Indian J. Genet., **67**(1): 51-55 (2007)

Genetic variability for trypsin inhibitor content in soybean [*Glycine max* (L.) Merrill.] and its correlation with oil and protein

J. G. Manjaya, T. Gopalakrishna, S. E. Pawar[1] and V. A. Bapat

Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400 085

(Received: April 2006; Revised: September 2006; Accepted: November 2006)

Abstract

Trypsin inhibitors are one of the most important antinutritional factors in soybean [*Glycine max* (L.) Merrill.]. They decrease the digestibility of protein and cause pancreatic hypertrophy. Fifty-five soybean genotypes including Indian cultivars were analysed for trypsin inhibitor activity (TIA). Mean TIA ranged from 14.49 mg g^{-1} seed meal to 29.05 mg g^{-1} seed meal. Genotypes PK-1042 (14.49) and EC-389178 (22.20) showed lower TIA mg g^{-1} seed meal. The studies showed no correlation of TIA with days to maturity, oil content, protein content and seed yield. Based on multivariate analysis, TIA showed maximum contribution to D^2 value indicating scope for selection of parents in a breeding programme.

Key words: Soybean, trypsin inhibitor, correlation, multivariate analysis

Introduction

Soybean is the most important grain legume in the world in terms of production and international trade. Its multivariate use as oil, feed, and other soy food products enable it to occupy a coveted place among the oil seed crops of the world. In India, soybean contributes about 10 per cent to the domestic edible oil pool and the country earns substantial foreign exchange through export of soy meal [1]. However, the presence of anti-nutritional factors in soybean exerts a negative impact on the nutritional quality of the protein [2]. Protease inhibitor is one of the important anti-nutritional factors that exert negative effect by causing pancreatic hypertrophy, hyperplasia that ultimately results in the inhibition of growth. Growth inhibition was observed in rats, chicks and mice when fed with purified extracts from soybean rich in trypsin inhibitors (TI) [3]. Huisman [4] reported high levels of trypsin inhibitor activity (TIA) in raw soybeans [50 trypsin inhibitor units (TIU mg^{-1} of dry matter)]. Thus, the presence of trypsin inhibitors limits the use of soybeans for human and animal consumption. Soybean needs to be treated with heat to reduce trypsin inhibitor concentration. However, thermal treatment does not eliminate the inhibitor completely and on the contrary over heating may affect the protein quality [5]. Hence, development of cultivars with low trypsin inhibitors will help to improve nutritional quality of soybean for export and domestic use. Genotypic variants for low trypsin inhibitor content have been reported globally [6-9].

In India, breeding efforts so far have been for increasing seed yield and resistance to various biotic and abiotic stresses. To facilitate the breeding of Indian cultivars for improved nutritional quality for export and domestic use, it was necessary to survey existing germplasm and released varieties for levels of TIA. Hence, the objectives of this study were to screen the soybean genotypes for levels of TIA and to determine the interrelationships among TIA, oil and seed protein content. A quantitative estimation of genetic diversity present among different genotypes helps the plant breeder for selecting diverse genotypes in hybridisation programme to develop useful plants. Therefore, attempts were also made to study magnitude of genetic divergence based on quality characters to facilitate the breeding of soybean cultivars for improved nutritional quality for export and domestic use.

Materials and methods

Plant material: The present investigation was carried out using 55 genotypes of soybean (Table 1). The foundation seeds of the released varieties were obtained from Dr. Punjabrao Deshmukh Krishi Vidyapeeth, Akola, and the seeds of germplasm lines from National Research Centre for soybean, Indore and National Bureau of Plant Genetics Resources, Akola. India. The field experiments were conducted at the experimental station of Bhabha Atomic Research Centre, Trombay, India. The experiments were conducted in replicated trials in a randomized block design with three replications. The matured seeds were used for biochemical studies. All chemicals used in this study

[1]Present address: CH2-44, Kendriya Vihar Coop. HSG Soc., Kharghar, Sector 11, Navi Mumbai 410 210

were of analytical grade and fine chemicals were procured from Sigma Chemical Company, USA.

