PLANT GROWTH PROMOTING ACTIVITY OF ANTAGONISTIC SOIL BACTERIA

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Abstract

Pseudomonas fluorescens and *Bacillus subtilis* isolated from rhizosphere of healthy groundnut plants were screened for their antagonistic activity towards the crown rot pathogen *Aspergillus niger* in *Arachis hypogaea* L. Invitro assay revealed that 10% of the isolates were antagonistic in nature and they were tested for their ability to produce growth promoting substances such as Auxin and Extracellular Phosphatase. Green house studies, with the application of *Pseudomonas fluorescens* 04 (80% inhibition of pathogen & 7.14mg/l auxin production) and *Bacillus subtilis* 03 (80% inhibition of pathogen & 79µg/l phosphatase production), to *Arachis hypogaea* L. showed increase in growth, biochemical constituents and yield of test plants than that of control.

Keywords: *Pseudomonas fluorescens*, *Bacillus subtilis*, *Aspergillus niger*, Auxin, Phosphatase.

I.INTRODUCTION

Protecting crop plants from vulnerable attack of phytopathogens using eco-friendly techniques is the need of the hour in farming practices. Understanding the diversity and beneficial activity of the plant-bacterial association is important to sustain agro-ecosystems for sustainable crop production (Germida *et al.,* 1998). Several bacteria thrive on abundant nutrients in the rhizosphere and some of these possess antagonistic action, which safeguard plants from pathogens and stimulate growth (Gray and Smith, 2005).

Arachis hypogaea [groundnut], an annual legume is known as peanut, earthnut, monkeynut and goobers. It is the 13th most important food crop and 4th most important oilseed crop of the world. Crown or collar rot caused by *Aspergillus niger* in groundnut leads to "patchy" crop stand and ultimately reduce the yields. Collar rot reported to cause 40 per cent loss in yield in India (Chohan, 1973).

Pseudomonas fluorescens and *Bacillus subtilis* are root colonizing bacterial biocontrol strains that suppresses soil-borne plant diseases caused by phytopathogenic fungi (Manjula and Podile, 2001; Nagarajkumar, 2004; Moataza and Saad, 2006; Vleesschauwer *et al.,* 2008). These antagonistic bacterial agents induce the immune mechanism of plants to produce significant levels of Plant Defense Enzymes (PDE) (Saravanakumar *et al.,* 2007; Latha *et al.,* 2009).

Auxins are a group of herbal hormones, in which Indole -3-Aceitic Acid (IAA) is the most important of them (Glick, 1995). IAA is produced through L-TRP metabolism by plants and many of soil microorganisms such as bacteria, fungi and algae (Sarwar and Kremer, 1995). Plant growth regulator produced by *Pseudomonas* spp. could play a critical role in plant growth promotion (Karnwal, 2009; Karthikeyan *et al.,* 2009).

Phosphorous is limiting nutrient in spite of its vast occurrence in natural environment since it its bound to or strongly adsorbed to silt and clay (De Souza *et al.,* 2000). The major forms of phosphorous are solubulized enzymatically or by acid hydrolysis and crops absorb phosphorous in the form of soluble orthophosphate ion (Ponmurugan and Gopi, 2006). Biological solubilization of rock phosphate is eco-friendly than acidulation (Taiwo and Ogundiya, 2008).

Protection of bacterial inoculated seedlings against soil borne pathogens was observed inseparable from the growth promoting activity of several of the reported PGPR (Plant growth Promoting Rhizobacteria) (Raupach and Kloepper, 2000; Guo *et al.,* 2004). The present work aims to study the auxin and phosphatase producing ability of the antagonistic bacteria *P.fluorescens* and *B.subtilis* respectively. Also an attempt was made to evaluate the growth stimulating activity of these organisms in *Arachis hypogaea* L. seedlings.

II.MATERIALS AND METHODS

A. Isolation and Characterization of Phytopathogen

The rotted seedlings with black mass of spores in collar 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).

B. Isolation and Characterization of Bacterial Antagonist

Pseudomonas fluorescens and *Bacillus subtilis* were isolated from rhizosphere of healthy groundnut plants and maintained in laboratory using Kings B and Nutrient agar respectively at 4°C. Antagonistic actions of these bacterial isolates were confirmed by performing Dual Plate Method (DPM).

