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Standardization of detached leaf assay to screen chickpeas for resistance to beet armyworm, *Spodoptera exigua* (Hübner, 1808)

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Abstract: The beet armyworm, *Spodoptera exigua* (Hübner, 1808) is an important pest of several economically important crops, and recently emerged as a serious pest of chickpea in South Central India. We standardized a detached leaf assay technique to evaluate chickpea germplasm and segregating populations for resistance to this pest under laboratory conditions. Two chickpea genotypes ICCL 86111 and ICC 3137 grown under field and greenhouse conditions were used for the detached leaf assay at the vegetative and flowering stages. The terminal branches were infested with 5, 10, 15, and 20 neonate larvae of *S. exigua*. The test genotypes were also infested with 2, 4, 6 and 8 third-instar larvae at the podding stage. At the vegetative stage, ICCL 86111 suffered less damage than ICC 3137 across infestation levels. The differences in larval survival between the genotypes were significant, and larval survival was lower on ICCL 86111 than on ICC 3137 across infestation levels. The results suggested that infesting the chickpea terminal branches with 10–15 neonate larvae per branch at the vegetative stage or six third-instar larvae at the podding stage can be used to evaluate chickpea genotypes for resistance to *S. exigua*.

Key words: *Spodoptera exigua*; chickpea; host plant resistance; screening technique; detached leaf assay

Standardizacija preiskusa z odtrganimi listi za preiskus odpornosti čičerike na pesno sovko (*Spodoptera exigua* (Hübner, 1808))

Izvleček: Pesna sovka (*Spodoptera exigua* (Hübner, 1808)) je pomemben škodljivec številnih ekonomsko pomebnih kulturnih rastlin in se zadnje čase pojavlja kot resen škodljivec čičerike v južni osrednji Indiji. Standardiziran je bil preiskus z odtrganimi listi za ovrednotenje genotipov čičerike na odpornost proti temu škodljivcu in razdelitev njenih populacij glede na odpornost v laboratorijskih razmerah. Dva genotipa čičerike, ICCL 86111 in ICC 3137, rastoča na prostem in v rastlinjaku sta bila uporabljena v poskusu z odtrganimi listi v vegetativni in reproduktivni fazi razvoja. Vršni poganjki so bili okuženi s 5, 10, 15, in 20 mladimi ličinkami pesne sovke. Preiskušani genotipi so bili okuženi še z 2, 4, 6 in 8 ličinkami tretje razvojne stopnje v razvojni fazi tvorbe strokov. V vegetativni razvojni fazi je imel genotip ICCL 86111 manj poškodb kot genotip ICC 3137 pri vseh jakostih okužbe. Razlike v preživetju ličink med genotipi so bile značilne in njihovo preživetje je bilo manjše na genotipu ICCL 86111 kot na genotipu ICC 3137 pri vseh jakostih okužbe. Rezultati nakazujejo, da bi se okužba vršnih poganjkov čičerike z 10–15 mladimi ličinkami na poganjek v vegetativni fazi ali s 6 ličinkami tretje razvojne stopnje v fazi tvorbe strokov lahko uporabila za ovrednotenje odpornosti genotipov na pesno sovko.

Ključne besede: *Spodoptera exigua*; čičerika; odpornost gostiteljske rastline; preiskus ugotavljanja; preiskus na odtrganih listih

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1 INTRODUCTION

Chickpea, *Cicer arietinum* L., is the third most important grain legume in the world, after dry beans and peas. It is cultivated in more than 42 countries in Asia, Eastern and Northern Africa, North and Central America, Mediterranean Europe and Australia. Chickpea yields have shown only a marginal increase over the past 50 years because of the heavy losses due to biotic and abiotic stress factors. Besides *Helicoverpa armigera* (Hübner, 1808), which is the major constraint to chickpea production in the Indian sub-continent (Sharma, 2005a), the beet armyworm, *Spodoptera exigua* (Hübner, 1808) (Lepidoptera: Noctuidae) is emerging as an important pest especially in South Central India. The young larvae of *S. exigua* initially feed gregariously on chickpea foliage and reproductive parts of the plant (Gutierrez et al., 1986; Sharma et al., 2007). As the larvae mature, they become solitary and continue to eat, producing large, irregular holes on the foliage (Ahmed et al., 1990; Sharma et al., 2007).

