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Indole-3-acetic Acid Production by *Streptomyces* sp Isolated from Rhizospheric Soils of Medicinal Plants

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A total of 50 *Streptomyces* sp. isolates were obtained from rhizospheric soil of six medicinal plants. All the isolates were screened for their potential to produce IAA and 32 (64 %) isolates were able to utilize tryptophan and produce the IAA in the range of 11.40±0.1-90.33±0.50 µg/ml. Highest amount of IAA was produced by *Streptomyces* TL-10 recovered from rhizospheric soils of *Ocimum sanctum* and *Streptomyces* CR-2, an isolate of *Cathranthus roseus* was producing minimum amount of IAA. Sequencing of 16S rDNA genes of the *Streptomyces* sp.TL-10 isolate had 16SrDNA gene with 99% nucleotides identity to that *Streptomyces* sp. VITTKGB available in Genbank database. The IAA production was maximum (110.3±1.5 µg/ml) when the strain TL-10 was cultivated in a yeast malt extract broth amended with 2 mg/ml of L-tryptophan at pH 7.0, and incubated at 30°C with shaking at 125 rpm for 3 d. The culture filtrate from the strain TL-10 was found to stimulate significantly the root elongation of chickpea and tomato. These results suggest that IAA-producing *Streptomyces* TL-10 could be a promising candidate for utilization in growth improvement of plants of economic and agricultural value.

Key words: Actinomycetes, indole-3- acetic acid, medicinal plants, Rhizosphere, *Streptomyces* TL-10

Plant growth promoting rhizobacteria (PGPR) are the soil bacteria that colonize the roots of plants and enhance plant growth. PGPR can directly or indirectly affect plant growth through various mechanisms. Direct stimulation may include fixation of atmospheric nitrogen (Soares *et al.* 2006), synthesis of various phytohormones and enzymes (Cheng *et al.* 2007) and solubilization of minerals in plants (Panhwar *et al.* 2012), while indirect stimulation includes inhibiting phytopathogens (Hao *et al*. 2011). Plant rhizospheric soil has a diverse microflora and *Streptomyces* is widely distributed group present in plant rhizosphere and produces plant growth promoter substances. Plant growth regulators are natural compounds that have shown far reaching effects on the growth and development of plants even at low concentration auxins, gibberellins (GA3) and kinetin being well known plant growth promoting hormones have shown to be involved in a variety of plant growth and development processes.

IAA is a natural auxin and Approximately 80% of rhizosphere bacteria can secrete IAA (Bhavdish *et al.* 2003). Substrates available in root exudates creates the potential for the *Streptomycetes* to synthesize and release IAA. Soils may be attractive sources of *Streptomyces* sp., capable of producing bioactive compounds related to plant growth promotion (Thangapandian *et al.* 2007). Several *Streptomyces* species have the ability to produce IAA and improve plant

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growth by increased seed germination, root elongation and root dry weight. Indole acetic acid is the main member of auxin family that controls many important physiological processes including cell enlargement and division.

The aim of this study was to investigate the production of IAA by *Streptomyces* species isolated from rhizospheric soils of medicinal plants and to evaluatethe optimization of IAA production by active isolate *Streptomyces* TL-10 .

MATERIALS AND METHODS

Isolation and colonial characterization of actinomycete isolates

Soil samples were collected from the rhizospheres of six medicinal plants (*Catharanthus roseus*, *Curcuma longa* L. *Cymbopogon citrates*, *Mentha arvensis*, *Ocimum sanctum* and Zingiber *officinalis*) from PAU herbal garden, Jalandhar, Moga, Faridkot, Patiala. *Streptomyces* strains were isolated by plating serially diluted samples on a Starch casein agar (SCA), pH 7.0 supplemented 50 µg/ml of cycloheximide (Taechowisan *et al*. 2003). After incubation at 30°C for 7-10 days the isolated colonies were subcultured on fresh agar medium until pure cultures were obtained. The microscopic morphology was studied by examining Gram stained smears and slide cultures. Physiological criteria such as ability to degrade casein, starch, esculin, tween 80, tyrosine, xanthine and hypoxanthine as substrates by the various actinomycete strains were also used for genus confirmation.

