IN- VITRO STUDIES IN KOKUM CV. KONKAN AMRUTA

By

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DIST. RATNAGIRI (M.S.) INDIA

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A thesis submitted to the

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In partial fulfillment of the requirements for the degree of

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CERTIFICATE

This is to certify that, the thesis entitled "*In-vitro* studies in kokum cv. KonkanAmruta"Submitted to the Faculty of Agriculture, Dr. BalasahebSawantKonkanKrishiVidyapeeth, Dapoli, Dist. Ratnagiri,Maharashtra State in partial fulfillment of the requirement for the degree of **MASTER OF SCIENCE (AGRICULTURE)** in **PLANT PHYSIOLOGY** embodies the result of a piece of bona-fide research carried out by **Mr. KulkarniAjinkya Ashok (Reg. No. ADPM/18/2620)** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma or published in other form. All the assistance and help received during this course of investigation and the sources of literature have been duly acknowledged by him.

Place: Dapoli
Date: October,2020

(R.S. DESHPANDE)
Chairman,
Advisory Committeeand
Research Guide

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I wish to end this note by expressing thanks to all those whom, I was able to recall here and also to those I might have left unknowingly.

Place: Dapoli

Date:

(Kulkarni Ajinkya Ashok)

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ABBREVIATIONS

Explant	: Excised Plant Part
BAP	:6-Benzylaminopurine
KIN	:Kinetin
IBA	:Indole-butyric acid
cm	:centimeter
ml	:mililitre
°C	:Degree Centigrade
g/l	:Gram per litre
HCl	:Hydrochloric Acid
$HgCl_2$	:Mercuric Chloride
NaOCl	:Sodium Hypochloride
TDW	:Triple distilled water
i.e	:that is
min	:minutes
rpm	:Revolutions pre minute
LAF	:Laminar Air Flow
NaOH	:Sodium Hydroxide
ppm	:Parts per million (mg/l)
PVP	:Polyvinyl pyrillidone
UV	:Ultra violet light
WPM	:Woody Plant Medium (1981)

DEPARTMENT OF AGRICULTURAL BOTANY,

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ABSTRACT

Kokum (*Garcinia indica*Choisy) belongs to family Clusiaceae. It is important spice crop in the konkan region of Maharashtra. It is popularly known as "Ratamba" and widely grown in tropical rain forests of western ghat. Kokum has got multifarious uses and therefore, finds inevitable place in the lifestyle of local population.

Micro propagation is promising method for clonal multiplication of plants on large scale. Establishment of an efficient technique for reducing microbial contamination and exudation of phenols to produce maximum sterile cultures of kokum, which are true-to-type is the most urgent need of the Konkan region. This study aims to standardize micro propagation of female cultures of Kokum cv. Konkan Amruta, hence focusing on the improvising sterilization techniques and minimize the effect of phenolic components for ensuring high survival rate of kokum cultures. Shoot tip and Nodal explants were collected from mature fruiting tree of kokum cv. Konkan Amruta. Kokum is proliferent producer of phenols and polyphenols and also prone to certain endogenous contamination. The treatment of the explants

with sterile distilled water, TWEEN-20, 10%Savlon, and pre-conditioning of explants with 0.1%PVP along with anti-fungal agent1% Bavistin fallowed by an aseptic treatment with 0.75% $HgCl_2$ for 6 min. resulted 81.66 per cent survival of healthy and sterile buds.

This study also conducted for induction of *in-vitro* shooting *and in-vitro* rooting using shoot tip and nodal explants on woody plant medium with different concentrations of plant growth regulator. From the result obtained, it is observed that the maximum shoot induction was showed on plant growth regulator combination WPM + 1mg kin + 2mg BAP + 1mg IBA for both the explants 73.33 and 70.00 percent respectively. Better shoot induction was observed in explants which were collected in January than any other season.

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CHAPTER I
INTRODUCTION

Kokum (*Garciniaindica* Choisy) is one of the native underexploited tree spice belongs to family Clusiaceae of order The ales and sub class Dilleniidae. A French botanist Laurence Garcin identified the trees of this genus during his stay in India and his valuable work in this field was responsible for naming these trees as Garcinia. *Garcinia* comprises over 400 species in the world and 40 species have been listed as edible species. Among those, about 30 species are known to occur in India.

Kokum is perennial, monopodial and tall growing tree mostly found in West Coast region of India and to some extent in the forests of Assam, Meghalaya, West Bengal. Especially, In the Western Ghats, the tree is mainly found along the coastal belt of Konkan region of Ratnagiri district of Maharashtra, Goa, Uttara Kannada, Udupi and Dakshina Kannada Districts of Karnataka and Kasaragod area of Kerala. It is slender evergreen tree with dense canopy and drooping branches of pyramidal or conical shape. Kokum flourishes very well up to an elevation of about 800m from sea level. It reaches heights up to 15m (50 ft). Its leaves are ovate or oblong, dark green above and pale beneath. The chromosome number of kokum is reported as 2n = 54 by Krishnaswamy and Raman (1949) and as 2n = 48 by Thombre (1964). The polygamodioecious tree flowers in November- February and fruits ripen in May-June. Fruits are globose or spherical 1-1.5 in diameter, dark red when ripe, and each fruit contain 5-8 large seeds.

G. *indica* is commonly known as Kokum Butter tree or Brindonia Tallow tree in English. The other vernacular names are Kokum, Birand, Amsol in Konkani and Marathi, Murugalu in Kannada Punurpuli in Malayalam and Vrikshamia, Amlabija, Amlapura, Amlashaka in Sanskrit.

In relation to Kokum, the precise statistics regarding area production and productivity is not available as kokum is not planted in an organized manner. As per a base line survey in 2010, kokum was cultivated on about 1000 ha area in the Konkan region and annual production of fruits was about 4500 MT. According to the survey conducted earlier by Chief Conservator of Forest out of the total 46,600 Kokum trees in the state of Maharashtra; 43,000 trees existed in Ratnagiri and Sindhudurg Districts. Up to now two improved kokum varieties i.e. Konkan Amruta and KonkanHatis are released by Dr. B. S. KonkanKrishi Vidyapeeth, Dapoli Konkan Amruta was released in the year 1997. The main distinguishing morphological and physicochemical characters of this variety are the medium sized, thick rind and attractive red coloured apple shape fruits. Fruits having long shelf life i.e. 15 days. Trees show early flowering and yield up to 140 kg fruits per tree.

Kokum has got multifarious uses and therefore, finds inevitable place in the lifestyle of local population. In kokum the fruit is the only edible part of the tree which has agreeable flavour and sweetish taste. Being a medicinal plant, Kokum fruits has many medicinal values such as anthelminthic, digestive-tonic, cardiotonic. They are useful against piles, dysentery and act as a heart tonic. Kokum fruits are chopped and steeped in sugar water to prepare "Amrut kokum", a healthy drink during the summer to relieve sunstroke (George *et al.,* 2002; Mishra *et al.,* 2006). The dried rind is soaked in water and boiled into a soup called solkadi. Kokum juice spiced and sweetened with jaggery, is a must for marriage feasts and functions in Uttara Kannada district of Karnataka and Goa (Korikanthimath and Desai, 2005). The normal shelf-life of fresh fruit is about five days (George *et al.,* 2002). The fruits can also be used in making of wines and liquor and good substitute for grapes in wine industry. The fruit rind has also been utilized as a pink and purple food colouring agent (Kaur*et al.,* 2012). Hydroxyl citric acid (HCA) extracted from

fruit is fat-reducing medicine and used against obesity. Life enhancing antioxidant found in kokum pericarp is known as Xanthone. Garcinol is a benzophenone derivative extracted from the fruit rind.

Apart from the different uses of fruit, Kokum seed is rich source of fat, which is called as Kokum butter. The seed kernel contributes 60 per cent weight of the seed, and yields 33 to 44 per cent oil. The total oil content of seed is about 23 to 26 per cent. This is the only solid oil at room temperature, which is used in manufacture of soaps, candles, ointments and cosmetics. Kokum butter is considered as nutritive demulcent, astringent and emollient. Ointments prepared from kokum butter are used against ulceration and cracks on lips, hands and feet in cold. It reduces the degradation of skin cells and restores elasticity of skin. The oil cake left after oil extraction can be used as manure and cattle feed. (Korikanthimath and Desai, 2012).

Seed or vegetative propagation such as softwood grafting and inarching is the conventional way to propagate kokum. But these propagation methods depend on season, requires space and availability of grafting material. Being polygamodioceous in nature, identification of female plants is a hurdle in the multiplication of this species. In Konkan, there is increasing demand for true to type of seedlings. This demand can be fulfilled with the help of micropropagation technique, within very short period by rapid multiplication of desired genotypes.

Apart from recalcitrance of *Garcinia*plant the two major but basic hurdles in the tissue culture technique in woody plants are the microbial contamination and exudation of phenolic compounds during early stages of tissue culture. Browning is generally due to the oxidation of phenolic compounds released from the cut end of the explants, by polyphenol oxidases, peroxidases or air. The oxidized products, quinones are highly reactive and inhibit enzyme activity leading to death of explants. The physiological status

of tree, age of tree and parameters like photoperiod, temperature, light are equally important in tissue culture of woody plants.

Establishment of an efficient technique for reducing microbial contamination and exudation of phenols to produce maximum sterile cultures which are true-to-type is the most urgent need of the Konkan region. Hence the research work "*In-Vitro* studies in kokum Cv. Konkan Amruta" was undertaken with following objectives.

1) Standardization of the surface sterilization treatment of Kokum explant for phenol alleviation and aseptic establishment.
2) Induction of the organogenesis in Kokum Explant

CHAPTER II
REVIEW OF LITERATURE

Vegetative propagation of Kokum at large scale by conventional methods such as grafting is limited due to the season boundness, non-availability of rootstock and labour intensiveness etc. The relatively new micropropagation technique has been developed for clonal multiplication of kokum. Manvi and Parasharami (2019) regenerated *Garcinia indica* plants by using shoot bud explants. Kulkarni and deodhar (2002) developed protocol for plant regeneration using immature seeds of *Garcinia indica.* Deodher*et al.* (2014) developed micro propagation technique of (*Garcinia indica)* using apical and axillary buds as explants.

