CHAPTER 3

MATERIALS AND METHODS
3.1 Materials

Most of the vegetative clones and seedling stocks released by Tocklai Experimental Station for commercial cultivation and some commonly used garden clones were selected for experimentation. There are three phenotypically distinct plant types amidst the tea populations:

(i) China Tea (*Camellia sinensis* var. *sinensis* (L.)).
(ii) Assam Tea (*C. sinensis* var. *assamica* (Masters)).
(iii) Cambod Tea (*C. sinensis* var. *assamica* sub. sp. *lasiocalyx* (planch, M.S.)).

They are indigenous to three different geographical regions of South East Asia, viz. China, Assam and Indo-China respectively (Wight 1962, Barua 1965). The Tocklai released clones and seed stocks have also been categorised as drought tolerant and drought susceptible (Barua and Bezbaruah 1970). Care was taken to include representatives of each category and plant type as and whenever necessary while conducting the experiments.

Description of the materials used regarding their plant types and category of drought tolerance is given in Table 1. Additional informations of the materials used are also provided under the respective experimental heads.
Table 1: Description of the Tocklai released clones and seed stocks used for experiments

<table>
<thead>
<tr>
<th>Clones/stocks</th>
<th>Parentage</th>
<th>Leaf size</th>
<th>Category of drought tolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td>* TV1</td>
<td>Sinensis</td>
<td>Medium erect</td>
<td>Good</td>
</tr>
<tr>
<td>TV2</td>
<td>Assamica</td>
<td>Medium</td>
<td>Poor</td>
</tr>
<tr>
<td>TV3</td>
<td>&quot;</td>
<td>Large</td>
<td>&quot;</td>
</tr>
<tr>
<td>TV4</td>
<td>&quot;</td>
<td>Medium</td>
<td>&quot;</td>
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<tr>
<td>TV5</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>TV6</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>TV7</td>
<td>Sinensis</td>
<td>Small erect</td>
<td>Good</td>
</tr>
<tr>
<td>TV8</td>
<td>Assamica</td>
<td>Medium</td>
<td>Poor</td>
</tr>
<tr>
<td>TV9</td>
<td>Cambodiensis</td>
<td>&quot;</td>
<td>Good</td>
</tr>
<tr>
<td>TV10</td>
<td>Assamica</td>
<td>&quot;</td>
<td>Fair</td>
</tr>
<tr>
<td>TV11</td>
<td>&quot;</td>
<td>Large</td>
<td>&quot;</td>
</tr>
<tr>
<td>TV12</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>TV13</td>
<td>&quot;</td>
<td>&quot;</td>
<td>Poor</td>
</tr>
<tr>
<td>TV14</td>
<td>Sinensis</td>
<td>Medium</td>
<td>Good</td>
</tr>
<tr>
<td>TV15</td>
<td>Assamica</td>
<td>Large</td>
<td>Poor</td>
</tr>
<tr>
<td>TV16</td>
<td>Sinensis</td>
<td>Medium</td>
<td>Good</td>
</tr>
<tr>
<td>TV17</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>TV18</td>
<td>Cambodiensis</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>TV19</td>
<td>&quot;</td>
<td>Large</td>
<td>&quot;</td>
</tr>
<tr>
<td>TV20</td>
<td>Assamica</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>TV21</td>
<td>&quot;</td>
<td>&quot;</td>
<td>Poor</td>
</tr>
</tbody>
</table>

contd.
3.2 Methods

3.2.1 Simulation of Drought and Waterlogging Conditions

In laboratory conditions, drought was imposed by withholding irrigation till wilting point, when soil moisture declined to around 6% or even below depending upon the type of soil used in pots and the leaf water potential decreased to about -15 bars.
Virgin top soil with predetermined field capacity generally between 20 to 25% moisture content was used for this study.

In case several drought cycles were required for the investigation, the first drought cycle was terminated by resuming watering which was continued till the relative leaf water content was built up to 80% or above. Before termination of each drought cycle, the relevant parameters were studied.

When needed, watering frequencies were adopted by withholding watering for several durations of time intervals.

In certain cases, drought was induced by maintaining the soil at predetermined moisture levels by compensating for the loss of moisture with measured amount of water to the soil. The soil moisture level was maintained at different fractions or percentages of field capacity or available moisture content.

