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In vitro propagation of *Satureja punctata* (Benth.) Briq. – A rare aromatic and medicinal plant from Ethiopia using shoot tip explant

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This is to certify that the thesis prepared by Indrias Teshome entitled: ***In vitro* propagation of *Satureja punctata* (Benth.) Briq. – A rare aromatic and medicinal plant from Ethiopia using shoot tip explant** and submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology complies with the regulations of the university and meets the standard with respect to originality and quality.

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Abstract

***In vitro* propagation of *Satureja punctata* (Benth.) Briq. – A rare aromatic and medicinal plant from Ethiopia using shoot tip explant**

Indrias Teshome

Addis Ababa University, 2015

Satureja punctata *is an important medicinal plant in Lamiaceae family. This plant is threatened mainly due to overgrazing, deforestation, over harvesting of the whole plant for medicinal purpose. This study was aimed to develop an efficient* in vitro *propagation protocol for* S. punctata *using shoot tips.* In vitro *germinated seedlings were used as mother plant for culture induction. Shoots excised from thirty-day-old seedlings were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of 6-benzyl amino purine (BAP) and Kinetin (KIN) for shoot induction and multiplication. Half strength MS medium containing different concentrations of Indole-3- butyric acid (IBA), Indole-3-acetic acid (IAA) and α-naphthalene acetic acid (NAA) were used for root induction. BAP at 0.5mg/l concentration and plant growth regulator free medium induced the highest frequency (100%) of shoot induction. Maximum mean number of shoot per explant (8.25±1.64) and shoot length (2.62±017 cm) were obtained on medium containing 0.5 mg/l BAP and PGRs free medium respectively. The combination of BAP with NAA found to be an optimum concentration producing 26.20±2.71 shoots per explant and 2.48±0.13 cm shoot length was obtained from PGRs free medium for shoot multiplication. MS medium supplemented with 1.0 mg/l IBA gave the highest rooting percentage (100%) with optimum root number (5.90±0.48) and root length (1.55±0.11cm). Up on acclimatization, 97.5% survival rate was observed on soil composed of garden soil, sand and farm yard manure at a ratio of 2:1:1, respectively. The results demonstrated that, this study was very important for mass propagation and ultimate conservation of this valuable plant.*

Key words; Acclimatization, i*n vitro* propagation, medicinal plant, plant growth regulators, *Satureja punctata*

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List of Abbreviations

BAP	6-Benzyl Amino Purine
IBA	Indole-3-Butyric Acid
IBC	Institute of Biodiversity Conservation
KIN	Kinetin
Kpa	kilo Pascal
MS	Murashige and Skoog
PGRs	Plant Growth Regulators
WHO	World Health Organization

1. Introduction

Medicinal and aromatic plants have been recognized since time immemorial and they are still used in medicine, food and cosmetic industry (Lahlou, 2004; Belay *et al*., 2011). Accessibility and affordability of the medicinal herbs have made them as fundamental part of many people's life all over the world. About 65-80% of the world's populations in developing countries mainly depend on medicinal plant for their primary health care and this is due to poverty and lack of access to modern medicine (Momtaz and Abdollahi, 2010). In Africa up to 80% of the population uses traditional medicine for primary health care (WHO, 2003). Similarly, according to the report of Kebede *et al*. (2006) and Ethiopian Institute of Biodiversity (2012), in Ethiopia around 85% of the people use medicinal plants as traditional medicines for their health care, and this is due to the cultural acceptability of healers and local pharmacopeias, the relatively low cost of traditional medicine and difficult access to modern health facilities.

The genus *Satureja* in Lamiaceae (Labiateae) family contains about 200 species of aromatic herbs and shrubs, which are used as flavoring agents and some for medicinal purposes (Neimere, 2010; Babadi *et al*., 2012).

In Ethiopia, the genus *Satureja* contains around 8 species (Belay *et al*., 2011). The aerial parts of these species are used as a traditional medicine to treat headache, stop menstruation, relieve stomach pains, for the treatment of liver diseases and leishmanias and improve the quality of milk (Tariku *et al*., 2010; Tesfaye *et al*., 2010). The essential oils obtained from the leaves and flowers of different *Satureja* species are commonly used in various industrial applications as flavoring material, medicine and perfume (Teklu *et al*., 1998).

Cultivation of most medicinal plants is very difficult because of low germination rates due to loss of viability within short period of time, very specific ecological requirements and they are collected from their natural habitat (Vines, 2004). According to Vasisht *et al*. (2002), in Asia and Africa, more than 80% of medicinal plant supply comes from the wild sources. About 90% of medicinal plants used by industries are collected from the wild and over 70% of collections involve destructive harvesting (Sharma *et al*., 2010). In Ethiopia, about 87% of medicinal plants are harvested from wild population except few herbs that are grown in the backyards (Lehoux *et al*., 2012; Malik *et al*., 2012). Although Ethiopia has been described as one of the most important sources of biodiversity in the world, much of the rich diversity is lost due to deforestation, land degradation, lack of documentation of species in some areas as well as of traditional cultural knowledge, and potential acculturation (D'avigdor *et al*., 2014).

In general, medicinal plants are disappearing from natural habitats by rapid agricultural development, urbanization, indiscriminate deforestation and uncontrolled collection. Industrialization coupled with urbanization is constantly putting pressure on natural and ecological resources. Due to over depletion and ruthless collection, medicinal plants are on the verge of extinction. The destructive harvesting leads to endangered genetic stocks and diversity of medicinal plants (Robert *et al*., 2012).

This problem can be overcome by *in vitro* propagation methods often used to produce clones of a plant at large-scale and to preserve endangered species (Aicha *et al.,* 2013). *In vitro* propagation refers to true-to-type propagation of selected genotypes. Different explants such as single cells, protoplasts, pieces of leaves or roots can be used to generate a new plant on culture media with required nutrients (Mohamed *et al*., 2010; Kher *et al*., 2014). *In vitro* propagation technique is a powerful tool to obtain a large number of high-quality clonal plants, for rapid large-scale

propagation of new genotypes, to generate pathogen-free propagules and for plant germplasm conservation. Hence, tissue culture is the only rapid process for the mass propagation of plants (Altman, 1999).

Satureja punctata propagates by seed and the seed is exposed to damage due to ecological change including climatic change, overgrazing, and deforestation which results in low availability of this valuable medicinal and aromatic plant to use it as cure as well as for industrial purpose. Therefore, the aim of this study was to develop *in vitro* propagation protocol for *S. punctata*.

2. Literature Review

2.1.Botanical description

Satureja punctata (Benth.) Briq. is a flowering dicotyledinary plant that contains annual or perennial herbs, sub shrubs, or shrubs, leaves entire or scarcely toothed, stem sometimes elongate or apiculate, smooth or muricate, sometimes hairy (Kubitzki, 2004). It is an erect perennial herb and grows to a height of 10–100 cm often with long unbranched branches and hairy stem (Belay *et al*., 2011; Hedberg *et al*., 2006). Leaves are sessile or shortly petiolate, blade often leathery, circular to ovate or narrow lanceolate, margin entire, often thickened in a white line, revolute with pleasant lemon-scented fragrance and distinctive purple or violet flowers with chromosome number of $2n = 30$ (Hedberg *et al*., 2006; Belay *et al*., 2011).

2.2. Ecology and Distribution

Satureja species are native to warm temperate regions and members of the genus need sun and well drained soil (Tepe, 2015). Many authors consider that Mediterranean Basin and Central Asiatic region as a center for the genus *Satureja* and distributed to Europe, Africa, Asia, and America (North and South) (Momtaz and Abdollahi, 2010; Tariku *et al*., 2010; Tepe, 2015). In Ethiopia, *Satureja punctata* is growing often on stony slopes and on limestone area or on dry and often on rocky ground, highly grazed grassland occurring at altitudes of 600-3840 m.a.s.l. and distributed in Shoa, Gondar, Tigray, Wello, Gojam, Gamo Gofa, Bale, Sidama, Harerge, Arssi and Keffa Regions (Hedberg *et al*., 2006; Teklu *et al*., 1998).

2.3.Taxonomy of Satureja

Labiateae or Lamiaceae family contains more than 4000 species which are grouped in approximately 220 genera (Kaufmann and Wink, 1994). Of this Lamiaceae family, the genus *Satureja* includes about 200 species of herbs and shrubs, mostly ornamental (Tepe, 2015). The classification of *Satureja* is still a matter of debate and with active research still proceeding, and in the future further changes expected. Satureja species commonly called savory that include the two main culinary savories are summer and winter savory. Summer savory (Satureja hortenensis) is an annual and is the most commonly used culinary savory. Winter savory (Satureja montana) is an evergreen perennial that is a well known aromatic and medicinal plant which produces essential oil (Chizzola, 2003). A satisfying separation of the African species into the genera *Acinos*, *Calamintha*, *Micromeria* and *Satureja* is very difficult (Hedberg *et al.*, 2006)

Satureja punctata is a flowering dicotyledinary plant that belongs to the Lamiaceae family, Nepetoideae subfamily and Mentheae tribe which consists of strongly aromatic plant, frequently used as traditional medicinal herb and spice for food and teas (Miladi *et al*., 2013).

Satureja punctata synonymus to *Micromeria punctata* (Benth.) (Teklu et al., 1998), *Micromeria biflora* (Benth.) and similar to *Micromeria abyssinica* (Benth.) (Chagonda and Chalchat, 2005).

2.4. Importance of Medicinal and Aromatic plants

Medicinal plants are important and major resources of the traditional medicine and have economic importance in herbal and pharmaceutical industries (Asha-Rani and Prasad, 2014). It is estimeted that close to 25% of the active compounds is currently prescribed synthetic drugs were first identified in plant resources and 20,000 plants have been used for medicinal porposes

among which 4000 have been used commonly and 10% of those are commercial (Momtaz and Abdollahi, 2010).

The food industry is becoming increasingly interested in aromatic herbs, mainly of the Lamiaceae family due to growing consumer demands for healthy natural foods. In addition to the food industry, Lamiaceae herbs are also of high demand in dyeing, fragrances, cosmetics, beverages and smoking industries. Lamiaceae family contains popular aromatic herbs such as rosemary, oregano, marjoram, sage, basil, satureja (savory) and thyme (Aicha *et al*., 2013).

2.5.Importance of *Satureja*

Satureja species have economic and medicinal importance because of their high essential oil content. With their pleasant fragrance, *Satureja* species are widely used as herbal teas and spices (Satil and Kaya 2007). Essential oils obtained from the leaves and flowers of different *Satureja* species are commonly used in various industrial applications as flavoring material, medicine and in perfumers (Teklu *et al*., 1998; Tariku *et al*., 2010).

