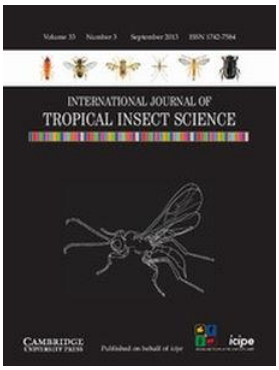


International Journal of Tropical Insect Science

# Expression of resistance to the pod borer Helicoverpa armigera (Lepidoptera: Noctuidae), in relation to high-performance liquid chromatography fingerprints of leaf exudates of chickpea

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**DOI: http://dx.doi.org/10.1017/S1742758413000234**



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# Expression of resistance to pod borer, *Helicoverpa armigera* (Lepidoptera: Noctuidae) in relation to HPLC fingerprints of leaf exudates of chickpea

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**Abstract.** The noctuid, *Helicoverpa armigera*, is the most damaging pest of chickpea worldwide, and plant resistance is an important component for managing this pest. To develop cultivars with resistance to insects, it is important to understand the role of different components associated with resistance to insects. Therefore, we characterized a diverse array of chickpea genotypes and for organic acid profiles in the leaf exudates that are associated with resistance to *H. armigera*. Chickpea leaf exudates contained five major organic acids, which were identified as malic acid, oxalic acid, acetic acid, citric acid, and fumaric acid. The high performance liquid chromatography (HPLC) profiles of the leaf exudates of nine chickpea genotypes showed that amounts of malic acid were

negatively correlated with leaf feeding by *H. armigera* larvae at flowering and maturity, and with pod damage. Oxalic acid showed a negative association with leaf damage in detached leaf assay, while the amounts of acetic acid were negatively correlation with larval weight, and damage rating at flowering and maturity. Citric acid levels were negatively associated with damage rating at flowering. Implications of using HPLC profiles of organic acid leaf exudates to breed for resistance to *H. armigera* have been discussed.

**Keywords:** Chickpea, Pod borer, Helicoverpa armigera, acid exudates, mechanisms of resistance

## Introduction

Chickpea is the third most important food legume, grown in 10.2 m ha with an annual production of 7.9 million tons, and an average productivity of 770 kg per hectare (FAO, 2005). It is grown in over 45 countries in Asia, North and Eastern Africa, Australia, and North America. More than 80 % of the world's chickpea area is in India, and it ranks first among the food legumes (10.6 million ha) (Chabbra *et al*., 1990). 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 (Sharma, 2005; Chen *et al*., 2011). It causes an estimated loss of US $328 million in chickpea in the semi-arid tropics. Its control is largely based on insecticides. However, with the development of resistance to insecticides in *H. armigera* populations (Kranthi *et al*., 2002), there has been a renewed interest in developing alternative methods of pest control, of which plant resistance to *H. armigera* is an important component (Sharma et al. 2005a).

Low to moderate levels of resistance to *H. armigera* has been identified in the chickpea germplasm (Das *et al*., 1983; Lateef, 1985); Lateef and Sachan, 1990). Acidic exudates produced by the trichomes on the surface of chickpea plants, of which malic acid and oxalic acid are the principal components, result in oviposition non-preference and antifeedant effects on *H. armigera* (Rembold et al. 1990; Yoshida et al., 1995). The present studies focused on estimation of acid exudates in the leaf samples of a diverse array of chickpea genotypes to assess the possibilities of using high performance liquid chromatography (HPLC) fingerprints for the organic acids as markers to breed for resistance to *H. armigera*.

