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Research Article

Genome-Wide Microsatellite Survey and Introgression of Null KTI Allele in Charcoal Rot Resistant Soybean

Pravin Vishwanathrao Jadhav[1]*, Akash Namdeo Shinde[1], Pranita Prabhakar Thakur[1], Umesh Dnyaneshwar Shinde[1], Arpita Mahobia[1], Snehal Chavan[1], Swarup Nagrale[1], Satish Subhashrao Nichal[2], Mangesh Moharil[1], Shyamsunder Mane[1], Raviprakash Govindrao Dani[3] and Torop Elena Alexandrovna[4]

[1]Department of Agricultural Botany, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, India

[2]Regional Research Centre, Amravati, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, India

[3]Gen Scan Inc Global Consultancy, Houston, Texas, USA and Namangan Engineering and Technology Institute, Uzbekistan

[4]Voronezh State Agrarian University, Voronezh, Russia

***Corresponding author**

Pravin V Jadhav, Department of Agricultural Botany, Biotechnology Centre, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola Maharashtra-44410, India, Tel: 8999434438

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- *M. phaseolina*
- Polymorphic information content

Abstract

Soybean though being a vegetable crop have not been used for table purpose due to various quality constraints. Kunitz trypsin inhibitor is a major ANF in soybean which interfere protein digestion and absorption. The objective of the experiment is formulated to address these issues with soybean that are preventing high-quality soybean production is necessary. For parental polymorphism, 388 SSR markers across all 20 linkage groups were examined in four parental combinations comprising four soybean genotypes: AMS MB-5-18, AMS MB-5-19, NRC 101, and NRC 127. Seventy-eight molecular markers showed polymorphism with 37 of them being highly polymorphic. Allelic frequency of 3.84, 4.3, 4.7 and 3.95 per marker was observed respectively for parental combinations 1,2,3 and 4 PIC value ranging from 0.11 to 0.75, 0.15 to 0.75, 0.25 to 0.75 and 0.13 to 0.75 respectively. Using a marker specific to the Null KTI gene, foreground selection was applied to the F1 progenies acquired from the experiment. Future soybean breeding programs will benefit from the obtained polymorphic markers.

INTRODUCTION

With high protein content, soybean is also referred to as functional food and is currently being highlighted for its potential to end malnutrition. According to the USDA, the worldwide soy food business was worth $44.7 billion in 2021. The soy food business is primarily driven by consumers' changing preferences for protein-rich diets over high-calorie diets. The soy food items including textured vegetable proteins, tofu, and soy milk, have been in high demand on the international market.

However, its popularity has been limited because of anti-nutritional components like Kunitz trypsin inhibitor (KTI), beany flavor, and the presence of allergens. The anti-nutritional substances in soybean seed, especially from the two families of Kunitz trypsin inhibitors (KTI) and Bowman-Birk inhibitors, prevent the activity of digesting proteases (BBI). High quantities of raw soybean meal in feed mixes can result in poor body weight and pancreatic anomalies by inactivating trypsin/chymotrypsin enzymes. Heat-treating soybean protein meal in order to disable inhibitors is common practice that can deplete some essential amino acids, is expensive and energy-intensive [1] and 80% of the overall trypsin inhibitor activity is accounted for by KTI [2]. Despite the fact that KTI is heat labile and heat treatment costs money and reduces protein solubility by about 20%. Similarly, beans must be boiled before being ground with wheat (1:9) to make chapatti flour with soy supplements [3].

Genotypes with a null allele of KTI have been identified in soybean germplasm. KTI is a monomeric protein containing 181

amino acid residues [4]. Ten independent differentially expressed KTI genes have been identified with KTI3 being seed specific [5]. Thirteen KTI3 protein isozymes have also been identified which are encoded by multiple KTI alleles [6]. A single dominant gene (Ti) controls the presence of KTI, whereas a recessive gene (titi) controls its absence which encodes for a truncated protein. One substitution and two deletions are present in recessive null kti allele which alters the translation process resulting in lower levels of KTI in seed embryos [7]. Genetic research indicates that numerous alleles, notably Ti a, Ti b, Ti c, and Ti d, are combined at a single locus controlling the KTI trait in soybean. These four soybean KTI electrophoretic forms are controlled by a variety of allelic series of co-dominance (Ti a, Ti b, Ti c and Ti d). A final version 'ti' that lacks Kunitz trypsin inhibitor activity is controlled by a recessive gene [8]. The gene has been located in linkage group (LG) A2 on the genetic linkage map of soybean [9]. Three SSR markers namely Satt409, Satt228 and Satt429 are closely linked with the ti locus at distances of 4.5, 0-3.7, and 5.1 cM, respectively [10].

Abiotic and biotic stresses pose significant challenges to soybean production. Charcoal rot, a broad-spectrum biotic stress caused by the soil-borne polyphagous fungus *Macrophomina phaseolina* that reduces seed quality and yield, is the second most important yield-reducing disease in soybean-growing countries worldwide after brown spot (*Septoria glycines*). This major disease is responsible for 80% of soybean yield loss. The fungus infects the roots and lower stems of soybeans, causing general root rot. Charcoal rot disease appearance looked like roots dipped in charcoal and hence its name. It infects wide range of hosts from sorghum, soybean to cucurbits and various weeds. This was first noticed in the United States in 1949.Every part of the sensitive soybean plant becomes infected as it travels from the roots up through the entire plant. The yield loss in extreme circumstances might be as high as 80%. It is drawing more attention from the breeders in present time because of changing global climatic conditions. The prevalence of recurrent droughts or drought-like circumstances, in particular, increases the susceptibility of soybean to this disease [11]. This issue must be addressed as soon as possible. Therefore, it is anticipated that the creation of KTI-free charcoal rot-resistant soybean cultivars, which are currently unavailable in India, will increase the use of soybean in food items [12].

