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Genetic Diversity, Phylogenetic Affinities and Multifarious Functions of Plant Growth-Promoting Rhizobacteria Associated with *Oryza sativa* Rhizosphere from Vaigai-Basin Located Paddy Fields in Madurai District, Tamilnadu, India

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ABSTRACT

Genetic and functional diversity of plant growth-promoting rhizobacteria (PGPR) associated with rice plant (*Oryza sativa* L.) in rhizosphere collected from the rice fields located in Vaigai basin (Sholavandhan, Thirumagor, and Karuppaiyurani) Madurai district, TamilNadu, India has been analyzed. A total of 11 *Pseudomonas* strains among 691 isolates were randomly selected for studies. Phylogenetic analysis based on 16S rDNA gene sequences generated 5 clusters showing more than 87% of the bootstrap value. Further, selected isolates were investigated for P-solubilization, IAA production, ammonia production, siderophore production, and HCN production.

INTRODUCTION:

Plant growth-promoting rhizobacteria (PGPR) are well studied in different agricultural crops but the interaction of PGPR in rice crop is not yet well understood. Complication due to scarcity of phosphate in the soil is a major limitation to crop production in India. Further, the level of inorganic phosphorus is very low in the soil as most of the phosphorous are present in insoluble forms. Rice crop land holds considerable accumulation of phosphorous due to the regular excessive applications of chemical fertilizers. Principally, increasing use of chemical inputs causes several negative effects - development of resistance in the pathogens and detrimental impacts in the non-target organisms. Secondly, the application of chemical fertilizers resulted in the decline of beneficial microbes in the soil.

Also, a large proportion of fertilizers added to the soil are converted to insoluble form generally unavailable to plants [1]. In recent years, the interest in soil microorganisms has increased as they are known to sustain soil fertility and alleviate different types of plant stress. PGPR enhances plant growth and augments agronomic practices towards the mobilization of plant nutrients in the soil [2]. PGPRs stimulate plant growth through mobilizing nutrients in soils, producing numerous plant growth regulators, protecting plants from phytopathogens by controlling or inhibiting them, improving soil structure and bioremediating the polluted soils by sequestering toxic heavy metals and degrading the xenobiotic compounds (chemical fertilizers/ pesticides) [3].

Pseudomonas is one among the best studied PGPR for its potential phosphate solubilizing activity, siderophore production, extracellular ammonia production, IAA production, soil nutrient recycling and several other plant-growth-promoting activities [4]. Cosmopolitan distribution of Pseudomonads is further ascertained by their involvement in the control of biotic and abiotic stresses as well as upholding water conservation status in plants [5]. Pseudomonads with plant growth-promoting traits were previously isolated from Sivagangai [6] and examined for their ability to produce secondary metabolites that promote specific activity in the rice plant rhizosphere.

The high degree of conservation of 16S rDNA gene led to a small number of informative sites in its sequence. To date, sequenced strains represent only a fraction of the diversity within the

Pseudomonads whereas much of the group's metabolic, ecological, and genetic diversity remains unexplored. In the present study, the phylogenetic relationship among the isolates from Madurai region was established based on the 16S rDNA gene. The isolates are characterized for their phosphate solubilising ability, IAA, ammonia, siderophore and HCN production.

MATERIALS AND METHODS:

Isolation

Soil sample was collected from Vaigai basin located paddy fields in Madurai District. Isolation of pseudomonads from the rhizosphere was performed as described earlier [6]. Briefly, soil suspension was obtained by shaking 10 g of soil sample having roots with tightly adhering soil in 90 ml of 0.1 M $MgSO_4.7H_2O$ buffer for 10 min at 180 rpm on a rotary shaker. The resulting suspensions were serially diluted and 0.1 ml aliquots of each dilution were spread onto King's medium B (KB) agar in triplicates. Purified single colonies were further streaked onto KB agar plates to obtain pure cultures. Stock cultures were made in Luria Bertani (LB) broth containing 50% (w/v) glycerol and stored at -80°C until further use.

16S rDNA gene amplification, sequencing and phylogenetic tree analysis

Amplification of 16S rDNA gene was performed from the genomic DNA of strains using universal primers fD1 (5'-GAGTTTGATCCTGGCTCA-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') as described previously by Nagajothi and Jayakumararaj. PCR cocktails (50 µl) contained 50 pM of primer, 50 ng of genomic DNA, 1× Taq DNA polymerase buffer, 1 U of Taq DNA polymerase (Promega, Madison, WI, USA), 0.2 mM of each dNTP, and 1.5 mM MgCl2. Amplification was performed in a DNA thermocycler (2400 cycler, Perkin Elmer International, Rotkreuz, Switzerland) at 94°C for 3 min, followed by 30 cycles of 10 s at 94°C, 1 min at 56°C and 30 s at 72°C with an extension of 72°C for 5 min. A 5 µl aliquot of each amplification product was electrophoresed on a 0.7% agarose gel in 1× TAE buffer at 50 V for 45 min, stained with Ethidium bromide and the PCR products were visualized with a UV transilluminator. PCR products were purified using Quick PCR purification column (Promega, Madison, USA). Purified PCR products were sequenced with an automated DNA sequencer with specific primers using the facility at Macrogen Inc. (Seoul, Korea).