S. exigua is a cosmopolitan species infesting >90 plant species in North America, many of which are crop plants (Pearson, 1982). Insecticides directed against the larvae are the primary method of control, but its high tolerance to most insecticides and associated environmental problems may jeopardize their continued use (Mascarenhas et al., 1996). Development of crop cultivars with resistance or tolerance to *H. armigera* (Hübner [1808]) and *S. exigua* has a major potential for use in integrated pest management (Fitt, 1989; Sharma and Ortiz, 2002), but there is very little information on identification and utilization of resistance to control *S. exigua*. However, interspecific derivatives of *Cicer reticulatam* Ladiz. (FLIP 84-92C - susceptible) x *Cicer arietinum* (PI 599072 - resistant) have shown high levels of resistance to this pest (Clement et al., 2010).

Large-scale screenings of *C. arietinum* germplasm accessions have not resulted in identification of high levels of resistance to insects (Clement et al., 1999). It is important to screen the test material for resistance to the target insect under optimum and uniform level of insect infestation at the most susceptible stage of the crop (Sharma et al., 1992; Smith et al., 1994). Of the several techniques used to screen for insect resistance, detached leaf assay is quite fast, precise, and requires minimum resources. It can be used to screen a large number of germplasm lines, mapping populations and segregating breeding material (Sharma et al., 2005b). Therefore, the present studies were undertaken to standardize detached leaf assay to screen for resistance to beet armyworm, *S. exigua* under uniform insect pressure.

2 MATERIALS AND METHODS

The test genotypes, ICCL 86111 and ICC 3137, which had shown resistant and susceptible reaction under field conditions, respectively (Shankar et al., 2013), were grown under field and greenhouse conditions at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana, India (latitude 17.53 ^{0}N, longitude 78.27 ^{0}E and an altitude of 545 m).

2.1 REARING *S. EXIGUA* ON ARTIFICIAL DIET

The larvae of *S. exigua* were reared on a chickpea flour based artificial diet, which was developed for rearing *H. armigera* (Armes et al., 1992). The egg masses and larvae of *S. exigua* were collected from chickpea plants from farmer's fields in Andhra Pradesh, India. The *S. exigua* culture was maintained under controlled environmental conditions (27 ± 2 °C and 65 to 75% RH) (Chitti babu et al., 2014). The neonates were reared in groups of 300-400 in 250 mL plastic cups (having 2 to 3 mm layer of artificial diet on the bottom and sides) for 7 days or up to third-instar. After seven days, the larvae were transferred individually to cell-well plates containing six cells (each cell with 3.5 cm diameter, and 2 cm in depth) or small plastic cups (3.5 cm diameter and 2.5 cm in depth) to avoid cannibalism. The pupae were removed from cell wells, sterilized with 2% sodium hypochlorite solution, and kept in groups of 50 in plastic jars containing moist vermiculite. After adult emergence, 25 pairs were released inside an oviposition cage (L x B x H: 30 x 30 x 30 cm). Adults were provided with 15% sucrose solution in a cotton swab for feeding. The adults laid eggs on the nappy liners hung inside the cage. The liners were removed daily and the eggs were sterilized with 10% formalin. The liners were then washed with tap water, dried under a fan, and placed inside the plastic cups (250 mL). After egg hatching, the larvae were transferred on to the artificial diet.

2.2 RAISING CHICKPEA PLANTS UNDER FIELD AND GREENHOUSE CONDITIONS

2.2.1 Field conditions

Two chickpea genotypes (ICCL 86111 - resistant, and ICC 3137 - susceptible) were sown in the field (latitude 17.53^{0}N, longitude 78.27^{0}E and an altitude of 545 m) during the post-rainy seasons of 2010-11 and 2011-12.

The experiment was laid in a randomized complete block design (RCBD) with five replications. The plot size was two rows, 2 m long, and row-to-row spacing of 60 cm and plant-to-plant spacing was 10 cm. Normal agronomic practices were followed for raising the crop. A basal dose of di-ammonium phosphate (100 kg ha^{-1}) was applied before sowing. There was no pesticide application in the field. The chickpea genotypes were evaluated for resistance to *S. exigua* using neonate and third-instar larvae in the detached leaf assay and pod bioassay, respectively. The plants were evaluated for resistance to *S. exigua* at the vegetative (30 days after seedling emergence, DAE) and flowering (45 days after seedling emergence) stages.

