IN VITRO PROPAGATION STUDIES ON *Citrus* spp.

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THESIS

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CITRUS FRUITS – PROPAGATION
POMOLOGY
THESIS – Ph.D. POMOLOGY
TISSUE CULTURE – CITRUS FRUITS

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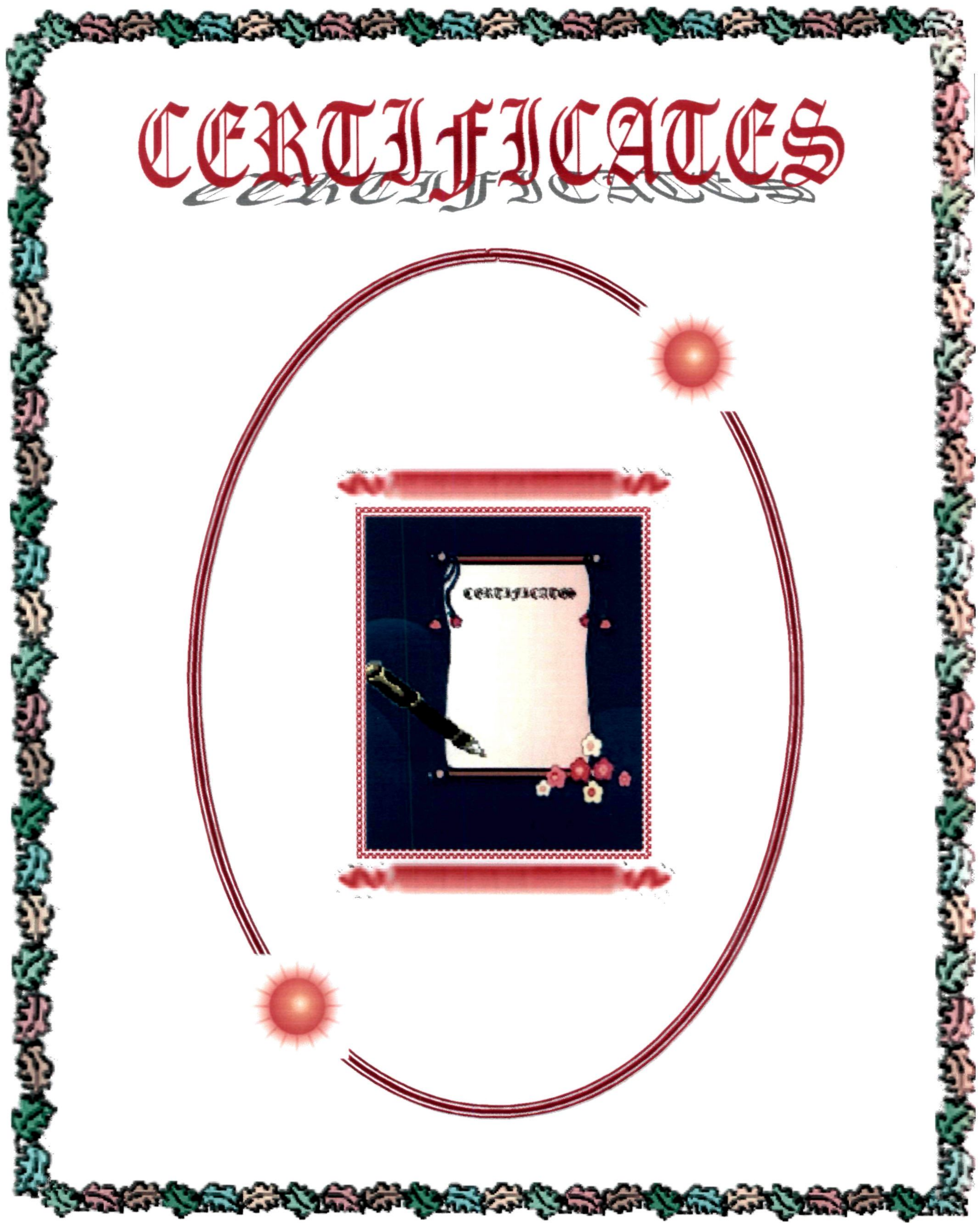
"One must regard the hyphen as a blemish to be avoided whenever possible"

Dedicated To

My Venerable Parents

"Any wide piece of ground is the potential site of a palace, but there's no palace till its built"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Certificate-I

This is to certify that the thesis entitled "***In vitro* propagation studies on *Citrus* spp.**" submitted in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Pomology and PHT** is a record of bonafide research, carried out by **Miss. Kiran Kour**, Registration No. **J-03-D-16**, under my supervision and guidance. No part of the thesis has been submitted for any degree or diploma. It is further certified that help and assistance received during the course of investigation have been duly acknowledged.

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There is no duty so sacred as doing good for others,
And there is nothing as ignoble as oppressing others.

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ABSTRACT

The present investigations were carried out at the Tissue Culture Laboratory, Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences and Technology, Udheywalla, Jammu during the year 2004-2006 to study the various aspects of *in vitro* propagation of *Citrus* spp. The nodal segments of 2-3 weeks old newly grown flushes from 10-12 year old mature trees were used as explant. Two step sterilization procedure involving 70 per cent ethanol and 0.1 per cent mercuric chloride for 30 seconds and 8 minutes respectively, resulted in maximum per cent culture establishment and minimum contamination in both *C. jambhiri* and *C. sinensis* cv. Mosambi. The time taken for culture establishment was less and per cent culture establishment was more in BAP 1.5 mg/l with malt extract for *C. jambhiri*, whereas BAP 1.0 mg/l with malt extract 500 mg/l proved to be best for *C. sinensis* cv. Mosambi.

In *Citrus jambhiri*, BAP 1.0 mg/l in combination with malt extract 500 mg/l; BAP 1.5 mg/l with malt extract 500 mg/l and BAP 1.5 mg/l along with Kinetin 0.5 mg/l showed 100 per cent multiple shoots in all the cultures during proliferation. However, BAP 1.5 mg/l with 500 mg/l malt extract showed the maximum number of shoots per culture (5.34), while maximum length of longest shoot (2.31 cm) and more

number of leaves (9.31) on longest shoot were recorded in BAP 1.0 mg/l with malt extract 500 mg/l, whereas in case of Mosambi BAP 0.5 mg/l with malt extract 500 mg/l; BAP 1.0 mg/l with malt extract 500 mg/l and BAP 1.0 mg/l in combination with Kinetin 0.5 g/l showed cent per cent multiple shoots during proliferation. BAP 1.0 mg/l with malt extract 500 mg/l showed maximum number of shoots and more number of leaves on longest shoot. The combination of BAP 1.5 mg/l with malt extract 500 mg/l showed maximum multiplication of shoots in all the three continuous sub-culturing done at 6 weeks interval in *C. jambhiri*, whereas BAP 1.0 mg/l along with malt extract 500 mg/l resulted in highest shoot multiplication in *C. sinensis* cv. Mosambi during sub-culturing.

The MS medium (half-strength) supplemented with IBA with NAA, 1.0 mg/l each, and gelled with agar 0.4 per cent took lesser days for root initiation (16.51) with highest rooted culture 83.33 per cent, while more number of roots per shoot 2.47, maximum length of longest root and shoot and more number of leaves per shoot (4.67) were recorded in same combination in *C. jambhiri*. Agar (0.4 per cent), sucrose (3.0 per cent) and pH 5.8 were proved to be best for rooting of *C. jambhiri* plantlets.

Covering the plantlets with glass beaker individually and kept in AC culture room proved to be the best hardening treatment, whereas a combination of soil:sand:FYM (1:1:1) was the best potting mixture as this resulted in the maximum survivability (93.33 per cent), height and more number of leaves per plantlet. Glycerol 50 per cent was proved to be the best anti-transpirant as it resulted in maximum per cent plant survival (83.33 per cent). The unit cost of plantlet upto polyhouse stage, based on the 1,00,000 plantlets capacity of the production unit was estimated to be Rs. 2.54, whereas the estimated production cost of a field-grown seedling of *C. jambhiri* was Rs. 4.48.

Signature of Major Advisor **Signature of Student**

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List of abbreviations used in this manuscript

ABA	abscissic acid
Ads	adenine sulphate
BAP	6-benzyl amino purine
^0C	degree celsius
cm	centimeter
2,4-D	2,4-dichlorophenoxyacetic acid
EDTA	ethylene diamine tetra acetic acid
FYM	farm yard manure
GA_3	gibberellic acid
HCl	hydrochloric acid
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
Kin	6-furfuryl amino purine
l	litre
ME	malt extract
µM	micromolar
mg	milligram
MS	Murashige and Skoog
MT	Murashige and Tucker
NAA	α-naphthalene acetic acid
ppm	parts per million
%	per cent
NaOH	sodium hydroxide

INTRODUCTION

Introduction

Citrus, often regarded as golden fruit or queen of all fruits is horticulturally very important comprising of 32 distinct species including mandarins, sweet oranges, lemons, limes, grapefruits and others. They belong to family Rutaceae and subfamily Aurantoideae. These crops are grown in tropical and subtropical climates extending 40^0 North and South latitudes of the equator where winter temperatures are moderate (Gmitter *et al.*, 1992). After grapes and olives, they are cultivated in larger area than any other fruits of these zones in the world. Considering the nutritional value, the magnitude of fruit production and an array of commercial products made, the citrus may be considered as a number one fruit of the world. Besides being a rich source of vitamin C and minerals, it is a rare source of vitamin P, the anti-haemorrhage vitamin. In India, it is the second most important commercial fruit crop occupying an area of 0.488 million hectare with a production of 4.575 million tonnes annually (Anonymous, 2004). It is also an important fruit crop of Jammu and Kashmir state grown in an area of 9,659 hectares with an estimated annual production of 9,111 metric tonnes (Anonymous, 2003).

Commonly citrus is not grown on its own roots. In all advance countries, citrus trees are now grown as budded plants composed of two components *viz.* rootstock and scion. Rootstock determines the success or failure of the scion cultivar grafted on it. It also affects the plant growth, fruit quality, yield as well as adaptation to different soil and climatic conditions. In citrus, rootstocks have been reported to influence

more than 20 horticultural and pathological characteristics of the tree and fruit (Fisher, 1989).

Citrus rootstocks are generally propagated for commercial use by growing open-pollinated seeds. Most of the rootstocks are highly polyembroynic and reproduce true-to-type via. nuceller polyembryony. Theoretically, all nuceller trees should be genetically identical with mother tree, however, phenotypical variability in nuceller seedlings are quite marked. Maheshwari and Rangaswamy (1958) reported that pollination and particularly the fertilization influences genome expression in nuceller embryo. The genetic inconsistency between the nuceller embryo is further suggested by Iglesias *et al.* (1974) and Moore and Castle (1988). They found that peroxidase zymograms vary qualitatively between the plants of same cultivars and even between the nuceller seedlings derived from the same seed.

Rough lemon (*Citrus jambhiri* Lush.) is the most widely used rootstock for various scion cultivars of citrus species all over the world and the only rootstock recommended for propagation in Jammu and Kashmir. The trees budded on this stock are vigorous, precocious, bear heavily, remain productive for longer period and produce high quality fruits (Vij and Kumar, 1990). It imparts resistance to tristeza and exocortis viroid, tolerant to salt and drought (Chadha and Singh, 1990), but it is relatively intolerant to *Phytophthora* and susceptible to citrus nematodes (Hutchinson and Grimm, 1972; Whiteside, 1973). Moreover being polyembryonic in nature, rough lemon seeds give rise to several vigorous and virus-free nuceller seedlings which are difficult to eliminate from zygotic seedlings, which necessitate the application of *in vitro* micro propagation (Edriss and Burger, 1984). The major

advantages offered by *in vitro* propagation techniques are increased rate of multiplication, disease-free and uniform material, rapid selection and multiplication of elite genotype and year round availability of planting material with low cost of production. Therefore, developing *in vitro* methods for propagating rough lemon as a rootstock is regarded as essential and long term effort aimed at improving citriculture world wide especially in state like Jammu and Kashmir where it is the most accepted rootstock for mandarin, sweet orange and lemon.

Mosambi [*Citrus sinensis* (L.) Osbeck.] also hold a significant place in the commerce of citrus and is an important scion cultivar. Its fruits have the added advantage of early maturity and reasonable juice quality. Thus, it is also necessary to standardize methodology and potential culture medium for rapid multiplication of this cultivar, which will be useful for *in vitro* grafting.

In order to achieve the above objectives, several workers have tried to develop tissue culture protocol for these crops (Raman *et al.*, 1992; Das *et al.*, 1995; Das *et al.*, 2000; Lauzada *et al.*, 2002). However, it is very important to standardize the technique for local conditions because the performance of plant tissue is dependent on a number of factors which are intimately connected with the physiological state of the donor explant.

The paucity of information on *in vitro* propagation of *Citrus jambhiri* Lush. rootstock and *Citrus sinensis* (L.) Osbeck. cv. Mosambi necessitated the present investigation with following objectives:

i. Standardization of suitable surface sterilent, culture media and growth regulators for *in vitro* shoot initiation and shoot proliferation from the nodal

explants of rough lemon (*Citrus jambhiri* Lush.) and sweet orange [*Citrus sinensis* (L.) Osbeck] cv. Mosambi.

ii. Standardization of suitable medium and growth regulator for *in vitro* rooting of rough lemon.

iii. Evolving standard procedure for hardening *in vitro* developed plantlets.

iv. To work out economics of *in vitro* propagation system of rough lemon.

Review of Literature

Chapter II

Review of Literature

Plant tissue culture has been used in plant science research since long back and is relatively a new technique in commercial horticulture. *In vitro* culture has found its best commercial application in the production of clonal plants at a very rapid rate as compared to conventional methods of propagation. It has also proved to be of immense practical value as an aid to the production and maintenance of disease free plants, germplasm conservation and a valuable adjunct to the conventional methods of plant improvement including plant genetic engineering. Thus, efforts have been made to scan the available literature to bring out desired information on the relevant aspects related to the objectives of investigation under the following sub-heads:

2.1 Surface disinfection of explants

2.2 Nutrient media

2.2.1 Proliferation of shoots

2.2.2 Rooting of micro shoots

2.3 Acclimatization/Hardening

2.3.1 *In vitro* hardening

2.3.2 *Ex vitro* hardening

2.4 Economics of *in vitro* plantlet production

2.1 Surface disinfection of explants

Explants must be free from micro-organisms when placed on nutrient media and this has usually been achieved by surface sterilization treatment. Fruit trees right from the seedling stage to senescent stage are continuously exposed to numerous field contaminations. They also harbour various insects, the activities of which further increase micro-organism contamination in the host-plant tissue.

DeFossard (1985) suggested a combination of physical methods (aimed at reducing the size of microbial population) and chemical methods (killing remaining microbes) leading to aseptic cultures. The physical methods include growing of mother plant in a clean potting mixture e.g., vermiculite, in a greenhouse for 1-3 months, avoiding overhead watering, treatment with pesticide and exposing the plants to relatively low humidity. The successful disinfection of explants is a pre-requisite for *in vitro* culture and often involves a standard set of treatments which vary with the type and species of explant (Thorpe and Patel, 1984). Moore (1986) sterilized internodal stem sections of citrus rootstocks with 1 N HCl for 0.5 minutes or a combination of 70 per cent ethanol for 2.5 minutes and 1.5 per cent sodium hypochlorite for 5 minutes and reported that treatment of explant with HCl in this manner during disinfection significantly reduced contamination of the cultures without harming the explant.

Contamination in tissue culture can originate from two sources, either through micro-organisms on the surface of explant or in the tissue itself. Although, in meristem culture, depending on meristem size, most of the micro-organisms are eliminated, whereas in leaf, petiole and stem explants, the infection is carried over to

the cultures (Cassells, 1991). Singh *et al.* (1994) disinfected *C. reticulata* Blanco. and *C. limon* Burm.f. with 2 per cent calcium hypochlorite solution for 15 minutes proved to be effective surface sterilent. Dwivedi and Bist (1999) found that surface sterilization of pear explants with 0.1 per cent mercuric chloride and 10 per cent sodium hypochlorite for 4 and 10 minutes, respectively gave the maximum proliferating aseptic cultures.

Al-Bahrany (2002) used 70 per cent ethanol followed by 1 per cent sodium hypochlorite for 30 seconds and 15 minutes, respectively as surface sterilent for *C. aurantifolia* (Christm.) Swingle. Rana and Singh (2002) disinfected the shoot tips of *C. aurantifolia* with 1 per cent sodium hypochlorite and 0.1 per cent mercuric chloride for different time intervals and reported that highest percentage of aseptic cultures were obtained with 10 minutes sodium hypochlorite and 5 minutes mercuric chloride sterilization combination.

2.2 Nutrient media

The concept of growing plants from individual cell was suggested by Haberlandt (1902), a German Botanist who tried to grow leaf cell cultures in simple mineral solutions. The composition of media used for culturing is an important factor in successful establishment of a tissue culture conditions favouring callus growth may not be suitable for organ differentiation. Many nutrient media have been used in the citrus tissue culture. However, various modification in the nutrient media have been reported particularly for *Citrus* species by Tuckey (1933), White (1943), Hildebrandt *et al.* (1946), Nitsch (1951), Heller (1953), Gautheret (1957), Murashige and Skoog (1962), Murashige and Tucker (1969) and Kitto and Young (1981). Virtually all

media contain inorganic salts and sucrose as the carbon source, typically at concentration between 1 and 3 per cent. It has been reported that 5 per cent sucrose is optimum for the culture of tissue and organ of citrus. Concentration of these ingredients in the basal medium depends on the plant to be cultured and stage of culture development (Murashige and Tucker, 1969; Rangaswamy, 1975; Nagao *et al.*, 1994). Murashige and Skoog (1962) medium has been widely used with appropriate modifications for culturing of many *Citrus* species and cultivars of regenerations and/or proliferations, sub-culturing and its subsequent rooting (Murashige and Tucker, 1969; Murashige, 1974; Chaturvedi and Mitra, 1975; Gamborg *et al.*, 1976; Kochaba and Spiegel-Roy, 1977; Altman and Goren, 1978; Styler and Chin, 1983; Yang, 1983; Grosser and Chandler, 1986; Nel, 1987). The main difference between the MS medium and other formulations was in the concentration and source of nitrogen. Most of the media contain only nitrate, while the MS medium contains a relatively high concentration of ammonium and nitrate ions in addition to high concentration of calcium than other media. Thiamine, inositol, nicotinic acid and pyridoxine are the most commonly used vitamins in the media. Out of these, thiamine is critical and it is usually provided in the range of 0.1-0.4 mg/l. Inositol is not essential, it has been found beneficial and has been used at the rate of 100 mg/l (Murashige, 1974). The commonly used media for *Citrus* species are Murashige and Skoog (MS), Murashige and Tucker (MT) and Gamborg (B_5).

Among the various media, MS medium supplemented with different growth regulators has been widely used for *in vitro* propagation from different explants such as shoot-tips/shoot primordial, hypocotyl (root meristem), epicotyl (shoot), nodal

cuttings/nodes, internodes and young leaves etc. in the cultures of different *Citrus* species namely, 'Carrizo' and 'Troyer' citrange (an intergeneric hybrid between *C. sinensis* and *Poncirus trifoliata*), trifoliate orange (*P. trifoliata* (L.) Raf.), Cleoptera mandarin (*C. reshni* Hort.), Rangpur lime (*C. limon* Osb.), sweet orange (*C. sinensis* (L.) Osb.) cvs. ('Symon', 'Trovita', 'Mosambi', 'Malta', 'Pera'), lemon (*C. limon* L.) cvs. 'Eureka', 'Assam lemon', mandarin (*C. reticulata* Blanco cv. 'Khasi'), Kinnow mandarin (*C. nobilis* Lour x *C. deliciosa* Ten.), Pummelo (*C. grandis* Osb.), *C. latipes* Swingle, *C. indica* Tanaka, rough lemon (*C. jambhiri* Lush.), Sour lime (*C. aurantifolia* Swingle cv. 'Kagzi lime'), *C. taiwanica* L. (Sauton *et al.*, 1982; Litz *et al.*, 1985; Shengeliya and Butenko, 1986; Nel, 1987; El-Gindy *et al.*, 1990; Song *et al.*, 1991; Otoni and Teixeira, 1991; Omura and Hidaka, 1992; Singh *et al.*, 1994; Gill *et al.*, 1995; Goh *et al.*, 1995; Maggon and Singh, 1995; Baruah *et al.*, 1996; El-Morsy and Millet, 1996; Harada and Murai, 1996; Ramos *et al.*, 1996; Normah *et al.*, 1997; Singh *et al.*, 1999; Shah *et al.*, 1999). In addition to MS medium, Sauton *et al.* (1982) regenerated explants on Murashige and Tucker (1969), Navarro *et al.* (1975) and Favre (1977) media.

The growth additive is a complex organic substance such as malt extract, which has been frequently incorporated with various combinations and concentration of growth regulators for initiating citrus tissue culture. Malt extract has generally been found to be either essential (Bitters *et al.*, 1970) or atleast beneficial to embryogenesis in nucellus cultures (Button and Bornman, 1971; Starrantino *et al.*, 1978). In some systems, malt extract can be replaced by combinations of orange juice, NAA and adenine sulphate (Rangan *et al.*, 1969) or by adenine sulphate and

Chaturvedi, 1972). Ovular callus proliferation has also been enhanced by 500 mg/l malt extract (Rangaswamy, 1959) but similar concentration was detrimental to the development of embryos within ovules and to the growth of plantlets in culture (Rangaswamy, 1961). In stem-callus systems of *C. madurensis* L. malt extract 500 mg/l suppressed callus proliferation, and was not beneficial to either bud formation or shoot growth (Grinblat, 1972). Chaturvedi and Mitra (1974) observed enhanced shoot vigour in the presence of malt extract 500 mg/l, BAP 0.25 mg/l and NAA 0.1 mg/l in stem segment derived callus of *C. grandis* L. Osb. Likewise, Raj-Bhansali and Arya (1978a) observed enhanced shoot bud development in *C. aurantifolia* (Christm.) Swingle stem and root segment derived from callus with the addition of malt extract (500 mg/l) in MS medium containing NAA (0.5 mg/l) along with Kinetin (0.25 mg/l) and 2,4-D (0.25 mg/l). The malt extract (500 mg/l) when used in MS medium containing 50 μM Kinetin and 0.16 M sucrose, have been found to control embryogenesis in citrus cell culture (Hidaka and Omura, 1989).

2.2.1 Proliferation of shoots

Various combinations and concentrations of growth regulators have been tested in citrus to initiate proliferation of shoots and callus formation. In general, the auxins at low concentrations stimulate callus growth (Schroeter and Spector, 1957; Murashige and Tucker, 1969) and at higher concentrations it promoted root development (Ohta and Furusto, 1957; Kochaba *et al.*, 1972). However, cytokinins such as Benzyl amino purine and 6-furfurylaminopurine (Kinetin) promotes shoot bud development, but at higher concentrations, it suppressed the root growth (Grinblat, 1972). It has been reported that shoot proliferation is influenced by

source, cytokinin, sucrose level and light intensity (Bouzid, 1975). Explants taken from adventitious shoots of sweet orange cv. 'Shamouti' developed multiple shoots with BA (10^{-5} M). At the higher levels, regeneration was lower but continued for a longer period (Altman and Goren, 1978). Among the other cytokinins, kinetin, adenine, adenine sulphate were ineffective to induce shoot bud formation and the effect of 2, 4-D on organogenesis was inhibitory. However, regeneration of buds was controlled by the action of different growth regulators added in the culture medium (Raj-Bhansali and Arya, 1978b). Barlass and Skene (1982) produced successful micro propagated plantlets using 10 mg/l of BAP for shoot proliferation and same concentration of NAA for rooting in case of Carrizo citrange, Trifoliate orange, Cleopatra mandarin, Rangpur lime and sweet orange. The optimum concentration of BA for shoot and bud proliferation was 3.0 mg/l and 1.0 mg/l for sweet orange and Citron respectively (Duran-Vila *et al.*, 1989).

MS medium containing 2.0 mg/l BAP with or without 4.0 mg/l GA_3 was optimum for shoot initiation in *Citrus aurantium* var. Brezilia. The highest percentage of shoot regeneration was obtained in *Jatti khatti* (*Citrus jambhiri*) and Baramasi lemon (*Citrus limon*) on half-strength MS medium supplemented with 5.0 mg/l BA alone or in combination with 3.0 mg/l GA by Raman *et al.* (1992). Parthasarathy and Nagaraju (1993) reported that BAP 0.75 mg/l alone induced number of multiple shoots in 3 *Citrus* species. The combination of two or more cytokinins was generally more effective than single cytokinin in producing shoots for micro propagation (Bowman, 1994). Singh *et al.* (1994) revealed that MS medium supplemented with 1 mg/l BAP, 0.5 mg/l Kinetin and 0.5 mg/l NAA resulted in multiple shooting in *Citrus*

reticulata Blanco. and *C. limon* Burm.f. Likewise, Baruah *et al.* (1995) cultured shoot explants of Pummelo [*Citrus grandis* (*C. maxima*)], obtained from *in vitro* grown seedlings on MS medium supplemented with different levels of either BAP or Kinetin and reported best proliferation on media supplemented with BAP 0.75 mg/l. Similarly, axillary buds from shoot explants of Kagzi lime (*Citrus aurantifolia*) were cultured on modified MS medium supplemented with various levels of BAP, NAA and/or 2, 4-D with or without malt extract. The best proliferation was observed on medium supplemented with BAP 0.25 mg/l + ME 200 mg/l.

Desai *et al.* (1996) revealed that highest number of shoots were produced in sub-cultures on MS media supplemented with 0.25 mg/l BAP and 200 mg/l ME in acid lime. The best shoot proliferation was observed in medium supplemented with BAP 0.5 mg/l, whereas BAP was superior to Kinetin for shoot proliferation (Baruah *et al.* 1996). Thirumalai and Thamburaj (1996) reported that the formation of shoots in *Citrus sinensis* Osbeck took place on MS medium containing 0.1 and 0.25 mg/l of NAA and BA respectively. Parthasarathy and Nagaraju (1996) reported that proliferation of shoots proportionately increased with increasing BAP level upto 0.75 mg/l, whereas both shoot number and shoot length reduced at concentration above 0.75 mg/l in studied three *Citrus* species.

Belarmino and Posas (1997) reported that for rapid shoot proliferation, the MS medium containing 0.5 mg/l BAP, 0.5 mg/l IBA and 40 mg/l adenine was optimum for *Citrus maxima*. Nodal segments from shoot explant of sour lime (*Citrus aurantifolia*) were cultured on MS medium supplemented with various concentrations of BAP and Kinetin. Best results for multiplication (8 shoots per node) were obtained

with BAP 1.0 mg/l and Kinetin 0.5 mg/l, whereas shoot elongation and leaf size were inhibited in response to high level of BAP (Al-Khayri and Al-Bahrany, 2001). Rana and Singh (2002) stated that multiple shoot formation in Kagzi lime was pronounced in medium supplemented with 2.0 mg/l BAP and 0.1 mg/l NAA. Kamble *et al.* (2002) observed highest percentage of shoot proliferation with nodal segments on MS medium supplemented with 0.1 mg/l NAA, 2.0 mg/l IBA and 0.5 mg/l GA_3 in acid lime. Murkute *et al.* (2004) reported that the best treatment for shoot proliferation in shoot tip and nodal segment of pomegranate was BAP 1.0 mg/l with NAA 0.5 mg/l.

2.2.2 Rooting of micro shoots

Number of factors have been observed to be associated with rooting of micro shoots that includes the nature of cuttings, rooting cofactor, synergistic role of exogenously applied growth hormone and endogenously present cofactors in the rooting, the relative efficiency of different auxins, combination and methods of application. In the micro propagation practice, usually natural (IAA) and synthetic (NAA and IBA) auxins were used for rooting, whereas higher concentration of cytokinins suppressed root growth (Singh, 2002).

