Research article

Indole acetic acid and ACC deaminase from endophytic bacteria improves the growth of *Solanum lycopersicum*

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Abstract

Background: Endophytic bacteria are ubiquitous in all plant species contributing in host plant’s nutrient uptake and helping the host to improve its growth. *Moringa peregrina* which is a medicinal plant, growing in arid region of Arabia, was assessed for the presence of endophytic bacterial strains.

Results: PCR amplification and sequencing of 16S rRNA of bacterial endophytes revealed the 5 endophytic bacteria, in which 2 strains were from *Sphingomonas* sp.; 2 strains from *Bacillus* sp. and 1 from *Methylobacterium* genus. Among the endophytic bacterial strains, a strain of *Bacillus subtilis* LK14 has shown significant prospects in phosphate solubilization (clearing zone of 56.71 mm after 5 d), ACC deaminase (448.3 ± 2.91 nM α-ketobutyrate mg⁻¹ h⁻¹) and acid phosphatase activity (8.4 ± 1.2 nM mg⁻¹ min⁻¹). The endophytic bacteria were also assessed for their potential to produce indole-3-acetic acid (IAA). Among isolated strains, the initial spectrophotometry analysis showed significantly higher IAA production by *Bacillus subtilis* LK14. The diurnal production of IAA was quantified using multiple reactions monitoring method in UPLC/MS. The analysis showed that LK14 produced the highest (8.7 μM) IAA on 14th d of growth. Looking at LK14 potentials, it was applied to *Solanum lycopersicum*, where it significantly increased the shoot and root biomass and chlorophyll (a and b) contents as compared to control plants.

Conclusion: The study concludes that using endophytic bacterial strains can be bio-prospective for plant growth promotion, which might be an ideal strategy for improving growth of crops in marginal lands.

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1. Introduction

The microbial flora (bacterial and fungal) found in plant species are known as endophytes. These are asymptptomatically occurring microbes found almost in all parts of living plant species and their organs [1,2,3]. Endophytes are pivotally important for the plant growth and defense mechanisms. Studying the endophytic mode of microbial life and their ecological function has attracted much attention in recent years. The endophytic microbial diversity of beneficial organisms may be more as there are nearly 300,000 terrestrial plant species, whereas each plant can host more than one type of endophyte [4]. Endophytes establish a mutualistic interaction, enhancing host plant’s nutrient uptake and helping host to counteract adverse effects of biotic and abiotic stresses [5]. Colonization of host with such beneficial endophytic bacteria can be used as valuable alternative to synthetic fertilizers to enhance crop productivity [6,7,8].

The beneficial effects of endophytic bacteria or fungi have been regarded for their potential to produce biologically active metabolites and substances [9]. Production of such substances can maintain their own metabolism as well as supports the growth of their hosts [8]. These substances can range from secondary metabolites to various enzymes. In case of metabolites, endophytic microbes have recently been also known to produce plant hormones such as indole-3-acetic acid (IAA), gibberellins and cytokinins etc. [10]. Enzymes include ACC (1-aminocyclopropane-1-carboxylate) deaminase, which are normally related to free-living soil bacteria/rhizobacteria [11,12]. However, for endophytic microbes, this potential has recently been known [11,12,13]. ACC deaminase promotes plant growth by lowering ethylene levels of the plant [10,11,13]. This enzyme catalyzes the conversion of ACC, the immediate precursor of ethylene synthesis in plants, to ammonia and α-ketobutyrate [12]. ACC is exuded from seeds or plant
roots and then metabolized by bacteria possessing ability to produce ACC deaminase. This stimulates plant ACC efflux, decreases the root’s ACC and ethylene concentration, thus increases root growth and development [10]. Some of the recent studies have shown that endophytic bacteria can also produce ACC deaminase [12,13], however, this potential has to be explored further.

Endophytes have also been known to produce various classes of secondary metabolites. In case of bioactive metabolites, phytohormones are natural substances mainly produced by plants to regulate its growth [13]. In case of phytohormones, IAA plays a crucial role in plant growth and development, while an additional supply can help the host to safeguard it from environmental stimuli. The production of IAA is widespread among various classes of bacteria/rhizobacteria. Varieties of genus such as Bacillus, Acinetobacter, Pseudomonas, Azospirillum, and Azotobacter [14] have been reported to produce auxins including IAA. Several different IAA biosynthesis pathways are used by these bacteria, with a single bacterial strain sometimes containing more than one pathway. However, recent studies show that IAA is synthesized through (i) indole-3-pyruvic acid, (ii) indole-3-acetamide and (iii) indole-3-acetonitrile pathways. In the plant growth-promoting bacterium Azospirillum, as in Agrobacterium and Pseudomonas, both the indole-3-pyruvic acid and the indole-3-acetamide pathways are present, as tryptophan (Trp)-dependent and -independent pathways [15].

