CHAPTER 5

General materials and methods

5.1. Experimental Site:

The experimental site is located in the nursery and green house of the Department of Ecology and Environmental Science, Assam University, Silchar. It is situated in the Southern Assam, India between $24^0 41^{//} 169^{/}$ N latitude and $92^0 45^{//} 129^{/}$ E longitude. On the other hand the micropropagation of experimental plants, i.e. *Smilax glabra* Roxb. and *Bulbophyllum careyanum* (Hook.) Spreng. were done in the Plant Tissue Culture Laboratory of the Department of Biotechnology, Assam University, Silchar and *Paphiopedilum spicerianum* (Rchb.f.). Pfitz. was done in the Sreedhar Apex Biotech Laboratory at Bagbahar, Cachar.

5.2. Collection and cultivation of the tested plants:

The tested plants i.e. *Smilax glabra* Roxb., *Homalomena aromatica* (Roxb.) Schott., *Bulbophyllum careyanum* (Hook.) Spreng. were collected from the different wild habitat of Barak Valley and they were cultivated in the garden and nursery of the Department of Ecology and Environmental Science, Assam University, Silchar as a mother plant, while *Paphiopedilum spicerianum* (Rchb.f.). Pfitz., specimen was collected from BSI, Shillong.

5.3. Materials:

5.3.1. Experimental soil:

The experimental soil used was top forest floor soil. Soil was collected from the disturbed forest of Rosekandy Tea Estate, Cachar, situated between $24^0 8^{/}$ N latitude and $29^0 15^{/}$ E longitude. After collection soil samples were allowed to air dry. The fine grained air dried soil was used as basic substratum for the vegetative propagation of *Smilax glabra* Roxb., *Homalomena aromatica* (Roxb.) Schott., *Bulbophyllum careyanum* (Hook.) Spreng.

5.3.2. Culture pot:

Both earthen pots and non transparent poly bags (black coloured) were used as culture pot in the vegetative propagation of the experimental plants.

5.3.3. Planting materials:

The planting materials of the experimental plants were rhizome in case of vegetative propagation. They were collected from the wild habitat of Barak Valley, Southern Assam.

5.3.4. Natural Substrate

Three types of natural substrate, soil, sand and leaf mould were used as the substratum on the vegetative propagation of the experimental plants.

5.3.5. Organic amendments:

Cow dung and vermicompost were used as organic amendments.

5.3.6. Inorganic amendments:

Urea (46% of N), single super phosphate (SSP, 16 % P_2O_5) and muriate of potash (MOP, 60% of K_2O_4) were used as inorganic amendments and as a source of nitrogen, phosphorus and potassium.

5.4. METHODS

5.4.1. Determination of the physicochemical properties of soil:

The physical and chemical characteristics of the experimental soil for the pot experiment and field experiments were analyzed by standard methods.

5.4.1.1. Soil p^H:

p^H of the soil samples were determined by using a digital p^H meter . It was analyzed in 1: 2.5 (w/v soil: water) solution. 20 gm of air dried soil samples were taken in 250 ml conical flasks and 50 ml of distilled water was added. It was kept it for 30 minutes. Then

the solution was shaken in a rotary shaker (REMI) for 30 minutes. After that the solution was kept for sometimes and then measured by digital p^H meter (**GeNei^{TM}**).

5.4.1.2. Moisture content:

20 gm of soil samples were taken in the moisture boxes of known weight. Then the samples were allowed to oven dry at 105^0 c for constant weight.

The moisture content was determined by the following formula

$$\text{Percentage of moisture content (\%)} = \frac{\text{Weight of the fresh soil} - \text{Weight of the oven-dried soil}}{\text{Weight of the fresh soil}} \times 100$$

5.4.1.3. Water holding capacity:

It has been determined by Keen's box method as described by Piper (1944). Weight of the empty keen's box was first measured. The filter paper of same diameter of keen's box was cut and soaked with water. The wet filter papers were weighted and put on the bottom of the box and weighted. The box was filled with air dried and 2mm sieved soil samples and partially immersed in water to absorb water. After 24 hours (saturation of the soil with water), the soil samples with box were measured. Then the soils samples were allowed to oven dry at 105^0 c for constant weight.

The water holding capacity was determined by the formula as

$$\text{Water holding capacity (\%)} = \frac{B-(C+D)}{C-A} \times 100$$

A= weight of the empty keen's box

B= weight of the saturated soil + keen's box

C= weight of the oven dried soil + keen's box

D= weight of the wet filter paper

5.4.1.4. Measurement of the available organic carbon:

Organic carbon has been determined by Walkley and Black's rapid titration method (Jackson, 1958). 1 gm finely air dried soil sample was taken in 500 ml conical flask. 10 ml of potassium dichromate solution was added to it followed by 20 ml conc. H_2SO_4. The flask was then shaken for about 1 minute rigorously and allowed to cool for 30 minutes. Then, 200 ml distilled water was added, followed by 10 ml orth-phoshoric acid and 1 ml of diphenyl amine indicator. The solution was then titrated against freshly prepared 0.5 N ferrous ammonium sulphate until the colour of the mixture flashes to green. A blank (without soil sample) was also analyaed following the same procedure.