Explants

Mathew *et al.* (2001) studied *in vitro* regeneration in kokum and camboge using shoot tip and nodal explants from actively growing shoots culturing on murashige and skoog (MS) medium.

Kulkarni and Deodher (2002) studied *in-vitro* micro propagation of *Garcinia indica*Chois. using seeds as explants on modified murashige and skoog(MS) medium.

Farzana *et. al.* (2010) reported *in-vitro* shoot bud regeneration of *Garciniaquaesita* using nodal explants and leaves obtained from shoots of *in-vitro* growing seedlings and greenhouse grown *in-vivo* 2-3 years old seedlings.

Baskaran and Krishnan (2011) studied protocol for high frequency plant regeneration by using seed segments of *Garcinia indica* for production of elite clones.

Mohan *et al.* (2011) studied *in-vitro* and *in-vivo* adventitious bud differentiation from mature seeds of three Garcinia spp. viz. *Garcinia indica, Garcinia tinctoria, Garcinia gummigutta*. by culturing on modified MS medium.

Chauhan *et al.* (2012) studied *in-vitro* regeneration of *Garcinia indica*Choisy through direct organogenesis using leaf explant procured from *in- vitro* developed shoots raised from the nodal explants comprising of axillary buds.

Shekhawat (2012) studied multiple shoot induction by proliferation of axillary buds or meristem of *Garcinia cambogia*L. on MS medium incorporated with 2mg/l BAP.

Deodher*et al.* (2014) reported micropropagation of female plants of Kokum (*Garcinia indica)* using apical and axillary buds as explants on WPM (Woody Plant Medium).

Joshi *et al.* (2015) reported successful initiation of tissue culture of *Garcinia indica* using immature seeds as explants on half MS supplemented with 1.5mg/l $GA_{3.}$

Patel *et al.* (2018) reported micropropagation of perennial fruit crop *Punicagranatum*L. cv. *Bhagva*using nodal segments as explant on Murashige and Skoog (MS) medium.

Manvi and Parasharami (2019) studied micropropagation of female cultures of Kokum *Garcinia indica*. They determined optimum condition for growth and multiplication of kokum by using shoot buds as explants from fruiting mother trees across nine different locations in Konkan and Maharashtra.

EXPLANT PRETREATMENT AND STERILIZATION

Mathew *et al.* (2001) reported multiple shoot regeneration of kokum and camboge using shoot tip and nodal explants. Explants treated with Bavistin (0.2%) for 30 min. and surface sterilization with 0.1% % $HgCl_2$ for 10 min.

Kulkarni and Deodher (2002) used seed as explants, these explants surface sterilized with 70% alcohol for 1 min. fallowed by 2% sodium hypochlorite for 5-7 min.

Chauhan *et al.* (2012) reported pre-treatment of kokum seeds with 1% solution of Tween 20 for 10 min and placed under running tap water for 1 hr. fallowed by surface sterilization of seeds with 0.1% $HgCl_2$ for 10 min.

Joshi *et al.* (2015) studied *In-vitro* propagation of *Garcinia indica*Choisy by using immature seed as explants. Seeds washed under running tap water for 5 min. and dipped in teepol (1:1 v/v) for 10 min. seeds treated with 1% Bavistin for 15 min. followed by surface sterilized with 0.1% $HgCl_2$ for 10 min.

Patel *etal.* (2018) reported among different treatment tested for sterilization, 300mg/l Bavistin for 18 min, 200mg/l both streptocycline and cefotaxime 18 min and 8 min found best to obtain cent per cent axenic culture development.

Manvi and Parasharami (2019) reported treatment of the explants with sterile distilled water, Tweens 20, 10%(v/v) savlon, along with 1% anti-fungal agent Bavistin followed by an aseptic treatment with 0.75% $HgCl_2$ and drying of shoots for 60minutes before inoculations resulted in 87% survival of healthy, sterile buds.

Antioxidant treatment for Phenols alleviation:

Wang *et al.* (1994) studied removing of phenolic compounds produced by leaching or adsorption with activated charcoal or Polyvinylpyrrolidone (PVP).

Raghuvanshi and Srivastav (1995) reported pre-treatment of mango explant (leaf discs) in a liquid medium supplemented with 1%PVP prior to subculture on agar gelled medium increased the survival of explants due to the control of phenolic leaching.

Abdelwahd*et al.* (2008) observed pre-treatment of faba bean seeds with (1000mg/l) PVP solution for 1 hr. gave best results for reducing lethal browning in explants and improved shoot regeneration.

Nahid*et al*. (2013) reported pre-treatment of explants with ascorbic acid, citric acid, polyvinylpyrrolidone (PVP), Bavistin and chloramphenicol (0.1%) for 9 hr. significantly reduced the shoot tip explants browning in *Curculigolatifoliain-vitro* cultures.

Deodher*et al.* (2014) reported micropropagation of female plants of Kokum (*Garcinia indica)* using apical and axillary buds. This study shows explants were pre-treated with 0.1% PVP for 30 min. reduces explant browning.

Ndakidemi*et al.* (2014) studied effect of antioxidant ascorbic acid in controlling lethal browning due to oxidized phenols in *in-vitro* culture of *Brachylaenahuillensis*using nodal segments. The treatments included four levels of ascorbic acid (0, 50, 100, 150, 200, & 250mg/litre) supplied into basal woody plant medium supplemented with Benzylaminopurine (BAP). The best control of lethal browning was achieved by supplying 200 - 250 mg/litre of ascorbic acid in the woody plant medium supplemented with BAP.

Shimelis (2015) optimized the appropriate concentration of Polyvinylpyrrolidone (0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 g/L) and Activated charcoal (0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 g/L) on (C86-12, C86-56) genotypes of sugarcane. They observed Murashige and Skoog medium supplemented with 0.2 g/L and 0.3 g/L of Poly vinylpyrrolidone has gave 100% and 80% survived explants of C86-56 and C86-12genotypes respectively, while 0.4 g/L and 0.3 g/L of activated charcoal resulted in 46% and 40% of survived explants of C86-56 and C86-12 genotypes respectively, after 30 days of culturing

Ahmad *et al.* (2016) studied use of pre-treatment of nodal explants of guava (Psidium guajava L.) with antioxidant solutions of citric acid, ascorbic acid, poly vinyl pyrrolidine (PVP) and charcoal in reduction of phenolic exudation.

Manvi and Parasharami (2019) reported pre-culture treatment of kokum explants (shoot tip) with 0.1% Polyvinylpyrrolidone (PVP) for 30 min. gave best results in control of explants and media browning.

Induction of organogenesis:

Goh *et al.* (1997) studied role of ethylene on direct shoot bud regenation from mangosteen (*Garcinia mangostana* L.) was studied by culturing leaf explants on Woody Plant Medium (WPM) supplemented with 20 benzyladenine ethylene inhibitor ($AgNO_3$) aminoethoxyvinylglycine (AVG) or ethylene precursor (ACC) under airtight condition. Addition of ACC to the medium delayed shoot regeneration and enhancing callus proliferation. $AgNO_3$ and(AVG) were effective in preventing delaying of shoot regenerations and callus formation.

Kulkarni and Deodher (2002) studied *In-vitro* micropropagation of *Garcinia indica*Chois. They reported multiple shoots were obtained from immature seed explants on MS basal medium supplemented with NAA (2.69

µM), BAP (8.9 µM) and KN (0.93 µM). Elongation of shoots was achieved on half MS medium supplanted with NAA (0.54 µM), BAP (0.44 µM) and KN (0.93 µM). The shoots were developed roots when they treated with 4900 µM IBA for 30 seconds and cultured on half strength MS basal medium.

Malik *et al*. (2005) reported the efficient method for rapid regeneration of plantlets via adventitious bud differentiation using mature seeds of Kokum. The maximum percentage of direct shoot proliferation was observed in seed segments cultured on Murashige and Skoog's (MS) medium supplemented with cytokinins such as BAP and KN and Thidiazuron (TDZ) alone and in combination with auxin (NAA). Seed segments cultured on MS basal medium showed development of shoot along with roots from S-end (emergence of shoot) and only roots from R-end (emergence of root), while the middle segments showed no response.

Thengane*et al.* (2006) reported plant regeneration via somatic embryogenesis. Immature seeds of *Garcinia indica* were excised from immature fruits and cultured on woody plant medium (WPM) with different combinations of cytokinins and auxins. The explants showed swelling within 1 week and initiation of small protuberances within 2–3 weeks of culture. After 3 weeks of culture, somatic embryos were seen in all over the seed surface. The highest percentage (80%) of somatic embryogenesis was obtained on media supplemented with 22.1µm BAP alone. While increase the BAP from 2.2–16.0 µm the number of somatic embryos per explants also increased.

Rostika*et al*. (2008) studied in vitro propagation of Mangosteen. Surface sterilized seeds was sliced to four pieces and cultured on MS medium supplemented with BA (1,3, and 5 mg/1) for bud/shoot inductions. Highest percentage of seed growth (100%) with the highest number of shoots per seed (2.7) and highest leaf number (2.9) was obtained on medium supplemented with 5 mg/L BA. For root induction, combinations of two basal media (MS

and WPM), two concentrations of media (full strength and l/5 strength) and two levels of IBA (5 and 10 mg/L) were used. Within MS media, 5 mg/L IBA showed best for rooting (75%) with 1.6 cm root length.

Farzana *et al*. (2010) studied direct shoot bud formation on *Garcinia quaesita* Pierre. Leaf explants from in vitro grown plant were cultured on MS medium supplemented with 5.0 and 10.0 mg/L BAP showed direct shoot bud regeneration. The highest number of 9-12 shoot buds was produced on the medium supplemented with 10 mg/L BAP. Shoot elongation was obtained on medium supplemented with 20mg/L BAP. Rooting could not be achieved on MS medium supplemented either with DBA (1.0, 5.0, 10.0 mg/L) or NAA (1.0, 5.0, 10.0 mg/L).

