Micropropagation of *Coleus edulis* Vatke (acc.230694)

M.Sc. Thesis

Belete Kebede Andarge

Jimma, Ethiopia

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Micropropagation of *Coleus edulis* Vatke (acc.230694)

BY: BELETE KEBEDE

Thesis submitted to the Department of Biology, School of graduate studies, College of Natural Science, Jimma University in Partial Fulfillment of the Requirement for the Degree of Master of Science in Biology (Botanical Sciences)

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STATEMENT OF AUTHOR

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Belete Kebede Andarge

E-mail address: beletekebede2005@yahoo.com

</div>

School of Graduate Studies, Jimma University

As Thesis Research advisor, I hereby certify that I have read and evaluated this thesis prepared, under my guidance, by Belete Kebede Andarge entitled Micropropagation of *Coleus edulis* and recommend it to be submitted as fulfilling the thesis requirement.

<u>Balcha Abera (Ph.D.)</u>   _____

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As member of the Board of Examiners of the M.Sc. Thesis Open Defense, we certify that we have read and evaluated the thesis prepared by Belete Kebede Andarge entitled 'Protocol Development for micropropagation of *Coleus edulis* and examined the candidate. We recommend that the thesis be accepted as fulfilling the requirement for the Degree of Master of Science in Botanical Science. _____

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BIOGRAPHICAL SKETCH

Belete Kebede Andarge was born on 20th of March, 1980 in Chelenko, East Hararge (Oromia Regional State), Ethiopia. He attended his Elementary and Junior secondary schools at Chelenko Elementary and Junior Secondary School from 1990 -1998. He pursued his secondary school education at Dire Dawa Comprehensive Secondary School from 1999 – 2002, in Dire Dawa town. After completing high school, he joined former Ambo College of Agriculture (now Ambo University) in 2003, and graduated in July, 2004 with Diploma in Biology Laboratory Technician. Soon after graduation, he was employed by Oromia Education Bureau as Biology Laboratory Technician in Jimma Teachers Training College, Southwestern Ethiopia, in Jimma Zone. Since then, he has been working first as a Biology Laboratory Technician for five years and now as a Biology Teacher at Jimma Teachers Training College. In 2012, he had joined the School of Graduate Studies, of the Jimma University, College of natural sciences as a candidate for Master of Science Degree in Botanical Science.

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LIST OF ABBREVIATIONS

ANOVA   Analysis of variance

BAP   6-Benzylamino purine

B5 medium   Gamborg medium

CRD   Completely Randomized Design

IBA   Indole 3-butyric acid

MS medium   Murashige and Skoog

NAA   Naphthaleneacetic acid

PGRs   Plant Growth Regulators

REGWQ   Ryan, Elinot, Gabriel, and Welsh model

ACKNOWLEDGMENT

ABSTRACT

Coleus edulis Vatke belongs to the family Lamiaceae which occurs both as a wild and cultivated species. The Major constraints in the cultivation of C. edulis through the conventional propagation methods are its low productivity due to Shortage of planting materials and a long maturation period, incidence of pests and diseases. Therefore, the objective of this study was to develop protocol of micropropagation that enables multiplication of C. edulis. Explants were sterilized using, different concentrations of NaOCL for different times of exposure. MS medium supplemented with different types and concentrations of auxin and cytokinins were used for culture initiation, shoot multiplication and root induction through nodal and shoot tip culture. Sodium hypochlorite (NaOCl) at a concentration of one percent (1 %) and exposure time of 5 minutes gave the highest percentages (74.50±0.50) of clean culture for nodal and 69.83±0.76 from shoot tip. BAP at 1.5 mg/l was found to be an optimum concentration for shoot induction, yielding 91.67±0.58 for nodal and 85.57±0.51 for shoot tip explants, respectively. The combination of 2.0 mg/l BAP with 1.0 mg/l NAA was found to be the optimum concentration yielding 10.28±0.06 and 6.12±0.01 shoots per explants for nodal and shoot tip respectively for shoot multiplication. Half strength MS medium with 2.0 mg/l IBA and 1.0 mg/l NAA gave the highest rooting percentage with optimum root number and length. Up on acclimatization and transplanting, 85 % survival efficiency was observed on soil mix ratio of 2:1:1 decomposed coffee husk, forest soil and sand respectively. There were no observable variations with respect to morphology and growth characteristics to the greenhouse raised parent plant. The results obtained in this study permit the development of mass propagation protocol that could enable large scale commercial production of this highly demanded Coleus edulis cultivar true-to type and provide a possible system towards genetic improvement of the crop using nodal as well as shoot tip explant sources. One advantage of micropropagation is production of virus free plantlets; in the future studies on virus indexing should also be given attention and incorporated.

Key words: *Explant, Micropropagation, nodal culture, Microshoots, Plant growth regulators, Plantlet*

CHAPTER ONE

1. INTRODUCTION

1.1. Background

Coleus edulis Vatke is said to have originated in Ethiopia (Greenway, 1944; Ryding, 2000), and is one of the traditional tuber crops indigenous to Ethiopia (Asfaw & Woldu, 1997; ENBSAP, 2005). *C. edulis* is an under-utilized and neglected tuber crop, belongs to the family Lamiaceae (Mekbib, 2007). The genus Coleus consists of over 350 tuber-bearing and non-tuber bearing species distributed predominantly in Africa, Asia and Australia (Codd, 1985). In the various growing areas of Ethiopia, different vernacular names are used. Among these are '*Dinicha oromoo*' in Oromia Regional State, meaning "potato of the Oromo people" (Abdissa, 2000), 'Wolaita *Dinich*' (potato of the wolayita people) around wolaita (Endale, 1997), 'Agew *Dinich*', (potato of the Agew people) in the northwest and '*Gurage Dinich*' (potato of the gurage people) around the Gurage zone (Westphal, 1975).

C. edulis Vatke is an indigenous annual tuber crop grown widely in mid and high altitude areas ranging from 1880 to 2200 meters above sea level in the central, southern, western, northwestern and southwestern parts of Ethiopia (Uphof, 1968; Westphal, 1975; Zeven Zhukovsky, 1975; PGRC/E, 1986; Edward, 1991; Edossa, 1996; Abdissa, 2000; GRIN, 2005). It is primarily cultivated on small scale basis for its tubers. These tubers are cooked and consumed where; the leaves are used as a cooked vegetable in some western Ethiopian regions (Westphal, 1975; Asfaw and Woldu, 1997; Taye *et al*., 2007).

There is a number of edible *Coleus* spp. reported from Ethiopia and is clearly an important cultigen in the southwest (Lemordant, 1971). *C. edulis* have starchy tubers and are cultivated for food in tropical Africa and Asia. It is an important root crop in local diets, cultural, and socioeconomic life of the people (Mekbib and Weibull, 2012). *C. edulis has* a long history of local usage, and is important to the cultural, social and economic life of households (Demissie, 1998). It is particularly important in local diets mainly between September and November since

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other food crops will not be ready for consumption. It is also reported that it has no impact on the stomach whatever amount is consumed (Mekbib and Weibull, 2012).

C. edulis is propagated vegetatively by tubers, and the tuber for planting obtained from individually selected tubers of the previous harvest or bought in the market. Preparation of land usually commenced around the month of January and involves a range of cultivation practices. Planting of the tubers takes place from March to April to take advantage of available moisture. The tubers are often grown in home gardens next to other crops that were grown for daily uses (Mekbib and Weibull, 2012).

Though the above species can be multiplied by tubers and stem cuttings, but the conventional propagation of this species is beset with problems of shortage of planting material, long maturation period, pests and diseases. These problems pose serious challenges to conservation efforts of this species. Therefore, to alleviate the above mentioned problems there is need to develop alternative propagation methods for this species. *In vitro* propagation methods offer a powerful tool for conservation programs of this species and to regenerate a large number of plants.

One of the most important application of tissue culture as a tool of biotechnology is its application in the production of disease free plant materials. Moreover, it enables production of large number of plantlets in short period of time as well as conservation of germplasm under controlled conditions in small spaces and with reduced labor requirement (Abraham, 2009). Therefore; the main purpose of this research is to develop a protocol for micropropagation of *c. edulis* (acc.230694).

1.2. OBJECTIVES

1.2.1. General Objective:

To develop micropropagation protocol for *Coleus edulis*

1.2.2. Specific Objectives:

➢ To determine the duration of exposure of explants to different sodium hypochlorite (NaOCl) concentrations for sterilization
➢ To determine the effect of different level of BAP and Kinetin on culture initiation of explants.
➢ To determine the effects of different concentrations of BAP and NAA on multiplication of shoots.
➢ To test the effect of different concentrations IBA and NAA on root induction
➢ To determine the percentage of plantlets survived after acclimatization.

CHAPTER TWO

2. LITERATURE REVIEW

2.1 Botanical Description of *Coleus edulis*

Coleus edulis is a tuber crop plant belongs to the family Lamiaceae, subfamily Ocimodeae and genus *Coleus*. The genus consists over 350 tuber bearing and non-tuber bearing species mainly distributed in Africa, Asia and Australia (Codd.1985). There is a number of edible *Coleus* spp. reported from Ethiopia and *is* an important cultigen in the southwest (Lemordant, 1971)**.** *C. edulis* occurs both as wild and cultivated species. The genus Coleus has about 30 wild species in Ethiopia. The cultivated species is grown in the wetter south and south western of Ethiopia whereas the wild species are found throughout the country (IBCR, 2007).

C. edulis is an annual plant, it has square stems, branched, and the nodes are often hairy. The leaves are usually pubescent, narrow into petioles. The flowers born on racemes are perfect, calyx is fine toothed and deflexed in front. The plant 0.5 m tall with thick tubers, the leaves and tubers have quite different odors, but quite different from gingers. The roots are tubers fasciculate up to 20 cm long and 0.5 to 2.5 cm thick. Coleus requires red sandy loam soils with pH 5.5 to 7.0 and temperature of 10^0c to 25^0C (Jalihal, 2009). Coleus is a small, hairy, rather succulent herb, with ovate and shallowly serrate leaves. The inflorescence is a raceme of small flowers which are usually purplish blue. The leaves vary from dark green to purplish green (Bekele, 2007).

2.2 Origin and Distribution of *Coleus edulis*

C. edulis is said to have originated in Ethiopia, and has been grown in different mid and high altitude areas ranging from 1880 to 2200 meters above sea level (Demissie, 1998; Greenway, 1944; Ryding, 2000). According to Siegenthaler (1963) *C. edulis* is a major highland tuber crop in the southwestern parts of Ethiopia, Westphal (1975) stated that *C. edulis* is an indigenous crop in the northern, southern and southwestern parts of Ethiopia.

