*Indian J. Genet*., 75(4): 490-496 (2015)
DOI: 10.5958/0975-6906.2015.00078.4

# Biochemical screening for trypsin inhibitor factors and morpho-molecular characterization of soybean (*Glycine max* L. Merr.)

**Khushbu Verma[1], Akshay Talukdar*, M. Shivakumar[2], Bhupender Kumar[3], S. K. Lal, Nidhi Srivastava[1], R. L. Sapra, G. D. Harish and Vanishree Girmilla[4]**

Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi 110 012; [1]Department of Bioscience and Biotechnology, Banasthali, Tonk 304 022; [2]ICAR-Directorate of Soybean Research, Khandwa road, Indore 452 001; [3]Cummings's Lab., ICAR-Indian Institute of Maize Research, Pusa Campus, New Delhi 110 012; [4]ICAR-Central Potato Research Institute, Shimla 171 001

(Received: June 2014; Revised: September 2014; Accepted: October 2014)

**Abstract**

**A set of 101 diverse soybean genotypes were biochemically analyzed for trypsin inhibitor (TI) contents, and were characterized with morphological and molecular markers. The TI content in the seeds ranged from 14.65 to 175.52 mg $g^{-1}$ soymeal. The Indian soybean varieties were found to contain higher amount of TI (58.79-126.78 mg $g^{-1}$ soymeal) while PI542044, an exotic germplasm was found to contain the least (14.65 mg $g^{-1}$ soymeal). Morphological characterization with 16 qualitative and quantitative traits revealed significant variations and grouped the genotypes in to three major groups with overall mean dissimilarity value of 0.30. Genetic diversity study conducted with 100 simple sequence repeat (SSR) markers detected higher level of polymorphism (71%). Of the total 221 alleles amplified, 65 were rare (frequency <0.2). The mean polymorphism information content (PIC) of the markers was 0.27 while the value for gene diversity was 0.44. UPGMA-based cluster analysis grouped the genotypes into four major clusters of which I, II and III were occupied by genotypes containing higher and moderate level of TI. PI542044, which was free from Kunitz trypsin inhibitor (KTI), grouped singly. Such genotype would be suitable for use in breeding program directed towards development of varieties with zero KTI.**

**Key words:** Soybean, trypsin inhibitor, simple sequence repeats, morphological diversity, genetic diversity

## Introduction

Soybean [*Glycine max* (L.) Merr.] ranks first among the oilseeds crops in India. Globally, India ranks fifth in terms of area and production of soybean. This "miraculous bean" contains about 37-42% good quality protein, 6% ash, 29% carbohydrate and 17-24% oil comprising 85% poly-unsaturated fatty acid with two essential fatty acids (linoleic and linolenic acid) (Balasubramaniyan and Palaniappan, 2003). However, it contains various anti-nutritional factors, which limit its straight utilization in food and feed uses (Streit et al. 2001). One of such major anti-nutritional factor present in soybean seed is trypsin inhibitor (TI) (Westfall and Hauge, 1948). The most common of the TIs is the Kunitz trypsin inhibitor (KTI-20kDa) which constitutes about 80 per cent of the trypsin inhibitor activity. Not single Indian soybean varieties are free from TI (Kumar et al. 2001). Therefore, it is important to identify soybean genotypes that will be useful in breeding program directed towards reduction of TI in soybean (Shivakumar et al. 2015).

Assessment of the genetic relationships facilitates selection of diverse parental combinations and introgression of desirable genes or chromosome segments from diverse sources into elite germplasm (Iqbal et al. 2008). Diversity, based on the morphological characters reveals important traits to the plant breeders (Singh, 1989); however, manifestation of the phenotypes is highly influenced by the environmental variables. Molecular characterization can provide additional information about degree of diversity and genetic constitution of the genotypes. Among many molecular marker systems, simple sequence repeats (SSRs) are suitably preferred for genetic analysis because of their

*Corresponding author's e-mail: akshay.talukdar1@gmail.com
Published by the Indian Society of Genetics & Plant Breeding, F2, First Floor, NASC Complex, PB#11312, IARI, New Delhi 110 012
Online management by indianjournals.com

abundance and uniform distribution in the genome (Matsuoka et al. 2002, Liu et al. 1996), high variability (Tautz 1989), co-dominant inheritance and multiallelic amplification (Chin et al. 1996). The assay is less laborious and relatively inexpensive (Pinto et al. 2003). The present investigation was therefore conducted for biochemical screening of 101 soybean genotypes for TI contents and to characterize it with morphological and molecular (SSR) markers.

