To study of the genetic variations among the *Azospirillum lipoferu* isolates using randomly amplified polymorphic DNA (RAPD) marker

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Abstract – Among free-living microorganisms, which can be practically used in agriculture, bacteria from the *Azospirillum* genus as well as other endophytes are nowadays thought of as the most active component of associative dinitrogen fixation. The investigation was carried out to study the characterization of *Azospirillum lipoferu* found in the soils of the ten agro-climatic zones which Karnataka, is classified. By using RAPD markers, 75 bands were scored out of which 78.6 % were found to be polymorphic. Statistical analysis of RAPD data enabled the classification of 10 *Azospirillum* isolates into two major groups. . In this, the cluster analysis based on 75 RAPD bands revealed that the ten *A. lipoferu* isolates examined clustered at a linkage distance of about 40 units on the dendrogram. There was no correlation between RAPD and geographical origin of isolates.

Key words: arbitrary primer, auxonography, squared Euclidean distance, principle component analysis

I. INTRODUCTION

Nitrogen is an essential part of many of the chemical compounds, such as proteins and nucleic acids, of all life forms. However, before its incorporation into a living system, Nitrogen must first be reduced chemically or biologically, commonly referred to as N-fixation .

Nitrogen is the nutrient element most frequently found limiting to the growth of green plants. The nitrogen reserve of agricultural soils must therefore be replenished periodically in order to maintain an adequate (non-growth limiting) level for crop production.

Among free-living microorganisms, which can be practically used in agriculture, bacteria from the *Azospirillum* genus as well as other endophytes are nowadays thought of as the most active component of associative dinitrogen fixation, particularly in tropical plant crops - sugar cane, oil-seed palm, rice, fodder grasses and cereals. (Baldani *et al*., 1997)

Azospirillum spp. are diazotrophs associated with several plants, including wheat and maize, and they are classified within the alpha subclass of the *Proteobacteria* by 16S rRNA sequence analysis. The genus comprises the species, *A. brasilense*, *A. lipoferum*, *A. amazonense*, *A. irakense*, *A. halopraeferens*, *A. largimobile*. *Azospirillum oryzae* sp. nov, (Cheng and Yokota, 2005) *Azospirillum melinis* sp. (*Melinis minutiflora* Beauv.) (Guixiang, 2006).

The geographical area of Karnataka, India, is classified into ten agro-climatic zones *viz.,* on the basis of annual rain fall, soil type, cropping pattern, and other climatic conditions which influence on the number and kinds of organisms present in soil. Genetic diversity can be estimated by protein analysis using electrophoresis or direct aminoacid sequencing. Electrophoretic analysis of proteins has been useful in distinguishing genera, species further it can also identify different compounds produced by different strains (Dodd *et al.,* 1996). Another method is DNA fingerprinting. 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 (Bert *et al.,* 1996).

The present investigation was carried out to isolate and identify *Azospirillum spp* from roots and rhizosphere soil of cereals and grasses from the agro-climatic zones of Karnataka.

II. MATERIAL AND METHODS

A. *Soil sampling and roots taken*

Four soil samples of 500g each were collected randomly from rhizosphere soil of each agro climatic one during 2004 to 2006. They were packed in polythene bags, transferred and stored at 4ºC at the Department of Biotechnology, University of Agricultural Sciences, GKVK Campus, Bangalore, India-560065.

B. *Isolation of Azospirillum*

Azospirillum was isolated by following the enrichment culture technique as Day and Dobereiner (1976). The roots of grasses were thoroughly washed in running water. Then the roots were cut into small bits (1-2cm) and washed aseptically with 0.1% mercuric chloride, 70% ethyl alcohol followed by frequent washes in sterile distilled water. The root bits were aseptically placed into tubes containing semi-solid malate medium (Nfb) (Baldani and Dobereiner, 1980). *Azospirillum* species were also isolated by employing serial dilution technique. All the tubes were incubated at 30^{o}C for 48hour and observed for characteristic white sub surface pellicle.

C. *Identification of the isolates*

Each isolate was streaked on two Petri plates containing Nfb malate agar incubated for seven days for typical small white translucent colonies observation (Baldani and Dobereiner, 1980). Similarly the isolates were also streaked on the plates containing Congo red medium and potato BMS agar. Typical scarlet (Rodríguez-Cáceres, 1982) and pink colour

colonies (Baldani and Dobereiner, 1980) in each medium respectively can be taken as preliminary indication of *Azospirillum*.

