African Journal of Biotechnology Vol. 11(45), pp. 10368-10372, 5 June, 2012
Available online at http://www.academicjournals.org/AJB
DOI:10.5897/AJB12.140
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Potential of *Azotobacter vinelandii* Khsr1 as bio-inoculant

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Accepted 30 March, 2012

The present study deals with the isolation and characterization of *Azotobacter vinelandii* Khsr1 from roots of the weed, *Chrysopogon aucheri*, commonly known as golden beard grass indigenous to Khewra salt range, Pakistan and its evaluation as bio-inoculant. The population of the isolate varied from 10^7 to 10^8 cfu/g fresh weight of root. The preliminary identification of the isolate was made on the basis of carbon/nitrogen source utilization pattern as revealed by QTS-24 miniaturized identification system test which placed the isolate to the genus *Azotobacter*. The 16S-rRNA partial sequence analysis confirmed the isolate as *A. vinelandii* strain Khsr1. The 556 long nucleotide sequence of the isolate showed 98% similarity with *A. vinelandii* DJ (accession no. 012560.1). The isolate was capable of producing phytohormones: indole-3-acetic acid, gibberellic acid, trans-zeatin riboside and abscisic acid in culture supernatant, stimulated growth of *Zea mays* L. seedlings and augmented proline content of roots and shoots both under normal and NaCl stressed conditions. However, the magnitude of stimulation is higher under un-stressed condition.

Key words: *Azotobacter vinelandii* Khsr1, phytohormones production, growth stimulation, salinity stress.

INTRODUCTION

Different plant growth-promoting bacteria including free living and associative bacteria have been used in agri-culture system as an alternative to chemical fertilizers. Bio-fertilizers are based on renewable energy resources and increase fertility and as well as plant growth (Rodriguez et al., 2006; Tilak et al., 1982) and they are the best alternative to vegetate saline fields where most of the chemical fertilizers failed (Naz et al., 2010).

The genus *Azotobacter* has role in plant growth promotion due to the production of hormonal substances - indole-3-acetic acid (IAA), gibberellic acid (GA_3) and cytokinin (Mirkovacki and Milic, 2001; Lee et al., 1970; Tallar and Wong, 1989), improvement of nutrient uptake by plants (Subba Rao, 1982), involved in vitamins production (Martinez-Toledo et al., 1996) and act as biocontrol agent (Neyra et al., 1999; Fatima et al., 2009). Zahir et al. (2000) reported efficient growth of maize seedlings by the application of IAA producing *Azotobacter* sp. The application of *Azotobacter* and arbuscular mycorrhiza on plants also enhanced the survival percentage of *Morus alba* on salt affected lands (Kashyap and Sharma, 2006).

In the present study, the grass species, *Chrysopogon aucheri*, was collected from area surrounding Khewra salt mine located in Khewra, Jhelum District, Punjab in Pakistan. The main aim of the study was to isolate, characterize and identify bacterial isolates from roots of grass species growing in natural saline soil and to analyze its effects as bio-inoculant on cereal crop (maize) irrigated with 20 dS/m NaCl.

MATERIALS AND METHODS

Isolation and characterization of bacteria from roots of *C. aucheri*

The root and soil samples of *C. aucheri* were collected from three different localities of Khewra salt range. The samples were immediately transferred to the laboratory and stored at 4°C for

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further analysis. Soil was used to analyze nutrient contents of soil, electrical conductivity (EC) and pH, and data regarding it has already been published (Naz et al., 2009). For isolation of bacteria, 1 g root was grounded in sterile distilled water followed by successive washing with tap water. The root extract was centrifuged at 3000 rpm for 10 min and an aliquot (100 µL) from decimal dilutions was used to inoculate Macartney's vials containing NF semi solid agar medium and then to NF solid agar medium for the growth of isolate. The plates were incubated at 30°C for 24 to 72 h. Colonies that showed resemblance with *Azotobacter* were isolated, sub-cultured and observed under light microscope. Viable cell count was measured at 10^7 and 10^8 dilutions using the formula:

$$\text{Viable cell count (CFU/g fresh root weight)} = \frac{\text{Number of colonies}}{\text{Volume of inoculum}} \times \text{Dilution factor}$$