## Materials and methods

### *Biochemical screening*

A set of 101 soybean genotypes comprising of Indian varieties, breeding lines and exotic germplasm lines were biochemically analyzed for estimation of TI following procedure as described by Shivakumar et al. 2015. The TI estimation was worked out as per Hamerstrand et al. (1981).

$$\text{TI (mg g}^{-1}\text{ sample)} = \frac{\text{Differential absorbance}}{0.019 \times 1000} \times \frac{DF}{100} \times \text{sample (ml); where, DF = Dilution factor}$$

### *Phenotyping*

The soybean genotypes were morphologically evaluated during *kharif* 2010 and *kharif* 2011 at the experimental field of the Division of Genetics, ICAR-Indian Agricultural Research Institute (IARI), New Delhi following augmented design of experiment. The entries were sown in raised rows each of 3m length with 45cm spacing between rows and 10cm between plants. Observations were recorded on 16 traits including both qualitative (seed testa colour, flower colour, leaf shape, leaf colour intensity, plant growth type, plant growth habit, pod hair colour and seed hilum colour) and quantitative characters (plant height, days to 50% flowering, days to maturity, number of pod per plant, number of seed per pod, number of primary branches, 100 seed weight and seed yield) from five randomly selected plants of each genotype. The genotypes were characterized as per the DUS guidelines (Anonymous, 2009).

### *Molecular genotyping*

For molecular characterization, total genomic DNA was isolated from 2-3 week old seedlings using CTAB procedure (Saghai-Maroof et al. 1984) with minor modification. Based on quantification results a working stock of 10ng/µl prepared using the mother stock. A set of 120 SSR markers selected from across the genome were used for analysis of genetic diversity. Only 100 markers (av. 5 markers/chromosome), which amplified clear and unambiguous bands were used for further studies. The sequences of the markers (primers) were downloaded from www.soybase.org and were synthesized locally. Purified DNA was subjected to PCR amplification in 20µl reaction mixture containing 5.0µl DNA (10ng/µl), 2.0µl PCR 10x buffer, 2.0µl dNTPs (25mM), 2.0µl each forward and reverse SSR primers (30ng/µl), 0.3µl *Taq* DNA polymerase (3U/µl) and 6.7µl doubled distilled water. Amplification of the template DNAs was performed in thermocycler (Applied Biosystem) as per the following profile: the DNA was denatured at $94^0$C for 2 min. followed by 35 cycles each consisting of denaturation at $94^0$C for 1 min., primer annealing at $49^0$C for 2 min. primer elongation at $72^o$C for 3 min. Final elongation of the amplicons was allowed to complete at $72^o$C for 10 min which was finally put on hold at $4^0$C. Amplified products so obtained were resolved on 3% metaphor agarose gel stained with ethidium bromide and analyzed in Gene Genius Gel Imaging System from Syngene.

### *Data analysis*

Phenotypic and genotypic data recorded were analyzed and grouping of genotypes was done using GGT 2.0 software (Van Berloo, 2008) to produce an agglomerative hierarchical classification by employing Unweighted Paired Group Method using Arithmetic Averages (UPGMA). Scoring of the SSR alleles was performed manually in terms of positions of the bands relative to the ladder sequentially from the smallest to the largest-sized bands. Diffused bands or bands revealing ambiguity in scoring were considered as missing data and designated as 'V' in comparison with 'A' for the presence of the most frequent alleles followed by 'B' for the second and 'C' for the third most frequent alleles etc. Genotypes showing two allelic bands with equal intensity were considered as heterozygous for the locus. The polymorphism information content (PIC) was determined as described by Senior and Heun (1993), given as PIC = $1-\Sigma P_{ij}^2$, where $P_{ij}$ is the frequency of jth allele at ith locus summed across all alleles in the locus. Alleles with frequency of less than 0.20 were considered as rare alleles. Jaccard's coefficient (J) (Jaccard, 1908) was used to calculate the genetic similarities among pair wise comparison of genotypes based on SSR data, as follows: J = N11/(N11 +N10 + N01) where N11 is the number of bands present in both genotypes; N10

is the number of bands present in one genotype (lane) and N01 is the number of bands present in the other genotype. Genetic distances between the genotypes were calculated using 1-coefficient of genetic similarity. Data from 100 SSR markers were collected for further analysis of major allele frequency, allele number, number of rare alleles, gene diversity and polymorphic information content (PIC) were estimated using PowerMarker V3.0 (Liu and Muse 2005).

