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RESEARCH ARTICLE

Lower Frequency and Diversity of Antibiotic-Producing Fluorescent Pseudomonads in Rhizosphere of Indian Rapeseed–Mustard (*Brassica juncea* L. Czern.)

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Abstract Fluorescent pseudomonads are one of the most important microbial communities which play a key role in rhizosphere to enhance plant growth-promotion and protection. The diverse groups of antibiotics viz. 2,4-diacetylphloroglucinol (DAPG), phenazine-1-carboxylic acid (PCA) and pyoluteorin (PLT) are produced by fluorescent pseudomonads inhibiting growth of fungal pathogens which results in health upliftment of plants. The present study, discusses about frequency and diversity of 138 antibiotic-producing fluorescent pseudomonads isolated from eight genotypes of rapeseed mustard rhizosphere (*Brassica juncea* L. Czern.). The plant growth promoting traits and antibiotics (DAPG, PCA and PLT) production of isolates were examined by using polymerase chain reaction (PCR), thin layer chromatography (TLC) and dot blot-hybridization. Among 138 isolates, 47, 25 and 9 % of isolates were positive in indole production, phosphate solubilization and antagonism potential against *Sclerotinia sclerotiorum* (causal agent of white mold disease in rapeseed mustard), respectively. PCR amplifications showed that none of the isolates had *phl*D (DAPG) and *phz*C (PCA) genes, but four isolates (UKA-2, UKA-8, UKA-11, UKA-66) had *plt*B (PLT) gene, which was further confirmed by TLC and DNA dot-blot hybridization. BOX profiles of *plt*B positive isolates were distinct, showing unique genetic diversity in the small population. The four *plt*B positive fluorescent pseudomonad isolates could be used as promising bio-control and plant growth-promoting inoculants for Indian rapeseed mustard.

Keywords Indian rapeseed–mustard · Fluorescent pseudomonads · Antibiotics · Plant growth-promoting rhizobacteria · Polymerase chain reaction · Dot-blot hybridization · BOX profile

Abbreviations

FP	Fluorescent pseudomonads
IRM	Indian rapeseed mustard
DAPG	2,4-Diacetyl phloroglucinol
PCA	Phenazine-1-carboxylic acid
PLT	Pyoluteorin

Introduction

During the last three decades it has been demonstrated that secondary metabolites produced by antagonistic bacteria play a key role in the suppression of various soil-borne plant pathogens. Among the members of plant growth-promoting rhizobacteria, fluorescent pseudomonads (FP) are frequently found to produce antibiotics like 2,4-diacetyl phloroglucinol (DAPG), phenazine-1-carboxylic acid (PCA), pyoluteorin (PLT) and pyrrolnitrin (PRN) etc. which have been recovered from rhizosphere of many crops. These bacteria have been studied extensively because they are highly effective biocontrol agents of a wide genotype of plant diseases when applied as seed or soil treatments [1].

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It was reported that there was considerable genetic diversity which exists amongst DAPG [2], PLT [3, 4] and PCA [5] producing FP isolates. It has been shown by amplified ribosomal DNA restriction analysis (ARDRA), whole cell repetitive sequence based PCR (rep-PCR) and restriction fragment length polymorphism (RFLP) analysis in many crops. The genetic diversity analysis for DAPG, PCA and PLT-producing FP have not been studied so far in Indian rapeseed mustard (IRM). Similarly, the relationship between plant genotype and the PGPR rhizosphere colonization is also not known in IRM.

Rape-seed mustard is a member of family Brassicaceae, an economically important edible oil seed crop in India which has been cultivated for a long time especially in the northern plains of the country. The presence of sulphur compounds (glucosinolate 2-phenylethylglucosinolate) in the root exudates of these plants influences the rhizospheric resident population [6], which exhibits negative influence on Arbuscular mycorrhiza (AM) fungi [7]. These information hypothesized that the plant may have a great influence on the fluorescent pseudomonads which may be reflected on population density and antibiotic production. Most of the work on antibiotic-producing fluorescent pseudomonads has been carried out on flax [8], pea [9], tomato [10], wheat [11, 12] and green pepper [3] rhizospheres. As there was not much information with mustard rhizosphere, the present investigation was carried out with special emphasize on antibiotic-producing plant growth promoting fluorescent pseudomonads of mustard.

