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Defensive Responses in Groundnut Against Chewing and Sap-Sucking Insects

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Abstract Induced resistance is one of the important components of host plant resistance to insects. We studied the induced defensive responses in groundnut genotypes with different levels of resistance to the leaf defoliator *Helicoverpa armigera* and the sap-sucking insect *Aphis craccivora* to gain an understanding of the induced resistance to insects and its implications for pest management. The activity of the defensive enzymes (peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, superoxide dismutase, ascorbate peroxidase, and catalase) and the amounts of total phenols, hydrogen peroxide, malondialdehyde, and proteins were recorded at 6 days after infestation. Induction of enzyme activities and the amounts of secondary metabolites were greater in the insect-resistant genotypes ICGV 86699, ICGV 86031, ICG 2271, and ICG 1697 infested with *H. armigera* and *A. craccivora* than in the susceptible check JL 24. The resistant genotypes suffered lower insect damage and resulted in lower *Helicoverpa* larval survival and weights than those larvae fed on the susceptible check JL 24. The number of aphids was significantly lower on insect-resistant genotypes than on the susceptible check JL 24. The results suggested that groundnut plants respond to infestation by *H. armigera* and *A. craccivora* in a similar way; however, the degree of the response differed across the genotypes and insects, and this defense response is attributed to various defensive enzymes and secondary metabolites.

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Introduction

There are about six million insect species in the world, of which 50 % are herbivorous (Chapman 2006) and are a major threat to crop production. They cause an estimated loss of over US$14 billion worldwide annually despite application of insecticides costing over $2 billion annually (Oerke 2006). Groundnut (*Arachis hypogaea* L.) is an important oilseed crop and is cultivated on 23.4 million ha with an annual production of 34.9 million metric tons globally (FAO 2007). In India, groundnut is one of the major oil seed crops with an area of 6.21 million ha, production of 6.74 million tons, and an average yield of 1,081 kg ha^{-1} (DGR 2011). A large number of insect pests damage this crop, including thrips, *Aphis craccivora* Koch, white grubs, leaf miner, leafhoppers, *Spodoptera litura* Fab., and *Helicoverpa armigera* (Hub.) (Sharma and others 2003).

Host plant resistance plays an important role in insect pest management resulting in reduced losses due to the herbivores, less insecticide use, better crop yields, and a safer environment, in addition to being cost effective (Sharma and others 2009; Wu and Baldwin 2010). Plants respond to herbivory through various morphological, biochemical, and molecular mechanisms to counter or offset the effects of herbivore attack. This form of defense (induced resistance) adversely affects insect feeding, growth, and survival (Howe and Jander 2008; Wu and Baldwin 2010; War and others 2011a). It is a key component of plant defense against insect herbivory (Chen and others 2009; Sethi and others 2009; Karban 2011). Induced

resistance in plants is mediated through various defensive enzymes such as peroxidases (PODs), polyphenol oxidases (PPO), phenylalanine ammonia lyase (PAL), superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase, and secondary metabolites, including phenols and condensed tannins, and through hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) (Gulsen and others 2010; Usha Rani and Jyothsna 2010; War and others 2011a, 2012).

The cotton bollworm/legume pod borer *H. armigera* is a polyphagous pest and is widely distributed in Asia, Africa, southern Europe, and Australasia (Sharma 2005). It is a major pest of cereals, grain legumes, cotton, vegetables, and fruit crops, including groundnut (Sharma and others 2005). The cowpea or groundnut aphid *A. craccivora* is a polyphagous pest and feeds on a number of crops worldwide (Minja and others 1999; Ahmed and others 2007). It causes severe damage to groundnut by sucking plant sap and by acting as a vector of at least seven viral diseases, including groundnut rosette virus and peanut stripe (Padgham and others 1990). Plants respond differently to insects with different modes of feeding. The chewing insects cause extensive damage to plant tissues and induce the defense system differently than the sap-sucking insects. The chewing insects (caterpillars) release a wide range of elicitors that induce specific defense responses that are different from general mechanical damage. On the other hand, aphids, with their piercing and sucking type of mouth parts, use stylets for feeding and cause minimum physical injury to the plant tissue. They cause serious losses in crops worldwide by draining plant nutrients, injecting plant elicitors, and transmitting pathogenic viruses (Han and others 2009; He and others 2011). Aphids probe into the leaf epidermis cells immediately upon infestation. Once the probe is initiated, they insert their stylets into the epidermis cell wall and membrane, inject saliva, and ingest the cell wall contents (Tjallingi 2006). The stylet is then inserted further and the insect feeds on phloem (and xylem) sap. Plant defense against herbivory is mediated through both salicylic acid (SA)- and jasmonic acid (JA)-dependent pathways (Walling 2000; Moran and others 2002). The present study was carried out to understand the defensive responses of groundnut genotypes with different levels of resistance to insect pests with different modes of feeding and its implications for pest management. The results obtained could serve as important biochemical markers for plant resistance against insect pests.

Material and Methods

Chemicals

The chemicals used in this study were of analytical grade. Ethylene diamine tetraacetic acid (EDTA), bovine serum albumin (BSA), guaiacol, polyvinylpyrrolidone (PVP), jasmonic acid, tannic acid, vanillin, linoleic acid, dithiothreitol (DTT), disodium hydrogen phosphate, sodium dihydrogen phosphate, nitroblue tetrazolium salt (NBT), methionine, L-phenylalanine, glucose, potassium iodide (KI), sodium carbonate (Na_2CO_3), and vanillin were obtained from Sigma Aldrich (St. Louis, MO, USA). Catechol was obtained from Glaxo Laboratories (Mumbai, India). Coomassie brilliant blue G250, Tris-HCl, and trichloroacetic acid (TCA) were obtained from Sisco Research Lab. (Mumbai, India). 2-Mercaptoethanol, gallic acid, and Folin-Ciocalteu reagent were obtained from Merck (Mumbai, India). Thiobarbituric acid (TBA) and linoleic acid were obtained from HiMedia Pvt. Ltd. (Mumbai, India). Ammonium sulfate was obtained from Qualigens Fine Chemicals (Mumbai, India).

Groundnut Plants

Five groundnut genotypes were grown under greenhouse conditions at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India, to study their defensive responses to *H. armigera*, a chewing type of insect, and *A. craccivora*, a sap-sucking type of insect. These genotypes included ICGV 86699, ICGV 86031, ICG 2271, and ICG 1697 (with moderate to high levels of resistance to insects) and JL 24 (susceptible check) (Sharma and others 2003). The plants were grown in plastic pots (30 cm in diameter and 39 cm deep) filled with a mixture of soil, sand, and farmyard manure (2:1:1). After 10 days of seedling emergence, only two seedlings of similar growth were retained in each pot. Desert coolers were used to maintain the temperature at 28 ± 5 °C and relative humidity (RH) at 65 ± 5 % in a greenhouse. Twenty-day-old plants were infested with ten newly emerged *H. armigera* larvae or 20 nymphs of *A. craccivora*. Ten replications were maintained for each treatment/genotype in a randomized block design.

Insect Infestation

Newly emerged larvae of *H. armigera* were obtained from the stock culture maintained on a chickpea-based artificial diet under laboratory conditions (26 ± 1 °C; 11 ± 0.5 h photoperiod, and 75 ± 5 % RH) from the insect-rearing laboratory. Ten larvae were gently placed on each 20 day-old plant by using a camel hair brush. The *A. craccivora* wingless adults were obtained from the culture maintained on groundnut plants in the greenhouse, and ten aphids were released on each plant using a moistened camel hair brush. The insects were allowed to feed on plants for 6 days, after which the leaves were collected randomly from plants for the biochemical assays.

