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Evaluation of Native Fluorescent Pseudomonads Against Major Wilt Pathogens of Vegetables in Meghalaya, India

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Abstract Fluorescent pseudomonads are an ecologically important group of rhizobacteria that are well accepted as biocontrol agent and plant growth promoter. Exploitation of such rhizobacteria for management of plant diseases reduces the hazardous effects of chemical to the environment as well as to human health. In this study, fifty fluorescent pseudomonad isolates obtained from 72 collected soil samples of Ri-Bhoi (mid-hills) and East Khasi Hills (mid and up-hills) districts of Meghalaya. All the isolates were screened for production of HCN and presence of 2 biosynthetic genes for antibiotics viz., diacetyl- phloroglucinol and pyrrolnitrin by using gene specific primer. HCN production test was found positive for 12 isolates, whereas 2 and 6 numbers of isolates were recorded for presence of diacetylphloroglucinol and pyrrolnitrin biosynthetic genes respectively. Antagonistic potential of 17 isolates positive for HCN and antibiotic production was tested against major wilt pathogens i.e. *Fusarium oxysporum* f.sp. *pisi* (pea) and *Ralstonia solanacearum* (brinjal and capsicum) under *in vitro* conditions. The radial growth of *F. axysporum* f.sp. *pisi* was found to inhibit most by isolate MRN 18, whereas isolate PC followed by USR 9.2 was found to have the highest inhibition zone against both *R. solanacearum* isolates from brinjal and capsicum.

Keywords Fluorescent pseudomonads, Meghalaya, Antibiotics, HCN, Wilt.

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Introduction

Rhizospheric fluorescent psendomonads are often predominant among all rhizobacteria that colonize the plants. They have been considered as an important group due to their biocontrol and bio fertilizing abil-

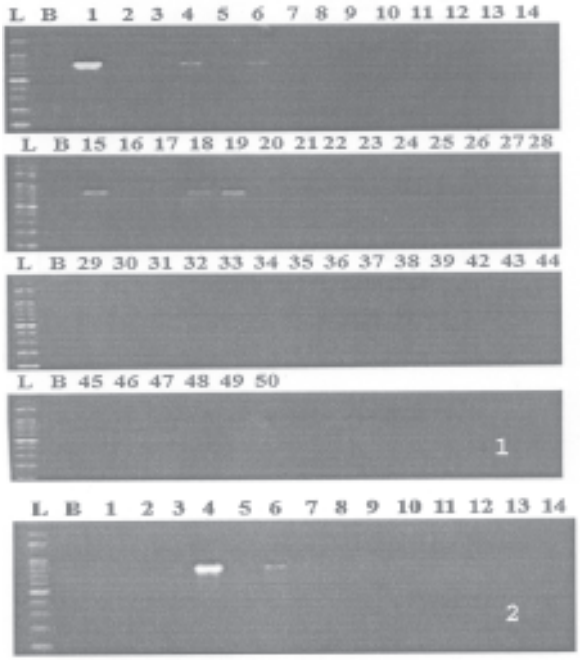

Fig. 1. Fluorescent pseudomonad isolates showing amplification using gene specific primer (Prncf-Prncr) for antibiotic pyrrolnitrin. Lane name with the respective isolates; L=100 bp ladder; B=blank; 1=PC; 4=MRN 11; 6=BF 2; 15=N 2; 18=MRN 4; 19=MRN 18. **Fig. 2.** Fluorescent pseudomonad isolates showing amplification using gene specific primer (Ph12a-Ph12b) for antibiotic DAPG. Lane name with respective isolates; L=100 bp lader; B=blank; 4=PC; 6=9.2.

ity. Plant-protection mechanisms on fluorescent pseudomonads as biocontrol agents are because of their catabolic versatility, their excellent root-colonising abilities and also due to combination of many useful traits in disease control through several mechanisms viz., production of antibiotics, siderophores, HCN, enzymes like proteases, glucanases and competition for space and nutrients [1]. Plant growth promoting abilities have been attributed to provision of nutrients, microelements, hormones to the plant.They take part in phosphate solubilization in the soil and make them accessible to plants. This rhizobacterial group also produce Phytohormone, indole-3-acetic acid (IAA) and I-aminocyclopropane-I-carboxylate (ACC) which helps in plant growth.

