Egypt. J. of Appl. Sci., 37 (1-2) 2022 *31-57*

ISOLATION AND CHARACTERIZATION OF GERANIUM *(PELARGONIUM GRAVEOLENS)* BACTERIAL ENDOPHYTES

Mahmoud S. Abdel-Hamid[1] ; H.M. Atta[2] ; H.K. Abo El-Ela[1] ; A.A. El-Ghamry[2] and A. Fouda[2*]

1- Soil Fertility and Microbiology Department, Desert Research Center, El-Mataria, Cairo, Egypt.

2- Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo 11884, Egypt.

***E-mail: amr_fh83@azhar.edu.eg**

ABSTRACT

In the current study, eleven bacterial endophytes were isolated from the shoot system of aromatic medicinal plant *Pelargonium graveolens .L.* that inhabits North Sinai-Egypt . Out of eleven isolates, two identified on the basis of high nitrogen fixation as *Bacillus subtilis* (G5s) and *Bacillus velezensis* (G6s). The bacterial endophytic isolates showed varied activities as plant growth promoting agents. These two bacterial isolates have activity for ammonia production, siderophores and HCN. They exhibited enhanced qualitative Nitrogen fixation activity as well as quantitatively (nitrogenase assay) 365.667±1.8 C_2H_4/ml/24h and 87.767±1.6 C_2H_4/ml/24h respectively. They showed no significant assessment in phosphorus solubilization. The ability of the two isolates for producing phytohormone, indole-3-acetic acid (IAA) was detected using Salkowski's reagent and confirmed their productivity for IAA, gibberellic acid, abscisic acid, benzyl, kinetin, and ziaten by HPLC, G6s isolate exhibited maximum productivity of IAA with recorded 74.0 ± 2.1 mg mL^{-1} in the presence of 5 mg mL^{-1} tryptophan after 14 days. These two isolates had a high potential enzymatic activity for amylase, protease, cellulase, chitinase, and catalase except G6s that don't release amylase enzyme.

Key Words: bacterial endophytes, Geranium, biofertilization, nitrogen fixation, IAA.

INTRODUCTION

An endophyte is an endosymbiont, often a bacterium or fungus, that lives within a plant for at least part of its life cycle without causing apparent disease.

Endophytes are ubiquitous and have been found in all species of plants studied to date; however, most of the endophyte-plant relationships are not well understood. Some endophytes may enhance host growth, nutrient acquisition and improve the plant's ability to tolerate abiotic stresses, such as drought, and decrease biotic stresses by enhancing plant resistance to insects, pathogens and herbivores.

Endophytes are organisms living as symptomless colony, maybe during a part of their life cycle, inside the host plants **(Stone *et al.,* 2000)**.

The term **'endophyte'** was coined by **de Bary (1866)** to distinguish the epiphytic organisms living on surface of plant. Endophytes belong to diverse taxa such as bacterial, fungal, protistic, archaeal and are generally considered as mutualists. Endophytes are defined also as organisms isolated from surface-sterilized explants or from within the plant tissue and produce no harm to the host plant **(Hallman *et al.,* 1997)**. Use of the term 'infection' thus should be avoided to describe endophytes in general, except those endophytes involved in diseases as causal agents of disease of the host plant.

The first method divides endophytes into two categories: systemic (true) and non-systemic (transient). These categories are based on the endophyte's genetics, biology and mechanism of transmission from host to host **(Wani *et al.,* 2015)**.

Systemic endophytes are defined as organisms that live within plant tissues for the entirety of its life cycle and participate in a symbiotic relationship without causing disease or harm to the plant at any point. Additionally, systemic endophytes concentrations and diversity do not change in a host with changing environmental conditions **(Wani *et al.*, 2015)**.

Non-systemic or transient endophytes on the other hand vary in number and diversity within their plant hosts under changing environmental conditions. Non-systemic endophytes have also been shown to become pathogenic to their host plants under stressful or resource limited growing conditions **(Wani *et al*., 2015)**.

However, in contrast to the plant response to phytopathogens, few defense mechanisms have been described in plant response to plant growth promoting bacterial endophytes (PGPBEs). Moreover, certain plants have been shown to change their chemical responses when interacting with PGPBEs compared with non-beneficial bacteria, indicating that the plant does not recognize the PGPBEs as harmful agents **(Miché *et al.* 2006 and Rocha *et al.* 2007)**. Thus, if entophytes can produce the same rare and important bioactive compounds as their host plants, this would not only reduce the need to harvest slow growing and possibly rare plants but also preserve the world's ever diminishing biodiversity as well the host plant and their endophyte have a very agriculture and pharmaceutical importance for mankind. Furthermore, it is recognized that a microbial source of a valued product may be easier and more economical to produce, effectively reducing its market price.

Endophytic microbes produce many bioactive compounds that have different biological activities that can be plant growth-promoting (PGP) directly or indirectly. Most plants harbor endophytes within their tissue; however, the information available on PGPE and its biological activities is not proportional to

the high distribution of endophytes. A superior understanding of the native microbial endophytes in plants may clarify their capabilities and their potential in promoting plant growth and creating a sustainable system for crop production **(Hassan, 2017)**.

A biofertilizer is an ingredient comprises living microorganisms which, when added to seed, plant surfaces, or soil, colonizes the rhizosphere or the inner of the plant and stimulates development by enhancing the resource or obtainability of prime nutrients to the host plant **(Vessey, 2003).** The expression plant growth promoting rhizobacteria (PGPR) was primary used by **Kloepper and Schroth, (1978).** Some plant growth promoting rhizobacteria (PGPR) can be deliberated as biofertilizers, though others that encourage plant development by regulating harmful creatures are biopesticides.

Biofertilizers encourage plant growth by nitrogen fixation, phytohormones, phosphate and potassium solubilization **(Bashan and de-Bashan, 2005)**. Phosphate solubilizing bacteria (PSB) can change the insoluble inorganic phosphate mixtures, such as tri-calcium phosphate, di-calcium phosphate, hydroxyapatite, and rock phosphate, to plant obtainable forms. Numerous bacterial genera were documented to have P-solubilization action as *Pseudomonas*, *Bacillus*, *Rhizobium*, *Erwinia* and *Klebsiella* **(Yao *et al.*, 2006 and Walpola *et al.*, 2014)**.

Pelargonium graveolens (*P. graveolens*), commonly known as rose geranium is one of more than 250 species within the Pelargonium genus and that are native to the southern parts of Africa **(Mativandlela *et al.*, 2005)**. However, not all species of the genus reside there. Some have branched out and thanks to the spice trade and medical plant collection by sailors some species can now be found growing naturally in Australia, eastern Africa, New Zealand, the Middle East, the islands of Helena, Tristan de Chuna and Madagascar **(Herb Society of America, 2006 and Ćavar and Maksimović 2011).**

In This study, we primarily aimed to emphasis on isolation, effectiveness assessment, and identification of effective bacterial strains from Geranium, which can fix nitrogen, solubilize phosphorus, production of Indole acetic acid (IAA), siderophores, ammonia, HCN and catalase production.

MATERIALS AND METHODS

Material Used

The reagents, chemicals and media constituents applied within this investigation were delivered from Desert research center chemical store, Cairo, Egypt. Distilled water was used for accomplish all reactions.

DNA sequence analysis (16S rRNA full gene sequences) The Kits and universal primers used in molecular identification were analyzed and conducted by Macrogen, Incorporation which is a South Korea public biotechnology company for research services especially in identification. 16S rRNA gene sequences obtained from bacterial isolates were analyzed

afterwards using BLASTn tool at the National Center for Biotechnology Information database (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) analysis tools **(Altschul *et al.*, 1990)** to identify the most similar 16S rRNA sequences available in the GenBank.

Plant sampling and microbial endophyte isolation

Medicinal plant of Geranium *(Pelargonium graveolens .L)* family *Geraniaceae* collected in November 2017 from area located from Balouza research station, North Sinai, Egypt (31°00'20.6"N 32°33'46.1"E).

For good plant sample collection aimed to bacterial endophytes isolation a group of notes must be taken in consideration **(Strobel and Daisy, 2003)**. Collection should be for Plant samples from a unique ecological environmental nature and growing in special habitats, especially those with an uncommon nature. Plants with traditional medicines history and widespread, having an unusual long life. Plants growing in areas of great biodiversity.

The samples were reserved and transported to the laboratory and subjected to selective isolation procedures within short period of collection. Plant samples kept for reserving in sterile polyethylene bags. The plant identification was recorded in the field with help of local Balouza Research Station agricultural engineer, and the botanical identification was carried out at the herbarium unit of Desert Research Center.

For isolation of bacterial endophytes from plants there are some recommendations must take in consideration, it is better to collect healthy green shoot and root parts of plants, after selection brought the plant sample in two sterile bags or falcon tubes for every plant individually as shoot in one sterile bag or tube and the root also in that manner and immediately subjected aseptically in the microbiology laboratory for processing and preserving the remaining of each plant as a copy in refrigerator in case if needed for using or retest.

