Asian Journal of Agricultural Sciences 4(4): 242-248, 2012
ISSN: 2041-3890

Submitted: February 07, 2012 Accepted: April 14, 2012 Published: July 15, 2012

Introgression of *Helicoverpa armigera* Resistance from *Cajanus acutifolius*-a Wild Relative from Secondary Gene Pool of Pigeon Pea (*Cajanus cajan)*

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Abstract: The aim of the study was to introgress *Helicoverpa armigera* resistance from wild relative *Cajanus acutifolius* into pigeonpea, (*Cajanus cajan* L.), an important grain legume in South Asia, East Africa and the West Indies. Pigeonpea grain yields on farmer's fields are quite low, largely because of damage by insect pests, of which legume pod borer *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is the important pest worldwide. Pod borer has developed high levels of resistance to chemical insecticides. Currently, there are no cultivars of pigeonpea with high levels of resistance to *H. armigera*. Therefore, there is a need to identify and introgress resistance genes from the wild relatives of this crop. Wild relative of pigeonpea, *Cajanus acutifolius* (ICPW 15613) and the interspecific derivatives *C. acutifolius* x *C. cajan* have shown resistance to *H. armigera*. The results showed that all the test lines and *C. acutifolius* had high levels of flavonoids such as chlorogenic acid, quercetin and rutin in the flowers and buds, which may have resulted in less damage due to *H. armigera* larvae. Most of the test lines had more than 15.00 g of seed weight (100 seed weight) and beige seed color. These lines can be used for pigeonpea improvement for resistance to *H. armigera.*

Keywords: Chlorogenic acid, flavonoids, *Helicoverpa armigera*, high seed weight, pigeonpea, pod borer, quercetin, resistance mechanisms, rutin

INTRODUCTION

Pigeonpea [*Cajanus cajan* (L.) Millspaugh] is an important grain legume in the semi-arid tropics and subtropical areas of the world. Asia accounts for the 90% of the world production and an important grain legume in India (FAO, 2009). Although ample morphological diversity is exhibited by pigeonpea as a crop, the same is not true at the molecular level (Yang *et al*., 2006). Low molecular/genetic diversity has resulted in the crop being susceptible to a range of diseases and insect pests.

Pigeonpea grain yields on farmers' fields are quite low, largely because of damage by insect pests, of which legume pod borer, *Helicoverpa armigera* (Hübner), also known as cotton bollworm/tomato fruit-worm, is a major pest of grain legumes in general and of pigeonpea in particular. Annual yield losses due to this pest have been estimated to be over USD 2 billion worldwide (Sharma, 2005) and 300-400 million per annum in India alone, this is apart from the expenses spent on insecticides to control the insect pest (Shanower *et al*., 1999). It is difficult to control this insect as it is polyphagous and develops insecticide resistance, both in the larval and adult stages (Forrester *et al*., 1993). As a result, chemical control of *H. armigera* has become difficult due to the development of resistance to the commonly used insecticide to control it Armes *et al*. (1996) and Kranthi *et al*. (2002). Widespread and injudicious use of insecticides to control *H. armigera* has not only led to the development of insecticide-resistant populations, but has resulted in detrimental effects on the farming community and the environment.

Screening of more than 15,000 accessions of pigeonpea germplasm for resistance to *H. armigera* has revealed very low levels of resistance to this pest (Sharma, 2005). Development of crop cultivars resistant to this pest has a greater potential for integrated pest management, particularly under subsistence farming conditions in the developing countries (Fitt, 1989). Incidentally, the crop has a rich source of variability in the form of wild relatives, which have played a major role in the introduction of disease resistance, good agronomic traits such as high protein content and identification and diversification of cytoplasmic base of Cytoplasmic Male Sterile (CMS) system, to name a few (Mallikarjuna *et al*., 2011).

Some of the wild relatives of pigeonpea have shown high levels and biochemical components of resistance to *H. armigera* (Sharma *et al*., 2009). *Cajanus acutifolius* (F.v. Muell.) van der Maesen comb. nov., is one such wild relative in the secondary gene pool of pigeonpea and has

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shown resistance to the pest (Sujana *et al*., 2008). A major initiative was undertaken to introgress *H. armigera* resistance from *C. acutifolius* into cultivated pigeonpea. As a result advance generation lines with high levels of resistance to *H. armigera* are now available. The stability of resistance was tested by screening advance generation derivatives under unprotected field conditions for five consecutive years. In all the screenings, resistant selections were identified and re-screened the following year, discarding a small percentage of plants with low levels of resistance.

