The Pharma Innovation Journal 2022; SP-11(3): 617-620

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; SP-11(3): 617-620

www.thepharmajournal.com
Received: 23-01-2022
Accepted: 25-02-2022

Thota Joseph Raju
University of Agricultural Sciences, Raichur, Karnataka, India

SN Vasudevan
University of Agricultural Sciences, Bengaluru, Karnataka, India

Basavae Gowda
University of Agricultural Sciences, Bengaluru, Karnataka, India

SR Doddagoudar
University of Agricultural Sciences, Raichur, Karnataka, India

TC Suma
University of Agricultural Sciences, Raichur, Karnataka, India

B Kisan
University of Agricultural Sciences, Raichur, Karnataka, India

RV Beladhadi
University of Agricultural Sciences, Raichur, Karnataka, India

Corresponding Author
Thota Joseph Raju
University of Agricultural Sciences, Raichur, Karnataka, India

SSR markers linked to hard seediness and electrolyte leakage in soybeans

Thota Joseph Raju, SN Vasudevan, Basavae Gowda, SR Doddagoudar, TC Suma, B Kisan and RV Beladhadi

Abstract
The objective of this research was to identify SSR markers associated with hard seediness (seed coat permeability) and electrolyte leaching in soybean genotypes. The polymorphism survey was carried out using five SSR markers, of which three were polymorphic. These markers were used for genotypic analysis of 20 soybean genotypes. To assess the hard seediness (seed coat permeability) and electrolyte leaching, seeds of 20 genotypes are selected phenotypically from 96 genotypes which were subjected to seed coat characterstics screening in laboratory. A total of three (Satt 371, Satt 281and Satt 434) independent SSR markers were significantly associated with hard seediness (seed coat permeability) and electrolyte leaching. Markers Satt 371, Satt 281and Satt 434 produced specific allelic bands with respect to seed coat permeability, testa colour and electrolyte leaching. Satt 371, Satt 281and Satt 434 produced allelic bands of 180 bp, 220 bp, 235 bp respectively for minimum seed coat permeability and electrolyte leaching, specific for black and brown colour testa soybean seeds. Satt 371, Satt 281and Satt 513 produced allelic bands of 120 bp, 200 bp, 183 bp respectively for maximum seed coat permeability and electrolyte leaching, specific for yellow colour testa soybean seeds.

Keywords: SSR markers, hard seediness, electrolyte leaching, soybeans

Introduction
Soybean (*Glycine max* (L.) Merrill) is an important oil seed crop in the world contributing 25 per cent to the global vegetable oil production and also serving as the major source of protein (40-42%) and oil (20-22%) for both human and animal consumption (Archana *et al.*, 2007) [1]. Globally, it is grown in an area of about 78.6 M ha with a production of 181 MT and productivity of 2297 kg per ha. Though it is comparatively a new crop to India, it occupies an area of 10.91 M ha with a production of 10.37 MT and productivity of 951 kg per ha. In Karnataka, it is cultivated in an area of 0.28 M ha with a production of 0.192 MT and the productivity is 758 kg per ha (Indianstat, 2020). In Karnataka the major soybean growing districts are Dharwad, Belgaum, Bidar, Bagalkot and Haveri.
The seed coat characteristics such as seed size, colour, hilum colour, hard seededness, seed coat thickness, permeability, seed coat percentage, oil content *etc* are reported to be associated with seed quality in soybean and are found to be genetically controlled. The larger the seeds produced larger the embryos, exhibited higher respiratory rates and possessed greater field emergence than small seeds (Burris, 1973) [3]. The knowledge of genetic control of seed longevity will help the breeders to develop varieties with good seed longevity. Among various molecular markers *viz.*, RAPD, SSR, AFLP *etc.* SSR marker which is highly polymorphic, efficient, economic and user friendly are the most appropriate one for this purpose. Recent advances in molecular marker (e.g. SSR) and high resolution genetic linkage maps provide powerful tool for genetic dissection and characterization of many quantitatively inherited seed quality traits including seed longevity in soybean. However, information pertaining to morphological and seed quality traits associated with seed longevity is highly limited in soybean genotypes therefore, selection of parents which possess afore said seed traits in breeding programme *vis-a-vis* application of molecular tools for development of varieties with good seed longevity assumes paramount importance.

Materials and Methods
SSR Markers associated with seed coat
Out of the 96 genotypes screened for seed coat characteristics phenotypically, 10 genotypes each with highest and lowest values were selected for genotypic evaluation (Table 1).

The methods used for genotyping are briefly described here under.

