Research Article

Open Access

Mahmoud Soliman Abdel-Hamid, Amr Fouda*, Hesham Kamal Abo El-Ela, Abbas A. El-Ghamry, Saad El-Din Hassan*

Plant growth-promoting properties of bacterial endophytes isolated from roots of *Thymus vulgaris* L. and investigate their role as biofertilizers to enhance the essential oil contents

<https://doi.org/10.1515/bmc-2021-0019>

received November 26, 2021; accepted December 19, 2021.

Abstract: The main objective of the current study was to improve the essential oil contents of *Thymus vulgaris* L. using bio-inoculation with bacterial endophytes. Therefore, out of fourteen endophytic bacterial isolates obtained from roots of *T. vulgaris*, five isolates were selected based on the highest nitrogen-fixation and phosphate solubilization activity and identified as: *Bacillus haynesii* T9r, *Citrobacter farmeri* T10r, *Bacillus licheniformis* T11r, *Bacillus velezensis* T12r, and *Bacillus velezensis* T13r. These five strains have been recorded as ammonia, hydrogen cyanide (HCN), siderophores, and indole-3-acetic acid (IAA) producers. These strains have the efficacy to fix-nitrogen by reduction of acetylene with values of 82.133 ± 1.4 – 346.6 ± 1.4 n-mole- C_2H_4 / ml/24 h. The IAA, gibberellic acid, abscisic acid, benzyl, kinten, and ziaten production were confirmed using HPLC. Two strains of T11r and T13r showed the highest plant growth-promoting properties and were selected for bio-inoculation of *T. vulgaris* individually or in a consortium with different mineral fertilization doses (0, 50, 75, and 100%) under field conditions. The highest growth performance was attained with the endophytic consortium (T11r+T13r) in the presence of 100% mineral fertilization. The GC-MS analysis of thyme oil contents showed the presence of

23 various compounds with varying percentages and the thymol fraction represented the highest percentages (39.1%) in the presence of the bacterial consortium.

Keywords: Thyme; Plant growth-promoting; Bacterial endophytes; Oil fraction; Biofertilizations.

Introduction

Thymus vulgaris L. or thyme (Family: *Lamiaceae*) is a flowering plant and is considered indigenous in Northern Africa, the Mediterranean, and various parts of Asia. Thyme plants are widely cultivated in Africa, for instance: Morocco, Egypt, Libya, Algeria, Cameroon, Tunisia, South Africa, and Nigeria [1]. The thyme plant is a common flavoring agent which has various importance in herbal medicine for the treatment of fever, cough, cold, diabetes, and chest infections. Also, it can be used to treat digestive disorders, calmate for throat infections due to its antibiotic, antiseptic, and antifungal characteristics [2]. Moreover, the thyme plant has been successfully displayed in the treatment of several intestinal infections including ascarids, hookworms, pathogenic Gram-positive and Gram-negative bacteria, uni- and multi-cellular fungi [3]. The importance of thyme plants is mainly related to their essential oils, which have antiviral, antimicrobial, and antioxidant activities along with their carminative actions [4]. According to European Pharmacopoeia, the minimum essential oil contents in the thyme plant is 12 ml kg⁻¹, and it contains mainly six chemotypes, geraniol, γ -terpineol, linalool, thymol, carvacrol, and thujan-4-ol [5]. Therefore, these findings raise the great necessity to improve the development and growth of the thyme plants for their essential oil contents.

Recently, the endophytic microbes and their metabolites have been integrated into various biomedical

*Corresponding authors: Amr Fouda, Soil Fertility and Microbiology Department, Desert Research Center, El-Matara, Cairo, Egypt, E-mail: amr_fh83@azhar.edu.eg; Saad El-Din Hassan, Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo 11884, Egypt, E-mail: saad.el-din.hassan@umontreal.ca
Mahmoud Soliman Abdel-Hamid, Hesham Kamal Abo El-Ela, Soil Fertility and Microbiology Department, Desert Research Center, El-Matara, Cairo, Egypt
Abbas A. El-Ghamry, Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo 11884, Egypt

and biotechnological applications [6, 7]. Bacterial endophytes are considered the major endophytic group consisting of approximately 200 genera belonging to 16 phyla, and most of them associated with Proteobacteria, Actinobacteria, and Firmicutes [8]. Although the existence of the bacterial endophytes in plants is variable and in some cases, it is in transit, it has the efficacy to make various physiological and biochemical changes that regulate plant growth and performance under different environmental conditions [9]. Bacterial endophytes act as plant growth-promoting bacteria (PGPB) that can modulate plant growth through different mechanisms such as the production of phytohormones (indole-3-acetic acid, gibberellic acid, cytokinin, etc.), secretion of lytic enzymes, phosphate solubilization, nitrogen fixation, siderophores production, hydrogen cyanide (HCN), and ammonia production [10-12]. Moreover, bacterial endophytes can promote plant growth through the production of ACC deaminase enzymes that control the amount of ethylene in plants and increase the resistance of the plant to phytopathogens [9]. Interestingly, the presence of bacterial endophytes increases the resistance of the plant to various biotic and abiotic stresses [13]. Recently, various bacterial endophytes are used to regulate the growth of different plants such as rice, canola, tomato, mung bean, *Vicia faba*, black pepper, *Phaseolus vulgaris*, and groundnut, to name of few [7, 11, 14].

The involvement of PGPB in the various bio-fertilizing systems started in the 1950s and now occupies approximately 5% of the fertilizer market, the majority of components of them are nitrogen-fixing bacteria [15]. The advantages of biofertilizers containing PGPB to agricultural sectors are improving soil fertility, providing plants with essential nutrition, enhancing the plant resistance against both biotic and abiotic stresses [15]. The main challenge of PGPB utilization for agronomic practice is the low efficiency of PGPB isolated from certain plant species to improve plant growth and performance in a wide range of other plant species [16]. Another complexity is the low stability of plant colonizing with PGPB under different environmental conditions along with the host plant specificity [17]. Therefore, the highest activity of PGPB can be achieved when these bacteria are applied to the same isolated plant and under the same conditions.

The main purpose of the current study is to improve the growth of *T. vulgaris* as oil-producing plants using bacterial endophytes. To accomplish this, bacterial endophytes were isolated from the healthy root of *T. vulgaris* collected from North Sinai. Five bacterial endophytes were selected based on their high potency

for nitrogen fixation and phosphate solubilization. The plant growth-promoting properties of selected bacterial isolates include quantitative nitrogen fixation (using acetylene reduction), quantitative phosphate solubilization, ammonia production, hydrolytic enzyme activity, siderophores and HCN production, and synthesis of various phytohormones (IAA, gibberellic acid, abscisic acid, benzyl, kintan, and ziaten) were investigated. Finally, a field experiment was conducted to evaluate the effect of an individual or a consortium of the most two potent strains (based on higher plant growth-promoting properties) on the growth performance and essential oil contents of *T. vulgaris* in presence of different concentrations of mineral fertilization (0, 50, 75, and 100%).

Material and method

Plant sample collection

The root of the medicinal plant *Thymus vulgaris* L. (family Lamiaceae) was collected from Balouza Research Station, North Sinai, Egypt (31°00'20.6"N 32°33'46.1"E). Four individual plants were collected per site. The collected plant samples were kept in sterile polyethylene bags and transported to the laboratory on the same day and subjected to isolation procedures within a short period of collection [18]. The plant identification was tentatively recorded in the field with help of a local Balouza Research Station agricultural engineer, and the botanical identification was carried out at the herbarium unit of Desert Research Center.

Isolation of endophytic bacterial strains

The collected roots samples of *T. vulgaris* were subjected to surface sterilization as follows: the plant samples were washed with tap water to exclude the soil debris and epiphytes adhered. After that, the plant samples were washed with sterilized distilled water for 60 sec. followed by 2.5% sodium hypochlorite for four min. and ethyl alcohol (70%, v/v) for 30 sec. Finally, the surface-sterilized plant samples were immersed in sterilized distilled water for 60 sec. To check the successful surface sterilization, approximately one ml of final distilled water was streaked onto Czapek Dox agar, nutrient agar, and starch nitrate agar for the growth of fungi, bacteria, and actinomycetes respectively. The success of surface sterilization was checked by the absence of microbial growth on the surface

of the previous inoculated media after an appropriate incubation period [19].

The sterilized roots were cut into small fragments (5mm) under aseptic conditions. Approximately twenty fragments (20 fragments per sterilized root) were loaded on the surface of four nutrient agar Petri dishes (8 cm, 5 fragment/plate) supplemented with 25 $\mu\text{g ml}^{-1}$ nystatin (as antifungal). The loaded plates are incubated at $30 \pm 2^\circ\text{C}$ and checked daily for bacterial growth. The appeared bacterial colonies from sterilized fragments were picked-up and re-streaked on the same media to confirm the purity. The purified colonies were inoculated into nutrient agar slants and kept at 4°C for further investigation. All obtained endophytic bacterial isolates were subjected to primary identification based on morphological, physiological, and biochemical tests according to standard keys [20, 21].

Selection of the most potent endophytic bacterial stains

The selection of the most potent endophytic bacterial isolates was achieved based on their efficacy in qualitatively fixing nitrogen and solubilizing phosphate. The endophytic bacterial isolates were inoculated on nitrogen-deficient Jensen's agar medium (containing g L^{-1} : sucrose, 20; K_2PO_4 , 1; MgSO_4 , 0.5; NaCl, 0.5; Fe_2SO_4 , 0.1; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.005; CaCO_3 , 2; Agar, 15; 1.0 L distilled H_2O) supplemented with bromothymol blue as an indicator. The positive nitrogen-fixing endophytic bacterial isolates were selected based on their efficacy to change media color to blue color [22].

The primary screening to investigate the efficacy of endophytic bacterial strains to phosphate solubilization was achieved using Pikovskaya agar media (containing g L^{-1} : glucose, 10; $\text{Ca}_3(\text{PO}_4)_2$, 5; $(\text{NH}_4)_2\text{SO}_4$, 0.5; NaCl, 0.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; KCl, 0.2; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002; yeast extract, 0.5; $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$, 0.002; agar, 15; 1.0 L distilled H_2O). Briefly, the endophytic bacterial isolate was inoculated as a spot on the center of the Pikovskaya agar plate and incubated at $35 \pm 2^\circ\text{C}$ for 48 h. The positive isolates for phosphate solubilizing were recorded as the diameter of the clear zone formed around each bacterial growth [23]. The experiment was achieved in triplicates.

Identification of the most potent endophytic bacterial isolates

The endophytic bacterial isolates that showed a positive result for nitrogen fixation and phosphate solubilization

(T9r, T10r, T11r, T12r, and T13r) were subjected to molecular identification based on amplification and sequencing of the 16S rRNA gene. The genomic DNA was extracted according to the modified method [24]. Briefly, a separate bacterial colony was picked up by a sterile toothpick and suspended in 50 μl of sterilized deionized water. The cell suspension was put in a water bath for 10 min at 97°C , after that, the suspension was centrifuged for 10 min at 15000 rpm to recover the upper layer that contains the DNA. The intensity of DNA in the collected layer was calculated by measuring its absorbance at 260 nm by UV-spectrophotometer. A bacterial universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') were used to PCR amplification of the 16S rDNA fragment. The PCR tube containing PCR buffer (1 x), MgCl_2 (0.5 mM), Tag DNA polymerase (2.5 U, QIAGEN Inc.), Deoxynucleoside triphosphate (dNTP, 0.25 mM), universal primer (0.5 μM), and bacterial DNA (5 ng/ μl). The PCR cycling conditions were 94°C for three minutes, followed by 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, and final extension at 72°C for ten minutes. The forward and reverse sequencing for PCR products was achieved using Applied Biosystems 3730xl DNA Analyzer technology at Sigma company, Cairo, Egypt.

