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# *Cajanus cajan* (cv. C11): An Important Non-Host Resource for Proteinase Inhibitors Active against Larval Midgut Proteinases of Lepidopteran Insect Pest *Achaea janata*

**Marri Swathi[1] • Soundappan S. Mohanraj[1] • Chilka Srinivas[1] • Elaprolu R. Prasad[1] • Vantaku Venkatrao[2] • Nalini Mallikarjuna[3] • Aparna Dutta-Gupta[2] • Kollipara Padmasree[4*]**

[1] Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad-500 046, India
[2] Department of Animal Sciences, School of Life Sciences, University of Hyderabad, Hyderabad-500 046, India
[3] International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad-502 324, India
[4] Department of Biotechnology, School of Life Sciences, University of Hyderabad, Hyderabad-500 046, India

*Corresponding author*: * **kpssl@uohyd.ernet.in; padmasree_kollipara@yahoo.com**

## ABSTRACT

Our previous studies indicated that seeds of various cultivars and wild relatives of pigeonpea possessed proteinase inhibitors (PIs) which were active against midgut trypsin-like proteinases of lepidopteran insects. In the present study a detailed analysis of PIs distribution in mature seeds of five different cultivars of *Cajanus cajan* and thirteen different accessions of *Cajanus platycarpus* was carried out. Further the inhibitory potential of these PIs against larval midgut trypsin-like proteinases of *Achaea janata* was also evaluated. Among the chosen cultivars and *C. platycarpus* accessions, the cultivar 'ICP 7118' also called as 'C11' showed highest inhibitory potential against *A. janata* larval midgut trypsin-like proteinases (AjPs). The inhibitory potential of PIs from 'C11' was 25 and 106 fold higher against AjPs, compared to bovine pancreatic trypsin and chymotrypsin, respectively. Further, the activity profile of PIs of 'C11' on gelatin-polyacrylamide gel demonstrated the presence of higher number of trypsin inhibitor isoforms as well as stronger inhibitory potential against AjPs, when compared with *C. platycarpus* accessions. Thus, the results from the present investigation confirmed that the cultivar 'C11' of *C. cajan* is the best non-host resource for PIs active against AjPs and these PIs can be readily exploited further as ecofriendly pesticide in the management of this lepidopteran insect pest *A. janata* using transgenic technology.

**Keywords:** *Achaea janata*, gelatin-PAGE, gut proteinases, pigeonpea, proteinase inhibitor
**Abbreviations: AjP**, *Achaea janata* larval midgut trypsin-like proteinase; **AjPI unit**, *Achaea janata* larval midgut trypsin-like proteinase inhibitor unit; **BAPNA**, *N*-α-Benzoyl-DL-arginine-*p*-nitroanilide; **CI unit**, chymotrypsin inhibitor unit; **Gelatin-PAGE**, gelatine-incorporated polyacrylamide gel electrophoresis; **GLUPHEPA**, *N*-glutaryl-L-phenylalanine-*p*-nitroanilide; **PI**, proteinase inhibitor; **TI unit**, trypsin inhibitor unit

## INTRODUCTION

Plants synthesize certain biologically active substances, which play a major role in defense against insect pests and microbial attacks. Some of these include defense proteins like proteinase inhibitors (PIs), amylase inhibitors, lectins and class of pathogenesis related proteins (Garcia-Olmedo *et al.* 1987; Chrispeels and Raikhel 1991; Tatyana *et al.* 1998). Over a decade, PIs are the most exploited class of plant defense proteins which could be used to develop insect-resistant transgenic crop plants (Ryan 1990; Jouanin *et al.* 1998; Prasad *et al.* 2011).

Castor (*Ricinus communis* L.) is an important non-edible oilseed crop cultivated predominantly in India, China and Brazil. Castor oil and its derivatives were found to have more than 700 industrial uses. For example, they are mainly used in paints and varnishes for surface coatings, cosmetics, textile dyeing, leather industry and as nylon type synthetic polymers, resins, lubricants for aviation engines, insecticides, and a host of similar products (Ogunniyi 2006). Among different pests that attack the castor, *Achaea janata* (castor semi-looper) is a voracious feeder that crop up during early and late stages of castor growth causing about 30-50% crop defoliation.