Estimation of oil and protein content: Oil content of seed samples was estimated by solvent extraction using Soxhlet apparatus [Soxtec system - HT (1043)]. The nitrogen content of the seed was determined by the micro-Kjeldahl method [11] and the amount of total protein was calculated from percent nitrogen content using a conversion factor of 6.25.

Protein extraction and estimation: Dry seeds were soaked in water overnight at room temperature and 1 g of cotyledons (without testa) was extracted in 5ml of 50mM Tris-HC1 buffer, pH 8. The slurry was centrifuged at 17000g for 30 minutes in a Kubota Centrifuge 6800. The clear supernatant was collected and used for protein estimation and trypsin inhibitor assay. Protein was estimated by the Biuret method [12].

Trypsin and trypsin inhibitor assay: Trypsin activity was measured by using N-α-benzoyl DL-arginine p-nitroanilide (BAPNA) essentially according to the method described by Erlanger *et al.*, [13]. Trypsin stock solution was prepared and the concentration was determined based on an absorbance of 15.2 at 280nm for a 1% solution. The stock was diluted with buffer (0.05 M Tris-HCl, pH 8 containing 20mM $CaCl_2$) to prepare a 1 mg/ml working solution.

Trypsin (5-30μg) in a volume of 0.3ml was incubated at 25° C with 1 ml of 1mM BAPNA. After 10 min the reaction was stopped by adding 0.2ml of 30% (V/V) acetic acid. The liberated p-nitroanilide was measured at 410nm. Trypsin was assumed to be 100% active. For measuring the trypsin inhibitor (TI) activity, 20μg of trypsin was mixed with a suitable quantity of inhibitor (so as to give 50-60% inhibition) and incubated at room temperature for 5 minutes before measuring the residual trypsin activity as above. TIA was expressed in mg g^{-1} of seed meal assuming equimolar inhibition of trypsin by the inhibitor.

Statistical methods. Significance of various observations was tested using standard statistical methods. Analysis of variance, co-variance, 't' test and Chi-square test were applied and correlation co-efficients were calculated as described by Panse and Sukhatme [14]. Multivariate analysis was done by using Mahalanobis generalized distance D^2 [15] and clustering of genotypes was done by Tocher's method [16].

Results and discussion

Analysis of variance showed highly significant variation among the 55 genotypes for all the characters studied (Table 1). Oil content ranged from 13.19% to 20.72%.

