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RESEARCH ARTICLE

Genetic Diversity Analysis of Cowpea Mutant (*Vigna unguiculata* (L.) Walp) as Revealed by RAPD Marker

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Abstract

The aim of present study is examine the effect of different doses gamma rays, ethyl methane sulphonate and combination of both gamma rays with EMS on cowpea seeds and to identify DNA polymorphism among the mutants through a RAPD marker analysis. The mutants showing the differences in morphological traits showed DNA polymorphism in PCR profile amplified by RAPD marker. Ten random decamer primers revealed a high DNA polymorphism among the mutant populations like tall, dwarf, leaf; flower and seed mutants were analyzed. Ten primers produced a total of 60 amplified products. Among these 48 were monomorphic with an average of 53.84 per cent polymorphism. Only five primers (PG-04, PG-05, PG-07, OPA-05 and OPA-07) showed highest polymorphism. The primer OPA-02 gave the lowest polymorphism (22.27%). Highest genetic coefficient similarity (0.76) was observed between leaf and tall; whereas least similarity (0.58) was observed between control and leaf. In a Dendrogram constructed based on genetic similarity coefficients, the mutants were grouped into three main clusters namely, cluster A, B and C.

Introduction

Cowpea is one of the most important pulse crops in tropical Africa. The seeds are a major source of dietary protein in most developing countries. Induced mutation breeding has been recognized as a valuable supplement to the conventional breeding in crop improvement programmes and has been least applied in grain legumes. The grain contains about 25% protein and 64% carbohydrate (Bressani, 1985). Protein in seed is rich in amino acids, lysine and tryptophan, compared to other cereal grains. Cowpea provides an extremely significant portion of the dietary protein of the people and plays an important nutritional role in developing countries of the tropics and subtropics, especially in sub-Saharan Africa.

Mutation breeding is one possible alternative to conventional breeding for crop improvement. Exposing plant genetic material to mutagens enhances the chance of isolating unique genetic material. In the past, induced mutations have effectively been utilized in development of new and valuable alterations in plant characteristics that have contributed to increased yield potential. Induced mutations can rapidly create variability in quantitatively and qualitatively inherited traits in crops (Maluszynski *et al.,* 1995; Muduli and Mishra, 2007). Induced mutagenesis has been used to obtain direct mutants or by using these mutants in hybridization (Ahloowalia *et al.,* 2004) to overcome yield plateaus and generate desirable horticultural traits. Mutation breeding has contributed significantly to plant improvement, resulting in release of at least 2250 varieties of different crops. In India, at least 300 cultivars have been developed in at least 55 plant species (Kharkwal *et al*., 2004).

Gamma rays belong to ionizing radiation and interact with atoms or molecules to produce free radicals in cells. These radicals can damage or modify important components of plant cells and have been reported to affect the morphology, anatomy, biochemistry and physiology of plants differentially depending on the irradiation level. These effects include changes in the cellular structure and metabolism of the plants e.g., dilation of thylakoid membranes, alteration in

photosynthesis, modulation of the antioxidative system and accumulation of phenolic compounds (Kim *et al.*, 2004; Kovacs and Keresztes, 2002; Wi *et al.*, 2005).

Alkylating agents such as ethyl methane sulphonate (EMS) induce chemical modification of nucleotides, which result in mispairing and base changes. Strong biased alkylation of guanine (G) residue results, forming O^6-ethyl guanine, which can pair with thymine (T) but not with cytosine (C). Through subsequent DNA repair, the original G/C pair can then be replaced with A/T. Ninety-nine percent of mutations from alkylation of guanine induced by EMS are reported as G/C-to-A/T transitions (Greene *et al.*, 2003).

The synergism among two mutagens may be firstly because of first mutagen treatment making accessible otherwise non-available sites for reaction to the second mutagen; and secondly pre-mutational lesions induced by the first mutagen becomes fixed due to an inhibitory effect of the second mutagen on repair enzyme (Payaz and Deering 1972; Kharkwal, 1998). Both these pathways should yield a frequency of mutations higher than the total of two mutagens applied individually (Makeen and Suresh Babu, 2010).

The Random Amplified polymorphic DNA (RAPD) marker technique (Welsh and Mcclelland, 1990; Williams *et al*., 1990) is quick, easy and requires no prior sequence information. A single random decamer primer is used to specify the sequence that is to be amplified. The homozygous presence of fragment is not distinguishable from its heterozygote, and such RAPDs are dominant markers. The technique has been used for identification purposes in many crops like cowpea (Mignouna *et al*., 1998) and mungbean (Lakhanpaul *et al*., 2000). Selected mutant varieties were analyzed at the molecular level and differences in variability could be determined. At the end of the induction of ionized radiation with mutation, the numbers of released mutant varieties were derived (Donini and Sanino, 1998). Mutagenic PCR (polymerase chain reaction) is often used to create libraries of mutated genes. PCR mutagenesis violates some of the assumptions critical to the models presented here (Sun, 1995; Drummond *et al*., 2005).

