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Molecular Characterization and Diversity Analysis of Soybean Varieties Against Soybean *Rhizoctonia* Aerial Blight Resistance

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ABSTRACT

Analysis of polymorphism among soybean varieties and determination of genetic relationship among soybean varieties differing in *Rhizoctonia* Aerial Blight (RAB) resistance, using Inter Simple Sequence Repeats (ISSR) marker and Random Amplified Polymorphic DNA (RAPD). In the present investigation carried out 20 ISSR primers amplified 174 markers. Out of them 122 loci were polymorphic 70.11% (6.1±0.52 average), while 52 loci were monomorphic 29.89% (2.6±0.27 average) and RAPD primer amplified 78 loci, out of them 61 loci were polymorphic 78.02% (6.1±0.44 average), while 17 loci were monomorphic 21.78% (1.7±0.44 average). A dendrogram was generated by UPGMA clusters analysis based on Jacquard's similarity coefficient. Estimated genetic similarity among 18 soybean varieties using 20 ISSR primers, were 0.71 – 0.95. In this study, observed high level of polymorphism because low level of monomorphic band scored this type of markers system. Out of these 20 primers all of them produce polymorphic bands which represented maximum diversity in between soybean varieties. In these markers system to evaluate the genetic similarity as well as genetic relationships among the soybean cultivars, these were able to differentiate each and every varieties In the used of the ISSR primers which generated genetic similarity in between BRAGG with NRC 37 have 94% genetic similarity and was observed different cluster with NRC 37 and HIMSO 1602 with SL 710 and MACS 756 in between having about 93% genetic similarity, it was clustered with SL 710 and MACS 756 cultivars. In soybean cultivars using of RAPD primers e.g. PS 1374 with BRAGG and NRC 37 having with represented much closed clusters in which differentiate with other varieties.

Keywords: • % ISSR, inter simple sequence repeat •% RAPD, Random Amplified polymorphic DNA•% UPGMA,(unweighted pair-group arithmetic mean) •% PIC, polymorphic information content. •%RAB, Rhizoctionia Aerial Blight.

INTRODUCTION

Soybean is one of the most important sources of the protein and oil. Soybean ranks

first among the major oilseed crops in the world and India, it's rank third. Soybean [*Glycine max* (L.) Merrill; family- *Leguminasae*, chromosome no. (2n=40) is the most important and valuable oil seed crops of the world after USA, Brazil, Argentina, and China. The major soybean growing states in India are Madhya Pradesh, Maharashtra, Karnataka, Andhra Pradesh and Chhattisgarh. In Madhya Pradesh state, which has lion share, contributes about 60% of the total area and around 57% of total production in the country (Indian economic survey, 2006). Soybean is susceptible to many diseases viz. *Rhizoctonia, Phytophthora* and different viruses under Indian conditions, but *Rhizoctonia* Aerial Blight assumes special importance because according to an estimate (Wrather *et al.* 1997) it is one of the major pathogenic disease responsible for up to 70% yield losses in Soybean. *Rhizoctonia* Aerial blight is a fungal disease caused by *Rhizoctonia solani* (*Thanatephorus cucumeris*). Using host plant resistance is the most effective way of controlling the damage caused by *Rhizoctonia* in soybean. Many varieties have been identified as resistant to aerial blight. However, only a few resistance sources have been used extensively in the development of *Rhizoctonia* Aerial blight resistant cultivars. Studies have shown that *Rhizoctonia* resistant cultivars have major resistance genes in common. Increasing available genetic diversity for *Rhizoctonia* resistance is critical for the long term stability of host plant resistance as a control strategy for *Rhizoctonia*. Prior knowledge of genetic relationship among *Rhizoctonia* resistant sources will facilitate the development of new cultivars with novel resistance genes and enhance the genetic base of *Rhizoctonia* resistance in these cultivars. The genetic base of Indian soybean varieties is extremely narrow which limits the ability of the breeder and biotechnologist to sustain genetic improvement (Gizlice *et al.,* 1993). At present soybean cultivation the problem of monoculture as wide spread cultivation of only single varieties i.e. JS 335 which is also susceptible for *Rhizoctonia.*

