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Alteration of enzyme-levels in tea leaves under attack of Tea Mosquito Bug, *Helopeltis theivora* (Hemiptera: Miridae)

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Abstract

Tea Mosquito Bug, *Helopeltis theivora* Waterhouse (Hemiptera: Miridae) is a serious pest of tea (*Camellia sinensis* L.) causes considerable damage to the crop. Severe outbreak of the pest and crop-loss were reported from time to time from Assam, Dooars and Tarai areas. The nymphs and adults of *Helopeltis* suck the sap of the young leaves, buds, and tender stems and while doing so they inject toxic saliva which causes the breakdown of tissues surrounding the puncture. The badly affected leaves become deformed and even curl up. In severe attack, bushes virtually cease to form shoots and may not flush for weeks together. To find out the changes at biochemical level in a tea leaf under attack of *Helopeltis theivora*, the present study is carried out with respect to the changes of oxidative enzyme level, acid phosphatase level, concerned in the phenolic metabolism since phenol compounds are the most important and characteristic components of tea leaves. The oxidative enzymes which catalyzed the oxidation of phenols (peroxidase and polyphenol oxidase) increased two times their levels of occurrence in *H. theivora* sucked plant leaves. Ascorbic acid oxidase and IAA-oxidase levels were higher in the un-attacked tissue than in the sucked tissue. Acid phosphatase level in the sucked tissue (4-6 hours after attack) was reduced than in the un-attacked tissue. Plant tissues produce phenol compounds in their cells as a response to the attack by parasite/ attacker to protect themselves from the invaders. High levels of polyphenol oxidase and peroxidase and moreover the amounts of phenol compound imply that quinones are produced in the sucked and surrounding tissues. The toxic action of quinones may be a cause of the permanent malformation of the leaf attacked by *Helopeltis.* The synthesis of quinones from flavanol may also influence the colour and quality of the tea. Beside the heavy loss in weight due to the necrosis of the tea leaves, the advanced polyphenol oxidase activity at higher level changes colour of the leaves and causes fermentation before the actual processing in the factory.

Keywords: *Helopeltis theivora*, Leaf enzyme, Pest, Tea, Tea Mosquito Bug.

Introduction

Darjeeling hill is known throughout the world as “the champagne of teas”. Later on tea cultivation has also spread in the foothills and adjacent plain, known as Darjeeling Terai and Dooars. The tea from Dooars and Terai has strong liquors and is immensely popular in the domestic market and neighbouring countries. This tea growing area falls under the warm humid agro-eco region with brown and red hill soils encompassing northern hilly parts of West Bengal. The region occupies an area of 9.6 million hectare representing 2.9% of the Geographical area of the country.

Each tea growing region has its own distinctive pest fauna though many species have been recorded from more than one country. About 230 species of arthropods are known to attack tea in India (Muraleedharan, 2007).

Increase in tea productivity requires extensive monoculture which leads to rise in pest attack. A steady loss of 10% due to overall pest attack is a generally accepted figure though it can be 40% in devastating attack by defoliators (Banerjee, 1983). Investigation had shown that 8-17% increase in crop could be obtained following efficient methods of control of pest (Rao and Subrahmaniam, 1968).

Considerable loss of crop due to damage caused by Tea Mosquito Bug (*Helopeltis theivora*) a serious pest of tea was recorded as early as in 1865 from Cachar when a company lost about 22,727 kg (50,000 lbs) of crop for which a commissioner was appointed by the Government in 1885 and 1887 to look into the problem (Watt and Mann, 1903). Severe outbreaks of the pest and crop-loss were reported from time to time from the Dooars. In 1958, the loss incurred by eleven gardens in the Nagrakata Sub-District, West Bengal was estimated to be about 7,50,000 kg of made tea (Das, 1965). During 1978, an estate in Upper Assam near Margherita suffered a loss of about 30,000 kg of made tea due to the attack of *Helopeltis*. In 1992-93 one estate lost about 50% of the annual crop in Central Dooars (Barbora and Singh, 1994). In recent years *Helopeltis* shows a discontinuous population due to heavy control measures by some gardens, although in general its population has spread to other regions to cause concern, economic crop losses are also reported from Assam and Darjeeling (Debnath, 2011).

