Research Article

Biochemical Quantification of Transgenic Chickpea Lines and Correlation between Resistance/Susceptibility to Pod Borer, *Helicoverpa Armigera*

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Abstract

There were no significant differences in protein content between the transgenic and non-transgenic chickpeas. Maximum amount of protein was recorded in ICC 506EB (6.0 mg/g).Among the transgenic lines, the protein content was highest in BS5A.1(T2) 18-2P1 (5.8 mg/g). The amounts of carbohydrates were significantly higher in the leaves of ICC 506EB (44.9%) as compared to that on transgenic chickpeas. Lowest carbohydrate content was recorded in Semsen (28.5%). There were no significant differences in lipid content between the leaves of transgenic and non transgenic chickpea lines. However, the amount of lipids were higher in BS5A.2(T2) 19-3P1 (20.6%) than in BS5A.2(T2) 19-3P2 (7.9%). There were no much significant differences in phenol content between the transgenic and non transgenic chickpea lines. The phenol content ranged from 1.0 mg/g to 1.1 mg/g and the tannins from 1.2 to 2.1 mg/g.

Keywords: *Transgenic chickpea lines, Helicoverpa armigera, Biochemical,* Correlation, Resistance and Susceptiblity

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Introduction

Chickpea (*Cicer arietinum* L.) is the third most important pulse crop, grown in an area of 8.21 m ha, with a total production of 7.48 m tonnes globally [1]. It is even more important for India as the country's production accounts for 67% of the global chickpea production and chickpea constitutes about 40% of India's total pulse production. It is a source of high quality protein for the poor people in many developing countries, including India. Chickpea yields are quite low, and have remained almost stagnant for the past 2 to 3 decades. It is damaged by over 50 insect species in different parts of the world, of which the pod borer, *Helicoverpa armigera* (Hubner) (Noctuidae: Lepidoptera) is the most damaging pest worldwide [2]. It has been estimated to cause chickpea losses worth more than US$325 million annually. Genetic transformation as a means to enhance crop resistance or tolerance to biotic constraints has shown considerable potential to achieve a more effective control of target insect pests for sustainable food production [3]. Its control is largely based on insecticides. However, with the development of resistance to insecticides in *H. armigera* populations [4], there has been a renewed interest in developing alternative methods of pest control, of which plant resistance to *H. armigera* is an important component. The δ-endotoxin genes from the bacterium *Bacillus thuringiensis* Berliner (*Bt*) have been deployed in several crops for pest management [5] Efforts are underway to develop chickpea plants with *Bt* δ-endotoxin genes for resistance to *H. armigera* [6] However concerns have been expressed that the trichome exudates in chickpea leaves and the pods, which are highly acidic in nature (pH 2.0 – 3.5) [7], may have a negative influence on the biological activity of *Bt* sprayed on chickpea or toxin proteins expressed in transgenic chickpea.

Material and Methods

Experimental material

The six transgenic chickpea lines, BS5A.1(T2) 18-1P1, BS5A.1(T2) 18-2P1, BS5A.2(T2) 19-1P2, BS5A.2(T2) 19-2P1, BS5A.2(T2) 19-3P1, BS5A.2(T2) 19-3P2 and two non transgenic chickpea lines, ICC506 EB (Resistant check) and Semsen (Control) were used to estimate the biochemical components in transgenic chickpea lines.

Estimation of biochemical constituents

Proteins

Sixty mg of the dried test sample was macerated in 10 ml of cold TCA (10%) for 30 min, kept at 4ºC for 24 h, and then centrifuged [8]. The supernatant was discarded and the resultant pellet was re-suspended in 5 per cent TCA (10 ml) and heated on a water bath at 80ºC for 30 min. The sample was cooled and re-centrifugated, and each time the supernatant was discarded. Finally pellet was washed with distilled water, centrifuged and the residue dissolved in 1N NaOH (10 ml), and left overnight at room temperature.

Total protein content was estimated in an aliquot of 1 ml sample extract [9]. A stock solution (1 mg/ml) of Bovine Serum Albumin (Sigma Chemicals Manufacturing Limited, St. Louis, Missouri, USA) was prepared in 1N NaOH, from which 0.1 to 0.9 ml of aliquots were dispensed in a series of test tubes. The volume was made up to 1 ml by adding distilled water. To each test sample, 5 ml freshly prepared alkaline solution (prepared by mixing 50 ml of 2% Na_2CO_3 in 0.1N NaOH and 1 ml of 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartrate) was added at room temperature and left undisturbed for a period of 10 min.

