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Micropropagation of violet tree (*Securidaca longepedunculata,* Fresen.), an Endangered Ethiopian medicinal plant from Shoot tip



A Thesis Submitted to the School of Graduate Studies, Addis Ababa University in Partial Fulfillment of the Requirement for the Degree of Master of Science in Biotechnology

By

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This is to certify that the thesis prepared by Teklebrihan Lijalem entitled: **Micropropagation of violet tree (*Securidaca longepedunculata,* Fresen), an endangered Ethiopian Medicinal plant, from shoot tip** 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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List of Abbreviations

ANOVA	Analysis of Variance
BAP	6-benzyl Amino Purine
GA_3	Gibberellic acid
IAA	Indole-3-acetic acid
IBA	Indole -3-butyric Acid
LSD	Least Significant Difference
MS	Murashige and Skoog formulated media
NAA	α-naphthalene Acetic Acid
PGRs	Plant Growth Regulators
SPSS	Statistical Package for Social Science
TDZ	Thidiazuron

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Abstract

*Micropropagation of Violet Tree (*Securidaca longipedunculata*, Fresen), an endangered Ethiopian medicinal plant from Shoot tip.*

Teklebrihan Lijalem

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Securidaca longipedunculata *Fresen, locally called Etse-manahi, is semi-deciduous shrub or small tree that belonging to the family Polygalaceae and found in rainfall and equatorial humid areas of Africa. It is an indigenous medicinal plant in Africa and has important place in the traditional medicine as well as modern medicine. This plant becomes endangered because of high seed dormancy, low germination rate, over exploitation and long growth period. Therefore, micropropagation method is important to address these problems. Thus, the objective of this study is to develop micropropagation protocol for* S. longipedunculata *from shoot tip explants. Seeds were de-coated and disinfected using 3%, 5%, 10% and 15% (v/v) Clorox and cultured on growth regulators-free solid MS medium. Shoot tips were initiated using various concentrations of 6-Benzyl Amino Purine (BAP) in combination with Thidiazuron. MS medium containing different concentrations of BAP in combination with IBA; TDZ in combination with IBA or NAA were used for shoot multiplication. Shoots were cultured on full and half strength MS medium containing IAA, IBA or NAA for root induction. The highest percentage of shoot initiation (87%) was obtained on MS medium containing 1.0 mg/l BAP. Maximum number of shoots (8.5 ± 0.69) was achieved on MS multiplication medium containing 1.5 mg/l BAP in combination with 0.1 mg/l IBA. Shoots gave highest number of roots (3.73 ± 0.69) on full strength MS rooting medium containing 2.0 mg/l IAA. Among 102 plantlets transferred to greenhouse, 61 plantlets survived on the pot containing soil, sand and compost in a ratio of 2:1:1 respectively.*

Key words/phrases: ex vitro, in vitro, Micropropagation, plant growth regulator, *Securidaca longipedunculata,* shoot initiation, shoot tip culture

1. Introduction

Securidaca longepedunculata is a woody plant belonging to the family *Polygalaceae*. It is a semi-deciduous shrub or small tree that grows to 12 m tall, with an often flattened or slightly fluted bole. The hatchet like appearance of the fruit is referred to in the generic name, *Securidaca* while the specific name, '*longepedunculata*', refers to the long, slender stalks of the flowers. *S. longipedunculata* flowers are hermaphrodite, have both male and female organs. It is an indigenous medicinal plant in Africa and has important place in the traditional medicine as well as modern medicine. There are many different medicinal uses for this tree used all around Africa. It is used to treat sicknesses ranging from mild headaches to severe arthritis. This tree is also commonly used as pesticide against beetles in stored grains (Ojewole, 2008).

This species has different common names in different areas, Este- manahi (Amhara), shotora (Tigray), fibre tree, Rhodesian violet, violet tree (English), alali, saggat (Arabic), muteya, mzigi (Swahili) and krinkhout (Afrikaans). It is distributed in a wide range of climates, from subtropical, hot and arid climate to summer rainfall and equatorial humid. It occurs in a broad range of vegetation, from semi-arid scrub to dense forest, including many woodland and bush habitats and gallery forests. It is resistant to bush fires and is frost sensitive (Beentje, 1994).

The exact origin of the plant is not clearly known. However, it is found in rainfall and equatorial humid areas of Africa and is Native to Angola, Benin, Botswana, Burundi, Cameroon, Chad, Cote d'Ivoire, Democratic Republic of Congo, Eritrea, Ethiopia, Gambia, Ghana, Guinea, Kenya, Malawi, Mali, Mozambique, Namibia, Niger, Nigeria,

Rwanda, Senegal, Sierra Leone, South Africa, Sudan, Tanzania, Uganda, Zambia, and Zimbabwe (Beentje,1994).

Securidaca longipedunculata has different chemical constituents like, methylsalicylate, flavonoids, alkaloids elymoclavine, and dehydroelymoclavine, an ergo line compound and cinnamonic acid (Wrobel *et al.,* 1996) and the xanthones: 1, 7-Dimethoxy-2-hydroxy-xanthone and 1, 4-dihydroxy-7-methoxy-xanthone (Rakuambo *et al*., 2004). Also, a number of fatty acids and triglycerols such as coriolic acid, trans-9-dienoic acid and 9-hydroxytetradeca-cis-5, trans-7-dienoic acid have been isolated from the seed oil of *S. longipedunculata* (Smith *et al*., 1979).

Due to the presence of different chemicals, which is important in traditional medicine , the root and bark are taken orally either powdered or as infusion for treating malaria, stomach problems, toothache, headache, sleeping sickness, cough, chest complaints, snakebite, and wound dressing and roots are used to kill seed storage pests. An infusion of the roots is used as a mouthwash in cases of toothache and is applied to cuts on the legs to treat inflammatory conditions. Powder from the burned roots is rubbed into small incisions made on the forehead to relieve headaches. Seeds are used for headache, fever and rheumatism; leaves for snakebite, venereal diseases and coughs; bark for stomach problems and as an arrow poison antidote (Leeuwenberg, 1987).

The medicinal importance of *S. longepeduncudata* has been recognized by the findings of different bioactive metabolites isolated from the bark yielding such as oleanolic acid, glycoside and alkaloid securiene, used in treating convulsion in children, increased blood pressure and paralysis following infectious disease (Oliver-Bever, 1986). It is used for

preparation of different medicines for neuromuscular blocking and negative inotropic and chronotropic cardiac effects (Ojewole *et al*., 2000).

Root extracts of the plant increased sodium ion currents and enhanced the contractile response elicited by durable depolarization thus suggesting the possibility of one or more of the constituents acting on the voltage-sensor of excitation-contraction coupling in rat skeletal muscles (Mouzou *et al*., 1999). The root bark of *S. longipedunculata* is popularly used for the treatment of rheumatic conditions and inflammation. The alkaloid securine confers activity against *Plasmodium falciparum*, the causative agent of malaria (Maiga *et al*., 2005). Some Flavonoids isolated showed activity against many micro-organisms and a methanol extract of the root material against three major stored product pests (Ajali and Chukwurah (2004).

Although S. *longipedunculata* has many benefits in medicine, it has problems associated with propagation of the plant due to high seed dormancy, low germination rate, over exploitation for its root and long growth period.

Viable seeds that do not germinate are said to be dormant. Dormancy can be regulated by the environment or by the seed itself. If a seed is not exposed to sufficient moisture, proper temperature, oxygen, and for some species light, the seed will not germinate. In *S. longipedunculata* seeds do not germinate because of some inhibitory factor of the seed itself. This particular kind of dormancy consists of two general types: seed coat or external dormancy and internal (endogenous) dormancy (Dirr and Heuser, 1987). There are different techniques to overcome seed dormancy; these are Seed Scarification, Seed Stratification and others. The *ex vitro* germination rates did not exceed 43%, whereas *in*

vitro procedures achieved 67 to 90% germination rates. *In vitro* germinated seedlings produced at least two re-sprouts and consistently showed vigorous growth (Zulu *et al*, 2011).

Securidaca longepedunculata is used for various purposes, often involving the roots of the plant. If the root of the plant is always being cut, the plant becomes endangered. In addition, *S. longipedunculata* has long growth period until it sets seeds.

As the fruits usually stay on the tree for a year or more, it has been suggested that seeds should not be sown until it is one year old; but the seeds seem to lose viability quickly and germination is erratic. It is best to sow a seed soaked in cold water for 24 hours in the soil, *in situ*, and cover with grass and water until the beginning of the rains. Because of having poor germination, it is difficult to cultivate in large number. Seedling growth is slow, and planting out is difficult because the taproot is broken easily (Mbuya *et al*., 1994).

Natural stands are under considerable pressure from harvesting of roots for its numerous uses (Ouedraogo *et al*., 2003). Natural regeneration is limited because this plant has low seed germination rates and slow seedling growth (Mbuya *et al*., 1994). Planting on farm land is thought to reduce pressure on the natural stands. However, little effort has also been made to develop propagation methods to facilitate planting of *S. longepedunculata* in agro forestry systems.

Securidaca longepedunculata is a threatened species because of the fact that roots are the target for people using this plant, which makes it difficult for the plant to survive constant harvesting. It suffers from uncontrolled harvesting for use in local medicines, as well as

periodic droughts and bushfires. It is considered vulnerable due to increasing threats to their habitats.