Material and Methods

Isolation of Fluorescent Pseudomonads (FP)

Rhizosphere of eight genotypes of IRM (Pusa Bold, Pusa Carinata, Pusa Mahak, Pusa Jaganath, Pusa Karishma, Pusa Vijay, NPJ-114 and Bio-3401) were collected from two locations (Entomology and Gentetics fields) of Indian Agricultural Research Institute, New Delhi (28°38′N, 77°09′E; 228.61 m above mean sea level), India. Their serial dilutions were made and plated on King's B medium (KBM) [13] and incubated at 30 ± 2 °C for 48 h. FP colonies were screened under ultra-violet transilluminator. These colonies were sub-cultured on KBM and pure cultures of 138 isolates were maintained on nutrient agar (NA) slants.

Indole Acetic Acid (IAA) Production

FP isolates were inoculated in 5 mL Luria–Bertani (LB) broth and incubated at 30 ± 2 °C at 180 rpm for 48 h. Two microlitre of log phase cultures were spot inoculated on both LB and LB agar supplemented with 5 mM L-tryptophan. Sterilized nylon membranes were overlaid on those spots after complete drying and incubated at 30 ± 2 °C for 48 h. The grown cultures on nylon membrane were soaked in Salkowski reagent (2 % of 0.5 M $FeCl_3$ in 35 % $HClO_4$) and pink color development around the colonies was observed and compared to reference strain *P. fluorescence* Pf-5 [14].

Phosphate Solubilization

The phosphate solubilization test was done on a solid medium described by Pikovaskya [15]. Agar plates were prepared and the FP isolates were spot inoculated on the grids and incubated for 5–6 days. A clear zone around the colony was taken as positive for P-solubilization.

Dual Plate Assay Against *Sclerotinia sclerotiorum*

Dual plate assay was carried out of FP isolates against *S. sclerotiorum,* a causal organism of white mold disease in rapeseed mustard. All screenings were carried out on potato dextrose agar (PDA) plates. An actively growing fungal agar plug (3 mm diameter) was placed at the centre of PDA plates. FP isolates were streaked 2 cm away from the fungal disk and incubated at 28 ± 2 °C for 7 days.

Bacterial Genomic DNA Extraction

FP isolates were grown in 5 mL LB broth and incubated at 30 ± 2 °C for 24 h. Genomic DNA was extracted by phenol–chloroform method [16].

Polymerase Chain Reaction (PCR) of Antibiotics Production Genes

Polymerase chain reactions were carried out to detect the presence of three antibiotic production genes viz. 2,4-diacetylphloroglucinol (DAPG: *phl*D gene), phenazine (PCA: *phz*C and *phz*D genes) and pyoluteorin (PLT: *plt*B gene). The detailed specification of primer, respective reference strains and source of these antibiotics are mentioned in Tables 1 and 2. Amplification of *phz*D and *phl*C genes was carried out by PCR using a thermal cycler (Eppendorf, Master cycler gradient). The amplification reactions were performed in a 25 µL volume by mixing 4 ng μL^{-1} of template DNA with polymerase reaction buffer (10×), 1.5 mM $MgCl_2$, 200 µM dNTPs, 20 pmol each primers of DAPG (Phl2a and Phl2b) and PCA (PCA2a and PCA3b) and 1.5 U Taq polymerase [17]. The following programme was used for thermocycling conditions, 94 °C denaturation for 90 s, followed by 35 cycles at 94 °C denaturation for 35 s, 53 °C annealing for 30 s and

