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Effect of Soybean Leaf Protease Inhibitor on the Mean Leaf Area Consumed by *Spodopetra litura* and *Spilosoma obliqua larvae*

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A B S T R A C T

The aim of the present investigation was to study the effect of soybean leaf protein and protease inhibitor content on the mean leaf area consumed by *Spodoptera litura* and *Spilosoma obliqua* larva. The activity of trypsin inhibitor was assayed by determining the residual trypsin activity by using BapNA as the substrate and bovine trypsin as the standard enzyme. The highest and lowest TIA activity exhibited by genotypes SL 979 and SL 688 (2.25 TUI.mg^{-1}protein) and CSB 904 (0.35 TUI.mg^{-1}protein), respectively. The highest gut trypsin inhibition activity ascertained in larvae fed on SL 688 (14.40 %) While the lowest intensity of gut trypsin inhibition activity was observed in genotypes NRC 94 (0.12%). A highly significant and negative correlation was observed between MLAC (cm^2) by *S. litura* and *S. obliqua* and protein content in 33, leaves (r= -0.728**) and (-0.674**) respectively and trypsin inhibitor in leaves (r= -0.909**) and (r= -0.913**). Thus it can be concluded that the genotypes which were having higher protein and trypsin inhibitor in their leaves offered resistance against *S. litura* and *S. boliqua* in soybean.

Introduction

Plant protease inhibitors (PIs) are small proteins which bind with proteolytic enzymes and are widely spread throughout the plant kingdom, these are generally present in high concentration in storage tissues (up to 10% of protein content), and also detectable in leaves during several physiological processes, such as reserve control and defense against pathogens and insect pests (Koiwa *et al.*, 1997). In the latter case, PIs have been shown to be developmentally expressed in seeds and reserve organs (Koiwa *et al.*, 1997) or induced by wounding in leaves (Schaller and Ryan, 1995). The most common of these plant inhibitors are those inhibiting serine proteases such as trypsin (Ahmad *et al.*, 1980; Sasaki and Suzuki, 1982; Hamad and Attias, 1987; Broadway, 1989; Houseman *et al.*, 1989; Johnston *et al.*, 1991). PIs efficiently inhibit elastase and trypsin-like activities from the larval midgut of *Spodoptera littoralis* leading to their starvation and subsequent death. Hence, mode of action and expression profile suggests that PIs is a factor of Soybean insect resistance. This fact can be used as a potential strategy for increasing the level of plant defnce against insects (Brik, 1995 and Koiwa *et al.*, 1997). Many reports shows that different serine protease inhibitors have negative effect on the growth and

development of lepidoptrous larvae (Shukle and Murdock). Therefore, it is important to biochemically characterized the protease inhibitors from various indigenous cultivated legumes and evaluate their insecticidal potential.

Soybean is a crop of global importance and is one of the most frequently cultivated crops worldwide. It suffers severe losses due to insect predation. Most of these losses caused by defoliators (*Spodopetera litura*). Gangrade (1976) reported over 99 insect species attacking soybean crop at Jabalpur. But now the situation has changed and as many as 275 insect species have been recorded attacking soybean crop in India. For this reason, it became important to assess the levels of protease inhibitors form soybean and there interaction with the gut protease of *S. litura*. In view of this the present work focus on the determination of the concentration of trypsin inhibitor in leaves of 33 genotypes of soybean, gut trypsin inhibition percent in *S. litura* and their correlation with the mean leaf area consumed by the larvae of *S. litura* and *S. obliqua*.

Materials and Methods

Plant material

Leaves of 33 verities viz. CSB-904, DS-2705, DS-2706, DS-2708, DSb-19, DSb-21, JS 20-41, JS-20-69, JS 20-71, KBS 22-2009, KDS-378, KDS-378, KDS-695, KDS-699, KDS-705, KDS-708, MACS-1340, MACS-1394, MACS-1416, MAUS-612, MAUS-614,NRC-92,NRC-93, NRC-94, PS-1518, PK-5113, RVS 2001-18, SL-958, SL-979, SL-982, SL-688, PS-1092, PS-1347, SL-688 (SC), PS-1092(SC), PS-1347(SC) of soybean were obtain from entomological block of Norman E. Borlaug Crop Research Centre of Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India.

