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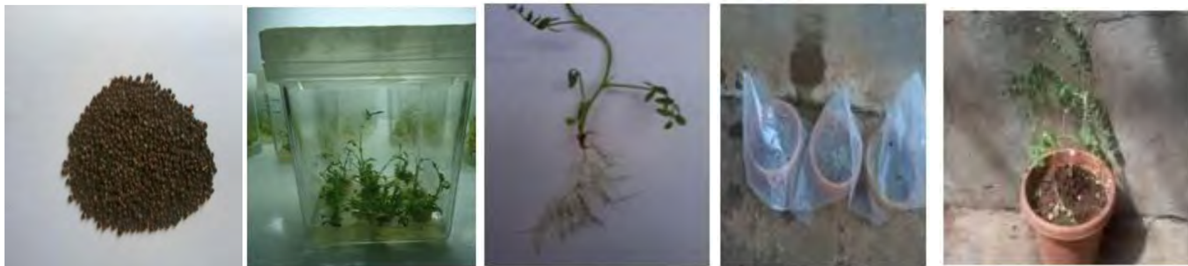
In vitro Propagation of *Taverniera abyssinica* A. Rich (Dingetegna)



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

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Abstract

In vitro Propagation of Dingetegna (*Taverniera abyssinica* A. Rich)

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Taverniera abyssinica *A. Rich, commonly known as 'Dingetegna' in Amharic in Ethiopia, belongs to family Fabaceae. This species has been threatened as it is used as a major traditional medicine for stomachache, headache and fever in many parts of Ethiopia.* In vitro *germination and propagation of* T. abyssinica *has immense value in rapid production and conservation of this species. Therefore, the objective of this study is to develop micropropagation protocol for* T. abyssinica *using seed as explant. Plant growth regulators (PGRs)-free Murashige and Skoog (MS) medium was used to initiate the growth of shoots from seeds. Shoots were multiplied on the same medium containing different concentrations and combinations of 6-Benzyl amino purine (BAP) in combination with gibberrelic acid (GA_3). The rooting experiment was done using half and full strength MS medium supplemented with 2g/l activated charcoal (AC) and 0.4mg/l IBA and NAA. Among the various concentrations of BAP (0.1, 0.2, 0.3, 0.4, and 0.5 mg/l) in combination with GA_3 (0.5 or 1 mg/l) used, highest rate of shoot multiplication (5.93±4.08) was obtained on MS medium containing 0.2 mg/l BAP combined with 1.0 mg/l GA_3. Moreover, shoots cultured on PGRs- free medium exhibited the highest mean shoot length (3.89±1.33).* In vitro *developed shoots were rooted best (2.30±1.39) in half strength MS medium supplemented with 2g/l AC and 0.4 mg/l IBA in combination with 0.4 mg/l NAA. Upon transfer to glasshouse, 83.33% of plants survived after six weeks of acclimatization.*

Key Words: Activated charcoal**,** Dingetegna, Medicinal plant, Plant growth regulators, Shoot multiplication

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Table of Contents

Abstract I

Acknowledgments III

Table of Contents IV

List of Figures VI

List of Tables VII

List of Appendices VIII

List of Abbreviations IX

1. Introduction 1

2. Literature Review 4

2.1 Description, origin and distribution of *Taverniera abyssinica* 4

2.2 Significance and use of *T. abyssinica*: Its role in traditional and modern medicine 5

2.3 Impact of market and overharvest on the quantity of *T. abyssinica* 6

2.4 Biotechnology and conservation of medicinal plants 8

2.4.1. Tissue culture 8

2.4.2. Micropropagation and its role for medicinal plants 9

3. Objectives 11

3.1 General objective 11

3.2 Specific objectives 11

4. Materials and Methods 12

4.1 Plant material 12

4.2 Media preparation 12

4.2.1 Murashige and Skoog medium stock preparation 12

4.2.2 Plant growth regulator stock preparation 12

4.2.3 Culture medium preparation 13

4.3 Surface sterilization of explants 13

4.4 *In vitro* seed germination 14

4.5 *In vitro* shoot multiplication 14

4.6 *In vitro* rooting 14

4.7 Acclimatization 15

4.8 Statistical analyses 15

5. Results 16

5.1. Shoot induction from seed 16

5.2. Shoot multiplication 16

5.3. Rooting 20

5.4. Acclimatization 21

6. Discussion 22

6.1. Seed germination 22

6.2 Shoot multiplication 22

6.3. Rooting 25

6.4. Acclimatization 26

7. Conclusions 28

8. Recommendations 29

9. References 30

10. Appendix 38

List of Figures

Figure 1. Multiple shoots of *T. abyssinica* on MS media supplemented with 1.0 mg/l GA_3 and different concentrations of BAP. (A) 0.2 mg/l; (B) 0.5 mg/l; (C) 0.3 mg/l. Bars represent 2 cm……………………………………………………………………….. ……………19

Figure 2. Multiple shoots of *T. abyssinica* on MS media supplemented with 0.5 mg/l GA_3 and different concentrations of BAP. (A) 0.2 mg/l; (B) 0.3 mg/l; (C) 0.1 mg/l. Bars represent 2 cm. 19

Figure 3. (A-F) Multiple shoots of *T. abyssinica* on PGRs free MS medium. Bars represent 2 cm. 19

Figure 4. Rooted shoot of *T.abyssinica* (A, B) on ½ MS medium containing 2 g/l AC and 0.5 mg/l IBA in combination with 0.5 mg/l NAA; acclimatization of in vitro rooted shoots in glasshouse (C) plantlets covered by polythene plastic bags (D) plantlets after six weeks 21

List of Tables

Table 1 Mean shoot number and length per explant on MS medium containing different concentrations of BAP 17

Table 2 Effect of combination of BAP with GA_3 on the rate of shoot multiplication and length 18

Table 3 Number and length of root on full strength MS medium containing 2.0 g/l activated charcoal and different concentration of IBA alone and IBA with NAA 20

