Mittal P *et al.* (2021)
Notulae Scientia Biologicae 13(1):10836
DOI:10.15835/nsb13110836
Research Article

Bowman-Birk inhibitor in soybean: Genetic variability in relation to total trypsin inhibitor activity and elimination of Kunitz trypsin inhibitor

Priyanka MITTAL[1], Vineet KUMAR[1]*, Anita RANI[1], S.M. GOKHALE[2]

[1]*ICAR-Indian Institute of Soybean Research, Khandwa road, Indore, M.P., 452001, India; mittal.priyanka9@gmail.com; vineetksahni@yahoo.com (corresponding author); anitavks@yahoo.co.in*
[2]*School of Biochemistry, Devi Ahilya Vishwavidhyalaya, Khandwa Road, Indore, Madhya Pradesh, 452001, India; gokhale.drsm@gmail.com*

Abstract

Bowman-Birk inhibitor (BBI) is a soybean seed serine protease inhibitor whose nutraceutical properties far exceed its anti-nutritional property arising from its trypsin and chymotrypsin inhibitor activity. High BBI soybean genotypes are sought for the commercial preparation of this nutraceutical biomolecule while low BBI content in Kunitz trypsin inhibitor (KTI)-free genetic background is desirable in soymeal manufacturing. In the present investigation, monoclonal antibody assay of 95 soybean genotypes revealed 11-fold genetic variation for BBI concentration. The study led to the identification of 3 very high (>20 mg/g defatted soy flour) and 5 very low BBI (< 4 mg/g defatted soy flour) soybean genotypes. Contribution of BBI to total trypsin inhibition activity ranged from 2.2 to 53.5% with average value of 11.6%. Genotypes with varying level of BBI raised consecutively for two years exhibited non-significant (p>0.05) effect of growing year on the trait. BBI content in BC_3F_2 derived KTI-free lines was at par with the recurrent parent. Low and high BBI content genotypes with diverse genetic background identified in the study may be exploited to develop mapping population to identify genomic regions underlying BBI in soybean.

Keywords: Bowman-Birk inhibitor; genetic variability; Kunitz trypsin inhibitor; total trypsin inhibitor

Introduction

Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor (BBI) are two major serine protease inhibitors in soybean seeds, accounting for nearly 6% fraction of total soluble protein. KTI, 21 kDa polypeptide with 181 amino acids, is an anti-nutritional factor (ANF) in soybean seeds as it inhibits trypsin to the magnitude of 2.51 times of its own concentration (Kumar *et al.*, 2018), thereby interfering with the digestion of proteins in human gastrointestinal tract (GIT). Various physical and biological methods, namely, boiling, autoclaving, pulse electric field, microwave irradiation and sprouting are being used by soy industries for the inactivation of this ANF (Dia *et al.*, 2012; Chen *et al.*, 2014; Kumar *et al.*, 2019). Though, plant breeders have developed KTI free soybean genotypes through marker assisted and traditional plant breeding using soybean germplasm accession with null allele of KTI (Peric *et al.*, 2011; Kumar *et al.*, 2015; AICRPS, 2018). BBI is a

Received: 26 Oct 2020. Received in revised form: 18 Feb 2021. Accepted: 24 Feb 2021. Published online: 05 Mar 2021.
From **Volume 13, Issue 1, 2021,** Notulae Scientia Biologicae journal will use article numbers in place of the traditional method of continuous pagination through the volume.

low molecular weight (8 kDa), double headed serine protease inhibitor, with 71 amino acids in its polypeptide chain which can inhibit trypsin and chymotrypsin simultaneously (Odani and Ikenaka, 1977). However, in view of several reports which demonstrated the role of BBI in suppressing carcinogenesis in animal models, BBI concentrate (BBIC) was given the status of Investigational New Drug (IND) by Food and Drug Association (FDA) way back in 1992 (Kennedy, 1993). Research works spanning across the past three decades have revealed chemo-preventive nutraceutical and cosmeceutical properties of BBI. BBIC has been reported to prevent the initiation of tumors and cause cell death in *in vivo* and *invitro* cancer models (Kennedy, 1998; Kennedy and Wan, 2002). Besides its chemo-preventive and anti-inflammatory properties (Clemente et al.,2011), BBI has been recently reported to be effective in the treatment of Alzheimer's disease (Akbari *et al.*, 2019) and inhibition of HIV replication in macrophages (Ma *et al.*, 2016). Further, in the backdrop of the fact that SARS-CoV-2, the causative agent of COVID-19 pandemic, requires the expression of self-encoded chymotrypsin-like protease for its replication in the host cell (Cascella *et al.*, 2020), BBI in soybean may show an inhibitory effect on the multiplication of this novel virus in human cell due to its chymotrypsin inhibiting property. Further, the cosmeceutical application of BBI stems from its role in preserving skin health, weight-loss and anti-alopecia (Losso, 2010). Therefore, soybean varieties with high level of BBI are much sought after commodity as raw material for manufacturing commercial formulations.

