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ORIGINAL ARTICLE

Protease Inhibitors from *Rhynchosia sublobata* (Wild Relative) and BDN1 (Cultivar) Varieties of *Cajanus cajan* in the Management of Castor Semilooper *Achaea janata*

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ABSTRACT

In agriculture crop production, India is ranked second, with around 4.5 million hectares involved in vegetable cultivation, producing ~75 million tons each year. Thus, agricultural crops hold an important position in the Indian economy. However, every year, various abiotic/biotic stresses, including insect pests, cause significant loss of agricultural crops. In response, plants produce several defensive molecules, including protease inhibitors (PIs), for their protection from various insect pests. The wild relatives are known to harbor an important gene pool for biotic stress, while non-host plants discourage insect invasion. Therefore, PIs from wild relatives and non-host plants are considered effective candidates for the management of various insect pests. In the present study, serine PIs are purified from '*Rhynchosia sublobata*', a wild relative and 'BDN1', a cultivar varietyof *C. cajan*. Both the varieties are non-hosts to *Achaea janata*. The PIs from these varieties are tested for their effect on the growth and development of *A. janata* larvae. The feeding of the PI to larvae was started at two different instar stages such as (i) the 'first instar' stage for up to 14 days and (ii) actively feeding 'third instar' stage for up to 7 days. This study helps to identify the appropriate larval stage at which PI should be applied to minimize the infestation of plants in the natural environment. PIs purified from both varieties caused a significant reduction in the weight of larvae and promoted mortality. Further, the PI treatment started at the first instar stage and showed a greater insecticidal impact on *A. janata* larvae than the PI treatment started at the third instar stage.

KEY WORDS: *Achaea janata*, Growth and Development, Insecticidal activity, Insect gut proteases, Protease inhibitors

INTRODUCTION

Worldwide, biotic stresses, including bacteria, viruses, insects, parasites, and fungi cause severe damage (~40% loss) to agricultural crops, which contributes to around $2T global loss (Saha *et al*., 2024). In India, the insect pests caused around 26% loss to agricultural crops (Lal *et al*., 2017). Castor (*Ricinus communis*) is an economically important agricultural crop, and India is the world's largest producer of castor oil seed. Castor seed oil is used to manufacture several industrial products, such as polymers, lubricants, paints, soap, perfumery, *etc.* (Kammili, 2014; Punnet *et al*., 2022). *Achaea janata* (Lepidoptera), commonly known as castor semilooper, a devastating insect pest of castor plants that defoliate entire crop within a short period of time and causes 30-50% yield loss (Punnet *et al*., 2022). In the field, farmers are mostly using either chemical pesticides such as dimethoate, triazophos, endosulfan, dichlorvos, *etc.,* or Bt toxin-expressing transgenic plants to manage the insects (Gahukae, 2015). However, frequent use of chemical pesticides is causing a harmful impact on the environment, while Bt toxin-induced stress resistance in Lepidopteran insect-pests (ffrench-Constant, 2013; Jin *et al*., 2013; Wei *et al*., 2015). In spite of the usage of different insect control strategies, the rapid development of resistance by insects towards insecticide is a major concern. Insects manage to develop resistance due to (i) larger clutch size, (ii) sperm mixing during mating, and (iii) less generation time. Insects are also developing resistance due to the recessive, dominant, and point mutations in genes (Ahmad, 2007; Fordyce, 2005; Karaaðaç, 2012).

In view of all the above, the usage of biopesticides is

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necessary for managing agricultural insect pests in an environmentally friendly way. One such natural plant defense compound is Protease-inhibitor (PI), a globular protein found to be either induced in vegetative parts of plants (*e.g.* leaves) as a result of abiotic/biotic stresses or constitutively expressed in the plant storage organs (*e.g.* tubers and seeds) (Drame *et al*., 2013; Grosse-Holz & Hoom, 2016). PIs mainly target insect digestive enzymes such as trypsin or chymotrypsin, hence reducing the availability of free amino acids for larvae, resulting in the retardation of insect growth and development (Mohanraj *et al.,* 2018). The wild plant varieties offer several advantages over the cultivar varieties in terms of better adaptation to various environmental stresses such as diseases, pests, drought, *etc.*, because among the wild populations, the structure of genetic diversity is found to be stronger than domesticated (Tirnaz *et al*., 2022). The PIs from the non-host or wild variety are found to be more effective in controlling the insect pests, as the insect digestive enzymes are not adapted to it (Jongsma *et al*., 1997). In our previous papers (Mohanraj *et al.,* 2018; Mohanraj *et al.,* 2019), serine PIs purified from *Rhynchosia sublobata* (a wild relative of *Cajanus cajan*) seeds and cloned Bowman-Birk inhibitor (BBI) from *R. sublobata* (rRsBBI) showed the significant inhibitory impact against *A. janata* gut proteases. Other papers from our laboratory showed that cultivars of *C. cajan* are enriched in PI(s)/BBIs (Prasad *et al.,* 2010a; Swathi *et al.,* 2014).

