Micropropagation of *Plectranthus edulis (*Vatke) Agnew

M.Sc. Thesis

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April, 2014

Jimma, Ethiopia

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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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JIMMA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
DEPARTEMENT OF BIOLOGY

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STATEMENT OF AUTHOR

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ACKNOWLEDGMENT

I thank and praise GOD ALMIGHTY for his immense grace and blessings, which guided me in taking up this research project and gave me the confidence and sustained ability to complete this work successfully.

First my sincere thanks with great joy to acknowledge my deep sense of gratitude and heartfelt thanks to my research advisor Dr. Balcha Abera, for his kindness, patience, encouragement, continual help and advice, for his unreserved assistance to realize my work, for all the effort you made to realize the completion of my work. I am really grateful for all activities including checking, analyzing, developing ideas and editing the papers.

My special thanks also go to Mr. Mezgebe Getahun and for his remarkable role in giving constructive comments from the very inception of the work and Mr. Folla Bekele for his unreserved effort in lab. Assistance .Much credit also goes to Jimma University for granting me the financial assistance needed to complete the study and accessibility of materials and computer utility. My special thanks to all staff members of Biology Department and Chemistry Department especially technical staffs, you all are the greatest blessing in my life, I love you all and thank you very much.

Lastly, I am deeply indebted to my beloved wife Kebebush Abera for her tolerance, patience, love, support and strength. My baby Daughter Hana Belete is additional source and spice of joy and enchantment both at home and away from home and also thank you for your long patience and for being so understanding to cope with situations that were sometimes beyond my handling. Finally, all other people who helped me during the study are also acknowledged, may God bless all of you!

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LIST OF ABBREVIATIONS

Acc.	Accession
ANOVA	Analysis of variance
BAP	6-Benzylamino purine
B5 medium	Gamborg medium
CRD	Completely Randomized Design
IAR	Institute of Agriculture Research
IBA	Indole 3-butyric acid
MS medium	Murashige and Skoog
NAA	Naphthaleneacetic acid
PGRs	Plant Growth Regulators
REGWQ	Ryan, Elinot, Gabriel, and Welsh model
SAS	Statistical Analysis Sostware

ABSTRACT

Plectranthus edulis (Vatke) Agnew belongs to the family Lamiaceae which occurs both as a wild and cultivated species. The Major constraints in the cultivation of P. edulis through the conventional propagation methods are shortage of planting materials and a long maturation period. Therefore, the objective of this study was to develop protocol of micropropagation that enables multiplication of P. 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, 69.83±0.76) of *clean culture for nodal and shoot tip, respectively. 6-(BAP) Benzylamino purine 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. 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 P. edulis and provide a possible system towards genetic improvement of the crop using nodal as well as shoot tip explant sources.*

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

CHAPTER ONE

1. INTRODUCTION

1.1. Background

P. edulis (Vatke) Agnew (synonym *Coleus edulis* (Vatke) belongs to the family Lamiaceae. The genus Plectranthus consists of over 350 tuber-bearing and non-tuber bearing species distributed predominantly in Africa, Asia and Australia (Codd, 1985 as cited in Taye *et al.*, 2006). *P. edulis* is said to have originated in Ethiopia (Greenway, 1944; Ryding, 2000 as cited in Taye et al., 2006), and is one of the traditional tuber crops indigenous to Ethiopia (Asfaw & Woldu, 1997; ENBSAP, 2005). 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).

P. edulis Vatke is an indigenous annual tuber crop grown widely in mid and high altitude areas ranging from 1880 m to 2200 m 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 parts of Ethiopia, particularly in Kefa area (Westphal, 1975; Asfaw & Woldu, 1997; Taye *et al*., 2007).

P. edulis have starchy tubers and are cultivated for food in tropical Africa and Asia. It is an important root crop in local diets, culture, and socioeconomic life of the people (Mekbib and Weibull, 2012). *P. 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 other food crops will not be ready for consumption (Mekbib & Weibull, 2012).

