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Resistance to beet armyworm in a chickpea recombinant inbred line population

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Abstract

Beet armyworm, *Spodoptera exigua* (Hübner), is an economic pest of chickpea, *Cicer arietinum* L., in Mexico and the Indian subcontinent. Larvae feed on the vegetative and reproductive stages of chickpea and the development of plant resistance is a priority in the management of this pest. Forty-two recombinant inbred lines (RILs) from a chickpea recombinant inbred line population (CRIL-7) developed from a cross between FLIP 84-92C (susceptible *C. arietinum*) and PI 599072 (resistant *C. reticulatum* Lad. accession) were rated resistant (nine lines with post-trial larval weights 0.42–0.59 mg), moderately resistant/susceptible (25 lines, larval weights 0.61–0.99 mg) and susceptible (eight lines, larval weights 1.01–2.17 mg) to beet armyworm larvae in a general glasshouse screening. Resistance and susceptibility of entries (RILs in the CRIL-7 population, parents, checks) was based on the average weight gain and fate of early-stage larvae on pre-flowering plants. In a growth chamber trial, early-instar larval weight gain differed significantly ($P < 0.0001$) among entries (12 RILs, parents, checks), with mean weights from 0.80 mg (resistant RIL) to 4.03 mg (susceptible kabuli cultivar). There were no significant differences ($P = 0.0836$) in larval mortality among the entries in the growth chamber trial, although mortality rates were 28.2–61.9%. Flavonoid and isoflavonoid extractions and analyses did not clarify the role played by these phytochemicals in chickpea resistance to *S. exigua*. The requisite high levels of resistance to *S. exigua* and other pests for breeding resistant culivars may reside in the CRIL-7 population.

Introduction

Chickpea (*Cicer arietinum* L.) is an important grain legume in the world with major production in southern Asia, eastern and northern Africa, North and Central America, Mediterranean Europe and Australia (Kelley et al. 2000). At least 55 species of insects are known to feed on chickpea worldwide (Reed et al. 1987), of which pod borers in the genus *Helicoverpa* (Lepidoptera: Noctuidae) are major constraints to production in the Indian subcontinent [*H. armigera* (Hübner)], Australia [*H. armigera* and *H. punctigera* (Wallengren)], and many other parts of the world (Zalucki et al. 1986; Clement et al. 2000; Sharma 2005; Yadav et al. 2006). Indeed, some kabuli chickpeas are so susceptible to *Helicoverpa* attack in India that few pods survive without insecticide applications (Reed et al. 1987). Also, the beet armyworm *Spodoptera exigua* (Hübner) (Lep.: Noctuidae) is an economic pest of chickpea, especially in Mexico and in parts of the Indian sub-continent where larvae feed on the vegetative and reproductive stages

(Gutierrez et al. 1986; Ahmed et al. 1990; Sharma et al. 2007). The development and use of chickpea cultivars with resistance to *S. exigua* and other lepidopterous pests will provide an environmentally safer option than contact insecticides for controlling these pests.

Entomologists and plant breeders usually search for insect resistance in the wild relatives of crop species after failing to locate good genetic variation for resistance in domesticated germplasm and/or to widen the arsenal of plant defensive traits for possible use in breeding (Clement 2002). This is the current approach with respect to developing insect-resistant chickpea cultivars because large-scale screenings of *C. arietinum* germplasm accessions have not identified high levels of insect resistance (Clement et al. 1999). For example, only moderate levels of resistance to *H. armigera* have been identified, and only in a few accessions among the more than 14 000 *C. arietinum* accessions that have been screened (Sharma et al. 2007). Thus, chickpea entomologists and breeders have expanded their searches for insect resistance to the wild relatives of *C. arietinum* (Singh et al. 1998; Kaur et al. 1999; Sharma et al. 2002, 2005b, 2006). Some of these screenings identified high levels of resistance to *H. armigera* in accessions of *C. reticulatum* Lad. (Sharma et al. 2002, 2005b), the putative wild progenitor that is cross compatible with *C. arietinum* (Ladizinsky 1975; Muehlbauer et al. 1994). This discovery suggests that conventional plant breeding could be used to endow chickpea cultivars with insect resistance traits in wild *Cicer*.

