CHAPTER III

Materials and Methods
Seven accessions of common buckwheat (*Fagopyrum esculentum* Moench), Viz. IC-18889, Kulugangri, PRB-8901, IC-13141, IC-13145, IC-13411 and BDS- were obtained from North Eastern Regional station of National Bureau of Plant Genetic resources, Shillong and maintained in the experimental fields of the Botany Department of North Eastern Hill University, Shillong. These accessions are native of different parts of North-Eastern States and Himalayan ranges. The geographical distribution of these accessions is shown in Fig. 1.
Regions of Buckwheat Cultivation in India.

1. Leh
2. Pahal gaon
3. Srinagar
4. Udhampur
5. Chamba
6. Kangra
7. Lahaul & Spiti
8. Kinnaur
9. Mandi
10. Kulu
11. Shima
12. Uttarkashi
13. Chamoli
14. Pauri
15. Almora
16. Pithoragarh
17. Darjeeling
18. Siliguri
19. Assam
20. Meghalaya
21. Arunachal Pradesh
22. Nagaland
23. Manipur
All the seven accessions were evaluated for their seed characters. Scanning electron microscopic photographs of the surface features of the seed coat of the accessions were taken using the method of Hayat (1974). Seeds were dehydrated by gradual and sequential passage through 30, 50, 70, 80, 90, 95 & 100 percent acetone. After dehydration the seeds were subjected to critical point drying, followed by fixing in a copper Stub for gold coating. Gold coating was done for about 5 minutes; soon after seed coat photograph was taken in SEM.

For the determination of weight per grain, 25 grains from each of the seven accessions were weighed in a monopan balance. The weight of each grain was calculated by dividing the total weight by the number of grains. For the determination of hull groat ratio, the hull fraction of the grains was separated from the grains manually and weighed. The weight of groat was calculated as the difference between total weight of the grain and the weight of the hull. The hull groat ratio has been calculated by dividing the weight of the hull by the groat weight.

Moisture content of the seeds has been determined by "Low Constant Temperature Oven Method". A known fresh weight of the grains was placed in the preweighed weighing bottles and kept in a forced draught oven at 110 ± 2°C for 24 ± 1 hours. At the end of the prescribed period the bottles were placed in a desiccator for cooling for 1 hour. After
cooling, the final weight of the bottles was determined. The moisture content of the grains, expressed as percentage by weight, has been calculated by the following formula:

\[
\frac{M_2 - M_3 \times 100}{M_2 - M_1}
\]

where \( M_1 \) = the weight in grams of the container and its cover

\( M_2 \) = the weight in grams of the container, its cover and its content before drying and

\( M_3 \) = the weight in grams of the containers, cover and contents after drying.

TISSUE CONSTITUENTS

For the estimation of grain tissue constituents, a known fresh weight (usually 1 gm) of the dehulled grains was fixed by plunging it into boiling 80 percent ethanol. After 24 hours the tissue was macerated in the alcohol in a tissue homogenizer. Separation into alcohol soluble and alcohol insoluble fractions was carried out by filtration under suction over Buchner funnel. The alcohol soluble fraction was made to volume and used for the estimation of soluble sugars, free amino acids, total phenolics and total lipids.

The alcohol insoluble residue was dried in a forced draught oven at 70°C for 72 hours over \( \text{P}_2\text{O}_5 \). The dried
material was weighed and used for the estimation of total starch and alcohol insoluble nitrogen.

Carbohydrates

**Starch**: The starch content of the grains has been determined by the method of McCready et al., (1950). A suitable quantity, generally 50 mg, of the alcohol insoluble material was transferred to a centrifuge tube containing a small volume of distilled water. The contents were heated over a water bath to gelatinize starch. After cooling, starch was extracted from the jelly by repeated trituration with 72 percent perchloric acid. The solution was filtered through a sintered funnel and the filtrate made to volume. To a suitable aliquot of the filtrate, 4ml of 0.1 percent anthrone in conc. HSO₄ was added; the solution was cooled for 10-15 minutes. Absorbance of the solution was measured at 700 nm in a spectronic 20 spectrophotometer.