All the sequences were aligned and saved as molecular evolutionary genetics analysis (MEGA) format in software MEGA v5.0. The pairwise evolutionary distances were computed using the Kimura 2-parameter model. To obtain the confidence values, the original data set was resampled 1000 times using the bootstrap analysis method. The bootstrapped data set was used directly for constructing the phylogenetic tree using the MEGA v5.0 program for calculating the multiple distance matrixes and analysis. The tree was constructed with Mega version 5 software package [7] by using the NJ method from distance calculated by the method of Kimura two-parameter model with a discrete Gamma distribution and multiple distance matrix obtained was used to construct phylogenetic trees using Neighbor-Joining (NJ) method [8].

Solubilization of organic phosphate

Rhizobacteria were determined quantitatively for phosphate solubilization according to the method described by [9]. Bacterial strains were tested by plate assay using Pikovskya medium (HiMedia, India). Bacterial culture (5 µl) was placed on the plates and then incubated at 28°C for 14 days. The formation of a clear zone around the bacterial growth spot was used as a signal of phosphatase activity.

Indole acetic acid (IAA) production

Bacterial cultures were grown for 7 days in 50 ml of CCM (ammonium chloride was added at 1.0 g/l). L Tryptophan (100 mg/l) was added to the media as a precursor of IAA biosynthesis. Cells were harvested at 10,000 rpm for 15 min. The pH of the supernatant was adjusted to 2.8 with hydrochloric acid and then extracted three times with equal volumes of ethyl acetate. The extract was evaporated to dryness and resuspended in 1 ml of ethanol. The samples were analyzed by high-performance liquid chromatography as described by Mehnaz and Lazarovits [10].

Hydrocyanic acid (HCN) production

To qualitatively determine the hydrocyanic acid (HCN) production, bacterial strains were streaked on LB medium. Filter paper saturated with an alkaline picrate solution (picric acid, 2.5 (g/l); Na2CO3, 12.5 (g/l); pH 13) was placed in the lid of a Petri plate containing a bacterial culture. Petri plate was sealed with Parafilm and incubated for four days. HCN production was assessed by the color change of yellow filter paper to brown/reddish brown [11].

Siderophore production

Siderophore production was detected by using the O-CAS method [12]. The pellets of overnight-grown bacterial cultures were suspended in sterilized water, and 10 µl of each culture was spotted on LB plates. The plates were incubated at 30^{o}C for 48 h. Ten ml of medium was used as overlay and applied on LB agar plate containing 48 h grown bacterial culture. A change in color from blue to orange (hydroxamate-type siderophore) or purple (catechol-type siderophore) was considered a positive reaction. Sterilized LB and water were used as negative control.

RESULTS AND DISCUSSION:

Genetic diversity and phylogenetic affinities among the isolates

Rice (*Oryza saliva* L.) is one of the oldest staple crops in the world, and the main source of calories for more than half of humanity. To meet global needs, a 40% increase in production of rice must be achieved in the next 20 years on limited and increasingly degraded arable lands and an unstable global climate context [13]. In the present study, eleven potential PGPR strains were isolated from *O. sativa* rhizosphere soil. The strains were selected based on the 16S rDNA sequence and the sequence data was analyzed by BLAST [14]. The nearest match from GenBank data was reported and the sequences were deposited in the GenBank. DNA sequencing and phylogenetic analysis revealed that all the isolates showed 97–99% similarity with GenBank sequences (Fig.1). As stated by Spiers and colleagues [15], the extraordinary phenotypic and genetic diversity within *Pseudomonads* showed no definite pattern of distribution that could precisely define any of the lineages.

Phosphate solubilization and ammonia production

Clear zone around the colony on Pikovskya's agar medium showed P-solubilization (Data not shown). 11 PGPR strains that were selected among the isolates from the paddy rhizosphere of Madurai district exhibited significant Phosphate solubilizing activity (Table 1). Out of eleven selected strains for Madurai, the average maximum zone of inhibition were exhibited by SM2 (18.3 mm) followed by SM4 (18 mm), SM11 (14.6 mm), TM1 (13 mm), KM3 (14 mm) and KM2 (12.3 mm) respectively, whereas the average minimum zone of inhibition was exhibited by

SM9 (7.6 mm), TM3 (8.6 mm) and KM1 (9 mm) strains. However, there was no zone formation observed by TM5 (Table 1).

