Chapter: 4

4.0: Micropropagation of Fig

Fig tree plantations cover 426.24 ha. area world wide with an overall yield of 1,070,676 million tones. The largest fig producing countries are Turkey, Egypt, Iran, Greece and Algeria (FAOSTAT 2006). The planting of species occurs predominantly with vegetative propagated plants especially by cuttings. Through micropropagation of fig tree it is possible to obtain pathogen free plantlets and this is one of the basic requirements for a successful commercial orchard. Furthermore, the technique has the advantage of large-scale production, providing plantlets whenever needed. Aside the advantages of micropropagation, *in vitro* culture has also supported the research on transgenic plants obtained by genetic transformation.

4.1A: Taxonomy

Family: Moraceae (Mulberry family) (Wagner *et al*. 1999).

Latin name: *Ficus carica* L.

Common names: Common fig, edible fig, fig tree (Bailey and Bailey 1976, Neal 1965).

4.1B: Description

Shrubs or small trees, deciduous, up to 5 m. Roots not adventitious. Bark grayish, slightly roughened. Branchlets pubescent. Leaves: stipules 1-1.2 cm; petiole 8-20 cm. Leaf blade obovate, nearly orbiculate, or ovate, palmately 3-5-lobed, 15-30 x 15-30 cm, base cordate, margins undulate or irregularly dentate, apex acute to obtuse; surfaces abaxially and adaxially scabrous-pubescent; basal veins 5 pairs; lateral veins irregularly spaced. Syconia solitary, sessile, green, yellow, or red-purple, pyriform, 5-8 cm, pubescent; peduncle ca. 1 cm; subtending bracts ovate, 1-2 mm; ostiole with 3 subtending bracts, umbonate (Flora of North America 2000).

4.1C: Cultivation

*F. carica*has been cultivated for a long time in various places worldwide for its edible fruit. Remnants of figs have been found in excavations of sites dating as far back as at least 5,000 B.C. (California Rare Fruit Growers, Inc. 1996). *F. carica*is presumed to be originated from Western Asia and spread to the Mediterranean by humans (California Rare Fruit Growers, Inc. 1996). It is an important world crop today. It grows in places with typically mild winters and hot dry summers (Tous and Ferguson 1996). Fruits can be eaten raw, dried, canned, or in other preserved forms (Neal 1965).

4.1D: Pests and diseases

California Rare Fruit Growers, Inc. (1996) reported following pests and diseases for *F. carica*. "Fig tree roots are a favorite food of gophers, who can easily kill a large plant. Birds can cause a lot of damage to the fruit. Nematodes, particularly in sandy soils, attack roots, forming galls and stunting of the trees. Dried fruit beetles can enter ripen fruits through the eye and cause damage by introducing fungi and rots. Euryphid mites are carriers of mosaic virus from infected to clean trees. Other pests include fig canker, *Rhizopus* rot, and *Aspergillus* rot."

4.1E: Distribution

Native range: *F. carica* is thought to be native to Western Asia (California Rare Fruit Growers, Inc. 1996). *F. carica*is cultivated for its fruits in warm and temperate parts of Europe, the Mediterranean countries, and the United States of America. According to Dehgan (1998), *F. carica*prefers full sun and various well-drained soils. It is slightly salt tolerant, though is not adapted to wet sites or compacted soils, and it is moderately drought tolerant. In most Mediterranean countries it grows wild (Grieve 2002). In the United States, *F. carica*is considered as an invasive plant species (California Exotic Pest Plant Council, 1999).

4.1F: Micropropagation

In vitro micropropagation is a complicated process requiring many steps or stages Murashige (1978), proposed four distinct stages that can be adopted for overall production technology of clones commercially. Stages I-III are followed under *in vitro* conditions where as stage IV is accomplished in greenhouse condition. Debergh and Maene (1981) suggested an additional stage O for various micropropagation systems. Establishment of a reproducible system with well characterised stages is a pre-requisite for promotion of projection targets and schedule in the commercial of plants.

1**. Stage O**

This is initial step of micro propagation in which stock plants used for culture initiation are grown for at least 3 months under carefully monitored conditions. Stock plants are grown at a relatively low humidity and watered either with irrigation tubes or by capillary sand beds or mats. This stock plant preconditioning stage also includes measures to be adopted for reduction of surface and systemic microbial contaminants.

2. Stage 1

Murashige (1962) defined this stage as the initiation and establishment of aseptic cultures. The main steps involved are preparation of the explant using followed by the establishment on a suitable culture medium. Cultures are initiated from explants several organs but shoot tips and auxillary buds are most often used for commercial micro propagation. Procedures to surface sterilise the explant and induce a healthy growth in the culture medium may be defined for each species. It may also be advisable to control microbial contaminantion within explant tissues in

case such efforts at stage O were not successful. Stage I lasts 3 months to 2 years and requires atleast four passages of the subculture.

