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Research article

EVALUATION OF PLANT GROWTH PROMOTING ATTRIBUTES AND BIOCONTROL POTENTIAL OF NATIVE FLUORESCENT *PSEUDOMONAS* SPP. AGAINST *ASPERGILLUS NIGER* CAUSING COLLAR ROT OF GROUND NUT

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ABSTRACT: Ground nut is an economically important oil seed crop, which is subjected to infection by a host of fungal, viral and bacterial pathogens. Fluorescent *Pseudomonas* has potential to synthesize diverse secondary metabolites with diverse PGPR activities which enhance soil fertility and promote plant growth. Fifty five (JS-1…….JS-55) isolates of fluorescent *pseudomonas* were isolated from the rhizosphere soil of ground nut fields in Rayalaseema region of Andhra Pradesh. Based on colony morphology and biochemical tests, out of 120 rhizosphere soil samples, only 55 isolates were identified as fluorescent *Pseudomonas.* These isolates were screened for their antagonistic activity towards the collar rot pathogen *Aspergillus niger* in *Arachis hypogaea* L. Out of 55, only 15 belonging to different regions showed remarkable antifungal activity against *Aspergillus niger* with different levels of inhibition pattern. These were tested for their ability to produce secondary metabolites such as Hydrogen cyanide (HCN), Ammonia, Salicylic acid (SA), Indole acetic acid etc. Maximum production of secondary metabolites (HCN – 0.094 Abs 625; SA – 4.68 mg/ml; IAA-22.5 µg/ml) was found with fluorescent *Pseudomonas*. In this study, our investigations clearly showed that isolates collected from this region of India can be better exploited to be used as prospective biocontrol agents in agriculture system.

Key Words: Plant growth promotion, biocontrol, *Aspergillus niger*, HCN, SA

INTRODUCTION

Arachis hypogaea, an annual legume. It is the 4th most important oilseed crop of the world. Various fungal, bacterial and viral pathogens reduce the crop yield. Collar rot caused by *Aspergillus niger* in groundnut cause 40% loss in yield in India [1]. Numerous bacteria thrive on rich nutrients in the rhizosphere and some of these possess antagonistic action, which safeguard plants from pathogens and stimulate growth [2]. Biological control of plant diseases using antagonistic microorganisms offers a highly effective, lucrative and environmental friendly alternative to the use of synthetic pesticides [3]. Bacterial species like *Bacillus*, *Pseudomonas*, have been proved in controlling the fungal diseases. Bacteria recognized as plant growth promoting rhizobacteria and biocontrol strains often belong to the following genera (i) *Bacillus* [4], (ii) *Pseudomonas* [5]. Several studies describe the use of fluorescent *pseudomonas* as effective biocontrol agents against plant diseases [6]**.** The mechanisms through which *Pseudomonas* spp. control plant diseases involve (i) competition for niches and nutrients, (ii) antibiosis, (iii) predation, and (iv) induction of plant defense responses. Production of secondary metabolites like antibiotics, Fe-chelating siderophores and hydrogen cyanide is most often associated with fungal suppression by fluorescent *pseudomonas* [7]. Their ability to colonize the roots and maintain a high population density is significant [8]. Salicylic acid (SA) plays an important role in plant defense response against pathogen show aggression and is essential for the development of SAR [9].

Indole acetic acid (IAA) is one of the main physiologically active auxins. IAA is a common product of L-tryptophan metabolism by numerous microorganisms including PGPR [10, 11]. Microorganisms inhabiting rhizospheres of various plants are likely to synthesize and release auxin as secondary metabolites because of the rich victuals of substrates exuded from the roots compared with non rhizospheric soils [12, 13]. The present study demonstrates the ability of *Pseudomonas* to produce lytic enzymes and secondary metabolites that serve as biochemical weapon against phytopathogens.

MATERIALS AND METHODS

Isolation of antagonists from the rhizosphere region of groundnut plants.

