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Original article

Fluorescent pseudomonads (FPs) as a potential biocontrol and plant growth promoting agent associated with tomato rhizosphere

Perumal Suresh [a,1], Shanmugaiah Vellasamy [a,*,1], Khalid S. Almaary [b], Turki M. Dawoud [b], Yahya B. Elbadawi [b]

[a] Department of Microbial Technology, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, Tamil Nadu, India
[b] Department of Botany and Microbiology, College of Science, King Saud University, P.O. 2455, Riyadh 11451, Saudi Arabia

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ABSTRACT

A total of 87 FPs have been isolated from tomato *(Lycopersicon esculentum)* rhizosphere and evaluated by dual plate assay method for the antimicrobial activity of *Ralstonia solanacearum*. Of which, 30 FPs showed antagonistic activity against *R. solanacearum* with the zone of inhibitions (ZOI) ranging from 19 mm to 28 mm. Similarly, antagonistic FPs significantly controlled various fungal phytopathogens such as *Rhizoctonia solani* (6.3 mm−20 mm), *Macrophomina phaseolina* (8.7 mm−19.3 mm), *Fusarium oxysporum* (7.3 mm−30.7 mm), and *Sclerotium rolfsii* (5.3 mm−21 mm). The cell-free culture filtrate of *Pseudomonas* sp VSMKU3054 significantly suppressed the fungal pathogens and increased the root and shoot length of the tomato seedlings compared to the control. Furthermore, the antagonistic FPs effectively produced lytic enzymes and antimicrobial traits such as amylase (6), protease (30), cellulase (28), pectinase (12), chitinase (22), gelatinase (27), siderophore (28), hydrogen cyanide (25), phosphate solubilization (30), and Indole acetic acid (IAA) (23). Genetic diversity of FPs was assessed by BOX-PCR with specific primers revealed that two distinct clusters were observed, whereas, RFLP analyses were showed that 5 to 16 bands ranged from 75 bp to 1.2 bp with two major clusters using restriction enzyme *Hae*III. The antibiotic encoding genes were detected from FPs, of which 10 FPs were positive for 2,4-diacetylphloroglucinol (DAPG), 9 FPs were positive for pyoleuterin (PLT), 10 FPs were positive for hydrogen cyanide (HCN) and none of them obtained pyrrolnitrin (PRN) with respective primers. Based on the superior antagonistic isolates against *R. solanacearum* and phytofungal pathogens and other antimicrobial traits, the isolate VSMKU3054 was selected for further studies. Based on the morphological, physiological and 98% of 16 s rDNA sequence similarity, the selected isolate VSMKU3054 was identified as *Pseudomonas fluorescens*. Further, the potential isolate *P. fluorescens* VSMKU3054 and cell free culture filtrate remarkably enhance tomato seedling growth such as root and shoot length, fresh and dry weight and vigor index compared to control. This study indicated that rhizospheric isolates of FPs have more potential for plant growth promotion and plant protection from plant pathogenic microbes.

1. Introduction

Beneficial microbes like *Pseudomonas* spp are colonizing plant roots and protect the plants from plant pathogens by the secretion of plant growth promoting substance, antimicrobial compounds and pathogenic related proteins (Ashajyothi et al., 2020; Agaras et al., 2020). Plant growth prompting rhizobacteria (PGPR) were includes *Azospirillum, Azotobacter, Pseudomonads, Bacillus, Streptomyces, Enterobacter, Clostridium, Burkholderia,* and *Stenotrophomonas* (Sheoran et al., 2015). FPs act as PGPR and produce a multiple level of plant growth-promoting substances like indole −3- acetic acid (IAA) (Ricci et al., 2019), aminocyclopropane-1-carboxylate (ACC) deaminase (Win et al., 2018) and insoluble phosphates are solubilized by phosphate solubilizing bacteria like FPs (Naik et al., 2008). Root associated FPs are controlled various phytopathogens with different mechanisms such as volatile substances, antibiosis and hydrolytic enzymes such as chitinase and protease production (Shanmugaiah et al., 2008; Köhl et al., 2019).

* Corresponding author.
E-mail address: shanmugaiahv@gmail.com (S. Vellasamy).
[1] These authors contributed equally to this work.
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Antagonistic microorganisms are mostly isolated from rhizosphere, among them *Pseudomonas* spp is a promising biocontrol agent without extensive studies (Xue et al., 2013). FPs naturally occurs in rhizosphere; they are non-pathogenic and produce secondary metabolites to control the soil-borne plant pathogens (Raaijmakers et al., 2002). *Pseudomonas* spp are plant growth prompting rhizobacteria (PGPR) and potential biocontrol agent to suppress various plant pathogenic bacteria like *R. solanacearum* (Lemessa and Zeller, 2007). Furthermore, FPs is a prominent group of rhizospheric microorganisms that are ecologically very important microorganism for plant growth promotion and biocontrol properties (Salman, 2010).

In recent days, due to high demand of tomato production based on its high potential both food and agriculture sector, tomato production has been increased. About 15 to 55% tomato crop loss has been reported by bacterial wilt vascular pathogen *R. solanacearum*. Whereas in India, on average up to 10 to 20% were seen in some cases 90% (Rai et al., 2017). Chemically synthesis antibiotics, fungicides, pesticides, and weedicides have been used for several years for the protection of plant fungi and bacterial pathogens. But many regulations on the use of chemicals have been enforced in recent days. Because, they are creating major environmental and public risks and health hazards. Significant problems have been caused by chemical-based treatments, such as decreased productivity, fruit taste and yield loss, because of chemical fungicide persistent in to soil and environment. Moreover, the chemical fungicide could not be degradable from the environment. Hence it will create more environment problems and health hazards (Pertot et al., 2008). In addition, chemical treatments for plant diseases are costly and harm to the environment and public health (Shanmugaiah et al., 2010, 2015).

In this contest alternative strategies were developed for the management of plant diseases and encourage plant growth instead of using chemical fertilizers, fungicides and pesticides for the development of environment friendly and sustainable ecosystem. Hence, the use of antagonistic microorganism for plant growth-promotions are wise choice with a broad spectral and widespread alternative approach to control plant pathogens (Shanmugaiah et al., 2006; Harikrishnan et al., 2014). Studies were shown that the management of bacterial wilt disease and other phytopathogens could be successfully controlled by the application of biocontrol agents like *Pseudomonas* sp. (Shanmugaiah et al., 2010; Nithya et al., 2020; Elsayed et al., 2020), *Bacillus* (Shanmugaiah et al., 2008; Rais et al., 2017; Wang et al., 2019), *Streptomyces* (Harikrishnan et al., 2016; Kaur et al., 2019) and *Trichoderma* (Shanmugaiah et al., 2009; Mazrou et al., 2020) to control various plant pathogenic bacterial and fungal organism (Vanitha and Umesha, 2011).

In addition, to understand molecular plant microbe interaction and ecological function in the rhizosphere and other various molecular and biotechnological applications for the rhizosphere and root residing bacteria and their biocontrol potential are most essential. Rice, banana, sugarcane, and tomato are most important food crops in the world and they are widely grown in developing countries. But, the bacterial and fungal pathogens are constrains and most important for the production of rice, banana, sugarcane, tomato, and other crops (Naik et al., 2008). Indiscriminate use of chemical fungicides and chemically synthesized antibiotics in agriculture is detrimental to the environment and lethal to other beneficial micro-organisms for the control of phytopathogens. (Giorgio et al., 2016). Hence, in this juncture need to develop antagonistic microbes are resistant towards pathogenic microbes. Further, the multiple applications of antibiotics and plant growth-promoting molecules and other substance produced by *Pseudomonas* spp are most important to study their diversity, through which to find out novel indigenous bio inoculants for sustainable and bioorganic fertilizer without causing any environmental pollution and harmful effect to farmers.

The indigenous antagonistic rhizobacteria are potentially enhanced the control of soil-borne plant pathogens because native isolates are more effective than exotic isolates (Cabanas et al., 2018; Chenniappan et al., 2019). *Pseudomonas* spp are well known producer of antimicrobial metabolites like DAPG, but as per our literature survey FPs from tomato rhizosphere and it also modify stress hormones and antioxidant expression in abiotically stressed plants that would be useful for initiation of plant defense mechanisms (Duke et al., 2017). Hence, the present study was carried out with the following objectives i) isolation and characterization of FPs for the effective control of phytopathogens ii) Detection of antibiotic encoding gens from FPs isolated from tomato rhizosphere iii) Functional characterization and plant growth promoting traits of FPs iv) To study, the genetic diversity of FPs by RFLP and BOX-PCR.