Together with *H. armigera* resistance, it was possible to identify lines with bold seeds with high seed weight in the early generations. Color of the seeds is an important trait while developing pigeonpea lines with *H. armigera* resistance and bold seeds. Seeds were selected which were light brown (beige) in color-the preferred color by the pigeonpea farmers. The main objective of this long term field evaluation was to identify and confirm resistance to *H. armigera* in the introgression lines derived from *C. acutifolius*.

MATERIALS AND METHODS

Glasshouse experiment: The experiment was carried out at International Crops Research Institute for Semi Arid Tropics (ICRISAT), India. During the rainy season of 2003, two pigeonpea cultivars (ICPL 85010 and ICPL 2) and the wild species *C. acutifolius* (ICP 15613) were grown in plastic pots (30 cm dia) and maintained in the glasshouse. Pots containing the black soil, sand and farm-yard manure (2:10:1) were steam sterilized. The temperature in the glasshouse was maintained by desert coolers @ 26±4ºC and 65% RH. Staggered sowings were made at fortnight intervals to synchronize flowering of both cultivated pigeonpea and wild relative *C. acutifolius*. Cultivated pigeonpea was used as the female parent and *C. acutifolius* as the pollen parent. Emasculations followed by pollinations were carried out in the morning and gibberellic acid (50 mg) was applied to the base of pollinated pistils to prevent pod-abscission and promote pod-formation from pollinated pistils. On maturity, the pods were collected, sun-dried and sown in pots. Introgression lines were developed by backcrossing the F_1 hybrid to cultivated pigeonpea parent used in the crossing program and selfing the progeny six times to obtain stable lines. The lines had uniform morphology and phenology.

Field trials: Field trials were carried out at International Crops Research Institute for Semi Arid Tropics (ICRISAT), India. Twenty-one lines derived from the crosses between pigeonpea cultivar ICPL 85010 and *C. acutifolius* along with parental material used as resistant and susceptible checks (ICPW 15613 and ICP 85010), respectively, were evaluated during the rainy seasons 2007-10 for resistance to pod damage by *H. armigera*. Seeds were sown in two replications in a randomized complete block design on the ridges 75 cm apart, each row 2 m long for each line/accession (comprising of 20 seeds), crop was thinned to a spacing of 30 cm between the plants after 21 days of seedling emergence. Standard agronomic practices were followed, with a basal fertilizer (N: P: K) application in the proportion of 100:60:40 kg/ha, which was applied in the furrows before planting. In addition, a basal dose of fungicide (metalaxyl 1.0 kg/ha) was also applied to control *Fusarium* wilt at the seedling stage. Subsequently, no other control measures were applied throughout the cropping season. The crop was planted in June at the start of the monsoon season and irrigated at regular intervals between December to mid-February. For the 21 lines derived from *C. acutifolius*, the annual yield data and pod damage (%) was recorded from 10 plants from each line including the susceptible and resistant checks and the three year means were calculated. Data were recorded at maturity on the number of pods/plant, 100-seed weight (g) and the number of healthy pods and pods damaged by *H. armigera*. Pod borer damage (%) was assessed by counting the total number of damaged pods from the total pods harvested at maturity. Selections were based on <10% pod damage and higher 100-seed weight in (g) compared to cultivated parent (ICPL 85010) and were re-screened in the following year.

HPLC analysis: HPLC analysis of flowers, buds, pods and seeds of both the parents and the test lines were performed at the Central Institute for Cotton Research, Nagpur, India, by employing the Shimadzu (Japan) liquid chromatograph system with a dual pump (LC-6A) binary system, UV detector (SPD 6AV), auto-injector (SIL-6A) with system controller. The compounds were separated at 254 nm on Phenomenex Luna RP, C18 column (4.6x250 mm, 4.5 μm particle) by using linear gradient of acetonitrile and water containing 1% acetic acid with a flow rate of 1 mL/min.

Data was integrated by C-R7A chromatography data station software and the results were obtained by comparison with standards. The mean values represent average of three replicates of each sample. All the samples and solutions were filtered through 0.45 μm nylon filters (millipore) before analysis by HPLC. The estimation of chlorogenic acid, quercetin and rutin was performed by comparing the retention time of analytes and reference compounds. The calibration curves were constructed for each flavonoid in the range of sample quantity and are presented in μg/mL of the extract.

