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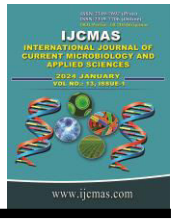

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Molecular Characterization of *Azotobacter* and PSB from Xerophytic Plants of Maharashtra State, India

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A B S T R A C T

The present study was conducted at Department of Plant Pathology and Agricultural Microbiology, MPKV, Rahuri during year 2021 to 2023 to characterize and screen different isolates of *Azotobacter* and PSB from Different Xerophytic plants from Different districts of Maharashtra State. 68 *Azotobacter* isolates were biochemically characterized and screened under *In vitro* conditions for their plant growth promoting properties. This isolates were biochemically characterized and screened *In-vitro* condition for their plant growth promoting properties. DNA polymorphism of isolates was studied using randomly amplified polymorphic DNA analysis. The genetic diversity among the bacterial strains was assessed by RAPD analysis. All the isolates showed reproducible DNA banding pattern. Diversity among the bacterial strains was assessed on the basis of variation of size number and intensity of bands.

Introduction

The *Azotobacter* and PSB organisms, genomic DNA can be analyzed molecularly to help distinguish bacterial strains at the inter species level. This technique also offers important information on the degree of genetic variation present in and among various species of organisms.

With the development of molecular techniques, a variety of arbitary primer based RAPD techniques have been utilized to type and identify several closely related bacterial species as well as to determine their genetic relationships. Its outcomes can be utilized to calculate the genetic distance because they are typically consistent with those of DNA-DNA homology investigations. Babalola *et al.,* (2002) analysed genetic diversity among three bacteria, namely *Pseudomonas spp., Enterobacter sakazakii* and *Klebsiella oxytoca* from Rhizospheres of sorghum by RAPD markers. Genetic diversity of rhizobacterial isolated from different countries were analysed using RAPDs (Hafeer *et al.,* 2006).

By using DNA markers like Restriction Fragment Length Polymorphism (RFLP) and PCR based randomly amplified polymorphic DNA (RAPD) which detect variation directly at DNA level and are not influenced by the environment, it is possible to overcome the limitation of bacterial strains on phenotypic markers. Additionally, PCR based RAPDs are fast laborious and therefore a

large number of samples can be handled in a very short period of time. These characteristics make DNA markers highly helpful for researching bacterial staining variations and identifying genetically varied species.

The most widely utilised techniques for differentiating between organisms is RAPD. An arbitary 10 base pair nucleotide sequence is used in this process to anneal to complementary template sequences throughout the genome and serve as a primer for extension by a heat stable Tag DNA polymerase, producing a series of amplified DNA fragments in each microorganisms. Each organisms has a unique amplification pattern, which may vary due to mismatches between various primer and template sequences.

The goal of current studies is to analyse the bacteria connected to moisture stress in the rhizosphere and root system and to explore how they could be used to enhance sorghum plant growth parameters and combat drought.

Therefore, in the present study, an attempt was made to elucidate the genetic diversity of *Azotobacter chroococcum* strains isolated from different xerophytic plants of Maharashtra state using RAPD markers.

Sharma *et al.,* (1999) PCR used to distinguish very closely related strain of pathogen without prior knowledge of the nature of polymorphic region by random amplified polymorphic DNA (RAPD) unlike specific (18-20 bp) primer of PCR in RAPD analysis short 10 mer primers of arbiter nucleotide sequence were used without any prior knowledge of genomic base pairs of the template.

The DNA fingerprints (banding patterns) so generated are compared for their relatedness using genetic similarity coefficients. Loganathan *et al.,* (1999) carried out total genomic DNA extracted from Nine *Acetobacter* isolates from ragi and one reference strain pal -5 were used as template DNA for RAPD fingerprinting.

In order to obtain a sufficient number of polymorphic bands to permit reliable comparisons 10 different 10-mer universal primers produce different informative banding patterns for all strains in amplification reaction.

The number of amplification product DNA fragments, irrespective of intensity used for cluster analysis were 93 of which 75 found polymorptric (80 %) when only the samples of the isolates were analyzed. The overall size of amplification products ranged from 0.2 to 0.5 kb. When a comparison was made between the type strain and the isolates a total of 95 amplification products were obtained of which 97 were polymorphic (81 %).