Antagonistic bacteria were isolated from the rhizosphere soil collected from different ground nut growing areas of Rayalaseema region. Antagonist bacteria were isolated by serial dilution plate method [14]. From the final dilutions of 10^{-5} and 10^{-6}, one ml of each aliquot was pipetted out, poured in sterilized Petri dish containing King's B medium and they were gently rotated clockwise and anti clockwise for uniform distribution and incubated at room temperature (28 ± 2^{o} C) for 24 hours. The colonies were viewed under UV light at 366 nm. Colonies with characteristics fluorescent *Pseudomonas* spp. were isolated individually and purified by streak plate method [15] on King's B medium. The pure cultures were maintained on king's B agar slants at 4°C. Fluorescent *Pseudomonas* spp. was characterized on the basis of morphological biochemical and physiological tests as prescribed in Bergey's manual of systematic bacteriology [16].

Isolation and Characterization of Phytopathogen

The rotted seedlings with black mass of spores in collar rot were collected from a farm and brought to the laboratory for further studies. The fungal pathogen namely *Aspergillus niger* was isolated from the collar of *Arachis hypogaea* L, using PDA and further characterized based on macroscopic and microscopic observations (LPCB staining).

Invitro Characterization of biocontrol features

Antifungal activity

a) Preliminary test

Four different bacterial species were streaked as thick bands on four opposite edges on the PDA plates. Then 4 mm diameter disc of tested fungus was cut from of an actively growing culture by a sterile cork borer and placed onto the center of above PDA plates. The Petri dishes were sealed by parafilm and incubated at room temperature in dark for 2-3 days. Where mycelia disc on PDA medium without bacteria was maintained as control. The above procedure was carried out to eight soil borne bacteria and four selected fungi separately, and antagonistic effect showed by bacteria was noted as strong, moderate and weak have no effect [17].The experiment was conducted in three replicates.

The percent inhibition was calculated using the formula:

$\%\ \text{inhibition} = (R - r) / R \times 100$

Where 'r' is radial growth of the fungal colony opposite the bacterial colony and,

R is the radial growth of the pathogen in control plate.

b) Agar disc method.

0.1 ml of the test bacterial suspension (108 CFU/ml) was transferred to the center of the PDA plate using sterile pipette and spread by sterile glass spreader separately. Then 4 mm diameter of each mycelia disc was cut using a sterile cork borer and placed in the center of the above PDA plate separately under aseptic condition. Mycelia disc on PDA medium without bacteria was used as control. The cultures were incubated at room temperature in dark for 3-5 days and diameter of the fungal mycelia growth was measured [18]. The experiments were carried out thrice.

Hydrogen cyanide (HCN) production

Qualitative assay

HCN production of fungal and bacterial biocontrol agents was tested qualitatively following the method [19]. The antagonistic bacteria were streaked on King's B medium amended with glycine at 4.4g/ l. sterile sodium picrate filter paper placed in the upper lid of the Petri plate. The dishes were sealed with Parafilm and incubated at 28^{o}C for 48 h. A change of colour of the filter paper from yellow to light brown, brown or reddish-brown was recorded as weak (+), moderate (++) or strong (+++) reaction respectively.

Quantitative assay by hanging method

Antagonistic bacteria were grown in King's B broth amended with glycine (4.4g/ l) and Uniform strips of filter paper (10 x 0.5 cm^2) were soaked in alkaline picrate solution and kept hanging inside the conical flask. After incubation at 28 ± 2^{o}C for 48 h, the sodium picrate in the filter paper was reduced to a reddish compound in proportion to the amount of HCN evolved. The colour was eluted by placing the filter paper in a test tube containing 10 ml of distilled water and its absorbance was read at 625 nm [20] Three replications were maintained for each isolate.

6) Ammonia Production

For the production of Ammonia method of Lata [21] was used. *Pseudomonas* spp. was grown in peptone water (5 ml) in tubes. Tubes were incubated at 30°C for 4 days. After 4 days, 1ml of Nessler's reagent was added to each tube. Presence of faint yellow colour (+) indicated small amount of ammonia and deep yellow (++) to brown color (+++++) indicated large amount of ammonia production, characterization of plant growth promoting.

