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Time-course accumulation of phenylalanine ammonia lyase, tyrosine ammonia lyase and polyphenol oxidase triggered by *Glomerella cingulata* in tea varieties

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ABSTRACT

Eighteen varieties of tea were evaluated for their response to *Glomerella cingulata*, causal agent of brown blight disease following artificial inoculation technique, and catagorised as resistant and susceptible. Time course activity profiles of defense enzymes involved in phenol metabolism, viz. phenylalanine ammonia lyase (PAL), tyrosine ammonia lyase (TAL) and polyphenol oxidase (PPO) were evaluated during the infection process in these varieties. PAL activity was found to be significantly higher in the infected tissues than in corresponding control and reached its peak 24 hours after inoculation in the resistant varieties, while for TAL the same occurred at 48 hours. Polyphenol oxidase, on the other hand, showed its higest activity at 72 hours. Isozyme analyses of PPO indicated that a specific isozyme is involved in resistance mechanism. These three enzymes acted in a co-ordinated manner to protect the tea plants from brown blight infection.

Keywords: *Camellia sinensis*, *Glomerella cingulata*, phenylalanine ammonia lyase, polyphenol oxidase, tyrosine ammonia lyase

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INTRODUCTION

Tea is the most popular and inexpensive beverage produced from young leaves of the commercially cultivated perennial plantation crop - *Camellia sinensis* (L.) O. Kuntze (tea plant). Tea leaves, besides providing the healthiest drink, are now-a-days being exploited for other innovative aspects like extraction of active principles that give protection from cancer and other diseases [1,2]. The antioxidant properties of tea have been largely attributed to the high amount of phenolics in tea leaves. Therefore, metabolism of the phenolics under naturally occurring biotic stress in tea plants is very important.

The enzymes such as Tyrosine ammonia lyase (TAL) and phenylalanine ammonia lyase (PAL) are involved in phenolic compound biosynthesis [3-5]. It is well-known that the resulting phenolics are often converted into more reactive species by polyphenol oxidases (PPO) [6,7]. Polyphenol oxidase catalyses the oxygen-dependent oxidation of phenols to quinines. Enhancement of phenylalanine ammonia lyase and polyphenol oxidase enzyme activities in response to the pathogen (*Rhizoctonia solani*) challenge under controlled conditions resulted in reduced symptom development and containment of disease in transgenic rice lines compared to non-transgenic control plants [8]. On the other hand, constitutive expression of a phenylalanine ammonia lyase gene in transgenic tobacco leads to higher disease resistance [9]. Higher PPO activity was related to higher contents of phenolic compounds, which have been shown to provide resistance against diseases [10]. In a perennial plantation crop, coconut, the induced resistance was found to be associated with significant increase in activities of PAL and PPO, as well as induction of new PPO isozymes [11]. In other perennial plantation crops like cassava [12] and citrus [13] high PAL activity has been found to be associated with incompatible plant-pathogen interactions.

Thus, presently, the specific activity of important enzymes - PAL and TAL that are involved in the phenolics biosynthesis on one hand and oxidation of phenolics on the other hand PPO have been determined in tea varieties at different time intervals following inoculation with *G. cingulata*. Besides, PPO isozyme analysis of anionic fractions was also done in order to find out whether there are any specific isoforms related to resistance mechanism.

MATERIALS AND METHODS

Plant material

Eighteen months old tea [*Camellia sinensis* (L.)O. Kuntze] saplings were obtained from cuttings made from shoots of the mother bush of the 18 tea varieties (TV-18, TV-22, TV-25, TV-26, TV-29, TV-30, T-17/1/54, CP- 1/1, BS/7A/76, P-312, AV-2, TS-449, UP-2, UP-3, UP-9, UP- 26, BSS-2, BSS-3) maintained in Tea Germplasm Bank, Department of Botany, University of North Bengal. The hardened saplings were grown in earthen pots of 50cm diameter containing a potting mixture with soil pH 4.8-5.0 and cow dung manure @ 50g per pot. These were maintained in glasshouse under controlled condition of 30 ± 5^{o}C, relative humidity 60-80%, 16h

photoperiod and irradiance of 400 μ m m^{-2} s^{-1}. Plants were watered daily with tap water and once a week with Hoagland and Knop's solution.