List of Appendices

Appendix 1 Stock solution for MS medium (Murashige and Skoog,1962) 39

Appendix 2 ANOVA table on shoot multiplication 40

List of Abbreviations

AC	Activated Charcoal
ANOVA	Analysis of Variance
BAP	6-Benzyl Amino Purine
EBC	Ethiopian Biodiversity Institute
EDTA	Ethylene Di-Amine Tetra acetic Acid
GA_3	Gibberrelic Acid
GEF	Global Environment Facility
IAA	Indole-3- Acetic Acid
IBA	Indole-3-Butyric Acid
KN	Kinetin
LSD	Least Significance Difference
MS	Murashige and Skoog
MSB_5	Murashige and Skoog + B_5 Vitamins
NAA	α-Naphthalene Acetic Acid
PGRC	Plant Genetic Resource Center
PGRs	Plant Growth Regulators
TDZ	Thidiazuron

1. Introduction

Taverniera abyssinica is a medicinal plant species belonging to a Fabaceae family and a small genus of 15 taxa. *T. abyssinica* is a shrub about 2 meters high. It displays flower clusters held on a stalk. The plant grows in Shewa and Tigrai regions of Ethiopia. It is known to occur in north-east Africa and south-west Asia. *T. abyssinica* is a threatened medicinal plant that usually grows in a bush land limestone areas with an altitude range of 1700 to 2300 above sea level (Berhanu Abegaz *et al*., 1999). The Ethiopian vernacular name of the plant, 'Dingetegna', tells the full story since it means `cure for sudden illness'. A small bundle of the roots is chewed and the juice swallowed for immediate relief of fever, discomfort and pain. The roots of this plant have yielded four compounds classified as isoflavonoid derivatives (formononetin and afrormosin) and pterocarpans like medicarpin and 3,4-dihydroxy-9-methoxypterocarpan (Dudeck *et al*., 1987).

'Dingetegna' is traditionally used to heal stomach pain. The reason behind the claimed healing effect of this plant may be due to its antispasmodic bioactivity. Those extract which are active towards the antispasmodic bioactivity test will show a decrement in the natural rhythmic and histamine induced contraction of smooth muscles. According to the analysis of the result medicarpin is the more active compound and these compounds are probably the ones most responsible for the well-known effect of the extract of the plant in combating stomach pain (Mekuriaw Assefa, 2010).

T. abyssinica is mainly propagated by seed, which is a slow process relative to other medicinal plants. It is mostly grown in the tropics. Getachew Addis (2003) reported that seeds from brownish-green and brown pods germinated successfully (98% - 100%) and could be stored for

at least 20 months in dry condition under 2°C-20°C without loss of viability. However, the seeds of *T. abyssinica* required pre-germination treatment. Mechanical or 98% sulphuric acid treatment for 10-40 minutes improved germination from 8% (control) to over 98%. Hot water (70°C) treatment for one hour also improved germination to 54%. Mechanical seed scarification is the most promising approach to overcome seed coat dormancy provided that cheap, safe, efficient and easily applicable devices are developed.

T. abyssinica is a threatened medicinal plant which is found as a remnant in the field condition because of high market demand and collection by consumers for the purpose of traditional medicine. *T. abyssinica* is posted in the red list of endemic trees and shrubs of Ethiopia and Eritrea (Vivero *et al*., 2005).

Although, the traditional medicinal values (Ermias Dangne *et al*., 1990), the chemical extracts, their effect (Naomesi *et al*., 1990; Stadler *et al*., 1994), the pre-germination treatments, bioassay (Mekuriaw Assefa, 2010), *in vitro* regeneration (Balcha Abera *et al*., 2010) and *in vitro* seed culture (Getachew Addis, 2003) of *T. abyssinica* has been reported, only few researches are done in attempting to conserve this endangered germplasm from the increasingly devastating man-made environmental conditions.

Some medicinal plant species of Ethiopia are reported to have been threatened by the overuse and overharvesting from the field for marketing purpose as medicine. *T. abyssinica* is one of the most important one whose slender roots are swathed and small coiled bundles presented for market. Nowadays, the tissue culture technique *i.e.* micropropagation has expanded its scope and potential on commercial scale. So that micropropagation is suitable for the rapid and large-scale clonal multiplication of elite germplasm. *In vitro* propagation techniques would constitute an

approach for the systematic germplasm conservation and genetic improvement. *In vitro* method of vegetative multiplication of *T. abyssinica* will have considerable benefits for its availability as planting material and germplasm conservation (since maintenance of plant germplasm requires the use of disease-free stocks). Therefore, the aim of this study is to develop micropropagation protocol for *T. abyssinica* using seed as explant.

2. Literature Review

2.1 Description, origin and distribution of *Taverniera abyssinica*

The genus *Taverniera* belongs to the family Fabaceae and includes sixty species. It is endemic to the Northeast African and Southwest Asian countries (Mangalorka, *et al.,* 2013).

It is a shrub or shrublet to 2 cm high; young stems rather densely appressed pubescent. Leaves 1-foliate, very rarely pinnately 3-foliate ones present as well; leaflets obovate-oblong, up to 20 (-23) x 10 (-13) mm, glabrous above, appressed pubescent beneath; petiole 1-10 mm long. Racemes 2-8 flowered; rhachis and peduncle together 3-25 mm long. Calyx 5-7.5 mm long, appressed pubescent outside; lobes equaling or longer than tube. Corolla 12-17 mm long, dark pink to purplish red. Pod with 1-3 segments, stipitate; segments 5-6.5 x 4.5-5 mm, finely pubescent and with spines to 1.5 mm long. It grows on limestone bush-land at altitudes of 1700-2150 m (Hedberg and Edwards 1989).

T. abyssinica is an important medicinal plant species threatened by collection and labeled as critically endangered in the Red List of Endemic Trees and Shrubs of Ethiopia. It is sold in the Addis Ababa market as a cure for stomach cramps and fever (Vivero *et al*., 2003). Among the major known Ethiopian plants of medicinal value are *Hagenia abyssinica*, *Glinus lotoides*, *Rumex spp*. which are used as a source of taeniacide and *T. abyssinica* for treating stomachache, headache and fever (IBC, 2007). Twenty-five species belonging to 21 families were claimed to be used in self-care. *T. abyssinica* A. Rich. (Fabaceae), *Ocimum lamiifolium* Hochst. ex Benth (Labiatae), *Allium sativum* (Alliaceae), *Ruta chalepensis* (Rutaceae), *Linum usitatissimum* L. (Linaceae), *Hagenia abyssinica* (Bruce) Gmel (Rosaceae), *Zingiber officinale* Rosc.

