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RESEARCH ARTICLE

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Physiological and Molecular Analysis of Soybean Seed Longevity and Validation of Candidate Markers

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ABSTRACT

Background: Seed longevity is a major constraint in soybean seed production. The major focus of this study is to analyze the physiological and molecular changes associated with seed longevity and identify promising germplasm which are good storers for soybean breeding program.

Methods: Nineteen genotypes were studied for seed longevity using accelerated ageing test and genetic integrity based on SSR marker data. Genotypes were clustered into distinct groups based on seed morphological and physiological parameters (Mahalanobis D^2 analysis). SSR markers for seed longevity were validated in the germplasm.

Result: Per cent reduction in germination after accelerated ageing was significantly and positively correlated with traits associated with seed storability such as seed length, seed width, seed thickness and 100 seed weight and negatively correlated with seedling vigour indices. Hence, it would be worthwhile to rely upon these parameters for enhancing the seed storability in soybean. Genetic integrity of the germplasm was evaluated based on SSR markers in accelerated ageing seeds. SSR markers (Satt 285, Satt 534, Satt 538, Satt 281, Satt 162, Satt 631 and Satt 371) revealed significant association for the seed longevity characters such as seed length, seed width, seed thickness and seed weight. Candidate markers (Satt 371, Satt 281, Satt162, Satt 285, Satt 534) which can differentiate the soybean genotypes for storability have been identified in this study. The genotypes were grouped into seven clusters with monogenotypic cluster III (PSPB23) having minimum reduction in germination after accelerated ageing.

Key words: Accelerated ageing, Genetic divergence, Seed longevity, Soybean, SSR, Storability.

INTRODUCTION

Seed ageing is commonly described as the loss of seed quality or viability under long storage conditions (Priestley, 1986, Coolbear, 1995). It is a complex biological activity and involves a network of physiological, biochemical, metabolic and molecular processes (McDonald, 1999; Zaheer *et al*., 2016). Seed physical and physiological traits like seed coat, seed thickness, germination percentage, vigour and seed leachate conductivity are the common considerations for seed longevity. Seed ageing affects germination (Rehman *et al*., 1999), emergence (Verma, *et al*., 2003; Basra 2003), shoot and root dry weight (Verma *et al*., 2003), seedling length (Verma *et al*. 2003), normal seedling percentage and seed size (Dell' Aquila and Di Turi, 1996). Accelerated ageing is a technique (AA) widely used to evaluate seed storability and is based on increased seed deterioration after exposure to high temperature and relative humidity (Mali *et al*., 2014). The decrease in germination, increase in abnormal seedlings and dead seeds after AA is the result of progressive loss of seed viability and vigor (Jain *et al*., 2006; Radha *et al*., 2016). Soybean [*Glycine max* (L.) Merrill] is an important legume crop of Fabaceae family, which meets both protein and oil requirements across the globe. Soybean seed deterioration is a major problem in agricultural production systems as seed rapidly deteriorates than most of the other crops (Shelar *et al*., 2008; Finch-Savage and Bassel, 2016) due to high temperature and high relative humidity during storage. Soybean is classified as a "poor storer" when compared to other grain crops (Jagadish *et al*., 2013). Loss of germination potential or viability is more severe in tropical and subtropical areas than in temperate habitats. Soybean germplasm identified with good storability traits can be utilized as parental lines in the breeding program.

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MATERIALS AND METHODS

Nineteen soybean genotypes with variable seed coat colour were used for this study (Table 1). The experiments were conducted at PJTSAU, Hyderabad. Soybean seed was categorized as black, brown, yellow, yellow green and green based on seed coat colour and on the basis of size as bold, medium and small seeded types. Three replications of 10 seeds each were randomly selected from each genotype to measure seed length, width and thickness by using digital vernier calipers. Weight of 100 seeds was measured for test weight/100 seed weight.

Freshly harvested soybean seeds (42 g) of each genotype were placed in ageing boxes and subjected to accelerated ageing for 72 hr at 42°C and 90% relative humidity (RH) in accelerated ageing chamber. Samples were drawn after 72 hr and immediately kept for germination test (ISTA, 2016). Seed physiological parameters such as germination (%), seedling length (cm), seedling dry weight (mg), seedling vigour index I and II, per cent reduction in germination, percent reduction in field emergence were recorded for both freshly harvested seeds and seeds subjected to AA.