The existence of trypsin and chymotrypsin inhibitors in several isoforms was reported in pigeonpea (Kollipara *et al.* 1994; Pichare and Kachole 1994, 1996). Further, several cultivars and wild relatives of pigeonpea were also found to possess PIs active against gut proteinases of *Helicoverpa armigera, A. janata* and *Spodoptera litura* (Chougule *et al.* 2003; Prasad *et al.* 2009). The red gram proteinase inhibitor (RgPI) purified from the cultivar 'ICPL-332 (Abhaya)' of *Cajanus cajan* possessed both trypsin and chymotrypsin inhibitory activity and was found to be stable in presence of larval midgut digestive proteinases of *A. janata* (Prasad *et al*. 2010). Thus, being aware of the significance of *C. cajan* PIs in inhibiting midgut proteinases of lepidopteran insects, the present study was intended to screen more cultivars (*C. cajan*) and wild accessions (*C. platycarpus*) of pigeonpea so as to identify the potential resource of PIs for the management of *A. janata*, which is a serious pest to castor but not of pigeonpea. Further, the present investigation also emphasizes the significance of the use of non-host PIs in combating insect pests.

## MATERIALS AND METHODS

### Plant material and insects

Mature dry seeds of thirteen *Cajanus platycarpus* accessions ICPW 60, ICPW 61, ICPW 62, ICPW 63, ICPW 64, ICPW 65, ICPW 66, ICPW 67, ICPW 68, ICPW 69, ICPW 70, ICPW 71, ICPW 72 and five cultivars of pigeonpea ICP 7118, ICP 7119, LRG 30, ICP 85063, ICP 87 were procured from the International

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Crops Research Institute for Semi Arid Tropics (ICRISAT), Patancheru, Hyderabad, India. The larvae of *A. janata* were maintained in an insect culture room on fresh castor leaves at 25 ± 1°C, 60 ± 5% relative humidity and 12-h photoperiod. Bovine pancreatic trypsin and chymotrypsin were procured from Sisco Research Laboratory (Mumbai, India). Substrates *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and *N*-glutaryl-L-phenylalanine-*p*-nitroanilide (GLUPHEPA) were purchased from Sigma (USA). All other chemicals and reagents were of analytical grade purchased from Fischer Scientific (India).

## Experiments to determine the potential PI's against *A. janata* gut proteinases

### *1. Extraction of crude protein from seeds*

Crude proteins were extracted from decorticated mature dry seeds of both cultivars and *C. platycarpus* accessions of pigeonpea according to the procedure described by Prasad *et al*. (2009). Mature dry decorticated seeds were ground to a fine powder, depigmented and defatted with several washes of acetone and hexane, respectively. After the solvents were removed, the air dried seed powder was extracted in 0.05 M Tris-HCl, pH 8.0 containing 1% PVP (molecular weight of 40,000) in 1:6 (w/v) with mild continuous stirring for overnight at 4°C. The clear supernatant obtained after centrifugation (twice) at 10,000 rpm for 20 min at 4°C, was used as crude protein. The protein content was estimated by Folin-Ciocalteau method using bovine serum albumin as a standard (Lowry *et al*. 1951).

### *2. Extraction of A. janata larval midgut proteinases*

The newly hatched larvae of *A. janata* were reared on fresh castor leaves. Larval midgut proteinases were prepared from actively growing fourth and fifth-instar larvae after narcotizing on ice for 15 min. The larvae were dissected using the insect Ringer's solution (0.13 M NaCl, 0.5 M KCl, 0.1 mM $CaCl_2$ and 1 mM phenylmethylsulfonyl fluoride) and the midguts were isolated into isoosmotic saline (0.15 M NaCl) and stored at -80°C, until further use (Prasad *et al*. 2009). When required, the mid-gut proteinases were extracted by homogenising gut tissue in two volumes of 0.05 M Tris-HCl containing 0.02 M $CaCl_2$ (pH 8.2) and centrifuging twice at 12,000 rpm for 20 min at 4°C. The resulting supernatant was collected and analyzed for trypsin-like proteinase activity.

### *3. Assay of A. janata larval midgut trypsin-like proteinases and chymotrypsin-like proteinases*

The assay for gut trypsin-like (pH 8.2) and chymotrypsin-like (pH 7.8) proteinases was carried out in 0.05 M Tris-HCl containing 0.02 M $CaCl_2$, using BAPNA and GLUPHEPA as chromogenic substrates at 1 mM concentration each (Budatha *et al*. 2008). One proteinase unit (trypsin-like or chymotrypsin-like) is defined as the amount of enzyme that increases the absorbance by 1.0 optical density after 45 min incubation at 37°C. The molar extinction coefficient ($M^{-1}$ $cm^{-1}$) for *p*-nitroanilide at 410 nm is equivalent to 8,800.