Malik *et al*. (2010) reported plantlet regeneration via adventitious bud differentiation on leaf explants from *in-vitro* raised seedling as well as mature trees. The explants were cultured on modified MS basal medium supplemented with cytokinins such as 6-benzylaminopurine (BAP), kinetin (KIN) and thidiazuron (TDZ) alone and in combination with auxin (a-napthaleneacetic acid (NAA), for direct shoot proliferation. Maximum number of shoots (2.67) was observed in 63.89% explants on 1.0 µM TDZ after 2-4 weeks of culture. Elongation of shoots was achieved on MS basal medium containing 0.2% activated charcoal. Rooting was achieved in shoots cultured on half-strength MS medium supplemented with 10 µM IBA. Rooted plantlets were transferred to pots containing soil, vermiculite and farmyard manure (FYM) 1:1:1 and recorded 90% of survival rate in pots

Baskaran and Krishnan (2011) studied high frequency plant regeneration by using seed segments of *Garcinia indicavia* direct organogenesis. A maximum number of 86.2 shoot buds per explant were induced from the mature seed segments cultured on Woody Plant Medium (WPM) supplemented with 2.0 mg dm^{-3} N^6 –benzyl adenine and 1mg dm^{-3}

Indol-3-acetic acid. Rooting was achieved on half strength of (WPM) supplemented with 3 mg dm^{-3} Indol-3-butyric acid.

Tembe and Deodhar (2011) studied *in-vitro* propagation of Kokum using apical and intercalary buds of the root as source explants. Multiple shoots were formed in woody plant medium supplemented with benzylaminopurine (BAP) and thidiazuron (TDZ). Elongation of shoots was achieved on half-strength WPM medium supplemented with BAP (0.22, 0.44, and 0.66µM) with 0.5g/L activated charcoal. Higher shoot elongation was observed on medium with 0.44 µM of BAP + 0.5 g/L activated charcoal. For rooting pulse, treatment of indole-3-butyric acid ranging from 4.9 to 19.6 µM for 30 seconds and 1 min was tried. About 65% root induction was obtained in shoots subjected to 19.6 µM of IBA for 30 seconds. Rooted plantlets were transplanted to pot containing coco-peat and it showed about 75% survival rate.

Chauhan *et. al* (2012) studied in vitro regeneration of *Garcinia indica*Choisy through direct organogenesis using leaf explant. Leaf explants were cultured on MS medium supplemented with different concentration of BAP. highest number of shoots per explant was obtained on the medium supplemented with 22.2 µM BAP. and highest number of rooting was obtained from on MS basal medium MS+ 0.45 µM BAP supplemented with 0.81-1.07 µM NAA, while maximum root length was recorded on the medium supplemented with 0.005 µM NAA.

Deodher*et al.* (2014) reported micro propagation of female plants of Kokum (*Garcinia indica)* using apical and axillary buds. Maximum healthy and sterile cultures were obtained on WPM basal fortified with 2% sucrose and 8.87 µM BAP. Multiple shoots were obtained with use of TDZ in the range 0.22 –4.54 µM along with BAP. Highest 63% rooting was obtained on WPM medium with 4.90 µM IBA.

Joshi *et al.* (2015) studied in-vitro propagation of *Garcinia indica*Choisy from seedling explants. This study shows successful initiation of tissue culture was achieved by using immature seeds. Germination was achieved on half MS supplemented with 1.5 mg /l GA_3. Highest shoot production on MS medium supplemented with (6-BAP) 3mg/l and 0.5mg/l NAA within 4-5 weeks. In-vitro rooting was obtained on half MS agar gelled medium supplemented with 2g/l AC, 0.5 mg/l NAA and 1.5 mg/l IBA.

Manvi and Parasharami (2019) studied in-vitro micropropagation of female cultures of *Garcinia indica* from shoot buds to overcome various difficulties that occurred during vegetative propagation of kokum by conventional methods. This study shows that shoot tip explants were capable to form shoots ensuring high survival of explant. Proliferation of shoots obtained on Woody Plant Medium (WPM) with different concentrations of auxin and cytokiknins. High shoot formation was obtained on WPM composed of Kin 1mg/L and BAP 2mg/L and IBA 1mg/L and 200mg/L Taxim and are regularly sub-cultured after 50-60 days until the S_4 stage.

Mulik*et al.* (2019) studied concentration and time exposure of rooting hormone of IBA (Indole-3-Butyric Acid) for efficient survival of tissue culture raised *Garcinia indica*Choisy plantlets for in-vitro and ex-vitro rooting techniques. Shoots were regularly sub-cultured in autoclaved jam bottles containing multiplication WPM media (Woody Plant Medium, Lloyd and McCown,1981) supplemented with growth hormones like BAP 2 mg/L, KIN 1mg/L, IBA 1 mg/L along with 3% sucrose (w/v) and 0.4% phytagel (w/v).Induction of rooting was observed within thirty days of treatment with IBA. It was observed that 500ppm of IBA gave 30% rooting for in-vitro rooting trials whereas 2000ppm of IBA induced 80% rooting for shoots given ex-vitro rooting treatments.

CHAPTER III
MATERIALS AND METHODS

The detailed information regarding material used and methods followed during the course of present investigation entitled as "*In-Vitro* studies in kokum Cv. Konkan Amruta" is given below. The investigation was carried out in the tissue culture laboratory of the Plant Biotechnology Centre, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Dist: Ratnagiri (MS) during the academic year 2019-2020.

3.1 MATERIALS

3.1.1 Genotype:

The experimental materials consist of one variety of Kokum (i.e. Konkan Amruta). Young and healthy segments containing shoot tip (apical bud) and nodal (axillary bud) explants were collected from mature Kokum tree, from orchard of Cv.Konkan Amruta, Department of Horticulture, Dr.Balasaheb sawant Konkan Krishi Vidyapeeth, Dapoli.

3.1.2 Chemicals:

The details of various laboratory chemicals and biochemical components used in the present investigation are given below:

a) Chemicals for media preparation:

i. Salts of macro and micro elements of analytical grade.
ii. Vitamins and amino acids.
iii. Sucrose as a carbon source.
iv. Myo-inositol.
v. Agar-agar as a gelling agent.
vi. 0.1N HCL and 0.1N NaOH for pH adjustment.

b) Chemicals for surface sterilization:

1. Savlon
2. Tween 20 (as surfactant)
3. Mercuric chloride
4. Sodium hypochlorite
5. Carbendazim (Bavistin)
6. Antibiotic
    i. Streptocycline
    ii. Cefotaxime

c)Plant growth substances

i. Auxins: IBA
ii. Cytokininns: BAP, KIN

3.1.4 Culture Vessels and Culture Apparatus:

i. Beakers of capacity 100, 250, 500, and 1000 ml
ii. Conical flasks of capacity 250, 500 and 1000 ml
iii. Petri plates of size 100 × 20 ml
iv. Micropipettes of capacity 20-100 µl and 200-1000µl
v. Pipettes of capacity 5 and 10 ml
vi. Measuring cylinders of capacity 5, 100, 250, 500, and 1000 ml
vii. Culture bottles with polyurethane polyvinyl cap.
viii. Reagent bottles

The culture vessels and culture apparatus were first soaked in detergent solution (Teepol 0.1%) overnight and cleaning solution were removed by washing under running tap water, then rinsed in double distilled water (DDW) and dried in oven at 105 ^{o}C. The petri plates and other glassware used for inoculation were covered with aluminium foil or packed in plastic bags

and then autoclaved at 15lbs/m^2 pressure for 20 minutes and then stored in dust- proof room.

3.1.4.Laboratory Equipment:

i. Nat Horizontal Steam sterilizer – Autoclaving glass wares, DDW and nutrient media.
ii. Hot air oven – Drying glass wares.
iii. Double distillation unit – obtaining double distilled water.
iv. Electronic Digital balance- Accurate weighing of chemicals.
v. pHmeter – testing pH of solutions and media.
vi. Refrigerator- For storing stock solution and plant growth regulators.
vii. Laminar air flow bench – All aseptic manipulations.
viii. Incubator – maintenance of temperature and light.

3.1.5.Others:

Test tube baskets, Test tube stands, Trays, caps, bags, aluminium foil,parafilm, non- absorbent cotton, spirit lamp or gas burner, forceps, scalpels, surgical blade of no.20 for maintaining the aseptic culture.

3.1.6.Experimental Conditions:

All *in vitro* studies were carried out aseptically in laminar air flow chamber. The experiments were conducted under well-defined conditions of culture room maintained at 25 ± 2^oC temperatures, uniform light (1600Lux) provided by fluorescent tubes (7200K) over a light and dark cycles of 16/8 hours.

3.1.7.Culture Media:

Success of tissue culture protocol depends upon appropriate composition of media. In the present study Woody Plant Medium (WPM), Lloyd and McCown (1981) were used with certain additions of various concentrations and combinations of plant growth substances. The

concentration of various nutrients while preparation of one litre media are given in (Table 1.)

3.2 METHODS

3.2.1. PREPARATION OF STOCK SOLUTION

It is practically, not possible to weigh each and every ingredient at the time of media preparation. Therefore, for the sake of convenience, concentrated stock solutions of basal WPM medium containing different ingredients were prepared in Triple Glass Distilled water (TDW) and then stored in Borosil reagent bottles at 5^{o}c temperature in refrigerator.

Major salt (20x concentration)

Minor salt (200x concentration)

Iron (200x concentration)

Organic nutrients except sucrose (200x concentration)

3.2.1.1 Salts and Vitamins

Stock solutions of major and minor inorganic salts of WPM medium were prepared as recommended and stored in refrigerator. similarly, stocks of vitamins used in WPM were also prepared and stored at 5^{o}C in refrigerator until further use.