2.3. Uses of *C. edulis*

2.3.1 Food values

 C. edulis has been cultivated for its edible tuber in different parts of the country, mainly in south and west and is a highland crop with the tubers of different shapes; color and size .It is one of the major sources of food and contributes significantly to household food security (Taye *et al*., 2006). According to the study carried out by EHNRI (1997) on the nutritional content of 100 gm edible portion of both raw and cooked tubers of *C. edulis* showed that it has ample amounts of macro and micro-nutrients. This study also revealed that the cooked tubers have more amounts of calorie, fiber and carbohydrate compared to the raw tuber. However, the raw tuber is richer in nitrogen, protein, calcium, phosphorous, iron and niacin than cooked ones.

2.4. Conventional Propagation of *C. edulis*

C. edulis is propagated vegetatively by tubers, and stem cuttings (Bekele, 2007) **.**The tuber for planting obtained from individually selected tubers of the previous harvest or bought in the market. Preparation of land usually started around the month of January and involves a range of cultivation practices. Planting of the tubers takes place from March to April to take advantage of available moisture .The tubers often grown in home gardens next to other crops that were grown for daily uses (Mekbib and Weibull, 2012).

2.5 Plant Tissue Culture and Its Application

Plant tissue culture is a collection of techniques used to maintain or grow plant cells, tissues or organs under sterile conditions on a nutrient culture medium of known composition. Plants cultured *in vitro* yield thousands of genetically identical plants (clones) from a single plant. This process is called micropropagation and is used to commercially propagate plants asexually (Bhojwani and Razdan, 1996). *In vitro* growth of plants is largely determined by the composition of the culture medium. The main components of most plant tissue culture media are mineral salts and sugar as carbon source and water. Other components may include organic supplements, growth regulators, a gelling agent, (Gamborg *et al*., 1968; Gamborg and Phillips**,** 1995). The amounts of the various ingredients in the medium vary for different stages of culture and plant

species; the basic MS (Murashige and Skoog, 1962) and LS (Linsmaier and Skoog, 1965) are the most widely used media.

There is no research that was conducted on *in vitro* propagation of *C. edulis*, but some reports of *in vitro* propagation from genus coleus are available in *Coleus forskohlii* (Reddy *et al*., 2001). Previous researches regarding the establishment of quick protocols for *Coleus Blumei* Blenth species multiplication include experiments using different kinds of inoculums: apical meristems on IAA medium (Smith and Murashige, 1982), leaves excerpts and knot segments on NAA and BA, (Gaurav *et al*., 2010), medium and knot segments (Rani *et al.*, 2006). Other studies focused on obtaining rosmarin acid in callus of *Coleus* spp. (Razzaque and Ellis, 1997; *Zenk et al*., 1977, Verpoorte *et al.*, 2002). Other studies were targeted to investigate the resistance of the species to the induced stress of several salts (Collins *et al*., 1990). *In vitro* Culture Studies on Medicinal Herb - *Coleus forskohlii* Briq (Praveena *et al.,* 2012), Micropropagation of *Coleus blumei* from nodal segments and shoot tips (Rani, 2006), *in vitro* micropropagation of *coleus blumei* benth species (Vasile *et al*., 2011) and Establishment of a rapid multiplication protocol of Coleus forskohlii Briq. and *in vitro* conservation by reduced growth (Pratibha *et al*., 2011).

2.5.1. Micropropagation

In vitro propagation of plants vegetatively by tissue culture to produce genetically similar copies of a cultivar is referred to as micropropagation (Rai, 2007). Micropropagation is the true to type propagation of selected genotype using *in vitro* culture techniques. This technique provides a rapid reliable system for production of large number of genetically uniform and disease free plantlets. Through this technology from a single shoot tip or axillary bud, a large quantity of uniform and disease free plants with good genetic potential can be produced within a short time (Vuylsteke and Langhe, 1985; Wong, 1986 and Akbar and Roy, 2006).

During the last thirty years, micropropagation and other *in vitro* techniques have become more widely used in commercial horticulture and agriculture for the mass propagation of crop plants and for conservation of genetic resources, particularly with those crops which are vegetatively propagated or have recalcitrant seeds which cannot be stored under conventional seed bank conditions (George and Sherrington, 1984; Dodds, 1991 and George, 1993). Likewise, *in vitro*

culture is being used in an increasing number of botanic gardens for the propagation and conservation of wild plant species (Fay, 1992).

2.5.2 Stages of micropropagation
Stage 0 Selection and Maintenance of Stock plants for culture initiation

The pre-propagation stage (also called stage 0) requires proper maintenance of the mother plants in the greenhouse under disease- and insect-free conditions with minimal dust. Collection of plant material for clonal propagation should be done after appropriate pretreatment of the mother plants with fungicides and pesticides to minimize contamination in the *in vitro* cultures. This improves growth and multiplication rates of *in vitro* cultures. The control of contamination begins with the pretreatment of the donor plants. They may be prescreened for diseases, isolated and treated to reduce contamination (George, 1993; Holdgate and Zandvoort, 1997).

A small tissue excised from any part of the plant is called explant which is the starting point. It can be initiated from any part of plant- root, stem, petiole, axillary shoots, shoot buds, nodal segments, leaf or flower, choice of explant varies with species. Meristems are more responsive and give better success as they are actively dividing. The physiological state of the plant also has an influence on its response to initiate tissue culture. Therefore, the parent plant must be healthy and free from obvious signs of disease or decay (Rai, 2007).

In some plants, leaf discs, intercalary meristems from nodes, small pieces of stems, immature zygotic embryos and nucellus have also been used as explants to initiate cultures. The apical meristem maintains itself, gives rise to new tissues and organs, and communicates signals to the rest of the plant (Medford, 1992). Shoot-tips and meristem-tips are perhaps the most popular source of explants to initiate tissue cultures. The physiological state of the plant also has an influence on its response to initiate tissue culture. Therefore, the parent plant must be healthy and free from obvious signs of disease or decay (Rai, 2007).

Stage I: Shoot Culture Initiation

This stage refers to the inoculation of the explants on sterile medium to initiate aseptic culture. Initiation of explants is the very first step in micropropagation. A good clean explant, once established in an aseptic condition, can be multiplied several times; hence, explant initiation in

an aseptic condition should be regarded as a critical step in micropropagation. More than often, explants fail to establish and grow, not due to the lack of a suitable medium but because of contamination. The explants are transferred to *in vitro* environment, free from microbial contaminants. The process requires excision of tiny plant pieces and their surface sterilization with chemicals such as sodium hypochlorite, ethyl alcohol and repeated washing with sterile distilled water for callus and plantlet before and after treatment with chemicals. After a short period of culture, usually 3 to 5 days, the contaminated explants are discarded. The surviving explants showing growth are maintained and used for further subculture ((Hartmann *et al*., 2002).

In certain *Coleus* species*, C. forskohlii* (Sen and Sharma, 1991) and *C. parviflorus* (Ponsamuel *et al*., 1994), BA alone was sufficient for shoot initiation from nodal segments and shoot tips. Similarly Hiregoudar *et al*., (2005) also reported that addition of BA (2 mg/l) alone to MS medium is responsible for shoot induction and multiplication in *in vitro* culture of hypocotyl and internodal explants of *Feronia limonia.*

However, in certain other previous studies BA and NAA were found to be useful in shoot induction from nodal segments and shoot tip explants of various other plants (Bhattacharya and Bhattacharya 1997; George and Ravishankar 1997*;* Manjula *et al*., 1997; Kannan and Jasrai 1998; Anand *et al*., 1999 and Bringmann *et al*., 1999). Similar combinations of plant hormones were also found to be responsible initiation in leaf discs of *C. blumei* (Ibrahim *et al*., 1992).

Stage II: Shoot multiplication

Shoot multiplication is a crucial step for *in vitro* clonal propagation of virus free plantlets Effective explants are sub cultured on to a fresh medium. The time and concentration of auxins and cytokinins in multiplication medium is an important factor affecting the extent of multiplication. In tobacco, high level of cytokinins initiates bud formation while high concentration of auxin favors rooting (Skoog and Miller, 1957). Shoot multiplication could also be optimized using combinations of different cytokines.

Hundred percent shoot induction from both explants was achieved on the medium containing BA (2 mg/l) and NAA (1 mg/l). Shoot tips were proved to be the better explant in comparison to nodal segments in having high rate of shoot induction and more number of shoots (Rani *et al*., 2006).

In some other studies, kinetin and IAA were also found to be suitable for shoot induction and multiplication from nodal segments and shoot tips of *C. forskohlii* (Sharma *et al.,* 1991, Bhattacharya and Bhattacharya, 2001). Zagrajski *et al.,* (1997) studied the shoot induction from nodal, internodal and leaf explants of *C. blumei* Benth with the use of BA alone or in combination with IAA. They reported that nodal explants were best for shoot induction and multiplication while internodal and leaf explants did not give consistent results.

Stage III: Root Induction

The *in vitro* shoots obtained at Stage II are rooted to produce complete plantlets and plants. If the proliferated material consists of bud-like structures (e.g. orchids) or clumps of shoots (banana, pineapple), they should be separated after rooting and not before. Many plants (e.g. banana, pineapple, roses, potato, chrysanthemum, strawberry, mint, several grasses and many more) can be rooted on half-strength-MS (Murashige and Skoog, 1962).

When used in low concentration, auxins induce root initiation and in high, callus formation occurs. Commonly used synthetic auxins are 1-naphthaleneacetic acid (NAA), 2, 4 –D dichlorophenoxyacetic acid (2, 4-D), indole-3 acetic acid (IAA), indole butyric acid (IBA) etc. Both IBA and IAA are photosensitive so the stock solutions must be stored in the dark. 2, 4-D is used to induce callus and regulate somatic embryogenesis (Rai, 2007). IBA at 2 mg /l is found to be best treatment for induction of roots (Rani *et al*., 2006). Similar findings on some other plants, *e.g. Elaeagnus angustifolia* (Iriondo *et al*., 1995), *Asparagus robustus* (Nayak and Sen 1998), *Eucalyptus tereticornis* (Sharma and Ramamurthy ,2000) and *Hemidesmus indicus* (Sreekumar *et al.,* 2000).

Stage IV: Acclimatization

The period of transition during the process of hardening after transfer from the *in vitro* to *ex vitro* environment is considered to be the most important step in plant tissue culture (Faisal *et al*., 2007).At this stage, the *in vitro* micropropagated plants are weaned and hardened. This is the final stage of the tissue culture operation after which the micropropagated plantlets are ready for transfer to the greenhouse. Steps are taken to grow individual plantlets capable of carrying out photosynthesis. The hardening of the tissue-cultured plantlets is done gradually from high to low humidity and from low light intensity to high intensity conditions. If grown on solid medium, most of the agar can be removed gently by rinsing with water. Plants can be left in shade for 15 to 30 days where diffused natural light conditions them to the new environment. The plants are then transferred to an appropriate substrate (sand, peat, compost, etc.), and gradually hardened (Thangavel, 2008).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Plant material

Healthy tubers of *C. edulis* were obtained from the Institute of Biodiversity Conservation (IBC), located in Addis Ababa (the capital city of Ethiopia). The tubers were planted in a tin pot contained a sterilized soil that had a mixture of loom soil, coffee husk and sand at a ratio of (2:1:1 respectively), kept and grown under greenhouse condition of the College of Natural Science , Jimma University until used for experiment. The growing plant materials were daily watered with tap water and also sprayed with 0.3 % Mancozeb at 15 days interval to control fungal infection. Very young, healthy and vigorous plantlets were used as a source of explants (Figure 1).

