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Research Article

Protease inhibitors of *Cajanus* conferring resistance to pod borer of pigeonpea (*Cajanus cajan* L. Millsp.)

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Abstract
Pigeonpea is susceptible to pod borer damage with resistance lacking in its primary gene pool. Many *Cajanus* species harbor high levels of resistance. Host plant resistance can play an important role in minimizing the extent of losses due to insects and pests as well as the use of insecticides/pesticides and thus protect the environment. A major initiative was undertaken to tap the defence genes from wild relatives of secondary and tertiary gene pool through wide hybridization and thereby introgress resistance to pod borer. A range of interspecific derivatives derived from *C. lanceolatus, C. cajanifolius, C. volubilis* and *C. platycarpus* along with their parents were screened for the pod borer resistance under unprotected field conditions at ICRISAT, Patancheru, India. Biochemical basis of resistance was also identified by studying the levels of defence proteins active against bovine pancreatic trypsin, chymotrypsin and trypsin-like enzymes of *H. armigera* mid-gut proteases. Protease inhibitor profiles of parents and interspecific derivatives differed in terms of activity units, number and intensities of activity bands visualized on gelatin-PAGE. As the protease inhibitors are anti-nutritional factors, parents and interspecific derivatives, which resulted in high levels of *Helicoverpa* gut protease inhibitor (HGPI) units were screened for Human pancreatic trypsin inhibitor (HPTI) activity levels. Samples with high ratio of HGPI/HPTI represent less or no effect on human pancreatic trypsin and high effect on insect gut proteases.

Key words
Chymotrypsin, trypsin, *Helicoverpa armigera*, human pancreatic trypsin, midgut trypsin-like proteases, pigeonpea, pod borer, protease inhibitors

Introduction

Pigeonpea [*Cajanus cajan* (L.) Millspaugh], also known as red gram, is the sixth most important grain legume crop grown in the semi-arid tropics of Asia, Africa and the Caribbean under a wide variety of cropping systems (Mula and Saxena, 2010). It occupies an important position as a protein source in the vegetarian diet. The most important constraint of pigeonpea is *Helicoverpa armigera* (pod borer) and it causes heavy losses every year. The current pest control measures are not high enough to protect pigeonpea from such a voracious feeder. Chemical controls with insecticides are costly and not environmentally friendly, in addition, insects develop high levels of resistance to conventional insecticides. Therefore, there is a need to focus on more sustainable, cost effective and environmentally friendly methods of pest control. In this context host plant resistance can play an important role in minimizing the extent of losses due to this pest. The levels of resistance to this pest in the cultivated germplasm are low to moderate, but the wild relatives of pigeonpea have shown high levels of resistance to this pest (Sujana *et al.* 2008, Sharma *et al*. 2009). Transfer of insect resistance genes from the wild into cultivated pigeonpea is now possible through wide hybridization (Jadhav *et al.* 2012).

Pigeonpea seeds contain proteinaceous inhibitors (PIs) of trypsin, chymotrypsin, and amylases as well as phytolectins, and secondary metabolites, which serve as a defense mechanism against the herbivores (Chougule *et al.* 2004). Pigeonpea PIs are Bowman-Birk Inhibitors (BBI) type PIs, having inhibitory activity against bovine pancreatic trypsin and chymotrypsin (Prasad *et al.* 2009). However, *H. armigera* has developed the ability to overcome the effect of host plant PIs either by producing a different suite of proteases or over-production of certain proteases to overcome the adverse effects of host plant PIs (Ranjekar *et al.* 2003). Nevertheless, PIs from the non-host plants have also been found to be effective against this pest (Parde *et al*. 2012). PIs from the wild relatives of pigeonpea, that have shown high levels of resistance to this pest, will be more effective as inhibitors of proteases in the insect gut (Swathi *et al.* 2014). Thus the present study was undertaken to evaluate the potential introgression of PIs from the wild relatives of pigeonpea to their interspecific derivatives to confer biochemical based resistance and field screening for pod borer damage under natural pest infestation conditions.

Materials and methods

Crosses were made between *C. cajan* and four wild relatives of pigeonpea from secondary (*C. cajanifolius* and *C. lanceolatus*) and tertiary (*C. platycarpus* and *C. volubilis*) gene pools. This resulted in a wide range of interspecific derivatives derived from the four wide crosses such as, I. *C.*

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cajan (ICPL 87119) x *C. cajanifolius* (ICP 29), II. *C. cajan* (ICPL 85010) x *C. lanceolatus* (ICPW 15639), III. *C. platycarpus* (ICPW 68) x *C. cajan* (ICPL 85010) and IV. *C. cajan* (ICPL 85010) x *C. volubilis* (ICPW 15774) at Legume Cell Biology Unit, Grain Legumes Program, ICRISAT.

Seeds of wild relatives of pigeonpea and their derivatives: Mature, dry seeds of four wild relatives of pigeonpea (ICPW 29, ICPW 15639, ICPW 68 and ICPW 15774), 12 inter specific derivatives from the cross I (*C. cajan* (ICPL 87119) x *C. cajanifolius* (ICP 29): 18-5, 37-1, 76-2, 82-2, 113-2, 129-1, 130-1, 131-1, 132-1, 133-1), ICPL 87119 X ICPW 29, 3-1-1); 12 from cross II (ICPL 85010 x *C. lanceolatus* (ICPW 15639): P-1, P-2, P-3 P-4, P-6 , P-7 P-10 , P-11, P-13 , P-14); 11 from cross III (*C. platycarpus* (ICPW 68) x *C. cajan* (ICPL 85010): A6-1,A6-12, B3-2 , B-6, C3-13, C7-13, C11-3, D1-10-1, D2-1, E5-8-1, E8-2) and one from cross IV (*C. cajan* (ICPL 85010) x *C. volubilis* (ICPW 15774)) were evaluated for TI, CI and PI activity against the larval mid gut proteases of pod borer, *H. armigera*, along with two genotypes of cultivated pigeonpea, *Cajanus cajan* (ICPL 87119 and ICPL 85010-susceptible parents as checks).