Subsequently, 0.5 ml of Folin-Ciocalteau reagent (prepared by diluting the reagent with distilled water in 1:2 ratio just before use) was added to each sample. The optical density (OD) of each sample was measured at 750 nm after 30 min in a spectrophotometer (Hitachi, Tokyo, Japan, U 2900). Three replicates of each sample were taken and their mean values were used to prepare the standard curve. The total protein content in each sample was calculated from the standard curve for Bovine Serum Albumin (BSA). Three replicates were examined for each treatment.

Carbohydrates

The dried sample (50 mg each) was macerated in a grinder with 20 ml of ethanol and left for 12 h. The samples were then centrifuged at 1200 rpm for 15 min, the supernatants were removed and concentrated on a water-bath. The volume of aqueous concentrates was made up to 50 ml with distilled water (Extract A) and processed to estimate total soluble sugars [10].

Residual pellet obtained by centrifugation was suspended in a mixture of 5 ml of 52 per cent perchloric acid and 6.5 ml of distilled water, shaken vigorously (5 min) and centrifuged at 2500 rpm. This step was repeated three times and the supernatants were collected and pooled. The volume was made up to 100 ml with distilled water (Extract B). An aliquot of 1 ml was used to estimate starch content [11].

One ml aliquot of the test sample from Extracts A and B were used for quantifying total carbohydrates using phenol-sulphuric acid method [12]. A standard curve was prepared using glucose. A stock solution of glucose (100 µg/ml) was prepared in distilled water, of which 0.1 to 0.9 ml aliquots were transferred to a series of test tubes and the volume made up to 1 ml with distilled water. To each of these, 1 ml of 5 per cent aqueous phenol was added quickly in an ice chest and shaken gently and then 5 ml of concentrated H_2SO_4 was added by agitating the test tube. The test tubes were kept in a water-bath (26º–30ºC) for 20 min and the optical density (ODs) of the yellow orange color thus developed was recorded at 490 nm in a spectrophotometer after setting the instrument for 100 per cent transmission against the blank. Four replicates of each sample were run and the mean values calculated. A regression was computed between known concentrations and their respective OD (based on Beer's Lamberts Law). The concentration (mg/g dry weight) of total soluble sugars was estimated from the standard curve for glucose. Three replicates of each sample were taken and their mean values recorded. The carbohydrate content in terms of glucose equivalent and the conversion factor (0.9) were used to convert values of glucose to starch in each case.

Standards with different concentrations (i.e., 25, 50, 75, 100 and 125 µg of glucose) were prepared from the working standard, and their absorbance was read by taking 1 ml aliquots.

Total soluble sugars were calculated by using the formula:

$$\frac{\text{Conc. of standard}}{\text{Absorbance of standard}} \times \text{Absorbance of 1 ml extract} \times \frac{1}{10{,}00{,}000} \times \frac{3\text{ ml}}{0.1\text{g}} \times 100$$

Lipids

One g of each of the dried and milled test sample was macerated in 10 ml distilled water [13]. To this, 30 ml of chloroform: methanol (2:1 v/v) was added and mixed thoroughly. The mixture was left overnight at room temperature; 20 ml each of chloroform and distilled water was added to the sample and centrifuged. Of the three layers, a clear lower layer of chloroform containing lipids was collected in a pre-weighted beaker. The solvent was

allowed to evaporate and the beaker was re-weighed and the amount of lipids were recorded and expressed as total lipids/g of the dried sample.

Phenols

Dried and milled test samples (200 mg) were homogenized in 80% ethanol (10 ml) for 2 h and left over night at room temperature. The samples were centrifuged and the supernatants were collected individually and the volume of each was made up to 40 ml with 80 per cent ethanol.

A standard curve of caffeic acid (phenol) was prepared. A stock solution (100 µg/ml) of caffeic acid was prepared in 80 per cent ethanol, from which 0.1 to 0.9 ml aliquots were transferred into a series of test-tubes and the volume was made up to 1 ml with 80 per cent ethanol. To each of these tubes, 1 ml of Folin–Ciocalteau reagent (1: 2 ratio) with 2 ml of 20 per cent Na_2CO_3 solution was added and the contents mixed vigorously. The samples were incubated in boiling water bath for 1 min, cooled and diluted to 25 ml with distilled water. The optical density (OD) was recorded at 750 nm using a spectrophotometer against a blank [14].