Markers are various characters present in different parental lines of cultivated species. Their closely and wild relatives that can be easily scored and it may be quantitative or qualitative in nature. Distinct markers are essential for varietals release, identification, characterization and intellectual property protection association with germplasm and purity determination. Morphological marker has been traditionally used to identify genotypes. But these markers suffer from the limitation of interaction with the environment in which the variety grow and subjectively in decision making. However, molecular markers are the alternative for unambiguous identification of the genotypes. Molecular markers are useful tools to estimate genetic relationship among *Rhizoctonia* resistant varieties. With the advent of PCR based DNA marker technology, several types of DNA marker like RAPD, ISSR, SSR etc. are now available. Among the various available techniques, the ISSR analysis is potentially simple, rapid, reliable and effective method for detecting polymorphism in soybean (Viering Ngugen, 1992).Through this marker system to asses the genetic variability.

MATERIALS AND METHODS

Plant materials and DNA extraction (Table 1) list the soybean genotypes collected from different parts of India that were used in the present study. DNA was extracted from young leaves using the method described by Saghai and Maroof (1984). The

concentration of the DNA samples was determined in a UV spectrophotometer at 260 and 280nm, and the DNA samples were diluted to 25-50ng ìl”1 for PCR amplification.

RAPD AMPLIFICATION

PCR amplification (Williams *et al.* 1990) was performed with random decamer primers obtained from Operon Technologies (Almeda, Calif., USA). Amplification was performed in a 25ìl reaction volume containing, 1x buffer containing 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 20 pmol RAPD primer, 50ng genomic DNA, and 1U Taq DNA polymerase (Bangalore Genei, Bangalore, India). Amplification was performed in a “Thermo-hybaid”programmable thermal cycler (P×2). Amplification conditions were an initial denaturation at 94°C for 4 min and 45 cycles at 94°C for 45 sec, 37°C for 1 min, 72°C for 2 min, followed by 10 min at 72°C. Amplified products were separated on 1.5% agarose gel in 1× TBE buffer (100 mM Tris-HCl, pH 8.3, 83 mM boric acid, 1 mM EDTA) at 75 V. The gels were stained with 0.5 ìg/ ml ethidium bromide solution and visualized by trans-illumination under UV light. The sizes of the amplification products were determined by comparison to ëDNA digested with EcoRI and HindIII.

Table 1: Name and location of Soybean cultivars

SI.No	Varieties	Location	Remarks
1.	BRAGG	USA	Susceptible
2.	KHSH-2	Karnataka	Susceptible
3.	PK-472	Pantnagar	Susceptible
4.	NRC-37	NRCS,	Susceptible
5.	PS-1374	Pantnagar	Susceptible
6.	MRSB-345	Myco	Susceptible
7.	SL-710	Ludhiyana	Resistant
8.	MACS-756	Pune	Susceptible
9.	NRC-66	NRCS,	Resistant
10.	JS 97-52	JNKVV,	Susceptible
11.	HIM-1602	HP	Susceptible
12.	DS 98-14	Delhi	Susceptible
13.	UPSM-534	Pantnagar	Susceptible
14.	JS –335	JNKVV	Susceptible
15.	VLS-64	Aulmora	Susceptible
16.	SL-96	Ludhiyana	Susceptible
17.	JS 93-05	JNKVV	Susceptible
18.	G. SOJA	Chinese (Wild)	Susceptible

ISSR amplification

ISSR amplification reactions were carried out in 25ìl volume containing 50ng template DNA, 1 U Taq DNA polymerase, 200 µM dNTP, 10 pmol primer (The University of British Columbia, Vancouver, Canada) in 1× reaction buffer that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, and 0.01% gelatin. Amplification was performed in “Thermo-hybaid” (P×2) programmable thermal cycler, Amplification

conditions were one cycle at 94°C for 4 min, and 94°C for 1 min, 50°C for 1min, followed by stepwise reduction of 1°C for the first five cycles, and 72°C for 2 min. In subsequent 40 cycles, annealing temperature was maintained at 50°C, followed by one cycle of 7 min at 72°C. Amplified products were loaded on 1.5% agarose gel and separated in 1× TBE buffer at 90 V. The gels were visualized under UV after staining with ethidium bromide and documented using a gel documentation and image analysis system (Syngene, UK). Data analysis The RAPD and ISSR bands were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity. Data analyses were performed using the NTSYS-pc (Numerical Taxonomy System, version 2.0, Rohlf 1990). The SIMQUAL program was used to calculate the Jaccard's coefficient, a common estimator of genetic identity and was calculated as follows.