### *4. Assay of proteinase inhibitors*

Inhibition of trypsin, chymotrypsin or AjPs was determined by using the appropriate volume of crude protein (PI sample) that yields a 40-60% decrease in corresponding enzyme activity. Assay mixture consists of PI sample in assay buffer 0.05 M Tris-HCl containing 0.02 M $CaCl_2$ either at pH 8.2 for trypsin and AjPs or pH 7.8 for chymotrypsin. An aliquot of trypsin (10 µg) or chymotrypsin (80 µg) or appropriate volume of AjPs, which gives 1.0 optical density with 1mM BAPNA or 1 mM GLUPHEPA was mixed with different concentrations of crude PI sample and incubated for 15 min at 37°C. Residual proteinase activity in the above mentioned sample was measured after incubating for 45 min at 37°C using 1 mM BAPNA as a substrate for trypsin/AjP (Erlanger *et al*. 1961) and 1 mM GLUPHEPA as a substrate for chymotrypsin (Mueller and Weder 1989). One TI or CI or AjPI unit was defined as the minimum amount of PI sample required to inhibit 50% of corresponding enzyme (trypin/chymortrypsin/AjP) activity under the optimal assay conditions. The activity of PIs was expressed as trypsin inhibitor (TI) units/mg protein, chymotrypsin inhibitor (CI) units/mg protein and *A. janata* larval midgut trypin-like proteinase inhibitor (AjPI) units/mg protein (Prasad *et al*. 2009).

### *5. Visualization of AjPIs*

PIs of different *C. platycarpus* accessions and cultivated varieties of pigeonpea were separated on 12.5% gelatin-PAGE by incorporating 0.1% (w/v) gelatin (Sigma, USA) into the polyacrylamide at the time of casting (Felicioli *et al*. 1997). After electrophoresis, native gels were incubated in 0.1 M Tris-HCl pH 8.2 containing AjPs (equivalent to 0.1 mg/ml trypsin activity) for 30 min at 4°C followed by 2 h at 37°C. After gelatin hydrolysis, the gel was washed with distilled water and stained with 0.1% Coomassie Brilliant Blue R-250 followed by destaining. The inhibitor activity of AjPIs was visualized as dark blue bands in a clear background due to complex formation of the unhydrolyzed gelatin with stain.

## Statistical analysis

All the experiments were carried out three times each with three replications, and the mean ± SE was reported by using Sigma plot 11.0 (Systat Software Inc., San Jose, CA).

# RESULTS AND DISCUSSION

## Trypsin-like proteinases are predominant over chymotrypsin-like proteinases in the larval midguts of *A. janata*

Serine proteinases contribute to about more than 95% of the total digestive activity in larval gut environment and they usually exist as an array of diverse proteinase isoforms. However, only few of them, for e.g., trypsin and chymotrypsin-like proteinases contribute mostly in the process of digestion (Patankar *et al*. 2001). In the present study we ascertained for the presence of trypsin-like and chymotrypsin-like proteinases in the midgut extracts of $4^{th}/5^{th}$ instar larval stage of *A. janata* larvae. Under the chosen experimental growth conditions, the activity of trypsin-like proteinases were found to be predominant (80 nmol $min^{-1}$ $mg^{-1}$ protein), compared with chymotrypsin-like proteinases (0.8 nmol $min^{-1}$ $mg^{-1}$ protein) in the midguts of *A. janata* larvae (**Table 1**). There was a 100 fold difference in the activity between trypsin-like and chymotrypsin-like proteinases of *A. janata* which was in agreement with those of Prasad *et al*. (2009). But the higher activities shown by both trypsin-like and chymotrypsin-like proteinases in Prasad *et al*. (2009) when compared to the activities observed in the present study could be due to variation in the batch of *A. janata* larvae from which midgut extracts were collected. Further, it is well known that the differential regulation of digestive proteinases activity in lepidopteran larvae depend on several factors: growth and developmental stage of the larvae; dietary composition on which the larvae feed; any change in host plant and/or variation in neuropeptide profile of insects (Huang *et al*. 1998; Harshini *et al*. 2002a, 2002b; Bown *et al*. 2004; Chougule *et al*. 2005).