Preparation of plant samples (shoot and root) is processed as follow whereas the samples cut into 3 cm to 5 cm fragments then washed in tap water for 3-5 minutes for removing soil particles, adhered remains and microbes, afterwards washed in tween or very light detergent and then washed or rinsed with distilled water for removing any foreign particles or microbes on surface (epiphytes) followed with drying in laminar flow.

To sterilize plant surface successfully to eliminate epiphyte a procedure is followed with little modification defined with **(Petrini, 1992 and Werner *et al.*, 1997)**. Surface sterilization processed whereas samples were immersed 2 times in 70% ethanol for 3 minutes and immersed twice in (<5%) 2-4% aqueous solution of sodium hypochlorite for 5 minutes and again immersed for 1 minute in 70 % ethanol.

Lastly, rinse samples 6-8 times with sterile water for 5 minutes and wash 2 times in sterile distilled water for 5 min to remove surface

sterilization agents with further drying in sterilized paper in a laminar flow hood then preserved in sterile falcon tubes. Individually, each plant was preserved in a tube.

After surface sterilization process attained it may be successful or failed so it must be followed with checking of sterility.

Sterility check

The last washing water was checked for sterility of surface sterilization. 1ml of the final sterile distilled water that used in the final rinse plated onto nutrient agar and Czapek Dox (CD) agar **(McInroy and Kloepper, 1994)**. The success of surface sterilization method was confirmed when no microbial growth was detected on the cultural media.

Bacterial endophytes isolation

Antifungal drops nystatin was added (4 to 8 drops) for 100 ml pre prepared ashby's agar medium and nutrient agar media. The isolation process was conducted with two methods:

A. Under aseptic conditions the surface-sterilized segments were cut into about 1 cm length pieces then cut longitudinal or horizontal or both into small parts and squeezed gently with care then placed on nutrient agar plates and ashby's medium plates then incubated at 35°C for 2 days.
B. Aseptically, surface-sterilized segments were aseptically squeezed in 100 ml of sterile saline cup by glass rod. About 200 − 400 micro liters of the squeezed mixture was pipetted by automatic pipette with sterile tips into nutrient agar and ashby's agar petri dishes then mixed gently by rotating the dishes. All plates were kept in an incubator at 35°C for 48 hrs.

Subsequently, the purity of bacterial growth of internal tissues or crushed segments were assured and processed. The morphologically different endophytic bacteria were further purified by repeated copying on nutrient agar (N.A.) plates. The pure cultures obtained were transferred to fresh N.A. slants as well as the subcultures then stored below 8 °C for further study.

Endophytes isolates characterization:

1. Nitrogen fixation.

a. Nitrogen fixation qualitative:

In this test the bacterial isolates developed for assaying nitrogen fixation ability qualitatively. Also, in this test the best effective isolates selected depending on this test results. The test is conducted by inoculating the isolates on nitrogen deficient of Modified Ashby's broth

medium (MABM; containing g/L: Mannitol, 10.0; Sucrose, 10.0; K_2HPO_4, 0.5; $MgSO_4{\cdot}7H_2O$, 0.2; NaCl, 0.2; $CaSO_4$, 0.1; $CaCO_3$, 5.0; $MnSO_4.7H_2O$, traces; $FeCl_3$, traces; $Na_2.MoO_4.2H_2O$, traces; distilledH_2O, 1L; pH, 7) **(Abd El-Malek and Ishac, 1968)** for 7 days at 35 ± 2°C then checking them to determine the best growth. The incubation period is observed for the growth.

After incubation period the growth of bacteria screened, the plates that showed bacterial growth indicates qualitative evidence for atmospheric nitrogen fixation by endophytic isolates, which is recorded as a positive test. On the other hand, isolates which cannot fix atmospheric nitrogen cannot grow on this medium indicating a negative result. Thereafter, best isolates according to their growth selected for the second step.

The best isolated bacterial endophytes that can grow well in modified Ashby's agar medium selected according to their nitrogen fixation qualitatively as mentioned before to pass the rest of tests

b. Nitrogen fixation quantitative (nitrogenase activity assay):

Acetylene reduction assay or nitrogenase activity was assayed for the greatest active isolate in N_2–fixation by acetylene reduction technique **(Schöllhorn and Burris,1967)**. The bacterial endophytic isolates inoculated in test tubes containing ashby's broth medium with single different colonies for each different isolate respectively. For this process the tubes closed with carful and tight and then incubated for 72 h at 35°C. After incubation period to come to the end ten percent of pure acetylene (C_2H_2) was inoculated into the tubes which were incubated for 4 hrs, also 0.1 of gas samples were quiet for the determination of C_2H_4 formed using Hewlett-Packard 5890 gas chromatography Series 2 plus, Standard pure ethylene was diluted with air in addition acetylene and mixed together in distinct containers to get concentrations including from 100 to 1000 ppm; this was used as reference for calculating the ethylene concentration in the sample. Results were calculated as ethylene produced/ml liquid culture /day.

2. Phosphate solubilization efficiency:

Phosphorus standard curve

Standard graph plotted to gradually different phosphorus concentrations according to the following method to determine phosphate dissolving efficiency. Different phosphorus concentrations are prepared earlier used as solution of phosphorus started from 0 microgram/ml to 200 microgram/ml whereas for each 1 ml of the standard solution. Reads are recorded within 10-30 minutes at 530 nm by spectrophotometer T60 UV-visible spectrophotometer. Standard graph is designed whereas

concentration of phosphorus in micrograms/ml on X axis versus optical density at 530 nm on Y axis.

A. Phosphate solubilization efficiency qualitatively:

Qualitative Phosphate solubilization capability is tested for the selected bacterial endophytic isolates whereas the isolates are inoculated as spots in *modified* pikoviskaya *agar medium.* A clear zone is shown for the isolates that had ability for phosphate solubilization. The clear zone is estimated around the growing colonies after the incubation period of 7 days at 35 ± 2˚C.

B. Phosphate solubilization efficiency quantitatively (soluble phosphorus):

Quantitative Phosphate solubilization efficiency was assessed by inoculating bacterial isolates endophytes in pikoviskaya broth medium, which was earlier supplemented with the addition of 0.9g rock phosphate per 100 ml medium, in 1 ml tube of bacterial suspension. The tubes are put in incubator for incubation ten days at 35 ± 2˚C. After incubation period, the incubation process followed with centrifugation at 6000 rpm for 15 minutes, the supernatant was used in to find the quantity of dissolved phosphorus with the aid of method designed by **Watanabe and Olsen (1965)**.

3. Indol acetic acid (IAA) production:

Indol acetic acid standard curve.

For IAA evaluation an IAA standard graph was plotted. Varied IAA concentrations are prepared earlier as a solution of IAA from 0 µg/ml to 200 µg/ml. To estimate IAA a standard graph of IAA was plotted as described by **Glickmann and Dessaux, (1995)**. For this process a different IAA concentration are made earlier as a solution of IAA including from 0 µg/ml to 200 µg/ml. Where for each 1 ml of the standard solution, 2ml of Salkowaski reagent (300 ml concentrated Sulfuric acid; 500 ml distilled water; 15ml 0.5 M $FeCl_3$) is added and the reads are recorded after 25 minutes at 530 nm by spectrophotometer. So standard graph is plotted where concentration of IAA in µg/ml on X axis versus optical density at 530 nm on Y axis.

A. Indol acetic acid qualitative production

For this purpose, suitable media (nutrient agar) complemented with different tryptophan conc. (0, 1, 2 and 5 mg/ml) was used to develop bacterial endophytic isolates. The incubation period adjusted for 15 days at 35± 2°C. After incubation the cultures were centrifugated at 3000 rpm for 30 minutes. Two milliliters of the supernatant were mixed with 2 drops of orthophosphoric acid and 4 ml of Salkowski's reagent (300 ml concentrated Sulfuric acid; 500 ml distilled water; 15ml 0.5 FeCl3). Appearance of a pink color proves the IAA production. Optical densities were read at 530 nm using T60 UV-visible spectrophotometer. Standard IAA graph **(Loper and Schroth, 1986)** was used for estimation of produced IAA amount.

B. Indol acetic acid quantitative production

Best tryptophan concentration is selected based on qualitative testing; the selected concentration is used to assess the efficacy of endophytic bacteria quantitatively at several interval times from 2 to 14 days. Bacterial isolates were developed in Nutrient Broth. Incubation is set for 24h. at 35± 2°C. Two ml of each bacterial broth was inoculated to the 20 ml of liquid medium containing 5 g/L of tryptophan (the best concentration of tryptophane for IAA production), incubation period for two weeks.