Table 1. Means of different characters of soybean genotypes

Genotypes	Oil %	Protein %	Soluble protein (mg/ml)	Trypsin inhibitor activity mg g^{-1} seed meal
*Ankur	17.63	40.10	133.22	56.88
*Co-1	18.44	40.67	115.37	74.82
*JS 335	17.43	39.46	74.83	64.92
*JS 80-21	18.69	40.90	49.22	54.39
*JS 90-41	17.67	39.10	67.63	55.23
*MACS-58	17.75	40.25	127.74	57.36
*Monetta	17.28	42.09	125.69	53.94
*NRC 7	19.79	37.03	162.79	59.73
*NRC 12	18.41	38.36	156.38	64.11
*PK 472	17.62	39.08	25.80	62.25
*PK 1024	16.34	41.75	121.39	57.24
*PK 1042	16.61	40.83	44.94	14.49
*Punjab 1	17.28	40.20	125.91	47.07
*VL Soya 2	19.62	39.03	133.76	54.15
*VL Soya 47	18.37	40.23	88.84	52.26
JAW A 16	16.76	33.29	137.62	63.99
JS 220	17.95	32.53	59.75	47.79
P 4-2	18.61	38.52	150.53	66.15
UM 21	17.46	42.08	57.20	47.64
IC-202	18.11	38.51	77.20	56.07
IC-18758	14.53	35.00	107.31	83.85
IC-96297	19.39	38.06	69.89	45.36
IC-96245	17.72	40.46	77.63	78.00
IC-96382	17.71	41.12	114.83	59.46
1C-118041	13.19	39.19	89.45	43.35
1C-118047	15.45	40.61	110.31	87.15
1C-118053	15.62	40.32	68.59	65.64
1C-118054	15.55	37.43	122.79	68.28
1C-118058	15.17	41.48	122.25	57.00
IC-118183	17.02	43.07	70.53	67.32
1C-118268	16.61	42.30	107.09	69.51
1C-118296	18.00	39.44	92.98	83.91
EC-18735	19.32	39.06	114.06	44.19
EC-39076	17.29	40.36	116.55	63.06
EC-7724	18.58	40.15	115.37	58.32
EC-106992	17.36	40.33	126.12	51.06
EC-113394	18.54	41.16	105.37	57.45
EC-241755	20.10	37.56	97.56	50.64
EC-251358	18.65	38.56	91.17	48.00
EC-251523	18.66	39.50	94.18	83.16
EC-280125	15.50	43.23	114.61	54.36
EC-280132	19.73	34.51	102.68	64.59
EC-341755	18.38	37.62	146.66	62.52
EC-389148	15.54	42.26	82.79	69.00
EC-389159	15.84	40.61	86.87	84.78
EC-389165	17.22	41.26	72.89	47.28
EC-389170	17.63	39.66	133.43	48.96
EC-389178	18.50	40.10	106.23	22.20
EC-389179	16.68	38.60	82.89	85.50
EC-389392	18.85	37.20	48.43	57.81
EC-389400	20.72	37.98	107.20	66.87
EC-390981	16.89	40.11	87.09	84.75
EC-391172	17.97	37.92	138.66	71.94
EC-391181	19.15	37.67	68.81	54.42
EC-389166	17.28	41.23	100.85	62.31
S. E.	0.11	0.29	1.94	0.90
CD 5%	0.31	0.83	5.45	2.52
CD 1%	0.41	1.10	7.20	3.33
CV %	1.10	1.31	3.28	2.60

*Released varieties

High oil content was observed in the genotypes EC 389400 (20.72%), EC 241755 (20.10%) and EC 280132 (19.73%). The low oil content was observed in the genotypes IC 118041 (13.19%) and IC 18758 (14.53%). Seed crude protein content in the genotypes ranged from 32.53 to 43.23 % with mean value of 39.44%. Most of the genotypes exhibited higher seed crude protein content. The genotypes EC 280125 showed highest seed crude protein content (43.23%) followed by IC 118183 (43.07%), IC 118268 (42.30%), EC 389148 (42.26%), Monetta (42.09%) and UM 21 (42.08%). The lowest protein was observed in the genotype JS 220 (32.53%). The soluble protein content ranged from 44.94 to 162.79 mg/ml and mean value was 102.37 mg/ml. The higher level of soluble protein content was observed in the genotypes NRC-7 (162.79 mg/ml), NRC 12 (156.38 mg/ml) and P-4-2 (150.53 mg/ml). The lowest seed soluble protein content was recorded in the genotype PK 1042 (44.94 mg/ml).

TIA ranged from 14.49 mg g^{-1} seed meal to 29.05 mg g^{-1} seed meal. Low trypsin inhibitor content was observed in the genotypes PK 1042 (14.49 mg g^{-1} seed meal) and EC 389178 (22.20 mg g^{-1} seed meal). All other genotypes showed higher trypsin inhibitor content. The highest trypsin inhibitor content was observed in the genotypes 1C 118047 (87.15 mg g^{-1} seed meal), EC 389179 (85.50 mg g^{-1} seed meal) and EC 389159 (84.78 mg g^{-1} seed meal). Genotypic variation for TIA in soybean ranging from 32.6 to 80.4 mg g^{-1} of soy meal has been reported [17]. Large number of variants for TIA has been reported among the soybean germplasm [6-8]. Vineet Kumar *et al.* [9] determined TIA levels of 45 Indian cultivars which ranged from 30.78 mg g^{-1} to 148 mg g^{-1} of defatted soy flour and lowest TIA was observed in the cultivar Hardee (30.70 mg g^{-1} seed meal). It was observed in soybean that TIA is not influenced significantly by location itself but there exists a genotype × location interaction [18]. Thus, the observed variation for TIA in our studies proves to be because of genotypic differences and the genotypes showing low TIA can be utilized for breeding low TIA lines.

