Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

J E Z S Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2016; 4(6): 184-190

Received: 25-09-2016
Accepted: 26-10-2016

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Plant defense response against grasshopper herbivory

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Abstract
The objective of the current work was to study the defense mechanism of Maize plant (*Zea mays* var.GKSF2) against the herbivory caused by grasshopper (*Choreodocus illustris*). Fifteen plants of the selected crop were exposed to adult grasshoppers in varying number (10, 20 and 40) and compared with the control plants protected from insect attack. Accumulation of proline in insect attacked plant increased linearly and significantly with increased population of insects (10, 20 and 40 adults). In response to the insect attack highest proline contents (34.2 mgg^{-1} of fresh weight) were recorded with 40 adults. But, protein content in the infested leaf decreased consistently with the increase in insect population and lowest of 8.3% observed in plants with 40 adults of grasshopper. The chlorophyll content, leaf number and leaf area also suffered damage in proportion to the insect population. The chlorophyll was lowest as 2.2 mgg^{-1} of fresh weight in plants attacked by 40 adults. Number of damaged leaves (27) and damaged leaf area (72 cm^2) was highest in the plants exposed to 40 adults of grasshopper. The correlation and linear regression analysis showed greater degree of dependence of protein content, chlorophyll content and leaf damage on the insect population. Moreover, proline accumulation in insect attacked plant also signaled the unattacked (control plant) for marginal proline accumulation.

Keywords: Proline, *Choreodocus illustris,* herbivory, plant defense, protein

1. Introduction
Herbivory is a key ecosystem process. The extent of plant damage caused by herbivory corresponds to the proportion of energy partitioned to second trophic level and depends on type and intensity of herbivore feeding [1]. Different types of herbivores feed on varied plant tissues, affecting primary production, translocation of photosynthates and accumulation of defensive by-products to varying degrees [1]. Grazing insects are smaller herbivores in many ecosystems and form a food web complex. Almost 80% of the plant material consumed is rebuild as secondary productivity by insect and vertebrate grazers [2]. Insects have been most significant herbivores and the evolution of land plants stimulated the co-evolution of insects also.
Herbivores are dependent on plants for food and shelter. The herbivores evolved their own mechanisms to obtain a restricted quantity of food from plants despite diverse ways of plant defenses [2]. Plants challenged by insects respond through changes in the composition and physical properties of the cell wall as well as the biosynthesis of secondary metabolites [3, 8]. Plant metabolites and macromolecules like proteins, peptides, enzymes, lignin, phenolic metabolites and cuticular waxes also serve as defense arsenals against herbivores [4]. Formation of wax barriers by plants also protects them from herbivory to some extent [5]. Herbivory of plants generally, stimulates accumulation of proline whereas total carbohydrate content decreases whereas there is no significant effect on the content of phenolics [3].
The proline and protein content plays a significant role in plant defense against different types of biotic and abiotic stress. Proline increases osmotic tolerance during drought conditions, but its specific role in plant growth is not completely clear [6]. Proline was found phagostimulatory to locusts when presented on an inert matrix [7]. Proline (or Valine) is detected by grasshoppers and serves as a cue for drought-stressed nitrogen-enriched plants [7]. Moreover, changes in plant compounds have large effects on the performance of herbivores [8]. There is also much evidence that relative shortage of resources, especially of nitrogen, determines the insect growth and reproductive success [9]. Available nitrogen in terms of proteins is the basic material for soft tissues and the integument of insects. As nitrogen increases in food plants, insects convert more plant material into body tissue [10]. Changes in phenol content (a plant defense

strategy) of food plants can affect herbivorous insects by reducing their feeding and oviposition [11].

The chemical plant defenses against herbivory require synthesis of secondary metabolites obviously at the cost of photosynthates, and in turn plant growth and productivity. There are fewer reports on the impact of herbivory on the alteration in plant growth with special reference to proline accumulation and protein content in *Z. mays*. In the present study, extent of increase in the accumulation of proline and reduction of protein content has been estimated in a selected cultivar of maize under grasshopper herbivory.

