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A study of the infestation on *Persea bombycina* kost by woolly beech aphids & its impact on host plant's secondary metabolite & lipooxygenase activity

Jyoti Wangnow, Sabita Gogoi*, Agu Pertin
Department of Life Sciences, Dibrugarh University, Dibrugarh, Assam, India

Abstract
From time immemorial, the cultivation of muga silkworm is very popular in Assam & adjoining part of North East India regardless of community and caste. In order to improve the quality and quantity of the golden silk produced by muga silkworm, one should have better knowledge about its host plant *Persea bombycina* Kost. Thus, present study report throws light on the changes in various biochemical parameters when this plant is infested by pest like woolly beech aphid or *Phyllaphis species.* On comparison between infested and non-infested leaves, it was found that the protein, tannin & phenol content (after 2 minutes & 2hours) in infested leaves are decreased by 131.1 mg/g/wet wt., 64.2 mg/g/wet wt., 199.12 mg/g/wet wt.(2 mins), 117.04 mg/g/wet wt.(2 hours) respectively. However, the lipoxygenase enzyme activity was found to be increased by 4.167 µg/min/gm wet wt. (after 15 seconds) & 7.734 µg/min/gm wet wt. (after 45 seconds).

Keywords: *Persea bombycina* kost, woolly beech aphid, secondary metabolite, protein, lipoxygenase

Introduction
Persea bombycina Kost, popularly known as "Som tree", is the primary host plant of Assam's pride Muga silkworm & is one of the most economical tree species of India. It is a perennial, nondecidous, aromatic tree belonging to the family Lauraceae [1]. The morphological feature of the plant includes rough grey coloured bark, presence of simple straight hairs on young shoots, lanceolate leaves with varying size & shapes, spreading branches & height up to 20-30 meters on maturity [2]. The species of Persea have a disjunct distribution to almost all parts of the world.

These plants are afflicted by a variety of pests. Any species or biotype of plant, animal, or pathogenic agent that causes harm to plants or plant products is considered a pest. One such pest which infest on *Persea bombycina* Kost is Woolly beech aphids or *Phyllaphis species.* Woolly beech aphids are small sized, 2-4mm long, oval shaped and pale yellowish green in colour. They have small eyes & fairly long antenna but are shorter in length as compared to the insect's body. It has mouth parts that are piercing and sucking type. They produce their young ones parthenogentically. One of the most distinguishing feature about the woolly beech aphids is that they produce filamentous waxy white covering around their body which resembles to cotton or wool, thereby they are known as woolly beech aphid [3, 4, 5]. These wax is produced by well-developed dorsal wax glands which are made up of greatly enlarged epidermal cells buried beneath a modified cuticle that forms distinct wax gland plates. The secreted wax, in the form of threads, exits the cuticle. The arrangement of these filaments in the cuticle above each epidermal cell provides rise to the distinct wax skin found in each species, whether hollow, solid, or honeycomb. Researchers suggested that the primary function of the secreted wax is to keep aphids from becoming contaminated by their own honeydew and that of other colony members [6, 7]. They feed on the undersides of matured leaves on both sides of the mid-rib.

Secondary plant metabolites are the bioactive chemical substances manufactured by plants via numerous metabolic pathways that are derived from primary metabolic pathways [8]. These substances aid a plant in important functions such as protection, competition and species interaction & are known to exert its effect on various physiological activities of plants such as flower induction, abscission, maintenance of growth & signal deciduous behaviour, fruit set etc. [9] Secondary metabolites are species-specific, and they affect different species in different ways. Secondary metabolites aid plants in maintaining a delicate balance with their surroundings, and they frequently alter to meet those needs. Terpenes, phenolics, and nitrogen-containing chemicals are the three types of secondary metabolites found in plants. Terpenes are the most common type of secondary metabolite. They are insoluble in water and play a role in plant growth; hence they are also classified as primary metabolites [10].

