Addis Ababa University
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In vitro regeneration of niger (*Guizotia abyssinica* Cass.) using cotyledon and hypocotyl explants

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By

Tesfaye Disasa

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List of abbreviations

BAP	6-Benzylaminopurine
cm	centimeter
Cv	Cultivar
Fig	Figure
IAA	Indol-3-Acetic acid
IBA	Indol-3-Butyric acid
l	liter
M	molar
mg	milligram
masl	meters above sea level
ml	milliliter
mm	millimeter
MS	Murashige and Skoog basal media
NAA	α-Naphthalene Acetic Acid
s	seconds
µ	micrometer

Abstract

Callus induction and shoot formation from hypocotyls and cotyledons of *Guizotia abyssinica* Cass. has been achieved in this experiment. The frequency of callus induction was influenced by the concentrations and types of growth regulators. Six to eight-day-old hypocotyl segments and cotyledons were cultured on MS medium containing different concentrations of NAA, IAA and BAP. Among the various concentrations tested, 0.5 mg/l NAA in combination with 1 mg/l BAP was found to be the best for maximum callus induction of hypocotyl explants. Furthermore, 2 mg/l IAA in combination with 1 mg/l BAP was the best for callus induction of cotyledonary explants. Highest percentage of shoot formation (60%) was obtained when cotyledons were cultured on medium supplemented with 3.0 mg/l IAA in combination with 1.0 mg/ l BAP. Maximum number of shoots per explant (20.3) was obtained from medium containing 0.1 mg/l NAA in combination with 1 mg/l BAP. The types of explant, growth regulator combinations and genotypes were showed significant effect on shoot regeneration. The elongated shoots were successfully rooted on media supplemented with IBA at a concentration of 0.5 mg/l. The shoots were established in soil where 65% of them survived. Morphologically aberrant plants were not observed. As plant regeneration protocol is a prerequisite for genetic transformations, this protocol can be used for such purposes and development of new varieties with desired traits by *in vitro* selection.

1. Introduction

Niger (*Guizotia abyssinica* Cass*)* is stout, erect moderately branched annual herb with attractive yellow flowers and small black glossy seeds. Niger is indigenous to Ethiopia and it is one of the most important oil crops of Ethiopia. About two third of the total edible oil in Ethiopia is obtained from niger (Rilay and Hiruy Belayneh, 1989). It is more commonly known by its local name, nug in Ethiopia (Eklund, 1971).

Niger is a naturally-cross-pollinated crop grown extensively in Ethiopia and India as an oil crops. A single plant may produce 20-40 flower heads (Rilay and Hiruy Belayneh, 1989). The height ranges from 35 cm to 2 m, with a well developed root system consisting of a sharp root with many laterals especially in the top 5 cm of the soil (Seegler, 1983). The branches per plant are usually limited when competition between plants is too high. The type of germination in niger is reported to be epigeal with fast growing hypocotyls. The cotyledons stay on the plant for a long time and grow considerably after germination.

Ethiopia is one of the major niger producing countries in the world. The annual production is estimated to be 200,000-250,000 tones (Weiss, 2000). However, its average seed yield per hectare remained very low (Hiruy Belayneh, 1986). The major reasons for low seed yields include low yielding capacity of the cultivars, self-incompatibility and susceptibility to abiotic stresses such as susceptibility to lodging, shattering as a result of heavy rain and wind at maturitry and so on. These problems necessitated the use plant tissue culture and genetic transformation in addition to conventional plant

breeding in order to improve the yield and the nutritional or oil content of the crop.

Therefore, it is needless to mention the importance of tissue culture methods which offer the possibility of large scale production of elite clones and also the induction and isolation of useful genetic variants. The use of tissue culture technology for the vegetative propagation of plants is most widely applicable in many plants.

Immature embryos, young inflorescence and young leaves have proved to be most suitable for the establishment of totipotent culture in many crops (Thorpe and Patel, 1994).

Niger crop improvement efforts have been limited to Ethiopia and India. *In vitro* regeneration of niger has not been reported with Ethiopian genotypes, although some works have been reported from Indian genotypes (Nikam and Shitole, 1993; Sarvesh *et al*., 1993; Sujath, 1997)). Therefore, this work was the first attempt to test the *in vitro* regeneration potential for Ethiopian cultivars of niger.

