Original Research Article

Isolation, Identification and Evaluation of the Plant Growth Promoting Activities of Endophytic *Stenotrophomonas maltophilia* to Stimulate Growth of Clover Plants under Salt Stress

ABSTRACT

Two endophytic bacterial isolates were obtained from root nodules of Clover plants grown in salt affected clay soil of Egypt. The isolates were closely linked to *Stenotrophomonas maltophilia* strains IPR-Pv696 and 262XG2 based on the sequencing and phylogenetic analysis of *16S rRNA* genes, and deposited in GenBank with accession numbers OM980221.1 (AM1) and OM980223.1 (AM2) respectively. The isolates were evaluated for their potential to promote plant growth. The results revealed that the two isolates of *S. maltophilia* strains (IPR-Pv696 and 262XG2) respectively exhibited production for indole-3-acetic acid (30.26 & 31.15 µg/ml), exopolysaccharides (13.57 & 13.68 g/l), nitrogen fixation activity and they solubilize the phosphate (278 & 208 mg/l) and potassium (33.5 & 32.9 µg/ml). In a field trial, these two isolates increased Clover plant growth, chlorophyll, carbohydrates content and nutrients uptake while lowering proline levels. Hence this highlights its application to be exploited as biofertilizer by leading to sustainable agriculture. This could be a promising inoculant for many other crops.

Keywords: Endophytic bacteria; Stenotrophomonas; PGPR; 16S rRNA; Phylogenetic tree

1. INTRODUCTION

Using biofertilizer as an alternative to chemical fertilizers is a comparatively safer, more cost-effective, and ecologically beneficial strategy [1]. These biopreparations are known to have a variety of plant growth-promoting (PGP) traits, which improve plant health, growth, and production [2]. Recently, endophytic bacteria from root nodules have drawn increased interest as unique resources for enhancing plant development. The endophytic bacteria reside inside the tissues of the plants and do not alter the morphology of their hosts in any way that can be seen. They have a significant impact on the plants' capacity to absorb nutrients because they are more efficient in nutrient transformation, mobilization, and solubilization [3,4]. They can benefit the host plants through nitrogen fixation, phosphate solubilization and by the production of indole-3- acetic acid phytohormone (IAA) [5]. It was thought that the only rhizobial bacteria found in the root nodules of leguminous plants were endophytic bacteria, however a variety of non-rhizobial bacteria are also housed in root nodules, and they have a noticeable impact on the crop's survival, nodulation, and grain yield [6,7,8]. Stenotrophomonas bacteria have attracted growing biotechnological attention because of their potential to serve as efficient bioinoculants for promoting plant development and controlling a variety of plant diseases [9]. *S. maltophilia*, a species of Stenotrophomonas, is recognized as a crucial species for boosting plant development in agriculture [10].In the family Xanthomonadaceae, the genus Stenotrophomonas contains gram-negative bacteria. The dominating member of the bacterial community linked with plants, Stenotrophomonas species are endophytes as well as free-living bacteria [11, 12]. In prior research, the Stenotrophomonas genera were shown to be effective phosphate solubilizers and biofertilizers [13]. According to [9], *S. maltophilia* can fix nitrogen in plants including peanuts, wheat, maize, and rice [14]. The Stenotrophomonas genus has been as a rhizospheric microbiota of different crops, including corn [15, 16]. [17] confirmed the plant growth promoting potential of *S. maltophilia* in wheat plants along with resistance against biotic and abiotic stress.

Sequencing the 16S rRNA gene, which is contained in the chromosome of the bacterium, is the most effective method for identifying unidentified bacteria. Genes of *16S rRNA* are found in all prokaryotes, code for the RNA component of the ribosomal 30S subunit, and these have essential role in translation [18]. The taxonomic origin pattern of the bacterial strains is represented by a root in a phylogenetic tree [19, 20].

Egyptian Clover (*Trifolium alexandrinum* L.) The main annual winter forage leguminous crop in Egypt. Berseem clover is high nutritional quality for animal field. Berseem also contributes to soil fertility and improves soil physical characteristic [21]. Berseem forage is superior to grasses in protein and mineral contents [22].

The present work intends to isolate, identify two bacterial isoletes using 16S rRNA technology and study of plant growth-promoting activities of *Stenotrophomonas maltophilia* isolated from the nodules of clover plants, as well as to determine the effect of their inoculation to plants under salinity stress conditions.

2. MATERIAL AND METHODS

2.1 Isolation of nodule endophytes

Nodules were chosen at random from each clover plant, washed with sterile distilled water to remove soil particles, and surface sterilized with 95 % alcohol for 30s and 0.1 % $HgCl_2$ (w/v) for 2 min before being rinsed 6-8 times with sterile distilled water to completely remove $HgCl_2$. For the isolation of endophytic bacteria, the surface sterilized nodules were crushed

and streaked on Congo red yeast-extract-mannitol agar (YEMA) plates as indicated by [23, 24] and Luria Bertani agar medium based on the modified method of [25]. Single colonies were purified further by streaking on the same medium repeatedly for 3 days at 30°C. The isolates were kept at -20°C in glycerol (20% v/v).

2.2 Molecular characterization

2.2.1 DNA isolation

The technique described in [26] was used for DNA isolation and purification. On a 1% Agarose gel, the quality of the extracted total genomic DNA from several bacterial species was assessed.

2.2.2 Identification of isolates by 16S rRNA sequencing

Isolated DNA was amplified with 16S rRNA. In PCR tube and 20 µl of reaction solution, the PCR amplification was performed using 27F/1492R universal primers [27] as shown in Table 1. The amplification took place during 35 cycles for 45 sec at 94° for denaturation, 55℃ for 60 sec for annealing, and extension at 72℃ for 60 sec. The PCR include a positive control (*E. coli* genomic DNA) and a negative control. Using a column-based technique and the Montage PCR Clean up kit (Macro gen), the PCR amplicon purification was completed. The purified PCR products were sequenced by using 2 primers as described in table 1. Big Dye Terminator Cycle Sequencing Kit was used for the sequencing, and an Applied Biosystems model 3730XL automated DNA sequencing device was used to resolve the results (Applied BioSystems, USA).

