



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

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Authors' contributions

This work was carried out in collaboration between both authors. Author AAA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author NHO managed the analyses of the study and managed the literature searches. Both authors read and approved the final manuscript.

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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)

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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 safer, cost-effective, and ecologically beneficial [1]. These biopreparations are known to have a variety of plant growth-promoting (PGP) traits, which improve plant health, growth, and production [2]. "Endophytic bacteria from root nodules have recently drawn increased interest as unique resources for enhancing plant development. The endophytic bacteria reside inside plant tissues without any observed alteration of the morphology of their hosts. They significantly impact the plants' capacity to absorb nutrients because they are more efficient in nutrient transformation, mobilization, and solubilization" [3,4]. "Additionally, they benefit host-plants by fixing nitrogen, solubilizing phosphate, and producing indole-3- acetic acid (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 have a noticeable impact on the crop's survival, nodulation, and grain yield [6,7,8]. *Stenotrophomonas* bacteria received increased attention because they could serve as efficient bioinoculants for promoting plant development and controlling various diseases [9]. *S. maltophilia*, a species of *Stenotrophomonas*, is recognized as a crucial species for boosting plant development in agriculture [10]. Gram-negative bacteria of the *Xanthomonadaceae* family. Endophytes and free-living bacteria, *Stenotrophomonas* species, have been identified as the major species in the bacterial community linked with plants [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 used 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.

The 16S rRNA genes are present in all prokaryotes and code for the RNA component of the ribosomal 30S subunit that has an essential role in translation [18]. Sequencing the 16S rRNA gene is the most effective method for identifying unknown bacteria. A root represents a phylogenetic tree's bacterial taxonomic origin pattern [19,20].

"Egyptian clover (*Trifolium alexandrinum* L.) is Egypt's primary annual winter forage leguminous crop. Berseem clover is highly nutritious for the animal field and improves soil fertility and its physical characteristics" [21]. "Berseem forage is superior to grasses in protein and mineral contents" [22].

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

2. MATERIALS 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 % HgCl₂ (w/v) for 2 min before being rinsed 6-8 times with sterile distilled water to completely remove HgCl₂. 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

Total genomic DNA was isolated and purified following the technique described in [26]. The quality of the extracted DNA from several bacterial species was assessed on a 1% Agarose gel.

2.2.2 Identification of isolates by 16S rRNA sequencing

“Isolated DNA was amplified with 16S rRNA in a PCR tube and 20 µl of the reaction solution. The PCR amplification was performed using 27F/1492R universal primers” [27], as shown in Table 1. The amplification took place over 35 cycles for 45 sec at 94° for denaturation, 55°C for 60 sec for annealing, and extension at 72°C for 60 sec. The PCR include a positive control (*E. coli* genomic DNA) and a negative control. The PCR amplicon purification was completed using a column-based technique and the Montage PCR Clean-up kit (Macro gen). The purified PCR products were sequenced using two 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

The 16S rRNA gene sequences of chosen isolates were compared to the 16S rRNA gene sequences using BLASTN in the GenBank database. A phylogenetic tree was constructed using the Neighbor joining method. 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

“To estimate 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 at 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 the amount of exopolysaccharides was calculated by dry-weight measurements as g/l according to” [31].

2.3.3 Nitrogen fixing activity

Nitrogen-fixing activity was examined using Jensen's medium and Bromothymol Blue (BTB) as a colour 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. 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)

Table 1. The sequences of the primers used for 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'

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 (dS/m)	Cations (meq ⁻¹)				Anions (meq ⁻¹)		
		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		
107.1	2.95	171.16	0.868	2.16	1.85	0.61		

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 seven 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 incubating for seven 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 a flame photometer” [37].

2.4 Field Experiment

To elucidate the role of the isolated bacteria in alleviating salt stress in clover plants grown in saline soil compared to *R.leguminosarum* bv. *trifolii* strain 102, specific to clover plants, a field experiment was carried out at El-Rowad village farm, Sahl El-Hussinia, El-Sharkia Governorate, Egypt, with a randomized complete block design and three replicates. 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 + a half dose of NPK
4. *S. maltophilia* strain 262XG2 + a 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, 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).

2.8 Statistical Analysis

Data were statistically analyzed using the general linear model's 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 colour when cultured on yeast extract mannitol agar (YEMA) containing Congo red.

3.2 Genotypic Characterization of the Bacterial Isolates

Using PCR to detect the 16S rRNA gene revealed the presence of a band of approximately 1,200 bp in length. Isolates AM1

and AM2 were subjected to the partial 16S rRNA gene sequences of 1274 and 1228 base pairs. The 16S rRNA gene sequencing of the chosen isolates AM1 (accession number OM980221.1) and AM2 (accession number OM980223.1) had 98% sequence similarity with the 16S rRNA gene of *Stenotrophomonas maltophilia* (IPR-Pv696) and (262XG2), respectively. The results indicated that rRNA gene sequencing is beneficial for bacterial categorization [44]. A phylogenetic tree of the 16S rRNA sequences was created (Fig. 1) showing the phylogenetic relationship of the selected isolates *Stenotrophomonas maltophilia* strains, IPR-Pv696 and 262XG2.

