RESEARCH PAPER

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# Study the genetic variations among the *Azotobacter chroococcum* isolates using randomly amplified polymorphic DNA (RAPD) marker

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The geographical area of Karnataka is classified into ten agroclimatic zones. The first species of the genus *Azotobacter* named *Azotobacter chroococcum,* was isolated from the soil.The investigation was carried out to study the genetic variation of *Azotobacter chrococcum* occur in the soils of the ten different agroclimatic zones of Karnataka. These isolates were characterized by using RAPD markers. A total of 103 bands were scored out of which 87 bands were found to be polymorphic (84.97%). Statistical analysis of RAPD data enabled the classification of 10 *A. chroococcum* isolates in to three major groups. The RAPD banding pattern of these isolates could easily distinguish the isolates of different zones. In this the cluster analysis based on 103 RAPD bands revealed that the ten *A. chroococcum* isolates examined, clustered at a linkage distance of about 55 units on the dendrogram.

Key words : *Azotobacter chrococcum*, Waksman No. 77 N-free medium, RAPD, OPB-12, Hind3 marker,OPD-05.

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## INTRODUCTION

The geographical area of Karnataka is classified into ten agroclimatic zones. Each zone has its own characteristic feature in relation to climatic condition, soil type, vegetation which has influence on the establishment of diversified flora and fauna. Soils form an excellent cultural media for the growth and establishment of many kinds of microorganisms. A gram of soil contains millions of microorganisms. The number and kinds of organisms present in soil depend on the nature of the soil, depth, season, state of cultivation, pH, organic matter content, temperature, moisture, aeration etc.

*Azotobacter* spp. are Gram negative, aerobic, free living nitrogen fixing bacteria that play an important role in improving plant growth and yield. The first species of the genus *Azotobacter* named *Azotobacter chroococcum,* was isolated from the soil. *Azotobacter* are widely distributed in non-acidic soils of India. *Azotobacter* have been used for studying nitrogen fixation and inoculation of plants due to their influence on growth and high level of nitrogen fixation. Several studies have revealed the beneficial effect of these bacteria in the improvement of crop growth and yield and Genetic diversity also estimated at molecular level. For genetic diversity estimation species characterization of DNA fingerprinting techniques are used for an extremely wide variety of applications. Molecular analysis of genomic DNA of the organism is useful for distinguishing the bacterial strains better at intra-species level. These techniques provide valuable information on the magnitude of genetic variability within and between organisms of different species. With the advent of molecular DNA techniques, several arbitrary primers based randomly amplified polymorphic DNA (RAPD) technique has been used for typing and identification of number of closely related species of bacteria and assessment of genetic relationships. Its results are usually consistent with those of DNA-DNA homology studies and can be used to estimate the genetic diversity.

RAPD uses the polymerase chain reaction to amplify DNA samples with short oligonucleotide primers that anneal randomly through out the genome. The result is a distinctive set of amplification products. Differences are visualized by staining the gel after electrophoresis of amplification products. Use of different oligonucleotide

sequences as primers brings different banding patterns and individuals or populations are characterized by set of banding patterns they produce for a number of different primers. RAPD analysis is faster, technically less demanding and more economical than the other genomic typing methods.

Hence, the study was undertaken to study the genetic variations among the *Azotobacter chroococcum* isolates using randomly amplified polymorphic DNA (RAPD) marker.

## RESEARCH METHODOLOGY

The present project was carried out for the following objectives. It was carried out in the Department of Biotechnology, University of Agricultural Sciences,G.K.V.K Campus, Bengaluru.

– To isolate and characterized the bacteria *Azotobacter chroococcum:*

– To study the genetic variations among the *Azotobacter chroococcum* isolates using randomly amplified polymorphic DNA (RAPD) marker.

The materials used and methods followed are described below.

**Location of soil sample:**

Karnataka state has different soil type and divided into ten agroclimatic zones on the basis of annual rain fall, soil type, cropping pattern and other climatic conditions. Preferably soil was collected in polythene covers and stored in cool place.

**Soil sampling:**

Four soil samples of 500 g each were collected randomly from top six inch layer of soil from each agroclimatic zone and packed in polythene bag. They were brought to the Department of Biotechnology, University of Agricultural Sciences, GKVK Campus, Bangalore.