Materials and Methods

Isolation of *Azotobacter* and Phosphate Solubilizing Bacteria

Azotobacter and PSB strains were isolated from the soil samples, collected from different xerophytic plants from Kolhapur, Sangli, Satara, Pune and Ahmednagar districts of Maharashtra, by serial dilution and pour plate technique, using Jensen's Agar Medium and Pikovskaya's Agar Medium.

DNA Extraction

The total genomic DNA of bacterial cultures was isolated by following method given with some modification.

Protocol for DNA extraction

1. Ten ml of bacterial culture was taken in centrifugal tubes and centrifused it at 10,000 rpm for 10 minutes at 4°C.
2. Resuspended the pellet in 10 mM Tris, 100 mM NaCl and centrifused it at 10,000 rpm for 10 minutes at 4°C.
3. Resuspended the pellet in 2.5 ml of TE (Tris 50mM, EDTA 20mM) buffer containing 500 µlit of lysozyme (100mg/ µlit).
4. Incubated at 37°C for 20 minutes containing 25 µlit of RNase A (10mg/ µlit).
5. Added 2.5 ml of 2 % SDS.
6. Incubated at 50°C for 45 minutes.
7. After incubation added 50 µlit of protenase k (20 mg/µ lit)
8. Again incubated at 55°C for 10 minutes.
9. Added equal volume of CIA and centrifuged it at 10,000 rpm for 10 minutes at 4°C.
10. After centrifugation an aqueous phase is obtained and in that added ice cold ethanol (double volume).
11. Centrifuged it at 10,000 rpm for 10 minutes at 4°C.
12. After centrifugation pellet is obtained.
13. Air dried the pellet and dissolved in 50-100 µlit of TE buffer.

DNA quantification and purity analysis

Following reagents were used for DNA quantification and purity analysis

1. TBE buffer (10X) 1M Tris base (pH 8.0)
2. TBE buffer (1X)
3. Ethidium bromide, 10 mg/ml

Protocol for DNA Quantification

1. Confirmation of DNA in the sample was carried out on 0.8% agarose gel containing ethidium bromide @ 0.5 mg/ml.
2. Five µlit of sample was loaded and after 5 cm run, gel was observed under UV light and the DNA yield and quality was confirmed.
3. After confirmation of the DNA integrity it was quantified using Spectrophotometer. The concentration of purified DNA were measured by measuring absorbance at 280 nm.
4. Five µlit of all DNA extracts were electrophoresed (Bio Rad sub cell model 96 USA) in 0.8% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide at 60 V/cm in TBE buffer.
5. After electrophoresis the band intensity of genomic DNA was visualized on gel documentation unit and compared to standard unit 2. DNA. These gels also provided a visual measure of the purity and integrity of the DNA.

Quality of DNA

The quality of DNA was observed by running 50ng DNA in 0.8 % agarose gel and measuring the absorbance at 280 nm.

Dilution of DNA

Dilution was made of the samples to reach the value up to 25 ng/ µlit. The dilution was also checked by comparing them with the DNA quantification standards in agarose gel.

DNA amplification by RAPD-PCR technique

RAPD amplification was performed as per protocol by Williams *et al.,* (1990). However, modifications were made to enhance reproducibility and consistency in RAPD profiles. Amplifications were performed in a 0.2 ml PCR tubes.

PCR Amplification

1. Master Mix prepared for required samples were added to PCR tubes and then template DNA added.
2. Mixed the samples properly.
3. PCR was run on the programmable thermal cycler with the following temperature profiles-

Profile 1

Initial one cycles of denaturation 94°C for 4 minutes

Profile 2

Denaturation at 94°C for 1 minutes.
Primer annealing at 38°C for 1 minute
Primer extention at 72°C for 1 minute

Profile 3

One cycle of final extention at the 72°C for 7 minutes.
After completion of the cycles the samples were kept at 4°C till electrophoresis.

Gel Analysis and Documentation

Amplicons were separated on 1.5% agarose gel prestained with ethidium bromide solution using 1X TBE buffer.

1. Agarose (0.6g) was added to 40 ml of 1x TBE buffer and agarose was melted by heating the solution in a microwave oven.
2. The solution was cooled to about 50°C and 2ulit of ethidium bromide was added.
3. The solution was poured in to the gel casting platform after inserting the gel comb in the trough.
4. After the gel solidified (20-30 minutes) the seal was removed from the gels.
5. The trough was then placed in the electrophoretic apparatus. Sufficient amount of electrophoresis buffer (1x TBE buffer) was added to over the wells completely.
6. The 210µlit PCR product to be analyzed were mixed with 2ulit of tracking dye and loaded carefully in the wells of gel.
7. Four µlit of DNA molecular weight marker Ecor/Hind III double digest was also loaded as a standard.
8. The unit was connected to a power pack and electrophoresis was performed at 50 volts. 9. The

power supply was switched off when the bromophenol dye reached almost to the end of the gel.