(Zingiberaceae) and *Lepidum sativum* (Crucifereae) are the most frequently used plants (Leporatti and Ivancheva, 2003).

The endemic species known for their medicinal purposes include: dengetegna (*Taverniera abyssinica*), kebericho (*Enchinops kebericho*), incense tree (*Boswellia pirottae*), gibbira (*Loblellia rhynchopetalum*) and koso tree (*Hagenia abyssinica*). These species are highly threatened with extinction because of unsustainable use and neglect (Mwebaza *et al*., 2009).

Due to over-exploitation, *T. abyssinica* is now found as a remnant of isolated and scattered populations in the Ethiopian Shewa, Tigray and Welo regions (Ensarmu Kelbessa *et al*., 1992; Fasil Kibebew and Getachew Addis, 1996).

2.2 Significance and use of *T. abyssinica:* Its role in traditional and modern medicine

T. abyssinica has been traditionally used for the treatment of various diseases in Ethiopia. A small bundle of the roots chewed and consequently the juice swallowed for immediate relief of fever, discomfort and pain (Kloos, 1978; Ermias Dagne *et al*., 1990). Root extracts of *T. abyssinica* were used locally, as antipyretic and analgesic (Ermias Dange *et al*., 1990). In a screening for nematicidal natural products, *T. abyssinica* extracts showed strong nematicidal activities towards *Caenorhabditis elegans*, from the extracts medicarpin and 4-hydroxy medicarpmn were isolated as nematicidal constituents (Stadler *et al*., 1994). It is reported that *T. abyssinica* is among the major known Ethiopian plants of medicinal value for treating stomachache, headache and fever in the traditional health care system (Leipzig, 1996; IBC, 2007).

This plant is so widely used that steps should be taken to produce standardized phytomedicines and formulate dosages. At the same time it is necessary to look into the conservation and cultivation aspects of the plant (Berhanu Abegaz *et al*., 1999).

Its use for the treatment of vomiting and dysentery was reported by Tilahun Teklehaymanot and Mirutse Giday (2007). Consequently, pharmacological studies were also conducted using root crude extracts on rats made hyperthermic with yeast injections (Ermias Dange *et al*., 1990). The aqueous extract of the roots was shown to antagonize the contractile responses of guinea pig ileum to acetylcolin and histamine. At least some of the analgesic properties of the root extracts have been attributed to the isoflavanoids, probably linked to the blocking of histamine receptors (Stadler *et al*., 1994).

2.3 Impact of market and overharvest on the quantity of *T. abyssinica*

Some medicinal plant species of Ethiopia are reported to have been threatened by the over harvesting for marketing as medicine. A good example is *T. abyssinica* whose slender roots are swathed and small coiled bundles presented for market. *T. abssynica* is a popular traditional medicine for what is known as sudden disease (Endashaw Bekele, 2007).

Endashaw Bekele (2007) also reported that there are some species that are frequently traded and applied in healing that are endemic. Such species need to be given top priority in conservation as they are subjected to multiple pressure of genetic erosion. *Taverniera abyssinica* and *Solanum maginatum* are two such species. It is reported that about 90% of the collectors sell plants to earn income. The economic role of the collectors play in medicinal plant sector in terms of employment and income generation is significant. The volume of sales of medicinal plants has increased over years and this has been taken that by some as the existence of disease prevalence

requiring traditional Medicare has caused increased demand which led to harvesting important medicinal plants. The expected increase in the cost of commercial drugs and their occasional impotency also increase demand of medicinal plants. The proportion of consumers who rely on harvesting medicinal plant is the highest in the rural area. This is reasonable since collecting from natural plantation is most accessible and cost effective particularly for the rural communities.

Medicinal plants, since times immemorial, have been used virtually all cultures as a source of medicine. It is estimated that 70-80% people worldwide relay chiefly on traditional, largely herbal, medicines to meet their primary health care needs (Srivastava *et al.,* 1995). Approximately 85% of traditional medicine preparations involve the use of plants or plant extracts (Vieira and Skorupa, 1993).

In the past few decades, there has been an ever-increasing global inclination towards herbal medicine, followed by a belated growth in international awareness about the dwindling supply of the world's medicinal plants (Bodeker, 2002). The plants used in the phyto-pharmaceutical preparations are obtained mainly from the naturally growing areas. The genetic diversity of medicinal plants in the world is getting endangered at alarming rate because of ruinous harvesting practices and over-harvesting for production of medicines, with little or no regard to the future. Also, extensive destruction of the plant-rich habitat as a result of forest degradation, agricultural encroachment and urbanization are other factors, thus challenging their existence (Gupta *et al.,* 1998).

A large sum of money is pumped every year to replenish the lost biodiversity and large numbers of protocols are available at present (Tripathi, 2008). However, limited capacity to conserve

biodiversity and especially medicinal plants in the wild and under cultivation will constrain conservation efforts. It is risky to store seeds of most wild medicinal plants in gene bank cold rooms due to lack of knowledge on their storage characteristic. Most importantly, in Ethiopia there is no institutional plan for implementation of conservation strategies linked to utilization that contributes to economic development (Endashaw Bekele, 2007).

2.4 Biotechnology and conservation of medicinal plants

Biotechnology has emerged as the most powerful weapon for the conservation of medicinal plants. It helps in conservation of plants without affecting their natural features. There are several techniques in biotechnology for conservation of medicinal plants. Some of these techniques are: - tissue culture, *in-vitro* regeneration, callus-mediated organogenesis, regeneration through somatic embryogenesis, and conservation through cryopreservation (Sharma and Dubey, 2011).

2.4.1. Tissue culture

The propagation of a plant by using a plant part or single cell or group of cells in a test tube under very controlled and sterile conditions is called "Tissue Culture". It plays an important role in conservation of medicinal plants by quickly producing multiples of plants in the absence of seeds or necessary pollinator to produce seeds. It also conserve by regenerating whole plants from plant cells that have been genetically modified or by producing plants from seeds that otherwise have very low chances of germinating and growing. Plant tissue culture is one area of biotechnology that has a dramatic impact on conservation and improvement of plants. Theoretically, a single cell or piece of plant tissue can produce an infinite number of new plants.

The main industrial goal of plant tissue culture is to produce a large number of plants in a month instead of years (Bera *et al.,* 2009).

