COMPARATIVE STUDY ON MICROPROPAGATION OF SOME CITRUS SPP. IN NEPAL

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CERTIFICATE

This is to certify that the dissertation work entitled, "**COMPARATIVE STUDY ON MICROPROPAGATION OF SOME CITRUS SPP. IN NEPAL"** submitted by **Mr. Kumar Prasai** for the partial fulfillment of M.Sc. degree in Botany, has been carried out under my supervision. To the best of my knowledge, the result of this work has not been submitted for any other degree. Therefore, I am pleased to forward this dissertation for the final approval and acceptance.

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APPROVAL LETTER

The dissertation work submitted by **Mr. Kumar Prasai** entitled **"COMPARATIVE STUDY ON MICROPROPAGATION OF SOME CITRUS SPP. IN NEPAL"** has been accepted as a partial fulfillment of M.Sc. in Botany.

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ABSTRACT

In this investigation, *in vitro* germination, multiplication and acclimatization of virus free citrus plants was done.

Lime seeds were germinated after 5 weeks of culture on CEM supplemented with 500 mg/l of GA_3. The germination of lemon seeds was found to be better than of lime. Lemon did not express ploymbryony but its seedlings gave multiple shoots which was not expressed by lime.

Better multiple shoots were obtained from the nodal explants of lime AO_1 (6) on MS- medium supplemented with 0.5mg/l BAP + 0.5 mg/l NAA. The best multiple shoots of lime BO_1 (15) were obtained from the nodal explants when cultured in MS-medium supplemented with 0.5 mg/l BAP + 1 mg/l NAA. Under similar conditions (MS-medium, supplemented with 1mg/l BAP + 0.1mg/l NAA) among lime AO_1 (6), lime BO_1 (15), lemon and Troyer Citrange, the best multiplication was observed in Troyer Citrange.

Basically, the main purpose of meristem culture was for virus elimination. The meristems were selected from the young shoot apices and cultured in filter paper bridge on MS-medium supplemented with BAP1mg + NAA0.1mg/l. Four meristems (16.16%) could be saved and multiplied for 6 months attaining the size of about 0.5 cm which eventually turned brown and died.

Rooting of lime AO_1 (6) in IBA 1 mg/l was found to be better on MS-medium. Similarly, 0.5 mg/l of NAA was also found to be optimum. Acclimatization was carried on non-sterile sand and soil mixture (1:1). About 37.71% single rooted plants and 46.15% multiple rooted plants were successfully acclimatized. The acclimatization was done inside screen house.

The whole protocol was developed for lime, lemon, mandarin orange and trifoliate orange under *in vitro* (controlled) condition. All initial explants were CTV- free and finally rooted and then successfully acclimatized. Lime AO_1 (6) was also found to be CTV free by DAS-ELISA test.

CONTENTS

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LIST OF TABLES

LIST OF FIGURES

LIST OF PHOTOGRAPHS

S.N. **Photoplate**

1. Photo plate - 1 = Seed Culture of Lime and Lemon
2. Photo plate - 2 = Lime AO_1 (6)
3. Photo plate- 3 = Lime BO_1 (15)
4. Photoplate-4 = Lemon
5. Photoplate-5 = Troyer Citrange & Mandarin Orange
6. Photoplate-6 = *in vitro* Rooting of Lime AO1 (6) in IBA & NAA.
7. Photo plate-7 = Acclimatization of *in vitro* - rooted Lime AO_1 (6)
8. Photo plate-8 = Main instruments used for micro propagation & CTV- detection.
9. Photo plate-9 = ELISA- Plates (1-3)

ABBREVIATION

%	=	Percent
μ	=	Micron
+	=	Plus (adding Factor)
<	=	Less than
>	=	Greater than
±	=	Plus or minus
≤	=	Less than or equal to
2, 4-D	=	2, 4- Dichlorophenoxy Acetic Acid.
ANOVA	=	Analysis of Variance.
atm	=	Atmosphere
B	=	BAP (6- Benzyl Amino Purine)
BA	=	6, Benzyl adenine.
CEM	=	Citrus Establishment media.
cm	=	Centimeter
CTV	=	Citrus Tristeza Virus
DAS-ELISA	=	Double Antibody Sandwich Enzyme- linked Immunosorbant Assay
DAT	=	Days after transplantation.
DOA	=	Department of Agriculture.
e.g.	=	Example gratia (for example)
et. al	=	et alebi
etc	=	et Cetera
FAO	=	Food and Agriculture Organization.
Fig.	=	Figure
g/gm	=	Grams
GA_3	=	Gibberellic Acid.
ha	=	Hectare
HDP	=	Horticulture Development Program.
i.e.	=	that is
IAA	=	Indole-3Acetic Acid
IBA	=	Indole- 3Butyric Acid.
JICA	=	Japan International Co- Operation Agency.

L	=	Litre
m	=	Meter
m^{-1}	=	Per meter
Max	=	Maximum
Mg/l	=	Milligram per litre
Min	=	minimum
ml	=	Millilitre
mm	=	milliliter
MS	=	Medium Murashige and Skoog medium (1962)
N	=	NAA (Naphthalene Acetic Acid)]
NARC	=	Nepal Agriculture Research Council
NAST	=	Nepal Academy of Science and Technology.
NCDP	=	National Citrus Development Project.
O.D.	=	Optical Density
^{o}C	=	Degree Celsius
P^{H}	=	Negative logarithm of hydrogen ion concentration.
PPM	=	Parts Per million
rpm	=	Revolution per minute.
S.D.	=	Standard Deviation.
SDC	=	Swiss Assistance for Development & Co- Operation.
sp	=	Specie
spp	=	Species
sq.m	=	Meter Square
T.	=	Ton.
Temp	=	Temperature
Viz.	=	Videlicet (nameıy)
Vol.	=	Volume
wt	=	Weight

CHAPTER ONE

INTRODUCTION

1.1. Background

Citrus plants of Nepal cover about 2.5% of land in all the cultivated areas. The cultivation of Citrus plants in Nepal has been started in 66 districts covering about 13,515 ha (NCDP, 1990) at newer data.

The mid- hills of Nepal are quite suitable for cultivation of Citrus, particularly Mandarin Orange(*Citrus reticulata*), Lime(*Citrus aurantifolia),* & lemon (*Citrus limon*). The subtropical climate persisting in this region together with favorable edaphic conditions is congenial for Citrus spp. The areas lying between 900 m and 1400 m above sea level have suitable temperature range. The pocket area with deep sandy loam soil and soil pH range of 5.0 to 6.5 is most suitable for the cultivation of Citrus fruits (Ranjit, M. & G.C. Gokarna; 1997)

All Citrus plants are small, spreading, evergreen trees to tall shrubs. Limes are the smallest trees of the groups. Trees may reach 20-30 ft in height, but most cultivated trees are below 15 ft. Stem are often armed with long thorns, particularly lime, and in all types when young. Leaves are relatively thick, ovate with acute to obtuse tips, having entire or crenulate margins, and stout. Flowers are fragrant, white solitary or in short cymes. Flowers are perfect, with 5 petals and 5 sepals. The petals are linear, sometimes curved, waxy and thick. The sepals are fused at the base, to form a small cup. Ovary is compound with 10-14 locules. Most cultivars of Citrus spp are self-pollinated. The fruit is a hesperidium. The endocarp is the edible portion that is divided into 10-14 segments separated by thin septa; each containing upto 8 seeds, but usually only one.

Citrus is unique plant because it forms nucellar embryos (maternal clones) in addition to the zygotic embryo produced through fertilization. Exception includes some tangerines and the pummelo, in which only a zygotic embryo forms. The condition is termed nucellar embryo or polyembryony, and it

allows clonal propagation of Citrus by seed which is rare in horticultural crops. This condition is exploited by nurserymen, but causes obvious difficulties in Citrus breeding. Multiple embryos ensure that high germination rate percentages simplifies clonal rootstock production greatly.

The climatic condition of the mid-hills of Nepal favours the cultivation of high quality Citrus fruits. With the growth of Citrus cultivation, and the lack of knowledge how on virus infections, CTV and other Citrus viruses diseases are also rapidly growing. The recognized diseases of viruses are very numerous. They all are transmissible by budding or grafting, some can also be disseminated by insect vectors, and a few can be mechanically transmitted via the sap of infected plants. The main virus disease in Nepal is CTV. CTV belongs to the Closterovirus group. It is single stranded RNA virus. CTV has been found in all Citrus growing areas of the world. (Price, W.C.1970). Vein clearing, leaf cupping, vein corking and stem pitting are some common symptoms of CTV. Enzyme Linked Immuno-Sorbant Assay (ELISA) is used for detection of CTV. Once an area becomes infected by this disease, it in impossible to eradicate because of the widespread distribution of tropical Citrus aphid. However, the manifestation of the disease can be minimized by raising future Citrus planting of tristeza tolterant rootstock. Elimination of virus in infected area is essential for increasing the productivity of citrus.

1.2. History and Description

1.2.1. History and Description of Lime

1.2.1.1. *Citrus aurantifolia* swingle' (Lime AO_1 (6) and Lime BO_1(15))

Apparently Abd- Allatif made the first mention of the lime in literature, in the thirteenth century. Gallesio (1811, p.30) stated that his," balm lemon of smooth skin the size of a pigeon's egg" was apparently identical with the species of lime. The first mention of the lime, under that name, according to T.W. Brown (1924, P.74) was apparently by sir Thomas Herbert (Travels.1677), who spoke of finding, " oranges, lemons, and limes" on the island Mohelia during a voyage that begun in 1626.Sulavaticus in the middle of the thirteenth century spoke of the fruit vulgarly called ' Lima' which apparently was what we know now as Lime

(Gallesio, 1811,P.268). Evidently, therefore, the Arabs also knew the lime, probably played the major role in preceding its culture through India to Persia, Palestine, Egypt and Europe.

Lime AO_1 (6) and Lime BO_1 (15) are two Cultivars of lime recommended by Institute of Agriculture and Animal Sciences (IAAS), Rampur.

1.2.1.2. Chemical Composition of Lime

Lime has volatile oil (0.36%) that contains more than 70% limonene. The limnoid glycosides such as, 3-P-glucopyranoside are reported from the peel. It is rich source of organic acids like vitamin 'E' and minerals. The essential oil of lime leaves contains nearly 17.27% geranial 23.30% and that of lemon contain 29.03% (Anonymous, 2000).

Lime has great dietery value. From 100 gm of edible portion of Lime fruit, we may get 37 calories of energy, protein 0.1%, fat 0.3%, carbohydrate (12.3%), vitamin-A 1%, vitamin –B, 3.1%, vitamin B_2 1.1%, vitamin C 8.8%, calcium 2.4%, phosphorus 1.9%, iron 2.6%, potassium 2.1%.

1.2.1.3. Economic Importance of Lime

The basic chromosome No. of Citrus spp is x = 9 (2n =18, 27) lime is very popular citrus plant of Nepal, Lime production in 2001 was found to be 14,949 tons from 3,221 hectors (Poudyal, 2003) .In tropical and subtropical regions of the world like Mexico, Brazil, Florida, lime production dominates other Citrus fruits. In the USA, the Florida produces 100% of the Lime (FAO, 2002).

In Nepal, lime is cultivated in 3447 ha (Shrestha and Verma, 1998). Lime fruits can be cultivated in waste barren areas and yards of house, so it has been popular among poor farmers (Rochester, 1996). From the recent survey of NARC, it is found that in Nepali agricultural market only 32% need of lime is met by Nepalese product. Lime is highly recognized for nutritional and medicinal values. The fruit juice is used in the treatment of leprosy. Since limonins are nontoxic to human, they become an attractive alternative to pesticides. It maintains good immune system and prevents from cold, ' scurvy;' and ' anemia'. Flavonoids have a broad spectrum of biological activities including anticarcinogenic and

antitumeric activities. Polymethoxylated flavonoids (PMF) such as tangeretin have anti-tumor activity (Anonymous,2000)

1.2.2. History and Description of Lemon

1.2.2.1. Lemon: *Citrus limon* (L) Burm. F. (2n=18)

From the middle of the first century A.D. to the middle of the second, Roman trade was most active. Gourmets in Rome paid fantastic sums for exotic delicacies, and it seems probable that lemons in the freshest state possible occasionally arrived in shipments to Rome. A mosaic tile floor found in a Roman Villa at Carthage, probably of the second century AD., shows recognizable branches of fruit bearing lemon trees (Tolkowsky, 1938). Although much is known with reference to the spread of the lemon into various parts of the world, the exact place and the mode of its origin are still in doubt. That it must have originated some where in the countries of south eastern Asia seems certain (Lauter; 1934).

1.2.2.2. Chemical Composition of Lemon

100gm of edible portion of lemon fruit provide 27 calories & consists of protein 1.1%, fat 0.3% carbohydrate 8.2%, vitamin A 1%, vitamin B 2.9%, vitamin B_2 1.2%, vitamin C 11.8%, calcium 3.2%, phosphorous 2% and iron 6%, potassium 2.9%.

Pericarp of lemon consists of 6% essential oil in which Limonin 9%, Citral 5%, Citronellal. α-terpineol, Linalyl, geranyl acetate are also found. Mesocarp consists of flavone glycosides and coumarin derivatives.

1.2.2.3. Economic importance of Lemon

Lemon production is dominating over lime in Mediterranean climates (Spain, Italy, and California); (USDA, 2002). In market survey of some researcher, it is found that the most selling market of lemon in Nepal is found to be Dharan. A mature plant can give about 147 kg fruits. In Nepal lemon is cultivated in 772 ha of land (Shrestha and Verma, 1998). Weight of lemon fruit

is 500-800gm, Length is 190 to 225 mm, breath-130to160 mm. The edible portion is 58 to65% (Poudel, 2003)

Lemon peel is used in cosmetic as a hair rinser and as mouth freshner; its juice is used in 'lemon tea'. Leaves can be used as pesticides etc especially for the storage of seeds. It is highly recognized for its nutritional and medicinal value. Fruits can be used for the preparation of pickle, squash lemon juice etc. The peel (Pericarp) of fruit is used as spice, also the fruit juice has culinarily value. Lemon is used to make lemon sauce.

1.2.3. History and Description of Orange

1.2.3.1. Orange

Some historians of Citriculture have maintained that no sweet oranges were grown in Europe until the Portuguese brought the first trees from India or the far East after they discovered the direct sea- route around the cape of Good Hope. Some say the tree has come originally from China, hence, it is sometimes called Chinese or sinensian tree.

The mandarin orange, which is native of China and south- east Asia, was not taken to Europe only recently. The first mandarin tree was brought to England from China in 1805, and the mandarin spread from England first to Malta, and then to Sicily and Continental Italy (Tolkowsky, 1938). Thus, Orange, might have originated in south East Asia and spread all over the world.

1.2.3.2. Chemical Composition of Orange

Pericarp of orange consists of limonin and flavoprotein. Edible portion of orange fruits can provide more calories than lemon and lime. Other constituents are protein, fat, carbohydrates vitamin A, thiamin, riboflavin, niacin, vitamin C, calcium, phosphorus, iron, potassium.

1.2.3.3. Economic Importance of Orange

Orange fruits are highly recognized for nutritional and medicinal values. Orange peel is used in cosmetics. It is great source of vitamins. It has

great dietery value. Only the orange has covered 12,276 ha, of cultivated land of Nepal. By 2001, the production of orange was 74,794 ton (Poudel, 2003)

The annual production of orange in world is 64,128,523 MT (FAO, 2002). Oranges are produced commercially in 114 countries worldwide, on about 9 million acres. The top ten countries of the world for the production of orange are Brazil (29%), USA (18%), Mexico (6%), China (6%), India (5%), Spain (4%), Italy (3%), Iran (3%), Egypt (3%), and Pakistan (2%).

Troyer Citrange is the cross product of *Citrus trifoliata* L. and *C. sinesis* Osbeck.

1.2.4 History and Description of CTV

Citrus Tristeza Virus is apparently originated in Asia where it existed for centuries unrecognized, possibly because the commonly grown citrus cultivars were highly tolerant. Citrus was first introduced to Europe and the New World as seed and since CTV is not seed transmitted, these trees were free of the virus. The major disease of concern at that time were *Phytopthora gummosis* and root rot, and consequently trees were grafted onto the highly adaptable Phytophthora-resistant sour-orange rootstock. This decision has contributed to the dramatic effect that CTV has had on world Citrus production. The first tristeza disaster was reported in the 1930's in Argentina, where 90% of the citrus was planted on sour orange.