**Processing of soil samples:**

The soil samples collected were dried inside the laboratory at $28^0$C. The four soil samples collected from each soil type were mixed well to get a pooled soil sample for a zone. Totally ten soil samples were obtained for the study. Each soil sample was sieved through 1000µ mesh to remove the bigger soil particles and debris. The sieved soil samples were used for the isolation of the *Azotobacter* species.

**Isolation:**

*Azotobacter* species were isolated using Waksman No. 77 N-free medium by employing serial dilution plate technique. Then plates were incubated for 72 hours at 28 $^o$C$\pm$ 2. The plates were checked for *Azotobacter chroococcum* growth and pigmentation on prolonged incubation. The isolated colonies of *Azotobacter chroococcum* were re-streaked for purification and the pure isolates thus obtained were maintained on the agar slants prepared with Waksman No.77 medium

**Characterization and identification of *Azotobacter chroococcum* isolates:**

Each isolate was identified by conducting the test according to specific characters described in Bergey's manual of systematic bacteriology (1994). Observations taken were as follows:

**Morphology:**

Cell shape was observed by simple staining smears of each isolate was made, air dried and stained with crystal violet for 30 sec. Stained smear was then washed, air dried and observed under oil immersion objective of the compound microscope and cell motility was observed by Hanging drop mounts technique.

**Gram reaction:**

Gram's staining was carried out for all the isolates grown on W-77 liquid medium for 36 hours. Crystal violet and safranine were used as primary and counter stains, respectively. Gram's Iodine was used as mordant and absolute ethanol was used as decolourizer. The gram reaction (Gram positive or Gram negative) for the isolates was recorded.

**Capsule:**

*Azotobacter chroococcum* isolates were grown on Waksman No. 77 N-free agar medium for 3 days at room temperature and capsules were observed under oil immersion objective of the compound microscope after negative staining with nigrosine.

**Cyst formation and pigmentation:**

The *Azotobacter chroococcum* isolates representing each agroclimatic zone were grown on W-77 N-free agar medium for 7 days. These isolates were simple stained with crystal violate for cyst and observed under oil immersion objective of the compound microscope. The *Azotobacter* isolates were streaked on W-77 agar medium and incubated for seven days. Change of colour from

white to dark brown was recorded.

**Carbohydrate utilization:**

The ten *Azotobacter* isolates were separately streaked on Waksman No. 77 N-free medium containing different carbon sources *viz.,* mannitol, glucose, fructose, sucrose, rhamnose, malate, glycolate, and citrate and observation for growth was recorded 7 days after incubation.

**Preparation of *A. chroococcum* inoculants:**

The isolates representing each zone along with a reference culture maintained in the Department of Biotechnology were separately inoculated in to Waksman No. 77 N-free broth in 250 ml conical flasks and incubated on a rotary shaker at room temperature for five days to reach maximum population. Thus, grown cultures were mixed into sterilized lignite powder till the moisture of the carrier reached to field capacity. The population of *Azotobacter chroococcum* stains in the carrier material was determined by serial dilution plate method Then these carrier based inoculants were used for the inoculation in the pot experiment.

**Estimation of nitrogen content of the plants:**

The total nitrogen in plants was determined by Microkjeldal digestion and distillation method as described by Jackson (1973) with minor modification, Dried shoot and root samples were powdered and sieved. 0.5g of sieved samples were digested with 5 ml of concentrate $H_2SO_4$ in the presence of 0.5g catalyst mixture ($K_2SO4$:$CuSO_4$:Selenium powder in the ratio of 100:10:1) in digestion tubes. The digestion was done initially keeping lower temperature (250$^0$C) for 30 minutes and then increased the temperature to 300$^o$C for 60 minutes in the digestion block. After ensuring the clear solution, the digest was cooled and diluted with an excess quantity of 40 per cent NaOH. The liberated ammonia was trapped in 2 per cent boric acid ($H_3BO_3$) containing 2-3 drops of mixed indicator of methyl red and methylene blue. This ammonium borate was titrated against 0.05 N $H_2SO_4$. From the volume of acid consumed, the per cent nitrogen in the sample was estimated as follows.

$$\% N = \frac{\text{Sample titre value (ml) x Concentration of } H_2SO_4 \text{ x 0.014 x 100}}{\text{Weight of the sample taken}}$$

**Statistical analysis:**

The data obtained in the pot experiments were subjected to one way analysis of variance using MSTAT-C soft ware.