Screening of the isolates for the production of IAA

All the *Streptomyces* spp. isolates were tested for IAA production according to the method of Gordon and Weber (1951). For this, the *Streptomyces* isolates were grown on yeast malt agar (YM) incubated at 30°C for 5 days. These were then inoculated into 5 ml of YM broth and incubated at 30°C while shaking at 125rpm for 7 days. Cultures were centrifuged at 11,000 rev/min for 15mins. One milliliter supernatant was mixed with Salkowski reagent; the appearance of a pink color indicated IAA production. Optical density (O.D.) was read at 530 nm using spectrophotometer. The level of IAA produced by the isolates was compared with standard IAA.

Effect of incubation time, L-tryptophan concentration, temperature and pH on IAA production *Streptomyces* TL-10

Streptomyces TL-10 which produced highest amount of IAA, was studied to identify the optimal conditions for IAA production. IAA concentration was measured at different parameters by taking one parameter at one time. The effect of incubation time was studied in YM broth supplemented with 2mg/ml of L-tryptophan. Samples were drawn every 24 h for 10 d. The effect of L-tryptophan concentration was studied by cultivating the strain in YM broth supplemented with different concentrations of L-tryptophan (0, 1, 2, 5, 7 and 9 mg/ml). The effect of temperature and pH on IAA production was studied by cultivating the strain in YM broth containing 2 mg/ml of L-tryptophan at different temperatures (15-45°C) and pH levels ranging from 4.0-9.0 for 3 d.

Effect of IAA production on seed germination and root elongation

The supernatant of strain TL-10 was filtered (Milipore filter, 0.45 mm) and the cell free filtrate was used for subsequent testing and assayed for IAA production. Chickpea and tomato seeds were surface-sterilized by soaking in a 10% sodium hypochlorite (NaOCl) and 10% ethanol solution for 1.5 min, followed by thorough rinsing in sterile distilled-water. The surface-sterilized seeds were separately soaked in 250 ml of the culture filtrate, sterile distilled water and IAA standard (50 mg/ml) for 24 h and then planted in pots of sterile soil. The measurements of the root length of the chickpea and tomato were made at 10 d after germination.

Extraction, purification and detection of IAA

The isolate TL-10 was cultivated in 200 ml of YM broth containing 2 mg/ml of L-tryptophan, pH 7.0 and incubated at 30ºC with shaking at 125 rpm for 3 days. Cultures were centrifuged at 11,000 rpm for 15 min. IAA was extracted from the supernatants with ethyl acetate according to the method described by Ahmad *et al*. (2005). Ethyl acetate fractions (10-20 ml) were applied to TLC plates (Silica gel G F254, thickness 0.25 mm, Merck, Germany) and developed in butanone-ethyl acetate-ethanol-water (3:5:1:1). Spots with Rf values identical to authentic IAA were identified by spraying with Salkowski reagent.

Molecular characterization of *Streptomyces* TL-10

Molecular identification of *Streptomyces* TL-10 was done by isolating DNA from *Streptomyces* TL-10. Its quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose Gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1414bp 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4.

RESULTS AND DISCUSSION

Isolation and colonial characterization of actinomycete isolates

A total of fifty *Streptomyces* isolates were obtained from 20 soil samples; the number obtained from rhizosphere soils from *Catharanthus roseus* being the highest, followed by *Mentha arvensis* (n=10), *Zingiber officinalis* (n=9), *Ocimum sanctum* (n=8), *Curcuma Longa L.*(n=5) and *Cymbopogon citrates* (n=4). (Table 1). It is possible that root exudates from *Catharanthus roseus* could promote the growth of *Streptomyces* sp. which then synthesizes antimicrobial compounds from the roots might decrease number of other soil bacteria and fungi thereby enhancing the diversity of *Streptomyces* from this soil which was higher than the other soils. These results are supported by studies of Khamna *et al* (2010) who reported 270 isolates of *Streptomyces* from 14 rhizospheric soil samples of medicinal plants. Thangapandian *et al* (2007) isolated *Streptomyces* from some medicinal plant rhizosphere soils and found that most of soil

