13. Rao and Williams, The ICRISATS Sorghum Workshop, ICRISAT, Hyderabad, 6–13 March 1977.
14. Travis, W. D., Known-Chung, K. J., Kleiner, D. E., Geber, A., Lawson, W., Pass, H. I. and Henderson, D., *Hum. Pathol.*, 1991, **22**, 1240–1248.
15. Halwig, J. M., Brueske, D. A., Greenberger, P. A., Dreissin, R. B. and Sommes, H. M., *Am. Rev. Respir. Dis.*, 1985, **132**, 186–188.
16. Chandrashekar, A., Bandyopadyay, R. and Hall, A. J., Proceedings of an International Consultation, ICRISAT, Hyderabad, 18–19 May 2000.
17. Huang, H. C. and Hoes, J. A. C., *Can. J. Bot.*, 1976, **54**, 406–410.
18. Sneath, P. H. A., in *Bergey's Manual of Systematic Bacteriology* (eds Sneath, P. H. A. *et al.*), Williams and Wilkins, Baltimore, 1986, vol. 2, pp. 1104–1207.
19. Barrows-Broaddus, J. and Kerr, T. J., *Can. J. Microbiol.*, 1981, **27**, 20–27.
20. Broekaert, W. F., Franky, R. G., Terras, Bruno, P. A, Cammue and Jos, *FEMS Microbiol. Lett.*, 1990, **69**, 55–60.
21. Frandberg, E. and Schnurer, J., *Can. J. Microbiol.*, 1998, **44**, 121–127.
22. Bradford, M. M., *Anal. Biochem.*, 1976, **72**, 248–250.
23. Duvick, J. P., Rood, T., Rao, A. G. and Marshak, D. R., *J. Biol. Chem.*, 1992, **267**, 18814–18820.
24. Teather, R. M. and Wood, P. J., *Appl. Environ. Microbiol.*, 1982, **43**, 777–780.
25. Chet, I., Ordentlich, A., Shapira and Oppenheim, A., *Plant Soil*, 1990, **129**, 85–92.

Genotyping of antifungal compounds producing plant growth-promoting rhizobacteria, *Pseudomonas fluorescens*

N. Ramesh Kumar, V. Thirumalai Arasu and P. Gunasekaran*

Center for Advanced Studies in Functional Genomics, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India

Plant growth-promoting rhizobacterial strains belonging to fluorescent pseudomonads were isolated from the rhizosphere of rice and sugarcane. Among 40 strains that were confirmed as *Pseudomonas fluorescens*, 18 exhibited strong antifungal activity against *Rhizoctonia bataticola* and *Fusarium oxysporum*, mainly through the production of antifungal metabolites. Genotyping of these *P. fluorescens* strains was made by PCR–RAPD analysis, since differentiation by biochemical methods was limited.

PLANT growth-promoting rhizobacteria (PGPR) improve plant growth in two different ways, directly or indirectly.

*For correspondence. (e-mail: pguna@eth.net)

The direct promotion of plant growth by PGPR is through production of plant growth-promoting substances[1] or facilitation of uptake of certain nutrients from the soil[2]. On the other hand, PGPR can also prevent the proliferation of phytopathogens and thereby support plant growth. One of the mechanisms involved here is through their ability to produce siderophores for sequestering iron[3]. The secreted siderophores binds to the Fe^{3+} that is available in the rhizosphere, and thereby effectively prevent growth of pathogens in that region. For example, *Pseudomonas fluorescens* belonging to the PGPR class produces siderophores and control *Pythium ultimum*[4]. Some other PGPR synthesize antifungal antibiotics, e.g. *P. fluorescens* produces 2,4-diacetyl phloroglucinol, which inhibits growth of phytopathogenic fungi[5]. Certain PGPR degrade fusaric acid produced by *Fusarium* sp. causative agent of wilt and thus prevents the pathogenesis[6]. Some PGPR can also produce enzymes that can lyse fungal cells. For example, *Pseudomonas stutzeri* produces extracellular chitinase and laminarinase which could lyse the mycelia of *Fusarium solani*[7]. In recent years, fluorescent *Pseudomonas* has been suggested as potential biological control agent due to its ability to colonize rhizosphere and protect plants against a wide range of important agronomic fungal diseases such as black root-rot of tobacco[8], root-rot of pea[9], root-rot of wheat[10], damping-off of sugar beat[11,12], etc.

