Biochemical characteristics and ISSR profiling of microorganisms present in rhizospheric soil of pea, chilli and carrot

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Abstract

The Rhizospheric soil bacteria are a group of bacteria which plays an important role in regulating organic matter, decomposition and nutrient cycle. They also play vital roles in plant nutrition, growth promotion and disease interaction. Hence these bacteria are very crucial for breeders and especially for leguminous crops. The present study was aimed to identify Rhizosphere soil bacteria using PCR based molecular markers like ISSR (Inter simple sequence repeats) markers and their biochemical characteristics. ISSR primer (UBC-812) showed polymorphic amplification between the two samples thus proving that each sample contain different bacterial species. Further these samples were set for PCR reaction using 16s RNA primer which showed the conserved monomorphic amplification in all the samples. After sequencing and NCBI blast two bacteria were identified as (*Acinetobacter calcoacetius, Bacillus arabhattai*). Identification of these bacteria will help breeders in future to create different types of biofertilizers which will increase the fertility of the soil, solubilization of minerals, fixation of nitrogen, production of growth promoting hormones and competitive suppression of pathogens in crops.

Introduction

The Rhizosphere is the area of soil around a plant root where the biology and chemistry of the soil are changed by the root. Although there is no clear margin, the zone is around 1 mm wide. The substances that the root exudes and the microorganisms that feed on those compounds have an impact on the biological and chemical activity in this region.

Pisum sativum is an herbaceous annual in the Fabaceae (formerly Leguminosae) family, It contains high percentage of digestible proteins, vit.- A and vit.- C. carrot is conventional medicine carrots, in the form of the freshly grated root or juice, are used mainly for the treatment of nutritional disorders in babies, vitam in A deficiency and threadworms.

Chilies are a good source of Vitamin C. They also contain vitamins B1 and B2, beta carotene, protein, calcium and phosphorous. The present study designed to screen certain rhizospheric isolates from vegetable growing in soils of Raipur , Chhattishgarh.

Materials & Methodology

Sample collection

Soil sample of pea, chili and carrot was collected from field of Aditya Biotech Research and Development, Raipur (C.G.).

Isolation

a. Serial dilution

1g of the collected soil sample was added to 9 ml of sterile water to make 1:10 dilution, then 1ml of the previously diluted sample added to 9ml of sterile water to make 1:100 dilution and so on for further dilutions.

1 mL of diluted sample plated in a media NA (Nutrient Agar) with the pour plate method and incubated at 37^{o}C for 48 hr.

b. Purification

Isolated bacteria grown in a NA media by the streak plate method and this process repeated until pure isolates were maintained.

Identification

Bergey's Mannual of Systematic Bacteriology was used to identify the isolates based on their morphological, cultural, and biochemical traits (Kandler and Weiss, 1986).

Morphological study

a. Gram staining

A smear of the bacterial culture was prepared on a clean grease free slide with a pre-sterilzed loop. The air dried and heat fixed smear was flooded with crystal violet for 1min after washing with distill water. Then, washed with alcohol after flooded with Gram’s iodine for 1 min. Finally, the slide was counterstained with saffranin for 30 seconds before being washed with distil water.

b. Colony morphology

The stained slide was then observed under microscope and the morphology of selected strain determined according to their shape, size and colour.

Biochemical

The isolated strains were biochemically identified using the procedures described below.

a. Mannitol Test

The bacteria able to ferment mannitol sugar, raises pH of the media by producing acids. The change of color from red to yellow shows positive result.

b. Citrate Test

In this test citrate is the only available carbon source and converted to oxaloacetate by the bacteria. Bacteria turns media into bright blue color gives +ve result.

c. Catalase

The presence of catalase enzyme was tested by inoculating a loopful of culture into tubes containing 3% of hydrogen peroxide solution. The formation of effervescence resulting from breakdown of hydrogen peroxide to O_2 and H_2O indicates positive result.