Polyethylene glycol (PEG-6000) was used in various concentration solutions for inducing osmotic stress.

Overhead shelter with provisions for sufficient light interception was used for controlling unwanted rain water while conducting the drought experiments.

Waterlogged situation was created by using large size tubs. Potted or sleeved plants were submerged in these tubs up to 5 cm above the ground level and this level was maintained by adding fresh water whenever required (Plate 1).

Under field conditions, the natural drought and waterlogging conditions were exploited to investigate their effects
PLATE 1: SIMULATION OF WATERLOGGED CONDITION
on the growth and development of the tea plants during those periods.

3.2.2 Measurement of Stomatal Diffusion Resistance and Transpiration Rate

Stomatal diffusion resistance \( (r_s) \) and transpiration rate \( (T) \) were measured with the help of LI-1600 steady state porometer (Lambda Instrument Corpn., USA). A standard broad leaf aperture having a circular opening with an exposure area of 2 cm\(^2\) was used which was attached to the censor head. A factory installed LI-190S-I quantum censor provides direct read out of photosynthetically active radiation (PAR), a chromel-constantan thermocouple in contact with the leaf surface measures leaf temperature, a Vaisala Humicap measures relative humidity, and a linearised thermistor gives the cuvette temperature (Plate 2).

The design and other procedures were the same as those outlined by Beardsell et al. (1972) and Parkinson and Legg (1972).

Since the stomata are on the under surface of the tea leaf, the censor was attached to the under surface of the leaves for determination of the stomatal diffusion resistance and transpiration rate. The instrument can maintain a steady state relative humidity in the cuvette and before every reading the null balance point was set to a particular value of relative humidity.
PLATE 2 : LI-1600 STEADY STATE POROMETER IN OPERATION, MEASURING STOMATAL DIFFUSION RESISTANCE AND TRANSPIRATION RATE
In all the experiments, a fully exposed leaf of identical status in respect of age and position was used and care was taken not to shade the leaf during observations.

3.2.3 Determination of Water Potential

Determination of water potential was done as a primary measure for the quantification of plant water status, which was performed with the help of a pressure chamber (plant water status console Model 3005, Soil Moisture Equipment Corp., USA) (Plate 3). The excised leaf or a leafy shoot, as the case may be, was put into the specimen holder immediately after excision and was put under compression by exposure to nitrogen gas. The pressure applied in the chamber to push the xylem sap back exactly to the cut end of the sample is an estimate of the original tension in the xylem prior to excision (Barrs 1968). It was extensively used by Scholander et al. (1964, '65, '66). Most of the observations were made between 12:00 h and 13:00 h, when the stress was at maximum. Identical growing two and a bud shoots and leaves of identical conditions with respect to age and position were used for this study.

3.2.4 Determination of Relative Turgidity and Water Saturation Deficit

Relative turgidity (RT), also referred to as relative leaf water content (RWC) (Namken and Lemon 1960, Ehlig and Gardner 1964) was determined following the method of Weatherley (1950)
PLATE 3: PRESSURE CHAMBER (PLANT WATER STATUS CONSOLE, MODEL 3005) FOR MEASUREMENT OF WATER POTENTIAL
with certain modifications. With cork borer, several leaf discs were punched to uniform size and area. Care was taken to avoid the main veins. Ten leaf discs were taken for each repeat and their initial fresh weights were taken. They were then floated in distilled water and stored at room temperature in dark for 4 hr. The discs were then removed from water, blotted dry and their turgid weight was recorded. Then the discs were dried in oven at 80°C for 24 hr before recording their dry weights. Relative turgidity was expressed in percentage and was determined using the following formula:

\[
RT = \frac{\text{Fresh wt.} - \text{Dry wt.}}{\text{Turgid wt.} - \text{Dry wt.}} \times 100
\]

Water saturation deficit (WSD) was determined from its simple relationship with RT as follows:

\[
WSD = 100 - RT \quad (\text{Barrs 1968}).
\]

