Asian Journal of Crop Science

ISSN 1994-7879

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OPEN ACCESS Asian Journal of Crop Science

ISSN 1994-7879
DOI: 10.3923/ajcs.2023.66.74

Research Article
Studies on the Salivary Enzymes of *Leptocoris augur* Fabr. (Heteroptera: Rhopalidae) Injuring Seeds of the Kusum Plant (*Schleichera oleosa*)

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Abstract

Background and Objective: *Schleichera oleosa*, a pest and feeder of the kusum plant, *Leptocoris augur* is a brilliant red polyphagous sap-sucking beetle. The salivary composition may change depending on the bug's life cycle. For instance, salivary amylase activity rose with instar in the herbivorous beetle (*L. augur*). Hence, in this study, the food intake of *L. augur* was measured and the corresponding salivary activity of enzymes was determined. **Materials and Methods:** The host bug, *L. augur*, were collected from the field HRI (Hydraulics Research Institute) Training Centre, Saharanpur in polythene bags. The specimens were brought to the laboratory at atmospheric temperature (28±2°C) and necessary relative humidity (70%) and restored in hurricane glass lantern chimneys. **Results:** Physiological examination of the salivary glands of this bug has revealed a pH of 6.7 and presence of enzymes- amylase, proteinase, polygalacturonase, phosphatase, invertase, lipase, esterase and polyphenol oxidase. Diet (fatty seeds of *S. oleosa*) influences the occurrence of these enzymes in saliva. **Conclusion:** Thus, the components and activity of the salivary enzyme in *L. augur* are interconnected with its damage to host plants in a complicated way and the damage of *L. augur* to a plant's seed is the comprehensive outcome of its saliva enzyme.

Key words: *Leptocoris augur*, salivary glands, enzymes, pH, polyphenoloxidase, *Schleichera oleosa*, amylase

Citation: Kumkum, 2023. Studies on the salivary enzymes of *Leptocoris augur* Fabr. (Heteroptera: Rhopalidae) injuring seeds of the kusum plant (*Schleichera oleosa*). Asian J. Crop Sci., 15: 66-74.

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Leptocoris augur, (Heteroptera: Coreidae: Rhopalidae), (A pest of kusum plant*, Schleichera oleosa* and gregarious feeder on seeds and bark), a bright red polyphagous sap-sucking bug. The kusum plant is of great economic value to mankind and the best quality of lac is cultivated on this plant by lac insects. Seeds are also eaten raw or roasted. Powdered seeds are also applied to remove maggots from the ulcers and wounds of animals. A fatty oil obtained from the seeds is known as Macassar oil. It could be utilized for soap manufacture and as a textile lubricant. The oil cake has good manurial value and is also used for fuel. It is used for external massage in rheumatism. The oil is a common adulterant of mustard oil[1]. The bark is used in external applications for pain in the back, inflammations and ulcers.

The seeds of this plant are rich in fatty oil contents and the oil has a positive chemotrophic response. The bug pierces the testa of the seed and the stylet reach to the cotyledons. Then saliva is poured which dissolves the food and the saliva mix food is sucked up, causing a variety of damage, including holes in growing tissue and complete loss of viability of seeds. Bugs feed on bark and seeds using the mandible's and maxillae's ends to destroy host plant cells while simultaneously excreting saliva into the seed through the stylet[2-4].

To know the kinds of salivary enzymes in *L. augur* present studies have been taken up because the secretary process of salivary enzymes in *L. augur* bugs is not always uniform and is affected by the dietetical nature. During feeding, this piercing and sucking bug inject saliva into host plants and seeds to break down host tissues[5]. This was mediated by enzymes in the saliva.

Since bug saliva is injected into plants, its components may be an important means by which particular bugs affect their hosts besides the direct damage caused by feeding, since enzymes and various organic compounds in the saliva of *L. augur* can induce a series of biochemical reactions in kusum plants and seeds[6].

Contents of saliva may vary with the life stage of the bug. For example, salivary amylase activity in the herbivorous bug (*L. augur*) increased with instar, with the least activity in the 1st instar and the most in the 3rd instar nymphs, with no significant difference between 3rd, 4th and 5th instar nymphs and adults[7,8].