The obtained sequences were analyzed using BLAST as compared with those deposited in the GenBank database. Multiple sequence alignment was performed using ClustalX 1.8 software package and a phylogenetic tree was constructed by the neighbor-joining method using MEGA (Version 6.1) software. The confidence level of each branch (1000 repeats) was tested by bootstrap analysis [25].

Characterization of bacterial endophytes as plant growth-promoting

Quantitative nitrogen fixation using acetylene reduction assay

The efficacy of five selected endophytic bacterial strains to fixing nitrogen was investigated quantitatively through detecting the nitrogenase activity by the acetylene reduction method. In this method, each endophytic bacterial strain was inoculated in a test tube closed by a rubber stopper and containing 10 ml of nitrogen-free Jensen's semi-solid medium and incubated for 72 h at $33 \pm 2^\circ\text{C}$. After that, approximately, one ml of acetylene gas was injected into the air of the headspace and incubated for 24 h at $33 \pm 2^\circ\text{C}$. At the end of the incubation period,

one ml of gas sample existing in the tube headspace was assayed for ethylene production (C_2H_4) using Hewlett-Packard 5890 gas chromatography Series 2 plus [26, 27]. The nitrogenase activity was assessed as a mol n of C_2H_4 /ml/24 h. The experiment was performed in triplicate.

Quantitative phosphate solubilizing assay

The phosphate solubilizations were analyzed quantitatively by measuring pH values and P concentration at interval times (2 - 10 days). Briefly, Pikovskaya's broth medium supplemented with 0.5% $Ca_3(PO_4)_2$ was inoculated with endophytic bacterial strain and incubated at $33 \pm 2^\circ C$ for 10 days on a rotary shaker at 180 rpm. Five ml samples were taken daily and centrifuged at 10,000 rpm for 10 min. The phosphomolybdate method was used for the determination of available soluble phosphate in the culture supernatant using a spectrophotometer (Jenway 6305 UV) measuring at O.D. 700 nm. The concentration of P was calculated from the slope of the standard curve of P. Also, the pH of the broth medium was measured daily with a digital pH meter [13].

Ammonia production

The ability of endophytic bacterial strains for producing ammonia was assessed in peptone water broth. Each bacterial strain was inoculated into tubes containing 10 ml peptone water and incubated at $33 \pm 2^\circ C$ for 48 h. At the end of the incubation period, approximately 0.5 ml of Nessler's reagent was added to the culture broth media. The development of yellow color was recorded as +, ++, +++ based on the color intensity [28].

Extracellular Enzymatic Activities

The production of extracellular enzymes (amylase, gelatinase, cellulase, and catalase) of the endophytic bacterial strains was investigated by inoculating the isolates in a mineral salt (MS) agar media (containing g L^{-1} : $NaNO_3$, 5; KH_2PO_4 , 1; K_2HPO_4 , 2; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.1; $CaCl_2$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.02; agar, 15; distilled H_2O , 1L) supplemented with specific additives, depending on the enzyme being tested. The MS agar media without bacterial inoculation was running with the experiment as a negative control. After incubation at $33 \pm 2^\circ C$ for 24 h, specific reagents were added and the size of the clear zone (mm) surrounding the bacterial colony was measured,

indicating extracellular enzymatic activities. All assays were performed in triplicates.

For amylolytic and cellulolytic activities, the bacterial strains were inoculated onto MS agar medium supplemented with 1% soluble starch and 1% carboxymethylcellulose (CMC) respectively. After an incubation period, the plates were flooded with 1% iodine.

For gelatinase activity, the MS agar medium containing 1% gelatin was used to determine the proteolytic activity of the bacteria. After an incubation period, gelatin degradation was checked using acidic mercuric chloride as an indicator [19].

For chitinase, the bacterial endophytes were spotted on MS agar media containing 1% of colloidal chitin formed according to Wen *et al.* [29]. The positive chitinase results were recorded as the diameter of the clear zone around bacterial growth.

For catalase activity, the pure bacterial colony from young culture (18-24h.) was put into a glass slide and mixed with a few drops of 3% H_2O_2 . The appearance of oxygen bubbles indicates the presence of catalase activity [30].

Siderophore production

Secretion of siderophore was qualitatively analyzed using king's B agar medium (containing g L^{-1} : proteose peptone, 20; K_2HPO_4 , 1.5; $MgSO_4$, 1.5; Agar, 15, 1.0 L distilled H_2O ; pH, 7.2 ± 0.2) supplemented with chrome azurol S (CAS), Fe^{3+} solution, and hexadecyltrimethylammonium bromide (HDTMA) as an indicator. The indicator preparation was achieved as follows: 60.5 mg of CAS was dissolved in 50 ml distilled H_2O , followed by mixing with 10 ml of Fe^{3+} solution (1 mmol L^{-1} dissolved in 10 mmol L^{-1} of HCl). Under the stirring condition, the previous mixture was dropped wisely added to the HDTMA solution (72.9 mg of HDTMA dissolved in 40 ml distilled H_2O). The prepared indicator was added to King's B media with a ratio of 1:15. The fresh bacterial culture was spotted onto a king's B agar plate and incubated at $28^\circ C$ for 72 h. The appearance of an orange halo around the endophytic bacterial growth indicates a positive siderophore production [14].

HCN production

The purified endophytic bacterial isolate was tested for HCN production through inoculation on king's B agar medium amended with glycine [31]. After that, a Whatman No. 1 filter paper was soaked in a solution of (2% sodium

carbonate mixed with 0.05% picric acid solution) and put on the lid of inoculated king's B petri dish. The plates were sealed with parafilm and incubated at $28 \pm 2^\circ\text{C}$ for 48 h. The change in the color of the filter paper from deep yellow to reddish-brown color indicates a positive result for HCN production.

IAA production

The most potent endophytic bacterial isolates were screened for qualitative IAA production. Briefly, the bacterial isolates are inoculated into nutrient broth media amended with different concentrations of tryptophan (0, 1, 2, and 5 mg ml⁻¹) and incubated at $33 \pm 2^\circ\text{C}$ for 15 days. At the end of the incubation period, the inoculated culture was centrifuged at 3000 rpm for 30 min. After that, two ml of the obtained supernatant was mixed with 2 drops of orthophosphoric acid and 4 ml of Salkowski's reagent (300 ml concentrated Sulfuric acid: 500 ml distilled water: 15 ml 0.5 M FeCl₃). The development of pink color indicates successful IAA production. The intensity of the formed color was measured at 530 nm using a T60 UV-visible spectrophotometer [32]. The amount of IAA was estimated via a standard IAA graph to detect the best tryptophan concentration for IAA production.

For quantitative IAA assay, the endophytic bacterial isolates were inoculated into nutrient broth media supplemented with 5 mg ml⁻¹ of tryptophan (the best concentration of tryptophan for IAA production based on the qualitative assay) and incubated at $33 \pm 2^\circ\text{C}$ for 14 days. Approximately two ml of inoculated culture was withdrawn after the 2nd day up to the 14th day with 2 days interval and centrifuged at 3000 rpm for 30 minutes. One ml of the supernatant was mixed with 1 drop of orthophosphoric acid and 2 ml of Salkowski's reagent. The intensity of development of pink color was measured at 530 nm using a T60 UV-visible spectrophotometer. The amount of IAA produced was estimated by the standard IAA graph to detect the best days for IAA production.

Detection of other phytohormones.

The efficacy of bacterial isolates to secrete acid and alkali side of phytohormones (IAA, gibberellic acid (GA₃), abscisic acid (ABA), benzyl, kinten, and ziaten) were evaluated by high-performance liquid chromatography (HPLC). The bacterial isolates were grown in Ashby's broth medium (containing g L⁻¹: mannitol, 10; sucrose, 5; K₂HPO₄, 0.5; NaCl, 0.2; MgSO₄·7H₂O, 0.2; CaCO₃, 0.5; CaSO₄,

0.1; FeCl₃, 0.001; MnSO₄·2H₂O, 0.001; Na₂MoO₄·2H₂O, 0.002; 1.0 L distilled H₂O) and incubated at $30 \pm 2^\circ\text{C}$ for 7 days. Then the ability of the growing cultures to produce phytohormones is well detected quantitatively by HPLC as previously reported [33].

Field experiment

The seeds of *Thymus vulgaris* (local species) were purchased from the farm of the Faculty of Pharmacy, Cairo University, Cairo, Egypt. The bacterial isolates T11r and T13r were selected as the most potent based on previous plant growth-promoting activities to investigate their efficacy on the growth trail and oil content of *T. vulgaris* under filed experiment conditions. The experiment was carried out at Alqantra Sharq Research Station, North Sinai, Egypt (GPS: 31°00'21.6" N and 32°33'48.1" E) using a completely randomized design that contains four blocks, each block containing four plots based on different treatments. The texture and chemical constituents of soil used in the field experiment are recorded in table 1. Before the experiment, the soil was well-milled and thoroughly mixed with organic fertilizer (20 m³/feddan). Moreover, the mineral fertilization (MF) is designated as calcium super-phosphate (containing 15.5% P₂O₅), calcium ammonium nitrate (containing 33.3% N), and Potassium sulfate (containing 48% K₂O) were added twice, immediately after planting and after 65 days. The mineral fertilization doses were added as follows: 100%, 75%, 50%, and 0.0% with amounts (150, 112.5, 75 and 0 kg/feddan) for calcium ammonium nitrate, (300, 225, 150 and 0 kg/feddan) for calcium ammonium nitrate, and (100, 75, 50 and 0 kg/feddan) for Potassium sulphate according to standard of Egyptian Agriculture Ministry. The addition of plant growth-promoting bacteria as biofertilizers (BF) was applied as follows: immerse plant seeds in bacterial culture for one hour before planting, the bacterial cell was suspended in saline solution and added to soil beside the plant root at 65 and 140 days of planting. The following treatment was conducted: A1: seeds planting on soil with 100% MF without BF; A2: seeds planting on soil with 75% MF without BF; A3: seeds planting on soil with 50% MF without BF; A4: seeds planting on soil with 0.0% MF without BF; A5: seeds treated with BF T11r and planting on soil with 100% MF; A6: seeds treated with BF T11r and planting on soil with 75% MF; A7: seeds treated with BF T11r and planting on soil with 50% MF; A8: seeds treated with BF T11r and planting on soil with 0.0% MF; A9: seeds treated with BF T13r and planting on soil with 100% MF; A10: seeds treated with BF T13r and planting on soil with

Table 1: Physical and chemical characters of soil used in the current study in a field experiment.

Soil analysis	
Characters	measurements
Physical characters	
Coarse sand (%)	56.58
Fine Sand (%)	31.04
Silt (%)	7.93
Clay (%)	4.45
Soil texture	Loamy sand
Chemical characters	
pH	7.86
EC (Ds/m)	0.29
O.M. (%)	0.59
O.C. (%)	0.34
T.N. (%)	0.3
C/N ratio	11.3
Soluble Cations (meq L⁻¹)	
Ca ⁺⁺	4.9
Mg ⁺⁺	2.2
Na ⁺	8.3
K ⁺	0.9
Soluble anions (meq L⁻¹)	
HCO ₃ ⁻	3.1
Cl ⁻	6.7
SO ₄ ⁻⁻	5.7

75% MF; A11: seeds treated with BF T13r and planting on soil with 50% MF; A12: seeds treated with BF T13r and planting on soil with 0.0% MF; A13: seeds treated with consortium BF (T11r+T13r) and planting on soil with 100% MF; A14: seeds treated with consortium BF (T11r+T13r) and planting on soil with 75% MF; A15: seeds treated with consortium BF (T11r+T13r) and planting on soil with 50% MF; A16: seeds treated with consortium BF (T11r+T13r) and planting on soil with 0.0% MF. All treatments were achieved in triplicates. The plant was harvested two times, at 65 days and 140 days.