3.2.5 Determination of Total Water Loss from a Tea Bush

Total water loss from a tea bush per day was calculated from the rate of transpiration measured with the help of a steady state porometer, in different hours of the day and in different leaf positions of the bush canopy. For this purpose, the entire canopy of the bush was divided into three parts, viz. top, middle and bottom and the volume of each part was determined separately. The volume of the entire canopy was determined
assuming it to be a cone and using the equation:

\[ V = \frac{1}{2} \cdot (A \times H) - V_1 \]

where \( V \) is the volume of the entire leaf canopy, \( A \) is the area of the top surface, \( H \) is the perpendicular height of the cone and the subtractable part \( V_1 \) is the volume of the bottom most part of the cone without leaves. Area of the canopy surface was calculated by determining the circumference of the surface and using the equation:

\[ A = \left(\frac{1}{2} \cdot \frac{C}{L} \right)^2 \]

where \( C \) is the circumference of the surface and \( \frac{1}{2} \cdot \frac{C}{L} \) stands for the radius of the circle with circumference \( C \).

The number of leaves in a particular unit of volume was calculated for each profile of the canopy separately and from this the total number of leaves was determined for each profile. The average leaf area for each canopy profile was determined from the total area of 100 leaves taken at random from each canopy and measured with the help of a LI-3000 portable Leaf Area Meter. Thus, the total leaf area for each canopy profile was determined after multiplying the total number of leaves with the average leaf area. From the total leaf area and transpiration rate, the approximate total water loss per day was estimated.

Altogether 15 plants were observed from each clone taking 5 plants per repeat.
3.2.6 Determination of Water Release Characteristic of Leaves

Water release characteristic was obtained by estimating the relative turgidity at different water potentials (Sandanam et al. 1981). For this purpose excised shoots were allowed to wilt for different lengths of time to attain different water potentials. Representative shoots were taken at intervals, water potential was measured on one leaf and relative turgidity on the adjacent leaf. The difference in water potentials between the two adjacent leaves was negligible.

3.2.7 Determination of the Rate Constant

The rate constant for the loss of moisture from the detached leaves was determined following the method given by Hari-krishnan and Sharma (1980), and was calculated from the following equation:

\[
K = \frac{\log a - \log (a - x)}{t}
\]

where 'K' is the rate constant, 'a' is the initial moisture percentage expressed on dry weight basis and '(a-x)' is the moisture at time 't' in hours expressed on dry weight basis. 'K' is independent of the unit in which the moisture is expressed and is thus a useful constant for comparing the rate of loss of moisture of different clones. In the initial period it is determined by the stomatal resistance and thereafter by the
cuticular resistance when the stomata close completely (Harikrishnan and Sharma 1980).

3.2.8 Measurement of Translocation of Assimilates Using $^{14}\text{CO}_2$

Exposure of leaves to radio-active $^{14}\text{CO}_2$ was done following the procedure of Hale and Weaver (1962) with little modifications. The 4th leaf from the top of the main stem with a growing apical bud was used for the exposure. 5$\mu$ci of carbon-14-labelled Na $^{14}\text{CO}_3$ in liquid form was taken in a petri dish with the help of a 10$\mu$lit capacity syringe fitted to a disposal tip and used as the source. The petri dish was then placed conveniently under the leaf, which was covered with a leak proof clear plastic bag. The open end of the bag was tied carefully around the petiole by putting some cotton wool around it. Three drops of 70% lactic acid was inserted to the source with a syringe by making a hole on the plastic cover which was sealed with a piece of cellotape. In the same way, after 30 min, 3 drops of 1 N HCl was inserted into it in order to accelerate and complete the reaction for evolution of $^{14}\text{CO}_2$ inside the bag. Care was taken to prevent leakage of the gas from the bag. Mixing of the $^{14}\text{CO}_2$ with air inside the bag was facilitated by squeezing the bag gently at regular intervals. The time for absorption of radio active $^{14}\text{CO}_2$ by the leaf was allowed for 1 hr after which the bags were removed. The treated plants were allowed to translocate the radio active $^{14}\text{CO}_2$ to different parts of the plant for 24 hr. The set up for this experiment is shown in plates 4(A) & 4(B).
PLATE 4: PROCEDURE FOR \( ^{14}\text{CO}_2 \) EXPOSURE IN LEAVES

(A) A SET UP  (B) \( ^{14}\text{CO}_2 \) INJECTED
(a) Preparation of Sample for Auto-Radiography

A single-stem plant with leaves and roots was severed into segments and arranged in order on a glazed herbarium sheet. The plant parts were glued to the sheet which was then set out on a piece of 10" x 12" size metal sheet. A \( \frac{1}{4} \)" thick piece of hard board had been put over the metal sheet which was further covered with a piece of blotting paper. Finally, the stack was covered with a 10" x 12" piece of \( \frac{1}{2} \)" thick size hardware mesh.