There are more than 30 viruses and virus-like diseases of citrus known in the world, of which Citrus Tristeza Virus (CTV) is arguably the most destructive. In 1981, the total world loss attributable to this disease was estimated at 50 million trees (Bar-Joseph et. al., 1981). There are different strains of CTV found in Nepal that brings symptoms of stem pitting and vein cleaning on lemon and lime. A study by the Biotechnology Laboratory of NAST (Nepal Academy of Science and Technology) in 1997-1998 was conducted. The samples were tested using DAS-ELISA of 750 from around the Country. The result showed that there was variation in the distribution of CTV in different areas. However, infection of citrus disease has caused low production of Citrus (Regmi, et. al., 1999).

Citrus Tristeza Virus belongs to the closterovirus group. It is a single stranded RNA virus. It is not transmitted by seed and soil (Tang et at. 1996, Roistacher 1984). It is transmitted by aphid vectors. CTV is spread in the field by aphid species, which transmit the disease with varying degrees of efficacy. The most efficient vector of CTV is the black citrus aphid (*Toxoptera citricida*).Very few diseases have caused such severe crop losses as tristeza in citrus. Often large areas of plantings have been completely destroyed shortly after outbreak of tristeza and the only solution to the problem has been to replant the trees on stock tolerant to the disease.

The most spectacular damage has been recorded in Argentina and Brazil. In Argentina little more than 10 years after the outbreak in 1930 the disease killed 10,000,000 trees (Fernandez et. al.,1936). In Brazil 6,000,000 trees of sour orange rootstock were killed between 1937 and 1948. This figure represents 75% of the total number of citrus is the area (Bennet, et. al., 1949). Tristeza virus has been found in all citrus growing areas of the world (Price, 1970).

1.2.5 Meristem Culture and DAS-ELISA

A meristem is a tissue in which under favourable conditions, new cells are more or less continually being formed as a result of repeated divisions of some or all of the cells. As a result of the subsequent further enlargement and structural differentiation of cells originating in a meristem, various tissues are developed according to a pattern which is more or less distinctive for each species.

The most important meristems in the body of the majority of vascular plants are the apical-root and apical-stem meristems, and the vascular cambium. Whether or not a meristem will be active at a given time depends upon both environmental and internal conditions. Most apical-stem meristem of the woody plants of temperate zones are inactive during the winter months largely due to unfavourable environmental conditions.

Growth which is initiated in apical-stem and root meristem is called primary growth. Primary growth results in the construction of primary tissues of the plant that accounts for all the increase in the length of the plant axis at

both stem and root tips, resulting in the development of the branching system of the stem and roots and is responsible for the formation of lateral appendages such as root hairs, leaves and floral parts.

Meristem tip culture has at least two advantages over other types of tissue culture methods for eliminating viruses. 1. The integrity of the original plant or clone is maintained with minimal genetic variation and 2. The methods developed for meristem tip culture are relatively straight forward and can be used directly or with slight modification for clonal propagation (Collins, 1982).

For the tissue culture of CTV infected Citrus plants, lime is the best indicator. Lime, particularly Mexican Lime is used as an indicator plant for CTV because it readily expresses classical symptoms upon inoculation (Ranjit, et. al., 1998).

DAS- ELISA is an acronym for double antibody sandwich enzyme-linked immunosorbant assay. It is used for the detection of several plant pathgens, especially viruses. It is a very sensitive, accurate and rapid detection method. It is especially effective whether a large number of samples must be tested, where results are needed rapidly and where suitable indicator plants and/ or green- house facilities are not radialy available (Garnsey and Cambra, 1991). Use of ELISA for virus detection involves certain substances called antigen, antibody, conjugated antibody and substrates. Antigens and antibodies are porteinous in nature. All are specific i.e. particular antibodies bind particular antigens (e.g. Virus). The ELISA Plates that are used for virus detection are mostly made up of Polystyrene of Polyvenyl chloride. Thus, the surface of the wells of the plate can be coated either with the antibody or with the antigen.

1.3. Objectives

i. To develop a standard protocol for disease free Citrus plants from the stage of seed germination to acclimatization

ii. Comparative analysis of different phytohormones on the *in vitro* multiplication of different citrus spp.

iii. To eliminate CTV from infected Citrus plant by meristem culture technique.

iv. Transfer of technology for nursery or to the level of farmer.

1.4. Justification of Study

- Virus-free lime can be used as the indicator plant. Orange generally does not show the virus symptoms but when grafted on lime, the part of lime expresses the symptoms whether it contains virus or not.
- Pure line culture is possible even from the seed isolating nucellar embryo from zygotic-one during the *in vitro* seed germination of Citrus spp.
- *In vitro* grown plants are generally disease free and disease resistant resulting more productivity.
- We can test the viability of germplasms during storage in under *in vitro* Condition.
- Though *In vitro*-culture is expensive, we can use this technique for mass culture in the limited time and space.

CHAPTER TWO

LITERATURE REVIEW

Price, W.C., (1970) reported that Tristeza virus has been found in all Citrus growing areas of the world.

Bar-Joseph et. al., (1981) recorded thirty viruses and virus- like disease of Citrus known in the world. They have mentioned Citrus Tristeza Virus (CTV) arguable as the most destructive. They have said in 1981, the total world loss attributable to this disease was estimated at 50 millions trees. According to them, CTV is spread in the field by Aphid species, which transmit the disease with varying degrees of efficacy and the most- efficient vector of CTV is the black Citrus aphid (*Toxeptera citricida*).

Hutchinson (1981) reviewed the *in vitro* techniques for rapid clonal propagation of fruit trees such as apple, plum, peach, almond etc. He concluded that 6-benzyl aminopurine (BAP) induces multiple shoots and Indole butyric acid (IBA) induces adventitious roots.

Karki and Rajbhandary (1983) observed plant regeneration from leaf culture of *Solanum lanciniatum* Ait, cultured on MS medium supplemented with cytokinins. The proliferation potential of the culture was maintained for 5 years by regular subcultures. The proliferated shoots were rooted in half concentration of MS-medium with growth hormones (IBA, NAA).

Gupta, P. (1986) eradicated mosaic disease for three cultivars of banana (*Musa acuminata*) by heat therapy (38-40^{0}c for 14 days) and meristem tip (1.5mm-2mm) cultured on MS+ thiamine HCL 2mg/l + Nicotinic acid 0.5 mg/l + BAP 07mg/l + kinetin 0.7 mg/l.

Suwal et.al (1986) observed the production of multiple shoot from cotyledonary node culture of *Dalbergia sissoo* Roxb. Ex, D.C. in the presence of BAP 1mg /l and NAA 0.1 mg / l.

Niroula and Rajbhandary (1988) included shoots of *Poncitrus trifoliate* in the MS-medium supplemented with BAP 0.1 mg/ l and NAA 0.1mg/l and

casein hydrolysate 1mg/l. Roots were produced when transferred on MS-medium supplemented with NAA 0.1mg/l

Shrestha and Rajbhandary (1988) observed swelling of meristem of *Cymbidium gigantum* on MS media + BAP 5 mg/l + NAA 1mg/l. The numbers of plantlets were 6-10 in initial culture. From the 2nd subculture the number increased up to 10-15. Complete seedlings were developed 6-8 months after initial excision of meristem. They also observed that 0.1mm was difficult to establish in culture where as 0.5 mm long piece survived.

Amatya and Rajbhandary (1992) included micro shoots of Ficus memorials from shoot tips explant on MS-medium supplemented with BAP 1 mg/l, kinetin 1.5 mg/ l and casein hydrolysate 1mg/l. Rooting was done in sand beds under polythene bags.

Niroula (1992) induced shoots from cotyledonary node of *Citrus limon* in the presence of BAP 1 mg/l and NAA 0.1 mg /l. These shoots proliferated on subculture in the same medium supplemented with lower contribution of BAP o.5 mg/ l and NAA 0.0l mg/ l. The micro-shoots are established successfully in field.

Pradhan and Rajbhandary (1992) proliferated shoots from cotyledonary node culture of *Ficus nerifolia* on MS medium in the presence of BAP 1 mg/l and kinetin 1.5 mg/l and rooting was observed on sand beds.

Pradhan (1993) included few shoots from nodal segments of 12 years old tree of *Eucalyptus citriodora* Hook on MS-medium supplemented with BAP 1 mg /l and coconut milk 10%. These shoots proliferated with lower concentration of BAP 0.25 mg/l and coconut milk 10% .The microshoot produced roots on non- sterile sand.

Sako and Takami (1993) eliminated onion yellow dwarf virus (OYDV) and garlic leaf meristem tips. They found that when a virus-free explant was cultured on MS + NAA 0.5 mg/l +BAP 10-20 mg/l; numerous shoots were produced. The multiple shoots which were divided in to many pieces and shoots which were subcultured on MS + BAP 2mgl/ multiplied at a higher rate. Roots

were induced from shoots subcultured two times on the MS-medium excluding BAP. After transplanting into sandy loamy soil, more than 90% of the plants easily grew.

Karki and Rajbhandary (1994) performed that shoot tip (2-4 mm) culture from the sprouts of mature tree of *Populas ciliata*. The media were supplemented with BAP 5 mg/l and NAA 0.1mg/l over a filter paper bridge in order to remove browning of shoot explant. After 4-6 weeks, green shoots were transferred in MS-solid medium, supplemented with BAP 5 mg/ l and NAA 0.2 mg/l, adenine sulfate 20mg/l and coconut milk 10%. On average 20-25 multiple shoots were observed within 10-12 weeks. These micro shoots were excised from the flask and rooted in sand beds. After 15-20 days, roots were developed in sand which were transferred in the pots.

Niroula (1994) regenerated shoots from the cotyledonary node of *Citrus sinensis* when cultured on MS-medium in the presence of BAP 1 mg/l and NAA 0.1mg/l with 15% coconut milk. The micro shoots produced roots when transferred to sand.

Rajmilic et at. (1994) developed a micro- propagation method for *Fabiana imbricata* Ruizet pav. by culturing shoots tips on MS- medium supplemented with BAP 1 mg/l and GA_3 0.01mg/l for rooting, MS-medium supplemented with IBA 0.5mg/l was the best.

Sugimura (1995) produced virus-free patchouli (*Pogostemon cablin)* by isolating and culturing meristem tips on medium supplemented with BAP 0-2 mg/l. Multiple shoots proliferation was initiated during culture. Transferring multiple shoots to a medium devoid of phytohormones regenerated complete plantlets. Plantlets thus produced were transplanted to the soil with a high rate of survival and tested by ELISA to check. Elimination of patchouli mild mosaic virus was confirmed.

Javed et al. (1996) propagated *Bauganvillea spectabilis* through shoot apex culture. MS- media with different concentrations and combinations of plants growth hormones were used. Shoot development was observed on BAP 0.25 mg/l +NAA 0.25 mg/l and maximum number of shoots included on the

medium with NAA 3 mg/l + BAP 2mg/l and glutamine 250 mg/l. The shoots produced higher percentage (70%) of roots on MS-medium supplemented with IBA 5 mg/l + NAA 5 mg/l.

Roistacher, (1996) recommended a general certification program for Citrus in Nepal with emphasis on the greening disease problem. Citrus is found grown in 62 of the 75 districts in mountainous regions from 600 to 1400m elevations. Nepal has been saved from destruction by the greening disease by the fact that Citrus is grown in many remote mountainous regions where the vector (*Diphorina citri*) has not as yet penetrated He reported CTV and its aphid vector *Toxoptera citricida* as endemic to Nepal. He also noted that growth of nursery plants are at about 12% efficient.

Ranjit, and Chhetri (1997) documented the Citrus research and development action plan and drafted a memorandum of understanding between Nepal Agriculture Research Council and the Development of Agriculture to share the excellent facilities of the Horticulture Research Development and Training Center at Kirtipur and the potato Research Program at Khumaltar in order to established and implement the coordinated National Citrus Improvement Program in Nepal.

Amatya and Rajbhandary (1998) micro propagated *Ficus auriculata* Locur, through cotyledonous nodes on MS-medium supplemented with BAP 1 mg/l and NAA 0.01mg /l. Rooting of micro-shoots occured in a mixture of sand and dry leaf powder (2:1 v/v). Rooted plantlets were established in the field.

Ranjit, M. et. al., (1998) found Lime, particularly Mexican lime to be the best indicator plant for CTV. Thus Mexican lime can be used as an indicator plant even in commercial scale because it readily expresses classical symptoms upon inoculation. CTV is not expressed by oranges. But branches grafted on CTV- free Mexican lime then immediately expresses the symptoms of viral diseases such as vein-clearing and stem pitting etc.

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Joshi et al. (1999) cultured shoot tips from two year old plants of kiwifruit tree *Actinidies chinensis* var. Bruno on ms-medium supplemented with BAP2.5mg/l and NAA 0.1 mg/l. The shoots buds were initiated after two weeks of culture. The micro- shoots developed once the shoot buds were sub- cultured in MS- medium with BAP 7 mg/l and NAA 0.0l mg/l.

Ranjit, (1999) produced multiple shoots in *Bauhinia variegata*, on MS-medium supplemented with BAP 1 mg/l and NAA 0.5 mg/l.

Ranjit, M. (1999) produced protocol for *In vitro* propagation of disease-free trifoliate orange and sweet orange, an indicator plant for indexing against Citrus greening disease (CGD) has been developed. Hardened plants of trifoliate orange had been made available to the nurserymen of Gorkha and Lamjung districts for shoot tip grafting with virus-free budwords of mandarin inside insect- proof screen tunnels. Successful grafts had been monitored against Citrus Tristeza Virus using DAS- ELISA and CGD using biological indexing, completely disease free saplings of grafted mandarin had been made available for transplanting in the orchards for the first time in Nepal

Regmi, et. al., (1999) mentioned the climatic conditions of mid- hills of Nepal as the best for cultivation of quality Citrus fruits especially mandarin orange, lime etc. Citrus cultivation is one the main professions in the mid-hills of Nepal. Citrus shares about 15.4% of the total fruits area. The infection of Citrus disease has caused low production of Citrus.

Joshi and Singh (2000) found that MS- medium supplemented with BAP 1 mg/l and NAA 0.5mg/l gave good result in Bauhinia variegate, and MS-medium with BAP 0.5mg/l and NAA 0.1 mg/l was found to be best in *Bauhinia purpurea* for shoot multiplication when nodal explants were taken for culture initiation.

Ranjit, C (2001) studied about the distribution of Citrus Tristeza virus (CTV) in various regions of Nepal and eliminated it. The virus from the meristem culture technique from those to the virus infected species of Citrus According to him; the central and western regions are more effected by CTV in comparison to mid-western region. Out of 100 meristem, he cultured from CTV

infected plant, only 25% could survive (0.2- 0.5mm). When those survived culture were tested later, it was found that 100% were -ve in DAS- ELISA.

Pahari, S. (2002) evaluated isozyme diversity of *Citrus reticulata* 'Blanco' and *C. limon* (L) Burm f. of Nepal. He selected 10 populations of *C. limon* and population of *C. reticulata* and evaluated four enzymes namely peroxides (PRX), malate dehydrogenase (MDH), Shikimate dehydrogenagese (SKD) and malic enzyme (ME).

Ranjit (2002) eliminated Citrus Tristeza Virus by meristem culture in different species of Citrus sp. Collected from different part of Nepal. Knorr and Shah (1971) first reported CTV on Citrus in Nepal.

Ranjit (2003) has successfully diagnosed on Hwanglongbing or Citrus greening disease (Candidatus liberibacter Asiaticus) by PCR amplification of 16s DNA and rpl β operon DNA using primers such as 011, 012c, A_2 an J_5. Virus-free bud woods of mandarin grafted on tissue culture rootstocks of trifoliate orange, raised inside insect-proof screen-house and transplanted in the orchards at elevation of more than 1000m above sea level have shown to be free from Hwanglongbing. Sweet orange in the open nursery has been found to be infected with Hwanglongbing.

Singh et al. (2003) regenerated multiple shoots from nodal explants of *in vitro* grown Plantlets of Bauhinia *purpurea* L. when culture on MS-medium containing BAP 1 mg/l and IAA 1mg/l.

Adhikari, et. al., (2004) propagated two varieties of potato (Malta and Petronese). The samples were brought from Bangladesh and tested for three-potato viruses, VIZ. PVX, PVY and PLRY by DAS- ELISA method. Virus infected samples were taken. The most effective result in survival of meristem was found in the meritstems of 0.4 mm size. For meristem culture, MS liquid medium with 2% sucrose, $2mgL^{-1}$ calcium pentothenate etc and 0.25 mg L^{-1} GA_3 was used. For thermo-therapy, potato virus were incubated at 35 ^{0}c for 21 days. Potato virus Y was found to be eliminated easily than Potato virus X.