The bacteria isolates were observed at microscope for cell morphology, gram reaction and motility.
Physiological analysis viz., enlisted for identification of *Azospirillum* by Bergey's manual were studied.

D. *Auxanography of isolates*

Azospirillum species differ in their sugar preference (Okon et al., 1976). Hence, the Nfb was replaced with malate, glucose or sucrose carbon source at one percent concentration. *Azospirillum* isolates grown overnight in nutrient broth was inoculated to tubes (0.1ml) with these medium previously sterilized. The tubes were incubated at 37 ^{o}C for 48 h. A typical sub-surface pellicle formation was taken as indication of the carbon source utilization (Okon et al. 1976).

E. *Biotin requirement*

The pH of the medium was adjusted to 7.0 with KOH (0.1N) solution. Biotin (0.0001g/L) was added to one portion of the medium (Tarrand et al. 1978). Both biotin containing and biotin free media were sterilized in 5ml screw cap tubes by autoclaving. A loopful culture grown in MPSS broth was inoculated into 25ml of one fourth strength nutrient broth and incubated at 37 ^{o}C for 24 h. The cells were harvested by centrifugation (10000 rpm for 5 min) washed twice with sterile distilled water and suspended in water to obtain a uniform density. Each 5 ml of the media (with and without biotin) was inoculated with 0.1 ml of this suspension and incubated for 48 hr at 37^{o}C. Where the growth occurs in the absence of biotin, a second serial transfer was made to the media with and without biotin, using 0.1 ml from the first culture for confirmation.

F. *DNA extraction*

DNA extraction protocol followed was according to Sambrook *et al.,* (1989). Bacterial isolates were grown in Lurea broth and incubated at 33 ^{o}C for overnight under shaking. About 1.5 ml of culture was taken in microcentrifuge tube, spun for 7 min and supernatant was decanted. To the pellet 567µl of TE Buffer, 3µl of 20 mg / ml proteinase-k, 30µl of 10% SDS were added and incubated for 1 h at 37 ^{o}C. Again 100µl of 5 M NaCl and 80 µl of CTAB solution were added and incubated for 10 min at 65 ^{o}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 ^{o}C. It was washed with chloroform: isoamyl alcohol until the clear supernatant was obtained. Then, equal volume of chilled propanol was added, mixed gently and kept at -20 ^{o}C overnight for precipitation of DNA. Later, it wascentrifuged at 10,000 rpm for 20 min at 4 ^{o}C to pellet the DNA. The pellet was washed with 70 % ethanol and air-dried. The DNA was dissolved in TE buffer.

PCR amplification condition Reagents used in the PCR:

Template DNA	**30 ηg/µl**
dNTPs	**2 mm**
***Taq* polymerase**	**1 U/µl**
Primer	**100µM**

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 25 µl containing 30 ηg of template DNA, 0.75 µl of 2mM dNTPs each, 2.5µl of 10X taq buffer, 0.36µl 1 unit of *Taq* DNA polymerase, 3 ml of 10 pico mole primer. Amplifications were achieved in MWG-Biotech primus thermocycler with the program consisting initial denaturation of 94 ^{o}C for 3 min followed by 45 cycles each consisting of denaturation at 94 ^{o}C for 1 min, primer annealing temperature at 37^{0} for 1 min, primer extension at 72 ^{o}C for 3 min, and a final extension of 72 ^{o}C for 10 min. These reactions were repeated to check the reproducibility of the amplification.

Primer screening was carried out using DNA obtained from the *A. lipoferum* isolates out of the 14 primers screened. From these, 8 primers producing sharp, intense bands were selected for the RAPD analysis (Table1).