Fungal material

Glomerella cingulata (Stoneman) Spauld and Schrenk was isolated from naturally brown blight infected tea leaves (TV-22) grown in Phytopathological Experimental Garden of the Department of Botany, NBU and subsequently was identified (W7659) from the Diagnostic and Advisory Service, CABI Bioscience UK Center, and routinely subcultured on Richard's medium agar grown under 12h photoperiod at 30°C.

Artificial inoculation technique

Artificial inoculation technique [14] was followed to evaluate response of tea varieties to *Glomerella cingulata*. Spore suspension was placed (2-4 drops $leaf^{-1}$) on the adaxial surface of each leaf with a hypodermic syringe on the wounds. In control sets drops of sterile distilled water were placed on the wounded leaves. Percent drops that resulted in lesion production were calculated after 48, 72 and 96 hours of inoculation and diameter of the lesions was noted [15].

Extraction and assay of enzymes

Activity of all the enzymes was measured at 0, 24, 48 and 72 hours after inoculation in healthy and *G. cingulata* infected tea leaves. Protein content in the enzyme extracts was determined following the standard procedure [16].

Phenylalanine ammonia lyase

Phenylalanine ammonia lyase (PAL; E.C. 4.3.1.5) was extracted [17] in chilled 0.1 M sodium borate buffer (pH 8.8) containing 2mM β-mercaptoethanol. The reaction mixture contained 0.3ml of 300μ M sodium borate buffer (pH 8.8), 0.3ml 30μ M L-phenylalanine and 0.5 ml of enzyme extract. Following incubation for 1h at 40 °C, the absorbance at 290nm was read using UV-VIS Spectrophotometer (Digispec 200GL) against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as μ g cinnamic acid liberated from L-phenylalanine mg^{-1} protein $min.^{-1}$.

Tyrosine ammonia lyase

For extraction of Tyrosine ammonia lyase (TAL; E.C. 4.3.1.5), leaves were crushed using mortar and pestle on ice in 0.1 M sodium borate buffer (pH 8.8) containing 2mM β-mercaptoethanol [18]. The slurry was centrifuged at 15,000 rpm for 20 min. at 0°C. The supernatant was collected, its final volume was measured and the extract used immediately for

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assay. TAL activity in the supernatant was determined by measuring the production of p-coumaric acid from L-tyrosine spectrophotometrically. The reaction mixture contained 0.3ml of 300□ M sodium borate buffer (pH 8.8), 0.3ml 30□ M L-tyrosine and 0.5 ml of supernatant in a total volume of 3ml. Following incubation for 1h at 40^{o}C, the absorbance at 290nm was read using UV-VIS Spectrophotometer (Digispec 200GL) against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as □ g p-coumaric acid liberated from L-tyrosine mg^{-1} protein min.$^{-1}$

Polyphenol oxidase

For extraction of Polyphenol oxidase (PPO; E.C. 1.14.18.1), leaf tissue was crushed on ice with mortar and pestle [19] with 5 ml of 0.1 M sodium phosphate buffer (pH 6.5). The slurry was immediately centrifuged at 4000 rpm for 30 min. at 4^{o}C. The supernatant was collected and after recording its volume was used immediately for assay and isozyme analysis. For assay of PPO (catecholase) activity, 100 □ l of freshly prepared enzyme extract was mixed with 1.9 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 1ml of 0.025 M catechol solution. The reaction mixture was incubated in dark at room temperature for preventing photooxidation. Further readings were taken at 1 min interval at 495nm. The blank was set with 3 ml of phosphate buffer and enzyme activity expressed as □ A_{495} mg $^{-1}$ protein min.$^{-1}$

Isozyme analysis of PPO by Native PAGE

The extraction of polyphenol oxidase was conducted as described earlier. Casting and running of the Native PAGE was performed according to standard procedure [20]. After electrophoresis, the gel was removed carefully from the glass plates and then stacking gel was cut off from the resolving gel and finally stained. After equilibration of the gel for 30min. in 0.1% p-phenylenediamine in 0.1M potassium phosphate buffer (pH 7.0) at 4^{o}C, it was stained in 10mM catechol solution dissolved in 0.1M potassium phosphate buffer (pH 7.0) for 1h [21]. Analysis of bands was done immediately after their appearance.

