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PLANT GROWTH PROMOTING STUDIES ON AGROBACTERIUM AND RHIZOBIUM SPECIES ISOLATED FROM ROOT NODULES OF VIGNA MUNGO (L. HEPPER)

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ABSTRACT

The present study ten bacterial strains were isolated from root nodules of Vigna mungo, commonly known as black gram. The seeds were collected from national scientific corporation, Guntur. The seeds were sown in the earthen pots of botanical garden of our college and the nodules were collected from every ten days interval. Maximum size of the nodules was selected for isolation. After the preliminary and biochemical characterization the ten strains were identified as 5 *Agrobacterium* and 5 *Rhizobium* species. All the isolates were tested for plant growth promoting characters like IAA, Gibberellic acid production, Siderophore production. Out of ten isolates tested, only one isolates i.e. *Agrobacterium Sp-5* showed potential activity of Indole Acetic Acid (35.5 µg/ml) Gibberellic acid (28.3 µg/ml) and Siderophore production (26.5 µg/ml) respectively. Various factors like suitable pH 7.0, optimum temperature 30°C and 48 h of incubation periods influence the production. These plant growth promoting abilities can make this isolate a potential PGPR candidate for its application in sustainable agriculture.

KEYWORDS: *Agrobacterium*, *Rhizobium*, Gibberellic acid (GA3), Indole Acetic Acid (IAA), Siderophore.

INTRODUCTION

Rhizobia have been isolated from nodules on leguminous plants and establish symbiosis with nitrogen fixing bacteria of the family *Rhizobiaceae*. The bacterial belongs to the genera

Rhizobium, Bradyrhizobium, Allorhizobium, Mesorhizobium and *Sinorhizobium* (renamed as *Ensifer*) *Agrobacterium,* are able to form nodules on their host plants inside of which they fix-nitrogen. Nitrogen fixing soil bacteria are studied extensively owing to their considerable agriculture and environmental importance.[1] The evolution of the *Rhizobial* diversity among the natural populations has become significant.[2] More than 80% of the bacteria isolated from the rhizosphere can produce (IAA) Indole Acetic Acid as secondary metabolite by obtaining tryptophan either through root exudates or from the proteins released by the dead bacteria cells.[3]

The metabolites obtained from microorganisms. Indole-3-acetic acid (IAA), a plant hormone compound, is a natural auxin produced by plants, bacteria, fungi and a diverse group of organisms. Indole Acetic acid is a metabolite derived from tryptophan by many tryptophan dependant and tryptophan independent mechanism in plants and bacteria. There is more than one mechanism could be present in a bacteria.[4]

MATERIALS AND METHODS

Isolation

Bacterial suspension was prepared by crushing these nodules with sterile glass rod using sterile distilled water. A loopful of suspension was prepared on media plates containing selective medium yeast extract mannitol agar medium (YEMA) with 0.1% Congo red and incubated at room temperature for 3 days. After incubation, the white translucent, convex, colonies with high mucilage were isolated and pure cultures were maintained after subculturing the same medium. Pure cultures of all the twenty one isolates were authenticated as *Rhizobium* by performing the appropriate biochemical tests[5] and nodulation ability on homologous hosts by plant infection tests.[6]

Indole Acetic acid production

To determine the production of IAA, culture suspension of the strain was inoculated in starch tryptone broth supplemented with 0.5% L-Tryptophan, adjusted to initial pH 7.0 and incubated for 48 h, at 30°C. After incubation, culture broth was centrifuged at 10,000 rpm for 20 min. The supernatant (5 ml) was mixed with 1ml of IN HC1 and 4 ml of Salkowski reagent and observed for the development of pink colour. Optical density was read at 530 nm in a spectrophotometer.[7] Standard Graph was prepared by using increasing concentration of authentic IAA.[8]

Gibberellic acid production

To determine the production gibberellic acid, the culture suspension was inoculated into starch broth supplemented with 60 mM concentration of mevalonic acid, adjusted with initial ph 7.0 and incubated at 30^0 C for 48 h. After incubation culture broth was centrifused 10000 rpm for 20 min. The supernatant was acidified to pH 2.5 with HCl and extracted using liquid-liquid (Ethyl acetate/NaHCO3) extraction.[9] Gibberellic acid in the ethyl acetate phase was measured by UV spectrophotometer at 254 nm.[10]

Siderophore production

Siderophore production by the plant growth promoting bacteria was estimated by the method described by.[11] Siderophore production was indicated by orange halos around the colonies after the incubation.