Moreover, there is no research publication with regards to the micropropagation of this species. But Zulu *et al*. (2011) recommended further detailed research is needed to identify the best media and culture conditions to achieve optimal multiplication rates while they conducted the propagation of the African medicinal and pesticidal plant *S. longepedunculata.* *In vitro* method of vegetative multiplication of *S. longepedunculata* has considerable benefits for its availability as planting material, in the grower and germplasm conservation (since maintenance of plant germplasm requires the use of disease-free stocks). The protocol can facilitate mass-propagation of elite genotypes for wider planting in agro forestry systems or in its natural habitat where restoration and conservation is envisaged. This will have its own contribution to medicine in Ethiopia, being one of the developing countries suffering from health related problems, by producing enough *S. longepedunculata* plants with relatively short period of time and limited space. Therefore, the aim of this study is to develop micropropagation protocol for *S. longepedunculata* using shoot tip as explant.

2. Literature review

2.1. Description and taxonomy of *Securidaca longipedunculata*

Securidaca longipedunculata is a medicinal herb belonging to the family Polygalaceae and genus *Securidaca*. It is commonly used in parts of Africa. The plant is a savanna shrub with twisted bole or slender erect branches and grows up to 30 ft high (Iwu, 1986). It is a semi-deciduous shrub or small tree with an often flattened or slightly fluted bole. It is spiny and much branched, with an open, rather straggly looking crown. Leaves alternate or clustered on dwarf, lateral branchlets, simple, variable in size and shape, broadly oblong to narrowly elliptic, 1-5 x 0.5-2 cm with very fine hairs when young but losing these by maturity; apex rounded; base narrowly tapering; margin entire; petiole slender, up to 5 mm long. Flowers rather small, about 10 mm long, pink to lilac or purple, sweetly scented, on long slender stalks produced in beautiful profusion in terminal axillary sprays 3-5 cm long, appearing with the very young leaves; bisexual; sepals 5, unequal, the lateral 2 being petalloid, large and winglike; petals 3, free, the medium petal hooked; stamens 8, joined to form a split tube (Schimidt *et al*., 2002).

Fruit is more or less a round nut, somewhat heavily veined, occasionally Smooth, bearing a single, oblong, rather curved, membranous wing up to 4 cm long; purplish-green when young, becoming pale, straw-colored when mature. It is found in various types of woodland and wooded grassland across sub-Saharan Africa. It is rarely gregarious and a very variable species in leaf-shape and flower-size as well as in habit and extent to which spines develop. It has been subdivided by various authors into a number of varieties. Specimens from Mozambique coastal regions tend to have longer, narrower leaves, while some of the smallest and roundest leaves occur on specimens from Angola. There is,

however, great variation in most parts of the range. This is known as the Violet Tree owing to the scent of the flowers (Hutchings, 1996).

2.2. Origin and description of *S. longipedunculata*

Securidaca longipedunculata is grown in a wide range of climates, from subtropical, hot and arid climate to summer rainfall and equatorial humid. It occurs in a broad range of vegetation, from semi-arid scrub to dense forest, including many woodland and bush habitats and gallery forests. *S. longipedunculata* is resistant to bush fires and is frost sensitive (Beentje, 1994).

Securidaca longipedunculata is native to Angola, Benin, Botswana, Burundi, Cameroon, Chad, Cote d'Ivoire, Democratic Republic of Congo, Eritrea, Ethiopia, Gambia, Ghana, Guinea, Kenya, Malawi, Mali, Mozambique, Namibia, Niger, Nigeria, Rwanda, Senegal, Sierra Leone, South Africa, Sudan, Tanzania, Uganda, Zambia, Zimbabwe (Bein, 1996).

It occurs within altitudinal range of 0-1800 m above sea level, mean annual rainfall: 600-1000 mm, soil type is usually acid and sandy or rocky (Bein E. 1996). In Ethiopia it grows at altitude between 0 to 1800m, in arid and semi arid zones of Tigray region (Tekeze River), Amhara region (Quara), Oromia region (Gibe River) and Abay Bereha.

2.3. Significance and uses of *S. longipedunculata*

The young leaves are eaten as a vegetable or in sauces (Booth and Wickens, 1988).

Apiculture: Suitable for honey production as bees frequently visit the flowers. In Eritrea, for example, the tree is one of the most valuable lowland honey sources, and planting to increase honey production is recommended (Booth and Wickens, 1988). *S. longipedunculata* can be burnt for firewood and charcoal and fine quality fibre is

obtained from inner bark of the straight, annual shoots, which when retted could possibly be useful for flax like textiles. It is appreciated by Western, Central and Southern African peoples because of its long, durable and tough fibres. It is used for making string and rope for fishing net and lines, bird and animal snares, for thread to sew bark cloth and as bead string for necklaces (Booth and Wickens, 1988).

Timber: Wood is pale, soft, spongy, very light, and brittle, and is regarded to have little value. Used for poles, hut construction, bows and brooms. It is resistant to termites and decay (Booth and Wickens, 1988).

Lipids: The flowers yield oil with many possible uses; oil extracted from the seeds is used for cosmetics and as a furniture stain (Booth and Wickens, 1988).

Poison: The trees, especially the roots, are toxic if taken in excess. A saponin found in the roots can cause severe damage to bone marrow and haemolysis when in contact with blood. The solid portion of the root is said to be the most lethal. The root bark also contains 0.42% methyl salicylate. Severe poisoning can result from ingestion of 10- 30 ml of methyl salicylate. Bark, roots and seeds are used in arrow poison, and root can be used as a snake repellent. Roots at 350 ppm are 100% effective as a molluscicide (Booth and Wickens, 1988).

Medicinal uses

Traditional medicine refers to health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being (WHO, 2003).

The root and bark are taken orally either powdered or as infusion for treating chest complaints, inflammation, abortion, ritual suicide, tuberculosis, infertility, venereal diseases and for constipation. Toothache can also be relieved by chewing the roots. Powdered roots are used to treat headache by rubbing them on the forehead. Infusions of the root are used for washing topical ulcers (Mouzou, *et al*., 1999). In Limpopo, the Venda takes roots for mental disorders and against children´s illness during breast feeding. The Venda people mix the powdered root with maize and sorghum beverages for sexually weak men (Rakuambo *et al*., 2004).

In Africa, up to 80% of the population uses traditional medicine for primary health care and the global market for herbal medicines currently stands at over US $ 60 billion annually and is growing steadily (WHO, 2003). The clinical success of quinine and quinidine isolated from the *Cinchona* tree bark and recently artemisinin from *Artemisia annua* in the treatment of malaria have rekindled interest in medicinal plants as potential sources of novel drugs (Igoli *et al*., 2005).

Securidaca longipedunculata is the most popular of all the traditional medicinal plants in South Africa and is used for almost every conceivable ailment (Hutchings, 1996). The folk herbal uses of *S. longipedunculata* in the treatment of diarrhea, boils, gonorrhea, and cough prompted photochemical analyses and antimicrobial activity screening of extracts of the root. Some flavonoids isolated showed activity against many micro-organisms. These Flavonoids were isolated using chromatographic methods (Ajali and Chukwurah, 2004).

In herbal medicine practice, the aqueous root extract of *S. longepedunculata* is used in religious rites due to their psychotropic effects (Hagreaves, 1986). In South Africa, the Chopi and Kung tribes use the roots as medicine for people "possessed by evil spirits" and in healing sessions (Winkleman and Rios, 1989). In South Africa, the roots are used for coughs and chest complaints, respiratory problems, rheumatism, toothache, and headaches (Watt and Brandwijk, 1962). The plant is used for the treatment of the envenimations by bite of snake. The powdered root bark is used against headaches sniffing. It is strongly sternutatory (causes sneezing). The root powder exhibited insecticidal activity (Jayasekara, 2005).

Root bark extract exhibited neuromuscular blocking and negative inotropic and chronotropic cardiac effects (Ojewole *et al.,* 2000) and demonstrated spasmolytic activity on vascular and extra vascular smooth muscles (Ojewole *et al*., 2000). Because of psychotropic effects, aqueous root extract is used in herbal medicine practice (Hagreaves, 1986). Powdered dried roots are also used as a pest control agent and have potential as a protectant against insect pests in stored grain (Belmain *et al*., 2001; Boeke *et al*., 2001).

Chemical composition

The yields of the essential oils obtained by hydro distillation of fresh roots bark of *Securidaca longipedunculata* collected in three locations of Benin are given below.

Table.1: Chemical compositions of essential oils of root barks of *S. longipedunculata* (Alitonou et al., 2011).

Component	sample (% w/w)
p-cymene	0.1
γ-terpinene	0.1
p-α-dimethyl styrene	0.1
methyl salicylate	98.7
Eugenol	0.1
NI	0.6
germacrene D	0.1
NI	0.1
benzyl salicylate	0.1

NI - not identified.

This major compound was reported as a characteristic constituent of the roots bark oil from trees growing in Senegal, Ghana and Burkina Faso and of leaf oil from trees growing in Nigeria. The chemical composition of essential oil of the bark from the roots of *S. longipedunculata* was described for the first time in Benin (Alitonou et al., 2011).