Assessment of the genetic variability of BBI in the soybean cultivars/ promising germplasm lines is the first prerequisite to initiate plant breeding programme focusing on the development of high BBI soybean varieties. On the other hand, heat treatment required to render chymotrypsin and trypsin inhibiting activity of BBI dysfunctional, along with trypsin inhibiting activity of KTI, incurs extra cost in soymeal manufacturing. This necessitates the development of low BBI soybean genotypes in the KTI-free genetic background for use in soymeal industry. Though, investigations regarding the estimation of BBI concentration in soybean have been reported but these studies are not only scarce but the genotypes undertaken in them were also very few. Brandon *et al.* (2004) reported BBI in the range of 0.7 - 5.5 mg/g soy meal in 11 wild soybean germplasm using sensitive monoclonal antibody-based ELISA method. Pesic *et al.* (2007) determined BBI concentration in 12 soybean cultivars, and reported a range of 0.6- 6.32% of total extractable protein using polyacrylamide gel electrophoresis. Kumar *et al.* (2018) determined BBI content in 7 Indian soybean varieties and reported a range of 7.4 - 23.4 mg/g defatted soy flour using monoclonal antibody-based ELISA method. KTI is primarily responsible for trypsin inhibitor activity (TIA) of soybean seeds; however, a wide genotypic variation was reported with regard to its contribution to total TIA (Kumar *et al.*, 2019). Therefore, a variation may also exist with regard to percent contribution of BBI to total TIA activity across 90 commercially released soybean varieties undertaken in the study. As mentioned above, KTI free soybean genotypes with low BBI serve as excellent raw material for the soymeal manufacturing. However, Gillman *et al.* (2015) reported enhancement in BBI concentration in a KTI free F_2line (E16) at early seed stage obtained by crossing 'PI68679' (null *KTI1*) with 'PI542044' (null KTI3), though the null KTI3 was solely responsible for the absence of KTI. This observation was untenable because a F_2 line may be segregating for several BBI loci. In contrast to the segregating F_2 line undertaken for investigation pertaining the effect of genetic removal of KTI on BBI by these authors, 5 BC_3F_2derived KTI free lines of the cross 'JS97-52' x 'PI542044' (exhibiting more than 96% recurrent parent genomic content) were analysed. Moreover, the seed composition of the soybean is reported to be influenced by the growing environment as suggested in multi-location and staggered planting date experiments (Kumar *et al.*, 2006a; Kumar *et al.*, 2006b). Therefore, in the present study it was thought worthwhile to determine BBI content in large number of soybean genotypes (95), to determine the genetic variability for the trait and identify soybean genotypes with high and low levels of BBI in different genetic background, and to investigate the effect of growing year on BBI content by analyzing the harvest of these high and low BBI soybean genotypes obtained in the cropping season of two consecutive years.

Materials and Methods

Experimental data

Ninety-five soybean genotypes were raised in cropping season (last week of June to mid week of October) 2018 in the fields of ICAR-Indian Institute of Soybean Research, Indore (N 22.7196° and E 75.8577°), Madhya Pradesh, India. The geographic coordinates of the site of field crop was N 22.7196° and E 75.8577° and located in the Central zone, which is the major agroclimatic zone for commercial cultivation of soybean in India. Of the 95 genotypes, 89 were the soybean varieties released for commercial cultivation in the country by All India Co-ordinated Research Project on Soybean, India. Five genotypes were KTI-free breeding lines derived from BC_3F_2 generation of the cross 'JS97-52' (recurrent parent) × 'PI542044', with > 96% recurrent parent genome content (RPGC). One genotype 'NRC101' was a KTI free breeding line developed through marker assisted forward breeding from the cross 'Samrat' × 'PI542044'. The soil of the field where the soybean genotypes were raised was vertisol. Genotypes were planted in triplicate in single row plot of 3 m length with row-to-row and plant-to-plant distance of 45 and 5 cm, respectively, in random block design using standard agronomic practices recommended for cultivation of soybean in Central India.

Extraction and estimation of BBI concentration through indirect ELISA

Finely ground soybean flour (60 mesh) was defatted using petroleum ether (boiling point 40-60 °C). Defatted soy flour (50 mg) was suspended in 1 ml of 50 mM Tris buffer (pH 8.2) and homogenized using Polytron homogenizer (Kinematica, Model PT2100, Switzerland) followed by ultra-sonication (Pci Analytics) for 1 h. During ultrasonication, samples were vortexed after every 15 min. The suspension so obtained was centrifuged at 20,000 g for 35 min at 4 °C and the supernatant was diluted 25,000 times using phosphate buffered saline. Standard curve ($y = 0.03x + 0.3146$, $R^2 = 0.996$) was generated using varying concentration of purified BBI.

Extracted samples were dispensed in triplicate in 96-well maxisorb nunc immunoplates (100µl in each well) for overnight (15-18 h) incubated at 4 °C. Incubation was followed by repeated washing (4-5 times) with buffer (PBS + 0.05% Tween 20). Subsequently, 250 µl of blocking buffer (5% bovine serum albumin + 1% Tween 20 in PBS) was added and incubated for 2 h (with interval shaking) at 37 °C followed by 4-5 washings. A 50 µl of diluted (1:1000 in 3% BSA buffer solution) BBI primary antibody was dispensed into each well and incubated for 2 h with l shaking after every 10 sec. After washing, 50 µl of diluted (1:1000 in 3% BSA buffer) alkaline phosphatase conjugated anti-mouse antibody was added to each well and incubated for 1 h at room temperature. Unbound conjugated anti-mouse antibodies were removed by repeated washing followed by addition of 100 µl p-nitrophenyl phosphate (pNPP). The plate was immediately sealed with black polyvinyl seal and incubated at 37 °C. Reaction was stopped at 30 min using 3N NaOH. Absorbance was recorded using ELISA plate reader (Multiscan Go-Thermo Scientific Pvt Ltd, United States) at 405 nm.