Significant proportions of *Satureja* species are plants that have an important role in the pharmaceutical industry due to their unique phytochemical contents as well as their excellent pharmacological (antibacterial, antifungal, antioxidant, cytotoxic, insecticidal, antidiabetic, anti-leishmanial) properties. Additionally, effect of these species on fertility treatment, blood level of sex hormones, sperm characteristics, reproductive stimulation, premature ejaculation, peripheral blood temperature and treatment of cardiovascular diseases were reported (Tepe, 2015). Chagonda and Chalchat (2005) reported that *S. punctata* is used as a medicinal herb to treat bronchitis, stomach ailments and as a febrifuge in different traditional systems in East Africa. Biological activity of essential oil extracted from *S. punctata* was investigated by Tariku

et al. (2010) showed leishmanicidal effect against promastigote that cause Leishmaniasis which is a significant and widespread disease among the Ethiopian population.

2.6. Essential oil extracted from *S. punctata*

Most studies done on *S. punctata* have been mainly focused on its essential oil. Teklu *et al*. (1998), identified 16 chemical components among which only seven components (geranial, neral, geranyl acetate, β-caryophyllene, nerolidol, caryophyllene oxide and a-bisabolol) were isolated and characterized. Among these, geranial (41.67%) and neral (30.25%) were found to be the major constituents of the oil.

Essential-oil composition, antileishmanial and toxicity study of *S. punctata* from Ethiopia were also reported (Tariku *et al*., 2010). Essential oil from the leaf of *S.punctata* was extracted and its composition was studied, from the leaf of *Satureja punctata* (Benth.) Briq. from Zimbabwe (Chagonda and Chalchat, 2005).

2.7.Tissue culture

Biotechnology offers several important tools for fast and efficient multiplication and production of plants with desired features (Vinothkumar *et al*., 2011). Among the different techniques of biotechnology, plant tissue culture is the one being applied in crop improvement programme (Velayutham *et al*., 2012). Plant tissue culture is an *in vitro* culture of cells, tissues, organs and their components under defined physical and chemical conditions (Thorpe, 2007). Plant tissue

culture is a suitable method for obtaining a large quantity of genetically homogenous and healthy plant materials for planting (Velayutham *et al*., 2012).

In vitro conservation can be possible through micropropagation which allows production of numerous clones of the plant by the application of tissue culture techniques (Kanungo and Sahoo, 2011). For mass propagation of medicinal plant species in which conventional methods possess limitations, *in vitro* multiplication provides the way out. *In vitro* plant regeneration is a complex phenomenon involving different biochemical mechanisms for its progression (Robert *et al*., 2012).

Advanced biotechnological methods of culturing plant cells and tissues should provide new means of conserving and rapidly propagating valuable, rare and endangered medicinal plants (Nalawade *et al*., 2003). Successful micropropagation protocols for various medicinal plants have been developed and their conservation has also become feasible through synthetic seeds and cryopreservation technologies (Jesmin *et al*., 2013).

2.8.Influence of plant growth regulators in plant tissue culture

Plant tissue culture or *in vitro* culture of cells, tissues and organs are generally dependent for their success on the inclusion of plant growth regulators in the medium. Plant growth regulators are numerous chemical substances that profoundly influence the growth and differentiation of plant cells, tissues and organs (Gasper *et al*., 1996).

Auxins, abscisic acid, cytokinins, ethylene, and gibberellins are commonly recognized as the five main classes of naturally occurring plant hormones. However, auxins, cytokinins, and auxin-

cytokinin interactions are usually considered to be the most important for regulating growth and organized development in plant tissue and organ cultures (Evans *et al*., 1981; Vasil and Thorpe, 1994).

Synthetic compounds that act like natural plant hormones are called "plant growth regulators" (Davies, 1995). Many of such plant growth regulators have been discovered with a biological activity which equals or exceeds that of the equivalent endogenous hormones (Gasper *et al*.,1996).

The effects of natural and synthetic plant growth regulators are rarely specific in their ultimate influence on growth, development and the responses of cells, tissues, and organs. *In vitro* culture can vary with conditions of the culture, the type of explant, and the genotype. Usually a combination of two or more growth regulators of different classes is required, either applied simultaneously or sequentially (Evans *et al*., 1981).

2.8.1. Auxins

Root initiation and cell division for rooting is dependent on either endogenous or exogenous auxins (Hartman *et al*., 1997). Generally, IAA is regarded as the major auxin in higher plants and plays a central role in adventitious root formation (Stefancic *et al*., 2005). The most commonly detected natural auxin is indole-3-acetic acid (IAA), but depending on the species, age of the plant, season, and the conditions under which it has been growing, other natural auxins have been identified such as 4-chloroindole-3-acetic acid, indole-3-acrylic acid, indole-3-butyric acid (IBA) (Gasper *et al*., 1996).

Commonly used synthetic auxins in tissue culture are 2,4-dichlorophenoxyacetic acid (2,4-D; often used for callus induction and suspension cultures), and α-naphthalene acetic acid

(NAA; when organogenesis is required); indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) are commonly used in plant culture media and tend to be denatured in media and rapidly metabolized within plant tissues. These attributes can be useful in rooting that requires less auxin concentration (Gaspar *et al*., 1994).

The levels of endogenous auxins depend on the rate of their anabolism, catabolism, transport, and conjugation (Bandurski *et al*., 1995). Synthetic auxins might affect the level of endogenous auxin by modifying directly (enzyme synthesis) and indirectly via effectors (IAA-oxidase) that can behave as auxin-antagonists (Gasper, *et al*., 1996). The responsiveness of tissues to auxins can also be modified by other hormones such as cytokinins (Aloni, 1995) and ethylene (Liu and Reid, 1992).

2.8.2. Cytokinins

Two major properties of cytokinins that are useful in culture are, stimulation of cell division (often together with auxins) and release of lateral bud dormancy. They can also induce adventitious bud formation (in cuttings and cultures) (Fabijan *et al.*, 1981; Krikorian, 1995). In intact plants, cytokinins promote lateral bud growth and leaf expansion, retard leaf senescence, promote chlorophyll synthesis, and enhance chloroplast development (Kuhnle *et al*., 1977; Nooden and Leopold, 1988).

The most commonly used naturally occurring cytokinins in plant tissue culture are zeatin, 6-dimethylaminopurine (2iP), dihydro-zeatin, and zeatin riboside. Synthetic purine cytokinins, such as kinetin, 6-benzylaminopurine (BAP) and 6-benzyladenine (BA) and, are potent and economic alternatives. Substituted urea compounds, such as thidiazuron does not occur in plants

but act as cytokinins in tissue culture and may be optimal for the particular species being studied (Gasper *et al*., 1996).

2.8.3. Influence of Cytokinins and Auxin Combination

Cell division is regulated by the joint action of auxins and cytokinins, each of which influences different phases of the cell cycle. Auxins affect DNA replication, whereas cytokinins seem to exert some control over the events leading to mitosis (Vesely *et al*., 1994). Thus, auxin and cytokinin levels in cultures need to be carefully balanced and controlled.

Many aspects of cell growth, cell differentiation, and organogenesis in tissue and organ cultures have been found to be controlled by an interaction between cytokinins and auxins. The requisite concentration of each phytohormone varies greatly according to the kind of plant being cultured, the cultural conditions, and the form of the phytohormone used. Although both auxin and cytokinin are usually required for growth and morphogenesis, auxin can inhibit cytokinin accumulation, whereas cytokinins can inhibit at least some of the actions of auxin (Gasper *et al.,* 1996).

Recently, all *Satureja* species have been in the focus of scientific researches due to their phytochemical contents which have excellent pharmacological properties. Since 1981 many groups identified essential oil composition and phytochemical content of the extracts of these species (Tepe, 2015). However, no efforts have been made so far for the micropropagation of *S. punctata*. Therefore, the present study has been undertaken with the following objectives.

3. Objectives

3.1.General objective

To develop an *in vitro* propagation protocol for *Satureja punctata* using shoot tip explants.

3.2. Specific objectives

- To induce multiple shoot proliferation from shoot tip explants
- To identify the optimal concentrations of cytokinins on shoot initiation and multiplication
- To determine the optimal concentrations of auxins for root induction
- To evaluate the survival rate of plantlets during acclimatization
- To determine synergistic effect of KIN and NAA on *S.punctata*
- To determine synergistic effect of BAP and NAA on *S.punctata*

4. Materials and Methods

4.1.Plant material collection, disinfection and germination

Matured seeds of *Satureja punctata* were collected from Oromia region, Arsi Zone, Amigna district, Sade-Wale Kebele from a particular place called Golja. Seeds were tightly sealed in cotton cloth during transportation to avoid seed loss due to its very small size. The seeds were washed with detergent under running tap water and disinfected as they were sealed within cotton cloth. The seeds were surface disinfected with 70% (v/v) alcohol for 8 minutes and rinsed five times by sterile distilled water, followed by sodium hypochlorite solution containing two drops of tween 20 for 16 min and consequently rinsed three to five times thoroughly with sterile distilled water.

Seeds were planted on 40ml of growth regulators-free MS medium in baby food jars (6 cm diameter with 9.5cm height) containing growth regulator-free MS medium and supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. Before addition of agar, pH of the medium was adjusted to 5.8 with 1 M NaOH or 1 M HCl and autoclaved at 121ºC with a pressure of 105 Kpa for 15min. The *in vitro* germinated seedlings were used as mother plant for culture commencements.

4.2. Media preparation

4.2.1. MS medium stock solution preparation

Murashige and Skoog (1962) medium stock solution was prepared by weighing all components (macro, micro, iron EDTA and vitamin) powder and dissolving them in distilled water. All

prepared nutrients (i.e. macronutrient, micronutrient and vitamins) were stored at -20ºC but iron EDTA was stored at +4ºC for further manipulation, (Appendix 1).

4.2.2. Growth regulators stock solution preparation

Stock solution of plant growth regulators (PGRs), Kinetin (KIN), 6-Benzyl amino purine (BAP), Indole-3-butyric acid (IBA), α-naphthalene acetic acid **(**NAA) and Indole-3-acetic acid (IAA) were prepared in mg/ml concentration and stored at +4ºC. Prior to dissolving in water, the growth regulators, which are in powder form, were dissolved in two to three drops of 1M NaOH.

4.2.3. Culture medium preparation

MS stock solutions were prepared by mixing the recommended amount (macro =100 ml/l, micro = 10 ml/l, iron EDTA =10 ml/l and vitamin =10 ml/l) in distilled water. The medium was supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar.

The pH of the medium supplemented with respective growth regulators was adjusted to 5.8 with 1M NaOH or 1M HCl before addition of agar. Thereafter, the medium was stirred by magnetic bars and boiled on hotplate until it became clear and well mixed. Finally, 50ml medium was poured into culture vessels and autoclaved at 121ºC with a pressure of 105 Kpa for 15 min. Root induction was conducted on half strength MS medium. For shoot initiation, multiplication and root induction, 50 ml medium was used per Magenta-GA7.

