IN VITRO MULTIPLE SHOOT INDUCTION FROM NODAL EXPLANT IN SEEDLESS LIME (*Citrus aurantifolia* Swingle)

By

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B. Sc. (Agril. Biotechnology)

MASTER OF SCIENCE
(AGRICULTURE)
IN
AGRICULTURAL BIOTECHNOLOGY

VILASRAO DESHMUKH COLLEGE OF AGRICULTURAL BIOTECHNOLOGY, LATUR
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PARBHANI - 431 402 (M.S.), INDIA

2018

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(Reg. No. 2016/BT/08/ML)

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DISSERTATION

Submitted to the

Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani

In partial fulfillment of the requirements

for the Degree of

MASTER OF SCIENCE
(AGRICULTURE)

IN

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~ 2018 ~

Dedicated to

My Beloved Father

Late Shri. Dashrath Jetnaware

&

V D C O A B, Latur

The most beautiful thing

in this world is to see my parents smiling,

and knowing that I am the reason

behind that smile

CANDIDATE'S DECLARATION

I hereby declare that this dissertation or part thereof has not been previously submitted by me for a degree of any other Institution or University

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C E R T I F I C A T E - I

This is to certify that **Mr.JETNAWARE PANDURANG DASHRATH** has satisfactorily prosecuted his course and research for period of not less than four semesters and that the dissertation entitled **"*IN VITRO* MULTIPLE SHOOT INDUCTION FROM NODAL EXPLANT IN SEEDLESS LIME (*Citrus aurantifolia* Swingle)"** submitted by him is the result of original research work and is of sufficiently high standard that warrants its presentation to the examination.

I also certify that the dissertation or part thereof has not been previously submitted by him for a degree of any university.

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Date: / /2018 **Research Guide**

C E R T I F I C A T E – I I

This is to certify that the dissertation entitled **"*IN VITRO* MULTIPLE SHOOT INDUCTION FROM NODAL EXPLANT IN SEEDLESS LIME (*Citrus aurantifolia* Swingle)"** submitted by **Mr. JETNAWARE PANDURANG DASHRATH (Reg. No. 2016/BT/08/ML)** to the Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani in partial fulfillment of the requirement for the degree of **MASTER OF SCIENCE (Agriculture)** in the subject of **AGRICULTURAL BIOTECHNOLOGY** has been approved by the student's advisory committee after oral examination in collaboration with the external examiner.

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ACKNOWLEDGEMENT

Writing this thesis has been fascinating and extremely rewarding. I would like to thank a number of people who have contributed to the final result in many different ways:

To commence with, I pay my obeisance to GOD, the almighty to have bestowed upon me good health, courage, inspiration, zeal and the light. After GOD, I express my sincere and deepest gratitude to my research guide ***Prof. K. M. Sharma****, (Assistant Professor- Dept. of Post-Harvest & Food Biotechnology, VDCOAB, Latur) and Incharge, Tissue Culture Project, VNMKV, Parbhani who ploughed through several preliminary versions of my text, making critical suggestions and posing challenging questions. His expertise, invaluable guidance, constant encouragement, affectionate attitude, understanding, patience and healthy criticism added considerably to my experience. Without his continual inspiration, it would have not been possible to complete this study.*

I owe my special thanks to my guru, my inspiration & motivation ***Prof. HEMANT PATIL*** *honorable Associate Dean & Principal, VDCOAB, Latur for giving me an opportunity to work in PTC Lab, Parbhani as well as I would like to express my sincere gratitude to my advisory committee member* ***Dr. A. A. Bharose*** *(Associate Professor- Dept. of Plant Biotechnology),* ***Dr. A. M. Dethe*** *(Associate Professor- Dept. of Crop Science),* ***Dr. R. L. Chavhan*** *(Assistant Professor- Dept. of Plant Biotechnology) who offered me guidance and support all along the completion of the project.*

I am highly thankful to ***Dr. B. Venketeshwarulu*** *and* ***Dr. A. S. Dhavan*** *honorable Vice-Chancellor, Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani for their constant support in the form of providing all the infrastructure of Lab and College. I extend my most sincere gratitude to my college professors* ***Dr. S. S. Shende****,* ***Prof. B. N. Aglave****,* ***Dr. Y. S. Bhagat****,* ***Dr. V. R. Hinge****,* ***Prof. R. N. Dhawale*** *,* ***Dr. M. S. Dudhare, Dr. V. D. Surve*** *(PG Incharge) and* ***Dr. U. S. Kadam*** *as well as librarians Mr. Gaikwad sir, Mr. Ugile sir, Mr. Raut sir, Mr. Kamble sir, Mrs. Amabatwad madam, Mr. Bhagawan Khating & technical-non technical team for their valuable suggestions that have been very helpful for this study.*

It is a matter of great pleasure to express my wishes and special genuine thanks to ***Dr. Kausik Chattopadhay*** *(Associate Professor, Biological Sciences- IISER, Mohali) and* ***Dr. B. M. Kalalbandi*** *(Associate Professor, Dept. of Horticulture, Parbhani) for keeping me on his kind attention, advise, support, help and companionship throughout my dissertation work.*

I am very much thankful to ***Mr. U. K. Sutar*** *and* ***Mr. R. N. Wadikar*** *who guide me and help during my project work as a Lab Guide. I give special thanks to Mr. V. S. Bhosale, Mr. V. J. Bonde, Mr. V. T. Boratkar, Mr. D. S. Deshmukh, Suresh Shinde, Kale Mama and all staff of PTC lab, Parbbhani for giving me their valuable time and guidance.*

It is great pleasure to acknowledge my dearest ***seniors*** *Namdev Ade Mama, Arun Kachave, Suresh Wadkute, Deepak Waghmare,* ***Prakash Thakare, Sunil Lahankar****, Kiran Chormule, Kanishk Divekar,* ***Sanjivani Jawale****, Rani Jadhav and thanking to my close friends* ***Vishwajeet, Amol****, Shashi, Pandurang, Ram, Antrushi,* ***Jayashri*** *and Shital etc. all UG-PG alumni VDCOAB, Latur.*

I sincerely admire the contribution of all my ***batch mates*** *Abhi, Rushi, Ramesh, Madhav, Namrata, Rasika, Pooja, Rohini, Shruti, Sayali, Priyanka, and Bharati etc. for extending their unstinted support, timely motivation, sympathetic attitude and unfailing help during the course of entire study. I sincerely express my gratitude from the core of my heart to all the* ***lovely juniors*** *Abhijit, Pramod, Dipak, Yogesh, Mrugendra, Someshwar, Vrushabh, Anil, Navnath, Tukaram, Pratiksha, Nalini, Nisarga, Likhita, Swathi and Hemlata.*

I feel a deep sense of gratitude to my late father ***Dashrath Jetnaware*** *who formed part of my vision and taught me the good things that really matter in life. The happy memory of my father still provides persistent inspiration for my journey in this life. Words prove a meagre media to write down my feelings for my mother* ***Smt. Kalinda Jetnaware****, sister Sonali and all my relatives for providing me constant encouragement, divine presence and supporting me spiritually throughout.*

I am thankful to VDCOAB, Latur and ***Vaidyanath PG Hostel****, VNMKV, Parbhani for providing accommodation, advanced infrastructure and excellent research environment. I acknowledge* ***Department of Biotechnology, New Delhi****,* ***India*** *for providing me research fellowship.*

I sincerely apologize to all those who have extended their good will and moral support during the tenure of my experiments whose, name have not been mentioned you will always be cherished.

Thank you….

Place: VDCOAB, Latur
Date: - -2018 ***(Jetnaware P.D.)***

C O N T E N T S

LIST OF TABLES

LIST OF FIGURES

LIST OF GRAPH

ABBREVIATIONS

AA	:	Ascorbic acid
AC	:	Activated charcoal
BA	:	Benzyl adenine
BAP	:	6-Benzylamino purine
2,4-D	:	2,4-dichlorophenoxy Acetic Acid
2-ip	:	2-isopentenyl adenine
CD	:	Critical difference
CRD	:	Completely Randomized Design
cv.	:	Cultivar variety
^{o}C	:	Degree Celsius
DAI	:	Days after inoculation
DDW	:	Double distilled water
et.al	:	Etalia (and associate)
FAO	:	Food and Agricultural Organization
i.e.	:	That is
mg	:	Milligram
GA_3	:	Gibberellic acid
HCl	:	Hydrochloric acid
$HgCl_2$	:	Mercuric chloride
IAA	:	Indole 3-acetic acid
IBA	:	Indole 3-butyric acid
pH	:	Log $[H^+]$ ion concentration
Kin	:	Kinetin
KCl	:	Potassium chloride
LAF	:	Laminar air flow
mg/l	:	milligram per litre
μM	:	Micro molar
ME	:	Malt extract
MS	:	Murashige and Skoog medium, 1962
MT	:	Murashige and Tucker medium, 1969
min	:	minutes

N	:	Normal
NAA	:	α-Naphthalene acetic acid
NHB	:	National Horticulture Board
NaOH	:	Sodium hydroxide
NaOCl	:	Sodium hypo chloride
PDKV	:	Punjabrao Deshmukh Krishi Vidyapeeth
PGR	:	Plant Growth Regulator
ppm	:	Parts per million
p.s.i.	:	pound per square inch
SE	:	Standard error of means
Viz	:	Namely
WPM	:	Woody Plant Medium
%	:	Percent
etc.	:	Etcetra
v/v	:	Volume by volume
w/v	:	Weight by volume
g	:	Gram
g/l	:	Gram per litre
Var.	:	Variety

Introduction

CHAPTER- I

INTRODUCTION

Fruits have been the man's food from time immemorial. There is tremendous scope of developing fruit industry in India. However, one of the bottlenecks in expanding fruit industry is non-availability of quality planting material on large scale during season. Citrus is one of the most widespread fruit crops consumed throughout the globe over 100 countries with high economic and medicinal values. Citrus is an important genus of evergreen aromatic small trees, mostly spines, and having fruits called hesperidium. It occupies a place of prime importance among the major fruit crops of India, ranking third after mango and banana (Fresh Plaza News, 2016).

Citrus is one of the largest and most important groups of fruit crops in tropical and subtropical regions. Citrus is native to a large area, which extends from India to China, Philippines, Burma, Thailand, Indonesia, Nigeria, Colombia, Guinea, Saudi Arabia, New Caledonia. There are some popular species of citrus group known as Kagzi lime (*Citrus aurantifolia*), Grapefruit (*Citrus paradisi*) and Pummelo (*Citrus grandis*), Sweet orange (*Citrus sinensis*), Mandarin (*Citrus reticulata*). Among the citrus fruits of commercial value, sweet orange (*Citrus sinensis* L. Osbeck), mandarin (*Citrus reticulate* Blanco) and sour orange (*Citrus auranticum* L.) contribute approximately 80% of the world citrus fruit production (FAOSTAT, 2016). Other commercially important fruits are lime (*Citrus aurantifolia* Swingle), lemon (*Citrus limon* Burm f.) and pummelo (*Citrus grandis* L. Osbeck), but they are grown on relatively smaller scales.

Eastern Asia, mainly the south-east Asia is considered to be the centre of origin of citrus, in this region, many citrus types are still found in their wild state. In India, citrus is grown in many states, but the states of Andhra Pradesh and Maharashtra have the largest production share. In India, among the fruit crops (31.5% share in horticulture) citrus species (Lime/lemon, orange and mosambi) covers an area of major fruit crops is 10% with an area of 897.79 thousand ha, with production of 1,10,40,250 MT, giving rise to productivity of 38.6 MT per ha. (NHB, 2016-2017).

Table 1: Basic scientific classifications of citrus

Order	Geraniales (21 families)
Sub-order	Graniinea (12 families)
Family	Rutaceae (7 sub-family)
Sub-family	Aurantoidae (2 tribes)
Tribe	Citrae (3 sub-tribe)
Group C	Citrus fruit trees (6 genera)
Genus	1. Poncitrus (1 species) 2. Fortunella (4 species) 3. Citrus (2 sub genera)
Chromosome number	2n = 18

Lime, lemon and citron belong to the citrus fruit group. This group is characterized by an elliptical to round shaped fruit with high citric acid content. Limes can be distinguished from other fruit in the citrus family as they have both acid and sweet varieties. Its attractive appearance, penetrating aroma of peel and excellent taste gives a remarkable position to acid lime which is grown widely throughout the world. The external part of the rind consists of several morphologically different tissues called flavedo because of the presence of flavonoid compounds (Ortiz, 2002). The whole surface of the fruit is covered by polygonal cells to form the isodiametric layer. This layer contains cuticles that are partially enclosed with a waxy substance to prevent excessive loss of water from the fruit. The edible part of the fruit is divided by carpel segments or locules. There are many juice vesicles within the carpels. The number of carpels vary, with acid lime fruit normally containing around 8–11 segments (Loussert, 1992).

The three main types of lime largely cultivated worldwide are Persian lime, Key lime (Mexican lime) and Makrut lime. Key limes are a small rounded fruit (*Citrus aurantifolia* (Christ.) Swingle), while the Persian limes bear a larger fruit (*Citrus latifolia* Tanaka) that is triploid and seedless.

Acid lime (*Citrus aurantifolia* Swingle) belongs to the family Rutaceae and sub family Aurantiodae. It is one of the important commercial fruits. It is cross pollinated crop. Production and quality of fruits are decreasing every year due to inbreeding depression, which may be the results of crossing within the citrus species.

Acid lime fruit contain vitamin A and C that is very useful for human nutrition. Their fruits are important source of volatile oils, limonene, α-terpinene, β-terpinene, citral cumarins, bioflavonoids, vitamins, and mucilage are used for squashes, pickles, syrups, cordials, and manufacture of citric acid for table purpose. It supplies high calories and its values not only for its nutritiona1 qualities, but also for medicinal purpose. It has high medicinal values and used for the prevention of various diseases like bones and joints, piles, dysentery, cold, influenza, constipation and scurvy. They also act as an aseptic, antirheumatic, antibacterial and antioxidant (Rathore *et al*., 2007). Being acidic in nature, acid lime fruits have great medicinal value. Acid lime is good appetizer, anti helmentic and it checks biliousness and stomach ache. For making refreshing drinks in summer, lime fruits are very much preferred.

Citrus can grow well in wide range of soils. Soil properties like soil reaction, soil fertility, drainage, free lime and salt concentrations etc. are some important factors that determine the success of citrus plantation. Citrus fruits flourish well on light soils with a good drainage. Deep soils with pH range of 5.5 to 7.5 are also considered good. It can be cultivated in pH range of 4 to 9. Presence of calcium carbonate concentration within feeding zone may adversely affect the growth habit. Light loam or heavier but well drained sub-soils appears to be ideal for citrus. In high rainfall areas, it is susceptible to bacterial canker. Dry and arid conditions coupled with well-defined summer having low rainfall (ranging from 75 cm to 250 cm) are most favorable for the growth of the crop. High yielding cultivars are Kagzi lime, Vikram (Ms), Pramalini (Ms), Chakradhar, PKM1, Selection 49, Seedless lime, Tahiti are popular in India and Maharashtra (Pawar *et al*., 2015).

Citrus can be propagated by both sexual and asexual methods; generally, rootstocks are propagated through seeds, while most of the commercial varieties are propagated through various asexual methods (Chaudhary *et al.,* 1994). Citrus propagation by conventional means is restricted to particular season and availability of plant material. Vegetative propagation methods like budding, cutting, layering are practiced during limited period of the year. Moreover, these methods tend to propagate pathogens present in mother explants. Axillary bud proliferation is widely practiced for *in vitro* propagation of citrus because it ensures maximum genetic uniformity of the resulting plants.