Table 1: List of selected genotypes for molecular analysis

Lane No	Genotypes	Lane No	Genotypes	Lane No	Genotypes	Lane No	Genotypes
1	Harasoya	6	Bragg	11	IVT 11	16	BNS-5
2	104-31	7	Pune 14	12	PK 1029	17	SL 979
3	Kalitur	8	Pune 30	13	AGS 48	18	MAUS 61-2
4	Birsa soya	9	JS 7105	14	NRC-2	19	JS 335
5	JS 93 05	10	ASRB-1	15	NRC 55	20	IVT 38

DNA extraction and purification: Genomic DNA from leaves was extracted following the method of Doyle and Doyla (1990) [5]. Ten days old leaves were selected and homogenized to fine powder in liquid nitrogen and transferred to 2 ml Eppendorf tube to this 100 µl of extraction buffer (200 mM Tris-HCL, pH8.0: 200 mM NaCl: 25 mM EDTA, and 0.5% SDS) was added. Later 400 µl CTAB solution (2% CTAB): 100 mM TRIS-HCL, pH 8.0: 20 Mm EDTA, pH 8.0: 104 M NaCl and 1% PVP were added to the tubes and were incubated at 65 ^{0}C for 1 h with intermittent shaking and swirling at 25 min intervals and cooled to room temperature, equal volumes of phenol: chloroform: isoamyl alcohol (25:24:1) were added and the mixture was centrifuged at 12,000 rpm in a micro-centrifuge at 4 ºC overnight for 30 min. The supernatant was transferred to new tubes. To this 2/3 volumes of chilled isopropanol was added and the tubes were incubated at 4 ºC overnight to precipitate the DNA. The contents were once again centrifuged at 12,000 rpm for five min and the supernatant was removed . The pellets with 70% ethanol, air dried and resuspended in 100 µl TE_{10} buffer. The extracted DNA was quantified by using Bio-photometer (Eppendroff) at A_{260} nm and A_{280} nm, The purity of DNA was determined by calculating in ng / µl using the in -built software programme of Bio-photometer.

Simple sequence repeats (SSR) analysis: Five SSR markers were used to screen seed samples of soybean and are presented in Table 2. The markers were selected from the linkage map of soybean chromosomes (Cregan *et al*., 1999) [4]. The sequence of forward and reverse primers were obtained from the Soya Base database (http://soybase.agron.iastate.edu). The PCR reaction was performed in a 0.2 ml reaction tube with total volume of 25 µl containing 25 ng template DNA assay buffer (Bangalore Genei) and 01 Unit *Taq* polymerase (Bangalore Genei). PCR amplifications were performed with initial denaturation at 94 ^{0}C for five min followed by 35 cycles of denaturation at 94 ^{0}C for one min, annealing for 30 sec at 55 ^{0}C, with extension for 30 sec at 72 ^{0}C with a final extension for seven min. The final product was electrophoresed on 2.5% agarose gel in 1X TBE buffer (100 mM, Tris, 100 mM Boric acid, 2 mM EDTA, pH 8.3) at 120 V for three h. The gel was stained with ethidium bromide and visualized under UV light. The informativeness of the SSR markers was measured based on Polymorpic Information Content (PIC) for each SSR marker, which was calculated according to the given formula (Botstein *et al*., 1980) [2].

Amplification of genomic DNA using Polymerase Chain Reaction (PCR): PCR reactions were performed in 10 µl volume [1 µl of 50 ng template DNA, 0.25 µl of 10 pmol primers (each forward and reverse), 1 µl of 10X PCR buffer containing 1.5 mM $MgCl_2$, 0.1 µl of 1.0 U of *Taq* DNA polymerase, 0.4 µl of 2.5 mM dNTPs and distilled water] with the PCR programme described below: initial denaturation at 95 ºC for 5 minutes, first cycle with denaturation at 94 ºC for 30 seconds, annealing at 55 ºC for 30 seconds, extension at 72 ºC for 1 minute followed by 29 cycles and the final extension of 72 ºC for 7 min. The PCR products were checked for amplification in agarose gel electrophoresis (3-4%). The SSR allelic data subjected to DARwin 6.0.14 software and unweighted neighbour joining method used for tree / dendrogram construction (Perrier and Jacquemoud-Collet, 2006) [9].