2. Materials and Methods

2.1 Selection of Host Plant and Herbivore Insect

The herbivory experiments were conducted at Department of Botany, Aligarh Muslim University, Aligarh. A commonly cultivated cultivar of *Z. mays* cv GKSF-2 (white) of family Poaceae was selected for this study. Five seeds (surface sterilized with 1% HCl for 1 minute and washed thrice with distilled water and soaked for 24 hours) of the selected maize cultivars were sown in each of 60 pots (15 cm diameter, filled with equal volume of composted garden soil in 1:3 V/V ratio). Seven days after germination, the thinning was done to leave only one seedling of equal growth and vigor in each pot. All 60 pots were maintained with adequate watering for 40 days and protected from herbivory under net house of fine mesh size. Maize is highly preferred by a most common species of grasshoppers (or bamboo locust) namely, *C. illustris*, order Orthoptera, suborder Caelifera, super family Acridoidea and family Acrididae. Adult male and female grasshoppers were collected from the fields and reared in the laboratory to acclimatize for feeding and other behavior under captivity. Before allowing the selected maize crop to herbivory, the grass hoppers were collected and reared to acclimatize them, the life cycle of the herbivore was closely monitored. Both adult grasshoppers as well as their nymphal stages were collected using sweeping net following standard insect collection procedure. They were then transferred to jars (nymphal stages) and specially designed wooden rearing cages (adult grasshoppers) and fed daily with fresh grass (Plate 1B). The life cycle of the grass hoppers was studied and adult insects were reared for further experiments.

2.2 Experiment Design

Four sets of small insect net houses of 185 x 100 x 125 Cm (L x W x H) supported with iron rods were prepared and covered from 5 sides with mosquito nets (of fine mesh size) leaving the ground side open (Plate 1A). On one side of the net house, one zip (about 1m long) was provided to enter inside the net for watering and data recording. The collected and reared grasshoppers prefer warmer hide-outs to escape night time temperature fall. In each net house, one 100 watts electric bulb was lighted every night under a card board box of approximately (45 x 25 x 30 Cm). In each net house, 15 pots with 40 days old maize plants were placed after estimating standing average proline, protein, chlorophyll contents and leaf area. In 3 sets of net houses, reared and acclimatized adult grasshoppers were transferred in varying numbers (10, 20 and 40). One set of net houses with 15 pots of maize plants was maintained without grass hoppers (control). After allowing the insect to feed on the plants for three days, the proline content, total protein, chlorophyll, leaf area damage, number of damaged leaves and total leaf area per plant were estimated.

2.3 Proline Content

The proline content in fresh leaf and root samples was determined by following Bates *et al*. 1973 [12]. Samples were extracted with sulphosalicylic acid. In the extract an equal volume of glacial acetic acid and ninhydrine solutions were added. The samples were heated at 100 ^{0}C, to which 5 ml of toluene was added. The absorbance of the toluene layer was read at 528 nm, on a spectrophotometer (Scientronic-20).

2.4 Total Protein

Total protein content was determined by the method of Lowry *et al*. 1951 [13]. In glass centrifuge tubes, 50 mg of oven dried leaf powder was transferred in 5 ml of 50% trichloroacetic acid. The material was centrifuged at 4000 rpm for 10 minutes and the supernatant was discarded. 5 ml of 1N sodium hydroxide was added to the residue and mixed well. After cooling for 15 minutes, the mixture was again centrifuged at 4000 rpm for 15 min and the supernatant containing protein fraction together with three washings with 1N NaOH was collected in 25 ml volumetric flask. 0.5 ml of Folin phenol reagent was added rapidly with immediate mixing until blue color developed. The test tube was left for 30 minutes for maximum color development. Absorbance of the solution was read at 660 nm (Scientronic-20 spectrophotometer). Bovine serum albumin was used for making standard curve.