Insects

S. litura larvae for isolation of gut protease and no choice feeding assay were done on an established colony of *S. litura* and *S. obliqa,* maintained at 25 ± 1^0C, 66% RH in Entomology Department, College of Agriculture G.B.P.U.A & T Pantnagar.

Chemicals

Extraction of trypsin protein

5gm leaves of soybean were grinded with the help of chilled morter and pestel and was shaken with 10ml of 50mM sodium phosphate buffer (ph 7.6) in a shaking water bath for 4 hr at room temperature and the suspension was centrifuged at 10000g for 30 min the supernatant thus obtain was used to determining the activity of trypsin inhibitor.

Assay of trypsin inhibitor activity

The activity of trypsin inhibitor was assayed by determining the residual trypsin activity following the method of Kakade *et al.,* (1969) with slight modification using BapNA as the substrate and bovine trypsin as the standard enzyme. The reaction mixture contained 0.3ml diluted trypsin inhibitor (leaf extract), 0.3ml trypsin(2 mg in 40ml 0.001M HCL) and 2.1 ml of BapNA (30 mg dissolved in minimum volume of DMSO and adjust its final volume to 100ml with 0.05 M Tris HCl, pH 8.2, containing 0.003 $McaCl_2$) in a final volume of 2.7 ml.

The final concentration of BApNA in the reaction mixture was 0.54mM and the number at the of trypsin units was 180. After incubating the mixture at 37^0C for 15 min in a shaking water bath, the reaction was stopped by adding 0.3 ml of 30% (v/v) glacial acetic acid. A blank and a trypsin control run simultaneously. The absorbance was recorded

at 410nm against the blank. Trypsin inhibitor activity (TIA) is defined as number of trypsin units inhibited (TUI)

No choice experiment

The antifeedant activity of 33 genotypes of soybean was evaluated against 4th instar larvae of *S. litura* and *S. obliqua* under laboratory conditions (29±5ºC, RH 83±5%) using 'no-choice' feeding technique (Belles *et al.,* 1985 and Kumar, 1993). The fresh and matured leaves of thirty three genotypes of soybean were plucked, thoroughly washed and dried with the help of filter paper and the leaf discs (area = 4 x 4 cm^2) were cut from them. The leaf discs were kept in centre of pre sterilized corning glass petridishes (dia. 9 cm) containing an inner lining of moist filter paper. All the treatments were replicated three times along with control. Prestarved (3 h) and freshly molted worms of uniform size were released in each petridish (n=2) and were allowed to feed until more than 75% leaf discs were eaten away in control. The observations were recorded on leaf area consumed with the help of graph paper in the various treatments.

Results and Discussion

The activity of trypsin inhibitor was present in all the varieties but showed slight inter-varietal variation and the result of Trypsin inhibitor activity (TIA) are presented in Table 1. The highest TIA activity exhibited was 2.25 TUI.mg-^{1}protein (SL 979) and (SL 688) followed by JS 20-41 and SL 982 with 2.23 and 2.22 TUI.mg^{-1} protein respectively. The lowest TIA were recorded in CSB 904 with 0.35 TUI.mg-^{1}protein followed by MACS 1394 and MACS 1340 with 0.37 and 0.38 TUI.mg-1 protein respectively.

The *S. litura* gut extracts were assayed for trypsin activity by using synthetic substrates with respect to their specificities towards the protease enzyme. Specific protease activity in different cultivars fed by 4th larval stages of *S. litura* has been summarized in Table 1. The higher gut trypsin inhibition activity ascertained in larvae fed on SL 688 with 14.40 (%) followed by SL 979 and JS 20-41, with (14.47 and 14.22 %) respectively. The lowest intensity of gut trypsin inhibition activity was observed in genotypes NRC 94 (0.12%) followed by KDS 378 (0.14) and KDS 378 (0.22) per cent.