Marker assisted selection has been widely used for the development of disease and insect resistance in a variety of crops [13]. Nutritional quality traits such as the absence of protease inhibitor or specific fatty acid profile) which cannot be scored visually at the field level, but can be scored effectively by the MAS (Marker assisted selection) technique over morphological traits. Marker-assisted backcross selection (MABS) can make it possible to successfully transfer the 'ti' allele responsible for NULL KTI character to superior soybean varieties from genotypes of soybean resistant to charcoal rot disease. In the current study, NRC101 and NRC 127, two superior soybean varieties with a null allele of KTI, were crossed with AMS-MB-5-18 and AMS-MB-5-19 to introduce the ti allele via marker-assisted selection. SSR markers were used to determine the purity of the F1 hybrids and the parental polymorphism. To do this, a null allele-specific marker must be utilized in the foreground selection approach to choose the positive plants. The objective of the current investigation was to assess the level of polymorphic SSR loci between the donor and recurrent parents.

MATERIALS AND METHODS

Plant material and fungal isolate

For the investigation's parental polymorphism survey, four genotypes were examined. The two soybean genotypes resistant to charcoal rot, AMS-MB-5-18 and AMS-MB-5-19, obtained from Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola were used as recurrent parents. The other two genotypes, NRC-101 and NRC-127 obtained from Indian Institute for soybean research, Indore provided a null KTI allele and were employed as a donor. The experiment was done in the year 2021-22 and the breeding population was maintained at the field of the Biotechnology Center, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola (Latitude: 20^{0} 42'34.45"N, Longitude : 76 ^{0} 59' 53.16" E) and all molecular work was carried out in the Molecular Breeding laboratory of the Biotechnology Centre, Post Graduate Institute. The experiments were carried out in medium textured loamy soil with a pH of 6.5 to 7.5 and low to medium organic carbon content. For raising the crop in the experiment, the recommended package of practices was followed.

Fungal isolates of *Macrophomina phaseolina* were collected from the charcoal rot infected soybean plants as well as from soil found in the experimental fields of Regional Research Center, Amravati (Latitude: 20.9320° N, Longitude: 77.7523° E) which is known hot spot for charcoal rot disease). The samples were fetched to the laboratory of Department of Plant Pathology, Dr. PDKV, Akola. The isolates brought were cultured and purified and then re-infected to soybean plants to confirm its identity and virulence. Identified pure culture of *M. phaseolina* was maintained in controlled conditions and used for *in-vitro* screening to inoculate the target genotypes.

Performance of parental genotypes in response to Macrophomina phaseolina under in-vitro condition

The lab has already developed methods for screening genotypes against charcoal rot. Out of which pot and *in-vivo* showed similar responses. So, the experimental trial for estimating the performance of parental genotypes in response to *M. phaseolina* was set up using earthen pots. A set of earthen pots was raised to screen the genotypes through artificial inoculation. The experiment was conducted in controlled conditions at the Department of plant pathology, Dr PDKV, Akola. The detailed procedure followed is given below (Figure 1). The scoring of the plants was done using 0-9 scale described by [14], where 0 and 9 indicated immune and highly susceptible, respectively (Table 1).

Confirmation of presence and absence of KTI peptide in selected genotypes

The parental seeds of soybean were biochemically tested

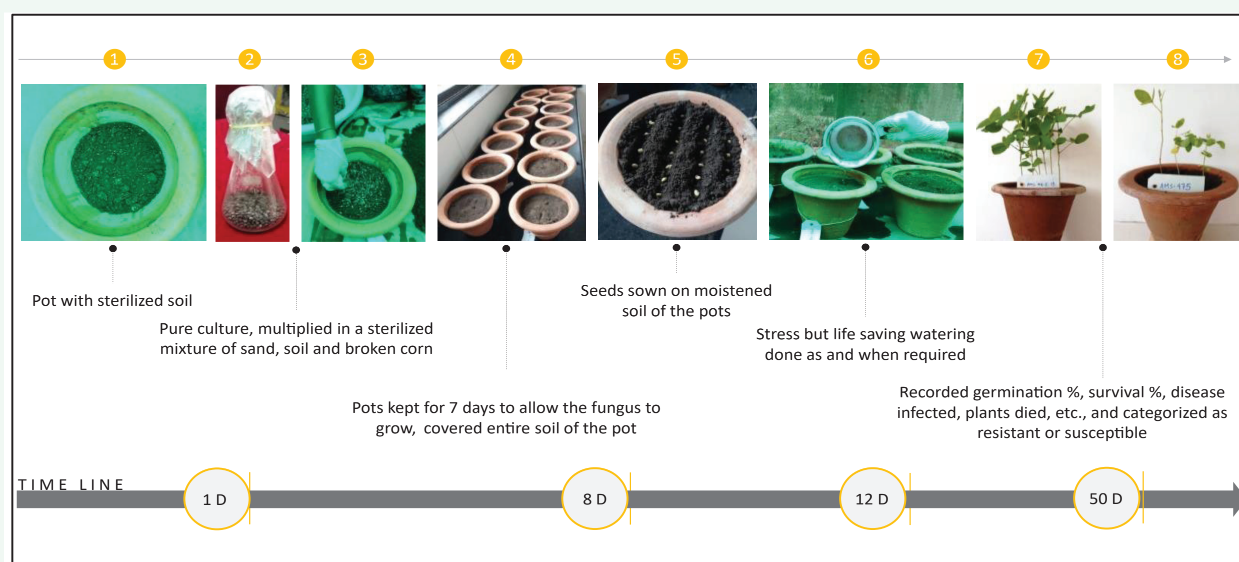

Figure 1 Procedure for screening of genotypes in controlled conditions

Table 1: Scoring of disease under study

Score	Category	Sign
0	Immune	I
1	Resistant	R
5	Moderately resistant	MR
7	Moderately susceptible	MS
9	Highly susceptible	HS

(Source: [15])

through native PAGE for confirming absence of KTI polypeptides. For this purpose, finely ground seed flour (100 mg) was incubated in 1 ml Tris–HCl buffer (pH 8.0) for 30 min. in 70^{0}C and then centrifuged. After this step, prepare 5 % stacking gel and pour and place comb immediately. After solidifying of gel, load the sample prepared having equal volumes of supernatant was added and 5× sample buffer and run at 35 mA for 2 h 30 min. Gels were stained overnight in 0.25 % aqueous solution of coomassie brilliant blue (R-250) in methanol, water and glacial acetic acid (45:45:10) followed by de-staining in methanol, water and glacial acetic acid (45:45:10) solution. Standard trypsin inhibitor protein (21.0 kDa) Thermo Fischer make was loaded in a separate lane for identification of KTI polypeptide.

