Materials & Methods

3.1. Plant Material

3.1.1. Collection

Fresh tea clones were collected mainly from two experimental stations of different geographical locations in India, which were maintained in the Germplasm Bank at Department of Botany, North Bengal University and were used for experimental purposes. The clonal cuttings were collected from a) Tocklai Experimental Station, Jorhat, Assam , and b) Darjeeling Tea Research Centre, Kurseong.West Bengal.

3.1.2. Propagation

Tea plants are generally propagated by cuttings or by seeds. For propagation by cuttings, plants were raised from the shoots of elite mother plants. Tea cuttings with good mature leaf and having a stem size of 2.5cm to 3.5cm were selected for planting. As soil preparation is the most important part in propagation technique, so immense care was taken to prepare soil appropriately.

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

Polythene sleeves (8"x6") were filled up with the prepared soil and stacked in rows in a bed and sprinkled with water thoroughly. All cuttings were planted directly into the sleeves after dipping them in rooting hormone. These cuttings (Plate III) were covered with a polethene cloche and watered every 3rd or 4th day as per requirement until the appearance of new leaf. The whole setup was kept under a green agro house.

3.1.3. Plantation

Experimental plots were prepared before plantation.Simazine@75gm /20lit water and Glyophosphate@1:200were used for weed control (Borpujari and Bannerjee, 1994). Then pits (.45m X .45 m X .45m) were dug at the intervals of 5cm between plants and 9 cm between rows to row. Planting mixture were prepared in the ratio of 4.5 kg well