The isolates SM2, SM11 exhibited strong production of ammonia whereas the isolates SM4, SM9, TM1, TM3, KM1, KM2 exhibited moderate production of ammonia. The strains of TM5, KM3 exhibited weak ammonia production (Table 1). Kuan *et al* [16] indicated that PGPR may provide a biological alternative to fix atmospheric N2 and delay N remobilization in maize plant to increase crop yield, based on an understanding that plant-N remobilization is directly correlated to its plant senescence.

Quantitative analysis of IAA, siderophore and HCN production

All the 11 isolates were subjected to spectrophotometric analysis to quantify the amount of IAA, siderophore and HCN production (Fig. 2). All the isolates exhibited IAA production, within the range of 1.02 to 1.3 OD value. Maximum value was observed in SM2, SM11, TM1, TM5, KM1 while SM4, SM9, TM3, KM2, KM3 recorded a minimum OD value (Fig. 2a). Tsavkelova *et al* [17] reported that production of auxin depends upon response of plant seedling and types of applied microbial inoculants. It has been pointed out that microbial strains with the ability to produce higher/ lower amount of IAA resulted from increase growth and yield of wheat. L-tryptophan, the precursor of IAA, is naturally present in root exudates of plants. Alternatively, it is also synthesized by hydrolysis of proteins of dead cells, and is converted into IAA by PGPRs [18].

All the isolates exhibited considerable siderophore and HCN production. Highest percentage of siderophore production (80-90%) was exhibited by SM2, SM4, SM9, SM11, KM2, KM3, moderate (70-80%) by TM1, TM5, KM1 and least (60-70%) by TM3 (Fig. 2b). Reports indicate that siderophore-producing bacteria contribute by increasing dry weight and maintain healthy soil that suppresses fungal growth [19]. Similarly, the isolates SM2, SM11 exhibited strong HCN production whereas the isolates SM4, TM3, KM2 exhibited moderate HCN production. However, the strains of SM9, TM1, TM5, KM1, and KM3 exhibited weak HCN production (Fig. 2c). HCN producing isolates have shown antagonistic activity against pathogenic fungi. It inhibits metalloenzyme cytochrome oxidase activity and thus plays a key role in biocontrol activity [20]. HCN producing Pseudomonas spp. has been well documented in the literature

regarding their biocontrol activity and their correlation with Phl (phenazine 1- carboxylic acid). In the present study, HCN production was found in lesser extent when compared with other traits.

CONCLUSION:

Study demonstrated that inoculation with locally isolated PGPR strains, mainly Pseudomonads could significantly increase plant- nutrient uptake, dry biomass and yield of rice crop in the study area.

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Table No. 1: Phosphate solubilisation and ammonia production by the isolates

Strains	Phosphate Solubilisation (diameter in mm)	Ammonia Production
SM2	18.33 ± 0.25	+++
SM4	18.00 ± 0.55	++
SM8	15.51± 0.51	+++
SM9	7.67 ± 0.27	++
SM11	14.67 ± 0.57	+++
TM1	13.00 ± 0.52	++
TM3	8.67 ± 0.62	++
TM5	0.00 ± 0.00	+
KM1	9.00 ± 0.51	++
KM2	12.33 ± 0.59	++
KM3	14.00 ± 0.26	+