C.Screening for Phosphate Solubilization

Phosphate solubulizing ability of the *Bacillus subtilis* possessing antagonistic action against *Aspergillus niger* was tested using Pikovs Kayas Agar (pH 7.0) containing calcium phosphate as source of phosphorous. Spread plate was performed using 0.1 ml of the inoculum and incubated at 37°C for 48 hrs. The colony showing halo zone around them was considered as phosphate solubulizer.

D.Partial Purification of Phosphatase

Bacillus subtilis was cultured in Pikovs Kayas broth at 37°C for 3 days. The culture broth was filtered using Whatman No.1 filter paper, followed by the addition of 2.5 ml of 70% ammonium sulphate to 10 ml of the filtrate. It was centrifuged at 15,000 rpm for 15 mins. The resulting pellet was collected and suspended in 5 ml of 0.1 M acetate buffer and then centrifuged at 15,000 rpm for 15 mins. The supernatant obtained was carefully separated and stored at 4°C for enzyme assay.

E.Phosphatase Assay

2 ml of 0.05 M disodium phenyl phosphate was added to 7 ml of acetate buffer (pH 4.0). 1 ml of enzyme supernatant, prepared by ammonium sulphate precipitation method was added to the mixture. Added 5ml of Folin-Ciocalteu reagent and incubated at room temperature for 30 mins. The chemical mixture was filtered through Whatman No.42 filter paper and 5ml of the aliquot of the filtrate was transferred to 15ml volumetric flask containing 1.5 ml of extra Folin-Ciocalteau reagent and 2.5ml of 20% sodium carbonate solution. The content of the flask was incubated at room temperature for 20 mins and the intensity of resulting blue color was measured at 660 nm (Rae and Eastcott, 2008). The standard was prepared by dissolving 0.1 mg of phenol in 10 ml of buffer solution, followed by the above procedure.

F.Auxin Production

Antagonistic *Pseudomonas fluorescens* was cultured using Yeast Peptone Dextrose (YPD) broth (Yeast – 10 g, Peptone – 20g, Dextrose – 20 g, Tryptophan – 3g, Distilled Water – 1000ml; pH – 7.0) at 35°C for 5 days. The culture broth was centrifuged at 4000 rpm for 30 mins (Kulanthaivel *et al.,* 2006). The supernatant containing auxin was detected by the method of Garden and Paleg (1957).

G.Auxin Assay

The supernatant was brought to pH 2.8 with 1 N HCl. Then it was extracted with diethyl ether. The ether phase was dried and dissolved in 2 ml of methanol. Auxin present in methanol extract was estimated using Salper's reagent (1 ml of 0.5 % ferric chloride in 50 ml of 35% perchloric acid). The intensity of resulting brick red to dark brown color was read at 535 nm using spectrophotometer.

H.Procuring Seeds and Raising Seedlings:

Surface sterilized groundnut seeds procured from Seed Science Department, TamilNadu Agricultural University (TNAU), Coimbatore, were used in the study. Seeds were sown in pots containing sandy red soil and the seedlings were raised in open field condition. The bacterial isolates were added to pot soil (T1- Control; T2 - *Bacillus subtilis*; T3 -*Pseudomonas fluorescens*; T4 - Combination of *Pseudomonas fluorescens* and *Bacillus subtilis*) after pelletization using effluent based carrier (Combination of cane molasses and liquid whey in the ratio 1:1). After 30 days of sowing, the morphological and biochemical contents of the seedlings were analyzed using standard protocols (Sadasivam and Manickam, 2003) as mentioned below.

I.Morphological Parameters

Morphological parameters include the estimation of root and shoot length, biomass, number of root nodules, number of primary roots and leaf surface area.

J.Total Protein

To 1 ml of the sample added 5 ml of alkaline copper sulphate solution, mixed well and incubated at room temperature for 10 minutes. After incubation 0.5 ml of Folin-Ciocalteau Reagent was added and incubated at room temperature for 30 minutes in dark. The intensity of blue color developed was measured at 660nm spectrophotometrically.