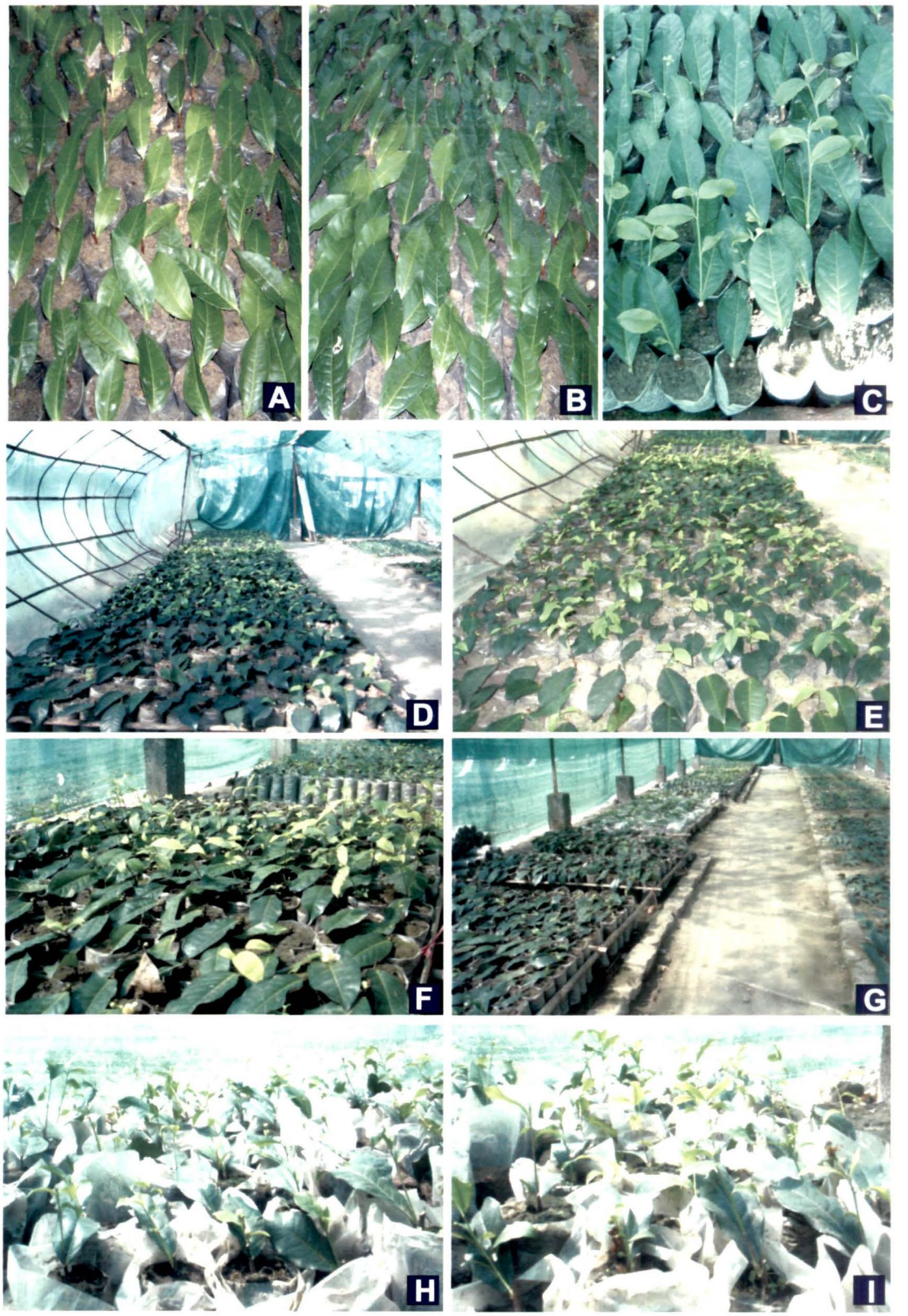

Plate III (A-I): Tea Nursery showing different stages of propagation.

rotten dry cattle manure, 30kg rock phosphate, 30kg superphosphate, and 2.5 gm phorate [O,O-diethyl S- (ethylthiomethyl) phosphorodithoioate]. Rock phosphate was placed at the bottom of each pit following which half portion was covered with cattle manure and soil mixture. Phorate was mixed with a portion of excavated soil and was applied approximately 5cm below the ground level.

Following the soil conditioning, the plants were inspected, selected and brought to the experimental field and planted in the prepared soil and pits were refilled with the conditioned soil upto the ground level. Well drained, deep and friable loam, heavily mulched rich in organic matter having pH 4 to 6, low in calciuim and generally rich in iron and manganese is considered ideal soil for the tea plantation.

Tea plants of different varieties were also grown in earthen pots (one plant per pot of 30cm diameter) each pot containing 5kg of prepared soil mixture (soil:planting mixture1:1).

All the pots were maintained in the glass house under natural condition (PlateIV).

Ten months old seedlings with well developed shoot and root system were transferred from the sleeves to the pots. Careful attention was paid to the seedlings to produce healthy plants.These were then maintained both in glasshouse and experimental garden under natural condition with regular watering.

3.1.4. Maintenance

Polythene cloche were removed from every bed when new shoots begin to appear, then they were treated with manure (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 upto 12 months once in 15 days. The mixture was dissolved @30gm in 1 lit of water and applied @50ml/plant.

The mature plants (1year and above) were maintained by using a manure of N,P,K consisting of 10 kg urea (46%N), 20kg ammonium phosphate (11% P_2O_5), 8kg muriate of potash (60%K_2O) in the soil at a regular interval . Miraculin (7ml /10lit) was

Plate IV (A-B): Tea varieties being grown in pots for experimental purposes

Plate V: Healthy tea bushes in experimental garden

sprayed in the field for the better growth of the bush. Watering was also applied at a regular interval. (Plate V).

In young plants of 3yrs tipping was done once in a year to promote lateral branching; but in case of mature plants two year of deep pruning cycle was maintained.

3.2. Application of chemicals

3.2.1. Heavy metals

A) Selection of heavy metals

The heavy metal compounds selected for the study were Copper sulphate 5-hydrate [$CuSO_4$, $5H_2O$] and Cadmium nitrate 4-hydrate [Cd (NO_3), 4 H_2O].

B) Application of chemicals

Solution of $CuSO_4$, $5H_2O$ and Cd (NO_3), 4 H_2O were prepared at concentration of 100µg / ml, 500 µg / ml and 1000 µg / ml. These solutions were applied in two ways either to detached shoots of well grown bushes i.e. *in vitro* or to the young intact seedlings i.e. *in vivo* of tea plants of different varieties at different intervals. For control plants water was applied.

3.2.1.1. *In vitro*

Heavy metal treatments were given to 10 varieties of tea i.e. TV-27, TV-23, TV-26, TV-30, TV-29, TV-28, TV-22, TV-18, HV-39, and T-78 collected from Tocklai Experimental Station, Jorhat, Assam, and Darjeeling Tea Research Centre; Kurseong.West Bengal.Very young shoots (first four leaves) were collected from healthy bushes of the experimental garden of Department of Botany and immeserd immediately with the different concentration of heavy metals and studied at 48 hr interval.