Oxidase test was performed to determine the presence of oxidase enzyme in bacterial isolates following the method of Steel (1961). Kovac's reagent (1% N,N,N,N-tetramethyl-*p*-phenylenediamine) was used for this purpose (Kovac's 1956). The oxidase positive colonies turned lavender-colored, and within 5 min these change to dark purple to black.

For catalase test, bacterial colonies (24 h old) were taken on glass slides and one drop of H_2O_2 (30%) was added. The appearance of gas bubble indicated the presence of catalase enzyme (MacFaddin, 1980). Also, physiological and biochemical tests for the isolate were performed using QTS-24 miniaturized identification system (DESTO Laboratories Karachi, Pakistan) using the method of MacFaddin (1980). For these tests, 24-h old bacterial cultures were used. The 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).

DNA extraction and 16S-rRNA sequence analysis

DNA was extracted from overnight grown culture of pure bacterial colony following the method of Chen and Kuo (1993) and further amplified adopting the method of Weisburg (1991). The polymerase chain reaction (PCR) was performed by using two primers rd1 and fd1 with the following nucleotide sequences AAGGAGGTGAT-CCAGCC and AGAGTTTGATCCTGGCTCAG, respectively. Each 25 µL reaction volume contained 1 µL of 50 µg of genomic DNA, 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 5 µL of 10 X *Taq* buffer, 1 U *Taq* DNA polymerase, 10 p moles of each primer and the rest of the volume was adjusted by sterile cold water. The reactions were carried out in thermocycler (Biometra, Germany). After denaturation at 95°C for 2 min, samples were cycled for 30 cycles through the following temperature profile: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 2 min plus one additional cycle for chain elongation at 72°C for 10 min.

The amplified PCR products were separated by gel electrophoresis on 1.2 % (w/v) agarose gel and visualized under UV trans-illuminator lamp (S. N. 76S/64069, Bio RAD, Italy) after staining with ethidium bromide (0.01 g/ml). DNA ladder (1kb) was used as molecular marker. The bands were excised from the gels and purified by JET QUICK gel extraction spin kit (GENOMED). The purified fragments were sequenced using CEQ DTCS kit (Beckman Coulter, USA). Standard procedure recommended by Beckman Coulter was performed on the automatic sequencer (CEQ-8800, Beckman Coulter Inc., Fullerton, USA). The comparison of sequence was performed via the internet at National Centre for Biotechnology Information (NCBI) database by employing Basic Local Alignment Search Tool (BLAST) software. The sequence obtained was deposited in NCBI Genbank (www.ncbi.nlm.nih.gov/ Genbank/submit.html) with the accession number (GQ849485).

Extraction and quantification of phytohormones from bacterial Isolates

For the extraction and quantification of phytohormones (indole-3-acetic acid, gibberellic acid, cytokinin and abscisic acid), pure *Azotobacter* colony was inoculated in 250 ml NF liquid media with three replicates containing tryptophan (100 mg/L) and ammonium chloride (1.0 g/L), and kept on a shaker (ECELLA E24, New Brunswick Scientific USA) at 100 rpm for five days at 30°C. Thereafter, the bacterial cells were harvested by centrifugation at 10,000 rpm for 15 min at 4°C and supernatant (cell-free liquid culture medium) was used for extraction of phytohormones using the method described by Tien et al. (1979). The pH of the samples was adjusted to 2.5 to 3 with the help of 1 N HCl/ acetic acid. The phytohormones were extracted with an equal amount of ethyl acetate and then evaporated to dryness on rotary thin film evaporator (BUCHI Rotavapor, R-210, Switzerland) at 35°C. The residues were dissolved in 1000 µL of pure methanol (Sigma Chemical Co). The samples were analyzed on a high performance liquid chromatography (HPLC; Agilent 1100) equipped with variable UV detector and C_{18} column (39 × 300 mm) [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 at of 1000 µL/min with an average run time of 20 min/sample.

