ASSESSMENT OF PHYTOHORMONES PRODUCING CAPACITY OF STENOTROPHOMONAS MALTOPHILIA SSA AND ITS INTERACTION WITH ZEA MAYS L.

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Abstract

In this paper we reported the isolation of Stenotrophomonas maltophilia SSA from roots of Solanum surrattense Burm. f., a weed growing at arid soil of district Attock, Pakistan. The isolate was characterized for morphological and biochemical attributes and was identified as Stenotrophomonas maltophilia SSA on the basis of 16S-rRNA partial sequence analysis. The population size varied from $10^6$ to $10^7$ cfu g$^{-1}$ fresh weight of root. Phytohormones: Indole-3-acetic acid, Gibberellic acid, trans-Zeatin riboside and Abscisic acid were found in culture supernatant as measured by high performance liquid chromatography. The bacterium used as inoculants on Zea mays L. seedlings showed significant increase in the growth and proline content of root and shoot both under normal and NaCl stressed conditions.

Introduction

The genus Stenotrophomonas phylogenetically resides within the γ-subclass of the Proteobacteria (Moore et al., 1997) and plays very important ecological role in the element cycle in nature. It is gram negative Bacillus (Gautam et al., 2009) and found in a wide variety of environmental and geographical regions including hospitals, extreme environment such as Soda Lake (Denton & Kerr, 1998), rhizosphere of plants (Berg et al., 1996) and aquatic habitats (Minkwitz & Berg, 2001).

Stenotrophomonas maltophilia has dual characteristics, on one side it act as nosocomial multidrug-resistant pathogen associated with immuno-suppressed patients (Denton & Kerr, 1998) and on the other side it is non-pathogenic to plants (Wolf et al., 2002) and aids beneficial effects in plant growth and development (Taghavi et al., 2009). The production of IAA, nitrogen fixing (Park et al., 2005) capability and production of anti-fungal metabolites (Minkwitz & Berg, 2001) by Stenotrophomonas maltophilia may reveal its bio-inoculant (Park et al., 2005) properties. It also showed tolerance to external osmolarity by the accumulation of certain osmoprotective compounds and can grow well under NaCl stress (Miller & Wood, 1996). Naz et al., (2009) reported isolation of Rhizobia from rhizosphere soil of Solanum surrattense growing in arid zone of Attock. PGPB implies positive effects on root biomass and yield of plants (Shahzad et al., 2008).

Weeds harbour PGPR which as bio-inoculant are the best alternative to vegetate agricultural soils (Naz et al., 2010) but very few reports are available on this aspect. Keeping this point in mind the present study was aimed to (1) isolate and characterize bacterial strain and to (2) estimate phytohormones viz., GA$_3$, ABA and trans-Zeatin riboside by this bacterial isolate besides IAA production by this bacterial isolate besides IAA production and (3) to evaluate its potential as bio-inoculant on Zea mays L., grown under normal and NaCl stressed (20 dS m$^{-1}$) conditions.

Materials and Methods

Sampling of the roots: The root samples were collected in five replicates from different localities of district Attock at vegetative phase of commonly growing weed: Solanum surrattense Burm. f. (Yellow berried night shade). The roots were stored at 4°C for further processing.

Isolation and biochemical characterization of bacterial isolate: Root (1g) was rinsed with tap water, subsequently washed with sterile distilled water and ground in a sterile mortar with 10 ml distilled water. Serial dilutions were prepared and 100 µl from decimal dilutions was used to inoculate Luria Bertani (LB) media. The plates were incubated at 30°C for 24-72 h. Colonies showed similarities with Stenotrophomonas were observed under light microscope (Nikon, Japan) and preserved in glycerol for further tests. The viable colonies were counted at $10^6$ and $10^7$ dilutions using the formula:

$$\text{Number of colonies} = \frac{\text{Volume of inoculum} \times \text{dilution factor}}{\text{Volume of inoculum}}$$