Sample preparation:

Extraction of chlorogenic acid, quercetin and rutin from the buds, flowers, pods and seeds of pigeonpea: Samples were oven-dried for 60 min, before being powdered. Hundred milligrams of the powder was extracted in 1 mL of 90% methanol incubated overnight.

The extractions were repeated with hexane to get rid of waxes and chlorophyll. After centrifugation, the methanolic extract (supernatant) was concentrated to dryness on a water bath. The residue was re-dissolved in 100 µL methanol and the mixture was taken for HPLC analysis.

Standards: Chlorogenic acid, rutin, quercetins were purchased from Sigma-Aldrich chemical company, USA. Standard solutions were prepared by dissolving in HPLC grade methanol and stored at -20°C between analyses. These primary stock solutions were subsequently diluted to prepare solutions with concentrations in the range of sample quantity. The HPLC grade solvents were purchased from Fisher Scientific (USA).

Statistical analysis: The data were analyzed by Analysis of Variance (ANOVA) using SPSS (Version 15.1) and Tukey's test was used to separate the means, when the treatment effects were statistically significant ($p \leq 0.05$).

RESULTS

Pod borer damage: A t-test was done to find out the significance at 5% level to determine the pod damage stability across the three cropping seasons from 2007-2010. Over 1,200 *C. acutifolius* derived lines were evaluated for pod borer damage, which ranged from 0-60%. Around 85% of the lines suffered <10% pod damage, which was significantly lower as compared to the susceptible check, ICPL 85010 and the plants with >10% pod damage were not evaluated in the next season. During 2007, the selected lines showed a range of 1-12% pod damage, while in the subsequent 2008 and 2009 season, most of these lines showed 3.5-6.5% pod damage. Pod damage in the susceptible check, ICPL 85010 was significantly higher (35-54%) than the interspecific derivatives in all the years. Pod borer damage during 2010 was low in most of the lines, including the susceptible check

Seed weight in (g): Selections were made based on high 100-seed weight in BC_1F_3 test lines, which consistently showed more than 15.0 g per 100 seeds, except for two lines, which had less than 11.0 g. The male parent ICP 15613 (wild relative; *C. acutifolius*) had a seed weight of 3.00 g (per 100 seed weight) and susceptible female parent cultivar ICPL 85010 had 10.05 g weight per 100 seeds. Seeds were round-pearly in shape and beige colored, except for one line with white seed color. Incidentally, all the high seed weight lines showed resistance to *H. armigera*, two lines with less than 15 g seed weight also showed *H. armigera* resistance (Table 1).

Flavonoids: The buds contained 1.70 ug/mL chlorogenic acid in the test lines as compared to 3.90 ug/mL in *C. acutifolius*. There were lower amounts of chlorogenic

Table 1: *H. armigera* pod damage between 2007-10 with 100 seed wt (g)

		Pod damage (%)		Pod damage (%)		Pod damages(%)		100 seed	
S. No:	Test lines	2007-08		2008-09		2009-10		wt (g)	
1	7018-26-8-1-9-1	5.50	fgh	2.79	fg	0.80	e	20.41	a
2	7018-26-9-2-9-1	6.52	fg	3.02	fg	0.22	e	20.00	ab
3	7018-39-12-5-5-4-1	7.94	cdef	4.53	def	0.60	e	19.35	abc
4	7018-40-1-1-9-16-1	10.62	bc	2.88	fg	0.35	e	18.40	bc
5	7018-40-2-1-10-7-1	10.81	b	5.13	def	0.91	e	18.15	c
6	7018-40-2-1-15-1	10.00	bcd	9.76	bc	7.05	cd	17.92	cd
7	7018-40-2-1-17-1-1	9.81	bcde	9.35	bc	0.74	e	17.85	cd
8	7018-40-2-2-8-1-1	6.00	fg	6.13	cdef	0.31	e	17.60	cd
9	7018-40-2-2-8-16-1	6.00	fg	7.53	bcd	1.72	e	17.55	cd
10	7018-40-2-2-10-1-1	6.00	fg	5.69	def	0.31	e	17.50	cd
11	7018-40-2-4-9-1-1	3.00	hi	5.50	def	2.87	de	17.25	cde
12	7018-40-26-2-19-1-1	7.27	defg	4.56	def	0.71	e	16.75	cdef
13	7018-40-26-2-19-16-1	1.31	i	10.11	b	9.94	bc	16.75	cdef
14	7018-40-26-6-9-16-1	7.27	defg	3.82	efg	1.00	e	16.35	def
15	7018-40-26-6-14-10-1	5.06	gh	0.71	g	3.58	de	16.30	def
16	7018-40-26-6-15-17-1	11.29	b	6.78	bcde	10.66	bc	16.25	def
17	7018-40-26-6-15-18-1	7.39	defg	6.78	bcde	0.50	e	15.75	ef
18	7018-40-26-6-18-9-1	7.25	efg	4.55	def	0.91	e	15.61	ef
19	7018-40-26-7-7-11-1	5.50	fgh	4.32	defg	0.31	e	15.42	f
20	7018-40-26-6-16-8-1	6.13	fg	4.03	defg	0.41	e	10.65	g
21	7038-12-21-3-3-11-1	6.11	fg	2.91	fg	12.57	b	10.50	g
Control (R = resistant)	ICP 15613 (R)	11.55	b	7.54	bcd	1.55	e	3.00	g
Control (S = susceptible)	ICP 85010 (S)	35.00	a	44.00	a	54.00	a	10.05	h
	Mean	8.41		7.06		4.87		15.88	
	LSD (0.01)	2.74		3.63		4.89		1.69	