For identification of hormones, 100 µL of sample filtered through a 0.45 millipore filter and 10 µL was injected into column. The growth hormones were identified on the basis of retention time of phytohormone standards (commercial grade, Sigma Chemical USA Company). IAA was eluted at a wavelength of 280 nm (Sarwar et al., 1992), while GA_3, abscisic (ABA) (Li et al., 1994) and t-zr were eluted at 254 nm respectively. The un-inoculated NF liquid medium was used as blank and three replicates were used.

Inoculation effects of *Azotobacter vinelandii* Khsr1 on *Zea mays* L.

The inoculation effects of isolated *Azotobacter* species was checked on *Z. mays* (advance germ plasm line: Islamabad Gold). The seeds were collected from the National Agricultural Research Council (NARC), surface-sterilized with 10% clorox for 1 to 2 min and then with 95% ethanol for 2 min, washed successively with water and soaked overnight in distilled water prior to soaking in 3-day old culture (10^7 to 10^8 cfu/g fresh weight of root) for 2 h. The seeds soaked in un-inoculated culture medium were treated as control. The soaked seeds were sown in pre-sterilized pots (17 cm × 15 cm^2) containing autoclaved soil and sand (2:1 ratio). The temperature of the growth room was maintained at 25°C ± 2 with a photoperiod of 16 h and the humidity varying from 75 to 80%. The plants were irrigated with ¼ strength of Hoagland nutrient solution added four to five days of germination.

Moreover, two weeks after inoculation, one set of inoculated seedlings were gradually exposed to NaCl by adding 5 dS/m NaCl per day until the final concentration of 20 dS/m was reached, and their control (un-inoculated seedlings) was also irrigated with NaCl in the same way. The second set of inoculated seedlings was un-exposed to NaCl and their control (un-inoculated seedlings) was also followed in the same pattern. Measurements including shoot length, root length, shoot dry weight, root dry weight and proline contents were made 40 days after inoculation. For proline contents, the method of Bates et al. (1973) was followed.

Statistical analysis

This was done by factorial analysis of variance (ANOVA) and two-factor completely randomize d design test (CRD) with least

Table 1. Biochemical characterization (QTS-24 miniaturized identification system) of bacterial isolate.

Test	Result	Test	Result
Oxidase	+	Indole	+
Catalase	+	Gelatin hydrolysis	-
Ortho nitro phenyl β-D-galactopyranoside	-	Acid from glucose	+
Sodium citrate	+	Acid from maltose	+
Sodium melonate	+	Acid from sucrose	+
Lysine decarboxylase	-	Acid from mannose	-
Arginine dihydrolase	-	Acid arabinose	-
Ornithine decarboxylase	+	Acid from rhamnose	+
H_2S production	+	Acid from sorbitol	+
Urea hydrolysis	+	Acid from inositol	-
Tryptophan deaminase	+	Acid from adonitol	-
Voges-Proskauer	-	Acid from raffinose	+
Organism identified	*Azotobacter*		

Table 2. Phytohormones (µg/ml) produced by the isolate in culture medium supplemented with (0.1 mg/ml) or without tryptophan.

Phytohormone (µg/ml)				
IAA (+Trp)	**IAA (-Trp)**	**GA_3**	**t-Zr**	**ABA**
2.7A	0.9^D	2.2^B	1.3^C	0.5^E

IAA (+Trp), IAA production upon addition of tryptophan into media; IAA (-Trp), IAA production without tryptophan.
All such values which share a common letter are non-significantly different, otherwise they differ at $P \leq 0.05$.

significance difference (LSD) using MSTAT C program, version 4.0.