Dohro [2] reported that native antagonists have been found to be more virulent to the soil borne pathogens because of their adaptability under soil and local climatic conditions of the region. Native potential strains of beneficial microorganisms of Meghalaya have not been effectively screened for production of different antimicrobial traits and also not yet commercialized for management of plant pathogens. Keeping this in view, this research work has been formulated to isolate the native fluorescent pseudomonads and to evaluate them against plant pathogens causing with disease in vegetable.

Materials and Methods

Soil sample collection

A total of 72 soil samples were collected from the rhizosphere of healthy maize, rice, banana, pea and pigeon pea plant from different locations of Meghalaya i.e. mid hills of Meghalaya (Ri-bhoi and

Table 1. Screened isolates positive for HCN and antibiotic production.

Tests	Isolates MRN 4	MRN 11	MRN 15	MRN 18	MRN 24	ENT 15	N 2	P 1	P7	USR 7	USR 9.2	USR 13.1	BF 1	BF 2	BF 3	BRS	PC
HCN test	–	–	+	–	+	+	–	+	+	+	+	+	+	–	+	+	+
DAPG production	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+
Pyrrolnitrin production	+	+	–	+	_	–	+	_	_	_	_	–	–	+	–	–	+

East Khasi Hills District) and up-hills of Meghalaya (East Khasi Hills District). The samples were kept in refrigerator at 4±1°C until use.

Isolation and characterization of fluorescent pseudomonads

Soil samples were plated in King's B medium (KMB) and fluorescent pseudomonad colonies were detected by viewing them in UV transilluminator (365 mm). The isolates were maintained by regular subculturing after every two months. Long term preservation was done in Luria Bertani (LB) broth containing 20% glycerol and stored at -20°C.

Morphological studies like shape, colony edges, colony elevation, colony appearance, optical property and water solubility were observed. The fluorescent pseudomonad isolates were also biochemically characterized for the identification and classification of the isolates [3], Biochemical tests like oxidase production, gelatin hydrolysis, levan production, hydrogen sulphide production, starch hydrolysis test, utilization of citrate, denitrification test and arginine dehydrolase were performed. Ability to grow at 4°C and 41°C were also recorded.

DNA extraction and 16S rRNA amplification

The genomic DNA of the 50 isolates was isolated by following the method described earlier [4, 5]. Amplification of 16S rRNA region was performed by using bacterial universal primers 27f and 1492r primer pairs. The reaction mixture (25µl) consisted of 50 ng/µl of g DNA, IX PCR buffer, 1.5 mM of $MgCl_2$, 200µM of dNTPs, 1µg/10µl of BSA, IU of Taq DNA, 100 nM of each forward and reverse primer.

Characterization for metabolite (HCN) production

HCN production as a secondary metabolite by fluorescent pseudomonads was performed by following the method already described by Lorck [4].

Characterization for antibiotic production by PCR based method

Presence of biosynthetic genes that specify the production of antibiotics viz., diacetylphloroglucinol (DAPG) and pyrrolnitrin were screened by using gene-specific primers in PCR based method. Primer pairs viz., Ph12a-Ph12b and Prncf-Prncr were used in this study for DAPG and pyrrolnitrin respectively. The reaction mixture (25µl) consisted of 50 ng/µl of g DNA, IX PCR buffer, 1.5 mM of $MgCl_2$, 200 µM of dNTPs, 1µg/10µl of BSA, IU of Taq DNA, 100 nM of each forward and reverse primer.

Sequencing and phylogenetic analysis

Sequencing and phylogenetic analysis was done only for those isolates with antagonistic properties. The reference 16S rRNA sequences used in the phylogenetic analysis was taken from NCBI GenBank.