Basnet, R.and B. Pant (2004) successfully developed the multiple shoot from nodal explant of *Citrus aurantifolia* ' Swingle ' on MS (1962) medium supplemented with BAP 1mg / l and NAA 0.5 mg /l. Explants were taken from *in vitro* germinated plantlets on hormone free MS- medium. Roots were developed on half strength MS- medium supplemented with IBA 1.5mg /l after 4 weeks of culture. These plants were transferred in half strength MS-medium supplemented with IBA 1mg /l by cutting its root apices. The average length was 1.5 cm after 4 weeks of culture. On half strength MS- medium supplemented with NAA 100mg /l, the average no. of roots was found to be 10.6 with average length 1.4cm. Basal callus mass was developed after 8 weeks of culture.

Vidalakis, G., (2004), studied the efficacy of bio-indexing for graft transmissible Citrus pathogens in mixed infection. He did biological indexing for graft transmissible pathogens of Citrus spp in the presence of additional pathogen. The probability for symptom expression, the efficacy of the bio-indexing tests and the number of Citrus indicators required for pathogen detection were statistically evaluated. Multiple infections did not preclude symptom expression or reduce the diagnostic efficacy of primary indexing shoots for Citrus Tristeza Virus (CTV) Citrus Psorosis Virus (CPsV), a Citrus tatter leaf virus. Symptoms of Citrus vein enation virus (CVEV) and the diagnostic efficacy of Mexican lime were suppressed by the T 30 group CTV isolates, but not by other CTV isolates testes. CPsV suppressed symptom expression and diagnostic effecting of sweet tangor and sweet orange for Concave gum. The application of alternate bioassay hosts for indexing was also investigated. In sweet orange, CVEV was detected using the alternative indicator *C. excelsa*.

Yoshimi, et. al., (2004) reported the effect of rootstock and crop load on sap-flow rate in branches of 'Shirakawa Satsuma' Mandarin (*Citrus unshiu* Marc). Mandarin trees were budded onto trifoliate orange. Diurnal sap flow rates in branches were measured continuously from September to December by 2002. Sap flow was different in trees on various rootstocks and the rootstock/ interstock combination as well as with the crop load applied.

CHAPTER THREE

MATERIALS AND METHODS

The present investigation was carried out at the laboratory of Central Department of Botany, Tribhuvan University, Kathmandu, Nepal and at the Green Research and Technology (GREAT) Baneshwor, Kathmandu, Nepal. The experiment was conducted over a time period of 14 months.

Healthy seeds of lime and lemon were obtained from the fruits of Kalimati Vegetable Market. Meristems of mandarin orange were harvested from virus positive plant found at GREAT, Baneshwor. Two cultivars of lime namely AO_1 (6) and BO_1 (15) and troyer citrange for protocol development were obtained from *in vitro* explants at GREAT, Baneshwor. Nodal cuttings of seedlings of lemon for research were obtained from those of the germinated seeds under *in vitro. In vitro* -rooted shoots were acclimatized inside screen house maintaining the humidity by Muslin clothes and CTV-testing before and after the research was done by DAS- ELISA method.

3.1. Materials

3.1.a. Sterilants:

i) Alcohol (Ethyl Alcohol) 60% v/v

ii) Mercuric Chloride

iii) Sodium hypochlorite

iv) Dettol

v) Tween 20.

vi) Laminar air flow.

vii) Autoclave.

viii) Hot Air Oven.

3.1.b. Stock Solutions and Chemical for MS-medium

I) Macronutrients- Stock 'A'

Chemicals	**10x (gm/litre)**
Potassium Nitrate (KNO_3)	19.00
Ammonium Nitrate ($NH_4 NO_3$)	16.50
Magnesium Sulphate ($MgSO_4$)	3.70
Potassium dihydrogen Phosphate (KH_2PO_4)	1.70
Calcium Chloride ($CaCl_2. 2H_2O$)	4.40

II) Micronutrients- Stock B'

Chemicals	**(100 x) mg/100 ml**
Boric Acid ((H_3BO_3)	620
Manganese Sulphate($MnSO_4.4H_2O$)	2230
Zinc Sulphate ($ZnSO_4. 7H_2O$)	860
Sodium Molybdate ($N_2MO_4.2H_2O$)	25
Copper Sulphate ($CuSO_4.2H_2O$)	2.5
Cobalt Chloride ($CoCl_2.6H_2O$)	2.5
Potassium Iodide (KI)	83

*Potassium Iodide was freshly prepared and stored.

III) Iron EDTA-Stock 'C'

Chemicals	**(10 x) mg/100ml**
Sodium Ethylene diamine tetracetate (Na_2 EDTA)	373
Ferrous Sulphate ($FeSO_4 .7H_2O$)	278

IV) Vitamins- Stock' D'

Vitamins	**(100 x) mg/ 100ml**
Glycine	200

Nicotinic Acid	50
Pyridoxin HCl	50
Thiamin HCl	10

V) Hormone Stocks

Hormones	**Concentration**
Indole-3- Butyric Acid (IBA)	1mg/ml
Naphthalene Acetic Acid (NAA)	0.1mg/ml
Gibberellic Acid (GA_3)	0.5mg/ml
Benzyl Aminopurine (BAP)	1mg/ml

VI) Carbon Source

Chemical	**gm/ litre**
Sucrose	20

VII) Solidifying Agent

Chemical	**gm/ litre**
Difco Bacto Agar	7

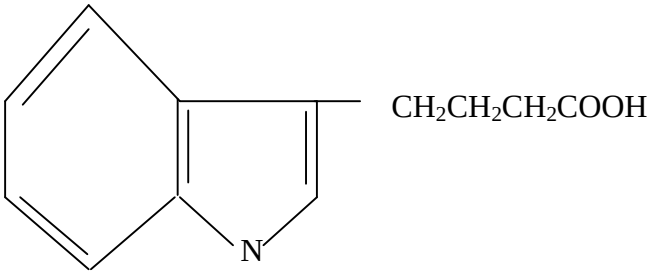
VIII) Other Chemical Used

Chemical	**Concentration**
Casein hydrolysate	4 mg/l
Myo- Inositol	100mg/ml

3.1.c Hormones Used for Investigation

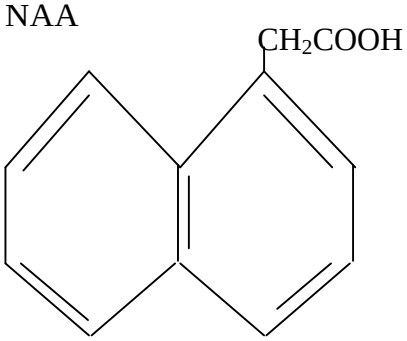
a. Auxins

i. IBA



Indole-3-Butyric Acid

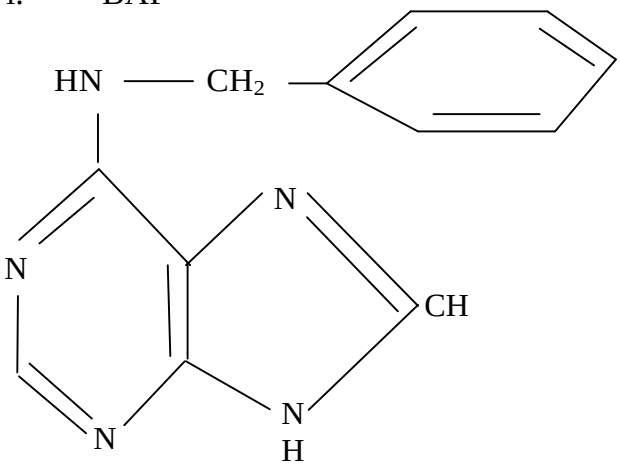
ii. NAA



Naphthalene Acetic Acid

b. Cytokinin

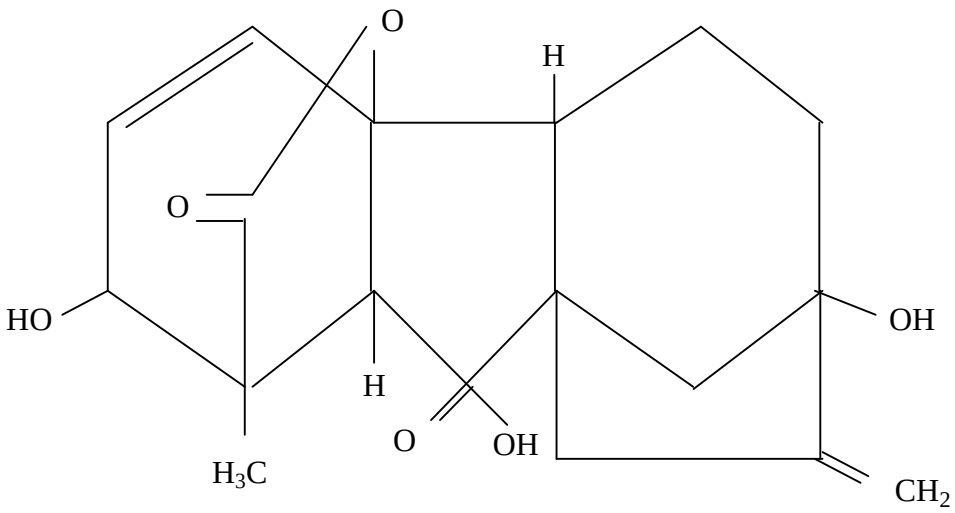
i. BAP



Benzyl Aminopurine

c. Gibberellin

i. GA_3



Gibberellic Acid

3.1.d. MS Media

After the preparation of MS media (Murashige and Skoog, 1962), was used as a basal medium.

1000ml Solution- P^H = 5.7

i. Stock A 100ml

ii. Stock B 1ml

iii. K1 1ml

iv. Stock C 10ml

v. Stock D 1ml

Myoinositol 1ml

iv. Hormones Used / Not Used.

v. Sugar 20 gm.

vi. Agar 7gm

3.1.e. Apparatus Required for *In vitro* Culture

Chemicals

Methylated Spirit, Ethyl Alcohol, Media etc.

Glassware's

R.B. flask, Beaker, Measuring Cylinder, Pipette, Volumetric flask, Bottles, Culture tubes, petridishes etc.

Other Instruments

Forceps, scisors, scalpel, needle, magnetic stirrer, electric balance, pH meter, aluminium foils, microscopes, autoclave, hot air ovan, laminar air flow, spirit lamp, match box, muslin cloth, micropipette, refrigerator.

Plant materials

Seeds, *In vivo* shoot tips *in vitro* explants to culture.

3.1.f. Buffer Solution for DAS-ELISA

I) Coating buffer (For 1 Liter)- $P^H = 9.6$

$Na_2 CO_3$	1.59g
$NaH CO_3$	2.93g

II) Phosphate Buffer saline (PBS), (For 1 liter) - $P^H = 7.4$

NaCl	8g
$K_2H PO_4$	0.2g
$Na HPO_4 12H_2O$	2.9g
KCl	0.2g

III) Washing Buffer- $P^{H=}$ 7.4

PBS	1 liter
Tween 20	0.5 ml

IV) Extraction Buffer - $P^{H=}$ 7.4

PBS	1 liter
Polyvinylpyrrolidone40 (PVP 40)	20 g

V) Conjugate Buffer- $P^{H=}$ 7.4

PBS	1 liter
PVP40	20 g
Ovalbumin (OVA)	2 g

VI) Substrate Buffer (1 liter) - P^{H} = 9.8

Diethanol amine	97 ml
Distilled water	800ml

VII) Reaction Stopping Solution (1liter)

NaOH	120g (3M)

The antibody and conjugated antibody for CTV and the substrate were received from BIO-RAD laboratories France (σ company, USA).

3.1.g. Apparatus required for DAS- ELISA

Chemicals:

Na_2Co_3, $NaHCO_3$, NaCl, K_2HPO_4, $Na_2HPO_4.12\ H_2O$, KCl, PBS, Tween- 20, PBS, PVP 40, Ovalbumin (Ova), NaOH, Diethanolamine, NaN_3 (optional), HCl, ELISA Kit etc.

Glassware's:

Round Bottom flask, Beaker, Measuring, Cylinder, Pipette, Volumetric, Flask etc.

Other instruments:

Forceps, magnetic stirrer, washing bottle, pH meter, spirit lamp, match box, spirit, scissor, micropipette, refrigerator, ELISA plate, centrifuge (300rpm), incubator (37^0c), Humareader (ELISA-plate reader), printer and paper etc.

3.1.h. Apparatus required for transplantation of In vitro - rooted plant in screen house

- Forcep-1
- Wooden stick-2
- Muslin cloth-2 meter
- Scissor-1
- Plastic pots-2
- Hazzari for irrigation-1
- Marker-1
- Soil & sand
- UV- sterile water.
- Plastic rope- about 3 meter.

3.2. Methods

3.2.1. Sterilization of seeds of lime and lemon

Lime and lemon were cut in laboratory and thus seeds obtained were kept in a bottle containing detergent. The seeds were rinsed in continuous flow of tap water for 10 minutes. The seeds were wased by sterile water. Again washed once

by sterile distil water. The seeds were kept in 70% ethyl alcohol for about one minutes and immediately soaked by 4% sodium hypochlorite + one drop of tween 20 for 10 minutes. Finally the seeds were washed by sterile distill water for three times inside laminar air flow the seeds were ready for culture in CEM (Citrus Establishment Media).

Once the contaminated seeds were also sterilized from the same process. But in addition to sodium hypochlorite, mercury chloride was also used.

3.2.2. Sterilization of *in vitro* Shoot tips of Mandarin Orange.

The shoot tips were washed in mild detergent (Beepol) 3 times. They were kept under continuously flow of tap water for 10 minutes taking in a beaker and covered with muslin cloth. Shoots tips were kept in 70% ethanol for 1 minute and immediately transferred to 1% Sodium hypochloride. After 10 minutes in sodium hypochlorite, they were washed with sterile distilled water 3 times inside laminar air flow. Then the shoot tips were ready for meristem excision and culture in filter paper bridge under aseptic condition.

3.2.3. Sterilization of Glassware's and metal Instruments

The entire glasswares and metal instrument used for the aseptic *in vitro* culture, especially used in laminar air flow, were firstly dried in hot air oven and in the autoclave. All the glasswares were dipped in detergent and water for 24 hours and washed with the help of bottlebrush. They were cleaned with distilled water. They were sterilized in hot-air oven at 160^0c for 2-3 hours. The forcep, knife, scalpel and petridishes were wrapped with aluminium foil, autoclaved at 121^0c for 15-20 minutes at 15-lb/ sq. inch pressure.

3.2.4. Preparation of Stock Solutions for MS- medium

Each stock solution was prepared in the given concentration by completely dissolving its components in the accurate amount in the given volume of distilled water. Each stock solution was kept in sterilized brown bottles with labeling and preserved in refrigerator.

3.2.5 Preparation of Hormone Stock Solutions

The Auxins for the present investigation were IBA, NAA, Gibberellin is (GA_3) and the cytokinin (BAP). Concentration of IBA and BAP stocks was 1 mg/ml, concentration of NAA was 0.1mg/l and the concentration of GA_3 was 0.5 mg/ml made by dissolving in sterile distilled water respectively. IBA was first dissolved in 95% ethyl alcohol and BAP, NAA and GA_3 were first dissolved in 0.5N NaOH then sterile distilled water was added for the final volume. pH of all the hormone stocks was adjusted to 5.5 by adding 0.1 N NaOH or 0.1 N HCl. All the stocks were kept in sterile brown bottles with labeling and preserved in refrigerator.

3.2.6. Preparation of MS-Media

To prepare 1 liter of MS-medium, 400ml of distilled water was taken in dry and clean conical flask. Then from the stock solutions, 100ml of macronutrients (stock-A), 1 ml of micronutrients (stock- B), 10ml of Iron EDTA (stock-C) and 1ml of vitamins (stock-D) were taken into the flask serially 100mg of myo-inositol was also added. Then, 20gm (2%) of sucrose was dissolved with the help of magnetic stirrer. The volume was then made 900ml adding sterile distilled water. The pH was adjusted 5.7 using 0.1 N NaOH or 0.1N HCl and the reading was noted by highly sensitive pH meter. 1gm of Casein Hydrolysate was added. Sterile water was added to make the final volume 1 liter. The hormone frees MS-medium was then supplemented with different concentrations of different hormones according to;

$S_1 V_1 = S_2 V_2$ gm/ liter

Where,

S_1 = Strength of hormone in stock solution.

V_1 = Volume of hormone to be added in the give medium from the stock solution.

S_2 = Strength of hormones require in medium.

V_2 = Volume of medium

7 gm of agar was added in 1 litre of medium. Solutions were then brought to boil using electric heater. Despensed into the bottles (about 50 ml in each) and culture tubes (about 10ml in each) and covered by lid with labelling.

3.2.7. Sterilization of Media for Culture

The media were autoclaved at 121^0C for 14- 20 minutes at 15 lb / sq. inch. The culture tubes were kept at slanting position (i.e around 45^0). If there was no sign of contamination even in 1 week, then it would be ready for use.

