

PLANT SCIENCE

Gall-induced stress in the leaves of *Terminalia arjuna*, food plant of tropical tasar silkworm, *Antheraea mylitta*

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Abstract

Tasar silkworm *Antheraea mylitta* Drury is reared on one of the important food plants, *Terminalia arjuna* (Arjun). Leaf gall *Trioza fletcheri* minor (Hemiptera: Psyllidae) is the most important gall forming insect on the leaves which has bearing on productive traits of tasar silkworm. Gall formation is the consequence of interaction between the offensive stimulus of the insect and the defensive response of the plant. Hence, the stress on Arjun leaves imparted due to gall formation was studied. There was significant decrease in photosynthesis rate ($P < 0.001$), transpiration rate ($P < 0.05$) and stomatal conductance ($P < 0.05$) in gall infested leaves in comparison to healthy ones while no change in leaf temperature. The oxidative stress assayed through lipid peroxidation and hydrogen peroxide production was found to be significantly higher ($P < 0.001$) in gall infested leaves than that of healthy ones. Non-enzymatic anti-oxidant, ascorbic acid content was found to be high in gall leaves while there was decrease in reduced glutathione content ($P < 0.01$). The total protein and moisture content values were recorded to be higher in gall infested leaves ($P < 0.01$) than the healthy ones indicating towards nutrient flux for the gall insect.

Key words: *Antheraea mylitta*, Gall, Oxidative stress, Photosynthesis rate, *Terminalia arjuna*

Introduction

Commercially important tropical tasar silkworm *Antheraea mylitta* Drury is reared outdoor on *Terminalia tomentosa* (Asan) and *T. arjuna* (Arjun). Wild tasar silkworm populations are also found in forests in tropical India on *Shorea robusta* (Sal), Asan and some other secondary plants. All these primary and secondary food plants are attacked by large number of insect pests. Leaf gall *Trioza fletcheri* minor (Hemiptera: Psyllidae) is the most important gall forming insect on the leaves of primary tasar food plant (Singh and Thangavelu, 1994). A major part of the life cycle of gall insect is closely associated with the formation of gall guild in which the insect almost completes its development by deriving its nutrition from the gall tissues.

Gall formation is the consequence of interaction between the offensive stimulus of the insect and the defensive response of the plant (Raman, 2007). Since insects derive their nutrition from gall tissue,

the gall becomes a sink for different nutrients and energy that will be vital for gall insect's growth. The induction and development of galls expose plant tissues to high oxidative stress (Schonrogge et al., 2000; de Oliveira and Isaias, 2010; de Oliveira et al., 2010). Plants have several scavenging mechanisms to limit the accumulation of harmful amounts of active oxygen species (Chaman et al., 2001; Ni et al., 2001). The antioxidants such as glutathione, ascorbic acid, etc. scavenge reactive oxygen radicals, and they are substrates for the antioxidant enzymes. Development of gall has a bearing on the photosynthetic activity of the plant. The attack of gall inducers may cause either negative (Gailite et al., 2005; Aldea et al., 2006; Fernandes et al., 2010) or positive effects on the photosynthesis of the host organs (Fay et al., 1993; Bogatto et al., 1996). Functioning of the gall involves an effect of mechanical damage to various degrees and/or arthropod-derived chemical signals, both affecting endogenous plant signals and, consequently, physiology of host plants (Raman 2007). Studies on the status of oxidants, antioxidants and photosynthetic efficiency in gall infested leaves of *T. arjuna* is scanty. Thus, the idea was coined to study the stress on Arjun leaves exerted due to gall formation.

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Materials and Methods

Plant material and sampling procedure

Tasar silkworm host plant *Terminalia arjuna* (Arjun) was selected for the study. The period of sampling was 1st week of June when the gall infestation attains the peak. Healthy leaves (fourth leaf from the tip) and counterpart gall infested leaves were used for photosynthesis, transpiration and other parameters. Leaf area and number of galls were counted in all sets of samples so that the variation in gall frequency was at the minimum (data not shown). Leaf samples were immediately stored in -80°C deep freezer for further biochemical analysis. Identical sets of leaf were kept for moisture analysis.

