



## SPATIAL DISTRIBUTION OF PROTEINASE INHIBITORS AMONG DIVERSE GROUPS OF SUGARCANE AND THEIR INTERACTION WITH SUGARCANE BORERS

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### ABSTRACT

Profiling of proteinase inhibitors (PIs) was done on the diverse group of sugarcane viz., exotic clones, hybrids and *Erianthus arundinaceus*. Their inhibitory activity against commercial trypsin enzyme and midgut protease of sugarcane borers was investigated. Proteinase inhibitors were extracted from leaf sheath, meristem and stalk tissue of selected groups. The results revealed that the PIs were in maximum quantity in meristem followed by leaf sheath and stalk tissue of hybrids and *E. arundinaceus*. The trypsin inhibition was maximum in leaf sheath (11.51- 32.26%), meristem (15.86- 90.94%) and stalk tissue (1.62- 39.62%) of *E. arundinaceus* compared to those of hybrids and exotic clones. Subsequently, extracted meristem and stalk tissue PIs were assayed against midgut protease of sugarcane early shoot borer (ESB) and internode borer (INB). The results showed that PIs from *E. arundinaceus* meristem significantly inhibited the midgut protease of ESB and INB up to >70 and >60%, respectively. However, the PI from the meristem of exotic clones and hybrids showed considerable inhibition to midgut proteases of ESB. The PIs from stalk tissue of all groups were ineffective as regards gut protease inhibition in the sugarcane borers.

**Key words:** Sugarcane, exotic clones, hybrids, *Erianthus arundinaceus*, meristem, stalk, leaf sheath, proteinase inhibitors, trypsin, *Chilo infuscatellus*, *Chilo sacchariphagus indicus*

Sugarcane (*Saccharum officinarum*. L) is one of the important industrial crops. Of late more than 200 insect pests are reported to attack sugarcane. In peninsular India, the early shoot borer, *Chilo infuscatellus* Snellen (ESB) and internode borer *Chilo sacchariphagus indicus* Kapur (INB) (Crambidae: Lepidoptera) together pose a major threat, causing serious economic losses. The estimated yield losses and sugar recovery with these pests account for 22-33% and 2% CCS and 34.88% and 1.7-3.07%, respectively (DAC, 2015). Although sugarcane borers can be efficiently checked with biocontrol using predators, parasites and pathogens, there is a residual damage of about 10%, which justifies the search for increased plant resistance. In this context, identification of resistance sources and deciphering the mechanism responsible for imparting resistance to major pests is an effective IPM strategy.

Plant proteinase inhibitors (PIs) are diverse group of proteins which have been extensively studied due to their potential for protecting plants against herbivorous insects by inhibiting digestive midgut proteases. These are classified mainly as serine, cysteine, aspartic and metallo PIs. The Kunitz (18-22 kDa proteins)

and Bowman-Birk inhibitors (8-10 kDa proteins) of serine PIs are well characterized and have gained importance as biopesticides (Laskowski and Qasim, 2000; Macedo et al., 2015). The PIs are known to influence the growth and development of insects by binding tightly and irreversibly to the active site of its digestive gut proteases, which are essential for various metabolic processes. The mechanism leads to critical amino acid deficiency and eventually insect death due to over production of digestive proteases by diverting the essential amino acids available for the production of other proteins. These herbivores challenged proteinase inhibitors have been identified in many agricultural crops. Their biological functions against important pests particularly *Helicoverpa armigera* and *Spodoptera litura* are known (Bown et al., 1997). However, quantitative variations of trypsin inhibition among different groups of sugarcane genotypes are yet to be documented. It is important to profile the proteinase inhibitors from *Saccharum* and its allied genera. Also, their insecticidal potential has to be evaluated against gut proteases of sugarcane borers. The study may facilitate to identify the elite cultivars from diverse groups which could be used as donors in future breeding of borer resistant sugarcane varieties.