This study researched plant hybridization for transferring insect resistance in wild annual *Cicer* to interspecific progeny for chickpea improvement programmes. Our evaluation of resistance was based largely on weight gain of early-stage *S. exigua* larvae on a recombinant inbred line (RIL) population (CRIL-7) derived from a cross between a resistant accession of a wild annual species (*C. reticulatum*) and a cultivated breeding line (*C. arietinum*), and on parents and suitable checks. Assessing the fate of neonate and other early-stage lepidopteran larvae is important because these stages establish the initial feeding sites for larvae on host plants (Zalucki et al. 2002). Consequently, standard measurements of *Cicer* resistance include the quantification of weight gain by early-stage larvae (e.g. Sharma 2005; Sharma et al. 2005a). A second objective quantified the levels of total extractable flavonoid and isoflavonoid molecules from foliage of four entries that exhibited different levels of susceptibility and resistance in screening trials. This phytochemistry work was undertaken because these molecules may play a role in chickpea resistance to *Spodoptera* and *Helicoverpa* larvae (Simmonds and Stevenson 2001; Stevenson et al. 2005).

Materials and Methods

Plants and insects

The CRIL-7 population was developed by the USDA, Agricultural Research Service (ARS), Grain Legume Genetics and Physiology Research Unit, Washington State University (WSU), Pullman, Washington, USA, from an interspecific cross between *C. arietinum* (FLIP 84-92C, kabuli type) and *C. reticulatum* (PI 599072). Seed of PI 599072 was acquired from the seed bank at the USDA, ARS Western Regional Plant Introduction Station, WSU, Pullman, Washington. The cross was advanced by single-seed descent from the F_2 to the F_6 in a glasshouse from 1995 to 1997 (Tekeoglu et al. 2000, 2002). The resulting population was designated as CRIL-7 population. This RIL population was designated as a reference population for chickpea genomics at the Indo-US Legume Genetics and Breeding Conference at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in India in March 2006.

Forty-five RILs from the CRIL-7 population, along with the parents and checks, were evaluated in 2006 (glasshouse) and 2007 (growth chamber) screenings at the USDA, ARS Plant Germplasm Introduction and Testing Research Unit, Pullman, Washington, USA. Checks were a desi chickpea (ICC 506) with moderate resistance to *H. armigera* (Lateef 1985; Sharma et al. 2005a) and a large seeded kabuli cultivar ('Sierra') (Muehlbauer et al. 2004), which is susceptible to *S. exigua* (S.L. Clement, unpublished data). In 2005 pilot studies, *S. exigua* larvae that fed on PI 599072 (resistant parent) were one-third and one-half the size of larvae that fed on 'Sierra' (susceptible cultivar) and FLIP 84-92C (susceptible parent), respectively (S.L. Clement, unpublished data).

Seeds of all entries were germinated following methods in Kaiser et al. (1997). Seeds were scarified by scoring testa with sandpaper before they were placed in labelled cheesecloth bags in 1000 ml beakers filled with distilled water that was aerated with laboratory-supplied air. Every 2 days, the water in each beaker was changed and any germinated seeds were removed for planting. Newly germinated seeds were treated with Captan® (Drexel Chemical Company, Memphis, TN) (1 g/kg of seed) and planted

individually in 15 cm pots containing a commercial soil-perlite (2 : 1) mix. Plants were grown in a glasshouse (natural light/dark cycles; 10–37°C) until used for screening trials and chemical analyses. The plants were fertilized when they reached 10 cm in height and again every 2 weeks with 100 ppm of Peters® (J.R. Peters, Inc., Allentown, PA) soluble fertilizer (21-7-7) in 100 ml of water. The plants were watered as required. No pesticides were applied.