Recently, plant growth promoting endophytic bacteria (PGPEB) has been found which colonizes plant roots and enhances plant growth by a wide variety of mechanisms like phosphate solubilization etc. The use of PGP Rhizobacteria is steadily increased in agriculture and offers an attractive way to replace chemical fertilizers, pesticides, and supplements. Bacteria belonging to the genus Bacillus are common rhizospheric microorganisms. These are isolated from various soil samples of various crop plants such as French bean, wheat, soybean, maize, red pepper, rice and lettuce [16]. The previous results support the potential of using Bacillus subtilis as biological control of seed pathogens, as well as promoting crop growth [17]. Endophytic mode of B. subtilis has been reported recently. It was isolated from the seeds of cacao plant and found greatly contributing to the developmental stages of the cacao plants. Besides this, it has also shown antimicrobial properties [18].

In present study, we isolated various endophytic bacterial strains from the bark of Moringa peregrine for the first time. M. peregrine, on the other hand, is a medicinal tree growing in arid land with an effective role in abdominal pain, constipation, and headache, and also used as vegetable [19]. Since the host was carrying medicinal importance, therefore, interest was developed to isolate bacterial endophyte from this tree. It was aimed to isolate and characterize the beneficial PGPEB, and to assess its potential in producing IAA and ACC deaminase. To quantify IAA, advance UPLC–MS/MS method was used. It was also aimed to assess whether it can be considered as PGP against crop plants such as tomato.

2. Materials and methods

2.1. Endophyte isolation and identification

The bark of Moringa peregrine Forssk. (Moringaceae), growing in the wild mountains of Jabal Al-Akhdr (23° 04′ 22.00 N, 57° 40′ 07.00 E°), Sultanate of Oman, was detached from the tree and transported to laboratory in sterile zip bags. To isolate endophytic microbes, a method of Arnold et al. [8] was adopted. Briefly, 20 bark samples were cut into 60 pieces (0.5 cm) and surface sterilized with 2.5% sodium hypochlorite (10 min in a shaking incubator at 120 rpm) and washed with autoclaved distilled water (DW) to remove the contaminants, rhizobacteria and mycorrhizal fungi. The bark pieces were carefully placed in petri-plates containing Hagem minimal media (0.5% of glucose, 0.05% of KH2PO4, 0.05% of MgSO4 × 7H2O, 0.05% of NH4Cl, 0.1% of FeCl3, and 1.5% of agar; pH 5.6 ± 0.2). The sterilized tissues were also imprinted on separate Hagem plates to ensure the effectiveness of the surface sterilization [8].

The bacterial isolates were identified on the basis of partial 16S ribosomal rRNA sequence. The 16S rRNA was amplified by PCR using the 27F primer (5’–AGAGTTTGATCCTGCGCAAGG–3’) and 1492R primer (5’–CCTACGGGATGCACCTT–3’), which were complementary to the 5’ end and 3’ end of the prokaryotic 16S rRNA, respectively. The amplification reaction was performed as previously described. The BLASTn search programs (http://www.ncbi.nlm.nih.gov/BLASTn) were used to compare the sequence homology of nucleotides. The closely related sequences obtained were aligned through CLUSTALW using MEGA version 4.0 software [19] and the maximum parsimony tree was constructed using the same software. The bootstrap replications (1 K) were used as a statistical support for the nodes in the phylogenetic tree.

