Research Article

Quantification of Organic Acids (on Fresh Weight Basis) Present on the Leaf Surface in Transgenic Chickpea Lines Using HPLC

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Abstract

Significantly higher amounts of oxalic acid were recorded in BS5A.2(T2) 19-1P2 and BS5A.2(T2) 19-3P1 (1.1 mg/g) than in BS5A.2(T2) 19-2P1 (0.8 mg/g). Highest malic acid content was recorded on BS5A.1(T2) 18-1P1 (2.3 mg/g) and lowest on BS5A.2(T2) 19-3P2 (1.5 mg/g). Among the non-transgenics, the maximum amount of oxalic acid was observed in ICC 506EB (2.2 mg/g), followed by Semsen (0.9 mg/g). Oxalic acid content was positively correlated with larval survival (r = 0.63) and larval weight (r = 0.60). A significant and negative association was observed between the amounts of the malic acid and leaf feeding (r = - 0.83), larval survival (r = - 0.93) and larval weight (r = - 0.95).

Keywords: Organic acids, Transgenic chickpea lines, HPLC

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Introduction

The legume pod borer, *Helicoverpa armigera* (Hubner) is one of the most important constraints to crop production globally. It is a polyphagous pest, and attacks more than 300 plant species [1]. In India it has been recorded from over 20 crops and 180 wild hosts [2]. It causes an estimated loss of US$927 million in chickpea and pigeonpea, and possibly over US$5 billion on different crops worldwide, despite application of pesticides costing over US$2 billion annually [2]. It is widely grown in South and West Asia, North and Eastern Africa, Australia, Mexico, and North America. Several chickpea genotypes with low to moderate levels of resistance have been identified [3]. The amount of acid exudates on leaves is an useful index for distinguishing relatively resistant genotypes from the susceptible ones [3]. Eight desi chickpea genotypes were and reported that low levels of acidity in the leaf extracts of different genotypes were associated with susceptibility to *Helicoverpa armigera* (Hubner) in ICC 3137, K 850, and ICC 1403 [4]. Chickpea exudates can be used to select for resistance to *H. armigera*, the main components being malate and oxalate, which are present in variable amounts in different genotypes of chickpea [5]. Genotypes resistant to *H. armigera* accumulated more oxalic acid on the leaves than the susceptible genotypes [6]. Oxalic acid showed significant growth inhibition of *H. armigera* larvae when incorporated into a semi-artificial diet. The effective accumulation of oxalic acid is considered to be one of the mechanisms of *H. armigera* resistance in chickpea. Inhibition of larval growth by oxalic acid was not caused by antifeedant effects, but was more likely attributable to antibiosis.

The oxalic and malic acids were detected by Shimadzu LC 6A liquid chromatograph (Shimadzu, Kyoto, Japan) with a supelcogel C610-H column, as major acid components in the leaf exudates of chickpea genotypes. Fumaric and citric acid were also detected, but as minor components at less than one per cent that of the major acids. The concentrations of oxalic acid was consistently higher in resistant (ICC 506 and ICCL 86102) than in susceptible (Annegeri and ICCX 730266-3-4) genotypes at both vegetative and flowering stages. Malic acid concentration did not appear to be related with resistance status. Oxalic acid when included in a semi-artificial diet, inhibition of larval growth and prolongation of larval period were observed, where as malic acid had no significant effect [6]. The malic acid content estimated through HPLC was significantly and negatively correlated with damage rating and pod damage, whereas oxalic acid was negatively and significantly correlated with damage rating. Acetic acid showed a negative correlation with larval weight and damage rating, whereas citric acid showed negative and significant correlation with damage rating [7].

Genetically engineered plants with resistance to insects has considerable potential to achieve more effective control of target insect pests for sustainable food production [8]. Novel genes, such as delta-endotoxins from the bacterium, *Bacillus thuringiensis* Berliner (*Bt*) need to be deployed through transgenic chickpea to make host plant resistance an effective weapon for the control of *H. armigera.* Chickpea cultivars ICCV 1 and ICCV 6, transformed with *cry* 1Ac gene, have been found to inhibit the development and feeding of *H. armigera* [9]. However, there is an apprehension that the acid exudates in chickpea leaves and pods may influence the effectiveness of *Bt* toxins produced in the transgenic plants.

Material and Methods

Experimental material

Six transgenic and two non transgenic chickpea lines were grown under greenhouse conditions (27 ± 5^0 C and 65 - 90% RH). The seeds were sown in a sterilized mixture of black soil (Vertisols), sand and farmyard manure (2:1:1) filled in medium sized plastic pots (30 cm in diameter, 30 cm in depth). The seeds were sown 5 cm below the soil surface and watered immediately and thereafter as and when required. Three plants with uniform growth were retained in each pot at 10 days after seedling emergence. Diammonium phosphate granules (DAP) were applied at 15 days after seedling emergence @ 20 g per pot. The experiment was laid out in a completely randomized design (CRD) with three replications.

