Studies on *in vitro* propagation of *Ocimum tenuiflorum* and testing of clonal fidelity of micro plantlets

A Dissertation

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Under The Guidance of

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CERTIFICATE

This is to certify that the dissertation **"Studies on *in vitro* propagation of *Ocimum tenuiflorum* and testing of clonal fidelity of micro plantlets"**, submitted by Nisha Neeti (Regd. No. 301101021) in the partial fulfillment of the requirement for the award of Master of Science in Biotechnology to Department of Biotechnology and Environment Sciences, Thapar University, Patiala. She has fulfilled all the requirements in completing this work under my supervision and guidance.

The work is an original contribution of the candidate and has not been submitted elsewhere for any other degree or diploma.

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Declaration

I, hereby declare that the work which is being presented in the dissertation entitled **"Studies on *in vitro* propagation of *Ocimum tenuiflorum* and testing of clonal fidelity of micro plantlets"**, in partial fulfillment of the requirement for the award of the degree of Masters of Science in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab; is an authentic record of my own work during a period of six months under the supervision of Dr. Anil Kumar, Assistant Professor, Department of Biotechnology and Environmental Sciences, Thapar University. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any other degree.

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Abstract

The present study was an attempt to develop the regeneration protocol from different explants of *Ocimum tenuiflorum*. The effect of media combinations on shoot multiplication, callusing, regeneration and rooting of microshoots of *Ocimum tenuiflorum* cultured on Murashige and Skoog (MS) medium were investigated. The best shoot proliferation in terms of shoot number and shoot quality was obtained on medium supplemented with 2.5 µM 6-benzylaminopurine (BA) and 5µM Naphthaleneacetic acid (NAA). Shoot regeneration was observed after 5-6 weeks of subcultured on MS medium supplemented with 5.0 µM BA and 2.5 µM NAA. The rooting was induced in microshoots by pulse treatment of 50 µM indole-3-butyric acid (IBA) for 36 hours. These shoots rooted on basal MS medium after 6 days of pulse treatment of IBA. The rooted plants were successfully acclimatized and transferred to the pots with about than 70 % survival. The testing of clonal fidelity of these plants using Random Amplified Polymorphic (RAPD) and Inter Simple Specific Repeats (ISSR) markers showed not a single polymorphic band. Thus these plants seem to be genetically uniform.

List of Abbreviations

µl	microlitre
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
NAA	Naphthaleneacetic acid
ABA	Abscisic acid
MS	Murashige and Skoog medium
2,4-D	2,4-Dichlorophenoxyacetic acid
BAP or BA	Benzylamino purine or benzyladenine
µM	micromolar
CTAB	Hexadecyltrimethylammonium bromide
dNTPs	deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
ISSR	Inter Simple Specific Repeats
L	litre
M	Molar
mM	Millimolar
mg	Milligram
mins	Minutes
ml	Mililitre

PCR	Polymerase Chain Reaction
PGR(s)	Plant growth regulator(s)
RAPD	Random Amplified Polymorphic DNA
TE	Tris EDTA
w/v	weight by volume

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Chapter 1

Introduction

Herbs are staging a comeback all over the globe and the herbal products are considered as a symbol of safety when compared with synthetics, which are usually considered unsafe for human consumption as well as for the environment (Jawla *et al.* 2009). Generally "medicinal plants" means those which are used for medicinal, perfumery, spices, condiments and for cosmetic purposes because they are related to the health (Shah 1997). Even though herbs had ancient importance; but easy availability, low cost and seasonal independence of synthetic products have made them more popular in modern ages. Realizing the importance of herbs, people are returning towards naturals with hope of safety and security (Jawla *et al.* 2009).

With Indian contribution of less than 1%, plant derived drugs contribute for about Rs 2 lakh crores to the world economy (Thomas *et al.* 1998). About 32 percent of plant species available on the earth are medicinal and about 15000-20000 having good medicinal properties (Thomas *et al.* 1998). From these valuable plants, about 700 species are used each by *Ayurveda* and *Unani* systems of medicine, 600 by *Siddha*, another 600 goes in the lap of *Amchi system of medicine* and a meagre amount, only 30 species are used in modern pharmacy (Thomas *et al.* 1998). The drugs and some other chemical intermediates from the medicinal important species are extracted either from the whole plant or from different organs (Thomas *et al.* 1998). Rise in population, inadequate supply of drugs, prohibitive cost of treatments, side effects of several allopathic drugs and development of resistance to currently used drugs for infectious diseases have attracted the interest of people in herbal drugs or derivatives of herbal molecules used in treatment of wide variety of human ailments (Murugesan *et al.* 2010).

Ocimum tenuiflorum, also known as Holy basil, tulsi or tulasi, is an aromatic plant belonging to family Lamiaceae which is native and widespread throughout the eastern world tropics as a cultivated plant or an escaped weed (Mehrotra *et al.* 2004). Being a subshrub, morphology include erect, branched, 30-60 cm tall hairy stems bearing strongly scented simple, opposite, green leaves (Banu and Bari 2007). Titled as "queen of the herbs" (Bhatnagar *et al.* 1993), the plant holds a sacred position in Hindu religion too (Sehgal and Singh 1999). It is also regarded as the "Elixir of life", due to innumerable health benefits possessed by it (Joseph and Nair 2013). Attracted by diverse medicinal properties of *Ocimum* such as antidiabetic (Mukerjee *et al.* 2006), antioxidant (Samson *et al.* 2007), cardioprotection (Sood *et al.* 2006), antifungal (Awuah and Ellis 2002), immunostimulant (Mukherjee *et al.* 2006), entrepreneurs had step into explore the potential of plant (Hussein *et al.* 2009). Importance of basil oil as a flavoring agent, made it suitable for use in confectionary, sausages and beverages production (Shahzad *et al.* 2012).

Ocimum is not only valued for its pharmaceutical importance but also for the aromatic oil it yields (Sahoo *et al.* 1997). Various advancement in selection of basils with high specific aromatic essential oil content has been made through traditional breeding methods (Morales and Simon 1997). The essential oils consist of linalool as the most abundant component (56.7-60.6%), followed by epi-α-cadinol (8.6-11.4%), α-bergamotene (7.4-9.2%) and γ- cadinene (3.2-5.4%) (Anwar *et al.* 2008). Eugenol imparts strong clove scent to sweet basil (Fandohan *et al.* 2008). The volatile aromatic oil present in the leaves consists mainly of thymol (32- 65%) and eugenol (Pino *et al.* 1996), xanthones, terpenes and lactone (Ljaduola *et al.* 1980). Extract of the plant also possess antimicrobial activity (Lemos *et al.* 2005). Purple varieties of basil have recently been shown to be rich source of anthocyanins and are potential source of antioxidants (Phippen and Simon 1998). Chemical composition of its essential oil depends on chemotypes, leaf and flower colors and aroma of the plants (Da-silva *et al.* 2003, Sajjadi *et al.* 2006). Basil leaves are also known to contain flavonoids, tannins, saponins, sterols, carbohydrates, proteins and triterpenoids, imparting various biological properties.

Ocimum cultivation

Most culinary and ornamental basils are cultivars of the species *Ocimum basilicum*, but other species are also grown and there are many hybrids between these species. Mostly annual but some perennial varieties growing in warm, tropical climates and sensitive to cold are also cited in literature including Holy basil and African blue basil (Maurya *et al.* 2008). Almost 160 cultivars are named till now but research continues to find new ones every year.

With a property of adaptation, basil behaves as an annual if there is any chance of a frost and may be sown in soil once chance of frost is past. Basil grows best outdoor, in well-drained sunny spots but also have potential to grow as an indoor herb.

Regeneration

Regeneration means to re-grow so in "Tissue culture terminology", it can be defined as "a process of growing an entire plant from a single cell or a group of cells". Two commonly followed methods for plant regeneration includes:

- Organogenesis
- Somatic embryogenesis

- **Organogenesis** entails the regulation of cell division, cell expansion, cell and tissue type differentiation, and patterning of the organ as a whole.
- **Somatic embryogenesis** is the developmental process by which theoretically any somatic cell behaves like a zygote that finally develops into complete embryo capable of growing to a complete plant.

  In the present study, organogenesis is used for development of regeneration protocol of *Ocimum tenuiflorum* using different concentrations of plant growth regulators (PGRs).

Micropropagation

Micropropagation is defined as growing whole plants from seed or small pieces of tissue on specially selected media under aseptic conditions. The technique of micropropagation is based on the concept of totipotency as proposed by Harberlandt. Within recent years micropropagation has evolved as a promising technique for rapid and large scale propagation of selected plants. Micropropagation is a complex process which can be achieved by any of the three approaches:-

- Multiplication by axillary and apical shoots.
- Induction of adventitious shoots via shoot organogenesis.
- Somatic embryogenesis.

The first two approaches lead to plant formation via organogenesis through production of unipolar shoots which must then be further multiplied, followed by rooting in a multistage process. In contrast, somatic embryogenesis leads to the formation of bipolar embryos through steps that are often similar to zygotic embryogenesis. Shoot multiplication is widely used for the clonal propagation using the above approaches.