Gram staining of the bacterial isolate was done by the method of Vincent (1970) and biochemically characterized on the basis of carbon/nitrogen source utilization pattern using QTS-24 miniaturized identification system (DESTO Laboratories Karachi, Pakistan) following the method of MacFaddin (1980). One day old bacterial culture was used for this purpose; results were noted after 18 h of incubation at 30°C and compared with standard species mentioned in Bergey’s Manual of Determinative Bacteriology (Holt et al., 1994).16S-rRNA gene amplification and sequencing DNA of the bacterial isolate was extracted using the method of Chen & Kuo (1993) and amplified following the method of Weisburg (1991). The polymerase chain reaction (PCR) was performed using two primers rdl (AAGGAGGTGATCCAGCC) and fdl (AGAGTTTGTCCGCCCTCAG). The reaction volume contained 1 µl of 50 µg of genomic DNA, 1.5 mM MgCl$_2$, 0.2 mM dNTP mix, 5 µl of 10 X Taq buffer, 1 U Taq DNA polymerase, 10 pmoles of each primer and
Finally, the volume was raised to 25 µl by autoclaved pure water. The reactions were carried out in thermocycler (Biometra, Germany). After denaturation at 95°C for 2 min, samples were passed for 30 min through the following temperature profile: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 2 min plus one additional cycle for chain elongation at 72°C for 10 min. Amplified PCR products were separated on 1.2% (w/v) agarose gel and visualized under UV trans-illuminator lamp and compared with 1 Kb DNA ladder. The band of almost 1.5Kb was excised from the gels, purified using JET QUICK gel extraction spin kit (GENOMED) and sequenced on the automatic sequencer (CEQ-8800 Genetic analysis system, Beckman Coulter, USA) using CEQ DTCS kit (Beckman Coulter, USA). The obtained sequence was compared with reference species available at National Centre for Biotechnology Information (NCBI) database by employing BLAST algorithm and deposited in NCBI Genbank (www.ncbi.nlm.nih.gov/Genbank/submit.html) with the accession numbers (GQ262775).

Quantification of growth regulating substances in the culture supernatant Luria Bertani (LB) media supplemented with or without tryptophan (10 mg/100 ml) was inoculated with single colony of 24 h old bacterial culture and kept on shaker (ECELLA E24, USA) at 100rpm for 5d, until OD of cultures became equivalent to 1 at 600nm and 10⁷ colony forming unit (CFU/g) was obtained on LB agar plates. The bacterial culture was centrifuged at 10,000 rpm for 15 min at 4°C and supernatant (cell-free liquid culture medium) was used for extraction of phytohormones following the method described by Tien et al., (1979). The extracted samples were analyzed on high performance liquid chromatography (Agilent 1100) equipped with variable UV detector and C₁₈ column (39 x 300mm) [BondaPack Porasil C-18, 37/50 µm, Waters, Eschborn, BRD). Methanol and water in the ratio of 30:70 v/v were used as mobile phase @ of 1500µl/min. with a run time of 20 min/sample. For identification of hormones, 100µl of sample filtered through a 0.45millipore filter, were injected into column. The growth hormones were identified on the basis of retention time of phytohormone standards (commercially grade, Sigma Chemical USA Company). IAA was eluted at 280nm wavelength while GA₃, t-Zr and ABA were eluted at 254nm respectively. The un-inoculated culture media was treated as blank.