The studies showed no correlation of TIA with maturity, oil content, seed protein content and seed yield, although there was a negative correlation between oil and protein content (Table 2). Negative correlation between TIA and seed yield as well as seed protein was also observed in field pea and grass pea [19].

Table 2. Correlation Coefficients (r) between different characters in soybean

Character	Days to maturity	Oil %	Protein %	Soluble protein	Trypsin inhibitor	Yield/ plant(g)
Days to maturity	1.000	0.3348**	0.2160	–0.1562	–0.1375	0.3784**
Oil %		1.000	–0.2818*	0.0823	–0.1912	0.0589
Protein %			1.000	–0.0991	–0.0697	0.2591
Soluble protein				1.000	0.1709	0.0492
Trypsin inhibitor					1.000	0.0579
Yield/plant(g)						1.000

*,**Significant at 1% and 5% level respectively

Multivariate analysis helps to determine the degree of genetic divergence among the parents. A set of 55 genotypes was evaluated and D^2 analysis was carried out to estimate the genetic divergence based on quality characters like oil content, protein content, soluble protein and TIA. The multivariate analysis revealed that all the genotypes could be grouped into 11 clusters (Fig. 1). The low TIA genotypes, PK-1042 and EC-389178 were grouped in cluster XI and IX respectively, indicating their distinctness from the other genotypes with respect to the traits considered. Grouping of soybean genotypes into different clusters based on multivariate analysis has also been reported [20]. Maximum intercluster value (51.54) was observed between low TIA genotype PK-1042 (cluster VII) and JS-220 (cluster XI) (Table 3) revealing existence of maximum diversity between them. Bhatt [21] reported that the crosses between genotypes with maximum intercluster distance would yield better recombinants. Thus, use of genotype PK-1042 in hybridisation programme will help to derive better

Table 3. Average intra and inter cluster distances (D^2 values) of 55 soybean genotypes

Cluster	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
I	16.78	24.14	22.90	20.97	22.79	25.29	23.84	25.40	27.83	30.75	38.85
II		13.89	25.51	31.04	25.30	31.36	38.22	29.73	42.06	27.13	44.64
III			00.00	14.12	13.14	7.95	38.63	18.65	24.30	23.22	22.03
IV				00.00	11.40	09.71	30.41	14.20	18.43	32.04	26.15
V					00.00	12.18	36.30	11.98	29.00	30.46	30.00
VI						00.00	38.59	17.26	21.42	29.37	20.24
VII							00.00	36.83	31.34	46.04	51.54
VIII								00.00	27.04	27.75	27.66
IX									00.00	34.36	23.64
X										00.00	29.06
XI											00.00

recombinants with low TIA. The character TIA (34.75) showed maximum contribution to D^2 values (Table 4) indicating scope for selection of parents in breeding programme. Study of cluster means (Table 4) indicates that the low TIA genotype PK-1042 (cluster XI) and EC-389178 (cluster IX) also posses high protein 40.84 and 40.11 percent respectively and will be good source of protein for human and animal consumption.

In India soy foods are yet to get recognition in daily diets as a protein rich economical food source because of antinutritional factors like TI. Though TI is heat labile, over heating can reduce both concentration and digestibility of much valued proteins [22]. Similarly, the animal feeding industry also cares about TIA of raw material, for their incorporation into animal diets [23]. In our studies the two genotypes PK-1042 and EC-3 89178 showed low TIA with high protein and can be used in breeding programme to develop low TI lines for domestic utilization and export purpose.