Proteases and Substrates: Bovine pancreatic trypsin and bovine pancreatic α-chymotrypsin were procured from Sisco Research Laboratory, Mumbai, India. *N*-α-benzoyl-DLarginine-*p*-nitroanilide (BAPNA), N-Glutaryl-L-phenyalanine p-nitroanilide (GLUPHEPA) and Human Pancreatic trypsin were purchased from Sigma Aldrich (St. Louis, MO).

Experiments to determine the potential PIs against H. armigera gut proteases: Extraction of crude protein from seeds: Crude proteins were extracted from decorticated mature, dry seeds of both parents and wide derivatives of pigeonpea according to the procedure described by Prasad *et al.* (2009). The protein content was estimated by the Folin-Ciocalteau method using bovine serum albumin as a standard (Lowry *et al.* 1951).

Extraction of Larval midgut proteases: Fourth to fifth instar larvae of *H. armigera* were collected from the pigeonpea fields of ICRISAT and were narcotized on ice for 15 min and dissected in an insect ringer solution (0.13M NaCl, 0.5M KCl, 0.1 mM $CaCl_2$ and 1mM Phenylmethylsulphonyl fluoride). The midgut was removed and placed on iso-osmotic saline (0.15 M NaCl) solution. Gut tissue was homogenized in 0.15 M NaCl and centrifuged twice at 12,000 rpm for 10 min at 4^{o}C. The supernatant was collected and stored frozen at -20^{o}C for further *in vitro* assays.

Assay of protease inhibitors: Trypsin or chymotrypsin inhibitory activity was determined by using appropriate volumes of crude extracts that resulted in 40-60% decrease in corresponding enzyme activity. Assay mixture (1.0 ml) consisted of PIs in assay buffer, 50 mM Tris-HCl, containing 20 mM $CaCl_2$ either at pH 8.2 for trypsin or pH 7.8 for chymotrypsin. 10 µg of trypsin or 80 µg of chymotrypsin was added to the assay mixture and incubated for 15 min at 37^{o}C. Residual protease activity in the above assay mixture was determined after incubating for 45 min at 37^{o}C using 1 mMBAPNA (1.0 ml) as a substrate (Erlanger *et al.* 1961) and 1 mMGLUPHEPA (1.0 ml) as a substrate for chymotrypsin (Mueller and Weder, 1989). The reaction was terminated by adding 0.2 ml of 30% acetic acid. The activity of PIs was expressed as trypsin inhibitor (TI) units/mg protein or chymotrypsin inhibitor (CI) units/mg protein. The molar extinction coefficient (M^{-1} cm^{-1}) for p-nitroanilide at 410 nm is equivalent to 8,800. One PI unit was defined as the amount of inhibitor required to inhibit 50% of the corresponding enzyme (trypsin/chymotrypsin/HGP) activity under the optimal assay conditions.

The effect of PIs on midgut trypsin like proteases of H. armigera: The effect of PIs on the midgut trypsin-like proteases was determined by incubating them in assay buffer (0.5 ml), 50mM glycine-NaOH (pH 10.5) with midgut extract of *H. armigera* enriched in trypsin-like proteases. After incubation with 1 mM BAPNA at 37°C for 45 min, the reaction was stopped with 30% acetic acid (v/v), and absorbance at 410 nm was recorded. All the assays were carried out in triplicates along with appropriate controls.

Visualization of inhibitor profiles against trypsin, chymotrypsin and insect midgut trypsin-like proteases: Gelatin-PAGE was performed by the incorporation of gelatin (0.1%, wt:vol, to final concentration) into polyacrylamide gels at the time of casting as described by Felicoli *et al.* (1997). Electrophoresis was performed at 50 V in stacking gel and 100 V in resolving gel. Following electrophoresis, the gel was processed and analysed using the procedure reported by Prasad *et al.* (2009). After hydrolysis of gelatin, the gel was washed with distilled water to remove the excess enzymes and stained with 0.1% Coomassie Brilliant Blue (CBB) R250. The presence of inhibitor activity was identified by the appearance of dark blue bands in a clear background due to complex formation of the unhydrolysed gelatin with stain. Commercially available soybean trypsin chymotrypsin inhibitor (soybean BBI) was used as marker in gelatin-PAGE.

Effect of PIs on Human Pancreatic trypsin (HPT): The effect of PIs on Human pancreatic trypsin was measured by using the BAPNA as a substrate. The assay mixture (0.2ml) consisted of 0.56µg of HPT in 40µl of 0.05 M Tris-HCl (pH 8.2) containing

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0.02M $CaCl_2$ and 2mM BAPNA which gave 1.0 optical density at 37^0C for 45 min. The inhibitory activity of PIs on human pancreatic trypsin was determined by using different concentrations of crude extracts of seeds to the assay mixture (above) and O.D were recorded on plate reader (TECAN, Switzerland) at 410 nm.

Statistical Analysis: All the experiments were carried out three times each with three replications, and the mean ± SE was reported by using Sigma plot 12.0 (Systat Software Inc., San Jose, CA).

Field screening under natural infestation: The stability of resistance was tested by screening advanced generations of interspecific derivatives (Table 1) along with their susceptible parents (ICPL 85010 and ICPL 87119) under unprotected field conditions. Field trials were carried out at International Crops Research Institute for Semi Arid Tropics (ICRISAT), India. All parents and interspecific derivatives were isolated by netted field area to avoid any cross pollinations. 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 (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 tocontrol *Fusarium* wilt at the seedling stage. Subsequently, no other control measures were applied throughout the cropping season.