Table 1 Primers for DAPG, phenazine and pyoluteorin antibiotics with corresponding reference strains and their respective amplicon sizes

Antibiotics	Primers	Sequence of primers	Amplicon size (bp)	Reference strains	References
DAPG	Phl 2a	5′GAGGACGTCGAAGACCACCA3′	745	Pf-5 and Ps-Q_2-87	[18]
	Phl 2b	5′ACCGCAGCATCGTGTATGAG3′			
Phenazine	PCA 2a	5′TTGCCAAGCCTCGCTCCACC3′	1150	Ps-2-79	[18]
	PCA 3b	5′CCGCGTTGTTCCTCGTTCAT3′			
Pyoluteorin	Plt Bf	5′CGGAGCATGGACCCCCAGC3′	773	Pf-5	[19]
	Plt BR	5′GTGCCCGATATTGGTCTTGACCGAG3′			

Table 2 Antibiotics and plant growth-promoting traits produced by reference and selected strains

Reference strains/isolates	Species	IAA	PS	Bioassay[a]	DAPG	PCA	PLT	Source[b]
Pf-5	PF	+	−	−	+	−	+	LT, USA
Ps-Q2-87	PF	+	−	−	+	−	−	LT, USA
Ps-2-79	PF	+	−	−	−	+	+	LT, USA
UKA-2	PF	+	−	−	−	−	+	OI
UKA-8	PF	+	−	+	−	−	+	OI
UKA-11	PF	−	−	−	−	−	+	OI
UKA-66	PF	+	+	−	−	−	+	OI
Fungal pathogen	SS	−	−	−	−	−	−	MB, India

PF, *Pseudomonas fluorescens*; SS, *Sclerotinia sclerotiorum*

[a] In vitro bioassay against *S. sclerotiorum* (causal agent of white mold disease in rapeseed mustard)

[b] LT: Linda Thomashow; OI: our isolate; MB: Mr. Basha, BHU, India

72 °C extension for 45 s and final extension at 72 °C for 30 s.

Similarly, the amplification of *plt*B gene was performed with primers pltBf and pltBR (20 pmol each). The PCR program consisted of a denaturation step at 94 °C for 120 s followed by 29 cycles at 94 °C for 60 s, 58 °C for 30 s, 72 °C for 60 s and a final extension at 72 °C for 120 s [19]. For every PCR reaction, a negative control (no template DNA) and a positive control (corresponding reference stains Pf-5, Ps-Q_2-87 and Ps-2-79) were invariably maintained (Table 1). The amplified product was run on 1.2 % agarose gel (containg 0.5 μL mL^{-1} EtBr) along with 100 bp and 1 kb ladders at a constant voltage (5 V cm^{-1}) for an hour and band was visualized under ultra-violet transilluminator.

Thin Layer Chromatography (TLC) of Pyoluteorin Antibiotic

Inocula were prepared from cells harvested from 3 days old cultures of fluorescent pseudomonads grown on KBM broth of 30 ± 2 °C on a rotary shaker at 180 rpm. The supernatant was collected by centrifugation at 3500 rpm for 5 min and transferred to micro-centrifuge tubes, vortexed for 30 s with 500 μL of ethyl acetate and again centrifuged. The ethyl acetate phase was collected and dried. The dried residue was dissolved in 10 μL methanol and proceeded for TLC analysis along with reference strain Pf-5 [19].

Dot-Blot Hybridization for Pyoluteorin Antibiotic

Dot-blots were probed with a α-^{32}P labeled *plt*B (~773 bp) purified fragment from reference strain Pf-5. Five microliter of purified DNA samples of 138 FP isolates along with 2 μL of the purified PCR product (~773 bp fragment) of pyoluteorin-producing reference strain Pf-5 were put onto nylon membrane and dot-blot hybridization was performed following the protocol of Udo and Dashti [20].

