MATERIALS & METHODS

3. 1. Plant Material

3.1.1 Collection

Fresh clonal cuttings of six Darjeeling varieties (BS/7A/76, T-78, B-157, T-135, HV-39, AV-2) generally grown in Darjeeling hills were collected from Darjeeling Tea Research Centre, Kurseong, West Bengal.

Seven TV clones (TV-9, TV-18, TV-20, TV-22, TV-23, TV-25, TV-26 and T-17/1/54), released by Tocklai Experimental Station, Jorhat, Assam, were collected from the clone house of Mohurgaon and Gulma Tea Estate, Sukna, W.B.

Cuttings of five clonal varieties (UPASI-2, UPASI-3, UPASI-8, UPASI-9, UPASI-26) and seeds of two varieties (BSS-1and BSS-2) were collected from UAPSI Tea Research Station, Valparai, Tamil Nadu.

3.1.2. Propagation

For propagation of tea plants by cuttings, sandy soil (sand 75% and soil 25%) with a pH ranging from 4.5-4.8 was used. Soil pH was adjusted by treating with 2% aluminium sulphate solution. It was followed by two waterings to remove excess aluminium sulphate. The treated soil was heated between 60^0-80^0C over a metal sheet with fire below to kill eelworms, if any, present in the soil.

Polythene sleeves were filled up with the prepared soil and stacked in rows in a bed and watered throughly. All cuttings were allowed for rooting in sleeves after dipping them in hormone. These cuttings (Plates II and III) were kept in a polythene cloche and watered every 4th day until the appearance of new leaf.

3.1.3. Plantation

Before planting, Simazine @ 75g/20lt.water and Glyophosphate @ 1:200 were used in the experimental plot for weed control (Borpujari and Banerjee 1994). Then pits (.45m x .45m x .45m) were dug at the intervals of 5cm between plants and 9cm between row to row. Planting mixture were prepared in the ratio of 4.5 kg of well rotten dry cattle manure, 30 kg rock phosphate, 30 kg. super phosphate and 2.5 g phorate [O,O- diethyl S-(ethylthiomethyl) phosphoro dithoioate]. At the bottom of each pit, rock phosphate was placed follwing which half portion was covered with

Plate II (figs. A-C) : Seedlings of tea varieties in sleeves

Plate III (figs. A & B) : Seedlings of tea in sleeves;
A- UPASI-3; B - Tocklai varieties

cattle manure soil mixture. Phorate was mixed with a portion of excavated soil and was applied approximately 5 cm. below the ground level.

Following soil conditioning, the plants were inspected, selected and brought to the experimental garden and planted in the prepared soil. Pits were prepared with conditioned soil.

Tea plants of all the varieties were also grown in earthen pots (one plant per pot of 30cm. diameter) each containing 5 kg soil mixture (soil: planting mixture 1:1). All these plants were maintained in the Glass House under natural condition.

3.1.4. Maintenance

In case of young plants, manuring (aluminium phosphate – 8 parts by weight, ammonium phosphate-sulphate 16:20 – 35 parts by weight, magnesium sulphate and zinc sulphate 3 parts by weight) was done after rooting following the method of Ranganathan and Natesan (1987) and continued up to twelve months once only in 15 days. The mixture was dissolved @ 30g in 1lt. of water and applied @ 50 ml/ plant.

The mature plants (1year and above) were maintained by applying a soluble mixture of N, P, K consisting of 10 kg urea(46% N), 20 kg ammonium phosphate (11% P_2O_5), 8 kg muriate of potash (60% K_2O) in the soil. Miraculin (7ml/10L) was sprayed at regular intervals for good growth of bush.

Only tipping was done once in a year to promote lateral branching in young plants (three years) but in case of mature plants two year of deep pruning cycle was maintained.

3.2. Application of temperature treatment

Tea plants were subjected to elevated temperatures in two ways- either to deteched leaves of well grown bushes, or to young intact seedlings . In all temperature treatments , ambient temperature of 31± 3^{0}C was taken as control.

