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Biochemical and molecular characterization of DAPG-producing plant growth-promoting rhizobacteria (PGPR) of groundnut (*Arachis hypogaea* L.)

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) thrive in the rhizosphere of plants and play a beneficial role in plant growth, and development along with biocontrol activities. The present study was undertaken with the aim of developing rhizobacterial inoculants for groundnut for enhancement of growth and yield and suppression of major soil-borne fungal diseases caused by *Sclerotium rolfsii* (stem rot) and *Aspergillus niger* (collar rot). Out of a total of 154 rhizobacterial isolates obtained from groundnut rhizosphere, 78 isolates were selected on the basis of *in vitro* antifungal activities against three major soil-borne fungal pathogens of groundnut, i.e. *Aspergillus niger, Aspergillus flavus* and *Sclerotium rolfsii*. The selected isolates were further screened for the production of 2,4-Diacetylphloroglucinol (2,4-DAPG) by the gene specific PCR amplification of *phlD* gene. A total of 11 rhizobacterial isolates were found to have DAPG-producing genes and selected for further studies. Gene specific primers were also used for characterization of the isolates for plant growth-promoting and biocontrol traits. The qualitative and quantitative estimation of the various attributes of the isolates were also carried out. Majority of the isolates showed production of IAA, siderophores and fluorescent pigments. The DAPG-producing rhizobacterial isolates have great potential as bio-inoculants for groundnut crop for suppressing soil-borne fungal pathogens and to enhance growth and yield.

Key words: Biocontrol, DAPG, Groundnut, Growth-promotion, Rhizobacteria.

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is a major oilseed crop of India with an estimated annual production of around 6.0 million tonnes. It contributes nearly 30% of the domestic edible oil production. In India, the crop is predominantly grown as a rainfed crop in nutrient-poor soils. Groundnut is also susceptible to a host of diseases and pests which limit the productivity along with factors like poor nutrition. The indiscriminate use of chemical fertilizers and herbicides, pesticides, etc. raises the concerns of environmental deterioration along with indirect effects on human and animal health. The productivity of groundnut can be enhanced in a sustainable way by the application of plant growth–promoting rhizobacteria (PGPR) for increasing the availability of plant nutrients and providing protection against plant pathogenic fungi. Plant growth promoting rhizobacteria (PGPR), inhabit plant roots and affect plant growth promotion by mechanisms ranging from a direct influence such as increased solubilization and uptake of nutrients like P (Richardson *et al.* 2009), Ca, K, Fe, Cu, Mn and Zn (Mantelin and Touraine 2004); production of plant growth regulators to indirect effects such as suppression of plant pathogens by producing siderophores, antibiotics (Dowling and O'Gara 1994; Dowling *et al.* 1996; Kloepper *et al.* 1989; Zahir *et al.* 2004), chitinase, β-1,3-glucanase, protease, or lipase (Chet and Inbar, 1994). Thus, the rhizospheric bacterial strains can be possible candidates for biological improvement of crops (Walia *et al.* 2014). Interest in the study of PGPR has increased due to their potential for improving growth and yields of various crops like groundnut (Dey *et al.* 2004).

Pseudomonads are common members of the plant growth-promoting rhizobacterial microflora in the rhizosphere of groundnut. As compared to other soil microorganisms, rhizospheric pseudomonads are more beneficial for production and survival of plants due to their high metabolic activity (production of enzymes, exo-polysaccharides, secondary metabolites, antibiotics, etc.). The ability of pseudomonads to suppress soil-borne fungal pathogens depends on their ability to produce antibiotic metabolites, such as pyoluteorin, pyrrolnitrin, phenazine-1-carboxylic acid, 2,4-diacetylphloroglucinol (DAPG) (Troppens *et al.* 2013) and biosurfactant antibiotics (Angayarkanni *et al.* 2005).

In this study, fluorescent pseudomonads were isolated from the groundnut rhizosphere and screened for plant growth-promoting traits besides assessing biocontrol abilities against major soil-borne fungal pathogens of groundnut. Biochemical and molecular characterization were

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carried out for ascertaining the production of growth promoting and anti-fungal metabolites by them. This will help in identifying potential rhizobacterial isolates for further testing in potted and field conditions for use as bioinoculants in groundnut cultivation for improvement of yield and suppression of soil-borne diseases.