Field emergence (%) =

$$\frac{\text{Number of seedlings emerged at 8 days after sowing}}{\text{Total number of seeds sown}} \times 100$$

$$\text{Germination (\%)} = \frac{\text{Number of normal seedlings}}{\text{Total number of seeds planted}} \times 100$$

Seedling vigour index I and II were calculated as suggested by Abdul-Baki and Anderson (1973).

Seedling vigour index I =

$$\text{Germination (\%)} \times \text{Seedling length (cm)}$$

Seedling vigour index II =

$$\text{Germination (\%)} \times \text{Seedling dry weight (mg)}$$

Genomic DNA was isolated from young leaves following the CTAB procedure as described by Saghai-Maroof *et al.,* 1984. A total of 21 SSR markers associated with seed longevity traits were used for PCR (Table S4 and S4a). Completely randomized design (CRD) was followed for the physiological experiments. Correlation analysis and genetic divergence was carried out using INDOSTAT software. A measure of group distance based on multiple characters was given by Mahalanobis (1936) using D^2 statistic by which, genetic divergence between genotypes was estimated (Windostat software). Correlations were computed according to Al-Jibouri *et al*., (1958). Single marker analysis was done with t-test and regression analysis to identify association between SSR markers and seed traits.

RESULTS AND DISCUSSION

Seed ageing can be defined as progressive deterioration of the structures and functions of the seed over time which ultimately leads to death of the organism. The quality of soybean seed during storage has been reviewed and seed deterioration has been identified as one of the basic reasons for low productivity in soybean (Shelar *et al*., 2008). Accelerated ageing is the most commonly used stress test to assess the storability of crop seeds in a short period of time (Mali *et al*., 2014). The genotypes were grouped on the basis of seed coat colour (Table 1).

Analysis of variance (ANOVA) and variability for seed physical and physiological traits

Seed physical traits of soybean genotypes were recorded in fresh seed lot, while seed physiological parameters were recorded in both freshly harvested seeds and seeds subjected to AA. ANOVA for seed physical parameters of fresh seed exhibited significant variation among all the genotypes for seed length, seed width, seed thickness and 100 seed weight. Significant variation was observed for the seed physiological traits viz percent reduction in germination, seedling length, seedling dry weight, seedling vigour index-I, seedling vigour index-II (Table S1, S2 and S3). Genotypic coefficient of variability (GCV) of soybean genotypes for seed, seedling traits and vigour ranged from 7.65% to 41.12%. The seed physical and physiological traits expressed high heritability estimates ranging from 82.3 to 99.5% (Table 2).

Correlation between seed physical/physiological traits and seed longevity

Physical parameters associated with seed storability like seed coat colour, seed size, seed weight can be helpful in making a decision on duration of soybean seed storage (Mosavi *et al*. 2011; Tubic *et al*., 2011). Seed physical and physiological traits were recorded before and after AA to study longevity of soybean germplasm and phenotypic correlation coefficients were computed (Table 3). Per cent reduction in germination after AA exhibited significant positive correlation with seed traits associated with storability such as seed length (0.40*), seed width (0.58***), seed thickness (0.51***) and 100 seed weight (0.52***). Significant negative correlation was observed with SV-I (-0.55***) and SV-II after ageing (-0.50**). SV-I displayed significant positive

Table 1: Classification of soybean genotypes based on colour of seed coat.

Seed coat colour	Genotypes
Black	CAT 1852 TG X 854-25D, TG X 849D-13-4, G 1922, IC 16572, UPSL 387, CAT 243 DE 201,
Brown	CAT 1622 TG X 302-2A, CAT 194 BR3, CAT 192 BR15, NRC 2755, NG 1142 CHICO, CAT 195 BR4
Yellow	G828, NRC-130, DS91-3
Green	PSPB-23, CAT 2059 GC 84058-18-4, KARUNE
Yellow green	EC 12503

correlation with seedling length (0.81 ***), while SV-II displayed significant positive correlation with seedling dry weight (0.55 ***) after AA (data not shown). Similar positive and significant correlations were reported by Kuchlan *et al*., (2010b) for seed size and 100 seed weight in soybean. Black-seeded genotypes were better than yellow-seeded genotypes. Traits such as hard seed coated seeds; small seed size and black seed coat were identified to have better storability in soybean (Subash *et al*., 2017). Negative correlation is observed in the present study for germination (Naik *et al*., 2019) and seedling vigour indices after AA (Pallavi *et al*., 2018).