2. Literature review

2.1. Taxonomy of *G. abyssinica*

G. abyssinica belongs to the family Compositeae and tribe Heliantheae (Baagøe, 1974). The genus *Guizotia* has been reported to contain six species of both domesticated and wild plants. All of them are native to tropical Africa, and five are found in Ethiopia. According to Kifle Dagne (1994), *G. abyssinica* is more closely related to *G. scabra spp. Schmperi,* which is distinguished from other species, belongs to the same genus by ovate outer phyllaries and large achene size. Furthermore, both *G. abyssinica* and *G. scabra spp. schmperi* are similar in morphology, karyotype and produced fertile hybrids with 95% of pollen fertility (Kifle Dagne, 1994). This strengthen the assumption that *G. abyssinica* may be evolved from *G. scabra spp. schmperi* through selection by Ethiopia farmers (Kifle Dagne, 1994). The two wild and cultivated species, can be distinguished from each other by the shape of the paleae and involucre bracts which are narrow and pointed in *G. scabra spp. schmperi* and broadily ovate in *G. abyssinica* (Rilay and Hiruy Belayneh, 1989). Furthermore, the seeds of niger is much bigger than the seeds of weedy species.

2.2. Origin and geographic distribution of *G. abyssinica*

G. abyssinica is originated in the northern high lands of Ethiopia where it grows wild and is cultivated (Baagøe, 1974; Weiss, 2000). According to Baagøe (1974), there are four indicators which strengthen this assumption. Firstly, there is highest concentration of *Guizotia* species found in the

region. Secondly, the species have been collected in Ethiopia both as a crop plant and as a weed, and in natural localities, but in India it has never been collected in natural localities. Thirdly, one may find similar distribution patterns in other genera containing cultivated species. Fourthly, the trade between India and the horn of Africa for long time explains how the seeds may have traveled from Africa to India. There are also reports that explain niger to be among the earliest of the domesticated crops in Ethiopia, along with teff (*Eragrostis tef*), Ensete (*Ensete ventricosum*), finger millet, and coffee (*Coffea arabica*) (Rilay and Hiruy Belayneh, 1989).

In addition to Ethiopia and India, niger is also cultivated, to some extent, in east African countries such as Tanzania, Uganda and Sudan (Kifle Dagne, 1994). In Zimbabwe it has been grown as a green manure and for silage (Vaughan, 1970).

2.3. Ecology and agronomy of *G. abyssinica*

Although, niger is adapted to both temperate and tropical climates, it prefers moderate climate for growth. It is reported to tolerate annual precipitation of 660 to 1790 mm, annual temperature of 13.6 to 27.5^{o}C. According to Weiss (2000) niger is adapted a wide range of soils from sandy to heavy clay soil but appears to grow best on clay loams to sandy clays. In East Africa, it grows at higher altitudes up to 2500 m.a.s.l. The principal regions of niger production in Ethiopia have an altitude of 1700 to 2200 m.a.s.l. The average daily temperature of the region where niger is grown is from 16 to 20^{0}C during the growing season. However, it gives high yield at lower altitudes ranging from 500-1600 m.a.s.l. (Duke, 1983). Smaller seedlings of niger do

not resist frost though night temperature to 9^0C had no significant effect on growth in the Ethiopian highlands (Weiss, 2000).

Niger requires adequate rainfall over the main growing period to produce a commercially accepted yield. It is not primarily a dry land crop though often it is grown in the low rainfall areas, but this is usually due to farmers preference to use more fertile land for food crops. A rainfall of 1000-1300 mm is the optimum; 800 mm will produce a reasonable yields if spread over the growing period, above 2000 mm usually depressed the growth of the crop. In addition, Prinz (1976) reported that as more water become available to the plant, there was an increase in height of the plant and of the reduction in number of branches, stem diameter, daily dry matter accumulation, ratio of dry matter in aerial parts to subterranean parts, and the number of heads per plant, but the number of florets per head or the length of the stem below the lower most green leaves were not affected.

In Ethiopia, there are cultivars that withstand water logging and are planted in high rainfall areas, or in soils with embedded drainage. There is no restrict sowing date for niger. It may be sown any time between May and September (Rilay and Hiruy Belayneh, 1989).