## Results and discussion

### *Trypsin inhibitor (TI) content*

Biochemical analysis revealed wider variability among the soybean genotypes for TI contents which ranged from 14.65 to 172.52 mg $g^{-1}$ seed meal. Lowest amount of TI (14.65 mg $g^{-1}$ seed meal) was found in PI542044, while highest TI content was detected in the genotype EC389392 (172.52 mg $g^{-1}$ seed meal) followed by EC25720 (170.68 mg $g^{-1}$ seed meal) and EC439606 (168.86 mg $g^{-1}$ seed meal) (Table 1). Popular Indian varieties used in this study *viz*., DS9712, DS9814, JS335, PK416, SL688 and BRAGG found to have higher TI levels i.e. 95.68, 126.78, 115.68, 77.46, 66.88 and 58.79 mg $g^{-1}$ seed meal, respectively. However, UPSL-573-B (41.95 mg $g^{-1}$ seed meal), MACS694 (45.62 mg $g^{-1}$ seed meal), UPSL-207 (45.80 mg $g^{-1}$ seed meal), SL528 (50.62 mg $g^{-1}$ seed meal), AVRDC5 (56.88 mg $g^{-1}$ seed meal) and UPSV-32 (58.37 mg $g^{-1}$ seed meal) contained moderate level of TI content. Kumar et al. (2001) and Manjaya et al. (2007) also reported moderate to higher level of TI contents in some other soybean genotypes of India. PI542044, an exotic germplasm contained least amount of TI (14.65 mg $g^{-1}$ seed meal), which is in agreement with the findings of Shivakumar et al. (2015). The amount of TI found in PI542044 represents the Bowman- birk fraction of TI, retention of which is considered to be desirable for health benefits of the consumer Rani et al. (2011). Thus, the genotype PI542044 is free from KTI and will be suitable donor in breeding program for developing genotypes with zero KTI. Applicability of this genotype in this respect has recently been established (Talukdar et al. 2014). Genotypes with moderate to lower level of TI would be highly preferred by soy-food industries as it would reduce the industrial cost by taking away the cost of pre-heat treatment of soy-flours. Further, such genotypes would be fit to eat raw or without pre-heat treatment. It would boost consumption of food grade soybean and thus open up vistas for economic benefit to the farmers.

### *Morphological characterization*

Analysis of the morphological data for 16 qualitative and quantitative traits over two years of experimentation indicated existence of significant variability among the genotypes. Genetic dissimilarity index among the genotypes was 0.30. Besides other traits, significant variation was observed for yield/plants (g). A set of genotypes *viz*., TGX1855-530 (29.95g), EC471975 (27.05g), EC457166 (23.55g), DS9814 (23.14g), DS2101 (22.81g), UPSL152 (21.86) and UPSL505 (21.62g) had higher yield than five nationally popular varieties such as JS335 (20.43g), SL688 (20.03g), DS9712 (19.76g), PK416 (19.65g) and BRAGG (17.71g). However, the genotypes had medium to higher content of TI.

The resulting dendrogram grouped the genotypes into three major clusters. Cluster I consisted of 44 genotypes while cluster II and III had 11 and 46 genotypes, respectively. PI542044, which is KTI free and susceptible to YMV diseases clustered together with other popular Indian varieties *viz*., DS9814, DS9712, Bragg, JS335 etc. in cluster III but in different sub-clusters. Genetic distance between PI542044 and other genotypes ranged from 0.20 to 0.73. The genotypes with high, moderate and low trypsin inhibitor contents overlapped in different phenotypic groups or sub-groups. Thus, phenotype of the genotypes seems to have no linkage with TI contents. Clusters based on phenotypic traits would help breeders to select diverse genotypes for breeding. Ojo et al. (2012) evaluated 40 soybean genotypes for seven morphological characters and obtained seven clusters. Groupings of the genotypes however, are highly influenced by environmental factors. Therefore, variations in grouping of genotypes are inevitable. It emphasizes need of analytical procedures like molecular characterization that are free from environmental influences.

