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 the salt-affected clay soil of Egypt. Based on the sequencing and phylogenetic analysis of *16S rRNA* genes, the isolates were closely linked to *Stenotrophomonas maltophilia* strains IPR-Pv696 and 262XG2 and deposited in GenBank with accession numbers OM980221.1 (Am1) and OM980223.1 (Am2), respectively. We evaluated the potential of both isolates to promote plant growth and found that the two isolates, IPR-Pv696 and 262XG2, respectively, produced indole-3- acetic acid (30.26 & 31.15 µg/ml) and exopolysaccharides (13.57 & 13.68 g/l). In addition, there was a difference in their nitrogen fixation activity and their ability to solubilize phosphate, 278 & 208 mg/l, and potassium, 33.5 & 32.9 µg/ml), respectively. Under salty soil field conditions, both isolates increased clover plant growth, chlorophyll, carbohydrate content, and nutrient uptake while lowering proline levels. Our results highlight the advantages of applying *Stenotrophomonas maltophilia* as a biofertilizer for clover and possibly for other crops in sustainable agriculture.

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 Stenotrophomonas genus are members 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. MATERIAL AND METHODS

2.1 Isolation of nodule endophytes

Nodules were collected randomly from each clover plant, washed with sterile distilled water to remove soil particles and surface sterilized with 95 % alcohol for 30 seconds and 0.1 % HgCl2 (w/v) for 2 min before being rinsed 6-8 times with sterile distilled water to remove HgCl2 completely. For the isolation of endophytic bacteria, the surface-sterilized nodules were crushed and streaked on 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 repeatedly streaking on the same medium for three days at 30℃. The isolates were kept at -20℃ 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℃ 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. 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.

Table 1. The sequences of the forward and reverse 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′

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℃. After incubation, bacteria were extracted using a centrifuge set at 3824 xg for 15 minutes, and 2 ml of the supernatant was mixed with two drops of orthophosphoric acid and 4 mL Salkowski's reagent, then 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℃ 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 exo-polysaccharides 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℃. 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 seven days at 28℃±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℃±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 follows:

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

Each plot's fresh yield was measured immediately after harvesting to prevent humidity loss. 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 a stable weight over three additional days, the weight obtained was scored as an approximation of the dry weight percentage of the dry matter produced by each treatment. Shoots' carbohydrate and chlorophyll contents were measured. Dry samples were ground and

digested utilizing the H2SO4 and HClO4 acid mixture [38]. The method described by [39] was used to determine the plants' N, P, and K content 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 air dried, sieved using 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. The pH of soil suspension (1: 2.5) was measured using a pH meter (Table 2). [38] measured the available nitrogen using the modified Kjeldahl method. Following 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 (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
PPM						
107.1	2.95	171.16	0.868	2.16	1.85	0.61

2.8 Statistical analysis

Data were statistically analyzed using the general linear model's procedure of SAS [43]. The significant differences between treatments were statistically tested using Duncan's multiple-range tests.