Results and discussion

Among all the interspecific derivatives derived from four different wild species, *C. platycarpus* x *C. cajan* derivatives showed higher level of TI units (22.12 to 113.84 TIU/ mg protein) compared to other interspecific derivatives derived from crosses *C. cajan* x *C. cajanifolius*, *C. cajan* x *C. lanceolatus* and *C. cajan* x *C. volubilis* which ranged from 22.5 to 81.7 TIU/ mg protein. Interspecific derivatives of the cross *C. cajan* x *C. cajanifolius* showed TI activity levels which ranged from 25.7 to 77.5 TIU (Fig 1A) and 0.79 to 1.19 CIU (Fig. 2A). Cultivar (ICPL87119) demonstrated littles higher 39.96 TIU compared to its wild partner ICPW 29 (25.71 TIU) in the cross 1. Few derivatives of this cross, 76-2 (32.5 TIU); 82-1 (44.37 TIU); 113-2 (41. 55 TIU); 129-1 (30. 76 TIU); 130-1 (37.3 TIU/mg proteins); 131-1 (35.13 TIU) and 3-1-1 (34.5 TIU) inherited TI activity levels more or less closer to cultivar parent (Fig 1A). Whereas, the derivatives 18-5 (26.1 TIU), 37-1(27.2 TIU) and 132-1 (28.5 TIU/mg proteins) showed TI activity levels closer to the *Cajanus* parent (ICPW 29-25.7TIU/mg proteins). Moreover two derivatives, (ICPL87119 x ICPW 29) F_1 (77.5 TIU and 1.1 CIU) and 131-1 (69.7TIU and 2.1 CIU) had exhibited two fold increased TI and CI activities compared with cultivated parent (ICPL 87119-0.76 CIU proteins) (Fig 1A and 1B). CI activity levels of these derivatives were more or less similar to the wild *Cajanus* parent (ICPW 29-1.0 CIU/mg proteins).

Trypsin inhibitory activity of F_1 hybrids (ICPL 85010 x ICPW 15639) ranged from 22.5 to 81.76 TIU (Fig 1B) and chymotrypsin inhibitory activity varied between 0.64 to 1.6 CIU (Fig 2B). The TI activity level of the cultivar ICPL85010 (22.6 TIU) was more than the *Cajanus* wild parent (ICP 15639-12.8 TIU). But all the derivatives of this cross exhibited 2 to 3 fold increase in TI activity when compared to cultivated parent. In two derivatives the TI activity was 60.9 TIU and 81.7 TIU which were 2.6 to 3.6 folds more than the activity levels seen in cultivated parent (Fig 1B). CI activity levels of the cultivated (ICPL 85010-0.57 CIU) and wild (ICP 15639-0.6 CIU) parents were similar. Except two interspecific derivatives P13 and P14 which exhibited CI activity comparable to their parents. All other derivatives exhibited >1.5 fold increase in CI activity when compared to their parents (Fig 2B).

Interspecific derivatives derived from the cross ICPW68 (*C. platycarpus*) x ICPL 85010 showed highest trypsin inhibitor activity (Fig 1C) against bovine pancreatic trypsin and moderate chymotrypsin inhibitory activity. Cultivated and wild parent showed showed 0.57 and 1.1 CIU respectively, whereas one of their derivatives B3-2 exhibited highest 3.04 CIU and remaining all ranged between 0.7 to 2.16 CIU/ mg protein (Fig 2C). Both the parents (ICPL 85010-22.6 TIU and ICPW 68- 22.7 TIU) of this cross and one interspecific derivative B3-2 (22.1 TIU) showed similar TI activity levels, whereas the remaining interspecific derivatives exhibited 1.5 to 3.3 fold more inhibitory activity against bovine pancreatic trypsin compared to both the parents. However, C7-13 of this cross displayed 113.8 TIU which was the highest TI activity among all the parents and derivatives (Fig 2C). F_2 hybrid derived from the cross ICPL 85010 X ICP15774 showed 63 TIU (Fig 1D) which was >2 fold increase than the wild *Cajanus* species parent (ICP 15774- 29.4 TIU). The CI activity of the F_2 hybrid was 1.51 CIU similar to the wild parent (ICP 15774-1.67 CIU) (Fig 2D).

Wild relatives exhibited 14.5 to 76 fold higher inhibition of *H. armigera* mid gut trypsin-like (HGPI) activity compared to cultivated pigeonpea (3 units/ mg in ICPL 85010) (Fig 3). However, tertiary gene pool species *C. platycarpus* (228 units/mg protein) exhibited highest HGPI activity than the other wild species, *C. volubilis*-66.63 units

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/mg protein (Fig 3D), *C. cajanifolius*- 27.2 units/mg protein (Fig 3A) and *C. lanceolatus*-74.9 units/mg protein (Fig 3B). The interspecific derivatives showed 3 to 35.3 fold more HGPI activity when compared to cultivated pigeonpea and four wild relatives of pigeonpea. Among all the interspecific derivatives and cultivars, two derivatives (ICPW 68 x ICPL 85010) C7-13 (106.2 units/ mg protein) and ICPL 85010 x ICP 15639 P8 (77.5 units/ mg protein) showed highest HGPIactivity (Fig 3B and 3C). These results demonstrate that the two derivatives, from the cross ICPL 85010 x ICP 15639, showed 100% introgression of resistance genes from the wild parent as they exhibited equal inhibitory activity with *C. lanceolatus* (74.9 units/ mg protein) against *Helicoverpa* gut trypsin-like proteases.

Interspecific derivatives, which expressed greater TI, CI and HGPI activities were further confirmed through activity staining studies. Interspecific derivatives and wild species showed greater variation in TI, CI and HGPI activities which was evident through activity profiles (Fig 4). In gelatin-PAGE under non-denaturing condition, the cultivar ICPL 85010 and *C. cajanifolius* (ICPW29) exhibited homomorphism in terms of TI and CI isoforms (Fig 4A and 4B) when the gels were incubated with bovine pancreatic trypsin or chymotrypsin, respectively. However, other *Cajanus* species and interspecific derivatives exhibited variation in number of TI, CI and HGPI isoforms (Fig 4A, 4B and 4C) and their banding pattern. All the genotypes, including parents and their derivatives showed two distinct TI, two to five CI and three to five HGPI bands on gelatin-PAGE.However, the interspecific derivative C7-13 which is derived from *C. platycarpus* showed TI, CI and HGPI bands with higher intensity when compared with other genotypes.Thus, activity staining studies also revealed that PIs from wild parents and their derivatives had more potential to inhibit the activity of insect midgut trypsin-like proteases.