2.2.2 Glasshouse conditions

Two chickpea genotypes (ICCL 86111 - resistant, and ICC 3137 - susceptible) were grown under glasshouse conditions (27 ± 5°C and 65 - 90% RH). The seeds were sown in a sterilized mixture of black soil (Vertisols), sand and farmyard manure (2:1:1) filled in medium sized plastic pots (30 cm in diameter and 30 cm in depth). The plants were watered as and when required. Six seeds were sown in each pot and three plants with uniform growth were retained at 10 days after seedling emergence. Di-ammonium phosphate (DAP) was applied at 15 days after seedling emergence (20 g per pot). There were five replications for each treatment in a completely randomized design (CRD). The chickpea genotypes were evaluated for resistance to *S. exigua* using neonate or third-instar larvae in the detached leaf assay at the vegetative (30 DAE) and flowering (45 DAE) stages, respectively.

2.2.3 Detached leaf assay to evaluate chickpea genotypes for resistance to *S. exigua*

The chickpea plants grown in the field and greenhouse were bioassayed under controlled conditions in the laboratory [27 ± 2°C temperature; 65 - 75% RH, and photoperiod of 12: 12 h (L: D)] to screen for resistance to *S. exigua* using detached leaf assay. Terminal branches of chickpea (three to four fully expanded leaves and a bud) were excised from the plants and inserted in 3% agar-agar in plastic cups (4.5 x 11.5 cm diameter) (Sharma et al., 2005b). The solidified agar-agar served as a substratum for maintaining the chickpea branches in a turgid condition for 5-7 days. The terminal branches were infested with 5, 10, 15 and 20 neonate larvae of *S. exigua* using a camel hairbrush, and then covered with a lid to keep the chickpea terminals in a turgid condition. The experiment was conducted in a CRD, and there were five replications for each treatment. The experiments were terminated when >80% of the leaf area was consumed in the susceptible genotype or when there were maximum differences between the resistant and susceptible genotypes (generally at 5 days after releasing the larvae on the leaves). The plants were scored for leaf feeding visually on a 1-9 damage rating scale (1 = <10 %, and 9 = >80 % leaf area consumed). Data were also recorded on larval survival and mass of the larvae 4 h after terminating the experiment.

At the podding stage, the plants raised under field conditions were used for the bioassays. The terminal branches (10 cm long) with pods (6 - 8 pods) were excised with a sharp knife and placed in agar-agar as described above in a 500 mL plastic jar. The terminal branches were infested with 2, 4, 6 and 8 third-instar (8 days old) larvae per branch. There were five replications for each treatment, and the cups were arranged in a completely randomized design (CRD). The experiment was terminated when >80% of the pods were damaged in susceptible control. Data were also recorded on pod damage rating (DR) on a 1-9 scale (1 = <10% and 9 = >80% pods consumed), larval survival, and larval mass.

2.2.4 Statistical analysis

Data were subjected to analysis of variance using GenStat version 14.0, (GenStat, 2010). The data on detached leaf assays were analyzed by factorial analysis with genotypes as the main treatment, and the infestation levels as the sub treatment. Significance of differences between the genotypes was tested by F-test, while the treatment means were compared by least significant differences (LSD) at p 0.05.

3 RESULTS

3.1 RESPONSE OF CHICKPEA GENOTYPES TO DIFFERENT LEVELS OF INFESTATION WITH NEONATE LARVAE OF *S. EXIGUA* IN PLANTS GROWN UNDER FIELD CONDITIONS

3.1.1 Leaf damage

The differences in leaf feeding across infestation levels at the vegetative stage, the differences between the genotypes and the interaction effects between the genotypes and the infestation levels were significant. Maximum differences in leaf feeding between ICCL 86111 and ICC 3137 were observed in branches infested with 10 (DR 2.6 in ICCL 86111 compared to 5.2 in ICC 3137)

and 15 larvae (DR 3.9 in ICCL 86111 compared to 6.7 in ICC 3137) per branch (Fig. 1a).