Enzyme Extraction

Fresh leaves (0.5 g) were ground in 3 ml of ice-cold 0.1 M Tris–HCl buffer (pH 7.5) containing 5 mM 2-mercaptoethanol, 1 % polyvinylpyrrolidone (PVP), 1 mM dithiothreitol (DTT), and 0.5 mM EDTA. The homogenate was centrifuged at 16,000×*g* for 25 min and the supernatant was used as an enzyme source. The supernatant was further processed for partial purification of proteins.

Precipitation of Proteins and Partial Purification

Proteins were precipitated by ammonium sulfate. To obtain 80 % of saturation, initially 40 % saturation was carried out followed by 80 %. Ammonium sulfate (1.2 g) was added to 5 ml of the protein extract to obtain 40 % saturation. The solution was kept overnight at 4 °C and then centrifuged at 12,000 rpm for 30 min. The pellet was collected, and the supernatant was used for further precipitation. For 80 % saturation, ammonium sulfate was added at the rate of 0.28 g ml^{-1}. The solution was stirred overnight at 4 °C and salt-precipitated proteins were collected after centrifugation at 12,000 rpm for 30 min. The pellets were pooled together and dissolved in buffer (0.1 M Tris–HCl buffer, pH 7.5, containing 0.5 mM EDTA and 1 mM DTT). The protein solution was dialyzed using a dialysis bag. For dialysis, the bag was washed with distilled water, sealed with a plastic clip on one end, and again washed with distilled water. The bag was filled with the precipitated protein sample and sealed on the other end with a plastic clip. The dialysis was carried out for 18 h in the preceding buffer at 4 °C. The buffer was changed after every 3 h.

Enzyme Assays

Peroxidase (POD) activity was estimated as per the method of Shannon and others (1966) with slight modification. Enzyme activity was expressed as IU g^{-1} FW. One unit of POD activity was defined as the change in absorbance by 0.1 unit per minute under conditions of assay. For the estimation of PPO activity, the method described by Mayer and Harel (1979) was followed with some modifications. Enzyme activity was expressed as IU g^{-1} FW. One unit of PPO was defined as the change in absorbance by 0.1 unit per minute under conditions of assay. The activity of SOD was assayed as described by Beauchamp and Fridovich (1971) with slight modifications. SOD activity was expressed as IU g^{-1} FW. One unit is the change in absorbance by 0.1 unit per minute. LOX activity was measured by following the method of Hildebrand and Hymowitz (1983) with slight modifications. One unit of enzyme activity was defined as the increase in absorbance by 0.01 per minute and was expressed as IU g^{-1} FW. Catalase activity was determined using the method of Zhang and others (2008). Phenylalanine ammonia lyase was estimated as described by Campos-Vargas and Saltveit (2002) with slight modifications and the activity was expressed as µmol cinnamic acid min^{-1} mg^{-1} protein.

Ascorbate Peroxidase Assay

To determine the APX activity, the method of Asada and Takahashi (1987) was followed with slight modifications. Leaf tissue (0.2 g) was homogenized using a mortar and pestle with 3 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 % PVP, and 1 mM ascorbic acid. After filtering through a double-layered cheese cloth, the homogenate was centrifuged at 15,000 rpm for 20 min at 4 °C. The supernatant after precipitation and dialysis was used as an enzyme source. The reaction mixture (1 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H_2O_2, and 0.2 ml of partially purified enzyme extract. Decrease in absorbance at 290 nm due to ascorbate oxidation was measured against the blank and the enzyme activity was expressed as IU g^{-1} FW, where 1 IU is the change in 0.1 unit of absorbance per minute.

Total Phenols, Condensed Tannins, H_2O_2, MDA, and Protein Contents

Phenolic content was estimated as per the Zieslin and Ben-Zaken (1993) method with some modifications. Phenolic concentration was determined from a standard curve prepared with gallic acid and was expressed as µg gallic acid equivalents g^{-1} FW (µg GAE g^{-1} FW). Condensed tannin content was estimated by the vanillin-hydrochloride method as described by Robert (1971) with some modifications. Catechin was used as the standard and the total amount of condensed tannins was expressed as µg catechin equivalents g^{-1} FW (µg CE g^{-1} FW). Hydrogen peroxide content was estimated by the method of Noreen and Ashraf (2009). H_2O_2 concentration was determined by using an extinction coefficient of 0.28 µM cm^{-1} and expressed as µmol g^{-1} FW. The level of lipid peroxidation was determined in terms of thiobarbituric acid-reactive substances (TBARS) concentration as described by Carmak and Horst (1991) with minor modifications. The concentration of TBARS was calculated using the absorption coefficient of 155 $mmol^{-1}$ cm^{-1} and expressed as µmol g^{-1} FW. Total protein content was determined according to the method of Lowery and others (1951), using bovine serum albumin as standard.

Plant Damage and Insect Biology

After 6 days of infestation, plants were assessed for *Helicoverpa* damage by visually rating them on a scale of 1 to 9, where 1 is <10 % damage and 9 is >80 % damage (Sharma and others 2003), and *A. craccivora* damage was evaluated visually on a scale of 1 to 5, where 1 is highly resistant and 5 is highly susceptible. The numbers of *Helicoverpa* larvae and aphids that survived were recorded. The *Helicoverpa* larvae collected were starved for 4 h and their weights (mg) recorded using a digital balance (Mettler Toledo, AB304-S).

Statistical Analysis

The data were subjected to analysis of variance (ANOVA) using SPSS v15.1 (SPSS, Inc., Chicago, IL, USA). Tukey's test was used to separate the means when the treatment effects were statistically significant ($P \leq 0.05$). The differences across the treatments and genotypes were shown by using Dunnett's *t* test.

Results

POD Activity

Infestation with *H. armigera* and *A. craccivora* resulted in significantly greater POD activity in all five groundnut genotypes (Fig. 1) compared to the noninfested control plants: ICGV 86699 ($F_{(2,8)} = 34.3$, $P < 0.001$), ICGV 86031 ($F_{(2,8)} = 25.4$, $P < 0.01$), ICG 2271 ($F_{(2,8)} = 28.2$, $P < 0.05$), ICG 1697 ($F_{(2,8)} = 19.3$, $P < 0.01$), and JL 24 ($F_{(2,8)} = 25.9$, $P < 0.05$). ICGV 86699 showed a strong induction of POD activity in all the plants infested with insects [*H. armigera* ($F_{(4,14)} = 45.4$, $P < 0.01$); *A. craccivora* ($F_{(4,14)} = 23.5$, $P < 0.05$), as well as the noninfested control plants ($F_{(4,14)} = 12.3$, $P < 0.05$)] than did the other genotypes. JL 24 also exhibited increased POD activity following insect infestation, but the activity was lower than in the insect-resistant genotypes. The POD activity was greater in the noninfested insect-resistant genotypes than in the susceptible check JL 24.

PPO Activity

Greater induction in PPO activity was observed in *H. armigera*- and *A. craccivora*-infested plants of all the groundnut genotypes than in the noninfested control plants (Fig. 2): ICGV 86699 ($F_{(2,8)} = 45.3$, $P < 0.001$), ICGV 86031 ($F_{(2,8)} = 89.4$, $P < 0.001$), ICG 2271 ($F_{(2,8)} = 32.3$, $P < 0.05$), ICG 1697 ($F_{(2,8)} = 19.5$, $P < 0.01$), and JL 24 ($F_{(2,8)} = 15.9$, $P < 0.05$). ICGV 86699 and ICGV 86031 plants infested with *H. armigera* showed significantly greater PPO activity ($F_{(4,14)} = 78.4$ and 67.2 for ICGV 86699 and ICGV 86031, respectively; $P < 0.001$) than did ICG 2271, ICG 1697, and JL 24. Insect-resistant genotypes had higher PPO activity ($F_{(4,14)} = 23.8$, $P < 0.05$) in *A. craccivora*-infested plants than did JL 24.

