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Plant Growth Promoting Characteristics of *Bacillus Licheniformis* DS3 Isolated from Agriculture Field Soil

D. Silpa, P. Brahmaji Rao, G. Kranthi Kumar, M. Raghu Ram*
Department of Botany & Microbiology, Acharya Nagarjuna University, Guntur, Andhra Pradesh , India

ABSTRACT

Plant growth promoting rhizobacteria (PGPR) are known to influence the plant growth by various direct or indirect mechanisms. In search of efficient PGPR strains with multiple activities, a total of 10 bacterial isolates belonging to Bacillus species isolated from different banana field soils, Tadepalli, Guntur district of Andhra Pradesh, India. Out of ten only one strain Bacillus licheniformis DS3 showed potential activity of producing IAA, Gibberellic acid production, Siderophore production and excretion of Ammonia. Bacillus licheniformis DS3 showed the maximum IAA and Gibberellic acid productions are 142 µg/ml and 74.75 µg/ml respectively. An orange halo appears around the Bacillus licheniformis DS3 on CAS agar media indicates the siderophore production. The optimum conditions for IAA production in Bacillus licheniformis DS3 are 0.1 % L-tryptophan, 48 h of incubation, Glucose (1%) and Ammonium sulphate (0.5%) were used as carbon and nitrogen sources. This strain has shown to exhibit multiple plant growth promoting characteristics. These plant growth promoting abilities can make this isolate a potential PGPR candidate for its application in sustainable agriculture.

Keywords : Ammonia**,** PGPR, IAA, Gibberellic acid (GA3), Siderophore

I. INTRODUCTION

Plant growth promoting Bacillus licheniformis (PGPB) are beneficial bacteria which have the ability to colonize the roots and either promotes plant growth through direct or indirect mechanism or via biological control of plant diseases (Kloepper and Schroth, 1978). They are associated with many plant species and are generally present in varied environments. Strains with PGPB characteristics, belonging to genera Bacillus licheniformis, have been reported (Hurek and Reinhold-Hurek, 2003). Bacteria that colonize the rhizosphere and plant roots, and enhance plant growth by any mechanism are referred to as PGPR. Plant growth promoters can exhibit a variety of characteristics responsible for influencing plant growth. The common traits include production of plant growth regulators (like auxin, gibberellins, and ethylene, siderophores, HCN and antibiotics (Arshad et al., 1992).

Gibberellic acid (GA3) is an important member of the gibberellins family and acts as a natural plant growth hormone, controlling many development processes, which is gaining great attention all over the world due to its effective use in agriculture, nurseries, tissue culture (Shukla et al., 2005). Gibberellic acid production was confirmed from B. pumilus and B. licheniformis [Gutierrez- Manero, 2001].

More than 80% of the bacteria isolated from the rhizosphere can produce (IAA) Indole Acetic Acid (Khalid et al., 2004) as secondary metabolite by obtaining tryptophan either through root exudates or from the proteins released by the dead bacteria cells (Patten and Glick, 1996). Indole-3-acetic acid (IAA), a plant hormone compound, is a natural auxin produced by plants, bacteria, fungi and a diverse group of organisms. It is a metabolite derived from tryptophan by many tryptophan dependant and tryptophan

independent pathways in plants and bacteria. There is more than one pathway could be present in a bacterium (Pattern and Glick, 2002).

Siderophores produced by some PGPR scavenge heavy metal micronutrients in the rhizosphere (e.g. iron) starving pathogenic organisms of proper nutrition to mount an attack of the crop. Antibiotic producing PGPR releases compounds that prevent the growth of the pathogens. Therefore, the present study was undertaken to isolate and characterize rhizospheric Bacillus licheniformis DS3 for its multiple plant growth promoting activities under in vitro conditions such as production, of ammonia and indole acetic acid, gibberellic acid and siderophore productions. For the first time we are reporting the amylolytic B. licheniformis DS3 exhibited the multiple PGP characters were isolated from banana field soils Guntur district of Andhra Pradesh, India.

II. MATERIALS AND METHODS

1. Soil sample collection

Soil samples were collected from various Banana field soils in Guntur region, Andhra Pradesh, India. From 3 to 4 cm depth with the help of sterile spatula and the soil samples were transferred to sterile plastic bags and maintained in aseptic conditions for future studies.