RESULTS

Assessment of inoculation infectivity and symptom development revealed that T-17/1/54, TV-22, BSS-3, UP-26, CP-1/1 and AV-2 were highly susceptible showing high percentage of lesion formation at each interval (Figure 1). On the other hand, resistant reaction was shown by TV-30 and BS/7A/76. Only one variety (UP-3) was moderately susceptible. The remaining varieties (TV-18, TV-25, TV-26, TV-29, BSS-2, UP-2, UP-9, TS-449 and P-312) showed moderately resistant reactions.

G. cingulata - infected tissues exhibited an increase in PAL activity 24h after inoculation with respect to control in all the varieties (Table 1). This was followed by a rapid decline. The highly susceptible varieties exhibited very low increase in activity on inoculation. Significantly

higher activities were observed in the inoculated leaf samples as tested by Student's t-test in the resistant varieties (Table 1) especially 24 h after inoculation.

The activity of TAL was lower than that of PAL and reached its peak 48 hours after inoculation. The increase was statistically significant as compared to uninoculated control tissues in case of the resistant varieties. In the susceptible lines there was a statistically very insignificant increase and the activity remained at the control level (Table 2).

In general, the activity profile of PPO was on the increasing trend in all the varieties. Significant differences between healthy and inoculated samples were found as tested by Student's t-test at 24 hours after inoculation in the resistant varieties (TV-29, TV-30, BSS-2, UP-2, UP-9, TS-449 and P-312) as evident from Table 3. On the other hand, at 48 hours after inoculation there was significant increase in all the varieties tested, irrespective of reaction with pathogen. However, activity reached its peak 72 hours after inoculation. High PPO activity in *G. cingulata* inoculated samples persisted or even increased further in the resistant varieties 72 hours after inoculation .

Karl-Pearson's correlation coefficient between percentage infection at 72 hours after inoculation and PPO activity was found to be - 0.64833, which was highly significant at 5% level, therefore, this point was selected for the detailed analysis of isozyme profile. There was loss of some isozymes of PPO on inoculation of *G. cingulata* especially in the compatible (susceptible) interactions (Table 4). Some susceptible varieties (TV-22, UP-26, CP-1/1 and AV-2) had lost one isozyme on inoculation with *G. cingulata*. No changes were observed in isozyme profiles on inoculation in three susceptible (T-17/1/54, BSS-3 and UP-3) and four moderately resistant (TV-26, TV-29, UP-9, TS-449) varieties. The isozymes with R_m=0.12 and 0.25 were present in all the healthy samples and are clearly constitutive. The isozyme with R_m=0.81 was present in 11 varieties constitutively and could not be associated with reaction towards pathogen. The prominent isozyme band with R_m=0.55 was induced only in the resistant tea leaf tissues (TV-30, TV-18, TV-25, BSS-2, UP-2, BS/7A/76 and P-312) on inoculation. This particular isozyme, therefore, is associated with defense reaction in tea.

DISSCUSSION

The present findings definitely confirm the reports on involvement of PAL in the incompatible reactions [21-26]. According to these reports, it is an early enzyme involved in stress and according to the present findings, it is the one induced as early as 24 hours after inoculation with *G. cingulata*. Significantly higher PAL activity in the incompatible interactions was also demonstrated in woody kiwifruit vines [27] and in tea plants [28].

TAL is a subsidiary enzyme in the phenylpropanoid pathway and it catalyses conversion of L-tyrosine to p-coumaric acid, which in turn is converted to caffeic acid, a phenolic precursor. The activity of TAL is not well-demonstrated in dicots [27]. However, Beaudoin-Eagan and

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Table 1: PAL (Phenyl alanine ammonia lyase) activities in tea varieties at various time intervals after inoculation with *G. cingulata*