2.4. Micropropagation and its advantage

Plant tissue culture is the science of growing plant cells, tissue or organs isolated from the stock plant on artificial media based on the cell doctrine that states a cell is capable of autonomy and is potentially totipotent (George, 1993). Tissue culture has been used by different scholars to propagate different species of plants in the past years. Micropropagation is one form of tissue culture which allows the production of large

number of plants from small pieces of the mother plant in relatively short period of time and limited space. It is an aseptic process which requires sophisticated laboratory procedures and special skills (Hartman *et al*., 2004).

In vitro culture has many applications like production of virus free plants, large scale micropropagation, plant improvement, and transformation of plant materials, production of metabolites, genetic improvement, preservation of germplasm, somatic hybridization, haploid and dihaploid production (Dong, 1987).

Micropropagation confers distinct advantages not possible with conventional propagation method. It is possible to multiply a single explant into several thousands in less than a year. Actively dividing cultures are continuous sources of plantlets without seasonal interruption. It has high commercial potential due to the speed of propagation, clonal propagation, germplasm conservation, genetic transformation and its high quality and ability to produce disease-free plants (Tileye Feyissa *et al*., 2005; Hartman *et al*., 2004).

3. Objective

3.1. General objective

The general objective of the present study is to develop micropropagation protocol for *S. longipedunculata* from shoot tip explants.

3.2. Specific objectives

- To determine seed sterilization with different Clorox concentrations
- To determine germination rate under *in vitro* and *ex vitro* conditions
- To determine shoots induction of *S. longipedunculata* in different growth regulators
- To optimize combinations and concentrations of different growth regulators for shoot multiplication of *S. longipedunculata*
- To optimize the concentrations and combinations of different auxin for rooting
- To optimize acclimatize protocol for plantlets

4. Materials and Methods

4.1. Plant material

Matured seeds were collected from Metema (Gendawuha), which is found in Amhara Regional State near the border, with Sudan, about 1,000 km West of Addis Ababa.

A four weeks old *in vitro* germinated and grown *S. longepedunculata* seedlings were used as a source of explants for micropropagation experiment.

4.2. Media preparation

4.2.1. MS medium stock solution preparation

Four different stock solutions of MS (Murashige and Skoog, 1962) basal medium were prepared. The stock solutions were components of MS medium (macronutrients, micronutrients, vitamins and iron EDTA).

4.2.2. Growth regulators stock solution preparation

Different growth regulators; 6-Benzyl Aminopurine (BAP), Thidiazuron (TDZ), α-naphthalene acetic acid (NAA), Indol acetic acid (IAA) and indol-3-butyric acid (IBA)

were used for this study. All growth regulator stock solutions were prepared by weighing and dissolving the powder in double distilled water at the concentration of 1.0 mg/ml. To begin the dissolving process, 3-4 drops of 1M NaOH, HCl or 94% ethanol were added based on the requirement of the growth regulators (NaOH for auxin, HCl for cytokinin, ethanol and gibbereline). Then, the volume was adjusted by adding double distilled water. Finally, growth regulators stock solutions were stored in a refrigerator at a temperature of 4 °C for use.

4.2.3. Culture medium preparation

Culture medium was prepared by taking proper amount of MS stock solution (50 ml/l macro, 5 ml/l micro including iron-EDTA and 5 ml/l vitamin). Sucrose (30 g) was weighed and dissolved in double distilled water in all cases. The shoot initiation medium containing BAP (0.5, 0.75,1, 1.25 and 1.5 in combination with TDZ (0.5) and TDZ (0.5, 0.75, 1, 1.25, 1.5 mg/l) was used.

The shoot multiplication medium was prepared using different concentrations of BAP (0.5, 1.0, 1.5, 2.0 and 2. 5 mg/l) in combination with IBA (0.01, 0.1 and 0.5 mg/l), TDZ (0.5, 1.0, 1.5, 2.0 and 2. 5 mg/l) in combination with IBA (0.01, 0.1 and 0.5 mg/l) and TDZ (0.5, 1.0, 1.5, 2.0 and 2. 5 mg/l) in combination with NAA (0.01, 0.1 and 0.5 mg/l).

The rooting media containing IBA (0.5, 1.0, 2.0 and 3.0mg/l), IAA (0.5, 1.0, 2.0 and 3.0mg/l) and NAA (0.5, 1.0, 2.0 and 3.0mg/l) in full and half-strength (1/2) MS medium were prepared.

After all these constituents including sucrose , were dissolved and thoroughly mixed in 1 L volumetric flask, and transferred to two liter beaker or bottle and 8 gm agar was added

after the pH was adjusted to 5.8 using 1M HCI and/or 1M NaOH and autoclaved at 121°C for 15 minutes. Then 20 ml medium was poured into sterile Petri-dishes and then kept until cooled to room temperature. In medium preparation, the medium was melted in microwave oven and 50 ml was dispensed in to each Magenta GA-7 vessel before it was autoclaved at 121^{o}C for 15 minutes.

4.3. Surface sterilization of seeds

Seeds were washed repeatedly with tap water and Omo, rinsed with double distilled water three times. Then, surface soaked using 70 % alcohol for 30 seconds and rinsed three times with sterile double distilled water followed by sterilization by using 3%, 5%, 10 % and 15% Clorox (NaOCl) containing three drops of Tween-20 for 10 min and 15 min. After sterilization, the seeds were rinsed three times with sterile double distilled water.

4.4. Evaluation of Germination rate

Seed germination was performed under four conditions using both coated and de-coated seeds. First, 20 of each coats and de-coated seeds were sown using wet filter paper on Petri dish. In the second experiment, the seeds were sterilized and cultured on growth regulator free MS medium. In third experiment, the de-coated seeds were cut half of the cotyledon transversally and sown on growth regulator free Ms Medium. In fourth experiment, seeds were sown using pot containing forest soil, compost and sand with the proportion of 2:1:1 in green house.

4.5. Shoot induction

Leaves were removed and shoot tip, were cut carefully by using sterile forceps and scalpels. Each shoot tips cultured on MS medium containing BAP (0.5, 0.75,1, 1.25 and

1.5) alone or combination with 0.5 mg/l TDZ or TDZ alone (0.5, 0.75, 1, 1.25, 1.5 mg/l). Growth regulators free media was used as a control.

Explants were cultured in baby food jars each containing 30 ml medium. For each treatment a total of 30 explants were used. There were six explants per jar with five replications. Numbers of dead shoots and number of shoots that were induced to shoots were recorded. Cultures were transferred to fresh media every four weeks. The cultures were maintained at a temperature of 25 ± 2°C under light intensity of 20 μmol m^{-2} s^{-1} and 16 hr photoperiod provided by cool-white fluorescent lamp.

4.6. Shoot multiplication

Initiated shoots were cultured on shoot multiplication medium. Shoot multiplication medium was MS medium containing different concentrations of BAP (0.5, 1.0, 1.5, 2.0 and 2. 5 mg/l) alone and combination with IBA (0.01, 0.1 and 0.5 mg/l), TDZ (0.5, 1.0, 1.5, 2.0 and 2. 5 mg/l) alone and combination with IBA (0.01, 0.1 and 0.5 mg/l) or TDZ (0.5, 1.0, 1.5, 2.0 and 2. 5 mg/l) in combination with NAA (0.01, 0.1 and 0.5 mg/l).

Shoots were cultured in Magenta GA-7 vessels containing 50 ml medium. For each treatment, six explants per jar, a total of 30 explants were used. The cultures were maintained at 25 o C ± 2 ° C and light intensity of 20 μmol m^{-2} s^{-1} under 16 hr photoperiod provided by cool white fluorescent light. Number of shoots per shoot explant was recorded every four weeks. The effects of different treatments were quantified on the basis of number of shoot per explants per treatment and length of shoots was also recorded. The whole experiment was repeated once. The two mean values were used for

analysis of variance (ANOVA) and mean value for each treatment were compared by least significance difference (LSD).

4.7. Rooting

Rooting was done using full and half strength MS medium containing IBA (0.5, 1, 2, and 3), IAA (0.5, 1, 2, and 3) or NAA (0.5, 1, 2, and 3). Growth regulator free MS medium was used as control. Six shoots per jar and 30 explants per treatment were used. Shoots were incubated for a week in darkness and then transferred to a 16-h photoperiod for three weeks. Rooting was evaluated in terms of rooting percentage, root number, the mean root length after one month of culture.

4.8. Acclimatization

After four weeks in rooting medium, *in vitro* rooted shoots were washed thoroughly under tap water to remove residual medium and transferred to plastic pots containing a mixture of Red soil, compost and sand in a ratio of 2:1:1, respectively. The washing was done in such a way that roots were damaged to a minimum level. Each pot was covered with polyethylene bag and kept in laboratory for one week before transferred to glasshouse. The polyethylene bags were removed after a week of transferring to glasshouse. The numbers of surviving plants in the glasshouse were recorded after a month.

4.9. Statistical analysis

Statistical analysis of quantitative data was carried out by SPSS computer software of version 20. A difference at probability level of $p \leq 0.05$ was considered significant for

analyses. Data were subjected to analysis of variance and variables that showed significant difference were compared by the LSD at 5 % probability.

5. Results

5.1. Seed sterilization

Highest percentage of decontaminated seeds (90%) was obtained at 15% Clorox, but the germination percentage was reduced to 20%.Among those Clorox concentrations, 10% Clorox concentration resulted in 85% decontaminated seeds and 80% germination of seeds. On the other hand, the least percentage of decontaminated seeds (15%) was obtained at 3% Clorox concentration. For each treatments 20 seeds were used.