Growth and Vegetative Parameters Measurement

The plant height after two harvests of 65 and 140 days was measured by cm. The shoots of 65 and 140-day-old thymus plants were separated from the plants and measured to detect the fresh weight. The collected shoots have dried in an oven at 70°C until constant weight, then, the dry weights of the shoots were measured.

Oil extraction

The extraction of oil was achieved for 100 g of *T. vulgaris* dry weight (collected from first and second harvest) using the oil distillation method according to British Pharmacopoeia [34]. Briefly, 100 g of the dry weight of the plant was added to a conical flask (1.0 L) and mixed with a sufficient water amount (4 – 5 times of dry weight). A proper oil trap connected to a condenser was attached to the conical flask followed by the addition of water to fill the trap. After that, the flask undergoes heating using an electric hot plate. The distillation process was continued for approximately 2.0-2.5 hours till separate the essential oil from water and no further increase in the content of oil was observed. The amount of the obtained essential oil was calculated in proportion to the plant dry weight used.

Oil Fraction by Gas chromatography-mass spectrometry analysis (GC-MS)

The GC-MS system (Agilent Technologies) was equipped with gas chromatography (7890B) and a mass spectrometer detector (5977A) at Central Laboratories Network, National Research Centre, Cairo, Egypt. Samples were diluted with hexane (1:19, v/v). The GC was equipped with an HP-5MS column (30 m x 0.25 mm internal diameter and 0.25 µm film thickness). Analyses were carried out using helium as the carrier gas at a flow rate of 1.0 ml/min at a split ratio of 1:10, injection volume of 1 µl, and the following temperature program: 40°C for 1 min; rising at 4°C /min to 150°C and held for 6 min; rising at 4°C/min to 210°C and held for 1 min. The injector and detector were held at 280°C and 220°C, respectively. Mass spectra were obtained by electron ionization (EI) at 70 eV; using a spectral range of m/z 50-550 and solvent delay 5 min. Identification of different constituents was determined by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data.

Statistical analysis

Split plot design was designed as a layout for the experiment with three replicates for each treatment. Data were analyzed statistically by a statistical program Sigma Plot v14. Analysis of variance (ANOVA) was used as a tool for calculating the mean difference comparison between the treatments and afterward by Tukey's test (honestly significant difference) at $p \leq 0.05$. Hierarchical two-way cluster analysis was conducted on the data matrix of oil content percentages using the centered method of the standardized data.

Results and discussion

Isolation of bacterial endophytes that inhabit the roots of *Thymus vulgaris* L.

Thymus vulgaris L. plant has medicinal and pharmacological importance including antibacterial, antifungal, antioxidant, antiviral, anti-inflammatory, and insecticidal activities [4]. The endophytic bacteria that inhabit *Thymus* tissues may be contributed to the biological activities of this plant; however, few investigations on the microbial communities of *Thymus vulgaris* have been recorded. In the current study, fourteen bacterial isolates designated as T1r to T14r were obtained from the sterilized root of *T. vulgaris* L. which was collected from Balouza Research Station, North Sinai, Egypt. The obtained endophytic bacterial species were subjected to primary identification based on morphological, physiological, and biochemical tests according to the standard keys. Data showed that the bacterial species were belonging to two phyla, *proteobacteria* (35.7%) and *Firmicutes* (64.2%). The *proteobacteria* species contains alpha (one isolate, T7r), beta (two isolates, T1r and T4r), and gamma (two isolates, T10r and T14r), and were identified as *Agrobacterium* sp., *Burkholderia* sp., *Burkholderia* sp., *Citrobacter* sp., and *Acinetobacter* sp., respectively. Whereas most species of *Firmicutes* were corresponding to *Bacillus* spp. (6 isolates designated as T3r, T8r, T9r, T11r, T12r, and T13r) and *Brevibacillus* spp. (three isolates designated as T2r, T5r, and T6r). Similarly, 117 endophytic bacterial isolates were obtained from leaves, stems, and roots of medicinal plant *T. vulgaris* collected from North Sinai, Egypt [35]. These 117 isolates were belonging to *Firmicutes*, *proteobacteria*, and *Actinobacteria*. In the recent study, the endophytic bacterial community isolated from the medicinal plant *Pulicaria incisa* were belonging to *proteobacteria* (α ,

β , and γ) and *Firmicutes* with percentages of 33.3% and 66.6%, respectively [32].

Selection of the most potent bacterial isolates

Phosphorus and nitrogen are the main nutrients for enhancement of the plant growth. In most cases, these elements are not available for plants [36]. Endophytic bacteria along with nitrogen-fixing and phosphate solubilizing bacteria facilitate nutrient requirement are widely used as biofertilizers [37]. Therefore, in the current study, the selection of the most potent bacterial isolates to be used as a biofertilizer was achieved based on higher nitrogen fixation and phosphate solubilization. Data represented in Table 2 showed that T3r and T8r to T13r had the ability of nitrogen-fixing with varying degrees according to the appearance of blue color on nitrogen-deficient Jensen's agar medium. The highest color intensity was recorded for isolates T9r, T10r, T11r, T12r, and T13r. The obtained data are compatible with those recorded by Abdelshafy and co-authors who reported that approximately 84% of the bacterial endophytes isolated from the tissues of *T. vulgaris* had the ability for fixing nitrogen when grown on Ashby's media [35].

Moreover, the bacterial isolates T9r, T11r, T12r, and T13r showed varied efficacy of phosphate solubilization designated as diameters of clear zone formed around bacterial growth on Pikovskaya agar media. Endophytic bacterial isolates T11r and T13r exhibited the highest phosphate solubilizing activity with clear zones of 7.5 ± 0.3 mm and 8.9 ± 0.2 mm, respectively. Similarly, among 13 endophytic bacterial strains isolated from medicinal plants *Fagonia mollis* and *Achillea fragrantissima* collected from Saint Katherine Protectorate, South Sinai, Egypt, only 9 strains exhibit phosphate solubilizing activity on Pikovskaya agar media with a diameter of clear zone ranging from 7.6 ± 0.3 mm to 9.6 ± 0.3 mm [38]. Based on the obtained data, the endophytic bacterial strains T9r, T10r, T11r, T12r, and T13r were selected for identification and further investigation on their efficacy as plant growth-promoting rhizobacteria.

Identification of the most potent endophytic bacterial isolates

Five bacterial isolates were selected based on their efficacy for nitrogen fixation and phosphate solubilization and were identified using PCR amplification and sequencing

Table 2: Nitrogen fixation and phosphate solubilization activities of bacterial endophytes isolated from the root of *T. vulgaris* L.

Endophytic bacterial strain	Nitrogen fixation	Phosphate solubilization (mm)	Endophytic bacterial strain	Nitrogen fixation	Phosphate solubilization (mm)
T1r	-	0	T8r	+	0 ^c
T2r	-	0	T9r	++	5.1±0.4 ^b
T3r	+	0	T10r	++	0 ^c
T4r	-	0	T11r	++	7.5±0.3 ^a
T5r	-	0	T12r	++	4.5±0.2 ^b
T6r	-	0	T13r	++	8.9±0.2 ^a
T7r	-	0	T14r	-	0 ^c

Values within the same column with different letters are significantly different ($p \leq 0.05$), values are means $\pm$ SE ($n = 3$).
 -, +, ++ denotes negative, moderate, and high nitrogen fixation based on color change.

Table 3: Molecular identification based on 16S rRNA sequencing of the most potent bacterial endophytes isolated from *T. vulgaris* L., and the quantitative evaluation of their nitrogen fixation and phosphate solubilization activities.

Bacterial endophytes code/ GenBank accession number	Homolog Sequences (%)	NCBI accession numbers	Nitrogen fixation (nitrogenase n-mole C ₂ H ₄ / ml/24h)	Ammonia production
T9r/ MZ617379	<i>Bacillus haynesii</i> (99.7%)	NR157609	82.133±1.4 ^d	+
T10r/ LC603783	<i>Citrobacter farmeri</i> (99.3%)	NR024861	88.067±1.4 ^d	+
T11r/ LC589065	<i>Bacillus licheniformis</i> (98.7%)	NR118996	322.4±2.1 ^b	++
T12r/ LC603784	<i>Bacillus velezensis</i> (99.9%)	NR075005	130.167±2.1 ^c	++
T13r/ LC89066	<i>Bacillus velezensis</i> (99.9%)	NR075005	346.6±1.4 ^a	+++

Values within the same column with different letters are significantly different ($p \leq 0.05$), values are means $\pm$ SE ($n = 3$).
 -, +, ++ denotes negative, moderate, and high production

of the 16S rRNA gene. The sequence analysis of strain T9r showed a similarity of 99.7% with the sequence of *Bacillus haynesii*, whereas the sequence of strains T10r and T11r showed 99.3% and 98.7% similarity percentages with *Citrobacter farmeri* and *Bacillus licheniformis*, respectively. Strains T12r and T13r showed 99.9% similarity percentages with the sequence of *Bacillus velezensis* (Table 3, Fig. 1). Moreover, the outgroup of Gram-positive and Gram-negative endophytic bacterial isolates was represented in Figures S1 and S2. Based on sequence analysis, *Bacillus* spp. were the most predominant endophytic bacteria isolated from medicinal plant *T. vulgaris* L. Hanna and co-workers reported that *Bacillus* spp. are the most endophytic bacterial strain isolated from annual and perennial plants collected from North Sinai, Egypt [39]. Consistent with our data, *Bacillus* spp. were widely distributed among the endophytic bacteria strains isolated from medicinal plants such as *Digitalis*

purpureae, *Leonurus heterophyllus*, *Pinellia ternata*, *Taxus yunnanensis*, *Achillea fragrantissima*, *Thymus vulgaris*, and *Pulicaria incisa* [32, 35, 38, 40].

Recently, endophytic *Bacillus haynesii* was isolated from *Ocimum tenuiflorum* and exhibits high antagonistic actions against different phytopathogenic fungi [41]. Also, *Bacillus velezensis* is identified as a novel endophytic *Bacillus* strain with high biological and plant growth-promoting activity as reported previously [42]. To the best of our knowledge, the obtained strains in this study were recorded for the first time as endophytic bacterial strains isolated from *T. vulgaris*.

Figure 1: Phylogenetic tree of 16S rRNA sequences for the most potent endophytic bacterial strains isolated from roots of *T. vulgaris*. The obtained sequences were compared with those retrieved from NCBI. The analysis was achieved by MEGA 6.1 using the neighbor-joining method with bootstrap value (1000 replicates).

***In-vitro* characterization of bacterial endophytes as plant growth-promoting rhizobacteria**

Quantitative nitrogen fixation using acetylene reduction assay

Nitrogen element (N) is considered the main component of enzymes, proteins, nucleic acids, and chlorophyll. Therefore, it is a fundamental element for adequate plant growth and hence increases the yield through its contribution within several routes affecting plant physiological and biochemical functions [43]. For example, certain bacterial endophytes have the ability to provide plants with their required nitrogen through the process of nitrogen fixation. From these bacteria, one group are identified as diazotrophic bacterial endophytes, they are considered the critical source for the input of nitrogen into the agricultural implements, and candidates as a promising alternative source for

chemical fertilizers [44]. In the current study, besides qualitative nitrogen fixation (Table 2), the quantitative nitrogen fixation efficacy of endophytic bacterial strains through reduction of acetylene was investigated. Data in Table 3 revealed that all selected bacterial endophytes have the ability to produce nitrogenase through the reduction of acetylene with a varied degree. *Bacillus licheniformis* T11r and *Bacillus velezensis* T13r showed the highest nitrogen fixation with amounts of 322.4 ± 2.1 and 346.6 ± 1.4 n-mole C_2H_4 / ml/24h, respectively. The lowest activity was recorded for *Bacillus haynesii* T9r with an amount of 82.133 ± 1.4 n-mole C_2H_4 / ml/24h. Similarly, out of 250 endophytic bacterial strains isolated from stems, mature seeds, roots, and leaves of rice, only 21 bacterial strains were positive for the acetylene reduction test with varied nitrogenase activity ranging from 7.6 to 225.9 n-mol C_2H_4 /h/mg/protein [45].