### *Polymorphism pattern*

A total of 100 SSR markers covering entire soybean genome were used to amplify genomic DNA of the soybean genotypes varying in trypsin inhibitor content and morphological characters. Among the markers used, 71 were polymorphic and rests were monomorphic. Thus, the level of polymorphism was 71%. Higher level of polymorphism was the result of inclusion of diverse soybean genotypes. The SSR markers used to amplify the genomic DNA segment had primarily dinucleotide and trinucleotide motifs. The

**Table 1.** Trypsin inhibitor contents in exotic lines, breeding lines and varieties of Soybean

| S.No. | Genotype | TI (mg $g^{-1}$) | S.No. | Genotype | TI (mg $g^{-1}$) | S.No. | Genotype | TI (mg $g^{-1}$) | S.No. | Genotype | TI (mg $g^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Exotic lines** | | | | | | | | | | | |
| 1 | EC439606 | 168.86 | 10 | EC456566 | 132.48 | 19 | EC456620 | 60.15 | 28 | EC471978 | 102.34 |
| 2 | EC39779 | 113.49 | 11 | EC471979 | 107.64 | 20 | EC472132 | 104.56 | 29 | EC456625 | 66.56 |
| 3 | EC109565 | 163.03 | 12 | EC483062 | 158.12 | 21 | EC457403 | 166.82 | 30 | EC44303 | 162.26 |
| 4 | EC457166 | 92.25 | 13 | EC439608 | 122.62 | 22 | EC471936 | 91.22 | 31 | EC439618 | 156.78 |
| 5 | EC389392 | 172.52 | 14 | EC471881 | 141.64 | 23 | EC457322 | 114.32 | 32 | EC471981 | 86.72 |
| 6 | EC471975 | 161.89 | 15 | EC472116 | 132.36 | 24 | EC471843 | 142.25 | 33 | EC14427 | 132.65 |
| 7 | EC25720 | 170.68 | 16 | EC472145 | 62.34 | 25 | EC471882 | 140.62 | 34 | PI542044 | 14.65 |
| 8 | EC114526 | 141.34 | 17 | EC18761 | 59.65 | 26 | EC439619 | 120.36 | 35 | EC472125 | 71.35 |
| 9 | EC471973 | 88.96 | 18 | EC50064 | 77.75 | 27 | EC1021 | 145.32 | | | |
| **Breeding lines and varieties** | | | | | | | | | | | |
| 36 | UPSL152 | 62.36 | 53 | SL528 | 50.62 | 70 | JUPITER | 92.36 | 87 | M-1094 | 97.58 |
| 37 | L-652 | 81.52 | 54 | PK1240 | 77.35 | 71 | TGX1855-530 | 87.14 | 88 | PUSA37 | 96.78 |
| 38 | KG-83-1 | 76.13 | 55 | PS1410 | 101.78 | 72 | UPSL505 | 62.95 | 89 | RAUS972 | 72.76 |
| 39 | TGX1873-14E | 77.14 | 56 | PK1251 | 96.78 | 73 | PK1135 | 80.95 | 90 | G2631 | 107.67 |
| 40 | MACS694 | 45.62 | 57 | BJJF-8 | 102.91 | 74 | UPSL-207 | 45.80 | 91 | G-2602 | 98.99 |
| 41 | M253 | 86.58 | 58 | M-53 | 106.78 | 75 | JS-SH-93-01 | 82.67 | 92 | KYUNG. NWOR2 | 111.59 |
| 42 | PK1225-A | 91.92 | 59 | AMSS34 | 66.32 | 76 | UPSE-2048 | 105.38 | 93 | G406 | 86.56 |
| 43 | E-20 | 100.52 | 60 | AVRDC5 | 56.88 | 77 | KHSB-3 | 97.89 | 94 | MACS450 | 66.78 |
| 44 | PKV-25 | 63.85 | 61 | NRC1180 | 73.16 | 78 | NLS-57 | 100.24 | 95 | DS9712 | 95.68 |
| 45 | TS40 | 62.82 | 62 | PS1374 | 97.98 | 79 | PLS-6A | 112.26 | 96 | DS9814 | 126.78 |
| 46 | MACS985 | 68.72 | 63 | SL432 | 70.97 | 80 | L377 | 107.33 | 97 | PK416 | 77.46 |
| 47 | JS-SH40 | 81.65 | 64 | SL295 | 66.86 | 81 | PS1392 | 103.56 | 98 | SL688 | 66.88 |
| 48 | PK564 | 78.32 | 65 | PK444 | 98.89 | 82 | BR-3 | 132.42 | 99 | BRAGG | 58.79 |
| 49 | DS2101 | 126.74 | 66 | UPSM-534 | 78.46 | 83 | MONETA | 111.92 | 100 | JS335 | 115.68 |
| 50 | PUSA16 | 82.19 | 67 | UPSV-32 | 58.37 | 84 | JS-SH-98-22 | 95.83 | 101 | M-1094 | 97.58 |
| 51 | HIS-01 | 101.36 | 68 | UPSL-29 | 69.05 | 85 | HIMSO-1598 | 123.34 | | | |
| 52 | LEE | 95.33 | 69 | UPSL-573-B | 41.95 | 86 | DS-76-1-2-2 | 105.68 | | | |