Tissue culture and micropropagation protocols have been described for a number of woody species (Starrantino and Caruso, 1988; Perez-Molphe-Balch and Ochoa-Alezo, 1997; Roussos *et al.,* 1999). Shoot proliferation and root induction of woody species have always been low and erratic. Many species have a slow rate of development in *in vitro* cultures and produce only a small number of shoots during the proliferation stage (Barlass and Skene, 1982; Moore, 1986). Many researchers have attempted to induce proliferation and rhizogenesis by adding specific growth regulators to the medium, e.g. cytokinins, auxins and gibberellins, in various concentrations and combinations (Parthasarathy and Nagaraju, 1996).

The *in vitro* micropropagation technology can overcome some constraints to citrus improvement and cultivation and can increase fruit quality and resistance to disease and environmental stresses (Grosser *et. al.,* 1993). The techniques such as meristem tip culture, nucellar embryogenesis and shoot tip grafting *in vitro* (Navarro *et. al.,* 1975) to provide virus free mother stock for budding has become more pronounced. Such material can then be used for international distribution and exchange without the risk of spreading disease or lengthy periods of quarantine (Button, 1977). Micropropagation offers the opportunity to reduce this interval and provide more reliable evaluation.

In vitro propagation has a useful tool to overcome problems related with the field culture of such species (Hidaka *et al.,* 1989). Multiple shoot induction and regeneration is potentially useful for the genetic improvement of fruit crop (Murashige *et al.,* 1972). It is known that the tissue obtained from young plant parts have relatively more regenerative capacity than old tissue. The root and bud

formation in tissue culture is dependent on a specific equilibrium between the auxin and cytokinin, gibberellins and cytokinin ratio which control the shoot and leaf development (Starrantino *et al.,* 1990).

There are great numbers of diseases and disorder that can adversely affect citrus plantation. There may be fungal (root rot, gummosis and dieback), insect and nutritional imbalance. This can be controlled by chemical treatment or cultural treatment or cultural practices. However, disease caused by virus and virus like agent such as trestiza and exocortis cannot be adequately controlled by such treatment. Micropropagation can contribute greatly to control diseases and disorder (Das, 2009).

The sustainable development of the citrus industry is mainly dependent on a continuous supply of new and improved cultivars (Tornero *et al*., 2010). For the citrus industry breeding objectives are to improve fruit quality and reduction in biotic and abiotic stresses (Wenwu *et al*., 2007). There are very few reports of direct regeneration of plants without callus initiation. There has been increasing interest in the use of vegetative material to produce adventitious buds from callus and directly from explant to provide a non-callus procedure for virus elimination and rapid multiplication (Barlass and Skene, 1982).

Micropropagation has many advantages over conventional methods of vegetative propagation (cutting or seed) and its application in horticulture, agriculture and forestry is expanding worldwide (Jeong *et al.*, 1995). The goal of micropropagation is to obtain a large number of genetically identical, physiologically uniform and develop normal plantlets preferably with a high photosynthetic potential to survive the *ex vitro* conditions, in a reduced time period and at a lowered cost.

Micropropagation technique in acid lime can increase rate of multiplication, disease free uniform planting material, easier transportation, improved crop yield, rapid selection and multiplication of elite planting material and round year availability of planting material. There are different strategies for micro propagation of citrus, *viz.*, somatic embryogenesis, adventitious shoot bud production and axillary enhancement which are routinely used. Among these, axillary enhancement using nodal segment as explant is most preferred as it does not involve a callus phase, thus minimize the risk of somaclonal variation and it offers economically optimum multiplication rate.

Seedless lime is devoid of seeds. Budding method is not economically feasible as it is time consuming and not suitable as it may induce tristeza virus disease. Juvenile characteristics and delayed bearing are disadvantages in production of true to type and virus free plants. Chakradhar is a thorn less and seedless lime. That was derived from Kagzi lime and has round fruit with thin papery rind and 60-66% juice content. It has a greater yield and vitamin C (118.2-140.8 mg/100g) and acid content (8.3-9.1%) than Kagzi lime. Seedless lime regarded as essential for long term efforts to improve citrus culture worldwide (Bagde and Patil, 1989).

Taking advantage of micropropagation technique, the research work entitled **"*In vitro* multiple shoot induction from nodal explant in Seedless lime (C*itrus aurantifolia* Swingle)"** with following objective,

1. To study *in vitro* shoot induction in seedless lime by using axillary bud
2. To study effect of different concentrations of growth regulators on *in vitro* multiple shooting in seedless lime

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Review of Literature

CHAPTER- II

REVIEW OF LITERATURE

Plant tissue culture is defined as the process in which plant parts there of are grown under aseptic conditions and in artificial environment. This technique reveals the unique capacity of plant cells irrespective of their ploidy level and form of specialization to give rise to whole plant. In recent years plant tissue culture has gained attention especially in agriculture, horticulture and forestry in relation to genetics, cell biology, plant physiology and plant pathology. It has been widely used in the field of genetic engineering and molecular biology.

In vitro culture has found its best commercial application methods of propagation. It has also proved to be immense practical value as an aid to the production and maintenance of disease free plants, germplasm conservation and a valuable adjunct to the conventional methods of plant development. The current tissue culture techniques are opening the door to a second green revolution.

2.1 A general review: Micropropagation

The concept of totipotency is the basis for micropropagation. Totipotency is a unique feature of plant cell and can be defined as the regeneration of intact and fertile plants from cultured tissues in nutrition medium supplied with plant growth regulators for appropriate stimuli. When Haberlandt (1902) attempted the first cell culture studies, his intention was to develop a versatile tool to explore morphogenesis and to demonstrate totipotency of plant cells. The first *in vitro* plant culture was reported with tomato root tip in liquid medium (White, 1934) and the first report of viable callus culture was reported by Gautheret (1939) in tobacco followed by White (1959) in carrot, respectively.

Micropropagation is a commercially viable branch of biotechnology which can be defined as *In vitro* regeneration of plants from organs, tissues, cells or protoplasts (Beversdorf, 1990) and the true to type propagation of a selected genotype using *in vitro* culture techniques (Debergh and Read, 1991).

Micropropagation has been successfully employed for production of uniform and superior quality planting material and also propagation of seedless fruit. The regeneration of plants can take place as extension or proliferation of shoot tips or axillary buds, direct plant regeneration of organs from explants i.e. organogenesis, indirect plant regeneration *via* callus induction or through production of somatic embryos i.e. embryogenesis. Recently *in vitro* shoot tip grafting technique has been found useful particularly for elimination of virus like pathogens (Starrantino, 1992).

2.2 Culture Medium

The different tissue culture media primarily consists of inorganic salts (macro and micro nutrients), carbohydrates, vitamins, amino acids, plant hormones *viz.*, auxins and cytokinins and other growth regulatory substances. The nitrogen can be applied in the form of nitrate or ammonia. Systematic studies for the requirement of inorganic nitrogen were done by Riker and Gutsche (1948) in sunflower tissue. They observed that nitrate was superior to nitrite and ammonia. The requirements of inorganic elements were systematically studied by Hilderbrandt *et al.* (1946) in sunflower and Murashige and Skoog (1962) in tobacco.

Plant growth regulators have a well-defined effect on growth and development and each phase requires a specific growth regulator or group of growth regulators. Auxins are a class of plant growth regulators which cause cell elongation, apical dominance and root initiation. The most frequently used auxins are 2, 4-D, NAA, IAA and IBA of which IAA presents naturally in plants. NAA and 2, 4-D are the most effective auxins to initiate callus. Cytokinins are phytohormones which promote cell division, proliferation and differentiation of tissues. The most widely used cytokinins are kinetin, BAP, Zeatin and 2-ip. Auxins or cytokinins alone cannot be effective for organogenesis through cell division or *in vitro* callus development but the relative combination of both (i.e. auxin and cytokinin) decides the plant morphogenesis *in vitro* (Skoog and Miller, 1957).

Murashige and Skoog (1962) published a defined medium for tobacco culture, which has probably been cited more than any other medium for culture of wide variety of plant species including both dicots and monocots. Vasil and Hildebrandt (1965) obtained differentiation of completely organized tobacco plant from a single cell.

Carbohydrates are essential for supply of carbon and energy to cultures. Though, a number of carbohydrates have been used in tissue culture media, the preferred carbohydrate is sucrose (Kumar and Kumar, 1998). Wolter and Skoog (1966) and Coffin *et al.* (1976) reported that galactose, mannitol and sorbitol were the best carbon sources of many other plant tissues.

Guha and Maheshwari (1966) for the first time cultured mature anthers of *Datura innoxia* on a nutrient medium containing kinetin and coconut water and demonstrated the possibility of raising large number of haploid plants from pollen grains. Bourgin and Nitsch (1967) confirmed the totipotency of pollen grains. Takebe *et al.* (1971) demonstrated the protoplasts isolated from mesophyll cells which could be cultured to generate into entire plants.

The various factors such as genotype, plant age, physiological (vegetative or reproductive) stage and age of explants, general health of plant, position of explant within plant size of the explant, method of inoculation etc. affect the *in vitro* plant growth while selecting a suitable explant for micropropagation (Otoni and Teixeira, 1991). Many fruit crops are sensitive to photoperiod; these are normally cultured *in vitro* under day night regime of light cycle of 16 hrs and darkness of 8 hrs. is the most preferred requirement. The optimum growth of tissue cultures has been observed at a temperature regime 24-26 °C (Pierik, 1987).

The environmental conditions and composition of culture media are known to be crucial for the growth of tissue cultures (Kobayashi *et al.* 1985; Duran *et al.* 1992).

Nagao *et al*. (1994) studied regeneration of *Poncirus trifoliata in vitro* culture of apical buds cultured on MS medium supplemented with BA 1.0 mg/l and NAA 1.0 mg/l with varying sucrose and inorganic nitrogen levels. The best results were obtained on medium supplemented with sucrose 30-45 g/l and double dose of inorganic nitrogen.

Siwach *et al.* (2012) reported that rate of maximum shoot proliferation enhanced by adding 25 mg/l glutamine, 50 mg/l adenine sulphate and 100 mg/l casein hydrolysate supplement in MS medium. Problems like defoliation, chlorosis and loss of vigour in shoots were observed at different stages of subculture. These problems were successfully controlled by SCM-4 having 1.5 mg/l of BAP, 0.5% charcoal, 25 mg/l glutamine, 50 mg/l adenine sulphate and 100 mg/l casein hydrolysate. Rooting was significantly enhanced by 100 mg/l casein hydrolysate.

2.3 Surface sterilization of explants

Khayri and Bahrany (2001) conducted an experiment on surface sterilization techniques of Lime *Citrus aurantifalia* (Christ. swing), using nodal explants of mature trees. The shoot branches about 10 cm long, were defoliated and washed with soapy water to remove any dust particles. These were treated for 30 seconds in 70% Ethanol followed by 15 min shaking in 1% w/v sodium hypochlorite (20 v/v chlorex) containing 3 drops of tween-20 per 100 ml of water. The twigs were rinsed three-four times in sterile distilled water.

Syamal *et al.* (2007) conducted an experiment on surface sterilization of nodal segments of field grown Kagzi lime. They found highest 82.51 % of aseptic culture with dipping in NaOCl (1%) for 20 min followed by dipping in $HgCl_2$ (0.1 %) for 10 min and finely in KCl (1 %) for one minute. Also the highest 72.43 % survival explants was recorded with the treatment of NaOCl (1%) for 10 minutes, $HgCl_2$ (0.1%) for 5 min KCl (1%) for 1 minute.

Kour *et al.* (2007) developed a protocol for surface sterilization of nodal segment of rough lemon *(Citurs jambhiri* Lush). Explants were collected from 8-10 year old mature tree. Explants were surface sterilized with 70 % ethanol for 30 seconds and 0.1 % mercuric chloride ($HgCl_2$) solution (w/v) for 8 minutes in laminar air flow cabinet and then were rinsed it for 3 minutes to sterile distilled water.

2.4 Micropropagation of Citrus

Chaturvedi and Mitra (1974) observed multiple shoots from callus regenerated shoot of *C. grandis* (L.) on MS medium supplemented with 0.25 mg/1 BA + 0.1 mg/1 NAA. Excised shoots were rooted on MS medium supplemented with 0.1 mg/1 to 0.5 mg/1 NAA.

Bhansali and Arya (1978) cultured stem and leaf explants of *C. paradisi* in Murashige and Tucker (MT) (1969) medium supplemented with BAP, NAA and malt extract at different concentration. The maximum shoots were formed on the medium with 1000 ppm ME + 0.5 ppm BAP + 0.15 ppm NAA. The leaves and stems were also vigorous and well developed. Roots were formed in medium with higher level of 2.5 ppm NAA.

Sauton *et al.* (1982) observed multiple shoot production in orange, lemon and citrange from both juvenile and mature trees on MS medium supplemented with 10 µM BA. Best rooting induced at 10 µM NAA.

Barlass and Skene (1982) studied *in vitro* plantlets formation from citrus varieties *viz.*, Carrizo citrange, Trifoliate orange, Cleopatra mandarin, Rangpur lime and sweet orange using internodal explant. Multiple shoots obtained on MS medium supplemented + 10µM/1 BA. *In vitro* grown shoots were rooted on basal medium with 10 µM/1 NAA (90 %) and 5 µM/1 NAA (60 %).

Edriss and Burger (1984) obtained shoot proliferation with epicotyl segment on MS medium with 0.25 mg/1 BAP and 1 mg/1 NAA in Troyer citrange. The best rooting was obtained with 2 mg/1 NAA.

Hutchinson and Zimmerman (1987) developed reliable and efficient protocols for *in vitro* plant regeneration through stimulation of axillary shoot proliferation from nodal stem segments and apical buds or through organogenesis or embryogenesis directly from various explants or callus for many important tropical and temperate fruit trees.

Starrantino and Caruso (1987) described *in vitro* propagation of citrus rootstocks Troyer citrange, Carrizo citranges, Flying Dragon trifoliate orange *(P. trifoliata)* and dwarf mutant seedling of Troyer citrange using shoot apices (0.5-1 cm)

as an explant. In Troyer and Carrizo best results obtained on MS medium supplemented with 1 mg/l BA, 0.5 mg/1 IBA and 40 mg/1 adenine, while the 'Flying Dragon' and the 'dwarf mutant' showed the highest multiplication rate with 0.5 mg/1 BA, 0.25 mg/1 IBA and 40 mg/1 adenine. Rooting was induced on MS with 1 mg/1 NAA.

Duran *et al*. (1989) studied morphogenesis and callus culture of sweet orange, citron and lime. The optimum concentrations of NAA to induce root formation on stem segment were 10 mg/l for sweet orange and lime and 3 mg/l for citron. The optimum BA concentration for shoot and bud proliferation was 3 mg/l for sweet orange and citron and 1 mg/l for lime.

Huang and Xiang (1992) reported that shoots multiplication in MT medium supplemented with BA 0.25-1.0 mg/l. Multiplication rate was highest with BA 0.5 mg/l and shoot proliferation rate was 3.2-4.2 in Jianshui Seedless tangerine.

Raman *et al*. (1992) observed that percent callus induction was more when the stem segments from 14-21 days old axenic seedling of *C. limon* and *C. jambhiri* were cultured on MS media supplemented with NAA 10 mg/l + kinetin 0.2 mg/l. The highest percentage of shoot regeneration was obtained on half MS medium supplemented with BA 5.0 mg/l alone or in combination with GA_3 3.0 mg/l. Regenerated shoots rooted most easily on half strength MS medium + NAA 1.0 mg/l + 2% sucrose.

Lin *et al.* (1992) reported *in vitro* propagation of *C. sinensis* Osbeck via lateral buds as an explant on MS medium containing different combination of BA, IAA, GA and activated carbon (AC). Far shoot proliferation best combination was 0.1 mg/1 BA, 0.05 mg/1 IAA and 0.1 mg GA_3. The highest rooting percentage (70%) was observed on half strength MS medium supplemented with 0.2 mg/1 NAA, 0.3% activated carbon and 15% sucrose.