Table 1. The occurrence of *Streptomyces* isolates from different rhizosphere soils of medicinal plants

Plant rhizospheric soils	No.of *Streptomyces* sp.
Curcuma Longa L.	5
Zingiber officinalis	9
Mentha arvensis	10
Cymbopogon citrates	4
Ocimum sanctum	8
Catharanthus roseus	14

Table 2. Effect of Incubation time on IAA production by *Streptomyces* TL-10

Incubation days	IAA produced (µg/ml)
1	16.3±0.2
2	29±0.2
3	110.0±0.2
4	95.7±0.5
5	93.7±0.4
6	91±0.2
7	90.3±0.50
8	80.3±0.2
9	80±0.1
10	79.2±0.1
CD@5%	0.53

*Average± standard error from triplicate samples

Table 3. Effect to L-tryptophan on IAA production

L-tryptophan concentration (mg/ml)	IAA produced (µg/ml) TL-10
0	11.16±0.2
1	16.33±0.4
2	110.3±1.5
5	95.8±0.7
7	21.63±0.4
9	16.33±2.1
CD@5%	0.52

*Average± standard error from triplicate samples

Table 4. Effect on temperature on IAA production

Temperature	IAA produced(µg/ml)
15ºC	24.7±1.18
30ºC	89.5±0.48
35ºC	74.4±0.71
45ºC	68.7±0.95
CD@5%	0.43

*Average± standard error from triplicate samples

pH was neutral. These confirmed that the distribution of rhizosphere soil *Streptomyces* sp. seemed to be related with pH of soil.

Screening of the isolates for the production of IAA

Thirty two out of 50 isolates were observed to produce the phytohormone indole acetic acid. The *Streptomyces* isolates were observed to produce IAA in range from 11.40±0.1-90.33±0.50 µg/ml (Fig 1). *Streptomyces* TL-10 , an isolate from rhizospheric soils of *Ocimum sanctum* was observed to produce highest amount of IAA

Table 5. Effect of pH on IAA production

pH	IAA produced (µg/ml)
4	-
5	-
6	40.2±0.08
7	89.9±0.60
8	75.6±0.39
9	48.8±0.24
CD@5%	0.32

*Average± standard error from triplicate samples

Table 6. Effect of IAA in culture filtrates of TL-10 potential isolate of *Streptomyces* on seed germination and root elongation in chickpea

	Root elongation (cm) (TL-10)		
L-Tryptophan Concentration	Sterile distilled water	Standard IAA	Culture filtrate
0	0.5±0.1	0.7±0.14	0.9±0.15
1	1±0.12	1.1±0.3	1±0.12
2	3.6±0.2	4±0.5	5.2±0.45
5	3.8±0.2	4.2±0.25	4.8±0.32
CD@5%	0.33	0.45	0.52
Seed germination(%)			
0	50.8	51.8	60
1	50.8	60.4	65.3
2	60.3	66.1	69.2
5	60.5	70.3	70.5

*Average± standard error from triplicate samples

Table 7. Effect of IAA in culture filtrates of TL-10 potential isolate of *Streptomyces* on seed germination and root elongation in Tomato

	Root elongation (cm) (TL-10)		
L-Tryptophan Concentration	Sterile distilled water	Standard IAA	Culture filtrate
0	0.3±0.12	0.8±0.16	0.8±0.16
1	1±0.18	1.2±0.21	1±0.18
2	2.4±0.24	3.6±0.45	4.2±0.5
5	3.4±0.35	4.0±0.48	4.8±0.51
CD@5%	0.38	0.47	0.55
	Seed germination(%)		
0	52.4	54.3	65
1	55.6	62.4	68.2
2	61.3	68.4	70.3
5	63.2	72.3	74.5