4.3.Shoot induction

After seeds were germinated, roots from thirty-day-old seedlings were removed and shoots were cultured on MS media containing different concentration of BAP (0.0, 0.5, 1.0, 1.5, 2.0 mg/l) and

Kinetin (0.0, 0.5, 1.0, 1.5, 2.0 mg/l). A total of 20 explants per treatment, 5 explant per culture vessel with 4 replications were used. Data were recorded after 4 weeks of culture.

4.4. Shoot multiplication

Young and healthy shoots were cultured on shoot multiplication medium containing different concentrations of BAP and kinetin.

- BAP alone (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mg/l),
- Kinetin alone (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mg/l),
- BAP (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mg/l) in combination with NAA (0.25mg/l),
- Kinetin (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mg/l) in combination with NAA (0.25mg/l),
- BAP (1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0mg/l) in combination with kinetin (0.25, 0.5 and 1.0mg/l) for each BAP concentration.

A total of 30 shoots per treatment and 10 shoots per culture vessel with 3 replications were used for each treatment. All five subsequent subcultures were done at 4 weeks intervals to fresh medium of the same composition. The number of shoots and shoot length per explant was recorded at each subculture stage.

4.5. Root induction

Well-developed micro-shoots obtained from shoot multiplication media were transferred to root induction medium containing various concentrations of IBA (0.0, 0.25, 0.5, 1.0, 1.5, 2.5 mg/l), IAA (0.0, 0.25, 0.5, 1.0, 1.5, 2.5 mg/l) or NAA (0.0, 0.25, 0.5, 1.0, 1.5, 2.5 mg/l). The cultures were maintained in the growth room with the same culture condition as for culture initiation and

shoot multiplication experiments. Number of roots and root length per shoot were recorded after 4 weeks of culture.

4.6. Culture condition

All cultures were maintained in culture room under light intensity of 40µmolm^{-2}s^{-1} and 16h photoperiod provided by cool-white fluorescent lamp with a temperature of 25±2 ºC.

4.7. Acclimatization

Plantlets with well-developed roots were isolated, gently washed under running tap water and transferred to plastic pots (20cm diameter) containing garden soil, sand and compost in ratio of 2:1:1, respectively. The potted plantlets were covered with polyethylene bags and kept in greenhouse at 25°C and 75-80% relative humidity upto 4 weeks. They were watered as necessary. After 15 days, the polyethylene bags were removed. The survived plantlets were recorded after 30 days of acclimatization.

4.8. Experimental design

A completely randomized design (CRD) was used for all experiments. The study was conducted at Addis Ababa University Plant Tissue Culture Laboratory of the Institute of Biotechnology. Data were subjected to analysis of variance (ANOVA) and significant differences among mean values were compared using least significant difference (LSD) test using statistical data analysis software SPSS version 22.0 at 5% probability level.

5. Results

5.1. Shoot induction

Well induced cultures were observed after 13 days of transfer to initiation media. All initiated shoots appeared to be of high quality except those initiated on 1.5 mg/l and 2.0 mg/l BAP, which resulted in vitrified shoots. The highest percentage of shoot initiation (100%) was obtained on the control and a medium fortified with 0.5mg/l BAP. The lowest initiation response (70%) was observed on MS medium supplemented with 1.5 mg/l and 2.0 mg/l BAP. The maximum mean number of shoots (8.25 ± 1.64) was obtained on MS medium supplemented with 0.5mg/l BAP. The minimum mean number of shoots (1.40±0.30) was recorded in the presence of 2.0 mg/l BAP, which were from vitrified shoots.

For KIN the highest percentage of shoot initiation (90%) was recorded at 0.5mg/l, 1.5mg/l and 2.0mg/l and maximum mean number of shoot per explant (5.55±1.28) was obtained on medium containing 2.0 mg/l KIN. The minimum initiation response (85%) and mean number of shoots (2.45±0.43) was recorded in the presence of 1.0 mg/l KIN.

The maximum shoot length (2.62± 0.17 cm) was recorded from PGRs free medium (Table 1, Fig. 1).

Table 1. Effect of BAP and KIN on shoot initiation from *in vitro* germinated seedlings.

Treatment (mg/l)		Response (%)	No. of shoots/explants Mean ± SE	Shoot length (cm) Mean ± SE
Kin	BAP			
0.0	0.0	100	1.90 ± 0.17^{c}	2.62± 0.17^{a}
0.5	0.0	90	3.75 ± 1.18^{bc}	1.72 ±0.28^{b}
1.0	0.0	85	2.45 ± 0.43^{c}	0.95± 0.13^{de}
1.5	0.0	90	3.75 ± 0.80^{bc}	1.05 ± 0.11^{cd}
2.0	0.0	90	5.55± 1.28^{b}	1.45 ± 0.20^{bc}
0.0	0.5	100	8.25 ± 1.64^{a}	1.00 ± 0.08^{de}
0.0	1.0	85	1.90 ± 0.29^{c}	0.62 ± 0.08^{de}
0.0	1.5	70	2.40 ± 0.56^{c}	0.60 ± 0.10^{e}
0.0	2.0	70	1.40 ± 0.30^{c}	0.57 ± 0.12^{e}

Means within columns having different letters (lower case) in superscript are significantly different at $p < 0.05$. Data are presented as mean ± SE.

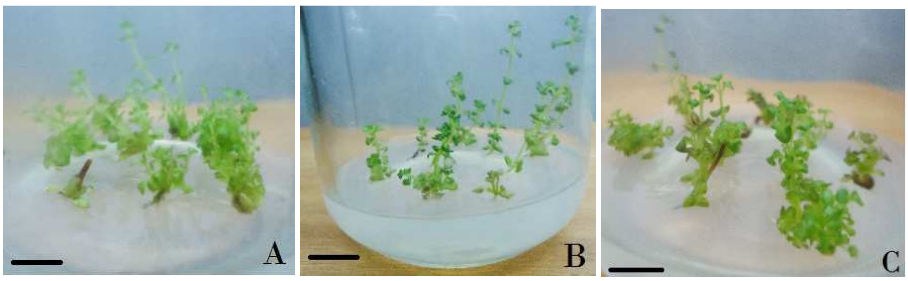

Figure 1. Shoot induction of *S. punctata* using shoots excised from *in vitro* sprouted seedlings. A. 0.5 mg/l BAP, B. 0.5 mg/l KIN, C. 1.5 mg/l KIN. Bars = 1 cm

5.2. Shoot multiplication

5.2.1. Influences of BAP and KIN on shoot proliferation

A significant difference ($P < 0.05$) was observed among the different concentrations of BAP and KIN in terms of shoot number per explant and shoot length after 4 weeks of culture. A maximum mean number of shoot per explant (10.80±2.26) was obtained on a medium containing 1.5 mg/l BAP. The lowest mean number of shoots (1.90±0.62, 2.73±0.65 and 2.30±0.29) were recorded from MS medium fortified with 3.5 mg/l BAP, 4.0 mg/l KIN and control (growth regulators free medium) respectively, and they were not statistically different. The maximum shoot length (2.48±0.13 cm) was obtained on PGRs free medium (Fig. 2). All shoots obtained on the medium containing different concentrations of growth appeared healthy (Table 2).

Table 2. Effect of BAP and KIN on shoot multiplication from shoot tip of *S. punctata*

Treatment (mg/l)		No. of shoot/explant	Shoot length (cm)
BAP	KIN	Mean ± SE	Mean ± SE
0.0	0.0	2.30±0.29^{d}	2.48±0.13^{a}
0.5	0.0-	7.56±2.73^{ac}	0.58±0.10^{cd}
1.0	0.0	5.50±1.02^{bcd}	0.51±0.06^{cd}
1.5	0.0	10.80±2.26^{a}	0.61±0.08^{c}
2.0	0.0	4.20±0.61^{c}	0.66±0.10^{c}
2.5	0.0	8.06±1.64^{ab}	0.53±0.06^{cd}
3.0	0.0	6.73±1.85^{b}	0.36±0.04^{d}
3.5	0.0	1.90±0.62^{d}	0.16±0.04^{d}
4.0	0.0	4.20±0.62^{cd}	0.38±0.05^{d}
0.0	0.5	3.40±0.54^{cd}	1.08±0.16^{b}
0.0	1.0	4.46±0.67^{cd}	1.21±0.24^{b}
0.0	1.5	3.66±0.55^{cd}	0.63±0.06^{c}
0.0	2.0	5.63±0.61^{bd}	0.71±0.10^{c}
0.0	2.5	6.33±0.65^{bc}	0.66±0.15^{c}
0.0	3.0	4.23±0.63^{cd}	0.60±0.09^{cd}
0.0	3.5	3.96±0.32^{cd}	0.58±0.04^{cd}
0.0	4.0	2.73±0.65^{d}	0.31±0.05^{d}

Means within columns having different letters (lower case) in superscript are significantly different at $p < 0.05$. Data are presented as mean ± SE.

5.2.2. Synergistic effect of KIN and NAA

Among the various combinations of KIN and NAA used, the MS media containing 0.5 mg/l KIN + 0.25 mg/l NAA, and 3.0 mg/l KIN + 0.25 mg/l NAA produced maximum mean number of shoots per explant (2.56±0.26 and 2.06±0.38) respectively. However, these data were not statistically different from shoots (2.30±0.29) obtained on control medium (Table 3). The highest mean shoot length (2.48±0.13 cm) was obtained on PGRs free MS medium. Shoots obtained from all combinations of KIN and NAA produced spontaneous roots, calli, dwarf shoots and showed poor multiplication rate (Fig. 2).

Table 3. Effect of combination of KIN and NAA on shoot multiplication on shoot tips of *S. punctata*

Treatment (mg/l)		Number of shoot/explant Mean ± SE	Shoot length (cm) Mean ± SE
KIN	NAA		
0.0	0.0	2.30 ± 0.29^{a}	2.48 ± 0.13^{a}
0.5	0.25	2.56 ± 0.26^{a}	0.98 ± 0.06^{b}
1.0	0.25	1.96 ± 0.33^{ab}	0.75 ± 0.05^{bc}
1.5	0.25	1.53 ± 0.27^{bc}	0.66 ± 0.11^{c}
2.0	0.25	1.06 ± 0.21^{c}	0.41 ± 0.07^{d}
2.5	0.25	1.03 ± 0.22^{c}	0.53 ± 0.10^{cd}
3.0	0.25	2.06 ± 0.38^{a}	0.45 ± 0.06^{cd}
3.5	0.25	0.40 ± 0.12^{c}	0.20 ± 0.06^{d}
4.0	0.25	0.63 ± 0.13^{c}	0.38 ± 0.07^{d}

Means within columns having different letters (lower case) in superscript are significantly different at $p < 0.05$. Data are presented as mean ± SE

5.2.3. Synergistic effect of BAP and NAA

MS medium supplemented with 3.5 mg/l BAP + 0.25 mg/l NAA produced the highest mean number (26.20±2.71) of shoots per explant (Table 4, Fig. 2). The minimum mean number of shoot (1.20±0.25) was obtained on medium containing 2.5 mg/l BAP + 0.25 mg/l NAA. Highest mean shoot length (2.48±0.13 cm) was recorded from plant growth regulators free medium. Calli were observed on shoots that were cultured on medium containing 1.5 mg/l BAP + 0.25 mg/l NAA, 3.0 mg/l BAP + 0.25 mg/l NAA and 4.0 mg/l BAP + 0.25 mg/l NAA, whereas both roots and calli were observed on shoots that were cultured on medium containing 1.0 mg/l BAP + 0.25 mg/l NAA, 2.0 mg/l BAP + 0.25 mg/l NAA and 2.5 mg/l BAP + 0.25 mg/l NAA.