Figure 1: Mother stock plants under greenhouse condition green house

3.2 Growth regulators stock preparation

Different growth regulators was prepared by weighing and dissolving the powder in double distilled water at the ratio of 1mg/ml including 6-benzyl amino purine (BAP), Kinetin ,α-naphthalene acetic acid (NAA), and indol-3-butyric acid (IBA). The dissolving process was carried out by adding 3-4 drops of 1N NaOH and 1N HCl based on the requirement of the growth regulators (NaOH for auxin, HCl for cytokinin). Then, the volume was adjusted by adding double distilled water. Finally, growth regulators stock solutions were stored in a refrigerator at a temperature of $+4^{o}C$.

3.3 Culture medium preparation

Prepared MS powder with vitamins $(4405.19gl^{-1}$ conc.) was weighed and dissolved, followed by adding sucrose to the double distilled water. The final volume was adjusted in a graduated cylinder/beaker by adding double distilled water. The pH of the solution was adjusted to 5.7-5.8 using 0.1 N HCl or 1N KOH. For solidification of the medium, agar powder (Tissue culture grade; agar-agar type) 0.8% (w/v) was added to the moderately warm solution and then, melted for proper dissolving. After the medium was dissolved very well, it was poured in glass vessels borosilicate test tubes. After plugging, the test tubes were covered with aluminum foil and autoclaved at $121^{0}C$ for 20 minutes in a vertical autoclave.

3.4 Tissue Culture Experiments

3.4.1 Experiment 1: Explant Preparation and sterilization

Young and juvenile explants were taken from *C. edulis* maintained in greenhouse .Both Shoot tip explants (1-1.5cm long) and nodal explants (1.5-2cm long) were excised and washed three times with tap water and detergent using sponge. Then after, the explants were kept under running water for 10 minutes and finally washed with double distilled water.

3.4.2. Experiment 2: Culture initiation

Sterilized explants were cultured on agar solidified (0.8% agar-agar). MS basal medium of full strength added with 3% sucrose was supplemented with different concentrations of BAP and Kinetin. The experiment was carried out with a treatment of four different concentrations of BAP (0, 0.5, 1.0, 1.5, and 2.0 mg/l) and three different concentrations of Kinetin (0, 1.0, 2.0, and 3.0

mg/l) to be tested for shoot induction rate of shoot tip and nodal explants in CRD design in 4x3x2 factorial combinations.

3.4.3. Experiment 3: Shoot multiplication

Shoot buds derived from those explants that had responded well to the prevailing culture conditions were transferred singly onto a shoot multiplication medium. In this experiment, the shoot multiplication media used were composed of MS basal medium added with 30g/l sucrose and different concentrations of BAP (0, 1.0, 1.5,2, and 2.5 mg/l) and interacting at each concentration with (0, 1, 2, and 3.0 mg/l) NAA). The experiment was thus being arranged in a 4x3x2 factorial in CRD. After two-three weeks, cultures proliferating shoot clumps were divided and sub cultured on to a fresh medium of similar composition.

3.4.4. Experiment 4: Root induction

Well-developed micro shoots obtained from shoot multiplication were transferred for rooting. For rooting experiment, agar solidified (0.8% agar-agar) half strength MS basal medium added with 3% sucrose and different concentrations of IBA, and NAA. Therefore, the experiment was laid with treatment of five concentrations for IBA (0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) and three concentrations for NAA (0, 0.5,1, and 1.5 mg/l) auxins in CRD in 5 x 3 factorial combination.

3.4.5 Experiment 5: Acclimatization

Plantlets from rooting media were isolated, washed and then treated in light polyethylene pot covered by shade net above it. The system was designed to give high humidity and to prevent desiccation for 15 days, prior to their transfer to a shade house. In the pot, the water was sprayed daily without creating water logging and meant to maintain relative humidity (RH) as high as possible. Starting from the 15th day, the RH within the system was reduced gradually at the end of the month. After the month, the plantlets were transferred to another shade net, where they were retained for a month. Later they were transferred to shade and maintained for three weeks. The numbers of survived plantlets were recorded in each step.

3.5 Experimental design and treatments

All experiments were laid in a Completely Randomized Design (CRD) with factorial treatment combinations, having three replications per treatment and five explants per jar under each replicate. All the experiments were repeated two times to ensure reproducibility of the results and the average of these two were considered for analysis. Prior to laying the multiplication and rooting experiments, sufficient explants were made to multiply till the desired numbers of explants are gained. At all times, explants were cultured on a PGR-free medium prior to their use for an experiment; so as to avoid any sort of carryover effects from the previous culture medium they were retained. Controls were set for each experiment with zero concentration of the analyte considered.

3.5.1 Data recording

After sterilization experiment, the number and percentage of explants affected by contamination and tissue death was recorded during the first two weeks of culture for shoot tip and nodal explants independently. For the second experiment, the number and/or percentage of explants forming shoot buds was recorded after four weeks for shoot tip and nodal explants independently. Number of shoots proliferated from each shoot bud on multiplication media was counted at three weeks interval during sub culturing. The number of roots (including the main roots and their branches), shoot length and the length of the roots was recorded after three weeks of culture for experiment four.

3.5.2 Data analysis

Average of the data collected from the two repetitions for each experiment were independently subjected to statistical analysis using the SAS statistical software (version 9.2) and ANOVA was constructed, followed by mean separation using appropriate procedures (REGWQ). When ANOVA indicated significant treatment effects (5%, 1% or 0.1%) based on the F-test, probability level of 0.05 ($p \leq 0.05$) was considered to determine which treatments were statistically different from the other treatments.

<p style="text-align:center;">**CHAPTER FOUR**</p>

4. RESULTS AND DISCUSSION

Results obtained from experiments on micro propagation of *C. edulis* shoot tip and nodal explants namely; sterilization, shoot initiation, shoot multiplication, and rooting of micro shoots and acclimatization of plantlets are presented and discussed in the following sections.

4.1 Effects of NaOCl concentrations and exposure time on sterilization of *C. edulis* explants

The interactive effect of different concentrations (amount of active chlorine) of sodium chlorite solutions and different time durations of explants exposure to the sterilants were evaluated for determining the most effective treatment combination on sterilization of shoot tip and nodal explants excised from greenhouse grown *C. edulis* plants.

The analysis of variance showed that the concentration of active chlorine in sodium hypochlorite solution, time duration of explants exposure to the sterilants and interaction of concentrations to time duration had very highly significant effect ($P < 0.0001$) on both of contamination and clean culture of shoot tip and nodal explants(appendix Ia-d). Very highly significant difference had also been revealed between the two types of explants (treatment * explants = $P < 0.0001$) indicating that the level of contamination and clean culture was influenced by the concentration of NaOCl and duration of exposure time and the mean average value for contamination and clean culture of node exceeded shoot tip.

The highest rate of clean culture 69.83±0.76 and 62.43±0.51 was obtained from treatment concentration of 1% active chlorine in NaOCl solution and five and seven minute exposure duration, respectively for shoot tip explants. For nodal explants 1% active chlorine and five minute exposure duration was found to be the most effective treatment combination with mean average result of 74.50±0.50 clean culture (Table 1).

<p style="text-align:center;">15</p>

Table 1: Interaction effect of Sodium hypochlorite concentrations and its time of exposure on sterilization off shoot tip and nodal explants of *C. edulis*.

Conc NaOCl	Duration (min)	% Shoot contamination		% Clean culture		% Tissue Death	
		Nodal	Shoot tip	Nodal	Shoot tip	Nodal	Shot tip
		(Mean ±Std Dev)	(Mean ±Std Dev)	(Mean ±Std Dev)	Mean ±Std Dev	(Mean ±Std Dev)	Mean ±Std Dev
0.5	3	89.76 ± 0.68^a	87.73 ± 0.64^a	10.24 ± 0.65^k	12.27 ± 0.63^i	0.00 ± 0.00^n	0.00 ± 0.00^l
0.5	5	79.83 ± 0.76^b	75.00 ± 0.50^b	17.17 ± 1.26^j	22.16 ± 0.76^g	2.50 ± 0.50^m	3.40 ± 0.52^k
0.5	7	68.40 ± 0.52^c	65.83 ± 0.76^c	25.60 ± 0.36^h	29.00 ± 1.00^f	5.76 ± 0.68^l	5.76 ± 0.68^j
0.5	9	59.76 ± 0.68^d	57.73 ± 0.64^d	32.24 ± 0.74^g	34.27 ± 0.86^e	8.40 ± 0.52^{kl}	8.50 ± 0.50^i
1	3	49.93 ± 0.90^e	48.76 ± 0.68^e	40.07 ± 1.10^f	42.00 ± 1.00^d	10.60 ± 0.52^k	9.40 ± 0.52^i
1	5	15.83 ± 0.76^f	20.83 ± 0.76^f	74.00 ± 0.40^a	69.16 ± 1.89^a	11.83 ± 0.76^k	10.43 ± 0.51^i
1	7	13.90 ± 0.85^g	18.60 ± 0.52^g	60.10 ± 1.15^b	62.00 ± 1.00^b	21.43 ± 0.51^j	19.73 ± 0.64^h
1	9	12.40 ± 0.52^g	16.43 ± 0.51^h	54.00 ± 0.80^c	53.57 ± 0.51^c	29.73 ± 0.64^i	29.76 ± 0.68^g
1.5	3	10.66 ± 0.76^h	$13..83\pm0.76^i$	51.34 ± 1.32^d	40.00 ± 1.00^d	37.83 ± 0.76^h	47.73 ± 0.64^f
1.5	5	10.60 ± 0.52^h	10.60 ± 0.52^j	43.40 ± 1.96^e	29.40 ± 1.21^f	55.90 ± 0.85^g	59.83 ± 0.76^e
1.5	7	$8.40\pm0.52i$	8.40 ± 0.52^k	20.00 ± 1.00^i	20.00 ± 0.30^{gh}	69.76 ± 0.68^f	72.50 ± 0.50^d
1.5	9	6.43 ± 0.51^j	6.43 ± 0.51^l	18.33 ± 1.04^{ij}	17.57 ± 1.24^h	76.50 ± 0.50^e	75.76 ± 0.68^c
2	3	5.60 ± 0.52^j	5.83 ± 0.76^l	9.40 ± 0.76^k	10.00 ± 0.50^j	85.83 ± 0.76^d	75.43 ± 0.51^c
2	5	$3..83\pm0.76^k$	2.60 ± 0.52^m	7.17 ± 0.64^l	$7.40\pm0.52j^k$	89.76 ± 0.68^c	89.73 ± 0.64^b
2	7	2.60 ± 0.52^k	2.50 ± 0.50	5.40 ± 0.50^l	$5.63\pm0.40k^l$	92.60 ± 0.52^b	92.40 ± 0.52^b
2	9	00.00 ± 0.00^l	0.00 ± 0.00^n	2.00 ± 0.20^m	3.00 ± 0.70^l	98.73 ± 0.64^a	96.40 ± 0.52^a
CV		2.37	2.18	3.15	3.90	2.05	1.77

Means with the same letters in a column are not significantly different from each other by Ryan - Einot - Gabriel - Welsch Multiple Range Test (REGWQ) at α= 5 %

As it was shown clearly in the above Table 1, the highest percentage of contamination from nodal and shoot tip explants were observed, this is due to the low concentration NaOCl (0.5%) at short exposure of time (three and five minute) for both types of explants tissue death is very low and contamination is highest. In the higher concentration of sodium hypochlorite (2% NaOCl) and long exposure of time (seven and nine minute) percentage of contamination is very low but tissue death is highest.

