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Identification of Fluorescent *Pseudomonas* Isolates with Potential Biocontrol activity from the Rhizosphere of Crops

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Native fluorescent *Pseudomonas* isolates from the rhizosphere of various crops grown in the state of Karnataka in southern India were isolated, identified and screened for their ability to act as biological control agents for economically important fungal plant pathogens. Ninety two *Pseudomonas* isolates were isolated from the rhizosphere soil of different crop plants from the farms of the University of Agricultural Sciences, Raichur (India) and surrounding villages, Dharwad and IIHR, Bangalore using specific medium. All the isolates were found to be gram-negative rods. Among these, 66 isolates produced the characteristic yellow pigmentation and showed fluorescence. The isolates were categorized into four groups based on siderophore production (high, moderate, low and no production) and evaluated for the presence of *pvdA*, *phl* and *plt* genes associated with siderophore production, DAPG and pyoluteorin respectively. None of them appeared to carry the *pvdA* and *plt* genes. However, one isolate RPF-13 was found to carry the *phl* gene based on gene specific PCR results. This DAPG^{+ve} isolate was identified as *Pseudomonas putida* by 16s r DNA sequence analysis. This paper reports the identification of one of the few highly effective DAPG producing *Pseudomonas* isolates from southern India that can be used biological control in addition to the identification and characterization of several other fluorescent *Pseudomonads* with potential to serve as bio-control agents against a number of important pathogenic fungal species.

Keywords: Fluorescent pseudomonads, characterization, 2,4-DAPG, siderophore.

Management of soil-borne pathogens has been a major concern in agriculture. Among bacteria with potential utility in bio-control, fluorescent pseudomonads show promise due to their ability to survive in the rhizosphere and inhibit numerous plant pathogens in diverse environments (Johri *et al* 1997). Certain strains suppress plant diseases by protecting roots from infection by soil-borne pathogens. Recently, considerable attention has been paid to plant growth-promoting rhizobacteria (PGPR), primarily fluorescent pseudomonads, such as *Pseudomonas fluorescens* and *Pseudomonas putida*. Both these species are aggressive root colonizers and play a major role in biological control of plant pathogens (Johri *et al* 1997).

The genus *Pseudomonas* consists of a large group of active bio-control bacterial strains with the general ability to produce antifungal metabolites such as siderophores, hydrogen cyanide (HCN), and proteases, possessing antagonistic activities against plant pathogens. Additionally, *P. fluorescens* is known to produce antibiotics such as phenazine and antimicrobial metabolites such as 2, 4-diacetylphloroglucinol

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(DAPG), pyoluteorin, HCN, and salicylic acid (SA), capable of inhibiting a broad spectrum of plant pathogenic fungi, bacteria, and nematodes (Boruah and Kumar 2002).

In iron-deficient environments, fluorescent pseudomonads such as *P. aeruginosa, P. putida*, and *P. fluorescens*, among others, produce different fluorescent peptidic siderophores, named pyoverdines (Pvd) (Meyer 2000). Siderophores are the low molecular weight compounds that chelate with ferric ions (Fe^{3+}) and get competitive advantage for fluorescent pseudomonads in association with plants. In addition to their role in iron acquisition, pvds also function as extracellular signaling molecules that induce the production of virulence factors in *P. aeruginosa* (Lamont *et al* 2002).

As previously mentioned, the antimicrobial compound DAPG is a major determinant in the biological control activity of a range of plant pathogens by many fluorescent *Pseudomonas* spp. (Fenton *et al* 1992). *PhlA, B, C,* and *D* genes are responsible for the production of DAPG in *P. fluorescens* (Abbas *et al* 2002). Since its distribution in the bacterial community is limited, *phlD* is used as a marker gene to identify DAPG producers. Probes and primers specific for a sequence within *phlD* have been used to monitor the population dynamics of 2, 4-DAPG producers in take-all decline disease-suppressive and -conducive soils (Raaijmakers *et al* 1997) and in rhizospheres of maize (Picard *et al* 2000). McSpadden-Gardener *et al* (2001) cloned and sequenced a major portion of the *phlD* open reading frame from five genotypically different strains and identified a primer pair (B2PF and BPR4) that could be used to detect as few as log 2.4 cells per sample.