Variety	Treatment	Phenyl alanine ammonia lyase activity (μg cinnamic acid mg^{-1} protein $min.^{-1}$)			
		Time after inoculation (h)			
		0	24	48	72
Resistant					
TV-30	H	16.3 ± 0.7	16.1 ± 0.5	11.3 ± 1.3	09.8 ± 1.2
	I	16.3 ± 1.2	**25.0 ± 2.5	14.2 ± 2.1	13.2 ± 1.7
BS/ 7A/ 76	H	16.8 ± 0.7	15.0 ± 1.6	14.3 ± 1.2	10.2 ± 0.9
	I	17.0 ± 0.7	**23.6 ± 2.9	15.0 ± 2.5	10.5 ± 3.4
Moderately resistant					
TV-18	H	16.6 ± 1.5	12.0 ± 1.3	09.5 ± 1.2	04.8 ± 1.9
	I	16.7 ± 1.7	**18.0 ± 2.2	11.1 ± 1.2	06.3 ± 2.4
TV-25	H	18.2 ± 1.1	18.6 ± 0.9	12.7 ± 1.3	12.1 ± 1.7
	I	18.5 ± 0.8	20.3 ± 2.1	**16.9 ± 2.6	15.8 ± 2.4
TV-26	H	11.4 ± 0.8	11.1 ± 1.5	09.3 ± 0.8	08.7 ± 1.6
	I	11.4 ± 1.7	**17.3 ± 2.5	09.9 ± 2.3	08.9 ± 2.8
TV-29	H	10.2 ± 1.1	10.1 ± 0.5	06.7 ± 0.7	06.3 ± 1.9
	I	10.5 ± 2.2	**19.4 ± 2.3	09.5 ± 1.2	09.2 ± 2.0
BSS-2	H	12.9 ± 1.2	13.2 ± 1.3	09.8 ± 2.1	09.6 ± 1.0
	I	13.1 ± 0.9	** 21.1 ± 1.2	10.6 ± 1.4	10.9 ± 2.4
UP-2	H	16.6 ± 1.3	13.3 ± 0.7	12.9 ± 1.1	12.2 ± 1.4
	I	16.6 ± 0.5	**18.8 ± 1.2	14.4 ± 2.1	13.7 ± 1.7
UP-9	H	11.9 ± 0.9	11.5 ± 0.7	08.0 ± 1.9	07.2 ± 2.1
	I	12.2 ± 0.7	13.2 ± 1.9	10.7 ± 2.5	10.3 ± 2.3
TS-449	H	12.1 ± 0.8	11.1 ± 1.3	10.1 ± 2.4	08.6 ± 2.4
	I	10.8 ± 1.1	**18.8 ± 2.4	11.0 ± 1.7	09.0 ± 2.5
P-312	H	13.5 ± 0.7	13.0 ± 0.5	12.3 ± 1.8	08.2 ± 2.8
	I	13.4 ± 0.5	16.0 ± 1.6	13.0 ± 2.4	10.1 ± 2.3
Moderately susceptible					
UP-3	H	11.7 ± 0.7	12.0 ± 1.2	08.7 ± 1.1	08.2 ± 0.9
	I	11.9 ± 0.5	14.8 ± 1.3	08.8 ± 1.0	06.8 ± 2.3
Susceptible					
TV-22	H	16.7 ± 0.9	12.5 ± 1.3	11.1 ± 1.6	10.6 ± 1.7
	I	17.0 ± 1.2	**18.8 ± 2.8	11.8 ± 2.3	11.6 ± 2.6
T-17/1/54	H	16.6 ± 0.8	16.5 ± 1.5	12.0 ± 0.7	11.9 ± 1.4
	I	16.7 ± 0.9	19.1 ± 1.9	12.5 ± 1.6	12.1 ± 2.3
BSS-3	H	13.3 ± 1.3	10.9 ± 1.2	10.3 ± 0.7	06.3 ± 1.4
	I	13.0 ± 1.2	13.8 ± 2.1	11.0 ± 2.1	06.8 ± 2.7
UP-26	H	13.8 ± 1.2	12.8 ± 1.1	10.4 ± 1.3	09.0 ± 2.1
	I	14.0 ± 1.6	14.5 ± 2.5	11.8 ± 2.4	09.9 ± 2.7
CP-1/1	H	15.3 ± 0.4	15.2 ± 0.7	11.3 ± 1.5	08.8 ± 2.1
	I	15.5 ± 0.5	16.6 ± 1.5	13.7 ± 1.9	12.9 ± 2.7
AV-2	H	06.9 ± 0.8	07.0 ± 1.1	04.0 ± 1.6	03.2 ± 2.7
	I	06.8 ± 0.9	**10.5 ± 1.3	06.0 ± 0.9	05.5 ± 0.8

H- Healthy control; I- Inoculated; Means ± S.E, n=3. ; ** Difference between healthy and infected Significant at 1% level as tested by Student's t-test

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Table 2: TAL (Tyrosine ammonia lyase) activities in tea varieties at various time intervals after inoculation with *G. cingulata*

