# Chapter: 3

## 3.0: Micropropagation of Lemon

### 3.1A: Micropropagation

Clonal propagation through tissue culture has the potential to provide high multiplication rates of uniform genotypes, resulting in short term gains (Gupta *et al*., 1993). Cloning allows for the immediate and total capture of genetic gain. Clonal propagation through tissue culture popularly called micropropagation can be achieved in short time and space. Micropropagation technique would play an important role in mass propagation of elite genotypes, conservation of germplasm, genetic improvement of plants and production of pharmaceuticals yet it has not replaced traditional methods of propagation, but has found its own niche in areas where it is clearly superior. Micro propagation involves following methods:

1. Establishment
2. Multiplication
3. Rooting
4. Hardening

**Establishment:**

Micropropagation begins with the selection of plant material to be propagated. Clean stock materials that are free of viruses and fungi are important in the production of the healthiest plants. Once the plant material is chosen for culture, the collection of explant(s) begins and is dependent on the type of tissue to be used; including stem tips, anthers, petals, pollen and others plant tissues. The explant material is then surface sterilized with alcohol washes and finally rinsed in sterilized water. This small portion of plant tissue, sometimes only a single cell, is placed on a growth medium, typically containing sucrose as an energy source and one or more plant growth regulators (plant hormones). Usually the medium is thickened with agar to create a gel which supports the explant during growth.

**Multiplication:**

Multiplication is the use of tissue samples produced during the first stage and increasing their number. Following the successful introduction and growth of plant tissue, the establishment stage is followed by multiplication. Through repeated cycles of this process, a single explant sample may be increased from one to hundreds or thousands of plants. Depending on the type of tissue grown, multiplication can involve different methods and media. If the plant material grown is callus tissue, it can be placed in a blender and cut into smaller pieces and recultured on the same type of culture medium to grow more callus tissue. If the tissue is grown as small plants called plantlets, hormones are often added that cause the plantlets to produce many small offshoots that can be removed and recultured.

**Rooting:**

This stage involves treating the plantlets/shoots produced to encourage the root growth. It is performed *in vitro*, or in a sterile test tube environment. Hardening refers to the preparation of the plants for a natural growth environment. Until this stage, the plantlets have been grown in ideal conditions, designed to encourage rapid growth.

**Hardening:**

In the final stage of plant micropropagation, the plantlets are removed from the plant tissue culture media and transferred to soil or (more commonly) potting compost for continued growth by conventional methods. Hardening typically involves slowly weaning the plantlets from a high-humidity, low light, warm environment to what would be considered a normal growth environment.

### 3.1B: Advantages

- Micropropagation has a number of advantages over traditional plant propagation techniques:

- The main advantage of micropropagation is the production of many plants that are clones of each other.
- Micropropagation can be used to produce disease-free plants.
- Micropropagation produces rooted plantlets ready for growth, saving time for the grower when seeds or cuttings are slow to establish or grow.
- It can have an extraordinarily high fecundity rate, producing thousands of propagules while conventional techniques might only produce a fraction of this a number.
- It is useful in multiplying plants which produce seeds in uneconomical amounts, or when plants are sterile and do not produce viable seeds or when seed can't be stored (recalcitrant seeds).
- Micropropagation often produces more robust plants, leading to accelerated growth compared to similar plants produced by conventional methods - like seeds or cuttings.
- Some plants with very small seeds, including most orchids, are most reliably grown from seed.
- Some woody plants that are difficult-to-propagate by cuttings, layers or grafts can be micropropagated.

## 3.2 : Results and Discusssion

### 3.2A: Surface Sterilization:

Sterilization is an essential step before placing the explants on establishment medium. Various types of treatments have been used for the sterilization of the explants. Sterilizing agents such as calcium and sodium hypochlorite, hydrogen peroxide, bromine water, silver nitrate, mercuric

chloride and antibiotics at various concentrations and treatment duration are useful to disinfect plant tissue (Bhojwani & Razdan, 1996).

In the present work the ex-plants collected from elite lemon tree containing apical buds, axillary buds from young branches and axillary buds from mature branches were tretaed 30 second with 70% Iso propyl alcohol, explants were separately surface sterilized with $HgCl_2$ (0.1%) solution for 5-7 minutes depending upon the tenderness of the tissue, followed by several rinses with sterile water. The nodal or apical stem sections were trimmed to 0.5 cm size under aseptic conditions and inoculated into the culture medium.Similar reports were reported by Hassan et al., (2008) surface sterilized *Eclipata alba*, explants with 0.1 % mercuric chloride for 5 min. followed by five rinses with sterile double distilled water in front of a laminar air flow cabinet. Mohapatra and Rout, (2005) reported 0.1 % mercuric chloride solution for 15 min for surface sterilization of *Geoderum purpureum* explants. Among the several chemicals, $HgCl_2$ is considered as a potent surface-sterilizing agent; however, its residual inhibitory effect is also greater than the others commonly used in plant tissue culture (Bhojwani and Razdan, 1996). Datta *et al.* (2007) collected nodal explants (2-3 cm in length) from the seven-month-old donor plants of *J.curcas*, kept them for 3 h in a systemic fungicide, Bavistin (BASF India Ltd.) and surface-sterilized in 0.1% $HgCl_2$ (w/v) for 20–25 minutes followed by repeated washing with sterile distilled water for 5 min.