The primary objective of the study described here was to collect and characterize fluorescent *Pseudomonas* isolates from the rhizospheres of various crops grown in the North-Eastern Dry Zone of the Indian state of Karnataka which harbors several different soil types (including black cotton and red soils in the drier regions and laterites in the transitional zone of the region) and assess the potential of these and two other previously collected isolates from this region to serve as bio-control agents.

MATERIALS AND METHODS

Isolation and characterization of indigenous fluorescent *Pseudomonas* isolates

Soil samples were collected at the end of the monsoon season (October) from the rhizosphere of numerous crops such as chilli, sunflower, redgram, groundnut, field bean, beans, greengram, egg plant, tomato, sunhemp, and cotton at a soil depth of 15-20 cm from the farms of UAS campus, Raichur (Karnataka) and surrounding farmers fields and also from farms of Dharwad and IIHR, Bangalore. Plants were removed from the soil by hand and 80-100 g of rhizosphere soil was collected along with portions of roots. The collected rhizosphere soil and roots were then transferred to 250 mL conical flasks containing 100 mL sterile distilled water, and were incubated with shaking for approximately 24 hr. Serial dilutions up to 10^{-4}, 10^{-5}, and 10^{-6} were prepared in 1 mL aliquots and approximately 0.1 mL of these suspensions was spread on plates with King's B medium (HIMEDIA, Mumbai, India). The plates were incubated at 28°C for 48 hr and subsequently evaluated for pigment production.

Isolates used

The isolates used in this study included 92 isolates collected from different hosts in the fields of UAS Raichur, Dharwad and another one from the Indian Institute of Horticultural Research, (IIHR) Bangalore, India as well as and two isolates Pf4 and Pf6 which had been isolated from the same region in a previous study (Anand *et al* 2010).

Characterization of the different isolates of fluorescent *Pseudomonas* was performed according to Schaad (1992) using 24 to 48 hr old cultures for each test. Tests used for identification of fluorescent pseudomonas include-

Gram staining

A loop full of bacterial suspension was smeared on to a glass slide in such way to get a thin smear. It was air-dried and heat fixed by passing the slide rapidly two to three times over Bunsen burner flame. The smear was flooded with crystal violet (primary stain) solution for 1 min. The slide was washed with a gentle stream of tap water, blotted dry and flooded with Lugol's iodine (moderant) for 1 min. The slide was washed again with water, blotted dry, and decolorized by rinsing

in a gentle stream of 95 % ethyl alcohol for 30 sec to remove excess stain, counter staining was done by flooding with safranin (secondary stain) for 20 sec. Finally, the slide was washed with tap water, blotted dry and evaluated microscopically for the identification of gram negative or gram-positive according to the pink-red or blue-violet staining respectively.

Pigment production

The bacterial colonies from the King's B plates were streaked on Pseudomonas Agar (HIMEDIA, Mumbai, India) and incubated at room temperature 28 ± 2°C for 48 h. These colonies were subsequently assessed for yellow pigmentation and fluorescence under the ultraviolet light.

Determination of spectrum of plant pathogen suppressed by different indigenous *Pseudomonas fluorescens* isolates

Dual culture test and screening of fluorescent *Pseudomonas* for biocontrol activity

Efficacy of two (Pf-4 and Pf-6) isolates of *Pseudomonas fluorescens* isolated in a previous study by Anand *et al* (2010) was evaluated against a range of pathogens predominant in the region using the dual culture technique described below. The fungal pathogens included in this test using Pf-4 and Pf-6 were *Fusarium solani, Rhizoctonia bataticola*, *Alternaria sesami*, *Colletotrichum gleoesporioides, Xanthomonas axonopodis* pv *punicae, Macrophomina phaseolina, Cercospora capsici* and *Sclerotium rolfsii*. The fluorescent Pseudomonads isolated as a part of this study were only evaluated against *Macrophomina phaseolina*, the dry root rot pathogen of many pulse crops in India.