DNA Extraction from leaf tissue

The chosen genotypes' young leaves were harvested for the extraction of genomic DNA using the CTAB (Cetyltrimethyl ammonium bromide) technique [15]. The DNA extraction solution (2% CTAB, 100 mM Tris-HCl, 50 mM ethylene diamine tetra acetate (EDTA), and 100 mM NaCl) was applied to crush 15 to 20-day-old soybean leaves [16]. A nanodrop spectrophotometer (IMPLEN make Munchen, Germany) was used to measure optical density for the quantity and purity of the DNA at 260 and 280 nm and agarose gel electrophoresis was also used to assess the quality of DNA. The dilution of genomic DNA was prepared at 40ng/µL for further experiment.

Source of genomic microsatellite sequences

The primer sequence and SSR sites were retrieved from the soybean database [17]. A total of 388 SSR markers from 20 linkage groups (LG) were used, with an average density of one SSR marker per 5 cM, to assess the level of polymorphism between different parental combinations for background selection (Figure 2). Null allele specific markers were used for screening of positive F1 plants.

SSR Profiling of donor and recurrent parents

The polymerase chain reaction was performed using 325 primers in an Eppendorf thermal cycler. In a 10 µL reaction mixture with 2.0 µL DNA (20 ng/l), 1.0 µL 10X buffer, 1.1 µL$MgCl_2$, 0.1 µL dNTPs (10 mM), 0.4 µL each of forward and reverse SSR primers, 0.068 µL *Taq* DNA polymerase (5U/ µL), and 4.932 µL nuclease-free water was subjected to PCR amplification. The template DNAs were amplified in a thermo cycler (Applied Bioscience by Thermo Fischer, Waltham, Massachusetts, United states) using the PCR profile: Initial denaturation at 94°C for 2 minutes. Followed by 32 cycles of denaturation at 94°C for 1 min, primer annealing at 49 to 52 °C for 2 minutes, and primer elongation at 72 °C for 3 minutes each are then performed.

The amplicons' final elongation was allowed to run its course at 72 °C for 10 min before being put on hold at 4 °C. After completion of amplification, PCR products were stored at –20°C and the amplified products were analyzed by electrophoresis using 8 % Polyacrylamide gel and visualized by silver stain. The silver nitrate staining solution was prepared at 0.18%

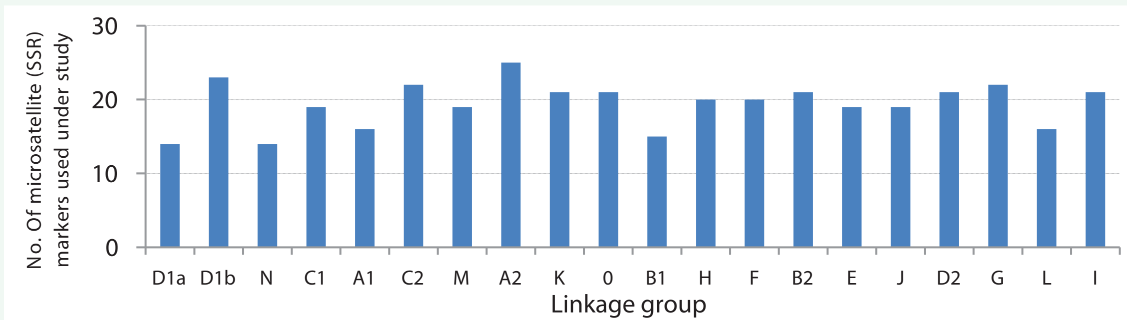

Figure 2 Histogram showing SSR markers throughout the linkage groups of soybean used under study

concentration and the gel was exposed for 10 minutes with continuous shaking and then washed foe 30s in distilled water. Then PAGE gel was treated with a developer solution consisting of 3 % sodium hydroxide with 0.3 % formaldehyde until the bands appeared clearly. Gel documentation unit (Biorad make, Hercules, California, United States) available at Biotechnology Centre, Dr. PDKV., Akola was used for the visualization of resolved PCR products.

Analysis of SSR polymorphism and Detection of informative markers

To assess four genotypes, genotyping by SSR marker was scored based on the presence or absence of the bands was used. The data was then organized as discrete variables into a binary matrix, where score '1' denoted the existence of the band and '0' denoted its absence. This data matrix was subsequently subjected to analysis [18]. The polymorphism information content (PIC) for each chromosomal SSR was calculated using the formula to assess the markers' in formativeness [19].

PIC=1-(Pi2)

Where Pi is the frequency of the ith plus allele among the group of 4 parental genotypes under investigation, and I is the total number of alleles discovered for the SSR marker. The SSR marker's discriminating power is estimated by the PIC value [20]. The PIC values are divided into three classes: PIC> 0.7 = highly informative, then 0.25> PIC> 0.7= moderate, and PIC < 0.25 = low. Markers with PIC value above 0.5 are informative in genetic studies and are extremely useful in determining polymorphism rate of specific locus [21]. The evaluation of genetic markers is based on DNA bands of PCR amplification results.

The polymorphism percentage was also calculated between the parents with the formula illustrated below:

$$Polymorphism\ percentage = \frac{Number\ of\ polymorphic\ markers}{Total\ number\ of\ markers\ used} X\,100$$

The polymorphism percentage of markers will be calculated as:

$$Polymorphism\ percentage\ of\ markers = \frac{Number\ of\ polymorphic\ allele}{Total\ number\ of\ alleles} X\,100$$

The higher the polymorphism percentage the more informative and discriminative it will be.