2.2.3 Nucleotide sequence accession numbers

Using BLASTN search technique, the *16S rRNA* gene sequences of chosen isolates were compared to the *16S rRNA* gene sequences in the GenBank database. Phylogenetic tree was constructed by using Neighbor joining method.

Table 1. Primers of 16S rRNA amplification and sequencing.

Primers	Sequences
Amplification primers	27F 3′ AGA GTT TGA TCM TGG CTC AG 5′
	1492R 3′ TAC GGY TAC CTT GTT ACG ACT T 5′
Sequencing Primers	518F 3′ CCA GCA GCC GCG GTA ATA CG 5′
	800R 3′ TAC CAG GGT ATC TAA TCC 5′

The evolutionary distances were computed using the Maximum Composite Likelihood method [28]. Evolutionary analyses were conducted in MEGA7 [29].

2.3 Plant growth promoting attributes

2.3.1 Indole acetic acid (IAA) production

Using the colorimetric technique outlined by [30], IAA production was quantified. In LB broth with L-tryptophan added, the isolated bacteria's pure colony grew for three days at 30 ± 2°C. Using a centrifuge set at 3824 xg for 15 minutes, bacteria were extracted after incubation. Then 2 ml of the supernatant was mixed with 2 drops of orthophosphoric acid, and 4 mL Salkowski's reagent, and measured at 530 nm by spectrophotometry.

2.3.2 Exopolysaccharides (EPS) production

For the estimation of EPS production, bacterial strain was inoculated into conical flasks containing 100 ml of YEM broth. The inoculated flasks were incubated at 30 ± 1°C on a rotary shaker at 200 rpm for 72 h. After incubation, the culture broth was centrifuged 3500 xg and the supernatant was mixed with two volumes of acetone. The crude polysaccharides developed were collected by centrifugation at (3500 xg) for 30 min. The EPS was washed with distilled water and acetone alternately, transferred onto a filter paper and weighed after overnight drying at 105^{o}C [31].

2.3.3 Nitrogen Fixing Activity

Nitrogen fixing activity was examined using Jensen's medium and Bromothymol Blue (BTB) as a color indicator according to [32].

2.3.4 Estimation of phosphate solubilization

Qualitative determination of phosphate solubilization was performed on Pikovskaya's agar plate (PVK) medium [33]. Isolate was spot inoculated and incubated at 28 ± 2°C, and the size of the halo corresponding to phosphate solubilization was measured after 3-7 days of incubation. Phosphate solubilization was expressed as solubilizing efficiency (SE %) [34, 35]. Quantitative estimation of tri-calcium phosphate solubilization was performed by growing the bacterial strain in Pikovaskya's broth. The concentration of the soluble phosphate was determined from the culture supernatant.

Phosphate solubilization efficiency = (Solubilization diameter (S) x 100)/(growth diameter)

2.3.5 Estimation of potassium solubilization

The spotting method was used to investigate potassium solubilization by bacterial isolates on Aleksandrov medium plates containing insoluble mica powder as a potassium source [36]. Plates were incubated for 7 days at 28°C±2. The ability of bacterial isolates to form solubilization zones was used to detect potassium solubilization. Quantitative Estimation of potassium release was performed by growing bacterial isolates in Aleksandrov broth medium and incubated for 7 days at 28°C±2. Following the incubation, the broth cultures were filtered through Whatman No. 1 filter paper and centrifuged for 20 minutes at 12,000 rpm. The soluble K content in the supernatant was measured using flame photometer [37].

2.4 Field Experiment

A field experiment was carried out at the farm El-Rowad village at Sahl El-Hussinia, El-Sharkia Governorate Egypt using a randomized complete block design with three replicates to elucidate the role of isolated bacteria in alleviating salt stress in Clover plants grown in a saline soil as compared to *R.leguminosarum* bv. *trifolii* strain 102 specific to Clover plants. Seeds of Clover plants were inoculated with gamma irradiated vermiculite-based inoculants. All treatments received the half-recommended dose of phosphorus, potassium and nitrogen. The treatments were as follow:

1. Control (Recommended dose of NPK)
2. *R. Leguminosarum* bv. *trifolii* strain 102 + half dose of NPK
3. *S. maltophilia* strain IPR-Pv696 + half dose of NPK
4. *S. maltophilia* strain 262XG2 + half dose of NPK

2.5 Plant growth parameters

For the purpose of preventing humidity losses, the fresh forage yield of each plot was calculated right away after harvest. A 500 g sample from each plot was dried at room temperature without exposure to sunlight to determine the forage's dry weight. After a few days, when samples showed an equal weight over the course of three additional days, the weight obtained was taken into account as an approximation of the dry weight percentage of the dry matter produced by each treatment. Shoots' carbohydrate and chlorophyll contents were measured. Utilizing an H2SO4 and HClO4 acid mixture, dry samples were ground and digested in accordance with [38]. The method described by [39] was used to determine the N, P, and K content of the plants in the plant digests.

2.6 Proline determination

Proline was extracted from the shoot and its concentration was determined using the [40]. Using a standard curve made from proline, the proline content was determined by spectrophotometer at 520 nm as µ mole proline / g of fresh weight material.

2.7 Soil analysis

A surface soil sample (0–30 cm) was taken, dried by air, sieved to pass through a 2 mm sieve, and thoroughly mixed. According to [41] calcium carbonate, soil organic matter (SOM), total soluble ions, and electrical conductivity (EC) were all measured in the saturated soil paste extract while pH was measured using a pH meter in soil suspension (1: 2.5) (Table 2). [38] measured the available nitrogen using the modified Kjeldahal method. In accordance with the methodology outlined by [42], the available phosphorus, potassium, and micronutrients (Mn, Fe, and Zn) were extracted using ammonium bicarbonate, and their concentrations were assessed using an ICP Spectrometer (model 400).