The bio-control agent and the test fungus were inoculated side by side on a single petri plate containing solidified PDA medium. The bacterial biocontrol agents were streaked one day earlier to the test pathogen. Three replicates were maintained for each isolate with one control containing only the pathogen. The plates were incubated at 28^0 C. Observations were recorded when there was full growth of pathogen in the control plate (6-7 days). The diameter of the colony of the pathogen was measured in both directions and average was recorded. Percent inhibition on growth of the test pathogen was calculated by using the formula I = (C-T)/C x 100, where I is Per cent inhibition, C is Radial growth of the pathogen in control and T is Radial growth of pathogen in treatment given by Vincent (1927).

Evaluation of fluorescent *Pseudomonas* isolates for synthesis of siderophore

Siderophore production was determined by the Chrome Azurol S (CAS) assay Schwyn and Nielands (1987) . The four solutions used to prepare CAS agar were sterilized separately before mixing to produce the final blue-colored medium. A change in color to orange or the presence of a light orange halo surrounding bacterial growth was used to indicate siderophore production by the bacterial isolates. This color change is based on the principle that the blue color of CAS agar medium is due to the iron-dye complex and when siderophores are produced, the iron is released from the complex by chelating with siderophores resulting in an orange color.

Molecular detection of *phl* (DAPG) gene from fluorescent Pseudomonas isolates

DNA was isolated from selected fluorescent Pseudomonas isolates using the HiPurATM Bacterial and Yeast Genomic DNA Purification Spin Kit (HIMEDIA, Mumbai, India). PCR amplification for *phl* (DAPG) and *plt* (pyoluteorin) was performed according to Naik *et al* (2008) and for the *pvdA* (siderophore) gene and it was performed according to Takase *et al* (2000). The procedure involved the use of 25 µL reaction mixtures containing approximately 40 ng of total DNA, 10X PCR buffer, dNTPs (200 µM each), 20 pmol of each forward and reverse primer and 3.0 U of Taq DNA polymerase enzyme (Bangalore Genei, Bangalore, India). PCR products (10µL aliquots) were separated on a 1.5% (w/v) agarose gel with ethidium bromide staining. Sequences of primers used in the study are provided in Table 1.

16s rDNA sequence analysis for identification of *Pseudomonas* species

Amplification of 16s rDNA was completed as described by Naik *et al* (2008). A 5 µL aliquot of amplified product was analyzed on a 0.7%

Table 1. Sequences of primers used in the study

Primer name	Sequence (5' to 3')
fD1	GAGTTTGATCCTGGCTCA
rP2	ACGGCTACCTTGTTACGACTT
Phl2a	GAGGACGTCGAAGACCACCA
Phl2b	ACCGCAGCATCGTGTATGAG

Table 2. Characterization of *Pseudomonas* isolates for fluorescence, siderophore production and antifungal activity against *M. phaseolina*.

