IN VITRO CHARACTERIZATION OF BACTERIAL ENDOPHYTES FROM TOMATO (*SOLANUM LYCOPERSICUM* L.) FOR PHYTOBENEFICIAL TRAITS

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Abstract. Bacterial endophytes, with plant beneficial traits, are being routinely isolated and investigated to address environmental impacts and low nutrient use efficiency of chemical fertilizers. The current study was framed with a hypothesis that local cultivar of tomato (*Solanum lycopersicum* L. cv. Nagina) harbors multi-trait endophytes with plant-beneficial attributes. From a collection of purified root endophytes, only two isolates *viz*. NgE3 and NgE4 with desired PGP traits were identified as *Bacillus subtilis* and *Paenibacillus* sp., respectively on the basis of 16S rRNA gene sequence analysis. Both the strains exhibited substantial nitrogenase activity, tricalcium phosphate-solubilizing ability and were positive for 1-aminocyclopropnae-1-carboxylate (ACC) deaminase synthesis. A considerable quantity of Indole-3-acetic acid was measured only in the culture supernatant of *Paenibacillus* strain NgE4, while N-acyl homoserine lactones (AHLs) production was found positive in *Bacillus subtilis* NgE3. Both the strains also revealed considerable antifungal activity (> 70%) against at least 3 out 5 fungal pathogens tested. The root colonization potential of *Bacillus* and *Paenibacillus* strains was confirmed by localizing the strains in the tomato root cortical cells as endophytes through transmission electron microscopy. Keeping in view very limited research reported on bacterial endophytes in Pakistan, both phytobeneficial strains are potential resources as bio-inoculants for the tomato to design greenhouse and field experiments.

Keywords: *isolation, plant-beneficial traits, 16S rRNA, transmission electron microscopy, antifungal activity*

Introduction

Maintenance of agricultural productivity for ever-increasing world population along with preservation of natural resources has become a major challenge in both developed and developing countries (Singh, 2017). Up to now, the farmer's strategy to increase crop productivity was totally based on the use of chemical fertilizer inputs; however, increasing the fertilizer inputs is no more boosting the agricultural yields in recent

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decades due to their low nutrient use efficiency (Yousaf et al., 2017). Therefore, this prompts the need to characterize the plant beneficial bacteria for their ultimate field inoculation as nutrient supplying agents (Hanif et al., 2015; Pii et al., 2015; Akram et al., 2016; Mahmood et al., 2017). Such beneficial bacterial agents, often known as plant growth-promoting rhizobacteria (PGPRs), establish a strong association with rhizosphere, the soil adhering to the root surfaces (Kloepper et al., 1989). They are also found in the direct attachment to the root surfaces as rhizoplanic bacteria, and tend to pierce root epidermis and cortex as endophytes (Sylvia et al., 2005; Shaid et al., 2015).

With the advent of advanced molecular techniques, it is now possible to sequence the genomes of both bacterial and fungal endophytes without isolation and purification on culture media. Thus, the more advanced definition of endophytes is the set of microbial genomes inside the plant organs (Bulgarelli et al., 2013). Endophytes are of three types depending upon plant inhabiting life strategies (Hardoim et al., 2008). Obligate endophytes transmit via seeds and are unable thrive outside the plants. For instance, *Xylella fastidiosa* is found in plant tissues as obligate endophyte and is capable of persisting inside the plant for long period of time (Hardoim et al., 2008). *Xylella fastidiosa* transmit through insect vectors. Facultative endophytes live freely in soil and cause infection and colonization as opportunity arrives (Hardoim et al., 2008); most endophytes known for plant growth promotion belong to this class, e.g. *Pseudomonas fluorescence* and *Azospirillum brasilense*. These endophytes enter in plants through cracks in lateral roots or wounds caused by nematodes or plant pathogens (Rosenblueth and Martinez-Romero, 2006). Many other rhizosphere competent bacterial genera are considered as endophytes at some stage of their life cycle. The third category, known as passive endophytes, is not competitive in terms of root colonization and plant growth promotion. Even though they lack the active machinery of plant infection and colonization, transmission via plant roots may occur as result of stochastic events (Verma et al., 2004; Rosenblueth and Martínez-Romero, 2006; Hardoim et al., 2008). After infection and transmission to roots, endophytes can exist both inter- and intracellularly (Hurek et al., 1994; Zakria et al., 2007). A variety of plant species bear bacterial endophytes as part of their root microbiome, with some being known to improve plant growth and productivity by exploiting a variety of mechanisms (Gaiero et al., 2013). Endophytic bacteria are also known to tolerate salt-stress and induce salinity tolerance mechanisms in plants (Damodaran et al., 2014; Yaish et al., 2015). Despite the extensive research on bacterial root endophytes to apply as inoculants for plant growth promotion (Thakore, 2006), a limited understanding exists about the drivers of endophytic communities and mechanisms involved for the success of inoculation in crop productivity. Bacterial endophytes, by synthesizing ACC deaminase enzyme, reduce ethylene concentration and help plants to thrive in adverse environmental conditions. Bacterial endophytes have also been described to produce phytohormones like indole-3-acetic acid (Hardoim et al., 2008). Moreover, different types of AHLs (C6, C8, C10, oxo-C12, 3-oxo-C6 etc.) are known to produce by endophytes for signal transduction during the association. These AHLs participate in plant signaling pathways to sense ecological dynamics (Hartmann et al., 2014).