An identical stack was made on the reverse side of the metal sheet with another herbarium sheet. The two opposite edges were clipped with large clips and dried in hot air (50°C) oven for 24 hr. After drying, the mounts were humidified in a moist chamber for about 2 hr in order to avoid crumbling of parts while pressing.

The mounts were put in plant press and after 2-3 days, were exposed to Kodak Royal Blue X-ray film in the dark for 3-7 days, the time depending upon the specific activity of \( ^{14} \)C in the tissues. The films were developed using Kodak liquid X-ray developer and fixer solution. The details of this procedure were the same as described by Crafts and Yamaguchi (1964).

(b) Grinding and Counting of Samples

The exposed plants were separated into various parts and dried in a draught-air oven at 55°C for 72 hr. The tissue samples were ground to 40 mesh with a small Wiley grinder and samples
weighing 10 mg were used for counting labelled carbon activity, using a LKB-1215 RACKBETA-II liquid scintillation counter with 90% efficiency (Plate 5), after suspending the samples in 10 ml of scintillation cocktail of PPO-POPOP-Dioxane mixture (5 gm PPO + 500 mg POPOP + 50 gm Naphthalene + 40 ml cellusolve per litre of Dioxane). The radioactivity in each plant part was taken as the measure of the fraction of the photosynthates present in it, ignoring the respiratory loss during or after exposure of the experimental plants to CO₂.

3.2.9 Measurement of Photosynthesis Using Infra-Red Gas Analyser

The infra-red gas analyser (IRGA) of Beckman, Model 865 (USA) (plate 6) was used to monitor the CO₂ concentration. Compressed air with a CO₂ concentration of 330 ppm was used as a source of CO₂. Cobalt coated 'Drierite' was used as a desiccant to filter the water vapour in the air circuit. The air was bubbled through water in a reagent bottle for humidification before entering the assimilation chamber. Tygon tubing of 0.5 cm inner diameter was used as the circuit line for air between the components in the set up.

(a) Assimilation Chamber

The assimilation chamber was a box constructed with plexiglass plates. Plexiglass tubular rods with perforations were used for the inlet and outlet of air in the chamber containing the plant. Top, bottom and side walls were sealed, and at the
PLATE 5: LKB-1215, RACKBETA-II LIQUID SCINTILLATION COUNTER FOR COUNTING RADIO-ACTIVITY IN VARIOUS PLANT PARTS
PLATE 6: BECKMAN MODEL-865, INFRA-RED GAS ANALYSER (IRGA) FOR MEASUREMENT OF PHOTOSYNTHESIS
bottom of a side wall one window was kept where the lid could be clamped with screws after inserting the plant inside the box. A small circulating fan attached to one of the side walls was used in order to mix the inside air.

The set up of the apparatus used was based on the principles and procedures outlined by Janac et al. (1977) and Lister et al. (1961). LI-COR 18C-90S quantum light meter was used to measure the PAR in $\mu$Em$^{-2}$S$^{-1}$. Leaf and ambient temperatures were determined with the help of thermistor probes and thermometers respectively using Cole Parma Temperature Recorder.

(b) Procedure

The treated plant was inserted in the assimilation chamber and the lid was closed carefully in order to ensure that the chamber was leak proof. The light intensity was measured at the leaf canopy level of the plant. The difference in CO$_2$ concentration in ppm was recorded from the IRGA.

CO$_2$ in nitrogen, in standard gas cylinders obtained from Indian Oxygen Ltd, was used to calibrate the concentration of gases and to check the zero reading in the analyser. The calibration of the scale was verified both at the beginning and at the end of the experiment with standard calibration gas. The area of the leaves of each plant was measured using the scanner of the LI-3000 Leaf Area Meter of LI-COR (USA).
Knowing the $\Delta CO_2$ in ppm, flow rate and area of the leaves, the flux or the $CO_2$ exchange in mg dm$^{-2}$h$^{-1}$ was calculated adopting the formula of Janac et al. (1971).

$$F = J \cdot \frac{\Delta C}{A}$$

where,
- $F =$ Flux of $CO_2$ in mg.
- $J =$ Flow rate in litre per hour.
- $\Delta C =$ Change in $CO_2$ concentration in CG.
- $A =$ Leaf area in dm$^2$.