**3.2B: Explant Type**

The field-grown plants are sometimes unsuitable to initiate cultures. This stage involved in preparation of mother plants and type of explant should be taken so that improved quality explants can be obtained for establishment of culture. Many features of the explants are known to affect the efficiency of culture initiation. Generally, younger, more rapidly growing tissue (or tissue at an early stage of development) is most effective.

Micropropagation through apical and axillary shoot proliferation is the most common technique for commercial mass production. The ability to rapidly multiply shoot cultures *in vitro* is necessary for establishment of economically feasible micropropagation systems (Harry and Thorpe, 1994).

In the present study apical buds and Axillary buds explants from fresh sprouts of mature trees were used for culture initiation on Murashige and Skoog (1962) medium containing combinations of NAA (0.1 mg/l) with 6-benzyl aminopurine (1.0 mg/l).The results from the Table.3.1 reveals that axillary buds from mature branch proved to be effective as highest percent survival was obtained.. Rapid clonal multiplication / propagation from mature explants of tree legumes were achieved in *Leucaena leucocephala* (Dhawan and Bhojwani, 1985).

For initiation of culture stage, development of explants is an important factor for which selection of an explant in the optimal physiological and developmental state is the most important parameter governing the success of shoot formation. The age of the stock plant, the physiological age of the explants and its development stage, as well as its size can also determine the success of a procedure. Younger tissue, such as terminal or axillary shoot apices or tips of adventitious shoot regenerate better than older and more mature tissue of the same plant. Frequency of sprouting is always higher in the buds taken from plant during its vegetative phase. Das and Pal, (2005) used nodal stem segment of *Bambusa balcooa* and *Bambusa tuda* with an axillary bud at the centre and internodes on either side were excised and used as explants after removal of the leaf segment. Different plant tissue viz., intermodal segments (Sujatha *et al*., 1995), shoot tip (Islam *et al.*, 1993) and apical and axillary buds (Gupta *et al*., 1980, Rout and Das, 1993) have been taken as explants. Apical buds of 8–10 mm were excised from 5 days old *in vitro* seedlings. These were used as explants in *Psoralea corylifolia* (Baskaran & Jayabalan, 2008). Dubey *et al*., (2004)has taken apical buds of 3 to 5 year old plants and 30 to 32 year old trees of *Adina cordifolia* as explants source.

Apical and nodal segments from 3-year-old green house grown important medicinal plant *Cordia verbenacea* (Lameira and Pinto,2006) and young leaves of 40-year-old ornamental tree oleaster (*Elaeagnus angustifolia*) (Karami and Piri, 2009) were employed as explants for micropropagation. Nodal segments from plants of different age have been employed to initiate shoot bud cultures. It has also been reported that selection of explants, composition of nutrient media, concentration of phytohormones and methods used for micropropagation have significant effects on shoot multiplication rates, rooting and quality of produced trees (Chalupa, 2002) levels of morphogenesis than apical ends. Nodal buds were collected from 15 - 20 year old trees of *Holarrhena antidysenterica* for *in vitro* propagation (Mallikaarjuna and Rajendrudu, 2007).

**Table 3.1: Morphogenesis response of different ex-plants, sprouting and percentage survival rate after (15 days) interval of *Citrus aurantifolia*. (Each experiment has 3 replicates)**

| Types of Ex-plant | Bud Break after (15 days) | % survival rate | % survival rate after (30 days) | Average survival rate (%) |
|---|---|---|---|---|
| Apical buds | 7<br>6<br>6 | 70<br>60<br>60 | 70<br>50<br>60 | 61.66 |
| Axillary buds from young branch | 6<br>5<br>4 | 60<br>50<br>40 | 60<br>50<br>30 | 48.33 |
| Axillary buds from mature branch | 8<br>7<br>9 | 80<br>70<br>90 | 80<br>70<br>80 | 78.33 |

Note: Number of ex-plant used in each treatment =10

### 3.2C: Effect of BAP and NAA on shoot formation

The axillary bud explants were placed on MS medium fortified with BAP and NAA with different concentration it was observed that the lower concentrations BAP (0.1) and NAA (0.1) resulted in 100 % response Table.3.2/Figure 3.1, (Plate 3.1, C-D).