High rain fall and wind during seed maturity cause sever shattering and reduce yield (Getinet Alemaw and Sharma, 1996). Furthermore, delayed harvesting time will cause heavy loss of seed through shedding (Duke, 1983). Therefore, appropriate harvesting time is an important practice in reducing shattering.

In Ethiopia, niger is mostly sown in areas with a poor soil fertility (Nema and Singh, 1965). Traditionally no fertilizer is used. This may affect the yielding potential of the crop.

Niger is usually planted in pure stand in rotation with *E. tef* (Getinet Alemaw and Sharma, 1996). However, in some places it is interplanted with *E. tef*. Niger is a good precursor for cereals because crops following niger have less weed infestation (Getinet Alemaw and Sharma, 1996).

2.4. Economic importance of *G. abyssinica*

The seed of niger is an important oil crops in Ethiopia and parts of India. In Ethiopia, niger seed provides about 50% to 60% of edible oil, while in India it accounts for almost 2% of total oil seeds produced (Rilay and Hiruy Belayneh, 1989). Traditionally the oil is prepared from slightly roasted seed, which is pounded or ground. The soft mass is placed in hot water and squeezed, as a result the oil librated and starts floating (Seegler, 1983).

The oil is used to prepare various types of foods, paints and soaps, and as an illuminant. It is used as an adulterant for the rape oil, sesame oil, and so on (Duke, 1983). Whole seeds can be used fried or as condiment, pressed with honey are made into cakes in Ethiopia, and the press- cake from oil extraction is used for livestock feed. In Ethiopia, crushed niger seed just like most other oilseeds, is mixed with pulses and used to prepare local foods, letlet. In Europe, the seed is used in the preparation of animal feed and is especially known in the feed cage birds. Plants are used as a ‘bee plant’. The oil of the seeds is also used in rheumatism (Duke, 1983).

Traditionally, niger sprouts mixed with garlic is used to cure cough. Niger oil is also used in birth control and, cooked with spices in the treatment of syphilis (Hiruy Belayneh, 1991).

Due to vigorous growth, even on poor soils, whole plants are used as green manure in the pre-flowering stage (Chavan, 1961). This plays an important role in improving the fertility of the soil. In addition, since the fresh plant is not eaten by cattle except sheep, it may therefore, be planted as a protective border along a field of a crop more favoured by animals.

2.5. Cytology and karyotype of *G. abyssinica*

All of the known taxa of *Guizotia* have 2n=30 (Hiremath and Murthy, 1992; Kifle Dagne, 1994). This shows that speciation within the genus did not involve changes in chromosome number (Kifle Dagne, 1994).

According to Kifle Dagne (1994), the number of satellited chromosomes varies between taxa of the same genus, which has occurred during speciation. Taxa with more symmetrical karyotype have higher numbers of satellited chromosomes. There are a number of chromosome banding techniques used for plant chromosome identification. The most commonly used are C-banding and silver staining (Vosa, 1985; Sumner, 1990 cited in Kifle Dagne, 1994). The identification of individual chromosomes of several plant species has been possible with the help of C-banding techniques; however, most of the chromosomes in the genus, *Guizotia* lack C-bands. In addition, most of the available bands are found at similar sites on different

chromosomes (Kifle Dagne, 1994). The difficulty in chromosome identification in this genus limits the detailed cytogenetical investigation of *G. abyssinica*.

Kifle Dagne and Heneen (1992) found eight major nucleolus organizing regions in the various types of tissues of niger, which is the same as the maximum number of satellited chromosomes observed in the tissue of *G. abyssinica* so far.

2.6. Oil content and fatty acid composition of *G. abyssinica*

Niger seeds contain edible oil which is important for human consumption. The oil has fatty acid composition typical for seed oils of the Compositeae. There is similarity in fatty acid composition among different taxa (Kifle Dagne and Johnson, 1997). However, there is a significant difference between Ethiopian niger and that of Indian regarding fatty acid composition. The difference mainly lies on the linoleic acid and oleic acids content (Table 1). For instance, the Ethiopian niger contains about 70% linoleic acid which is about 20 % higher than that of Indian niger. In addition, different cultivars of niger also showed variability in oil content (Getinet Alemaw and Adefris Teklewold, 1995). This indicates the oil content of the crop could be improved through simple selection. Niger seed contains about 26.0 to 30.6 % crude protein (Nairullah *et al*., 1982).