Eggs of *S. exigua* from a commercial vendor were express-mailed to Pullman, Washington. An egg cluster on parchment paper (*c.* 1×1 cm) was attached to a leaflet of a test plant with a micro-pin (fig. 1).

Screening protocols

The screenings were standardized with 4- to 6-week-old, pre-flowering potted plants. A cage enclosed beet armyworm eggs and larvae on a potted test plant with terminal branches supporting two to three fully expanded leaves, each with three to eight pairs of leaflets and a top leaflet. The cages were made from small plastic Petri dishes (6 cm diameter $\times$ 1.5 cm high). A hole (3 cm diameter) was made in the top and bottom of a Petri dish and covered with fine-mesh organdy to facilitate air circulation. Adhesive foam (5 mm thick) was attached to the rim of each top and bottom, which increased the height of a cage to 2.5 cm when enclosing experimental plant material. Cages, held together with a rubber band (size 16), were positioned on inverted 15 cm plastic pots in screening trials (fig. 1).

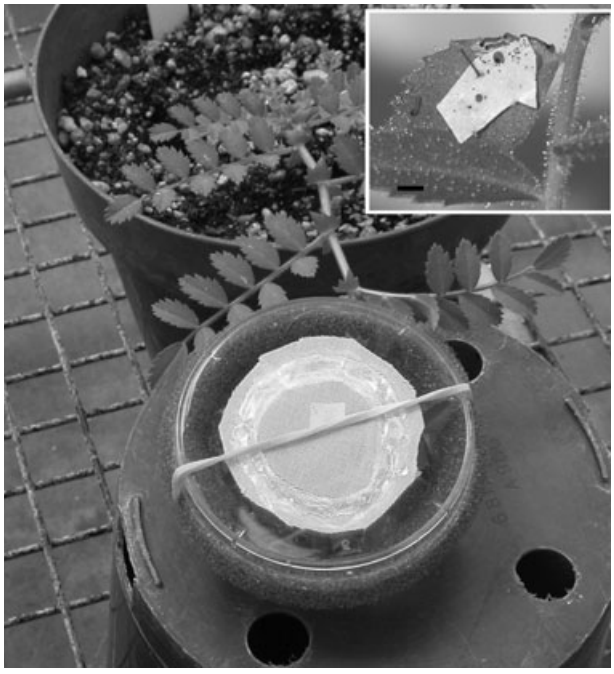

Fig. 1 Plastic Petri dish cage used to screen *Cicer* entries and recombinant inbred lines for resistance to *Spodoptera exigua* and egg-infestation method (insert showing hatched eggs on parchment paper) used in the screenings. Scale bar: 2 mm.

The 2006 glasshouse trial was a general screening [42 RILs from the CRIL-7 population, parents (PI 599072, FLIP 84-92C), checks (ICC 506, 'Sierra')] to rate RILs for relative resistance and susceptibility to *S. exigua*. There were nine groups of plants in this trial and groups were setup one after another over approximately 4 months (11 April–9 August). These groups had variable numbers of entries and plants per entry; three groups did not have plants of both parents and checks. This approach was necessary because repeated seed germinations were required over a 4-month period to obtain at least five test plants of several RILs for screening. Despite these efforts, poor germinations (0–4 germinated seeds) precluded the screening of 16 RILs in 2006. The availability of seed and seed germination rates determined the number of plants (5–13) tested per RIL.

In the 2006 glasshouse trial (16-h photoperiod provided by supplemental lighting, 10–37°C, 30–70% relative humidity), test entries were arranged on benches in a completely randomized design with five to17 replications (potted plants). Larval mortality was not recorded because test plants were infested with different numbers of eggs (20–40 per plant).

The 2007 growth chamber trial evaluated three RILs per resistance rating from the 2006 screening (see table 1), along with parents, checks and three RILs not screened in 2006. Plants were placed in a growth chamber (16-h photoperiod, 26.7 ± 0°C, 50% relative humidity) and arranged in a completely randomized design with eight to 11 replications (potted plants) per entry (12 CRIL-7 lines, two parents, two checks). For this trial, more attention was given to removing the natural layer of whitish scales over *S. exigua* egg masses so each test plant would receive 20 eggs. The number of hatched eggs and larval mortality rates per test plant were recorded.