PAL Activity

A strong induction of PAL activity was observed in response to insect infestation (Fig. 3). Plants infested with *H. armigera* and *A. craccivora* had greater PAL activity than the noninfested control plants: ICGV 86699 ($F_{(2,8)} = 34.5$, $P < 0.001$), ICG 2271 ($F_{(2,8)} = 12.6.7$, $P < 0.001$), ICG 1697 ($F_{(2,8)} = 18.9$, $P < 0.05$), and JL 24 ($F_{(2,8)} = 11.5$, $P < 0.05$). However, in ICGV 86031, *H. armigera* infestation elicited a significantly greater PAL activity ($F_{(2,8)} = 33.3$, $P < 0.01$) than did *A. craccivora* infestation and noninfested control plants. ICGV 86699 and ICGV 86031 plants infested with *H. armigera* exhibited greater PAL activity ($F_{(4,14)} = 23.2$, $P < 0.05$) than the other genotypes. The PAL activity in *A. craccivora*-infested plants of ICGV 86699, ICG 2271, and ICG 1697 was significantly higher ($F_{(4,14)} = 18.6$, $P < 0.05$) than that of the susceptible check JL 24. The constitutive levels of PAL in insect-resistant genotypes were significantly greater than in the susceptible genotype JL 24.

CAT Activity

Plants infested with *H. armigera* and *A. craccivora* had significantly greater CAT activities than the noninfested control plants (Fig. 4: $F_{(2,8)} = 12.2$, 18.9, 17.7, and 9.5 for ICGV 86699, ICGV 86031, ICG 2271, and ICG 1697, respectively; $P < 0.05$). ICGV 86699 and ICGV 86031 showed significantly greater CAT activity in plants infested with *H. armigera* ($F_{(2,8)} = 23.6$, $P < 0.05$) and *A. craccivora*-infested plants ($F_{(2,8)} = 14.2$, $P < 0.05$) than did ICG 2271, ICG 1697, and JL 24. Constitutive CAT activity was higher in insect-resistant genotypes than in the susceptible check JL 24.

SOD Activity

Infestation with *H. armigera* and with *A. craccivora* increased the SOD activity in all the groundnut genotypes (Fig. 5). The induction was significantly greater in *H. armigera*-infested plants of genotypes ICGV 86699 ($F_{(2,8)} = 68.7$, $P < 0.01$) and ICG 2271 ($F_{(2,8)} = 23.5$, $P < 0.05$) than in *A. craccivora*-infested plants and noninfested control plants. There were no significant differences in SOD activity between *H. armigera*- and *A. craccivora*-infested plants of ICGV 86031, ICG 1697, and JL 24.

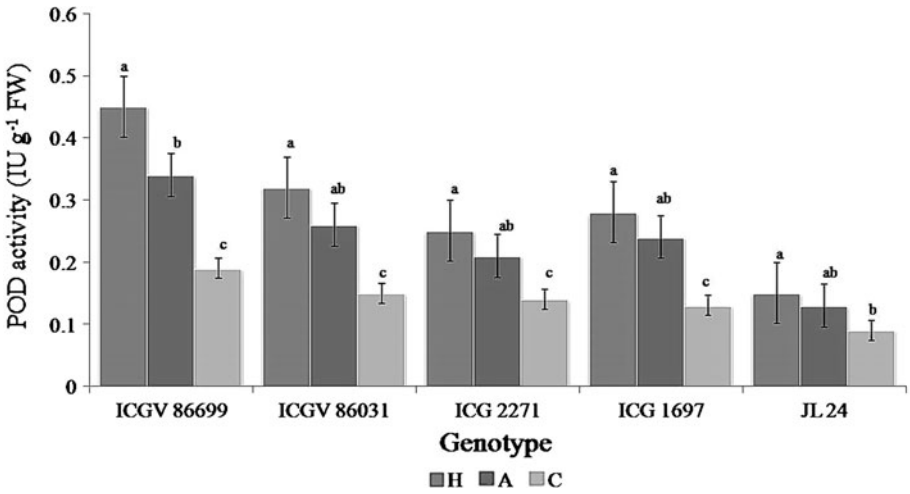

Fig. 1 POD activity (IU g^{-1} FW) of groundnut genotypes infested with *H. armigera* and *A. craccivora. Bars* (mean ± SE) of the *same color* with the *same letters* are not statistically different at $P < 0.05$. *H H. armigera*-infested, *A A. craccivora*-infested, *C* noninfested control, *FW* fresh weight of leaf tissue

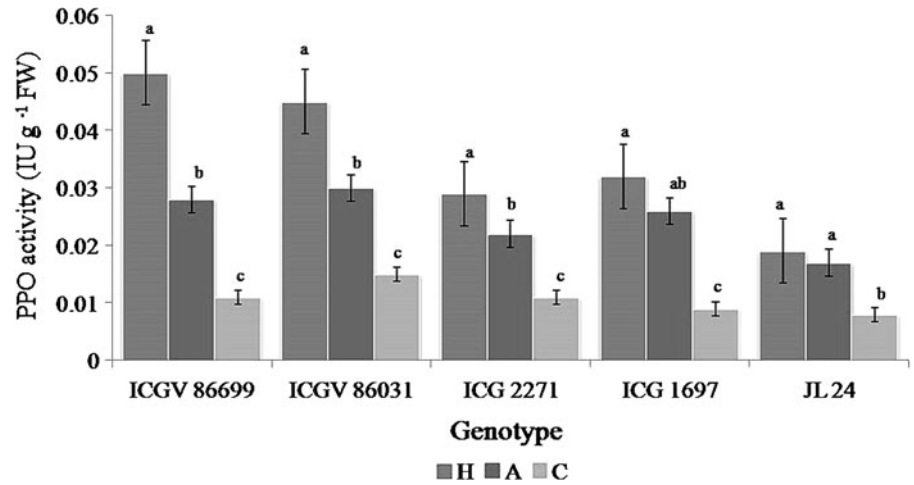

Fig. 2 PPO activity (IU g^{-1} FW) of groundnut genotypes infested with *H. armigera* and *A. craccivora. Bars* (mean ± SE) of the *same color* with the *same letters* are not statistically different at $P < 0.05$. *H H. armigera*-infested, *A A. craccivora*-infested, *C* noninfested control, *FW* fresh weight of leaf tissue

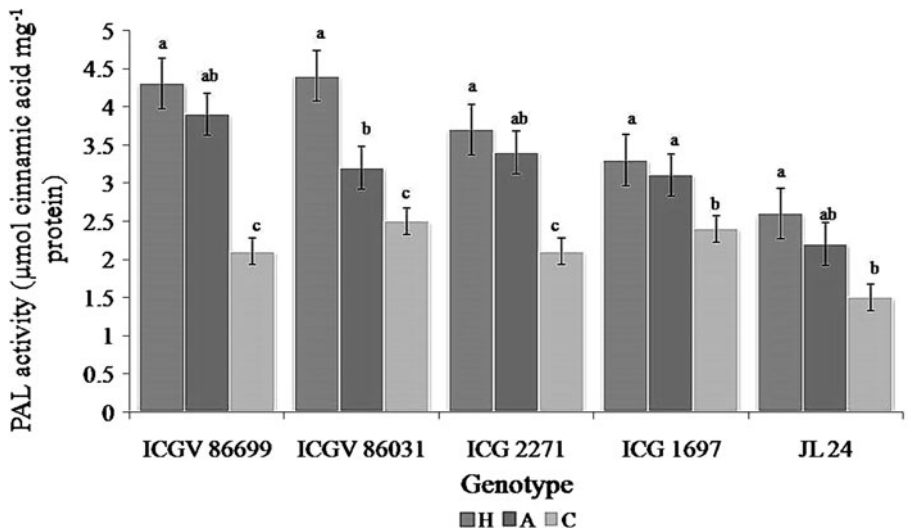

Fig. 3 PAL activity (µmol cinnamic acid mg^{-1} protein) of groundnut genotypes infested with *H. armigera* and *A. craccivora. Bars* (mean ± SE) of the *same color* with the *same letters* are not statistically different at $P < 0.05$. *H H. armigera*-infested, *A A. craccivora*-infested, *C* noninfested control

Constitutive levels of SOD activity were lower in JL 24 than in the insect-resistant genotypes. *H. armigera*- ($F_{(4,14)} = 98.1$, $P < 0.001$) and *A. craccivora*-infested plants ($F_{(4,14)} = 34.7$, $P < 0.05$) of ICGV 86699 and ICG 1697 had greater SOD activity than those of ICGV 86031, ICG 2271, and JL 24.