Murashige (1977) emphasized that high light intensity also induces better rooting and causes hardening of plants which renders them more tolerant to moisture stress and diseases. Raj-Bhansali and Arya (1978b) reported that roots were formed in 2,4-D free medium with higher level of NAA (2.5 ppm). Kitto and Young (1981) observed better rooting in 'Carrizo' citrange with NAA as compared to IBA or IAA, whereas Barlass and Skene (1982) reported 10 per cent rooting (1 root per shoot) with IBA (5 µM), 25 per cent (1 root per shoot) with IBA (0.5 µM) and 80 per cent rooting

(5-9 roots per shoot) with NAA (5 μM) in 'Carrizo' citrange. Rooting of cultured shoots from mature tree of sweet orange and sour orange was achieved on MS medium supplemented with 0.5 mg/l NAA (Bouzid, 1986). Duran-Vila *et al.* (1989) tested different concentrations of NAA for rooting of micro-shoots of sweet orange, citron and lime. The optimal concentration to induce root formation on stem segments was 10 mg/l for sweet orange and lime and 3.0 mg/l for citron. Koc *et al.* (1992) revealed that best rooting was obtained with 4.0 mg/l (NAA and IBA) each. Lin *et al.* (1992) observed highest percentage of rooting on half-strength MS medium supplemented with 0.2 mg NAA per litre, 0.3 per cent activated charcoal and 15 per cent sucrose. The rooting of regenerated shoots of *Jatti khatti* (*C. jambhiri*) and Baramasi lemon (*C. limon*) was induced on half-strength MS medium containing NAA 1 mg/l and sucrose 2 per cent.

Can *et al.* (1992) obtained best rooting of micro-shoots of sour orange (*C. aurantium* var. Brezilia) in the medium containing 1.0 mg/l both (IBA and NAA). NAA alone was also effective on rooting but to a less extent than the combination with IBA. A combination of 0.5 mg NAA, 0.5 mg IBA and 0.25 mg BAP per litre was found best for root development of micro-shoots of Assam lemon (*C. limon*) and Khasi mandarin (*C. reticulata*) cvs. of citrus (Singh *et al.*, 1994). Das *et al.* (1995) stated that the regenerated shoots of *C. sinensis* cv. Mosambi rooted well in half-strength basal medium with NAA and IBA (each 0.5 mg/l). Rooting was best on half-strength of MS medium supplemented with 0.1 mg/l NAA (Desai *et al.*, 1996). Harada and Murai (1996) reported that rooting of regenerated shoots of *Poncirus trifoliata* enhanced on half-strength MS medium supplemented with 0.5 or 5 μM IBA.

Parthasarathy and Nagaraju (1996) found that medium supplemented with NAA 0.05 mg/l induced good rooting of micro cuttings on three *Citrus* species, while for *C. sinensis* cv. Mosambi medium supplemented with NAA 0.2 mg/l was best. Rooting percentage and the days taken for rooting were dependent on the size of the shoot placed for rooting. Large sized shoot showed higher rooting percentage and took less time for rooting in *C. assamensis*, *C. latipes* and *C. indica* (Baruah *et al.*, 1996). Root development from proliferated shoots was best on MS medium supplemented with BA (0.1 mg/l), Ads (0.1 mg/l), NAA (0.5 mg/l) and IBA (0.1 mg/l) (Singh *et al.*, 1999).

Das *et al.* (2000) reported that regenerated shoots rooted best in MS medium supplemented with either NAA (0.75 mg/l) or NAA (0.5 mg/l) and IBA (2.0 mg/l). Kaya and Gubbuk (2001) stated that culture media supplemented with BA and NAA were more successful for rooting than those with BA alone. Al-Khayri and Al-Bahrany (2001) reported that highest percentage of rooting was observed on MS medium supplemented with 1.0 mg/l IAA in *C. aurantifolia* Christm. Swingle. and also observed that higher levels of IBA reduced root number and the longest roots were found in media containing 2.0 mg/l IAA. Al-Bahrany (2002) stated that highest rooting percentage was obtained on medium containing either 1.0 mg/l NAA alone or 0.5 mg/l NAA combined with 2.0 mg/l IBA, whereas highest number of roots were produced on treatment containing both 2.0 mg/l NAA and IBA each. IBA 2.0 mg/l took minimum days for root initiation with highest rooting percentage (Rana and Singh, 2002). For rooting of *in vitro* shoots, half strength of MS salts fortified with 0.1-1.0 mg/l NAA, IBA and IAA were used. The best rooting was observed on 0.1

mg/l NAA (Begum *et al.*, 2003). Karwa (2003) revealed that MS medium without auxin resulted in very poor or no rooting. IBA alone induced upto 50 per cent rooting, whereas highest rooting percentage (78 per cent) and average number of roots per shoot were obtained in 4.92 μM IBA and 1.11 μM BA.

A carbon energy source is inevitable in any culture medium. Sucrose is the most widely accepted carbon source. The growth of the culture is not only affected by the particular type of carbon source used, but also by its concentration (Mehta, 1980). Shah (1983) found that sucrose, dextrose and ordinary sugar were equally effective for *in vitro* growth and survival of *Glycyrrhiza glabra* plantlets. A study conducted by Amin and Jaiswal (1989) on the effect of sucrose on growth and proliferation of shoots as well as on *in vitro* rooting of guava revealed that sucrose in medium remarkably influenced the growth and multiplication of shoots. In the absence of sucrose, cultures could not survive after 3 weeks of incubation. In the sucrose containing media, 30-40 g/l gave the best results. Addition of 15 g/l sucrose to the medium increased the rooting percentage from 62 to 88, but further increase of sucrose upto 45 g/l could improve the rooting quality but not the rooting frequency. Hazarika *et al.* (2004) studied the influence of *in vitro* preconditioning of micro shoots of *Citrus* species with sucrose on their *ex vitro* establishment and reported that maximum values for shoot height, root length and new leaf number were obtained in all the studied *Citrus* species after transferring them to *in vivo* conditions when previously conditioned with 3 per cent sucrose.

The physical form of the medium viz., solid or liquid plays an important role in *in vitro* growth and differentiation. Since solid cultures are easier to handle, the

media for *in vitro* propagation are generally solidified with 0.8 to 1.0 per cent agar (Bhojwani and Razdan, 1983). Kitto and Young (1981) reported that agar affected rooting with increasing response as its concentration decreased from 2.0 to 0.5 per cent. In guava, Amin and Jaiswal (1989) reported that number of shoots per culture, fresh weight per shoot, shoot length and quality rating increased significantly with the increase in agar concentration from 5.0 to 8.0 g/l. Further, an increase of agar upto 15 g/l sharply decreased all these parameters. Loh and Rao (1989) used gelrite instead of agar in Vietnamese pear cv. of guava for *in vitro* establishment and proliferation.

The pH of the culture media is also a critical factor; nevertheless it has been much neglected in *in vitro* studies. The usual practice is to adjust the initial pH of the medium within the range of 5.5 to 5.8. Unfortunately, pH drifts occur during the growth of culture (Gautheret, 1947) and little is known of the influence of actual pH value on the development of culture. Rao (1966) studied the *in vitro* growth of mango leaf gall tissue at different levels of pH ranging from 3.0 to 11.0. Best growth of the tissue was obtained at a pH of 6.0, while the growth decreased at both lower and higher pH values. Murashige (1977) stressed on the importance of pH and observed that the precipitation of nutrients was associated with higher pH and on the other hand poor gelling of agar was resulted with lower pH. Shah (1983) studied the effect of different pH levels on *in vitro* growth and rooting of *Glycyrrhiza glabra* and found the survival of plantlets at pH 5.5 as best under green house conditions. Amin and Jaiswal (1989) stated that comparatively less acidic medium (pH 5.5-6.0) was better for *in vitro* rooting than more acidic medium (pH 4.5-5.0).

2.3 Acclimatization/Hardening

The survival and growth of *in vitro* propagated plants after removal from culture has been a major problem in the micro propagation of citrus. Hardening of shoots prior to placement in the rooting beds increase survival rates of the transferred plantlets. So, plantlets or shoots produced *in vitro* must be acclimatized gradually to withstand the harsh natural environment. Misting, spraying or covering the pots may serve to fulfill the above objective. Different types of substrates have been used during the acclimatization period such as soil-vermiculite mixture (Goyal and Arya, 1981), sterilized soil (Raj-Bhansali *et al.*, 1988) and soil (Kurten *et al.*, 1990). During acclimatization the temperature of root zone is important for better root growth. The medium should be warmer than the air for good root activity and to increase the humidity around the cuttings (Dunstan and Turner, 1984; McCown, 1986). Plants growing under heterotrophic conditions *in vitro* have low rates of photosynthesis. This is due to low light intensities, low carbon dioxide concentration (Infante *et al.*, 1989) and inhibition of photosynthesis by high sugar concentration in the medium (Sheen, 1990; Lees *et al.*, 1991; Reuther, 1991). Nevertheless, after transfer to *ex vitro* conditions, most of the micro propagated plants develop a functional photosynthetic apparatus. Although, the increase in light intensity is not linearly translated in an increase in photosynthesis (Kozai, 1991). In *in vitro* regenerated plantlets, the response of photosynthesis to light is similar to that of shade plants characterized by low photosynthetic rates, low light compensation and saturation points (Chaves, 1994). Deficiencies in chloroplast structures namely grana development (Wetzstein and Sommer, 1983) and at the biochemical level, the low

Rubisco activity (Grout, 1988) also contribute to a limited photosynthetic activity. Relative humidity only influences the survival of plantlets after their removal from culture vessels with stable high humidity (Murashige, 1974). *In vitro* regenerated plantlets exposed to low light intensities acquire shade characteristics and when they are transferred to *ex vitro* conditions at higher light irradiance, light stress can occur including photo-inhibition or even photo-oxidation of chlorophyll, the later being revealed by chlorotic and dry spots appearing in the leaf blade. The control and optimization of light is essential for successful acclimatization, to decrease the period of acclimatization, to increase the survival rate and for the proper development of new structures in leaves (Donnelly and Vidaver, 1984). Transfer of plants from *in vitro* conditions to the green house is still problematic for most of species. When plants are removed from culture vessels they undergo rapid water loss leading to desiccation and death if not handled properly during acclimatization phase (Sutter *et al.*, 1988). The shading of plantlets is also necessary because firstly the strong solar light itself may directly damage the plantlets and secondly the fluctuating solar light intensity with time leads to the fluctuation of temperature and relative humidity and hence an excess water loss from the plantlets (Kozai, 1991). Quatrini *et al.* (2003) stated that arbuscular mycorrhizal fungi have been successfully used to improve acclimatization of many micro propagated fruit species. Techniques that are most satisfying for acclimatization address the changes towards lower relative humidity, higher light levels, autotrophic growth and septic environment that are characteristics of green house and field.

2.3.1 *In vitro* hardening

The process of acclimatization can begin, while the plantlets are still *in vitro*. Although anti-transpirants have been used successfully to decrease water loss from fruit (Davenport *et al*., 1974) and whole plants (Davies and Kozlowski, 1974), their application to cultured plants at the critical stage of transfer to green house remains limited. Application of anti-transpirant on leaves at the time of transfer were found to reduce transpiration in almond (Davenport *et al*., 1974) and orange (Albrigo, 1972). A commercially available kit for *in vitro* hardening of micro-propagated plants was suggested by Smith *et al*. (1991). The kit consisted of cellulose plugs (sorbarods), which support and protect the roots during transfer to soil and ventilated culture vessels to improve the resistance of plantlets to desiccation thereby suggesting general applicability of technique for a diverse range of plant species. The regulatory control of exogenously applied ABA on stomatal resistance and on maintaining better tissue hydration through minimum water loss was noticed by Venkataramana and Naidu (1993). Hazarika *et al*. (1995) developed an acclimatization protocol of citrus. The protocol involves direct culturing of 4-week-old *in vitro* grown proliferated shoots in soilrite topped over sterile FYM. The shoots rooted within 7-15 days depending upon species. These plantlets were rooted in the above carriers in sterile tissue culture bottles, after 4-6 weeks caps were loosened and plantlets were taken to mist house for transfer. Rooting and acclimatization of micro-propagated citrus plantlets (mandarin, *C. volkameriana* and *C. nobilis* x mandarin) was carried out simultaneously by Hazarika *et al*. (1999). The micro-propagated plants of two sugarcane varieties Co. 419 and Co. 806 were hardened by Dhaliwal *et al*. (1997)

with different chemicals and/or growth regulators for one hour before transferring to glass house. They found that ABA treated plants during water stress exhibited a regulatory control on stomatal resistance, maintained better tissue hydration through minimal water loss and a pronounced increase in epicuticular wax content. They concluded that the hardening could be associated with the reduced cuticular transpiration.

2.3.2 *Ex vitro* hardening

The growing medium to which *in vitro* rooted plantlets are to be transplanted and exposure of plantlets to gradually changing environmental conditions are important for good survival of plantlets. Inhibitors or a dramatic shift in medium pH can adversely affect root growth and thus transplanting success. The cultures in closed vessels are with stable high humidity and the survival of plantlets is influenced by the relative humidity when they are removed from culture vessels.

The potted plants were retained in the culture room for 20 days and later on transferred to the shade house by Chaturvedi and Mitra (1974). Before transfer to field, plants were covered by bell jars for first 10 days to prevent them from desiccation. Raj-Bhansali and Arya (1978b) treated the plants with Hoagland solution and incubated under different light regimes for 10 days, before transfer of plantlets to soil. They omitted sucrose to encourage autotrophy and thereby the state of subsequent survival. Kitto and Young (1981) successfully established plants in soil under mist (10 second every 10 minutes for 10 hours a day). Under intermittent mist, humidity was gradually decreased to allow the plants to adjust to normal conditions. Starrantino and Caruso (1987) transplanted the plants to plastic pots filled with

sterilized or virgin soil and transferred them to a glasshouse with high humidity and temperature between 25-27^{0}C for hardening whereas, Starrantino and Caruso (1988) raised the transplanted young plantlets in a hot bed frame for acclimatization.

To acclimatize micro propagated plants of citrus, a number of workers (Bhat *et al.*, 1992; Raman *et al.*, 1992; Singh *et al.*, 1994; Das *et al.*, 1995) transplanted them to pots containing sterilized sand or soil, which were covered with polythene bags or jars. Some other workers (Gill *et al.*, 1995; Normah *et al.*, 1997) transferred the plantlets to pots containing sterilized mixture of sandy soil, peat and organic manure (2:1:1) or soil, sand and organic matter (1:1:1) mixture. Similarly, Sim *et al.* (1989), Goh *et al.* (1995) and Baruah *et al.* (1996) weaned the plants by transferring to vermiculite, whereas Harada and Murai (1996) acclimatized under intermittent mist for 20 second after every 40 minutes. Acclimatization of tissue culture grown plants of citrus has been a slow and difficult process. *In vitro* proliferated shoots were directly cultured in different media (soilrite, FYM, garden soil, sand, soilrite and FYM, soilrite and garden soil, soilrite and sand, FYM and garden soil, FYM and sand, garden soil and sand). Soilrite alone and in combination with other media gave the highest rooting percentage (Singh, 2002).

The effect of several kind of soil/soil less media in different combination on acclimatization of sugarcane was studied by Dhillon (2001). They concluded that addition of burnt rice husk improved aeration in substratum, besides, it absorbed harmful substances released in soil, leading to better growth of plants.

2.4 Economics of *in vitro* plantlet production

It is very important to evaluate the economics of *in vitro* grown plants for their mass multiplication. However, very few workers have worked out the economic analysis of *in vitro* produced plantlets.

Debergh and Maene (1981) reported that the entire process of *in vitro* rooting and hardening has been estimated to account for approximately 35 to 75 per cent of total cost of micro propagation. Rajmohan (1985) reported that by *in vitro* shoot tip culture technique in Jackfruit, on an average 65.38 plantlets could be produced per year from a single explant. The unit cost of production of one Jackfruit plantlet including one month hardening was estimated to be Rs. 9.09. Rajeevan and Pandey (1986) reported that by *in vitro* shoot tip culture in papaya, on an average 19,200 plants could be produced in every 3 weeks cycle from 250 initial shoot tips. They also found that *in vitro* rooting and green-house stages are the most expensive items and estimated that the average cost of papaya plantlet ready for field planting would be Rs. 0.85.

Pachauri and Dhawan (1989) proposed a structure for estimating total cost per annum for tissue culture plantlet production system.

$$T_c = (K_c + K_b)(i + d) + E + S + LK$$

Where, T_c = Total annual cost of production

K_c = Total cost of equipment

K_b = Total cost of building and infrastructure

i = Annual rate of interest applied to investment

d = Annual rate of depreciation

E = Annual cost of power consumed

S = Annual cost of consumable supplies

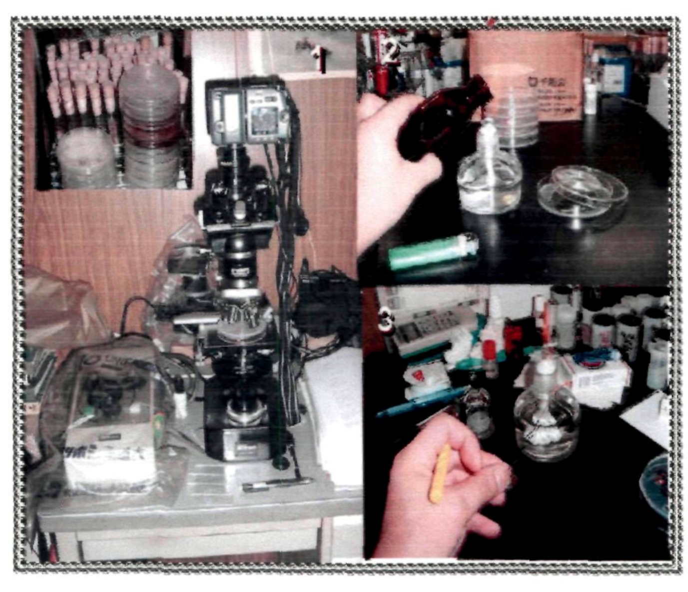
LK = Annual cost of labour in K^{th} activity

K = Activities such as inoculation, media preparation, transfer of plantlets etc

The unit cost of tissue culture plantlets of the Tata Energy Research Institute was estimated to be Rs. 5.33 according to the proposed structure. Raghunath (1989) reported that cost of one cardamom plantlet was Rs. 4.90 including one month hardening period. Shah (1989) estimated the unit cost of herb as Rs. 0.73, shrubs Rs. 1.15 and trees Rs. 1.53 including 2 weeks green house stage in a theoretical commercial scale production. Babylatha (1994) estimated the unit cost of banana plantlet as Rs. 2.22 including 4 weeks green-house stage.

In guava cv. Sardar, the unit cost upto polygreen house stage, based on the 1,00,000 plantlets capacity of the production unit was estimated to be Rs. 1.95 in case of seedling explant and Rs. 2.05 in case of mature tree explant (Wali *et al.*, 1996). Matsumoto *et al.* (1998) reported that one shoot explant of *C. reticulata* produced 1,80,000 shoots per year, while the cost of one *in vitro* grown plantlet was estimated to be 165 Yen.

MATERIALS AND METHODS



Materials and Methods

The present investigation entitled "*In vitro* propagation studies on *Citrus* spp." was conducted in the Tissue Culture Laboratory, Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences and Technology, Udheywalla, Jammu during the year 2004-2006. Materials and Methods used for conducting the study under report are given in this chapter.

On the geographical map, Udheywalla, (Jammu) is situated at 32^0 39' latitude and 74^0 54' East longitude at an elevation of about 332 meters above mean sea level. This region has a typical subtropical climate characterized by fairly hot summer and cold winters, with an average annual rainfall of 128 mm. The meteorological data pertaining to rainfall, temperature and relative humidity for the period under study are presented in Appendix XI.

3.1 Plant Material

Plant material of *Citrus jambhiri* Lush. and *Citrus sinensis* cv. Mosambi were taken from newly grown flushes (2-3 weeks old) of selected 10-12 year old mature trees grown in the orchard of University located at Udheywalla. The nodal segments were used as the experimental plant material.

3.2 Culture Media

In order to study the morphogenic response of *Citrus jambhiri* and *Citrus sinensis* cv. Mosambi the most widely accepted MS (Murashige and Skoog, 1962)

medium was used as basal medium. The composition of MS medium is presented in Table 1 containing only the basal salts (macro- and micro-nutrients), vitamins, sucrose and agar as reported in the original publication. The basal medium was modified with different concentration of growth regulators and malt extract.

3.2.1 Chemicals

Analytical grade chemicals obtained from British Drug House (BDH), Sisco Research laboratories (SRL), Loba or Hi-Media were used in the preparation of media.

3.2.2 Preparation of nutrient media

The stock solutions (I, II, III and IV) of MS basal medium were prepared by dissolving the required amount of salts in measured quantity of distilled water as mentioned in Table 1. These stock solutions were stored in dark amber coloured glass bottles at 5 ± 1^0C. The stock solutions were prepared afresh after every 4-6 weeks and that of growth regulators were prepared afresh after every week.

The basal medium was prepared by mixing required quantity of each of four stock solutions in 700 ml of distilled water by continuous stirring. Myo-inositol (100 mg/l) and sucrose (3 per cent) were added to this solution. Quantity of all the four stock solutions added to the medium was reduced to one half for preparing half-strength MS medium, while remaining ingredients of medium were kept same. The basal medium was modified by adding growth regulators as well as malt extract (whenever required).The final volume was adjusted to one litre by adding more distilled water. The pH of medium was adjusted to 5.8 with a systronic meter using either 0.1 N NaOH or 0.1 N HCl before adding agar.

TABLE 1. Composition of Murashige and Skoog (1962) medium

Stock Solution No.	Strength	Constituent Salts	Quantity Used	Stock Solution used (ml/l)	Actual amount in the culture medium (mg/l)
		1 litre			
1	X 10	Ammonium nitrate (NH_4NO_3)	16.5 g	100	1650
		Potassium nitrate (KNO_3)	19.0 g		1900
		Calcium chloride ($CaCl_2$. $2H_2O$)	4.4 g		440
		Magnesium sulphate ($MgSO_4$)	3.7 g		370
		Potassium dihydrogen orthophosphate (KH_2PO_4)	1.7 g		170
		1 litre			
2	X 100	Manganese sulphate ($MgSO_4$ .7 H_2O)	2.23 g	10	22.3
		Zinc sulphate ($ZnSO_4$ .7 H_2O)	0.86 g		8.6
		Boric acid (H_3BO_3)	0.62 g		6.2
		Potassium iodide (KI)	83 mg		0.83
		Copper sulphate ($CuSO_4.5H_2O$)	2.5 mg		0.025
		Cobaltous chloride ($CoCl_2$. $6H_2O$)	2.5 mg		0.025
		Sodium molybdate ($Na_2MoO_4.2H_2O$)	25 mg		0.25
		500 ml			
3	X 20	Ferrous sulphate ($FeSO_4$ .7 H_2O)	557 mg	25	27.8
		Ethylene diaminetetraacetic acid and disodium salt (Na_3EDTA. $2H_2O$)	745 mg		37.28
		200 ml			
4	X 100	Glycine	0.2 g	2	2.0
		Thiamine HCl	10 mg		0.1
		Nicotinic acid	50 mg		0.5
		Pyridoxine HCl	50 mg		0.5

Addendum: Myo-inositol 100 mg/l; Sucrose 30 g/l and Agar 8 %.

The $FeSO_4.7H_2O$ was dissolved in approximately 50 ml of distilled water and heated. The Na_2EDTA.$2H_2O$ was dissolved in approximately 50 ml of distilled water separately and mixed, while heating (under continuous stirring) with $FeSO_4.7H_2O$ solution. After cooling, the volume was adjusted to 500 ml. Heating and stirring resulted into a stable FeNaEDTA complex.

Agar at the rate of 8 g per litre was dissolved by placing the medium on heater. The medium was stirred regularly to avoid formation of agar clumps till it started boiling. Then it was allowed to cool for few minutes at room temperature and was poured into culture vessels.

3.2.3 Preparation of growth regulator solution

Depending upon the nature of experiment, different concentration of the growth regulators were added to the basal medium. The stock solutions of growth regulators were prepared as follows.

The stocks of different growth regulators were prepared by dissolving them in few drops of appropriate solvent (Table 2) and final volume of each dissolved growth regulator was made 20 ml by adding distilled water gently to avoid precipitation. The stock solutions thus prepared were stored in glass bottles and kept in refrigerator at 4 -5 ^{0}C temperature.

TABLE 2. Stock solutions of growth regulators

Type of growth regulator	Name	Quantity (mg)	Solvent	Final volume (ml)
Cytokinins	6-Benzyl amino purine	20	1N NaOH	20
	6-furfuryl amino purine	20	1N NaOH	20
Auxin	α-Naphthalene acetic acid	20	Absolute ethyl alcohol	20
	β-Indole butyric acid	20	Absolute ethyl alcohol	20

3.2.4 Culture vessels

Erlenmeyer flasks (100 and 150 ml) and test tubes (25 mm x 150 mm) made of Borosil glass were used as culture vessels. All culture vessels and glassware used in the preparation of media and for other purposes were cleaned in chromic acid

(Potassium dichromate in sulphuric acid). The acid was removed by prolonged rinsing in tap water. They were then washed with detergent Teepol (BDH) and traces removed by thorough washing with running tap water. The glassware were finally rinsed with distilled water and dried in an oven at 70^0C.

3.2.5 Sterilization of media and culture vessels

After adjustment of pH, known volumes of media were distributed in culture vessels (30 ml in culture flasks and 20 ml in test tubes) for autoclaving. The mouth of culture vessels were plugged with non-absorbent cotton plugs. The mouth of culture vessels were further covered with ordinary paper or aluminium foil to protect them from condensing water vapour during autoclaving at a pressure of 15 lb/in^2 for 20 minutes at approximately 121^0C. After sterilization, the culture vessels were immediately transferred to an air conditioned culture room.

3.2.6 Glass apparatus

Different types of glass apparatus such as culture jars (screw cap bottles, 400 ml), petri dishes (90 mm and 150 mm), measuring cylinders (500 ml) and graduated pipettes (1 ml, 5 ml and 10 ml) were cleaned with Teepol detergent followed by washing under running tap water and finally rinsed with distilled water before drying. Double wrapped petri dishes with newspaper and half filled bottles with distilled water were also autoclaved at same pressure for 25 minutes. Autoclaved petri dishes were used during culturing. Autoclaved distilled water was used for giving various treatments and washings.

3.3 Aseptic Techniques

All inoculations and manipulations involving sterile cultures or media were carried out under aseptic conditions in a laminar air flow cabinet (Klenzaid's model Ultraklenz). The floor of cabinet was thoroughly cleaned with cotton dipped in ethanol. All the instruments such as surgical scalpel handle (size no. 3), disposable surgical blade (size no. 11), forceps (20 cm) etc. were also cleaned with cotton dipped in ethanol and sterilized (red hot) by flaming for repeated use. This was done at the start of inoculation and also several times during the operation. During inoculation, first the cotton plugs of the culture vessel was removed and the neck of vessel was flamed over the burner kept in the cabinet. The sterile and trimmed explants were quickly transferred to the culture vessel containing suitable culture medium using sterilized forceps. The neck of the culture vessel was again flamed and quickly stopped by the cotton plug.

3.4 Culture Conditions

All the cultures were incubated in culture room at 25 $\pm$ 2^0C with relative humidity of 55 $\pm$ 5 per cent and were exposed to 16 hour photoperiod by 40 W cool white fluorescent tubes kept 50 cm above bench surface.

3.5 *In vitro* Cloning Procedure

The general *in vitro* cloning procedure adopted in the studies is presented in Fig. 1.