3.2.1. Detached leaves

Elevated temperature treatment was given to twenty tea varieties collected from three experimental stations i.e. UPASI-2, UPASI-3, UPASI-8, UPASI-9, UPASI-26,

BSS-2, BSS-3, T-17/1/54, TV-9, TV-18, TV-22, TV-23, TV-25, TV-26, BS/7A/74, T-78, T-135, B-157, HV-39 & AV-2.

For elevated temperature treatment of detached leaves, the method of Mason-Apps *et al.*, (1990) were followed with some modifications. Very young tea leaves (first four leaves) were collected from healthy bush of the experimental garden of Department of Botany and immediately immersed in temperature controlled water bath at desired temperature ranging from 40^0C – 60^0C, at 5^0C interval, for a period of two hours.In all experiments, ambient temperature of around 35^0C, was taken as control.

3.2.2. Intact plant

Intact plants were subjected elevated temperatures by keeping young plants (one and half year old nursery seedlings) in temperature regulated humid chambers for a period of four hours. Ten varieties of seedlings were selected from the above twenty varieties,ie., UP-3, UP-9, BSS-2, T-17/1/54, TV-18, TV-23, TV-25, TV-26, T-78 & HV-39. Temperature treatment was given from 40^0C onwards, at 5^0C intervals.

3.3. Application of water stress

Six young tea varieties, UP-3, UP-26, TV-18, TV-26, T-78 and HV-39, were selected for the application of drought stress. One-year-old nursery seedlings of different tea varieties were transferred to earthen pots. Regular maintenance schedules was followed for next one year. Water stress treatment was applied in these seedlings following the methods of Sinha *et al.*, (1999). Water was withheld from the pots completely for a period of 4, 8 and 12 days. After each period, sampling was done, morphological changes noted and soil moisture content determined.

3.4. Determination of moisture content after water stress treatment

Miosture content of soil was determined by direct method of Gardner (1965). Soil samples (30g) were taken in weighing bottles with tight fitting lids. Samples were weighed, lids were removed, placed in hot air oven, and dried it to constant weight. After that, samples were removed from the oven, placed in a desiccator until cooled and final weights were taken. Percentage of moisture content in the soil was then

obtained by dividing the difference between the wet and dry masses by the mass of the dry sample multiplied by 100.

3.5. Chemical sprays

One each of commonly used fungicide and insecticide were selected for studies on chemical sprays.

3.5.1. Fungicide

Hexaconazole is the most common fungicide which is applied to tea for the prevention of various kind of fungal diseases, of which blister blight is the most remarkable one. Two TV varieties (two year old), namely TV-18 and TV-26 were selected for the application of this stress. The seedlings were transferred to earthen pots after one year and routine maintenance were done for the next one- year and finally 0.1% solution of hexaconazole (normally applied by the planters in nursery) was sprayed with mist sprayer at an interval of 7 days. After three consecutive sprays, visible morphological effects were noticed and no more sprays were applied.

3.5.2. Insecticide

Monocil is one of the most common insecticide which is used by the planters of the Terai area for the prevention of various kind of insecticidal problems. Following the same procedure discussed above, 0.1% solution of monocil was applied three times to TV-18 and TV-26.

3.6. Collection of blister infected leaves

Fresh blister infected and healthy leaf samples were collected from different tea estates in Darjeeling hills namely Margaret's Hope Tea Garden (MarH TG), Diram Tea Garden (Dil TG), Singel Tea Garden (Sin TG), Castleton Tea Garden (Cas TG), Mokaibari Tea Garden (Mok TG) and Marrionbarie Tea Garden (Mar TG). In the Terai region, healthy and blister infetced samples were collected from Hansqua Tea Estate. Following collection, samples were immediately immersed in distilled water and brought to the laboratory for analyses. During collection, various stages of blisters were separately collected – young, mature and old.