Based on the research on *L. augur* food intake at different stages, enzymes were extracted in this study from *L. augur* saliva by placing nutrient solutions between parafilm layers. It is now possible to characterize the salivary components that help to trigger plant responses in more detail. It will be easier to create new control plans for this important pest if we are aware of the salivary enzymes in *L. augur*. Future studies will examine the salivary gland enzymes produced by kusum bugs (*Leptocoris augur*) that consume meals. It is now possible to characterize the salivary components that help to trigger plant responses in more detail.

In this study, the food intake of *L. augur* was measured and the corresponding salivary activity of pectinase, amylase, cellulase, protease, polyphenol oxidase and peroxidase was determined. In recent years, *Leptocoris augur* has caused increasing damage to the kusum seed and bark of kusum trees in Uttar Pradesh, India. The salivary enzymes secreted by *L. augur* when sucking on host plant seeds induce a series of biochemical reactions in plants and the pre-oral digestion benefits the bug feeding. The study's goal was to evaluate the levels of these enzymes in the saliva of *L. augur* bug life stages and correlate these levels with stage-specific food intake. Such measures should help clarify the relative importance of feeding at different bug life stages relative to plant damage to control the bug at an appropriate period.

MATERIALS AND METHODS

Experimental insects and determination of instars: The observation period was from 2014 to 2016 (from the month of May to August). For the laboratory experiment host, *Leptocoris augur* was collected along with fresh leaves and seeds of the kusum plant by hand picking method from the field (Horticulture Experiment and Training Centre, Saharanpur) in polythene bags. Collected bugs were reared in the laboratory, in hurricane glass lamp chimneys (24×36 cm) covered at the top by fine muslin cloth (Fig. 1). The fresh food supply was maintained daily and stale food was replaced. To maintain the necessary relative humidity (70%), a cotton swab was placed in a water-filled glass vial and the temperature was maintained at 28±2°C.

Each such rearing jar was initiated with 30-40 newly emerged nymphs and provided with food (green crushed leaves and crushed seeds, each about 0.0001 g). To avoid water stress, food was replaced with fresh material every other day. Nymphs were categorized to instar based mainly on the size and colour of wing buds. First instars, second instar, third instar, fourth instar, fifth instar and adult *L. augur*, respectively. For assaying enzymatic activity, samples were taken in instar nymphs adults and collected from the first day. Any *L. augur* nymphs that died during the experiment were replaced with fresh individuals of the same instar. The whole experiment was replicated ten times.

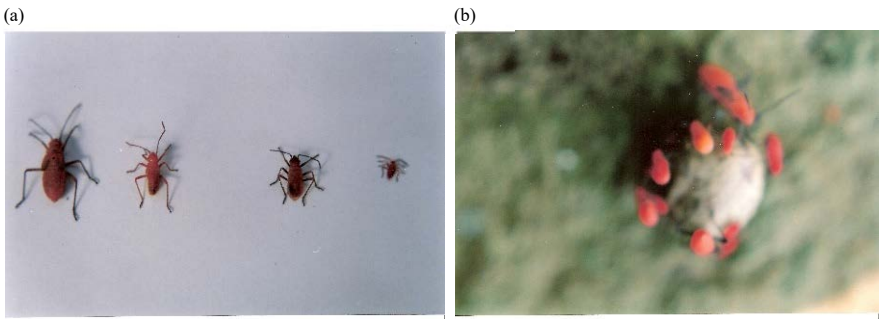

Fig. 1(a-b): (a) Nymphal instars (1st to 4th) of bug, *Leptocoris augur* and (b) Bugs, gregarious feeding on the seeds of *Schleichera oleosa*

Preparation of salivary enzyme solution: The bugs were dissected in a physiological salt solution (Ringer's solution) under a dissection microscope (10X) (MV tex DM-1, India). The exposed salivary glands were taken out. According to the object of the test, 12 or more pieces of glands were taken in distilled water and preserved at 2°C for one day. Then placed in 1 mL of phosphate buffer (pH 7.2) for all assays[9]. The enzyme solution was prepared by homogenizing the tissues of the salivary glands in the phosphate buffer solution (pH 7.2, having a pair of salivary glands/mL solution). This homogenate (Remi 8000 RPM, Mumbai) was then centrifuged for 15 min at 5,000 rpm at 4°C to remove debris. It was filtered through filter paper. The supernatant was used as the salivary enzyme source for the experiment.