K.Total Carbohydrate

To 1 ml of the sample added 3 ml of DNS reagent. The contents were heated in a boiling water bath for 5 minutes and added 1 ml of 40% Rochelle salt solution at luke-warm heat, brought to room temperature. The intensity of orange-red color developed was measured at 510nm using spectrophotometer.

L.Chlorophyll Estimation (Sadasivam and Manickam, 2004)

One gram of matured leaf was collected and washed with distilled water to remove the adhering dust. The tissue was ground to a fine pulp with the addition of 20 ml of 80% acetone. The contents were centrifuged at 5000 rpm for 5 minutes and transferred the supernatant to a 100 ml volumetric flask. The procedure was repeated until the residue turn colorless. The absorbance of the supernatant was read at 663 and 645 nm using 80% acetone as blank in a spectrophotometer. The Total Chlorophyll (TC)

content of the leaf was calculated using the formula,

V mg total chlorophyll / g tissue = 20.2 (A_{645}) + 8.02 (A_{633}) × 1000 × W

A = Absorbance at specific wave length

V = Final volume of chlorophyll extract

W = Fresh weight of tissue extracted

GC-MS: The supernatant of Pikovskayas broth cultured with B.subtilis was subjected to GC-MS to detect the acids that possibly aid in phosphate solubilization.

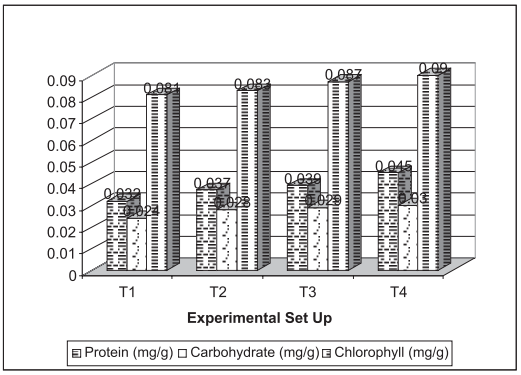

Fig .1. Biochemical Parameters of *A.hypogaea* seedlings inoculated with Anatagonistic Bacteria

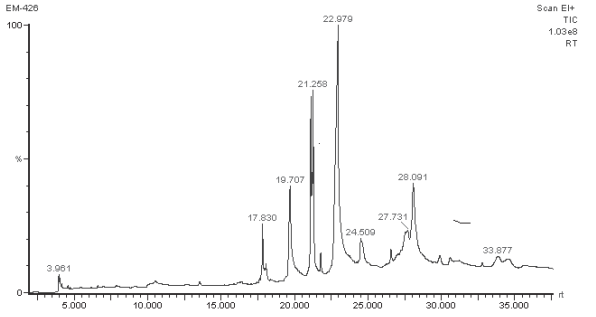

Fig. 2. GC-MS of Pikovskayas broth supernatant cultured using *B.subtilis*

Table 1. Effect of Antagonistic Bacteria on Seed Germination and Yield of *Arachis hypogaea*

S.No	Exptl. Set Up	Seed Germination (%)	Yield (Number of Pods p^{-1})
1	Pot 1	80	05
2	Pot 2	90	10
3	Pot 3	100	16
4	Pot 4	100	21

Table 2. Effect of Antagonistic Bacteria on Morphological Parameters of Arachis *hypogaea*

S.No	Exptl. Set Up	Root Length (cm)	Shoot Length (cm)	Root Biomass (g/plant)	Shoot Biomass (g/plant)	No. of Root Nodules (Per plant)	No. of Primary roots (Per plant)	Leaf Surface Area (Sq.cm)
1	Pot 1	13	7	0.20	0.13	8	3	6.30
2	Pot 2	16	9	0.27	0.16	18	6	7.56
3	Pot 3	20	9	0.29	0.17	20	7	8.40
4	Pot 4	23	13	0.31	0.20	21	11	10.92

Table 3. Characterization of Antagonistic Bacterial Strains

S.No	Organism	Antagonistic activity (%)	Auxin Production (mg/lit)	Phosphatase (µg/ml)
1	*B.subtilis* 01	35	-	54
2	*B.subtilis* 02	42	-	-
3	*B.subtilis* 03	80	-	79
4	*B.subtilis* 04	50	-	58
5	*B.subtilis* 05	62	-	64
6	*P.fluorescens* 01	50	4.96	-
7	*P.fluorescens* 02	45	6.41	-
8	*P.fluorescens* 03	60	-	-
9	*P.fluorescens* 04	80	7.14	-
10	*P.fluorescens* 05	48	-	-

