Austin Journal of Biotechnology & Bioengineering

Austin Publishing Group

Open Access Full Text Article

Research Article

Genetic Fingerprinting of Antimicrobial Fluorescent Pseudomonads Associated with Banana Rhizosphere

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Received: July 02, 2014; **Accepted:** July 28, 2014; **Published:** July 30, 2014

Abstract

In recent years, fluorescent pseudomonads are well recognized for their plant growth promoting and bio-control activities. This study was design to study the distribution of potential antimicrobial fluorescent pseudomonads associated with banana rhizosphere by using ERIC and BOX-PCR fingerprinting. For this purpose, 32 fluorescent pseudomonads were isolated from banana rhizosphere collected from three different major grown banana locations and screened against three fungal phytopathogens. Promising isolates were selected for genetic fingerprinting by using ERIC and BOX-PCR to establish clonal relationship among the antifungal fluorescent pseudomonads. ERIC-PCR fingerprinting was found to be a potential tool to differentiate among the fluorescent and non fluorescent bacterial isolates as compare to BOX-PCR fingerprinting. A subset of 25 antagonistic fluorescent pseudomonads were characterized by ERIC and BOX-PCR and showed a high degree of DNA heterogeneity. Four groups were identified based on ERIC-PCR and three of them showed homogeneity containing fluorescent *pseudomonas* with a group of non fluorescent *bacillus* sp (PF 7). A unique band of 450 bp was generated by ERIC-PCR profiling among all the fluorescent isolates, which can be used as a diagnostic tool, whereas non fluorescent bacteria will have an adjacent band of 430 bp, which was absent in all identified fluorescent *pseudomonas* isolates. Further, 16S rDNA based identification of randomly selected isolates proved that ERIC-PCR fingerprinting is better DNA based tool to differentiate fluorescent *pseudomonas* with other bacterial isolates. We conclude and recommend ERIC-PCR banding pattern could be potential tool to differentiate among fluorescent and non fluorescent group of bacteria.

Keywords: Fluorescent pseudomonads; Genetic fingerprinting; Antibacterial; ERIC-PCR; BOX-PCR; Repetitive sequences

Abbreviations

PDA: Potato Dextrose Agar; PCR: Polymerase Chain Reaction; ERIC-PCR: Entrobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction; EDTA: Ethylene Diamine Tetra Acetic acid; SDS: Sodium Dodecyl Sulfate; TAE: Tris Acetate EDTA; UPGMA: Unweighted Pair Group Method with Arithmetric average; 16S rDNA: 16S ribosomal DNA; NCBI: National Centre for Biotechnology Information; BLAST: Basic Local Alignment Search Tool

Introduction

The fluorescent pseudomonads, exist as one of the major group within the rRNA homology group I of genus *pseudomonas,* are characterized by the production of fluorescent pigment, produced on media containing low iron content and are responsible for the biodegradation of diverse range of compounds [1-3]. The group includes species for the production of bioactive compounds like, bio control agents of animal and plant diseases, promote plant growth, and control soil born diseases and production of plethora of biotechnologically important compounds [4-6]. Fluorescent pseudomonads were widely distributed which suggests their range of adaptability with the nature. This group of bacteria is preferably matched as soil inoculants due to aggressive colonization with the plant roots. This feature alone is suggested as a disease control mechanism by preventing the invasion of soil pathogens onto the root surface [7-11]. To apply these organisms in practice, it is very much important to understand the molecular diversity and metabolic versatility associated with different rhizosphere [12-14].

Banana is considered as one of the important food item due to its high nutritional and commercial values throughout the tropics of the world. It is the one of the major tropical fruit crop in 120 countries with annual production of 102 million tonnes [15]. Northeast India is considered as a rich source for the diversity of banana varieties which are believed to be hybrids of Indian subcontinents and Southeast Asia [16]. Wild and cultivated bananas are abundantly available in Mizoram, one of the states in Northeast India [17]. Henceforth, sustaining and enhancing the growth and productivity of banana have become a major focus of research. The growth and yield of banana in field, is greatly influenced by wide range of fungal and bacterial diseases. Control of diseases by the use of various chemicals and use of resistance cultivars are the important methods to improve the yield of crop plants. However, it is not possible to have resistant cultivar for every disease. Moreover, the availability of chemical fungicides is costly and has adverse effects on human health and is found to be lethal for the rhizospheric microorganisms. It has been recognized that the rhizospheric microorganisms are natural antagonistic

Citation: Singh BP. Genetic Fingerprinting of Antimicrobial Fluorescent Pseudomonads Associated with Banana Rhizosphere. Austin J Biotechnol Bioeng. 2014;1(2): 6.

towards important phytopathogens and offers us as an alternative tool against chemical fertilizers.

