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Biochemical and molecular studies on effect of polymer seed coating of soybean

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Abstract
The present study was conducted at the Research Farm Adhartal, Department of Plant Breeding and Genetics, JNKVV, Jabalpur (M.P.) during *Kharif* season of 2016-17. Research experiment was laid out in a Randomized block design with five replications and four treatments i.e T0 No treatment or (Water + Thiram), T1- Polymer seed coating (Disco AG SP Red L-200) + Thiram + Carboxine, T2- Polymer seed coating (Disco AG SP Red L-200) + Thiram + Genius coat, T3- Polymer seed coating (Disco AG SP Red L-200) + Thiram + Mycorhiza comprised of soybean genotype JS JS 20-29. Five plants were randomly selected from each treatment and replications for Phenological and Physiological Parameters. Maximum protein % was registered in Treatments Polymer seed coating (Disco AG SP Red L-200) + Thiram + Genius coat (T2) (40.64), Treatments T3 (20.04) possessed the maximum fat % and showed differed significantly among them. The DNA amplification pattern revealed that a total number of 42 RAPD loci were amplified with an average of 7.0 loci per RAPD primer comprising 30 polymorphic loci (71.43%) and 12 monomorphic loci (28.57 %). The range of polymorphism was 50 % (OPA-01&OPA 02) to 89.0 % (OPA 07). Amplified product (band) size ranged from 300 bp (OPA-03) to 2800 bp (OPA-05). The no of bands ranged from 04 (OPA-02) to 09 (OPA -07).

Keywords: Soybean, biochemical studies, molecular studies, RAPD, DNA finger printing

Introduction
Soybean is one of the most important protein and oil seed crops throughout the world. Its oil is the largest component, highly nutritive and every legume with 43% of biologically effective protein and 20% of edible oil ranks first among the oil seed crops in India. Soybean is native of China. It has emerged as one of the important commercial crops in many countries. Soybean is also known as the “Golden bean” or “Miracle crop” because of its multiple uses. Soybean is a crop of multiple qualities as it is both a pulse and oilseed crop. In India, It contributes around 25% of total edible oil pool of the country.

Seed coating with natural or synthetic polymers have gained rapid acceptance by the seed industry as a much safer coating material. The stability of poly coated seeds has also to be investigated in order to determine the viability of seeds for long term. Polycoated seeds can be stored for long term, if adequate storage condition are provided (Giang and Gowda, 2007) [7]. The polymer coating with the negligible 84 micron over the seed coat provide protection from the imposed accelerated ageing, which include fungal invasion. It reduce chemical; wastage, helps to make room for including all required ingredients protect the nutrients, oxygen suppliers and protect seed from fungal invasion and insects attack. But encasing the seed with thin film of biodegradable polymer, the adherence of seed treatment to the seed is improves, ensures dust free handling making treated seed both useful and environment friendly Kaushik *et al.* (2012) [5]. The polymer keeps the seed intact, as it acts binding material and corers the miner cracks and aberration on the seed coat and blocking the fungal invasion as well act as a physical barrier which reduces leaching of inhibiters from seed coverings and restrict oxygen movement and thus reducing the respiration of embryo there by reducing the ageing effect on seed. The polymer also prevents moisture content fluctuations during storage (West *et al.* 1985) [10].

DNA fingerprinting is also known as DNA profiling. It is the genetic variation among genotypes using Molecular markers. In the last two decades, a rapid progress has been made towards the development and application of molecular marker technology in plant genome