2. Materials and methods

2.1. Isolation of fluorescent pseudomonads

Samples of the tomato rhizosphere were collected from various locations in the Madurai district, Tamil Nadu, India. 10 g of tomato rhizosphere samples were shaken in 90 ml of sterilized water at 120 rpm for 30 min (Shanmugaiah et al., 2006). The soil suspension was diluted serially and spread on King's B agar (King et al., 1954). The plates were incubated for 48 h at 37 °C and viewed at 365 nm under a UV illuminator. The purified single colonies were preserved in 30% glycerol at −80 °C.

2.2. Screening of FPs against phytopathogens

The antagonistic behaviors of all FP isolates were screened towards bacterial tomato wilt pathogen *R. solanacearum* on Nutrient Agar (NA) by dual plate assay method (Zhou et al., 2012). The *R. solanacearum* (10^7 CFU/ml), similarly all FPs isolates (10^7 CFU/ml) inoculated on Nutrient Agar with three replicates on the periphery of Petri plate. After 2 days of incubation at 28 °C, the inhibition zone was measured.

Similarly, FPs isolated from tomato rhizosphere were assayed against phyto fungal pathogens such as *R. solani, S. rolfsii, M. pheaseolina,* and *F. oxysporum* on PDA medium in triplicate by dual plate assay method as per Shanmugaiah et al. (2010). Nine millimeters of mycelial discs were cut from the young growing edge of the fungus with a sterilized cork borer and placed in the centre of the Petri dish. The 24 bacterial isolates were punctiform inoculation aseptically at the periphery of the PDA plate and incubated for 5 days at 28 ± 2 °C. For every isolate, there were three duplications kept. The inhibition zone was measured in the nearest millimeter between the two cultures.

2.3. Biochemical tests of FPs

All FPs were chosen for further characterization by standard biochemical tests based on the promising antagonistic behavior compared to the control (Nithya et al., 2019). The following tests were conducted for Gram's reaction, catalase and oxidase activity, Indole, VP, MR, citrate utilization and shape of the isolates. Outcomes of these assessments were recorded as either positive or negative.

2.4. Production of lytic enzyme and antimicrobial traits

All antagonistic isolates of FPs were tested for hydrolytic enzyme production by the qualitative assay method

(Shanmugaiah et al., 2008). The hydrolytic enzyme production assay was performed to determine the production of protease, gelatinase, amylase, cellulase, chitinase, and pectinase for all isolates of FPs on nutrient agar supplemented with 1% of the respective substrates (Ayyadurai et al., 2006). A clear zone around the colonies of FPs was showed hydrolytic enzyme activity after 48 h of incubation.

2.5. Antimicrobial metabolites characterization

2.5.1. Hydrogen cyanide (HCN)

HCN development was accomplished as stated by Lorck (1948). Briefly, fresh isolates of FPs were grown in nutrient sucrose medium (NSM) supplement with 4.4% of glycine. Sterilized Whatman No1 filter paper soaked with a solution containing 1% picric acid and 2% sodium carbonate and placed on the lid of the petri dish. To prevent gas exchange from the inside of the Petri dish, the plates were tightly sealed with parafilm and incubated for 48 h. A change in the color of the filter paper from yellow to orange, indicating the production of HCN.

2.5.2. Siderophore

Siderophore creation by FPs was determined by Chrome Azurole S (CAS) assay by the method of Alexander and Zuberer (1991). The FPs was inoculated in cetrimide agar and incubated for 24 h. After incubation, 0.7% of CAS agar was superimposed on cetrimide agar and the plates were incubated at 28 °C for 24 h. The production of siderophore was indicates the change of color from blue to brownish orange.

2.5.3. Indole acetic acid (IAA)

The development of indole acetic acid by the FPs isolates was evaluated using the method of Bric et al. (1991). In King's B broth, the FPs were inoculated with 0.4 percent

L-tryptophan supplementation and incubated for 24 h. After 10,000 rpm centrifugation for 15 min, the 2 ml of cell-free culture filtrate was combined with 4 ml of reagent from Salkowski (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$). The mix was incubated for 20 min at room temperature.

2.5.4. Phosphate solubilization

Each isolates of FPs were punctiform inoculation on Pikovskaya's agar (Pikovskaya, 1948). At 28 ± 2 °C for 48 h, the plates were incubated. The creation of a clear zone around the colony was considered as positive for solubilization of insoluble phosphate compared to control.

2.6. DNA extraction

By the heat lysis method, genomic DNA was extracted from FPs (Keel et al., 1996). Briefly, a loopful of fresh colonies of FPs were suspended in 100 µl of lysis solution (50 mM KCl, 0.1% Tween 20, 10 mM Tris HCl, pH-8.3) and incubated at 99 °C for 10 min. After that bacterial cell suspension was centrifuged at 5000 rpm for 1 min. For 30 min, the heat-lysed bacterial suspension was frozen at −20 °C.

2.7. BOX – PCR and RFLP analysis for genomic fingerprinting

All the antagonistic isolates of FPs were subjected to BOX-PCR and RFLP analysis was performed with the procedure followed from previous studies (Nithya et al., 2020; Rana et al., 2014; Saikia et al., 2011) BOX-PCR are carried out with BOX-A1R primer (5′-CTACGGCAAGG CGACGCTGACG-3′). To perform RFLP analysis, amplified 16S rDNA product are digest with *Hae* III restriction enzyme. BOX PCR and RFLP genomic fingerprinting computer assisted analysis were performed by GelClust software to construct phylogenetic tree, for which neighbor-joining method and unweighted pair group with mathematical average (UPGMA) algorithms were used (Moin et al., 2020).

2.8. Detection of antimicrobial encoding genes by PCR

Antibiotic encoding genes such as 2, 4-diacetylphloroglucinol (*phlD*), pyoluteorin (*pltB*), pyrrolnitrin (*prnA*), and hydrogen cyanide (*hcnBC*) were detected from 30 FPs and amplified by PCR (Smart PCR, Cyber Lab, USA) using previously reported gene-specific respective primers (Table 1). As reference strains, positive controls were used, *P. fluorescens* CHAO, and *P. fluorescens* Pf5. 25 µl of PCR reaction was carried out included 25 ng of template DNA, 1x thermo-DNA buffer, 1.5 $mmol^{-1}$ $MgCl_2$, 0.2 $mmol^{-1}$ of each dNTP, 20 pmol of each primer, 1 U of *Taq* DNA polymerase (GeNet Bio, India). A 5 µl of aliquot of each amplification product was electrophoresed on a 1 percent agarose gel in 1x Tris-acetate- EDTA (TAE) buffer at 50v for 45 min, stained with ethidium bromide, and PCR products were visualized with Gel documentation.

2.9. 16 s rDNA identification of Pseudomonas sp. VSMKU3054

The selected isolates of *Pseudomonas* sp VSMKU3054 genomic DNA was amplified with 16S rDNA primer 27F (5′-AGAGTTT GATCCTGGTCAGAACGCT-3′) and 1492R (5′- TACGGCTA CCTTGTTACGACTTCACCCC-3′) (Rana et al., 2014). Phylogenetic tree was constructed by unweighted pair group of arithmetic mean analysis method and tree was evaluated by the bootstrap method using the software MEGA 4.

2.10. Influence of P. fluorescens VSMKU3054 on tomato seed germination

Tomato seeds germination was assessed treated with *P. fluorescens* VSMKU3054 under *in vitro* conditions. Bacterial wilt susceptible tomato seeds (PKM1) were purchased from Horticultural College & Research Institute, Periyakulam, Theni District, Tamil

Table 1
List of gene-specific primers used in this study.

Primers	Encoding gene	Sequence (5′ → 3′)	PCR Conditions	Product size (bp)	References
B_2BF	*phlD*	ACCCACCGCAGCATCGTTTATGAGC	95 ℃ for 3 min, 30 cycles at 94℃ for 60 sec, 57.5 ℃ for 60 sec, 72℃ for	629	Immanuel et al.,
BPR_4		CCGGTATGGAAGATGAAAAAGTC	60 sec, 72 ℃ for 5 min, 4 °C-∝.		2012
PltB -F	*pltB*	CGGAGCATGGACCCCCAG	95 ℃ for 2 min, 30 cycles at 94 ℃ for 60 sec, 67 ℃ for 45 sec, 72 ℃ for	850	Mavrodi et al., 2001
PltB -R		GTGCCCGATATTGGTCTTGACC	60 sec, 72 ℃ for 10 min, 4 °C-∝.		
PrnA -F	*prnA*	GTGTTCTTCGACTTCCTCGG	95 ℃ for 3 min, by 30 cycles at 94 ℃ for 30 sec, 63 ℃ for 45 sec, 72 ℃ for	1050	de Souza and
PrnA -R		TGCCGGTTCGCGAGCCAGA	60 sec, 72℃ for 5 min, 4 °C-∝.		Raaijmakers 2003
Hen -F	*hcnBC*	ACTGCCAGGGGCGGATGTGC	95 °C- 3 min; 94 °C for 30 s, 63 °C – 45 s; 72 °C for 1 min for 30	600	Ramette et al., 2006
Hen –R		ACGATGTGCTCGGCGTAC	cycles and 72 °C for 5 min, 4 °C-∝		

phlD: 2, 4-diacetylphloroglucinol; *pltB*: pyoluteorin; *prnA*: pyrrolnitrin and *hcnBC*: hydrogen cyanide.