Egg clusters on 2006 and 2007 test plants were observed twice daily (morning, afternoon) and date and time of egg hatch was recorded. After a 4-day feeding period, the branch supporting caged plant material was cut and the cage with plant material was brought to a laboratory to immediately record the number of surviving larvae and their weights.

Table 1 Summary of four *Cicer* entries and 42 chickpea recombinant inbred lines (RILs) evaluated for resistance to *Spodoptera exigua* larvae, glasshouse screening, 2006

Larval weight class (mg)[1]	Resistance rating	Placement and number of entries in each resistance rating				
		FLIP 84-92C[2]	PI 599072[3]	'Sierra'[2]	ICC 506[2]	RILs[4]
0.42–0.59	Resistant	–	1	–	–	9
0.61–0.99	Moderately resistant/susceptible	–	–	–	1	25
1.01–2.17	Susceptible	1	–	1	–	8

[1]Weight classes were arbitrarily established using mean weights of larvae reared on 46 entries, with each class established around a low and high mean weight value.
[2]*Cicer arietinum*.
[3]*Cicer reticulatum*.
[4]Individual RILs from a chickpea recombinant inbred line population (CRIL-7) developed from interspecific cross between FLIP 84-92C and PI 599072.

This feeding period was selected because 2005 pilot studies found that larvae consumed most if not all of the caged plant material within 4 days.

Flavonoid and isoflavonoid extraction and analysis

Plant materials

Four entries that exhibited different levels of resistance and susceptibility in the screening trials provided foliar and stem material: PI 599072 (resistant parent), FLIP 84-92C (susceptible parent), CRIL-7-1 (resistant RIL) and 'Sierra' (susceptible cultivar) (tables 1 and 2). The plant material was obtained from potted glasshouse plants (five or six per entry) of similar age (4- to 6-week-old) and growth stage (pre-flowering).

General analytical methods

Phenolic and flavonoid purification and quantification of chickpea species was performed using procedures modified from Fellman et al. (2000), Warren et al. (2002, 2003) and Thines et al. (2007). Chickpea stems and leaves (*c.* 2.5 g) were extracted three times with a solution of ethanol (80%) and formic acid (1%), with each extract decanted into fluted filter paper (Whatman 4, Whatman International Ltd., Maidstone, England). The ethanol extract was partitioned with an ethyl acetate/phenolic solution (20% ammonium sulphate, 20% ethanol, 2% metaphosphoric acid) and separated into the acetate (flavonoids) and aqueous phase (anthocyanins). The solvent was evaporated from the flavonoid fraction, and the remaining residue was dissolved in 2 ml HPLC grade methanol, syringe filtered (cellulose) and stored below 0°C until analysis.

Flavonoid extracts (10 μl) were injected onto an HPLC system (Varian, Inc., Walnut Creek, CA) equipped with a 5μM C-18 Zorbax-SB column and guard column (Agilent, Technologies, Avondale, PA) (250 × 4.6 mm with detection at λ254 nm). The

Table 2 Mean larval weights (2006) and means for larval mortality and weights (2007) of *Spodoptera exigua* on *Cicer* entries and recombinant inbred lines (RILs) from a chickpea RIL population (CRIL-7) in resistance screenings