analysis. Molecular markers are considered best for the analysis of genetic diversity and cultivar identification since they are indifferent to development stage or environment. Molecular markers are also called DNA markers**.** It is a DNA sequence that is readily detected and whose inheritance can be easily monitored. The use of molecular markers is based on naturally occurring DNA polymorphism, which forms the basis for designing strategies to exploit applied purpose. Random Amplified Polymorphic DNA (RAPD) is a type of molecular marker. This marker system was developed by Welsh & McClelland in 1991 [9]. RAPD is performed in conditions resembling those of PCR using genomic DNA from the target fungal pathogen and a single short oligonucleotide (generally 10 mer). The DNA amplification product is generated from a region that is flanked by a part of 10 b.p priming site in the appropriate orientation. A particular fragment generated for one genotype but not for other represents DNA polymorphism and can be used as a genetic marker. Molecular marker technology is an integral part of Agriculture Biotechnology. PCR based molecular markers are considered as boon for development of agriculture. (Yogendra Singh 2010) [12]. Due to ample applications of Agriculture Biotechnology, it is considered as a weapon for scientist to fight hunger, malnutrition and poverty. (Yogendra Singh, 2009) [11]. Molecular approaches have been found more reliable for assessment of genetic divergence in rice (Yogendra Singh, 2011) [13] and these are being used by various workers time to time (Yogendra Singh *et al*, 2013) [14] Keeping in view of the above facts the main objectives of this study were (i) To study the effect of polymer seed coating of soybean on biochemical constituents and (ii) To investigate the variation in DNA of treated population using RAPD Profiling.

Materials and Methods

The present study was conducted at the Research Farm Adhartal, Department of Plant Breeding and Genetics, JNKVV, Jabalpur (M.P.) during *Kharif* season of 2016-17. Research experiment was laid out in a Randomized block design with five replications and four treatments i.e T0 No treatment or (Water + Thiram), T1- Polymer seed coating (Disco AG SP Red L-200) + Thiram + Carboxine,T2- Polymer seed coating (Disco AG SP Red L-200) + Thiram + Genius coat,T3- Polymer seed coating (Disco AG SP Red L-200) + Thiram + Mycorhiza comprised of soybean genotype JS JS 20-29. Five plants were randomly selected from each treatment and replications for Phenological and Physiological Parameters

1. Biochemical estimations

The soybean seed were analyzed for the biochemical constituents as follows:

a. Estimation of protein % in seeds: The nitrogen content was estimated by micro kjeldhal method (A.O.A.C., 1965)

b. Total crude fiber (%): Total crude fibre was calculated as following

% Crude fibre = W2 – W1 / Weight of sample x100
W1= initial weight of crucible, w2= final weight of crucible

c. Determination of fat percentage: The fat content in the sample was estimated by pelican equipment socs plus based on principle of Soxhlet's extraction method as described in AOAC (1980) [15].

d. Total Ash (%): The ash content in the seed sample was estimated accordig to AOAC (1980) [15].

2. Molecular (DNA) profiling

a. Germination of Seeds for DNA Isolation: Healthy seeds with identical dimensions were selected by visual observation and dipped in distilled water overnight.The healthy seeds were kept in distilled sterilized water and after that seeds were placed in petri plates and then kept in germinator at 35°C for germination. After one week, the etiolated leaves were harvested using a sharp sterilized blade. Leaves surface sterilized with70% ethanol followed by distilled sterilized water.

b. DNA Isolation*:*The isolation of genomic DNA from leaf sample was undertaken following the method describe by Mukherjee (1999) [6] with slight modifications.

c. DNA purification: The purification of DNA was carried out in order to remove the impurities like RNA, proteins and polysaccharides. These are considered as inhibitors in DNA amplification during PCR.

d. Quantification of DNA: Quality of DNA was determined by horizontal submarine gel electrophoresis on 0.8% agarose gel. Purity of DNA was checked by taking the ratio of optical density (OD) using spectrophotometer, at 260 nm to that of 280 nm. DNA concentration of sample was calculated using following formula:

$$\text{Conc. in } \mu g/\ \mu l = \frac{O.D._{260\ nm} \text{ x } 50 \text{ x Dilution factor}}{1000}$$

e. Dilution of DNA: The quantified DNA was diluted according to the DNA quantity in each sample for PCR amplification in nuclease free water. Dilutions were carried out according to the following formula