Determination of pH values of salivary gland: The gland was teased directly on BDH [Brand name pH Test Strip BDH(R) 0 to 14.0 Manufacturer BDH 35309.606] pH paper and the debris of the gland was removed. Then the colour of the paper was matched with the standard colour. The pH indicators used were (Bromothymol Blue) BTB (5.8-7.4), (Bromocresol Purple) BCP (5.4-7.0), CPR (5.0-6.8), (Narrow range pH paper) MR (4.4-6.2), (Johnson test papers) BCG (4.8-6.2) and (Bromothymol Blue) BTB (3.0-4.6).

Assay for enzyme activity: Equal volumes of the salivary enzyme preparation, substrate and buffer were used in microtubes. The quantities varied from 1.0 to 2.0 mL, depending upon the type of substrate. Sorensen's buffers were used[10]. A few drops of toluene were added to the reaction mixture in each tube as antiseptic and the tubes were plugged by surgical wool plugs. Before incubation, these tubes were chilled. The incubation was done at 37°C for 20-24 hrs after which the reacted mixture was subjected to different chemical tests for the presence or absence of salivary enzymes[11]. Each test was repeated four times.

Tests for the following enzymes were carried out chemically and were summarised as under:

Amylase: The substrate used for the detection of this enzyme was a 1% solution of soluble starch (Merck's) containing a little one percent NaCl solution. The activity of the enzyme was tested at pH 6.7. The reaction mixture was in an incubator for 48 to 72 hrs to give time for the complete hydrolysis of starch. At the end of incubation, the mixture was tested for the presence of reducing sugars as the end products and the enzyme was tested in three ways:

- **Potassium iodide, iodine test:** One drop of potassium iodide solution was added to the incubated mixture and the appearance or absence of blue colour was noted
- **Fehling's test:** Two drops of Fehling A+B solutions were heated with the incubated mixture for a few minutes over a water bath. The presence or absence of brown or reddish-brown precipitate was noted
- **Picric acid test:** Two drops of saturated aqueous picric acid solution and one drop of 10% solution of NaOH were mixed in a drop of the incubated mixture. The tube was then kept in an electric oven at 60°C and the change from yellow to reddish brown colour was examined

Polygalacturonase: Pectic acid (Mercrk's) was used as a substance for polygalacturonase. The assays were performed at 37°C and the incubation mixture usually contained 1 mL each of 2% pectin acid, Macilvaine when buffer pH 5.0 and salivary gland extract. Toluene was added into this mixture as antiseptic and the mixture was incubated for 48-72 hrs. Determine the incubator mixture using the Somogyi-Nelson method[12]. Polygalacturonase gave normal activity in the salivary extract.

Proteinase: Due to the presence of at least two types of proteinases, the proteolytic activity was assayed separately at two different pH values. A 1% solution of casein (Merck's) was used as the substance and the extract substrate mixture was buffered to pH 6.5 and 7.5 Macilvaine buffer was used. The incubation time was kept at 5-7 days. At the end of incubation, 1% acetic acid was added drop by drop to the mixture.

Phosphatase: The 4-nitrophenyl phosphate disodium salt (Merck) was used as a substrate and 2.0 mL of saturated sodium carbonate solution for phosphatase activity at 37°C.

Invertase: As 4% solution of sucrose was taken as substrate and then the activity of the enzyme was tested at pH 6.0. Incubation was carried on up to 40°C to increase the rate of hydrolysis and after 48-72 hrs, the enzyme was tested by Fehling's solution.

Lipase: As 0.3% emulsion of neutral olive oil was prepared and a few drops of 1% solution of sodium carbonate were added to it. In the reaction mixture, a few drops of phenol red were added and incubation was done for 48 hrs.

Esterase: An emulsion of ethyl butyrate was employed as substrate and a rest test was performed like that of lipase.

Polyphenol oxidase: The activity was determined by using a (Holmarc's HO-SPE-3000S UV) spectrophotometer[13], at 37°C. For the determination of polyphenol oxidase activity, Catechol is used as a substrate.

A salivary enzyme secreted into nutrient solution by instar nymph and adult of *L. augur* used for assaying enzymatic activity.