BBI activity

The trypsin inhibition activity of BBI was calculated as half of the BBI concentration as per the information provided in product analysis note in BBI standard (T9777) procured from Sigma Aldrich Pvt Ltd India.

Statistical analysis

All the steps and assays were performed in triplicate samples with satisfactory repetition of values. Data presented in Tables 1 and 2 are mean ± standard deviation of three independent replicates. All the statistical analyses were carried out through *SAS* 9.3 with significance at $p<0.05$.

Results

Table 1 presents the data of BBI concentration in 90 soybean genotypes, comprising of 89 commercial soybean varieties and one advanced breeding KTI–free line ('NRC101'), grown in India in cropping season of 2018, while Table 2 gives BBI concentration in 5 KTI-free BC_3F_2 derived lines, which included first KTI-free soybean variety NRC127, developed by introgression null allele of KTI in variety 'JS97-52' through marker assisted backcrossing, released for commercial cultivation in India (AICRPS, 2018). BBI concentration in 90 varieties ranged from 2.2 ('RAUS5') to 24.5 mg/g defatted soy flour ('JS79-81'), with 11-fold variation and average value of 9.07 mg/g defatted soy flour. Further, the frequency distribution of BBI concentration across 90 genotypes showed skewness and kurtosis of 0.595 and -2.5, respectively, indicating that the data obtained was fairly symmetrical with least number of genotypes in extreme ranges. Further, this range was divided into 5 different classes: very low (<4 mg/g defatted soy flour), low (4-8 mg/g defatted soy flour), moderate (8-15 mg/g defatted soy flour), high (15-20 mg/g defatted soy flour) and very high (>20 mg/g defatted soy flour) (Figure 1). This classification showed that the highest number (39) of cultivars was in moderate BBI category followed by low BBI category (38). However, only three cultivars, namely, JS79-81 (24.5 mg/g defatted soy flour), JS90-41 (23.5 mg/g defatted soy flour) and PS1241 (22.0 mg/g defatted soy flour) exhibited very high BBI concentration while 5 cultivars, namely, RAUS5 (2.2 mg/g defatted soy flour), VLS47 (2.7 mg/g defatted soy flour), VLS2 (3.1 mg/g defatted soy flour), PUSA16 (3.6 mg/g defatted soy flour) and DS228 (3.7 mg/g defatted soy flour) exhibited very low BBI concentration (< 4.0 mg/g defatted soy flour).

Table 1. Genotypic variation of BBI conc. (mg/g defatted soy flour) in soybean varieties and its contribution to TIA

Variety	Zone	BBI[1]	BBI activity	% contribution to TIA*	Variety	BBI[1]	Zone	BBI activity	% contribution to TIA*
ADT-1	SZ	6.0^{a} ± 0.3	3	8.5	MAUS 32	10.1^{b} ±0.48	SZ	5.1	18.6
Alankar	NPZ	9.6^{b}±0.43	4.8	14.4	NRC2	11.3^{b} ±0.50	CZ	5.65	24.1
Birsa soybean	NPZ	4.5^{a} ±0.27	2.25	3.9	NRC 37	19.7^{d} ±0.78	CZ	9.85	53.5
Bragg	CZ	5.8^{a} ±0.23	2.9	8.5	NRC7	9.9^{b} ±0.34	CZ	4.95	14.1
Co-1	SZ	5.1^{a} ±0.31	2.55	5.8	NRC12	13.5^{c}±0.43	CZ	6.75	14.6
Co Soya-2	SZ	9.01^{b}±0.39	4.51	11.2	NRC86	12.4^{c} ±0.60	CZ	6.2	13.3
Co-3	SZ	7.6^{b} ±0.33	3.8	11.3	NRC101	7.5^{a}± 0.42	CZ	3.75	23.4
DS 228	SZ	3.7^{a} ±0.09	1.85	4.3	PRS 1	16.4^{c} ±0.69	CZ	8.2	23.4
Pusa97-12	NPZ	5.2^{a} ±0.30	2.6	4.5	Pusa16	3.6^{a} ±0.17	NPZ	1.6	2.56
Davis	SZ	11.1^{b}±0.51	5.55	14.2	Pusa20	5.0^{a} ±0.20	NPZ	2.5	4.6
Gujrat soybean 1 (J231)	SZ	10.7^{b}±0.49	5.35	16.4	Pusa24	6.2^{a} ±0.30	NPZ	3.1	9.6
Gujrat soybean 2 (J202)	SZ	17.5^{d}±0.53	8.75	22.3	Pusa 98-14	7.2^{b} ±0.33	NPZ	3.6	6.7
Harit soy (Hims1563)	NHZ	12.6^{c}±0.56	6.3	12.7	PK471	5.4^{a} ±0.22	NPZ	2.7	6.9
Hardee	SZ	9.8^{b} ±0.63	4.9	12.8	Palam soya	8.5^{b} ±0.51	NHZ	4.25	9.9
Indira soya 9	NEZ	7.44^{b}±0.40	3.72	7.5	PK 327	10.2^{b} ±0.46	NPZ	5.1	6.8
Improved Pelican	SZ	5.2^{a} ±0.30	2.6	5.6	Pratap Soya 2	7.7^{b} ±0.32	NEZ	3.85	6.1
JS20-34	CZ	12.5^{c}±0.20	6.25	9.7	Pusa22	13^{c} ±0.44	NPZ	6.5	8.5
JS 71-05	CZ	7.4^{b} ±0.32	3.7	8.6	Punjab1	9.9^{b} ±0.37	NPZ	4.95	26.6
JS72-44	CZ	4.7^{a} ±0.19	1.7	4.5	Pusa40	13.4^{c} ±0.72	NPZ	6.7	13.8
JS76-205	CZ	9.5^{b} ±0.43	4.75	8.3	PK416	12.5^{c} ±0.50	NPZ	6.25	12.2
JS79-81	SZ	24.5±1.07	12.25	31.6	PS1042	6.1^{a} ±0.28	NPZ	3.1	6.4
JS90-41	CZ	23.5±0.76	11.75	31.8	PS1024	5.1^{a} ±0.25	NPZ	2.6	6.6
JS93-05	CZ	12.9^{c}±0.39	6.45	17.6	PS 1225	12.7^{c} ±0.40	NHZ	6.35	18.4
JS95-60	CZ	12.7^{c}±0.52	6.35	16.9	PS1029	6.3^{a} ±0.31	NPZ	3.15	6.2
JS97-52	CZ	7.4^{b} ±0.55	5.1	7.6	PK472	13.4^{c} ±0.60	NPZ	6.7	16.6
JS 335	CZ	6.9^{a} ±0.33	3.45	7.9	Ps564	7.4^{b} ±0.22	NPZ	3.7	7.2
JS2029	CZ	12.44^{c}±0.59	6.22	16.1	PS1092	13.7^{c} ±0.48	NPZ	6.85	13.4
Kalitur	CZ	8.4^{b} ±0.54	4.2	12.4	PS1241	22.0^{e}±0.76	NHZ	11.0	19.4
KHSb2	SZ	5.9^{a} ±0.32	2.95	6.1	RVS2001-4	16.4^{c} ±0.73	CZ	8.2	16.4
KB79	SZ	7.2^{b} ±0.34	3.6	7.9	RKS24	11.0^{b} ±0.50	CZ	5.5	13.5
Lee	NHZ	13.3^{c} ±0.61	6.65	15.5	RAUS5	2.2^{a} ±0.13	NEZ	1.1	2.2