Even if it is a known fact that wild relatives of pigeonpea possess considerable insect resistance, the biochemical mechanism involved in the resistance has not been investigated. Few interspecific derivatives derived from the wild relatives (*C. cajanifolius, C. lanceolatus, C. platycarpus* and *C. volubilis*) of pigeonpea along with their cultivated parents (*C. cajan*- ICPL 85010 and ICPL87119) were screened for protease inhibitors (PIs) activity levels. The success has been achieved in obtaining the resistance to *H. armigera*. PIs were identified in interspecific derivatives and wild relatives of pigeonpea and showed higher inhibitory potential against bovine pancreatic trypsin, chymotrypsin and insect (*H. armigera*) trypsin-like proteases when compared to susceptible cultivated parents. In agreement with the previous reports (Prasad *et al.* 2009, Parde *et al.* 2012) wild *Cajanus* parents exhibited strong inhibitory potential against *H. armigera* mid gut trypsin-like proteases. Similar observation has been reported in chickpea, where high variation in PIs was recorded in the mature seeds of wild relatives than in the cultivated ones (Patankar *et al.* 1999). Swathi *et al.* 2014, identified the presence of trypsin inhibitors conferring resistance to *H. armigera* in *Cajanus platycarpus*. This opens up new avenues to look for components of *H. armigera* resistance, the trypsin inhibitors being reported in the present investigation as well as by Swathi *et al.* (2014).

The presence of higher TI and HGPI activites with parents and most of their derivatives warned to examine the inhibitory activity also against human pancreatic trypsin as pigeonpea is used for human consumption. Crude protein extracts of cultivars and wild parents along with their interspecific derivatives cv.ICPL 85010; *C. cajanifolius*; *C. lanceolatus*; *C. platycarpus*;*C. volubilis*, (ICPL 87119 X ICPW 29)133-1; (ICPL 87119 X ICPW29) F_1, ICPL (85010 X ICP 15639) P-1, P-8 , (ICPW 68 X ICPL 85010) C7-13, B6 instead of (ICPL 85010 X ICP 15774) HYB -3) which expressed high levels of HGPIs were screened for the human pancreatic trypsin inhibitor (HGTI) levels (Fig 5). Pigeonpea PIs (PPIs) showed the least inhibitory effect against human pancreatic trypsin when compared with the mid gut trypsin like proteases of Helicoverpa. HPT was weakly inhibited by crude extracts of pigeonpea PIs. HGPI inhibitor units (43.7 to 228 U/ mg protein) were more than the human pancreatic trypsin inhibitor (HPTI) units (11.1 to 53.13U/mg protein) in *Cajanus* species and interspecific derivatives.The HGPI to HPTI ratio of more than 1.0 indicate that there is less or no effect on the human pancreatic trypsin when compared to the effect on insect mid gut trypsin-like proteases.Since human pancreatic trypsin requires more amount of protease inhibitors (which expressed in units) for its inhibition when compared with the HGPIs.The PIs expressed in the interspecific derivatives can be ruined upon adequate heating for human consumption.

Interspecific derivatives were planted in the normal infested field along with their parents to evaluate further for *Helicoverpa armigera* resistance (Fig 6). Susceptible cultivars ICPL 87119 and ICPL 85010 showed 44.5% and 47.4% pod damage respectively. But most of the interspecific derivatives suffered less than 20% damage which was significantly lower as compared with susceptible cultivars (Table 1). Furthermore, in concurrence with the increase in HGPI activity, C7-13 (Fig 7a) showed only 2.9% pod damage with high yield (Fig 7b). The results indicate that the resistant interspecific derivatives were not being adversely affected by pod borer

Helicoverpa compared to susceptible parents, possibly due to introgression of defense genes from wild species.

As the trypsin inhibitors are known to cause reduced digestion and mainly are anti-nutritional and insecticidal, their presence in the pod from juvenile to mature stages could be an important component of the biochemical basis of resistance to podborer. The levels of these anti-nutritional factors have been determined in pigeonpea whole seed (Prasad *et al.*, 2009; Swathi *et al.*, 2014; Singh and Jambunathan, 1981). However, these inhibitors were thoroughly studied in soybean among all legume species and concluded as anti-nutritional and even their residual activities in processed human foods lead to concern for human health. Even though, it well known that pigeonpea PIs can be reduced by cooking, germination or fermentation, yet there are significant differences found in their electrophoretic forms in the wild and cultivated species. Nonetheless, these crude proteins were tested against human pancreatic trypsin to address human health concern and no significant inhibition against them was found. Surprisingly, the inhibition levels are more active against *Helicoverpa* mid gut trypsin-like enzymes when compared to human pancreatic trypsin which was evident through the enzymatic assays. This is because major protease inhibitors in the seed were found specific to mammalian serine proteases. The pod borer larval midgut has a serine type proteas, and these inhibitors are potent and could effectively inhibit the protease from insect gut.

The development of pod borer resistant lines has opened up new perspective in pigeonpea improvement program and these 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. The present study clearly demonstrated that it is possible to introgress pod borer resistance trait into cultivated pigeonpea and is advantageous to select for low damage varieties with high ratio of HGPI to HGTI, to gain high levels of resistance to *H. armigera*. Main objectives of the present experiment, to introgress *H. armigera* resistance from wild relatives into the cultivated pigeonpea, evaluate for biochemical basis of resistance and develop pre-breeding lines for pigeonpea improvement were successfully achieved.

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Table 1. Comparision of HGPI activity (fold increase) with pod damage by *H. armigera* (Percent of total yield) in the infested field