Phenolics are the most ubiquitously found secondary metabolite in plant kingdom. Some are water soluble, whereas others are only soluble in organic solvents. Phenols provide a range of They also play role in pollination and seed dispersal mechanism as pollinators and seed dispersers are attracted to the phenolic colourful pigments carotenoids and flavonoids [11]. Tannins are a type of phenolic polymer found in plants. They are broad-spectrum poisons that can impede the growth and survival of a variety of herbivores. Tannins also operate as a feeding

deterrent for a wide range of animals. Plant tannins also act as a defence against a variety of herbivores and diseases [12]. Alkaloids and cyanogenic glycocides are the most common nitrogen-containing chemicals. Recent studies highlighted a new trend in utilization of secondary metabolites in plant defence mechanism to combat bacterial, fungal and viral diseases [13]. Another important enzyme for stress tolerance is Lipoxygenase enzyme whose main function is initiation of heydroperoxidation of polysaturated fatty acids [14].

No study has been carried out about pest status of the woolly beech aphid with respect to *Persea bombycina* Kost. Besides the post infestation may cause biochemical changes in the host plants which may hamper to the economically important golden yellow silk producing muga silkworm *Antheraea assamensis.* Therefore, the present work was undertaken to study the infestation on *Persea bombycina* kost by woolly beech aphids and its impact on host plant with respect to secondary metabolites and lipoxygenase enzyme activity. To achieve the goal the following objectives were considered-

1. To study the life cycle of woolly beech aphids.
2. To estimate the population density of woolly beech aphid.
3. To evaluate the impact of woolly beech aphids on secondary metabolites and lipoxygenase enzyme of infested leaves.

Species Profile

Fig 1: Woolly aphids present on midrib of leaves

Systematic Position of Woolly Beech Aphid

Kingdom: Animalia
Phylum: Arthopoda
Class: Insecta
Infraclass: Neoptera
Subclass: Pterygota
Order: Hemiptera
Suborder: Sternorrhynca
Superfamily: Aphidoidea
Family: Aphidida
Subfamily: Phyllaphidinae
Genus: *Phyllaphis*

Materials and Methods

Study of life cycle of woolly beech aphid: By observing the development period of each instar, the life cycle of woolly beech aphid was studied. The number of days taken by each instar to moult into the next stage was recorded. Later the mean of all the days were calculated.

Estimation of population density: To estimate the population density of woolly beech aphid, five plants were considered. From each plant one leaf was taken to observe and count its population. Since the infestation by woolly beech aphid is small and in few plants only one leaf is infested so one leaf from each plant were taken to observe and estimate the population density of woolly beech aphid.

Population density is calculated as = total number of species /total no of leaves studied.

Biochemical analysis of leaves of infested plants

Leaves taken for biochemical analysis

1. Control leaf- they are the fresh leaves where it is not infested by woolly beech aphid and any other insects.

2. Early infested leaf- they are the leaves where 1st instar and 2nd instar nymph are found to attack the leaf. The early instar nymph just started to feed on the leaves.
3. Mid infested leaf- the leaves where 3rd and 4th instar nymph are found.
4. Late infested leaf- the leaves where 4th instar nymph and adult are found to infest on it and the pupa like substance are also found. The leaves are yellowish or brownish in colour.

Leaf extraction and determination of samples

Preparation of leaf extract for protein analysis: Protein was extracted in tris buffer and estimated by Lowry *et al*., method. 0.5 gm. of leaf sample was crushed in 10 ml buffer solution. Then it was centrifuge in 12000rpm for 10 minutes. The pellet obtained was discarded and the Supernatant was taken and suspended in 10% TCA in the ratio 1:1. It was then kept in -20C for 45minutes. It was again centrifuge for 10mins at 1000rpm at 4^0 C and the supernatant was discarded and the pellet was taken for further analysis. The pellet was suspended in 0.2 N NaOH and it was homogenised using glass rod and kept overnight. The clear sample observed is taken for protein extraction

Protocol

Table 1

Sample	Distilled water	Extract	reagent	interval	Follin reagent
Control	1 ml	00	5 ml	10 min	0.5 ml
Early infested	0.9 ml	0.1 ml	5 ml	10 min	0.5 ml
Mid infested	0.9 ml	0.1 ml	5 ml	10 min	0.5 ml
Late infested	0.9 ml	0.1 ml	5 ml	10 min	0.5 ml

Wait for 30 minutes and then O.D taken at 640nm

Preparation of sample extract & tannin determination

The tannin was determined by Follin Ciocalteu method. For extraction of tannin 250 gm of crude leaves from each type of leaves - non infested, early infested, mid infested and late infested were measured and taken. The leaves were then crushed in 15ml of 40% ethanol. Total volume made up to 25 ml by adding distilled water. The solution was then filtered with the help of whatman paper. The Clear solution was taken for determination of tannin.