In vitro characterization of endophytes for plant beneficial properties is the first key step towards the selection of potential bacterial inoculants and investigation of the mechanism involved in phytostimulation (Jasim et al., 2014). So far, most of the studies on bacterial endophytes from tomato were either aimed at their characterization for PGP traits (Amaresan et al., 2012) or biocontrol of phytopathogens (Munif et al., 2013;

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Upreti and Thomas, 2015). The present piece of work is conducted to target multi-trait bacterial endophytes from a local tomato cultivar bearing not only PGP characteristics but also antagonism against plant pathogenic fungi. The study will go a long way in the elucidation of the role of these endophytes for vigor and yield improvement of the tomato crop. Furthermore, the baseline information of these endophytic bacterial associations of tomato will steer to more comprehensive studies in terms of exploring the biochemical and genetic basis of this association.

Materials and methods

Isolation and morphological studies

Tomato plants (cv. Nagina), grown at experimental field of Nuclear Institute of Agriculture and Biology (NIAB, 31°23'42.13 N, 73°1'37.24 E), Faisalabad, Pakistan were uprooted followed by excision of aerial parts with sterilized a knife for isolation of bacteria from endosphere portion of the plant. This cultivar is considered as salt tolerant genotype (Amjad et al., 2014a; 2014b), and thus, selected for isolation of root endophytes. The samples were carried in sterilized polythene bags (25×30 cm) to the Microbial Physiology Laboratory, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan. Root portions were subjected to washing with tap water followed by sterile distilled water to detach adhered soil. Root surfaces were disinfected by dipping in ethanol (70%) for 1 min and sodium hypochlorite (5 % [w/v]) for another 10 min, followed by 5-6 washings with sterilized distilled water. For bacterial isolation, 1 g of macerated roots were put in 9 ml of saline solution (0.85% (w/v) NaCl) by serial dilution technique described by Somasegaran and Hoben (1994). Aliquots (100 µl) from two dilutions (10^{-4} and 10^{-6}) were spread on Luria-Bertani (LB) agar plates using a sterilized glass spreader, followed by 48 h incubation in a microbial incubator (Memmert, Germany) preset at 28 ± 2. Bacterial colonies were selected on the basis of prolific growth and colony morphology and purified through repeated streaking. The purified isolated were maintained at LB-agar plates to carry out further experiments. Five copies of each purified isolate were preserved in 20% (v/v) glycerol at −80 °C. Various morphological characteristics like the colony and cell morphology, motility and Gram's reactions were studied under the light microscope as described by Vincent (1970).

Nitrogen fixation

Nitrogen fixation ability of isolates (NgE3 and NgE4) was assessed by acetylene reduction assay (ARA) using GC-FID as described by Hardy et al. (1968). The purified isolates were individually inoculated into 30 ml semisolid nitrogen free malate (NFM) medium (In 1 l distilled water: 5g l^{-1} malic acid, 0.5 g l^{-1} K2HPO4, 0.2 g l^{-1} MgSO4.7H2O, 0.02 g l^{-1} CaCl2, 0.1 g l^{-1} NaCl, 0.002 g l^{-1} NaMoO4.2H2O, 5 ml 0.5% (w/v) bromothymol blue (BTB), 10 µg biotin, 0.2% (w/v) agar, pH 7 was adjusted with KOH (Okon et al., 1977), in Erlenmeyer flasks (100 ml) and allowed to grow in an orbital shaker (150 rpm, Gallenhamp, UK) at 28 ± 2 °C for 72 h. A well-grown culture (4 ml) was further inoculated to glass vials (12 ml) containing 4 ml NFM medium and followed by incubation at 28 ± 2 °C for 24 h. After replacing the steel caps of vials with rubber septa, air 10% (v/v) was exchanged with the same volume of acetylene gas with the help of a sterilized syringe followed by incubation at 28 ± 2 °C for 24 h. Estimation

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of acetylene (C_2H_2) reduction to ethylene (C_2H_4) was made with Gas chromatograph (Thermoquest, Trace GC, Model K, Rodono Milan, Italy) fitted with a Porapak N column (2 mm × 2 m) and a flame ionization detector (FID). The operating conditions of GC were: oven temperature 80 °C, right inlet 100 °C, right detector 180 °C, hydrogen flow rate 30 ml min^{-1}, nitrogen flow rate 42 ml min^{-1} and air flow rate was maintained at 300 ml min^{-1}. Standard ethylene (200 µl, Spancan Calibration Gas, Spantach Products, England) was injected before analyzing samples and peak area was recorded followed by same volume of each sample. Protein concentration in vial mixture was determined by the method described by Bradford (1976).