Mas *et al.* (1994) observed best multiplication rate of 5:1 in Citremon 1452 *(Poncirus trifoliata* x *Citrus Union),* when 1 cm stem segments with 2 buds from *in vitro* plants were cultured on MS medium supplemented with 0.5 mg/1 BA and 0.5 mg/1 NAA. A survival rate 95% was obtained when shoots > 1.5 cm were

rooted in liquid MS medium with 25 g/1 sucrose, 0.2 mg/1 thiamine and 0.15 mg/1 NAA.

Singh *et al.* (1994) obtained multiple shoots from shoot tips (2-3 cm long) from mature plants (5 to 6 years old) of Mandarin cv. Khasi and Lemon cv. Assam lemon when cultured on MS medium supplemented with 1.0 mg/1 BA, 0.5 mg/1 kinetin and 0.5 mg/1 NAA. Root induction was noticed when seven week old single shoot (2 cm long) of both citrus species were cultured on MS medium supplemented with 0.25 mg/1 BA, 0.5 mg/1 NAA and 0.5 mg/1 IBA.

Baruah *et al.* (1996) observed the shoot proliferation from shoot tips of *C. assamensis, C. latipes* and *C. indica* as an explant with MS media containing different concentration of BAP or kinetin, MS medium combined with 0.5 mg/1 BAP was superior to kinetin for shoot proliferation in all species.

Desai *et al.* (1996) cultured axillary buds from shoot explants of Kagzi lime *(C. aurantifolia)* on modified MS medium supplemented with various levels of BAP, NAA, 2,4-D with or without malt extract (ME), shoot proliferation was best (2.63 shoots/explant) on MS medium supplemented with 0.25 mg/1 BAP + 200 mg/1 ME. The highest number of shoots was produced on MS medium supplemented with 0.25 mg/1 BAP + 200 mg/1 ME. Rooting was best on half strength MS medium fortified with 2 mg/1 IBA.

Parthasarathy and Nagaraju (1996) studied shoot proliferation with eight citrus species *viz., C. volkameriana, C. limon, C. grandis, C. jambhiri, C. aurantifolia, C. reticulata, C. reshni* and *C. sinensis* via shoot tips of axenic seedlings. Amongst these species, *C. sinensis* and *C. reshni* produced maximum number of shoots at 0.25 mg/1 BAP on MS medium. *C. limon* recorded longest shoot. BAP at 0.75 mg/1 produced maximum number of shoots.

Harada and Murai (1996) reported *P. trifoliate* shoot regeneration on MS solid medium supplemented with BA 44.4 µM, 3% sucrose and 0.8% agar. Regenerated shoots rooted on half strength MS basal medium with or without IBA 0.5 mg/l.

Belarmino and Posas (1997) reported rapid shoot proliferation in pummelo using shoot tip and single node stem segments on MS medium containing 0.5 mg/1 BA, 0.5 mg/1 IBA and 40 mg/1 adenine.

Normah *et al.* (1997) observed maximum number of shoots by using hypocotyl explants cultured on MS medium supplemented with 2.2-11.1 µM BA in wild *C. halimii.* Rooting of regenerated shoots was highest on MS medium supplemented with 2.7 µM NAA. Survival of regenerated plantlets was 83.30 % after transfer to a soil mixture in the green house.

Thirumalai and Thamburaj (1996) reported plantlets production through axillary bud culture in sweet orange cv. Sathgudi and acid lime cv. PKM-1. The explants were cultured on MS medium supplemented with BA, GA_3 and kinetin at different concentration. In sweet orange maximum number of shoots (5-6) produced on MS medium fortified with 3 mg/1 BA while in acid lime 5 shoots were produced on MS with 1.5 mg/1 BA. In sweet orange at 3 mg/1 higher shoot length obtained from axillary bud culture whereas in acid lime better length of shoots were obtained at 1.5 mg/1 BA + 0.1 mg/1 GA_3. Rooting of the shoots was achieved on $3/4^{th}$ strength MS medium along with 3 mg/l NAA.

Perez *et al.* (1997) studied *in vitro* regeneration by organogenesis using internodal stem segments from seedling of Mexican lime and Mandarin on MS medium with vitamins from B5 medium, sucrose 5%, 3.3 uM BA and 5.4 µM NAA and incubated at 25±2 °C for 21 days in darkness followed by 29 days on 15 h/8h light/dark cycle. They obtained an average 7-8 well differentiated shoots per explant in Mexican lime and 5 in mandarin. Rooting of 70% of shoots was achieved in culture medium with NAA (2.7-5.4 µM*)* or IBA (2.5-4.9 µM) of the rooted plants, 85% were well adapted to soil conditions.

Singh *et al.* (1999) studied *in vitro* propagation *via* shoot tip explant (1-2 cm) collected from Rough lemon trees by culturing it on modified MS medium supplemented with various combinations of BAP, NAA or IBA. Shoot proliferation was best (80%) on MS medium supplemented with 1.0 mg/1 BA and 0.25 mg/1 NAA. Root development from shoots was best on MS medium supplemented with 0.1 mg/1 BA, 0.5 mg/1 NAA and 0.1 mg/1 IBA. Regenerated plantlets (80%) were successfully established in soil.

Das *et al.* (2000) standardized protocol for *in vitro* propagation of sweet orange (cv. Mosambi) through nucellar embryo culture. The (5-6) shoots were obtained from collar region of embryos in MS medium supplemented with 1 mg/1 BAP within 60 days of inoculation. The number of plantlets was double after transfer in same medium and culture for another 30 days. Higher dose of BAP initiated callus directly from the embryos. The regenerated shoots (2-3 cm) could be rooted in MS medium supplemented with 0.5 mg/1 NAA + 2 mg/1 IBA.

Paudyal and Haq (2000) studied *in vitro* propagation using shoot tip explants on MS medium from pummelo seedlings. They reported that the most adventitious shoots / explant were cultures, (average 5.2) supplemented with 1.8 µM/l BA. Additions of 5.8µM/1 GA_3 in shoot proliferation medium improved shoot elongation. Over 75 % of the shoots developed roots when transferred to half strength MS medium supplemented with 1.3, 2.7 or 5.4 NAA µM/l.

Khayri and Bahrany (2001) developed a complete protocol for *in vitro* regeneration of lime (*C. aurantifolia*) cv. Binzahir using nodal explants of mature trees. Nodes were cultured on MS medium containing different concentration of BAP, IBA and kinetin. Maximum number of shoots (8) per node was obtained with 1 mg/1 BAP and 0.5 mg/1 kinetin. IBA had little effect on shoot multiplication. They further reported that combination of 0.25 mg/1 BAP with 1 mg/1 kinetin produced elongated multiple shoots. Shoot elongation and leaf size were inhibited in response to high levels of BAP. Excised shoots were rooted on MS medium supplemented with 1 mg/1 IAA (56 %).

Abdulaziz *et al.* (2002) studied the effect of phytohormones on *in vitro* shoot multiplication and rooting of *Citrus aurantifolia*. They produced multiple shoots from nodal explants on MS media. The highest numbers of shoots (9) shoots per node were produced on a medium containing 2 mg/l BAP, 1 mg/l kinetin and 1 mg/l NAA produced complete plantlets. The highest rooting percentage was obtained on media containing 0.5 mg/l NAA combined with 2 mg/l IBA. Plantlets that survived acclimatization, 82% exhibited normal growth in soil under greenhouse conditions.

Usman *et al.* (2005) worked on regeneration of citrus plant by culturing nodes and internodes of seedlings germinated *in vitro*. Both shoot and root initiations were positively influenced by hormones NAA and BAP, such that the number of roots and shoots per explant increased proportionately with increase in hormone concentration of 1mg/l BAP and 10 mg/l NAA.

Kamble *et al.* (2005) developed protocol for micropropagation of acid lime (*C. aurantifolia* S.) var. Sai-Sharbati using shoot tip and nodal segment explants obtained from axenically grown seedlings of 21 to 30 days age. In both the explants, 100% shoot proliferation was induced on MS + BA 0.25 mg/l + malt extracts 200 mg/1 media. The shoot proliferation rate was more (5.0) in nodal segment as compared to the shoot tip explant (4.3). Half strength MS medium supplemented with IBA 1.0 mg/1 was best for root initiation within 15.66 days. A higher Kin/IAA ratio favored stem elongation more than root formation whereas a lower Kin/IAA ratio favored root formation and inhibited stem elongation.

Rathore *et al.* (2007) developed a protocol for *in vitro* cloning of mature plants of *Citrus limon* using nodal segments. Three to four shoots were induced on MS medium supplemented with 9 µM BAP. Repeated transfer of the initial explants for up to six passages on MS medium with 0.22µM BAP yielded crops of shoots. The cloned shoots rooted *in vitro* on half strength MS medium + 27 µM NAA+ 0.1% activated charcoal about 95% of rooted plantlets survived in hardening and transplanting.

Saini *et al*. (2010) established an efficient *in vitro* regeneration protocol for rough lemon (*Citrus jambhiri*) from 1 month old seedlings. Higher numbers of elongated shoots were obtained on MS medium having BAP (0.5 mg/l) + GA3 (1.0 mg/l). The elongated shoots were then rooted on MS medium containing NAA (1.0 mg/l) + IBA (1.0 mg/l) with high (77%) rooting percentage. The plantlet survival was 96% when plantlets were transferred to plastic bags containing mixture of garden soil and vermiculate (1:1). The hardened plants were successfully established in the soil.

Tornero *et al*. (2010) developed an efficient protocol for micropropagation of lemon (*Citrus limon*) from mature nodal segments. The number of shoots was dependent on the BAP and GA_3 concentrations and the best results were obtained with 2 mg/l BAP and 1 mg/l GA_3. The highest rooting percentages were obtained on media containing 3 mg/l IBA alone or IBA in combination with 1 mg/l IAA. The highest numbers of roots were produced with this combination of media. Root length was more along with 3 mg/l IBA rooting medium a better appearance, with greener and larger leaves. The success during the acclimatization was about 100% and the plantlets exhibited normal growth in soil under greenhouse conditions.

Samarina *et al.* (2010) worked on the regeneration and micropropagation of lemon cultivars *in vitro* by using nodal explant. Several combinations of BAP with NAA were used for optimization of cell proliferation and micropropogation and the maximum effect at the stage of cell proliferation was obtained on MS basel medium containing 0.1 mg/l BAP and 0.5 mg/l NAA. Cultural media consisted of MS components with 1 mg/l BAP and 0.1 mg/l NAA stimulated multiplication of shoot. Rooting of regenerates was obtained on half MS supplemented with 0.5 mg/l NAA.

Taha *et al.* (2011) studied *in vitro* propagation of lemon tree (*Citrus aurantafolia*) by using various plant parts *viz*. shoot tips, nodes and internodes the best result in the multiplication of shoots was obtained on MS medium with 1.5 mg/l BAP + 1 mg/l GA_3. At rooting stage they studied the effect of 1 and 2 mg/l IBA and NAA respectively and achieved the highest percentage of rooting i.e. 60% in 1 mg/l NAA added to half MS medium.

Misra *et al.* (2012) developed protocol for micropropagation of kinnow from nodal explants. These nodal explants were cultured on MS medium fortified with different concentrations and combinations of BAP and NAA. Best shoot multiplication was obtained on MS medium supplemented with BAP (3 mg/l) and NAA (0.5 mg/l). Transfer of shoots to a rooting medium induced highest percentage of rooting on MS supplemented with NAA (2.0 mg/l).

Goswami *et al.* (2013) developed a micropropogation protocol for multiplication of Seedless lemon (*Citrus limon* L. cv. Kaghzi Kalan) by using nodal explants. The maximum number of shoots per explant was observed on MS + 0.1 mg/l BAP and 0.5 mg/l Kin. Shoot proliferation increased with the concentration of BAP up to 10 mg/l. None of the treatments including BAP or kinetin alone or BAP in combination with NAA produced significantly more shoots for commercial exploitation. In case of a combination of BAP + kinetin + IBA, the maximum (5.5 shoots per explants) proliferation was observed on MS medium containing 1.0 mg/l IBA. Regenerated shoots showed root induction on MS Basel medium or on MS medium containing 1.0 mg/l IBA.

Chamandoosti *et al.* (2017) performed shoot multiplication in *Citrus latifolia* Tan. (Persian lime) using nodal segment explants of young one year old trees by two different pathways containing with and without callusing phase. The best result for multiple shoot formation and regenerated shoot formation was 3.2 and 2.6 shoots per explants with 4.44 µM BA + 0.053 µM NAA and 4.44 µM BA plus 0.049 µM IBA respectively. Alike shoot regeneration, shoot elongation was occurred in medium with 4.44 µM BA and 0.049 µM IBA.

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Material and Methods

CHAPTER- III

MATERIALS AND METHODS

This experiment was performed at Tissue Culture Project, Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani during the period from 2017 - 2018 to establish protocol of micropropagation of seedless lime (*Citrus aurantifolia* Swingle) cv. PDKV Chakradhar from axillary bud (nodal explant).

3.1 Materials:

3.1.1 Experimental Plant Material:

The cultivars of seedless acid lime (*Citrus aurantifolia* S.) selected for this study were collected from of PDKV Akola. Axillary bud (Nodal explant) from healthy mature (2-3 year old) seedless lime plant of cultivar 'PDKV Chakradhar' was used as explants source for this study.

3.1.2 Equipment's, Chemicals and Glassware's:

a) Equipment's:

1) Laminar air flow cabinet (LAF)
2) Autoclave
3) Hot air oven
4) Microwave oven
5) pH meter

b) Glassware's:

1) Jam bottles
2) Reagent bottles
3) Beakers
4) Measuring cylinder
5) Petridishes
6) Pipettes
7) Shaker

The test tubes, culture bottles, glass bottles of varying capacities, beakers and measuring cylinder of varying capacities, trays, micro-pipettes and tips of different capacities, stirrers etc. were used during different stages of micropropagation.

Inoculation material required for initiation and shoot multiplication of explants *viz.*, scalpels, forceps, blades, petri plates, tissue paper, surgical cotton, spirit lamp, wash bottle, coupling jar etc. were used during experiment *in vitro* study. The glasswares used during the course of the experiments, namely jam bottles, reagent bottles and petri plates were procured from M/s. Borosil Glassware Ltd., Chennai, India.

3.2 Methodology:

3.2.1 Selection and preparation of explant:

Axillary buds are the most regenerable explants. Young axillary buds are physiologically very active and affected by environmental factors such as exogenous plant growth regulators (Muralikrishna and Gowda, 1994). Success in *in vitro* micropropagation of lime from nodal explants of mature trees was reported by (Khayri *et al*., 2001) by using nodal segments as the source of explants.

The axillary bud separated from mother plant was used as explant. The nodal segments of 1.0-1.5 cm with at least one axillary bud were surface sterilized and inoculated in to the MS media by aseptic practices.

3.2.2 Surface sterilization of explants:

The axillary bud derived from healthy mature plants of seedless lime (*Citrus aurantifolia* S.) cv. Chakradhar was used as an explant. The explants were thoroughly washed with tween 20 under running tap water for 15-20 minutes properly followed by 2-3 successive washing with sterile double distilled water. The explants were further treated with 1% cetrimide solution with 0.1% bavistin for 20 minutes on rotating shaker. This facilitates proper action of treated chemicals on the explants taken. The explants were then washed properly with double distilled water (DDW).