Preparation of leaf extract for phenol & its determination

For preparation of leaf extract for protein estimation, at first 250 mg of leaves crude were crushed in 15ml of 40% ethanol and Sonicate for 30 mins. The total volume was made up to 25ml mixing with water. The solution was then filtered with whatman paper & obtained clear solution for phenol extract.

The concentration of phenolic compounds present in plants was determined by using spectrophotometric method. Follin ciocalteu assay was used for determination of total phenolic content in plant.

Determination of lipoxygenase enzyme

1. **Phosphate buffer preparation:** At first 800ml of Distilled water was prepared. To it added 20.209 g of $Na_2HPO_4.7H_2O$. Then 3.394g of sodium phosphate monobasic was added to the solution. The pH was adjusted to 5.8-7.4
2. **Linoleic acid preparation:** At first 10 milimole stock solution of linoleic acid was taken. Where it is converted to mg, the amount is equal to 28mg linoleic acid. Then 4ml of distilled water taken. 28mg of tween 20 was added to it to make a total volume to 10 ml. Milky emersion was produced. To the solution 0.55ml of 0.5 M NaOH was added. Then 5.45 ml of distilled water was added to the solution to make a total volume of 10 ml.
3. **Determination of lipoxygenase enzyme activity:** For determination of lipoxygenase enzyme 10 ml of sample extract were taken. To it add 9.8 ml of phosphate buffer solution. To the mixture solution 10 ml of linoleic acid was added. Then absorbance was taken after 15 seconds and 45 seconds at 234 nm in spectrophotometer.

Calculation

Working formula for protein, tannin and phenol estimation = concentration of BSA at 1 O.D *optical density of sample *dilution of sample Working formula for enzyme estimation =absorbance * total volume*1000000/molar extension coefficient*1*time of enzyme activity.

Result and Discussion

Life cycle: Life cycle of woolly beech aphid completes through following stages

Egg: The eggs are deposited on the underside of branches and forks of some shoots. Newly laid eggs are bright, slightly covered with wax. And with time the eggs become black.

Nymph: The eggs are hatched to nymph on March and April. They show mobility and nymph remain active during these period. They started feeding on the underside of leaves. The nymphs are almost similar to the adult except in their size.

1st instar nymph: The first instar nymph measures about 1 to 1.5 cm in length. It lasts for 2-3 days to moult to 2nd instar nymph. Only a slight amount of wax are present.

2nd instar nymph: The second instar nymph measures about 1.5 to 2.5 cm in length. It takes 3-4 days to moult to 3rd instar nymph. Waxes are clearly seen as a pair of waxy filaments on the terminal end of the abdomen.

3rd instar nymph: The third instar nymph measures about 2.5 to 3.5 cm. Wax filaments fully cover the abdomen region and are present on other body parts like head, thorax and sides of their body. It takes 5-6 days to moult to 4th instar nymph.

4th instar nymph: Fourth instar nymph measures about 3.5 to 4.5 cm. It lasts for 7-8 days to become an adult. Wax filaments are very long and they almost cover every part of their body, more densely on the terminal end of abdomen.

Wingless adult: they are of length 4.5 cm, and they are yellow- green oval body. Waxes are fully present on its body.

Winged adult: presence of wings on their body. Wax filaments almost cover their whole body. It measures about 5mm in length. The wax forms protective insulation against fungus, parasites and predators.