9. The amplified PCR products were visualized under UV transilluminator in gel documentation system and image was captured.

RAPD data analysis

The genetic diversity among the bacterial stains was assessed by RAPD analysis. All the isolates showed reproducible DNA banding pattern. Diversity among the bacterial strains was assessed on the basis of variation of size number and intensity of bands. The strains with identical DNA fingerprints were placed in one group.

Results and Discussion

DNA number in bacterial strain on the basis of RAPD PCR analysis using random primers of OPB series

The genomic DNA of *Azotobacter* and PSB strains was analyzed using RAPD primer set obtained from OPB series. In the RAPD analysis total 10 random primers were used *viz*; OPB- 01, OPB- 02, OPB- 03, OPB- 04, OPB- 05, OPB- 06, OPB- 07, OPB- 08, OPB- 09, OPB- 10 for their ability to yield clear amplification patterns.

The results presented in Plate 9 and 10. The analysis showed variation in the profiles depending on the primer and genomic DNA used. In the same way Bacillus spp strains shared different DNA banding pattern. All the random primers yielded polymorphic amplified products with average band size ranging from 5500 bp to 250 bp when compared with DNA molecular weight marker of 1 kb in all 10 random primers. The RAPD profile shows the change in the polymorphism with primers screene. Some primers amplified unique bands.

RAPD technique has been frequently used for identification and differentiation of bacterial strain such as *Rhizobium* (Hebb *et al.,* 1998). The random and genomic wide nature of the RAPD technique is able to indicate over all genetic relatedness or dissimilarity than sequence analysis of single genome of the genome (Achenbach *et al.,* 1996). The total genomic DNA of bacterial strain was isolated by following the method given by Ivanovo *et al.,* (2000).

Molecular Characterization

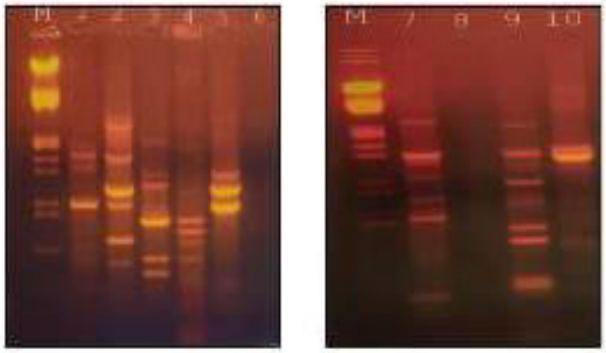
The random and genomic wide nature of RAPD technique is able to indicate over all genetic relatedness or dissimilarity than sequence analysis of a single region of the genome. This assay is rapid independent of gene expression and proving to be beneficial for the grouping of bacterial strains. In DNA fingerprinting, the present challenge is to compile standardized pattern in a database for inter laboratory use and future reference.

In the present study, RAPD markers analysis was conducted to detect the genetic relatedness and variation between bacterial strains. With some modifications giving higher yield and pure DNA.

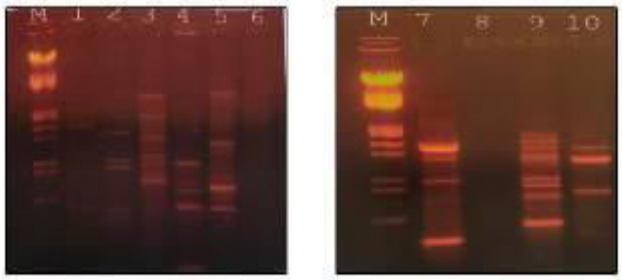
Table.1 Following RAPD primer were used with their sequences

Sr. No.	Primer code	5' to 3' Sequence
1	RBa -1	AAAACCGGGC
2	RBa -2	ACAGGGCTCT
3	RBa -3	ACAGGGGTGT
4	RBa -4	ACCGGGTTTC
5	RBa -5	AGGGGCGGCA
6	RBa -6	ATCCTGCCTG
7	RBa -7	ATCGGGTCCT
8	RBa -8	ATCGGGTCGA
9	RBa -9	ATCTGCGAGC
10	RBa -10	CCCGCCTTCC

