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Antioxidant enzymes in *Solena amplexicaulis* (Lam.) Gandhi plants against *Aulacophora foveicollis* Lucas

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ABSTRACT

We investigated the induced defensive responses in *Solena amplexicaulis* (Lam.) Gandhi (Cucurbitaceae) plants caused by feeding of *Aulacophora foveicollis* (Lucas) (Coleoptera: Chrysomelidae) adults at different time intervals. The activity of defensive enzymes [catalase (CAT), superoxide dismutase (SOD), peroxidases towards phenolic substances {pyrogallol (PPX), syringaldazine (SPX) and guaiacol (GPX)}, ascorbate peroxidase (APOX) and glutathione reductase (GR)], hydrogen peroxide (H_2O_2), total phenols, thiols and proteins were recorded in undamaged plants and in plants 6, 12 and 24 h after *A. foveicollis* feeding. Induction of enzyme activities (CAT, SOD, PPX, SPX, GPX APOX and GR) and the phenols content were higher in plants 24 h after insect feeding than plants 6 h after insect feeding. While the total amounts of H_2O_2, proteins and thiols were higher in plants 6 h after insect feeding followed by plants 12 and 24 h after insect feeding. The results suggested that differential induction of CAT, SOD, peroxidases to phenolic substances and APOX rapidly reduced the H_2O_2 content in insect damaged plants. The increase in activity of these enzymes matched their function to control the accumulation of H_2O_2 and detoxify this reactive oxygen product, when *S. amplexicaulis* plants were fed on *A. foveicollis*.

Key words: Antioxidant response, *Aulacophora foveicollis*, enzymes, hydrogen peroxide, herbivory, peroxidases, phenols, *Solena amplexicaulis*.

INTRODUCTION

All plants are attacked by insects and pathogens which reduce the growth and development of plant. The insects and pathogens damages cause physiological and biochemical changes along with oxidative stress in plants. Insect feeding on plants leads to over production of reactive oxygen species (ROS), which causes the oxidative damage (5, 12, 23). In undamaged plants, synchronization occurs between the production of antioxidant enzymes and ROS, but the equilibrium is disrupted due to production of additional ROS caused by insect feeding (3). Excess ROS is detoxified by antioxidative system comprising of non-enzymic as well as enzymic antioxidants. Superoxide dismutase (SOD), catalase (CAT), activity of peroxidases towards phenolic substances [syringaldazine peroxidase (SPX), guaiacol peroxidase (GPX) and pyrogallol peroxidase (PPX)], ascorbate peroxidase (APOX) and glutathione reductase (GR) are enzymic antioxidants; whereas the nonenzymic antioxidants include ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols and phenolics (34, 36). SOD is the first line of defense against oxygen free radicals, which catalyses the disputation of superoxide radicals into oxygen and H_2O_2; whereas CAT catalyses the disputation of H_2O_2 into oxygen and water,

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*Correspondence author, [1]Plant Physiology and Biochemistry Research Laboratory, Department of Botany but CAT is not able to scavenge H_2O_2 at low concentration (4,15). Peroxidative damage in plant cells are prevented by peroxidases (PPX, GPX and SPX) by detoxifying H_2O_2 as well as oxidizing a variety of phenolic compounds (47). PPX, GPX and SPX enzymes influence physiological process such as somatic embryogenesis, auxin catabolism, lignification and degradation of the cell wall (2,22,35). Furthermore, APOX acts at low concentration to scavenge H_2O_2. Another important enzyme, GR reduces the oxidised form of glutathione (GSH) (14).

Aulacophora foveicollis Lucas (Coleoptera: Chrysomelidae) is an important herbivore pest of *Solena amplexicaulis* (Lam.) Gandhi (syn: *Melothria heterophylla*) (Cucurbitaceae), commonly known as creeping cucumber in India, Bangladesh, China and Taiwan (Figure 1) (30, 31). The crop is cultivated for production of fruits and young leaves are also consumed as vegetables in developing countries (32). *A. foveicollis* also feeds on leaves of squash, pumpkin, cucumber, bottle gourd, luffa, spine gourd and water melons (Cucurbitaceae) (27,28,29,32). We have previously demonstrated that allelochemicals (alkanes, free fatty acids and volatile organic compounds) from *S. amplexicaulis* plants might act as attractant of *A. foviecollis* adults and subsequently, might be used for development of baited traps in integrated pest management program (19,20,21,38). But, there are no reports on the antioxidant enzymes in *S. amplexicaulis* plants in the literature.

Figure 1. *Aulacophora foveicollis* adults feeding on *Solena amplexicaulis* plants.

Reactive oxygen species (ROS) signalling pathway is strongly linked with plant-insect interactions. Hence, determination of antioxidant enzymes in *S. amplexicaulis* plants would serve as a quantitative indicator of stress following *A. foveicollis* feeding. Therefore, this study is aimed to determine antioxidant enzymes (CAT, SOD, PPX, SPX, GPX, APOX and GR), hydrogen peroxide (H_2O_2), total phenols, thiols and proteins in *S. amplexicaulis* plants after continuous feeding by *A. foveicollis* for 6 h, 12 h and 24 h in comparison to undamaged plants.