To reduce phenolic compound, ascorbic acid (150 mg/l) used for 20 min with gentle shaking followed by two times washed with sterile double distilled water. Streptomycin (100 mg/l) treatment to avoid bacterial contamination for 10 min followed by repeated washing with DDW was performed. The explants were then subjected to different surface sterilants i.e. 1% NaOCl and 0.1% $HgCl_2$ and 1% KCl for different durations respectively (Table 3.1). The explants were dipped in 70%

ethanol for 30 seconds and finally rinsed thrice with double distilled water. Different sterilizing agents and time taken for explant surface sterilization and preparation are mentioned below:

Table 3.1: Sterilizing agents and time for surface sterilization

Sr. No.	Sterilizing agent	Concentrations	Time taken (min)
1.	Cetrimide solution	1%	20
2.	Bavistin	0.1%	
3.	$HgCl_2$	0.1%	5
4.	Ethanol	70%	0.5
5.	NaOCl	1%	5
6.	KCl	1%	1
7.	Ascorbic acid	150 mg/l	20
8.	Streptomycin	100 mg/l	10

All operation for *in vitro* culture was carried out inside a laminar air flow cabinet under aseptic condition using sterilized plants material, equipments, glass materials and chemicals. The cabinet was sterilized by germicidal ultraviolet light for at least 20 min prior to use. The cabinet surface was wiped and cleaned with medicated cotton with 70% ethanol.

3.2.3 Cleaning and disinfection of glassware's:

The glassware required for this experiment was washed with Labolin detergent followed by disinfection in solution containing potassium dichromate and concentrated H_2SO_4. The disinfection solution was prepared by dissolving 60 gm of potassium dichromate in 350 ml of distilled water, heated for 2-3 minutes and final volume was made to 1 litre by adding H_2SO_4. On subsequent day, disinfected glassware were thoroughly washed in to diluted solution for overnight. Finally the cleaned and disinfected glassware were allowed to dry on draining racks, until their sterilization.

3.2.4 Sterilization of equipment's and glassware's:

The glassware *viz.*, culture bottles, petridishes, pipettes, beakers, measuring cylinder, etc. were procured for sterilization. The culture bottles were closed with caps and kept for sterilization. Petridishes, pipettes, beakers, measuring cylinder etc. were sterilized by wrapping with aluminium foil and kept in wire mesh basket prior to autoclaving. The wire mesh basket containing glassware were autoclaved at 15 lbs at 121^0C for 20 min followed by drying in hot air oven at 80-100^0C for 1hr to remove excess moisture. Forceps and scalpel were sterilized by flame sterilized technique. The cultures showing unwanted microbial growth (contaminated cultures 2%) were discarded after autoclaving in order to destroy the source of contaminants.

3.2.5 Media composition:

The two type of culture medium used for this present study in that WPM (Hi-Media Laboratories) and Murashige and Skoog (1962) basel medium. Woody Plant Medium was originally formulated by Lloyd and McCown (1981) for the culturing of shoot tips of Mountain Laurel (*Kalmia latifolia*). Since then, it is widely used for the propagation of many woody plant species. The formulation is a nutrient blend of inorganic salts, vitamins and amino acid.

Media was prepared as a basal medium supplemented with organic acids and vitamins. Sucrose was added at 30.0 g/l, MS media supplement (Hi-Media Laboratories Pvt. Ltd. Mumbai) 3.55 g/l and myo-inositol at 0.1 g/l. Specific quantities of stock solutions of major and minor nutrients, organic constituents and growth regulators were pipetted out in a beaker. Sucrose and myo-inositol were added and dissolved. The volume was made up to 1 litre using double distilled water and the pH of the medium was adjusted to 5.8-5.9 using 0.1 N NaOH or 0.1 N HCl. The medium was then boiled and agar 0.8 percent was added in the boiling medium and dissolved to homogenize the medium. It was poured hot at the rate of 40-50 ml per sterilized bottle and 10-15 ml per sterilized culture tubes (150 mm x 25 mm). Growth regulators and sucrose were added before autoclaving.

Following quantities of stock solution, vitamins, sucrose and agar were taken to prepare half strength and full strength MS basel media.

Table 3.2: Chemical composition of MS (Murashige and Skoog, 1962) medium

Stock	Constituents	Concentration in stock solution g/L	Volume of stock in final volume mg/litre (for 1 L)	Final concentration in medium (mg/L)
A	NH_4NO_3	82.50	20 ml	1650.00
B	KNO_3	95.00	20 ml	1900.00
C	H_3BO_3	1.24	5 ml	6.20
	KH_2PO_4	34.00		170.00
	KI	0.166		0.83
	$Na_2MoO_4.2H_2O$	0.050		0.25
	$CoCl_2.6H_2O$	0.005		0.025
D	$CaCl_2.2H_2O$	88.00	5 ml	440.00
E	$MgSO_4.7\ H_2O$	74.00	5 ml	370.00
	$MnSO_4.4\ H_2O$	4.46		22.30
	$ZnSO_4.7\ H_2O$	1.72		8.60
	$CuSO_4.5\ H_2O$	0.005		0.025
F	Na_2 EDTA	7.40	5 ml	37.55
	$FeSO_4.7\ H_2O$	5.57		27.85
G	Thiamine HCl	0.02	5 ml	0.10
	Nicotinic acid	0.10		0.50
	Pyridoxine HCl	0.10		0.50
	Glycine	0.40		2.0
	Myo-inositol	-	100 mg	100 mg
	Sucrose	-	30 g	30 g
	Agar	-	8.0 g	8.0 g

*$FeS0_4.7H_2O$ was dissolved in approximately 40 ml of distilled water. Na_2.EDTA was dissolved in approximately 40 ml of distilled water. Heating separately and mixed (under continuous stirring) with $FeS0_4.7H_20$ solution. After cooling, the volume was adjusted to 100 ml. heating and stirring resulted in a more stable FeNa-EDTA complex.

3.2.6 Procedure for preparation of MS culture media:-

1. One liter of MS basal media was prepared by taking 20 ml of each stock (A & B) of macronutrient (Table 3.2) in a clean and sterile beaker.
2. To the beaker, 5 ml of stock of micronutrient (Table 3.2) was added and stirred well with the help of magnetic stirrer.
3. Five ml of stock of iron source was added in the beaker and stirred well with the help of magnetic stirrer.
4. The major source of carbon (sucrose) was added at a concentration of 30 g/l (3%) and dissolved to the medium with continuous stirring and add 3.55 g/l MS media supplement and the volume of solution was made up to 900 ml with doubled distilled water.
5. The pH of the medium was adjusted to 5.8 by using 0.1 N HCl and 0.1 N NaOH before sterilization.
6. The final volume of solution was made to one liter by using sterile doubled distilled water.
7. The solidifying agent, agar-agar was added to the medium at 0.8% concentration (8 g/l) and mixed thoroughly to the medium by gentle heating to the media.
8. The required quantity of media i.e. about 25 ml per test tube and 40 ml per sterilized jam bottle for establishment and proliferation stage was dispensed, capped with cotton and labelled properly.
9. The bottles were plugged with autoclavable screw caps and culture tubes were plugged with non-absorbent cotton and autoclaved at 15 lbs (1.06 kg/cm^2) pressure a temperature of 121ºC for 20 minutes (Bhojwani and Razdan, 1983).
10. After sterilization, the media was allowed to cool and then required (As per experiment) volumes of growth regulators (BAP, NAA and Kinetin) were added separately in each bottle in front of laminar air flow cabinet under aseptic condition.
11. Cefotaxime sodium salt (Lupin Pvt. Ltd. Pune) antibiotics were added to the culture medium by using micropipette inside laminar air flow cabinet at concentration 60 µl/40 ml media.
12. The jam bottles were sealed with *Para film* and stored at 25^0C until use.

3.2.7 Precaution and care taken while preparing media:

- Appropriate volume of stock of micro and macro nutrients were measured and mixed thoroughly and added in appropriate concentration.
- Sucrose was used as carbon source added at 3% concentration (30 g/l).
- Growth hormones like BAP, NAA and Kinetin were added to the medium in adequate amounts as mentioned in (Table 3.3, 3.4 and 3.5).
- Appropriate pH of the medium was ensured before addition of gelling agent as acidic pH will lead to decreased gelation resulting in semi-solid flowing gel while alkaline pH will lead to formation of hardened gel.
- We had tested five level of pH (5.2, 5.4, 5.6, 5.8, and 6.0) for influence of pH on shoot induction.
- Agar was added in the specified amount, and then dissolved through heating on a gas burner. Further, media was dispensed in Jam bottle and autoclaved at 15 1bs pressure, 121^0C temperature for 15 minute and kept for cooling in media storage room till further use.

3.2.8 Preparation of stock solution of growth hormones:

The growth hormones (BAP, NAA and Kinetin) were dissolved in a few drops of the solvent (NaOH) or absolute ethanol and then volume was made up to the required level with double distilled water and then filter sterilized with syringe filter. The stock of growth hormones was prepared in 1 mg/ml and stored at refrigerator at 4^0C until further use.

3.2.9 Transfer area and aseptic manipulations:

All the aseptic manipulations such as surface sterilization of explants, preparation and inoculation of explants and subsequent subculturing were carried out under aseptic conditions in the hood of a clean laminar airflow chamber. The working table of the laminar air flow chamber was first surface sterilized with absolute alcohol and then by switching on the U.V. light for 20-30 minutes before work started. The Petri dishes as well as instruments used for inoculation were earlier steam sterilized in an autoclave at 15 lbs pressure and 121ºC for 20 minutes were flame sterilized before each inoculation. Hands were also swabbed in 70 % alcohol before inoculation.

3.2.10 Inoculation of Explants for Shoot Initiation, Multiplication:

1) The surface sterilized small young branches collected in glass plate and the axillary bud (2-3 cm) was cut with help of sterile forcep and scalpel blade (size-15).
2) The separated explants were inoculated in culture bottle with the help of sterilized forcep.
3) The initial incubation of explants was exposed in darkness and subculturing to fresh medium.

3.3 Culture room

All inoculation steps were performed in laminar air flow cabinet under aseptic conditions. The inoculated cultures were incubated at 25±2ºC in an air-conditioned culture room with a light intensity of 2000-3000 Lux by cool while fluorescent tubes. Photoperiod was maintained 16 hours daily. The following various treatments were employed for optimization protocol in Seedless lime listed below by using growth medium along with different concentration of auxin and cytokinin.

3.4 Experiment Details for Shoot Proliferation:-

MS medium with various concentrations of auxin and cytokinin tested for *in vitro* multiple shoot induction of seedless acid lime cv. Chakradhar based on the different treatments applied to the experiment. For establishment stage, BAP 0.1 to 1.0 mg/l were used while, for proliferation Stage NAA 0.1 to 0.5 mg/l and kinetin 0.25 to 1.0 mg/l along with BAP (0.25, 0.5, 0.75 and 1.0 mg/l) were tested for the cultivar Chakradhar.

Experiment- I

The present treatment was carried out on MS basal medium with different concentration of cytokinin (BAP) to standardize the most suitable shoot initiation medium with nodal explant. Standardization of shoot establishment medium there were various concentration of BAP used. The treatments are given as below -

Table 3.3: Standardization of shoot induction and proliferation medium

Treatments	No. of explant cultured	Replication	Hormone concentrations for shoot proliferation
T1	15	3	Only full MS (control)
T2	15	3	MS+ BAP 0.10 mg/l
T3	15	3	MS+ BAP 0.20 mg/l
T4	15	3	MS+ BAP 0.25 mg/l
T5	15	3	MS+ BAP 0.30 mg/l
T6	15	3	MS+ BAP 0.40 mg/l
T7	15	3	MS+ BAP 0.50 mg/l
T8	15	3	MS+ BAP 1.00 mg/l

Experiment- II

The experiment II was carried out on MS basal medium with different concentration of BAP along with kinetin to standardize the most suitable shoot multiplication medium and to study effect of different combinations of growth regulators on *in vitro* multiple shooting in seedless lime using nodal explant are given as below-

Table 3.4: Details of the multiplication medium (BAP + Kinetin)

Treatments	BAP (mg/l)	Kin (mg/l)
BK-1	0.0	0.50
BK-2	0.0	1.00
BK-3	0.25	0.50
BK-4	0.25	1.00
BK-5	0.50	0.50
BK-6	0.50	1.00
BK-7	1.00	0.50
BK-8	1.00	1.00

Experiment- III

The experiment III was carried out on MS basal medium with different concentration of BAP along with NAA to study effect of different concentration of growth regulators on *in vitro* multiple shooting in seedless lime using nodal explant are given as below-

Table 3.5: Details of the multiplication medium (BAP + NAA)

Treatments	BAP (mg/l)	NAA (mg/l)
BN-1	0.0	0.25
BN-2	0.25	0.10
BN-3	0.25	0.25
BN-4	0.25	0.50
BN-5	0.50	0.10
BN-6	0.50	0.25
BN-7	0.50	0.50
BN-8	0.75	0.25
BN-9	1.00	0.25

Table 3.4 and 3.5: MS medium (1962) was used throughout the study. Growth regulators values were in mg/l.

3.4.1 Observation to be recorded:

Observations were taken frequently for each of the stage of shoot proliferation i.e. shoots initiation, multiple shooting. The observations were recorded on basis of the following points.

a) Study on multiple shoot induction:

Responses of axillary bud explant for shoot induction on different media concentration in seedless lime cv. Chakradhar was recorded. The observations were noted based on the following points.

1. Effect of culture medium and pH on shoot proliferation
2. Days to shoot initiation and completion of shoot regeneration (Mean)

   Mean number of days taken by each of the inoculated explant to initiate the multiple shoots were counted from the date of inoculation.
3. Effect of different concentrations of growth regulators on multiple shoot induction
4. Number of shoots per explant (mean)

   The mean number of shoots developed from each explant were numerically counted separately for each media and recorded as number of shoots per explant during subculture.
5. Length of shoot (cm)
6. Percent regeneration (%) and shooting response (%)

b) Statistical Analysis:

Completely randomized design (CRD) was employed to analyze the "*In vitro* multiple shoot induction from nodal explant in seedless lime". Each of the treatment was replicated thrice with 15 samples (culture bottles) for each media treatment (phytohormones). The data in percentage were transformed to arc sin values for statistical analysis. The data were subjected to ANOVA as suggested by Panse and Sukhatme (1967) to evaluate their responses in different treatment in respective experiments. The mean, standard error and critical difference (5% probability) values were tabulated.

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Results and Discussion

CHAPTER- IV

RESULTS AND DISCUSSION

The experimental findings on "*In vitro* multiple shoot induction from nodal explant in seedless lime (*Citrus aurantifolia* Swingle)" cv. PDKV Chakradhar conducted at the Tissue Culture Project, Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani during the period of 2017 – 2018.

The aim of this experiment was to develop *in vitro* multiple shoot induction protocol in seedless lime (cv. Chakradhar) and investigate the effect of BAP, Kinetin and NAA with possible combinations for shoot proliferation, shoot multiplication from the axillary bud explants of seedless lime. Plant tissue culture technique has been successfully used for commercial production of microbe free plants and to conserve the germplam (Silva and Jumin, 2003). During this experiment the following steps were performed *i. e.* explant collection, explant isolation, sterilization, inoculation and standardization of protocol for efficient multiple shoot induction.

4.1 Study of culture medium and pH:

In this study, in order to test the influence of the culture media on shoot induction, two formulations were evaluated; MS (Murashige and Skoog, 1962) and woody plant medium (WPM) (Lloyd and McCown, 1980). Woody plants have low responsiveness to exogenous growth regulators.

During *in vitro* study of seedless lime Murashige and Skoog (1962) medium was used as basal medium. The stock solution of major and micronutrient and vitamins as A, B, C, D, E, F and G were prepared and stored at 4^0C (Table 3.2). Media was prepared by using stock solution, 3% sucrose, 0.8% agar agar, 3.55g MS media supplement and variable concentration of hormones. The pH was adjusted to 5.8 by using 0.1N NaOH and 0.1N HCl, Agar agar was dissolved in medium and final volume was adjusted with sterile water.