The nymph and adults of *Helopeltis* suck the sap of the young leaves, buds and tender stems and while doing so they inject toxic saliva which causes the breakdown of tissues surrounding the puncture. Within 2-3 hours of sucking, a circular spot is formed around the sucking points and in 24 hours the inside portion of the ring becomes translucent, light brownish and within a few days the spots appear as dark brown sunken spots which subsequently dry up. The badly affected leaves become deformed and even curl up. In a severe attack, bushes virtually cease to form shoots and may not flush for weeks together (Majumdar, 2015).

There have been a lot of works concerning the effect of toxic substances produced by injured plant tissue on the parasite and the plant tissue itself. Along with the attack of parasites or pests on a plant, the amount of phenolic compounds increases in the cells of the injured parts, followed by the production of the substances promoting necrosis of tissue. The necrosis promoting substances in the cells destroy rapidly the cells themselves and their neighbouring cells, releasing polyphenol oxidase and peroxidise (Hori, 1973). These oxidases catalyze the oxidation of phenols to produce quinones, which prevent parasites from attacking the plant tissue by the toxic reaction. However, quinones are transformed into insoluble brown non-toxic substances (polymers, melanine like substance and coagulative protein complexes) by the oxidation owing to the catalysis of polyphenol oxidase (from the plant or parasites). Some sedentary insects have polyphenol oxidase system in their saliva and can transform quinones, which are toxic to the insects, into non-toxic substances (Miles, 1968, 1969).

The leaf tissue of tea attacked by *Helopeltis* sp. accumulates brown coloured materials around the sucking spots (Majumdar, 2015) like that of *Lygus disponsi* when feeding on sugar beet leaf (Hori, 1971). Similar brown materials are found in the lesion of plants attacked by mirid bugs (Sarkar, 2006; Miles, 1968). The injured leaf of tea shows various malformations in external appearance like that of the sugar beet leaf when attacked by *L. disponsi* (Hori, 1967).

The most important and characteristic components of tea leaves are the polyphenolic compounds; they are mainly responsible for the unique character of processed teas (Roberts, 1962). Out of the polyphenolic compounds identified in fresh tea flush, flavanols are oxidized by polyphenol oxidase during processing of tea and they are the major determinates of the colour of tea brews.

The determination of the quality and quantity of tea leaves is expected resulting from the underlying histochemical changes due to extensive exploitation of the leaf tissue by *Helopeltis*. There are only few studies on the physiology of the injury caused by mirid bugs (Strong, 1970), which therefore, leaves an opportunity to investigate such an interesting area of insect-plant relationship, especially when it concerns the tea leaves. To find out the changes at biochemical

level in a tea leaf under attack of *Helopeltis theivora* and in order to learn the phenolic changes that possibly take part in the "physiology of the injured tea leaves", the present study was carried out at the enzyme level with respect to levels of enzymes like, acid phosphatase, peroxidase, polyphenol oxidase, ascorbic acid oxidase, IAA oxidase, which are directly or indirectly connected with the phenolic metabolism since phenol compounds are the most important and characteristic components of tea leaves.

Occurrence and depredation of *Helopeltis theivora*:

A. Description:

The adult Tea Mosquito Bug (*Helopeltis theivora* Waterhouse) is a tiny insect with head black or olive green; thorax pale yellow and black to greenish black. Abdominal colour of Darjeeling specimen is often reddish as a result of sucking up leaf juices with high anthocyanin content. Antennae long and the drum-stick shaped horn strongly recurved to the rear and terminated by a relatively large knob.

The freshly hatched, dirty yellow nymphs are with bright pink antennae and eyes. General colour of the first and second instar nymphs is greenish yellow which turns green in the advanced instars (Roy, 2015).

