Biochemical responses of tea plants induced by foliar infection with *Exobasidium vexans*

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ABSTRACT: Biochemical response of tea plants growing in Darjeeling hills exposed to biotic stress due to blister blight infection caused by *Exobasidium vexans* Massee in the levels of proteins, proline, phenols and enzyme activities such as phenyl alanine ammonia lyase, polyphenol oxidase and peroxidase were studied. Activity of phenylalanine ammonia lyase registered a decline in infected leaves whereas peroxidase and polyphenol oxidase activity was higher. Analysis of peroxidase isozymes by polyacrylamide gel electrophoresis using benzidine as substrate showed four isozymes in healthy and five in infected leaves. One of the most significant changes was observed in proline content which increased markedly in blister infected leaves. Protein showed a decline at very early stages of infection. SDS-PAGE analysis revealed the loss of several proteins following infection. Total phenols were significantly higher in the infected leaves in comparison to the healthy ones. Antifungal activity of phenols from both healthy and blister infected leaves were detected following agar cup bioassay. Antifungal activity was higher in healthy leaves. HPLC analysis of catechin extract from healthy and infected leaves showed little qualitative difference but, some quantitative changes.

Key words: Tea, blister blight, enzymes, phenols

Darjeeling produces world's finest quality tea on the steep hill slopes of eastern Himalayas up to an elevation of 2000m. Blister blight disease of tea is economically the most important fungal disease in the major tea growing areas like Darjeeling and southern peninsular parts of India. This disease is caused by *Exobasidium vexans* Massee, an obligate pathogen, which mainly attacks juvenile shoots, affecting harvestable economic crop; yet for this pathogen, alternate host is not known. Blister blight could cause 50% loss in crops (Agnihothrudu, 1995); in addition, affected shoots produce flaky teas, resulting in inferior cup quality.

It is well known that plants respond in a variety of ways to attacks by pathogens and other types of stresses. Polyphenols are the major components of tea leaves, which play an important role in the plant's metabolism. Major enzymes involved in phenol metabolism are polyphenol oxidase (PPO), peroxidase (PO) and phenylalanine ammonialyase (PAL). PPO is involved in producing characteristic flavour of tea leaves (Yamanishi, 1991). In the present study, changes in the major biochemical constituents of tea plants following infection by the pathogen has been worked out.

MATERIALS AND METHODS

Fresh samples of healthy and blister infected leaves were collected from six tea gardens spread out in the Darjeeling hills viz. Margaret's Hope, Dilaram, Singel, Castleton, Makiahari and Marrionbarie designated as Sections 1, 2, 3, 4, 5 and 6, respectively. The above tea estates were all located in the hilly terrain of Darjeeling, and the bushes as such, did not show any outward differences. All plants were of 'China' variety. In infected leaf samples, blisters were, in general, at the fully mature stage (6-7 day old blister). Twenty samples were taken from each section and the samples were of same age (first two leaves) and variety.
Extraction and assay of PAL activity

Phenylalanine ammonia lyase (EC 4.3.1.5.) was extracted from tea leaves in 0.1M sodium borate buffer (pH 8.8) containing 2 mM B-mercaptoethanol and assayed as described by Chakraborty et al. (1993).

Extraction and assay of PPO

Polyphenol oxidase (EC 1.10.3.2.) was extracted from tea leaves and estimated as described by Mahadevan and Sridhar (1982), using pyrogallol as substrate.

Extraction and assay of POD

Peroxidase (EC 1.11.1.7) was extracted in 0.1M sodium borate buffer following the method of Chakraborty et al. (1993) with modification. Assay buffer consisted of 0.2 M sodium phosphate buffer (pH 5.4), 4 mM \( \text{H}_2\text{O}_2 \), O-dianisidine (5 mg ml\(^{-1}\) methanol) and enzyme extract. Peroxidase activity was expressed as D.O.D. at 460 nm g\(^{-1}\) fresh weight of tissue min\(^{-1}\) as suggested by Sadasivam and Manickam (1996) with modifications.

Isozyme analysis of peroxidase by PAGE

Peroxidase enzyme extract was prepared in 0.1M sodium phosphate buffer (pH 7). Polyacrylamide gel electrophoresis was performed according to the method of Davis (1967), followed by staining of the gel.

Extraction and estimation of free proline

Proline was extracted from the leaves of tea as described by Bates et al. (1973) in 3% sulphosalicylic acid.

Extraction and estimation of protein

Soluble proteins were extracted from tea leaves as described by Chakraborty et al. (1995). Protein content was estimated following Bradford's (1976) method using B.S.A. as standard.

Analysis of protein

Analysis of crude protein extract was carried out on 10% SDS-PAGE gels as described by Laemmli (1970). Protein solutions (50 \( \mu \)g) as well as standard molecular weight markers were loaded on the gel and separated for 5 h at 200 V and 30 mA. Following electrophoresis the gel was fixed, stained in a coomassie brilliant blue (R 250, Sigma) staining solution and finally destained.