**Table 1** Activity of larval midgut proteinases from *A. janata*.

| *Achaea janata* midgut proteinases | Activity (µmol $min^{-1}$ $mg^{-1}$ protein) |
|---|---|
| Trypsin-like enzymes | 0.08 ± 0.01 |
| Chymotrypsin-like enzymes | 0.0008 ± 9 × $10^{-5}$ |

## Differential responses of *C. platycarpus* accessions and *C. cajan* cultivars against *A. janata* larval midgut trypsin-like proteinases

In the present study, the activities of PIs from mature dry seeds of *C. platycarpus* accessions and cultivars of *C. cajan*

**Table 2** Inhibitory potential of PIs in wild accessions of *C. platycarpus* and cultivars of *C. cajan* against bovine pancreatic trypsin, chymotrypsin and AjPs.

| Wild accession/ Cultivar variety | TI units/mg protein* (±SEM) | CI units/mg protein* (±SEM) | AjPI units/mg protein* (±SEM) |
|---|---|---|---|
| ICPW 60 | 17.0 ± 0.5 | 4.8 ± 1.0 | 28.0 ± 3.5 |
| ICPW 61 | 15.0 ± 0.6 | 5.8 ± 1.3 | 31.0 ± 5.4 |
| ICPW 62 | 8.0 ± 0.5 | 3.8 ± 0.6 | 16.0 ± 2.5 |
| ICPW 63 | 19.0 ± 1.0 | 7.6 ± 1.3 | 44.0 ± 4.1 |
| ICPW 64 | 7.8 ± 0.3 | 2.4 ± 0.1 | 10.0 ± 3.1 |
| ICPW 65 | 7.0 ± 0.5 | 2.2 ± 0.1 | 12.0 ± 4.1 |
| ICPW 66 | 6.6 ± 0.4 | 2.6 ± 0.1 | 8.0 ± 2.2 |
| ICPW 67 | 15.0 ± 0.7 | 3.9 ± 0.1 | 39.0 ± 5.3 |
| ICPW 68 | 22.0 ± 1.3 | 3.7 ± 0.7 | 41.0 ± 3.4 |
| ICPW 69 | 5.4 ± 0.3 | 2.3 ± 0.3 | 13.0 ± 3.4 |
| ICPW 70 | 6.1 ± 0.3 | 2.8 ± 0.2 | 23.0 ± 5.1 |
| ICPW 71 | 21.0 ± 0.2 | 4.9 ± 0.6 | 29.0 ± 3.6 |
| ICPW 72 | 23.0 ± 0.2 | 3.8 ± 0.3 | 31.0 ± 5.6 |
| ICP 7118 | 27.0 ± 3.5 | 8.5 ± 1.1 | 686.0 ± 33 |
| ICP 7119 | 23.0 ± 1.0 | 4.6 ± 0.2 | 451.0 ± 19 |
| LRG 30 | 13.0 ± 1.1 | 3.5 ± 0.1 | 374.0 ± 20 |
| ICP 85063 | 21.0 ± 0.3 | 6.4 ± 0.8 | 466.0 ± 21 |
| ICP 87 | 27.0 ± 0.3 | 6.8 ± 1.3 | 574.0 ± 38 |

*One TI, CI and AjPI unit is defined as the amount of crude protein required to inhibit 50% of BAPNA hydrolysis by trypsin, chymotrypsin and trypsin-like proteinases of *A. janata* midgut, respectively.

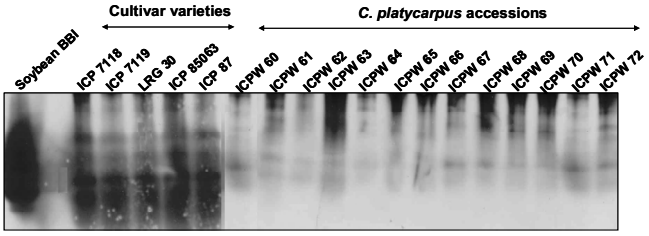


**Fig. 1 PIs profile of *C. platycarpus* accessions and *C. cajan* cultivars separated on Gelatin-PAGE (12.5%).** Lane 1 was loaded with 5 µg soybean BBI (molecular weight 8 kDa) as control. Crude proteins of different seed extracts (50 µg) were separated in each lane under non-reducing conditions. After electrophoresis gel was incubated in *A. janata* mid gut extract as described in materials and methods.

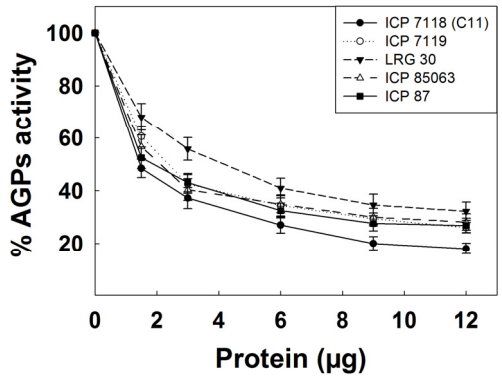


**Fig. 2 Effect of PIs from different cultivars of *C. cajan* on the activity of AjPs.** Other details of the assay are as described in materials and methods. The values are averages of three different independent assays run in triplicates. Values represent mean ± S.E.