Table 1. Ranges of fatty acid composition (%) of Ethiopian and Indian niger Source: Getinet Alemaw and Sharma (1996)

Fatty acid	Ethiopian niger		Indian niger	
	Range	Mean	Range	Mean
Palmitic	7.6-8.7	8.2	6.0-9.4	8.2
Stearic	5.6-7.5	6.5	5.0-7.5	6.7
Oleic	4.8-8.3	6.5	13.4-39.3	28.4
linoleic	74.8-79.1	76.6	45.4-65.8	56.0
Linolenic	0.0-0.9	0.6	-	-
Arachidic	0.4-0.8	0.5	0.2-1.0	0.6
Behenic	0.4-1.5	0.7	-	-

2.7. Production and research constraints of *G. abyssinica*

The yield of niger is considerably low mainly due to its cultivation on very shallow soil of poor fertility, lack of suitable package practices (Ganguli *et al*., 1992). In addition, insect pests are also responsible for the low yield although insects and diseases are not usually serious pests in either Ethiopian or Indian niger (Rilay and Hiruy Belyaneh, 1989).

2.7.1. Genetic factors

Maintenance of pure line is a problem because of self incompatibility nature of the crop and therefore, genetic improvement of niger has been limited

(Rilay and Hiruy Belayneh, 1989). The self incompatibility system in the Ethiopian population was studied and detailed genetic analysis was performed by Sileshi Nemomissa *et al.* (1999). According to these authors, the self incompatibility system in niger is under the direct influence of a saprophytic system. It is too complex to control pollination for the purpose of keeping pure lines of niger (Getinet Alemaw and Adefris Teklewold, 1995). Selfing results in little or no seeds. Besides, natural selfing results 100 % sterility in niger crop (Naik and Panda, 1968).

2.7.2. Pest, fertility and management problem

Duke (1993) reported that many fungi species such as *Alternaria pori, Alternaria carthami, Alternaria tenuis, Cercospora guizoticola, Cercospora guizotiae, Chaetomium guizotiae, Epicoccum nigrum* (leaf spot), *Macrophomina phaseoli, Puccinia guizotiae, Rhizoctonia bataticola, Rhiwctonia solani*, and *Scleitinia scleitiorum* have been isolated from different parts of niger plant, although niger has no serious pests and diseases. Many of the important pests of sesame and safflower may also attack niger (Weiss, 2000). Niger may be more resistant to many common pests attacking other crops since it often appears to suffer less damage than other crops when interplanted (Weiss, 2000).

There is no detailed description on niger diseases but it may generally said that the plant is resistant to some of the more wide spread pathogenic fungi infecting other oil seeds (Weiss, 2000).

The other factors that hinder the yielding capacity of niger crop in Ethiopia is the poor management system by the farmers. The crop has been classified as minor oil crop, although about two third of the total oil seed production is obtained from niger.

Niger is relatively a low–input crop, its yield potential show low response to fertilizer like phosphorus and nitrogen which only act on vegetative part. Too much fertilizer promotes vegetation and in some areas decreases seed yield as a result of lodging (Hiruy Belayneh and Nigussie Alemayehu, 1992). However, the effect of fertilizer is more pronounced when sowing is delayed. In most cases the fertilizer requirement of niger are ignored, the crop benefiting from fertilizer applied to suit the major component of the mixed crop (Weiss, 2000), but when the crop is sown in a rotation with a monocrop, it receives the minimum of added nutrients and residual fertility from previous crops. In addition, small growers do not consider niger important enough to warrant the use of precious cash resources needed more urgently elsewhere, with the result that yields often extremely low. Niger is generally grown as inter crop, lands are cultivated to favour the most important component, as a result niger frequently suffers from unsuitable land preparation (Weiss, 2000). It is important to control weed in niger to ensure high yield. However, weed control has not been practiced in many areas of Ethiopia. Substantial weed growth can reduce niger yield and this may be a major reason for the low average yields.

