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RESEARCH ARTICLE

COMPARATIVE STUDIES OF GENETIC VARIABILITY IN SOYBEAN (*GLYCINE MAX.* (L.) MERRILL) DERIVED GENOTYPES USING MOLECULAR MARKER.

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Abstract

The vast Mutagenic effect showed by application of mutagens (Sodium Azide -3mM, 5mM, 7mM; Ethyl Methane Sulphonate -5mM, 10mM, 15mM; And Gamma Irradiation -100kr, 200kr, 300kr).That clearly showed in field as well as laboratory testing; by using molecular markers, PCR (Polymerase chain reaction) ISSR(Inter Simple Sequence Repeat). Mutagenic variation founded in all the mutagens, but selected Genotypes were obtained from 100kr and 10mM Mutagenic lines. We demonstrate these genotypes for phenotypic screening and associated maturity, morphology, pigmentation traits. ISSR analysis done by two Primers and it was detected polymorphisms. For analysis, two primers were designed from consensus sequence regions on protein homolog genes, and used to amplify the genomic region. PCR samples were sequenced. The total number of polymorphic ISSR bands showed specific range and scored on all genotypes and their homology was measured.

Introduction:-

The genus Glycine consists of two subgenera, i.e. Glycine and Soja. Glycine max (L).Merr. And G.soja Sib. & Zucc. Belongs to Soja subgenus. Glycine max is the cultivated soybean while Glycine Soja is its wild progenitor. Soybean (Glycine max (L) Merrill) is an important crop as a source of protein and oil. Charles and Morse (1923) reported that the origin of soybean is china, Manchuria and Korea. Soybean is one of the best valued crops for its ability to fix nitrogen and provide seed protein and oil. In this importance the species are needed to develop new strain by mutagenesis to prove key step for identifying gene function in cultivar. A number of mutagen sources exist for introducing genomic variation. These include chemical, radiation, and transformation-induced mutagenesis of plant genomes (Waugh et al; 2006; Kuromori et al; 2009).Each of these methods results in a signature footprint of structural variation across the genome (Alonso and Ecker, 2006).

Molecular markers have become new tools and technique to help reduces workers effort(in identifying genotypes , save time i.e. work performed in short period of time, in most plant breeding programme, including introduction, selection and certification. Among them, SSR also known as micro satellite or short tandem repeat or simple sequence length polymorphisms is a repeated nucleotide sequence of 2-7 base pair units. Repeatability of fragment resulted from slippage in replication (Schlotterer and Tautz, 1992) and unequal crossover (Smith, 1976). The SSR technique uses PCR to amplify DNA fragments by repeated cycles of DNA denaturing, annealing and extension using DNA polymerase enzyme. The resulting DNA was separated by banding on gel (Akkaya et al; 1992).This

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method has many advantages such as rapid, reliable (Diwan and Cregan,1997), abundance (Lagerkrantz et al;1993),co-dominant(Akkaya et al;1992), high heterozygosity (Powell et al;1996), and high polymorphism(Akkaya et al;1995).

SSR has been used in the construction of genetic linkage map of human(Gyapay et al;1994),soybean shoe-marker Morgante, 1994; Akkaya et al;1995;Maughan et al;1995)and also used in DNA fingerprint of soybean (Yanagisawa et al;1994;Diwan and Cregan,1997).

SNP is single base variation between two identical DNA sequences. Brooks (1999) reported that SNP was single base pair positions in genomic DNA at which different sequence alternatives (alleles) exist in normal individuals of natural populations. This technology allows greater number of tests to be run at a significantly lower cost than existing technology. This technology has application in genome sequencing, SNP identification and typing, screening for genetically-linked diseases, identification of genetic drug targets, screening for individuals for potential drug side effects, gene cloning, screening potential tissue donors, screening cancer cells for genes conferring chemotherapy resistance. This technique has more advantages than markers such as isozyme, RFLP, RAPD, SSR. These advantages include very large number of polymorphic loci which distributed throughout the genome, marker present within coding regions, introns and regions that flank genes, simple and unambiguous assay techniques, high level of polymorphism in the population, stable Mendelian inheritance and low levels of spontaneous mutation (Brown, 1999).