RESULTS

The colonies of the isolated plant growth-promoting rhizobacteria (PGPR) were smooth, convex, glistening and opaque on media and their population size in roots ranged from 10^7 to 10^8 cfu/g fresh weight of root. The isolate was Gram-negative, motile rods, positive for catalase and oxidase test. The biochemical characterization (QTS-24 miniaturized identification tests) based on carbon/nitrogen source utilization pattern (Table 1) revealed that the isolate can utilize more carbon sources than that of nitrogen and indicated highest (98%) similarity to the genus *Azotobacter* when compared with Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). The isolate was positive for tryptophan deaminase, sodium citrate, sodium melonate, ornithine decarboxylase, H_2S production, urea hydrolysis, indole acid from glucose, acid from maltose, acid from sucrose, acid from rhamnose, acid from sorbitol and acid from raffinose.

The 16S-rRNA sequence analyses revealed sequence information of 556 nucleotides. The comparison of this sequence depicted 98% similarity (identities 545/552 positions) with the sequence of *A. vinelandii* DJ (accession no. 012560.1) already deposited in Gene Bank. The bacterium was active in the conversion of tryptophan into IAA. The production of IAA was almost 3 times higher in supernatant supplemented with tryptophan, although the bacterial isolate showed potency to produce IAA even in absence of tryptophan (Table 2 and 1). Besides, IAA, GA_3, ABA and t-zr were also extracted and quantified on HPLC. In culture medium free from tryptophan, the production of GA_3 was comparatively higher than other three phytohormones followed by trans-zeatin production. The ranking order for the production of phytohormones was in the following order: GA_3 > t-zr > IAA > ABA.

Furthermore, the bacterium was used to inoculate *Z. mays* L. (advance germplasm line Islamabad Gold) seedlings irrigated with 20 dS m^{-1} NaCl and seedlings un-exposed to NaCl. The result showed significant increases in length and dry weight of shoot and root of seedlings grown under normal and salt stressed conditions as compared to uninoculated un-stressed and un-inoculated stressed (control) plants. The extent of stimulation was distinctly greater under inoculated un-stressed condition while un-inoculated stressed (control) plants affected severely. The level of proline contents was also higher in inoculated plants and further augmented when irrigated with NaCl, while proline contents were less in inoculated

Table 3. Shoot length, root length, shoot dry weight, root dry weight and Proline contents (mg/g) of shoots and roots of 40 days old *Zea mays* plants (growing in presence and absence of 20 dS/m NaCl stress) inoculated with *Azotobacter vinelandii* Khsr1.

Treatment	Shoot length (cm)		Root Length (cm)		Shoot weight (g)		Root weight (g)		Proline contents of shoot (mg/g)		Proline contents of root (mg/g)	
	-NaCl	+NaCl	-NaCl	+NaCl	-NaCl	+NaCl	-NaCl	+NaCl	-NaCl	+NaCl	-NaCl	+NaCl
T1	46.7^{A}	40.4^{B}	29.3^{A}	21.3^{C}	10.4^{A}	7.1^{B}	7.6^{A}	5.2^{B}	13.2^{B}	18.2^{A}	15^{B}	22.7^{A}
T2	40^{B}	31^{C}	24^{B}	16.5^{D}	7.5^{B}	4.5^{C}	4.5^{B}	2.6^{C}	10.7^{C}	14.9^{B}	12^{C}	17^{B}

-NaCl, Seedlings not treated with 20 dS/m NaCl; +NaCl, seedlings treated with 20 dS/m NaCl. T1 represents the inoculation treatment with isolate while T2 represents un-inoculated plant. All such values which share a common letter are non-significantly different otherwise; they differ at $P \leq 0.05$.

plants un-exposed to NaCl and least in un-inoculated un-stressed (control) plants (Table 3). On the whole, the magnitude of stimulation was higher in roots of the seedlings, which is indicative of better osmoregulation.