Usually explants carrying a priformed vegetative bud are suitable for enhanced auxillary branching. When objective is to produce virus free plants from an infected individual, it becomes obligatory to use cultures derived from submillimetre shoot tips. If stock plants are tested virus-free, the most suitable explants are nodal cuttings. These are some advantages in using small sized explants for micro propagation. Small shoot-tip explants have low survival rate and show slow initial growth. Meristem- tip cultures may also result in the loss of certain horticultural traits exhibited by the presence of virus. Therefore sub-terminal or slightly older segments are desirable which can withstand the toxic effects of sterilization agents much better than the terminal cuttings. For rhizomatic plants, runner tips are commonly used.

3. Step I

This stage takes up the bulk of micropropagation activity using a defined culture medium that stimulates maximum proliferation of regenerated shoots. Various approaches followed for micro propagation include:

a) Multiplication through the growth and proliferations of meristems excised from apical and axillary shoots of the parent plant.

b) Induction and multiplication of adventitious meristems through a process of organogenesis or somatic embryogenesis directly on explants.

c) Multiplication of calli derived from organs, tissues, cell or protoplasts and their subsequent expression of either organogenesis or somatic embryogenesis in serial subculture. Shoots obtained from these calli can be further multiplied.

Passage or harvest cycle generally requires 4 weeks. Shoots are harvested from the multiplying culture to either be sold as a Stage II product or carried onto Stage III. Generally stage II lasts to 10-36 months with large number of subcultures of similar age.

4. Stage III

Shoots proliferated during stage II are transferred to a rooting medium. Sometimes shoots are directly established in the soil as micro cuttings to develop roots. Since such a possibility depends on the particular species and at present, a large number of species cannot be handled in this manner. The shoots are generally rooted in vitro. When the shoots or plantlets are prepared for soil, it may be necessary to evaluate the survival factors such as i) Dividing the shoots and rooting individually ii) Hardening the shoots to increase their resistances to moisture stress and diseases. iii) Rendering the plants capablr of autotrophic development in contrast to the heterotropic state induced by culture and iv) Fulfilling requirement of breaking dormancy, especially of bulb crops. Stage III requires 1-6 weeks.

5. Stage IV

Steps taken to ensure successful transfer of the plantlets of Stage III from the aseptic environment of the laboratory to the environment of greenhouse comprise stage IV. Unrooted stage II shoots are also acclimatised in Suitable compost mixture or soil in pots under control conditions of light, temperature and humidity inside the greenhouse. In such cases stage III is skipped. Supplying bottom head-aids to pots with plantlets or cuttings and maintenance of a dense fine- particle fog system, within the greenhouse enhances the rooting process. Complete plants can also be established in the artificial growing media such as soilless mixes, rockwood plugs or even sponges. It takes 4-16 weeks for the finished product to be ready for sale or shipment.

4.2: Results and Discussion

4.2A: Surface Sterilization

One or two days prior to collection of explants, selected mother plants were sprayed with fungicide solution (1gm/l of Carbendazim). Collected explants (axillary buds), (Plate 4.1, A-D) were washed thoroughly under running tap water for 30 min., followed by immersion in 1% sodium hypochlorite solution containing APSA 2-3 drops(Amway product, USA) for 20 minutes. After rinsing with water, treatment with a mixture of fungicide and bactericide, i.e. Bavistin 1mg/l and Streptomycin 40 mg/l was given for 45 min. Explants were surface sterilized with 0.1 % $HgCl_2$for 5 minutes and washed several times with sterile water. Final immersion was done in solution containing 150mg/l L-ascorbic acid and 100mg/l citric acid.

4.2B: Effect of individual cytokinin

When axillary bud explants were inoculated in presence of individual cytokinin in MS and WPM medium the MS medium + BAP (0.5mg/l) resulted in highest response with highest number of shoots (Table 4.1). Rashid *et al*. (2009) reported multiple shoot induction from shoot tip and nodal segments of *Scopariadulcis*that were cultured on MS medium supplemented with different concentrations of cytokinins. They found highest number of shoots in nodal segments cultured on MS medium supplemented with 1.0 mg/lBAP.