IAA Production

IAA was measured by using the following method described by Patten and Glick (2002), [22] with slight modification *Pseudomonas* spp. isolates were cultured in King's broth supplemented with tryptophan 0.5 mM for 48 h. The measurement of IAA was done by spectrophotometer at 520 nm using Salkowsky reagent (150 ml H_2SO_4, 7.5 ml $FeCl_3$. $6H_2O$, 0.5 M and 250 ml aquadest). The detection of IAA was determined by the development of pink color. IAA concentration of each sample was estimated by compared to IAA standard curve.

Salicylic Acid production

Pseudomonas isolates were grown at room temperature (28±2ºC) for 48 hours on a rotary shaker in 250 ml conical flask containing 50 ml of the King's B broth. Cells were then collected by centrifuging at 10,000 rpm for 10 minutes and 4 ml of cell free culture filtrate was acidified with 1N HCl to pH 2.0 and salicylic acid was extracted with chloroform (2x2 ml). To the pooled chloroform extracts, 4 ml of distilled water and 5 ml of 2M $FeCl_3$ were added. The absorbance of the purple iron-salicylic acid complex, which was developed in the aqueous phase, was read at 527 nm in a spectrophotometer [23]. A standard curve was prepared with salicylic acid dissolved in King's B broth. The quantity of salicylic acid in the culture filtrate was expressed as mg ml-1.

RESULTS AND DISCUSSION

Interest in biological control has recently intensified because of imminent bans of chemical controls such as methyl bromide, widespread development of fungicide resistance in pathogens and a general need for a more effective and safer alternative disease control strategies.

Table-1 Morphological, Cultural and Biochemical Characteristics of fluorescent *pseudomonas* Isolates

S.No	Name of the Test	JS – 7	JS - 16	JS - 24	JS – 31	JS – 52
1	Gram reaction	-	-	-	-	-
2	Cell Shape	Rod	Rod	Rod	Rod	Rod
3	Motility test	Motile	Motile	Motile	Motile	Motile
4	Growth at 41°C	+	+	-	-	+
5	Growth at 4°C	+	-	+	-	+
6	Fluorescent pigment production	+	+	+	+	+
7	Oxidase	+	+	+	+	+
8	Catalase	+	+	+	+	+
9	KOH String Test	+	+	+	+	+
10	Indole	-	-	-	-	-
11	Methyl Red Test	-	-	-	-	-
12	Voges Prokaur	-	-	-	-	-
13	Citrate Utilization	+	+	+	+	+
14	Glucose fermentation test	-	-	-	-	-
15	Starch hydrolysis	-	-	-	-	-
16	Gelatin hydrolysis	+	+	+	+	+
17	H_2S Production	+	-	+	+	+

Certain plant associated bacteria particularly fluorescent *pseudomonas has* been exploited for suppression of crop diseases [24]. *Pseudomonas* were identified as biocontrol agents of groundnut stem rot and other soil-borne diseases [25]. Fifty five (JS1…….JS 55) isolates of fluorescent *pseudomonas* were isolated from rhizosphere soil. A strong correlation was observed between the chitinolytic potential of different bacterial strains and *invitro* lysis of fungal mycelium [26], Broad-spectrum activity of *Pseudomonas* spp. contributes to their *invitro* antifungal activity and *invivo* disease control [27]. Among these 15 isolates showed antagonism against the 6 different pathogenic *Aspergills niger* and 5 isolates were effective.

Table-No-2 Antagonistic activity of *pseudomonas* isolates

S.No	*Pseudomonas* isolates	Antifungal activity against *Aspergillus niger*	
		Qualitative	Quantitative (Avg. diameter of zone of inhibition in mm)[#]
1	JS -7	+++	56.8
2	JS -16	+++	48.4
3	JS -24	+	22.5
4	JS -31	++	26.8
5	JS -52	+++	44.6

Weak inhibition: + (Fungal growth was slightly inhibited by bacteria)
Average inhibition: ++ (Loosely arranged mycelial growth over the bacteria)
Strong inhibition: +++ (Fungal growth was completely inhibited by bacteria)
Average zone of inhibition of isolates against 6 pathogenic *Aspergillus niger* from 3 replicates.