Nadu, India. The seeds were sterilised with 1 percent sodium hypochlorite for 2 min and washed three times with sterile distilled water, followed by blot drying. *P. fluorescens* VSMKU3054 and *R. solanacearum* were grown in the respective medium for 24 h and bacterial cells were collected by centrifugation at 10,000 rpm for 20 min. The pellets were resuspended by distilled water and the bacterial cell suspension was adjusted to 0.1O.D. at 600 nm (≈1 X 10^8 CFU). The seeds were immersed with bacterial cultures for 30 min and seeds were germinated on moist blotter paper placed with 25 Petri dishes. About 25 seeds per plate were placed with the standard procedure as described by the International Seed Testing Association. The plates were incubated at 28 ± 2 °C for 10 days. The seeds were treated with distilled water was considered as negative control (Vanitha and Umesha, 2011).

2.11. Statistical analysis

Statistical analysis was carried out with statistical tools from SPSS 16.0 (Statistical Software for the Social Sciences). The data in all groups were analyzed by one-way analysis of variance (ANOVA). If the P-values were < 0.05 the findings were deemed statistically important.

3. Results

3.1. Isolation and antagonistic activity of FPs

A total of 87 FPs was isolated from tomato rhizosphere. Of which, 30 FPs were exhibited potential antagonistic activity against *R. solanacearum*. Among them an isolate *Pseudomonas* sp VSMKU3054 was chosen for additional studies based on the more antibacterial activity against *R. solanacearum* (28 mm) as compared to control (Table 2 and Fig. 1a & b). All the 30 antagonistic isolates of FPs were exhibited remarkable antifungal activity with various levels of the zone of inhibition (ZOI) against different fungal phytopathogens such as *R. solani* (20 mm), *S. rolfsii* (21 mm), *M. phaseolina* (19.3 mm), and *F. oxysporum* (30.7 mm) compared to control tested on dual plate. The ZOI ranged from 5 to 31 mm (Table 2 and Fig. 1c–f).

3.2. Morphological and physiological characterization

All the antagonistic isolates of FPs were tested for phenotypic characterization by morphological and various biochemical tests. All antagonistic isolates were Gram-negative, rod shape, motile, positive for catalase, oxidase and citrate consumption, whereas negative for IMVIC (Table 3). The productions of hydrolytic enzymes by antagonistic FPs were showed positive with different levels of the zone of clearance such as amylase (6), protease (30), cellulose (28), pectinase (12), gelatinase (27) and, chitinase (22) compared to control (Table 4).

3.3. Production of antimicrobial and plant growth-promoting traits by FPs

Among 30 FPs, 27 and 25 FPs were showed positive for siderophore and hydrogen cyanide production. All 30 FPs were showed positive for phosphate solubilization. Of the 30 FPs, 23 isolates of FPs were showed positive for the production of Indole acetic acid (IAA) (Table 4).

3.4. Genotypic fingerprinting analysis

3.4.1. Box PCR

The cluster analysis was performed based on the pair-wise constant similarity with UPGMA of BOX-PCR. A total of two distinct genomic clusters and 85% similarity coefficient generated 16 distinct BOX profiles with 160 bp to 6 kb (Fig. 2a and b). A total of

Table 2
Antagonistic activity of FPs against phytopathogens.

Isolates of FPs	*R. solanacearum* (mm)	*R.solani* (mm)	*S. rolfsii* (mm)	*M. phaseolina* (mm)	*F. oxysporum* (mm)
VSMKU3001	26.7 ± 1.15	10 ± 1	14.3 ± 1.15	8.7 ± 0.58	8.7 ± 2.08
VSMKU3003	22 ± 0	8 ± 1	11.3 ± 0.58	11 ± 1.15	11 ± 2
VSMKU3004	23.3 ± 0.58	9.3 ± 0.58	9 ± 1	11.7 ± 1.15	8.3 ± 2.08
VSMKU3005	19 ± 1	8 ± 0	7.7 ± 0.58	11 ± 2.65	7.3 ± 0.58
VSMKU3007	22.7 ± 0.58	–	8 ± 0	–	10 ± 1
VSMKU3009	25.7 ± 2.08	7.3 ± 0.58	5.3 ± 0.58	9.7 ± 0.58	8.3 ± 0.58
VSMKU3011	22.7 ± 0.58	8.3 ± 0.58	10.7 ± 0.58	10 ± 1.7	8 ± 1
VSMKU3012	24.3 ± 0.58	12 ± 1	14 ± 0	15.3 ± 2.08	21.7 ± 1.53
VSMKU3021	19.7 ± 0.58	12.7 ± 0.58	13.3 ± 1.15	20 ± 1	25.7 ± 1.53
VSMKU3022	20.3 ± 1.15	12.7 ± 0.58	10.7 ± 0.58	19.3 ± 0.58	26 ± 1
VSMKU3024	20 ± 1	13.3 ± 1.53	14 ± 1	17.7 ± 1.53	27 ± 1
VSMKU3025	19.3 ± 0.58	12.7 ± 1.53	11.7 ± 1.15	18.7 ± 1.53	25 ± 1
VSMKU3029	25 ± 1	12.3 ± 0.58	13.3 ± 0.58	18.7 ± 1.53	25.7 ± 1.15
VSMKU3030	21 ± 1	11 ± 0	14.3 ± 1.15	17.7 ± 1.15	27.7 ± 1.15
VSMKU3032	21 ± 1	10 ± 1	10.7 ± 0.58	16 ± 1	26.3 ± 0.58
VSMKU3034	24.7 ± 1.15	8.7 ± 1.15	10.3 ± 1.53	18 ± 1	26.3 ± 1.15
VSMKU3035	22 ± 1	6.3 ± 1.15	13 ± 0	18.7 ± 0.58	30.7 ± 0.58
VSMKU3036	20.7 ± 0.58	15 ± 1	15.3 ± 0.58	13 ± 1	16.3 ± 2.31
VSMKU3038	21.7 ± 0.58	15.7 ± 1.15	21 ± 1	11 ± 1	16.7 ± 0.58
VSMKU3044	23.4 ± 0.58	14 ± 1	16.6 ± 0.58	14.7 ± 1.53	16 ± 1.73
VSMKU3049	24.3 ± 0.58	16 ± 0	16.7 ± 0.58	14.7 ± 1.53	15 ± 1
VSMKU3054	28 ± 1	16.3 ± 3.21	13.7 ± 1.53	11 ± 1	11.7 ± 0.58
VSMKU3055	22.7 ± 0.58	15.7 ± 0.58	10 ± 0	13.7 ± 0.58	20.3 ± 1.53
VSMKU3062	22.7 ± 2.08	12.7 ± 0.58	13.3 ± 0.58	11.7 ± 0.58	20 ± 1
VSMKU3063	21.7 ± 1.15	12.3 ± 0.58	10 ± 1	13 ± 2.65	20 ± 2.65
VSMKU3065	20.7 ± 2.30	17 ± 1	9.3 ± 1.53	11.7 ± 1.53	14 ± 2.65
VSMKU3066	21 ± 1	15.3 ± 1.53	10.3 ± 1.53	12.3 ± 1.15	15.3 ± 2.08
VSMKU3076	22.7 ± 0.58	18 ± 2	13 ± 0	13.7 ± 1.53	12.7 ± 2.08
VSMKU3078	23.7 ± 0.58	20 ± 0	12.7 ± 0.58	12.3 ± 2.08	13.7 ± 1.53
VSMKU3080	22.3 ± 0.58	18 ± 1	7 ± 1	15.3 ± 1.53	15 ± 1.73

Values are triplicates with standard deviation.