## Materials and methods

*Evaluation of chickpea genotypes for resistance to* Helicoverpa armigera

Nine chickpea genotypes (eight desi and one kabuli type) were selected (based on earlier evaluation for resistance to *H. armigera* (Lateef and Sachan 1990; Sharma *et al*. 2005a), to study the biochemical mechanisms of resistance to pod borer. Amongst these, ICC 12475 (ICC 506EB), ICC 12476, ICC 12477, ICC 12478, ICC 12479, and ICCV 2 (ICC 12968) were relatively less susceptible (Lateef,

1985; Sharma *et al*., 2005a); while ICCC 37 (ICC 12426), ICC 3137, and ICC 4918 were used as susceptible checks. The chickpea genotypes were raised on a sterilized mixture of black soil (Vertisols), sand, and farmyard manure (2: 1: 1). The soil was filled into the medium sized pots (30 cm in diameter, and 30 cm in depth). The seeds were sown 5 cm below the soil surface and watered as and when required. Ten seeds were sown in each pot, and 5 plants with uniform growth were retained in each pot at 10 days after seedling emergence. The plants were fertilized with diammonium phosphate (DAP) at 20 g per pot at 15 days after seedling emergence. There were five pots for each genotype. The plants were raised in the greenhouse, which was cooled by desert coolers ($27 \pm 5^{o}$C and 65 to 90% RH). There was no pesticide application on the test plants. The test material was evaluated for resistance to *H. armigera* under natural infestation in the field, and detached leaf assay under laboratory conditions (Sharma et al. 2005a,b).

*Sample collection and estimation of organic acids*

Chickpea plants grown in the greenhouse were used for collection of acid exudates. Glass vials of 15 ml capacity were used for collecting the acid exudates. The weight of the vial along with 5 ml of distilled water was recorded ($W_1$), and then ten fully expanded leaflets were collected from each genotype at the flowering stage and placed in the vials. The weight of the vial + leaves was recorded ($W_2$), and fresh weight of the leaves was computed by subtracting $W_1$ from $W_2$. The vials were Vortexed for 1 min, and the leaves were taken out from the vials and placed on a filter paper. The leaf samples were dried at $55^{0}$C for 3 days, and then, the dry weight of the leaves was also recorded. The water-extracted chemicals were filtered through 0.45 µ Millipore filter, and 2 ml of extract was taken into a screw top vial (12 × 32 mm) with an injection needle. The contents were sonicated for 10 min for dissolving the solutes and degassing of solvents, and then used for HPLC analysis.

The HPLC fingerprinting of the organic acids was carried out by using Waters 2695 Separation Module with photodiode detector, and Atlantis dC-18 column (4.6 x 250 mm, 5 µm). Mobile phase consisted of 25 mM $KH_2PO_4$ pH 2.5. Flow rate 0.8ml $min^{-1}$, Run time 20 min per sample. Injected sample volume was 20µl. Three samples of each test genotypes were run through the HPLC to obtain as estimate of the organic acids present in water-soluble leaf exudates of different chickpea genotypes. Standard samples of known organic acids (oxalic, malic, citric, fumaric, and acetic acids) were used to spike the HPLC peaks to identify different acids. After identification of peaks corresponding to different organic acids, a range of concentrations for each organic acid were run through the HPLC to obtain a normal curve. The amounts of different organic acids present in the leaves of different chickpea genotypes were estimated from normal curves based on peak areas.

*Statistical analysis*

The data were subjected to analysis of variance. The amounts of different acids were correlated with survival and weights *H. armigera* larvae in detached leaf assay, and plant damage rating at the flowering and podding stages of the crop in the field. Diversity among the chickpea genotypes was assessed using similarity matrix analysis.

## Results

*HPLC fingerprints of different chickpea genotypes*

Maximum numbers of HPLC peaks were recorded in leaf exudates of ICC 12476 and ICC 12477 (13 peaks), followed by ICC 506EB, ICC 12478, ICC 3137, and ICCV 2 (12 peaks) (Table 1). The lowest numbers of peaks (6) were recorded in the susceptible check, ICCC 37 (Fig. 1). The peak at RT 4.7 was observed in all the genotypes, except in ICC 12478 and ICCC 37, while the peak at RT 4.9 was observed in all the genotypes, except in Annigeri and ICCC 37. Peak 8 at RT 9.4 was observed in ICC 12476 and ICC 12479, while the peak at RT 12.8 was observed in all the genotypes, except ICCC 37. The resistant check, ICC 506EB had additional peak at RT 15.5. The genotypes ICC 506EB, ICC 12476, ICC 12478, ICC 12479, and ICCC 37 had 3 major peaks for oxalic acid, malic acid, and acetic acid. ICC 12477 and ICCV 2 had an additional peak at RT 3.5, while ICC 3137 had a peak for fumaric acid. ICC 4918 had major peaks for oxalic acid, malic acid, and fumaric acid.