	2006		2007		
Entry[1]	n[2]	Wt (mg)[3,4]	n[2]	% mortality	Wt (mg)[3]
'Sierra'	15	2.17 a	10	28.20	4.03 a
FLIP 84-92C	17	1.50 b–e	10	28.60	2.14 bc
CRIL-7-9	5	0.82 g–k	8	38.75	2.13 bc
ICC 506	17	0.93 f–k	10	32.50	2.04 b
CRIL-7-65	5	1.90 ab	10	38.80	1.66 b–d
CRIL-7-45	–	–	10	30.00	1.48 b–e
PI 599072	14	0.53 h–k	11	31.27	1.34 c–g
CRIL-7-82	–	–	8	32.25	1.23 b–f
CRIL-7-2	12	0.42 k	8	31.25	1.20 d–g
CRIL-7-59	5	0.82 g–k	10	45.00	1.18 d–g
CRIL-7-51	8	1.51 b–d	10	45.30	1.12 d–g
CRIL-7-57	12	1.13 c–g	10	47.60	0.98 g
CRIL-7-34	–	–	8	61.88	0.89 d–g
CRIL-7-87	11	0.50 h–k	10	50.70	0.87 e–g
CRIL-7-1	13	0.45 jk	10	38.60	0.82 e–g
CRIL-7-42	5	0.84 g–k	9	42.78	0.80 fg
F-values (d.f.)	–	6.28 (45, 304)***	–	1.59 (15, 136) ns	5.86 (15, 136)***

[1]Entries are those in the 2007 growth chamber trial and 13 of 46 entries in the 2006 glasshouse screening. Each trial included *Cicer arietinum* (FLIP 84-92C) and *C. reticulatum* (PI 599072) parents of the CRIL-7 population, plus susceptible ('Sierra') and moderately resistant (ICC 506) *C. arietinum* checks (see text).
[2]Number of plants tested.
[3]Least squares means followed by the same letter in a column are not significantly different ($LSD_{0.05}$) (ns, no significant difference; ***<0.0001).
[4]F-value at the bottom of the column computed from data of 46 entries.

mobile phase consisted of a solvent gradient of 90 : 10 (0.5% phosphoric acid, 100% methanol) that was increased linearly to 30 : 70 over 40 min, maintained at 30 : 70 for 5 min, cleaned with 100% methanol for 13 min, and then re-equilibrated to 90 : 10 for 2 min at a flow rate of 1.0 ml/min.

Quantification was accomplished with detection at λ 254 nm by comparing sample retention times and using response factors generated from standards. Many flavonoid compounds have a bimodal absorption pattern in methanol (MeOH) with the first peak in the UV-B or UV-C range (λ 230–300 nm) and the second peak often above 300 nm (Mabry et al. 1970). External calibration was used, and major flavonoid compounds used in our laboratory (Fellman et al. 2000) and known to exist in these plants were purchased from Sigma-Aldrich Corp. (St Louis, MO) and Indofine Chemical CO. (Belle Mead, NJ).

Qualitative analysis was performed for all standards to determine the spectrophotometric absorbance profile pertaining to a given peak. Analysis was performed using an Agilent 1100 series HPLC system equipped with a tertiary pump, auto sampler, degasser and internal Diode Array Detector (DAD). The column was held at 40°C, using the same column listed above as well as the solvents and gradient. Additionally, chickpea samples from the ether and ethyl acetate fractions were DAD analysed. Samples were subjected to DAD analysis to continuously monitor and record UV-Vis spectra in the λ 200–700 nm range. Individual HPLC peaks were assigned wavelength maximums and shoulders (data not shown).

Data analysis

The data were expressed as mean weight of larvae (2006 and 2007) and mean percentage larval mortality (2007). The 2006 mortality data were not analysed statistically because test plants were infested with different numbers of eggs (20–40 per plant). Data (larval weights, 2007 mortality data, flavonoid and isoflavonoid concentrations) were analysed by one-way ANOVA and the generalized linear model procedure of SAS for unbalanced data sets (SAS Institute Inc. 2003). Pearson correlation coefficients were computed to determine the degree of relationship between variables (SAS Institute Inc. 2003). Flavonoid and isoflavonoid data (ether fraction) and mortality data were subjected to appropriate transformations (log, arcsine) to satisfy ANOVA assumptions. Pre-transformed data are presented. Mean values were compared by Fisher's least significant difference test ($LSD_{0.05}$; SAS Institute Inc. 2003).