$$\text{Organic carbon (\%)} = \frac{10\ (\text{Blank reading} - \text{Titration reading}) \times 0.003 \times 1.32 \times 100}{\text{Blank reading} \times \text{Weight of the reading}}$$

5.4.1.5. Available nitrogen:

Available nitrogen of the soil sample was determined by semi-micro Kjeldahl method (Anderson and Ingram, 1993) in pelican semi-automatic N- analyser (Kel plus). 1gm air dried and 0.5 mm sieved soil samples was taken in digestion tube and 5gm sulfate mixture catalyst (K_2SO_4, $CuSO_4$, Se) (50:10:1) was added followed by 15 ml conc. H_2SO_4. The mixtures were digested at 420^0 C for about 1 hour or until the mixture colour turned whitish. Then the digested samples were distilled in presence of excess alkali (40% NaOH) for about 8 minutes. The distillate was collected in 20 ml of 4% boric acid added with the mixed indicator and was continued until the colour of the solution turned green. The solution was then titrated against 0.05 N HCl for the end point where the colour changed to pink. The available nitrogen was calculated by the following formula

$$\text{Available nitrogen (\%)} = \frac{14 \times (\text{sample titer} - \text{blank titer}) \times \text{Normality of acid} \times 100}{\text{Weight of the soil samle} \times 1000}$$

5.4.1.6. Available Phosphorus:

Available phosphorus is determined by Ammonium molybdate stannous method (Anderson and Ingram, 1993). 37 gm of ammonium fluoride was dissolved in double distill water and the volume was make upto 1 litre and this gave 1 N ammonium fluoride solution, 20.2 ml conc. HCl was added to 500 ml volumetric flask and volume was made

upto 500 ml with double distilled water. This gives 0.5 N HCl. 15 ml of ammonium fluoride solution (1N) and 25 ml 0.5N HCl was added to 460 ml double distilled water. This gives a phosphorus extracting solution of 0.03 N ammonium fluoride and 0.25 N HCl.

5 gm of air-dried and 2 mm sieved soil sample was taken in 250 ml conical flask. 35 ml phosphorus extracting solution was added to it and then shaken for 30 minutes at 120 rpm on a rotatory shaker. The solution was then filtered through Whatman No. 44 filter paper. 5ml of the filtered aliquot was taken in 50 ml volumetric flask and 10 ml double distilled water was added to it, followed by 2ml ammonium molybdate and 1 ml fresh stannous chloride solution. The volume was made up to 50 ml with double distilled water. Then the optical density of the blue colour between was measured by using spectrophotometer (UV-1800 Shimadzu) at 680 nm within 30 minutes. It was calculated by the following formula:

$$\text{Available phosphorus (ppm)} = \frac{\text{X (mg)} \times \text{volume of the extractant (ml)}}{\text{Volume of aliquot (ml)} \times \text{air dried weight of soil}}$$

X (mg) = Phosphorus concentration was calculated from the standard graph. Standard curve was prepared using potassium dihydrogen phosphate.

5.4.1.7. Available potassium:

Potassium content of soil sample was determined by the flame photometer method. 5 gm of air dried and 2 mm sieved soil sample was taken in 100 ml conical flask and 25 ml of neutral normal ammonium acetate was added to it and then shaken for 30 minutes in a rotator shaker. The solution was filtered through Whatman No. 44 filter paper. Then the potassium content of the alliquit was measured by using flame photometer (Systronics 129). Standard curve for available potassium was prepared by using KCl solution.

5.4.2. GROWTH PARAMETERS:

The growth parameters of the experimental plants were recorded at 15 days interval from the date of potting the rhizome up to one year.

5.4.2. 1. Plant height:

The maximum length of the shoot from the soil surface to the tip of experiment plants was measured with the help of a scale and the measuring tap in centimeter. In case of *Homalomena aromatica* (Roxb.) Schott. length of shoot from the soil surface to the tip of the longest leaf was recorded.

5.4.2. 2. Number of leaves:

The total number of leaves present per plant were counted and recorded at the time of observation.

5.4.2. 3. Number of sprouting:

The total number of sprouting per tested rhizome was counted and recorded.

5.4.2. 4. Fresh weight and Dry weight:

The fresh weight and dry weight per plant were determined on an average basis of three using destructive harvest method (Misra, 1968). The plants were uprooted by taking precaution not lose roots during the operation. Roots were carefully washed to remove the soil. Then the shoots and roots were carefully separated and spread over the laboratory table just to remove the surface water. The fresh weight of shoots, root and rhizomes were measured with the help of electronic balance. The separated parts of the experimental plants were allowed to dry at 70^{o} c hot air oven till constant weight. The fresh weight and dry weight per plant is expressed as gram per plant.

5.4.3: Data analysis:

The collected data were expressed as mean ± SD and analyzed by using the software Microsoft Excel 2007, SPSS 16.0 and Graph Pad Prism. The significance between the different treatments was done by Kruskal Wallis Non parametric test. The correlations between the different growth parameters in different treatments were calculated by Spearman's rank correlation coefficient (r_s).