f. PCR amplification with RAPD markers: The PCR reaction was performed in a volume of 20 µl reaction set up having 30 ng of template DNA, 800µM of dNTPs mix, 1.0 U of Taq DNA polymerase, 1x Reaction buffer, 0.3 µM of Primer and rest of deionized water. The PCR amplification was achieved in M.J Research Thermo cycler (PTC 200), The PCR conditions were initially 5 min denaturing step at 94 ^{0}C, followed by 41 cycles having denaturing at 94 ^{0}C for 1 min, annealing at 52 ^{0}C for 1 min, polymerization at 72 ^{0}C for 2 min and incubation at 72 ^{0}C for 5 min. A set of 10-25 mer nucleotides RAPD primers was used for PCR amplification.

g. Electrophoresis: Horizontal submerged gel electrophoresis unit was used for fractionating RAPD markers on Agarose gel (1.8 %), prepared by dissolving appropriate amount of Agarose in 1x TAE/TBE buffer as per Sambrook *et al.*, 1981 and adding ethidium bromide stain (1.5 µg/ml). For each well, DNA sample and DNA loading dye (6x) were mixed in ratio of 5:1, v/v and loaded with a micropipette. Electrophoresis was done at 80 V for 4 h in 1xTAE electrophoresis buffer. The gel image was viewed and stored in gel documentation (Syngen) system.

h. Data Analysis: Data analysis of RAPD primer based fingerprinting was done using Gel Compar-II, version 3.5 (Applied Maths. U.S.A. The dendrogram based on all 06 RAPD markers was generated using Jaccard coefficient of similarity and UPGMA cluster analysis.

Table 1: RAPD Primers used in study (with sequence)

S.N	Primer code	Primer sequence (5'-3')
1	OPA01	CAGGCCCTTC
2	OPA02	TGCCGAGCTG
3	OPA03	AGTCAGCCAC
4	OPA05	AGGGGTCTTG
5	OPA07	GAAACGGGTG
6	OPA12	TCGGCGATAG

Results and Discussion

Biochemical estimation

The biochemical investigations revealed that the significant variation among different treatmnts for various biochemical constituents quantified as represented in (Table 02).

a. Protein (%)

Maximum protein % was registered in Treatments Polymer seed coating (Disco AG SP Red L-200) + Thiram + Genius coat (T2) (40.64), it showed significant variation among them. Lowest was noted inT0 (38.26).

Table 2: Variation in biochemical constituents in seeds of different soybean Treatments

Treatments	Protein %	Fat %	Fibre %	Moisture %	Ash %
No treatment or (Water + Thiram) (T0)	38.26	18.06	3.52	9.52	5.28
Polymer seed coating (Disco AG SP Red L-200) + Thiram + Carboxine (T1)	39.3	18.96	4.1	11.3	3.936
Polymer seed coating (Disco AG SP Red L-200) + Thiram + Genius coat (T2)	40.64	19.08	4.2	12.52	3.844
Polymer seed coating (Disco AG SP Red L-200) + Thiram + Mycorhiza (T3)	40.44	20.04	4.1	10.6	4.176
MEAN	39.66	19.035	3.98	10.985	4.309
SEM±	0.377	0.225	0.074	0.076	0.248
C.D 5%	1.175	0.702	0.23	0.236	0.773

b. Crude fibre (%)

The treatment Polymer seed coating (Disco AG SP Red L-200) + Thiram + Genius coat (T2) (4.2) showed the significantly higher % fiber over the remaining treatments. The No treatment or (Water + Thiram) T0 (3.52) showed the lowest value.

c. Fat (%)

Treatments T3 (20.04) possessed the maximum fat % and showed differed significantly among them. Significant minimum value was found in to (18.06).

d. Ash (%)

The No treatment or (Water + Thiram) T0 (5.28) significantly superseded remaining treatment. The minimum was registered in Polymer seed coating (Disco AG SP Red L-200) + Thiram + Genius coat (T2) (3.84).