Indole-3-acetic acid (IAA) production

The isolates were inoculated into Erlenmeyer flasks (500 ml) containing 100 ml LB broth supplemented with tryptophan (100 mg l^{-1}) and subjected to incubation at 28 ± 2 °C for 48 h in an orbital shaker at 150 rpm (Gallenhamp, UK). Supernatant, collected in separate tubes after spinning at 13000 *g*, was acidified (pH 2.8) with HCl. Supernatant was mixed with equal volume of ethyl acetate for IAA extraction in separating funnel (Tien et al., 1979). The IAA-containing upper layer of separating funnel was collected in separate sterilized tubes followed by removal of excessive ethyl acetate under vacuum at 45 °C, using a rotary evaporator (EYELA, Tokyo, Japan). This extract was dissolved in methanol (1 ml) and filter sterilized through 0.2 µm nylon filter (Millipore, USA) prior to analyze by HPLC (λ = 260 nm) (Perkin Elmer, USA) equipped with Turbochrom software and C_{18} column (150 mm length, 4 mm diameter and 120Å pore size) at a flow rate of 0.5 ml min^{-1} using 30:70 (v/v) methanol:water as mobile phase.

Phosphate solubilization and detection of organic acids

Each of the purified isolates was spot-inoculated on Pikovskaya's agar (Pikovskaya, 1948) plates, incubated at 28 ± 2 °C in a microbial incubator (Memmert, Germany) and observations were taken to 168 h for halo-zone formation. For quantitative analysis of phosphate solubilization and organic acid production, 100 ml of Pikovskaya's broth was inoculated with single purified colonies of each isolate in Erlenmeyer flasks (500 ml) and incubated at 28 ± 2 °C for 240 h in an orbital shaker (Gallenhamp, UK) at 150 rpm. An aliquot of 20 ml of pure bacterial culture from each flask was harvested after after 72 h, 120 h and 168 h and centrifuged at 13000 *g* for 10 min to collect the supernatant. The quantification of phosphate solubilization was accomplished according to phosphomolybdate blue color method (Murphy and Riley, 1962) using spectrophotometer (JENWAY6305, UK) (λ = 882 ηm). For the determination of the organic acids, the cell-free supernatant of isolates was filtered through nylon filters (0.2 µm, Millipore, USA) and 20 µl was injected into HPLC (λ = 210 ηm) equipped with Turbochrom software (Perkin Elmer, USA) and C_{18} column (150 mm length, 4 mm diameter and 120Å pore size). A flow rate of 0.6 ml min^{-1} was maintained using methanol: acetic acid (30:70 (v/v)) as a mobile phase. The standards of organic acids (gluconic, malic, lactic, acetic, citric, and tartaric acid) were commercially purchased (Sigma-Aldrich, Germany).

ACC deaminase activity

The ability of endophytes to use 1-aminocyclopropane-1-carboxylic acid (ACC) as sole nitrogen source was determined in 5 ml DF salt minimal media (Penrose and Glick,

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2003) containing 3 µl of 0.5 M ACC. The cultures were incubated at 28 ± 2 °C at constant shaking (150 rpm) for 48 h. The turbidity of cultures in comparison with non-inoculated control indicated the ability to utilize ACC.

Screening for N-acyl homoserine lactone (AHL) production

The purified isolates (NgE3 and NgE4) were streaked onto the center of a Tryptone Yeast extract (TY) (Beringer, 1974) agar plates and were allowed to grow overnight in a microbial incubator (Memmert, Germany) preset at 28 ± 2 °C. The indicator strain *Chromobacterium violaceum* CV026 (obtained from John Innes Centre, Norwich, UK) was grown separately in LB broth at 28 ± 2 °C with constant shaking overnight up to an OD of 10^6 CFU ml^{-1}. Detection of AHLs was made by the plate overlay assay as described earlier by McLean et al. (2004). The reference strain A34 (obtained from the John Innes Centre, Norwich, UK) was used as the positive control. A34 is a *Rhizobium leguminosarum* 8401 containing *p*RLIJ1. Plates were incubated at 30 ± 2 °C overnight, and purple pigmentation produced by the indicator strain on the plates was an indicator of AHL production.

Antifungal activity

The antifungal activity was tested using a dual-culture assay as described by Sakthivel and Gnanamanickam (1986). A drop of the exponentially grown bacterial culture (approx. 20 µl) was spotted onto potato dextrose agar (PDA) plates close to the walls of the Petri dish on both sides and the plates were allowed to dry in laminar air flow cabinet. A 6-mm agar disk of each of three fungal species, namely, *Fusarium solani*, *F. oxysporum* and *F. moniliforme*, *Aspergillus niger* and *A. flavus* (obtained from Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan), were separately placed at the center of PDA plates. Fungal disc was grown separately over Petri plate containing PDA to serve as a control. The incubation of plates was made at 28 ± 2 °C in microbial incubator (Memmert, Germany) for 5 days to measure the inhibition of radial fungal growth between fungal and bacterial colonies.

$$\text{Inhibition } (\%) = \left(1 - \left[\text{fungal growth / Control growth}\right]\right) \times 100$$