B. Habits:

The nymphs and adults are active in the morning and afternoon, but when sun becomes hot, they seek shelter in shaded twigs or descend to sheltered places, however; a few early nymphs may remain on damaged leaves. On a dull cloudy day, the bug population may be found on the top hamper of the bushes at any time of the day. The adults rarely migrate unless distributed and the spread is mainly affected by wind, carrying them long distances. They remain persistently in some localised areas, particularly adjacent to jungle and abandoned tea plantation from which they often spread when condition are favourable (Roy, 2015).

C. Alternate hosts:

Melastoma malabathricum (wild rhododendron), *Maesa ramentacea*, *Eurya acuminata*, *Jasminum scandens*, *Mikania micrantha* (a common creeper) are recorded as alternative hosts of tea mosquito bug. *Acalypha* sp. (an ornamental plant) has recently been observed to be another host plant on which the insect breeds throughout the year in Terai (Roy, 2015).

Figure: 1. A & B. *Helopeltis theivora* on tea leaves.

D. Seasonal Incidence:

Adults and nymphs of *Helopeltis* could be seen on tea bushes almost throughout the year but peak of the incidence is noticed during June-July and August-September. With the advent of winter the population of *Helopeltis* dwindles down gradually, reappearing again in February-March (Roy, 2015).

E. Nature of damage:

Both nymph and adults of *Helopeltis* suck the sap of the young leaves, buds and tender stems and while doing so, it injects toxic saliva which causes the breakdown of tissues surrounding the puncture. Within 2-3 hours of sucking, a circular spot is formed around the sucking point and in 24 hours the inside portion of the ring becomes translucent, light brownish and within a few days the spots appear as dark brown sunken spots which subsequently dry up. The badly affected leaves become deformed and even curl up. In a severe attack, bushes virtually cease to form shoots and affected area may not flush for weeks together (Majumdar, 2015; Roy, 2015).

A nymph of the 1st instar and the adults produce highest number of sucking spots (106) followed by 2nd (74), 4th (72) and 3rd instar (28) within 24 hours. Moreover, total number of spots produced by an adult was recorded to be 2358 within a period of 21-26 days (Barbora and Singh, 1994).

Figure: 2. Tea leaves under attack of *Helopeltis theivora.* (A) Within 2-3 hours of sucking, a circular spot is formed around the sucking points. (B) In 24 hours the inside portion of the ring becomes translucent, light brownish. (C) The badly affected leaves become deformed and even curl up. (D) In a severe attack, bushes virtually cease to form shoots.

F. Susceptibility of Tea:

Recent studies on the Tocklai varieties, have revealed that TV1 and TV26 are very susceptible to *Helopeltis* attack followed by TV4, TV5, TV6, TV9, Dehing12, S3A3 and TeenAli 17 while TV18 and P126 are relating tolerant (Barbora and Singh, 1994).

Materials and Methods

A. Selection of plant variety:

Tests were carried out using only one variety of tea, TV1 leaves. The changes in the level of a group of enzymes in response to the attack of *Helopeltis theivora* were estimated. TV1 is one of the earliest and

standard clones released by "Tocklai Experimental station", Assam, India in 1949. TV1 is characterized by high yield potential and high quality. It has a compact frame with acute branch angle (50^0) leaves are erect, medium sized with pubescence on lower surface and sunken stomata, surface matty in nature. It is a hybrid of Assam and China in origin.

B. Biochemical Studies:

Although some literature is available on the population study of *Helopeltis* and its attack on tea plants and also on its control measures, yet very little is known about the feeding impact of the pest at histochemical level. For comparison, observations were made on control tea leaf and freshly sucked tea leaf with respect to the important enzymes.

C. Collection of leaf material:

Both the freshly sucked and control leaves were collected from field, i.e. from the Sannyasisthan Tea Estate (Upper Bagdogra, Siliguri, West Bengal, 26.4^0 N, and 88.26^0 E.), the location belongs to the foothills of the district of Darjeeling. The heavily sucked leaves (the young leaves and buds) were collected just within 12 hours +/- 2 hours of sucking from the healthy bushes of TV1 clone. At the same time young control leaves (of same age) were also collected from similar portions of the same bushes having no infestation. Just after collection, the leaves were then immediately stored in an ice box and taken to the laboratory for preventing any biochemical changes.