Clonal fidelity

Clonal fidelity is a technique used to test the genetic uniformity amongst individuals by their respective DNA profiles. Although 99.9% of DNA sequences of an organism are the same in every person, even then enough of the different DNA is available to distinguish one individual from another, unless they are monozygotic twins. In plants, RAPD markers were used to check genetic fidelity by (Ghosh *et al*. 2012). The major hurdle in the pharmaceutical use is genetic and biochemical heterogenecity. For the confirmation of clonal fidelity of micropropagated plants, two PCR-based molecular marker techniques, RAPD (Random amplified polymorphic DNA) and ISSR (Inter simple sequence repeat) (Alizadeh and Singh 2009) are commonly used.

The standard RAPD technology utilize short oligonucleotides of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperature by PCR. At an appropriate annealing temperature during the thermal cycle, oligonucleotides primers of random sequence bind several priming site(s) (if any) on the complementary sequences in the template genomic DNA and produce discrete DNA fragments if these priming sites are within an amplifiable range (Bardakci 2001). The profile of amplified DNA primarily depends on nucleotide sequence homology between the template DNA and oligonucleotides primer at the end of each reaction.

ISSR (Inter simple sequence repeats), a powerful dominant molecular marker system used for studying genetic diversity (Ansari *et al.* 2012) achieved through amplification of DNA by a single 16-18 bp long primer composed of a repeated sequence anchored at the 3’or 5’end of 2-4 arbitrary nucleotides. The ISSR amplification assay established by (Zietkiewicz *et al.* 1994) was employed for genomic DNA amplification.

RAPD and ISSR markers have been extensively used for DNA fingerprinting (Moreno *et al*. 1998), genetic diversity (Sanchez *et al.* 1996; Esselman *et al.* 1999), population genetic (Wolfe *et al.* 1998a; Nebauer *et al.* 1999) and phylogenetic studies (Hess *et al.* 2000). Consequently, ISSR markers help in revealing a much higher level of polymorphism than RAPD (Random Amplified Polymorphic DNA) markers. In addition, the ISSR reaction is more specific than RAPD reaction (Williams *et al.* 1990; Fang & Roose 1997; Wolfe *et al.* 1998 a, b) and thus is reproducible marker.

The key objectives of study are-

- Development of shoot organogenesis protocol from different explants of *Ocimum tenuiflorum.*
- To achieve *in vitro* shoot multiplication of *Ocimum tenuiflorum*.
- To achieve rooting of microshoots and acclimatization of plantlets.
- To test the clonal fidelity of regenerated plants.

Chapter 2

Review of literature

Ocimum is commercially produced as a culinary herb and dried flavoring or spice in the USA and as a rich source of essential oils (Simon *et al.* 1984). The current commercial basil varieties show a tremendous range of leaf shape, texture and color, plant height, structure and fragrance (Simon *et al.* 1999). Recent discoveries prove that anthocyanins rich, purple varieties of basil exist as potential source of antioxidants (Phippen and Simon 1998). The oil of basil is also used for various purposes like scenting dental oral preparation and in certain perfumery formulations, notably jasmine blends, to impart strength and smoothness (Shahzad *et al.* 2012). Little practical experience has been acquired in "*in vitro*"propagation of *Ocimum* species. The volatile aromatic oil obtained from the leaves of basil consists of many active compounds such as thymol (32-65%) and eugenol (Pino *et al.* 1996). This oil also contains xanthones, terpenes and lactone (ljaduola *et al.* 1980). *Ocimum* also possesses various therapeutic properties which include the wound healing properties (Bhatia *et al.* 2010).

In vitro propagation of *Ocimum tenuiflorum*

The problem associated with conventional methods of propagation such as poor germination of seeds (<10%) and response from cuttings (Sulistiarini *et al*. 1999). These constrains have been overcome through micropropagational methods, known for its time and cost effectiveness.

Micropropagation is now a well established technique used commercially all over the globe for rapid clonal production of large number of commercially important plants (Yoshimatsu 2008). *In vitro* propagation stands out as an effective means for rapid multiplication of species in which conventional methods had failed (Nehra and Kartha 1994, Sudha and Seeni 1994, Sehgal and Singh 1999, Martin 2006). First report describing the efficient, *in vitro*, micropropagation of *Ocimum* (sweet basil) through axillary shoot multiplication on MS medium containing $N^{6'}$-benzyladenine (BA) was reported by (Sahoo *et al.* 1997). These authors reported successfully procedure for the micropropagation of *Ocimum* species using nodal explants from field grown plants and subsequent establishment of these plants in soil. The effect of different PGRs concentrations and medium composition on micropropagation through shoot multiplication, shoot elongation, rooting of microshoots, acclimatization and even regeneration of *Ocimum* sp. were also reported (Siddique and Anis 2007).

Shoot organogenesis of *Ocimum*

Through callus, shoot organogenesis for basil is a successful and reliable method (Phippen and Simon 2000). Effect of different concentration of PGRs (BA and NAA) and source of explant on shoot organogenesis of basil has also been reported (Asghari *et al.* 2012). Progeny with high levels of uniformity can be obtained by impressive means of shoot organogenesis (Asghari *et al.* 2012). It has been shown that highest percentage of shoot formation (90%) and number of shoots can be obtained from shoot tip explants (Banu and Bari 2007).

Due to the difference in the level of endogenous plant growth regulators in the plant tissues, the explant shows different responses (Grattapaglia and Machado 1998). For the shoot organogenesis and multiplication of basil, cytokinins in culture medium play an important role (Galiba *et al.* 1986). Dode *et al.* (2003) reported the higher efficiency of shoot organogenesis using cotyledonary leaf on MS medium supplemented with different concentrations of PGRs. Murashige (1974) identified several factors that should be considered in explants selection including explant choice, the physiological and ontogenetic age of the organ, and overall quality of the donor plant. Other group of factors such as medium composition, physical factors such as light quality and intensity, temperature and nutritional factors were also studied (Thorpe and Patel 1984; Brown and Thorpe 1986). It had been reported by Siddique and Anis (2008) that the rate of shoot multiplication and elongation on the PGR-free MS medium increases by the end of third subculture. The highest frequency (90.00%) of organogenesis callus induction was observed by Banu and Bari (2007) on MS medium containing 1.0 mg/l BA. Even highest regeneration frequency (85%) with an average of 5.1 shoots per explant can be obtained from one month old seedlings (Phippen and Simon 2000), with increase in age of explant the organogenic response was found to decrease.

Rooting of microshoots and Acclimatization of plantlets

In many plants, formation of roots is a very difficult step (Custodio *et al.* 2004) and is regulated by a number of factors such as physiological, biochemical and genetic (Pawlicki and Welander 1995). Thorpe (1982) indicated that growth and initiation of roots were high energy requiring processes that requires the metabolic substrates, mainly the carbohydrates. Microshoots of *Ocimum* were transferred from stage II axillary shoot cultures to stage III rooting media containing indole-3-butyric acid (IBA) for four weeks to determine optimal conditions for rooting. (Siddique and Anis 2007) observed the maximum frequency of rooting of shoots in regenerated shoots on the basal MS medium containing 1.0 µM indole butyric acid. The root initiation from the excised shoots on the half strength MS medium in the absence of auxin was not observed even after 30 days and IBA was found most effective out of the tested auxins (Sahoo *et al.* 1997). (Daniel *et al.* 2010) reported that 6.25 µM in half strength MS was optimal for the root induction out of the different concentrations of IBA tested. In another combination, well developed shoots were transferred to half strength MS medium supplemented with either IBA singly or in combination with NAA. The supplementation of auxin either singly or in combination was also reported to be beneficial for root induction in many plant species (Gopi *et al.* 2006; Baksha *et al.* 2007; Kalidass *et al.* 2008; Kalidass and Mohan 2009).

Dode *et al.* (2003) reported that the presence of NAA inhibited the root formation even when combined with different concentrations of auxin. In cotyledons and nodal explants, with the increasing concentration of BA, root formation decreased whereas in hypocotyle explant, with the increasing concentration of BA, root formation also increased and also the highest root formation was observed on the culture medium containing 2.85 µM IAA (Asghari *et al.* 2012). About 90% of *in vitro* regenerated shoots rooted on PGR-free MS medium within 2-3 weeks of culture and 85% of the micropropagated plantlets could be successfully established in soil, where they grew normally by Sehgal and Singh (1999).

Clonal fidelity of Ocimum

For micropropagation, clonal uniformity is considered as an important feature. When plant tissue passes through different stages of *in vitro* culture; many of the regenerated plantlets appear different from the mother plant. Due to the existence of many mysterious genetic changes and development of somaclones, the broader utility of any micropropagation system may be limited (Rani and Raina 2000). Genetic fidelity is maintained by the micropropagated plants regenerated from preformed structures like shoot tips, axillary buds and tissues of hard wood shoot cuttings but plant growth regulators especially synthetic ones at sub- and super- optimal levels are responsible for the occurrence of somaclonal variations (Martin *et al.* 2006).

Thus screening the micropropagated plants and identifying variants at an early stage is essential as genetic uniformity is one of the most important prerequisites for the successful micropropagation of any crop species.

Nevertheless, a major problem encountered in cells grown *in vitro* is the occurrence of genetic variation due to change in either DNA sequences (point mutation, activation of transposons), chromosomal structures (duplications, translocations) or in chromosomal numbers (leading to polyploidy). Furthermore, abnormalities in tissue culture particularly caused by growth regulators often increase the frequency with increasing number of cycles (Sheeja *et al*. 2013).