2.2. Screening for IAA production

Estimation of IAA in the culture broth was done using colorimetric assay [20]. The endophytes cultured in 20 mL of LB broth without (0 g/L) and with 0.1 g/L of L-tryptophan and incubated at 30 ± 2°C in shaking incubator at 200 rpm for 7 d. The bacterial cultures were then centrifuged at 10,000 × g for 10 min at 4°C and the cell free cultures were filtered through 0.45 μm cellulose acetate filter (DISMIC®, Denmark). The filtrates were acidified to pH 2.8 with 1 N of HCl and extracted 3 times with 20 mL of ethyl acetate. The ethyl acetate fractions were combined and evaporated under vacuum at 45°C in a rotary evaporator. The residue was re-suspended in 3 mL 50% methanol. One millilitre of supernatant was mixed with 2 mL of Salkowski reagent (12 g of FeCl3/L, 7.9 M of H2SO4) and kept in dark for 30 min. The resultant reddish color was read after 30 min at 535 nm in ELISA Spectrophotometer (BioRad, USA). The amount of IAA was calculated with standard of pure IAA (Sigma-Aldrich Ltd, Korea,) prepared separately.

2.3. IAA quantification by UPLC/MS MS

After finding positive results of endophyte, we selected one strain which was showing highest IAA producing ability. Bacterial IAA was quantified using Waters Ultra Performance Liquid Chromatography (UPLC) system (Acquity UPLC, Waters Inc. USA). The analytical column used was Waters Acquity BEH HILIC (2.1 × 50 mm 1.7 μ) and the column oven temperature was set to 40°C. The injection volume was set at 10 μL and the injection mode was partial loop with needle overfill (PLUNO). The flow rate was set at 0.2 mL/min using channel A, 0.5% of formic acid in water; and channel B, 0.5% of formic acid in acetonitrile. The mobile phase was run on a gradient profile with a starting point of 95% A. This was held for 3 min then changed to 10% and held for 1 min. At 4.1 min the starting composition was introduced. With the pre-equilibration time of 1.9 min the total analysis time was 6 min.

The Acquity UPLC was interfaced with a Waters Quattro Premier XE Tandem Quadrupole system (Waters Inc., USA). The tuning parameters were optimized using a standard solution of approximately 4 ng/μL (ppm) of IAA. For these measurements, electrospray ionization (ESI+) was used with the source operation parameters set as follows: Capillary voltage: 3.0 kV, Cone voltage: 12.0 V, Collision Energy: 13 eV, source temperature: 150°C and desolvation temperature: 400°C. The desolvation gas was set at 1000 L/h and cone gas at 20 L/h. The Precursor ion was optimized at 175.65 and the Product ion 129.8.
2.4. Verification and quantification of IAA by LC–MS/MS

For this experiment the IAA standard was dissolved in 100% water. The weak reverse phase solvent as well as the gradient mobile phase helped to produce good peak shape. The quantification of IAA was carried out using two external standard preparation and average response factor for the two standards. This was done by integrating the area under the peak for each Multiple Reaction Monitoring (MRM) trace which was obtained from transition of Precursor ion 175.65 to 129.8 Product ion for both the standards and the samples. The purity of the standard (98% w/w) was also taken into consideration during the response factor calculations.

2.5. Phosphate solubilization and acid phosphatase activity

To detect the phosphate solubilizing bacteria, strains were streaked onto Pikovskaya’s agar medium, which contains (per liter): 0.5 g of yeast extract, 10 g of dextrose, 5 g of Ca₃(PO₄)₂, 0.5 g of (NH₄)₂SO₄, 0.2 g of KCl, 0.1 g of MgSO₄·7H₂O, 0.0001 g of MnSO₄·H₂O, 0.0001 g of FeSO₄·7H₂O and 15 g of agar. After 3 days of incubation at 28°C, strains that induced clear zone around the colonies were considered as positive [20]. Strains were collected from the cultures and immediately resuspended in water. This suspension was centrifuged (5000 × g, 5 min at 4°C) and the pellet was washed twice in water and once in 50 mM of sodium acetate buffer, pH 5.5, before being re-suspended in the same buffer. The preparation was then mixed with glass beads in an ice bath and fragmented in a vortex mixer at maximum speed for 30 s, followed by immediate cooling in ice bath for 2 min (this procedure was repeated five times). The glass beads were removed by sedimentation and the supernatant was centrifuged (5000 × g, 5 min, 4°C) to remove unbroken cells and cell debris. The supernatant was collected for enzyme analysis.

Acid phosphatase activity was assayed by measuring p-nitrophosphophosphate (pNPP) hydrolysis [21] the assay mixture contained sodium acetate buffer (50 mM, pH 5.5), 3 mM of pNPP and 5-10 μg of protein from the homogenate, in a final volume of 325 μL. After 10 min at 37°C, the reaction was stopped by adding 1 mL of 0.2 M NaOH. The activity was expressed in nano moles of p-nitrophenol (pNP) formed/mg of protein/min, based on an extinction coefficient of 18,400 M⁻¹ cm⁻¹ at 410 nm.