Genetic typing techniques based on conserve repetitive regions have been shown to be more accurate and discriminatory than morphological and phenotypic methods for typing *pseudomonas* isolates [18-20]. Rep-PCR fingerprinting utilizes primers from highly conserved repetitive sequences of the bacterial genome. Two of such type of elements is entrobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) and BOX-PCR more common to gram negative enteric bacteria [21]. The fingerprinting generated by using ERIC-PCR shows characteristic pattern and could be used to differentiate bacterial genomes [22].

Mizoram has been known for its diversified nature in the field of plant and animal biodiversities and can offer a wide range of research objectives in near future. Very limited research had been done so far in the local microbial biodiversity [23-25]. Majority of the population is living in rural areas and depends on agriculture. Lack of proper methods of agricultural farming and extensive use of chemical fertilizers are rapidly depleting the fertility of soils in cultivated lands. The use of chemical fertilizers and conventional agricultural practices such as jhumming could not sustain the fertility of the soil for long terms [26]. As a result, farmers are not able to get the worth of their hard works with fertility of soil rapidly decreasing and the yield of crop plants lowering day by day. Henceforth, the use of native microorganism to fight against soil born diseases will be a best alternative tool to increase crop production. This study was design to search for a potential antifungal fluorescent *pseudomonas* to develop a crop specific bio-fertilizer and to understand the genetic relationship among them by using repetitive sequence markers.

Materials and Methods

Isolation of fluorescent pseudomonads

Fluorescent pseudomonads were isolated from the rhizosphere of selected banana cultivated places in Aizawl around Mizoram, Northeast India. Three places were selected based on the production of banana in Mizoram and samples were collected randomly and pooled together before processing. Roots were collected with adhering soil and homogenized in washing buffer (0.1 M potassium phosphate, pH 7) solution followed by serial dilution and plating on King's B agar medium. After spreading, plates were incubated at 30^{O}C for 24 hours, and colonies those fluoresced under UV light (λ= 356 nm) were selected and were further purified on same media by repetitive streaking.

Antimicrobial screening of fluorescent pseudomonads

Fluorescent pseudomonads were isolated from the rhizosphere of banana cultivated laces in Aizawl around Mizoram, Northeast India (23.44N 92.57E). All the isolates were screened for antifungal activities against three fungal phytopathogens viz. *Fusarium oxysporum* (CABI-293942), *Fusarium udum* (MTCC-2755) and *Fusarium graminearum* (MTCC-1893) by dual plate assay [27]. All the pathogens were grown on PDA media and agar plug (4 mm diameter) was taken by using sterilized cork borer and placed on PDA agar plates. At the same time, bacterial cultures were streaked about 3 cm away from the agar plug towards the edge of the plates. A plate alone with fungal plug was used as a control and the plates were incubated at 28^{O}C for 5 days. Inhibition diameter was recorded in mm against each pathogen.

Genomic DNA extraction and quantification

A single purified bacterial colony of the potential isolates showing significant antifungal activity at least against two phytopathogens was inoculated in 10 ml nutrient broth (Hi-Media, Mumbai, India) and incubated in orbital shaker for 24 hours at 30^{O}C. Cells were precipitated by centrifugation and re-suspended in 2ml of 25% sucrose, 50mM EDTA (pH 8.0). Saturated lysozyme solution (150 µl) and 10 µl of 20 mg ml^{-1} RNAse A were added and incubated at 37^{O}C for 3 hours. Lysis was done by using 150 µl of 20% SDS, mixed gently and incubated at 37^{O}C for 10 minutes and suspension was incubated in water bath at 65^{O}C for 2 hours. Other macromolecules like proteins and carbohydrates were removed by extraction with chloroform/ isoamyl alcohol (24:1). The resulting suspension was mixed gently and centrifuges at 10,000 rpm for 10 minutes and upper clear layer was transferred in new autoclaved tubes. DNA was precipitated by adding absolute alcohol and DNA was spooled out and dissolved in 200 µl of TE buffer (10mM Tris-Cl, pH-8.0; 1mM EDTA). Quantification of DNA was done by using UV-VIS spectrophotometer by taking the ratio at OD_{260}/OD_{280}.