Lack of any phenotypic variation do not necessarily signifies lack of genetic variation among regenerants (Larkin an Scowcroft 1981) and therefore it is important to assay *in vitro* raised plants at genetic level. With the help of informative DNA markers such as RAPD and ISSR, any DNA variations raised in tissue culture derived plants can be detected (Modgil *et al.* 2009). Among various polymorphism detection techniques, RAPD is widely used to study clonal integrity, genetic and somaclonal variations in several crops (Martin *et al.* 2006). Polymerase chain reaction (PCR) has been previously used to assess the genetic stability of micropropagated grape (Singh *et al.* 2005), MM 106

Apple rootstock (Modgil et al. 2005), peach (Hashmi *et al.* 1997) and strawberry (Boxus *et al.* 2000). (Aggarwal *et al.* 2010, 2012) has successfully used the RAPD and ISSR markers in testing the clonal fidelity of micropropagated and regenerated shoots of *Eucalyptus tereticornis*. Aggarwal *et al.* (2010) successfully used these markers in testing the clonal fidelity of regenerated plants of chlorophytum. Ghosh *et al.* (2012) used the RAPD marker for genetic fidelity of *Ocimum gratissimum.*

Chapter 3

Materials and Method

3.1 Chemicals and glasswares

Glassware used for the culture work comprised of culture bottles (300 ml) (Kasablanka, Mumbai) covered with propylene cap containing 50 ml of medium, measuring cylinders (100 ml, 1000 ml), conical flasks (150 ml, 250 ml, 500 ml, 1000 ml), beakers and a range of pipettes (20 µl, 100 µl, 1 ml). All chemicals used routinely were purchased from HiMedia Laboratories (Mumbai, India), growth regulators and antibiotics were purchased from Sigma Chemical Co. (St Louis, MO, USA).

3.2 Washing and sterilization of glasswares

All contaminated culture bottles were autoclaved in an autoclave at a pressure of 15 lb/in^2 at 121˚C for 15-20 minutes. All contents were discarded before washing of culture bottles. Cleansing of glasswares was carried out by soaking these in chromic acid solution followed by thorough washing with tap water. All the glasswares were then washed with teepol (1% v/v) detergent solution. They were then cleansed under running tap water and then washed glasswares were inverted in a clean tray and were dried in an oven for 1 or 2 hours at 72˚C.

3.3 Plant material and surface disinfection of explant

Explants were taken from the healthy plant growing under *in vivo* conditions. These were placed in bottles and covered with net and washed for 30 minutes under running tap water. The explants were then washed with detergent (1% v/v) for another 10-15 minutes followed by distilled water to remove the detergent. The explants were then pretreated with commercial Bavistein (0.1% w/v) for 10-14 minutes and then washed properly to remove the fungicide. In the laminar air flow, the explants were treated with 0.1% (w/v) mercuric chloride solution for 3-5 minutes.

The explants were then thoroughly washed (4-5 washings) with sterilized distilled water to remove the traces of mercuric chloride. Those explants were then planted on MS medium.

3.4 Preparation of media and culture conditions

The most universally used high salt medium developed by Murashige and Skoog, 1962 was also used in this study. It is also referred as MS medium consisting of MS macro and micro elements and supplemented with MS vitamins, 7.5 g/l agar and 30 g/l sucrose (Annexure 1). Different plant growth regulators such as BA, NAA, IBA and IAA were added to the basal media with different concentrations either alone or in various combinations for each specific experiment. All media were adjusted to pH 5.8 with HCl or NaOH. Then 0.7-0.8% (w/v) agar was added in the conical flask along with media and volume made up with the distilled water. Medium thus prepared was sterilized by autoclaving at a pressure of 15lb/in^2 for 15-20 minutes. All plant growth regulators (PGRs) were added before autoclaving. Cultures were incubated in growth room for 16/8 hour (day/night) under cool white fluorescent light (50 µmol/m^2/s) provided by fluorescent lamps (Crompton, India) at temperature of 25±2°C with 55 to 66% relative humidity. Stocks of major and minor elements and vitamins were prepared with high concentration and were stored in refrigerator for future use. The stock solutions of PGRs were also prepared by dissolving these in a few drops of sodium hydroxide (1.0 N) if auxin or in hydrochloric acid (1.0 N) if cytokinin and made to desired volume for 2.5 mM with distilled water and stored at 4°C.

3.5 Culture establishment and multiplication of shoots

Aseptic cultures were established from young disease free plant in the growth room under specific conditions. These explants were subcultured on MS basal media containing 3% (w/v) sucrose, 0.7% (w/v) agar supplemented with BA (2.5 µM). An optimum concentration of BA for continuous production of shoots was investigated under specific growth conditions.

3.5 Regeneration of shoots from leaf explants

Experiment was performed with leaf explant to achieve shoot regeneration. Leaves in good condition of size (7-9 mm) from actively growing microshoots (35-45 days) were used as explants. The leaves were excised under aseptic conditions in laminar air flow cabinet and cut in to transverse segments (2-3 mm wide) and inoculated with their adaxial surface towards medium. The effect of different concentration of BA (1.0-12.5 µM) in combination with different concentration of NAA (2.5-25 µM) was studied on shoot regeneration of *Ocimum tenuiflorum.*

3.6 Rooting and acclimatization of plantlets

Microshoots with 2-3 cm long shoot tips were used for the root induction. For the purpose of root induction, a pulse treatment was given on MS medium supplemented with IBA (50 µM) for 12h, 24h and 36 h and subsequent cultured on the basal MS medium.

3.6 Clonal fidelity of micropropagated plants

3.61 DNA isolation

Total genomic DNA was extracted from the leaf tissues of the mother plants and randomly selected micropropagated plants following a 4 week acclimatization period using the Cetyl-trimethyl ammonium bromide (CTAB) method.

Reagents required

1. CTAB buffer –

2% CTAB	20 g CTAB
20 mM EDTA	40 ml EDTA stock
100 mM Tris-HCl pH 8.0	100 ml Tris-HCl stock (1M)
1.4 M NaCl	280 ml NaCl stock (5M)

Total volume made upto 1 litre using distilled water, pH adjusted within range of 7.5 – 8 and autoclaved mercaptoethanol (0.2 % v/v) was added into buffer just before use.

2. Isopropanol
3. Chloroform
4. Isoamyl alcohol
5. Saturated alcohol
6. Sodium acetate 3M
7. T.E buffer contain 20 mM EDTA and 100 mM Tris-HCl (pH 8.0) obtained by mixing required amount from the stocks: EDTA stock (0.5 M) and Tris-HCl stock (1M).

Procedure

- 1.5 g of fresh leaves were taken from each microshoot ground in liquid nitrogen to fine powder, followed by immediate transfer to 50 ml centrifuge tube. To this prewarmed CTAB extraction buffer was added to make slurry and incubated at 60°C for 1 h in water bath.

- Equal volume of Chloroform and Isoamylalcohol (24:1 v/v) was added to the above slurry and mixed for about 3 mins followed by centrifugation at 5000 x g for 10 mins.

- Aqueous phase was removed with the help of wide-bore pipette and transferred to clean tube. Chloroform extraction step was repeated again in case extract was coloured.

- DNA was precipitated with 0.66 volume of cold isopropanol followed by incubation for 1 h at -20°C.

- After centrifugation (10000 rpm for 15 mins) the supernatant was discarded and the pellets were dissolved in 1 ml TE buffer and transferred to microfuge tube.

- To the above solution, pre-heated 2 µl RNase solutions (10 mg/ml stock) was added and incubated at 37°C for 1 hour.

- After incubation, equal volume of phenol and chloroform was added (1:1 v/v), gentle shaking for 5 mins was followed by centrifugation at10000 rpm for 10 mins.

- Aqueous layer was retained. To this aqueous solution 0.3 volume of 3M sodium acetate and 0.6 volume of chilled isopropanol was added and incubated for 1 h at -20°C.

- Following incubation centrifugation was carried out at 10000 rpm for 10 mins. The pellets were retained, dried and dissolved in T.E buffer and stored at -20°C.

3.62 Checking of DNA quality

Quality of DNA was checked on 0.7% (w/v) agarose gel and the concentration determined using a nanodrop 1000 spectophotometer (thermo scientific, Wilmington, DE) by evaluating absorbance ratio 260/280.