MATERIALS AND METHODS

Collection of samples and isolation of rhizobacteria: Rhizosphere soil samples were collected from the groundnut growing fields of the Directorate of Groundnut Research, Junagadh during (*kharif* 2010). Serial dilution and spread-plating methods were used for isolation of groundnut rhizobacteria, followed by purification and characterisation. King's medium B (King *et al.* 1954) was used for isolation and culturing of the isolates. A total of one hundred and fifty four rhizobacterial isolates were obtained. The isolates were incubated at 28°C during culturing and maintained as glycerol stocks (35% glycerol-KB broth) at -20°C.

Screening for antifungal activities: The 154 rhizobacterial isolates were screened for the production of antifungal antibiotics on King's B medium following the standard procedure of Hebbar *et al*. (1992). Bacterial isolates were spotted on King's B medium by spotting 5 µl of 24h old culture of fluorescent pseudomonads. The Petri plates were incubated for 48 h after which the bacterial growth was scrapped and three ml of chloroform was kept in the lid of each Petri plate and kept in inverted position for 30 min. Fungal spores were sprayed (*Aspergillus niger* and *Aspergillus flavus*, as per treatment) onto the agar surface of these Petri plates to screen the antifungal activity of the rhizobacterial isolates. The antifungal activities of the rhizobacterial isolates were measured in the form of zone of inhibition and expressed in mm. In case of *Sclerotium rolfsii*, 2-3 mm disc of mycelial growth of *S. rolfsii* was placed at the center of Petri plates containing King's B medium followed by spotting of a young culture of rhizobacterial isolate at the edge. After 72 h, the zone of inhibition of the mycelial growth of *S. rolfsii* by the rhizobacterial isolate was measured.

Screening for DAPG production by PCR amplification of *phlD* gene: The rhizobacterial isolates which showed antifungal activity against any/all of the the three major fungal pathogens, i.e., *A. niger*, *A. flavus* or *S. rolfsii* were screened for the presence of *phlD* activity in a PCR based assay. The method of Ahmadzadeh *et al.* (2006) was followed for the detection of *phlD* gene. A total of 78 rhizobacterial isolates which showed antifungal activity were screened for the presence of *phlD/phlA* gene(s). The bacterial DNA was isolated using Axygen Bacterial DNA isolation kit (Axy Prep™ Bacterial Genomic DNA Mini prep Kit, Axygen, USA) following the protocol of the manufacturer. The DNA, thus isolated, was used as template for the detection of *phlD*/ *phlA* genes in the rhizobacteria having antifungal activities against major soil-borne fungal pathogens. PCR amplification of *phlD* gene was performed using forward primer phl2a (5'-GAG GAC GTC GAA GAC CAC CA-3') and reverse primer phl2b (5'-ACC GCA GCA TCG TGT ATG AG-3'), which were developed from the *phlD* sequence of *Pseudomonas fluorescens* Q2-87 (Raaijmakers *et al*. 1997). PCR amplification was carried out in 20 µl reaction mixtures. PCR cycling program was used as described by Wang *et al*. (2001), but with minor modification of the annealing temperature. The cycling program included an initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 60s, 53.2°C for 60s, 72°C for 60s, and then a final extension at 72°C for 5 min. Amplification was performed using a Takara PCR thermal cycler (Takara TP600 gradient, Takara, Japan). The primers phlA-1f (5'-TCA GAT CGA AGC CCT GTA CC-3') and phlA-1r (5'- GAT GCT GTT CTT GTC CGA GC-3') were used to amplify a 418 bp fragment of *phlA* as described by Rezzonico *et al.* (2003) with slight modification. The cycling program included an initial denaturation at 94°C for 5 min followed by 25 cycles of 94°C for 30s, 62°C for 30s, 72°C for 45s, and final extension for 5 min at 72°C. The PCR amplified products were resolved in 1.2% (w/v) agarose gel using 100 bp DNA step ladder as molecular size marker. *PhlD* amplified product of *Pseudomonas aeruginosa* AMAAS 57 (NCBI accession no. JN391537) was used as positive control.