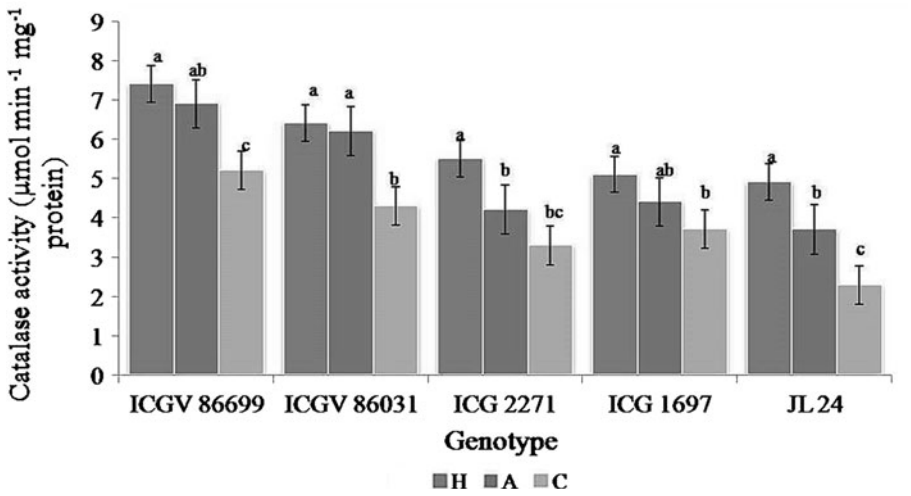

Fig. 4 Catalase activity (µmol min^{-1} mg^{-1} protein) of groundnut genotypes infested with *H. armigera* and *A. craccivora*. *Bars* (mean ± SE) of the *same color* with the *same letters* are not statistically different at $P < 0.05$. *H H. armigera*-infested, *A A. craccivora*-infested, *C* noninfested control

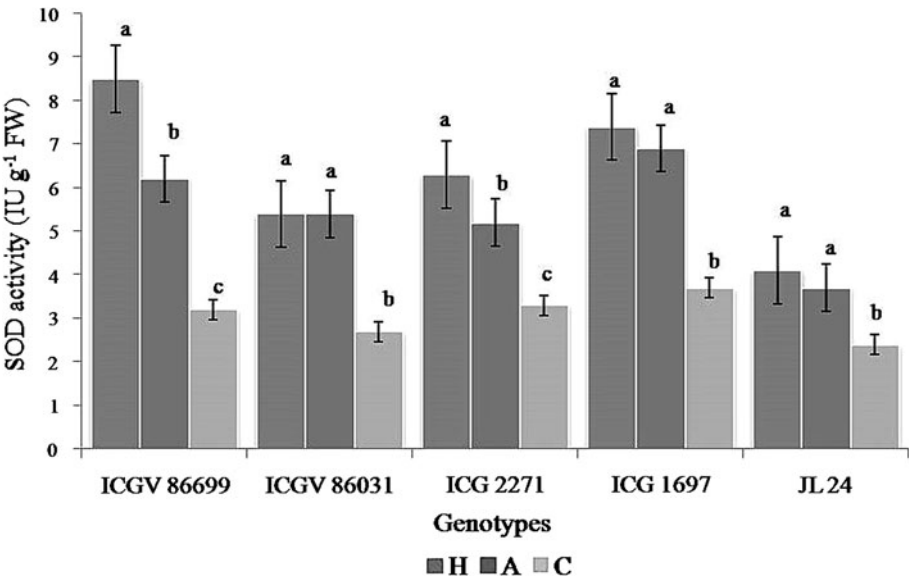

Fig. 5 SOD activity (IU g^{-1} FW) of groundnut genotypes infested with *H. armigera* and *A. craccivora*. *Bars* (mean ± SE) of the *same color* with the *same letters* are not statistically different at $P < 0.05$. *H H. armigera*-infested, *A A. craccivora*-infested, *C* noninfested control, *FW* fresh weight of leaf tissue

APX Activity

The APX activities were significantly greater in *H. armigera*-infested plants of ICGV 86699 ($F_{(2,8)} = 43.8$, $P < 0.01$), ICGV 86031 ($F_{(2,8)} = 27.8$, $P < 0.01$), and ICG 1697 ($F_{(2,8)} = 12.3$, $P < 0.05$) than the APX activities of *A. craccivora*-infested plants; and the noninfested control plants (Fig. 6). The *H. armigera*- and *A. craccivora*-infested plants of ICG 2271 and JL 24 had greater APX activities (both with $P > 0.05$) than their noninfested control plants ($P < 0.05$). *H. armigera*-infested plants of ICGV 86699, ICGV 86031, and ICG 1697 showed significantly greater APX activity ($F_{(4,14)} = 32.4$, $P < 0.05$) than that of ICG 2271 and JL 24. ICGV 86699 plants infested with *A. craccivora* had higher APX activities ($F_{(4,14)} = 19.1$, $P < 0.001$) than the *A. craccivora*-infested plants of ICGV 86031, ICG 2271, ICG 1697, and JL 24. Constitutive levels of APX were significantly high in ICGV 86031 and ICG 2271, followed by ICGV 86699, ICG 1697, and JL 24.

LOX Activity

Insect infestation resulted in increased levels of LOX in all the genotypes (Fig. 7). The induction was significantly greater in plants infested with *H. armigera* and *A. craccivora* in resistant genotypes [ICGV 86699 ($F_{(2,8)} = 6.8$, $P < 0.01$), ICGV 86031 ($F_{(2,8)} = 8.9$, $P < 0.05$), and ICG 1697 ($F_{(2,8)} = 11.6$, $P < 0.05$)] than the noninfested control plants. In ICGV 2271, LOX activity in *H. armigera*-infested plants was significantly greater ($F_{(2,8)} = 18.5$, $P < 0.01$) than that of *A. craccivora*-infested plants and the noninfested control plants. Insect-resistant genotypes showed a greater increase in LOX activity in plants infested with *H. armigera* ($F_{(4,14)} = 9.1$, $P < 0.05$) and *A. craccivora* ($F_{(4,14)} = 5.2$, $P < 0.05$) than in JL 24.