Soybean genotypes were grown for said phenotypic characters indicating the different genetic variability among 12 genotypes. The objective of this study was to evaluate genetic variability and relationship among 12 soybean genotypes. by using PCR-ISSR .

Materials and Methods:-

Plant material and DNA isolation:-

Fresh seeds of soybean genotypes were used in this study for DNA extraction and isolation. The genotypes of soybean were grown in Aurangabad district (MS). The analyzed samples included dried soybean seeds that were used directly for DNA extraction. They found to yield DNA's comparable in quality and quantity when using EZI nucleic acid isolation analyzer (QIAGEN, 2007), CTAB method. Plant genomic DNA purification kit method, and DNA extraction with phenol purification and liquid nitrogen method, a soybean seed bulk sample was ground to fine powder. The experiment was carried out under the same conditions for all DNA samples.

Molecular material:-

DNA was extracted from seeds of soybean genotypes (*Glycine max* (L.) Merrill.) were grown in Aurangabad (MS) and isolated genomic DNA was amplified with two primers.

Table 1:- Primers for PCR amplification of Soybean (*Glycine max* (L.) Merrill.) Used in the study

Primer	Annealing Tm	Primer bp	Primer Sequence
SPA	54°c	320	5'-CCACTATCCTTCGCAAGACCCTTCC-3'
SPB	54°c	320	5'-CTTCTGTGCTGTAGCCACTGATGC-3'
LE5	53°c	180	5'-TCAACGAAAACGAGTCTGGTG-3'
LE6	53°c	180	5'-GGTGGAGGCATCATAGGTAAT-3'

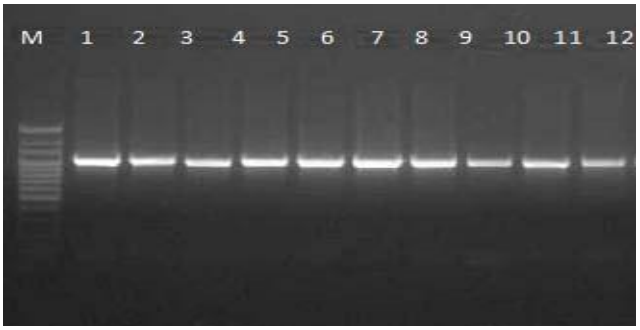

Fig.1:- Agarose gel electrophoresis of genomic DNA isolated from soybean genotypes were grown in Aurangabad (MS) .DNA loaded in 1% Agarose gel and separated by electrophoresis for 90 min at 50V, then visualized by ethidium bromide staining with transillumination. Respectively; Lane I-1kb M, Lane II to Lane XII-genomic DNA isolated from soybean genotype viz. 1. (GENO-1), 2. (GENO-2), 3. (GENO-3), 4. (GENO-4), 5. (GENO-5), 6. (GENO-6), 7. (GENO-7), 8. (GENO-8), 9. (GENO-9), 10. (GENO-10), 11. (GENO-11), 12. (GENO-12)

Digestion of DNA:-
The restriction enzyme recognizes a unique, specific sequence of, usually, 4-6 base pairs (bp) in length, termed a restriction site, where the enzyme cuts (restricts) the DNA. In general, restriction sites will occur throughout the genomic and, consequently, application of the enzyme to total genomic DNA (Restriction of DNA) results in its conversion into millions of fragments. The frequency of restriction sites will vary depending on both the restriction enzyme and on the genome.

Polymerase Chain Reaction Amplifications:-
Here, in study two primer pairs were used for PCR amplification. We performed different PCR optimizations of the PCR reaction mix and conditions for each primer pair.