Biochemical characterization of the isolates for antifungal activities: *In vitro* screening was done to detect the possible biocontrol characters of the selected rhizobacteria. A total of 12 isolates (11 isolates which showed *phlD* activity and a known standard culture *P. aeruginosa* AMAAS 57) were biochemically characterised for antifungal activities. The isolates were tested for the production of chitinase, on a medium (Frandberg and Schnurer, 1994) with 0.15% colloidal chitin from crab shell. The bacteria were spot inoculated on the plates & incubated at 28°C for 3-4 days. Hydrocyanic acid (HCN) production was detected by the method of Bakker and Schippers (1987). King's B medium, after sterilization, was amended with glycine (filter sterilized separately with 0.1 µ sterile syringe filter) in such a manner to make the final concentration of glycine at 4.4 g/l). In each plate 25 ml of the medium was poured and bacteria were streaked. Whatman No.1 filter paper disk (9 cm in diameter) soaked in 0.5 % picric acid in 2 % sodium carbonate was placed in the lid of each Petri plate. The Petri plates were sealed with parafilm and incubated at 28°C for 4 days while keeping an uninoculated control. Cyanogenesis from glycine resulted in the production of HCN which is volatile in nature. The reaction of HCN with picric acid in presence of Na_2CO_3 resulted in the colour change of the filter paper from deep yellow to orange and finally to orange brown to dark brown. In case of negative tests the deep yellow colour of the filter paper remained unchanged even after the growth of bacteria.

Detection and quantification of plant growth-promoting attributes: The selected DAPG-positive groundnut rhizobacteria with antifungal activities were characterized for the presence of plant growth-promoting traits and quantified for the positive traits. For the detection of ammonia production, all the rhizobacterial isolates were grown in peptone water (Dye, 1962) broth (peptone-10.0 g; NaCl-5.0 g; d/w-1000 ml; pH 7.0). The tubes were inoculated after sterilization and incubated at 30°C for 4 days. The accumulation of ammonia was detected by addition of Nessler's reagent @ 1 ml per tube. A faint yellow colour indicated small amount of ammonia, deep yellow to brownish colour indicated maximum production of ammonia.

For detecting and quantifying the production of indole acetic acid (IAA) like substances, the rhizobacterial isolates were grown in 100 ml Erlenmeyer flasks containing 25 ml of Tryptone yeast extract (TY) broth and incubated on a rotary shaker in the dark at 28°C for 4 days (Sarwar and Kremer, 1995). Then 25 ml of the culture broth was centrifuged at 1000 rpm for 10 min to remove bacterial cells. For detection, 1 ml of the supernatant was mixed with 1 ml of Salkowsky reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 3.5% $HClO_4$) with continuous agitation and the reaction mixture was incubated in the dark for one h. Development of pink colour confirmed the production of IAA or IAA like substances. For the quantification of IAA production, 1 ml of the supernatant was mixed with 1 ml of the reagent with rapid but continuous agitation. The reaction mixture was incubated in the dark for 60 min. Absorbance was read in UV spectrophotometer at 535 nm. The amount of IAA produced was measured from the standard curve.

Solubilization of insoluble phosphate by the rhizobacterial isolates was tested in Pikovskaya's medium (Pikovskaya, 1948), containing tri-calcium phosphate. Spot inoculation was done with the rhizobacterial isolates and plates were incubated at 28°C for 4-7 days. Observation was taken for clearing or solubilization zone around the colonies. The solubilization zone was measured in mm (diameter). The solubilization of tri-calcium phosphate was quantified in Pikovskaya's broth (Pikovskaya, 1948), following the protocol described by Dey *et al.* (2004). The change in pH of the medium, as a result of solubilization, was measured at 3-day intervals.

Molecular characterization of selected *phlD*$^+$ rhizobacterial isolates of groundnut: The selected rhizobacterial isolates were also characterized at molecular level targeting specific genes responsible for imparting biocontrol abilities against soil-borne plant pathogenic fungi and plant growth promotion (Table 1).

The polymerase chain reactions were performed in Eppendorf MasterCycler Gradient 3113 using the primers mentioned in Table 1 and the bacterial chromosomal DNA were isolated using Axygen Bacterial DNA isolation kit. The reaction mixture of 20 µl was used. The PCR products were resolved on a 1.2% agarose gel (w/v) to check the presence of a particular trait in a particular isolate.