Materials and Methods

Plant material

Six genotypes (five mutants and one parent) of cowpea variety CO7 were analyzed in the study. The mutants were resulting from gamma rays, ethyl methane sulphonate (EMS) and combination treatment (Gamma rays with EMS) in M_6 generation.

Mutagenic treatment

Mutation was induced using physical gamma rays, chemical ethyl methane sulphonate (EMS) and combination treatment gamma rays with EMS to find out genetic variation of DNA base analogues. For physical gamma rays, five sets of two hundred, well-matured seeds of cowpea were subjected to irradiation. Seeds were treated with 0, 5, 10, 15, 20, 25 and 30KR of gamma ray irradiation. Irradiation was accomplished in the Sugarcane Breeding Institute (ICAR) at Coimbatore, TN, India. The labeled Cobalt (^{60}CO) was used as source of gamma ray irradiation. For chemical EMS treatment, four hundred, well matured healthy seeds were used. Prior to treatment seeds were pre-soaked in distilled water for 5 hours at room temperature (28 ± 2 °C) prior to treatment. After pre-soaking, the excess moisture in the seeds was removed by filter paper. The seeds were then soaked in the freshly prepared aqueous solution in a volume three times larger than the volume of seeds with corresponding concentrations of EMS *viz*, 0, 5, 10, 15, 20, 25 and 30mM for 6 hours at room temperature (28±2°C) with 1 hour intermittent shaking. After the treatment, seeds were washed thoroughly with distilled water. For combination treatments two hundred seeds each were first irradiated with gamma rays at 0, 5, 10, 15, 20, 25 & 30KR doses and then followed by EMS, only one concentration of EMS (25mM) was used in combination with 5, 10, 15, 20, 25 & 30 KR gamma rays. After the EMS treatment, the treated seeds were washed thoroughly for 1h in running tap water and followed by distilled water to terminate the residual effect of the mutagenic chemicals. The untreated seeds pre-soaked in water were used as parent. Both treated and parent (control) seeds were sown in the field by randomized black design (RBD) with three replications to rise the M_1 generation. M_1 plants were harvested and grown in successive seasons and developed M_2, M_3, M_4, M_5, and M_6 generations.

Isolation of mutants

The mutants scored in M_5 generation were bulked and raised in Petri plates to obtain the M_6 generation. The five mutants selected by morphological and biochemical (protein profile) criteria, namely, tall, dwarf, leaf, flower and seed mutants derived from

gamma rays, EMS and combined treatments. The well-matured and untreated seeds were used as control (parent) and subjected to RAPD study along the mutants.

Isolation of DNA

Five gram of fresh, young leaf tissue (mutants and parent) was ground to fine powder in a pestle and mortar containing liquid nitrogen. Total genomic DNA was extracted using the CTAB method of Doyle and Doyle (1987).

PCR amplification

Twenty random, arbitrary decamer primers were selected and screened to produce polymorphic bands for this investigation obtained from Operon Technologies Inc. (Alameda, CA, USA). The polymerized chain reaction PCR) amplification was performed in each 25 µl reaction mixture (Eppendorf Pvt. Ltd., Germany) containing 10 mM of Tris HCl (pH-8.3), 50mM KCl, 0.5U of Taq DNA polymerase, 0.01% gelatin, 0.2 mM each dNTPs, 2.5 mM MgCl2, 50pmol µl-1 of random primer, and 20 ng µl-1 of genomic DNA. The amplifications were performed in TECHNE Master Cycler System (TECHNE Ltd., Cambridge, UK) with the following sequential thermal cycling: initial denaturation step at 94°C for 4 min, followed by 40 cycles at 94 °C for 1 min, an annealing step at 37°C for 1 min and an extension at 72°C for 1 min, with a final extension stepat 72°C for 5 min. The final products were held at 3°C. The amplified products were run on 1.5% agarose gel (Bangalore Genie Pvt. Ltd., India) using with 1X TAE buffer at 6.5 Vcm^{-1} for 1 hour. The gel was stained with (5 mg ml-1) ethidium bromide for 15 min and visualized under UV light at 290 nm. The size of amplified fragments was determined by co-electrophoresis low range marker (Bangalore Genie, Pvt. Ltd., India).