Sl.NO.	**Sequence**
1	5'-GGT GCG GGA A-3'
2	5'-GTT TCG CTC C-3'
3	5'-GTA GAC CCG-3'
4	5'-AAG AGC CCG T-3'
5	5'-AAC GCG CAA C3'
6	5'-CCC GTC AGC A-3'
7	5'-GAA CGG ACT C-3'
8	5'GAG AGC CAA C3'

Table 1. RAPD primers with sequences chosen for analysis

Agarose gel electrophoresis was performed to resolve the amplification product using 1.4 % agarose in 1X TBE buffer, 0.5µg/ml of ethidium bromide, and loading buffer (0.25% Bromophenol Blue in 40% sucrose). 5 µl of the loading dye was added to 25µl of PCR products and loaded to the agarose gel. Electrophoresis was carried at 65 V for 4.5 h. The gel was visualized under UV light and documented using Hero Lab Gel Documentation unit.

G. *Standardization of protocol for RAPD analysis*

For fingerprinting and diversity analysis, PCR amplification conditions were optimized according to 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, and primer annealing and primer extension were tried. The PCR reaction was evaluated for 30, 40 and 45 cycles using standard buffer. The optimum conditions for each cycle of PCR were developed for obtaining high

amplification levels. The optimum PCR conditions consisted of the following steps which were repeated for 45 times.

The genetic relationships between the genotypes were estimated with the principal component analysis (PCA) (Ludwig and Reynolds, 1988) was done for 75 RAPD bands generated by 10 decamer random primers. A dissimilarity matrix was developed, and the genotypes were clustered on the first three axes and the pattern of clustering or separation of genotypes from one another was also analyzed.

H. *Statistical analysis*

The data obtained in the pot experiments were subjected to one way analysis of variance using MSTAT-C software The manually scored band data of RAPD (presence or absence) was subjected to cluster analysis using Statistica version xx (Statsoft, Inc. Tulsa, OK USA)

III. RESULT AND DISCUSSION

A. *Isolation and identification*

Isolation of Azospirillum was made from soil and plant roots from different agro climatic zones by the enrichment culture technique in Nfb malate medium. All the isolates were subjected to various tests to confirm identity

Figure: 1 Image showing Results

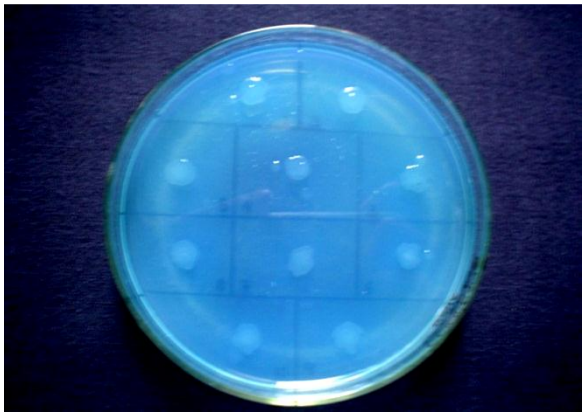

Figure: 2 Image showing internal portion

All the isolates formed sub surface pellicle in semi solid Nfb malate media. The pellicle formation was about 1 to 2 mm below the surface of semi solid Nfb malate media. All isolates turned green colour of the BTB to brilliant blue . All isolates appeared as small white translucent colonies on Nfb agar . Further confirmation of Azospirillum isolates were done by plating it on Congo red medium where the Azospirillum isolates appeared as small red coloured (scarlet) colonies after five to six days of incubation in light .The isolates can be easily identified on potato infusion agar, a differential media where the colonies appeared as light pink and smooth some of them appeared as pale pink and lobbed after seven to nine days of incubation.

Colony morphology was also checked on Luria agar and growth was observed on overnight incubation, some of the isolates appeared as dull white colonies with lobed edges while others appeared as cream with lobbed edges .

B. *Microscopic observation*

Azospirillum isolates were further examined for their gram reaction shape and motility and presence of poly hydroxyl butyrate granules (PHB) characteristically all the isolates were gram negative vibroid and exhibited spiral (cork screw) movement when observed under the hanging drop technique. All the isolate had the characteristically PHB granules.

C. *Biochemical and physiological characters*

The test isolates were further examined for their biochemical properties . All the isolates were checked for sub surface pellicle formation in Nfb malate media.

D. *Production of acid from glucose peptone broth*

Among the 20 isolates 16 showed to produce acid from the glucose peptone broth , a character of *Azospirillum lipoferum* and hence these strains were identified as *Azospirillum lipoferum* and the rest which failed to produce acid, were tentatitively identified as *Azospirillum brasilense*.

