

**"MICROPROPAGATION IN ROUGH LEMON  
(*Citrus jambhiri* Lush.)"**

A

THESIS  
SUBMITTED TO  
NAVSARI AGRICULTURAL UNIVERSITY  
NAVSARI

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS  
FOR  
THE AWARD OF THE DEGREE

OF

**MASTER OF SCIENCE  
(HORTICULTURE)**

IN

**FRUIT SCIENCE**

BY

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FEBRUARY – 2011

# MICROPROPAGATION IN ROUGH LEMON

(*Citrus jambhiri* Lush.)

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## ABSTRACT

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## **ABSTRACT**

The present investigation was carried out at the Department of Biotechnology, ASPEE College of Horticulture and Forestry, Navsari Agricultural University, Navsari, Gujarat on “Micropropagation in rough lemon (*Citrus jambhiri* Lush.)” during the year 2008-2010.

The surface sterilization of explants, treatment involving HgCl<sub>2</sub> (0.1 %) for 5 minutes gave better sterilization of nodal segment and shoot tips. Moreover, better response of the sterilant treatment was reported in nodal segment than shoot tip explants with respect to establishment of explants and growth of shoots, besides, contamination control. The use of mercuric chloride alone was more effective in reducing the contamination per cent as compared to sodium hypochloride.

Maximum per cent establishment of shoot tips and nodal segment explants was observed on treatment MS medium containing 1.0 mg/l BAP + 0.5 mg/l NAA.

MS medium supplemented with 1.0 mg/l BAP + 500 mg/l ME exhibited maximum multiplication rate of shoot with higher length of shoots in nodal segment explant. In serial sub cultures, subsequently, multiple shoots and length of shoot were reported higher in the same treatment up to four sub cultures.

Maximum proliferation of culture was recorded in 3% sucrose in the medium followed by 4% sucrose.

Media pH had much influence on growth of regenerated shoots. Among the different pH levels tested, 5.7 pH was found to be the best for maximum number of shoots and length of shoot followed by 6.0 pH.

Maximum number of shoots, length of shoot and growth rate were observed at 3000 lux light intensity.

*In vitro* rooting in regenerated shoots was found the best with full strength MS medium supplemented with 1.0 mg/l IBA + 1.0 mg/l NAA treatment.

*In vitro* plantlets showed better survival in FYM + soil + sand (1:1:1 v/v) medium. *In vitro* raised plantlets were acclimatized successfully under the climatic condition of plantlets covered with plastic cup individually and kept under net house condition.



**Dr. R. M. Patel**  
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## **C E R T I F I C A T E**

This is to certify that the thesis entitled, **“Micropropagation in rough lemon (*Citrus jambhiri* Lush.)”** submitted by **Mr. RANA JIGARKUMAR SANMUKHLAL** in partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE (Horticulture)** in the subject **FRUIT SCIENCE** of Navsari Agricultural University is a record of bonafide research work carried out by him under my guidance and supervision and that the thesis has not been previously formed the basis for the award of any degree, diploma or has been published for other similar title. All the assistance and help received during the course of the investigation have been duly acknowledged by him.

**Place: Navsari**

**Date : 28<sup>th</sup> February, 2011**

  
**(R. M. Patel)**

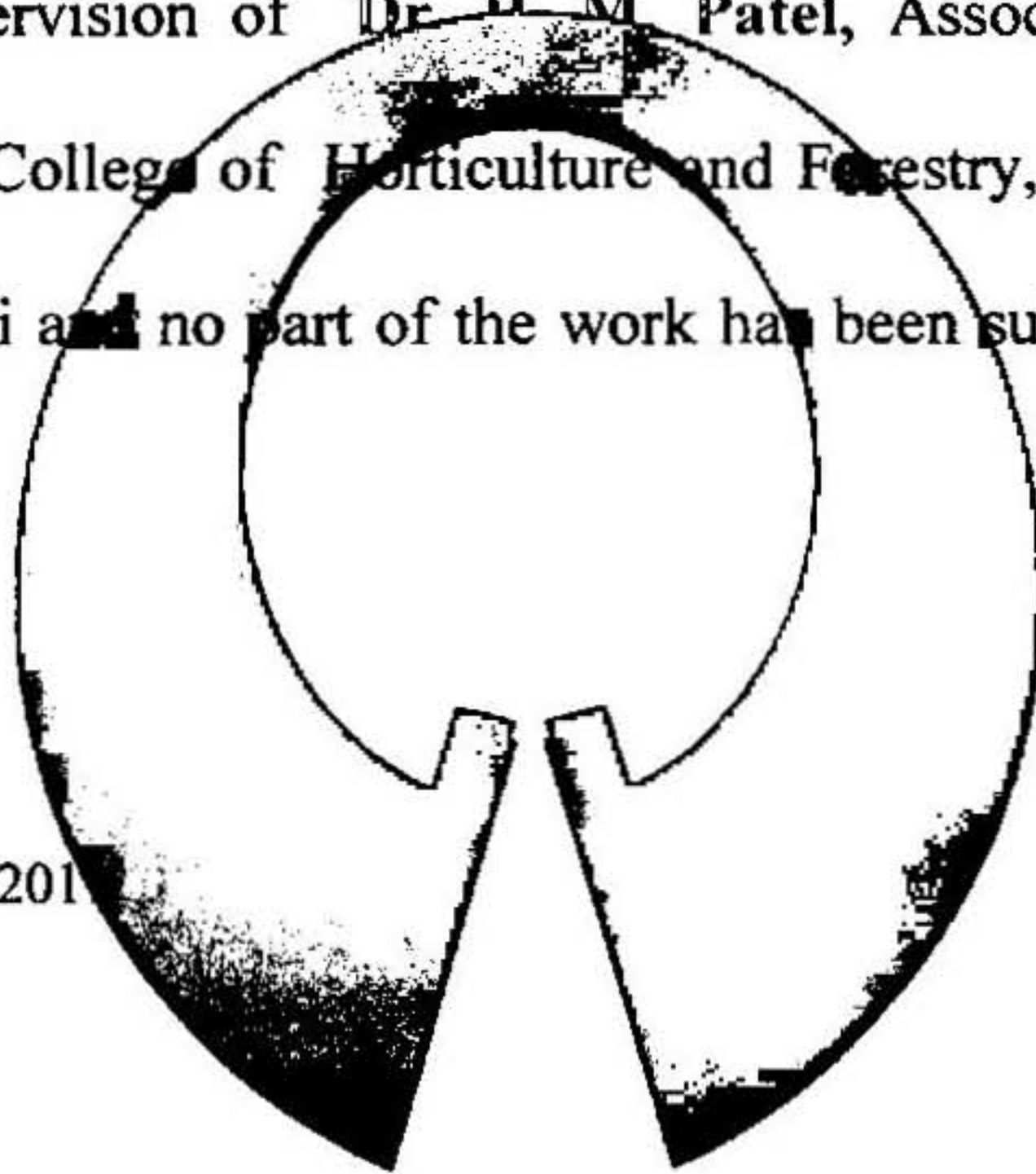
**Major Advisor**

# DECLARATION

This is to declare that the whole of the research work reported here in the thesis for the partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (HORTICULTURE) in FRUIT SCIENCE** by the undersigned is the result of investigation done by him under direct guidance and supervision of **Dr. P. M. Patel**, Associate Professor (Fruit Science), **ASPEE College of Horticulture and Forestry**, Navsari Agricultural University, Navsari and no part of the work has been submitted for any other degree so far.

**Place:** Navsari

**Date:** 28<sup>th</sup> February, 201



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**ACHFA**  
Let's Bloom Human Fruition **NAU**

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## *ACKNOWLEDGEMENT*

*At last I write the beginning. My feelings are of exhilaration and exhaustion I felt only one month ago is gone.*

*Foremost I express my infinite sense of gratitude to the Lord "Shree Krishna" for continually providing me spiritual energy, which has inspired me to reach at this point during my academic career.*

*I wish to offer my sincerest thanks to my major advisor Dr. R. M. Patel, Associate Professor (Fruit Science), ASPEE College of Horticulture and Forestry, Navsari Agricultural University, Navsari for his most valuable and inspiring guidance, constant encouragement, enormous help and constructive criticism throughout the course of this investigation and preparation of this manuscript. I will always cherish his friendly nature, love, affection and magnanimous attitude.*

*I feel a great pleasure in getting this proud privilege offering my sincerest and devoted thanks to my minor advisor Mr. R. B. Patel, Associate Professor (Floriculture), ASPEE College of Horticulture and Forestry, Navsari Agricultural University, Navsari.*

*I consider my self fortunate for excellent opportunity to work under inspiring and fruitful guidance and my gratitude toward the member of my advisory committee, Late Dr. R. R. Shah, Professor of Plant Physiology, ASPEE College of Horticulture and Forestry, for deeply interest in shaping my research work and giving me a valuable time when ever and where ever needed during the study.*

*I am thankful to the other member of my advisory committee, Mr. H.N. Chhatrola, Assistant Professor, Agricultural Statistics, ASPEE College of Horticulture and Forestry, Navsari*

*Agricultural University, Navsari. For his worthy suggestions, ever willing help and unbiased attitude throughout the course of this investigation.*

*I would like to express my deep gratitude to Dr. B. K. Dhaduk, Professor and Head, Department of Horticulture, ASPÉE College of Horticulture and Forestry and Director of Student Welfare, Navsari Agricultural University, Navsari. for taking me into his laboratory family and helping me during my work.*

*I am sincerely thankful to the teachers of Navsari Agricultural University, specially Dr. Sanjay Jha, Dr. Maheshkumar Mahatma, Dr. Lalitkumar Mahatma, Dr. Suman Jha and Mr. Taslim Ahmed, Assistant Professor, for their valuable help, constant attention and useful suggestions.*

*I would like to express my greatest sense of gratitude to Vice Chancellor, Dr. A. R. Pathak, Director of Research & Dean P. G. Studies, Dr. H. C. Pathak and Principal of ASPÉE College of Horticulture and Forestry, Dr. N. L. Patel for approving my thesis and providing necessary facilities during my studies.*

*"A journey is best measured in friends rather than miles" and I traveled along these miles in the fantabulous company of my dear friends Ankur Prajapati, Rakesh Swami, Sankalp Patel, Tejash Ahir, Dharendra Rathod, Ashutosh Chhatrola, Rashmikant Gujjar, Yaghyadevsinh Rana, Abhijit C., Shailendra B., Ashish G., Rajshree, Hansha, Smita, Raina, and other my seniors and colleagues Jaimin Patel, Kiran Shinde, Santnath Shinde, Jayesh Pathak, Rajveer, Dilshwar, Minal Tandel, Aditi Joshi, Shital and played a multi-faceted role; fantastic friends, great helping hands, sink to all my problems, partners to all my failures and achievements, rocking traveling and shopping companions and what not.*

Though thank is a taboo in a friendship, my conscience does not permit me refrain myself from expressing my heartfelt feelings towards my beloved friends Sandip, Paresh, Rohan, Vivek, Bahubali, Pawan, Indrajeet, Pushpraj, Vishal, and other my juniors Nishtha, Rajesh, Himesh, Mahesh, Mithil and Saurabh - all other friends who gave me direct and indirect sympathetic touch for completion of my research work. Friends, your jolly company has made my life richer.

Thanks are due to the non-teaching staff of lab, Arunaben, Premilaben, Ritesh, Jatin, Jayesh, Tarpan and for their guidance in all the official work, for helping in lab maintainence and organization, which make it a better place to work in. I sincerely thank you for all your help and support.

My vocabulary fails to get words to express deep sense of gratitude and indebtedness to my parents, my papa Late Shri Rana Sanmukhlal, my mummy Smt. Rana Daxaben, and also thankful for my Late grand father and grand mother, my dear sister Divyadidi, also motivation for my Uncle Dave Hariprasad, Rana Rajubhai, Rana Shaileshbhai and my all family members for their everlasting love, constant encouragement, prayer support and sacrifice, without which this dream could not have become a reality. I also wish to express my deepest gratitude and respect to my family members especially for there everlasting love and support.

Last, but far from the least, I could like to place on record my sincere regard, deepest gratitude, Soul full respect and a million thanks to 'God' the almighty, who helped me on every path of my life and made every step a great success.

Place : Navsari

Date : 28/02/2011

  
(JUGAR RANA)

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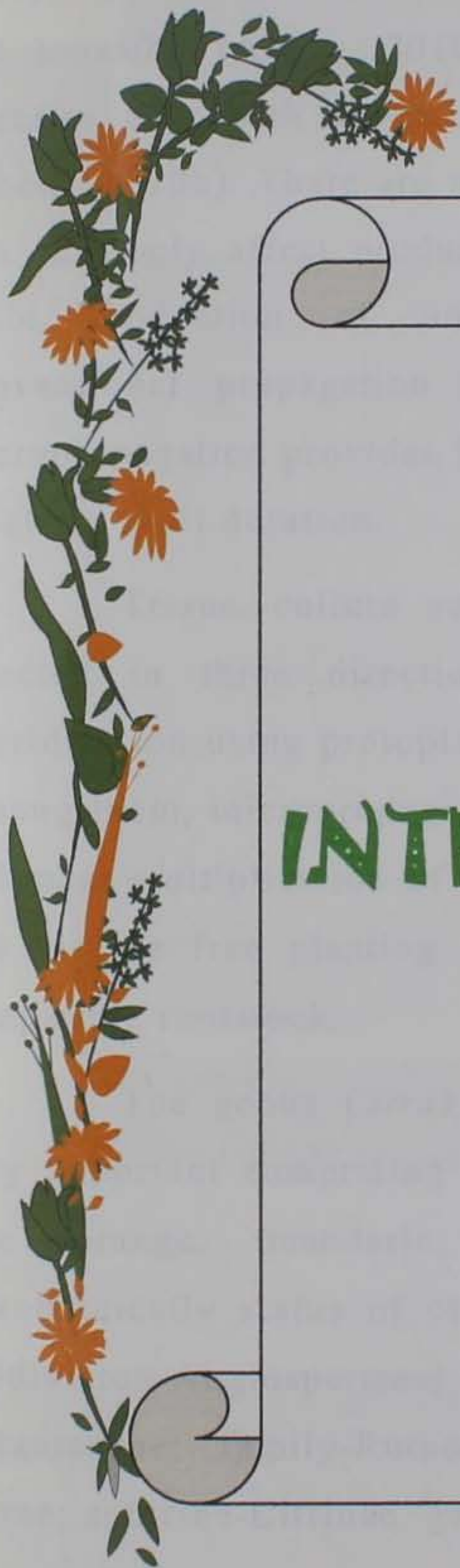
## **LIST OF ABBREVIATIONS AND SYMBOLS**

Abbreviations	Meaning
%	Per cent
$\mu M$	Micro molar
mM	Mili molar
AC	Activated charcoal
Anon.	Anonymous
ANOVA	Analysis of variance
B5	Gamborg medium
BAP	6-Benzyl amino purine
C. D.	Critical difference
C. V.	Co-efficient of variance
cm	Centimeter
Conc.	Concentration
cv.	Cultivar
EDTA	Ethylene diamine tetra acetic acid
<i>et al.</i>	Et alibi (and others)
etc.	And the rest
Fig.	Figure
FYM	Farm yard manure
GA <sub>3</sub>	Gibbrellic acid
g	Gram
ha	hectare
HgCl <sub>2</sub>	Mercuric chloride
hrs	Hours
i.e.	That is

Contd...

Contd....

Abbreviations	Meaning
IBA	Indole-3-butyric acid
Kin	kinetin
ME	Malt extract
mg/l	Milligram per litre
Min.	Minutes
ml	milli Litre
MS	Murashige and Skoog (1962) medium
MT	Metric tone
NAA	$\alpha$ - Naphthalene acetic acid
NaOCl	Sodium hypochloride
No.	Number
°C	Degree Celsius
RT	Room temperature
S.Em. $\pm$	Standard error of mean
sub-cult	subculturing
v/v	Volume / volume (concentration)
Viz.	Namely
w/v	Weight / volume (concentration)
WPM	Woody plant medium



# INTRODUCTION



## I. INTRODUCTION

In India, citrus ranks third in area and production next to mango and banana with an estimated production of 86.08 lakh tones fruits from 9.23 lakh hectares, having the productivity of 9.3 tones/ha (Anon., 2010a). Gujarat State occupies 35,300 hectares area with an estimated productivity of 9.9 tones/ha. (Anon., 2010b). There are number of diseases and disorders that can adversely affect productivity of citrus orchards. The large scale production of uniform planting material through conventional propagation methods is not possible; whereas micropropagation provides large number of uniform, disease free plants in short duration.

Tissue culture research in citrus has been mainly directed in three directions viz. micropropagation, somatic hybridization using protoplast fusion and genetic transformation. Among them, micropropagation technique is applicable for rapid and mass multiplication of desirable genotype to set true to type and disease free planting materials. It also helps to find out compatible rootstock.

The genus *Citrus* and its relatives are horticulturally very important comprising of many important citrus fruit crops like orange, mandarin, lemon, lime, grapefruit, etc. Taxonomically status of citrus is in the division-Embryophyta; subdivision-Angiospermae; order-Geraniales; suborder-Geraniales; family-Rutaceae; subfamily-Aurantioideae; tribe-Citreae; subtribe-Citrinae, genus-*Citrus*. These crops are grown in

tropical and subtropical climates and have wide distribution in the world. It is long-lived perennial crop which comprises 140 genera and 1300 species. It is often regarded as golden fruit or queen of all fruits. (Nito, 1996)

The classifications of important citrus species are as under.

➤ **Acid group (Citron, Lemon, Lime):**

- *Citrus medica* L. : Citron
- *Citrus limon* (L) Burm : Lemon
- *Citrus jambhiri* Lush : Rough lemon
- *Citrus karna* Raf. : Kharna Khatta
- *Citrus aurantifolia* Swingle (L): Lime
- *Citrus latifolia* Tanaka : Tahiti or Persian lime
- *Citrus limettioides* Tanaka : Sweet lime

➤ **Orange group**

- *Citrus aurantium* L.: Sour orange
- *Citrus myrtifolia* Raf. : Myrtle-leaf orange
- *Citrus bergamia* Risso : Bergamot orange
- *Citrus natsudaidai* Hayata : Japanese summer grapefruit
- *Citrus sinensis* (L.) Osbeck : Sweet orange

➤ **Pummelo – grapefruit group**

- *Citrus maxima* Osbeck : Chakotra
- *Citrus paradisi* Macf. : Grapefruit

➤ **Mandarin group (loose skin orange)**

- *Citrus reticulata* Blanco : Sangtra
- *Citrus unshiu* Marc. : Satsuma mandarin

- *Citrus deliciosa* Tenore : Willow leaf mandarin
- *Citrus rashni* Tanaka: Billi kichili or Cleopatra mandarin
- *Citrus nobilis* Loureiro : King of Kunembo
- *Citrus tangerine* Hort. (Tanaka) : Tangerins

➤ **Papeda group**

- *Citrus inchangensis* Swing : Ichang papeda
- *Citrus junos* Siebold : Yuzu of Japan
- *Citrus latipes* Swing : Khasi papeda
- *Citrus macroptera* Montr. : Melanesian papeda
- *Citrus hystrix* DC : Mauritius papeda
- *Citrus macrophylla* Wester : Alemow

➤ **Other species**

- *Citrus limonia* Osbeck : Rangpur lime
- *Citrus limetta* Risso: Limettas
- *Citrus maderaspatana* Tanaka : Guntur Sour orange
- *Citrus madurensis* Loureiro : Calamondin
- *Citrus indica* Tanaka : Indian wild orange
- *Citrus pennivesiculata* Tanaka : Gajanimma of India

Citrus fruit is consumed as a fresh fruits or processed products. The cultivation of citrus has globally flourished due to its fruit and ornamental value. The fruit possesses nutritive,

refreshing and medicinal value. Rough lemon is a commercially important crop in the tropical and subtropical regions. Slices of rough lemon are served as a garnish on fish or meat or with iced or hot tea, to be squeezed for the flavoured juice. Rough lemon is locally well adapted and dominant rootstock. It has extreme variability and different strains (Altaf and Khan, 2008).

Considering the nutritional value, the magnitude of fruit production and an array of commercial products made, citrus is considered as the number one fruits in the world (Chaturvedi *et al.*, 2001) Citrus varieties are propagated by both sexual and asexual methods.

Conventional propagation of citrus for new plantings of improved material relies on bud-wood selection and grafting for scion varieties and rooted cuttings or more commonly nucellar seed propagation for rootstocks. Citrus seedlings are not usually raised for commercial planting unless, they are polyembryonic one. There is considerable variation in the performance of gametic seedlings raised using seeds of different trees and that they do not exhibit true to type. However, the seedlings are most desirable as rootstock. The rootstock is a vital component of grafted fruit tree and determines the success or failure of the orchard. Most common rootstocks in India are rough lemon, rangpur lime, karna khatta and trifoliate orange. Selection of bud-wood or seeds from trees that have indexed negatively for virus infection is of course a priority. However, in view of the only limited success of heat treatment of bud-wood *in vivo* for virus

### *Introduction*

elimination, the importance of using techniques such as meristem tip culture, nucellar embryogenesis, and shoot tip grafting *in vitro* to provide virus-free mother stock for budding has become more pronounced (Navarro *et al.*, 1975). Such material can exchange without the risk of spreading disease or lengthy procedures in quarantine.

Similarly, propagation of hybrid citrus seedlings from breeding programmes aimed at improved rootstock characters e.g. disease resistance, salt tolerance etc., depends on striking cuttings or raising nucellar seedlings. Each of these methods involves a long interval between the production of a hybrid seedling and the replicated evaluation of its tolerance to disease or stress. Micropropagation offers the opportunity to reduce this interval and provide a more rapid evaluation. Use of reproductive material of citrus (e.g. nucellus) to establish callus lines has provided the impetus for research in a number of areas including clonal propagation, plant improvement, virus elimination and nutritional studies.

Generally, rootstocks are propagated sexually through seeds (Chaudhary, 1994). Micropropagation is an important asexual method that can be used for the production of virus-free rootstock plants (Roistacher *et al.*, 1976). In citrus, the rootstock (on which the scion is grafted) influences essential growth, nutrition, longevity of the tree and other characteristics of scion's performance, there by playing an important role in the success of any orchard. Micropropagation of selected rootstocks would be

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able to provide a solution to bring about qualitative and quantitative improvement in citrus production (Sen and Dhawan, 2009). The large scale production of uniform planting material through conventional propagation methods is not possible. Citrus propagation by conventional means is restricted to particular season and availability of plant material (Usman *et al.*, 2005).

Most Citrus species exhibit polyembryony and large percentage of seedlings are nucellar in origin (Frost and Soost, 1968). This most useful character for clonal multiplication *in vivo* has been exploited *in vitro* to produce highly embryogenic callus (Rangaswamy, 1958). This approach was later extended to monoembryonic citrus species (Rangan and Murashige 1968). However, the method is tedious. Moreover, the right stages of ovules are available only for a very short period during the year. Juvenile characteristics and delayed bearing are other disadvantages of nucellars warranting such for some other method for producing true to type and virus free citrus plants.