Table 2. Physical and chemical properties of the soil sample before planting

Coarse sand (%)	Fin sand (%)	Silt (%)	Clay (%)	Texture	O.M (%)	$CaCO_3$ (%)
4.2	15.2	35.5	45.3	Clay	0.47	11.5

pH (1:2:5)	EC	Cations (meq^{-1})				Anions (meq^{-1})		
	(dS/m)	Ca^{++}	Mg^{++}	Na^{+}	K^{+}	HCO^{-}_{3}	Cl^{-}	SO^{--}_{4}
8.12	12.82	22.61	35.61	68.5	1.48	4.61	95.61	27.98
N	P	K	Zn	Mn	Fe		Cu	
				PPM				
107.1	2.95	171.16	0.868	2.16	1.85		0.61	

2.8 Statistical analysis

The obtained results were statistically analyzed using the general linear models procedure of SAS [43]. The differences were statistically tested using Duncan's multiple range tests.

3. RESULTS AND DISCUSSION

3.1 Appearance of bacterial isolates

Two endophytic Gram-negative, rod-shaped bacterial isolates recovered from root nodules of Clover plants. Isolates did not absorb red color when cultured on yeast extract mannitol agar (YEMA) containing Congo red.

3.2 Genotypic characterization of the bacterial isolates

The PCR technique was used for detecting the *16S rRNA* gene. Gel electrophoresis was performed for screening of DNA isolate, the results show the presence of approximately 1,200 bp band. Isolates AM1 and AM2 were subjected to the partial *16S rRNA* gene sequences of 1274 and 1228 base pairs.

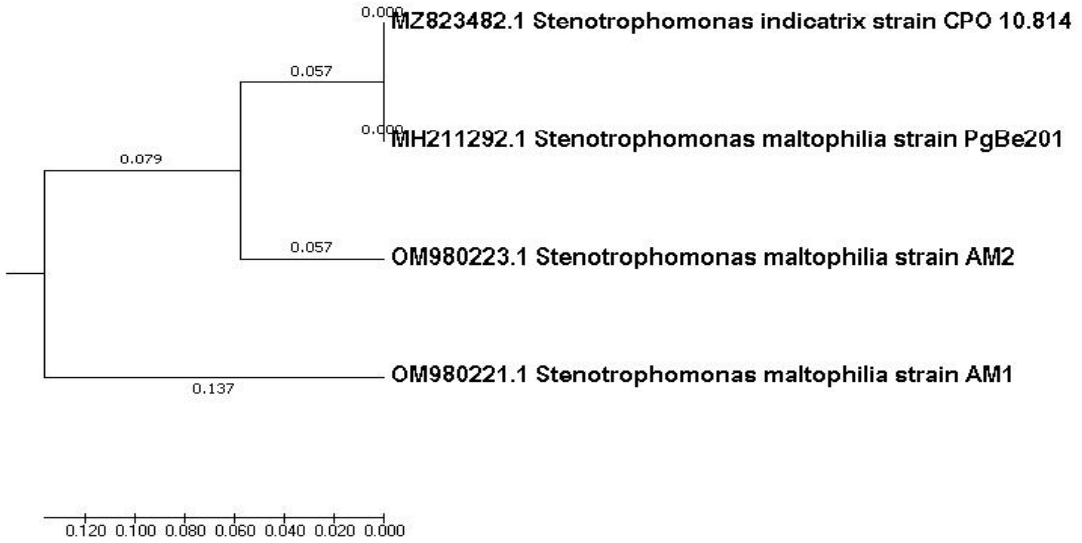

Fig. 1. Phylogenetic tree indicates the phylogenetic relationship of the two bacterial isolates

16S rRNA gene sequencing of the chosen isolates AM1 (accession number OM980221.1) and AM2 (accession number OM980223.1) had 98% similarity with 16S rRNA gene of *Stenotrophomonas maltophilia* (IPR-Pv696) and (262XG2) respectively based on nucleotides identity. The results indicated that rRNA gene sequencing is very helpful for bacterial categorization [44]. To confirm the phylogenetic position of *S. maltophilia*, a phylogenetic tree of 16S rRNA sequences was created (Fig. 1). Phylogenetic tree indicates the phylogenetic relationship of the selected isolates *Stenotrophomonas maltophilia* strains (IPR-Pv696 and 262XG2. With the use of a frequency filter included into the ARB (from the Latin arbour, tree) software package, a neighbor-joining tree was generated using partial *16S rRNA* gene sequences [45]. By Neighbour-Joining algorithm, the phylogenetic tree has been divided into clusters and with values ranging from 0.057 to 0.137. These clusters verified the AM1 and AM2 bacterial isolates as Stenotrophomonas, and are closely with *Stenotrophomonas maltophilia* strain PgBe201. These results agree with [46], who reveald that for phylogenetic analyses of the genus Arcobacter, the *16S rRNA* gene sequence analysis has proven to be a very useful technique.

3.3 *In Vitro* assessment of plant-growth-promoting activities

As shown in Table 3 and Fig. 2, two bacterial isolates, *Stenotrophomonas maltophilia* strains (IPR-Pv696) and (262XG2), were discovered to produce IAA at concentrations of 30.26 µg $ml^{-1}$and 31.15 µg ml^{-1} in the cultural filtrates, respectively. These support the findings of [47], who established that *S. maltophilia* can produce IAA and encourage plant growth. The extent of IAA production was dependent on the isolates, according to [48]. According to [49], *S. maltophilia* isolate from cucumber rhizosphere produced 26.78 µg /ml of IAA.

Regarding the amounts of EPS released (Table 3 and Fig. 2), it was discovered that the bacterial isolates can produce EPS at rates of 13.57 and 13.68 g/l for the *S. maltophilia*

strains (IPR-Pv696) and (262XG2), respectively. These findings support the assertion made by [50] that *S. maltophilia* produced a high yield of EPS.

Jensen's medium, which is nitrogen-free, is intended for the discovery and cultivation of bacteria that fix N_2 where bacterial growth on this medium suggests that the bacteria can fix nitrogen. Additionally, a large number of nitrogen-fixing endophytic bacteria produce acid, as evidenced by the colour change from greenish blue to yellow. The growth of the two isolates in the current study on N free medium demonstrated their capacity to fix atmospheric nitrogen. These findings support those made by [51], who discovered that a new strain of Stenotrophomonas can fix atmospheric nitrogen. Also by analyzing the entire genome sequences and an annotation of *Stenotrophomonas maltophilia* JVB5 isolated from the sunflower root endosphere in the North West province of South Africa, [52] were able to identify the nitrogen fixation genes, nifSF, amt, and aztF coding for cysteine, desulfurase, flavodoxin, ammonium transport, and allophanate hydrolase.

