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Research article

BIOCHEMICAL DEFENCE MECHANISM IN *CAMELLIA SINENSIS* AGAINST *HELOPELTIS THEIVORA.*

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ABSTRACT: The state of Assam located in the north-eastern part of India is the world's largest tea growing region**.** *Helopeltis theivora* is a major pest of tea which causes damage to two and a bud of the plant from which the actual tea beverage is prepared. Hence an attempt is made to understand its biochemical changes and hence its defence mechanism. The biochemical parameters such as protein, carbohydrate, phenol, flavonoid, photosynthetic pigment (total chlorophyll, chlorophyll-a, chlorophyll-b), antioxidant enzymes viz; polyphenol-oxidase and peroxidase of non- infected and infected tea leaves were analysed.The infected and non-infected two and a bud of tea clones- TV1, TV23 (most susceptible), S3A3, Tinali (moderately susceptible) collected from 5 leading tea gardens located in Dibrugarh district, Assam, India was selected for the study. The results revealed that all varieties have varying levels of infectivity. With infection total protein, carbohydrate, phenol, chlorophyll, flavonoid decreases while oxidative enzymes viz; peroxidase, polyphenol-oxidase increases. The results showed that biochemical changes in host might be the outcome of oxidative stress and biochemical defence mechanism of *helopeltis* infested tea leaves.

Keywords: *Camellia sinensis*, *Helopeltis theivora*, biochemical changes, defence mechanism.

INTRODUCTION

Tea (*Camellia sinensis*) the most popular drink worldwide is prepared from the young shoot of the plant. *Helopeltis theivora* Waterhouse(Hemiptera : Miridae) is the major insect pest of tea which causes heavy loss to the tea crop every year [1], since it attacks the young shoots i.e, two and a bud which are the actual crop of tea [2].In North-East India, major parts of tea plantation are infested by *helopeltis* causing each year a loss of around 15-20 lakhs of made tea while in South India, around 40,000 acres annually are under the attack of this notorious pest [3]. Depending on severity, yield losses ranges from around 10-50 % [4]. This pest suck the sap from young leaves by injecting their labial stylet containing saliva into soft plant tissue. The water soak lesion around the site of puncture turns into brown spot and in severe damage leaves curl up and ultimately dry thereby reducing the yield of the plant [5].In some cases of severe infestation, the affected young bushes may not flush for several weeks thereby not forming shoots [6]. In recent years, this pest has become a major threat all throughout as it has developed resistance to commonly used insecticides [7]. Hence a thorough insight of defence mechanism of the plants becomes a must to combat against the pest, so that alternate plant resistance approaches other than insecticide could be used.

Plants defend themselves from pathogens by employing different mechanism. By understanding the host pathogen defence mechanism may help in establishing novel approaches to enhance the plant resistance against this pathogen. Several biochemical changes such as phenols, secondary metabolites, oxidative enzymes play important role in defence mechanism [8].Biochemical study is a must at the grass-root level to understand complex interaction between host and the pathogen. But according to previous literature, defence mechanism of tea plants against *Helopeltis theivora* is very limited. Considering this fact in mind, the present research is carried out to understand host pathogen defence mechanism.

MATERIALS AND METHODS

Sample collection

Tea leaves (Fig 1&2), two and a bud (healthy and *helopeltis* infected) of clones TV1, TV23, S3A3, Tinali was collected from 5 leading tea gardens, Ethelwood, Borborooah, Moran, Deohal and Tippuk tea estates located in Dibrugarh district, Assam, India (Fig 3).For the experimental study, healthy and naturally infected young two and a bud from the tea bushes were collected.