Measurement of food consumption by instar: The food consumption (salivary enzymes) of *L. augur* in various 1st to 5th nymphal instar and adults and their potential relation to bug feeding was measured using a method by Tan *et al.*[14]. In the laboratory 25 instar nymph and adult bugs were taken in the hurricane glass lantern chimney along with their food (fresh tender leaves and moist crushed seeds of kusum plants) at 28±2°C with 70% relative humidity. The stale food was replaced daily with fresh ones and nymphs, adult bugs were examined daily. Moreover, the change in weight of the fresh food due to evaporation was taken into consideration by measuring the food weights of blank controls at the same time points.

Collection of *L. augur* saliva: The adult and instar nymphs were maintained on 2% honey solution (nutrient solution) in a hurricane glass lantern chimney which in turn was covered by a fine muslin cloth on the top to allow circulation of air. The saliva of *L. augur* was collected as follows. The upper end was covered with two layers of parafilm with 200 µL of nutrient solution sandwiched between them (10% sugar, 80 mmol L^{-1} L-serine, 85 mmol L^{-1} methionine, 90 mmol L^{-1} aspartic acid, 25 min pressure high-pressure steam sterilization, cooled and kept in the refrigerator at 6°C) and the other end covered with gauze to ensure ventilation.

The tubes were held at 26±0.5°C, 70±1% relative humidity. After 6-8 hrs, bugs feed on a nutrient solution, saliva was collected for the detection of enzymes. For this purpose saliva was centrifuged at 15000 rpm for 25 min and the temperature was 4°C. After centrifugation, the supernatant was used for further experiments.

Statistical analysis: The relationship was analyzed with Excel software (Microsoft Office Professional Plus 2019, version 2208) and analyzed with SPSS 20.0 by One-way Analysis of Variance (ANOVA) followed by the LSD multiple range test. Significance among factors was considered at the level of $p<0.05$. Also using Pearson's correlation coefficient (R) and linear regression analysis.

RESULTS

Hydrogen ion concentration: The pH of salivary gland enzymes of *L. augur* ranges from 6.5 to 6.9 and the mean of 25 observations has been calculated as pH 6.7. No differences in pH values of nymphal instars or adult sexes were noticed.

Enzymes and their tests: Tests for the following enzymes were carried out chemically and results were summarised as under:

Amylase: As mentioned in the materials and methods section, out of three tests, in the first case no blue colour appeared, in the second test brown precipitate appeared while in the third test yellow colour was changed to radish brown. These confirmed the presence of strong amylase activity in salivary glands.

Polygalacturonase: As mentioned in the materials and methods section, the result of the tests, was the presence of the polygalacturonase enzyme and gave normal activity in the salivary extract.

Proteinase: As mentioned in the materials and methods section, the result of tests, no precipitate appeared in the mixture which indicates the digestion of casein by the proteinase and the presence of this enzyme hence remained in normal activity.

Phosphatase: As mentioned in the materials and methods section, the result of tests, was the presence of phosphatase enzyme (saliva) and strong activity was observed.

Invertase: As mentioned in the materials and methods section, the results of tests, were the presence of invertase. Weak enzyme activity was observed.

Lipase: As mentioned in the materials and methods section, the result of the tests, was colour of the incubated mixture changed from pink to yellow which indicates the presence of a lipase.

Esterase: As mentioned in an earlier section, the results of the tests, were the presence of esterase. The enzyme was found in normal activity in the salivary gland.

Polyphenol oxidase: As mentioned in the materials and methods section, the result of the tests, was the presence of the polyphenol oxidase enzyme in the saliva of *L. augur*. Polyphenoloxidase was determined in normal activity in salivary gland extract.

Digestive enzyme levels in the saliva of *L. augur* from different life stages: Amylase and phosphatase activity quantitatively was also lowest in the 1st and 2nd nymphal instars, then rose in the 3rd nymphal instar and remained at statistically identical levels thereafter, through the adult *L. augur* stage (Fig. 2). According to the result shown in figure, proteinase and polygalacturonase activity sample mean values were lowest in the 1st nymphal instar, reached their highest in the 4th instar and then decreased in the nymphal instar and adult *L. augur*.