Biotechnological tools have been increasingly applied for mass propagation, conservation of germplasm, study and production of bioactive compounds and for genetic improvement of the medicinal plants. Tissue culture is useful for multiplying and conserving the species, which are difficult to regenerate by conventional methods and save them from extinction. Micropropagation has superiority over conventional method of propagation because of high multiplication rate. Most of the plants raised through seeds are highly heterozygous and show great variations in growth, habit and yield and may have to be discarded because of poor quality of products for their commercial release. Likewise, majority of plants are not amenable to vegetative propagation through cutting and grafting. Moreover, many plants propagated by vegetative means contain systemic bacteria, fungi and viruses (Murch *et al.,* 2000).

The *in vitro* propagated medicinal plants are genetically pure elite. Micropropagation techniques can be used for conservation of an endangered medicinally important species within short period and limited space. The plants produced from this method are independent of climatic changes or soil condition (Kuldeep *et al.,* 2012). Generally tissue culture has immense value in rapid production, eradication of pathogens and conservation of genetic diversity.

2.4.2. Micropropagation and its role for medicinal plants

Micropropagation is defined as production of miniature planting materials (seeds, somatic embryos or plantlets) in large number by vegetative multiplication through regeneration. Micropropagation provides a rapid, reliable system for the production of large numbers of genetically uniform plantlets. It offers a method to increase valuable genotypes rapidly and

expedite the release of improved varieties. In addition, micropropagation ensures mass production of elite clones from hybrid or specific parental lines. Micropropagation ensures healthy seedlings with desirable characters. This method is used routinely for many species to obtain a large number and high quality of plants. It has great potential as a tool for rising best plants which provide raw material for traditional medicines. It is carried out in aseptic and favorable conditions on growth media, using various plant tissue culture techniques (Zhou and Wu, 2006).

In conventional cultivation many plants do not germinate, flower and produce seed under certain climatic conditions or have long periods of growth and multiplication. Micropropagation ensures a good regular supply of medicinal plants, using minimum space and time (Prakash and Van Staden, 2007).

One of the substantial advantages of micropropagation over traditional clonal propagation is the potential of combining rapid large-scale propagation of new genotypes, the use of small amounts of original germplasm (particularly at the early breeding and/or transformation stage, when only a few plants are available), and the generation of pathogen-free propagules (Altman, 1999).

3. Objectives

3.1 General objective

- The general objective of this study is to develop micropropagation protocol for *T. abyssinica* from seed explant.

3.2 Specific objectives

- To induce shoots from seed of *T. abyssinica* using growth regulator free media;
- To determine the optimum plant growth regulators concentration for shoot multiplication;
- To identify optimum growth regulators concentrations and combinations for *in vitro* rooting;
- To identify an optimal acclimatization condition for *T. abyssinica* in greenhouse.

4. Materials and Methods

4.1 Plant material

Seeds of *Taverniera abyssinica* were collected from Melka Konture area, Kersana Malima district, East Shoa zone, Oromia Regional State which is 65 km south of Addis Ababa on the way to Butajira.

4.2 Media preparation

4.2.1 Murashige and Skoog medium stock preparation

Murashige and Skoog (1962) MS nutrient was used throughout the experiments. Full strength stock solution of macronutrients, micronutrients, Fe-Na-EDTA and FeSO4 mixture and vitamins were prepared separately. To do so, appropriate amount of each nutrient was weighed in grams per liter (Appendix 1) and dissolved in double distilled water. After all the components were completely dissolved using magnetic stirrer, finally the solution was dispensed in to 100 ml plastic bottles and stored at -20°C until used.

4.2.2 Plant growth regulator stock preparation

Different growth regulators; 6-benzyl aminopurine (BAP), Gibberrelic acid (GA_3), α-naphthalene acetic acid (NAA) 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 used based on the requirement of the growth regulators (NaOH for auxin, HCl for cytokinin and ethanol for gibbereline were used). 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 immediate use.

4.2.3 Culture medium preparation

Culture medium was prepared by taking proper amount of MS stock solution containing 50 ml/l macro, 5 ml/l micro, 5 ml/l iron-EDTA and 5 ml/l vitamin with different concentrations and combinations of plant growth regulators. Sucrose (30 g) was used.

Medium for seed culture was prepared using growth regulator-free MS medium, 30 g/l sucrose and 8 g/l agar was added after the pH was adjusted to 5.75 using 1M HCl and/or 1M NaOH and autoclaved at 121°C for 15 minutes. Then 10 ml medium was poured into sterile test tubes and the medium was kept until cooled to room temperature.

MS medium containing different concentrations of BAP (0.1, 0.2, 0.3, 0.4, or 0.5 mg/l) in combination with GA_3 (0.5 or 1 mg/l) was used for shoot multiplication. In media preparation, media were melted in microwave oven and 40 ml was dispensed in to each Magenta GA-7 vessel and baby food jars before it was autoclaved at 121°C for 15 minutes.

For rooting, full and half-strength (1/2) MS medium containing 0.4 mg/l IBA combined with 0.4 mg/l NAA in combination with 2 g/l of activated charcoal were prepared and poured into sterile Magenta GA-7 vessel.

4.3 Surface sterilization of explants

Seed explants were washed twice with tap water and detergent, and soaked in double distilled water for 5 minutes and rinsed twice. Following a 5 min treatment with 70% ethanol, seeds were surface-sterilized with an aqueous solution of 10% sodium hypochlorite solution (NaClO)

containing 1-2 drops of Tween 20 for 10 min and then rinsed five times with sterile double distilled water in a laminar air flow hood.

4.4 *In vitro* seed germination

Single seed was cultured per test tube containing 20 ml growth regulator-free MS medium. The cultures were maintained at a temperature of 25 ± 2°C under light intensity of 20 µmol m^{-2} s^{-1} and 16 h photoperiod provided by cool-white fluorescent lamp.

4.5 *In vitro* shoot multiplication

Young and healthy micro-shoots were excised aseptically from seedlings that were obtained from seed culture were cultured on shoot multiplication medium. Shoot multiplication medium was full strength MS medium containing different concentrations of BAP (0.1, 0.2, 0.3, 0.4, or 0.5 mg/l) in combination with GA_3 (0.5 or 1 mg/l). Shoots were cultured in Magenta GA-7 vessels and baby food jars containing 40 ml medium. Nine shoots per culture vessel with three replications were used. For each treatment, a total of 27 explants were used. The cultures were maintained at 25 ± 2°C with light intensity of 20 µmol $m^{-2}s^{-1}$ under 16 h photoperiod provided by cool white fluorescent light. Subculturing was carried out every four weeks. PGRs-free medium was used as a control.