When shoots were multiplied on multiplication medium having different concentrations of BAP and NAA in MS medium it was observed that optimum multiplication of shoots was obtained with (1.0 mg/l BAP and 0.1 mg/l NAA)(Figure 3.1), (Plate 3.1 E-F). Enhancement in the induction of shoots by the synergy of BAP and auxins has been documented in *Stevia rebaudiana* (Sivaram and Mukundan, 2003), *Aloe vera* (Liao *et al*., 2004), *Murraya koeningii* (Rout, 2005) and *Euphorbia nivulia* (Martin *et al*., 2005). Pandey *et al*. (2006) reported that shoot multiplication from nodal explants of *T. arjuna* occurred on Murashige and Skoog (MS) medium containing different concentrations of 6-Benzyladenine (BA), Thidiazuron or Kinetin, or BA in combination with a-Naphthaleneacetic acid (NAA).

**Table 3.2: Effect of BAP and NAA on shoot formation from axillary buds after 8 weeks**

| Conc. of cytokinin and auxin(mg/l) | Average No. of shoot | Average shoot length (cm.) |
|---|---|---|
| BAP0+NAA0.1 | 0.3 | 0.03 |
| BAP0.1+NAA0.1 | 1 | 0.1 |
| BAP0.3+NAA0.1 | 1 | 0.5 |
| BAP0.5NAA0.1 | 4 | 0.66 |
| BAP0.8+NAA0.1 | 0.4 | 0.3 |
| BAP1+NAA0.1 | 6 | 0.9 |
| BAP1.5+NAA0.1 | 5 | 0.7 |
| BAP2+NAA0.1 | 4 | 0.5 |

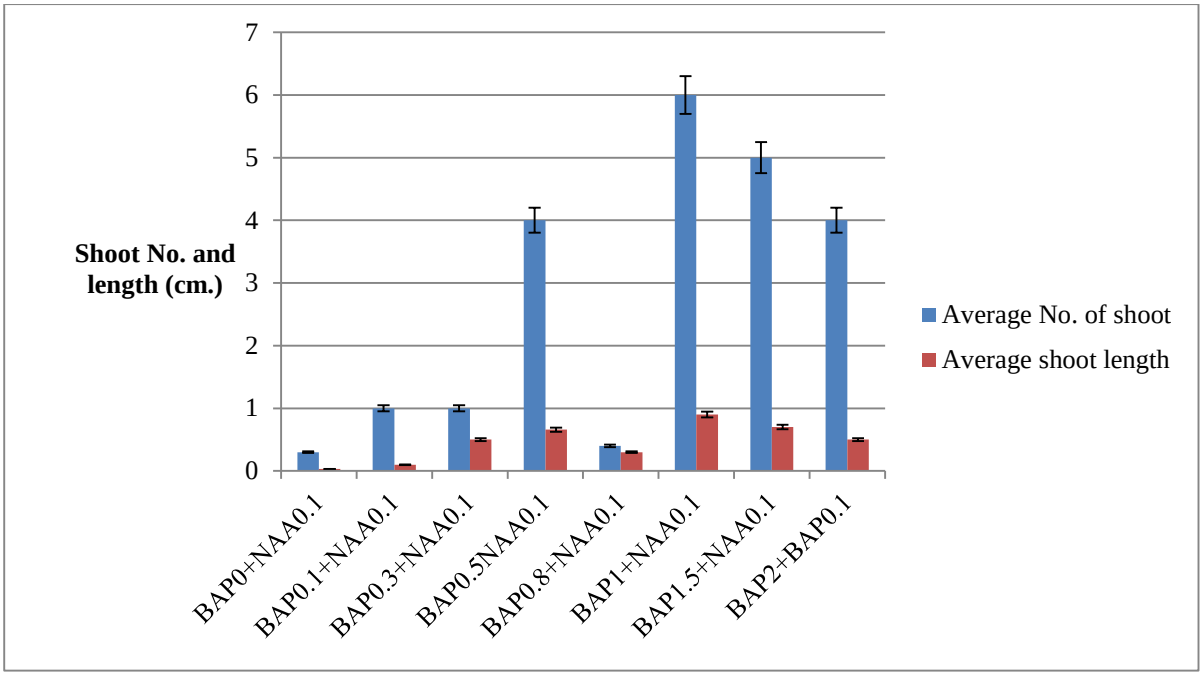



**Figure3.1: Effect of BAP and NAA on shoot formation from axillary buds after 8 weeks.**

### 3.2D: Effect of BAP and GA on shoot formation

In the present work the effect of Gibberllic acid on shoot multiplication was also evaluated for which GA was added with BAP. But not much improvement in terms of enhancement of number of shoots was obtained (Table 3.3).The highest number shoots were produced on medium containing 2 mg $l^{-1}$ BAP and 1or 2 mg $1^{-1}$ Gibberellin. Addition of Gibberellic acid (1.45 µM) to the same medium, promoted elongation of adventitious buds. Dubey *et. al*. (2004) reported *in vitro* plant regeneration from apical buds of *Adina cordifolia*.