The commercial citrus fruit crops grown in India are generally propagated through "T-budding". Rough lemon is the common rootstock used for the propagation of these fruit crops. The rootstock is tolerant to salt, drought and viral diseases like tristeza, exocortis etc., and the plants raised on it are vigorous and produce fruits of good quality. Citrus being propagated by budding, demands for true to type and quality rootstock material in ample number. In India, about 80 per cent citrus is being raised on *C. jambhiri*. Citrus sp. budded on rough lemon are

### *Introduction*

vigorous, precocious, bear heavily remain productive for longer period and produce high quality of fruits. Micropropagation technique is one of the most important techniques for rapid and mass multiplication having of true to type, disease free and uniform plantlets (Desai *et al.*, 1996). Further, the demand of quality planting material of important rootstocks in ample number necessitates for their *in vitro* propagation (Sharma *et al.*, 2009). However, lacking of information on micropropagation in rough lemon in our climatic condition, the present study is carried out to attempt the standardization of protocol for micropropagation in rough lemon.

The major advantages offered by *in vitro* propagation technique in rough lemon are increased the rate of multiplication, disease free uniform propagules, easier transportation, improved crop yield, rapid selection and multiplication of elite genotypes and year round availability of planting materials. Therefore, developing and improving *in vitro* methods for propagating rough lemon is regarded as essential for long term efforts aimed at improving citrus culture world wide; especially in a state like Gujarat where rough lemon is one of the major citrus rootstocks.

The present investigation with this context aims to standardize the micro propagation technique for rough lemon with the use of the existing research based information, the experiments were designed to study the various aspects of *in vitro* propagation in rough lemon as follows:

*Introduction*

- 1) To standardize the medium for culture establishment.
- 2) To standardize the optimum multiplication medium.
- 3) To standardize the medium for *in vitro* rooting of regenerated shoots.
- 4) To standardize the technique for acclimatization of *in vitro* produced plantlets.

## II. REVIEW OF LITERATURE



# REVIEW OF LITERATURE



## II. REVIEW OF LITERATURE

A clonal propagation through tissue culture can be achieved in small space and short time. Rapid propagation is one of the benefits provided by this technique for economically important fruit crops, especially plants such as citrus that are so many problems to propagate conventionally. Achievements made by various scientists in the field of plant tissue culture and different plant species is reviewed in this chapter.

### 2.1 History of plant tissue culture research

*In vitro* culture has proved to be of immense practical value as an aid to plant breeding, plant propagation, raising and maintenance of disease free plants, germplasm storage as well as creating novel types through plant genetic engineering. Considering the importance and progress made during the last two decades, the National Biotechnology Board has chosen tissue culture as one of the areas of immediate interest.

India, with its amazing diversity of horticultural crops offers tremendous scope for exploiting this technique for mass multiplication of large number of elite and rare plants. So, for micropropagation techniques, over 51 fruit crops have been standardized to the extent that it can be utilized on commercial scale (Litz, 2005). However, there are several problems and limitations which must be overcome in a routine method of plant propagation. The key to successfully overcome the major problems lies with the standardization of correct combinations of medium composition, cultural and environmental condition, explants, acclimatization, etc. for local condition.

Many crops are being propagated commercially through tissue culture. The recent trends towards liberalization of Indian economy have a profound impact on the future development of biotechnology industry. Micropropagation of plants through tissue culture technique has proved to be one of most successful and popular techniques for economic development. Micropropagation techniques have been commercialized globally, especially in the nation with industrialized agriculture. It is reported that over 796 commercial companies are engaged in such activities all over the world and about 900 million plants are being produced worldwide. In India, 120 companies are registered, however, only about 14 companies are engaged in micropropagation of fruit crops. The quality planting material of elite genotypes for the horticulture crops is provided through tissue culture regeneration (Chandra and Mishra, 2010).

Plant micropropagation is a powerful and potential tool for producing the required number of propagules of desired characteristics. Until three decades ago, plant tissue culture was regarded as merely an experimental tool for highly specialized botanical investigation; but with the progress in research, it has now emerged as dynamic and important field of endeavor. *In vitro* culture has found its best commercial application in Agriculture, Horticulture and Forestry.

Plant tissue culture has an important role to play in the production of horticultural plants and in the manipulation of plant for improved agronomic performance. Plant tissue culture research

is a multi-dimensional science that offers exciting prospects for improvements in crop productivity. While, most nurserymen have introduced the technique of micropropagation, other dimensions of tissue culture research have been less publicized. For example, the potential for selecting pathogen-free or stress-resistant plant clones, the creation of novel genetic combinations through somatic hybridization, etc. are techniques that have been frequently transposed to the nursery industry.

Preview work carried out by various scientists in relation to plant tissue culture of different citrus species is reviewed and discussed in the subsequent pages.

Some of the landmarks in the field of plant tissue culture since the event start are reviewed in Table 2.1.

The concept of totipotency was given by Haberlandt (1902) who was the first person to attempt the culture of plant cell *in vitro* on a nutrient media.

Using the above methods, innumerable plants species have been cloned which suggest that it is possible to multiply most of the plant species through micropropagation. Some horticultural plant species in which, micropropagation is demonstrated are listed in Table 2.2.

## **2.2 General review on *in vitro* propagation techniques in citrus**

A review of work done in various techniques of *in vitro* propagation of citrus is presented below. The important *in vitro* propagation techniques are dealt under 7 major heads:

**Table 2.1: Historical events in plant tissue culture**

Year	Events	Workers
1902	First attempt of plant tissue culture	Habelandt
1904	Embryo culture of selected crucifers attempted	Hanning
1922	Asymbiotic germination of orchid seeds <i>in vitro</i>	Knudson
1922	<i>In vitro</i> culture of root tips	Robbins
1925	Use of embryo culture technique in interspecific crosses of <i>Linum</i>	Laibach
1934	<i>In vitro</i> culture of the cambial tissue of a few trees and shrubs, although failed to sustain cell division	Gautheret
1934	Successful culture of tomato roots	White
1939	Successful establishment of continuously growing callus cultures	Gautheret, Nobecourt and White
1940	<i>In vitro</i> culture of cambial tissues of <i>Ulmus</i> to study adventitious shoot formation	Gautheret
1941	Use of coconut milk containing a cell division factor for the first time in <i>Datura</i>	Van Overbeek
1941	<i>In vitro</i> culture of crown gall tissues	Braun
1944	<i>In vitro</i> adventitious shoot formation in tobacco	Skoog
1946	Raising of whole plant of <i>Lupinus</i> and <i>Tropaeolum</i> by shoot tip culture	Ball
1950	Regeneration of organ from callus tissue of <i>Sequoia sempervirens</i>	Ball
1952	Use of meristem culture to obtain virus-free dahlias	Morel and Martin
1952	First application of micrografting	Morel and Martin
1953	Production of haploid callus of the gymnosperm <i>Ginkgo biloba</i> from pollen	Tulecke
1954	First plant from single cell	Muir <i>et al.</i>
1955	Discovery of kinetin, a cell division hormone	Muir <i>et al.</i>
1957	Discovery of regulation of organ formation by changing the ratio of auxin: cytokinin	Skoog and Miller
1958	Regeneration of somatic embryos <i>in vitro</i> from the nucles of <i>Citrus</i> ovules	Maheshwari and Rangaswamy

Contd.... Table – 2.1

Year	Events	Workers
1959	Regeneration of embryos from callus clumps and cell suspensions of <i>Daucus carota</i>	Reinsert and Steward
1959	Publication of first handbook on plant tissue culture	Gautheret
1960	First successful test tube fertilization in <i>Papaver rhoeas</i>	Kanta
1960	Use of the micro culture method of growing single cells in hanging drops in a conditioned medium	Jones <i>et al.</i>
1960	Enzymatic degradation of cell walls to obtain large number of protoplasts	Cocking
1960	Filtration of cell suspensions and isolation of single cells by planting	Bergmann
1962	Development of Murashige and Skoog nutrition medium	Murashige and Skoog
1964	Production of first haploid plant from pollen grains of <i>Datura</i>	Guha and Maheshwari
1970	Selection of biochemical mutants <i>in vitro</i> by the use of tissue culture derived variation	Carlos
1970	First achievement of protoplast fusion	Power <i>et al.</i>
1970	Discovery of first restriction endonuclease from <i>Haemphillus influenzae</i> Rd, later purified and named <i>Hind-II</i>	Smith
1971	Regeneration of first plants from protoplasts	Takabe <i>et al.</i>
1972	First report of interspecific hybridization through protoplast fusion in two species of <i>Nicotiana</i>	Carlson <i>et al.</i>
1973	Cytokinin found capable of breaking dormancy in excised capitulum explants of Gerbera	Pierik <i>et al.</i>
1974	Regeneration of haploid <i>Petunia hybrida</i> plants from protoplast	Binding
1976	Shoot initiation from cyropreserved shoot apices of carnation	Seibert
1978	Somatic hybridization of tomato and potato resulting in pomato	Melchers <i>et al.</i>
1981	Introduction of the term "Somaclonal variation"	Larkin and Scowcroft
1982	Development of root, shoot, embryo and callus <i>in vitro</i>	Thorpe
1985	Large scale propagation of plants through suspension culture	Ammirato
1987	Axillary bud proliferation and propagation in ornamental and woody plants	Pierik

Contd.... Table – 2.1

Year	Events	Workers
1992	Micropropagation in ornamental and woody plants	Chu
1993	Plant tissue culture takes place by two ways, organogenesis and embryogenesis.	Thorpe
1999	Studied mesocotyl culture of lemon grass	George and Subramanian
1999	Tissue culture of <i>Boerhaavia diffusa</i>	Phukan <i>et al</i>
1999	Studied multiple shoot formation in sindur plant through indirect development	Ramamurthy <i>et al</i>
1999	Studied leaf culture of <i>Cymbopogon citratus</i> and its oil content in different regenerants	Saraswati and Chandra
2000	Studied micropropagation of <i>Largesroemia reginae</i>	Sumana and Kaveriappa
2001	Studied micropropagation of <i>Salvadora persica</i>	Batra <i>et al</i>
2001	Studied callus culture of mulberry	Chandra and Satyanarayana
2001	Studied multiple shoot formation from rhizome of <i>Agave americana</i>	Hazra <i>et al</i>
2001	Studied micropropagation and organogenesis in <i>Withania somnifera</i>	Pawar <i>et al</i>
2002	Studied somatic embryogenesis and plantlet formation in <i>Pinus</i> species	Deb and Tandon
2002	<i>In vitro</i> seed germination of <i>Arnebia benthamii</i>	Ganai and Nawchoo
2002	Culture of different explants of nutmeg	Iyer <i>et al</i>
2002	Studied micropropagation in <i>Kaempferia galanga</i>	Jose <i>et al</i>
2002	Studied frequent plant formation in <i>Solanum</i> species and its alkaloid solasodine exploitation	Manjula and Nair
2002	<i>In vitro</i> culture of <i>Lilium mackliniae</i>	Mao <i>et al</i>
2002	Studied cloning of <i>Jatropha curcus</i>	Rajore <i>et al</i>
2002	Studied plant formation through axillary bud culture in <i>Cassia alata</i>	Ramamurthy and Savitramma
2002	Studied embryogenesis and plantlet formation in callus culture of <i>Gloriosa superba</i>	Sachdev <i>et al</i>
2002	Developed protocol for <i>Azadirachta indica</i>	Shekhawat <i>et al</i>

(Trivedi, 2006)

**Table:2.2 Micropropagation of some important fruit and plantation crops**

Family	Fruit crop
Actinidiaceae	<i>Actinidia deliciosa</i>
	<i>Actinidia chinensis</i>
Anacardiaceae	<i>Anacardium occidentale</i>
	<i>Mangifera indica</i>
	<i>Pistachio vera</i>
Annonaceae	<i>Annona cherimola</i>
	<i>Annona squamosa</i>
Arecaceae	<i>Cocos nucifera</i>
	<i>Phoenix dactylifera</i>
Bromeliaceae	<i>Ananas comosus</i>
Caricaceae	<i>Carica papaya</i>
Guttiferae	<i>Garcinia mangostana</i>
Eridaceae	<i>Vaccinium spp.</i>
Fagaceae	<i>Castanea spp.</i>
Juglandaceae	<i>Carya illinoensis</i>
	<i>Juglance regia</i>
Lauraceae	<i>Persea Americana</i>
Moraceae	<i>Ficus carica</i>
	<i>Artocarpus heterophyllus</i>
	<i>Morus alba</i>
Musaceae	<i>Musa accuminata</i> × <i>M. Balbisiana</i>
Myrtaceae	<i>Psidium guajava</i>
	<i>Syzygium cumini</i>

Contd...

Contd... Table - 2.2

Family	Fruit crop
Oleaceae	<i>Olea europaea</i>
Oxalidaceae	<i>Averrhoa carambola</i>
Pasifloraceae	<i>Pasiflora edulis</i>
Punicaceae	<i>Punica granatum</i>
Rosaceae	<i>Fragaria × ananassa</i>
	<i>Malus × domestica</i>
	<i>Prunus amygdalus</i>
	<i>Prunus armeniaca</i>
	<i>Prunus persica</i>
	<i>Pyrus communis</i>
	<i>Cydonia oblonga</i>
Rhamnaceae	<i>Rubus spp.</i>
	<i>Zizyphus mauratiana</i>
Rutaceae	<i>Indian jujube</i>
	<i>Citrus aurantifolia</i>
	<i>Citrus limon</i>
	<i>Citrus jambhiri</i>
	<i>Citrus limonia</i>
	<i>Citrus limettoides</i>
Sapindaceae	<i>Citrus reticulata</i>
	<i>Litchi chinensis</i>
Sterculiaceae	<i>Euphorbia longan</i>
	<i>Theobarma cocoa</i>
Vitaceae	<i>Vitis vinifera</i>

( Litz, 2005 )

- 1) Organogenesis
- 2) Embryogenesis
- 3) Anther culture
- 4) Embryo culture
- 5) Callus culture
- 6) Protoplast culture
- 7) Germplasm conservation

### 2.2.1 Organogenesis

Kumar *et al.* (2001) studied *in vitro* culture of the sweet orange cvs. Mosambi and Jaffa using epicotyls segments from nucellar seedlings. Murashige and Skoog (1962) (MS) medium supplemented with 1.0 mg/l 6-benzyle aminopurine (BAP) was found good for maximum shoots/explants. However in Jaffa, maximum shoot proliferation was observed with MS + 2 mg/l BAP. Root development was maximum in MS + 0.5 mg/l  $\alpha$ -naphthalene acetic acid (NAA) + 0.5-1.0 mg/l indole 3-butyric acid (IBA). The complete *in vitro* plantlets were hardened and transferred to pots containing sterilized sand and soil (2:1 v/v) with 67 per cent survival rate.

Almeida *et al.* (2002) reported the best result for bud induction in epicotyl segments with Murashige and Tucker (MT) (1969) medium supplemented with 1.0 mg/l BAP in Natal, Valencia and Halmin cultivars of sweet orange while, in case of rangpur lime 3.5 mg/l BAP gave better response.

Mukhtar *et al.* (2005) cultured Kinnow mandarin (*Citrus reticulata*) *in vitro* shoot tip and nodal segment explants on MS

medium supplemented with different concentrations of kinetin (Kn) and BAP to produce multiple shoots. The results revealed that the shoot tip explants cultured on MS medium supplemented with 1 mg/l of BAP and 1.5 mg/l of Kn showed maximum shooting percentage. Average number of shoots per explant was noticed to be the highest at 1.5 mg/l BAP and 0.5 mg/l Kn. However, nodal segments cultured on MS medium containing 2 mg/l NAA produced more roots.

Silva *et al.* (2006) stated that the organogenesis was induced in internode explants of sweet orange cv. Bahia in DBA<sub>3</sub> (Basal MT medium + 20 mg/l coconut water) medium supplemented with 1.0 to 3.0 mg/l BAP + 0.5 mg/l NAA.

Laskar *et al.* (2009) studied *in vitro* propagation of *Citrus indica* Tanaka by shoot organogenesis from leaf derived callus. Regenerative calli were induced on MS medium supplemented with 0.01 mg/l thidiazuron (TDZ) and 0.1 mg/l NAA. Shoots were regenerated on woody plant medium (WPM) supplemented with 0.5 mg/l BAP, 0.25 mg/l TDZ and 0.25 mg/l NAA. Regenerated shoots were rooted on MS medium supplemented with 1.0 mg/l NAA. Sixty per cent of the rooted plantlets were acclimatized successfully under *ex situ* condition.

### 2.2.2 Embryogenesis

Nagasava *et al.* (1999) found that 20 mg/l adenine, 500 mg/l malt extract (ME) and 2g gellen gum in basal MT medium induced embryogenesis in immature ovules of 'Ohta' ponkan (*Citrus reticulata*).

Vijayakumari and Singh (2001) reported that maximum cotyledonary shaped embryos (58.20%) were produced in *Citrus reticulata* Blanco cv. Nagpur Mandarin on MS medium supplemented with 500 mg/l ME + 0.1 mg/l NAA. While, torpedo shaped (43.91%) and globular shaped (50.68%) embryos were obtained on MS medium containing 500 mg/l ME + 0.5 mg/l NAA. Further, it was reported that maximum percentage of embryoids (68.5%) were observed on MS medium containing 0.1 mg/l NAA + 0.1 mg/l Kn, where as, 0.1 mg/l Kn was superior for plantlets production (39.66%).

Miah *et al.* (2002) reported that higher percentage of somatic embryogenic callus (73.33%) in citrus macroptera was obtained from nucellus tissue. Callus initiation was recorded earlier in 7-12 days after inoculation on MS medium containing 500 mg/l malt extract.

### 2.2.3 Anther culture

Germana *et al.* (1994) studied androgenesis in two clementine cultivars (Nules and S.R.A. 63) and two mandarin cultivars (Avana and Tardivo di Ciaculli) with the aim too obtaining haploids. The anthers, collected at the uninucleate stage were cultured first on 11 media, differing either in the basic medium, the growth regulators, the carbon source and concentration, or the presence of activated charcoal. Calli, shoots, embryos and then, plantlets were obtained. Significant differences were observed among the different cultural conditions and genotypes. Cytological observations on calli and plantlets from

'Nules' anthers revealed the haploid chromosome number, while mandarin calli and plantlets, and S.R.A. 63 calli had the diploid chromosome number. Electrophoretic analysis on calli and leaf tissues showed that 'Nules' had a homozygous genotype and confirmed that these tissues had developed

#### 2.2.4 Embryo culture

Parthasarathy and Nagaraju (2000) studied embryogenesis by using excised ovules (8-10 weeks old) of citrus species, viz. Khasi mandarin (*Citrus reticulata* Blanco), Cleopatra mandarin (*Citrus reshni* Tanaka) and Calamondin (*Citrus madurensis* Lour) on MS medium supplemented with yeast extract and 500 mg/l ME each. Observations recorded 12 weeks after culture initiation showed differential responses of the organic supplements used. Malt extract was superior to yeast extract in inducing more number of cotyledonary, torpedo and globular embryoids formation in Khasi mandarin and cluster of undifferentiated embryogenic callus in Cleopatra mandarin.

Altaf *et al.* (2008) studied ovule culture in rough lemon in which immature ovules responded to shoot, root, callus and embryogenesis. The callus was formed on MS medium supplemented with 0.2 mg/l Kn + 2 mg/l 2,4-Dichlorophenoxy acetic acid (2,4-D). Embryos were obtained on MS + 0.5 to 500 mg/l ME + 25 mg/l adenine sulphate (AdSO<sub>4</sub>).

#### 2.2.5 Callus culture

Desai *et al.* (1996) studied *in vitro* propagation of acid lime (*Citrus aurantifolia* Swingle) var. 'Kagzi lime'. Callus was

initiated from nodal regions excised from young tender growth of mature tree. Callus initiation occurred within 4 days on modified MS medium containing 0.5 mg/l Kn, 1 mg/l NAA and 0.25 mg/l 2,4-D. After 30 days, callus pieces were transferred to regeneration on modified MS medium, in which shoots initiated within 30 days of incubation. After 60 days, about 10-12 shoots per callus piece were obtained. Rooting was obtained on half strength modified MS medium supplemented with 0.1 mg/l NAA and the rooted plantlets were successfully established to natural environment.

Ali and Mirza (2006) studied *in vitro* propagation in rough lemon (*Citrus jambhiri*) through callus induction. Callusing was observed on MS medium supplemented with 1.5 mg/l 2,4-D from leaves and stem segment explants, with stem explants showing the highest response (92%). Maximum shoot regeneration response (70%) from callus was observed on MS medium supplemented with 3 mg/l BAP. Direct shoot regeneration was highest in stem segment explants on MS medium with 3 mg/l BAP. MS medium supplemented with 0.5 mg/l NAA provided 70% rooting response.

Sharma *et al.* (2009) attempted to standardize a protocol for micropropagation of citrus rootstocks viz. rough lemon, cleopatra mandarin, pactinifera and troyer citrange. They found that shoot tip explant was better for callus induction of these rootstocks than nodal segment. Maximum callus formation was obtained in shoot tip explants of cleopatra mandarin (40%) followed by pactinifera (40%) and minimum in rough lemon (23.3%) and troyer citrange (23.3%) on MS medium supplemented with 0.05 mg/l Kn + 2.0 mg/l NAA + 2.0 mg/l 2,4-D. Further,

maximum shoots were regenerated through callus in pachtinifera, cleopatra mandarin and rough lemon on MS medium supplemented with 1.0 mg/l BAP. Maximum rooting was noticed in rough lemon, cleopatra mandarin and pachtinifera on half strength MS medium supplemented with 10 mg/l IBA. Soil, sand and FYM (1:1:1::v/v) was found to be the best potting mixture supplemented with maximum survival rate of plantlets.

Altaf *et al.* (2009) reported tissue culture of Kinnow mandarin (*Citrus reticulata*), rough lemon (*Citrus jambhiri*), Gada Dehi (*Citrus aurantium*), Washington Naval (*Citrus sinensis*), Grape fruit (*Citrus paradisi*), Sweet lime (*Citrus aurantifolia*) and Feutrell's early mandarin (*Citrus reticulata*), Where the calli were successfully produced from different somatic explants. Nucellus culture of Gada Dehi responded to embryogenesis and callusing on MS medium supplemented with BAP, Kn, 2,4-D and NAA (0-0.4 mg/l). Best callusing was noticed in 0.1-0.3 mg/l Kn + 0.5-2.0 mg/l 2,4-D and nucellus embryogenesis in 0.5 g/l ME + 25 mg/l adenine sulphate in MS medium in both rough lemon and Gada Dehi. MS medium supplemented with 0.25 mg/l BAP + 1.5 mg/l 2,4-D was good for nucellar embryogenesis in Kinnow mandarin.

#### 2.2.6 *In vitro* propagation

Desai (1994) studied *in vitro* propagation in cv. Kagzi lime and reported that the axillary buds gave cent per cent regeneration of shoots on MS medium supplemented with 0.25 mg/l BAP and 200 mg/l malt extract. Further, maximum multiple shoots were obtained on MS medium supplemented with 0.25 mg/l BAP +

200 mg/l malt extract at the end of third subculture. Half strength MS medium supplemented with 0.1 mg/l NAA took less days (17.5 days) for initiation of roots. Plantlets were successfully transferred to pots containing mixture of sand, soil and vermiculite (1:1:1 v/v).