For Exotic lines: Mean= 118.47; Range=14.65-172.52; SE=±6.74; SD=39.8. For Breeding lines and varieties: Mean= 87.02; Range=41.95-132.42; SE=±2.61; SD=21.39

dinucleotides repeat motif SSRs were found to be more polymorphic than the trinucleotides repeat motif SSRs. The 21 out of 25 dinucleotide repeat motif SSRs (84%) and 47 out of 69 trinucleotide repeat motif SSRs (68.11%) were found to be polymorphic. The markers amplified 221 alleles across the genotypes evaluated. The number of alleles per marker locus varied from two to five with an average of 2.21. The gene or genetic diversity value ranged from 0.05 for Satt386 (Chr. 17) to o.78 for Sat_420 (Chr. 20). The average gene diversity value was found to be 0.44. Only five markers viz.Sat_420, Sat_413, Sat_389, Satt175 and Satt114 located on chromosome 20, 7, 1 and 13 had gene diversity value more than 0.70. The PIC value of the markers ranged from 0.04 for Satt386 to 0.75 for Sat_420 with an average of 0.27. In this study, 51 out of 100 SSR markers (51%) had PIC value more than 0.3 highlighting discriminating power of the markers. A set of 17 SSR markers had PIC value more than 0.50. Only four markers *viz*., Sat_413, Sat_389,

Satt175 and Sat_420 had PIC value more than 0.70 (Fig. 1). Thus, the distribution of alleles per marker locus and corresponding PIC values was random. Amplification pattern of SSR alleles as resolved through metaphor gel has been shown in Fig. 2. The level of diversity and PIC values reported by various workers was found to vary with the experimental material as well as with type of markers used. Therefore, it was hard to establish a specific relationship between the alleles/locus and the PIC values observed. Kaga et al. (2012) observed gene diversity value (0.28) among 96 genotypes using 963 SNP markers which is less than the value observed in the present study. Kumar et al. (2014) analyzed genetic diversity in 96 genotypes using 121 SSR markers and observed 2.36 alleles/ locus and average PIC value 0.32 which is comparable with the values obtained in the present study. In general, Indian soybeans are less diverse and their genetic base is extremely narrow (Karmakar and Bhatnagar, 1996), hence, their inclusion in large numbers might reduce the variability. Indian soybean germplasm constitutes direct introductions from USA and China (Taiwan), selection from the introduced germplasm and the single cross hybrids (two parent crosses) developed through crossing among these introductions (Tiwari, 2014). Further, the process of domestication and dissemination also contributes towards reduction of genetic diversity (Gepts, 2004; Zeder et al. 2006). Alleles with a frequency of less than 0.2 were considered as rare alleles. In this investigation, 65 rare alleles were produced by 47 markers. The rare alleles are useful for plant breeders and geneticists as a rich source of genetic diversity for crop improvement (Lapitan et al. 2007).