Extraction and estimation of total phenols

Total phenols were extracted from the tea leaves following the method of Harborne (1973) and estimated as described by Mahadevan and Sridhar (1982).

Extraction and assay of antifungal phenolics from fresh tea leaves

Extraction: Free and glycosidically linked phenolics were extracted from tea leaves following the method of Daayf et al. (1995). Acid hydrolysis of the aqueous fraction (yielding phenolic aglycones) was performed according to the method of Daayf et al. (1997). Aglycones were recovered by partitioning hydrolysates against ethyl acetate.

Bioassay

Antifungal activity of all the fractions were tested using agar cup bioassay techniques. Richard's (\( \text{KNO}_3-10g; \text{KH}_2\text{PO}_4-5g; \text{MgSO}_4 \cdot 7\text{H}_2\text{O}-2.5g; \text{Sucrose}-30gL}^{-1} \)) agar medium was poured in petridishes and four wells were made in each plate. Aliquots (40 \( \mu l \)) corresponding to 1.5 g of fresh leaf tissue of individual fractions and ethyl acetate (as control) were dispersed in each of the four wells. A 5mm-agar block of \textit{Glomerella cingulata} was deposited in each plate at equal distance from the wells and incubated at 30°C for 7 days.

Extraction of catechin from fresh leaf tissue

Catechin was extracted from leaf tissue according to the method of Obanda and Owuor (1994).

HPLC analysis of catechin extract

Catechin analysis of the extract was carried out on C 18 hypersil column using linear gradient elution system as follows: mobile phase A 100% acetonitrile; mobile phase B 2% acetic acid in water; elution: 88% B for 6 min. and then linear gradient to 75% B over 5 min; the elution was complete after a total of 25 min. Flow rate
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RESULTS AND DISCUSSION

Change in enzyme activities after blister blight infection

Blister infection resulted in a contrasting change in the case of two important enzymes in phenol metabolism namely PAL and PPO. Assay of PAL activity, which is generally considered to be involved in defense mechanism, revealed a decreased activity in the infected leaves. But, an increase though not very significant of PPO activity, was noticed after blister infection. Assay of peroxidase enzyme showed significant increase after infection. In some cases, peroxidase activity almost doubled after infection (Table 1). Rajalakshmi and Ramarethinam (2000), also reported increased activities of PPO and POD and decreased activity of PAL in Tea assamica leaves infected with blister. However, Sudhakaran et al. (2000) observed increased activity of PPO and POD enzymes in tea leaves only when infestation with Helopeltis theivora was mild to moderate. Native PAGE analysis, using benzidine showed at least three very prominent peroxidase isozyme bands, in healthy leaf sample. In the first stage of infection, leaf with pinkish translucent spot, the isozyme pattern was same as the healthy one. With the increase in disease severity, leaf with chlorotic area and with white mature blister, two additional isozyme bands were observed below the previous three isozyme bands. One additional band of low intensity and low Rm was prominent in the final stage of infection. The appearance of new bands following infection can be correlated with the induction of the catalytic activity of more isozymes, leading also to an overall increase in peroxidase activity. Previous study has shown the existence of multiple molecular form of peroxidase in different tea clones irrespective of the substrate used (Takeo and Kato, 1971; Gunasekar et al. 1996).

Effect of blister blight infection on biochemical constituents

Free proline, protein and total phenol content was measured from the healthy and infected leaf tissue. Significant increase of proline content was noticed following infection. About 27-80% increase in free proline content was detected (Table 2). Protein content showed a decline, while phenol content increased following blister infection (Table 2). SDS-PAGE analysis of protein pattern showed similar trend as protein content. Intensity of many bands having low, intermediate and high molecular weights decreased in comparison to the healthy leaf sample (Fig.1). Recovery of some of intermediate molecular weight proteins was noticed in 3rd and final stages of infection (Fig.1, Lane 6). No new protein band was visualised in different stages following infection. Almost no variation was observed between the leaves collected from different tea gardens. Jones and Hartley (1999)

Table 1. Enzyme activities of healthy and blister infected tea leaves

<table>
<thead>
<tr>
<th>Replicate Sampling*</th>
<th>PAL activity (µg cinnamic acid/g tissue/ min)</th>
<th>PPO activity (Δ O.D./g tissue/min)</th>
<th>POD activity (Δ O.D./ g tissue/ min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>I</td>
<td>H</td>
</tr>
<tr>
<td>Section1</td>
<td>408</td>
<td>276</td>
<td>3.8</td>
</tr>
<tr>
<td>Section2</td>
<td>552</td>
<td>294</td>
<td>5.1</td>
</tr>
<tr>
<td>Section3</td>
<td>696</td>
<td>264</td>
<td>4.8</td>
</tr>
<tr>
<td>Section4</td>
<td>480</td>
<td>180</td>
<td>5.8</td>
</tr>
<tr>
<td>Section5</td>
<td>540</td>
<td>302</td>
<td>4.7</td>
</tr>
<tr>
<td>Section6</td>
<td>420</td>
<td>238</td>
<td>4.1</td>
</tr>
<tr>
<td>LSD 5% =</td>
<td>109.13</td>
<td>1.37</td>
<td>3.80</td>
</tr>
<tr>
<td>LSD 1% =</td>
<td>171.15</td>
<td>0.87</td>
<td>2.41</td>
</tr>
</tbody>
</table>