Siderophore assays

For the detection of Siderophore production in Bacillus sp. PB-3 was grown on the medium containing 0.5 µM of iron, and incubated for 24 h on rotary shaker at 200 rpm at room temperature. A clear orange hallo zone around the colonies appears on Chrome Azurol S (CAS) agar medium which indicate the siderophore positive.

Chrome Azurol S (CAS) Agar medium

For the detection of Siderophore, Agrobacterium sp-5 isolate was grown in synthetic medium, containing 0.5 µM of iron and incubated for 24 h on a rotary shaker at 200 at 30^0 C. Culture supernatant was added to the wells made on the CAS agar plates and incubated at room temperature for 24 h. Formation of yellow to orange coloured zone around the well indicates siderophore production (Schwyn and Neiland's, 1987). All glassware used to store stock solution of the medium were treated with concentrated HNO_3 and left to overnight. After 24 h, the acid was removed and the glassware was rinsed thoroughly with double distilled water.[12]

Optimization for Indole acetic acid, Gibberellic acid and Siderophore production

Different factors like Incubation, pH, Temperature, effect of carbon and nitrogen sources influence the Siderophore production. Quantitative estimations were done by using spectrophotometer (480 nm) method.

Effect of incubation period, pH, temperature

For the production of IAA, GA3 and Siderophore by different incubation periods (24, 48, 72, 96, 120, 144 and 168 h), pH (5,6,7,8,9,10,11) temperature (20,25,30,35,40 °C) were carried out in this study.[13,14]

Effect of carbon sources

To study the IAA, GA3 and siderophore production by using different carbon sources (Mannitol, glucose, sucrose, Succinate and citrate) were studied. The *Agrobacterium* sp-5 were inoculated in the basal medium for 168 h of incubation and estimated the siderophore production.[15]

Effect of nitrogen sources

To study the IAA, GA3 and Siderophore production by using different nitrogen sources (ammonium sulphate, sodium nitrate, urea, glutamine and glycine) were replaced with 0.1% yeast extract. The Agrobacterium were inoculated in the basal medium for 48 h of incubation and estimated the IAA, GA3 and Siderophore production.[16]

RESULTS AND DISCUSSION

A total of 20 isolates were obtained from chilli rhizosphere in the vicinity of Guntur, Andhra Pradesh. The preliminary characterization like cultural and biochemical characteristics of rhizobium was done by Bergey's manual of systemic bacteriology. All the isolates belong to Bacillus species according to their preliminary and biochemical studies. All the isolates were designated as *Agrobacterium* sp-1 to *Agrobacterium* sp-5 and *Rhizobium* sp-1 to *Rhizobium* sp-5 tested for plant growth promoting study (Table-1). All the isolated showed that the IAA, gibberellic acid and Siderophore productions. Isolate *Agrobacterium* sp-5 showed maximum IAA and gibberellic acid productions. Tryptophan is the main precursor of IAA biosynthesis.[16] The other reports related that L- tryptophan was more active for IAA production, though bacteria were able to produce IAA in absence of tryptophan.[17] Five Agrobacterium and five rhizobium species showed the IAA (35.5 µg/ml), GA3 (28.3µg/ml) and Siderophore production (26.5 µg/ml). Bacillus and Paenibacillus species showed the growth promoting catechol type of Siderophore production was reported. *Agrobacterium* strains showed better plant growth promoting characters was reported previously.[6,18,19]

Table 1: Plant growth promoting characteristics of *Agrobacterium* and *Rhizobium* species.

Isolates	Indole Acetic Acid production (µg/ml)	Gibberellic acid production (µg/ml)	Siderophore production (µg/ml)
Agrobacterium sp.1	12.3	5.5	12.0
Agrobacterium sp.2	14.2	12.5	14.2
Agrobacterium sp.3	17.5	8.7	15.5
Agrobacterium sp.4	18.8	6.8	13.5
Agrobacterium sp.5	35.5	28.3	26.5
Rhizobium sp.1	22.5	15.3	17.1
Rhizobium sp.2	23.4	16.8	18.9
Rhizobium sp.3	21.8	22.5	15.5
Rhizobium sp.4	15.5	21.2	20.2
Rhizobium sp.5	17.5	23.1	22.5

*****Each data is an average of three replicates

Effect of incubation period

IAA, GA3 and Siderophore productions was started after 24 h of incubation time showed by *Agrobacterium* sp-5. Maximum zone was observed after 168 h of incubation (Table-2). Maximum production was obtained at 48 h of incubation. Further increase in incubation period there is no change in the production. Incubation period plays a major role in the production was reported.[21]

Table 2: Effect of incubation period.