**DNA extraction protocol:**

DNA extraction protocol was followed was according to Sambrook *et al.* (1989) and some modification also done. Bacterial isolates were grown in W-77 nitrogen free medium and incubated at 28$^0$C for over night under shaking. About 1.5 ml of culture was taken in microcentrifuge tube, spun for 7 minutes and supernatent was decanted. To the pellet 450µl of TE buffer, 3µl of 20 mg / ml proteinase-k, 30µl of 10 per cent SDS were added and incubated for one hour at 37$^0$C. Again 100µl of 5 M NaCl and 80 µl of CTAB solution were added and incubated for ten minute at 65$^0$C. Further it was extracted with equal volume of chloroform: isoamyl alcohol and the aqueous phase was transferred to the fresh tube and to this equal volume of phenol :chloroform:isoamyl alcohol was added and subjected to centrifugation at 8,000 rpm for 5 min at 4$^0$C. It was washed with chloroform:Isoamyl alcohol until the clear supernatant was obtained. Then equal volume of chilled isopropanol was added, mixed gently and kept at -20$^0$C overnight for precipitation of DNA. Later centrifuged at 10,000 rpm for 20 min at 4$^0$C to pellet the DNA. The pellet was washed with 70 per cent ethanol and air-dried. The DNA was dissolved in TE buffer.

**PCR amplification conditions:**

30 ηg of genomic DNA was used as the template for the standardization of PCR reactions and the PCR conditions were optimized to produce the reproducible and fine fingerprints. PCR reactions were performed in a final volume of 20 µl containing 30 ηg of template DNA, 200 µM dNTP, 2µl of 1X assay buffer, 1 unit of *Taq* DNA polymerase, 2.5ml of 5 pico mole primer and 1.5 mM $MgCl_2$. Amplifications were achieved in MJ Research thermocycler (PTC 100) with the program consisting initial denaturation of 94$^0$C for 3 min followed by 45 cycles each consisting of denaturation at 94$^0$C for 1 min, primer annealing temperature at 40$^0$ for 1 min, primer extension at 72$^0$C for 3 min, and a final extension of 72$^0$C for 10 min (Table 1). These reactions were repeated to check the reproducibility of the amplification.

**Agarose gel electrophoresis:**

Agarose gel electrophoresis was performed to

**Table 1: Optimum concentration and conditions for RAPD analysis**

| Variable | Conditions/ concentration | |
|---|---|---|
| | Evaluated | Optimum |
| PCR amplification | | |
| Hot start (94 $^{o}$C) | 2min, 3min, 4 min, 5min | 3min, |
| Denaturation (94 $^{o}$C) | 30sec, 1min, 1.5min. | 1min, |
| Annealing (40 $^{o}$C) | 1min, 1.5min, 2.0min | 1min, |
| Extension(72 $^{o}$C) | 1min, 1.5min, 2.0min, 3min | 3min, |
| Number of cycles | 30, 40, 45 cycles | 45 cycles |
| RAPD Protocol | | |
| Template DNA | 10-15ng, 25-30ng, 40-50ng | 25-30ng |
| $MgCl_2$ | 1.0mM, 1.5mM, 2.0mM, 2.5mM | 1.5mM, |
| DNTPs | 150µM, 200µM, 215µM, 225µM, | 200µM |

resolve the amplification product using 1.4 per cent agarose in 1X TBE buffer, 0.5µg/ml of ethidium bromide, and loading buffer (0.25% Bromophenol Blue in 40% sucrose). 4 µl of the loading dye was added to 20µl of PCR products and loaded to the agarose gel. Electrophoresis was carried at 65 V for 4.5 hour. The gel was visualized under UV light and documented using Hero Lab Gel Documentation unit.

**Analysis of RAPD data:**

The bands were manually scored '1' for the presence and '0' for the absence and the binary data were used for statistical analysis. The scored band data (Presence or absence) was subjected to cluster analysis using STATISTICA.