2.5 Chlorophyll Content

The chlorophyll content (a, b and total) in leaf tissues was estimated following the method of Arnon 1951 [14]. Fresh 500 mg of leaf tissue was ground in a mortar with 5-10 ml 80% acetone, and less than a pinch of calcium carbonate. The extract was filtered with Whatman filter paper No. 1. The residue was washed with 5-10 ml of acetone. The filtrate or supernatant was collected in a volumetric flask and volume of filtrate was made to 50 ml with 80% acetone. The % transmittance of chlorophyll solution was read at 645 nm, and 663 nm wavelength with the help of a spectrophotometer (Scientronic-20).

3. Results

3.1 Life cycle of insect in laboratory

The life cycle of *C. illustris* is illustrated in (Fig. 1). The individuals of *C. illustris* took about 5-15 days to become sexually mature. The copulation lasted for 1-2 hours and the fertilized female then probed for the suitable sites for ovipositing the eggs. Oviposition occurred for 1-6 hours (Fig. 1). The average incubation period was (27.25 ± 2.24) days. A single female laid on an average about 59 eggs per egg-pod (Table 1). Newly hatched I instar young ones (called Hatchings), stretched their body and started feeding voraciously on the grass. Hatchlings moult 5 times to become fully winged adult male or female (Table 1). The hopper instars took (37.62 ±0.46) days to become adult (Table 1). Hence, the reared *C. illustris* took in total about (74.14 ±3.9) days to complete their life cycle at 33±2 ^{0}C temperature and 65±5% R.H (Fig.1; Table 1).

Table 1: Life cycle of *Choroedocus illustris* feeding on grass at 33±2 ^{0}C and 65±5% R.H

Process	Days	Development of Hoppers	
		Duration	Instar
Pre-Copulation Period	5.15 (8.15±1.06)	I	6-9 (6.75±0.41)
Copulation Period	0.04-0.08 (0.06±0.01)	II	7-9 (7.87±0.29)
Pre-oviposition Period	0.66-0.83 (1.02±0.11)	III	6-9 (7.37±0.42)
Oviposition period	0.04-0.25 (0.09±0.02)	IV	6-9 (7.04±0.46)
Incubation Period	19-40 (27.25±2.24)	V	7-10 (8.62±0.42)
Hopper Duration	33-43 (37.62±0.46)	Total Hopper Duration	33-43 (37.62±0.46)