4.6 *In vitro* rooting

Before culturing of shoots on root induction media, since GA_3 inhibit root formation, shoots from shoot multiplication media were sub cultured on PGRs-free medium for a month to reduce the carryover effect of growth regulators in the shoot multiplication media and to elongate the shoots. These shoots were then cultured on half and full strength MS basal media containing 2.0

g/l activated charcoal and the same concentrations of (0.4 mg/l) IBA and NAA. Nine explants per Magenta GA-7 vessel and baby food jars in three replications were used for each treatment.

The cultures were maintained under the same conditions as for shoot multiplication for six weeks. Rooting was evaluated in terms of root number and root length.

4.7 Acclimatization

Rooted shoots were removed from rooting medium and gently washed under running tap water and then plantlets were transferred to pots containing a mixture of soil, compost and sand in a ratio of 2:1:1 respectively. Each plastic pot was labeled and covered with polyethylene bags to ensure high humidity and kept in the glasshouse. The plastic bags were removed after two weeks. Thirty plantlets from each medium (half and full medium) were acclimatized. The numbers of surviving plants in the glasshouse were recorded after six weeks.

4.8 Statistical analyses

Statistical analysis of quantitative data was carried out by SPSS computer software of version 20.

A difference at probability level of $p = 0.05$ was considered significant for all analyses. Data were subjected to analysis of variance and variables that showed significant difference were compared by least significance difference (LSD) at 5 % probability.

The effects of different treatments were quantified on the basis of number of shoot and shoot length per explant per treatment. Mean value was used for analysis of variance (ANOVA) and mean values for each treatment were compared by LSD.

5. Results

5.1. Shoot induction from seed

The use of growth regulator-free solid MS medium resulted in the development of shoots from seeds. Growth was continued up to a month, and 30 seeds were used for shoot induction, resulting in 80% primary shoot establishment.

5.2. Shoot multiplication

The shoots on solid MS medium containing different concentrations of BAP alone and in combination with GA_3 resulted in different responses (Tables 1 and 2). Shoot number was highly influenced by concentration and type of the growth regulators. Among different concentrations and combinations of growth regulators used, the highest mean number of shoots per shoot was obtained on MS medium supplemented with 0.2 mg/l BAP and 1.0 mg/l GA_3 which was 5.93±4.08 shoots per explant (Table 2 and Fig.1 A).

However, higher concentration of BAP (0.5 mg/l) alone and in combination with 1.0 mg/l GA_3 could only induce a few numbers of shoots per explant compared to cultures with moderate or lower concentration of BAP and GA_3 which was 2.33 ± 1.76 and 2.33 ± 1.12 respectively. As the concentration of GA_3 increased from 0.5 to 1.0 mg/l, the mean shoot length increased (Fig.1 B and C). At 0.5 mg/l GA_3, as the concentration of BAP increased from 0.0 to 0.5 mg/l, the mean number of axillary shoot proliferation increased and the shoot length decreased (Fig. 2 A, B and C).

The multiplication rate of T. abyssinica on PGRs free medium (control) was relatively less than that of the medium containing PGRs and produced the lowest mean number of axillary shoot per explant (1.80±1.03) (Fig. 3 A-F).

Table1. Mean shoot number and length per explant on MS medium containing different concentrations of BAP

PGRs (mg/l)			
BAP	GA_3	Mean number of shoot per explant	Mean shoot length (cm)
0.0	0.0	1.80 ± 1.03^{m}	3.89 ± 1.33^{a}
0.1	0.0	3.67 ± 1.56^{fj}	1.22 ± 0.67^{cfh}
0.2	0.0	2.73 ± 1.41^{hijm}	1.49 ± 0.63^{c}
0.3	0.0	5.30 ± 2.35^{ac}	0.48 ± 0.25^{ijkl}
0.4	0.0	5.47 ± 3.29^{ab}	0.65 ± 0.64^{gk}
0.5	0.0	2.33 ± 1.76^{klm}	0.80 ± 0.42^{gi}

Means followed by the same letter within a column are not significantly different at 5 % probability. Values are presented as mean ± SD.

Table2. Effect of BAP with GA_3 on the rate of shoot multiplication and length

PGR(mg/l)			
BAP	GA_3	Mean number of shoots per explant	Mean shoot length (cm)
0.1	0.5	3.17 ± 2.23^{gijk}	1.22 ± 1.24^{ce}
0.2	0.5	3.83 ± 2.03^{fgh}	0.87 ± 0.71^{g}
0.3	0.5	4.53 ± 2.20^{bcef}	0.87 ± 0.39^{gh}
0.4	0.5	4.20 ± 2.53^{cdegij}	0.47 ± 0.47^{ijkl}
0.5	0.5	5.17 ± 2.24^{ad}	0.64 ± 0.27^{gl}
0.1	1.0	3.80 ± 2.09^{fi}	1.86 ± 0.64^{b}
0.2	1.0	5.93 ± 4.08^{a}	1.24 ± 0.35^{c}
0.3	1.0	5.10 ± 1.56^{ae}	0.94 ± 0.37^{defg}
0.4	1.0	2.97 ± 1.12^{hijl}	1.34 ± 0.42^{c}
0.5	1.0	2.33 ± 1.12^{klm}	0.79 ± 0.35^{gj}

Means followed by the same letter within a column were not significantly different at 5 % probability. Values are presented as mean ± SD.

After one month of culture, most of the shoots cultured on PGRs- free medium produced small number of shoots (1.80±1.03) with highest mean shoot length of 3.89±1.33 cm (Table 1 and Fig. 3). Following shoots cultured on PGR free medium, the second longest shoots (1.86±0.64 cm) were produced on medium containing 0.1 mg/l BAP combined with 1.0 mg/l GA_3. The shortest mean shoot length was observed on medium containing 0.4 mg/l BAP + 0.5 mg/l GA3

(0.47±0.47 cm) (Table 2). Shoots cultured on medium containing higher BAP concentration (0.5 mg/l) produced shoots with lower shoot length compared to those cultured on lower BAP (0.1 mg/l) concentration.