Statistical analysis

The fragments obtained from amplification were scored as the presence or absence of each single fragment for a binary data matrix. The binary data were used to compute a pair-wise similarity/ distance matrix using the Jaccard's distance index. The similarity matrix was subjected to cluster analysis using the UPGMA (Unweighted Pair Group Method with Arithmetic average) algorithm on NTSYS-pc version 2.10 software (Rohlf, 2000). The genetic distance was analyzed between mutants and parent as per Nei and Li (1979).

Results

The present investigation was undertaken in order to study the artificial inducement of mutation in cowpea variety CO7 by using Gamma rays, EMS, and combined mutagens through the biological changes in M_3, M_4, M_5 and M_6 generations. This was aimed to find out the economic potentialities of the viable mutant and the nature of induced variability in the qualitative and quantitative traits in all generation.

DNA from the isolated mutants and their parents were used for RAPD analysis. Due to advances in molecular biology techniques, large numbers of highly informative DNA markers have been developed for the identification of genetic polymorphism. In the last decade, the random amplified polymorphic DNA (RAPD) technique based on the polymerase chain reaction (PCR) has been one of the most commonly used molecular techniques to develop DNA markers. RAPD markers are amplification products of anonymous DNA sequences using single, short and arbitrary oligonucleotide primers, and thus do not require prior knowledge of a DNA sequence. Low expense, efficiency in developing a large number of DNA markers in a short time and requirement for less sophisticated equipment has made the RAPD technique valuable although reproducibility of the RAPD profile is still the centre of debate.

Six genotypes (five mutants and one parent) were subjected to RAPD assay to analyze for identifying DNA polymorphism. Ten random decamer primers revealed a high DNA polymorphism among the mutant populations like tall, dwarf, leaf, flower and seed mutants were analyzed. Ten random decamer primers revealed a high DNA polymorphism among the genotypes. Ten primers produced a total of 60 amplified products. Among these 48 were monomorphic with an average of 53.84 per cent polymorphism. Only five primers (PG-04, PG-05, PG-07, OPA-05 and OPA-07) showed highest polymorphism. The primer OPA-02 gave the lowest polymorphism (22.27%). The number of bands ranged from 1 (OPA-02) to 10 (PG-07) with an average of 6.0 bands per primer (Table-A).

Genetic similarity coefficient ranged from 0.58 to 0.76. Highest genetic coefficient similarity (0.76) was observed between leaf and tall; whereas least similarity (0.58) was observed between control and leaf (Table-B). Further, the dendrogram constructed from the pooled data (Fig. A) clearly showed, three major clusters namely, cluster A, B

and C. Cluster A was divided into a control and dwarf mutant. Cluster B was divided into leaf, tall and flower mutant. Cluster C was divided into a seed mutant. It was a single group.

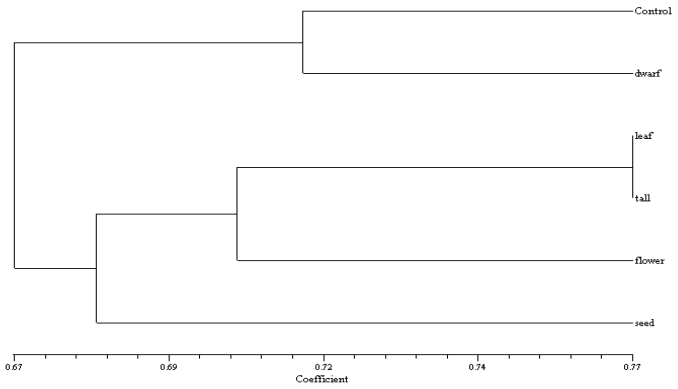
Table A: Analysis of RAPD banding pattern for cowpea genotypes

Primer code	GC content	Primer sequence 5^1 to 3^1	Total Number of Bands	Number of Polymorphic Bands	Number of Monomorphic Bands	% of Polymorphism
OPA-01	70%	5^1-CAG GCC CTT C- 3^1	10	4	6	40.00
OPA-02	70%	5^1-TGC CTA GGT G- 3^1	11	3	8	27.27
OPA-03	60%	5^1-CGT CTA GGT G- 3^1	9	4	5	44.44
OPA-05	60%	5^1-CGT CTA GGT G- 3^1	8	4	4	50.00
OPA-07	70%	5^1-CGT CTA GGT G- 3^1	11	7	4	63.63
OPC-11	60%	5^1-CGT CTA GGT G- 3^1	10	4	6	40.00
OPC-15	60%	5^1-CGT CTA GGT G- 3^1	11	5	6	45.45
PG-04	60%	5^1-CGT CTA GGT G- 3^1	13	9	4	69.23
PG-05	60%	5^1-GCA GGC TAA C-3^1	14	12	2	85.71
PG-07	60%	5^1-GCT GCA GTA G- 3^1	11	8	3	72.72
			Total	60	48	53.84
			Average	6.0	4.8	5.38