Table 1:Composition of Woody Plant Medium (Lloyd and McCown 1981)

Nutrients Constituents	(mg/L)
Macronutrient	
Potassium nitrate (KNO_3)	400
Calcium nitrate ($Ca(N0_3)_2.4H_20$)	556
Calcium chloride ($CaCl_2.2H_20$)	96
Magnesium sulphate ($MgS0_4.7H_20$)	370
Potassium dihydrogen phosphate (KH_2P0_4)	170
Potassium sulphate (K_2SO_4)	990
Micronutrient	
Boric acid (H_3BO_3)	6.20
Manganese sulphate ($MnS0_4.4H_20$)	22.30
Zinc sulphate ($ZnS0_4.4H_2O$)	8.60
Copper sulphate ($CuS0_4.5H_2O$)	0.25
Sodium molybdate ($Na_2MoO_4.2H_20$)	0.25
Iron sources	
Ferrous sulphate ($FeS0_4.7H_2O$)	27.8
Sodium ethylene diamine tetra acetic acid (Na_2 EDTA. $2H_20$)	37.3
Vitamins and amino acid	
Thiamine HCL	0.1
Nicotinic acid	0.5
Pyridoxine HCL	0.5
Glycine	2
Myo- inositol	100
Supplements	
Sucrose	30 g
Agar Agar	8 g
Auxins	As per requirements
Cytokinin	

3.2.1.2 Auxin

Requisite amount of auxin (IBA) was dissolved in minimum amount of 0.1 NaOH (1 ml for each 10 mg auxin) and final volume made up by adding Triple Distilled Water (TDW). The quantity of auxin(IBA)in stock solution was adjusted such that each ml of stock solution contained 1 mg of auxin.

3.2.1.3 Cytokinin

Requisite amount of Benzyl Amino Purine (BAP) and kinetin (KIN) was dissolved in minimum amount of 0.1 NaOH (1ml for each 10 mg of cytokinin) and final volume made up by adding Triple Distilled Water (TDW). The quantity of cytokinin (BAP) and (KIN) in stock solution was adjusted such as each 1ml of stock solution contained 1 mg of cytokinin.

At the time of media preparation, Plant growth substances were added before pH adjustment and autoclaving of media while antibiotics were added after pH adjustment and autoclaving.

3.2.2 PREPARATION OF CULTURE MEDIA

Correct formulation of the culture medium is necessary for successful differentiation of tissues. The basal medium developed by Lloyd and McCown (1981) i.e. Woody Plant Medium was partially modified for rapid growth and development by adding different concentration of plant growth substances.

For preparation of one litre medium first 30 g sucrose was dissolved in optimum quantity of water in the 1000 ml beaker. Then requisite quantities of major and minor salts, vitamins, and growth substances were taken with the help of pipette and added to the beaker containing dissolved sugar. After adding all ingredients pH of medium was adjusted to 5.8 by using 0.1 NaOH and 0.1 HCl.

Final volume was adjusted and 8g/ litre agar-agar was added to the medium and dispense into 1000 ml conical flask. Autoclaving was done by using Nat Horizontal Steam sterilizer at 121^{o}Cand 1.5 kg/cm^{2} pressurefor 20 min. After sterilization media was allowed to cool down and 200mg/l cefotaxime was added to media then poured in sterilized test tubes and was allowed to solidify at room temperature then stored into dust proof room to check for any contamination occur.

3.2.3. ASEPTIC TECHNIQUES

As suggested by Street (1977) the standard sterilization techniques were followed for inoculation and sub culturing of explants. Inoculation of explants was carried out under aseptic conditions in laminar air flow bench. All the surgical appliances were immersed in alcohol, incinerated on gas burner and cooled before use while transfer of explants.

3.2.4.PREPARATION OF EXPLANTS FOR CULTURE ESTABLISHMENT

Shoot tip and Nodal explants:

The explant material was obtained from orchard of cv. Konkan Amruta located at Horticulture department. Young and healthy nodal segments (2-4 cm) of 10-12 years old mature kokum tree containing axillary or apical bud was collected in DDW with sterile scalpel.

Explants were soaked in sterile distilled water before surface sterilization. Then these explants were washed with TWEEN-20 as surfactant and followed by washing with 10% Savlon as an antiseptic. This was followed by 0.1% (w/v) PVP(poly vinyl pyrrolidone) for 60 min. for reducing phenols oxidation. Then explants were treated with 1%(w/v) Bavistin for 60 min. After each treatment explants were washed twice with sterile distilled water. All further operations were carried out in laminar air flow bench.

Table 2 : Details of various treatments given for sterilization of explant material:

Treatments	Sterilizing agents	Conc.	Time exposure (min)
S_0	In triple distilled water (Control)	--	10
S_1	Mercuric Chloride ($Hgcl_2$)	0.1%	10
		0.5%	08
		0.75%	06
S_2	Sodium hypochlorite (NaOCl)	0.75%	12
		1%	10
		1.5%	10
S_3	Streptocycline	0.1%	15
		0.2%	10
		0.3%	10

3.2.5. Inoculation of explant on medium

Surface sterilizedaxillary or apical buds were inoculated in the test tubes containing WPM medium Lloyd and McCown (1981) supplemented with different growth regulators using aseptic culture techniques. And culture vessels were incubated in culture room at 25 ± 2°C temperature and under uniform light (1600Lux). The explants in test tubes observed every day.

Table 3: Media combinations used for culture establishment and induction of organogenesis:

Treatment	Media compositions
M_1	WPM (Control) without growth regulator
M_2	WPM+ 0.5mgKin +1mg BAP+ 0.5mg IBA
M_3	WPM+ 0.75mg kin+ 1.5mg BAP+ 1.75mg IBA
M_4	WPM + 1mg kin + 2mg BAP + 1mg IBA
M_5	WPM+ 1mg kin+ 1.25mg BAP +1.25mg IBA
M_6	WPM + 1.25mg kin+ 1.75mg BAP+ 0.75mg IBA
M_7	WPM+ 1.75mg kin+ 2.25mg BAP +1.75mg IBA

3.2.6. OBSERVATION RECORDED

1. Establishment of aseptic culture.
2. % aseptic culture.
3. Days to appearance of contamination.
4. Browning intensity of media.
5. Days to sprouting.

3.2.7. STATISTICAL ANALYSIS

As all the studies were done in laboratory under well-defined conditions of the medium, growth, temperature, light. Completely randomized design(CRD) was employed for the experiment.

CHAPTER IV

EXPERIMENTAL RESULTS

In-Vitro propagation is reliable and routine method for rapid multiplication of plants on a large scale, which is based on the cultivation of organs defined under aseptic condition in plant tissue and tissue culture media. Now a days, *in-vitro* plant regeneration is suitable for overcoming the problems that encounter during conventional methods.

The account of results obtained from the investigation entitled "*In- Vitro* studies in kokum cv. Konkan Amruta" is presented below:

1. Standardization of Protocol

A) Explant:

1) **Stage of explants:**

The data on effect of explants on per cent shoot induction is presented in (Table 4). Among various stages of explants, in this experiment two stages of explants i.e. mature and immature were used to know the effect of stage of the explant on establishment of culture. Mature explants were green in colour and of leaves with apical leaf primordia. While immature explants were reddish in colour with brown coloured leaves. The mature explants registered highest (82.33%) and immature explants gave lowest (36.60%) shoot establishment.

Table 4: Effect of explant on percent shoot induction

Explant maturity	Per cent shoot induction
Immature	36.60
Mature	82.33

2) Explant collection season:

The data on effect of season and stage of tree on aseptic culture establishment is presented in (table 5). The occurrence of the rate of contamination occurred and bud break or sprouting washighly dependent on the season in which explants were collected. It is observed that, explants which were collected during flowering stage (January- March) scored greater bud break and less contamination than the explant which were collected during its vegetative stage (August-November). It is further noted that, the explants collected during rainy season results in high rate of contamination.

Table 5: Effect of season and stage of tree on aseptic culture establishment of kokum explant

Month of collection of explants	Stage of tree	Contamination %	Aseptic culture %
August	Vegetative	83.33	16.66
September	Vegetative	75.00	25.00
October	Vegetative	70.00	30.00
November	Vegetative	63.33	36.66
December	Flowering	60.00	41.66

January	Flowering	16.66	83.33
February	Flowering	18.33	80
March	Flowering	24.66	75.00

3) Length of explants:

The data on effect of length of explants on shoot induction is presented in (Table 6). To determine the best suitable length for shoot induction, the mature greenish explants having different length were studied. It is evident from data that, the explant having length 3 cm registered highest percentage of shoot establishment than explants having length 2cm and 4 cm.

Table 6: Effect of length of explant on shoot induction

Length	Percent shoot Induction
2cm	67.84
3cm	83.33
4cm	75.60

B) Surface Sterilization:

The data on effect of surface sterilizing agents on *in-vitro*survival of different type of explants is presented in (Table 7). The explants were obtained from mature kokum tree throughout the experiment. Establishment of aseptic culture was major hurdle in the development of regeneration protocol from mature trees. Major constraints occurred were contamination and phenolic exudation at the time of culture initiation. Explant sterilization treatments were given in three steps. In primary sterilization, the explants were rinsed in sterile distilled water for 1 hr. and then washed with TWEEN- 20 for 10 min.

followed by 10%Savlon for 10 min. In secondary sterilization, explants were treated with 1% Bavistin and 0.1% polyvinylpyrrolidone for1 hr. The solution was kept on shaker at 100-110 rpm for1 hr. After that, the explants were rinsed with DDW twice. In the tertiary sterilization, the explants were treated with antimicrobial agents like $HgCl_2$, NaOCl, and Streptocycline with different concentrations and duration.