Invertase activity was lowest in 1st and 3rd nymphal instars, peaked in the 4th nymphal instar and decreased in adult *L. augur*. Lipase activity sample mean values were lowest in the 1st nymphal instar, reached their highest in the 3rd instar and then decreased in the 4th to 5th nymphal instar and adult *L. augur*. Esterase and

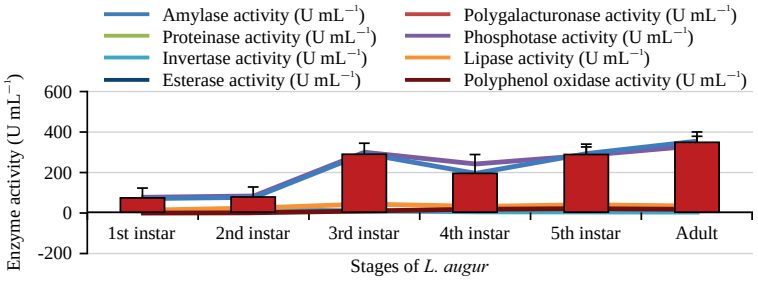

Fig. 2: Enzyme activity present in the saliva of *L. augur*

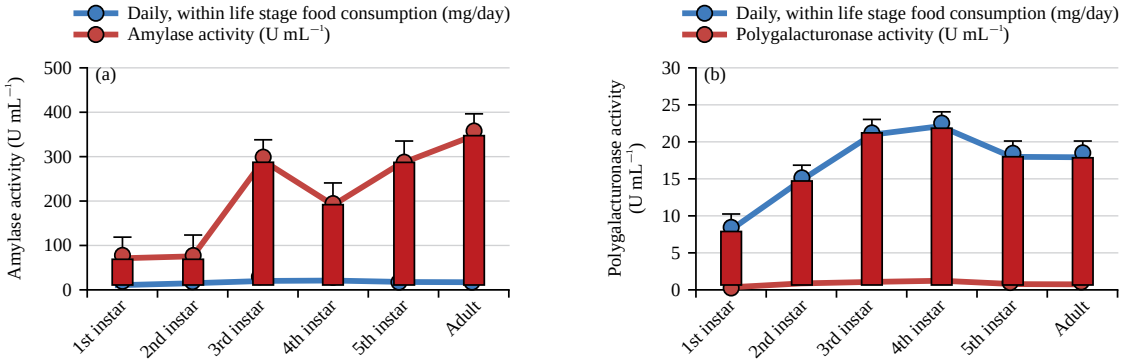

Fig. 3(a-b): Correlation between (a) Amylase and (b) Polygalacturonase activity with daily food consumption in *L. augur*

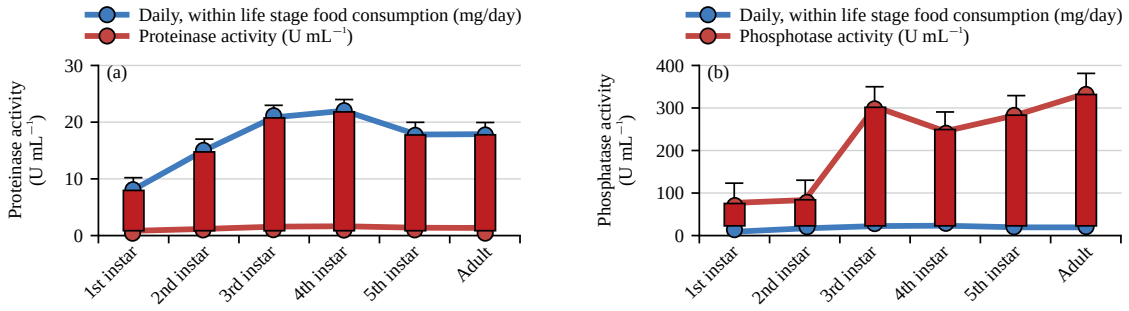

Fig. 4(a-b): Correlation between (a) Proteinase and (b) Phosphatase activity with daily food consumption in *L. augur*

