*Eco. Env. & Cons. 20 (3) : 2014; pp. (1031-1035)*
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*ISSN 0971–765X*

# Antagonistic activity and molecular characterization of local isolates of fluorescent *Pseudomonads*

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(Received : 7 February, 2014; accepted 15 March, 2014)

**ABSTRACT**

Plant growth promoting rhizobacterial isolates belonging to fluorescent *Pseudomonads* were isolated from the rhizosphere of redgram and maize. Among 15 isolates that were confirmed a *Pseudomonas fluorescens* three exhibited strong antifungal activity against *Macrophomina phaseolina*. Genotyping of these *P. fluorescens* isolates was made by PCR-RAPD analysis.

***Key words :*** *Fluorescent pseudomonads, Antagonistic activity, Molecular, Characterization*

*Pseudomonads* which are present predominantly in the soil have the ability to colonize rhizosphere of a wider variety of crops including cereals, pulses, oil seeds and vegetables (Johri *et al.*, 1997). These properties collectively lead to suppression of pathogens and help in improvement of crop yields. This study focuses attention on the exploitation of a fluorescent *Pseudomonas* as a potent biological control agent for an important soil-borne plant pathogen *Macrophomina phaseolina*, the incitant of charcoal stalk rot of maize. The important antibiotic compounds which contribute to biocontrol activities have been demonstrated to the 2,4-diacetylphloroglucinol (DAPG), pyoluteorin, phenazines, pyrrolnitrin, cyclic lipopeptides and hydrogen cyanide (Haas and Keel, 2003 and Haas and Defago, 2005). The diversity of *Pseudomonads* present in the soil also depends on the crop in the field and the type of metabolites that produce to arrest the growth of pathogens. Further differentiation within the same class of *Pseudomonads* based on the chemical component can be done with the help of molecularmarkers.

Many molecular methods are used to detect the presence of soil borne pathogens and also to assess the genetic variability among the different isolates (Mavrodi *et al.* 2001 and Kumar *et al.* 2002). Application of molecular marker techniques has been useful for studying the genetic changes in the pathogen populations. In another study, Picard *et al.* (2000) differentiated 64 genotypes based on random amplified polymorphic DNA (RAPD) from a single ARDRA group of phlD-containing isolates from the roots and rhizosphere of maize.

## Materials and Mehods

### Isolation of fluorescent *Pseudomonads*

Fluorescent *Pseudomonads* were isolated from the rhizosphere of redgram and maize plants using King's B medium as described by Vidhyasekaran and Muthamilan (1995).

### *In vitro* antibiosis

Fifteen isolates of fluorescent *Pseudomonads* grown on pseudomonas agar plates for two days at 28 ± 2$^0$ C. Loopful of bacterial suspension was streaked on the Potato dextrose agar plate, which was

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preinoculated with mycelial discs of test pathogen at one side. In the control, plate only mycelial disc was placed in the center without bacteria.

The assay plates were incubated at 28 ± 1° C for four days and observations were made on inhibition of mycelial growth of the test pathogens. Three replicates with suitable controls were maintained for each test and per cent inhibition over control was calculated by using the formula:

$$I = \frac{C - T}{C} \times 100$$

Where,

I = Per cent growth reduction of test pathogen

C = Radial growth of test pathogen in control (mm)

T = Radial growth of test pathogen in treatment (mm)

Note: The percent inhibition in control was taken as zero per cent

**Molecular characterization**

***Chemicals***

All the chemicals used in this study were of molecular biology grade. Tris, CTAB, Proteinase K and Agarose were obtained from Sigma Chemical Co. (USA), dNTPs from GE Healthcare (USA) and Taq Polymerase from Genei (Bangalore, India). Standard solutions and buffers were prepared according to the procedures given by Ausubel *et al.* (1999).

***Bacterial genomic DNA isolation***

Genomic DNA was isolated following the method of Ausubel *et al.* (1999). DNA samples were quantified by running on agarose gels along with standard DNA and staining with ethidium bromide. Samples were mixed with appropriate amount of 6X loading dye and electrophoresis on 0.8% agarose gel along with varying concentrations of λ DNA (New England Biolabs, USA). The ethidium bromide stained gels were placed on a UV transilluminator (Syngene, USA) and visual comparisons were made with the standards to estimate the DNA concentration in samples.