Kobayashi *et al.* (2003) found that WPM is best for vegetative growth compared to MS and MT medium in sweet orange cv. Pera by using mature tissue as an explant source. However, in contrast MS medium has been proved to produce best vegetative growth characteristics compared to WPM medium (Patil *et al*. 2011). He compared both WPM and MS medium with different levels of BAP and NAA but WPM medium showed poor proliferation response compared to MS. The composition of the culture media used for citrus tissue culture is usually based on the nutrients and vitamins of MS and MT media, although WPM has been successfully used for tissue culture of recalcitrant woody species (Lloyd and McCown, 1980). In present experiment WPM has not given that much response for shoot proliferation.

pH is the measure of the intensity of acidity or alkalinity and measure the concentration of hydrogen ions in water. During media preparation, the pH is a critical factor. We had tested five level of pH (5.2, 5.4, 5.6, 5.8, and 6.0) for influence of pH on shoot induction. The lower media pH (5.2 to 5.4) didn't result proper solidification while higher pH (5.8 to 6.0) result in solidification media. The desired solidification of media was best achieved with the pH 5.6 to 5.8. We have tried different pH range of the media to observe the effect of pH on solidification of media from 5.2 to 6.0. Acidic pH will lead to decreased gelation resulting in semi-solid flowing gel while alkaline pH will lead to formation of hardened gel.

MS medium has been frequently used for micropropagation of large number of plant species including fruit plants (Bouza *et al*., 1994). Although the composition of the media used for *in vitro* culture of citrus is usually based on the nutrients and vitamins of the MS medium (Abdulaziz and Bahrany, 2002; Begum *et al*., 2004).

The use of adenine sulphate, glutamine and casein hydrolysate increases the rate of shoot proliferation supplemented with 2.5 mg/l BAP (Siwach *et al.,* 2012). Shoot elongation was also enhanced by the presence of silver nitrate during the proliferation stage; it was considered to stimulate multiple shoot production and elongation (Roussos *et al.,* 1999) and inhibit ethylene action (George, 1993). In this study, adenine sulphate, glutamine, casein hydrolysate and silver nitrate were not used during establishment stage, although we have obtained good multiplication of shoots at proliferation stage.

4.2 *In vitro* shoot regeneration of seedless lime:

4.2.1 Explant preparation and sterilization:

(a) Explant selection and isolation

Axillary buds (nodal segment) were the most regenerable explants; young axillary buds are physiologically very active and may be the most easily affected by environmental factors such as exogenous plant growth regulators. Axillary bud proliferation is widely practiced for *in vitro* propagation of citrus because it ensures maximum genetic uniformity of the resulting plants.

There were different vegetative material to produce adventitious buds from callus as well as directly from explant to provide a non-callus procedure for virus elimination and rapid multiplication (Barlass and Skene, 1982). The type of explants also influences the *in-vitro* regeneration. Nodal explant has been found superior to shoot explants to initiate the culture of various mature fruit and forest trees (Bhojwani and Razdan, 1983).

Responsive nodal explant was obtained by pruning the mother plant. Our finding support the suggestion those in woody plants, axillary meristem from shoot of a severely pruned source plant are more amenable to physiological reinvigoration and respond better to tissue culture (Hackett, 1985; Bonga *et al.,* 1992).

The experimental material comprised seedless acid lime cultivar Chakradhar. Axillary bud (nodal explant) was used as explant for *in vitro* studies, these shoot pieces were collected in the sterilized polythene bag and brought to the laboratory. From this freshly sprouted shoots axillary bud was cut by sterilized surgical blade for the separation of explant. The nodal explants were prepared containing single node of 1.0 -1.5 cm.

(b) Explant source

In this study, axillary bud was selected as explant for the *in vitro* multiple shoot induction in *Citrus aurantifolia* cv. Chakradhar. As per previous study (Syamal *et al*., 2007; Rathore *et al*., 2007; Samarina *et al*., 2010; Tornero *et al*., 2010; Goswami *et al*., 2013; Chamandoosti, 2017) axillary bud proliferation from nodal explant excised from mature trees are desirables explants for regeneration. They also reported that dormant bud must be collected prior to appearance of new leaves and

branches on the trees, but a complete study on seasonal variation and establishment of culture from dormant buds would clarify the most appropriate stage of bud for culture establishment.

For *in vitro* studies, axillary bud from seedless lime was used as explant for inoculation. Axillary branches of 5-10 cm long from new growth flushes (2-3 week old) were carefully taken from the mature tree (1 to 2 year old) from the Central Nursery Scheme, VNMKV, Parbhani.

In citrus, the best results of rapid proliferation are usually obtained using juvenile explants (Perez- Molphe and Ochoa-Alejo, 1997). It has previously been reported that differently from most juvenile explants, explants from mature citrus plants require balanced combinations of BAP and NAA in the culture media for maximum shoot regeneration (Almeida *et al*., 2003; Rodriguez *et al*., 2008).

(c) Microbial contamination and Exudation of phenolic compounds:

One of the most important factors limiting the use of mature explants for tissue culture is microbial contamination. Microbial contamination from the explants leads to the contamination on the medium and reduces development of explants. To avoid and minimize this problem, various workers tried the use of fungicide (M - 45), streptomycin and mercuric chloride etc. and such compounds have been suggested to control microbial contamination (Kour *et al*., 2007). Microbial contamination was successfully restricted by using fungicide (0.1% bavistin), streptomycin and mercuric chloride. About 85 to 90 percent reduction in microbial contamination was observed using this treatment as compared with control (explant without treatment).

The most severe problems associated with the establishment of shoot cultures and callus cultures is the extensive release of phenolic compounds, which leads to browning of the medium, necrosis of the explants, and eventual death of the explants. In woody plant species, phenolic compounds are produced in response to wounding; these can be deleterious to growth of *in vitro* cultures. The initial incubation of explants was exposed in darkness and subculturing to fresh medium was followed at 24 hours interval up to eight days of initial inoculation. This had overcome the harmful effects of phenol exudation. The use of antioxidant solution

(ascorbic acid and citric acid; 150 mg/l and 100 mg/l respectively) reduced the phenol secretion and transferred the explants were transferred to fresh medium.

The addition of antibiotics to the culture medium had helpful to eliminate bacterial contamination, which hinder the *in vitro* establishment of explants from mature tissues. Beta-lactam antibiotics, such as carbenicillin and cefotaxime, are the most commonly used antibiotics in plant transformation protocols, since they have a broad spectrum of activity against bacteria and a low toxicity to eukaryotes (Bhau and Wakhlu, 2001; Yu *et al*., 2001). We used cefotaxime sodium salt 60 µl/40 ml media for our experiment had given best results.

(d) Surface sterilization of explant

To avoid the problem of contamination, present study was carried out with axillary bud of seedless lime as explant. The nodal explants were surface sterilized with 1% NaOCl (5 min), 0.1% Mercuric chloride (5 min), 70 % ethanol (0.5 min), 0.1% bavistin (20 min) and cultured aseptically on MS medium containing different concentrations on phytohormones.

The beneficial effect of $HgCl_2$ and NaOCl had reported earlier by several investigators. In addition to $HgCl_2$ and NaOCl one min treatment with 1% KCl improves survival percentage of explant as KCl in known to remove excess Hg^{++} ions from explant tissue. Increase in exposure time of the above surface sterilants drastically affected explant survival. One reason for the death of explants when exposed to surface sterilants for longer duration may be due to heavy metal contamination of mercury present in $HgCl_2$. The explants obtained were in conformity by (Syamal *et al.,* 2007) that used NaOCl and $HgCl_2$ as surface sterilants for sterilizing citrus explants.

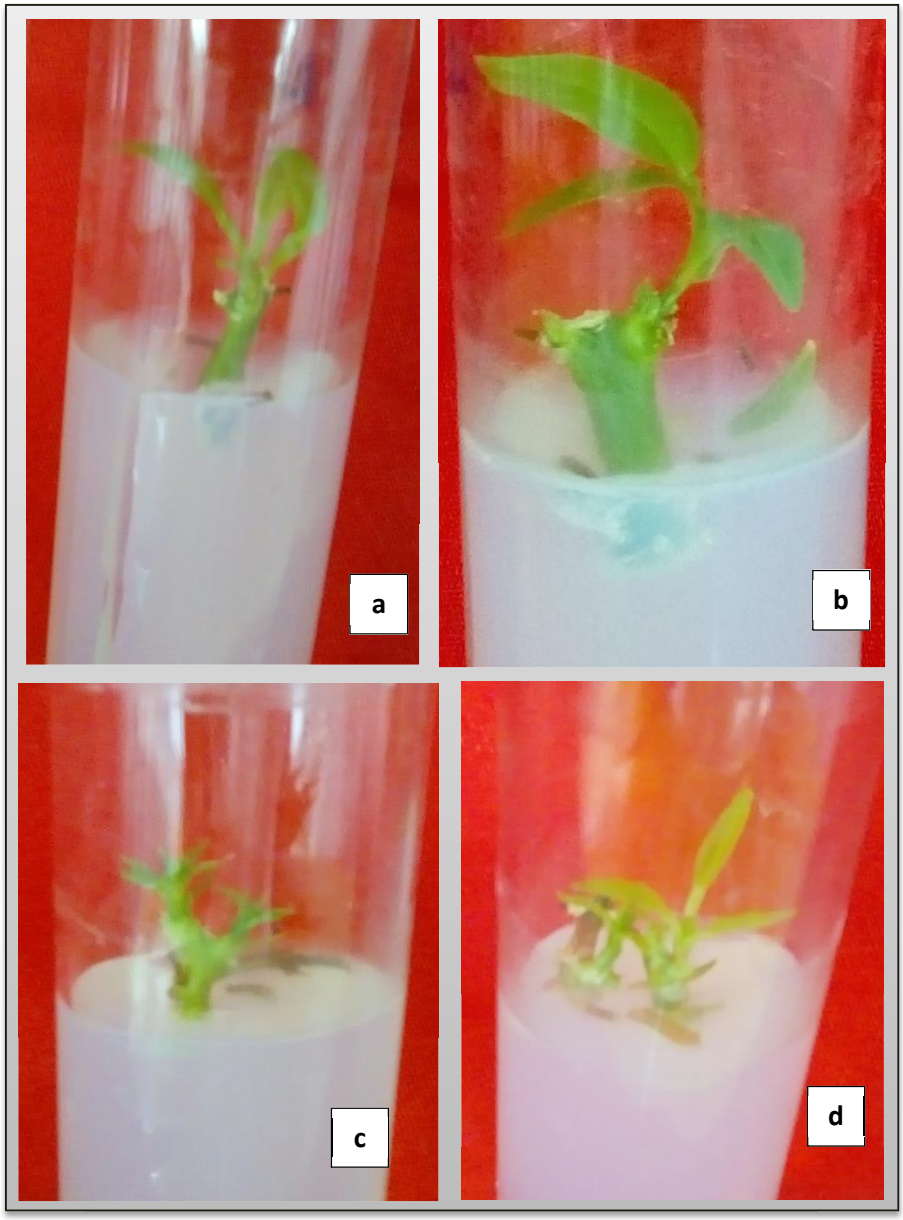


Fig. 4.1: Shoot initiation stage of seedless lime (*Citrus aurantifolia)* after 30 days. Shoot initiation by using 0.30 mg/l BAP (a & b) and 0.25 mg/l BAP (c & d).

4.3 Shoot establishment

In vitro citrus plant regeneration via organogenesis has been described from several tissues, including roots (Bhat *et al.,* 1992; Sauton *et al.,* 1982; Sim *et al.,* 1989), leaf sections (Chaturvedi and Mitra, 1974) and stem internodes (Barlass and Skene, 1982; Duran *et al.,* 1989; Moore, 1986). Previous studies have been shown differences in the responses to culture conditions. They detect effect of various combinations and concentrations of growth regulators from nodal explants on adventitious shoot formation.

The efficiency of nodal bud with different concentration of BAP hormones for shoot initiation was investigated based on the hormone treatments used by the previous workers on seedless acid lime. Nodal explants were inoculated on MS media supplemented with different concentrations of BAP for optimization of the shoot induction protocol from axillary bud and concentrations for shoot multiplication (Table 3.3). Effect of various concentrations of BAP growth regulators to shoot establishment and induction from nodal explant are given below (The data recorded 30 days after inoculation).

4.3.1 Number of days required for shoot initiation

The effect of different BAP concentrations and their interactions for number of days required for shoot initiation are presented in Table 4.1 and are graphically illustrated in Graph 1. The effects are shown in Fig 4.1.

It is evident from the data presented in table 4.1 that the nodal explant has significant effect on the number of days required for shoot initiation.Treatments T5 (0.3 mg/l) and T4 (0.25 mg/l) was statistically superior as required lesser number of days 8.6 and 8.8 respectively for shoot initiation than the other treatments. MS medium containing BAP (0.25 and 0.3 mg/l) was found best for shoot initiation (Fig. 4.1; Table 4.1). Similarly, Shah *et al*. (1999) reported that earliest shoot initiation in *Citrus spp.* took place on MS medium + BAP 0.25 mg/l. Maximum days taken for shoot initiation were noted with BAP 1.0 mg/l (17.40 days) whereas, control was (22.40 days) (Graph 1).

Table 4.1: Standardization of BAP medium for shoot establishment using Nodal segment

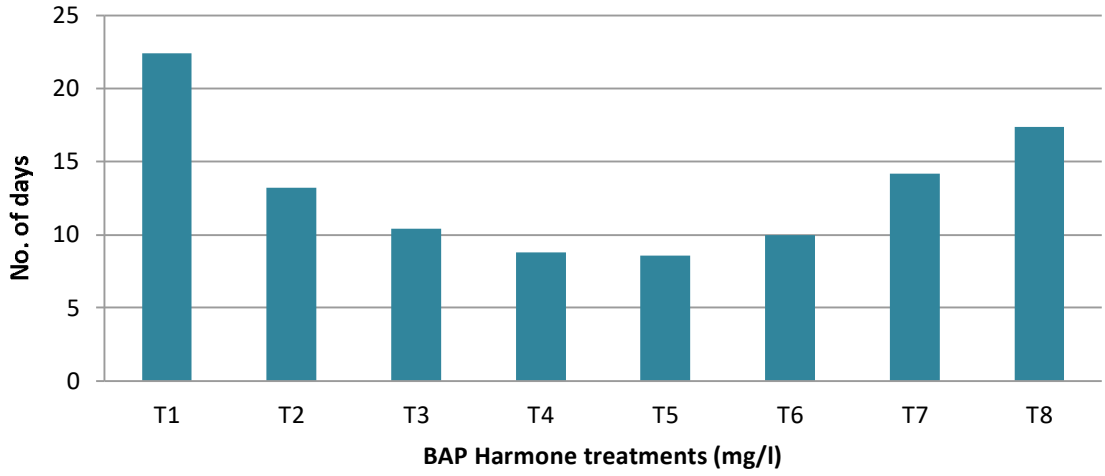
Shoot induction treatment	Hormone supplemented in mg/l (BAP) with full MS	Days for shoot initiation (Mean)	Days for completion of shoot regeneration (Mean)	Percent regeneration (Mean)
T1	Full MS (control)	22.40	41.20	26.42 (30.93)
T2	0.10	13.20	32.00	78.05* (62.06)
T3	0.20	10.40*	28.40*	82.00* (64.90)
T4	0.25	8.80*	25.80*	88.18* (69.89)
T5	**0.30**	**8.60***	**25.20***	**90.00* (71.57)**
T6	0.40	10.00*	27.60*	84.34* (66.69)
T7	0.50	14.20	34.00	68.00 (55.55)
T8	1.00	17.40	40.20	52.60 (46.49)
(Mean)		13.13	31.80	71.20
± SE		0.64	0.76	0.82
CD at 5%		1.93	2.32	2.50

Figures shown in brackets denote arc sin transformed values

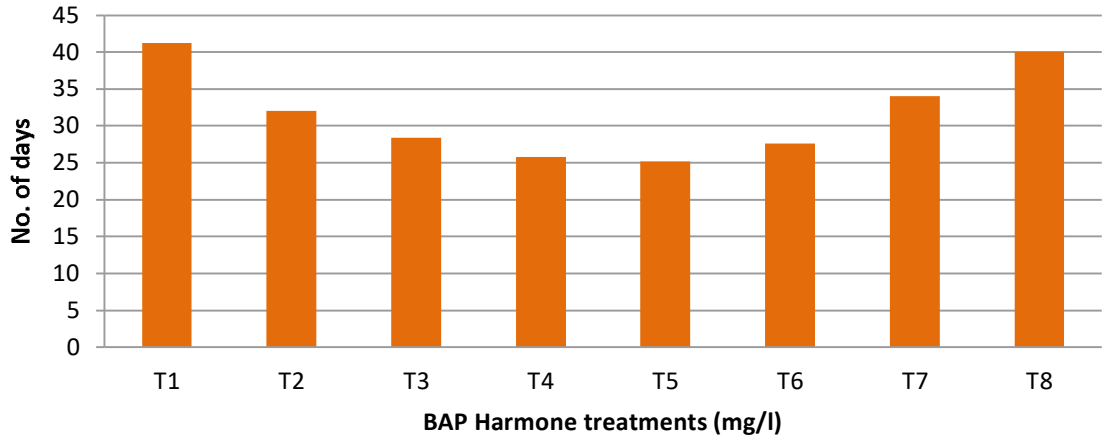
** Significance at 0.05 probability level.*

Note: The values represent the mean of 3 separate replications/ experiments for statistical analysis such as estimation of mean and standard error.