In no choice feeding method for *S. litura* the minimum feeding was observed with SL 979 (1.42 cm^2) and maximum in CSB 904 (17.48 cm^2) over check (Bragg=18.77 cm^2), while the minimum and maximum feeding was found with SL 979 (1.71 cm^2), and CSB 904 (18.03 cm^2) respectively against larvae of *S. obliqua* over control (MLAC=18.76 cm^2). On the basis of preference index DS 2708, JS 20-41, JS 20-69, KDS 693, KDS 705, NRC 93, RKS 113, RVS 113, RVS 2001-18, SL 979, SL 982, SL 688 and PS 1347 genotypes were found to be extremely antifeedant while DSb 19, DSb 21, MACS 1407,MACS 1416, MAUS 614, NRC 92, PS 1518, SL 958 and PS 1092 were found strongly antifeedant and DS 2706, KBS 22-2009, KDS 708 and MAUS 612 were found to be moderately antifeedant, while the remaining genotypes where found slightly antifeedant (Tables 2 and 3).

In the present study a fairly high degree of association was found between mean leaf area consumed and with some of important biochemical constituents in soybean genotypes (Table 4). A highly significant and negative correlation was observed between MLAC (cm^2) by *S. litura* and *S. obliqua* and protein content in 33, leaves (r= -0.728**) and (-0.674**) respectively and trypsin inhibitor in leaves (r= -0.909**) and (r= -0.913**). Thus it can be concluded that the

genotypes which were having higher protein and trypsin inhibitor in their leaves offered resistance against *S. litura* and *S. boliqua* in soybean.

Table.1 Total Protein content, trypsin inhibitor activity in leaves of soybean genotypes and activity of larval gut protease of *S. litura*

Sr. No.	Genotypes	Protein (g/100g) Total	Trypsine inhibitor in leaves (TUI.mg^{-1} protein)	Inhibition of *S.litura* guts protease (%)
1	**CSB 904**	21.393 ± 0.106	0.35±0.23	0.21±0.29
2	**DS 2705**	22.669 ± 0.022	0.64±0.19	0.51±0.12
3	**DS 2706**	31.750 ± 0.017	0.96±0.09	2.02±0.16
4	**DS 2708**	31.840 ± 0.044	2.19±0.11	13.62±0.23
5	**DSb 19**	33.638 ± 0.040	1.61±0.02	7.03±0.09
6	**DSb 21**	34.375 ± 0.013	1.79±0.21	8.83±0.17
7	**JS-20-41**	32.953 ± 0.006	2.23±0.13	14.22±0.11
8	**JS-20-69**	28.274 ± 0.026	1.98±0.07	11.04±0.05
9	**JS-20-71**	24. 051 ± 0.037	0.71±0.03	0.77±0.12
10	**KBS-22-2009**	24.177 ± 0.028	0.92±0.14	1.78±0.07
11	**KDS-378**	24.750 ± 0.037	0.45±0.19	0.14±0.13
12	**KDS-693**	31.562 ± 0.015	2.18±0.09	13.47±0.10
13	**KDS-699**	23.449 ± 0.071	2.06±0.07	12.03±0.13
14	**KDS 705**	28.500 ± 0.017	0.79±0.12	1.14±0.08
15	**KDS 708**	23.378 ± 0.017	0.42±0.15	0.22±0.19
16	**MACS 1340**	22.598 ± 0.034	0.38±0.06	0.30±0.14
17	**MACS 1394**	22.373 ± 0.079	0.37±0.03	0.32±0.18
18	**MACS 1407**	26.677 ± 0.002	1.87±0.04	9.75±0.08
19	**MACS 1416**	27.469 ± 0.009	1.49±0.03	5.93±0.11
20	**MAUS 612**	26.151 ± 0.170	1.12±0.11	3.03±0.04
21	**MAUS 614**	27.033 ± 0.003	1.36±0.18	4.82±0.07
22	**NRC 92**	32.930 ± 0.028	1.55±0.08	4.91±0.11
23	**NRC 93**	28.156 ± 0.030	1.37±0.02	6.49±0.16
24	**NRC 94**	24.254 ± 0.019	0.50±0.05	0.12±0.08
25	**PS 1518**	23.597 ± 0.182	2.03±0.05	11.64±0.17
26	**RKS 113**	29.625 ± 0.003	1.69±0.05	7.81±0.15
27	**RVS 2001-18**	35.455± 0.029	2.12±0.25	12.74±0.20
28	**SL 958**	26.438 ± 0.108	2.16±0.15	13.24±0.19
29	**SL 979**	36.761 ± 0.032	2.25±0.22	14.47±0.17
30	**SL 982**	32.114 ± 0.010	2.22±0.12	14.07±0.24
31	**SL 688**	36.954 ± 0.056	2.25±0.23	14.50±0.14
32	**PS 1092**	30.060 ± 0.046	1.20±0.12	3.61±0.21
33	**PS 1347**	35.035 ± 0.021	2.20±0.18	13.80±0.12

