ISOLATION AND CHARACTERIZATION OF N_2 FIXING BACTERIA FROM CURCUMA RHIZOSPHERE SOIL OF WESTERN GHATS.

SATHEESH CHANDRAN.J.G
Research Scholar, Center for Marine Science and Technology, Rajakkamangalam.

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ABSTRACT: *The rhizome adhering soil of turmeric has high microbial population in comparison to the bulk soil. The rhizosphere of the plant is hot spots for the plant- microbe interaction. Plant associated microbes sometimes give physiological and environmental advantages to their host. The microbes are isolated by growing in selective media. 4 strains PRI, PRII, PF and PFII are selected and from these strains PRII (Rhizobium pusense) was identified as N2 fixing bacteria and characterized by means of next generation sequencing. Rhizobium pusense strains were used as bio inoculants to demonstrate their effects on curcuma plant growth. In pot studies, it was found that leaf parameters such as length, number, breadth, leaf area, chlorophyll content, shoot length, shoot biomass and root characteristics such as length and biomass, rhizome yield, size, weight all the growth parameters and yield found maximum in plants which was inoculated with Rhizobium pusense.*

Key Words: *PGPR, Turmeric, Rhizosphere, Jensens Media, Next Generation Sequencing, Pot trials*

Introduction

Curcuma genus is classified under the family Zingiberaceae. The genus contains about 80 species (Larsen K 2005). The rhizome is commonly used as spices and traditional medicine in Indian households. It is a herbaceous plant with thick fleshy rhizomes, pseudo stem and leaf blades.Soil parameter includes PH, electrical conductivity, available nitrogen, Phosphorus, potassium and organic carbon contents. Two dominant types of turmeric found in the World market includes 'Madras' and 'Alleppy' and they are named after the production regions of India. .The important turmeric production states in India are Andhra Pradesh, TamilNadu, Orissa, Maharashtra, Assam, Kerala, Karnataka and West Bengal. Tamil Nadu accounts for about 17% of total area under turmeric production in India.Turmeric is available in two seasons in India i.e., February to May and August to October. The microbes attain shelter and nutrients from the host plants (Reiter and Sessitsch 2006). Rhizosphere is the most prominent zone for the interaction of microbes. The rhizosphere region is highly favorable habitat for the multiplicationand metabolism of various types of microorganisms. Among rhizospheric and non-rhizospheric soil the population of microorganisms differs both qualitatively and quantitatively. Turmeric possess innumerable diverse microbes in the rhizosphere. Rhizosphere bacteria influence plant growth both by direct and indirect mechanism. These bacteria are also called as plant growth promoting rhizobacteria (PGPR). Approximately 80% of rhizosphere bacteria can secrete IAA (Indole Acetic Acid) a common natural auxin and is a product of L-Tryptophan metabolism in microorganisms by phosphate soloubilization or by N_2 fixation.

It is necessary to study the rhizosphere micro flora with more advanced culture-independent techniques such as next generation sequencing (NGS) with the aim of discovering novel bacteria with plant growth promoting traits. The automated Sanger method is considered as a "first-generation" technology, and the newer methods are referred to as next-generation sequencing (NGS) (Pettersson et al., 2009). Next generation sequencing technologies (NGS) have enabled whole genome sequencing of bacteria and other organisms (Schuster SC, 2008). Recently, NGS has been employed to study genomes of several PGPRs.

The objective of the study was to isolate beneficial N_2 fixing bacteria from curcuma rhizosphere of Western Ghats. Western Ghats is one of the global hotspots of biodiversity. It is also a rich germplasm center of number of wild relatives of many medicinal plants including *Curcuma*. The enumerated beneficial bacterial strains was used to retrieve their plant growth promotion potential, soil parameters, microbial profiling and also the impact of PGPR inoculation on growth of curcuma by checking their parameters after potting.

Materials and Methods

Soil sampling and bacterial isolation

Bacterial strain were isolated from the rhizospheric soil of young and healthy turmeric plants grown in various farmers field of Kanyakumari district which comes under region of Western Ghats using

standard microbiological techniques (soil serial dilutions or spread-plate methods). Rhizospheric soil (1g) was dissolved in10ml of sterile distilled water, making 10^{-1} dilution. This dilution was further diluted to10^{-7}. 1 ml of each dilution 10^{-6} and 10^{-7} was allowed to grow on selective media called Jensen's media for N_2 fixing bacteria (K_2HPO_4 – 1g, $MGSO_4$ – 1g, Nacl – 0.5 g, $FesO_4$ -0.1 g, Sucrose – 20 g, $Ca(CO_3)_2$ – 2 g and Agar – 20 g). The plates were incubated (48h)at 30° C and colonies showing morphologicaldifference isolated for further analysis. The parameters of rhizosphere soil collected from fields such as organic carbon, electrical conductivity pH, available nitrogen, phosphorus and potassium were determined.