## RESULTS AND ANALYSIS

The results of the study have been discussed in detail as under:

**RAPD analysis:**

Lane 1,2,3,4,5,6,7,8,9 and 10 are isolates from zone

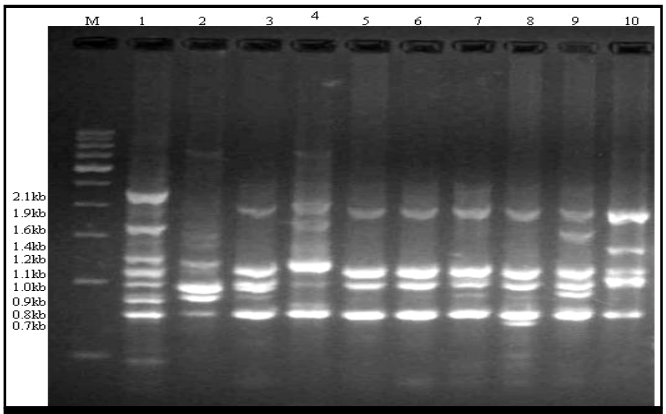


**Fig. 1: RAPD GEL profile of *Azotobacter chroococcum* isolates generated using 10-mer random primer OPD 05.**

**Table 2: Characters of *Azotobacter Chrococcum* isolates grown on Waksman No.77 N free medium**

| *Azotobacter chroococcum* isolates | *Cultural Characters | * Pigmentation | Oxygen requirement |
|---|---|---|---|
| Zone – 1 Isolate | Good growth, raised slimy colony | Light brown | Aerobic |
| Zone – 2 Isolate | Moderate growth, flat entire slimy colony | Black | Aerobic |
| Zone – 3 Isolate | Good growth, flat entire slimy colony | Pale brown | Aerobic |
| Zone – 4 Isolate | Good growth, raised slimy colony | Dark brown | Aerobic |
| Zone – 5 Isolate | Good growth, raised slimy colony | Light brown | Aerobic |
| Zone – 6 Isolate | Good growth, flat entire slimy colony. | Light brown | Aerobic |
| Zone – 7 Isolate | Moderate growth, flat entire slimy colony | Dark brown | Aerobic |
| Zone – 8 Isolate | Moderate growth, raised slimy colony. | Black | Aerobic |
| Zone – 9 Isolate | Moderate growth, flat entire slimy colony | Pale brown | Aerobic |
| Zone – 10 Isolate | Moderate growth, flat entire slimy colony. | Light brown | Aerobic |

* Cultural characters were observed 3 days after incubation.

* Pigmentation observed 7 days after incubation.

1 to zone 10, respectivly. Lane M is 5 Kb lambda/Hind III marker and respectively use different primer.

For fingerprinting and diversity analysis, PCR amplification conditions were optimized based on the protocol outlined by William *et al*. (1990) and Welsh and Mcclelland (1990) with minor modifications.

In order to obtain high amplification rate and reproducible banding pattern, different duration for hot start, denaturation, primer annealing and primer extension were tried. The PCR reaction was evaluated for 30, 40 and 45 cycles using standard buffer as outlined in Material and Methods. The optimum conditions for each cycle of PCR was developed for obtaining high amplification levels. The optimum PCR conditions consisted of the following steps. Which were repeated for 45 times.

Initial strand separation or hot start at $94^0$C for one minute followed by, 45 cycles of

- Denaturation at $94^0$C for one minute.
- Primer annealing at $40^0$C for one minute
- Primer extension at $72^0$C for one minute
- Final extension period at $72^0$C for one minute.

**Principal component analysis (PCA):**

To visualize the genetic relatedness among the *Azotobacter chroococcum* isolates in detail principal component analysis (PCA) was zone for 103 RAPD bands generated by 10 decamer random primers. The description of the data was done using three dimensions and the same is presented.The results of PCA showed that the isolates from zone 3, zone 5, zone 6, zone 7, zone 8, zone 9 and zone10 isolates were grouped together. Isolates from zone1 and zone 2 were grouped separately and isolate from zone 4 was quite, distinct forming a separate entity.