Table 4. Cluster means and percent contribution of characters towards divergence in soybean

Cluster	Oil %	Protein %	Soluble protein	Trypsin inhibitor activity (mg g^{-1} seed meal)
I	17.90	39.49	111.75	60.30
II	15.95	40.08	87.40	78.51
III	17.46	42.09	57.20	47.64
IV	19.39	38.07	69.89	45.36
V	18.86	37.20	48.43	57.81
VI	18.69	40.90	49.22	42.39
VII	19.80	37.03	162.79	59.73
VIII	17.95	32.53	59.76	47.79
IX	18.57	40.11	106.23	22.20
X	13.19	39.19	89.46	43.35
XI	16.61	40.84	44.94	14.49
Per cent contribution	23.84	5.59	35.82	34.75

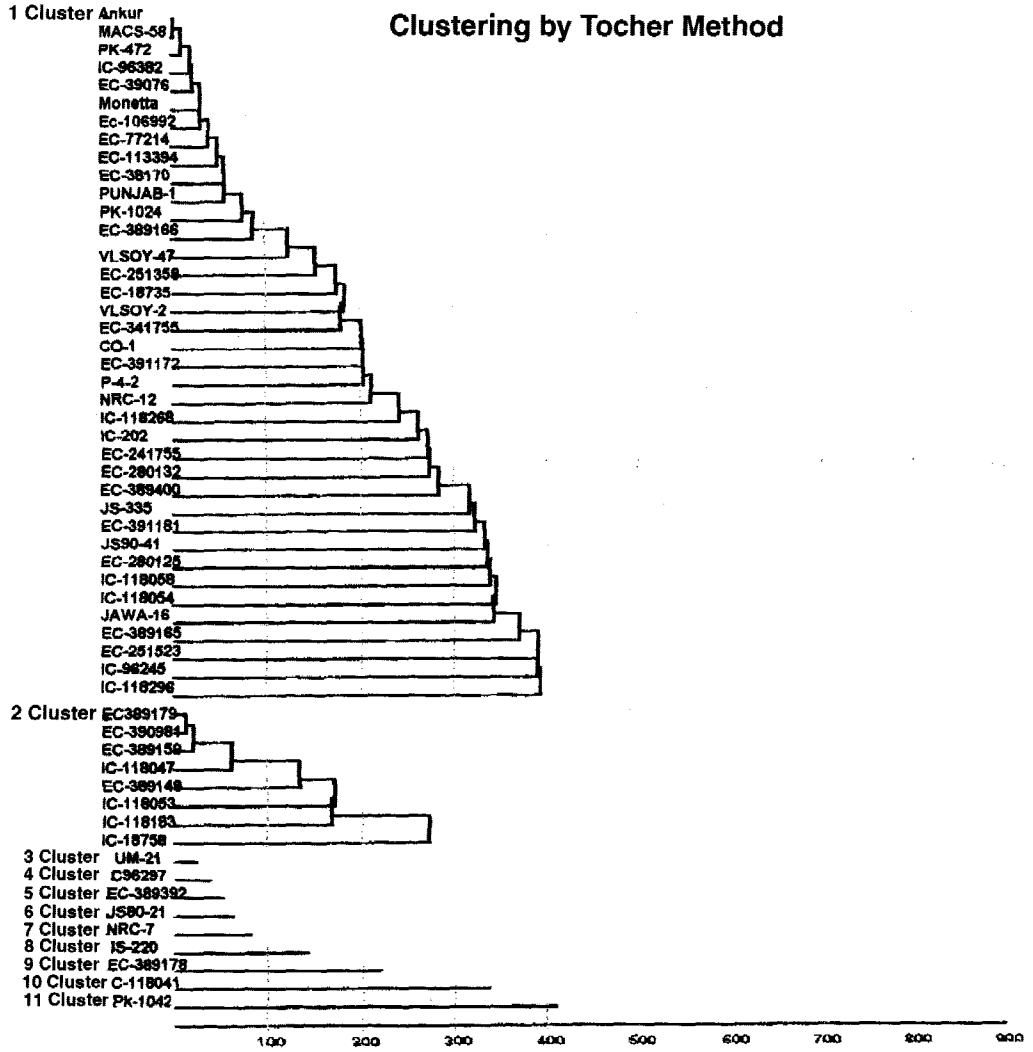

Fig. 1. Clustering of 55 soybean genotypes based on Tocher's method

References

1. **Joshi O. P.** 2003. Future perspectives of soybean in India. Soybean Res., **1**: 29-42.
2. **Liener I. E.** 1994. Implications of antinutritional components in soybean foods. Crit. Rev. Food Sci. Nutr., **34**: 31-37.
3. **Liener I. E. and Kakade M. L.** 1980. Protease inhibitors. *In*: Toxic-Constituents of Plant Foodstuffs, 2nd ed (ed. I. E. Liener). Academic Press, New York: 7-71.
4. **Huisman J.** 1993. Effects of tiypsin inhibitors on monogastric animals. European Association for Grain Legume Research. Grain Legumes, **1**: 20-21.
5. **McNiven M. A., Grimmelt B., MacLeod J. A. and Voldeng H.** 1992 Biochemical characterization of a low trypsin inhibitor soybean. J. Food Sci., **57**: 1375-1378.
6. **Yang L. J., Li S. F., Zhang Y., Huo G. and Yang Q.** 1999 The content of main antinutritional factors in thirteen soybean variants planted in the Heilongjiang area. Soybean Sci., **18**: 77-80.
7. **Wang K. I., Kaizuma N., Takahata Y. and Hatakeyama S.** 1996. Detection of two new variants of soybean Kunitz. trypsin inhibitor through electrophoresis. Breed. Sci., **46**: 39-44.
8. **Bernard R. L., Hymowitz T. and Cremeens C. R.** 1991. Registration of 'Kunitz' soybean. Crop Sci., **31**: 232-233.
9. **Vineet Kumar, Anita Rani and Tiwari S. P.** 2001. Comparative activity of trypsin inhibitor among released soybean varieties/strains of India. Ind. J. of Nut. Diet., **38**: 437-440.
10. **A. A. C. C.** 1976. Approved methods. American Association of Cereal Chemists, St. Paul, Minnesota, USA.