Table 2: Stages of woolly aphid's life cycle

1	Egg	1st instar	2nd instar	3rd instar	4th instar	adult
2	-	2-3days	4 days	4-5 days	6-7 days	Up to death

Some of the pictures depicting woolly beech aphid's life cycle are

Fig 2: wingless adult

Fig 3: winged adult

Fig 4: Dorsal view of woolly beech aphid

Fig 5: Ventral view showing piercing & Sucking type mouth part

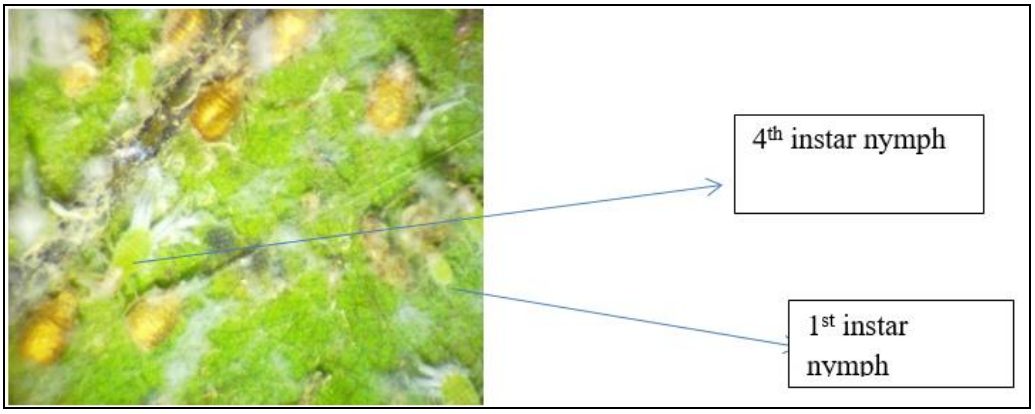

Fig 6: 1st and 4th instar nymph

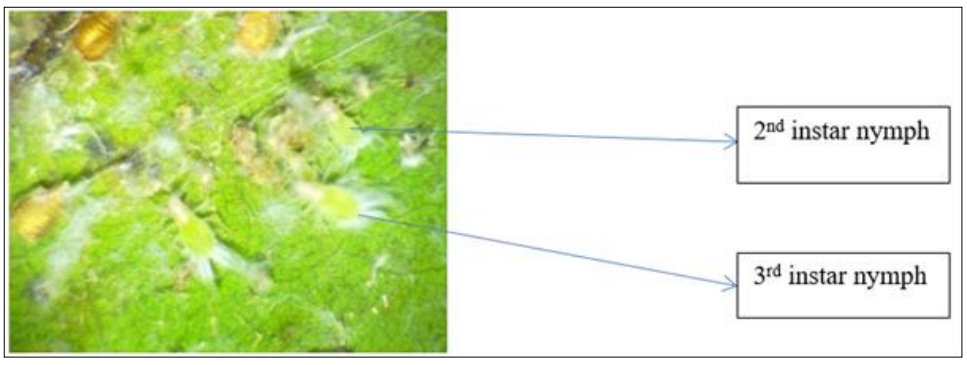

Fig 7: 2nd and 3rd instar nymph

Leaf infestation

Effect on leaf due to woolly beech aphid infestation

Fig 8: Small back spot seen in

Fig 9: Yellowing of leaf after 3 days early infestation.

Fig 10: Fully yellowing of leaf after 6-7 days

Fig 11: Brown spots developed after infection after 9-10 days

Fig 12: Brownish spots become enlarge

Fig 13: The leaf become completely after 12-13 days of infection yellow & completely dries up & wither

Table 3: Depicting total population of each instar in the sample plants

SI. No.	Plant	1st instar	2nd instar	3rd instar	4th instar	Adult wingless	Adult winged
1	1	147	113	84	42	12	2
2	2	94	61	59	48	8	2
3	3	72	65	60	28	7	1
4	4	149	138	134	58	6	5
5	5	63	51	42	17	3	1
total		525	428	379	193	36	11

Table 4: depicting the density of all the instar and adult of woolly beech aphids

Stages	1st instar	2nd instar	3rd instar	4th instar	Adult wingless	Adult winged
Density	105	85.6	75.8	38.6	7.2	2.2