Table 3. Production of IAA, EPS, nitrogen fixation, solubilization of phosphate and potassium of the isolates

Isolates No	IAA ($\mu g\ ml^{-1}$)	EPS (g/l)	Nitrogen fixation	Phosphate solubilization		Potassium solubilization	
				Solubilization efficiency (SE %)	P-liberated (mg/l)	Solubilization efficiency (SE %)	K-liberated (µg/ml)
AM1	**30.26^{b}**	**13.57^{b}**	++	**100**	**278^{a}**	**208**	**33.5^{a}**
AM2	**31.15^{a}**	**13.68^{a}**	+	**75**	**208^{b}**	**192**	**32.9^{b}**

Means in the same column followed by the same letters are not significantly different (P<0.05) according to Duncan's test.

AM1: Stenotrophomonas maltophilia strain (IPR-Pv696) and AM2: Stenotrophomonas maltophilia strain (262XG2).

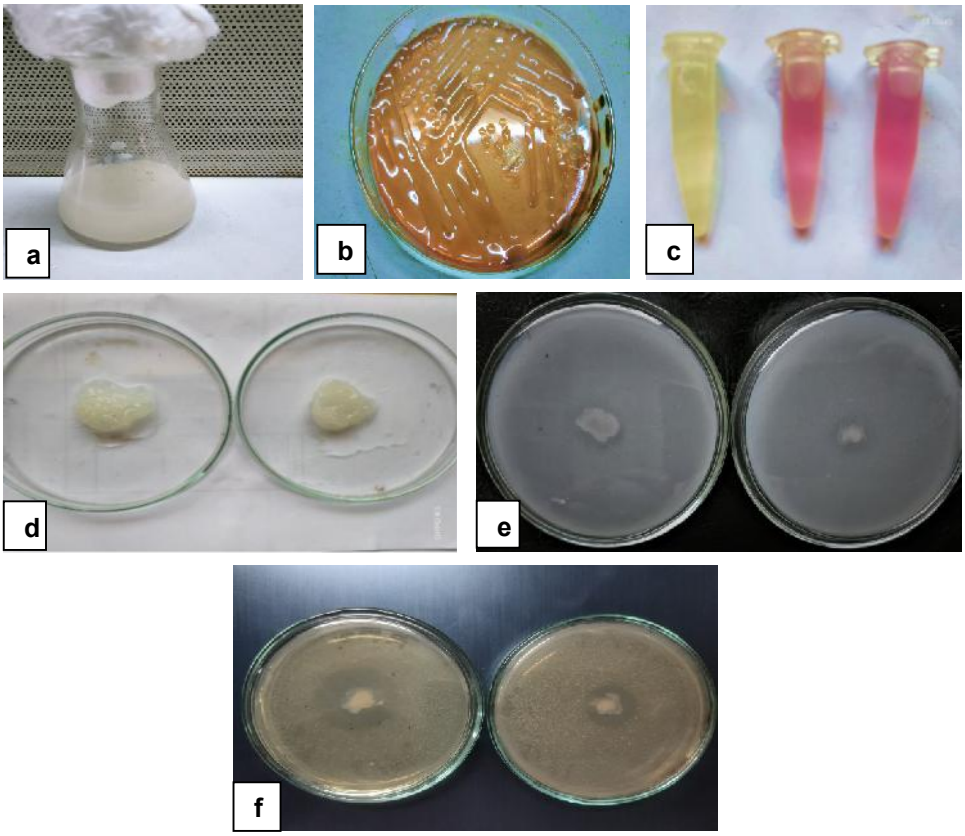

Fig. 2. Plant growth promoting activities of the bacterial isolates *in vitro*
(a) Pure isolate grown in YEM broth media. (b) Isolate grown in Congo red YEMA medium. (c) IAA production by isolates. (d) EPS production by isolates. (e) In vitro assay for phosphate solubilization by bacterial isolates. (f) Potassium solubilization by bacterial isolates.

The ability of P solubilization is also a desired attribute in rhizobacteria [53]. In this study both isolates of *Stenotrophomonas maltophilia* strains (IPR-Pv696) and (262XG2) grew on PVK agar medium with solubilization efficiencies of 100% and 75%, and P liberated in the broth medium was 278 and 208 mg/l, respectively as shown in Table (3) and Fig. (2). These findings are in agreement with those of [54] and [55] who demonstrated that the phosphate solubilizing values of the *S. maltophilia* strains CA158, 79, and AVP 27 are 222.43, 216.38, and 818 g/ml, respectively. Moreover endophytic *S. maltophilia* strains, namely, SEN1 [56], B11 [57], SY-2 [58] have been identified as phosphate solubilizers with the potential of enhancing plant growth.

Another vital trait of PGPR, that may ultimately effect the plant growth, is the solubilization of potassium. Both of the two isolates grew in Aleksandrov's medium recorded solubilization efficiency 208 and 192 % and the amount of K liberated in the broth medium were 33.5 and 32.9 µg/ml in *S. maltophilia* strain (IPR-Pv696) and (262XG2) respectively as indicated in (Table 3 and Fig. 2). Some authors associate the solubilization of potassium with the production of acids of microbial origin [59].

2.4 Plant growth parameters

Proline builds up in response to salt stress to protect the cell membrane, stabilize the protein's structure, and scavenge free hydroxyl radicals [60]. In the current study, plants inoculated with isolates had lower proline levels than plants with rhizobia and uninoculated control plants (Fig. 3). This decrease in proline levels of inoculated plants suggested that plants inoculated with bacterial isolates were less affected by salinity. These findings are in agreement with [61], who confirmed that the presence of lower proline content in the plant with the bacteria reflects the role of *S. maltophilia* in aiding the plant in overcoming the salt stress.