Quantitative phosphate solubilizing assay

Approximately, 95 to 99% of phosphorus levels in soils are unavailable for plant uptake because they present either as immobilized or precipitated, or insoluble forms. This leads to an increase in applying chemical phosphorus fertilization and harmfully affects the environment and soils [46]. Bacterial endophytes can increase the availability of phosphorus for plant uptake by various mechanisms, for instance, chelation, producing organic acids, exopolysaccharide secretion, ion exchange, and synthesis of different phosphate solubilizing enzymes such as phytase, phosphatase, and C-P lyase [9]. In the current study, the most potent endophytic bacteria showed varied phosphate solubilizing activity through detecting the amount of soluble phosphate in Pikovskaya's broth medium supplemented with $\text{Ca}_3(\text{PO}_4)_2$ that was measured by the phosphomolybdate method. Analysis of variance showed that the highest P amount was liberated after nine days with values of 123.9 ± 2.8 , 149.5 ± 0.9 , 149.5 ± 1.01 , and $159.5 \pm 0.9 \mu\text{g ml}^{-1}$ for bacterial endophytes *Bacillus haynesii* T9r, *Bacillus licheniformis* T11r, *Bacillus velezensis* T12r, and *Bacillus velezensis* T13r, respectively (Fig. 2A). The lowest P secretion was recorded for bacterial isolate *Citrobacter farmeri* T10r and these data were compatible with preliminary screening (Table 2). The pH value of Pikovskaya's inoculated broth media was decreased with time increase as compared with control. In the present study, the medium pH was decreased from 6.6 on the first day to reach 4.5 on the ninth day as compared with control (pH=7.1 on the first day and reach 6.8 on the ninth day) (Fig 2B). The reduction in pH values was acidified in the medium and hence the Ca^{2+} is replaced by H^+ and liberates the phosphate ions [47]. Various organic acids such as malic, gluconic, citric, oxalic, salicylic, fumaric, and tartaric acid are the main ones involved in phosphate solubilization [9]. Among selected endophytic bacterial strains, the strains *Bacillus licheniformis* T11r and *Bacillus velezensis* T13r possess the highest activity for phosphate solubilization.

Ammonia production

Bacterial endophytes can enhance plant growth through the production of ammonia, which significantly improves the shoot and root growth; moreover, ammonia enhances the growth of plants by the formation of different biomolecules containing nitrogen [48]. In the current study, all bacterial endophytes differed in their efficacy to produce ammonia through a color change of inoculated

Figure 2: Quantitative phosphate solubilizing assay in Pikovskaya's broth medium supplemented with $\text{Ca}_3(\text{PO}_4)_2$. A is denotes the amount of liberated phosphorus ($\mu\text{g mL}^{-1}$) at interval times; B is denotes the pH values of Pikovskaya's media at interval times. Values within the same time interval with different letters are significantly different ($p \leq 0.05$), values are means $\pm$ SE ($n = 3$).

peptone water broth media after adding Nessler's reagent. Data showed the highest color change was recorded for the bacterial isolate of T13r followed by T11r and T12r (Table 3). Among 52 bacterial endophytes isolated from roots, stems, and leaves of *Clerodendrum colebrookianum*, 51 isolates (98%) exhibited ammonia production [28]. Endophytic bacteria possibly produce ammonia from different routes such as the hydrolysis of amino acids containing nitrogen or through deamination of by-products resulting from the breakdown of various macromolecules, or from the breakdown of urea to produce CO_2 and NH_4 [32, 49].

Extracellular Enzymatic Activities

Data represented in Table 4 showed that all selected bacterial isolates were positive for protease and chitinase, whereas *Bacillus licheniformis* T11r, *Bacillus velezensis*

Table 4: Plant growth-promoting activities including enzyme activity, siderophores, and HCN production of the most potent selected endophytic bacterial isolates.

Bacterial strain	Enzyme activity (mm)					Siderophores	HCN
	Amylase	Cellulase	Protease	Chitinase	Catalase		
T9r	0.0 ± 0.0 ^c	31.3±0.9 ^b	35.0±0.6 ^a	16.0 ± 1.5 ^d	+	+	++
T10r	0.0 ± 0.0 ^c	0.0 ± 0.0 ^c	19.7±1.2 ^c	17.5±1.7 ^{cd}	-	-	+
T11r	16.0±1.73 ^b	32.7±0.9 ^{ab}	28.3±1.2 ^b	20.0±1.4 ^b	+	+	+++
T12r	16.7±1.45 ^b	31.3±0.8 ^b	35.6±1.5 ^a	19.6±1.6 ^c	+	+	+
T13r	19.0±1.53 ^a	35.0±0.6 ^a	35.7±1.5 ^a	24.0±0.7 ^a	+	+	+++

Values within the same row with different letters are significantly different ($p \leq 0.05$), values are means ± SE (n = 3).

-, +, ++ denotes negative, moderate, and high production

T12r, and *Bacillus velezensis* T13r were positive for amylase. Moreover, out of the 5 most potent endophytic bacterial isolates, four isolates designated as T9r, T11r, T12r, and T13r are cellulase and catalase producers. In general, the highest activity for enzymes production was recorded for endophytic bacterial isolate *Bacillus velezensis* T13r with clear zones of 19.0±1.53, 35.0±0.6, 35.7±1.5, and 24.0±0.7 mm for amylase, cellulase, protease, and chitinase, respectively. The bacterial isolate *Citrobacter farmeri* T10r showed the lowest protease and chitinase enzymes activity with a clear zone of 19.7±1.2 and 17.5±1.7 mm, respectively. In a similar study, out of 52 endophytic bacterial isolates, 45 isolates (86.5%), 44 isolates (84.6%), and 47 isolates (90.3%) were positive for cellulase, amylase, and protease, respectively [28].

The ability of bacterial endophytes to penetrate the cells of their host can be related to their efficacy to secrete various extracellular lytic enzymes. Moreover, these lytic enzymes protect plant hosts from the attack by phytopathogenic fungi [50]. It has been reported that the endophytic bacterial strains characterized by catalase production are more resistant to different mechanical, environmental, and chemical stresses [30]. This phenomenon can be attributed to the catalase enzyme which is responsible for the scavenging of free radicals raised due to exposure to biotic and abiotic stresses. Also, the chitinase enzyme has a critical role in the hydrolysis of chitin which is the main component of the fungal cell wall, and hence protects the plant from infection by phytopathogenic fungi [7].

Microbes with amylase, cellulase, and protease enzymes activity have different advantages including organic matter degradation, plant growth promotion, and plant protection from diseases caused by soil-borne pathogens [51]. Amylase enzymes are considered the major industrial enzyme group represented by 25-30% of

the total market enzyme [52]. Bacterial cellulase enzymes are not only important for depolymerization of cellulose, which is the major component in the plant cell but also assists and facilitate bacterial penetration to plant host [53, 54]. Also, protease enzymes secreted by bacterial endophytes have primary importance to suppress phytopathogens injury and protect the plant from nematodes infection. The protease enzyme secreted by endophytic bacterial strain *Bacillus cereus* BCM2 showed high activity against *Meloidogyne incognita* (plant-parasitic nematodes) through the degradation of chemical components of nematodes cuticle and their eggshells [55]. The finding that *Bacillus* spp. are considered the highest endophytic strains producing various lytic enzymes has been previously reported [13, 28], and these data are in harmony with the current study. Based on enzymatic activity, bacterial endophytes can provide the plant with their requirement of nutrients and protect their host from phytopathogen infection by secretion of different lytic enzymes [28].

Siderophores

Siderophores are defined as secondary metabolites with a low molecular weight that has the ability to iron-chelating from complex substance. Siderophores are synthesized by different endophytic bacterial species and promote plant growth through the availability of Fe³⁺ for iron-deficient plants [56]. In the current study, four bacterial strains of the selected most potent isolates can synthesize siderophores by forming an orange halo around the bacterial growth on king's B agar medium (Table 4). Similarly, out of five endophytic bacterial strains isolated from *Cassia tora* L. root, only three isolates produced siderophores [57]. Brígido and co-author reported that although the few

bacterial endophytes isolated from chickpea root can be produced siderophores, most of them are isolated from a plant collected from soil contaminated with manganese [58]. Besides the critical role of siderophores in the high scavenging of insoluble iron, siderophores secreted by various bacterial species can bind with other non-iron metals, and hence reduce the toxicity of these metals in the environment [59]. Accordingly, bio-inoculation of metal-sensitive plants with siderophore-producer bacterial endophytes could possibly acclimate plants within metal contaminated soils.

HCN production

Various plant-associated microbes can produce volatile substances as secondary metabolites known as hydrogen cyanide (HCN). This volatile substance has a crucial role in suppressive the growth of plant pathogenic microbes [60]. In the current study, all selected bacterial endophytes have the capacity to change the color of filter paper from deep yellow to reddish-brown with varying degrees. The highest bacterial strains producing HCN were *Bacillus licheniformis* T11r and *Bacillus velezensis* T13r, followed by *Bacillus haynesii* T9r (Table 4). As shown, the highest producing strain was *Bacillus* spp. which is compatible with a previous study [28]. In addition to the capacity of HCN to suppress phytopathogen, it is also used to control nematodes infection [61]. The endophytic bacterial strains that produce HCN along with siderophores are considered a good candidate in biocontrol investigation as previously mentioned [62].

IAA production

The main feature of plant growth-promoting microbes is their potency in the synthesis of various phytohormones such as indole-3-acetic acid (IAA). Various processes involved in plant growth such as cell division, stem elongation, geotropisms, root development and hence nutrient uptake, biomass production, apical dominance, phototropism, and cell differentiation are regulated by secretion of IAA [63, 64]. In the current study, the efficacy of isolated endophytic bacterial strains to produce IAA was assessed after 15 days of incubation in the presence and absence of tryptophan (0, 1, 2, and 5 mg mL⁻¹) as the precursor for IAA. Data analysis showed that the ability of various endophytic bacterial strains for the synthesis of IAA in absence of tryptophane was non-significant compared with control. At tryptophane of 1 and 2 mg mL⁻¹,

Figure 3: Indole-3-acetic acid (IAA) production by the most endophytic bacterial strains isolated from *T. vulgaris*. A is denotes the qualitative IAA assay at different tryptophan concentrations (0, 1, 2, and 5 mg mL⁻¹), B is denoted the quantitative IAA assay in broth media amended with 5 mg mL⁻¹ tryptophan at various time courses (2 to 14 days). Values with different letters are significantly different ($p \leq 0.05$), values are means $\pm$ SE ($n = 3$).

the bacterial strains T9r, T10r, T11r, and T13r produced IAA with values of 16.9 ± 1.1 , 14.1 ± 0.3 , 28.7 ± 1.9 , and 48.3 ± 1.5 mg mL⁻¹ and $(20.3 \pm 0.2, 16.4 \pm 0.7, 43.2 \pm 2.9, \text{ and } 50.4 \pm 1.9 \text{ mg mL}^{-1})$, respectively. These values are significant ($p \leq 0.001$) different as compared with control (2.8 ± 0.4 and 3.8 ± 0.5 mg mL⁻¹) and those produced by endophytic strain T12r (2.1 ± 0.3 and 4.1 ± 0.1 mg mL⁻¹) (Fig. 3A). The concentration of IAA was increased by increasing tryptophan concentration. At 5 mg mL⁻¹ tryptophan, the concentration of IAA was increased to reach 29.6 ± 1.3 , 18.1 ± 0.6 , 74.2 ± 1.8 , 16.2 ± 0.8 , and 81.8 ± 1.9 mg mL⁻¹ for strains T9r, T10r, T11r, T12r, and T13r, respectively (Fig. 3A). It was reported that 48 out of 52 endophytic bacterial strains isolated from the roots of medicinal plant *Clerodendrum colebrookianum* showed efficacy to produce IAA in nutrient broth media supplemented with 2 mg mL⁻¹ tryptophan [28]. The authors

Table 5: Detection and quantification of various phytohormones (GA₃, IAA, ABA, benzyl adenine, kinten, and ziaten) produced by the most potent endophytic bacterial strains using HPLC analysis.