Based on the literature and our previous research, in this study, firstly, we have screened the insecticidal potential of crude PI extracts of BDN1 (cultivar) and *R. sublobata* (wild-relative) of *C. cajan* against midgut proteases of six different lepidopteran larvae. Based on the significant inhibitory activity against midgut proteases of *A. janata*, we have chosen it to compare the insecticidal impact of PIs from wild relative or cultivar of *C. cajan*. Thus, trypsin-specific PI has been purified from both these varieties to evaluate their effect on *A. janata* larvae growth and development. Also, we have attempted to identify the appropriate stage of larvae at which the PI treatment should be started such that it shows maximum damage to the larvae in a cost-effective manner. The purified PI from both sources has been supplemented to *A. janata* larvae at the first instar stage (neonatal) and third instar (actively feeding) stage. Thus,the study aims to compare the insecticidal potential of PIs from wild and cultivar varieties of *C. cajan* and identify the appropriate infestation stage at which PI(s) should be used as a bioinsecticide in the field.

MATERIALS AND METHODS

Materials

The seed materials of *R. sublobata* (ICP 15868) and cultivar variety (BDN1) of *C. cajan* were obtained from the International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, India. Water bath Model F12 (Julabo, Germany) and UV-1700 Spectrophotometer (Shimadzu, Japan) were used. The bicinchoninic acid reagent was procured from Thermo Scientific, India. DEAE Sepharose, CNBr activated Sepharose 4B, and Sephadex G-50 were procured from GE Healthcare Biosciences Pvt. Ltd., India. Benzylarginylpara nitroanilide (BApNA) was procured from Sigma (Bangalore, India). Tris base, trypsin enzyme, and sodium chloride were procured from Sisco Research Laboratory, Mumbai, India.

Insect rearing and larval gut protease preparation

The *A. janata* larvae were fed on fresh castor leaves (castor is a host plant for *A. janata*), and the pupae were kept in moist sand for generation of adults. The adults were fed through a cotton swab immersed in a 20% honey solution containing 200 mg of vitamin E and allowed to lay eggs on fresh castor leaves. Once the eggs were hatched, this generation was used for *in vivo* feeding experiments. *A. janata* insects were reared in an insect culture room at 26 ± 1°C and 65 ± 5% relative humidity with a 14:10 hour light: dark photoperiod. The larvae of *Helicoverpa armigera, Spodoptera litura, Papilio demoleus, Amsacta albistriga,* and *Daphnis nerii* were obtained from the fields surrounding the University of Hyderabad. The gut trypsin-like proteases were preparedaccording to Prasad *et al*. (2010a). Briefly, the 5th instar larvae were dissected in 0.15M saline solution. The guts were homogenized in the same solution by using the plastic homogenizer stick. After the homogenizing, the samples were centrifuged at 11,000g for 20 minutes at 4°C, and the supernatant was collected, labeled as gut protease extract, and stored at -20°C for further use.

Crude extract preparation

The crude PI extract was prepared according to Prasad *et al*. (2010a). Briefly, the seeds of both varieties were ground properly. The seed powders were then de-pigmented and de-fatted using acetone and hexane, respectively, by washing them 3-4 times. After air-drying the seeds powder, it was added into the 50 mM Tris-HCl, pH 8.0, containing 1% PVP (1:6 W/V) and stirred overnight at 4°C. The next day, samples were centrifuged at 11,000 g for 10 mins, and the supernatant was collected and labeled as a crude PI extract.