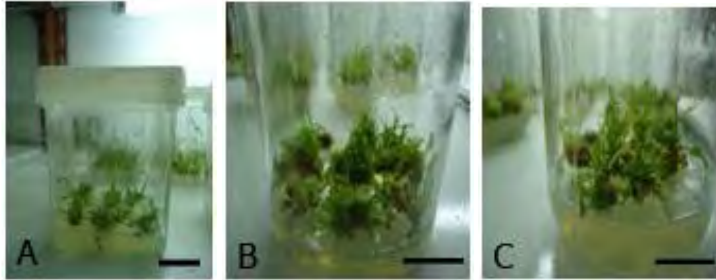

Figure 1. Multiple shoots of *T. abyssinica* on MS media supplemented with 1.0 mg/l GA_3 and different concentrations of BAP. (A) 0.2 mg/l; (B) 0.5 mg/l; (C) 0.3 mg/l. Bars represent 2 cm.

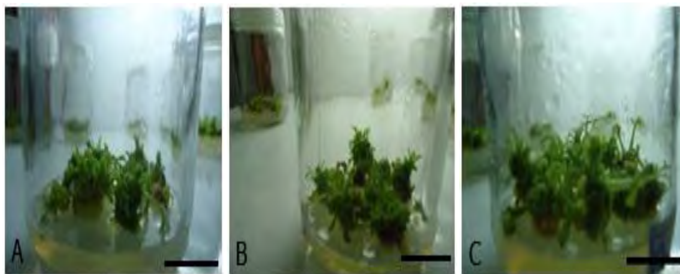

Figure 2. Multiple shoots of *T. abyssinica* on MS media supplemented with 0.5 mg/l GA_3 and different concentrations of BAP. (A) 0.2 mg/l; (B) 0.3 mg/l; (C) 0.1 mg/l. Bars represent 2 cm.

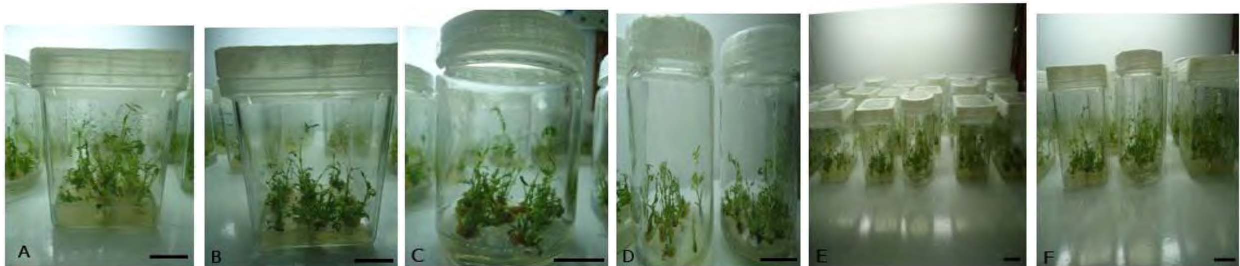

Figure 3. (A-F) Multiple shoots of *T. abyssinica* on PGRs free MS medium. Bars represent 2 cm.

5.3. Rooting

Rooting was observed six weeks after culturing the shoots on root induction medium containing 2.0 g/l activated charcoal. Out of the two different strength MS medium, the highest mean root length (2.66±1.31cm) and the highest mean root number (2.30±1.39) was observed on half strength MS basal medium containing 2.0 g/l AC supplemented with the same concentrations (0.4 mg/l) of IBA with NAA. However, no roots were observed on both full and half strength growth regulators free medium as well as on other hormone combinations (Table 3 and Fig. 4). Moreover, lowest number of roots (2.03±1.27) was obtained on full strength medium containing 0.4 mg/l IBA + 0.4 mg/l NAA.

Table 3. Number and length of root on full and half strength MS medium containing 2.0 g/l activated charcoal and different concentration of IBA alone and IBA with NAA

PGRs(mg/l)		Mean no. of root/explant		Mean root length (cm)	
IBA	NAA	Full strength	Half strength	Full strength	Half strength
0.0	0.0	0.0	0.0	0.0	0.0
0.5	0.0	0.0	0.0	0.0	0.0
1.0	0.0	0.0	0.0	0.0	0.0
1.5	0.0	0.0	0.0	0.0	0.0
2.0	0.0	0.0	0.0	0.0	0.0
0.1	0.4	0.0	0.0	0.0	0.0
0.2	0.3	0.0	0.0	0.0	0.0
0.3	0.2	0.0	0.0	0.0	0.0
0.4	0.1	0.0	0.0	0.0	0.0
0.4	0.4	2.03±1.27	2.30±1.39	2.53±1.26	2.66±1.31

Means followed by the same letter within a column were not significantly different at 5 % probability. Values are presented as mean ± SD

5.4. Acclimatization

After six weeks of acclimatization, approximately 30 plantlets from each full and half strength MS medium were harvested and transferred to greenhouse; 83.33 % plants from half strength and 66.6 % from full strength was survived with vigorous growth while the rest showed aberrant phenotypes and stunted growth as compared to the wild type (Fig. 4).

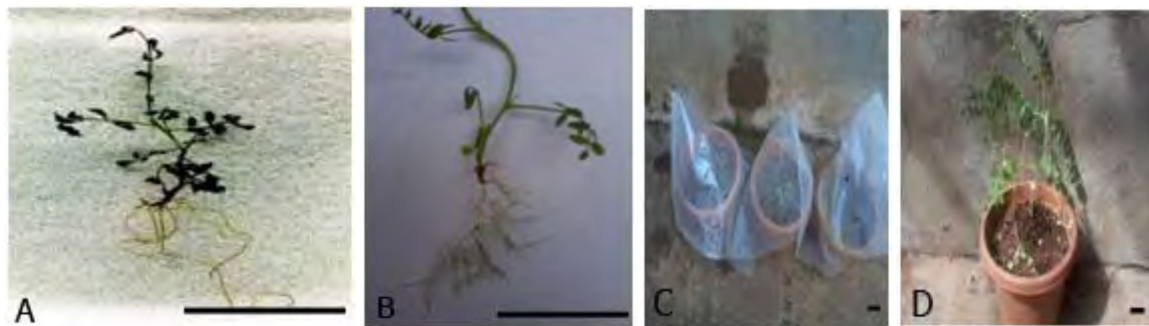

Figure 4. Rooted shoot of *T. abyssinica* (A, B) on ½ MS medium containing 2 g/l AC and 0.4 mg/l IBA in combination with 0.4 mg/l NAA; acclimatization of *in vitro* rooted shoots in glasshouse (C) Plantlets covered by polythene plastic bags (D) plantlets after six weeks. Bars represent 5 cm for A & B 1cm for C & D.