Table 2: Effect of Clorox concentrations and 15 min exposure time on decontamination and germination rate of seeds of *S. longipedunculata*.

Clorox Concentration (%)	Decontaminated seeds (%)	Germinated seeds (%)
3	15	0
5	75	55
10	85	80

15	90	20

ANOVA result showed that Clorox concentrations significantly affected percentage of decontaminated seeds and germinated seeds ($P \leq 0.05$). However, no significance difference was observed among 5%, 10% and 15% Clorox concentration with respect to seed decontamination, but the increasing concentration of Clorox towards 15% negatively affected seed germination. There was no significance difference on germinated seeds on 3 % and 15% Clorox concentrations.

5.2. Germination rate of seeds

The germination rate was investigated using three media (wet filter paper, MS medium and soil). When the de-coated seeds were cut transversally, germination percentage was enhanced. None of the coated seeds were germinated in all media.

Among those media, the highest germination percentage (100%) was observed within 7-10 days on MS medium, when the seeds were de-coated and transversally cut at the tip end. As shown in Table 3, 10% of the de-coated but not transversally cut seeds germinated on all media.

The root part of the seedling showed faster growth than the aerial part at the first week of germination, but later the aerial part showed rapid growth and was elongated. 20 seeds for each treatment were used.

Table 3: Germination rate results in different growth conditions.

Medium	Seeds	Germination	Germination rate

		time (day)	(%)
Filter paper	Coated	-	0
	De-coated	90	10
MS medium	Coated	-	0
	De-coated	30	10
	De-coated and cut tip of cotyledon	7-10	100
Soil	Coated	-	0
	De-coated	90	10

Figure1: *S. longipedunculata* seeds with coats (A) and De-coated seeds (B). Bars represent 2cm.

Figure 2: Seeds cultured on MS medium after de-coating (A). Germinated seedlings on MS medium (B). The Bars represent 2 cm.

5.3. Shoot initiation

The use of solid MS medium containing BAP or in combination with TDZ and TDZ alone was resulted in the induction of shoots from shoot tips. The shoot tips induced micro shoots within a week. The highest percentage (87%) of explants survived on MS medium containing 1.0 mg/l BAP followed by 67 % of shoot initiation in 1.0 mg/l BAP in combination with 0.5 mg/l TDZ (Table 4). The micro- shoots that were initiated on MS medium containing 1.0 mg/l BAP was higher in number, but shorter in length than the shoots obtained from 1.0 mg/l BAP in combination with 0.5 mg/l TDZ. The lowest percentage of shoot number percent was obtained on growth regulators-free MS medium and the medium supplemented with 1.25 or 1.5 mg/l TDZ (10%, 23% and 20%) respectively. These were very short and stunted.

Table 4: Effects of different concentrations of BAP in combination with TDZ on shoot induction from shoot tip of S. longipedunculata on MS medium after 4 weeks of culture.

PGRs (mg/l)		
BAP	TDZ	Shoot initiation (%)
0.0	0.0	10
0.5	0.0	50
0.75	0.0	53
1.0	0.0	87
1.25	0.0	57
1.5	0.0	47
0.5	0.5	53
0.75	0.5	50

1.0	0.5	67
1.25	0.5	47
1.5	0.5	43
0.0	0.5	43
0.0	0.75	40
0.0	1.0	30
0.0	1.25	23
0.0	1.5	20

Figure 3: Shoot induction from shoot tip culture of *S. longipedunculata* using various concentrations of BAP and TDZ on MS medium, 0.5 mg/l BAP combined with 0.75 mg/l

TDZ (A); shoots cultured on MS medium containing 1.0 mg/l BAP alone (B); 1.0 mg/l BAP combined with 0.5 mg/l TDZ (C) and 0.5 mg/l BAP alone (D). Bars represent 2 cm.

5.4. Shoot multiplication

Shoot number was highly influenced by concentration and type of the growth regulators. Different combinations of BAP, IBA, TDZ and NAA resulted in different response. There were three different hormone combinations. The use of BAP in combination with IBA with different concentrations induced best shoot number and length per explant. The medium containing 1.5 mg/l BAP with 0.1 mg/l IBA had better shoot inducing response (8.50±0.69) as compared to other concentrations (Table 5). However, the lowest shoot number was recorded on growth regulators-free MS medium, which had mean of 2.10±0.12 shoot number.

In terms of shoot length, the highest mean shoot length (2.11±0.45 cm) was obtained on 2.5 mg/l BAP in combination with 0.1 IBA. But no significance difference was observed between shoots that were produced on 2.5 mg/l BAP in combination with 0.1 IBA and 2.5 BAP in combination with 0.01 mg/l IBA. The lowest shoot length was recorded on MS medium containing 0.5 mg/l BAP with 0.5 mg/l IBA, which had 0.96±0.40 cm mean of shoot length (Table 5).

Table 5: The effect of different concentrations of BAP and IBA on shoot number and length of *S. longipedunculata*

PGR((mg/l)		Number of shoots per explant	Shoot length (cm)
BAP	IBA		
0.0	0.0	2.10 ± 0.12^{j}	1.22 ± 0.12^{fh}
0.5	0.0	5.40 ± 0.54^{efh}	1.58 ± 0.54^{ce}
1.0	0.0	4.63 ± 0.24^{gh}	1.52 ± 0.24^{cd}
1.5	0.0	3.60 ± 0.22^{hi}	1.11 ± 0.22^{gh}
2.0	0.0	4.06 ± 0.37^{h}	1.13 ± 0.37^{gi}
2.5	0.0	7.13 ± 0.42^{bce}	1.42 ± 0.42^{de}
0.5	0.01	5.73 ± 0.30^{dg}	1.84 ± 0.30^{b}
0.5	0.1	5.80 ± 0.39^{df}	1.51 ± 0.39^{ce}
0.5	0.5	6.73 ± 0.40^{cd}	0.96 ± 0.40^{hi}
1.0	0.01	4.56 ± 0.26^{gi}	1.10 ± 0.26^{gi}
1.0	0.1	5.33 ± 0.32^{efg}	1.31 ± 0.32^{eg}
1.0	0.5	6.23 ± 0.37^{dg}	1.18 ± 0.37^{fh}
1.5	0.01	8.13 ± 0.53^{ab}	1.33 ± 0.53^{ef}
1.5	0.1	8.50 ± 0.69^{a}	1.62 ± 0.69^{c}
1.5	0.5	7.46 ± 0.38^{ad}	1.01 ± 0.38^{hi}
2.0	0.01	3.40 ± 0.15^{i}	1.18 ± 0.15^{fg}
2.0	0.1	6.30 ± 0.50^{de}	1.22 ± 0.50^{fh}
2.0	0.5	7.60 ± 0.56^{ac}	1.99 ± 0.56^{ab}
2.5	0.01	5.43 ± 0.28^{efh}	2.08 ± 0.28^{a}
2.5	0.1	5.76 ± 0.45^{dg}	2.11 ± 0.45^{a}
2.5	0.5	5.50 ± 0.32^{efh}	1.24 ± 0.32^{fh}

Means followed by the same letter within a column were not significantly different at 5 % probability level.

In cases where TDZ or TDZ in combinations with IBA was used, the highest mean number of shoots (6.20±0.43) was produced on MS medium containing 0.5 mg/l TDZ alone followed by shoots cultured on MS medium containing 2.5 mg/l TDZ in combination with 0.5 mg/l IBA, which was 5.23 ± 0.49. However, there was no significant difference between mean shoot number per explants of 2.5 mg/l TDZ in combination with 0.1 mg/l and 0.5 mg/l IBA.

MS medium containing 0.5 mg/l TDZ had better shoot inducing response than other concentrations of it or in combinations with IBA. On the other hand, the lowest mean shoot number (2.10±0.12) was recorded on growth regulators- free MS medium (Table 6).

In terms of shoot length, the highest mean shoot length (1.55±0.09 cm) was obtained on MS medium containing 0.5 mg/l TDZ while the shortest mean shoot length (0.31±0.02cm) was produced on MS medium containing 1.0 mg/l TDZ in combination with 0.5 mg/l IBA. Following 0.5 mg/l TDZ, better mean shoots length (1.22±0.03 cm) was obtained on growth regulator-free MS medium than other concentrations (Table 6).

In most cases, MS medium containing 0.5 mg/l TDZ had better shoot length response (1.55±0.09 cm) as compared to other concentrations. The increasing or decreasing concentration of TDZ alone or in combination with IBA didn't show any correlation with respect to mean shoot length.

Table 6: The effect of different concentrations of TDZ and IBA on shoot number and length of *S. longipedunculata*.