RESULTS AND DISCUSSION

In the present investigation, with the aim to identify potential rhizobacterial inoculants which can enhance growth and yield of groundnut along with suppression of major soil-borne fungal pathogens of groundnut using DAPG-producing fluorescent pseudomonads, a total of 154 rhizobacterial isolates were screened for antifungal activities against the three major soil-borne fungal pathogens (Figure 1) of groundnut, i.e. *Aspergillus niger*, *Aspergillus flavus* and *Sclerotium rolfsii* causing collar rot, aflaroot and stem rot. Out of the 154 isolates, only 78 isolates (nearly 50%) showed antifungal activity against one or two or all the three pathogens. Out of the 78 isolates, 72 isolates showed antifungal activity against *A. niger*, 68 against *A. flavus*, 75 against *S. rolfsii* and 64 against all the three pathogens. Against *A. niger*, the inhibition zone ranged from 6 mm to 40 mm. Against *A. flavus*, the zone of inhibition ranged from 6.7 mm to 17.3 mm. However, in case of *S. rolfsii*, the zone of inhibition ranged from 10 mm to 35 mm.

A total of 78 rhizobacterial isolates showing antifungal activity were screened for the presence of *phlD* gene in a PCR based assay along with a standard *phlD* positive isolate, *Pseudomonas aeruginosa* AMAAS 57 and

TABLE 1: Genes targeted for characterizing the isolates at molecular level

Gene	Activity	Primers used		Reference
acc	ACC deaminase	Acc F	Acc R	Sarvanankumar & Samiyappan (2007)
fur	Siderophore production	Fur F	Fur R	Tsujibo *et al.* (2000)
phl D	DAPG	Phl 2a	Phl 2b	Ahmadzadeh *et al.* (2006)
hcn BC	HCN	Aca	Acb	Ramette *et al.* (2003)
fpv	Pyoverdine	599 F	599 R	Pacheco *et al.* (2006
pfe	Pyocyanine	2134 F	2134 R	Pacheco *et al.* (2006)
*iaa*M	IAA	iaaMF	iaaMR	Yang *et al.* (2007)
chi C	Chitinase	chiCF	chi CR	Arzu *et al.* (2013)
amo A	Ammonification	AMO F	AMO R	Christopher *et al.*(1995)
plt	Pyoluteorin	plt F	pltR	Nowak-Thompson *et al.* (1999)
Prn	Pyrrolnitrin	PrnF	PrnR	Selin *et al.* (2010)
phlA	DAPG	phlA-1f	phlA-1r	Rezzonico *et al.* (2003)

FIG 1: *In-vitro* inhibition of fungal pathogens by DAPG-producing fluorescent pseudomonads: left: *A. flavus*; right: *S. rolfsii*

eleven isolates were found to possess *phlD* genes and produced a band of nearly 745 bp on amplification (Figure 2). Biocontrol fluorescent *Pseudomonas* spp. living in the rhizosphere can protect plants from soil-borne fungal pathogens, and often this involves the production of antimicrobial compounds, such as 2,4-diacetylphloroglucinol (Phl) (Defago and Haas, 1990; keel *et al.* 1990; Weller *et al.* 2002). DAPG inhibits growth of several phytopathogenic fungi including *Thielaviopsis basicola* (tobacco black root rot), *Gaeumannomyces graminis* var. *tritici* (wheat take-all) and *Pythium ultimum* (cotton damping-off) (Howell and Stipanovic, 1980). Besides, DAPG can also act by altering multiple basic cell processes (Kwak *et al*. 2011; Troppens *et al*. 2013). In addition, molecular and statistical analyses have shown the importance of Phl production ability in plant protection against diseases (Couillerot *et al*. 2009).

A total of 11 isolates were selected on the basis of the *phlD* activity (Figure 2) for further studies. All these 11 isolates showed antifungal activities against the major soil-borne fungal pathogens of groundnut.

The 16S rRNA gene of the DAPG-producing fluorescent pseudomonads (DAPG2, DAPG3, DAPG4 and DAPG7) were sequenced to authenticate the identity on the basis of blast search at NCBI site for identifying maximum match with the available 16S rRNA sequence database. On this basis, these isolates were identified as *Pseudomonas putida* DAPG2, *P. putida* DAPG3, *P. putida* DAPG4 and *P. putida* DAPG7 (Table 2). All other DAPG-producing rhizobacterial fluorescent pseudomonads were identified by biochemical tests and using BIOLOG carbon utilization patterns (data not shown). They were identified as *P. fluorescens* FP86, *P. fluorescens* FP20, *P. putida* FP46, *Pseudomonas fluorescens* FP93, *Pseudomonas fluorescens* FP94, *Pseudomonas fluorescens* FP121 and *Pseudomonas aeruginosa* FP 133 (Table 2).