Determination of total protein

Total protein was estimated following the method of Lowry [9].A gram of fresh green tea leaves were washed with deionised distilled water and were homogenised in 4.5mL of 0.1 M phosphate buffer of pH 6.5 for 20 min. The homogenate was centrifuged at 10,000 rpm for 15 min (Sigma 3-30 K, Germany).The absorbance was recorded at 660 nm using UV-VIS Spectrophotometer (TCC-240A, Shimadzu corporation, Kyoto Japan).Bovine serum albumin (BSA) was used as standard for the assay. Protein content expressed as BSA equivalent (mg/gm of leave tissue) was obtained from the standard curve (Table 1).

Determination of total carbohydrate

Total carbohydrate was estimated based on the method of anthrone [10]. For the estimation, reagent mixture containing suitably diluted plant extract and anthrone was recorded spectrophotometrically at 630nm.Glucose (1mg/mL) was used as standard for the assay. Carbohydrate content expressed as glucose equivalent (mg/gm of leave tissue) was obtained from the standard curve (Table 2).

Determination of total phenol

Total phenol was estimated following Gallic acid Equivalence method [11].1 gm of leaves was homogenised with 10 time volume of 80% ethanol and the homogenate was centrifuged at 10,000 rpm for 20 min. Reagent mixture containing 1 mL of the plant extract and Folin-Ciocalteau reagent was measured spectrophotometrically at 650 nm for total phenol against a reagent blank. Gallic acid (1mg/mL) was used as standard for the assay. Phenol content expressed as gallic acid equivalent (mg/gm of leave tissue) was obtained from the standard curve (Table 3).

Determination of total flavonoid

Total flavonoid was estimated by aluminium chloride method with some modification [12].For the estimation, reagent mixture containing 1mL of plant extract, 2 mL of methanol, 0.2 mL of 10% aluminium chloride, 0.2 mL of 1M potassium acetate was measured spectrophotometrically at 420 nm. Quercetin (1mg/mL) was used as standard for the assay. Flavonoid content expressed as quercetin equivalent (mg/gm of leave tissue) was obtained from the standard curve (Table 4).

Determination of photosynthetic pigment

Photosynthetic pigment viz; total chlorophyll, chlorophyll a, chlorophyll b was estimated by acetone method [13].Chlorophyll was extracted from 1 gm of fresh leaves using 80% acetone and measured spectrophotometrically at 645nm and 663nm against acetone blank. Chlorophyll amount present in the extract is calculated according to the formula [14] and is expressed in mg/gm of leave tissue (Fig 4).

Determination of antioxidant enzymes

Antioxidant enzymes viz; peroxidase and polyphenol oxidase was extracted from healthy and infested tea leaves. Peroxidase activity (Table 5) was estimated [15] by taking 0.1 mL of plant extract in 0.1 mL o-dianisidine (1 mg/mL) maintained at 28^{o}C for 2min (Sartorius Stedium biotech, Certomat BS-1, Germany) and the reaction was stopped by adding 0.2 mL of H_2O_2 (30%).The change in absorbance recorded for every 30 seconds for 5 min was measured spectrophotometrically at 430 nm. Polyphenol oxidase activity (Table 6) was estimated [16] by taking 0.1mL of plant extract,2 mL of 2M carbonate-bicarbonate buffer (pH 10), 0.15 M of catechol maintain at 25^{o}C for 2 min and the reaction was stopped by adding 0.5 mL of H_2SO_4 (5%).The absorbance was measured spectrophotometrically at 420 nm. The specific activity of the enzymes was expressed in units/mg of protein /mL of reaction mixture.

All the chemicals used in the study were procured from Merck India Pvt. Ltd.

Statistical Analysis

The values reported are the mean of three independent determinants. The significance in variation of the means was determined by student's t-test at $P \leq 0.05$.

RESULTS AND DISCUSSION

In the present study, infestation of tea leaves by *helopeltis theivora* causes reduction in the level of total protein (Table 1) and total carbohydrate (Table 2) unlike the healthy plants. This might be due to taking up of assimilates by the insect or decrease in the biosynthetic pathway. The result was in accordance with the study of feeding of plants by aphidoidae who reported that insects draw nutrients from the host plant for their food [17].