2. Isolation of Bacteria

The Amylolytic bacteria (Bacillus licheniformis DS3) was isolated from banana fields and screened for α-amylase production. The starch medium used for the isolation of bacteria contained (g/L): Starch , 10.0; yeast extract, 5.0; peptone, 2.0; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 0.5; NaCl, 1.5; $CaCl_2$, 0.1; Agar, 20.0. Initial pH was adjusted to 7.0. One gram of each soil sample was suspended in 9.0 ml of sterile water and 0.1 ml of suitably diluted suspension was spread on the agar plates. The plates were incubated at 35^0C, for 24 to 48 hours. The isolated colonies were flooded with iodine solution. Colonies with good colourless halos around them were picked and maintained on starch agar slants at 4^0C and further assessed for enzyme production in liquid medium. The characterization and identification of the isolate was made following Bergey's Manual of Systemic Bacteriology (Sneath, 1986).

3. Amylase production

The medium for enzyme production comprised (gl-1): starch, 10.0; yeast extract, 5.0; peptone, 5.0; KH_2PO_4, 0.12; $CaC1_2.2H_20$, 0.12; $MgSO_4.7H_20$, 0.12; $MnSO_4.4H_20$, 0.02. Initial pH of the medium was adjusted at 7.0 and 50 ml of medium in 250 ml of Erlenmeyer flasks were inoculated with a cell suspension of optical density 0.5 (prepared from 24 h old culture). All the flasks were incubated for four days on a rotary shaker at 200 rpm at 45^0C. Samples were drawn after a time interval of 12 h, centrifuged at 8000 x g for 10 minutes and cell free culture supernatant fluid used as enzyme source.

4. Plant growth promoting characteristics

Plant growth promoting characteristics including Ammonia, IAA, Gibberellic acid and siderophore productions were studied.

5. Production of ammonia

For ammonia excretion, freshly grown bacterial culture was inoculated in 5 ml sterilized peptone water containing tubes and incubated at 30^0C for 48h at stationary conditions. After 48 h, one ml of Nessler's reagent (0.5 ml) was added to peptone water and shaked thoroughly. Culture broth (1.5 ml) from this tube was centrifuged at 12000 rpm for 15min. Supernatant was taken in a cuvette and absorbance was measured at 450 nm in a spectrophotometer (Cappuccino and Sherman, 1992).

6. Estimation of Gibberellic acid (GA3)

The gibberellic acid production by plant growth promoting rhizobacteria was determined by the method of Borrow et al. 1955.

7. Siderophore production

Siderophore production by the plant growth promoting bacteria was estimated by the method described by Schwyn and Neilands (1987). Siderophore production was indicated by orange halos around the colonies after the incubation.

8. Production of Indole acetic acid

Indole acetic acid (IAA) production was detected as described by Brick et al. (1991). Bacterial cultures were grown for 48 h of incubation and 35^0C temperature at pH 7.0. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was mixed with two drops of ortho phosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). Development of pink colour indicates IAA production. The amount of IAA produced was calculated by using the standard graph of authentic IAA (Gordon and Weber, 1951).

9. Optimization for IAA production by Bacillus licheniformis DS3

Various factors including Incubation period, L-tryptophan concentration, pH, carbon and nitrogen sources effected the IAA production.

10. Effect of incubation period on IAA production

For the production of indole acetic acid different incubation periods (12, 24, 36, 48 and 60 h) were maintained in the production medium. For the estimation of IAA by using spectrophotometer at 540 nm.

11. Effect of L-Tryptophan Concentration on IAA production

Different concentrations of (50, 100, 150, 200 and 250 mg) of L-Tryptophan was added to the production medium after sterilization and inoculated with B. licheniformis DS3 and incubated for 48 h (optimum time for maximum IAA production) on rotatary shaker 200 rpm at room temperature. Growth and IAA were measured by using spectrophotometer at 540 nm.

12. Effect of Carbon sources on IAA production

In the production medium, starch was replaced with 5 different carbon sources (Mannitol, glucose, lactose, sucrose and maltose,) at 1 % concentration inoculated with B. licheniformis DS3 and incubated for 48 h on rotatary shaker at 200 rpm at room temperature. Control was maintained without carbon source. Growth and IAA production was measured by using spectrophotometer at 540 nm.

13. Effect of Nitrogen sources on IAA production

In the production medium, sodium nitrate was replaced with different Nitrogen sources (Ammonium sulphate, Potassium chloride, L-Asperagine, Peptone and Beef extract) at 0.5 % level along with L-Tryptophan. Growth and IAA was measured by using spectrophotometer at 540 nm.