Jacquard's coefficient=NAB/(NAB + NA + NB)

Where N_{AB} is the number of bands shared by samples, N_A represents amplified fragments in sample A, and N_B represents fragments in sample B. Similarity matrices based on these indices were calculated. Correlation between the two matrices obtained with two marker types was estimated by mean of the Mantel matrix correspondence test (Mantel 1967). This test yields a product moment correlation (r) that is one measure of the relatedness between the two matrices. In this instance, the matrix correlation corresponds to two independently derived dendrograms. For matrix correlation of this type, a correlation value (r) greater than 0.58. Similarity matrices were utilized to construct the UPGMA (unweighted pair group method with arithmetic average) dendrograms. Cophenetic matrices for each marker and index types were computed and compared using the Mantle test. Finally, a principal coordinate analysis was performed in order to highlight the resolving power of the ordination. Polymorphism information content (PIC) was also calculated according to Anderson *et al.* (1993) using the following equation

n

$$PIC_i = 1- \Sigma P_i^2$$

Where P_{ij} was the fraction of the jth allele for marker and summation extends over n alleles.

RESULTS

Amplification of genomic DNA of the 18 genotypes, using 10 primers for RAPD analysis, yielded 78 fragments that could be scored. All the chosen primers amplified fragments across the 18 genotypes studied, with the number of amplified fragments ranging from 1 (OPB- 20) to 13 (OPA-16) and which varied in size from 200 bp to 5000 bp. Of the 78 amplified bands, 61(78.02%) and remaining 7(21.78%) were monomorphic. Average numbers of bands per primer were 7.8±1.19**,** while average number of polymorphic bands 6.1±0.44. Out of 10 RAPD primers used in this study, 9 primers amplified polymorphic bands, while other 1 showed monomorphic banding pattern. The average polymorphic information content (PIC) was 0.65±0.09 ranging from 0.36-0.90 and the lowest and highest PIC value were recorded for primer OPB-

17 and OPA-16, respectively Figure 1 is the representative of the extent of polymorphism observed among the soybean genotypes as revealed by OPB-08 RAPD primers were resolved on 1.5% agarose gel. A dendrogram based on UPGMA analysis grouped the 18 genotypes into 5 main clusters, with Jaccard's similarity coefficient ranging from 0.59 to 0.94 (Fig.3a). The first group included soybean genotypes BRAGG, PK 472 and PS 1374. The PK 472 showed genetic similarity of 94% with PS 1374, while both these cultivars shared 87% genetic similarity with BRAGG. Cultivars MACS 756, JS 93-05 NRC 66 and JS 97-52 (genetic similarity 91% with JS 97-52) formed the second group. The cultivars MACS 756 and JS 93-05 shared 88 % genetic similarity, while NRC 66 and JS 97-52 were 91% similar with each other. The third major group included UPSM 534, JS 335 and VLS 64, with JS 335 and VLS 64 showing genetic similarity of 87% with each other, while average genetic similarity of both these cultivars with UPSM 534 was 83%. The fourth major cluster grouped MRSB 345, HIMSO 1602, DS 98-14 and SL 710 with genetic similarity of 80 to 86%. The fifth major group placed KHSB 2 with SL 96 with genetic similarity of 74%. Genotypes NRC-37 did not form a cluster and remain ungrouped. The wild relative of soybean *G. soja* showed maximum diversity (41%) with other cultivars and was placed at the extreme end of the cluster.