*Average± standard error from triplicate samples

of 90.33µg/ml. *Streptomyces* Cr-2 an isolate from rhizospheric soils of *Catharanthus roseus* was observed to produce the minimum amount of IAA (11.40 µg/ml).Our results are supported by Khamna *et al* 2010 who reported 30 isolates of *Streptomyces* sp. had this ability and several reports have shown that *Streptomyces* sp.from many crop rhizosphere soils have this ability (El-Tarabilya and Sivasithamparamb 2006, El-Tarabily 2008, Ameor and Ghoul 2012, Abd-Alla *et al* 2013). The most active isolates were isolated from the rhizospheric soil of *Ocimum sanctum*. It is possible that the high levels of tryptophan will be present in root exudates of *Ocimum sanctum* and enhance IAA biosynthesis in actinomycetes isolated from the rhizosphere. These reports are consistent with other researcher (Abd-Alla *et al* 2013). It could be inferred that IAA, a plant growth hormone can promote plant growth in rhizosphere soils.

Effect of incubation time, L-tryptophan concentration, temperature and pH on IAA production by *Streptomyces* TL-10

IAA production by *Streptomyces* TL-10 commenced after 24 h, reaching a maximum after 3 d and then decreasing slowly (Table 2). This decrease might be due to the release IAA degrading enzymes such as IAA oxidase and peroxidase as was reported earlier in *Rhizobium* sp. from *Cajanus cajan* (Datta and Basu 2000). L-tryptophan at 2 and 5 mg/mL concentration was the best for IAA production by the isolates, whereas at higher concentrations tryptophan exerts an adverse effect on production. Most of the organisms produce IAA in presence of tryptophan. In present study it was observed that as the concentration of tryptophan in the medium increases, the amount of IAA produced increased. *Streptomyces* TL-10 from *Ocimum sanctum* showed maximum

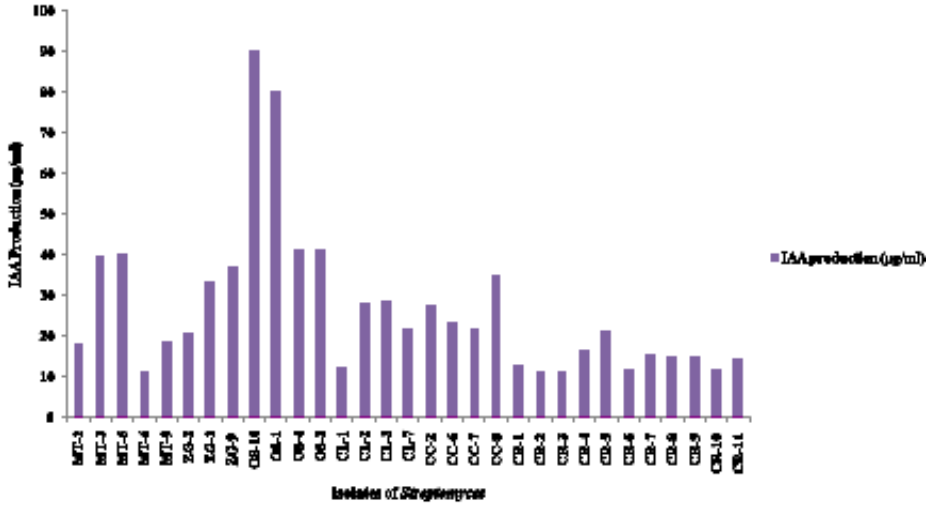

Fig. 1. IAA production by different *Streptomyces* isolates

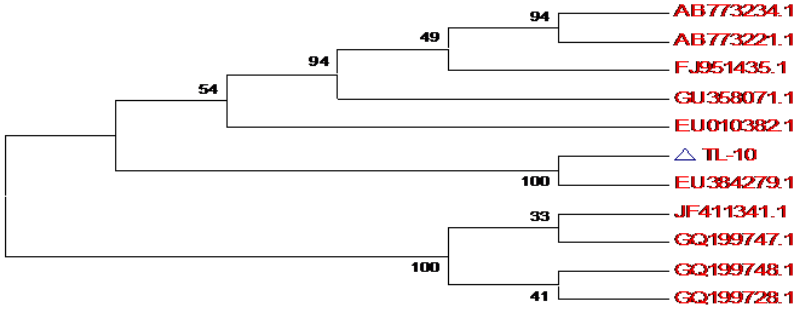

Fig. 2. The phylogenic tree obtained by applying the neighbor-joining method. The neighbour-joining tree based on 16S rDNA gene sequences showing the positions of isolate TL-10 and related strains. Phylogenetic analyses were conducted in MEGA4