P. 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 & 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 (Mekbib, 2007). 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 *P. edulis*.

1.2. OBJECTIVES

1.2.1. General Objective:

The main objective of this study is to develop micropropagation protocol that contributes mass cultivation and in- situ conservation of *P. edulis*

1.2.2. Specific Objectives:

- To determine the optimum concentration and time of exposure to sodium hypochlorite (NaOCl) concentration
- 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. Origin and distribution of *P. edulis*

Plectranthus edulis (Vatke) Agnew belongs to the Lamiaceae family and the genus *Plectranthus* are widely distributed in Africa, Madagascar, India, Australia and a few Pacific islands (Lukhoba, 2006). Some of the African native *Plectranthus* species were, probably, brought to the New World after the pioneer voyages of the Portuguese discoverers in the 16th century, a few being used, for example, in the traditional medicine of Brazil .The genus *Plectranthus* was first described by the French botanist L´Heritier in 1788 and since then the number of species there included have been increasing. Nowadays, *Plectranthus* plants are known all over the world due to their horticultural uses since they are fast-growing, produce lovely flowers (Jaarsveld, 2006).

P. edulis (Vatke) Agnew also known as Ethiopian potatoes are believed to have been domesticated and originated in Ethiopia (IBC, 2001; Greenway, 1944; Ryding, 2000).According to Siegenthaler (1963) & Westphal (1975), *P. edulis* is a major highland and indigenous tuber crop in southern, northern and southwestern parts of Ethiopia. In Ethiopia *P. edulis* occurs both as wild and cultivated species. The wild species which are 30 in numbers are found throughout the country where as the cultivated species are grown in wetter south and south western Ethiopia (IBCR, 2007).

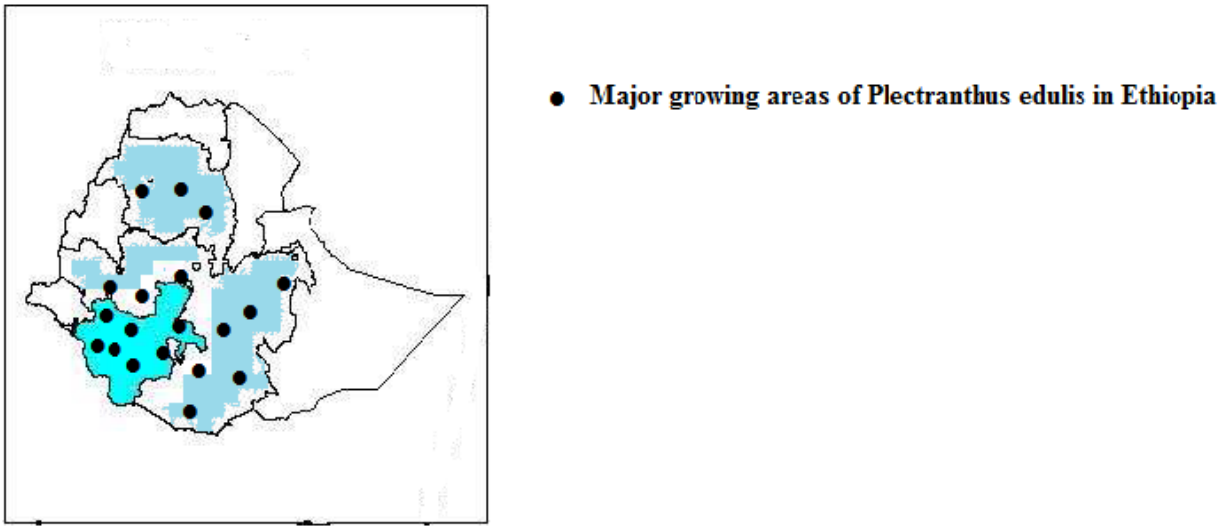

Figure 1 Source :(Mekbib, 2007) Map of growing areas of *P. edulis* in Ethiopia

2.2. Botanical description of *P. edulis*

2.2.1. Taxonomy

The taxonomical placement of *P. edulis* is Kingdom: Plantae, Phylum: Magnoliophyta, Class: Magnoliopsida, Order: Lamiales, Family: Lamiaceae, Genus: Plectranthus, Species: edulis (APG, 2009)