LSb1	SZ	13.3^{c} ±0.67	6.65	13.0	Shilajeet	8.1^{b} ±0.38	NHZ	4.1	8.5
MACS13	SZ	4.0^{a} ±0.28	2	6.3	SL96	6.6^{a} ±0.31	NPZ	3.3	9.4
MACS57	SZ	5.8^{a} ±0.30	2.9	6.2	SL 688	6.4^{a} ±0.30	NPZ	3.2	6.6
MACS58	SZ	10.1^{b} ±0.49	5.1	11.2	Shivalik	5.2^{a} ±0.20	NHZ	2.6	7.4
MACS124	SZ	3.3^{a} ±0.12	1.65	4.3	SL295	11.9^{c} ±0.54	NPZ	5.95	16.2
MACS450	SZ	11.7^{c} ±0.44	5.85	11.7	SL525	10.3^{b} ±0.42	NHZ	5.2	12.4
MAUS1	SZ	9.6^{b} ±0.43	4.8	13.9	TAMS 38	18.0^{d} ±0.48	CZ	9	21.8
MAUS2	SZ	9.0^{b} ±0.37	4.5	9.5	TAMS 98-21	4.2^{a} ±0.18	CZ	2.1	4.5
MAUS61	CZ	9.4^{b} ±0.44	4.7	11.1	VLS1	4.7^{a} ±0.20	NHZ	2.4	5.7
MAUS61-2	CZ	4.7^{a} ±0.23	2.35	6.5	VLS2	3.1^{a} ±0.18	NHZ	1.6	2.3
MAUS71	CZ	4.2^{a} ±0.21	2.1	3.3	VLS21	12.3±0.54	NHZ	6.15	10.1
MAUS158	SZ	12.5^{c} ±0.57	6.25	17.0	VLS47	2.7^{a} ±0.34	NHZ	1.4	3.4
MAUS47	CZ	4.7^{a} ±0.22	2.35	5.0	VL S63	7.1^{b} ±0.21	NHZ	3.6	6.7
MAUS81	SZ	7.1^{b} ±0.47	3.55	7.8	VLS 65	5.5^{a} ±0.30	NHZ	2.8	8.2

*TIA value extracted from previously published study Kumar et al.2019

[1]Values given are mean of triplicate samples ± SD. Values given with different superscripts are significantly ($p < 0.05$) different from each other

Table 2. BBI concentration (mg/g defatted soy flour) in KTI-free BC_3F_2lines

BC_3F_2	BBI	KTI
JS97-52 (RP)	7.4^{a}±0.32	KTI+ve
NRC127	11.0^{a}±0.40	KTI-ve
JSKTi9	7.7^{a}±0.30	KTI-ve
JSKTi10	10.8^{a}±0.41	KTI-ve
JSKTi11	10.4^{a}±0.40	KTI-ve
JSKTi12	9.4^{a}±0.44	KTI-ve

*Note: Values given are mean of triplicate samples ± standard deviation. Values given with different superscripts in the same column are significantly ($p < 0.05$) different from each other