3.6 Culture Technique

3.6.1 Culture Establishment (STAGE-I)

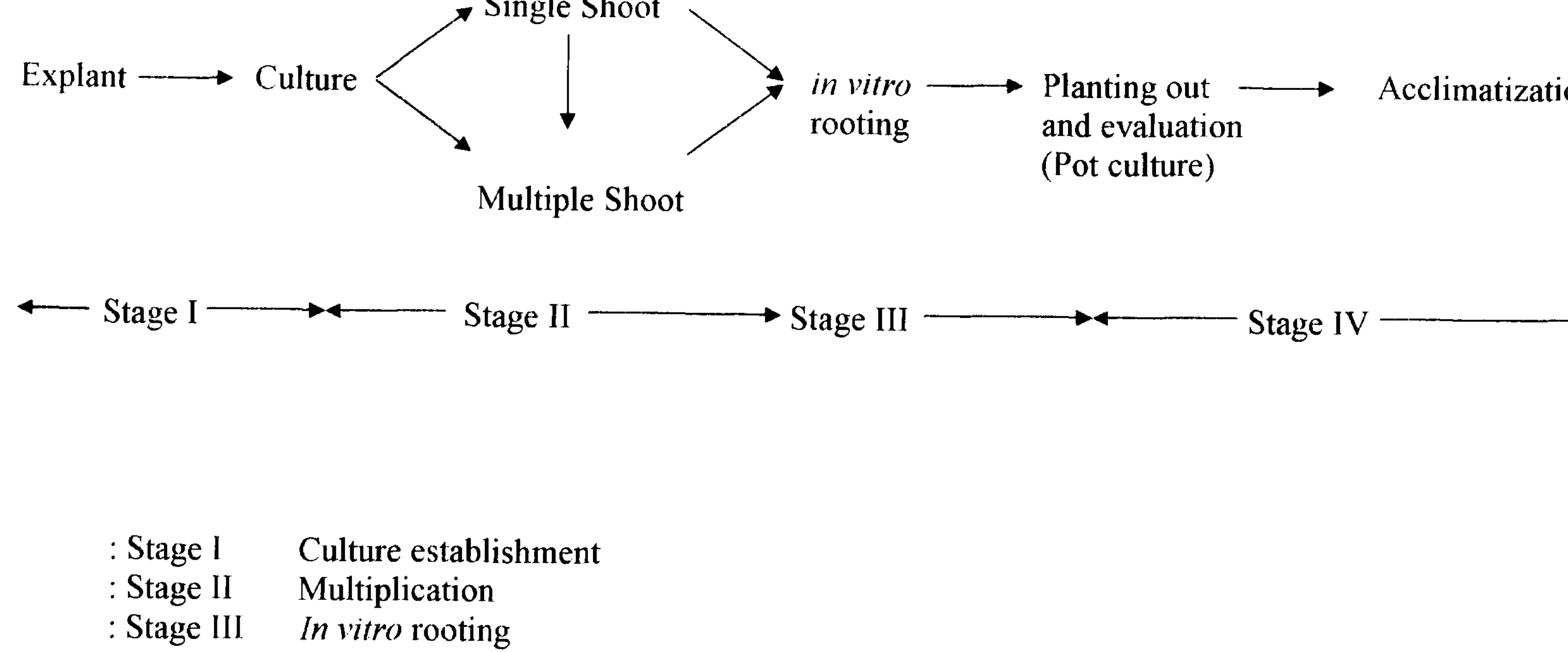


Fig.1: *In vitro* cloning procedure in *C. jambhiri*

3.6.1.1 Explant preparation

Apical shoot pieces of 5-10 cm long rough lemon (*Citrus jambhiri* Lush.) and sweet orange (*Citrus sinensis* (L.) Osbeck) were collected from current season growth. These shoot pieces were collected in a polythene bag and brought to the laboratory. Leaves were removed carefully from the shoots with the help of secateur/scissor. These shoots were cut for preparing nodal segments of size 2-3 cm long each having atleast one node. These shoot segments were washed under running tap water for about 30 minutes to remove all adherent dirt and then treated with 10 per cent solution of detergent Teepol for 10 minutes.

All the traces of detergent Teepol were removed by repeated washing in distilled water. Further, sterilization procedures were carried out under aseptic conditions in a laminar air flow cabinet. These nodal segments were subjected to surface sterilization using ethanol 70 per cent for 30 seconds and 0.1 per cent mercuric chloride ($HgCl_2$) solution (w/v) for 8 minutes. Then these segments were thoroughly washed with sterile (autoclaved) distilled water before inoculation.

The surface sterilized shoot pieces were then cut into small pieces of 1-1.5 cm size and these explants were then quickly inoculated on a suitable nutrient medium.

3.6.1.2 Standardization of surface sterilization method for *Citrus jambhiri* Lush. and *Citrus sinensis* cv. Mosambi

In order to identify the most effective surface sterilization method, a trial was conducted to isolate contamination free explants for culture establishment by using different chemicals as mentioned below:

Sterilant	Concentration	Duration
1. Mercuric chloride	0.05 %	5 minutes
2. Mercuric chloride	0.05 %	8 minutes
3. Mercuric chloride	0.1 %	5 minutes
4. Mercuric chloride	0.1 %	8 minutes
5. Ethanol and subsequent dipping in mercuric chloride	70 %, 0.05 %	30 sec., 5 minutes
6. Ethanol and subsequent dipping in mercuric chloride	70 %, 0.05 %	30 sec., 8 minutes
7. Ethanol and subsequent dipping in mercuric chloride	70 %, 0.1 %	30 sec., 5 minutes
8. Ethanol and subsequent dipping in mercuric chloride	70 %, 0.1 %	30 sec., 8 minutes
9. Sodium hypochlorite	5 %	10 minutes
10. Sodium hypochlorite	10 %	10 minutes

The nodal stem segment explants after initial treatment with detergent followed by thorough washing with distilled water as described in section 3.6.1.1 were subjected to surface sterilization using different chemicals mentioned above. The sterilization treatments were given under aseptic conditions in a laminar air flow cabinet. After surface sterilization the explants were inoculated on suitable nutrient medium.

3.6.1.3 Standardization of culture establishment medium

Nodal segment explants were taken from newly grown flushes (2-3 weeks old) of selected 10-12 years old mature trees for culture establishment trial. The trials were conducted on MS medium supplemented with different growth regulators with or without malt extract. The details of which are given below:

1. 4 levels of BAP (0.5, 1.0, 1.5, 2.0 mg/l) each.
2. 4 x 1 combination of BAP (0.5, 1.0, 1.5, 2.0 mg/l) and malt extract (500 mg/l) each.

3. 4 x 2 combination of BAP (0.5, 1.0, 1.5, 2.0 mg/l) and kinetin (0.5, 1.0 mg/l) each
4. 2 x 2 combination of BAP (0.5, 1.0 mg/l) and NAA (0.25, 0.5 mg/l) each.
5. 2 x 2 x 1 combination of BAP (0.5, 1.0 mg/l), NAA (0.25, 0.5 mg/l) and malt extract (500 mg/l) each.
6. 4 levels of kinetin (0.5, 1.0, 1.5, 2.0 mg/l) each.
7. 4 x 1 combination of kinetin (0.5, 1.0, 1.5, 2.0 mg/l) and malt extract (500 mg/l) each.
8. 2 x 2 combination of kinetin (0.5, 1.0 mg/l) and NAA (0.25, 0.5 mg/l) each.
9. 2 x 2 x 1 combination of kinetin (0.5, 1.0 mg/l), NAA (0.25, 0.5 mg/l) and malt extract (500 mg/l) each.
10. MS (Basal) medium.

In all, 41 treatments each of *Citrus jambhiri* Lush. and *Citrus sinensis* (L.) Osbeck cv. Mosambi were tried.

3.6.2 Shoot Proliferation (STAGE-II)

The *in vitro* shoots obtained by culturing the nodal segments on MS medium supplemented with various growth regulators and malt extract which showed culture establishment more than 85 per cent served as material for proliferation, the details of treatments tested for *C. jambhiri* and *C. sinensis* cv. Mosambi are given in Table 3 and Table 4 respectively. The multiplication rate (the number of shoots produced per culture) during the observation period of shoot cultures were examined for 3 continuous sub-cultures done at 6 weeks interval. Single shoot from proliferated cultures were used for this study. BAP 1.0 and 1.5 mg/l, kinetin 1.0 and 1.5 mg/l alone or in combination with malt extract 500 mg/l and BAP 1.5 mg/l with kinetin

TABLE 3. Composition of different media used for shoot proliferation of *Citrus jambhiri* Lush. during the investigation

S. No.	Composition
1.	2 levels of BAP (1.0, 1.5 mg/l) each
2.	2 x 1 levels of BAP (1.0, 1.5 mg/l) and malt extract (500 mg/l) each
3.	2 levels of kinetin (1.0, 1.5 mg/l) each
4.	2 x 1 levels of kinetin (1.0, 1.5 mg/l) and malt extract (500 mg/l) each
5.	1 x 1 levels of BAP (1.5 mg/l) and kinetin (0.5 mg/l) each
6.	2 x 1 x 1 levels of BAP (1.0, 1.5 mg/l), NAA (0.25 mg/l) and malt extract (500 mg/l) each

TABLE 4. Composition of different media used for shoot proliferation of *Citrus sinensis* cv. Mosambi during the investigation

S. No.	Composition
1.	2 levels of BAP (0.5, 1.0 mg/l) each
2.	2 x 1 levels of BAP (0.5, 1.0 mg/l) and malt extract (500 mg/l) each
3.	2 levels of kinetin (0.5, 1.0 mg/l) each
4.	2 x 1 levels of kinetin (0.5, 1.0 mg/l) and malt extract (500 mg/l) each
5.	1 x 1 levels of BAP (1.0 mg/l) and kinetin (0.5 mg/l) each
6.	2 x 1 x 1 levels of BAP (1.0, 1.5 mg/l), NAA (0.25 mg/l) and malt extract (500 mg/l) each

0.5 mg/l were tried for *Citrus jambhiri*, whereas for *Citrus sinensis* cv. Mosambi BAP (0.5 and 1.0 mg/l), kinetin (0.5 and 1.0 mg/l) alone or in combination with malt extract 500 mg/l and BAP 1.0 mg/l with kinetin 0.5 mg/l were used. Thus in all, 9 different treatment combinations were tried for both *Citrus jambhiri* and *Citrus sinensis* cv. Mosambi.

3.6.3 *In vitro* rooting of regenerated shoots (STAGE-III)

After 6 weeks of culturing on shoot proliferation media, the regenerated shoots were taken out with the aid of sterilized forcep under aseptic condition. The dried and under sized leaves were removed and multiple shoots were cut with the help of sterilized scalpel blade. The regenerated explants with single shoot (1.5 cm) were transferred to root induction media. Solid MS media (half strength) was employed for this trial. The different treatments tested are as following:

S. No.	Composition
1.	4 levels of IBA (0.25, 0.50, 0.75, 1.0 mg/l)
2.	4 levels of NAA (0.25, 0.50, 0.75, 1.0 mg/l)
3.	4 combinations of IBA + NAA (0.25, 0.50, 0.75, 1.0 mg/l) each
4.	MS basal (half strength) medium

In all, 13 different combinations were tried.

3.6.3.1 Effect of supporting media on rooting characteristics and survival of *Citrus jambhiri* Lush. plantlets

Experiments were conducted to observe effect of different supporting media on rooting characteristics of rough lemon plantlets, the details of treatments are given below:

S. No.	Treatments
1.	MS + Agar (0.4 %)
2.	MS + Agar (0.8 %)
3.	MS + Whatman No.1 filter paper
4.	MS + filter paper (ordinary)
5.	MS + brown paper

MS medium (half strength) was used for the trial. Shoots of approximately 1.5 cm length excised from proliferated cultures were utilized.

3.6.3.2 Standardization of optimum and best carbohydrate source for *in vitro* rooting

Trials were conducted to study the effect of different sources of carbohydrates on *in vitro* rooting characteristics and survival of *Citrus jambhiri* plantlets. Shoots approximately 1.5 cm in length excised from proliferating cultures were utilized for this trial. MS medium (half strength) supplemented with 0.4 per cent agar was used. The details of carbohydrate sources tested for *in vitro* rooting are as under:

S. No.	Carbohydrate source	Concentration (%)
1.	Sucrose	3.0
2.	Commercial sugar	3.0
3.	Glucose	2.0
4.	Fructose	2.0
5.	Lactose	2.0
6.	Maltose	2.0

3.6.3.3 Standardization of sucrose concentration for *in vitro* rooting

Trials were conducted to study the effect of different sucrose concentrations on *in vitro* rooting characteristics and survival of *Citrus jambhiri* plantlets. Shoots approximately 1.5 cm in length excised from proliferating cultures were utilized for this trial. MS medium (half strength) supplemented with 0.4 per cent agar was used. The details of sucrose concentration tested for *in vitro* rooting were as under:

S. No.	Sucrose concentration (%)
1.	1.5
2.	3.0
3.	4.5

3.6.3.4 Standardization of optimum pH for *in vitro* rooting

Experiment was conducted to study the effect of different levels of pH on *in vitro* rooting. The different levels of pH tried were given below:

S. No.	pH
1.	5.4
2.	5.6
3.	5.8
4.	6.0
5.	6.2

MS medium (half-strength) supplemented with 0.4 per cent agar was used. The required pH level of the medium was adjusted with 0.1 N HCl or 0.1 N NaOH as found necessary before autoclaving.

3.6.4 Acclimatization and planting out of plantlets (STAGE-IV)

3.6.4.1 Standardization of hardening treatment (Primary hardening)

In order to acclimatize the *in vitro* produced rough lemon plantlets to natural environment, a study on different hardening treatments was carried out. Uniformly rooted plantlets were taken out from culture vessels with the help of forcep. The roots were washed thoroughly with distilled water to remove the traces of nutrient media. Dead and decayed parts of plantlets were removed and then

transplanted in pots containing different substrates viz. soil, sand and FYM (1:1:1) mixture. Then they were subjected to the following hardening treatments:

S.No.	Treatment
1.	Covering the plantlets individually with glass beaker and keeping in culture room
2.	Covering the plantlets individually with polythene bag and keeping in culture room
3.	Covering the plantlets with glass beaker individually and keeping in open
4.	Covering the plantlets individually with polythene bag and keeping in open
5.	Keeping the plantlets in open without any cover.

The plantlets were covered with glass beaker/polythene bags continuously for 6 days in all treatments except 5th treatment in which plantlets were kept without cover. For the initial 3 days no air was allowed to enter and then the little air space was provided into the cover. In case of first and second treatments, the plantlets were kept in continuous light in culture room at a temperature of $26 \pm 2\ ^0C$. The cover was gradually removed after 6 days initially for 3 hours followed by 6 and 12 hours in next 3 days. Then they were kept at room temperature during night hours (by putting off lights) for next 3 days. Subsequently, the period of keeping plantlets at room temperature was gradually increased after 15 days and then they were brought outside culture room. In case of third and fourth treatments, the plantlets were kept at room temperature. After six days, the covers were removed during night hours for next 3 days. Subsequently, the period of removing the cover gradually increased and after 15 days the cover was completely removed. The plantlets were kept in laboratory for 6

days more, after which they were brought to natural environment. All the plantlets were watered everyday.

3.6.4.2 Influence of potting mixture on growth and establishment of plantlets

To study the influence of potting mixture on growth and establishment of plantlets, a trial was carried out using various kinds of potting mixtures made of different ingredients as detailed below:

S. No.	Treatment
1.	Soil
2.	Soil:Sand (1:1)
3.	Soil:Sand:FYM (1:1:1)
4.	Soil:Sand:Vermiculite (1:1:1)

Uniformly rooted plantlets were removed from culture vessels. Their roots were washed in distilled water and then planted in pots filled with different potting mixtures. The constituents of different substrates were mixed volume/volume. Just before transplanting and at weekly interval afterwards, the potting mixtures were drenched with Bavistin (0.1 per cent). The plantlets were watered after every alternate day.

3.6.4.3 Influence of anti-transpirants on establishment of plantlets (Secondary hardening)

Anti-transpirants were applied to randomly selected plants. The stem and leaves of the hardened plants were dipped into the solution of glycerol for one minute

before planting them in pots. Care was taken to avoid dipping of roots in the solution. The abscissic acid (ABA) was sprayed on the plants with the help of hand sprayer.

S. No.	Anti-transpirants	Concentration
1.	Glycerol	10 per cent
2.	Glycerol	25 per cent
3.	Glycerol	50 per cent
4.	ABA	7 ppm
5.	ABA	10 ppm
6.	ABA	15 ppm
7.	Control	

3.6.5 OBSERVATIONS RECORDED

3.6.5.1 Surface sterilization of nodal segment explants

Observations with regard to the percentage of contaminated explants (fungal/bacterial) were recorded after 3 weeks of incubation of cultures. There were 20 explants per treatment and for calculation of contamination following formula was applied:

$$\text{Contaminated explant (per cent)} = \frac{\text{Number of contaminated explants}}{\text{Total number of explants cultured}} \times 100$$

3.6.5.2 Culture establishment/initiation (Stage I)

Observations were recorded on the number of days taken for culture establishment (as exhibited by fresh green colour and actively growing culture-visual observation) and percentage of culture establishment (12 explants per treatments after 3 weeks of incubation).

The multiplication rate (number of shoots produced per plant during observation period) of shoot culture was also recorded for three continuous sub culture done at 6 weeks interval.

3.6.5.4 *In vitro* rooting (Stage III)

Observations on number of days taken for root initiation were recorded. In addition the percentage of cultures rooted, number of roots per shoot, length of longest root, length of shoot and number of leaves per shoot were recorded. These observations were recorded on 10 cultures (one explant per culture tube) per treatment at the time of transplanting.

3.6.5.5 Acclimatization and planting out (Stage IV)

Observations were recorded on 10 plantlets per treatment on percentage of plantlets survival, height of plantlets and number of leaves per plantlet after four weeks of planting out.

3.6.5.6 Economics of *in vitro* propagation system

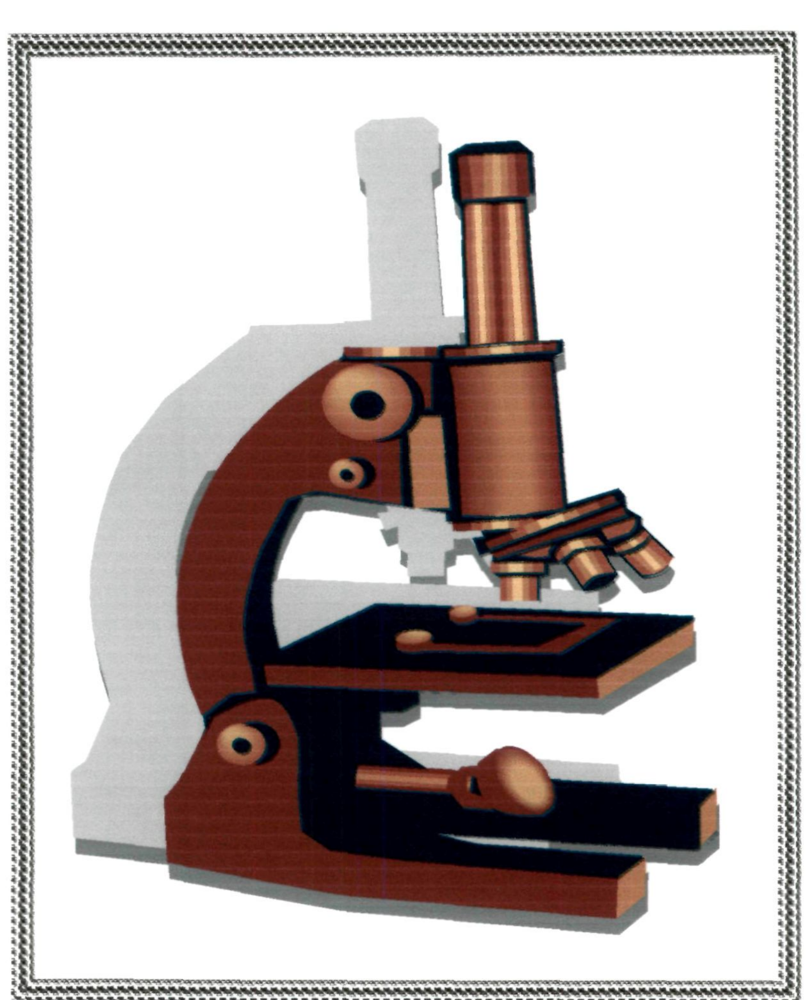
The cost of production of rough lemon plantlets using the technological information as per the protocol developed in the present investigation was computed based on the facilities available at Tissue Culture Laboratory, Faculty of Agriculture, Udheywalla, SKUAST-Jammu based on the rate of culture establishment, rate of multiplication, rooting response of shoots, survival of plantlets, maximum capacity of the laboratory and the number of initial explant capable of producing 1,00,000 plantlets was calculated. The total cost involved per cycle was worked out, which involves the cost of building, equipments, glassware, chemicals and miscellaneous items, having been distributed over the years according to their potential durability.

The cost of production of one plantlet of rough lemon grown *in vitro* upto polyhouse stage as well as field grown plant was eventually calculated.

3.7 Statistical analysis

The data generated from the various experiments were subjected to statistical analysis in Completely Randomized Design wherever necessary as prescribed by Panse and Sukhatme (1985).

Experimental Results



Experimental Results

The results of the experiments on *in vitro* propagation studies of *Citrus jambhiri* and *Citrus sinensis* cv. Mosambi conducted at the Tissue Culture Laboratory, Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Udheywalla during the period 2004-06 are presented in this chapter.

4.1 Culture establishment

4.1.1 Surface sterilization of nodal segment explants

The results of the surface sterilization of nodal segment explants taken from 10-12 year old mature trees of *Citrus jambhiri* Lush. and *Citrus sinensis* (L.) Osbeck. cv. Mosambi are presented in Table 5 and Table 6 respectively.

4.1.1.1 *Citrus jambhiri* explants

The perusal of data in Table 5 showed that a combination of ethanol 70 per cent and mercuric chloride 0.1 per cent for 30 seconds and 8 minutes respectively, resulted in least contamination 11.67 per cent and highest culture establishment 75.00 per cent as compared to all other treatments. Inspite of that, this combination also showed more death of culture 13.33 per cent followed by 11.67 per cent in both mercuric chloride 0.1 per cent for 8 minutes and sodium hypochlorite 10 per cent for 10 minutes. Higher concentration of mercuric chloride 0.1 per cent for 8 minutes resulted in 46.66 per cent culture establishment and 41.67 per cent contamination which varied significantly with all other treatments of mercuric chloride alone. The

Table 5. Standardization of surface sterilization treatments for nodal segment explants of *Citrus jambhiri*

Medium: MS + BAP (1.5 mg/l) + malt extract (500 mg/l)

Incubation: 3 weeks

S.No.	Sterilants	Duration	Contamination (%)	Death of culture (%)	Culture establishmen (%)
1.	Mercuric chloride (0.05 %)	5 minutes	68.33 (55.75)[+]	0.00 (0.00)	31.67 (34.22)
2.	Mercuric chloride (0.05 %)	8 minutes	61.67 (51.73)	0.00 (0.00)	38.33 (38.23)
3.	Mercuric chloride (0.1 %)	5 minutes	51.67 (45.94)	8.33 (16.50)	40.00 (39.19)
4.	Mercuric chloride (0.1 %)	8 minutes	41.67 (40.18)	11.67 (19.88)	46.66 (43.07)
5.	Ethanol (70 %) + Mercuric chloride (0.05 %)	30 seconds + 5 minutes	38.33 (38.23)	0.00 (0.00)	61.67 (51.73)
6.	Ethanol (70 %) + Mercuric chloride (0.05 %)	30 seconds + 8 minutes	31.67 (34.22)	0.00 (0.00)	68.33 (55.75)
7.	Ethanol (70 %) + Mercuric chloride (0.1 %)	30 seconds + 5 minutes	31.67 (34.22)	10.00 (18.04)	58.33 (49.80)
8.	Ethanol (70 %) + Mercuric chloride (0.1 %)	30 seconds + 8 minutes	11.67 (19.88)	13.33 (21.33)	75.00 (59.98)
9.	Sodium hypochlorite (5 %)	10 minutes	81.67 (64.67)	6.67 (14.75)	11.67 (19.88)
10	Sodium hypochlorite (10 %)	10 minutes	73.33 (58.91)	11.67 (19.88)	15.00 (22.78)
S.E. m (±) :			1.08	1.46	2.90
C.D. $_{(P = 0.05)}$:			3.22	4.33	3.48

[+] Figures given in parenthesis are transformed (angular) values

treatment of explant with sodium hypochlorite (5-10 per cent) resulted in lower culture establishment 11.67-15.00 per cent due to higher contamination rate 73.33-81.67 per cent.

4.1.1.2 *Citrus sinensis* cv. Mosambi explants

The results of surface sterilization of nodal segments of *Citrus sinensis* cv. Mosambi (Table 6) indicated that out of the various treatments tried, the treatment combination of ethanol 70 per cent for 30 seconds and mercuric chloride 0.1 per cent for 8 minutes resulted in minimum contamination 11.67 per cent and maximum explant survival 80 per cent which was statistically at par with combination of ethanol 70 per cent for 30 seconds and its subsequent treatment with mercuric chloride 0.05 per cent for 8 minutes. Sodium hypochlorite (5 and 10 per cent) each for 10 minutes proved to be weak surface sterilent as it resulted in less culture establishment (13.33 to 18.33 per cent) and higher contamination (78.33 to 68.33 per cent).

4.1.2 Culture establishment medium

In order to standardize a suitable culture establishment medium for nodal segment explants of 10-12 year old trees of *Citrus jambhiri* and *Citrus sinensis* cv. Mosambi, detailed trials were conducted using different treatments alone as well as in combinations of BAP, kinetin, malt extract and NAA. In all, 41 treatments were tried. The results on the number of days taken for culture establishment and per cent culture establishment obtained in each treatment for nodal segment explants of *Citrus jambhiri* and *Citrus sinensis* cv. Mosambi are presented in Table 7 and Table 8 respectively.