3.2.1.2. *In vivo*

Intact young plants maintained in pot (two year old nursery seedlings) of 8 varieties of TV-27, TV-23, TV-26, TV-30, TV-29, TV-28, HV-39, and T-78 were subjected to different concentration of heavy metal solutions at a definite time interval.

3.2.2. Fungicide/ insecticide

A) Selection of Chemicals

One each of commonly used fungicide and insecticide applied in tea garden were selected for the study.

Fungicide

Hexaconazole is the one of the most common fungicide applied in the tea garden for the prevention of various types of fungal diseases.

Insecticide

Acephate is one of the most commonly used organophosphate foliar insecticides in tea garden for the prevention of different kind of insecticidal problems like, aphids, leaf miners, caterpillars, sawflies and thrips.

B) Application of chemicals

Fungicide

Hexaconazole ,the common fungicide applied at 0.1% (normally applied by the planters in tea garden) concentration in intact young plants maintained in pot (two year old nursery seedlings) of 8 varieties of TV-27, TV-23, TV-26, TV-30, TV-29, TV-28, HV-39, and T-78 with mist sprayer at an interval of 7 days.

Solutions were also sprayed at the full grown bushes of two varieties TV-23 and, HV-39 with mist sprayer at an interval of 7days.

Insecticide

Acephate was sprayed at 1:400 ratios on the potted plants and full grown bushes of the said varieties in the same way as mentioned above.

3.3. Extraction and quantification of phenols

3.3.1. Extraction

Phenols were extracted from tea leaves following the method of Mahadevan and Sridhar (1982). 1gm of leaf tissues were cut into pieces and immersed immediately into 10ml boiling absolute alcohol for 15min. After boiling it was cooled and crushed in

mortar with pestle. The extract was passed through two layers of cheesecloth and then filtered through Whatman No.1 filter paper. Final volume was adjusted with 80% ethanol (5ml / gm fresh weight of leaves). The total extraction procedure was done in dark to prevent any light induced degradation of phenol.

3.3.2. Estimation

3.3.2.1. Total phenol

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

3.3.2.2. O-dihydroxy phenol

The O-dihydroxy phenol was estimated following the method of Mahadevan and Sridhar (1982). 1ml of alcholic extract was mixed with with 2ml of 0.05 N HCl , 1ml of Arnow's reagent ($NaNO_2$ -10gm, Na_2MO_4 -10gm , distilled water -100ml) ,and 2ml of 1N NaOH were mixed thoroughly, following which the volume of the reaction mixture was raised to 10ml. Absorbance of pink coloured solution was recorded at 515nm . Quantity of the O-dihydroxy phenol was estimated by using caffeic acid solution as standard.

3.4. Extraction and estimation of free proline

3.4.1. Extraction

For the extraction of free proline the method of Bates *et al.* (1973) was followed. 1gm of plant tissue was crushed with 3% sulphosalycylic acid in mortar with pestle. The slurry was then filtered through Whatman No.1 filter paper at room temperature. The filtrate was collected and stored at 4^0C for further analysis.

3.4.2. Estimation

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

3.5. Protein analysis

3.5.1. Quantification of protein

Extraction

Soluble protein was extracted from healthy and treated leaves, following the method of Chakraborty *et al.* (1995).Plant tissues (1gm) were ground in liquid nitrogen and crushed with 0.05M Sodium phosphate buffer (pH 7.2) containing 10mM $Na_2S_2O_5$,0.5 mM $MgCl_2$, 2mM soluble Polyvinyl pyrolidone (PVPP 10,000M), and 2mM poly methyl sulphonyl fluoride (PMSF),sea sand , insoluble PVPPwas added during crushing in mortar with pestle at ice cold condition .The homogenate was centrifuged at 4^0C for 20min at 10,000 r.p.m. The supernatant was used as crude protein extract and stored immediately in ice cold condition for the further analysis.

Estimation

Soluble protein content was estimated by following the method of Lowry *et al* (1951).To 1ml test solution 5ml of alkaline reagent (1ml of 1% $CuSO_4$, and 1ml of 2% sodium potassium tertarate, was added to 100 ml of 2% Na_2CO_3 in 0.1N NaOH) was added . This mixture was incubated at room temperature for 15 min then 0.5 ml of 1N Folin Ciocalteau reagent was added and mixed thoroughly and was measured at 720nm. Soluble protein content was estimated from the standard curve made with BSA (bovine serum albumin).

