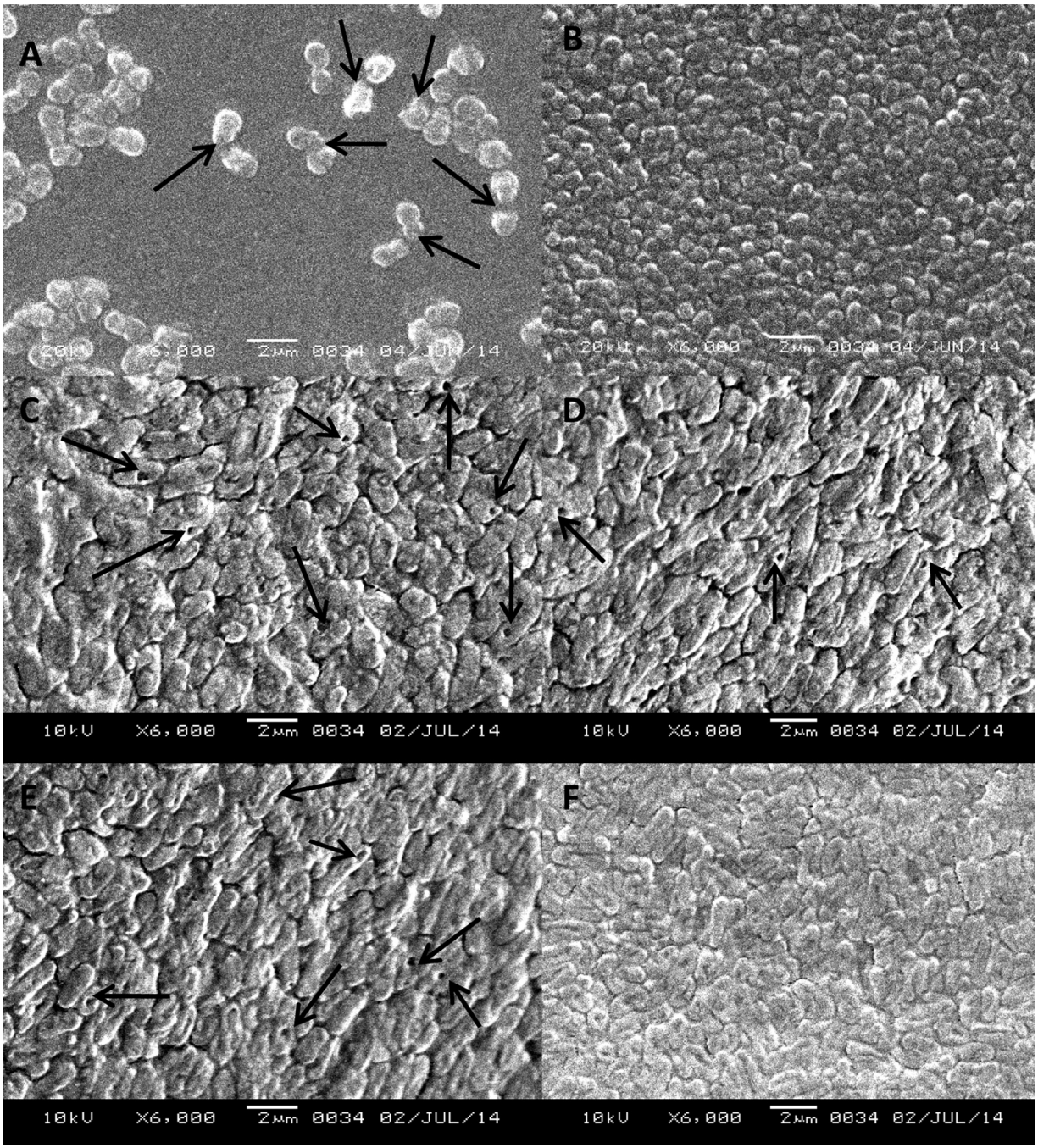

Fig. 6. Scanning electron micrographs reveal morphological changes in the growth of Gram-positive pathogen, *S. pyogenes* (A) caused by endophytic, *Serratia marcescens* methanolic extract compared to normal cell growth at the unaffected region (B). Morphological changes in the growth of Gram-negative pathogen, *S. enterica ser. paratyphi* caused by endophytic methanolic extract of *S. marcescens* (C), *Bacillus subtilis* (D) and *B. methylotrophicus* (E) compared to its normal growth (F). Arrows indicate the morphological changes in the pathogens.

5. Conclusion

The study included both, microscopic evidence of endophytic colonization and isolation of endophytes from surface-disinfected tissues. TEM has provided a comprehensive understanding of endophyte colonization within ethnomedicinal plants at the ultrastructural level. The study revealed the different capacities of bacterial endophytes to colonize various plant compartments and provides a better prediction on bacterial interaction with their host and whether they are likely to establish themselves in the natural plant environment. SEM study has given insight into the morphological damages and defor-

mities in pathogens caused by the culture extracts of endophytes.

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