3.2.8. Use of Inoculation Chamber

Before using, laminar air flow chamber or clean bench was properly cleaned by 95% ethanol. The culture tubes and bottles with media, metal instruments like scalpel, forceps needle, scissor, sterilizing box containing autoclaved glassware's were cleaned by spraying ethanol (95%). All the necessary materials (spirit, ethanol, sodium hypochorite, mercuric chloride, sterile distilled water, glasswares, metal instruments etc.) used in clean bench, except plant materials were exposed to UV-light for about 45 minutes. By closing UV- light, air- blower was opened for 30 minutes then, laminar air flow. (Clean bench) was ready for use.

3.2.9. Seed Culture of Lime

Four different conditions were maintained for the seed culture of lime for: 1.CEM (Citrus Establishment Media), which was MS-media without stock-D, sugar and hormone, was used and 26 seeds were cultured in the culture tubes, 2. CEM was used and the seeds were without seed coat. The total 34 seeds were cultured in 34 culture tubes, 3. CEM was used adding 1 mg/l BAP and the seeds were with seed coat. The total seeds were 28, which were cultured in 28 culture tubes and 4. CEM was used. The seeds were treated with 500mg/l GA_3 for 24 hrs before culture of sterilization 14 seeds with seed coat were culture in 14 culture tubes. All the culture tubes were maintained at 21± 2°C and kept in light.

3.2.10. Seeds culture of lemon:

Sixty- five sterilized seeds were cultured in 13 bottles (5seeds per bottle) in CEM. All the sterile seeds were cultured inside laminar air flow under aseptic condition. All the culture bottles were maintained at 21 ± 2^0C and kept in light.

3.2.11. Nodal Culture of Lime AO_1 (6), Lime BO_1 (15), Lemon and Troyer Citrange

Explants of node culture (single node for each culture was taken from *in vitro* grown seedling of lemon. Similarly, the explants of lime AO_1 (6), lime BO_1 (15) and troyer citrange (single node for each culture) were taken from *in vitro* grown seedlings. Explants were aseptically excised and inoculated on MS with different concentrations and combinations of BAP and NAA. Five nodes were planted in each bottle. Data were taken after 4 weeks, 8weeks, 12 weeks & 16 weeks respectively.

3.2.12. Meristem Culture of Mandarin Orange

The sterile shoot-tips of mandarin were placed on a petridish under a microscope of 30x magnification, the protective leaves on the bud were removed with a scalpel and the apical dome of meristem less than 0.5mm were separated. The meristem was placed on a filter paper bridge in culture tubes with the liquid medium. The whole process was done inside laminar air flow chamber under aseptic condition. The incubation was under low light at 21± 2^0C.

3.2.13. *In vitro* rooting of shoots of lime AO_1 (6)

The *in vitro* proliferated young shoots of lime AO_1(6),size of about 1cm,were transferred aseptically in MS-media with different concentration of rooting hormone IBA and NAA. For rooting, five shoots were planted in each bottle and the single shoots were transferred in each culture tube. The cultures were mainted at 21±2°C .Average no. of Node per shoot per cm, percentage (%) of rooting and average no. of. root per shoot were noted for analysis.

3.2.14. Acclimatization of *In vitro* rooted plants of lime AO_1 (6)

Mixed soil and sand, in the ratio of 1:1 was used for the acclimatization of *in vitro* plants well and made holes all in equal distances(about 7cm) in two pots named as pot-1 and pot-2.

The *in vitro* rooted plants were planted, removing media properly, in such a way that plants with multiple roots (26 plants) were planted in pot-1 and plants with single root (28 plants) were planted in pot-2. Both the pots were of about the diameter of 50cm. Both plastic pots were covered with muslin cloths and watered properly. There were small pores at bottom to remove excess water while watering.

Plants were kept moistly watering once in a day in first week, once in two days in second week and continued up to six weeks. After six-weeks, the muslin cloths were removed and data were taken. These plants were then pulled from the pot and transferred to the field in the evening time. Plants were watered properly and covered with plastic to save the plant from low humidity of air. After 4 weeks of field transfer, the plants were ready for virus testing of plantation in the farmers field.

Aphid is the main vector to transmit CTV from infected plant to young and healthy plant. So, we most do acclimatization inside screen house.

3.2.15. DAS- ELISA for detection of CTV in different Citrus spp:

Coating with Antibodies:

Coating buffer was prepared as shown in material required. Antibody was mixed with the coating buffer at 1:500 ratio. 200µl was poured into each well of the ELISA plate. The plate was incubated at 37^0C for 3 hours. After the incubation period, the plate was washed three times with a washing buffer.

Sample Preparation and loading

Leaf and pedicel samples of *in vitro* plants of lime AO_1 (6), lime BO_1 (15), lime (Zygotic), lemon and Troyer Citrange *in vivo* plants of lime, lemon and mandarin were collected from the screen house of GREAT, Baneshwor,

tissue culture laboratory of GREAT Central Department of Botany, Kirtipur Kathmandu and *in vivo* seedling of Chitwan lime. Plant samples were each of 500mg. Samples were ground by mortar and pestle using extraction Buffer and centrifuged in 2000rpm for 2-3 minutes.

Each sample was loaded in to each well with a different micropipette tip. 200µl was poured into each well. The plate was then incubated at 4°C for 16 hrs. After the incubation period, the plate was washed 3 times with a washing buffer.

Conjugate loading

Conjugate buffer was prepared as shown in material required. The conjugate antibody was mixed with the buffer at 1:500 ratio. 0.2 ml of sample was poured into each well. The plate was then incubated at 37^0 for 3 hrs. and again washed three times with washing buffer.

Substrate Loading

Substrate was prepared as shown in materials required. 200 µl was poured into each well. 30-60 minutes was allowed for colour reaction.

Result Reading

After the colour formation in 60 minutes, the optical density of each well was calculated with the help of an ELISA plate reader. The lens was set at 405 nm. The reading of ELISA plate reader (Huma reader) was printed out with the help of printer.

Reaction stopping

The colour reaction was stopped after plate reading this was done by adding 50 µl (3M)of NaOH Solution to each well.

3.2.16 Statistical Analysis

Standard Deviation (σ) and Duncan Multiple Range Test (DMRT), was done applying raw data through the use of SPSS computer programming.

CHAPTER-IV

RESULTS

4.1 Micropropagation of Lime

4.1.1 Seed Germination of Lime

Seeds started to germinate after five weeks of culture. There was no branching in seedling plumule. The polyembryony was expressed giving two seedlings (maximum) from single seed. The average height of seedling after 16 weeks was 7.5 cm. The present germinations of seeds differed with or without seed coats. With seed coat, seeds germination is fully (100%) in presence of GA_3 CEM (Fig. 4.1.1)

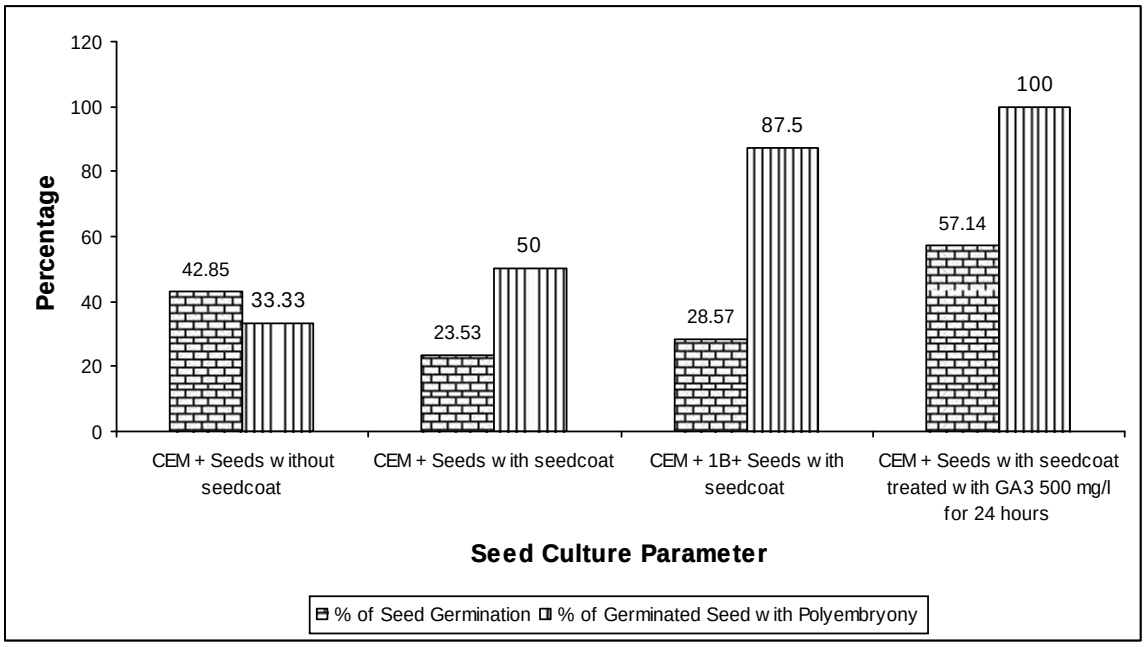


Fig 4.1.1 Seed Germination of Lime in 16 weeks

4.1.2 Shoot Multiplication from Nodal Explant of Lime AO_1 (6)

Explants cultured on MS supplemented with combination of BAP 0.5 mg/l and NAA 0.5 mg/l produced maximum 6 number (individual) of shoots from single node in 4 weeks. After 16 weeks, some of the plants which were not showing the response, produced yellowish masses.

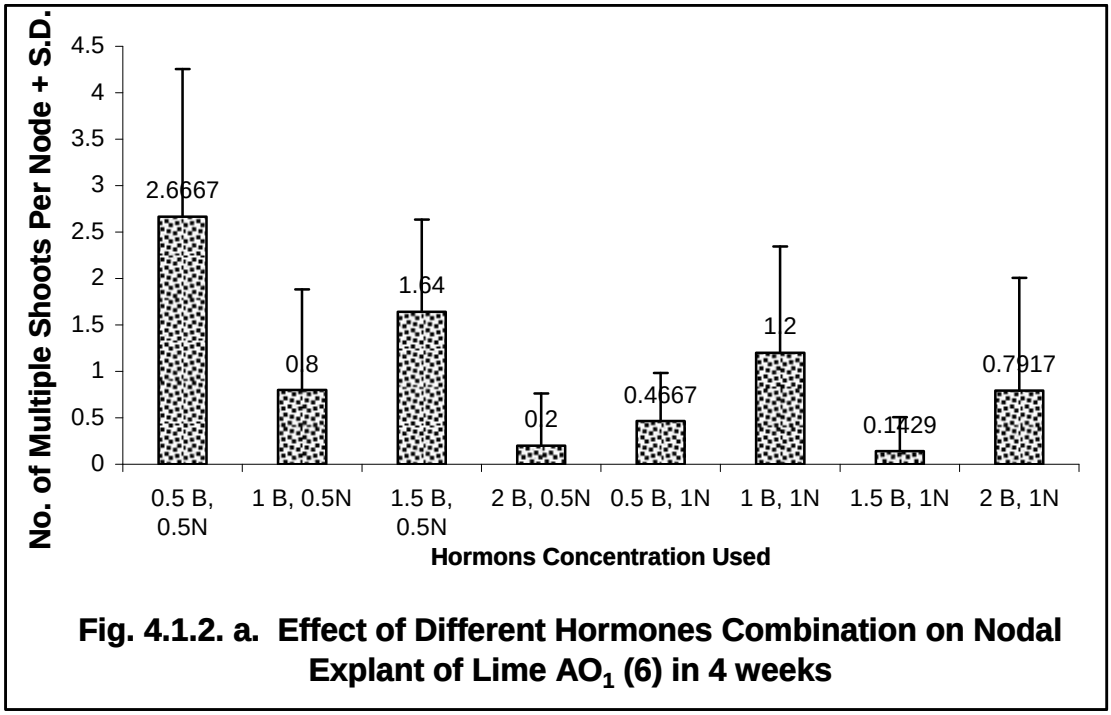


Fig. 4.1.2. a. Effect of Different Hormones Combination on Nodal Explant of Lime AO_1 (6) in 4 weeks

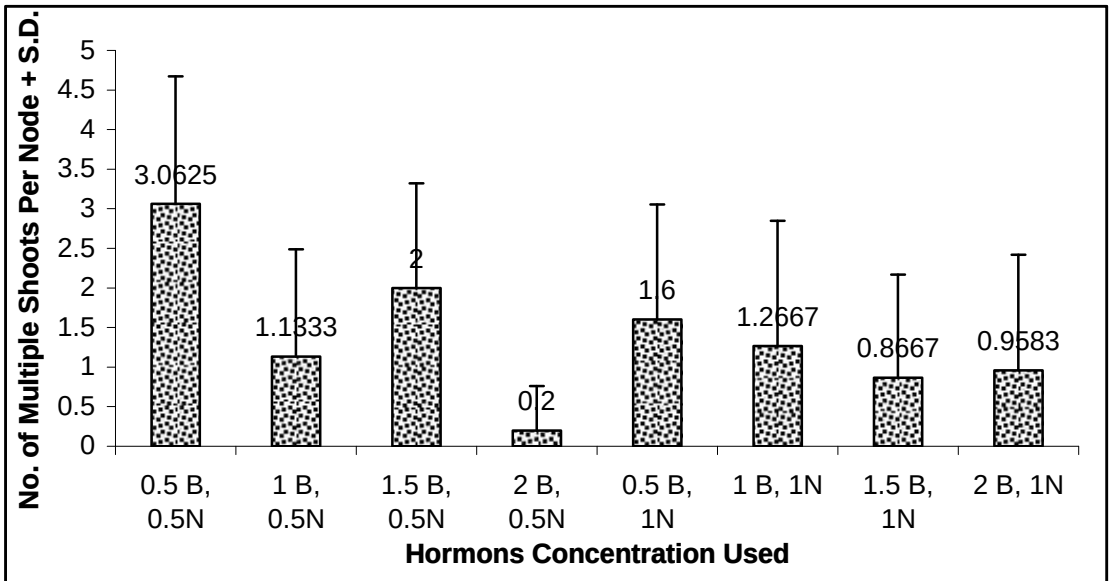


Fig. 4.1.2. b. Effect of Different Hormones Combination on Nodal Explant of Lime AO_1 (6) in 8 weeks

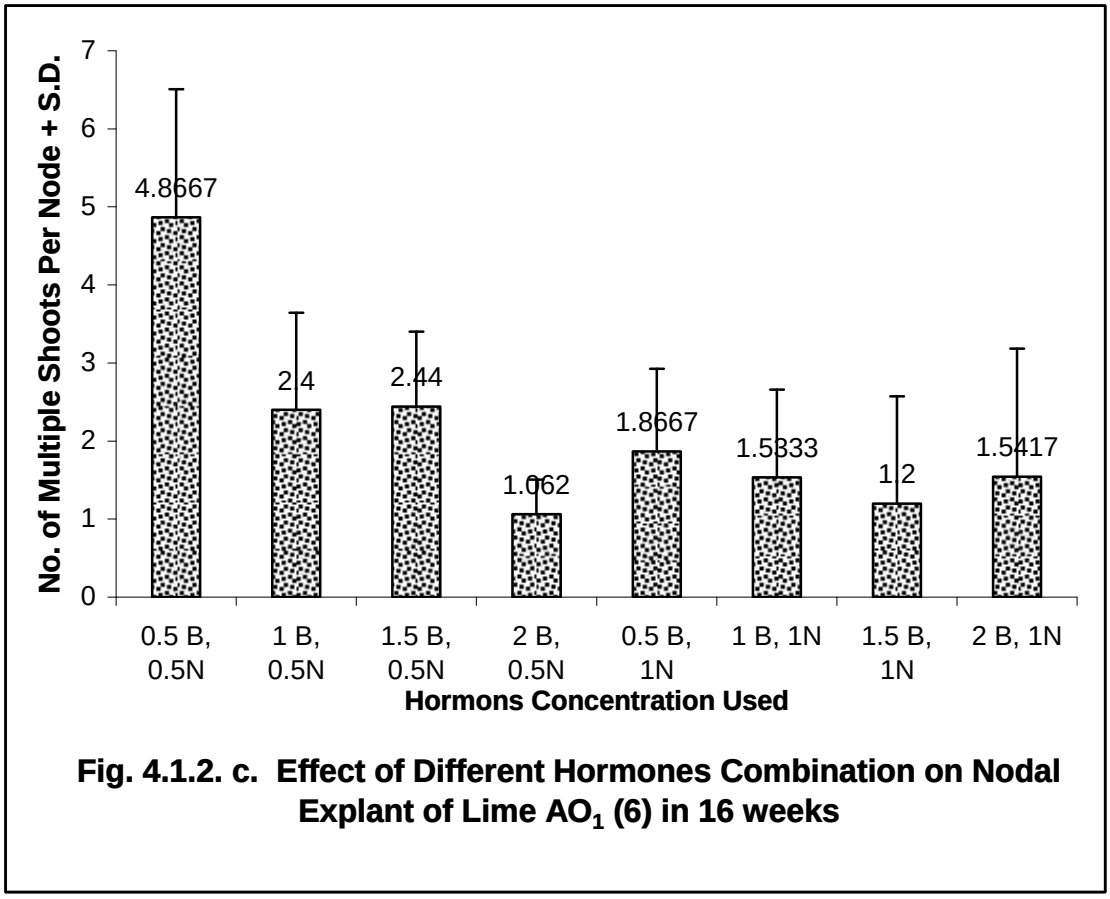


Fig. 4.1.2. c. Effect of Different Hormones Combination on Nodal Explant of Lime AO_1 (6) in 16 weeks

4.1.3. Shoot Multiplication from Nodal Explant of Lime BO_1 (15)

Explants cultured on MS supplemented with combination of BAP 0.5 mg/l and NAA 0.5 mg/l produced maximum 12, number (individual) of shoots from single node in 16 weeks. In 16 weeks, maximum 40% callus was observed in MS with BAP 2 mg/l + NAA 0.5 mg/l. Similarly, BAP 1.5 mg/l +NAA 1 mg/l induced callus in 13.33% nodes cultured and 26.66% in BAP 2 mg/l + NAA 1mg supplement.