Protease inhibition assay against gut trypsin-like proteases

The protease inhibitory activity of crude PI extract of *R. sublobata* and BDN1 were evaluated against gut

trypsin-like proteases of *A. janata, H. armigera, S. litura, P. demoleus, A. albistriga,* and *D. nerii* according to Prasad *et al*. (2010a). This experiment was performed as a preliminary study to select the larvae against which crude PI extract shows the maximum inhibition. The trypsin-protease assay was performed using the substrate BAPNA, which yields yellow-colored *p* nitroanilide with maximum absorption at 410 nm on cleavage by trypsin present in larval gut protease extract. The assays were performed in 50 mM Tris-HCl (pH 8.2) containing 20 mM $CaCl_2$. First, the concentration of larval gut protease extract at which it yields 1.0 OD with substrate BAPNA was taken in respective assay buffers *i.e.* 50 mM Glycine NaOH, pH 10.5 for *S. litura, A. albistriga, H. armigera, D. nerii* and *P. demoleus*; and 50 mM Tris HCl containing 20mM $CaCl_2$, pH 8.2 for *A. janata*. After that for the assay of trypsin-inhibitory activity of crude PI extract, it was mixed with insect gut protease extracts and incubated for 15 min at 37 °C. After incubation, 1mM BAPNA was added into the sample and incubated for 45 min at 37°C, followed by the addition of 30% acetic acid to stop the reaction after 45 mins. The reduction in OD was monitored at 410 nm. The molar extinction coefficient ($M^{-1}cm^{-1}$) *p*-nitroanilide is equivalent to 8,800 at 410 nm.

Purification of PIs from the crude PIs extract of *R. sublobata* and BDN1

The crude PI extract (mentioned above) of *R. sublobata* and BDN1 were used for the purification of PIs, as described in Prasad *et al*. (2010a). The crude PI extracts were subjected to 20-60% ammonium sulfate fractionation, followed by dissolving the obtained pellet in 50 mM Tris-HCl pH 8.0 and dialysis against the same buffer to remove salts from the sample. The ammonium sulfate fractions were passed through different chromatography columns: (1) ion exchange DEAE-Sepharose (IEC) column, which was pre-equilibrated with 50 mM Tris HCl pH 8.0, followed by elution of bound protein with a linear gradient of 0.5-1.5 M NaCl contained in the same buffer. The eluted protein was dialyzed against 50 mM Tris-HCl, pH 8.0, and further applied onto a (2) trypsin-Sepharose 4B affinity column (TAC). The unbound protein was first washed by wash buffer (50 mM Tris-HCl, pH 8.0 containing 100 mM NaCl), followed by protein elution with 0.01 N HCl. The eluted protein fractions were neutralized with 50mM Tris-HCl, pH 8.0, and subjected to (3) Sephadex G-50 fine gel filtration columns (GFC), which were pre-equilibrated. Finally,the protein was eluted with 50 mM Tris-HCl, pH 8.0, and labeled as RsPI (PI purified from *R. sublobata* seeds) and BDN1PI (PI purified from BDN1 seeds). The concentration of purified PIs was determined by the Bicinchoninic acid method, and the purified PI was stored at -20 °C. The purity of purified proteins was visualized in 18% Tricine SDS-PAGE followed by silver nitrate staining using the protocol as described in Mohanraj *et al*. (2018).

In vivo feeding experiment

No-choice *in vivo* feeding experiment was performed to evaluate the insecticidal impact of RsPI and BDN1PI on *A. janata* larvae. Fresh castor leaves were coated with respective PIs (at concentrations 2,4 and 8 µg/cm^2) and air dried at room temperature. The PI-coated leaves were then fed to both the first and third instar larvae of *A. janata* for 14 and 7 days, respectively. Once feeding with PIs were completed, larvae were allowed to complete their life cycle on castor leaves without PI. The control group of larvae was fed continuously on 50 mM Tris-HCl (pH 8.0) coated castor leaves. The weight of the larvae was measured on alternative days, and photographs were taken. The reduction in the growth of treated insect groups was calculated by considering the average weight of the control group as 100%.

Statistics

All the experiments were repeated thrice, and the mean±SE was represented. Statistical differences were determined by one-way ANOVA followed by the Post hoc Tukey test of multiple comparison analysis using Sigma-Plot, version 12.5, software (San Jose, CA, USA).

RESULTS

In vitro assay of crude PI extract from *R. sublobata* and BDN1 seeds against gut trypsin-like proteases of different lepidopteran larvae

The preliminary experiment was performed to examine the inhibitory potential of crude PIs against the gut trypsin-like protease of six different Lepidoptera larvae: *H. armigera, S. litura, P. demoleus, A. albistriga, D. nerii,* and *A. janata.* Crude PI extract of both seeds *i.e. R. sublobata* and BDN1,showed significant inhibitory activity against *A. janata* larval gut enzyme with 820±187 TIU and 534±108 TIU, respectively. Contrarily, a marginal inhibitory effect on larval gut enzymes of *S. litura, P. demoleus, A. albistriga,* and *D. neeri* with (1) RsPI: 20±3, 13±1, 19±1.5 and 50±2 TIU, respectively and (2) BDN1PI: 2.7±0.7, 1.9±0.2, 19±1.2 and 0.5±0.1 TIU, respectively was observed (Fig. 1). Though, the crude PI extract of *R. sublobata* also showed a significant inhibition against *H. armigera* gut proteases (550±30 TIU), a marginal inhibition was shown by BDN1 crude PI extract (4±2TIU). As both the crude PI extracts showed a significant inhibitory effect on *A. janata* gut trypsin-like enzymes, *A. janata* was selected in further studies.