Table 4.1: Effect of individual cytokinin on shoot bud initiation from axillary bud explants after 4 weeks in MS Medium

Conc. of Cytokinin (mg/l)		**% Response**	**Average No. of shoots (Mean ± S.E)**
BA	**Kn**		
0	-	38	0.4± 0.2
0.5	-	88	2.2±0.2
1	-	75	1.6±0.6
-	0	25	0.3±0.2
-	0.5	50	1.1±0.5
-	1	75	1.3±0.4

Values represent mean ± S.E of each experiment consist of eight replicates conducted twice. Data were analysed using one way ANOVA and were significant at 0.05 level of significance ($P<0.05$)

Table 4.2: Effect of individual cytokinin on shoot bud initiation from axillary bud explants after 4 weeks in WPM Medium

Conc. of Cytokinin (mg/l)		% Response	Average No. of shoots (Mean ± S.E)
BA	**Kn**		
0	-	40	0.4± 0.2
0.5	-	50	1.2±0.2
1	-	75	2.0±0.6
-	0	50	0.5±0.2
-	0.5	50	0.5±0.5
-	1	60	1.6±0.4

Values represent mean ± S.E of each experiment consist of eight replicates conducted twice. Data were analysed using one way ANOVA and were significant at 0.05 level of significance ($P<0.05$)

Since MS medium was proved to be effective in presence of individual cytokines as compared to WPM medium hence MS medium was used for further experiments. The MS salts have been employed for culture of several more species such as *Jatropha curcas* (Misra *et al*. 2010), *Punica granatum* (Kanwar *et al*, 2010), *Eucalyptus tereticornis* (Aggarwal *et al*. 2010), *Pueraria tuberose* (Rathore and Shekhawat, 2009), *Pistacia vara* (Onay, 2000), *Swertia chirata* (Wawrosch *et al.* 1999) etc.

4.2C: Effect of BAP and NAA on shoot formation

In present work nodal segments containing axillary buds from a mature Fig tree, were induced to produce large number of multiple shoots by culturing on MS medium with different

concentrations of 6-benzyl amino purine (BAP) in combination with naphthalene acetic acid (NAA) was used.

For induction of multiple shoots, explants were implanted on MS medium supplemented with BAP (1-5 mg/l) and NAA (0.1 to 0.5 mg/l). Cultures were incubated at 25± 1 ˚C under 14 hours photoperiod by cool white fluorescent tubes. Then *in-vitro* regenerated shoots were subcultured every 2 weeks on fresh medium.

Table 4.3: Morphogenesis response of sprouting and percentage survival rate of *Ficus carica* grown MS media supplemented with different concentration of BAP with 0.1mg/l NAA. (Values determined after four weeks from three individual experiments).

S.No.	BAP(mg/l)	NAA(mg/l)	Bud Break after 15 days intervel (% Survival)	Average no. Of shoot/ex-plant	Average shoot length ex-plant (cm)
1	1.0	0.1	45	3±0.2	0.5±0.1
2	2.0	0.1	75	5±0.3	0.8±0.2
3	3.0	0.1	55	4±0.3	1.3±0.5
4	4.0	0.1	45	3±0.1	1.2±0.3
5	5.0	0.1	35	3±0.5	0.9±0.5

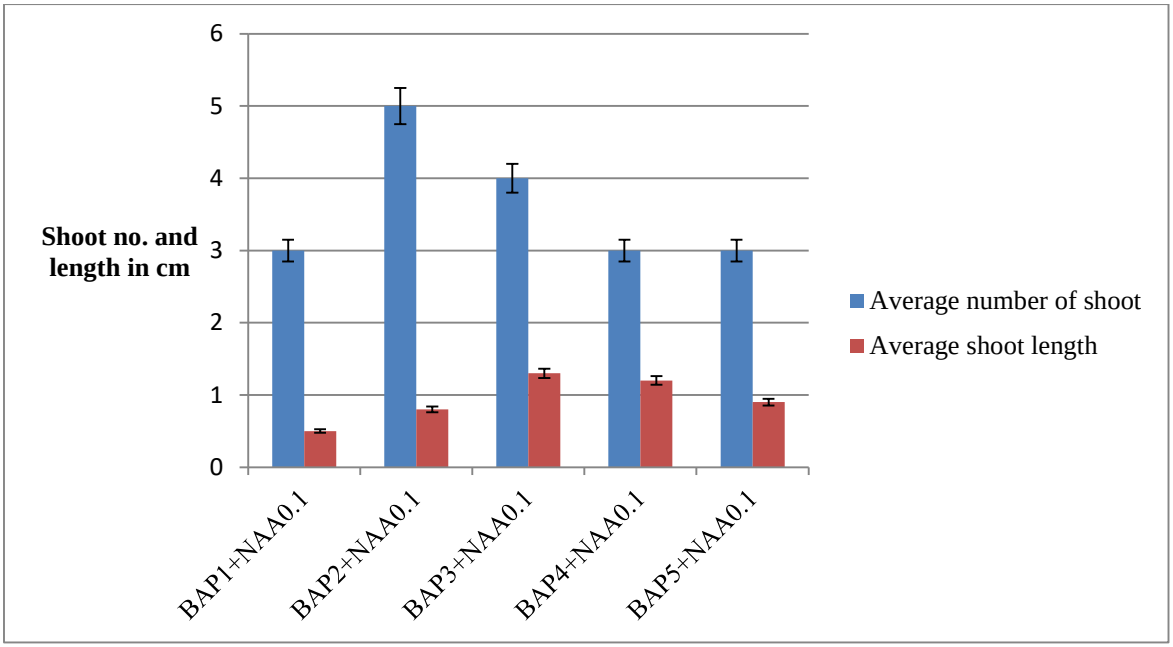

Figure 4.1: Morphogenesis response of sprouting and percentage survival rate of *Ficuscarica* grown MS media supplemented with different concentration of BAP with 0.1mg/l NAA.