### *Genotyping and cluster analysis*

The genomic DNA extracted from the leaf samples of the genotypes were subjected to molecular genotyping with SSR markers and the data so obtained were used to estimate genetic co-relatedness among the genotypes. The pair-wise mean genetic dissimilarity value as calculated by Jaccard's coefficients was 0.50, which indicated existence of enormous genetic variability among the genotypes. The dendrogram constructed with the molecular data had four major clusters (Fig. 3). Cluster I was the major one consisting of 46 genotypes. However, it had three sub-clusters within it. Cluster II and III consisted of 22 and 27 genotypes, respectively. Cluster IV had only one genotype i.e. PI542044, which is free from KTI. The genetic distance between PI542044 and other genotypes ranged from 0.37 to 0.58. The average genetic dissimilarity between the genotypes in Cluster I, II and III with PI542044 were found to be more than 50%.

While comparing grouping of the genotypes based on morphological and molecular data, it was

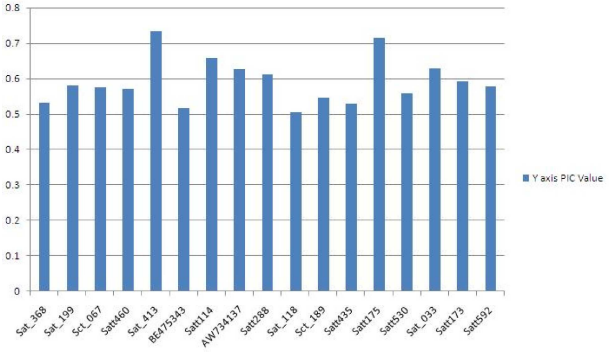


**Fig. 1. Distribution of PIC values (>0.50) of a set of SSR markers used in the study**

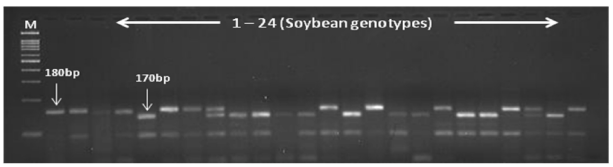


**Fig. 2. Gel showing amplification of genomic DNA by SSR marker (Sct_067). Lane 1-24: different genotypes, M = 1.5 Kb Ladder**

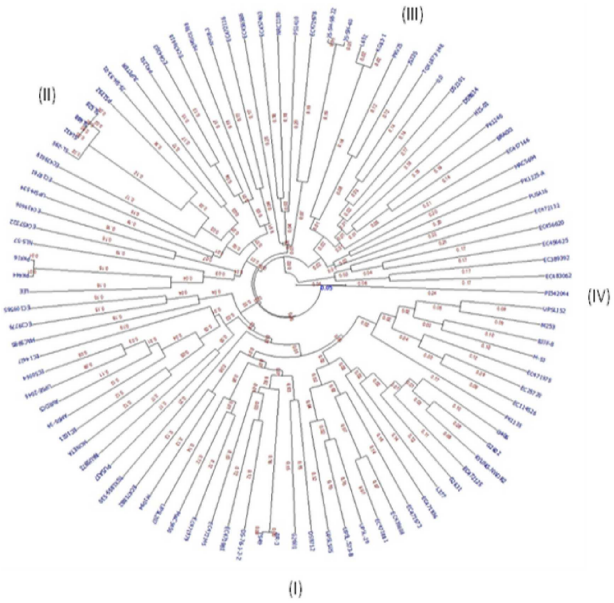


**Fig. 3. Clustering of the genotypes based on molecular data. Jaccard's coefficient value on the nodes indicates dissimilarity between the genotypes**

observed that numbers of groups formed were more with molecular data. As the molecular profile of a genotype do not change with the environment, hence finer and accurate groupings of the genotypes were obtained with the marker data. It clustered PI542044 separately in an independent cluster. However, in morphological analysis, it clustered with other genotypes. It thus revealed that genetic variability is analyzed more precisely with molecular markers than morphological data.