SPA/SPB Primer Pair:-
Works carried in two tubes of a total volume of 25µl. The tubes contained 2µl of genomic DNA, 0.2µl of each primer, 2.5µl 10x PCR Buffer, 3.5µl of 50mmol/1MgCl2, 2.5µl of 10mmol/1 dNTPs, and 0.3µl of Taq DNA polymerase. The program was initiated on Bio-Rad PCR amplifier with 5 min of denaturation, followed by 10 cycles of amplification with denaturation for 15 sec at 94ºc first annealing for 20 sec at 54ºc and extension at 72ºc for 15 sec; followed by 40 cycles of amplification with denaturation for 1.5 min, second annealing for 1.5 min at 61ºc,an extension at 72ºc for 1min, and final elongation at 72ºc for 5min.

LE5/LE6 Primer Pair:-
Works carried in two tubes of a total volume of 25µl. The tubes contained 2µl of genomic DNA, 0.2µl of each primer, 2.5µl 10x PCR Buffer, 3.5µl of 50mmol/1MgCl2, 2.5µl of 10mmol/1 dNTPs, and 0.3µl of Taq DNA polymerase. The program was initiated on Bio-Rad PCR amplifier with 5 min of denaturatuion, followed by 10 cycles of amplification with denaturation for 15 sec at 94ºc first annealing for 20 sec at 55ºc and extension at 72ºc for 15 sec; followed by 40 cycles of amplification with denaturation for 1.5 min, second annealing for 1.5 min at 53ºc,an extension at 72ºc for 1min, and final elongation at 72ºc for 5min

All amplification products (10µl) were electrophorised in 1.5% (w/v) TAE Agarose gels containing 1 g/ml ethidium bromide. The electrophoresis was run in TBE at 100v for 40min and DNA bands were observed under UV light and photographed by image system.

Fig.2. Specificity of the PCR method using the primer pairs SPA/SPB and LE5/LE6 to identify the DNA. Agarose Gel Electrophoresis of PCR products amplified from genomic DNA of soybean genotypes.
Lane I-100bp ladder size standard;
Lane II-Lane XIII- sample Genomic DNA PCR products of Primer Pairs Respectively as above.

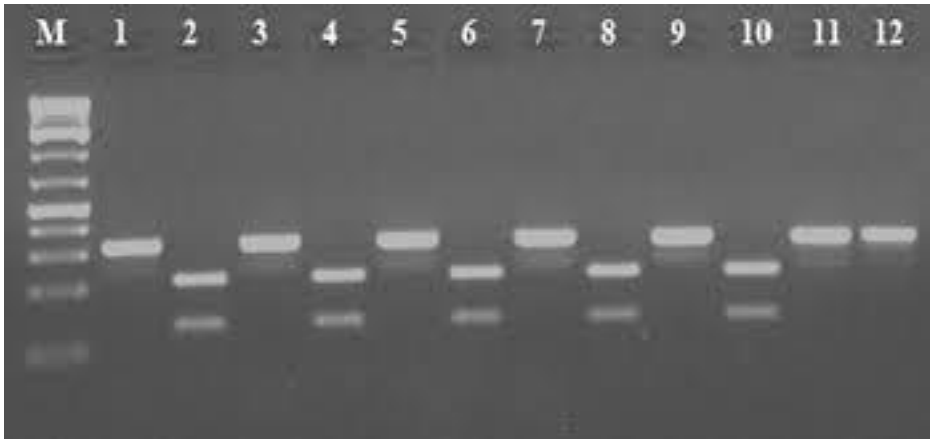

Fig 2:- Specificity of the polymerase chain reaction method using the primer pairs SPA/SPB and LE5/LE6 to identify the DNA Agarose gel electrophoresis of PCR products amplified from genomic DNA of Soybean genotypes. *Lane I-100bp ladder size standard(M); Lane I,III,V,VII,IX,XI and XII sample genomic PCR product of SPA/SPB; Lane II,IV,VI,VIII and X sample genomic PCR product of LE5/LE6.*

PCR-RFLP Data analysis:-

The PCR-RFLP analysis were performed in Agarose gel electrophoresis and its makes the banding pattern with high molecular weight DNA running fast in the lane; while low molecular weight run slow shown in the lane. The high molecular weight DNA was digested with an appropriate restriction endonuclease and the digest was fractioned by electrophoresis in 1% Agarose gel by Shiraishi et.al. The banding patterns obtained from the ISSR markers were scored as present (1) and absent (0). The PCR-RFLP method enabled to identify nucleotides at each of the variable sites clearly reliable.