Kingdom	Plantae
Phylum	Magnoliophyta
Class	Magnoliopsida
Order	Lamiales
Family	Lamiaceae
Genus	Plectranthus
Species	edulis

2.2.2. Morphological description

P. edulis is an annual plant, it has square stems, branched, and the nodes are often hairy. The leaves are usually pubescent, narrow into petioles (Figure 1A). The flowers born on racemes are perfect, calyx is fine toothed and deflexed in front. The *P. edulis* flowers are cross pollinated by means of wind and insects. 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. Plectranthus requires red sandy loam soils with pH 5.5 to 7.0 and temperature of 10^0c to 25^0C P. edulis is an annual herb, with the root-fibres thickened at the end into oblong tubers. Stems herbaceous, erect, pubescent, 2–3 ft. long, simple or slightly branched. Leaves are sessile, oblong, membranous, crenate, slightly pubescent, 3–6 in. long, Whorls many-flowered, remote, forming a simple terminal racemose panicle 5–6 in. long; pedicels pubescent, longer than the calyx. Calyx are pubescent, 1/8– 1/6 in. long; upper tooth ovate, as long as the tube; lower lanceolate-deltoid. Corollas above 1/2 in. long; lower lip deeply concave, oblong-navicular, nearly as long as the tube. *Plectranthus* 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 (Figure 1B). The leaves vary from dark green to purplish green (Baker, 1900, Bekele, 2007, Jalihal, 2009).

Figure 2 *P. edulis* plant (A): leaf (B): flower

2.3. Uses of *P. edulis*

2.3.1 Food values

P. 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 *P. 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 (Table 1).

According to market observation made by the researcher one kilo of *P. edulis* was sold six birr by local people in Jimma town. But this price varies during production and planting period. For instance from September to November the supply of tuber to the market is very high and the price per kilo is also constant, but from March to April the price per kilo became twice of the price during production period. This is because of the shortage of tuber in the market.

Table1. Food composition of *P. edulis* tubers (Data per 100 g edible portion).

	Plectranthus edulis		**Irish potato**
	Raw	Boiled	Boiled with skin
Energy (calories)	69	101	84
Moisture (%)	81.9	73.8	77.2
Nitrogen (g)	0.30	0.24	0.24
Protein (g)	1.50	1.00	1.50
Fat (g)	0.20	0.20	0.20
CHO (incl. fibre) (g)	15.3	23.7	19.8
Fibre (g)	0.70	1.00	0.70
Ash (g)	1.10	1.30	1.30
Calcium (mg)	29.0	19.0	18.4
Phosphorous (mg)	90.0	62.0	74.3
Iron (mg)	9.30	1.10	3.60

Source: Food composition table for use in Ethiopia, Ethiopian Health and Nutrition Research Institute [EHNRI, 1997 (*Plectranthus* data) and 1998 (potato data)].

2.4. Conventional Propagation of *P. edulis*

P. 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 & 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 & 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 & Phillips**,** 1995). The amounts of the various ingredients in the medium vary for different stages of culture and plant species; the basic MS (Murashige & Skoog, 1962) and LS (Linsmaier & Skoog, 1965) are the most widely used media. The type and concentration of these nutrient components and PGRs needed in either stock solution or medium are based on genetic makeup of the plant for the regulation of the growth and development of the plant (Abera *et al*., 2010).