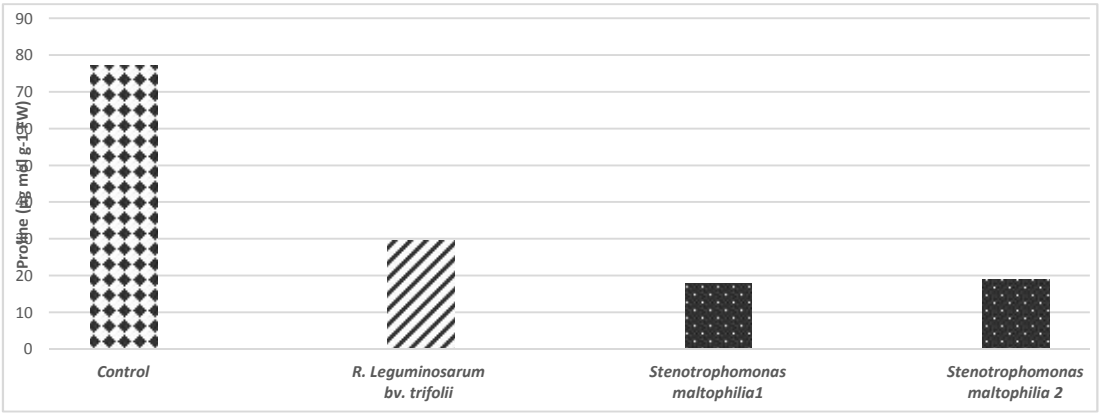

Fig. 3. proline as affected by the inoculation of bacterial isolates

The data shown in Table (4) revealed that the two isolates significantly increased the N and K contents when compared to the control and rhizobial inoculated plants, but that there was no significant difference in the P content when using the new bacterial isolates*, Rhizobium* sp. and the control. Several previous studies have demonstrated that IAA primarily increases the size and distribution of roots and the number of root hairs, resulting in better nutrient uptake from the soil [48].

Table 4. Effect of inoculation with bacterial isolates on N, P and K content in shoot after cutting

Treatments	N (%)	P (%)	K (%)
Control (Recommended dose of NPK)	2.36^{b}	0.10^{a}	3^{ab}
R. Leguminosarum bv. *trifolii* strain (102)	2.23^{c}	0.09^{a}	2.35^{b}
S. maltophilia strain IPR-Pv696 (AM1)	2.90^{a}	0.11^{a}	3.55^{a}
S. maltophilia strain 262XG2 (AM2)	2.92^{a}	0.10^{a}	2.95^{ab}

The Duncan's test indicates that the means in the same column and following the same letters are not significantly different ($P < .05$).

The photosynthetic process is also hampered by salt stress due to chlorophyll peroxidation [62, 63]. However, inoculation of bacterial isolates significantly improved the contents of leaf chlorophyll as compared to uninoculated control or rhizobial inoculated plants (Table 5). The inoculation of bacterial isolates also caused an increase in the content of carbohydrates, which followed a similar pattern (Table 5). These findings support [17] research, which showed that *S. maltophilia* SBP-9 inoculation significantly increased the leaf chlorophyll

content compared to the uninoculated control under both non saline and salinity conditions, demonstrating the strain's capacity to mitigate salinity stressors.

Fresh weight of yield significantly increased to 40.53 and 42.68 tons fed-1 in addition to the yield dry weight that increased to 6.92 and 7.18 tons fed-1 by inoculation of *S. maltophilia* strains (IPR-Pv696) and (262XG2) respectively in Compared to corresponding uninoculated control or rhizobial inoculated plants. The increase in yield can be attributed to increased photosynthetic activity as well as effective nutrient and water use [64]. The ability of the bacterial isolates to synthesis IAA, which is associated with enhancing root proliferation, could account for these effects [65]. By promoting root growth and enhancing plant mineral absorption, this indirectly promotes plant growth [66]. Many studies proved that *Stenotrophomonas* sp. can enhance plant productivity by several mechanisms, including the production of the plant growth hormone (IAA) [13] and nitrogen fixation, as suggested by [14]. [67] confirmed that IAA is the most common plant hormone, which stimulates plant growth and reproduction.

Table 5. Chlorophyll (a+b), carbohydrate and yield of clover plants as affected by isolates of *S. maltophilia*

Treatments	Chlorophyll (a+b) (mg /g. f.w.)	Carbohydrate (%)	Fresh yield (tons fed^{-1})	Dry yield (tons fed^{-1})
Control (Recommended dose of NPK)	**32.61^{c}**	**48.34^{c}**	**39.75^{c}**	**6.76^{c}**
***R. Leguminosarum* bv. *trifolii* strain (102)**	**32.28^{d}**	**47.39^{d}**	**39.25^{d}**	**6.67^{d}**
***S. maltophilia* strain IPR-Pv696**	**36.93^{b}**	**49.08^{b}**	**40.53^{b}**	**6.92^{b}**
***S. maltophilia* strain 262XG2**	**37.12^{a}**	**59.19^{a}**	**42.68^{a}**	**7.18^{a}**

The Duncan's test indicates that the means in the same column and following the same letters are not significantly different (P< .05).

4. CONCLUSION

[In conclusion, *S. maltophilia* was highly effective in promoting the growth of Clover plants due to its IAA, EPS, and solubilization of phosphate and potassium as well as its ability to fix nitrogen. This emphasizes its potential use as biofertilizer, which will promote sustainable agriculture. For many other crops, this may be a promising inoculant.

DATA AVAILABILITY

Data generated or analyzed during this study are provided in the manuscript.