- On medium supplemented with 2 mg/l BAP with 0.1 mg/l NAA(Table 4.3)/Figure.4.1, axillary buds started sprouting from second week onwards (Plate 4.2, A-B). An average of 5 shoots developed from each axillary buds which was optimal among all the concentrations (Plate 4.2, C-E). Lower or higher levels 1, 3 and5 mg/l of BAP, the number of shoots was less formed.Rout *et al.*,(2001) obtained clonal propagation from nodal explants of *Paulownia tomentosa*by manipulating the cytokinin and auxin. Inclusion of NAA (0.53-1.34 µM) in the culture medium enhanced the rate of multiplication. The shoot length was attained 3-4 cm on MS medium supplemented with 4.44 µM BA + 0.53 µM NAA + 3% (w/v) sucrose, within 4 weeks of culture. Mansor *et al.* (2003) developed procedures for micropropagation of *Balanitiesaegyptiaca*using axillary bud explants obtained from mature trees. Cultures were established in Murashige and Skoog (MS) medium supplemented with 2.5 mg/l (BAP) and 0.1 mg/l (NAA).

4.2D: Effect of TDZ and IBAin shoot formation

The axillary bud explants were placed on MS medium fortified with TDZ and IBA, observations revealed that only single shoots were obtained (Table.4.4). The results were in contrast as reported by Murthy *et al*. (1998) where TDZ at lower concentration favored high frequency shoot regeneration.

Table 4.4: Effect of TDZ and IBA on shoot formation from axillary bud explants after 4 weeks in MS Medium

Conc. of Cytokinin (mg/l)	Conc. of Auxin (mg/l)	% Response	Average No. of shoots (Mean ± S.E)
TDZ	IBA		
0.1	1	38	0.6±0.3
1	1.5	38	0.4±0.2
1.5	2	25	0.3±0.2

4.2E: Effect of BAP and GA on shoot multiplication

BAP and GA had been given results in *Ficus carica* as reported by Hepaksoy and Aksoy (2006). Hence in the present studies it was taken to see its effect on shoot multiplication.It was observed that BAP (10mg/l)+GA(0.3mg/l) resulted in highest number of shoots with 88 % response(Table.4.5), (Plate 4.3, A-D).

Table 4.5: Effect of BAP and GA on shoot multiplication from axillary bud explants after 8 weeks in MS Medium.

Conc. of Cytokinin (mg/l)	Conc. of GA (mg/l)	% Response	Average No. of shoots (Mean ± S.E)
BAP	GA		
1	0.1	63	1.9±0.7
2	0.2	75	1.8±0.5
2.5	0.25	75	1.9±0.6
5	0.3	63	2.1±0.8
7.5	0.3	50	0.9±0.4
10	0.3	88	2.8±0.8

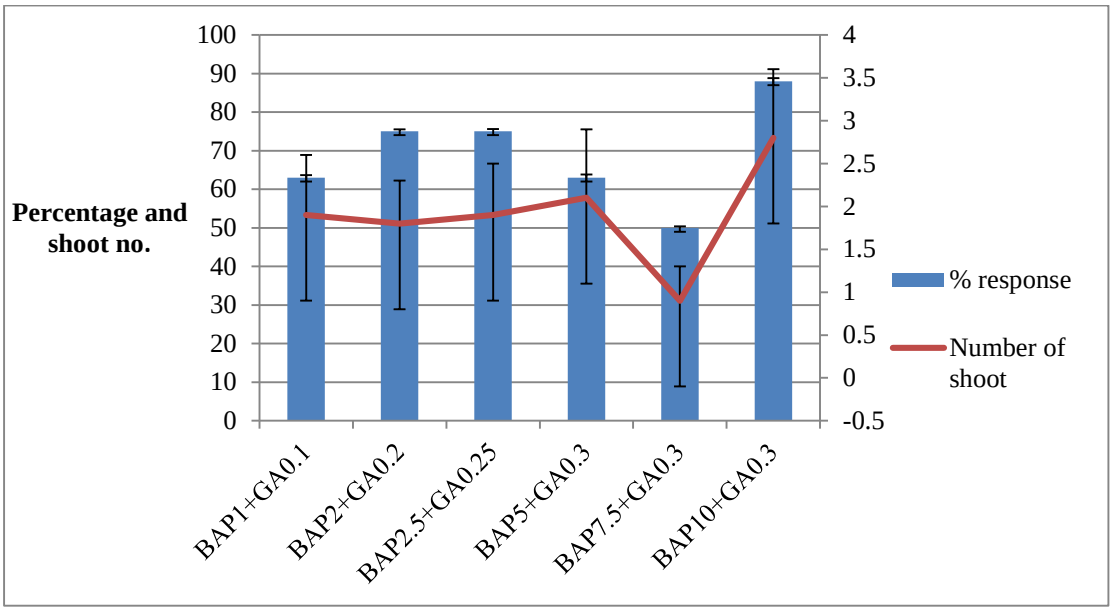

Figure4.2 : Effect of BAP and GA on shoot multiplication from axillary bud explants after 8 weeks in MS Medium.