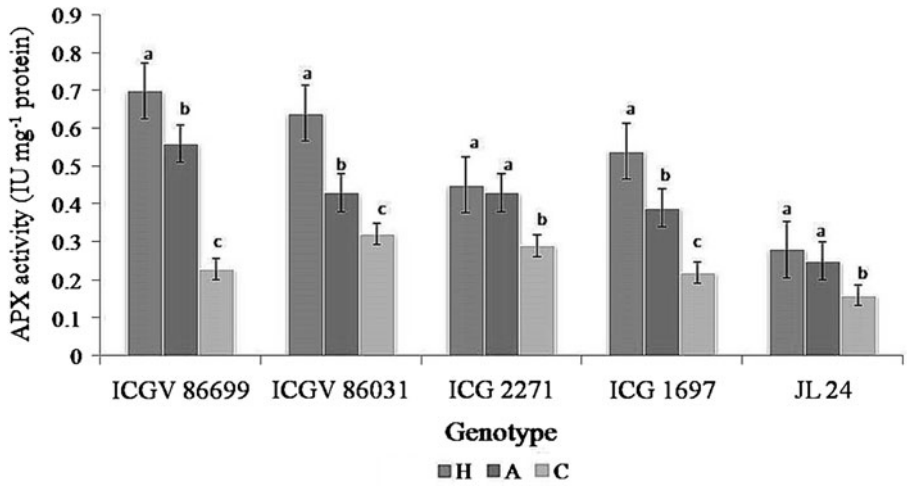

Fig. 6 APX activity (IU mg^{-1} protein) of groundnut genotypes infested with *H. armigera* and *A. craccivora*. *Bars* (mean ± SE) of the *same color* with the *same letters* are not statistically different at $P < 0.05$. *H H. armigera*-infested, *A A. craccivora*-infested, *C* noninfested control

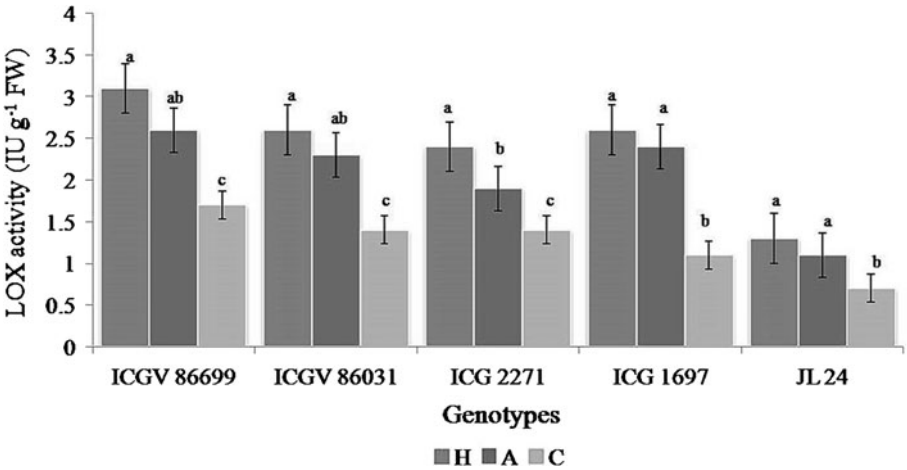

Fig. 7 LOX activity (IU g^{-1} FW) of groundnut genotypes infested with *H. armigera* and *A. craccivora*. *Bars* (mean ± SE) of the *same color* with the *same letters* are not statistically different at $P < 0.05$. *H H. armigera*-infested, *A A. craccivora*-infested, *C* noninfested control, *FW* fresh weight of leaf tissue

Total Phenols

Insect damage resulted in a tremendous increase in the amounts of phenolic compounds as compared with the noninfested control plants (Fig. 8). The increase in phenolic content was significantly greater in *H. armigera*-infested plants than in *A. craccivora*-infested plants ($F_{(2,28)} = 39.4$, 16.8, 28.1, and 13.6 for ICGV 86699, ICGV 86031, ICG 2271, and ICG 1697, respectively; all $P < 0.001$). No significant differences were observed between *H. armigera*- and *A. craccivora*-infested plants of the susceptible check JL 24 ($P > 0.05$). The insect-infested plants of ICGV 86699 showed higher phenolic content [*H. armigera*-infested ($F_{(4,14)} = 16.2$, $P < 0.01$, and *A. craccivora*-infested ($F_{(4,14)} = 14.3$, $P < 0.01$)] than those of ICGV 86031, ICG 2271, ICG 1697, and JL 24. Constitutive levels of phenolic compounds were similar among the resistant genotypes, but significantly higher ($F_{(4,14)} = 9.3$, $P < 0.05$) than the susceptible genotype JL 24.

Condensed Tannins

The *H. armigera*-infested plants had greater amounts of tannins in the insect-resistant genotypes ($F_{(2,28)} = 13.7$, 21.1, 7.4, and 11.6 for ICGV 86699, ICGV 86031, ICG 2271, and ICG 1697, respectively; all $P < 0.001$) than did the *A. craccivora*-infested plants and the noninfested control plants (Fig. 9). Insect-resistant genotypes, in general, had significantly higher tannin content [*H. armigera*-infested ($F_{(4,14)} = 11.4$, $P < 0.01$, *A. craccivora*-infested ($F_{(4,14)} = 18.3$, $P < 0.05$), and noninfested controls ($F_{(4,14)} = 21.4$, $P < 0.05$)] across the genotypes.

H_2O_2 Content

Greater amounts of H_2O_2 were observed in insect-infested plants of all the genotypes (Fig. 10). H_2O_2 content was significantly greater in *H. armigera*-infested plants of ICGV 86031, ICG 1697, and JL 24 than in the *A. craccivora*-infested and noninfested control plants ($F_{(2,28)} = 11.2$, 14.4,

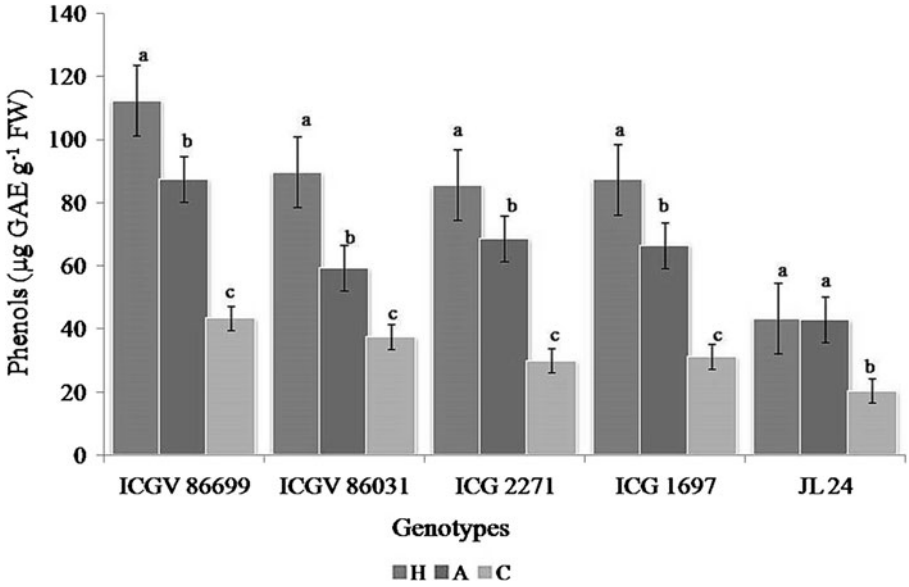

Fig. 8 Total phenols (µg GAE g^{-1} FW) of groundnut genotypes infested with *H. armigera* and *A. craccivora*. *Bars* (mean ± SE) of the *same color* with the *same letters* are not statistically different at $P < 0.05$. *H H. armigera*-infested, *A A. craccivora*-infested, *C* noninfested control, *GAE* gallic acid equivalents, *FW* fresh weight of leaf tissue