Collection of diseased samples

Vegetables like pea (*Pisum sativum*), capsicum (*Capsicum* spp.) and brinjal (*solanum melongena*) show-

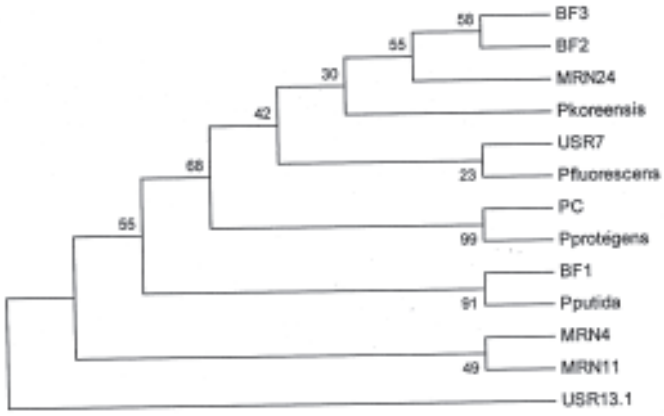

Fig. 3. Molecular phylogenetic analysis by maximum likelihood method.

ing wilted symptoms were collected from experimental field of ICAR Complex for North Eastern Hill (NEH) Region, College of Post Graduate Studies, Umiam and vegetable belt of Nongpoh area of Ri-Bhoi district, Meghalaya and were used for isolation of pathogens like *Fusarium oxysporum* f.sp. *pisi* sp. and *Ralstonia solanacearum.*

In vitro screening for
antagonistic activity

Isolates found positive for HCN and antibiotics (DAPG and pyrrolnitrin) production were tested for their antagonistic activity against wilt pathogens viz., *F. oxysporum* f.sp. *pisi* and R. solanacearum by following dual culture technique [6].

Results and Discussion

Fluorescent pseudomonads are known for its aggressive root colonization character which has been also reported to play an important role in thizosphere competence and associated biocontrol activity [1]. The present study confirmed the geographical distribution of fluorescent pseudomonads in different elevations (mid and up hills) of the state. Out of 50 isolates obtained, 28 isolates were associated with mid-hills rhizospheric soil, whereas 22 were from up hills. Hence, result of the present study indicated their wide distribution and adaptability. Availability of rhizobacteria in large number in all major natural environments viz., terrestrial, fresh water, marine is well known. This universal distribution is attributed to the remarkable degree of physiological and genetic adaptability of this group of rhizobacteria [7].

Identification and characterization
of fluorescent pseudomonads

From the soil samples, 50 isolates were identified as fluorescent pseudomonads based on morphological properties as well as biochemical tests performed. Biochemical test revealed that all the 50 isolates belonged to the genus Pseudomonas. Morphological characters pertaining to colony pigmentation, colony edges, elevation, appearance, optical property and solubility in water were studied. All the isolates were found positive for florescent pigment production, however some isolates were observed with less fluorescent color. All isolates were also found to be soluble in water. Colony edges, elevation, appearance (shiny or dull) and optical property of the isolates differed among them. Maximum (43 numbers) fluorescent pseudomonad isolates showed smooth edges, except few i.e. 7 numbers were recorded with wavy edges. 27

Table 2. Dual culture test of screened fluorescent pseudomonads. Values within the column followed by different letters are statistical significant at $p<0.01$.

Isolates	Marginal mean of the radial diameter (cm) of *Fusarium oxysporum* f.sp. *pisi*	Zone of inhibition against *R. solancearum* (brinjal) (mm)	Zone of inhibition against *R. solancearum* (capsicum) (mm)
MRN 4	3.54^{abc}	ND	ND
MRN 11	3.71^{bcd}	ND	ND
MRN 18	3.32^{a}	ND	ND
P 7	4.43^{gh}	ND	ND
USR 13.1	4.29^{fgh}	ND	ND
BRS	3.83^{bcde}	12.33^{a}	12.67^{ah}
BF 3	3.76^{bcd}	$12,33^{a}$	12.00^{a}
N 2	3.75^{bcd}	12.33^{a}	12.00^{A}
P 1	4.25^{efgh}	13.33^{ab}	13.00^{ab}
BF 1	4.26^{cfgh}	13.67^{ab}	13.00^{ab}
BF 2	3.41^{ab}	13.67^{ab}	13.67^{bc}
MRN 24	3.91^{cdef}	14.00^{ab}	13.33^{b}
MRN 15	3.77^{bcd}	14.00^{ab}	13.33^{b}
ENT 15	4.11^{defg}	14.33^{b}	12.67^{ab}
USR 7	4.04^{dcfg}	14.33^{b}	14.67^{cd}
USR 9.2	3.49^{abc}	16.00^{c}	15.00^{d}
PC	4.09^{dcfg}	17.33^{c}	15.67^{d}
Control	4.57^{h}	ND	ND

isolates were observed with raised colony surface and shiny appearance, whereas 24 had flat surfaces with dull appearance. Studies on optical properties of 50 fluorescent pseudomonad isolates revealed 40 colonies as opaque, 8 as translucent and 2 as transparent.