***RAPD analysis***

RAPD analysis was performed following the method of Williams *et al.* (1990) with necessary modifications. A total number of 20 primers (OPL) supplied by Operon Technologies, USA were used in this study. Genomic DNA (25-50 ng/µL) of the *P. fluorescens* isolated was used as template and PCR amplification was performed in a 20 µL reaction mixture containing 2 µL template, 2 µL of 10X PCR buffer, 2mM $MgCl_2$, 0.2mM dNTPs, 1 µL (10 pmol) RAPD primer, 1 U Taq Polymerase (Genei, Bangalore). PCR reaction was carried out in a DNA thermocycler (Eppendorf, Germany) with a heated lid. The amplification conditions were as follows: Initial denaturation at 94° C for 4 min followed by 35 cycles of denaturation at 94° C for 1 min, annealing at 37° C for 2 min and extension at 72° C for 2 min. A final extension at 72° C was carried out for 8 min. After PCR, the samples were loaded onto a 1.5% agarose gel along with a standard marker of 100bp (New England Biolabs, USA)

***Data analysis***

The experiments were repeated a minimum of three times to confirm the banding pattern and only those consistent bands on the gels were scored for data analysis. The gels were scored for the presence (1) or absence (0) of the corresponding band in the different local isolates. A score of '1' was given for the presence and '0' for the absence of bands. The binary data generated was analyzed for genetic similarity using unweighted pair group arithmetic mean (UPGMA) program of Ntsyspc software, v. 2.1. The dendrograms obtained served as the basis for assessing the genetic relatedness of the *P. fluorescens* strains within the species.

## Results and Discussion

**Isolation of fluorescent *Pseudomonads***

Fifty soil samples were collected from different places of Rangareddy district for the isolation of fluorescent pseudomonads. The soil samples were mainly collected from the rhizosphere of redgram and maize crop plants. After collection of soil samples, the bacterial isolates were obtained by following the dilution plate technique on *Pseudomonas* agar. Out of thirty isolates, fifteen (nine from redgram and six from maize) were identified as fluorescent pseudomonads based on their morphological, cultural characters and fluorescence nature under UV- light which were labelled as RPF-1 to RPF-9 and MPF-1 to MPF-6. All the isolates produced different kinds of fluorescent pigments on Pseudomonas agar and they were found to be

Gram-negative. Production of fluorescent pigments by the strains of pseudomonads was reported by King *et al.* (1954). Jayashree *et al.* (2000), isolated fluorescent pseudomonads from the rhizosphere of blackgram, carrot, banana, pepper, rice and forest trees grown in several geographical areas of Tamil Nadu and confirmed them based on the fluorescent colonies by viewing under UV-light. Shivani *et al.* (2005), reported that, ten strains of fluorescent pseudomonads were isolated from the rhizosphere of sunflower, potato, maize and groundnut. Which were confirmed as fluorescent *Pseudomonads* by observing them under UV-light and also by the production of fluorescent pigments.

### *In vitro* antibiosis

The data on the effect of fluorescent pseudomonads on the radial growth of *Macrophomina phaseolina* is presented in Table 1. Three isolates of fluorescent *Pseudomonads* (PF) viz., RPF-5, MPF-6 and RPF-8 formed inhibition zone against the test pathogen, the remaining PF isolates did not produce zones of inhibition and the pathogen has over grown on the bacterial colony.

**Table 1.** Antagonistic potential of fluorescent *Pseudomonas* isolates on the radial growth of *Macrophomina phaseolina* under *in vitro* conditions.

| S. No. | Isolates | *Inhibition zone (mm) | *Percent inhibition |
|---|---|---|---|
| 1. | RPF-1 | 00 | 27.78 (31.79) |
| 2. | RPF-2 | 00 | 25.92 (30.58) |
| 3. | RPF-3 | 00 | 25.18 (30.10) |
| 4. | RPF-4 | 00 | 27.41 (31.55) |
| 5. | RPF-5 | 7.66 | 40.00 (39.21) |
| 6. | RPF-6 | 00 | 26.66 (31.07) |
| 7. | RPF-7 | 00 | 23.70 (29.11) |
| 8. | RPF-8 | 6.66 | 38.89 (38.56) |
| 9. | RPF-9 | 00 | 26.66 (31.07) |
| 10. | MPF-1 | 00 | 24.44 (29.61) |
| 11. | MPF-2 | 00 | 28.52 (32.26) |
| 12. | MPF-3 | 00 | 27.41 (31.55) |
| 13. | MPF-4 | 00 | 28.15 (32.03) |
| 14. | MPF-5 | 00 | 26.66 (31.07) |
| 15. | MPF-6 | 7.00 | 39.26 (38.77) |
| 16. | Control | 00 | 00 (0.00 ) |
| (SEm±) | | | 0.42 |
| CD 0.05 | | | 1.22 |

*Mean of the 3 replications
Figures in the parenthesis are angular transformed values.