Morphological characteristics

The identification and characterization of bacterial isolates were performed based on morphology and biochemical screening according to Bergey's manual of systematic bacteriology (Holt et al.,1994). From that it was identified that the strain PRII belongs to Rhizobium species. To confirm this further molecular works such as next generation sequencing is done.

Identification ofbacterialisolates using Molecular biology techniques

16s rRNAgeneamplification andsequencing

Genomic DNA was isolated using Genei Pure™ bacterial DNA purification kit (GeNei™, Bangaluru,India) according to the manufacture's protocol. Universal eubacterial primers F-D1-5′-ccgaattcgtcgacaacagagtttgatcctggctcag-3′ and R-D1-5′-cccgggatc-caagcttaaggaggtgatccagcc-3′ (Kumaretal.,2015), were used to amplify the 1500bp region of 16Sr RNA gene using a thermal cycler(BioRad,USA). Amplified products were resolved by electro phoresisinagarose(1%), and visualized in the gel documentation system.The amplicons were purified using Gene iPure™ quickPCRpurification kit (GeNei™, Bangaluru,India).Thepurified partial16SrDNA amplicon was sequenced in Applied Biosystems 3130 Genetic Analyzer.

Analysis sequenced data with BLAST

The 16s rRNA gene partial sequences of the isolated strains were sequenced and compared with the RNA databases. The resulting nucleotide sequence were assigned for bacterial taxonomic affiliation based on the closest match to the sequences available at National Center for Biotechnology Information (NCBI)BLAST server using Nucleotide Basic Local Alignment Search Tool (BLAST) program. The sequences showing 92% similarity were retrieved. The cluster analysis of the sequence was done using the multiple sequences alignment tool, Clustal X2.1 version. The phylogenetic and molecular analyses were conducted using MEGA5.1(Kumaretal.,2015). The strain PRII was identified as *Rhizobium pusens* and its inoculam was prepared for further works.The population density that resulted in formation of 10^8 cfu/ml of bacterial isolate was used for preparation of liquid formulation.

Methods of bacterial inoculation

Young and healthy rhizotomus buds of turmeric collected were surface sterilized by washing with 70% ethanol(4–5 times) followed by incubation in 1% sodium hypochlorite (5min). The solution was suctioned off and the rhizome is thoroughly rinsed with sterilized distilled water at least five times.The rhizome were inoculated with10^8 CFU/ ml of *Rhizobium pusens* along with 1% Carboxy methyl cellulose (CMC) as the adhesive. The rhizome coated with 1% slurry without the bacterial strain used as control. After 24h, rhizome planted in the soil collected from the field and mixed with (soil: sand: compost) in the ratio of (2:1:1). The pots were arranged with treatment and control under natural conditions and irrigated from time to time. (Ambardar and Vakhlu,2013; Kumaretal.,2014a,b).

Shoot and root parameters such as leaf number, length, leaf breadth, biomass, leaf area, chlorophyll content, root length, root biomass, rhizome weight, rhizome size, rhizome yield, height of plant and harvest index etc. are noted in control and treatment plants.

Result and discussion

A total of 40bacteria were isolated from the rhizospheric soils of turmeric (as shown in fig. 1) and most of them were Gram negative (70%), rod shaped (100%) and positive for catalase and oxidase.Most of the bacterial strains belonged to genus Rhizobium and Agrobacterium.

Fig1: Isolated Bacterial colonies on media

Table 1: Biochemical characteristics of selected bacterial isolate

Characteristics	Results
Gram staining	-
Shape	Rod
Catalase	+
Starch hydrolysis	+
P - solubilization	+

Further confirmations of the species levels of the isolates were carried out through biochemical and 16SrRNA gene sequence analysis and the Isolate PRII was identified as *Rhizobium pusense*as mentioned in table 2.

Table 2: Closest relative of the isolated strains as revealed by 16S rRNA gene sequencing.

Description	Max. Score	Total Score	Query cover	E value	Ident	Accession
Rhizobium pusense strain A1143 16S ribosomal RNA gene, partial sequence	1947	1947	99%	0.0	92%	JX266311.1

Sequence:

CACGGCTACTGTTGACTTCACCCCAGTCGCTGACCCCTACCGTGGTTAGCTGCCT
CCCTTGCGGTTAGCGCACTACCTTCAGGTGAAACCAACTCCCATGGTGTGACGGG
CGATGTACAGGCCAGAGACGTATTCACCGCAGCATGCTGATCTGCGATTACTAGC
GATTCCAGCTTCATGCACTCGAGTTTTGAGTGCAATCGAACTGAGATGGCTTTTG
AGATTAGCTCGACATCGCTGTCTCTTTGCCCACTGTCACCACCATTGTAGCACGT
GTGGCCCAGCCCGTAAGGGCCATGAGGACTTGACGTCATCCCCCACCTTCCTCTC
GGCTATCACCGGCGGTCCCGCCGTGCCCAACTAAATGCTGGCAACTTCAGGGCG
AGGGTTGCTCGTTGCGGGACAACCCAACATCTCACGACACGAGCTGACGACAAC
CATGCAGCACCTGTTCTGGGGCCAGCCTAACTGAAGGACATCGTCTCCAATGCCC
ATACCCCGAATGTCAGAGCTGTGTGGAGTTCTGCGCGTTGCTTCGGGTAAACCAC
ATGCTCCACCGCTTGTGCGGGCCCCGTCAATTCGCTTTGAGTTTTAATCTTGCATG
CCCCGGGCGGAATGTTTGTGCGTTGGCTGCGCCGCCGAACAGTATACTGCCCGACGGCTAGCATTCATCGTTTACGG
CATTAGACTACCAGGGTATCTAATCCTGTTTGC
TCCCCCACGCTTTCGCACCTCCAGCGTCAGTAATGGACCAGTAAGCCGCCTTCGC
CCTGGTGTTCCTCGAATATCTACGAATTTCACCTCTACACTCGAATTCCGCCTCTT
CCATACTCAAGATACCCAGTATCAAGGCAGTTCCGCAGTTGAGCTGCGGGATTTC
ACCCTGACTTAAATATCCGCCTACGTGCGCTTTACGCCCAGTATTCCGAACAACG
CTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAGATTAGCCGAACTGTGCTC
TCCGACTACCGTCATTATCTTCATCGGTGAAGGAGCTTTACAACCTAAGACCTTC
ATCACTCACGCGGCATGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCAC
TGCTGCTCCGTAGGGGTTTTGGGCCCGTGTCTCAGTCCCAAGTATGGCTGATCAT
CCTCAGACCAGCTATGGATCGTCGCCTTGGTAGGCCTTTTACCAACTAGCTAATC
CAACGCGGGCCAATCGCTGATAAATCTTTCCCCCGTAGGGCGGTAATGCGGTATT
AATTCCAGTTTCCCGGGCTATTCCGCAGGAAGGTATGTTCACGCGTTACTCACCC
GTCTGCCACTCCCCTTGCGGGGCGTTCATTTGCATGTGTTAAGCCTGCCGCCAGC

GTTCGTTCTGAGCCAGGGTCAAACT
Organism: *Rhizobium pusense*

Soil parameters

The physico chemical properties of the rhizospheric soil collected from farmers field such as organic carbon, available N, P, K, soil pH are measured. The parameters are again checked for soil in the potting mixture after bacterial inoculation.The data revealed that the soil was slightly acidic with pH 6.34;EC (0.48) was safe for crops and medium in organic carbon (0.92).The available N (297.70) and K (285.21) contents was in mediumrange, however, available P (45.0) content was in high range and the values are interpreted in table 3.

Table3: Parameters of Rhizospheric soil

Soil Properties	Values of field soil sample	Values after treatment
PH	7.1	6.34
Organic carbon	0.63	0.92
Electrical conductivity	0.62	0.48
Available N2(kg ha-1)	225	297.70
Available Phosphorus (kg ha-1)	22.55	45
Available Potassium (kg ha-1)	218.54	285.21

Pot trials

The various morphological parameters measured in control and *Rhizobium pusens* inoculated turmeric at the time intervals. *Rhizobium pusens* inoculated turmeric reflected better growth and yield in comparison to control. PGPR inoculation increased the average number of leaves, length of leaves, breadth of leaves, leaf area and chlorophyll content. In case of root and yield also the same level of increase in inoculam treated strain can be observed and are shown in table 2.

Table 3: Various growth and yield parameters of *R.pusens*PRII inoculated turmeric

Parameters	Control (without bacterial inoculam)	T1(Bacterial strain Inoculated)
Leaf Number	3.20	6.10
Length (cm)	52.07	60.05
Leaf Breadth	10.21	13.75
Biomass (g/ plant)	22.50	27.24
Leaf Area (cm2)	10.78	13.75
Chlorophyll content (%)	2.54	3.84
Root Length (cm)	14.32	17.19
Root biomass (g/ plant)	4.58	6.94
Rhizome weight (g/ plant)	179.25	218.90
Size of Rhizome (cm^2)	10.12	13.70
Rhizome Yield (g)/ plant	178.35	224.07
Height of plant (cm)	35.47	43.9
Harvest Index	57.98	65.61

Fig 2: Effect of rhizome microbial treatments on potting with isolated R.pusens

Conclusion

This study demonstrates the isolation and characterization of rhizobacterial isolates from turmeric, in terms of N2 fixing activity by growing in selective Jensens media. On the basis of pottrials, it may be concluded that *R.pusense* PRII would be one of the best option, as plant inoculants for N_2 fixing activity in sustainable turmeric production because when compared to control the treatment T1(bacterium inoculated) showed high yield .

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