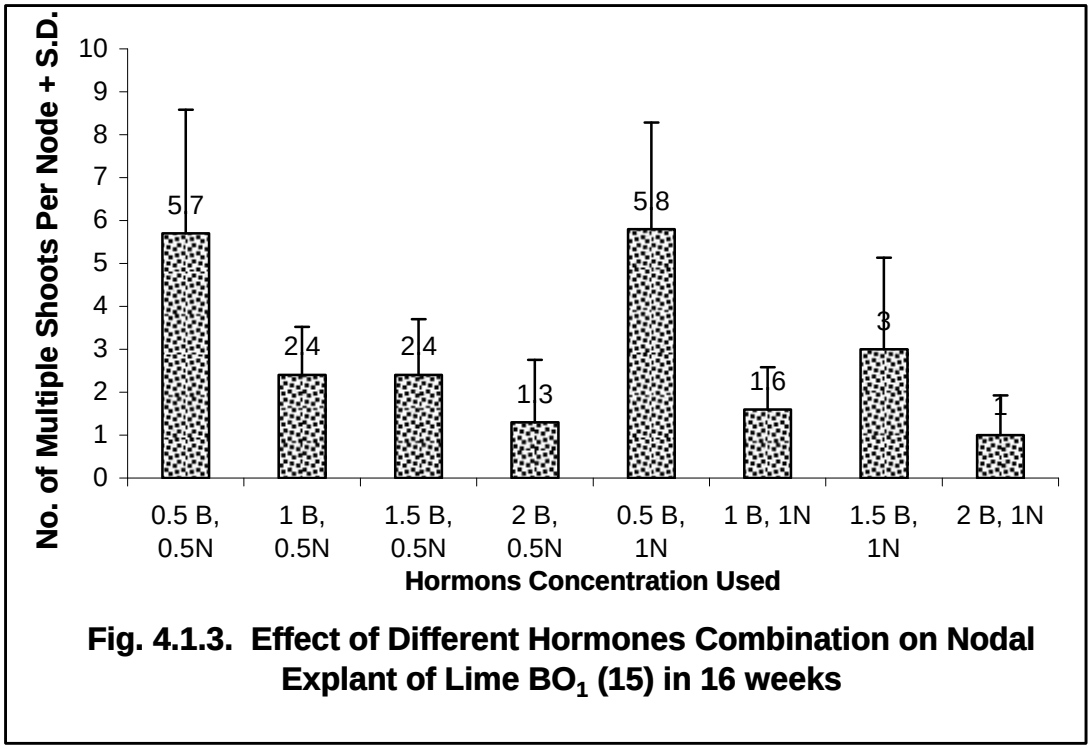


Fig. 4.1.3. Effect of Different Hormones Combination on Nodal Explant of Lime BO_1 (15) in 16 weeks

4.1.4. *In vitro* rooting of lime AO_1 (6)

Aseptically transferred young shoot each of about 1 cm in different concentrations of rooting media started producing root after 20 days of culture. First root initiation was in MS supplemented with IBA 1 mg/l in 20 days. Similarly in MS supplemented with NAA 0.5 mg/l the root initiation was observed in 26 days of culture. This was first record when NAA was used as rooting hormone. The maximum no. of node per shoot per cm was recorded 16 (individual) in MS supplemented with IBA 0.5 mg/l. And the maximum no. of root per shoot was noted to be 28 (individual) in MS supplemented with NAA 2 mg/l.