Means within a column with same letter(s) are not significantly different at 0.05%

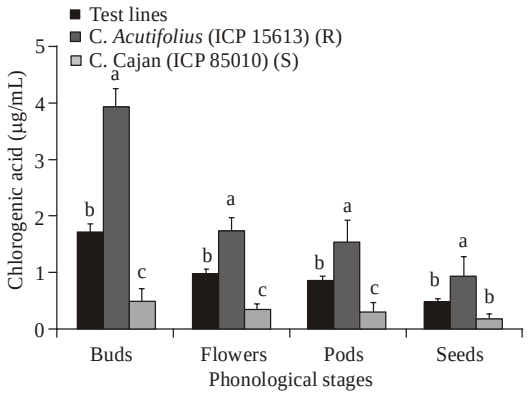

Fig. 1: Chlorogenic acid content at different phenological stages in test lines and resistant check (ICP 15613) of *C. acutifolius* and susceptible check (ICP 85010) of *C. cajan*. Bars (Mean±SEM) with same letter(s) are not significantly different by Tukey's test ($p \leq 0.05$)

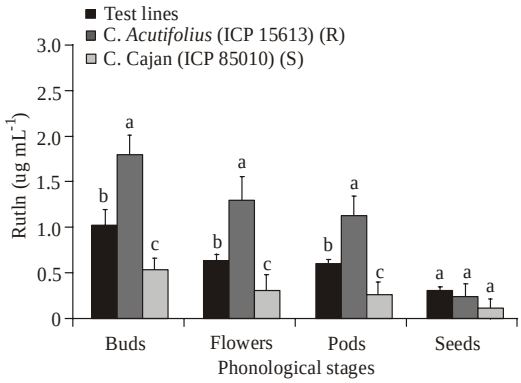

Fig. 2: Rutin content at phenological stages in test lines and resistant check (ICP 15613) of *C. acutifolius* and susceptible check (ICPL 85010) of *C. cajan*. Bars (Mean±SEM) with same letter(s) are not significantly different by Tukey's test ($p \leq 0.05$)

acid in the flowers of test lines (0.98 ug/mL) as compared to the wild parent, *C. acutifolius* (1.74 ug/mL) and least in ICPL 85010 flowers (0.33 ug/mL). The chlorogenic acid content was high in the pods of *C. acutifolius* (1.65 ug/mL), followed by the test lines (0.86 ug/mL) and least amount in ICPL 85010 (0.30 ug/mL). However, the seeds contained the lowest concentration of chlorogenic acid (0.95 ug/mL) in *C. acutifolius*, followed by the test lines (0.48 ug/mL) and least amount in ICPL 85010 (0.15 ug/mL) (Fig. 1).