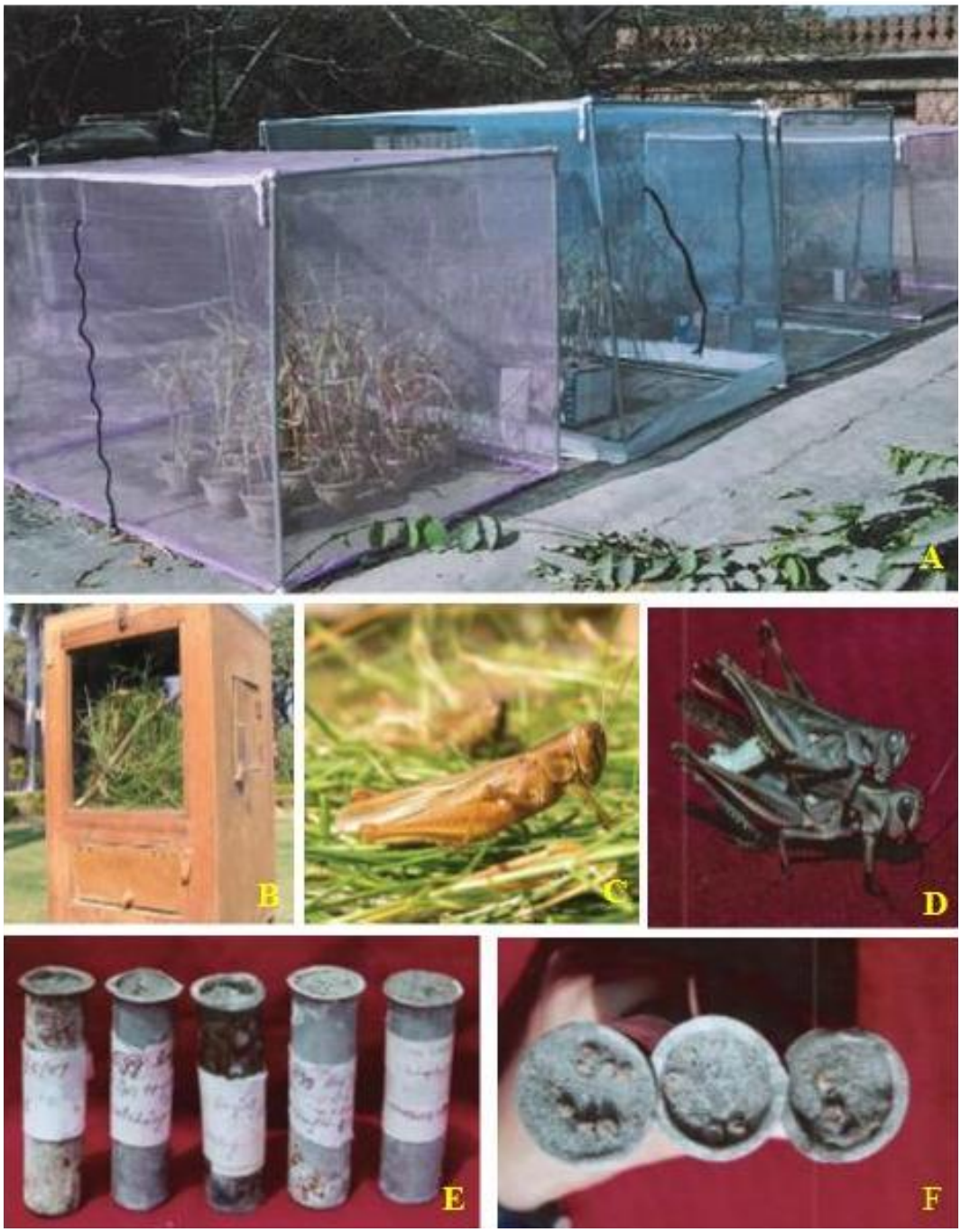

Fig 1: Experimental setup (A) and rearing cage (B) of grass hoppers. Adult *Choroedocus illustris* (C) and showing copulation (D). Egg laying tubes (E) containing egg pods (F) of *Choroedocus illustris*

3.2 Proline and protein levels of attacked plant

The proline content in the leaves of *Z. mays* increased significantly at all three levels of herbivory caused by 10, 20, 30 and 40 adult *C. illustris* ($p<0.05$; Fig. 2A). The proline also increased marginally in the control plant as compared to the content estimated 10 days before the herbivory experiment (Fig. 2A). The proline content in the leaf (estimated after the insect feeding) linearly increased in proportion to the number of attacking insects (Fig. 2A). There was a high degree of positive and linear correlation between proline content and

herbivore population. Statistically, proline content had 96.4% dependence on number of insects attacking the crop (Fig. 2C). The protein content in the control plants was higher as compared to the plant under varying levels of insect herbivory (Fig. 2B). The horizontal dotted bar (Fig. 2B) indicates the protein content in the *Z. mays* crop 10 days before herbivory. The protein content in control plants increased during the 10 days period before herbivory experiment. But after three days of herbivory, the protein content consistently decreased in the leaves of grazed plants as compared to control and below the protein level estimated 10 days before the herbivory (Fig. 2B). This trend clearly indicate that not only the protein synthesis was impaired on herbivory but also the protein already present in the leaves (before herbivory) denatured (Fig. 2B). The correlation between protein content and herbivore population was linear and negative (Fig. 2D) showing a high degree of dependence (92.1%) of protein loss on insect population.