Production of HCN

HCN, a volatile metabolite is attention to play a major role in biological control of some soil born diseases [28]. HCN is the common secondary metabolite produced by rhizosphere *Pseudomonas* [29]. Exposing plants to the volatile metabolites of antagonist causes a significant increase in peroxides activity, which may contribute to induction of disease resistance [30]. In quantitative estimation, JS1 and JS5 recorded the maximum OD value of 0.094 and 0.085 respectively but JS-10 recorded least OD value (0.015).

Salicylic acid

Salicylic acid is known to play a critical role in the activation of plant defense response [31, 32].
These mechanisms, such as suppression of harmful microorganisms and induced systemic resistance (ISR), are usually recognized as having a role in biological control [33]. PGPR-elicited induced systemic resistance (ISR) is phenotypically similar to pathogen-induced systemic acquired resistance (SAR) [34]. Many studies have indicated that salicylic acid (SA) plays an important role in plant defense response against pathogen attack and is essential for the development of SAR [35]. A maximum concentration of 4.68 mg/ml of salicylic acid was produced by JS -7 in our investigation. Increased resistance was correlated with accumulation of pathogenesis-related (PR) proteins, which are generally considered to be markers of SAR [36].

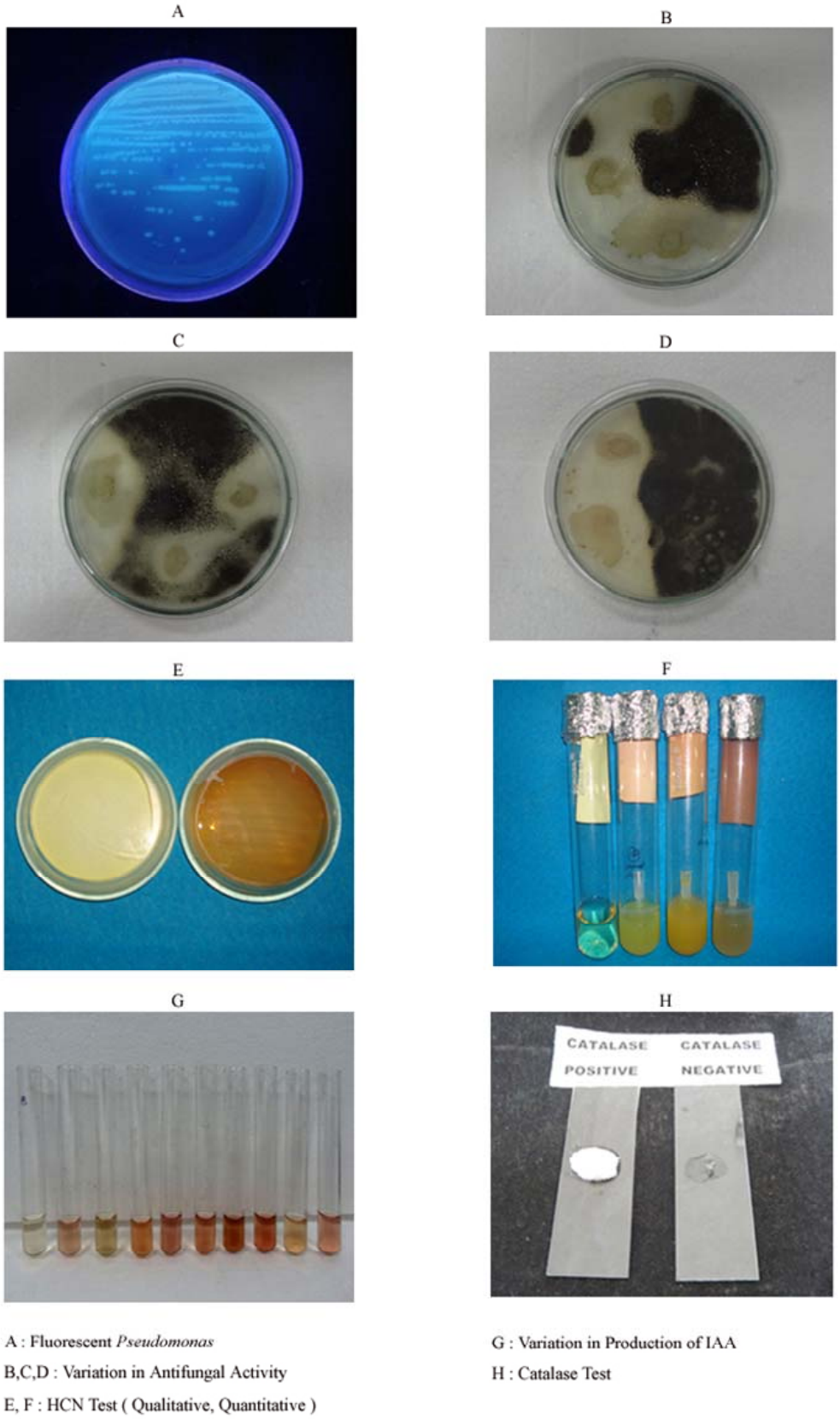
Production of Ammonia

Development of yellow-brown color was observed after addition of Nessler's reagent indicating a positive test for ammonia production. It has been reported that ammonia production circuitously influences the plant growth. *Pseudomonas* was efficient in ammonia production and significantly increased biomass of medicinal and aromatic plant [37]. In this study 80% *pseudomonas* produced ammonia.

Table no- 3 Plant Growth Promoting Characteristics and hydrolytic enzyme production of rhizobial isolates