Table 1: Effect of RsPI and BDN1PI on *A. janata* after *in vivo* feeding, started from first and third instar larval stage.The data shown was the mean ± SE of at least three different samples performed on different days.

Stage	PI concentration (µg per square cm leaf area)		Average weight of each larvae (mg)	Reduction in larval growth (% control)	Survival rate (% control) [c]	Mortality rate (% control) [c]
Control		0	505 ± 6	0	100	0
First instar [a]	RsPI	2	139 ± 51	73 ± 10	47 ± 7.10	53 ± 6.45
		4	115 ± 51	77 ± 8	48 ± 2.85	52 ± 4.29
		8	101 ± 63	80 ± 12	33 ± 3.42	67 ± 2.57
	BDN1	2	100 ± 5	80 ± 7	30 ± 2.85	70 ± 3.15
		4	62 ± 7	88 ± 1	20 ± 3.42	80 ± 2.50
		8	58 ± 13	89 ± 3	19 ± 2.15	81 ± 1.19
Control		0	888 ± 61	0	100	0
Third instar [b]	RsPI	2	329 ± 3	63 ± 2	73 ± 6.65	27 ± 7.52
		4	312 ± 23	65 ± 3	52 ± 6.50	48 ± 2.23
		8	350 ± 12	61 ± 4	50 ± 2.52	50 ± 1.05
	BDN1	2	474 ± 153	45 ± 21	87 ± 1.35	13 ± 2.00
		4	337 ± 17	62 ± 3	73 ± 1.65	27 ± 2.01
		8	302 ± 27	66 ± 3	60 ± 6.65	40 ± 7.34

[a] reduction in larval growth after 14 days of feeding from the first instar
[b] reduction in larval growth after 7 days of feeding from the third instar
[c] rate after respective days of feeding with respective PIs

Purification of RsPI and BDN1PI

The crude extracts of *R. sublobata* and BDN1 seed were subjected to ammonium sulfate fractionations followed by ion exchange, trypsin affinity, and gel filtration chromatography for the purification of RsPI and BDN1PI, and the purity was visualized on 18% Tricine SDS-PAGE (Fig. 2). The purified RsPIexisted as two bands of MW 10kDa and 20kDa (Fig. 2A), while Purified BDN1PI existed as multiple bands of ~16 kDa, 24 kDa and 32 kDa in tricine SDS-PAGE after silver staining (Fig. 2B).

Effect of RsPI and BDN1PI on *A. janata* larvae: feeding started from the first instar (neonatal) stage

The impact of purified PIs was examined after feeding *A. janata* larvae on castor leaves coated with 2,4 and 8µg/cm^2 of RsPI/BDN1PI, from the first instar to up to 14 days. RsPI induced significant weight reduction and mortality in larvae compared to a control group of larvae in all three concentrations mentioned above. The weight reduction caused by RsPI was 73±10, 77±8 and 80±12%, respectively, and the mortality rate was 53±6, 52±4 and 67±2%, respectively (Fig. 3A1 and Table 1). Similarly, BDN1PI also caused significant weight reduction and mortality in larvae as compared to controls when fed at respective concentrations. The weight reduction caused by BDN1PI was: 80±7, 88±1, 89±3% respectively, while mortality rate was 70±3, 80±2 and 81±1%, respectively (Fig. 3B1 & Table