Response of Zea mays L. seedlings to Stenotrophomonas maltophilia SSA

The seeds of the maize (advance germ plasm line: Islamabad Gold) were surface sterilized with 95% ethanol for 2-3 min and soaked overnight in distilled water prior to soaking in 5d old culture of Stenotrophomonas maltophilia (10⁶-10⁷ cfu g⁻¹ fresh weight of root) for 2 h. The seeds soaked in un-inoculated culture media were treated as control. The soaked seeds were sown in pre-sterilized pots (19cm x 17cm) containing sterilized soil and sand (2:1 ratio). The temperature of growth room was maintained at 25 ± 2°C with the photoperiod 16h and the humidity varying from 65-70%. The plants were irrigated with Hogland nutrient solution. Two weeks after inoculation, seedlings were gradually exposed to NaCl by adding 5 dS m⁻¹ NaCl per day until the final concentration of 20 dS m⁻¹ was reached. Measurements were made 30d after inoculation. Three replicates (five plants / replicate) were used for measurement. Proline contents of shoots and roots were measured following the method of Bates et al., (1973).

Statistical analysis: Factorial analysis of variance (ANOVA) and two factor completely randomized design test (CRD) with least significance difference (LSD) was applied using MSTAT C program, version 4.0.

Results

The colonies of Stenotrophomonas maltophilia SSA isolated from roots of Solanum surattense grown in arid area of Attock appeared round, yellow and with smooth margin on LB medium. The number of viable colonies of isolate ranged from 10⁶-10⁷ cfu/g fresh weight of root. The isolate was gram -ive, rod shaped, motile in nature and characterized by biochemical tests (QTS-24 miniaturized identification system, DESTO Laboratories Karachi, Pakistan) based on carbon/nitrogen source utilization pattern (Table 1) which revealed the isolate was positive for the tests including sodium citrate, sodium melanone, lysine decarboxylase, ornithine decarboxylase, H₅S production, tryptophan deaminase, indole production, gelatin hydrolysis, acid from glucose, acid from maltose, acid from inositol, acid from sorbitol and acid from raffinose. The isolate was negative for tests viz: ortho nitro phenyl β-D-galactopyranoside, arginine dihydrolase, urea hydrolysis, Voges proskauer, acid from sucrose, acid from mannose, acid from arabinose, acid from rhamnose and acid from adonitol.

<table>
<thead>
<tr>
<th>Tests</th>
<th>Result</th>
<th>Tests</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ortho nitro phenyl β-D-galactopyranoside</td>
<td>-</td>
<td>Gelatin hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>+</td>
<td>Acid from glucose</td>
<td>+</td>
</tr>
<tr>
<td>Sodium melanate</td>
<td>+</td>
<td>Acid from maltose</td>
<td>+</td>
</tr>
<tr>
<td>Lysine decarboxylase</td>
<td>+</td>
<td>Acid from sucrose</td>
<td>-</td>
</tr>
<tr>
<td>Arginine dihydrolase</td>
<td>-</td>
<td>Acid from mannose</td>
<td>-</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>+</td>
<td>Acid arabinose</td>
<td>-</td>
</tr>
<tr>
<td>H₅S production</td>
<td>+</td>
<td>Acid from rhamnose</td>
<td>-</td>
</tr>
<tr>
<td>Urea hydrolysis</td>
<td>-</td>
<td>Acid from sorbitol</td>
<td>+</td>
</tr>
<tr>
<td>Tryptophan deaminase</td>
<td>+</td>
<td>Acid from inositol</td>
<td>+</td>
</tr>
<tr>
<td>Voges proskauer</td>
<td>-</td>
<td>Acid from adonitol</td>
<td>-</td>
</tr>
<tr>
<td>Indole</td>
<td>+</td>
<td>Acid from raffinose</td>
<td>+</td>
</tr>
</tbody>
</table>

**Stenotrophomonas sp.**
The 16S-rRNA sequence analyses revealed sequence information of 527 nucleotides. The comparison of this sequence depicted 95% similarity (identities 487/512 positions) with the sequence of *Stenotrophomonas maltophilia* R551-3 (Accession No. 011071) already deposited in GenBank.