Table 4. Combined effects of BAP and NAA on shoot multiplication of *S. punctata*

Treatment (mg/l)		Shoot number/explant Mean ± SE	Shoot length (cm) Mean ± SE
BAP	NAA		
0.0	0.0	2.30 ± 0.29^{d}	2.48 ± 0.13^{a}
0.5	0.25	4.13 ± 0.53^{cd}	0.53 ± 0.04^{cd}
1.0	0.25	6.56 ± 1.25^{bc}	0.61 ± 0.05^{c}
1.5	0.25	7.30 ± 0.94^{b}	0.70 ± 0.07^{c}
2.0	0.25	4.10 ± 0.59^{c}	0.71 ± 0.09^{c}
2.5	0.25	1.20 ± 0.25^{d}	0.16 ± 0.03^{d}
3.0	0.25	2.26 ± 0.43^{d}	0.51 ± 0.07^{cd}
3.5	0.25	26.20 ± 2.71^{a}	1.08 ± 0.05^{b}
4.0	0.25	1.93 ± 0.32^{d}	0.36 ± 0.04^{d}

Means within columns having different letters (lower case) in superscript are significantly different at $p < 0.05$. Data are presented as mean ± SE.

5.2.4. Synergistic effect of BAP and KIN

The highest mean numbers of shoots per explant (18.60 ± 1.85 and 18.10 ± 1.43) were obtained on media supplemeted with 3.5 mg/l BAP + 0.25 mg/l KIN and 4.0 mg/l BAP+0.25 mg/l KIN respectively. While the lowest mean number of shoots per explant (0.50 ± 0.18) was recorded on medium fortified with 2.0 mg/l BAP+1.0 mg/l KIN. The highest mean length of shoots (2.47 ± 0.16cm) was obtained from shoots cultured on growth regulators free medium (control) followed by 1.75 ± 0.28 cm from shoots cultured on medium containing 0.5 mg/l BAP+0.25 mg/l KIN. The lowest mean length of shoots (0.27 ± 0.05cm) was recorded from shoots cultured on medium supplemented with 4.0 mg/l BAP+1.0 mg/l KIN (Table 5). Cultures under this combination were appeared healthy and better in shoot proliferation rate as compared to BAP and KIN alone.

Table 5. Combined effects of BAP and KIN on shoot multiplication of *S. punctata*

Teatment (mg/l)		No.of shoot/explant Mean ± SE	Shoot length (cm) Mean ± SE
BAP	KIN		
0.0	0.0	2.40 ± 0.42^{ef}	2.47 ± 0.16^{a}
0.5	0.25	4.00 ± 0.54^{e}	1.75 ± 0.28^{b}
0.5	0.5	6.70 ± 0.56^{de}	1.16 ± 0.09c
0.5	1.0	4.50 ± 0.63^{e}	1.71 ± 0.35^{b}
1.0	0.25	4.80 ± 0.52^{e}	1.17 ± 0.12^{c}
1.0	0.5	5.50 ± 0.50^{e}	1.22 ± 0.12^{c}
1.0	1.0	9.55 ± 1.16^{cd}	0.50 ± 0.00^{d}
1.5	0.25	14.75 ± 2.22^{bc}	0.50 ± 0.00^{d}
1.5	0.5	7.60 ± 0.59^{d}	0.98 ± 0.06^{c}
1.5	1.0	7.35 ± 0.73^{d}	0.78 ± 0.05^{d}
2.0	0.25	8.25 ± 0.71^{d}	1.16 ± 0.07^{c}
2.0	0.5	2.30 ± 0.34^{f}	0.52 ± 0.04^{d}
2.0	1.0	0.50 ± 0.18^{f}	0.30 ± 0.09^{d}
2.5	0.25	11.05 ± 1.32^{c}	1.11 ± 0.05^{c}
2.5	0.5	11.25 ± 1.29^{c}	1.02 ± 0.08^{c}
2.5	1.0	11.65 ± 1.57^{c}	1.06 ± 0.08^{c}
3.0	0.25	11.05 ± 1.09^{c}	0.93 ± 0.04^{c}
3.0	0.5	9.35 ± 1.03^{cd}	0.93 ± 0.05^{c}
3.0	1.0	16.20 ± 2.46^{ab}	0.60 ± 0.04^{d}
3.5	0.25	18.60 ± 1.85^{a}	0.75 ± 0.04^{d}
3.5	0.5	15.85 ± 2.07^{ab}	1.02 ± 0.06^{c}
3.5	1.0	17.40 ± 1.62^{ab}	1.02 ± 0.06^{c}
4.0	0.25	18.10 ± 1.43^{a}	0.88 ± 0.05^{c}
4.0	0.5	0.90 ± 0.23^{f}	0.30 ± 0.05^{d}
4.0	1.0	0.55 ± 0.11^{f}	0.27 ± 0.05^{d}

Means within columns having different letters (lower case) in superscript are significantly different at $p < 0.05$. Data are presented as mean ± SE.

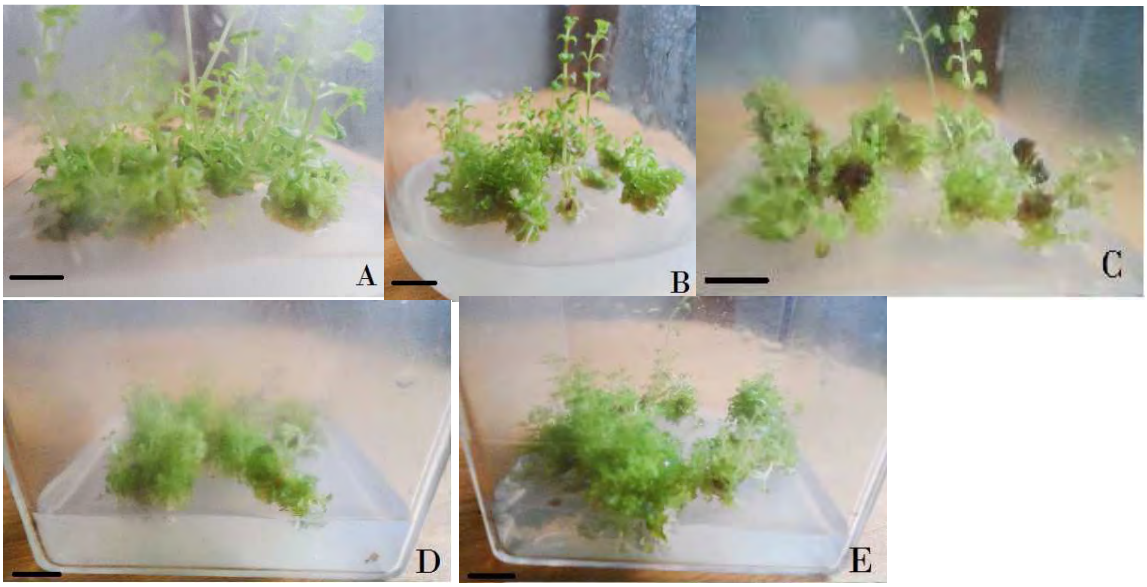

Figure 2. *In vitro* propagation of *S. punctata*. **A**. 3.5 mg/l BAP + 0.25 mg/l NAA, B. 2.0 mg/l BAP +0.25 mg/l NAA, **C**. 2.5 mg/l KIN, **D.** 3.5 mg/l BAP + 0.25 mg/l KIN, E. 4.0 mg/l BAP + 0.25 mg/l KIN. Bars = 2 cm

5.3.Root induction

Shoots cultured on half strength of MS medium containing different concentrations of IBA, IAA and NAA showed a significant difference in all measured parameters of rooting. After 4 weeks of culture, MS medium supplemented with 1.0 mg/l IBA resulted in highest mean number (5.90±0.48) of root per explant with 100% root formation frequency. The next highest root number (5.60±0.71) was obtained on the medium containing 0.5 mg/l IBA with 100% root induction response. However, roots were not produced by shoots cultured on MS medium containing NAA (0.5, 1.0, 1.5, 2.5 mg/l) or IAA (1.5, 2.5 mg/l). The shoots cultured on MS medium containing 1.5 mg/l and 2.5 mg/l IAA resulted in production of compact calli at the base of shoots. All shoots cultured on MS medium containing NAA, produced callus except those cultured on the medium containing 0.25 mg/l (Table 5). The highest root lengths (1.55±0.11 cm,

1.45±0.11 cm) were obtained on medium supplemented with 1.0 mg/l and 0.5 mg/l IBA respectively (Fig. 3).

Table 6. Root induction efficiency of *S. punctata* shoots cultured on MS medium containing IBA, IAA and NAA after 4 weeks of culture.

Treatment (mg/l)	Frequency of root formation (%)	No. of root/explant Mean ± SE	Root length (cm) Mean ± SE
IBA			
0.0	75	1.70 ± 0.30^{ef}	0.50 ± 0.08^{c}
0.25	75	3.55 ± 0.81^{cd}	0.80 ± 0.11^{b}
0.5	100	5.60 ± 0.71^{ab}	1.45 ± 0.11^{a}
1.0	100	5.90 ± 0.48^{a}	1.55 ± 0.11^{a}
1.5	70	4.05 ± 0.89^{c}	0.53 ± 0.05^{c}
2.5	55	2.25 ± 0.65^{df}	0.27 ± 0.05^{d}
NAA			
0.25	90	4.25 ± 0.49^{bc}	0.45 ± 0.03^{c}
0.5	00	0.00^{g}	0.00^{e}
1	00	0.00^{g}	0.00^{e}
1.5	00	0.00^{g}	0.00^{e}
2.5	00	0.00^{g}	0.00^{e}
IAA			
0.25	75	4.20 ± 0.68^{bd}	0.37 ± 0.04^{cd}
0.5	85	3.35 ± 0.53^{ce}	0.42 ± 0.04^{c}
1	40	2.15 ± 0.70^{de}	0.22 ± 0.06^{d}
1.5	00	0.00^{g}	0.00^{e}
2.5	00	0.00^{g}	0.00^{e}

Means within columns having different letters (lower case) in superscript are significantly different at $p < 0.05$. Data are presented as Mean ± SE.