3.63 PCR amplification

Table 1: Sequences of RAPD primers and characterization of the amplified markers using these primers:

Primer No.	Primer sequence	No. of bands	Size range (bp)
RAPD-5	ACCGCGAAGG	2	500-1000
RAPD-8	TCTGGTGAGG	3	700-2000
RAPD-10	ACCTGAACGG	-	-
RAPD-11	TTGGCACGGG	4	500-1000
RAPD-12	GTGTGCCCAA	4	500-1500

RAPD-15	GGGGTGACGA	3	750- 2000
RAPD-20	ACCCGGTCAC	2	1000-1500
RAPD-40	GTTGCGATCC	4	500- 2000
RAPD 38	TCTGTGCTGG	4	500-2000
RAPD 33	CAGCACCCAC	5	1000-1500

Table 2: Sequences of ISSR primers and characterization of the amplified markers using these primers:

Primer No.	Primer sequence	No. of bands	Size range (bp)
ISSR-1	$(CA)_8CG$	2	750-1200
ISSR-11	$(GC)_8AT$	-	-
ISSR-14	$(CT)_8AG$	3	750-1500
ISSR-15	$(GT)_8A$	2	500-1000
ISSR-19	$(AT)_8GC$	-	-
ISSR-20	$(AT)_8$	-	-
ISSR-24	$(GA)_8CA$	2	500-1000
ISSR-25	$(GA)_8CC$	2	750-1000
ISSR-26	$(GA)_8T$	2	500-1000
ISSR-27	$(CT)_8T$	2	750-1000

Table 3: PCR reaction mixture comprises of following components:

Components	**Stock solution**	**Vol/Rxn**
DNA	50ng/µl	2µl (100mg)
Taq DNA polymerase	5.0U/ µl	0.3µl (1U)
DNTPs	10µM	2µl (1.0 µM)
PCR buffer	10X	2µl

Primer	10nmol	1µl
Sterile H_2O	-	12.7µl
	Total rxn volume	20µl

PCR conditions

PCR tubes were placed in thermal cycler (Applied Biosystems, Model Gene Amp2700 USA) and amplified using temperature profile mentioned in Table 4.

Table 4: PCR amplification parameters:

Temperature (°C)	Time	No. of cycles
94	5 min	1
94	1 min	41
35/55*	45 sec	
72	1.5 min	
72	5 min	1

*Annealing temperature for RAPD is 35˚C.

*Annealing temperature for ISSR is 55˚C.

3.9.3 Agarose gel electrophoresis

Amplified products were separated in 1.5% agarose gel containing ethidium bromide using 1x TAE buffer. A constant voltage of 55 was provided for 4-5 h. DNA fragments were visualized under UV light and documented using Geldoc system (Vilber Loumart, France).

Chapter 4

Results

4.1 Multiplication of shoots

Property of "*in vitro"* clonal propagation to produce large number of clones in a short period of time from a single individual proves very beneficial in tissue culture technology. Clonal multiplication of *Ocimum tenuiflorum* was carried out to increase explant reservoir and effect of concentration of plant growth regulator such as BA (2.5 µM) on the multiplication of shoots was studied by inoculating the microshoots on basal MS medium containing different concentrations of BA (0- 10 µm) and results recorded after 2 months showed that the highest numbers of shoots multiplied on medium supplemented with 2.5 µM BA. Four multiplied shoot segments were transferred to each tissue culture bottle (as shown in figure 1A and 1B) and results were visualized after 4 weeks of subculture.

4.2 Shoot regeneration via organogenesis

Young, expanded (1.5-2 cm in size) leaves of *Ocimum tenuiflorum* from microshoots were taken as potential explant. These were given superficial transverse cuts along midrib to generate 3-4 mm segments and culturing (cut side up) on MS medium supplemented with plant growth regulators (BA and NAA) with their adaxial side facing the medium and experiments were carried out for evaluating the shoot regeneration via organogenesis on different concentrations of BA and NAA.

In the first experiment, three concentrations of BA (1 µM, 5 µm and 12.5 µM) and four concentrations of NAA (2.5 µM, 5 µM, 12.5 µM, and 25 µM) were used for the purpose of investigation. Data were recorded as percentage of shoot formation, average number of shoots per explant, callus initiation after time interval of 4 weeks.

It was interpreted from the data obtained (Fig. 1C Table 1) that maximum number of explants showed callus formation (86.2%) at concentration of 5.0 µM each BA and NAA but these calluses do not showed any shoot regeneration. Maximum number of shoots developed on medium supplemented with BA (5 µM) and NAA (2.5 µM). The maximum percent of explants (26) on this medium differentiated shoots.

Table 1: Effect of different concentrations of BA and NAA on shoot regeneration of *Ocimum tenuiflorum*

Plant Growth Regulator (µM)		**Percent explants showing callus formation**	**Percent explants showing shoot regeneration**
BA(µM)	**NAA(µM)**		
0.0	0.0	0.0	-
1.0	2.5	76	-
1.0	5.0	78	15
1.0	12.5	82.3	-
1.0	25.0	79	-
5.0	2.5	83	26
5.0	5.0	86.2	-
5.0	12.5	81	-
5.0	25.0	77	-
12.5	2.5	81	-
12.5	5.0	68	21
12.5	12.5	66.6	-
12.5	25.0	66.6	23

Values are mean of three experiments consisting 5 leaves in each experiment

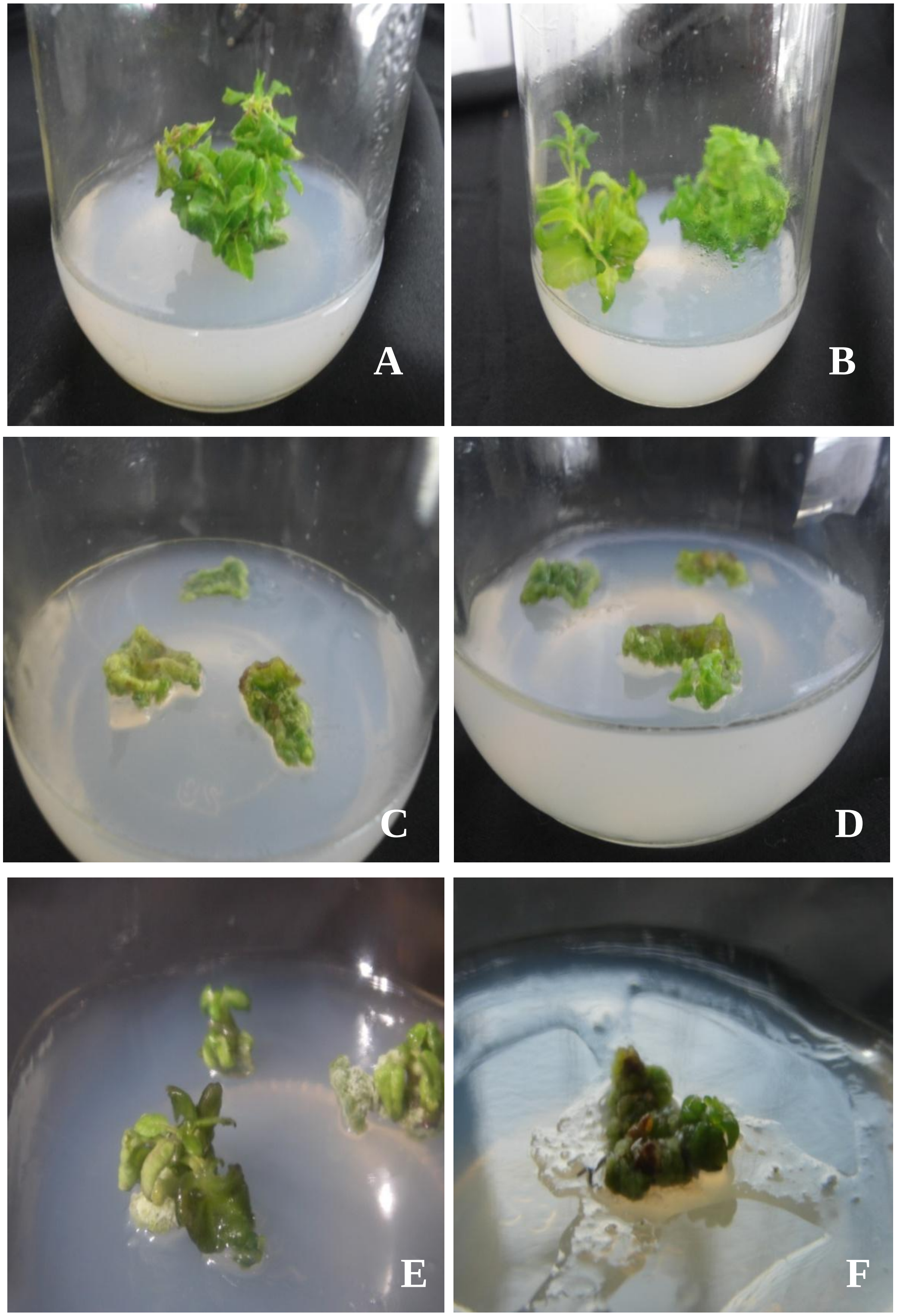

Fig: 1 Shoot multiplication, callus initiation and shoot regeneration from *Ocimum tenuiflorum*. (A) and (B) Shoot multiplication on MS medium supplemented with 2.5 µM BA. (C) Callus induction from leaf explant on MS medium supplemented with 5.0 µM NAA (D) Shoot regeneration on MS medium supplemented with 1.0 µM BA and 5.0 µM NAA (E) with 5.0 µM BA and 2.5 µM NAA (F) with 12.5 µM BA and 5 µM NAA

In the 2nd experiment, three concentrations of NAA (0, 0.2 µM, 1 µM and 3 µM) were used for investigation purpose without any BA in media. Data was recorded after 4 weeks. Callus thus formed, were transferred on the basal MS media containing three concentrations of BA (1 µM, 5 µM and 12.5 µM) and data was recorded after 4 weeks.