Results

For the 2006 glasshouse screening conducted under a wide range of temperatures and variable egg-infestation rates and numbers of test plants per entry, we used mean larval weights, albeit without regard to statistical separations, to arbitrarily establish three larval weight classes to rate entries for resistance and susceptibility (table 1). Therefore, this trial was conducted as a general glasshouse screening of RILs. Using this approach, 9, 25 and 8 RILs in the CRIL-7 population were rated resistant, moderately resistant/susceptible and susceptible respectively (table 1). As previously stated, three RILs from each rating were selected for the 2007 trial. Expected levels of resistance and susceptibility were exhibited by PI 599072, FLIP 84-92C, 'Sierra' and ICC 506 (tables 1 and 2).

The weights of 4-day-old larvae on pre-flowering plants differed significantly ($P < 0.0001$) among all entries in the 2006 glasshouse screening. Instead of presenting the values for all 46 entries in this screening, only the values for nine RILs, parents and checks are shown in table 2. This approach is used to conserve space while illustrating the 2006 and 2007 results for the 9 RILs. In the 2006 screening, mean weights ranged from 0.42 (CRIL-7-2) to 2.17 mg ('Sierra') (table 2).

Table 2 also shows the results from the 2007 growth chamber trial. Although mortality levels among the entries were not significantly different ($P = 0.0836$), there was a significant inverse correlation ($r = -0.56$, $P < 0.05$) between weight and mortality variables because larval mortality was generally higher on entries that produced smaller larvae. This correlation was muted by a single outlier (CRIL-7-34). Larval mortality was lowest on the susceptible check ('Sierra') and susceptible parent (FLIP 84-92C), whereas mortality trended higher on the RILs in the CRIL-7 population (table 2). Also, large numbers of dead neonate larvae were observed on leaflet trichomes of RIL plants (fig. 2). Early-instar weight gain over a 4-day period differed significantly ($P < 0.0001$) among the 2007 entries, with mean larval weights ranging from 0.80 (CRIL-7-42) to 4.03 mg ('Sierra') (table 2).

Although mean larval weights in the 2007 trial were generally higher than those recorded in 2006, the weights from the 13 entries in both trials

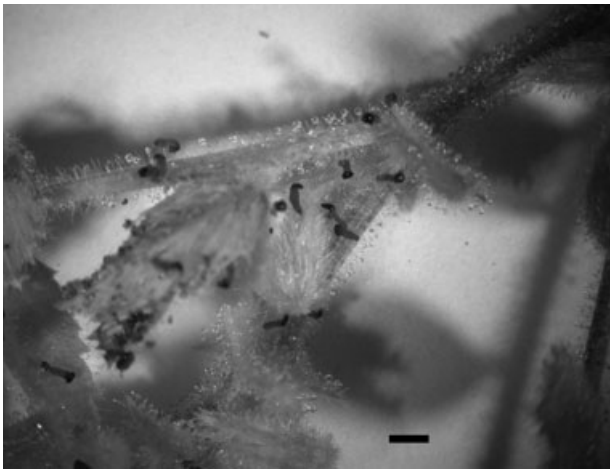

Fig. 2 Dead neonate larvae of *Spodoptera exigua* on and near leaflet trichomes and trichome exudates of a plant from a chickpea recombinant inbred line population (CRIL-7). Scale bar: 1 mm.

(table 2) were significantly correlated ($r = 0.674$, $P < 0.05$). This suggests that the two screening approaches yielded fairly similar results. The high larval weights in the 2007 trial were a likely consequence of the high static temperature in the growth chamber, compared to the widely fluctuating temperatures in the 2006 glasshouse screening. Importantly, some RILs (CRIL-7-1, CRIL-7-87) in both screenings produced some of the smallest larvae (table 2).

Table 3 lists the total extracted flavonoid and isoflavonoid molecules in the foliage and stems of four entries that exhibited different levels of susceptibility and resistance to *S. exigua*. There were significant differences (table 3) in total flavonoid and isoflavonoid concentrations among the entries, although there was statistical concordance among pairs of entries in both soluble fractions (table 3). The entry rankings for total contents in both ethyl acetate and ether fractions, while similar ('Sierra' followed by increasing amounts in PI 599072, CRIL-7-1 and FLIP 84-92C) (table 3), were not predictive of a resistance ranking for the four entries in which 'Sierra' was the most susceptible, followed by FLIP 84-92C, PI 599072 and CRIL-7-1 (most resistant) (table 2).