There is no research that was conducted on *in vitro* propagation of *P. edulis*, but some reports of *in vitro* propagation from synonym genus *Coleus* are available in *C. forskohlii* (Reddy *et al*., 2001). Previous researches regarding the establishment of quick protocols for *C. Blumei* Blenth species multiplication include experiments using different kinds of inoculums: apical meristems on IAA medium (Smith & 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 & 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 – *C. forskohlii* Briq (Praveena *et al.*, 2012), Micropropagation of *C. blumei* from nodal segments and shoot tips (Rani, 2006), *in vitro* micropropagation of *C. blumei* benth species (Vasile *et al*., 2011) and establishment of a rapid multiplication protocol of *C. 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 most genetically identical clones 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 period of time (Vuylsteke & Langhe, 1985; Wong, 1986 & Akbar & 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 & Sherrington, 1984; Dodds, 1991 & 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 cost. 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 with fungicide (0.3% Mancozeb). They may be prescreened for diseases, isolated and treated to reduce contamination (George, 1993; Holdgate & Zandvoort, 1997).

Stage I: 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 a*l., 2002).

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 & 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 & 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 & 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 & Sen 1998), *Eucalyptus tereticornis* (Sharma & 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 healthy plants are then transferred to an appropriate substrate (sand, peat, compost, etc.), in the ratio of 2:1:1 to mixture for acclimatization and gradually hardened (Thangavel, 2008).Hardened plantlets were transferred to a greenhouse. Immediately after planting, the plantlets were irrigated and adequate soil moisture was maintained through daily watering.

CHAPTER THRE

3. MATERIALS AND METHODS

3.1 Plant material

Healthy and vigorously young plants of *P. edulis* (acc.230694) were obtained from the Ethiopian Institute of Biodiversity Conservation and Research (EIB) to be used as stock plant for study. The stock plants were transplanted in to pot and 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 3).

Figure 3: Mother stock plants under greenhouse condition green house

3.2. Growth regulators stock solution 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 initiated by adding 3-4 drops of 1N NaOH and 1N HCl (NaOH for auxin, HCl for cytokinin); then the volume adjusted by adding double distilled water. Finally, growth regulators stock solutions were stored in a refrigerator.

3.3. Culture medium preparation

Prepared MS powder with vitamins *(*4405.19g/l.) was weighed and dissolved, followed by adding 30 g/l 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. Agar powder (Tissue culture grade; agar-agar type) 0.8% (w/v) was added to the moderately warm solution and melted properly. 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 121^0C for 20 minutes in a vertical autoclave.

3.4. Micropropagation Experiments

3.4.1. Explant Preparation and sterilization

Young and juvenile explants were taken from *P. 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 tap water for 10 minutes and finally washed with double distilled water.

This experiment was laid in a 5x4 factorial combination in CRD. The factors evaluated in this experiment were five concentrations (0,0.5,1,1.5, and 2% active chlorine) of sterilant solution, four durations (3, 5, 7 and 9 minute) of explants exposure to the stated chemical concentrations and two types of explants (shoot tip and nodal).

3.4.2. Culture initiation

Sterilized explants were cultured on agar solidified (0.8% agar-agar) with 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 five different concentrations of BAP (0, 0.5, 1.0, 1.5, and 2.0 mg/l) and four 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 Completely Randomized Design in 5x4 factorial combinations.

3.4.3. 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 full strength 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 NAA (0, 1, 2, and 3.0 mg/l). The experiment was thus being arranged in a 5x4 factorial in Completely Randomized Design. After two-three weeks, cultures proliferating shoot clumps were divided and sub cultured on to a fresh medium of similar composition.

3.4.4. 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 6 x 4 factorial combination.

3.4.5 .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 at each stage. The whole experiment takes three months or twelve weeks. Two weeks for initiation, three weeks for multiplication, three weeks for rooting and four weeks for acclimatization.

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 replication for each experiment were independently subjected to statistical analysis using the SAS (Inc.,Cary,NC,USA,2002-2008) 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.

CHAPTER FOUR

4. RESULTS AND DISCUSSION

Results obtained from experiments on micro propagation of *P. 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 *P. edulis* explants

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

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 2).