2.6. ACC deaminase activity

ACC deaminase activity was assayed according to a modified method of [22,23] which measures the amount of α-ketobutyrate produced upon the hydrolysis of ACC. The number of μmol of α-ketobutyrate produced by this reaction was determined by comparing the absorbance at 540 nm of a sample to a standard curve of α-ketobutyrate ranging between 10 and 200 μmol. A stock solution of 100 mmol L⁻¹ α-ketobutyrate was prepared in 0.1 mol L⁻¹ of Tris–HCl (pH 8.5) and stored at 4°C. Just prior to use, the stock solution was diluted with the same buffer to make 10 mmol L⁻¹ of solution from which a standard concentrations curve was generated. In a series of known α-ketobutyrate concentrations, 2 mL of the 2,4-dinitrophenyl-hydrazine reagent (0.2% 2, 4-dinitrophenyl-hydrazine in 2 mol L⁻¹ HCl) was added, the contents were vortexed and incubated at 30°C for 30 min, during which the α-ketobutyrate was derivatized as aphenylhydrazine. The color of phenyl hydrazine was developed by the addition of 2 mL, 2 mol L⁻¹ of NaOH, the absorbance of the mixture was measured after mixing by using spectrophotometer at 540 nm.

For determining ACC deaminase activity, endophytes strains were grown in rich Tryptic Soy Broth medium (TSB) for 18 h at 28°C. The cells were then harvested by centrifugation, washed with 0.1 M Tris–HCl (pH 7.5), and incubated for another 18 h in minimal medium containing 3 mM ACC as the sole source of nitrogen. The bacterial cells were collected by centrifugation [20] and suspended in 5 mL of 0.1 mol L⁻¹ of Tris–HCl, pH 7.6, and transferred to microcentrifuge tube. The contents of the tubes were centrifuged at 16,000 rpm for 5 min and supernatant was removed. The pellets were suspended in 2 mL 0.1 mol L⁻¹ Tris HCl, pH 8.5. Thirty microliters of toluene was added to the cell suspension and vortexed for 30 s. 200 μL of the toluenized cells were placed in a fresh microcentrifuge tube, 20 μL of 0.5 mol L⁻¹ ACC was added to the suspension, vortexed, and then incubated at 30°C for 15 min, following the addition of 1 mL of 0.56 mol L⁻¹ HCl, the mixture was vortexed and centrifuged for 5 min at 13,000 rpm at room temperature. 2 mL of the supernatant was vortexed together with 1 mL of 0.56 mol L⁻¹ HCl. Thereupon, 2 mL of the 2,4-dinitrophenylhydrazine reagent (0.2% 2, 4-dinitrophenylhydrazine in 2 mol L⁻¹ HCl) was added to the glass tube, and the contents were vortexed and then incubated at 30°C for 30 min. Following the addition and mixing of 2 mL of 2 mol L⁻¹ of NaOH, the absorbance of the mixture was measured by using spectrophotometer at 540 nm [23]. The cell suspension without ACC was used as negative control and with (NH₄)₂SO₄ (0.2% w/v) as positive control.

2.7. Effect of endophyte inoculation on tomato plant growth

Among endophytic bacterial strains, only B. subtilis showed highest IAA production capacity. Therefore, to assess its potential in plant growth regulation, the bacterial strain was applied on tomato plants. Briefly, bacteria culture suspension was incubated for 3 days at 30°C in a shaking incubator at 200 rpm to an estimated cell density of 10⁶ CFU/mL. Tomato (Solanum lycopersicum cv.) seeds (Seminis Korea Co. Korea) were surface sterilized with NaOCl (5%) for 10 min and thoroughly rinsed with autoclaved distilled water. Seeds were sown in autoclaved soil (peat moss (12–18%), perlite (8–12%), coco-peat (62–68%) and zeolite (6–8%), while the macro-nutrients present were as follows: NH₄⁺~90 mg/L, NO₃⁻~205 mg/L, P₂O₅~350 mg/L, and K₂O~100 mg/L) plastic pots under controlled greenhouse conditions at 28 ± 2°C. Tomato seedlings were treated with 5 mL of bacterial suspension 2 weeks after sowing and the growth attributes i.e. shoot length, shoot and root fresh weight, shoot and root dry weight, and chlorophyll contents were recorded after 21 d of treatment. The experiment comprised three treatments and four replicates. Each replicate consists of 24 plants. Distilled water and NB media were used as negative and positive controls respectively during the experiment. Photosynthetic pigments were extracted from leaves of Tomato plants ground with 80% acetone. The chlorophylls and carotenoid were estimated according to the method of Lichtenthaler. The absorbance for chlorophylls a and b and carotenoid was recorded at 663, 645, and 470 nm, respectively. Chlorophyll content was calculated using the following formulae:

Chlorophyll a (mg/g FW) = [(12.7 × A₆₆₃) − (2.69 × A₆₄₅)] / 1000 × W] × V

Chlorophyll b (mg/g FW) = [(22.9 × A₆₄₅) − (4.68 × A₆₆₃)] / 1000 × W] × V

where W is the fresh weight and V is the extraction volume [19].

2.8. Statistical analysis

The data were analyzed statistically for standard deviation and error by using GraphPad Prism (Ver 5.0; CA USA). The mean values were compared using Duncan’s multiple range tests at p < 0.05 (SAS, Cary, NC, USA).
3. Results and discussion

3.1. Isolation of endophytic bacteria from the bark of M. peregrina

We isolated five endophytic bacterial strains from the bark of M. peregrina. The isolates were grouped upon morphological analysis such colony shape, size, color, pattern and texture. The five isolates were subjected to DNA extraction, PCR amplification, sequencing and phylogenetic analysis using universal primers for 16S rRNA region. Phylogenetic analysis of the bacterial strain was performed and a neighbor joining (NJ) tree was made using MEGA 6.0. Consensus tree was constructed using reference and homologues sequences aligned with 16S rRNA sequence (statistical support of 1000 bootstrap replications; Supplementary information). Results of BLASTn search (National Center of Biotechnology Information — NCBI) revealed that the sequences of isolated endophytic bacteria from M. peregrina had ~90% sequence homology. The results showed that among 5 endophytic bacteria, 2 strains were from Sphingomonas sp.; 2 strains from Bacillus and 1 from Methyllobacterium genus (Table 1). The sequence data was submitted to Gen-Bank with the accession number previously. Number of the few endophytic microbes isolated from M. peregrina. Among reported endophytic micro flora Aspergillus caesipitosus LK12 and Phoma sp. LK13 [19], however, there is no report of endophytic bacterial diversity from this medicinal tree.

3.2. ACC deaminase production by endophytic bacteria

Enzymes such as ACC (1-aminocyclopropane-1-carboxylate) deaminase are produced by the various bacterial strains and its production is mostly related to free-living soil bacteria and some fungi. However, more recently, it has been reported from rhizobacteria and endophytic bacteria [10,11,12,13]. ACC deaminase-producing bacteria promote plant growth under stress conditions and also playing a very important role in the process of nodulation, specific to plant species. According to Onofre-Lemus et al. [24] the bacterial enzyme ACC (1-aminocyclopropane-1-carboxylate) deaminase promotes plant growth by lowering plant ethylene levels. ACC deaminase was observed in PGPEB strains by their growth on Dworking and Foster (DF) minimal medium containing ACC, MPB2.1 ACC deaminase was observed in PGPEB strains by their growth on DF minimal medium containing ACC, MPB2.1. The growth of the MPB2.1 strain showed the highest value in ACC deaminase. Different letters in the column show that values are significantly different (p < 0.05) from each other as evaluated from DMRT (Duncan’s Multiple Range Test) test.

Fig. 1. ACC deaminase by the isolated endophytes in (DF) minimal medium containing ACC MPB2.1 strain had the highest value in ACC deaminase. Different letters in the column show that values are significantly different (p < 0.05) from each other as evaluated from DMRT (Duncan’s Multiple Range Test) test.