d. Starch hydrolysis or amylase production test

This test is used to detect the disappearance of starch. Iodine combines with the amylase starch fraction to form an intense, deep color complex. Inoculated A starch plate was inoculated with the organism to be tested and incubated at 28°C for at least 24 hours. A clear zone indicates hydrolysis while blue color indicates no hydrolysis.

e. Hydrolysis of gelatin

Gelatinases are proteases secreted extracellularly by some bacteria which hydrolyze or digest gelatin. This process takes place in two sequential reactions. In the first reaction, gelatinases degrade gelatin to polypeptides.

f. IMViC TEST

The IMViC test consists of four different tests:

1. Indole production
2. Methyl- red
3. Voges- Proskauer, and
4. Citrate utilization.

Physiological test
Estimating of pH**:** Tubes of NAM broth were made with different pH (4, 5, 6, 7, & 8) inoculated with bacterial culture and kept at 37°C for 24 hrs for growth.

Molecular characterization

Isolation of genomic DNA**:** Isolation of genomic DNA was done by CTAB (cetyltrimethylammonium bromide) method referred from Aditya Biotech Agricon Research and Development Centre lab manual.

Procedure

2 ml overnight culture is taken and the cells are harvested by centrifugation (5000 rpm) for 10 minutes. Remove the supernatant and repeat this step again. 750 µl of extraction buffer is added to the cell pellet and the cells are resuspended in the buffer by gentle mixing vortex the bacterial pallet with extraction buffer. The above mixture is incubated at 50^{o}C for 15 minutes in water bath. 1 ml of chloroform is added to the contents, mixed well by inverting and incubated at room temperature for 5 minutes. The contents are centrifuged at 10,000 rpm for 10 minutes. Transfer the supernatant to a new tube and add 2-3 volume of ice cold isopropanol. Mix very gently till you see cotton fiber like growth. Store at -20^{o}C for 5 to 10 min and spin 10000 rpm for 10 min. Discard supernatant and collect DNA pellet. Wash pellet with 75 % ethanol by centrifuge 10000 rpm for 5 min, dry the ethanol and pellet and dissolve the pellet with autoclave D/W or M.Q. Leave overnight at room temp. Add 1/10 volume of 3M sodium acetated and 2-3 volume of ethanol. Mix gently and incubate at -20^{o}C for 10-15 min. Spin 10000 rpm for 5 to 10 min. Discard supernatant and was the pellet with 70% ethanol by centrifuge 10000 rpm for 2-3 min. Dry the ethanol and pellet in room temp. Dissolve the pellet in T.E. Buffer.

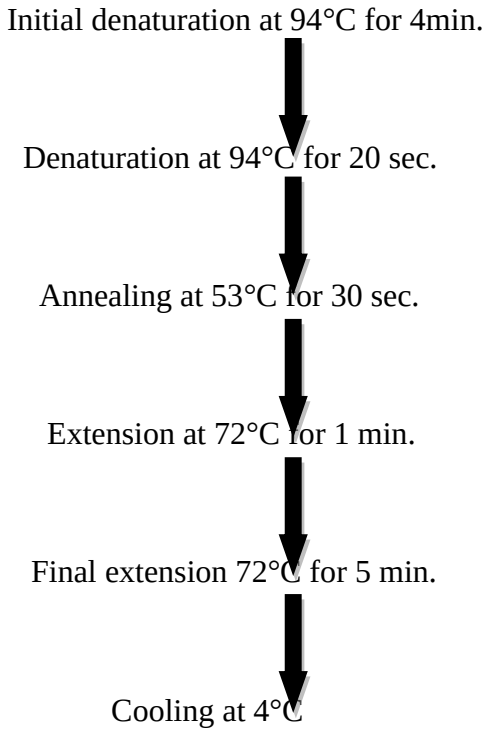
Quantification of extracted DNA

Quantification of DNA sample done by using 0.8% agrose gel. 2µl of EtBr solution added on it. The electrophoresis tank filled with 1x TAE buffer. Prepared samples (5µl of DNA sample, 5µl of MQ & 3µl of gel loading dye) was loaded and run it at 50-100 volts for 20 min. Place the gel on the UV- transilluminator tray and checked DNA bands by using Gel doc.