PGR (mg/l)		Number of shoots per explant	Shoot length (cm)
TDZ	IBA		
0.0	0.0	2.10±0.12^{l}	1.22±0.03^{b}
0.5	0.0	6.20±0.43^{a}	1.55±0.09^{a}
1.0	0.0	3.53±0.18^{efgi}	0.56±0.03^{f}
1.5	0.0	3.06±0.13^{gjk}	0.64±0.04^{e}
2.0	0.0	2.93±0.11^{ijk}	0.76±0.04^{de}
2.5	0.0	3.46±0.19^{efgj}	0.85±0.06^{d}
0.5	0.01	3.23±0.15^{fgj}	0.65±0.04^{ef}
0.5	0.1	2.86±0.14^{jk}	0.60±0.05^{f}
0.5	0.5	3.20±0.20^{fgjk}	0.42±0.02^{g}
1.0	0.01	3.60±0.17^{efgh}	0.59±0.05^{f}
1.0	0.1	3.66±0.18^{efgh}	0.39±0.03^{g}
1.0	0.5	2.56±0.11^{kl}	0.31±0.02^{g}
1.5	0.01	2.83±0.11^{jk}	0.75±0.04^{df}
1.5	0.1	3.16±0.13^{gjk}	0.98±0.04^{c}
1.5	0.5	3.03±0.20^{hijk}	0.42±0.03^{g}
2.0	0.01	3.70±0.19^{efg}	0.44±0.03^{g}
2.0	0.1	3.83±0.27^{ef}	0.32±0.02^{g}
2.0	0.5	4.50±0.32^{cd}	0.35±0.02^{g}
2.5	0.01	4.03±0.23^{de}	0.58±0.04^{f}
2.5	0.1	5.10±0.36^{bc}	0.65±0.04^{e}
2.5	0.5	5.23±0.49^{b}	0.76±0.03^{df}

Means followed by the same letter within a column were not significantly different at 5 % probability level.

In case of TDZ and NAA combinations, the highest mean number of shoots (4.70±0.32) was obtained on MS medium containing 1.5 mg/l TDZ in combination with 0.5 mg/l NAA followed by shoots on MS medium containing 1.5 mg/l TDZ in combination with 0.1 mg/l NAA, which was 4.56 ± 0.24. However, there was no significance difference between mean shoot numbers per explant of 1.5 mg/l TDZ in combination with 0.1 mg/l NAA and 0.5 mg/l TDZ with 0.01 mg/l NAA.

MS medium containing 1.5 mg/l TDZ in combination with 0.5 mg/l NAA had better shoot inducing response (4.70±0.32) than other TDZ in combination with NAA concentrations. On the other hand, the lowest mean shoot number (2.10±0.12) was recorded from growth regulators- free MS medium (Table 7).

In terms of shoot length, the use of TDZ in combination with NAA produced relatively shorter shoots. Among those treatments, the highest mean shoot length (1.22±0.03 cm) was obtained on growth regulator-free MS medium, while the shortest mean shoot length (0.16±0.01cm) was produced on MS medium containing 2.5 mg/l TDZ in combination with 0.01 mg/l NAA (Table 7).

Most of the shoots produced on MS medium containing TDZ in combination with NAA were vitrified showing greenish-white color, formed calli like materials at the bottom end of the explants and they were dwarf. The calli didn't regenerate new micro shoots when they were transferred to new fresh medium. The use of TDZ in combination with NAA produced lower shoot number and shorter shoots.

Table 7: The effect of different concentrations of TDZ and NAA on shoot number and length of *S. longipedunculata.*

PGR (mg/l)		Number of shoots per explants	Shoot length (cm)
TDZ	NAA		
0.0	0.0	2.10±0.12^{f}	1.22±0.03^{a}
0.5	0.01	4.50±0.34^{ab}	0.41±0.02^{de}
0.5	0.1	3.60±0.20^{bc}	0.36±0.02^{eg}
0.5	0.5	3.13±0.20^{cd}	0.30±0.02^{fg}
1.0	0.01	2.50±0.12^{ef}	0.19±0.01^{hi}
1.0	0.1	2.96±0.18^{de}	0.43±0.04^{df}
1.0	0.5	2.56±0.11^{df}	0.34±0.02^{eg}
1.5	0.01	4.46±0.24^{ab}	0.68±0.04^{b}
1.5	0.1	4.56±0.24^{ab}	0.46±0.02^{d}
1.5	0.5	4.70±0.32^{a}	0.29±0.01^{g}
2.0	0.01	2.93±0.21^{df}	0.18±0.01^{hj}
2.0	0.1	4.06±0.26^{b}	0.57±0.03^{c}
2.0	0.5	3.66±0.21^{bc}	0.38±0.03^{ef}
2.5	0.01	2.70±0.16^{df}	0.16±0.01^{ij}
2.5	0.1	2.90±0.14^{df}	0.26±0.02^{gi}
2.5	0.5	3.26±0.15^{e}	0.25±0.03^{gh}

Means followed by the same letter within a column were not significantly different at 5 % probability level.

Among all used growth regulators in shoot multiplication treatments, the maximum mean shoot number (8.5± 0.69) was obtained on MS medium containing 1.5 mg/l BAP in combination with 0.1 mg/l IBA. On the other hand, the minimum mean shoot number (2.10±0.12) was obtained on growth regulator-free MS medium. MS medium containing 2.5 mg/l BAP in combination with 0.1 mg/l IBA resulted in the highest mean shoot length (2.11±0.45 cm) while the shortest mean shoot length (0.16±0.01 cm) was obtained on MS medium containing 2.5 mg/l TDZ in combination with 0.01 mg/l NAA. The explants showed best shoot number and length on BAP or in combination with IBA at used concentrations. However, most of the shoots produced callus like material at the bottom end and vitrified leaves with green-whitish color.

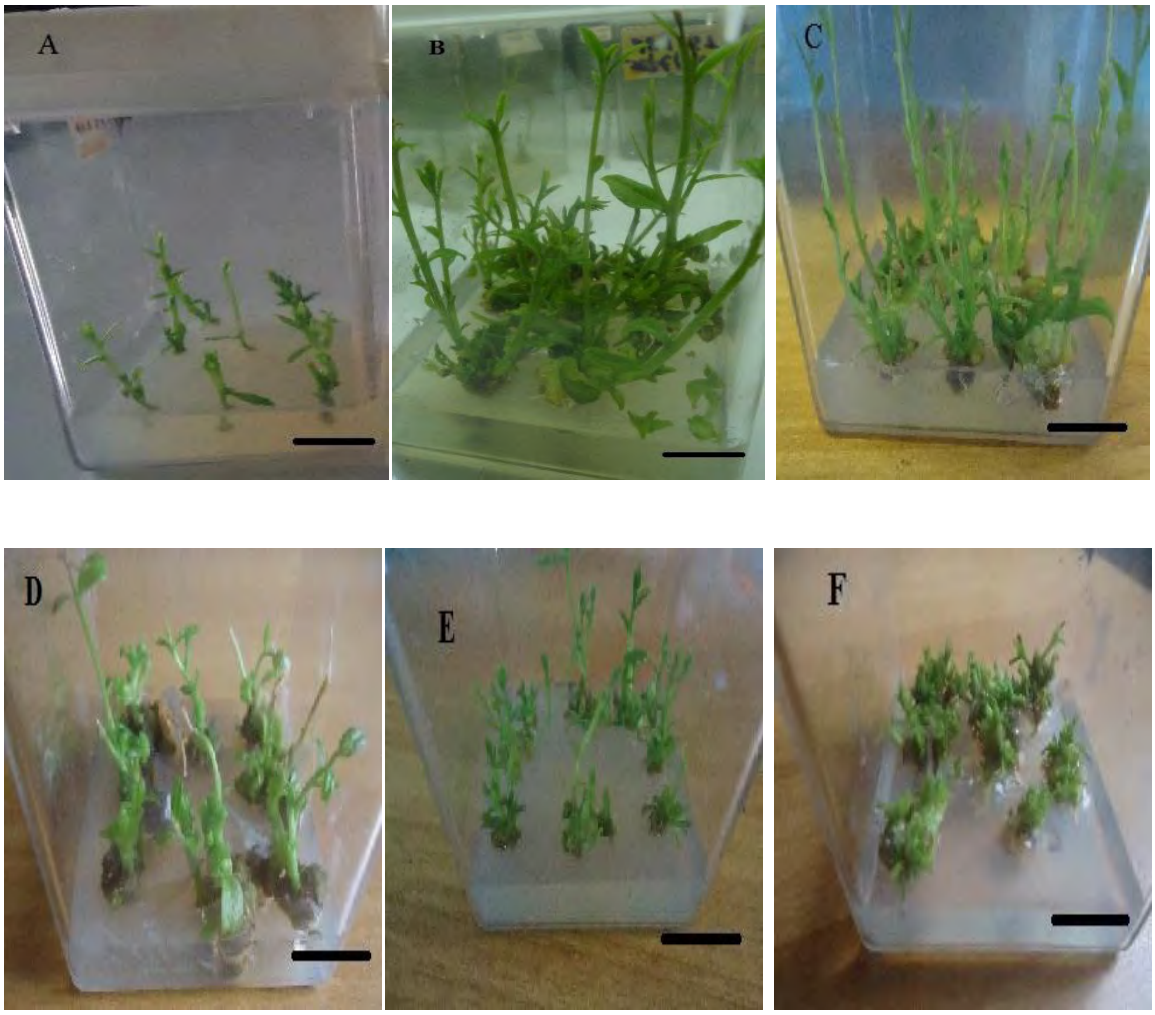

Figure.4. Shoots cultured on different concentrations of growth regulators on MS medium, Control (A); 2.5 mg/l BAP (B); 1.5 mg/l BAP in combination with0.1 mg/l IBA (C); 0.5 mg/l TDZ (D); 2.5 mg/l TDZ in combination with0.5 mg/l IBA (E); 1.5 mg/l TDZ in combination with 0.5 mg/l NAA (F). Bars represent 2 cm.