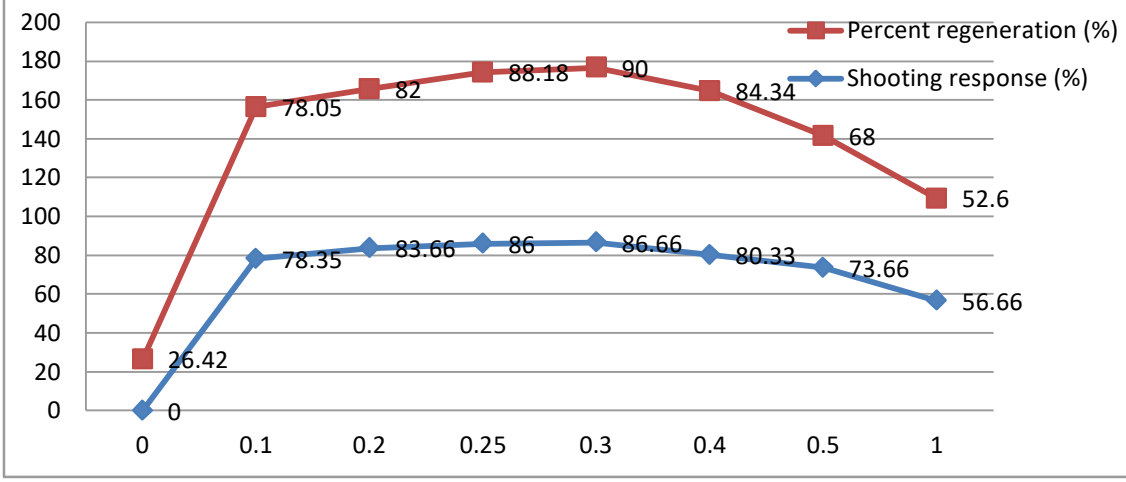
Graph 1: Number of days required for shoot initiation



Graph 2: Number of days required for completion of shoot regeneration



Graph 3: Effect of BAP concentration (mg/l) on percent shoot regeneration and shooting response (%)



The present results indicate that the hormone treatment differences in respect of number of days required for shoot initiation. As regard to the media BAP 0.25 mg/1 and 0.30 mg/l concentration were found effective in reducing the number of days required for shoot initiation as compared to the lower BAP 0.10 mg/l and higher BAP 1.00 mg/l concentrations. Results obtained in experiment were similar with Desai *et al.,* (1996) who obtained 100% culture proliferation with maximum multiple shoot per explant of acid lime on modified MS media supplementary with BAP 0.25 mg/1 and ME 200 mg/l.

4.3.2 Number of days required for shoot regeneration

The data indicated that there were significant differences among various concentrations of BAP in respect of number of days required for shoot regeneration.The total mean number of days taken for shoot regeneration (2.5 cm in height) was counted numerically from the inoculation to subculture. The effect of different levels of BAP on number of days required for completion of regeneration is presented (Table 4.2). Maximum days were taken for completion of regeneration on MS medium with BAP 1.0 mg/l (40.20 days). Treatments T1 (control) and T8 found inferior however, in treatment T5 (BAP 0.3 mg/l) the regeneration was completed within 25.20 (Mean) days that was superior. Treatments T4 (0.25 mg/l) and T5 (0.30 mg/l) were *at par* with each other (Graph 2).

4.3.3 Percent shoot regeneration

The number of cultures which responded to shoot regeneration was counted in each treatment medium excluding the contaminated (about 2%) cultures inoculated and multiplied with 100 and represented as percent shoot regeneration.

$$\text{Percent shoot regeneration} = \frac{\text{Number of culture regenerated}}{\text{Total number of cultures inoculated}} \times 100$$

From table 4.1, it was revealed that the 90% shoot regeneration took place by using 0.30 mg/l and 88.18% by 0.25 mg/l BAP treatments, which were significantly superior for the shoot establishment. Whereas, the least result of 52.60% (Mean) was obtained with 1.0 mg/l BAP on MS medium as well as control (Graph 3).

Fig. 4.2: Transfer of shoot during subculture of seedless lime (*Citrus aurantifolia*)

These results were found in agreement with Goswami *et al.* (2013), who observed 62.66% bud break in low BAP 0.1 mg/l, which was highest than high level BAP concentrations. Thus, BAP was required for shoot bud activation and multiple shoot formation from nodal explant. Results indicated that up to certain concentration of growth hormones axillary bud supports good response for shooting but after saturated concentration the response in term of shooting is declined. Regenerated plants were transferred to MS nutrient medium with the addition of BAP, Kinetin and NAA in different combinations to further multiplication.

4.4 Shoot proliferation and multiplication

In present studies, the regeneration of shoot was demonstrated from nodal segment excised from axenic shoot culture on MS medium. The shoot regeneration was achieved on MS basal medium containing BAP at different concentration. The *in vitro* shoot proliferation was not only influenced by the type of growth regulators; but also with their concentrations.

Multiplication of shoots of seedless lime (*Citrus aurantifolia*) was achieved by repeated transfer of original explants and subculture of *in vitro* produced shoots. Repeated transfer of mother explant has been suggested as one of the effective approaches for *in vitro* cloning of woody plants (Deora *et al*., 1995). Multiplication of shoots *in vitro* by repeated transfer of mother explant or subculture of shoots is always effected by the concentration of growth hormones used.

As mentioned earlier, the interaction between plant growth regulators have significant effects on induction of shoots also number of shoot multiplied. Three subcultures for the shoot multiplication were performed for three weeks duration. A protocol for multiple shoot induction has successfully been developed for clonal propagation of seedless lime (*Citrus aurantifolia*) cv. Chakradhar.

The explant with control and 0.25 mg/l NAA on MS culture media as well as WPM medium had not showed any development of shoots for the same period of culture despite repetitions of the experiment.

4.4.1 Hormonal Effect of BAP on multiple shoot induction

In seedless lime shoot proliferation and multiplication was performed by using nodal segment on MS media supplemented with BAP concentrations. Subculturing was performed on establishment medium and after 4 weeks the explants were transferred to a fresh medium having the same composition for further proliferation (Fig. 4.2). Data regarding number of days to shoot induction, number of shoots per explant, number of shoot length and shooting response were recorded.

In this study, the separate concentrations of BAP for shoot multiplication. Sprouting of bud was mostly observed (Fig.4. 3 a, b) and single shoot observed in very few (1 or 2) culture vessel. The sprouting of bud on the nodal segment was affected by the doses of BAP.

The average number of shoot buds initiated from nodal segment explant ranged from 2.10 to 5.66 shoots buds per explant (Table 4.2; Fig. 3 a, b). Explant were numerically counted separately for each media and recorded as number of shoots per explant during subculture. Nodal segment produced maximum number of shoots on MS medium with BAP 0.3 mg/l (5.66) which is *at par* with BAP 0.25 mg/l (5.0) showed in (Graph 4). The minimum number of shoots was observed on MS medium with BAP 1.0 mg/l (2.10).

The results obtained (Graph 4) indicated that BAP attains saturation level with the increased concentration during the *in vitro* shoot multiplication studies. The maximum (5.66) average number of shoots observed in treatment T5 (BAP 0.3 mg/l), which was significantly different with the other. Treatments T3 (BAP 0.2 mg/l) and T7 (BAP 0.5 mg/l) were observed *at par* with each other. Whereas, the least responsive treatment was T1 reported control (without BAP). Das *et al.* (2000) reported that the higher concentration of BAP (2 mg/1) induced callus formation and suppressed shoot proliferation and shoot length.

The present investigation indicates that *in vitro* shoot multiplication does not respond to the higher level of BAP but attains the saturation level at a specific concentration of BAP. Cytokinin induce bud break by activation of meristems and cause shoot proliferation (Murashige, 1974). Outgrowth of axillary

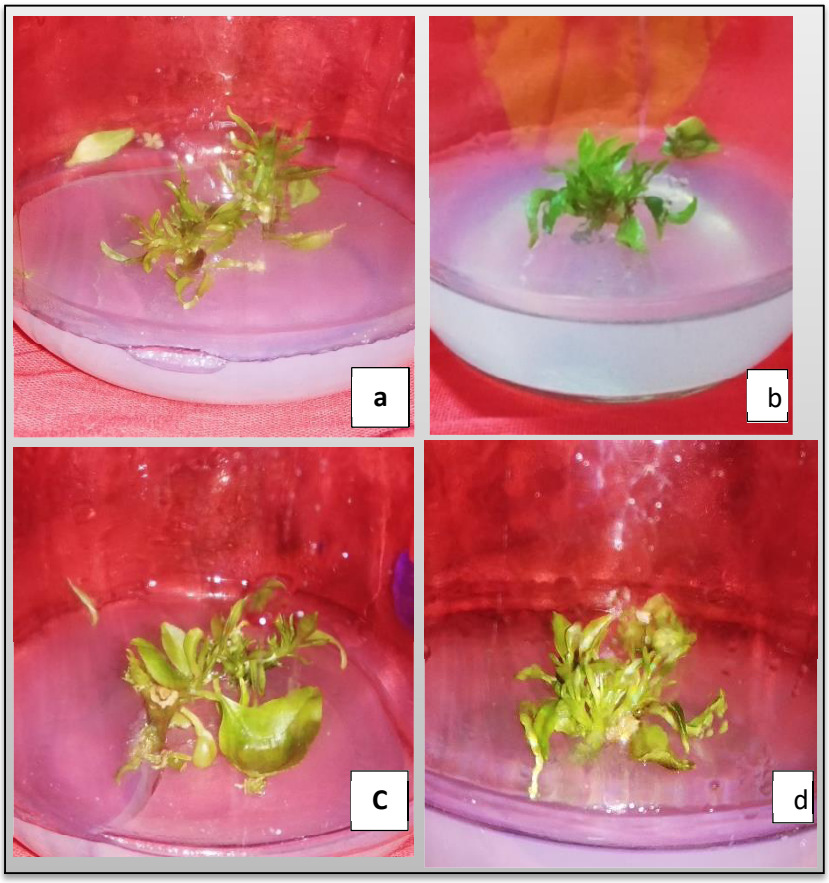


Fig. 4.3: Maximum bud sprouting on medium supplemented with BAP 0.25 and 0.30 mg/l (a & b respectively) and (c) 1.0 mg/l BAP+ 0.50 mg/l Kinetin (d) 0.25 mg/l BAP + 0.25 mg/l NAA of seedless lime.

buds is well correlated with the cytokinin level in the bud. It has been suggested that cytokinin independently regulates axillary buds proliferation (Shimizu *et al.,* 2001).

Table 4.2: Effect of BAP on MS medium for shoot proliferation and multiplication

Shoot induction treatment	Hormones supplements in mg/l (BAP) added to full MS	Avg. No. of Shoots/ explant	Length of shoots (cm)	Shooting response (%)
T1	Only full MS (Control)	0	0	0 (1.01)
T2	MS + BAP 0.10	3.00	1.95	73.60 (59.08)
T3	MS + BAP 0.20	3.66	2.43	83.56* (66.17)
T4	MS + BAP 0.25	5.00*	3.05*	86.00* (68.05)
T5	MS + BAP 0.30	5.66*	3.20*	86.66* (68.60)
T6	MS + BAP 0.40	4.33*	2.86*	80.33* (63.67)
T7	MS + BAP 0.50	3.80	2.74	78.35* (62.28)
T8	MS + BAP 1.00	2.10	2.24	56.66 (48.25)
Mean		3.44	2.31	68.03
± SE		0.24	0.13	0.81
CD at 5%		0.72	0.38	2.37

Figures shown in brackets denote arc sin transformed values

** Significance at 0.05 probability level.*

Note: All data collected in this experiment (60 days after inoculation) is obtained from three biological replications used for statistical analysis such as estimation of average data (mean) and standard error.

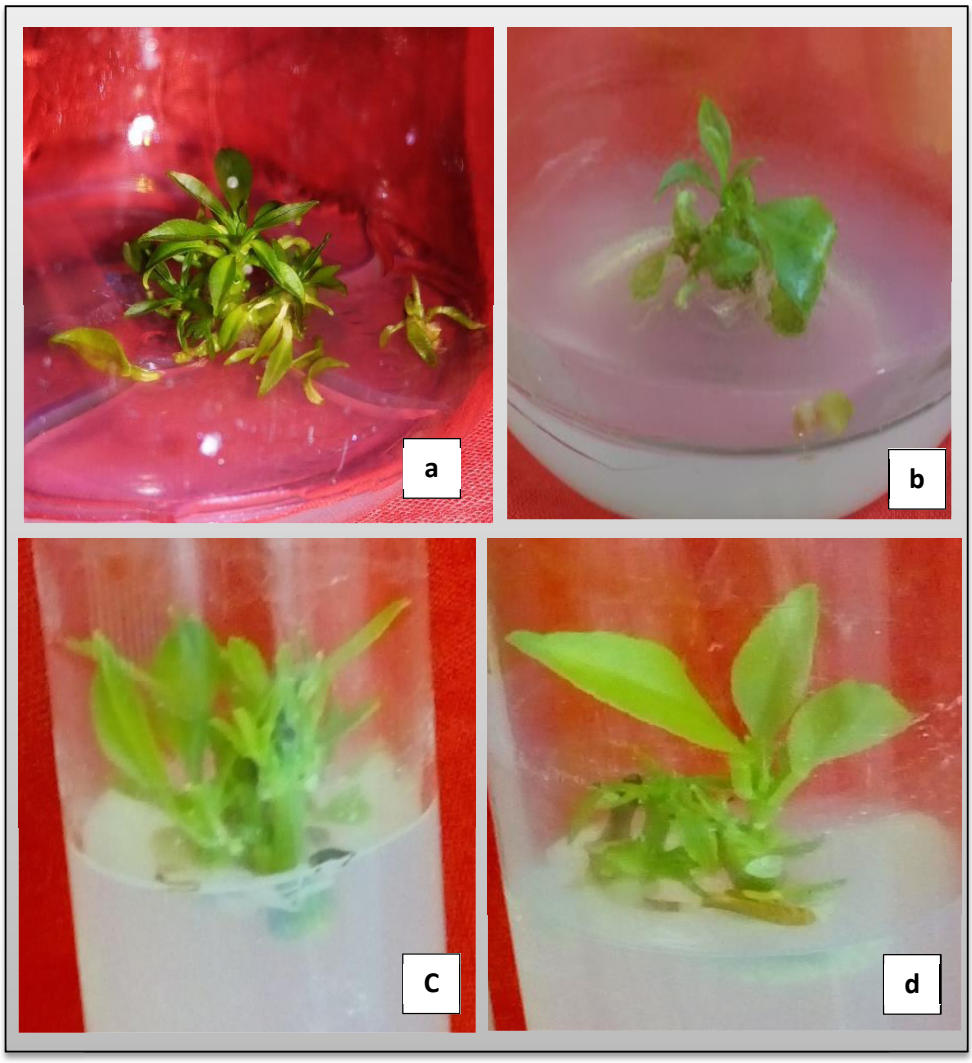


Fig. 4.4: Shoot multiplication of seedless lime by using (a) 0.30 mg/l BAP (b) 0.25 mg/l BAP (c) 1.0 mg/l BAP+ 0.50 mg/l Kinetin and (d) 0.25 mg/l BAP + 0.10 mg/l NAA supplemented to MS medium.