MATERIALS AND METHODS

Preparation of plant materials

Seeds of *S. amplexicaulis* were germinated on moist filter papers (Whatman No.1 filter paper, 12.5 cm diameter, GE Healthcare). Each seed with cotyledon was planted in a pot (diameter 20 cm and height 16 cm) containing ca. 1500 cm^3 of sterilized soil [organic matter 5.3, pH 7.7, collected from the Crop Research Farm of our University of Burdwan (23°16′ N, 87°54′ E), West Bengal, India] and kept in natural conditions (photoperiod 13 L: 11 D at 30-35° C) for two months **(**June - July, 2016). Insect attack and unintentional infection on each experimental plant was prevented by covering with fine mesh nylon net [100 cm (height) × 60 cm (dia)]. Plants were irrigated (350 ml on first day) and 250 ml subsequently. Five to six-weeks old plants (about 100-120 cm tall) with 10 leaves were used for insect feeding experiment.

Test insect and infestation

Adults of *A. foveicollis* males and females were collected and reared at 27 ºC and 80 ± 5 % relative humidity (RH) under a 14 h : 10 h (light : dark) photoperiod in a biochemical oxygen demand (BOD) incubator and were fed on *S. amplexicaulis* leaves. Newly emerged larvae were fed on young roots of *S. amplexicaulis* plants and subsequently, emerged males and females were separated. Males and females (8-10 days old) were used for feeding experiment. For feeding assay, an experimental plant containing 10-leaves were housed in a nylon screen cage [100 cm (height) × 60 cm (dia)] and fed with 10 adults (5 females and 5 males). There were 3-types of feeding damage treatments: (i). adults were allowed to feed on 5 separate plants for 6 h, (ii) adults were allowed to feed on 5 separate plants for 12 h and (iii). adults were allowed to feed on 5 separate plants for 24 h. Each plant was fed with 10 adults for each feeding damage treatment, and adults were discarded. After insect feeding for 6 or 12 or 24 h, infested leaves were abscised from each experimental plant followed by removal of main veins from the leaves and the plant was discarded. Leaves from each undamaged plant were sampled as control and 5 undamaged plants were used. A total of 15 separate plants [*i.e*, five separate plants were used for each type of feeding damage treatment (plants 6 or 12 or 24 h after insect feeding)] were individually assayed for feeding damage treatments. To determine all the antioxidant enzymes, same leaf sample from a plant after insect feeding or undamaged plant was used and the plant was discarded. Further, five separate leaf samples were collected from 5-different plants for each type of feeding damage treatment to determine antioxidant enzymes for five times.

Determination of hydrogen peroxide (H_2O_2)

Extraction and estimation of hydrogen peroxide was done by the spectrophotometric method (24). Fresh leaves (1 g) were extracted with cold acetone (5 mL) followed by filtering through Whatman No.1 filter paper and distilled water was added to this filtrate to make the volume 10 mL. One mL of 5% titanic sulfate (in 20% H_2SO_4) was added to this followed by addition of concentrated NH_4OH (2 mL). The reaction mixture was centrifuged at 6000 rpm for 10 min and the pellet obtained was washed with acetone (5 mL) thrice, which was again centrifuged (cold) at 5000 rpm for 10 min and the pellet was dissolved in 2(N) H_2SO_4 (3 mL). The absorbance was measured at 420 nm against a blank in the UV-visible spectrophotometer (Shimadzu, UV-1800240V).

Extraction and determination of enzymatic antioxidants

(i). **Catalase:** The catalase (CAT) was estimated by the procedure of Snell and Snell (42) with some modifications. Hundred mg fresh leaf tissues were homogenized with 0.1 M sodium phosphate buffer (pH 7.0) containing 1% polyvinylpolypyrillidone (PVPP) followed by centrifugation at 5000 rpm for 10 min at 4 °C and the supernatants were used for enzyme assays. One mL of enzyme extract was added to 1 mL of 0.5 mM H_2O_2 followed by incubation for 15 min at 37 °C. The reaction mixture was stopped by adding 2 mL 1% $TiSO_4$ (in 25% H_2SO_4). The assay mixture was centrifuged again at 5000 rpm and the absorbance of the supernatant was measured at 420 nm in the UV-visible spectrophotometer (Shimadzu, UV-1800240V). The enzyme activity was expressed as enzyme unit min^{-1} g^{-1} dry leaf tissue according to Fick and Qualset (13).