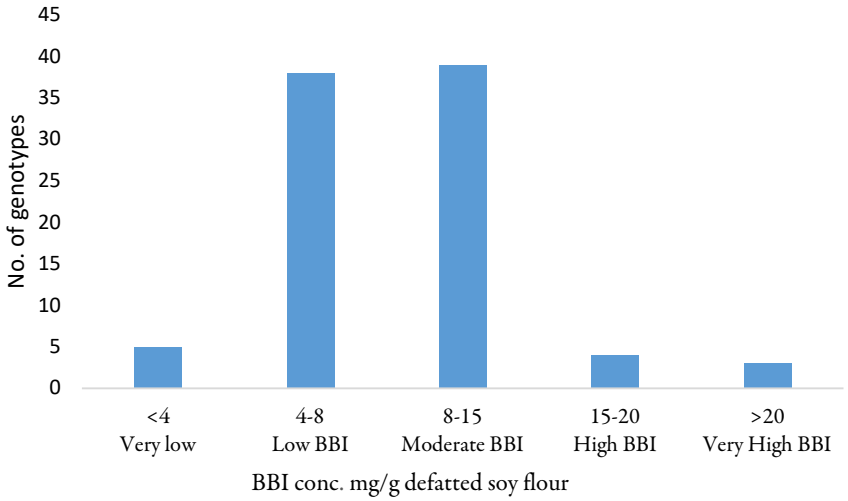

Figure 1. Distribution of 90 soybean varieties based upon varying BBI concentration

Soybean cultivation area in India is divided into 5 major agroclimatic zones, namely, North East Hill Zone, Northern Hill Zone, North Plain Zone, Central Zone and Southern Zone, though majority of this area under soybean cultivation is Central Zone followed by Southern Zone. The varieties developed for different agroclimatic zones are best adapted to biotic and abiotic factors prevailing there. Average BBI content of varieties developed from North East Hill Zone was found minimum (5.16 mg/g defatted soy flour) while average BBI content of from Central Zone was the highest (12.6 mg/g defatted soy flour). The varieties developed from Northern Hill Zone and Northern Plain Zone exhibited almost same BBI concentration (7.5 and 7.7 mg/g defatted soy flour). Average BBI concentration of soybean varieties from Southern zone was slightly higher than North Plain zone and Northern Hill zone but significantly (p<0.05) lower than the Central Zone. Further, BBI content among the most popular 7 Indian soybean varieties, namely, 'JS335', 'JS20-34', JS20-29', 'JS95-60', 'JS93-05', 'RVS2001-4' and 'JS20-69', which account for 84% of the total soybean breeder seed indent in the country (AICRPS, 2018) varied from 6.9 mg/g defatted soy flour (JS335) to 14.7 mg/g defatted soy flour ('JS20-69').

Results presented in Table 2 gives BBI concentration in KTI free BC_3F_2 derived lines from the cross 'JS97-52' (the recurrent parent) ×'PI542044' (the donor of null allele of KTI). Range of 9.4-11.0 mg/g defatted soy flour for BBI concentration was noted among these 5 recombinant lines, thereby showing increase in BBI content compared to recurrent parent (7.4 mg/g defatted soy flour) but this increase was statistically not significant (p>0.05).

Table 3 gives BBI concentration of 7 soybean genotypes selected from very low (below 4), low (4-8), moderate (8-15) and high BBI concentration (15-20 mg/g defatted soy flour) category raised for 2 years consecutively. Across all the 7 genotypes, no significant (p>0.05) differences were noted for BBI concentration across two growing years despite the differences in weather parameters (refer Table 4). DS228 was in very low BBI concentration category in both first and second cropping year with non-significant differences for BBI concentration across two years. 'JSKTI9' in both the years was in low concentration category with no significant differences across 2 growing years. 'NRC127', which is a KTI free soybean variety, in moderate BBI concentration category did not register any significant change in BBI across two growing years. 'RVS2001-4' remained under high BBI concentration category with no significant changes in BBI concentration in both 2018 and 2019. On the other hand, 'NRC101', which is the KTI-free germplasm line developed from the cross 'Samrat' × 'PI542044', registered slightly higher value of BBI in 2019 than 2018, but statistically the values were not significantly different from each other. Both 'RAUS5' and 'VLS47' remained in very low concentration category in both the growing years. These results showed that the effect of growing year on BBI content was non-significant (p>0.05).

Table 3. BBI concentration (mg/g defatted soy flour) of selected soybean genotypes across different growing years

Genotype	2018	2019
NRC101	7.5^a± 0.42	9.6^a±1.0
RAUS 5	2.2^a±0.14	3.9^a± 0.38
NRC127	11.0^a±0.87	11.4^a±1.2
DS228	3.2^a±0.26	3.9^a±0.30
RVS2001-4	16.4^a±1.3	15.7^a±1.3
VLS47	2.7^a±0.24	3.8^a±0.41
JSKTI 9	7.7^a±0.36	7.5^a± 0.67

*Note: Values given are mean of triplicate samples ± standard deviation. Values given with the same superscript in a row are statistically at par with each other (p >0.05).

Table 4. Average maximum, minimum and mean temperature (unit °C), and rainfall (inches) in cropping season (last week of June to mid week of October) of 2018 and 2019.