RESULTS AND DISCUSSION

Response of parental genotypes against Macrophomina phaseolina under *in vitro*

The isolates of *Macrophomina phaseolina* were collected from the charcoal rot infected soybean plants as well as from soil found in the experimental fields of RRC, Amravati. The isolates were cultured, purified and re-infected the soybean plants to confirm its identity and virulence. Pure culture of *M. phaseolina* was maintained in controlled condition and used for inoculating the target genotypes.

A set of four genotypes was subjected to screening in pots under artificial inoculated conditions as proposed by [14]. The genotypes were selected based on the experiments conducted earlier. The experiment was conducted as per procedure in the net-house of Department of Plant Pathology, PGI, Dr. PDKV Akola (Table 2).

To permit disease development in the potted plants, limited supply of nutrients and water was done. Based on the disease score, 2 genotypes viz., AMS MB 5-19 and AMS MB 5-18 were identified as resistant ones. Similarly, one genotypes NRC 101 was found to be moderately resistant and NRC 127 was found highly susceptible (Figure 3). Therefore, these genotypes were used for developing the mapping population for studying the genetics of charcoal rot resistance.

Confirmation of presence and absence of KTI peptide in selected genotypes

The parental seeds were checked biochemically through NATIVE PAGE for confirming the absence of KTI polypeptides. The samples were loaded in NATIVE PAGE gel and it has been observed that the bands similar to KTI standard were present in genotypes AMS MB 5-18 and AMS MB 5-19 but NRC 101 and NRC 127 does not have. This indicates the absence of KTI allele in donor genotypes NRC 101 and NRC 127 (Figure 4).

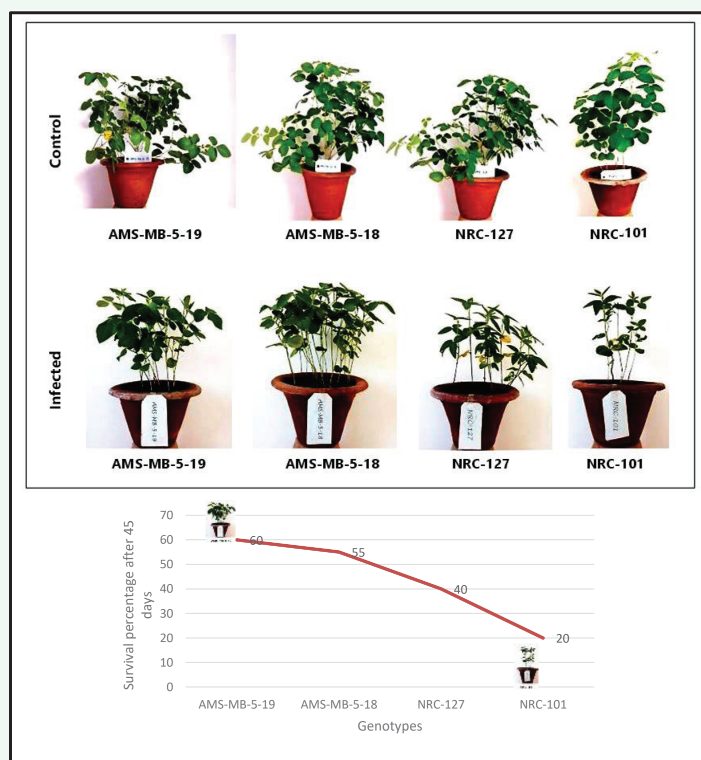

Figure 3 Results obtained by screening of the soybean genotypes through screening in pots along with the graphical representation of the survival % of the plants after 45 days.

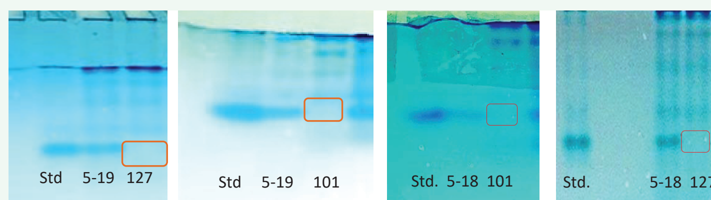

Figure 4 Confirmation for presence of Null KTI peptide

Table 2: Summary of charcoal rot disease reaction under *in-vitro* condition

S.N	Genotype	Response
1	AMS MB 5-19	R
2	AMS MB 5-18	R
3	NRC-101	MR
4	NRC-127	S

R=Check Resistant, S=Check Susceptible

Microsatellite marker-based profiling and polymorphism

78 of the 388 SSR markers that were tested were found to be polymorphic collectively for four parental combinations of selected genotypes under investigation among them 46 markers were highly polymorphic (Table 3,4). Three markers Satt144, Satt543, and Satt 301 present on the F, D2, and L linkage groups respectively, were found highly polymorphic in all the parental combinations with the highest PIC value of > 0.75. The representative gel picture showing parental polymorphism of SSR markers is depicted in Figure 5 and monomorphic markers in Figure 6.

The level of polymorphism (PIC) is required to select markers that can differentiate between the lines/hybrid used. Markers that produce fewer alleles have a smaller ability to distinguish samples tested. Other investigations have likewise shown low polymorphism [22,23]. In order to produce a polymorphism, it is encouraged to use genetically diverse genotypes in hybridization programs.