Principal components analysis (PCA)

Using RAPD markers generated three dimensional scaling of 18 soybean genotypes (Fig.4a).The soybean cultivars MRSB 345, HIMSO 1602, DS 98-14, SL 710, NRC 66 and BRAGG were placed closely. The other group included genotype PK-472, JS 97-52, VLS-64 and PS-1374. The cultivars JS 335, JS 93-05, UPSM 534 and MACS 756 were placed closely. Rests of the three genotype (NRC 37, KHSB 2 and SL 96) were not grouped with any other genotype, while *G.soja* was placed at the extreme end.

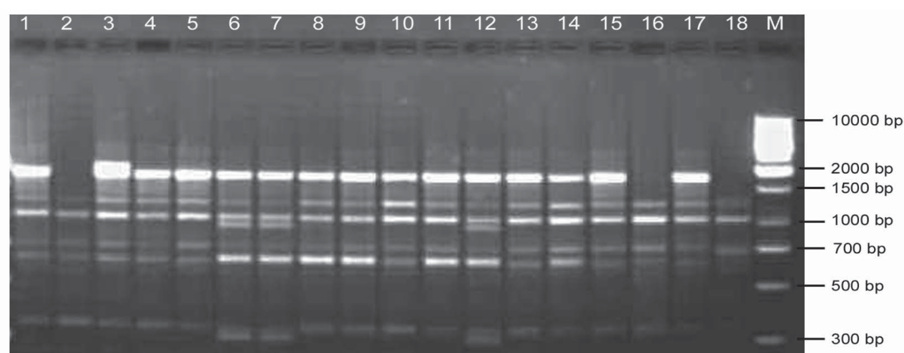

Fig. 1: The random amplified polymorphic DNA (RAPD) profile of soybean genotypes, using the random primer OPB-08. Lane M ë DNA marker. Lanes 1–18 soybean genotypes as listed in Table 1.

The PCR amplification using 20 32 -anchored ISSR primers had di-nucleotide repeat motifs rather than tri or penta nucleotide motifs. Out of 20 ISSR primers, 17 had di-nucleotide, while other three had tri (866), tetra (876) and penta (881) nucleotide repeat motifs, respectively. Twenty ISSR primers amplified 174 ISSR markers loci. The size of the amplified markers ranged from less than 250 bp-4000 bp. The lowest band size of less than 250 bp was amplified with primer 822, while highest band size of 4000-bp band was amplified with primer 842. Maximum numbers of bands i.e. 15 were scored by primer 812, while minimum numbers of band i.e. 2 were obtained with primer 810. Out of total 174 loci, 122 loci were found polymorphic (70.11%) across all the cultivars. The percentage polymorphism ranged from 33.33% (primer 866) to as high as 100% (primer 842). Average number of bands per primer was 8.7±0.49, while average number of polymorphic bands per primer was 6.1±0.52. The average polymorphic information content (PIC) was 0.66±0.04 ranging from 0.29-0.89 and the lowest and highest PIC value were recorded for primer 835 and 876, respectively (Fig. 2). Among the total 17 primers (with di-nucleotide repeat), 6 primer having GA repeat, 2 primers having AC repeat and 5 having TC repeat produced higher number of bands, averaging 8.5±1.15 and 7.5±0.50 and 8.4±0.68 per primer, respectively as compared to the primers having CT,TG and AG repeat. The ISSR bands were scored for presence or absence among the genotypes and used for the UPGMA cluster analysis divided the soybean germplam into 3 major clusters. It ranged from 0.71 to 0.95 (Fig.3b). In the first group, BRAGG and NRC 37 showed 94% genetic similarity with each other, while showed 91 % and 90 % genetic similarity with PS 1374 and MRSB 345, respectively. The second group included MACS 756 and NRC 66, which were 95% similar with each other. The other cultivars in this group, SL 710 and HIMSO 1602 showed 93% genetic similarity with each other, MACS 756 and NRC-66. Genotypes UPSM 534, JS 97-52, PK472, VLS 64, DS 98-14 and KHSB2 did not form a cluster and remain ungrouped. In the third group, cultivars SL96 and JS 93-05 shared genetic similarity of 79% with each other and showed 78% similarity with JS335. The wild relative of soybean *G. soja* showed maximum diversity (29%) with other cultivars and was placed at the extreme end of the cluster. Principal components analysis using ISSR markers generated two and three dimensional scaling of 18 genotypes. In this analysis also BRAGG, NRC 37, PS 1374 and MRSB2 were placed closely. Similarly, MACS 756, NRC 66 and SL 710 were placed together. The cultivars SL96, JS 93-05 and JS335 were placed together but were placed distantly from above two groups, while *G.soja* was placed at the extreme end.(Fig.4b).