6. Discussion

6.1. Seed germination

In the present study shoots of *T. abyssinica* were successfully induced (80%) on PGRs-free MS medium without using any pre-germination treatment. Balcha Abera *et al*. (2010) obtained 96 ± 0.6% seed germination of *T. abyssinica* after 30 days of culture on MS medium without sucrose in the presence of pre-germination treatment.

According to Poonam (2013) germination rate of *Taverniera cuneifolia* was higher in those seeds which were treated with concentrated sulphuric acid with the germination percentage to be 80. The second highest germination percentage was 62.67% seen in boiling water and the third highest percentage germination was seen in lukewarm water with 57.33%.

6.2 Shoot multiplication

T. abyssinica responded differently on different concentrations of BAP and GA_3. At higher concentrations of BAP, stunted shoots were obtained. Malik *et al.* (2012) also reported, when the concentration of BAP was increased beyond optimal level, there was suppression of sprouting of axillary buds of *Cissus quadrangularis* (Vitaceae). Moreover, increasing the concentration of BAP (above 0.2 mg/l) in the culture medium, resulted in basal end callusing and browning of the growing shoots and this is in agreement with Rout (2005), but contrasting with Balcha Abera *et al* (2010) where both shoot formation frequency and shoot number per callus increased with increasing concentration of BAP.

In GA_3 supplemented medium, the shoot multiplication was increased as compared to BAP alone. The medium containing 0.1 mg/l BAP combined with 1.0 mg/l GA_3 showed good

response on shoot elongation. Poonam (2013) also obtained a good amount of growth and increased shoot length at low level of GA_3. This result is in contrast with the result of Rout (2005) where medium containing BAP 1.6–2.8 mg/l alone showed good response on shoot elongation. Unlike Rout (2005), Malik *et al.* (2012) reported that BAP + GA_3 was found more effective than KN+GA_3 for producing multiple shoots from nodal explants of *Clitoria ternatea*. Similarly, the result of this study showed that the mean number of shoots produced per explant increased when GA_3 was combined with BAP.

According to Ayob *et al*. (2012), the highest number (5.33 ± 0.33) of shoots was recorded when nodal explants of *Justicia gendarussa* were cultured on both types of media (MS and MS + vitamin B5) treated with 2.0 mg/l BAP. However, there was no significant difference in the number of shoots produced between MS and MSB5 media for all treatments. As the BAP was increased (up to 2.0 mg/l), number of shoots was raised from 2 to 5; the later values with BAP at 0.0 and 2.0, respectively. Beyond these values, the mean number of shoots dropped. In contrast, other researchers indicated that 3.0 mg/l BAP produced shoot regeneration (67 %) compared to 2.0 mg/l BAP produced shoot regeneration (52 %) from nodal explants of *Justicia gendarussa* (Hernandez *et al*., 2008).

Cytokinins were shown to be the most critical growth regulators for shoot elongation of many medicinal plant species (Jha and Jha, 1989; Sharma *et al*., 1993; Chen *et al*., 1995; Saxena *et al.,* 1998; Rout *et al.,* 2000). Among the cytokinins used, BAP was highly suitable compared to other cytokinins (Hwang, 2005). According to Agrawal and Sardar (2003), 0.2 mg/l BAP was found best to induce multiple shoots in cotyledonary node. However, at higher concentration of BAP (2.5 mg/l), a decreasing trend in response in terms of percentage of responding explants, average shoot number per explant as well as average shoot length was observed.

Malik *et al.* (2012) obtained efficient shoot regeneration from axillary buds on MS medium with lower levels of BAP than on media supplemented with higher concentrations of BAP. These findings were consistent with the results of this experiment. Malik *et al.* (2012) also reported that the promontory effect of BAP added to MS medium on bud break and multiple shoot formation in *Tridax procumbens* is comparable to the reports published in other medicinal plants (Debnath *et al*., 2006; Bhat *et al*., 1995).

Goyal and Bhadauria (2006) reported highest frequency of shoot formation (85 %) from nodal explant using MS basal medium containing BAP (1.1 mg/l) with GA_3 (0.2 mg/l) and NAA (0.5-2.0 mg/l).

The highest shoot multiplication rate (20.65 ± 0.20 shoots/explant) of *Ceropegia juncea* Roxb was achieved on MS medium supplemented with 2.2 mg/l BAP + 1.0 mg/l TDZ (Krishnareddy *et al.*, 2011). Murthy and Kondamudi (2011) established a thin cell layer of nodes and internodes of *Ceropegia spiralis* L. cultured on MS medium supplemented with 3.3 mg/l BAP + 0.09 mg/l NAA induced 17.34 ± 0.55 shoots showing extensive growth.

T. abyssinica that was cultured on PGR-free medium (control) exhibited strong apical dominance with minimum tendency to branch. Due to this, most of the shoots produced one or two shoots and mean length of shoots was the highest which may be because of apical dominance. This is in agreement with Folla Bekele *et al*. (2013) who reported that shoots cultured on PGRs-free medium resulted in low multiplication rate on *Coccinia abyssinica*. Rout (2005) also reported that nodal explants cultured in media without PGRs did not show any sign of growth.

The result of this study revealed that with increasing the concentration of BAP number of shoots increased. However, when the concentration of BAP is supra-optimal, the shoots became stunted

and the addition of GA_3 increased the mean shoot length. In addition to these as the concentration of GA_3 increased mean shoot length also increased.

Kalidass *et al.* (2010) also obtained maximum length of shoots (5.88±0.44) and the highest percentage of shoot induction on MS medium containing 1 mg/l BAP and 1 mg/l GA_3. Some authors have also reported combination of BAP and GA_3 for achieving multiple shoot formation (Sarker *et al.,* 2003; Sakila *et al*., 2007).