3.6.1. Artificial inoculation of blister blight

Blister blight disease causing organism is an obligate fungus of basidiomycetes group. The pathogen is an obligate one. Artificial inoculation was done in the following procedure:

3.6.1.1. Material collection

Tea shoots containing blister infected leaves were collected from tea gardens with sharp scalpel and immediately transferred to 2% sucrose solution and brought to the laboratory.

3.6.1.2. Spore collection

For collection of blister spores, twigs with blister infected leaves were put into 2% sucrose solution as described earlier. The infected portions were positioned at the mouth of a sterile beaker and the set up was placed around 18^0C. Spores were collected at the bottom of the beakers. Spore suspension was prepared after two days by dissolving the spores in sterile distilled water.

3.6.1.3. Inoculation of plant material

Young nursery seedlings of TV-26 were selected for the inoculation by the pathogen. The healthy sleeves were placed in beakers and the leaves were surface sterilized with sterile distilled water. With the help of a brush the spores were painted on the both surfaces of the leaves of the seedlings. The whole set up was covered with polythene sheets and tightly tied at the bottom to prevent moisture loss. Under humid condition, the sleeves were placed inside the BOD adjusted to 22^0C.

3.7. Extraction of enzymes from tea leaves

Three enzymes – phenylalanine ammonia lyase, polyphenol oxidase and peroxidase – involved in phenol metabolism were extracted from tea leaves to determine their activites.

3.7.1. Phenylalanie ammonia lyase

For the extraction of phenylalanine ammonia lyase (PAL), the method of Chakraborty *et al.*, (1993) was followed. Leaves (1g each) were crushed in a mortar with pestle in 5 ml of 0.1 M sodium borate buffer (pH8.8) containing 2mM β-mercaptoethanol in ice. The slurry was centrifuged at 15,000 r.p.m. for 20 min. at 4^0C. The supernatant was collected and after recording its volume, was used immediately for assay or stored at -20^0C.

3.7.2. Polyphenol oxidase

For the extraction of polyphenol oxidase the method of Mahadevan and Sridhar (1982) was followed with a little modification. Leaf tissues were cut into pieces and then crushed in mortar with pestle on ice with 5 ml of 0.2 M sodium phosphate buffer (pH 6.6). The slurry was immediately centrifuged at 4000 r.p.m. for 30 min. at 4^0C. The supernatant was collected and after recording its volume was used immediately for assay or stored at -20^0C.

3.7.3. Peroxidase

To extract peroxidase, the method of Chakraborty *et al.*, (1993) was followed with modification. Tea leaf samples were crushed with 0.1 M sodium borate buffer (pH 8.8) containing 2mM β-mercaptoethanol in mortar with pestle on ice. The homogenate was centrifuged immediately at 15,000 r.p.m. for 20 min. at 4^0C. After centrifugation the supernatant was collected and after recording its volume was used immediately for assay or stored at -20^0C.

3.8. Assay of enzyme activities

Enzyme activities were assayed following specific procedure in each case.

3.8.1. Phenylalanine ammonia lyase

PAL activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The reaction mixture contained 0.3 ml 300μM sodium borate (pH8.8), 0.3ml 30 μM L-phenylalanine and

0.5 ml of supernatant in a total volume of 3 ml. Following incubation for 1 h at 40^{0}C the absorbance at 290 nm was read against a blank without the enzyme in the assay mixture. The enzyme activity was expressed at as μg cinnamic acid produced in one minute / g fresh weight of tissue.

3.8.2. Polyphenol oxidase

Polyphenol oxidase activity were assayed by the method of Mahadevan and Sridhar (1982) with slight modification. 1 ml of freshly prepared enzyme extract was mixed with 2 ml of 0.2 M sodium phosphate buffer (pH 6.0) containing 0.01 M pyrogallol in the dark. Initial absorbance was noted at 495 nm immediately. The reaction mixture was incubated at room temperature in the dark for the prevention of photo oxidation of the enzyme. Further reading were taken after 30 min. at 495 nm. The blank was set with only 3 ml of phosphate buffer. PPO activity was assayed as Δ O.D. at 495nm per min., when the substrate pyrogallol, was oxidised due to the enzyme activity from 1 g of tissue.