Isolates codes	Acid side mg/100 ml			Alkaline side mg/100 ml		
	GA ₃	IAA	ABA	Benzyl adenine	Kinten	Ziaten
T9r	2.31±0.05 ^c	0.7±0.12 ^b	0.02±0.006 ^a	0.15±0.06 ^c	0.15±0.06 ^b	0.2±0.05 ^d
T10r	4.21±0.06 ^b	1.86±0.19 ^a	0.03±0.003 ^a	0.28±0.09 ^b	0.43±0.07 ^a	0.94±0.06 ^a
T11r	7.08±0.15 ^a	2.06±0.08 ^a	0.02±0.005 ^a	0.21±0.05 ^b	0.3±0.07 ^a	0.48 ± 0.02 ^c
T12r	2.61±0.09 ^c	0.79±0.08 ^b	0.02±0.004 ^a	0.12±0.04 ^c	0.16±0.07 ^b	0.35±0.03 ^{cd}
T13r	6.59±0.69 ^a	1.93±0.09 ^a	0.02±0.004 ^a	0.3±0.1 ^a	0.19±0.06 ^b	0.6 ± 0.01 ^b

Values within the same row with different letters are significantly different ($p \leq 0.05$), values are means $\pm$ SE ($n = 3$).

reported that the highest production was recorded for isolates *Bacillus* sp. BPSAC3 and *Bacillus* sp. BPSAC6 with values of 78.4 and 80.6 mg ml⁻¹. Besides the importance of IAA for plant growth, the secretion of this phytohormone displays a crucial key in the relationship between plant and endophytic microbes [65].

Based on the qualitative screening, the best concentration of tryptophan used for quantitative screening at interval times was 5 mg ml⁻¹. Data represented in Figure (3B) showed that the productivity was time-dependent, increased by increasing incubation times. The highest IAA production was still recorded for bacterial strains T11r and T13r with amounts of 75.6 $\pm$ 3.7 and 80.8 $\pm$ 3.9 mg ml⁻¹ after 14 days, respectively. The high IAA production can be attributed to the enzymes responsible for the conversion of tryptophan [66]. Previous studies that harmony with the current investigation showed that the production of IAA by bacterial and fungal endophytic strains is dependent on the concentration of tryptophan in liquid media and incubation times [13, 19, 67].

Detection of phytohormones using HPLC.

The secreted acidic (GA₃, IAA, and ABA) and alkaline (Benzyl, Kinten, and Ziaten) plant hormones were quantified for the selected bacterial endophytes (T9r, T10r, T11r, T12r, and T13r) using HPLC (Table 5). For gibberellic acid (GA₃), the bacterial isolates *Bacillus licheniformis* T11r and *Bacillus velezensis* T13r exhibited the highest productivity with values of 7.08±0.15 and 6.597±0.69 mg/100 ml, respectively. Whereas the lowest production was recorded for bacterial isolate *Bacillus haynesii* T9r with a value of 2.31±0.05 mg/100 ml. On the other hand, the highest production of IAA was recorded for endophytic bacterial isolates T11r and T13 followed by T10r, and these data are compatible with a qualitative and

quantitative assay using Salkowski's reagent. In a similar study, among four endophytic bacterial strains isolated from *Zingiber officinale* and identified as *Bacillus* sp. ZoB1, *Pseudomonas* sp. ZoB2, *Stenotrophomonas* sp. ZoB3, and *Staphylococcus* sp. ZoB4, only one strain ZoB2 has the efficacy to produce IAA and confirmed their productivity by HPLC [68]. Absciscic acid (ABA) has a critical role in ameliorating the various stresses and enhancing plant adaptation [69]. Moreover, under normal conditions, ABA at adequate concentration has an important role in the development and growth of the plant [70]. In the current study, all selected bacterial isolates have the potency to secrete ABA at low concentration ranging between 0.02±0.004 to 0.03±0.003 mg/100 ml. The HPLC analysis showed the activity of selected endophytic bacteria to secrete other phytohormones including benzyl adenine, kinten, and ziaten. The isolates T10r, T11r, and T13r still possess high activity in phytohormones production. Compatible with our study, the HPLC analysis for ethyl acetate extract of *Bacillus* sp. BPSAC6 isolated from roots of medicinal plant *Clerodendrum colebrookianum* exhibit varied activity for production of IAA, kinten, and benzyl adenine with values of 31.2, 12.3, and 3.24 µg ml⁻¹, respectively [28]. Based on plant growth-promoting traits of selected five endophytes bacterial strains, two strains designated as T11r and T13r were selected to investigate their activity on the growth and oil content of *T. vulgaris* under field conditions.

Field experiment

Effect of bacterial inoculation and mineral fertilization treatment on *T. vulgaris* growth

The improvement of medicinal and agronomical plants for integrating into various life sectors is being an important

Table 6: Effect of bacterial inoculation of *Bacillus licheniformis* T11r and *Bacillus velezensis* T13r on the growth and oil content of *T. vulgaris* under different concentrations of mineral fertilization.

Treatment*	Plant height (cm)		Shoot fresh weight (g)		Shoot dry weight (g)		Oil content (ml)/ 100 gm dry weight
	65 days	140 days	65 days	140 days	65 days	140 days	
A1	28.7±0.8 ^e	39.7±0.9 ^e	71.7±0.8 ^d	107.3±0.9 ^b	49.6±0.2 ^a	66.2±0.3 ^b	2.13 ^a
A2	22.7±0.8 ^f	33.5±0.9 ^g	66.7±0.8 ^e	98.0±0.9 ^c	47.7±0.2 ^b	63.8±0.3 ^c	1.57 ^b
A3	17.3±0.8 ^h	26.1±0.9 ^h	60.3±0.8 ^f	86.7±0.9 ^d	45.8±0.2 ^c	60.5±0.3 ^d	1.13 ^b
A4	10.0±0.8 ⁱ	12.7±0.9 ⁱ	53.0±0.8 ^h	71.3±0.9 ^f	43.6±0.2 ^d	56.1±0.3 ^e	0.35 ^c
A5	42.3±0.8 ^a	55.9±0.9 ^a	97.0±0.8 ^a	110.3±0.9 ^{ab}	50.9±0.2 ^a	67.9±0.3 ^{ab}	2.27 ^a
A6	34.7±0.8 ^c	43.1±0.9 ^d	69.7±0.8 ^d	100.0±0.9 ^c	48.4±0.2 ^a	64.8±0.3 ^c	2.12 ^a
A7	27.0±0.8 ^e	32.7±0.9 ^g	65.3±0.8 ^e	88.0±0.9 ^d	47.4±0.2 ^b	61.0±0.3 ^d	1.28 ^b
A8	20.3±0.8 ^g	24.5±0.9 ^h	58.7±0.8 ^g	78.0±0.9 ^e	45.4±0.2 ^c	57.9±0.3 ^e	0.51 ^c
A9	33.0±0.8 ^{cd}	51.6±0.9 ^b	80.0±0.8 ^b	111.3±0.9 ^a	51.0±0.2 ^a	67.6±0.3 ^{ab}	2.31 ^a
A10	26.9±0.8 ^e	42.3±0.9 ^d	72.3±0.8 ^c	101.7±0.9 ^c	49.0±0.2 ^a	64.4±0.3 ^c	2.16 ^a
A11	23.1±0.8 ^f	31.6±0.9 ^g	67.7±0.8 ^e	88.3±0.9 ^d	47.4±0.2 ^b	60.6±0.3 ^d	1.29 ^b
A12	20.0±0.8 ^g	24.0±0.9 ^h	61.0±0.8 ^f	77.3±0.9 ^e	45.7±0.2 ^c	57.7±0.3 ^e	0.52 ^c
A13	38.9±0.8 ^b	55.7 ±0.9 ^a	83.0±0.8 ^b	113.6±0.9 ^a	52.1±0.2 ^a	68.3±0.3 ^a	2.39 ^a
A14	31.4±0.8 ^d	46.2±0.9 ^c	74.6±0.8 ^c	101.0±0.9 ^c	49.8±0.2 ^a	65.2±0.3 ^{bc}	2.18 ^a
A15	26.9±0.8 ^e	35.8±0.9 ^f	69.6±0.8 ^d	89.5±0.9 ^d	48.3±0.2 ^{ab}	61.8±0.3 ^d	1.31 ^b
A16	20.2±0.8 ^g	26.0±0.9 ^h	62.1±0.8 ^f	78.6±0.9 ^e	46.2±0.2 ^{bc}	58.6±0.3 ^e	0.54 ^c

Values within the same column with different letters are significantly different ($p \leq 0.05$), values are means $\pm$ SE ($n = 3$). * Description of various treatment (A1 – A16) are listed in the material and method section.

challenge to cope with the overpopulation growth [71, 72]. Bacterial endophytes can enhance plant growth by various direct and indirect mechanisms, such as the production of phytohormones, phosphate solubilization, nitrogen fixation, ammonia production, secretion of lytic enzymes, production of siderophores and HCN, and protect the plant against phytopathogens [9, 35]. In the current study, all selected bacterial endophytes exhibited various plant growth-promoting traits with varying degrees. The two bacterial endophytic strains *Bacillus licheniformis* T11r and *Bacillus velezensis* T13r possess the highest activity as plant growth-promoting rhizobacteria. Therefore, it can be used as biofertilizers to enhance *T. vulgaris* growth performance and the oil content and quality. The efficacy of the two most potent bacterial strains T11r and T13r and their consortium as bio-inoculants or biofertilizers (in presence and absence of different concentrations of mineral fertilization) on the growth performance of *T. vulgaris* was investigated at two stages, after 65 days and 140 days under field conditions. The height of *T. vulgaris* in the absence of bacterial endophytes (control) was (10.0±0.8 cm and 12.7±0.9 cm) after 65 and 140 days in

the absence of mineral fertilization (A4), this height was significantly increased to (28.7±0.8 cm and 39.7±0.9 cm) after adding 100% mineral fertilization (A1). On the other hand, the fresh weight and dry weight of the control (A4) was (13.0±0.8 g and 21.3±0.9 g) and (3.6±0.2 g and 6.1±0.3 g) after 65 and 140 days, respectively, and these weights were increased after adding 100% mineral fertilization (A1) to (31.7±0.8 g and 57.3±0.9 g) and (9.6±0.2 g and 16.2±0.3 g), respectively. Hence, the addition of mineral fertilization positively increased the growth parameters of *T. vulgaris*. These positive impacts were increased in the presence of bacterial endophytes (*Bacillus licheniformis* T11r and *Bacillus velezensis* T13r) individually or in a consortium (Table 6). For instance, the maximum plant heights (42.3±0.8 cm and 55.9±0.9 cm) were recorded for seeds treated with *Bacillus licheniformis* T11r (A5) in the presence of 100% mineral fertilization followed by those treated by the bacterial consortium in presence of 100% mineral fertilization (38.9±0.8 cm and 55.7 ±0.9 cm). On the other hand, the maximum fresh and dry weights of shoots were reported for seeds treated by the bacterial consortium (A13) followed by seeds treated by *Bacillus velezensis*

T13r (A9) and those treated by *Bacillus licheniformis* T11r (A5) (Table 6). Based on the obtained results, it can be concluded that the presence of bacterial endophytes promotes plant growth in the presence of mineral fertilization as compared with control. The potency of two selected endophytic strains to improve plant growth can be attributed to their plant growth-promoting properties and hence increase the availability of nutrients required for plant growth as reported previously [73]. Ullah and co-authors reported that the enhancement of plant morphological and physiological properties (root and shoot length, number of root tips responsible for nutrient uptake, plant biomass, and chlorophyll contents) due to microbial endophytes treatment are mainly because of their efficacy in producing phytohormones and phosphate solubilization [74]. Recently, the endophytic bacterial strains *Bacillus cereus* strain PI-8 and *Bacillus subtilis* strain PI-10 isolated from *Pulicaria incisa* leaves enhanced the morphological characteristics (plant heights, fresh shoots, and roots weight, and dry shoots and roots weight) of *Zea mays* compared with control (un-inoculated plants) [32]. Similarly, out of three bacterial endophytes, one strain identified as *Bacillus* sp. strain BPSAC6 exhibited high efficacy to improve the root length, height of shoots, and weight of tomato plants after 45 and 60 days after planting as compared with control (un-inoculated seeds) [28].