One ml of each liquid medium was separated and preserved from 20 ml incubating broth from 2nd day up to 14th day with 2 days interval and passed for separation by centrifugation at 3000 rpm for 30 minutes. One ml of the supernatant was mixed with 1 drop of orthophosphoric in addition to 2 ml of Salkowski's reagent. Development of a pink color indicated the evidence of IAA production. Optical density was read at 530 nm using T60 UV-visible spectrophotometer. According to **Ahmad *et al*, (2005)** the amount of IAA produced was obtained by the standard IAA graph.

4. Ammonia production:

For ammonia production test a qualitative method was used for this purpose, peptone water medium used to detect ammonia production efficacy for bacterial isolates. In the beginning, bacterial isolates developed for a day to use as a fresh growth, then followed by the inoculation in a tube containing 10 ml peptone water. The tubes were incubated at 35°C for two days. After incubation the bacterial isolates directed for ammonia production test after incubation whereas the test is processed by the addition from 5 to 10 drops of Nessler reagent as a colorimetric reagent, development a yellow color from faint yellow to yellow color in the tube bottom of the peptone water broth medium after adding the drops is an indication for ammonia production positive result, and if not developed is an indication for a negative result.

5. Testing the extracellular enzymatic activities for bacterial isolates.

To study some phycological behaviors of bacterial endophytes such as enzymatic activities an agar diffusion method employed for that. In this study some an extracellular enzyme (amylase, cellulase and protease) is assayed by using a mineral salt (MS) agar media (MSA; containing g/L: $NaNO_3$, 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; distilledH_2O, 1L) enhanced with related varied additives according to the tested enzyme, amylase and cellulase activity were examined developing the bacterial endophytes on MS agar medium complemented with 1% soluble starch and 1% cellulose or carboxy-methylcellulose (CMC) respectively. The result is observed after incubation period whereas the petri dishes were swamped or covered with 1% iodine. MS agar medium complemented with 1% gelatin was used to check bacterial proteolytic behavior or activity. The degradation of gelatin

was highlighted using acidic mercuric chloride as an indicator (**Lv *et al.*, 2010**). Control for all treatments composed of the same test media without bacterial inoculation. The results are observed after incubation period ranging from day to two days depending on the growth ranges of the bacterial endophytes isolates at temperature 35 ± 2 °C. For screening the outcomes, the test specific reagents were added up and the clear zone size closely encircling the bacterial colony was assessed or measured, indication extracellular enzymatic activities. As well catalase enzyme examined where it is induces the separation of hydrogen peroxide to water and oxygen. The assessment is conducted by adding drops ranging from 1 to 4 of hydrogen peroxide to bacterial broth or suspension (bacterial growth developed in nutrient broth for 24 hours) about 100 u on slide. Evolution of air bubbles is an indication for the activity of catalase enzyme which is considered a positive result. The stable state for the surface without bubbling is considered a negative result (**Rørth and Jensen, 1967**).

6. Siderophores production

For siderophores production detection Blue Chrome Azurol S (CAS) agar medium was used. Inoculation of five µl of fresh cultures was added onto a plate then incubated at 28 °C for three days. The positive results are indicated by appearing a halo from yellow to orange color around the bacterial colony which is changing in color from dark blue to yellow-orange **(Lacava *et al.*, 2008).**

7. HCN production

For HCN production (HCN) Kings B agar medium is used as described by **Geetha *et al.*, (2014)**. The test is performed by streaking the bacterial isolates on Kings B agar medium premixed with glycine, a filter paper Whatmann No.1 soaked in (2% sodium carbonate in 0.05% picric acid solution) was positioned firmly in the lid of petri dishes, Then the plates were sealed with parafilm and the incubation period set to ten days at 35 ± 2°C. The positive result for HCN production is indicated by changing the color of filter paper from deep yellow to reddish-brown as mentioned by **Bakker and Schipperes (1987)**.

8. Identification of the bacterial endophyte isolates with 16S rRNA gene amplification and sequencing

Endophytic bacterial isolates were identified based on routine cultural and morphological characteristics and microscopical features ordinary basis. In molecular identification step, the DNA was extracted by following subsequential procedures for identification.

Molecular identification was carried out on the basis of amplification and sequencing of bacterial 16S rRNA gene, where genomic DNA was extracted according to practical method and the PCR protocol.

The genetic identifications of bacterial isolates were processed by grown the isolates on nutrient agar plates for 2 days then sent to Macrogen

Scientific Services Co. to accomplish process of DNA extraction and 16s sRNA sequencing identification.

8.1 Nucleotide sequence accession numbers

The 16S rRNA gene sequences obtained in this study were deposited in NCBI GeneBank/EMBL/DDBJ under the accession numbers for the strains. The accession numbers for the closely related strains are illustrated in the results section. Sequences were then compared with 16S rRNA sequences in the GenBank database using BLAST (Basic Local Alignment Search Tool).

RESULTS AND DISCUSSION

Isolation of endophytic bacteria from Geranium (*Pelargonium graveolens* .L) roots in Sinai soil

Rose Geranium (*Pelargonium graveolens .L*) plant common in Sinai soil that inhabits several areas at Sinai. *Pelargonium graveolens .L* (Rose Geranium) has importance in medicinal and pharmacological field where is a novel therapeutic agent for antibacterial, antioxidant, antifungal and diabetics **(Hamidpour *et al*., 2017)**. Also, rose geranium has many uses in food industry beside its effect as pesticide. The endophytic microbes inhabit rose geranium tissues may be contributed to the biological activities of this plant. The studies about the microbial communities of rose Geranium up to now are few.

In the present study, a count of eleven bacterial isolates seven from shoot encoded as G1s to G7s and four from root encoded as G8r to G11r where attained from sterilized shoot and root parts of *Pelargonium graveolens .L* which gathered from Balouza Research Station, North Sinai, Egypt. The isolated endophytic bacterial strains were subjected to primary identification based on morphological, physiological, and biochemical test according to standard keys.

Selection and determination the most potent endophytic bacterial strain

Nitrogen and phosphorus are the main element of nutrient for plant growth. In general events, these important nutrients are not obtainable for plants. Bacterial endophytes playing important role to facilitate the uptake of these nutrients for plants **(Kalayu, 2019).** Endophytes accomplish this by increasing the uptake of valuable land limited nutrients from the soil such as phosphorus and making other plant nutrients available to plants such as rock phosphate and atmospheric nitrogen which are normally trapped informs that are inaccessible to plants, **(Rai *et. al*, 2014)**. So, in the present investigation, the most potent endophytic bacterial isolate is basically established on the maximum nitrogen fixation and phosphate solubilization. The Data in the Table (1) showed that all the bacterial isolates G1s to G7s and G8r to G11r able to fix atmospheric nitrogen with varied degree according to blue color of nitrogen deficient ashby's agar medium. It was

observed that the maximum color alteration is for G5s and G6s. on the other hand all isolates not able to solubilize phosphate intended as clear zone on pikoviskaya agar media whereas all isolates give negative result. After reviewing the previous data, the bacterial endophytic isolates G5s and G6s were selected for identification and examining their ability as plant growth-promoting.

Table 1. Selection of the most potent bacterial endophytes based on nitrogen fixation and phosphate solubilization activity.

Endophytic bacterial strain	Nitrogen fixation	Phosphate solubilization clear zones	Endophytic bacterial strain	Nitrogen fixation	Phosphate solubilization clear zones
G1s	++	0	G8r	++	0
G2s	+	0	G9r	+	0
G3s	+	0	G10r	++	0
G4s	+	0	G11r	++	0
G5s	+++	0			
G6s	+++	0			
G7s	++	0			

-, +, ++ denotes negative, moderate, and highly nitrogen fixation based on color change.

Identification of the most potent endophytic bacterial strain

The chosen G5s and G6s were classified as most potent bacterial isolates according to nitrogen fixation were identified with molecular identification through 16S rRNA gene sequencing.

G5s isolate sequences indicated 99.4% similarity with *Bacillus subtilis* sequences where G6s isolate sequences showed similarity 99.8% with *Bacillus velezensis* (Table 2, Fig. 1). According to the previous results *Bacillus* spp. Were common bacterial isolates from *Pelargonium graveolens .L.* Romero et al. and co-workers reported that *Bacillus* is the most common bacterial species identified as endophytes, **(Romero *et al*., 2014)**.

Bacillus velezensis growth-promoting capacity described in some works as a PGPR which considered as an important characteristic of the bacterium, **(Teixeira *et al*., 2021).**

Table 2. Two most potent bacterial endophytes identification isolated from *Pelargonium graveolens .L* using 16S rRNA sequence analysis and quantitative evaluate of their nitrogen fixation.