Table 6. Standardization of surface sterilization treatments for nodal segment explants of *Citrus sinensis* cv. Mosambi

Medium: MS + BAP (1.0 mg/l) + malt extract (500 mg/l)

Incubation: 3 weeks

S.No.	Sterilants	Duration	Contamination (%)	Death of culture (%)	Culture establishment (%)
1.	Mercuric chloride (0.05 %)	5 minutes	66.67 (54.73)+	0.00 (0.00)	33.33 (35.24)
2.	Mercuric chloride (0.05 %)	8 minutes	48.33 (44.02)	0.00 (0.00)	51.67 (45.95)
3.	Mercuric chloride (0.1 %)	5 minutes	50.00 (44.98)	5.00 (12.92)	45.00 (42.10)
4.	Mercuric chloride (0.1 %)	8 minutes	33.33 (35.24)	6.67 (14.75)	60.00 (50.77)
5.	Ethanol (70 %) + Mercuric chloride (0.05 %)	30 seconds + 5 minutes	31.67 (34.22)	0.00 (0.00)	68.33 (55.75)
6.	Ethanol (70 %) + Mercuric chloride (0.05 %)	30 seconds + 8 minutes	21.67 (27.70)	0.00 (0.00)	78.33 (62.27)
7.	Ethanol (70 %) + Mercuric chloride (0.1 %)	30 seconds + 5 minutes	23.33 (28.84)	6.67 (14.75)	70.00 (56.82)
8.	Ethanol (70 %) + Mercuric chloride (0.1 %)	30 seconds + 8 minutes	11.67 (19.88)	8.33 (16.59)	80.00 (63.52)
9.	Sodium hypochlorite (5 %)	10 minutes	78.33 (62.27)	8.33 (16.59)	13.33 (21.33)
10	Sodium hypochlorite (10 %)	10 minutes	68.33 (55.75)	13.33 (21.33)	18.33 (25.30)
S.E. m (±)			1.39	1.25	1.64
C.D. (P = 0.05)			4.14	3.17	4.86

+ Figures given in parenthesis are transformed (angular) values

4.1.2.1 *Citrus jambhiri* explants

The data presented in Table 7 on per cent culture establishment and the number of days taken for culture establishment indicated that the establishment of explant was affected by BAP, Kinetin, malt extract and NAA concentrations. The per cent culture establishment was more, while the time taken for culture establishment was less in medium supplemented with malt extract as compared to treatments without malt extract. The explants recorded least time 4.34 days for culture establishment with highest establishment of 94.45 per cent on MS medium supplemented with BAP 1.5 mg/l and malt extract 500 mg/l (Plate I) followed by MS medium containing BAP 1.0 mg/l with malt extract 500 mg/l and BAP 1.5 mg/l recording 91.67 per cent culture establishment which was at par with BAP 1.5 mg/l and malt extract 500 mg/l. Out of two cytokinins tried, BAP took significantly less time (7.12-12.15 days) for culture establishment than Kinetin (9.15-14.30 days). However, the highest level of BAP tested 2.0 mg/l registered only 58.33 per cent establishment of the cultures as compared to 91.67 and 88.89 per cent with BAP 1.5 and 1.0 mg/l respectively. It was observed that BAP (0.5, 1.0, 1.5 and 2.0 mg/l) in combination with lower levels of Kinetin (0.5 and 1.0 mg/l) took more time for culture establishment than the treatment containing BAP or Kinetin alone. The medium containing lower levels of BAP 0.5 and 1.0 mg/l in combination with NAA (0.25 and 0.5 mg/l) showed that the combination containing lower level of NAA (0.25 mg/l) took lesser time (9.04-9.54 days) and more culture establishment 69.94-75.00 per cent than those containing 0.5 mg/l NAA. Similar trend was followed when BAP or Kinetin in combination with NAA and malt extract was used. Culture

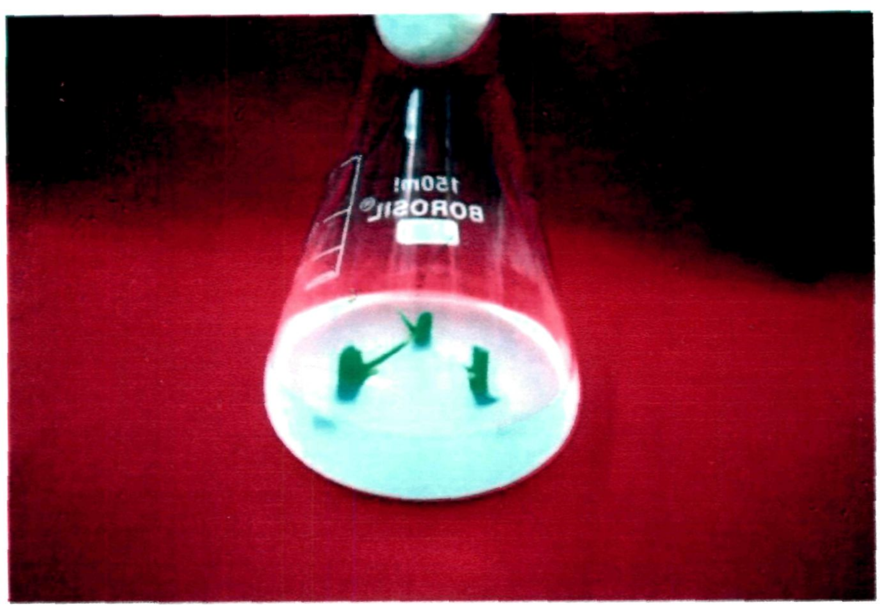


Plate I (a). Innoculation of nodal segments explants of *Citrus jambhiri* on MS medium supplemented with BAP 1.5 mg/l and malt extract 500 mg/l.

Plate I (b). Shoot initiation from nodal segment explants of *Citrus jambhiri* on MS medium supplemented with BAP 1.5 mg/l and malt extract 500 mg/l (After 2 week of incubation at 26 $\pm$ 2°C and exposed to 16 hours photoperiod).

Plate I (c). Shoot initiation from nodal segment explants of *Citrus jambhiri* on MS medium supplemented with BAP 1.5 mg/l and malt extract 500 mg/l (After 3 week of incubation at 26 $\pm$ 2°C and exposed to 16 hours photoperiod).

Table 7. Effect of cytokinins, malt extract and naphthalene acetic acid on culture establishment of nodal segments of *Citrus jambhiri*

Medium: MS

Incubation: 3 weeks

S.No.	Treatment	Time taken for culture establishment (days)	Percent Culture establishment
1	BAP (0.5 mg/l)	9.32	77.78 (61.94)$^{+}$
2	BAP (1.0 mg/l)	8.24	88.89 (70.76)
3	BAP (1.5 mg/l)	7.12	91.67 (76.36)
4	BAP (2.0 mg/l)	12.15	58.33 (49.82)
5	Kinetin (0.5 mg/l)	11.17	69.45 (56.47)
6	Kinetin (1.0 mg/l)	10.17	86.11 (68.32)
7	Kinetin (1.5 mg/l)	9.15	88.89 (70.76)
8	Kinetin (2.0 mg/l)	14.30	44.45 (41.79)
9	BAP (0.5 mg/l) + Malt extract (500 mg/l)	9.15	83.33 (66.35)
10	BAP (1.0 mg/l) + Malt extract (500 mg/l)	6.48	91.67 (76.36)
11	BAP (1.5 mg/l) + Malt extract (500 mg/l)	4.34	94.45 (78.80)
12	BAP (2.0 mg/l) + Malt extract (500 mg/l)	10.33	66.67 (54.82)
13	Kinetin (0.5 mg/l) + Malt extract (500 mg/l)	10.29	77.78 (61.94)

Contd...

14	Kinetin (1.0 mg/l) + Malt extract (500 mg/l)	8.28	86.11 (68.32)
15	Kinetin (1.5 mg/l) + Malt extract (500 mg/l)	6.45	88.89 (70.76)
16	Kinetin (2.0 mg/l) + Malt extract (500 mg/l)	12.32	55.55 (48.18)
17	BAP (0.5 mg/l) + Kinetin (0.5 mg/l)	11.34	69.45 (56.47)
18	BAP (1.0 mg/l) + Kinetin (0.5 mg/l)	9.86	77.78 (61.94)
19	BAP (1.5 mg/l) + Kinetin (0.5 mg/l)	7.43	86.11 (68.32)
20	BAP (2.0 mg/l) + Kinetin (0.5 mg/l)	12.62	50.00 (44.98)
21	BAP (0.5 mg/l) + Kinetin (1.0 mg/l)	13.70	50.00 (44.98)
22	BAP (1.0 mg/l) + Kinetin (1.0 mg/l)	12.80	72.22 (58.22)
23	BAP (1.5 mg/l) + Kinetin (1.0 mg/l)	11.44	77.78 (61.94)
24	BAP (2.0 mg/l) + Kinetin (1.0 mg/l)	14.70	44.45 (41.79)
25	BAP (0.5 mg/l) + NAA (0.25 mg/l)	9.54	69.94 (56.79)
26	BAP (0.5 mg/l) + NAA (0.5 mg/l)	13.00	41.67 (40.14)
27	BAP (1.0 mg/l) + NAA (0.25 mg/l)	9.04	75.00 (60.19)
28	BAP (1.0 mg/l) + NAA (0.5 mg/l)	11.70	47.22 (43.38)
29	BAP (0.5 mg/l) + NAA (0.25 mg/l) + Malt extract (500 mg/l)	8.68	86.11 (68.32)
30	BAP (0.5 mg/l) + NAA (0.5 mg/l) + Malt extract (500 mg/l)	11.40	44.45 (41.79)

Contd...

31	BAP (1.0 mg/l) + NAA (0.25 mg/l) + Malt extract (500 mg/l)	8.23	88.84 (70.76)
32	BAP (1.0 mg/l) + NAA (0.5 mg/l) + Malt extract (500 mg/l)	10.90	50.00 (44.98)
33	Kinetin (0.5 mg/l) + NAA (0.25 mg/l)	9.62	52.78 (46.58)
34	Kinetin (0.5 mg/l) + NAA (0.5 mg/l)	12.60	47.22 (43.38)
35	Kinetin (1.0 mg/l) + NAA (0.25 mg/l)	10.90	72.22 (58.22)
36	Kinetin (1.0 mg/l) + NAA (0.5 mg/l)	13.40	44.45 (41.79)
37	Kinetin (0.5 mg/l) + NAA (0.25 mg/l) + Malt extract (500 mg/l)	9.08	63.89 (53.07)
38	Kinetin (0.5 mg/l) + NAA (0.5 mg/l) + Malt extract (500 mg/l)	12.70	41.67 (40.14)
39	Kinetin (1.0 mg/l) + NAA (0.25 mg/l) + Malt extract (500 mg/l)	9.02	80.55 (63.91)
40	Kinetin (1.0 mg/l) + NAA (0.5 mg/l) + Malt extract (500 mg/l)	11.60	61.10 (51.42)
41	Control	14.80	33.33 (35.14)
S.E. m (±)	:	0.08	2.92
C.D. (P = 0.05)	:	0.22	8.24

[+] Figures given in parenthesis are transformed (angular) values

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establishment was slow on basal medium alone (control) requiring maximum days (14.80) for establishment with minimum culture establishment 33.33 per cent as compared to other treatments.

4.1.2.2 *Citrus sinensis* cv. Mosambi explants

The data pertaining to the effect of different cytokinins, malt extract and NAA on per cent culture establishment and number of days taken for culture establishment (Table 8), revealed that nodal segment explants of Mosambi recorded maximum establishment 97.22 per cent and took minimum days 5.22 for culture establishment when inoculated on MS medium supplemented with BAP 1.0 mg/l and malt extract 500 mg/l (Plate II) which was significantly superior to all other treatments. Further, the lower doses of both BAP (0.5 and 1.0 mg/l) and Kinetin (0.5 and 1.0 mg/l) alone or in combination with malt extract 500 mg/l took less time and resulted in more culture establishment as compared to higher levels of BAP (1.5 and 2.0 mg/l) and Kinetin (1.5 and 2.0 mg/l). The results also revealed that out of two cytokinins (BAP and Kinetin) used, BAP was superior to Kinetin as on an average BAP registered 84.72 per cent establishment as compared to 73.61 per cent with Kinetin. Increase in concentration of BAP and Kinetin above 1.0 mg/l i.e. (1.5 and 2.0 mg/l) resulted in callus induction (Plate Va) with poor culture establishment. The lower levels of Kinetin (0.5 and 1.0 mg/l) with malt extract 500 mg/l took lesser time (7.26-7.60 days) for 88.89-91.67 per cent culture establishment as compared to higher levels of Kinetin (i.e. 10.31-12.23 days for 61.11 to 69.45 per cent culture establishment). Out of various combinations of BAP (0.5, 1.0, 1.5 and 2.0 mg/l) and Kinetin (0.5 and 1.0 mg/l), the combination of BAP 1.0 mg/l with Kinetin 0.5 mg/l took less time 7.73

Table 8. Effect of cytokinins, malt extract and naphthalene acetic acid on culture establishment of nodal segments of *Citrus sinensis* cv. Mosambi

Medium: MS

Incubation: 3 weeks

S.No.	Treatment	Time taken for culture establishment (days)	Percent Culture establishment
1	BAP (0.5 mg/l)	7.42	91.67 (76.36)$^{+}$
2	BAP (1.0 mg/l)	6.43	94.45 (78.80)
3	BAP (1.5 mg/l)	9.41	80.55^{++} (63.91)
4	BAP (2.0 mg/l)	12.27	72.22^{++} (58.22)
5	Kinetin (0.5 mg/l)	9.42	86.11 (71.95)
6	Kinetin (1.0 mg/l)	8.43	88.89 (73.92)
7	Kinetin (1.5 mg/l)	11.40	69.45^{++} (56.47)
8	Kinetin (2.0 mg/l)	13.36	50.00^{++} (44.98)
9	BAP (0.5 mg/l) + Malt extract (500 mg/l)	6.12	94.45 (78.80)
10	BAP (1.0 mg/l) + Malt extract (500 mg/l)	5.22	97.22 (84.40)
11	BAP (1.5 mg/l) + Malt extract (500 mg/l)	8.20	80.55^{++} (63.91)
12	BAP (2.0 mg/l) + Malt extract (500 mg/l)	11.22	75.00^{++} (60.19)
13	Kinetin (0.5 mg/l) + Malt extract (500 mg/l)	7.60	88.89 (70.76)

Contd...

14	Kinetin (1.0 mg/l) + Malt extract (500 mg/l)	7.26	91.67 (76.36)
15	Kinetin (1.5 mg/l) + Malt extract (500 mg/l)	10.31	69.45^{++} (56.47)
16	Kinetin (2.0 mg/l) + Malt extract (500 mg/l)	12.23	61.11^{++} (51.42)
17	BAP (0.5 mg/l) + Kinetin (0.5 mg/l)	9.85	83.33 (66.35)
18	BAP (1.0 mg/l) + Kinetin (0.5 mg/l)	7.73	88.89 (70.76)
19	BAP (1.5 mg/l) + Kinetin (0.5 mg/l)	12.56	72.22^{++} (58.22)
20	BAP (2.0 mg/l) + Kinetin (0.5 mg/l)	12.85	69.45^{++} (56.47)
21	BAP (0.5 mg/l) + Kinetin (1.0 mg/l)	9.63	80.55 (63.91)
22	BAP (1.0 mg/l) + Kinetin (1.0 mg/l)	12.40	83.33 (66.35)
23	BAP (1.5 mg/l) + Kinetin (1.0 mg/l)	13.30	66.67^{++} (54.82)
24	BAP (2.0 mg/l) + Kinetin (1.0 mg/l)	13.50	50.00^{++} (44.98)
25	BAP (0.5 mg/l) + NAA (0.25 mg/l)	9.35	75.00 (60.19)
26	BAP (0.5 mg/l) + NAA (0.5 mg/l)	12.50	44.45 (41.79)
27	BAP (1.0 mg/l) + NAA (0.25 mg/l)	8.72	80.55 (63.91)
28	BAP (1.0 mg/l) + NAA (0.5 mg/l)	11.20	47.22 (43.38)
29	BAP (0.5 mg/l) + NAA (0.25 mg/l) + Malt extract (500 mg/l)	8.20	88.89 (70.76)
30	BAP (0.5 mg/l) + NAA (0.5 mg/l) + Malt extract (500 mg/l)	11.00	47.22 (43.38)

Contd...

31	BAP (1.0 mg/l) + NAA (0.25 mg/l) + Malt extract (500 mg/l)	7.34	88.89 (70.76)
32	BAP (1.0 mg/l) + NAA (0.5 mg/l) + Malt extract (500 mg/l)	10.30	50.00 (44.98)
33	Kinetin (0.5 mg/l) + NAA (0.25 mg/l)	10.40	61.11 (51.42)
34	Kinetin (0.5 mg/l) + NAA (0.5 mg/l)	13.10	41.67 (40.1)
35	Kinetin (1.0 mg/l) + NAA (0.25 mg/l)	9.23	72.22 (58.22)
36	Kinetin (1.0 mg/l) + NAA (0.5 mg/l)	12.20	44.45 (41.79)
37	Kinetin (0.5 mg/l) + NAA (0.25 mg/l) + Malt extract (500 mg/l)	8.67	69.44 (56.79)
38	Kinetin (0.5 mg/l) + NAA (0.5 mg/l) + Malt extract (500 mg/l)	12.20	44.45 (41.79)
39	Kinetin (1.0 mg/l) + NAA (0.25 mg/l) + Malt extract (500 mg/l)	8.33	83.33 (66.35)
40	Kinetin (1.0 mg/l) + NAA (0.5 mg/l) + Malt extract (500 mg/l)	11.10	66.67 (54.82)
41	Control	13.27	36.11 (36.90)
S.E. m (±)	:	0.07	3.67
C.D. $_{(P = 0.05)}$	:	0.19	10.34

[+] Figures given in parenthesis are transformed (angular) values

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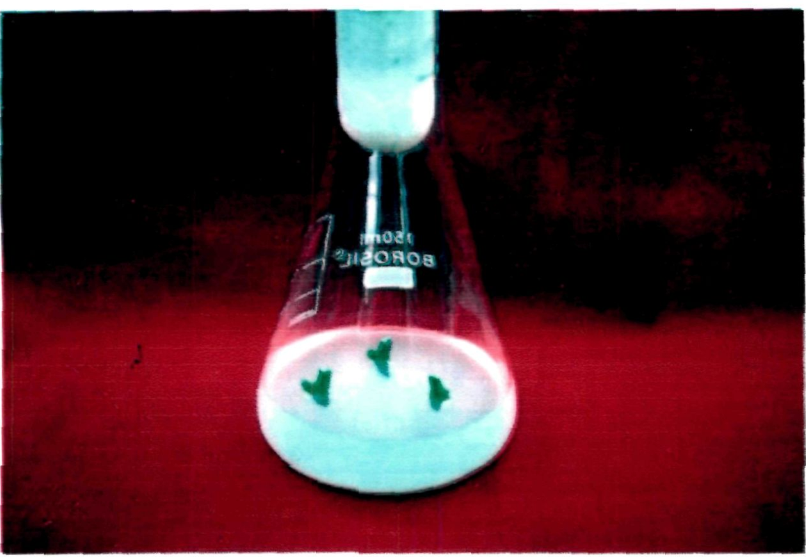


Plate II (a). Innoculation of nodal segments explants of *Citrus sinensis* cv. Mosambi on MS medium supplemented with BAP 1.0 mg/l and malt extract 500 mg/l.

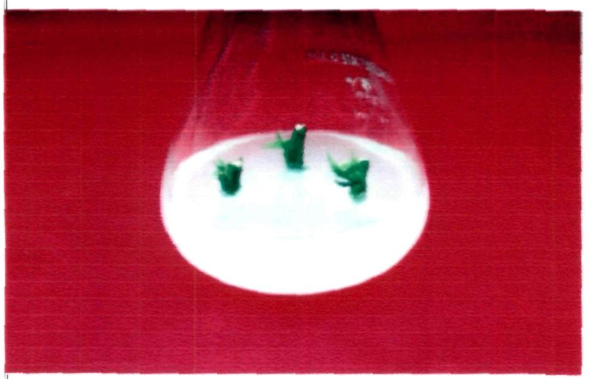


Plate II (b). Shoot initiation from nodal segments explants of *Citrus sinensis* cv. Mosambi on MS medium supplemented with BAP 1.0 mg/l and malt extract 500 mg/l (After 1 week of incubation at 26 $\pm$ 2°C and exposed to 16 hours photoperiod).

Plate II (c). Shoot initiation from nodal segments explants of *Citrus sinensis* cv. Mosambi on MS medium supplemented with BAP 1.0 mg/l and malt extract 500 mg/l (After 2 weeks of incubation at 26 $\pm$ 2°C and exposed to 16 hours photoperiod).

days which varied significantly with all treatments of this combination. BAP 1.0 mg/l and NAA 0.25 mg/l took minimum time 8.72 days for establishment of 80.55 per cent culture which was at par with BAP 0.5 mg/l and NAA 0.25 mg/l as it resulted in 75.00 per cent culture establishment. Similar trend was observed when NAA 0.25 mg/l was used in combination with BAP (0.5 and 1.0 mg/l) and malt extract 500 mg/l. Likewise, Kinetin with NAA and Kinetin with NAA and malt extract showed similar trend as that of BAP, while it took more number of days and less culture establishment than BAP with NAA and BAP with NAA and malt extract. The culture establishment was least 36.11 per cent on basal medium alone (control) requiring maximum days 13.27 for establishment as compared to other treatments.

4.2 Shoot proliferation

4.2.1 Shoot proliferation medium for nodal segments taken from *C. jambhiri* Lush.

4.2.1.1 Effect of cytokinins, malt extract and naphthalene acetic acid on shoot proliferation

In order to fix the optimum growth regulator concentration for shoot proliferation medium, the nodal segments which showed culture establishment above 85 per cent were cultured on the selected treatments from establishment trial for their proliferation. The results on the frequency of explants showing multiple shoots, number of shoots per culture, length of longest shoot (cm) and number of leaves on the longest shoot are presented in Table 9.

The influence of BAP 1.0 and 1.5 mg/l with malt extract 500 mg/l and BAP 1.5 mg/l with Kinetin 0.5 mg/l was found to be significantly superior to BAP 1.0 mg/l and Kinetin 1.0 and 1.5 mg/l alone or BAP in combination with NAA and malt

Table 9. Effect of cytokinins, malt extract and naphthalene acetic acid on shoot proliferation from nodal segments of *Citrus jambhiri*

Medium: MS

Incubation: 3 weeks

S.No.	Treatment	Per cent multiple shoots	No. of shoots per culture	Length of longest shoot (cm)	Number of leaves on longest shoot
1	BAP (1.0 mg/l)	83.33 (66.17)[+]	3.88 (2.21)[++]	1.89	8.75 (3.12)[++]
2	BAP (1.5 mg/l)	91.67 (76.18)	4.41 (2.33)	1.39	8.23 (3.04)
3	Kinetin (1.0 mg/l)	66.67 (54.80)	3.20 (2.05)	1.77	7.53 (2.92)
4	Kinetin (1.5 mg/l)	87.50 (73.08)	3.88 (2.21)	1.26	7.13 (2.85)
5	BAP (1.0 mg/l) + Malt extract (500 mg/l)	100.00 (90.00)	4.50 (2.35)	2.31	9.13 (3.18)
6	BAP (1.5 mg/l) + Malt extract (500 mg/l)	100.00 (90.00)	5.34 (2.52)	1.96	8.88 (3.14)
7	Kinetin (1.0 mg/l) + Malt extract (500 mg/l)	79.17 (63.07)	4.34 (2.31)	2.19	8.79 (3.13)
8	Kinetin (1.5 mg/l) + Malt extract (500 mg/l)	83.33 (66.17)	4.67 (2.38)	1.87	8.37 (3.06)
9	BAP (1.5 mg/l) + Kinetin (0.5 mg/l)	100.00 (90.00)	4.68 (2.38)	1.34	8.38 (3.06)
10	BAP (1.0 mg/l) + NAA (0.25 mg/l) + Malt extract (500 mg/l)	41.67 (40.16)	2.36 (1.83)	1.17	7.17 (2.86)
11	BAP (1.5 mg/l) + NAA (0.25 mg/l) + Malt extract (500 mg/l)	50.00 (44.98)	2.46 (1.86)	1.06	7.08 (2.84)
S.E. m (±)	:	3.76	0.11	0.02	0.10
C.D. $_{(P=0.05)}$	:	11.13	0.33	0.05	0.30

[+] Figures given in parenthesis are transformed (angular) values

[++] Figures given in parenthesis are transformed (sq. root) values

extract. The frequency of the cultures showing multiple shoots ranged from 83.33 to 100 per cent in treatment containing BAP (1.0 and 1.5 mg/l) alone or in combination with malt extract 500 mg/l, whereas it ranged from 66.67 to 87.50 per cent in treatments containing Kinetin 1.0 and 1.5 mg/l alone or in combination with malt extract 500 mg/l.

The treatment combination of BAP 1.5 mg/l with malt extract 500 mg/l gave maximum number of shoots 5.34 (Plate III) with cent per cent cultures showing multiple shoots followed by 1.5 mg/l BAP with 0.5 mg/l Kinetin and 1.0 mg/l BAP with malt extract 500 mg/l. However, BAP 1.5 mg/l with malt extract 500 mg/l was significantly superior to all other treatments. Incorporation of NAA with BAP in combination with malt extract had negative influence on the induction of multiple shoot, as the number of shoots produced by using the same concentration of either BAP alone, in combination with malt extract or Kinetin was significantly higher than those treatments where NAA was incorporated.

The effect of different treatments on the length of longest shoot and number of leaves on longest shoot in proliferation medium was significant. The maximum length of longest shoot 2.31 cm was obtained on the medium containing BAP 1.0 mg/l in combination with malt extract 500 mg/l which varied significantly with all other treatments. On the other hand, maximum number of leaves on the longest shoot 9.13 were recorded in the same treatment combination which was followed by BAP 1.5 mg/l with malt extract 500 mg/l and Kinetin 1.0 mg/l with malt extract 500 mg/l, and these were significantly superior to all other treatments. In general, addition of malt extract to BAP and Kinetin or Kinetin to BAP increased the length of longest

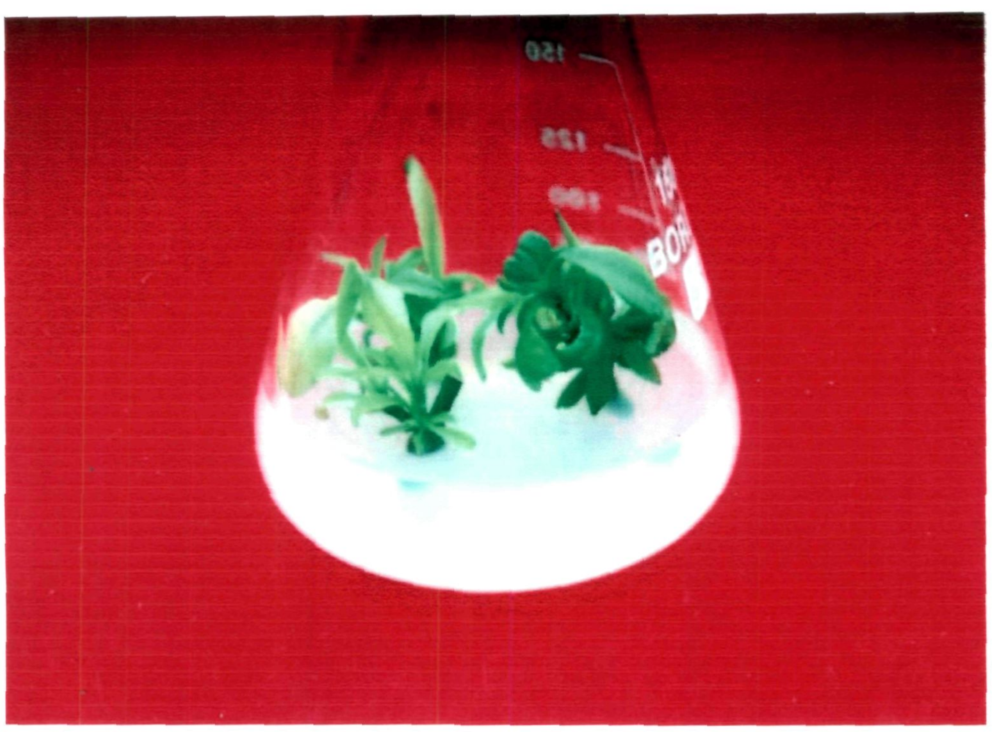


Plate III. Shoot proliferation from nodal segment explants of *Citrus jambhiri* on MS medium supplemented with BAP 1.5 mg/l and malt extract 500 mg/l (after 6 weeks of incubation at 26±2°C and exposed to 16 hours photoperiod).

shoot as well as number of leaves on longest shoot, whereas NAA in combination with BAP and malt extract reduced it.

4.2.1.2 Effect of continuous sub-culturing

In order to study the multiplication rate during serial sub-culturing, a trial was conducted by supplementing MS medium with different concentration of BAP 1.0 and 1.5 mg/l and Kinetin 1.0 and 1.5 mg/l alone or in combination with malt extract 500 mg/l and BAP 1.5 mg/l with Kinetin 0.5 mg/l. Since NAA did not show any significant effect on shoot proliferation, the treatment combination containing BAP, NAA and malt extract was discarded during further sub-culturing. Thus, in all 9 treatment combinations were tried. The results presented in Table 10 revealed that in general, the rate of multiplication decreased gradually with the increase in number of sub-culturing done at 6 weeks interval in all the treatments tried. The maximum number of shoots was produced by treatment combination containing BAP 1.5 mg/l and malt extract 500 mg/l. In all, 1576.33 shoots were produced from an initial explant in about 24 week time. This was far superior to all other treatments. Further, the reduction in the growth and vigour of the cultures was observed during continuous sub-culturing in all the treatments tried (Plate IV).

