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SM Naik
University of Agriculture Sciences, Dharwad, Karnataka, India

K Madhusudan
National Seed Projesct, University of Agriculture Sciences, GKVK, Bangalor, Karnataka, India

BM Motagi
College of Agriculture, Vijayapura, Karnataka, India

Suma Mugali
University of Agriculture Sciences, Dharwad, Karnataka, India

HL Nadaf
University of Agriculture Sciences, Dharwad, Karnataka, India

Correspondence
SM Naik
University of Agriculture Sciences, Dharwad, Karnataka, India

Molecular characterization of seed longevity and associated characters using SSR markers in soybean [*Glycine max* (L.) Merrill]

SM Naik, K Madhusudan, BM Motagi, Suma Mugali and HL Nadaf

Abstract
The soybean is economically the most important oilseed in the world, unfortunately have poor competence for seed longevity. This study include set of 21 soybean genotypes grouped into good and poor seed longevity were evaluated phenotypically and same were analysed by 15 SSR marker with53.33% polymorphism. The marker Satt 281 gave distinct banding pattern by produced an amplicon size of 184 bp for those having poor seed longevity with higher electrolyte conductivity of the seed leachet and seed coat permeability across the genotypes, on the contrary other amplicon of size 235bp was produced for good seed longevity genotypes with electrolyte conductivity and seed coat permeability. This distinct banding pattern and genetic similarity coefficients obtained through SSR data analysis grouped the genotypes into two major clusters representing the good and poor seed longevity genotypes gave a clear cut differentiation between the two extreme of seed longevity at the molecular level.

Keywords: soybean, seed longevity, SSR, germination, EC

Introduction
Soybean is one of the richest and cheapest source of protein and staple in the diet of people in numerous parts of the world. Soybean, otherwise known as a 'miracle crop' with over 40 per cent protein and 20 per cent oil and for its multiple uses and is one of the world's most important economic oilseed crops and is recognized as the most important grain legume in the world in terms of total production and international trade. Seed is a basic input for agriculture productivity and germplasm conservation. Seed quality predominantly comprises of germination and vigour which are quintessential for successful stand establishment. On the other hand, seed industries are thriving to improve the crop yield through control of seed vigour [1]. The wide occurrence of unfavorable weather during soybean harvest, results in poor quality soybean seed and further speedy deterioration during storage under Indian conditions. Planting of such seed leads to poor emergence, hence, poor crop stand and reduced productivity of soybean crop. High quality seed that provides adequate plant stand is the basis for profitable production and expansion of soybean crop. In order to increase the production of soybean, a source of high quality, disease free seed must be established and maintained. Loss of viability and vigour under high temperature and relative humidity conditions is a common phenomenon in many crop seeds, but it is well marked in soybean [2].

The variation in speed of seed deterioration of soybean varieties is a genetic character. Soybean genotypes differ in their ability to maintain seed longevity [3]. The longevity of seeds in storage is influenced by four major factors *viz.*, Genetic constitution, Quality of the seed at the time of storage, Moisture content of seed or ambient relative humidity, Temperature of storage environment. Research carried out on soybean seed quality during storage and reported that the germination of soybean varieties decreased during storage irrespective of varieties, threshing and processing methods and storage containers. Genetics provides a powerful approach to identify the physiological and molecular basis of phenotypic traits such as seed longevity and other quality factors. Seed longevity is a quantitative trait and is strongly affected by the environment during seed formation, harvest and storage [4]. A number of seed characters such as seed size, percent hard seededness, seed coat thickness and permeability, electrical conductivity of the seed leachet, hull percentage, oil content etc., are associated with seed quality in soybean and were shown to be under genetic control [5, 6].

Molecular gene markers have brought phenomenal changes in the area of plant biotechnology by their ability to produce unique DNA profiles in various crops. Availability of molecular marker technology has made possible the genetic dissection and characterization of many quantitatively inherited seed quality traits in soybean.

Restriction fragment length polymorphisms (RFLPs) were utilized to identify several major quantitative trait loci (QTLs) in soybean influencing hard seededness in an interspecific soybean population [7]. Molecular markers were utilized to identify several genomic regions significantly associated with seed protein, oil, seed weight and sucrose content in different intraspecific soybean population [8, 9] and molecular studies support the complex genetic nature of seed longevity.