Estimation of Organic acids

A standard protocol for collection and analysis of organic acids from chickpea leaf exudates was followed, with a slight modification [7] and [10].
Standards: Oxalic acid, malic acid, fumaric acid, and citric acid.
Reagents: Potassium phosphate (KH_2PO_4), phosphoric acid (H_3PO_4), and millipore water.

Preparation of standards and sample collection

Two replicates of each standard organic acid were prepared by mixing 2 to 10 mg of standard organic acid in 10 ml of water to get concentrations of 200 to 1000 ppm. The chickpea leaf samples were collected early in the morning (before 9 am) in 25 ml centrifuge tubes containing 5 ml double distilled water/millipore water. The tubes were labelled for each genotypes, and weight of the tube and water was recorded (initial weight). First fully expanded leaf from three plants was excised with scissors and placed in the respective tubes containing double distilled millipore water for 10 to 15 min. The weight of tube with water and the leaves was recorded (final weight). Based on the initial and final weights, the fresh weights of the leaves were recorded. After extraction of the exudates, the leaves were removed from the tubes and placed on a filter paper for 1 h to remove the excess water. Later, the leaf area was measured using a leaf area meter. The dry weight of the leaves was recorded by placing the leaf samples in an oven at 45^0 C for three days.

The leaf exudates extracted in water were filtered through 45 µm hydrophilic PVDF millipore millex-HV filters using a 5 ml luer lock syringes. Approximately 3 ml sample solution was taken in 5 ml luer lock syringe from the centrifuge tubes. The needle was removed from the syringe and attached to millipore filter to dispense 1.5 ml of the filtrate into the HPLC vials. There were three replicates for each sample.

Quantification of organic acids in leaf exudates of chickpea by high performance liquid chromatography (HPLC)

For preparing 2 L of 25 mM KH_2PO_4 of pH 2.5 with H_3PO_4, 6.805 g of KH_2PO_4 was weighed and transferred in a 2 L conical flask and mixed with 1 L of millipore water until KH_2PO_4 was completely dissolved. Then added 4 ml of H_3PO_4 and the volume made up to 1.8 L, adjusted the pH to 2.5 by adding drop-by-drop H_3PO_4, and finally made up the volume to 2 L.

After priming, the mobile phase was run for 1 h. The vials containing leaf exudates of different chickpea genotypes were arranged in a carousel. Analysis was carried out by using Atlantis dC-18 column (4.6 x 250 mm, 5 µm). The samples (20 µl) were chromatographed singly on a Waters Atlantis C_{18} column (4.6 × 250 mm) with 5-µm pore size (A Waters HPLC 2695 separations module (alliance) system consisting of a PCM 11 reciprocating piston pump and a 2996 photodiode array detector in the range of 210 to 400 nm was used in a isocratic solvent system (25

Mm KH_2PO_4)). Chromatographic separation was done using mobile phase with a flow rate 0.8 ml min^{-1}, and the injected volume was 20 µl with 20 min run time per sample.

Based on the standards, retention time and peak areas of different organic acids present in the samples were identified and quantified. From the known concentrations of the standards, linear curve was plotted against concentration on the X-axis and absorbance on Y-axis. From the linearity curve, unknown concentrations of different organic acids from the samples were plotted and the amounts estimated. Amounts of organic acids present in a sample were expressed in mg g^{-1} fresh or dry weight or µg cm^{-2} leaf area.

Results and Discussion

Amounts of organic acids on fresh weight basis

During 2011-12, there were no significant differences in amounts of organic acids between the transgenic and non-transgenic chickpea lines. Maximum amount of oxalic acid was recorded on non-transgenic ICC 506EB (2.5 mg/g) and lowest on BS5A.2(T2) 19-2P1 (0.8 mg/g). Among the transgenics, highest amount of oxalic acid was recorded on the leaf surface of BS5A.2(T2) 19-3P2 (1.5 mg/g). High amounts of malic acid were observed in BS5A.1(T2) 18-1P1 (2.8 mg/g), ICC 506EB (2.7 mg/g), BS5A.2(T2) 19-3P1 (2.5 mg/g), BS5A.1(T2) 18-2P1 (2.4 mg/g), BS5A.2(T2) 19-1P2 (2.3 mg/g), BS5A.2(T2) 19-3P2 (2.2 mg/g) and BS5A.2(T2) 19-2P1 (2.1 mg/g) and lowest on Semsen (0.4 mg/g) (**Table 1, Figure 1**).