Bacterial endophytes Code/accession number	Homologue Sequences (%)	NCBI Accession Numbers	Nitrogen fixation (nitrogenase n-mole C_2H_4/ml/24h)	Ammonia production
G5s/MZ617398	*Bacillus subtilis* (99.4%)	NR027552.1	365.667 ± 1.8	+++
G6s/LC603785	*Bacillus velezensis* (99.8%)	NR075005.2	87.767 ± 1.6	+++

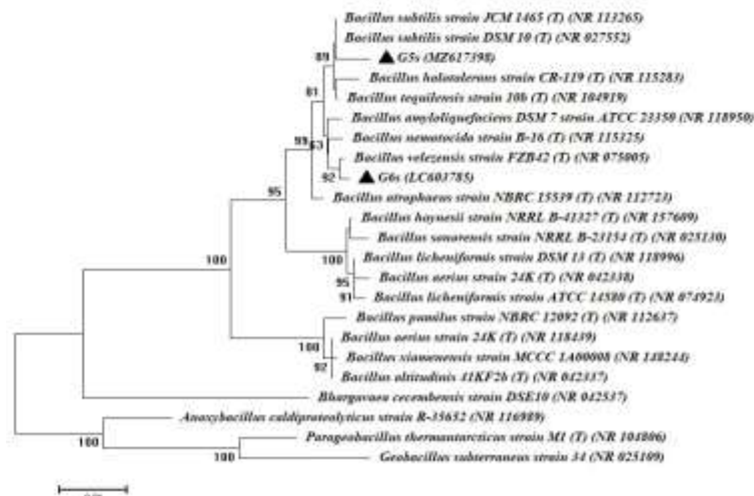

Fig (1). Phylogenetic tree of 16S rRNA sequences of two most potent endophytic strains isolated from *Pelargonium graveolens .L*, the sequences of the isolated bacterial endophytes compared with those recalled from NCBI.

Characterization of the endophytic bacterial isolates as potential plant growth-promoting agents

Nitrogen assay through acetylene reduction

Nitrogen is one of the most important elements in biological systems, comprising the main building blocks of nucleic acids, enzymes and proteins among its multiple functions. In nature, it exists primarily in the gaseous form and constitutes approximately 78% of the atmosphere. Despite its abundance, nitrogen (N) is one of the most growth-limiting nutrients in terrestrial and aquatic ecosystems **(Dalton and Krammer, 2006)**. Biological N fixation (BNF), is process in which certain prokaryotic microorganisms such as endophytes, known as diazotrophs also bacterial endophytes, fix N by breaking down the triple bond of dinitrogen using a highly specialized enzyme complex called nitrogenase enzyme and convert it to ammonia, **(Myrold and Bottomley 2007)**.

In the presented study, quantitative nitrogen fixation is assayed beside qualitative nitrogen fixation (Data in Table 1). Nitrogenase or reduction of acetylene used to assay quantitative nitrogen fixation. The data represented in the Table 2 showed G5s and G6s have the efficacy to release nitrogenase enzyme with varied degree. *Bacillus subtilis* G5s showed higher amount than *Bacillus velezensis* G6s in nitrogenase assay through reduction of acetylene. *Bacillus subtilis* G5s showed activity with capacity 365.667 ± 1.8 C_2H_4/ml/24h whereas *Bacillus velezensis* G6s showed activity with capacity 87.767 ± 1.6 C_2H_4/ml/24h. In similar manner, six strains of *Pseudomonas aeruginosa* grown on LGI media showed efficient reduction of acetylene, ranging from 23.34 ± 1.8 to 28.91±3.5 nmol C2H4 h-1 mg protein-1 **(Gupta *et al.* 2013)**.

Testing of Phosphate-solubilization qualitatively and quantitatively

Phosphorus is one of the macronutrients of which a high amount is required for plant growth promotion. In most cases, phosphorus is present in the soil as insoluble inorganic forms; interestingly, different rhizospheric and endophytic fungal strains have the efficacy to convert it from an unavailable to available source for plant uptake **(Patle *et. al.*, 2018)**.

In the current investigation, the phosphate solubilizing activity for the two bacterial endophytes strains (*Bacillus subtilis* G5s and *Bacillus velezensis* G6s) were qualitatively evaluated on pikoviskaya media amended with tri-calcium phosphate as a source for inorganic phosphate. Unfortunately, none of the studied isolates (*Bacillus subtilis* G5s and *Bacillus velezensis* G6s) were able to create clear zones, (Table 1). Also, phosphate solubilizing activity were quantitively evaluated for the two bacterial endophytes strains through Pikovskaya's broth medium supplemented with $Ca_3(PO_4)_2$ by measuring the amount of soluble phosphate in the medium. The results showed that the amount of liberated phosphorus at ninth day for *Bacillus subtilis* G5s and *Bacillus velezensis* G6s with values 12.41 ± 0.797 µg/ml and 11.065 ± 0.936 µg/ml compared with control 28.643 ± 0.472 µg/ml respectively which is an indication for none significant result which meaning in other words that G5s and G6s unable to solubilize phosphorus. Fig (2), some reports had mentioned that not all endophytic bacterial isolates able to solubilize phosphate, **(Munir *et al* 2019)** reported that among the six isolates, there isolates (EPS36, EPS37, and EPS41) exhibited phosphate solubilization activity.

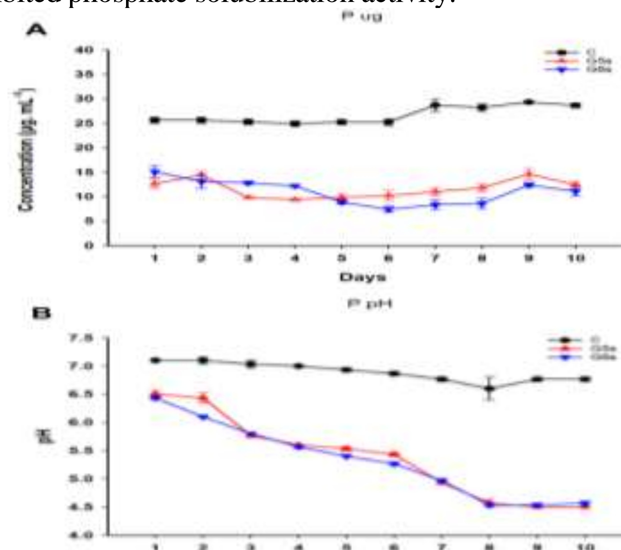

Fig (2). Quantitative phosphate solubilizing assay in Pikovskaya's broth medium supplemented with $Ca_3(PO_4)_2$. A is denoting the amount of liberated phosphorus (µg mL^{-1}) at interval times; B is denoting the pH values of Pikovskaya's media at interval times.

Ammonia production

Endophytic bacteria have the ability to promote plant growth through ammonia production and solubilization of phosphates (**Afzal *et. al*, 2017**). Endophytic bacteria that develop beneficial metabolites for the plant such as ammonia, can sustain plant root and shoot, beside maximize plant fresh weight of the plant inoculated with bioinoculants (**Passari *et. al*, 2016**). Ammonia can assist meet the nitrogenous demands of the plant and also aids the plant to minimize colonization and invasion by plant pathogens (**Rodrigues *et al*, 2016).** The presented study showed that bacterial endophytes *Bacillus subtilis* G5s and *Bacillus velezensis* G6s have similar results about ammonia production through testing ammonia in which the color change is happened in inoculated tubes with peptone water broth medium treated with adding Nessler's reagent. Microorganisms also produce ammonia from the hydrolysis of urea into ammonia and carbon dioxide. **Eid, (2021)** reported that frequent nitrogen input in the soil primarily raises crop production cost; hence, ammonia production by endophytes being a desirable trait for plant growth promotion and soil fertility.

Testing of extracellular enzymatic efficacy

Choi and co-authors reported that extracellular enzymatic production through endophytes it may involve in a resistance strategy of the host plant against pathogenic microorganisms beside improving plant nutritional conditions (**Choi *et al.*, 2005**).

Extracellular enzymatic efficacy of isolated bacterial endophytes was qualitatively assessed with the agar plate method. The results revealed that *Bacillus subtilis* G5s has positive results for all tested enzyme than *Bacillus velezensis* G6s whereas *Bacillus velezensis* G6s showed negative result for amylase. Clearly, G5s expressed higher activity than G6s for cellulase enzyme production whereas it recorded clear zone with 39.7±4.4 mm. on the other hand *Bacillus velezensis* G6s showed activity higher than *Bacillus subtilis* G5s for protease and chitinase production in which it was recorded 35.00±0.0 mm and 23.2 ± 1.2 mm respectively. Moreover, *Bacillus velezensis* G6s clearly recorded catalase activity than *Bacillus subtilis* G5s where it recorded (++) against (+) for G5s. Catalase enzyme is known to be primary line associated in defense strategy in microbes, which is it share in protection whereas its action against harmful free radicals, the free radicals arises from biotic and abiotic stresses, therefore, catalase indirectly promote plant growth indirect (**Kumar *et al.*, 2012**), similarly. **Amirita *et al.* (2012)** reported that 72% of endophytic isolates from several medicinal plants were good in amylase and protease production. Extracellular enzymatic activity of endophytes plays potential role in degrading macromolecules like protein and polysaccharides throughout plant growth. From another view of

biotechnology, amylolytic and proteolytic enzymes of bacterial endophytes are being interestingly studied to improve industrial processes for polysaccharides and protein biodegradation. The results are in harmony with previous published studies that reported *Bacillus* spp. are thought involved as highest endophytic isolates that produce various lytic enzymes (**Passari *et al.*, 2016 and Mahgoub *et al.*, 2021**).