Though plant growth promoting rhizobacteria employ an array of mechanisms for enhancement of plant growth and yield, both direct and/or indirect mechanisms would be involved in such plant-growth promotion by rhizobacteria (Pal *et al.* 2000; Pal *et al.* 2001; Pal *et al.* 2003; Dey *et al.* 2004). One of the mechanisms of plant growth promotion is biocontrol activity. However, several factors are thought to be responsible for the antifungal property of a strain. These include production of siderophore, hydrogen

TABLE 2: Identification and zone of inhibition of selected rhizobacteria against fungal pathogens (after 72 h; dia. in mm) (data mean of three replications; figures in parentheses indicate standard deviation from mean)

Isolate	Identity as per 16S rRNA gene sequence data and biochemical traits	*Aspergillus niger*	*Aspergillus flavus*	*Sclerotium rolfsii*
DAPG2	*Pseudomonas putida*	26.7 (±0.6)	12.7 (±1.5)	17.5 (±0.7)
DAPG3	*Pseudomonas putida*	25.3 (±1.0)	12.0 (±0.0)	14.3 (±0.6)
DAPG4	*Pseudomonas putida*	29.0 (±1.2)	13.0 (±1.0)	15.0 (±0.0)
DAPG7	*Pseudomonas putida*	20.7 (±0.7)	-	14.3 (±0.6)
FP20	*Pseudomonas fluorescens*	13.7 (±3.2)	10.0 (±0.1)	14.3 (±1.5)
FP46	*Pseudomonas putida*	13.0 (±0.0)	13.7 (±2.1)	14.0 (±1.0)
FP86	*Pseudomonas fluorescens*	13.3 (±1.5)	10.0 (±1.0)	11.3 (±0.6)
FP93	*Pseudomonas fluorescens*	12.0 (±1.0)	10.0 (±1.0)	12.7 (±0.6)
FP94	*Pseudomonas fluorescens*	13.0(±1.0)	10.0 (±1.0)	13.3 (±2.1)
FP121	*Pseudomonas fluorescens*	16.5 (±0.0)	13.7 (±0.6)	14.0 (±1.0)
FP133	*Pseudomonas aeruginosa*	16.3 (±4.9)	13.5 (±0.7)	16.0 (±0.0)

TABLE 3: Detection of antifungal/plant-growth promoting factors of selected *phlD*+ groundnut rhizobacteria

Isolates	HCN	IAA	Chitinase	Ammonia	Production of fluorescent pigment on King's B
DAPG2	-	+	-	+	+
DAPG3	+	+	-	+	+
DAPG4	-	+	-	+	+
DAPG7	-	+	-	+	+
FP20	-	+	-	++	+
FP46	-	+	-	-	+
FP86	-	+	-	-	+
FP93	-	+	-	-	+
FP94	-	+	-	-	+
FP121	+	+	-	-	+
FP133	-	+	-	-	+
AMAAS 57	+	+	+	++	+

+ indicates positive activity; - indicates no activity; ++ indicates strong activity

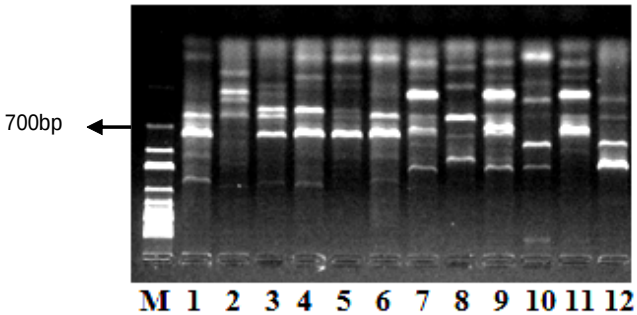

FIG 2: *PhlD* gene amplification of selected fluorescent pseudomonads: M=100 bp ladder; 1=DAPG2; 2=DAPG3; 3=DAPG4; 4=DAPG7; 5=FP20; 6=FP46; 7=FP86; 8=FP93; 9=FP94; 10=FP121; 11=FP133; 12=AMAAS 57

