Chapter - 3

Material and Methods
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The Material

Apple (*Malus domestica* Borkh)

The material used in the present studies is the *Malus domestica* Borkh and its natural associated mycorrhizal roots. An Apple tree has three main vegetative parts - the root, the stem and the leaf. Even though the roots are not visible but the effect of root damage or injury is exhibited on the aerial parts.

Roots can be classified on the basis of their size: viz, scaffold roots, which are long and thick and fibrous roots which are short, not more than a few centimeter long, and thin 1.3 mm. They can also be less parallel to the surface, and vertical roots which grow straight downward, going as deep as 6 to 12 m. The scaffold roots which may be vertical and horizontal produce the fibrous roots which form mycorrhizal association and are the main feeding roots of the plants. The aerial portion of an apple tree initially grows as a single stem, which then bears shoots. Some of these shoots become branches. It can only grow on end or terminally.

An apple tree conjures up an image of a very large tree, perhaps 8 to 10 m high, with a diameter spread of 9 m. This impression is correct as far as the conventional apple trees goes because over the centuries very large apple trees have been grown all over the world. These trees are raised from the seeds and grafted with
vigorously growing selected scion cultivars. Practically all orchard in India are grown in this manner. Orchards containing large tree are classified as low density or chards. They maintain a planting distance of 6-9 m accommodating 150 to 300 trees per hectares.

In the recent past, however, there has been a shift from large tree to medium/small sized trees in the advanced apple growing regions of the world, where the planting density ranges between 300 to 750 trees per hectares. A high density orchard is 2000 to 5000 trees per hectares.

Large trees are usually referred to as vigorous trees while the medium and small are called semidwarf and dwarf, respectively. An apple a day keeps the doctor away this old adage focusses man’s attention on the importance of apple in the daily diet. Apple has long been the staple fresh fruit in the temperate parts of the world. Eating apples is believed to reduce the incidence of dental caries, helps to control obesity and supply extra energy for heavy exercise.

The germination of apple seed under natural condition takes place during March and April because seeds require a cold treatment for a period of 60-80 days. But even under favourable conditions a number of seedlings may die from one cause or the other. Therefore, for artificial regeneration seedlings of apple are raised either in nursery beds or in polythene bags.
A search into literature reveals that no specific attention has been paid to ascertain the role of ectomycorrhiza in artificial regeneration of apple seedling. Grafting of seedling at the stage 6-10 mm diameter which is usually achieved after 1 year of age. Therefore, the present studies were undertaken to determine and ascertain the effect of artificial mycorrhization on the seedlings of apple with pure inoculum which was isolated from its natural mycorrhizal roots.
The Methodology

1. Sampling Technique:-

In Himachal Pradesh different varieties of apples are grown. Most commonly Royle Delicious (Starking Delicious) Red Delicious, Golden Delicious, Richa Red and Tiedman’s Early Worcester: Among these starking Delicious is the most preferred and popular cultivar of apple in Himachal Pradesh. Preference for this is because of its attractive colour, tastes, aroma and market values, therefore, the studies have been carried out on the root system of starking delicious. Root samples of apple plants from five orchards located at different agroclimatic conditions were collected.

2. Morpho-anatomical studies of natural mycorrhiza:-

Root samples from apple plants from different orchards were collected from the top 10-15 cm (feeder roots) of the soil. Morphological characters with following features were recorded following Zak (1971).

i. Form of the mycorrhizae.

ii. Colour of the fungal mantle.

iii. Thickness of mantle.

iv. Texture of the fungal mantle.

v. Surrounding mycelium presence or absence.

vi. Odour and Taste.

vii. Microscopic examination such as
(a) Degree of development of Hartig net.
(b) Features of surrounding hyphae.

To confirm the formation of ectomycorrhizae, short roots exhibiting visual morphological changes were collected from lateral roots. The segments were washed with tap water; fixed in Formaline Acetic Acid (F.A.A.*) for 24 hours and then preserved in 70% alcohol. For characterization and identification of mycorrhizal types, root sections were cut and stained in safranin, fast green combination (Marx and Davey, 1969) Cotton blue** (Philips and Hayman, 1970) and finally examined under microscope (Getner Binocular Research microscope GTRAbA) at 10X, 40X and 100X- magnification. Presence of "Hartig net" of fungal mantle was considered evidence of ectomycorrhizal development. Plants grown in glass house were also assessed for the presence of mycorrhizal association and development at 1-1/2, 3, 6 and 12 months of plant age.