At the flowering stage, the differences in leaf feeding across infestation and the interaction effects between the genotypes and the infestation levels were significant. However, the differences between the genotypes were non-significant. Maximum differences in leaf feeding were observed when the terminal branches were infested with 20 neonate larvae per branch (DR 5.0 in ICCL 86111 compared to 6.8 in ICC 3137) (Fig. 1a).

3.1.2 Larval survival

The differences in larval survival across infestation levels at the vegetative stage were non-significant while the differences between the genotypes and the interaction effects between the genotypes and the infestation levels were significant. Maximum differences in larval survival were observed when terminals were infested with 10 larvae (28% on ICCL 86111 and 62% on ICC 3137) (Fig. 1b).

At the flowering stage, the differences in larval survival across infestation levels and the interaction effects between the genotypes and the infestation levels were non-significant. However, the differences between the genotypes were significant (Fig. 1b).

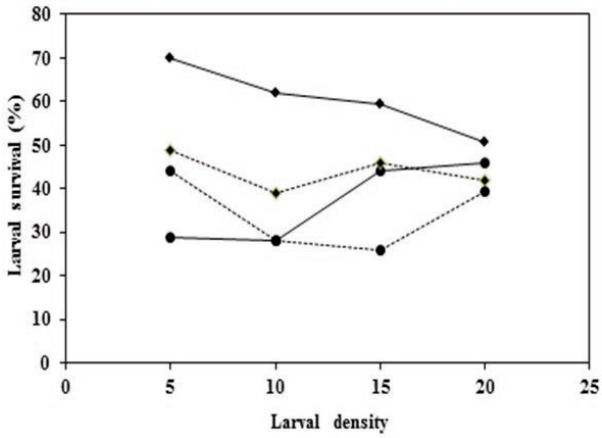

Fig. 1b: Survival of neonate larvae of *S. exigua* on two chickpea genotypes (ICC 3137 (♦) and ICCL 86111(●)) at the vegetative (solid line) and flowering (dotted line) stages in plants grown under field conditions using detached leaf assay (Vegetative stage SE ± 3.35, Flowering stage: SE ± 2.60)

3.1.3 Larval mass gain

The differences in larval mass across infestation levels at the vegetative stage, differences between the genotypes, and the interaction effects were non-significant. Maximum differences in larval mass were recorded when the chickpea terminal branches were infested with 10 neonate larvae. (Fig. 1c).

At the flowering stage, the differences in larval mass across infestation levels and the differences between the genotypes were non-significant. However, the interaction effects were significant (Fig. 1c).

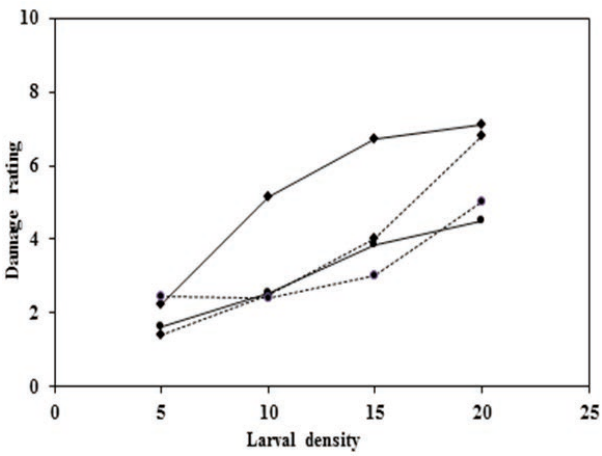

Fig. 1a: Leaf damage ratings in two chickpea genotypes (ICC 3137 (♦) and ICCL 86111(●)) infested with different densities of neonate larvae of *S. exigua* at the vegetative (solid line) and flowering (dotted line) stages in plants grown under field conditions using detached leaf assay (Vegetative stage SE ± 0.19, Flowering stage SE ± 0.17). Damage rating (1 = <10% leaf area, and 8 = > 90 % leaf area damaged)

3.2 RESPONSE OF CHICKPEA GENOTYPES TO DIFFERENT LEVELS OF INFESTATION WITH NEONATE LARVAE OF *S. EXIGUA* IN PLANTS GROWN UNDER GREENHOUSE CONDITIONS

3.2.1 Leaf damage rating

The differences in leaf feeding across infestation levels and between the genotypes were significant (Fig. 2a). However, the interaction effects between infestation levels and the genotypes were non-significant. Maximum differences in leaf feeding were observed when the terminals were infested with 20 neonates (Fig. 2a).