cyanide, antifungal metabolites, chitinase, ammonia, fluorescent pigments, etc. (Pal and Gardener 2006). All the isolates were tested for these characters (Table 3). It was observed that all the selected isolates showed production of IAA in TY broth and fluorescent pigment on King's B medium. Isolates DAPG3 and FP121 also exhibited HCN production. However, none of the 11 isolates showed chitinase activity. The isolates FP20, DAPG2, DAPG3, DAPG4 and DAPG7 produced ammonia. The reference strain AMAAS 57 exhibited multiple characteristics of HCN, IAA, chitinase, ammonia and fluorescent pigment production.

All the selected rhizobacterial isolates were found to produce IAA like substances *in vitro* in TY broth. The IAA production ranged from 3.62 mg/L broth to 17.21 mg/L broth and the maximum production was found in case of isolate FP86, after 72 h of growth. Contrary to this, the standard culture AMAAS 57 produced only 1.72 mg of IAA /L of TY broth (Table 4). *In vitro* screening of the 11 isolates for phosphate solubilization indicated that all the isolates could solubilize tri-calcium phosphate (TCP) onto Pikovskaya's agar (Table 4). The solubilisation zone ranged from 10.6 mm in case of DAPG2 to a maximum of 18.6 mm

TABLE 4: Quantification of IAA production and zone of phosphate solubilization by *phlD*+ groundnut rhizobacteria (data mean of three replications; figures in parentheses indicate standard deviation from mean)

Isolates	IAA (mg/L broth)	P-solubilization zone (diameter in mm) after 72 h at 28°C
DAPG2	9.77 (±0.04)	10.6 (±0.6)
DAPG3	5.40 (±0.04)	17.3(±0.3)
DAPG4	14.39 (±0.01)	18.6(±0.1)
DAPG7	12.11 (±0.03)	15.3(±0.4)
FP20	3.62 (±0.03)	14.6(±0.4)
FP46	13.99 (±0.02)	17.7(±0.6)
FP86	17.21 (±0.07)	15.7(±0.7)
FP93	16.53 (±0.04)	18.3(±0.7)
FP94	13.88 (±0.01)	16.7(±0.3)
FP121	3.97 (±0.02)	14.3(±0.6)
FP133	14.64 (±0.06)	16.7(±0.4)
AMAAS 57	1.72 (±0.07)	19.7(±0.1)

in DAPG4, after 96 h of incubation as compared to the solubilized zone produced by AMAAS 57 (19.7 mm).

Quantification of phosphate solubilization in liquid medium (Table 5) was carried out after 3 days, 6 days and 9 days of incubation. Isolate FP133 was most efficient in TCP solubilisation and solubilized (179.6 mg P_2O_5/100 ml) followed by DAPG4 (206.8 mg P_2O_5/100 ml), FP94 and FP86 (193 mg P_2O_5/100 ml), respectively. The reference culture *Pseudomonas aeruginosa* AMAAS 57 solubilized 177.3 mg P_2O_5/100 ml after 9 days of incubation at 28°C. During phosphate solubilisation pH of the broth also reduced significantly as compared to control. It was found that lowering of pH was more pronounced in case of isolate DAPG2 (4.60) and FP20 (5.04) and in other isolates the pH was around 6.0 after 9 days of incubation (Table 5).

Three of the isolates, i.e., *P. putida* DAPG3, *P. putida* DAPG7 and the standard culture *P. aeruginosa* AMAAS 57 exhibited multiple plant growth promoting attributes. The presence of multiple plant growth promoting

TABLE 5: Quantification of phosphate solubilisation capacity of *phlD*$^+$ groundnut rhizobacteria in liquid medium