Evaluation of genetic integrity using SSR markers

Genetic integrity is also of equal concern as much as the physiological integrity during seed storage. Decline in germination percent after accelerated ageing was observed in all genotypes and was drastic in NRC 130 and Karune (Table S2). Variation in the SSR profiles after ageing was observed and four markers *viz*., Satt 434, Satt 281, Satt 286, Satt 390 were able to detect polymorphisms among AA seeds of soybean. A drastic decrease in the number of alleles has been observed after AA indicating loss of DNA integrity (Table 4). Only one allele was detected for SSR markers Satt 523, Satt 371 and SOYGPATR after AA, while 4-5 alleles were observed in freshly harvested seeds. Loss of alleles due to failure of PCR amplification may be attributed to loss in genetic integrity during AA. It is interesting to note that most of the genotypes with black/brown seed coat have amplified few alleles after AA, indicating loss of genetic integrity was more prominent in green and yellow green seeded genotypes. Reduction in the number of alleles after AA has also been reported in several crops such as rice (Adeboye *et al*., 2015), barley (Parzies *et al*., 2000), rye (Chebotar *et al*., 2003), bread wheat (Fu *et al*., 2015), Siberian wild rye (Huang *et al*., 2019), soybean and safflower (Vijay *et al*., 2009). Assessment of DNA integrity combined with a germination test efficiently characterizes the vigour of seeds after AA and may be a promising tool for long term conservation in seed banks.

Validation of SSR markers for seed longevity

SSR markers associated with seed longevity such as seed length, seed width, seed thickness and 100 seed weight (single marker analysis) were validated in our study. Seven markers (Satt 285, Satt 534, Satt 538, Satt 281, Satt 162, Satt 631, Satt 371) having significant association with seed storability traits have been identified (Table 5). SSR marker Satt 285 was significantly associated with seed physical parameters such as seed length, seed width, seed thickness and seed weight, while Satt 534 was significantly associated with seed thickness, seed width and 100-seed weight. Despite the small sample size and minimum number of markers used in our study we observed significant

Table S1: Mean values of soybean genotypes for seed physical parameters.

Genotypes	Seed length (mm)	Seed width (mm)	Seed thickness (mm)	100 seed weight (gm)
CAT 195 BR4	7.73	6.10	4.96	15.50
CAT 192 BR15	7.61	5.79	4.61	13.82
CAT 1622 TG X 302-2A	7.72	5.81	4.60	13.63
CAT 194 BR3	7.94	5.57	4.42	13.26
NG 1142 CHICO	7.46	5.91	4.91	14.12
NRC 2755	7.97	5.79	4.61	14.18
UPSL 387	6.73	5.13	4.10	9.86
TG X 849D-13-4	6.29	4.97	3.63	7.45
IC 16572	5.85	4.44	3.36	5.98
EC 12503	5.85	4.77	3.94	7.51
G828	6.56	5.63	4.56	10.96
NRC-130	6.72	6.40	5.28	15.23
KARUNE	9.26	8.04	6.46	28.48
PSPB-23	7.17	5.06	4.53	10.80
CAT 2059 GC 84058-18-4	6.48	5.22	4.52	10.47
CAT 243 DE 201	7.37	5.21	4.18	11.17
G1922	6.33	4.91	4.13	8.27
CAT 1852 TG X 854-25D	6.28	4.90	4.17	8.20
DS91-3	6.64	5.25	4.49	10.18
Max.	9.25	8.03	6.45	28.48
Min.	5.84	4.44	3.35	5.98
Mean	7.05	5.52	4.49	12.05
C.V. %	1.34	1.17	1.17	2.78
C.D. 5%	0.19	0.13	0.11	0.70

association of seven SSR markers with seed longevity. This is corresponds with previous findings (Singh *et al*., 2008a, b; Mian *et al*., 1996). We also identified a few candidate SSR markers that discriminated the good storer soybean genotypes from the rest of the genotypes based on allele size. These markers (Satt 371, Satt 162, Satt 463, Satt 281, Satt 285) were associated with seed specific traits such as seed coat colour, seed size and storability. Our results are in conformity with earlier reports where SSR markers Satt 371, Satt 453 and Satt 618 produced specific allelic bands making them candidate markers for linkage with seed storability and testa colour (Jagadish *et al*., 2013a). SSR marker Satt 281 exhibited distinct banding pattern that could clearly differentiate good and poor seed

Table S2: Mean values of soybean genotypes for physiological parameters before and after accelerated ageing.