Effect of bacterial inoculation and mineral fertilization treatment on oil content and fractions using GC-MS analysis

The oil content of *T. vulgaris* due to different treatments was analyzed using GC-MS. The obtained results revealed that the thymus plants inoculated with the mixture of endophytic bacterial strain *B. licheniformis* T11r and *B. velezensis* T13r mostly gave the highest oil volume in comparison to that individually inoculated in the presence and absence of chemical fertilizer ratios, which gave slightly lower oil content. Data of oil extraction showed that the treatment of *T. vulgaris* with bacterial endophytes have plant growth-promoting properties are stimulate and improvement the oil contents as compared with those untreated. Data showed that the amount of oil/100 g dry weight (mixture of first and second harvest) of *T. vulgaris* treated with T11r, T13r and consortium (T11r+T13r) were (2.27, 2.12, 1.28, 0.51 ml), (2.31, 2.16, 1.29, 0.52 ml), and (2.39, 2.18, 1.31, 0.54 ml) in presence of 100, 75, 50, and 0% mineral fertilization respectively (Table 6). Whereas the amount of oil in the untreated plant was 2.13, 1.57, 1.13,

0.34 ml in presence of various concentrations of mineral fertilization (100, 75, 50, 0% respectively) (Table 6). Hence, the bacterial endophytes have the efficacy to improve oil contents even though the absence of mineral fertilization. The obtained results are in harmony with those recorded that the dry weight, fresh weight, and content of oils in *T. vulgaris* were the highest in soil fortified with phosphate solubilizing bacteria plus manure, Nitroxin biofertilizer, and phosphate at full doses [75]. The effect of biofertilizations, mineral fertilizations, and/or organic fertilization on the oil contents was previously reported [76, 77].

The GC is considered the main technique used for the separation of volatile and semi-volatile compounds in herbs and identifying each one. The attachment of MS with GC apparatus enables the fragmentation of each separated compound and compared these fragments with available database and identified it without using markers [78, 79]. In the current study, the GC-MS analysis was achieved for sixteen different treatments of *T. vulgaris* to investigate the effects of bacterial endophytes in the presence and absence of mineral fertilization on thyme contents (Fig. 4). GC-MS data analysis showed that the thyme herb oil samples containing twenty-three (23) different compounds were identified based on retention times and authentic substances. The identification of these 23 compounds and their concentrations in each treatment were represented in Table 7 and Figure 5. The major components of thyme oil were α -Thujene, Sabinene, β -Myrcene, α -Terpinene, o-Cymene, γ -Terpinene, Terpinen-4-ol, α -Terpineol, Caryophyllene, and Thymol (Table 7) with varied contents according to treatments. Most of these fractions, especially Thymol, have medicinal and biotechnological importance [80, 81]. The obtained data are compatible with that recorded by Al-Asmari and co-authors [82], who showed that the GC-MS analysis of *T. vulgaris* oil contains 24 various compounds and is identified based on their retention time. Moreover, the major compounds recorded in the current study are matched with those recorded in various published studies [82, 83].

Our data recorded that the thymol compounds are the highest value (39.1%) when the plants were treated with a full dose of mineral fertilizers with the consortium of endophytic bacteria (*B. licheniformis* T11r and *B. velezensis* T13r), whereas the lowest value (29.85%) was recorded in plants which un-treated with chemical or bacterial fertilizations. In all cases, uninoculated thyme plants gave the lowest thymol compound value. Two-way cluster analysis showed that the different treatments were clustered in two groups. The first group included the control untreated plant in presence of various

Figure 4: Total Ion Chromatograms (TIC) of GC-MS analyses of oils extracted from *T. vulgaris* plants treated or not with bacterial endophytes (T11r, T13r, and their consortium) under different mineral fertilization doses. The description of symbols A1 to A16 was recorded in the material and method section.

Table 7: List of compounds and their percentages existing in the extracted oil from *T. vulgaris* plant treated or not with bacterial endophytes (T11r, T13r, and their consortium) under different mineral fertilization doses.

Peak No.	compound	Different treatment/ percentages of oil contents (%)															
		Control				T11r				T13r				T11r+T13r			
		0% MF	50% MF	75% MF	100% MF	0% MF	50% MF	75% MF	100% MF	0% MF	50% MF	75% MF	100% MF	0% MF	50% MF	75% MF	100% MF
1	α -Thujene	2.93	2.95	2.85	2.92	2.60	2.58	2.54	2.57	3.49	2.58	2.90	2.72	2.90	2.74	2.70	2.65
2	α -Pinene	0.84	0.84	0.80	0.81	0.74	0.75	0.74	0.76	1.00	0.73	0.84	0.77	0.84	0.79	0.79	0.72
3	Sabinene	1.25	1.28	1.25	1.24	1.09	1.07	1.04	1.08	1.52	1.24	1.60	1.45	1.57	1.44	1.41	1.26
4	β -Myrcene	1.46	1.44	1.42	1.43	1.24	1.22	1.23	1.20	1.49	1.20	1.41	1.33	1.38	1.32	1.25	1.21
5	α -Terpinene	8.38	8.38	8.37	8.28	7.14	7.20	7.05	7.11	7.42	6.36	5.89	5.96	5.93	5.93	5.96	6.20
6	o-Cymene	4.23	4.20	4.13	4.14	3.64	3.57	3.57	3.57	4.77	4.15	4.36	4.19	4.32	4.17	4.09	3.95
7	Cyclohexanol, 1-methyl-4-(1-methylethenyl)-, acetate	1.98	2.00	1.72	1.98	1.84	1.78	1.85	1.81	1.69	1.87	1.69	1.72	1.78	1.77	1.72	1.66
8	γ -Terpinene	23.29	23.26	23.5	23.50	20.62	20.69	20.48	20.67	22.2	20.61	18.06	18.39	17.97	18.18	18.28	19.19
9	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	1.60	1.61	1.59	1.58	1.28	1.27	1.27	1.27	0.98	0.93	0.92	0.91	0.89	0.91	0.90	0.95
10	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1- α ,2- β ,5- α)-	3.36	3.28	3.34	3.30	6.76	6.71	6.76	6.75	8.50	8.71	8.94	8.63	9.00	8.72	8.65	8.26
11	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, cis-	0.73	0.74	0.74	0.73	0.68	0.64	0.66	0.66	0.48	0.46	0.56	0.49	0.49	0.49	0.49	0.47
12	Terpinen-4-ol	13.32	13.26	13.31	13.21	11.18	11.01	11.13	11.08	7.91	8.49	8.86	8.72	8.80	8.68	8.70	8.27
13	α -Terpineol	4.95	4.74	4.92	4.80	4.85	4.83	4.95	4.74	1.64	4.56	5.21	4.99	5.23	5.06	4.69	4.54
14	Thymol	29.85	30.11	30.58	30.6	35.01	35.26	35.29	35.31	33.31	36.7	37.81	38.16	37.94	38.02	38.87	39.10
15	Caryophyllene	1.16	1.14	1.13	1.13	1.01	0.99	0.99	0.98	0.63	0.71	0.81	0.85	0.80	0.86	0.85	0.94
16	Aromandendrene	0.24	0.24	0.24	0.00	0.19	0.18	0.17	0.18	0.00	0.00	0.15	0.00	0.16	0.00	0.00	0.00
17	1,3,6-Octatriene, 3,7-dimethyl-, (E)-	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
18	Linalyl acetate	0.10	0.30	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
19	Caryophyllene oxide	0.10	0.10	0.15	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
20	tau-Cadinol	0.11	0.12	0.12	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
21	Terpineol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.89	0.00	0.00	0.00	0.00	0.00	0.00	0.00
22	2- β -PINENE	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.34	0.00	0.00	0.00	0.00	0.00	0.00	0.00
23	2,5-Diethylphenol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.74	0.72	0.00	0.71	0.00	0.92	0.64	0.62

Figure 5: Percentages of oil fractions based on GC-MS analysis in the presence and absence of mineral fertilization (0, 50, 75, and 100%) and bacterial inoculation. C is a control (presence of mineral fertilization and absence of endophytic bacteria); T11r is *B. licheniformis*; T13r is *B. velezensis*; BC is an endophytic bacterial consortium (T11r+T13r). The description of each peak number is listed in Table 7.

concentrations of mineral fertilization (0, 50, 75, and 100%), whereas the second group included all other bacterial inoculated plants under mineral fertilization range of 0-100%. However, cluster analysis discriminated against plants inoculated with *B. licheniformis* T11r than other groups (Fig. 6 and S3).

It is clear that cooperation between both bacterial endophytes and mineral fertilizers activated biosynthesis reactions of thyme plants resulting from the highest value of the main component. This result agrees with Sharaf EL-Din *et al.*, [84], who reported that the use of biofertilization can improve the biosynthesis of different constituents in thyme plants. Moreover, the application of plant growth-promoting bacteria increases the morphological characteristics and oil contents of *Ocimum basilicum* L. as compared with un-treated plants [85]. Based on obtained data, can be concluded that the plant

growth-promoting bacterial endophytes as biofertilizers could serve as a potential treatment to increase the growth, yield, and oil fractions of the thymol plant. Further, the activity of thymol contents to incorporate into various biomedical applications will be assessed *in-vitro* and *in-vivo*. The ecological roles of these bacterial endophyte need to be studied in the future because they might be important in supporting plant survival in different regions. The benefits derived from the plant– endophytic bacteria interaction should be considered at genetic levels below the species. Overall, the bacterial endophytes of *T. vulgaris* might depend on several environmental factors that may structure their communities, more research is required to identify the functional and ecological significance of these bacterial endophytes. Some of the species identified have been described potential characteristics to promote plant growth. Therefore, a better understanding of this

Figure 6: Two-way cluster analysis to group the different treatments into a number of clusters based on the observed values of the compounds and their percentages existing in the extracted oil from *T. vulgaris* plant treated or not with bacterial endophytes (T11r, T13r, and their consortium) under different mineral fertilization doses. The description of each peak number is listed in Table 7.

complex network of interactions between the plant and bacterial endophytes would help to enhance productivity and sustainability.