**Table 3.3:Effect of BAP and GA on shoot multiplication after 8 weeks in MS Medium**

| Conc of BAP(mg/L) | Conc of GA(mg/L) | % Response | Average No. Of shoots (Mean ± S.E) |
|---|---|---|---|
| 0.5 | 0.2 | 40 | 0.8±0.3 |
| 1 | 0.2 | 50 | 1.0±0.2 |
| 2 | 0.2 | 63 | 1.8±0.7 |

### 3.2E: Effect of BAP , NAA and GA on shoot formation

The effect of GA along with BAP and NAA was also evaluated but only single shoots were obtained,there was not much enhancement observed in terms of shoot number(Table 3.4). In contrast the GA resulted in highest number of shoots in *Citrus limonia* (Eed *et al*. 2011).

**Table 3.4: Effect of BAP, NAA and GA on shoot multiplication after 8 weeks in MS Medium**

| Conc of cytokinin(mg/L) | Conc of auxin(mg/L) | Conc of GA(mg/L) | % Response | Average No. of shoots (Mean ± S.E) |
|---|---|---|---|---|
| BAP | NAA | GA | | |
| 0.5 | 0.1 | 0.2 | 40 | 0.5±0.5 |
| 0.1 | 0.1 | 0.2 | 50 | 1±0.5 |
| 2 | 0.1 | 0.2 | 63 | 1±0.3 |

### 3.2F: *In vitro* rooting

The elongated shoots developed (Plate 3.2, A-B) were transferred to rooting medium for induction of roots in presence of IBA along with BAP (Figure 3.2)/Table 3.5. In the present study ½ MS + BAP (1.0) +IBA(0.1) was optimum combination for root induction as highest number of roots was obtained with highest root length (Plate 3.2, C-D).BAP and IBA have been proved to be effective in *Citrus sinensis* and *C.limonia* as reported by de Almeida *et al.* (2002). Half MS medium has been found suitable for rooting in many species like *Cleistanthus collinus* (Quraishi *et al*., 1996; Quraishi and Mishra,1998), *Artocarpus heterophyllus* (Amin and Jaiswal,1993), *Withania somnifera* (Wadegaonkar *et al*., 2006) and *Rhodiola fastigiata* (Liu *et al*., 2006).

IBA is a most commonly used auxin for *in vitro* and *ex vitro* rooting of micro-cuttings (George, 1996). Generally an auxin is added in nutrient for inducing root in microshoot of hardwoods.

Indloe-3-butyric (IBA) was found more effective in root production compared to others (Hossain *et al*., 2003). Nearly 95% shoots developed roots on half strength MS medium supplemented with 10 µM IBA. Ravanfar *et al*. (2009) reported 100% rooting in shoots of *Brassica oleracea* placed on MS medium with 0.2 mgl-1 IBA.

**Table 3.5: Effect of different concentration of IBA on rooting with combination of BAP (0.1mg/l) of in-vitro regenerated *Citrus aurantifolia* roots in ½ MS medium.**

| BAP(mg/l) | IBAmg/l) | No. of roots/shoot | Root length/shoot(after 15 days)(cm) | Average root length/shoot (%) |
|---|---|---|---|---|
| 0.1 | 0 | 0.0±0.0 | 0.0±0.0 | 0 |
| 0.1 | 0.5 | 1.3±0.7 | 0.2 ±0.2 | 23.33 |
| 0.1 | 1 | 4.7±0.3 | 0.7±0.4 | 70 |
| 0.1 | 1.5 | 2.7±0.8 | 0.3±0.1 | 26.66 |
| 0.1 | 2 | 1.7±0.9 | 0.2±0.1 | 20 |