Genotypes	Fresh seed				Accelerated ageing				
	Seedling length (cm)	Seedling dry weight (mg)	Seedling vigour index-I	Seedling vigour index-II	Seedling length (cm)	Seedling dry weight (mg)	Seedling vigour index-I	Seedling vigour index-II	Percent reduction in germination
CAT 195 BR4	24.53	437.00	1864.29	33214	16.84	390.00	757.80	17550	40.7
CAT 192 BR15	25.42	462.50	1829.87	30546.5	19.23	424.50	1076.60	25900	22.2
CAT 1622 TG X 302-2A	25.37	419.50	1852.01	30636	18.67	380.00	671.94	13673	50.6
CAT 194 BR3	24.88	410.50	1791.00	29556	17.67	350.50	618.45	12267.5	51.3
NG 1142 CHICO	26.69	466.00	1627.79	28426	17.52	319.50	472.90	8629.5	55.7
NRC 2755	27.73	480.00	2384.78	41045.5	24.15	480.00	1327.97	25400	36.0
UPSL 387	27.38	408.50	2381.63	35539.5	16.12	292.50	1128.05	20475	19.5
TG X 849D-13-4	26.04	306.00	2005.00	23554	19.40	269.50	1202.49	16715.5	19.4
IC 16572	26.27	250.00	1891.44	17993	22.37	220.50	1207.71	11907	25.0
EC 12503	26.33	333.00	1895.76	23976	16.48	275.00	922.60	15400	22.2
G828	24.93	406.50	1620.12	26419	14.85	289.00	623.70	12132	35.3
NRC-130	22.89	450.50	1510.74	29742.5	10.34	189.00	175.69	3213	74.2
KARUNE	20.47	712.00	1248.37	44868	10.34	474.00	186.12	8538	71.4
PSPB-23	24.98	390.50	1698.30	26554	14.46	294.00	939.57	19120	4.4
CAT 2059 GC 84058-18-4	23.78	400.00	2187.30	36808	17.34	328.00	1074.77	20336	32.6
CAT 243 DE 201	23.30	422.50	1677.60	30420	12.83	270.00	474.71	9990	48.6
G1922	24.51	339.50	2082.92	28847	17.66	281.50	882.75	14075	41.1
CAT 1852 TG X 854-25D	24.97	357.50	1672.99	23960	15.89	303.50	921.60	17595.5	13.4
DS91-3	20.31	380.50	1421.70	26610	12.82	301.50	640.75	15075	28.5
Max.	27.7	712	2384.75	44868	24.1	480	1327.97	25400	74.2
Min.	20.3	250	1248.36	17993	10.3	189	175.69	3213	4.5
Mean	24.77	412.2	1823.3	29963.3	16.5	322.63	805.59	15210.1	38.4
C.V.	3.54	4.25	4.89	4.30	2.05	2.49	3.47	4.71	4.81
C.D. 5%	1.84	36.70	185.71	2677.66	0.71	17.04	53.5	1376.24	4.02

Table S3: Analysis of variance for seed physical and physiological parameters.

Parameters	Mean sum of squares			
	Fresh seed		Accelerated ageing	
	Treatment d.f=18	Error d.f=18	Treatment d.f=18	Error d.f=18
Seed length (mm)	1.50**	0.009	-	-
Seed width (mm)	1.25**	0.004	-	-
Seed thickness (mm)	0.86**	0.002	-	-
100 seed weight (gm)	46.17**	0.113	-	-
Seedling length (cm)	7.96**	0.77	25.35**	0.11
Seedling dry weight (mg)	17017.64**	305.19	13034.68**	65.82
Seedling vigour index-I	180134.79**	7814.34	182347.29**	649.28
Seedling vigour index-II	83541632.75**	1624400.40	56223841.28**	429114.60
Percent reduction in field emergence	132.40**	1.93	263.73***	1.29
Percent reduction in germination	-	-	272.94***	3.63

Table S4: List of reported SSR markers associated with seed longevity in soybean.