Table B- Similarity index of six mutants and control

	Control	**Leaf**	**Flower**	**Seed**	**Tall**	**Dwarf**
Control	1.000					
Leaf	0.580	1.000				
Flower	0.702	0.757	1.000			
Seed	0.677	0.650	0.669	1.000		
Tall	0.622	0.765	0.650	0.727	1.000	
Dwarf	0.714	0.702	0.702	0.663	0.693	1.000

Fig. A. Dendrogram



Discussion

DNA from the isolated mutants and their parents were used for RAPD analysis followed by Doyle and Doyle (1987). RAPD assay revealed very low level of polymorphism among cultivated types, while abundant polymorphism among wild diploid species (Halward *et al.,* 1991, 1992 and Subramanian *et al*., 2000; Dwivedi *et al.,* 2001). Sufficient diversity in DNA profiles of some of the disease resistant germplasm using RAPD has also been reported (Dwivedi and Gurtu, 2002). Random amplified polymorphic DNA (RAPD) markers, which can quickly detect a large number of genetic polymorphisms, have led to the creation of genetic map in a number of crops (Luo *et al.,* 2002) and RAPD markers, have been used to detect mutations and DNA damage (Atienzar *et al*., 2002).

A total of 10 primers were used to screen polymorphism between the mutagenic treatments in both mutant and parents. Gamma rays, EMS and combined treatments of M_6 generation were subjected to RAPD analysis using 10 random primers. The

DNA fragments with different molecular weights were amplified and the product size ranged between 250-1000Kb (Fig. B and C). Ten primers produced a total of 60 amplified products. Among these 48 were monomorphic with an average of 53.84 per cent polymorphism. Only five primers (PG-04, PG-05, PG-07, OPA-05 and OPA-07) showed highest polymorphism. The primer OPA-02 gave the lowest polymorphism (22.27%). Similar varied levels of polymorphism have been reported by Multan and Lyon (1995) in cotton; Encheva *et al*., (2005) in sunflower; Subramanian *et al.*, (2000) and Silvanacrieste *et al.*, (2005) in groundnut.

Fig. B. Primer-PG04

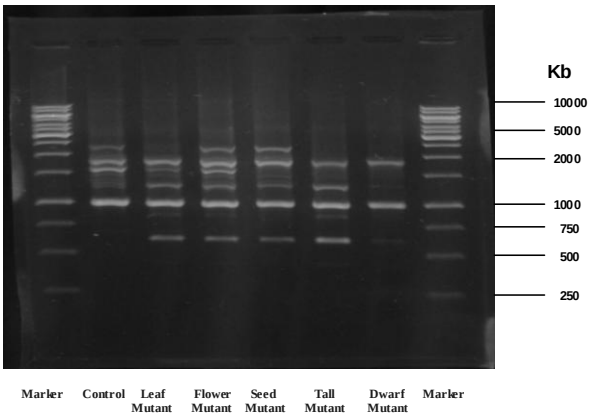
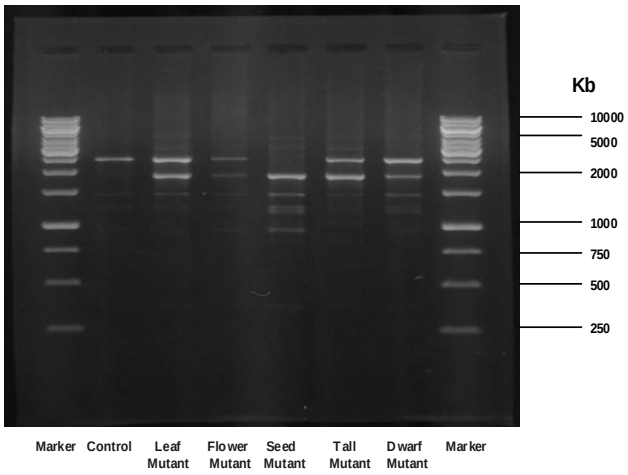


Fig. C. Primer-PG05



Mutations are the tools used to study the nature and function of genes which are the building block and basis of plant growth and development, thereby producing raw materials for genetic improvement of economic crops (Adamu and Aliyu, 2007). Mutation methodology has been used to produce many cultivars with improved economic value and study of genetics and plant developmental phenomena (Van *et al.*, 1990 and Bertagne- Sagnard *et al.*, 1996). It has been demonstrated that genetic variability for several desired characters can be induced successfully through mutations and its practical value in plant improvement programs has been established.