**Isolation of symbiont from mycorrhizal roots:**
Mycorrhizal roots were washed in running tap water for 30 minutes, surface sterilised with 0.02 percent murcuric chloride (Hgcl2) for 30 seconds, washed in sterilized distilled water repeatedly to remove any traces of Hgcl2. Gently pressed between the folds of a sterilized filter paper to remove any extraneous water and finally aseptically planted on PDA*** slants (Rawling, 1933). Incubated at 22+- 10C in a B.O.D. incubator.
Inoculum multiplication:- For inoculum multiplication, wheat grain substrate was used. In this method 10 kg of wheat grain in 15 litre of water was boiled for 15 minutes initially and then allowed to soak for another 15 minutes (without heating). The water was decanted and grains were spread on sieves to dry, turning them several times with a wooden spoon. The boiled grains (9 kg) were mixed with (120g) Gypsum (CasO₄·2H₂O) and whiting (CaCO₃ 30g). Gypsum prevents the grains from sticking together (Stoller, 1962) and whiting is necessary to adjust a correct pH. The grains were filled into polypropylene bags (150-200g bag) and sterilized for 2 hours at 15 p.s.I pressure. After sterilisation the material had a dry matter content of about 50% and a pH of 6.5-6.7. The bags were incubated at 22+-1oC in B.O.D. incubator (Cattan Make). Spawn was ready for inoculation after 20-25 days.

Apple Seeds Collection:- Seeds of apple (Malus domestica Borkh.) were collected from healthy fruit of cultivar Golden Delicious in the month of October-November, 1995. The seeds were carefully washed in running tap water, dried in shade and stored in refrigerator.

Surface sterilization:- Before using the seeds for experiments, these were washed in running tap water for half an hour and surface sterilised with 30% H₂O₂ solution for 30 minutes. Stratification procedure:- Apple seeds donot germinate immediately. They require a special temperature treatment, known as stratification. During this treatment, certain chemical and other
changes take place which initiate the dormant embryo into growth. The soaked seeds were placed in alternate layers of moist sand filled in a small tub. Tub was stored in a refrigerator (5c) for 60 days. The sand was kept moist by watering occasionally. Stratification is complete only when germinating seeds start showing emergence of radicle.

**Inoculation Technique:** Two methods viz column and bottom layer were tested to find out a convenient system of seedling inoculation with mycorrhizal fungus. To compare these two methods empty ice cream cups, 6 cm in diameter and 4 cm height, were selected. To facilitate water drainage and aeration small pin prick holes were made at the bottom of cups which were covered with a layer of fine gravel (autoclaved) before filling the sterilized soil in following two methods.

1. **Preparation of column:** To make the column, 200g sterilized soil was gently pressed in the above mentioned cups. 3cm deep columns were made with the help of cork borer 0.80mm diameter. The columns were filled with wheat grain inocula (5 grains) and covered with layer of sterilized sand. A single stratified seed was kept on each column in such a way that germinating radicle passes through the column. Seeds were covered with fine layer of sterilized sand. A filter paper (No-I) cover was provided over the cup for a week so that it may help in maintaining ideal moist conditions for seed germination as well as to avoid seed dislocation while watering.
2 Preparation of layer method:- Over the layer of fine gravels, 100g (by volume) autoclaved soil was poured and spread out evenly in the cup. A layer of grain inocula (20 grains) was made over it and covered with a layer of 100 g sterilized soil. Stratified seeds, five in number were placed over the soil layer and covered by a fine cover of sterilized sand. A filter paper cover was also provided over the cup.

Collection and preparation of soil:- Two types of soil i.e. natural orchard soil (NS) and soil collected from the sites of uprooted apple tree pits here after named as basin soil (BS) were selected. To investigate the establishment of mycobiont and its impact on apple seedlings w.r.t. growth characters and interaction with diseases. These soils were collected from apple orchard site at Dr. Y.S. Parmar UHF-Regional Fruit Research Station Mashobra, District Shimla. Physical characteristics of these soils were also recorded. Soils were sieved through a coarse wire mesh to remove gravels etc. following four sets were made with each type.

1st set: Natural orchard soil (NS):- Soil was collected from the 20-25cm depth at the site of unplanted orchard area.

2nd set: Natural sterilised soil (NSS):- The collected natural soil (NS) was sterilised with 2% formaline. Soil was first saturated with 2% formaline and then covered with polythene sheet for 20 days. The sheet was removed and soil was exposed to air, frequently pulverised till all the vapours escaped from the soil and no formaline smell was detectable.
3rd set: Natural sterilised soil plus mycorrhiza (NSSM): - The mycorrhiza grown as wheat grain inoculum was added as detailed in column inoculation method.

4th set: Natural sterilised soil plus mycorrhiza plus farmyard manure: (NSSMF): - To enhance microbial activities in the rhizosphere of seedlings, farmyard manure was added. Well decomposed FYM was first sieved through a course wire mesh. 500mg was added in each ice cream cup over the wheat mycobiont inocula.

Similarly for basin soil: four set viz. BS, BSS, BSSM and BSSMF were made. For each treatment 40 earthen pots of 20 cm diameter were maintained in the glass house. These were used to transplant apple seedlings and finally utilized for laying out different experiments.

Transplantation: - Though apple seedlings cannot be transplanted during active growth stage, therefore ice cream cup technique was devised. Twenty days old seedlings were transplanted in the pots. For this purpose a soil cavity equal to the size of ice cream cup was made at the centre of the earthen pot and seedlings with intact cup soil was gently placed in the cavity after dissecting away the ice cream cups.