SSR marker	Associated traits	Reference
Satt 285	Seed size, seed viability	Cregan *et al.* (1999), Singh *et al.* (2008b)
Satt 534	Poor storability, seedling dry weight	Jagadish *et al.* (2013a)
Satt 281	Seed coat permeability, electrolyte leakage, seed weight	Cregan *et al.* (1999), Singh *et al.* (2008a), Jagadish *et al.* (2013a), Naik *et al.* (2019)
Satt 538	Seed weight, seed viability, Seed coat permeability	Cregan *et al.* (1999), Singh *et al.* (2008a), Singh *et al.*(2008b)
Satt 162	Seed coat permeability, seed coat colour (black), seed weight	Cregan *et al.* (1999), Pawar *et al.* (2017)
Satt 371	Seed storability and testa colour (black)	Jagadish *et al.* (2013a)
Satt 286	Storability	Pawar *et al.* (2017)
Satt 565	Good storability and black seed coat colour	Jagadish *et al.* (2013a)
Satt 184	Good storability and black seed coat colour	Jagadish *et al.* (2013a)
Satt 619	Good storability and black seed coat colour	Jagadish *et al.* (2013a)
Satt 481	Good storability	Jagadish *et al.* (2013a)
Satt 463	Good storability	Jagadish *et al.* (2013a)
Satt 193	Good storability	Jagadish *et al.* (2013a)
Satt 175	Poor storability	Jagadish *et al.* (2013a)
Satt 598	Seed coat permeability and poor storability	Singh *et al.* (2008a), Jagadish *et al.* (2013a)
Satt 389	Poor storability	Jagadish *et al.* (2013a)
Satt 600	Seed longevity	Singh *et al.* (2008b)
Satt 434	Seed coat permeability	Singh *et al.* (2008a,b)
Satt 523	Seed storability and black testa colour	Pawar *et al.* (2017)
SOYGPATR	Storability	Pawar *et al.* (2017)
Satt 631	Seed width, seed thickness	Song *et al.* (2004)

Table S4a. Primer sequences and annealing temperature of SSR markers associated with seed longevity in soybean.

Primer name	Forward primer	Reverse primer	Annealing temp. (${}^{238}_{92}C$)
Satt 286	GCGGCGTTAATTTATGCCGGAAA	GCGTTTGGTCTAGAATAGTTCTCA	55
Satt 534	CTCCTCCTGCGCAACAACAATA	GGGGGATCTAGGCCATGAC	60
Satt 565	GCGCCCGGAACTTGTAATAACCTAAT	GCGCTCTCTTATGATGTTCATAATAA	55
Satt 371	TGCAAACTAACTGGATTCACTCA	GAGATCCCGAAATTTTAGTGTAACA	55
Satt 184	GCGCTATGTAGATTATCCAAATTACGC	GCCACTTACTGTTACTCAT	51
Satt 619	GGCAGAACTAGTACGCTTCTGATT	GCGGTTAAGCATAATAGATCAGCCT	53
Satt 481	GGGTTAACCGTCCACACATCTATT	GACGGTTTTAAACGGTAAGAAAAT	48,50
Satt 463	TTGGATCTCATATTCAAACTTTCAAG	CTGCAAATTTGATGCACATGTGTCTA	52
Satt 193	GCGTTTCGATAAAAATGTTACACCTC	TGTTCGCATTATTGATCAAAAAT	48
Satt 175	GACCTCGCTCTCTGTTTCTCAT	GGTGACCACCCCTATTCCTTAT	53
Satt 598	CGATTTGAATATACTTACCGTCTATA	CACAATACCTGTGGCTGTTATACTAT	48,50
Satt 281	AAGCTCCACATGCAGTTCAAAAC	TGCATGGCACGAGAAAGAAGTA	50
Satt 389	GCGGCTGGTGTATGGTGAAATCA	GCGCCAAAACCAAAAGTTATATC	55
Satt 538	GCAGGCTTATCTTAAGACAAGT	GGGGCGATAAACTAGAACAGGA	51
Satt 600	GCGCAGGAAAAAAAAACGCTTTTATT	GCGCAATCCACTAGGTGTTAAT	52
Satt 285	GCGACATATTGCATTAAAAACATACTT	GCGGACTAATTCTATTTTACACCAACAAC	50
Satt 434	GCGTTCCGATATACTATATAATCCTAAT	GCGGGGTTAGTCTTTTTATTTAACTTAA	50
Satt 162	GGGAAGAAGTATATGCTACATCAA	GGGTTAATTTTTATCTTCTAATAGTTT	48
Satt 523	GCGATTTCTTCCTTGAAGAATTTTCTG	GCGCTTTTTCGGCTGTTATTTTTAACT	53
SOYGPATR	GGAAGAAAGTATTGGTCTGT	AGGAGAGAGTGGAGAGATTA	54
Satt 631	GGTAGATCCAGGAGCTTGAGTCAG	GCGCATCTCACTGCACTTGATTTT	55