S. No.	Plant number	HGPI ± S.E (fold change)		Pod damage (%)
1	ICPL 87119	2.96	±0.0833 (1.0)	44.51
2	ICPW29	43.7	±0.548 (14.7)	3.0
3	(18-5) BC_1F_2	16	±0.178 (5.4)	8.11
4	(37-1) BC_1F_2	18	±0.293 (6.08)	9.67
5	(76-2) BC_1F_2	24	±0.0567 (8.1)	10.8
6	(82-2) BC_1F_2	20	±0.0306 (6.75)	8.96
7	(113-2) BC_1F_2	22	±0.177 (7.43)	6.53
8	(129-1) BC_1F_2	20	±0.05 (6.75)	4.35
9	(130-1) BC_1F_2	21	±0.08 (7.09)	4.5
10	(131-1) BC_1F_2	18	±0.123 (6.08)	7.05
11	(132-1) BC_1F_2	20	±0.113 (6.75)	5.3
12	(133-1) BC_1F_2	74.27	±0.389 (25)	3.18
13	ICPL87119xICPW29 F_1	46.53	±0 (15.7)	8.11
14	ICPL 85010	2.993	±0.00882 (1.0)	47.45
15	ICPW 15639	74.9	±0.16 (25)	0
16	P1 BC1F1	67.8	±0.217 (22.6)	3.95
17	P2 BC1F1	16	±0.106 (5.5)	7.32
18	P3 F2	11	±0.0926 (3.6)	6.42
19	P4 BC1F1	49	±0.573 (15.3)	1.89
20	P6 BC1F1	40.2	±0.0321 (13.4)	5.28
21	P7 BC1F1	20	±0.07 (6.68)	1.51
22	P8 F2	77.5	±0.0524 (25.9)	2.03
23	P10 BC1F1	40	±0.0869 (13.3)	4.2
24	P12 BC1 F1	43	±0.288 (14.8)	2.63
25	ICPL 85010	2.993	±0.00882 (1.0)	47.45
26	ICPW 68	228	±1.214 (76.25)	0
27	F1 BC6 A6-1	19	±0.234 (6.5)	4.16
28	F1 BC6 A6-12	14	±0.246 (4.8)	0
29	F1BC5B6	69.3	±0.547 (23.8)	3.84
30	F3BC3C7-13	106.2	±0.12 (36.55)	3.88
31	F3BC3C11-3	25	±0.0463 (8.3)	3.12
32	F1 BC6 D1-10-1	11	±0.0393 (3.7)	8.56
33	F3 BC3 D2-1	9	±0.118 (3.1)	11.1
34	F1 BC6 E5-8-1	11	±0.0133 (3.7)	6.52
35	F1 BC6 E8-2	11	±0.0437 (3.7)	4.04

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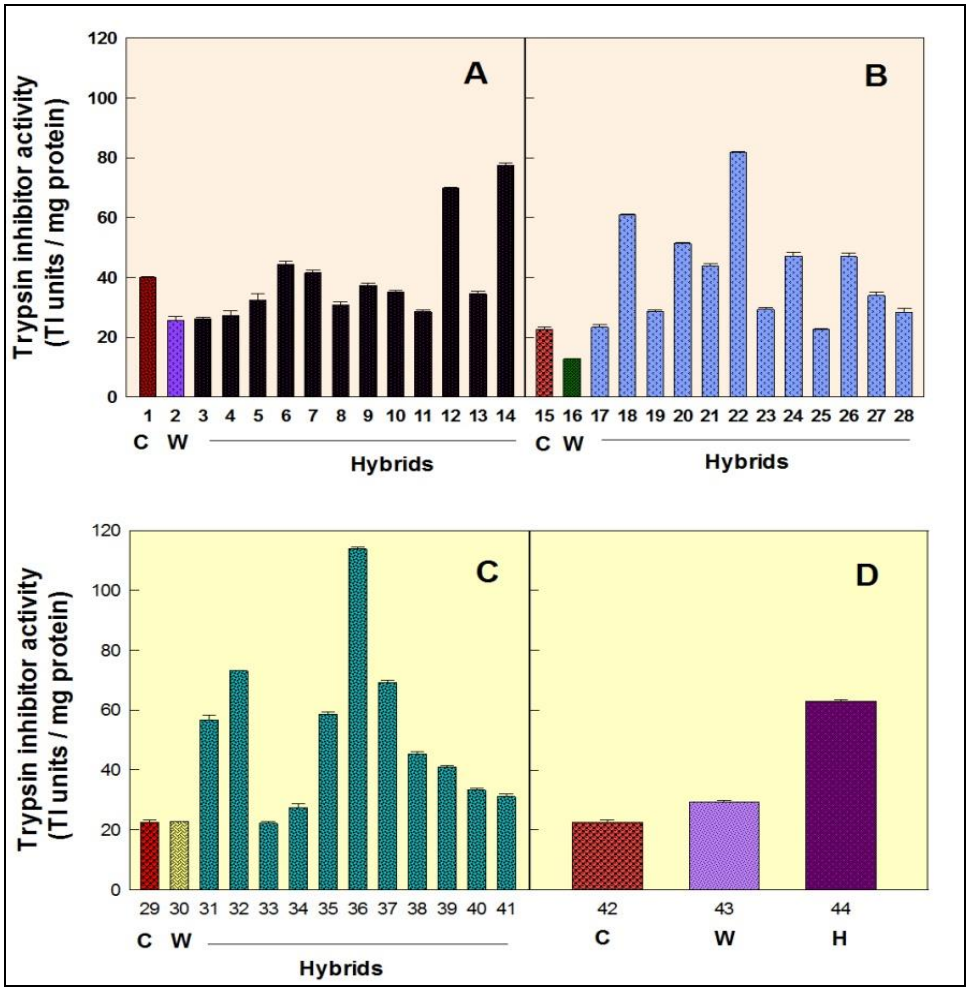

Fig. 1. Inhibition of bovine pancreatic trypsin by crude extracts of protease inhibitors from the parents (cultivated and wild) and their derivatives of pigeonpea

A. ***C. cajan*** **(ICPL 87119) x** ***C. cajanifolius*** **derivatives and their parents (1- pigeonpea; 2-*C. cajanifolius*; 3 to 14 - derivatives)**

1. ICPL 87119 (Asha) 2. ICPW 29 (*C. cajanifolius*) 3. (ICPL 87119 X ICPW 29)18-5 BC_1F_2 4. 37-1 BC_1F_2
5. 76-2 BC_1F_2 6. 82-2 BC_1F_2 7. 113-2 BC_1F_2 8. 129-1 BC_1F_2
9. 130-1 BC_1F_2 10. 131-1 BC_1F_2 11. 132-1 BC_1F_2 12. 133-1 BC_1F_2
13. ICPL 87119 x ICPW 29 F_1 14. 3-1-1 F_5

B. ***C. cajan*** **(ICPL 85010) x** ***C. lanceolatus*** **derivatives and their parents (15- pigeonpea; 16-*C. lanceolatus*; 17 to 28 - derivatives)**