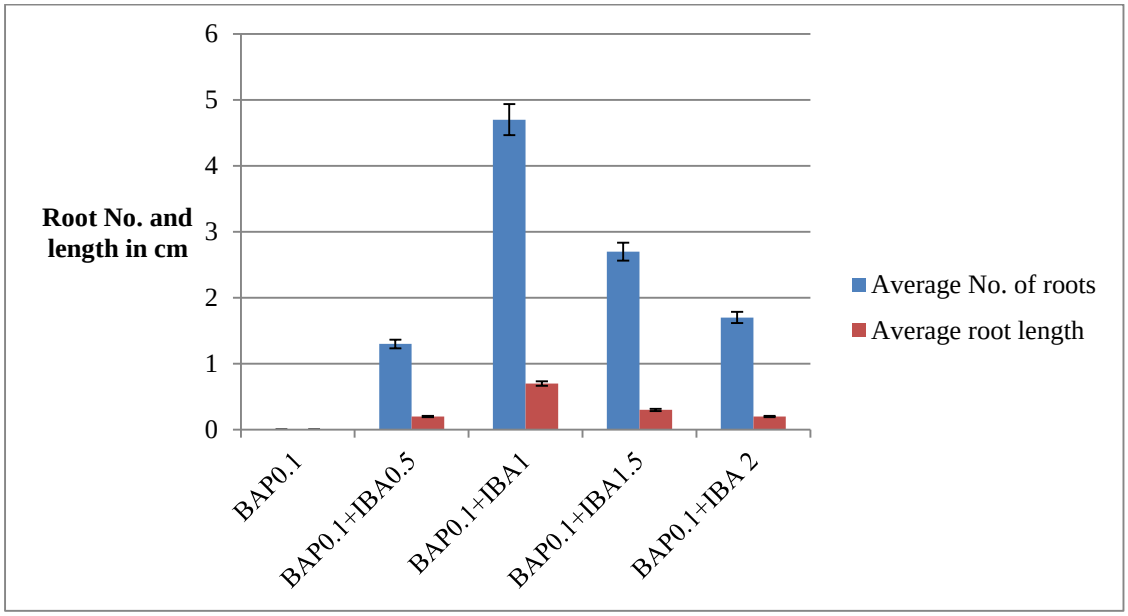



**Fig 3.2: Effect of different concentration of IBA on rooting with combination of BAP (0.1mg/l) of in-vitro regenerated *Citrus aurantifolia* roots in ½ MS medium.**

Das and Pal, 2005 for rooting, they tried half strength liquid MS media supplemented with 0.2 mg l-1 IBA. Isikalan *et al*.,(2008) obtained rooting in microshoots of *Amygdalus commiunis* cultured on half-MS medium supplemented with (8 mgl-1) IAA. The tissue culture plants acclimatized in a growth room were successfully transferred to the field. A dilute basal medium is generally used for rooting of microshoots. MS medium with half-strength of macronutrient has been used for rooting the microshoot of *Balanites aegyptiaca* (Siddique and Anis, 2009), *Mitragyna parvifolia* (Roy *et al*., 1988), *Dalbergia latifolia* (Rai andChandra, 1989). Purkayastha *et al*.,(2008) obtained rooting in 94% micro shoots of *Andrographis paniculata* cultured on MS medium supplemented with 2.5 μM IBA within a week.

**3.2G:Hardening**

Acclimatization phase is critical, because the plantlets undergo a great change in the form of nutrition and in environmental conditions. During this process plants have to adapt to the new environmental conditions such as high light intensity, lower relative humidity, fluctuation of temperatures and microbial attack (Preece and Sutter, 1991). *In vitro* raised plants develop under perfect aseptic condition and high relative humidity (50-70%). When these plantlets are transferred to the field, they sometime die. To overcomes this problem; special pretreatment is necessary to harden plantlets. A variety of potting mixture such as peat, perlite, polystyrene beads, vermiculite, fine bark, coarse sand or their mixture in different proportions are used for transplantation. Acclimatization of micropropagated plants on a large scale requires a polyethylene house or glasshouse where misting or fogging system maintains a gradation of high to low humidity. Amin *et al*.,(2003) transferred *Paederia foetida* plantlets to plastic pots maintained within a polythene tent for two weeks and then transferred the acclimatized plants to open environment where 80% of the plantlets survived. Ravanfar *et al*.,(2009) successfully acclimatized rooted plants of *Brassica oleracea* in potting medium containing peat moss, perlite, and vermiculite (3:1:1). Shukla *et al*.,(2009) transferred the rooted plantlets of

*Stereospermum personatum* to net pods containing mixture of sand: soil: farmyard manure (1:1:1) or coco- peat and placed in green house for primary hardening. After 30 days, survival percentage of tissue culture plants was 83.3 on coco-peat and 50.0 on mixture of sand: soil: farmyard manure.

In the present study plantlets with well developed root system, i.e. 70% of primary hardened-off and successfully acclimatized through these methods. Primary hardening was done in pro-trays in a mixture of soil: vermiculite (1:1) in poly tunnel kept in the green house. Secondary hardening was done in 50% shade net nursery by transferring plants in poly bags filled with soil: farmyard manure mixture (2:1). Total hardening cycle was 3 months - one month of primary hardening and two months of secondary hardening (Plate 3.2, E-G).