Total chlorophyll, chlorophyll a and b (Fig 4) was found to be less in infected tea plants than the healthy ones. Such reduction might be due to imbalanced pigment synthesis due to passing of nutrients towards the insect from the host plant or might be the effect of reactive oxygen species [18]

The infestation of tea leaves by *helopeltis theivora* causes an increase in the oxidative enzymes viz; peroxidase(POX) (Table 5) and polyphenol oxidase(PPO) (Table 6).The findings are in accordance with the study of chocolate spot disease of broad bean where increase in peroxidase is considered as indicator for resistance [19]. It has also been reported that increased PPO in infected S.lycopersicum leaves lead to disease resistance [20]. Host pathogen reaction of the host to insect result in oxidative state of the plant which produces reactive oxygen species that are removed by oxidative enzymes [21].It has been reported that increase in POX and PPO activities increases the reactive oxygen species which act as scavenger to prevent the spread of infection [22]. Both these oxidative enzymes plays role in defense mechanism by oxidation of phenolic compounds to quinones [23].Quinones are toxic to the pathogen causing cell death in affected area which prevent further spread of infection to nearby sites [24].Quinones being highly reactive intermediate compound react with amino acid and cross-link proteins thus reducing the protein content [25]. The secondary metabolite produced by the plants are the phenolics which defend themselves from the pathogen flavonoid is one of the largest classes of phenolics [26].In the present study, total phenol(Table 3) was found to be lesser in infected plant than the healthy one. With the increase of POX and PPO activities, more phenol is used as it act as substrate for antioxidant enzymes.This lead to the decrease of phenols in the infected plant. Similar result has been recorded in the study of cabbage against aphid [27] stating that phenol oxidation by antioxidant enzyme is a potential defense mechanism in plants against insect attack. Phenols activate defensive enzymes by reduction of reactive oxygen species and play a role in host pathogen reaction against herbivore and insects [28-30].Several studies also reported PPO to play a vital role in plant defence against insect attack [31,32].

In the current study, the flavonoid content (Table 4) of helopeltis infected leaves was found in lesser amount than the healthy plant. The result was in accordance with the findings in the leaves of cluster bean [33].Flavonoids scavenges the reactive oxygen species by chelating the metal thus protecting the plant against insect [34].

Fig 1: Healthy tea leaves (two and a bud) of a Tea Estate located in Dibrugarh district, Assam, India.

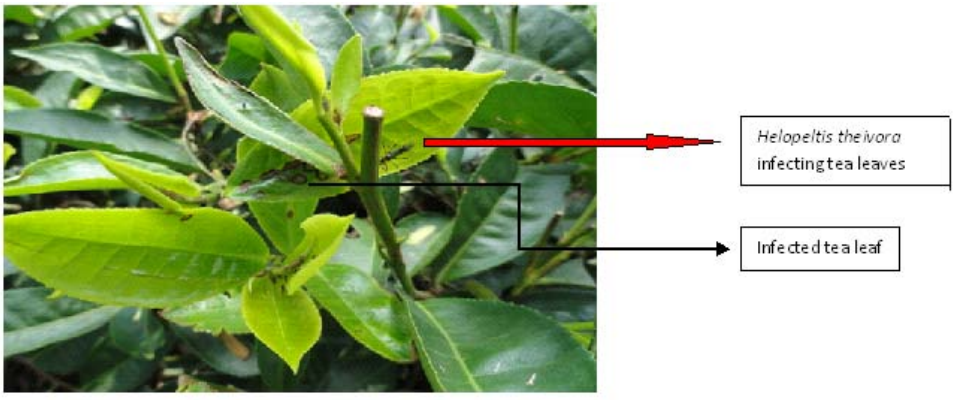

Fig 2: *Helopeltis* infested tea leaves of a Tea Estate located in Dibrugarh district, Assam, India.