Erdem and Oldacay, (2004) recommended that radiation is one of the best known physical mutagens. It dissociates the atoms of water molecule and causes the generation of hydroxyl radicals that are the most reactive. They react with most of the biomolecules including DNA and scavenge the electrons from them. The oxidation of biomolecules by the radicals in turn damages the DNA structure and biological activity. EMS treatment causes alkylation of guanine bases (G) leading to mispairing or mismatch pairing in the DNA of a treated organism. Under these conditions, an alkylated G pairs with T (thymine) in place of C (cytosine), causing a G/C to A/T transition in the backbone of the DNA. EMS treatments can cause allelic mutations, small deletions and other chromosomal rearrangements. These mutations can be used to activate morphometric and reproductive changes in plants; further selection of mutant plants through a number of generations can be used to eliminate deleterious mutations, resulting in introduction of new traits into a treated population (Acharya *et al*. 2007).

A synergistic effect may cause if the sites of action protected during treatment with first mutagen are exposed to the action of the second (Arnason *et al.*, 1963). An additive effect or even lowering of the effect may result, in case their actions are independent or the two mutagens compete for the same site (Aastveit, 1967). Khan (1981) reported that less additive effect was observed in the combination treatments, because of the fact that both the mutagens compete for the same site. Thus it is obvious that combination of mutagenic agent is another useful method for explaining the mechanism of their action.

Changes the DNA bands, where the main changes in the RAPD profiles of the appearance or disappearance of different bands with variation in their intensity. These effects might be due to the structural rearrangements in DNA caused by different types of DNA damages (Hegazi and Hamideldin., 2010). Thirteen accessions of germplasm collection of *Vigna* species were characterized using randomly amplified polymorphic DNAs (RAPD). After screening them with ten random primers, five were found to produce clear PCR amplification profiles. Out of a total of 134 bands generated with the five

random primers chosen, 104 were polymorphic. Thirteen accessions could be separated into two major groups, one comprised of six accessions of *V. trilobata* and the other included rest of the accessions. One *V. trilobata* accession showed a deviation in RADP pattern and morphology from the other *V. trilobata* collections (Samarajeewa *et al*., 2002).

In RAPD analysis, the velvet bean genotypes were grouped into two sub clusters revealing sufficient amount of diversity within the cluster. Similar results have been found by Dikshit *et al.,* (2007) for *Vigna umbellata* accessions and estimated 53 per cent of genetic similarity based on RAPD and SSR markers data also. Dhakshanamoorthy *et al.,* (2010) reported that the effect of different doses of gamma irradiation on seed germination, flowering, fruit and seed traits of *Jatropha curcas* and to identify DNA polymorphism among the mutants through a Randomly Amplified Polymorphic DNA (RAPD) marker analysis.

The mutants showing the differences in morphological traits showed DNA polymorphism in PCR profile amplified by RAPD marker. The main changes in the RAPD profiles of the present investigation were the appearance or disappearance of different bands with variation in their intensity. These effects might be due to the structural rearrangements in DNA caused by different types of DNA damages. RAPD method is applicable for the detection of changes in the DNA structure after different genotoxical treatments (radiation). Thus, the variation in band intensity and disappearance of some bands may correlate with the level of photoproducts in DNA template after radiation, which can reduce the number of binding sites for Taq polymerase. Appearance of new bands can be explained as a result of different DNA structural changes (Breaks, transpositions, deletions, etc) (Danylchenko and Sorochinsky, 2005). Thus, the estimate on the existence of mutation and structural alterations in plant DNA after impact of radiation on the bases of DNA patterns could be obtained after RAPD with the set of primers. In this present investigation DNA polymorphism detected by RAPD analysis offered a useful molecular marker for the identification of mutants in different mutagenic treated plants.

Conclusion

In the present investigation the genetic diversity among the cowpea cultivars investigated was large and the RAPD proved to be a useful technique to characterize it. Based on the molecular variance, the fixation index suggests a large differentiation of cowpea cultivars. The RAPD markers are useful in identifying the mutant at DNA level, which is not influenced by the environment. These molecular markers are helpful in assessing genetic purity at a shorter time. A large number of useful mutants for different characters were observed in different concentrations. These mutants are to be evaluated to assess their stable performance, involve them in crop improvement programme and to isolate productive mutants.

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