At the flowering stage, the differences in leaf feeding across infestation levels and the differences in leaf feeding between the genotypes were significant (Fig. 2a). The interaction effects between the genotypes and the infestation levels were non-significant. Maximum differences in

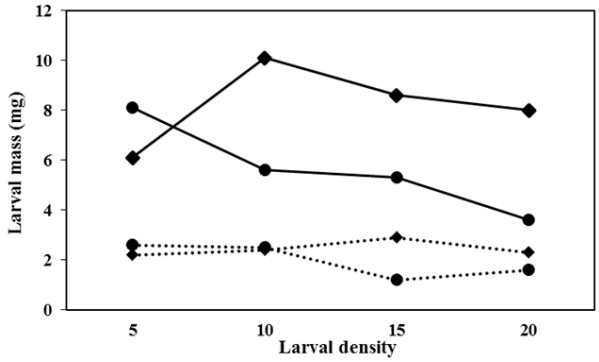

Fig. 1c: Mass gain by neonate larvae of *S. exigua* on two chickpea genotypes (ICC 3137 (♦) and ICCL 86111(●)) at the vegetative (solid line) and flowering (dotted line) stages in plants grown under field conditions using detached leaf assay (Vegetative stage SE ± 0.97, Flowering stage SE ± 0.19)

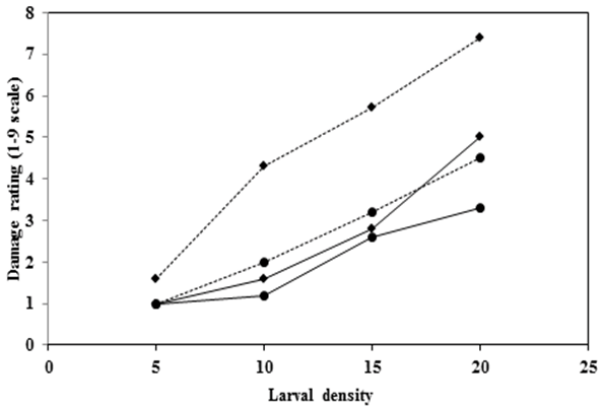

Fig. 2a: Leaf damage ratings in two chickpea genotypes (ICC 3137 (♦) and ICCL 86111(●)) infested with different densities of neonate larvae of *S. exigua* at the vegetative (solid line) and flowering (dotted line) stages in plants grown under greenhouse conditions using detached leaf assay (Vegetative stage SE ± 0.16, Flowering stage SE ± 0.41). Damage rating (1 = < 10 % leaf area, and 8 = > % leaf area damaged)

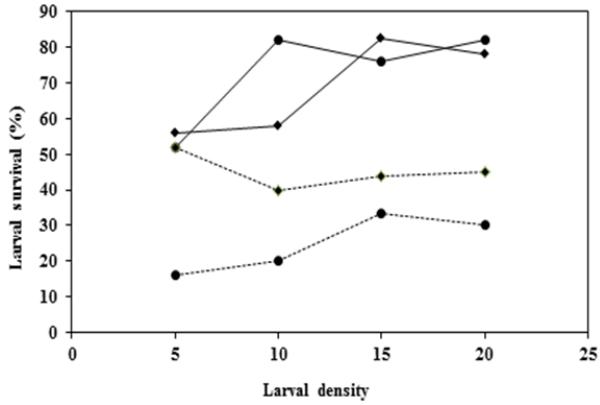

Fig. 2b: Survival of neonate larvae of *S. exigua* on two chickpea genotypes (ICC 3137 (♦) and ICCL 86111(●)) at the vegetative (solid line) and flowering (dotted line) stages in plants grown under greenhouse conditions using detached leaf assay (Vegetative stage SE ± 4.07, Flowering stage SE ± 3.94)