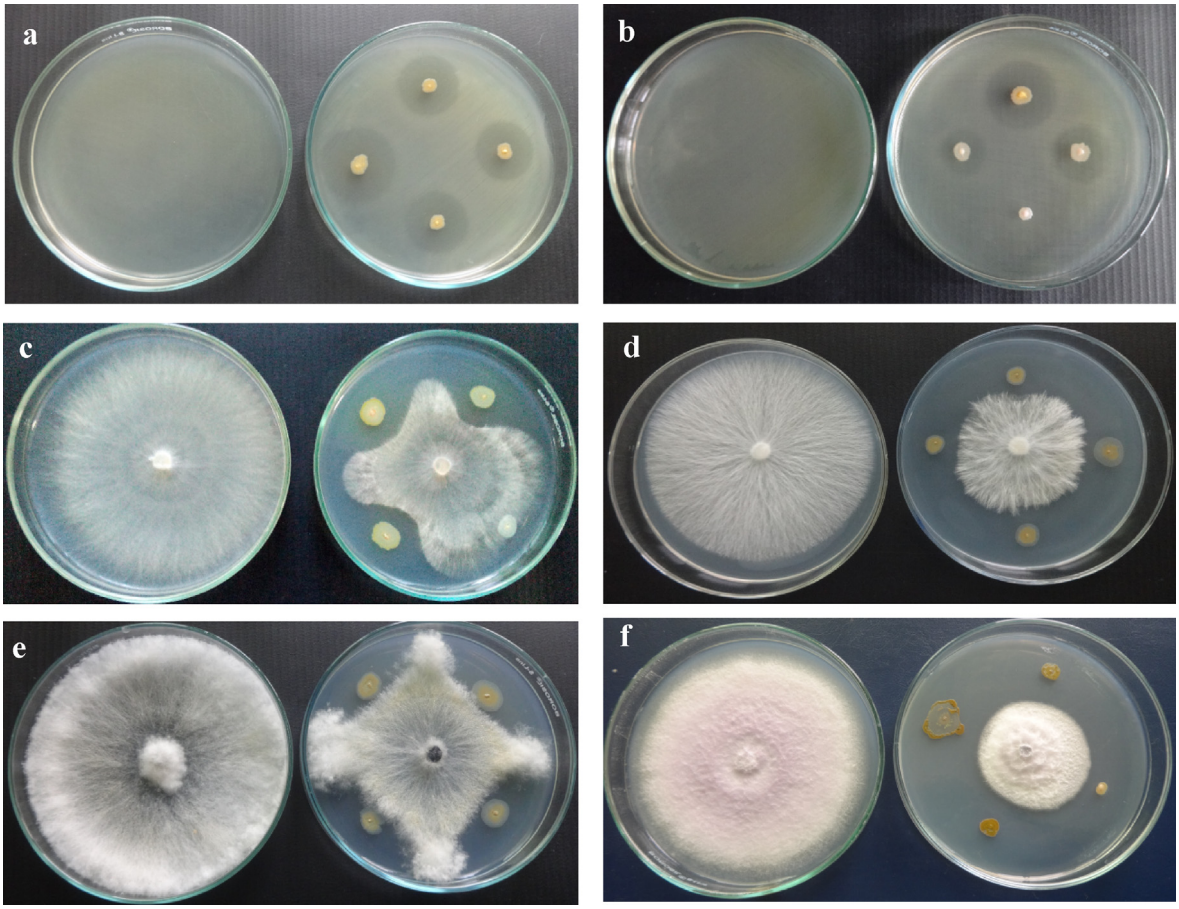

Fig. 1. Antagonistic activity of FPs against *R. solanacearum* and fungal plant pathogens: a & b - *R. solanacearum* + FPs, c - *R. solani* + FPs, d - *S. rolfsii* + FPs, e - *M. phaseolina* + FPs and f- *F. oxysporum* + FPs.

Table 3
Biochemical Characterization of FPs.

FP's	Gram's Reaction	Shape	Indole	VP	MR	Citrate Utilization	Catalase	oxidase
VSMKU3001	–	Rod	–	–	–	+	+	+
VSMKU3003	–	Rod	–	–	–	+	+	+
VSMKU3004	–	Rod	–	–	–	+	+	+
VSMKU3005	–	Rod	–	–	–	+	+	+
VSMKU3007	–	Rod	–	–	–	+	+	+
VSMKU3009	–	Rod	–	–	–	+	+	+
VSMKU3011	–	Rod	–	–	–	+	+	+
VSMKU3012	–	Rod	–	–	–	+	+	+
VSMKU3021	–	Rod	–	–	–	+	+	+
VSMKU3022	–	Rod	–	–	–	+	+	+
VSMKU3024	–	Rod	–	–	–	+	+	+
VSMKU3025	–	Rod	–	–	–	+	+	+
VSMKU3029	–	Rod	–	–	–	+	+	+
VSMKU3030	–	Rod	–	–	–	+	+	+
VSMKU3032	–	Rod	–	–	–	+	+	+
VSMKU3034	–	Rod	–	–	–	+	+	+
VSMKU3035	–	Rod	–	–	–	+	+	+
VSMKU3036	–	Rod	–	–	–	+	+	+
VSMKU3038	–	Rod	–	–	–	+	+	+
VSMKU3044	–	Rod	–	–	–	+	+	+
VSMKU3049	–	Rod	–	–	–	+	+	+
VSMKU3054	–	Rod	–	–	–	+	+	+
VSMKU3055	–	Rod	–	–	–	+	+	+
VSMKU3062	–	Rod	–	–	–	+	+	+
VSMKU3063	–	Rod	–	–	–	+	+	+
VSMKU3065	–	Rod	–	–	–	+	+	+
VSMKU3066	–	Rod	–	–	–	+	+	+
VSMKU3076	–	Rod	–	–	–	+	+	+
VSMKU3078	–	Rod	–	–	–	+	+	+
VSMKU3080	–	Rod	–	–	–	+	+	+

+ indicates positive reaction; – indicates negative reaction.

Table 4
Characterization of hydrolytic enzymes, secondary metabolites and plant growth hormone production of FPs.

FP's	Amylase	Protease	Cellulase	Pectinase	Chitinase	Gelatinase	Siderophore Production	HCN	IAA	Phosphate Solubilization
VSMKU3001	–	+	+	–	+	+	+	–	+	+
VSMKU3003	–	+	+	–	+	+	+	–	+	+
VSMKU3004	–	+	+	–	+	+	+	–	–	+
VSMKU3005	–	+	+	–	+	+	+	–	–	+
VSMKU3007	–	+	+	–	+	+	+	+	+	+
VSMKU3009	–	+	+	–	–	+	+	+	+	+
VSMKU3011	–	+	+	–	+	+	–	–	+	+
VSMKU3012	–	+	+	–	+	+	+	+	+	+
VSMKU3021	–	+	+	–	+	+	+	+	+	+
VSMKU3022	–	+	+	–	+	+	–	+	+	+
VSMKU3024	–	+	+	–	+	+	+	+	+	+
VSMKU3025	–	+	+	–	+	+	+	+	+	+
VSMKU3029	–	+	–	–	+	+	+	+	+	+
VSMKU3030	–	+	+	–	+	+	+	+	+	+
VSMKU3032	–	+	+	–	+	+	+	+	+	+
VSMKU3034	–	+	+	–	+	+	+	+	–	+
VSMKU3035	–	+	+	–	+	+	+	+	+	+
VSMKU3036	–	+	+	+	+	+	+	+	+	+
VSMKU3038	–	+	–	+	–	+	+	+	–	+
VSMKU3044	+	+	+	+	+	+	+	+	+	–
VSMKU3049	+	+	+	+	+	+	+	+	+	+
VSMKU3054	+	+	+	+	+	+	+	+	+	+
VSMKU3055	–	+	+	+	+	+	+	+	–	+
VSMKU3062	–	+	+	+	–	–	+	+	+	+
VSMKU3063	–	+	+	–	–	–	+	+	+	+
VSMKU3065	–	+	+	+	–	+	+	+	–	+
VSMKU3066	+	+	+	+	–	–	+	+	+	+
VSMKU3076	+	+	+	+	+	+	+	+	+	+
VSMKU3078	–	+	+	+	–	+	+	+	–	+
VSMKU3080	+	+	+	+	+	+	+	+	+	+

+ indicates positive reaction; – indicates negative reaction.