Weather parameter	Growing year	
	2018	2019
Average maximum temperature	31.4	30.9
Average minimum temperature	23.0	22.7
Average mean temperature	27.25	26.8
Average rainfall	31.4	53

Discussion

Estimation of BBI concentration in large number of genotypes has not yet been reported, however, a few studies concerning genetic variation for the levels of BBI in selected number of soybean genotypes are available in the literature (Brandon *et al.*, 2004; Pesic *et al.*, 2007' Kumar *et al.*, 2018). Among 11 wild soybean germplasms, Brandon *et al.* (2004) reported 7.8-fold variation in the concentration of this polypeptide with a range of 0.7 - 5.5 mg/g defatted soy flour. Pesic *et al.* (2007) investigated 12 soybean cultivars for BBI concentration including one KTI free genotype and the BBI concentration reported in the study was 1.79% of total extractable protein. Assuming average 39% protein content and maximum 80% extractable protein in soybean, 1.79% of extractable protein will be equivalent to 5.6 mg/g of flour which is much lesser than the average BBI content (9.07 mg/g defatted soy flour) in our study. Pauchar-Menacho *et al.* (2010) reported 5.9 mg/g flour BBI concentration in the variety BRS133 which corresponds to the low BBI category in the present study. Kumar *et al.* (2018) investigated 7 Indian soybean cultivars for BBI content, and reported a range of 7.4 - 23.4 mg/g defatted soy flour. In the present study, we identified 5 soybean genotypes which exhibited BBI concentration below 4 mg/g defatted soy flour and 3 genotypes with BBI concentration between 22-25 mg/g defatted soy flour. Introgression of null allele of KTI in very low BBI genotypes through marker assisted backcrossing (MABC) may be used for the development of soybean cultivars with the lowest TIA value, which would be excellent raw material for soymeal manufacturing industry.

Contribution of BBI to total TIA

Though, KTI has been reported to account for 1-79.8% of the total TIA with average value of 52.8% recently (Kumar *et al.*, 2019), however, no information pertaining to the contribution of BBI to total TIA is available in literature. As both soybean cultivars (90) and the seed lot from which they were drawn for the investigation of BBI concentration in the present study and Kumar *et al.* (2019) were same, the TIA data reported in the latter study was used to compute the contribution of BBI to total TIA. As evident from Table 1, significant (p<0.05) varietal differences were observed for contribution of BBI to total trypsin inhibitor activity (TIA), which ranged from 2.2 ('RAUS5') to 53.5% ('NRC37') with 11.6% average contribution to TIA. High BBI varieties, namely, 'JS79-81' and 'JS90-41' contributed 31.6 and 31.8% while low BBI genotypes 'RAUS5', 'VLS2' and 'Pusa16' contributed 2.2, 2.3 and 2.6% to total TIA, respectively. Further, 'Punjab1' showed low BBI concentration to the magnitude of 4.95 mg/g defatted soy flour but contributed 26.6% of TIA, while 'NRC37' which has same level (4.95 mg/g defatted soy flour) of BBI activity but accounted for 14% of TIA. This may be attributed to very low level of KTI activity in 'Punjab1' as reported in our recent study (Kumar *et al.,* 2019). Further, higher BBI concentration in cultivar 'NRC37' (14.8 mg/g defatted soy flour) than its parents cultivars 'JS72-44' (4.7 mg/g defatted soy flour) and 'Punjab1'' (9.9 mg/g defatted soy flour) and similarly, higher BBI concentration of 'PK1092' (13.7 mg/g defatted soy flour) than its parents 'PK327' (10.2 mg/g defatted soy flour) and 'PK416' (12.5 mg/g defatted soy flour) showed the transgressive segregation for this trait (Deshimaru *et al.*, 2004).

Effect of genetic removal of KTI on BBI

In international market, soybean meal is traded with stringent norms pertaining to the level of total trypsin inhibitor activity, which is brought down to the desirable benchmark (<10 mg/g soymeal) by heat-treatment during manufacturing. To obviate the need of this cost-incurring in heat treatment, soybean genotypes genetically free from KTI, which contributes primarily to TIA, have been developed by the plant breeders (Peric *et al.*, 2011; Kumar *et al.*, 2015; AICRPS 2018). However, in these KTI free genotypes, BBI is also sought to be low so that total TIA is below the international limit of soymeal in the seed (raw material) itself which would save the cost incurring in the heat treatment. This necessitates investigation pertaining to the effect of genetic removal of KTI on the concentration of BBI. Friedman *et al.* (1991) investigated BBI in a KTI free cultivar derived from 'Williams 82' through back crossing and showed BBI concentration at par with the parent genotype ('Williams-82'). Gillman *et al.* (2015) investigated the effect of genetic removal of the KTI from 'Williams 82'. The authors reported overproduction of BBI in a KTI free F_2line 'E 16' compared to wild type and suggested that the tot proteome was rebalanced itself by a dramatic increase in the levels of BBI on the genetic removal of KTI. Therefore, both the above-mentioned studies gave contrasting results. Five BC_3F_2-derived KTI free soybean genotypes developed by introgression of null allele of KTI from 'PI542044' in Indian soybean variety 'JS97-52' (Kumar *et al.*, 2015) were analyzed for BBI content in the present study and the results are presented in Table 2. Numerically, the data showed higher BBI content in BC_3F_2 derived KTI free breeding lines compared to recurrent parent, however statistically the values of the former were not significantly different from the latter. Therefore, assessment of 5 BC_3F_2 derived KTI free lines along with the recurrent parent showed that the genetic removal of KTI did not impact BBI concentration significantly in contrast to the study of Gillman *et al.* (2015) but in consonance with Friedman *et al.* (1991).