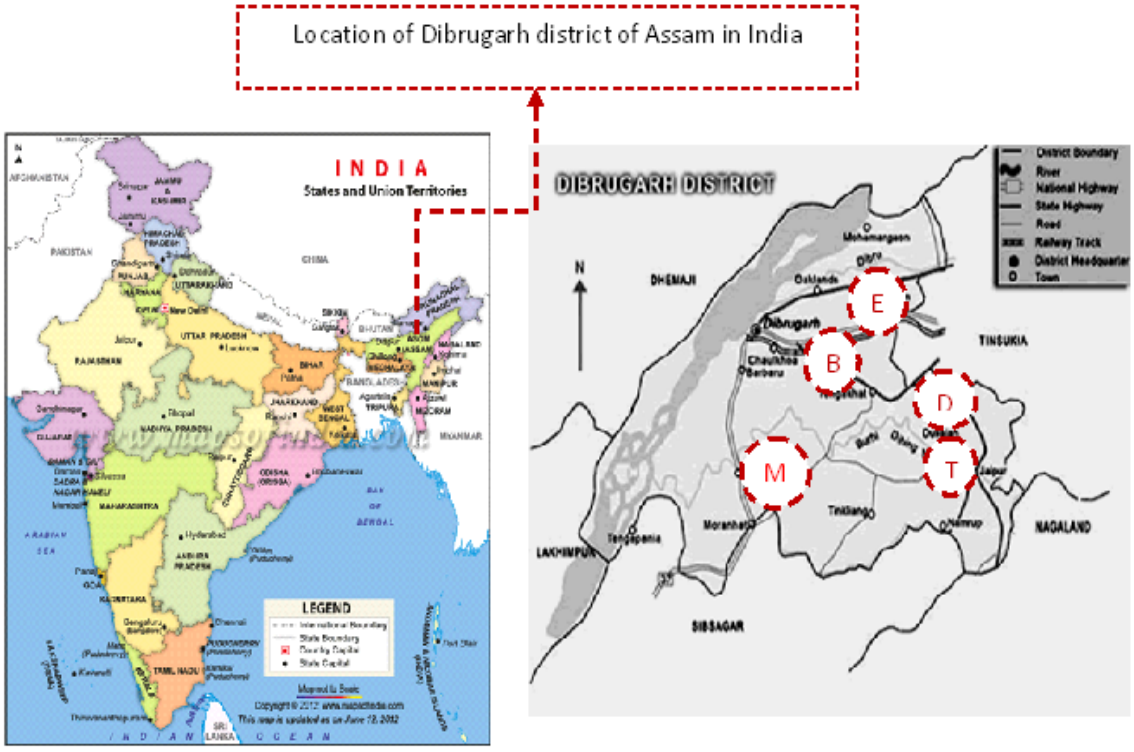

Fig 3: Location of Dibrugarh district in India and the location of the 5 tea gardens from which the samples were collected (E= Ethelwood Tea Estate, B= Borborooah Tea Estate, M= Moran Tea Estate, D= Deohal Tea Estate and T= Tippuk Tea Estate).