BOX A1R-PCR for Genetic Diversity of Pyoluteorin-Producers

Amplification of 156 bp box element was carried out by PCR using a thermal cycle (Eppendorf, Master cycler

gradient) with BOX-A1R Primer (5′CTACGGCAAGGCG ACGCTGACG3′) [21]. The amplification reactions were performed in a 50 μL volume by mixing template DNA (40 ng μL^{-1}) with polymerase reaction buffer (10×), 1.5 mM $MgCl_2$, 200 μM dNTPs, BOXA1R-primer (15 pmol μL^{-1}) and 3 U *Taq* polymerase. The program consisting of a denaturation step at 95 °C for 7 min followed by 30 cycles at 94 °C for 60 s, 53 °C for 60 s and 65 °C for 8 min was used to eliminate additional fragments resulting from final extension at 65 °C for 16 min. The amplified product was run on 1.5 % agarose gel (containing 0.5 μL mL^{-1} Etbr) along with 100 bp and 1 kb ladder at a constant voltage (5 V cm^{-1}) for 6 h and bands were visualized under ultra-violet transilluminator.

Data Analysis

Pyoluteorin producing fluorescent pseudomonads isolates were identified using biochemical-based PibWin software [22]. For genetic diversity, BOX-PCR fingerprints were converted into two-dimensional binary matrix (1, presence of a given band; 0 absence of a given band) and analyzed with NTSYS-PC (Applied Biostatistics Inc. NY). Dendrogram was generated by using the SIMQUAL (Similarity for Qualitative data) and SAHN (Sequential Agglomerative Hierarchial and Nested) clustering subroutines of NTSYS-PC [23].

Results and Discussion

One hundred and thirty eight FP were isolated from rhizosphere of eight different genotypes of IRM. Among them, 71.4 % of isolates of genotype BIO-3401 were non-functional in growth-promoting traits viz. indole, P-solubilization, antagonistic against *S. sclerotiorum*, antibiotics production (DAPG, PCA and PLT) whereas it was 57.1, 55.8, 38.4, 35.7, 28.5 and 22.7 % in NPJ 114, Pusa Bold, Pusa Carinata, Pusa Jaganath, Pusa Mahak and Pusa Karishma, respectively. The frequency of FP from rhizosphere of Pusa Vijay was found to be predominant (data not shown). It clearly indicated the varietal effect. This was amply demonstrated by the lower frequencies of the isolates in genotype BIO-3401 rhizosphere which was strongly inhibitory towards the rhizospheric FP. This might be due to root exudates containing sulphur compounds causing allelopathic effect against rhizospheric microbes [6] and also variation of root exudation in different plant species [24]. The above reasons were also corroborated with the earlier findings [8, 25].

Plant-Growth Promoting Traits

A qualitative test of IAA production indicated that out of 138 FP, 47 % were found to be positive with variable IAA production. Most of the isolates were found to produce IAA constitutively in the absence of the precursor tryptophan (Figs. 1a, b, 2). Twenty five percent of isolates showed positive in P-solubilization (Fig. 2). Against *S. sclerotiorum*, 9 % of isolates showed antagonistic activity (Fig. 2). Among the isolates, there was a wide variation in functional diversity of 6 growth promoting traits. Only 4 isolates possessed three functional traits (IAA, antagonistic activity and antibiotic production), 29 had two functions (IAA and antagonistic activity) and others possessed only a single function (IAA) (Fig. 2).

FP often predominates among the bacteria of plant rhizosphere, and some can have beneficial effects on plants, either by direct stimulation of plant growth or by exerting antagonism towards soil borne pathogens [26, 27]. Rhizobacteria from the rhizosphere of different *Brassica* species varied greatly in their auxin production [28]. It has been shown that host genotype influences the enhancement of plant growth by auxin producing strains. In the present study, 47 % of isolates were positive for IAA production. Similarly, the host genotype had a drastic influence in selecting phosphate (P)-solubilizers. Only 35 of the 138 isolates were found to solubilize insoluble P, corresponding to a frequency of 25.3 %. The P requirement of rapeseed mustard is low or may be the root exudates do not stimulate P-solubilizers in the vicinity of roots of mustard.