5.5. Rooting

Rooting was observed after four weeks culturing of the shoots on root induction medium. However, most of the shoots developed roots by week five. Shoots were rooted best on full strength MS medium. Full MS medium containing IAA had better rooting response (3.73±0.69) as compared to IBA and NAA. On the other hand, none of the shoots induced root on growth regulators-free MS medium (Table 8). Although, the growth regulators have significant effect on root induction, there was no significant difference among roots induced from full strength MS medium containing 0.5, 1.0, 2.0 mg/l IAA and 1.0 mg/l NAA or those were the maximum number of roots.

The highest mean root length (0.34 ± 0.02) was observed on full strength MS medium containing 3.0 mg/l IAA. However, no significant difference was observed among roots developed on full strength MS medium containing 3.0 mg/l IAA, 1.0 mg/l NAA and half strength MS medium containing 0.5 mg/l IAA. Among all concentrations, the minimum mean root length was observed on half strength MS medium containing 0.5 mg/l NAA and 0.5 mg/l IBA (i.e.0.07±0.01).

Full strength MS medium containing different concentrations of IAA (0.5 and 1.0 mg/l) NAA (1.0 and 2.0 mg/l) and IBA (2.0 and 3.0 mg/l) produced enlarged callus like symptoms on and around the root tip. This structure had an influence on reducing the number and length of roots compared to treatments without callus like growth. The color of the swelling of shoots were dark green and at some treatments deep green to white.

Table 8: Effect of MS salt strength and different auxin concentrations on number of roots per shoot and root length of *S. longipedunculata*.

Salt strength	Growth regulators (mg/l)			Mean no. of root	Mean length of root (cm)
	IAA	NAA	IBA		
Full strength	0	0	0	0.0 ± 0^{g}	0.0 ± 0^{h}
	0.5	0	0	3.53 ± 0.44^{a}	0.21 ± 0.17^{bde}
	1	0	0	3.06 ± 0.33^{ab}	0.19 ± 0.01^{cd}
	2	0	0	3.73 ± 0.69^{a}	0.26 ± 0.02^{b}
	3	0	0	3.10 ± 0.33^{ab}	0.34 ± 0.02^{a}
	0	0.5	0	0.83 ± 0.13^{f}	0.08 ± 0.01^{f}
	0	1	0	3.56 ± 0.58^{a}	0.33 ± 0.04^{a}
	0	2	0	2.10 ± 0.25^{be}	0.15 ± 0.01^{def}
	0	3	0	1.46 ± 0.36^{e}	0.12 ± 0.02^{eg}
	0	0	0.5	0.76 ± 0.13^{f}	0.12 ± 0.02^{eg}
	0	0	1	1.33 ± 0.21^{e}	0.22 ± 0.03^{bc}
	0	0	2	1.13 ± 0.19^{e}	0.18 ± 0.03^{ce}
	0	0	3	1.76 ± 0.26^{cde}	0.23 ± 0.03^{bde}
Half strength	0.5	0	0	2.43 ± 0.22^{bd}	0.33 ± 0.0^{3a}
	1	0	0	2.30 ± 0.16^{be}	0.17 ± 0.01^{cf}
	2	0	0	2.66 ± 0.34^{b}	0.19 ± 0.01^{cf}
	3	0	0	2.36 ± 0.23^{be}	0.26 ± 0.02^{b}
	0	0.5	0	0.50 ± 0.09^{fg}	0.07 ± 0.01^{g}
	0	1	0	2.50 ± 0.29^{bc}	0.26 ± 0.03^{bd}
	0	2	0	1.73 ± 0.16^{cdf}	0.12 ± 0.01^{eg}
	0	3	0	0.83 ± 0.11^{f}	0.09 ± 0.01^{fg}
	0	0	0.5	0.50 ± 0.09^{f}	0.07 ± 0.01^{g}
	0	0	1	0.96 ± 0.13^{ef}	0.14 ± 0.02^{de}
	0	0	2	0.73 ± 0.16^{f}	0.09 ± 0.02^{fg}
	0	0	3	1.20 ± 0.06^{e}	0.12 ± 0.01^{eg}

Means followed by the same letter within a column were not significantly different at 5 % probability level.

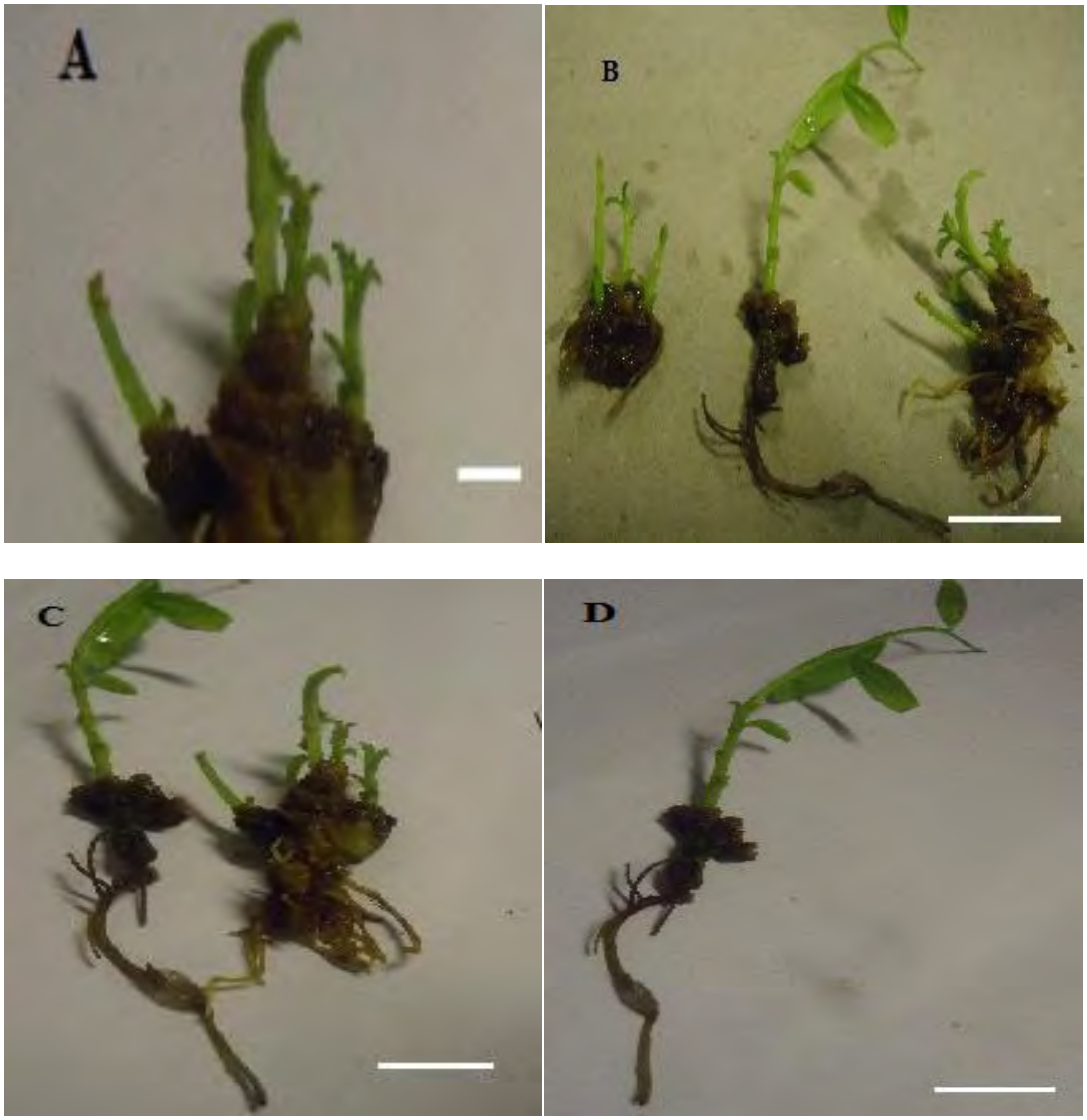

Figure 5: Rooted shoots of *S. longipedunculata* on different rooting MS medium. Rooted shoots on PGR-free medium (A); rooted shoots on 2.0 mg/l IAA (B); rooted shoots on 1.0 mg/l NAA (C); rooted shoots on ½ MS + 2.0 mg/l IAA (D). Bars represent 2 cm.

5.6. Acclimatization

Out of 102 plantlets which were planted in pots containing red soil, compost and sand (2:1:1) placed in green- house, 60% were survived after a month.

Most of the dead plantlets were due to small root number or hairs, fungal contamination and rotting. None of the plantlets survived on pots which were not covered by plastic bags and those containing unsterilized soil, compost and sand. The growths of the plantlets were slow.