Highest percentage of clean culture from shoot tip explants 69.83±0.76 % was obtained on one percent (1%) NaOCl for five minutes and for nodal 74.50±0.50 % clean culture was obtained and highest tissue death was observed by 2% at nine minute for both types of explants.

In the present study, one percent of sodium hypochlorite within five minute exposure of time is optimum for sterilization of *C. edulis* nodal and shoots tip explants (Table 1, Figure 2A). Similar results were reported by Yanagihava *et al*. (1996) for another Coleus species of *Coleus forskohlii* recommended that young shoot segments were surface sterilized with two per cent sodium hypochlorite for 10 minutes. Different scholars use mercuric chloride for explant surface sterilization (Bhattacharyya and Bhattacharya, 2000; Vasile *et al*., 2011; Rani *et al*., 2006) but using mercuric chloride for surface sterilization is not environmentally friendly so that it is not recommendable.
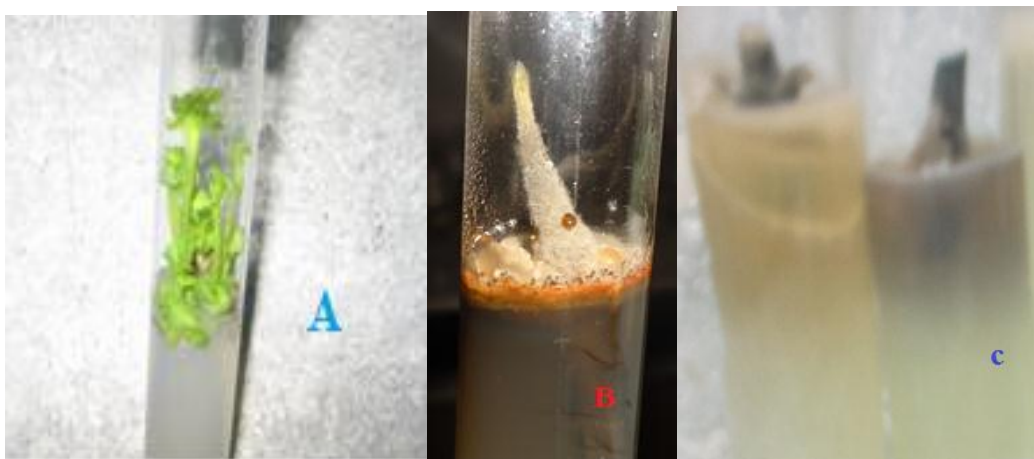
Figure 2: Response of cultures to different concentrations of NaOCl, (A) 1% sodium hypochlorite concentration and 5 minute exposure time results for clean culture (B) 0.5% sodium hypochlorite concentration and three minute exposure time results for contamination (C) 2% sodium hypochlorite concentration and nine minute exposure time results for tissue death.

4.2 Effect of different concentration and combination of BAP and Kinetin on shoot initiation of *C. edulis*

Aseptic shoot tips and nodal cultures were transferred on to MS media fortified with different concentrations of BAP in combination with kinetin for four weeks to determine optimum medium for shoot induction of *C. edulis.*

The analysis of variance obtained from the data (appendix II) showed that the interaction with BAP and type of explant had very highly significant effect (P < 0.0001) on the shoot induction rate. Interaction effect of explants type with BAP on rate of shoot induction was found to be highly significant (BAP*Explant).The response of shoot tip and nodal explants to a given concentration of BAP was not the same that, the nodal explants gave greater response than shoot tip explants (Table 2).

The highest rate of shoot induction 91.67±0.58 was achieved on an MS medium supplemented with 1.5 mg /l BAP from nodal and 85.57±0.51 on 1.5 mg /l BAP from shoot tip on MS media (Table 2). For both shoot tip and nodal explants, MS basal media added with 1.5 mg /l BAP used alone were found to be optimum media for *in vitro* shoot initiation of *C. edulis* (Fig. 3a and c). From all given treatments the minimum rate of shoot induction was observed on MS medium containing 2.0 mg /l BAP and 3.0 mg /l Kinetin 53.37±0.54 and 50.40±1.21 for nodal and shoots tip explants respectively.

Table 2 Effects of different concentrations of BAP and Kinetin alone and in combination on shoot induction of *C. edulis* from shoot tip and nodal explants

CONC. OF PGRS		Explant	
		Nodal	Shoot tip
BAP[mg/l]	Kin[mg /l]	Mean±STD	Mean±STD
0	0	10.56 ± 0.51^l	9.60 ± 0.52^o
0	1	40.92 ± 0.88^k	35.62 ± 1.07^n
0	2	41.87 ± 0.26^{jk}	40.86 ± 1.02^m
0	3	43.62 ± 0.67^j	42.65 ± 0.56^m
0.5	0	81.25 ± 0.90^c	74.32 ± 0.58^c
0.5	1	53.08 ± 0.88^i	50.18 ± 1.04^l
0.5	2	57.57 ± 0.38^h	53.72 ± 0.62^k
0.5	3	58.33 ± 0.32^h	57.66 ± 0.57^j
1	0	85.50 ± 0.50^b	77.76 ± 0.67^b
1	1	61.58 ± 0.51^g	60.58 ± 1.41^{hi}
1	2	64.78 ± 1.07^f	62.35 ± 1.52^{hg}
1	3	66.67 ± 1.15^f	63.56 ± 1.25^{fg}
1.5	0	91.67 ± 0.58^a	85.57 ± 0.51^a
1.5	1	70.68 ± 1.00^e	66.00 ± 0.99^{ef}
1.5	2	$71.47\pm1.04d^e$	67.38 ± 1.19^e
1.5	3	73.20 ± 1.05^d	70.56 ± 1.39^d
2	0	66.63 ± 0.54^f	64.58 ± 1.00^{fg}
2	1	61.25 ± 1.56^g	59.68 ± 0.58^{ij}
2	2	56.65 ± 0.56^h	54.65 ± 0.56^k
2	3	53.37 ± 0.54^i	50.40 ± 1.21^l
CV		1.34	1.70

Means within a column followed by the same letters are not statically significant at p< 0.01 *by Ryan - Einot - Gabriel - Welsch Multiple Range Test (REGWQ)*

In combination of BAP and kinetin the highest percentage of induction were observed at 1.5 mg/l BAP and 3.0 mg /l kinetin 73.20±1.05 and 70.56±1.39 for both nodal and shoots tip explants, respectively (Table 2). From the given concentrations, high concentration of BAP and kinetin and a medium with free growth regulator resulted in low percentage of shoot induction. Therefore, BAP proved a more effective than Kinetin for multiple shoot induction of *C. edulis*.

The present result was in accordance with the result of Vasile *et al*. (2006) achieved high regeneration shoot induction from *C. blumei* Benth, using 1.5 mg /l of BAP. Similar result was also reported by Pratibha *et al*. (2011) BAP 1.5 mg/l was found to be optimum. High BAP concentration decreased the shoot production either by inhibition of shoot initiation or by encouraging callusing (Fig. 3.B).
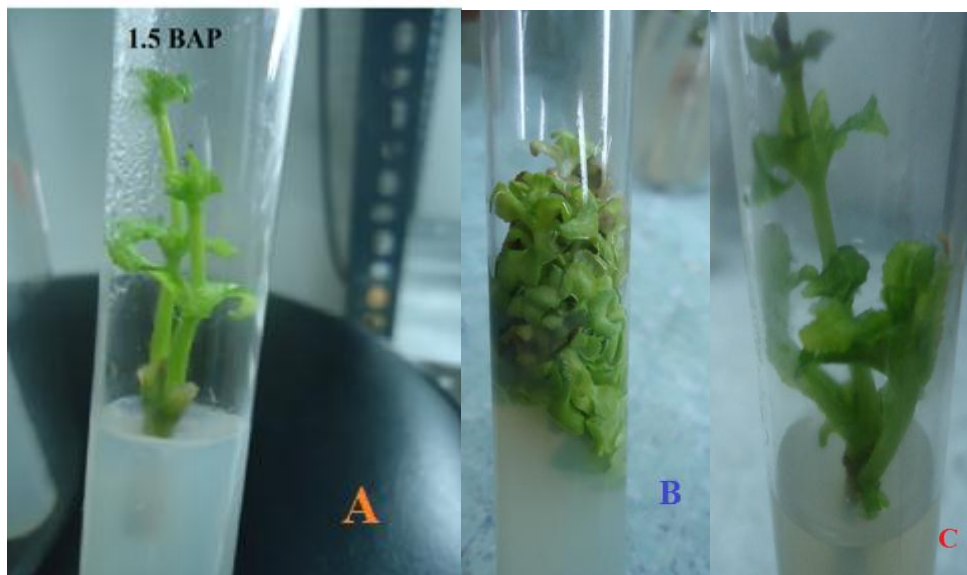
Figure 3: Effect of BAP on Shoot induction, (A) Shoot induction on 1.5 mg/l of BAP (B) unnecessary callus formation at the base of explant and (c) shoot induced on 1.5 mg/l BAP

4.3 Effect of different concentration and combination of BAP and NAA on shoot multiplication of *C. edulis*

Those shoot buds induced well on the prevailing shoot induction medium were transferred to MS media supplemented with BAP (1.0-2.5 mg/l) alone and in combination with (1.0-3.0 mg/l) NAA. Cultures were sub cultured twice and the effect of hormones on *in vitro* shoot multiplication of *C. edulis* cultivar was evaluated.

In this study, the significance of BAP and its interaction with NAA were considered. The ANOVA revealed that the concentration of BAP both alone and in combination with NAA had very highly significant effect (P < 0.0001) on shoot multiplication rate. Shoot buds raised from nodal explants responded exceeds shoot tip shoot multiplication and this indicating the significant effect of explants at this stage.

Table 3 Effects of different concentrations and combinations of BAP and NAA treatments on shoot multiplication of *C. edulis*.