The factor ($\frac{x_{ppm}}{1000} \times 1.85$) was used to convert ppm of $CO_2$ into mg of $CO_2$.

3.2.10 Determination of Evapotranspiration Rate and Water Use Efficiency

Evapotranspiration (ET) was determined from the water balance equation as follows: Initial Soil Moisture + precipitation = Final Soil moisture + ET + Deep drainage + Run off

The experiment was conducted in potted plants under one soil moisture deficit condition within field capacity range and water was replenished at a regular interval as and when needed. Hence deep drainage and run off water were ignored while calculating ET. The amount of water added to the individual pots during the experimental period was considered as the precipitation required for the equation. As the experimental period
was less than three months, the metabolic use of water was considered to be negligible and hence was not adjusted.

Percentage of initial and final soil moisture was determined gravimetrically. The sampling depth of the soil was recorded and the bulk density (g/cc) was determined for conversion of per cent soil moisture into millimeter soil moisture by using the following equation:

\[
\text{Soil moisture (mm)} = \frac{\text{Depth of soil} \times \text{Bulk density} \times \% \text{ soil moisture}}{100}
\]

The distribution of the added water was calculated from the area of the soil surface in the pot.

Water use efficiency (WUE) was calculated as:

\[
\text{WUE} = \frac{\text{Yield}}{\text{ET}}
\]

Here 'yield' refers to the total dry matter gained during the experimental period with respect to leaf, stem and root. WUE was expressed in centigram per millimeter water used.

3.2.11 Determination of Stomatal Frequency and Size

A positive nail polish replica of the lower leaf surface was made by painting the surface with clear commercial nail polish diluted to 1:1 with acetone (Beakbane and Majumder 1975, Miller and Ashby 1978). Density and size of stomata were estimated from epidermal impressions using a microscope with a
calibrated graticule in the eye piece. Sizes of the stomata and stomatal pore were evaluated by measuring the maximum length and breadth of guard cells and pores respectively (Sandanam et al. 1981).

The leaves were first washed thoroughly with distilled water to remove dirts and then allowed to dry. The imprints were prepared from the abaxial surface only using a glass rod or a fine brush. The drop of liquid was spread on to the leaf to make a thin, transparent film upon drying. The films were stripped off with a pair of forceps without damaging the tissue and was mounted on a clear slide in water with impressed face down and sealed with varnish or paraffin wax (Williams 1973).

Area (A) and perimeter (P) of stomatal apparatus and pore were calculated assuming it to be an ellipse and hence using the formulae:

\[
A = \pi ab \quad \text{and} \quad P = 2\pi \sqrt{\frac{a^2 + b^2}{2}}
\]

where 'a' is the major semi-axis and 'b' is the minor semi-axis, '2a' refers to the maximum length and '2b' refers to the maximum width of the stomatal apparatus and pore (Turrell 1946).

3.2.12 Determination of Specific Leaf Weight
Specific leaf weight (SLW) was measured as dry weight per unit leaf area. It was determined on selected days and at
each position 5 leaves were sampled by taking 4 punches each with a sharp cork borer of known diameter between the mid-rib and leaf margin. Care was taken to avoid the large veins. This technique showed a correlation coefficient of 0.99 to 0.95 to the whole leaf method (Porpiglia and Barden 1980).

The discs were then oven dried at 70°C for 48 hr before recording dry weight and finally SLW was determined in mg/cm² (Castel and Fereres 1982).

3.2.13 Determination of the Per Cent Withering Rate

Per cent withering rate was calculated after Ulla et al. (1979). Ignoring the loss of dry matter during withering, the equation for per cent withering was obtained as:

\[ P_w = \frac{100}{100 - M_w} \times (100 - M_g) \]

where 'Pw' is the per cent wither, 'Mg' is the per cent moisture in green leaf and 'Mw' is the per cent moisture in withered leaf.

