International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2019; 7(5): 3070-3073

Received: 22-07-2019
Accepted: 24-08-2019

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Molecular characterization of soybean genotypes in response to charcoal rot disease by using RGA markers

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Abstract
Charcoal rot disease caused by *Macrophomina phaseolina* is one of the most damaging diseases of soybean resulting to 70% losses and till date no immune genotype is known for the same.Charcoal rot (CR) disease caused by *Macrophomina phaseolina* is responsible for significant yield losses in soybean production. The present study helped to evaluate soybean genotypes for identifying promising genotypes which proved to be resistant to charcoal rot. The present study was carried out at Department of Agricultural Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola during the year 2018-19 to evaluate various genotypes of soybean for charcoal rot resistance. Among the methods available for controlling this disease, breeding for resistance is the most promising. Molecular characterization of these genotypes was done by using RGA markers. Molecular profiles revealed remarkable polymorphism and observations showed that in total 38 amplicons were tested with an average of 5.42 alleles per locus. Out of the total screened alleles 13 were monomorphic alleles with an average of 1.85 and 25 were polymorphic alleles with an average of 3.57. Results showed an average of 65.03 polymorphism percent. The PIC (Polymorphic information content) value ranged from 0.43 to 0.83 with an average value of 0.62, these studies will help in mapping studies and breeding program for development of charcoal rot resistance in soybean genotypes which will be of utmost importance.

Keywords: Soybean, charcoal rot, inheritance, RGA, validation

Introduction
Soybean [*Glycine max* (L.) Merrill] designated as miracle bean established its potential as an industrially vital and viable oilseed crop in many areas of India. It is the cheapest source of vegetable oil and protein. It contains about 40 percent protein, well balanced in essential amino acids, 20 percent oil rich with poly unsaturated fatty acid specially omega 6 and Omega 3 fatty acids, 6-7 percent total mineral, 5-6 percent crude fiber and 17-19 percent carbohydrates (Chauhan and Opena, 1988) [4]. It is not only used for human consumption, but also used to produce lowcost, high protein feed ingredients. It also finds wider application in industry to produce numbers of products and services for human uses.

Among the biotic challenges, charcoal rot disease is the most serious one. It is caused by fungus *Macrophomina phaseolina* (Tassi) Goid, a soil borne pathogen distributed worldwide with a host range of more than 500 plant species of both monocots and dicots (Mihail and Taylor, 1995) [9]. The destructive attack of M phaseolina has been more pronounced during the drought/ drought like situations that often prevails during crop growing period due to early withdrawal of the monsoon. The disease can attack the soybean plants at any stage of development- from the seedling stage all the way through maturity. After attack, the plant loses its vigor; turn yellow, wilt and drop leaves early. It results in poor pod setting, improper seed filling and eventual loss of yield. It can create a yield loss of 10-50% in years with prime weather conditions. However, it may go up to 70% in severe cases (Almeida *et al.*, 2001; Yang and Navi, 2005).

Control of charcoal rot disease through cultural and chemical means was found neither effective nor economical. The genome of soybean has been fully sequenced and various classes of molecular markers are in abundance. The most abundant markers developed for soybean includes RFLP markers (Apuya *et al.*, 1988; Keim *et al.*, 1989) [3, 7] simple sequence repeat (SSR) (Akkaya *et al.*, 1995), amplified fragment length polymorphism (AFLP) markers (Keim *et al.*, 1997) [6] and single nucleotide polymorphism (SNP) markers (Choi *et al.*, 2007) [5]. However, the SSR markers have been widely used in gene and QTL mapping studies in

soybean because of its higher level of polymorphism, user-friendly nature, multiple allele per locus and specificity (Netu *et al.*, 2007) [10]. Genetic resistance has therefore been promoted through deployment of resistant or tolerant genotypes. However, genotype with higher level of resistance is not available yet for commercial cultivation (Mengistu *et al.* 2011) [8]. Breeding for charcoal rot resistance met with little success primarily due to absence of robust screening technique and unclear inheritance pattern of the disease resistance in the host plants. It indicates importance of finding linked molecular markers for effective and efficient screening. In this study, attempt was made to study the inheritance pattern and mapping of charcoal rot resistance in soybean.