ERIC and BOX-PCR fingerprinting

Universal primers based on ERIC sequences were used to generate fingerprinting were ERIC1 (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') while those used for BOX-PCR was BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3') [28]. The reaction mixture for ERIC-PCR after standardization consisted of 1 µl of DNA template, 0.6 µl dNTP mix (15mM), 100 pmol of primer ERIC1R and ERIC 2, 1 µl of $MgCl_2$ (50mM) and unit of Taq DNA polymerase (Invitrogen- life technologies, USA), final volume was makeup to 25 µl by adding remaining sterilized water. Negative control reaction without template DNA was used for each amplified set. Amplification was conducted in Veriti Applied Bio-system thermal cycler with an initial denaturation step at 95^{O}C for 7 min, followed by 30 cycles of denaturation at 94^{O}C for 1 min, annealing at 51^{O}C for 1 min, extension at 65^{O}C for 8 min., followed by final extension at 65^{O}C for 16 min. The reaction mixture for BOX-PCR is similar with ERIC-PCR except the annealing temperature for BOX-PCR was 50^{0}C for 1 min. The products were analyzed by running on 1.5% agarose gel in 1X TAE buffer, 1kb DNA ladder (Merk pvt. Ltd) was used as size standard. The gel was stained by ethidium bromide (0.5 µg/ml), and visualized under a gel documentation system (Bio-Rad Gel Doc XR+, USA).

PCR amplification of 16S rDNA and sequencing

Randomly ten isolates were selected for identification by amplification of 16S rDNA region of bacterial rRNA genes with universal *E. coli* primers, forward from position 7 to 26 (5'-AGAGTTTGATCCTGGCTCAG-3') [29] and reverse primer from position 1,513-1492 (5'-ACGGCTACCTTGTTACGACTT-3') [30]. Amplification was performed in 20µl reaction mixture consisting of 2µl of 10X PCR assay buffer (10X), 2.0µl of dNTPs (2.5mm), 0.6µl primers (10 pmol), 0.2µl of Taq polymerase (1U/µl), 1.5µl of template DNA (50ng/ µl), and remaining of Millipore distilled water. Amplification was conducted in Veriti Applied Bio-system thermal cycler with an initial denaturation step at 95^{O}C for 5 min, followed by

Table 1: Biochemical and antifungal screening of fluorescent pseudomonads of banana rhizosphere.

Isolate No.	NCBI Accession Number	Biochemical Characterization				Antifungal Screening (mm*)		
		Grams Reaction	Shape	Motility	Spores	*FG*	*FO*	*FU*
PF1	**KM112023**	-	Rod shaped	Motile	-	++	++	+++
PF2	NS	-	Rod shaped	Motile	-	+	++	-
PF3	**KM112030**	-	Rod shaped	Motile	-	++	+++	++
PF4	NS	-	Rod shaped	Motile	-	+	++	++
PF5	NS	-	Rod shaped	Motile	-	+	++	-
PF6	**KM112031**	-	Rod shaped	Motile	-	++	+++	++
PF7	**KM112032**	-	Rod shaped	Motile	-	+	-	++
PF8	NS	-	Rod shaped	Motile	-	++	++	++
PF9	**KM112024**	-	Rod shaped	Motile	-	+	++	++
PF10	NS	-	Rod shaped	Motile	-	+	-	++
PF11	**KM112025**	-	Rod shaped	Motile	-	++	++	+++
PF12	NS	-	Rod shaped	Motile	-	-	++	+
PF13	NS	-	Rod shaped	Motile	-	+	++	++
PF14	NS	-	Rod shaped	Motile	-	+	++	++
PF15	**KM112026**	-	Rod shaped	Motile	-	+	++	++
PF16	NS	-	Rod shaped	Motile	-	+	-	++
PF17	**KM112027**	-	Rod shaped	Motile	-	+++	+	++
PF18	NS	-	Rod shaped	Motile	-	++	++	-
PF19	**KM112028**	-	Rod shaped	Motile	-	++	+	++
PF20	NS	-	Rod shaped	Motile	-	+	-	++
PF21	**KM112029**	-	Rod shaped	Motile	-	++	++	++
PF22	NS	-	Rod shaped	Motile	-	+	++	++
PF23	NS	-	Rod shaped	Motile	-	+++	+++	++
PF24	NS	-	Rod shaped	Motile	-	++	++	-
PF25	NS	-	Rod shaped	Motile	-	++	++	++

NS: Not sequenced; FG: *Fusarium graminearum*; FU: *Fusarium udum*; FO: *Fusarium oxysporum*.
*+: 5-8 mm inhibition zone
*++: 9-12mm inhibition zone
*+++: 13-18mm inhibition zone.