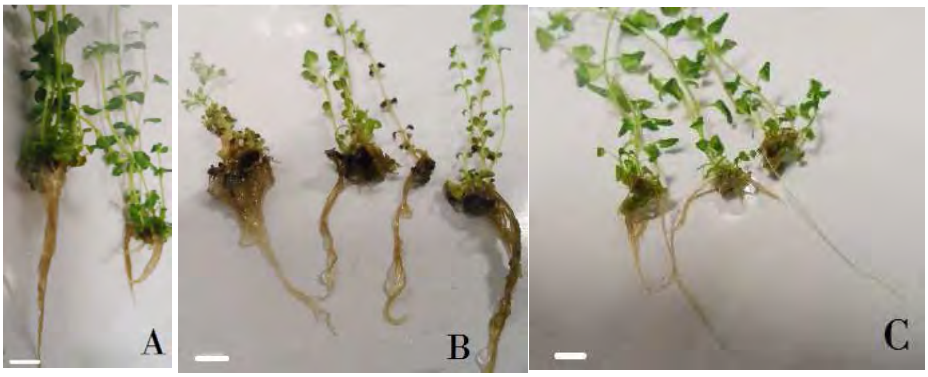

Figure 3. *In vitro* rooting of *S. punctata*. A. 1.0 mg/l IBA, B. 0.25 mg/l NAA, C. 0.25 mg/l IAA. Bars = 2 cm

5.4.Acclimatization

For acclimatization, 120 plants were transferred to pots and the percentage of survival rate was recorded after 4 weeks of planting. The highest survival rate (97.5%) was obtained. Plantlets with multiple shoots easily survived than those with a single shoot (Fig. 4).

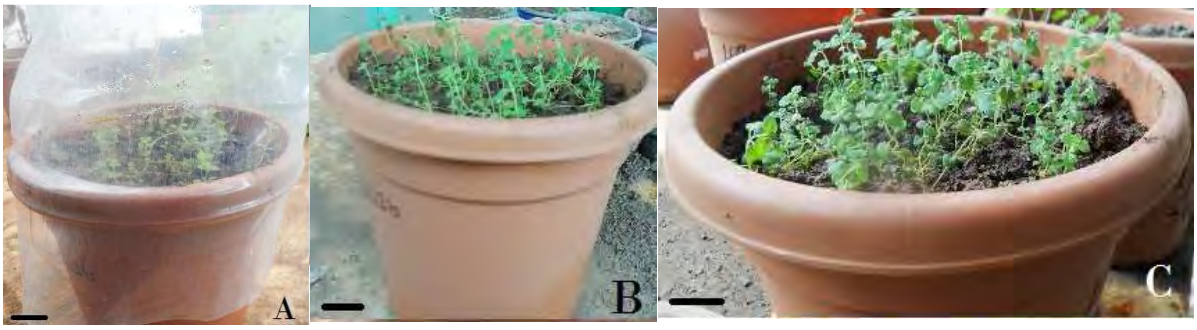

Figure 4. *Ex vitro* acclimatization of *S. punctata*. A. 10-day-old, B. 20- day-old, C. 30- day-old. Bars =2 cm

6. Discussion

6.1. Shoot induction

Cytokinins such as BAP and KIN are generally known to reduce the apical meristem dominance and induce both axillary and adventitious shoots formation from meristematic explants and the effectiveness of BAP over other cytokinins in inducing multiplication of shoot tip cultures (Sudharson *et al*., 2014).

Results of the analysis of variance revealed that different level of BAP and KIN had a highly significant ($p<0.05$) effect on shoot initiation percentage, number of shoot and shoot length. The shoot induction efficiency of BAP was better than KIN in this species. This was due to BAP mostly induce more shoots compared to KIN and similar results were reported on shoot induction of *Sida cordifolia* (Sivanesan and Jeong, 2007), in *Podophyllum hexandrum* (*Chakraborty et al.*, 2010) and *Curculago orchioides* (Shende *et al*., 2012), on *Satureja abyssinica* (Shiferaw Teshome and Teshome Soromessa, 2015), on *Glinus lotoids* (Shiferaw Teshome and Tileye Feyissa, 2015b).

On the other hand, poor shoot initiation percentage (70%) and lower number of shoots was recorded at 2.0 mg/l BAP. This result showed that shoot induction response of this plant decreased as the concentration of BAP increased. Alam *et al*. (2010) on *Ricinus communis*, Seyoum and Mekbib (2014) on *Cordeauxia edulis*, Shiferaw Teshome and Teshome Soromessa (2015) on *S. abyssinica*, Shiferaw Teshome and Tileye Feyissa (2015b) on *G. lotoids*, similarly reported that higher concentration of BAP reduce the number of shoots. However, at higher

concentration of KIN (2.0 mg/l) the number of shoots per explant increased. This is in agreement with Shiferaw Teshome and Teshome Soromessa (2015) on *S. abyssinnica.*

Raising the amount of cytokinin in medium resulted in reduction of shoot length, because cytokinins promote axillary shoot formation by decreasing apical dominance (Sudherson et al., 2014). Similarly in this investigation as BAP concentration increased, shoot length decreased and mean number of shoot per explant increased. Similar results have been reported by Seyoum and Mekbib (2014) on *Cordeauxia edulis*, Shiferaw Teshome and Teshome Soromessa (2015) on *S. abyssinica*, Shiferaw Teshome and Tileye Feyissa (2015b) on *G. lotoids.*

In this study, the effects of different concentrations of KIN on shoot initiation were investigated. Of the two cytokinins tested, BAP was more effective than KIN in percentage of shoot induction and multiple shoot proliferation, but KIN was more effective in shoot length. Low concentration of BAP promotes multiple shoot induction. Similar findings were reported by Velayutham, *et al*. (2012) on *Hybanthus enneaspermus*, Shiferaw Teshome and Teshome Soromessa (2015) on *Satureja abyssinica*. In contrast, Arunkumar and Jayaraj (2011) reported that KIN was more effecive in producing more shoots of *Ionidium suffruticosum.*

Vitrified cultures were induced at higher concentrations (1.5 and 2.0 mg/l) of BAP and this is parallel with previous report on *Satureja abyssinica* (Shiferaw Teshome and Teshome Soromessa, 2015).

6.2. Shoot multiplication

The concentration of exogenous cytokinin appears to be main factor affecting shoot multiplication. BAP has a marked effect in stimulating the growth of axillary and shoot tip cultures (Sudharson *et al*., 2014).

Different concentrations of BAP and KIN on shoot multiplication were investigated. The present results revealed that BAP had highly significant ($p<0.05$) effects on shoot multiplication of *S. punctata.* Among different BAP concentrations used, the highest number of shoots per explant was recorded on medium containing 1.5 mg/l BAP. Multiple shoot production capability of BAP was also reported in *G. lotoides* (Shiferaw Teshome and Tileye Feyissa, 2015a, 2015b). in this particular plant (1.5 mg/l) BAP was responded better than other concentrations, this might be the endogenous and exogenous level was optimized at this particular concentration, however, the induction of multiple shoots per explant declined with increased concentration of BAP beyond the optimal level. Similar result was reported on *Gloriosa superba* (Venkatachalam *et al*., 2012), *G. lotoides* (Shiferaw Teshome and Tileye Feyissa, 2015a, 2015b), *S. abyssinica* (Shiferaw Teshome and Teshome Soromessa, 2015).

The highest mean shoot length (2.48 ±0.13 cm) was obtained from PGRs free medium while lowest mean shoot length was recorded from media supplemented with BAP and KIN. This result showed that PGRs free medium was more effective in achieving better shoot length because application of cytokinins reduce apical dominance and induce lateral axillary shoots (Sudharson *et al*., 2014; Shiferaw Teshome and Tileye Feyissa, 2015b). This is in disagreement with Shiferaw Teshome and Teshome Soromessa (2015) on *S. abyssinica*.

BAP was the most suitable cytokinin for *S. punctata* micropropagation to obtain multiple shoots. These results are in agreement with those obtained by some researchers, Baba and Yurekli (2000) worked on *Thymus sipyleus*, Fraternale *et al*. (2003) worked on *Thymus mastichina* and Aicha *et al*. (2013) on *Thymus satureioides.* Nevertheless, this is in disagreement to earlier report on *S. abyssinica* (Shiferaw Teshome and Teshome Soromessa, 2015).

6.2.1. Synergistic effect of KIN and NAA

In this investigation, KIN in combination with NAA produced relatively less number of shoots than KIN alone. Thirupathi *et al*. (2013) reported that the number of shoots per explant was less in comparison to KIN alone on *Paederia foetida*. There was the negative interaction effect between KIN and NAA that resulted in reduction of mean number of shoots per explant. Whereas, contrasting result was reported by Jesmin *et al*. (2013) on *Tridax procumben,* which responded more number of shoots from combination of KIN and NAA. But, in this study mean number of shoots under this combination was not significantly different from the mean number of shoots on PGRs free medium. The mean number of shoots in some of the combinations was lower than on control medium and this is in agreement with Shiferaw Teshome and Teshome Soromessa (2015) report on *S. abyssinica.*

Spontaneous root and callus formation was observed almost on all media supplemented with KIN in combination with NAA. This result might be due to the presence and interaction of NAA with KIN. Many authors observed that presence of auxin in medium is necessary for root and callus induction in *P. minima* (Farhana *et al*., 2009; Sheeba *et al*., 2010; Mungole *et al*., 2011) , in *Physalis peruviana* (Otroshy *et al.,* 2013), in *S. abyssinica* (Shiferaw Teshome and Teshome Soromessa, 2015).

6.2.2. Synergistic effect of BAP and NAA

Among all BAP + NAA combinations used, medium supplemented with 3.5 mg/l BAP + 0.25 mg/l NAA resulted in highest mean number of shoots per explant. BAP in combination with NAA produced higher number of shoots than BAP alone. BAP in combinations with NAA was

found to be effective in many species (Ndoye *et al*., 2003; Ahmad *et al*., 2002). But in case of *Tridax procumbens* L. (Jesmin *et al.,* 2013) using BAP alone was considered more effective.

Spontaneous rooting and callus formation were observed on some media fortified with BAP and NAA. Several authors observed that NAA was more effective PGR for callus induction than any other auxins (Rehman *et al*., 2003; Velayutham *et al*., 2006; Velayutham *et al*., 2012).

6.2.3. Synergistic effect of BAP and KIN

MS medium supplemented with combination of BAP and Kinetin in different concentrations was found to be effective in enhancing shoot number per explant and shoot length and several authors reported that the synergistic combination of two cytokinins was more effective for shoot proliferation (Selvaraj *et al.,* 2006; Velayutham *et al*., 2006).