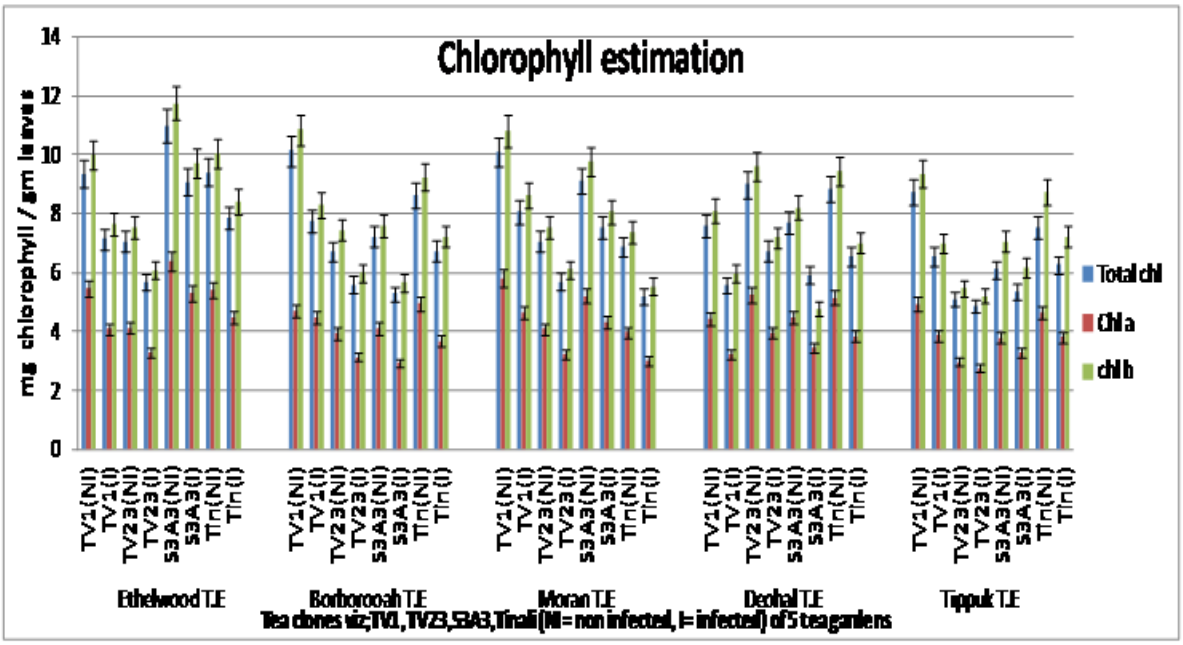

Fig 4: Chlorophyll estimation (total chlorophyll, chlorophyll a, chlorophyll b) in healthy and infected tea leaves (tea clones=TV1,TV23,S3A3,Tinali) of 5 tea gardens located in Dibrugarh district, Assam, India (data are averages ± SD).

Table 1: Protein estimation in healthy and infected tea leaves (tea clones= TV1, TV23, S3A3, Tinali) of 5 tea gardens located in Dibrugarh district, Assam, India (data are averages ± SD).

	Ethel wood Tea Estate		Borborooah Tea Estate		Moran Tea Estate		Deohal Tea Estate		Tippuk Tea Estate	
Tea clones	Non-infected	Infected leaves	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected
	Protein expressed in (mg/gm leaves)±SD									
TV1	1.496 ±0.019	0.704 ±0.002	0.942 ±0.004	0.760 ±0.002	1.037 ±0.003	0.879 ±0.004	1.478 ±0.004	1.059 ±0.002	1.449 ±0.002	1.269 ±0.004
TV23	1.422 ±0.004	1.003 ±0.0138	1.014 ±0.003	0.864 ±0.005	1.122 ±0.004	0.807 ±0.003	1.190 ±0.003	0.868 ±0.003	1.512 ±0.002	0.909 ±0.002
S3A3	1.237 ±0.004	0.769 ±0.007	0.882 ±0.003	0.762 ±0.006	0.922 ±0.003	0.672 ±0.002	1.397 ±0.002	1.100 ±0.006	1.449 ±0.002	1.190 ±0.002
Tinali	0.920 ±0.005	0.114 ±0.006	1.050 ±0.003	0.783 ±0.003	0.996 ±0.003	0.690 ±0.003	1.158 ±0.003	0.576 ±0.004	1.480 ±0.003	1.145 ±0.001

Table 2: Carbohydrate estimation in healthy and infected tea leaves (tea clones =TV1, TV23, S3A3, Tinali) of 5 tea gardens located in Dibrugarh district, Assam, India (data are averages ± SD).