Three replicates were taken for each concentration, and the average OD was plotted against the respective concentrations to prepare the standard curve. Each test sample was processed in a similar manner. Total amount of phenols was estimated from (with reference to caffeic acid) the standard curve.

The standard curve was prepared by plotting the average absorbance readings of the duplicate determinations of catechin concentrations and the catechin equivalents (CE) calculated by using the following formula.

$$\text{CE (\%)} = \frac{\text{mg catechin/ml}}{\text{Vol. of extract taken}} \text{ X } \frac{\text{Volume made up}}{\text{Wt. of sample}} \text{ X } 100$$

Tannins

The amounts of condensed tannins present in the leaves of chickpea were estimated by Vanillin – hydrochloride assay [15]. The following reagents were used in the present study.

Reagents

- Vanillin-hydrochloride reagent: Mixture of equal volumes of 8% hydrochloric acid in methanol and 4% vanillin in methanol.
- 8% concentrated HCl in methanol (8 ml of HCl add to 92 ml of methanol).
- 4% Vanillin in methanol (4 g of Vanillin brought to 96 ml of methanol).
- Mixed 2 and 3 in equal volumes just before use.

Standard solution

A stock solution was prepared by dissolving 1 mg of catechin in 1 ml of methanol. The stock solution was diluted ten times (10 times dilution: 1 ml stock + 9 ml of methanol) and 10 ml to 100 ml (100 µg/ml).

Chickpea leaves were collected from the field at 30 DAE and placed in paper bags. These were initially shade-dried and kept in an oven at 50^0C for complete drying. These samples were ground to a fine powder in a blender from which 0.5 g of leaf powder was taken in 25 ml methanol. It was mixed by swirling occasionally and the sample kept at room temperature for 24 h, and centrifuged for 20 min at 4500 rpm.

From the above extract, 1 ml aliquot was pipetted out into a test tube to which freshly prepared vanillin – hydrochloride reagent was added slowly. An individual blank was prepared for each extract by adding 5 ml of vanillin – hydrochloride to 1 ml aliquot. These tubes were incubated in the water bath for 20 min. The absorbance was recorded at 500 nm against the reagent blank in a Spectrophotometer. Standard curve was prepared by plotting the average absorbance readings of the duplicate determinations of catechin concentrations. The catechin equivalents were calculated by using the formula.

$$\text{Catechin Equivalents (\%)} = \frac{\text{mg catechin/ml}}{\text{Vol. of extract taken}} \text{ X } \frac{\text{Volume made up}}{\text{Wt. of sample}} \text{ X } 100$$

Results and Discussion

Biochemical profile of different transgenic chickpea lines

There were no significant differences in the protein content between the transgenic and non-transgenic chickpea lines. Protein content was highest in the leaves of BS5A.2(T2) 19-1P2 (5.8 mg/g dw), followed by 5.5 mg/g in Semsen, 5.3 mg/g in BS5A.1(T2) 18-2P1, BS5A.2(T2) 19-3P1, 5.2 mg/g in BS5A.1(T2) 18-1P1, BS5A.2(T2) 19-3P2, 4.9 mg/g in BS5A.2(T2) 19-2P1 and 4.8 mg/g in ICC 506EB. Highest amounts of carbohydrates were recorded in the leaves of ICC 506EB (55.0%), whereas the leaves of Semsen (24.3%) had the lowest amount of carbohydrates. The amount of carbohydrates ranged from 34.0 to 49.3% in transgenic chickpea lines. Among the transgenic chickpea lines tested, the amount of carbohydrates was significantly greater in the leaves of BS5A.2(T2) 19-3P1 (49.3%) than in BS5A.1(T2) 18-1P1 and BS5A.2(T2) 19-1P2 (34.0%) (**Table 1**).