With the use of a frequency filter included in the ARB (from the Latin arbour tree) software package, a neighbour-joining tree was generated using partial 16S rRNA gene sequences [45]. The Neighbour-Joining algorithm divided the phylogenetic tree into clusters with values ranging from 0.057 to 0.137. These clusters verified the AM1 and AM2 bacterial isolates as *Stenotrophomonas*, and are close to *Stenotrophomonas maltophilia* strain PgBe201. These results agree with [46], who revealed that for phylogenetic analyses of the genus *Arcobacter*, the 16S rRNA gene sequence analysis has proven to be a beneficial 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 N₂ 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.

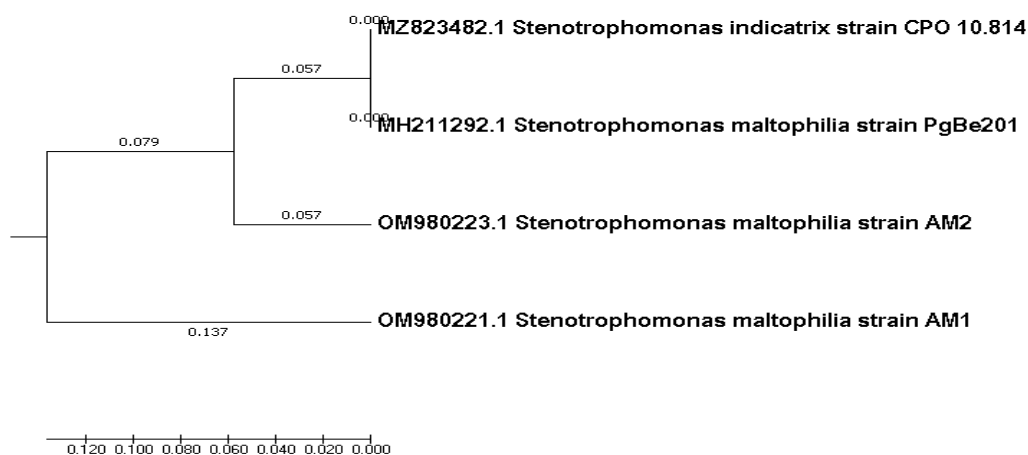


Fig. 1. Phylogenetic tree showing the phylogenetic relationship of the two bacterial isolates

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

Isolates No	IAA ($\mu\text{g ml}^{-1}$)	EPS (g/l)	Nitrogen fixation	Phosphate solubilization		Potassium solubilization	
				Solubilization efficiency (SE %)	P-liberated (mg/l)	Solubilization efficiency (SE %)	K- liberated ($\mu\text{g/ml}$)
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)