The grouping of the genotypes however did not follow any specific pattern with respect to its place of collection or geographical origin. All the exotic collections from Indonesia, Japan, Korea, Brazil, USA and Taiwan overlapped with Indian cultivars in different groups or sub-groups. Das et al. (2001) also reported non-parallelism between genetic divergence and geographical distribution of the genotypes. Such mismatch may be due to the inadequate pedigree records or genetic admixture encountered in breeding programs (Kumar et al. 2008). Sihag et al. (2004) obtained no relationship between genetic diversity and geographic diversity. Uncontrolled movement of the genotypes between various geographical locations might have resulted in mixing of the genotypes leading to loss of geographical identity of the genotypes.

Thus, the present study could generate information regarding TI contents of a number of soybean genotypes including Indian varieties. It could also identify soybean genotype containing least amount of TI. The genotypes were further characterized through morphological and molecular markers so as to establish their genetic interrelationship. The genotypes identified in this study would be good starting materials for developing soybean varieties free from KTI.

**Acknowledgements**

Financial support received from DBT, Govt. of India and the facilities provided by ICAR-Indian Agricultural Research Institute including National Phytotron Facility and V. Kumar (DSR, Indore) is duly acknowledged.

**References**

Anonymous. 2009. The Soybean Processors Association of India, (SOPA), Indore, M.P. PP. 2-6.

Balasubramaniyan P. and Palaniappan S. P. 2003. Principles and practices of agronomy. Pub. By agrbios (India). Field crops. An overview. Chapter 1. PP. 45-46.

Chin E. C. L., Senior M. L., Shu H. and Smith J. S. C. 1996. Maize simple repetitive DNA sequences: abundance and allele variation. Genome, **39**: 866-873.

Das S. P., Harer P. N. and Biradar A. B. 2001. Genetic divergence and selection of genotypes in soybean. J. Maharashtra Agricultural Universities, **25**: 250-252.

Gepts P. 2004. Crop domestication as a long term selection experiment. *In*: J. Janick (ed). Plant Breed Rev., **24**: 1-44.

Hamerstrand G. E., Black L. T. and Glover J. D. 1981. Trypsin inhibitors in soy products: Modification of the standard analytical procedure. Cereal Chemistry, **58**: 42-45.

Iqbal Z., Arshad M., Ashraf M., Mahmood T. and Waheed A. 2008. Evaluation of soybean (*Glycine max* (L.) Merrill) germplasm for some important morphological traits using multivariate analysis. Pak. J. Bot., **40**: 2323-2328.

Jaccard P. 1908. Nouvelles researches sur la distribution florate. Bull. Soc. Vaudolse Sci. Nat., **44**: 223-270.

Kaga A., Shimizu T., Watanabe S., Tsubokora Y., Katayose Y., Harade K., Vaughan D. A. and Tomooka N. 2012. Evaluation of soybean germplasm conserved in NIAS genebank and development of mini core collections. Breed Sci., **61**: 566-592.

Karmakar P. G. and Bhatnagar P. S. 1996. Genetic improvement of soybean varieties released in India from 1969 to 1993. Euphytica, **90**: 95-103.

Kumar B., Rakshit S., Singh R. D., Gadag R. N., Ravindra N., Paul A. K. and Wasialam. 2008. Genetic diversity of early maturing Indian maize (Zea mays L) inbred lines revealed by SSR markers. J. Pl. Biochem Biotech., **17**: 133-140.

Kumar B., Talukdar A., Verma K., Girimilla V., Bala I., Lal S. K., Singh K. P. and Sapra R. L. 2014. Screening of soybean [*Glycine max* (L.)Merrill.] genotypes for yellow mosaic virus(YMV) disease and their molecular characterization using RGA and SSRs markers. Australian J. Crop Sci., **8**: 27-34.

Kumar V., Rani A., Mourya V., Reena R., Khushbu V., Shivakumar M., Lal S. K. and Talukdar A. 2011. Maker assisted selection for development of kunitz trypsin inhibitor free soybean varieties: parental polymorphism study, a few considerations. Indian J. Genet., **71**: 372-376.

Kumar V., Rani A. and Tiwari S. P. 2001. Comparative activity of trypsin inhibitor among released soybean varieties/strains of India. Ind. J. Nut. Diet., **38**: 437-440.