Material and Methods

Plant material

A set of 14 diverse soybean genotypes were used for screening. The collected genotypes included promising varieties, indigenous, mutants, few pre released collections, advanced breeding lines as well as obsolete varieties. It varied in maturity, seed color, flower colour, seed size, and reaction to charcoal rot disease as well as other yield attributing traits. Specific features of the genotypes are presented in Table 1.

Table 1: Soybean genotypes included in the study

S.N	Genotypes	Parents	Remarks
1	AMS MB 5-19	Mutant of Bragg	Developed by Mutation breeding and characteristically fixed at M8 generation.
2	AMS MB 5-18	Mutant of Bragg	Developed by Mutation breeding and characteristically fixed at M8 generation.
3	AMS – 1001	Mutants	Pre released variety
4	AMS – 77	Mutant of JS 93-05	Developed by Mutation breeding and characteristically fixed at M5 generation.
5	AMS – 353	Mutants	Pre released variety
6	AMS – 358	Mutant of JS 93-05	Developed by Mutation breeding and characteristically fixed at M5 generation.
7	BRAGG	Parental genotype	Parental genotypes
8	AMS – 243	Mutant of Bragg	Developed by Mutation breeding and characteristically fixed at M8 generation.
9	JS - 93-05	Parental genotype	Parental genotypes
10	AMS 99-33	Mutants	Pre released variety
11	AMS 38-24	TAMS 38 x RKS 24	Recombinant breeding, entry fixed at F2 generation.
12	AMS -475	Mutant of JS 93-05	Developed by Mutation breeding and characteristically fixed at M5 generation.
13	JS – 335 (R)	(Check-Resistant)	High yielding variety, most popular
14	TAMS -38 (S)	(Check-Susceptible)	Highly susceptible variety

R=Check Resistant; S=Check Susceptible

Selection of markers for polymorphism and genotyping

RGA markers are being extensively validated in scientific literature and extensively used in genome studies and marker assisted selection and are well-known for their versatility in providing a quick assay and for their highly informative data. In the light of above facts and the hypothesis that molecular markers are more efficient than morphological markers for verification of soybean varieties, a set of total 8 RGA markers were used in this study. The markers were selected from across the soybean genome. The sequences of the markers were downloaded from soybase (www.soybase.org) and synthesized through local vendors (www.idtdna.com)The sequences and related information about the RGA primers have been given in Table 2.