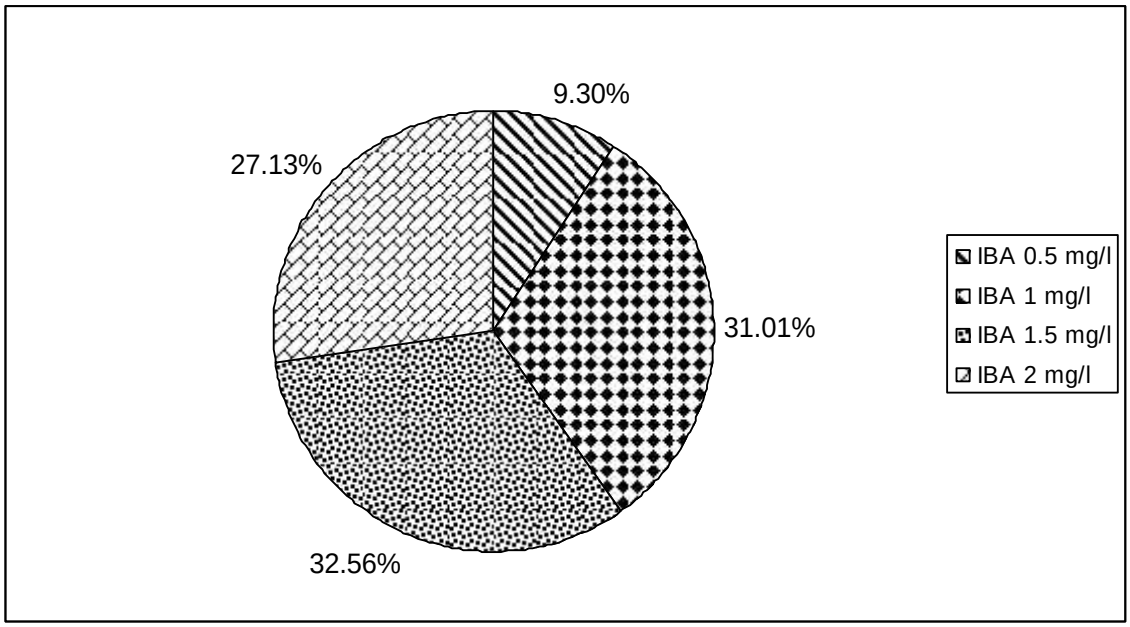


Fig. a. Pie Diagram Showing the % of Rooting of Lime $AO_1(6)$ in IBA

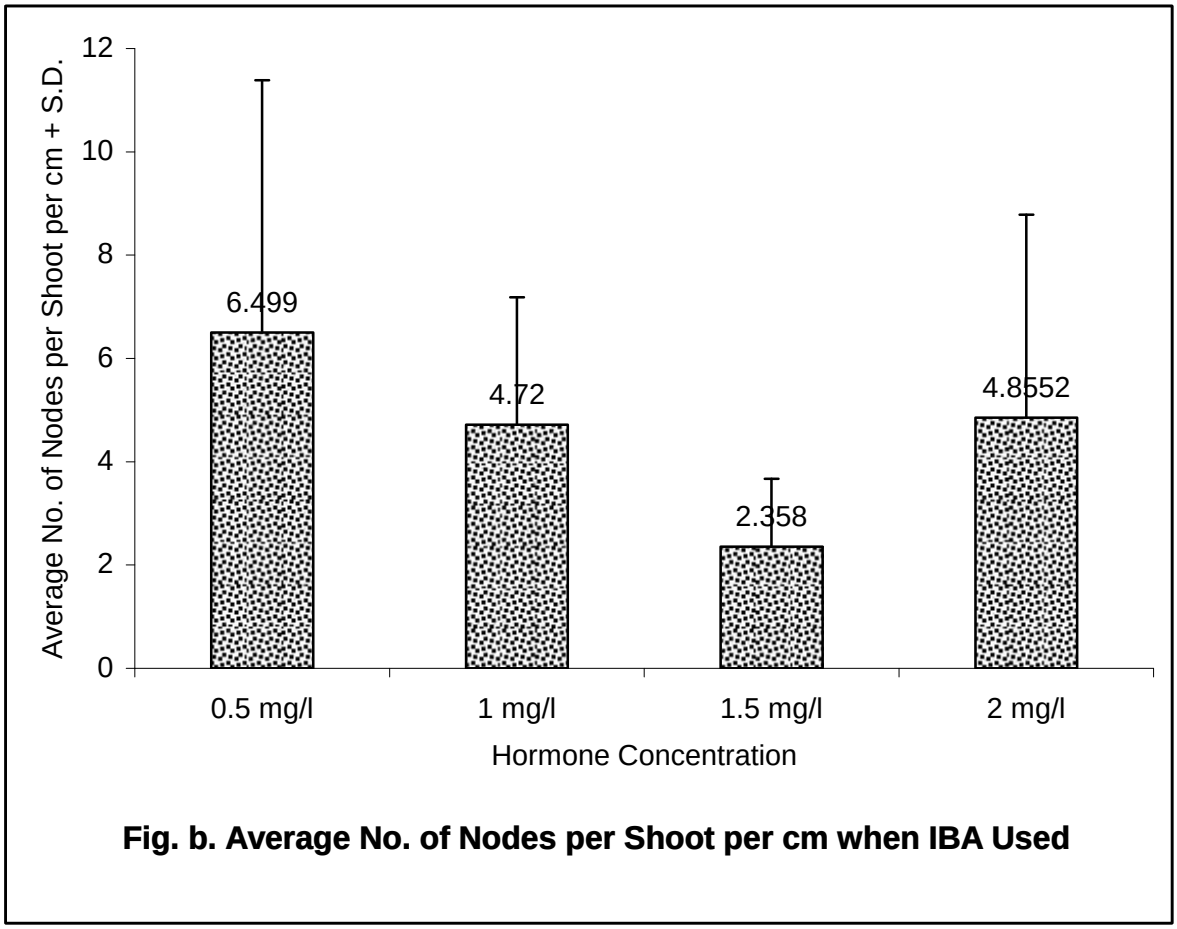


Fig. b. Average No. of Nodes per Shoot per cm when IBA Used

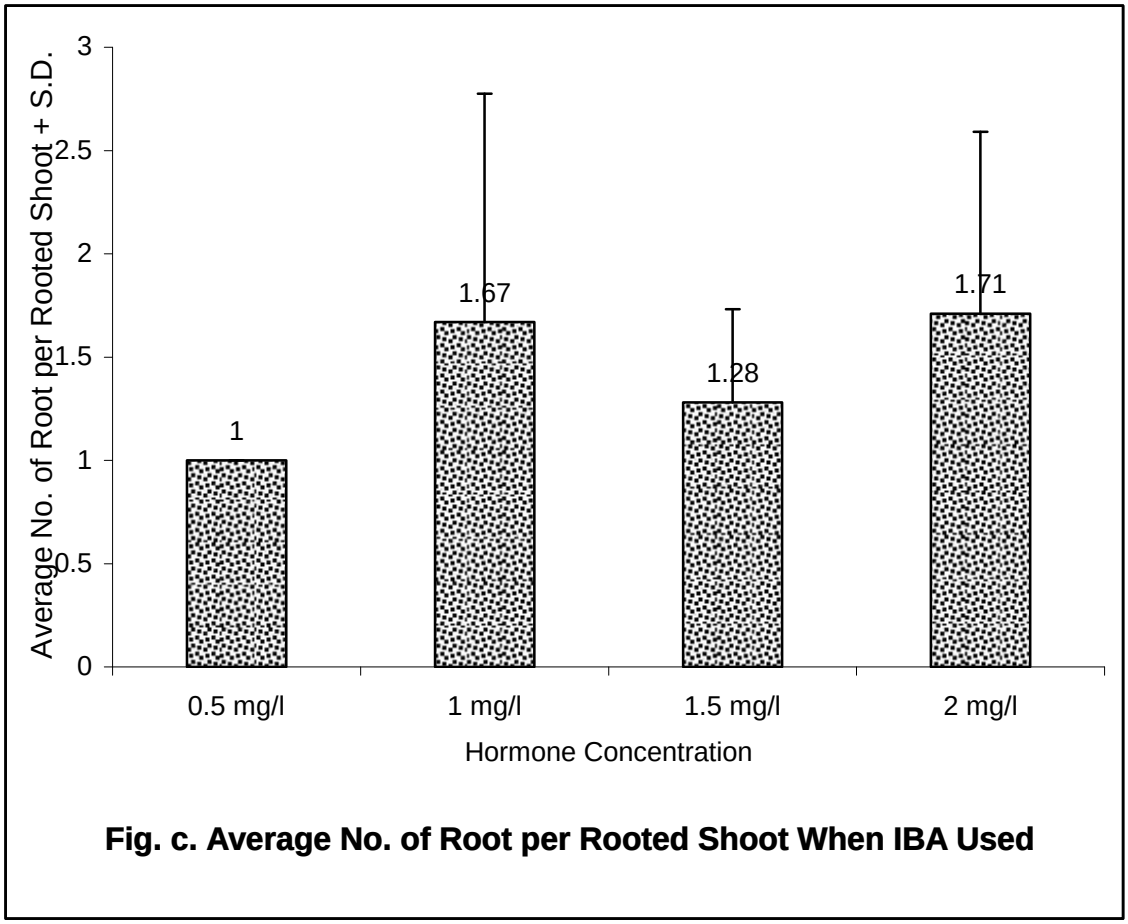


Fig. c. Average No. of Root per Rooted Shoot When IBA Used

Fig. 4.1.4.1 a,b,c, Effect of IBA on Root Formation of Lime AO_1 (6) in 16 Weeks

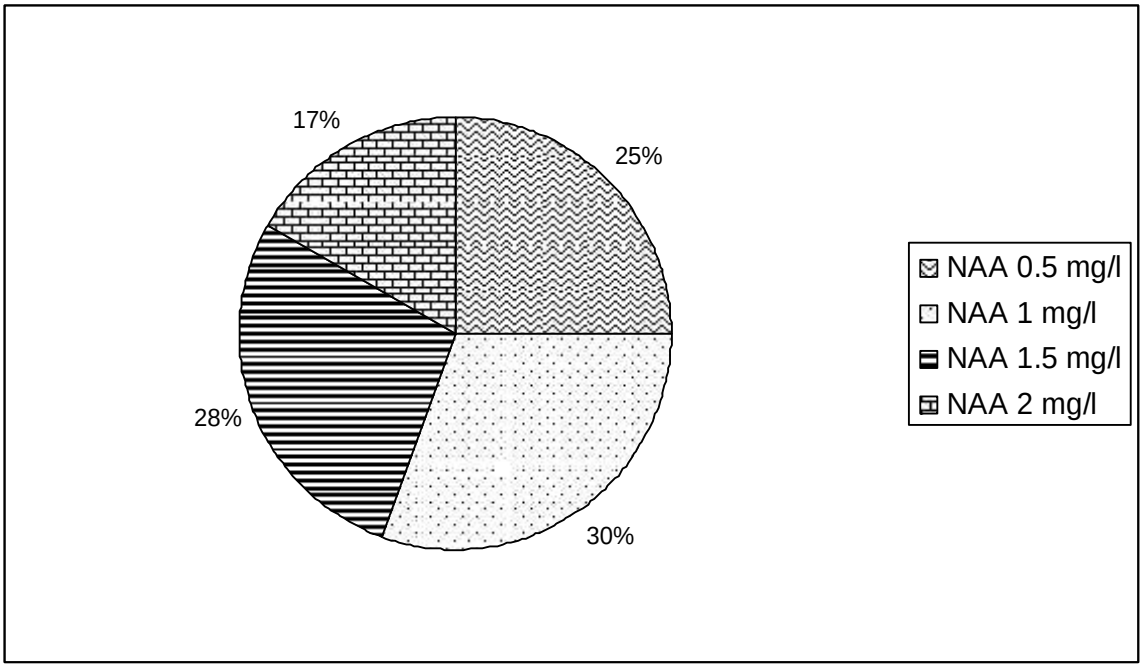


Fig a. Pie Diagram Showing % of Rooting in NAA Used

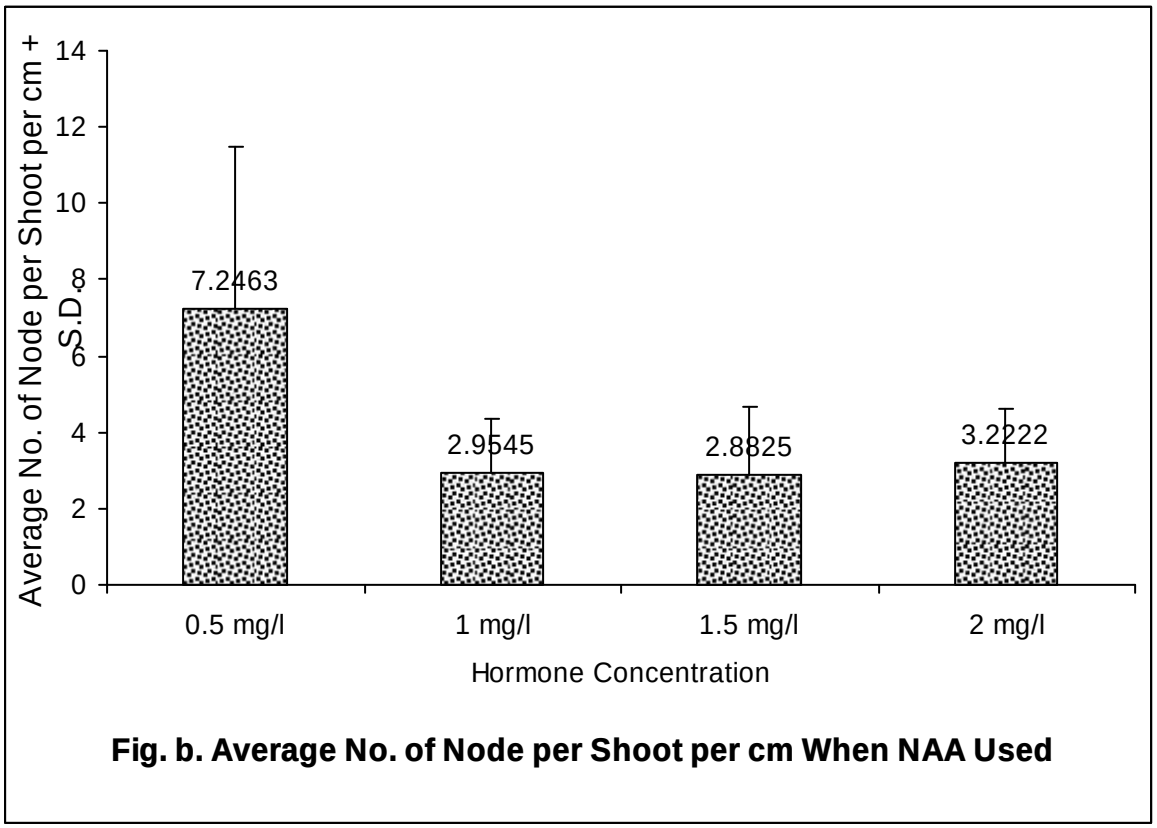


Fig b. Average No. of Node per Shoot per cm

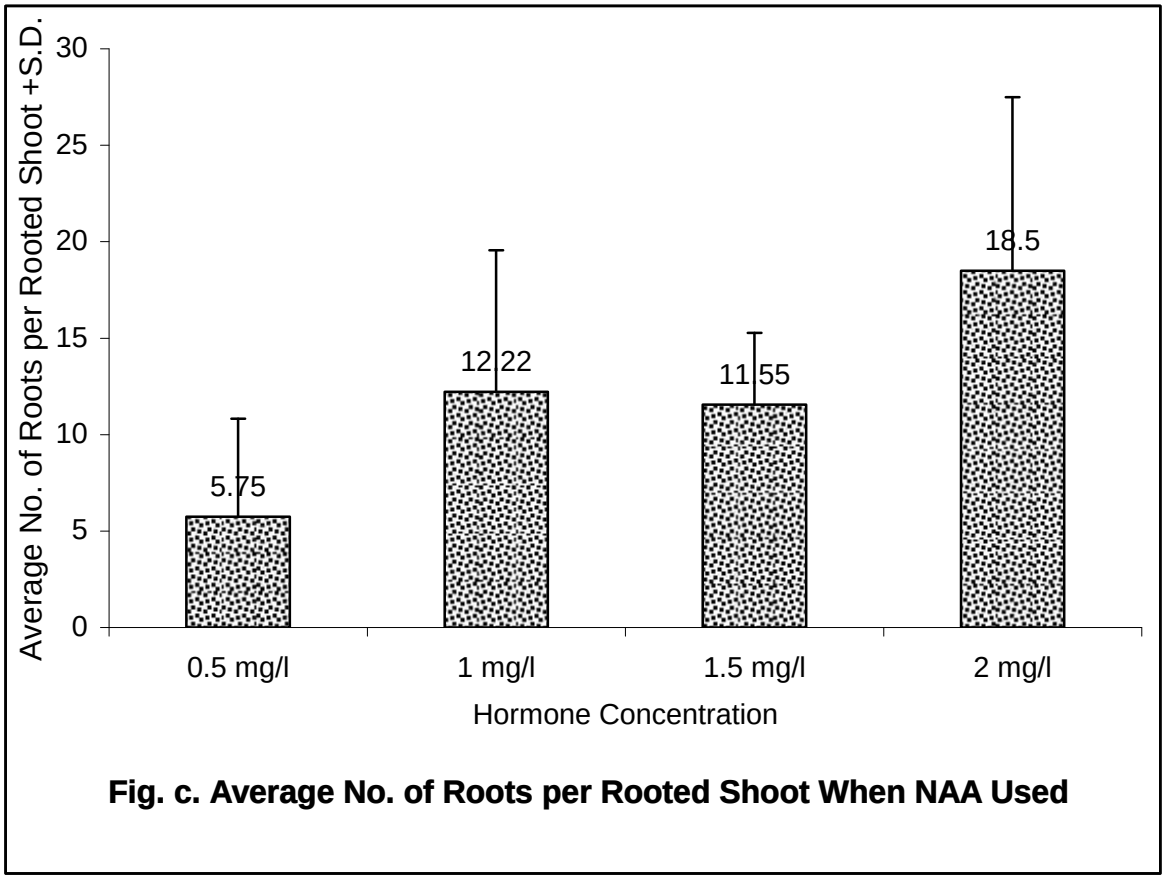


Fig. 4.1.4.2 a,b,c Effect of NAA on Root Formation of Lime AO_1 (6) in 16 Weeks

4.1.5. Acclimatization of *In vitro* Rooted Lime

After six- weeks of transplantation (in screen house) the *in vitro* grown plants were found to acclimatize well that 12 (46.15%) plants with multiple root and 10 (53.71%) plants with single root were acclimatized. When these plants were transferred to the field, only 12 plants (54.54% of those of acclimatised plants) were alive after 8 weeks of field trial (transfer). Out of 54 *in vitro* rooted plants only 12(22.22%) could be saved in field trial.

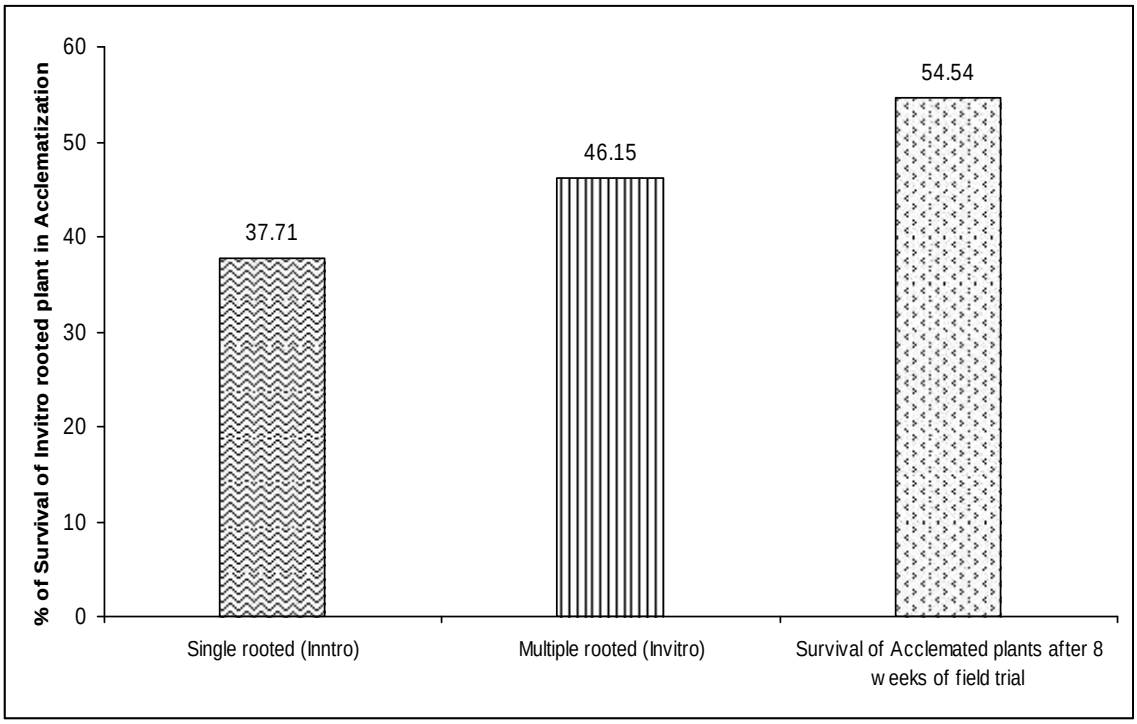


Figure 4.1.5 Acclimatization of *In vitro* Rooted Lime AO_1 (6)

4.2. Micro-propagation of Lemon

4.2.1. Seed Germination of Lemon:

The healthy i*n vitro* cultured seeds of lemon started to germinate from the five weeks (35 days) of culture. In 8 weeks, 18 seeds (27.69%) were germinated out of 65 total cultured seeds. Similarly, in 12 week, 16 weeks and 20 weeks, the percentage of germination increased by to 36.92% (24 seeds), 61.43% (40 seeds) and 83.07% (54 seeds) respectively. None of the other seeds were found to be germinated later. Polyembryony was not expressed in seed germination but multiple branching in single seeding was observed in few cases

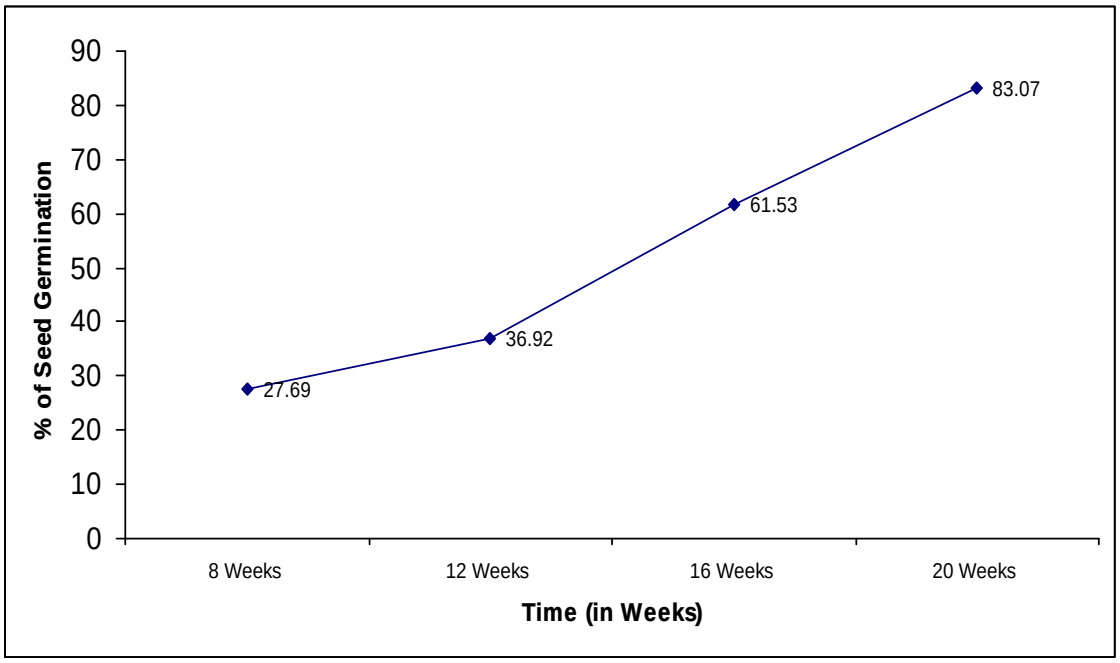


Fig. 4.2.1 Seed Germination of Lemon

4.2.2. Shoot Multiplication from Nodal explants of Lemon:

Explant cultured on MS supplemented with combination of BAP 1.5 mg/l and NAA 1 mg/l produced maximum 7 (individual) numbers of shoots from single node in 16 weeks. In this culture condition the callus was observed in 6.66% of explants. Callus producing shoots also produced healthy multiple shoots.

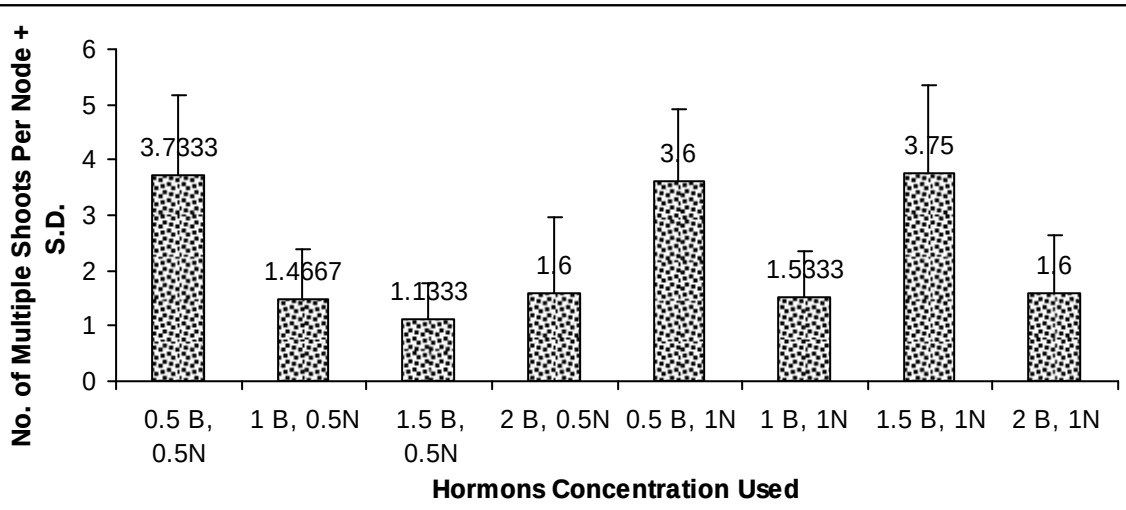


Fig. 4.2.2. Effect of Different Hormones Combination on Nodal Explant of Lemon in 16 weeks

4.3. Comparative Analysis of multiple shoot production from node culture among lime AO_1 (6), lime BO_1 (15), lemon and Troyer Citrange under similar condition maintained

Nodal explants of lime AO_1 (6), lime BO_1 (15) lemon and Troyer Citrange were cultured for their *in vitro* study. Nodal explants cultured on MS supplemented with BAP 1 mg/l and NAA 0.1 mg/l.They expressed very good response for multiple shoot production. Among all the varieties, Troyer Citrange showed good response producing more multiple shoots than other in the same interval of time. Lime AO_1 (6) and lime BO_1 (15) did not produce callus even after 16 weeks of culture. Lemon and Troyer Citrange both expressed the callus. In 8 weeks of culture, lemon expressed 5.45% callus and that reached to 16.16% in 10 weeks. Troyer Citrange developed 28.18% callus in 8 weeks and reached 36.036% in 16 weeks.