The study revealed that among all the stages of life cycle, the population density of woolly beech aphid on the *Persea bombycina* Kost is highest in 1st instar larvae which is about 105 aphids per leaf and the lowest density is found in winged adult comprising 2.2 aphids per leaf.. Their population are not found to be constant. This

population fluctuation may depend on various environmental parameters like temperatures, humidity, and rainfall. It was observed that there is decrease in woolly aphid population size at high temperature. The presence of predators like beetles and some hymenopteran insects are also responsible to decrease the population of woolly beech aphids. Some coccinelids beetles, hemipterans and hymenopteran insects found on the same plants may also consume the nymph of woolly beech aphid and thus decrease the woolly aphid's population. The nymphs especially, the 3rd and 4th instar larvae are found to travel to other leaves before moulting to adult, which may be in search of food materials or in search of suitable site for oviposition. The latter phenomenon may also lead to reduction of aphid population.

Table 5: Showing biochemical analysis of leaves of Persia bombycina kost before & after infestation by woolly beech aphids.

Sl. No	Biochemical	Control leaf	Early infested	Mid- infested	Late infested
1	Protein (mg/g/wet wt.)	450.3	129.96	139.65	319.2
2	Tannin (mg/g/wet wt.)	165.6	133.8	124.2	101.4
3	Phenol (after 2 mins) (mg/g/wet wt.)	443.08	133.76	213.56	243.96
4	Phenol final (after 2 hours)	468.92	147.44	352.92	351.88
5	Lipoxygenase enzyme activity (15 secs) (µg/min/gm wet wt.)	1.856	5.568	4.64	6.023
6	Lipoxygenase enzyme activity (45 secs) (µg/min/gm/wet wt.)	1.546	2.01	2.629	9.28

On comparison between non-infested leaves and late infested leaves the Proteins, tannin, and phenol content (after 2 minutes and 2 hours) in infected leaves are reduced by 131.1 mg/g/wet wt., 64.2 mg/g/wet wt., 199.12 mg/g/wet wt.(2 mins), 117.04 mg/g/wet wt.(2 hours), respectively. However, after 15 seconds, the lipoxygenase enzyme activity increased by 4.167 g/min/gm wet wt. and 7.734 g/min/gm wet wt (after 45 seconds).

The result obtained from biochemical analysis of infested and non-infested leaves of *Persea bombycina* Kost shows that the overall protein content in infested leaf is decreased as compared to control leaf (non infested). But it was also observed that among infested leaves there is an increasing trend in protein content from early infestation to late infestation. However, opposite scenario was observed in Lipoxygenase activity & it was found to be increased in infested leaves. While lipoxygenase activity enhancement may indicate defensive response of the infested leaves, the reduced protein level may indicate adverse effect caused on the plant due to infestation by the woolly beech aphids. Further analysis of the secondary metabolites revealed that the phenol and tannin level both decreased in all the stages of infestation. This further indicates the deleterious effect of the infestation by woolly peach aphid *Persea bombycina* as enhancement of phenol or polyphenol is reported in several cases to be related to plant defence mechanism.

The study therefore indicated woolly beech aphid as a potential pest *of Persea bombycina* Kost which may reduce the nutritional quality of som plants to be fed to muga silkworms. However further research is required to understand the effect on other primary and secondary metabolites.

Conclusion

Persea bombycina Kost constitute the sole food of muga silkworm *Antheraea assamensis*, it is therefore obvious that the quality of Persea sp. has a predominant influence on the growth and development of the silkworm and the quality of the cocoon. Though frequently harvesting and pruning up the shoot restricts the attack of pest, they still find enough time for their feeding and breeding thus contributing towards bringing down the foliage both in terms of quantity and quality. Though climatic conditions favour the growth and development of this som plant in North East India, the pest infection in turns retards its growth and productivity. Thus it is very much essential to control these pests through suitable effective strategies. Conservation and preservation of existing natural indigenous enemies of the pest and providing alternating hosts for the pest can be one of such measures to overcome such noxious organisms to a tolerable level. Effective and handy methods should be encouraged to reduce these harmful pests so as to minimise the severity of the damage cause to this host plant.

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