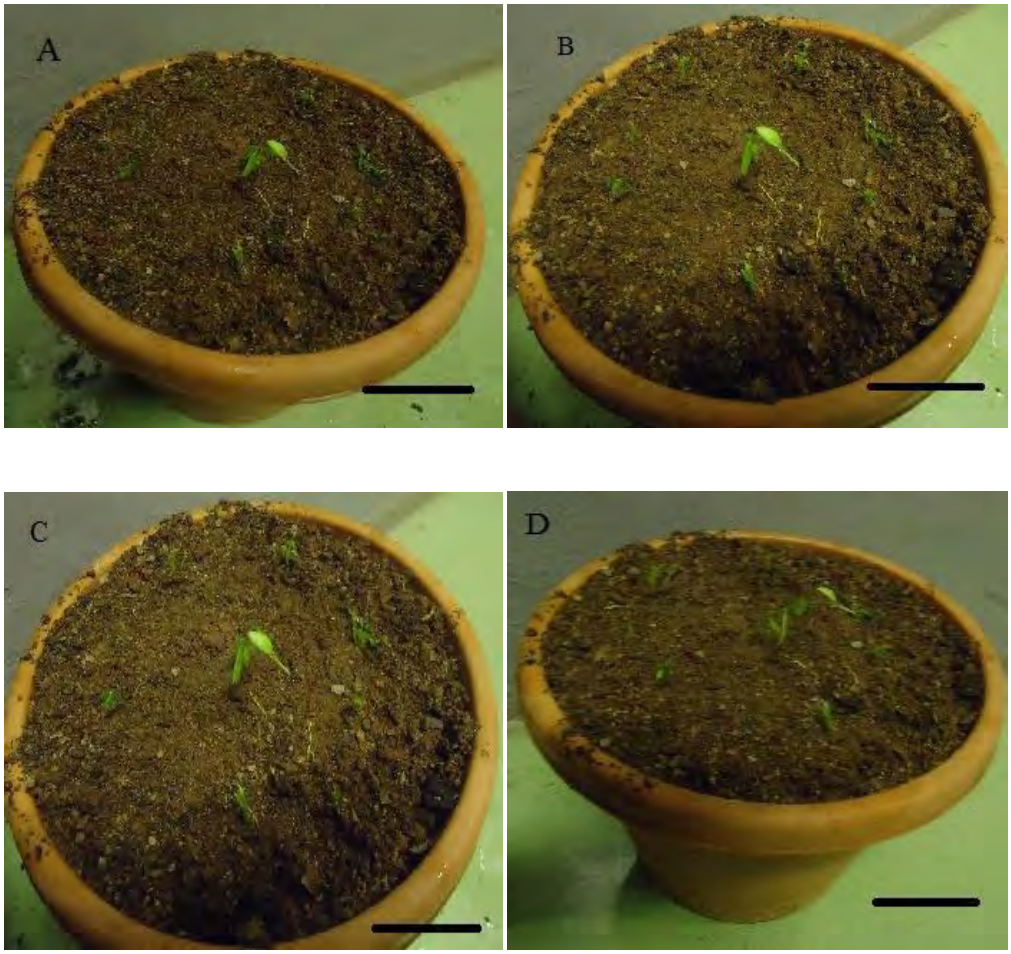

Figure 6: Acclimatized plants, (A) after one week of planting, (B) after two weeks of planting,(C) after three weeks of planting and (D)after four weeks of planting. Bars represent 2 cm.

6. Discussion

6.1. Seed sterilization

The result showed that the increasing of Clorox concentration increases decontamination of seeds, but decreases viability of the seeds. Guanih *et al*. (2004) stated that the percentage of commercial Clorox needs to be varied depending on the species and environment. On the other hand, the work is in disagreement with that of Srivastava *et al*. (2010) who found that sterilization with NaOCl didn't give acceptable sterilization even on increasing concentration over *Aconitum heterphyllum* (medicinal herb) seeds. According to our results, the higher percentage of decontaminated seeds was observed at 15% sodium hypochlorite (Clorox) for 15 min, but low germination percentage. Guanih, *et al*. (2004) found that sterilization of de-coated seeds of a medicinal plant, *Dryobalanops lanceolata* with 30% Clorox concentration for 5 min and cultured on MS medium showed less than 20% contamination, but sterilization of coated seeds with 50% Clorox concentration for more than 20 min would break the seed coat and reduce seed viability.

Among those Clorox concentrations, 10% Clorox concentration resulted in 85% decontaminated seeds and higher germination percentage (80%) in 15 min . This suggest that the lower the Clorox concentration, the higher the contaminated seeds and germination percentage. As Talie *et al*. (2011) tested on a medicinal herb, *Andrographis pancuatanee,* no significant correlation was found between contamination and germination percentages. Even though all safety measures were taken with asepsis, microorganism growth in the culture medium can happen and might have affected germination of the seeds (Grattapaglia and Machado, 1990).

Seeds germinated at higher concentrations of Clorox were thin, dwarf with folded leaves. The work of Sen *et al*. (2013) on medicinal herb, *Anchyranthes aspera*, showed that at higher concentration all sterilizing agents including NaOCl showed maximum effect against microbial contamination, but the survival percentage was low. When Clorox concentration decreases, the seeds became decayed and contaminated.

6.2. Germination rate of seeds

The de-coated seeds showed better germination than coated seeds. As Zulu *et al*. (2011), reported that removing the seed coat could reduce contamination sources while improving the imbibitions of water and subsequent germination in *S. longipedunculata*. In addition, Legesse Negash (2004) reported successful germination (91±2.7%) of de-coated seeds of a tree plant, African cherry (*Prunus africana*) in the laboratory.

The germination of de- coated seeds on full strength MS medium was best among media tested. According to Pandey *et al*. (2014), the highest percentage of seed germination (95%) from an important medicinal plant, *Psoralea corylifolia,* was recorded on full strength MS medium. The result showed that none of the coated seeds germinated in all media tested. The reason may be the presence of seed coat hampered the imbibitions of water. Similarly, Pandey *et al*. (2014) who worked on *Psoralea corylifolia* suggested as the presence of seed coat challenged the entry of water and oxygen into the embryo. In contrast, Zulu *et al*. (2011) obtained (<45%) germination rates of intact seeds of *S. longepedunculata* using GA_3.

Among all media, the highest germination percentage (100%) of seeds was observed within 7-10 days on MS medium, when the seeds were de-coated and transversally cut at

the tip end. This is partly due to the removing of the seed coat facilitated the uptake of water and cutting at the tip end reduce the size to use the limited amount of moisture on the MS medium. Similarly, Ganaie *et al*. (2011) reported maximum germination percentage (96.66%) on *Arnebia benthamii* by removing the seed coat within mean germination time of around 4 days.

6.3. Shoot initiation

The effect of PGR on shoot induction from shoot tip was investigated. Sixteen different treatments including the control were used to induce shoot from *S. longipedunculata* shoot tip. These media, with various combinations of cytokininns (BAP and TDZ), triggered direct shoot initiation from the shoot tip. Similar results were obtained in S. *longipedunculata* for shoot induction from shoot tip by Zulu *et al*. (2011).

However, there was difference in the type of the growth regulators, combination and concentrations in the same plant. According to Verma and Bansal (2014), the percentage response of explants for shoot induction, shoot number and shoot length varied according to the type and concentration of cytokinins used. Biological activity of any one substance not only varies with the dosage, but depends greatly on the other conditions in which it is placed. Combination of plant growth hormones exerted growth stimulatory effects.

From our results, the shoot tips induced micro shoots within a week on full strength MS medium. On the other hand, Zulu *et al*. (2011) reported that shoot tips of *S. longipedunculata* induced microshoots using B_5 medium supplemented with 3 mg/L 2iP in four weeks.

The highest percentage (87%) of explants survived on MS medium containing 1.0 mg/l BAP followed by 67 % of shoot initiation on 1.0 mg/l BAP in combination with 0.5 mg/l TDZ. Tiwari *et al*. (2010) proved that MS medium containing 1.0 mg/l BAP was better for establishment of *Trichosanthes dioica* from nodal explants. The micro shoots that resulted from MS medium containing 1.0 mg/l BAP was higher in number, but shorter in length than those shoots obtained from 1.0 mg/l BAP in combination with 0.5 mg/l TDZ. This was in contrary to the work of Victorio *et al.* (2012) who reported that TDZ was less effective compared to BAP because shoots from any TDZ concentration failed to elongate.

The lowest number (10%) of shoots explants was initiated on growth regulator-free MS medium. Not only growth regulator free MS medium, but also the MS medium containing 1.25 and 1.5 mg/l TDZ resulted in lowest amount of initiated shoots (23% and 20%) respectively. The induced micro shoots of those media were very short and stunted. Ahmed and Anis (2012) reported that exposure of the culture to TDZ had an adverse affect.

The shoots were initiated better on MS medium containing BAP alone than MS medium containing TDZ alone or in combination with BAP. According to Kumar and Singh (2010) BAP react best for shoot formation as they tested on the multipurpose desert tree, *Prosopis cineraria*. Similarly, Malik and Saxena (1992) showed that the advantageous outcome of BAP for shoot induction as they observed on tissue culture of grain legumes, *Phaseolus vulgaris.*

6.4. Shoot multiplication

Application of BAP in combination with IBA with different concentrations induced highest shoot number and length per explants. From our result, the MS medium containing 1.5 mg/l BAP in combination with 0.1 mg/l IBA produced the highest mean number of shoots (8.50±0.69). As Pierik (1987) stated medium supplemented with auxin at low concentrations in combination with cytokinin promote the growth and formation of new shoots, consequently, increasing multiplication rate. In contrast, the micropropagation study of Fraternale *et al*. (2002) showed that high concentration of auxin (IBA) with BAP (1.5 mg/l) in MS medium was suitable for shoot multiplication of *Bupleurum fruticosum*. According to Askari-Khorasgani *et al*. (2013), in comparison to the application of BAP alone, the combination of BAB along with auxins (IBA), led to more shoot formation. In addition, Ahmad *et al.* (2010) stated that the response of the explants is a communal result of endogenous and exogenous PGRS concentrations. Similarly, Tsay *et al.* (1988); and Rout (2004) indicated that the inclusion of a low concentration of an auxin along with cytokinin activates much higher rate of shoot multiplication.