*Organic acids in chickpea genotype in relation to expression of resistance to* H. armigera

Highest amounts of organic acids were present in ICC 4918 (66.33 μg $g^{-1}$), followed by ICC 12477 (47.38 μg $g^{-1}$), and ICC 506EB (36.90 μg $g^{-1}$) on wet weight basis, (Table 2). Malic acid amounts were >45 μg $g^{-1}$ in case of ICC 506EB, ICC 12479, and ICCV 2 compared to 24.08 μg $g^{-1}$ in the susceptible check, ICCC 37; while the acidic acid amounts were high (>20 μg $g^{-1}$) in the leaf exudates of ICC 12476, ICC 12479, ICC 3137, and ICCV 2, but very low in ICCC 37 (9.71 μg $g^{-1}$). The citric acid amounts were high in case of ICC 506EB, ICC 12477, ICC 4918, and ICCV 2, while the fumaric acid amounts were high in case of ICC 506EB, ICC 12479, ICC 3137, ICC 4918, and ICCV 2. On dry weight basis, ICC 4918 had the highest amounts of oxalic acid (547.06 μg $g^{-1}$), followed by ICC 12477 (316.9 μg $g^{-1}$), and ICC 506EB (209.2 μg $g^{-1}$) (Table 3). Highest amounts of malic and acetic acids were recorded in ICC 12476 (362.79 μg $g^{-1}$) and ICC 12479 (230.47 μg $g^{-1}$), respectively, while highest amounts of citric acid were recorded in ICC 506EB (69.38 μg $g^{-1}$), followed by ICC 4918 (68.38 μg $g^{-1}$), and ICC 12476 (48.62 μg $g^{-1}$). Highest amounts of fumaric acid were observed in ICC 3137 (157.73 μg $g^{-1}$). Peaks at RT 3.52 and 3.72 min showed a negative and significant association with larval weight (r = -0.26* and –0.28**), while the peak at 3.72 min showed a negative and significant correlation with larval survival (r = -0.230*) (Table 4). Oxalic and acetic acids were negatively associated with larval weight (r = -0.28** and -0.27*, respectively). Malic acid showed a

negative and significant correlation with damage by *H. armigera* at the flowering (r = -0.28**) and maturity (r = -0.32**) stages, and pod damage (r = -0.22*) under field conditions.

*Diversity among chickpea genotypes based on HPLC fingerprints*

The UPGMA (un-weighted pair group method with arithmetic averages) dendrogram based on peak areas of compounds at different retention times (RT) placed the test genotypes into five groups at 85% similarity (Fig. 2). Amongst these, the Ist group comprised of ICC 506 EB, ICC 12476, and ICC 12479, and all these genotypes are resistant to *H. armigera*. Group II consisted of ICC 12477 (moderately-resistant) and ICCV 2 (moderately susceptible), while another moderately resistant line ICC 12478 was placed independently in group III. The susceptible check, ICC 3137 was placed independently in group IV, while ICCC 37 and ICC 4918 were placed in group V. The HPLC fingerprinting of the water-soluble leaf surface exudates differentiated between the resistant and susceptible genotypes, and these were placed in separate groups. Some of the resistance lines were also placed in separate groups, indicating the presence of diversity in the sources of resistance to *H. armigera*.