Liu K. and Muse S. V. 2005. PowerMarker: integrated analysis environment for genetic marker data. Bioinformatics, **21**: 2128-2129.

Liu Z. W., Biyashev R. and Saghai-Maroof M. 1996. Development of simple sequence repeats DNA

markers and their integration into a barley linkage map. Theor. Appl. Genet., **93**: 869-876.

Manjaya T., Gopalakrishna S. E., Pawar and Bapat V. A. 2007. Genetic variability for trypsin inhibitor content in soybean [*Glycine max* (L.) Merrill.] and its correlation with oil and protein. Indian J. Genet., **67**: 51-55.

Matsuoka Y., Mitchell S. E. and Kresovich S. 2002. Microsatellites in Zea-variability, patterns of mutations and use for evolutionary studies. Theor. Appl. Genet., **104**: 436-450.

Ojo D. K., Ajayi A. O. and Oduwaye O. A. 2012. Genetic Relationships among Soybean Accessions Based on Morphological and RAPDs Techniques. Pertanika J. Trop. Agric. Sci., **35**: 237-248.

Pinto L. R., Vieira M. L. C., De Souza C. L. et al. 2003. Genetic diversity assessed by microsatellites in tropical maize population submitted to high density reciprocal recurrent selection. Euphytica, **134**: 277-286.

Rani A., Kumar V., Mourya V., Singh R. K. and Husain S. M. 2011. Validation of SSR markers linked to null kunitz trypsin inhibitor allele in Indian soybean [*Glycine max* (L.) Merrill.] population. J. Plant Biochem. Biotechnol., **20**: 258-261.

Saghai-Maroof M. A., Soliman K. M., Jergensen R. A. and Allard R. W. 1984. Ribosomal DNA spaces-length polymorphism in barley Mendelian inheritance, chromosomal location, and population dynamics. ProcNatlAcadSci USA, **81**: 8014-8018.

Senior M. L. and Heun M. 1993. Mapping maize microsatellites and polymerase chain reaction confirmation of the targeted repeats using a CT primer. Genome, **36**: 884-889.

Shivakumar M., Verma K., Talukdar A., Srivastava N., Lal S. K., Sapra R. L. and Singh K. P. 2015. Genetic variability and effect of heat treatment on trypsin inhibitor content in soybean [*Glycine max* (L.) Merrill]. Legume Res., **38**: 60-65.

Sihag R., Hooda J. S., Vashishtha R. D. and Malik B. P. S. 2004. Genetic divergence in soybean [*Glycine max* (L.) Merrill]. Ann. Biol., **20**: 17-21.

Singh S. P. 1989. Pattern of variation in cultivated dry bean. Annual Rep. Bean improvement. Coop., **31**: 180-182.

Streit L. G., Beach L. R., Register III J. C., Jung R. and Fehr W. R. 2001. Association of the Brazil Nut Protein Gene and Kunitz Trypsin Inhibitor Alleles with Soybean Protease Inhibitor Activity and Agronomic Traits. Crop Sci., **41**: 1757-1760.

Talukdar A., Shivakumar M., Verma K., Kumar A., Mukherjee K., Lal S. K. 2014. Genetic elimination of Kunitz trypsin inhibitor (KTI) from DS 9712, an Indian soybean variety. Indian J. Genet., **74**(4): Suppl. 608-611.

Tautz D. 1989. Hypervariablity of simple sequences as a general source of polymorphic DNA markers. Nucleic Acids Res., **17**: 6463-6471.

Tiwari S. P. 2014. Raising the yield ceiling in soybean - an Indian overview. Soybean Res., **12**(2): 1-43.

Van Berloo R. 2008. GGT 2.0: Versatile Software for Visualization and analysis of genetic data. J. Heredity, **99**: 232-236.

Westfall R. L. and Hauge S. M. 1948. The nutritive quality and the trypsin inhibitor content of soybean flour heated at various temperatures. J. Nutrition, **35**: 379-389.

Zeder M. A., Emshwiller E., Smith B. D. and Bardley D. G. 2006. Documenting domestication: the intersection of genetics and archeology. Trends Genet., **22**: 138-155.