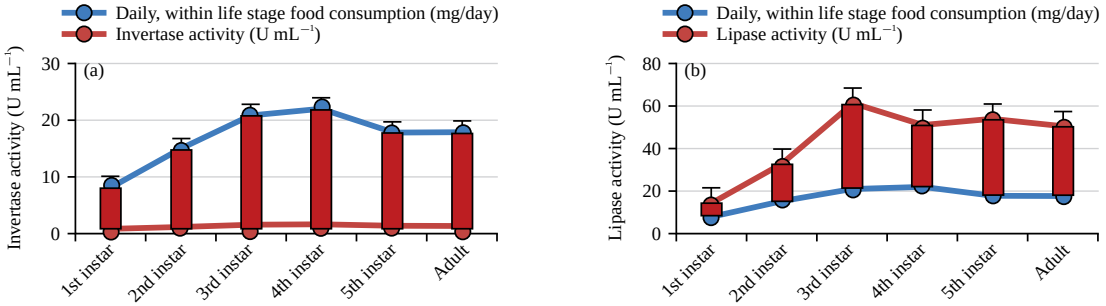

Fig. 5(a-b): Correlation between (a) Invertase and (b) Lipase activity with daily food consumption in *L. augur*

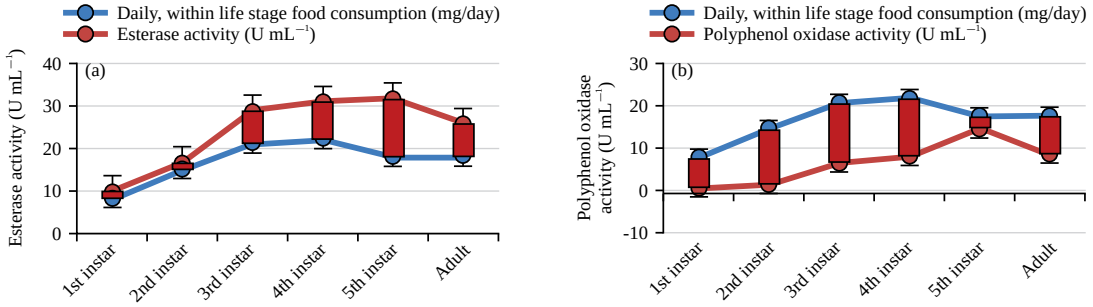

Fig. 6(a-b): Correlation between (a) Esterase activity and (b) Polyphenol oxidase with daily food consumption in *L. augur*

polyphenol oxidase activity was lowest in the 1st and 2nd nymphal instars, peaked in the 5th instar and decreased in adult *L. augur* (Fig. 3-6).

Food consumption by different nymphal instar stages and adults of *L. augur*: Both total and daily within-stage food intake of (*L. augur*) bugs differed significantly among instars Total food intake corresponded to the average daily food intake before the 4th nymphal instar, both increasing with larval instars. Daily food intake peaked at the 4th instar and declined thereafter. However, because of the longer period of older stages, total intake of food per stage continued to increase, through the adult stage. The result of the correlation was 0.930 (showing a high degree of positive correlation) between total stage-specific food consumption with the length of the instar stage. Value of correlation (0.567 showing positive correlation) between daily life stage food consumption with the length of the instar stage. Value of correlation (0.820 showing a high degree of positive correlation) between daily life stage food consumption with total stage-specific food consumption of the instar stage and adult bug. Regression value was (Total: df = 5, p = 0.00 and Daily: df = 5, p = 0.000). It shows a relationship between daily consumption positively correlated with the length of

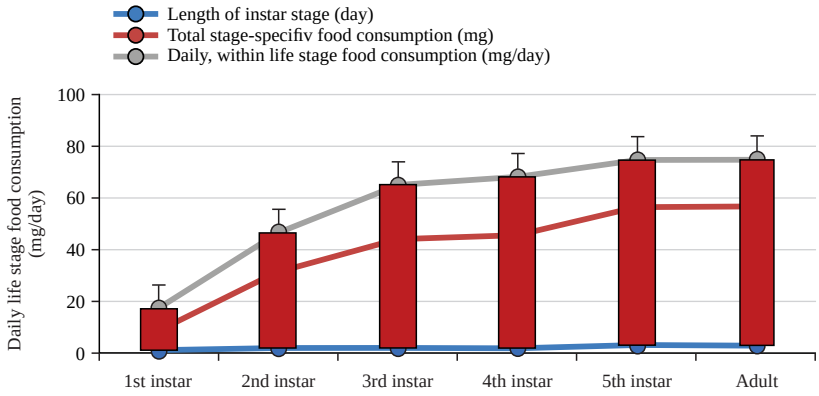

Fig. 7: Food consumption by different nymphal instar stages and adult of *L. augur*

the instar stage and highly positively correlated with total stage-specific food consumption (*L. augur*). Food consumption by different instars and adults of *L. augur* (Fig. 7).