3.2.14 Anatomical Studies of Leaf, Stem and Root

Sampled leaves, stems and roots from various treatments were first fixed in formalin - acetic acid - alcohol solution for anatomical studies.

(a) Determination of the Proportion of Xylem and Phloem in Stem and Root Sections

Transverse sections cut from a fixed identical position of preserved internodes and roots were examined under the
microscope. Camera-lucida drawings were made and the area of xylem and phloem of the individual sections were determined with the help of a planimeter.

(b) Determination of the Thickness of Leaf Cuticle, Palisade and Spongy Parenchyma Tissue

The thickness of cuticle, palisade layer and the spongy parenchyma was determined by examining a 15μ thick transverse microtome section of a leaf. The measurements were made with the help of a calibrated graticule mounted in the eye-piece of the microscope.

(c) Determination of Cell Size and Number of Cells Per Unit Area in Leaf Sections

Cell size and number of cells per unit area were determined from cross sections of leaf. Average cell size was determined from camera-lucida drawings of individual cells with the help of a planimeter. Number of cells per unit area was determined using a calibrated micrometer grid mounted in the eye-piece of a microscope.

(d) Determination of the Volume of Vascular Tissues in the Leaf Petiole

The volume of the vascular elements inside the leaf petioles was determined from cross-sectional areas of the xylem and phloem multiplied by the length of petioles and was expressed in cc (Meidner 1965).
(e) Determination of Intercellular Air Space in Leaf

Intercellular air space in leaves was determined from camera-lucida drawings of transverse leaf sections. Since the area of the cellular space within a unit area of the leaf section was estimated, the area of the intercellular space could be determined by subtracting it from the total area of the portion taken for measurement.

3.2.15 A Simple Method for Measuring Leaf Volume, Density, Thickness and Internal Gas Volume

A simple method as suggested by Raskin (1983) was adopted to measure the above parameters which was based on the principle of Archimedes. A good correlation was found between the observed data of leaf thickness and intercellular air spaces determined by this method and the earlier stated microscopic method. The principle behind this method is that the buoyant force acting upon a body immersed in a fluid is equal to the weight of the fluid displaced by the body.

Ten mature identical leaves were sampled and the leaf surfaces were rubbed with 0.05% (v/v) Triton solution with cotton wool in order to eliminate air bubbles that might be trapped onto the surface of the submerged leaves. Excess triton solution was removed with a blotting paper. Leaf discs twenty in number from identical positions were punched with a cork borer of known area. The fresh weight of the leaf discs
was recorded (Wair). A holder consisting of a Hoffman clamp with sufficient weight to keep the leaf material submerged in water was clipped to two hypodermic needles which pierced the leaf. The holder with the leaf material was then suspended from the pan hook of an analytical balance and submerged in distilled water and the weight of the leaf discs under water was determined (Wwater).

The plant material fixed to the holder was placed inside a desiccator partially filled with triton solution so that the tissue was submerged totally. Agitation of the desiccator assisted the infiltration process. The vacuum of about 500 mm Hg was applied and released at least 4 times, with every evacuation lasting for about 40 seconds. The infiltrated tissue attached to the holder was then weighed in water as described above (Winfil).

Now from these data the following parameters of a leaf were calculated using the equations as stated:

(I) Volume of leaf (Vleaf) = (Wair - Wwater) mm$^3$.

(II) Density of leaf ($\rho$ leaf) = (Wair/Vleaf) mg mm$^{-3}$.

(III) Volume of internal air space (Vair) = (Winfil - Wwater) mm$^3$.

(IV) Volume of nongaseous leaf content (Vnongas)

\[ = (Vleaf - Vair) \text{ mm}^3 \]

(V) Density of nongaseous leaf content ($\rho$ nongas)

\[ = (Wair/Vnongas) \text{ mg mm}^{-3} \]