4.2.2 Shoot proliferation medium for nodal segments taken for *Citrus sinensis* cv. Mosambi

4.2.2.1 Effect of cytokinins, malt extract and naphthalene acetic acid on shoot proliferation

In order to standardize a suitable shoot proliferation medium, 11 treatment combinations comprising BAP, Kinetin, malt extract and NAA were selected from that establishment trial. Nodal segments established on MS medium supplemented

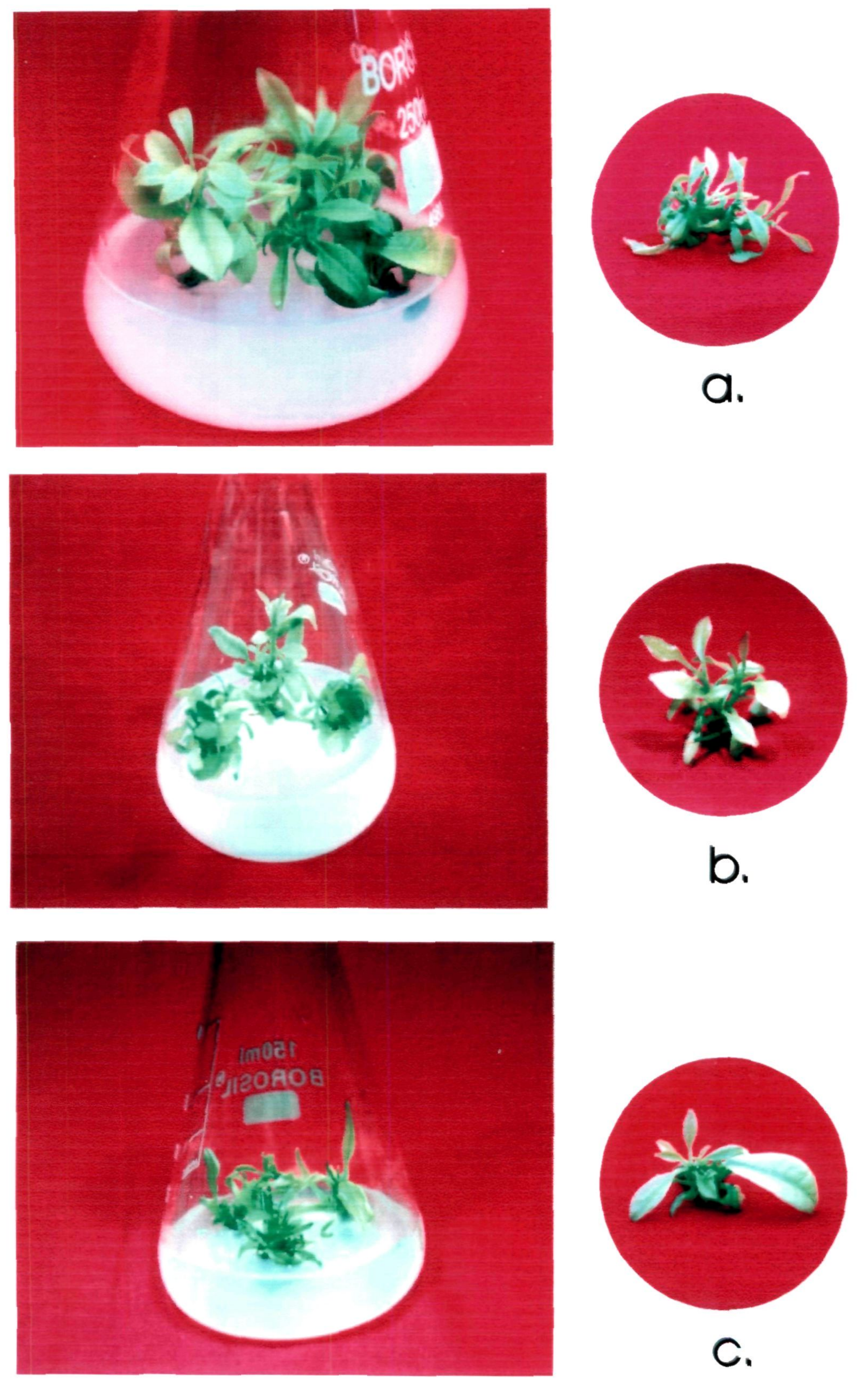


Plate IV. Serial sub-culturing for shoot multiplication from nodal segment explants of *Citrus jambhiri* on MS medium supplemented with BAP 1.5 mg/l and malt extract 500 mg/l (after 6 weeks of incubation at 26±2°C and exposed to 16 hours photoperiod).

a. After Ist sub-culture. b. After IInd sub-culture. c. After IIIrd sub-culture.

Table 10. Effect of cytokinins and malt extract on shoot multiplication of *Citrus jambhiri* from nodal segments

S.No.	Treatment	Number of shoots per sub culture			Number of total shoots produced per initial explant
		I	II	III	
1	BAP (1.0 mg/l)	8.36 (3.06)+	6.58 (2.75)	4.46 (2.34)	829.25
2	BAP (1.5 mg/l)	8.46 (3.08)	6.65 (2.77)	4.86 (2.42)	1205.78
3	Kinetin (1.0 mg/l)	6.40 (2.72)	5.02 (2.45)	2.92 (1.98)	300.20
4	Kinetin (1.5 mg/l)	6.94 (2.82)	5.14 (2.48)	3.08 (2.02)	426.29
5	BAP (1.0 mg/l) + Malt extract (500 mg/l)	8.54 (3.09)	6.70 (2.77)	4.64 (2.38)	1194.71
6	BAP (1.5 mg/l) + Malt extract (500 mg/l)	8.79 (3.13)	6.73 (2.78)	4.99 (2.45)	1576.33
7	Kinetin (1.0 mg/l) + Malt extract (500 mg/l)	7.07 (2.84)	5.19 (2.49)	3.12 (2.03)	496.86
8	Kinetin (1.5 mg/l) + Malt extract (500 mg/l)	7.30 (2.88)	5.34 (2.52)	4.04 (2.25)	735.47
9	BAP (1.5 mg/l) + Kinetin (0.5 mg/l)	8.49 (3.08)	6.70 (2.78)	4.92 (2.43)	1309.77
S.E. m (±)	:	0.01	0.00	0.00	
C.D. $_{(P = 0.05)}$	:	0.02	0.01	0.01	

+ Figures given in parenthesis are transformed (sq. root) values

with BAP 1.0 mg/l in combination with malt extract 500 mg/l were recultured on these media. The results obtained are presented in Table 11.

BAP 0.5 and 1.0 mg/l in combination with malt extract 500 mg/l, BAP 1.0 mg/l and Kinetin 0.5 mg/l showed cent per cent multiple shoots followed by BAP 1.0 mg/l which was at par with these treatment combinations. The maximum number of shoots 7.71 were obtained in treatment combination of BAP 1.0 mg/l and malt extract 500 mg/l which varied significantly with all other treatments (Plate Vb). However, the length of longest shoot was significantly more 2.40 cm in treatment combination of BAP 0.5 mg/l with malt extract 500 mg/l. Regarding the number of leaves on the longest shoot, BAP 1.0 mg/l and malt extract 500 mg/l showed maximum number of leaves 9.84 followed by BAP 0.5 mg/l and malt extract 500 mg/l. Addition of NAA to BAP and malt extract reduced the per cent multiple shoots and number of shoots per explant ranging from 41.67-54.17 and 3.20-3.63 respectively, which were significantly lower than other treatments tried. Moreover length of longest shoot and number of leaves on longest shoot also showed a decline with incorporation of NAA.

4.2.2.2 Effect of continuous sub-culturing

To study the effect of three continuous sub-culturing done at an interval of 6 weeks are presented in Table 12 with BAP (0.5 and 1.0 mg/l), Kinetin (0.5 and 1.0 mg/l) alone or in combination with malt extract 500 mg/l and BAP 1.0 mg/l in combination with Kinetin 0.5 mg/l. The rate of multiplication gradually decreased with the increase in number of sub-cultures. The number of shoots in Ist, IInd, and IIIrd sub-cultures were 9.48, 7.42 and 6.84 respectively obtained from the significantly superior treatment combination containing BAP 1.0 mg/l with malt extract 500 mg/l.

Table 11. Effect of cytokinins, malt extract and naphthalene acetic acid on shoot proliferation from nodal segments of *Citrus sinensis* cv. Mosambi

Medium: MS
Incubation: 3 weeks

S.No.	Treatment	Per cent multiple shoots	No. of shoots per culture	Length of longest shoot (cm)	Number of leaves on longest shoot
1	BAP (0.5 mg/l)	87.50 (73.08)[+]	4.92 (2.43)[++]	1.98	9.38 (3.22)[++]
2	BAP (1.0 mg/l)	95.83 (83.09)	5.13 (2.48)	1.45	8.44 (3.07)
3	Kinetin (0.5 mg/l)	79.17 (63.07)	4.05 (2.24)	1.76	8.96 (3.16)
4	Kinetin (1.0 mg/l)	83.33 (66.17)	4.75 (2.40)	1.53	8.38 (3.06)
5	BAP (0.5 mg/l) + Malt extract (500 mg/l)	100.00 (90.00)	6.21 (2.69)	2.40	9.79 (3.29)
6	BAP (1.0 mg/l) + Malt extract (500 mg/l)	100.00 (90.00)	7.71 (2.95)	2.12	9.84 (3.29)
7	Kinetin (0.5 mg/l) + Malt extract (500 mg/l)	83.33 (66.17)	5.67 (2.58)	2.02	9.13 (3.18)
8	Kinetin (1.0 mg/l) + Malt extract (500 mg/l)	87.50 (73.08)	6.25 (2.69)	1.89	8.75 (3.12)
9	BAP (1.0 mg/l) + Kinetin (0.5 mg/l)	100.00 (90.00)	5.11 (2.47)	1.56	8.88 (3.14)
10	BAP (0.5 mg/l) + NAA (0.25 mg/l) + Malt extract (500 mg/l)	41.67 (40.16)	3.20 (2.05)	1.28	7.29 (2.88)
11	BAP (1.0 mg/l) + NAA (0.25 mg/l) + Malt extract (500 mg/l)	54.17 (47.39)	3.63 (2.15)	1.05	7.17 (2.86)
S.E. m (±) :		4.73	0.05	0.02	0.03
C.D. (P = 0.05) :		13.95	0.15	0.07	0.10

[+] Figures given in parenthesis are transformed (angular) values
[++] Figures given in parenthesis are transformed (sq. root) value

Table 12. Effect of cytokinins and malt extract on shoot multiplication of *Citrus sinensis* cv. Mosambi from nodal segments

S.No.	Treatment	Number of shoots per sub culture			Number of total shoots produced per initial explant
		I	II	III	
1	BAP (0.5 mg/l)	8.96 (3.16)[+]	6.97 (2.82)	4.42 (2.33)	1358.09
2	BAP (1.0 mg/l)	9.14 (3.19)	7.12 (2.85)	4.51 (2.35)	1505.64
3	Kinetin (0.5 mg/l)	6.82 (2.80)	5.56 (2.56)	3.30 (2.07)	506.7
4	Kinetin (1.0 mg/l)	7.76 (2.96)	5.84 (2.62)	3.58 (2.14)	770.64
5	BAP (0.5 mg/l) + Malt extract (500 mg/l)	9.28 (3.21)	7.26 (2.87)	4.66 (2.38)	1949.68
6	BAP (1.0 mg/l) + Malt extract (500 mg/l)	9.48 (3.24)	7.42 (2.90)	6.84 (2.79)	3709.56
7	Kinetin (0.5 mg/l) + Malt extract (500 mg/l)	7.05 (2.84)	5.32 (2.51)	3.64 (2.15)	774.08
8	Kinetin (1.0 mg/l) + Malt extract (500 mg/l)	7.54 (2.92)	5.68 (2.59)	4.26 (2.29)	1140.27
9	BAP (1.0 mg/l) + Kinetin (0.5 mg/l)	9.24 (3.20)	7.21 (2.87)	4.58 (2.36)	1559.17
S.E. m (±)	:	0.02	0.00	0.06	
C.D. $_{(P = 0.05)}$	:	0.06	0.01	0.18	

[+] Figures given in parenthesis are transformed (sq. root) values

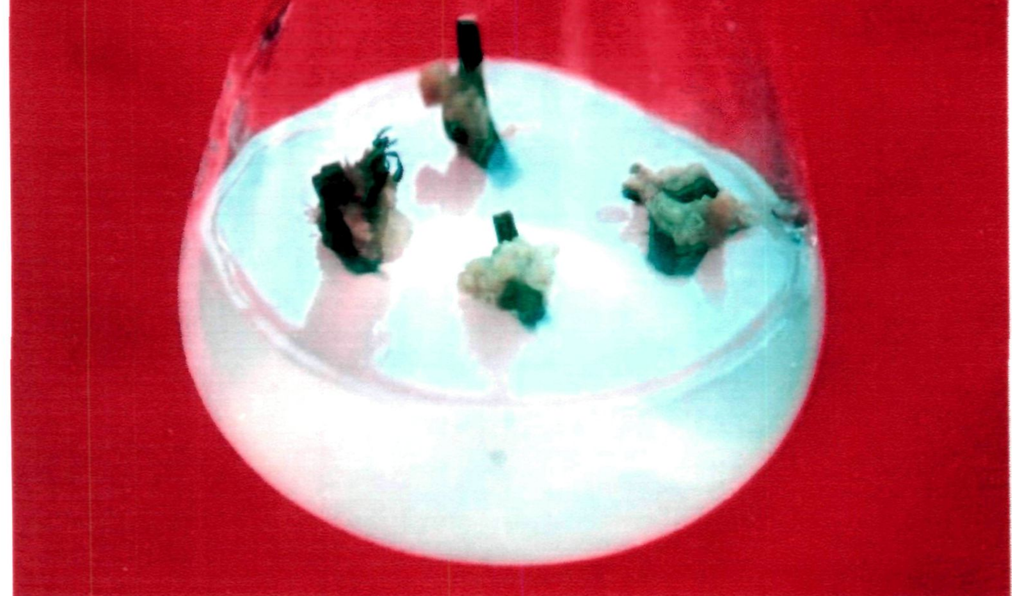


Plate V(a). Callus induction on the cut ends of the explants from *Citrus sinensis* cv. Mosambi on MS medium supplemented with BAP 2.0 mg/l and malt extract 500 mg/l

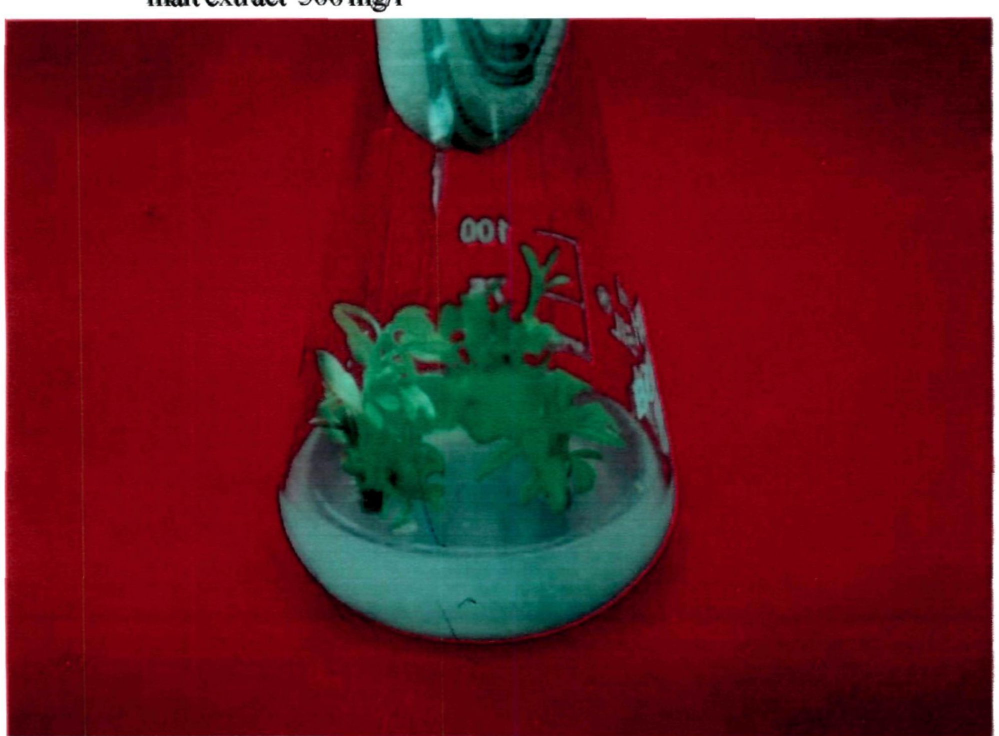


Plate V(b). Shoot proliferation from nodal segment explants of *Citrus sinensis* cv. Mosambi on MS medium supplemented with BAP 1.0 mg/l and malt extract 500 mg/l (after 6 weeks of incubation at 26$\pm$2°C and exposed to 16 hours photoperiod).

Thus, 3709.56 shoots were produced from an initial explant in same treatment combination which showed maximum number of shoots per culture and also it was superior to all other treatments. A reduction in the growth and vigour of the cultures during sub-culturing was also observed in all treatments tried (Plate VI).

4.3 *In vitro* rooting

4.3.1 Effect of auxins on *in vitro* rooting and shoot growth

The data pertaining to the response of different auxins (IBA and NAA) concentrations alone as well as in combination on *in vitro* rooting of *Citrus jambhiri* shoots are presented in Table 13.

MS medium with half strength of salt concentrations was used as a basal medium. Out of the 13 treatments tried, only IBA (0.25 mg/l) and control (which was devoid of hormones) failed to produce rooting in the shoot. The root initiation from the *in vitro* derived shoots of rough lemon started within 16-34 days. The results indicated that half strength MS medium fortified with 1 mg/l NAA and IBA each was earlier (16.51 days) to show the root induction (Plate VII) with highest per cent rooting (83.33), whereas IBA 0.5 mg/l took more time (34.28 days) for root initiation. Lowest percentage of rooting (13.33 per cent) was observed on medium supplemented with NAA 0.25 mg/l which was followed by IBA 0.5 mg/l.

Maximum number of roots per shoot (2.47) and length of longest root (3.57 cm) was observed in the medium supplemented with NAA and IBA (1.0 mg/l) each followed by a combination of (0.75 mg/l) NAA and IBA each; while length of shoot (2.91 cm) and number of leaves per shoot (4.67) were also higher in 1.0 mg/l NAA and 1.0 mg/l IBA combination (Plate VIII).

Table 13. Effect of auxin concentration on *in vitro* rooting characters of *Citrus jambhiri*

Medium: MS (half-strength)
Incubation: 5 weeks

S.No.	Treatment	Root initiation (days)	Per cent Culture rooted	No. of root per shoot	Length of longest root (cm)	Length of shoot (cm)	Number of leaves per shoot
1	IBA (0.25 mg/l)	0.00	0.00 (0.00)*	0.00 (1.00)**	0.00	1.25	2.09 (1.76)**
2	IBA (0.50 mg/l)	34.28	23.33 (28.77)	1.56 (1.60)	1.05	1.48	2.86 (1.96)
3	IBA (0.75 mg/l)	30.85	43.33 (41.14)	1.62 (1.62)	1.10	1.57	3.11 (2.03)
4	IBA (1.0 mg/l)	29.80	53.33 (46.90)	1.62 (1.62)	1.26	1.83	3.43 (2.11)
5	NAA (0.25 mg/l)	33.50	13.33 (21.14)	1.17(1.47)	0.90	1.37	3.66 (2.16)
6	NAA (0.50 mg/l)	26.36	30.00 (32.99)	1.64 (1.62)	1.25	1.71	3.93 (2.22)
7	NAA (0.75 mg/l)	21.21	50.00 (44.98)	1.68 (1.64)	1.41	1.90	4.16 (2.27)
8	NAA (1.0 mg/l)	19.29	73.33 (58.98)	1.92 (1.71)	1.55	2.12	4.33 (2.31)
9	IBA (0.25 mg/l) + NAA (0.25 mg/l)	32.44	30.00 (32.99)	1.28 (1.51)	1.38	1.92	4.06 (2.25)
10	IBA (0.50 mg/l) + NAA (0.50 mg/l)	22.27	50.00 (44.98)	1.86 (1.69)	1.74	2.12	4.35 (2.31)
11	IBA (0.75 mg/l) + NAA (0.75 mg/l)	19.21	63.33 (52.75)	2.17 (1.78)	2.84	2.34	4.54 (2.35)
12	IBA (1.0 mg/l) + NAA (1.0 mg/l)	16.51	83.33 (66.12)	2.47 (1.86)	3.57	2.91	4.67 (2.38)
13	Control	0.00	0.00 (0.00)	0.00 (1.00)	0.00	1.15	2.02 (1.74)
S.E. m (±)	:	0.19	2.55	0.03	0.03	0.03	0.01
C.D. $_{(P = 0.05)}$	:	0.55	7.45	0.08	0.08	0.08	0.03

* Figures given in parenthesis are transformed (angular) values

** Figures given in parenthesis are transformed (sq. root) values

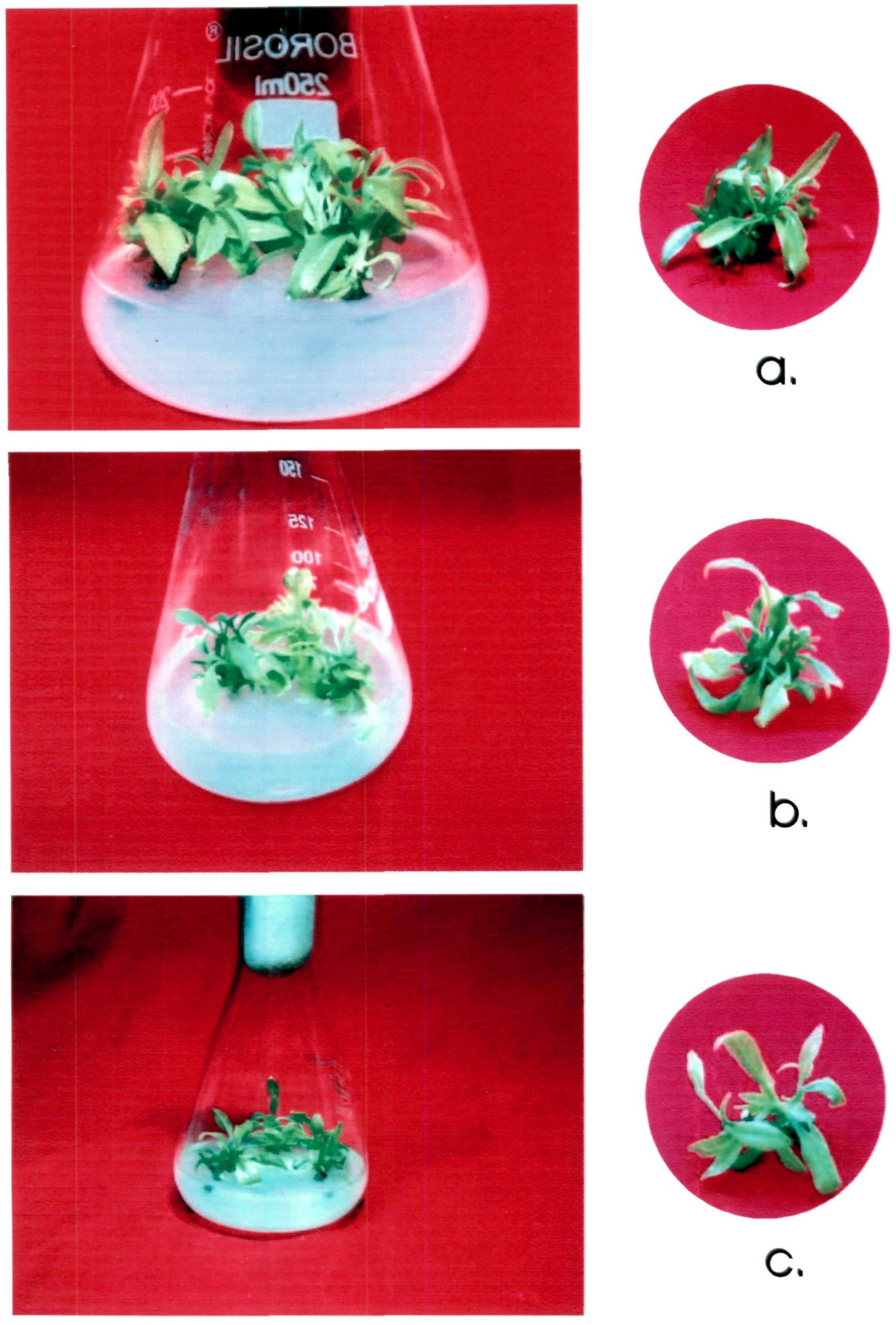
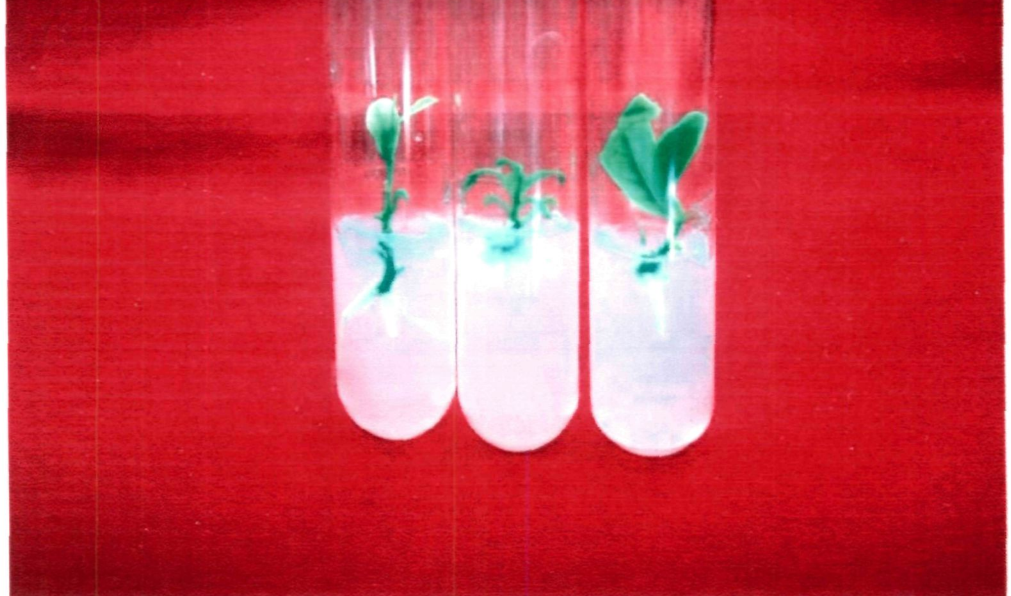


Plate VI. Serial sub-culturing for shoot multiplication from nodal segment explants of *Citrus sinensis* cv. Mosambi on MS medium supplemented with BAP 1.0 mg/l and malt extract 500 mg/l (after 6 weeks of incubation at 26$\pm$2^{0}C and exposed to 16 hours photoperiod).

a. After Ist sub-culture. b. After IInd sub-culture. c. After IIIrd sub-culture.

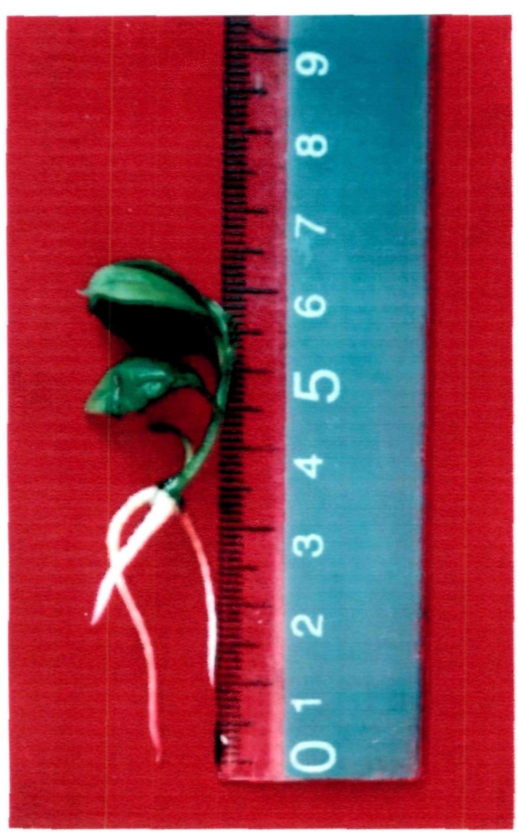


a. Root initiation of *Citrus jambhiri* in half strength medium supplemented with NAA and IBA (1.0 mg/l) each (after 3 weeks of incubation)

b. *In vitro* rooting of *Citrus jambhiri*

Plate VII. Effect of auxin concentration on rooting of *Citrus jambhiri*

a. *In vitro* rooting of shoots on MS (half strength medium) containing NAA and IBA (1.0 mg/l) each



b. Length of longest root of *in vitro* produced plantlet of *Citrus jambhiri* on MS (half strength) medium supplemented with NAA and IBA (1.0 mg/l) each

c. Length of root of *in vitro* produced plantlet of *Citrus jambhiri* on MS (half strength) medium supplemented with NAA and IBA (0.5 mg/l) each

Plate VIII. Effect of rooting medium on root and shoot character of *Citrus jambhiri* plantlets from nodal segment explant source at 26±2°C and exposed to 16 hours photoperiod

4.3.2 Effect of supporting media on *in vitro* rooting and shoot growth

The data related to the effect of supporting media on *in vitro* rooting and shoot growth characteristics of *Citrus jambhiri* as shown in Table 14 revealed that 86.67 per cent cultures showed rooting on medium gelled with 0.4 per cent agar which varied significantly with 0.8 per cent agar, whereas all other supporting media such as Whatman No. 1 filter paper, ordinary filter paper and brown paper were found unsuitable for rooting.