Table 2: Details of SSR markers used in the present study

S. No		Forward Sequences		Reverse Sequences	
1	Satt281	AAGCTCCACATGCAGTTCAAAAC	23	TGCATGGCACGAGAAAGAAGTA	22
2	Satt434	GCGTTCCGATATACTATATAATCCTAAT	28	GCGGGGTTAGTCTTTTTATTTAACTTAA	28
3	Satt371	TGCAAACTAACTGGATTCACTCA	23	GAGATCCCGAAATTTTAGTGTAACA	25
4	Satt598	CGATTTGAATATACTTACCGTCTATA	26	CACAATACCTGTGGCTGTTATACTAT	26
5	Satt618	GCGGTGATATTACCCCAAAAAAATGAA	27	GCGCTAGTTTCTAGTGGAAAGATGAGT	27

Results and Discussions

Out of 96 genotypes, 20 genotypes were selected based on highest and lowest values for seed coat characteristics like per cent proportion of seed coat, seed coat permeability, testa colour and electrical conductivity in each 10 genotypes. These were tested for DNA profiling to know the extent of genetic relatedness for hard seediness (seed coat permeability) and electrolyte leaching using SSR markers. Set of 5 SSR primers were used. Among them, 3 primers (Satt 281, Satt 434, Satt 371) generated polymorphic banding pattern and clearly produced a specific band for hard seediness (seed coat permeability) and electrolyte leaching. A set of three polymorphic SSR primers amplified 5 alleles from 20 soybean genotypes. The number of alleles per SSR locus varied from 1 to 2 with an average of 1.6 alleles, among them 2 primers were found bi-allelic and 3 primers were tri-allelic. A few SSR markers clearly discriminated the two groups of genotypes. Markers Satt 371, Satt 281and Satt 434 produced specific allelic bands with respect to seed coat permeability, testa colour and electrolyte leaching.

Satt281 produced two alleles *viz.*, Satt 281_{220} and Satt 281_{200}. Satt 281_{220} was present only in the less electrolyte leaching while, Satt 281_{200} was present in more electrolyte leaching genotypes. Thus, the allele Satt 281_{220} can be said to have close linkage with electrolyte leaching as well as black seed coat colour. On the other hand, marker allele Satt 371_{180} and

Satt 434_{235} were present for slow rate of seed coat permeability and black colour testa and Satt 371_{120} and Satt 434_{195} making it to be candidate marker for linkage with high rate of seed coat permeability and black colour testa trait. Therefore, these markers may be good candidate for identifying marker linked with seed coat permeability and black seed coat colour, indicating their possible association with the hard seedness and may be used for developing field weathering resistance trait (Fig 1a to 1c).

Markers Satt 371, Satt 281and Satt 434 produced specific allelic bands with respect to seed coat permeability, electrolyte leaching and testa colour. This is in agreement with the earlier reports stating that black (KALITUR, BIRSA SOYA 1, JS 93 05, PUNE 14, PUNE 30) and few yellow seeded (ASRB 1, PK 1029, BNS 5, MAUS 61 -2, JS 335) landraces of tropical and subtropical regions had better seed hardness than yellow seeded (NRC 55, NRC-2, AGS 48, JS 7105, BRAGG) temperate varieties (Kueneman and Wein, 1981 and Singh R. K. and Ram, 1986, Kuchlan, 2006 and Singh *et al.*, 2008) [8, 11, 7, 10]. Cluster analysis was made based on an allelic data, subjected to DARWIN (6.0) to estimate similarity / dissimilarity and to construct dendrogram, in order to depict the genetic relationship among the genotypes of soybean (Table 3). The genotypes were divided into two major clusters (Fig 2).

Table 3: Distribution of soybean genotypes based on molecular markers into different clusters

Sl. No.	Main cluster	Sub cluster	Number of genotypes	Name of the genotypes
1.	I	I	5	NRC 55, NRC-2, AGS 48, JS 7105, BRAGG
		II	2	IVT-38, HARASOYA
2.	II	I	10	KALITUR, BIRSA SOYA 1, JS 93 05, PUNE 14, PUNE 30, ASRB 1, PK 1029, BNS 5, MAUS 61 -2, JS 335
		II	2	SL 979, IVT11
		III	1	104-31

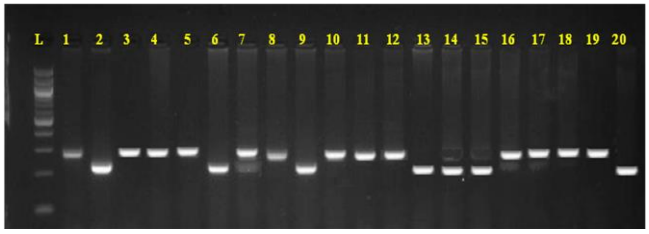

Fig 1a: Amplification pattern of the soybean genotypes obtained by using the SSR marker Satt 281, Lane 1, 3-5, 7,8, 10-12 and 16-19 genotypes are having slow rate of seed coat permeability as well as electrolyte leaching. Lane 2.6.9,13,14,15 and 20, genotypes are having high rate of seed coat permeability as well as electrolyte leaching, L-Ladder