Maximum rutin content of 1.75 ug/mL was recorded in the buds of *C. acutifolius* compared to 0.51 ug/mL in ICPL 85010. The test lines showed 1.0 ug/mL of rutin in the buds. *C. acutifolius* had 1.30 ug/mL of rutin in the flowers compared to 0.30 ug/mL in ICPL 85010. The test lines had intermediate amount of 0.63 ug/mL of rutin in the flowers. Pods of *C. acutifolius* had 1.10 ug/mL of

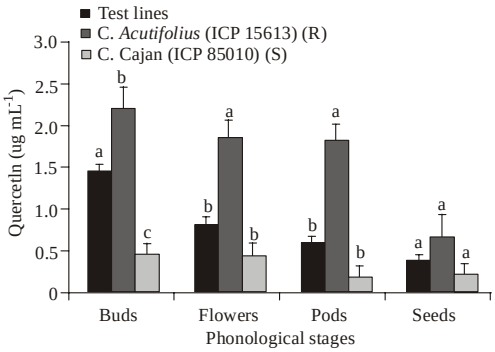

Fig. 3: Quercetin content at different phenological stages in test lines and resistant check (ICP 15613) of *C. acutifolius* and susceptible check (ICP 85010) of *C. cajan*. Bars (Mean±SEM) with same letter(s) are not significantly different by Tukey's test ($p \leq 0.05$)

rutin, test lines had 0.60 ug/mL of rutin and ICPL 85010 had 0.25 ug/mL of rutin in the pods. Least quantity of rutin was present in the seeds and maximum rutin was observed in *C. acutifolius* and minimum in ICPL 85010, with intermediate levels in test lines (Fig. 2).

The concentration of quercetin was higher in buds in general, followed by flowers, pods and seeds. There was 2.15, 1.81, 1.77 and 0.65 ug/mL of quercetin in buds, flowers, pods and seeds of *C. acutifolius*, respectively. In ICPL 85010, the quercetin content was 0.44 ug/mL in the buds and flowers and 0.20 ug/mL and in the pods and seeds. The test lines showed a maximum of 1.40 ug/mL in the buds, 0.80 ug/mL in the flowers and 0.59 ug/mL in the pods and 0.37 ug/mL in the seeds (Fig. 3).

DISCUSSION

With the identification of higher quantities of chlorogenic acid, quercetin and rutin in the resistant parent, *C. acutifolius* and their minimal quantities in the susceptible pigeonpea cultivar, ICPL 85010 has shed new light on the components of resistance to *H. armigera* and the plants' defensive chemistry with higher quantities of flavonoids. Since the test lines were selected for resistance to *H. armigera* with minimal damage, they had intermediate levels of flavonoids between the resistant and susceptible parents. It is now known that resistance to insect pests in grain legumes, cotton, maize, rice and wheat is under polygenic control (Panda and Khush, 1995; Smith, 2005).

The HPLC analysis for the estimation of chlorogenic acid, quercetin and rutin contents indicated lower concentrations of these compounds in the cultivated pigeonpea as compared to the wild relative, *C. acutifolius* and its derivatives. The chlorogenic acid content varied not only between the cultivars and wild species, but also in different plant parts.

Although it is known that *C. acutifolius* has pod borer resistance, the present study clearly demonstrated that it is possible to introgress this trait into cultivated pigeonpea. The present study also indicated that it is advantageous to select for low damage in each evaluation to have high levels of resistance to *H. armigera*. *C. acutifolius* is endowed with many useful traits such as disease and pest resistance, cytoplasmic nuclear male sterilty (Mallikarjuna *et al*., 2011) and *H. armigera* resistance (Stevenson *et al*., 2005; Sharma *et al*., 2008; Kumari *et al*., 2010; Mallikarjuna *et al*., 2011).

Flavonoids chlorogenic acid, quercetin and rutin were selected as candidates for *H. armigera* resistance based on the report by Stevenson *et al*. (1993a, b), Tomczyk and Gudej (2003) and Niranjaan and Tewari (2008). These three flavonoids act as deterrents to another lepidopteran insect, *Spodoptera litura* F., which is an insect pest on groundnut. It was possible to introgress resistance to *S. litura* from the wild relative of groundnut, *Arachis kempffmercadoi*, which had the flavonoids chlorogenic acid, quercetin and rutin in larger quantities than the susceptible control (Mallikarjuna *et al*., 2004; Treutter, 2006). Simmonds and Stevenson (2001) also found flavonoids to be effective against *H. armigera* in *Cicer* spp. The bioassays/feeding experiments with *H. armigera* by feeding different concentrations of pure chlorogenic acid, quercetin and rutin and found that the above mentioned flavonoids had deterrent effect on both *S. litura* and *H. armigera* (Jadhav D, unpublished data).