Only 13 isolates, corresponding to a frequency of 9.4 % were able to inhibit *S. sclerotiorum* which is an important soil-borne fungal pathogen of mustard causing white mold disease in the crop. Since the three reference strains were not able to inhibit this fungus in vitro, it could be possible that the three antibiotics, phenazine, pyoluteorin and DAPG have no effect. This finding indicates that 13 isolates may produce some other metabolite which could

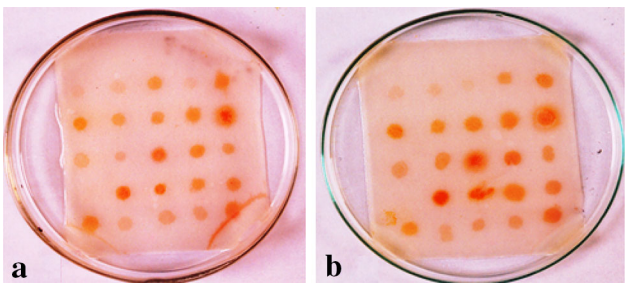

Fig. 1 Visualization of IAA producing colonies of fluorescent pseudomonads on nylon membrane without tryptophan (**a**); with tryptophan (5 mM mL^{-1}) (**b**)

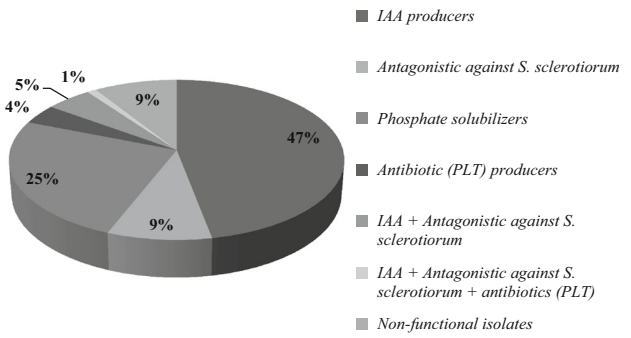

Fig. 2 Pie-diagram of percentage functional diversity in terms of plant growth promotion of fluorescent pseudomonads

suppress the pathogen [29]. The low frequency of antibiotic producing fluorescent pseudomonad isolates in rapeseed mustard rhizosphere is gaining much importance as no chemical control measures are so effective against white mold disease.

PCR Screening of FP for DAPG, PCA and PLT

The three reference strains Pf-5, Ps-Q2-87 and Ps-2-79 were PCR amplified for their respective antibiotic genes corresponding to *phl*D (DAPG) with 745 bp, *phz*C (phenazine) with 1150 bp and *plt*B (pyoluteorin) with 773 bp (Fig. 3a). None of the isolates showed the presence of the *phl*D and *phz*C codes for DAPG and phenazine genes (Fig. 3b, c). However, 4 isolates i.e. UKA-2, UKA-8, UKA-11, and UKA-66 showed the *plt*B gene amplification (~773 bp) (Fig. 3d). These four isolates were identified by biochemical profiles and the data was computed with PibWin software. All isolates were 95–99 % similar to fluorescent pseudomonads (Table 3).

It is generally accepted that pathogen inhibition by antibiotic metabolites is one of the primary mechanisms of biocontrol provided by these root colonizing bacteria [30]. In the rhizosphere of lily (*Lilium candidum* L.), *phl*D$^+$ pseudomonads were not detected, although it supported in an average the highest population densities of fluorescent *Pseudomonas* [31]. It is also reported that host plant species has significant influence on the composition and activity of specific indigenous antagonistic *Pseudomonas*. Raaijmakers et al. [17] could not detect any phenazine producer in roots of wheat grown in three soils. Similarly, in the present study, only four fluorescent isolates amplified the *plt*B gene, which indicated the lower frequency for this antibiotic in mustard rhizosphere.