Levels of PGR		Nodal		Shoot tip	
BAP (mg/l)	NAA (mg/l)	Shoot Number Mean ± Std Dev	Shoot Length Mean ± Std Dev	Shoot Number Mean ± Std Dev	Shoot Length Mean ± Std Dev
0	0	2.41±0.01[kl]	2.12±0.06[i]	2.49±0.07[gh]	1.99±0.01[e]
0	1	2.70±0.10	2.96±0.07[hi]	2.78±0.05[e]	2.02±0.02[e]
0	2	2.68±0.02[fgh]	2.15±0.03[hi]	2.51±0.01[gh]	2.07±0.01[de]
0	3	2.88±0.07[d]	2.25±0.06g[hi]	2.85±0.02[cde]	2.13±0.02[de]
1	0	2.54±0.06[ijk]	2.97±0.03[c]	2.43±0.03[hij]	2.07±0.06[de]
1	1	2.38±0.04[l]	2.56±0.06[e]	2.65±0.02[f]	2.62±0.10[c]
1	2	2.62±0.01	2.19±0.04[ghi]	2.19±0.04[m]	2.80±0.10[bc]
1	3	2.52±0.06[ijk]	2.75±0.07[d]	2.31±0.01[kl]	2.19±0.08[de]
1.5	0	2.85±0.01[de]	2.48±0.06[ef]	2.80±0.04[de]	2.91±0.06[b]
1.5	1	2.55±0.01[hijk]	2.51±0.04e	2.35±0.02[jkl]	2.75±0.12[bc]
1.5	2	2.46±0.06[jkl]	3.18±0.05[b]	2.53±0.03[gh]	2.70±0.03[c]
1.5	3	2.57±0.01[ghij]	2.14±0.04[hi]	2.91±0.01[c]	2.15±0.04[de]
2	0	3.35±0.05[b]	2.30±0.10[gh]	3.58±0.03[b]	2.27±0.06[d]
2	1	10.28±0.06[a]	4.51±0.04[a]	6.12±0.01[a]	3.45±0.09[a]
2	2	3.23±0.05[c]	1.70±0.05[j]	2.60±0.02[gf]	2.92±0.02[b]
2	3	3.18±0.06[c]	2.21±0.04[ghi]	2.90±0.05[cd]	2.10±0.07[de]
2.5	0	2.71±0.04[f]	2.35±0.02[fg]	2.25±0.06[ml]	2.03±0.02[e]
2.5	1	2.52±0.02[ijk]	2.28±0.11[ghi]	2.45±0.05[hij]	2.17±0.09[de]
2.5	2	2.75±0.02[ef]	2.52±0.04[e]	2.57±0.04[gf]	2.15±0.08[de]
2.5	3	2.45±0.04[jkl]	2.89±0.05[cd]	2.39±0.02[ijk]	2.20±0.09[de]
CV		1.58	2.36	1.38	2.98

Means within a column followed by the same letters are not statically significant at p< 0.01 *by Ryan - Einot - Gabriel - Welsch Multiple Range Test (REGWQ)*

In this study, maximum number of shoot multiplication 10.28±0.06 and 6.12±0.01 was obtained on MS medium containing 2.0 mg/l BAP and 1.0 mg/l NAA from nodal and shoot tip explants respectively (Table 3). Similar results were reported by Rani *et al.* (2006). Some of the previous studies, where BA and NAA were found to be useful in shoot induction from nodal segments and shoot tip explants of various other plants, *e.g. Jasminum officinale* (Bhattacharya and Bhattacharya 1997), *Vanilla planifolia* (George and Ravishankar, 1997), *Aristolochia indica* (Manjula *et al.*, 1997), *Vitex negundo* (Kannan and Jasrai, 1998), *Syzygium travancoricum* (Anand *et al.,* 1999) and *Ancistrocladus abbreviatus* (Bringmann *et al.,* 1999). However, in certain other *Coleus* species*, C. forskohlii* (Sen and Sharma 1991) and *C. parviflorus (*Ponsamuel *et al.,* 1994), BA (2.0 mg/l) alone was sufficient for formation of multiple shoots from nodal segments and shoot tips. Similarly Hiregoudar *et al.* (2005) and Velmurugan *et al*. (2009) also reported that addition of BA (2.0 mg/l) alone to MS medium is responsible for shoot induction.

In the present study, among all the combinations and concentrations, the longest shoots 4.51±0.04 cm and 3.45±0.09 cm were observed on the medium containing 2.0 mg/l BAP with 1.0 mg/l NAA for both nodal and shoot tip explants, respectively. A medium free growth regulators and a medium with high concentration of BAP alone and in combination with NAA resulted in low multiplication rate (Table 3). Length of shoots that were obtained from nodal explants was longer than observed from shoot tip explants.
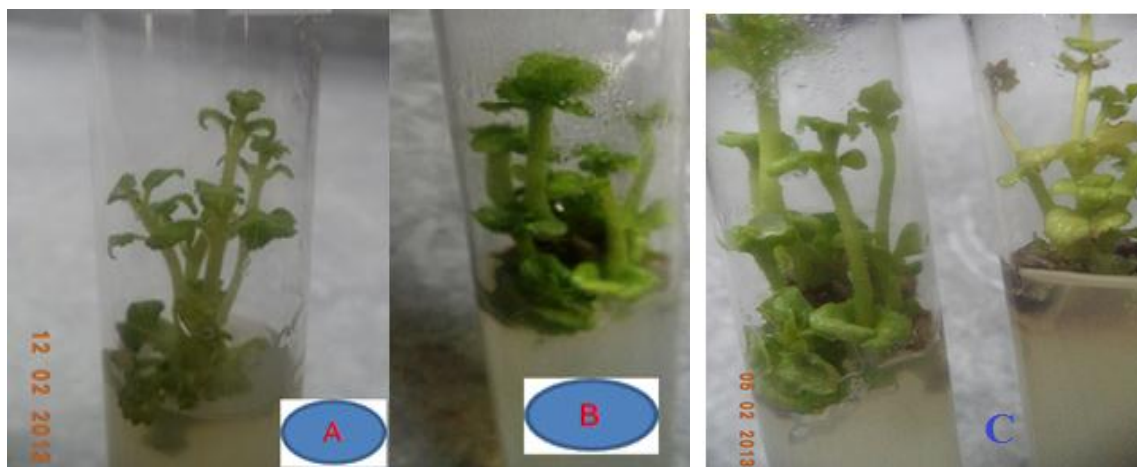
Figure 4: *In vitro* shoot multiplication of *C. edulis,* (a, b and c) Shoots multiplied on 2.0 mg/l of BAP and 1.0 mg/l NAA.

4.4 Effect of different concentrations of IBA, and NAA for *in vitro* root initiation of *C. edulis*

Table 4: Effect of various concentrations of IBA and NAA on rooting of shoots of *C. edulis* cultured on half - strength MS medium.

Conc. of PGRs (µM)		Rooting Percent (Mean ± SD)	Shoot height (cm) (Mean ± SD)	Root number (Mean ± SD)	Root length(cm) (Mean ± SD)
IBA(mg/l)	NAA(mg/l)				
0	0	49.71 ± 0.47^n	2.70 ± 0.26^h	4.60 ± 0.10^m	1.07 ± 0.04^{jk}
0	0.5	70.95 ± 0.39^f	3.17 ± 0.22^{fgh}	8.30 ± 0.43^{gh}	1.45 ± 0.09^{ghi}
0	1	85.25 ± 0.97^c	3.32 ± 0.24^{fg}	19.32 ± 0.71^e	1.59 ± 0.20^{fgh}
0	1.5	51.17 ± 0.67^{mn}	2.71 ± 0.14^h	$4.83 \pm 0.20l^m$	1.52 ± 0.17^{ghi}
0.5	0	59.32 ± 0.88^i	3.48 ± 0.19^{ef}	7.80 ± 0.26^{ghi}	2.11 ± 0.14^{cde}
1	0	84.39 ± 0.61^c	4.65 ± 0.30^{bcd}	26.07 ± 0.55^c	2.15 ± 0.08^{bcd}
1.5	0	90.76 ± 1.11^b	4.90 ± 0.18^b	30.57 ± 0.24^b	2.47 ± 0.04^b
2	0	97.00 ± 0.28^a	5.90 ± 0.09^a	32.73 ± 0.14^a	2.95 ± 0.08^a
2.5	0	68.55 ± 0.71^g	2.60 ± 0.07^i	17.37 ± 0.01^f	1.75 ± 0.01^{efg}
CV		0.99	5.43	2.90	6.73

(±). Means within a column followed by the same letters are not statically significant at **α**= 5 % by Ryan - Einot - Gabriel - Welsch Multiple Range Test (REGWQ).

The highest rooting percentage (97.00± 0.28 %) was obtained on half - strength MS medium at 2.0 mg/l of IBA followed by 85.25±0.97 at 1.0 mg/l of NAA (Table 4). Among the given concentrations, auxins with higher concentration resulted in less rooting percentages. Naphthalene acetic acid (NAA) at a concentration of 1.5 mg/l resulted in less percentage of rooting (51.17±0.67) that was less than the root induced from all the rest at high and low concentration.

Regenerated shoots with average height of 2-4cm cultures were sub cultured on a medium prepared for rooting purpose on half-strength MS medium of the above different auxins at different concentrations. The longest shoots 5.90±0.09 cm was obtained from a medium that contained 2.0 mg/l 1BA followed by 3.32±0.24cm from 1.0 mg/l of NAA. Smallest shoot height 2.00±0.23cm were obtained from 2.5 mg/l of IBA.

The highest mean number 32.73±0.14 of roots were obtained from 2.0 mg/l of IBA followed by 19.32±0.71 on 1.0 mg/l NAA. Highest concentrations of auxins resulted in less number of root. Relatively, less number of roots 2.60±0.07 was obtained from IBA at 2.5 mg/l (Table 4). Similar results were reported by Rani *et al*. (2006) observed that half-MS with 2 mg/l IBA was found to be the best treatment for induction of roots. Root induction decreased with increase in concentration of IBA. NAA resulted in comparatively lesser number of roots.
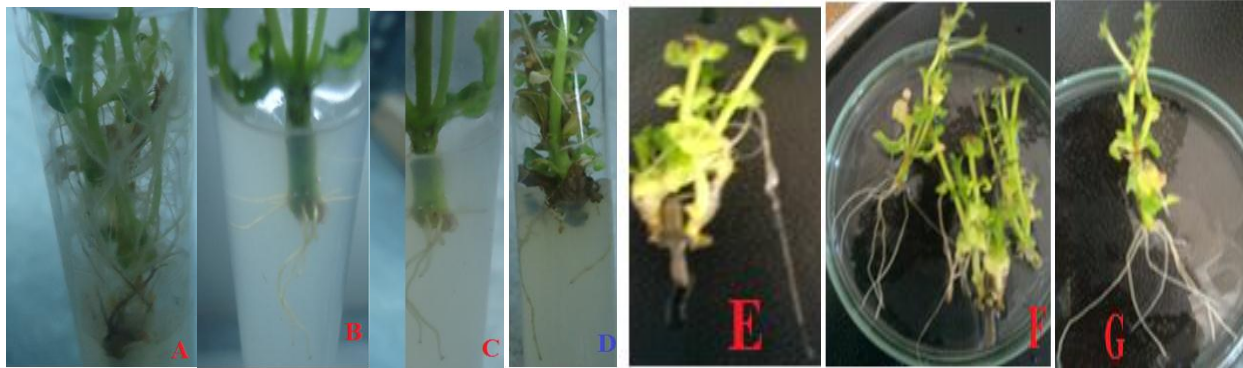
Figure 5: Root induced on different auxins (a-c) rooting on half - strength medium with 2 mg/l IBA; (d) rooting on half - strength medium with 1.0 mg/l NAA, (e-g) plantlets ready to transfer on sterile soil mix.