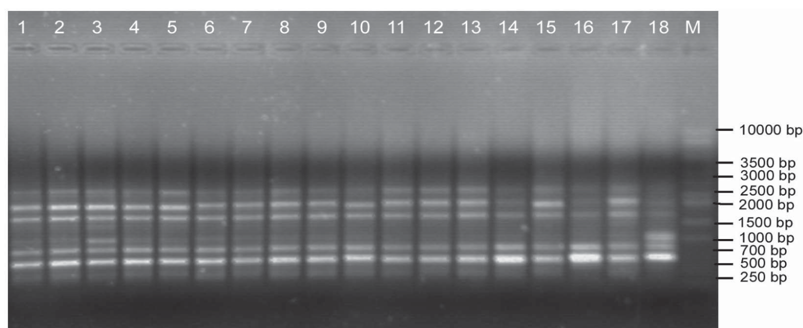

Fig. 2: Inter-simple sequence repeat (ISSR) polymorphism in soybean genotypes, using the ISSR primer UBC 835. Lane M ë DNA EcoRI and HindIII double-digest marker. Lanes 1–18 soybean genotypes as listed in Table 1.

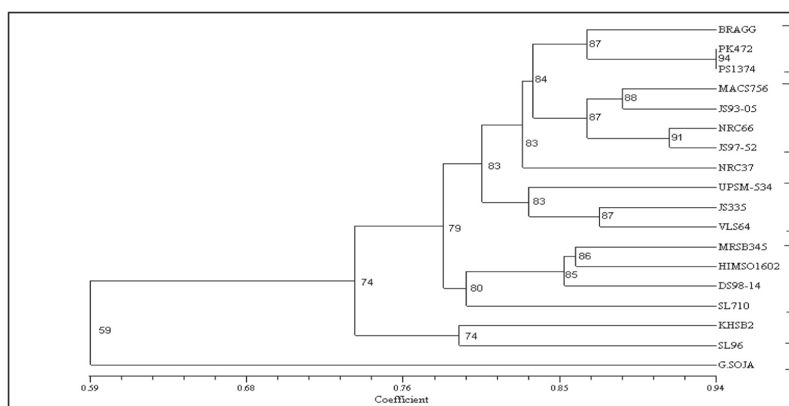

Fig.3a: Dendrogram generated using unweighted pair group method with arithmetic average analysis, showing relationships between soybean genotypes, using RAPD, The numbers at the forks indicate the confidence limits for the grouping of those species in a branch occurred.

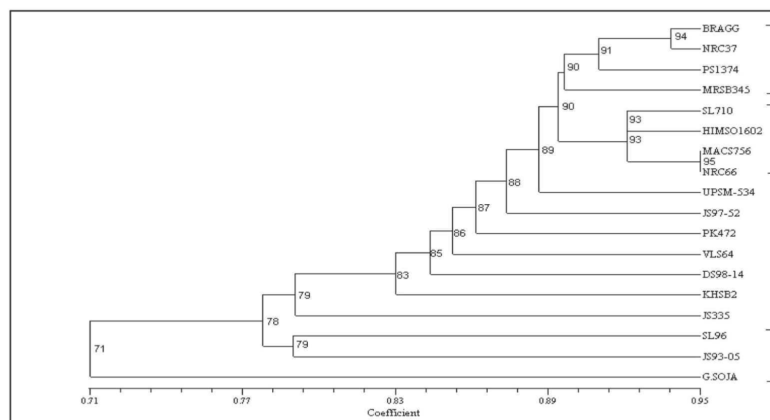

Fig. 3b: Dendrogram generated using unweighted pair group method with arithmetic average analysis, showing relationships between soybean genotypes, using ISSR, The numbers at the forks indicate the confidence limits for the grouping of those species in a branch occurred.