Table.2 Effect of 33 genotypes of soybean on feeding behaviour of 10 d old larvae of *S. obliqua*, Bihar hairy caterpillar

SR. NO.	Genotype name	MLAC (cm^2)	Feeding Percentage (%)	Antifeedant Activity (%)	Feeding Inhibition %	Preference Index (C)	Antifeedant Category
1	CSB 904	18.03 (4.30)	72.1	0.79	2.01	0.98	Slightly antifeedant
2	DS 2705	13.00 (3.67)	51.98	6.155	18.165	0.82	Slightly antifeedant
3	DS 2706	9.13 (3.10)	36.5	10.28	34.57	0.65	Moderately antifeedant
4	DS 2708	2.17 (1.63)	8.66	17.705	79.315	0.21	Extremely antifeedant
5	DSb 19	4.22 (2.17)	16.86	15.515	63.315	0.36	Strongly antifeedant
6	DSb 21	4.07 (2.13)	16.28	15.67	64.355	0.35	Strongly antifeedant
7	JS 20-41	1.94 (1.56)	7.76	17.94	81.26	0.18	Extremely antifeedant
8	JS 20-69	2.73 (1.79)	10.92	17.1	74.62	0.25	Extremely antifeedant
9	JS 20-71	12.71 (3.63)	50.84	6.46	19.24	0.81	Slightly antifeedant
10	KBS 22-1009	9.94 (3.23)	39.76	9.41	30.745	0.69	Moderately antifeedant
11	KDS 378	15.92 (4.05)	63.66	3.04	8.215	0.92	Slightly antifeedant
12	KDS 693	2.21 (1.64)	8.84	17.655	78.925	0.21	Extremely antifeedant
13	KDS 705	2.27 (1.66)	9.08	17.59	78.42	0.21	Extremely antifeedant
14	KDS 708	10.15 (3.26)	40.58	9.195	29.825	0.70	Moderately antifeedant
15	KDS 99	15.95 (4.05)	63.8	3	8.11	0.92	Slightly antifeedant
16	MACS 1340	16.27 (4.09)	65.06	2.665	7.14	0.93	Slightly antifeedant
17	MACS 1394	17.00	67.98	1.89	4.95	0.95	Slightly antifeedant