Table 3. Enzyme activity, siderophores, and HCN production of *Bacillus subtilis* G5s and *Bacillus velezensis* G6s isolates.

Bacterial strain	Enzyme activity (mm)				Catalase	Siderophores	HCN
	Amylase	Cellulase	Protease	Chitinase			
G5s	8.33 ± 1.2	39.7 ± 4.4	35.00 ± 0.0	23.2 ± 1.2	+	+	+++
G6s	0.0 ± 0.0	32.0 ± 0.6	36.67 ± 1.2	22.2 ± 0.9	++	+	+++

Siderophores activity

Saha et al., (2016) reported that The soil with iron deficiency, several microorganisms able to produce and secrete metabolite called siderophores with low molecular, which bind Fe3+, Fe2+, and other divalent metal ions, these divalent metal ions are very important for plants. Besides delivery to the plants, siderophores also have an imoprtant role in scavenging undesired metal ions that present in the rhizosphere layer to stop uptake by the roots. These undesired metal ions include Zn, Cd, Cr, Al, and Pb and, also radioactive ions like U or Np to make reduction in toxicity for the plants **(Neubauer *et al.*, 2000)**.

Rana *et al.* (2020) and Fouda *et al.* (2021) described that siderophores have the ability to stimulate plant-induced systematic resistant response to provide relieve trace metals toxicity to plant growth. In the present study, bacterial isolates *Bacillus subtilis* G5s and *Bacillus velezensis* G6s able to synthesis and produce siderophores through creating orange halo zone surrounding the bacterial spots growing on king's B agar medium. The process of plant detoxification from undesired metal ions in soil by inoculating the plants with endophytic bacteria that is promising and helpful practice to enhancing plant growth.

HCN production

HCN is a secondary metabolite that produced by PGPR, is considered a biological control agent against phytopathogens. Among 650 PGPB, production of HCN is comparable with SD (siderophore) and PS (phosphate solubilization) activity. Exploitation expenditure of HCN-producing rhizobacteria will assume an important role in sustainable agriculture **(Pandey *et al.*, 2021)**. In the present investigation, *Bacillus subtilis* G5s and *Bacillus velezensis* G6s have the ability to produce HCN through altering the color of filter paper from deep yellow to reddish-brown in approximately similar result. As mentioned previously *Bacillus* spp. Producing HCN in high capacity and this in harmony with previous study **(Passari *et al.*, 2016)**. Several PGPB strains have potential ability to

synthesize hydrogen cyanide and ammonia. This synergetic effect is considered leads to the regulation process of growth rate and metabolites of the plant (**Agbodjato *et al.*, 2015**).

Assaying of phytohormones using HPLC

Phytohormones are considered an important growth regulator. They synthesized naturally in defined plant organs. They have a prominent impact on metabolism of the plant **(Kazan, 2013)** and play an important role in the process of mitigation of abiotic stresses **(Teale *et al.*, 2006 and Hu *et al.*, 2013).**

Gibberellin is an important plant growth regulator, which has a vital role in seed dormancy, formation of floral organs, and lateral shoot growth **(Olszewski *et al.*, 2002).** The available literature clearly reveals the ameliorative impact of gibberellic acid against salinity. Gibberellic acid was found to stimulate plant growth and development under various abiotic stress conditions (**Ahmad, 2010**).

Indole- 3-acetic acid (IAA) is an auxin phytohormones. It was revealed to promote many growths and developmental consequences, as cell division, plant elongation, and differentiation **(Asgher *et al.*, 2015).** Tryptophan is the precursor and similar to IAA is synthesized from and chemically similar to tryptophan.

ABA has an important role in plants life by bettering adaptation and stress responses. It involved in the regulation of growth. Many reports encouraging the role of ABA in integrating signaling at the time of stress exposure with subsequent control of downstream responses **(Wilkinson *et al.*, 2012**). It was revealed to promote several growth and developmental events, such as cell division, elongation, and differentiation **(Asgher *et al.*, 2015).**

6-Benzylaminopurine or benzyl adenine, is considered from the first-generation synthetic cytokinin that help in eliciting plant growth beside development responses, setting blossoms as well as help in stimulating fruit richness through stimulating of cell division (**Siddiqui *et al.*, 2011**).

Kinetin (/ˈkaɪnɪtɪn/) is a type of cytokinin, a class of plant hormone that promotes cell division. Fifty years ago, scientists from the Botany and Biochemistry departments at the University of Wisconsin announced the isolation, crystallization, characterization, and synthesis of 6-furfurylaminopurine (**Miller *et al.*, 1955a**,**b and Miller 1956**), a plant hormone in a class now referred to as cytokinins (**Skoog *et al.*, 1965**). They proposed the trivial name kinetin for this substance, and described its ability to promote cell division in test tissues from tobacco (*Nicotiana tabacum*).

Zeatin is a cytokinin derived from adenine, which occurs in the form of a cis- and a trans-isomer and conjugates. Zeatin was discovered in immature corn kernels from the genus Zea. Zeatin and its derivatives occur

in many plant extracts and are the active ingredient in coconut milk, which causes plant growth **(David *et al.*, 1994**).

Phytohormones production ability for both *Bacillus subtilis* G5s and *Bacillus velezensis* G6s was assayed by using HPLC for both acidic side (GA3, IAA, and ABA) and alkaline side (Benzyl, Kinetin, and Zeatin). For the first one gibberellic acid (GA3), the bacterial strain *Bacillus velezensis* G6s produced higher amount 1.89 ± 0.262 mg/100ml than *Bacillus subtilis* G5s with amount 1.27 ± 0.12 mg/100ml. Similarly, in IAA test *Bacillus velezensis* G6s exhibited higher value 0.36 ± 0.08 mg/100ml than *Bacillus subtilis* G5s value 0.18 ± 0.012 mg/100ml. lastly, in acidic side Abscisic acid (ABA) *Bacillus velezensis* G6s gave value 0.03 ± 0.00 mg/100ml higher than *Bacillus subtilis* G5s value 0.02 ± 0.003 mg/100ml. On the other hand, alkaline side phytohormones (Benzyl adenine, Kinetin, and Zeatin) *Bacillus velezensis* G6s gave amounts higher than *Bacillus subtilis* G5s. In Benzyl adenine, *Bacillus velezensis* G6s yielded 0.32 ± 0.004 mg/100ml whereas *Bacillus subtilis* G5s gave 0.24 ± 0.015 mg/100ml. Secondly, Kinetin test for *Bacillus velezensis* G6s yielded 0.12 ± 0.008 mg/100ml whereas *Bacillus subtilis* G5s gave 0.11 ± 0.009 mg/100ml. Thirdly, Zeatin test *Bacillus velezensis* G6s gave 0.74 ± 0.03 mg/100ml but *Bacillus subtilis* G5s gave 0.44 ± 0.01 mg/100ml. In the current study, the two isolate G5s and G6s have the ability to release ABA at low concentrations. Similarly , 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, kinetin, and benzyl adenine with values of 31.2, 12.3, and 3,24 µg mL^{-1} respectively **(Passari *et al.*, 2016)**.

Table 3. phytohormones activity assessment (GA3, IAA, ABA, benzyl adenine, kinetin, and zeatin) by HPLC analysis *Bacillus subtilis* G5s and *Bacillus velezensis* G6s.

isolates codes	acid side mg/100ml			alkaline side mg/100ml		
	GA3	IAA	ABA	Benzyl adenine	Kinetin	Zeatin
G5s	1.27 ± 0.12	0.18 ± 0.012	0.02 ± 0.003	0.24 ± 0.015	0.11 ± 0.009	0.44 ± 0.01
G6s	1.89 ± 0.26	0.36 ± 0.08	0.03 ± 0.00	0.32 ± 0.004	0.12 ± 0.008	0.74 ± 0.03

Production of IAA

The plant growth phytohormones like gibberellins, IAA and cytokinin have an important role in the interactions between bacteria and plants **(Dobbelaere *et al.*, 2003)**. A wide range of variation existed in the IAA producing ability between the endophytic isolates. IAA production by numerous rhizobacteria and *Azospirillum sp.* in the absence of external supply of tryptophan was described by **Wang *et al.* (1984) and Thuler *et al.*, (2003)**. The produced amount of IAA by the different isolates was enhanced by the adding supplement tryptophan which is a precursor in the medium, **Karthigai and Purushothaman (1996)**.