longevity in soybean genotypes (Naik *et al*., 2019). Markers Satt 162, Satt 523 and Satt 453 which are either linked with seed coat colour or seed permeability exhibited a specific size allelic fragments in soybean genotypes and crosses revealing better seed longevity (Pawar *et al*., 2018). On the other hand, marker Satt 285 showed allelic variation based on seed size.

Candidate markers identified for seed storability

SSR marker Satt 371 amplified in black seed genotypes exclusively at 268-278 bp. In our study, 10 out of 11 good storer genotypes amplified within this size range. Thus, preliminary studies indicate that Satt 371 may have close linkage with good storability that may be attributed to black seed coat. Few other markers such as Satt 162 amplified at 300 bp in 10 out of 11 good storer genotypes and Satt 463 amplified five out of seven black seed genotypes at 110 bp. Eight out of 11 good storer genotypes were amplified

Table S5. Percent contribution of characters towards genetic divergence in soybean genotypes.

Characters	Times ranked 1st	Contribution %
Seedling length before AA	0	0.00
Seedling length after AA	0	0.00
Seed vigour-I before AA	1	0.58
Seed vigour-I after AA	0	0.00
Seedling dry weight before AA	0	0.00
Seedling dry weight after AA	32	18.71
Seed vigour-II before AA	0	0.00
Seed vigour-II after AA	0	0.00
Seed length	0	0.00
Seed width	9	5.26
Seed thickness	18	10.52
100-seed weight	2	1.16
% reduction in germination	59	34.50
% reduction in field emergence	50	29.23

Table 2: Estimates of GCV, PCV, heritability in broad sense (h^2) and genetic advance over mean (GAM) for different characters in soybean.

Characters	Mean	Range	PCV (%)	GCV (%)	h^2	GAM
Seed length	7.05	5.84-9.25	12.32	12.25	98.8	25.08
Seed width	5.52	4.44-8.03	14.36	14.36	99.3	29.39
Seed thickness	4.50	3.35-6.45	14.61	14.56	99.4	29.90
100-seed weight	12.1	6.05-28.17	39.80	39.80	99.5	81.58
Seedling length before AA	24.78	20.30-27.73	8.43	7.65	82.3	14.30
Seedling length after AA	16.57	10.33-24.15	21.53	21.43	99.1	43.95
Seed vigour-I before AA	1806.48	1289.37-2385.25	16.97	16.25	91.7	32.05
Seed vigour-I after AA	733.1	134.35-1328	41.26	41.12	99.3	84.40
Seedling dry weight before AA	410.12	250-712	22.69	22.29	96.5	45.10
Seedling dry weight after AA	324.76	189-480	24.92	24.92	99.0	50.82
Seed vigour-II before AA	29620.11	17993-480	22.03	22.03	96.2	43.65
Seed vigour-II after AA	13891.95	2457-26400	38.31	38.02	98.5	77.73

Table 3: Correlation between seed longevity parameters and percent reduction in germination.

	HSW	ST	SW	SL	SV IIAA	SV IAA	PRG
HSW	1.00000	0.95127***	0.97539***	0.88780***	-0.20651	-0.59057***	0.52140***
ST		1.00000	0.95381***	0.77097***	-0.26039	-0.67550***	0.51596***
SW			1.00000	0.81934***	-0.26010	-0.61948***	0.58814***
SL				1.00000	0.04814	-0.36789*	0.40384*
SV IIAA					1.00000	0.81326***	-0.50121**
SV IAA						1.00000	-0.55985***

HSW= 100 Seed weight; ST= Seed thickness; SW= Seed width; SL= Seed length; AA= Accelerated ageing; SV-I= Seedling vigour index-I; SV-II= Seedling vigour index-II; PRG= % Reduction in germination.

Significance levels 0.05; 0.01; 0.005; 0.001.