3.3. Phosphate solubilization and acid phosphatase potentials of Endophyte

The isolated endophytic culture filtrates from M. peregrina were screened for phosphate solubilization and acid phosphatase potentials. The screening results showed that phosphate solubilization by MPB 2.1 strain produced a clear zone around the colonies which shows the highest phosphate solubilization potential on NBRIP medium. Various researchers have documented acid phosphatase activity in the different strains of Bacillus species. The previous study showed that Enterobacter asburiae and Bacillus cereus enhanced phosphate solubilization (165–415 g L−1) upon 4 d of growth [17,18]. In our results the growth of MPB 2.1 showed the phosphate clearing zone of 56.716 mm after 5 d of growth. Similarly the research work of Almoneafy et al. [28] showed that three strains of Subtillus D29, Am1 and H8 have capability to solubilize phosphate. The results of Rodríguez and Fraga [25] showed that Pseudomonas striata and Bacillus polymyxa solubilized 156 and 116 mg P L−1, respectively. In our results MPB 2.1 strain showed prominent results for phosphate solubilization (8.4 mM mg−1 Min−1) followed by MPS1.2 (6.3 mM mg−1 Min−1) and MPB6.1 (5.9 mM mg−1 Min−1) Fig. 2. In this way the present results are in conformity with the previous data of phosphate solubilization.

3.4. IAA quantification from endophytic bacteria

Plants regulate its developmental process by producing phytohormones in its various parts, while endophytic microbial flora

Table 1

Endophytic bacteria isolated and identified from the bark of M. peregrina and their potential to produce IAA using spectrophotometer.

<table>
<thead>
<tr>
<th>Code name</th>
<th>Endophytic bacteria</th>
<th>μM in 50 mL broth</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPB 4.1</td>
<td>Sphingomonas sp. LK18</td>
<td>35.19 ± 0.38b</td>
</tr>
<tr>
<td>MPS 21</td>
<td>Methyllobacterium radiotolerans LK17</td>
<td>31.13 ± 0.48c</td>
</tr>
<tr>
<td>MPB 2.1</td>
<td>B. subtilis LK14</td>
<td>165.53 ± 1.01a</td>
</tr>
<tr>
<td>MPB 6.1</td>
<td>B. subtilis LK15</td>
<td>32.01 ± 0.81c</td>
</tr>
<tr>
<td>MPS 1.2</td>
<td>Sphingomonas sp. LK16</td>
<td>25.46 ± 0.13cd</td>
</tr>
</tbody>
</table>

± shows the standard error of three replications. Different letters in the column show that values are significantly different (p < 0.05) from each other as evaluated from DMRT test.

Fig. 2. Acid phosphatase activity of different endophytic bacterial strains of M. peregrina. Different letters in the column show that values are significantly different (p < 0.05) from each other as evaluated from DMRT test.
also contributing in the production of these phytohormones. It is now a matter-of-fact that IAA can be produced by variety of endophytes [9,15]. Similarly, in our study endophytic bacteria *B. subtilis* LK14 was isolated from *M. peregrina*, and were applied on tomato plants. As IAA plays a vital role in plant development and at the same time its additional supply can support the host in stress conditions such drought [22] and pathogenic attacks [14].

There are various types of rhizospheric bacteria which are documented for the production of IAA, exploit different IAA biosynthesis pathways and single bacterial strain sometimes encompassing more than one pathway [14,15]. With reference to earlier data, IAA production by bacteria can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability [1]. In our results the MPB 2.1 strain of *B. subtilis* LK14 showed the highest results (165.53 μM in 50 mL culture broth) for IAA production, while *B. subtilis* LK15 produced a little amount (32.01 μM in 50 mL culture broth) as compared to *B. subtilis* LK14 (Fig. 3; Table 1). Similarly, the study of Fuentes-Ramirez et al. [19] showed that various strains of *Acetobacter diazotrophicus* produced IAA in defined culture medium and their HPLC analyses revealed that *A. diazotrophicus* strains produced from 0.14 to 2.42 μg IAA mL⁻¹ in culture medium.

In our studies, for the estimation of IAA in culture broth sophisticated methodologies were used as Colorimetric assay in addition with L-tryptophan which favored the high amount of IAA. Then PGPEB IAA was quantified by using Waters UPLC system and further verification and quantification of IAA was brought about by LC-MS/MS. Our results of mass spectroscopy showed that the strains of *B. subtilis* LK14 give a high value of IAA on 9th day in their culture medium which is 165.53 mMol/50 mL LB broth (Fig. 4). The greater amount of IAA produced by PGPEB in LB broth is because of L-tryptophan, which is also confirmed from the results of Idris et al. [5] revealed that IAA secretion can be increased by providing tryptophan. In this way, the Inoculation of IAA producing bacteria...
induce the development of tomato plants. In the present study IAA production PGPEB are in agreement with previous reports.