35 cycles of denaturation at 94^{O}C for 1 min, annealing at 55^{O}C for 1 min, extension at 72^{O}C for 1 min 20 sec, followed by final extension at 72^{O}C for 6 min. Gel visualization was done as stated earlier. Sequencing was done commercially for the amplified products. Bioinformatics tool BLAST was used to locate the closest relative and sequences were submitted to NCBI Gen Bank.

Data analysis

The banding pattern generated by using ERIC and BOX-PCR were scored as absent (0) or present (1) and the resulting bands were used to generate similarity matrix for the construction of phylogenetic tree by NTSYS-pc analysis package [31]. Jaccard's coefficient of similarity index was used to calculate similarity distances [32]. Cluster analysis was done using unweighted pair-group method with arithmetic average (UPGMA).

Results and Discussion

Isolation and antifungal screening of fluorescent pseudomonads

In total 32 isolates with characteristic fluorescent pigment were isolated from rhizosphere of banana collected from three different locations. All the isolates were screened for their antifungal potential against three fungal phytopathogens. Among them, 25 isolates, were shown significant activity against at least two fungal pathogens were selected for genetic fingerprinting by using ERIC and BOX-PCR and designated as PF1- PF25 (Table 1). Sixteen isolates inhibited all three pathogens whereas remaining nine isolates were inhibiting two pathogens. The inhibition zone was ranging from 5 to 18 mm in diameter (Table1). Fluorescent pseudomonads are one of the common culturable soil bacteria known to promote plant growth either by enhancing plant growth or suppressing the soil-borne pathogens. Their predominance in the rhizosphere of various plants is being reported, but genetic fingerprinting of potential fluorescent pseudomonads is in its initial phase [33-35]. This group is of interest due to their abundance in soil and their role in controlling different fungal pathogens [36]. A significant number of isolates (25 out of 32) exhibited antifungal activity against selected phytopathogenic fungi. Similar findings were reported by Neelamegam, 2012 [15] in

the fluorescent pseudomonads isolated from sugarcane rhizosphere. Plant growth promoting traits of fluorescent pseudomonads was well studied under normal and saline conditions [6].

Biochemical characterization of fluorescent pseudomonads

Phenotypic characterization of the banana rhizosphere isolates shows that all are gram-negative, non spore forming, rod shaped motile organisms. All the selected antifungal isolates were exhibiting catalase, oxidase and arginine hydrolysis activities (Table 1). Based on these characteristics, isolates could be identified as Fluorescent *pseudomonas* sp. Ten isolates based on ERIC-PCR pattern were randomly selected for identification by using 16S rDNA sequencing [37,35]. Biochemical characterization of banana rhizosphere isolates indicates that most of the isolates expected to be *pseudomonas*, but biochemical and phenotypic methods are not mare enough to distinguish and identified closely related isolates till species level [38,18]. This study is also shows the similar findings that biochemical and phenotypic methods could identify fluorescent pseudomonads till genus level, which is proved by 16S rDNA sequencing [39,40].

ERIC and BOX- PCR fingerprinting of fluorescent pseudomonads

Whole genomic DNA, ERIC-PCR amplification of the antifungal fluorescent pseudomonad isolates yielded unique genomic fingerprinting pattern consisting of 10-31 products ranging in size from 150 bp to 3000 bp (Figure 1), which shows this technique was useful to differentiate the isolates. Dendrogram analysis was divided the isolates into four clusters (A-D) (Figure 2). Cluster A was the major cluster containing 19 isolates, out of which 7 were randomly selected for identification, among which two were identified as *P. braccicacearum* (PF 17:KM112027 and PF 21: KM112029) and one each as *P. aeruginosa* (PF 1: KM112023), *P. Syringae* (PF 9: KM112024), *P. koreensis* (PF 11: KM112025), *P. Putida* (PF 15: KM112026), *Pseudomonas* sp.(PF 19: KM112028) Cluster B was the second large cluster consists of 3 isolates, which were belongs to *Pseudomonas* sp. Cluster C and D contains 1 isolates each which were belongs to *P. mendocina* (PF 6: KM112031) and *Bacillus* sp. (PF 7: KM112032) respectively. Interestingly, isolate no PF 7, which was not having any similarity with other isolates was identified as *Bacillus* sp. We found that ERIC-PCR fingerprinting could make very clear-cut differentiation among the fluorescent *pseudomonas* and other genus as all the *pseudomonas* isolates were fall in other clusters and *Bacillus* was placed in a separate cluster.