3.8.3. Peroxidase

For the determination of peroxidase activity, 100μl of freshly prepared crude enzyme extract was added to the reaction mixture containing 1 ml of 0.2 M sodium phosphate buffer (pH 5.4), 100μl of 4 mM H_2O_2, 100μl of O- dianisidine (5mg/ml of methanol) and 1.7 ml of distilled H_2O. Peroxidase activity was assayed spectrophotometrically at 460 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2. Specific activity was expressed as the increase in absorbance at 460 nm /g tissue /min.

3.9. Extraction and quantification of chlorophyll

Chlorophyll was extracted from leaves following the method of Harborne (1973) with modification. Crushing of leaf tissue (1g) was done in a mortar with pestle using 80% acetone in the dark to prevent the photo oxidation of chlorophyll. The extract was filtered through Whatmann no. 1 filter paper, adding sufficient amount of acetone and the final volume was made up 10 ml. For the estimation of chlorophyll,

O.D. values of the 10^{-1} dilution of the crude cholrophyll sample was measured directly at 645 nm and 663 nm in a spectrophotometer. The amount of total chlorophyll, chlorophyll a and chlorophyll b was calculated by the following formula:

Total chlorophyll = $(20.2\ A_{645} + 8.02A_{663})$ µg/ml

Chlorophyll a = $(12.7A_{663} - 2.69\ A_{645})$ µg/ml

Chlorophyll b = $(22.9\ A_{645} - 4.68\ A_{663})$ µg/ml

3.10. Extraction of proteins

Soluble proteins were extracted from healthy and treated leaves following the method of Chakraborty *et al.* (1995). Leaf tissues (1g) were homogenised with 0.05 M sodium phosphate buffer (pH 7.2) containing 10 mM $Na_2S_2O_5$, 0.5 mM $MgCl_2$, 2 mM soluble Polyvinyl pyrrolidone (PVPP 10,000 M) and 2 mM poly mehtyl sulphonyl fluoride (PMSF) in mortar with pestle at 4^0C with sea sand and insoluble PVPP. The homogenate was centrifuged at 4^0C for 20 min. at 10,000 r.p.m. and the supernatant was used as crude protein extract and immediately stored at -20^0C for further use.

3.10.1. Estimation of protein content

Protein estimation was done following the method of Bradford (1976). The Bradford's reagent was prepared in the following way: 100 mg of Comassie Brilliant Blue G_{250} (Biorad) was dissolved in 50 ml of 95% ethanol, followed by addition of 100 ml of concentrated phosphoric acid and deionized water upto a volume of 200 ml. This stock solution was diluted 5 times with distilled water and filtered through Whatman No. 1 filter paper during estimation. A standard curve was prepared with Bovine Serum Albumin (Sigma). The standard curve showed linearity from 20-150 µg of protein sample in 100µl. To the 100 µl of test protein sample 5 ml of Bradford's reagent was added, mixed in a cyclomixture and incubated at room temperature for 5 min. for blue color development, following which O.D. was measured at 595 nm by UV-spectrophotometer (SICO, model Digispec 200 GL). The O.D. values were plotted on the standard curve prepared for the purpose and the quantity of protein determined from this.

3.10.2. SDS-PAGE analysis of total soluble protein

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for the detailed analysis of protein profile following the method of Laemmli (1970).

3.10.2.1. Preparation of stock solutions

For the preparation of gel following stock solutions were prepared :

A) Acrylamide and N'N' –mehtelene bis acrylamide:

A stock solution containing 29% acrylamide and 1% bis acrylamide was prepared in warm water. As both of them are slowly deaminated to acrylic and bis acrylic acid by alkali and light the pH of the solution was kept below pH 7.0 and the stock solution was filtered through Whatman No. 1 filter paper and was kept in brown bottle, stored at 4^0C and used within one month.

B) Sodium Dodecyl Sulphate (SDS):

A 10% stock solution of SDS was prepared in warm water and stored at room temperature.