(ii). **SOD:** The activity of SOD was determined by homogenizing 500 mg of fresh leaf tissue in pre-chilled l0 mL of 100 mM Na-phosphate buffer (pH 6.8) containing 1% w/v PVPP followed by centrifugation at 6000 rpm at 4°C for 10 min. The enzyme activity was determined using the supernatant by measuring the photochemical reduction ability of nitroblue tetrazolium (NBT) according to Giannopolitis and Ries (16). Reaction mixture (3.2 mL) contained 0.05 M Na_2CO_3, 0.1 mM EDTA, 63 mM NBT and 13 mM riboflavin, which was added at last. The assay mixture was kept under 40W florescent lamp at a distance of 30 cm at 25 °C for 30 min and absorbance was measured at 560 nm against the non-irradiated sample (control) in the UV-visible spectrophotometer (Shimadzu, UV-1800240V). The enzyme activity was expressed as enzyme unit min^{-1} g^{-1} dry leaf tissue according to Fick and Qualset (13).

Extraction and estimation of peroxidase to phenolic substrates

(iii). **Peroxidase:** Frozen leaves (500 mg) were homogenized at 4 °C with a mortar and pestle in 3 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 10 % (w/v) PVPP followed by centrifugation at 15,000 g for 30 min at 4°C and the supernatants were used for enzyme assays. Peroxidases activity was determined spectrophotometrically using phenolic substrates such as pyrogallol, syringaldazine and guaiacol and was expressed as enzyme unit min^{-1} g^{-1} dry leaf tissue. Peroxidase activity towards pyrogallol (PPX) was determined by measurement of the purpurogallin, which is a product of pyrogallol oxidation (37). The reaction mixture was 50 mM sodium phosphate buffer (pH 7.0), 40 µL enzyme extract, 180 mM pyrogallol and 2 mM H_2O_2. The absorbance was read at a wavelength of 430 nm in the UV-visible spectrophotometer (Shimadzu, UV-1800240V).

Peroxidase activity to syringaldazine (SPX) was determined by measurement of the colored product of syringaldazine oxidation according to Imberty et al. (18) with minor modifications. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 60 µL enzyme extract, 41.6 mM syringaldazine and 4 mM H_2O_2. Absorbance was read at 530 nm in the UV-visible spectrophotometer (Shimadzu, UV-1800240V).

Peroxidase activity to guaiacol (GPX) was determined according to the modified method described by Maehly and Chance (25) via determination of the tetraguaiacol - a colored product of guaiacol oxidation. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 100 µL enzyme extract, 20 mM guaiacol and 6 mM H_2O_2. Absorbance was measured at 470 nm in the UV-visible spectrophotometer (Shimadzu, UV-1800240V).

Extraction and estimation of ascorbate peroxidase (APOX)

(iv). **Ascorbate peroxidase** : Its (APOX) activity was determined as per Nakano and Asada (33). The assay mixture contained 1 mL of enzyme extract, 0.5 mM ascorbic acid and 0.1 mM EDTA. Parallel experiments in the presence of p-chloromercuribenzoate (50 M) were done to stop interference from guaiacol peroxidases. Absorbance was measured at a wavelength of 265 nm in the in the UV-visible spectrophotometer (Shimadzu, UV-1800240V) and activity of APOX was expressed as enzyme unit min^{-1} g^{-1} dry leaf tissue.

Extraction and estimation of glutathione reductase (GR)

(v). **Glutathione reductase**: Its (GR) activity was determined by the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}) for 3 min in 1 mL of an assay mixture containing 50 mM potassium phosphate buffer (pH 7.8), 2 mM Na_2EDTA, 0.15 mM NADPH, 0.5 mM oxidized glutathione (GSSG) and 200 µL of enzyme extract. The reaction was initiated by adding NADPH. Corrections were made for the background absorbance at 340 nm, without NADPH (39) and activity of GR was expressed as enzyme unit min^{-1} g^{-1} dry leaf tissue.

Determination of protein: Protein content in leaves was determined by the method described by Bradford (7) using bovine serum albumin as a standard.

Extraction and estimation of total phenol content: Total phenol content in leaves was measured by the procedure of Bray and Thorpe (8) using catechol as a standard.

Extraction and estimation of total thiol content : Total thiol content in leaves was estimated as per the procedure of Tietze (44). Total -SH content was assayed in acid soluble extract (500 mg of fresh leaf tissue in 3%, w/v TCA solution) followed by a brief centrifugation. The supernatant was then diluted 10-fold in 100 mM phosphate buffer (pH 7.5) and thiol content was determined measuring the absorbance at 412 nm in the presence of 0.5 mM 5,5′ - dithiobis 2-nitrobenzoic acid (DTNB), 0.5 U mL^{-1} GR and 0.2 mM NADPH.

Statistical analysis

All data on the enzymic and nonenzymic antioxidants in undamaged and insect damaged *S. amplexicaulis* were analyzed using one way analysis of variance (ANOVA) by SPSS software. Means associated with the data for each variable were separated using the Tukey test when significant values were obtained (48).

RESULTS AND DISCUSSION

The effects of feeding by *A. foveicollis* on *S. amplexicaulis* plants varied in the content of endogenous hydrogen peroxide (H_2O_2) in leaves of *S. amplexicaulis* plants during experimental time ($F_{3,\,16}$= 95.368, P <0.0001) (Figure 2). An early remarkable increase of H_2O_2 generation was recorded in plants 6 h after continuous insect feeding followed by plants 12 and 24 h after continuous insect feeding. The lowest content of H_2O_2 was recorded in the undamaged *S. amplexicaulis* plants.