## Discussion

Host plat resistance to *H. armigera* in chickpea has largely been attributed to antixenosis for oviposition, antibiosis, and recovery resistance (Narayanamma *et al*., 2007, 2008), and is influenced by the organic acids in the leaf exudates (Rembold, 1981; Bhagwat *et al*., 1985; Srivastava and Srivastava, 1989; Rembold *et al*., 1990). However, resistance expressed by PDE 2-3, PDE 7-3, and ICC 506 has been attributed to factors other than acidity, while that of PDE 7-2 is due to high acidity. Malic acid and oxalic acid in the acid exudates are known to play a considerable role in genotypic susceptibility to *H. armigera*. The genotypes ICC 506EB, ICC 12476, ICC 12478, ICC 12479, and ICCC 37 had 3 major peaks, while the kabuli genotype, ICCV 2 had 4 major peaks. Malic acid content was found to be significantly and negatively associated with *H. armigera* damage at the flowering and maturity stages, while oxalic acid was negatively associated with leaf damage rating in the detached leaf assay. Malic acid acts as a deterrent to the *H. armigera* larvae, and the pod borer-resistant lines have more amounts of malic acid than the susceptible lines (Bhagwat *et al*., 1985). Oxalic acid inhibits the growth of *H. armigera* larvae when incorporated in artificial diet, while malic acid shows no such effects (Yoshida et al., 1995, 1997). Acetic acid showed a negative association with larval weight gain, and *H. armigera* damage rating at flowering and at maturity, while citric acid showed a negative and significant association with leaf damage at flowering. Leaves at the flowering and early podding stages would be the most appropriate for chemical analysis as the differences in organic acid levels between resistant and susceptible genotypes were most marked at this growth stage. Antifeedant and/or antibiotic properties of organic acids may influence the host selection and

feeding behavior, and thus, influence the growth and development of *H. armigera* larvae and determine the extent of damage on a particular genotype (Rembold and Winter, 1982; Rembold *et al*., 1990). The present studies indicated that in addition to oxalic acid and malic acid, citric acid, acetic acid, and fumaric acid also play an important role on genotypic resistance to *H. armigera*. Monitoring the amounts of organic acids through HPLC can be used to select chickpea genotypes for resistance to *H. armigera*. The HPLC fingerprinting placed the resistant and susceptible lines on different groups, while some of the lines showing resistant reactions were placed in different groups, indicating that these lines have different profiles of leaf surface exudates that contribute to resistance/susceptibility to *H. armigera*. The lines showing resistance to *H. armigera*, but placed in different groups can be used to increase the levels of resistance to this pest.

**Acknowledgements**

The authors are thankful to staff of entomology for their help in these studies, and Dr Rajiv Varshney for reviewing the manuscript. We also thank Mr Suraj P Sharma for help in revising the manuscript

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**Table 1.** Relative amounts (peak area %) of different compounds in water soluble leaf exudates of nine chickpea genotypes (ICRISAT, Patancheru, India)

| Genotypes | No of peaks | Retention time (min) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 3.5 | 3.9 | 4.7 | 5.9 | 6.8 | 9.3 | 12.9 | 15.5 | 16.0 |
| | | Unknown | Oxalic acid | Unknown | Malic acid | Acetic acid | Unknown | Citric acid | Unknown | Fumaric acid |
| ICC 12476 | 13 | 6.6 | 12.7 | 1.7 | 25.8 | 16.8 | 0.8 | 4.6 | 9.3 | 3.8 |
| ICC 12477 | 13 | 15.1 | 28.4 | 1.7 | 20.9 | 11.5 | 1.1 | 4.3 | 6.1 | 2.1 |
| ICC 12478 | 12 | 7.3 | 22.1 | 1.1 | 33.7 | 16.6 | 1.8 | 3.4 | - | 6.3 |
| ICC 12479 | 11 | 7.2 | 17.9 | 1.1 | 25.4 | 24.5 | 1.1 | 2.7 | - | 12.1 |
| ICC 3137 | 12 | 3.3 | 15.2 | 0.2 | 17.9 | 27.8 | 0.7 | 3.4 | - | 24.9 |
| ICC 4918 | 10 | 8.1 | 47.1 | 0.2 | 10.5 | 9.5 | 0.7 | 6.9 | - | 12.5 |
| ICCV 2 | 12 | 20.4 | 16.4 | 0.8 | 21.1 | 12.4 | 0.3 | 4.2 | - | 9.7 |
| ICC 506EB (R) | 12 | 2.6 | 23.2 | 0.64 | 24.2 | 12.4 | 1.3 | 9.1 | 8.7 | 5.3 |
| ICCC 37 (S) | 6 | 2.6 | 46.3 | - | 30.4 | 14.3 | - | - | - | 5.9 |
| SE ± | 0.72 | 2.0 | 4.31 | 0.20 | 2.28 | 2.05 | 0.15 | 0.71 | 0.57 | 2.30 |

R = Resistant check. S = Susceptible check.