C) Tris Buffer:

a) 1.5 M Tris buffer was prepared for resolving gel. The pH of the Tris was adjusted to 8.8 with conc HCl and stored at 4^0C for use.

b) 1.0 M Tris buffer was prepared for use in the stacking and loading buffer. The pH of this Tris was adjusted to 6.8 with concentrated HCl and stored at 4^0C.

D) Ammonium Persulphate (APS):

Fresh 10% APS solution was prepared with distilled water each time before use.

E) Tris –Glycine electrophoresis buffer:

This running buffer consists of 25 mM Tris base, 250 mM glycine (pH8.3) and 0.1% SDS. A 1X solution can be made by dissolving 3.02 g Tris base, 18.8 g glycine and 10 ml of 10% SDS in 1L of distilled water.

F) SDS gel loading buffer:

This buffer contains 50 mM Tris Cl (pH 6.8), 10 mM β Mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol. A 1X solution was made by dissolving 0.5ml of 1M tris buffer (pH6.8), 0.5 ml of 14.4 M β mercaptoethanol, 2 ml of 10% SDS, 10 mg bromophenol blue, 1 ml glycerol in 6.8 ml of distilled water.

3.10.2.2. Preparation of gel

Slab gels of two sizes were prepared for the analysis of protein patterens by SDS-PAGE i.e. big gel (plate size 17cm x 19 cm) and mini gel (8 cm.x 10 cm.). For both types of slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5 mm thick spacers were placed between the glass plates at the three sides and the three sides of glass plates were sealed with high vacuum grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. Resolving and stacking gels were prepared by mixing compounds in the following order by pasture pipette leaving sufficient space for comb the stacking gel (comb + 1 cm.).

Composition of solutions for 10% resolving gel:

Name of the compound	Mini gel	Big gel
Distilled water	2.85 ml	11.9 ml
30% acrylamide mix	2.55 ml	10.0 ml
1.5 M Tris (pH8.8)	1.95 ml	7.5 ml
10% SDS	0.075 ml	0.30 ml
10% APS	0.075 ml	0.30 ml
TEMED	0.003 ml	0.012 ml

Composition of solutions for 5% stacking gel

Name of the compound	Big gel	Mini gel
Distilled water	6.8 ml	2.1 ml
30% acrylamide mix	1.7ml	0.5 ml
1.5 M Tris (pH6.8)	1.25 ml	0.38 ml
10% SDS	0.10 ml	0.03 ml
10% APS	0.10 ml	0.03 ml
TEMED	0.01 ml	0.003ml

After pouring the resolving gel solution, itwas immediately overlayered with isobutanol and kept for polymerization for 2 hours. After polymerization of the resolving gel was complete overlay was poured off and washed with water to remove

any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and comb was inserted immediately and overlayered with water. Finally the gel was kept for polymerization for 30 minutes. After polymerization of the stacking gel the comb was removed and washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris- glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble, trapped at the bottom of the gel, was removed very carefully with a bent syringe.

3.10.2.3. Sample preparation

Sample (32µl) was prepared by mixing the sample protein with 1X SDS gel loading buffer (16µL) in cyclomixture. All the samples were floated in boiling water bath for 3 min. to denature the protein sample. The samples were immediately loaded in a pre- determined order into the bottom of the wells with a microliter syringe. Along with the samples, protein markers consisting of a mixture of six proteins ranging in molecular weight from high to low molecular weight (Phosphorylase b - 97,400; Bovine Serum Albumin – 68,000; Ovalbumin – 43,000; Carbonic Anhydrase 29,000; Soyabean Trypsin inhibitor – 20,000; Lysozyme – 14,300) was treated as the other samples and loaded in a separate well.

3.10.2.4. Electrophoresis

Electrophoresis was performed at constant 15 mA current for a period of 3 hours in case of mini gel and at a constant 30mA for a period of about 6 hours for large gel until the dye front reached the bottom of the gel.