Catalase (CAT) enzyme activity differed significantly ($F_{3, 16}$= 149.851, P <0.0001) in the undamaged *S. amplexicaulis* plants and plants after continuous feeding by *A. foveicollis* at different time intervals. The highest activity of catalase was recorded in plants 24 h after continuous feeding by *A. foveicollis* followed by plants 12 h after continuous insect feeding, plants 6 h after continuous insect feeding and undamaged plants (Figure 3).

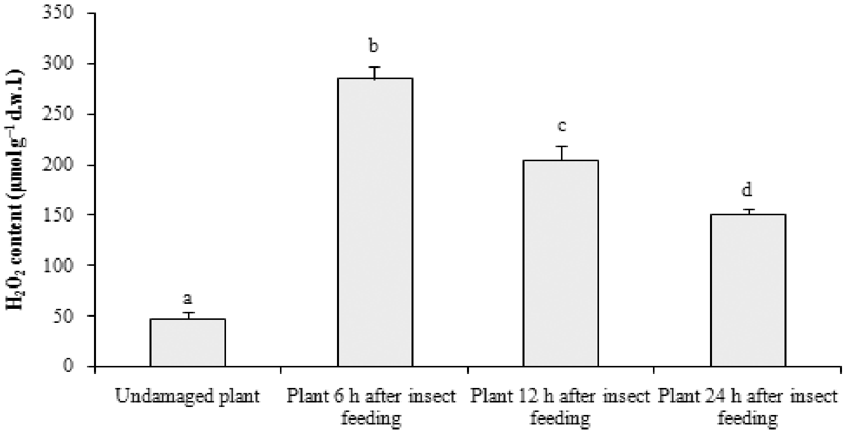

Figure 2. H_2O_2 content (µmol g^{-1} d.w.l.) of a damaged/undamaged *Solena amplexicaulis* plant and by feeding of *Aulacophora foveicollis* adults at different time intervals. Bars (N=5, Mean ± SE) with similar alphabets are not statistically different at $P < 0.05$; d.w.l = dry weight of leaf tissue.

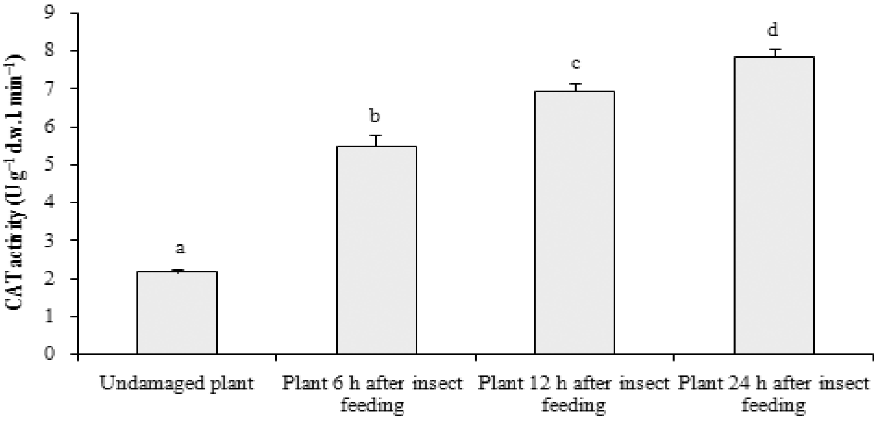

Figure 3. Catalase activity (U g^{-1} d.w.l. min^{-1}) of a damaged/undamaged *Solena amplexicaulis* plant and by feeding of *Aulacophora foveicollis* adults at different time intervals. Bars (N=5, Mean ± SE) with similar alphabets are not statistically different at $P < 0.05$; d.w.l. = dry weight of leaf tissue.

Feeding by *A. foveicollis* on *S. amplexicaulis* plants had a significant activity on SOD ($F_{3,\ 16}$= 131.868, P <0.0001). SOD activity exhibited a significant increase for insect feeding treatments compared to the undamaged plants. There was a maximum increase in SOD activity in plants 24 h after continuous feeding by *A. foveicollis* followed by plants 12 h after continuous insect feeding and plants 6 h after continuous insect feeding (Figure 4).