Pedigree analysis

Pedigree of low BBI genotypes, namely, 'DS228', 'VLS47', 'VLS2', 'RAUS5' was scrutinized. 'RAUS5' was developed from 'Pusa16' × 'JS335'. It was interesting to note that Pusa16, the female parent of 'RAUS5', also showed low BBI activity (1.6 mg/g defatted soy flour) (Table 1). 'DS228' is a genotype developed from the cross 'JS335' × 'DS181', 'VLS47' is a selection from 'KHSF3-1' and 'VLS2' is selection from 'VHC 856007'. Therefore, 'DS228' and 'RAUS5' both the low BBI genotypes are genetically close, as both have a common parent ('JS335'). Two high BBI content varieties, namely, 'JS79-81' and 'JS90-41' are genetically diverse as the former was developed from the cross 'Bragg' × 'Harosoy-Deciduous' while the latter from the cross 'PS73-7' × 'Hark'. Further, the information regarding the identification of genomic regions governing the biosynthesis of BBI content in soybean seeds is scarce (de Almeida Barros *et al.*, 2012). Therefore, the crossing between high and low BBI genotypes, such as 'DS228'/'RAUS5' × 'JS79-81'/ 'JS90-41', 'VLS2'/'VLS47' × 'JS79-81'/'JS90-41' may be effected to develop mapping population to identify QTLs associated with BBI in soybean and to develop novel genotypes which may have BBI more than the maximum value (24.5 mg/g defatted soy flour) observed in the present study. Furthermore, BBI in soybean have been reported to have several isoforms, which differ in molecular weight and substrate specificity (Tan-Wilson *et al.*, 1987). Of the 5 BBI isoforms, namely, BBI-A, BBI-B, BBI-CII, BBI-DII and BBI-E1 identified in soybean cultivars (Deshimaru *et al.*, 2004), BBI-A, BBI-CII and BBI-DII are the major isoforms with different substrate specificity as both BBI-B and BBI-A possess similar amino acid composition and specificity while BBI-EI and BBI-DII lack nine amino acid residue at N-terminal region. It would be interesting to investigate which isoforms correspond to low and high BBI content genotypes identified in the present study and exploit the information in designing breeding programme for developing novel high and low BBI soybean genotypes.

Conclusions

In brief, this is the first report pertaining to the estimation of BBI in large number of soybean genotypes to assess its genetic variation and contribution to total TIA. The investigation revealed 11-fold genetic variability for the trait and led to the identification of very low and very high BBI content soybean genotypes, which did not show significant (p>0.05) variation across two growing years. The magnitude of contribution of BBI to total TIA was genotype-dependent. Our results also showed that genetic removal of KTI in soybean did not impact BBI content significantly. High BBI genotypes identified in the study can serve as excellent raw material for the nutraceutical industry while low BBI genotypes may be crossed with null KTI genotypes to develop genotypes with negligible level of total TIA required for soymeal industry. The genotypes with extreme values of BBI content and diverse genetic background may be used for developing mapping population to identify genomic regions underlying BBI content in soybean.

Authors' Contributions

Conceptualization: VK and AR; Investigation: PM; Data curation: VK and PM; Methodology: VK, AR and PM; Supervision: SMG and VK; Writing original draft: VK and PM; Writing-review and editing: PM, VK, AR and SMG. All authors read and approved the final manuscript.

Acknowledgements

We thank ICAR-Indian Institute of Soybean Research, Indore for providing the farmland and laboratory facilities. This work was financially supported by University Grant Commission of India [421/ (CSIR-UGC NET DEC. 2018). Authors also thank Dr. Surendra Kumar, Information and Documentation officer, ICAR- Indian Institute of Soybean Research, for providing the local weather data of years 2018 and 2019

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

AICRPS (2018). Proceeding of 48th annual group meet of the 'All India Coordinated Research Project on Soybean (AICRPS)' held from March 15th to March 18th, 2018 at Raipur.

Akbari S, Akrami H, Mostafaei A, Kiani S (2019). Bowman-Birk inhibitor modifies transcription of autophagy and apoptosis genes in an *in vitro* model of Alzheimer's disorder. Journal of Cellular Biochemistry 120(7):11150-11157. *https://doi.org/10.1002/jcb.28391*

Brandon DL, Bates AH, Friedman M (2004). Immunoassays for Bowman-Birk and Kunitz soybean trypsin inhibitors in infant formula. Journal of Food Science 69(1):11-15. *https://doi.org/10.1111/j.1365-2621.2004.tb17849.x*

Cascella M, Rajnik M, Cuomo A, Dulebohn SC, Napoli RD (2020). Features, evaluation and treatment of Coronavirus (COVID-19). Statpearl Publishing.

Chen Y, Xu Z, Zhang C, Kong X, Hua Y (2014). Heat induced inactivation mechanism of Kunitz trypsin inhibitor and Bowman-Birk inhibitor in soymilk processing. Food Chemistry 154:108-116. *https://doi.org/10.1016/j.foodchem.2013.12.092*

Clemente A, Sonnante G, Domoney C (2011). Bowman-Birk inhibitors from legumes and human gastrointestinal health: current status and perspectives. Current Protein and Peptide Science 12(5):358-373. *https://doi.org/10.2174/138920311796391133*

De Almeida Barros B, da Silva W, Moreira M, de Barros E (2012). In silico characterization and expression analysis of the multigene family encoding the Bowman-Birk protease inhibitor in soybean. Molecular Biology Reports 39(1):327-334.