In the present study, the isolates *Bacillus subtilis* G5s and *Bacillus velezensis* G6s were examined for their ability to produce IAA through 15 days of incubation at conditions of absence and presence of tryptophan (0, 1, 2, and 5 mg mL^{-1}) based on it is the precursor of IAA. Based on the collected data as a result from the test it is clear that the capability of the two isolates for producing IAA is no significant in the absence of tryptophan compared to control. In the case of tryptophan concentrations 1, 2 and 5 mg mL^{-1} the bacterial isolate *Bacillus velezensis* G6s producing higher IAA qualitatively with amounts (49.0 ± 0.9 mg mL^{-1}, 47.1 ± 0.2 mg mL^{-1} and 92.7 ± 2.1 mg mL^{-1}) respectively, which is significant indicator ($p \leq 0.001$) as compared with control with amounts (14.1 ± 0.2 mg mL^{-1}, 16.4 ± 0.4 mg mL^{-1} and 18.1 ± 0.4 mg mL^{-1}) respectively and *Bacillus subtilis* G5s (2.3 ± 0.1 mg mL^{-1}, 3.3 ± 0.2 mg mL^{-1} and 14.1 ± 0.7 mg mL^{-1}) for 1, 2 and 5 tryptophan concentrations respectively.

Passari *et al.*, (2016) describes that out of 52 bacterial endophytes isolates from *Clerodendrum colebrookianum* medicinal plant, only 48 isolates revealed activity for IAA production in a media of nutrient broth amended with tryptophan concentration 2 mg mL^{-1}.

In accordance with qualitative assessment results, 5 mg mL^{-1} is selected for assaying quantitively at interval periods because it being the best concentrations for producing IAA. The data in Figure 3 revealed that the values of IAA production on basis of dependence of time is increased as long as increasing in time of incubation. *Bacillus velezensis* G6s still had the maximum IAA production quantitively with records 74.0 ± 2.1 mg mL^{-1} after 14 days of incubation.

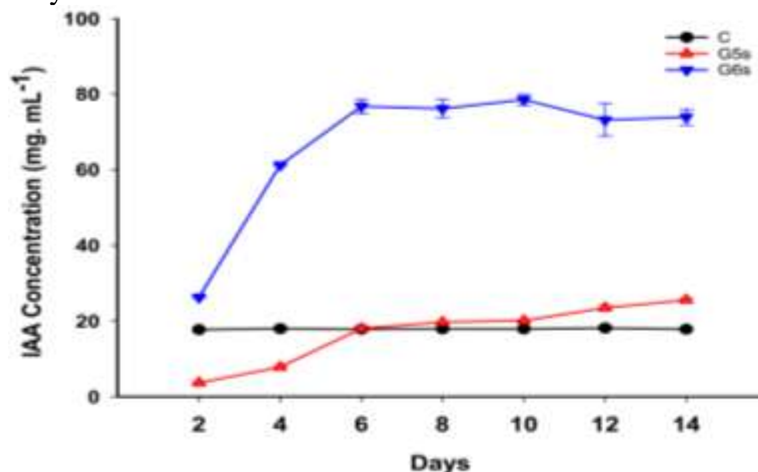

Fig. 3. Indole-3-acetic acid (IAA) production by *Bacillus subtilis* G5s and *Bacillus velezensis* G6s isolated from *Pelargonium graveolens .L.* The quantitative IAA assay in broth media supplemented with 5 mg mL^{-1} tryptophan at various time courses (2 to 14 days).

Dawwam *et al.*, (2013) ; Fouda *et al.*, (2015) and Mahgoub *et al.*, (2021) and co-workers reported studies compatible with these results of the current investigation where they revealed that the IAA production by some bacterial and fungal endophytic isolates are depending on tryptophan concentration in specially on liquid media plus incubation times.

Lin and Xu (2013) described that microbial endophyte that able to produce IAA play a significant role in the process of mutualistic interactions that happen between the host plant plus endophytes and, thus, able to regulate the plant growth. **Hassan *et al.* (2017)** reported that *Bacillus cereus* and *Bacillus subtilis* strains isolated from shoot of *Teucrium polium* L. collected from the same area possessed the efficacy to produce IAA with maximum values ranging between 4.1 and 23.4 µg mL-1 in the presence of 5 mg mL^{-1} tryptophan. Also, the collected data in this investigation were in harmony with the result of **Al - Kahtani *et al.* (2020)**, the author cleared that the maximum IAA production utilizing bacterial endophytes linked with the two medicinal plants, *Fagonia mollis* and *Achillea fragrantissima*, was achieved in the state of 5 mg mL^{-1} tryptophan being present after incubation period of 10 days.

CONCLUSION

The current study revealed that the aromatic and medicinal plant *Pelargonium graveolens .L.* which inhabits Sinai, Egypt, is generally considered an ecological niche for various reputed endophytic bacteria. The isolated bacterial endophytes of *Pelargonium graveolens .L.* belonging to *Bacillus subtilis* and *Bacillus velezensis*. According to the study these bacterial endophytes showed several mechanisms for plant growth promoting, these mechanisms include nitrogen fixation, ammonia production, extracellular hydrolytic enzyme activity, siderophores production, HCN production, phytohormones production as well as IAA production.

Therefore, these bacterial endophytes can be used as biofertilizers or used as a promising agent for extracting several of metabolites which is play an important role in our life specially in agriculture, biotechnology or in pharmaceutical field. Lastly, the using of these bacterial endophytes in the agriculture sector as bioinoculants have a potential role for improving plant growth and hence the production beside plant health and improving soil fertility plus quality.

REFERENCES

Abd-el-Malek, Y. and Y.Z. Ishac(1968). "Evaluation of methods used in counting azotobacters." J. Appl. Bacteriol., 31(3): 267-275.

Afzal, I. ; I. Iqrar ; Z.K. Shinwari and A. Yasmin (2017). Plant growth-promoting potential of endophytic bacteria isolated from roots of wild *Dodonaea viscosa* L. Plant Growth Regul., 81: 399–408.

Agbodjato, N.A. ; P.A. Noumavo ; F. Baba-Moussa ; H.A. Salami ; H. Sina ; A. Sèzan and H. Bankolé (2015). A. Adjanohoun, and L. Baba-Moussa. 2015. Characterization of potential plant growth promoting rhizobacteria isolated from maize (*Zea mays* L.) in central and Northern Benin (West Africa). Appl. and Envi. Soil Sci., 901656.

Ahmad, P. (2010). Growth and antioxidant responses in mustard (*Brassica juncea* L.) plants subjected to combined effect of gibberellic acid and salinity. Arch. Agron. Soil Sci., 56: 575–588.

Ahmad, F. ; I. Ahmad and M.S. Khan (2005). Indole acetic acid production by indigenous isolates of Azotobacter and fluorescent Pseudomonas in the presence and absence of tryptophan. Turk. J. Biol., 29: 29-34.

Al - Kahtani, M.D. ; A. Fouda ; K.A. Attia ; F. Al-Otaibi ; A.M. Eid ; E.E-D. Ewais ; M. Hijri ; M. St-Arnaud ; S.E-D. Hassan and N. Khan (2020). Isolation and characterization of plant growth promoting endophytic bacteria from desert plants and their application as bioinoculants for sustainable agriculture. Agronomy, 10: 1325.

Altschul, S.F. ; W. Gish ; W. Miller ; E.W. Myers and D.J. Lipman (1990). "Basic local alignment search tool." J. Molecular Biol., 215 (3): 403-410.

Amirita, A. ; P. Sindhu ; J. Swetha ; N.S. Vasanthi and K.P. Kannan (2012). Enumeration of endophytic fungi from medicinal plants andscreening of extracellular enzymes. World J. Sci. Technol. 2 (2): 13–19.

Asgher, M. ; M.I.R. Khan ; N.A. Anjum and N.A. Khan (2015). Minimising toxicity of cadmium in plants-role of plant growth regulators. Protoplasma, 252: 399–413.

Bakker, A.W. and B.O.B. Schippers (1987). "Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp-mediated plant growth-stimulation." Soil Biol. and Biochem., 19(4): 451-457.

Bashan, Y., and L.E. De-Bashan(2005). "Plant growth-promoting." Encyclopedia of Soils in the Envi., 1: 103-115.

Ćavar, S. and M. Maksimović (2011). Antioxidant activity of essential oil and aqueous extract of *Pelargonium graveolens* L'Her. Food Control, 23: 263-267.

Choi, Y.W. ; I.J. Hodgkiss and K.D. Hyde (2005). Enzyme production by endophytes of *Brucea javanica*. J. Agric. Technol. 1, 55–66.

Dalton, D.A. and S. Kramer (2006). Nitrogen-fixing bacteria in non-legumes. In Springer Netherlands, Dordrecht, pp. 105-130

David W. ; S. Mok ; Machteld and C. Mok (1994). Cytokinins: Chemistry, Activity, and Function. CRC Press. p. 8. ISBN 0-8493-6252-0.