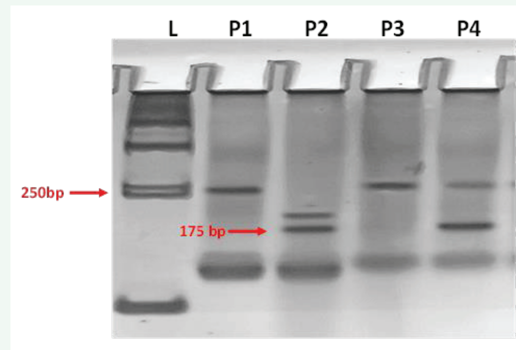

Figure 5 Representative images of polymorphic marker for selected genotypes
L- Ladder 100kb, P1 - AMS MB 5-18, P2 =AMS-MB-5-19, P3=NRC-101 and P4=NRC-127

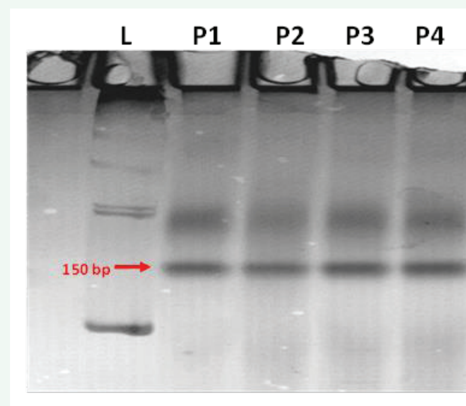

Figure 6 Representative images of monomorphic markers for selected genotypes
L- Ladder 100kb P1 - AMS MB 5-18, P2 =AMS-MB-5-19, P3=NRC-101 and P4=NRC-127

The Polymorphic Information Content (pic) value of 31 polymorphic markers for parental combination 1 (AMS MB 5-18 X NRC 101) ranged from 0.11 to 0.75 with 100 polymorphic alleles and average polymorphic percentage of 63.7% among identified polymorphic markers for parental combination 1. The polymorphism percentage for different polymorphic markers ranged from 25 to 100 % and seven markers showed 100 % polymorphism with 0.75 PIC value for parental combination 1. Similarly, 48 markers for parental combination 2 (AMS MB 5-18 X NRC 127) were found polymorphic with PIC value ranging from 0.15 to 0.75. The polymorphism percentage of markers ranged from 20 to 100 % with 220 polymorphic alleles detected out of total 339. 57 polymorphic markers for parental combination 3 (AMS MB 5-19 X NRC 101) has been observed with PIC value ranging from 0.13 to 0.75 and 91 polymorphic alleles out of total 143 alleles with average polymorphism percentage of 63.6 %. In addition, 67 markers have been found polymorphic for parental combination 4 (AMS MB 5-19 X NRC 127) whose PIC value ranged from 0.25 to 0.75 and polymorphism percentage ranged from 25 to 100% with 226 polymorphic alleles (Table 5).

The graph showing the PIC value of all the parental combinations have been shown in Figure 7. The location of each SSR marker found polymorphic has been depicted in Figure 8. The SSR markers which are more informative with greater than 0.30 pic value will be selected for further screening in the breeding program.

Among highly polymorphic SSR markers, three markers namely, Satt 144, Satt 543 and Satt_301 were found polymorphic in all the four parental combinations (Figure 9) with high PIC value of 0.75 and these markers are located on chromosome 13 (Satt 144) and 17 (Satt 543 and Satt_301).

The average allele frequency for parental combination 1 (AMS MB 5-18 X NRC 101) obtained was 3.84 per marker, for parental combination 2 (AMS MB 5-18 X NRC 127) allelic frequency was 4.3 per marker. Similarly, an allelic frequency of 3.95 per marker was observed for parental combination 3 (AMS MB 5-19 X NRC 101) and parental combination 4 (AMS MB 5-19 X NRC 127) have an allelic frequency of 4.7 per marker.

Table 3: List of 78 markers polymorphic to 4 parental combinations

Sr. No	Linkage Group	Chr. no.	PC1	PC2	PC3	PC4
1	D1a	1	Satt179,Satt507	Satt198, Satt507	Satt179, Satt507	Satt198, Satt507
2	D1b	2	Satt095,Satt558,Satt 189	Sat_227, Satt095, Satt558	Satt095,Satt558, Sat_135, Satt644, Satt 643, Satt 202, Satt558, Satt644, Satt 189	Sat_227, Satt095, Satt 202 Satt558, Satt644
3	N	3	Satt152	Sat_379, Satt152	Satt152	Sat_379, Satt152
4	C1	4	Satt476	Sct_186, Sat_337,Satt661, Satt195, Satt713	Satt476, Satt195, Satt713, Satt682. Satt 524	Sct_186, Sat_337, Satt661, Satt476, Satt195, Satt713, Satt682. Satt 524
5	A1	5	Sat_265	Sat_265, Satt 591, Satt 545, Satt 258	Sat_265, Satt258, Satt211, Satt236	Sat_265, Satt591, Satt545, Satt258, Satt211, Satt236
6	C2	6	Satt277	Satt457, Satt170,Satt277, Satt557	Satt277	Satt457, Satt170, Satt277, Satt557 Satt 640
7	M	7	Satt201	Satt201, Satt636,Sat_244,Sat_003,Sat_147, Sat_276	Satt201, Sat_147, Sat_276, Sat_330	Satt201, Satt636,Sat_244, Sat_003, Sat_147, Sat_276, Sat_330
8	A2	8	Satt378	Sat_406, Satt315, Satt424, Sat_199	Satt378, Sat_232	Sat_406, Satt315, Satt424, Sat_199, Sat_232, Satt378
9	K	9	Satt725	Sat_325, Satt725	Satt725, Satt 087, Satt 242	Sat_325, Satt725, Satt 087, Satt 242
10	O	10	Satt478	Satt 358, Satt 345	Satt478	Satt358,Satt345
11	B1	11	Satt519,Satt444	Sat_272, Satt519	Satt519, Satt444, Satt332	Sat_272, Satt519,Satt332
12	H	12	Satt142, Satt314	Satt142, Satt629	Satt142, Satt314	Satt142, Satt629
13	F	13	Satt335,Satt144	Satt144	Satt335, Satt144	Satt144, Satt395
14	B2	14	Satt126, Satt 070	Satt126	Satt126, Satt 070, Satt 556	Satt126
15	E	15	SSR1766, Satt 691	SSR1766, Satt231	SSR1766, Satt 691, Satt 483, Satt 230	SSR1766, Satt231, Satt 483, Satt 230
16	J	16	Sct_046, Satt414	Sct_046	Sct_046, Satt414, Satt 183	Sct_046
17	D2	17	Satt543	Satt543	Satt543	Satt543
18	G	18	Satt564	Satt564, Satt 038	Satt564, Satt 130	Satt564, Satt 038, Satt 130
19	L	19	Sat_301	Sat_301	Sat_301	Sat_301
20	I	20	Sat_268, Satt 451, Satt 354	Sat_268	Sat_268, Satt 451, Satt 354, Satt 049	Sat_268
Total Polymorphic Marker			**31**	**48**	**57**	**67**