Isolate	Rhizosphere source	Fluorescent (F) or Non-fluorescent (NF)	Siderophore production*	Antifungal activity against *M. phaseolina* (% inhibition)
Pf-1	Chilli	F	-	31-40
Pf-2	Chilli	F	-	0-10
Pf-3	Chilli	F	+	31-40
Pf-4*	Chilli	F	+	11-20
Pf-5	Chilli	F	+	21-30
Pf-6*	Chilli	F	-	11-20
Pf-7	Chilli	F	-	0-10
Pf-8	Chilli	F	+	31-40
Pf (PGPR)	Chilli	F	-	21-30
Pf (RCH)	Chilli	F	-	11-20
Pf (m)	Chilli	F	+	31-40
RPF-1	*Cynodon dactylon*	NF	-	11-20
RPF-2	*Cynodon dactylon*	NF	-	21-30
RPF-3	Ground nut	F	+	31-40
RPF-4	Ground nut	F	+	21-30
RPF-5	Ground nut	F	+	11-20
RPF-6	Chilli	F	+	11-20
RPF-7	Chilli	F	+	31-40
RPF-8	Greengram	F	+	31-40
RPF-9	Greengram	F	+++	21-30
RPF-10	Greengram	NF	-	11-20
RPF-11	Field bean	F	-	31-40
RPF-12	Field bean	F	+	11-20
RPF-13	Brinjal	F	+	>40
RPF-14	Field bean	F	+	21-30
RPF-15	Field bean	F	+	21-30
RPF-16	Field bean	F	+	11-20
RPF-17	Field bean	F	++	11-20
RPF-18	Field bean	F	-	21-30
RPF-19	Field bean	F	++	11-20
RPF-20	Field bean	F	+++	0-10
RPF-21	Field bean	NF	-	11-20
RPF-22	Field bean	F	++	11-20
RPF-23	Field bean	F	++	11-20
RPF-24	Field bean	F	++	0-10
RPF-25	Tomato	NF	+	11-20
RPF-26	Tomato	F	+++	11-20
RPF-27	Tomato	F	+++	31-40
RPF-28	Tomato	F	+++	31-40
RPF-29	Ground nut	NF	-	11-20
RPF-30	Sesame	F	+++	11-20
RPF-31	Tomato	F	+	31-40
RPF-32	Tomato	F	+	11-20
RPF-33	Tomato	F	+	11-20
RPF-34	Chilli	F	-	11-20
RPF-35	Bean	F	-	21-30
RPF-36	Bean	F	+	11-20
RPF-37	IIHR, Bangalore	NF	-	0-10

RPF-38	Bean	F	+	11-20
RPF-39	Bean	F	-	11-20
RPF-40	Bean	F	-	0-10
RPF-41	Bean	F	+	0-10
RPF-42	Sorghum	F	+	11-20
RPF-43	Sorghum	F	+	0-10
RPF-44	Sorghum	F	-	11-20
RPF-45	Okra	F	+	21-30
RPF-46	Okra	F	+	11-20
RPF-47	Okra	F	-	11-20
RPF-48	Egg Plant	F	-	21-30
RPF-49	Egg Plant	F	+	11-20
RPF-50	Egg Plant	F	+	31-40
RPF-51	Field bean	F	+	11-20
RPF-52	Egg Plant	F	+	21-30
RPF-53	Egg Plant	F	+	11-20
RPF-54	Egg Plant	F	++	21-30
RPF-55	Sunflower	F	+	11-20
RPF-56	Pigeon pea	NF	+	0-10
RPF-57	Paddy	NF	-	21-30
RPF-58	Paddy	NF	-	11-20
RPF-59	Paddy	NF	+	0-10
RPF-60	Paddy	NF	+	11-20
RPF-61	Paddy	NF	-	21-30
RPF-62	Paddy	NF	+	0-10
RPF-63	Paddy	NF	-	11-20
RPF-64	Paddy	NF	-	11-20
RPF-65	Paddy	NF	-	11-20
RPF-66	Paddy	NF	-	11-20
RPF-67	Paddy	NF	-	11-20
RPF-68	Paddy	NF	+	0-10
RPF-69	Paddy	NF	-	0-10
RPF-70	Paddy	NF	-	11-20
RPF-71	Paddy	NF	-	21-30
RPF-72	Paddy	F	-	31-40
RPF-73	Paddy	NF	-	21-30
RPF-74	Paddy	NF	-	11-20
RPF-75	Paddy	NF	+	11-20
RPF-76	Groundnut	F	++	21-30
RPF-77	Pigeon pea	F	-	21-30
RPF-78	Pigeon pea	F	+	0-10
RPF-79	Paddy	F	+	11-20
RPF-80	Sunflower	F	-	21-30
RPF-81	Chilli	F	+	0-10

*<2 mm orange zone = + (low producer); 2-4 mm orange zone = ++ (moderate producer); >4 mm orange zone = +++ (high producer); - = No siderophore production,
*Initially reported by Anand et al., 2010

(w/v) agarose gel which was stained with ethidium bromide. PCR products were purified using a commercial kit (Sigma-Aldrich, Bangalore, India) and then sequenced by Bangalore Genei, Bangalore, India.