Table 3 Amounts of total extractable flavonoid and isoflavonoid molecules from foliage of three *Cicer* entries and one recombinant inbred line (RIL)

	Mean μg/g fresh weight[1]		
Entry[2]	Ethyl acetate[3]	Ether[3]	Resistance rating
FLIP 84-92C	41257.99 a	89948.28 a	Susceptible parent
CRIL-7-1	36514.69 a	50788.03 b	Resistant RIL
PI 599072	17981.02 b	44929.00 b	Resistant parent
'Sierra'	13559.18 b	29214.76 c	Susceptible cultivar
$F_{3,19}$	7.83 (P = 0.0013)	11.21 (P = 0.0002)	–

[1]Least squares means followed by the same letter in a column are not significantly different ($LSD_{0.05}$).
[2]*Cicer arietinum* (FLIP 84-92C, 'Sierra'), *C. reticulatum* (PI 599072) and one RIL (CRIL-7-1) from a cross between FLIP 84-92C and PI 599072.
[3]Soluble fraction.

Discussion

This study shows firstly that RILs from a *Cicer* CRIL-7 exhibited different levels of susceptibility and resistance, thereby confirming the transfer of insect resistance in a wild parent (*C. reticulatum*) to interspecific progeny. The required genetic variation to breed both beet armyworm and *Helicoverpa* resistant chickpeas might reside in the CRIL-7 population. In this context, field and laboratory research at ICRISAT will evaluate genetic variation in the CRIL-7 population for breeding noctuid-resistant cultivars, with initial emphasis on *H. armigera*. Secondly, several RILs in the CRIL-7 population were significantly more resistant to *S. exigua* than ICC 506 (table 2), a desi chickpea that has become the standard resistant or moderately resistant check for host plant resistance studies involving *H. armigera* (e.g. Sharma et al. 2005a, 2006; Cotter and Edwards 2006; Narayanamma et al. 2008). Therefore, an important finding from our research is that highly resistant chickpea RILs might be more promising breeding material for chickpea improvement programmes than previously identified germplasm that exhibit only moderate levels of resistance (including ICC 506) to pod borers (Clement et al. 1999; Sharma et al. 2002; Sharma 2005).

The theory that chickpea cultivars containing low concentrations of isoflavonoids are highly susceptible to insect attack (Simmonds and Stevenson 2001; Stevenson et al. 2005) applies to one entry in this limited study. This susceptible kabuli cultivar ('Sierra') (tables 1 and 2) contained the lowest amounts of total flavonoid and isoflavonoid molecules (table 3). By contrast, the susceptibility of the kabuli cultivar FLIP 84-92C to *S. exigua* (tables 1 and 2) was not matched by low levels of the putative defensive chemicals. Instead, FLIP 84-92C contained higher levels of these molecules than the resistant entries PI 599072 and CRIL-7-1 (table 3). Therefore, we cannot make an association between extractable con-

centrations of flavonoids and isoflavonoids in PI 599072 and CRIL-7-1 and their strong resistance to *S. exigua*. Interestingly, Simmonds and Stevenson (2001) found that larvae of *S. exigua* were not deterred from feeding by four isoflavonoids.

We cannot discount a possible plant defensive role for specific isoflavonoids and other compounds in other RILs and plants in the *Cicer* primary and secondary gene pool. Phytochemicals such as oxalic and malic acid, *H. armigera* resistance factors in chickpea (Yoshida et al. 1997), might prove to be important mechanisms of resistance to *S. exigua*. At this point, more research is required to determine the defensive role played by different *Cicer* phytochemicals and the value of genetic variation for insect resistance in the CRIL-7 population for chickpea breeding programmes.

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