Ten *Azotobacter* species from different agroclimatic zones of Karnataka were isolated on W-77 N-free agar medium. Observations for growth characters on W-77 N free agar medium were recorded and the isolates from zone 1, 3, 4, 5, 6 showed good growth while isolates from zone 2, 7, 8, 9, 10 showed moderate growth on the medium. Similarly, raised slimy colonies were observed in the zone 1, 4, 5 and 8 isolates, while flat entire slimy colonies were obsereved in the zone 2, 3, 6, 7, 9 and 10 isolates. Since the colonies were growing on the surface of agar medium, they are considered as aerobic. One week old cultures showed pigmentation. Among these isolates, isolate from zone 1, zone 5, zone 6 and zone 10 showing light brown colour, zone 2 and zone 8 showing black colour, zone 3, zone 9 showing pale brown colour and isolates from zone 4 and zone 7 showing dark brown colour. (Table 2) The morphological characters studied under microscope and most of the colonies were found to be oval to round in shape while some are blunt ended long cells. (Table 3) Cells were motile, Gram negative and formed capsule and microcyst. All the isolates representing each zone were tested for growth on different carbon source *viz.,* mannitol, glucose, fructose, sucrose, rhamnose and glycolate. The data pertaining to growth of *A.chroococcum* on different carbon source and all the 10 isolates grew on media containing mannitol, glucose, fructose, sucrose and glycolate but not on rhamnose as carbon source. Based on the colony characters, cell shape, presence of cyst, capsule, Gram reaction and utilization of different carbohydrates tested, the isolates were confirmed as *Azotobacter chroococcum* species (Table 4).

**RAPD analysis reaction parameters:**

It is important to optimize the concentration of PCR mixture, in order to produce informative and reproducible RAPD fingerprints. Hence, different concentrations and

**Table 3: Characters of *Azotobacter* isolates as observed under microscope**

| Isolates of different agro climatic zones | Shape | Motility | Capsule | Microcyst | Gram reaction |
|---|---|---|---|---|---|
| Zone – 1 isolate | Ovoid | + | + | + | Gram negative |
| Zone – 2 isolate | Ovoid | + | + | + | Gram negative |
| Zone – 3 isolate | Round | + | + | + | Gram negative |
| Zone -4 isolate | Blunt ended | + | + | + | Gram negative |
| Zone -5 isolate | Ovoid | + | + | + | Gram negative |
| Zone – 6 isolate | Ovoid | + | + | + | Gram negative |
| Zone -7 isolate | Round | + | + | + | Gram negative |
| Zone -8 isolate | Blunt ended | + | + | + | Gram negative |
| Zone -9 isolate | Ovoid | + | + | + | Gram negative |
| Zone – 10 isolate | Ovoid | + | + | + | Gram negative |

Note: + = Presence of Motility, Microcyst and Capsule

**Table 4: Effect of different carbon sources on growth of *Azotobacter chroococcum* isolates**

| *Azotobacter chroococcum isolates.* | Growth of *Azotobacter* isolates on different Carbon Sources | | | | | |
|---|---|---|---|---|---|---|
| | Mannitol | Glucose | Fructose | Sucrose | Rhamnose | Glycolate |
| Zone – 1 Isolate | + | + | + | + | - | + |
| Zone – 2 Isolate | + | + | + | + | - | + |
| Zone – 3 Isolate | + | + | + | + | - | + |
| Zone – 4 Isolate | + | + | + | + | - | + |
| Zone – 5 Isolate | + | + | + | + | - | + |
| Zone – 6 Isolate | + | + | + | + | - | + |
| Zone – 7 Isolate | + | + | + | + | - | + |
| Zone – 8 Isolate | + | + | + | + | - | + |
| Zone – 9 Isolate | + | + | + | + | - | + |
| Zone – 10 Isolate | + | + | + | + | - | + |