REFERENCES

1. Khaitov B, Kurbonov A, Abdiev A, Adilov M. Effect of chickpea in association with *Rhizobium* to crop productivity and soil fertility. Eurasian Journal of Soil Science. 2016;5(2):105–12. https://doi.org/10.18393/ejss.2016.2.105-112

2. Guo DJ, Singh RK, Singh P, Li DP, Sharma A, Xing YX, et al. Complete genome sequence of *Enterobacter roggenkampii* ed5 a nitrogen fixing plant growth promoting endophytic bacterium with biocontrol and stress tolerance properties isolated from sugarcane root. Front Microbiol. 2020;11: 580081. https://doi.org/10.3389/fmicb.2020.580081

3. Hayat R, Ali S, Amara U, Khalid R, Ahmed I. Soil beneficial bacteria and their role in plant growth promotion: a review. Ann Microbiol. 2010;60:579–598. https://doi.org/10.1007/s13213-010-0117-1

4. Zewide I. Role of bio-fertilizer for maximizing productivity of selected horticultural crops. J Agri Res Adv. 2019;0102:01–18.

5. Aeron A, Maheshwari DK, Meena VS. Endophytic bacteria promote growth of the medicinal legume *Clitoria ternatea* L. by chemotactic activity. Arch Microbiol. 2020;202:1049–1058. https://doi.org/10.1007/s00203-020-01815-0

6. Pandya M, Kumar GN, Rajkumar S. Invasion of rhizobial infection thread by non-rhizobia for colonization of *Vigna radiata* root nodules. FEMS Microbiol Lett. 2013; 348: 58-65. https://doi.org /10.1111/1574-6968.12245

7. Tariq M, Hameed S, Yasmeen T, Ali A. Non-rhizobial bacteria for improved nodulation and grain yield of mung bean [*Vigna radiata* (L.) Wilczek]. Afr J Biotechnol. 2012; 11(84): 15012–15019.

8. Rajendran G, Patel MH, Joshi SJ. Isolation and characterization of nodule associated *Exiguobacterium* sp. from the root nodules of Fenugreek (*Trigonella foenum-graecum*) and their possible role in plant growth promotion. Int J Microbiol. 2012;436:1–8. https://doi.org/10.1155/2012/693982

9. Alexander A, Singh VK, Mishra A, Jha B. Plant growth promoting rhizobacterium *Stenotrophomonas maltophilia* BJ01 augments endurance against N2 starvation by

393 modulating physiology and biochemical activities of Arachis hypogea. PLoS ONE. 2019;149:
394 e0222405. https://doi.org/10.1371/journal.pone.0222405

395 10. Kumar NP, Audipud AV. Exploration of a novel plant growth promoting bacteria
396 *Stenotrophomonas maltophilia* AVP27 isolated from the chilli rhizosphere soil. Int J Eng Res
397 Gen Sci. 2015;3:265–276.

398 11. Berg G, Egamberdieva D, Lugtenberg B, Hagemann M. Symbiotic plant–microbe
399 interactions: stress protection, plant growth promotion, and biocontrol by *Stenotrophomonas*.
400 In: Seckbach J, Grube M (eds) Symbioses and Stress. Cellular Origin, Life in Extreme
401 Habitats and Astrobiology, vol 17. Springer, Dordrecht. 2010; pp 445-460.
402 https://doi.org/10.1007/978-90-481-9449-0_22

403 12. Naz I, Bano A. Assessment of phytohormones producing capacity of *Stenotrophomonas*
404 *maltophilia* SSA and its interaction with Zea mays l. Pak J Bot. 2012;44(1):465–469

405 13. Suckstorff I, Berg G. Evidence for dose dependent effects on plant growth by
406 *Stenotrophomonas* strains from diffffferent origins. J Appl Microbiol. 2003;95:656–663.
407 https://doi.org/10.1046/j.1365-2672.2003.02021.x

408 14. Park M, chungwoo K, Jinchul Y, Hyoungseok L, Wansik S, Seungwan K, et al. Isolation
409 and characterization of diazotrophic growth promoting bacteria from rhizosphere of
410 agricultural crops of Korea. Microbiol Res. 2005;160:127–133. https://doi.org
411 /10.1016/j.micres.2004.10.003

412 15. Sanchez AC, Gutierrez RT, Santana RC, Urrutia AR, Fauvart M, Michiels J,
413 Vanderleyden J. Effects of co-inoculation of native Rhizobium and Pseudomonas strains on
414 growth parameters and yield of two contrasting Phaseolus vulgaris L. genotypes under
415 Cuban soil conditions. European J Soil Biology. 2014;62:105-112.
416 https://doi.org/10.1016/j.ejsobi.2014.03.004

417 16. Granada-Mora KI, González R, Alvarado Y, Robles AR, Torres R. Caracterización de
418 rizobacterias y estimulación de parámetros morfológicos y biomasa en maíz (*Zea mays* L.).
419 Centro de Biotecnología. 2015;4(1):14–22.

420 17. Singh RP, Jha PN. The PGPR *Stenotrophomonas maltophilia* SBP-9 augments
421 resistance against biotic and abiotic stress in wheat plants. Frontiers in Microbiology.
422 2017;8:1945. https://doi.org/10.10.3389/fmicb.2017.01945

423 18. Xin Wang, I. King Jordan, Leonard W Mayer. A phylogenetic perspective on molecular
424 epidemiology. In: Yi-Wei Tang, Max Sussman, Dongyou Liu, Ian Poxton, Joseph
425 Schwartzman, editors. Molecular medical microbiology. 2nd ed. pp 517-536; 2015.
426 https://doi.org/10.1016/B978-0-12-397169-2.00029-9

427 19. Ciccarelli FD, Doerks T, von Mering C, Creevey CJ, Snel B, Bork P. Toward automatic
428 reconstruction of a highly resolved tree of life. Science. 2006;311:1283–1287.
429 https://doi.org/10.1126/science.1123061

430 20. Sayers EW, Barrett T, Benson DA, Bryant SH, Canese K, Chetvernin V, et al. Database
431 resources of the National Center for Biotechnology Information. Nucleic Acids Res Vol 37
432 (Database issue), 2009; D5–D15. https://doi.org/10.1093/nar/gkn741

433 21. Graves WL, Wilhams WA, Thomsen CD. Berseem clover: A winter annual forage for
434 California agriculture. University of California Division of agriculture and natural resources;
435 publication No. 21536, 1996;pp: 12.