HPLC analysis showed that maximum amounts of flavonoids were present in the buds, followed by flowers, pods; and least amount in seeds. The *H. armigera* females lay eggs on the buds, which hatch and devour the flowers and then bore into the pods and eat the seeds. The rationale behind the presence of maximum amounts of flavonoids (chlorogenic acid, quercetin and rutin) in the buds of resistant species, *C. acutifolius* and the test lines is well placed as buds are the first organs to come in contact with the insect and act as a deterrent for the insect to lay eggs on the buds.

Swathi *et al*. (2011) identified the presence of trypsin inhibitors conferring resistance to *H. armigera* in another wild relative, *Cajanus platycarpus*. This opens up new avenues to look for components of *H. armigera* resistance in the form of flavonoids mentioned in the present investigation and the trypsin inhibitors which Swathi *et al*. (2011) have reported.

Wild relative in the compatible gene pool of pigeonpea namely, *C. acutiofolius* showed higher levels of resistance to *H. armigera* than the cultivated germplasm, which can be introgressed through sexual hybridization. Crossability with *C. autifolius* is successful as a one way cross when used as a male parent than when used as a female parent (Mallikarjuna and Saxena, 2002).

The aims of the present experiment to introgress *H. armigera* resistance from *C. acutifolius* into the cultigen and develop pre-breeding lines for use in pigeonpea improvement was successfully achieved. Studies in 2010 showed that some of the lines with *H. armigera* resistance also had Fusarium wilt (Patancheru isolate) and/or sterility mosaic (Patancheru isolate) disease resistance. This is an added advantage of utilizing wild species to transfer multiple pest resistance into the cultivated germplasm. As a spillover, lines with *H. armigera* resistance showed high seed weight, a desirable character in pigeonpea breeding. High seed weight was consistently recorded across three seasons. Based on this observation it can be concluded that seed size may be a recessive trait. This is in consistence with the observation of Singh and Pandey (1974) who have reported that small seed size is dominant over large seed size. Large seed size may be due mutational event changing the dominant small seed size into recessive large seed size (Saxena, 2008; Saxena *et al*., 2011).

Utilization of wild relatives for wheat and rice improvement has yielded lines with low disease incidence and high yield. Experience of utilizing wild reltives of pigeonpea has been promising with the development of cytoplasmic male sterile lines (Saxena *et al*., 2010a) and lines with high protein content have also been obtained (Saxena *et al*., 2010b). The research program to introgress pod borer resistance from *C. acutifolius* into the cultigen has been rewarding.

Pod borer is a major biotic constraint of pigeonpea with low levels of resistance in cultivated germplasm, which completely succumb to the pest under high insect pressure. The development of pod borer resistant lines has opened up new vistas in pigeonpea improvement program. Development of pod borer resistant lines will have a major impact on the pigeonpea producers as they need not depend heavily on synthetic chemicals to control this insect and thus, saving farmer's resources and protecting the environment.

Many of the lines with high seed weight had beige color, a favorable seed color in pigeonpea, preferred by farmers in India and Africa. In Africa, farmers prefer medium duration pigeonpea lines with high seed weight, round shape and beige seed color (Ranga Rao G.V., personal communication)

It was interesting to note that some of the lines with low pod borer damage, high seed weight and beige seed color had resistance to Fusarium wilt and sterility mosaic disease. These lines can be used for pigeonpea improvement in future.

CONCLUSION

C. acutifolius, a wild realtive from the secondary gene pool of pigeonpea, is a good source of *H. armigera* resistance which can be introgressed successfully. Stable lines derived fropm *C. acutifolius* showed high level of resistance to the insect with majority of the lines showing

higher 100 seed weight. Most of the lines had beige seed coat color. All the lines with *H. armigera* resistance had higher levels of chlorogenic acid, quercetin and rutin (flavonoids) in their buds, as seen in pollen parent *C. acutifolius* when compared to the buds of cultivated parent ICPL 85010. In general the flavonoids were in higher quantity in the buds followed by flowers, pods and least amount in the seeds. *C. acutifolius* had the maximum quantity of flavonoids flowed by the hybrid lines with *H. armigera* resistance. Least amount of flavonoids was present in cultivated pigeonpea ICPL 85010. The report concludes that pre-breeding for *H. armigera* is successful in pigeonpea.

ACKNOWLEDGMENT

The authors thank Mr. Srinivasu Kurella of Resilient Dryland Systems and the support staff of Legume Cell Biology Unit of Grain Legumes Program, ICRISAT, for technical assistance. We wish to thank Dr. CLL. Gowda, Program Director, Grain Legumes, ICRISAT, for the facilities to carry out the experimental study.

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