Table 1 Biochemical profile of different transgenic chickpea (2011-2013)

Genotype	2011-2012					2012-2013				
	Proteins (mg/g)	Carbo hydrates (%)	Lipids (%)	Phenols (mg/g)	Tannins (mg/g)	Proteins (mg/g)	Carbo hydrates (%)	Lipids (%)	Phenols (mg/g)	Tannins (mg/g)
BS5A.1(T2) 18-1 P1	5.2^{ab}	34.0^{b}	13.9^{a}	1.1^{a}	2.2^{d}	5.2^{ab}	35.0^{a}	16.6^{ab}	0.9^{a}	1.5^{a}
BS5A.1(T2) 18-2 P1	5.3^{ab}	44.6^{cd}	10.6^{a}	1.0^{a}	0.5^{a}	6.3^{cd}	38.8^{a}	16.7^{ab}	1.0^{a}	2.0^{a}
BS5A.2(T2) 19-1 P2	5.8^{b}	34.3^{b}	7.8^{a}	1.1^{a}	1.6^{c}	5.4^{bcd}	30.6^{a}	14.0^{ab}	1.1^{a}	1.4^{a}
BS5A.2(T2) 19-2 P1	4.9^{a}	38.00^{bc}	16.4^{a}	1.2^{a}	2.1^{d}	6.4^{de}	31.3^{a}	8.2^{a}	0.9^{a}	1.6^{a}
BS5A.2(T2) 19-3 P1	5.3^{ab}	49.3^{de}	11.9^{a}	0.9^{a}	1.2^{b}	6.1^{bcd}	38.0^{a}	29.4^{c}	1.2^{a}	1.7^{a}
BS5A.2(T2) 19-3 P2	5.2^{ab}	36.0^{bc}	8.8^{a}	1.2^{a}	3.2^{e}	5.4^{bc}	28.1^{a}	7.0^{a}	0.9^{a}	1.1^{a}
ICC 506 EB (Resistant check)	4.8^{a}	55.0^{e}	11.5^{a}	1.0^{a}	1.0^{b}	7.2^{e}	34.8^{a}	13.7^{ab}	1.0^{a}	1.8^{a}
Semsen (Control)	5.5^{ab}	24.3^{a}	13.7^{a}	1.1^{a}	0.8^{b}	4.5^{a}	32.8^{a}	20.1^{b}	1.1^{a}	1.6^{a}
SE $\pm$	0.2	2.8	3.2	0.1	0.1	0.2	3.7	2.9	0.1	0.4
Fp	0.191	<0.001	0.641	0.695	<0.001	<0.001	0.502	0.003	0.80	0.901
Vr	1.6	11.5	0.7	0.6	60.5	9.0	0.9	5.7	0.5	0.3
LSD (P 0.05)	NS	8.69	NS	NS	0.34	0.84	NS	8.947	NS	NS

*Figures followed by the same letter within a column are not significantly different at $P \leq 0.05$

There were no significant differences in lipid content between the transgenic and non transgenic chickpea lines. Among the transgenics, BS5A.2(T2) 19-2P1 leaves had the highest lipid content (16.4%), followed by 13.9% in BS5A.1(T2) 18-1P1, 11.9% in BS5A.2(T2) 19-3P1, 10.6% in BS5A.1(T2) 18-2P1 and 8.8% in BS5A.2(T2) 19-3P2. The lowest lipid content was detected in BS5A.2(T2) 19-1P2 (7.8%). Among the non-transgenic chickpea lines, Semsen and ICC 506EB had 13.7 and 11.5% lipid content, respectively (Table 1).There were no significant differences in phenol content in the leaves between the transgenic and non-transgenic chickpeas. Phenol content (mg/g dw) of leaves was highest in BS5A.2(T2) 19-2P1 and BS5A.2(T2) 19-3P2 (1.2 mg/g), while the leaves of BS5A.2(T2) 19-3P1 had the lowest phenol content (0.9 mg/g). Leaves of BS5A.2(T2) 19-3P2 had the highest tannin content (3.2 mg/g), followed by 2.2 mg/g in BS5A.1(T2) 18-1P1, 2.1 mg/g in BS5A.2(T2) 19-2P1, 1.6 mg/g in BS5A.2(T2) 19-1P2, and 1.2 mg/g in BS5A.2(T2) 19-3P1. Tannin content was lowest in BS5A.1(T2) 18-2P1 (0.5 mg/g). Amounts of tannins in Semsen and ICC 506EB were 0.8 and 1.0 mg/g, respectively (Table 1).