## MATERIALS AND METHODS

Three groups of sugarcane genotypes viz., exotic clones, hybrids and *E. arundinaceus* were selected. Based on the field screening (2013-2015), promising borer resistant genotypes from each group were selected for the profiling of proteinase inhibitors. The seed material of three groups were brought from the Regional station of Sugarcane Breeding Institute (SBI-RC), Kannur, Kerala. Two budded setts were planted in a randomized block design with three replications with spacing of 6x 0.9 m. The crop was raised following recommended agronomic practices without any plant protection measures. Healthy leaf sheath, meristem and stalk (cane tissue) samples were collected randomly from 3- and 5-months old plants for proteinase inhibitor (PI) extraction. The methodology of PI extraction and assay protocols were followed as per Ramesh Babu et al. (2012). Proteinase inhibitory activity of the extracted PIs were evaluated against trypsin from bovine pancreas under laboratory conditions. The trypsin inhibitory assay was performed using BApNA (N $\alpha$ -Benzoyl-DL-arginine 4-nitroanilide hydrochloride) as substrate. 0.3ml of sample extract was added to 60  $\mu$ g of bovine trypsin in 0.6 ml of assay buffer and incubated at 37°C in a water bath for 10 min. Residual trypsin activity was measured by adding 3ml of 1mM BApNA in pre-warmed (37°C) assay buffer and incubated at 37°C for 10 min. Reactions were arrested by adding 0.6 ml of 30% glacial acetic acid. After centrifugation at 5000 rpm for 6 min, the liberated p-nitroaniline in the clear solution was measured at 410 nm. All assays were performed in triplicate with sample and reagent blank.

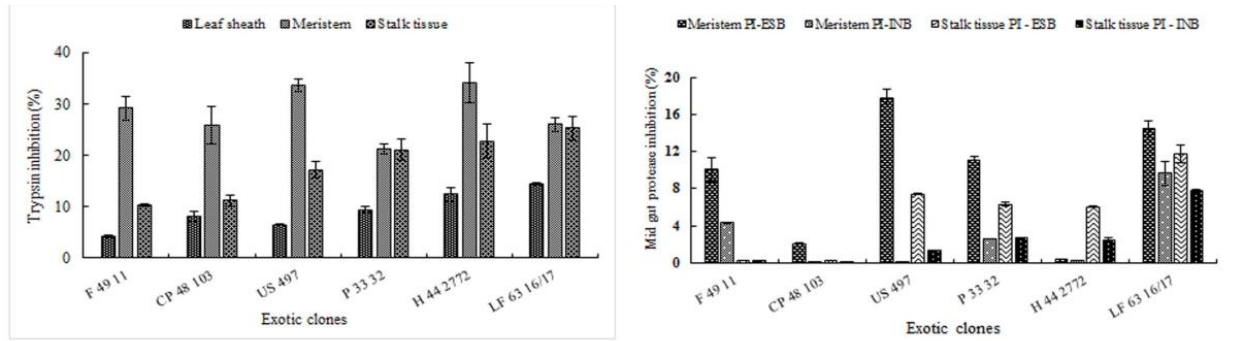
To assess the potential effects of the extracted PIs from the exotic clones, hybrids and *E. arundinaceus* on the digestive proteinases of ESB and INB, midguts of the fifth instar larvae were removed by carefully dissecting and storing at -20°C. Protease enzyme from the midgut tissue were extracted separately in equal volume of ice-cold 0.2M glycine-NaOH buffer (pH 10) (containing 2mM DTT and 10% PVP) for 2 hr at 4°C and then centrifuged at 8000 rpm for 15 mins at 4°C (Telang et al., 2003). The resulting supernatant was collected and analyzed for gut protease inhibition against the PIs extracted from meristem and stalk tissues. The procedure for ESB and INB gut protease inhibition was as similar to the plant proteinase inhibition except for the gut protease in place of trypsin. The data obtained were statistically analyzed in a complete randomized block design and different parameters observed were subjected to ANOVA and means were compared with

Duncan's Multiple Range Test (DMRT,  $p=0.05$ ). The analysis based on students' "t" test was also done.