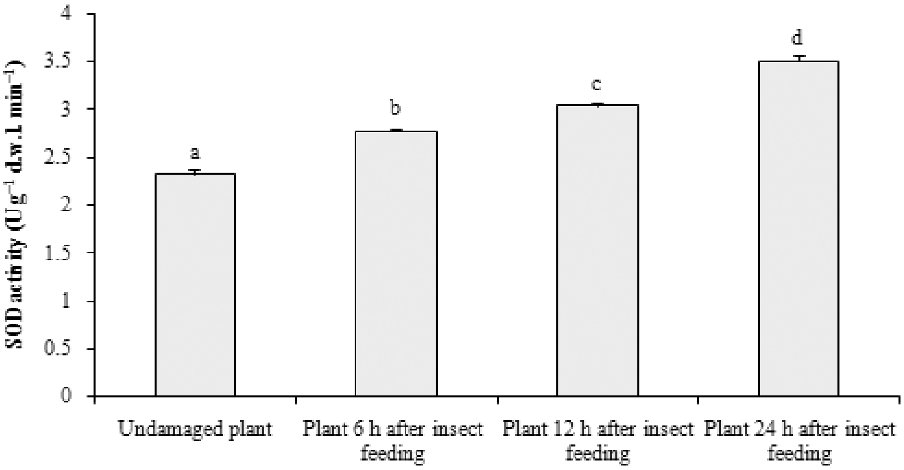

Figure 4. SOD activity (U g^{-1} d.w.l. min^{-1}) of a damaged/undamaged *Solena amplexicaulis* plant and by feeding of *Aulacophora foveicollis* adults at different time intervals. Bars (N=5, Mean ± SE) with similar alphabets are not statistically different at $P < 0.05$; d.w.l. = dry weight of leaf tissue.

Activity of peroxidase-oxidized phenolic substrates such as pyrogallol (PPX), syringaldazine (SPX) and guaiacol (GPX) in leaves of *S. amplexicaulis* plants were differently enhanced after continuous feeding by *A. foveicollis* following the evaluated time points compared to undamaged plants (Figure 5). The activity of SPX increased slightly in plants 6 h after continuous feeding by *A. foveicollis* and reached a peak in plants 24 h after continuous feeding by *A. foveicollis* ($F_{3,\ 16}$ = 156.233, P <0.0001) (Figure 5a). The maximum activity of SPX in the infested plants 24 h after continuous feeding by *A. foveicollis* was 0.619 U g^{-1} d.w.l. min^{-1}, which was 2.5-fold higher than that in undamaged plants (Figure 5a). Following the accumulation of SPX, there was an enhancement in the activity of both PPX (Figure 5b) and GPX (Figure 5c) in the damaged *S. amplexicaulis* plants, which increased from plants 6 h after continuous feeding by *A. foveicollis*, reached at the highest levels in plants 24 h after continuous feeding by *A. foveicollis* (PPX: $F_{3,\ 16}$ = 549.884, P <0.0001; GPX: ($F_{3,\ 16}$ = 123.353, P <0.0001). Activities of these two enzymes (PPX and GPX) in insect damaged plants were significantly higher compared to undamaged plants (Figure 5b and 5c).

Insect feeding had a significant effect on APOX activity in *S. amplexicaulis* plants compared to undamaged plants ($F_{3,\ 16}$= 117.334, P <0.0001) and activity of APOX was highest on plants 24 h after continuous feeding by *A. foveicollis* followed by plants 12 h after continuous insect feeding and plants 6 h after continuous insect feeding (Figure 6).

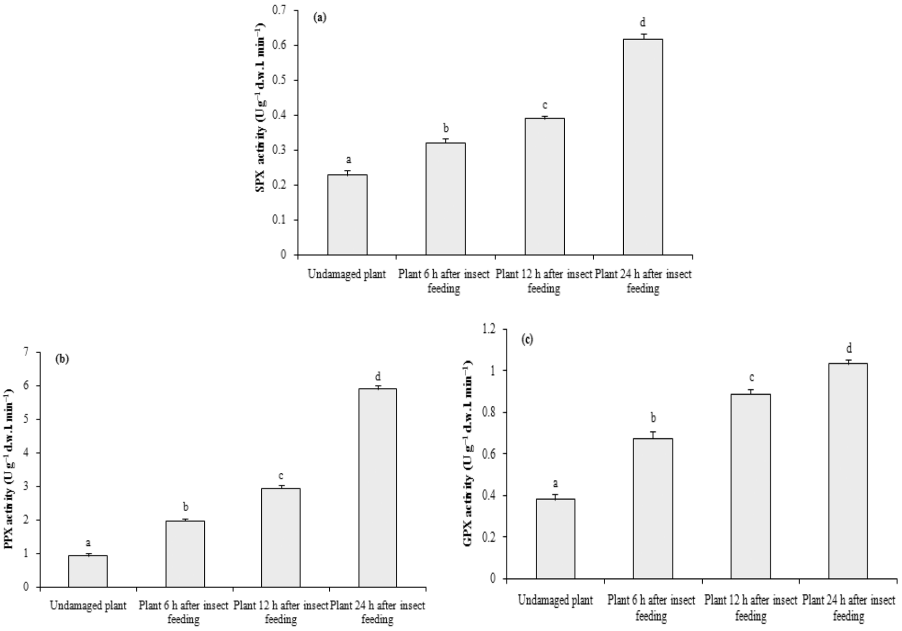

Figure 5. SPX (a), PPX (b) and GPX (c) activity (U g^{-1} d.w.l. min^{-1}) of a damaged/undamaged *Solena amplexicaulis* plant and by feeding of *Aulacophora foveicollis* adults at different time intervals. Bars (*N*=5, Mean ± SE) with similar alphabets are not statistically different at $P < 0.05$; d.w.l. = dry weight of leaf tissue.