Variety	Treatment	Tyrosine ammonia lyase activity (μg coumaric acid mg^{-1} protein $min.^{-1}$)			
		Time after inoculation (h)			
		0	24	48	72
Resistant					
TV-30	H	4.17 ± 0.17	3.87 ± 0.13	3.65 ± 0.35	3.94 ± 0.22
	I	4.46 ± 0.29	4.85 ± 0.55	**8.19 ± 0.59	4.66 ± 0.75
BS/ 7A/ 76	H	4.41 ± 0.16	4.17 ± 0.28	4.08 ± 0.21	3.76 ± 0.16
	I	4.40 ± 0.16	4.25 ± 0.24	**5.93 ± 0.31	3.60 ± 0.24
Moderately resistant					
TV-18	H	7.21 ± 0.15	6.32 ± 0.34	6.24 ± 0.20	6.15 ± 0.27
	I	7.93 ± 0.74	6.41 ± 0.23	**9.39 ± 0.27	**6.75 ± 0.45
TV-25	H	4.50 ± 0.17	4.72 ± 0.41	4.92 ± 0.33	4.45 ± 0.51
	I	4.32 ± 0.43	4.54 ± 0.18	*5.98 ± 0.65	4.07 ± 0.72
TV-26	H	4.16 ± 0.08	4.43 ± 0.50	4.92 ± 0.12	4.61 ± 0.22
	I	4.23 ± 0.20	4.55 ± 0.52	*5.89 ± 0.83	4.30 ± 0.81
TV-29	H	4.23 ± 0.21	4.14 ± 0.23	3.78 ± 0.22	3.37 ± 0.29
	I	4.31 ± 0.25	**4.98 ± 0.58	**5.94 ± 0.52	**4.63 ± 0.68
BSS-2	H	5.52 ± 0.22	5.24 ± 0.30	4.75 ± 0.19	4.53 ± 0.31
	I	5.67 ± 0.25	5.38 ± 0.48	**6.67 ± 0.45	4.63 ± 0.41
UP-2	H	2.77 ± 0.36	2.54 ± 0.19	2.46 ± 0.25	2.48 ± 0.41
	I	2.80 ± 0.25	2.94 ± 0.19	**4.49 ± 0.23	2.37 ± 0.34
UP-9	H	2.25 ± 0.09	2.21 ± 0.08	2.80 ± 0.11	2.64 ± 0.09
	I	2.08 ± 0.45	3.52 ± 0.51	**3.78 ± 0.42	2.86 ± 0.36
TS-449	H	3.70 ± 0.25	3.61 ± 0.22	3.51 ± 0.12	3.41 ± 0.14
	I	3.72 ± 0.53	3.96 ± 0.41	**5.14 ± 0.70	**3.98 ± 0.51
P-312	H	1.32 ± 0.11	1.07 ± 0.09	1.22 ± 0.08	1.73 ± 0.14
	I	1.46 ± 0.18	1.35 ± 0.16	*2.65 ± 0.25	2.11 ± 0.32
Moderately susceptible					
UP-3	H	2.23 ± 0.17	2.15 ± 0.18	1.98 ± 0.13	1.86 ± 0.18
	I	2.26 ± 0.15	2.56 ± 0.32	2.36 ± 0.33	2.19 ± 0.38
Susceptible					
TV-22	H	4.44 ± 0.45	4.18 ± 0.35	4.35 ± 0.41	4.89 ± 0.56
	I	4.71 ± 0.24	4.57 ± 0.86	4.28 ± 0.39	4.41 ± 0.60
T-17/1/54	H	3.21 ± 0.08	3.54 ± 0.40	3.82 ± 0.45	2.82 ± 0.09
	I	3.76 ± 0.28	3.53 ± 0.65	3.68 ± 0.52	3.87 ± 0.49
BSS-3	H	5.35 ± 0.32	5.14 ± 0.24	5.07 ± 0.15	5.53 ± 0.24
	I	5.46 ± 0.33	5.65 ± 0.25	5.09 ± 0.26	5.69 ± 0.35
UP-26	H	3.07 ± 0.19	2.64 ± 0.33	2.57 ± 0.32	2.34 ± 0.25
	I	3.24 ± 0.26	2.66 ± 0.25	** 4.75 ± 0.24	2.43 ± 0.27
CP-1/1	H	3.53 ± 0.14	3.34 ± 0.17	3.45 ± 0.13	2.90 ± 0.11
	I	3.61 ± 0.15	3.64 ± 0.26	3.38 ± 0.39	3.25 ± 0.48
AV-2	H	2.75 ± 0.08	2.56 ± 0.21	2.27 ± 0.26	2.09 ± 0.17
	I	2.66 ± 0.21	2.26 ± 0.33	2.67 ± 0.50	2.19 ± 0.47

H- Healthy control; I- Inoculated; Means ± S.E, n=3. ; Difference between healthy and infected significant at *5% and at **1% level as tested by Student's t-test.