3.10.2.5. Fixing and staining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid : methanol : water (10:20:70) for overnight.

The staining solution was prepared by dissolving 250 mg of coomassie brilliant blue (Sigma R_{250}) in 45 ml of methanol. After the stain was completely dissolved,45 ml of water and 10 ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No. 1 filter paper.

The gel was removed from fixer and stained in this staining solution for 4 hours at 37^{0}C with constant shaking at very low speed. After staining the gel was finally destained with destaining solution containing methanol, water and acetic acid (4.5:4.5:1) at 40^{0}C with constant shaking until the back ground became clear.

3.11. Extraction of free proline

For the extraction of free proline the method of Bates *et al.*, (1973) was followed. 1 g of leaf was crushed with 5 ml of 3% sulphhosalicylic acid in mortar with pestle. The slurry was centrifuged at 5000 r.p.m. at room temperature for 15 min. The supernatant was collected and stored at 4^{0}C for further work.

3.12. Estimation of free proline

Proline content of the extract was estimated as described by Bates *et al.*, (1973) with modification. To 1 ml of extract, 3 ml of distilled water and 1 ml of ninhydrine solution (2 g in 50 ml of acetone and water mixture) were added. Then the mixtures were kept on a boiling water bath for 15 min. After cooling, the reaction mixture was poured in a separating funnel and 5 ml of toluene was added and mixed vigorously. Lower colored layer was taken and O.D. values were measured at 520 nm. Quantification was done from a standard curve of proline.

3.13. Peroxidase isozyme analysis by Poly Acrylamide Gel Electrophoresis (PAGE)

Extract for peroxidase isozymes analysis was prepared by crushing 1g of leaf tissue in mortar with pestle in 2M sodium phosphate buffer (pH7) on ice as described by Davis (1964) and immediately used for the gel.

3.13.1. Preparation of the stock solution

Solution A. Acrylamide stock solution (Resolving gel):

For the preparation of acrylamide stock solution for resolving gel 28 g of acrylamide and 0.74 g of N'N' methelene bis acrylamide was dissolved in 100 ml of

warm distilled water. The stock solution was filtered with Whatman No.1 filter paper and stored at 4^0C in dark bottle.

Solution B. Acrylamide stock solution (Stacking gel):

For the preparation of acrylamide stock solution for stacking gel 10 g of acrylamide and 2.5 g of bis acrylamide was dissolved in 100 ml of warm distilled water. The stock solution was filtered and stored at 4^0C in dark bottle.

Solution C. Tris –HCl (Resolving gel):

36.6 g of Tris base was mixed with distilled water and 0.25 ml of TEMED was added. The pH was adjusted to 8.9 with concentrated HCl. The volume of the solution was made up to 100 ml with distilled water. The solution was stored at 4^0C for future use

Solution D. Tris -HCl (Stacking gel):

5.98 g of tris base was mixed with distilled water and 0.46 ml of TEMED and the pH was adjusted to 6.7 with concentrated HCl. The volume of the solution was made up to 100 ml with distilled water. The solution was stored at 4^0C for future use.

Solution E: Ammonium persulfate solution:

Fresh solution of ammonium persulphate was prepared by dissolving 0.015 g of ammonium persulphate in 10 ml of distilled water.

Solution F: Riboflavin solution:

Fresh solution of riboflavin was prepared by dissolving 0.4 mg of riboflavin in 10 ml of distilled water. The solution was kept in dark bottle to protect from light.

Solution G: Electrode buffer:

Electrode buffer was prepared freshly by dissolving 0.6 g of Tris base and 2.9 g Glycine in 1 L of distilled water.

3.13.2. Preparation of gel

For the polyacrylamide gel electrophoresis of peroxidase isozymes mini slab gel was prepared. For slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any trace of grease and then dried. 1.5 mm thick spacers were placed between the glass plates on three sides and these were sealed with high vacuum grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. 7.5% resolving gel was prepared by mixing solution A: C: E: dH_2O in

the ration of 1: 1: 4: 1 by pasture pipette leaving sufficient space for (comb + 1 cm.) for the stacking gel.