**Sugars**: Reducing sugars were determined colorimetrically according to the method of Nelson (1944). From a suitable aliquot of the alcohol soluble fraction, alcohol was removed by keeping the tubes in a boiling water bath till the odour of alcohol disappeared completely. The sample was allowed to cool and then the sample was made to volume with
distilled water. To a suitable aliquot of the aqueous extract, 1 ml of mixed copper reagent was added and the solution heated in a boiling water bath for 20 minutes. After cooling, 1 ml of arsenomolybdate reagent was added and the final volume made to 20 ml. Absorbance of the solution was measured at 490 nm in a spectronic 20 spectrophotometer. A calibration curve was prepared with glucose as the standard.

Total sugars were estimated as reducing sugars after hydrolysing enzymatically by 0.2 percent invertase (yeast). The solution was allowed to stand overnight, protected by layering a drop of toluene on top of the solution. Values for non-reducing sugars have been obtained as the difference between total and reducing sugars.

Total Nitrogen

Nitrogen was estimated from the alcohol insoluble fraction by semi-micro Kjeldahl's method. A suitable amount of the alcohol insoluble powder was transferred to a Kjeldahl digestion flask and digested with concentrated H₂SO₄ using selenium-copper catalyst, according to the method of Chiball et al., (1943). Digestion was continued till the solution was faint blue in colour.

The digests were made to volume. Ammonia was estimated from the digests titrimetrically. Ammonia was steam distilled in a Markham's apparatus into boric acid buffer
and estimated by titration against N/140 $\text{H}_2\text{SO}_4$ containing phenol red-bromocresol green indicator (Conway and O’malley, 1942). Anhydrous ammonium sulphate was used as the standard. The percent of protein has been calculated by multiplying the content of nitrogen determined by Kjeldahl’s-titrimetric method with a constant 6.25.

**Total Free Amino Acids**

Total free amino acid were estimated from the alcohol soluble fraction as $\alpha$-amino nitrogen by the method of Rosen (1957). A suitable aliquot of the alcohol soluble fraction; from which alcohol had been completely removed by heating over a water bath, was made to 1 ml by distilled water. 0.5 ml of 0.002 M acetate-cyanide buffer, pH 5.4 and 0.5 ml of 3 percent ninhydrin were added to the solution in succession. The mixture was heated in a boiling water bath for 15 minutes followed by the addition of 4 ml of isopropyl alcohol-water diluent (1:1). After cooling absorbance of the coloured complex was recorded on a Spectronic-20 spectrophotometer at 550 nm with glycine as the standard.

**Total Lipids**

The lipids were extracted from the alcohol soluble fraction with Methanol-Chloroform mixture (1:1), after the alcohol was removed from the sample by gentle heating over a water bath. The solution was centrifuged at 1000 x g for 10
minutes and the supernatant transferred to preweighed petriplates. The solution was allowed to evaporate and the petriplates weighed again. The total lipid content was determined as the difference in the initial and final weight.

Total Phenolics

Total phenolics were estimated from the alcohol soluble fraction according to the method of Swain and Hills (1959). A suitable aliquot, usually 0.5ml of the ethanolic extract was diluted to about 7 ml with distilled water, followed by the addition of 0.5 ml Folin-Dennis reagent. After 3 minutes, 1 ml of a saturated solution of Na$_2$CO$_3$ was added and the mixture made to 10 ml with distilled water. Absorbance was measured after one hour at 700 nm in a spectronic-20 spectrophotometer with gallic acid as the standard.

GROWTH ANALYSIS

All the seven accessions of common buckwheat were assessed for the periodicity of their growth behaviour by conventional growth analysis under field conditions. To carryout the field experiment, a 7x4 metre field was selected in the campus of North-Eastern Hill University, Shillong. 14 raised beds, prepared as rows along the breadth of the field, were prepared; the distance between each row was approximately 50 cm and two rows were allotted for each accession.
Healthy seeds from each of the seven accessions were selected and washed thoroughly under running tap water. The seeds were germinated in petriplates in the laboratory in an incubator. The germinated seeds were transferred to a growth chamber maintained at 25°C and 65 percent R.H with constant illumination. The seeds were maintained in the growth chamber for 7 days till the cotyledonary leaves unfolded completely. The 7 day old seedlings from each of the seven accessions were transferred to the allotted raised beds in the field; 40 seedlings were sown in each row. The crop was watered periodically. Mild dressings of FYM were applied to the field twice, one at the time of sowing and the other after 30 days of transplanting.