Each sample has amplified by using two primers for each fragment and it was treated as rearrangement in genome. The primers shows scoring and reproducibility were considered for the scoring of bands. The gel pictures were taken and documented to the computer by using alpha imager gel documentation system and each amplicon were measured by software. The dendrogram was plotted by using phylogeny free and tree view of DNA fingerprinting analysis tool (Dendrogram).

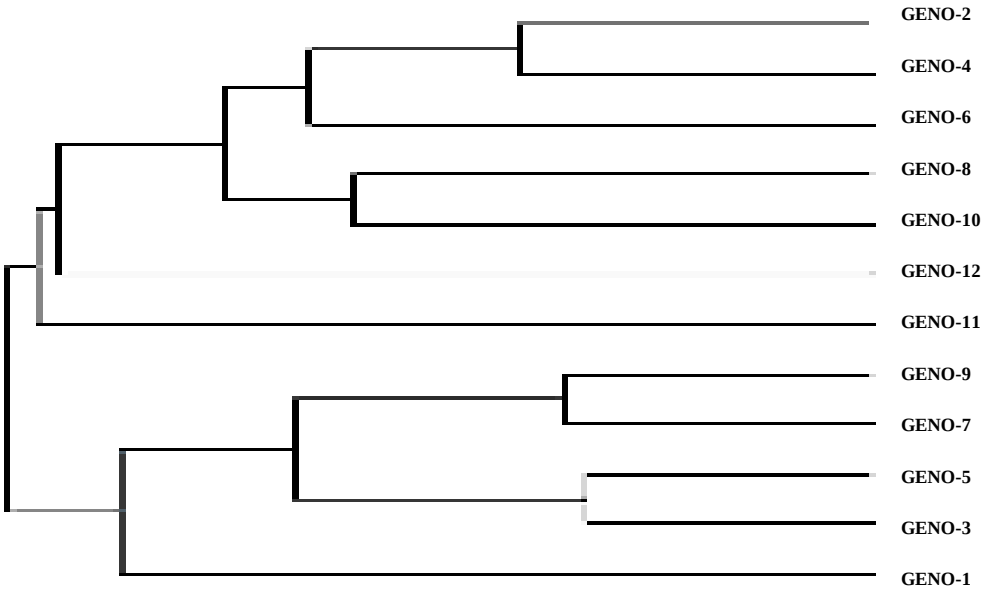

Fig 3:- Dendrogram based on bioinformatics phylogeny tool Free Tree and tree view of DNA fingerprint analysis.

Result and Discussion:-

The selected samples of isolated DNA was run on 1 % w/v Tris-Acetate EDTA gel were to check the quality of DNA sample have shown high molecular weight of DNA band (Fig.1). The amplification of genomic DNA of twelve selected genotypes using ISSR marker yielded total amplified 207 fragments were obtained while analysis of ISSR some fragments showed monomorphic and polymorphic in nature (Fig.2). In ISSR number of amplified fragments were ranged from 118bp to 320 bp. The PCR amplification using ISSR primers have potential to reproducibility of amplification of the product. The dendrogram analysis was carried out by using bioinformatics phylogeny tools free tree and tree view of DNA fingerprint analysis (Fig.3). Earlier the comparative analysis of genetic diversity was also attempted by using SNP, ISSR and RAPD markers in soybean genotypes, Vigna genotypes and Mung bean genotypes. The twelve *Glycine max. (*L.) Merrill derived genotypes were clustered in three groups. Cluster-I, II and III. The narrow genetic diversity identified among the soybean genotypes which derived from variety of JS-335. The present study may be the serve as the source of new alleles in soybean breeding programme.

Conclusion:-

The derived genotypes were identified for qualitative and quantitative characteristics. In cross breeding between the superior genetically different individuals which are having different gene of interest characteristics rather than involving individual belonging close relative genetic group. The analysis reported that in the present study could be useful to the selection of screened individuals for cross breeding purpose.

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