16S rRNA gene sequencing

Total genomic DNA of isolates (NgE3 and NgE4) was isolated by the alkaline lysis method (Maniatis et al., 1982) and quantified by ultraspec™ 3100 ($OD_{260,\ 260/280}$). This DNA was used as template to amplify the 16S rRNA gene with primers fD1 and rD1 as described earlier by Weisburg et al. (1991) with slight modifications as: for 50 µL reaction in ultra-pure water, 5 µl of Taq buffer (Fermentas, USA), 3 µl of 25 mM $MgCl_2$ (Fermentas, USA), 5 µl of 2 mM dNTPs (Fermentas, USA), 0.5 µl of 100% DMSO, 1.5 µl each of forward and reverse primer, 0.75 µl of 5 U μl^{-1} Taq DNA polymerase (Fermentas, USA) and 40 ηg of template DNA. Polymerase chain reaction (PCR) was conducted in thermal cycler (PeQLab, advanced Primus 96, Germany) with modified temperature conditions as: 30 cycles of 95 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min. The initial denaturation and final extension steps were performed at 95 °C for 5 min and 72 °C for 10 min, respectively. Amplicons were purified with QIAquick PCR purification kit (Qiagen, USA) according to manufacturer's protocol and sequenced

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directly on both sides (Macrogen, Korea). The gene sequence was analyzed using sequence scanner software package. Forward and reverse sequenced strands were joined by Caps 3 assembly online software and compared with others in the GenBank database using the NCBI BLASTn tool. Final sequences were deposited in Genbank and accession numbers were obtained.

Ultrastructure studies

For ultrastructure-studies, tomato (cv. Nagina) seeds were surface sterilized by immersing them in an aqueous solution of sodium hypochlorite (5% (v/v)) for 10 min followed by 5-6 washings with sterilized distilled water. The seeds were soaked in the inoculum (1×10^8 CFU ml^{-1}) for 30 min and allowed to germinate on water-agar (1.5% (w/v)) plates. Root hairs of ten days old seedlings were cut into pieces (approximately 1-3 cm) and embedded in water-agar (1.5% (w/v)) again followed by cutting of approximately 2-3 mm small agar cubes. The cubes were put in 1.5-ml tubes in the presence of 5 % (v/v) gluteraldehyde (made in 0.2 M PIPES buffer, pH 8.0) as a fixative. After 16-18 h, the fixative was replaced with 0.2 M PIPES buffer [0.58 g NaCl, 3 g PIPES {piperazine-*N*,*N*′ bis(2-ethanesulfonic acid)}, 1M NaOH, 0.2 g $MgCl_2.6H_2O$, pH 6.8] (Salema and Brandao, 1973) and samples were washed 2 × 1 h in fresh buffer. Afterwards, these samples were treated with aqueous osmium tetra oxide (0.2 % (w/v) made in PIPES buffer solution (0.2 M, pH 6.8) for 16-18 h and washed 2 × 30 min with sterilized distilled water. After treating with aqueous uranyl acetate (5 % (w/v) for 16-18 h, the samples were washed with sterilized distilled water for 2 × 30 min. First dehydration was carried out with absolute alcohol for 2 × 30 min and then with propylene oxide (100% (v/v)) for 1 × 30 min. Infiltration of samples was carried out with propylene oxide in ratio 1:1 for 24-48 h and then with spur-resin for further 24-72 h. The accelerator Benzyl Di-Methyl Amine (BDMA) was used in all infiltration steps. Samples were placed on flat embedding molds for polymerization for 72 h at 65-70 °C in the oven (Memmert, Germany). Polymerized resin blocks were left at room temperature for at least 24 h before cutting ultra-thin sections. Ultra-thin sections (150-200 nm) were cut on ultra-microtome (RMC-7000) and the sections were carefully placed on copper grids. The sections were double stained with uranyl acetate and lead citrate for 30 min and 10 min, respectively. The grids were washed with deionized water and observed under Transmission Electron Microscope (TEM, JEOL 1010, Japan).

Results

Morphological and physiological characterization

Isolate NgE3 was appeared with round and whitish colonies on agar medium and its cells were found as long rods under a light microscope. In addition, it showed 73.93 ± 4.23 ηmol mg^{-1} protein h^{-1} nitrogenase activity and 4.92 ± 0.64 μg ml^{-1} IAA production ability (*Table 1*). Isolate NgE3 was also found positive for AHLs production and demonstrated substantial (> 79%) inhibition of three fungal pathogens *viz*. *Fusarium oxysporum*, *Aspergillus niger* and *A. flavus*. On the other hand, isolate NgE4 had curled and off-white colonies with cell shapes appeared as short rods under a light microscope. Nitrogenase activity of NgE4 was measured as 63.00 ± 6.35 ηmol mg^{-1} protein h^{-1}. It also synthesized 1.66 ± 0.11 μg ml^{-1} IAA in the culture medium (*Table 2*). In addition,

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isolate NgE4 also showed biocontrol potential (> 79%) against *Fusarium solani*, *F. oxysporum* and *Aspergillus flavus* (*Table 3*), but was found negative found for AHLs production. was ACC deaminase and catalase enzyme activity was found positive in both isolates. Moreover, both the isolates were found Gram-positive showing the cell motility glass slide. Isolates NgE3 and NgE4 were unable to show the antifungal activity against a panel of fungal pathogens tested and also lacked AHL production.