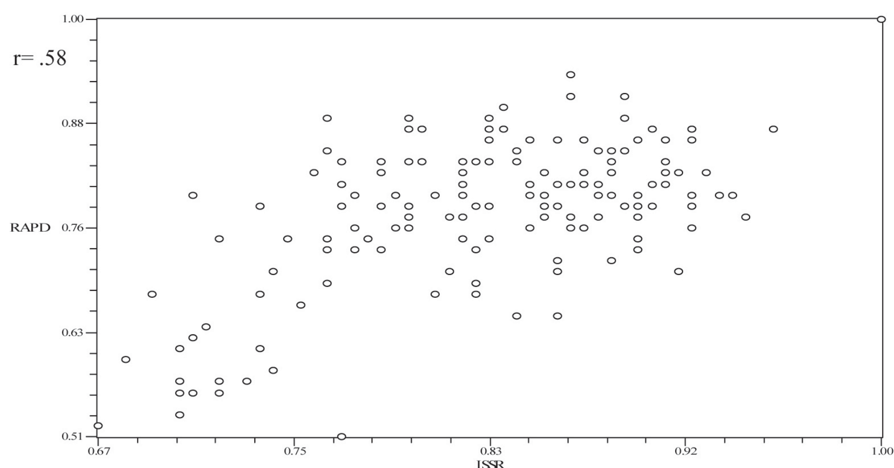

Fig. 4c: Positive correlation between RAPD, ISSR

Correlation between ISSR and RAPD

In order to study the correlation between the two markers systems used under present investigation the distance matrices obtained using Jaccard's coefficient in the ISSR and RAPD analysis were compared using correlation analysis. The two matrices showed a positive correlation (r= 0.58) as shown in Fig. 4c.

DISCUSSION

Determination of genetic diversity and relationship among individuals and populations are important considerations for genetic conservation and utilization of plant genetic resources (Cuartero *et al.,* 2006; Yan *et al.,*2005)**.** Present study of genetic diversity analysis in soybean, using ISSR and RAPD markers would help to achieve the goal for selection of suitable cultivars for *Rhizoctonia* aerial blight resistance. The use of genetically diverse resistance sources is important in breeding for durable disease resistance. Detection and evaluation of resistance genes by conventional inheritance experiments, however, often require laborious screening and genetic testing. In the present study, a marker-assisted screening for resistance sources was initiated in soybean (Yu *et. al.* 1994).It is therefore surprising to find significant levels of polymorphism among the 18 genotypes of soybean in RAPD (78.02%) and ISSR (70.11%) markers. The RAPD technique has been applied to assess molecular polymorphism in Vigna (Kaga *et al.* 1996), mung bean (Santalla *et al.* 1998; Lakhanpaul 2000), chickpea (Sonnante *et al.* 1997), pea (Simioniuc *et al.* 2002), pigeonpea (Ratnaparkhe *et al.* 1995), and in cowpea (Mignouna *et al.* 1998). The success of our study in identifying polymorphism for RAB is due to the use of a number of randomly selected pre-screened highly informative primers. The ISSR technique has been used in genetic relationships in genus soybean (Ajibade *et al.* 2000) and in several other crops (Reddy *et al.* 2002). ISSR markers were more efficient than the RAPD. Similar results were obtained for several other plant species like wheat (Nagoaka and Ogihara 1997) and groundnut (Raina *et al.* 2001). ISSR markers