Table 7: Effect of surface sterilizingtreatments on *in-vitro* survival of different types of kokum explant

Sr. No.	Treatments	Time	% Survival		Mean
			Shoot tip	Nodal	
T_1	TDW	10	0.00	0.00	0.00
T_2	0.1%$Hgcl_2$	10	63.33	53.33	58.33
T_3	0.5%$Hgcl_2$	08	46.66	63.33	54.99
T_4	0.75%$Hgcl_2$	06	83.33	80.00	81.66
T_5	0.75%NaOCl	12	43.33	30.00	36.66
T_6	1%NaOCl	10	30.00	36.66	33.33
T_7	1.5%NaOCl	10	33.33	26.66	30.16
T_8	0.1%Streptocycline	15	23.33	16.66	19.99
T_9	0.2%Streptocycline	10	16.66	20.00	18.33
T_{10}	0.3%Streptocycline	10	13.33	16.66	14.99
	Mean		**35.33**	**34.33**	**34.99**
	SE±		0.40	0.5	
	CD		1.89	2.32	

Degeneration of culture due to contamination is major concern in micro propagation technique. To minimize this, ten treatments were imposed, among which the treatment with 0.75% $HgCl_2$ for 6 min. gave the maximum survival of explant (81.66 per cent). It was followed by the treatment with 0.1% $HgCl_2$that gave(58.33 per cent) aseptic culture establishment. $HgCl_2$ is a potential toxic for microbial contamination and helps to kill the almost all thriving bacteria. Although 0.1% $HgCl_2$ showed a greater number of aseptic cultures but, it failed to express further proliferation and growth was ceased.

B) Days to appearance of contamination:

The data on effect of various surface sterilizing treatments on days to appearance of contamination of kokum cultures is presented in (Table 8)**.** The effective treatment of sterilizing agents to check contamination in culture was observed for shoot tip and nodal explants. Treatment T_4 (0.75% $HgCl_2$for 6 min.) was found superior to check contamination in cultures of shoot tip and nodal to the extent of 5.33 and 5.00 days respectively.

C) Phenols alleviation treatment:

The data on effect of various media combinations on phenols alleviation on different type of explant is presented in (Table 9).One of the most common problem associated with *in-vitro* establishment of many woody species is the deleterious effects of oxidized phenols. To control problem of phenol alleviation and browning of explants the treatment of PVP was imposed on the explants. Pre-conditioning of 0.1% PVP for 60 min prior to inoculation of explant scored minimum browning when, media combination was, WPM + 1mg kin + 2mg BAP + 1mg IBA (M_4) for both type of explants. The minimum browning was recorded 26.67 per cent and 30.00 per cent for shoot tipandn odalexplant respectively. The per cent browning in kokum cultures ranged from 26.67 to 86.96 per cent.It was noticed that phenol exudation was prominent when media was without supplemented with growth regulator.

Table 8: Effect of various surface sterilizing treatments on days to appearance of contamination of kokum cultures

Sr. No.	Treatments	Time	Shoot tip	Nodal
T_1	TDW	10	1.33	1.66
T_2	0.1%$Hgcl_2$	10	3.0	3.66
T_3	0.5%$Hgcl_2$	08	3.33	3.33
T_4	0.75%$Hgcl_2$	06	5.33	5.0
T_5	0.75%NaOCl	12	3.0	3.66
T_6	1%NaOCl	10	3.6	3.33
T_7	1.5%NaOCl	10	3.0	2.66
T_8	0.1%Streptocycline	15	2.6	2.0
T_9	0.2%Streptocycline	10	2.0	2.33
T_{10}	0.3%Streptocycline	10	2.3	2.66
	Mean		**2.96**	**3.02**
	SE±		0.31	0.46
	CD		1.47	2.16

Table 9: Effect of various media combinations on phenols alleviation on different type of kokum explants

Sr. No.	Media Combinations	%Browning (Shoot tip)	%Browning (Nodal)
M_1	WPM (Control) without growth regulator	82.61	86.96
M_2	WPM+ 0.5mgKin +1mg BAP+ 0.5mg IBA	60.00	66.67
M_3	WPM+ 0.75mg kin+ 1.5mg BAP+ 1.75mg IBA	46.67	50.00
M_4	WPM + 1mg kin + 2mg BAP + 1mg IBA	26.67	30.00
M_5	WPM+ 1mg kin+ 1.25mg BAP +1.25mg IBA	36.67	56.67
M_6	WPM + 1.25mg kin+ 1.75mg BAP+ 0.75mg IBA	56.67	40.00
M_7	WPM+ 1.75mg kin+ 2.25mg BAP +1.75mg IBA	33.33	36.67
	Mean	**48.94**	**52.42**
	SE±	0.37	0.50
	CD	1.59	2.12

D) Effect of various media combination on days to Sprouting:

The data on effect of various media combinations on days to sprouting is presented in (Table 10). The different plant growth regulator combinations were used to study the effect of media combinations on days to sprouting of kokum cultures. The minimum days to sprouting 6.33 and 7.67 in shoot tip and nodal explants respectively, on plant growth regulator combination (M_4)WPM + 1mg kin + 2mg BAP + 1mg IBA. The range of days to sprouting

was from 6.33 to 8.67 days for shoot tip explants while 7.67 to 9.67 days for nodal explant.

Table 10: Effect of various media combinations on days to sprouting on different type of kokum explant

Sr. No.	Media Combinations	Shoot tip	Nodal
M_1	WPM (Control) without growth regulator	0.00	0.00
M_2	WPM+ 0.5mgKin +1mg BAP+ 0.5mg IBA	8.00	9.33
M_3	WPM+ 0.75mg kin+ 1.5mg BAP+ 1.75mg IBA	7.33	8.33
M_4	WPM + 1mg kin + 2mg BAP + 1mg IBA	6.33	7.67
M_5	WPM+ 1mg kin+ 1.25mg BAP +1.25mg IBA	8.00	9.67
M_6	WPM + 1.25mg kin+ 1.75mg BAP+ 0.75mg IBA	7.33	9.33
M_7	WPM+ 1.75mg kin+ 2.25mg BAP +1.75mg IBA	8.67	9.00
	Mean	**6.52**	**7.62**
	SE±	0.39	0.28
	CD	1.67	1.18

In the present investigation gelling agents namely agar agar (8gm/l) and clerigel (4gm/l) were used in media. it was noticed that agar agar provided better culture proliferation with lesser contamination as compare to its counterpart clerigel. Further cracking of media and contamination was more with clerigel than the gelling agent agar agar.

2. Induction of organogenesis:

The effect of media combinations and type of explant on organogenesis of kokum cultures was studied by using WPM media and two types of explants i.e. shoot tip and nodal explants. For this investigation Woody Plant Medium was used with seven combinations of plant growth regulator. plant growth regulators kinetin, BAP was used for shoot induction and IBA for root induction. It is evident from the data, by using two types of explants on

various media combination shoot tip explants were found better for the initial establishment of the kokum culture.

A)Establishment of culture:

1)Shoot tip explants:

The data on establishment of culture using shoot tip explants of kokum is presented in (Table 11). Seven media combinations were studied for establishment of kokum culture. The maximum establishment (86.67 per cent) was recorded by treatment M_4 (WPM + 1mg kin + 2mg BAP + 1mg IBA). Lowest establishment of shoot tip explants was observed whenever WPM media was devoid of plant growth regulator.

Table 11: Effect of various media combinations on establishment of shoot tip explant of kokum

Sr. No.	Media Combinations	% Establishment (Shoot tip)
M_1	WPM (Control) without growth regulator	0.00
M_2	WPM+ 0.5mgKin +1mg BAP+ 0.5mg IBA	23.33
M_3	WPM+ 0.75mg kin+ 1.5mg BAP+ 1.75mg IBA	43.33
M_4	WPM + 1mg kin + 2mg BAP + 1mg IBA	86.67
M_5	WPM+ 1mg kin+ 1.25mg BAP +1.25mg IBA	53.33
M_6	WPM + 1.25mg kin+ 1.75mg BAP+ 0.75mg IBA	50.00
M_7	WPM+ 1.75mg kin+ 2.25mg BAP +1.75mg IBA	60.00
	Mean	**45.23**
	SE±	0.54
	CD	2.31

2) Nodal explants:

The data on establishment of culture using nodal explants is presented in (Table 12). The seven media combinations of plant growth regulator were used for establishment of axillary bud explants. The maximum establishment (73.33 per cent) was observed on treatment M_4 (WPM + 1mg kin + 2mg BAP + 1mg IBA). lowest establishment of nodal explants was observed whenever, WPM media was devoid of plant growth regulator.

Table 12: Effect of various media combinations on establishment of nodal explant of kokum.

Sr. No.	Media Combinations	% Establishment (Nodal)
M_1	WPM (Control) without growth regulator	0.00
M_2	WPM+ 0.5mgKin +1mg BAP+ 0.5mg IBA	26.67
M_3	WPM+ 0.75mg kin+ 1.5mg BAP+ 1.75mg IBA	53.33
M_4	WPM + 1mg kin + 2mg BAP + 1mg IBA	73.33
M_5	WPM+ 1mg kin+ 1.25mg BAP +1.25mg IBA	56.67
M_6	WPM + 1.25mg kin+ 1.75mg BAP+ 0.75mg IBA	60.00
M_7	WPM+ 1.75mg kin+ 2.25mg BAP +1.75mg IBA	43.33
	Mean	**44.76**
	SE±	0.67
	CD	2.85

B) Shoot induction

1. Shoot tip explants:

Table 13: Effect of various media combinations on organogenesis on shoot tip explant of kokum.

Sr. No.	Media Combinations	% Shooting	% Rooting
M_1	WPM (Control) without growth regulator	0.00	0.00
M_2	WPM+ 0.5mgKin +1mg BAP+ 0.5mg IBA	23.33	0.00
M_3	WPM+ 0.75mg kin+ 1.5mg BAP+ 1.75mg IBA	43.33	0.00
M_4	WPM + 1mg kin + 2mg BAP + 1mg IBA	73.33	0.00
M_5	WPM+ 1mg kin+ 1.25mg BAP +1.25mg IBA	53.33	0.00
M_6	WPM + 1.25mg kin+ 1.75mg BAP+ 0.75mg IBA	46.67	0.00
M_7	WPM+ 1.75mg kin+ 2.25mg BAP +1.75mg IBA	60.00	0.00
	Mean	**42.86**	**0.00**
	SE±	0.41	-
	CD	1.75	-

The data on effect of various media combinations on organogenesis of shoot tip explants of kokum is presented in (Table 13). Seven treatments of plant growth regulators were used for shoot induction in kokum explants. The maximum percentage of shoot induction (73.33 per cent) was observed in the shoot tip explants in the treatment M_4 (WPM + 1mg kin + 2mg BAP + 1mg IBA). The range of shoot induction was 23.33 to 73.33 per cent in shoot tipexplants. Minimum shoot induction was observed in the treatment M_1(WPM without growth regulators).