Paudyal and Haq (2000) studied the *in vitro* propagation of pummelo (*Citrus grandis* L. Osbeck) using shoot tip explants from seedlings. MS medium supplemented with a concentration of 1.8  $\mu$ M BAP was used to determine the rate of shoot proliferation. Addition of 5.8  $\mu$ M gibberellic acid (GA<sub>3</sub>) in shoot-proliferation medium during the second subculture improved shoot elongation significantly. Shoot multiplication increased 3.5 fold in each successive subculture. NAA was superior to IBA for *in vitro* root induction. Over 75% of the shoots developed roots, when transferred to half strength MS medium supplemented with 1.3, 2.7, or 5.4  $\mu$ M NAA.

Khayri and Bahrany (2001) studied micropropagation technique for lime, *Citrus aurantifolia* Swingle, using nodal explants of mature trees. Best results for multiple shoots formation (8 shoots/explants) were obtained on MS medium supplemented with 1 mg/l BAP and 0.5 mg/l Kn. Shoot elongation appeared at 0.25 mg/l BAP + 1 mg/l Kn. Further, shoots inoculated to a rooting medium induced the highest percentage of rooting (56%) on MS medium supplemented with 1 mg/l IAA. Plantlets survived successfully in soil and exhibited normal growth in a greenhouse. Over ninety per cent of plantlets (76 out of 83 plantlets) survived

during acclimatization and grew normally in soil with development of new leaves.

Usman *et al.* (2005) observed that in citrus cv. Kinnow, Sweet lime and Succari multiple shoots and roots induction were found directly proportionate to increase in the levels of BAP and NAA. The highest shoot induction was found on the modified MS medium supplemented with 1.0 mg/l BAP and 0.1 mg/l NAA. Maximum roots per shoot were observed on modified MS medium + 10 mg/l NAA.

Agisimanto *et al.* (2005) studied the multiplication of shootlets with BAP concentrations on MS medium supplemented with 500 mg/l malt extract, 146 mM sucrose and 10 g/l agar-agar in *Citrus sinensis* cv. Pacitan. Application of 2.5  $\mu$ M BAP in the medium produced higher number of shootlets from shoot tip explants rather than uninode explants. About 3-6 fold of multiplication was observed from medium containing 2.5  $\mu$ M BAP. Levels of BAP ranging from 2.5 to 5  $\mu$ M induced axillary and adventitious shootlets of shoot tip and uninode as well.

Miah *et al.* (2008) conducted trial on *in vitro* propagation in *Citrus macroptera* Mont. The nodal and shoot tip explants taken from *in vitro* grown seedlings were cultured on MS medium supplemented with different concentrations of BAP and Kn either singly or in combinations. Maximum number of shoots was obtained from nodal explants on MS medium supplemented with 1.0 mg/l BAP. The best treatment for multiplication and shoot induction was found at 1.0 mg/l BAP.



Kour *et al.* (2008) conducted trial for micropropagation of *Citrus jambhiri* rootstock. The apical nodal segments of 2-3 cm from newly grown flushes from 8 years old mature trees served as explants. The MS medium supplemented with 1.5 mg/l BAP and 500 mg/l ME was found to be optimum for shoot proliferation as compared other treatments. The days taken for root initiation were minimum (16.51 days), while the maximum number of roots/shoot (2.47) was in MS medium supplemented with NAA and IBA (1.0 mg/l each). The *in vitro* regenerated shoots rooted on this medium also showed maximum survival in potting mixture of soil: sand: FYM (1:1:1 v/v).

Tornero *et al.* (2009) observed that in *Citrus limon*, the number of shoots was dependent on the BAP and GA<sub>3</sub> concentrations. The best results were obtained with 2 mg/l BAP and 2 mg/l GA<sub>3</sub>. Explant length was shorter with the higher BAP concentration in all genotypes while, shoot length was higher with 2 mg/l GA<sub>3</sub>. The best results for productivity (number of shoots × the average shoot length) were obtained with 2 mg/l BAP and 2 mg/l GA<sub>3</sub>. The highest rooting percentage was obtained on MS medium containing 3 mg/l IBA or IBA in combination with 1 mg/l IAA. The highest number of root and root length were noticed, when 3 mg/l IBA was used in rooting medium. Plantlets that survived acclimatization exhibited normal growth in soil under greenhouse conditions. Plantlets were transferred to 250 cm<sup>3</sup> pots containing a mix of peat and perlite (2:1v/v) for their acclimatization.

### 2.2.7 Germplasm conservation

Normah and Siti Dewi Serimala (1997) successfully cryopreserved *Citrus aurantifolia* seeds after desiccating them to a moisture content of 12.93% (50% viability) while seed of *Citrus halimii* exhibited only 25% viability after cryopreservation at moisture content of 9.5%. Seeds of *Citrus hystrix*, however, were sensitive to desiccation as they failed to germinate when the moisture content was reduced to 27% and thus, did not survive cryopreservation. The embryonic axis of the three citrus species gave higher percentage of survival after cryopreservation. Survival was 100% in *Citrus aurantifolia* and *Citrus halimii* embryonic axis with moisture content of 9-11% and 16.6%, respectively. With *Citrus hystrix*, the highest survival obtained was 60% at a moisture content of 11.40%. Cryopreservation methods (encapsulation, dehydration, slow freezing and vitrification) were further employed for the embryonic axis of *Citrus hystrix*; however, there was no improvement in the survival percentage.

Van Le *et al.* (1999) reported a new efficient regeneration method leading to a high frequency of plant regeneration by using transverse thin cell layer (tTCL) explants excised from the stem internodes of 1-year-old trifoliolate orange (*Poncirus trifoliata*). The optimal shoot regeneration was obtained with MS medium supplemented with 10  $\mu$ M BAP and 1  $\mu$ M N-phenyl-N-1, 2,3-thidiazol-5ylurea (thidiazol, TDZ), leading to 90% of responsive tTCL forming on average 37 buds per tTCL. The cent per cent of shoot elongation was obtained in MS medium

supplemented 1  $\mu M$  GA<sub>3</sub>, 60% of shoots formed roots on MS medium containing 5  $\mu M$  NAA.

Parthasarathy and Nagaraju (1996b) found that 0.05 mg/l NAA in MS medium induced rooting of *Citrus reticulata*, *Citrus madurensis*, *Citrus sinensis* and *Citrus limon*, while for Mosambi, the best treatment was MS medium + 0.2 mg/l NAA.

Perez *et al.*, (1997) found that best regeneration system and adventitious shoots were obtained from 96 per cent and 88 per cent of the explants in Mexican lime and Mandarin in normal MS medium, respectively. 70 per cent rooting was achieved and 85 per cent rooted plants adapted to soil conditions.

Filho *et al.* (2001) observed that *Citrus sinensis* thin section explants (1-2 mm) from seedlings of produced adventitious shoot buds on MS medium supplemented with 0.5-5  $\mu M$  BAP. Optimal shoot induction was achieved on 2-5  $\mu M$  BAP. Higher number of elongated shoots were reported on MS medium supplemented with 0.5  $\mu M$  BAP. The elongated shoots were rooted on half strength MS medium without growth regulators and transferred to soil for acclimatization.

Gill and Gosal (2004) studied the citrus rootstock *viz.*, pectinifera (*Citrus depressa* Hayata). The epicotyl segments (0.5-1 cm) excised from 3 weeks-old *in vitro* raised seedlings, served as explant. MS medium supplemented with 1 mg/l BAP was found to be optimum for shoot bud induction. Supplementation of bud induction medium with 2 mg/l GA<sub>3</sub> caused excessive shoot regeneration. Average number of shoots per explant on the liquid

medium after 90 days of culturing were higher ( $36.4 \pm 0.85$ ) as compared to the solid medium ( $20.4 \pm 0.66$ ). The average number of roots per shoot was maximum on the MS medium supplemented with 2 mg/l NAA.

Sen and Dhawan (2009) reported that in Troyer citrange (*Poncirus trifoliata* Rat.  $\times$  *Citrus sinensis*), multiple shoots could be obtained from single nodes of field grown trees cultured on MS medium containing BAP 1.11  $\mu$ M, Kn 1.1625  $\mu$ M and 3% sucrose. A shoots multiplication fold of 3.86 every four weeks was achieved. Proliferated shoots were rooted on half strength MS medium with NAA 0.5  $\mu$ M.

Murkute *et al.* (2009) studied *in vitro* regeneration for trifoliolate orange (*Poncirus trifoliata*) and reported that 40 mg/l adenine sulphate improved culture response including percentage forming shoot buds, days required for shoot initiation, shoot length and number of resultant shoots per explants. Maximum root initiation in half strength MS medium supplemented with 0.5 mg/l IBA + 0.5 mg/l NAA.

### 2.3 Review on Rooting, Acclimatization and planting out of plantlets

Acclimatization is necessary in the case of *in vitro* produced plantlets because such plantlets did not adopt for *in vivo* conditions (Brainerd and Fuchigami, 1981). The success in acclimatization of *in vitro* produced plantlets largely dependent upon not only the post-transfer growth conditions but also the pre-transfer culture conditions (Ziv, 1986). *In vitro* grown plantlets are

very poorly adopted to resist the low humidity, high light levels and more variable temperatures prevailing outside (Wainwright, 1988). Thus, light, temperature and relative humidity are the major factors to be controlled during acclimatization to natural environment.

Desai (1994) obtained 95 per cent survival of Kagzi lime plantlets in culture room at  $26 \pm 2$  °C covered with a glass beaker. The potting mixture containing soil, sand and vermiculite/FYM (1:1:1, v/v) gave best survival under lath house condition.

Perez *et al.*, (1997) found that (85%) *in vitro* regenerated plantlets of Mexican lime and Mandarin were adapted to soil conditions successfully.

Hazarika *et al.* (1997) described a protocol for *in vitro* acclimatization of citrus plantlets for up to 97 per cent *ex vivo* survival of plants following their transfer to polythene bags in the green house. They reported that addition of paclobutrazol in the growth medium causes a reduction in stem and root elongation.

Filho *et al.* (2001) reported that the *Citrus sinensis in vitro* rooted shoots from cvs. Pera, Valencia and Folha Murcha were successfully acclimated (100%) under mist condition.

Gill and Gosal (2004) studied hardening of citrus rootstock *viz.*, Pectinifera (*Citrus depressa* Hayata). The *in vitro* regenerated plantlets showed the highest plantlet survival upon transfer to soil.

Sen and Dhawan (2009) reported that in Troyer citrange (*Poncirus trifoliata* Rat. × *Citrus sinensis*), plantlets were transferred to a mixture of soil and agropeat. Cent per cent survival was observed during hardening of the rooted plantlets.

Murkute *et al.* (2009) studied hardening of *in vitro* regenerated plants of trifoliolate orange (*Poncirus trifoliata*). The hardening medium consisted of cocopeat and soilrite (2:1 v/v) in which the survival rate of hardened plant was found to be 82.2% after one month.



# MATERIALS & METHODS



### **III. MATERIALS AND METHODS**

The present investigation entitled “Micropropagation in rough lemon (*Citrus jambhiri* Lush.)” was carried out at the Department of Biotechnology, ASPEE College of Horticulture and Forestry, Navsari Agricultural University, Navsari, Gujarat, India during the year 2008-2010. The chapter contains the detail regarding the experimental materials used and methodology adopted during the course of investigation.

#### **3.1 Source of explants materials**

The experiment was conducted with two different sources of explants material. Out of which, shoot tip and nodal segment (axillary bud) explants were collected from 3-4 weeks old juvenile stage of rough lemon plants of one year age grown at Horticulture Nursery, ASPEE College of Horticulture and Forestry, Navsari Agricultural University, Navsari, Which were used as mother (donor) plants (Plate – I).

#### **3.2 Culture media**

Three different media viz. MS medium (Murashige and Skoog, 1962), B5 (Gamborg, 1968) and WPM (Lloyd and Mc.Cown, 1981) (Woody Plant Medium) were used as basal medium. White medium (White, 1963) and MS medium were used for rooting of plants. The composition of MS, B5, WPM and White Medium is presented in Table 3.1, 3.2, 3.3 and 3.4 respectively, containing only the basal salts (Macro and Micro nutrients), vitamins, sucrose and agar as reported in the original publication.



**A. Juvenile stage plant**



**B. Shoot tip**



**C. Nodal segment**

**Plate I : Selection of explant**

Table 3.1: Composition of Murashige and Skoog (MS) (1962) medium

	Constituents	Cone. in stock solution (g/100ml)	Final concentration in medium (mg/l)
A)	<b>Macronutrient</b>		
	NH <sub>4</sub> NO <sub>3</sub>	8.25	1650.00
	KNO <sub>3</sub>	9.50	1900.00
	MgSO <sub>4</sub> .7H <sub>2</sub> O	1.85	370.00
	KH <sub>2</sub> PO <sub>4</sub>	0.85	170.00
B)	CaCl <sub>2</sub> .2H <sub>2</sub> O	4.40	440.00
C)	<b>Micronutrient</b>		
	H <sub>3</sub> BO <sub>3</sub>	0.062	6.20
	KI	0.0083	0.83
	Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.0025	0.250
	CoCl <sub>2</sub> .6H <sub>2</sub> O	0.00025	0.025
	MnSO <sub>4</sub> .4H <sub>2</sub> O	0.223	22.30
	ZnSO <sub>4</sub> .7H <sub>2</sub> O	0.086	8.60
	CuSO <sub>4</sub> .5H <sub>2</sub> O	0.00025	0.025
D)	<b>Iron sources *</b>		
	FeSO <sub>4</sub> .7H <sub>2</sub> O	0.278	27.80
	Na <sub>2</sub> . EDTA	0.373	37.30
E)	<b>Vitamins and Organics</b>		
	Thiamine HCl	0.001	0.1
	Pyridoxine HCl	0.005	0.5
	Nicotinic acid	0.005	0.5
	Glycine	0.02	2.0
	<i>myo</i> -Inositol	-	100.0
	<b>Carbon sources</b>		
	Sucrose	-	30000.0

**Table 3.2: Composition of Gamborg's (B5) (1968) medium**

	Constituents	Conc. in stock solution (g/100ml)	Final concentration in medium (mg/l)
A)	<b>Macronutrient</b>		
	KNO <sub>3</sub>	12.50	2500.00
	MgSO <sub>4</sub> .7H <sub>2</sub> O	1.25	250.0
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.67	134.0
	NaH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O	0.75	150.0
B)	CaCl <sub>2</sub> .2H <sub>2</sub> O	1.50	150.0
C)	<b>Micronutrient</b>		
	H <sub>3</sub> BO <sub>3</sub>	0.03	3.00
	KI	0.0075	0.75
	MnSO <sub>4</sub> .H <sub>2</sub> O	0.10	10.00
	Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.0025	0.25
	CoCl <sub>2</sub> .6H <sub>2</sub> O	0.00025	0.025
	ZnSO <sub>4</sub> .7H <sub>2</sub> O	0.02	2.00
	CuSO <sub>4</sub> .5H <sub>2</sub> O	0.00025	0.025
D)	<b>Iron sources *</b>		
	FeSO <sub>4</sub> .7H <sub>2</sub> O	0.278	27.80
	Na <sub>2</sub> . EDTA	0.373	37.30
E)	<b>Vitamins and Organics</b>		
	Thiamine HCl	0.10	10.00
	Pyridoxine HCl	0.01	1.0
	Nicotinic acid	0.01	1.0
	<i>myo</i> -Inositol	-	100.0
	<b>Carbon sources</b>		
	Sucrose	-	20000.0

Table 3.3: Composition of Lloyd and Mc.Cown (WPM) (1981) medium

	Constituents	Conc. in stock solution (g/100ml)	Final concentration in medium (mg/l)
A)	<b>Macronutrient</b>		
	NH <sub>4</sub> NO <sub>3</sub>	2.00	400.00
	K <sub>2</sub> SO <sub>4</sub>	4.95	990.00
	MgSO <sub>4</sub> .7H <sub>2</sub> O	1.85	370.00
	KH <sub>2</sub> PO <sub>4</sub>	0.85	170.00
	Ca(NO <sub>3</sub> ) <sub>2</sub> .4H <sub>2</sub> O	2.78	556.00
B)	CaCl <sub>2</sub> .2H <sub>2</sub> O	0.96	96.00
C)	<b>Micronutrient</b>		
	H <sub>3</sub> BO <sub>3</sub>	0.062	6.20
	Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.0025	0.25
	MnSO <sub>4</sub> .H <sub>2</sub> O	0.223	22.30
	ZnSO <sub>4</sub> .7H <sub>2</sub> O	0.086	8.60
	CuSO <sub>4</sub> .5H <sub>2</sub> O	0.0025	0.25
D)	<b>Iron sources *</b>		
	FeSO <sub>4</sub> .7H <sub>2</sub> O	0.278	27.80
	Na <sub>2</sub> .EDTA	0.373	37.30
E)	<b>Vitamins and Organics</b>		
	Thiamine HCl	0.016	1.60
	Nicotinic acid	0.005	0.50
	<i>myo</i> -Inositol	-	100.0
	<b>Carbon sources</b>		
	Sucrose	-	20000.0

Table 3.4: Composition of White (1963) medium

	Constituents	Conc. in stock solution (g/100ml)	Final concentration in medium (mg/l)
A)	<b>Macronutrient</b>		
	KNO <sub>3</sub>	0.40	80.0
	MgSO <sub>4</sub> .7H <sub>2</sub> O	3.75	750.0
	Ca(NO <sub>3</sub> ) <sub>2</sub> .4H <sub>2</sub> O	1.50	300.0
	Na <sub>2</sub> SO <sub>4</sub>	1.00	200.0
	NaH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O	0.095	19.0
	KCl	0.325	65.0
B)	CaCl <sub>2</sub> .2H <sub>2</sub> O	-	-
C)	<b>Micronutrient</b>		
	H <sub>3</sub> BO <sub>3</sub>	0.015	1.50
	MnSO <sub>4</sub> .4H <sub>2</sub> O	0.05	5.0
	ZnSO <sub>4</sub> .7H <sub>2</sub> O	0.03	3.0
	CuSO <sub>4</sub> .5H <sub>2</sub> O	0.0001	0.01
	KI	0.0075	0.75
	MoO <sub>3</sub>	0.00001	0.001
D)	<b>Iron sources</b>		
	Fe(SO <sub>4</sub> ) <sub>3</sub>	0.025	2.50
E)	<b>Vitamins and Organics</b>		
	Thiamine HCl	0.0001	0.01
	Pyridoxine HCl	0.0001	0.01
	Nicotinic acid	0.0005	0.05
	Glycine	0.03	3.0
	<i>myo</i> -Inositol	-	100.0
	<b>Carbon sources</b>		
	Sucrose	-	20000.0

Media were supplemented with cytokinins with and without auxins at various concentrations; the details of which are given at the appropriate places.

The  $*\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  was dissolved in approximately 40 ml of double distilled water and heated. The  $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$  was dissolved in approximately 40 ml of double distilled water, separately and mixed while heating (under continuous stirring) with  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  solution. After cooling, the volume was adjusted to 100 ml heating and stirring resulted in a more stable FeNaEDTA complex.

### **3.2.1 Chemicals**

Analytical grade chemicals, obtained from Hi Media Laboratory – Mumbai (Maharashtra) and Bengaluru (Karnataka), British Drug House (BDH) – ENGLAND and Sisco Research Laboratory (SRL) Mumbai (Maharashtra) were used in the preparation of media.

### **3.2.2 Preparation of the medium**

The media were prepared either from commercially prepared media package (Hi Media Laboratory) or from the stock solutions. Standard procedures were followed as described below.

#### **3.2.2.1 Culture vessels**

Bottles (250 ml) with screw caps and borosil glass test tubes (25 mm x 150 mm) of autoclavable glass were used as culture vessels.

The culture vessels and glass-wares used in the preparation of media and the concomitant purposes were cleaned in chromic acid (Potassium dichromate in sulphuric acid). The acid was removed by prolonged and thorough washing in tap water. The glass wares and culture vessels were then, washed with detergent (Teepol, BDH) followed by thorough washing with excess tap water. The glass wares and culture vessels were finally rinsed with double distilled water and dried in an oven at 70°C temperature. They were then, used for experiments.

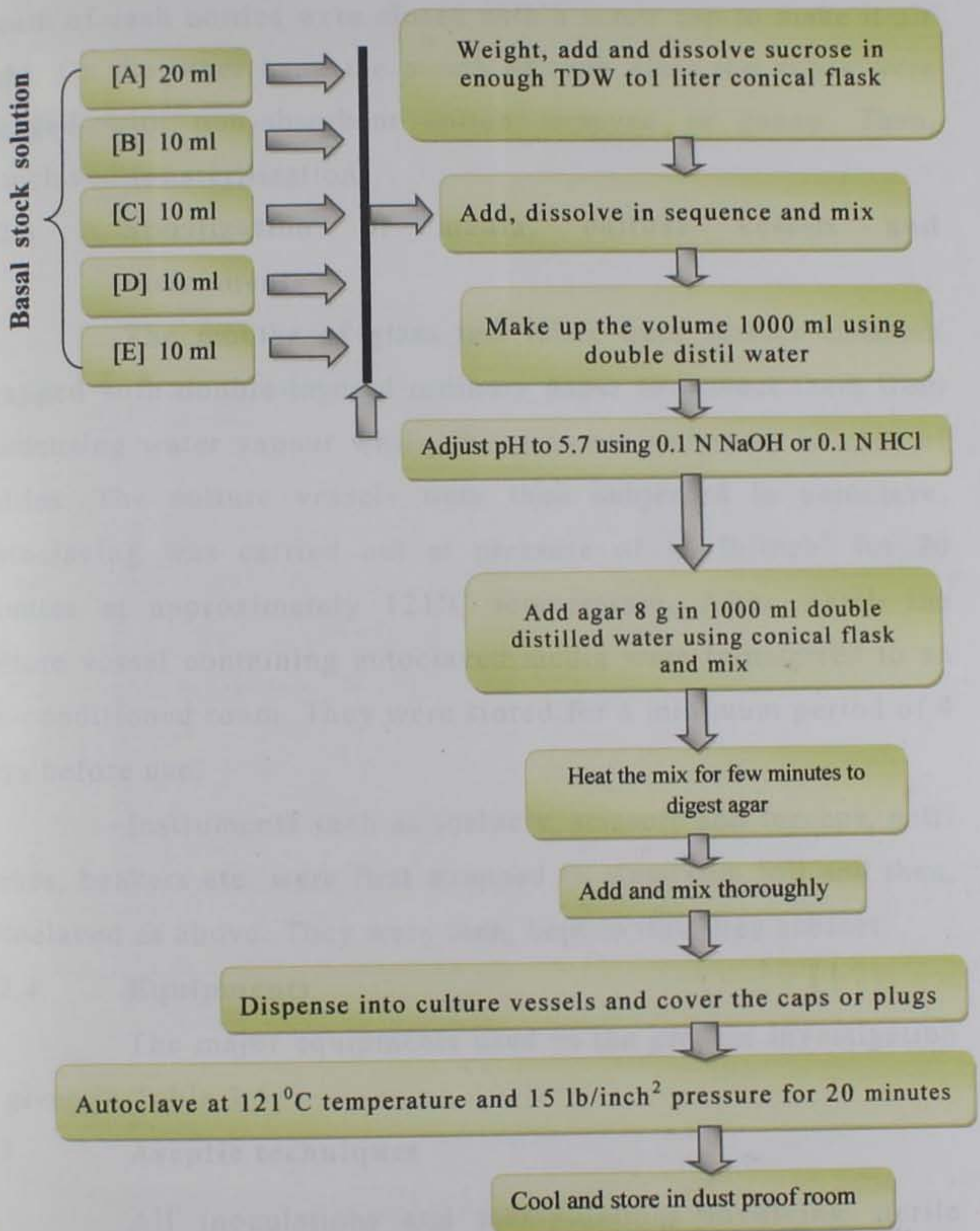
#### **3.2.2.2 Preparation of the stock solutions**

The stock solutions were prepared by dissolving the required quantity of each chemicals in double distilled water and stored under refrigerated condition. The stock solutions were prepared fresh after every 4 to 6 weeks and that of the plant growth regulators were prepared fresh after every week.