All the seven accessions were sampled in a fixed order at random. The first harvest was made on 3rd day after planting and the subsequent harvests were made on 7, 19, 31, 43, 55 and 67 days after planting. 10 plants from each accession were harvested on a given sampling date. The harvested plants were washed in running tap water, blotted dry with the help of a filter paper and separated into stem, leaves and root portions. The length of the stem was measured by measuring tape. The leaf area was calculated by measuring the imprints of the leaves made on the ferrostate paper with the help of planimeter.
After recording their fresh weights, the samples of leaves, stem and root were allowed to dry for 48 hours in an oven at 80°C. The dried samples were cooled in a desiccator and then weighed for recording their dry weight. In the later part of the field experiment the observations were made regarding the time of flowering and number of grains.

The data recorded were used to derive the growth components or indices which were interpreted with reference to the differences amongst accessions. The growth components like Net Assimilation Rate (NAR), Relative Growth Rate (RGR) and Leaf Area Ratio (LAR) were calculated with the formula given by Watson (1950).

\[
RGR = \frac{\frac{1}{t_2} - \frac{1}{t_1}}{W_2 - W_1} \text{ mg/mg dry weight/day}
\]

\[
NAR = \frac{\frac{1}{LA_2} - \frac{1}{LA_1}}{\frac{1}{n_2} - \frac{1}{n_1}} \times \frac{W_2 - W_1}{LA_2 - LA_1} \text{ mg/cm}^2 \text{ leaf area/day}
\]

\[
LAR = \frac{(S_2 - S_1)}{(W_2 - W_1)} \frac{(\ln W_2 - \ln W_1)}{(\ln S_2 - \ln S_1)} \frac{1}{cm^2/mg \text{ dry weight}}
\]

where \(W_1\) & \(W_2\) are the initial and final dry weight of whole plant, \(LA_1\) & \(LA_2\) are initial and final leaf area of the harvest, \(t_1\) & \(t_2\) represents initial and final harvest dates, between two consecutive sampling intervals and \(S_1\) & \(S_2\) are
the initial and a final dry weight of leaf between two consecutive sampling intervals.

NITRATE UPTAKE

Seeds of common buckwheat (*Fagopyrum esculentum* Moench) were washed for one hour under running tap water followed by rinsing with deionized water. The washed grains were germinated for 48 hours in darkness at 27±2°C. The germinated seeds were transferred to a solution of 0.2 mM CaSO$_4$ for 24 hours. After 24 hours the seedlings were transferred to a modified full strength nitrate free Hoagland’s nutrient solution (Arnon and Hoagland, 1940) having the following composition.

<table>
<thead>
<tr>
<th>Salt</th>
<th>gram/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(SO$_4$)$_2$</td>
<td>0.492</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.23</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>0.49</td>
</tr>
<tr>
<td>H$_2$BO$_3$</td>
<td>0.00286</td>
</tr>
<tr>
<td>MnCl$_2$·4H$_2$O</td>
<td>0.00181</td>
</tr>
<tr>
<td>CuSO$_4$·5H$_2$O</td>
<td>0.00008</td>
</tr>
<tr>
<td>ZnSO$_4$·7H$_2$O</td>
<td>0.00022</td>
</tr>
<tr>
<td>H$_2$MOO$_4$·H$_2$O</td>
<td>0.00009</td>
</tr>
<tr>
<td>FeSO$_4$·7H$_2$O</td>
<td>0.5%</td>
</tr>
<tr>
<td>0.6 ml Tartaric acid 0.4%</td>
<td></td>
</tr>
</tbody>
</table>
The germinated seedlings were suspended in the solution through a stainless steel mesh, so that the root portion dipped into the solution. The seedlings were maintained in a growth chamber under continuous white fluorescent light 30 mmol cm\(^{-1}\)/ S\(^{-1}\) at 27±2\(^\circ\)C and 65 percent R.H. The solution was aerated to provide ample Oxygen and solution mixing. For this purpose a peristaltic pump was used, in which continuous air flow was maintained.