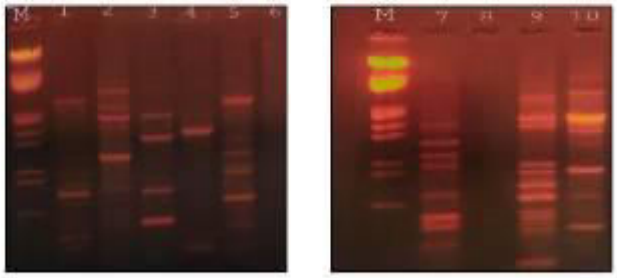
Figure.1 DNA Amplification profile of Azotobacter using different primers



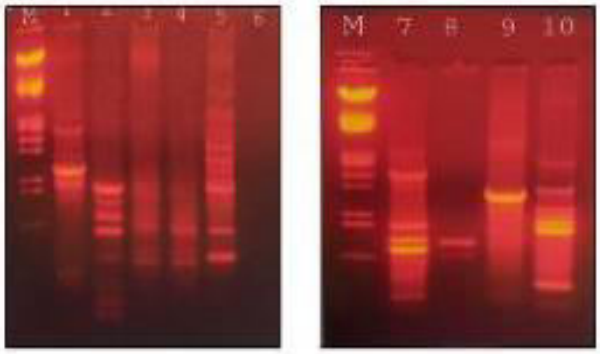
a. DNA Amplification profile using primer - 1



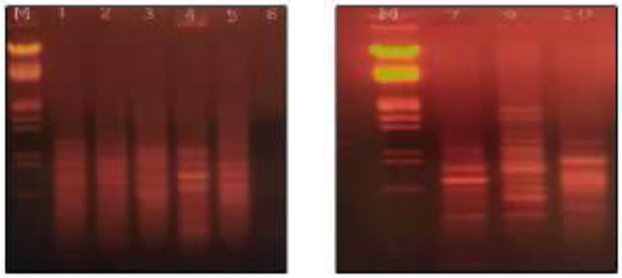
c. DNA Amplification profile using primer -3



d. DNA Amplification profile using primer - 4

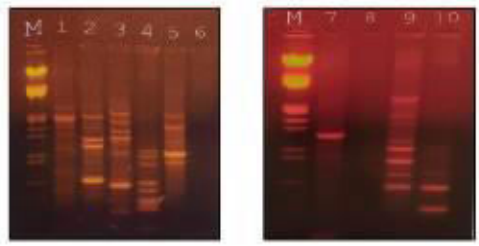


b. DNA Amplification profile using primer - 2

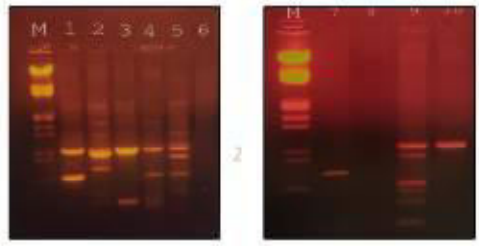


e. DNA Amplification profile using primer - 5

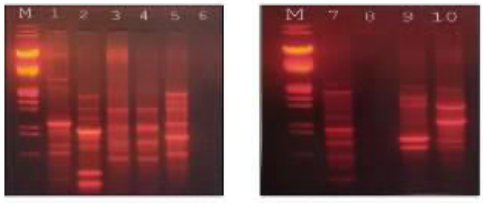
Figure.2 DNA Amplification profile of PSB using different primers



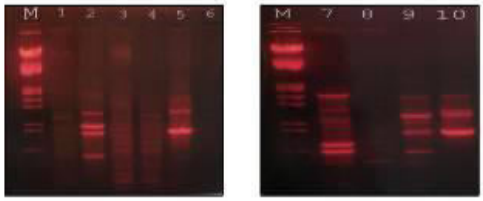
a. DNA Amplification profile using primer -1



b. DNA Amplification profile using primer -2



c. DNA Amplification profile using primer - 3



d. DNA Amplification profile using primer - 4

e. DNA Amplification profile using primer - 5

Author Contribution

Monika Suresh Barge: Investigation, formal analysis, writing—original draft. T. K. Narute: Validation, methodology, writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

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Ethical Approval: Not applicable.

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

Conflict of Interest: The authors declare no competing interests.

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