2. Nodal explants:

The data on effect of various media combinations on organogenesis of nodal explants of kokum is presented in (Table 14). The nodal explants of kokum showed highest shoot induction 70.00 percent in the treatment M_4(WPM + 1mg kin + 2mg BAP + 1mg IBA) and followed by the treatment M_7 (WPM+ 1.25mg kin+ 1.75mg BAP+ 0.75mg IBA) (56.67%). The range of shoot induction in nodal explants was 16.67 to 70.00 per cent.

Table 14: Effect of various media combinations on organogenesis of nodal explant of kokum

Sr.No.	Media Combinations	%Shooting	%Rooting
M_1	WPM (Control) without growth regulator	0.00	0.00
M_2	WPM+ 0.5mgKin +1mg BAP+ 0.5mg IBA	16.67	0.00
M_3	WPM+ 0.75mg kin+ 1.5mg BAP+ 1.75mg IBA	36.67	0.00
M_4	WPM + 1mg kin + 2mg BAP + 1mg IBA	70.00	0.00
M_5	WPM+ 1mg kin+ 1.25mg BAP +1.25mg IBA	46.67	0.00
M_6	WPM + 1.25mg kin+ 1.75mg BAP+ 0.75mg IBA	56.67	0.00
M_7	WPM+ 1.75mg kin+ 2.25mg BAP +1.75mg IBA	40.00	0.00
	Mean	**38.10**	**0.00**
	SE±	0.33	--
	CD	1.40	--

The shoot tip explants scored maximum induction of shoots in the treatment M_4 ((WPM + 1mg kin + 2mg BAP + 1mg IBA) as compared to the nodal explants when those were used in the same media combination.

C) Root Induction:

The data on effect of various media combinations on *in-vitro* induction of rooting in kokum cultures is presented in (Table 13 and 14). Induction of *in-vitro* rooting in kokum was attempted using six levels of IBA. However, all the treatment imposed were not effective for inducing rooting in established kokum cultures of shoot tip and nodal explants.

D) Effect of culture vessel on growth of explant:

Maximum response given by the explants in test tubes than the glass bottles using WPM media was observed. It may be due to the vessels containing cotton plug provides more suitable micro-environment for the growth of kokum explant. While in glass bottles the leaves remained stunted for longer period and only partially opened. Further leaf fall was seen after 10-15 days after inoculation. Cotton plugs in test tubes permits the gaseous exchange and evaporation of moisture produced by the media. It was observed that average (87.13%) explants were proliferated in test tubes while (12.87%) only proliferated in glass bottles.

E) Multiple shoot production from the kokum explants on WPM medium:

Established cultures of shoot tip and nodal explants were sub-cultured after20 days for the multiple shooting purpose. This step ensures surplus nutrition supply for growing kokum culture. The media combination used for induction of shooting was WPM + 1mg kin + 2mg BAP + 1mg IBA. Same media combination was used for sub-culturing of kokum cultures. Established cultures were sub-cultured after 20 days of inoculation and second subculture was executed after 30 days after inoculation. Sub-culturing with short time interval showed fast growth and less phenol exudation.

CHAPTER VI

SUMMARY AND CONCLUSION

The present investigation entitled "*In-vitro* studies in kokum cv. Konkan Amruta" was conducted for induction of *in-vitro* shooting and rooting using shoot tip and nodal explants on woody plant medium with different combinations of growth regulator. The experiments were conducted in the Tissue culture laboratory of plant biotechnology centre,Dr.Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli. The experiments were conducted using completely randomized design (CRD) with three replications. The aseptic cultures were obtained with controlled condition of light and temperature. The salient features of result obtained as summarised below:

1. Among the explants used of various growth stages, mature explants of both shoot tip and nodal explants showed better performance for induction of organogenesis than the immature i.e. reddish coloured explants. Mature explant showed 83.33 per cent of shoot establishment as a highest shoot establishment.

2. The shoot explants having length 3 cm posted highest shoot induction 83.33 as compared 75.60 posted by shoot tip explants having length of 4 cm.

3. The explants which were collected during month of January showed highest 83.33 per cent aseptic culture as compared to 16.66 per cent in the month of August.

4. Among the different sterilizing treatments, the treatmentT_4(0.75% $HgCl_2$ for 6 minutes) found to be better for surface sterilization as survival % for shoot tip and nodal explant was 83.33 and 80.00 per cent respectively.

5. The range of days to appearance of contamination was 1.33 to 5.33 days. The treatment T_4(0.75% $HgCl_2$for 6 min.)delayed the contamination upto5.33 and 5.0 days in shoot tip and nodal explant respectively.

6. Lowest level of browning was recorded towards shoot tip and nodal explants 26.70 and 36.00 per cent respectively on the media combination containing WPM + 1mg kin + 2mg BAP + 1mg IBA(M_4).

7. Days to sprouting also showed variation on WPM medium. The range for days to sprouting for shoot tip explant was 6.33 to 8.67days and 7.67 to 9.67 days for nodal explant. The plant growth regulators combination WPM + 1mg kin + 2mg BAP + 1mg IBA (M_4) scored comparatively early sprouting for both type of explants than other combinations. Shoot tip and nodal explant took minimum 6.33 and 7.37 days respectively for bud sprouting.

8. The growth regulator combination gave variable response for per cent establishment of culture. The maximum establishment of culture was observed on the growth regulator combination WPM + 1mg kin + 2mg BAP + 1mg IBA(M_4) for both shoot tip and nodal explants. Shoot tip explants recorded maximum and minimum establishment of 86.66 per cent and23.33 percent respectively. while nodal explants showed maximum and minimum establishment of 73.33 and 26.67 per cent respectively.

9. The wide range of variation was observed for per cent shoot induction in kokum explants on WPM media. The maximum shoot induction was showed on growth regulator combination WPM + 1mg kin + 2mg BAP + 1mg IBA(M_4) for both the explants. Maximum shoot induction of

73.33 and 70.00 percent were registered by shoot tip and nodal explant respectively. Minimum shoot induction was observed on WPM (without growth regulator)(M_1) in both the explants.

10. For induction of *in-vitro* rooting prolonged period of sub culturing is necessary to trigger the process of cyto differentiation and to accelerated morphogenetic potential. This might be due to the inherent genetic potential of slow growth and devolopement of the species in its early growth stages.

Conclusion:

From the present investigation, it is concluded that, matured greenish to red coloured explants having length 3 cm were found best for the culture establishment of kokum explant. Treatment T_4 (0.75% $HgCl_2$ for 6 min.) performed better for the surface sterilization of explants. Browning of media was prevented by imposing the antioxidant treatment of 0.1% PVP which results more aseptic culture. The woody plant medium with (1mg kin + 2mg BAP + 1mg IBA) (M_4) found to be better for establishment and shoot induction in kokum explants. Better shoot induction was observed in explants which were collected in January than any other season.

Implications of the present investigations on future tissue culture work of kokum:

Kokum is an important spice crop in India. Generally, it grows near road side or in hilly areas hence this crop is not produced commercially as like mango and cashew. Kokum has variability in their sex forms and is a highly cross-pollinated crop hence, the propagation of kokum is not recommended by seeds. Seed propagated plants are not identified till the flowering either it is male or female plant. Also, the other conventional methods such as soft wood grafting etc.have their own limitations like season boundness, labour

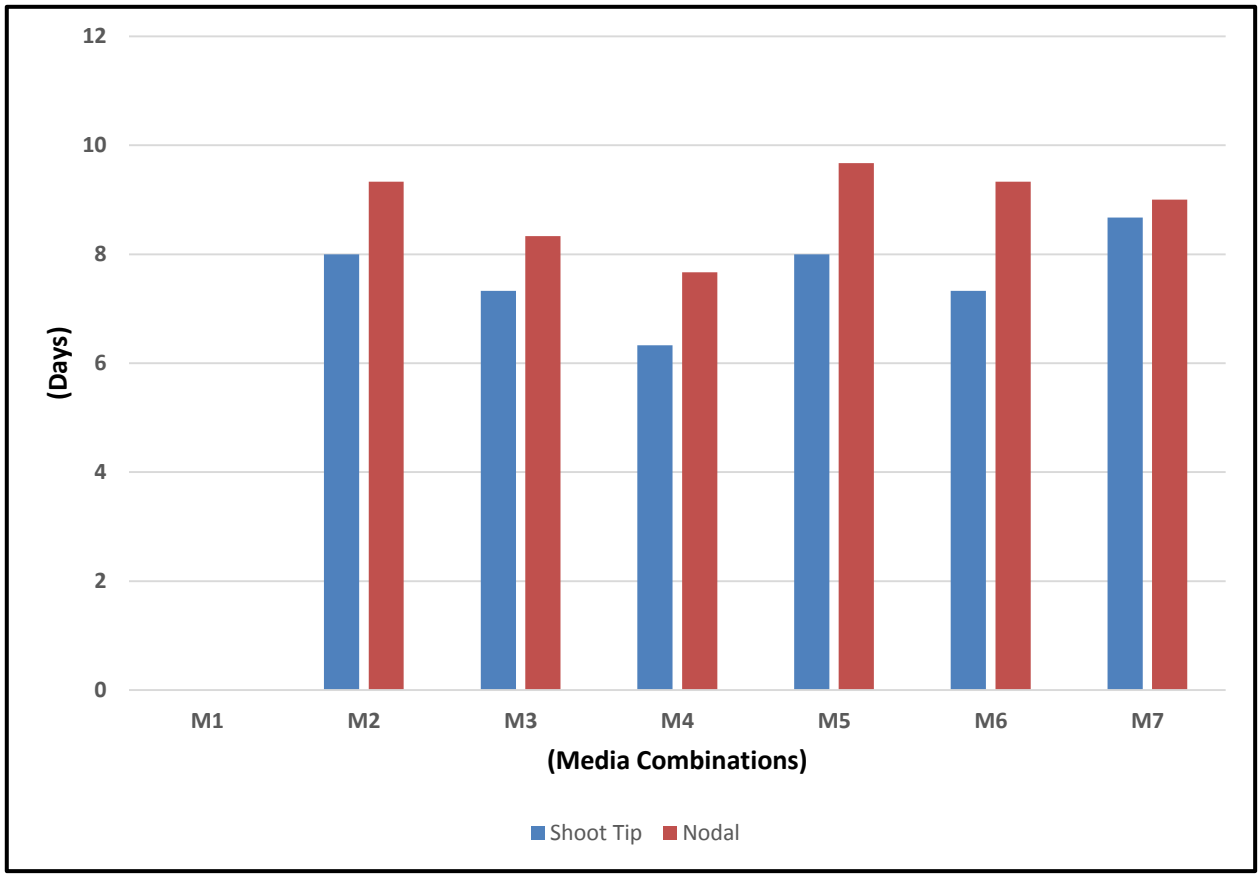
requirement, high mortality rate etc. Therefore, it is necessary to consider all these hurdles to develop suitable method of propagation in kokum var. Konkan Amruta to provide true-to-type seedlings of kokum to the farmers. In this respect tissue culture technique is used for the rapid multiplication of prominent genotype. To supply true to type seedlings micropropagation is much faster than the other conventional propagation methods.