D. Preparation of test solution:

The sample leaves collected were extracted with 10 ml distilled water per gram of fresh weight in mortar and pestle with quartz sand and then in the glass cell tissue homogenizer. After refrigerated centrifugation at 15,000 rpm the supernatant was used for various assays or analyses (modified after Hori, 1973).

E. Determination of enzyme level:

I. Peroxidase:

The test solutions were diluted 20 fold for determination of peroxidase level. The determination of the enzyme level was based on the method of Chance and Maehly (1955). The reaction mixture consisted of 0.5 ml phosphate buffer (pH 7.0, 0.07M), 0.5 ml H_2O, 1.0 ml enzyme solution, 1.0 ml Guaiacol (0.02M) and 0.5 ml.H_2O_2 (0.3%). The enzyme reaction was initiated by adding H_2O_2 to the reaction mixture in a glass cell of spectrophotometer. The enzyme level was then determined and represented as (Optical Density) OD units at 470 nm, using a Hitachi 101 spectrophotometer against an appropriate blank.

II. Polyphenoloxidase:

For the determination of polyphenoloxidase level, the test solutions were diluted 20 fold. The determination of the enzyme level was carried out by modifying the method of Ponting and Joslyn (1948). The reaction mixture consisted of 2.0 ml phosphate buffer (pH 7.0, 0.07 M), 1.0 ml Catechol (1.6%) and 1.0 ml enzyme solution. The enzyme reaction was initiated by adding the enzyme solution to the reaction mixture in a glass cell. The enzyme level was then recorded and represented as OD units at 470 nm against an appropriate blank.

III. Acid Phosphatase:

Acid phosphatase level was determined by p-nitrophenol method (Omori, 1937) with the test solution diluted 80 fold. The reaction mixture was composed of 0.5 ml Acetic acid buffer (pH 5.5, 0.1M), 1.0 ml p-nitro phenylphosphate disodium salt (0.001M) and 0.5 ml enzyme solution. After the incubation for 30 min at 37 C, 2.0 ml of saturated Sodium carbonate solution was added to the reaction mixture. The enzyme level was then determined and represented as OD units at 405 nm against an appropriate blank.

IV. IAA- Oxidase:

IAA-oxidase level determination was performed by the method of Gordon and Weber (1951), the method of quantitative analysis of IAA (3- indoleacetic acid). The reaction mixture consisted of 1.0 ml phosphate buffer (pH 5.0, 0.05 M), 1.0 ml IAA solution (0.02%) and 1.0 ml enzyme solution. After the reaction for 2 hours at 30 C, 3.0 ml trichloroacetic acid solution (5.0%) was added to the reaction mixture in order to deproteinize. After the filtration, 3.0 ml Salkowski reagent ($FeCl_3$ reagent) was added to 1.5 ml of the filtrate, which was then incubated for 30 min at 30 C. The IAA oxidase level in the sucked tissue was compared with that in the control tissue by the amount of remnant of IAA (expressed as OD units at 530 nm against an appropriate blank).

V. Ascorbic Acid Oxidase:

Ascorbic acid oxidase level was represented as the amount of remnant ascorbic acid after the oxidation of ascorbic acid by the enzyme. The reaction mixture was composed of 1.0 ml phosphate buffer (pH 6.0, 0.07 M), 1.0 ml H_20 and 1.0 ml enzyme solution (Ponting, 1948). After the incubation for 10 min at 25 C, the reaction mixture was deproteinized by $ZnS0_4$ and $Ba(OH)_2$ solutions, and percolated through Toyo No.3 filter paper. OD units of the filtrate were determined at 260 nm against an appropriate blank.