		(4.18)					
18	MACS 1407	3.55 (2.01)	14.18	16.235	68.275	0.32	Strongly antifeedant
19	MACS 1416	5.60 (2.46)	22.4	14.04	54.035	0.46	Strongly antifeedant
20	MAUS 612	6.77 (2.69)	27.08	12.795	46.98	0.53	Moderately antifeedant
21	MAUS 614	5.78 (2.50)	23.1	13.855	52.935	0.47	Strongly antifeedant
22	NRC 93	2.32 (1.68)	9.28	17.54	77.995	0.22	Extremely antifeedant
23	NRC 92	5.47 (2.44)	21.86	14.185	54.89	0.45	Strongly antifeedant
24	NRC 94	14.86 (3.91)	59.42	4.17	11.63	0.88	Slightly antifeedant
25	PS 1518	5.89 (2.52)	23.56	13.73	52.225	0.47	Strongly antifeedant
26	RKS 113	2.25 (1.65)	8.98	17.615	78.63	0.21	Extremely antifeedant
27	RVS 2001-18	1.84 (1.52)	7.34	18.055	82.185	0.17	Extremely antifeedant
28	SL 979	1.71 (1.48)	6.82	18.195	83.34	0.16	Extremely antifeedant
29	SL 982	2.11 (1.61)	8.42	17.765	79.83	0.20	Extremely antifeedant
30	SL 958	6.08 (2.56)	24.3	13.535	51.095	0.49	Strongly antifeedant
31	SL688	2.00 (1.58)	7.98	17.885	80.78	0.19	Extremely antifeedant
32	PS1092	4.17 (2.16)	16.66	15.57	63.635	0.36	Strongly antifeedant
33	PS1347	2.05 (1.59)	8.18	17.835	80.345	0.19	Extremely antifeedant
34	**BRAGG**	18.77 (4.38)	75.06	0.00	0.00	1.00	Preferred plant
	CD at 5%	0.496					
	F value	**					

Table.3 Effect of 33 genotypes on feeding behaviour of 10d old larvae of tobacco caterpillar, *S. litura* (Fab.)

SR. NO.	Genotype name	MLAC (cm^2)	Feeding Inhibition %	Feeding Percentage (%)	Antifeedant Activity (%)	Preference Index (C)	Antifeedant Category
1	CSB 904	17.49 (4.24)	3.57	69.94	1.38	0.97	Slightly antifeedant
2	DS 2705	12.48 (3.60)	20.17	49.92	6.72	0.80	Slightly antifeedant
3	DS 2706	7.42 (2.81)	43.40	29.68	12.11	0.57	Moderatly antifeedant
4	DS 2708	1.89 (1.54)	81.76	7.54	18.01	0.18	Extremely antifeedant
5	DSb 19	3.78 (2.06)	66.55	15.10	16.00	0.34	Strongly antifeedant
6	DSb 21	3.48 (1.99)	68.81	13.92	16.31	0.31	Strongly antifeedant
7	JS 20-41	1.64 (1.46)	83.98	6.54	18.28	0.16	Extremely antifeedant
8	JS 20-69	2.15 (1.62)	79.45	8.60	17.73	0.21	Extremely antifeedant
9	JS 20-71	11.82 (3.50)	22.74	47.28	7.42	0.77	Slightly antifeedant
10	KBS 22-1009	7.82 (2.88)	41.20	31.28	11.69	0.59	Moderately antifeedant
11	KDS 378	13.81 (3.78)	15.27	55.24	5.29	0.85	Slightly antifeedant
12	KDS 693	1.97 (1.56)	81.05	7.86	17.93	0.19	Extremely antifeedant
13	KDS 705	2.08 (1.60)	80.10	8.30	17.81	0.20	Extremely antifeedant
14	KDS 708	8.47 (2.99)	37.85	33.88	10.99	0.62	Moderately antifeedant
15	KDS 99	15.07 (3.94)	11.00	60.28	3.95	0.89	Slightly antifeedant
16	MACS 1340	15.19 (3.96)	10.56	60.76	3.83	0.90	Slightly antifeedant
17	MACS 1394	15.55 (4.00)	9.42	62.20	3.44	0.91	Slightly antifeedant
18	MACS 1407	2.51 (1.73)	76.46	10.02	17.35	0.24	Extremely antifeedant
19	MACS 1416	4.65 (2.26)	60.36	18.58	15.07	0.40	Strongly antifeedant
20	MAUS 612	5.95	51.87	23.80	13.68	0.48	Strongly antifeedant