3.5.2. SDS- PAGE analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for detail analysis of protein profile. Total soluble proteins were extracted in 0.05 M sodium phosphate buffer, used as crude protein extract for 10% SDS-PAGE analysis following the method of Sambrook *et al.*(1989). Protein samples were loaded on the well of the gel and run for 3hrs at 200V and 15-20mA current. After completion of electrophoresis the gel was kept in fixer, stained in Coomassie Brilliant Blue (R-250) solution and finally kept in destain solution of methanol, glacial acetic acid and water (4.5: 4.5: 1).

3.5.2.1. Preparation of stock solution

For the preparation of gel the following stock solutions were prepared.

(A) Acrylamide and N'N'-methelene bis acryl amide

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

(B) Sodium Dodecyl sulphate (SDS)

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

(C) Tris buffer

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

b) 1.0 M Tris buffer was prepared for use in the stacking and loading buffer. The pH of this buffer was made 6.8 by using conc HCl and stored at 4^0 C.

(D) Ammonium Persulphate (APS)

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

(E) Tris –Glycine electrophoresis buffer

Tris –Glycine running buffer consists of 25mM Tris base, 250mM Glycine (pH8.3) and 0.1% SDS. A 1X solution was made by dissolving 3.02gm Tris base, 18.8 gm Glycine and 10 ml of 10%SDS in 1L of distilled water.

(F) SDS gel loading buffer

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

3.5.2.2. Preparation of gel

For the analysis of protein patterns by SDS-PAGE mini slab gel (plate size 8cm X 10 cm) was prepared. For the preparation of gel first two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and dried. Then 1.5 mm thick spacers were placed between the glass plates at three sides and sealed thoroughly with high vacuum grease and clipped tightly to prevent any leakage of the gel solution during pouring. Resolving and stacking gel solution were prepared by mixing compounds in the following order and the solution were poured by Pasture pipette leaving sufficient space for comb in the stacking gel(comb + 1cm).

Composition of solutions

10% resolving gel

Name of the compound	Amount (ml)
Distilled water	2.85
30% acrylamide	2.55
1.5 M Tris (pH8.8)	1.95
10% SDS	0.075
10% APS	0.075
TEMED	0.003

5% stacking gel

Name of the compound	Amount (ml)
Distilled water	2.10
30% acrylamide	0.50
1.5M Tris (pH6.8)	0.38
10% SDS	0.030
10% APS	0.030
TEMED	0.003

After pouring the resolving gel solution, it was overlaid immediately with isobutanol and kept 2hrs for polymerization .After complete polymerization of resolving gel the overlay was poured off and washed with water to remove any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and the comb was inserted immediately and overlaid with water. Finally the gel was kept for polymerization for 30-45min. After polymerization the comb was removed carefully and washed thoroughly with water. The gel was then mounted in the electrophoresis apparatus. Tris –Glycine buffer was added sufficiently in both upper and lower reservoir of the gel apparatus. Any bubble trapped at the bottom of the gel was removed very carefully with a bent syringe.

3.5.2.3. Sample preparation

Sample solutions were prepared by mixing the sample protein with 1xSDS gel loading buffer (final volume 40 µl). All the samples were floated in a boiling water bath for 3min. After boiling 40 µl of each sample was loaded in a predetermined order into the bottom of the well carefully with a micropipette.

3.5.2.4. Electrophoresis

Electrophoresis was performed at constant 15mA current for a period of 3hrs until the dye front reached the bottom of the gel.

3.5.2.5. Fixing and staining

After electrophoresis the gel was removed carefully from the glass plates and then stacking gel was cut off from the resolving gel and finally kept in fixer solution of glacial acetic acid: methanol: water (10: 20: 70) for over night. The gel was removed from fixer and stained with Coomassie blue for 4 hrs at 37^{O} C with constant shaking at low speed. The stain was prepared by dissolving 250mg of Coomassie brilliant blue (Sigma R 250) in 45ml of methanol .After dissolving the stain completely ,45ml of water and 10ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No.1 filter paper.

After staining the gel was finally destained with destaing solution,containing methanol,water and acetic acid (4.5:4.5:1) with continuous shaking at 40^0 C until the background became clear.