4.2F: Effect of BAP, NAA and GA on shoot formation

The GA was added along with BAP and NAA to see its effect on shoot multiplication.The highest percent response was obtained in BAP (0.2)+ NAA(0.2)+GA(0.2) with highest number of shoots(Table .4.6).

Table 4.6: Effect of BAP, NAA and GA on shoot multiplication from axillary bud explants 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		
0.2	0.2	0.1	88	2.6±0.6
0.2	0.2	0.2	63	1.6±0.6

4.2G: Rooting of shoots

After 5 subculture of multiple shoot cultures, all cultured transferred in rooting medium for initiation of roots.

In 4 weeks, roots induction and elongation were observed, average root induction occurred in 3 weeks and root length.The highest root length was observed in half strength MS basal salt concentrations(Table 4.7), (Plate 4.4, A). It was observed in Jack fruit that application of auxin , was essential for adventitious root formation (Roy *et al*., 1990).

Table4.7: Effect of Basal Salt Concentrationson *in vitro* plants.

MS Salt Concentration	Average shoot/culture	Shoot Length (cm)	% Plant showing Roots	Average roots/plant	Leaves
½ MS Salt	1.6 ±0.2	0.8±0.1	66	1.8±0.2	Normal
Full MS Salt	1.66±0.2	0.75±0.1	46	1.2±0.1	Normal

Note: 1. All media contain 2mg/l IBA.

2. Number of cultures inoculated 175 to 250

In the present work roots showed rapid elongation on transffered to ½ MS medium with 2 mg/l IBA with 0.25 mg/l activated charcoal (Table.4.8). Cheruvathur *et al*., (2010) reported highest frequency of rooting (96%) and mean number of roots per shoot (3.3) in microshoots of *Malaxis acuminata* on MS medium supplemented with 4.0 mgl-1 IBA and 1.5 mgl-1 activated charcoal.The initiation of root development in in-vitro regenerated individual shoots was achieved with 85% success after 4 weeks on ½ MS medium with 2 mg/l IBA with activated charcoal (0.25 mg/l).They observed when basal region of shoot explants were dipped in IBA solution for 30 min and inoculated on MS basal medium with 0.25% activated charcoal, gave 100% rooting response. Nath *et al*., (2005) reported in *Adhatoda vasica*, rooting of elongated

shoots was assessed by transferring them to full strength MS basal medium containing activated charcoal and 2-3 mg IBA. Activated charcoal when added to the culture medium was found to have a remarkable positive influence on the rooting efficiency.

Table 4.8: Effect of Activated Charcoal in full strength MS medium

Treatment	Average shoot/culture	Shoot Length (cm)	% Plant showing Roots	Average roots/plant	Leaves
Activated Charcoal	1.66	0.86	100	2.0	Normal
Without Activated Charcoal	1.6	0.8	46	1.8	Normal

The rooting experiments were also done with different combinations of IAA, IBA, NAA in full MS strength medium.It was observed that the highest number of roots was obtained in IBA 10 µM.Hussain *et al*. (2008) studied rooting in *Crotalaria verrucosa*. Hussain *et al*. (2008) transferred *in vitro* shoots of *Sterculia urens* to full strength, half strength and quarter strength MS medium, supplemented individually with IBA, IAA or NAA. Mitra and Pal, (2007) obtained 90% rooting from regenerated shoots of *Stevia rebaudiana*on half strength MS semi solid medium supplemented with 1.0 mgl-1 IAA within 4 weeks.Hossain *et al*. (2003) used MS medium at half strength with IBA, IAA, or NAA for root induction in *Zyziphus jujuba*. They found that IBA was more effective in root production compared to others. In *Zehneria scabra* (Anand and Jeyachandran, 2004) found MS medium for rooting on in various subculture.

Thomas and Philip, (2005) when transferred multiple shoots of *Tylophora indica* to the half MS medium supplemented with IBA, NAA or IAA, the optimum rooting response was observed in the presence of IBA. Rashid *et al*. (2009) revealed that highest number of roots per shoot in *Scopariadulcis*in half MS medium having 0.5 mg/lNAA followed by 1.0 mg/lIBA; on the other

hand, the lowest number of roots was observed in the full MS medium supplemented with 1.0 mg/lNAA.