Determination of net photosynthesis rate, transpiration rate, stomatal conductance and leaf temperature

Net photosynthesis rate, transpiration rate, stomatal conductance and leaf temperature were recorded in the healthy and gall infested leaves in the field under bright sunlight condition during the first week of June. These parameters were recorded with handheld photosynthesis system (CI-340) from CID Bioscience (USA). Each observation was repeated thrice for all the leaf samples.

Estimation of lipid peroxidation (LPX)

The level of lipid peroxidation (LPX) was measured in terms of malondialdehyde (MDA), a product of LPX estimated by thiobarbituric acid (TBA) reaction (Heath and Packer, 1968). Both in control and gall infestation, ten leaves each were taken as samples (n=10). Leaf sample (0.5 g) was homogenized in 10 ml of 0.1% (W/V) trichloroacetic acid (TCA), and the homogenate was centrifuged at 7000 x g for 10 min. The supernatant was mixed with 0.5% TBA solution in 20% TCA. The mixture was then heated at 95°C for 45 min and cooled under room temperature. The supernatant was read at 532 nm after removal of any interfering substances by centrifuging at 4000x g for 10 min. The amount of thiobarbituric acid reactive substances (TBARS) formed was calculated by using an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (Wills 1969), and expressed as nmol TBARS/g weight (wt) tissue.

Estimation of hydrogen peroxide (H2O2)

Hydrogen peroxide content was determined according to the method of Sergiev et al. (1997). Both in control and gall infestation, ten leaves each were taken as samples (n=10). Leaf sample (0.5 g) was homogenized with 10 ml of 0.1% (W/V) TCA

in an ice bath. The homogenate was centrifuged at 7000 x g for 10 min, and the supernatant was added with 50 mM potassium phosphate buffer (pH 7.0) and 1 M potassium iodide (KI). The absorbance was read at 390 nm. H₂O₂ was used as standard and expressed as nmol H₂O₂/g wt tissue.

Estimation of ascorbic acid, reduced glutathione and total proteins

For ascorbic acid assay, plant material was homogenized in an ice bath with 10 ml of 0.1% (W/V) TCA and centrifuged at 7000 x g for 10 min. The deproteinised supernatant was used as assay for ascorbic acid following the stoichiometric reduction of phosphomolybdate by ascorbic acid (Mitusi and Ohata, 1961). Ascorbic acid was used as the standard, and results were expressed as µg AsA/g wt tissue. Reduced glutathione content was estimated using Ellman's (DTNB) reagent (Ellman, 1959). Aliquots of supernatant were mixed with 0.6 mM DTNB and incubated for 30 min at room temperature in dark. The sample was finally centrifuged at 2,000 x g for 10 min at room temperature and the absorbance of the supernatant was measured at 412 nm. GSH was taken as standard and expressed as µmol GSH/g tissue. Protein concentration of samples was estimated according to the method of Lowry et al. (1951).

Statistical analysis

Data were analyzed for mean, standard error of mean (SEM), coefficient of variation (CV) and Student's t test using SPSS-11 (SPSS-Inc, Chicago, USA).

Results

For the comparison of healthy and gall infested leaves, *T. arjuna* plants in the economic plantation of field laboratory, CTR&TI, Ranchi were selected. Care was taken to select the position of leaves on the twig where one side was healthy and the other side was infested with gall in order to avoid the variation due to leaf position. Net photosynthesis rate in healthy Arjun leaves was found to be significantly more ($P < 0.001$) than that of gall infected leaves (Table 1). The coefficient of variation (CV) was found to be identical. Transpiration rate and stomatal conductance also showed the similar trend. Transpiration rate was significantly high ($P < 0.05$) in healthy leaves than the gall infected leaves (Table 1). The stomatal conductance also recorded to be high ($P < 0.05$) in healthy leaves in comparison to the gall infested leaves (Table 1). However, no significant difference was observed for the leaf temperature in both types of leaves (Table 1).