#### **3.2.2.3 Preparation of medium (MS, B5, WPM and White Medium)**

The composition for preparing culture medium is given in Table 3.1 to 3.4. The basal medium was prepared from the concentrated stock solutions (Fig.1). Cytokinin and/or auxin, gibberellin to be incorporated into the basal medium were added before the final adjustment of the volume. Double distilled water was used for the preparation of media. The pH of the medium was adjusted at 5.7 with a systronic make pH meter using either 0.1 N NaOH or 0.1 N HCl and then, medium solidified with bacteriological agar (8 gm/l) boiled, dispensed into suitable culture

**Fig. 1 : Schematic procedure for preparation of different media**



**Operation to make one litre medium**

vessels and autoclaved. Known volumes of media were distributed in culture vessels (30 ml in bottles and 20 ml in test tubes). The mouth of each bottles were closed with a screw cap to make it air tight. On the other hand the mouth of each glass test tubes were plugged with non-absorbent cotton wrapped in gauze. Then, autoclaved for sterilization.

### **3.2.3 Sterilization of media, culture vessels and instruments**

The mouths of glass test tubes were further covered/ wrapped with double-layered ordinary paper to protect them from condensing water vapour while, this was not necessary in case of bottles. The culture vessels were then subjected to autoclave. Autoclaving was carried out at pressure of 15 lb/inch<sup>2</sup> for 20 minutes at approximately 121°C temperature. After which the culture vessel containing autoclaved media were transferred to an air-conditioned room. They were stored for a minimum period of 4 days before use.

Instruments such as scalpels, scissors and forceps, petri dishes, beakers etc. were first wrapped in aluminum foil and then, autoclaved as above. They were then, kept in dust free cabinet.

### **3.2.4 Equipments**

The major equipments used in the present investigation is given in Table 3.5.

### **3.3 Aseptic techniques**

All inoculations and manipulations involving sterile culture media were carried out under aseptic condition in a laminar air flow cabinet. The interior of the laminar flow was swabbed

Table 3.5: Major equipments used in present investigation

Sr. No.	Equipments	Company
1.	<i>In vitro</i> studies	
	Magnetic stirrer	MAC, India.
	Temperature controlled oven	MAC, India.
	Laminar air flow cabinet	MAC, India.
	Analytical balance	Sartorius, Germany
	pH meter	Systronic
	Water purification system	Millipore, USA
	Autoclave	MAC, India
2.	<b>Histological studies</b>	
	Light microscope	Leica, DM 2500
	Camera	Canon power shot 570
	Spectrophotometer	Nanodrop, USA

with 70 per cent ethanol. The instruments and other materials and the culture vessels containing medium were sprayed with 70 per cent ethanol and placed inside the laminar air flow cabinet. The cabinet door was closed and UV light was switched on for 30 minutes prior to working. The plant material was not kept in the cabinet during the UV irradiation. The hands and arms were washed with soap and water and then, swabbed with 70% ethanol before carrying out plant manipulations inside the cabinet. The instruments such as forceps, scalpels, blade handle etc. were sterilized by dipping them in 70 per cent absolute alcohol followed by flaming and cooling. This was operated carried out at the start of inoculation and also several times during the operation. During inoculation, first the cap or cotton plug of the culture vessel was removed and then immediately the neck of the vessel was flamed over a spirit lamp kept in the cabinet. The sterilized and trimmed explants were quickly transferred to the culture vessels containing suitable culture medium using sterilized forceps. The neck of the culture vessel was once again flamed and quickly closed up by cotton plug.

Care was also taken to avoid any obstruction of the laminar air flow by placing nothing between the working area and the source of air flow. Further, crossing over of hands and arms was avoided. If any plant material fell on to the floor of the cabinet, it was discarded assuming that it was contaminated. After completion of work, the cabinet was sprayed with 70 per cent ethanol then, switched off.

### 3.4 Culture conditions

All the cultures were incubated in a culture room at a temperature of  $26 \pm 2^{\circ}\text{C}$  with relative humidity at  $55 \pm 5$  per cent. Cultures were provided with light using fluorescent tubes with 16:8 hours light/dark cycle, kept 50 cm above bench surface (3000 lux).

### 3.5 Micropropagation procedure

In general, micropropagation procedure adopted in the present investigation is illustrated in Fig. 2.

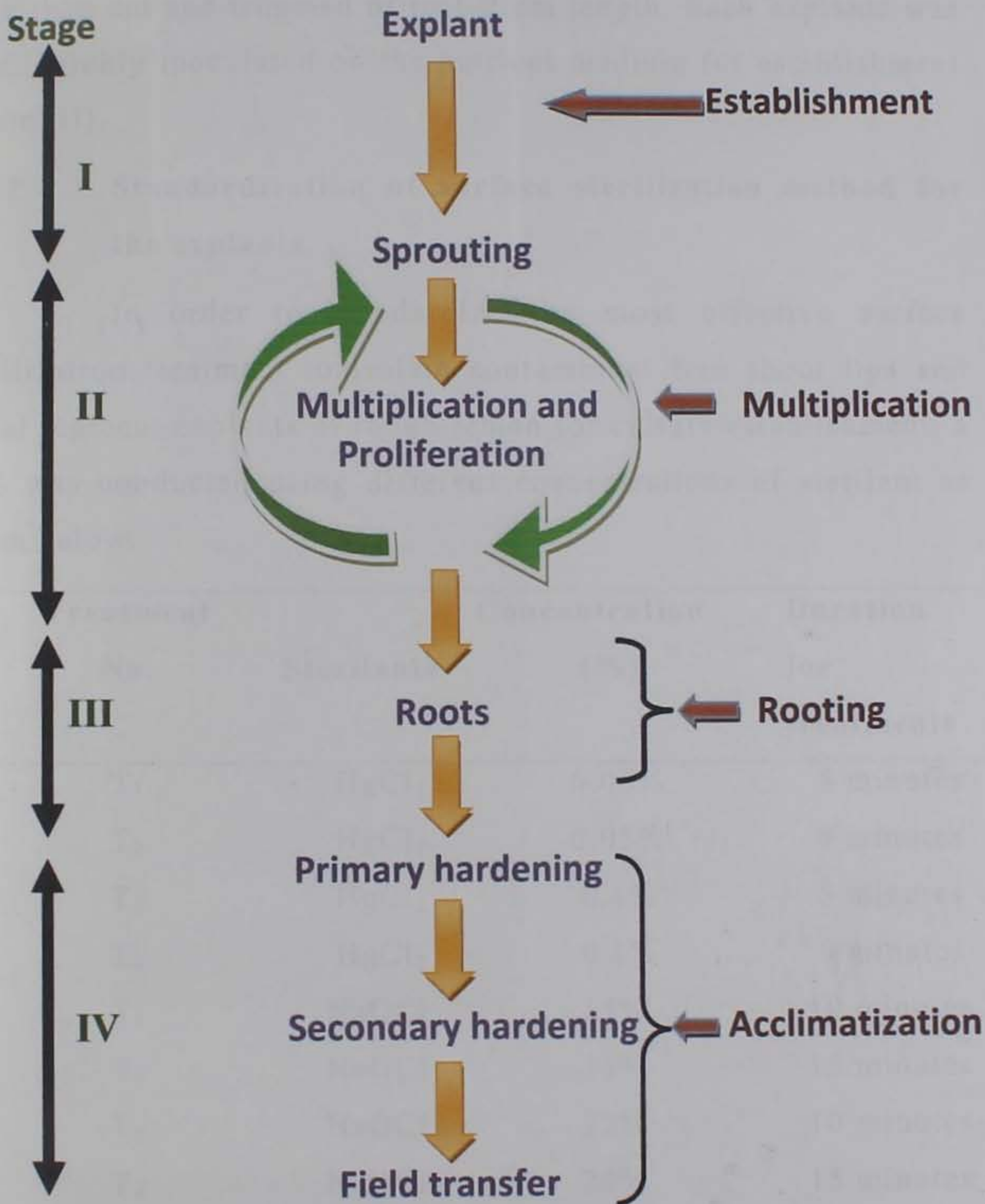
#### 3.5.1 Citrus

##### 3.5.1.1 Preparation and inoculation of explants

Shoot tips, and nodal segments, were collected from 3-4 weeks old juvenile stage of rough lemon plant from new growth flushes. Leaves were removed leaving the petioles. They were washed thoroughly in running tap water for 2 hours to remove traces of dirt. The explants were then, kept in a solution of 0.05 per cent bavistin (carbendazim 50 per cent WP) and 0.01 per cent streptomycin for two hours. The solution was then removed and explants were treated with 10 per cent solution of Teepol for 10 minutes. All traces of detergent were removed by repeated washing in double distilled water.

Further, sterilization procedures were carried out under aseptic conditions in laminar air flow cabinet. The surface sterilization was made using mercuric chloride ( $\text{HgCl}_2$ ) solution and sodium hypochloride ( $\text{NaOCl}$ ) separately at different

**Fig. 2 : Micropropagation in rough lemon**



concentrations with different durations for the explants. They were then, thoroughly rinsed at least three times with autoclaved de-ionized distilled water. The sterilized shoot tip and nodal segments were then cut and trimmed of to 1-2 cm length. Each explants was then, quickly inoculated on the nutrient medium for establishment (Plate- II).

### 3.5.2 Standardization of surface sterilization method for the explants

In order to standardize the most effective surface sterilization treatment to isolate contaminant free shoot tips and nodal segment explants of rough lemon for culture establishment, a trial was conducted using different concentrations of sterilant as given below.

Treatment No.	Sterilants	Concentration (%)	Duration for treatments
T <sub>1</sub>	HgCl <sub>2</sub>	0.05%	5 minutes
T <sub>2</sub>	HgCl <sub>2</sub>	0.05%	8 minutes
T <sub>3</sub>	HgCl <sub>2</sub>	0.1%	5 minutes
T <sub>4</sub>	HgCl <sub>2</sub>	0.1%	8 minutes
T <sub>5</sub>	NaOCl	15%	10 minutes
T <sub>6</sub>	NaOCl	15%	15 minutes
T <sub>7</sub>	NaOCl	25%	10 minutes
T <sub>8</sub>	NaOCl	25%	15 minutes

Treatments were repeated three times.



**A. Shoot tip explant**



**B. Nodal segment explant**

**Treatment E1 - MS + 1.0 mg/l BAP + 0.5 mg/l NAA**

**Plate II : Establishment of different explants of rough lemon  
(3 weeks after inoculation)**

The shoot tip and nodal segment explants after initial treatment as described in this chapter section 3.5.1.1 were subjected to surface sterilization using different concentrations of sterilant mentioned above. Thus, eight treatments were repeated thrice. The traces of sterilants were washed out with sterilized double distilled water. Then, the explants were inoculated on MS, B5, WPM medium. The observations on per cent contamination, non responsive explants (per cent), length of shoot (cm) were recorded.

### 3.5.3 Standardization of establishment medium

MS, B5 and WPM media were used in the trial. The medium supplemented with 1.0 mg/l BAP + 0.5 mg/l NAA was tried to standardize the most suitable culture establishment medium for rough lemon. The treatment details are given below.

Treatment No.	Treatments
E <sub>1</sub>	MS + BAP 1.0 mg/l + NAA 0.5 mg/l
E <sub>2</sub>	B5 + BAP 1.0 mg/l + NAA 0.5 mg/l
E <sub>3</sub>	WPM + BAP 1.0 mg/l + NAA 0.5 mg/l

Treatments were repeated seven times.

### 3.5.4 Standardization of multiplication medium

Best medium found in establishment (W) was used in the trials. Different growth regulators were tried to standardize the most suitable culture multiplication medium for rough lemon. The treatment details are as under.

Treatment No.	Treatments
M <sub>1</sub>	W + BAP 1.0 mg/l
M <sub>2</sub>	W + BAP 1.5 mg/l
M <sub>3</sub>	W + BAP 2.0 mg/l
M <sub>4</sub>	W + BAP 1.0 mg/l + Malt extract 500 mg/l
M <sub>5</sub>	W + BAP 1.5 mg/l + Malt extract 500 mg/l
M <sub>6</sub>	W + BAP 2.0 mg/l + Malt extract 500 mg/l

Treatments were repeated four times.

#### 3.5.4.1 Standardization of optimum sucrose for *in vitro* shoot growth

Experiment was conducted to study the effect of different levels of sucrose on proliferation rate of shoot. Best medium for proliferation (S) was used in the trial and solidified with 0.8 per cent agar. The different levels of sucrose are as under.

Treatment No.	Sucrose (%)
S <sub>1</sub>	1.0
S <sub>2</sub>	2.0
S <sub>3</sub>	3.0
S <sub>4</sub>	4.0
S <sub>5</sub>	5.0

Treatments were repeated four times.

#### 3.5.4.2 Standardization of optimum pH for *in vitro* shoot growth

Experiment was conducted to study the effect of different pH levels on proliferation rate of shoot. Best medium for

proliferation (P) was used in the trial and solidified with 0.8 per cent agar. The different pH levels are appended below.

Treatment No.	pH level
P <sub>1</sub>	4.5
P <sub>2</sub>	5.0
P <sub>3</sub>	5.5
P <sub>4</sub>	5.7
P <sub>5</sub>	6.0
P <sub>6</sub>	6.5

Treatments were repeated four times.

The pH was adjusted with 0.1N HCl or 0.1N NaOH as found necessary before autoclaving.

#### 3.5.4.3 Standardization of light intensity for shoot growth

A trial was conducted to study the effect of different intensities of light on proliferation rate of shoot. Best medium for proliferation (L) was used in the trial and solidified with 0.8 per cent agar. The different levels of intensity of light are given as under.

Treatment No.	Light intensity (Lux)
L <sub>1</sub>	1000
L <sub>2</sub>	2000
L <sub>3</sub>	3000

Treatments were repeated six times.

### 3.5.5 Standardization of *in vitro* rooting medium

The trial on *in vitro* rooting was conducted on half and full strength MS medium and White medium gelled with 0.8 per cent agar. Each medium was supplemented with different combinations of IBA and NAA. The treatment details are as follow.

Treatment No.	Treatments
R <sub>1</sub>	1/2 MS + IBA 1.0 mg/l
R <sub>2</sub>	1/2 MS + IBA 1.0 mg/l + NAA 1.0 mg/l
R <sub>3</sub>	Full MS + IBA 1.0 mg/l
R <sub>4</sub>	Full MS + IBA 1.0 mg/l + NAA 1.0 mg/l
R <sub>5</sub>	White medium + IBA 1.0 mg/l
R <sub>6</sub>	White medium + IBA 1.0 mg/l + NAA 1.0 mg/l

Treatments were repeated four times.

### 3.5.6 Standardization of potting mixture for hardening of *in vitro* plants

The different potting mixtures (H) used for *in vitro* raised plantlets in the experiment, are appended below.

Treatment No.	Treatments
H <sub>1</sub>	Vermicompost
H <sub>2</sub>	Soil
H <sub>3</sub>	Coco Peat
H <sub>4</sub>	Vermicompost : Soil (1:1v/v)
H <sub>5</sub>	FYM : Soil : Sand (1:1:1v/v)

Treatments were repeated five times.

Rooted plantlets were taken out from the culture vessels with the help of forcep. The nutrient medium was gently removed and washed thoroughly in tap water ensuring that all agar particles were completely removed without damaging the roots. The rooted plantlets were then, dipped in 0.05 per cent bavistin, (carbendazim 50 per cent WP) and planted in earthen pots containing pretreated cocopeat. They were covered with plastic cup continuously for 6-7 days and kept in air conditioned room. The cover was gradually removed after 7 days, initially for 3 hours followed by 6 hours and 12 hours in next 3 days. The cover was removed during night and lights put-off for next 3-4 days. Subsequently, the period of keeping the plantlets without any cover was gradually increased and after 15 days they were brought outside the room in shade. Within next 15 days by gradually exposing them to sun, they were acclimatized to natural environment.

### 3.5.7 Standardization of climate control for hardening of *in vitro* plants

The trial to standardize the climate control for hardening of *in vitro* raised plantlets, was conducted on different situations of climate. The treatment details are given below.

Treatments	Control conditions
A. Individual plants	a. Net house b. Air condition
B. Group of plants	a. Net house b. Air condition
C. Low tunnel net house	---

Treatments were repeated six times.

**3.6 Observations recorded****3.6.1 Surface sterilization of explants**

- 1) Per cent of culture establishment after 3 weeks inoculation.
- 2) Per cent of non responsive explants after 3 weeks inoculation.
- 3) Length of shoot (cm) after 3 weeks inoculation.
- 4) Per cent of culture contamination after 3 weeks inoculation.

A contamination of explants was calculated by using the following formula.

$$\text{Contaminated explants (\%)} = \frac{\text{No. of Contaminated explants}}{\text{Total no. of explants used}} \times 100$$

**3.6.2 Standardization of establishment medium**

- 1) Days taken for establishment
- 2) Establishment of explants (per cent)
- 3) Length of internode per shoot (cm)
- 4) Number of internodes per explant

**3.6.3 Standardization of multiplication and proliferation medium**

- 1) Number of shoots per culture
- 2) Length of internode per shoot (cm)
- 3) Number of internodes per shoot

- 4) Multiplication of shoot in sub culturing
- 5) Proliferation of culture (%)
- 6) Length of shoot (cm)

#### 3.6.4 *In vitro* rooting

- 1) Culture rooted (per cent)
- 2) Days taken for root initiation
- 3) Length of root (cm)
- 4) Number of roots per shoot in rooting medium
- 5) Length of shoot (cm)

#### 3.6.5 Acclimatization of *in vitro* rooted plantlets

- 1) Establishment of plantlets (per cent)
- 2) Days taken for new sprouting
- 3) Length of shoot (cm)

#### 3.7 Statistical analysis

Statistical methods were used for comparison of treatment means during optimizing parameters for micropropagation. Completely randomized design (CRD) was used for all the experiment. The data were subjected to analysis of variance (ANOVA) and treatment means were compared (Panse and Sukhatme, 1985).



# EXPERIMENTAL RESULTS



## **IV. EXPERIMENTAL RESULTS**

Citrus is one of the important fruit crops of commercial value to be propagated *in vitro* tissue culture. Micropropagation techniques require the organs and tissues to be passed through a sequence of steps in which different cultural and environmental conditions are provided. The stage I being physiological preconditioning of explants and culture establishment, stage II rapid multiplication of shoots, stage III *in vitro* rooting and stage IV acclimatization of *in vitro* raised plantlets and then, transplantation to field condition.

The results of experiment entitled, "Micropropagation in rough lemon (*Citrus jambhiri* Lush.)" conducted at the Department of Biotechnology, ASPEE College of Horticulture and Forestry, Navsari Agricultural University, Navsari during the period 2008-2010 are presented in this chapter.

### **4.1 Standardization of protocol Micropropagation technique**

While standardizing the method of micropropagation of rough lemon, various factors such as surface sterilization agents, explant sources, plant growth regulators, pH, sucrose, light, etc. were considered for optimum response. Media for establishment, multiplication, proliferation, rooting and hardening were standardized.

#### **4.1.1 Surface sterilization treatments for rough lemon**

The results on surface sterilization of shoot tip and nodal segment explants from 3-4 weeks old juvenile stage as raised

plant are presented in Table 4.1 and Table 4.2, and graphically presented in Fig. 3 and Fig. 4.

It is evident from the results presented in (Table 4.1), that the shoot tip explants reported highest establishment (69.50 per cent) in T<sub>3</sub> (HgCl<sub>2</sub> 0.1 per cent for 5 minutes), as it showed less contamination of culture (27.93 per cent) and maximum length of shoot (1.71 cm) followed by T<sub>1</sub>, T<sub>2</sub>, and T<sub>4</sub>. Similarly, in case of nodal segment explants (Table 4.2), treatment T<sub>3</sub> (HgCl<sub>2</sub> 0.1 per cent for 5 minutes) recorded highest establishment of explants (73.73 per cent) with the less contamination (21.06 per cent) and maximum length of shoot (2.31 cm) followed by treatment T<sub>2</sub>, T<sub>1</sub>, and T<sub>4</sub>.

Hence, the sterilant treatment with HgCl<sub>2</sub> 0.1 per cent for 5 minutes was found to be better surface sterilizing treatment for shoot tip and nodal segment as well as for better culture establishment and growth of explants.

#### **4.2 Standardization of medium for establishment of explants**

In order to standardize a suitable culture establishment medium for shoot tip explants of juvenile stage plant by using MS, B5 and WPM media, each supplemented with 1.0 mg/l BAP + 0.5 mg/l NAA. The results revealed that maximum establishment of explants (47.56 %) was achieved on MS medium supplemented with 1.0 mg/l BAP + 0.5 mg/l NAA, in which, shoot tip and nodal segment recorded 47.56% and 65.78%, respectively (Table 4.3 and Fig. 5).