Table 2: Interaction effect of Sodium hypochlorite concentrations and its time of exposure on sterilization off shoot tip and nodal explants of *P. edulis*.

Conc NaOCl (%)	Time (min)	% Shoot contamination		% Clean culture		% Tissue Death	
		Nodal (Mean ±Std Dev)	Shoot tip (Mean ±Std Dev)	Nodal (Mean ±Std Dev)	Shoot tip Mean ±Std Dev	Nodal (Mean ±Std Dev)	Shot tip 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}	74.50±0.50^{a}	69.83±0.76^{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±0.76^{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±0.52i	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±0.76^{k}	2.60±0.52^{m}	7.17±0.64^{l}	7.40±0.52j^{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±0.40k^{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 of the steriliant solution .NaOCl at (0.5%) with short exposure of time (three and five minute) for both types of explants tissue death is very low and contamination is very high. 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 very high.

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 *P. edulis* nodal and shoots tip explants (Table 1, Figure 4A). Negash *et al*. (2000) used one percent NaOCl for surface sterilization for *Ensete ventricosum*. Similar results were reported by Yanagihava *et al*. (1996) & Bekele *et al*. (2013) for another *Coleus* species of *C. forskohlii* and *Coccinia abyssinica* recommended that young shoot segments were surface sterilized with 2 per cent sodium hypochlorite for 10 minutes. Different Scientists use mercuric chloride for explant surface sterilization (Bhattacharyya and Bhattacharya, 2001; Vasile *et al*., 2011; Rani *et al*., 2006).

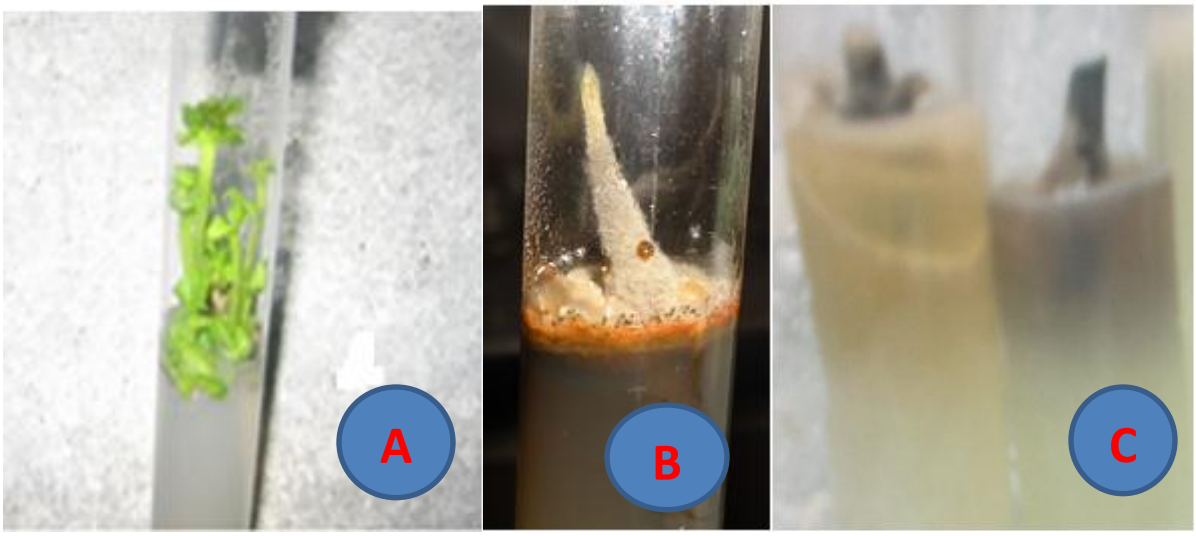

Figure 4: Response of cultures to different concentrations of NaOCl, (A) 1% sodium hypochlorite concentration and 5 minute exposure time results for clean culture (B) 0.5% sodium hypochlorite concentration and three minute exposure time results for contamination (C) 2% sodium hypochlorite concentration 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 *P. 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 *P. edulis.*

The analysis of variance obtained from the data **(**appendix II**)** showed that the interaction with BAP and nodal explant had very highly significant effect ($P < 0.0001$) on the shoot induction rate. Interaction effect of nodal explants with BAP on rate of shoot induction was found to be highly significant .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 3).