### 3.2H:Effect of AM fungi on seedling height and number of leaf in lemon

To see the effect of AM fungi,thelemon seeds were grown in normal soil used as control and lemon seeds were grown in sterilised soil with AM.The results on seedling height and number of leaves were recorded after 7 days and 15 days.(Fig.3.3)It was observed that the seedling height and number of leaves were more in seedlings containing AM after 15 days which proves AM fungi is beneficial for growth of plants (Plate 3.3, A-F).

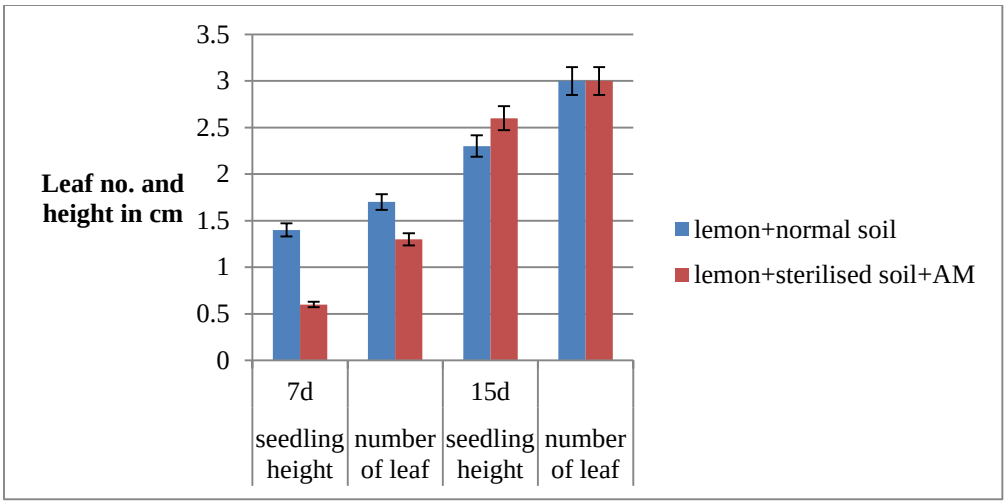
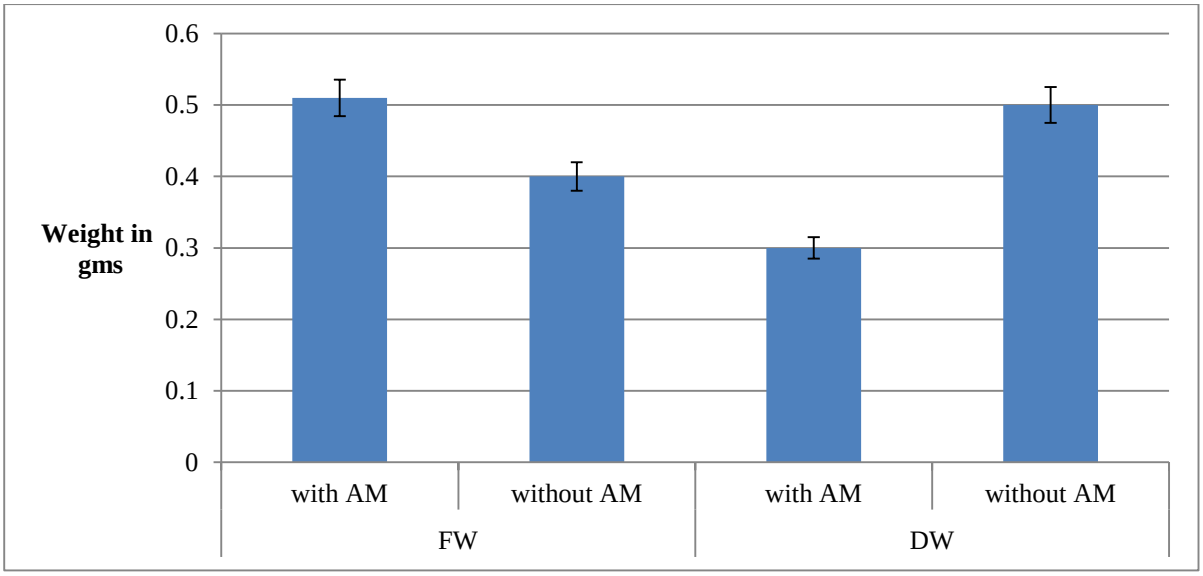



**Fig 3.3: Effect of AM fungi on seedling height and number of leaf in lemon**

### 3.2I:Effect of AM fungi on biomass of lemon plant

In this the lemon seeds were grown in pots with AM and without AM.After one month seedlings were taken for fresh weight and dry weight.For dry weight the seedlings were kept in hot air oven for 48 hour at 80ºC ± 5 ºC.The fresh weight and dry weight of leaf+stem and root with AM and without AM were taken in five samples .It was observed that the samples containing AM fungi obtained highest fresh weight and dry weight(Fig.3.4).This proves that AM fungi is beneficial for growth of plants.