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Table 3: PPO (Polyphenol oxidase) activities in tea leaf tissues of different varieties at various time intervals after inoculation with *G.cingulata*

Variety	Treatment	Polyphenol oxidase activity (x0.01 ΔA_{495nm} mg^{-1} protein $min.^{-1}$)			
		Time after inoculation (h)			
		0	24	48	72
Resistant					
TV-30	H	0.60 ± 0.07	0.69 ± 0.05	0.74 ± 0.03	0.65 ± 0.02
	I	0.65 ± 0.02	**2.36 ± 0.02	**3.50 ± 0.01	**7.30 ± 0.07
BS/ 7A/ 76	H	1.10 ± 0.07	1.02 ± 0.06	0.90 ± 0.02	0.60 ± 0.09
	I	1.00 ± 0.07	0.82 ± 0.09	**1.26 ± 0.05	** 5.02 ± 0.04
Moderately resistant					
TV-18	H	1.40 ± 0.02	1.65 ± 0.04	1.48 ± 0.04	1.20 ± 0.05
	I	1.30 ± 0.09	1.80 ± 0.04	**2.10 ± 0.02	**5.90 ± 0.07
TV-25	H	0.55 ± 0.10	0.42 ± 0.09	0.47 ± 0.03	0.38 ± 0.07
	I	0.58 ± 0.08	0.76 ± 0.10	*0.83 ± 0.06	**2.56 ± 0.04
TV-26	H	0.63 ± 0.08	0.82 ± 0.05	1.00 ± 0.08	0.57 ± 0.06
	I	0.65 ± 0.07	0.83 ± 0.05	**1.70 ± 0.03	**2.74 ± 0.08
TV-29	H	0.40 ± 0.01	0.36 ± 0.05	0.32 ± 0.07	0.30 ± 0.09
	I	0.41 ± 0.02	**1.23 ± 0.02	**1.56 ± 0.02	**2.36 ± 0.05
BSS-2	H	0.64 ± 0.02	0.69 ± 0.03	0.56 ± 0.10	0.59 ± 0.06
	I	0.68 ± 0.09	**1.30 ± 0.02	**1.50 ± 0.04	**3.10 ± 0.04
UP-2	H	3.51 ± 0.03	2.32 ± 0.07	2.52 ± 0.10	2.40 ± 0.04
	I	3.57 ± 0.05	**4.05 ± 0.02	** 4.85 ± 0.12	**3.60 ± 0.07
UP-9	H	0.58 ± 0.09	0.88 ± 0.07	0.40 ± 0.09	0.22 ± 0.01
	I	0.63 ± 0.07	**1.48 ± 0.09	** 3.16 ± 0.05	** 1.50 ± 0.03
TS-449	H	0.63 ± 0.08	0.60 ± 0.03	0.50 ± 0.02	0.52 ± 0.04
	I	0.65 ± 0.10	1.00 ± 0.04	**1.54 ± 0.07	**2.87 ± 0.05
P-312	H	0.56 ± 0.03	0.55 ± 0.05	0.42 ± 0.08	0.58 ± 0.08
	I	0.50 ± 0.05	**1.20 ± 0.06	**2.80 ± 0.04	**5.74 ± 0.03
Moderatelysusceptible					
UP-3	H	3.20 ± 0.07	3.50 ± 0.02	3.00 ± 0.11	2.45 ± 0.09
	I	3.00 ± 0.05	3.50 ± 0.03	** 3.90 ± 0.01	**3.70 ± 0.03
Susceptible					
TV-22	H	0.52 ± 0.09	0.56 ± 0.03	0.54 ± 0.06	0.47 ± 0.07
	I	0.51 ± 0.02	0.59 ± 0.08	**1.90 ± 0.03	**1.30 ± 0.06
T-17/1/54	H	1.30 ± 0.08	1.00 ± 0.05	0.90 ± 0.07	0.40 ± 0.04
	I	1.32 ± 0.09	1.50 ± 0.09	**1.73 ± 0.06	**1.20 ± 0.03
BSS-3	H	0.54 ± 0.03	0.58 ± 0.02	0.55 ± 0.07	0.49 ± 0.04
	I	0.56 ± 0.02	0.59 ± 0.01	* 1.00 ± 0.14	0.68 ± 0.07
UP-26	H	0.49 ± 0.02	0.47 ± 0.01	0.46 ± 0.03	0.51 ± 0.10
	I	0.52 ± 0.06	0.69 ± 0.05	**1.05 ± 0.04	* 0.85 ± 0.07
CP-1/1	H	2.30 ± 0.04	2.00 ± 0.07	1.90 ± 0.05	1.70 ± 0.10
	I	2.60 ± 0.05	2.05 ± 0.05	**2.67 ± 0.09	2.09 ± 0.09
AV-2	H	0.60 ± 0.08	0.58 ± 0.10	0.54 ± 0.06	0.49 ± 0.07
	I	0.61 ± 0.10	0.64 ± 0.03	0.98 ± 0.09	0.52 ± 0.08