Conclusion

In the current study, seven bacterial endophytes were isolated from the collected roots of *T. vulgaris* and showed varied qualitative activities for phosphate solubilization and nitrogen fixation. Five endophytic strains identified as *Bacillus haynesii* T9r, *Citrobacter farmeri* T10r, *Bacillus licheniformis* T11r, *Bacillus velezensis* T12r, and *Bacillus velezensis* T13r were selected as the most potent strains. These strains showed varied plant growth-promoting properties including acetylene reduction (nitrogenase), phosphate solubilization (in ranging of 123.9 ± 2.8 - 159.5 ± 0.9 $\mu\text{g ml}^{-1}$), various hydrolytic enzyme activity, siderophores and HCN production, and the synthesis

of different phytohormones. The strains *Bacillus licheniformis* T11r and *Bacillus velezensis* T13r were further selected based on the highest plant growth-promoting activity to be used as bioinoculant for *T. vulgaris* under different mineral fertilization doses (0, 50, 75, and 100%). Data analysis showed that the growth characteristics (plant heights, fresh and dry weight of shoots) of inoculated thyme plants with the bacterial consortium (T11r + T13r) in presence of 100% mineral fertilization were high as compared with single endophytic strains and control. Moreover, the GC-MS analysis for thyme oil revealed that the presence of 23 various compounds, and the maximum percentages were recorded for the bacterial consortium in presence of 100% mineral fertilization. This study provides an efficient method to improve the oil contents of the thyme plant using plant growth-promoting bacterial endophytes. However, the biomedical activity of various oil fractions obtained in the current study has been proposed for further investigation. This study needs

more investigation to show the efficacy of the obtained plant growth-promoting bacterial endophytes on the oil contents and growth performance of others agronomical plants under different environmental conditions. Also, the association between bacterial endophytes and plants needs more study.

Author Contribution: Amr Fouda and Saad El-Din Hassan: Conceptualization, Data curing, Formal analysis, Investigation, methodology, Project administration, Resources, Software, Validation, Visualization, Writing an original draft, Writing -review and editing; Mahmoud Soliman Abdel-Hamid: Data curing, Formal analysis, methodology, Resources, Software, Validation, Visualization, Writing original draft; Hesham Kamal Abo El-Ela and Abbas Ahmed El-Ghamry: Conceptualization, Data curing, Investigation, Resources, Validation, Visualization, Writing an original draft.

Conflict of Interest: Authors state no conflict of interest.

Data Availability Statement: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

References

- Kuete V. Chapter 28: *Thymus vulgaris*. In: Kuete V, editor. *Medicinal Spices and Vegetables from Africa*. Academic Press; 2017. pp. 599–609.
- Ekoh SN, Akubugwo EI, Ude VC, Edwin N. Anti-hyperglycemic and anti-hyperlipidemic effect of spices (*Thymus vulgaris*, *Murraya koenigii*, *Ocimum gratissimum* and *Piper guineense*) in alloxan-induced diabetic rats. *Int J Biosci*. 2014;4:179–87.
- Prasanth Reddy V, Ravi Vital K, Varsha P, Satyam S. Review on *Thymus vulgaris* traditional uses and pharmacological properties. *Med Aromat Plants* 2014;3(164):2167-0412.1000164.
- Mandal S, DebMandal M, Chapter 94 - Thyme (*Thymus vulgaris* L.) Oils. In *Essential Oils in Food Preservation, Flavor and Safety*, Preedy, V. R., Ed. Academic Press: San Diego, 2016; pp 825-834.
- Commission EP, Medicines ED. Healthcare, *European pharmacopoeia*. Volume 1. Council of Europe; 2010.
- Salem SS, El-Belely EF, Niedbala G, Alnoman MM, Hassan SE, Eid AM, et al. Bactericidal and In-Vitro Cytotoxic Efficacy of Silver Nanoparticles (Ag-NPs) Fabricated by Endophytic Actinomycetes and Their Use as Coating for the Textile Fabrics. *Nanomaterials* (Basel). 2020 Oct;10(10):E2082.
- de Almeida Lopes KB, Carpentieri-Pipolo V, Oro TH, Stefani Pagliosa E, Degrassi G. Culturable endophytic bacterial communities associated with field-grown soybean. *J Appl Microbiol*. 2016 Mar;120(3):740–55.
- Golinska P, Wypij M, Agarkar G, Rathod D, Dahm H, Rai M. Endophytic actinobacteria of medicinal plants: diversity and bioactivity. *Antonie van Leeuwenhoek*. 2015 Aug;108(2):267–89.
- Eid AM, Fouda A, Abdel-Rahman MA, Salem SS, Elsaied A, Oelmüller R, et al. Harnessing Bacterial Endophytes for Promotion of Plant Growth and Biotechnological Applications: an Overview. *Plants*. 2021 May;10(5):935.
- Santoyo G, Moreno-Hagelsieb G, Orozco-Mosqueda MC, Glick BR. Plant growth-promoting bacterial endophytes. *Microbiol Res*. 2016 Feb;183:92–9.
- Ismail MA, Amin MA, Eid AM, Hassan SE, Mahgoub HA, Lashin I, et al. Comparative Study between Exogenously Applied Plant Growth Hormones versus Metabolites of Microbial Endophytes as Plant Growth-Promoting for *Phaseolus vulgaris* L. *Cells*. 2021 Apr;10(5):1059.
- Khalil AM, Hassan SE, Alsharif SM, Eid AM, Ewais EE, Azab E, et al. Isolation and Characterization of Fungal Endophytes Isolated from Medicinal Plant *Ephedra pachyclada* as Plant Growth-Promoting. *Biomolecules*. 2021 Jan;11(2):140.
- Mahgoub HA, Fouda A, Eid AM, Ewais EE, Hassan SE. Biotechnological application of plant growth-promoting endophytic bacteria isolated from halophytic plants to ameliorate salinity tolerance of *Vicia faba* L. *Plant Biotechnol Rep*. 2021;15(6):819–43.
- Kotasthane AS, Agrawal T, Zaidi NW, Singh US. Identification of siderophore producing and cynogenic fluorescent *Pseudomonas* and a simple confrontation assay to identify potential bio-control agent for collar rot of chickpea. *3 Biotech* 2017;7(2):137.
- Timmusk S, Behers L, Muthoni J, Muraya A, Aronsson AC. Perspectives and Challenges of Microbial Application for Crop Improvement. *Front Plant Sci*. 2017 Feb;8(49):49.
- Vejan P, Abdullah R, Khadiran T, Ismail S, Nasrulhaq Boyce A. Role of Plant Growth Promoting Rhizobacteria in Agricultural Sustainability-A Review. *Molecules*. 2016 Apr;21(5):E573.
- Dessaux Y, Grandclément C, Faure D. Engineering the Rhizosphere. *Trends Plant Sci*. 2016 Mar;21(3):266–78.
- Fouda A, Hassan SE, Abdo AM, El-Gamal MS. Antimicrobial, Antioxidant and Larvicidal Activities of Spherical Silver Nanoparticles Synthesized by Endophytic *Streptomyces* spp. *Biol Trace Elem Res*. 2020 Jun;195(2):707–24.
- Fouda AH, Hassan SE, Eid AM, Ewais EE. Biotechnological applications of fungal endophytes associated with medicinal plant *Asclepias sinaica* (Bioss.). *Ann Agric Sci*. 2015;60(1):95–104.
- Garrity GM, Bell JA, Lilburn T. Phylum XIV. Proteobacteria phyl. nov. In: Brenner DJ, Krieg NR, Staley JT, Garrity GM (eds), *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2 (The Proteobacteria), part B (The Gammaproteobacteria). New York, NY, USA: Springer; 2005.
- De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, et al., editors. *The Firmicutes*. 2nd ed. Vol. 3, *Bergey's manual of systematic bacteriology*. New York, NY, USA: Springer; 2011.
- Ahmad F, Ahmad I, Khan MS. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiol Res*. 2008;163(2):173–81.
- Jasim B, John Jimtha C, Jyothis M, Radhakrishnan EK. Plant growth promoting potential of endophytic bacteria isolated from *Piper nigrum*. *Plant Growth Regul*. 2013;71(1):1–11.

24. Miller DN, Bryant JE, Madsen EL, Ghiorse WC. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Appl Environ Microbiol.* 1999 Nov;65(11):4715–24.
25. El-Belely EF, Farag MM, Said HA, Amin AS, Azab E, Gobouri AA, et al. Green Synthesis of Zinc Oxide Nanoparticles (ZnO-NPs) Using *Arthrospira platensis* (Class: Cyanophyceae) and Evaluation of their Biomedical Activities. *Nanomaterials (Basel).* 2021 Jan;11(1):E95.
26. Kifle MH, Laing MD. Isolation and Screening of Bacteria for Their Diazotrophic Potential and Their Influence on Growth Promotion of Maize Seedlings in Greenhouses. *Front Plant Sci.* 2016 Jan;6(1225):1225.
27. Muangthong A, Youpensuk S, Rerkasem B. Isolation and Characterisation of Endophytic Nitrogen Fixing Bacteria in Sugarcane. *Trop Life Sci Res.* 2015 Apr;26(1):41–51.
28. Passari AK, Mishra VK, Leo VV, Gupta VK, Singh BP. Phytohormone production endowed with antagonistic potential and plant growth promoting abilities of culturable endophytic bacteria isolated from *Clerodendrum colebrookianum* Walp. *Microbiol Res.* 2016 Dec;193:57–73.
29. Wen CM, Tseng CS, Cheng CY, Li YK. Purification, characterization and cloning of a chitinase from *Bacillus* sp. NCTU2. *Biotechnol Appl Biochem.* 2002 Jun;35(3):213–9.
30. Kumar A, Kumar A, Devi S, Patil S, Payal C, Negi S. Isolation, screening and characterization of bacteria from Rhizospheric soils for different plant growth promotion (PGP) activities: an in vitro study. *Recent Research in Science and Technology* 2012;4:(1).
31. Geetha K, Enkatesham EV, Amballa H, Hadraiah BB. Isolation, screening and characterization of plant growth promoting bacteria and their effect on *Vigna radita*(L.) R.Wilczek. *Int J Curr Microbiol Appl Sci.* 2014;3:799–809.
32. Fouda A, Eid AM, Elsaied A, El-Belely EF, Barghoth MG, Azab E, et al. Plant Growth-Promoting Endophytic Bacterial Community Inhabiting the Leaves of *Pulicaria incisa* (Lam.) DC Inherent to Arid Regions. *Plants (Basel, Switzerland)* 2021;10:(1).
33. Rizzolo A, Baldo C, Polesello A. Application of high-performance liquid chromatography to the analysis of niacin and biotin in Italian almond cultivars. *J Chromatogr A.* 1991;553:187–92.
34. Pharmacopoeia B. Determination of volatile oil in drugs. The Pharmaceutical Press. 17 Bloomsburg, Square. W.C.I. London; 1963. pp. 112–25.
35. Abdelshafy Mohamad OA, Ma JB, Liu YH, Zhang D, Hua S, Bhute S, et al. Beneficial Endophytic Bacterial Populations Associated With Medicinal Plant *Thymus vulgaris* Alleviate Salt Stress and Confer Resistance to *Fusarium oxysporum*. *Front Plant Sci.* 2020 Feb;11(47):47.
36. Kalayu G. Phosphate Solubilizing Microorganisms: Promising Approach as Biofertilizers. *Int J Agron.* 2019;2019:4917256.
37. Ha TQ, Chu TT. Selection of nitrogen fixation and phosphate solubilizing bacteria from cultivating soil samples of Hung Yen province in Vietnam. *J Vietnam Environ.* 2020;12(2):162–8.
38. Alkahtani MD, Fouda A, Attia KA, Al-Otaibi F, Eid AM, Ewais EE, et al. Isolation and Characterization of Plant Growth Promoting Endophytic Bacteria from Desert Plants and Their Application as Bioinoculants for Sustainable Agriculture. *Agronomy (Basel).* 2020;10(9):1325.
39. Hanna AL, Youssef HH, Amer WM, Monib M, Fayed M, Hegazi NA. Diversity of bacteria nesting the plant cover of north Sinai deserts, Egypt. *J Adv Res.* 2013 Jan;4(1):13–26.
40. Miller KI, Qing C, Sze DM, Roufogalis BD, Neilan BA. Culturable endophytes of medicinal plants and the genetic basis for their bioactivity. *Microb Ecol.* 2012 Aug;64(2):431–49.
41. Pramod KS, Pramod KS, Shailendra S, Abhay Raj G, Amrita G, Udai BS, et al. Endophytic bacilli from medicinal-aromatic perennial Holy basil (*Ocimum tenuiflorum* L.) modulate plant growth promotion and induced systemic resistance against *Rhizoctonia solani* in rice (*Oryza sativa* L.). *Biological control* 2020;150:104353.
42. Wang C, Zhao D, Qi G, Mao Z, Hu X, Du B, et al. Effects of *Bacillus velezensis* FKM10 for Promoting the Growth of *Malus hupehensis* Rehd. and Inhibiting *Fusarium verticillioides*. *Front Microbiol.* 2020 Jan;10(2889):2889.
43. Leghari SJ, Wahocho NA, Laghari GM, HafeezLaghari, A.; MustafaBhabhan, G.; HussainTalpur, K.; Bhutto, T. A.; Wahocho, S. A.; Lashari, A. A., Role of nitrogen for plant growth and development: a review. *Adv Environ Biol.* 2016;10:209.
44. Puri A, Padda KP, Chanway CP. Nitrogen-fixation by endophytic bacteria in agricultural crops: recent advances. *Nitrogen in agriculture. IntechOpen.* London: GBR; 2018. pp. 73–94.
45. Rangjaroen C, Rerkasem B, Teaumroong N, Noisangiam R, Lumyong S. Promoting plant growth in a commercial rice cultivar by endophytic diazotrophic bacteria isolated from rice landraces. *Ann Microbiol.* 2015;65(1):253–66.
46. Gouda S, Kerry RG, Das G, Paramithiotis S, Shin HS, Patra JK. Revitalization of plant growth promoting rhizobacteria for sustainable development in agriculture. *Microbiol Res.* 2018 Jan;206:131–40.
47. Alori ET, Glick BR, Babalola OO. Microbial Phosphorus Solubilization and Its Potential for Use in Sustainable Agriculture. *Front Microbiol.* 2017 Jun;8(971):971.
48. Afzal I, Iqar I, Shinwari ZK, Yasmin A. Plant growth-promoting potential of endophytic bacteria isolated from roots of wild *Dodonaea viscosa* L. *Plant Growth Regul.* 2017;81(3):399–408.
49. Lovell R, Harvey DG. A preliminary study of ammonia production by *Corynebacterium renale* and some other pathogenic bacteria. *J Gen Microbiol.* 1950 Sep;4(3):493–500.
50. Castro RA, Quecine MC, Lacava PT, Batista BD, Luvizotto DM, Marcon J, et al. Isolation and enzyme bioprospection of endophytic bacteria associated with plants of Brazilian mangrove ecosystem. *Springerplus.* 2014 Jul;3(1):382.
51. Kavamura VN, Santos SN, Silva JL, Parma MM, Ávila LA, Visconti A, et al. Screening of Brazilian cacti rhizobacteria for plant growth promotion under drought. *Microbiol Res.* 2013 May;168(4):183–91.
52. Azad MA, Bae JH, Kim JS, Lim JK, Song KS, Shin BS, et al. Isolation and characterization of a novel thermostable alpha-amylase from Korean pine seeds. *N Biotechnol.* 2009 Oct;26(3-4):143–9.
53. Li Xh, Yang Hj, Roy B, Wang D, Yue Wf, Jiang Lj, et al. The most stirring technology in future: cellulase enzyme and biomass utilization. *Afr J Biotechnol.* 2009;8(11):2418–22.
54. Hassan SE. Plant growth-promoting activities for bacterial and fungal endophytes isolated from medicinal plant of *Teucrium polium* L. *J Adv Res.* 2017 Nov;8(6):687–95.