PC1- Parental combination 1 (AMS MB 5-18X NRC 101)
PC2 - Parental combination 2 (AMS MB 5-18X NRC 127)
PC3- Parental combination 3 (AMS MB 5-19X NRC 101)
PC4 - Parental combination 4 (AMS MB 5-19X NRC 127)

Table 4: Number of monomorphic and polymorphic SSR markers under investigation

	Parental Combinations			
	PC 1	PC 2	PC 3	PC 4
Total markers	388	388	388	388
Monomorphic markers	357	340	331	321
Polymorphic markers	31	48	57	67

PC1- Parental combination 1 (AMS MB 5-18X NRC 101)
PC2 - Parental combination 2 (AMS MB 5-18X NRC 127)
PC3- Parental combination 3 (AMS MB 5-19X NRC 101)
PC4 - Parental combination 4 (AMS MB 5-19X NRC 127)

The number of alleles per marker was found to range from 2 to 5, only. It reflected the poor variability among the soybean genotypes used in the study. The kind of motif (di, tri, tetra, etc.) and the number of repeats of the motif i.e. n=5, 10, 15, etc., in the SSRs have a significant impact on the detection of polymorphism. In the present investigation, it was shown that tri-nucleotide repeat SSR motifs were more polymorphic than other SSR motifs. Ten of the 19 highly polymorphic markers had tri-nucleotide repeat motifs, and seven had di-nucleotide repeat patterns. The SSR motif (TA) 13-58, which has 13–58 repeats, seems to be more variable than other di-nucleotide repeats. In the case of tri-nucleotide repeats, the four cross combinations revealed increased variation in motifs with 5-32 repeats (Table 6, Figure 10). The markers which have been found to be having highest PIC value and polymorphism percentage in all the four parental combinations have trinucleotide motif (TAT) and (ATT) and only one marker have dinucleotide repeat of (AT) motif with varying number of repeats. In parental combination 1, 4 out of 7 markers have trinucleotide motif, similarly 7 out of 11 in case of parental combination 2, 4 out of 6 in PC3 and 7 out of 12 in PC4 have trinucleotide motif which detected more polymorphism among the four cross combinations. Similar results have already been reported in soybean [24].

The exploitation of informatics markers for hybrid confirmation

There are two possible approaches for adding extra SSR markers to the genome's sparsely polymorphic regions. The first

Table 5: Features of markers with high pic values of different cross combinations

S.N.	Primers	Parental combination	Motif	Product size	Primer sequence
1	Satt142	1,4	(TTA)20	151	F- GGACAACAACAGCGTTTTTAC R- TTTGCCACAAAGTTAATTAATGTC
2	Satt144	1,2,3,4	(TAA)18	224	F- CGTCGCCATCACTATGAGAA R- CCATCTTGAGCAGAGTTTGAAGTT
3	Sct_046	1,2	(GA)11	157	F-AAAAAGGAAACTTCGTCA R-AAACTAAACAGTGTCCATAAGA
4	Satt543	1,2,3,4	(ATT)19	171	F-GCGGATCTAAGGATAATTCATTAA R-GGGAGCGGATCATTCGGTGAAA
5	Satt564	1,2	(AAT)22	164	F-GCGCTTCCACCACAATAACA R-GCGGCAGAGGACTGACAGCTA
6	Sat_301	1,2,3,4	(AT)21	155	F-CGCACAGGACTTAGTGTTATCATTCAATGT R-CGGGGTTCCCATATTCTTGGTATGAAACTA
7	Sat_268	1,3,4	(AT)26(AC)6	253	F-GCGTGCAACATATGACACCATAAAT R-GCGTGAGGAGGTTCAAAAATAACAT
8	Satt227	2	(GTT)5	144	F-GCTCTGCCAAATAGTGTT R-CACCCTGGCACATAGA
9	Sat_003	2,4	(TA)33	160	F-TGATTTTTGGTGTAGAACTC R-CAAATTGGTTAGCTTACTCCA
10	Sat_325	2,4	(TA)58	292	F-CGATTCTCAATTAAGTCAGTTCAATGATGA R-GCGTTGTCTCCTTATGTTTGTTCTCCC
11	Satt345	2	(ATT)27	248	F-CCCCTATTTCAAGAGAATAAGGAA R-CCATGCTCTACATCTTCATCATC
12	Satt629	2	(TAT)13	215	F-GCGGGACTACACCATTTTTTTTAACAG R-GCGGAGGCCTTGATTATTAGACAATAG
13	SSR1766	2,4	(ATT)17	235	F- TCAGGGGTGGACCTATATAAAATA R-CAGTGGTGGCAGATGTAGAA
14	Sat_337	4	(TA)26	263	F-GCGCATGTTTTACAAATTTTGAAGCCTTAG R-GCGATCAATCCATTTATGAGGTTAGTTTCTT
15	Satt713	4	(TTAT)3	251	F-GCGAAACGTATTAATTATGTGTCTTTCTTTA R- GCGGTTTGCAGTGTGATATTACAATG
16	Satt457	4	(TAA)20	262	F-GTCCGTGTATTTTGTTTTGC R-TTATCCATTTTCCCTTTAGTCC
17	Sat_244	4	(AT)27	222	F- GCGTCAACCGGTGAAAAAACCTA R-GCGTGGCTGGCAGTAGTCTATATCA
18	Sat_147	4	(TA)13	268	F- GTGCGACGTCATGCCTTACTCAAT R- GCGCTCCGTACACTTAAAAAAGAA
19	Satt231	4	(TAT)32	242	F- GCGTGTGCAAAATGTTCATCATCT R- GGCACGAATCAACATCAAAACTTC