**Table 2.** Amounts (μg $g^{-1}$) of organic acids in the exudates of nine chickpea genotypes (on fresh weight basis) (ICRISAT, Patancheru, India)

| Genotypes | Oxalic acid | Malic acid | Acetic acid | Citric acid | Fumaric acid |
|---|---|---|---|---|---|
| CC 12476 | 16.15 | 36.84 | 20.47 | 4.94 | 4.49 |
| ICC 12477 | 47.38 | 39.19 | 18.42 | 6.15 | 3.34 |
| ICC 12478 | 23.87 | 41.12 | 17.31 | 3.21 | 6.42 |
| ICC 12479 | 29.74 | 47.58 | 39.16 | 3.88 | 18.77 |
| ICC 3137 | 15.04 | 19.95 | 26.41 | 2.84 | 23.13 |
| ICCV 2 | 31.55 | 45.71 | 23.09 | 6.85 | 17.60 |
| ICC 4918 | 66.33 | 16.66 | 12.87 | 8.29 | 16.58 |
| ICC 506EB (R) | 36.90 | 43.23 | 18.93 | 12.24 | 7.94 |
| ICCC 37 (S) | 32.58 | 24.08 | 9.71 | - | 3.92 |
| SE ± | 5.32 | 3.88 | 2.84 | 1.04 | 2.53 |

R = Resistant check. S = Susceptible check.

**Table 3.** Amounts (μg $g^{-1}$) of organic acids in the exudates of nine chickpea genotypes (on dry weight basis) (ICRISAT, Patancheru, India)

| Genotypes | Oxalic acid | Malic acid | Acetic acid | Citric acid | Fumaric acid |
|---|---|---|---|---|---|
| ICC 12476 | 159.11 | 362.79 | 201.56 | 48.62 | 44.25 |
| ICC 12477 | 316.94 | 262.14 | 123.19 | 41.14 | 22.35 |
| ICC 12478 | 143.14 | 246.60 | 103.83 | 19.26 | 38.48 |
| ICC 12479 | 175.01 | 279.98 | 230.47 | 22.81 | 110.48 |
| ICC 3137 | 102.57 | 136.01 | 180.10 | 19.38 | 157.73 |
| ICC 4918 | 547.06 | 137.40 | 106.16 | 68.38 | 136.73 |
| ICCV 2 | 162.77 | 235.80 | 119.13 | 35.31 | 90.77 |
| ICC 506EB (R) | 209.20 | 245.05 | 107.32 | 69.38 | 45.00 |
| ICCC 37 (S) | 152.48 | 112.67 | 45.46 | - | 18.32 |
| SE ± | 45.6 | 27.02 | 19.21 | 6.8 | 17.21 |

R = Resistant check. S = Susceptible check.

**Table 4.** Association of peak area with larval survival, larval weights, and plant damage in nine chickpea genotypes (ICRISAT, Patancheru, India)