TLC and Dot-Blot Hybridization of PLT-Producing Isolates

The four positive pyoluteorin producing isolates were further confirmed through TLC with reference strains Pf-5. Visualization of TLC plates under ultra violet light and spraying with DSA revealed only one isolate, UKA-66 showing the brown spot corresponding to PLT (Fig. 4a) and other three were undetectable by TLC.

To validate the negative PCR results a DNA dot blot experiment was carried out with all 138 FP onto nylon

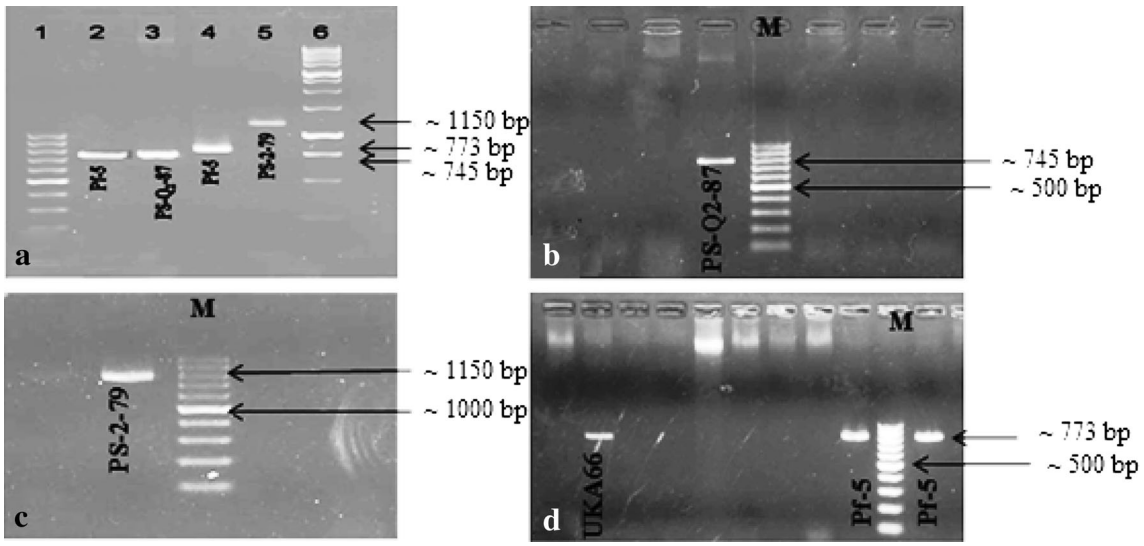

Fig. 3 PCR amplification of antibiotic genes for of 2,4-diacetyl phloroglucinol (DAPG), phenazine-1-carboxylic acid (PCA) and pyoluteorin (PLT). **a** Amplification of reference strains. *Lane 1* 100 bp DNA ladder; *Lane 2* Pf-5 (~745 bp); *Lane 3* Ps-Q_2-87 (~745 bp); *Lane 4* Pf-5 (~773 bp); *Lane 5* Ps-2-79 (~1150 bp); *Lane 6* 1 kb DNA ladder. **b** Gel picture of DAPG with reference strain Ps-Q_2-87. **c** Gel picture of phenazine with reference strain Ps-2-79. **d** Amplification of *pltB* gene (pyoluteorin)with reference strain Pf-5

Table 3 Biochemical profile of pyoluteorin antibiotic producing fluorescent pseudomonads