Microsatellites or Simple Sequence Repeat (SSR) markers are highly polymorphic, abundant and distributed throughout the genome [10]. In one of the study SSR markers are reported to be associated with seed coat permeability and electrolyte leaching and seed longevity in an $F_{2:3}$ soybean population in a cross involving good and poor storer genotypes [11, 12]. Marker assisted selection (MAS) using DNA markers instead of phenotypic assays reduces cost and increases the precision and efficiency of subsequent selection steps applied in breeding. In this context, the present study made an attempt to characterize a set of good and poor seed longevity soybean genotypes with SSR markers.

Material and Methods

Experimental material

The experimental material comprised set of 21 genotypes grouped as good and poor seed longevity, derived from a core set of 225 genotypes of soybean which includes released varieties in India and mutant genotypes of varieties JS-335 and KHSb-2, collected from NSP/BSP, UAS Dharwad.

Seed germination

Germination test was conducted in two replications of 100 seeds each by adopting between paper method [13]. Seeds were incubated at slanting position in Walk-in germination room in growth cabinets. The temperature of 25 ± 1^0C and RH of 95 per cent was maintained during the germination test.

Seed coat permeability test

The seed coat permeability was measured as amount of water absorbed per unit of seed weight and expressed as percent water absorbed. Two replicates of 25 seeds were weighed and soaked in 50 ml distilled water for one hour. Seeds were taken out from water, excess water was removed and weight was determined.

Electrical conductivity (dSm^{-1})

Five grams of seeds were weighed in two replications from each selected genotypes and soaked in 50 ml distilled water in a beaker and kept at 25±1ºC temperature. 50 ml of distilled water was used as control. After 24 hours of soaking, the leachets were stirred using a glass rod, poured into another beaker and the volume was made up to 25 ml by adding distilled water. The electrical conductivity of the leachet was measured using digital conductivity meter and the mean of two replicates were expressed in dSm^{-1}.

Accelerated ageing test

The seed material was subjected to accelerated ageing by controlled deterioration test. First the chamber was sterilized with alcohol to prevent the fungal contamination to the seed material. Individual genotypes were taken in separate petriplate and placed on the wire mesh and incubated in temperature and relative humidity control chamber at 40^0C temperature and 94 to 100 per cent RH for 72 hours continuously.

DNA extraction and molecular assay

Based on seed longevity measured as reduction in germination, EC and seed coat permeability, 21 soybean genotypes were selected out of 225 genotypes and grouped as good and poor seed longevity. The leaf samples were collected and genomic DNA was extracted based on a previously reported cetyl-trimethyl ammonium bromide (CTAB) method [14] with some modification. Polymorphism survey was carried out with 15 SSR markers distributed across the soybean genome [10]. The markers were selected based on the earlier studies [12, 15] and are linked to the seed quality traits.

SSR markers were amplified in a PCR reaction mixture of 10X Assay buffer (2µL/tube), 2mM dNTP (1µL/tube), 3pM of forward primer and Reverse primer (1µL/tube), *Taq* DNA polymerase (5U/µL), nano pure water (17.8µL/tube) and 20-40ng template DNA (2µL/tube)of genomic DNA for the total volume of 25µL using a thermal cycler. The PCR reactions were consists of initial denaturation at 94°C for 5mins for 1cyclefollowed by 34 cycles of denaturation at 94°C for 2 mins, annealing at 58°C to 62°C for 1min and primer extension at 72°C for 2min and final extension for 7mins at 72°C and stored at 4ºC.

The DNA fragments were scored in a binary form with '1' for the presence and '0' for the absence of bands and scored data was used for constructing a dendogram by NTSYS-Pc software. Pair wise genetic similarities between genotypes were estimated by DICE similarity coefficient. Clustering was done using the symmetric matrix of similarity coefficient and clusters obtained based on Unweighted Pair Group Arithmetic Mean(UPGMA) using SHAN module of NTSYS-Pc version 2.0 [16].