Isolates	Incubation (in days)					
	3		6		9	
	Phosphate solubilization (mg P_2O_5/100 ml)					
	pH	P_2O_5	pH	P_2O_5	pH	P_2O_5
Control	7.00	-	7.00	-	6.80	-
DAPG2	5.88	33.5 (±0.10)	4.58	110.1 (±0.02)	4.60	116.3 (±0.11)
DAPG3	5.86	20.0 (±0.04)	6.34	106.7 (±0.13)	5.88	98.9 (±0.05)
DAPG4	4.62	34.7 (±0.03)	4.77	68.2 (±0.08)	4.78	206.8 (±0.03)
DAPG7	6.12	7.3 (±0.03)	6.09	49.1 (±0.06)	6.07	177.3 (±0.03)
FP20	5.16	21.2 (±0.03)	5.15	52.8 (±0.08)	5.04	96.9 (±0.10)
FP46	6.07	27.0 (±0.03)	6.09	158.8 (±0.11)	6.02	174.4 (±0.02)
FP86	5.55	29.1 (±0.03)	5.29	57.4 (±0.05)	5.12	193.0 (±0.21)
FP93	4.60	36.2 (±0.02)	4.68	72.4 (±0.05)	4.94	159.5 (±0.05)
FP94	5.73	21.8 (±0.04)	5.20	44.2 (±0.06)	6.15	193.5 (±0.10)
FP121	6.50	21.0 (±0.02)	6.45	51.0 (±0.03)	6.10	90.6 (±0.02)
FP133	5.75	26.3 (±0.02)	5.06	77.3 (±0.03)	5.07	279.6 (±0.03)
AMAAS 57	6.10	35.9 (±0.02)	6.00	65.8 (±0.07)	5.00	177.3 (±0.20)

Data mean of three replications; figures in parentheses indicate standard deviation from mean

attributes among the DAPG producing strains of groundnut rhizosphere pseudomonads is in consistence with earlier observations reported in *Pseudomonas putida* GR12-2 (Jacobson *et al*. 1994), *Pseudomonas fluorescens* Pf-5, *Pseudomonas fluorescens* 2-79, *Pseudomonas fluorescens* CHA0 (Wang *et al*. 2000), *Pseudomonas fluorescens* PGPR2 (Dey *et al*. 2004). The mechanisms of PGPR mediated enhancement of plant growth and yields may include the production of ACC deaminase (Glick *et al*. 1995); IAA like substances (Patten and Glick, 2002); asymbiotic nitrogen fixation; production of siderophores, α-1,3-glucanase, chitinases, antibiotics, fluorescent pigment, and cyanide (Scher and Baker, 1982; Voisard *et al*. 1989; Renwick *et al*. 1991; Catellan *et al*. 1999); and solubilisation of mineral phosphates and mineralization of other nutrients (De Freitas *et al*. 1997).

The presence of different genes responsible for exhibiting different plant growth promoting traits and biocontrol attributes by different rhizobacteria was evaluated by gene specific primers for PCR amplification of targeted genes. Several genes are responsible for activities like ACC deaminase (*acc*), siderophore (*furA*), chitinase (*chiC*), HCN (*hcnBC*), ammonia (*amoA*), pyoverdine (*fpv*), pyocyanine (*pfe*), IAA (*iaaM*), pyoluteorin (*plt*), DAPG (*phlD* and *phlA*), etc. (Table 6). Gene specific primers as mentioned in the materials and methods (Table 1) were used for PCR amplification of the targeted genes. All the eleven isolates and the standard culture AMAAS 57 were positive for *phlD* activity but only isolate DAPG4 and FP46 exhibited the presence of *phlA* gene. PCR amplification revealed that only four isolates *viz. P. putida* DAPG7, *P. fluorescens* FP93, *P. fluorescens* FP94 and *P. aeruginosa* AMAAS57 had genes for ACC deaminase (using AccF and AccR primers) (Table 6). It was also found that *P. putida* DAPG3, *P. putida* DAPG4, *P. putida* DAPG7, *P. fluorescens* FP20, *P. fluorescens* FP93, *P. fluorescens* FP94 and *P. aeruginosa* AMAAS 57 had genes

TABLE 6: Molecular characterisation of selected DAPG-producing groundnut rhizobacteria