Dawwam, G.E. ; A. Elbeltagy ; H.M. Emara ; I.H. Abbas and M.M. Hassan (2013). Beneficial effect of plant growth promoting bacteria isolated from the roots of potato plant. Annals of Agric. Sci., 58(2): 195-201.

De Bary, A. (1866) Morphologie und Physiologie Pilze, Flechten, und myxomyceten. Hofmeister's Handbook of Physiological Botany, vol. 2. Leipzig

Dobbelaere, S. ; J. Vanderleyden and Y. Okon (2003). Plant growth promoting effects of diazotrophs in the rhizosphere. Crit. Rev. Plant Sci., 22:107-149.

Eid, A.M. ; A. Fouda ; M.A. Abdel-Rahman ; S.S. Salem ; A. Elsaied ; R. Oelmüller ; M. Hijri ; A. Bhowmik ; A. Elkelish and S.E-D. Hassan (2021). "Harnessing bacterial endophytes for promotion of plant growth and biotechnological applications: an overview." Plants, 10(5): 935.

Fouda, A.H. ; S.E-D. Hassan ; A.M. Eid, and E.E-D. Ewais (2015). Biotechnological applications of fungal endophytes associated with medicinal plant *Asclepias sinaica* (Bioss.). Annal. Agric. Sci., 60(1): 95-104.

Fouda, A. ; A.M. Eid ; A. Elsaied ; E.F. El-Belely ; M.G. Barghoth ; E. Azab ; A.A. Gobouri and S.E-D Hassan (2021). "Plant growth-promoting endophytic bacterial community inhabiting the leaves of *Pulicaria incisa* (Lam.) DC inherent to arid regions." Plants, 10 (1): 76.

Geetha, K. ; E. Venkatesham ; A. Hindumathi and B. Bhadraiah (2014). "Isolation, screening and characterization of plant growth promoting bacteria and their effect on *Vigna Radita* (L.) R. Wilczek." Int. J. Curr. Microbiol. Appl. Sci., 3(6): 799-803.

Glickmann, E. and Y. Dessaux (1995). "A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria." Appl. and Envi. Microbiol., 61(2): 793-796.

Gupta, G. ; J. Panwar and P.N. Jha (2013) Natural occurrence of *Pseudomonas aeruginosa*, a dominant cultivable diazotrophic endophytic bacterium colonizing *Pennisetum glaucum* (L.) R. Br. Appl. Soil Ecol., 64: 252–261.

Hallman, J. ; A. Quadt-Hallman ; W.F. Mahafee and J.W. Kloepper (1997). Bacterial endophytes in agricultural crops. Canadian J. Microbiol., 43: 895–914

Hamidpour, R. ; S. Hamidpour and M. Hamidpour (2017). *Pelargonium graveolens* (Rose Geranium) – A Novel Therapeutic Agent for Antibacterial, Antioxidant, Antifungal and Diabetics. Arch Can Res. 2017, 5: 1.

Hassan, S.E-D. (2017). Plant growth-promoting activities for bacterial and fungal endophytes isolated from medicinal plant of *Teucrium polium* L. J. Adv. Res., 8(6): 687-695.

Herb Society of America (2006). Pelargoniums: An Herb Society of America Guide. Kirtland, Ohio

Hu, Y.F. ; G. Zhou ; X.F. Na ; L. Yang ; W.B. Nan and X. Liu (2013). Cadmium interferes with maintenance of auxin homeostasis in Arabidopsis seedlings. J. Plant Physiol., 170: 965–975.

Pandey K.D. ; A.K.Patel ; M.S. Vandana and A.K. Jalaluddin (2021). Chapter 2 - Secondary metabolites from bacteria and viruses. Natural Bioactive Compounds, Pp: 19-40.

Kalayu, G. (2019). "Phosphate solubilizing microorganisms: promising approach as biofertilizers." Int. J. Agro., Article ID 4917256, 7 pages.

Karthigai, S.P. and D. Purushotham (1996). Production of indole acetic acid (IAA) by *Acetobacter diazotrophicus*. Proc. Nat. Acad. Sci. Ind., 66: 273-276.

Kazan, K. (2013). Auxin and the integration of environmental signals into plant root development. Ann. Bot., 112: 1655–1665.

Kloepper, J.W. and M.N. Schroth (1978). Plant growth-promoting rhizobacteria on radish. Proc. Fourth Intern. Conf. Pl. Path. Bacteria, II: 879–882.

Kumar, A. ; A. Kumar ; S. Devi ; S. Patil ; C. Payal and S. Negi (2012). Isolation, screening and characterization of bacteria from rhizospheric soils for different plant growth promotion (PGP) activities: An in vitro study. Recent Res. Sci. Technol., 4: 1–5

Lacava, P.T. ; M.E. Silva-Stenico ; W.L. Araújo ; A.V.C. Simionato ; E. Carrilho ; S.M. Tsai and J.L. Azevedo (2008). "Detection of

siderophores in endophytic bacteria *Methylobacterium* spp. associated with *Xylella fastidiosa* subsp. pauca." Pesquisa Agropecuária Brasileira, 43(4): 521-528.

Lin, L. and X. Xu (2013). Indole-3-Acetic Acid Production by Endophytic Streptomyces sp. En-1 Isolated from Medicinal Plants. Curr. Microbiol., 67, 209–217.

Loper, J.E. and M.N. Schroth (1986). "Influence of bacterial sources of indole-3-acetic acid on root elongation of sugar beet." Phytopathol., 76(4): 386-389.

Lv, Y. ; F. Zhang ; J. Chen ; J. Cui ; Y. Xing ; X. Li and S. Guo (2010). "Diversity and antimicrobial activity of endophytic fungi associated with the alpine plant Saussurea involucrata." Biological and Pharmaceutical Bulletin, 33(8): 1300-1306.

McInroy, J.A. and J.W. Kloepper (1994) Studies on indigenous endophytic bacteria of sweetcorn and cotton. In: O'Gara F, Dowling DN, Boesten B (eds) Molecular ecology of rhizosphere microorganisms. VCH, Weinheim, pp 19–28

Mahgoub, H.A.M. ; A. Fouda ; A.M. Eid ; E.E-D. Ewais and S.E-D. Hassan (2021). Biotechnological application of plant growth-promoting endophytic bacteria isolated from halophytic plants to ameliorate salinity tolerance of *Vicia faba* L. Plant Biotechnology Reports.

Mativandlela, S.P. ; N. Lall and J.J. Meyer (2005) Antibacterial, antifungal and antitubercular activity of (the roots of) *Pelargonium reniforeme* (CURT) and *Pelargonium sidoides* (DC) (Geraniaceae) root. South African J. Botany, 72: 232-237.

Miché, L. ; F. Battistoni ; S. Gemmer ; M. Belghazi and B. Reinhold-Hurek (2006). Upregulation of jasmonate-inducible defense proteins and differential colonization of roots of *Oryza sativa* cultivars with the endophyte *Azoarcus* sp. Molecular Plant-Microbe Interactions, 19: 502– 511.

Miller, C.O. ; F. Skoog; M.H. Von Saltza and F. Strong (1955 a) Kinetin, a cell division factor from deoxyribonucleic acid. J. Am. Chem. Soc., 77: 1392.

Miller, C.O. ; F. Skoog ; Okumura F.S. ; M.H. von Saltza and F.M. Strong (1955b) Structure and synthesis of kinetin. J. Am. Chem. Soc.,78 : 2662–2663.

Miller, C.O. (1956). Similarity of some kinetin and red light effects. Plant Physiol., 31 : 318–319.

Munir, E. ; J. Mamangkey and A. Lutfia (2019). Antibacterial and phosphate solubilization activity of endophytic bacteria isolated from Pterydophyta (*Tectaria barberi*). In IOP Conference Series: Earth and Envi. Sci., 305(1): 012019.

Myrold, D.D. and P.J. Bottomley (2007). Biological N. Inputs. In: Paul EA, editor. Soil Microbiology, Ecology and Biochemistry. Burlington: Elsevier Academic Press, pp: 365-388

Neubauer, U. ; G. Furrer ; A. Kayser and R. Schulin (2000). Siderophores, NTA, and Citrate: Potential Soil Amendments to Enhance Heavy Metal Mobility in Phytoremediation. Int. J. Phytoremediation, 2: 353–368,

Olszewski, N. ; T.P. Sun and F. Gubler (2002). Gibberellin signaling, biosynthesis, catabolism, and response pathways. Plant Cell, 14: 561–580

Passari, A.K. ; V.K. Mishra ; V.V. Leo ; V.K. Gupta and B.P. Singh (2016). Phytohormone production endowed with antagonistic potential and plant growth promoting abilities of culturable endophytic bacteria isolated from *Clerodendrum colebrookianum* Walp. Microbiol. Res., 193: 57–73.