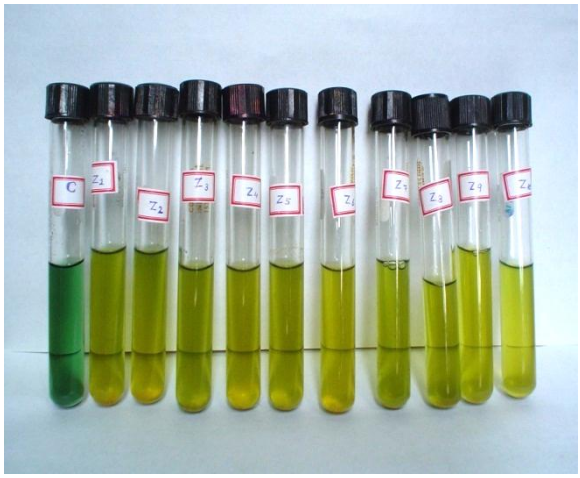

Figure: 3 Image showing isolates to produce acid

E. *Requirement of biotin for growth*

Azospirillum brasilense and *Azospirillum lipoferum* differ in their ability to utilize biotin. Hence growth on biotin supplemented media was recorded. Semi solid malate media was supplemented with biotin favored the growth of 16 isolates. However media with biotin did not support growth of 4 isolates.

F. *Utilization of carbon source*

Among the carbon sources malate was found to be utilized for growth and nitrogen fixation by all isolates where as glucose was found to be utilized by 16 isolates characteristic of Azospirillum *lipoferum* and others which did not utilize glucose were tentatively identified as *Azospirillum brasilense.* All isolate studied could not utilize sucrose as sole carbon source .Ten *Azospirillum lipoferum* strains were taken for further studies. Mention in table format biochemical analysis and colony morphology.

G. *RAPD analysis*

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

1) *Denaturation at 94^0C for one minute.*
2) *Primer annealing at 37^0C for one minute*
3) *Primer extension at 72^0C for two minute and*
4) *Final extension period at 72^0C for ten minute.*

H. *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 template DNA (10-15ηg, 25-30ηg and 40-50ηg) were tried with similar amplification conditions . A concentration of 25-30ηg of template DNA and 2mM of dNTPs per reaction were found to be optimum for obtaining intense, clear and reproducible banding pattern in *Azospirillum lipoferum* isolates. In all these cases, 3 µl of 10 pico moles of primer and 0.36 µl of 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.

I. *RAPD characterization*

A total of 75 RAPD bands produced from the selected 8 primers were used for fingerprinting and for estimation of genetic diversity among ten isolates of *Azospirillum lipoferum.* For the purpose of illustration, the RAPD fingerprints/electrophoregram generated for ten *Azospirillum lipoferum* isolates using eight primers . The number of bands scored for each primer varied from 1 to 8 with on average of 9.3 bands per primer. Out of 75 amplification bands, 11 bands (14.66%) were monomorphic, 5 bands (6.66%) were unique and 59 bands (78.6%) were polymorphic, which were informative in revealing the relationship among the genotypes .