Table 1. Net photosynthesis, transpiration rate, stomatal conductance and leaf temperature of healthy and gall infected Arjun leaves.

Parameters	Status	Mean± SEM	Coefficient of variation (CV)	t value	p value
Net photosynthesis rate ($\mu\text{mol}/\text{m}^2/\text{sec}$)	H	11.31 ± 0.469	13.825	4.679**	0.001
	G	8.535 ± 0.311	12.132		
Transpiration rate ($\mu\text{mol}/\text{m}^2/\text{sec}$)	H	2.41 ± 0.114	15.794	2.359*	0.015
	G	2.02 ± 0.108	17.742		
Stomatal conductance ($\text{nmol}/\text{m}^2/\text{sec}$)	H	149.3 ± 8.642	19.292	1.831*	0.041
	G	126.85 ± 7.787	20.461		
Leaf temperature ($^{\circ}\text{C}$)	H	31.262 ± 0.147	1.566	1.751	0.098
	G	30.915 ± 0.117	1.262		

H – Healthy, G – Gall infested *P < 0.05, **P < 0.01

Insect herbivory of *T. arjuna* by *Trioza fletcheri* minor exerted a significant increase in lipid peroxidation ($P < 0.05$), measured as malondialdehyde, as compared to the healthy ones (Table 2). Infested Arjun leaves recorded higher level of hydrogen peroxide than the control healthy leaves which was statistically highly significant ($P < 0.001$) (Table 2). The coefficient of variation among data set was also comparable. Infested Arjun leaves had higher content of ascorbic acid as compared with their respective controls (Table 2). Stressed Arjun leaves showed significantly less ($P < 0.001$) reduced glutathione (GSH) in comparison to healthy counterpart (Table 2). Protein content in gall infested Arjun leaves was found to be significantly more ($P < 0.001$) in comparison to the healthy ones (Table 2). Similar type of observation was also recorded for the moisture content of leaf where the difference in the mean was statistically significant ($P < 0.01$) (Table 2).

Discussion

Net photosynthesis rate in healthy Arjun leaves was found to be significantly more than that of gall infected leaves. Transpiration rate and stomatal conductance also showed similar trend. This may be due to the reduction of net chlorophyllous area of leaf due to gall formation, besides, higher level of reactive oxygen formation and oxidative damages of cell membranes. However, no significant difference was observed for the leaf temperature in both types of leaves. Recent studies have shown lower photosynthetic rate (de Oliveira et al., 2011) and pigment contents in galls (Yang et al., 2003) which support our findings. In other studies, deficiency of several pigment-protein complexes of the light-harvesting complex of photosystem II in gall tissues could account for decreased photosynthetic function at the level of light-harvesting, energy transfer and photochemical energy conversion (Yang et al. 2007; Huang et al. 2009).

Table 2. Oxidative stress related and other parameters in healthy and gall infected Arjun leaves.

Parameters	Status	Mean± SEM	Coefficient of variation (CV)	t value	p value
Lipid peroxidation (LPX) nmol malondialdehyde (MDA)/g fresh tissue	H	151.42 ± 2.94	28.483	2.174*	0.022
	G	188.79 ± 9.93	17.523		
Hydrogen peroxide (H_2O_2) nmol /g fresh tissue	H	120.16 ± 4.61	12.789	3.528**	0.001
	G	149.52 ± 6.41	14.289		
Ascorbic acid content $\mu\text{g}/\text{g}$ fresh tissue	H	305.33 ± 36.75	40.116	0.645	0.263
	G	343.03 ± 41.51	40.334		
Reduced glutathione (GSH) $\mu\text{mol}/\text{g}$ fresh tissue	H	79.61 ± 1.231	5.153	7.349**	0.001
	G	62.51 ± 1.833	9.777		
Protein content mg/g fresh tissue	H	23.086 ± 1.248	18.011	3.814**	0.001
	G	29.428 ± 0.966	10.938		
Moisture content (%)	H	42.457 ± 1.474	11.573	2.634	0.01
	G	48.634 ± 1.515	10.381		