**Table 4.1: Effect of surface sterilization agents on establishment, growth and contamination of rough lemon**

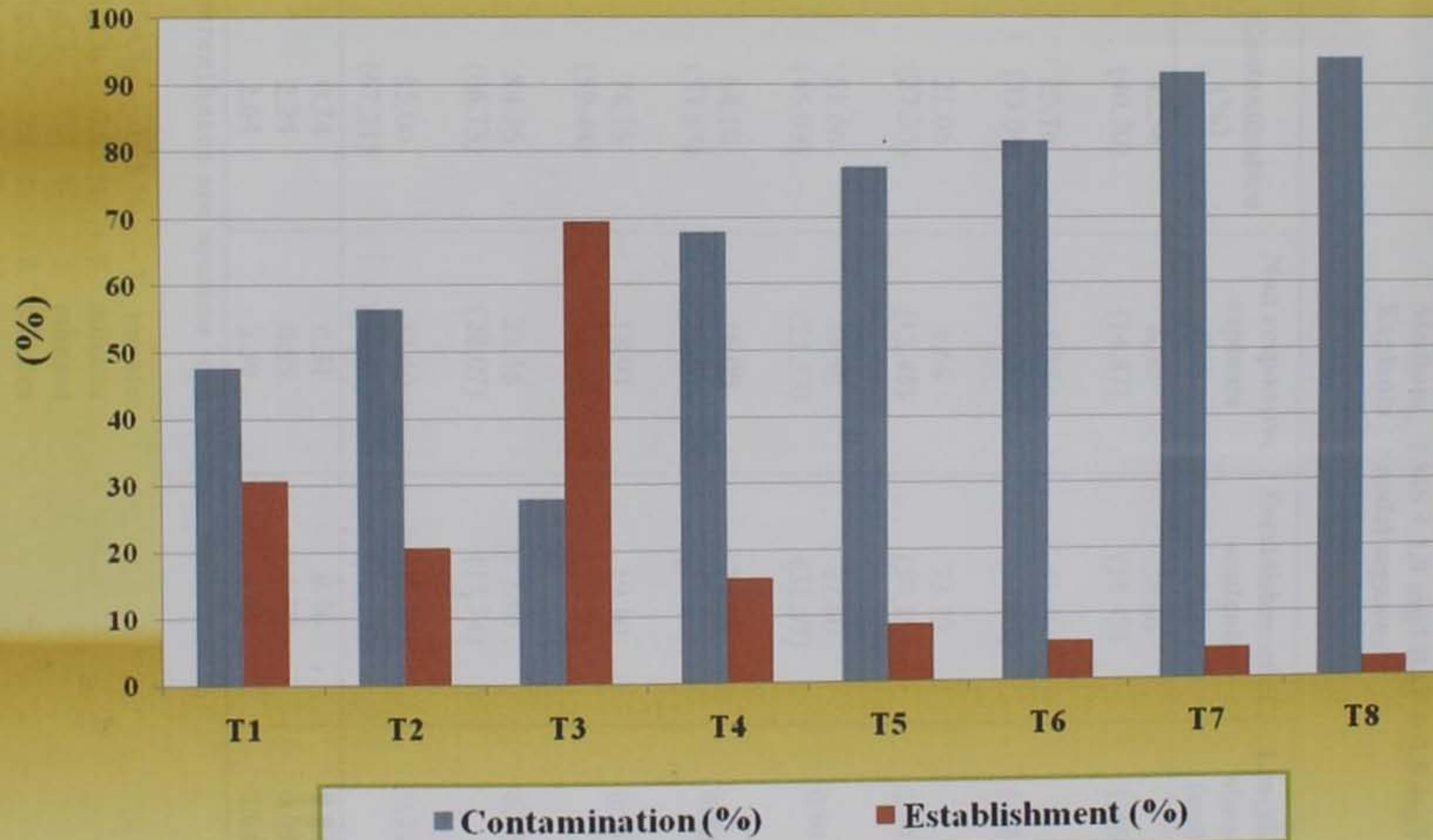
Medium : MS + 1.0 mg/l BAP + 0.5 mg/l NAA  
 Explants : Shoot tip  
 Incubation: 4 weeks

Treatment No.	Contamination (%)	Non responsive explants (%)	Establishment of explants (%)	Length of shoot (cm)
T <sub>1</sub>	47.66 (43.64)	9.83 (18.24)	30.73 (33.64)	1.30
T <sub>2</sub>	56.40 (48.65)	10.50 (18.89)	20.63 (26.99)	1.02
T <sub>3</sub>	27.93 (31.89)	6.50 (14.75)	69.50 (56.45)	1.71
T <sub>4</sub>	67.80 (55.41)	11.30 (19.63)	15.76 (23.38)	0.76
T <sub>5</sub>	77.63 (61.75)	13.53 (21.56)	8.56 (17.00)	0.53
T <sub>6</sub>	81.50 (64.50)	15.00 (22.77)	5.83 (13.90)	0.36
T <sub>7</sub>	91.83 (73.42)	20.90 (27.19)	4.53 (12.23)	0.20
T <sub>8</sub>	94.00 (75.87)	23.66 (29.09)	2.80 (9.57)	0.15
S.Em. ±	0.71	0.39	0.62	0.03
C.D. at 5 %	2.14	1.19	1.88	0.11
C.V. %	2.18	3.20	4.52	8.31

\*Figure in parentheses are arcsine transformed value

Treatments: T<sub>1</sub>: HgCl<sub>2</sub> - 0.05 % - 5 minutes  
 T<sub>2</sub>: HgCl<sub>2</sub> - 0.05 % - 8 minutes  
 T<sub>3</sub>: HgCl<sub>2</sub> - 0.1 % - 5 minutes  
 T<sub>4</sub>: HgCl<sub>2</sub> - 0.1 % - 8 minutes  
 T<sub>5</sub>: NaOCl - 15 % - 10 minutes  
 T<sub>6</sub>: NaOCl - 15 % - 15 minutes  
 T<sub>7</sub>: NaOCl - 25 % - 10 minutes  
 T<sub>8</sub>: NaOCl - 25 % - 15 minutes

**Fig. 3: Effect of surface sterilants on contamination (%) and establishment (%) of rough lemon  
Explants : Shoot tip**



**Table- 4.2: Effect of surface sterilization agents on establishment, growth and contamination of rough lemon**

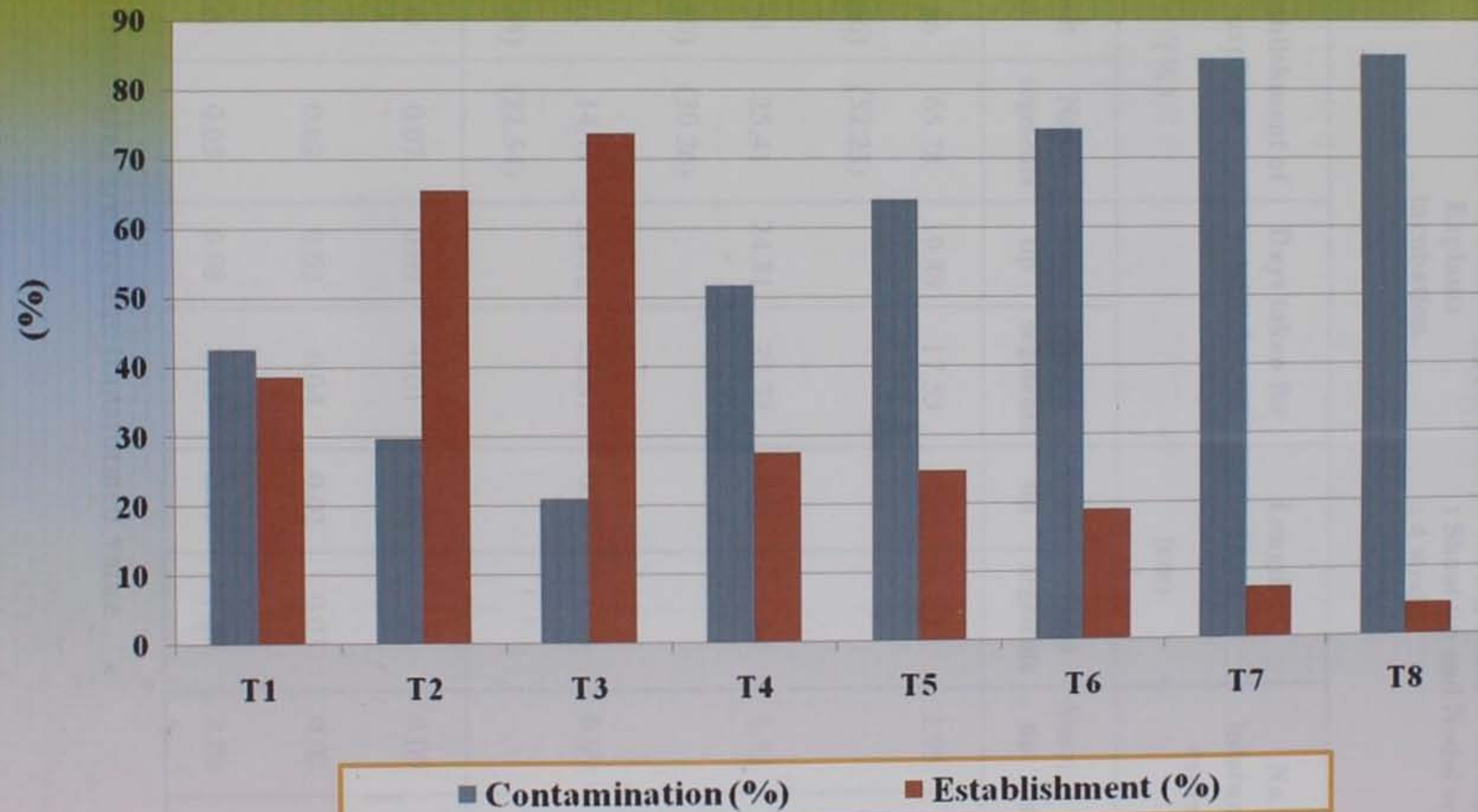
Medium : MS + 1.0 mg/l BAP + 0.5 mg/l NAA  
 Explants : nodal segment  
 Incubation : 4 weeks

Treatment No.	Contamination (%)	Non responsive explants (%)	Establishment of explants (%)	Length of shoot (cm)
T <sub>1</sub>	42.56 (40.70)	6.60 (14.87)	38.66 (38.43)	1.40
T <sub>2</sub>	29.76 (33.04)	5.33 (13.34)	65.56 (54.65)	1.45
T <sub>3</sub>	21.06 (27.30)	4.66 (12.45)	73.73 (59.15)	2.31
T <sub>4</sub>	51.66 (45.93)	13.50 (21.53)	27.60 (31.67)	0.91
T <sub>5</sub>	64.10 (53.17)	16.00 (23.56)	24.86 (29.89)	0.83
T <sub>6</sub>	74.16 (59.44)	18.93 (25.78)	19.10 (25.90)	0.81
T <sub>7</sub>	84.36 (66.73)	22.16 (28.07)	7.40 (15.74)	0.65
T <sub>8</sub>	85.00 (67.21)	25.40 (30.25)	4.70 (12.49)	0.37
S.Em. ±	0.74	0.31	0.46	0.02
C.D. at 5 %	2.24	0.95	1.39	0.08
C.V. %	2.64	2.59	2.41	4.68

\*Figure in parentheses are arcsine transformed value

Treatments: T<sub>1</sub>: HgCl<sub>2</sub> - 0.05 % - 5 minutes  
 T<sub>2</sub>: HgCl<sub>2</sub> - 0.05 % - 8 minutes  
 T<sub>3</sub>: HgCl<sub>2</sub> - 0.1 % - 5 minutes  
 T<sub>4</sub>: HgCl<sub>2</sub> - 0.1 % - 8 minutes  
 T<sub>5</sub>: NaOCl - 15 % - 10 minutes  
 T<sub>6</sub>: NaOCl - 15 % - 15 minutes  
 T<sub>7</sub>: NaOCl - 25 % - 10 minutes  
 T<sub>8</sub>: NaOCl - 25 % - 15 minutes

**Fig. 4: Effect of surface sterilants on contamination (%) and establishment (%) of rough lemon  
Explants : Nodal segment**



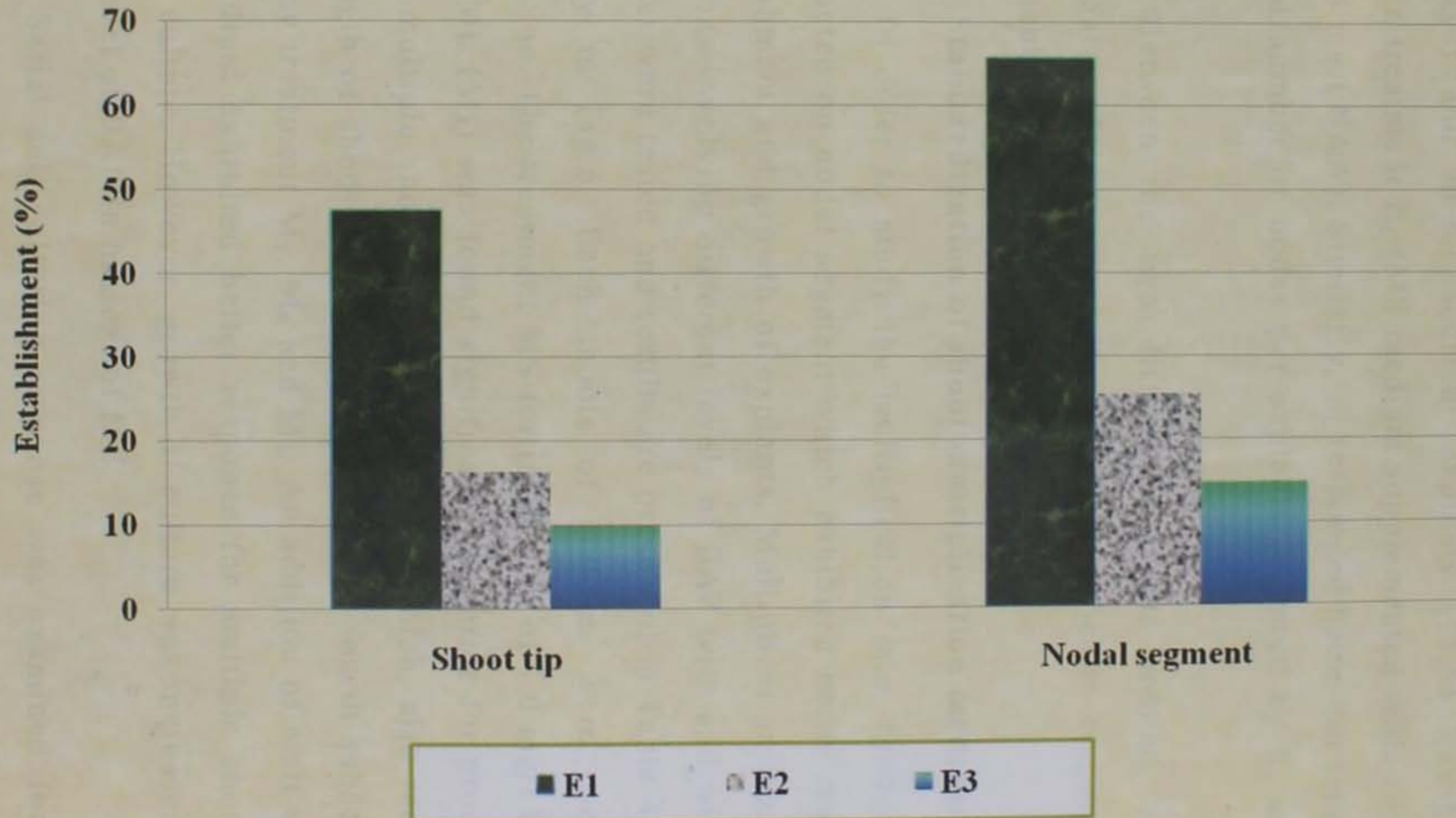
**Table 4.3: Effect of different media on establishment and growth of explants of rough lemon**

Mediums supplemented: 1.0 mg/l BAP + 0.5 mg/l NAA  
 Explants : Shoot tip and Nodal segment  
 Incubation : 4 weeks

Media	Establishment of explants (%)		Days taken for establishment		Length of Shoot (cm)		No. of internodes / explants	
	Shoot tip	Nodal segments	Shoot tip	Nodal segments	Shoot tip	Nodal segments	Shoot tip	Nodal segments
E <sub>1</sub> - MS	47.56 (34.66)	65.78 (52.23)	19.89	17.53	1.72	2.27	1.95	2.62
E <sub>2</sub> - B5	16.31 (23.80)	25.41 (30.26)	24.81	22.78	0.92	1.68	1.12	1.86
E <sub>3</sub> - WPM	9.81 (18.24)	14.71 (22.54)	25.12	23.81	0.57	1.08	0.69	1.15
S.Em. ±	0.09	0.07	0.09	0.01	0.08	0.01	0.09	0.01
C.D. 5 %	0.02	0.02	0.02	0.04	0.02	0.03	0.02	0.03
C.V. %	0.08	0.05	0.08	0.13	2.19	1.89	2.06	1.81

\*Figure in parentheses are arcsine transformed value

**Fig. 5: Effect of media on establishment of explants of rough lemon**  
**Explants : Shoot tip and Nodal segment (juvenile stage)**



The minimum days taken for establishment of shoot tip (19.89 days) and nodal segment explants (17.53 days) was recorded in treatment E<sub>1</sub> (MS medium supplemented with 1.0 mg/l BAP + 0.5 mg/l NAA). Similarly, E<sub>1</sub> responded better for length of shoots and number of nodes per explants followed by E<sub>2</sub> and E<sub>3</sub> (Plate III).

Between the two different explants studied, results showed that nodal segment explants gave better response to establishment and growth of explants.

#### **4.3 Standardization of shoot multiplication medium**

In order to study the multiplication rate, further trial was conducted on nodal segment which exhibited better response to establishment and growth of explants. Multiplication treatment combinations involving different levels of BAP with and with out malt extract were tested and results are presented in Table 4.4 and graphically in Fig.6. Each shoots of cultures were used for multiplication. The treatment MS fortified with of 1.0 mg/l BAP + 500 mg/l ME (M<sub>4</sub>) was found significantly superior for producing maximum multiple shoots (3.78) per culture which, also recorded higher length of shoot (1.82 cm) in culture at fourth sub culture followed by treatment M<sub>5</sub>, M<sub>6</sub> and M<sub>3</sub>. An addition of malt extract in the medium exhibited better response for multiple shoot and growth of culture. However, growth of culture was increased even at lower level of BAP in present of ME.

Serial sub culturing response was examined for each treatment in the multiplication medium for four subcultures

**A**



**B**



**C**



**A - E1 - MS + 1.0 mg/l BAP + 0.5 mg/l NAA**

**B - E2 - B5 + 1.0 mg/l BAP + 0.5 mg/l NAA**

**C - E3 - WPM + 1.0 mg/l BAP + 0.5 mg/l NAA**

**Plate III : Effect of different media on best establishment of nodal segment explants**

**Table 4.4: Effect of BAP and Malt Extract for serial subculturing on shoot multiplication rate from nodal segment of rough lemon**

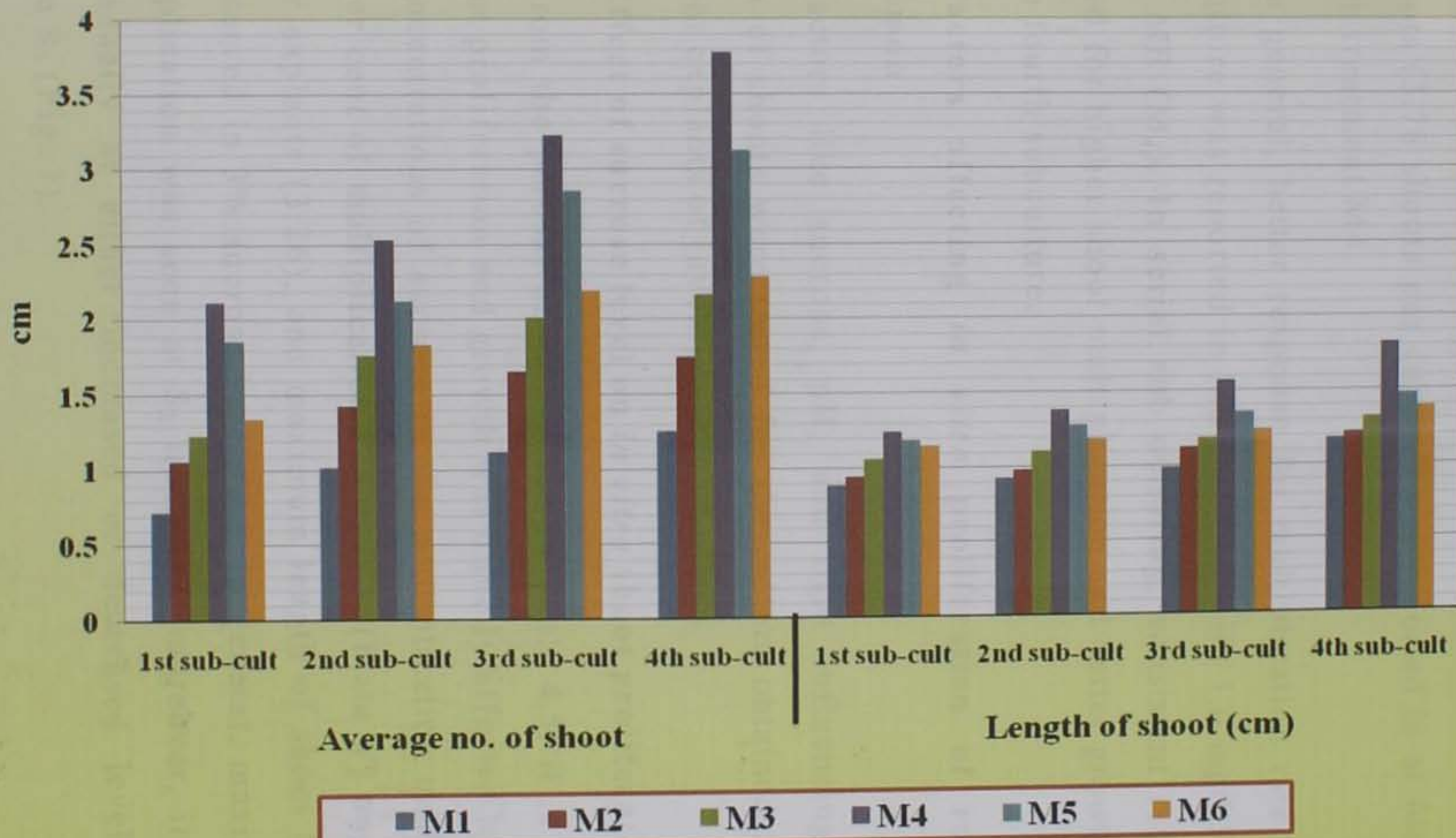
Medium : MS Medium  
Incubation : 3 weeks

Treatment No.	Average no. of shoots				Length of shoot (cm)			
	1 <sup>st</sup> sub culturing	2 <sup>nd</sup> sub culturing	3 <sup>rd</sup> sub culturing	4 <sup>th</sup> sub culturing	1 <sup>st</sup> sub culturing	2 <sup>nd</sup> sub culturing	3 <sup>rd</sup> sub culturing	4 <sup>th</sup> sub culturing
M <sub>1</sub>	0.72	1.02	1.12	1.26	0.89	0.93	0.99	1.18
M <sub>2</sub>	1.06	1.43	1.66	1.75	0.94	0.98	1.12	1.22
M <sub>3</sub>	1.23	1.77	2.02	2.17	1.06	1.11	1.19	1.32
M <sub>4</sub>	2.12	2.54	3.23	3.78	1.24	1.38	1.57	1.82
M <sub>5</sub>	1.86	2.13	2.86	3.13	1.19	1.28	1.36	1.48
M <sub>6</sub>	1.34	1.84	2.19	2.29	1.15	1.19	1.24	1.39
S.Em. ±	0.01	0.07	0.09	0.08	0.02	0.02	0.02	0.02
C.D. at 5 %	0.03	0.02	0.02	0.02	0.04	0.06	0.06	0.05
C.V. %	0.80	0.68	1.06	1.29	2.13	3.48	3.49	2.43

\*Figure in parentheses are arcsine transformed value

Treatments: M<sub>1</sub>: + 1.0 mg/l BAP  
M<sub>2</sub>: + 1.5 mg/l BAP  
M<sub>3</sub>: + 2.0 mg/l BAP  
M<sub>4</sub>: + 1.0 mg/l BAP + 500 mg/l ME  
M<sub>5</sub>: + 1.5 mg/l BAP + 500 mg/l ME  
M<sub>6</sub>: + 2.0 mg/l BAP + 500 mg/l ME

**Fig. 6: Effect of serial subculturing (sub-cult) on multiplication rate from nodal segments of rough lemon**



(Plate IV). It is evident from the data given (Table 4.4), that the multiplication rate of shoots and growth of shoots in all the treatments were increased in all subsequent subcultures. Maximum multiple shoots (3.78 shoots per culture) was reported in at fourth sub culture in treatment M4.