In this study, half - strength MS medium supplemented with IBA (0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) and NAA (0.5, 1.0 and, 1.5 mg/l) were evaluated and relatively 2.O mg/l and 1.0 mg/l IBA and NAA respectively gave good rooting percentage. The concentrations beyond these led to a decrease in the number of roots and root length per rooted explant and rooting rate. Similar findings on some other plants, *e.g. Elaeagnus angustifolia* (Iriondo *et al.* 1995), *Asparagus robustus* (Nayak and Sen 1998), *Eucalyptus tereticornis* (Sharma and Ramamurthy ,2000) and *Hemidesmus indicus* (Sreekumar *et al.* 2000). The root elongation phase is very sensitive to auxin concentration, and it is inhibited by high concentration of auxin in the rooting medium. Daffala *et al*. (2011) reported that roots may require a less concentration of auxin to grow, but root growth is strongly inhibited by its higher level because at this level, auxin induces the production of ethylene, a root growth inhibitor.

4.5 Acclimatization in *vitro* derived *C. edulis* plantlet

The establishment of *in vitro* plantlets under different environmental conditions was greatly affected in terms of survival percentage of plantlets. In the present study, employing the acclimatization procedure indicated in the methodology, the plantlets showed 85% survival efficiency. The plantlets transferred under net house conditions resulted in the best establishment, and all the regenerated plants exhibit normal morphology with respect to growth habit, leaf shape, flower shape and size when compared with the mother plant (Fig. 6).

Figure 6: Acclimatization on 2:1:1 top soil, coffee husk and sand soil mix (a) plantlets under polystyrene plastic tube and (b) plantlets on polyethylene tray (c) four weeks old seedling of *C. edulis*

CHAPTER FIVE

5. CONCLUSION AND RECOMMENDATIONS

In the course of micropropagation, it is essential to make use of all the steps that are indispensable to mass propagate *Coleus edulis*. The first of these is sterilization of selected explants, which is a key step for removing any sorts of microbial contaminants from the explant surface; thereby attain successful *in vitro* establishment that is the basis to subsequent growth and development of the tissues.

From different concentration of sterilant used for sterilizing *Coleus edulis* nodal and shoot tip explants one percent sodium hypochlorite with five minute exposures of time was optimum for nodal and shoot tip explants . So that optimum sterilants' concentration and exposure of time is crucial for micropropagation of *Coleus edulis*.

The combined effects of different concentrations of BAP, as a source of cytokinin, together with several concentrations of kinetin were evaluated for enhancing *Coleus edulis* shoot induction. The use of MS basal medium containing 1.5mg/l for nodal and for shoot tip explants was found to be the best giving rise to 91.67±0.58 and 85.57±0.51 shoots per explant respectively on the average after three weeks of culture.

For mass propagation of *Coleus edulis*, multiplication protocol development is indispensible. To do this BAP and NAA combination of the two at concentration of (2.0 and 1.0mg/l) for both nodal and shoot tip were optimized in this study.

The present study is of significant importance in developing a rapid method for multiple shoot proliferation along with efficient root development, and successful plant development, as well. To this end, the availability of this protocol is so pertinent to step up *Coleus edulis* production in Ethiopia. In this research, an efficient protocol for the micropropagation of a commercial *Coleus edulis* genotype was developed.

The present finding could also be of paramount importance as it avoids the need for using the imported, hazardous and costly mercuric chloride solution for sterilizing explants. Instead, it had

devised effective ways for using the locally available bleach, Sodium hypochlorite (*Berekina)*, at a rate of 1% active chlorine.

❖ One percent concentration of NaOCl solution for five minute exposure time were found to be optimum treatment combinations for sterilization of shoot tip and nodal explants of *Coleus edulis*.

❖ The maximum percentage of shoot induction (91.67±0.58) and (85.57±0.51) was observed on an MS medium supplemented with 1.5mg/l BAP from nodal and shoot tip explants respectively.

❖ For both nodal and shoot tip explants, MS basal media containing 1.5 mg/l BAP were found to be optimum media for *in vitro* shoot initiation of *Coleus edulis* .

❖ When BAP concentration increased, the shoot regeneration frequency was also increased up to optimal level and further increasing concentration of BAP decreased the shoot regeneration frequency.

❖ The number of shoots also increased with the increase in the concentration of BAP in the medium up to optimal level.

❖ Nodal explants gave best shoot induction response than shoot tip.

❖ BAP combined with Kinetin resulted in very low shoot induction.

❖ MS basal medium supplemented with 2.0 mg/l BAP and 1.0 mg/l NAA resulted in 10.28±0.06 shoot number with best and vigor morphological appearance.

❖ Best rooting percentage was achieved on half strength MS basal media containing 2.0 mg/l IBA which resulted mean values of 97.00± 0.28 with 32.73±0.14 root number, followed by half strength MS basal media containing 1 mg/l NAA which resulted 85.25±0.97 with 19.32±0.71 root number, 1.59±0.20 cm root length and 3.32±0. 24 shoot length.

❖ Those plantlets well performed *in vitro* showed 85% survival efficiency after hardening and acclimatization on soil mix ratio of 2:1:1 decomposed coffee husk, top forest soil and sand respectively.

❖ There were no observable variations with respect to morphology and growth characteristics to the greenhouse raised parent plant.

❖ Thus, this protocol could be useful for large-scale production of highly demanded *Coleus edulis* cultivar true-to-type and provide a possible system towards genetic improvement of the crop using nodal as well as shoot tip explants sources.

RECOMMENDATIONS

❖ For surface sterilization sodium hypochlorite was the best. Shoot explants may not able to tolerate others.

❖ Applying the protocol developed to address the current development needs with regard to expansion of *Coleus edulis* plantations in the country, thereby responding to the overwhelming demands of the producers.

❖ One advantage of micropropagation is production of virus free plantlets so; in the future studies on virus should also be given attention.

❖ *In vitro* multiplication of *Coleus edulis* plants can be tried with other explant and culture media for high rate of multiplication.

❖ Callus development in leaf base and tuber explants can be tried by using other growth regulators in different concentrations and combinations.

❖ Proper hardening procedure can be tried to achieve maximum field establishment in *Coleus edulis* plantlets raised through *in vitro* technique.

❖ Field performance of tissue culture raised *Coleus edulis* plantlets can be tried during off season *i.e.* when *Coleus edulis* tubers are in dormant condition.

REFERENCES

Abdissa, G. (2000). Root and tuber crops as compliments to sustainable livelihood of the farm family in the west Ethiopia .*AgriTopia, Quarterly Newsletter of* EARO. Addis Ababa, Ethiopia.**15**: 2-3.

Abraham, A. (2009). A review on Agricultural biotechnology research and development in Ethiopia. *African Journal of Biotechnology*, **8**: 7196-7204.

Akbar, A.M. and Roy, K.S. (2006). Effect of Liquid medium on rooting and acclimation of regenerated microshoots of Banana (*Musa sapientum* L.) cv. Sagar. *Plant Tiss. Cult. and Biotech.*, **16**(1): 11-18.

Amsalu, N. and Tesfaye, A. (2004). Exploration and collection of root and tuber crops in southwestern Ethiopia: Its Implication for Conservation research .In: Asfaw, Z., , Hailu, T., Gemechu ,G., Amsalu ,A., Tesfaye ,T.,Solomon, A., Dessalegn ,D. and Belay ,S.(eds.) /Proceeding of the Eleventh conference of the Crop Sciences Society of Ethiopia ,26-28 April 2004, Addis Ababa ,Ethiopia.

Anand, A., Rao, C.S. and Balakrishna, P. (1999) .In *vitro* propagation of *Syzygium travancoricum* Gamble – an endangered tree species. *Plant Cell, Tissue and Organ Culture*, **56**: 59-63.

Asfaw, Z. and Woldu, Z. (1997). Crop associations of home-gardens in Wolaita and Gurage in Southern Ethiopia. SINET. *Ethiopian J. Sci*., **20**:73-90.

Bekele, E. (2007). Study on Actual Situation of Medicinal Plants in Ethiopia.

Bhattacharya, S. and Bhattacharya, S. (1997). Rapid multiplication of *Jasminum officinale* L. by *in vitro* culture of nodal explants. *Plant Cell, Tissue and Organ Culture*, **51**: 57-60.

Bhattacharya, R. and Bhattacharya, S. (2001). *In vitro* multiplication of *Coleus forskohlii* Briq. An approach towards shortening the protocol. *In vitro Cell. Dev. Biol. Plant*, **37**: 572-575.

Bhojwani, S. S. and Razdan, M. K. (1996). Plant tissue culture: theory and practic*e.* Developments in Crop Science, Vol. 5. Elsevier, Amsterdam.

Bringmann, G., Rischer, H., Schlauer, J., and Aké, A. L. (1999). *In vitro* propagation of *Ancistrocladus abbreviatus* Airy Shaw (Ancistrocladaceae). *Plant Cell, Tissue and Organ Culture,* **57:**71-73.

Codd, L.E. (1985). Flora of Southern Africa: Lamiaceae. Botanical Research Institute, Pretoria. 28:4.

Collins, J.C., Ibrahim, K.M. and Collin, H.A. (1990). *In vitro* Selection for Salt Tolerance in *Coleus Blumei*. *Acta Hort*., **280**:431-434.

Daffalla, H.H., Abdellatef, E., Elhadi, E.A. and Khalafalla, M.M. (2011). Effect of growth regulators on *in vitro* morphogenic response of *Boscia senegalensis* (Pers.) Lam. Poir. using mature zygotic embryos explants. *Biotech. Res. Int.,* **10:** 1–8

Demissie, A. (1988). Potentially valuable crop plants in a Vavilovian center of diversity: Ethiopia. Pp. 89-98 in Crop Genetic Resources of Africa. Proceedings of the international conference on crop genetic resources of Africa, Nairobi (Kenya), 26-30 Sep 1988. Edited by F. Attere, H. Zedan, N.Q. Ng & P. Perrino. International Board for Plant Genetic Resources, Rome.

Dodds, J.H. (1991). In *vitro methods for conservation of plant genetic resources*. Chapman and Hall, London.

Edossa, E. (1996). Root and tuber crops: Potential as food crops in the humid areas of Ethiopia. IAR Newsletter of Agricultural Research 2(1).

Edwards, S. (1991). Crops with wild relatives found in Ethiopia. In: Engles, J.M.M.; Hawkes, J.G. and Melaku, W. (eds.).*Plant Genetics Resource of Ethiopia*. Cambridge.42-74.