Table 1 Concentration of organic acids (on fresh weight basis) present on the leaf surface of transgenic chickpea lines

Genotype	2011-2012		2012-2013	
	Oxalic acid (mg/g)	Malic acid (mg/g)	Oxalic acid (mg/g)	Malic acid (mg/g)
BS5A.1(T2) 18-1 P1	0.9^{a}	2.8^{b}	1.2^{ab}	1.8^{bc}
BS5A.1(T2) 18-2 P1	1.0^{a}	2.4^{b}	0.8^{a}	1.3^{ab}
BS5A.2(T2) 19-1 P2	1.3^{abc}	2.3^{b}	1.0^{ab}	1.2^{ab}
BS5A.2(T2) 19-2 P1	0.8^{a}	2.1^{b}	0.7^{a}	1.1^{ab}
BS5A.2(T2) 19-3 P1	1.3^{ab}	2.5^{b}	0.9^{a}	1.6^{b}
BS5A.2(T2) 19-3 P2	1.5^{abcd}	2.2^{b}	0.5^{a}	0.9^{ab}
Semsen (Control)	1.2^{a}	0.4^{a}	0.7^{a}	0.2^{a}
ICC 506 EB (Resistant check)	2.5^{bd}	2.7^{a}	2.0^{b}	2.9^{c}
SE ±	0.3	0.3	0.2	0.3
Fp	0.132	0.004	0.095	0.022
LSD (P 0.05)	**NS**	1.1*	0.9*	1.1*

*Figures followed by the same letter within a column are not significantly different at P≤ 0.05.

During 2012-13, among the transgenic chickpea lines, the amounts of oxalic acid and malic acid were highest on BS5A.2(T2) 18-1P1 (1.2 and 1.8 mg/g, respectively) and lowest on BS5A.2(T2) 19-3P2 (0.5 and 0.9 mg/g, respectively). Among the non-transgenics, maximum amounts of oxalic acid and malic acid were observed on ICC 506EB (2.0 mg/g and 2.9 mg/g), followed by Semsen (0.7 and 0.2 mg/g, respectively) (Table 1, **Figure 2**).

Correlation between resistance/susceptibility to pod borer and the amount of organic acids

During 2011-12, oxalic acid content was positively correlated with larval survival (r = 0.63) and larval weight (r= 0.60). A significant and negative association was observed between the amounts of the malic acid and leaf feeding (r= -0.83), larval survival (r = -0.93) and larval weight (r = - 0.95) (**Table 2**).

During 2012-13, there was a positive and significant correlation between the oxalic acid and mean larval weight (r = 0.56). However, a positive non-significant relationship was observed with leaf damage (r = 0.19) and larval survival (r = 0.47). Further, the amounts of malic acid had positive non-significant correlation with leaf damage (r = 0.18), larval survival (r = 0.23) and larval weight (r = 0.27) (Table 2).

Oxalic acid and malic acid were detected as major components in the leaf surface exudates of transgenic and non-transgenic lines [6]. A low amounts of acids in the leaf exudates (21.1 and 4.9 meq./100 gm) of genotypes (ICC

14665) were detected [11]. A diverse array of chickpea genotype was characterized for organic acid profiles in the leaf exudates. Chickpea leaf exudates contained malic acid, oxalic acid, acetic acid, citric acid and fumaric acid [7].

