CHAPTER-III
MATERIALS AND METHODS

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3.1 MATERIALS

To study the effect of PSM on growth and metabolism of *Arachis hypogaea* L. an experiment was carried out with different treatements as follows.

1) Uninoculated control (soil)
2) Soil + PSM (*Aspergillus awamori*)
3) Soil + Rock Phosphate
4) Soil + Rock Phosphate + PSM (*A.awamori*)

Red clay loam soil having pH8.1), organic carbon 0.22 kg per hectare, available phosphate 28kg per hectare and potassium 90 kg per hectare was used for this study.

The plants were raised in . polythene bags , filled with 5kg soil. 500 mg. rock phosphate per polythene bag was applied wherever the treatment had rock phosphate.

The pure culture of *Aspergillus awamori* was obtained from NCL. Pune. It was multiplied in petridishes containing Sabouraude's agar.

The Sabouraude's agar was prepared by dissolving 4g of glucose, 1g of

peptone and 2.5g of agar in 100ml of distilled water.

The seeds of SB-11 var. of groundnut were obtained from Agricultural Research Centre, Digraj. The seeds were surface sterilized with 0.1% $HgCl_2$

Surface sterilized seeds were introduced into the petridish containing culture of *A. awamori*, it was shaken slowly so the spores of *A.awamori* got uniformly spread on the surface of the seeds. These inoculated seeds were then sown into the polythene bags wherever the treatment had PSM. Uninoculated seeds were sown in control as well as soil + R.P. sets.

The experiment was conducted using a completely randomized design with 10 pots (polythene bags)for each treatment. Three seeds per polythene bag were sown. Germinating seedlings were thinned to two seedlings per Polythene bag. Fifteen days after sowing regular weeding was carried out. Plants were watered regularly. On 40th day after sowing the plants were uprooted, washed thoroughly and carefully to remove adhering soil particles. The leaves, stems and roots were separated and dried in a hot air oven at 80° c for 7 days to constant weight. The dried plant parts were powdered and were used for the study of mineral nutrition. For the study of nitrate reductase activity, chlorophyll content, leghaemoglobin content the fresh plant material was used.

3.2 METHODS

3.2a Growth analysis

Fresh plants were used for recording length of root and shoot. The nodules were separated from the root. The number of nodules per plant and fresh wt. of

nodules per plant were recorded.

The plants kept in hot air oven at 80° C for 7 days were used for recording the dry matter production per plant.

3.2b Estimation of chlorophyll

Chlorophyll was estimated by using method of Arnon (1949). Young and mature leaves were washed first with tap water and then with distilled water, blotted to dry and cut into small pieces. 0.5 g of the material was homogenized in 80% acetone and filtered through No. 1 Whatman filter paper. The residue was washed thoroughly 3-4 times with 80% acetone, collecting all the washings in the same container. Final volume was made to 100 ml. with 80% acetone. Absorbance was read at 663 nm and 645 nm for chlorophyll a and chlorophyll b respectively on spectrophotometer. The total chlorophyll content was estimated using the calculations as follows.

Chl a = 12.7 x A 663 - 2.69 X A 665 = X

Chl b = 22.7 x A 645 - 4.68 X A 663 = Y

$$\text{Chl a/b mg/100g fresh tissue} = \frac{X/Y \times \text{Vol of extract} \times 100}{100 \times \text{wt of plant material (g)}}$$

3.2c Optical estimatoin of leghaemoglobin in root nodules.

Leghaemoglobin estimation was carried out according to the procedure

described by Appleby and Bergerson (1980).

A known weight of fresh clean root nodules (1g) were ground in a mortar and pestle with about 5 ml. of distilled water. After preparing a fine paste, contents were transferred to centrifugation tubes using little more quantity of distilled water. They were centrifuged for 10 minutes at 9000 rpm and centrifugation was repeated once again to get a clear and nearly pink supernatant. The supernatant was transferred to a 25 ml volumatric flask and volume was made upto the mark with alkaline pyridine reagent. One g of sodium dithionite was added to all samples in large tubes and shaken slowly without frothing. Within few minutes the greenish yellow colour developed was read using a spectrophotometer at 556 nm and using universal range filter after adjusting the blank(all reagents+distilled water) to 100 percent transmitance The O.D. per g of nodule was recorded.

3.2d Nitrate reductase

Nitrate reductase was determined (in vivo) by the leaf disc method (Evans, 1982). The known quantity of leaf tissue was suspended in a 5 ml reaction mixture containing 0.1 M KNO_3 ,1% (v/v) n-propanol, and 0.1 M phosphate buffer at pH 7.5 after cutting them once or twice into pieces. The test tube containing reaction mixture and pieces of leaves were then sealed and kept in darkness for incubation for 60 min. The enzyme activity was measured by treating 0.4 ml. of incubation mixture with 0.3 ml of 1% sulfanyl amide and 0.3ml of 0.02% NEEDA (N-1-naphthyl ethylene diamine dihydrochloride). To this 2ml. of distilled water was added and the absorbance of colour developed was read at 540 nm on spectrophotometer expressed in terms of µg NO_2.h.$^{-1}$g^{-1} fresh tissue.

sometime till the initial reaction subsided. Then it was heated till all the particles of plant material got dissolved. 10 ml. of perchlroic acid was added to it and the mixture was heated strongly till 3-4 ml of colourless extract was obtained. Then the mixture was cooled and diluted to 100 ml. with distilled water, in 100ml. volumetric flask. This acid digest was then cooled and stored in stoppered bottles.

3.2g Estimation of potassium.

Potassium was estimated using 902 Double beam at omic abosorption spectrophotometer.

3.2h Estimation of phosphate:

Phosphate was estimated by using method by Peterson (1978).[Modified Fiske and Subbarow's method.(1924)]

The ammonium molybdate reagent was prepared by dissolving the compound (4.4g) in water (500 ml) adding concentrated sulphuric acid (14 ml) and making upto 1 litre with distilled water. The reducing reagent consists of sodium bisulphate (2.5g), sodium sulphite (0.5g) and 1 amino-2naphthol-4-sulphonic acid (0.042 g) which were dissolved in water (250 ml) and allowed to stand in dark for several hours. The solution was filtered in brown bottle and stored in refrigerator.

A standard solution of 0.5 m M sodium dihydrogen phosphate was also prepared for calybration purpose.

To 1ml of acid digest (sample) ammonium molybdate reagent (2.4 ml) was added followed by reducing reagent (2.4 ml). The solution mixture was mixed thoroughly and was heated on a boiling water bath for 10 min. for colour development. On cooling the absorbance was read at 830 nm.

The amount of phosphorus in the unknown sample was read from the calybration curve prepared at the same time by performing the reaction on known amount of standard phosphate solution.