In general, better response for multiplication rate and growth of culture was reported in MS fortified with 1.0 mg/l BAP + 500 mg/l ME (M<sub>4</sub>). In serial sub culturing, consistent results were obtained for higher shoot multiplication and better growth of culture up to fourth subculture.

#### 4.4 Factors affecting *in vitro* proliferation of rough lemon

Factors like sucrose, pH, and light influencing on proliferation of shoots were tested for the culture obtained from nodal segment explants of juvenile stage of plants.

##### 4.4.1 Effect of sucrose level on *in vitro* shoot proliferation

From the perusal of data given in Table 4.5, it is seen that the shoot proliferation and growth was directly affected by the different concentrations of sucrose in the multiplication medium. Maximum per cent of multiplication (73.00 %), (Plate V) number of shoot per explants (3.26), and maximum length of shoot (2.57 cm) was observed in 3% sucrose level (S<sub>3</sub>). In general, maximum shoot multiplication was seen at S<sub>3</sub> treatment; moreover, it was reduced gradually at either increased or decreased levels of sucrose from S<sub>3</sub> (Fig. 7).

**1<sup>st</sup> subculturing**



**2<sup>nd</sup> subculturing**



**3<sup>rd</sup> subculturing**



**4<sup>th</sup> subculturing**



**Treatment M4 - MS + 1.0 mg/l BAP + 500 mg/l Malt Extract**

**Plate IV : Effect of serial subculturing on multiplication of nodal segment explant**

**(3 weeks after culturing)**

**Table-4.5: Effect of different level of sucrose on *in vitro* shoot proliferation of culture of rough lemon**

**Medium** : MS Medium + 1.0 mg/l BAP + 500 mg/l ME

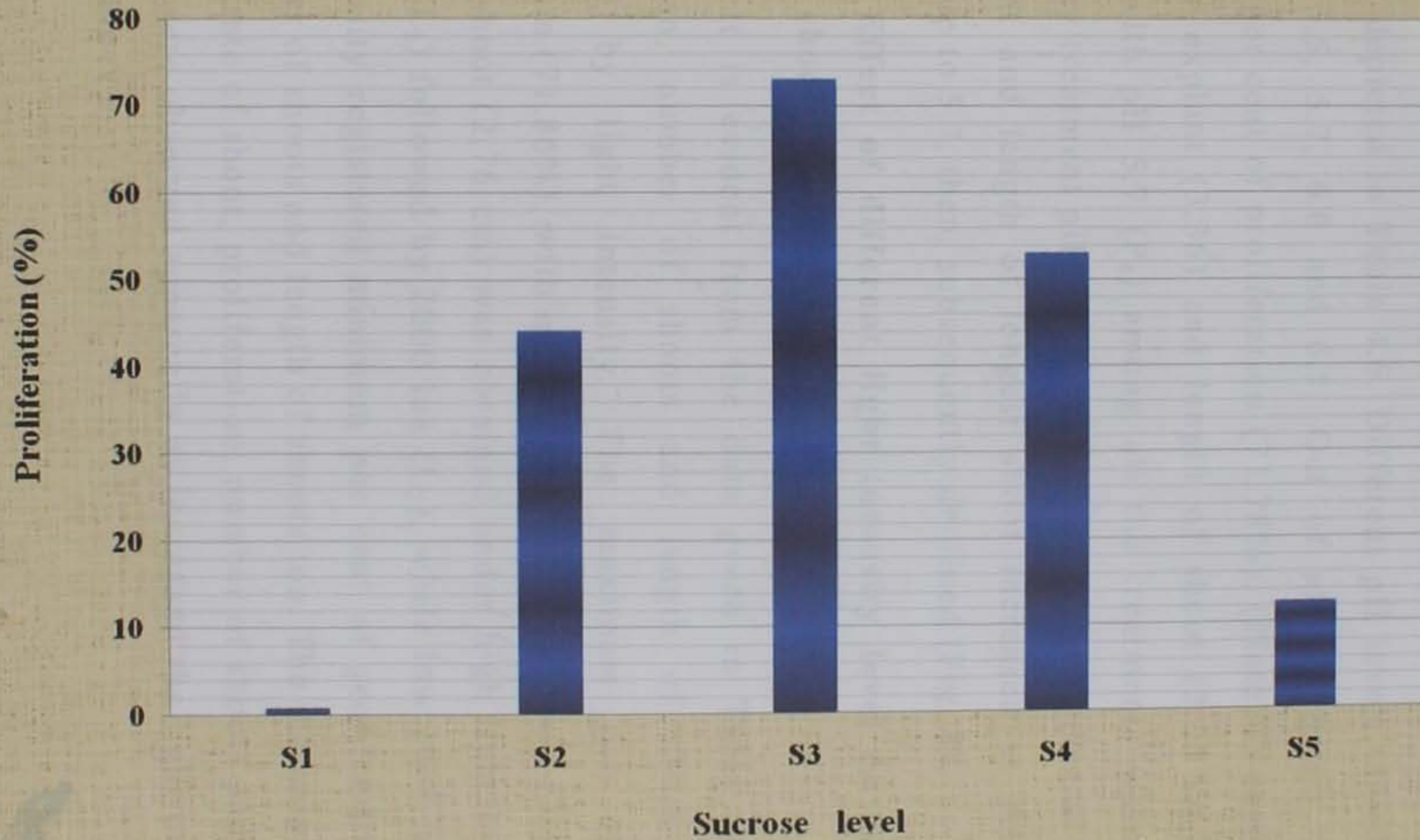
**Incubation** : 3 weeks

**Explants** : Nodal segment

<b>Treatment No.</b>	<b>Proliferation (%)</b>	<b>No. of shoot / culture</b>	<b>Length of shoot (cm)</b>
S <sub>1</sub> – Sucrose 1%	0.75 (2.33)	0.12	0.15
S <sub>2</sub> – Sucrose 2%	44.20 (41.65)	1.49	1.12
S <sub>3</sub> – Sucrose 3%	73.00 (58.67)	3.26	2.57
S <sub>4</sub> – Sucrose 4%	52.97 (46.68)	1.75	1.30
S <sub>5</sub> – Sucrose 5%	12.51 (20.70)	1.16	1.04
S.Em. ±	0.35	0.21	0.14
C.D. at 5 %	1.06	0.65	0.45
C.V. %	2.08	2.76	2.40

\*Figure in parentheses are arcsine transformed value

**Fig. 7: Effect of levels of sucrose on shoot proliferation (%) of rough lemon  
Explants : Nodal segment**



#### 4.4.2 Effect pH of the medium on *in vitro* shoot proliferation

The influences of pH on proliferation and growth of shoots are depicted in Table 4.6. Different pH levels tested were 4.5, 5.0, 5.5, 5.7, 6.0 and 6.5. Out of all pH levels tested, maximum per cent of proliferation (71.78%), (Plate V) number of shoots per explant (3.36) and length of shoot (2.25 cm) were recorded with pH 5.7 (P<sub>4</sub>) among all the treatments which was followed by treatment pH 6.0 (P<sub>5</sub>). The trends of number of shoots per explant and length of longest shoot increased as pH level increased up to 5.7, then, subsequently declined (Fig. 8).

#### 4.4.3 Effect of different light intensity level on *in vitro* shoot proliferation

It is evident from the data given in Table 4.7 that proliferation, number of shoots and length of shoots were influenced by light intensity. The maximum per cent of proliferation (71.86%), with maximum number of shoots (3.53) and length of shoot (2.76 cm) was obtained under high light intensity 3000 lux (L<sub>3</sub>) followed by 2000 lux (L<sub>2</sub>), while low 1000 lux (L<sub>1</sub>) light intensity registered minimum per cent of proliferation and less number of shoots and length of shoots too. The increased trend of growth rate of shoot, proliferation, number of shoots and length of shoots were observed apparently as increased the light intensity (Fig. 9, Plate-V).

**Table 4.6:Effect of initial pH of the medium on *in vitro* shoot proliferation of rough lemon**

Medium : MS Medium + 1.0 mg/l BAP + 500 mg/l ME

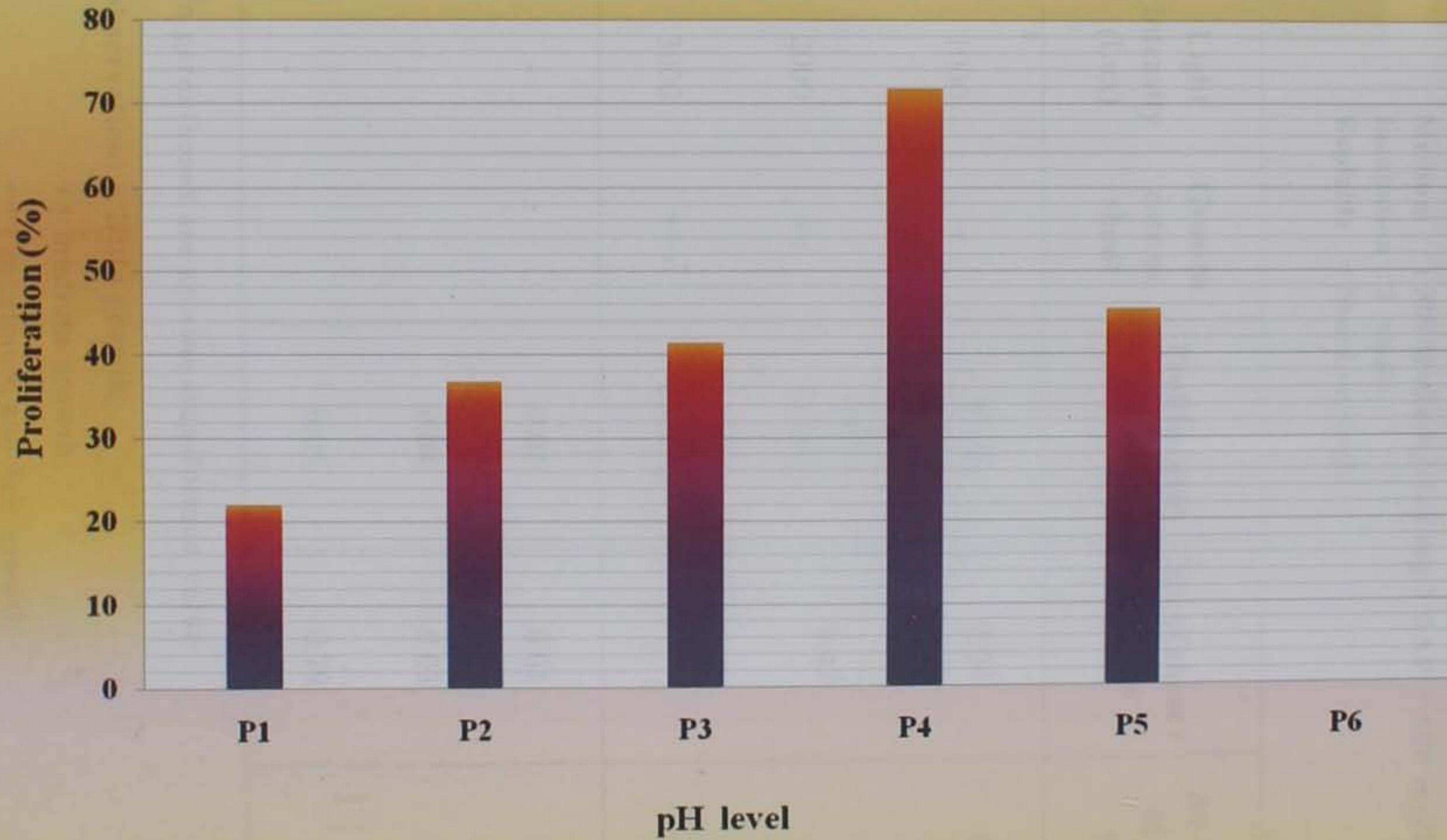
Incubation : 3 weeks

Explants : Nodal segment

Treatment No.	pH level	Proliferation (%)	No. of shoot / culture	Av. length of shoot (cm)
P <sub>1</sub>	4.5	21.98 (27.94)	1.04	0.77
P <sub>2</sub>	5.0	36.84 (37.35)	1.47	1.07
P <sub>3</sub>	5.5	41.43 (40.04)	1.90	1.39
P <sub>4</sub>	5.7	71.78 (57.89)	3.36	2.25
P <sub>5</sub>	6.0	45.35 (42.31)	2.15	1.67
P <sub>6</sub>	6.5	0.06 (1.45)	0.03	0.02
S.Em. ±	-	0.23	0.01	0.09
C.D. at 5 %	-	0.68	0.03	0.02
C.V. %	-	1.34	1.34	1.55

\*Figure in parentheses are arcsine transformed value

**Fig. 8: Effect of levels of pH of medium on shoot proliferation (%) of rough lemon  
Explants : Nodal segment**



**Table - 4.7: Effect of different light intensity on *in vitro* shoot proliferation of culture of rough lemon**

Medium : MS Medium + 1.0 mg/l BAP + 500 mg/l ME

Incubation : 3 weeks

Explants : Nodal segment

Treatment No.	Light intensity (Lux)	Growth rate of shoot	Proliferation (%)	No. of shoot / culture	Av. length of shoot (cm)
L <sub>1</sub>	1000	+	41.26 (39.95)	2.04	0.72
L <sub>2</sub>	2000	++	53.57 (47.02)	2.47	1.47
L <sub>3</sub>	3000	+++	71.86 (57.94)	3.53	2.76
S.Em. ±	-	-	0.01	0.01	0.08
C.D. at 5 %	-	-	0.04	0.03	0.02
C.V. %	-	-	0.05	0.79	1.08

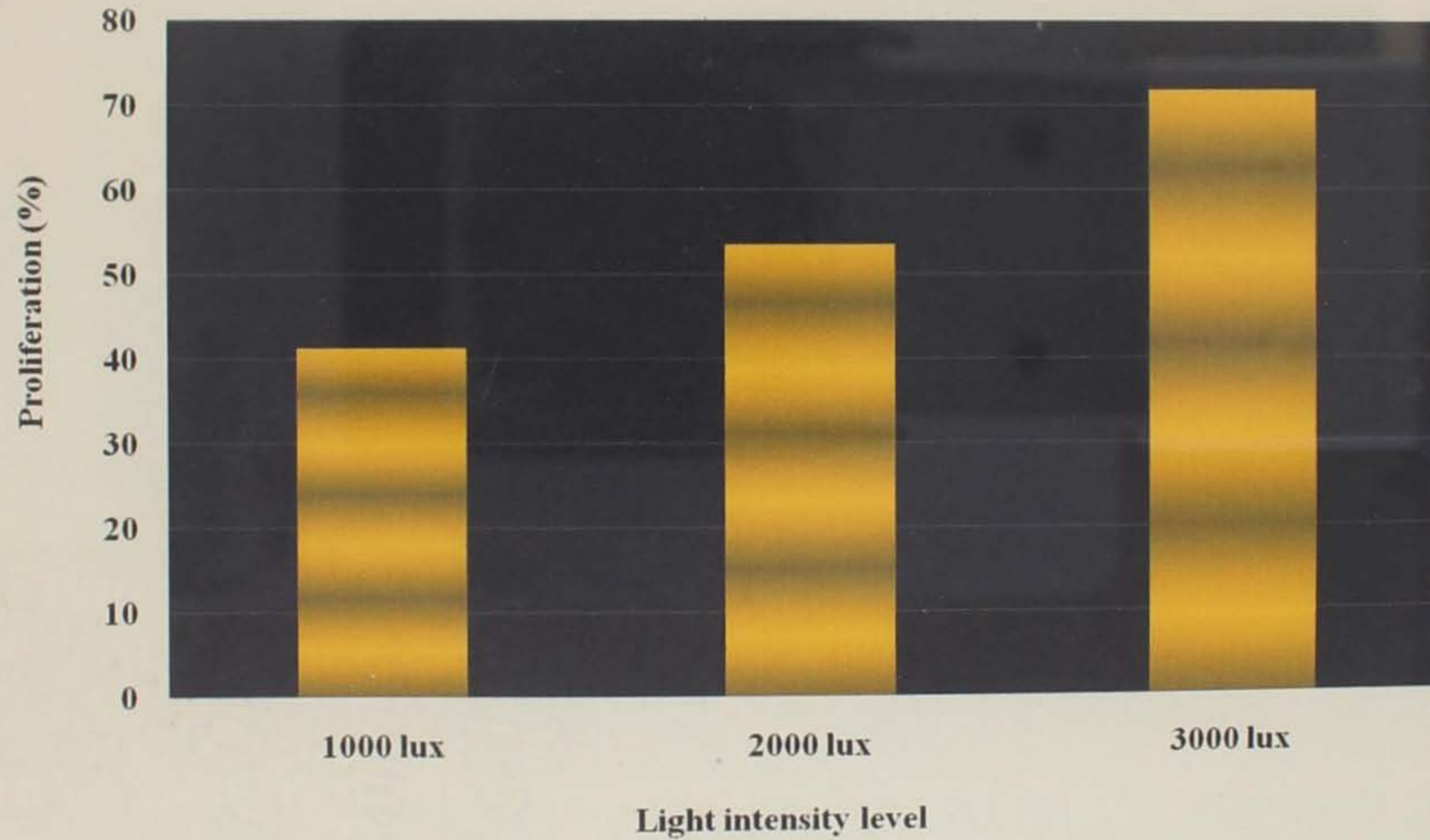
\*Figure in parentheses are arcsine transformed value

Visual observation: + little growth,

++ medium growth,

+++ maximum and fair growth.

**Fig. 9: Effect of light intensities on shoot proliferation (%) of rough lemon  
Explants : Nodal segment**





**Sucrose 3% (S3)**



**pH 5.7 (P4)**



**Light 3000 Lux (L3)**

**Treatment M4 : MS + 1.0 mg/l BAP + 500 mg/l Malt Extract**

**Plate V : Best treatment for factors affecting on proliferation of rough lemon culture**

**(3 weeks after culturing)**

#### 4.5 Effect of IBA, NAA and strength of the media on *in vitro* rooting in shoot of rough lemon

The data on rooting response to different levels of IBA and IAA supplemented in half and full strength of MS and White medium are presented in Table 4.8. It was noticed that rooting of *in vitro* shoot on full strength MS medium was found significantly better in respect to all the rooting characters than that observed on half strength MS medium and White medium. The best rooting per cent (73.23%) (Fig.10) with minimum days to root initiation (18.19), maximum length of roots (4.53 cm), maximum length of shoots (3.57 cm) and maximum number of roots (3.43) were observed in treatment full MS + 1.0 mg/l IBA + 1.0 mg/l NAA (R<sub>4</sub>) followed by treatment (R<sub>2</sub>) (Plate VI).

In general, the response of rooting was observed maximum in full strength MS medium supplemented with 1.0 mg/l IBA + 1.0 mg/l NAA. It is an interesting to note that efficacy of IBA was increased in present of NAA for rooting.

##### 4.5.1 Effect of different potting mixtures on survival of *in vitro* raised plantlets of rough lemon

The survival rate of plantlet was significantly influenced by potting mixtures Table 4.9. The significantly maximum survival per cent of plantlet (70.86%) with minimum days for new sprouting (10.86 days) and maximum length of shoots (4.15 cm) was observed in treatment FYM:soil:sand (1:1:1v/v) followed by Vermicompost:Soil (1:1v/v) and Vermicompost (Fig.11, Plate VII).

**Table 4.8: Effect of IBA, NAA and strength of medium on induction of rooting in rough lemon**

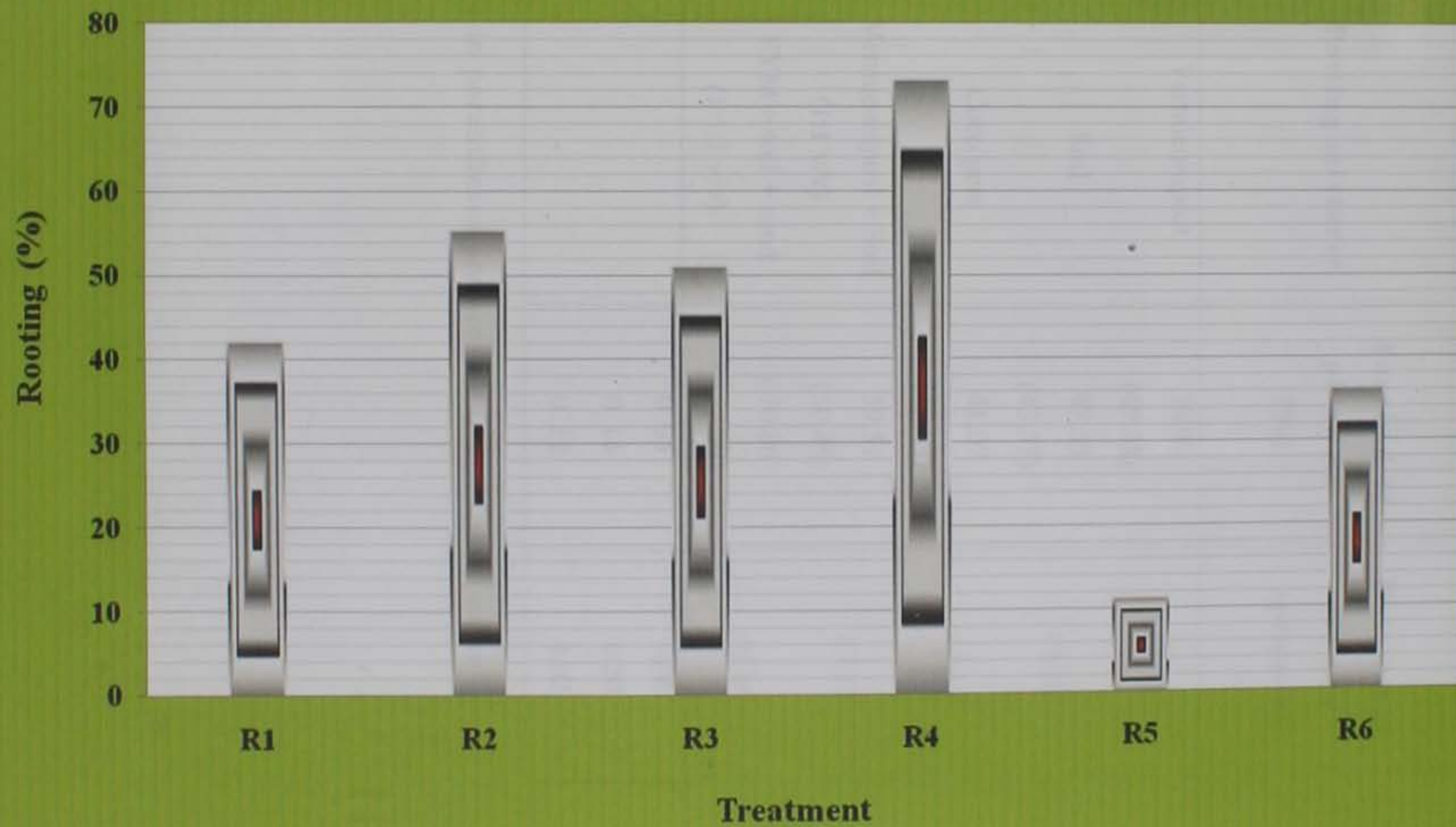
Medium : MS Medium + White Medium  
Incubation : 4 weeks