Patle, P.; N. Navnage and P. Ramteke (2018). Endophytes in plant system: Roles in growth promotion, mechanism and their potentiality in achieving agriculture sustainability. Int. J. Chem. Stud., 6: 270–274.

Petrini, O.(1992). "Fungal endophytes of bracken (*Pteridium aquilinum*), with some reflections on their use in biological control." Sydowia, 44: 282-293.

Rai, M. ; R. Rathod ; G. Agarkar ; M. Dar ; M. Brestic ; G.M. Pastore and M.R.M. Junior (2014). "Fungal growth promotor endophytes: a pragmatic approach towards sustainable food and agriculture". Cite this Article, 62: 63–79.

Rana, A. ; K. Yadav and S. Jagadevan (2020). "A comprehensive review on green synthesis of nature-inspired metal nanoparticles: Mechanism, application and toxicity." J. Cleaner Production, 272: 122880.

Rocha, F.R. ; F.S. Papini-Terzi ; M.Y. Nishiyama ; R.Z.N. Vêncio ; R. Vicentini ; D. Rdc ; V.E. Jr De Rosa ; F. Vinagre ; C. Barsalobres ; A.H. Medeiros ; F.A. Rodrigues ; E.C. Ulian ; S.M. Zingaretti ; J.A. Galbiatti ; R.S. Almeida, F. Avo ; A.S. Hemerly ; M.C. Silva-Filho ; M. Menossi and G.M. Souza

(2007). Signal transduction-related responses to phytohormones and environmental challenges in sugarcane. BMC Genomics 8(1):1

Rodrigues, A.A. ; M.V. Forzani ; R.D.S. Soares ; S.T. Sibov and J.D.G. Vieira (2016). Isolation and selection of plant growth-promoting bacteria associated with sugarcane. Pesqui. Agropecuária Trop, 46: 149–158.

Romero, F.M. ; M. Marina and F.L. Pieckenstain (2014). The communities of tomato (*Solanum lycopersicum* L.) leaf endophytic bacteria, analyzed by 16S-ribosomal RNA gene pyrosequencing. FEMS Microbiol. Lett., 351: 187–194.

Rørth, M. and P.K. Jensen (1967). "Determination of catalase activity by means of the Clark oxygen electrode." Biochimica et Biophysica Acta (BBA)-Enzymology, 139(1): 171-173.

Saha, M.; S. Sarkar ; B. Sarkar ; B.K. Sharma ; S. Bhattacharjee and P. Tribedi (2016). Microbial siderophores and their potential applications: A review. Environ. Sci. Pollut. Res., 23: 3984–3999.

Schöllhorn, R. and R.H. Burris (1967). "Acetylene as a competitive inhibitor of N-2 fixation." Proceedings of the National Academy of Sciences of the United States of America, 58(1): 213.

Siddiqui, M.W.; A. Bhattacharjya ; I. Chakraborty and R.S. Dhua (2011). 6-benzylaminopurine improves shelf life, organoleptic quality, and health-promoting compounds of fresh-cut broccoli florets. J. Scientific and Industrial Res., 70 (6): 461–465.

Skoog, F. ; F.M. Strong and C.O. Miller (1965). Cytokinins. Sci., 148: 532–533.

Stone, J.K. ; C.W. Bacon and J.F. White (2000). An overview of endophytic microbes: endophytism defined. In: Bacon CW, White JF (eds) Microbial endophytes. Dekker, New York, pp 3–30.

Strobel, G. and B. Daisy (2003). "Bioprospecting for microbial endophytes and their natural products." Microbiol. and Mol. Biol. Rev.,67(4): 491-502.

Teale, W.D. ; I.A. Paponov, and K. Palme (2006). Auxin in action: signalling, transport and the control of plant growth and development. Nat. Rev. Mol. Cell Biol., 7: 847–859.

Teixeira, G.M. ; M. Mosela ; M.L.A. Nicoletto ; R.A. Ribeiro ; M. Hungria ; K. Youssef and A.Y. Higashi (2021). "Genomic insights into the antifungal activity and plant growth-promoting ability in *Bacillus velezensis* CMRP 4490." Frontiers in Microbiol., 11: 3495.

Thuler, D.S. ; E.I.S. Floh ; W. Handro and H.R. Barbosa (2003). Plant growth regulators and aminoacids released by *Azospirillum* sp. in chemically defined media. Lett. Appl. Microbiol., 37: 174-178.

Vessey, J.K. (2003). Plant growth promoting rhizobacteria as biofertilizers. Plant Soil, 255:571–586

Walpola, B.C. ; K.K.I.U. Arunakumara and Y. Min-Ho (2014). "Isolation and characterization of phosphate solubilizing bacteria (*Klebsiella oxytoca*) with enhanced tolerant to environmental stress." African J. Microbiol. Res., 8 (31): 2970-2978.

Wang, T.L. ; E.A. Wood and N.J. Brewin (1984). Growth regulators of rhizobium and nodulation in peas, IAA from culture medium of nodulating and nonnodulating strains of *Rhizobium leguminosarum*. Planta., 155: 345-349.

Wani, Z.A. ; N. Ashraf ; T. Mohiuddin and S. Riyaz-Ul-Hassan (2015). "Plant-endophyte symbiosis, an ecological perspective." Appl. Microbiol. and Biotechnol., 99(7): 2955-2965.

Watanabe, F.S. and S.R. Olsen (1965). "Test of an ascorbic acid method for determining phosphorus in water and NaHCO3 extracts from soil." Soil Sci. Soc. America J., 29(6): 677-678.

Werner, C. ; O. Petrini and M. Hesse (1997). "Degradation of the polyamine alkaloid aphelandrine by endophytic fungi isolated from *Aphelandra tetragona*." FEMS Microbiol. Letters, 155(2): 147-153.

Wilkinson, S. ; G.R. Kudoyarova ; D.S. Veselov ; T.N. Arkhipova and W.J. Davies (2012). Plant hormone interactions: innovative targets for crop breeding and management. J. Exp. Bot., 63: 3499–3509.

Yao, A.V. ; H. Bochow ; S. Karimov ; U. Boturov ; S. Sanginboy and A.K. Sharipov (2006). Effect of FZB 24® *Bacillus subtilis* as a biofertilizer on cotton yields in field tests. Arch. of Phytopathol. and Plant Protection, 39(4): 323-328.

عزل و وصف للبكتريا النامية داخل أنسجة نبات العطر (بيلارجونيم جرافيولنس)

محمود سليمان عبد الحميد[1]; حسام محمد عطا[2]; هشام كمال أبو العلا[1];

عباس أحمد الغمري[2]; عمرو فودة[2]

1. قسم خصوبة وميكروبيولوجيا الأراضي، مركز بحوث الصحراء، المطرية، القاهرة، مصر.
2. قسم النبات والميكروبيولوجي، كلية العلوم، جامعة الأزهر، القاهرة 11884، مصر.

في الدراسة الحالية ، تم عزل إحدى عشر عزلة من البكتريا النامية داخل انسجة النباتات والتي تعيش في المجموع الخضري للنبات الطبي العطري نبات العطر (بيلارجونيم جرافيولنس) و الذي يتواجد في شمال سيناء – مصر، ومن بين إحدى عشر عزلة، تم تحديد اثنين على

أساس الأعلى في تثبيت النيتروجين وهما باسيلس ساتلس و باسيلس فيليزينسيس حيث أظهرت العزلات البكتيرية النامية داخل انسجة النبات أنشطة متنوعة كعوامل تعزز نمو النبات، حيث أن السلالاتين لهما قدرة إنتاج الأمونيا، السيدروفورس، سيانيد هيدروجين.

حيث أن العزلتين أظهرت قدرة علي تثبيت النيتروجين نوعيا (+++) لكلا منهما وكميا (اختبار النيتروجينيز) 365.667±1.8 C_2H_4/ml/24h و 87.767±1.6 $_2H_4$/ml/24h على التوالي، العزلتين لم يكن لديهم نتيجة تذكر في إذابة الفسفور، تم قياس قدرة العزلتين البكتيريتين علي إنتاج هرمون الاإندول اسيتيك اسيد بإستخدام محلول سالكوسكي وتم التأكيد على الإختبار لإنتاج الإندول اسيتيك اسيد، جيبريلك اسيد، ابسيسيك اسيد، البنزيل، الكينيتين، والزياتين بإستخدام HPLC، حيث أظهرت العزلة البكتيرية باسيلس فيليزينسيس أعلى إنتاجية للإندول اسيتيك اسيد حيث سجلت 2.1 ± 74.0ملليجرام/ مللي في وجود تريبتوفان بتركيز 5 ملليجرام/مللي بعد مرور 14 يوم.

السلالاتين لديهما نشاط إنزيمي وقدرة على إنتاج انزيمات إلأميليز، السيليوليز، الكيتينيز، الكتاليز ما عدا السلالة البكتيرية باسيلس فيليزينسيس ليس لها القدرة علي انتاج إنزيم الأميليز.