During 2012-13, the protein content was significantly higher in ICC 506EB (7.2 mg/g) than in Semsen (4.5 mg/g). Among the transgenic chickpea lines tested, the maximum amount of protein was observed in BS5A.2(T2) 19-2P1 (6.4 mg/g) and BS5A.1(T2) 18-1P1 had the lowest protein content (5.2 mg/g). There were no significant differences in carbohydrate content in the leaves between the transgenic and non transgenic chickpeas. The amounts

of carbohydrates were highest (38.8%) in the leaves of BS5A.1(T2) 18-2P1 and BS5A.2(T2) 19-3P1. The leaves of BS5A.2(T2) 19-3P2 had the lowest (28.1%) of carbohydrates. Highest amounts of lipids (29.4%) were recorded in BS5A.2(T2) 19-3P1, followed by BS5A.1(T2) 18-2P1 (16.7%), BS5A.1(T2) 18-1P1 (16.6%), BS5A.2(T2) 19-1P2 (14.0%), BS5A.2(T2) 19-2P1 (8.2%) and BS5A.2(T2) 19-3P2 (7.0%). The lipid content in Semsen and ICC 506EB was 20.1 and 13.7%, respectively. There were no significant differences in phenol content between the transgenic and non-transgenic chickpeas. Highest phenol content was recorded in BS5A.2(T2) 19-3P1 (1.2 mg/g) and the lowest in BS5A.2(T2) 19-2P1, BS5A.2(T2) 19-3P2 and BS5A.1(T2) 18-1P1 (0.9 mg/g). Transgenic and non-transgenic chickpea lines differed significantly in tannin content in the leaves. BS5A.2(T2) 18-2P1 had the highest (2.0 mg/g), while BS5A.2(T2) 19-3P2 had lowest tannins (1.1 mg/g) (Table 1).

Correlation between resistance/susceptibility to pod borer and the amounts of biochemical composition of chickpea lines

During 2011-12, the protein content was negatively correlated with larval survival (r = -0.25), larval weight (r = -0.27) and leaf damage rating (r = -0.45). Significant positive correlation was observed between carbohydrate content and leaf damage (r = 0.4). Negative, but non-significant relationship of phenols was observed with leaf damage (r = -0.24), larval survival (r = -0.27) and larval weight (r = -0.17). There was a negative significant association of tannins with leaf feeding damage (r = -0.41), larval survival (r = -0. 40) and larval weight (r = -0.42) (**Table 2**).

Table 2 Correlation between resistance/susceptibility to pod borer, *H. armigera* and the amounts of biochemical components in transgenic chickpea (on dry weight basis 2011-13)

	2011-12					**2012-13**				
	Proteins	**Carbo hydrates**	**Lipids**	**Phenols**	**Tannins**	**Proteins**	**Carbo hydrates**	**Lipids**	**Phenols**	**Tannins**
HDR	-0.45*	0.40*	0.02	-0.24	-0.41*	0.31	0.25	0.05	-0.33	-0.47*
Larval survival (%)	-0.25	0.15	0.08	-0.27	-0.40*	-0.23	0.23	0.00	-0.40*	-0.45*
Mean larval wt. (mg)	-0.27	0.10	0.09	-0.17	-0.42*	-0.29	0.22	0.07	-0.23	-0.43*

,* Significant at P≤ 0.05 and 0.01, respectively

During 2012-13, the correlation co-efficients between the protein content and damage rating (r = 0.31) was positive but non-significant and there was a negative association with the larval survival (r = -0.23), larval weight (r = -0.29). Amounts of carbohydrates were positively correlated with leaf damage (r = 0.25), larval survival (r = 0.23) and larval weight (r = 0.22). There was a negative and significant association of the phenols with larval survival (r = -0.40). However, a negative but non-significant correlation was observed with leaf damage (r = -0.33) and larval weight (r = -0.23). Association between tannins and leaf damage (r = -0.47), larval survival (r= -0.45) and larval weight (r = -0.43) was found to be negative and significant (Table 2).

These results are in accordance with the earlier reports, wherein tannins have been shown to inactivate insecticidal crystal proteins of *B. thuringiensis* [16]. Tannin chemistry has been implicated in variation in host plant resistance to insects. Tannins, an important constituent of many plants, reacts strongly with the proteinaceous insecticidal proteins of *B. thuringiensis*. Commercial tannin preparation inhibits the activity of activated δ-endotoxin. Interaction between host plant tannins and δ-endotoxins might be one of the factors affecting the field efficacy of *B. thuringiensis* preparations or of *Bt*-transgenic crops [17].

Conclusions

There were no much significant differences in proteins, carbohydrates, lipid content and phenols between the transgenic and non transgenic chickpea lines. Transgenic and non-transgenic chickpea lines differed significantly in tannin content in the leaves. BS5A.2(T2) 18-2P1 had the highest (2.0 mg/g), while BS5A.2(T2) 19-3P2 had lowest tannins (1.1 mg/g).

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