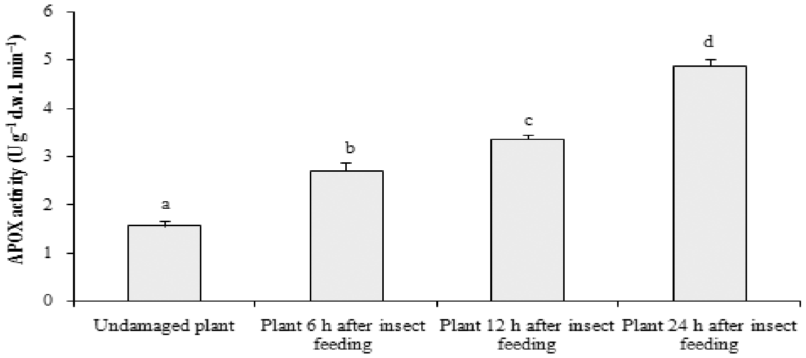

Figure 6. APOX activity (U g^{-1} d.w.l. min^{-1}) of a damaged/undamaged *Solena amplexicaulis* plant and by feeding of *Aulacophora foveicollis* adults at different time intervals. Bars (*N*=5, Mean ± SE) with similar alphabets are not statistically different at $P < 0.05$; d.w.l. = dry weight of leaf tissue.

GR activity in *S. amplexicaulis* plants was significantly affected by insect feeding treatment compared to undamaged plant ($F_{3,\ 16}$= 32.245, P <0.0001). There was no significant change in the GR activity in plants when fed by *A. foveicollis* insects for 6 and 12 h. However, the activity of GR was highest in plants 24 h after continuous feeding by *A. foveicollis* (Figure 7).

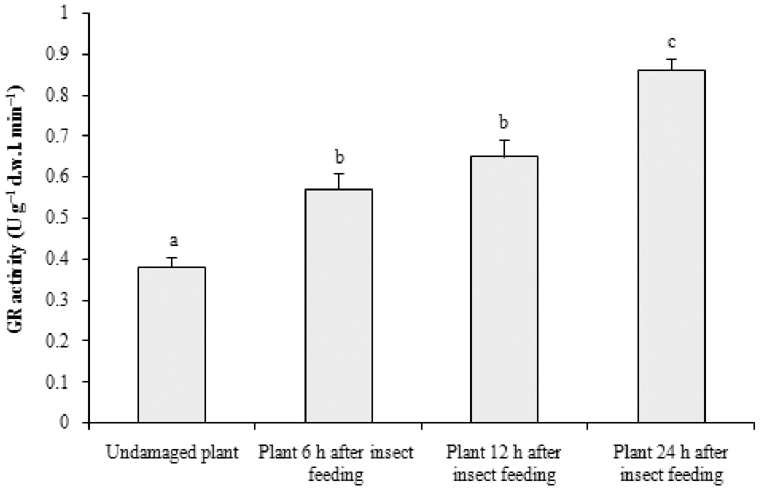

Figure 7. GR activity (U g^{-1} d.w.l. min^{-1}) of a damaged/undamaged *Solena amplexicaulis* plant and by feeding of *Aulacophora foveicollis* adults at different time intervals. Bars (N=5, Mean ± SE) with similar alphabets are not statistically different at $P < 0.05$; d.w.l. = dry weight of leaf tissue.

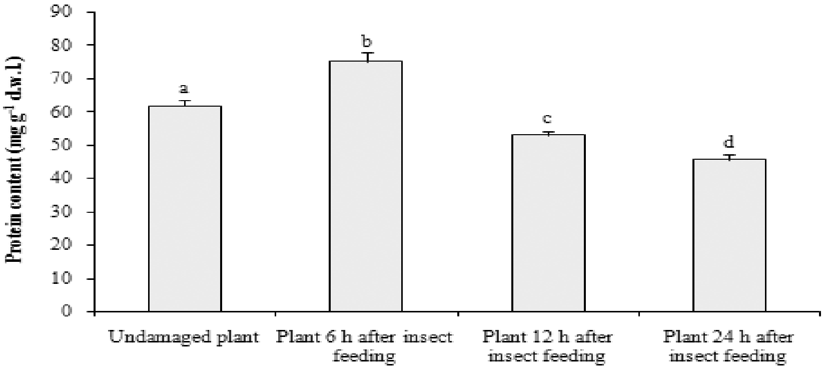

Figure 8. Protein content (mg g^{-1} d.w.l.) of a damaged/undamaged *Solena amplexicaulis* plant and by feeding of *Aulacophora foveicollis* adults at different time intervals. Bars (N=5, Mean ± SE) with similar alphabets are not statistically different at $P < 0.05$; d.w.l. = dry weight of leaf tissue.