In this experiment, high frequency of callus formation was observed in both 1 µM and 3 µM NAA concentrations with maximum percentage of callus formation in medium with 3 µM NAA (Fig 2 A and B, Table 2). After four weeks, when callus were transferred to BA containing media, shoot regeneration was notified on medium supplemented with 5 µM BA (Fig 2C).

Table 2: Effect of different concentration of NAA on the callus formation form leaf explant of *Ocimum tenuiflorum*

Concentration of NAA (µM)	Callus morphology	Percentage of callus formation
0 µM	-	0
0.2 µM	Green, smooth	79
1 µM	Creamish, green	85
3 µM	Green, smooth	88

Values are mean of three experiments consisting 5 leaves in each experiment

In the 3rd experiment, three concentrations of only BA (1 µM, 2.5 µM and 5 µM) were used in the investigation. Data was recorded after 3 weeks. Here shoot was directly regenerated from the leaf explant on the medium supplemented with BA (2.5 µM) as seen in (Fig 2 D).

Table 3: Effect of different concentrations of BA on the shoot regeneration of *Ocimum tenuiflorum*

Concentration of BA (µM)	**Callus morphology**	**Percentage of shoot formation**
1 µM	Green, smooth	10.2
5 µM	Creamish, green	33.3
12.5 µM	Green, smooth	15.5

Values are mean of three experiments consisting 5 leaves in each experiment

As analyzed from all three above mentioned experiments, during the initial stage (1-2 weeks of incubation), there was some proliferation of cells at the cut surface of leaf and no callus formation occurred but after four weeks of culture, leaf segments of *Ocimum* developed into callus with various visible changes such as globular structure formation on MS medium supplemented with different BA concentrations. The callus thus formed were subcultured on MS medium supplemented with various concentrations of (1-12.5 µM) BA and (2.5-25 µM) NA and after some interval of time, shoot regeneration was observed. Data was recorded and average number of shoots per explant initiated from callus was calculated after 4 week of interval. Here we also observed the shoot regeneration directly from the leaf explant on the MS media containing the concentration of BA.

4.3 Rooting of microshoots and acclimatization of plantlets

Here the effects of concentrations of PGRs (IBA) with respect to incubation period on the rooting of microshoots were studied. It showed that the rooting of maximum number of shoots was achieved by pulse treatment of 50 µM IBA with 36 hours incubation time. All the incubation periods for rooting with IBA have significantly effect on the rooting of *Ocimum tenuiflorum*. The maximum numbers of roots were observed on 50 µM with incubation time 36 hr (Fig 2E and F Table 4).

Acclimatization of plantlets was carried out in a poly- house under controlled temperature and humidity (Fig 2F). In this purpose, 25 microshoots along with roots were transferred in a polythene bags and kept in a polyhouse. Data recorded after four weeks showed 66% survival rate through survival of only 17 microshoots out of 25 microshoots transferred.

Table 4: Effect of different concentrations of IBA on rooting of microshoots of *Ocimum tenuiflorum*

Concentration of IBA (µM)	**Incubation time (hr)**	**% of shoots showing rooting**	**No. of roots on each microshoot**
50 µM	12	76	4
50 µM	24	82	5
50 µM	36	83	7

Values are mean of three experiments consisting 10 shoots in each experiment

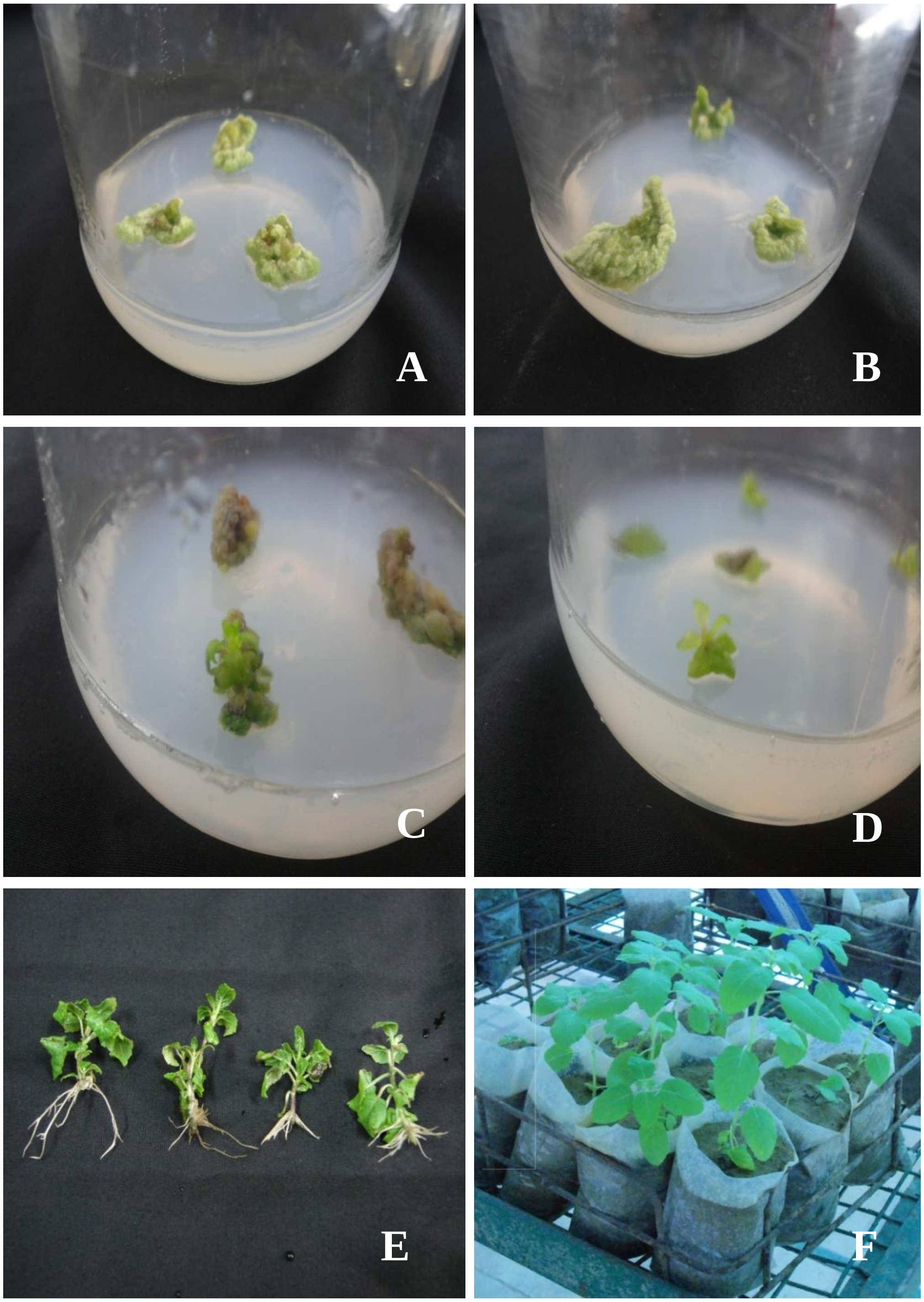

Fig 2. Callus formation, shoot regeneration, rooting and acclimatization of *O. tenuiflorum*. (A) Callus formation on MS media supplemented with 1 µM NAA (B) with 3 µM NAA (C) Shoot regeneration from callus on MS media supplemented with 5 µM BA (D) Shoot regeneration from leaf explant on MS media supplemented with 2.5 µM BA. (E) Microshoots rooted on MS medium after pulse treatment of 36 hrs on 50.0 µM IBA (F) Acclimatized plantlets

4.4 Clonal fidelity of micropropagated plantlets

Genetic fidelity of micropropagated plants and the mother plant was assessed using RAPD (Random amplified polymorphic DNA) and ISSR (Inter simple sequence repeats) techniques for their genetic variations detection. Leaves from micropropagated plants were taken for assessing the clonal fidelity randomly. 10 primers for each RAPD and ISSR were used for PCR and reproducible band profiles were obtained.

Out of the 10 RAPD primers used, 9 primers resulted in 31 scorable bands ranging 250 to 1500 bp in size. The number of bands for each primer varied from minimum of 2 to maximum of 5 with an average of 2 bands per RAPD primers. Out of the 10 ISSR primers, 7 primers produced 14 clear, scorable band. Bands from each primer varied from 1 to 3 with an average of 2 bands per ISSR primer. The screening of 7 ISSR primers generated 14 scorable bands, ranging from 250 to 1500 bp. A total of 37 bands were generated which were monomorphic in nature indicating the clonal nature of the micropropagated plants.