Table 4.9: Effect of Auxins (IAA, IBA, NAA) in full strength MS medium for root induction.

	Auxin Concentration	No.of roots/culture	Root length (cm)	% rooting	Leaves
IAA	5 µM 10 µM 15 µM	3 2 1	1.5 1.0 0.8	15.62 6.85 7.81	Normal
IBA	5 µM 10 µM 15 µM	5 8 4	0.7 1.3 1.0	21.4 9.84 5.07	Normal
NAA	5 µM 10 µM 15 µM	2 2 1	0.5 0.7 0.9	16.66 4.03 1.56	Normal

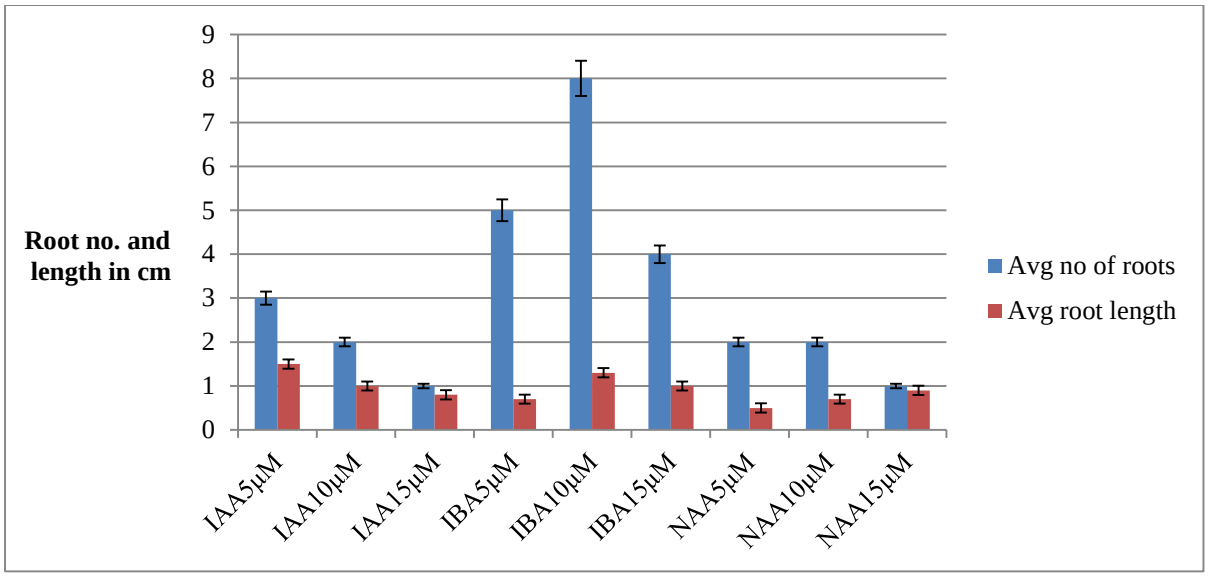

Figure 4.3: Effect of Auxins (IAA, IBA, NAA) in full strength MS medium for root induction

4.3:Transfer of Plantlets to the Green house

Plantlets with well developed root systems, transffered for primary hardening in different combination of Cocopeat, Soil, Vermiculate and Sand. Primary hardening experiment were done in plastic poly tray and maintained humidity 80% by covering plastic sheet on the tray. In this experiment got best result by using cocopeatand 25 % sand with 78.57% success. (Table 4.10). After 4 weeks interval healthy primary hardened plantlets (plugs),transffered for secondary hardening in 50% shade net house (Plate 4.4B).

Table 4.10: Use of different substrates for Primary Hardening Experiments:

Sr. No	Potting Mixture	Inoculate plantlets	After 15 days	After 21 days	After 29 days	Survival Rate %
1	Coco peat	28	7	3	18	64.28
2	Coco peat + 25% Vermiculate	28	8	4	16	57.14
3	Coco peat + 25% Vermicompost	28	12	5	11	39.28
4	Coco peat + 25% Sand	28	4	2	22	78.57

Shukla *et al*. (2007) acclimatized tissue culture plantlets of *Curcuma angustifolia* on net pots containing coco- peat substrate and obtained healthy plants. Davood *et al*. (2008) found the rooted plants of *Aloe vera* were gradually acclimatized in plastic pots containing a mixture of cocopeat and perlite (1:1) covered with transparent plastic. About 95% of the transplanted plantlets survived. Park *et al*. (2009) found rooted plants of *Rehmannia glutinosa* were hardened and transferred to soil with a 73% survival rate. Yusuf *et al*. (2005) reported that the plantlets were hardened and acclimatized by transferring to autoclaved soilrite moistened with one fourth strength liquid MS mineral salts in glass bottles and then to soil mixture containing sandy soil, black soil and pond soil (1:1:1 v/v) in *Anogeissus sericea*. To acclimatize the plantlets of

Artemisia absinthium, Zia et al.,(2007) transferred *in vitro* rooted plants to pots filled with soil and peat moss (3:1) under high humid condition until maturation of leaves and then transferred to green house.