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 3). 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 *P. edulis (*Figure. 5A 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 3 Effects of different concentrations of BAP and Kinetin alone and in combination on shoot induction of *P. 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±0.26j^{k}	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±1.04d^{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 3). 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 *P. 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 (Figure. 5. B).

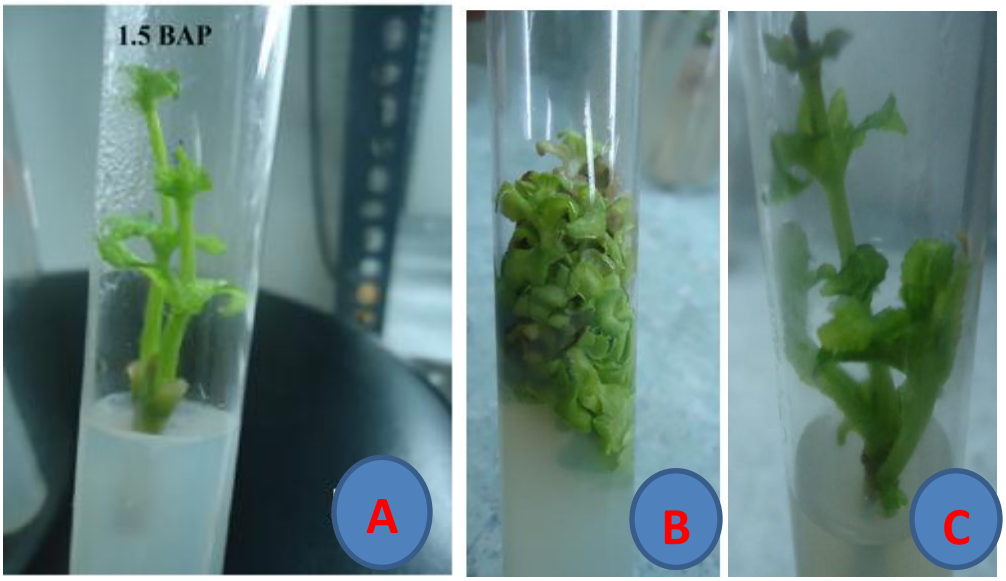

Figure 5: Effect of BAP on Shoot induction, (A) Shoot induction on 1.5 mg/l of BAP (B) unnecessary callus formation at the base of explant and (c) shoot induced on 1.5 mg/l BAP

4.3 Effect of different concentration and combination of BAP and NAA on shoot multiplication of *P. 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 *P. edulis* 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 (appendix III). Shoot buds raised from nodal explants response exceeds shoot tip shoot multiplication and this indicating the significant effect of explants at this stage.

Table 4 Effects of different concentrations and combinations of BAP and NAA treatments on shoot multiplication of *P. 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 (Figure 6 A,B and C, Table 4). Similarly, Rani *et al.,* (2006) reported proliferation of 4.5±0.6 microshoots on MS + 2.0 mg/l BAP and 1.0 mg/l NAA. 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 & Bhattacharya 1997), *Vanilla planifolia* (George & Ravishankar, 1997), *Aristolochia indica* (Manjula *et al.*,

1997), *Vitex negundo* (Kannan & Jasrai, 1998), *Syzygium travancoricum* (Anand *et al.,* 1999) and *Ancistrocladus abbreviatus* (Bringmann *et al.,* 1999). However, in certain other *Coleus* species, *C. forskohlii* (Sen & 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) & 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.