Deshimaru M, Yoshimi S, Shioi S, Terada S (2004). Multigene family for Bowman-Birk type proteinase inhibitors of wild soja and soybean: the presence of two BBI-A genes and pseudogenes. Bioscience, Biotechnology and Biochemistry 68(6):1279-1286. *https://doi.org/10.1271/bbb.68.1279*

Dia VP, Gomez T, Vernaza G, Berhow M, Chang YK, Gonzalez de Mejia E (2012). Bowman-Birk and Kunitz protease inhibitors among antinutrients and bio-actives modified by germination and hydrolysis in Brazilian soybean cultivar BRS133. Journal of Agricultural and Food Chemistry 60:7886-7894. *https://doi.org/10.1021/jf301926w*

Friedman M, Brandon DL, Bates AH, Hymowitz T (1991). Comparison of a commercial soybean cultivar and an isoline lacking the Kunitz trypsin inhibitor: composition, nutritional value, and effects of heating. Journal of Agricultural and Food Chemistry 39(2):327-335. *https://doi.org/10.1021/jf00002a022*

Gillman J, Kim W, H.B. Krishnan (2015). Identification of a new soybean Kunitz trypsin inhibitor mutation and its effect on Bowman-Birk protease inhibitor content in soybean seed. Journal of Agricultural and Food Chemistry 63(5):1352-1359. *https://doi.org/10.1021/jf505220p*

Kennedy AR, Szuhaj BF, Newberne PM, Billings PC (1993). Preparation and production of a cancer chemopreventive agent Bowman-Birk inhibitor concentrate. Nutrition and Cancer 19:281-302. *https://doi.org/10.1080/01635589309514259*

Kennedy AR (1998). The Bowman-Birk inhibitor from soybeans as an anticarcinogenic agent. The American Journal of Clinical Nutrition 68(6):1406S-1412S. *https://doi.org/10.1093/ajcn/68.6.1406S*

Kennedy AR, Wan XS (2002). Effects of the Bowman-Birk inhibitor on growth, invasion, and clonogenic survival of human prostate epithelial cells and prostate cancer cells. The Prostate 50(2):125-133. *https://doi.org/10.1002/pros.10041*

Kumar V, Rani A, Pandey V, Mande P (2006a). Compositional traits of soybean seeds as influenced by planting date in India. Experimental Agriculture 42(1):19-28. *https://doi.org/10.1017/S0014479705003042*

Kumar V, Rani A, Solanki S, Hussain SM (2006b). Influence of growing environment on the biochemical composition and physical characteristics of soybean seed. Journal of Food Composition and Analysis 19:188-195. *https://doi.org/10.1016/j.jfca.2005.06.005*

Kumar V, Rani A, Rawal R, Mourya V (2015). Marker assisted accelerated introgression of null allele of Kunitz trypsin inhibitor in soybean. Breeding Science 65(5):447-452. *https://doi.org/10.1270/jsbbs.65.447*

Kumar V, Rani A, Shuaib M, Mittal P (2018). Comparative assessment of trypsin inhibitor vis-à-vis Kunitz trypsin inhibitor and Bowman-Birk Inhibitor activities in soybean. Food Analytical Methods 11:2431-2437. *https://doi.org/10.1007%2Fs12161-018-1227-9*

Kumar V, Rani A, Mittal P, Shuaib M (2019). Kunitz trypsin inhibitor in soybean: Contribution to total trypsin inhibitor activity as a function of genotype and fate during processing. Journal of Food Measurement and Characterization 13(2):1593-1590.

Losso JN (2010). The biochemical and functional food properties of Bowman Birk inhibitor. Critical Reviews in Food Science and Nutrition 48(1):94-118. *https://doi.org/10.1080/10408390601177589*

Ma TC, Zhou RH, Wang X, Li JL, Sang M, Zhou L, Hou W, Ho WZ (2016). Soybean-derived Bowman-Birk Inhibitor (BBI) inhibits HIV replication in macrophages. Scientific Reports 6:34752. *https://doi.org/10.1038/srep34752*

Odani S, Ikenaka T (1977). Studies on soybean trypsin inhibitors. X. Isolation and partial characterization of four soybean double-headed proteinase inhibitors. The Journal of Biochemistry 82:1513-1522. *https://doi.org/10.1093/oxfordjournals.jbchem.a131845*

Pauchar-Menacho LM, Berhow MA, Mandarino JMG, de Mejia EG, Chang YK (2010). Optimization of germination time and temperature on the concentration of bioactive compounds in Brazilian soybean cultivar BRS 133 using response surface methodology. Food Chemistry 119:636-642. *https://doi.org/10.1016/j.foodchem.2009.07.011*

Peric V, Zilic S, Srebric M, Mikic A (2011). Nutritional value of the grain of Kunitz-free soybean cultivars. Biotechnology in Animal Husbandry 27(4):1537-1542. *https://doi.org/10.2298/BAH1104537P*

Pesic M, Vucelic-Radovic B, Barac M, Stanojevic S, Nedovic V (2007). Influence of different genotypes on trypsin inhibitor levels and activity in soybeans. Sensors 7(1):67-74.

Tan-Wilson AL, Chen JC, Duggan MC, Chapman C, Obach RS, Wilson KA (1987). Soybean Bowman-Birk trypsin isoinhibitors: classification and report of a glycine rich trypsin inhibitor class. Journal of Agriculture and Food Chemistry 35:974-981.