Soil samples were collected from 31 different locations representing rhizosphere of mainly rice and sugarcane around Madurai. The rhizosphere samples were plated on King's B-medium (*Pseudomonas* isolation agar medium, Hi-Media, Mumbai) and the plates were incubated at 37°C for 24 h. Colonies that fluoresced under UV light ($\lambda = 356$ nm) were selected and further purified on the same medium. Among the isolates, 40 were confirmed as *P. fluorescens* based on biochemical tests such as arginine hydrolysis, catalase activity, production of fluorescing compounds, gelatin liquefaction and growth at 4°C and 42°C. Plant growth-promoting properties of the selected 18 strains were confirmed with their ability to produce indole acetic acid, phosphate solubilization and inhibition of fungal phytopathogens.

Preliminary screening for antifungal activity was performed on PDA medium without supplementation of $FeCl_3$. Under this condition, fungal growth inhibition could be either due to production of siderophores or antifungal metabolites. Pathogen used were *Fusarium oxysporum* Fox1 and *Rhizoctonia bataticola* Rba1 (obtained from Tamil Nadu Agricultural University, Coimbatore) and antagonism was tested on PDA with and without $FeCl_3$ (100 μg ml^{-1}).

An agar plug (4 mm dia) taken from an actively growing fungal culture was placed on the surface of the PDA plate. Simultaneously, *P. fluorescens* strains were streaked 3 cm away from the agar plug at sides towards the edge of petri plates. Plate inoculated with fungal agar

Table 1. Comparison of antifungal activity of *Pseudomonas fluorescens* strains on *R. bataticola* and *F. oxysporum* in the presence and absence of FeCl₃

Strain	R. bataticola		F. oxysporum	
	Presence of FeCl₃	Absence of FeCl₃	Presence of FeCl₃	Absence of FeCl₃
JAI1	+++ (12)	+++ (12)	+ (2)	+ (2)
JAI2	+++ (11)	+++ (11)	+ (2)	+ (2)
JAI3	–	+++ (13)	–	++ (10)
JAI5	+++ (12)	+++ (12)	+ (3)	+ (3)
JAI6	+++ (12)	+++ (12)	+ (4)	+ (4)
JAI8	+++ (14)	+++ (14)	+ (4)	+ (4)
JO5	–	–	++ (9)	++ (9)
MKU3	+++ (11)	+++ (11)	–	–
PAON2	+++ (12)	+++ (12)	+++ (13)	+++ (13)
PAON3	+ (5)	+ (5)	+ (3)	+ (3)
PNC1	+++ (11)	+++ (11)	+ (3)	+ (3)
PUJA1	+++ (14)	+++ (14)	+++ (11)	+++ (11)
PUJA3	+++ (11)	+++ (11)	+++ (11)	+++ (11)
SOCO6	+++ (13)	+++ (13)	+++ (12)	+++ (12)
SOCO12	++ (10)	++ (10)	+ (3)	+ (3)
TS4	+ (2)	+ (2)	+ (4)	+ (4)
TVM2	–	–	++ (8)	++ (8)
V35	++ (10)	++ (10)	–	–

Numbers in the parentheses indicate the zone of inhibition in mm.
'–', No inhibition; '+', Low inhibition (1 to 5 mm); '++', Medium inhibition (6 to 10 mm); '+++', Strong inhibition (11 to 15 mm).

plugs alone was used as control. The plates were incubated at 37°C until fungal mycelia completely covered the agar surface in control plate. Strains that inhibited mycelial growth of *F. oxysporum* and *R. bataticola* on plates with FeCl₃ were identified.

Among 40 strains of *P. fluorescens*, 17 inhibited the mycelial growth of *R. bataticola* and *F. oxysporum* on PDA in presence and absence of FeCl₃ (Table 1). Strains JAI3 inhibited fungal growth only in the absence of FeCl₃, suggesting siderophore mediation. Greater number of strains inhibited growth of *R. bataticola* than *F. oxysporum*. Strains PAON2, PUJA1, PUJA3 and SOCO6 strongly inhibited growth of both fungal pathogens. Strains MKU3 and V35 inhibited growth of *R. bataticola* only, while strains TVM2 and JO5 inhibited growth of *F. oxysporum* only. This suggested that various isolates of *P. fluorescens* produce different antifungal compounds. These strains were not further distinguishable based on biochemical tests; therefore genotyping was performed by PCR–RAPD analysis.