15. cv. ICPL 85010 16. ICP 15639 (*C. lanceolatus*) 17. (ICPL 85010 X ICP 15639)P-1 BC_1F_1 18. P-2 BC_1F_1,
19. P-3 F_2 20. P-4 BC_1F_1 21. P-6 BC_1F_1 22. P-7 BC_1F_1
23. P-8 F_2 24. P-10 BC_1F_1 25. P-11 F_2 26 P-12 BC_1F_1
27. P-13 F_2 28. P-14 F_2

C. ***Cajanus platycarpus*** **x** ***Cajan cajan*** **(ICPL 85010) derivatives and their parents (29-pigeonpea; 30-*C. platycarpus*; 29 to 41 - derivatives)**

29. cv.ICPL 85010 30. ICPW 68 (*C. platycarpus*) 31. (ICPW 68 X ICPL 85010)A6-1 F_1BC_6 32. A6-12 F_1BC_6
33. B3-2 F_1 BC_5 34. B-6 F_1 BC_5 35. C3-13 F_3 BC_3 36. C7-13 F_3 BC_3
37. C11-3 F_3 BC_3 38. D1-10-1 F_1 BC_6 39. D2-1 F_3 BC_3 40. E5-8-1 F_1 BC_5
41. E8-2 F_1BC

D. ***C. cajan*** **(ICPL 85010) x** ***C. volubilis*** **derivative and its parents**

42 cv.ICPL 85010 43 ICP 15774 (*C. volubilis*) 44 (ICPL 85010 X ICP 15774) HYB -3 F_2

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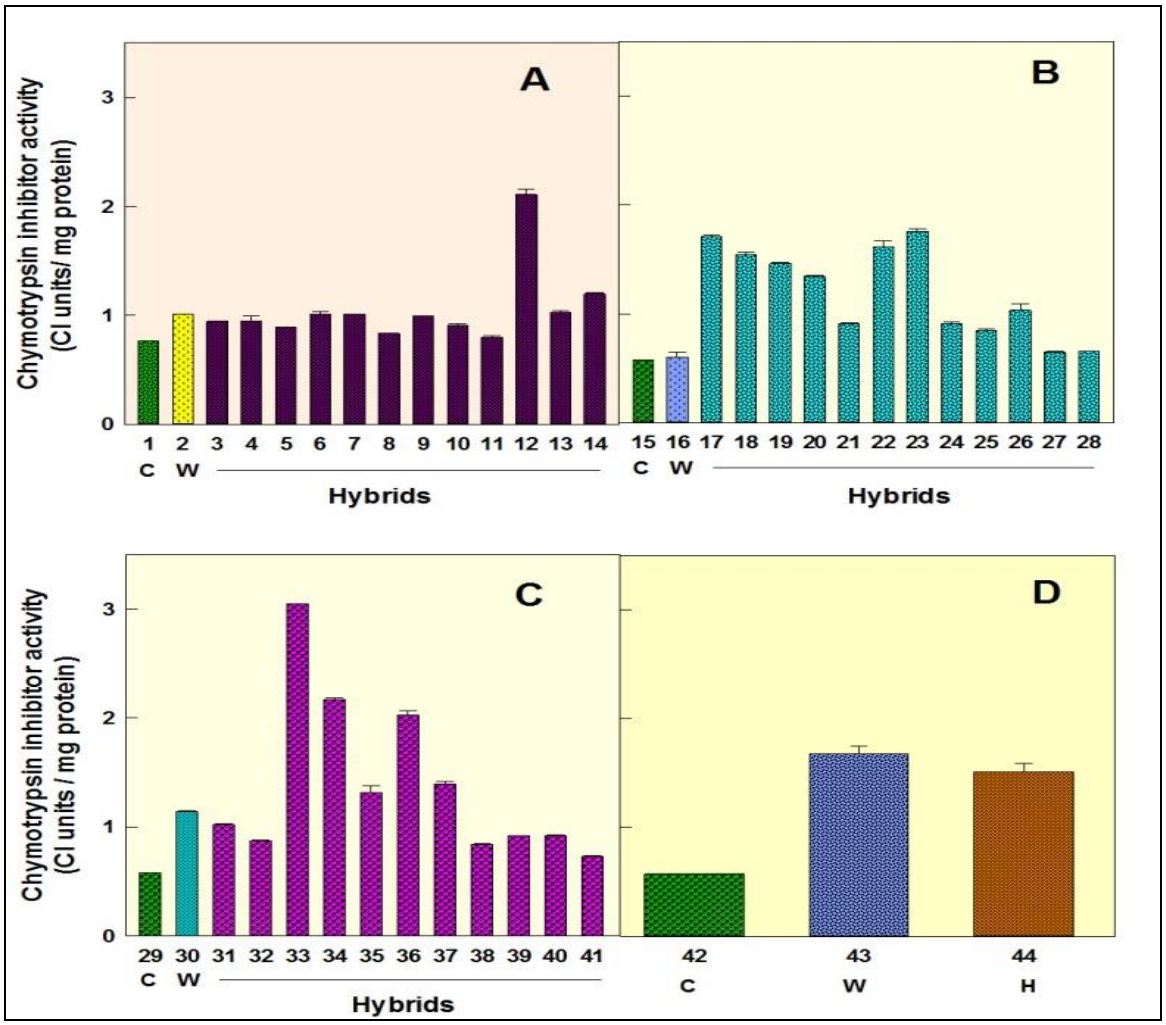

Fig. 2. Inhibition of bovine pancreatic chymotrypsin by crude extracts of protease inhibitors from the parents (cultivated and wild) and their derivatives of pigeonpea

***A. C. cajan* (ICPL 87119) x *C. cajanifolius* derivatives and their parents (1- pigeonpea; 2-*C. cajanifolius*; 3 to 14 - derivatives)**

1. ICPL 87119 (Asha) 2. ICPW 29 (*C. cajanifolius*) 3. (ICPL 87119 X ICPW 29)18-5 BC_1F_2 4. 37-1 BC_1F_2
5. 76-2 BC_1F_2 6. 82-2 BC_1F_2 7. 113-2 BC_1F_2 8. 129-1 BC_1F_2
9. 130-1 BC_1F_2 10. 131-1 BC_1F_2 11. 132-1 BC_1F_2 12. 133-1 BC_1F_2
13. ICPL 87119 x ICPW 29 F_1 14. 3-1-1 F_5