* Different sections indicate different tea estates in Darjeeling hills

Difference between values of healthy and infected significant at P=0.05 and 0.01
Table 2. Effect of blister infection on important biochemical constituents of tea leaves

<table>
<thead>
<tr>
<th>Replicate sampling*</th>
<th>Proline content (mg/g dry wt.tissue)</th>
<th>Protein content (mg/g dry wt.tissue)</th>
<th>Total Phenol content (mg/g dry wt.tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>I</td>
<td>H</td>
</tr>
<tr>
<td>Section 1</td>
<td>13.0</td>
<td>16.4</td>
<td>44.0</td>
</tr>
<tr>
<td>Section 2</td>
<td>11.1</td>
<td>16.2</td>
<td>40.0</td>
</tr>
<tr>
<td>Section 3</td>
<td>16.0</td>
<td>19.5</td>
<td>32.0</td>
</tr>
<tr>
<td>Section 4</td>
<td>13.0</td>
<td>17.4</td>
<td>24.0</td>
</tr>
<tr>
<td>Section 5</td>
<td>16.2</td>
<td>22.2</td>
<td>22.0</td>
</tr>
<tr>
<td>Section 6</td>
<td>13.0</td>
<td>24.0</td>
<td>21.8</td>
</tr>
<tr>
<td>LSD 5% =</td>
<td>2.41</td>
<td>2.66</td>
<td>9.71</td>
</tr>
<tr>
<td>LSD 1% =</td>
<td>1.54</td>
<td>4.17</td>
<td>6.19</td>
</tr>
</tbody>
</table>

Difference between values of healthy and infected significant at P=0.05 and 0.01

* Different sections indicate different tea estates in Darjeeling hills

Antifungal phenol extracted both from healthy and blister infected leaves showed a different trend in comparison to the total phenol. Antifungal property was noticed only in Fraction III in the case of both the samples. In petridish bioassay, partial inhibition in mycelial growth of *Glomerella cingulata* was noticed with 10 ml sample from Fraction III while complete inhibition was observed with 40 ml sample for Fraction III. The inhibition zone was relatively bigger in the healthy extract than the infected one. Chakraborty and Saha (1994) reported the presence of antifungal catechins in healthy leaf extracts, which, they averred, had been broken down to catechol in the infected leaves. Nagahulla *et al.* (1996) also reported the production of antifungal compounds in tea leaves following infection with the blister pathogen. It seems probable in the present study, that, antifungal phenols present constitutively in the healthy leaf tissues are metabolized by the pathogen during the course of its infection and once infection is established, the quantity of antifungal phenolics show a decline. HPLC analysis of the catechins extracted from healthy and infected tea leaf samples, showed almost the same profile; with differences in the peak height in some of the components (Fig. 2). Two major peaks showed lesser peak height in the infected extract, in comparison to the healthy ones.

![Fig. 1. SDS-PAGE analysis of proteins from healthy and blister infected tea leaf tissues. Lanes 1-3: Blister infected leaves from different tea gardens; 1-Makaibari; 2-Margaret's Hope and 3-Marrionbarie; 4-7: Castleton; 4- Healthy; 5-7: Young, mature and old stages of infection, respectively.](image-url)

proposed a protein competition model (PCM) for predicting total phenolic allocation and concentration in leaves of terrestrial higher plants. They suggested that protein and phenol synthesis competes for the common limiting resource phenyl alanine, and hence, protein and phenolic allocations are inversely correlated. The observed increase in phenolic concentration and decrease in protein concentration in the present study could be explained by the above model. However, Kumaravadivelu *et al.* (1996) and Sudhakaran *et al.* (2000) obtained decreased levels of both phenolics and protein in tea leaves infested with flushworm and *Helopeltis theivora*, respectively.
In conclusion, it might be stated that the tea plant responds to blister infection by adjustment of its metabolic processes. Increased levels of PPO and POD would be needed to neutralise the peroxide radical formed during pathogenesis. Similarly, it also responds by increasing levels of phenolic and proline accumulation with decreased levels of protein. Such biochemical changes, manifested by the plant as a stress response, would, ultimately affect the quality of tea made from these infected leaves.

ACKNOWLEDGEMENT

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