Table 1. *Morphological and physiological characterization of isolates NgE3 and NgE4*

Isolate**	Colony morphology	Cell morphology	IAA production (µg ml^{-1})	AHLs production
NgE3	Round and whitish	Long rods	4.92 (0.64)	+
NgE4	Curled and off-white	Short rods	1.66 (0.11)	-

**Both isolates were Gram-positive and motile. Isolates NgE3 and NgE4 were also found positive for catalase and ACC deaminase activity. Standard error of three replicates is presented in parentheses

Table 2. *The antifungal activity of bacterial endophytes from the tomato against different fungal pathogens*

Isolate	% inhibition				
	Fusarium			*Aspergillus*	
	solani	*oxysporum*	*moniliforme*	*niger*	*flavus*
NgE3	46 (5)*	78 (4)	51 (11)	76 (5)	79 (3)
NgE4	77 (6)	83 (6)	42 (3)	66 (10)	86 (13)

*Standard error of three replicates is presented in parentheses

Table 3. *Phylogenetic identity of `16S rRNA gene sequences after BLASTn analysis*

Isolate	Sequence length (bp)	Similarity %	Closest GenBank match	Strain identified	Accession #
NgE3	1057	99	*Bacillus subtilis* subsp. *subtilis* strain DDKRC2	*Bacillus subtilis*	KC202298
NgE4	857	98	*Paenibacillus* sp. IHB B 3310	*Paenibacillus* sp.	KC202299

Relationship between phosphate solubilization and organic acid production

Isolate NgE3 solubilized 44.71 ± 2.25 µg ml^{-1} insoluble tricalcium phosphate (TCP) after 168 h of incubation with the drop in the pH of the medium up to 5.3, which was found directly correlated ($P \leq 0.001$, $r = 0.97$) to synthesis of lactic acid in the culture medium (21.89 ± 1.66 µg ml^{-1}). Similarly, isolate NgE4 converted 54.40 ± 1.92 µg ml^{-1} of TCP to soluble form with the total lowering of pH up to 4.2. The amount of TCP solubilized by NgE4 was positively correlated to both gluconic acid ($P \leq 0.001$, $r = 0.99$) and lactic acid ($P \leq 0.001$, $r = 0.99$). The total amount of gluconic and lactic

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acids produced by NgE4 after 168 h of incubation were measured as 19.40 ± 0.82 µg ml^{-1} and 14.06 ± 0.85 µg ml^{-1}, respectively (*Fig. 1*).

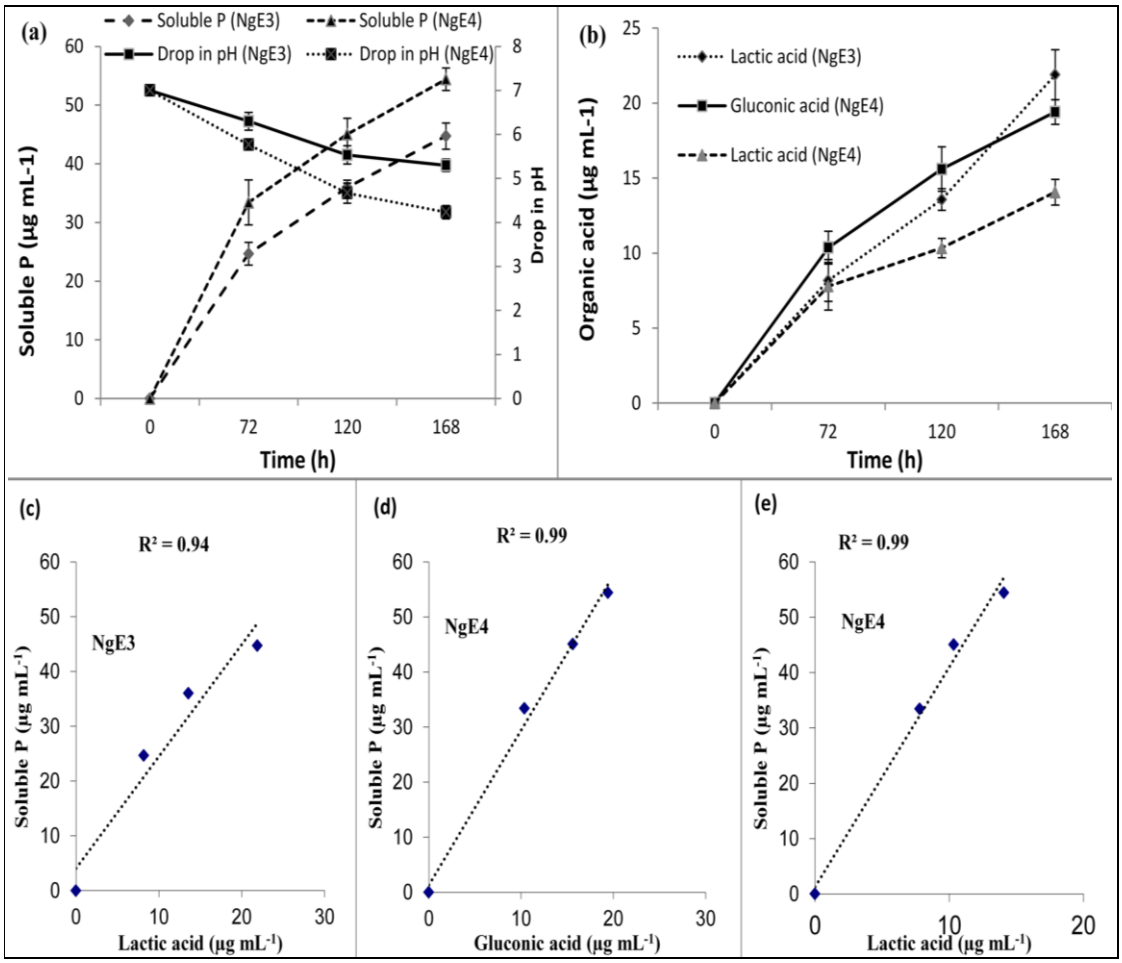

Figure 1. *Phosphate solubilized by isolates NgE3 and NgE4 with respect to drop in pH of medium (a), organic acids produced by the isolates at time intervals (b) and relationship between organic acid production and phosphate solubilization as determined through regression correlation analysis (c, d, e)*