Treatment No.	Rooting (%)	Days Taken for Root initiation	Length of root (cm)	No. of roots / shoot	Length of shoot (cm)
R <sub>1</sub>	42.15 (39.47)	22.84	2.15	1.64	2.36
R <sub>2</sub>	55.36 (48.06)	20.51	2.86	1.96	2.93
R <sub>3</sub>	51.04 (45.57)	22.08	2.53	1.75	2.53
R <sub>4</sub>	73.23 (58.81)	18.19	4.53	3.43	3.57
R <sub>5</sub>	11.20 (19.54)	25.25	0.65	0.74	0.24
R <sub>6</sub>	36.34 (28.05)	23.54	1.92	1.33	1.05
S.Em. ±	0.01	0.12	0.08	0.09	0.01
C.D. at 5 %	0.03	0.32	0.03	0.02	0.03
C.V. %	0.05	0.89	0.81	1.00	1.01

\*Figure in parentheses are arcsine transformed value

Treatments: R<sub>1</sub>: + ½ MS + 1.0 mg/l IBA  
R<sub>2</sub>: + ½ MS + 1.0 mg/l IBA + 1.0 mg/l NAA  
R<sub>3</sub>: + Full MS + 1.0 mg/l IBA  
R<sub>4</sub>: + Full MS + 1.0 mg/l IBA + 1.0 mg/l NAA  
R<sub>5</sub>: + White medium + 1.0 mg/l IBA  
R<sub>6</sub>: + White medium + 1.0 mg/l IBA + 1.0 mg/l NAA

Fig. 10 : Effect of IBA , NAA and strength of media on *in vitro* rooting of rough lemon

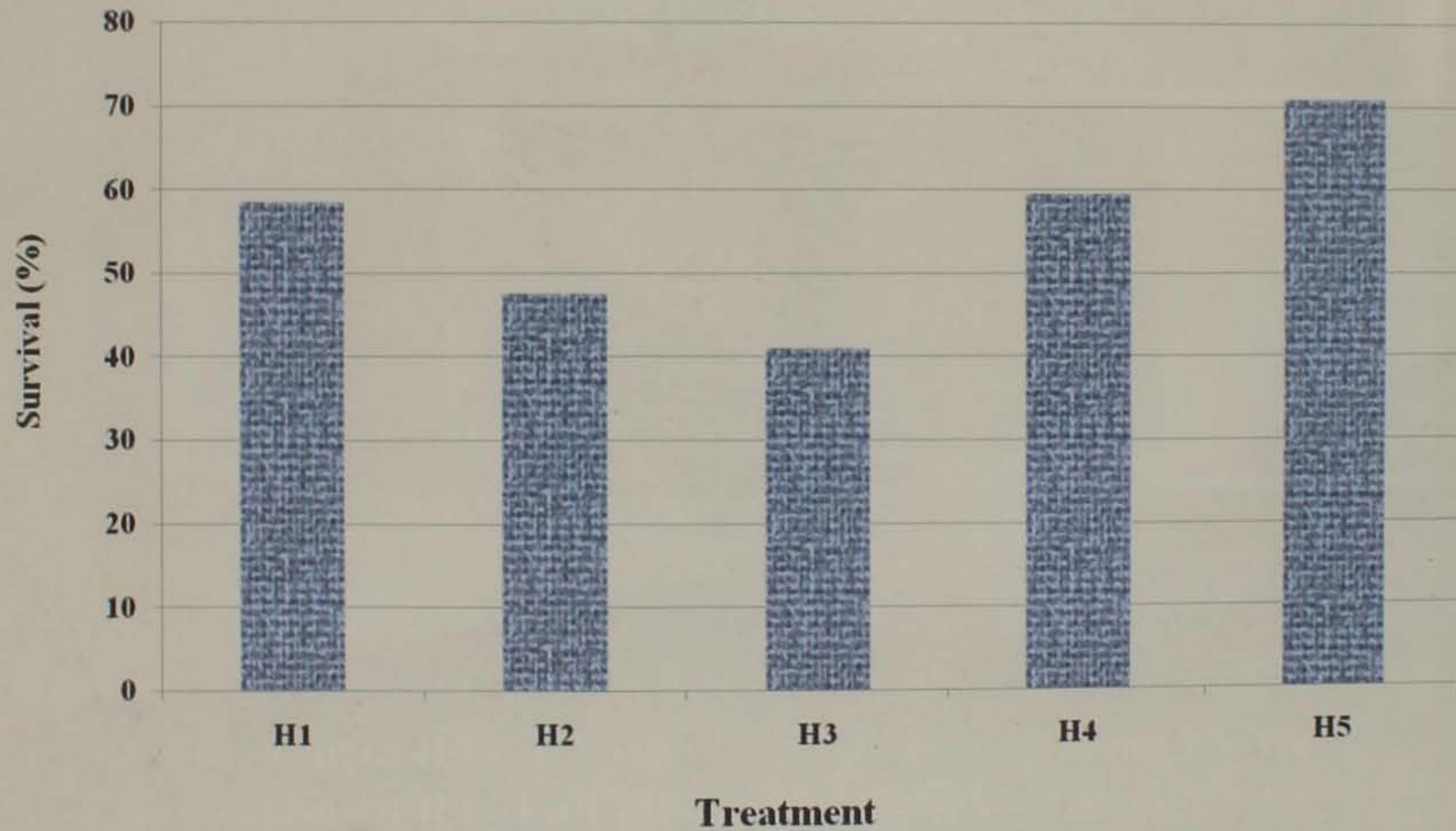


**Table -4.9: Effect of different potting mixtures on hardening of rough lemon**

Treatment No.	Potting mixtures	Survival of plantlets (%)	Days taken for new sprouting	Length of shoot (cm)
H <sub>1</sub>	Vermicompost	58.46 (49.85)	13.31	3.37
H <sub>2</sub>	Soil	47.55 (43.57)	14.24	2.83
H <sub>3</sub>	Coco peat	41.07 (39.84)	14.68	2.55
H <sub>4</sub>	Vermicompost + Soil (1:1 v/v)	59.51 (50.46)	13.19	3.42
H <sub>5</sub>	FYM + soil + sand (1:1:1 v/v)	70.86 (57.30)	10.86	4.15
S.Em. ±	-	0.05	0.06	0.01
C.D. at 5 %	-	0.16	0.20	0.04
C.V. %	-	0.22	1.02	0.89

\*Figure in parentheses are arcsine transformed value

Fig. 11: Effect of different potting mixtures on survival of *in vitro* plantlet of rough lemon



A



3 weeks old

B



3 weeks old

A - Treatment R2 -  $1/2$  MS + 1.0 mg/l IBA + 1.0 mg/l NAA

B - Treatment R4 - Full MS + 1.0 mg/l IBA + 1.0 mg/l NAA

**Plate VI : Effect of strength of medium on *in vitro* rooting in shoots of rough lemon**



**H5 : FYM + Soil + Sand (1:1:1 v/v)**



**[A] (a<sub>1</sub>) : Individual plantlet in net house**

**Plate VII : Acclimatization of plantlets**

**(2 week old plantlets)**

#### 4.5.2 Effect of different climatic conditions on acclimatization of *in vitro* raised plantlets of rough lemon

The survival rate of plantlet was significantly influenced by different climatic conditions Table 4.10. Maximum survival per cent of plantlet (72.55%) with minimum days taken for new sprouting (11.35 days) and maximum length of shoots (4.37 cm) was observed in treatment individual plants in net house [A] ( $a_1$ ), which was followed by group plants in net house [B] ( $b_1$ ) (Fig. 12, Plate VII).

#### 4.6 Protocol developed for micropropagation in rough lemon

The protocol developed in the present study on "Micropropagation in rough lemon (*Citrus jambhiri* Lush.)" using shoot tip and nodal segment explants from juvenile stage plants is given below in working laboratory (Plate VIII).

##### A) In working laboratory

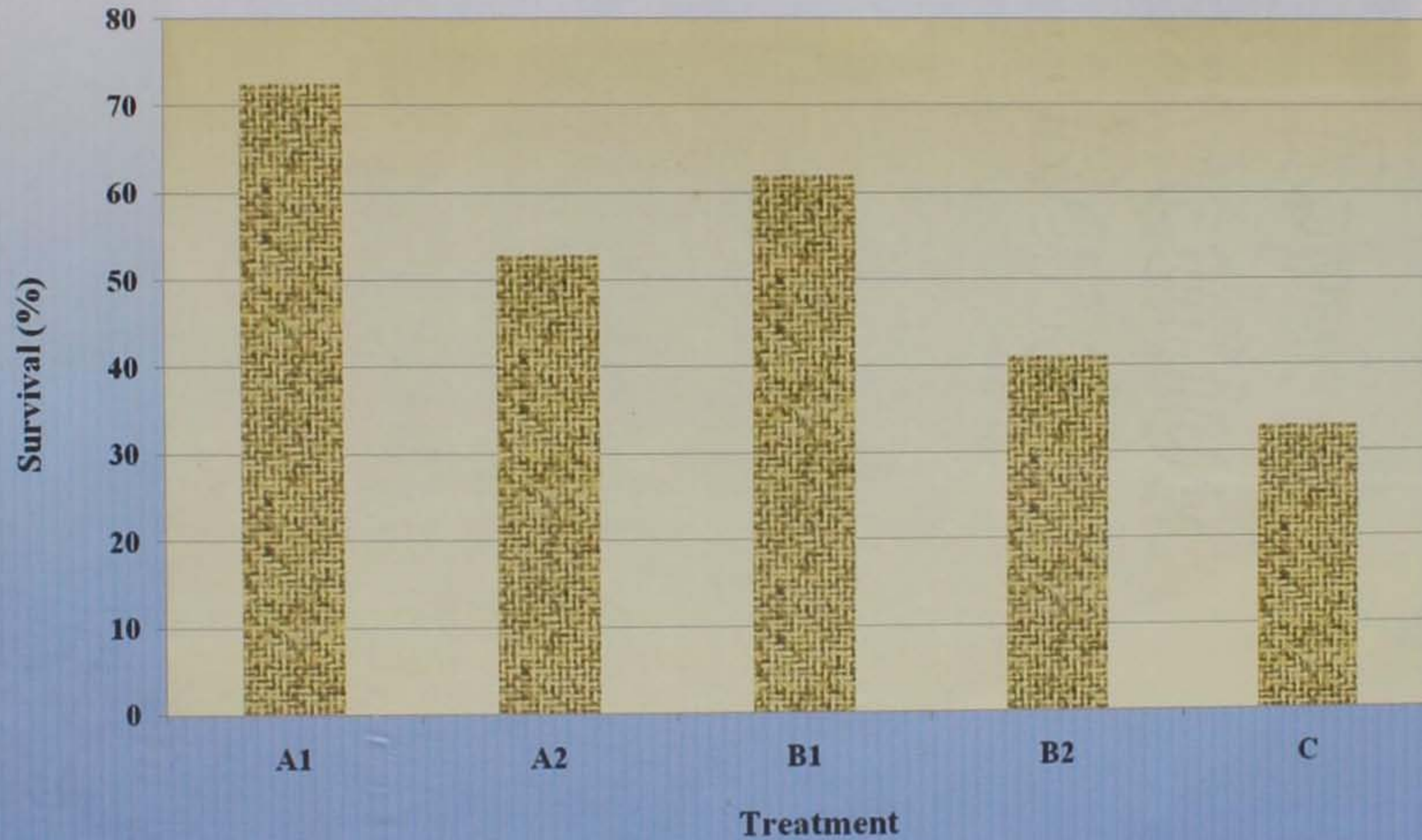
1. Shoot tip and nodal segment explants were collected from 3-4 weeks old juvenile stage plants of rough lemon. Remove the leaves with stainless steel knife then, washed thoroughly in running tap water for 2-3 hours to remove dirt. Treat the explants with 0.05 per cent Bavistin and 0.01 per cent Streptocyclin for 2 hours.
2. Remove the solution and treat explants with 10 per cent solution of detergent (Teepol) for 10 minutes.

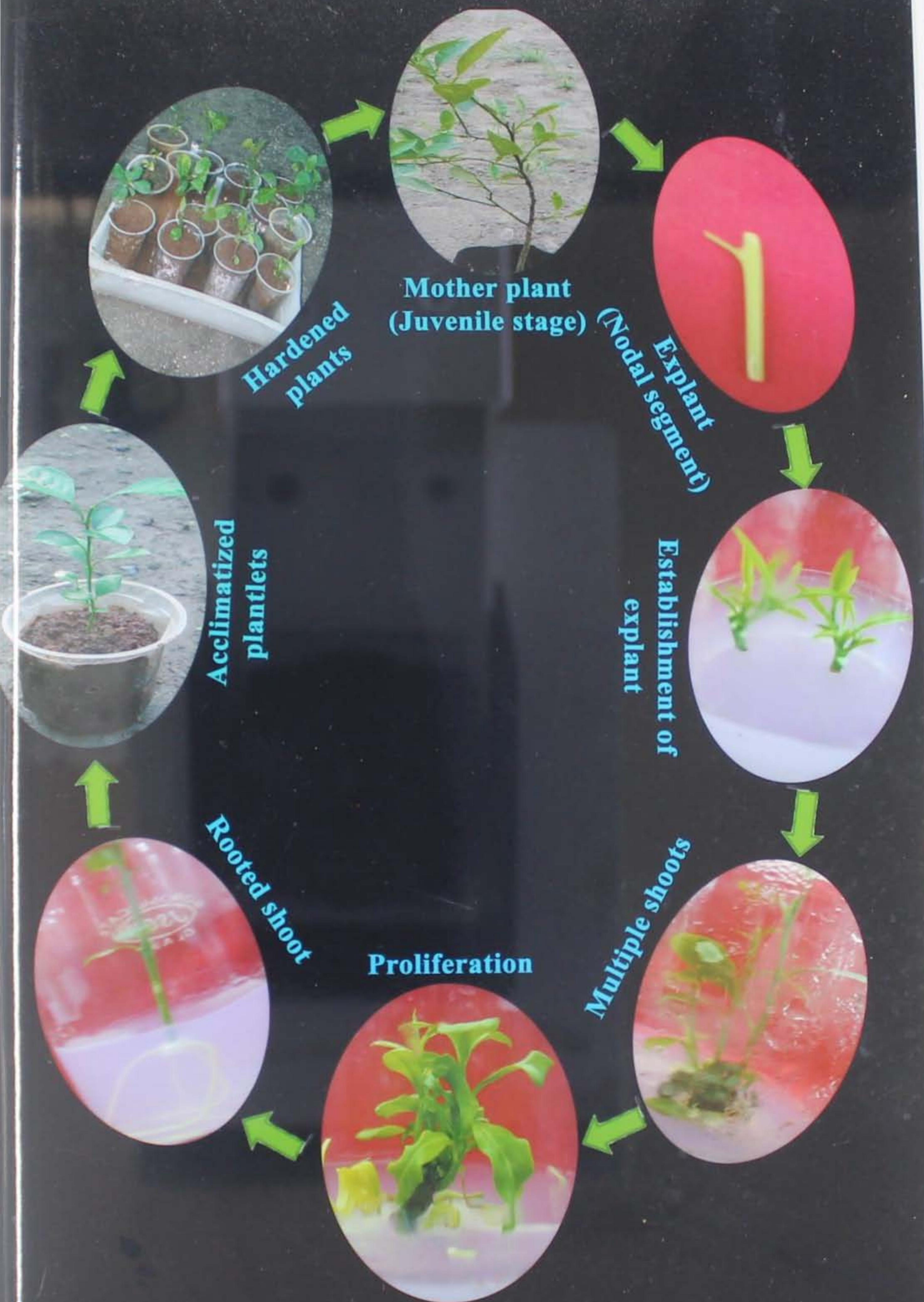
**Table-4.10:Effect of different climatic control conditions on acclimatization of *in vitro* raised plantlets of rough lemon**

Climatic conditions	Survival of plantlets (%)	Days taken for new sprouting	Length of shoot (cm)
<b>A. Individual</b>			
a <sub>1</sub> . Net house	72.55 (58.38)	11.35	4.37
a <sub>2</sub> . Air condition	52.97 (46.68)	13.68	3.35
<b>B. Group</b>			
b <sub>1</sub> . Net house	62.00 (51.92)	13.31	3.53
b <sub>2</sub> . Air condition	41.21 (39.92)	14.24	2.74
<b>C. Low tunnel net house</b>	33.01 (35.04)	14.94	2.02
S.Em. ±	0.26	0.04	0.01
C.D. at 5 %	0.80	0.14	0.03
C.V. %	1.15	0.73	0.65

\*Figure in parentheses are arcsine transformed value

Fig. 12: Effect of climate control on survival of *in vitro* plantlets of rough lemon





**Plate VIII : Protocol for micropropagation in rough lemon**

3. Remove all the trace of detergent by repeated washing in double distilled glass water.

**B) In sterile room (Laminar air flow chamber)**

1. Surface sterilize the shoot tip and nodal segment explants collected from 3-4 weeks old juvenile stage plants of rough lemon using 0.1 per cent  $\text{HgCl}_2$  for 5 minutes. Remove traces of  $\text{HgCl}_2$  by thorough rinsing with doubled distilled glass water.

2. Inoculate the explants in culture bottles containing MS medium gelled with 0.8 per cent agar and supplemented with 1 mg/l BAP + 0.5 mg/l NAA; adjust the pH 5.7 and 30 gm/l sucrose. The basal end of the explants should be inserted in the medium in such a way that the nodal segment of explants remains above the surface of the medium.

**C) Incubation**

1. Incubate the culture bottles in an air condition culture room at  $26 \pm 2$  °C temperature with relative humidity  $55 \pm 5$  per cent and 16 hours photoperiod in which, light is supplied with 40-W cool white fluorescent tubes at intensity of approximately 3000 lux.

2. After four weeks of incubation, transfer the *in vitro* established explants for further multiplication in MS medium supplemented with 1.0 mg/l BAP + 500 mg/l ME.

3. Separate the individual shoot and then, transfer for rooting on full strength MS medium containing 1.0 mg/l IBA + 1.0 mg/l NAA.

4. Approximately after four weeks, the well rooted plantlets are ready for acclimatization.

#### D) Acclimatization

1. Carefully take out the plantlets from culture bottles, remove the adhering agar from roots by thorough washing in tap water.
2. Transplant well developed *in vitro* grown plantlets for hardening to plastic glass containing FYM:soil:sand (1:1:1v/v). Drench potting mixture with 0.05 per cent bavistin at weekly interval.
3. Acclimatize the plantlets individually under net house (72.55 per cent) condition by covering them with plastic glass and remove gradually for 2 hours then, gradually increased the interval in a day for 15 days. After 15 days glass are removed and plants are kept in natural condition.

# V. DISCUSSION



## DISCUSSION



## V. DISCUSSION

The present investigation was done for protocol development of "Micropropagation in rough lemon (*Citrus jambhiri* Lush.)". The investigations were carried out at the Department of Biotechnology, ASPEE College of Horticulture and Forestry, Navsari Agricultural University, Navsari, Gujarat during 2008-2010.

Micropropagation in plant is a powerful and potential tool for producing the large number of propagules of desired characteristics at a competitive price, as and when the customer or farmer requires. Large scale propagation of elite clones from hybrids or specific parental lines through commercial micropropagation holds the promise of alleviating problems of shortage of healthy seeds and planting material and lack of disease resistance clones which have been affecting the economy of various countries of the world. At present, Plant Tissue Culture technique is being successfully employed for rapid production of uniform and superior quality planting material. Rapid and large scale clonal production of many horticultural species is now possible, outstanding example being large scale cloning of orchids, banana, rose, anthurium, gerbera, carnation, oil palm, date palm, etc. producing unlimited number of quality planting material. A wide range of tissues can be used as explants from different crops as source for micropropagation. The regeneration can take place as extension or proliferation of explants, direct organogenesis or somatic embryogenesis.

Rough lemon is commercially propagated by seeds, which show considerable variation in the performance of gametic seedlings raised, using seeds of different trees and that they do not exhibit true to type, although, the seedlings are most desirable as rootstock in citrus. Some of these vegetative propagation methods like air layering, cutting etc. are impractical in practice. Number of plants that could be obtained through these methods are less and slow process. So, clonal propagation is an urgent necessity for improvement in yield and quality of citrus. The technique of tissue culture has made clonal propagation a possibility as reported by many workers in rough lemon (Ali and Mirza, 2006; Murkute *et al.*, 2008; Altaf *et al.*, 2008; Kour *et al.*, 2008; and Sharma *et al.*, 2009;) and in citrus (Desai *et al.*, 1996; Paudyal and Haq, 2000; Khayri and Bahrany, 2001; Filho *et al.*, 2001; Kobayashi *et al.*, 2003; Gill and Gosal, 2004; Devy *et al.*, 2004; Usman *et al.*, 2005; Agisimanto *et al.*, 2005; Altaf, 2006; Rathore *et al.*, 2007; Murkute *et al.*, 2008; Miah *et al.*, 2008; Tornero *et al.*, 2009; Sen and Dhawan, 2009; Almeida *et al.*, 2002; Khayri and Aziz, 2001; Kumar *et al.*, 2001; Silva *et al.*, 2006).

In micropropagation, the organs and tissues are carried out through a sequence of steps in which, differential cultural and environmental conditions are provided. Murashige (1974) grouped this sequence of steps into different stages as under.

Stage: I : Explant establishment including selection of  
mother plant and collection of explants

Stage: II : Rapid multiplication of shoots through increased axillary branching / somatic organogenesis

Stage: III : *In vitro* rooting

Stage: IV : Acclimatization and planting out

However, in certain cases, the large number of shoots formed may be more but the growth of individual shoots remain arrested. This necessitates an additional *in vitro* step of shoot elongation before they can be tested for rooting Lakshmi Sita *et al.*, (1979) and Parthasarathy and Nagaraju (1996).

## 5 Culture establishment

### 5.1 Surface sterilization of explants

The explants collected from field grown stock harbor fungi and bacteria in addition to adhered soil and dust particles. Thus, it is necessary to go through an effective surface sterilization procedure for the explants before culturing. The results on effectiveness of surface sterilization of shoot tip and nodal segment explants from (3-4 weeks) old juvenile stage grown plants are presented in chapter IV, (section 4.1.1, Table 4.1 and Table 4.2, respectively). Treating nodal segment and shoot tip explants with  $\text{HgCl}_2$  (0.1%) for 5 minutes were found to be very effective for sterilization and survival of explants. Kour *et al.*, (2008) reported surface sterilization successfully made to nodal segment explants of rough lemon plant by using 0.1%  $\text{HgCl}_2$  for 8 minutes. Rathore *et al.*, (2007) was also able to sterilize effectively nodal segment explants of *Citrus limon* plant by using

alone 0.1% HgCl<sub>2</sub> for 3-5 minutes. Altaf (2006) sterilized successfully axillary bud explants of citrus cv. kinnow by using 0.1% HgCl<sub>2</sub> for 10 minutes. Usman *et al.*, (2005) reported surface sterilizing of nodal segment explants of citrus cv. Kinnow, Sweet lime and Succari successfully, by using 0.1% HgCl<sub>2</sub> for 3 minutes. Sharma *et al.*, (2009) observed surface sterilizing of shoot tip and nodal segment explants of citrus rootstocks viz. Pectinifera, Troyer citrange, rough lemon and Cleopatra mandarin effectively by using 0.1% HgCl<sub>2</sub> for 8 minutes.