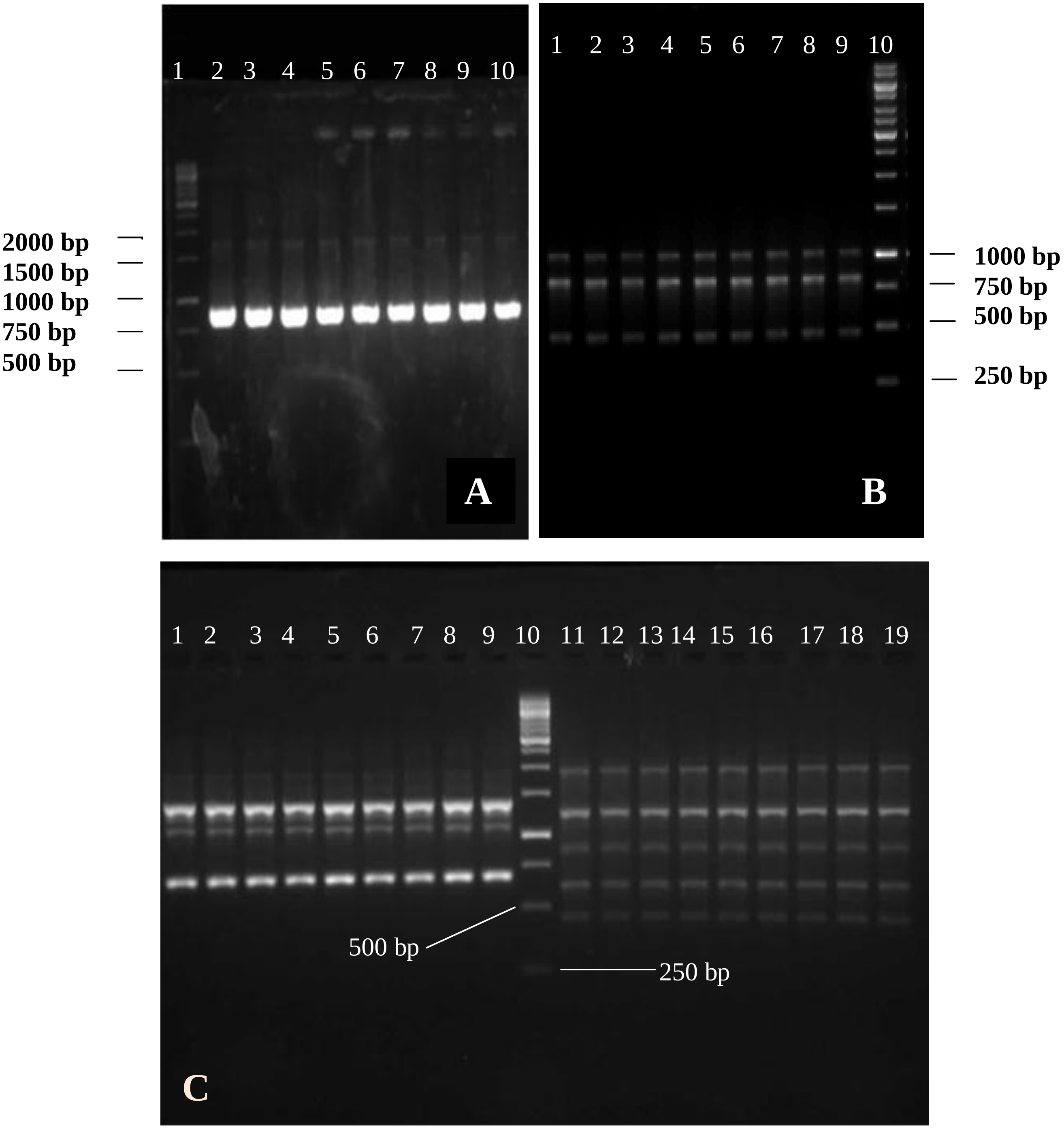

Figure 3: RAPD and ISSR analysis of micropropagated and mother plant of *O. tenuiflorum* **A.** Primer ISSR-26, Lane -1: 1 kb DNA ladder, Lane-2: Mother Plant, Lane -2-9: Micropropagated plants **B.** Primer ISSR-14, Lane-1: Mother plant, Lane -2-9: Micropropagated plants; Lane-10: 1kb DNA ladder. **C**. RAPD primer 38 (lane 1-9) and 33 (lane 11-19), Lane 1 and 11: Mother plant, Lane 2-9 and 12-19: micropropagated plants, Lane-10: 1 kb DNA ladder.

Chapter-5

DISCUSSION

Plant tissue culture techniques are essential for many types of academic inquiries, as well as to many applied aspects of plant sciences. Plant tissue culture techniques are regarded as center for innovative areas of applied plant sciences, including plant biotechnology and agriculture. Tissue culture techniques are often used for commercial production of plants as well as for plant research. It includes the techniques such as micropropagation, somatic embryogenesis, regeneration of plants etc. Micropropagation has a great commercial potential due to the speed of propagation, decreased space requirement for production and the ability to multiply elite clones exhibiting superior growth (Kane *et al*., 1989). In India, micropropagation protocol of many plant species have been developed (Devi *et al.* 1994).

Plant biotechnology along with conventional breeding methods is able to develop biotic and abiotic stress resistance in various cultivars and even seed quality, plant architecture and reproduction modes could be altered with the purpose of crop improvement (Veltcheva and Svetleva 2005). The present study was carried out to investigate the various factors involved in *in vitro* propagation of *Ocimum tenuiflorum*. Study includes the *in vitro* micropropagation, regeneration, rooting of microshoots and acclimatization of plantlets and clonal fidelity of plantlets. The main aim was to establish the regeneration protocol for Ocimum tenuiflorum and to test the clonal fidelity of regenerated plants.

Various aspects including shoot multiplication shoot elongation, leaf production or rooting of *Ocimum tenuiflorum* were significantly influenced by composition of plant growth regulators (Siddique and Anis 2008; Sahoo *et al.* 1997).The success in the micropropagation of *Ocimum* depends upon the conditions such as type of explant used, time of collection of explant, accuracy in sterilization procedure, composition of nutrient medium, type of hormones used, control over oxidative browning after inoculation and hardening of the plants produced.

Although there are many research papers published on the work on the *Ocimum,* but still there is no report available on the shoot multiplication and regeneration via organogenesis of *Ocimum tenuiflorum.* The main objective of *in vitro* multiplication phase is to produce large number of plants in a short period of time. To obtain good results, apart from the plant growth regulators and their concentration, it is necessary that the culture medium meet the demand for nutrients, essential for in *vitro* plants growth and their development. The regeneration protocol is the prerequisite for taking up any improvement programme based on plant genetic engineering.

In this study, we observed the effect of different concentrations of plant growth regulators on the shoot multiplication of *Ocimum.* For this purpose, basal media containing 3.0% (w/v) sucrose and 0.7% agar (w/v) was supplemented with different concentrations of BA. Maximum shoot multiplication occurred when shoots were inoculated on MS medium supplemented with 2.5 µM. The utility of BA on shoot multiplication has been reported in many plants.

In this present study, we established the regeneration protocol for the *Ocimum tenuiflorum* and observed the effect of different concentrations of plant growth regulators on the regeneration of *Ocimum* when cultured on the basal MS media consisting different concentration combination of BA (1 µM, 5 µM and 12.5 µM) and NAA (2.5 µM, 5 µM, 12.5 µM and 25 µM). The PGR concentration of BA (1 µM, 5 µM) and NAA (2.5 µM, 5 µM and 25 µm) was found to be best for the regeneration of *Ocimum tenuiflorum.* The effect of these PGRs on shoot regeneration has been earlier reported in Eucalyptus (Aggarwal *et al.* 2012). Important factors that influence the regeneration included media composition, age, genotype and orientation of the explant and incubation conditions (James *et al.* 1988). The regeneration medium that has generally produced the greatest shoot regeneration is the MS medium (1962) supplemented with 2-3% sucrose and standard vitamins complements.

For rooting, IBA (Indole butyric acid) is the best auxin and maximum rooting was observed at concentration 50 µM. Here an experiment was carried out with a pulse treatment with IBA. The microshoots were cultured on the 50 µM IBA with incubations of (12 hr, 24hr and 36 hr) and then transferred on the simple basal media without any auxin. Maximum rooting percentage is due to the optimum concentration of IBA with different incubation periods (hr). Earlier the rooting of difficult-to-root microshoots was attained by the pulse treatment of microshoots with IBA for 12 hours (Aggarwal *et al.* 2010). Auxin is clearly involved in morphogenesis since it regulates plant cell division, elongation and differentiation. Thus, IBA is by far the most commonly used auxin to obtain root initiation.

For acclimatization of plantlets, many parameters such as effect of light source, temperature and soil texture plays very important role. In our experiment, acclimatization was carried out in a polyhouse with controlled temperature (25-28˚C) and humidity (90-95%) and then plantlets were planted in a mixture of soil and agropeat (3:1, w/v) in polythene bags and kept in polyhouse. After 4 weeks, data was recorded and microshoot survivals were observed under *in vivo* conditions. After that, plants were transferred to green house in pots.

Analysis of individual primers revealed that RAPD patterns were similar for both the *in vitro* raised plantlets and their respective mother plants (control plant), which indicated that there was no genetic variation in the regenerated plantlet population.

Amplification with RAPD and ISSR markers established that a high level of genetic uniformity exists amongst the micropropagated plants and with that of mother plant. All scorable markers amplified (both RAPD and ISSR) were monomorphic thus supporting clonal fidelity. The utility of these markers in assessing the clonal fidelity is well documented (Aggarwal *et al.* 2010; Kumar *et al.* 2010). The high degree of genetic uniformity observed in the micropropagated plants could be due to stability of genome during aseptic manipulations and culture conditions, which could be critical for longer-term commercial propagation of elite clones.