Note: + : Growth - : No growth

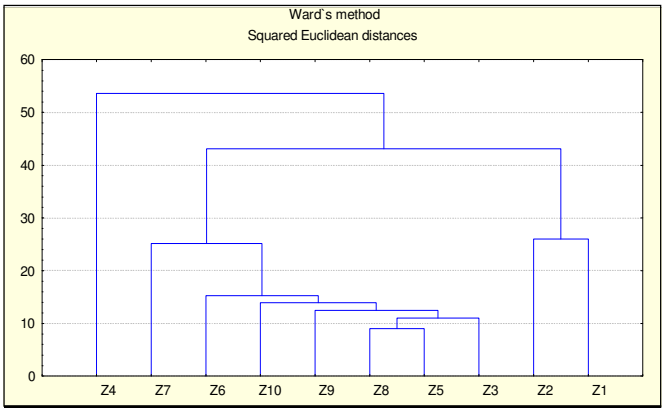


**Fig. 2: Jaccard's coefficient matrix for 10 different *Azatobacter chroococcum* isolates**

template DNA (10-15µg, 25-30µg and 40-50µg), $MgCl_2$ (1.0 mM, 1.5 mM, 2.0 mM and 2.5 mM) and dNTPs (150 µM, 200 µM, 215 µM and 225 µM) were tried with similar amplification conditions . A concentration of 25-30çg of template DNA, 1.5 mM of $MgCl_2$ and 200 µM of dNTPs per reaction were found to be optimum for obtaining intense, clear and reproducible banding pattern in *Azotobacter chroococcum* isolates. In all these cases, 5 pico moles of primer and 1 unit of *Taq* polymerase per reaction were used. However, fluctuation in the concentration of template DNA did affect the amplification, with too little DNA (10-15ηg) causing either reduced or no amplification of small fragments and higher concentration of DNA (40-50 ηg) producing a smear.

A dNTP concentration of 200 mM was found adequate for generating reproducible RAPDs. At lower concentrations (150µM) the intensity of stained bands in the gel became progressively weaker and at higher concentrations smearing of the bands was evident. Magnesium ion concentration did affect the relative intensity of amplified bands. As the magnesium ion concentration increased, some DNA segments were amplified more efficiently while others are amplified less efficiently. For generating reproducible RAPD band, 1.5 mM $MgCl_2$ was found optimum.

**Table 5: Oligonucleotide primers that showed genetic variation among the *A. chroococcum* isolates**

| Primers | No. of amplified fragments | No. of polymorphic bands | No. of Monomorpic bands | No. of unique bands. |
|---|---|---|---|---|
| OPD-05 | 10 | 09 | 01 | 02 |
| OPD-20 | 12 | 12 | 00 | 03 |
| OPH-13 | 12 | 08 | 04 | 08 |
| OPH-5 | 09 | 06 | 03 | 02 |
| OPB-08 | 04 | 02 | 02 | 00 |
| OPB-01 | 11 | 11 | 00 | 04 |
| OPB-12 | 14 | 12 | 02 | 08 |
| OPA-11 | 10 | 09 | 01 | 04 |
| OPD-03 | 13 | 11 | 02 | 05 |
| OPD-13 | 08 | 07 | 01 | 02 |
| Total | 103 | 87 | 16 | 38 |
| Percentage | | 84.46% | 15.53% | 36.89% |