16 isolates, 60 percent identical to other strains, were grouped into cluster I. Cluster II consisted of 14 isolates which, compared to other strains, were 40% identical. Due to their high degree of genetic heterogeneity, all antagonistic isolates of FPs displayed substantial variations in fingerprinting patterns and distributed into different clusters. The current study showed a high degree of genetic heterogeneity and distribution between different isolates of FPs in different clusters. The present study detected a high degree of genetic variability among different species of FPs based on our findings.

3.4.2. Restriction fragment length polymorphism (RFLP)

The PCR product dependent RFLP (16 s rDNA) was performed using a *Hae* III restriction enzyme with genomic fingerprinting process. A total of 30 FPs and reference strains were amplified with primers 27F and 1492R. Gel electrophoresis of the undigested PCR products were exposed that all the isolates produced a single band, about 1500 bp (Fig. 3a and b). Genetic fingerprinting analysis exhibited massive variation among the isolates of FPs**.** The fingerprinting pattern after digestion with *Hae* III exposed 16 different restriction patterns in the range of 75 bp to 1.2kp. A dendrogram was constructed and divided into two major clusters. A total of 28 isolates were grouped in Cluster I and further they were divided into four subs clads. Sub-clads 1 comprises nine isolates, 2 comprising fifteen isolates, 3 comprising one isolate, and 4 comprising four isolates. The cluster II presented only two isolates. The data analysis of RFLP similarities was done using Jaccard's coefficient.

3.5. Detection of antibiotic encoding genes in FPs

The presence of phenolic and polyketide related genes *phlD*, *pltB*, *prnA,* and *hcnBC* were detected using specific primers. The amplification of DAPG encoding gene with size 629 bp in 10 FPs out of 30 isolates (Fig. 4a), In case of PLT encoding gene, about 12 isolates were identified with an amplicon size of 850 bp out of 30 isolates (Fig. 4b), Similarly, the amplicon size of HCN encoding genes was 600 bp from 26FPs among 30 FPs (Fig. 4c), whereas none of the isolates of FPs did not showed the presence of PRN gene.

3.6. 16 s rDNA sequence analysis

16 s rDNA gene sequence was carried out subjected to BLAST analysis. The sequence was submitted to NCBI with the GenBank accession no. MH443348. The numerous sequence arrangements was done using CLUSTALW for phylogenetic tree construction, a phylogenetic tree was created using the neighbor-joining method by MEGA4 software. Based on the morphology, physiochemical and 16 s rDNA analysis, the isolate VSMKU3054 was identified as *P. fluorescence* with 98% similarity comparison with other isolates of *Pseudomonas* spp nucleotide sequence obtained from NCBI (Fig. 5).

3.7. Effect of P. fluorescens VSMKU3054 on tomato seed germination

P. fluorescens VSMKU3054 and their cell-free culture filtrate significantly enhanced seed germination of tomato at 10^8 CFU and 5 ml of cell-free culture filtrate compared to control. Besides, the isolate *P. fluorescens* VSMKU3054 and their cell-free culture filtrate significantly promoted the length of shoot and root in tomato plants. Fresh and dry weight also increased upon treated with *P. fluorescens* VSMKU3054 and their cell-free culture filtrate compared to controls (Table 5). Shoot length was remarkably reduced ($P < 0.05$) in *R. solanacearum* when compared with control and culture. Similarly, root length and vigor index also strangely decreased in *R. solanacearum* inoculated tomato seedling compared to control.

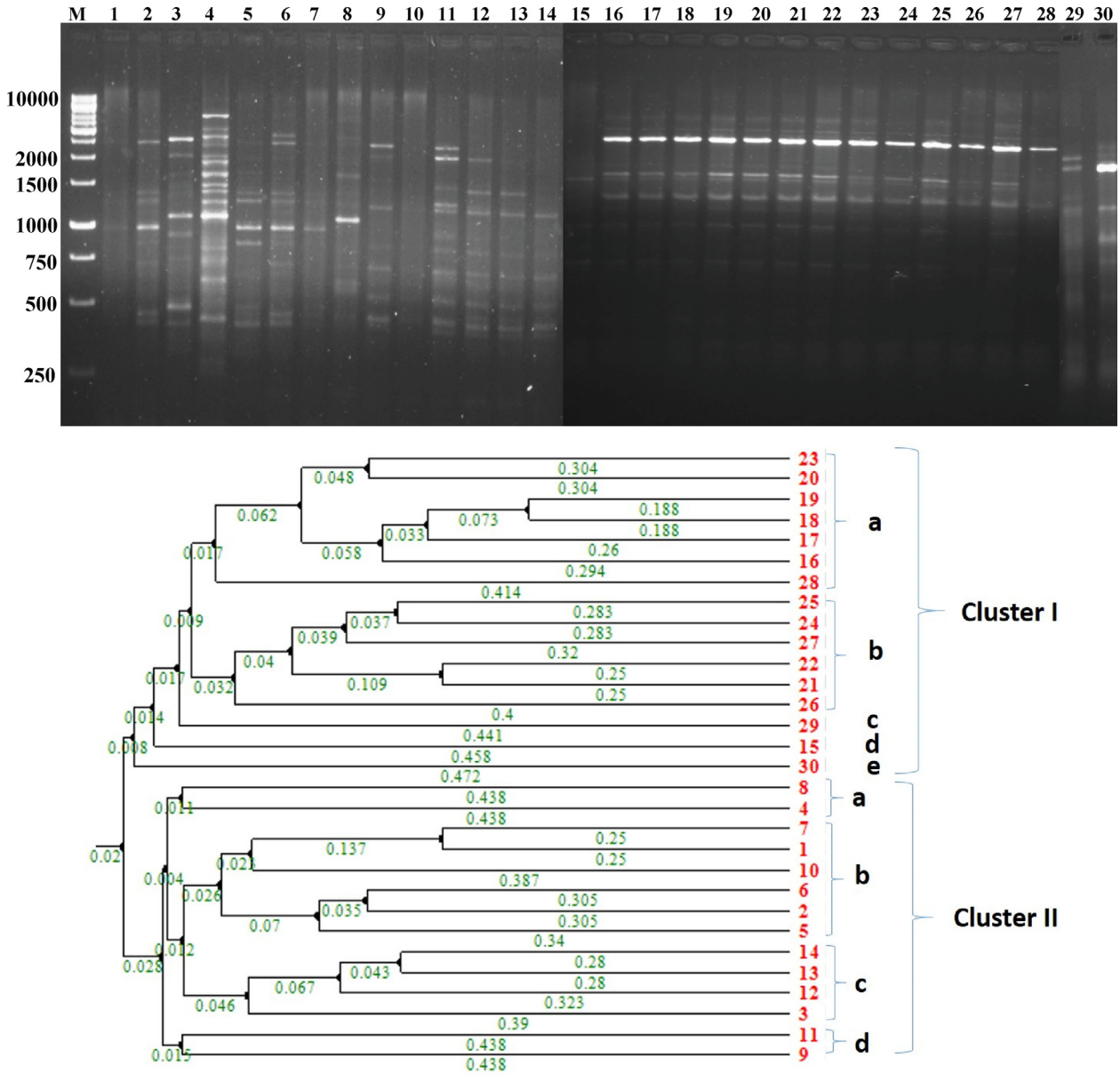

Fig. 2. BOX-PCR analysis of FPs. Fig. 2a and b. Dendrogram of BOX-PCR by Neighbor-joining metheod: 1-VSMKU3001, 2-VSMKU3003, 3-VSMKU3004, 4-VSMKU3005, 5-VSMKU3007, 6-VSMKU3009, 7-VSMKU3011, 8-VSMKU3012, 9-VSMKU3055, 10-VSMKU3062, 11- VSMKU3063, 12-VSMKU3065, 13-VSMKU3066, 14-VSMKU3076, 15-VSMKU3078, 16- VSMKU3080, 17-VSMKU3025, 18-VSMKU3029, 19-VSMKU3030, 20-VSMKU3032, 21- VSMKU3034, 22-VSMKU3035, 23-VSMKU3036, 24-VSMKU3038, 25-VSMKU3044, 26- VSMKU3049, 27-VSMKU3054, 28-VSMKU3024, 29-VSMKU3021, 30-VSMKU3022.