III. RESULTS AND DISCUSSION

The selection of plant growth promoting isolate involved in screening of over 10 bacterial isolates from banana field soil samples. The isolate was identified by B. licheniformis DS3 after 16 S rRNA sequencing analysis. The sequences were deposited in the Gen bank (NCBI) data base under accession number MG 870112. B. licheniformis DS3 was screened for their ability to produce plant growth regulators like IAA, GA3, Ammonia production and Siderophore production (Table-1). This strain showed the maximum IAA production of (142 µg/ml) at 0.1 % of L-tryptophan concentration and 48 h of incubation at pH 7.0. The ability of Bacillus species isolate to use tryptophan supplemented in the cultivation medium is one of the important points to determine IAA production. Tryptophan is the main precursor of IAA biosynthesis (Patten and Glick, 1996). Gibberellic acid production by B. licheniformis DS3 is most important

in this study. The present strain showed the maximum amount of gibberellic acid (74.75 µg/ml). Ammonia production was determined to produce 1.03 % of ammonia. An orange halo zones around the colony on CAS agar media are confirmed as siderophore positive. In this paper we focussed mainly optimum conditions for IAA production.

Table 1. Plant growth promoting characteristics of Bacillus licheniformis DS 3

Strain name	Indole acetic acid production	Gibberellic acid production	Ammonia production	Siderophore production
Bacillus licheniformis DS 3	142	74.75	1.03	Positive (+)

1. Optimization for IAA production

For the production of IAA in B. licheniformis DS3 strain was applied in various optimum conditions like Effect of incubation period, tryptophan concentration, and carbon and nitrogen sources.

2. Effect of incubation period

IAA production was started at 12 h of incubation period and it reached maximum at 48 h of incubation (Table-2). IAA production was increased with increasing incubation period up to 48 h. The maximum production of IAA (142 µg/ml) at 48 hours of incubation. Further increase in incubation the IAA production was decreased.

Table 2. Effect of incubation period on IAA production

Incubation periods (Hours)	IAA production (µg/ml)
12	22
24	56
36	120
48	142
60	42

* The F- Value for incubation period and interactions are all significant with $p<0.05$.

3. Effect of L-tryptophan concentration

Tryptophan is the main precursor of the bacteria. The IAA production was increased with increasing L-tryptophan concentration up to 200 mg/ml. The maximum production of IAA (220 µg/ml) at 200 mg/ml concentration of L-tryptophan. Above 200 mg concentration of L-tryptophan the IAA production was declined (Table-3). Tryptophan in the medium is an important factor for maximum IAA (174.72 µg/ml) in Bacillus licheniformis ML3 (Kayasth, et al., 2013). Lee et al., (2004) have reported that L- tryptophan was more active for IAA production, though bacteria were able to produce IAA in absence of tryptophan (Jayaprakashvel et al., 2014).

Table 3. Effect of L-tryptophan concentration on IAA production

L-tryptophan concentration (mg/g)	IAA production (µg/ml)
50	56
100	142
150	184
200	220
250	135

*The F- Value for L-tryptophan and interactions are all significant with $p<0.05$.

4. Effect of carbon and nitrogen sources

Glucose containing the medium the strain produced maximum amount of IAA (142 µg/ml) followed by mannitol (130 µg/ml). (Table-4). The other carbon sources maltose and lactose produced IAA was 70 and 75 µg/ml respectively. Mohite (2013) reported that the most suitable carbon source for IAA production was glucose for Bacillus megaterium br1 isolated from banana rhizosphere . Basu and Ghosh (2001) have reported that Glucose and KNO_3 as the best carbon and nitrogen sources of IAA production by Rhizobium spp. Shilts et al. (2005) have reported high IAA

production in medium containing mannitol and galactose. The results (Table-5) showed that the nitrogen source (0.5%) Ammonium sulphate induced the maximum IAA production (120 µg/ml). Ammonium sulphate was replaced with different nitrogen sources, maximum IAA production was observed in peptone (112 µg/ml) and beef extract (102 µg/ml). The suitable nitrogen source for IAA production was different with the isolate type as $NaNO_3$ for br1, KNO_3 and peptone for Lactobacillus casei br2 isolated from banana rhizosphere (Mohite 2013).

Table 4. Effect of carbon sources on IAA production

Carbon sources	IAA production (µg/ml)
Control	07
Sucrose	25
Mannitol	130
Glucose	142
Maltose	75
Lactose	70

*The F- Value for carbon sources and interactions are all significant with $p<0.05$ and at $p<0.01$.

Table 5. Effect of nitrogen sources on IAA production

Nitrogen sources	IAA production (µg/ml)
Control	12
Potassium chloride	36.5
L- Aspargine	42
Ammonium sulphate	120
Peptone	112
Beef extract	102

*The F- Value for nitrogen sources and interactions are all significant with $p<0.05$ and at $p<0.01$.

IV. CONCLUSION

From the present study it was concluded that the plant growth promoting B. licheniformis DS3 have the capacity to produce Ammonia, IAA, GA3 and siderophore production. The indole acetic acid producing B. licheniformis DS3 will promote the growth at the field level to development of liquid Bioinoculant for sustainable agriculture.

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