	Ethelwood Tea Estate		Borborooah Tea Estate		Moran Tea Estate		Deohal Tea Estate		Tippuk Tea Estate	
Tea clones	Non-infected	Infected leaves	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected
	Carbohydrate expressed in (mg/gm leaves)±SD									
TV1	0.146 ±0.005	0.088 ±0.006	0.223 ±0.011	0.183 ±0.010	0.167 ±0.008	0.108 ±0.011	0.182 ±0.016	0.117 ±0.023	0.160 ±0.009	0.075 ±0.013
TV23	0.190 ±0.005	0.083 ±0.005	0.230 ±0.007	0.144 ±0.009	0.187 ±0.011	0.132 ±0.009	0.201 ±0.013	0.090 ±0.020	0.202 ±0.013	0.120 ±0.012
S3A3	0.177 ±0.006	0.118 ±0.006	0.143 ±0.008	0.122 ±0.012	0.191 ±0.011	0.113 ±0.010	0.163 ±0.017	0.096 ±0.012	0.154 ±0.007	0.097 ±0.008
Tinali	0.157 ±0.006	0.093 ±0.006	0.176 ±0.009	0.114 ±0.009	0.184 ±0.010	0.110 ±0.011	0.150 ±0.016	0.092 ±0.020	0.186 ±0.013	0.129 ±0.013

Table 3: Phenol estimation in healthy and infected tea leaves (tea clones= TV1, TV23, S3A3, Tinali) of 5 tea gardens located in Dibrugarh district, Assam, India (data are averages ± SD).

	Ethelwood Tea Estate		Borborooah Tea Estate		Moran Tea Estate		Deohal Tea Estate		Tippuk Tea Estate	
Tea clones	Non-infected	Infected leaves	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected
	Phenol expressed in (mg/gm leaves)									
TV1	0.350 ±0.030	0.243 ±0.024	0.323 ±0.023	0.190 ±0.024	0.336 ±0.033	0.172 ±0.024	0.342 ±0.039	0.258 ±0.034	0.333 ±0.029	0.220 ±0.028
TV23	0.329 ±0.042	0.206 ±0.030	0.262 ±0.025	0.172 ±0.026	0.352 ±0.030	0.199 ±0.021	0.408 ±0.034	0.307 ±0.022	0.338 ±0.030	0.185 ±0.039
S3A3	0.307 ±0.043	0.193 ±0.033	0.275 ±0.031	0.177 ±0.041	0.262 ±0.029	0.195 ±0.017	0.340 ±0.026	0.257 ±0.043	0.391 ±0.030	0.190 ±0.035
Tinali	0.326 ±0.030	0.195 ±0.042	0.324 ±0.033	0.187 ±0.036	0.289 ±0.028	0.175 ±0.035	0.372 ±0.044	0.247 ±0.033	0.259 ±0.024	0.189 ±0.034

Table 4: Flavonoid estimation in healthy and infected tea leaves (tea clones= TV1, TV23, S3A3, Tinali) of 5 tea gardens located in Dibrugarh district, Assam, India (data are averages ± SD).

	Ethelwood Tea Estate		Borborooah Tea Estate		Moran Tea Estate		Deohal Tea Estate		Tippuk Tea Estate	
Tea clones	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected
	Flavonoid expressed in (mg/gm leaves)									
TV1	1.178±0.003	1.073±0.004	1.226±0.006	0.885±0.004	1.144±0.003	0.901±0.002	1.341±0.003	1.120±0.005	1.295±0.008	0.933±0.007
TV23	1.125±.0.002	0.928±0.006	1.150±0.003	0.990±0.005	1.100±0.005	0.887±0.006	1.256±0.006	1.019±0.007	1.336±0.006	1.059±0.004
S3A3	1.151±0.004	0.845±0.007	1.195±0.004	0.813±0.006	1.168±0.004	0.975±0.003	1.218±0.005	0.992±0.003	1.311±0.005	0.981±0.006
Tinali	1.318±0.003	0.0984±0.005	1.169±0.002	0.799±0.003	1.210±0.002	1.014±0.005	1.310±0.004	1.058±0.006	1.249±0.006	0.922±0.005

Table 5: Peroxidase activity in healthy and infected tea leaves (tea clones= TV1, TV23, S3A3, Tinali) of 5 tea gardens located in Dibrugarh district, Assam, India (data are averages ± SD).