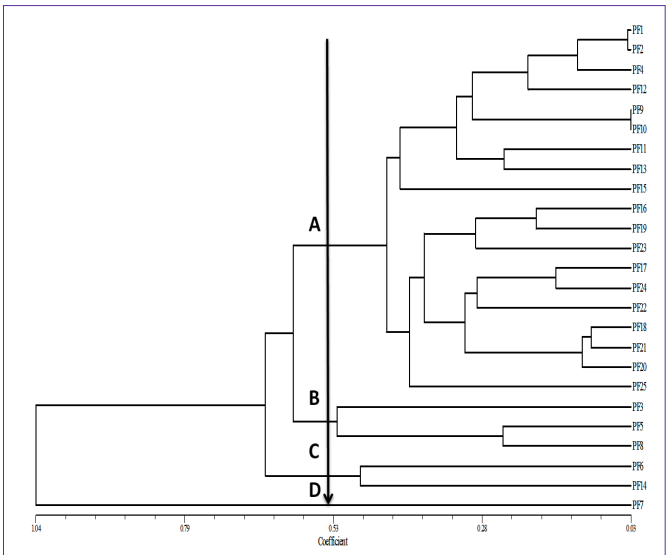

Figure 2: ERIC-PCR generated Dendrogram showing relationship among fluorescent pseudomonad isolates. Cluster analysis was performed by UPGMA with a matrix calculated with Jaccard's coefficient.

BOX-PCR fingerprinting pattern of the isolates differentiated the isolates belongs to banana rhizosphere, genomic fingerprinting pattern consisting of 06-25 products ranging in size from 250 bp to 2000 bp. However, it was not as discriminatory as ERIC-PCR fingerprinting (Figure 3). Dendrogram analysis divides the isolates into 3 clusters (A-C) (Figure 4). Cluster A was the largest cluster consists of 20 isolates and cluster B and C consists of 3 and 2 isolates respectively. In the box proofing the fluorescent *pseudomonas* isolates were mixed in different clusters and non fluorescent *bacillus* sp. (PF 7) also mixed with other fluorescent isolates (PF 11 & PF 15). We concluded that the BOX-PCR fingerprinting could not able to differentiate between fluorescent and non fluorescent pseudomonads.

Repetitive sequence analysis like rep, ERIC and BOX were useful to understand the diversity among the antifungal fluorescent

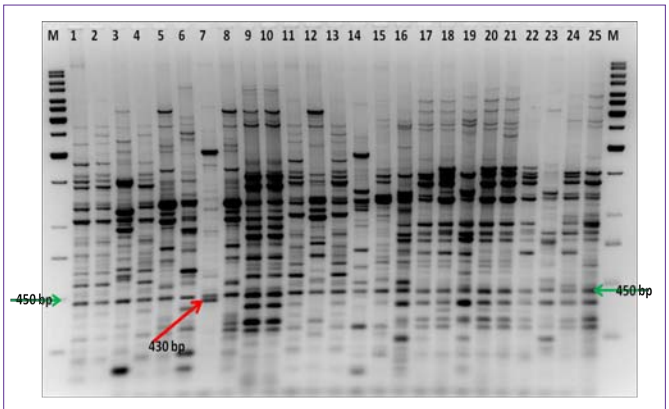

Figure 1: ERIC-PCR fingerprinting pattern of fluorescent pseudomonad isolates isolated from banana rhizosphere (Lane 1 to 25 are represented as isolate no PF 1 to PF 25); Lane M is a standard 1 kb DNA ladder; Highlighted unique bands of 450 bp and 430 bp.

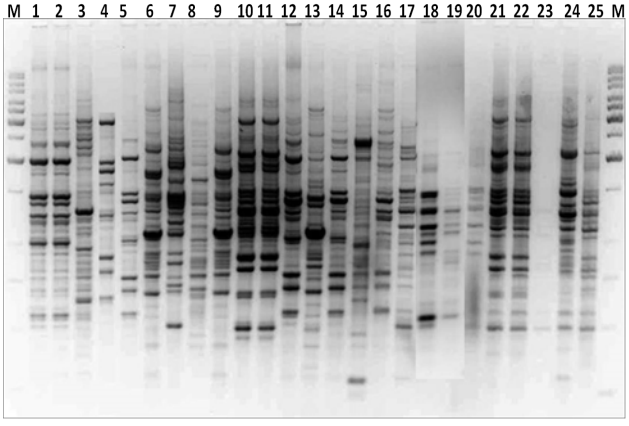

Figure 3: BOX-PCR fingerprinting pattern of fluorescent pseudomonad isolates isolated from banana rhizosphere (Lane 1 to 25 is represented as isolate no PF 1 to PF 25); Lane M is a standard 1 kb DNA ladder.