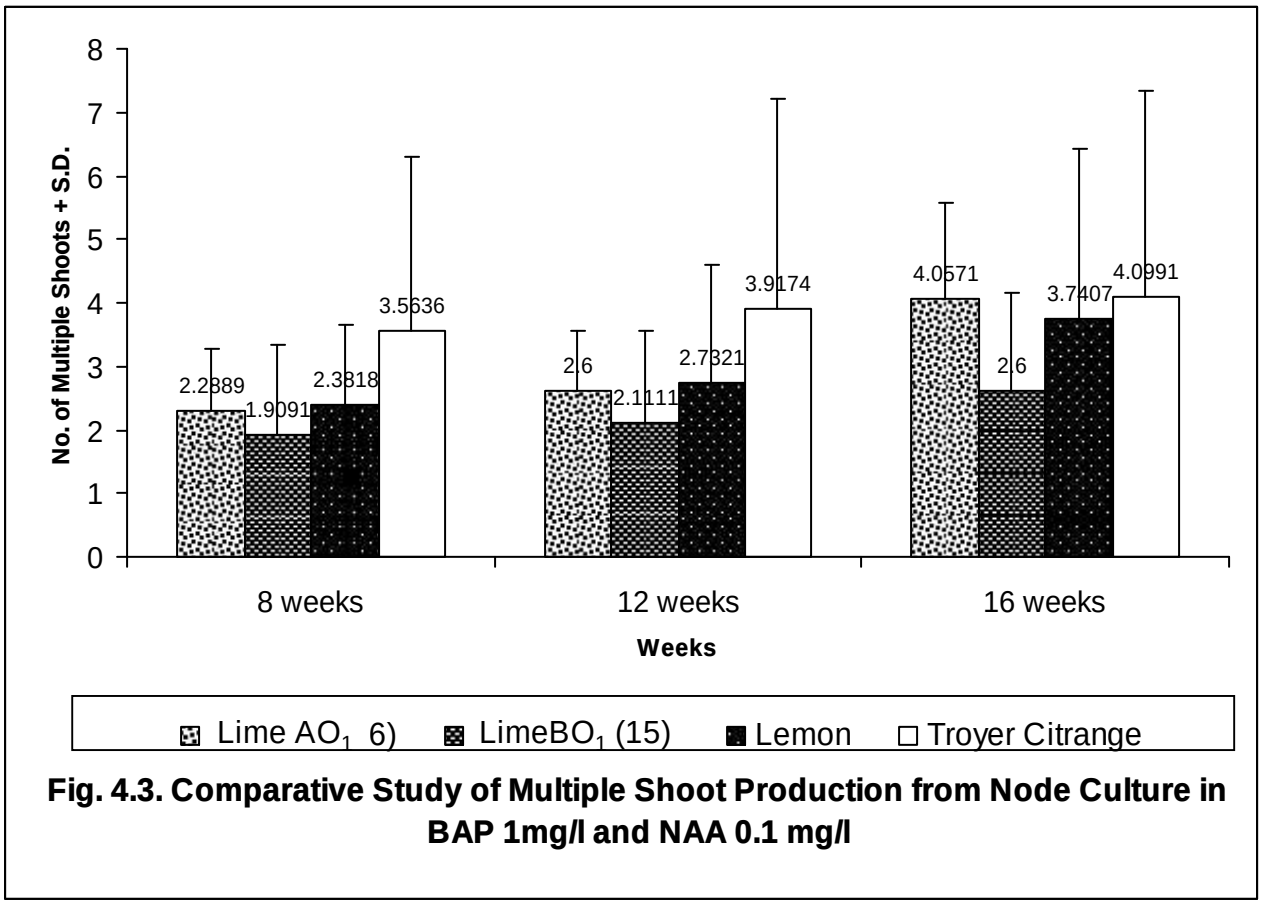


Fig. 4.3. Comparative Study of Multiple Shoot Production from Node Culture in BAP 1mg/l and NAA 0.1 mg/l

4.4. Meristem Culture of Mandarin Orange

Out of 24 apical meristem (size < 0.5mm) cultured in filter Paper Bridge of 12 culture tubes, four meristem (16.66%) were alive.. These four meristems increased upto 0.5 to 0.7 cm in 24 weeks

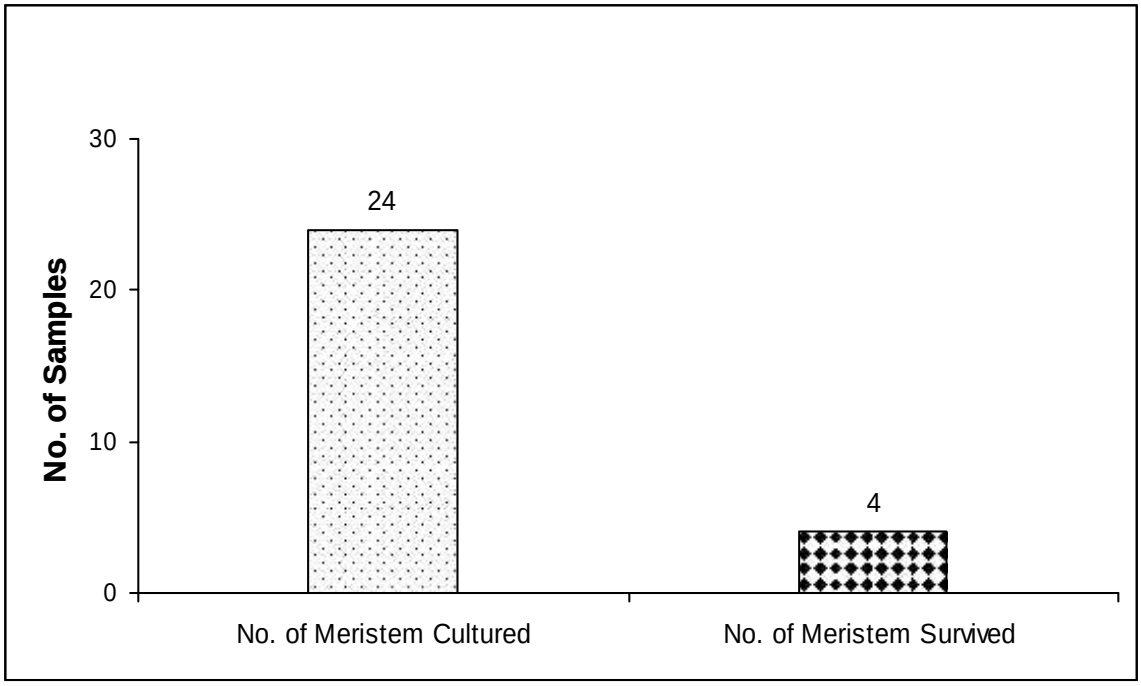


Figure 4.4 Meristem Culture of Mandarin Orange

4.5. ELISA- plate reading of DAS- ELISA

In vitro grown seedling of lime brought from Chitwan (two samples) were found to be virus negative. *In vitro* grown seedling- derived plants obtained from CDB, TU (three samples) also found to virus negative. *in vitro* grown lime AO_1 (6) (Four samples) also found to be virus negative. Already known virus positive samples used for reference gave positive result on test.

Virus negative lime AO_1 (6), lime BO_1 (15), Troyer Citrange, seedling derived lemon were used for research. Finally, tissue culture- derived of then acclimatized lime AO_1 (6), as well as *In vitro* grown lime tested whether there is CTV or not. All gave negative result for CTV- test using DAS-ELISA. Already known virus positive mandarin orange also gave positive result, from which

apical meristem was excised and cultured under *in vitro* for virus elimination. Virus test of meristem was not done.

Table- 4.5.1

Optical Density Reading by DAS-ELISA

(Test plate-1)

Sample No. In test plate	Sample used	A_{405}	Location	Reference
10	CTV +ve. Lime	1.316	GREAT, Baneshwor	CTV +ve
11	CTV +ve. Lemon	1.120	GREAT, Baneshwor	CTV +ve
22-25	Lime AO1(6), *In vitro*	0.175, 0.219, 0.236, 0.211	GREAT, Baneshwor	CTV-ve
26-28	*In vitro* Lime of CDB	0.180,0.267,0.330	Tissue culture lab. CDB, Kirtipur	CTV-ve

Maximum O.D. of blank well= 0.351

Table- 4.5.2

Optical Density Reading by DAS- ELISA

(Test- plate-2)

Sample No. In test plate	Sample used	A_{405}	Location	Reference
6	CTV +ve. Lime (Invivo)	0.573	GREAT, Baneshwor	CTV +ve
29	Seedling derived lime (Invivo)	0.104	Chitwan Nursery, (NGT)	CTV -ve
30	Seedling derived lime (Invivo)	0.075	Chitwan Nursery, (NGT)	CTV-ve

Maximum O.D. of blank well= 0.247

Table- 4.5.3

Optical Density Reading by DAS-ELISA

(Test- Plate-3)

Sample No. In test-plate	Sample used	A_{405}	Reference
1	*In vitro* lime $AO_1(6)$	0.252	CTV-ve
2	*In vitro* lime $AO_1(6)$	0.202	CTV-ve
3	*In vitro* lime $AO_1(6)$	0.115	CTV-ve
4	*In vitro* lime $BO_1(15)$	0.151	CTV-ve
5	*In vitro* lime $BO_1(15)$	0.225	CTV-ve
6	*In vitro* lime $BO_1(15)$	0.171	CTV-ve
7	In vitro lemon	0.129	CTV-ve
8	In vitro lemon	0.144	CTV-ve
9	In vitro lemon	0.170	CTV-ve
10	In vitro Troyer-	0.077	CTV-ve
11	In vitro Troyer Citrange	0.233	CTV-ve
12	In vitro Troyer Citrange	0.205	CTV-ve
13-15	CTV +Ve Mandarin Orange (Invivo)	0.448	CTV+ve
16-18	CTV +Ve Mandarin Orange (Invivo)	0.365	CTV+ve
22-24	CTV +Ve Mandarin Orange (Invivo)	0.941	CTV+ve
43-45	CTV-ve lime $AO_1(6)$	0.164	CTV-ve
46	Acclimatized lime $AO_1(6)$	0.166	CTV-ve
47	Acclimatized lime $AO_1(6)$	0.189	CTV-ve
48	Acclimatized lime $AO_1(6)$	0.099	CTV-ve
49	Acclimatized lime $AO_1(6)$	0.143	CTV-ve
50	Acclimatized lime $AO_1(6)$	0.168	CTV-ve
37-42	CTV +ve Unknow sample	0.705	CTV+ve

Maximum O. D. of blank well = 0.281

Location of sample used = GREAT

CHAPTER FIVE

DISCUSSION

5.1 Seed Germination of Lime

When the seeds with seed coat were cultured in CEM., the seeds were not treated with any dormancy breaking as well as ploymbryony inducing hormones , the germination is less (i.e. only 23.53%) in respect to the other three conditions. When the seed coats were removed and cultured in CEM, the % of germination is more than before (seeds with seed coat). The percentage of polyembryony expressed was reduced from 50% to 33.33%. This may be due to removal of embryo on removing seedcoat. Addition of BAP 1 mg/l in CEM did not alter the percentage of germination more but percentage of plyembryony was increased significantly. This may be due to the effect of cytokine (BAP). When the seeds were treated with GA_3 500 mg/l for 24 hours before culture, the percentage of germination of seeds as well as percentage of polyembroyony increased signinficantly because GA_3 breaks seed dormancy and induces seed germination (Davies, 1995; Mauseth, 1991; Raven, 1992; Salisbury and Rose, 1992)

5.2 Seed Germination of Lemon

The number of embryos per seed varies greatly even on one tree, the average number differs greatly according to variety. There is no consistency on polyembryony expression. In lemon, there is low percentage of *nucellar* seedlings (Frost, 1926)

The percentage of germination of lemon was found to be high in comparison to lime. Due to the presence of less phenolic compound, the percentage of germination of lemon seed is generally more. Since, citrus seeds contain antioxidant like ascorbic acid, it relatively gets free from endogenous organic inhibitor.

The length of dormancy of Citrus varies from one month to as long as six months. It is due to the fact that GA can be employed to break natural dormancy in Citrus (Cooper and Peynado, 1958).

5.3 Shoot Multiplication from Nodal Ex-plant of Lime AO_1(6)

In the observation of shoot multiplication every step from 4 weeks up to 16 weeks, the best multiplication was found in MS supplemented with BAP 0.5 mg/l and NAA 0.5 mg/l among all eight combinations of BAP and NAA. Grinblat (1972) reported that maximum shoot production in citrus culture has been achieved more through the use of BAP than other cytokinin. The best combination of BAP and NAA for Citrus Spp was found to be BAP 1 mg/l and NAA 0.1 mg/l where as optional concentration of BAP was 1 mg/l (Ranjit and Karki, 1999; Niroula, 1994). There was no development callus during the shoot multiplication. This hormone combination may be the appropriate one. The no. of multiple shoots increased from 2.66 m/s in 4 weeks to 4.86 m/s on 16 weeks which is significant.

5.4 Shoot multiplication from nodal explant of lime BO_1(15);

MS-supplemented with either BAP 0.5mg/l + NAA 0.5 mg/l or BAP 0.5 mg/l was found to be similar and the best among all hormone combination. There was no callus development during shoot multiplication though some of other remained combination of BAP and NAA induced the maximum number of multiple shoots i.e. 5.8 m/s in BAP 0.5 mg/l + NAA 0.5 mg/l and 5.7m/s in BAP 0.5 mg/l + NAA 1 mg/l . Increase in level of Auxin (NAA) and decrease in level of Cytokinin (BAP) might be the reason that both hormone combination did not induce callus. Grinblat (1972) reported that more shoot production of citrus culture has been achieved through the use of BAP than other cytokinin.

5.5 Shoot Multiplication from the Nodal Explant of Lemon

MS-supplemented with either BAP 0.5 mg/l + NAA 0.5 mg/l or BAP 0.5 mg/l + NAA 1 mg/l or BAP 1.5 mg/l + NAA 1 mg/l, all these three conditions was found to be better than the other five combinations of BAP and NAA applied in MS media. The shoot growth was also very good compared to other *in vitro* studied citrus spp. The maximum multiple shoots achieved 3.75 m/s when BAP 1.5mg/l + NAA 1 mg/l were used. Similarly, other rates of growth such as 3.7m/s in BAP 0.5 mg/l + NAA 0.5 mg/l and 3.6 m/s on BAP 0.5 mg/l + NAA 1 mg/l were achieved. There was callus induced in BAP 1.5 mg/l + NAA

1mg/l, this may be due to high concentration of cytokinin (BAP). The callus induction might be suppressed due to high concentration of NAA. In other conditions too, absence of callus and good growth as well as multiple shoot production may be due to appropriate but not less (0.5mg/l) use of BAP and due to more use of Auxin (NAA) i.e. 0.5 mg/l and 1 mg/l. Pradhan (1999) obtained green callus from shoot tip of *Santalum album* L. cultured on MS medium supplemented with BAP 1mg/l + NAA 0.1 mg/l. Niroula (1992) obtained multiple shoots from cotyledon node culture of *Citrus limon* in MS medium in the presence of BAP1mg/l + NAA 0.1 mg/l.

5.6 Comparative Study of Multiple Shoot Production from Nodal Culture among Lime AO_1 (6), Lime BO_1 (15), Lemon and Troyer Citrange:

Among four Citrus spp all started giving m/s after 5 weeks of culture. Troyer Citrange expressed better result inducing 3.56 m/s growth in 8 weeks. This is maximum in comparison to other three citrus spp. Another interesting result was that, there was not more significant growth of Troyer Citrange from 8 weeks to 16 weeks. In 16 weeks there were only 4.09 m/s per node cultured. In other hand, though previously there was not good growth of lime AO_1 (6) that was 2.20 m/s in 8 weeks and 2.6 m/s in 12 weeks that surprisingly increased to 4.11 m/s in 16 weeks, that was the second better result after Troyer Citrange. In case of lemon, that was in third position to give multiple shoot i.e. 3.74m/s in 16 weeks but multiple shoot i.e. BO_1(15) couldn't give better result comparing with the other four. Only 2.6 m/s were found in 16 weeks. Shoots also were not good.

Lime AO_1 (6) and Lime BO_1 (15) did not develop callus. This may be due to very young and *in vitro* grown shoots used for study. Lemon and Troyer Citrange developed callus under study that may be due to either more cytokinin (BAP1 mg/l) and less auxin (NAA 0.1 mg/l) used or due to hard as well as old tissues cultured.

5.7 Meristem Culture of Mandarin Orange

The meristem cultured in one season was not successful, that may be due to more sterilant (ethanol and sodium hypochlorite) effect or due to filter paper

which did not provide nutrients to delicate meristem properly over which meristems were cultured supplementing nutrient on its touching; resulting the death of meristem after 24 weeks of culture. If the shoot tip of the meristem that is removed is small enough (0.1mm to 0.5 mm) the disease agent will not have had sufficient time to infect this rapidly growing tips. So theoretically disease is not present in the meristem.

In the test, it was found that the *in vivo* mandarin orange from which apical meristem was excised for virus elimination from meristem culture were severely attacked from CTV (ELISA plate-3). Obtaining disease free propagation materials is essential but not always easy; Samson(1977).