It is very clear from present investigation that although, the best surface sterilant treatment for shoot tip and nodal segment both explants was same, response for shoot tip and nodal segment explants was different. This may be possible because the difference in nature and intensity of contaminants present at different physiological stage from where, explants were collected. The research work of our department on furcraea (Patel, 2006), anthurium (Sunila Kumari, 2006), citrus (Desai, 1994), guava (Kadam, 2010), pomegranate and (Singh, 2010) also supported this view where, all together a new procedure is reported or effective sterilization of explants suitable to local condition.

## **5.2 Establishment of shoot tip and nodal segment explants in different media**

Medium is the formulation of inorganic salts and organic compounds with an energy source necessary for nutrition of plant cultures. As such no single medium can be suggested as being entirely satisfactory for all types of tissues and organs.

There are many formulations of media which have been used in *in vitro* studies. In the present investigation, three different basal media (MS, B5 and WPM medium) were tried for establishment of culture for rough lemon. The results obtained on the culture establishment of rough lemon are presented in the chapter IV (section 4.2, Table 4.3). Shoot tip and nodal segment explants at juvenile stage of plants exhibited better response for establishment and growth of shoots in MS medium as compared to B5 and WPM.

Many workers viz., Laskar *et al.*, (2009) stated that regenerative calli and rooting were induced on MS medium, while shoots were regenerated on WPM medium in *Citrus indica* Tanaka. Parthasarathy, (2004); Sharma *et al.*, (2009) obtained regeneration of plantlets of rough lemon, Cleopatra mandarin, Pectinifera and Troyer citrange by using the MS medium. While, Almeida *et al.*, (2002) regenerated plantlets through *in vitro* organogenesis in Murashige and Tucker (MT) medium in *Citrus sinensis* and *Citrus limonia*. Usman *et al.*, (2005) reported maximum callus induction in MT medium. Silva *et al.*, (2006) obtained highest callusing on DBA3 culture media. Different workers recommended different culture media formation of same/or different explants for citrus. However, Altaf *et al.*, (2009); Altaf *et al.*, (2008); Filho *et al.*, (2001); Altaf (2006); Paudyal and Haq, (2000); Sharma *et al.*, (2009); Almeida *et al.*, (2002); Parthasarathy *et al.*, (2001); Kumar *et al.*, (2001); Parthasarathy and Nagaraju *et al.*, (1996); all these workers registries that MS medium was best for establishment of citrus. The research work of our laboratory on guava

(Kadam, 2010), and pomegranate (Singh, 2010) also supported this view. This may be due to response of different genotypes.

Among all three media MS medium (Table 3.1) showed best results because of rich in salts, ammonium concentration in addition to higher nitrate and calcium level when compared to other media. Gamborg's medium (Table 3.2) other hand, contains more sulphate in the form of ammonium sulphate which is not desirable, because a medium with high ammonium sulphate has a greater tendency to become acidic. Similarly, Mengel and Kirkbly (1982) also observed the growth to be affected adversely in potato with concentration of sulphate exceeding  $5 \mu M$  per litre.

### 5.3 Shoot multiplication

The basic phenomenon involved in explants establishment, multiplication and subsequent plantlet formation *in vitro* are reported to be due to the action of plant hormones. The requirement of plant growth regulators varies with the species as well as the mode of development desired i.e. organogenesis or embryogenesis. However, there is one universally accepted concept that morphogenetic response is regulated by the relative concentrations of auxins and cytokinins in the medium as pointed out by Skoog and Miller (1957) and it should serve as guidelines in plant tissue culture work. It was observed that stunted shoot growth with increasing concentration of BAP. This may be due to the fact that cytokinins at higher concentrations led to oxidation of polyphenols at a rapid rate. The role of cytokinins in shoot organogenesis is well established by Skoog and Miller (1957) and

Evans *et al.*, (1981). Cytokinins are used in combination with auxin for initiation as well as maintenance of shoot and bud development. During the present investigation cytokinins were found to be essential for establishment (Parthasarathy *et al.*, 2001; Kour *et al.*, 2008; Desai *et al.*, 1996; Silva *et al.*, 2006; Altaf *et al.*, 2008; and Sharma *et al.*, 2009).

Since, the multiplication of shoot is a major criteria successful commercial tissue culture propagation. Shoots regeneration were tested for multiplication with different treatments of BAP and combination with or without malt extract (chapter III, section 3.5.4). The data on multiplication rate of shoots are presented in chapter IV, (section 4.3; Table 4.4) shows that maximum multiplication rate of shoots is obtained on MS medium supplemented with 1 mg/l BAP + 500 mg/l ME. In general, the addition of malt extract with BAP in the medium has exhibited better response for multiple shoot and growth of culture. Growth of culture is increased even at lower level of BAP in present of ME, however, higher levels of BAP inhibits the growth. These results are in agreement to those of Kour *et al.*, 2008; Baruah *et al.*, 1996; Bowman, 1994, Usman, *et al.*, 2005; and Desai *et al.*, (1996) had also reported promontory effect of malt extract on shoot multiplication and growth of shoots in citrus. Malt extract is an ideal supplement and most obligatory component as earlier observed by Kochba *et al.*, (1982) and Das *et al.*, (2000). Miah *et al.*, (2008) reported that 1.0 mg/l BAP was found superior to 0.5 mg/l Kn shoot multiplication in citrus. These results are in close conformity with the findings of Begum *et al.*, (2001) and

Otoni and Teixeira (1991) also noted that BAP is superior to other hormones for shoot proliferation from nodal segment explants in citrus. Similarly, Sharma *et al.*, (2009) also observed maximum proliferation in different rootstocks was recorded in treatment 1.0 mg/l BAP for nodal segment explants maximum proliferation in different rootstocks of citrus species was recorded in treatment 1.0 mg/l BAP for nodal segment explants.

The result on shoot multiplication with serial sub-culturing of nodal segment explants given in chapter IV (section 4.3; Table 4.4) apparently indicated that regeneration of multiple shoots at the rate of 30 folds, besides the growth of shoots was observed in treatment M<sub>4</sub> (MS + 1.0 mg/l BAP + 500 mg/l ME) up to four subcultures.

The increased trend of multiple shoots and growth of shoot was also reported by Desai *et al.*, 1996, in citrus, Kadam, 2010 in guava and Patel, 2006 in furcraea. The effect of cytokinin on axillary bud brusting and multiplication shoot production have been demonstrated by Murashige (1974). Cytokinin which could be used to release apical dominance in the lateral buds. The dormant buds of vegetative apex are stimulated to grow and elongated into presence of cytokinin and also produce new axes.

### 5.3.1 Effect of sucrose on shoot proliferation

Each tissue has its own requirement and sometime less defined adjuvant play a part in organogenesis. The phenomenon is dependent upon physiological factors such as source of the explants, genotypes and age, endogenous hormone levels and

various physical factors such as quality and quantity of light, temperature, photoperiod, pH and concentration of carbohydrates. chapter IV (Section 4.4, 4.4.1)

The culture growth is influence by the source of carbon energy. Sucrose is the most widely accepted source of carbon energy in most of *in vitro* studies. Sucrose is usually the carbohydrates of choice in most of the cell culture media. Attempts were made to find out optimum concentration of sucrose as a carbon source for *in vitro* proliferation of culture. It is seen from the results given in chapter IV (section 4.4.1, Table 4.5) that among the six levels of sucrose tested, sucrose 3 per cent gave high shoot proliferation. Several workers have stressed the importance and concentration of a particular source of carbohydrate for getting desirable response like Shinde (2008) in grape and Patel (2008) in papaya, achieved better response at 3 per cent sucrose. Kumar and Kumar (1998) reported that level of sucrose was maintained between 2 to 3 per cent in majority of medium. However, optimum response to growth in citrus was obtained at 3 per cent sucrose level in the medium. The requirement may be related to the specific carbohydrates metabolism through which, water relations and endogenous phytohormones are regulated.

### 5.3.2 Effect of pH on shoot proliferation

The result obtained on influence of pH on multiplication and growth of shoots is presented in chapter IV (Section 4.4.2; Table 4.6). Though, the importance of pH in tissue

culture studies was reported by Gauthert as early as 1947 by observing drift in pH during the growth of a culture. Tissue culture of majority of fruit crops are grown satisfactory at pH 5.6 to 5.8 (Conger, 1987 and Skirvin, 1981). In the present investigation, maximum proliferation and growth of shoots were observed at pH 5.7, which decreased at lower as well as higher pH value. This work is supported by earlier work carried out in our laboratory in guava (Kadam, 2010); pomegranate (Singh, 2010); papaya (Patel, 2008) and in grape (Shinde, 2008). The effects different of pH of culture medium on the growth of *in vitro* culture as obtained in present investigation may be due to the well known effect of pH on the availability of nutrients from the medium. Such effect of pH on the availability of nutrients has been reported by many workers using sand water culture technique (Wallihan *et al.*, 1997 and Hurley *et al.*, 1981). According to Street (1966), the ambient pH could be desired for absorption of various nitrogen sources: pH growth response curves indicated that nitrate N when pH is acidic (4.7-4.9 pH approx.), ammonical N at neutral pH (7.0-7.2 pH approx.) and nitrate at pH 5.0 to 6.0 supported maximum growth. The result obtained in present investigation may be considered in presence of nutrients for growth; growth at pH 5.8 suggesting favorable effect of nitrate N rather than ammonical N.

### **5.3.3 Effect of Light intensity on *in vitro* shoot proliferation**

Light is an important factor which influencing the growth of culture in micropropagation. It is the ultimate substrate

for photosynthetic energy conversion. Light intensity exhibited more effective role on the multiplication and growth of shoots by earlier workers in pomegranate, Singh (2010); Singh and Khawale (2006); and Sharon and Sinha (2000). The results clearly indicate that present findings in Chapter IV (section 4.4.3, Table 4.4) highest rate of multiplication and shoot growth were recorded at 3000 lux light intensity). Light intensity in the range of 1000 - 3000 lux shows gradually increasing in the shoot growth. These findings are supported by earlier workers carried out in our laboratory by Kadam (2010) in guava; Patel (2008) in papaya and Shinde (2008) in grape. This result may be due to the interaction between light intensity and internal factors which directly affect plant growth. Thus, suitable light intensity and duration of light are very important factors for better *in vitro* plantlet production of rough lemon.

#### 5.4 *In vitro* rooting response in shoots

MS medium at half strength and full strength and white medium with auxin was found to favour root induction from the *in vitro* regenerated individual shoots. The total omission of cytokinin in the medium did not affect root differentiation and induction. Even then, the role of cytokinin in rooting could be overlooked. The exogenous auxin might have combined with endogenous level of cytokinin resulting in the induction of roots.

Addition of IBA and NAA to the culture medium enhanced rooting. The root induction ability of auxin is universally accepted. These results are supported by earlier workers Miah

*et al.*, (2008); Kumar *et al.*, (2001); Sen and Dhawan, (2009); Sharma *et al.*, (2009); and Altaf (2006) in many citrus species and varieties. Maximum rooting and average number of roots were found at full strength in MS medium 1.0 mg/l IBA + 1.0 mg/l NAA. Chapter IV (section 4.5, Table 4.8), further increase same concentration of the auxin for the rooting percentage and also number of roots. These results are in accordance with the finding of those of Tapati *et al.*, (1995) and Murkute *et al.*, (2008) in citrus. Kour *et al.*, (2008) reported the half strength media for best rooting treatment in rough lemon.

In general, auxins like IBA and NAA were used widely for inducing the roots in *in vitro* raised shoots by Bhojwani and Razdan (1992). Similarly, Prasad (1999) stated that IBA, IAA as well as NAA were found effective for induction of roots. The data on rooting response to different levels of IBA and IAA supplemented in half and full strength of MS and White medium is illustrated in chapter IV (section 4.5, Table 4.8). Maximum response of rooting is observed in full strength MS medium supplemented with 1.0 mg/l IBA + 1.0 mg/l NAA. The efficacy of IBA was increased in present of NAA for rooting. The result of rooting in, *in vitro* raised shoots of citrus observed at optimum nutrient status and with the combination of IBA and NAA of NAA.

Similar results were also reported by Kour *et al.*, (2008) in which half strength MS medium supplemented with 1.0 mg/l IBA + 1.0 mg/l NAA resulted better rooting than IBA and NAA used alone. Thus, efficacy of IBA was enhanced in present of NAA

for rooting rough lemon. The root induction ability of auxin is universally accepted. The present findings are supported by Murkute *et al.*, (2008) those reported the percentage of rooting and roots per shoot were increased markedly when NAA was used in addition to the IBA in medium in *Citrus jambhiri* and *Citrus karna*. Similar results were also reported by earlier workers Tornero *et al.*, (2009) in *Citrus limon*, Kumar *et al.*, (2001) in sweet orange.

### 5.5 Hardening of the *in vitro* raised plantlets

Finally, goal of *in vitro* plant propagation is to obtain a large scale of plantlets in a short period to the natural environment with high survival rate. Hardening was done when the *in vitro* raised plantlets had 3-4 well developed roots.

In present investigation, the obtained on survival rate of plantlets as influenced by potting mixtures is depicted in chapter IV (Section 4.5.1; Table 4.9). The potting mixture containing FYM:soil:sand (1:1:1v/v) was found to be the most suitable for better growth and survival of plantlets followed by Vermicompost:soil (1:1v/v). Physical, chemical and biological properties of potting mixture are important in the establishment of *in vitro* produced plantlets. Hence, FYM:soil:sand resulted to giving grip for roots and retention of optimum moisture with adequate aeration to provide plantlets. The lowest percentage was recorded when the regenerated plantlets were transferred to community pots containing coco peat as the substrate. It may be due to high water holding which did not favour proper *ex vitro*

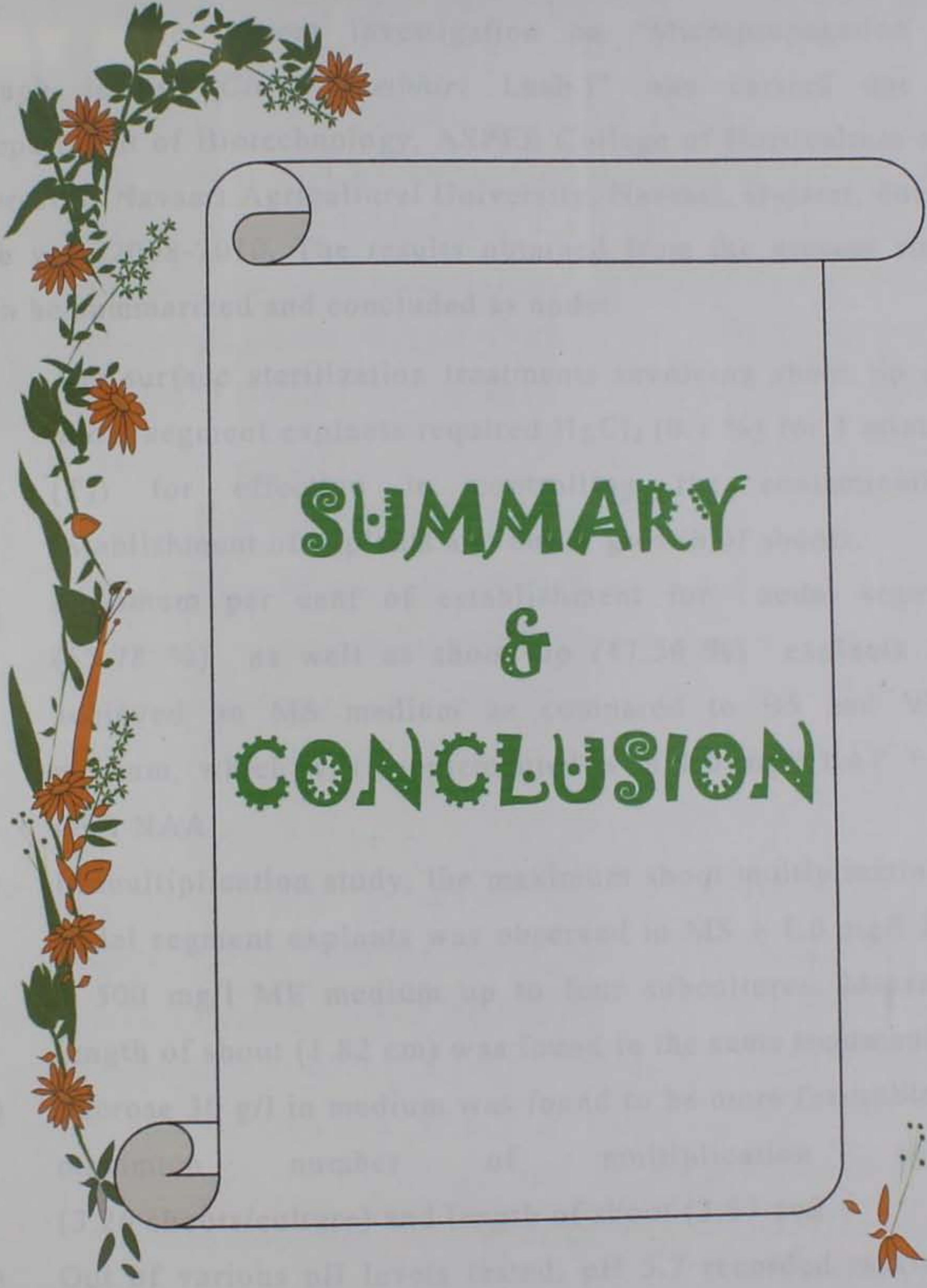
growth of the plantlets. Many workers studied on different potting mixture for better establishment of rough lemon plantlets. Present results are in conformity with earlier workers those of Sharma *et al.*, (2009) in citrus rootstocks, Kour *et al.*, (2008) in rough lemon, both reported the most suitable FYM:soil:sand (1:1:1v/v) potting mixture for better growth and survival of plantlets. Rathore *et al.*, (2007) reported better establishment in polybags containing a mixture of organic manure, garden soil and sand (1:1:1 v/v) in *Citrus limon*. Similarly, results were also reported by Normah *et al.*, (1997) in *Citrus halimil* and Sen and Dhawan, (2009) in citrus for maximum survival of hardening plants.

Hardening the *in vitro* raised plantlets, so as to make them to adopt the natural environment, it is a critical process as these plants are very delicate and generally lack epicuticular development (Murashige, 1979) leading to desiccation and wilting. On transplanting, excessive water loss from the plantlets was recorded which was attributed to the improper critical and slowness of stomatal response to water stress (Brainerd and Fuchigami, 1981 and Fabbri *et al.*, 1984). Therefore, a period of humidity in acclimatization was considered necessary for the newly transferred plantlets to adapt to the natural environment, during which the plantlets undergo a morphological and physiological adoption enabling them to develop typical terrestrial plant water control mechanism (Grout and Aston, 1977 and Sutter *et al.*, 1985). The results obtained in our study on effect of climate control on survival and growth of plantlets is presented in chapter IV, (Section 4.5.2; Table 4.10). Maximum per cent plantlets

(72.55%) survived when they covered by plastic glass and kept individually under net house. Method of covering the newly transferred plantlets with plastic glass cover allowed by misting in greenhouse, poly house, and net house for initial period and subsequently removing the cover in a gradual process was successfully adapted by number of earlier workers for hardening the plantlets (Murashige, 1974; Rajeevan and Pandey, 1986; Rout *et al.*, 1989; Jyothi, 1991 and Hazarika *et al.*, 1995). According to them, plantlets developed their stomatal control mechanism during this period.

The fore going discussion clearly leads to the possibilities of efficient micropropagation in rough lemon nodal segment for proliferation. However, before the protocol developed is being utilized as commercial method, there is need to examine the performance of *in vitro* propagules in the field.

## VI. SUMMARY AND CONCLUSION

A decorative scroll frame with a floral border on the left side and a small floral sprig at the bottom right. The scroll is unrolled, revealing the title text.

# SUMMARY & CONCLUSION

## VI. SUMMARY AND CONCLUSION

The present investigation on “Micropropagation in rough lemon (*Citrus jambhiri* Lush.)” was carried out at Department of Biotechnology, ASPEE College of Horticulture and Forestry, Navsari Agricultural University, Navsari, Gujarat, during the year 2008-2010. The results obtained from the present study can be summarized and concluded as under.

- 1) The surface sterilization treatments involving shoot tip and nodal segment explants required  $\text{HgCl}_2$  (0.1 %) for 5 minutes ( $T_3$ ) for effective in controlling the contamination, establishment of explants and better growth of shoots.
- 2) Maximum per cent of establishment for nodal segment (65.78 %) as well as shoot tip (47.56 %) explants was achieved on MS medium as compared to B5 and WPM medium, which was supplemented with 1.0 mg/l BAP + 0.5 mg/l NAA.
- 3) In multiplication study, the maximum shoot multiplication in nodal segment explants was observed in MS + 1.0 mg/l BAP + 500 mg/l ME medium up to four subcultures. Maximum length of shoot (1.82 cm) was found in the same treatment.
- 4) Sucrose 30 g/l in medium was found to be more favorable for maximum number of multiplication shoots (3.26 shoots/culture) and length of shoot (2.57 cm).
- 5) Out of various pH levels tested, pH 5.7 recorded maximum numbers of shoots (3.36 shoots/culture) and maximum length of shoot (2.25 cm) followed by pH 6.0.

- 6) High light intensity (3000 lux) was found to be most suitable for proper growth and regeneration of shoots. Low light intensity (1000 lux) resulted in stunted growth.
- 7) Rooting treatment consisting of full strength MS medium supplemented with 1.0 IBA + 1.0 mg/l NAA was found to be the best for early induction of roots (18.19 days), maximum number of roots/shoot (3.43) and length of root (4.53 cm) also as compared to half strength MS rooting treatment. Efficacy of IBA for rooting was increased in present of NAA.
- 8) FYM: soil: sand (1:1:1v/v) was found better potting mixture for maximum survival of plantlet (70.86%).
- 9) The *in vitro* plantlets obtained were acclimatized successfully in climate control treatment [A] (a<sub>1</sub>) (individually in net house condition) with individual plastic cup covered to maintain high humidity.

## CONCLUSION

The present investigation on "Micropropagation in rough lemon (*Citrus jambhiri* Lush.)" has clearly demonstrated its potentiality for rapid clonal propagation. It was estimated that using the present protocol of micropropagation, large number of plantlets can be produced in a year starting from single nodal segment explants. This protocol may be prepared commercially viable provided some work is intensified to increase the vigour and growth of the plantlets in the initial stage after transplanting and testing the plantlets in field conditions. Besides some scaling up techniques for large scale production. The results obtained would

be very much useful for mass multiplication of rough lemon (*Citrus jambhiri* Lush.) using nodal segment explants under local condition and provide guidelines for setting commercial unit for propagation of rough lemon.



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## REFERENCES

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\* Original not seen



# APPENDIX



# CERTIFICATE

This is to certify that I have no objection for supplying to any scientist only one copy or any part of this thesis at a time through reprographic process, if necessary for rendering reference services in a library or documentation centre.

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