In the present investigation from the shoot tip and nodal explants shoot establishment was obtained from the various growth regulator combinations of auxins and cytokinins. The investigation revealed that, 0.75% $HgCl_2$ performed as the best surface sterilization treatment for getting maximum aseptic cultures. PVP reduced browning in the media which results in less contamination and death of explant. The results obtained from the present investigation promotes to intensify *in-vitro* shooting and *in-vitro* rooting in speedy manner. But the kokum species itself has certain limitations which needs to be discussed and debated scientifically for commercial-scaling-up of micro propagation technology.

Limited genetic variability, polygamodioecious nature, long juvenile stage, lower physical and physiological growth and season bound grafting have complicated the issue of commercial propagation of the kokum. In the current past enormous efforts were focused to induct *in-vitro* shooting and *in-vitro* rooting by deploying varied types and sizes of explants on permutationand combination of media and growth regulators. However, slow growth of culture, prolonged period of sub culturing and phenol exudation have put limitations on research efforts. In the present investigation *in-vitro*shoot induction and phenol alleviation was successful but, *in-vitro*rooting could not make way ahead due to want of adequate experimental period. Therefore, efforts should be channelised to induct *in vitro* rootingby using results of present investigation. For induction of *in-vitro*rooting etiolated

incubation of the cultures, addition of organic supplements and use of different carbon sources in media be attempted.

Fig. 3 : Effect of media combinations on days to sprouting on different type of kokum explants



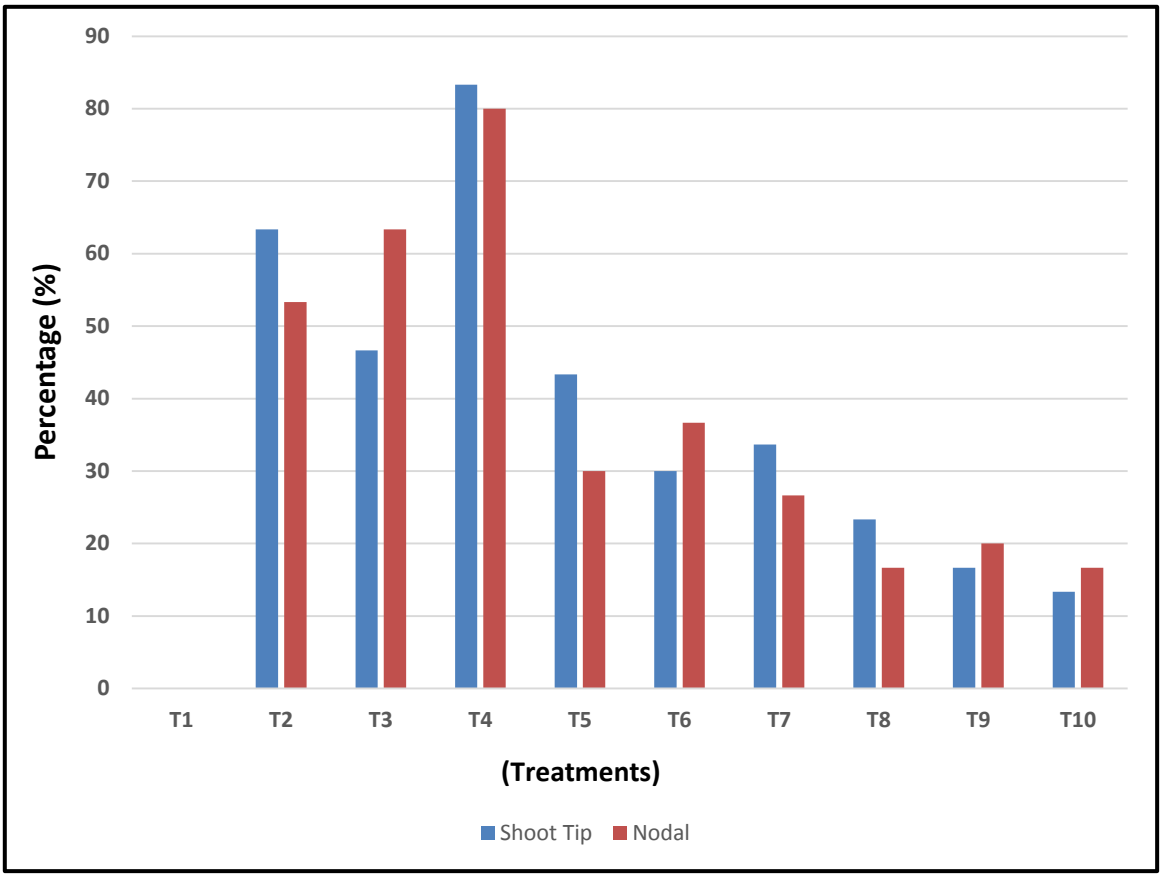


Fig. 1 : Effect of surface sterilizing treatments on *in-vitro* survival of different type of kokum explant