3.6. Extraction and quantification of pigments

3.6.1. Chlorophyll

Chlorophyll was extracted from leaves following the method of Harborne (1973). Leaf tissues (1gm) were crushed by using 80% alcohol in a mortar with pestle in the dark to prevent the photo oxidation of chlorophyll. The extract was then filtered through Whatman No.1 filter paper by adding 80% acetone from the top till the the residue became colourless. The filtrate was collected and the final volume was made up to 25ml,following which the chlorophyll content was measured by taking the O.D values at 645nm and 663nm respectively in a UV-VIS spectrophotometer (DIGISPEC-200GL) and calculation was done by using the following formulae described by Arnon(1949).

Total chlorophyll: (20.2 A_{645} +8.02 A_{663}) µg/ml

Chlorophyll a: (12.7 A_{663}- 2.69 A_{645}) µg/ml

Chlorophyll b: (22.9 A_{645}-4.68 A_{663}) µg/ml

3.6.2. Carotenoids

Carotenoids were extracted and estimated by following the method of Lichtenthaler (1987). 1gm leaf tissue were crushed in a mortar with pestle in O.D. values of the filtrate were taken at 480nm , 645nm, 663nm in a UV- VIS spectrophotometer and carotenoid content was estimated by using the following the standard formula.

A_{480}- (0.114X A_{663}) – 0.638(A_{645})µg /mlfresh weight.

3.7. Extraction and quantification of carbohydrates

3.7.1. Extraction of total Sugar

For extraction of total soluble sugars method of Harborne (1973) was followed. Fresh leaf tissues were crushed with 95% ethanol and filtered. Then the alcoholic

fraction was evaporated on boiling waterbath.The aqueous fraction was centrifuged by using table centrifuge and supernatants were collected. Finally the volume was made up with double distilled water.

3.7.2. Estimation total sugar

Total sugar estimation was done following using of anthrone, (Plummer 1978). To 1ml of test solution 4ml of Anthrone reagent was added and mixed thoroughly. Then the mixture was placed in a boiling water bath for 10min. The reaction mixture was cooled under running tap water. The absorbance was measured in Systronic photoelectric Colorimeter Model 101 at 570nm. Total sugar was then calculated from the standard curve of Dextrose solution.

3.7.3. Estimation of reducing sugar

For the estimation of reducing sugar Somogyi method as described by Plummer (1978) was followed.1ml of alkaline copper tartarate solution (prepared by dissolving 4gm $CuSO_4.5H_2O$,24 gm anhydrous Na_2CO_3, 16gm of Sodium Potasium tartarate and 180 gm anhydrous Na_2SO_4 in distilled water and made the volume upto 1liter.) was added to 1ml of test solution .After mixing the reaction mixture was kept in a boiling water bath for 15min.The reaction mixture was cooled under running tap water and then1ml of Nelson's ArsenoMolybdate reagent and 2ml distilled water were added . Absorbance was measured in a colorimeter at 540nm .Finally; concentration of reducing sugar was determined by plotting the O.D values on the standard curve of dextrose solution.

3.8. Extraction of enzymes

3.8.1. Phenylalanine ammonia lyase (PAL, EC:4.3.1.5)

For the extraction of phenylalanine ammonia lyase (PAL) method of Chakraborty *et al.* (1993) was followed. Leaves (1gm) were ground to powder in liquid nitrogen and extracted in 0 .1M Sodium borate buffer (pH8.8) containing 2mM β-mercaptoethanol under ice cold condition. The slurry was centrifuged at 15000 rpm for 20min at 4^0C. After centrifugation the supernatant was collected, the final volume was meuasured and used immediately for assay or stored for the further use at 20^0C.

3.8.2. Peroxidase (POX; EC.1.11.1.7)

For the extraction of peroxidase the method of Chakraborty *et al.* (1993) was followed. The leaves (1gm) were powdered in liquid nitrogen and extracted in 0.1M sodium borate buffer (pH 8.8) in a mortar with pestle in an ice cold condition. The homogenate was centrifuged immediately at 15000rpm for 20min at 4^0C. After centrifugation the supernatant was collected, the volume was measured and used immediately for assay or stored at 20^0C.

3.8.3. Polyphenol oxidase (PPO, EC: 1.14.18.1)

For the extraction of PPO the method of Mahadevan and Sridhar (1982) was followed with some modifications. Leaf tissues (1gm) were powdered in liquid nitrogen and extracted with 0.2M Sodium phosphate buffer (pH 6.6). The slurry was centrifuged at 4000 rpm for 30min at 4^0C. After centrifugation the supernatant was collected, volume was measured and the enzyme assay was done immediately or stored at-20^0C.