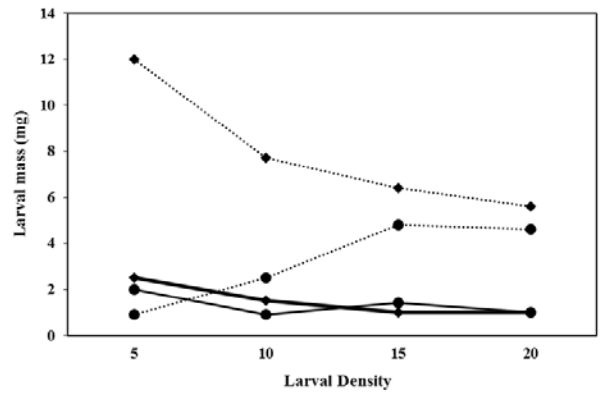

Fig. 2c: Mass gain by the neonate larvae of *S. exigua* on two chickpea genotypes (ICC 3137 (♦) and ICCL 86111(●)) infested at the vegetative (solid line) and flowering (dotted line) stages in plants grown under greenhouse conditions using detached leaf assay (Vegetative stage SE ± 0.11, Flowering stage SE ± 0.93)

leaf feeding were observed when the terminal branches were infested with 15 neonates (Fig. 2a).

3.2.2 Larval survival

Differences in larval survival across infestation levels at the vegetative stage were significant, but the interaction effects, differences between the genotypes and the interaction effects between the infestation levels and the genotypes were non-significant (Fig. 2b). Differences in larval survival across infestation levels were non-significant. However, the differences between the genotypes were significant.

3.2.3 Larval mass gain

Differences in larval mass across infestation levels at the vegetative stage were significant, but the interaction effects and the differences between the genotypes were non-significant (Fig. 2c).

At the flowering stage, the differences in larval mass across infestation levels were non-significant. However, the differences between the genotypes (Fig. 2c) and the

interaction effects between the genotypes and the infestation levels were significant.

3.3 RESPONSE OF CHICKPEA GENOTYPES TO DIFFERENT LEVELS OF INFESTATION WITH THIRD-INSTAR LARVAE OF *S. EXIGUA* AT THE PODDING STAGE

At the podding stage, the differences in leaf feeding across infestation levels and the differences between the genotypes were significant (Fig. 3a). However, the interaction effects between the infestation levels and the genotypes were non-significant.

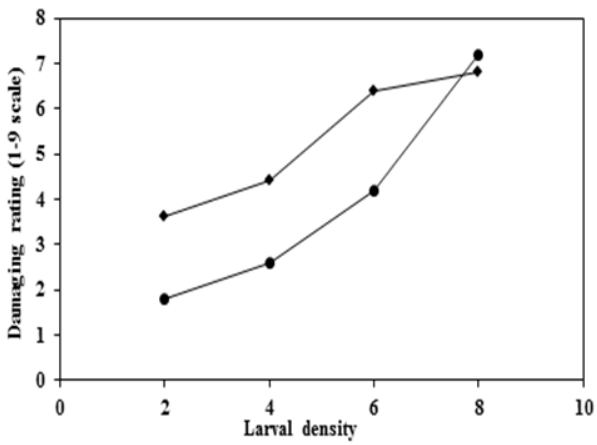

Fig. 3a: Leaf damage ratings in two chickpea genotypes (ICC 3137 (♦) and ICCL 86111(●)) infested with different densities of third-instar larvae of *S. exigua* at the podding stage in plants grown under field conditions using (SE ± 0.52). Damage rating (1 = < 10 % leaf area, and 8 = > 90 % leaf area damaged)

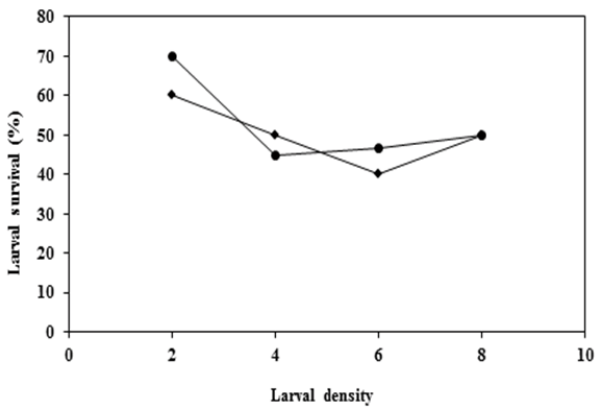

Fig. 3b: Survival of third-instar larvae of *S. exigua* on two chickpea genotypes (ICC 3137 (♦) and ICCL 86111(●)) at the podding stage in plants grown under field conditions using (SE ± 14.3)