This resolving gel was immediately overlayered with water and kept for polymerization for 2 hrs. After polymerization of the resolving gel was complete overlay was poured off and washed with water to remove any unpolymerized acrylamide. The stacking gel solution was prepared by mixing solution B, D, F and dH_2O in the ratio of 2: 1: 1: 4 .

Stacking gel solution was poured over the resolving gel and comb was inserted immediately and overlayered with water. Finally the gel was kept for polymerization for 30 minutes in strong sunlight. After polymerization of the stacking gel the comb was removed and washed thoroughly. The gel was now finally mounted in the electrophoresis apparatus. Tris- glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble, trapped at the bottom of the gel, was removed very carefully with a bend syringe.

3.13.3. Sample preparation

Sample (32µl) was prepared by mixing the sample enzyme (20µl) with gel loading dye (40% Sucrose and 1% bromophenol blue in distilled water) in cyclomixture in ice. All the solutions for electrophoresis were cooled. The samples were immediately loaded in a predetermined order into the bottom of the wells with a microliter syringe.

3.13.4. Electrophoresis

Electrophoresis was performed at constant 15 mA current for a period of 4 hrs at 4^0C until the dye front reached the bottom of the gel.

3.13.5. Fixing and staining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally stained. Staining of the gel was performed following the method of Reddy and Gasber (1973). The gel was incubated in the aqueous (80 ml) solution of benzidine (2.08g), acetic acid (18 ml), 3%

H_2O_2 (100 ml) for 5 minutes. The reaction was stopped with 7% acetic acid after the appearance of clear blue colored bands. Analysis of isozyme was done immediately.

3.14. Extraction of phenols from leaves

Phenols were extracted from fresh tea leaves following the method of Mahadevan and Sridhar (1982). 1 g of leaf tissues were cut into pieces and immediately immersed in 10 ml of boiling alcohol. After 15 minutes of boiling it was cooled and crushed in mortar and pestle thoroughly in room temperature. The extract was passed through two layers of cheesecloth and then filtered through Whatmann No. 1. Final volume was adjusted with 80% ethanol (5 ml /g fresh weight of leaves). The whole extraction of phenol was done in dark to prevent light induced degradation of phenol.

3.15. Estimation of phenol contents

3.15.1. Total phenol

The total phenol content was estimated by Folin–Ciocalteau's reagent, following the method of Mahadevan and Sridhar (1982). To 1 ml of the alcoholic extract, 1 ml of 1 N Folin –Ciocalteau reagent followed by 2 ml of 20% sodium carbonate solution was added in a test tube. The test tube was shaken and heated on a boiling water bath for 1 min.. After cooling, the volume of the reaction mixture was raised to 25 ml. Absorbance of the blue colored solution was measured in a systronic photoelectric colorimeter Model 101 at 650 nm. Quantity of the total phenol was estimated using caffeic acid as standard.

3.15.2. O-dihydroxy phenol

The O-dihydroxy phenol was estimated following the method of Mahadevan and Sridhar (1982). 1 ml of the alcoholic extract was mixed with 2 ml of 0.05 N HCl, 1 ml of Arnow's reagent ($NaNO_2$ – 10g, Na_2MoO_4 – 10 g, distilled water – 100 ml), and 2 ml of 1 N NaOH and mixed thoroughly in room temperature following which the volume of the reaction mixture was raised to 10 ml. Absorbance of the pink colored solution was recorded by a systronic photoelectric colorimeter Model 101 at

515 nm. Quantity of the O – dihydroxy phenol was estimated using caffeic acid as standard.