55. Hu H, Gao Y, Li X, Chen S, Yan S, Tian X. Identification and Nematicidal Characterization of Proteases Secreted by Endophytic Bacteria *Bacillus cereus* BCM2. *Phytopathology*. 2020 Feb;110(2):336–44.
56. Maheshwari R, Bhutani N, Suneja P. Screening and characterization of siderophore producing endophytic bacteria from *Cicer arietinum* and *Pisum sativum* plants. *J Appl Biol Biotechnol*. 2019;7(5):7–14.
57. Kumar V, Kumar A, Pandey KD, Roy BK. Isolation and characterization of bacterial endophytes from the roots of *Cassia tora* L. *Ann Microbiol*. 2015;65(3):1391–9.
58. Brígido C, Singh S, Menéndez E, Tavares MJ, Glick BR, Félix MD, et al. Diversity and Functionality of Culturable Endophytic Bacterial Communities in Chickpea Plants. *Plants*. 2019 Feb;8(2):E42.
59. Schalk IJ, Hannauer M, Braud A. New roles for bacterial siderophores in metal transport and tolerance. *Environ Microbiol*. 2011 Nov;13(11):2844–54.
60. Rezzonico F, Zala M, Keel C, Duffy B, Moënné-Loccoz Y, Défago G. Is the ability of biocontrol fluorescent *pseudomonads* to produce the antifungal metabolite 2,4-diacetylphloroglucinol really synonymous with higher plant protection? *New Phytol*. 2007;173(4):861–72.
61. Babalola OO. Beneficial bacteria of agricultural importance. *Biotechnol Lett*. 2010 Nov;32(11):1559–70.
62. Rijavec T, Lapanje A. Cyanogenic *Pseudomonas* spp. strains are concentrated in the rhizosphere of alpine pioneer plants. *Microbiol Res*. 2017 Jan;194:20–8.
63. Remans R, Beebe S, Blair M, Manrique G, Tovar E, Rao I, et al. Physiological and genetic analysis of root responsiveness to auxin-producing plant growth-promoting bacteria in common bean (*Phaseolus vulgaris* L.). *Plant Soil*. 2008;302(1):149–61.
64. Soundar Raju C, Aslam A, Thangadurai D, Sangeetha J, Kathiravan K, Shajahan A. Indole acetic acid (IAA) producing endophytic bacteria on direct somatic embryogenesis and plant regeneration of *Exacum travancoricum* Bedd. *Vegetos*. 2020;33(4):690–702.
65. Khan Chowdhury MD, Jeon J, Ok Rim S, Park YH, Kyu Lee S, Bae H. Composition, diversity and bioactivity of culturable bacterial endophytes in mountain-cultivated ginseng in Korea. *Sci Rep*. 2017 Aug;7(1):10098.
66. Taghavi S, Garafola C, Monchy S, Newman L, Hoffman A, Weyens N, et al. Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of poplar trees. *Appl Environ Microbiol*. 2009 Feb;75(3):748–57.
67. Dawwam GE, Elbeltagy A, Emara HM, Abbas IH, Hassan MM. Beneficial effect of plant growth promoting bacteria isolated from the roots of potato plant. *Ann Agric Sci*. 2013;58(2):195–201.
68. Jasim B, Joseph AA, John CJ, Mathew J, Radhakrishnan EK. Isolation and characterization of plant growth promoting endophytic bacteria from the rhizome of *Zingiber officinale*. *3 Biotech*. 2014 Apr;4(2):197–204.
69. Egamberdieva D, Wirth SJ, Alqarawi AA, Abd Allah EF, Hashem A. Phytohormones and Beneficial Microbes: Essential Components for Plants to Balance Stress and Fitness. *Front Microbiol*. 2017 Oct;8:2104–2104.
70. Sah SK, Reddy KR, Li J. Absciscic Acid and Abiotic Stress Tolerance in Crop Plants. *Front Plant Sci*. 2016 May;7(571):571.
71. Badawy AA, Abdelfattah NA, Salem SS, Awad MF, Fouda A. Efficacy Assessment of Biosynthesized Copper Oxide Nanoparticles (CuO-NPs) on Stored Grain Insects and Their Impacts on Morphological and Physiological Traits of Wheat (*Triticum aestivum* L.) *Plant. Biology (Basel)*. 2021 Mar;10(3):233.
72. Fouda A, Hassan SE, Saied E, Hamza MF. Photocatalytic degradation of real textile and tannery effluent using biosynthesized magnesium oxide nanoparticles (MgO-NPs), heavy metal adsorption, phytotoxicity, and antimicrobial activity. *J Environ Chem Eng*. 2021;9(4):105346.
73. Puri A, Padda KP, Chanway CP. Evidence of nitrogen fixation and growth promotion in canola (*Brassica napus* L.) by an endophytic diazotroph *Paenibacillus polymyxa* P2b-2R. *Biol Fertil Soils*. 2016;52(1):119–25.
74. Ullah I, Khan AR, Park GS, Lim JH, Waqas M, Lee IJ, et al. Analysis of phytohormones and phosphate solubilization in *Photobacterium* spp. *Food Sci Biotechnol*. 2013;22(1):25–31.
75. Yadegari M, Farahani GH, Mosadeghzad Z. Biofertilizers effects on quantitative and qualitative yield of Thyme (*Thymus vulgaris*). *Afr J Agric Res*. 2012;7(34):4716–23.
76. Gharib F, Moussa L, Massoud O. Effect of compost and bio-fertilizers on growth, yield and essential oil of sweet marjoram (*Majorana hortensis*) plant. *ISSN OnlineMFA*. 2008;10:1560–8530.
77. El-Wahab A, Ellabban H, Moghith W. Combined effect of organic and biofertilizer on herb yield and essential oil production of *Origanum vulgare* L. plants under sandy soil conditions. *J. Agric. Res. Kafr El-sheikh Univ*. 2016;42:144–1592016.
78. Fouda A, Hassan SE, Abdel-Rahman MA, Farag MM, Shehaldeen A, Mohamed AA, et al. Catalytic degradation of wastewater from the textile and tannery industries by green synthesized hematite ($\alpha\text{-Fe}_2\text{O}_3$) and magnesium oxide (MgO) nanoparticles. *Current Research in Biotechnology*. 2021;3:29–41.
79. Fouda A, Hassan SE, Saied E, Azab MS. An eco-friendly approach to textile and tannery wastewater treatment using maghemite nanoparticles ($\gamma\text{-Fe}_2\text{O}_3$ -NPs) fabricated by *Penicillium expansum* strain (K-w). *J Environ Chem Eng*. 2021;9(1):104693.
80. Satyal P, Murray BL, McFeeters RL, Setzer WN. Essential Oil Characterization of *Thymus vulgaris* from Various Geographical Locations. *Foods*. 2016 Oct;5(4):70.
81. Lee JH, Lee K, Lee DH, Shin SY, Yong Y, Lee YH. Anti-invasive effect of β -myrcene, a component of the essential oil from *Pinus koraiensis* cones, in metastatic MDA-MB-231 human breast cancer cells. *J Korean Soc Appl Biol Chem*. 2015;58(4):563–9.
82. Al-Asmari AK, Athar MT, Al-Faraidy AA, Almuhaiza MS. Chemical composition of essential oil of *Thymus vulgaris* collected from Saudi Arabian market. *Asian Pac J Trop Biomed*. 2017;7(2):147–50.
83. Mancini E, Senatore F, Del Monte D, De Martino L, Grulova D, Scognamiglio M, et al. Studies on Chemical Composition, Antimicrobial and Antioxidant Activities of Five *Thymus vulgaris* L. Essential Oils. *Molecules*. 2015 Jul;20(7):12016–28.
84. Sharaf El-Din MN, Ahmed HA, Shalan MM, Hussien HM; The Effect of Organic and Bio-Fertilization on Vegetative Growth and Yield of Thyme. *Thymus vulgaris*, L. *Journal of Plant Production*. 2019;10(12):1175–85.
85. Ahmad SK, Hammam KA, Amer AA. Effect of bio-fertilization and some plant extracts on the growth, yield and chemical constituents of basil plant. *Journal of Plant Production*. 2014;5(2):193–210.