The results (Table 2) revealed that the level of IAA was 5 folds greater in the culture medium supplemented with tryptophan in contrast, the production of GA3, t-Zr and ABA were six, four and five times respectively, greater in without tryptophan when compared to with tryptophan medium. The isolate was used to inoculate seedlings of *Zea mays* L., (advance germplasm line Islamabad Gold) to evaluate its performance under normal and induced salt stress (20 dS/m NaCl) conditions (Table 3). Significant increases were observed in root length, shoot length, root dry weight and shoot dry weight of inoculated *Zea mays* L., plants growing under normal and salt stressed conditions in contrast to un-inoculated (control) plants. The magnitude of stimulation was markedly greater under unstressed condition.

![Image](https://example.com/image)

**Table 3. Inoculation effects of Bacterial isolate on shoot and root length (cm) and dry weight (g) of 40 days old *Zea mays* L. plants growing under induced salt stress (20 dS m⁻¹ NaCl) and unstressed conditions.**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Shoot length (cm)</th>
<th>Root Length (cm)</th>
<th>Shoot weight (g)</th>
<th>Root weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+NaCl -NaCl</td>
<td>+NaCl -NaCl</td>
<td>+NaCl -NaCl</td>
<td>+NaCl -NaCl</td>
</tr>
<tr>
<td>T1</td>
<td>39.4B 48.2A</td>
<td>20C 30.6A</td>
<td>6.7 B 11.2 A</td>
<td>5 B 8.2 A</td>
</tr>
<tr>
<td>T2</td>
<td>28.8C 41.2B</td>
<td>15.4D 25.3B</td>
<td>4.00 C 8.6 B</td>
<td>2.3 C 5.1 B</td>
</tr>
</tbody>
</table>

+NaCl= Seedlings treated with 20 dS m⁻¹ NaCl, -NaCl = Seedlings not treated with 20 dS⁻¹ NaCl. T1 represents the inoculation treatment with *Stenotrophomonas maltophilia* SSA while T2 represents un-inoculated plant.

The proline content of root was significantly increased in presence of NaCl both in the un-inoculated and seedlings inoculated with *S. maltophilia* SSA (Table 4). The basal level of proline was also higher in both roots and shoots of inoculated plants, comparatively the roots of inoculated plants accumulated more proline than the shoots.

**Table 4. Proline contents (mg g⁻¹) of shoots and roots of 40 days old *Zea mays* plants (growing in presence and absence of 20 dS/m NaCl stress) inoculated with *Stenotrophomonas maltophilia* SSA.**

<table>
<thead>
<tr>
<th>Treatments</th>
<th><em>Zea mays</em> shoots</th>
<th><em>Zea mays</em> roots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- NaCl + NaCl</td>
<td>- NaCl + NaCl</td>
</tr>
<tr>
<td>T1</td>
<td>12.4 C 16.2 A</td>
<td>14.3 C 19.6 A</td>
</tr>
<tr>
<td>T2</td>
<td>10 D 13.9 B</td>
<td>11 B 16.3 B</td>
</tr>
</tbody>
</table>

+NaCl= Seedlings treated with 20 dS m⁻¹ NaCl, -NaCl = Seedlings not treated with 20 dS⁻¹ NaCl. T1 represents the inoculation treatment with *Stenotrophomonas maltophilia* SSA while T2 represents un-inoculated plant. All such values which share a common letter are insignificantly different otherwise, they differ at p≤0.05.

**Discussion**

The morphological and biochemical analyses indicated highest (98%) similarity of the isolate with the genus *Stenotrophomonas* when compared with Bergey’s Manual of Determinative Bacteriology (Holt et al., 1994). In addition the phenotypic characteristic of this species correlate well with the molecular analyses based on 16S-rRNA partial sequence analyses. Naz et al., (2010) identified three phosphate solubilizing bacteria up to genera level by QTS-24 kit as belonging to genera *Pseudomonas* and these three strains were identified as *Pseudomonas mendocina* Khsr2, *Pseudomonas stutzeri* Khsr3 and *Pseudomonas putida* Khsr4 by 16S-rRNA.