11. **A. O. A. C.** 1984. Official methods of Analysis. Association of Official Agricultural Chemistry, Washington, D. C.
12. **Layne E.** 1957. Spectrophotometric and turbidometric methods for measuring proteins. *In*: Method in Enzymology, (ed. S. P. Colowick and N. O. Kaplan). New York: 447-457.
13. **Erlanger B. F., Kokowsky N. and Cohen W.** 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem, Biophys., **95**: 271.
14. **Panse V. G. and Sukhatme P. V.** 1967. Statistical methods for agricultural worker ICAR, New Delhi.
15. **Mahalanobis P. C.** 1936. On the generalized distance in statistics. Proc. Natl. Inst. Sci., India, **2**: 49-55.
16. **Rao C. R.** 1952. Advance statistical methods in biometrical research John Wiley and Son, New York.
17. **Kedlec M., Michele P., Letae J., Baranek M. and Vachun M.** 2000. Genetic resources of soybean with respect to quality of seeds. Acta-Universatatis-Agriculture-et Silviculture-Mendeliance-Brunensis, **48**: 55-59.
18. **Vineet Kumar, Anita Rani, Chanda Tindwani and Mukti Jain.** 2003. Lipoxygenase isozymes and trypsin inhibitor activities in soybean as influenced by growing location. Food Chem., **83**: 79-83.
19. **Wang X., Warkentin T. D., Briggs C. J., Oomah B. D., Campbell C. G. and Woods S.** 1998. Trypsin inhibitor in field pea (*Pisum sativum* L.) and grass pea (*Lathyrus sativus* L.). J. Agric. Food Chem., **46**: 2620-2623.
20. **Dobal V. K.** 1995. Genetic divergence in soybean. Legume research, **18**: 29-34.
21. **Bhatt G. M.** 1970. Multivariate analysis approach to selection of parents for hybridisation aiming at yield improvement in self-pollinated crop. Aust. J. Agri. Res., **21**: 1-7.
22. **Friedman M., Brandon D. L., Bates A. H. and Hymowitz T.** 1991. Comparison of a commercial soybean cultivar and an isoline lacking the Kunitz trypsin inhibitor: composition, nutritional value and effects of heating. J. Agri. Food Chem., **39**: 327-335.
23. **Kakade M. L., Rackis J. J., McGhee J. E. and Puski G.** 1974. Determination of trypsin inhibitor activity of soy products: a collaborative analysis of an improved procedure. Cereal Chem., **51**: 376-382.