Chapter 6

Conclusion

- Maximum number of shoots was achieved on MS medium supplemented with 2.5 µM of BA.
- Callus induction was achieved on MS medium supplemented with 5.0 µM of NAA.
- Shoot regeneration was achieved on MS medium supplemented with 2.5 µM of BA.
- Microshoots were rooted on MS medium after pulse treatment of 50.0 µM of IBA.
- Rooted plantlets were successfully acclimatized under green house condition.
- RAPD and ISSR primers confirm the clonal fidelity of micropropagated plants with mother plant.

Chapter 7

References

Aggarwal D, Kumar A, Sharma J and Reddy MS (2012). Factors effecting micropropagation and acclimatization of an elite clone of *Eucalyptus tereticornis*. *In Vitro* Cellular Developmental Biology DOI: 10.1007/s11627-012-9446-z.

Alizadeh M and Singh KS (2009). Molecular assessment of clonal fidelity in micropropagated grape (Vitis spp.) rootstock genotypes using RAPD and ISSR Markers. Iranian Journal of Biotechnology 7: 37 -44.

Ansari AS, Narayanan C, Wali AS, Kumar R, Shukla N and Rahangdale KS (2012). ISSR markers for analysis of molecular diversity and genetic structure of Indian Peak (*Tectona grandis* L.) population. Annals of Forest Research 55: 11-23.

Anwar F, Tufail S, Sherazi H, Hussain IA and Prybylski R (2008). Chemical composition, anti-oxidant and antimicrobial activities of basil (*Ocimum basilicum*) essential oils depends on seasonal variations. Food Chemistry 108: 986 – 995.

Asghari F, Hossieni B, Hassani A and Shirzad H (2012). Effect of explants source and different hormonal combinations on direct regeneration of *Ocimum basilicum*. Australian Journal of Agricultural Engineering 3: 12-17.

Awuah RT and Ellis WO (2002). Effects of some groundnut packaging methods and protection with *Ocimum* and *syzygium* powders on kernel infection by fungal. Mycopathologia 154: 264-269.

Baksha R, Miskat AAJ, Khatun R and Munshi JL (2007). *In vitro* rapid clonal propagation of *Rauvolfia serpentina* L. Benth. Bangladesh Journal of Scientific and Industrial Research 42: 37-44.

Banu LA and Bari MA (2007). Protocol establishment for multiplication and regeneration of *Ocimum sanctum* L. Journal of Plant Science 2: 530-537.

Bardakci F (2001). Random Amplified Polymorphic DNA (RAPD) marker. Turkish Journal of Biology 25: 185-196.

Bhatia KA, Goel A and Kumar S (2010). Wound healing potential of *Ocimum sanctum* L. with induction of tumor necrosis factor α. Indian Journal of Experimental Biology 48: 402-406.

Bhatnagar M, Kapur KK, Jalees S and Sharma SK (1993). Laboratory evaluation of insecticidal properties of *Ocimum basilicum* L. and *Ocimum. sanctum* L. plant's essential oils and their major constituents against vector mosquito species. Entomology Research 17: 21–29.

Boxus PH, Jemmali A, Terzi JM and Arezki O (2000). Drift in genetic stability in micropropagation: The case of strawberry. Iranian Journal of Biotechnology 7: 155-162.

Brown DCW and Thorpe TA (1986). Plant regeneration by organogenesis. In: Vasil IK (ed) Cell Culture and Somatic Cell Genetics of Plants 3: 46-65.

Custodio L, Loucao MA and Romano A (2004). Influence of sugars on *in vitro* rooting and acclimatization of carob tree. Biologia Plantarum 48: 469-472.

Da- silva F, Santos RHS, Diniz ER, Barbosa LCA, Casali VMD and De-Lima RR (2003). Content and composition of basil essential oil at two different hours in the day and the season. Revista Brasileira de Plantas Medicinais 6: 33-38.

Daniel A, Kalidass C and Mohan VR (2010). In vitro Multiple Shoot induction through Axillary Bud of *Ocimum basilicum* L. International Journal of Biological Technology 1: 24-28.

Devi SY, Mukherrjee BB, Gupta S (1994). Rapid cloning of elite teak (*Tectona grandis* Linn.) by *in vitro* multiple shoot production. Journal of Indian Experimental Biology 32: 668-671.

Dode B, Bobrowski LV, Braga BJE, Seixas KF and Schuch WM (2003). *In vitro* propagation of *Ocimum basilicum* L. Acta Scientiarum Biological Sciences 25: 435-437.

Esselman EJ, Jianqiang L, Crawford DL, Windus JL and Wolfe AD (1999). Clonal diversity in the rare Calamagrostis porter spp. Insperata (Poaceae): Comparative result for allozymes RAPD and ISSR. Molecular Ecology 8: 443-451.

Fandohan P, Gnonlonfin B, Laleya A, Gbenou JD, Darboux R, Moudachirou M (2008). Toxicity and gastric tolerance of essential oils from *Cymbopogan citratus, Ocimum gratissimum* and *ocimum basilicum* in Wistar rats. Food and chemical Toxicology 46: 2493-2497.

Fang and Roose M (1997). Identification of closely related citrus cultivars with ISSR. Theoretical and Applied Genetics 95: 408-417.

Galiba G, Kovacs G and Sutka J (1986). Substitution analysis of plant regeneration from callus culture in wheat. Plant Breeding 97: 261-263.

Ghosh P, Saha S, Abdul K and Sengupta C (2012). *In vitro* propagation of *Ocimum gratissimum L.* and its evaluation of genetic fidelity using RAPD marker. American Journal of Plant Sciences 3: 64-74.

Gopi C, Sekhar NJ and Ponmurugan P (2006). *In vitro* multiplication of *Ocimum gratissimum L.* through direct regeneration. African Journal of Biotechnology 5: 723-726.

Grattapaglia D and Machado MA (1998). Micropropagation. In: Cultura de Tecidos e Transformacao Genetica de Plantas. Embrapa-cnph, Brasilia. 183-247.

Hashmi G, Huettel R, Meyor R, Krusberg L and Hammerschlag F (1997). RAPD analysis of somaclonal variants derived from embryo cultures of peach. Journal of Plant Cell 16: 624-627.

Hess J, Kadereit JW and Vargas P (2000). The colonization history of *Oleo Europea* L. in Macaronesia based on internal transcribed apacer 1 (ITS-1) sequence, randomly amplified polymorphic DNAs (RAPD) and ISSR. *Mol Ecol. 9:857-868.*

Hussein S, Ling KPA and Lim XZ (2009). Callus induction of *Ocimum sanctum* and estimation of its total flavonoids content. Asian Journal of Agricultural Sciences 1: 55-61.

James DJ, Passey AJ and Rugini E (1988). Factors affecting high frequency plant regeneration from apple leaf tissues cultured in vitro. Journal of Plant Physiology 132: 148-154.

Jawla S, Gupta AK, Singla R and Gupta V (2009). General awareness and relative popularity of allopathic, ayurvedic and homeopathic systems. Journal of Chemical and Pharmaceutical Research 1: 105-112.

Joseph B and Nair MV (2013). Ethanopharmacological and phytochemical aspects of *Ocimum sanctum* L. - The Elixir of Life. British Journal of Pharmaceutical Research 3: 273-292.

Kalidass C and Mohan VR (2009). *In vitro* rapid clonal propagation of *Phyllanthus urinaria* L. (*Euphorbiaceae*) -A medicinal plant. Researcher 1: 56-61.

Kalidass C, Manickam VS and Glory M (2008). *In vitro* studies on *Leptadenia reticulata* (Retz.) Wight & Arn. (Asclepiadaceae). Indian Journal of Multidisciplinary Research 4: 221– 225.

Kane ME, Sheehan TJ, Dehgan B and Philman NL (1989). *In vitro* propagation of Florida native plants: *Stryx americana* and *Persea palustris*. Proceedings of the Florida State Horticultural Society 102: 264-268.

Kumar A, Aggarwal D, Gupta P and Reddy MS (2010). Factors effecting the *in vitro* propagation and field establishment of *Chlorophytum borivilianum.* Biologia Plantarum 54: 601-606.

Larkin PJ and Scowcroft WR (1981). Somaclonal variation a novel source of variability from cell cultures for plant improvement. Theoretical Apple Genetics 60: 197-214.

Lemos JA, Passos XS, Fernades OFL, Paula JR, Ferri PH, Souza LKH, Lemos AA and Silva MRR (2005). Antifungal activity from *Ocimum gratissimum* L. towards *cryptococcus neoformans*. Memorias do Institute Oswaldo Cruz, Rio de Janeiro 100: 55-58.

Ljaduola G, Anyiwo I and Thomas C (1980). *Ocimum gratissimum* and blood coagulation. Journal of Research Ethinomedian 1: 19-21.

Martin KP, Pachathusdikandi S, Zang CL, Slater A and Madassery J (2006). RAPD analysis of a variant banana (*Musa sp.*) cv.Grande naine and its propagation by shoot tip culture. In Vitro Cell Development and Biology of Plants 42: 188- 192.

Maurya P, Sharma P, Mohan L, Batabyal L, Srivastava CN (2008). Evaluation of toxicity of different phytoextracts of *Ocimum basilicum* against *Anopheles stephensi* and *Culex Quinquefasciatus.* Journal of Asia-Pacific Entomology 12: 113-115.