5.8. *In vitro* Rooting of Shoots of Lime AO_1 (6)

In this study, the rooting hormone IBA and NAA were widely used for Citrus spp. The hormones were used in four concentrations from 0.5 mg/l to 2 mg/l respectively in MS. The time period of root initiation as well as the best rooting was important. For the selection of best rooting hormone concentrations, it was assumed three parameters of rooting 1. Average no. of node per shoot per cm, 2. percentage of rooting and 3. Average no. of. Roots per rooted shoot. The best concentration of IBA used was 1 mg/l and the best concentration of NAA used was 0.5 mg/l. The data was taken in late period of culture i.e. 16 weeks because root initiation was only in 20 days when IBA (1 mg/l) used and in 26 days when NAA (0.5 mg/l) used. The slow rooting was may be due to low temperature 21ºC maintained.

For IBA, though there was more average no. of node per shoot i.e. 4.88 in 0.5 mg/l, percentage of rooting was only 20%. Average number of roots per shoot did not bring any variation. Average number of node per shoot per cm in IBA 0.5 mg/ l and IBA 1mg/l use was found to be the same.. % of rooting was also not bad (66.66%) in IBA 1 mg/l use. So, IBA 1 mg/l found to be the best rooting hormone concentration.

In NAA use, average no. of roots per shoot did not bring any variation. Though the percentage of rooting is highest among all in 1 mg/l use but the no. of node per shoot per cm is no significantly maximum as 0.5 mg/l use, in which

7.24 node per shot per cm was obtained. Thus considering all three parameters of study, NAA 0.5mg/l found to be the best rooting hormone concentration.

As far as *in vitro* rooting of the shoot tips is concerned, NAA at 1 mg/l is most optimum compared to other concentrations for Citrus spp. When concentration of NAA is reduced by 50%, the number of roots also reduces almost by one half. In corporation of Cytokinin, BAP even at 0.1 mg/l in the medium prohibits rooting completely in both *Poncirus trifoliate* and *Citrus sinensis* (Ranjit, 1999).

Kukreja and Mathur (1985) induced roots on MS + NAA 0.5 mg/l of Duboisia myoporoides. Karki and Rajbhandary (1983) rooted *Solanum lanciniatum* Ait. In half concentration of MS media with IBA and NAA.

5.9 Acclimatization of *In vitro* Rooted Lime AO_1(6)

The less percentage of survival of both multiple rooted and single rooted plants may be due to no fertilizer used, no hormone treatment on acclimatization, very low humidity of environment and not proper maintained the humidity due to lack of practices in myself. Some roots were very long, *in vitro* developed roots are more or less not functional as usual of *in vivo* plant. We should maintain plants very carefully. Even from the single mistake, the acclimatization is not successful. *In vitro* plants do not have properly developed epidermis and the waxy layer over it to check the ranspiration. Day time is also one of the major factor for acclimatization.

Pradhan (1993) transferred *in vitro* rooted plants of *Eucalyptus citriodora* on non-sterile sand.

5.10 ELISA- Plate reading of DAS-ELISA

CTV is not transmitted from seed. Thus seedling of *In vitro* and *in vivo* were found to be CTV free by DAS-ELISA. CTV is easily graft transmissible because phloem tissues are contained with the inoculum of viruses (Rocha-Pena et al, 1991). Movement of nursery material which has been propagated from a CTV- infected bud source is the mechanism of most long distance movement of

the virus. The virus can be mechanically transmitted with partially purified inoculums by slash inoculation (Garnsey and Muller, 1988).

In the various ELISA plates that were tested for the virus, different positive and negative indicators were present. When the resulted ODs were compared with that of the indicators, it was portrayed that the samples with OD results equal to or more than that of the positive indicators indicated positive CTV infections. The OD results of the samples that were equal to or less than that of the negative indicators indicated no CTV infections.

Due to the chemical and climatic differences, each ELISA plate had different readings for the standard positive and negative indicators for CTV. Thus, for the reference; the bridge line of virus infection i.e. in between OD reading of Virus negative and virus positive was taken from the maximum OD reading of well without sample by Humareeder (Spectrophotometer) or ELISA plate reader. More OD indicates virus-positive and less OD indicates the virus negative.(Ranjit et al, 1998)

CHAPTER SIX

CONCLUSION

Seed germination of lime was found to be best, when planted on citrus establishment media (CEM) after treating those seeds with GA_3 (500 mg/l) for 24 hrs. Lime seeds showed the dormancy as well as polyembryony. There was more expression of polyembryony by use of cytokinin (BAP). Gibberellin (GA_3) broke the dormancy as well as induced germination and polyembryony both. The expression of polyembryony by lime due to use of GA_3 was found to be 100%.

The germination of lemon seeds was found to be more better than the lime when equal conditions were maintained. Lemon did not express the polyembryony. Lemon seedling gave much more branching from the base. Dormancy also was expressed by lemon; little bit more than lime in germination.

The best multiplication of lime $AO_1(6)$ was found in MS-supplemented with BAP 0.5 mg/l + NAA 0.5 mg/l among all the eight different combinations of BAP and NAA used.

MS supplemented with both BAP 0.5 mg/l + NAA 0.5 mg/l and BAP 0.5 mg/l + NAA 1 mg/l was found to be same and the best for multiple shoot production from nodal explant of lime $BO_1(15)$ among all eight different combinations of BAP and NAA applied. Explant of lemon, cultured on MS-supplemented with three different combinations of BAP and NAA i.e. BAP 0.5 mg/l + NAA 0.5 mg/l, BAP 0.5 mg/l + NAA 1 mg/l and BAP 1.5 mg/l + NAA 1 mg/l found to have the same result and the best for multiple shoot production from nodal explant among all eight combination of BAP and NAA supplement.

Under the same MS-media and same hormones (BAP 1mg/l + NAA 0.1 mg/l) supplement, the response of nodal explant, for multiple shoot production of lime $AO_1(6)$, lime $BO_1(15)$, lemon and Troyer Citrange was different. This was varying from one species to another species and even in the two cultivars of same species too. The no. of multiple shoot production of Troyer Citrange was

better than the other three. There was not any significant increase in no. of shoot from eight weeks to 16 weeks. In case of lime AO_1 (6), though that was in third position in the rate of multiple shoot production but surprisingly the no. of multiple shoots increased from 12 weeks to 16 weeks and reached in the 2nd position. The multiple shoots produced due to lemon were healthy and with good growth than rest three. Lime BO_1 (15) did not show good response in BAP 1 mg/ l + NAA 0.1 mg/l hormone supplement.

Rooting of Lime AO_1 (6) in IBA 1 mg/l and 0.5 mg/l of NAA supplement was found to be better on MS medium.

In vitro rooted lime AO_1 (6) were successfully acclimatized in sand and soil (1:1). The time period of humidity maintenance covering by muslin cloths and watering over regularly was about one and half month.

Seedlings derived from small (young) lime, under *in vitro* and in vivo grown were found virus negative.

The study indicated that MS-medium was good for seed germination, in promoting no. of multiple shoots, for *In vitro* -rooting when supplemented with auxins and cytokinins on different citrus spp.

Elimination of plant viruses by tissue culture technique is now new in Nepal. It is most effective in most cases of virus infection. In citrus, if we think to eliminate virus – seed culture may be selected. If we think for homogeneity (pureline culture), vegetative propagation may be used. But if we think to eliminate viruses, for pure line culture as well as for production of disease resistant plant the meristem culture is the only way. The nodal explant is found to be more suitable for multiple shoot production in *Citrus aurantifolia* 'Swingle'.

CHAPTER SEVEN

RECOMMENDATION

I. Government should Identify Potential Citrus research and development activities that can be carried out by the private sector.

II. Government should interact with potential donor agencies representatives (JICA, FAO, USAID, SDC etc) regarding the citrus development in Nepal.

III. NARC, DOA should extend the facility as collaborative approach to Citrus research and development including detection and elimination of CTV from Citrus spp for production, supply and distribution of disease-free planting material to certified nurseries and individual farmers.

IV. The specialist should examine the adequacy of national seed act and plant quarantine act to restrict the free movement of disease within the country and the entrance from the outside of country.

V. Public Awareness to viral disease and disease free planting materials is necessary.

VI. It is better that we give emphasis for pureline culture especially in Citrus spp. Which produces both zygotic and *nucellar* embryo in seed.

VII. Researches in botany, especially in plant biotechnology should be addressing the farmers problems.

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APPENDEX I

Citrus Species found in Nepal

S.N.	Local Name	Common Name	Scientific Name
1	Kagati	Acid lime	*C. aurantifolia* swingle
2	Junar/Mausami	Sweet orange	*C. sinensis*
3	Nibuwa/Chasme Kagti Eureka	Hill lemon/Nepali oblong lemon/Eureka lemon	*C. pseudolimon* Tanaka *C. limon* (L.) Burn.f.
4	Bhogate	Pummelo	*C. grandis* Osbeck *C. maxima*
5	Kali jyamir	Sour orange	*C. aurantium* L.
6	Keep	Bitter orange	*C. aurantium* L**.**
7	Seti jyamir	Rough lemon	*C. jambhiri* Lush.
8	Suntala/Kamala	Mandarin/tangerine	*Citrus reticulata* Blanco *C. tangerine*
9	Bimiro	Citron	*C. medica* L.
10	Chaksi	Sweet lime	*C. limettioides* Tanaka
11	Sankhatro	Possible hybrid of shaddock or pummelo	
12	Chaku paw	Possible hybrid of grapefruit	
13	Tinpate suntala	Trifoliate orange	*Poncirus trifoliate* L.
14	Muntala	Kumquat	*Fortunella japonica Swingle/F. margarita*
15	Kinnow suntala	Kinnow mandarin	*C. nobilis x C. deliciosa* Hybrid
16	Satsuma suntala	Satsuma orange	*C. unshiu* M.

Source: Nepal Citrus Development Programme, Kirtipur, Kathmandu (2002)

APPENDIX-II

ELISA-Plate reading before *In vitro* Study

Humareader : H SN 1453 (85) Date: 02.06.05
Absorbance mode 12 PAGE 1
LOT NUMBER: EXP. DATE: ANALYST:
WAVELENGTHS = 405 NM

	1	2	3	4	5	6	7	8	9	10	11	12
1A	0.269	0.293	0.259	0.202	0.225	0.284	0.280	0.235	0.260	0.299	0.273	0.221
1B	0.241	0.755	1.291	0.956	0.789	0.870	0.303	0.485	0.257	0.211	1.316	0.241
1C	0.154	1.120	0.225	0.226	0.251	0.353	0.340	0.262	0.191	0.229	0.189	0.300
1D	0.271	0.165	0.180	0.248	0.242	0.266	0.223	0.215	0.307	0.190	0.720	0.256
1E	0.262	0.497	1.167	0.634	0.785	0.677	0.398	0.406	0.247	0.213	0.893	0.268
1F	0.249	1.046	0.130	0.171	0.151	0.209	0.181	0.201	0.160	0.202	0.197	0.259
1G	0.217	0.093	0.170	0.191	0.230	0.156	0.138	0.320	0.354	0.242	0.904	0.263
1H	0.318	0.243	0.337	0.298	0.287	0.351	0.220	0.241	0.214	0.219	0.240	0.263

APPENDIX-III

ELISA-Plate reading before *In vitro* Study

Humareader : H SN 1453 (85) Date: 05.08.05
Absorbance mode 12 PAGE 1
LOT NUMBER: EXP. DATE: ANALYST:
WAVELENGTHS = 405 NM

1	2	3	4	5	6	7	8	9	10	11	12
0.132	0.127	0.133	0.243	0.225	0.240	0.225	0.247	0.230	0.213	0.206	0.182
0.149	0.338	0.427	0.755	0.089	0.762	0.648	0.842	0.118	0.718	0.128	0.178
0.151	0.719	0.657	0.297	0.749	0.239	0.305	0.395	0.431	0.493	0.535	0.221
0.218	0.275	0.521	0.120	0.077	0.092	0.088	0.077	0.005	0.104	0.075	0.228
0.179	0.386	0.380	0.699	0.206	0.459	0.499	0.528	0.132	0.461	0.142	0.194
0.230	0.432	0.446	0.200	0.385	0.166	0.165	0.175	0.178	0.248	0.231	0.172
0.232	0.371	0.431	0.165	0.159	0.173	0.178	0.129	0.173	0.105	0.076	0.175
0.067	0.032	0.093	0.143	0.145	0.109	0.191	0.150	0.194	0.077	0.119	0.192

APPENDIX-IV

ELISA-Plate reading after *In vitro* Study

Humareader : H SN 1453 (85) Date: 20.09.06
Absorbance mode 12 PAGE 1
LOT NUMBER: EXP. DATE: ANALYST:
WAVELENGTHS = 405 NM

	H	G	F	E	D	C	B	A
1	0.213	0.211	0.249	0.217	0.201	0.199	0.211	0.252
2	0.249	0.172	0.547	0.228	0.280	0.233	0.252	0.194
3	0.211	0.108	0.438	0.168	0.966	0.205	0.202	0.186
4	0.208	0.175	0.171	0.174	0.927	0.442	0.115	0.230
5	0.189	0.149	0.150	0.185	0.930	0.434	0.151	0.228
6	0.244	0.149	0.171	0.230	0.202	0.470	0.225	0.235
7	0.214	0.165	0.166	0.224	0.186	0.330	0.171	0.264
8	0.183	0.183	0.189	0.849	0.198	0.405	0.129	0.253
9	0.138	0.194	0.099	0.772	0.143	0.361	0.144	0.280
10	0.208	0.214	0.143	0.819	0.145	0.314	0.170	0.281
11	0.257	0.185	0.168	0.807	0.174	0.233	0.077	0.247
12	0.224	0.194	0.124	0.252	0.260	0.241	0.199	0.279

APPENDIX V

BIORAD ELISA TEST KIT DETAILS

Bio-Rad
Laboratories

3, boulevard Raymond Poincaré
92430 Marnes la Coquette France
Téléphone : +33 (0) 1 47 95 60 00
Fax : +33 (0) 1 47 41 91 33

REACTIF ELISA - *ELISA REAGENT*

Produit / *Product*: CTV DAS ELISA

Anticorps / *Antibodies*

N° de lot / *Batch n°* :	00403 glycérolé / glycerolated
Vol. pour/*for* 500 tests ; Dilution :	500 µl ; 1/100
Conservation / *Storage* :	-20 C
Péremption / *Use by* :	OCT. 2001
Nature des anticorps / *Antibodies* :	Polyclonal (chèvre/goat)
Souche d'origine / *Isolate* :	agrume / citrus

Conjugué / *Conjugate*

N° de lot / *Batch N°* :	00403 glycérolé / glycerolated
Vol. pour/*for* 500 tests ; Dilution	500 µl ; 1/100
Conservation / *Storage* :	-20 C
Péremption / *Use by* :	oct. 2001
Nature des conjugués / *Conjugate* :	Polyclonal (chèvre/goat)
Souche d'origine / *Isolate* :	agrume / citrus

Autres caractéristiques / *General information* :

tampon d'extraction / *extraction buffer* : PBS Tween-PVP

broyage / grinding : 1g / 10 ml

spécificité / specificity : pas de spécificité particulière / no particular specificity

Contrôle qualité et validation / *Quality control and validity*

	N° de lot *Batch n°*	Valeur DO *OD value*	Norme qualité *Quality standards*
Témoin sain *Negative control*	Témoin de référence *Control Reference*	0,001	< 0,10
Témoin positif *Positive control*	Témoin de référence *Control Reference*	2,832	> 0,70
Tampon de broyage *Extraction buffer*		0,003	< 0,05
Substrat *Substrate*		0,065	0,06< - <0,09

Valeur de DO sur lecteur de microplaques LP 400 à 405 nm, avec des microplaques NUNC maxisorp certifiées, après 1 h d'incubation et déduction de la DO du substrat.
O.D. value on LP 400 microplate reader at 405 nm, with certified maxisorp NUNC microplates, after incubation of one hour and deduction of substrate O.D. value.

Réactif certifié conforme aux normes qualité / *Conformity with quality standards*

Approbation du technicien / *Technician approval*

Date : 22/08/00

Bio-Rad
S.A. au capital de 26 440 100 F - RCS Nanterre B 313 293 150 - N° intracommunautaire FR [illegible] 313 293 150 - Code APE 741 D

APPENDIX VI

How to operate HUMAREADER?

PRESS	DISPLAY
0 Power On	SELECT MODE
Load Plate	Date Time
1. ABS 1	SELECT FILTER
	1=405etc
2. 1	405 nm
3. ENTER	SELECT DIFFERTIAL
	FILTER
	1=405etc
4. O	No DIFF FILTER
5. ENTER	ABSORBANCE MODE
	OFFSET BLAN'S ABS Y/N
6. N	ABSORBANCE MODE
	Load Next Plate- READ
7. READ	Reading & Recording Plate