Isolates	Presence of genes									
	acc (700 bp)	*fur* (192 bp)	*chi C* (205 bp)	*hcn* BC (587 bp)	*amo* A (665 bp)	*iaa* M (900 bp)	*plt* (560 bp)	*prn* (740 bp)	*fpv* (1000 bp)	*phl* A (418 bp)
DAPG2	-	-	-	-	+	-	-	-	+	-
DAPG3	-	+	-	+	+	-	-	-	-	-
DAPG4	-	+	-	-	+	-	-	-	-	+
DAPG7	+	+	-	-	+	-	-	-	+	-
FP20	-	+	-	-	+	-	-	-	-	-
FP46	-	-	-	-	-	-	-	-	+	+
FP86	-	-	-	-	-	-	-	-	+	-
FP93	+	+	-	-	-	-	+	-	+	-
FP94	+	+	-	-	-	-	+	-	+	-
FP121	-	-	-	+	-	-	+	-	+	-
FP133	-	-	-	-	-	-	-	-	+	-
AMAAS57	+	+	+	+	+	-	-	-	+	-

for siderophore production (using FurF and FurR primers). The presence of *chiC* could be detected in only the reference strain, *P. aeruginosa* AMAAS 57 by using *chiC* specific primers (chiCF and chiCR). *Pseudomonas putida* DAPG3, *P. fluorescens* FP121 and *P. aeruginosa* AMAAS57 had genes for HCN production (using gene specific primers Aca and Acb). *Pseudomonas putida* DAPG2, *P. putida* DAPG3, *P. putida* DAPG4, *P. putida* DAPG7, *P. fluorescens* FP20 and *P. aeruginosa* AMAAS 57 showed *amoA* activity as confirmed by gene specific primers AMO-F and AMO-R. Though all the isolates tested positive for IAA production *in vitro*, none of the isolates showed *iaaM* activity using iaaMF and iaaMR primers. This could be attributed to non-alignment of the primers used in the *iaa* genes of these organisms. While *P. fluorescens* FP93, *P. fluorescens* FP94 and *P. fluorescens* FP121 showed *plt* activity as confirmed by primers pltF and pltR, none of the isolates produced pyrrolnitrin *(prn*) tested by the primers prnF and prnR. Except *P. putida* DAPG3, *P. putida* DAPG4 and *P. fluorescens* FP20, all other isolates showed presence of *fpv* gene using primers 599F and 599R. Both *P. putida* DAPG4 and *P. fluorescens* FP46 showed *phlA* activity using primers PhlA-1f and PhlA-1r. Fluorescent pseudomonads were reported to produce a number of metabolites including DAPG for suppression of fungal pathogens (Weller *et al*. 1988). PCR amplification of the targeted genes using specific primers confirmed the presence of these genes in these plant growth promoting isolates. The observation was consistent with the earlier observations made with other pseudomonad strains (Sarvanankumar and Samiyappan, 2007; Ahmadzadeh *et al*. 2006; Pacheco *et al*. 2006).

Ahmadzadeh *et al*. (2006) used PCR analysis to detect 2,4-DAPG-producing *Pseudomonas* populations based on the amplification of *phlD* and *phlA* genes sequences. Occurrence of the *phlD* gene was demonstrated through the detection of bands of around 745 bp DNA fragment in three strains P-5, P-32 and P-47 from Iran as well as the control strain CHA0, using a PCR assay with primers phl2a and phl2b. They were also able to demonstrate the occurrence of the *phlA* gene in a DNA fragment of approximately 418 bp in four bacterial strains P-5, P-18, P-34 and P-35 from Iran as well as the control strain CHA0, using primers phlA-1f and phlA-1r. In the present investigation, similar observation has been made. The presence of *phlD* was confirmed in all the 11 isolates through the detection of bands of around 745 bp DNA fragment in these strains. Only with two isolates, *P. putida* DAPG4 and *P. fluorescens* FP46, PCR amplification of 418 bp was obtained for *phlA* gene. This indicated the presence of *phlA* gene in these two strains in addition to *phlD* gene.

The rhizospheric fluorescent pseudomonads described in this study possess multiple plant growth promoting and biocontrol attributes. The biochemical and molecular characterization of the isolates complement each other. This study indicates the potential of the isolated rhizospheric pseudomonads which could be used in future for testing and validation for enhancing growth and yield of groundnut along with the suppression of major soil-borne fungal pathogens. These DAPG-producing fluorescent pseudomonads are being used for amending soils which will become naturally suppressive to soil-borne fungal pathogens of groundnut like *S. rolfsii* and *A. niger* causing stem and collar rot in groundnut, respectively. Application of DAPG-producing fluorescent pseudomonads would be a part of management strategies for combating the menace of soil-borne fungal pathogens of grounndut.

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