There were significant effects of protein concentration in *S. amplexicaulis* plants when fed by *A. foveicollis* ($F_{3,\ 16}$= 52.782, P <0.0001). The protein concentration was significantly higher in plants 6 h after continuous feeding by *A. foveicollis* followed by undamaged plants, plants 12 h after continuous insect feeding and plants 24 h after continuous insect feeding (Figure 8).

The effects of feeding on accumulation of phenols differed significantly among the treatments ($F_{3,\ 16}$= 50.385, P <0.0001). The concentration of phenol did not differ significantly between plants 12 h after continuous feeding by *A. foveicollis* and plants 24 h after continuous insect feeding. The phenol concentration was significantly higher in plants 12 h or 24 h after continuous feeding by *A. foveicollis* followed by plants 6 h after continuous insect feeding and undamaged plants (Figure 9).

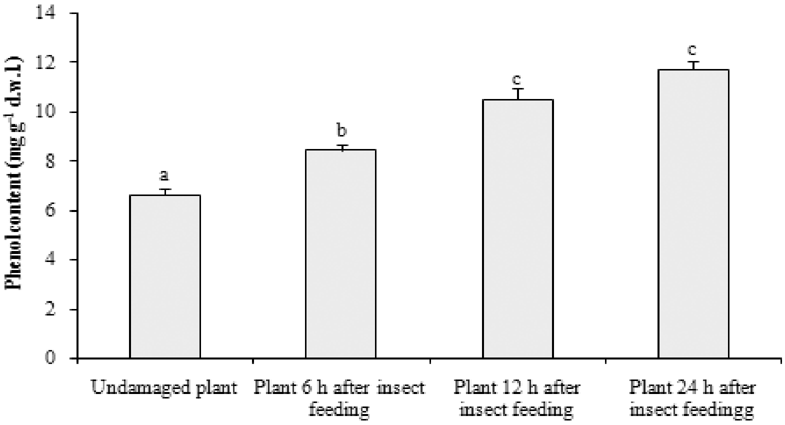

Figure 9. Total phenols (mg g^{-1} d.w.l.) of a damaged/undamaged *Solena amplexicaulis* plant and by feeding of *Aulacophora foveicollis* adults at different time intervals. Bars (N=5, Mean ± SE) with similar alphabets are not statistically different at $P < 0.05$; d.w.l. = dry weight of leaf tissue.

The thiol content differed significantly among the treatments ($F_{3,\ 16}$= 102.239, P <0.0001). The concentration of thiol content decreased significantly from undamaged plants to plants after continuous feeding by *A. foveicollis*. The concentration of thiol content was higher in plants 6 h after continuous feeding by *A. foveicollis* compared to plants 12 h and 24 h after continuous feeding by *A. foveicollis* (Figure 10).

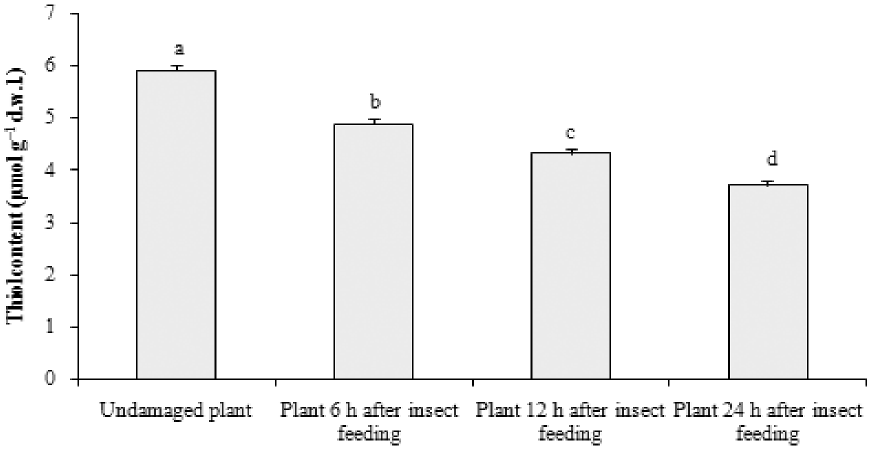

Figure 10. Total thiol content (µmol g^{-1} d.w.l) of a damaged/undamaged *Solena amplexicaulis* plant and by feeding of *Aulacophora foveicollis* adults at different time intervals. Bars (*N*=5, Mean ± SE) with similar alphabets are not statistically different at $P < 0.05$; d.w.l. = dry weight of leaf tissue.