If correlation r =>0.32019; 0.41281; 0.44608; 0.51260.

at 190-225 bp using Satt 281 marker. Marker Satt 619 amplified at 125 bp in 8 out of 11 good storers, which were black seeded suggesting that Satt 619 is specific to seed coat colour. Marker Satt 534 amplified at 175 bp in extremely poor storer genotypes, while Satt 656 amplified in 8 out of 11 good storer genotypes at around 150 bp indicating its possible association with storability.

Genetic divergence (D^2 statistics) of soybean germplasm

The genotypes were grouped into seven clusters on the basis of seed physical and physiological attributes with cluster I being the largest comprising of ten genotypes. Five out of six brown seeded genotypes were grouped in cluster I (Table 6). The average inter-cluster distances varied from 378.08 to 56,445.64 while intra cluster distance ranged from 0.00 to 1300.32. The highest inter-cluster distance was recorded between clusters IV and VII indicating greater diversity (Table 7). Cluster means were computed for six seed traits and four seedling traits (before and after AA) (Table 8). Monogenotypic cluster V (NRC 2755) recorded maximum cluster mean for seedling length, seedling vigour-I & II, seedling dry weight both before and after AA indicating better storability of the germplasm. The monogenotypic cluster III (PSPB-23 [green]) recorded lowest per cent

Table 4: SSR markers showing variation in number of alleles before and after accelerated ageing.

SSR marker	Before AA		After AA			Genotypes amplified
	No. of alleles	No. of genotypes amplified	No. of alleles	No. of genotypes amplified		
				Br/B	G/YG	
Satt 434	6	19	3	5	3	CAT 195 BR4, CAT 243 DE 201, KARUNE, NRC-130, CAT 192 BR15,G828, NRC 2755, CAT 1622 TGX302-2A
Satt 281	5	19	2	5	3	CAT 243 DE 201, TGX849D-13-4, NRC-130, EC 12503, UPSL 387, G828, CAT 192BR15, CAT 1622 TGX302-2A
Satt 286	4	19	2	9	1	CAT 243 DE 201, G1922, TGX849D-13-4, EC 12503, CAT 192 BR15, NRC 2755,CAT 194 BR3, CAT 1852 TGX854-25D, CAT 1622 TGX302-2A
Satt 390	3	19	2	7	0	CAT 243 DE 201, G1922, TGX849D-13-4, CAT 192 BR15, G828, NRC 2755,CAT 194 BR3, CAT 1622 TGX302-2A
Satt 523	5	19	1	4	4	TGX849D-13-4, NRC-130, EC 12503, PSPB-23, CAT 2059 GC 84058-18-4, CAT 194 BR3, CAT 1852 TGX302-2A, CAT 1622 TGX302-2A
Satt 371	5	19	1	10	3	CAT 195 BR4, NG 1142 CHICO, CAT 243 DE 201, GI922, KARUNE, TGX849D-13-4, NRC-130, EC 12503, UPSL 387, CAT 192 BR15,G828, NRC 2755, CAT 1622 TGX302-2A
SOYGPATR	4	19	1	5	5	CAT 243 DE 201, KARUNE, TGX849D-13-4, NRC 130, EC 12503, UPSL 387, PSPB-23, CAT 2059 GC 84058-18-4, CAT 192 BR15, CAT 1622 TGX302-2A
Satt 162	2	19	-	-	-	-
Satt 538	4	19	-	-	-	-
Satt 038	4	19	-	-	-	-

Br: Brown; B: Black; G: Green; YG: Yellow green.

Table 5: SSR markers associated with seed longevity in soybean.

Trait	Seed length		Seed width		Seed thickness		100-Seed weight	
Marker	p value	R^2 value	p value	R^2 value	p value	R^2 value	p value	R^2 value
Satt 285	0.004	0.0078*	0.00015	0.0146**	0.0004	0.022**	0.00002	0.011**
Satt 538	0.04	0.055*	-	-	-	-	-	-
Satt 281	0.04	0.038*	-	-	0.0300	0.009*	-	-
Satt 534	-	-	0.004	0.041*	0.0010	0.124**	0.009	0.053**
Satt 162	0.03	0.0048*	-	-	-	-	-	-
Satt 631	0.04	0.0041*	-	-	-	-	-	-
Satt 371	-	-	-	-	-	-	0.05	0.039*

Note: **significant at 1%, *significant at 5%.

Table 6: Distribution pattern of soybean genotypes based on seed longevity traits.