H- Healthy control; I- Inoculated; Means ± S.E, n=3. ; Difference between healthy and infected significant at *5% and at **1% level as tested by Student's t-test.

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Table 4: Isozymes (PPO-1, PPO-2, PPO-3, PPO-4) of polyphenoloxidase expressed constitutively and on *G.cingulata* inoculation of tea leaves of different varieties.

Disease reaction	Variety	Treatment	Number of isozymes	PPO-1 R_m=0.12	PPO-2 R_m=0.25	PPO-3 R_m=0.55	PPO-4 R_m=0.81
Resistant							
	TV-30	H	3	(+)	(+)	(-)	(+)
		I	4	(+)	(+)	(+)	(+)
	BS/ 7A/ 76	H	2	(+)	(+)	(-)	(-)
		I	4	(+)	(+)	(+)	(+)
Moderately resistant							
	TV-18	H	2	(+)	(+)	(-)	(-)
		I	3	(+)	(+)	(+)	(-)
	TV-25	H	2	(+)	(+)	(-)	(-)
		I	3	(+)	(+)	(+)	(-)
	TV-26	H	3	(+)	(+)	(-)	(+)
		I	3	(+)	(+)	(-)	(+)
	TV-29	H	3	(+)	(+)	(-)	(+)
		I	3	(+)	(+)	(-)	(+)
	BSS-2	H	3	(+)	(+)	(-)	(+)
		I	4	(+)	(+)	(+)	(+)
	UP-2	H	3	(+)	(+)	(-)	(+)
		I	4	(+)	(+)	(+)	(+)
	UP-9	H	3	(+)	(+)	(-)	(+)
		I	3	(+)	(+)	(-)	(+)
	TS-449	H	3	(+)	(+)	(-)	(+)
		I	3	(+)	(+)	(-)	(+)
	P-312	H	3	(+)	(+)	(-)	(+)
		I	4	(+)	(+)	(+)	(+)
Moderately susceptible							
	UP-3	H	2	(+)	(+)	(-)	(-)
		I	2	(+)	(+)	(-)	(-)
Susceptible							
	TV-22	H	3	(+)	(+)	(-)	(+)
I	2	(+)	(+)	(-)	(-)		
	T-17/1/54	H	2	(+)	(+)	(-)	(-)
		I	2	(+)	(+)	(-)	(-)
	BSS-3	H	2	(+)	(+)	(-)	(-)
		I	2	(+)	(+)	(-)	(-)
	UP-26	H	2	(+)	(+)	(-)	(-)
		I	1	(-)	(+)	(-)	(-)
	CP-1/1	H	3	(+)	(+)	(-)	(+)
		I	2	(+)	(+)	(-)	(-)
	AV-2	H	3	(+)	(+)	(-)	(+)
		I	2	(+)	(+)	(-)	(-)