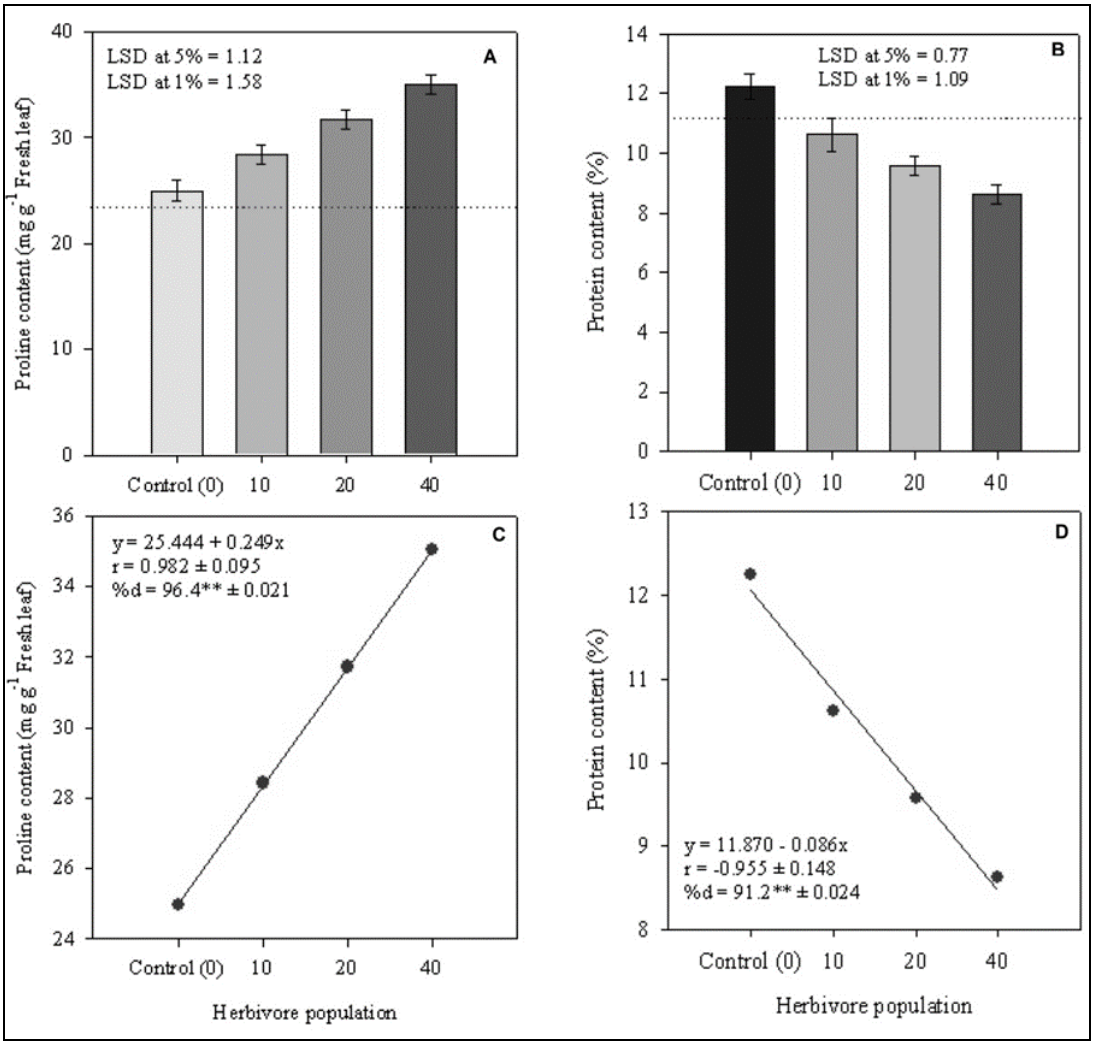

Fig 2: Histograms showing proline (A) and protein content (B) in leaves of *zea mays* (mean ± SD), 3 days after herbivory. Horizontal dotted bar indicate the content in healthy plants 10 days before herbivory treatments. Regression line showing the correlation between proline (C) and protein contents (D) against herbivore population.