The assay of nitrate uptake was carried out on 8 day old seedlings from each of the seven accessions. The seedlings were transferred separately to beakers containing Hoagland nutrient solution supplemented with 5 mM KNO\(_3\). The concentration of KNO\(_3\) in the solution was determined in an aliquot of the solution at 0 and one hour after the experiment was raised (t\(_\infty\) minutes). The difference between the nitrate level represented the amount of nitrate taken up by the seedlings.

Experiments were initiated by placing 8 day old seedlings (grown as described above) in 50 ml pyrex beakers containing 25 ml of Hoagland solution with 5 mM Nitrate given as KNO\(_3\). For the determination of nitrate uptake as a function of time, samples in triplicate were drawn from each treatment solution at 15, 30, 45, 60, 120, 180, 240, and 300 minutes intervals and nitrate levels in 0.1 ml aliquot
of the test solution were determined using the Brucin reduction method, described by Nicholas and Nason (1957). According to this method, 0.1 ml of sample was taken in a test tube and the total volume made up to 5 ml with H₂O, 5 ml of H₂SO₄, 1 ml of sodium chloride solution and 0.25 ml of Brucine Sulphanalic acid mixture were added to the above solution and the test tube heated for 25 minutes in boiling water bath. The tubes were allowed to cool. The optical density of the cooled solution was read at 410 nM. Standard graph was prepared with KNO₃ using the same procedure.

For the determination of nitrate uptake as a function of NO₃⁻ concentration in the nutrient medium, an experiment was raised in which the 8 day old seedlings were transferred to Hoagland nutrient solution containing varying levels of KNO₃⁻. In each solution the amount of nitrate taken up was determined by drawing 0.1 ml aliquot from the test solution before the start of the experiment and 15 minutes after the seedlings were transferred to the solution. NO₃⁻ levels in each aliquots were determined by the Brucine reduction method described above. The difference in the nitrate levels in the ambient medium between t₀ and t₁₅ minutes represented the amount of nitrate taken up by the seedling. For the determination of effect of pH on the uptake of nitrate, the seedlings were transferred to Hoagland nutrient solution containing 5mM NO₃⁻; each of the solution
having a different pH. Uptake of nitrate under such conditions was determined in the same way as described above. For the determination of the effect of glucose, sucrose, DCMU, KClO$_4$ and NH$_4^+$ on the uptake of nitrate by the seedlings, the nutrient solution containing 5mM KNO$_3$ was supplemented with varying amounts of the particular metabolite/compound. Uptake of the nitrate in the solution was determined in the same way as described above.

In order to determine the effect of depleting substrate concentration on the uptake of nitrate, an experiment was raised in which the level of nitrate in the ambient nutrient solution was kept constant throughout the duration of the experiment. This was done by constant replenishment of NO$_3^-$ depleted from the nutrient solution. The amount of nitrate depleted from the solution was determined by measuring the nitrate content in suitable aliquots from the test solution at regular intervals. The drop in the nitrate content during two successive tests gave the amount of nitrate depleted during a particular time period. The uptake of nitrate from such solutions was determined by drawing suitable aliquot (0.1 ml) in triplicate at 15, 30, 45, 60, 120, 180, 240, and 300 minutes intervals and determining the level of nitrate in the test solution by Brucine reduction method (as described above).
The values for uptake rate expressed as \( \mu\text{mol NO}_3^- \) taken up mg dry weight root\(^{-1}\)min\(^{-1}\), were computed from the values of nitrate at each sampling time, i.e., the difference between final and initial concentration of \( \text{NO}_3^- \) in the nutrient medium after a definite time interval has been expressed as amount of \( \text{NO}_3^- \) taken up by the plant. The cumulative uptake has been expressed as \( \mu\text{mol nitrate taken up per 100 mg dry weight root during a particular time interval.} \)