**Note:**FW: Fresh weight, DW: Dry weight

**Fig 3.4:Effect of AM fungi on biomassof lemon plant**

# Plate:3.1


**Plate 3.1: A. Fruits of Lemon, B. Cut fruits of Lemon (Kagji Lime), C. Sprouted culture of Lemon, D, E and F. Multiplied culture of Lemon.**

# Plate : 3.2

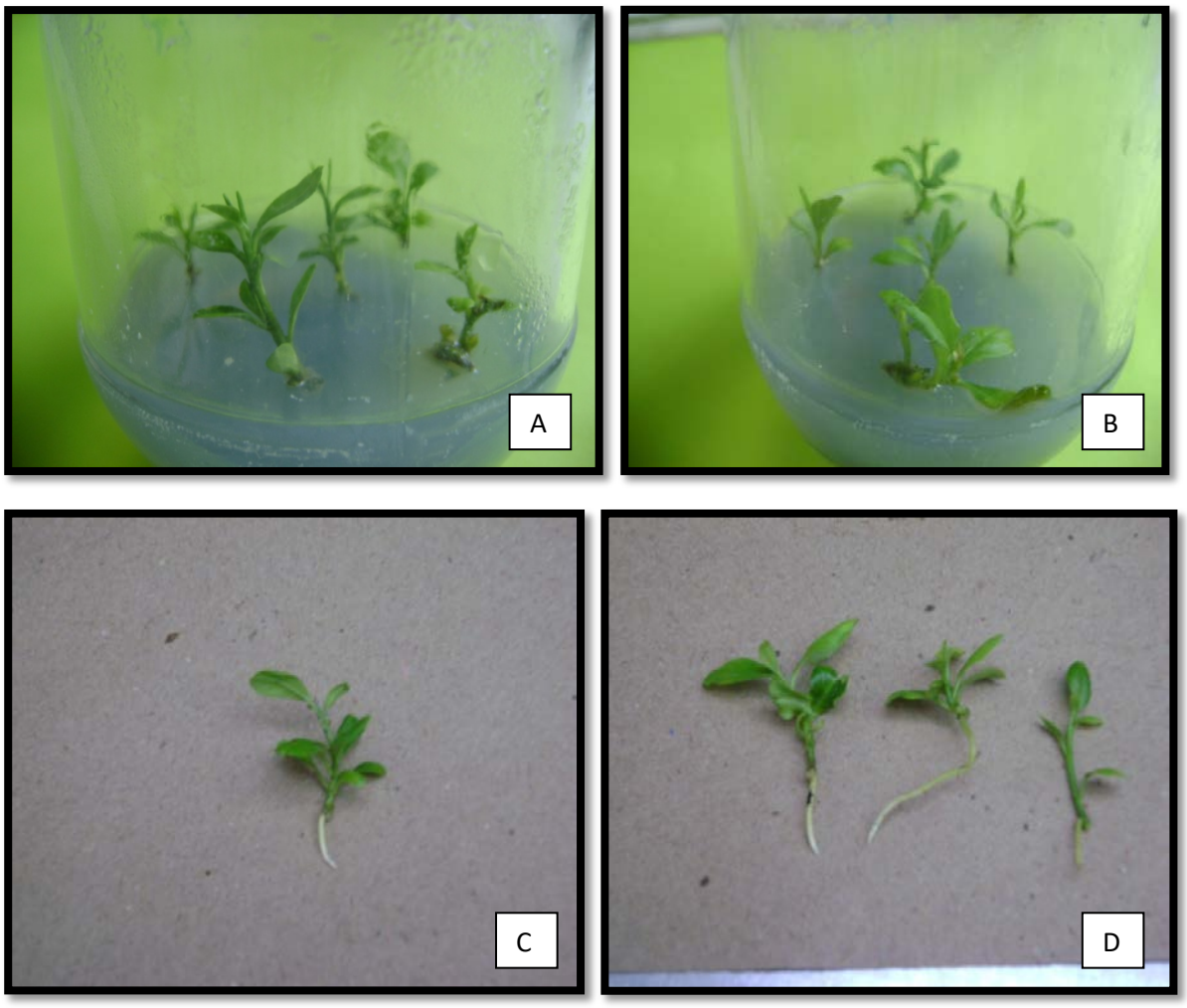



**Plate 3.2:Aand B. Elongated cultures of Lemon, C and D. Rooted cultures of Lemon,**

# Plate : 3.3


**Plate:3.3E and F. Lemon plantlets in poly tray for primary hardening, G. Secondary hardened plants of lemon in black polybeg, H. Numbers of secondary hardened plants of Lemon in tunnels.**