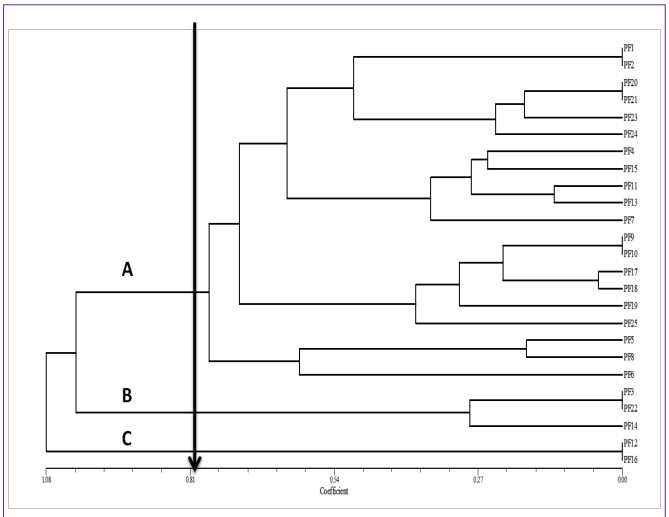

Figure 4: BOX-PCR generated Dendrogram showing relationship among fluorescent pseudomonad isolates. Cluster analysis was performed by UPGMA with a matrix calculated with Jaccard's coefficient.

pseudomonads associated with banana rhizosphere. Although in this study BOX sequences have their own level of genetic resolution to distinguish the organisms, they were not sufficient to differentiation among the fluorescent and non fluorescent pseudomonads. A positive correlation was observed among the phenotypic and ERIC-PCR fingerprinting, this is in contradiction with other findings [18,41]. ERIC-PCR fingerprinting pattern clearly differentiate among the fluorescent pseudomonads falls in one major cluster and *bacillus* sp. being non fluorescent was clearly located in a separate cluster, which was not true with the case of BOX-PCR, which was in agreement with the findings of Bruijn [22]. On the whole, the ERIC-PCR fingerprinting could be a potential and reliable tool for the detection and differentiating among fluorescent pseudomonads. A unique band of 450 bp generated by ERIC-PCR fingerprinting could also used as a detection method for the primary detection of fluorescent pseudomonads, though it needs to be verify by some other molecular techniques whereas non fluorescent bacteria generates an extra band of 430 bp, which was not present in any of the fluorescent *pseudomonas* (Figure 3).

Conclusion

In this study we concluded that fluorescent *pseudomonas* isolates obtained from banana rhizosphere could be differentiated based on DNA typing tool ERIC-PCR fingerprinting as compare to BOX-PCR banding pattern. ERIC-PCR profiling clearly differentiates the non fluorescent bacterial isolate PF 7, identified as *bacillus* sp. which falls under a different cluster. We also found a unique band of about 450 bp in all the fluorescent *pseudomonas* whereas in non fluorescent *pseudomonas* an extra band of 430 bp was also present, which was not present in any of the fluorescent *pseudomonas* (PF 7). Henceforth, we also concluded that band of 450 bp in ERIC-PCR profiling could be used as an identification tool to differentiate between fluorescent *pseudomonas* with other bacterial group. Further, these findings describe the diversity, plant-growth promoting traits, and antagonistic potential of banana rhizosphere-associated fluorescent *pseudomonas* and exploitation of their plant beneficial traits for sustainable agriculture.

Acknowledgment

BPS is thankful to Indian Council of Agricultural Research for funding under National project on Application of Microorganisms in Agriculture and Allied Sectors (AMAAS) to National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau, India. We are also thankful to Department of Biotechnology, Government of India, New Delhi for establishment of State-Biotech Hub and Bioinformatics facility at Department of Biotechnology, Mizoram University. The authors thank Mr. Hardayanto Sulisto Putro, Research Associate, University of Aberdeen, Scotland, UK for the critical reading and the language editing of the manuscript.

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Citation: Singh BP. Genetic Fingerprinting of Antimicrobial Fluorescent Pseudomonads Associated with Banana Rhizosphere. Austin J Biotechnol Bioeng. 2014;1(2): 6.