The \( K_m \) and \( V_{\text{max}} \) values have been calculated from the data on uptake rate v/s \( \text{NO}_3^- \) concentration in the nutrient medium using Lineweaver and Burk plot (Lineweaver and Burk, 1934). The data of \( 1/v \) and \( 1/s \) were subjected to regression analysis by using the formula

\[
Y = a + bX
\]

The two seedlings which were used for nitrate uptake study were harvested from the nutrient medium, washed thoroughly in deionized water and separated into root and shoot portions. After measuring the fresh weight of the shoot and root one of the seedlings was used for NR activity study, as per the method of Jaworski (1971). According to this method suitable quantity (usually 100 mg) of freshly harvested tissue was chopped into small pieces and incubated in a test tube in 4.5 ml of substrate solution containing 3.9 ml phosphate buffer (pH 7.5), 0.5 ml of 5 percent isopropanol, and 0.1 ml of 0.02 M \( \text{KNO}_3^- \). Incubation was done in
dark for one hour at 30°C in a water bath. After one hour from each sample, 0.8 ml aliquot was taken in sterilized test tubes to which, 0.6 ml of NEDH and 0.6 ml of sulphanilamide were added. Optical density of the solution was recorded at 450 nm in Spectronic 20 spectophotometer. Measurement of in vivo nitrate reductase activity was carried out separately in shoot and root portions of the harvested seedlings.

The root and shoot portion of the other seedling was dried in a forced draught oven at 80°C for 48 hours. The dried sample was weighed and used for the estimation of the tissue content of total and nitrate nitrogen. For the estimation of total nitrogen a suitable dry weight of the sample was digested as described earlier. The total nitrogen content was estimated titrimetrically by semi-micro Kjeldhal method as described earlier. The nitrate content of the dried sample was estimated after extraction with HCl. The solution was filtered through Whatman No. 1 filter paper. The nitrate content of the sample was estimated from the filtrate according to the method of Nicholsen and Nasson (1957).

From the titter value of total nitrogen, the content of total nitrogen per 100 mg dry weight root and per 100 mg dry weight shoot as well as per shoot and per root have been determined. Similarly the content of nitrate nitrogen per 100 mg dry weight shoot and per 100 mg dry weight root as well as per shoot and per root have been
determined from the data of absorbance for nitrate. The content of reduced nitrogen has been determined as the difference between total and nitrate nitrogen.

For the determination of nitrate uptake by excised roots the 8 day old seedlings, raised as described earlier, were washed in deionised water and dried on a filter paper. The root portion was excised from the seedlings by cutting with a sharp blade. A suitable fresh weight of the root tissue was transferred to Hoagland nutrient solution supplemented with 5 mM nitrate to determine the uptake, following the same protocol as that for intact seedlings.

PARTITIONING OF NITRATE IN WHOLE PLANT

For nitrate partitioning study the seedlings of common buckwheat were raised in the laboratory condition. Eight day old, seedlings were transplanted into pots, each containing 2.6 Kgs of fine sand washed with 0.3 per cent of \( \text{H}_2\text{SO}_4 \). The pots were arranged in 4 sets representing four treatments. The plants were maintained in the net house with four plants in each pot and 10 pots for each treatment. The pots were regularly supplied with full strength Hoagland solution, containing 5, 20 and 50 mM KNO\(_3\), which represented the 3 levels of nitrate supplied to the plant. The untreated controls, representing one set on treatments, received Hoagland nutrient solution which did not contain any
nitrogen. The plant were harvested at random from each set of treatments, on 7, 19, 31, 43, 55 and 67 days after planting. At each interval 5 plants from each treatment were harvested. The harvested plants washed under running tap water and blotted dry on sheets of filter paper. The length of the shoot was measured and the plants were separated into stem, leaf, petiole and root segments. The stem was further divided into 2, 3 or 4 segments according to length. The fresh weight of each segment was determined and the segments dried in an oven for 72 hours at 80°C. From the oven dried materials, the dry weight of each segment was determined. Based on dry weight data of different samples and leaf area, the growth indices, viz., RGR, NAR and LAR were calculated from the plants for each treatment and each samplings. In order to determine the partitioning of Nitrogen between various tissue segments of the plant, harvested plants were separated to shoot, leaves, petiole and stem. The leaves and petiole were numbered in the acropetal order. The stem was divided into 2, 3, or 4 segments from the base and each segment numbered in an acropetal order. In each segment the amount of various nitrogenous components viz. total nitrogen, nitrate nitrogen and reduced nitrogen and the activity of nitrate reductase was determined. In figure the values for various nitrogenous constituents have been expressed with illustration in µg per total amount of dry weight of the particular unit. The units for NR activity have
been expressed as μmol nitrate reduced/100 mg fresh weight/hour.