Molecular identification and phylogenetic analysis

Isolate NgE3 was identified as *Bacillus subtilis* (KC202298) on the basis of 16S rRNA gene sequence analysis (*Table 3*). In phylogenetic tree, constructed to compare strain NgE3 with type strains of genus *Bacillus*, it clustered itself with *Bacillus subtilis* NCDO 1769^T and *Bacillus subtilis* IAM 12118^T. On the other hand, NgE4 was identified as *Paenibacillus* sp. as it demonstrated 98% 16S rRNA gene sequence similarity with more than one *Paenibacillus* spp. Phylogenetic tree showed its grouping with *P. pabuli* JCM 9074^T and *P. illinoisensis* JCM 9907^T (*Fig. 2*).

Ultrastructure studies for root colonization

When observed by TEM, both *Bacillus subtilis* NgE3 and *Paenibacillus* sp. NgE4 infected the tomato root hair cells under gnotobiotic conditions. The colonization

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pattern of both strains apparently looked similar with the only difference in the number of bacterial cells infected the tomato root hair cells was greater in case of *Paenibacillus* sp. NgE4. Both the strains were found potential bacterial endophytes through ultrastructure TEM studies (*Fig. 3*).

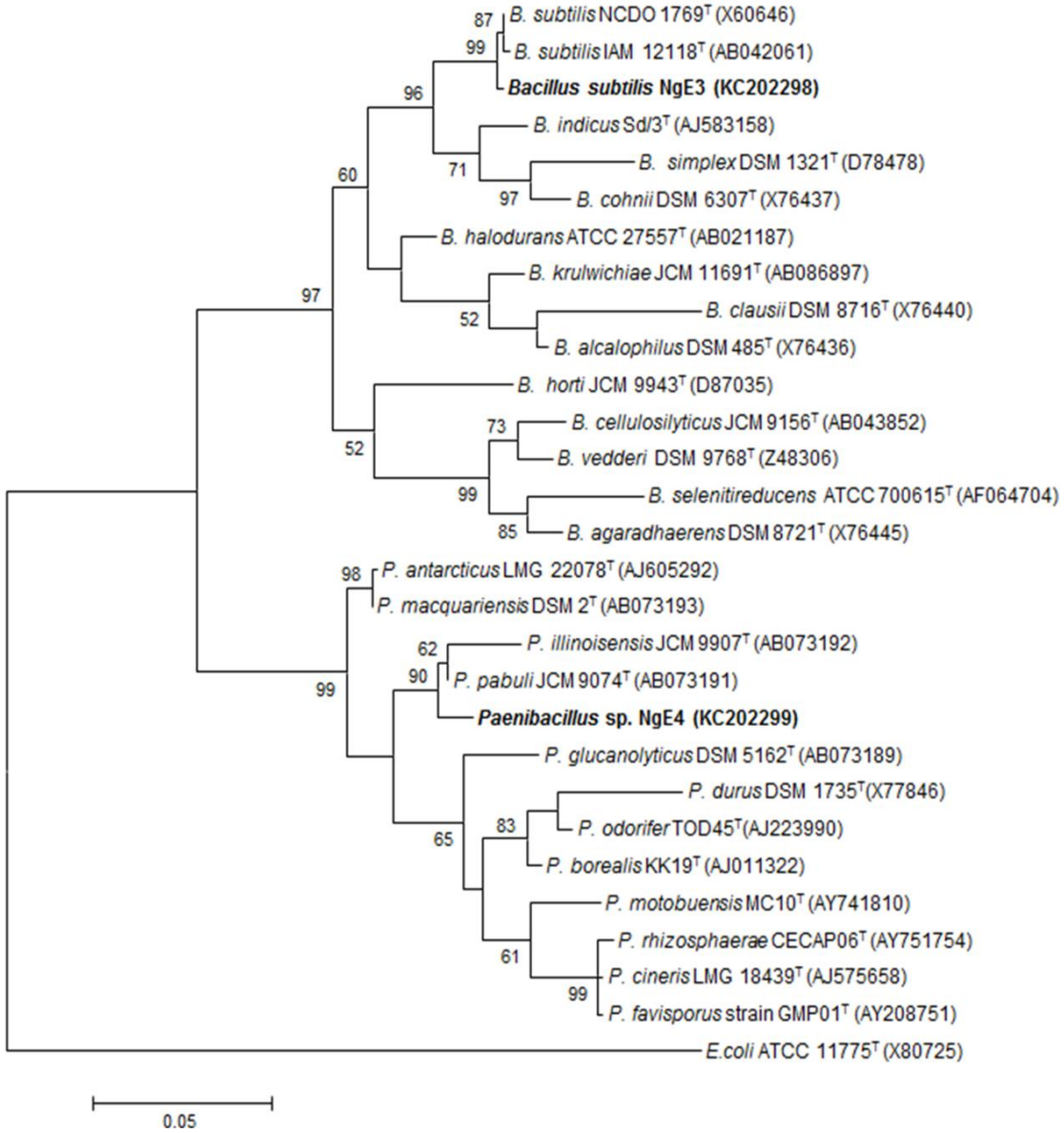