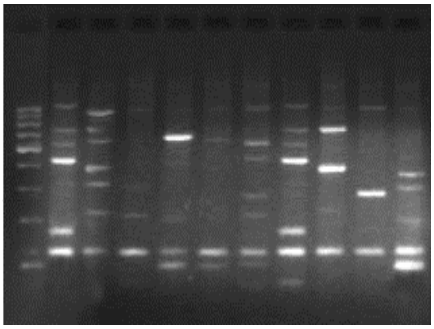

Figure : 4 RAPD analysis and characteristics-1

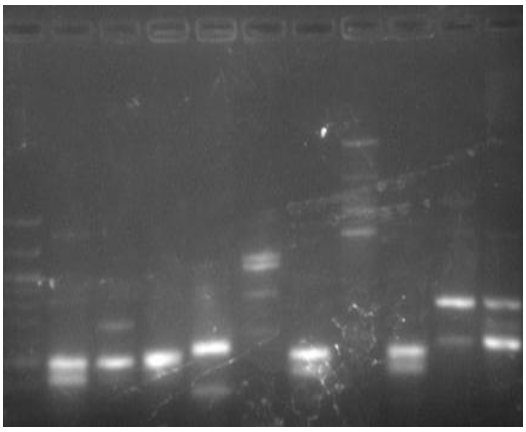

Figure:5 RAPD analysis and characterstics-2

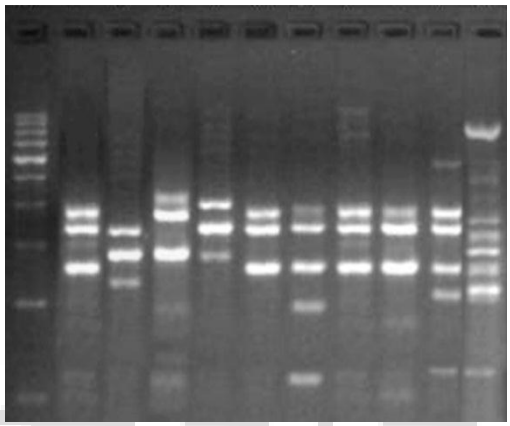

Figure :6 RAPD analysis and characterstics-3

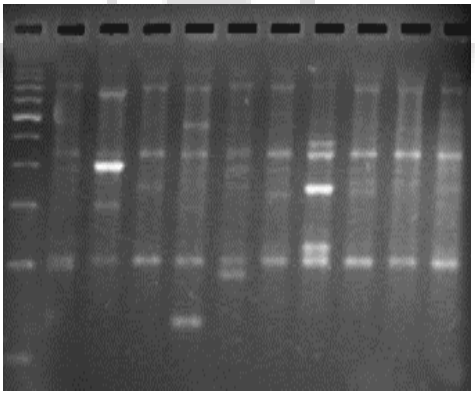

Figure :7 RAPD analysis and characterstics-4

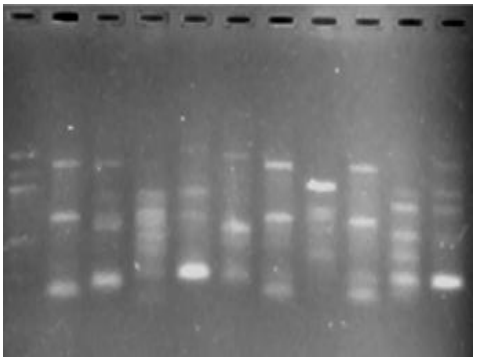

Figure :8 RAPD analysis and characterstics-5

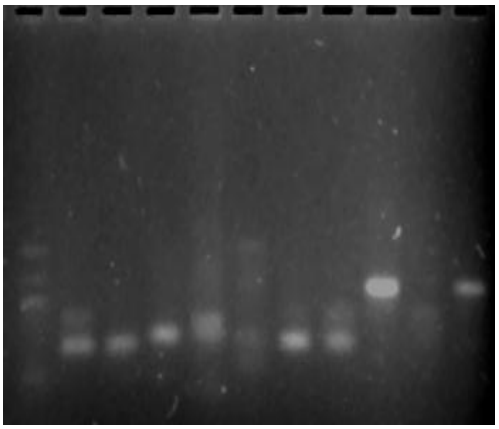

Figure :9 RAPD analysis and characterstics-6

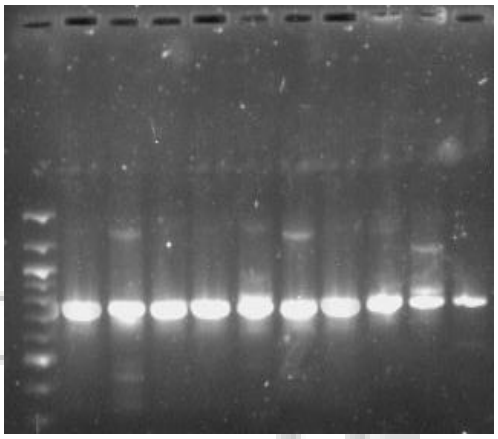

Figure:10 RAPD analysis and characterstics-7

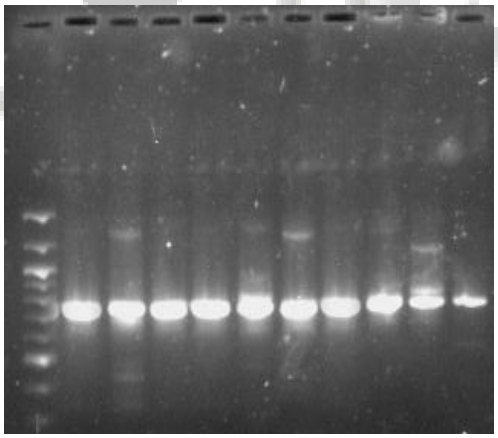

Figure:11 RAPD analysis and characterstics-7

J. *Cluster analysis and genetic dissimilarity matrix of 10 Azospirillum lipoferum isolates*

The Cluster analysis based on 75 RAPD bands revealed that the ten *Azospirillum lipoferum* isolates examined, clustered at a linkage distance of about 40 units on the dendrogram with Zone 4 and Zone 1 isolate spanning the extremes. The dendrogram has clearly depicted that all the 10 *Azospirillum lipoferum* isolates formed two major clusters. Among the two major groups, isolates from zone 2, zone 4, zone 8, and zone 10 formed the first group and the isolate from zone 1, zone 3, zone 5, zone 6, zone 7 and zone 9 the second group.