HCN and antibiotics production

In the present study, 12 isolates were found to produce HCN. Production of HCN by many plant associated fluorescent pseudomonads has been found to play a role in biological control of soil borne phytopathogens as well as plant growth promotion [8]. It was on these lines, that the 12 isolates positive for HCN in the present study was further evaluated for their biocontrol activity. Production of antibiotics like DAPG, pyrrolnitrin, PCA and pyoluteorin have been shown to be an important mechanism of biological control of a wide variety of plant pathogens by fluorescent pseudomonads. In the present investigation, antibiotic production by 50 flourescent pseudomonad isolates were studied. The results of the PCR analysis with primer pair Prncf-Prncr for antibiotic pyrrolnitrin was found to amplify six isolates with the expected size of 719 pb (Fig. 1). Pyrrolnitrin is known for its have strong antifungal activity against pathogens [9]. Amplification with primer phl2a-phl2b showed positive result for two isolates i.e. PC and USR 9.2 with an amplicon size of 745 bp (Fig. 1). These results corresponded to those of Ahmadzadeh et al. [10] and Wang et al. [11]. Antibiotic DAPG producing *Pseudomonas* spp. have been studied intensively during the last decades, and has been given special attention because of their ability to control a wide variety of soil-borne plant pathogens [10, 11].

Screening of fluorescent pseudomonads for evaluation of their biocontrol potentiality

A total of 17 isolates which were found positive for both HCN and antibiotic production (Table 1) were further screened *in vitro* for evaluation of their biocontrol potential against wilt pathogens of vegetables.

Phylogenetic analysis

Blast analysis of 16S rRNA sequences revealed different species within the Genus pseudomonas of which a phylogenetic tree was constructed using reference strains from NCBI (Fig. 3). Phylogenetic analysis was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [12].

Dual culture test

In the present study (Table 2) isolate MRN 18 was found to be the most effective in inhibiting the radial growth of the pathogen *F. oxysporum* f.sp. *pisi*. Isolate MRN 18 was isolated from maize rhizosphere, belonged to *P. fluorescens* and had the ability to produce antibiotic pyrrolnitrin only. This study revealed that the biocontrol activity of the isolate might be attributed to the production of antibiotic pyrrolnitrin which helps in suppressing the wilt disease. Ability of fluorescent pseudomonads to produce pyrrolnitrin has already been correlated with biocontrol activity

against fungal plant pathogens [11]. However isolates which produced HCN only, did not play major role in inhibition of the fungal pathogen.

Dual culture test of the screened fluorescent pseudomonads against *R. solanacearum* of brinjal and capsicum revealed that isolate PC was the most effective in suppressing the pathogen followed by USR 9.2. Isolate PC belonged to *P. protegens* and was found associated with pea rhizosphere. This was the only isolate which was found positive for the production of all of the three antimicrobial metabolites i.e. HCN and antibiotic DAPG and pyrrolnitrin. Isolate USR 9.2 which was isolated from rice rhizosphere, belonged to *P. fluorescens* and had the ability to produce the antibiotic DAPG and HCN. Therefore, successful inhibition of *R. solanacearum* by these two isolates could be correlated with production of these three secondary metabolites which were already known for their role in disease suppression of broad range of plant pathogens [13].

Conclusion

Overall findings of the study revealed that antibiotic pyrrolnitrin qwas effective against *F. oxysporum* f.sp. *pisi* whereas antibiotics DAPG, pyrrolnitrin along with secondary metabolite HCN was found best in inhibiting *R. solanacearum*. Screening of isolates based on presence of biosynthetic gene for antibiotic production can be successfully used for studying their biocontrol activity against pathogenic strains of *F. oxysporum* f.sp. *pisi* and *R. solanacearum*.

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