Observations and Results

Enzyme Levels in the freshly sucked and unattacked tissues of tea leaves:

The sucked leaf tissues used for this investigation showed heavy damage symptoms, externally marked by round necrotic brown spots. The results are shown in the table 1 in which the average values have been calculated based on 6 replicates from different sucked and unattacked (control) tea leaves. In general, the colour of the extract from the sucked tissue (pale brown) differed from that of the unattacked (control) tissue (pale yellow).

Table 1: Enzyme levels in the extracts obtained from the freshly sucked (12 +/- 2 hours) and unattacked (control) tea leaf tissues by *Helopeltis theivora* (n = 6).

Assayed materials	Units of measurement	Measurements (as per gram of fresh weight)		Relative value (Taking control as 100)	T-test (p<0.001)
		Sucked leaf tissue	Un-attacked leaf tissue (Control)		
Peroxidase	OD units at 470 nm	0.015 ± 0.003	0.0081 ± 0.0007	185	S
Polyphenoloxidase	OD units at 470 nm	0.152 ± 0.003	0.0815 ± 0.0018	186	S
Acid Phosphatase	OD units at 405 nm	0.053 ± 0.0015	0.131 ± 0.002	40	S
IAA- Oxidase	OD units at 530 nm	0.124 ± 0.0009	0.513 ± 0.0029	24	S
Ascorbic Acid Oxidase	OD units at 260 nm	2.153 ± 0.009	2.235 ± 0.006	96	S

The oxidative enzymes which catalyzed the oxidation of phenols (peroxidase and polyphenoloxidase) increased their levels of occurrence as the result of sucking by *Helopeltis theivora* to the plant leaf. Peroxidase and polyphenoloxidase levels in the sucked tissue were about two times as high as those in the unattacked (control) tissue respectively. Ascorbic acid oxidase and IAA-oxidase levels were higher in the unattacked tissue than in the sucked tissue. Acid phosphatase level in the sucked tissue (4-6 hours after attack) was rather reduced than in the un-attacked tissue.

Discussion

Plant tissues produce phenol components in their cells as a response to the attack by parasites/ attacker to protect themselves from the invaders. The attacked cells turnout the factors accelerating necrosis and destroying rapidly their own neighbouring cells (hypersensitive reaction) to prevent the parasites from further invasion. Here, polyphenoloxidase and peroxidase are released from the broken cells. By catalysing the oxidation, they produce quinones which give a toxic action to the plant tissue itself (hypersensitive reaction) and an antibiotic action to the parasites as well. However, quinones may be reversed to phenols by quinone-reductase or may be, polymerized by the catalysis of polyphenol oxidase, into melanin like substances, or may sometimes be combined with amino acids or proteins to form coagulative protein complexes. Thus, phenols may get transformed into some non-toxic substances (Uritani, 1963). Some sedentary insects seem to detoxify quinones by polyphenoloxidase contained in their saliva and thus succeed in making permanent attack on their host plant (Sarkar, 2006 and Miles, 1969).

In the study, it was found that polyphenoloxidase and peroxidise activities increased markedly in the tissue of the tea leaf attacked by *Helopeltis theivora.* Ishaaya (1971a) and Hori (1973) found phenomenons similar to this in lemon buds infested by *Aceria sheldoni* and sugar beet leaf by *Lygus disponsi* respectively. The fact that polyphenoloxidase and peroxidise activities in the sucked tissue remained at high level might be attributed to some of the following causes:

(a) Certain fungi invade through the feeding punctures of the bugs and accelerate continuously the production of the oxidative enzymes in the plant tissues.
(b) The Change in environmental condition of the sucked part of plant tissue (Change in concentration and composition of amino acids and sugars) causes the production system of the enzyme to be altered.
(c) Certain phytotoxin in the saliva of the bug remains in the sucked tissue for long period and promotes the production of the oxidative enzymes (Schaller, 1968).
(d) It is also possible that this phenomenon is not due to a single cause but to the complex of various factors.