The colony count of *S. maltophilia* SSA appeared to be in the range reported earlier. Tsavkelova et al., (2007) quantified 10⁶ *Stenotrophomonas* colonies per g root of terrestrial and epiphytic tropical orchids. The population density of bacteria in the rhizosphere and roots depend on nature of root exudates, root morphology, stages of plant growth, physical and as well as chemical properties of soil (Vessey, 2003).

The amount of IAA produced (250 µg/ml) by the isolate in presence of added tryptophan was almost 2x higher than the IAA produced by *Stenotrophomonas maltophilia* PM-1 (112.8 µg/ml) and *Stenotrophomonas maltophilia* PM-26 (139.2 µg/ml) reported by Park et al. (2005) and 8X more than *Stenotrophomonas* sp. (31 µg/ml) isolated by Tsavkelova et al., (2007). The PGPR strains have been reported to produce IAA either with or without the tryptophan supplement in culture media (Fatima et al., 2009; Mehnaz et al., 2001). Greater production of IAA obtained during the present study might be due to the presence of tryptophan deaminase enzyme in *Stenotrophomonas maltophilia* that utilized tryptophan as precursor of IAA. Auxin biosynthesis is wide spread among soil and plant associated bacteria (Verma et al., 2001) including *Stenotrophomonas maltophilia* (Suckstorff & Berg, 2003).

Literatures are documented regarding the GA₃, ABA and trans-Zeatin riboside production by PGPR (Perrig et al., 2007; Boiero et al., 2007; Cohen et al., 2007) but there is no evidence describing the production of GA₃, ABA and trans-Zeatin by *Stenotrophomonas maltophilia*. This is the first report regarding the GA₃, t-zr and ABA.
production by *Stenotrophomonas maltophilia* in culture supplemented with or without tryptophan.

The increase in IAA production but decrease in GA3, t-zr and ABA concentration in culture media supplemented with tryptophan represents antagonistic interaction of IAA with GA3, ABA and t-zr as reported earlier (Naz et al., 2010) Present results evaluated that plant growth affected badly by salt stress. Negative effects of salt stress on germination, seedling growth, plant bio mass and some physiological activities of many plant species have been investigated (Hussain et al., 2008; Hussain et al., 2009; Shah et al., 1987; Ashraf et al., 1994) but the growth of NaCl stressed plants inoculated with *Stenotrophomonas maltophilia* SSA was better then un-inoculated plants. This is due to *Stenotrophomonas maltophilia* SSA isolated from arid zone of Attock which exhibited the ability to adapt under saline condition and provided tolerance to maize seedlings. Miller & Wood (1996) demonstrated that *Stenotrophomonas* strains have the ability to adapt themselves under salt stress perhaps by the accumulation of osmoprotective compounds. This view was further supported by the finding that proline (an osmoregulant) content was higher in maize plants inoculated with *Stenotrophomonas maltophilia* SSA. Among the mechanism of adaptation by *Stenotrophomonas maltophilia* SSA under saline condition are the ability to produce ABA in culture medium and the ability to augment the production of proline in inoculated plants. PGPR-induced the proline accumulation, membrane integrity and antioxidant enzyme activities but it has not been widely studied for plants which are exposed to salt stress (Kohler et al., 2009; Nadeem et al., 2007; Paul & Nair, 2008). Microbial inoculation enhanced proline accumulation in the roots thus provides tolerance to plant under salinity stress (Sharifi et al., 2007). The link between proline and ABA production has been reported under salinity (Woodward & Bennett, 2004).

Evaluation of this isolate under the field condition and thorough investigation of bioactive metabolites production to unravel its use as a biocontrol agent constitute future research.

Acknowledgement

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References


Nadeem, S. M., Z.A. Zahir, M. Naveed and M. Arshad. 2007. Preliminary investigations on inducing salt tolerance in maize through inoculation with rhizobacteria containing


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