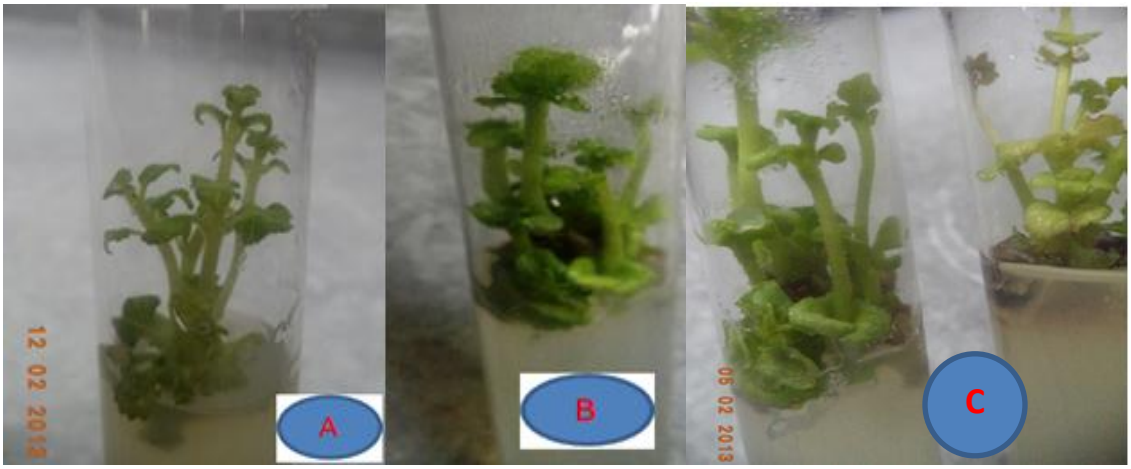

Figure 6: *In vitro* shoot multiplication of *P. edulis,* (a, b and c) Shoots multiplied on 2.0 mg/l of BAP and 1.0 mg/l NAA.

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

The ANOVA had indicated a very high significant effect of interaction of IBA with NAA on *in vitro* rooting of C. edulis micro cuttings (appendix IV).

Table 5: Effect of various concentrations of IBA and NAA on rooting of shoots of *P. edulis* cultured on half - strength MS medium.

Conc. of PGRs (mg/l)		Rooting Percent (Mean ± SD)	Root 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}	$3.32\pm0.\ 24^{fg}$	19.32 ± 0.71^{e}	1.59 ± 0.20^{fgh}
0	1.5	51.17 ± 0.67^{mn}	2.71 ± 0.14^{h}	$4.83\pm0.20l^{m}$	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 2.60±0.07cm 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. The roots formed in half MS basal media were normal longer thicker and with many branches (Figure 5A, B and C). 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 5D).

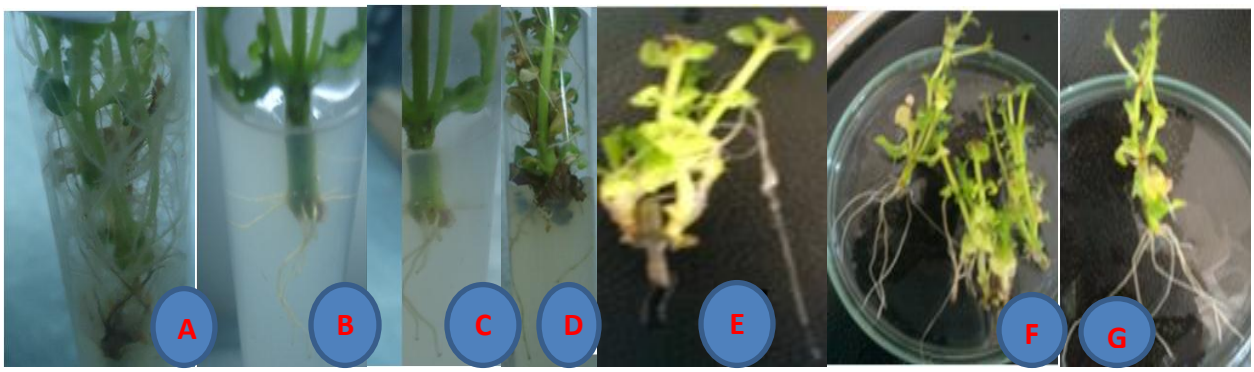

Figure 7: Root induced on different auxins (A-C) rooting on half - strength medium with 2 mg/l IBA; (D) rooting on half - strength medium with 1.0 mg/l NAA, (E-G) plantlets ready to transfer on sterile soil mix.