Results and Discussion

The per cent reduction the soybean calculated by difference between initial germination % of freshly harvested seeds and final germination % after accelerated ageing test were comparable for seed longevity. The initial germination was above 90% for both the good and poor seed longevity genotypes however, significant decline in the germination was noticed in all the genotypes. Based on the difference in the reduction in germination % among the genotypes, they were grouped into good and poor genotypes. The reduction in germination % was also comparable with EC of the seed leachet and seed coat permeability measured as water absorbed (%) by the seed coat, both have significant negative correlation with good seed longevity [17, 18, 19]. The average reduction in germination was 15% and 56% good and poor storer respectively and the same pattern was holding good with EC (1.83 d Sm^{-4} and 3.86 d Sm^{-4}) and seed coat permeability(41.66% and58.69%) (Table 1).

SSR analysis was performed using 15 markers, out which 8 markers showed polymorphism by giving 28 alleles with an average of 1.86 alleles per locus across the genotypes with 53.33% polymorphism. Among fifteen primers Satt 281 exhibited distinct banding pattern that could clearly differentiate good and poor seed longevity. This particular primer produced an amplicon size of 184bp for those 6 genotypes which are having poor seed longevity phenotypically. As a counterpart same primer produced amplicon size of 235bp for the remaining 15 genotypes which belongs to good seed longevity as measured phenotypically (Fig 1). Other primers like Satt 434 and Satt 538 were slightly able differentiate the good and poor seed longevity genotypes

with less occuracy compre to Satt 281. This particular SSR locus Satt 281 is already reported to be linked to the seed longevity measured through electrical conductivity of seed leachates which is negatively correlated with the seed longevity [12]. Thus confirmed the phenotypic evaluation. However, other Satt primers did not showed clear distinct banding pattern, even though they are reported to be linked to the seed longevity [20], further validation of these markers are needed with large number of genotypes.

A dendrogram based on UPGMA analysis grouped the twenty one genotypes into six clusters, with two major Clusters, Cluster I and Cluster II at 79 per cent similarity. In the relationship dendrogram constructed from the SSR data, two distinctive groups (I and II) are apparent. There was clear clustering of the 21 genotypes in 2 major Clusters I and II, which were grouped as good and poor seed longevity based on phenotypic data, where Cluster I included the genotypes with good seed longevity. However, Cluster II included genotypes with poor seed longevity (Fig. 2). The similarity coefficient ranged from 0.67 to 0.96. Cluster I had six genotypes, *viz.*, Hara Soy, Indira Soy 9, Gaurav, Shivalik, EC 241696 and JS 71-05, three each in sub cluster I (a) and I (b) respectively, which are having poor seed longevity. While Cluster II comprised of remaining fifteen genotypes which are having good seed longevity. Cluster II (a) included three genotypes *viz.*, NRC – 37, JS-97-52 and JS 80-21. Cluster II (b) included Samrat, AGS 91, 1-28, PI 284815, SL 48-40, DSb 21, DSb-12, JS 93-05, JS-335, Birsa Soy 1, PS 1241 and Kalitur.

This study demonstrate the utility of molecular markers to study seed longevity, the primer Satt 281 can be utilized for validation in different mapping population since limited number of markers were used in this study, there is a need of more number of markers need to be evaluated to get complete expression of seed longevity and for identifying QTLs. On the other hand electrolyte conductivity can be used as simple and easy but indirect measure of seed longevity in soybean.

Table 1: List of soybean genotypes with phenotypic observations

S. No	Genotype	Reduction in Germination (%)	Seed coat permeability (%)	EC (dSm^{-1})
1	Birsa Soy 1	13.64	44.38	2.08
2	JS-97-52	14.77	43.28	2.14
3	JS 80-21	24.47	45.90	1.98
4	Samrat	7.95	42.10	1.28
5	AGS 91	6.38	45.72	0.83
6	28-1	9.18	9.96	1.91
7	PI 284815	4.49	46.68	1.75
8	SL 48-40	2.11	42.81	1.56
9	DSb-21	21.98	42.29	2.29
10	DSb-12	23.96	46.67	1.99
11	JS 93-05	17.14	35.90	2.14
12	JS-335	20.22	45.87	1.82
13	Kalitur	17.78	46.59	1.21
14	NRC - 37	24.73	52.94	2.62
15	PS 1241	27.00	33.88	1.99
16	Hara Soy	32.26	54.68	3.80
17	Indira Soy 9	50.60	64.01	3.08
18	Gaurav	71.88	53.53	3.59
19	Shivalik	56.03	56.36	5.74
20	EC 241696	71.43	54.32	3.35
21	JS 71-05	55.75	69.21	3.60