DISCUSSION

The grass species, *C. aucheri*, and other weeds growing in Khewra salt range have higher concentration of Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Cl^{1-} and $HCO3^{2-}$ ions in their rhizosphere. The PGPRs surviving in such a high EC i-e 2.3 dS m^{-1} showed salt tolerant when tested on culture medium (Naz et al., 2009). The bacterial isolate from roots of *C. aucheri* was identified as *Azotobacter* sp. on the basis of QTS-24 miniaturized identification system based on carbon/ nitrogen source utilization pattern. The isolate was positive for tryptophan deaminase test and this enzyme have role in the IAA biosynthesis. The identification of isolate on the basis of biochemical tests showed positive correspondence with molecular analysis of isolate based on 16S-rRNA partial sequence. QTS-24 miniaturized identification system is one of the techniques that help in the identification of bacterial genus (Mirza et al., 2007). The isolated bacterial strain: *A. vinelandii* Khsr1 efficiently converted tryptophan into IAA, also excreted GA3, t-zr and ABA in tryptophan free culture super-natant that showed its plant growth promoting ability. Fatima et al. (2009) reported IAA production in *Azotobacter* culture supplemented with tryptophan. Production of trans-zeatin, isopentenyl adenosine and isopentenyl adenine had been reported in culture of *A. vinelandii* OP (Taller and Wong, 1989). GA_3 production also had been reported in *Azotobacter* cultures (Lee et al., 1970) but ABA had not been detected in the culture supernatant of *A. vinelandii.* The IAA, GA_3, t-zr and ABA production by *A. vinelandii* Khsr1 was higher than phytohormones produced by PGPR isolated from rhizospheric soil of same grass species grown in same area as reported by Naz et al. (2009).

In present study, the isolated *A. vinelandii* Khsr1 strain showed positive effects when inoculated on maize plants and marked difference in growth was observed when compared with control (un-inoculated) plants. The plant growth was badly affected in un-inoculated (control) plants when irrigated with salt stress, but the health status of inoculated plants was better under NaCl stress, the soil EC and pH of these plants was also low. This may be due to the presence of *A. vinelandii* Khsr1 inoculation that helps the plant to maintain its growth under salt stress by reducing pH and EC of soil and production of microbial phytohormones particularly IAA and ABA. In addition, the proline contents were higher in plants inoculated with *A. vinelandii* Khsr1 and further increased when exposed to NaCl, and it helped the plant to survive under salinity along with microbially produced ABA. Root colonizing plant growth promoting bacteria circumvent the problem of high salinity of soil, thus improving the growth of plant in saline agriculture soil (Egamberdieva and Kucharova, 2009). *Azotobacter* stimulated growth and yield of corn grown under field conditions (Biari et al., 2008). A better response was observed also by Kumar et al. (2001) in wheat varieties upon inoculation with *Azotobacter chroococcum*.

The isolated *A. vinelandii* Khsr1 strain was capable of producing phytohormones and promotes plant growth under salinity. The plants exposed to NaCl also have higher proline contents. These results depict the use of this strain as bio-inoculant for saline soils. Further exploitation of strain for the production of other osmoregulants like glycine betaine, anti-oxidants production, 1-aminocyclopropane-1-carboxylate (ACC) deaminase production and volatile organic compounds (VOC) production is necessary for its commercial utilization as biofertilizer for saline fields.

ACKNOWLEDGEMENTS

We thank the National Agriculture Research Council (NARC), Islamabad, for providing seeds of Islamabad Gold to carry out the inoculation experiment and Dr. Wasim Ahmad (Department of Biochemistry, Quaid-i-Azam University, Islamabad) for providing technical support in the sequence analysis of bacterial isolate. We are also grateful for the cooperation of Dr. Mushtaq Ahmad (Department of Plant Sciences, Quaid-i-Azam University, Islamabad) in the taxonomic identification of plant species.

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