PCR amplification

Bacterial DNA samples were subjected to PCR using a variety of primers, including the universal primers UBC 807 and UBC 812. The thermal cycle profile was as follows: initial denaturation for 1 cycle lasting 4 minutes at 94°C, 35 cycles of denaturation for 20 seconds each, annealing for 35 cycles lasting 30 seconds each at 53°C, extension for 35 cycles lasting 1 minute each at 72°C, and a final extension for 1 cycle lasting 5 minutes. Storage of PCR products at 4 °C.Gel electrophoresis (Tarson M1-01) 2% agrose gel stained with ethidium bromide was used to analyse the PCR products. Gel Documentation System (Bio Rad Gel doc EZ imager) was used to take pictures of the gels.

Thermal profile of PCR



Amplification of 16S r-RNA gene

Pure culture of the target bacteria was grown overnight on nutrient broth for the isolation of DNA. The DNA was isolated from bacteria using the CTAB method and 16S rRNA was amplified by Thermocycler (Eppendroff) using the primers,

Universal Primer

16S F – 5’ GAG TTT GAT CCT GGC TCA G

16S R – 5’ AGA AAG GAG GTG ATC CAG CC

Each reaction mixture (20ul) contained 2µl primer (F- 1µl and R 1µl) , 0.2µl Taq DNA polymerase , 2µl 10X Buffer , 1µl DNTP’s , 2µl isolated DNA and Nuclease free water until final volume reaches 20µl. thermocycling condition were set up as follows: 1 cycle for 4 min initial denaturation at 94°C, 35 cycle of 20 sec denaturation at 94°C, 35 cycle for 30 sec

annealing at 53°C, 35 cycle for 1 min extension at 72°C, followed by a final extension 1 cycle for 5 min. Storage at 4°C for PCR product. After amplification, 3µl PCR product was migrated in 1.2% agarose gel and visualized (figure no.) using Gel Documentation system (Bio Rad- Gel doc EZ imager).

Phylogenetic and Bioinformatics analysis

The PCR products were sent to BIOSERVE BIOTECHNOLOGIES HYDRABAD (INDIA) PVT. LTD. for sequencing. The data of 16S rRNA gene sequences were compared with database at Gene Bank using BLAST-N search program in National Center Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Results and Discussion

Few biochemical tests (Starch hydrolysis test, gelatinase test, and phosphatesolubilising test and catalase test), Physiologicaland Gram staining were performed. Bacterial genomic DNA extraction by C-TAB method and its PCR Amplification were performed.

Test performed on the microbes present in rhizospheric soil around the pea soil

Isolation and colony characteristics

The sample of soil was collected from the field where Pea was grown, sample of soil was then serially diluted up 10^{-1} to 10^{-7} and dilution was transferred to the NAM plates and incubated at 37°C for 48 hrs.

Morphological characteristics

The three colonies (P1, P2, and P3) were observed after the desired period of incubation. The colonies were first identified on the basis of different morphological feature viz. shape, size, color, margin, elevation, transparency and consistency.

Table 1. Colony characterization of bacteria isolated from rhizospheric soil around Pea

Colony	Shape	Size	Color	Margin	Elevation	Transparency	consistency
P1	Cocci	Tiny	White	Entire	Concave	Non-transparent	Wet
P2	Cocci	Tiny	White	Smooth	Concave	Non-transparent	Wet

Gram staining

Two colonies were recorded as Gram negative and one were found as Gram positive in Pea soil sample. The obtained microbial flora was observed and result showed in table no. 6.2.

Table 2. Morphological characterization of bacteria isolated from rhizospheric soil around Pea

Colony name	Shape	Color	Gram nature
P1	Short & thick rod	Pink	-ve
P2	Cocci	Pink	-ve

Biochemical tests:

The following test had been done on the three microbial strains isolated from the soil:

1. Starch hydrolysis
2. Hydrolysis of gelatin
3. Catalase test
4. Casein test
5. IMViC test

All microbes gave positive result in starch hydrolysis test. But some microbes are gave negative result in gelatin hydrolysis as shown in table no.