3.3 Plant losses to herbivory

The chlorophyll content (a, b and total) in the control plant was higher as compared to the plant under varying levels of insect herbivores. The chlorophyll content in all plants was slightly higher 10 days before insect attack (Figs. 3A, B, and C). The correlation between chlorophyll content and herbivore population was linear and inverse showing a high degree of dependence of chlorophyll damage on the insect population (Figs. 3D, E and F). The leaf number and area damage in *Z. mays* increased significantly with the level of herbivory caused by the grasshoppers (Figs. 4A, B and C). The number of leaves attacked and damaged increased consistently with the herbivore population and showing a high degree of correlation (Figs. 4D). The regression line between herbivore population and leaf area damage (Fig. 4E) was positive and significant having a high degree of linear correlation and per cent dependence (86.3%). Forty adult grasshoppers attacked on 26.33% of leaves of all 15 plants in three days. The total number of leaf in control plants were as high as 40 and in plants attacked by 20 and 40 insects, 30.67 and 33.33 leaves per plant, respectively. As evident from the analysis of variance and test of significance, the leaf number was statistically similar in control plants and those attacked by 10 insects indicating plant could defend herbivores only at lower intensity of insect attack. The grasshoppers preferred young leaves mainly on its apical portion. The grasshoppers also damaged the shoot apex and male inflorescence of the crop. The insect attack not only damaged leaves but also suppressed emergence of new leaves.