Fifteen PF isolates screened against *Macrophomina phaseolina*, exhibited significant difference in their inhibition capability. The isolate RPF-5 recorded the highest percentage of inhibition (40.00%) of test pathogen compared to the control plate, with 7.66 mm of inhibition zone against pathogen followed by MPF-6 with 39.26 per cent (7mm inhibition zone) and RPF-8 with 38.89 per cent inhibition. Though the per cent inhibition of the pathogen is significantly higher in the isolates RPF-5, it was on par with RPF-8 and MPF-6.

The findings of present investigation are in agreement with the findings of Sendhilvel *et al.* (2005) who screened five isolates (SVPF1, SVPF2, SVPF3, SVPF3, CPPF1 and CPPF2) of *Pseudomonas fluorescens* against *M. phaseolina*, the causal organism of cowpea root-rot, and it was reported that SVPF2 isolate, showed the maximum inhibition of mycelia growth compared to the control. Further, these findings are also in conformity with Gupta *et al.* (2001), who isolated fluorescent *Pseudomonas* GRC2 from potato rhizosphere and showed necrotrophic antibiosis against two major plant pathogens *Macrophomina phaseolina* and *Sclerotium rolfsii*. Paramasivan *et al.* (2007) also reported that, *P. fluroscens* isolate inhibited the mycelial growth of *Macrophomina phaseolina* with 58.7 per cent, when compared to control under *in vitro* conditions.

### Molecular diversity of fluorescent *Pseudomonas* isolates

The present investigation was carried out to analyze the genetic diversity of fluorescent *Pseudomonas* isolates to reveal their polymorphism using PCR-based RAPD technique.

Twenty OPL series primers were used for appropriate amplification. Banding pattern was observed on 1% agarose gel electrophoresis among twenty primers, out of which eight primers were selected to test the repeatability of the method.

Genomic DNA of fifteen isolates of fluorescent *Pseudomonads* was analyzed and the results revealed cent per cent polymorphism with all the primers. Screening of the entire set of samples was done twice to assess the repeatability of the RAPD profiles and identical patterns were obtained.

The percentage of polymorphism observed for each isolate with all the primers is calculated, which is cent per cent for all the isolates, individually. The primers which gave maximum number of polymorphic bands are OPL-3 & OPL-1 with 17 bands (Table

2) and the primer with minimum number of polymorphic bands is OPL-13 with only nine bands. The number of bands generated with all the primers ranged from 9 to 17. The number of loci and polymorphic bands vary widely between primers and isolates. A high level of genetic diversity was revealed when cluster analysis was carried out with fluorescent *Pseudomonas* isolates based on similarity matrix using Ntsys version 2.1

The Dendrogram (Fig. 1) analysis revealed that, at 61% similarity level 6 clusters were distinguished with 15 isolates of fluorescent *Pseudomonas* by RAPD using twenty primers (OPL series). All the genotypes were divided into two major classes at 55% similarity level. In the 1st major class RPF-1, RPF-6, RPF-8, RPF-9, MPF-1, MPF-3, RPF-7 and MPF-2 formed three clusters. RPF-1 and RPF-6 belonged to one cluster, RPF-7 and MPF-2 belonged to one cluster. In the 2nd major class isolate RPF-5 formed a separate major subgroup with 59 per cent similarity coefficient. RPF-5 was best isolate in the present study, it has the ability of antagonistic activity against *Macrophomina phaseolina*. Another best isolate MPF-6, it belongs to 2nd cluster. Then RPF-2 formed a separate one with 61 per cent similarity coefficient. 2nd Cluster consists of five isolates. In that MPF-4 and MPF-5 are almost similar. RPF-8 also considered the best isolate because it also inhibited the growth of the pathogens. RPF-8 belongs to 5th cluster. RPF-2 was separate one in the 2nd major cluster. At 55 per cent similarity coefficient, all the fifteen isolates are genetically related.