3.16. Extraction of antifungal phenolics

The extraction method used was adopted from that of Daayf *et al.*, (1995) with modification for the determination of free and glycosidically linked phenolics. Leaf samples (10 g) were mixed with 80% methanol at 10 ml /g tissue and homogenised by blending for about 1 min. Samples were extracted for 48 hrs on a rotary shaker in a conical flask at 40 r.p.m. covered with aluminium foil for protection from light. Methanolic extracts were then collected by filtration on a Wahtmann No.1 filter disc and concentrated by evaporation to a final volume of 20 ml (aqueous fraction). Concentrates were first partitioned against equal volume of anhydrous diethylether three times. The ether fraction were stored and termed as Fraction I. The aqueous fraction was partitioned secondly against equal volume of ethyl acetate also for three times and the ethyl acetate portion was considered as Fraction II.

Acid hydrolysis, with 4N HCl, of the remaining aqueous fraction (yielding phenolic aglycones) was performed according to the method of Daayf *et al.*, (1997). Aglycones were recovered by partitioning hydrolysates against equal volume of ethyl acetate also for three times and labeled as Fraction III. All the fractions were evaporated to dryness and finally dissolved in 3 ml of respective solvent.

3.17. Thin Layer Chromatography (TLC)

For thin layer chromatography, clean, grease free glass plates were coated evenly with Silica Gel G (E Merck) . After air drying, the plates were activated at 80-100^{0}C before spottong the respective compounds.

3.18. Bioassays of antifungal phenols

3.18.1. *TLC plate bioassay*

All the fractions derived from the extraction procedure were analysed by TLC on silica gel G. All the three fractions were spotted on TLC plates and the development of the chromatogram was carried out at the room temperature using a

ethyl acetate : chloroform (9:11) solvent mixture as suggested by Chakraborty and Saha (1994). After development, chromatogram inhibition assay was performed as devised by Hofmans and Fuchs (1970) using *Curvularia lunata* as the test organism. Spore suspension in 2% sucrose supplemented with Richard's Medium were sprayed on the developed TLC plates and incubated in a sterile humid chamber at 25^{0}C for 6 days. Fungitoxicity was ascertained by the appearance of inhibition zone, which was visualised as white spots surrounded by a deep black background of mycelia. Diameter of inhibition zones and Rf values were noted.

3.18.2. Petriplate bioassay

10 ml 3% Richard's medium (KNO_3-10g; KH_2PO_4-5g; $MgSO_4$, $7H_2O$-2.5g; Sucrose-30g; distilled water 1L.) was poured in sterile 9 cm petridish. After solidification of the medium 5 mm wells were made in each plate with sterile cork borer. Antifungal phenolics (10-40µl) were added to each well. Aliquots (40µl) of individual fractions and ethyl acetate (as control) were dispersed at each of three wells made in each plate. A 5 mm agar slice of *Glomerella cingulata* was deposited in equal distance from the wells. Inhibition zones were seen after 7 days at 30^{0}C.

3.19. UV-spectrophotometric analysis

Antifungal phenol extracts were diluted with spec. methanol and taken for UV-spectrophotometric anlysis at a range of 200-400 nm.

3.20. Extraction of catechins from tea leaves

Catechin was extracted from tea leaf tissue following the method of Obanda *et al.* (1994) with modification. Leaf samples (10g) were extracted with 100 ml of 80% acetone at 45^{0}C in water bath for 30 min. Extracts was decanded and filtered through Whatman No. 1 filter paper. Acetone extract was concentrated to dryness and finally the residue was dissolved in 20 ml distilled water. Water solution was extracted with equal volume of chloroform for four times. The pH of the water layer was adjusted to 2 by2 drops of 2 N HCl and finally extracted with methyl isobutyl ketone. The extractw as concentrated to dryness and finally dissolved in 3 ml of 2% acetic acid.

The samples were finally filtered through milipore filter (Millipore 0.4 μm HA filter paper).

3.21. HPLC analysis of catechins

Catechin analysis of the extract was carried out on HPLC (Waters M 486) using C18 hypersil column with 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. then linear gradient to 75% B over 5 min. the elution was complete after a total of 25 min. Flow rate was fixed as 1 ml/min. with sensitivity of 0.5 aufs, injection volume 40 μl and monitored at 278 nm.