Biochemical profile	Fluorescent pseudomonads isolates			
	UKA 2	UKA 8	UKA 11	UKA 68
IMVIC				
a. Indole	+	+	+	+
b. MR	−	+	+	+
c. VP	−	−	+	−
d. Citrate	+	+	+	+
Mannitol motility				
a. Mannitol utilization	+	+	−	+
b. Motility	+	+	+	+
Urease	+	+	+	+
Gelatinase	+	+	+	+
Caesinase	+	+	+	+
Amylase	+	−	−	−
Chitinase	−	−	−	−
Nitrate reduction	−	+	+	−
Catalase	+	−	+	+
Oxidase	+	+	+	+
Aesculin hydrolysis	+	+	+	+
Lecithinase	−	−	−	−
Tributyrine hydrolysis	+	+	+	−
Tween 80 hydrolysis	+	+	+	−
Arginine dihydrolase	+	+	−	+
Cellulase	−	−	−	+
Carbon sources				
a. Fructose	+	+	+	+
b. Glucose	−	+	−	+
c. Sucrose	−	+	−	+
d. Mannose	+	+	+	+
e. Lactose	−	−	+	−
f. Raffinose	+	+	+	+
g. Rhamnose	+	+	+	+
h. Trehalose	+	−	+	+
i. Adonitol	−	−	+	+
j. Sorbitol	+	+	+	+
k. Inositol	+	−	−	+
l. Dulcitol	+	−	−	−

membranes. Dot blots were probed with a α-^{32}P labeled *plt*B (~773 bp) purified fragment from reference strain Pf-5. Strong signals were observed in PCR positive strains along with reference strain Pf-5; however 7 more isolates, UKA-32, UKA-33, UKA-68, UKA-71, UKA-83, UKA-124 and UKA-128 gave good detectable signals (Fig. 4b, c). DNA blotting experiments increased this number to 11, confirming that negative PCR is not always confirmatory. Eight blots were scored to have strong detectable signal. Two PCR positive isolates UKA-2 and UKA-11 gave faint signals, quite possibly because of heterologous sequences. However, TLC of the 4 PCR positive isolates gave the appropriate band only in one isolate (UKA-66) probably due to little concentration of the antibiotics produced by other isolates (since extraction was carried out with 5 mL of culture) so as to be undetectable by TLC.

Genetic Diversity of Pyoluteorin Producing Isolates

In the present study DNA fingerprints were generated for total chromosomal DNA for all the four isolates positive for the antibiotic pyoluteorin using BOX A1R primer. Distinct banding patterns were generated using the 22-mer oligonucleotide primer in combination with PCR conditions that favored the simultaneous amplification of multiple different sized DNA fragments. The range of amplified bands were found between 300 bp and >10 kb. Total number of bands scored were 31. No single band was uniformly present in these isolates showing a high degree of heterogeneity (Fig. 5a). Dendrogram generated of the BOX profiles confirmed the presence of polymorphism. Two clusters were generated with the similarity coefficient being less than 40 %. UKA-66 and UKA-2 were more similar to each other and UKA-8 was closer to UKA-11 (Fig. 5b).

It has been postulated that the genotypic diversity within a group of microorganisms that share the same antagonistic trait provides a largely untapped resource for improving biological control of soil borne pathogens [32]. Studies have supported the hypothesis that certain indigenous *phl*D$^+$ genotypes preferentially colonize the roots of specific crop plants. Similar type of findings were reported by other researchers [17, 18, 33]. In the present study, BOX-PCR distinguished 4 isolates amplifying *plt*B gene. The result showed that the isolate UKA-8 was positive for *plt*B, IAA production and antagonistic activity. Isolate UKA-66 was positive for *plt*B, IAA production and P-solubilization, isolate UKA-2 was positive for IAA and *plt*B, but UKA-11was positive only for *plt*B. However, the BOX profiles of each of these were unique and different indicating the genetic heterogeneity existing in these isolates. The present findings will further help to identify the efficient PGPR strains from the mixed heterogenous rhizosphere population.