Chromosomal DNA from cultures grown in nutrient broth at 37°C for 16 h was extracted according to the method of Byun *et al.*[13]. To confirm strains as fluorescent *Pseudomonas*, 16S–23S rRNA intervening sequence-specific primers ITS1F (AAGTCGTAACAAGGTAG); ITS2R (GACCATATATAACCCCAAG) were used to get an amplicon size of 560 bp. The random primers pgs2 (GTTTCGCTCC), pgs3 (GTAGACCCGT) and pgs4 (AAGAGCCCGT) were used for RAPD analysis. All primers were synthesized and obtained from Microsynth,

Switzerland. PCR reactions were carried out in 20 µl reaction containing 10 × buffer (with 2.5 mM MgCl₂), 2 µl; 2 mM dNTP mixture, 2 µl; 2 µM primer, 5 µl; *Taq* DNA polymerase, 3U; H₂O, 8 µl, and 50 ng of template DNA samples were amplified on DNA thermalcycler (DNA engine, MJ Research, USA) using the PCR conditions 92°C for 4 min, 28°C for 1 min and 72°C for 2 min. The total number of cycles was 40, with the final extension time of 10 min. The PCR products were resolved on 2% agarose at 50 V, stained with ethidium bromide (0.5 µg ml⁻¹) and photographed and analysed using Gel documentation system (Biorad, USA, model 2000, Quantity One software).

PCR–RAPD analysis was repeated at least three times and fingerprints were compared. The RAPD bands which appeared consistently were evaluated. Calculation of the pair-wise coefficient similarity based on the presence and absence of bands and cluster analysis with unweighed pair group method arithmetic mean (UPGMA) were used to generate similarity matrix.

The 16S–23S ITS region from all 18 strains of *P. fluorescens* (one strain sid⁺, others ant⁺, sid⁺) gave a single amplicon of size of 560 bp, which confirmed that all 18 strains were *P. fluorescens*. Similar PCR protocol has been employed for the detection of 16S rRNA genes of the genus *Pseudomonas* in environmental samples[14].

The pgs4 primer did not provide sufficient RAPD bands for analysis and therefore they were not taken into further consideration. The primer pgs3 provided several bands, including a unique band with size of 800 bp (Fig-

ure 1 a). Sequence analysis of this unique band from a type strain *P. fluorescens* NRRL B10 (obtained from Northern Regional Research Laboratory, Peoria, USA) revealed that it belonged to 23S rRNA gene. The sequence was submitted to GenBank (Accession No. AF369903). Other amplicons obtained with pgs3 primers were too small for analysis, while amplification with primer pgs2 gave greater number of clear bands (Figure 1 b).