4. Discussion

Fluorescent pseudomonad's (FPs) are prevalent group of microorganism among plant rhizosphere associated bacteria. In recent days, FPs are eco-friendly weapons towards agricultally important plant pathogens, because of their antagonistic potential with abundance presence of antibiotic encoding genes and the secretion of plant growth promoting substance, hydrolytic enzymes and other antimicrobial traits (Shanmugaiah et al., 2006). In the present study focused on, biocontrol potential of phytopathogens, finger printing analysis and plant growth promoting potential of antagonistic FPs. Among 87 FPs, 34.4% (30) FPs were exhibited substantial antagonistic action towards *R. solanacearum* with different level of ZOI from 19 mm to 28 mm. In addition to that, 30 FPs were effectively control various phytopfungal pathogens from 6.3 mm to 30.7 mm compared to control (Table 2). Similarly, different researchers have also been documented that rhizospheric isolates have shown different levels of inhibition zone (ZOI) against various soil-borne fungal and bacterial pathogens, such as *R. solanacearum, R. solani* and *F. oxysporum* (Shanmugaiah et al., 2010; Elsayed et al., 2020). It has been documented that *Pseudomonas* spp is effective against a broad range of plant pathogens. Due to treatment of *Pseudomonas* spp against fungal pathogens indicated that, the remarkable morphological and physiological changes were take place in all tested fungal hyphae. The hyphal morphology and physical changes including disintegration, bulge, knotting, crumpling, destruction, contracting, bursting of mycelium and leakage of cytoplasm (Singh et al., 2016).

All the isolates of fluorescent pseudomonades were identified up to genus level by various physiological and biochemical analysis such as Grams reaction negative, shape of the all the isolates rod, indole, MR &VP negative, citrate utilization positive, catalase

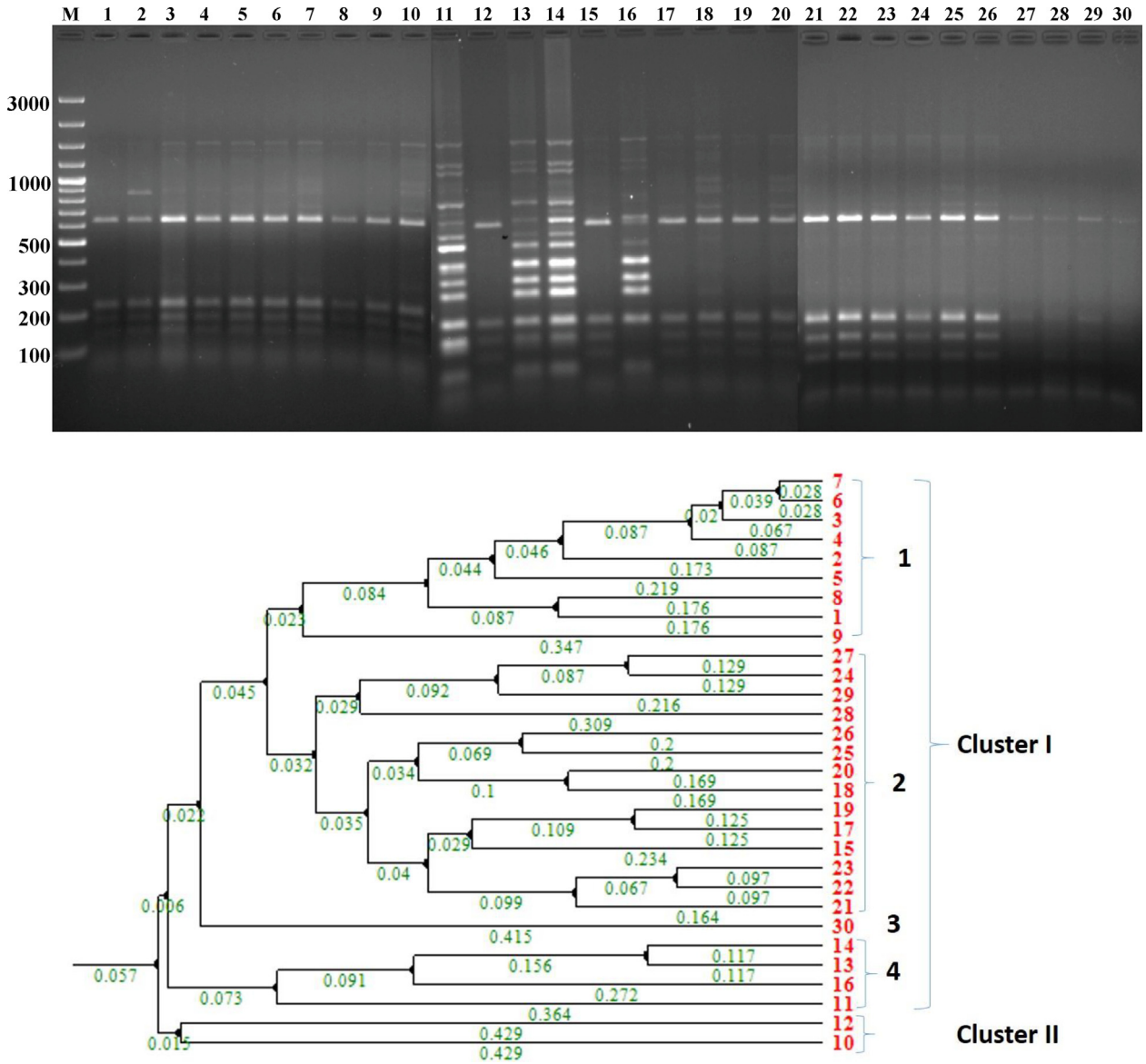

Fig. 3. RFLP analysis of FPs. Fig. 3a and b. Dendrogram of RFLP by UPGMA –Pearson correlation: M–Marker (100 bp), 1-VSMKU3001, 2-VSMKU3003, 3-VSMKU3004, 4-VSMKU3005, 5-VSMKU3007, 6-VSMKU3009, 7-VSMKU3011, 8-VSMKU3012, 9- VSMKU3035, 10- VSMKU3036, 11- VSMKU3021, 12- VSMKU3022, 13- VSMKU3024, 14-VSMKU3025, 15- VSMKU3029, 16- VSMKU3030, 17- VSMKU3032, 18- VSMKU3034, 19- VSMKU3038, 20- VSMKU3044, 21- VSMKU3049, 22– VSMKU3054, 23– VSMKU3055, 24- VSMKU3062, 25- VSMKU3063, 26- VSMKU3065, 27- VSMKU3066, 28- VSMKU3076, 29- VSMKU3078, 30- VSMKU3080.

positive and oxidase positive (Table 3). In accordance with our results (Eladawy et al., 2020) reported that for identification of *Pseudomonas* spp based on their Grams's reaction, shape, physiological and biochemical analysis. Moreover, the main characteristic future of *Pseudomonas* spp is pyocyanine pigments are presence on King's B agar indicated that 100% comes under *Pseudomonas* spp (Nithya et al., 2019; Varatharaju et al., 2020).

There are various mechanism involved for the suppression fungal and bacterial pathogens by rhizosphere beneficial microbes. The prime mechanism is production of metabolites, hydrolytic enzyme, defense enzymes, hydrogen cyanide, siderophore and phosphate solubilization (Antoun and Kloepper, 2001; Harikrishnan et al., 2014; Nithya et al., 2019). In this context, from our study we assessed various hydrolytic enzymes of which, 20% amylase, 100% protease, 91% cellulose, 40% pectinase, 80% chitinase and 90% gelatinase was produced by our tomato rhizosphereric FPs (Table 4). Likewise, earlier research stated that FPs are generated by fungal cell wall degrading enzymes such as protease, cellulase and chitinase that are primarily involved for the control of phytopathogenic microbes and insects (Dunne et al., 1997).

Zhou et al. (2012) stated that *Pseudomonas brassicacearum* J12 was effectively control *Ralstonia solanacearum* by the production of protease. Hence, the assortment of rhizosphere isolates like *B. subtilis, B. cereus, B. thuringiensis* and *Pseudomonas* spp *and* many more rhizobacteria are possible to produce hydrolytic enzymes and metabolites to control phytopathogens like *R. solani, F. oxysporum, S. rolfsii, P. ultimum* etc. by swelling in the hyphae and at the hyphal tip, hyphal curling or bursting of the hyphal tip. The structural integrity of the cell wall of the targeted pathogens affects by hydrolytic enzymes produced by antagonistic microbes. In this view, biocontrol phase is more significant to inhibit phytopathogenes and makes them more important (Shanmugaiah et al., 2006; Jadhav and Sayyed, 2016). Interestingly, our potential antagonistic isolate *Pseudomonas* sp VSMKU3054 could produce all tested hydrolytic enzymes such protease, gelatinase, cellulase,