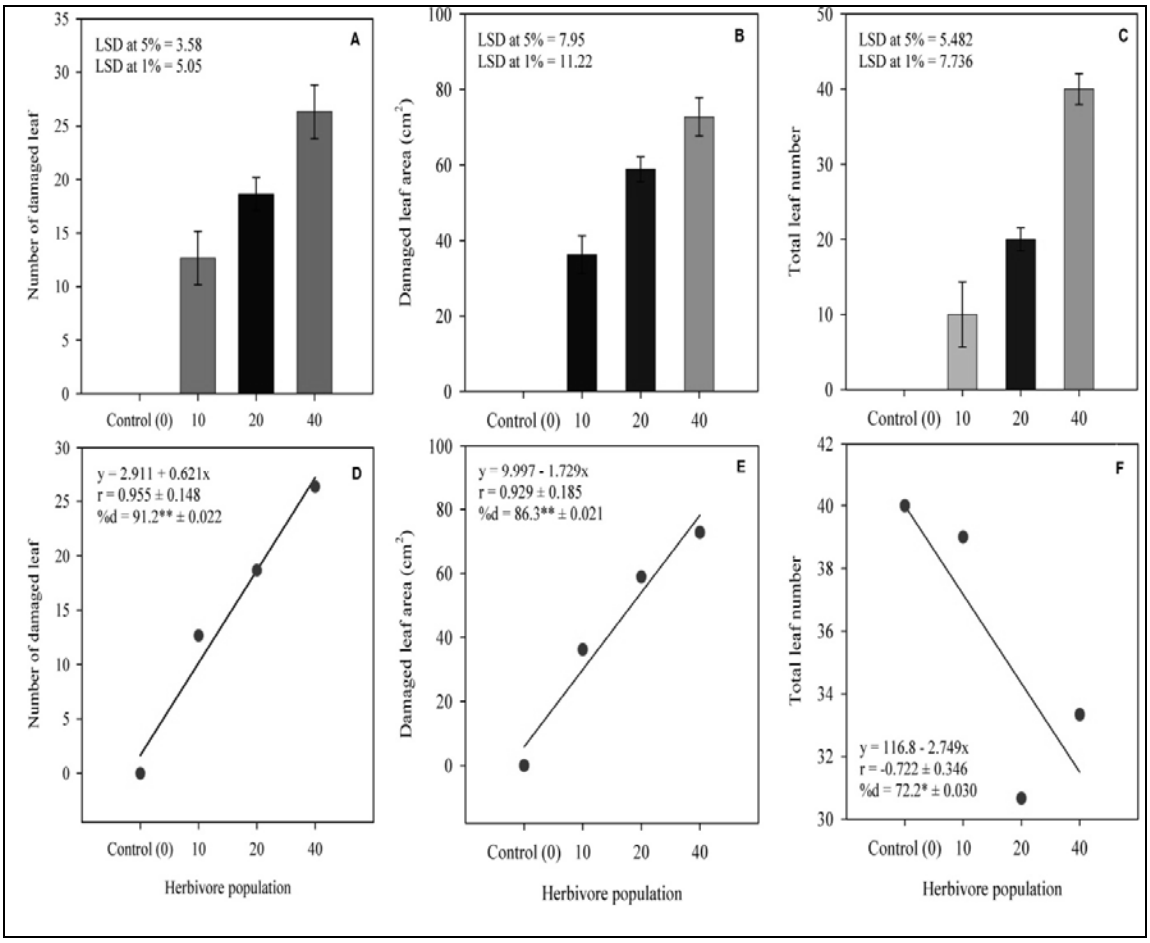

Fig 4: Histograms showing number of damaged leaves, damaged leaf area and total leaf number (A, B and C respectively; mean ± SD) of *Zea mays*, 3 days after herbivory. Regression line showing the correlation between number of damaged leaves, damaged leaf area and total leaf number (D, E and F) of *Zea mays* against variable herbivore population.

4. Discussion

Synthesis and release of these chemical signals are active physiological process triggered by chemical elicitors or substances contained in the oral secretion of herbivores. Certain chemicals contained in the saliva of grazing insect (herbivores) activate the synthesis and release of the plant volatiles [8]. The process of attracting predatory insects involves the interaction of specific blends of plant volatiles with highly sensitive receptor molecules of the predators [8, 12, 17]. Significant amounts of proline accumulated in selected maize plants exposed to the insect herbivores. The proline quantity had direct dependence on the number of insects attacking on the crop. In the present experiment, all net houses including control were relatively closer to each other. The proline content in control plants also increased marginally during the course of insect attack in adjacent net houses. It is reported that the proline synthesis gets stimulated on grasshopper herbivory and when grasses were treated with proline [7]. Free proline accumulation is reported in infested cabbage leaves [3]. Higher proline content in xylem-feeding eucalypt leaves is also reported earlier [15]. Thus, proline content increased on herbivory caused by grasshoppers, phloem feeding insects and treatments of grasses with proline [7, 15].

Proline (a universal osmolyte), accumulates in plants under several types of stresses and defends plant [16, 17]. The excessive proline accumulation in the insect attacked maize plants may have stimulated the control plants in adjacent net houses for marginal increase in proline content feasibly through volatiles signaling. The proline usually accumulates in drought stressed plants. In the present experiment, all plants were adequately irrigated to avoid drought stress. Under stress condition, protein breaks down into amino acid and gets converted into proline for storage [18]. The proline accumulation may also result from physiological and pathological stresses. The negative and strong linear relationship with high degree of per cent dependence on insect population in the present study indicates that proline accumulation resulted due to insect herbivory as defensive arsenal and tissue repairing metabolites. Total soluble protein content has also been found inversely proportion to proline content in aphid infested cabbage leaves [3]. It has been observed that decreased proteolysis and reduced protein synthesis enhanced proline accumulation in 3 cultivars of *Ziziphus mauritiana* [19]. The increased proline accumulation with corresponding decrease in protein content indicates that proline accumulated at the cost of protein which reduced the forage quality of the crop for the insect also recorded similar trends of proline and increased cellular tolerance [20].