(VI) Leaf thickness (Tleaf) = (Vleaf/Aleaf) mm.
3.2.16 Estimation of Chlorophyll

Leaf discs of 5 mm diameter were punched from mature leaves of identical position and age avoiding large veins. Leaf discs were then immediately weighed and immersed in 10 ml of 80% aqueous acetone in pyrex glass tubes wrapped with aluminium foil and kept in a refrigerator at 4°C for three days. The supernatant was then decanted and the volume was made upto 10 ml with fresh acetone. The absorbancy was recorded at 663 and 645 nm against 80% acetone blank using a Beckman spectrophotometer. Chlorophyll a and Chl b were calculated adopting the equations suggested by Arnon (1949) based on the existing coefficients for the pigments. The equations adopted are given below:

\[
\text{Chlorophyll a (mg/g)} = \frac{12.7 (A_{663}) - 2.69 (A_{645})}{1000} \times \frac{V}{W}
\]

\[
\text{Chlorophyll b (mg/g)} = \frac{22.9 (A_{645}) - 4.68 (A_{663})}{1000} \times \frac{V}{W}
\]

where \(V\) = Volume of the extract in ml, and,

\(W\) = Fresh weight of the sample in gm

A 663 and A 645 are optical densities at respective wavelengths

Chlorophyll concentrations were expressed in mg per gm of fresh weight of the sample.
3.2.17 Estimation of Starch

Estimation of starch was carried out following the method suggested by McCreedy et al. (1950) with certain modifications. Oven dried and ground samples were passed through a 60-80 size wire mesh and 100 mg powder was taken in a 100 ml volumetric flask. Few ml of 80% ethyl alcohol was added in order to wet the powder, stirred thoroughly and then allowed to stand for 5 min. Distilled water (5 ml) was added to it, stirred thoroughly and allowed to stand for another 5 min. Then added to it 25 ml of hot 80% ethyl alcohol, stirred thoroughly and again allowed to stand for 5 min. Then the extract was transferred to 50 ml centrifuge tubes and centrifuged at 2000 RPM for 15 min. The extract was then decanted and the alcoholic solution was discarded. Again 30 ml of hot 80% ethyl alcohol was added, stirred, and centrifuged as before discarding the alcoholic solution. This process was repeated 4-5 times until the last trace of glucose was removed, which was confirmed by anthrone negative test.

The residue was taken for starch determination. Distilled water (5 ml) was added to the residue, cooled in an ice bath and stirred thoroughly. Then added to it 6.5 ml of 52% perchloric acid, stirred with a glass rod for 15 min, while keeping the mixture cool in the ice bath. Added to this starch solution 20 ml of distilled water and centrifuged as before. The filtrate was then transferred into a 100 ml volumetric
flask. Then 5 ml distilled water was added to the residue, cooled in ice bath, which was followed by addition of 6.5 ml of 52% perchloric acid and stirred thoroughly as before. After centrifuging or filtering, the filtrate was poured and washed into the 100 ml volumetric flask containing the previous extract. The final volume was made upto 100 ml with distilled water, after filtering the whole solution through Whatman No. 42.

After shaking thoroughly, 5 ml of the filtered starch solution was taken in a 100 ml volumetric flask and diluted to 100 ml. Of this diluted starch solution 5 ml was pipetted out into a test tube and added to it 10 ml of freshly prepared anthrone reagent. The solution was then cooled in an ice bath and thoroughly mixed with the help of a glass rod. It was then heated for 7.5 min in a boiling water bath, and then immediately cooled to 25°C in a water bath. Optical density was measured at 630 nm with the help of a Beckman spectrophotometer, against reagent blank.

Sugar content was calculated in terms of glucose equivalent and a conversion factor of 0.9 was used to convert the values of glucose to starch.

3.2.18 Estimation of Total Soluble and Reducing Sugars

(a) Total Soluble Sugars : Estimation of total soluble sugars was done following the method suggested by Dev Choudhury and Bajaj (1976) with little modifications.
Oven dried ground samples were passed through a 60-80 size wire mesh and 5 gm of powdered sample was extracted with 200 ml of boiling water for 15 min using a reflux condenser. The infusion was filtered through cotton wool and the residue was washed repeatedly with hot water for complete removal of sugars. The extract was collected in a 250 ml marked volumetric flask and the volume was made upto 250 ml after cooling. From this extract 20 ml was taken in a beaker and about 3 drops of aqueous basic lead acetate was added to remove the polyphenols. Then added to it 3 drops of ammonia solution to raise the pH of the solution to 8.5. Complete removal of polyphenols was ensured by adding a few more drops of lead acetate solution. After 20 min, the precipitate was separated by centrifuging the extract at 5000 RPM for 25 min followed by decantation of the supernatant liquid. The filtrate was collected, the residue was washed repeatedly with water till the final washing did not respond to anthrone reagent. The combined sugar solutions were treated with 1 gm sodium oxalate for about 40 min to remove the excess of lead present in the solution. The extract was then filtered through Whatman No. 42 and the filtrate was collected in a 100 ml volumetric flask. The residue was washed and filtered as before and the final volume of the total sugar extract was made up to 100 ml.