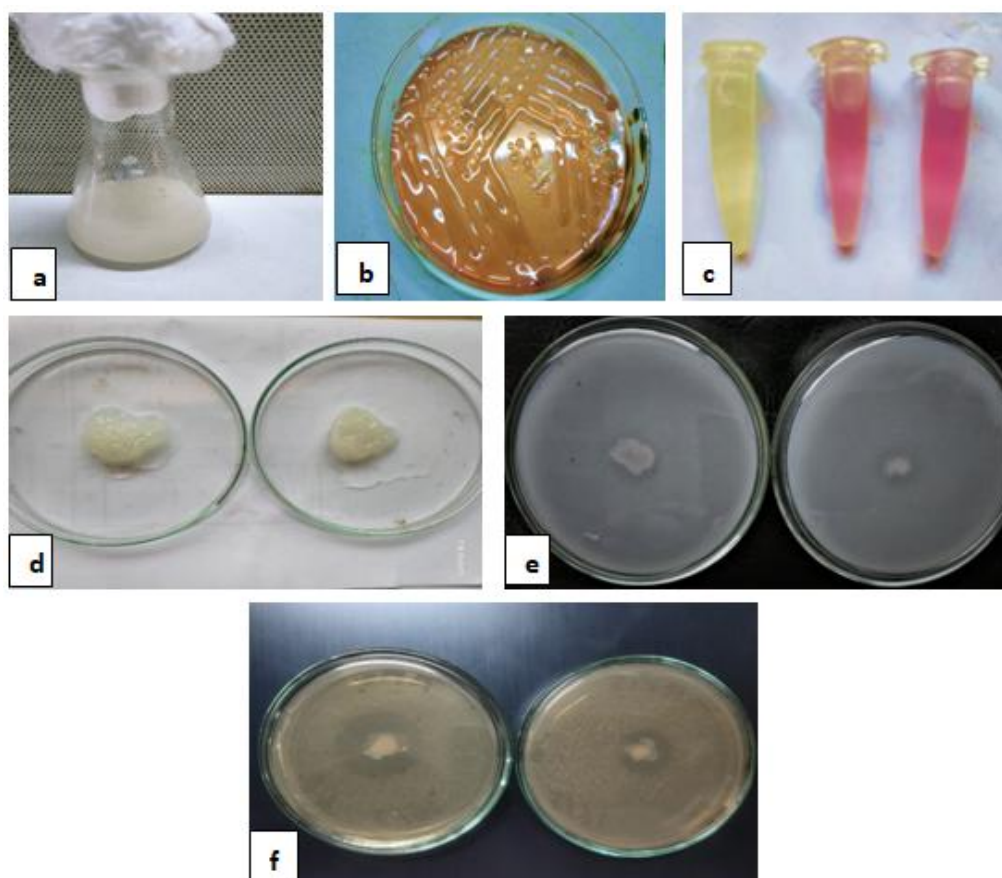


Fig. 2. Plant growth-promoting activities of the bacterial isolates *In vitro*
(a) Pure isolate was grown in YEM broth media. (b) Isolate grown in Congo red YEMA medium.
(c) IAA production by isolates. (d) EPS production by isolates. (e) *In vitro* assay for phosphate solubilization by bacterial isolates.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

The ability of P solubilization is also the 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%. Phosphate 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].

3.4 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]. Our results showed that 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.

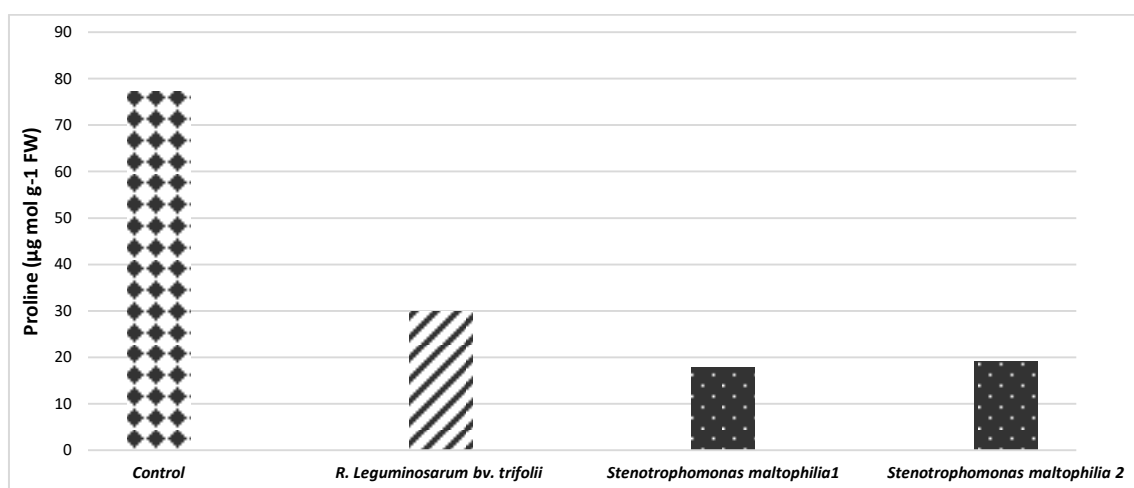


Fig. 3. Proline as affected by the inoculation of bacterial isolates

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

Treatments	N (%)	P (%)	K (%)
Control (Recommended dose of NPK)	2.36 ^b	0.10 ^a	3 ^{ab}
<i>R. Leguminosarum</i> bv. <i>trifolii</i> strain (102)	2.23 ^c	0.09 ^a	2.35 ^b
<i>S. maltophilia</i> strain IPR-Pv696 (AM1)	2.90 ^a	0.11 ^a	3.55 ^a
<i>S. maltophilia</i> 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 data shown in Table 4 revealed that the two isolates significantly increased the N and K contents compared to the control and rhizobial inoculated plants. However, there was no significant difference in the P content between 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].

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
<i>R. Leguminosarum</i> bv. <i>trifolii</i> strain (102)	32.28 ^d	47.39 ^d	39.25 ^d	6.67 ^d
<i>S. maltophilia</i> strain IPR-Pv696	36.93 ^b	49.08 ^b	40.53 ^b	6.92 ^b
<i>S. maltophilia</i> 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 the potential use of *S. maltophilia* as biofertilizer. Altogether, we propose *S. maltophilia* to promote sustainable clover agriculture and as a promising inoculant for many other crops.

DATA AVAILABILITY

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

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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