# Plate : 3.4

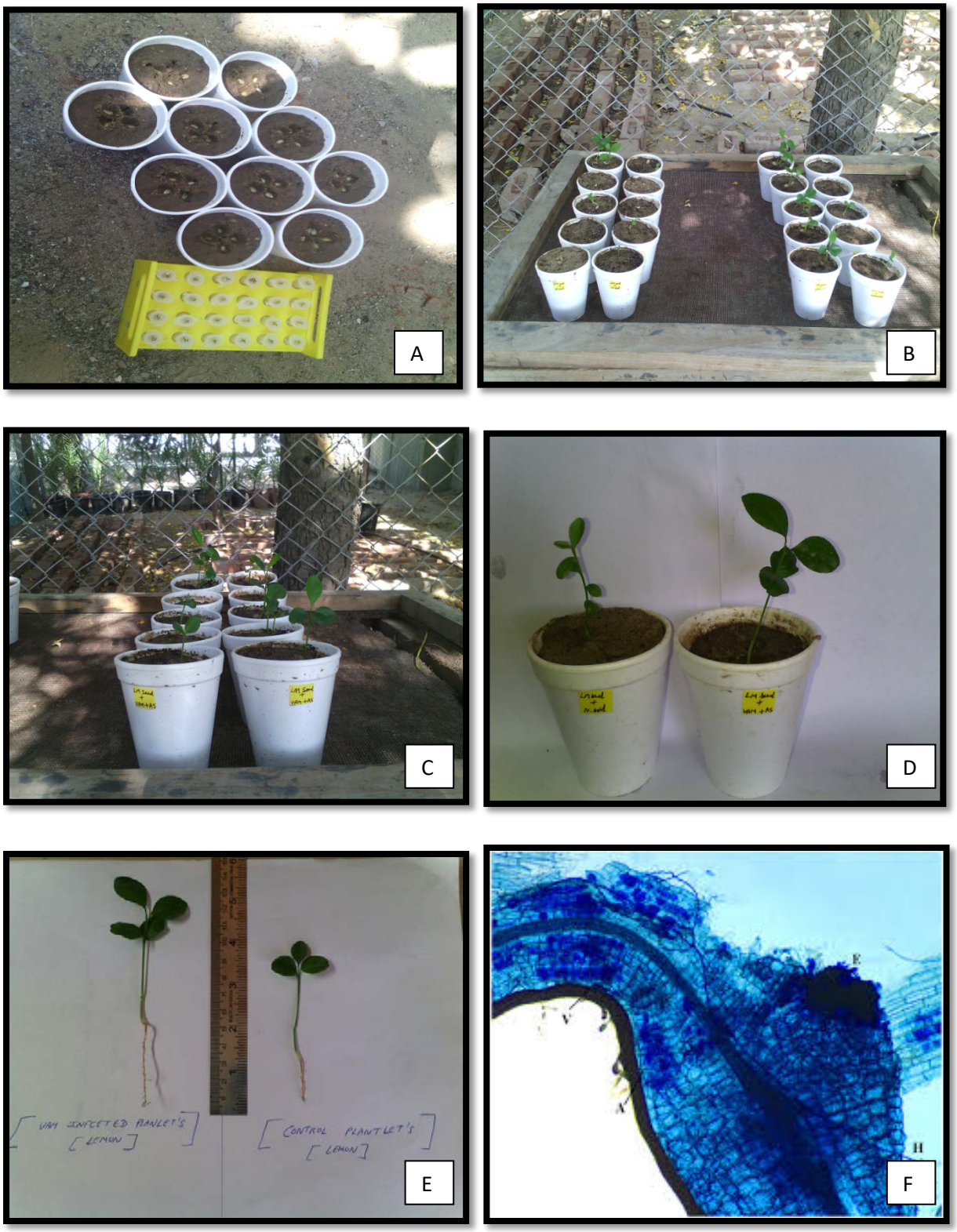



**Plate:3.3A. Posts having seeds with AM spores, B. Lemon seed germination with and without AM spores, C and D. Seedling of Lemon after 15 days, E. Rooted seedlings of lemon showing effect of plant growth after AM interaction, F. Section of lemon root showing presence of arbuscules.**