have emerged as most efficient markers for genetic analysis in plants with the broad taxonomic applications over isozymes, RFLPs and RAPDs. Because of large amount of repeated regions, large primer length and more stringent temperature at the annealing step (Joshi *et al.*, 2000 and Blair *et al.*, 1999), Secondly ISSR is informative about many loci and is suitable to discriminate closely related genotype variants (Fang *et al.,* 1997, Akagi *et al.,* 1996, Wiesner and Wiesnerova 2003, Saini *et al.,*2004).Thirdly ISSR markers have great potential for studies of natural populations (Wolfe *et al.,* 1998). The number of total polymorphic and discriminant fragments is higher for ISSRs than RAPD. Similar results were observed in Nothofagus spp. (Mattioni *et al.* 2002). Xu and Gai (2003) obtained similar results in which a total of 177 bands were amplified by 20 primers and 66 (37.3%) of which were polymorphic in soybean. Similarly, Thompson *et al.*, (1998) found that genetic similarity between ancestral soybean lines and northern plant introduction was 0.69% as determined on the basis of RAPD marker analysis. Similar results were observed by Maughan *et al* (1996), McGregor *et al.*, (2000), Jin *et al.*, (2003), Medina *et al.,* (2006). 8 primers i.e. 835, 852, 834, 814, 876, 881, 841 and 822 amplified specific bands in one or two cultivars. These primers can be used to differentiate the specific cultivars from other cultivars.

The correlation between RAPD and ISSR Jaccard's similarity coefficient value was low in magnitude. The ability to resolve genetic variation among different genotype may be more directly related to the number of polymorphisms detected with each marker technique rather than a function of which technique is employed. The number of polymorphisms detected among genotypes influences the standard errors of the genetic diversity estimates. The correlation between Jaccard's similarity values generated from RAPD and ISSR marker techniques was low (r =0.58).

In this study, two markers systems (RAPD and ISSR) were used for comparison and assessment of genetic diversity. There was not much difference in the amount of polymorphism produced by ISSR (70.11 %,) and RAPD primers (78.02 %.). In the present study, not a single marker was found that could differentiate the susceptible cultivar from resistant cultivars. Among the markers, ISSR and RAPD markers had similar PIC values indicating good consensus between both marker systems. Similar

Fig.4a: Three dimensional picture of RAPD Fig.4b.Three dimensional picture of ISSR

results were observed in by Patzak, 2001, Raina *et al.* 2000. The Mantel Z-test statistic was significant for ISSR and RAPD markers which conclude that both ISSR and RAPD markers provide consistent information for germplasm identification, because the main clusters in the dendrograms were consistent for all marker systems. The molecular marker technique used in this study has proved to be successful in elucidation of the relationships among the 18 soybean genotypes, the identification of species-specific markers and the generations of a fingerprinting key are important resources for the breeding and management of soybean germplasm and some of these markers will be used for MAS in future soybean breeding programmes. The putatively similar bands originating for RAPDs in different individuals are not necessarily homologous, although they may share the same size in base pairs. This situation may lead to wrong results when calculating genetic relationships (Fernandez *et al.,* 2002). Dendrograms did not indicate any clear pattern of clustering according to the location in which they were collected. Similar results were obtained in Azuki bean (Yee *et al.* 1999) and in groundnut (Dwivedi *et al.,* 2001). The genetic closeness among the cultivars can be explained by the high degree of genetic distance in their pedigree. 5 genotypes grouped in cluster with RAPD primer. Similar results were observed in soybean (Gaffor *et al.* 2001) and mungbean (Lakhanpaul *et al.,* 2000). The level of observed polymorphism is very high, and the ability of the ISSR technique to effectively distinguish species in the genus *Vigna* was reported by Ajibade *et al.* (2000). The primer containing the CT repeats was one of those, which did not give interpretable phenotype analyzed, while primers with GA and CA repeats revealed polymorphism (Ajibade *et al.,* 2000). Genotype with the most distinct DNA profiles are likely to contain the greatest number of novel alleles, It is the genotypes that are likely to uncover the largest number of unique and potentially agronomic useful alleles. This strategy has resulted in a high proportion (H"50%) of new and useful quantitative trait loci alleles in rice and tomato (Tanksley and McCouch 1997). Our results indicate the presence of great genetic variability among elite genotypes of soybean. ISSR markers are useful in the assessment of soybean diversity, the detection of duplicate sample in germplasm collection, and the selection of a core collection to enhance the efficiency of germplasm management for use in soybean breeding and conservation.

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