The number of shoots produced per explant in the present experiment was same as reported by Baruah (1996) who found best shoot proliferation in three citrus species with 0.3 mg/l BAP. Desai *et al.* (1996) also obtained maximum shoot proliferation on MS medium supplemented with BAP 0.25 mg/l and ME 200 mg/1. The results obtained in the present investigation were significant with the previous studies reported. The concentration of BAP 0.30 mg/1 was found to be optimum concentration.

Minimum four shoots per explant were obtained on the MS medium containing BAP 0.30 mg/l (Fig. 4.4 a). BAP alone has significant effect on shoot multiplication at lower concentration contradicted with findings of Kour *et al.* (2007) reported 3.88 (Mean) shoots per explant at 1 mg/l BAP. In accordance with our findings, the role of only BAP was also found to be highly significant for most of the citrus cultivars as reported by Dewan *et al*., (1992) and Pereira *et al*., (1995).

There were many studies that report shoot proliferation decreased with increasing concentration of BAP (Goswami *et al*., 2013). This was accordance with ours experiment concluding the frequency of response from nodal explants decreased with a progressive increase in the level of BAP. The result presented earlier indicate that treatment T5 (0.30 mg/l) superior 3.20 cm (mean) length of longest shoots over all other BAP concentrations. The findings were similar to Kamble *et al*. (2005) reported that maximum shoot length was observed on MS medium containing BAP 0.25 mg/l in acid lime cv. Sai-Sharbati.

Data pertaining to mean number of shooting response per treatments ranged from 0 to 86.66% with nodal explant. Highest shooting response (86.66%) was observed in treatments T5 (0.3 mg/l BAP) followed in T4 (0.25 mg/l BAP) 86 %. Control did not show any shooting response (Graph 3).

Table 4.3: Effect of (BAP + Kin) with MS medium on shoot proliferation and multiplication

Shoot induction treatment	Hormones supplements in mg/l added to full MS	Avg. No. of Shoots/ explant	Length of shoots (cm)	Shooting response (%)
BK-1	BAP 0 mg/l + Kin 0.5 mg/l	1.90	1.80	56.30 (48.82)
BK-2	BAP 0 mg/l + Kin 1.0 mg/l	1.40	1.60	52.66 (46.52)
BK-3	BAP 0.25 mg/l + Kin 0.5 mg/l	2.90	2.75*	80.00* (63.44)
BK-4	BAP 0.25 mg/l + Kin 1.0 mg/l	3.45*	2.25	76.50 (61.00)
BK-5	BAP 0.50 mg/l + Kin 0.50 mg/l	1.50	1.65	60.00 (50.77)
BK-6	BAP 0.50 mg/l + Kin 0.50 mg/l	2.33	1.50	65.00 (53.73)
BK-7	**BAP 1.0 mg/l + Kin 0.50 mg/l**	**6.00***	**2.56***	**86.30* (68.28)**
BK-8	BAP 1.0 mg/l + Kin 1.0 mg/l	4.35*	2.20	78.50* (62.40)
Mean		2.98	2.04	69.40
± SE		0.36	0.17	0.66
CD at 5%		1.10	0.51	2.03

Figures shown in brackets denote arc sin transformed values

** Significance at 0.05 probability level.*

Note: All data collected in this experiment (60 days after inoculation) is obtained from three biological replications used for statistical analysis such as estimation of average data (mean) and standard error.

4.4.2 Effect of (BAP + Kin) with MS medium on multiple shoot induction

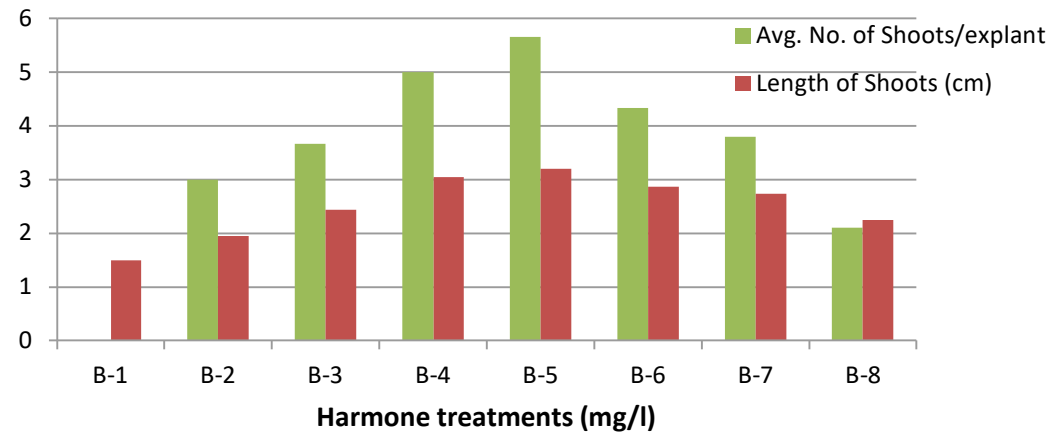
The morphogenic response of nodal segments of seedless lime inoculated on MS medium supplemented with various combinations of BAP and Kin are presented (Table 4.3). A significant effect was observed due to different concentrations of BAP and Kin. From the perusal of table 4.3 revealed that, 1.0 mg/l BAP + 0.5 mg/l kinetin resulted better shoot multiplication in minimum (10 days) while only 0.5 mg/l kinetin completed shoot induction in 15 days.

In present investigation nodal segment produced maximum (6.0) number of shoots per explant on MS medium containingBAP 1.0 + Kin 0.50 mg/l. The minimum (1.4) average number of shoots was observed on MS media supplemented with only Kin 1.0 mg/l.The sprouting of bud on nodal segment affected by the doses of BAP with kinetin combinations.The maximum sprouting of bud mostly observed as the shooting response given 86.30 % (Fig. 4.4 c; Graph 5).The least sprouting occurred on 1.0 mg/l Kin i. e. 52.66% and 56.30 % with 0.5 mg/l Kin.

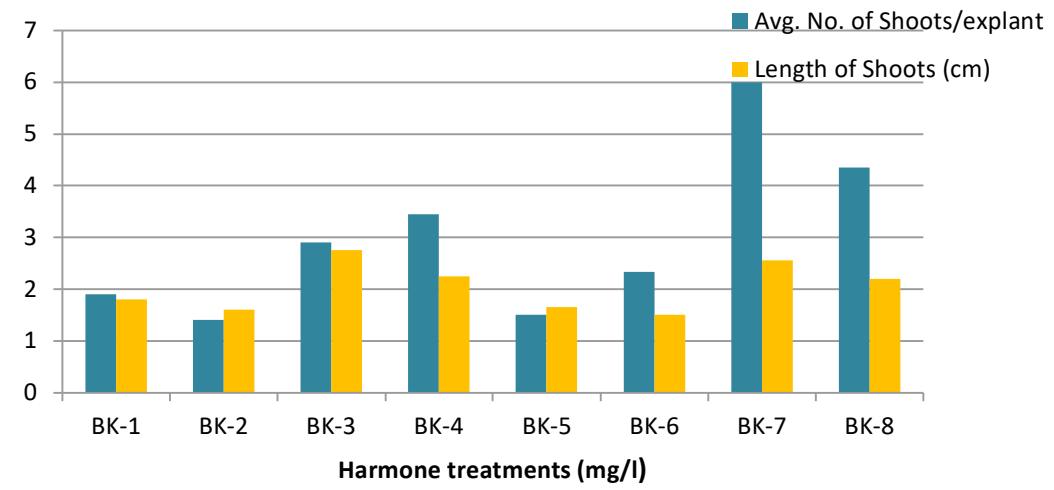
Goswami *et al*. (2013) also obtained maximum multiple shooting (5.5 shoots per explants) in MS medium supplemented with 1 mg/l BAP + 0.5 mg/l Kin using nodal segment. Similar results have been also reported by Mas *et al*. (1994) with the best multiplication rate of 5:1 achieved by culturing on MS medium + BA 1.0 + Kin 0.5 + NAA 0.5 mg/l. Khayri and Bahrany (2001) expressed the best results for multiple shoot formation with 8 shoots per node, using 4.44 μM BA and 2.32 μM kinetin.

Shoot length is considered as growth parameter of multiple shoots regenerated from different explants. Multiple shoots regenerated from nodal segment explant recorded maximum length on MS medium with BAP 0.25 + Kin 0.5 mg/l (2.75 cm) which was *at par* with BAP 1.0 mg/l + Kin 0.25 (2.56 cm) and minimum length1.5 cm and 1.6 cm observed on BK-6 (BAP 0.5 + Kin 0.5 mg/l) and BK-2 (Kin 1.0 mg/l) graphically presented in Graph 5. It clearly indicated that lower concentration of Kin with BAP had given better result.

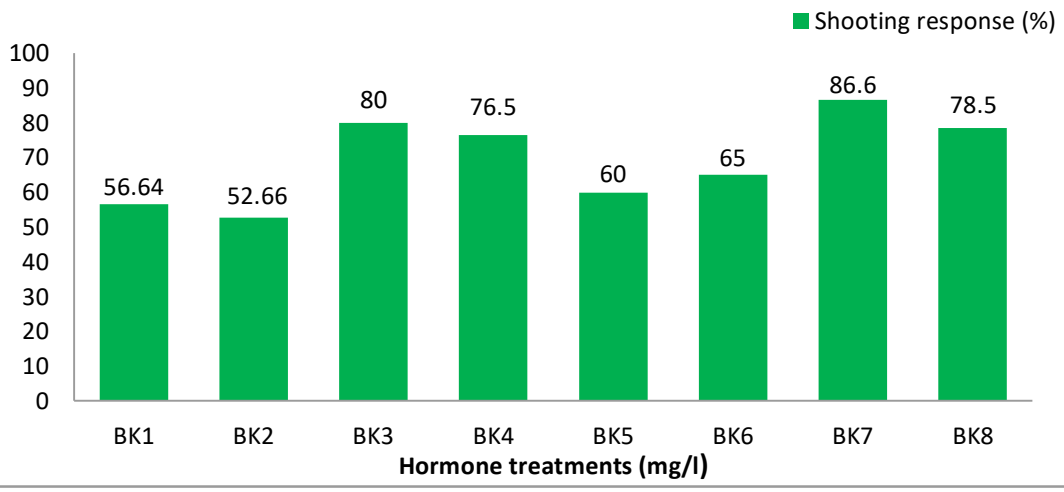
Graph 4: Length of shoots and No. of shoots per explant in different BAP concentration



Graph 5: Length of shoots and No. of shoots per explant in different BAP + Kin concentration



Graph 6: Multiple shooting response produced in different BAP + Kin concentration



4.4.3 Effect of (BAP + NAA) with MS medium on multiple shoot induction

In vitro shoot induction takes place on the shoot establishment medium supplemented by the various concentrations of BAP.These explants were transferred to the multiplication medium supplemented with the BAP and NAA at appropriate concentration (Table 10).

Data pertaining to the effect of BAP and NAA combinations on percent shooting response were presented (Table 4.4; Fig. 4.4). The data indicated that the best treatment for shoot multiplication from nodal segment explant was observed on MS with 0.1 mg/1 NAA + 0.25 mg/1 BAP with 88.33% shooting response in 11 days and maximum number of shoots per explant was 3.80 (Fig 4.4; Graph 7). Our finding support by Thirumalai and Thamburaj (1996) obtained higher percentage of shoot bud differentiation in sweet orange cv. Sathgudi and acid lime cv. PKM-1 on MS medium supplemented with NAA 0.1 mg/1 + BA 0.25 mg/1.

From the table 4.4 it revealed that, average number of shoots per explant, length of shoots and shooting response vary with treatments. In case of average number of shoots treatment BN-2 (BAP 0.25 + NAA 0.10 mg/l) was significant and *at par* with BN-3 (BAP 0.25 + NAA 0.25 mg/l). However, in case of length of shoots BN-3 and BN-5 found superior than BN-9 and BN-1. Multiple shooting responses showed highest in treatments BN-2 and BN-3 whereas, BN-1 (0.25 mg/l NAA) was least responsive illustrated in Graph 8.

The benefit of using a combination of cytokinin and auxin was that it stimulates shoot proliferation and organogenesis. The usefulness of BAP and NAA in culture medium in producing more number of shoots in sweet orange and acid lime was also reported by Duran *et al.* (1989). Similar results were reported by Desai *et al.* (1996) from axillary bud explant of Kagzi lime *(C. aurantifolia)*. From the table 4.4 found that the concentration of NAA increased than BAP there were decrease in average number of leaves, shoots and shoot length as well as shooting response.

The concentration of NAA 0.25 mg/l did not have any result, it means only NAA cannot used for *in vitro* shoot regeneration. In shoot regeneration studies of seedless acid lime, it is observed that combination of NAA and BAP in MS basal media was resulted in callus induction.