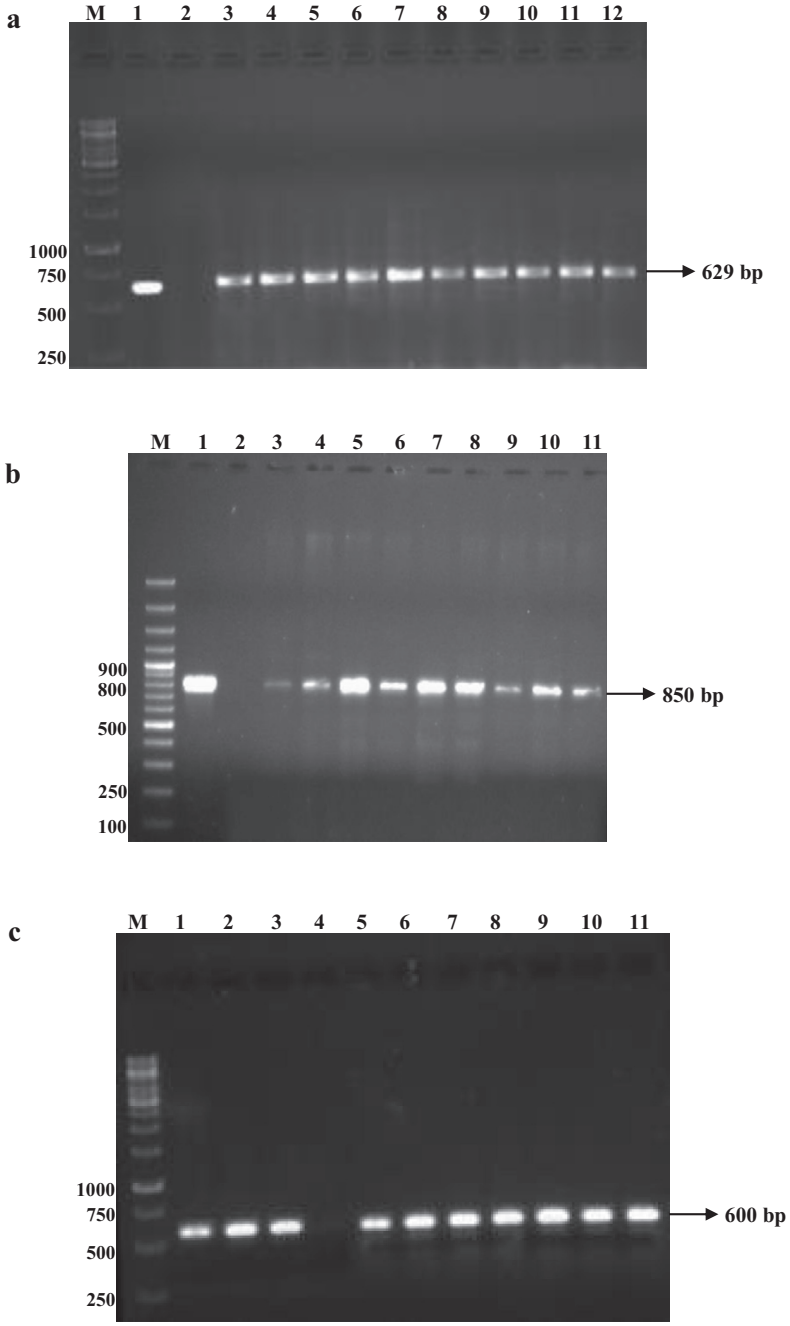

Fig. 4. Detection of antibiotic encoding genes from antagonistic FPs by PCR. a – DAPG, b. PLT and c. HCN. 4a - M–Marker (1kp), 1-CHAO, 2-Negative control, 3-VSMKU3021, 4-VSMKU3022, 5- VSMKU3024, 6-VSMKU3025, 7-VSMKU3029, 8-VSMKU3030, 9-VSMKU3032, 10- VSMKU3034, 11- VSMKU3049, 12-VSMKU3054. 4b - M–Marker (100 bp), 1-Pf5, 2-Negative Control, 3-VSMKU3065, 4-VSMKU3066, 5-VSMKU3080, 6- VSMKU3036, 7-VSMKU3038, 8-VSMKU3049, 9-VSMKU3054, 10-VSMKU3055, 11-VSMKU3062. 4c - M–Marker (1kp), 1-VSMKU3021, 2-VSMKU3022, 3- VSMKU3024, 4-Negative Control, 5-VSMKU3025, 6- VSMKU3029, 7-VSMKU3030, 8-VSMKU3032, 9- VSMKU3049, 10-VSMKU3054, 11-VSMKU3055.

amylase, chitinase and pectinase (Table 4). More recent research has shown that most of the antagonistic microbes are capable of degrading the fungal and bacterial cell membrane, cell membrane protein and regulate extracellular virulence factor of phytopathogens by antagonistic FPs due to the production of hydrolytic enzymes (Palaniyandi et al., 2013). The growth of *R. solanacearum* and development of wilt symptoms in tomatoes was arrested by the production of antimicrobial substance and hydrolytic enzymes like chitinase and glucanase related to substantial action and suppression of disease development by *Bacillus thuringiensis* (Elsharkawy et al., 2015).

Biocontrol potential against different phytopathogens by rhizobacterium *Pseudomonas* spp by the production of several secondary metabolites like Phenazine-1-carboxamide, 2,4-diacetylpholoroglucinol, pyrrolnitrin, pyoluteorin, siderophore, hydrogen cyanide, cell wall degrading enzymes and bio surfactants (Saikia et al., 2009; Shanmugaiah et al., 2010). The results were indicating in our isolates of *Pseudomonas* spp produced various metabolites like, siderophore (27), HCN (25), phosphate solubilization (30), indole acetic acid (23) compared to control (Table 4). In corroborate with our results, the production of Indole acetic acid (IAA), siderophore and phosphate solubilization evidently suggests

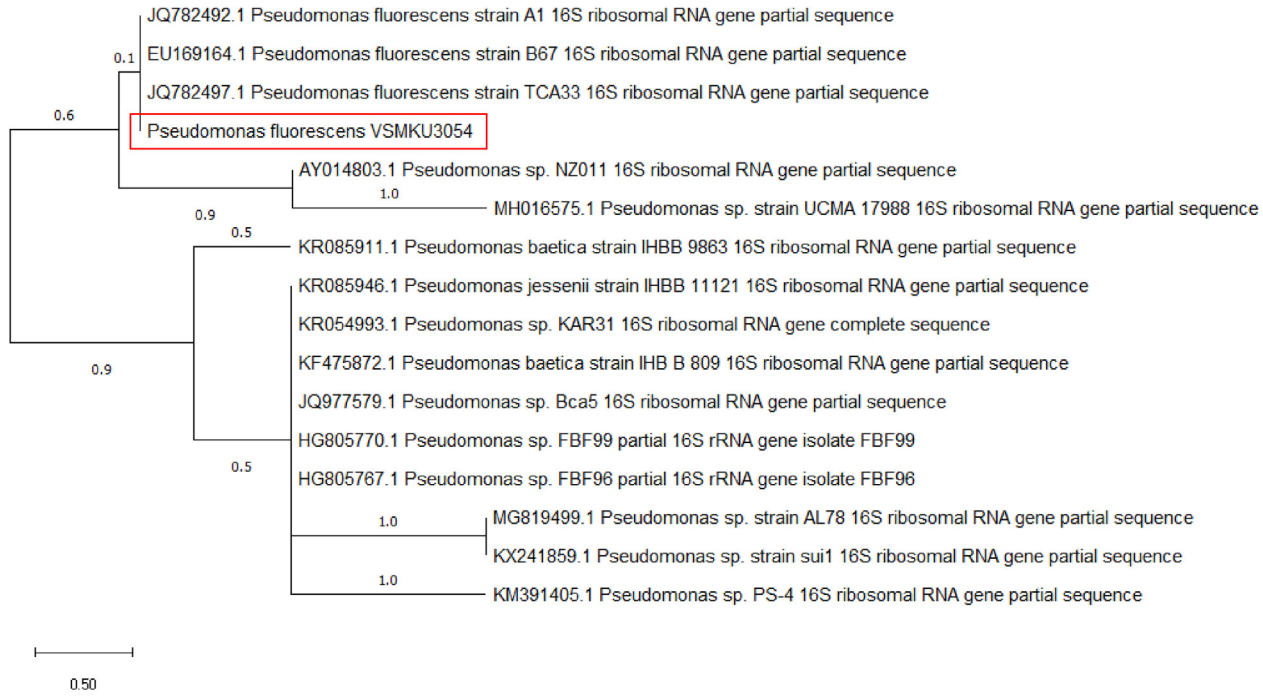

Fig. 5. Phylogenetic tree based on 16 s rRNA gene sequence of *P. fluorescens* VSMKU3054 depicted with other *Pseudomonas* sp. and GenBank accession no. MH443348.