436 22. Laghari HH, Channa AD, Solangi AA, Soomro SA. Comparative digestibility of different
437 cuts of berseem (*Trifolium alexandvinum*) in sheep. Pak J Biol Sci. 2000;3:1938-1939.
438 https://doi.org/10.3923/PJBS.2000.1938.1939

439 23. Vincent JM. The cultivation, isolation and maintenance of rhizobia in: A Manual for the
440 Practical Study of the Root-Nodule Bacteria. Blackwell, 1970;pp 1-13.

441 24. Kshetri L, Pandey P, Sharma GD. Rhizosphere mediated nutrient management in *Allium*
442 *hookeri* Thwaites by using phosphate solubilizing rhizobacteria and tricalcium phosphate
443 amended soil. J Plant Interact. 2018;13(1):265–269.
444 https://doi.org/10.1080/17429145.2018.1472307

445 25. Khamwan S, Boonlue S, Riddech N, Jogloy S, Mongkolthanaruk W. Characterization of
446 endophytic bacteria and their response to plant growth promotion in *Helianthus tuberosus* L.
447 Biocatalysis and Agricultural Biotechnology. 2018;13:153–159.
448 https://doi.org/10.1016/j.bcab.2017.12.007

449 26. Sambrook J, Russell D. Molecular cloning a laboratory manual. Vol. 2, 3rd ed. Cold
450 Spring Harbor Laboratory Press, New York; 2001.

451 27. Yang J, Benyamin B, McEvoy BP, Gordon S, Henders AK, Nyholt DR, et al. Common
452 SNPs explain a large proportion of the heritability for human height. Nat Genet. 2010;42:565-
453 569. https://doi.org/10.1038/ng.608

454 28. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the
455 neighbor-joining method. Proceedings of the National Academy of Sciences (USA):
456 2004;101:11030-11035. https://doi.org/10.1073/pnas.0404206101

457 29. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis
458 version 7.0 for bigger datasets. Molecular Biology and Evolution. 2016;33:1870-1874.
459 https://doi.org/10.1093/molbev/msw054

460 30. Glickmann E, Dessaux Y. A critical examination of the specificity of the Salkowski
461 reagent for indolic compounds produced by phytopathogenic bacteria. Appl Environ
462 Microbiol. 1995;61:793–796. https://doi.org/10.1128/aem.61.2.793-796.1995

463 31. Damery J, Alexander M. Physiological differences between effective and ineffective
464 strains of *Rhizobium*. Soil Sci. 1969;103:209-215. https://doi.org/10.1097/00010694-
465 196909000-00010

466 32. Ahmad F, Ahmad I, Khan MS. Screening of free-living rhizospheric bacteria for their
467 multiple plant growth promoting activities. Microbiol Res. 2008;163:173–181.
468 https://doi.org/10.1016/j.micres.2006.04.001

469 33. Pikovskaya RI. Mobilization of phosphorous in soil connection with the vital activity of
470 some microbial species". Microbiologiya. 1948;17:362-370.

34. Nguyen C, Yan W, Le T, Lapeyrie F. Genetic variability of phosphate solubilizing activity by monocaryotic and dicaryotic mycelia of the ectomycorrhizal fungus *Laccaria bicolor* (Maire) P.D. Orton Plant Soil. 1992; 143:193–199. https://doi.org/10.1007/BF00007873

35. Srivastava S, Yadav KR, Kundu BS. Prospects of using phosphate solubilizing Pseudomonas as biofungicide. Indian J Microbiol. 2004;44:91-94.

36. Sindhu SS, Gupta SK, Dadarwal KR. Antagonistic effect of Pseudomonas spp. on pathogenic fungi and enhancement of plant growth in green gram (*Vigna radiata*). Biology and Fertility of Soils. 1999;29:62-68. https://doi.org/10.1007/s003740050525

37. Sugumaran B, Janarthanam BS. Solubilization of potassium-containing minerals by bacteria and their effect on plant growth. World J Agric Sci. 2007;3(3):350–355.

38. Black CA. Methods of soil analysis. Part I, American Society of Agronomy. Madison, Wisconsin, USA. 1572 p; 1965.

39. Rebecca B. Soil survey laboratory methods manual. Soil Survey Investigations Report No. 42. Natural Resources Conservation Services; 2004.

40. Bates LS, Waldren RP, Teare JD. Rapid determination of free proline for water stress studies. Plant Soil. 1973;39:205-207. https://doi.org/10.1007/BF00018060

41. Page AL, Miller RH, Keeney DR. "Methods of Chemical Analysis". Part 2: "Chemical and Microbiological Properties" 2nd ed. American Society of Agronomy, Inc. and Sci., Soc of America, Inc Publishers, Madison, Wisconsin USA; 1982.

42. Soltanpour N, Schwab A. A new soil test for simultaneous extraction of macro and micronutrients in alkaline soils. Commun Soil Sci plant Anal. 1977;3:195. https://doi.org/10.1080/00103627709366714

43. SAS. “SAS user's guide”, Statistical Analysis System Institute, Inc. Cary, N. C. 1999.

44. Mignard S, Flandrois J. 16S rRNA sequencing in routine bacterial identification: a 30-month experiment. J Microbiol Methods. 2006;67:574–581. https://doi.org/10.1016/j.mimet.2006.05.009

45. Ludwig F, Kroon H D, Berendse F, Prins H. The influence of savanna trees on nutrient, water and light availability and the understorey vegetation. Plant Ecology. 2004;170:93–105. https://doi.org/10.1023/B:VEGE.0000019023.29636.92

46. Abdel Ghaffar L, Eissa AE, Elhariri1 M, Abdelsalam M, Al-Amry K. Comparative analysis of 16S rRNA based phylogeny, antibiotic susceptibility, and virulence traits of *Arcobacter* species recovered from domestic fowl and the Nile tilapia. Egyptian Journal of Aquatic Biology & Fisheries. 2021;25(3):263–279. https://doi.org/10.21608/EJABF.2021.175523

47. Ambawade MS, Pathade GR. Production of Indole Acetic Acid (IAA) by Stenotrophomonas maltophilia BE25 isolated from roots of banana (*Musa* spp.). Intl J Sci Res. 2015;4(1):2644-2650.