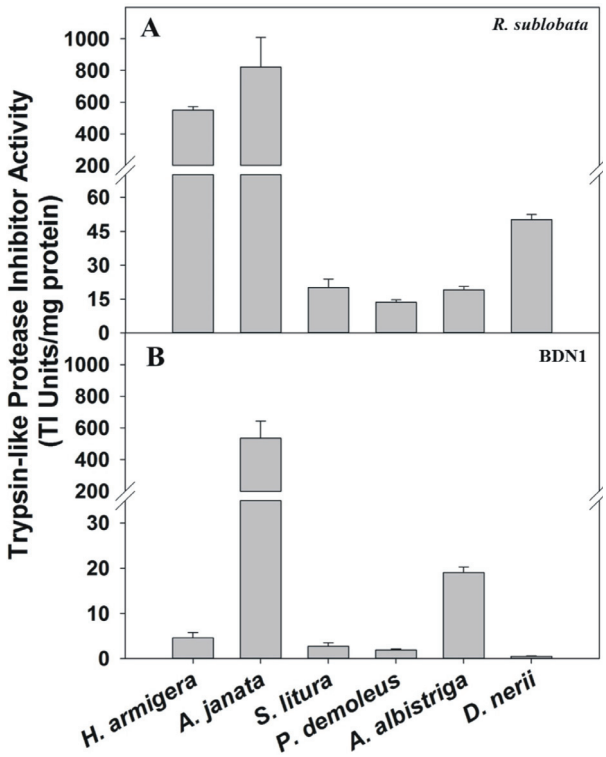

Fig.1. *In vitro* inhibition of gut trypsin-like protease of *H. armigera, A. janata*, *S. litura*, *P. demoleus*, *A. albistriga,* and *D. nerii* by the crude PI extracts of (A) *R. sublobata* and (B) BDN1 seeds. The data shown was the mean ± SE of at least three different samples performed on different days.

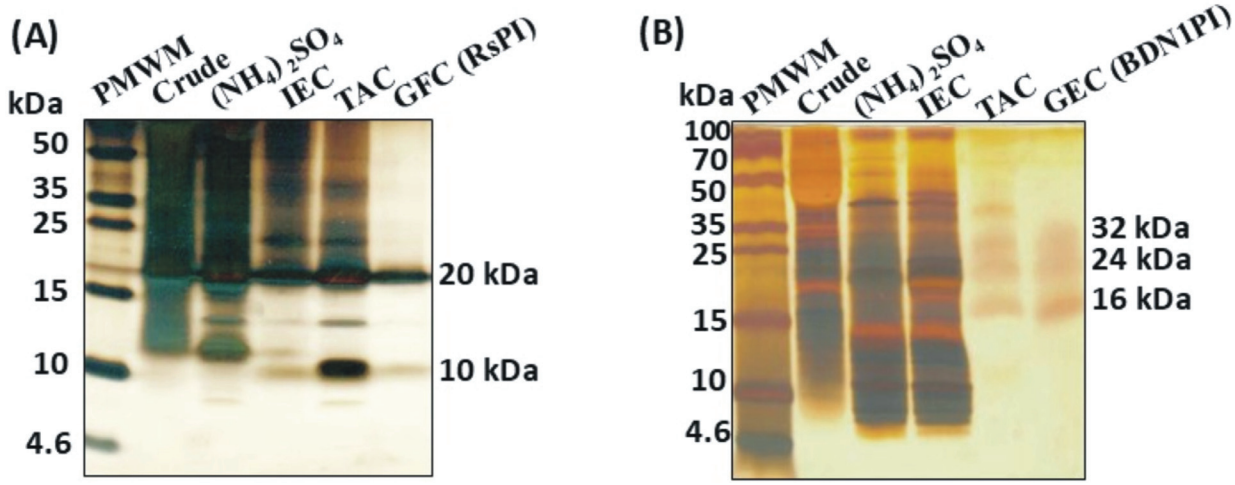

Fig. 2. Tricine SDS-PAGE (18%) showing the different fractions of purification protocol to purify RsPI and BDN1PI from the seeds of *R. sublobata* (A) and BDN1(B). Lanes 1-6 are loaded with PMW marker, crude PI extract (10 µg), $(NH_4)_2SO_4$ fraction (10 µg), active fraction pool from ion exchange column (IEC-5 µg), trypsin affinity column (TAC-10 µg) and RsPI/BDN1PI from gel exclusion column (GEC-5 µg). The gel was stained with silver nitrate.