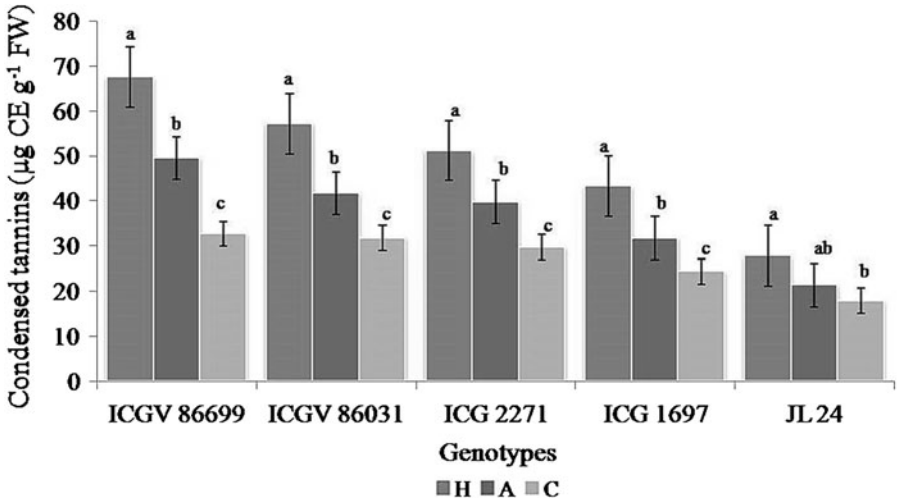

Fig. 9 Condensed tannins (µmol CE g^{-1} FW) of groundnut genotypes infested with *H. armigera* and *A. craccivora*. *Bars* (mean ± SE) of the *same color* with the *same letters* are not statistically different at $P < 0.05$. *H H. armigera*-infested, *A A. craccivora*-infested, *C* noninfested control, *CE* Catechin equivalents, *FW* fresh weight of leaf tissue

and 23.1 for ICGV 86031, ICG 1697, and JL 24, respectively; all $P < 0.05$). ICGV 86699 and ICG 2271 had greater H_2O_2 content in *H. armigera*- and *A. craccivora*-infested plants than in the noninfested controls ($F_{(2,28)} = 17.5$ and 9.6 for ICGV 86699 and ICG 2271, respectively; all $P < 0.01$). Similarly, greater induction of H_2O_2 was recorded in *A. craccivora*-infested plants of resistant genotypes ($F_{(4,14)} = 13.3$, $P < 0.05$) than in those of the susceptible check JL 24. Constitutive levels of H_2O_2 were greater in the insect-resistant genotypes than in JL 24.

MDA Content

A significant increase in MDA content was observed in insect-infested plants compared to the noninfested controls (Fig. 11). Greater MDA content was observed in *H. armigera*-infested plants ($F_{(2,28)} = 12.5$, 17.3, and 45.5 for ICGV 86031, ICG 2271, and JL 24, respectively; all $P < 0.01$) than in *A. craccivora*-infested plants and the noninfested control plants. JL 24 had greater amounts of MDA in *H. armigera*-infested plants ($F_{(4,14)} = 78.3$, $P < 0.05$) than did ICGV 86699, ICGV 86031, ICG 2271, and ICG 1697. No significant differences were recorded in MDA content of *A. craccivora*-infested plants among the genotypes tested.

Protein Content

A significant increase in protein content was observed in insect-infested plants as compared to the control plants (Fig. 12). There were no significant differences between the plants infested with *H. armigera* and those infested with *A. craccivora*. Insect-resistant genotypes had higher protein content in the insect-infested plants [*H. armigera*-

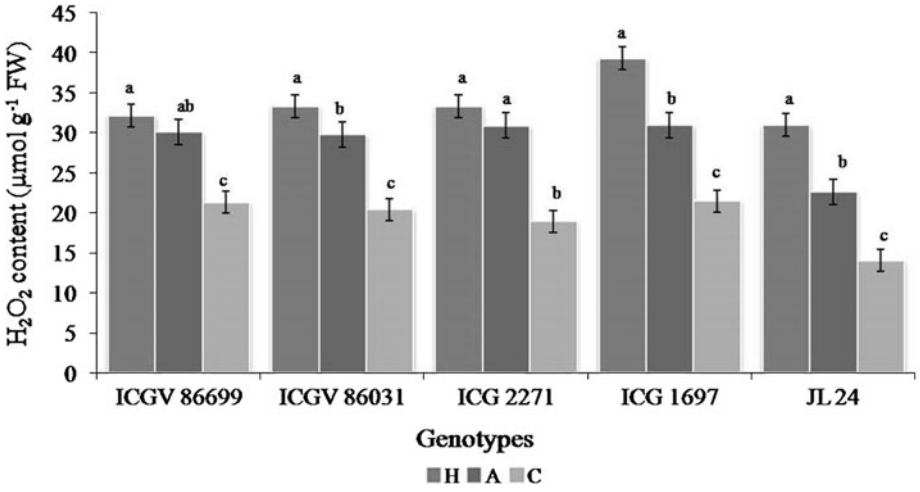

Fig. 10 H_2O_2 content (μmol g^{-1} FW) of groundnut genotypes infested with *H. armigera* and *A. craccivora*. *Bars* (mean ± SE) of the *same color* with the *same letters* are not statistically different at $P < 0.05$. *H H. armigera*-infested, *A A. craccivora*-infested, *C* noninfested control

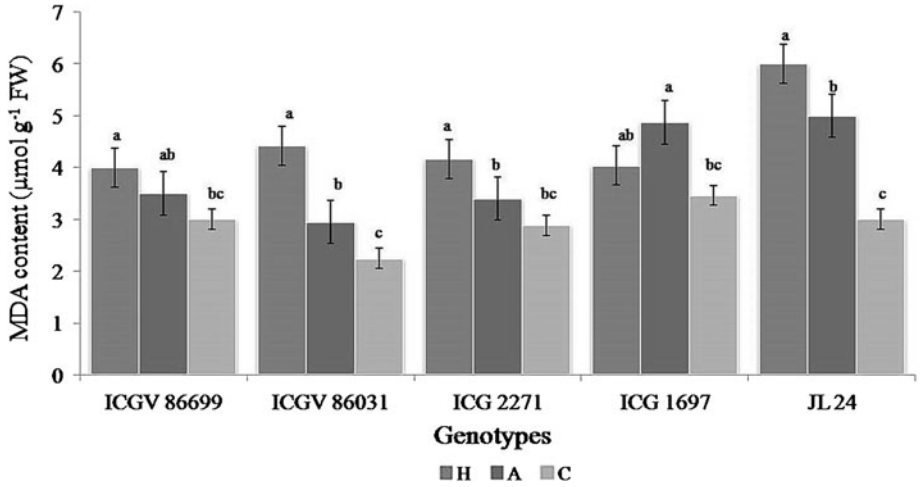

Fig. 11 MDA content (μmol g^{-1} FW) of groundnut genotypes infested with *H. armigera* and *A. craccivora*. *Bars* (mean ± SE) of the *same color* with the *same letters* are not statistically different at $P < 0.05$. *H H. armigera*-infested, *A A. craccivora*-infested, *C* noninfested control

infested ($F_{(4,14)} = 24.3$, $P < 0.01$, *A. craccivora*-infested ($F_{(4,14)} = 19.4$, $P < 0.05$)] than did the susceptible genotype JL 24.

Plant Damage and Insect Biology

Greater leaf damage by *H. armigera* was observed in the susceptible check JL 24 as compared to that in ICGV 86699, ICGV 86031, ICG 2271, and ICG 1697. Six days after infestation (DAI), the leaf damage rating due to *H. armigera* ranged from 2.8 in ICGV 86699 to 7.5 in JL 24 (Table 1). Survival of *H. armigera* larvae was significantly lower in resistant genotypes ICGV 86699 (33.5 %), ICGV 86031 (39.4 %), ICG 2271 (45.6 %), and ICG 1697 (48.3 %) than in the susceptible check JL 24 (77.5 %). The genotypes exhibiting low susceptibility to *H. armigera* were also less susceptible to the aphid *A. craccivora*, and the least aphid damage was recorded in ICGV 1697 (DR = 2.0) as compared the susceptible check JL 24 (DR = 4.2). A similar trend was observed in terms of numbers of aphids. ICG 1697 had the lowest number of aphids (19 per plant), whereas the susceptible check JL 24 had the highest number of aphids per plant (56.5). Weights of *H. armigera* larvae were significantly lower (55.5–68.9 mg/5 larvae) on ICGV 86699, ICGV 86031, ICG 2271, and ICG 1697 than those fed on the susceptible check JL 24 (95.5 mg/5 larvae).