One ml of the sugar extract was taken in a loosely stoppered thickwalled pyrex glass tube and was layered with 10 ml of anthrone reagent. The tubes were then cooled by
immersing in ice or ice cold water and stirred properly for 5 min. Then the tubes were heated for 7 min in a boiling water bath followed by immediate cooling in ice cold water. A blank was maintained simultaneously using 1 ml distilled water in lieu of sugar extract.

Absorbancy was measured at 625 nm using a Beckman spectrophotometer. Total soluble sugars were estimated in terms of glucose by referring to a standard curve obtained similarly.

(b) Reducing Sugars : The same extract prepared for estimation of total soluble sugars may be used for determination of reducing sugars in tea using Nelson's modification of Somogyi's method (Nelson 1944), with some additional modifications.

Of the extract, 50 ml was taken in a beaker and concentrated to 5 ml by evaporation in a boiling water bath. One ml of the aliquot was taken in a test tube and added to it 1 ml of freshly prepared Nelson's A + B mixture. The tubes were then heated for 20 min in a boiling water bath and then cooled rapidly in ice cold water. Then 1 ml arsenomolybdate reagent was added to it and shaken well. After 5 min, 7 ml of distilled water was added to it.

A reagent blank was allowed to run simultaneously using 1 ml of distilled water in lieu of 1 ml of the sugar extract. Finally, the absorbancy was measured at 500 nm and the amount
of reducing sugar was determined using a standard curve prepared from glucose.

3.2.19 Estimation of Proline

Free proline was estimated following the method suggested by Bates et al. (1973).

Fully expanded leaves exposed to full sunlight were sampled and 500 mg was homogenised in 10 ml of 3% aqueous sulfosalicylic acid and the homogenate was filtered through Whatman No. 2 filter paper. The filtrate was allowed to pass through a small column of insoluble polyvinyl pyrrolidone (BDH Chemicals) for removal of polyphenols.

To 2 ml of the filtrate to be analysed, added 2 ml of glacial acetic acid and 2 ml of the acid ninhydrin mixture in a test tube and the test tubes were capped. Then the extract was allowed to react for 1 hr at 100°C in a constant temperature bath, after which the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene, mixed vigorously with a test tube stirrer for 15-20 sec. The chromophore containing toluene was aspirated from the aqueous phase and then warmed to room temperature.

The absorbancy was read at 520 nm using toluene for blank, in a Beckman spectrophotometer. The volume stated above may be changed after approximate dilution with toluene to meet the requirement of the available spectrometric
equipment in the laboratory, but the proportion mentioned above must be maintained.

The proline concentration was determined from a standard curve and calculated on a fresh weight basis using the following formula:

\[
\frac{(\mu \text{g proline ml} \times \text{ml toluene})}{(115.5 \mu \text{g/ M mole})} \times \frac{\text{(g sample/5)}}{\mu \text{mole proline per gm FW}}.
\]

3.2.20 Estimation of Epicuticular Wax Content in Leaves

Determination of the epicuticular wax content in leaves was done following the method of Silva Fernandez et al. (1964). Area of the leaf samples for wax determination was first determined and then allowed to wilt for few minutes to avoid penetration of the solvent which might leach out internal components. Individual leaves were then immersed with agitation for 5-10 sec in four successive portions of chloroform taking 5 ml for each wash. When required, the waxes from the upper and the lower surfaces of leaves were isolated separately by allowing successive portions of the solvent to run over the respective surfaces in turn, with fine orifice burettes. The residual chloroform was then allowed to evaporate out and the weight of the deposited wax could be measured and finally the epicuticular wax content was expressed in terms of \( \mu \text{g/cm}^2 \) of leaf area.

Other specific methods used in the experiments are described in the body of the text as and whenever necessary.