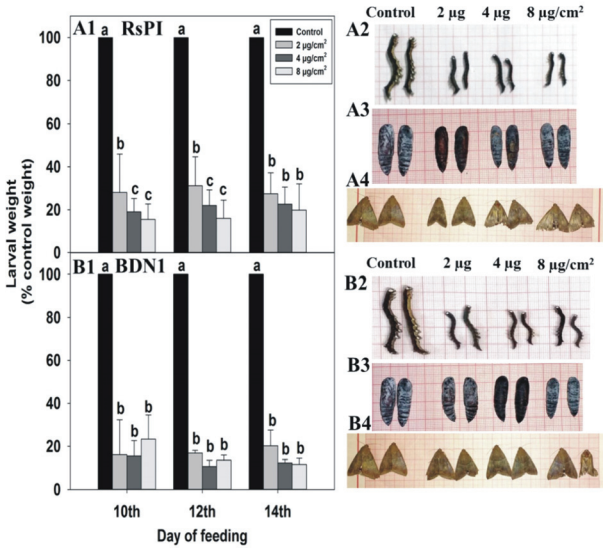

Fig.3. Growth retardation studies of RsPI and BDN1PI on *A. janata* larvae where feeding started at first instar. (A1) Average weight of *A. janata* larvae fed up, from first instar to up to 14 days, on RsPI coated (2, 4, and 8 µg/cm^2) castor leaves. The pictures of (A2) larvae after the 14th day of feeding on RsPI; (A3) pupal and (A4) adult pictures of *A. janata* developed from survived larvae after 14 days of feeding on RsPI. Likewise, (B1) average larval weight and (B2) larval pictures of *A. janata* on the 14th day up on feeding on BDN1PI; (B3) pupal and (B4) adult pictures of *A. janata* developed from survived larvae after 14 days of feeding on BDN1PI from their first instar stage. All the experiments were performed thrice, and the results are mean±SE of three different experiments. Different lowercase alphabetical letters indicate statistically significant differences (ANOVA test, $P \leq 0.05$).

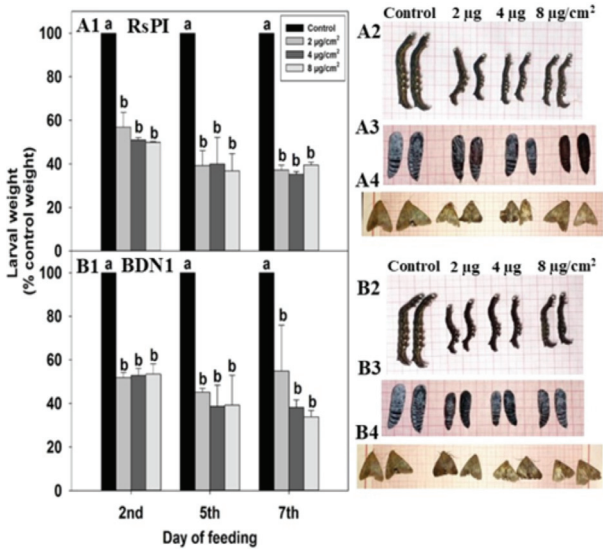

Fig. 4. Growth retardation studies of RsPI and BDN1PI on *A. janata* larvae where feeding started at third instar. (A1) Average weight of *A. janata* larvae fed up from third instar to up to 7 days, on RsPI coated (2, 4, and 8 µg/cm^2) castor leaves. The pictures of (A2) larvae after the 7th day of feeding on RsPI; (A3) pupal and (A4) adult pictures of *A. janata* developed from survived larvae after 7 days of feeding on RsPI. Likewise, (B1) larval weights and (B2) larval pictures of *A. janata* on 7th day up on feeding on BDN1PI; (B3) pupal and (B4) adult pictures of *A. janata* developed from survived larvae after seven days of feeding from their third instar stage, on BDN1PI. All the experiments were performed thrice, and the results are mean±SE of three different experiments. Different lowercase alphabetical letters indicate statistically significant differences (ANOVA test, $P \leq 0.05$).

1). After 14 days, the survived larvae were allowed to grow on non-coated castor leaves to complete their life cycle. The pictures of pupae and adults developed from survived larvae after feeding on RsPI and BDN1PI were depicted in Figures 3A2-A4 and Figures 3B2-B4, respectively.

Effect of RsPI and BDN1PI on *A. janata* larvae: feeding started from the third instar (actively feeding) stage

The impact of purified PIs was examined after feeding *A. janata* larvae on castor leaves coated with 2,4 and 8µg/cm^2 of RsPI/BDN1PI from the third instar to up to seven days. RsPI induced significant weight reduction in larvae compared to the control group of larvae. The weight reduction caused by RsPI was 63±2, 65±3 and 61±4%, and mortality rates were 27±7, 48±2 and 50±1%, respectively (Fig. 4A1 & Table 1). Similarly, weight reduction induced by BDN1PI was 45±21, 62±3 and 66±3%, while the mortality rate was 13±2, 27±2 and 40±7%, respectively, to *A. janata* larvae after feeding at the above-mentioned concentrations of PIs (Fig. 4B1 & Table 1). After seven days, the survived larvae were allowed to grow on non-coated castor leaves to complete their life cycle. The pictures of pupae and adults developed from survived larvae after feeding on RsPI and BDN1PI were depicted in Fig. 4A2-A4 and Fig. 4B2-B4, respectively.