Table 3. Result of biochemical tests performed on all three pure culture of microbial colonies

Colony	**Catalase test**	**Starch hydrolysis**	**Gelatin hydrolysis**	**Casein test**	**IMViC test**			
					Indole	**MR**	**VP**	**Citrate utilization**
P1	+ve	+ve	+ve	+ve	-ve	-ve		-ve
P2	+ve	+ve	+ve	+ve	-ve	-ve		-ve

Test performed on the microbes present in rhizospheric soil around the carrot soil

Isolation and colony characteristics

The sample of soil was collected from the field where Carrot was grown, sample of soil was then serially diluted up 10^{-1} to 10^{-7} and dilution was transferred to the NAM plates and incubated at 37°C for 48 hrs.

Morphological charecterstics

The three colonies (C1 & C2) were observed after the desired period of incubation. The colonies were first identified on the basis of different morphological feature viz. shape, size, color, margin, elevation, transparency and consistency.

Table 4. Colony characterization of bacteria isolated from rhizospheric soil around Carrot

Colony	Shape	Size	Color	Margin	Elevation	Transparency	consistency
C1	Cocci	Medium	White	Smooth	Concave	Non-transparent	Wet
C2	Cocci	Medium	White	Rough	Concave	Non-transparent	Wet
C3	Cocci	Tiny	White	Smoth	Concave	transparent	Dry

Gram staining

All three colonies were found as Gram negative in Carrot soil sample.The obtain microbial flora was observed and result showed in table 5.

Table 5. Morphological characterization of bacteria isolated from rhizospheric soil around Carrot

Colony name	Shape	Color	Gram nature
C1	Cocci	Pink	-ve
C2	Short & thick rods	Pink	-ve
C3	Cocci	Pink	-ve

Biochemical tests

The following test had been done on the three microbial strains isolated from the soil around Carrot:

1. Starch hydrolysis
2. Hydrolysis of gelatin
3. Catalase test
4. Casein test
5. IMViC test

Two microbes gave positive result in starch hydrolysis test. But some microbes are gave negative result in gelatin hydrolysis as shown in table no.

Table 6. Result of biochemical tests performed on all three pure cultures of microbial colonies

Colony	**Catalase test**	**Starch hydrolysis**	**Gelatin hydrolysis**	**Casein test**	**IMViC test**			
					Indole	**MR**	**VP**	**Citrate utilization**
C1	**-ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**-ve**
C2	**+ve**	**-ve**	**-ve**	**-ve**	**+ve**	**+ve**	**-ve**	**+ve**
C3	**+ve**	**-ve**	**-ve**	**+ve**	**-ve**	**-ve**	**-ve**	**-ve**

Test performed on the microbes present in rhizospheric soil around the chili soil

Isolation and colony characteristics

The sample of soil was collected from the field where Chili was grown, sample of soil was then serially diluted up 10^{-1} to 10^{-7} and dilution was transferred to the NAM plates and incubated at 37°C for 48 hrs.

Morphological characteristics

The three colonies (Ch1, Ch2, & Ch3) were observed after the desired period of incubation. The colonies were first identified on the basis of different morphological feature viz. shape, size, color, margin, elevation, transparency and consistency.

Table 7. Colony characterization of bacteriaisolated from rhizospheric soil around Chili

Colony	Shape	Size	Color	Margin	Elevation	Transparency	consistency
Ch1	Cocci	Tiny	White	Erose	Raised	Opaque	Wet
Ch2	Cocci	Medium	White	Entire	Flat	Opaque	Wet
Ch3	Bacillus	Medium	White	Entire	Flat	Opaque	Wet

Gram staining:- Two colonies were recorded as Gram negative & one was found as Gram positive in chili soil sample. The obtain microbial flora was observed and result showed in table no.