4.4: Secondary Hardening

In secondary hardening used potting mixture of Cocopeat: sand: soil (1:1:1).In this experiments got 82.08% survival rate of primary hardened plants. Fully hardened plants were ready for field planting after two months. The regenerated plants did not show any detectable variation in growth as morphological characteristics of leaves and fruits when compared with donor plants.Due to constraints in conventional propagation methods in *Ficuscarica*, there is a shortage of planting stock for commercial propagation. This could be overcome by following the protocol developed in the present study (Plate 4.4, C-D).

Potting mixture	**Total transferred plantlets**	**Mortality rate**	**Total survived plants**	**% survival**
Cocopeat:sand:soil	67	12	55	82

Plate :4.1

Plate4.1 : A. and C. Ripe fruits of fig obtained from an orchard in Pune , B. Cut fruits of Fig (Poona red), D. Fig plantation at Saswad, Pune.

Plate :4.2

Plate 4.2: A. Axillary buds of Fig plant, B. Apical buds of Fig plant, C. Leaf disc of Fig plant, D. axillary and apical buds of fig plant.

Plate :4.3

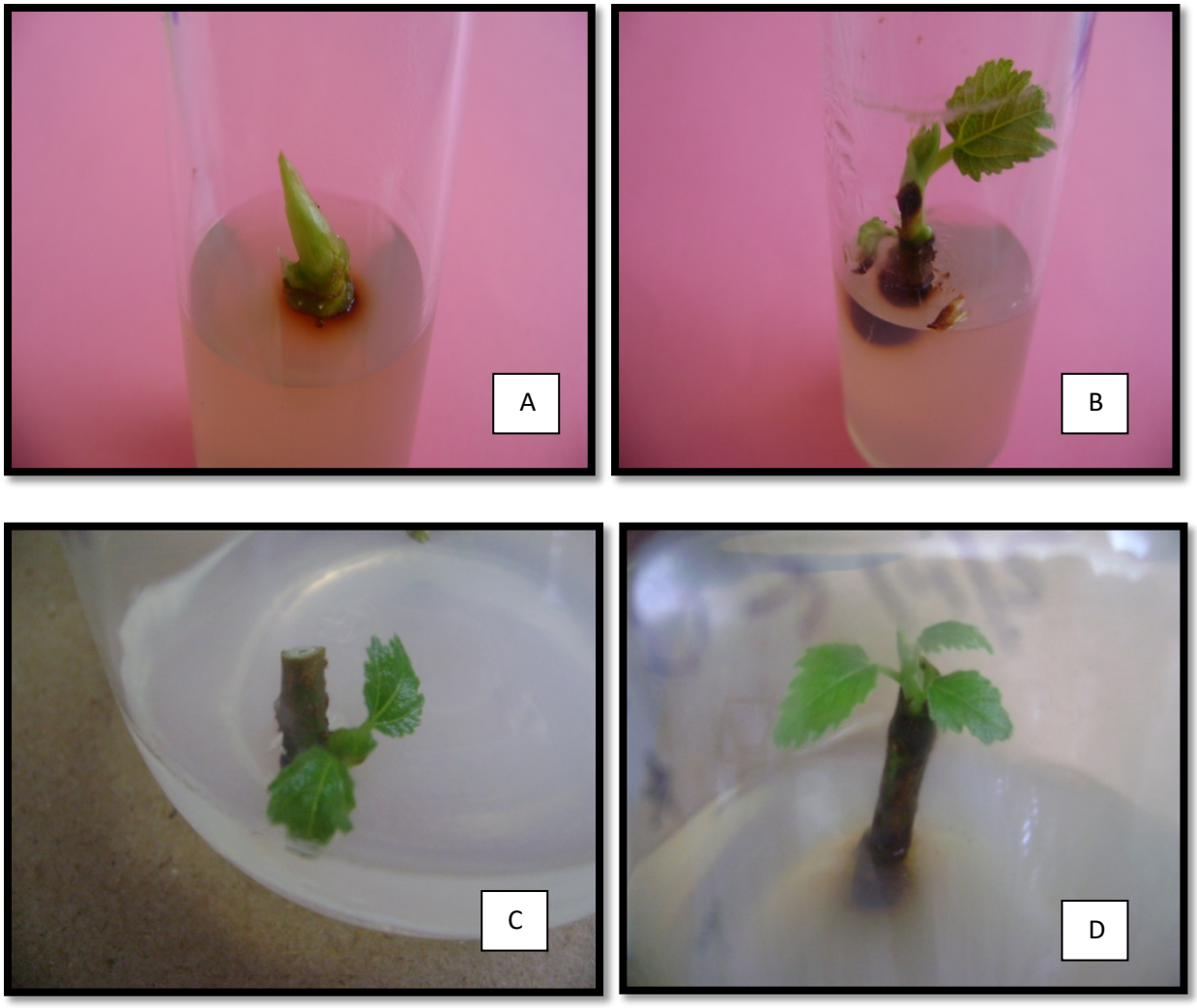

Plate4.3 : A,B,C and showing sprouted explants of Fig.