RESULTS AND DISCUSSION

Isolation and characterization of fluorescent *Pseudomonas* isolates

Ninety two bacterial isolates collected from the rhizosphere of different crops were

Table 3. Inhibition of mycelial growth of plant pathogens by *Pseudomonas fluorescens* as determined by a dual culture

Pathogens	% of mycelial growth inhibition over control	
	Pf-4	Pf-6
Macrophomina phaseolina	21.30 (27.35)	14.44 (22.26)
Rhizoctonia bataticola	24.07 (28.59)	23.33 (28.82)
Rhizoctonia solani	32.96 (34.83)	26.85 (30.94)
Fusarium oxysporum f. sp. *Udum*	48.14 (43.93)	46.30 (42.85)
Colletotrichum gloeosporioides	41.67 (39.87)	32.22 (34.57)
Fusarium solani	24.07 (29.34)	31.48 (34.10)
Sclerotium rolfsii	40.74 (39.61)	11.11 (19.06)
Cercospora capsici	21.67 (27.63)	21.48 (27.53)
Alternaria sesame	20.00 (26.46)	19.26 (25.53)
Mean	30.51	25.16

	Pathogen	*P. fluorescens* isolates	Interaction
S.E±	10.73	8.31	4.79
CD at 1%	40.37	31.27	18.05

Figures in the parenthesis are arc sine transformed values.

characterized, all isolates were found to be Gram-negative and rod shaped. Sixty six isolates produced pigment on the Pseudomonas Agar medium and also fluoresced under UV light. The source, designation, and characterization of the different *Pseudomonas* isolates used in this study are presented in Table 2.

Determination of the spectrum of bio-control activity of fluorescent Pseudomonas isolates against different plant pathogens

It has been reported that isolates Pf-4 and Pf-6 possess good bio-control activity and induced high systemic resistance to Fusarium wilt in chilli Anand *et al* (2010). Therefore, the efficacy of Pf-4 and Pf-6 isolates against a range of pathogens such as *Fusarium solani* f. sp. *udum, Rhizoctonia bataticola*, *R. solani, Alternaria sesami Colletotrichum gloeosporioides, Xanthomonas axonopodis* pv. *punicae, Macrophomina phaseolina, Cercospora capsici,* and *Sclerotium rolfsii* was evaluated using a dual culture technique. Both these isolates were found to be capable of inhibiting the growth of the different pathogens used in this study though to varying degrees. Overall, the Pf4 isolate was found to be more effective than the Pf 6 isolate. The results of the dual culture technique are presented below. (Table 3, Figure 1)

Evaluation of fluorescent *Pseudomonas* isolates for siderophore production

A scale based on the size of the orange zone created due to siderophore production was developed to determine the ability of fluorescent *Pseudomonas* isolates for siderophore production on the CAS agar medium, among 92 isolates obtained in this study, 6 isolates were observed to be high producers of siderophore (>4 mm orange zone surrounding bacterial growth), 7 isolates were moderate producers (2-4 mm orange zone), 41 isolates were low producers (<2 mm orange zone), and 38 isolates did not produce any siderophore. (Table 2 and Figures 2 and 3). These variations in the ability to produce siderophores is consistent with earlier reports (Dave and Dube 2000; Yeole *et al* 2001).