Table 8. Morphological characterization of bacteria isolated from rhizospheric soil around Chili

Colony name	Shape	Color	Gram nature
Ch1	Cocci	**Pink**	**-ve**
Ch2	Cocci	**Pink**	**-ve**
Ch3	Bacillus	**Purple**	**+ve**

Biochemical tests: The following test had been done on the three microbial strains isolated from the soil around Chili:

1. Starch hydrolysis
2. Hydrolysis of gelatin
3. Catalase test
4. Casein test
5. IMViC test

All three microbes gave positive result in starch hydrolysis test. But some microbes are gave negative result in gelatin hydrolysis as shown in table no.

Table 9. Result of biochemical tests performed on all three pure cultures of microbial colonies

Colony	**Catalase test**	**Starch hydrolysis**	**Gelatin hydrolysis**	**Casein test**	**IMViC test**			
					Indole	**MR**	**VP**	**Citrate utilization**
Ch1	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**
Ch2	**-ve**	**+ve**	**-ve**	**+ve**	**+ve**	**-ve**	**-ve**	**-ve**
Ch3	**-ve**	**-ve**	**-ve**	**+ve**	**-ve**	**-ve**	**+ve**	**-ve**

Molecular Characterization

Genetic diversity analysis: Genetic diversity analysis was performed by ISSR based markers. Total 8 markers used for the genetic diversity analysis.

UBC 807 – Using ubc primer 807 observed amplification size 480bp in Ch2, C1, C2, & absent in P2, P3, 500bp in C1, C2, & P2 and absent in Ch2, & P3 and 690bp observed in only P3. 1000bp observed in only C1. Dose it conform that both the bacteria different from each other (Figure No.9.).

UBC 812 – Using ubc primer 812 observed amplification size 790bp in C1, 820bp & 890bp in Ch2. dose it conform that both the bacteria different from each other (Figure No.10).

Molecular Identification of Rhizosphere Bacteria

The DNA fragment containing partial 16S rRNA gene of the three isolates were amplified and sequenced. The sequenced obtained were matched with those available in Gene Bank, which revealed maximum identification of these rhizophere bacterial strains.

The isolate were identified using the maximum aligned sequence through BLAST search. The result have shown that **C2** had highest homology (95%) ***Acinetobacter calcoacetius,*** **Ch1** had highest homology (93%) with ***Bacillus arabhattai***

	Bacteria Name	Ident.	Accession
Ch1	***Bacillus arabhattai***	**93%**	**KT719705.1**
C2	***Acinetobacter calcoacetius***	**95%**	**EU921460.1**

Sequence ID	Organism Name	Isolation source
Ch1	***Bacillus arabhattai***	Rhizosphere

1 GTGGGCAACC TGCCTGTAAG ACTGGGATAA CTTCGGGAAA CCGAAGCTAA
51 TACCGGATAG GATCTTCTCC TTCATGGGAG ATGATTGAAA GATGGTTTCG
101 GCTATCACTT ACAGATGGGC CCGCGGTGCA TTAGCTAGTT GGTGAGGTAA
151 CGGCTCACCA AGGCAACGAT GCATAGCCGA CCTGAGAGGG TGATCGGCCA
201 CACTGGGACT GAGACACGGC CCAGACTCCT ACGGGAGGCA GCAGTAGGGA
251 ATCTTCCGCA ATGGACGAAA GTCTGACGGA GCAACGCCGC GTGAGTGATG
301 AAGGCTTTCG GGTCGTAAAA CTCTGTTGTT AGGGAAGAAC AAGTACGAGA
351 GTAACTGCTC GTACCTTGAC GGTACCTAAC CAGAAAGCCA CGGCTAACTA
401 CGTGCCAGCA GCCGCGGTAA TACGTAGGTG GCAAGCGTTA TCCGGAATTA
451 TTGGGCGTAA AGCGCGCGCA GGCGGTTTCT TAAGTCTGAT GTGAAAGCCC
501 ACGGCTCAAC CGTGGAGGGT CATTGGAAAC TGGGGAACTT GAGTGCAGAA
551 GAGAAAAGCG GAATTCCACG TGTAGCGGTG AAATGCGTAG AGATGTGGAG
601 GAACACCAGT GGCGAAGGCG GCTTTTTGGT CTGTAACTGA CGCTGAGGCG
651 CGAAAGCGTG GGGAGCAAAC AGGATTAGAT ACCCTGGTAG TCCACGCCGT
701 AAACGATGAG TGCTAAGTGT TAGAGGGTTT CCGCCCTTTA GTGCTGCAGC
751 TAACGCATTA AGCACTCCGC CTGGGGAGTA CGGTCGCAAG ACTGAAACTC
801 AAAGGAATTG ACGGGGGCCC GCACAAGCGG TGGAGCATGT GGTTTAATTC
851 GAAGCAACGC GAAGAACCTT ACCAGGTCTT GACATCCTCT GACAACTCTA
901 GAGATAGAGC GTTCCCCTTC GGGGGACAGA GTGACAGGTG GTGCATGGTT
951 GTCGTCAGCT CGTGTCGTGA GATGTTGGGG TTAAGTCCCG CAACGAGCGC