Mehrotra S, Vartika RV, Vajpayee P and Singh NK (2004). Effect of chromium accumulation on photosynthetic pigments, oxidative stress defense system, nitrate reduction, proline level and eugenol content of *Ocimum tenuiflorum* L. Plant Science 167: 1159–1169.

Modgil M, Gupta R and Chakrabarti SK (2009). Assessment of genetic fidelity of micropropagated apple rootstock plants, EMLA, using RAPD markers. Indian Journal of Experimental Biology 47: 925-928.

Modgil M, Mahajan K, Chakrabarti SK, Sharma DR and Sobti RC (2005). Molecular analysis of genetic stability in micropropagated apple rootstock MM106. Scientia Horticulturae 104: 151-160.

Morales MR and Simon JE (1997). “Sweet Dani”: A new culinary and ornamental lemon basil. Horticulture Sciences 32: 148-149.

Moreno S, Martin JP, Ortis JM (1998). Inter simple sequence repeats PCR for characterization of closely related grapewine germplasm. Eucalyptica 110:117-125.

Mukerjee PK, Maitib K, Mukerjee K and Houghton PJ (2006). Leads from Indian medicinal plants with hypoglycemic potentials. Journal of Ethanopharmacological 106: 1-28.

Murashige T (1974). Plant propagation through tissue culture. Annual Review of Plant Physiology 25: 135–166.

Murugesan AG, Geetha K and Narayanan KR (2010). Antimicrobial efficiency of *Achyranthes asp*era L. against selected pathogenic organisms. Journal of Bioscience Research 1: 187-190.

Nebauer SG, Agudo LDC, Segura J (1999). RAPD variation within and among natural populations of outcrossing willow-leaved foxglove (*Digitalis obscura* L.). Theoretical and Applied Genetics 98: 985-994.

Nehra NS and Kartha KK (1994). Plant cell and tissue culture. In: Vasil IK and Thorpe TA (eds). Dordrecht: Kluwer Academic Publishers, pp 37–70.

Pawlicki N and Welander M (1995). Influence of carbohydrate source, auxin concentration and time of exposure on adventitious rooting of the apple rootstock Jork 9. Plant Science 106: 167-176.

Phippen BW and Simon JE (2000). Shoot regeneration of young leaf explant from *Ocimum basilicum*. In Vitro Cellular Development Biology- Plant 36: 250-254.

Phippen WB and Simon JE (1998). Anthocyanins in basil. Journal of Agriculture and Food Chemistry 46: 1734-1738.

Pino JA, Rosado A and Fuestes V (1996). Composition of essential oils from the leaves and lowers of *Ocimum gratissimum* L. grown in Cuba. Journal of Essential Oil Research 8: 139-141.

Rani V and Raina SN (2000). Genetic fidelity of organized meristem derived micropropagated plants: A critical reappraisal. Journal of In Vitro Cell Development and Biology of Plants 36: 319-330.

Sahoo Y, Pattnaik SK and Chand PK (1997). *In vitro* clonal propagation of an aromatic medicinal herb *Ocimum basilicum L.* (Sweet Basil) by Axillary Shoot proliferation. In Vitro Cell Development Biology Plant 33: 293-296.

Sahoo Y, Pattnaik SK and Chand PK (1997). *In vitro* clonal propagation of an aromatic medicinal herb *Ocimum basilicum L.* (Sweet Basil) by Axillary Shoot proliferation. In Vitro Cell Development Biology-Plant 33: 293-296.

Sajjadi SE (2006). Analysis of the essential oils of two cultivated basil (*Ocimum basilicum)* from Iran. Daru Journal of Pharmaceutical Sciences 14: 128-130.

Samson JR, Sheeladevi and Ravindran R (2007). Oxidative stress in brain and antioxidant activity of *Ocimum sanctum* in noise exposure. Neurotoxicology 28: 679-685.

Sanchez MP, Davilla JA, Loarce Y and Ferrer E (1996). Simple sequence repeat primers used in polymerase chain reaction amplification to study genetic diversity in barley. Genome 39: 112-117.

Sehgal and Singh NK (1999). Micropropagation of Holy Basil *(Ocimum sanctum L.)* from young inflorescences of mature plants. Plant Growth Regulator 29:161-166.

Shah NC (1997). Conservation of wild medicinal plants: Need for a comprehensive strategy. Kurukshetra XLVI: 15-18.

Shahzad A, Faisal M, Ahmad N and Anis M (2012). An efficient system for *In vitro* multiplication of *Ocimum Basilicum* through node culture. African Journal of Biotechnology 11: 6055-6059.

Sheeja TE, Anju PR, Shalini RS, Siju S, Dhanya K and Krishnamoorthy B (2013). RAPD, SCAR and conserved 18SrDNA markers for a red-listed and endemic plant species, *Knema andamanica (Myristicacaea)*. Physiology and Molecular Biology of Plants 19: 245-250.

Siddique I and Anis M (2007). Rapid micropropagation of *Ocimum basilicum* using shoot tip explants pre- cultured in thidiazuron supplemented liquid medium. Biologia Plantarum 51: 787 – 790.

Siddique I and Anis M (2008). An improved plant regeneration system and ex vitro acclimatization of *Ocimum basilicum* L. Acta Physiologiae Plantarum 30: 493 – 499.

Simon JE, Craker LE and Chadwick A (1984).The scientific literature on selected herbs, aromatic and medicinal plants of the temperature zone. Archon Books, Hamden. 7-9

Simon JE, Morales MR, Vieira RF, Phippen WB and Basil HZ (1999). A source of raw aroma chemicals and a popular herb for culinary and ornamental market. In: J. Janick (ed). Perspectives on new crops and new uses. 499-505.

Singh AK, Singh M, Singh AK, Singh R, Kumar S and Kalloo G (2005). Genetic diversity within the genus *Solanum (Solanaceae)* as revealed by RAPD markers. Current Science 90: 711-714.

Sood SD, Narang MK, Thomas, Gupta YK and Maulik SK (2006). Effects of *Ocimum sanctum* L. on cardiac changes in rats subjected to chronic restraint stress. Journal of Ethanopharmacological 108: 423-427.

Sudha CG and Seeni S (1994). In vitro multiplication and field establishment of Adhatoda beddomei CB Clarke, a rare medicinal plant. Plant Cell Reports 13: 203–

207.

Sulistiarini D, Oyen LPA and Nguyen Xuan Dung (1999). *Ocimum gratissimum* L. In. Plant resource of South East Asia. *Essential oil plants.* Prosea Foundation Bogor, Indonesia. 19: 140-142.

Thomas J, Joy PP, Mathew S and Skaria PS (1998). Medicinal plants, Kerala Agricultural University, Aromatic and Medicinal Plants Research Station.

Thorpe T (1982). Carbohydrate utilization and metabolism. In: Cell and Tissue Culture in Forestry, Martinus Nijhoff Dordrecht 89-112.

Thorpe TA and Patel KR (1984). Clonal propagation: adventitious buds. In: Vasil IK (ed) Cell Culture and Somatic Cell Genetics of Plants 1: 49–60.

Veltcheva MR and Svetleva DL (2005). In vitro regeneration of *Phaseolus vulgaris* L. via organogenesis from petiole explants. Journal of Central European Agriculture 6: 53-58.

Williams JSK, Kubelik AR ,Livak KJ ,Rafalski JA and Tingey SV (1990). DNA Polymorphisms amplified by arbitary primers are useful as genetic markers. Nucleic acids Research 18: 6531-653

Wolfe AD, Xiang QY and Kephart SR (1998a). Diploid hybrid speciation in Penstemon. Proceeding of National Academy of Sciences, USA 95: 5112-5115.

Wolfe AD, Xiang QY and Kephart SR (1998b). Assessing hybridization in national populations of Penstemon (Scrophulariaceae) using hypervariable inter simple sequence repeat markers. Molecular Ecology 7: 1107-1125.

Yoshimatsu K (2008). Tissue culture of medicinal plants: Micropropagation, transformation and production of useful secondary metabolites. Studies in natural products chemistry (Part N) - Bioactive Natural Products 34: 647-752.

Zietkiewicz E, Rafalski A and Labuda D (1994). Genome fingerprinting by simple sequence repeat (SSR) - anchored polymerase chain reaction amplification. Genomics 20: 176-183.

Annexure 1

Media Composition

Murashige and Skoog (1962) Medium

1. Macronutrients	**mg/l**
NH_4NO_3	1650
KNO_3	1900
$CaCl_2\cdot 2H_2O$	440
$MgSO_4\cdot 7H_2O$	370
KH_2PO_4	170
2. Micronutrients	
$MnSO_4\cdot 4H_2O$	16.90
$FeSO_4\cdot 7H_2O$	27.80
$ZnSO_4\cdot 7H_2O$	08.60
H_3BO_3	06.20
KI	00.83
$Na_2MoO_4\cdot 2H_2O$	00.25
$CoCl_2\cdot 6H_2O$	00.025
$CuSO_4\cdot 5H_2O$	00.025
NaFeEDTA	30.00
3. Vitamins	
Myoinositol	100.00
Glycine	2.0
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Sugar	3000
4. **Agar**	7000