Conclusion

Overall, the present study revealed the lower frequency and diversity of plant growth promoting traits of FP in IRM. It was also demonstrated that out of three antibiotics (DAPG, phenazine and pyoluteorin), none of the isolates produced DAPG and phenazine and only 4 isolates produced

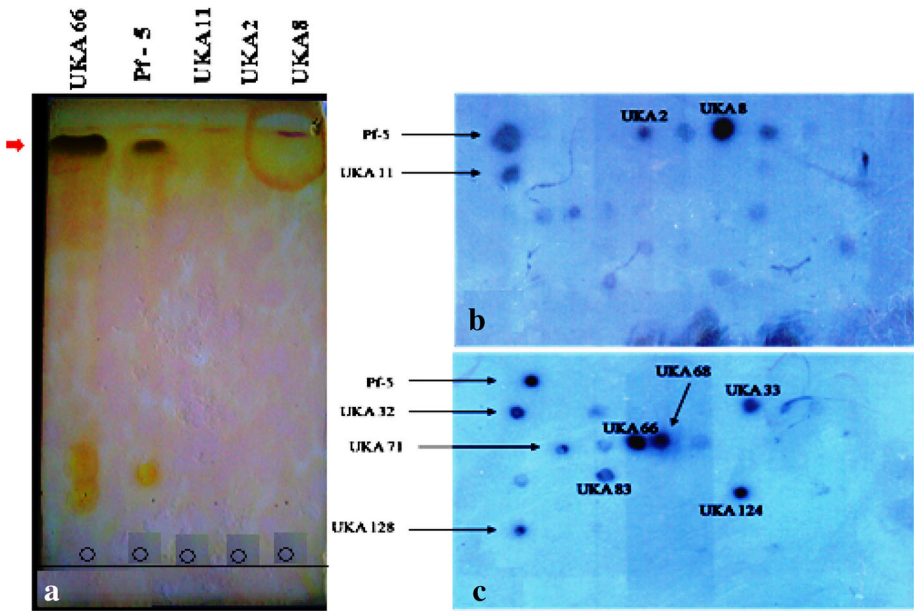

Fig. 4 Thin layer chromatography for pyoluteorin producing strains; UKA-66 showed a spot reactive with DSA with retardation factor (Rf): 0.65 similar to reference strain Pf-5 spot for pyoluteorin (**a**). Dot blot hybridization for detecting *plt*B gene in fluorescent pseudomonad isolates (**b**, **c**)

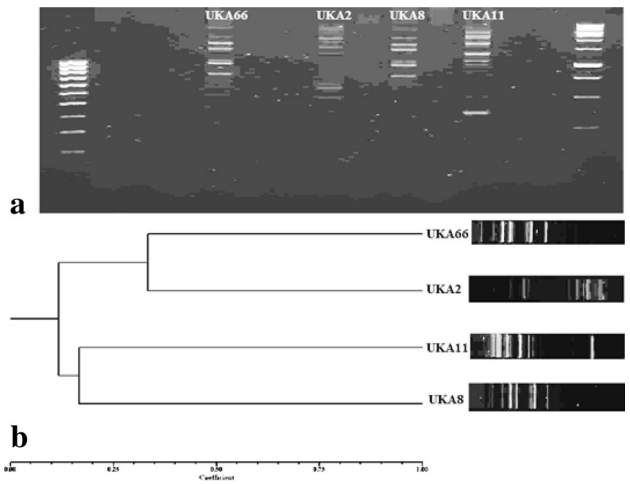

Fig. 5 BOX-PCR based genomic fingerprinting of pyoluteorin producing isolates. *Lane 1* 100 bp DNA ladder; *Lane 2* UKA66; *Lane 3* UKA 2; *Lane 4* UKA 8; *Lane 5* UKA 11; *Lane 6* 1 kb DNA ladder (**a**). Dendrogram showing the genetic relationship among PLT producing isolates (**b**)

pyoluteorin which further confirmed the lower frequency of antibiotic producing FP in IRM rhizosphere.

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Compliance with Ethical Standards

Conflict of interest All authors declares that there are no conflicts of interest.

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