| Retention time (min) | Water soluble organic acids | Larval survival | Larval weight | Pod borer damage rating[a] | |
|---|---|---|---|---|---|
| | | | | Flowering stage | Maturity |
| 3.52 | Unknown | -0.04 | -0.26* | 0.02 | -0.05 |
| 3.72 | Unknown | 0.23* | -0.22* | -0.12 | 0.01 |
| 3.90 | Oxalic acid | 0.20 | -0.28** | -0.19 | -0.12 |
| 4.98 | Unknown | 0.08 | -0.02 | -0.07 | -0.01 |
| 5.92 | Unknown | 0.03 | 0.14 | 0.10 | 0.16 |
| 5.93 | Malic acid | -0.13 | -0.03 | -0.28** | -0.32** |
| 6.89 | Acetic acid | 0.07 | -0.27* | -0.09 | 0.06 |
| 6.82 | Unknown | 0.07 | -0.08 | -0.23* | -0.28** |
| 10.33 | Unknown | 0.42** | -0.24* | -0.08 | -0.02 |
| 12.95 | Citric acid | -0.25* | -0.23* | -0.03 | 0.09 |
| 16.00 | Fumaric acid | 0.00 | 0.11 | -0.16 | -0.07 |
| 16.76 | Unknown | 0.07 | 0.05 | 0.04 | 0.23* |

*, ** = Correlation coefficients significant at P 0.05 and 0.01, respectively.
[a] Pod borer damage rating (1 = <10% lead area and/or pods damaged, and 9 = >80% leaf area and/or pods damaged).