The time taken for root initiation was minimum (16.00 days) in medium gelled with agar 0.4 per cent which varied significantly with agar 0.8 per cent. More number of roots (2.43) per culture with maximum length of root (3.35 cm) was observed in the cultures rooted on medium gelled with agar 0.4 per cent (Plate IX). Similar trend was obtained regarding length of shoot and number of leaves per shoot.

4.3.3 Standardization of carbohydrate source for *in vitro* rooting

4.3.3.1 Effect of different carbohydrates on *in vitro* rooting and shoot growth

The effect of five different carbohydrate sources i.e. sucrose (3 per cent), commercial sugar (3 per cent), glucose (2 per cent), fructose (2 per cent) and maltose (2 per cent) on rooting and shoot growth of rough lemon under *in vitro* conditions were compared (Table 15).

Root initiation was observed within 2 weeks after transfer to different carbohydrates. Plantlets cultured on MS medium fortified with sucrose (3 per cent) were the earliest (14.96 days) to show root induction followed by commercial sugar (15.60 days). All the treatments differed significantly from each other. The percentage of rooting

Table 14. Effect of supporting media on *in vitro* rooting and shoot growth of *Citrus jambhiri* plantlets

Medium: MS (half-strength) + NAA and IBA (1.0 mg/l) each

S.No.	Treatment	Root initiation (days)	Per cent Culture rooted	No. of root per shoot	Length of longest root (cm)	Length of longest shoot (cm)	Number of leaves per shoot
1	Agar (0.4 %)	16.00	86.67 (68.83)*	2.43 (1.85)**	3.35	2.84	4.53 (2.35)**
2	Agar (0.8 %)	19.01	73.33 (58.98)	2.16 (1.78)	2.81	2.40	4.36 (2.31)
3	Whatman No. 1 filter paper	0.00	0.00 (0.00)	0.00 (1.00)	0.00	0.00	0.00 (1.00)
4	Ordinary filter paper	0.00	0.00 (0.00)	0.00 (1.00)	0.00	0.00	0.00 (1.00)
5	Brown paper	0.00	0.00 (0.00)	0.00 (1.00)	0.00	0.00	0.00 (1.00)
S.E. m (±) :		0.09	1.57	0.00	0.04	0.06	0.01
C.D. $_{(P = 0.05)}$:		0.38	4.99	0.01	0.15	0.23	0.02

* Figures given in parenthesis are transformed (angular) values

** Figures given in parenthesis are transformed (sq. root) values

Table 15. Effect of different carbohydrates on *in vitro* rooting and shoot growth of *Citrus jambhiri* plantlets.

Medium: MS (half-strength) + NAA and IBA (1.0 mg/l) each

S.No.	Treatment	Root initiation (days)	Per cent Culture rooted	No. of root per shoot	Length of longest root (cm)	Length of shoot (cm)	Number of leaves per shoot
1	Sucrose (3 %)	14.96	90.00 (74.98)*	2.24 (1.80)**	3.34	2.92	4.65 (2.38)**
2	Commercial sugar (3 %)	15.60	90.00 (74.98)	2.02 (1.74)	2.79	2.48	4.37 (2.32)
3	Glucose (2 %)	16.83	73.33 (58.98)	1.99 (1.73)	2.02	2.17	4.07 (2.25)
4	Fructose (2 %)	16.43	83.33 (66.12)	1.81 (1.68)	1.98	2.19	4.07(2.25)
5	Maltose (2 %)	17.67	70.00 (56.98)	1.64 (1.62)	1.81	2.10	4.05(2.25)
S.E. (m) ± :		0.14	5.47	0.01	0.02	0.02	0.01
C.D. $_{(P = 0.05)}$:		0.44	NS	0.04	0.07	0.06	0.03

* Figures given in parenthesis are transformed (angular) values

** Figures given in parenthesis are transformed (sq. root) values

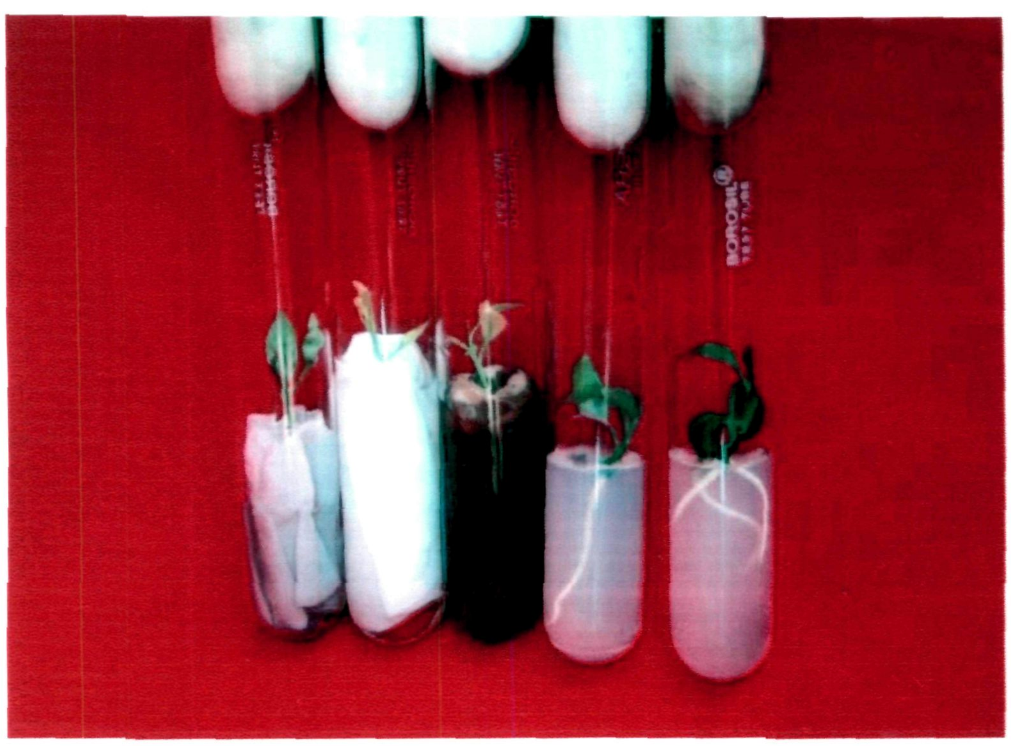


Plate IX. Effect of supporting medium on *in vitro* rooting of *Citrus jambhiri* obtained from nodal segment explants

From Left : 1. Ordinary filter paper
2. Whatman No. 1filter paper
3. Brown paper
4. Agar 0.8 %
5. Agar 0.4 %

ranged from 70 to 90 per cent. The maximum rooting 90 per cent was observed in medium supplemented with sucrose (3 per cent) and commercial sugar (3 per cent) which were statistically at par to all other treatments. Maximum numbers of roots per shoot were observed in medium fortified with 3 per cent sucrose (2.24) which was significantly higher than all other treatments.

Root length ranged from 1.81-3.34 cm. The length of longest root (3.34 cm) was observed in medium supplemented with sucrose 3 per cent followed by 2.79 cm in 3 per cent commercial sugar, while it was minimum 1.81 cm in maltose 2 per cent. However, length of shoot ranged between (2.10-2.92 cm) which was maximum in sucrose (3 per cent) and minimum in 2 per cent maltose. Similar trend was observed in number of leaves per shoot as all these treatments varied significantly with each other.

4.3.4 Effect of sucrose concentration on *in vitro* rooting and shoot growth

Effect of sucrose concentration on rooting behaviour and shoot growth of *Citrus jambhiri* was studied in this experiment (Table 16). The sucrose was added in three concentrations (1.5, 3.0 and 4.5 per cent) to half strength MS medium. Keeping remaining ingredients of media same, MS medium supplemented with 3 per cent sucrose was earliest to initiate roots in 14.96 days followed by 17.50 days in medium with 1.5 per cent sucrose (17.50 days), while medium supplemented with 4.5 per cent sucrose was the last to show root initiation (19.07 days) among all the treatments.

Percentage of rooting ranged from 33.33 to 90.00 per cent, whereas maximum rooting (90.00 per cent) was observed on medium supplemented with 3.0 per cent sucrose followed by (53.33 per cent) rooting in medium supplemented with

Table 16. Effect of sucrose concentration on *in vitro* rooting and shoot growth of *Citrus jambhiri* plantlets

Medium: MS (half-strength) + NAA and IBA (1.0 mg/l) each

S.No.	Treatment	Root initiation (days)	Per cent Culture rooted	No. of root per shoot	Length of longest root (cm)	Length of shoot (cm)	Number of leaves per shoot
1	Sucrose (1.5 %)	17.50	53.33 (46.90)[+]	1.80 (1.67)[++]	2.29	1.57	4.23 (2.29)[++]
2	Sucrose (3.0 %)	14.96	90.00 (74.98)	2.24 (1.80)	3.30	2.72	4.65 (2.38)
3	Sucrose (4.5 %)	19.07	33.33 (35.20)	1.50 (1.58)	2.39	1.12	4.03 (2.24)
S.E. m (±)	:	0.09	4.82	0.03	0.17	0.02	0.01
C.D. $_{(P = 0.05)}$	:	0.33	16.99	0.10	0.61	0.07	0.03

[+] Figures given in parenthesis are transformed (angular) values
[++] Figures given in parenthesis are transformed (sq. root) values

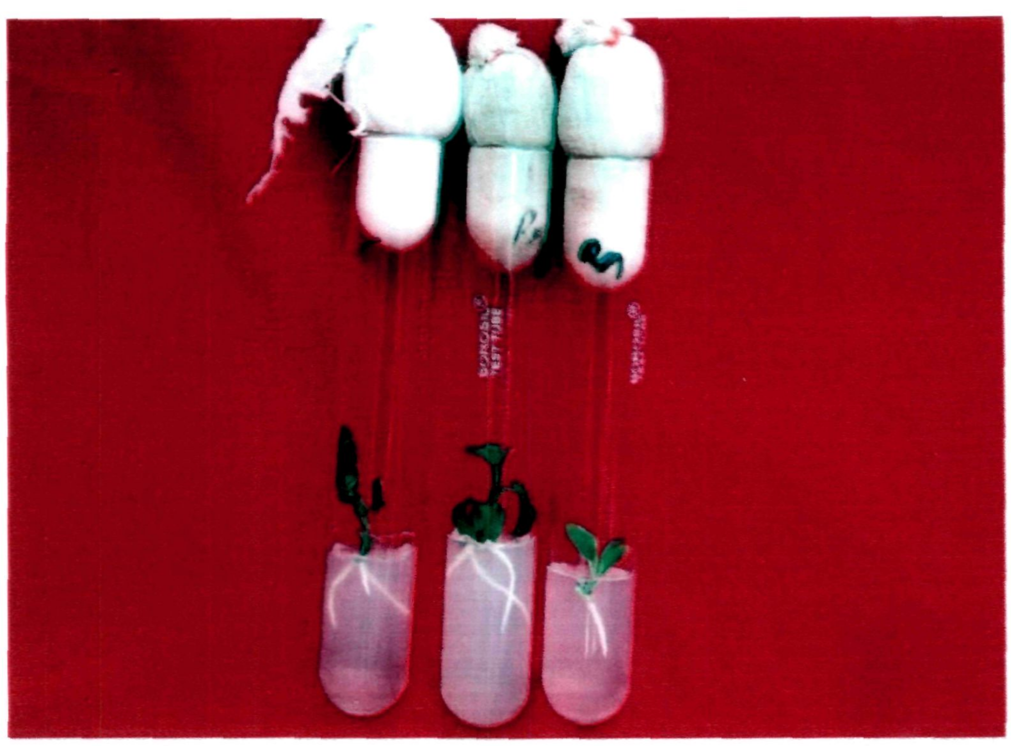


Plate X. Effect of sucrose concentration on rooting of *Citrus jambhiri*

From Left : 1. 1.5 %
2. 3.0 %
3. 4.5 %

1.5 percent sucrose (Plate X). Similar trend was observed for root number per shoot, root length (cm), length of shoot (cm) and number of leaves per shoot. Root number per shoot was significantly higher (2.24) on 3.0 per cent sucrose supplemented medium as compared to the other treatments. Root length was also significantly influenced by the sucrose concentration of the medium. The maximum length of shoot 2.72 cm and more number of leaves per shoot 4.65 was recorded in sucrose 3 per cent supplemented medium, which varied significantly with all other treatment.

4.3.5 Effect of pH on *in vitro* rooting and shoot growth

The influence of different pH levels viz. 5.4, 5.6, 5.8, 6.0 and 6.2 on rooting and shoot growth of rough lemon under *in vitro* conditions was studied (Table 17). The half strength MS medium having pH 5.8 took minimum of 15.40 days for root initiation, while those having pH 5.4 was last to show root initiation by taking 19.50 days. All these treatments took significantly more days (17.82-19.50) for rooting at highest and lowest levels of pH. The percentage of rooting was also maximum (80.00 per cent) in medium having pH 5.8 which was at par with medium having pH 6.0 and these two treatments varied significantly with all other treatments. However, minimum percentage of rooted culture was obtained in medium with pH 5.4 (Plate XI).

The maximum number of *in vitro* roots per shoot (2.37) was obtained at pH 5.8 which was significantly superior to all other treatments, while it was minimum in pH 6.2. The maximum root length (3.09 cm) of the longest root was recorded at pH 5.8 followed by pH 5.6 (2.59 cm).

Table 17. Influence of pH on *in vitro* rooting and shoot growth of *Citrus jambhiri* plantlets.

Medium: MS (half-strength) + NAA and IBA (1.0 mg/l) each

S.No.	Treatment	Root initiation (days)	Per cent Culture rooted	No. of root per shoot	Length of longest root (cm)	Length of shoot (cm)	Number of leaves per shoot
1	pH 5.4	19.50	33.33 (35.20)[+]	1.50 (1.58)[++]	1.78	1.39	4.01 (2.24)[++]
2	pH 5.6	17.12	53.33 (46.90)	1.56 (1.60)	2.59	1.68	4.31 (2.31)
3	pH 5.8	15.40	80.00 (63.90)	2.37 (1.84)	3.09	2.82	4.62 (2.37)
4	pH 6.0	16.37	70.00 (56.98)	1.68 (1.64)	2.32	2.01	4.45 (2.34)
5	pH 6.2	17.82	43.33 (41.14)	1.38 (1.54)	1.93	1.56	4.11 (2.26)
S.E. m (±)	:	0.12	2.93	0.03	0.06	0.02	0.01
C.D. $_{(P = 0.05)}$	:	0.40	9.36	0.08	0.19	0.08	0.01

[+] Figures given in parenthesis are transformed (angular) values
[++] Figures given in parenthesis are transformed (sq. root) values

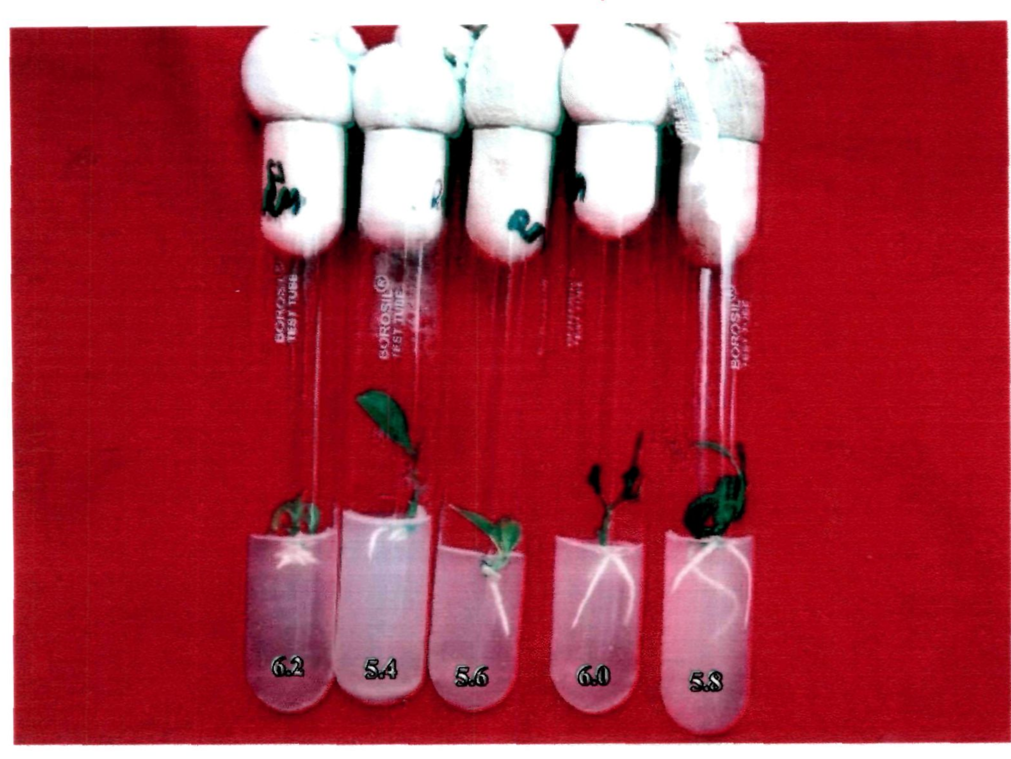


Plate XI. Effect of pH on *in vitro* rooting of *Citrus jambhiri* on MS (half strength medium) supplemented with NAA and IBA (1.0 mg/l) each

The length of *in vitro* shoot was also significantly affected by different pH levels. The maximum length (2.82 cm) was recorded at pH 5.8 which was followed by pH 6.0 (2.01 cm). In case of number of leaves, the medium with pH 5.8 showed maximum leaves (4.62), whereas it was minimum (4.01) in pH 5.4.

4.4 Acclimatization and planting out

In order to find out the most appropriate hardening procedure as well as to find out the influence of different potting mixtures on survival and growth of *Citrus jambhiri* plantlets, the plantlet developed 4 weeks after root initiation were studied. The results obtained are shown in Table 18 and 19.

4.4.1 Standardization of hardening treatments

The data pertaining to the survival and growth of plantlets under different hardening treatments are presented in Table 18. The different hardening treatments influence the survival percentage of the plantlets after 4 weeks of transplanting. Covering the plantlets individually with glass beaker (Plate XIIa) and keeping them in culture room at 26 $\pm$ 2^{0}C with low light intensity resulted in 96.67 per cent survival, while all the plantlets dried when kept in laboratory at room temperature without cover. The treatment of covering plantlets individually with glass beaker and keeping them in culture room recorded maximum height (5.01 cm) which was closely followed by 4.75 cm height in treatment covering plantlets individually with polythene bags and keeping them in culture room (Plate XIIb), however, all the treatments varied significantly with each other. Similar trend was obtained the number of leaves per plantlet.

Table 18. Effect of hardening treatments on survival and growth of *in vitro* produced *Citrus jambhiri* plantlets

S.No.	Treatment	Survival (%)	Height (cm)	No. of leaves per plantlet
1	Covering the plantlets with glass beaker individually and kept in AC room	96.67 (83.84)[+]	5.01	6.19 (2.68)[++]
2	Covering the plantlets with polythene bag individually and kept in AC room	83.33 (66.12)	4.75	5.55 (2.56)
3	Covering the plantlets with glass beaker individually and kept in open	80.00 (63.90)	4.33	5.28 (2.51)
4	Covering the plantlets with polythene bag individually and kept in open	63.33 (52.75)	4.15	5.05 (2.46)
5	Keeping the plantlets in open without cover	0.00	-	-
S.E. m (±) :		4.11	0.05	0.02
C.D. $_{(P = 0.05)}$:		13.60	0.15	0.06

[+] Figures given in parenthesis are transformed (angular) values
[++] Figures given in parenthesis are transformed (sq. root) values

a. Covering the plantlet with glass beaker individually and kept in culture room

b. Covering the plantlet with polythene bag individually and kept in culture room

Plate XII. Acclimatization of plantlets

4.4.2 Effect of different potting substrates

The study was undertaken to find a suitable medium ensuring better survival and growth of micro propagated plants on transfer to *ex vitro* conditions (Table 19). Four potting mixtures viz. soil, soil and sand (1:1), soil, sand and FYM (1:1:1) and soil, sand and vermiculite (1:1:1) were tried as different substrate to study their relative efficacy in enhancing the survival of micro propagated plantlets (Plate XIII). Maximum survival (93.33 per cent) was observed in potting mixture containing soil, sand and FYM (1:1:1) which was at par with a mixture containing soil, sand and vermiculite (1:1:1), while lowest survival of 73.33 per cent was recorded in soil alone. The maximum height of plantlets 5.29 cm and more number of leaves per plantlet (6.07) were recorded in mixture of soil, sand and FYM (1:1:1) followed by (4.93 cm and 5.86), respectively in soil, sand and vermiculite (1:1:1). The acclimatized micro-propagated plantlets were then planted in field (Plate XIV).

4.4.3 Effect of anti-transpirant on plants

The experiment was conducted to study the effect of anti-transpirants *viz.*, glycerol (10, 25 and 50 per cent) and ABA (7, 10 and 15 ppm) on *ex vitro* survival of micro propagated plants (Table 20). Among all the treatments glycerol 50 per cent emerged best with maximum plant survival (83.33 per cent) followed by glycerol 25 per cent (55.56 per cent), while control showed the least plant survival (22.21 per cent).

Table 19. Influence of potting substrates on survival and growth of *in vitro* produced *Citrus jambhiri* plantlets

S.No.	Treatment	Survival (%)	Height (cm)	No. of leaves per plantlet
1	Soil	73.33 (58.98)*	4.29	5.19 (2.49)**
2	Soil:Sand (1:1)	76.67 (61.20)	4.69	5.63 (2.57)
3	Soil:Sand:FYM (1:1:1)	93.33 (77.69)	5.29	6.07 (2.66)
4	Soil:Sand:Vermiculite (1:1:1)	83.33 (66.12)	4.93	5.86 (2.62)
S.E. m (±) :		3.71	0.02	0.04
C.D. $_{(P = 0.05)}$:		12.83	0.07	0.01

* Figures given in parenthesis are transformed (angular) values
** Figures given in parenthesis are transformed (sq. root) values

Table 20. Effect of anti-transpirants on survival of *Citrus jambhiri* plantlets

S.No.	Treatment	Survival (%)
1	Glycerol (10 %)	38.87 (38.48)[+]
2	Glycerol (25 %)	55.56 (48.23)
3	Glycerol (50 %)	83.33 (70.20)
4	ABA (7 ppm)	33.33 (34.77)
5	ABA (10 ppm)	27.76 (31.52)
6	ABA (15 ppm)	38.87 (38.48)
7	Control	22.21 (27.80)

S.E. m (±) : 5.40

C.D. $_{(P = 0.05)}$: 16.54

[+] Figures given in parenthesis are transformed (angular) values

Plate XIII. Effect of different potting mixtures on survival of micro propagated plantlets of *Citrus jambhiri*

Plate XIV. Acclimatized micropropagated plantlets of *Citrus jambhiri*

4.5 Protocol developed for *in vitro* propagation of *Citrus* spp.

The protocols developed in the present study for *in vitro* propagation of *Citrus jambhiri* and *Citrus sinensis* cv. Mosambi are given below and illustrated in Plate XV.

4.5.1 Axillary bud culture of *Citrus jambhiri*

A) In the working laboratory

1. Collect 5-10 cm long shoot pieces from newly grown flushes (2-3 week old) during the period from March to May.
2. Remove the leaves and petiole using a stainless steel knife and divide these shoot pieces into segments of 2-3 cm length. Wash thoroughly in running tap water (approximately 30 minutes) to remove all the adhering dirt.
3. Treat the explant with 10 per cent solution of detergent (Teepol) for 10 minutes.
4. Remove all the traces of detergent by repeated washing in double glass distilled water.

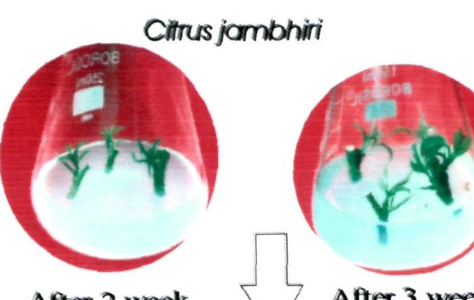
B) In sterile room (Laminar air flow chamber)

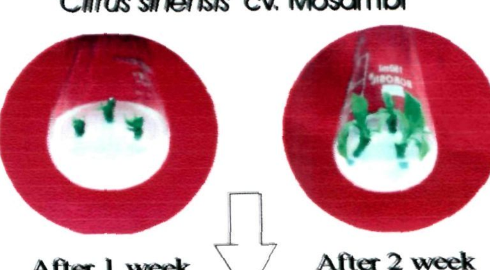
5. Surface sterilize the explants using 70 per cent ethanol for 30 seconds and 0.1 per cent mercuric chloride for 8 minutes which is followed by thorough rinsing with autoclaved distilled water. Cut the explants further into segments containing single node and trim the stem portion further to the extent that the final size of explant is about 1 cm.
6. Inoculate the explant in 150 ml Erlenmeyer flask/250 ml culture bottles containing MS medium gelled with 0.8 per cent agar and supplemented with BAP 1.5 mg/l and malt extract 500 mg/l and sucrose 30 g/l. The basal end of the explant should

Culture establishment

Citrus jambhiri

Citrus sinensis cv. Mosambi





After 2 week

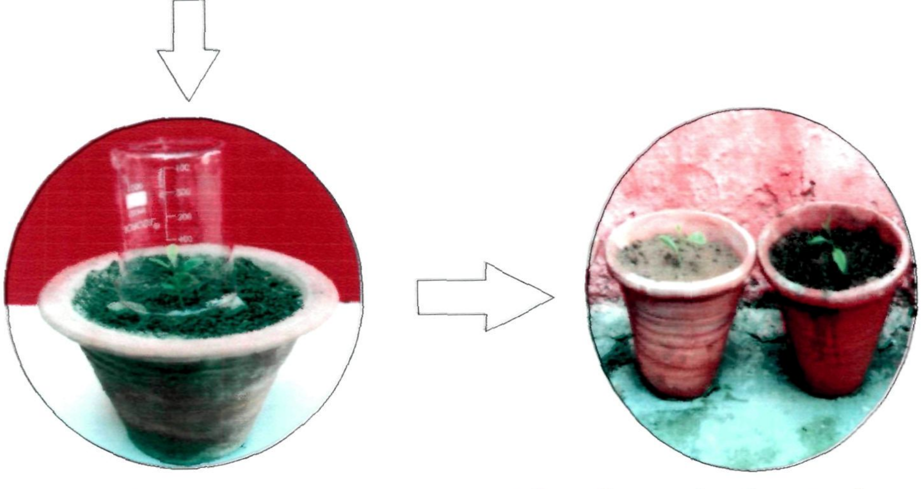
After 3 week

After 1 week

After 2 week

Culture proliferation

In vitro rooting



Acclimatization

Hardened plants in pot

Plate XV. Steps involved in *in vitro* propagation of *Citrus* spp. using nodal segment explants (axillary bud culture)

be inserted in the medium in such a way that the axillary bud remain above the surface of the medium.