The protein accumulation was significantly affected by cultivars and agroclimatic conditions (Galova *et al*., 1999) [4]. Salmani *et al*. (2012) [4] reported in ten vegetable soybean genotypes along with the control that the protein content was ranged from 12.32-14.96%. Salmani *et al*. (2012) [7] reported in ten vegetable soybean genotypes along with the control that the fiber content was ranged from 1.89-2.69%. Popinigis and Andrews (1972) observed that percentage of completely broken seeds and seeds with cracked seed coats increased with decreased levels of seed moisture content in soybean. Virendra and Shrivastava (2002) [8] observed that the genotypes exhibited significant variation in biochemical constituents such as moisture, protein, ether extractives, ash and carbohydrates (7.59-8.23%, 19.19-22.86%, 4.85-6.37%, 2.22-2.77% and 69.40-73.34%, respectively)

Molecular (DNA) Profiling

DNA fingerprinting was carried out using randomly selected 10 Operon Technologies decamer RAPD primers. Out of 10 RAPD primers after screening based on DNA amplifications through PCR, we found that only 06 primers amplified from all the treated population in a reproducible manner. Variation was present in DNA profile of all treated population both in no of bands and molecular size of bands (amplified product). The DNA amplification pattern revealed that a total number of 42 RAPD loci were amplified with an average of 7.0 loci per RAPD primer comprising 30 polymorphic loci (71.43%) and 12 monomorphic loci (28.57 %) (Table 03) The range of polymorphism was 50 % (OPA-01&OPA 02) to 89.0 % (OPA 07). Amplified product (band) size ranged from 300 bp (OPA-03) to 2800 bp (OPA-05). The no of bands ranged from 04 (OPA-02) to 09 (OPA -07) (Fig.no 01)

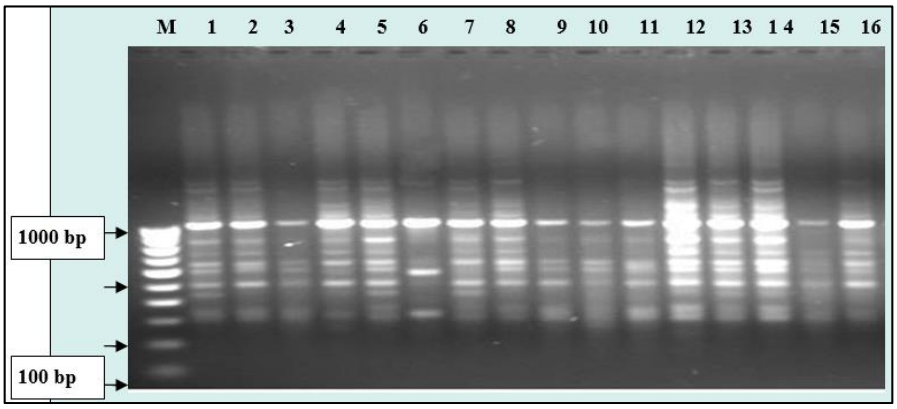

Fig 1: PCR Amplification with RAPD primer OPA01

Table 3: Number of RAPD loci and percentage of polymorphism in treated population genomic DNA.

S.N	Primer Code	Range of loci scored (bp)	Total loci	Mono morphic loci	Polymorphism	
					No. of loci	%
1	OPA01	350-2000	06	03	03	50.0
2	OPA02	450-1000	04	02	02	50.0
3	OPA03	300-2500	06	02	04	67.0
4	OPA05	320-2800	08	02	06	75.0
5	OPA07	500-2400	09	01	08	89.0
6	OPA12	300-2150	09	02	07	78.0

RAPD is a valuable tool for assessing genetic diversity levels in soybean (Khare *et al* 2013) [2]. It detects polymorphism at the DNA level and thus is efficient in discrimination between varieties (Chowdhury *et al*., 2001) [1]. In present study in field results treatment no 16 showed best result but we could not find any specific band directly correlated with this best performance. Although RAPD markers proved in the current study to be useful for DNA finger printing to evaluated variation in soybean, use of other molecular marker techniques such as AFLP, ISSR and SSRs may be preferred for finer results.

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