Effect of RsPI and BDN1PI on pupal weights of larvae

The weight of the pupae developed from survived larvae after feeding with RsPI (2,4 and 8µg/cm^2) from the first instar to 14 days was 70±9, 61±16 and 87±3%, respectively, of control pupal weight. Similarly, the weight of the pupae developed from survived larvae after feeding with BDN1PI on respective concentrations was 99±1, 90±1 and 93±1% of control pupal weight (Fig. 5A). The picture of malformed and abnormal larval-pupal intermediate survived from larvae fed on RsPI was shown in Figure 5B.

Whereas, the pupae developed from the survived larvae after feeding with RsPI (2,4 and 8µg/cm^2) from the third instar to up to 7 days showed a weight of 83±8, 89±16 and 70±2% of control pupal weight. Likewise, the weight of the pupae developed from survived larvae after feeding with BDN1PI for 7 days at the above-mentioned concentrations was: 80±8, 74±3, and 80±1%, respectively, of control pupal weight (Fig. 5A).

DISCUSSION

Among the main agricultural important insects, Lepidopterans are found to cause significant damage to several crops and are responsible for huge economic loss, with an estimated loss of ~30-40% to the vegetable crops. Among the four development stages of insects (*i.e.* egg,

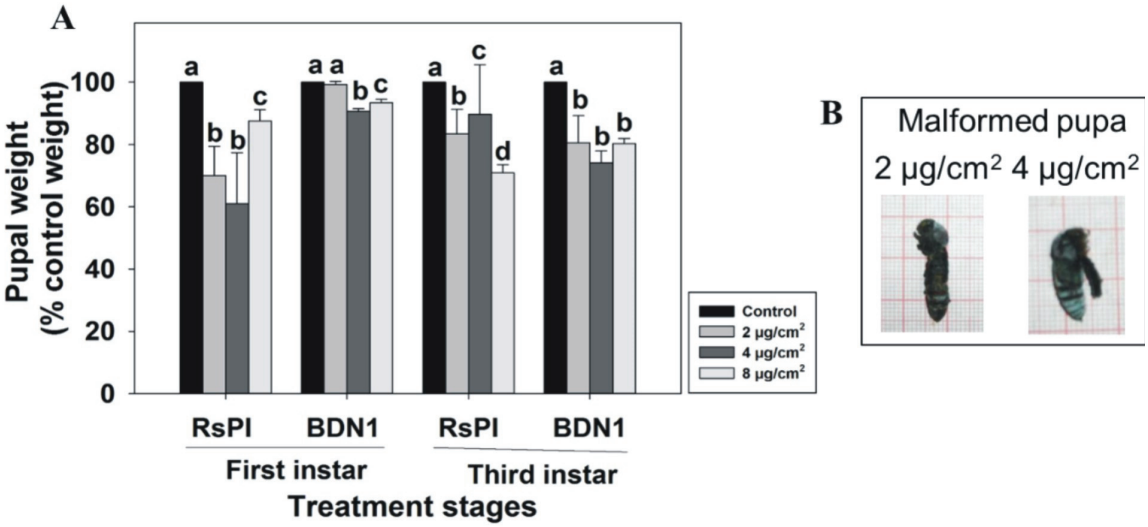

Fig. 5. (A) Average weight of pupa developed from the survived *A. janata* larvae after feeding with RsPI or BDN1PI from first/third instar to 14/7 days respectively. (B) A representative of larval-pupal intermediate and malformed pupa formed upon feeding of *A. janata* with RsPI (2 and 4 µg/cm^2) from first instar larvae. The results are the mean±SE of three different replicates. Different lowercase alphabetical letters indicate statistically significant differences (ANOVA test, $P \leq 0.05$).