C) Incubation

7. Incubate the culture flasks/bottles in an air-conditioned culture room at 26 $\pm$ 2^{0}C temperature and 16 hour photoperiod in which light is supplied by cool white fluorescent tubes at an intensity of approximately 1000-2000 lux.
8. After 3 weeks of incubation, transfer the established nodal segments to above mentioned medium and incubate for another 3 weeks.
9. After 6 weeks of incubation, separate the multiple shoots produced and transfer the individual shoot to rooting medium i.e., MS basal medium (half strength) supplemented with 30 g/l sucrose and gelled with 0.4 per cent agar. It takes 16 days incubation upto root initiation. A portion of multiple shoots can be maintained in proliferation medium MS medium with BAP 1.5 mg/l and malt extract 500 mg/l by serial sub-culturing done at every 6 weeks interval.

D) Acclimatization

10. 3 weeks after root initiation, transplant the plantlets to pots/polythene bags containing soil:sand:FYM (1:1:1 v/v) mixture. Drench the potting mixture with 0.1 per cent Bavistin at weekly interval.
11. Acclimatize the plantlets to natural environment in air-conditioned room by covering them individually with glass beaker for initial 6 days and removing the cover gradually with in 15 days. The plantlets should be then kept in polyhouse for 1 week for further hardening.

4.5.2 Axillary bud culture of *Citrus sinensis* cv. Mosambi

A) In the working laboratory

1. Collect 5-10 cm long shoot pieces from newly grown flushes (2-3 week old) during the period from March to May.
2. Remove the leaves and petiole using a stainless steel knife and divide these shoot pieces into segments of 2-3 cm length. Wash thoroughly in running tap water (approximately 30 minutes) to remove all the adhering dirt.
3. Treat the explant with 10 per cent solution of detergent (Teepol) for 10 minutes.
4. Remove all the traces of detergent by repeated washing in double glass distilled water.

B) In sterile room (Laminar air flow chamber)

5. Surface sterilize the explants using 70 per cent ethanol for 30 seconds and 0.1 per cent mercuric chloride for 8 minutes which is followed by thorough rinsing with autoclaved distilled water. Cut the explants further into segments containing single node and trim the stem portion further to the extent that the final size of explant is about 1 cm.
6. Inoculate the explant in 150 ml Erlenmeyer flask/250 ml culture bottles containing MS medium gelled with 0.8 per cent agar and supplemented with BAP 1.0 mg/l and malt extract 500 mg/l and sucrose 30 g/l. The basal end of the explant should be inserted in the medium in such a way that the axillary bud remain above the surface of the medium.

4.6 Economics of *in vitro* propagation system of *Citrus* spp.

The economic feasibility of *in vitro* propagation is an important consideration for the commercial exploitation. Thus, an attempt is being made to calculate the cost of production of one plantlet upto polyhouse stage considering the protocol developed during this investigation; using nodal segments of 2-3 week old flushes from 10-12 year old *Citrus jambhiri* trees. This calculation is based on following assumptions.

4.6.1 *In vitro* grown *Citrus jambhiri* Lush. plantlets

The production capacity of tissue culture laboratory is over 1,00,000 plantlets at a time. As per the protocol developed in the present investigation, the multiplication rate was 5.34, 8.79, 6.73 and 4.99 in I^{st}, IInd, IIIrd and IVth sub-cultures, respectively. The culture establishment is 88 per cent, while 80 per cent rooting is recorded. The contamination was 12 per cent in stage I and 5 per cent in stage II and stage III. It is assumed that the production may be started with 113 nodal stem segments. They are inoculated on the establishment media (stage I, 3 weeks). The established nodal segments are continuously multiplied to produce a total 1,27,075 shoots (stage II, 21 weeks). Since rooting was recorded in 80 per cent of shoots thus 1,05,472 shoots are transferred to rooting media and 1,00,198 rooted plantlets are produced (stage III, 5 weeks). The plantlets are transferred to bags containing a mixture of soil, sand and FYM and are initially kept in air-conditioned room for hardening and then shifted to polyhouse (stage IV, 3 weeks). Thus, cost of 1 plantlet is calculated for a total of 32 week cycle.

Technician can inoculate sufficient number of cultures in each stage in approximately 1195 hours. It is also assumed that approximately 280 hours are necessary for media preparation. Thus, the total time required for media preparation and inoculation is considered to be about 1475 hours. It is also assumed that a total of about 1475 hours are required from helper who assists the technician in all these works. A supervisor can supervise these works in about 295 hours.

It is estimated that 30 ml of medium is required per culture vessel in stage I and stage II, whereas 50 ml of medium is required per culture vessel in stage III. Thus, it is calculated that approximately 0.85 litres of nutrient medium is required for stage I, 237 litres for stage II and 878.93 litres for stage III. Thus, a total of 1116.78 litres are required for producing 1,00,198 *in vitro* plantlets. The cost of nutrient medium for stage I and stage II is Rs. 23.74 per litre. Half strength MS medium gelled with 0.4 per cent agar and supplemented with 1.0 mg/l IBA and NAA each and 3 per cent sucrose is used for rooting as per protocol developed and the cost of this rooting medium is Rs. 18.06 per litre as per the current market rates (2004-05) of the chemicals.

The glassware costs are calculated for the approximate number required and is amortized over the normal life i.e. 25 cycles. The equipment cost is based on the approximate price for year 2004-05 are amortized over 15 years. The laboratory cost is estimated to be Rs. 7,20,000 and is amortized over 25 years. The overhead charges are calculated to be Rs. 31,950 for 29 week cycle in the laboratory. The total cost for laboratory stage is about Rs. 2,06,575 and the unit cost is estimated to be Rs. 2.06.

From the analysis of the cost of hardening and raising plantlets for 3 weeks (air conditioned room 2 weeks and polyhouse 1 week) it is found that approximately 2,330 hours are required for preparation of potting mixture, filling bags, transplanting and other works. Cost of polythene bag is estimated to be Rs. 84 per kilogram. It is estimated that approximately 100 kg of polythene bags are required for transplanting 1,00,000 plantlets. The cost of potting mixture and fungicides are calculated based on the approximate quantity needed for 1,00,000 plants. The cost of construction of air-conditioned room is calculated to be Rs. 2,16,000 and is amortized for 25 years. The cost of glass bottles required for covering the plantlets to maintain high humidity and other equipments required for hardening the plantlets in air-conditioned room are calculated to be Rs. 6,62,500 and is amortized for 15 years. The polyhouse cost is based on the area needed for keeping 1,00,000 polythene bags with plantlets with necessary passage for easy approach to the plantlets. It is assumed that approximately 70 kg of polythene is required to cover the framework of polyhouse, which is amortized over 3 years. The cost of polythene carpet is calculated at the rate of Rs. 170 per kg. The total cost for polyhouse stage is about Rs. 47,509 and unit cost is assumed to be 0.48. All these calculations are given in Appendix-I to X.

The summary cost of producing 1,00,000 plantlet of *Citrus jambhiri* upto polyhouse stage is presented in Table 21. The total cost is found to be Rs. 2,54,084. The unit cost of rough lemon plantlet raised from nodal segment explant source is calculated to be Rs. 2.54.

4.6.2 Field grown seedling of *Citrus jambhiri*

The cost of cultivation for producing 1,00,000 *Citrus jambhiri* seedlings from

Table 21. Summary of the cost for producing 1, 00, 000 plantlets upto polyhouse stage

S.No.	Item	Laboratory stage			Polyhouse stage		
		Total cost (Rs.)	Cost per plantlet (Rs.)	Percent age	Total cost (Rs.)	Cost per plantlet (Rs.)	Percent age
1.	Salary and wage	50, 150	0.50	24.28	26, 795	0.27	56.40
2.	Nutrient medium	21,520	0.22	10.42			
3.	Glassware	9, 425	0.09	4.56			
4.	Equipments	47, 353	0.47	22.92			
5.	Laboratory	46, 177	0.46	22.35			
6.	Transplanting material				12, 400	0.12	26.10
7.	Plant hardening cost				4, 565	0.05	9.61
8.	Polyhouse				249	0.003	0.52
9.	Overhead charges	31, 950	0.32	15.47	3, 500	0.04	7.37
	Total	2, 06, 575	2.06		47, 509	0.48	

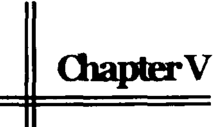
Cost of producing 1 plantlet of *Citrus jambhiri* = 2.06 + 0.48 = **Rs. 2.54**

seed under field condition is also worked out so as to compare the cost of cultivation of producing one seedling under laboratory as well as field condition and to find out the most economical method. The economics is worked out on the basis of capital investment and operational cost. In capital investment, the total cost comes out to be Rs. 4,21,072, while the operational cost includes Rs. 27,334 (Table 22). Thus, the total cost for producing 1,00,000 seedlings under field condition is Rs. 4,48,406. The unit cost of raising and transplanting of rough lemon seedling is Rs. 4.48.

Table 22. Economics for producing 1, 00, 000 *Citrus jambhiri* seedlings from seeds under field conditions

A. Capital investment	
a) Land required for nursery and transplanting @ Rs. 400 per kanal on lease rate for 30 kanal for 20 years	= Rs. 12, 000
b) Land preparation including preparation of beds and channels (64 x 4 labour days @ Rs. 70 per MD)	= Rs. 17, 920
c) Sowing of seeds and irrigation (120 labour days)	= Rs. 8, 400
d) Regular year round maintenance of nursery with 3 labour per day (4, 380 labour days) and farm equipment	= Rs. 3, 16, 600
e) Barbed wire fencing (Rs.75,000) assumed life (10 years)	= Rs. 7,500
f) Bore well and pump set (Rs. 1, 00, 000) assumed life (10 years)	= Rs. 10,000
g) Store room 10'x15' @ Rs. 600 per sq.ft. assumed life (15 years)	= Rs. 6,000
h) Labour shed 10'x10' @ Rs. 400 per sq.ft. assumed life (15 years)	= Rs. 2,667
i) Farm equipments (Foot sprayer, spade etc.)	= Rs. 2, 000
j) Power tiller with accessories (Rs. 1,61,000) assumed life (20 years)	= Rs. 8,050
Total	= Rs. 3, 91, 137
Interest @ 7.5 per cent per year	= Rs. 29, 935
Total cost including interest	= Rs. 4, 21, 072
B. Operational cost	
a) Fertilizer	= Rs. 5, 000
b) Chemical	= Rs. 5, 000
c) Weedicide	= Rs. 5, 000
d) Electricity	= Rs. 1, 000
e) Seed cost	= Rs. 3, 334
f) Supervision	= Rs. 8, 000
Total	= Rs. 27, 334
Total cost required (capital investment and operational)	= 4, 21, 072 + 27, 334 = Rs. 4, 48, 406 for 1 lakh seedlings
Cost of producing 1 seedling	= **Rs. 4.48**

DISCUSSION



Discussion

The principle of micro propagation is based on the phenomenon that shoot tip when cultured on tissue culture medium, can develop large number of shoots identical to the parent plant. In general, the response of explant cultured *in vitro* is influenced by several *in vitro* and *ex vitro* factors. These include the genotype of the donor plant, nature of the initial inoculum, age of the culture, composition of the culture medium and physical conditions like temperature, duration and intensity of the light and relative humidity prevailing during the growth of cultures. It has been demonstrated that citrus can be propagated through tissue culture techniques. These techniques provides many advantages over conventional propagation methods such as product development in highly speedy manner, uniformity in product development, large population can be produced in small growing space, quick multiplication of elite and genetically engineered products, *in vitro* storage of germplasm and disease free plant material can be obtained through out the year.

In all the advanced countries, citrus trees are now grown as budded plants composed of two components i.e., rootstock and scion. Rootstock is an integral part of the budded fruit plant which provides tolerance/resistance to biotic and abiotic stresses besides influencing the yield, quality as well as shelf life of the fruit. In Citrus, the rootstocks are propagated commercially through seeds. However, the occurrence of polyembroyony in *Citrus* species, hinder the production of uniform rootstock (Maheshwari and Rangaswamy, 1958; Humes, 1966; Iglesias *et al.*, 1974; Gill and Gosal, 2002). Juvenile characteristics and delayed bearings are other

disadvantages of nucellars. On the other hand, vegetative methods of propagation like budding, layering and cuttings are tedious, time consuming, season dependent and also the rate of multiplication by these methods is rather slow. The prevalence of disease problem and need for generating disease free uniform planting stock in large quantity have stimulated an upsurge of interest in the rapid clonal propagation of *Citrus* spp. by applying tissue culture technique (Raj-Bhansali and Arya, 1978a; Bhat *et al.*, 1992; Desai *et al.*, 1994). The results reported by these workers suggested that *in vitro* techniques will not only provide clonal material in large number but will also aid the breeding programmes. Therefore, exploiting such a technique for commercial purpose or as an aid to breeding programmes, is essential to develop *in vitro* methods for propagation of citrus plant species which suits to the local conditions, as *in vitro* performance of plant tissue is dependent on a number of factors which is intimately connected with the physiological state of the donor.

The present investigation was mainly aimed to standardize the various aspects of *in vitro* propagation technique of *Citrus* species since the first report of *in vitro* studies with *Citrus* species using unpollinated ovaries (Mitra and Chaturvedi, 1972), various explant sources such as shoot tip (Raj-Bhansali and Arya, 1978b; Duran-vila *et al.*, 1989; Desai *et al.*, 1994), root segment (Raj-Bhansali and Arya, 1978a; Bhat *et al.*, 1992), leaf sections (Chaturvedi and Mitra, 1974), and root meristems (Sauton *et al.*, 1982) have been used from seedlings and/or mature trees, these studies showed that axillary bud culture is the most promising one. Attempts were therefore centered around to standardize the various aspects of *in vitro* propagation using nodal stem segments (axillary bud culture) in rough lemon and sweet orange cv. Mosambi.

In the *in vitro* propagation, the organs and tissues are carried through a sequence of steps in which differential cultural and environmental conditions are provided. These steps have been grouped in 4 different stages namely stage I: explant establishment; stage II: rapid multiplication of shoot through increased axillary branching, somatic organogenesis and somatic embryogenesis; stage III: *in vitro* rooting and stage IV: acclimatization and planting out (Murashige, 1974). The experimental results obtained on different aspects of *in vitro* propagation of *Citrus* spp. have been discussed in this chapter in the light of available literature.

5.1 Culture establishment (stage I)

5.1.1 Surface sterilization of explant

The explants collected from field harbour fungi and bacteria in addition to adhered soil and dirt particles. Thus; it necessitates a thorough and effective surface sterilization of explant before culturing. The results of surface sterilization of nodal segment explant obtained from 12 year old mature trees of *Citrus jambhiri* and *Citrus sinensis* cv. Mosambi are presented in Table 5 and 6 respectively (Chapter IV section 4.1). Treating the explants of both *Citrus jambhiri* and *Citrus sinensis* with 70 per cent ethanol for 30 seconds and 0.1 per cent mercuric chloride for 8 minutes gave best sterilization and survival. In explant sources of both species, sterilization with mercuric chloride or sodium hypochlorite alone was not found effective in controlling contamination problem satisfactorily. These results thus clearly indicated that two step sterilization procedure for mature tree explants was effective to isolate contamination free explant under local conditions. Moore (1986) used HCl, ethanol and sodium hypochlorite for disinfection of sour orange, Cleopatra mandarin and

Carrizo citrange rootstocks, while Dwivedi and Bist (1999) and Rana and Singh (2002) sterilized explants of pear and *C. aurantifolia*, respectively with a combination of two surface sterilent mercuric chloride and sodium hypochlorite for different time interval. Both the species treated with higher concentration of mercuric chloride (0.1 per cent) alone or in combination with ethanol (70 per cent) showed death of culture. One probable reason for the death of explants when exposed to surface sterilents for longer duration may be due to heavy metal contamination of mercury present in the mercuric chloride, proving detrimental for the survival of explant. These results confirm the findings of Dwivedi (1995); Singh (1995) and Rana and Singh (2002).

It is evident from the present investigation that response of *Citrus jambhiri* and *Citrus sinensis* cv. Mosambi explants to surface sterilization treatments was different. This may possibly be because of the difference in nature and intensity of contaminants present in two locations from where explants were collected and also due to species variation.

5.1.2 Culture initiation/establishment medium

The results obtained in the culture establishment trials on *Citrus jambhiri* and *Citrus sinensis* cv. Mosambi are presented in Chapter IV, section 4.1.2. In all, 41 treatments alone as well as in combination were tried using different cytokinins (BAP and/or Kinetin), malt extract and NAA in MS medium. The study indicated that the nodal segment explants of *Citrus jambhiri* showed highest culture establishment (94.45 per cent) in the treatment containing BAP 1.5 mg/l and malt extract 500 mg/l with least time taken (4.34 days) for culture establishment, whereas in case of Mosambi BAP 1.0 mg/l in combination with malt extract 500 mg/l showed maximum

maximum culture establishment 97.22 per cent in less time (5.22 days). This increase in culture establishment in media supplemented with malt extract showed that malt extract is an ideal supplement and the most important obligatory component as observed by earlier workers (Kochaba and Spiegel-Roy, 1973; Parthasarathy and Nagaraju, 1994; Das *et al.*, 1995). Irrespective of explant type, these results are in confirmation to those of Gill *et al.* (1994) who reported that MS medium containing malt extract along with cytokinins (BAP and Kinetin) and/or NAA gave significantly more culture establishment, than the medium containing cytokinins and/or auxins lacking only malt extract. The beneficial effect of malt extract on culture establishment has also been reported by (Raj-Bhansali and Arya, 1978a; Moore, 1985). The difference obtained in the requirement of cytokinins for shoot organogenesis in both the species may be attributed to the difference in the levels of endogenous phytohormones, nutrients, metabolites and interaction between various factors. The requirement of different concentration of cytokinin for culture establishment of different *Citrus* species has also been reported by Kitto and Young (1981); Raman *et al.* (1992) and Kumar *et al.* (2001).

At higher doses of BAP (i.e. above 1.0 mg/l), callus induction was observed which suppressed the shoot initiation in Mosambi, whereas Maggon and Singh (1995) reported that BAP (upto 2.0 mg/l) had a promotive effect, while higher concentration of BAP had a depressive effect, which may be due to endogenous levels of growth regulators. Das *et al.* (2000) reported that at higher doses of BAP callusing was induced suppressing shoot initiation which are in close conformity with the present investigation.

The role of cytokinin in shoot organogenensis is well established (Skoog and Miller, 1957; Evans *et al.* 1981). However, endogenous levels of cytokinin and auxin play a very important role in this response. Depending on the endogenous levels, requirements of the exogenous applications vary to get the optimum response. In the present investigation, the reduction in the culture establishment percentage due to higher levels of auxin (NAA 0.5 mg/l) indicated that endogenous level of auxin was near to optimum. Moreover, it is a specific auxin-cytokinin ratio which controls root and shoot formation in tissue culture (Engelke *et al.* 1973). In general, the basal MS medium lacking growth regulators did not exhibit plant regeneration in any of the explants viz. leaf, epicotyl, cotyledon and roots. This observation clearly indicated that the supplementation of regeneration media with auxins and/or cytokinins is essential for obtaining plantlet differentiation (Gill *et al.*, 1995).

5.2 Shoot proliferation (Stage II)

The results of shoot proliferation trials on *Citrus jambhiri* and *Citrus sinensis* cv. Mosambi are presented in Chapter IV section 4.2. Cent per cent culture proliferation with maximum multiple shoot per culture were produced on MS medium containing BAP (1.0 and 1.5 mg/l) with malt extract 500 mg/l and BAP 1.5 mg/l with Kinetin 0.5 mg/l in case of *Citrus jambhiri*, whereas MS medium supplemented with BAP (0.5 and 1.0 mg/l) in combination with malt extract 500 mg/l and BAP 1.0 mg/l with Kinetin 0.5 mg/l showed highest multiple shoots in case of *Citrus sinensis* cv. Mosambi. The effects of cytokinins on axillary bud bursting and multiple shoot production have been demonstrated by Murashige (1974). In the present study, the treatment combinations involving BAP and malt extract were more

effective than BAP or Kinetin alone or in combination with NAA in case of both the species. From the results obtained, two points have become clear. First, the requirement of cytokinins for multiple shoot induction was different for two species. Second, the requirement of cytokinins was low for *Citrus sinensis* cv. Mosambi as compared to *Citrus jambhiri*. The basic phenomenon involved in the explant establishment, induction of multiple shoot and subsequent plantlet production *in vitro* are reported due to action of plant hormones. Little is known about how hormones evoke the particular pattern of morphogenesis (Thorpe, 1978). One hypothesis is that hormone treatment starts the cell on a specific developmental pathway, the alternative view is that hormones responsive cells are already determined and hormones evoke the expression of the committed state. Hence, the observed difference is the requirement of growth hormones for shoot proliferation of explant used by Desai *et al.* (1994) and the explants used in the present investigation could be attributed to the differential requirement of the growth hormones. Parthasarathy and Nagaraju (1996) reported that in certain *Citrus* spp. proliferation of shoot proportionately increased with the increasing BAP level upto 0.75 mg/l, whereas both shoot number and shoot length reduced at concentration above 0.75 mg/l. In the present investigation, BAP was superior to Kinetin for shoot proliferation and these results are in agreement to those of Baruah *et al.* (1996).

Since multiplication is the major economic criterion for successful commercial tissue culture propagation, the proliferation rate of stage II determines the feasibility of *in vitro* propagation of a given species (Bhojwani and Razdan, 1983). The effect of different cytokinins alone or in combination with malt extract on shoot multiplication

was also looked into (Table 10 and 12). The study indicated that MS medium supplemented with BAP 1.5 mg/l and malt extract 500 mg/l was best for shoot multiplication in *Citrus jambhiri*, while in case of *Citrus sinensis* cv. Mosambi BAP 1.0 mg/l with malt extract 500 mg/l showed highest shoot multiplication rate per sub-culture. It was found that when sub-culturing was done at a 6 weeks interval, the multiplication rate of first sub-culture increased tremendously. However, there was a gradual reduction in the multiplication rate in each subsequent sub-culturing in both the species. This might be due to altered physiological stage of the cells with sub-culturing.

Al-Kharyi and Al-Bahrany (2001) stated that best results for shoot multiplication were obtained in BAP 1.0 mg/l and Kinetin 0.5 mg/l in sour lime. The results obtained in the present study are in contradiction to those of Rana and Singh (2002) who reported pronounced multiple shoot formation in medium supplemented with 2.0 mg/l BAP and 0.1 mg/l NAA as in present investigation BAP in combination with NAA and malt extract showed least multiple shoots.

5.3 *In vitro* rooting (Stage III)

Stage III involves the regeneration of adventitious roots from the shoots obtained in stage II. The results on the influence of different auxins (IBA and NAA) on *in vitro* root and shoot characters of *Citrus jambhiri* are presented in Chapter IV, section 4.3.1. In rooting, half strength MS medium was used with respect to root initiation, percentage of rooting, average number of roots per shoot and root length.

Improved rooting in lower strength media was attributed to reduction in nitrogen concentration by Hundman *et al.* (1982). They found that lowering of

concentration of salts in the media to a certain limit leads to more favourable nitrogen concentration. Rooting of shoots of *Citrus junos* and *Citrus jambhiri* were also achieved easily on half strength MS medium by Oh *et al.* (1991) and Rahaman *et al.* (1996), respectively. The treatment involving MS medium (half strength) supplemented with IBA (1.0 mg/l) and NAA (1.0 mg/l) was best with respect to initiate roots in maximum cultures (83.33 per cent) within shortest period of time (16.51 days). Maximum root and shoot length was also recorded in this treatment. The effect of IBA and NAA on rooting of micro shoots was found significant and synergistic. There was no rooting when none of the auxins were supplied to the media, which showed that exogenous application of auxins was necessary for rooting. These results are in close conformity with the findings of Starrantino and Caruso (1984) and Can *et al.* (1992) who also obtained maximum rooted shootlets of trifoliate orange and sour orange respectively, on medium containing IBA and NAA (1.0 mg/l) each. Karwa (2003) reported that MS medium lacking auxin showed very poor or no rooting in Nagpur mandarin. NAA alone was effective on rooting but to a less extent than its combination of IBA. Das *et al.* (1995) obtained rooted shootlets of *Citrus sinensis* cv. Mosambi on half strength basal medium supplemented with NAA and IBA 0.5 mg/l each. These results are contradictory to Parthasarthy and Nagaraju (1996) as they revealed that NAA at 0.05 mg/l supplemented in MS media was best for *C. reticulata* and *C. limon*, while for Mosambi, best concentration was 0.2 mg/l. Also, the low concentration of NAA was identified to be the best for rooting in Kinnow mandarin (Parveen *et al.*, 2003).

The results on the effect of different supporting media on *in vitro* rooting and shoot growth of rough lemon plantlets are presented in Chapter IV, section 4.3.2. Out of various supporting media *viz.* Whatman No. 1 filter paper, ordinary filter paper and brown paper were found unsuitable for *in vitro* rooting of rough lemon shoots. However, the reduction of agar concentration from 0.8 to 0.4 per cent in the medium was found to improve the *in vitro* rooting and shoot characters. These results are in confirmation with those of Kitto and Young (1981) who reported increased response with the decrease in agar from 2.0 to 0.5 per cent. The number of rooted cultures and plantlets survival increased with reduction in agar concentration in the medium. This may be attributed to (1) the better absorption of nutrients from semi-solid (0.4 per cent agar) than solid (0.8 per cent agar) medium, (2) the plantlets could be taken out from the culture vessels with ease at the time of transplanting in pots and (3) the nutrient medium adhered to the roots could be removed easily without causing damage to the root system.

Review of literature on different *in vitro* rooting experiments indicated that the concentration of agar varies from 0 to 0.9 per cent, usual being 0.6-0.8 per cent. Lowering of agar content makes the availability of nutrients better. Since agar generally serves to support the propagules, Anderson (1980) considered that its concentration should be as low as possible. Lowering the agar content from 5 to 6 grams per litre resulted in better rooting of plant (Skirvin *et al.*, 1980). The better response of rough lemon shoots on semi-solid medium for *in vitro* rooting is quite encouraging as far as commercialization of the technique for mass multiplication was concerned, as agar is the costliest component in nutrient medium composition.

A carbon energy source is inevitable in any culture medium. Sucrose is the most widely accepted carbon energy source in most of *in vitro* studies. Attempts were made to find the best carbon source for *in vitro* rooting of rough lemon shootlets. The results obtained in the carbohydrate trail are presented in Chapter IV, section 4.3.3.2. In general, the increased response was obtained in terms of rooting and survival of plantlets with the increase in concentration of carbohydrate source. Out of various carbohydrate sources, sucrose (3 per cent) was the best as it resulted in 90 per cent rooted culture in least time of 14.96 days followed by commercial sugar. The positive effect of increased level of sucrose on *in vitro* rooting has been reported for sour cherry (Snir, 1983) and walnut (Driver and Kuniyuki, 1984). Shah (1983) had similar observations for *Glycyrrhiza glabra*, where 1 per cent level of different carbohydrates tried responded poorly. The tissues grew well in different carbohydrates tested in several cereal plants (Mascarenhas *et al.*, 1969) and in mango leaf gall tissues (Rao, 1966); whereas normal shoot development was obtained in mulberry only when fructose or glucose was used (Oko and Ohyma, 1982). These workers have stressed on the importance of concentration of a particular source of carbohydrate for a particular species for getting desired response. This requirement may be related to the specific carbohydrate metabolism through which water relations and endogenous phytohormones are regulated.