Strains JAI1, JAI2, JAI5, JAI6 and JAI8 isolated from the same rhizosphere showed similar antifungal activity; however there was a wide genetic variation (Figure 1 b). However, isolates from different locations showed strong similarity. For example, strains JAI6 and MKU3 isolated from different locations showed similar RAPD pattern. Strain JAI5 was found to be similar to strain PAON3, and JAI2 similar to PNC1. RAPD analysis revealed that
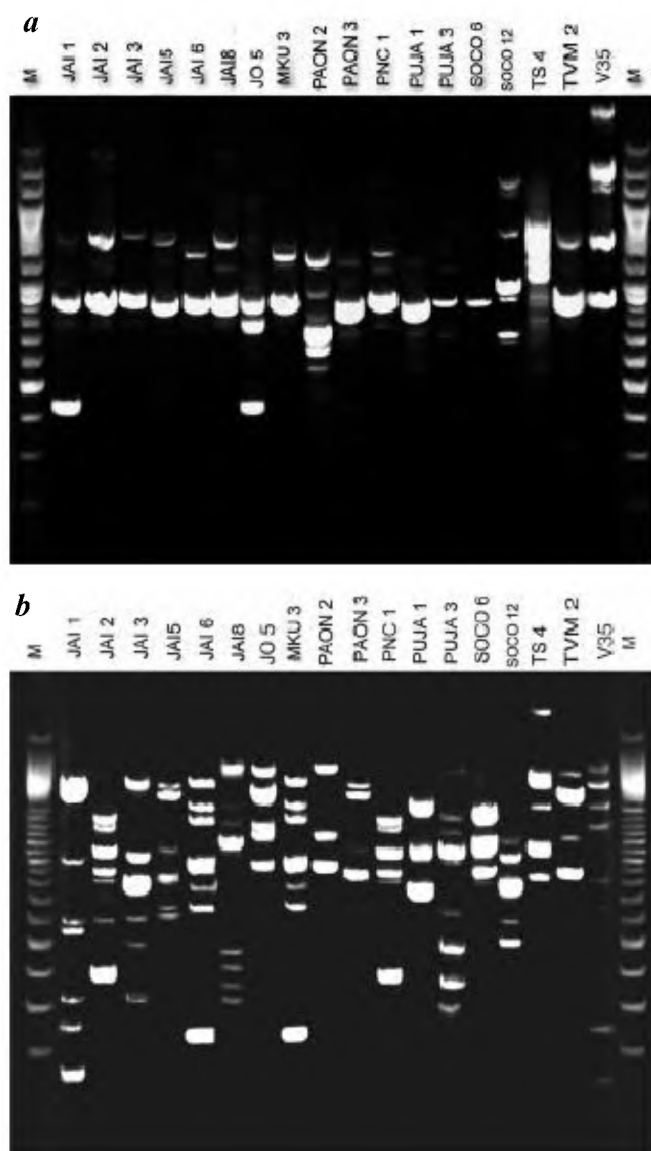
Figure 1. RAPD analysis of (*a*) primer pgs3 and (*b*) primer pgs2 amplified DNA fragments of antifungal compound producing *Pseudomonas fluorescens*.
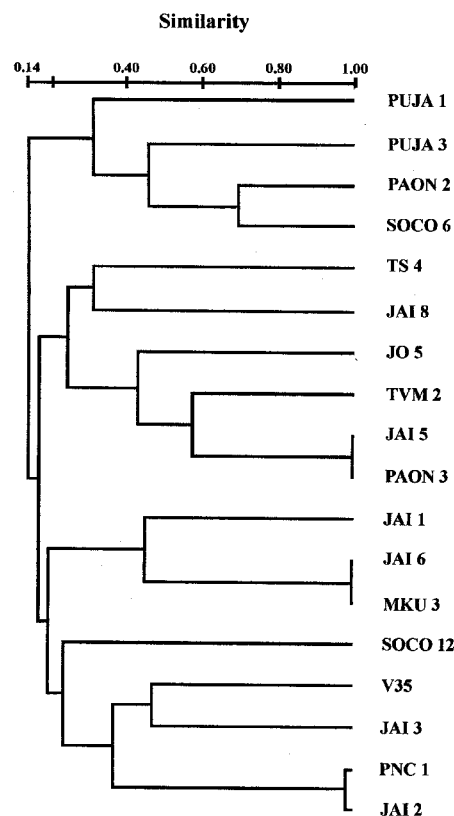
Figure 2. Genetic relatedness between antifungal compounds producing *Pseudomonas fluorescens*.

strains JAI2, JAI5 and JAI6 formed distinct groups compared to other strains and showed maximum similarity with strains PNC1, PAON3 and MKU3 respectively, which were isolated from different locations (Figure 2). Similarly, strains SOCO6 and SOCO12 isolated from the same area were placed in separate subgroups. Strain SOCO6 shared greater similarity (> 50%) with strain PAON2, thus confirming that bacteria isolated from entirely different geographical areas can also share some genetic relatedness. Ellis *et al.*[15] used a collection of 29 fluorescent *Pseudomonas* involved in the suppression of *P. ultimum* to fatty acid profiling and RFLP analysis of the ribosomal DNA operon (ribotyping), to determine the genetic diversity of the organism possessing similar functions. A small group of genetically related *Pseudomonas* sp. was identified; each isolate was confirmed to produce diffusible bioactive product; therefore organisms having similar properties may share some genetic relationship among themselves irrespective of geographical location. In the present study, PAON2 and PAON3 isolated from the same ecological niche were strongly antagonistic to phytopathogens, but shared genetic homology with SOCO6 and JAI5, isolated from entirely different regions. Although there are several environmental factors which can modulate the production of antifungal compounds, the producers cannot be differentiated based on biochemical tests. Molecular analysis using PCR-based

RAPD method is thus useful to differentiate such strains at the intraspecific level.

The production of antimicrobial agents in soil is influenced by several environmental factors such as soil chemistry and microbial population. For example, presence of Zn^{2+} in soil increases the production of Phenazine (PCA)[16] and low temperature (12°C) influences production of 2,4-diacetyl phloroglucinol (DAPG) by *Pseudomonas* sp.[5]. It is however not possible to differentiate the strains based on the antimetabolites production. According to Manceau and Harvais[17], the relationship among strains at the genetic level can be analysed by genomic fingerprinting techniques such as PCR–RAPD, repetitive sequence-based PCR (rep-PCR), and amplified 16S ribosomal DNA restriction analysis (ARDA). For example, the genetic diversity of *P. syringae* var tomato has been studied by PCR–RFLP analysis of rRNA operon[18].