Table 4.4: Effect of BAP + NAA with MS medium on shoots proliferation and multiplication

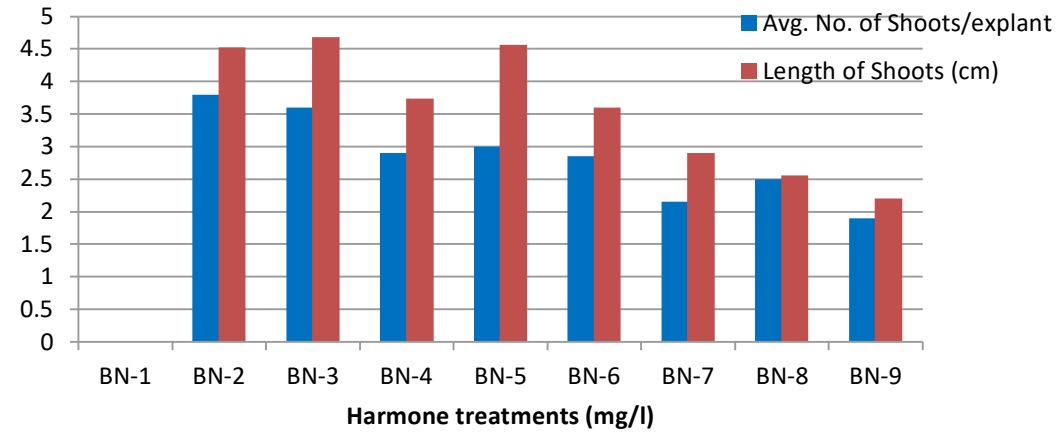
Shoot induction treatment	Hormones supplements in mg/l added to full MS	Avg. No. of Shoots/ explant	Length of shoots (cm)	Shooting response (%)
BN-1	BAP 0.0 mg/l + NAA 0.25 mg/l	0.0	0.0	0.0 (0.95)
BN-2	**BAP 0.25 mg/l + NAA 0.10 mg/l**	**3.80***	**4.52***	**88.33* (70.06)**
BN-3	BAP 0.25 mg/l + NAA 0.25 mg/l	3.60*	4.68*	86.00* (68.03)
BN-4	BAP 0.25 mg/l + NAA 0.50 mg/l	2.90	3.74*	68.40 (55.87)
BN-5	BAP 0.50 mg/l + NAA 0.10 mg/l	3.00*	4.56*	73.50* (59.02)
BN-6	BAP 0.50 mg/l + NAA 0.25 mg/l	2.85	3.60*	56.66 (48.83)
BN-7	BAP 0.50 mg/l + NAA 0.50 mg/l	2.15	2.90*	64.30 (53.31)
BN-8	BAP 0.75 mg/l + NAA 0.25 mg/l	2.50	2.56*	55.00 (47.87)
BN-9	BAP 1.0 mg/l + NAA 0.25 mg/l	1.90	2.20	50.45 (45.26)
Mean		2.52	3.20	60.30
±SE		0.24	0.27	1.00
CD at 5%		0.72	0.81	2.73

Figures shown in brackets denote arc sin transformed values

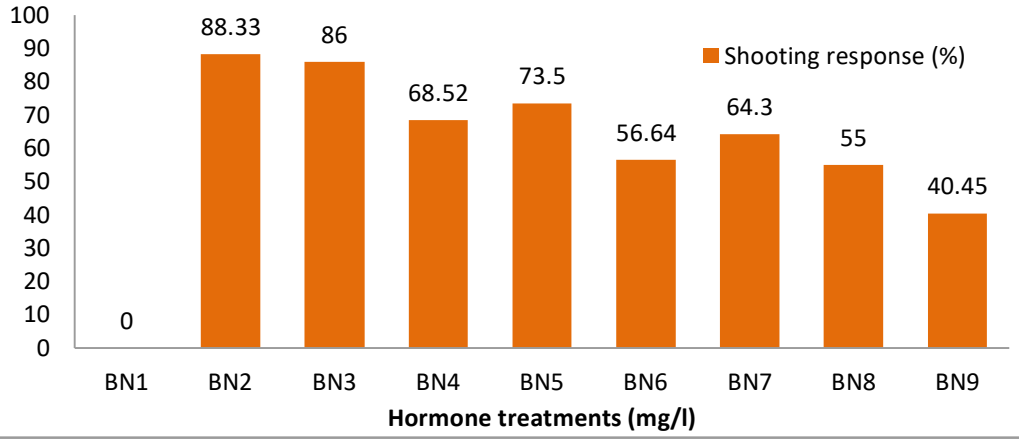
** Significance at 0.05 probability level.*

Note: The values represent the mean of 3 separate replications/ experiments for statistical analysis such as estimation of mean and standard error.

Graph 7: Length of shoots (cm) and No. of shoots per explant in different BAP + NAA concentration



Graph 8: Multiple shooting response produced in different (BAP + NAA) concentration



It indicated that the lower value of BAP and NAA showed the better result. For the shoot proliferation there should be appropriate concentration otherwise the callus induction obtained. In shoot proliferation process, supplementation with lower concentration of BAP (0.30 mg/l) resulted in higher number of shoots (Table 4.2). However, increasing concentration of BAP with NAA above 0.25 mg/l showed no beneficial results. It was correlated with Desai *et al.,* (1996) reporting that NAA inhibits shoot proliferation and medium containing NAA developed callusing at the base of the explants. The findings of Otoni and Teixeira (1991) and Baruah *et al.* (1995) were also confirmed to present result that lower levels of auxin and cytokines were beneficial in shoot regeneration of citrus species.

Table 4.5: Comparative study of growth regulators on shoot proliferation in seedless lime using nodal explant

Sr. No.	Growth Parameters	Growth Regulators (mg/l) with MS medium (Mean ± SE)		
		BAP	BAP + Kinetin	BAP + NAA
1)	No. of Shoots/ explant	3.44 ± 0.24	2.98 ± 0.36	2.52 ± 0.24
2)	Length of shoots (cm)	2.31 ± 0.13	2.04 ± 0.17	3.20 ± 0.27
3)	Shooting response (%)	68.03 ± 0.81	69.40 ± 0.66	60.30 ± 1.0

The data presented in table 4.5 revealed that, the BAP individually had effect on shoot multiplication and maximum multiplication was 68.03% (Mean) shooting response and mean number of shoots per explant 3.44±0.24 (Mean ± SE) as well as length of shoots (2.31±0.13 cm) was obtained on the medium supplemented with BAP. The concentration of BAP and lower NAA combination recorded better than the BAP and Kin in terms of mean length of shoot while BAP with Kin had better for shooting response and number of shoots per explants. The above table indicated that length of shoot (3.20±0.27) was more in BAP with NAA combinations than other treatments whereas highest mean number of shoots (3.44±0.24) on BAP medium. From (Fig. 4.3 & 4.4) showed that the leaves and stems were also vigorous and well developed.

The shoot multiplication is always affected by the concentration of growth hormones. Superiority of BAP over kinetin has been recorded in many woody systems. BAP was more stable, less expensive than the others and the only that can attain autoclave conditions (Bonga *et al*., 1992; Deora *et al.*, 1995). Whereas Drateza *et al.,* (1997) observed complementary effect of cytokines and auxins on production of shoots.

In the present study, it was found that the nodal segment explants were more responsive. This was probably due to the physiological states of explants. The present results are in confirm with Barlass and Skene (1982) who reported nodal material (axillary bud) provided the best sources of shoot cultures capable of continuous subculture. Further, Kamble (2002) also reported that 0.25 mg/1 BAP nodal explants in seeded acid lime *(C. aurantifolia)* produced more shoots than shoot tip explant. However, these results were contradictory to that of Kitto and Young (1981) who reported shoot tip explants proliferated significantly more than nodal sections using 5 mg/1 BAP.

The role of only BAP was found to be highly significant for most of the citrus cultivars which in stimulating multiple shoot formation in seedless lime.These studies will be foundation for further micropropagation of seedless lime cv. Chakradhar using axillary bud as explant.

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Summary and Conclusion

CHAPTER- V

SUMMARY AND CONCLUSIONS

Micropropagation technique has been successfully used for many horticultural fruit trees (Das *et al.*, 1996) and a powerful tool for large-scale multiplication. It is a rapid technique, where mass multiplication of ornamental, timber and fruit trees can be achieved in a relatively short time with high fidelity index. It is also an ideal system for production of disease-free plants.

The callus induction and plantlet regeneration is a complex phenomenon than that of direct regeneration by using nodal segment on MS culture medium. The callus induction influenced by a number of factors like genotypes (species), type of explant, growth status of the mother plant, time of explants harvest and quality of micro flora present on the explants as well as its age, ploidy level, added hormones and cultural conditions etc. The conventional plant breeding method has its own limitations. The only way to get true to type and pathogen free plants in a large scale is the *in vitro* technique of micropropagation.

Summary

The present study entitled, "*In vitro* multiple shoot induction from nodal explant in seedless lime (*Citrus aurantifolia* Swingle)" was conducted during the year 2017-18 with a view to develop a protocol for direct regeneration of cultiver Chakradhar by using axillary bud.

Nodal explants was inoculated on MS media supplemented with BAP for shoot initiation. The initiation shoots were multiplied by using BAP, kinetin and NAA with their possible combinations. The observations on percentage of sprouted bud, shooting percentage, number of shoots per explant and length of shoots were recorded. The results obtained from the present study are summarized and concluded as follows.

- Micropropagation establishment of seedless lime is very critical process as it met with an array of different problems such as microbial contamination, phenol exudation. To avoid and minimize this problem, explants were

sterilized by using 1 mg/l fungicide (0.1% bavistin), 100 mg/l streptomycin and 0.1% mercuric chloride. Antioxidant solution treatment (150 mg/l ascorbic acid) was also applied to reduce the phenol secretion.

- Acid lime *in vitro* cultures utilized juvenile source of explants. These explants more responsive than that of other explants taken from mature trees and it were more preferable to propagate mature plants selected for desirable characteristics.
- MS and WPM medium was used during this *in vitro* study, MS medium gives best shoot proliferation than the WPM (woody plant medium). The pH of the medium should be 5.8 to 6.0 for best results.
- An attempt was made for direct regeneration of shoot induction without intervention of callus from axillary bud explant on basal medium containing BAP at different concentration.

5.1 Shoot initiation:

The young axillary bud explants was inoculated on MS media containing various concentration of BAP as 0.1 to 1.0 mg/l for optimization of shoot establishment and proliferation protocol. Lower level of BAP induced the highest frequency of shoot regeneration in the nodal explants compared to their respective higher concentrations. The treatments 0.25 mg/l and 0.30 mg/l found best treatment in case of earliest shoot initiation and more shooting response shows by the explants. BAP attains saturation level with the increased concentration during *in vitro* shoot induction and multiplication.

The highest shoot regeneration (90%) of explant was observed on medium containing 0.30 mg/l BAP. Minimum 8.60 (Mean) days required for shoot initiation and completion of shoot regeneration took 25.20 (Mean) days. The similar results observed in another 0.25 mg/l BAP treatment. BAP was essential for shoot bud activation and multiple shoot formation from nodal explant. Concentration of BAP was found directly proportional to shoot initiation and completion of plant regeneration while inversely proportional with respect to shooting response.

5.2 Shoot multiplication:

- The multiplication rate of shoot on MS medium supplemented with BAP 0.30 mg/l was significant in respect of response on sprouted bud in shoot proliferation experiment, multiple shooting responses (86.66%). Average number of shoot per explant (5.66) and length of shoot (3.20 cm) was recorded. BAP has given better result than other combinations as it have ability to cell division and stimulates shoot proliferation.
- The interaction of BAP and kinetin had given more proliferation whereas, 1.0 mg/l BAP + 0.50 mg/l Kin was found to be significant combinations and reported to produce maximum of 6 (mean) shoots per explant and 86.30 % shooting response in multiplication. Minimum concentrations of kinetin along with BAP had given better result. The highest average length of shoot was 2.75 cm and least 1.5 cm in these given treatment on MS medium with 0.25 mg/l BAP + 0.5 mg/l Kin and 0.5 mg/l BAP + 1 mg/l Kin observed respectivley. Kinetin can give best shoot proliferation on MS medium but not that much better comparative to BAP with kinetin combinations.
- Effect of BAP in combination with NAA on regeneration capacity of shoots per explant was found to be dependent on concentration of BAP. MS medium supplemented with BAP 0.25 mg/l + NAA 0.10 mg/l found best treatment for shoot multiplication. The highest shooting response (88.33%) and length of shoot (4.52 cm) had given by this treatment. Only NAA produced callusing in the medium. Treatment BN-3 (BAP 0.25 + NAA 0.25 mg/l) also found superior than others for shoot multiplication.
- Frequency of shoot regeneration from nodal explants decreased with a progressive increase in the level of cytokinins and produce negative effect on all parameters. Auxins do not promote shoot proliferation that was required in culture medium to promote growth of shoots by counteracting suppressive effect of high cytokinin on shoot elongation.
- The MS medium + BAP 0.25 mg/l (T4) was found another to be the most effective one for morphogenic growth parameters like number of shoot (5.0) and shoots length (3.05 cm).

- ❖ The number of shoots emerging ranged 1.5 to 6.0 shoots for nodal segment explant from all treatment. 0.25 mg/1 and 0.30 mg/l BAP was an optimal dose while more than these were responsible for excessive fast callus growth which reduced shoot number and shoot length.
- ❖ The maximum length of shoot was 4.68 cm on the MS medium + BAP 0.25 + NAA 0.25 mg/l regenerated from nodal segment explants.

Conclusions

This report provides a simple protocol and best combinations of growth hormones for the multiple shoot induction of *Citrus aurantifolia* cv. Chakradhar. The best treatment for establishment of shoot induction medium were 0.25 mg/l BAP and 0.30 mg/l BAP and also for multiplication and proliferation of shoot. The nodal segments has showed best multiplication rate and multiple shooting responses found best results also with phytohormones BAP 1.0 mg/l + Kin 0.50 mg/l and NAA 0.10 mg/l + BAP 0.25 mg/l combinations. *In vitro* propagation method has a great potential and promise for clonal propagation of citrus trees. The optimization of different concentrations of growth regulators could also be helpful to increase the number of shoots per explant in regenerated plantlets from the axillary bud explants. This protocol can be used for further root initiation and rapid clonal propagation of seedless lime.

1. Genotype, growth regulators, basal media and cefotaxime antibiotics affect the morphogenetic response in mature tissues of seedless acid lime.
2. BAP was observed to be superior to kinetin in taking less time for induction of shoot regeneration and for number of shoots per explant.
3. The absolute value of cytokinin and auxin was found to be important rather than their ratio.
4. BAP had given maximum shooting response comparative to another combination with respect to number of shoots, length of shoots as well as leaves and stem were also found vigorous and well developed.

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LITERATURE CITED

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** Original are not seen*

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Thesis Abstract

THESIS ABSTRACT

Title of research topic	:	***"In Vitro* Multiple Shoot Induction from Nodal Explants in Seedless Lime (*Citrus aurantifolia* Swingle)"**
Name of the student	:	**Jetnaware Pandurang Dashrath**
Degree to be awarded	:	M. Sc. Agricultural Biotechnology
Major subject		Agricultural Biotechnology
Total number of pages in thesis	:	54
Number of words in thesis abstract	:	382
Signature of the student	:	**Jetnaware P. D.** (Reg. No. 2016/BT/08/ML)
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ABSTRACT

Acid lime (*Citrus aurantifolia* Christm. Swing.); (2n=18) is commercially important crop in the tropical and subtropical regions. Acid lime contains vitamin C, which is very useful for human nutrition. The experiment was conducted to get reliable and reproducible protocols to produce healthy plantlets from axillary bud explants of seedless lime (*Citrus aurantifolia* S.) cv. Chakradhar. Nodal segment of 1.0-1.5cm were collected and cultured on MS medium. To test influence of the culture medium on shoot bud induction, MS and woody plant medium (WPM) formulations were evaluated. The treatment, NaOCl (1%) 7 min + $HgCl_2$ (0.1%) 5 min + KCl (1%) 1 min + ethanol (70%) 0.5 min was found to be best sterilization treatment. Direct regeneration of multiple shoots without intervention of callus was produced from node explants. The growth medium used was MS medium supplemented with 3% sucrose with different concentrations of BAP, Kinetin and NAA. BAP (0.25 and 0.30 mg/l) + MS medium was found to be promising treatments for establishment medium with respect to maximum sprouting, minimum mean days of 8.6 for sprouting with highest number of shoots per explant i.e. 86.66 %. The leaves and stems were also vigorous and well developed. The maximum shoot regeneration (90%) was observed on optimum level of BAP (0.3 mg/l) compared to lower (0.10 mg/l) and higher (1.0 mg/l) level. Shoot proliferation decreased with increasing concentration of BAP. MS medium + BAP 1 mg/l + Kin 0.5 mg/l and MS + BAP 0.25 mg/l + NAA 0.25 mg/l were observed to be best treatments for multiplication medium with maximum average shoot length and highest number of leaves with shooting response 86.30% and 86%, respectively. Shoot multiplication was performed repeatedly by subculturing the sprouted bud with newly formed shoot on a fresh batch of the same medium. The highest numbers of shoots (6) were produced on a medium containing BAP 1 mg/1 + Kin 0.5 mg/l at par with 0.30 mg/1 BAP. Maximum shoot length (4.68 cm) was obtained from treatments containing BAP 0.25 mg/1 +NAA 0.25 mg/l on MS medium. The MS medium containing 0.3 mg/l BAP and BAP 0.25 mg/l + 0.10 mg/l NAA emerged as the best combination for shoot proliferation that can be advocative for root induction and refinement of efficient micropropagation protocol of seedless acid lime cv. Chakradhar.

...pages: 1-54

Keywords: Seedless lime, Chakradhar, Micropropagation, Nodal segment, Proliferation