Acid phosphatase activity in the freshly sucked tea leaf tissue was rather lower than that in the unattacked tissue. A similar situation was observed in sugar beet leaf infested by another mirid, *Lygus disponsi* (Hori, 1973). Ascorbic acid oxidase level was lower in sucked tissue than that in the unattacked tissue. Ascorbic acid is required in the metabolism of tyrosine, tryptophan and phenylalanine. The biosynthesis of polyphenol may take place by an alternative route which involves phenylalanine as an intermediate (Neish, 1960; Stafford, 1974), further a shikimic acid-phenylalanine-flavanol pathway has also been suggested by Zaprometov et al. in 1963. It has also been established (Iwasa, 1976) that shikimic acid is phenylalanine 14_C itself. It is possible therefore; that in tea leaves the pathway of flavanol biosynthesis may not necessarily include phenylalanine as an obligate intermediate.

In the sucked tissue, polyphenoloxidase and peroxidase levels and moreover the amount of phenol compounds, which takes part in energy metabolism necessary for phenol metabolism, were at high levels. These facts imply that quinones are produced contentiously in the sucked and surrounding tissues. Since *H. theivora* is not a sedentary insect like *L. disponsi*, the bug may not be able to contentiously transform quinones into non-toxic substances by polyphenoloxidase present in the saliva unlike some sedentary insects (Miles 1968, 1969; Ishaaya 1971b). In addition to the peculiar sucking spots on the leaf and the destruction of tissue by polygalacturonase (usually present in the saliva) and the toxic action of quinones may be a cause of the permanent malformation of the leaf attacked by *Helopeltis*.

Evidence of increased levels of oxidative enzymes and phenolic compounds in co-ordination with the enzymatic digestion by salivary polygalacturonase has been recorded in the damaged area as a cause of feeding by various hemiptera insects (Strong, 1970; Hori, 1973, 1975; Hori and Atalay, 1980). Further, due to the action of these chemicals, the feeding areas are considered as regions of increased metabolic activities (Way and Cammell, 1970) characterised by an action-imbalance of growth promoting substances (Hori, 1975). The tissue surrounding the part sucked by *H. theivora* possibly suffers the toxic action of quinones produced by the plant tissue itself, and necrosis occurs because quinones inactivate amino acids, IAA and various enzymes (Schaller, 1968). Thus, it is likely that the causes of the injury in the tea leaf attack by the bug *H. theivora* are firstly there peculiar feedings punctures, secondly the destructions of the tissue by polygalacturonase in the saliva, thirdly the factor which keep polyphenoloxidase and peroxidase in the sucked and surroundings tissues at high levels (phytotoxin in the saliva and the alteration of the concentration and composition of amino acids and sugars in the plant tissue), and fourthly the long lasting presence of the quinones at higher level in the sucked and neighbouring tissues. The brown substances produced around the feeding cavity of mirids are possibly polymers, melanin like substances or coagulative protein complexes originating from quinones (Awati, 1914; Smith, 1926; Leach and Smee, 1933; Hori, 1971; Sarkar, 2006).

Polyphenolic compounds are mainly responsible for the unique character of processed tea (Roberts, 1962). The main effect is a progressive decline in total phenolic material along with an alteration in the proportions of flavanols. Out of the polyphenol compounds, flavanols are oxidised by polyphenoloxidase during processing of tea and they are the major determinants of tea colour of tea brews. Enzymatic oxidation of catechins (polyphenol compound) and their subsequent modifications to a great extent determine the quality of the brew.

The amino acid has particular relevance in the manufacture of green tea as it protects enzymes from inactivation by polyphenolic products and is primarily responsible for the quality of green tea. Increased polyphenoloxidase also helps in fermenting tea leaves.

So, beside the heavy loss in weight due to the necrosis of the tea leaves, the advanced polyphenoloxidase activity at higher level changes colour of the leaves and causes fermentation before the actual processing in the factory. Possibly higher activity of polyphenoloxidase and peroxidase causes the synthesis of quinones from flavanol which may influence the colour and quality of the tea.

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