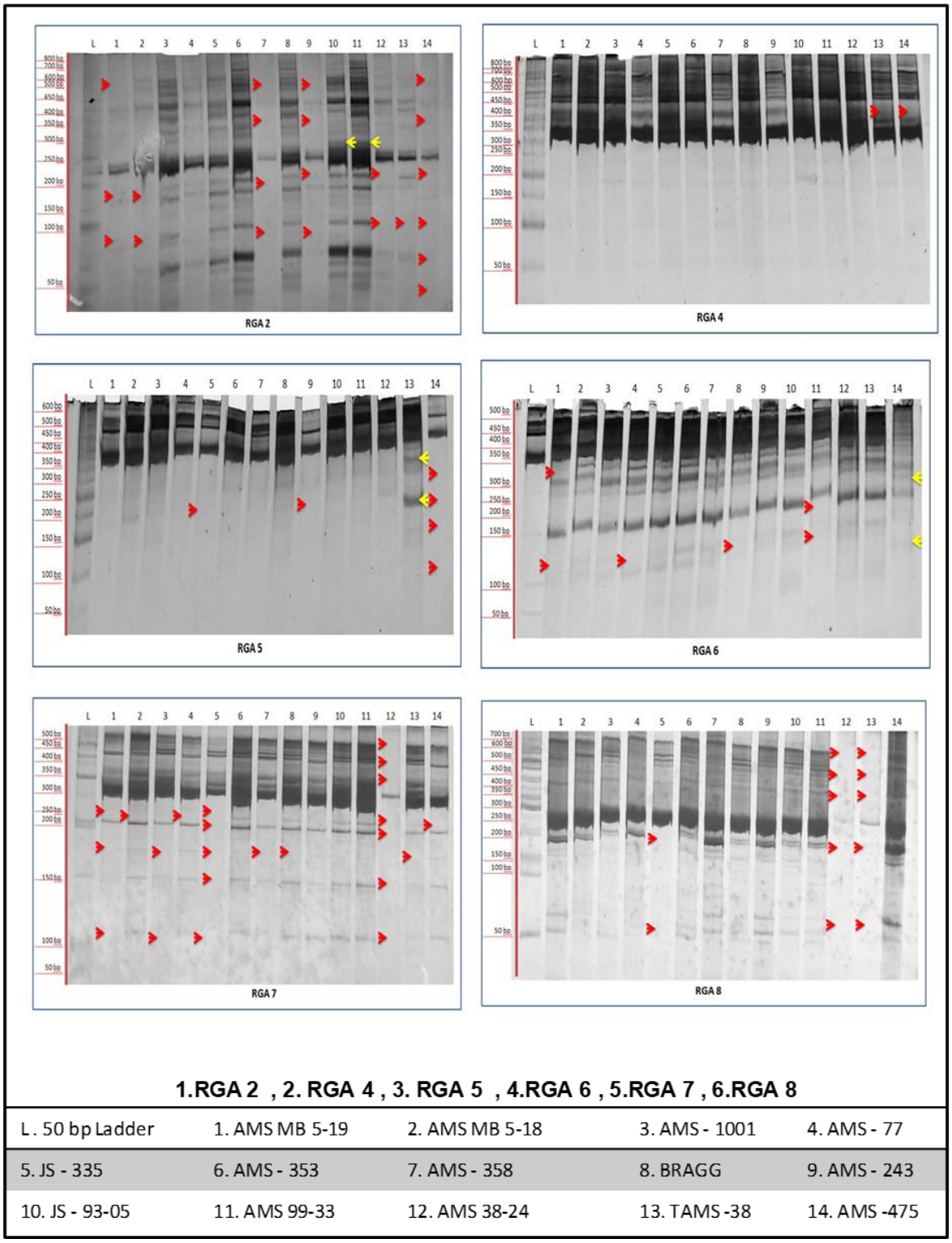
Table 2: List of RGA primers used in experiment

S.N	Primer	Forward sequence	Reverse sequence	Base pair
1	RGA 1	AGTTTATAATTCCATTGCT	ACTACGATTCAAGACGTCCT	39
		AGTTTATAATTCGATTGCT		39
		AGTTTATAATTTCATTGCT		39
		AGTTTATAATTTGATTGCT		39
2	RGA 2	AGTTTATAATTCCATTGCT	CACACGGTTTAAAATTCTCA	39
		AGTTTATAATTCGATTGCT		39
		AGTTTATAATTTCATTGCT		39
		AGTTTATAATTTGATTGCT		39
3	RGA 3	AGTTTATAATTCCATTGCT	CTCTCGATTCAAAATATCAT	39
		AGTTTATAATTCGATTGCT		39
		AGTTTATAATTTCATTGCT		39
		AGTTTATAATTTGATTGCT		39
4	RGA 4	TGTTACTGCTTTGTTTGGTA	TACATCATGTGTTACCTCT	39
5	RGA 5	TGCTAGAAAAGTCTATGAAG	TCAATCATTTCTTTGCACAA	40
6	RGA 6	AGCCAAAGCCATCTACAGT	AACTACATTTCTTGCAAGT	38
7	RGA 7	AGTTTATAATTCCATTGCT	CCGAAGCATAAGTTGCTG	37
		AGTTTATAATTCGATTGCT		37
		AGTTTATAATTTCATTGCT		37
		AGTTTATAATTTGATTGCT		37
8	RGA 8	AGCGAGAGTTGTATTTAAG	AGCCACTTTTGACAACTGC	38