Molecular detection of 2, 4-DAPG gene from indigenous fluorescent *Pseudomonas* isolates

Initial screening of fluorescent *Pseudomonas* isolates obtained in this study for their bio-control activity was performed against

M. phaseolina using the dual plate technique. Among all isolates tested, 38 were observed to reduce mycelial growth by 25 to 44% under *in vitro* conditions (Table 2). Further, to assess their ability to produce DAPG, genomic DNA of 38 fluorescent *Pseudomonas* spp. isolates demonstrating 25 to 44% reduction in mycelia growth of *M. phaseolina* were analyzed for the presence of *phlD*. The results of the PCR analysis conducted using primers *Phl2a* and *Phl2b* led to the amplification of a DNA fragment of the desired size (approximately 745 bp) from only one isolate, RPF-13 which had been isolated from the rhizosphere of egg plant (Figure 4). This isolate was also the one that showed maximum inhibition of mycelial growth in the *in-vitro* assays (Table 2). The fact that only one isolate tested positive for the presence of *phlD* is, perhaps, expected based on what others have observed previously. The presence of DAPG-producing *Pseudomonas* spp. was first reported by Velusamy *et al* (2006) where only 27 out of the 637 strains isolated were found to produce 2, 4-DAPG. Moreover, out of 47 isolates assessed by Ahmadzadeh *et al* (2006) only three isolated *Pseudomonas* were found to produce DAPG. These studies highlight the fact that the occurrence of DAPG producers in Indian soils is very low. This study is the one of the report in which *phlD* was detected in an isolate of fluorescent pseudomonad from a particular ecological region of Raichur.

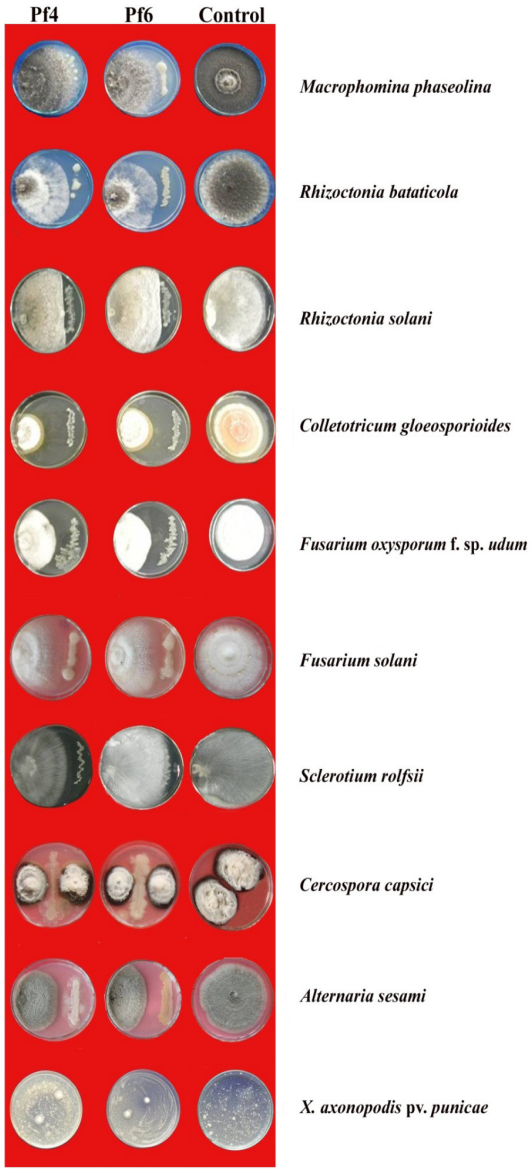

Fig. 1. Spectrum of pathogens inhibited by *Pseudomonas fluorescens* isolates

16s rDNA sequence analysis

A 1316 bp fragment of the 16S rDNA gene of RPF-13 was amplified using primers fD1 and rP2 (Bangalore Genei, Bangalore, India). The PCR products were purified and sequenced. The resulting sequence was compared to sequences in the GenBank using BLASTN and the RPF-13 isolate was identified as *P. putida* on the basis of 16S rRNA sequence homology. The sequence of the 16S rDNA is provided below and has been deposited in the Genbank (accession number HM439957).

CONCLUSION

This article is one of the reports of an indigenous fluorescent *Pseudomonas* isolate that can be effectively used for bio-control of pathogenic fungal species in a largely agricultural region of India where the use of chemicals is rampant. This would provide growers in the region an invaluable tool for disease control in a number of economically important crops. Further characterization of the phenotypic and biochemical properties of the anti-microbial compounds produced by this isolate are in progress and need to be completed prior to commercialization.