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 & Sen 1998), *Eucalyptus tereticornis* (Sharma & 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 *P. edulis* plantlet

The establishment of *in vitro* plantlets under different environmental conditions was greatly affected in terms of survival percentage of plantlets. The plantlets showed 85% survival efficiency. The plantlets transferred under net greenhouse conditions resulted in the best establishment, and all the regenerated plants exhibit normal morphology with respect to growth characteristics when compared with the mother plant (Figure. 6B, C).

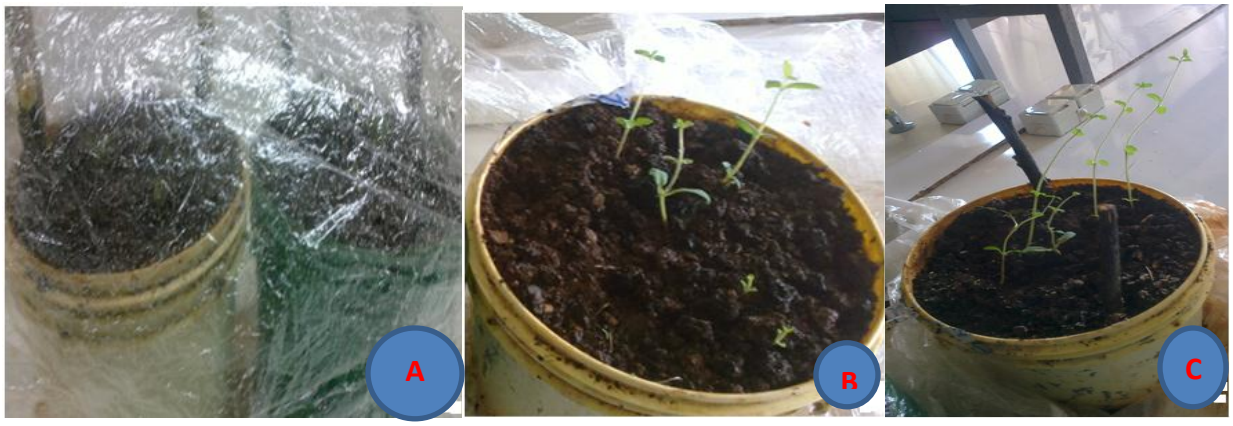

Figure 8: Acclimatization on 2:1:1 top soil, coffee husk and sand soil mix (a) plantlets under polystyrene plastic tube and (b) plantlets on polyethylene tray (c) four weeks old seedling of *P. edulis*

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 *P. edulis*. Based on the findings of the study, the following conclusion points were drawn:

- 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 *P. 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 *P. edulis.*
- Nodal explants gave best shoot induction response than shoot tip.
- MS basal medium supplemented with 2.0 mg/l BAP and 1.0 mg/l NAA were found to be optimum media for shoot multiplication of *P. edulis*.
- Half strength MS basal media containing 2.0 mg/l IBA and 1 mg/l NAA were found to be optimum for rooting.
- Thus, this protocol could be useful for large-scale production of highly demanded *P. 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.
- *In vitro* multiplication of *P. edulis* plants can be tried with other explant and culture media for high rate of multiplication.
- Callus development in leaf base and tuber explants could be tried.

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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 *P. 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 *P. 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 *P. 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 *P. 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 *P. 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 *P. 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 *P. 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 *P. 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 *P. 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 *P. 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 *P. 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 *P. 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 *P. 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 *P. 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 *P. 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 %