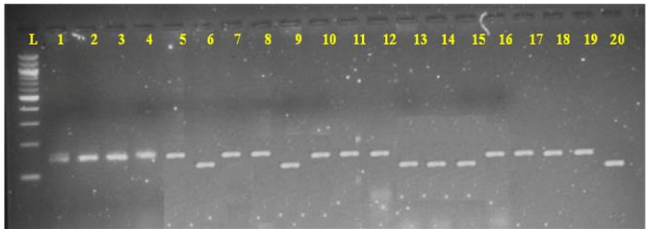

Fig 2b: Amplification pattern of the soybean genotypes obtained by using the SSR marker Satt 371 Lane 1-5, 7,8, 10-12 and 16-19 genotypes are having slow rate of seed coat permeability. Lane 6.9,13-15 and 20 genotypes are having high rate of seed coat permeability, L-Ladder

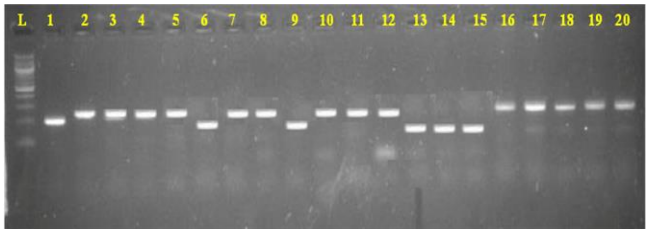

Plate 3c: Amplification pattern of the soybean genotypes obtained by using the SSR marker Satt4 34, Lane 2-5, 7,8, 10-12 and 16-20 genotypes are having slow rate of electrolyte leaching, Lane 6.9,13-15 and 20 genotypes are having high rate of electrolyte leaching, L-Ladder

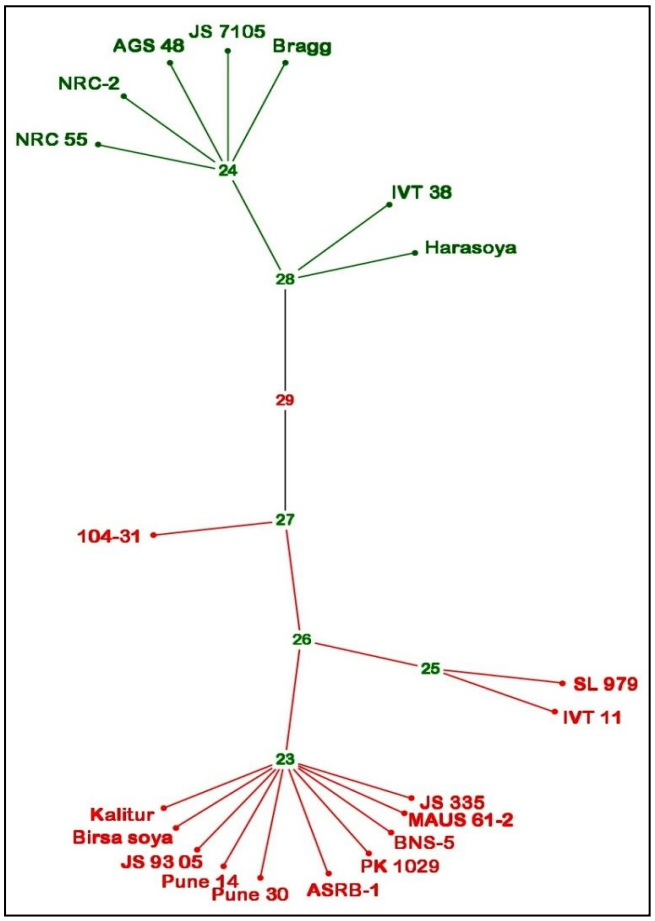

Fig 2: Dendrogram showing relationship among 20 genotypes of soybean based on seed coat characterstics according to unweighted neighbour joining method

Conclusion: This is the report on the identification of SSR markers associated to hard seediness and electrolyte leaching in soybean. This study demonstrates the utility of species-specific molecular markers to study quantitative traits related to hard seediness and electrolyte leaching in this important oil seed crop. In the present investigation, a limited number of polymorphic markers used, which did not allow complete

expression of the trait. As a result, more polymorphic markers with a wide distribution among linkage groups are needed to find new QTLs, particularly those with a large influence on trait expression, and to use them effectively in marker-assisted selection.

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