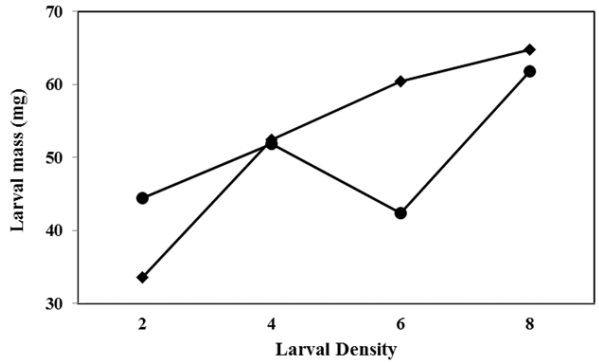

Fig. 3c: Mass gain by the third-instar larvae of *S. exigua* on two chickpea genotypes (ICC 3137 (♦) and ICCL 86111(●)) infested at the podding stage in plants grown under field conditions using (SE ± 9.29)

Differences in larval survival across infestation levels, genotypes, and the interaction effects were non-significant (Fig. 3b).

The differences in larval mass across infestation levels, genotypes, and the interaction effects were non-significant (Fig. 3c).

4 DISCUSSION

Screening for host plant resistance to insect pests under natural conditions is a long-term process because of variations in insect population in space and time. As a result, it is difficult to identify stable sources of resistance under natural infestation (Sharma et al., 1997, Devetak et al., 2014). Is important to develop techniques to screen for resistance to insects under uniform insect pressure. Therefore, careful consideration should be given to use an optimum insect density that results in maximum differences between the resistant and susceptible genotypes. The detached leaf assay not only gives an idea of the relative feeding by the larvae on different cultivars but also provides useful information on antibiosis component of resistance in terms of larval mass (Sharma et al., 2005c; Jaba et al., 2017). In this context, the detached leaf assay can be used to evaluate the test material under uniform insect pressure at the seedling, flowering and podding stages under laboratory conditions.

In the crop raised under field conditions, the differences in leaf feeding, larval survival, and larval mass between ICCL 86111 and ICC 3137 were greater at the vegetative stage than at the flowering stage. Maximum differences in leaf feeding between ICCL 86111 and ICC

3137 were observed in branches infested with 10 and 15 larvae per branch at vegetative stage. Across infestation levels, larval survival was lower on ICCL 86111 than on ICC 3137, maximum differences were observed when the terminal branches were infested with 10 neonates of *S. exigua* at vegetative stage and 15 neonates at flowering stage. Heavy rains in the 2010-11 post-rainy season possibly washed out the organic acids on the chickpea leaves (Sharma et al., 2010), resulting in reduced differences in leaf feeding and larval survival between the genotypes tested. Lower leaf feeding, larval survival and larval mass gain were recorded on EC 583260, EC 583264 and ICC 12475 (Shankar et al., 2014). Narayanamma et al., 2007 reported low larval survival and mass gain on ICC 12475. In other study (Jaba et al*.,* 2017), significantly low *H. armigera* larval mass and maximum percent mass gain were recorded in chickpea genotypes, ICCV 097105 and ICCV 07306 respectively (101.9 mg (88.5%) and 382.3 mg (317.4%), respectively.

In plants raised under greenhouse conditions, leaf feeding was maximum when the plants were infested with 20 neonate larvae at vegetative and flowering stages. The differences in leaf feeding, larval survival and mass varied across plant growth stages and infestation levels possibly because of differences in plant growth and accumulation of secondary metabolites (Sharma et al., 2005b; War et al., 2013), that affect leaf feeding, growth and development of insects.

At the podding stage, the differences in leaf feeding between the genotypes were significant. Maximum differences in leaf feeding were recorded in branches infested with 4 and 6 larvae per branch. The results suggested that infesting the chickpea terminal branches with 10-15 neonate larvae per branch at the vegetative stage or six third-instar larvae at the podding stage could be used to evaluate chickpea genotypes for resistance to *S. exigua*.

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6 CONFLICTING INTEREST

The authors declare no conflict of interest

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