Discussion

The evolutionary race between plants and insects has resulted in the development of an elegant defense system in plants. The defense system recognizes the nonself molecules, the signals from the damaged plant parts/cells, or the insect regurgitants and in turn activates the plant defense response against the herbivores (Howe and Jander 2008; Karban 2011; Smith and Clement 2012). When damaged by herbivorous insects, plants produce increased amounts of antinutritive and toxic proteins and secondary

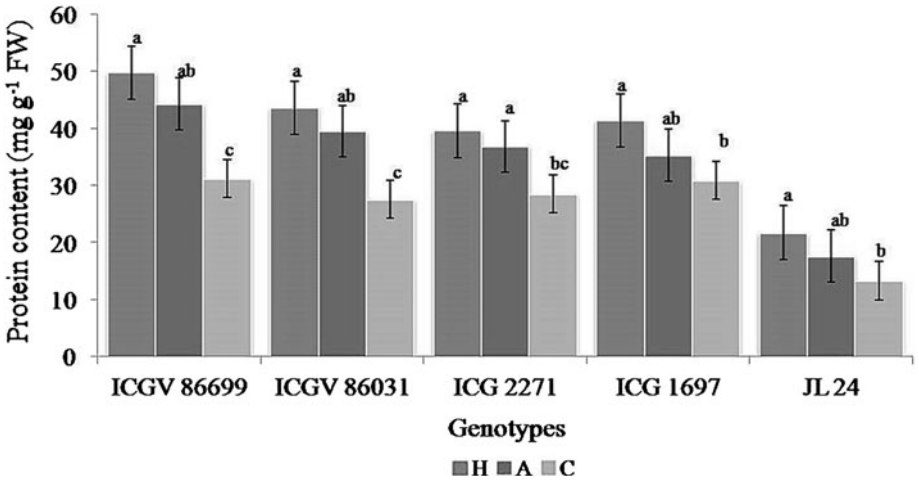

Fig. 12 Protein content (mg g^{-1} FW) of groundnut genotypes infested with *H. armigera* and *A. craccivora*. *Bars* (mean ± SE) of the *same color* with the *same letters* are not statistically different at $P < 0.05$. *H H. armigera*-infested, *A A. craccivora*-infested, *C* noninfested control

Table 1 Plant damage, larval survival, and weight of *H. armigera* and *A. craccivora* after feeding on groundnut genotypes

Genotypes	*Helicoverpa* DR*	Larval survival (%)	Larval weight (mg)†	Aphid DR‡	No. of aphids
ICGV 86699	2.8^{bc}	33.5 ± 2.4^{c}	55.5 ± 3.1^{bc}	2.5^{b}	31.5 ± 3.5^{b}
ICGV 86031	3.5^{b}	39.4 ± 2.8^{bc}	68.9 ± 6.9^{b}	2.6^{b}	27.8 ± 2.8^{b}
ICG 2271	4.2^{b}	45.6 ± 4.6^{b}	65.6 ± 5.2^{b}	2.3^{b}	37.8 ± 4.6^{b}
ICG 1697	3.8^{b}	48.3 ± 3.4^{b}	67.4 ± 4.7^{b}	2.0^{b}	19.0 ± 3.3^{c}
JL 24	7.5^{a}	77.5 ± 7.6^{a}	95.5 ± 6.8^{a}	$4.\ 2^{a}$	56.5 ± 6.2^{a}

Values (mean ± SEM) with the same letter(s) within a column are not significantly different at $P < 0.05$

* DR = *Helicoverpa* damage rating on a scale of 1–9 ($1 \leq 10$ % and $9 \geq 90$ %) 6 days after infestation

† Weight per five larva at the time of recovery

‡ Aphid damage rating on a scale of 1–5 (1 = highly resistant and 5 = highly susceptible)

metabolites that interfere with oviposition, feeding, digestion, and absorption of essential nutrients by the insects (Howe and Jander 2008; He and others 2011; Wu and Baldwin 2010; Smith and Clement 2012). The successful defense of plants against the biotic stresses depends on their ability to quickly perceive the incoming stimuli, decode it, and build a strong morphological, physiological, and/or biochemical shield against the invaders. The oxidative state of the host plants, an important component of host plant resistance to insects, results in the production of ROS and toxic secondary metabolites (Howe and Jander 2008; Zhao and others 2009; Wu and Baldwin 2010; He and others 2011). Different defensive systems are activated in response to different modes of feeding by insects.

Antioxidative enzymes such as POD, PPO, LOX, SOD, PAL, and CAT are induced in plants in response to herbivory (Felton and others 1994; Zhao and others 2009; He and others 2011). Infestation of groundnut plants by *H. armigera* and *A. craccivora* resulted in a strong induction of defensive enzymes, including POD, PPO, PAL, CAT, SOD, APX, and LOX in all the genotypes; however, the strength of induction varied across insects and genotypes. There were no significant differences in the activities of POD, PAL, and CAT in groundnut genotypes infested by *H. armigera* and *A. craccivora*, except in ICGV 86699 and ICGV 86031, where the *H. armigera*-infested plants exhibited greater POD and PPO activities, respectively, than the *A. craccivora*-infested plants. In the susceptible check JL 24, the *H. armigera*-infested plants had greater induction of CAT activity than the *A. craccivora*-infested plants and the noninfested control plants. The *H. armigera*-infested plants of ICGV 86699, ICGV 86031, and ICG 2271 exhibited greater PPO activity than did plants infested by *A. craccivora*.

In general, greater SOD activity was observed in insect-infested plants than in the noninfested control plants across the genotypes. However, ICGV 86699 and ICG 2271 showed significantly greater SOD activity in *H. armigera*-infested plants than in *A. craccivora*-infested plants and noninfested control plants. Overall, the insect-resistant genotypes exhibited greater CAT activity in *H. armigera*- and *A. craccivora*-infested plants than in the noninfested control plants. Greater APX activity was observed in *H. armigera*-infested plants than in *A. craccivora*-infested plants and the noninfested control plants, except in ICG 2271 and JL 24. The LOX activity increased significantly

in both *H. armigera*- and *A. craccivora*-infested plants in all the genotypes, and there were no significant differences in the levels of LOX activity between the two treatments in all the genotypes, except ICG 2271. Overall, the insect-resistant genotypes exhibited greater induction of LOX activity than the susceptible check JL 24.

The enzymes POD, PPO, PAL, CAT, SOD, APX, and LOX play an important role in plant defense against different stresses, including insect herbivory (Bhonwong and others 2009; Chen and others 2009; Zhao and others 2009; Gulsen and others 2010; Usha Rani and Jyothsna 2010, He and others 2011; War and others 2011a, b). The role of POD in the production of semiquinone free radicals and subsequent formation of quinones has been attributed to its direct postingested toxicity against insects (Zhu-Salzman and others 2008; Barbehenn and others 2010). In addition, it also mediates the oxidation of hydroxylcinnamyl alcohols into free radical intermediates, oxidation of phenols, cross-linking of polysaccharides and monomers, lignification, and suberization (Zhang and others 2008; Chen and others 2009), which in turn lead to the production of antinutritive compounds (Gulsen and others 2010; He and others 2011). Induction of POD activity was greater in the insect-resistant genotypes than in the susceptible check JL 24. PPO plays an important role in plant defense against insect herbivory as an antinutritional enzyme and it reduces the food quality (Mahanil and others 2008; Bhonwong and others 2009). It oxidizes phenols to highly reactive and toxic quinines that interact with the nucleophilic side chain of amino acids, leading to cross-linking of proteins and thereby reducing their availability to insect pests (Zhang and others 2008; Bhonwong and others 2009). In addition to their role in digestibility and palatability of plant tissues, melanin formation by PPOs increases the cell wall resistance to insects and pathogens (Zhao and others 2009).