	Ethelwood Tea Estate		Borborooah Tea Estate		Moran Tea Estate		Deohal Tea Estate		Tippuk Tea Estate	
Tea clones	Non-infected	Infected leaves	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected
	Peroxidase activity expressed in (units/mg protein/min)									
TV1	0.013 ±0.001	0.021 ±0.001	0.023 ±0.001	0.057 ±0.004	0.018 ±0.003	0.035 ±0.001	0.014 ±0.002	0.025 ±0.001	0.018 ±0.002	0.038 ±0.007
TV23	0.010 ±0.0004	0.055 ±0.002	0.016 ±0.002	0.021 ±0.002	0.013 ±0.001	0.021 ±0.001	0.016 ±0.004	0.063 ±0.006	0.020 ±0.002	0.032 ±0.001
S3A3	0.012 ±0.0005	0.027 ±0.002	0.015 ±0.001	0.040 ±0.001	0.013 ±0.001	0.029 ±0.004	0.017 ±0.002	0.028 ±0.001	0.019 ±0.002	0.038 ±0.003
Tinali	0.021 ±0.0035	0.045 ±0.004	0.021 ±0.002	0.054 ±0.002	0.020 ±0.001	0.034 ±0.003	0.018 ±0.001	0.025 ±0.001	0.021 ±0.001	0.023 ±0.001

Table 6: Polyphenol oxidase activity in healthy and infected tea leaves (tea clones= TV1, TV23, S3A3, Tinali) of 5 tea gardens located in Dibrugarh district, Assam, India (data are averages ± SD).

	Ethelwood Tea Estate		Borborooah Tea Estate		Moran Tea Estate		Deohal Tea Estate		Tippuk Tea Estate	
Tea clones	Non-infected	Infected leaves	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected
	Polyphenoloxidase activity expressed in (units/mg protein/min)									
TV1	0.007 ±0.013	0.009 ±0.019	0.005 ±0.022	0.007 ±0.019	0.006 ±0.013	0.008 ±0.018	0.006 ±0.016	0.008 ±0.020	0.007 ±0.023	0.010 ±0.015
TV23	0.006 ±0.019	0.008 ±0.017	0.006 ±0.019	0.008 ±0.031	0.005 ±0.017	0.007 ±0.020	0.006 ±0.015	0.007 ±0.023	0.008 ±0.033	0.010 ±0.031
S3A3	0.006 ±0.020	0.008 ±0.016	0.006 ±0.018	0.008 ±0.021	0.006 ±0.014	0.008 ±0.024	0.006 ±0.014	0.008 ±0.019	0.008 ±0.013	0.011 ±0.013
Tinali	0.005 ±0.017	0.010 ±0.022	0.005 ±0.014	0.007 ±0.015	0.005 ±0.021	0.007 ±0.015	0.005 ±0.018	0.008 ±0.019	0.007 ±0.023	0.010 ±0.019

CONCLUSION

From the present finding, it can be inferred that *Helopeltis theivora* induces an oxidative stress in the plant system which it reduces by increasing the activities of antioxidant enzymes viz; peroxidase and polyphenol-oxidase. This study also indicates total phenols and flavonoids to be the important biochemical constituent which may impart resistance to *Camellia sinensis* against *Helopeltis theivora.*

Further research in this direction may provide an insight of the complex host-pathogen interaction which can be utilized for developing more stable genotype by incorporating desirable trait of resistance through transgenic approach in the susceptible genotypes of tea. This eco-friendly and cost efficient approach will further reduce our dependency on synthetic insecticides which ultimately will benefit our agro-industry to a much wider extent.

Conflict of Interest: The authors declare that they have no conflict of interest and do not have any financial relationship with the organization that sponsored the research in the manuscript.

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