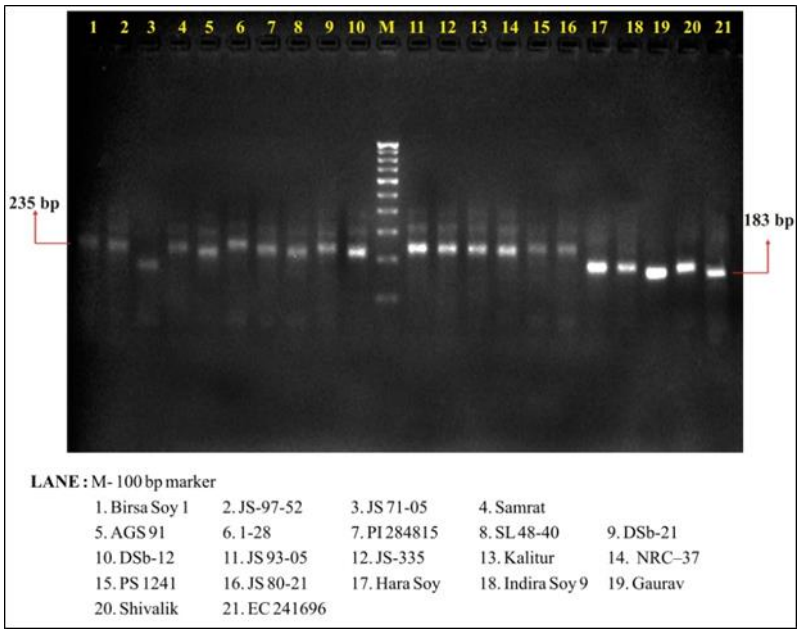

Fig 1: PCR amplification generated by marker Satt 281

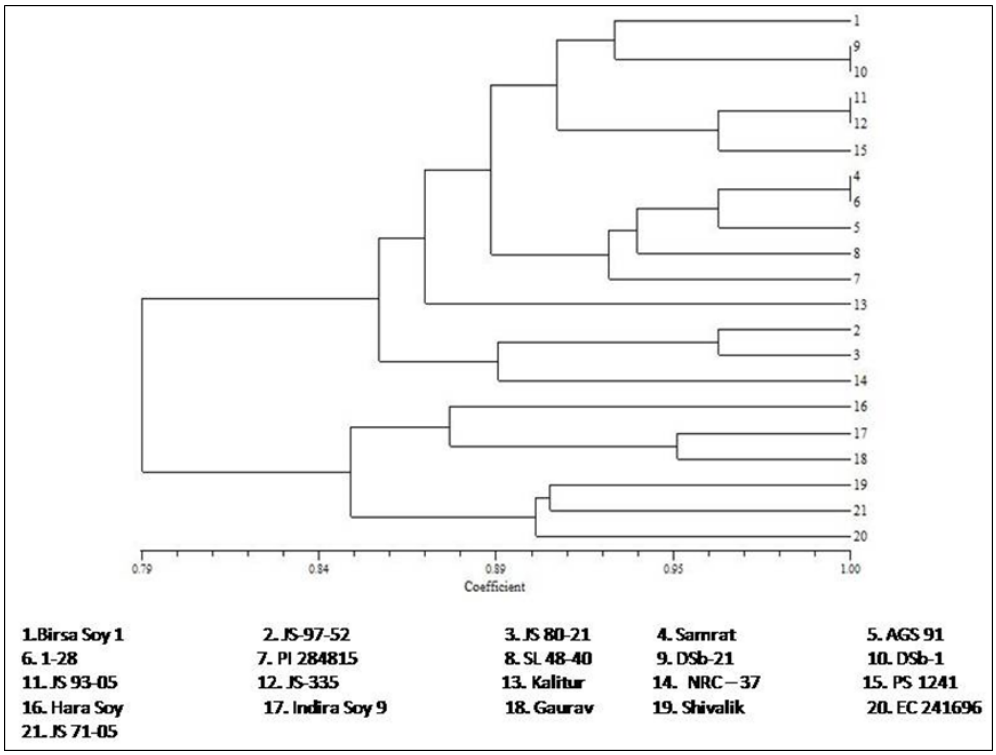

Fig 2: Dendrogram of selected varieties of soybean (SSR)

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