1001 AACCCTTGAT CTTAGTTGCC AGCATTCAGT TGGG

Sequence ID	Organism Name	Isolation source
C2	***Acinetobacter calcoaceticus***	Rhizosphere

1 CTTCGGACCT TGCGCTAATA GATGAGCCTA AGTCGGATTA GCTAGTTGGT
51 GGGGTAAAGG CCTACCAAGG CGACGATCTG TAGCGGGTCT GAGAGGATGA
101 TCCGCCACAC TGGGACTGAG ACACGGCCCA GACTCCTACG GGAGGCAGCA
151 GTGGGGAATA TTGGACAATG GGCGCAAGCC TGATCCAGCC ATGCCGCGTG
201 TGTGAAGAAG GCCTTATGGT TGTAAAGCAC TTTAAGCGAG GAGGAGGCTA
251 CTTTAGTTAA TACCTAGAGA TAGTGGACGT TACTCGCAGA ATAAGCACCG
301 GCTAACTCTG TGCCAGCAGC CGCGGTAATA CAGAGGGTGC AAGCGTTAAT
351 CGGATTTACT GGGCGTAAAG CGCGCGTAGG CGGCTAATTA AGTCAAATGT
401 GAAATCCCCG AGCTTAACTT GGGAATTGCA TTCGATACTG GTTAGCTAGA
451 GTGTGGGAGA GGATGGTAGA ATTCCAGGTG TAGCGGTGAA ATGCGTAGAG
501 ATCTGGAGGA ATACCGATGG CGAAGGCAGC CATCTGGCCT AACACTGACG
551 CTGAGGTGCG AAAGCATGGG GAGCAAACAG GATTAGATAC CCTGGTAGTC
601 CATGCCGTAA ACGATGTCTA CTAGCCGTTG GGGCCTTTGA GGGCTTTAGT
651 GGCGCAGCTA ACGCGATAAG TAGACCGCCT GGGGAGTACG GTCGCAAGAC
701 TAAAACTCAA ATGAATTGA

Conclusion

Soils are regarded as dynamic living environment. Soil and potting media provide plants and other organisms with nutrients and habitats. Microorganisms in soils and potting media are constantly vying for food, water, and space. Microbes in these substrates obtain nutrients by competing with each other for dead organic matter, feeding on other living organisms (including each other), and/ or interacting with other organisms. Seasonal changes in the microbes affect the plant Zou *et al.*, reported similar result in the microbial populations.

Goverdarica and Rovira in the year 2000 suggested that soil containing greater organic matter will have greater microbial population. Wanger *et al.*, in 2004 have found that the plants deficient of major catalase isoform not only suggests a wide range of pathogens but also poor quality of yield. As a wide variety of catalase positive bacteria were present around the grass, we can conclude that the microbes present around the soil were not pathogenic and it will not affect the ruminants which will graze these crops.

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