The ANOVA revealed that highest mean number of shoots per explant was obtained at optimal concentration of BAP in combination with KIN (3.5 mg/lBAP+0.25 mg/l KIN and 4.0 mg/l BAP +0.25 mg/l KIN). Shoot proliferation was increased as concentrations of BAP and KIN increased. This is in agreement with well-documented reports for medicinal plants such as *Swertia corymbosa* (Mahendran and Bai, 2014), *S. chirata* (Balaraju *et al.,* 2009), and *Bauhinia racemosa* (Rajanna *et al.,* 2011). Similar synergistic effect of the two cytokinins for enhanced shoot multiplication was also reported in *Acalypha indica* (Saikia and Pratap, 2014) and in *S. abbysinica* (Shiferaw Teshome and Teshome Soromessa 2015). However, supra-optimal concentrations of BAP and KIN showed significant reduction in multiple shoot formation, which agrees with Baskaran *et al*. (2005) in *Eclipta alba,* Balaraju *et al*. (2008) in *Vitex agnus-castus,* Rasool *et al*. (2009) in *Prunella vulgaris,* Loganathan and Bai (2015) in *Enicostema axillare* and Shiferaw Teshome and Tileye Feyissa (2015b) in *Glinus lotoides*.

However, the average shoot length of *S. punctata* decreased as the concentration of BAP increased from 0.5mg/l to 4.0mg/l. This finding was in agreement with the work of Zuraida *et al*. (2014) in *Melicope lunu-ankenda.*

6.3. Root induction

6.3.1. Effect of IBA, NAA and IAA on root formation of *S. punctata*

Chhun *et al*. (2003) have shown that exogenous application of auxins results in increased initiation of lateral roots and that lateral root development was highly dependent on auxin and auxin transport.

Maximum rooting success was observed when elongated shoots were cultured on MS medium containing 0.5 and 1.0 mg/l IBA. But increasing IBA concentration to 2.5 mg/l resulted in a reduction of rooting response. This investigation was in agreement with the work on *in vitro* regeneration and mass propagation of *Hybanthus enneaspermus* (Velayutham, *et al*., 2012) and on *Melicope lunu-ankenda* (Zuraida, *et al*., 2014).

The highest number of roots per explant and the longest roots were obtained on MS medium containing 0.5 mg/l and 1.0 mg/l IBA respectively, while the lowest number of roots was obtained on growth regulators free medium (control), and the lowest shoot length was recorded at 2.5 mg/l IBA. This result indicated that the increased concentration reduced mean number of roots as well as root length. This was in agreement with studies carried out on other plant species such as *Dendrobium orchid* (Rafique *et al.*, 2012), *Dalbergia sissoo* (Ali *et al*., 2012) and

Tridax procumbens L. (Jesmin *et al*., 2013). Contrasting result was reported by Velayutham *et al*. (2012).

Besides declined mean number and mean length, higher concentrations of IBA (1.5, 2.5 mg/l) showed callus formation at basal end of micro-shoot accompanied with rooting, because callus tissue inhibited the vascular supply to rooting so the mean number and mean length was also declined as compared to lower concentration of IBA. The same result was reported by Saha *et al*. (2010) on two plant species *Ocimum basilicum* L. and *Mentha piperita* L.

On the other hand, the addition of IAA to any of the concentrations used resulted in a reduction of rooting as compared to controls that did not receive auxins. Lower concentration of IAA favored increase in root number and length. This also indicated that increased concentration of IAA resulted in poor rooting and induced spontaneous root accompanied with callus at cut ends of micro-shoots. The present investigation was directly coinciding with other studies (Thirupathi *et al*., 2013; Kanungo *et al*., 2012; Arunkumar *et al*., 2011 and Chinnamadasamy *et al.*, 2010).

Among all NAA concentrations (0.25, 0.5, 1.0 1.5 and 2.5 mg/l) used in the present study, the best rooting response, mean number of root and root length were accounted only on medium fortified with 0.25 mg/l NAA. Similar results on root formation were obtained in *Portulaca grandiflora* (Jain and Bashir, 2010). Higher level of NAA initiated callus formation at basal end of micro-shoots rather than root formation. In contrast, high concentration of NAA (2.0 mg/l) required to produce maximum number of root and root length were reported in *Paederia foetida* L. (Thirupathi, 2013).

Spontaneous rooting was observed in the explants cultured on medium without any PGRs (control). This might be due to the presence of endogenous PGRs in the explant, thus it required

optimal endogenous levels of PGRs for rooting as reported in *Spilanthes mauritian* (Shahzad, *et al.*, 2009).

In the present experiment, the highest response of root formation, mean number and mean length of root were seen in the presence of IBA. This result showed that IBA was superior and more effective for root induction than other auxins (NAA and IAA). Similar responses were observed in different plant species *Gymnema sylvestre* (Komalavalli and Rao, 2000), *Gloriosa superba* (Sivakumar and Krishnamurthy, 2000) and *Tridax procumbens* L. (Jesmin *et al*., 2013), *S. abyssinica* (Shiferaw Teshome and Teshome Soromessa, 2015), *G. lotoides* (Shiferaw Teshome and Tileye Feyissa, 2015a).

7. Conclusion

S.punctata is a multi-purpose plant where most parts especially the areal parts of the plant are usable. Although the species has multiple uses, it is threatened with extinction. This has led to poor or none natural regeneration which leads to rescue rare, endemic and endangered species through mass and continuous plantlet production within short period of time. In the present study, an efficient protocol of *S.punctata* for *in vitro* propagation of shoots derived from seedlings was established. The results from the study demonstrated that a high rate of shoot induction and root formation of *S. punctata* could be achieved by modifying growth regulator (cytokinins and auxins) concentration in the culture medium. Medium fortified with 0.5mg/l BAP was highly responded for shoot initiation and maximum mean number of shoots was obtained. The presence of BAP and NAA (3.5 mg/l BAP+0.25 mg/l NAA) in the medium was important for the development of shoots and highest mean shoot number per explant was obtained. Effective root formation was observed in shoots cultured on MS medium containing 0.5 mg/l and 1.0mg/l IBA. *In vitro* regenerated shoots were acclimatized to a greenhouse and plantlets were survived successfully. This is the first attempt *in vitro* propagation of *S. punctata*, which can be used for the large scale multiplication. Hence, the aforementioned potential benefits of the outputs of this study could be used in the areas of future conservation, research and development of elite genotypes for further pharmacological evaluations of this important medicinal plant species.

8. Recommendations

- Since the *in vitro* propagation protocols of this valuable medicinal and aromatic herb, *S. punctata* has been developed, anyone interested for commercial cultivation and domestication is recommended to utilize this protocol effectively.
- Further *in vitro* propagation method can be developed using direct and indirect embryogenesis.
- Since this study was in a particular laboratory condition on particular genotype, reproducibility of the results must be checked or tested.

References

Ahmad, S., Amin, M.N., Azad, M.A.K, and Mosaddik, A.M. (2002). Micropropagation and plant regeneration of *Rauvolfia serpentina* by tissue culture technique. *Pakistan Journal of Biological Sciences.* **5:** 75-79.

Aicha N., Abdelmalek, El. M. and Rachida, C.T. (2013). Micropropagation of *Thymus satureioides* Coss. an endangered medicinal plant of Morocco. *Journal of Agricultural Technology*. **9:** 487-501.

Alam, I., Sharmin, S.A., Mondal, S.C., Alam, M., Khalekuzzaman, M. and Anisuzzaman, M., (2010). *In vitro* micropropagation through cotyledonary node culture of caster bean (*Riceinus communis* L.). *Australian Journal of crop science*. **4**:81-84.

Ali A., Muhammad R., Abdul, M., Asif, S. and Naima, H.N. (2012). Effect of media type and explants source on micropropagation of *Dalbergia sissoo*: A tree of medicinal importance. *Journal of Medicinal Plants Research*. **6:** 1742-1751.

Aloni, R. (1995). The induction of vascular tissues by auxin and cytokinin. **In:** *Plant Hormones* pp.531-546 (Davies, P. J., ed.). Kluwer Academic Publishers, Dordrecht.

Altman, A. (1999). Plant biotechnology in the 21st century: the challenges ahead. *Electronic Journal of Biotechnology* **2**:51-55.

Arunkumar, B.S. and Jayaraj, M. (2011). Effect of auxins and cytokinins on *in vitro* propagation of *Ionidium suffruticosum* Ging- A seasonal multipotent medicinal herb.*International Journal of Research in Ayurveda and Pharmacy*. **2:**198-203.

Asha-Rani, N.S. and Prasad, M.P. (2014). Auxin effect on high frequency root induction and callus generation from *Atropa belladonna. Indian Journal of Advances in Plant Research.***1**:57-60.

Baba Erdag, B. and Yurekli, A.K. (2000). *In vitro* propagation of *Thymus sipyleus* Boiss. (Lamiaceae). *Turkish Journal of Biology.* **24**:81–86.

Babadi, E.S., Pirbalouti, A.G.,Nourafcan, H. and Hamedi, B. (2012). Bioactivity of Essential Oil of Bakhtiari Savory (Lamiaceae). *Electronic Journal of Biology*. **8:** 73-78.

Balaraju, K., Agastian, P., Preetamraj, J.P., Arokiyaraj, S. and Ignacimuthu, S. (2008). Micropropagation of *Vitex agnus-castus*, (Verbenaceae) — a valuable medicinal plant. *In Vitro Cell. Dev. Biol. – Plant.* **44**:436–441.

Balaraju, K., Agastin, P. and Ignacimuthu, S. (2009). Micropropagation of *Swertia chirata* Buch.-Hams. ex Wall. a critically endangered medicinal herb. *Acta Physiol. Plant*. **31**: 487-494.

Bandurski, R. S., Cohen, J. D., and Slovin, J., (1995). Auxin biosynthesis and metabolism. **In**: *Plant Hormones*, pp.39-65, (Davies, P. J., ed.). Kluwer Academic Publishers, Dordrecht.

Baskaran, P. and Jayabalan, N. (2005). An efficient micropropagation system for *Eclipta alba* - A valuable medicinal herb. *In Vitro Cell. Dev. Biol. — Plant.* **41**:532–539.

Belay, G., Tariku, Y., Hymete, A. and Yalemtsehay, M. (2011). Ethnopharmacological investigations of essential oils isolated from five Ethiopian medicinal plants against eleven pathogenic bacterial strains. *Phytopharmacology*. **1:** 133-143.

Chagonda, S.K. and Chalchat J.C. (2005). The composition of the leaf essential oil of *Satureja punctata* (Benth.) Briq. from Zimbabwe. *Flavour Fragr. J.* **20:** 316–317.

Chakraborty, A., Bhattacharya, D., Ghanta, S. and Chattopadhyay, S. (2010). An efficient protocol for in vitro regeneration of *Podophyllum hexandrum*, a critically endangered medicinal plant. *Indian Journal of Biotechnology*. **9**:217-220.

Chhun, T., Taketa, S., Tsurumi, S. and Ichii, M. (2003). "The efects of auxin on lateral root initiation and root gravitropismin a lateral rootless mutant Lrt1 of rice (*Oryza sativa* L.)," *Plant Growth Regulation*. **39**:161–170.