		(2.53)					
21	MAUS 614	5.03 (2.35)	57.74	20.12	14.66	0.43	Strongly antifeedant
22	NRC 93	2.13 (1.62)	79.67	8.50	17.76	0.21	Extremely antifeedant
23	NRC 92	4.32 (2.19)	62.69	17.26	15.42	0.37	Strongly antifeedant
24	NRC 94	13.05 (3.68)	18.03	52.20	6.11	0.82	Slightly antifeedant
25	PS 1518	5.18 (2.38)	56.76	20.72	14.50	0.31	Strongly antifeedant
26	RKS 113	2.04 (1.59)	80.45	8.14	17.85	0.20	Extremely antifeedant
27	RVS 2001-18	1.54 (1.42)	84.84	6.16	18.38	0.15	Extremely antifeedant
28	SL 979	1.42 (1.38)	85.98	5.66	18.52	0.14	Extremely antifeedant
29	SL 982	1.84 (1.52)	82.20	7.34	18.07	0.18	Extremely antifeedant
30	SL 958	5.72 (2.49)	53.30	22.88	13.92	0.47	Strongly antifeedant
31	SL688	1.75 (1.49)	83.00	6.98	18.16	0.17	Extremely antifeedant
32	PS1092	3.51 (2.00)	68.58	14.04	16.28	0.39	Strongly antifeedant
33	PS1347	1.78 (1.50)	82.73	7.10	18.13	0.23	Extremely antifeedant
34	**BRAGG**	18.78 (4.38)	0.00	75.10	0.00	1.00	Preferred plant
	CD at 5%	0.554					
	F value	**					

Table.4 Simple correlation coefficient between biochemical constituents of soybean genotypes leaves and mean leaf area consumed

Chemical compounds	Mean leaf area consumed by *S. litura*	Mean leaf area consumed by *S. obliqua*
Proteins	-0.728**	-0.674**
Trypsin inhibitor	-0.909**	-0.913**

** Highly significant at 1%

Other authors also found that protease inhibitors play important role in plant defense mechanism by preventing proteolysis in the midgut of insect larvae leading to their starvation and subsequent death (Gatehouse *et al.,* 1999). This fact can be interpreted as a potential strategy for increasing the level of plant defense against insects (Kansal *et al.,* 2008).

Manus and Burgess (1995) reported that the extracts from the digestive tract of final instar larvae of *Spoduptera litura,* reared on artificial diet, contained trypsin activity with a pH optimum of 10.5, an elastase activity with a pH optimum of 10.5 and leucine aminopeptidase activity with a pH optimum of 8.5. No chymotrypsin amidase activity could be detected, but chymotrypsin esterase activity was measured with a pH optimum of 8.5. Titration, in uitvo of the soybean (Kunitz) trypsin inhibitor (SBTI) against each enzyme at its optimal pH revealed that the inhibitor was most effective at retarding the trypsin-like activity, only slightly effective against the elastase-like and chymotrypsin esterase-like activity, but completely ineffective against leucine aminopeptidase. Incorporation of SBTI into the diet of neonate larvae at 0.2% (w/v) and 0.5% (w/v) retarded growth rate when compared with larvae fed artificial diet only. Franco *et al.,* (2004) reported that Enzymatic assays using gut extracts from larval and adult boll weevil have demonstrated the presence of digestive serine proteinase-like activities and in vitro assays showed that soybean Kunitz trypsin inhibitor (SKTI) was able to inhibit these enzymes.

Gholamzadeh *et al.,* (2013) investigated that, the Proteolytic activity of azocasein as a protein substrate in the gut of *C. nemorana* was 7.267 ± 0.37 µmol/min/mg protein. In addition, the trypsin and chymotrypsin activity was 1.53 ± 0.03 and 1.42 ± 0.1 µmol/min/mg protein, respectively. The azocasein hydrolysis by the midgut extract of *A. janata* resulted in the specific activity of 1200 ± 90 nmol/ min/mg protein (Budatha *et al.,* 2008).

A highly significant negative correlation was observed between mean leaf area consumed (cm^2) by *S. litura* and *S. obliqua* and protein content in 33 genotypes leaves (r= -0.728**) and (-0.674**) respectively and trypsin inhibitor in leaves (r= -0.909**) and (r= -0.913**). Thus it can be concluded that the genotypes which were having higher protein and trypsin inhibitor in their leaves offered resistance against *S. litura* and *S. boliqua* in soybean crop.

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