Figure 2. *Phylogenetic analysis based on 16S rRNA gene sequences of strains NgE3 and NgE4 with their respective type strains. The evolutionary history was inferred using the Neighbor-Joining method. Only greater than 50% of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in Phylmyl software package. E. coli strain ATCC 11775^T was included as an outgroup*

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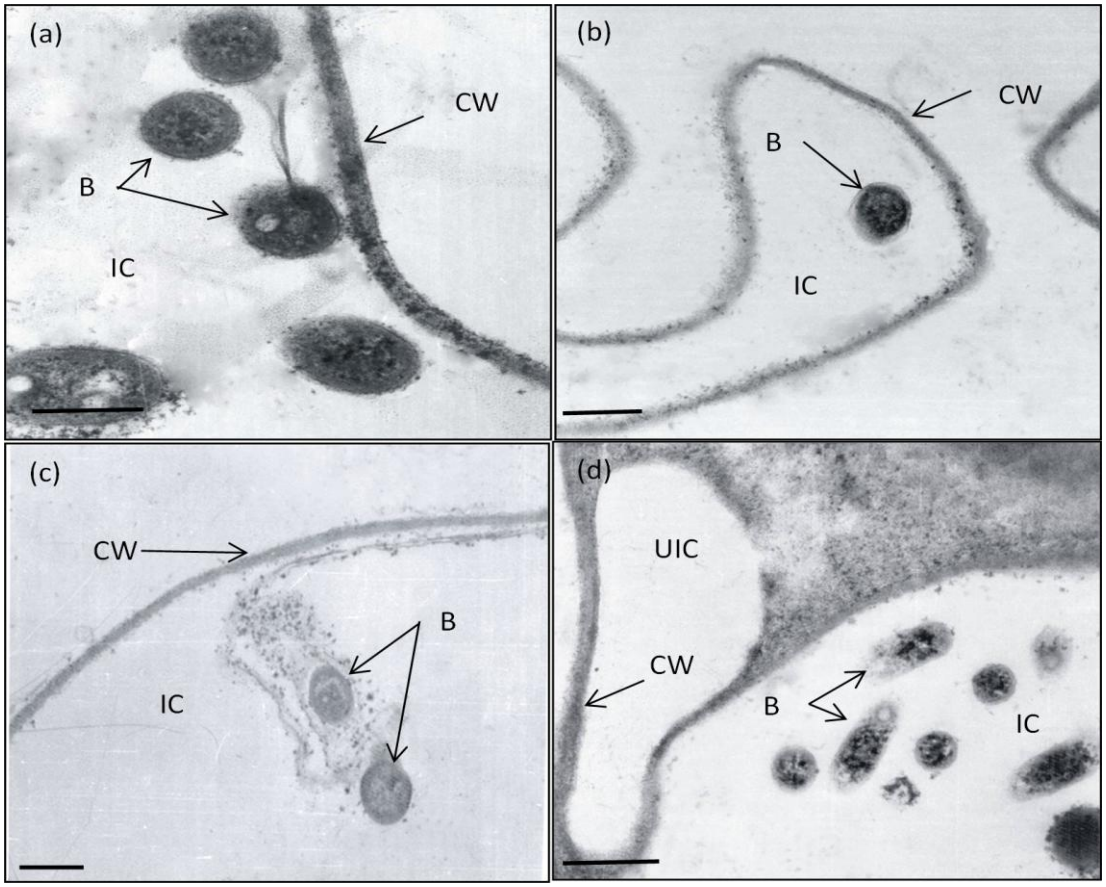

Figure 3. *Tomato root endosphere colonization of strains Bacillus subtilis NgE3 (a, b) and Paenibacillus sp. NgE4 (c, d). B Bacteria, CW Cell wall, IC Infected cell, UIC Uninfected cell. Scale bar: 1μm*

Discussion

Recently, endophytic bacteria have gained significant attention due to their interesting features related to plant growth and health issues. A very limited research is available on exploiting the potential of bacterial endophytes in Pakistan. The present study demonstrated the in vitro efficiency of *Bacillus subtilis* NgE3 and *Paenibacillus* sp. NgE4 in terms of nitrogen fixation, P-solubilization through organic acid production, synthesis of IAA, ACC deaminase activity, AHLs production and biocontrol potential. Globally, bacterial endophytes have already been reported to exhibit phytobeneficial characteristics (Gyaneshwar et al., 2001; Miliūtė and Buzaitė, 2011; Oteino et al., 2015) and stress tolerance (Khan et al., 2015).