The production of H_2O_2 caused by herbivore feeding might have several effects such as microbiocidal and mediating excess linking of cell wall polymers along with expression of genes encoding proteins involved in defensive and antioxidant processes (5, 6, 10, 12, 17). H_2O_2 has been implicated as a key factor mediating programmed cell death (PCD) that occurs during hypersensitive reactions in plants due to herbivore feeding (26, 37). Feeding by the larvae of *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) on soybean leaves (cv. Forrest or Hutcheson) indicated significant increase of H_2O_2 (5). Sing *et al.* (41) recorded an increase of peroxidases content in cowpea, cotton and tomato plants when fed by larvae of *S. litura* for 48 h compared to undamaged plants. This study also revealed an increase in H_2O_2 content in *S. amplexicaulis* leaves infested by adults of *A. foveicollis* during early feeding damage. Endogenous level of H_2O_2 is an important indicator of redox status of plant tissues and its ability to diffuse freely allows H_2O_2 to play as a control role in plant defence responses (3, 41).

The changes in antioxidative defence of *S. amplexicaulis* plants when attacked by *A. foveicollis* were assessed in terms of activities of SOD, CAT, APOX, GR and total thiol content of the plants within 6-24 h after continuous feeding by *A. foveicollis* adults. The present study revealed significant increment in the activity of the antioxidative enzymes particularly CAT, APOX and GR in insect damaged *S. amplexicaulis* plants compared to undamaged plants. However, the total thiol content was comparatively higher in undamaged *S. amplexicaulis* plants indicating that H_2O_2 might inactivate enzymes by oxidizing their thiol groups (5). The depletion of thiol compounds is one of the biochemical markers of oxidative stress.

The SOD serves as the first line of defense by catalyzing the superoxide into oxygen and H_2O_2 (36). It scavenges toxic free radicals generated in plants during herbivory (41). CAT, an important component of the oxygen-scavenging systems, converts the toxic and unstable ROS into less toxic and more stable components such as O_2 and water (3). A number of studies indicate that increased CAT activity in plants increases cell wall resistance and also serves as a signal for induction of defensive genes (11). The increased activity of APOX in insect damaged plants results a reduction in the availability of ascorbate in leaf tissues, which inhibits insect growth and development (1, 9, 10). Furthermore, scarcity of ascorbate in insect midgut causes an increase of the oxidative stress which results in generation of highly unstable ROS, including semiquinone, peroxides and hydroxyl radicals (1). APOX had a higher affinity for H_2O_2 and scavenges H_2O_2 to water and oxidizes phenolic compounds to quinones, which deters insect feeding (1, 12).

This study revealed that feeding by *A. foveicollis* on *S. amplexicaulis* plants resulted in an early increase in protein content in damaged leaf tissues of plants 6 h after continuous feeding by *A. foveicollis*. Increase in protein concentration in the damaged *S. amplexicaulis* plants is due to *A. foveicollis* feeding (37).

Generally, plants produce phenol compounds in the leaf tissue as a potential defense mechanism in plants against feeding by herbivorous insects (41, 43, 45). This study also indicated a significant increase of phenol content in the insect damaged leaf tissue within 6-24 h after insect feeding compared to undamaged plant tissue. Peroxidases and other oxidative enzymes oxidize phenols or their derivatives in the damaged tissues to form reactive quinones, which bind covalently to leaf proteins and inhibit the protein digestion in insect herbivores (4). Furthermore, quinones generate oxidative stress to insect herbivores by their direct toxicity to insects. Phenols also serve an important role in reduction of ROS (superoxide anion and hydroxide radicals, H_2O_2 and singlet oxygen), which in turn activate a cascade of reactions leading to the activation of defensive enzymes (26). Simple phenolics (salicylates) present in *Salix* leaves act as antifeedant to insect herbivores such as *Operophtera brumata* (L.) and a negative correlation was recorded between the salicylate levels and the larval growth (40).

Cell wall peroxidase oxidizes NADH and catalyzes the formation of O_2 and might serve as a major enzymatic system to control cellular damage (46). This enzyme employs H_2O_2 during the oxidation of NADH to NAD^+, which in turn reduces toxicity of H_2O_2 to water (3). The present study revealed a significant increase of class III peroxidases (SPX, GPX and PPX) in *A. foveicollis* damaged *S. amplexicaulis* leaves, which was in parallel with the accumulation of H_2O_2 content after *A. fovecollis* damage.

CONCLUSIONS

There was increase of CAT, SOD, peroxidases (SPX, GPX and PPX) and GR activity of the plants within 6-24 h after continuous feeding by *A. foveicollis* adults. The induced antioxidant responses are important components of plant-herbivore relationships. A significant increase in the APOX activity was recorded in *S. amplexicaulis* plants with an increase of feeding time by *A. foveicollis* adults, whereas a significant decrease in the accumulated H_2O_2 was observed in the damaged leaves with an increase of feeding time by the adults. The relation between an early enhancement of APOX activity and reduction of H_2O_2 content in the *A. foveicollis* damaged *S. amplexicaulis* plants implicates a protective role of this enzymatic antioxidant in the defence mechanisms of *S. amplexicaulis* plants. It is widely known that redox processes influence the chemical mediation of interspecific competition among herbivores sharing the same food plant. The production of ROS and scavenging mechanisms are inter-linked and the balance between them will determine the defence signalling output. However, it remains to be seen whether induced defence response helps to improve the competitive ability of *A. foveicollis* to displace other herbivores.

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