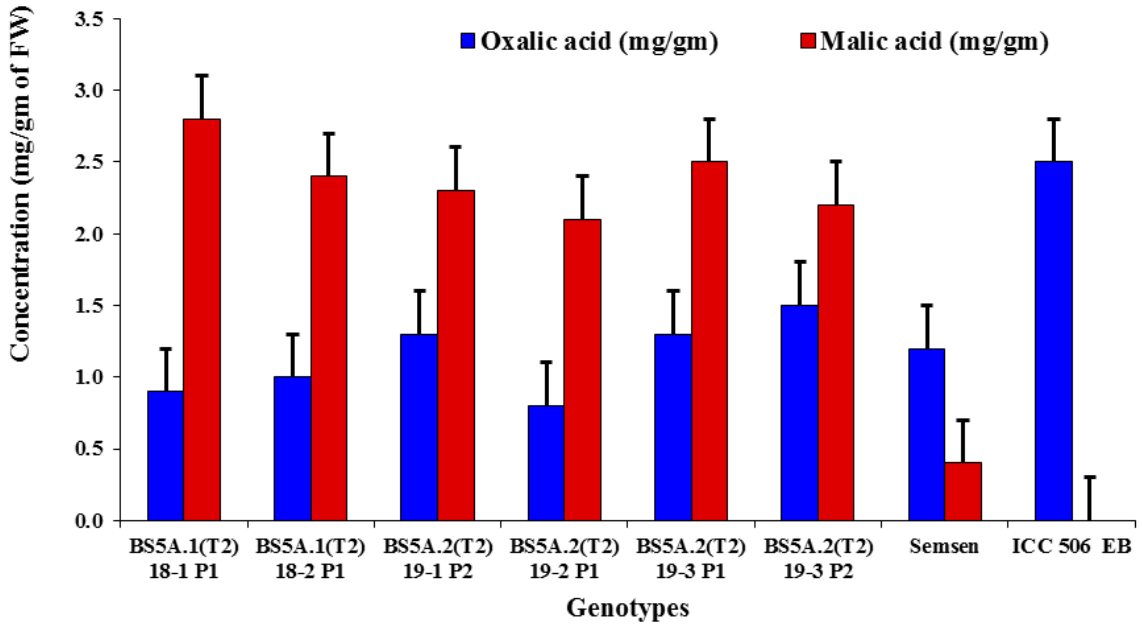

Figure 1 Concentration of organic acids (on fresh weight basis) present on the leaf surface of transgenic chickpea lines 2011-2012)

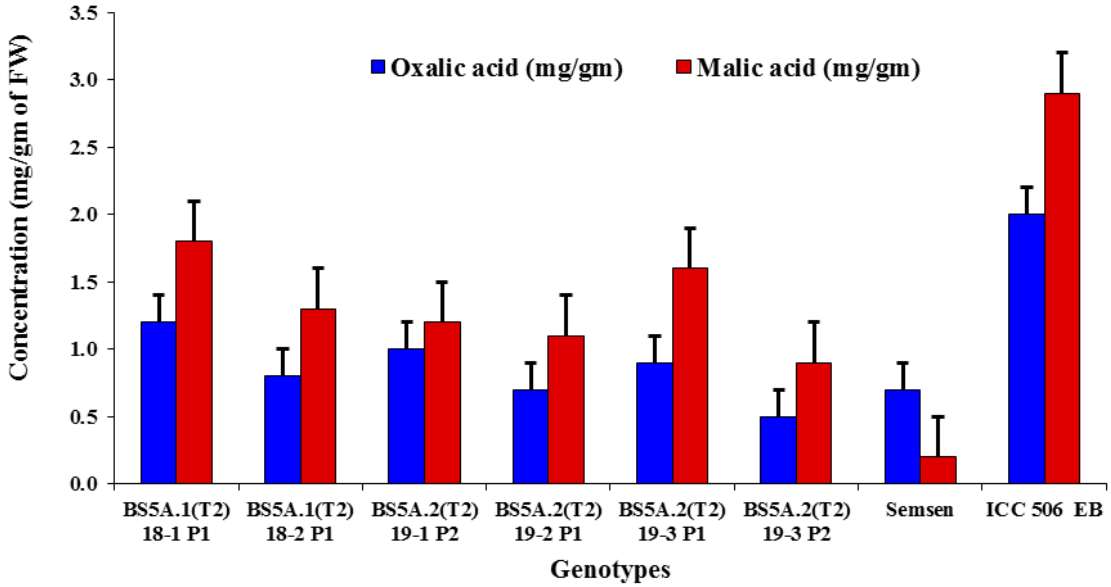

Figure 2 Concentration of organic acids (on fresh weight basis) present on the leaf surface of transgenic chickpea lines 2012-2013)

Table 2 Correlation between resistance/susceptibility to pod borer, *H. armigera* and the amounts organic acids in transgenic chickpea (on fresh weight basis)

	2011-12		**2012-13**	
	Oxalic acid	**Malic acid**	**Oxalic acid**	**Malic acid**
HDR	0.32	-0.83**	0.19	0.18
Larval survival (%)	0.63**	-0.93**	0.47	0.23
Mean larval wt. (mg)	0.60*	-0.95**	0.56*	0.27

,* Significant at P≤ 0.05 and 0.01, respectively

Conclusions

A significant variation in organic acid composition among the transgenic and non-transgenic chickpea lines were observed in the present studies. Significantly higher amounts of oxalic acid were recorded in BS5A.2(T2) 19-1P2 and BS5A.2(T2) 19-3P1 (1.1 mg/g) than in BS5A.2(T2) 19-2P1 (0.8 mg/g). Highest malic acid content was recorded on BS5A.1(T2) 18-1P1 (2.3 mg/g) and lowest on BS5A.2(T2) 19-3P2 (1.5 mg/g). Among the non-transgenics, the maximum amount of oxalic acid was observed in ICC 506EB (2.2 mg/g), followed by Semsen (0.9 mg/g). Oxalic acid content was positively correlated with larval survival (r = 0.63) and larval weight (r = 0.60). A significant and negative association was observed between the amounts of the malic acid and leaf feeding (r = - 0.83), larval survival (r = - 0.93) and larval weight (r = - 0.95).

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