2.8. Breeding of *G. abyssinica*

Niger oil is the most widely used in Ethiopia due to its high quality oil. Therefore, its yield and oil content must be improved (Getinet Alemaw and Sharma, 1996). According to the authors, variety with single head, dwarf types must be developed in order to minimize the loss of seed by shattering. Shattering may be the result of non uniformity in maturity date. Production of single headed plant is more desirable than many heads per plant due to variation among many heads. Niger plants have many leaves which reduce the harvest index. Furthermore, dwarf plants have the potential of using fertilizer more efficiently than taller plants thus seed yields could be improved through fertilizer application (Getinet Alemaw and Sharma, 1996). The application of fertilizer to taller niger plants some times may result in negative response as it favoures vegetative growth which in turn promotes lodging of the crop.

Population improvement methods have been initiated by breeders both in Ethiopia and India. In Ethiopia, a number of populations are improved through simple mass selection (Rilay and Hiruy Belayneh, 1989).

2.8.1. Selection

Mass selection is a powerful tool for crop improvement. It is effective when it is designed to improve an already established cultivar for certain traits that are controlled by additive genes (Getinet Alemaw and Adefris Teklewold, 1995). The selection was designed to improve desirable traits such as dwarf type plant height, lodging resistance, synchronicity of maturity and seed yield per plant. Different types of genotypes have been developed by this type of selection.

Recurrent selection may also be used which involves selecting the best plants for desirable traits, mixing uniform size seeds and growing them in isolation for possible intercrossing, selecting as many plants as possible at maturity, and growing progeny rows of the selected plants.

2.8.2. Crossing technique

Niger has similar pollination behavior with sunflower (Getinet Alemaw and Sharma, 1996). It is considered to be an excellent candidate for the development of hybrid variety. Therefore, one can produce crossed seed by rubbing flowers derived from two parents together (Rilay and Belayneh, 1989). However, rubbing the florets may cause some selfing. Thus, it is advisable to make specific crosses by removing all the disc florets, just as the bud begins to open, but leaving the ray female florets intact.

2.9. Biotechnological approach for *G. abyssinica* improvement

2.9.1. Tissue culture of *G. abyssinica*

Most methods of plant transformation applied to genetically engineered crops require a whole plant regeneration from the isolated plant cell or tissue which has been genetically transformed. Hence, *in vitro* culture system plays an important role in the development of a reproducible transformation protocol. For these, the growth environment and culture medium should be manipulated to ensure a high frequency of regeneration. In addition to a high frequency of regeneration, the regenerable cell must be accessible to gene transfer by what ever technique is chosen (gene transfer methods).

Tissue culture response in many species depends on media composition, growth regulator combinations, genotype, explant type, organic component, developmental stage of the explants at the time of culture initiation and culture conditions (Vasil, 1983; Tang *et al*., 2003). Auxin and cytokinin were proved to be essential in callus induction and shoot formation. BAP is widely employed for the *in vitro* cultures. It has been used for the induction of callus as well as shoot regeneration in many plant species including oil crops (Sarvesh *et al*., 1993; Sujatha, 1997; Gurel and Kazan, 1998; Tang *et al*., 2003; Zhang *et al*., 2003 and Qin *et al*., 2006).

Most oil crops have been reported to be amenable to tissue culture although the degree of response varies from species to species. Among various species of oil crops, *Brassica* species are found to be the most responsive to tissue culture. In addition, there are reports on the development of transgenic

niger plants through *Agrobacterium* genetic transformation (Murthy *et al*., 2003). However, plant tissue culture and genetic transformation have not been reported for Ethiopian cultivars of niger to improve the productivity of the crop.

A number of protocols for oil crop plant regeneration through tissue culture have been developed by different researchers at different times. For instance, in niger (Nikam and Shitole, 1993; Sarvesh *et al*., 1993; Sujath, 1997), in sunflower (Gurel and Kazan, 1998), in brassica (Tang *et al*., 2003; Zhang *et al*., 2003), in safflower (Babbar *et al*., 2005) and in cauliflower (Qin *et al*., 2006).

Plant production using anther or microspore culture plays a vital role to develop homozygous mutant types and inbred lines in a short time. Microspore culture technology could also provide the production of recessive, simply inherited and easily identifiable marker traits, which are important for niger seed production to ensure genetic purity of varieties (Getinet Alemaw and Sharma, 1996).