Observation on growth and development of seedlings: - Five pots were randomly selected from each set to record observation on growth. Single seedling was randomly uprooted from each pot after moistening the soil at 45, 90, 180 and 360 days of growth. Obser-
Observation on shoot height, root length, collar diameter, root volume, fresh and dry weight of shoot and root were recorded at different growth stages. The root samples were also assessed for association of mycorrhiza.

Observation on the association of mycorrhiza: Seedling roots from all samples were fixed in F.A.A. and then preserved in 70% alcohol. The morphology and anatomy of the short and long roots was studied as detailed earlier to observe different stages of mycorrhizal development.

Estimation of Macro and Micronutrients of seedling: Nutrient status of different plant parts of inoculated and uninoculated seedlings, were analysed for macro and micronutrients after twelve months of age in the Central Potato Research Institute (CPRI) at Shimla.

Estimation of Nitrogen (N): Leaf, stem and root samples were collected separately, dried in oven at 50°C and fine powder was prepared in the grinder. Estimation of nutrients was done as detailed below. 0.2g. dry weight of sample was taken in a flask, one tablet (special kjeltab 3.5g. k2So4, 0.4g. Cu So4) was added and 5 ml conc. H2 SO4 was poured in it. It was kept overnight and heated on hot plate (350°C) till green colour appears. Then autolized in Kjelteck autolizer and readings were recorded.

Estimation of phosphorus (P): To 0.2g sample, 3ml of Triacid was added for digestion, then it was kept overnight at room
temperature and after that it was heated on hot plate till white colour appears. The final volume was made to 25ml with the addition of distilled water. From this extract 2ml of solution was taken and 2ml of ammonium molybdate solution was added and 1/1ml of 1/4th reagent. Again the volume was made to 25ml (with distilled H2O). After half an hour our readings were recorded in spectronic 20.

Estimation of Potassium (K):- To 0.2g sample, 3ml of triacid was added for digestion, it was kept overnight at room temperature. It was heated on hot plate till white colour appears. The final volume was made to 25ml with the addition of distilled water. Readings were read in flame photometer (Corning Model-400) after calibration.

Estimation of Iron (Fe), Zinc (Zn), Copper (Cu), Magnesium (Mg):- To 0.2g sample, 3ml of triacid was added for digestion, it was kept overnight at room temperature. It was heated on hot plate till white colour appears. The final volume was made to 25ml with the addition of distilled water. Digested sample were directly read in Atomic Absorption Spectrophotometer for estimation of Fe, Zn, Cu and Mg.

Estimation of Nitrogen (N), Phosphorus (P) and Potassium (K) and pH of Natural soil (NS) and Basin soil:- N, P, K of the above mentioned soil was also analysed and pH was also recorded.

Phosphorus (P):- 2 gm soil sample was taken in a flask, 21 ml of
Bray solution was added (extracting solution 0.03N in ammonium floride and 0.25 N in HCL). It was shaken for one minute and then filted it. 2ml of extract and 2ml of ammonium molybdate solution and one ml of stenus cloride were mixed. Colour was measured photometrically using 660nm between 6 to 20 minutes after mixing.

Potassium (K):- To 5g soil sample25 ml(IN Ammonium acetate) was added. It was Shaken for five minutes, filtered it then reading was observed in flame photometer.

Nitrogen (N):- It was calculated from 1:10 Nitrogen and organic carbon ratio.

pH:- To 10g. soil, 20 ml of distilled water was poured. It was kept undisturbed for 2 hours and reading were recorded in pH metre.

Observation on powdery mildew interaction:- Five pots each with five seedlings were selected from each set and placed in a isolated chamber in the glass house. Seedlings were inoculated by dusting conidia of powdery mildew (Podosphaera leucotricha) collected from naturally infected apple plants. Mildew infection was recorded with 0-IV rating scale and percent disease index was computed as given by Mckinney (1923):

Percent disease index (PDI):- \[ \frac{Env \times 100}{N \times V} \]

where
- n= number of plants under each rating
- v= disease rating
- N= Total number observed.
- V= Maximum disease rating
- E= Sum of all the numerical rating.
Rating scale:-
0= No visible mildew symptom.
I= Infection visible (<10% leaf area).
II= Mildew growth scanty but easily visible (<25% leaf area).
III= Mildew growth medium (<50% leaf area).
IV= Mildew growth profused (<75% leaf area).

White root rot:- Seedling with and without mycobiont association were challenge inoculated with white root rot fungus (Dematophora necatrix) at variable disease pressure. The disease gradient was created by varying the inoculum as 25, 50, 100 and 200 wheat grain inocula per pot. Uninoculated pots served as check. Observations were recorded on disease initiation and successive development.

Statistical analysis:- The data obtained were statistically analysed. To see the significance of differences between means of different parameters, F-Values were used. The statistical analysis was carried out with the help of computers (Pentium-133).