were examined against bovine pancreatic trypsin, chymotrypsin and AjPs. The cultivars of *C. cajan* showed significant inhibitory potential (374-686 AjPI units/mg protein) against AjPs, compared with the accessions of *C. platycarpus* (8-44 AjPI units/mg protein). Similarly, the cultivars of *C. cajan* showed highest inhibitory activity of 13 to 27 TI unis/mg protein against bovine pancreatic trypsin and 3.5 to 8.5 CI units/mg protein against bovine pancreatic chymotrypsin, compared with *C. platycarpus* accessions which showed 5.4 to 21 TI units/mg protein and 2.2 to 7.6 CI units/mg protein, respectively (**Table 2**).

The earlier demonstration indicated that cultivars as well as few wild relatives of pigeonpea possessed stronger inhibitory activity against AjPs when compared with bovine pancreatic trypsin or chymotrypsin (Prasad *et al*. 2009). However, in the present study, we have chosen a different set of cultivars of *C. cajan* as well as accessions of *C. platycarpus*, with a view to identify and characterize a PI with stronger inhibitory potential against AjPs. When the cultivars were compared with accessions of *C. platycarpus*, there was a 9 to 86 fold difference in AjPI activity (**Table 2**). On the other hand, there was only a 2- to 6-fold difference in AjPI activity between cultivars and wild relatives examined by Prasad *et al*. (2009). Taken together the results from the present study and Prasad *et al*. (2009) clearly demonstrated that the PIs from cultivars were more effective than wild relatives / accessions in inhibiting the trypsin-like gut proteinases of *A. janata*.

A comparison of the activity profile of PIs from cultivars and accessions of *C. platycarpus* on gelatin-Native PAGE under non-reducing conditions indicated that cultivars possessed 6-7 stronger PI bands against AjPs, while there were few low intensity PI bands in *C. platycarpus* accessions (**Fig. 1**). Thus the differences in the activity staining studies also further confirmed that the PIs from cultivars have more potential to inhibit the activity of AjPs compared with *C. platycarpus* accessions.

Differences observed between PI analysis of pigeonpea cultivars and those of *C. platycarpus* for the insecticidal potential against *A. janata* is although surprising were not totally unexpected as *A. janata* is a pest of castor and not of pigeonpea. The reason behind the selection of *C. platycarpus* accessions in the present investigation was because the *C. platycarpus* has shown high level of resistance to another insect pest *H. armigera* which is an economically important pest of pigeonpea (Mallikarjuana *et al*. 2011). Biochemical analysis also evidenced that, PIs of *C. platycarpus* shown high inhibitory activity against *H. armigera* midgut proteinases (unpublished data).

## Inhibitory potential of different cultivars of *C. cajan* against AjPs activity

To strengthen the defense system in plants by producing transgenic crops, identification of high potential PIs against a particular target insect is extremely important (Koiwa *et al*. 1998). As it is well known that the insects adapt to host defense system by producing inhibitor insensitive or inhibitor degrading proteinases, the present study was intended to identify potential PIs active against AjPs from pigeonpea, a non-host plant to *A. janata*.

The inhibitory potential of PIs from different cultivars chosen in the present study was further evaluated by examining the effect of different concentrations of crude protein against AjPs activity (**Fig. 2**). With increasing concentration of PIs from the five cultivars: ICP 7118, ICP 7119, LRG 30, ICP 85063 and ICP 87, there was a steep decrease in the activity of AjPs. At 9 µg concentration of crude protein, 'ICP 7118' inhibited > 82% activity of AjPs, while the cultivars ICP 7119, LRG 30, ICP 85063 and ICP 87 inhibited < 74% activity of AjPs. However, at the same concentration, 'ICP 7118' inhibited < 13% activity of bovine pancreatic trypsin (data not shown). These results clearly indicate that the PIs from 'ICP 7118' have higher (6.3 fold) inhibitory potential against AjPs when compared with bovine pancreatic trypsin.

## CONCLUSIONS

The PIs from cultivars of *C. cajan*, particularly 'ICP 7118 or C11' had stronger potential in inhibiting the activity of AjPs compared with accessions of *C. platycarpus*. We suggest that the PI genes from 'C11' cultivar of *C. cajan* which

is a non-host plant to *A. janata* are the best candidates for engineering through transgenic technology to strengthen the defense system of any of the host plant, which is seriously affected by this lepidopteran pest. It was interesting to note that candidate genes can sometimes emerge from totally unrelated plants and not necessarily from related genera/species.

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