S.No	*Pseudo-monas* Isolates	Production of HCN by glycine supplimentation	Production of HCN without glycine supplimentation	HCN O.D Values at 625nm	IAA productio n µg/ml (24hr)	Salicylic acid Production mg/ml (48hr)	Ammonia production
1	JS -7	+++	+++	0.094	22.5	4.68	Brown
2	JS -16	+++	++	0.078	18.2	3.80	Brownish Yellow
3	JS -24	+++	+	0.059	19.8	3.58	Light Brown
4	JS -31	++	_	0.030	16.6	3.05	Brown
5	JS -52	+	_	0.022	18.5	2.15	Yellow

HCN production: - negative, + weak, ++ moderate, +++ strong



A : Fluorescent *Pseudomonas*

B,C,D : Variation in Antifungal Activity

E, F : HCN Test (Qualitative, Quantitative)

G : Variation in Production of IAA

H : Catalase Test

Figure-1: Antagonistic and PGP variation of fluorescent *pseudomonas* illustrated fig. a, b, c, d, e, f, g, h.

PGP traits of the selected isolates

Pseudomonas spp., belonging to plant growth promoting *Rhizobacteria* has received prominent attention because of the dual role of these bacteria in plant growth promotion and diseases control [38]. In the present study all the five selected isolates were positive for plant growth promoting hormone indole acetic acid (IAA) production. The amount of IAA produced varied from 10µg/ml to 35µg/ml, JS-42 produce maximum and least production was observed in JS-20. All the selected strains were strong IAA, Ammonia and Catalase producers, thus indicating their potential for plant growth promoting effects. Results also revealed that the antifungal activities and other plant beneficial traits appear to be the general and genetically dispersed traits of fluorescent *pseudomonas*.

CONCLUSION

Since *fluorescent pseudomonas* acquire multiple mechanism of antagonism and stable production of antifungal agents under inconsistent growth conditions to sustain antagonism during plant root development, it can be explored as one among the best biocontrol agent against phytopathogens. It can be easily and stably integrated into the accessible production practices.

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