The two different endophytic species of *Sphingomonas* LK18 and LK16 produce 35.19 and 25.46 μM in 50 mL broth respectively while, *Pseudomonas aeruginosa* LK17 encodes for 31.13 μM in 50 mL culture broth. By comparing the IAA amount produced by endophytic *B. subtilis* LK14 and *B. subtilis* LK15, the former strain produce 52% greater amount of IAA in LB broth medium.

While the results of mass spectroscopy revealed that some strains of *B. subtilis* give a high value on 9th d in their culture medium which is 14.02 μM/50 mL (Fig. 4). According to Srinivasan et al. [27] the different strains of *Bacillus* showed significant difference in the amount of IAA varying from 0.40 to 4.88 μg/mL, for the quantification of IAA they used thin-layer chromatography, high-performance liquid chromatography, gas chromatography, mass spectrometry, and the *Avena Coleoptile bioassay*. In our results the greater amount of IAA produced by these endophytes in 20 mL LB broth is because of L-tryptophan which was incubated at 30 ± 2°C in shaking incubator at 200 rpm for one week. Auxin production by *Bacillus* spp. in L-broth medium supplemented with 1000 μg/mL L-tryptophan ranges from 0.60 to 3.0 μg IAA ML-1 as revealed by gas chromatography and mass spectrometric (GC-MS) analysis [1]. While the results of Idris et al. [5] also showed that IAA secretion can be increased up to fivefold by providing 5 mM of tryptophan in the culture medium.

3.5. Effect on the tomato plant growth

The screening results showed that application of endophytic bacterial strain MPB 2.1 culture significantly enhanced the development of tomato seedling. The Shoot length, Shoot weight, Root length, and chlorophyll contents were significantly boosted by the application of MPB 2.1 culture (Fig. 5a). The length of shoot and root were paralleled with control and it is deciphered from the results that the tomato plantlet treated with endophytic bacterial strain MPB 2.1 showed upsurge in length 2.5 cm and 3.5 cm respectively. Almoneafy et al. [28] noted a positive correlation (0.777 and 0.686) between the tomato plantlet treated with endophytic bacterial strain MPB 2.1 and 470 nm respectively. Almoneafy et al. [28] noted a positive correlation (0.777 and 0.686) between the tomato plantlet treated with endophytic bacterial strain MPB 2.1 and 470 nm respectively. Almoneafy et al. [28] noted a positive correlation (0.777 and 0.686) between the tomato plantlet treated with endophytic bacterial strain MPB 2.1 and 470 nm respectively. Almoneafy et al. [28] noted a positive correlation (0.777 and 0.686) between the tomato plantlet treated with endophytic bacterial strain MPB 2.1 and 470 nm respectively. Almoneafy et al. [28] noted a positive correlation (0.777 and 0.686) between the tomato plantlet treated with endophytic bacterial strain MPB 2.1 and 470 nm respectively.

Chlorophylls (a and b) and carotenoid were recorded at 663, 645, and 470 nm, respectively. As tomato seedlings were treated with bacterial suspension and chlorophyll contents were recorded after three weeks of the treatment. The chlorophyll contents were significantly enhanced by the application of MPB 2.1 culture after 9th d. By comparing the results chlorophyll a contents showed an increase of 0.4 mg/g while 0.34 mg/g in chlorophyll b contents were recorded. In this way, the total carotenoid presented the pragmatic results with the chlorophyll contents of tomato plants. Different letters show that values are significantly different (p < 0.05) from each other as evaluated from DMRT test.

In conclusion, endophytes are the microbial flora which are residing asymptomatically in all plants and enhancing host plant’s nutrient uptake and helping host to counteract adverse effects of biotic and abiotic stresses by producing a variety of secondary metabolites. In present study various endophytic bacterial strains were isolated from the bark of *M. peregrine* and were evaluated for their potential in producing IAA, phosphate solubilization and ACC deaminase activity. Advance and sophisticated strategies were used for the evaluation of all these activities. And finally, the plant growth promoting effects of these isolated strains were tested on tomato plants. Among all tested strains the endophytic *B. subtilis* LK14 showed prominent results and is capable to be utilized as PGP in crop plants.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ejbt.2016.02.001.

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Conflict of interest
The authors declare no conflict of interest.

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