Table 5
Effect of *P. fluorescens* VSMKU3054 for plant growth promotion of tomato.

Treatments	Root length (cm)	Shoot Length (cm)	Fresh Weight (g)	Dry Weight (g)	Vigor Index
Control	5.73 ± 0.09	12.9 ± 0.1	0.058 ± 0.0009	0.0025 ± 0.00011	536.23 ± 8.75
Culture	6.5 ± 0.12 ^{acd}	13.6 ± 0.1 ^{acd}	0.063 ± 0.0017	0.0029 ± 0.00015	623.77 ± 23.96 ^{acd}
Cell free culture filtrate	5.93 ± 0.12 ^{bd}	10.33 ± 0.15 ^{abd}	0.056 ± 0.003	0.0021 ± 0.0001	540.5 ± 23.09 ^{bd}
R. solanacearum	3.9 ± 0.08 ^{abc}	3.2 ± 0.1 ^{abc}	0.033 ± 0.004 ^{abc}	0.00092 ± 0.00066 ^{abc}	259.63 ± 19.38 ^{abc}

Date are average of 3 replications; The data in all groups were analyzed by one-way analysis of variance (ANOVA). If the P-values were < 0.05 the findings were deemed statistically important.

a potential plant growth promoting ability of the isolates of *Pseudomonas* spp (Ayyadurai et al., 2006) and enhancement of growth and yield of canola, tomato, soybean and wheat (Cattelan et al., 1999). Moreover, our results of the *in vitro* study suggested that potential antagonistic isolate *Pseudomonas* sp VSMKU3054 with prominent inhibitory activity was the only isolate be able to produce siderophore, HCN, phosphate solubilization, indole acetic acid, antibiotic encoding genes and hydrolytic enzymes confirming the hypothesis of biocontrol (Kheirandish and Harighi, 2015). Hydrogen cyanide is a toxic gas capable of forming metal complexes with functional groups of different enzymes that inhibit processes such as CO2 and nitrate assimilation; disturbance of oxygen reduction in the respiratory cytochrome chain; and transport of electrons in photosynthesis by the way antagonistic microbes to control phytopathogens by the production may antimicrobial substance like HCN (Lanteigne et al., 2012). Kheirandish and Harighi, (2015) reported that different *P. fluorescens* could be linked to their production of plant growth stimulating substances that increase biomass production. (Lanteigne et al., 2012; Lemessa and Zeller, 2007) reported that plant biomass was significantly increased by strains of fluorescent pseudomonads. This may be the outcome of structural resistance induction, siderophore development and the competitive advantage of antagonist root colonization.

PGPR antagonistic to the pathogen must be tested and integrated as a biocontrol agent into effective disease management. The ability to adapt to the rhizosphere and actively colonize the host roots is a key characteristic of PGPR (Dunne et al., 1997). It was also proposed that they should be separated from various environments where they would be needed to work in order to achieve greater efficiency of biocontrol agents (Cook, 1993). *R. solanacearum* morphology and physiological function was regulated by the effectiveness of some of the naturally antagonistic isolates like *Pseudomonas fluorescence*, *Bacillus* spp and (Xue et al., 2009), *Stenotrophomonas maltophilia* (Messiha et al., 2007), *Streptomyces setonii* (Lemessa and Zeller, 2007). A considerable amount of attention has been paid to the priority of the diversity of fluorescent pseudomonads to explore their biocontrol and biofertilization capabilities in recent scenarios (Naik et al., 2008; Saikia et al., 2011). In this context our results, among 16 distinct BOX-PCR prototypes two different clusters were find out with 60% and 40% similarity of *Pseudomonas* spp (Fig. 2a and b). Due to their high degree of genetic diversity, all our FPs isolates were shown different variations of fingerprinting patterns and distributed into different clusters.

Microbial diversity from rice and bean rhizosphere has been explored worldwide using a variety of molecular techniques (Koskey et al., 2018). Among different molecular tool, As a useful technique PCR-RFLP has been identified for the assessment of the genetic diversity of rhizobacteria among different groups of microorganisms (Moin et al., 2020; Nithya et al., 2020). Our results of DNA printing analysis showed that huge variation among the isolates of FPs. Among two major clusters, cluster 1 contained 90% of FPs, of which sub clad 2 consisting about 50% of FPs. whereas, the sub clad 1, 3, 4 and cluster 2 contain remaining of 50% FPs. Our finding specified that antagonistic FPs are highly

diverse even 10 km distance and a single crop rhizosphere (Fig. 3a and b). Hence, the expression of genetic properties is greatly influenced by soil environment (Nowak-Thompson et al., 1994). Genetic variation was observed among FPs in our study, because of mutation, recombination and SOS response for release of DNA damage free radical by various metabolic reactions. Our research findings conformed that studies on diversity within a targeted bacterial population, to screen genotypes that are best adapted in a particular environmental stress or ecological habitat with our constrain objectives.

Antimicrobial compounds produced by FPs were involved for the control of various bacterial and fungal pathogens. *Pseudomonas* spp (Ayyadurai et al., 2007), *P. aeruginosa* MML2212 (Shanmugaiah et al., 2010), *P. corrugate* GFBP 5454 and *P. mediterranea* GFBP 5447 (Strano et al., 2017), *Pseudomonas* sp VSMKU4036 (Varatharaju et al., 2020), and *P. aeruginosa* VSMKU1 (Nithya et al., 2020) are significantly control the growth of different bacterial and fungal plant pathogens by the secretion of diffusible compounds like 2,4-diacetylphloroglucinol (2,4-DAPG), phenazine-1-carboxamide (PCN). In the present study, we detected 10 FPs obtained 2,4-diacetylphloroglucinol encoding gene. Among 30 antagonistic FPs, 26 FPs and 12 FPs were observed the presence of hydrogen cyanide and pyoluteorin encoding gene with detection of specific size of the DNA along with their specific primers (Fig. 4b and c). Whereas none of the isolates FPs did not obtained pyrrolnitrin encoding gene isolated from tomato rhizosphere. Our result was concurrence with previous report for the production of 2,4-DAPG and PRN have been found to exist alone in one bacterium. However, *Pseudomonas* spp are rarely produced PLT antibiotics and no isolate has been obtained that only produces PLT (Liu et al., 2006). Pholoroglucinol assist and act as a signal molecule to induce the expression of pyrrolnitrin biosynthesis. *P. fluorescence* VSMKU3054 produced array of antimicrobial metabolites, and 2,4 diacetylphloroglucinol (2,4 DAPG) is a major encoding gene for the control of various plant pathogens. Moreover DAPG was effectively control *R. solanacearum* by *in vitro*. Guasp et al., (2000) demonstrated the 16 s rRNA is highly conserved primer binding sites and their gene sequences were hypervariable regions with high bacteria. The present study 16 s rRNA sequence compared with databases and identified as *P. fluorescens* with 98% similarity.

Based on the production of fluorescent pigments, rod shape, motile, catalase and oxidase and many carbon and nitrogen source utilization was positive by our isolate *Pseudomonas* sp VSMKU3054 and other various biochemical analysis were positive. After analysis of 16 s rDNA sequence analysis comparison with constructed phylogenetic tree, about 98% similarities coincide with that of *Pseudomonas fluorescence* (Fig. 5). Similar kind exercise done by Nithya et al., 2020.

Höfte et al. (1991) described that *P. aeruginosa* and *B. cepacia* enhanced seed germination and promoted plant growth. *Pseudomonas* sp MML2212 significantly enhances rice growth by the secretion of IAA (Shanmugaiah et al., 2006). Many rhizobial microbes like *Trichoderma* sp, *Pseudomonas* sp, *Bacillus* sp and *Streptomyces* sp VSMKU1014 are effectively enhance the growth of rice, cotton, green gram and sorghum (Harikrishnan et al., 2014; Mazrou et al., 2020). Similarly, our study has proven that, *P. fluorescens* VSMKU3054 remarkably enhance tomato seedling growth through remarkable increase of root and shoot length, fresh and dry weigh and vigor index compared to control (Table 5).

5. Conclusion

Our potential isolate *P. fluorescens* VSMKU3054 showed significant antagonism against phytopathogens. The isolate VSMKU3054 may prove the capacity to produce lytic enzymes and secondary metabolites. The effect of VSMKU3054 significantly enhance the seed germination of tomato seedlings. Hence, our isolate *P. fluorescens* VSMKU3054 could be used as a possible biocontrol agent for the management of soil borne plant pathogens.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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