H- healthy control; I- inoculated

(+) – band present; (-) – band absent

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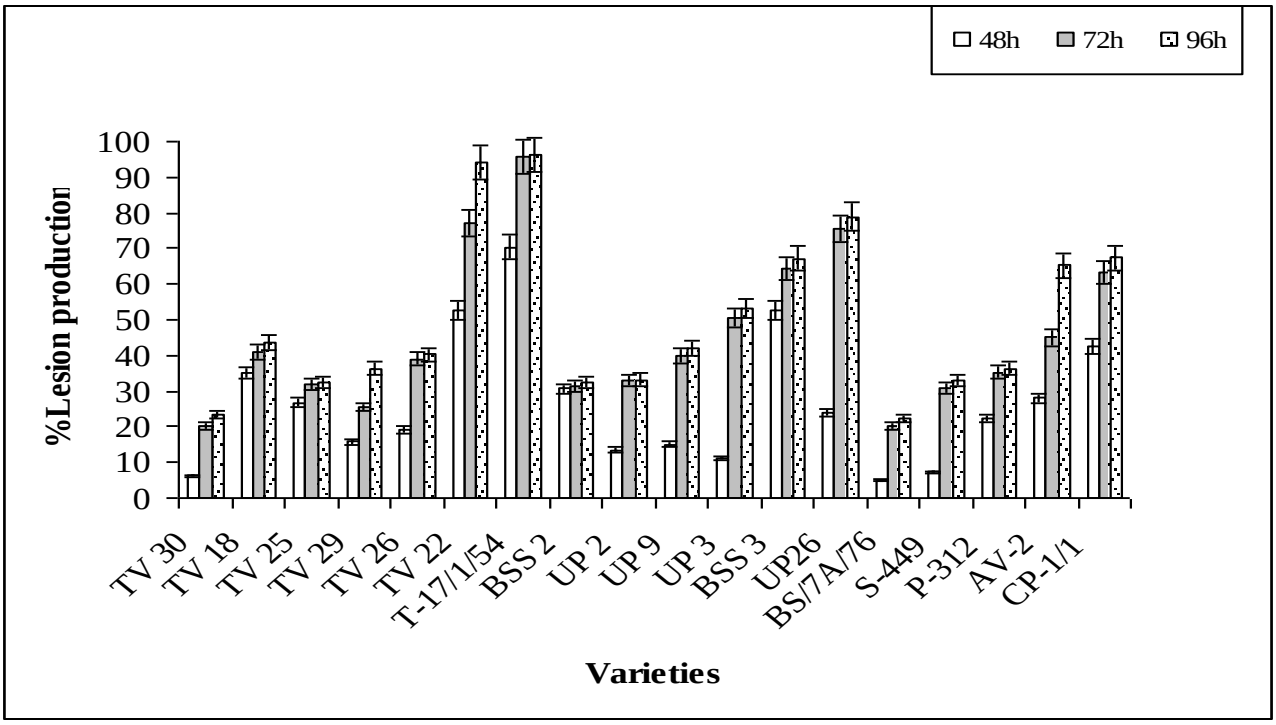

Figure 1. Brown blight disease assessment of tea varieties caused by *G. cingulata* at different time intervals.

Thorpe [3] had clearly demonstrated the activity of TAL during shoot initiation in tobacco callus cultures. TAL activity was definitely lower than PAL in the present findings similar to the results obtained in wheat tissues by Guerra *et al* [29], who also used the same tissue extract for estimation of both the enzymes. Notably, similar results on inoculation with pathogens were obtained in rubber [30] and tea [31].

Similar to the present report, PPO activity was definitely associated with resistance in many studies [10, 32-34]. In the resistant reaction in case of a woody perennial date palm, there was a five-fold increase in PPO activity [35]. Vanitha *et al* [36] have found that PAL and PPO activity increased significantly in the resistant cultivars of tomato at 12 h and 15h after pathogen inoculation. Thus, time course accumulation of PPO in disease reaction was more useful than estimation at only one point of time.

Isozyme pattern analysis of PPO indicates that there were varietal differences in the number of isozymes. Inoculation often reduced the intensity of the bands as well as their number probably due to destruction of tissue and symptom expression especially in the susceptible varieties. However, the isozyme with R_m= 0.55 was definitely induced only in the resistant varieties. Polyphenol oxidase isozymes had been studied earlier and particular isoforms were related to resistance in perennial crops like cocoa [37] and coconut [11].

Therefore, the time-course activity profile indicate that the three defense enzymes - PAL, TAL and PPO prevent the infection by *G. cingulata* in the resistant tea varieties, in a sequential manner. PAL is induced first, followed by TAL and then PPO, during biotic stress induced by *Glomerella cingulata* in tea plants.

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