DNA isolation and PCR reactions

Genomic DNA of the 14 genotypes was extracted from seed powder using the Dellaporta method described by Stephen L. Dellaporta 1983 with minor modifications. All PCR reactions were performed within a total volume of 20ul in 96-well plates using Eppendorf thermocycler. PCR reaction mixture containing 10X PCR buffer (Himedia), 10mM of each deoxyribonucleotide triphosphate (Himedia), 5U of Taq

polymerase (Himedia), and 10 pcm of primer. The PCR amplifications of the genotypes were performed in a 20µl reaction volume. Each reaction contained template genomic DNA. A standard PCR cycle was used with an initial denaturation step at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 50°-60 °C for 30 sec, and 72 °C for 1 min; the final extension at 72 °C was held for 5 min and hold at 4 °C. The annealing temperatures however, varied from primer to primer; hence optimization was done wherever required. Analysis of the amplified PCR products were further analyzed with the help of PAGE (Plate 1)



L . 50 bp Ladder	1. AMS MB 5-19	2. AMS MB 5-18	3. AMS - 1001	4. AMS - 77
5. JS - 335	6. AMS - 353	7. AMS - 358	8. BRAGG	9. AMS - 243
10. JS - 93-05	11. AMS 99-33	12. AMS 38-24	13. TAMS -38	14. AMS -475

Plate 1: Electrophoresis banding pattern of PCR amplified product resolved on 10% PAGE

Results and Discussions

Molecular characterization was done by using RGA primers and amplicons were scored as present (1) and absent (0) or as a missing observation for each genotype. Genotypes were assigned a null allele for a locus, whereas, an amplification product could not be decreased for a particular genotype. The reaction of the marker was measured and the Polymorphism Information content (PIC) and polymorphic% were calculated using software available at (www.liverpool.ac.uk.)The frequency of the null allele was not included in the calculation of PIC value and polymorphic percentage as given in Table 3.

Table 3: Molecular characterization of selected soybean genotypes using RGA primers

SN	Primer	No. of amplicon	Monomorphic alleles	Polymorphic alleles	Polymorphism (%)	PIC value
1	RGA 1	5	1	4	80	0.4416
2	RGA 2	7	2	5	71.43	0.7203
3	RGA 4	4	3	1	25	0.7031
4	RGA 5	5	1	4	80	0.5926
5	RGA 6	4	1	3	75	0.4388
6	RGA 7	6	2	4	66.67	0.6449
7	RGA 8	7	3	4	57.14	0.8397
•	Total	38	13	25	455.24	4.381
•	Average	5.42	1.85	3.57	65.03	0.62

Highest polymorphism was seen in primer RGA 1 and RGA 5(80%) followed by RGA 2 (71.43%). Lowest polymorphism was seen in primers RGA 4(25%). observations showed that in total 38 amplicons were tested with an average of 5.42 alleles per locus. Out of the total screened alleles 13 were monomorphic alleles with an average of 1.85 and 25 were polymorphic alleles with an average of 3.57. Results showed an average of 65.03 polymorphism percent. The PIC (Polymorphic information content) value ranged from 0.43 to 0.83 with an average value of 0.62.

Conclusion

Selective genotyping may be useful to see the association between genetic diversity and phylogenetic data, otherwise segregating population will have to screen. However, point mutations cannot be/very rarely detected by the RGA marker, considering this different approaches like single stranded confirmation polymorphism (SSCP), Endonucleolytic Mutation Analysis by Internal Labelling (EMAIL), High resolution melting (HRM), Heteroduplex, should be used to investigate the important point mutation in functional gene. The polymorphic marker identified in the present investigation for the characterization of promising genotypes can be further explored to see the association with any desired character.

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