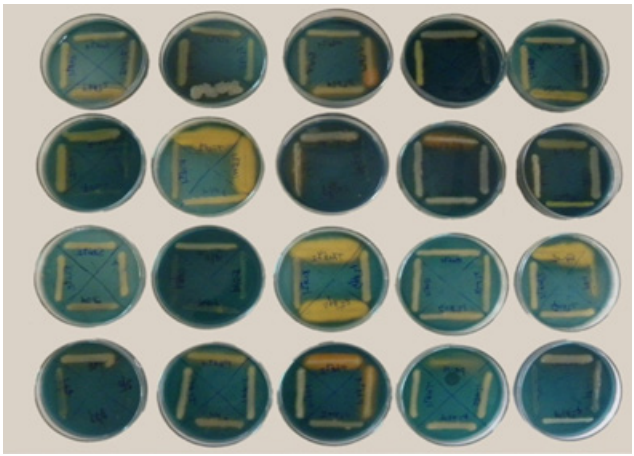

Fig. 2. Siderophore production by Flurescent Pseudomonas isolates on CAS agar medium

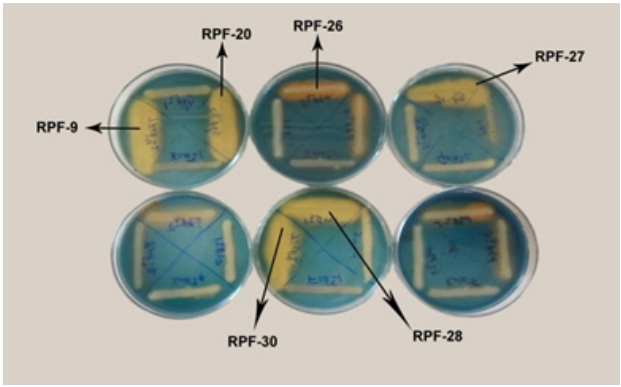

Fig. 3. Six fluorescent Pseudomonas isolates producing more Siderophore on CAS agar medium

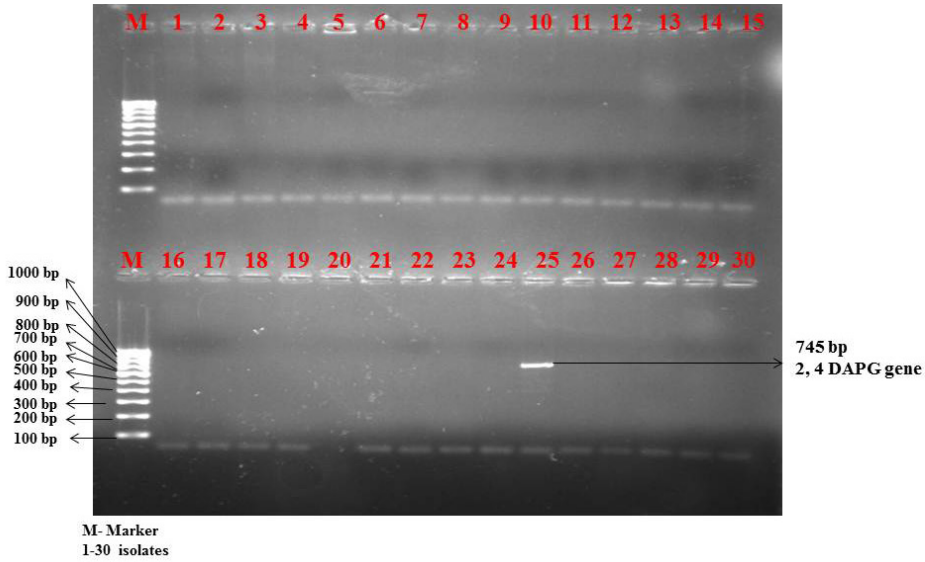

Fig. 4. Amplification of the DAPG gene in the RPF13 isolate

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