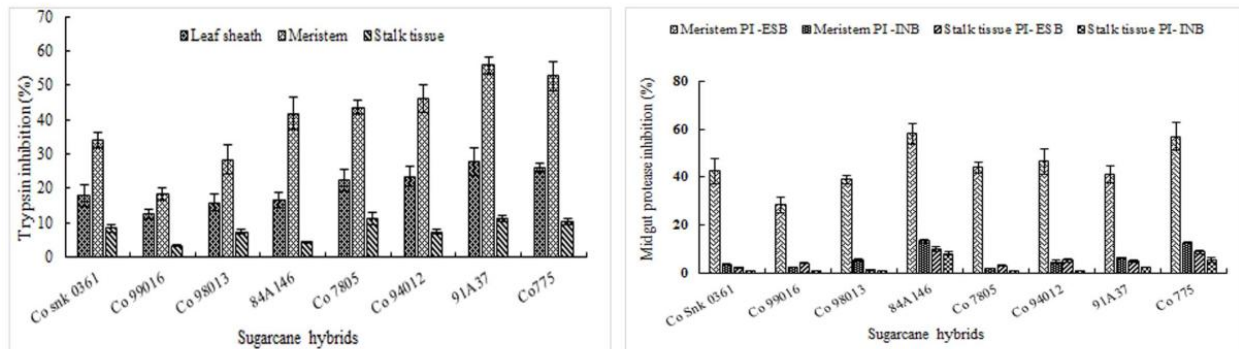
## RESULTS AND DISCUSSION

The results obtained on the extraction of the PIs from different plant parts of exotic clones revealed that the meristem showed maximum trypsin inhibition (>30%) followed by those from stalk tissue (>10-20%) (Fig. 1a). The stalk tissue of the genotypes H 44 2772 and US 497 followed next. In sugarcane, increased level of trypsin inhibitor had been detected in the preferential feeding sites of sugarcane borer *Diatraea saccharalis* (Falco et al., 2001). Many studies report that the PIs inhibit the larval gut proteases conferring resistance to insect pests (Amarjit et al., 2015). The present results show that the PIs from meristem of exotic clones considerably inhibit the midgut proteases of ESB than INB. On the contrary, PIs extracted from stalk tissues reveal only meagre inhibition of midgut protease in both ESB and INB. In general, cultivated plants have a relatively lower level of resistance to biotic stresses when compared to wild relatives of crops (Wang et al., 2015). Among exotic clones screened for PIs, those from meristem of US 497 and LF63 16/17 exhibited significantly more inhibition of midgut protease of ESB compared to other evaluated genotypes. Allsopp et al. (1996) identified PIs in many resistant sugarcane clones and found that the clones fed to the Australian cane grub reduced their growth and survival significantly. The PIs from *Theobroma* seeds showed adverse effect on fecundity and survival rate of velvet bean caterpillars (*Anticarsia gemmatilis*) and sugarcane borer (*D. saccharalis*) (Paulillo et al., 2012).

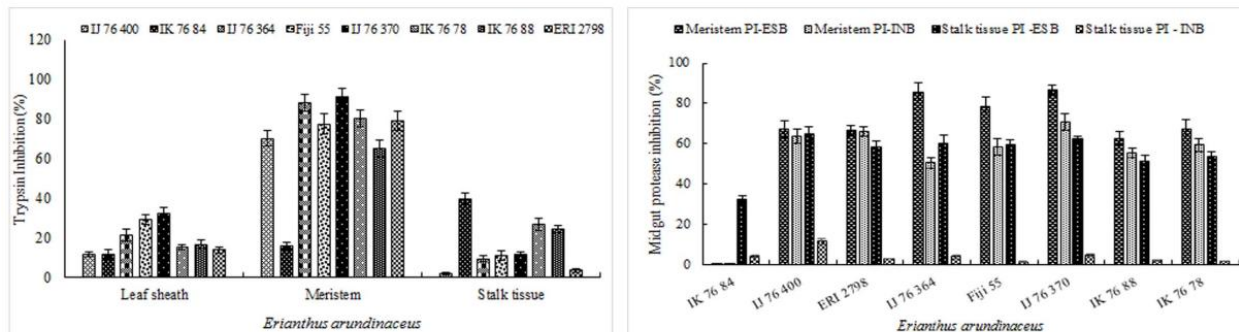
Proteinase inhibitors (PIs) quantified from different plant parts of eight borers promising sugarcane hybrids are presented in Fig 1b. The results showed that the amount of trypsin inhibition in leaf sheath, meristem and stalk tissues differed significantly among the chosen genotypes. However, it was comparatively higher in meristem (35- 55%) followed by leaf sheath (20- 25%) and stalk tissues (5-10%) in the evaluated genotypes. The trypsin inhibition was maximum with the PIs extracted from the meristem of genotypes 91A37 and Co 775 (55.78 and 52.67%, respectively), and was the least with that from the meristem of the genotype Co 99016 (18.36%). None of the genotypes showed marked increase in trypsin inhibition in stalk tissues. While selecting candidate PIs for use in developing insect resistant plants, their stability against digestion by insect gut proteinases is very important. The PIs extracted from meristem and stalk tissue were