production at 2 and 5mg/ml of tryptophan concentration (Table 3) after which amount of IAA produced decreased. IAA production in absence of tryptophan was 11.16±0.2 by *Streptomyces* TL-10, which concludes the requirement of tryptophan as a precursor for the synthesis of IAA. Ahmad *et al*. (2005) also reported that rhizosphere *Azotobacter* sp. and *Pseudomonas* sp. produced a high level of IAA when theses bacterium were cultured in a nutrient broth amended with 2 and 5 mg/ml of tryptophan. IAA production by the tested isolate reached maximum amount at concentration of 5 mg tryptophan per ml of basal medium. Higher concentration of L-tryptophan above 5 g/l decreased the production both of IAA and mycelia (Abd-Alla *et al* 2013). The effect of temperature on IAA production was studied. *Streptomyces* TL-10 produced maximum IAA when it was grown using a YM broth at 30°C (Table 4), this temperature was found suitable for growth and IAA production of both the isolates. Abd-Alla *et al* (2013) found that the optimum temperature for IAA formation and mycelia growth by *Streptomyces atrovirens* ASU1 4 was at 30°C. Aldesuquy *et al* (1998) found that temperatures in the range 25-30°C, were suitable for growth and IAA production of *Streptomyces* sp. IAA yield of TL-10 from rhizospheric soil of *Ocimum sanctum* was maximal at a pH of 7.0 (Table 5). Acidic or high alkaline pH is unsuitable for IAA production because *Streptomyces* grow poorly in these conditions. Usually, the distribution of *Streptomyces* sp. from acidic soils is lower than neutral soils (Shirokikh *et al* 2007). The pH affects the function of enzyme systems and also the solubility of many substances that are important for bacteria growth. Yurekli *et al*. (2003) reported that the synthesis of the highest IAA level was determined in cultures cultivated in an alkaline media at a pH of 7.0.Hence optimum pH for *Streptomyces* was found to be 7.0.

Effect of IAA production on seed germination and root elongation

The culture filtrates of *Streptomyces* TL-10 could promote plant-growth based on root elongation in both chickpea (Table 6) and tomato (Table 7) plants, in comparison with the control. IAA has an important role in plant growth promotion. El-Tarabily (2008) reported that *Streptomyces* spp. from a tomato rhizosphere had the ability to produce IAA and improve tomato growth. Tomato and chickpea roots inoculated with IAA-producing isolates has significantly increased root surface as compared with the control.

Extraction, purification and detection of IAA

Standard IAA showed Rf value of 0.64. The same Rf value was obtained from IAA produced by the isolate. Culture filtrates of isolate TL-10 was used to extract IAA for characterization by TLC. Chromatograms of culture extracts and standard IAA, showed almost the same Rf values. The TLC findings are in agreement with other reports (Khamna *et al.*, 2010; Abd-Alla *et al.*, 2013).

Molecular characterization of *Streptomyces* TL-10

The culture, which was labeled as *Streptomyces* TL-10 was found to be *Streptomyces sp. A515 Ydz-FQ* (GenBank Accession Number: EU384279.1) based on nucleotide homology and phylogenetic analysis. The phylogenetic tree was constructed using MEGA 4 (Fig. 2).

Rhizospheric soils has rich source of IAA producing *Streptomyces* sp. *Streptomyces* TL-10 can be very effective and is potential microbial inoculant for production of IAA that can be used as plant growth promoting rhizobacteria for enhancing soil health and crop yield.

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