The decrease in the photosynthetic pigment in the present study may be due to the inhibition of pigment biosynthesis which may result from the alteration in mineral nutrition or lack of assimilates which drain towards the insect [21]. The insect attack in the present study reduced the chlorophyll content in proportion to their population. The number of leaf attacked had a direct relationship with the herbivore population. The amount of leaf area consumed by varying population of the selected insect (*C. illustris*) was also in

proportion to the population of attacking insects. As evident from the visual observations, the grasshoppers mainly preferred young emerging leaves along with shoot apex and male inflorescence. It has been reported that aphid herbivory results in significant reductions in chlorophyll a and b contents in cabbage [3]. Mammalian (groundhog) herbivory on wild mustard (*Brassica kaber*) damaging leaf area and apical meristems impaired the growth and fitness of the plant [22]. The grasshoppers are also fast leaf biomass consuming insects and attacked the apical portions like groundhogs in our study. The chemical defense of plant was of lesser use against fast biomass consuming groundhogs [22]. The same observation may have been found in case of present crop attacked with the voraciously feeding grasshoppers and instead of any volatile deterrent, proline may have been relatively more effective plant defensive and injury repairing metabolites. Shoot organogenesis and regeneration is also mediated by proline [23]. The unloading of synthesized proline from effector to target cells may be for both these reasons (Fig.5). In a crucifer, proline accumulation enhanced on severing certain plant parts and also on herbivory by chewing and mining insects but not due to sap suckers [24]. The proline provides energy to flight muscle of 8 insects and enzymes of proline synthesis have also been found in their flight muscles [25]. The modified pathway of proline synthesis and catabolism proposed by Szabados & Savoure 2009 [26] (Fig. 4) and its unloading in target tissues may briefly enumerate our understanding of excessive proline accumulation in insect attacked tissues (Fig. 4).

4.1 Proline cycle as defence mechanism against insect herbivory

Proline synthesis occurs in the chloroplast and cytosol and degradation in the mitochondria of effective tissues (Fig.5). The excessive loss of chlorophyll and resulting stress may have enhanced respiration and have brought chloroplasts and mitochondria under stress. The excessive synthesis of proline may have resulted in the cytosol and at the expense of protein (as evident in the present study) and resulted into the accumulation of excessive proline in the tissues as defensive arsenal in proportion to severity of herbivores. Proline is known for its roles in plant development as metabolite and signal molecule [27]. Also, proline acts as antioxidants defense system rather than osmolitic mediator alone [28]. Plants under stress conditions require more effective antioxidant system. Proline estimates of 8 maize cultivars have been found in proportion to salinity [29]. The excessive proline accumulation in proportion to number of attacking insects suggest that proline estimates may be used to determine the extent of herbivory in maize and other grasses. Further studies with other voracious herbivores and larger number of crops are warranted to establish that proline estimate may be used to quantify the intensity of herbivory.

5. Conclusion

Herbivory caused by *C. illustris* reduced plant growth, damaged leaves and chlorophyll content and led to proline accumulation at the cost of protein. The increase in proline accumulation in control plants and much more higher amounts in plants under herbivory indicated that there happened to be some signaling mechanism for stimulation of proline synthesis in unattacked plants on one hand and higher amounts in attacked plants as defensive metabolite for sudden and fast attack of herbivores. Further studies are required to establish the utility of proline estimates as plant defensive and herbivory level. It would be important that, this work advances the field of insect interaction and plant defense mechanism.

6. Acknowledgement

Authors acknowledge the Department of Botany and Zoology, Aligarh Muslim University for necessary facilities, and funding agencies for financial assistance. Farha-Rehman acknowledges U.G.C. for research fellowship. S.M.A. Badruddin thanks ICAR for funding through the "Network Project on Insect Biosystematics".

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