The dissimilarity matrix for *Azospirillum lipoferum* isolates revealed that within the *Azospirillum lipoferum* isolates used in the present investigation, the highest dissimilarity was observed between zone 2 and zone 7 isolates followed by zone 2 and zone 5 isolates. Least dissimilarity was observed between zone 1 and zone 8 isolates, followed by zone 1 and zone 7 isolates.

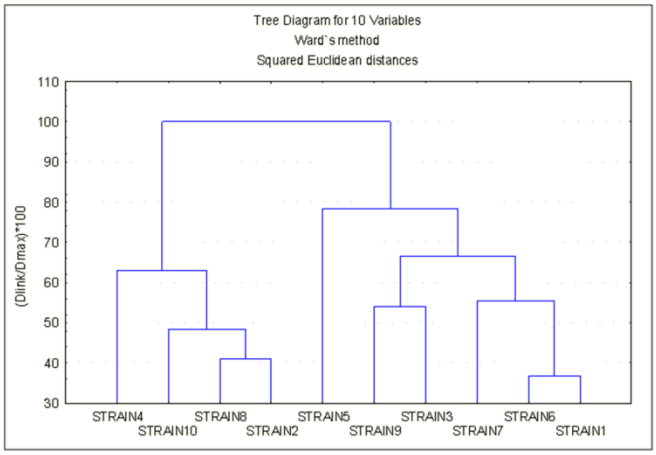

Fig 13: Dendrogram based on RAPD profile of 10 *A. lipoferum* isolates from different agroclimatic zones of Karnataka

K. *Principal component analysis (PCA)*

To visualize the genetic relatedness among the *Azospirillum lipoferum* isolates in detail principal component analysis (PCA) was done for 75 RAPD bands generated by 10 decamer random primers. The results of PCA showed that the isolates from zone 2, zone 3, zone 4, zone 8, and zone10 isolates were grouped together. Isolates from zone1 zone 6, zone 7 and zone 9 were grouped separately and isolate from zone 5 was quite, distinct forming a separate entity.

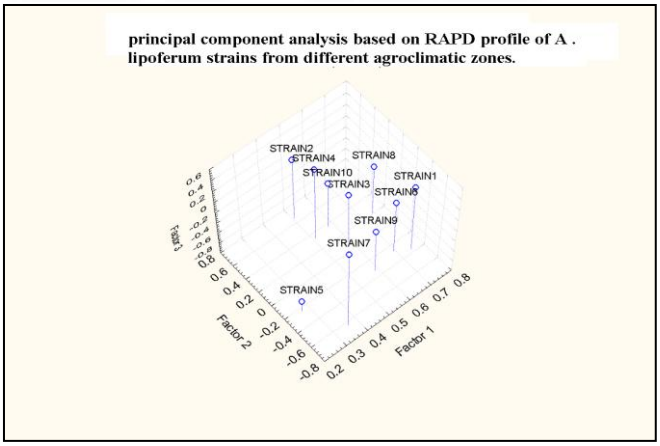

Figure : 14 PCA based on the RAPD profile

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