The results on the effect of sucrose concentration on *in vitro* rooting and shoot growth of rough lemon plantlets are presented in Chapter IV, section 4.3.4. The treatment involving MS medium (half strength) supplemented with sucrose (3.0 per cent) was best with respect to initiate roots in maximum cultures (90.00 per cent)

with in shortest period of time 14.96 days. The results are in consonance to Hazarika *et al.* (2004). Increase in sucrose concentration with decrease in salt concentration of media was found inhibitory to the root initiation and growth by Varidemoorele *et al.* (1993) in *Brassica oleraceae* L. var. botrytis. They further found that root formation required a low medium osmolarity but very low osmotic potential also had a negative effect on rooting. This may be the reason for lower response in medium supplemented with 1.5 and 4.5 per cent sucrose as compared to medium supplemented with 3.0 per cent sucrose.

Attempts were also made to study the effect of pH of the medium on *in vitro* root and shoot characters of *Citrus jambhiri*. The results obtained are presented in Chapter IV, section 4.3.5. The percentage of rooting was maximum in medium having pH 5.8, which was at par with medium having pH 6.0. Though, the importance of pH in tissue culture studies was reported by Gautheret (1947), who observed pH drifts during growth of a culture. The usual practice is to adjust the pH of the medium with in the range of 5.5 to 6.0 during the preparation of the medium. However, there are few reports on the effect of media pH on the growth of culture. According to Rao (1966), the best growth of the culture was observed at a pH of 6.0; while the growth decreased at both the lower (pH 3.0) and higher pH values (pH 11.0). According to Shah (1983), who also studied the effect of different pH levels on *in vitro* growth and rooting of *Glycyrrhiza glabra*, found that *in vitro* shoot and root growth was best at pH 5.5. In guava, Amin and Jaiswal (1989) observed that comparatively less acidic (pH 5.5-6.0) medium was better than more acidic (pH 4.5-5.0) medium for *in vitro* rooting.

5.4 Acclimatization and planting out (Stage IV)

Hardening the *in vitro* raised plantlets, so as to make them adapted to the natural atmosphere is a critical process due to their anatomical and physiological peculiarities. On transplanting, excessive water loss from the plantlets had been recorded which was attributed to the improper development of cuticle and slowness of stomatal response to water stress (Brainerd and Fuchigami, 1981; Fabbri *et al.*, 1984). The problem may be aggravated if the vascular connection between the shoot is improper. Therefore, a period of humidity for acclimatization was considered necessary for the newly transferred plantlets to adapt to the natural environment during which the plantlets undergo a morphological and physiological adaptation enabling them to develop typical terrestrial plant water control mechanism (Grout and Aston, 1977; Sutter *et al.*, 1985).

The results of the effect of hardening treatments on survival and growth of plants are presented in Chapter IV, section 4.4. Excessive water loss from plantlets was prevented by giving various treatments. These treatments were found to influence greatly the survival and growth of plantlets. Out of the different treatments adopted, 96.67 per cent plantlets survived when they were kept covered individually with glass beaker and kept in culture room at a temperature of 26 $\pm$ 2^{0}C under continuous light. Similar results have been obtained in rose (Skirvin and Chu, 1979); citrus (Desai *et al.*, 1994; Singh *et al.*, 1994); guava (Wali *et al.*, 1996), when young rooted plantlets from culture tube were potted and covered with glass beaker under continuous light. The continuous light provided in air-conditioned room as well as constant and low temperature may have favourable effect on morphological and

physiological adaptation for developing typical terrestrial water contact mechanism. The method of covering the *in vitro* developed plantlets followed by misting in green houses/polyhouse for initial period and subsequently removing the cover is a gradual process, was successfully adopted by a number of workers for hardening the plantlets (Murashige, 1974; Rajeevan and Pandey, 1986; Singh and Pandey, 1988; Raghunath, 1989; Rout *et al.*, 1989; Babylatha, 1994; Karwa, 2003). According to them, plantlets developed their stomatal control mechanism during this period. In the present study, the covering of plantlets individually with polythene bag and keeping them in culture room at a temperature of 26 $\pm$ 2^0C under continuous light was focused to reduce the plantlet survival as compared to the treatment to plantlets covered with glass beaker. This lowering down of survival of plantlets may probably be because of lesser penetration of light through the polythene sheet.

Among various potting mixtures tried, the mixture containing soil, sand and FYM (1:1:1 v/v) was found to be the most suitable. Physical, chemical and biological properties of the potting mixture are important for the establishment of *in vitro* produced plantlets. Better performance of FYM may be attributed to its ability to improve biological properties of the soil. On the other hand, sand may be responsible for providing sufficient aeration. Hence mixing soil, sand and FYM might have helped in giving better grip for the roots, ample aeration and sufficient organic matter. The participation of organic matter for better establishment of citrus plantlets have been described by many workers. Starrantino and Caruso (1988) reported successful establishment of various citrus rootstock plantlets in pots containing steam sterilized mixture of light volcanic soil and peat moss. However, Desai *et al.* (1994)

established *in vitro* rooted shoots of acid lime in soil, sand and vermiculite (1:1:1 v/v). Ling and Iwamasa (1997) reported better establishment of citrus related genera in pot containing sterile vermiculite and soil (1:1 v/v). Rana and Singh (2002) successfully established *in vitro* rooted shoots of Kagzi lime in sand:soil:compost (1:1:1 v/v) mixture. The probable reason could be the better soil aeration, water holding capacity of media and nutrient uptake. Rai *et al.* (2005) used vermiculite, sand and soil (1:1:1 v/v) as potting mixture for maximum survival of *in vitro* grown shootlets of Natal plum.

Among the anti-transpirants tried, glycerol 50 per cent was found to be the best as it resulted in maximum survival. The direct effect of anti-transpirants on plant let survival was found to be promotive. This may be due to closing of stomata or formation of film by the anti-transpirants on leaf and stem surface resulting in reduction of cuticular transpiration, which helps the plant to withstand stress conditions associated with acclimatization (Dhaliwal *et al.* 1997). Mishra *et al.* (2005) reported that glycerol was proved to be more effective than ABA for hardening of plantlets of tuberose.

5.5 Economics of *in vitro* propagation system of *Citrus* species

The cost of producing citrus plantlets using axillary bud culture was worked out by considering the available facilities of Tissue culture Laboratory with a potential of maintaining about 1,00,000 plantlets at a time. Based on the rate of culture establishment, rate of multiplication, rooting response of shoots and the survival of the plantlets at various stages, it was found that about 1,00,000 plantlets could be produced in 29 weeks time from 113 initial nodal segments of 2-3 week old

flushes from 10-12 year old *Citrus jambhiri* trees. The total cost involved was worked out based on the labour charges, cost of building, equipments, glassware, chemicals and miscellaneous items having been distributed over the years according to their potential/durability. The cost of producing one *in vitro* grown *Citrus jambhiri* plantlet including 3 weeks hardening (2 weeks in air-conditioned room and 1 week in polyhouse) was found to be Rs. 2.54.

A further period of 6 weeks may be required in natural environment before it is planted in fields, which will increase the cost of plantlet i.e., polyhouse facilities. The cost is projected cost and may vary depending particularly on the size of operation and efficiency of the technicians and other staff, besides, prevailing land and construction cost in the area where production unit is installed.

Debergh and Maene (1981) pointed out that *in vitro* rooting was the most labour intensive part which consumes larger amount of the total nutrient medium requirements for *in vitro* propagation system. In the present investigation also, *in vitro* rooting stage was found to be the most labour consuming and expensive part. Yeoman (1986) suggested that wherever possible, rooting and hardening process may be combined and if practicable rooting of shoot may be attempted by direct planting into a conventional medium so as to eliminate a further costly transfer to a sterile medium. There is however, a need to examine this possibility to effectively reduce the cost of production. The cost can be reduced further by augmenting the physical facilities, by further improving the rate of multiplication of propagules and by reducing the contamination of various stages of propagation system. The analysis of cost of media indicated that cost could further be reduced by replacing analytical

grade sucrose with commercial sugar and also by gelling the medium with 0.4 per cent agar instead of 0.8 per cent agar for *in vitro* rooting. Shah (1989) suggested the use of liquid medium instead of solid medium as an aid to reduce the cost of production considerably.

However, further work is needed to find out the suitability of liquid medium and also the use of commercial grade sugar in place of analytical grade sucrose for culture establishment and proliferation stages, which can remarkably reduce the unit cost of plantlet. The current price of *Citrus jambhiri* plant obtained through conventional method of propagation at Research Orchard; Udheywalla Campus, SKUAST-Jammu is Rs. 4.48. So, considering even the six week period cost before the plants are ready for transplanting in soil, tissue culture plants are more economical as compared to current rate prevailing in government and private nurseries. Therefore, the method of rapid multiplication of *Citrus jambhiri* as developed in the present investigation using nodal segments culture offers a great promise.

The foregoing discussion clearly showed that possibilities for developing nodal stem segment as a commercial method of propagation of *Citrus jambhiri* in Jammu (Jammu and Kashmir). However, before the protocol developed is utilized as a commercial method there is a need to examine the performance of *in vitro* propagules in the field.

Further, studies are needed to standardize optimum physical environmental requirements (light and temperature) which are critical factors affecting the induction of multiple shoots and rooting *in vitro*. Emphasis should be given to the use of liquid

medium for improving rate of multiplication and replacement of analytical grade sucrose with commercial sugar for all stages of *in vitro* propagation.

Thus, the research findings and protocol developed for *in vitro* propagation of *Citrus* spp. in the present investigation are the first series of steps towards commercialization of the technique under local conditions of Jammu region (Jammu and Kashmir). If the tissue culture plants are produced at the estimated rate, it is beyond doubt that this technique will become commercially viable for propagation of citrus.

Conclusion

The summary of Thesis is

Summary and Conclusion

The present investigations on the various aspects of "*In vitro* propagation studies on *Citrus* spp." were carried out at the Tissue Culture Laboratory, Faculty of Agriculture, Udheywalla, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu (J&K) during 2004-06. The results obtained from the present study can be summarized and concluded as follows:

1. The surface sterilization treatment involving ethanol 70 per cent and mercuric chloride 0.1 per cent for 30 seconds and 8 minutes respectively was found to give best sterilization of nodal segment explants taken from 10-12. year old mature trees of both *Citrus jambhiri* and *Citrus sinensis* cv. Mosambi.
2. The treatment, MS medium supplemented with BAP 1.5 mg/l in combination with malt extract 500 mg/l was found to be best for *Citrus jambhiri* as it took least time for maximum culture establishment, while in case of *Citrus sinensis* cv. Mosambi BAP 1.0 mg/l in combination with malt extract 500 mg/l gave more culture establishment in lesser time.
3. The treatment, MS medium containing BAP 1.0 and 1.5 mg/l with malt extract 500 mg/l and BAP 1.5 mg/l with Kinetin 0.5 mg/l in case of *Citrus jambhiri*, while BAP 0.5 and 1.0 mg/l with malt extract 500 mg/l and BAP 1.0 mg/l with Kinetin 0.5 mg/l in *Citrus sinensis* cv. Mosambi showed cent per cent multiple shoots during proliferation .

4. The treatment combination of BAP 1.5 mg/l with malt extract 500 mg/l showed maximum number of shoots per culture (5.34), while BAP 1.0 mg/l with malt extract 500 mg/l showed maximum length of longest shoot with higher number of leaves on longest shoot (9.13) in case of *Citrus jambhiri*. On the other hand, in case of *Citrus sinensis* cv. Mosambi, BAP 1.0 mg/l with malt extract 500 mg/l resulted more number of shoots per culture (7.71) with maximum number of leaves on longest shoot (9.84).
5. In general, incorporation of NAA had a negative influence on multiple shoot induction. It reduced both number of shoots per culture and length of longest shoot in both *Citrus jambhiri* and *Citrus sinensis* cv. Mosambi.
6. In general, the rate of multiplication decreased with the increase in number of sub-cultures in all the treatments tried in both *Citrus jambhiri* and *Citrus sinensis* cv. Mosambi.
7. The maximum number of shoots were obtained in the treatment containing BAP 1.5 mg/l with malt extract 500 mg/l in all the three continuous sub-culturing done at 6 weeks interval (8.79, 6.73 and 4.99) in case of *Citrus jambhiri*, while in *Citrus sinensis* cv. Mosambi, BAP 1.0 mg/l with malt extract 500 mg/l resulted in maximum number of shoots in all the three sub-culturing (9.48, 7.42 and 6.84).
8. Out of different auxins (IBA and NAA) tried for *in vitro* rooting of *Citrus jambhiri*, maximum rooting (83.33 per cent) was obtained in MS (half strength) medium with IBA and NAA (1.0 mg/l) each.
9. Liquid medium with supporting structures *viz*., Whatman No. 1 filter paper, ordinary filter paper and brown paper were found to be ineffective for *in vitro*

rooting of *Citrus jambhiri*. On the other hand, reduction in the concentration of agar from 0.8 to 0.4 per cent improved *in vitro* rooting characters of shoots. The survival of plantlets produced from nodal segment of explant increased from 73.33 to 86.67 per cent by reducing agar concentration from 0.8 to 0.4 per cent.

10. 3.0 per cent sucrose with half strength MS medium proved better than 1.5 and 4.5 per cent sucrose concentration for early root initiation (14.96 days) with maximum rooting percentage (90.00 per cent). The highest number of roots per shoot (2.24) and maximum length of the longest root (2.24) were observed on 3.0 per cent sucrose.
11. Among all the pH levels tested (5.4, 5.6, 5.8, 6.0 and 6.2), the rooting percentage was maximum in medium having pH 5.8. The minimum time for root initiation with longest length of root was obtained in the same treatment and it was found to be best for *in vitro* rooting of shoots obtained from *Citrus jambhiri* explant.
12. *In vitro* rooting and survival of plantlets were found to be influenced by different sources of carbohydrates. Out of the different sources of carbohydrates used, 3.0 per cent sucrose and commercial sugar recorded 90.00 per cent rooting of shoots obtained from *Citrus jambhiri* explant. Sucrose 3.0 per cent was proved to be best as it resulted in maximum number of roots (2.24) with highest length of longest root (3.34 cm). The minimum per cent of *in vitro* rooting was observed in 2.0 per cent maltose.
13. Covering the plantlet individually with glass beaker and keeping in air-conditioned room resulted in 96.67 per cent survival of plantlets. Keeping the

plantlets in laboratory at room temperature without any cover resulted in zero per cent survival.

14. The survival percentage was maximum (93.33 per cent) in the potting mixture containing soil, sand and FYM (1:1:1 v/v) which was superior to all other potting mixtures used.
15. Glycerol improved plant survival under *ex vitro* condition. The maximum plant survival was observed in plants treated with 50 per cent glycerol. The ABA as anti-transpirant had little effect on plant survival.
16. The unit cost of one *in vitro* grown *Citrus jambhiri* plantlet obtained from nodal segment explant source of 10-12 year old mature tree was estimated to be Rs. 2.54, whereas one field grown seedling costs about Rs. 4.48.

From the present study, it can be concluded that micro propagation in *Citrus jambhiri* can be exploited commercially for mass multiplication at a very rapid rate as compared to conventional methods, whereas the shootlets of *Citrus sinensis* cv. Mosambi can be utilized for future shoot-tip grafting. The results have suggested that this protocol may be made commercially viable. However, some work needs to be intensified to make the developed protocol commercially feasible besides testing tissue culture plants in the field. There are 4 main steps in protocol developed *viz.*, establishment, proliferation, rooting and acclimatization. It is estimated that by using the protocol it could be possible to produce approximately 1,00,000 plantlets of *Citrus jambhiri* from 113 initial nodal segments at a cost of Rs. 2.54. The results obtained would be very useful for mass multiplication of *Citrus jambhiri* plantlets

using nodal segment (axillary bud culture) under local conditions and may provide guidelines for setting up commercial unit for propagation of *Citrus* spp.

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APPENDICES

Appendix-I

Salary and wage of supervisor, technician and helper for *in vitro* production of *Citrus jambhiri* plantlets from nodal segments

(a) Time required for inoculation

Stage	No. of explants or culture		Rate of inoculation per hour	Total hours
I. Culture establishment (3 weeks)	113		100	1 hr 08 min
	-14	(Contamination)		
	99			
II. Culture proliferation (Cycle I - 3 weeks)	99		100	0 hr 59 min
	-5	(Contamination)		
	94			
	x 5.34*			
	502			
II. Cycle II – 6 weeks	502		100	5 hrs 01 min
	-25	(Contamination)		
	477			
	x 8.79*			
	4193			
II. Cycle III – 6 weeks	4193		100	41 hrs 56 min
	-210	(Contamination)		
	3983			
	x 6.73*			
	26806			
II. Cycle IV – 6 weeks	26806		100	268 hrs 04 min
	-1340	(Contamination)		
	25466			
	x 4.99*			
	127075			
III. Rooting (5 weeks)	105472		120	878 hrs 56 min
	-5274	(Contamination)		
	100198			
	100198			1195 hrs (approx.)

* Actual rate of multiplication
Total time required for inoculation = 1195 hrs (approx.)

(b) Time required for media preparation

Stage	Total quantity of media required (litres)	Approximate time required (hours)
I. Culture establishment	0.85	0 hr 13 min
II. Culture proliferation		
(i) Cycle I	0.74	
(ii) Cycle II	3.77	59 hrs 20 min
(iii) Cycle III	31.45	
(iv) Cycle IV	201.05	
III. Rooting	878.93	220 hrs 13 min
	Total	280 hrs (approx.)

Total time required for media preparation = 280 hrs

Total time required for inoculation & media preparation = 1195 + 280 = 1475 hrs

(A) Total labour hours of technician = 1475 hrs

(B) Total labour hours of helper for washing glassware, helping in media preparation = 1475 hrs

(C) Total labour hours of supervisor (20 % of technicians time) = 295 hrs

(c) Total amount of salary and wage

S.No.	Item	Hours of labour	Rate/hour (Rs.)	Total amount (Rs.)
1.	Technician	1475	20	29,500.00
2.	Helper	1475	9	13,275.00
3.	Supervisor	295	25	7,375.00
			Total	50,150.00

Total cost of Salary and wage = **Rs. 50,150**

Appendix-II

Quantity and cost of nutrient medium required for production of 1,00,000 plantlets

Stage	Number of expl -ant or culture	Number of explant or culture per vessel	Quantity of media required per culture vessel (ml)	Total quantity of media required (lts.)	Cost per litre (Rs.)	Total cost (Rs.)
I. Culture establishment	113	4	30	0.85	23.74	20.18
II. Culture proliferation	31,600	4	30	237.00	23.74	5,626.38
III. Rooting	1,05,472	6	50	878.93	18.06	15,873.48

Total quantity of medium required = 1116.78 litres

Total cost of nutrient medium = Rs. 21,520.04 i.e. **Rs. 21,520**

Appendix-III

Total glassware required for final production of 1,00,000 jambhiri plantlets

S.No.	Item	Approximate No. required	Rate/piece (Rs.)	Total cost (Rs.)
1.	Culture bottles	18,000	5	90,000
2.	Other glassware (flasks, cylinder, pipette etc.)	-	-	25,000
			Total	1,15,000

Total cost of glassware = Rs. 1, 15, 000

Appendix-IV

Total requirement of equipments for *Citrus jambhiri* plantlet production

S.No.	Item	Number required	Rate/piece (Rs.)	Total cost (Rs.)
1.	Air-conditioner	2	20,000	40,000
2.	Autoclave	1	1,25,000	1,25,000
3.	Culture racks	30	4,750	1,42,500
4.	Electronic balance	1	43,000	43,000
5.	pH meter	1	14,850	14,850
6.	Refrigerator	1	18,000	18,000
7.	Hot air oven	1	20,000	20,000
8.	Laminar air-flow	2	79,000	1,58,000
9.	Distillation unit	1	18,000	18,000
10.	Furniture	-		15,000
11.	Miscellaneous	-		5,000
			Total	5,99,350

Total cost of equipments = Rs. 5, 99, 350

Appendix-V

Laboratory building requirements and investments for *Citrus jambhiri* plantlet production

S.No	Item	Area (sq. ft.)
1.	Culture room (18′ x 20′)	360
2.	Inoculation room (8′ x 12′)	96
3.	Media preparation room (15′ x 20′)	300
4.	Office cum library (10′ x 15′)	150
5.	Washing room (8′ x 10′)	80
6.	Store room (8′ x 10′)	80
7.	Toilet block and passage	134
	Total	1200

Total cost of laboratory building @ Rs. 600/sq.ft. = Rs. 7, 20, 000

Appendix-VI

Overhead charges for producing 1, 00, 000 *Citrus jambhiri* plantlets

S.No.	Item		Approximate charges for 29 weeks cycles (Rs.)
1.	Electricity		30,000
2.	Gas		650
3.	Water		300
4.	Miscellaneous		1,000
		Total	31,950

Total overhead charges = **Rs. 31, 950**

Appendix-VII

Calculation of charges for capital investment

A. Glassware charges:

Total cost as per Appendix III	= Rs. 1, 15, 000
Life	= 25 cycles
For *C. jambhiri* production	= 25 x 29 weeks
	= 725 weeks
Interest @ 7.5 % per year for 725 weeks	= Rs. 1, 20, 252.39
	i.e. Rs. 1, 20, 252
Total cost including interest	= Rs. 1, 15, 000 + 1, 20, 252
	= Rs. 2, 35, 252 for 725 weeks
For 1 week	= Rs. 325
For 29 weeks	= **Rs. 9, 425**

B. Equipment charges:

Total cost as per Appendix IV	= Rs. 5, 99, 350
Life	= 15 years i.e. 780 weeks
Interest @ 7.5 % per year for 15 years	= Rs. 6, 74, 269
Total cost including interest	= Rs. 5, 99, 350 + 6, 74, 269
	= Rs. 12, 73, 619 for 780 weeks
For 1 week	= Rs. 1, 632.85
For 29 weeks	= Rs. 47, 352.65 i.e. **Rs. 47, 353**

C. Laboratory facility charges:

Total cost as per Appendix V	= Rs. 7, 20, 000
Life	= 25 years i.e. 1300 weeks
Interest @ 7.5 % per year for 25 years	= Rs. 13, 50, 000
Total cost including interest	= Rs. 7, 20, 000 + 13, 50, 000
	= Rs. 20, 70, 000 for 1300 weeks
For 1 week	= Rs. 1, 592.31
For 29 weeks	= Rs. 46, 176.99 i.e. **Rs. 46, 177**

Appendix-VIII

Summary of the estimated *in vitro* cost for producing 1, 00, 000 *Citrus jambhiri* plantlets

S.No.	Item	Total cost (Rs.)	Cost per plant (Rs.)	Percentage
1.	Salary and wage	50, 150	0.50	24.28
2.	Nutrient medium	21,520	0.22	10.42
3.	Glassware	9, 425	0.09	4.56
4.	Equipments	47, 353	0.47	22.92
5.	Laboratory	46, 177	0.46	22.35
6.	Overhead charges	31, 950	0.32	15.47
	Total	2, 06, 575	2.06	

Estimated *in vitro* cost per plantlet = **Rs. 2.06**

Appendix-IX

Estimated cost for transplanting and hardening 1, 00, 000 plantlets produced *in vitro*

A. Salary and wage

1. Time required for transplanting 1, 00, 000 plantlets @ 80 plantlets per hour	= 1250 hrs
2. (a) Time required for preparation of potting mixtures, filling the bags etc.	= 1000 hrs
(b) Time required for transferring 1, 00, 000 plantlets from air-conditioned room to polyhouse	= 80 hrs
3. Labour charges @ Rs. 9 per hour	= Rs. 20, 970
4. Supervisory charges, 233 hours @ Rs. 25 per hour upto polyhouse stage	= Rs. 5, 825
5. Total cost of salary and wage	= 20, 970 + 5, 825
	= **Rs. 26, 795**

B. Transplanting material cost

1. Cost of polythene bags (approximately 100 kg @ Rs. 84 per kg)	= Rs. 8, 400
2. Cost of potting mixture, fungicide etc.	= Rs. 4, 000
Total transplanting material cost	= Rs. 8, 400 + 4, 000
	= **Rs. 12, 400**

C. Cost of hardening *Citrus jambhiri* plantlets in air-conditioned room

1. Cost of construction for air-conditioned room (360 sq. ft.) @ Rs. 600 per sq. ft. = Rs. 2, 16, 000

Life = 25 years i.e. 1300 weeks

Interest @ 7.5 % per year for 25 years = Rs. 4, 05, 000

Total cost including interest = Rs. 2, 16, 000 + 4, 05, 000

= Rs. 6, 21, 000 for 1300 weeks

For 1 week = Rs. 447.69

For 2 weeks = Rs. 955.38

i.e. Rs. 955

2. Equipment/glassware cost

S.No.	Item	Number required	Rate/piece (Rs.)	Total cost (Rs.)
1.	Air-conditioner	1	20, 000	20, 000
2.	Racks fitted with tube lights	30	4, 750	1, 42, 500
3.	Glass bottles	1, 00, 000	5	5, 00, 000
			Total	6, 62, 500

Total cost of equipment/glassware = Rs. 6, 62, 500

Life = 15 years i.e. 780 weeks

Interest @ 7.5 % per year for 15 years = Rs. 7, 45, 313

Total cost including interest = Rs. 6, 62, 500 + 7, 45, 313

= Rs. 14, 07, 813 for 780 weeks

For 1 week = Rs. 1, 804.89

For 2 weeks = Rs. 3, 609.78

i.e. Rs. 3, 610

Total cost of hardening in air-conditioned room = 955 + 3, 610

= **Rs. 4, 565**

D. Polyhouse facilities cost

1. Cost of polyhouse with mist facilities	= Rs. 70, 000
Life	= 25 years i.e. 1300 weeks
Interest @ 7.5 % per year for 25 years	= Rs. 1, 31, 250
Total cost including interest	= Rs. 70, 000 + 1, 31, 250
	= Rs. 2, 01, 250 for 1300 weeks
For 1 week	= Rs. 154.80
	i.e. Rs. 155
2. Cost of polythene (approximately 70 kg @ Rs. 170 per kg)	= Rs. 11, 900
Life	= 3 years i.e. 156 weeks
Interest @ 7.5 % per year for 3 years	= Rs. 2, 678
Total cost including interest	= Rs. 11, 900 + 2, 678
	= Rs. 14, 578 for 156 weeks
For 1 week	= Rs. 93.45
	i.e. Rs. 94
Total polyhouse facilities cost	= 155 + 94
	= **Rs. 249**

E. Overhead charges for hardening plantlets in air-conditioned room (2 weeks) and keeping them in polyhouse (1 week)

(Electricity, water etc.) = Rs. 3, 500 (approx.)

Appendix -X

Summary of estimated costs for transplanting and hardening 1, 00, 000 *in vitro* produced plantlets

S.No.	Item	Total cost (Rs.)	Cost per plant (Rs.)	Percentage
1.	Salary and wage	26, 795	0.27	56.40
2.	Transplanting material cost	12, 400	0.12	26.10
3.	Plant hardening cost	4, 565	0.05	9.61
4.	Polyhouse cost	249	0.003	0.52
5.	Overhead charges	3, 500	0.04	7.37
	Total	47, 509	0.48	

Estimated cost for transplanting and hardening of one *C. jambhiri* plantlet = **Rs. 0.48**

APPENDIX-XI

Meteorological data for the period of study (2004-06)

Month	Temperature (^{0}C)		Relative humidity (%)		Rainfall (mm)
	Max	Min	Morning	Evening	
Oct-04	29.1	15.0	93	47	46.9
Nov-04	26.1	9.4	92	45	4.2
Dec-04	20.9	9.9	95	58	0.0
Jan-05	17.0	4.2	96	66	105.4
Feb-05	17.9	7.4	94	70	138.8
Mar-05	25.5	11.6	91	59	97.4
Apr-05	32.2	15.0	79	31	10.3
May-05	35.6	19.8	55	30	10.5
June-05	40.2	24.8	53	30	8.0
July-05	33.4	24.5	89	68	288.5
Aug-05	33.9	24.6	91	68	120.2
Sep-05	33.0	23.5	91	63	40.8
Oct-05	31.8	16.4	89	41	0.0
Nov-05	26.8	9.0	86	35	0.0
Dec-05	21.3	3.7	92	41	0.0
Jan-06	18.3	6.3	92	62	58.7
Feb-06	25.8	10.8	90	51	8.0
Mar-06	26.3	11.7	87	50	35.2
Apr-06	34.5	15.9	66	26	10.0

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