Source : Soybase (https://www.soybase.org/)

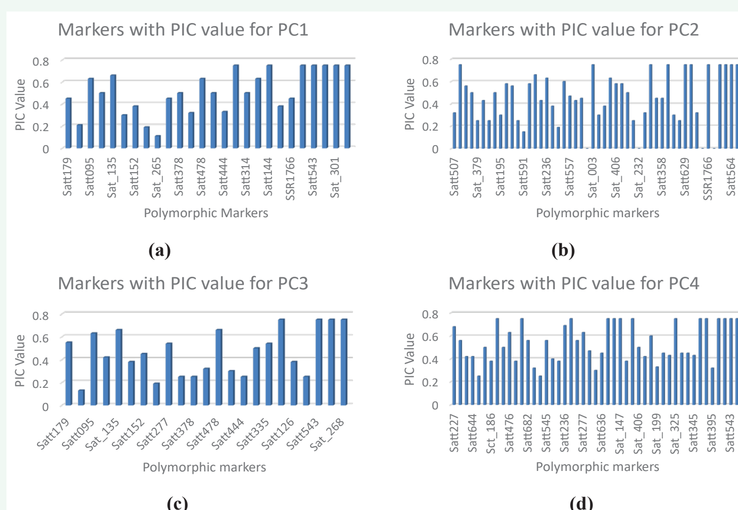

Figure 7 PIC value of different polymorphic markers in parental combinations PC1, PC2, PC3 and PC4

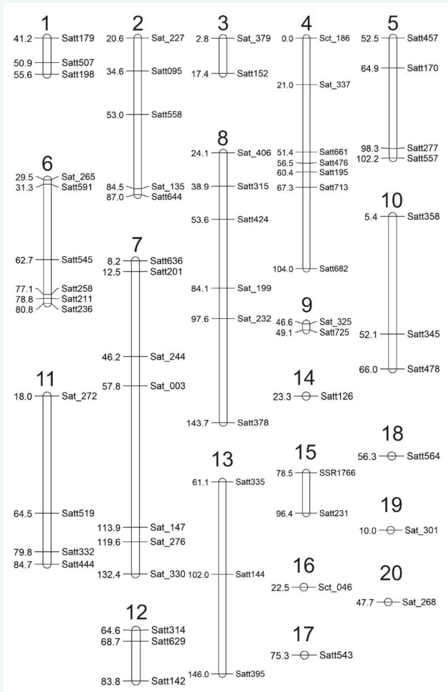

Figure 8 Chromosome wise distribution of polymorphic SSR markers of soybean.

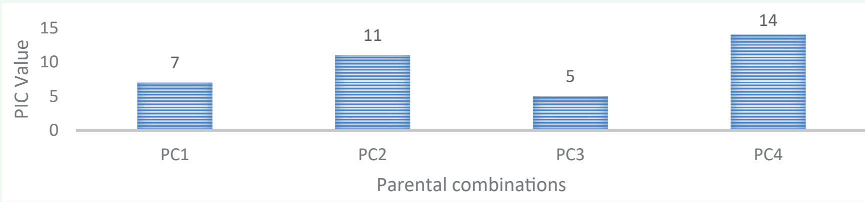

Figure 9 Histogram depicting number of highly polymorphic markers for different parental combinations with highest PIC value

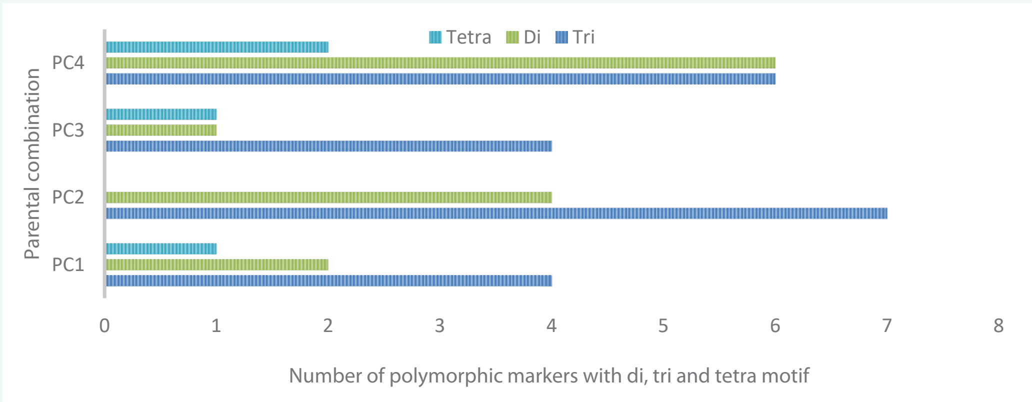

Figure 10 Histogram depicting number of highly polymorphic markers with different number of motifs in different parental combinations

strategy would include choosing SSR markers from previously existing resources, such as a map released in 2008 by the USDA, and testing them for polymorphism. The second strategy would involve creating new SSRs using the clonal sequences of those locations. In this instance, clone identification and the development of new primers would be made easier by the physical map and the soybean genome's whole sequence. There have already been reports of similar outcomes in soybean. Null KTI allele-specific marker was used for foreground selection of hybrids (Figure 9) and the selected progenies will be used for further generation advancement. In the future, this information will be helpful for selecting and/or creating SSR primers.

CONCLUSION

Soybean improvement basically revolves around the development of food grade soybean which involves reduction of potent compounds affecting food quality and yield increase by addressing biotic and abiotic stress. This experiment helps in inferring some set of markers polymorphic to specific four soybean genotypes which can be involved in the development of KTI free soybean with charcoal rot disease resistance. Precision molecular breeding in soybeans would benefit from the use of these techniques.