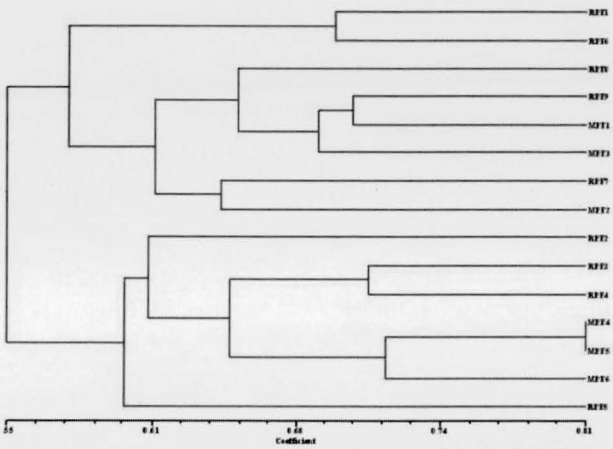

**Fig. 1.** Dendrogram of fifteen fluorescent *Pseudomonas* isolates

Moura *et al.* (2008) analyzed 15 non-fluorescent *Pseudomonad* strains by RAPD using 19 primers. Numerical analysis of the RAPD band patterns allowed the differentiation of two major groups corresponding to *P. corrugate* and *P. mediterranea* isolates sharing approximately 50% similarity. At 80% similarity level, 4 clusters were distinguished. Cluster II, the largest one, had 6 strains closely related to *P. corrugate* type strain. Cluster III, included 5 strains closely related to *P. mediterranea* type strain. Cluster I was represented by 2 strains shared 65% similarity with Cluster II, which contained the *P. corrugate* type strain. One unique strain that showed a slightly different banding pattern from the rest of the *Pseudomonas* strains was alone in Cluster IV. The 19 primers used in the RAPD study provided evidence for the great heterogeneity with in *P. corrugata* and *P. mediterranea* species with the presence of divergent strains.

DNA amplification products obtained from PCR analysis using random primers was proposed as an alternative method in targeting DNA sequences for genetic characterization and mapping (Williams *et al.*, 1990). Nandakumar *et al.* (2002) reported that, *Pseudomonas* spp. isolated from different geographical locations presented more differences than simi-

**Table 2.** Level of polymorphism among 15 isolates of fluorescent *Pseudomonas* with RAPD Primers

| Primer code | Primer sequence | Total bands | Polymorphic bands | Percentage of polymorphism |
|---|---|---|---|---|
| OPL-1 | GGCATGACCT | 17 | 17 | 100 |
| OPL-2 | TGGGCGTCAA | 16 | 16 | 100 |
| OPL-3 | CCAGCAGCTT | 17 | 17 | 100 |
| OPL-4 | GACTGCACAC | 14 | 14 | 100 |
| OPL-5 | ACGCAGGCAC | 14 | 14 | 100 |
| OPL-7 | AGGCGGGAAC | 14 | 14 | 100 |
| OPL-8 | AGCAGGTGGA | 16 | 16 | 100 |
| OPL-10 | TGGGAGATGG | 11 | 11 | 100 |
| OPL-13 | ACCGCCTGCT | 9 | 9 | 100 |

larities in their electrophoretic pattern of total cell proteins. In another study conducted by Reddy *et al.* (2004) on genotyping of antifungal compounds producing *Pseudomonas fluorescens* strains, it was reported that, molecular analysis using PCR based RAPD technique is useful in differentiating fluorescent Pseudomonads at intraspecific level.

Prasanna reddy and Rao (2009) isolated PGPR strains belonging to fluorescent pseudomonads from the rhizosphere of rice. Thirty isolates that were confirmed as *P. fluorescens*, were characterized by PCR-RAPD analysis and biochemical methods. Charan *et al* (2011) isolated fifteen isolates of *P. fluorescens* from the soil rhizosphere and other sources with a view to use them as biological control agents for different crops. The genetic diversity of *P. fluorescens* strains was assessed by two PCR- based molecular techniques, RAPDs and Rep-PCR. Both methods effectively assessed the diversity among the *P. fluorescens* isolates.

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