larvae, pupa, and adult), the larval stage causes maximum devastation of the crops and causes considerable damage to the plants' pods, flowers, buds, leaves, and sometimes stems (Maish, 2019). Successfully controlling these insect pests is necessary to increase the crop yield. Scientists have applied a variety of control measures, starting from the use of plant's own defense molecules, such as PIs. However, as the host and pests are known to co-evolve over the evolutionary time, a continuous screening of wild-relatives/non-host-based PIs would be a great way to manage these economically important insect pests (Jamal *et al.*, 2013; Losvik *et al.*, 2017). Therefore, in this paper, we have first examined the inhibitory potential of crude PIs extract from the seeds of wild relative (*R. sublobata*) and cultivar (BDN1) of *C. cajan* on six different lepidopteran larvae. Among them, *A. janata* mainly feeds on castor. Apart from castor, it also feeds on rose, guava, ber, pomegranate, coconut, and cocoa. *etc.* (Basappa, 2013). While, *S. litura* and *A. albistriga* are polyphagous pests that feed on castor upon choice. *D. nerii* and *P. demoleus* are the insect pests of toxic oleander leaves and cultivated citrus trees, respectively. *H. armigera* mainly damages cotton, tomatoes, chickpeas, soybeans, and pigeon peas (Aman *et al*., 2022). In our initial screening experiment against lepidopteran insects, the crude PI extract of both *R. sublobata* and BDN1 causes significant inhibition of *A. janata* gut trypsin-like proteases. Therefore, *A. janata* larvae were selected for the present study. However, among both varieties, crude PI extract of *R. sublobata* was more effective than BDN1 against *A. janata* gut trypsin-like proteases (Fig. 1).

From the literature, the effect of several serine PIs was examined against lepidopteran insect pests. PIs from bitter gourd, winged bean, soybean, and Capsicum showed an insecticidal effect on the growth and development of *H. armigera* (Johnston *et al*., 1993; Tamhane *et al*., 2005; Telang *et al*., 2003; Telang *et al*., 2009). The PI purified from another red gram cultivar variety ICPL 332, when fed to the first instar lepidoptera larvae for 6 days, it caused significant larval weight reduction and mortality (Fig. 3 & 4; Table 1; Prasad *et al.,* 2010b). Though plant-based PIs have shown potent insecticidal impacts, some insects have also overcome the effects of PIs either by expressing the different PIs or by overproducing the PI-sensitive proteases (Bown *et al*., 1997; Macedo *et al*., 2015; Wu *et al*., 1997; Zhu-Salzman & Zeng, 2015). Therefore, to counter insect resistance, some other strategies can also be used, such as gene pyramiding, gene fusion, or expression of multiple PIs genes, *etc.*(Abdeen *et al.,* 2005; Dunse *et al.,* 2010; Senthilkumar *et al*., 2010; Pardo López *et al*., 2009; Zhu-Salzman *et al.,* 2003). However, these strategies require the identification of insect-resistance genes.

Therefore, after the initial screening, an attempt was made to compare the insecticidal potential of PIs purified from the seeds of *R. sublobata* and BDN1, on the growth and development of *A. janata* larvae. For this purpose, PIs were first purified from both seeds using chromatography techniques. On the tricine SDS-PAGE RsPI showed two bands of mass of 10 kDa and 20 kDa, whereas BDN1PI showed three bands of mass of ~16 kDa, 24 kDa, and 32 kDa (Fig. 2). Purified proteins are considered serine protease inhibitors as they are trypsin-specific PIs purified using a trypsin-bound affinity chromatography column (Fig. 2; Swathi *et al.,* 2016). The purified PIs were fed to the *A. janata* larvae in two ways: 1) PIs treatment starts from first instar larvae to up to 14 days, and 2) PIs treatment starts from actively feeding third instar larval stage to up to 7 days. When purified PIs were fed to the first instar *A. janata* larvae for 14 days, PIs from both varieties showed a similar impact of reducing the larval weight, though the mortality rate was higher in the larvae fed on cultivar variety BDN1PI (Fig. 3 & Table 1). Whereas, purified PIs, when fed to the actively feeding third instar stage for 7 days, both BDN1PI and RsPI showed similar impacts on insect weight reduction, though the mortality rate was marginally higher in RsPI than in BDN1PI (Fig. 4 & Table 1). However, after comparing the two larval stages, when PIs feeding started from the first instar stage, it caused a more potent insecticidal impact than feeding started from the third instar stage (Fig. 3 & 4; Table 1). Besides, feeding with RsPI (or) BDN1PI also caused a reduction in pupal weight and also led to the formation of malformed pupae (Fig. 5), as shown in Prasad *et al.* (2010b).

CONCLUSION

The management of insect pests is a major concern in increasing crop yield. Thus, in our study, we have shown that PIs purified from wild relatives as well as cultivars of *C. cajan* are both useful in the management of *A. janata* larvae when the feeding starts at the first instar stage. However, BDN1PI caused more reduction in weight and mortality over RsPI when feeding started at the first instar stage. Contrarily, RsPI caused more mortality than BDN1PI when feeding started at the third instar stage.

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