The de novo synthesis and increased activity of PAL is an initial plant defensive response to insect damage (Campos-Vargas and Saltveit 2002) that leads to accumulation of phenolic compounds that are sequestered in the cell vacuole (Zhao and others 2009) and form toxic compounds upon oxidation (Bhonwong and others 2009). A negative correlation has been observed between PAL activity and growth and development of insect pests (Sethi and others 2009). SOD acts as the first line of defense by catalyzing the dismutation of superoxide into oxygen and H_2O_2 (Raychaudhuri and Deng 2000). It scavenges the toxic free radicals produced in plants on account of stresses, including herbivory (Khattab and Khattab 2005; Usha Rani and Jyothsna 2010). CAT is an important component of oxygen-scavenging systems. It scavenges the toxic and unstable ROS and converts them into less toxic and more stable components such as O_2 and water (Khattab and Khattab 2005). Increased CAT activity in plants increases cell wall resistance and also acts as a signal for the induction of defensive genes (Chen and others 1993). Higher levels of APX activity decrease the availability of ascorbate in plant tissues, which in turn reduces insect growth and development (Barbehenn and others 2005). In addition, nonavailability of ascorbate in the insect midgut increases the oxidative stress that leads to the generation of highly unstable ROS, including semiquinone, peroxides, and hydroxyl radicals (Barbehenn and others 2005). APX also reduces excessive H_2O_2 to water and oxidizes phenolic compounds to quinones, which inhibit insect feeding (Felton and others 1994; Barbehenn and others 2005). LOX catalyzes hydroperoxidation of polyunsaturated fatty acids resulting in the formation of fatty acid hydroperoxides, which are degraded to unstable and highly reactive aldehydes, γ-ketols, and epoxides (Bruinsma and others 2009). These interact with proteins and form protein-protein cross-links and also cause amino acid damage (Maffei and others 2007). Lipid peroxidation end products act as insect repellents (Bruinsma and others 2009) and are directly toxic to insect pests (Maffei and others 2007; Bhonwong and others 2009). *Nicotiana attenuata* (Torr. ex Wat.) plants deficient in LOX have been found to be susceptible to *Manduca sexta* (L.) (Rayapuram and Baldwin 2007). Greater induction of plant defensive enzymes in groundnut plants in response to *H. armigera* infestation could be attributed to more tissue damage by the chewing insect.

The amounts of total phenols and condensed tannins were greater in *H. armigera*-infested plants than in *A. craccivora*-infested plants of the insect-resistant genotypes. The increase in the amounts of phenols and condensed tannins were higher in insect-resistant genotypes than in the susceptible check JL 24. This could be ascribed to the extensive tissue damage caused by the chewing insects. Phenolic compounds induced in plants are either directly toxic to insects (Walling 2000; Bhonwong and others 2009) or mediate the signaling of various transduction pathways, which in turn produce toxic secondary metabolites and activate defensive enzymes (Walling 2000; Maffei and others 2007; Bhonwong and others 2009). Quinones formed by oxidation of phenols bind covalently to leaf proteins and inhibit protein digestion in herbivores (Bhonwong and others 2009). Tannins have been reported to reduce the growth and survivorship in many insect pests (Grayer and others 1992; Bernards and Bastrup-Spohr 2008; Sharma and others 2009). They precipitate proteins nonspecifically (including the digestive enzymes of herbivores) by hydrogen bonding or covalent bonding of protein–NH_2 groups, thereby reducing nitrogen mineralization and/or digestion in the herbivore midgut (Bernards and Bastrup-Spohr 2008). Sharma and others (2009) reported a higher quantity of polyphenols and condensed tannins in insect-resistant genotypes of pigeonpea that are resistant to *H. armigera*.

Greater amounts of H_2O_2 were recorded in insect-infested plants, and the insect-resistant genotypes responded more strongly than the susceptible check JL 24. H_2O_2 acts directly as a toxicant to the insects or as a secondary messenger, where it serves as an important component of intra- and intercellular signal transduction pathways, which in turn lead to the formation of various defensive proteins (Maffei and others 2007; Howe and Jander 2008; Torres 2010). H_2O_2 induces various defense signaling pathways in plants in response to insect attack (Maffei and others 2007; Torres 2010). Induction of H_2O_2 in plants in response to herbivory could be highly advantageous because the timing of induction of defensive responses is an important factor for defending the plants against subsequent insect and pathogen invasion (Barbehenn and others 2010; Torres 2010; He and others 2011). An increase in MDA content was observed in plants infested with insect pests; however, *H. armigera*-infested plants showed greater induction of MDA than the *A. craccivora*-infested plants. An important lipid oxidation, MDA is involved in signaling the plant's defense against a variety of stresses (Huang and others 2007). Lipid peroxidation stimulates green leaf volatile emission in plants in response to herbivory that attract the natural enemies of the herbivores (Arimura and others 2009).

The present findings indicated that feeding by *H. armigera* and *A. craccivora* resulted in an increase in protein content. An increase in protein concentration due to *H. armigera* and *A. craccivora* feeding might be partly due to the increase in antioxidative enzyme activities after herbivory. Protein-based compounds mediate wide-ranging defense responses in plants. On insect infestation, the production of defensive protein-based compounds following insect infestation, including enzymes, is one of the important strategies of plant defense (Ni and others 2001; Chen and others 2009). However, there were considerable differences in protein content in *H. armigera*- and *A. craccivora*-infested plants, which might be due to the extent of the stress caused by the insects due to different modes of feeding.

Genotypes with insect resistance affect both growth and development of herbivores (Sharma and others 2003). Insect-resistant genotypes suffered less leaf damage by *H. armigera* larvae. The *H. armigera* larvae that were fed on resistant genotypes exhibited lower larval survival and lower weights than those fed on the susceptible check JL 24. The rate of increase of the *A. craccivora* population was significantly lower on the insect-resistant genotypes than on the susceptible check JL 24. Among these, ICG 1697 suffered the lowest aphid damage because of a dense covering of trichomes on the leaves (War and others, unpublished data). Furthermore, reduced plant damage and high larval mortality on insect-resistant genotypes could be due to increased enzyme activities (Mahanil and others 2008; Bhonwong and others 2009; Gulsen and others 2010; Usha Rani and Jyothsna 2010; He and others 2011) and greater amounts of secondary metabolites (Bhonwong and others 2009; Chen and others 2009; Sharma and others 2009; Usha Rani and Jyothsna 2010; War and others 2011a, b).

Conclusions

Plant damage by *H. armigera* feeding induced a stronger response than the sucking pest *A. craccivora*. Although many reports have suggested that plants respond differently to chewing and sap-sucking insects, our results revealed that groundnut plants respond in a similar manner to both the chewing and sap-sucking insects, although the degree of the induced response varied among genotypes and between the insects. Lower induction of plant defensive compounds by *A. craccivora* infestation compared to that by *H. armigera* might be due to the greater tissue damage in leaves caused by *H. armigera* larvae. However, defensive responses induced by *A. craccivora* could be due to the damage caused by stylet probing and the elicitors in the oral secretions released on the leaf. There is a need for in-depth studies on plant responses to arthropod herbivores to gain a better understanding of signal transduction, coevolution between plants and insects, and the mechanisms of plant resistance to insects and use this information for crop protection and sustainable crop production.

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