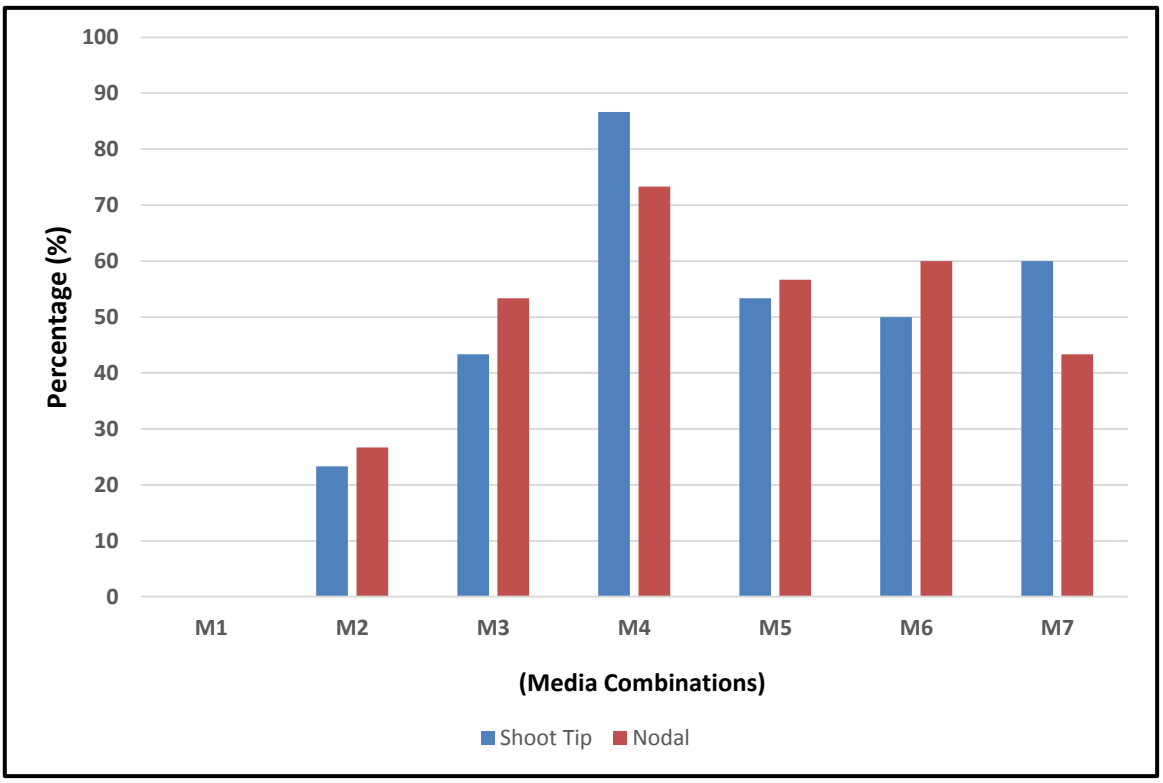


Fig. 4: Effect of various media combination on establishment of kokum explant

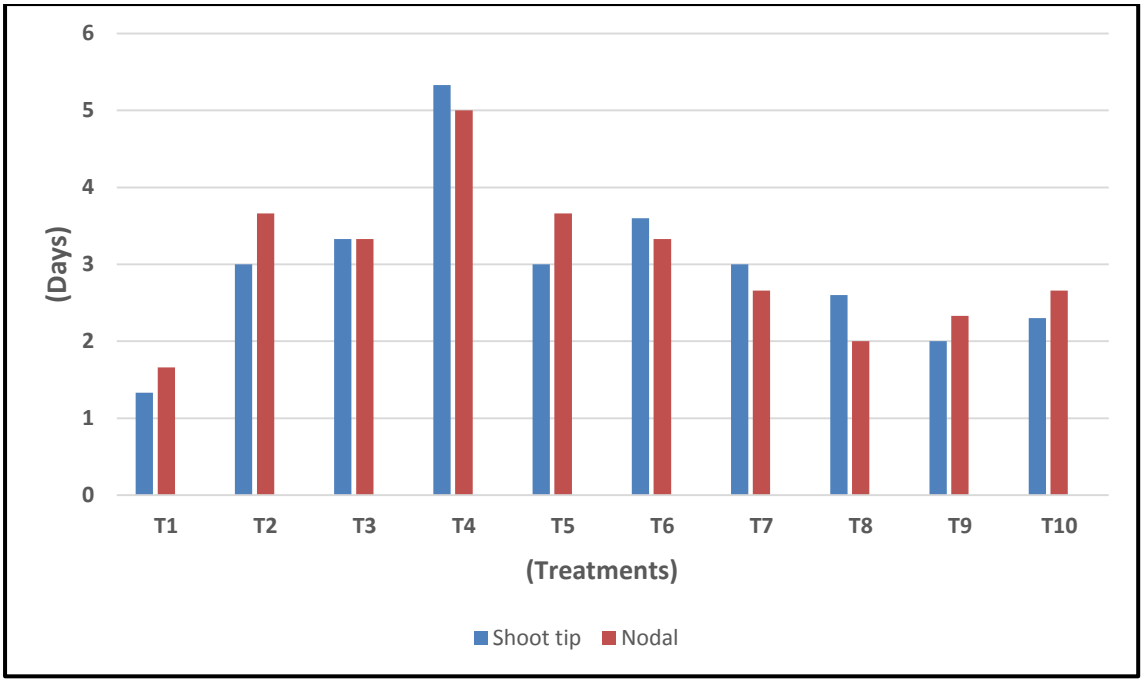


Fig. 2: Effect of various surface sterilizing treatments on days to appearance of contamination.

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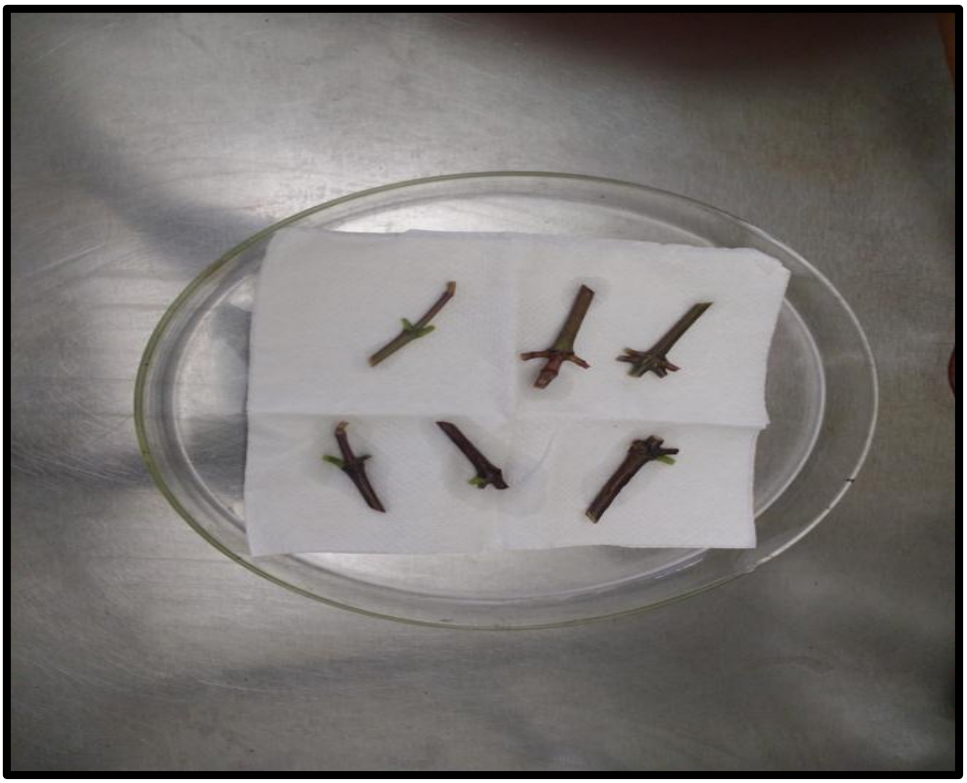
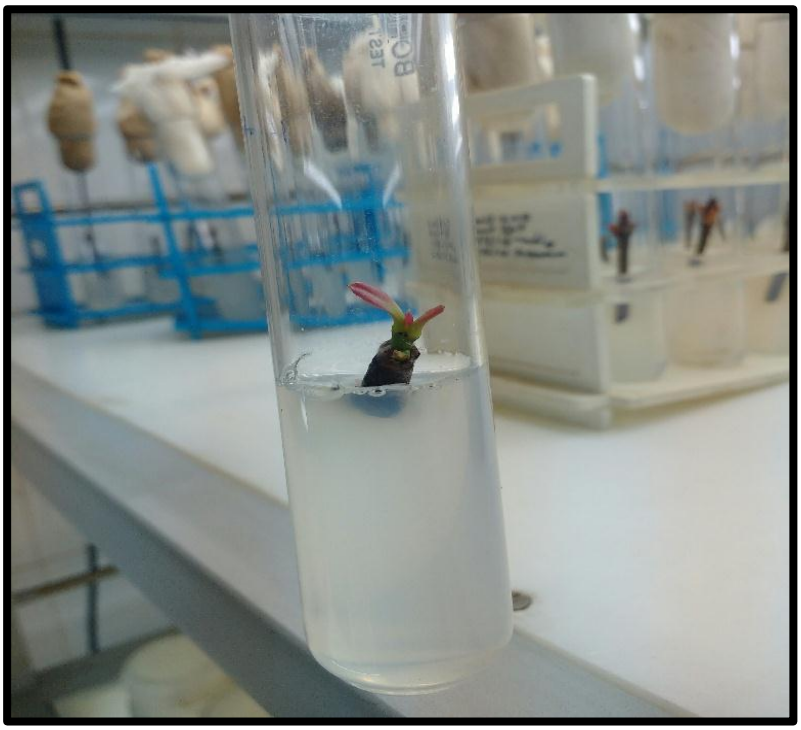
1. Kokum Tree Cv. Konkan Amruta

2.A) Selection of explant



B) Cutting of explant



3.A) Sprouting in shoot tip explant on media combination M_4 (WPM + 1mg kin + 2mg BAP + 1mg IBA)

B) Sprouting in nodal explant on media combination M_4 (WPM + 1mg kin + 2mg BAP + 1mg IBA

4.A) Establishment of shoot tip explant on media combination M_4

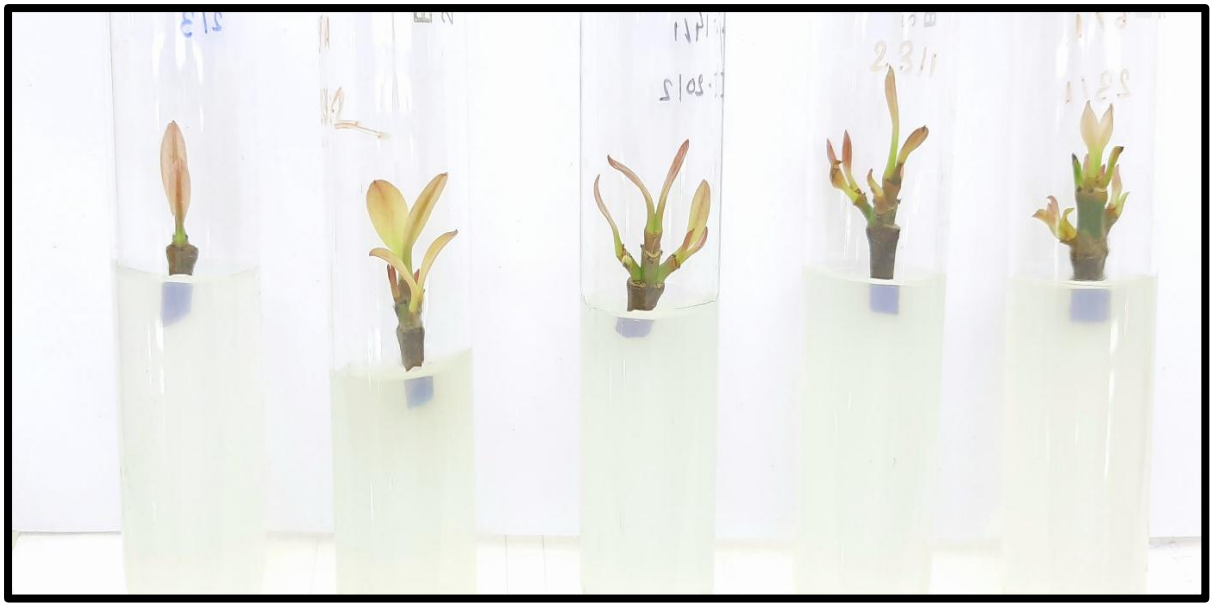
B)Establishment of nodal explant on media combination M_4

5.A)Shoot induction in shoot tip explant on media combination M_4(WPM + 1mg kin + 2mg BAP + 1mg IBA)

B)Shoot induction in nodal explant on media combination M_4(WPM + 1mg kin + 2mg BAP + 1mg IBA)

6. Multiple Shoot Production in kokum explant on media combination

M_4(WPM + 1mg kin + 2mg BAP + 1mg IBA)



7. A) *In-vitro* shooting in shoot tip explants of kokum

B) *In-vitro* shooting innodal explants of kokum

8. Growing Kokum Cultures