H – Healthy, G – Gall infested *P < 0.05, **P < 0.01

It has been shown that insect herbivory induces oxidative responses in plants (Chaman et al., 2001; Ni et al., 2001). Plants have several scavenging mechanisms to limit the accumulation of harmful amounts of reactive oxygen species. The antioxidants such as glutathione, ascorbic acid, phenolics, carotenoids and tannins scavenge reactive oxygen radicals, and they are substrates for the antioxidant enzymes (Valko et al., 2007). Insect herbivory of *T. arjuna* by *Trioza fletcheri* minor exerted a significant increase in lipid peroxidation, measured as malondialdehyde, compared with healthy ones. Biotic and abiotic stresses stimulate the production of active oxygen and subsequently lipid peroxidation of the cell macromolecules (Baker and Orlandi, 1996). The increase in lipid peroxidation may be due to the un-capability of antioxidants to capture all the reactive oxygen species produced by this biotic stress. Oxidation of unsaturated fatty acids by singlet oxygen produces distinctly different products such as MDA (Devasagayam et al., 2003). In the present study, MDA content as an expression of lipid peroxidation was increased by gall infestation.

Infested Arjun leaves had higher level of hydrogen peroxide than the control healthy leaves. According to Rosseti and Bonatti (2001) and Zentgraf (2007) one of the early events activated by the hypersensitive response (HR) is the production of ROS, including hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-). Stressed Arjun leaves showed significantly less reduced glutathione (GSH) in comparison to healthy counterpart. Ascorbate and glutathione play essential roles in plant metabolism and stress tolerance. Glutathione is a water soluble antioxidant which reacts directly or indirectly with the reactive oxygen species so, reduces stress injurious effects on membrane. Glutathione play an important role in the protection against oxidative stress. It is involved in the ascorbate/ glutathione cycle and in the regulation of protein thiol–disulphide redox status of plants in response to abiotic and biotic stress (Mullineaux and Rausch, 2005). The reduced glutathione content in the present study indicates their utilization during stress condition.

In the present study ASA level was found to be higher in gall infested leaves (Table 2). In contrast to ASA, reduced glutathione level significantly decreased in gall infested leaves when compared to healthy ones. In plants, ascorbic acid and glutathione are two important free radical scavengers and their formation is interrelated (Noctor and Foyer, 1998). Enhancement of H_2O_2 in the present study might have resulted in induction

of ASA synthesis and their conversion to protect cells against oxidative damage. Similar observations were made by Khattab and Khattab (2005) in Eucalyptus trees infested with gall.

Protein content in gall infested Arjun leaves was significantly more. Similar type of observation was also recorded for the moisture content of leaf. Singla and Grover (1994) recorded that the rate of protein synthesis declines during stress condition. The increase in protein may be due to the fact that the gall acts as a nutrient sink for the gall insects to develop. In our study the leaf sample was prepared including the gall and the insect residing in it. Dorchin et al. (2006) reported the photosynthesis and sink activity of wasp-induced galls in *Acacia pycnantha* where they found substantial increase in the availability of plant resources for the development of wasps in galls. So for the growth and metabolism, the protein flux might have been channelized to gall area. High moisture content also supports this hypothesis. However, during the process of leaf maturation, the galls fall apart and there is the chance of loss of protein content in the leaves, which needs further studies. Further studies on carbohydrate metabolism are required as there is indication of photosynthesis stress in gall infested leaves.

Conclusions

The study has come out with the finding that, due to gall infestation, the Arjun leaves face different types of stress. There is reduction in photosynthesis rate, respiration rate and stomatal conductance. Oxidative stress also increases as expressed through higher content of hydrogen peroxide. Higher content of lipid peroxidation indicates the damaging effects of ROS produced by gall infested plants. Furthermore, less glutathione indicates their utilization in scavenging action for free radicals. For completion of life cycle of the gall insect, there is a flux of protein content associated with increase in moisture. However, as a whole the leaf comes under stress condition.

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