48. Mohite B. Isolation and characterization of indole-3-acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. Journal of Soil Science and Plant Nutrition. 2013;13:638-649. https://doi.org/10.4067/S0718-95162013005000051

49. Islam S, Akanda AM, Prova A, Sultana F, Hossain MM. Isolation and identification of plant growth promoting rhizobacteria from cucumber rhizosphere and their effect on plant growth promotion and disease suppression. Front Microbiol. 2015;6:1360.

50. Abd Alla MH, Bashandy SR, Nafady NA, Hassan AA. Enhancement of exopolysaccharide production by *Stenotrophomonas maltophilia* and *Brevibacillus parabrevis* isolated from root nodules of *Cicer arietinum* L. and *Vigna unguiculata* L. (Walp.) plants Rendiconti Lincei. Scienze Fisiche e Naturali. 2018;29:117–129. https://doi.org/10.1007/s12210-018-0671-1

51. Reinhardt E, Patrícia L, Ramos Gilson P, Manfio Heloiza R, Barbosa Crodowaldo Pavan, Carlos A, Moreira F. Molecular characterization of nitrogen fixing bacteria isolated from Brazilian agricultural plants at Sao Paulo state. Braz J Microbiol. 2008;39:no.3. https://doi.org/10.1590/S1517-83822008000300002

52. Adeleke BS, Ayangbenro AS, Babalola OO. Effect of endophytic bacterium *Stenotrophomonas maltophilia* JVB5 on sunflowers. Plant Protection Science. 2022;58(3):185-198. https://doi.org/10.17221/171/2021-PPS

53. Khan Z, Kim SG, Jeon YH, Khan HU, Son SH, Kim YH. A plant growth promoting rhizobacterium, *Paenibacillus polymyxa* strain GBR-1, suppresses root-knot nematode. Biores. Technol. 2008;99:3016-3023. https://doi.org/10.1016/j.biortech.2007.06.031

54. Xiao C, Chi R, He H, Zhang W. Characterization of tricalcium phosphate solubilization by *Stenotrophomonas maltophilia* YC isolated from phosphate mines. J Central South Univ Technol. 2009;16(4):581-587. https://doi.org/10.1007/s11771-009-0097-0

55. Gonzalez AM, Victoria DE, Gomez Merino FC. Efficiency of plant growth promoting rhizobacteria (PGPR) in sugarcane. Terra Latinoamericana. 2015;33:321-330.

56. Etesami H, Alikhani HA. Suppression of the fungal pathogen *Magnaporthe grisea* by *Stenotrophomonas maltophilia*, a seed-borne rice (*Oryza sativa* L.) endophytic bacterium. Archives of Agronomy and Soil Science. 2016;62:1271–1284. https://doi.org/10.1080/03650340.2016.1139087

57. Kasim WA, Osman ME, Omar MN, Salama S. Enhancement of drought tolerance in *Triticum aestivum* L. seedlings using *Azospirillum brasilense* NO40 and *Stenotrophomonas maltophilia* B11. Bulletin of the National Research Centre. 2021;45:95. https://doi.org/10.1186/s42269-021-00546-6

58. Liaquat F, Munis MFH, Arif S, Haroon U, Shengquan C, Qunlu L. Cd-tolerant SY-2 strain of *Stenotrophomonas maltophilia*: A potential PGPR, isolated from the Nanjing mining area in China. 3 Biotech. 2020;10:519. https://doi.org/10.1007/s13205-020-02524-7

59. Singh S, Ram B, Bahadur I. Solubilization of potassium containing various K-mineral sources by K-solubilizing bacterial isolates on Aleksandrov medium. International Journal of Current Microbiology and Applied Sciences. 2018;7(3):1142–1151. https://doi.org/10.20546/ijcmas.2018.703.136

60. Claussen W. Proline as a measure of stress in tomato plants. Plant Sci. 2005; 168:241–248. https://doi.org/10.1016/j.plantsci.2004.07.039

550 61. Alexander A, Singh VK, Mishra A. Halotolerant PGPR *Stenotrophomonas maltophilia*
551 BJ01 induces salt tolerance by modulating physiology and biochemical activities of *Arachis*
552 *hypogea*. Front Microbiol. 2020;11:568289. https://doi.org/10.3389/fmicb.2020.568289

553 62. Tuna AL, Kaya C, Dikilitas M, Higgs D. The combined effects of gibberellic acid and
554 salinity on some antioxidant enzyme activities, plant growth parameters and nutritional status
555 in maize plants. Environ Exp Bot. 2008;62:1–9.
556 https://doi.org/10.1016/j.envexpbot.2007.06.007

557 63. Barry CS. The stay-green revolution: recent progress in deciphering the mechanisms of
558 chlorophyll degradation in higher plants. Plant Sci. 2009;176:325-333.
559 https://doi.org/10.1016/j.plantsci.2008.12.013

560 64. Baset MA, Shamsuddin ZH, Wahab ZH, Marziah M. Rhizobacteria as bioenhancer and
561 biofertilizer for growth and yield of banana (*Musa* spp. cv. 'Berangan'). Sci Hortic.
562 2010;126(2):80-87. https://doi.org/10.1016/j.scienta.2010.06.005

563 65. Spaepen S, Jos V, Roseline R. Indole-3-Acetic Acid in microbial and microorganism
564 plant signaling. FEMS Microbiology Review. 2007;31(4):425-448.
565 https://doi.org/10.1111/j.1574-6976.2007.00072.x

566 66. Lambrecht M, Okon Y, Vande Broek A, Vanderleyden J. Indole-3-acetic acid: a
567 reciprocal signalling molecule in bacteria–plant interactions. Trends in Microbiology.
568 2000;8(7):298-300. https://doi.org/10.1016/S0966-842X(00)01732-7

569 67. Taghavi S, Garafola C, Monchy S, Newman L, Hoffman A, Weyens N. Genome survey
570 and characterization of endophyt bacteria exhibiting a beneficial effect on growth and
571 development of poplar. Appl Environ Microbiol. 2009;75:748-757.
572 https://doi.org/10.1128/AEM.02239-08

573