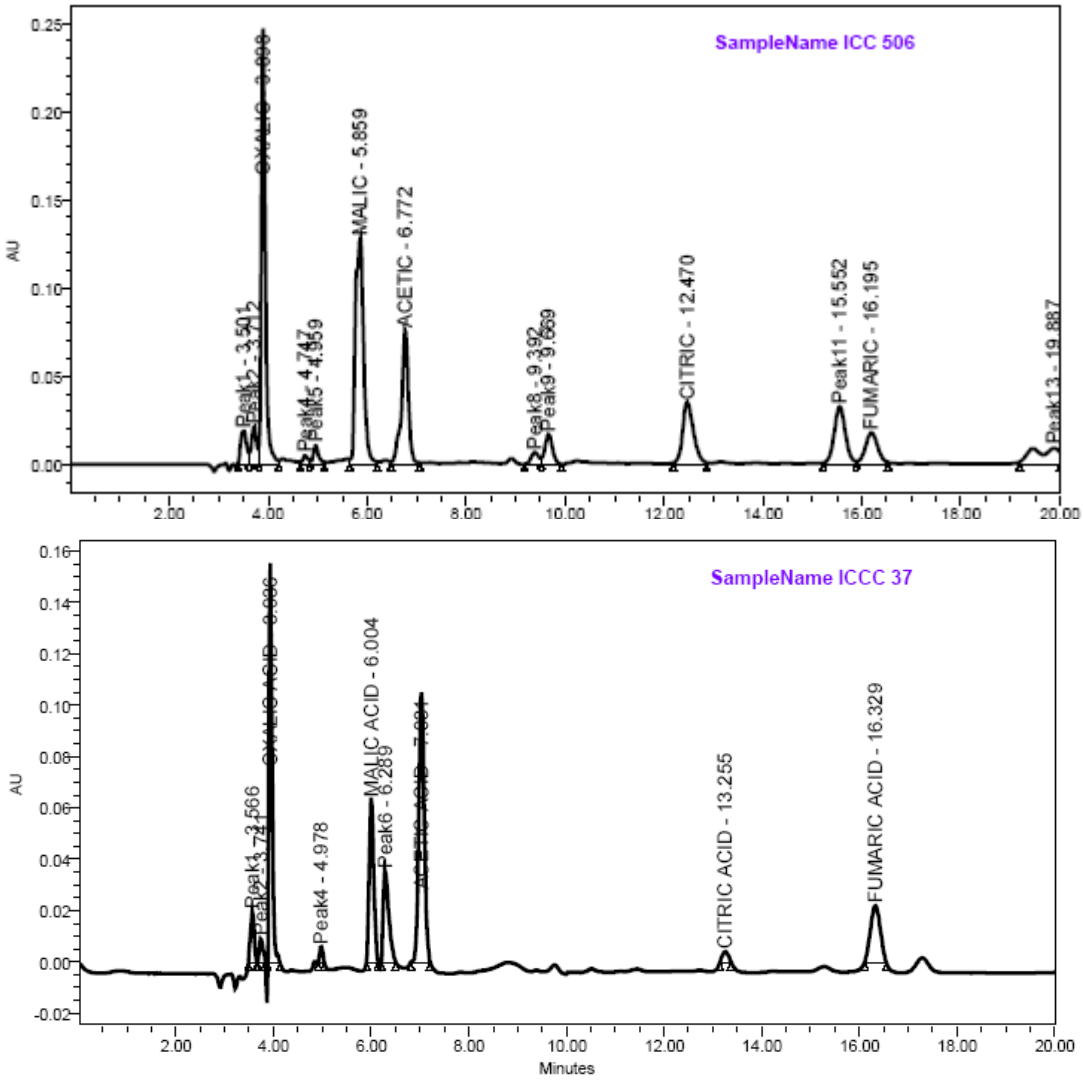


**Fig. 1.** HPLC fingerprints of water soluble leaf components of the leaf exudates of ICC 506EB (resistant) and ICCC 37 (susceptible) genotypes of chickpea.

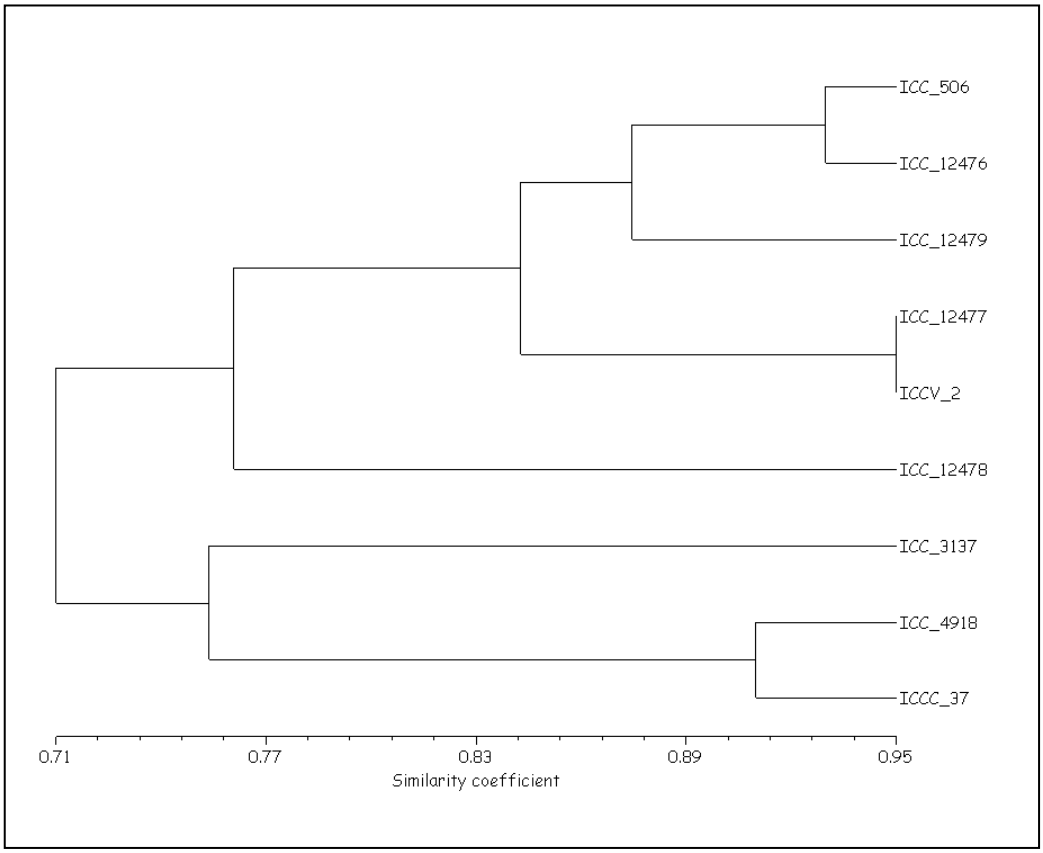


**Fig. 2.** Dendogram (based on UPGMA) depicting similarity between nine chickpea genotypes based on HPLC fingerprints of the water-soluble leaf exudates.