Based on the information collected, an attempt has been made to determine the relationship between the nitrogen content of the plant and growth behaviour. The data thus obtained has been fitted to the models proposed by Greenwood et al. (1986; 1991). The theory, which backs the model attempts to relate the percentage nitrogen in plant dry matter to growth rate and to plant dry weight per unit area. It covers all stages of growth until senescence, of crops grown at optimal and sub-optimal N-nutrition.

MODEL I

This model attempts to test the effects of applied nitrogen on the growth and photosynthetic activity with plants of common buckwheat (*Fagopyrum esculentum*) using the following equations;

\[
\frac{dW(F,t)}{dt} = \frac{[Kx(F)] \times [W(F,t)]}{X + W(F,t)}
\]

where \(W(F,t)\) is the dry weight of the plant (excluding root) per unit area

\(Kx(F)\), is the growth rate coefficient.

\(x\) is a constant
Integration of the above equation gives,

\[ [Kx(F)] [T - T_o] = X \ln W(F,t) + W(F,t) \\
- X \ln W(F,t_o) + W(F,t_o) \]

where, \( W(F,t) \) is the dry weight of the sample (excluding root) at time \( t \) and \( W(F,t_o) \) is the dry weight of sample (excluding root) at time \( t_o \). \( T_o \) represents the time of initial harvest and \( T \) represents the time of next harvest.

For specific conditions where there is just sufficient N-fertiliser to permit maximum growth, Greenwood et al., (1991) have defined \( Kx(F) \) as \( Kc \) and \( W(F,t) \) as \( Wc(t) \), so that

\[ \frac{d Wc(t)}{dt} = \frac{Kc \times Wc(t)}{X + Wc(t)} \]

where \( Kc \) is the critical growth constant and \( Wc(t) \) is critical dry weight at critical nitrogen level.

Greenwood et al., (1986) have assumed that there is a relationship between critical percent N in the plant dry matter \( Nc(t) \) and plant dry weight \( Wc(t) \) and have defined the critical nitrogen constant in a plant by the equation

\[ Nc(t) = 1.33 + \exp [1.4 - 0.26 Wc(t)] \]
MODEL II

In this Model the relationship between percent N and photosynthesis has been derived by using the formula derived by Greenwood et al., (1986). This has been done by regression of the rate of photosynthesis, $P_L$, against the concentration of $(N_L)$ nitrogen in the leaf. It is, therefore, written as

$$P_L = M_L N_L + C_L$$

where, $P_L = $ Leaf Photosynthesis.

$N_L = $ Nitrogen in the leaf

$M_L$ and $C_L$ are coefficients

There is a linear relationship between percent N in the leaf $(N_L)$ and percent N in the whole plant $(N_w)$. Therefore, it is written as,

$$N_L = M_w N_w + C_w$$

where, $M_w$ and $C_w$ are coefficients which have positive values.

The integration of above equation gives

$$P_L = M_L M_w N_w + M_L C_w + C_L$$

For a given crop of a given size, $M_L C_w + C_L$ are constants so that $P_L$ is linearly related to $N_w$. 
The model based on the linear regression equation \( Y = a + b X \), can therefore be used to relate photosynthetic efficiency of the leaf to the total nitrogen content within the plant. In the plant under study the leaf photosynthetic capacity defined as net assimilation rate has therefore been related to percent nitrogen in the whole plant at various stages of growth under varying levels of external nitrate supply.

The entire data presented in the present study represents experiments carried out over three consecutive years under laboratory and/or field conditions as applicable. The data represents the mean of at least three independent replicates for each study. The entire data has been subjected to statistical treatments and the LSD value depicting the level of significance at 5 percent probability determined using a computer programme for ANOVA.