3.9. Assay of enzyme activities

3.9.1. Phenylalnineammonia lyase

PAL activity was determined spectrophotometrically by measuring the production of cinnamic acid from L-phenylalanine. The reaction mixture contained 0.3ml 300µM Sodium borate buffer (pH8.8), 0.3ml 30µM L-phenylalanine and 0.5ml of supernatant in a total volume of 3ml. Reaction mixture was incubated at 40^0C for 1hr the absorbance was read at 290 nm against the blank i.e. assay mixture with out the enzyme. The enzyme activity was measured as µg cinnamic acid produced in 1min / g fresh leaf tissue.

3.9.2. Peroxidase

For the estimation of peroxidase enzyme activity, the reaction mixture was prepared by mixing 1ml of 0.2M Sodium phosphate buffer (pH 5.4), 100µl of 4mM H_2O_2 , 100µl of O-dianisidine(5mg /ml methanol) ,1.7ml distilled water and 100µl of freshly prepared enzyme extract was added . Peroxidase activity was estimated spectrophotometrically in UV –VIS spectrophotometer (DIGISPEC-200GL) at 460 nm by monitering the oxidation of O-dianisidine in presence of H_2O_2 (Chakraborty *et.al.*, 1993). Specific activity was expressed as the increase of absorbance at 460nm/g tissue/min.

3.9.3. Polyphenol oxidase

Polyphenol oxidase activity was measured by the method of Mahadavan and Sridhar *et al.* (1982) with slight modification. 1ml of freshly prepared enzyme extract was mixed with 2ml of 0.2M sodium phosphate buffer (pH6.0) and 0.01M pyrogallol was added to it in the dark. Reading was noted every 1min interval at 495nm. The blank was set with 3ml of phosphate buffer. PPO activity was measured at $\triangle$ O.D.A_{495} /g tissue/min.

3.10. Isozyme analysis by polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) for isozyme analysis of different enzymes the method of Davis(1964) was followed, using 8% resolving gel and 5% stacking gel in Tris-glicine buffer (pH8.3) . The different solutions were prepared for the analysis as follows:

3.10.1. Preparation of the stock solution

Solution A : Acrylamide stock solution (Resolving gel)

Acrylamide stock solution was prepared for the resolving gel , by dissolving 28g of acrylamide and 0.74 g of N'N'methelene bisacrylamide dissolved in100ml warm distilled water. The stock solution was filtered with Whatman No.filter paper in dark and stored in dark bottle at 4^{O}C.

Solution B: Acrylamide stock solution (stacking gel)

For the preparation of acrylamide stock solution for stacking gel 10gm of acrylamide and 2.5g of bis-acrylamide was dissolved in 100ml warm distilled water. The stock solution was then filtered withWhatman No.1 filter paper and stored at4^{O}C in a dark bottle.

Solution C: Tris-HCl (Resolving gel)

Tris-HCl buffer was prepared by dissolving 36.6.g of Tris base in distilled water and 0.25ml of TEMED was added. The pH of the solution was adjusted to 8.9 with conc. HCl. The final volume of the solution was made upto 100ml with distilled water. The solution was then stored at 4^{O}C for further use.

Solution D: Tris –HCl (Stacking gel)

5.98g of Tris base was mixed with distilled water and 0.46 ml of TEMED was added to it .Finally the pH of the solution was adjusted to 6.7 with conc. HCl. The final volume of the solution was made upto 100ml with distilled water. The solution was stored at 4^{O} C for further use.

Solution E: Ammonium persulphate solution (APS)

Fresh solution of ammonium persulphate was prepared by dissolving 0.15g of APS in 10ml of distilled water.

Solution F: Riboflavin solution

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

Solution G: Electrode buffer:

Fresh electrode buffer was prepared by dissolving 0.6g of Tris base and 2.9gm of Glycine in 1L distilled water.

3.10.2. Prepartion of gel

For native anionic PAGE mini slab gel was prepared. For slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any trace of grease from the plates and then dried. 1.5mm thick spacers were placed between the glass plates on three sides and 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: distilled water in the ratio of 1:1:4:1 by pasture pipette leaving sufficient space for (comb +1cm) the stacking gel. This resolving gel was immediately overlayed with water and kept for polymerization for 2hrs. After complete polymerization of the resolving gel, the overlay was poured off and washed with water to remove any unpolymerized acrylamide.