Value represents mean of triplicates; +++-High; ++ - Moderate; + - Low

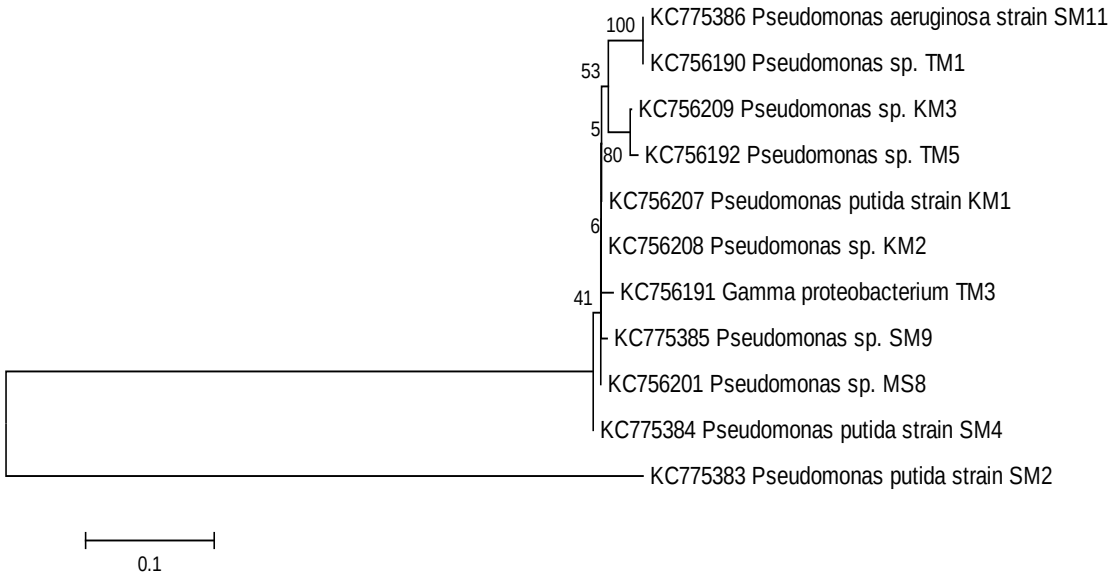

Figure No. 1: Evolutionary relationship of taxa using Neighbor-Joining method

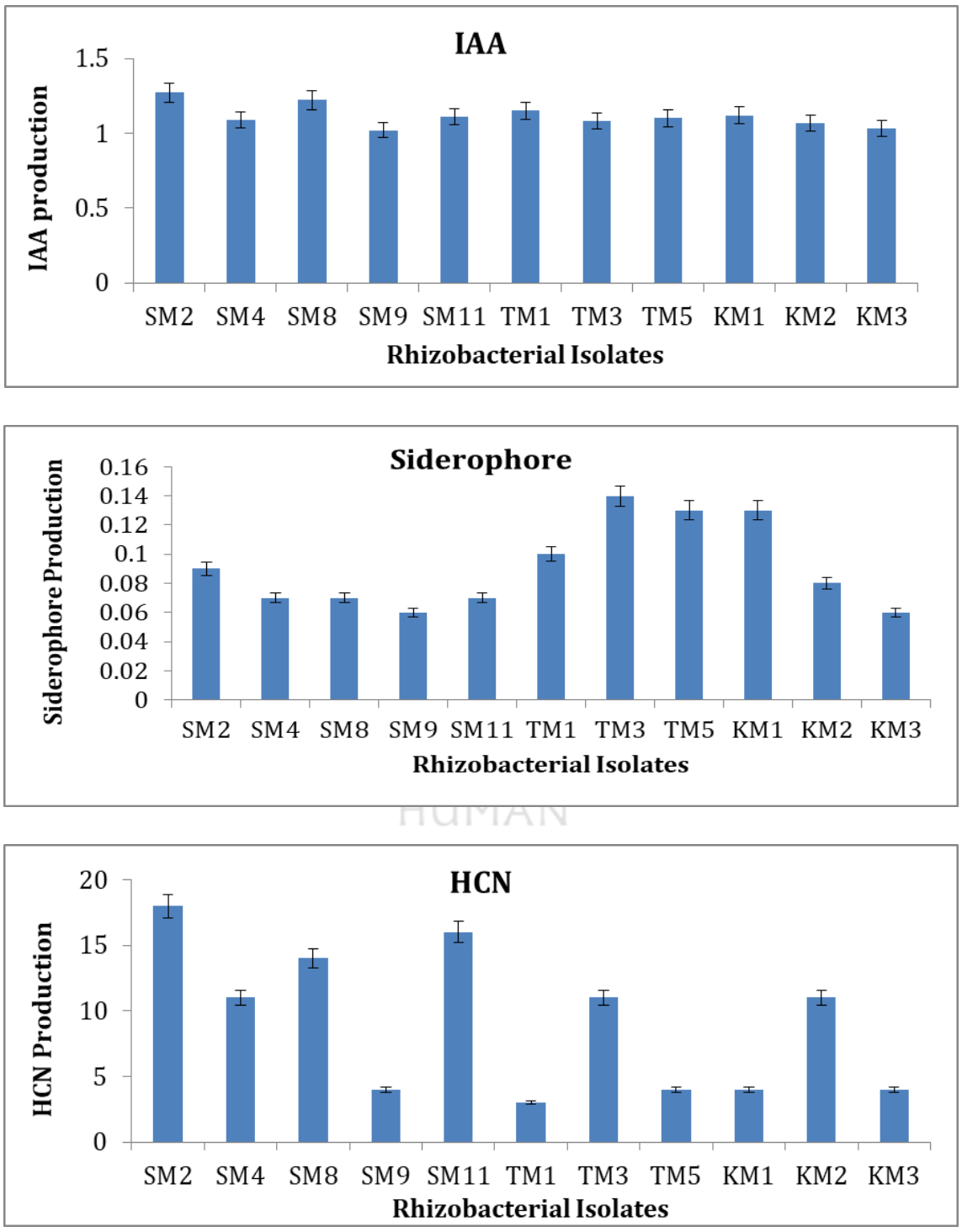

Figure No. 2: (a) IAA production, (b) Siderophore production and (c) HCN production by the isolates