***B. C. cajan* (ICPL 85010) x *C. lanceolatus* derivatives and their parents (15- pigeonpea; 16-*C. lanceolatus*; 17 to 28 - derivatives)**

15. cv. ICPL 85010 16. ICP 15639 (*C. lanceolatus*) 17. (ICPL 85010 X ICP 15639)P-1 BC_1F_1 18. P-2 BC_1F_1,
19. P-3 F_2 20. P-4 BC_1F_1 21. P-6 BC_1F_1 22. P-7 BC_1F_1
23. P-8 F_2 24. P-10 BC_1F_1 25. P-11 F_2 26 P-12 BC_1F_1
27. P-13 F_2 28. P-14 F_2

***C. Cajanus platycarpus* x *Cajan cajan* (ICPL 85010) derivatives and their parents (29-pigeonpea; 30-*C. platycarpus*; 29 to 41 - derivatives)**

29. cv.ICPL 85010 30. ICPW 68 (*C. platycarpus*) 31. (ICPW 68 X ICPL 85010)A6-1 F_1BC_6 32. A6-12 F_1BC_6
33. B3-2 F_1BC_5 34. B-6 F_1BC_5 35. C3-13 F_3BC_3 36. C7-13 F_3BC_3
37. C11-3 F_3BC_3 38. D1-10-1 F_1BC_6 39. D2-1 F_3BC_3 40. E5-8-1 F_1BC_5
41. E8-2 F_1BC

***D. C. cajan* (ICPL 85010) x *C. volubilis* derivative and its parents**

42 cv.ICPL 85010 43 ICP 15774 (*C. volubilis*) 44 (ICPL 85010 X ICP 15774) HYB -3 F_2

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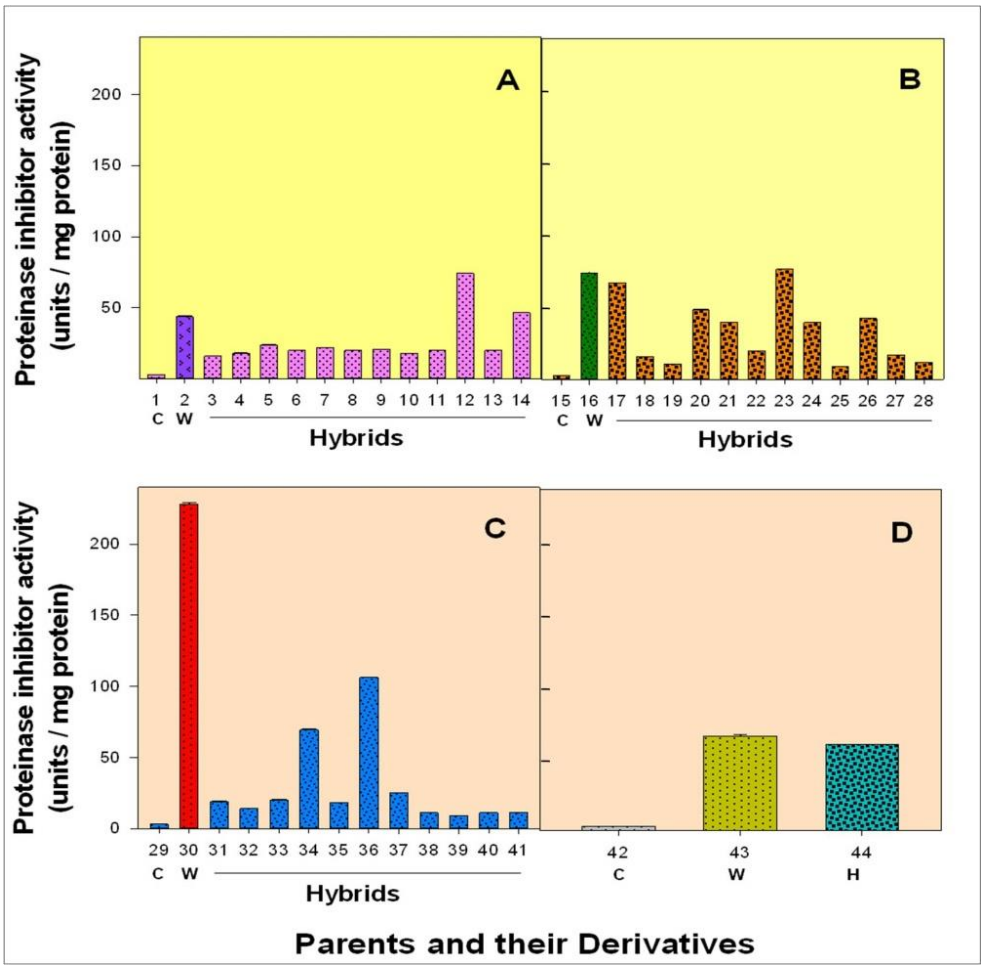

Fig. 3. Inhibition of midgut trypsin- like proteases of *Helicoverpa armigera* by crude extracts of protease inhibitors from parents (cultivated and wild) and their derivatives of pigeonpea. The results are shown as mean ± SE of three independent experiments done in triplicates

***A.* *C. cajan* (ICPL 87119) x *C. cajanifolius* derivatives and their parents (1- pigeonpea; 2-*C. cajanifolius*; 3 to 14 - derivatives)**

1. ICPL 87119 (Asha) | 2. ICPW 29 (*C. cajanifolius*) | 3. (ICPL 87119 X ICPW 29)18-5 BC_1F_2 | 4. 37-1 BC_1F_2
5. 76-2 BC_1F_2 | 6. 82-2 BC_1F_2 | 7. 113-2 BC_1F_2 | 8. 129-1 BC_1F_2
9. 130-1 BC_1F_2 | 10. 131-1 BC_1F_2 | 11. 132-1 BC_1F_2 | 12. 133-1 BC_1F_2
13. ICPL 87119 x ICPW 29 F_1 | 14. 3-1-1 F_5

***B.* *C. cajan* (ICPL 85010) x *C. lanceolatus* derivatives and their parents (15- pigeonpea; 16-*C. lanceolatus*; 17 to 28 - derivatives)**