The stacking gel solution was made by mixing solution B: D: F: distilled water in the ratio of 2:1:1:4. The stacking gel solution was poured over the resolving gel and comb was inserted immediately and overlayered with water. Finally the gel was kept for

30-45min in strong sunlight for polymerization. After polymerization the comb was removed carefully and washed carefully with water. The gel was then finally mounted in a electrophoresis apparatus. Tris –Glycine buffer was poured sufficiently in the both upper and lower reservoir. Any air bubble, was trapped at the bottom of the gel was removed very carefully with a bent syringe.

3.10.3. 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 used for electrophoresis were cooled. The samples were immediately loaded in a predetermined order into the bottom of the well with a microliter syringe.

3.10.4. Electrophoresis

Electrophoresis was performed at a constant 15mA current for a period of 3-4 hrs at $4^{O}C$ until the dye font reached at the bottom of the gel.

3.10.5. Fixing and Staining

After electrophoresis the gel was removed from the glass plates and then the stacking gel was cut off from the resolving gel and finally stained with suitable dye.

3.10.5.1. Peroxidase

Extraction for peroxidase isozyme analysis was done by grinding 1gm of leaf tissues in liquid nitrogen in pre-chilled mortar and pestle and finally extracted with 0.1M Sodium phosphate buffer (pH 7.0) as described by Davis (1964). The staining of the gel for the peroxidase isozyme pattern was performed following the method of Reddy and Gasber (1973). The gel was stained with the solution of Benzidine dye in acetic acid water mixture consisting of Benzidine (2.08gm), Acetic acid (18 ml), 3% H_2O_2 (100ml) for 5min . The reaction was stopped with 7% acetic acid after the appearance of clear blue coloured bands. Analysis of isozyme was done immediately.

3.10.5.2. Polyphenol oxidase

Extraction for polyphenol oxidase isozyme analysis was done by powdering 1gm of leaf tissues in liquid nitrogen in pre-chilled mortar and pestle and was extracted with 0.1M sodium phosphate buffer (pH 7.0) as described by Davis(1964). After the electrophoresis the gel was equilibrated in 0.1% p-phenylenediamine in 0.1M potassium phosphate buffer (pH 7.0) for 30min.This was followed by the addition of 10mM Catechol solution in the same buffer. The gel was shaken in this buffer until the appearance of brown discreate band, analysis of the isozyme pattern was done immediately.

3.11. Extraction of catechins from tea leaves

Catechin was extracted from tea leaf tissues following the method of Obanda and Owuor (1994) with some modifications. 10gm leaf samples were taken and extracted with 100ml of 80% acetone and kept in a water bath at 45^{O}C for 30 min. Extract was then filtered through Whatman No.1 filter paper. Acetone extract was concentrated to dryness and the residue was dissolved in 20ml of distilled water. Water solution was separated with equal volume of chloroform for four times in a separating funnel. The pH of the final extract i.e. water layer was adjusted to 2 by adding 2N of HCl and finally extracted with methyl isobutyl ketone. Finally the extract was concentrated to dryness and dissolved in 3ml of 2% acetic acid. The samples were finally filtered through milipore filter (Milipore 0.4 µm HA filter paper).

3.12. HPLC analysis of catechins

High performance liquid chromatography (HPLC) of the extract was performed for the anlysis of catechin. The HPLC analysis was done on a Shimadzu Advanced VP Binary gradient system with a C-18 hypersil column using 50% acetonitrile as the mobile phase, in isocratic mode. Injection volume was 20µl and the flow rate 1ml min^{-1}. Detection was done at 278 nm and the total run time was 25min.

3.13. Determination of heavy metal contents in tea leaf

Heavy metal content was estimated with the help of Atomic Absorption Spectrophotometer (AAS). Tea leaves were oven dried to a constant dry weight and

digested in a Ternary acid mixture of Nitric acid: Perchloric acid: Sulphuric acid (10: 4:1). One gm of leaf was digested at a temperature of 180^{O}Cfor 15min. After complete digestion the volume was made upto 100ml with distilled water and the AAS (Perklin Elmer A Analyst 200) was done and the reading was noted.

3.14. Statistical Analysis

Standard error of mean was calculated in all cases. Significance of difference between mean was analysed by ANOVA.