Chinnamadasamy, K., Arjunan, D. and Ramasamy, M. V. (2010). Rapid micropropagation of *Plumbago zeylanica* L. - An important medicinal plant. *Journal of American Science.* **6:** 1027-1031.

Chizzola, R. (2003). Volatile Oil Composition of Four Populations of *Satureja Montana* L. from Southern France, *Acta Hort.* **598:** 143-147.

D'Avigdor, E., Wohlmuth, H., Zemede A. and Tesfaye A. (2014).The current status of knowledge of herbal medicine and medicinal plants in Fiche, Ethiopia. *Journal of Ethnobiology and Ethnomedicine*. **1:**10:38.

Davies, P. J. (1995). Plant Hormones, pp13-38. Kluwer Academic Publishers, Dordrecht.

Evans, D. A., Sharp, W. R., and Flick, C. E. (1981). Growth and behavior of cell cultures: embryogenesis and organogenesis. **In:** Plant Cell Culture: Methods and Applications in Agriculture, pp 45-113, (Thorpe, T. A., ed.). Academic Press, New York.

Fabijan, D. Taylor, J. S. and Reid, D. M. (1981). Adventitious rooting in hypocotyls of sunflower (*Helianthus annum*) seedlings. II. Actions of gibberellins, cytokinins and ethylene. *Physiol. Plant.* **53:**589-597.

Farhana, A., Hassan, S.AKM., Shamroze, B.L., Sultana, R., Begum, N., Jahan, MAA, and Khatun, R. (2009). *In vitro* shoot proliferation and plant regeneration of *Physalis minima* L.- a perennial medicinal herb. *Bangladesh J Sci Ind*. **44**: 453−456.

Fraternale, D., Giamperi, L., Ricci, D., Rocchi, M.B.L., Guidi, L., Epifano, F. and Marcotullio, M.C. (2003). The effect of triacontanol on micropropagation and on secretory system of *Thymus mastichina. Plant Cell Tissue and Organ Culture.* **74**:87-97.

Gaspar, T., Kevers, C. and Hausman, J. (1994). Peroxidase activity and endogenous free auxin during adventitious root formation. **In**: Physiology, Growth and Development of Plants in Culture, pp289-298, (Lumsden, P. J. Nicholas, J. R. and Davies, W. J., ed.). Kluwer Academic Publishers, Dordrecht.

Gasper, T., Kevers, C., Penel, C., Greppin, H., Reid, D.M. and Thorpe, T.A. (1996). Plant hormones and plant growth regulators in plant tissue culture. *In Vitro Cell. Dev. Biol. Plant*. **32:**272-289.

Hartman, H.T., Kester, D.E., Davies, F.T. and Geneve, R.L. (1997). Plant Propagation: Principles and Practice. 6th ed., pp.770. Prentice hall, Upper Saddle River, New Jersey.

Hedberg, I., Kelbessa, E., S. Edwards, S., Demissew, S. and Persson, E. 2006. Flora of Ethiopia and Eritrea. Vol. 5, Addis Ababa University, Addis Ababa, pp. 516–517.

IBC (Institute of Biodiversity Conservation) (2007). Ethiopia: Second Country Report on the State of Plant Genetic Resources for Food and Agriculture to FAO.

Jain, A.K. and Bashir, M. (2010). Efficient micropropagtion protocol for *Portulaca grandiflora*. Hook. using shoot tip explants. *New York Science Journal*. **3**: 112-116.

Jesmin, S., Alam M. F., and Sarker, A.Q. (2013). Multiple shoot proliferation in *Tridax procumbens* L. through in vitro method. *Int. J. Biosci***. 3:**177-187.

Kanungo, S. and Sahoo, S.L. (2011). Direct organogenesis of *Withania somnifera* L. from apical bud. *Int. Res. J. Biotechnol.* **2:**58-61**.**

Kanungo, S., Pradhan, C., Sahoo, S.L. and Sahu, R.K. (2012). Role of auxins in the in vitro rooting and micropropagation of *Holarrhena antidysenterica* Wall., a woody aromatic medicinal plant, through nodal explants from mature trees. *Journal of Medicinal Plants Research*. **6:** 4660-4666.

Kaufmann, M. and Wink, M. (1994). Molecular Systematics of the Nepetoideae (family Labiatae): Phylogenetic Implications from rbcL Gene Sequences. Heidelberg, Bundespublik Deutschland.

Kebede, D. K., Alemayehu A., Binyam G., and Yunis, M. (2006). A historical overview of traditional medicine practices and policy in Ethiopia. *Ethiop.J.Health Dev*. **20:**127-134.

Kher, M.M., Joshi, D., Nekkala, S., Nataraj, M. and Raykundaliya, D.P. (2014). Micropropagation of *Pluchea lanceolata* (Oliver & Hiern.) using nodal explant. *Journal of Horticultural Research.* **22:** 35-39.

Komalavalli, N. and Rao, M.V. (2000). *In vitro* micropropagation of *Gymnema sylvestre* – A multipurpose medicinal plant. *Plant Cell Tiss. Org. Cult.* **61:** 97-105.

Krikorian, A.D. (1995). Hormones in tissue culture and microprupagation. **In:** *Plant Hormones,* pp.774-796, (Davies, P. J., ed.). Kluwer Academic Publishers, Dordrecht.

Kubitzki, K. 2004. The Families and Genera of Vascular Plants. **In**: Flowering Plants • Dicotyledon; Lamiales (except Acanthaceae including Avicenniaceae), pp. 239, (Kadereit, J.W. eds). Springer-Verlag, Berlin.

Kuhnle, J. A. Fuller, G. and Corse, J. (1977). Antisenescent activity of natural cytokinins. *Plant Physiol.* **41**:14-21.

Lahlou, M. (2004). Methods to study the Phytochemistry and bioactivity of essential oils. *Phytotherapy Research.* **18:** 435–448.

Lehoux, H. and Chakib, A. (2012). Non wood forest products. Ethiopia. http://www.fao.org/forestry/nwfp/78836/en/eth/.

Liu, J. H.; Reid, D. M. (1992). Auxin and ethylene-stimulated adventitious rooting in relation to tissue sensitivity to auxin and ethylene production in sunflower hypocotyls. *J. Exp. Bot.* **43:**1191-1198.

Loganathan, K. and Bai, N.V. (2015). Root culture of *Enicostema Axillare* (Lam.) Raynal ssp. *littoralis* (Blume) Raynal: An important medicinal plant. *Res. Med. Arom. Plants.* **1:**1-10.

Mahendran, G. and Bai, N.V. (2014). Micropropagation, antioxidant properties and phytochemical assessment of *Swertia corymbosa* (Griseb.) Wight ex C. B. Clarke: a medicinal plant. *Acta Physiol Plant.* **36:** 589-603.

Malik, C. P., Garg, P., singh, Y. and Grover, S. (2012). Medicinal uses, chemical constituents and micropropagation of three potential medicinal plants. International Journal of Life Science and Pharma Research. **2:**57-76.

Miladi, H., Slama, R.B., Mili, D., Zouari, S., Bakhrouf, A. and Ammar E. (2013). Chemical composition and cytotoxic and antioxidant activities of *Satureja montana* L. Essential Oil and its antibacterial potential against *Salmonella* spp. strains. *Journal of Chemistry*. **1:** 1-10.

Mohamed I.E., Nur E.l, Abdelrahman M.E. (2010). The antibacterial, antiviral activities and phytochemical screening of some Sudanese medicinal plants, *Journal Bio Sci*. **4:** 8-16.

Momtaz, S. and Abdollahi, M. (2010). An update on pharmacology of satureja species: From antioxidant, antimicrobial, antidiabetes and antihyperlipidemic to reproductive stimulation. *International journal of pharmacology*. **6**:454-461.

Mungole, A.J., Doifode, V.D., Kamble, R.B., Chaturvedi, A. and Zanwar, P. (2011). *In-vitro* callus induction and shoot regeneration in *Physalis minima* L. *Ann Biol Res* **2**: 79−85.

Nalawade, S.M., Sagare, A.P., Lee, C.Y., Kao, C.L. and Tsay, H.S. (2003). Studies on tissue culture of Chiness medicinal plant resources in Taiwan and their sustainable utilization. *Botanical Bulletin of Academia Sinica*. **44:** 79-98.

Ndoye, M., Diallo, I. and Gassama, Y.K. (2003). *In vitro* multiplication of the semiarid forest tree, *Balanites aegyptiaca* (L.) Del. *The American Journal of Botany.* **2:** 421-424.

Niemeyer, H. M. (2010). Composition of essential oils from *Satureja darwinii* (Benth.) Briq. and *S. muitifiora* (R. et P.) Briq. (Lamiaceae). Relationship between chemotype and Oil Yield in satureja spp. *Journal of Essential Oil Research.* **22**:477-482.

Nooden, L. D.; Leopold, A. C. (1988). Senescence and Aging in Plants. Academic Press; San Diego .

Otroshy, M., Mokhtari, A., Mohammad, S., Khodaee, M. and Bazrafshan, A.H. (2013). Direct regeneration from leaves and nodes explants of *Physalis peruviana* L. *International Journal of Farming and Allied Science.* **1**: 214-218.

Rafique, R., Fatima, B., Mushtaq, S., Shahid Iqbal M., Rasheed, M. Ali M., and Zia Ul Hasan S. (2012). Effect of indole-3-butyric acid (IBA) on in vitro root induction in dendrobium orchid *(Dendrobium sabin* H.*). African Journal of Biotechnology*. **11**: 4673-4675.

Rajanna L.N., Sharanabasappa G., Seetharam Y.N., Aravind B., Mallikharjuna P.B. 2011. *In vitro* regeneration of cotyledonary node explant of *Bauhinia racemosa. Bot. Res. Int.* **4**: 75-80.

Rasool, R., Kamili, A.N., Ganai, B.A. and Akbar, S. (2009). Effect of BAP and NAA on shoot regeneration in *Prunella vulgaris. Journal of Natural Sciences and Mathematic.* **3**:21-26.

Rehman, R.U., Israr, M., Srivastava, P.S., Bansal, K.C. and Abdin, M.Z. (2003). *In vitro* regeneration of witloof chicory (*Cichorium intybus* L.) from leaf explants and accumulation of esculin. In vitro *Cellular Developmental Biology Plant*. **39**: 142-146.

Robert, J., Xavier ravi, B. and Louis, C. (2012). An efficient *in vitro* plant regeneration of *Dipteracanthus prostratus* (Poir.) Nees.- a medicinal herb. *Asian Pacific Journal of Tropical Biomedicine*. 484-487.

Saha,S. Ghosh, P. D. and Sengupta, C. (2010). An Efficient Method for Micropropagation of *Ocimum basilicum L. Indian J. Plant Physiol.* **15**: 168-172.