15. cv. ICPL 85010 | 16. ICP 15639 (*C. lanceolatus*) | 17. (ICPL 85010 X ICP 15639)P-1 BC_1F_1 | 18. P-2 BC_1F_1,
19. P-3 F_2 | 20. P-4 BC_1F_1 | 21. P-6 BC_1F_1 | 22. P-7 BC_1F_1
23. P-8 F_2 | 24. P-10 BC_1F_1 | 25. P-11 F_2 | 26 P-12 BC_1F_1
27. P-13 F_2 | 28. P-14 F_2

***C.* *Cajanus platycarpus* x *Cajan cajan* (ICPL 85010) derivatives and their parents (29-pigeonpea; 30-*C. platycarpus*; 29 to 41 - derivatives)**

29. cv.ICPL 85010 | 30. ICPW 68 (*C. platycarpus*) | 31. (ICPW 68 X ICPL 85010)A6-1 F_1BC_6 | 32. A6-12 F_1BC_6
33. B3-2 F_1 BC_5 | 34. B-6 F_1 BC_5 | 35. C3-13 F_3 BC_3 | 36. C7-13 F_3 BC_3
37. C11-3 F_3 BC_3 | 38. D1-10-1 F_1 BC_6 | 39. D2-1 F_3 BC_3 | 40. E5-8-1 F_1 BC_5
41. E8-2 F_1BC

***D.* *C. cajan* (ICPL 85010) x *C. volubilis* derivative and its parents**

42 cv.ICPL 85010 | 43 ICP 15774 (*C. volubilis*) | 44 (ICPL 85010 X ICP 15774) HYB -3 F_2

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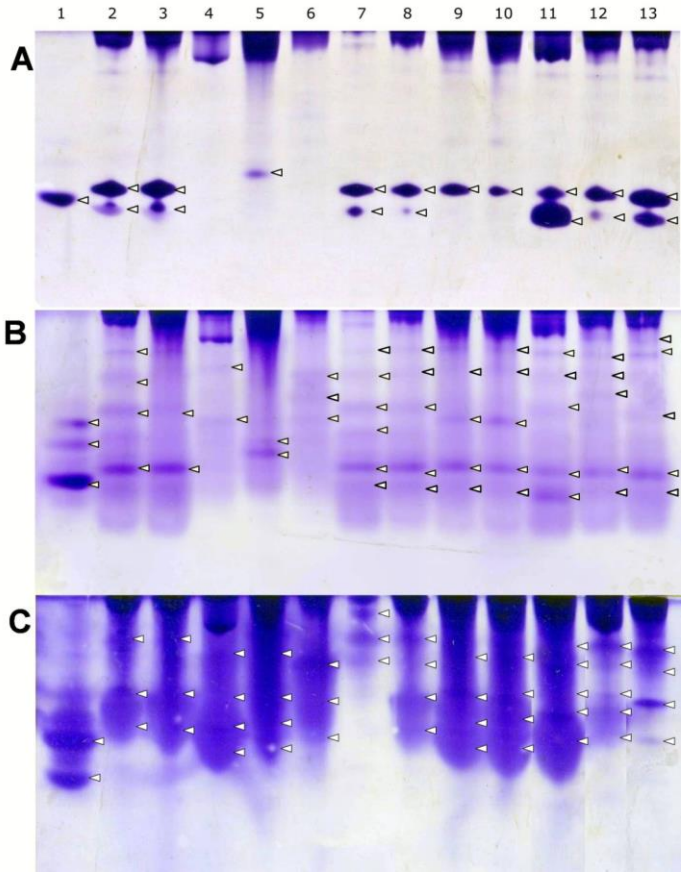

Fig. 4. Inhibition profiles of parents (wild and cultivated) and their derivatives of pigeonpea against pancreatic trypsin (A), chymotrypsin (B) and *Helicoverpa armigera* midgut trypsin-like proteases (C).

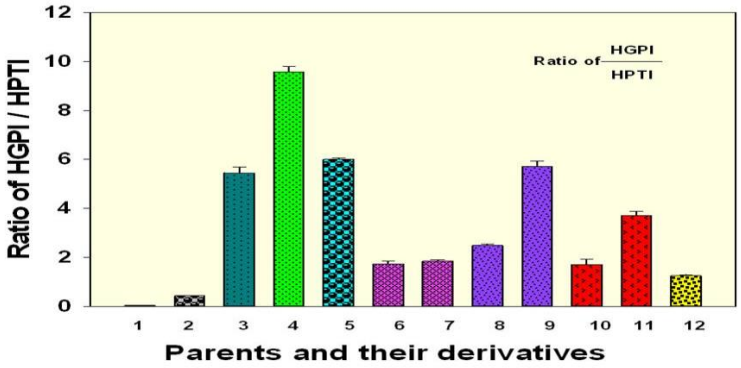

Fig. 5. Effect of protease inhibitors from parents (cultivated & wild) and their derivatives of pigeonpea on *Helicoverpa* midgut trypsin-like proteases and Human pancreatic trypsin as indicated by ratio of HGPI/HGTI

In lane 1: 9µg of soybean Bowman-Brick inhibitor (8 k Da) was loaded. Arrow heads indicate the number of inhibitor bands in each lane.; Lane 2 to 13: 2 ICPL 85010 (*C. cajan*). 3 ICPW 29 (*C. cajanifolius*). 4 ICP 15639 (*C. lanceolatus*). 5 ICPW 68 (*C. platycarpus*). 6 ICP 15774 (*C. volubilis*). 7 (ICPL 87119 X ICPW 29)133-1 BC_1F_2. 8 ICPL 87119 X ICPW29 F_1. 9 (ICPL 85010 X ICP 15639) P-1 F_1 BC_1. 10 P-8 F_2. 11 (ICPW 68 X ICPL 85010) C7-13 F_3 BC_3. 12 B-6 F_1 BC_5. 13 (ICPL 85010 X ICP 15774) HYB -3 F_2 respectively.

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Fig. 6. Interspecific derivatives at flowering stage in natural pod borer infested field of ICRISAT in the year 2012

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Fig. 7. *Cajanus platycarpus* derivative and its pods. a *Cajanus platycarpus* derivative F_3 BC_3C7-13 plant with healthy pods. b Healthy and damage pods from the same plant