2.9.2. Application of tissue culture

In vitro plant regeneration is an important step in plant biotechnology as it facilitates rapid vegetative multiplication (micropropagation) of valuable plant material for agriculture, horticulture and forestry. A single explant can be multiplied into several thousand plants in less than a year. This allows fast commercial propagation of new cultivars. It can be used to produce disease free plants, disease and pest resistant plants and so on. Plants grown

from tissue culture that pass through callus phase are subjected to variation. These may include some important agronomic characteristics like tolerance to pests, diseases, etc. In addition, rare and endangered plants can be cloned safely and plant ‘tissue banks’ can be frozen, then regenerated through tissue culture. Explants chosen from superior plants can be cloned. Plant cultures in media are easier to export than soil-grown plants, as they are pathogen free and take up little space (nowadays many companies use this system). Tissue culture, especially haplodization increase selection efficiency for crop improvement Moreover, tissue culture clones are ‘true to type’ as compared with seedlings, which show great variability.

3. Objectives

3.1. General objective

The objective of this study was to establish plant regeneration protocols in three genotypes of niger.

3.2. Specific objectives

The specific objectives are:

- to compare the regeneration potential among the different genotypes.
- to study the effect of various combinations of growth regulators on callus induction and shoot regeneration.
- to study the response of hypocotyls and cotyledons to different combinations of treatments in terms of callus induction and shoot formation.
- to examine the rooting potential of shoots at the different concentration of IBA.
- to acclimatize the plantlets in green house.

4. Materials and methods

4.1. Establishment of aseptic seedlings

Three improved varieties of niger namely Fogera, Shambu and Esete were obtained from Holeta Agricultural Research Center (HARC). The seeds were washed with 70% ethanol for about two minutes followed by surface sterilization in 10% (w/v) calcium hypochlorite for 15 minutes. The seeds were rinsed three times with sterile double distilled water. The sterilized seeds were aseptically plated on growth regulator free MS (Murashige and Skoog, 1962) basal medium. Glass Petri dishes were used and all have equal sizes (90 mm diameter). The Petri dishes were properly sealed with sealing tap and distributed on clean culture shelf. All the experiments were carried out in plant tissue culture laboratory (Plant physiology lab), Department of biology, Addis Ababa University.

4.2. Media

4.2.1. Stock solution preparation

In this experiment, MS medium was selected because it is the most widely used in various plant tissue culture experiments. The stock solutions of the media were prepared by weighing the recommended amount of macronutrients, micronutrients and vitamins (Table 2). The crystals were dissolved using double distilled water. The solutions were poured into labeled plastic bottles and stored at +4^{o}C until used. Stock solution for growth regulators were prepared by adding 1 mg powder in 1 ml double

distilled water. Auxins such as α-Naphthalene acetic acid (NAA), Indole-3-acetic acid (IAA) and Indole-3-butryric acid (IBA) were weighed and three to four drops of 1M NaOH were added until the powders were dissolved. The dissolved solution was poured into labeled volumetric flask. In addition, a cytokinin, 6-Benzyl aminopurine (BAP) was prepared in a similar way to that for auxin stocks except that 1 M HCl was used to dissolve the powders. The growth regulators were stored at +4^{o}C.

Table 2. Nutrient composition and concentration of MS basal medium used in the induction and regeneration media

Components	Concentration (g/l)
Macronutrients	
NH_4NO_3	16.5
KNO_3	19.0
$CaCl_2.2H_2O$	4.4
$MgSO_4.7H_2O$	3.7
KH_2PO_4	1.7
Micronutrients	
Fe-Na-EDTA	4.0
$ZnSO_4.7H_2O$	0.86
H_3BO_3	0.62
$MnSO_4.H_2O$	2.23
$CuSO_4.5H_2O$	0.0025
KI	0.083
$Na_2MoO_4.2H_2O$	0.025
$CoCl_2.6H_2O$	0.0025
Organic supplements	
Myo-inositol	1.0
Glycin (glycocoll)	0.2
Nicotinic acid	0.05
Pyridoxin (B6)	0.05
Thiamin (B1)	0.01

4.2.2. Culture media and culture conditions

The culture medium consisted of MS salts and vitamins containing 3 % (w/v) sucrose, and various concentrations of NAA, IAA and BAP (Table 3) were prepared. The pH of the solution was adjusted to 5.7 using 1 M HCl or 1 M NaOH. Then, 0.7% (w/v) agar was added into the solution and the media were covered with aluminum foil and autoclaved at 121^{o}C for 15 minutes. The media were then taken out and allowed to cool down in the laminar air flow cabinet. For seed germination, callus induction and shoot induction experiments, about 25 ml of the media was poured onto each 90 mm Petri dish. About 40 ml of the media was poured into each 50 mm diameter baby food jars and the media were used for shoot multiplication and elongation. About 60 ml of the media was poured in to magenta culture vessels and used for rooting purposes.