Saha,S. Ghosh, P. D. and Sengupta, C. (2010). *In Vitro* Multiple Shoot Regeneration of *Mentha piperita.Journal of Tropical Medicinal Plants*. **11:** 89- 92.

Saikia, M. and Pratap, J.H. (2014). *In vitro* Propagation of *Acalypha indica* Linn.: a medicinally important plant. *Int. J. Life Sci. Res*. **3**: 86-93.

Satil, F. and Kaya, A. (2007). Leaf anatomy and hairs of Turkish *Satureja* L. (Lamiaceae). *Acta Biologica Cracoviensia Series Botanica*. **49:** 67–76.

Selvaraj, N.,Vasudevan, A., Manickavasagam, M. and Ganapathi, A. (2006). *In vitro* organogenesis and plant formation in cucumber. *Biol. Plant*. **50**: 123–126.

Seyoum, Y. and Mekbib, F. (2014). *In vitro* germination and direct shoot induction of Yeheb *(Cordeauxia edulis* Hemsl.*). Agriculture, Forestry and Fisheries.;* 3: 452-458.

Shahzad, A., Sharma, S., Jan, N. and Sahai, A. (2009). *In vitro* studies on shoot regeneration through variuos explants and alginate-encapsulated nodal segments of *Spilanthes mauritian* DC.An endagered medicinal herb. *International journal plant developmental biology*.3: 56-61.

Sharma, S., Rathi, N., Kamal, B., Pundir, D., Kaur, B. and Arya, S. (2010). Conservation of biodiversity of highly important medicinal plants of India through tissue culture technology- a review. *Agric. Biol. J. N. Am.* **1:**827-833.

Sheeba, E., Parvathy, S. and Palanivel, S. (2010). Direct Regeneration from leaves and nodes explants of *physalis Minima* Linn. *Europ. J. App.l Sci.* **2:**58−61.

Shende, C.B., Undal, V.S., and Chaudhari, U.S. (2012). *In vitro* propagation of *Curculago orchioides* from rhizome bud. *Journal of Agricultural Technology*. **8:** 353-362.

Shiferaw Teshome and Teshome Soromessa (2015). *In Vitro* Propagation of *Satureja abyssinica* (Benth.) Briq. – A valuable medicinal plant. *Advances in Life Science and Technology*, **34**:100-109.

Shiferaw Teshome and Tileye Feyissa (2015a). *In vitro* callus induction and shoot regeneration from leaf explants of *Glinus lotoides* (L.)—An important medicinal plant. *American Journal of Plant Sciences*, **6**:1329-1340.

Shiferaw Teshome and Tileye Feyissa (2015b). Micropropagation of *Glinus Lotoides* L.: An endangered medicinal plant. *Advances in Life Science and Technology*, **34**:32-41.

Sivakumar, G. and Krishnamurthy, K.V. (2000). Micropropagation of *Gloriosa superba* L. – an endangered species of Asia and Africa. *Current Science*, **78**: 30–32.

Sivanesan, I. and Jeong, B.R. (2007). Direct shoot regeneration from nodal explants of *Sida cordifolia* Linn. *In Vitro* Cellular and Developmental Biology- *Plant* **43**:436-441.

Stefancic, M., Stampar, F. and Osterc, G. (2005). Influence of IAA and IBA on root development and quality of *Prunus* ' GiSeIA5' leafy cuttings. *Hortscience.* **40**:2052-2055.

Sudharson, S., Anbazhagan, M., Balachandran, B. and Arumugam K. (2014). Effect of BAP on *in vitro* propagation of *Hybanthus enneaspermus* (L.) Muell, an important medicinal plant *Int.J.Curr.Microbiol.App.Sci* **3:** 397-402.

Tariku, Y., Hymete, A., Hailu, A. and Rohloff, A. (2010). Essential-oil composition, antileishmanial, and toxicity study of artemisia abyssinica and *Satureja punctata* ssp. punctata from Ethiopia. *Chemistry & Biodiversity*. **7:** 1009-1018.

Teklu, S., Alemayehu, G. and Abegaz, B. M. (1998). Chemical composition of the essential oil of Satureja punctata. *J. Essent. Oil Res.* **10:**339-341.

Tepe, B. (2015) Inhibitory effect of satureja on certain types of organisms. *Rec. Nat. Prod.* **9:** 1-18.

Tesfaye, W., Ephrem, E., Kaleab, A. and Wondwossen, E. (2010). Evaluation of hepatoprotective activities of *Satureja punctata* (Benth.) Briq. and *Solanecio angulatus*

Vahl Jeffrey in ferric nitrillotriacetate induced hepatotoxicity in rats. *Ethiop. Pharm. J.* **28**: 63-74.

Thirupathi, M., Reddy, K.J. and Srinivas, D. (2013). High frequency of multiple shoots induction in *Paederia foetida* (L.)- A rare medicinal plant. *Plant.* **1**: 60-65.

Thorpe, T. A. (2007). History of plant tissue culture. *Mol Biotechnol*. **37**:169–180.

Vasil, I. K. and Thorpe, T. A. (1994). Plant Cell and Tissue Culture. Kluwer Academic Publishers, Dordrecht.

Vasisht, K. and Kumar, V. (2002). Medicines and Natural Health Products Trade and Production of Herbal. International Centre for Science and High Technology, Italy.

Velayutham, P., Karthi, C., Nalini, P. and Jahirhussain, G. (2012). *In vitro* Regeneration and mass propagation of *Hybanthus enneaspermus* (L.) F. Muell. from the stem explants through callus culture. *Journal of Agricultural Technology*. **8**: 1119-1128.

Velayutham, P., Ranjithakumari, B.D. and Baskaran, P. (2006). An efficient *in vitro* plant regeneration system for *Cichorium intybus* L. an important medicinal plant. *J. Agri.Tech*. **2**:287-298.

Venkatachalam, P., Ezhili, N. And Thiyagarajan, M.(2012). *In Vitro* Shoot Multiplication of *Gloriosa superba* L. – An Important Anticancer Medicinal Herb. International Conference on Biotechnology, Biological and Biosystems Engineering, Thailand.

Vesely, J., Havlicek, L. and Strnad, M. (1994). Inhibition of cyclin-dependent kinases by purine analogues. *Eur. J. Biochem*. **224:**771-786.

Vines, G. (2004). Herbal harvests with a future: towards sustainable sources for medicinal plants. Plantlife International, www.plantlife.co.uk.

Vinothkumar, D., Murugavelh, S. and Senthilkumar, M. (2011). Clonal propagation of *Wattakaka volubilis* through nodal explant culture. *Cey. J. Sci. (Bio. Sci.)***. 40**: 53-58.

WHO. Fact Sheet, Traditional Medicine, Geneva, May 2003.

Zuraida, A.R, Fatin, L. K. AND Ayu, N. O. (2014). *In vitro* plant propagation for rapid multiplication of *Melicope lunu-ankenda*: A plant species of high medicinal value. *Int J Pharm Bio Sci*. **5:**1148 – 1156.

Appendices

Appendix 1. Full MS basal medium stock solution composition

Components	Concentration (g/L)	Media preparation
Micronutrient		
$ZnSO_4.7H2O$	1.72	5ml/l
H_3BO_3	1.24	
* $MnSO_4.H2O$	3.38	
* $MnSO_2.4H2O$	4.46	
$CuSO_2.5H2O$	0.05	
KI	0.166	
$NaMoO_4.2H2O$	0.05	
$CoCl_2.6h2O$	0.05	
Na_2 EDTA	7.472	
$FeSO_4.7H_2O$	5.56	
Macronutrients		
NH_4NO_3	33	50ml/l
KNO_3	38	
$Cacl_2.2H_2O$	8.8	
$MgSO_4.7H_2O$	7.4	
KH_2PO_4	3.4	
Vitamins		
Myo-inositol	20	5ml/l
Glycin(glycocoll)	0.4	
Nicotinic acid (NaOH)	0.1	
Pyridoxin (B6)	0.1	
Thiamin (B1)	0.02	

* Alternatives

Appendix 2. ANOVA table for initiation

		Sum of squares	df	Mean square	F	Sig.
No.of shoot/explant	b/n groups	774.600	8	96.825	6.109	.000
	Within groups	2710.350	171	15.850		
	Total	3484.950	179			
Shoot length	b/n groups	71.411	8	8.926	17.667	.000
	Within groups	86.400	171	.505		
	Total	157.811	179			

Appendix 3. ANOVA table for BAP and KIN

		Sum of squares	df	Mean square	F	Sig.
No.of shoot/explant	b/n groups	2538.969	16	158.686	3.702	.000
	Within groups	21133.167	493	42.866		
	Total	23672.135	509			
Shoot length	B/n groups	130.782	16	8.174	22.341	.000
	Within groups	180.370	493	.366		
	Total	311.152	509			

Appendix 4. ANOVA table for BAP+NAA

		Sum of squares	df	Mean square	F	Sig.
No.of shoot/explant	B/n groups	14517.200	8	1814.650	49.805	.000
	Within groups	9509.467	261	36.435		
	Total	24026.667	269			
Shoot length	B/n groups	111.141	8	13.893		.000
	Within groups	43.233	261	0.166		
	Total	154.374	269			

Appendix 5. ANOVA table for KIN + NAA

		Sum of squares	df	Mean square	F	Sig.
No.of shoot/explant	B/n groups	140.519	8	17.565	8.506	.000
	Within groups	538.967	261	2.065		
	Total	679.485	269			
Shoot length	B/n groups	112.480	8	14.060	58.387	.000
	Within groups	62.850	261	.241		
	Total	175.380	269			

Appendix 6. ANOVA table of BAP+KIN

		Sum of square	df	Mean square	F	Sig.
No. of shoot/explant	B/n groups	15614.332	24	650.597	22.423	0.000
	W/n groups	13781.850	475	29.014		
	Total	29396.182	499			
Shoot length	B/n groups	116.701	24	4.863	17.812	0.000
	W/n groups	129.674	475	0.273		
	Total	246.376	499			

Appendix 7. ANOVA table for rooting

		Sum of squares	df	Mean square	F	Sig.
No.of roots	B/n groups	1382.250	15	92.150	17.416	.000
	Within groups	1608.500	304	5.291		
	Total	2990.750	319			
Root length	B/n groups	24.962	15	1.664	27.815	.000
	Within groups	18.188	304	.060		
	Total	43.149	319			
No.of shoot	B/n groups	597.872	15	39.858	11.670	.000
	Within groups	1038.250	304	3.415		
	Total	1636.122	319			
Shoot length	B/n groups	182.149	15	12.143	18.545	.000
	Within groups	199.063	304	.655		
	Total	381.212	319			

Declaration

I the undersigned, declare that this thesis is my original work and has not been presented for any academic degree in any other university and all sources of materials used for the thesis has been correctly acknowledged.

Name: Indrias Teshome Signature _____ Date_____

This thesis has been submitted for examination with my approval as an advisor:

Dr. Tileye Feyissa Signature_____ Date_____