Cluster No.	No. of genotypes	Name of the genotype
I	10	CAT 1622 TG × 302-2A, CAT 194 BR3, CAT 195 BR4, CAT 192 BR15, NG 1142 CHICO (brown)TG × 849D-13-4 (black) NRC-130, G828 (yellow) KARUNE, CAT 2059 GC 84058-18-4 (green)
II	1	G1922 (black)
III	1	PSPB-23 (green)
IV	4	UPSL 387, CAT 1852 TG × 854-25D, CAT 243 DE 201 (black)EC 12503 (yellow green)
V	1	NRC 2755 (brown)
VI	1	IC 16572 (black)
VII	1	DS91-3 (black)

*Seed coat colour included in parenthesis.

Table 7: Average inter and intra cluster distances (D^2).

Cluster	I	II	III	IV	V	VI	VII
I	1297.73	2252.71	3524.77	3990.40	3131.84	10327.22	35064.54
II		0.00	378.08	901.79	4296.24	16623.42	49726.26
III			0.00	739.56	5479.46	19745.61	55838.88
IV				1300.32	5469.82	21312.70	56445.64
V					0.00	17322.21	40037.23
VI						0.00	14391.03
VII							0.00

Values with bold letters indicate intra cluster distances.

Table 8: The mean values of 14 parameters in seven clusters of soybean genotypes.

Cluster	SL before AA	SL after AA	SV-I before IAA	SV-I After AA	SL dry wt before AA	SL dry wt after AA	SV-II before AA	SV-II After AA	Seed length	Seed width	Seed thickness	100-seed weight	PRG	PRFE
I	24.37	16.54	1786.24	669.25	410.65	337.25	29985.05	13716.75	7.18	5.54	4.54	12.19	32.80	33.45
II	26.33	16.48	1896.56	773.88	333.00	275.00	23976.00	12940.00	5.85	4.77	3.94	7.40	25.00	25.00
III	24.97	15.89	1675.19	921.60	357.50	303.50	23960.00	17595.50	6.28	4.90	4.17	8.36	9.00	9.50
IV	26.17	18.08	1925.70	973.12	338.75	269.13	24834.88	14712.25	6.51	4.90	3.90	8.66	19.00	21.00
V	27.73	24.15	2385.25	1328.01	477.50	480.00	41045.50	26400.00	7.97	5.79	4.61	14.08	31.00	35.00
VI	22.89	10.34	1511.45	134.36	450.50	189.00	29742.50	2457.00	6.72	6.40	5.28	15.11	53.00	50.00
VII	20.47	10.34	1289.37	186.11	712.00	474.00	44868.00	8538.00	9.26	8.04	6.46	28.18	45.00	40.00

AA= Accelerated ageing; SL= Seedling length; SV-I= Seedling vigour index-I; SV-II= Seedling vigour index-II; PRG= % Reduction in germination ; PRFE= % Reduction in field emergence.

reduction in germination and field emergence after accelerated ageing followed by cluster IV. Crosses may be attempted between genotypes of cluster III and IV with cluster V for enhanced seedling vigour and storability traits. Monogenotypic cluster (VII) consisted of bold seeded germplasm DS91-3 (black seeded) that recorded highest value for seed traits studied such as seed length, width, thickness and 100-seed weight. The genotype NRC 2755 with diverse genetic base is the most prominent contributor for seedling vigour- I and II after AA. The robust vigour after AA is attributed to seedling length and dry weight.

NRC 2755 can be used in crop improvement program for seedling vigour trait. Percent reduction in germination showed maximum contribution (34.5%) towards genetic divergence (Table S5).

CONCLUSION

Significant variation in seed physical and physiological traits was observed in fresh and AA seed among soybean genotypes with varying seed coat colour. D^2 analysis grouped the genotypes into seven clusters and monogenotypic cluster V (NRC 2755) recorded maximum cluster mean for seedling length, seedling vigour-I and II, seedling dry weight both before and after AA indicating better storability of the germplasm. Significant positive correlation was observed between seed storability traits (seed length, seed width, seed thickness and 100 seed weight) and per cent reduction in germination, while negative correlation with seed vigour-I and II. Loss of genetic integrity after AA (decreased number of alleles) has been identified using SSR markers. Single marker analysis confirmed potential SSR markers associated with seed longevity parameters (seed size and seed weight). Candidate markers for seed coat colour and storability differentiated the soybean genotypes.

Conflict of interest

All authors declared that there is no conflict of interest.

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