1. (a) Proteinase inhibitors of exotic clones vs gut proteases of ESB and INB



1. (b) Proteinase inhibitors of sugarcane hybrids vs gut proteases of ESB and INB



1. (c) Proteinase inhibitors of *Erianthus arundinaceus* vs gut proteases of ESB and INB

Fig. 1. Proteinase inhibitor profiles and their interaction with sugarcane early shoot borer and internode borer

screened in vitro for their inhibitory activity against gut proteases (Fig 1b). The PIs extracted from meristem of all the hybrids showed significantly more inhibition in ESB. According to Balaji et al. (2012), inhibitors from *Saccharum* spp. stem tissue extracts inhibited >30% gut proteinase activity of *C. infuscatellus*. The midgut protease inhibition in ESB was observed to be maximum with the PIs extracted from the meristem of 91A37 and Co 775 (reduction of 58.25 and 56.96%, respectively). However, PIs extracted from meristem and stalk tissue were ineffective as regards mid gut protease inhibition of IB.

The PIs extracted from eight field promising *E.*

*arundinaceus* genotypes viz., IJ 76 400, IK 76 84, IJ 76 364, FIJI 55, IJ 76 370, K 76 78, IK 76 88 and ERI 2798 were studied (Fig. 1c). There existed inter-variety differences among the plant parts of these genotypes. Results showed that the trypsin inhibition was significantly maximum with PIs extracted from meristem; it was > 85% with those from IJ 76 364 and IJ 76 370 followed by > 75% in IK 76 78, Fiji 55, ERI 2798 and IJ 76 400. This inhibition was the least with those PIs extracted from leaf sheath and stalk tissues of *E. arundinaceus* genotypes. Allsopp and Cox (2002) identified a number of clones of wild species *Saccharum spontaneum* and *E. arundinaceus* as sources of enhanced resistance to sugarcane cane grub.

Several *Erianthus* clones were observed to be resistant to nematodes and root parasites (Stirling et al., 2011).

PIs extracted from all the *E. arundinaceus* genotypes revealed significant inhibition of midgut proteases in both ESB and INB indicating their insecticidal potential (Fig. 1 c); PIs extracted from meristem provide significant inhibition; the PIs from the stalk tissue were effective only in ESB. This inhibitory activity towards general proteolysis would give more pronounced effects on larval growth and physiology (Srinivasan et al., 2005). The PIs extracted from meristem and stalk tissue of IJ 76 370 and IJ 76 364 gave maximum inhibition of midgut proteases. Daniels and Roach (1987) reported that hybrids derived from *E. arundinaceus* showed exceptional tolerance to salinity, drought and imparted resistance to pest and diseases. In an earlier study, significant reduction of shoot borer larval and pupal survival along with extended larval duration was observed with *E. arundinaceus* (Punithavalli and Jabamalaimary, 2019). The PIs are abundant in *E. arundinaceus* in comparison to that of hybrids and exotic clones. Besides, *E. arundinaceus* PIs showed strong inhibitory activity against the proteolytic activity of ESB and INB, proving their insecticidal potential. In the current sugarcane breeding programme, allied genera of *Saccharum* complex like *Erianthus* spp. are extensively used for the development of intergeneric hybrids adapted to diverse environments, resistance to insect pests and diseases, and tolerant to abiotic stress. Hence, the results of the present study could be of use in the breeding for borer resistant sugarcane varieties.

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