Plate:4.4

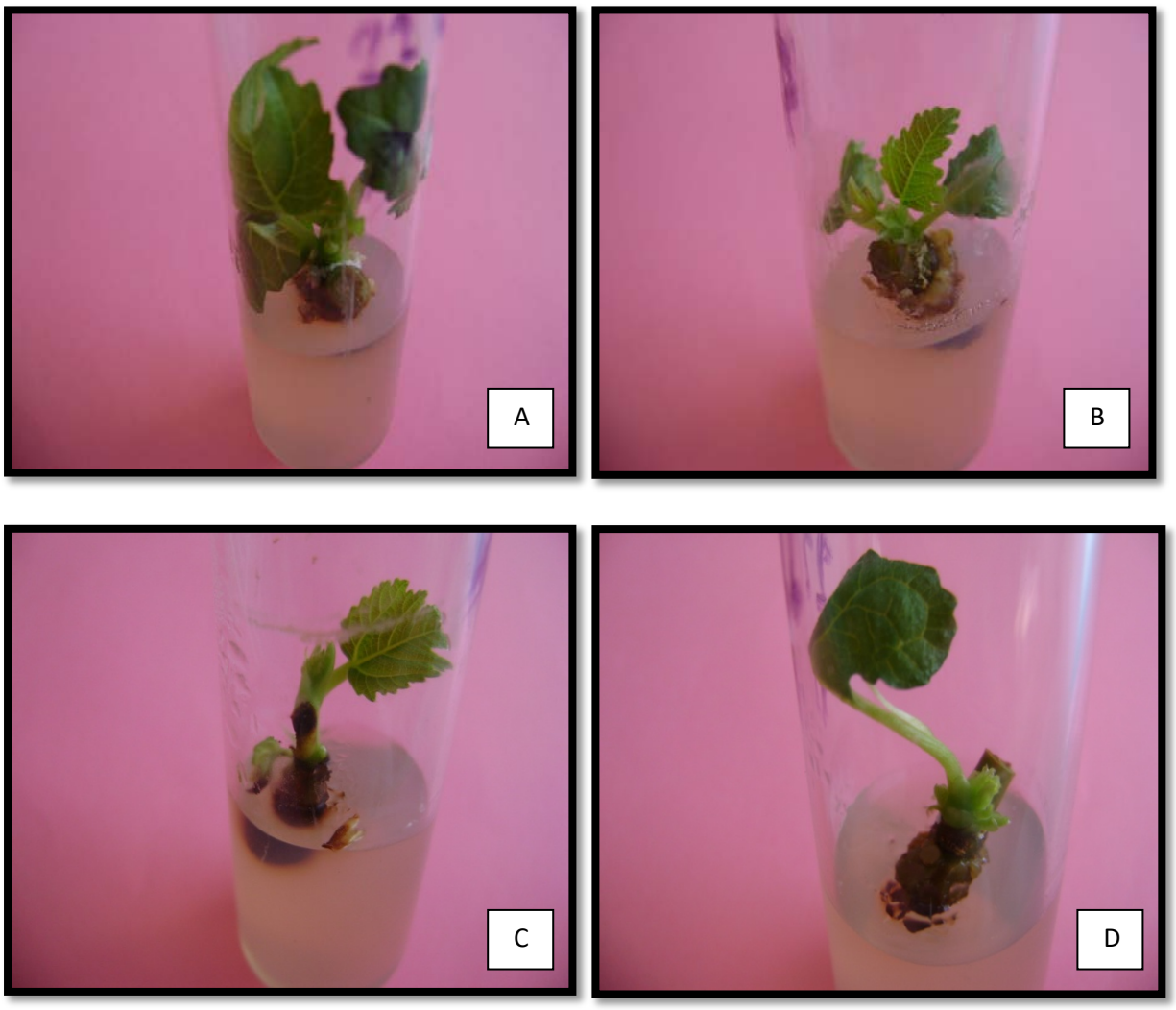

Plate 4.4: A,B,C and D showing multiplication stage of Fig.

Plate :4.5

Plate4.5: A. Rooted cultures of Fig, B. Fig plantlets in poly tray placed for primary hardening, C. Secondary hardened plants of Fig in black polybeg, D. Number of secondary hardened plants of Fig.