I. RESULTS AND DISCUSSION

Combined inoculation of *Pseudomonas fluorescens* and *Bacillus subtilis* was proved to enhance seed germination, when compared to control (Table-1). The production of growth hormone auxin by *P.fluorescens* could be responsible for the vigor induction and seedling growth. Similar reports were documented by earlier workers (Karnwal, 2009). Bacterization of peanut seeds with *P.fluorescens* was found to promote seed germination in the investigation performed by Gupta *et al.,* (2005) and Murugalakshmi *et al.,* 2009). Our results are line with these findings, since *P.fluorescens* inoculated seeds attained 100% germination.

Microbial communities that exert beneficial effects on plant growth upon root colonization were termed as Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper and Scroth, 1978). Plant growth enhanced by PGPR is quantified as an increase in seedling emergence, vigor, biomass, proliferation of root system and yield in various plant species (Zahir *et al.,* 2004; Ping and Boland, 2004; Khakipour *et al.,* 2009). Our results substantiate this statement in terms of profound growth pattern of seedlings supplied with *B.subtilis* and *P.fluorescens* (Table-2). Similar results were reported by Kishore *et al.,* (2005).

B.subtilis was compatible with other PGPR strains native to soil and its efficiency was reported to increase many a folds by synergistic activity (Taiwo and Ogundiya *et al.,* 2008; Elizabeth *et al.,* 1994). Our results authenticate these findings, since morphological and biochemical

contents of seedlings inoculated with *B.subtilis* and *P.fluorescens* was found to be phenomenal than control. Above all thermo-tolerant strains of phosphate solubulizing *B.subtilis* were reported by Gaind and Gaur (1991), which could be assert for tropical regions.

Estimation of biochemical components in test plants treated with antagonistic bacterial strains, when compared with control revealed higher concentration of total proteins, carbohydrates and chlorophyll (Fig-1). The growth promoting effect of antagonistic bacteria could be correlated with induction of defense enzyme and secretion of growth factors (Kamalakannan *et al.,* 2009). Production of growth promoting substances in the form of auxins, phosphate solubilization by secretion of extracellular phosphatase, in addition to growth factors may be responsible for growth promoting activity. Similar findings were reported by early workers (Kloepper and Beauchamp, 1992; Gupta *et al.,* 2005; Ponmurugan and Gopi, 2006).

Antagonistic bacteria isolated from rhizosphere of healthy groundnut plants were evaluated for their antagonistic potential by Dual Plate Method. Out of 60 *P.fluorescens* and 40 *B.subtilis* isolated, 10 strains (5 of each) were found to be antagonistic towards the crown rot pathogen of groundnut, *Aspergillus niger* at varied levels ranging from 35 to 80%. Antagonistic strains *P.fluorescens* 04 and *B.subtilis* 03 were selected fort studies since these organisms exerts potential antagonistic action comparatively with elevated level of auxin (7.14mg/l) and phosphatase(79µg/ml) secretion respectively (Table-3).

In addition to the enzyme phosphatase, several acids produced by the organism were reported to involve in phosphate solubilization. Analyzing the culture supernatant of Pikovskayas broth inoculated with *B.subtilis* through GC-MS revealed the presence of acetic, hepta, octa and undecanoic acid (Fig-2), possibly involved in the degradation of complex form of metal bound phosphate in to soluble monobasic and dibasic ions, a process know as mineral phosphate solubilization (Gyaneshwar et al.,2002).

In our study *P.fluorescens* species were found to be negative for phosphatase production. In contrast it was reported to possess the same by Souza *et al.,* (2000), yet they are halophilic strains isolated from marine sediments, that would be an advantage for salty soil. From the overall results it can be concluded that *B.subtilis* 03 and *P.fluorescens* 04 could afford protection against *A.niger* and efficiently promote growth of *A.hypogaea* when applied in combination. Further studies in this scenario could help us to understand the molecular mechanism behind the interactions between antagonistic soil flora and plant growth.

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