In summary, strains JAI1, JAI2, JAI5, JAI6 and JAI8 isolated from the same rhizosphere and strains PNC1 and SOCO12 isolated from different locations showed similar antifungal properties. Similarly, strains PAON2, PUJA1, PUJA3 and SOCO6 isolated from three different locations showed similar antifungal properties. Such strains synthesizing similar antifungal compounds could not be differentiated by biochemical tests; however they could display considerable genotypic differences. Therefore, differentiation of these strains was possible with the use of PCR–RAPD methodology.

1. Patten, C. L. and Glick, B. R., *Can. J. Microbiol.*, 1996, **42**, 207–220.
2. Glick, B. R., *ibid*, 1995, **41**, 107–109.
3. Neilands, J. B. and Leong, S. A., *Annu. Rev. Plant Physiol.*, 1986, **37**, 187–208.
4. Loper, J. E., *Phytopathology*, 1988, **78**, 166–172.
5. Nowak-Thompson, B., Gould, S. J., Kraus, J. and Loper, J. E., *Can. J. Microbiol.*, 1994, **40**, 1064–1066.
6. Toyoda, H. and Utsumi, R., US Patent 1991, #4, 988, p. 586.
7. Mauch, F., Mauch-Mani, B. and Boller, T., *Plant Physiol.*, 1988, **88**, 936–942.
8. Voisard, C., Keel, C., Haas, D. and Defago, G., *EMBO J.*, 1989, **8**, 351–358.
9. Papavizas, G. C. and Ayers, W. A., A Review, US Department of Agriculture, Washington DC, 1974.
10. Garagulya, A. D., Kiprianova, E. A. and Boiko, O. I., *Mikrobiol. Zh. (Kiev)*, 1974, **36**, 197–202.
11. Fenton, A. M., Stephens, P. M., Crowley, J., O'Callaghan, M. and O'Gara, F., *Appl. Environ. Microbiol.*, 1992, **58**, 3873–3878.
12. Shanahan, P., O'Sullvan, D. J., Simpson, P., Glennon, J. D. and O'Gara, F., *ibid*, 1992, **58**, 353–358.
13. Byun, M. O. K., Kaper, J. B. and Ingram, L. O., *J. Indian Microbiol.*, 1986, **1**, 9–15.
14. Widmer, F., Seidler, R. J., Gillevet, P. M., Watrud, L. S. and Di Giovanni, D., *Appl. Environ. Microbiol.*, 1998, **64**, 2545–2553.
15. Ellis, R. J., Timms-Willson, T. M. and Bailey, M. J., *ibid*, 2000, **2**, 274–284.
16. Schniger, P. J. and Jackson, M. A., *Appl. Microbiol. Biotechnol.*, 1992, **37**, 388–392.
17. Manceau, C. and Harvais, A., *Appl. Environ. Microbiol.*, 1997, **63**, 498–505.
18. Redecker, D., Feder, I. S., Vinuesa, P., Batinic, T., Schulze, U., Kosch, K. and Werner, D., *Z. Naturforsch., C*, 1999, **54**, 359–370.

Pollination ecology and fruiting behaviour in *Acacia sinuata* (Lour.) Merr. (Mimosaceae), a valuable non-timber forest plant species

A. J. Solomon Raju* and S. Purnachandra Rao
Department of Environmental Sciences, Andhra University, Visakhapatnam 530 003, India

Acacia sinuata shows leaf-fall, flushing and flowering during dry season. The flowers are small, massed into globose heads and function as units of reproduction. The plant is self-incompatible and exhibits synchronous flowering to facilitate cross-pollination. The flowers are hermaphroditic and monostylous, offering both pollen and nectar as floral rewards. Foragers included bees, wasps, flies, butterflies and thrips. Thrips acted as mere foragers, while all others acted as pollinators while foraging different conspecific flowers in succession. Additionally, sunbirds made occasional visits for feeding on extra-floral nectaries located at the base of leaf petioles. The pod-set rate was 2% only. The low pod-set rate is attributed to a variety of factors. The study suggests that pod and seed yields may be enhanced by introducing manageable bees together with their nesting requirements.

THE forests in India yield a large number of products which play an important role in the economy of the country. Among these, timber and firewood are known as major forest products. The minor forest products may not be of great economic importance individually, but collectively their value is immense. Large quantities of these products are consumed locally. They support a large number of indigenous industries besides finding their way into external trade. There are a number of other forest products which have not come into the market so far; but are likely to gain economic importance, if properly