EHNRI (Ethiopian Health and Nutrition Research Institute). (1997). Food composition table for use in Ethiopia, part III. Ethiopian Health and Nutrition Research Institute, Addis Ababa.

ENBSAP. (2005). *Ethiopian National Biodiversity Strategy and Action Plan*. Institute of Biodiversity Conservation, Addis Ababa.

Endale, T. (1997). Morphological characterization of enset (*Ensete ventricosum* (welw) Cheesman) clones and the association of yield with different traits. M.Sc. Thesis, Alemaya University of Agriculture. Alemaya, Ethiopia.

Faisal, M., Ahmad, N. and Anis, M. (2007). An efficient micropropagation system for *Tylophora indica*: an endangered, medicinally important plant. *Plant Biotech. Rep*., **1**: 155–161.

Fay, M.F. (1992). Conservation of rare and endangered plants using *in vitro* methods. *In vitro Cell. Dev. Biol Pl*., **28**: 1-4.

Gamborg, O.L., Miller, R.A. and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res*, **50:**151-158.

Gamborg, O.L. and Phillips, G.C. (1995). Media preparation and handling. In: Plant Cell, Tissue and Organ Culture - Fundamental Methods. Gamborg, O.L. and G.C. Phillips (Eds.). Springer-Verlag, Berlin. 21-34.

Gaurav, K., Reddy, P. S., Nair, N. A., Ramteke, P. W. and Bhattacharya, P. S. (2010). *In vitro* direct shoot regeneration from proximal, middle and distal segment of *Coleus forskohlii* leaf explants, *Physiology and Molecular Biology of Plants*,**16(2):**195-200.

George, E.F. (1993). Plant propagation by tissue culture. Part 1. The technology. Exegetics Ltd., Basingstoke, UK.

George, E.F. and Sherrington, P.D. (1984). *Plant propagation by tissue culture. Handbook and directory of commercial laboratories*. Exegetics Ltd., Basingstoke, UK.

George, P.S. and Ravishankar, G.A. (1997). *In vitro* multiplication of *Vanilla planifolia* using axillary bud explants. *Plant Cell Rep.*, **16**: 490-494.

Greenway, P. J. (1944). Origins of some of East African food plants (Part 1). *East African Agricultural Journal,* **10**:34–39.

GRIN (Germplasm Resource Information Network). (2005). National genetic resource program. National germplasm resource laboratory, Beltssoille, Maryland USDA, ARS. [http://www.ars-grin.gov 2/cgi-bin/npgs/html/taxon.].

Hartmann, H.T., Kester, D.E., Davies, F.T., and Geneve, R.L. (2002). Cell and tissue culture propagation In: *Plant Propagation Principles and practices* .7th Edition. Prentice Hall. New Jersey,pp.367-374.

Hiregoudar, L.V., Kumar, H.G.A., Murthy, H.N. (2005). *In vitro* culture of *Feronia limonia* (L.) Swingle from hypocotyls and internodal explants. *Biol. Plant*., **49**: 41-45.

Holdgate, D.P and Zandvoort, E.A. (1997). Strategic considerations for the establishment of microorganism free tissue cultures for commercial ornamental micropropagation. In: Pathogen and Microbial Contamination Management in Micropropagation. Cassells, A.C. (Ed.) Kluwer Acad. Pub. Dordrecht. 15-22.

IBCR. (2007). Institute for Biodiversity Conservation and Research (Now called IBC). Ethiopia: Second country Report on the State of Plant Genetic resources for Food and Agriculture to FAO.

Ibrahim, K.M., Collins, J.C. and Collin, H.A. (1992). Characterization of progeny of *Coleus blumei* following an *in vitro* selection for salt tolerance. *Plant Cell, Tissue and Organ Culture*, **28**: 139-145.

Iriondo, J.M., De la Iglesia, M. and Perez, C. (1995). Micropropagation of *Elaeagnus angustifolia* from mature trees. - *Tree Physiol*., **15**: 691-693.

Jalihal, S. (2009). Contract farming in medicinal plants– a case of coleus in Karnataka.

Kannan, R. and Jasrai, Y.T. (1998). Micropropagation of medicinal plant – *Vitex negundo. - J. med. arom. Plant Sci*., **20**: 693-696.

Lemordant, D. (1971). Contribution a L'Ethnobotanique Ethiopienne. *Journal d'Agriculture Traditionelle et de Botanique Appliquée*, **18**:1-35.

Linsmaier, E.M. and Skoog, F. (1965). Organic growth factor requirements of tobacco tissue cultures. *Physiol. Plant*., **18**: 100-127.

Manjula, S., Thomas, A., Daniel, B., Nair and G.M. (1997). *In vitro* plant regeneration of *Aristolochia indica* through axillary shoots multiplication and organogenesis. *Plant Cell Tissue Organ Cult*. , **51**: 145-148.

Medford, J.I. (1992). Vegetative apical meristems. *Plant Cell*, **4:** 1029-1039.

Mekbib, Y. (2007). Phenotypic variation and local customary use of Ethiopian potato (*Plectranthus edulis* (Vatke) Agnew.

Mekbib, Y. and Weibull, J. (2012). Local Customary Use and Management of Ethiopian Potato (*Plectranthus edulis* (Vatke) Agnew in Sodo Zuria District, South Ethiopia.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*, **15**: 473-497.

Nayak, S. and Sen, S. (1998). Regeneration of *Asparagus robustus Hort. - J. Herbs Spices med. Plants*, **5**: 43-50.

PGRC/E (Plant Genetic Resource Center/Ethiopia). (1986). Ten Years of Collection, Conservation and Utilization (1976-1986). Addis Ababa.

Ponsamuel, J., Samson, N.P. and Anderson, K.P. (1994). *In vitro* clonal propagation of country potato – an underexploited tuber plant. - *In vitro,* **30**:71.

Pratibha, D., Gangopadhyay, M., Dewanje, S. and Ali, N. (2011). Establishment of a rapid multiplication protocol of Coleus forskohlii Briq. and *in vitro* conservation by reduced growth. *Indian Journal of Biotechnology*, **10**:228-231.

Praveena, R., Pandian, S.A. and Jegadeesan, M. (2012). *In vitro* Culture Studies on Medicinal Herb - *Coleus forskohlii* Briq.

Rai, R. (2007). Introduction to plant tissue culture, National Research Centre on Plant Biotechnology. New Delhi. 1-44.

Rani, G., Talwar, D., Nagpal, A. and Virk, G.S. (2006). Micropropagation of *Coleus blumei* from nodal segments and shoot tips.

Reddy, P.S., Rodrigues, R. and Rajasekharan, R. (2001).Shoot organogenesis and mass propagation of *Coleus forskohlii* from leaf derived callus. *Plant Cell, Tissue and Organ Culture,* **66**: 183-188.

Ryding, O. (2000). A new species of *Plectranthus* (Lambiaceae) from Ethiopia and Kenya. *Nordic Journal of Botany,* **20**:43–46.

Razzaque, A. and Ellis, B.E. (1997).Rosmarinic acid production in Coleus cell cultures. *Planta* **137**:287–291.

Sen, J. and Sharma, A.K. (1991). *In vitro* propagation of *Coleus forskohlii* Briq. For forskolin synthesis. *Plant Cell Rep.*, **9**: 696-698.

Sharma, N., Chandel, K.P.S. and Srivastava, V.K. (1991). *In vitro* propagation of *Coleus forskohlii* Briq, a threatened medicinal plant. *Plant Cell Rep.*, **10**: 67-70.

Sharma, S.K. and Ramamurthy, V. (2000).Micropropagation of 4-year-old elite *Eucalyptus tereticornis* trees. *Plant Cell Rep*, **19**: 511- 518.

Siegenthaler, I. E. (1963).Useful plants of Ethiopia. *Experiment Station Bulletin* 1(14*)*. Alemaya: Imperial Ethiopian College of Agricultural and Mechanical Arts.

Skoog, F. and Miller, C. O. (1957).Chemical regulation of growth and organ formation in plant tissue cultures *in vitro*. Symp. *Soc. Exp. Biol.*, **11**:118–131.

Smith, R.H. and Murashige, T. (1982). Primordial leaf and phytohormone effects on excised shoot apical meristems of *Coleus blumei* Benth. - *Amer. J. Bot.*, **69**: 1334-1339.

Sreekumar, S., Seeni, S. and Pushpangadan, P. (2000). Micropropagation of *Hemidesmus indicus* for cultivation and production of 2- hydroxy-4-methoxybenzaldehyde. - *Plant Cell Tissue Organ Culture*, **62**: 211-218.

Sujatha, G. and Ranjithakumari, B.D. (2007). High frequency shoots multiplication in *Artemisia vulgaris* L. using thidiazuron. *Plant Biotech. Rep.*, **1**: 149-154.

Taye, M., Lommen, W.J.M., and Struik P. C. (2006). Indigenous cultivation practices of *Plectranthus edulis*. Experimental Agriculture, Cambridge. In Press

Taye, M., Lommen W.J.M., and Struik P.C. (2007).Indigenous multiplication and production practices for the tuber crop *Plectranthus edulis* in Chencha and Wolaita, southern Ethiopia. *Experimental Agriculture*, **43**:381-400.

Thangavel, P. (2008). *In vitro* Propagation of *Plectranthus barbatus* Andrews – A Valuable Medicinal Plant in the Palni Hills of the Western Ghats, South India.

Uphof, J.C.T.H. (1968). *Dictionary of economic plants*. 2nd edition.

Vasile, L., Ioana, V. S., Zapârtan, M., and Agud, E. (2011)**.** *In vitro* micropropagation of *coleus blumei* benth species.

Velmurugan, M., Rajamani, K., Paramaguru, P., Gnanam, R., Babu, J.R.K. and Harisudan, C. (2009).Standardization of protocol for direct organogenesis of medicinal coleus (*Coleus forskohlii* Briq.) *J. Swamy Bot. Club,* **26(1):** 22-22.

Verpoorte, R., Contin, A., and Memelink, J. (2002). Biotechnology for the production of plant secondary metabolites. Phytochem. Rev. 1, pp. 13–25

Vuylsteke, D. and De Langhe, E.A.L. (1985). Feasibility of *in vitro* propagation of bananas and plantains. *Tropical Agri*., **62**: 323-328.

Westphal, E. (1975). Agricultural Systems in Ethiopia. Agricultural Research Reports 826. Centre for Agricultural Publishing and Documentation, Wageningen, Netherlands.

Wong, W.C. (1986). *In vitro* propagation of banana (*Musa* spp): Initiation, proliferation and development of shoot tip cultures on defined media. *Plant Cell, Tiss. and Org Cult.*, **6**: 159-166.

Yanagihava, H., Sakota, R., Shoyama, Y. and MurakamI, H. (1996). Rapid analysis of small samples containing forskolin monoclonal antibodies. *Planta Medicine*, **62**(2): 169-172.