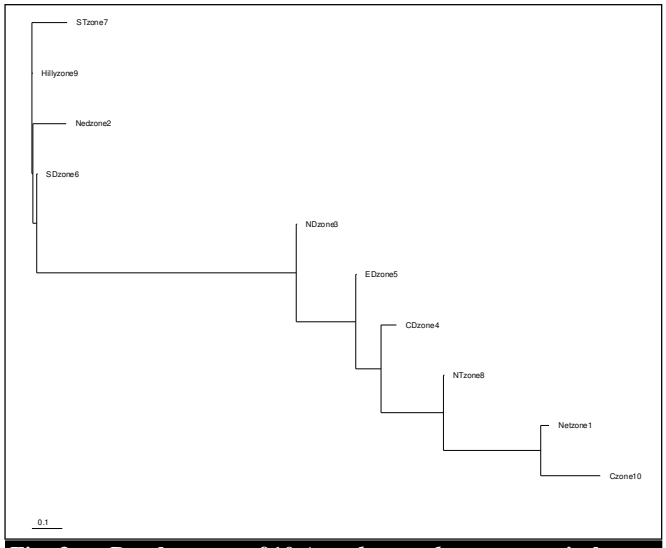


**Fig. 3: Dendrogram of 10 *Azatobacter chroococcum* isolates from different agroclimatic zones of Karnataka**

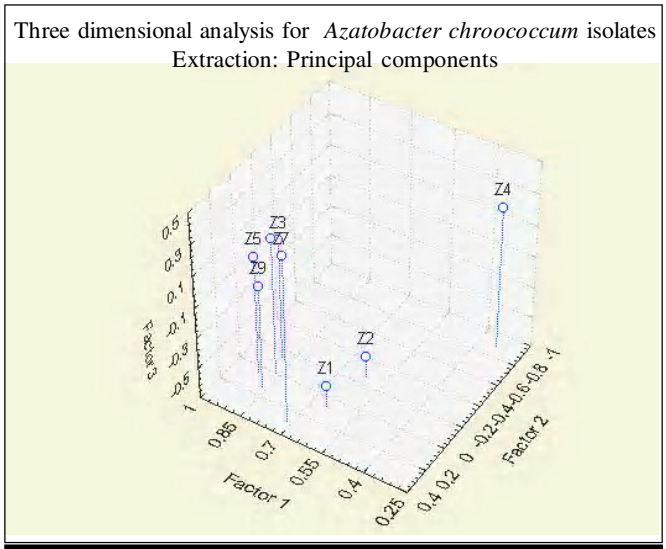


**Fig. 4: Principal component analysis of *Azotobacter chroococcum* isolates**

## RAPD characterization:

A total of 103 RAPD bands produced from the selected 10 primers were used for fingerprinting and for estimation of genetic diversity among ten isolates of *Azotobacter chroococcum.* The number of bands scored for each primer varied from 4 to 14 with on average of 10.3 bands per primer. Out of 103 amplification bands, 16 bands (15.53%) were monomorphic, 38 bands (36.89%) were unique and 87 bands (84.46%) were polymorphic, which were informative in revealing the relationship among the genotypes (Table 4). Among the selected primers OPB-12 and OPD-20 produced maximum number of polymorphic bands followed by OPB-01 and OPD-03.

## Cluster analysis and genetic dissimilarity matrix of 10 *Azotobacter chroococcum* isolates:

The Cluster analysis based on 103 RAPD bands revealed that the ten *A. chroococcum* isolates examined, clustered at a linkage distance of about 55 units on the dendrogram with Zone 4 and Zone 8 isolate spanning the extremes. The dendrogram has clearly depicted that all the 10 *Azotobacter chroococcum* isolates formed three major clusters. Among the three major groups, isolate from Zone 4 alone formed a separate group, isolates from zone 3, zone 5, zone 6, zone 7, zone 8, zone 9, and zone 10 formed a second group and the isolate from zone 2 and zone 1 formed the third group (Table 6).

The dissimilarity matrix for *Azotobacter chroococcum* isolates revealed that within the *A.chroococcum* isolates used in the present investigation, the highest dissimilarity was observed between zone 1 and zone 4 isolates followed by zone 4 and zone 7 isolates. Least dissimilarity was observed between zone 5 and zone 9 isolates, followed by zone 5 and zone 8 isolates .

**Table 6: Cluster analysis and genetic dissimilarity matrix of 10 *Azotobacter chroococcum* isolates**

| | Z1 | Z2 | Z3 | Z4 | Z5 | Z6 | Z7 | Z8 | Z9 | Z10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Z1 | 0 | 0.440678 | 0.471698 | 0.647059 | 0.436364 | 0.465517 | 0.532258 | 0.442308 | 0.410714 | 0.410714 |
| Z2 | | 0 | 0.454545 | 0.567164 | 0.448276 | 0.45 | 0.560606 | 0.454545 | 0.45 | 0.45 |
| Z3 | | | | 0.534483 | 0.244444 | 0.255319 | 0.415094 | 0.238095 | 0.291667 | 0.291667 |
| Z4 | | | | | 0.571429 | 0.546875 | 0.642857 | 0.583333 | 0.61194 | 0.61194 |
| Z5 | | | | | 0 | 0.26 | 0.351852 | 0.204545 | 0.1875 | 0.22449 |
| Z6 | | | | | | 0 | 0.296296 | 0.255319 | 0.301887 | 0.301887 |
| Z7 | | | | | | | 0 | 0.352941 | 0.385965 | 0.385965 |
| Z8 | | | | | | | | 0 | 0.255319 | 0.255319 |
| Z9 | | | | | | | | | 0 | 0.269231 |
| Z10 | | | | | | | | | | 0 |

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