Reduction of acetylene (C_2H_2) into ethylene (C_2H_4) is an indirect approach to authenticate the diastrophic nature of bacteria. Conversion of a significant amount of acetylene by NgE3 and NgE4 (73.93 ± 4.23 μg ml^{-1} and 63.00 ± 6.35 μg ml^{-1}, respectively) into ethylene confirmed the nitrogen-fixing ability of the strains. Gyaneshwar et al. (2001) reported bacterial endophyte *Serratia marcescens* IRBG500 as diazotroph on the basis its acetylene reduction ability and nifH gene sequence analysis. Bacteria make inorganic soil phosphates available to plants through the secretion or organic acids in plant vicinity (Richardson et al., 2009; Shahid et al., 2015). Secretion of low molecular weight organic acids in soil by phosphate solubilizing bacteria is the major

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mechanism that works through acidification of soil or media (Gyaneshwar et al., 1999; Puente et al., 2004; Khan et al., 2009). Through their hydroxyl and carboxyl groups, these organic acids play their role in detaching the cations bound to soil phosphates (Al-phosphates, Ca-phosphates and Fe-phosphates) transforming them into primary and secondary ortho-phosphates (Kpomblekou and Tabatabai, 1994). Moreover, extracellular oxidation of glucose to GA via quinoprotein glucose dehydrogenase is the most effective mineral phosphate solubilization phenotype in Gram-negative bacteria (Rodríguez et al., 2000). In our study, phosphate solubilization by strains NgE3 and NgE4 was found significantly correlated with gluconic acid ($P \leq 0.001$, $r = 0.99$) and lactic acid ($P \leq 0.001$, $r = 0.99$) (*Fig 1*). The amount of IAA produced by stains NgE3 and NgE4 (4.92 ± 0.64 μg ml^{-1} and 1.66 ± 0.11 μg ml^{-1}) is also similar to the findings of Miliūtė and Buzaitė (2011) who isolated 9 bacterial endophytes from apple tree buds and all of them were able to produce IAA in vitro. The positive reaction of both strains for ACC deaminase enzyme activity aided their significance for plant growth under stress conditions (Penrose and Glick, 2003; Glick et al., 2007; Mahmood et al., 2017). Similarly, biocontrol potential of isolates NgE3 and NgE4 against major phytopathogenic fungal strains is in agreement with the previous findings (Senthilkumar et al., 2009; Wang et al., 2013), which revealed antifugal activity of screened bacterial endophytes against many fungal pathogens including *Fusarium* and *Aspergillus* spp. *N*-acylhomoserine lactone (AHL)-based quorum sensing (QS) systems have been described in many bacterial endophytes (Liu et al., 2011), but in current study one of the two isolates NgE3 demonstrated AHLs production trait. Besides other plants, bacterial endophytes have also been isolated and characterized for plant growth-promoting attributes (Amaresan et al., 2012), biocontrol activity (Munif et al., 2013; Upreti and Thomas, 2015) and production of AHLs (Singh et al., 2015) from tomato plant.

The in vitro demonstration of plant-beneficial attributes and biocontrol efficiency led to the identification of NgE3 and NgE4 on molecular basis through the sequencing of the most accepted taxonomic marker, the 16S rRNA gene. Isolate NgE3 was identified as *Bacillus subtilis* and isolate NgE4 as *Paenibacillus* sp. with 99% and 98% sequence identity, respectively (*Table 3*). In the neighbor-joining phylogenetic tree, *Bacillus subtilis* NgE3 clustered itself with *Bacillus subtilis* NCDO 1769^T and *Bacillus subtilis* IAM 12118^T. On the other hand, *Paenibacillus* sp. NgE4 grouped itself with *P. pabuli* JCM 9074^T and *P. illinoisensis* JCM 9907^T (*Fig. 2*). Earlier, endophytes from genus *Bacillus* and *Paenibacillus* have been reported to carry plant beneficial characteristics. Malfanova et al. (2011) isolated *Bacillus subtilis* HC8 from the giant hogweed Heracleum sosnowskyi Manden, which significantly promoted plant growth and protected tomato against tomato foot and root rot. Many *Paenibacillus* spp. with positive effects on plant growth have been isolated by Ulrich et al. (2008) from poplar, larch and spruce. Similarly, it was concluded that inoculation of *Paenibacillus* sp. strongly affected the metabolic composition of in vitro-grown plants (Scherling et al., 2009).

Root colonization efficiency of *Bacillus subtilis* NgE3 and *Paenibacillus* sp. NgE4 was confirmed through ultra-structure studies (*Fig. 3*). Both strains infected tomato root hair cells. *Paenibacillus* sp. NgE4 densely colonized the endosphere of tomato root hair cells compared to *Bacillus subtilis* NgE3. Both the strains were found potential bacterial endophytes through ultrastructure TEM studies. Plant beneficial bacteria, as epiphytes and endophytes have been routinely localized in rhizosphere and endosphere with TEM and immunogold labeling techniques (Schloter et al., 1997; Hameed et al., 2005; Jeun et al., 2008; Yasmeen et al., 2012; Shahid et al., 2015).

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Conclusion

The bacterial endophytes NgE3 and NgE4 had great in vitro potential for major plant beneficial traits. Prior to their addition to indigenous biofertilizer regimes, the strains might be inoculated directly to tomato plants under pot and field experiments to check their effect on plant growth and disease suppression.

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