6.3. Rooting

First root was observed after six weeks of culture. Transfer of *in vitro* multiplied shoots to half strength MS medium with 0.4 mg/l IBA and 0.4 mg/l NAA showed maximum root induction. Agrawal and Sardar (2006) examined the effectiveness of various auxins on rooting of *Cassia angustifolia* microshoots and found that 2.0 mg/l IBA was superior to IAA or NAA. However 40 mg/l IBA was best for *ex vitro* rooting in *C. angustifolia* (Parveen and Shahzad, 2011). According to Parveen *et al*. (2010), 0.5 mg/l IBA gave the maximum roots in *C. siamea*.

In this study the shoots cultured on half strength MS basal media supplemented with the same concentrations (0.4 mg/l) of IBA with NAA showed better rooting responses. Sreelatha *et al*. (2007) also reported that NAA (1.0 mg/l) + IBA (0.25 mg/l) produced long and well developed roots in *C. siamea*. No roots were observed on both full and half strength growth regulators free medium as well as on other hormone combinations. This is may be because of the effect of high level of endogenous cytokine in the plant that probably result in inhibition of root formation.

Different plant species might vary in their requirement of auxin type for adventitious root formation. Hwang (2005) reported that IBA was more suitable for root induction than NAA. The

effectiveness of IBA in rooting has been reported for medicinal plants like *Hemidesmus indicus* (Sreekumar *et al*., 2000). *Decalepis hamiltonii* (Giridhar *et al*., 2003), *Melia azedarach* (Thakur *et al*., 1998), and *Taxus mairei* (Chang *et al*., 2001). The addition of NAA alone or IBA with NAA facilitated callus formation.

In present study, the highest number of roots produced per explants was obtained on medium containing 0.4 mg/l IBA and 0.4 mg/l NAA. A study also indicated that shoot explants of *T. abyssinica* produced the maximum number of roots (4.0 ± 0.44) in medium containing 2.0 mg/l IBA (Balcha Abera *et al*., 2010). The rooting of in vitro developed shoots of *T. cuneifolia* (4~6 cm length) was best in MS medium containing 3% sucrose and 2 mgl/l NAA (Poonam, 2013).

Benmahioul *et al.* (2012), Yadav *et al.* (2013), Mehrizadeh *et al*. (2014) and many other scholars reported that rooted shoots gave better results in medium with activated charcoal as compared with media without activated charcoal. The use of activated charcoal and auxins to improve rooting was also supported by the results of Sairkar *et al*. (2009) as there was significant increase in percentage of rooting in media containing activated charcoal when compared with media lacking the earlier. The use of auxins in medium for rooting was also supported by the results of Arya *et al*. (2012). Their results of study showed that no rooting occurred without plant growth regulators and presence of auxins increased the percentage of rooting in multiplied shoots. These findings are in agreement with present study.

6.4. Acclimatization

The *in vitro* grown plantlets were successfully acclimatized in the glasshouse with 83.33% and 66.6% of survival those derived from half and full strength MS medium respectively.

In *Cassia siamea*, Sreelatha *et al*. (2007) reported that when micropropagated plantlets were transferred to pots containing 3:1 vermiculite: sand under greenhouse conditions, about 40 % of the plants survived. Higher survival of 85 % was recorded when plantlets of *C. siamea* were transplanted into 1:1 sterilized garden soil and garden manure (Parveen *et. al*., 2010). Approximately 70 % of rooted plants of *Cassia obtusifolia* survived in pots containing a 1:1:1 mixture of sterile sand, soil and farmyard manure (Hasan *et al*., 2008).

7. Conclusions

- Results achieved in this study demonstrate that shoots of *T. abyssinica* were successfully established from seeds on PGRs free MS medium.
- MS medium containing 0.2 mg/l BAP + 1.0 mg/l GA_3 induced maximum axillary bud proliferation.
- The combination of 0.1 mg/l BAP and 1.0 mg/l GA_3 showed best response for obtaining better shoot length than BAP alone.
- Half strength MS medium is better for obtaining higher number of roots than the full strength.
- The higher acclimatization percentage (83.33%) was exhibited by plantlets derived from half strength MS medium supplemented with activated charcoal than those derived from full strength MS medium.
- The micropropagation protocol developed in this study provides a basis for germplasm conservation and for further investigation and improvement of this important and threatened medicinal plant.

8. Recommendations

- Based on the research findings of this study, the following recommendations are forwarded
- The different regeneration and micropropagation systems which have been developed need to be field tested and the field data is collected so that the complete technology packages could be ready for commercialization and transfer to the user agencies.
- Pharmaceutical companies and traditional medicine producers depend largely upon materials extracted from naturally occurring stands that are being rapidly depleted because of the use of parts like roots, bark, wood, stem and the whole plant in the case of herbs. This poses a specific threat to the gene pool and to the diversity of such medicinal plants so that, further investigation of the plant should be done to produce the bioactive compounds under *in vitro* conditions to conserve this threatened medicinal plant.

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10. Appendix

Appendix 1 Stock solution for MS (Murashige and Skoog1962)

Components	Concentration (g/L)
Macro nutrients	
NH_4NO_3	33
KNO_3	38
$CaCl_2.2H_2O$	8.8
$MgSO_4.7H_2O$	7.4
KH_2PO_4	3.4
Micro nutrient	Concentration (mg/500ml)
$Zn\ SO_4.7H_2O$	430
H_3BO_3	630
$MnSO_4.4H_2O$	2230
$CUSO_4.5H_2O$	2.5
KI	83
$Na_2Mo_4.2H_2O$	25
$CoCl_2.6H_2O$	2.5
Vitamins	Concentration (mg/500ml)
Myo-inositol	10000
Nicotinic acid(NaOH)	50
Pyridoxine(B6)	50
Thiamin(B1)	10
Glycin	200
Fe-Na-EDTA and $Feso_4$ mixture	Concentration (mg/500ml)
Fe-Na-EDTA	3730
$FeSO_4.7H_2O$	2780

To prepare 1L of medium, take 50ml of Macro, 5 ml of Iron-EDTA, 5 ml of Micro and 5ml of Vitamin.

Appendix2. ANOVA table on shoot multiplication

ANOVA

		Sum of Squares	Df	Mean Square	F	Sig.
Shtn	Between Groups	727.258	15	48.484	10.136	.000
	Within Groups	2219.533	464	4.783		
	Total	2946.792	479			
Shtl	Between Groups	301.388	15	20.093	47.538	.000
	Within Groups	196.117	464	.423		
	Total	497.505	479			