Significance statement

Soybean being a crop with high nutritional value and potential to eradicate malnutrition, improvement is of great significance. Agronomical traits for soybean improvement have been widely studied in soybean and now research is focused to improve its quality. This research experiment was based on addressing the charcoal rot disease caused by *M. phaseolina* which accounts for 80% yield loss and improving food quality by removing antinutritional factor (KTI) from soybean. The soybean genotypes selected for the research work included two charcoal rot resistant genotypes along with two genotypes with Null KTI allele. The purpose was to introgressed Null KTI allele in the background of charcoal rot resistant genotype. This research article represents first key findings of the experiment in which *in vitro* screening was done for detecting resistant genotypes and polymorphism detection of all the four genotypes with different parental combinations. The results showed 78 markers out of 388 to be highly polymorphic which will be used for screening in further generation advancement.

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REFERENCES

1. Gillman JD, Won-Seok Kim, Krishnan HB. Identification of a New Soybean Kunitz Trypsin Inhibitor Mutation and Its Effect on Bowman–Birk Protease Inhibitor Content in Soybean Seed. J Agric Food Chem. 2015; 63: 1352-1359.
2. Kumar V, Rani A, Mittal P, Mohd Shuaib. Kunitz trypsin inhibitor in soybean: contribution to total trypsin inhibitor activity as a function of genotype and fate during processing. J Food Meas Charact. 2019; 13: 1583-1590.
3. Andrade JC, Mandarino JMG, Kurozawa LE, Ida EI. The effect of thermal treatment of whole soybean flour on the conversion of isoflavones and inactivation of trypsin inhibitors. Food Chem. 2016; 194: 1095-1101.
4. Roychaudhuri R, Sarath G, Zeece M, Markwell J. Reversible denaturation of soybean Kunitz trypsin inhibitor. Arch Biochem Biophys. 2003; 412: 20-26.
5. Moraes R de, Soares T, Colombo L, Salla M, Barros J de A, Piovesan N, et al. Assisted selection by specific DNA markers for genetic elimination of the Kunitz trypsin inhibitor and lectin in soybean seeds. Euphytica. 2006; 149: 221-226.
6. Wang KJ, Xiang HL, Yamashita T, Yoshihito T. Single nucleotide mutation leading to an amino acid substitution in the variant Tik soybean Kunitz trypsin inhibitor (SKTI) identified in Chinese wild soybean (Glycine soja Sieb. & Zucc.). Plant Syst Evol. 2012; 298:1-7.
7. Jofuku DK, Schipper RD, Goldberg RB. 1989. A frameshift mutation prevents kunitz trypsin inhibitor mRNA accumulation in soybean embryos. Plant Cell 1: 427-435.
8. Orf JH, Hymowitz T. Inheritance of the absence of the Kunitz trypsin inhibitor in seed protein of soybeans. Crop Science. 1979; 19: 107-109.
9. Cregan PB, Jarvik T, Bush AL, Shoemaker RC, Lark KG, Kahler AL, et al. An integrated genetic linkage map of the soybean genome. Crop Science. 1999; 39:1464-1490.
10. Kim MS, Park MJ, Jeong WH, Nam KC and Chung J. SSR markers tightly linked to the ti locus in soybean [Glycine max (L.) Merr.]. Euphytica. 2006; 152: 361-366.
11. Kumar BNH, Bhat S, Borphukan B and Fakrudin B. Association analysis of charcoal rot disease component traits in sorghum minicore germplasm with EST-SSR markers. Indian J Genet Plant Breed. 2017; 77: 74-82.
12. Kumar V, Rani A, Rawal R, Mourya V. Marker assisted accelerated introgression of null allele of Kunitz trypsin inhibitor in soybean. Breed Sci. 2015; 65: 447-452.
13. Maroof S, Jeong SC, Gunduz I, Tucker DM, Buss GR, Tolin SA. Pyramiding of soybean mosaic virus resistance genes by marker-assisted selection. Crop Science. 2008; 48: 517-526.
14. Smith GS, Carvil ON. Field screening of commercial and experimental soybean cultivars for their reaction to Macrophomina phaseolina. Plant Dis. 1997; 81: 363-368.
15. Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 1980; 8: 4321-4325.
16. Siva Kumar G, Aruna Kumari K, Durga Rani CV, Sundaram RM, Vanisree S, Jamaloddin Mdet al. Study of simple sequence repeat (SSR) polymorphism for biotic stress resistance in elite rice variety JGL 1798. AJB. 2013; 12: 5833-5838.
17. Anonymous. 2023.
18. Guo X, Elston RC. Linkage information content of polymorphic genetic markers. Hum Hered. 1999; 49: 112-118.
19. Serrote CML, Reiniger LRS, Silva KB, Rabaiolli SMDS, Stefanel CM. Determining the Polymorphism Information Content of a molecular marker. Gene. 2020; 726: 144175.

20. Dalimunthe SR, Siregar LAM, Putri LAP, Chairunnisa T, Hairmansis. Polymorphism levels of some SSR markers (Simple Sequence Repeat) for parental line identification on low temperature tolerance. 2020; 454: 012165.

21. Akkaya M, Buyukunal-Bal E. Assessment of genetic variation of bread wheat varieties using microsatellite markers. Euphytica.2004; 135: 179-185.

22. Singh RK, Raipuria RK, Bhatia VS, Rani A, Pushpendra, Husan SM, et al. SSR markers associated with seed longevity in soybean. Seed Science and Technology. 2008; 36: 162-167.

23. Talukdar Akshay, Verma Khushbu, Gowda D, Lal Sanjay, Sapra Ramesh, Singh KP et al. Molecular breeding for charcoal rot resistance in soybean I. Screening and mapping population development. Indian J Genet Plant Breed. 2009; 69: 367-370.

24. Kumar B, Talukdar A, Verma K. Diversity analysis in soybean genotypes using SSR markers.In: Book of Abstracts, National Seminar on “Contemporary approaches to crop improvement”, 2011, UAS, Bangalore. 126. 2011.