Zagrajski, N., Leljak-Levanić, D., and Jelaska, S. (1997). Organogenesis and callogenesis in nodal, internodal and leaf explants of *Coleus blumei* Benth. - *Periodicum Biol.*, **99**: 67-76.

Zenk, M.H., El-Shagi, H. and Ulbrich, B. (1977). Production of rosmarinic acid by cell suspension cultures of *Coleus blumei*. Naturwissenschaften 64, pp. 585–586.

Zeven, A.C.and Zhukovsky, P. (1975). *Dictionary of cultivated plants and Their Center of Diversity.* Center for Agricultural Publishing and Documentation, Wageningen.

APPENDICES

I. Sterilization experiment SAS result

Summary of analysis of variance for the effect of concentration of Sodium hypochlorite (NaOCl 1%) and duration of explants exposure to different concentration of NaOCl on contamination percent of explants after a week.

Appendix I: Summary of analysis of variance for the interaction effect of NaOCl with Exposure time on percentage of contamination of *Coleus edulis* shoots tip and nodal explants

Appendix I a: Summary of ANOVA table for the interaction effect of NaOCl with Exposure time on percentage of clean culture from nodal explant of *C. edulis.*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Na	3	16684.89695	5561.63232	6454.29	<.0001**
Dur	3	598.54195	199.51398	231.54	<.0001**
Na*Dur	9	4617.96810	513.10757	595.46	<.0001**

R - square = 0.99 CV % = 3.15, CCL mean =29.37, ** = highly significant at α = 5 %, DF = degree of freedom, CCL = clean culture

*= shows the interaction effects of Sodium Hypochlorite (NaOCl) with exposure time.

Appendix I b: Summary of ANOVA table for the interaction effect of NaOCl with Exposure time on percentage of clean culture from shoot tip of *C. edulis.*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Na	3	15571.98729	5190.66243	4173.15	<.0001**
Dur	3	249.05116	83.01705	66.74	<.0001**
Na*Dur	9	2806.68512	311.85390	250.72	<.0001**

R - square = 0.99 CV % = 3.90, CCL mean =28.59, ** = highly significant at a = 5 %, %, DF = degree of freedom, CCL = clean culture

*= shows the interaction effects of Sodium Hypochlorite (NaOCl) with exposure time.

Appendix I c: Summary of ANOVA table for the interaction effect of NaOCl with Exposure time on percentage of contamination for nodal explant of *C. edulis*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Na	3	37976.67729	12658.89243	30021.1	<.0001**
Dur	3	2532.57063	844.19021	2002.03	<.0001**
Na*Dur	9	2017.27354	224.14150	531.56	<.0001**

R - square = 0.99 CV % = 2.37, CONFN mean =27.37, ** = highly significant at a = 5 %, DF = degree of freedom, CONFN = contamination for nodal

*= shows the interaction effects of Sodium Hypochlorite (NaOCl) with exposure time.

Appendix I d: Summary of ANOVA table for the interaction effect of NaOCl with Exposure time on percentage of contamination for shoot tip explant of *C. edulis*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Na	3	34400.71617	11466.90539	31647.2	<.0001**
Dur	3	2413.01617	804.33872	2219.87	<.0001**
Na*Dur	9	1312.26052	145.50672	402.41	<.0001**

R - square = 0.99 CV % = 2.18, CONFS mean =27.55, ** = highly significant at a = 5 %, %, DF = degree of freedom, CONFS = contamination for shoot

*= shows the interaction effects of Sodium Hypochlorite (NaOCl) with exposure time.

Appendix I e: Summary of ANOVA table for the interaction effect of NaOCl with Exposure time on percentage of tissue death from nodal explant of *C. edulis.*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Na	3	55802.246555	18600.748885	22920.1	<.0001**
Dur	3	3020.88305	1006.96102	1240679	<.0001**
Na*Dur	9	1230.09900	136.67767	168.42	<.0001**

R - square = 0.99 CV % = 2.05, TDZ mean =43.89, ** = highly significant at α = 5 %, DF = degree of freedom, TDZ = tissue death

*= shows the interaction effects of Sodium Hypochlorite (NaOCl) with exposure time.

Appendix I f: Summary of ANOVA table for the interaction effect of NaOCl with Exposure time on percentage of tissue death   from shoot tip explant of *C. edulis.*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Na	3	55787.27707	18595.75902	31403.1	<.0001**
Dur	3	2754.43867	918.14622	1550.50	<.0001**
Na*Dur	9	684.97417	76.10824	128.53	<.0001**

R - square = 0.99 CV % = 1.77, TDZ mean =43.25, ** = highly significant at α = 5 %, DF = degree of freedom, TDZ = tissue death

*= shows the interaction effects of Sodium Hypochlorite (NaOCl) with exposure time.

II. Shoot initiation experiment SAS result

Appendix II: Effect of different levels of BAP and kinetin on shoot induction of nodal and shoot tip *Coleus edulis* cultured on MS medium after three weeks.

Appendix II a: Summary of ANOVA table for the interaction effect of BAP with Kinetin on percentage of shoot initiation   from nodal explant of *C. edulis*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BAP	4	12441.38988	3110.34747	4676.01	<.0001**
KIN	3	899.03109	299.67703	450.53	<.0001**
BAP*KIN	12	5073.22279	422.76857	635.58	<.0001**

R - square = 0.99 CV % = 1.34, mean of shoot initiation =60.49, ** = highly significant at α = 5 %, DF = degree of freedom, PERSHINN= % shoot initiation, explant = nodal and shoot tip explants

* = shows the interaction of the treatments, *** = highly significant at α = 5 %,

Appendix II b: Summary of ANOVA table for the interaction effect of BAP with Kinetin on percentage of shoot initiation from shoot tip explant of *C. edulis*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BAP	4	11253.66578	2813.41644	2944.14	<.0001**
KIN	3	545.28374	181.76125	190.21	<.0001**
BAP*KIN	12	4230.52204	352.54350	368.92	<.0001**

R - square = 0.99 CV % = 1.70 mean of shoot initiation =57.38, ** = highly significant at a = 5 %, DF = degree of freedom, PERSHINN= % shoot initiation, explant = nodal and shoot tip explants

* = shows the interaction of the treatments, *** = highly significant at α = 5 %,

III. Shoot multiplication experiment SAS result

Appendix III: shoot multiplication

Appendix IIIa: Summary of ANOVA table for the interaction effect of BAP with NAA on percentage of shoot number of from nodal explant of *C. edulis*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BAP	4	55.97932333	13.99483083	5851.50	<.0001**
NAA	3	20.22187333	6.740062444	2818.38	<.0001**
BAP*NAA	12	91.71161000	7.64263417	3195.53	<.0001**

R - square = 0.99 CV % = 1.58, mean of shoot number =3.08, ** = highly significant at α = 5 %, DF = degree of freedom, PERSHN= % of shoot number

* = shows the interaction of the treatments, *** = highly significant at α = 5 %,

Appendix III b: Summary of ANOVA table for the interaction effect of BAP with NAA on percentage of shoot length of from nodal explant of *C. edulis*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BAP	4	0.67340667	0.16835167	46.27	<.0001**
NAA	3	3.52193833	1.17397944	322.67	<.0001**
BAP*NAA	12	15.26908667	1.27242389	349.73	<.0001**

R - square = 0.99 CV % = 2.36, mean of shoot length =2.55
** = highly significant at α = 5 %, DF = degree of freedom, PERSHL= % of shoot length

* = shows the interaction of the treatments, *** = highly significant at α = 5 %

Appendix III c: Summary of ANOVA table for the interaction effect of BAP with NAA on percentage of shoot number of from shoot tip explant of *C. edulis*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BAP	4	16.25999333	4.06499833	2725.14	<.0001**
NAA	3	5.21523333	1.73841111	1165.42	<.0001**
BAP*NAA	12	19.26590000	1.60549167	1076.31	<.0001**

R - square = 0.99 CV % = 1.38, mean of shoot number percentage =2.78
** = highly significant at a = 5 %, DF = degree of freedom, PERSHN= % of shoot number

* = shows the interaction of the treatments, *** = highly significant at α = 5 %

Appendix III d: Summary ANOVA table for the interaction effect of BAP with NAA on percentage of shoot length from shoot tip explant of *C. edulis*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
BAP	4	3.86211000	0.96552750	190.82	<.0001**
NAA	3	2.07088500	0.69029500	136.42	<.0001**
BAP*NAA	12	3.54129000	0.29510750	58.32	<.0001**

R - square = 0.99 CV % = 2.98, mean of shoot length =2.38, ** = highly significant at α = 5 %, DF = degree of freedom, PERSHL= % of shoot length

* = shows the interaction of the treatments, *** = highly significant at α = 5 %

IV. Root induction experiment SAS result

Appendix IV: ANOVA table for the interaction effect of IBA, and NAA on percentage of rooting, root number, root length and shoot height of *C. edulis*

Appendix IV a: Summery of ANOVA table for the interaction effect of IBA, and NAA on percentage of rooting of *C. edulis*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
IBA	4	10217.59731	2554.39933	4916.91	<.0001***
NAA	3	110.65392	36.88464	71.00	<.0001***
IBA*NAA	12	1826.17248	152.18104	292.93	<.0001***

R - square = 0.99 CV % = 0.99, mean of rooting percentage =66.58, *** = highly significant at α = 5 %, DF = degree of freedom, PERR= percentage of rooting

Appendix IV b: ANOVA table for the interaction effect of IBA, and NAA on root number of *C. edulis*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
IBA	4	4088.939673	1022.234918	7373.04	<.0001***
NAA	3	35.744458	11.914819	85.94	<.0001***
IBA*NAA	12	1013.746567	84.478881	609.32	<.0001***

R - square = 0.99 CV % = 2.90, mean of root number= 12.83, *** = highly significant at α = 5 %, DF = degree of freedom, PERRTN= % of root number

* = shows the interaction of the treatments, *** = highly significant at α = 5 %

Appendix IV c: ANOVA table for the interaction effect of IBA, and NAA on root length of *C. edulis*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
IBA	3	10.07332667	2.51833167	173.46	<.0001***
NAA	3	1.91857833	0.63952611	44.05	<.0001***
IBA*NA A	9	2.90044667	0.24170389	16.65	<.0001***

R - square = 0.96 CV % = 6.73, mean of root length = 1.78, *** = highly significant at α = 5 %, DF = degree of freedom, PERRTL= % of root length

* = shows the interaction of the treatments, *** = highly significant at α = 5 %

Appendix IV d: ANOVA table for the interaction effect of IBA, and NAA on shoot length of *C. edulis*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
IBA	4	23.28395667	5.82098917	133.05	<.0001***
NAA	3	0.91924500	0.30641500	7.00	<.0001***
IBA*NAA	12	15.73683000	1.31140250	29.97	<.0001***

R - square = 0.95 CV % = 5.43, mean of shoot length =3.84 *** = highly significant at α = 5 %, DF = degree of freedom, PERSHL= % of shoot length

* = shows the interaction of the treatments, *** = highly significant at α = 5 %