DISCUSSION

The quantitative tests in the present study indicated that the saliva of *L. augur* contains a good number of enzymes such as amylase, polygalacturonase, proteinase, phosphatase, invertase, lipase, esterase and polyphenoloxidase. Reporting only a few enzymes in the salivary gland of a single bug, Peiffer and Felton[15] investigated the brown marmorated stink bug's (Pentatomidae: Halyomorpha halys) salivary gland structure and created techniques for the separate collection of watery saliva and sheath saliva.

Lomate and Bonning[16] mention the optimal pH for protease activity was acidic in the gut, where cysteine proteases were the predominant proteases and alkaline in the saliva and salivary glands, where serine proteases were the predominant proteases. Silva and Terra[17] reported α-galactosidase activity in ingested seeds and the midgut of *Dysdercus peruvianus*. Agusti and Cohen[18] suggested evidence of feeding adaptations by the salivary and midgut digestive enzymes in *Lygus hesperus* and *L. lineolaris* while Schaefer and Panizzi[19] recorded possible causes of disease symptoms from the feeding of phytophagous. Silva *et al.*[20] studies were done to assess the brown-winged stink bug, Edessa meditabunda, eating behaviour and surface damage to soybean seed Anhe *et al.*[21] reported acid phosphatase activity distribution in the salivary glands of Triatomines. Mehrabadi *et al.*[22] reported salivary digestive enzymes of the wheat bug, *Eurygaster integriceps*.

Thus, it is well clear that there exists a variation in the salivary enzymes of Heteroptera. The food of *L. augur* chiefly consists of fat besides carbohydrates and protein. Hence both lipase and esterase are found in the saliva of *L. augur*. It completes its requirement of carbohydrates and protein from the seed endosperm also and hence amylase, invertase and proteinase are also present in the saliva. Swart *et al.*[23] reported optimum activities of amylase and proteases in the salivary glands of giant waterbugs, *Lethocerus uhleri* and *Belostoma lutarium*, were determined to occur at a pH of 7.5. The bug quenches its thirst by sucking the sap of adjacent herbs and shrubs. The sap meets the requirement of water, minerals and sugar. Damage to growing maize, *Zea mays* L., ears caused by the brown stink bug, *Euschistus servus* (Say) (Heteroptera: Pentatomidae), was investigated by Ni *et al.*[24] and Cohen[25] also reported salivary trypsin-like enzymes in a predaceous heteropteran and solid-to-liquid feeding of the insect. Depieri and Panizzi[26] studies were done to compare how long the Southern green stink bug, *Nezara viridula* (L.), Neotropical brown stink bug, *Euschistus heros* (F), red-banded stink insect and other species feed on and damaged soybean (*Glycine max*) seeds.

These findings will help inform the outbreak mechanism of *L. augur* by elucidating the levels of salivary enzymes related to the damage on host plants within each nymphal instar and adult, suggesting the optimal timing on the bug management. Thus, the present investigation has been taken which will arm the economic zoologist or entomologists to devise a suitable control measure for the insect.

CONCLUSION

These findings will help inform the outbreak mechanism of *L. augur* by elucidating the levels of salivary enzymes related to the damage on host plants within each nymphal instar and adult, suggesting the optimal timing on the bug management.

Such measures should help clarify the relative importance of feeding at different bug life stages relative to plant damage to control the bug at an appropriate period.

SIGNIFICANCE STATEMENT

This study considers the assessment of the salivary gland enzyme of *Leptocoris augur* (A pest of the kusum plant, *Schleichera oleosa* and gregarious feeder on seeds and bark) and by decapping habit loses the viability of seeds. This study helped in understanding the physiology of *L. augur* (Hemipteran bug) digestive systems and potentially lead to the development of novel strategies to manage these insect pests. Designing new control plans for this important pest will be made easier with an understanding of the salivary enzymes of *L. augur*.

ACKNOWLEDGMENTS

We are extremely thankful to the principal and head of the Zoology Department of M.S. College Saharanpur for providing the necessary facilities and to the Deputy Director cum in charge of HRI and Training Centre, Saharanpur for field studies. The authors wish to thank Dr. S.C. Dhiman for useful discussions on this work.

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