Incubation periods	IAA Production(µg/ml)	Gibberellic Acid Production (µg/ml)	Siderophore production (µg/ml)
24	15.1	18.5	2.40
48	35.5	28.3	26.5
72	22.2	22.1	11.8
96	17.5	19.8	14.4
120	18.2	17.4	19.6
144	22.7	15.2	23.0
168	14.7	11.2	23.0

* The overall model is significant with $p<0.05$

Effect of pH

The pH of the medium showed a significant influence on the production of IAA, GA3 and Siderophore production. The maximum IAA GA3 and Siderophore productions was observed at pH 7 (Table-3). Many autours state that the optimum pH for IAA production is between 6 to 11.[20,21]

Table 3: Effect of pH.

pH	IAA Production(µg/ml)	Gibberellic Acid Production (µg/ml)	Siderophore production (µg/ml)
5	22.2	15.3	2.40
6	20.5	22.1	5.20
7	35.5	28.3	26.5
8	21.0	25.4	14.4
9	23.5	22.1	19.6
10	12.2	10.6	23.0
11	10.2	8.5	23.0

* The overall model is significant with $p<0.05$

Effect of temperature

The effect of different temperatures on IAA, GA3 and Siderophore production was observed by Agrobacterium sp-5. The present strain showed maximum production on 30 °C temperature [table-4]. Previous reports reveals that the temperature effects the enzyme and compound productions.[16,17]

Table 4: Effect of temperature.

Temperature (°C)	IAA Production(µg/ml)	Gibberellic Acid Production (µg/ml)	Siderophore production (µg/ml)
4	2.2	3.5	2.40
15	10.5	5.8	5.20
20	18.2	10.8	11.8
25	20.3	16.7	14.4
30	35.5	28.3	26.5
35	22.5	17.5	23.0
40	15.4	12.9	23.0

* The overall model is significant with $p<0.05$

Effect of carbon sources

Among the 5 carbon sources tested, maximum Siderophore concentration was observed in Glucose containing medium, *Agrobacterium* sp-5 showed the maximum siderophore production with mannitol containing the medium (35.5 µg/ml) (Table-5). The amount and the type of the siderophore produced by an organism depend on the availability of organic and inorganic nutrients (Neilands 1982; Abd-Alla, 1998). Glucose and Mannitol proved the most suitable carbon source for hydroxamate type of siderophores in *Pseudomonas aeruginosa*, *Aspergillus nidulans*, *Pseudomonas chrysogenum* and *Bradyrhizobium japonicum*.[21]

Table 5: Effect of carbon sources.

Carbon sources	IAA Production(µg/ml)	Gibberellic Acid Production (µg/ml)	Siderophore production µg/ml
Control	10.2	8.5	1.20
Mannitol	35.5	28.3	26.5
Glucose	22.7	22.1	14.0
Sucrose	28.8	19.5	16.8
Succinate	18.5	15.3	11.4
Citrate	21.5	21.5	8.20

* The overall model is significant with $p<0.05$

Effect of Nitrogen sources

IAA, GA3 and Siderophore production by the tested microorganisms was affected by different nitrogen sources (Table -6). According to this Urea proved to be the most suitable nitrogen source for *Agrobacterium* sp-5.

Table 6: Effect of nitrogen sources.

Nitrogen sources	IAA production (µg/ml)	Gibberellic Acid production (µg/ml)	Siderophore production (µg/ml)
Control	2.5	3.8	3.0
Ammonium sulphate	12.7	8.7	14.2
Sodium nitrate	22.9	16.5	14.6
Urea	35.5	28.3	26.5
Glutamine	23.7	24.5	12.0
Glycine	15.8	20.7	13.2

* The overall model is significant with $p<0.05$

CONCLUSIONS

Out of twenty isolates there are 5 *Agrobacterium* and 5 *Rhizobium* strains showed maximum IAA, GA3 and Siderophore productions and the optimum conditions are 48 h of incubation periods, pH 7.0 and 30 °C temperature. There were several plant growth promoting rhizobacterial (PGPR) inoculants that seems to promote plant growth through different mechanism such as plant growth hormone production, nutrient acquisition and plant disease suppression. Thus the optimized microbial inoculants may be useful for the production of multifunctional biofertilizers.

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