However, the lowest mean shoot number (2.10±0.12) was recorded on growth regulators-free MS medium. Even the study of Kumar and Singh (2010) showed that PGRs-free MS medium produced only one shoot per explants of *Prosopis cineraria*.

The highest mean shoot length (2.11±0.45 cm) was obtained on 2.5 mg/l BAP in combination with 0.1 mg/l IBA whereas the lowest shoot length (0.96 ± 0.40 cm) was recorded on MS medium containing 0.5 mg/l BAP in combination with 0.5 mg/l IBA. This may be due to equal concentration of both cytokinin (BAP) and auxin (IBA) on MS

medium suppressed the action of one of another, but the presence of low concentration of auxin (IBA) over relatively higher concentration of cytokinin (BAP) increases the length of the shoot. This is in line with the work of Sadeq *et al*. (2014) who reported that to overcome the growth inhibition effect of BAP, low concentration of auxins (IBA) was combined to trigger shoot multiplication efficiency at the same time to enhance shoot length.

The explants cultured on MS medium containing higher concentration of BAP or in combination with IBA accumulates callus like structure. This callus was found to reduce use of growth regulators and nutrients from the medium and gives relatively lower number and length of shoots. According to Askari-Khorasgani *et al*. (2013) worked on an endangered medicinal plant, *Kelussia odoratissima* , when medium fortified with BAP alone, the concentration above 2 mg/l induced high amount of callus, which is not suitable for direct organogenesis.For instance, the use of MS medium containing 2.5 mg/l BAP on a perennial herb, *Rheum emodi,* produced callus (Parveen *et al.,* 2012).

In TDZ or in combinations with IBA, the highest mean number of shoots (6.20±0.43) was produced on MS medium containing 0.5 mg/l TDZ alone. As Mirici (2004) reported that TDZ alone prompted reasonable shoot multiplication in *Astragalus polemoniacus*. In addition, the micropropagation study of Guo *et al.* (2011) showed that among other agents with cytokinin activity, comparatively low amount of TDZ promote shoot multiplication.

In terms of length, the highest mean shoot length (1.55 ± 0.09 cm) was obtained on MS medium containing 0.5 mg/l TDZ. The micropropagation result of Fenugreek by Aasim

et al. (2009) showed that the maximum number of shoots was recorded on MS medium containing 0.4 mg/l TDZ with highest shoot length of 1.20 cm. On the other hand, the shortest mean shoot length (0.31 ± 0.02cm) was produced on MS medium containing 1.0 mg/l TDZ in combination with 0.5 mg/l IBA. Bisht *et al*.(2011) worked on *Polygonatum verticillatum*, suggested that the promoter effect of TDZ regarding growth is due to its own biological activities similar to N- substituted cytokinin or it may induce the synthesis or accumulation of an endogenous cytokinin.

In case of TDZ and NAA combinations, the highest mean number of shoots (4.70 ± 0.32) was produced on MS medium containing 1.5 mg/l TDZ in combination with 0.5 mg/l NAA. As He *et al.* (2007) used on a medicinal plant, *Hydrastis canadensis*, shoot organogenesis of explants was further enhanced by TDZ in combination with NAA.

Most of the explants produced on MS medium supplemented with TDZ in combination with NAA were vitrified with greenish-white in color, formed calli like materials at the bottom end of the explants. According to Huetteman and Preece, (1993), higher concentrations of TDZ can stimulate callus induction as reported in many woody species.

Generally, among all used growth regulators in shoot multiplication treatments, the maximum mean shoot number (8.5 ± 0.69) was obtained on MS medium containing 1.5 mg/l BAP in combination with 0.1 mg/l IBA. On the other hand, the minimum mean shoot number (2.10±0.12) was obtained on growth regulator-free MS medium. MS medium containing 2.5 mg/l BAP in combination with 0.1 mg/l IBA produced the highest mean shoot length (2.11±0.45 cm) -while the shortest mean shoot length (0.16±0.01 cm)

was produced on MS medium containing 2.5 mg/l TDZ in combination with 0.01 mg/l NAA.

6.5. Rooting

Shoots were rooted best on full strength MS medium. In general, full MS medium containing 2.0 mg/l IAA had better rooting response (3.73±0.69) as compared to IBA and NAA in most concentrations. Alvarez *et al.* (1989) analyzed the effectiveness of IAA on rooting in *Malus pumila*. In addition, Alagesaboopathi (2012) found 55.14% of rooting using IAA from *Andrographis macrobotrys*. Unlike to our study, the study of Shen *et al*. (2010) confirm that IAA failed to induced root formation on a medicinal tree plant, *Casuarina cunninghamiana.* On the other hand, none of the shoots induced root on growth regulators-free MS medium. Similar results were reported by Shen *et al*. (2010) who worked on *Casuarina cunninghamiana,* where no root formation was observed on the control medium. The highest mean root length (0.34±0.02cm) was observed on full strength MS medium containing 3.0 mg/l IAA. As Dharmendra *et al*. (2010) stated that i*n vitro* shoots (4-5 cm long) were rooted only upon transfer to half strength MS medium containing1.5mg/l IAA.

The lowest mean root length was observed on half strength MS medium containing 0.5 mg/l NAA and 0.5 mg/l IBA (i.e.0.07±0.01). As Jirakiattikul et al. (2013) worked on medicinal plant, *Smilax corbularia*; the Shoots grown on half strength MS medium supplemented with 0.5 mg /l NAA had the shortest roots. In addition, as Zhang et al. (2012) who worked on *Psammosilene tunicoides*, a rare and endangered medicinal plant, suggested that fast growing and highly branched roots in high ratio of NAA to IBA relatively.

6.6. Acclimatization

In this study, 60%of the plantlets survived in glasshouse after a month .As Dharmendra *et al*. (2010) who worked on medicinal plant, *Oxystelma secamone,* and normal growth of the potted plantlets which rooted on MS medium with IAA were visible after transfer to field conditions and the success was about 80%.

The dead plantlets might be due to small root number or hairs and rotting. According to Bohidar et *al* (2008) who worked on medicinal plant, *Ruta graveolens* the dead plantlets were due to improper development of root system in the culture. None of the plantlets survived on pots which were not covered with plastic bags.

7. Conclusions

Based on the results of this study, the following conclusions were reached:

- Results achieved in this study demonstrate that seeds sterilized with 10% Clorox with 15 min, resulted in higher decontaminated seeds (85%) and germination percentage (80%).

- Seeds that were de-coated and transversally cut at the top end and cultured on MS medium showed the highest germination percentage (100%) within 7-10 days.

- The highest percentage of shoot initiation from shoot tips was obtained on MS medium containing 1.0 mg/l BAP.

- The highest shoot number per explant was obtained on MS medium containing 1.5 mg/l BAP in combination with 0.1 mg/l IBA.

- The highest number of roots per explant was obtained on full strength MS medium containing 2.0 mg/l IAA and the longest root was obtained on full strength MS medium containing 3.0 mg/l IAA.

- After acclimatization, 60% of plants survived in glasshouse.

8. Recommendations

Based on the results of this study, the following recommendations were forwarded:

- The seed of *S. longipedunculata* didn't germinate in pot containing soil, sand and compost due to its high seed dormancy period. Thus, different treatments to break dormancy like GA_3 should be incorporated to break the seed dormancy.
- As the components of medium for shoot initiation, multiplication and rooting are expensive, low cost micropropagation methods such as using table sugar instead of sucrose should be used for large scale micropropagation protocol.
- The currents study focused on micropropagation of *S. longipedunculata*, and therefore, it is recommended to study *ex vitro* rooting efficiency of the plant using different PGRs so that it can serve as alternative source in solving shortage of the medicinal part.

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10. Appendix

Appendix I. Stock solution for MS (Murashige and Skoog's, 1962) basal medium nutrient composition and concentration.

	Compositions	**Concentrations**
Macro Nutrients (g/l)	NH_4NO_3	33
	KNO_3	38
	KH_2PO_4	3.4
	$CaCl_2.2H_2O$	8.8
	$MgSO_4$	7.4
Micro nutrients (mg/500 ml)	$CoCl_2.6H_2O$	2.5
	$CuSO_4.5H_2O$	2.5
	H_3BO_3	630
	$MnSO_4.\ 4H_2O$	2230
	$NaMoO_4.2H_2O$	25
	$ZnSO_4.7H_2O$	860
	KI	83
	$FeSO_4.\ 7H_2O$	2.78
	Na EDTA. $2H_2O$	3.736
Vitamins (mg/500 ml)	Myo-inositol	10000
	Glycine	200
	Nicotnic acid	50
	Pyridoxin HCl	50
	Thiamin HCl	10