Table 3. Growth regulator combinations used for callus induction, regeneration and rooting media (the concentration of treatments is expressed in mg/l)

Callus induction media				Regeneration media				Rooting media	
Treat't	NAA	IAA	BAP	Treat't	NAA	IAA	BAP	Treat't	IBA
1	0.1	-	1.0	1	-	-	0.5	1	0.5
2	0.5	-	1.0	2	0.1	-	1.0	2	1.0
3	1.0	-	1.0	3	-	2.0	1.0	3	2.0
4	0.1	-	5.0	4	-	3.0	1.0		
5	0.5	-	5.0	5	-	5.0	1.0		
6	1.0	-	5.0						
7	-	2.0	1.0						
8	-	2.0	2.0						
9	-	3.0	1.0						
10	-	5.0	1.0						
11	-	5.0	2.0						
12	-	10.0	2.0						

4.3. Callus induction and proliferation

In this experiment, the effects of twelve combinations of IAA, NAA and BAP concentrations (Table 3) on callus induction from two types of explants; hypocotyl segments and cotyledons were compared. Cotyledons and hypocotyl segments excised from 6 to 8-day-old aseptic seedlings were used as explants. Hypocotyls were sectioned into 3 to 4 segments at the length of 8mm-10mm so that from a single seedlings three to four hypocotyl segments and the two cotyledons were used (Fig. 1 C, D). Cotyledons were separated from each other and each cotyledon was cultured by placing up side down onto the medium. Growth regulators free MS basal medium was used as control to compare the callus induction frequency.

About thirty explants were used for each treatment. Ten explants were placed on each Petri dish and sealed with sealing tape before transferring to incubator. The Petri dishes were kept in the dark at a temperature of $21\pm2^{o}C$ for the first five days and after the cultures were transferred to continuous cool white fluorescent light (40 $\mu molm^{-2}s^{-1}$) at a temperature of $25\pm 2^{o}C$.

4.4. Shoot regeneration and multiplication

After two subcultures carried out every three weeks on the original callus induction medium, well developed, green and non-friable calli were isolated and transferred onto MS media containing various concentrations of growth regulators (Table 3) in order to examine the regeneration potential of each genotype. Furthermore, growth regulators free MS basal medium was used as a control to test the response of callus to shoot regeneration. The

regenerated shoots were separated and further cultured onto media containing 0.5 mg/l BAP only for multiplication of shoots. Multiple shoots were transferred onto growth regulators free MS basal medium for elongation. The experiments were repeated twice.

4.5. Rooting and acclimatization

The isolated shoots (3-8 cm long) were collected and transferred to MS medium supplemented with IBA at different concentration (0.5, 1 and 2 mg/l).

Plantlets with well developed shoot and roots were separated from the culture medium, washed gently under running tap water and transferred to pots containing 2:1 loam to sand ratio. Potted plantlets were covered with thin transparent polythene plastic to ensure high humidity. The pots were transferred to green house of plant physiology (AAU) and watered every two days. Polythene plastic were gradually removed after one week and completely removed after two weeks in order to acclimatize plants.

4.6. Data analysis

Percent of germination, callus induction, shoot regeneration, average number of shoots per explants, percent of root formation, number of roots per plantlet and survival rate of plantlets transferred to green house were calculated. Completely Randomized Design (CRD) was used and ANOVA table was constructed to analyze the significance difference at or below 0.05 probability level.

5. Results

5.1. Seed germination

Surface sterilized seeds of all the three genotypes cultured directly onto growth regulators free MS basal medium showed 94-100% germination after five days of culturing (Fig. 1 A). The highest percentage (100%) of seed germination was obtained from variety Shambu (Fig. 1 B). All the genotypes showed elongated root and hypocotyls of similar length.

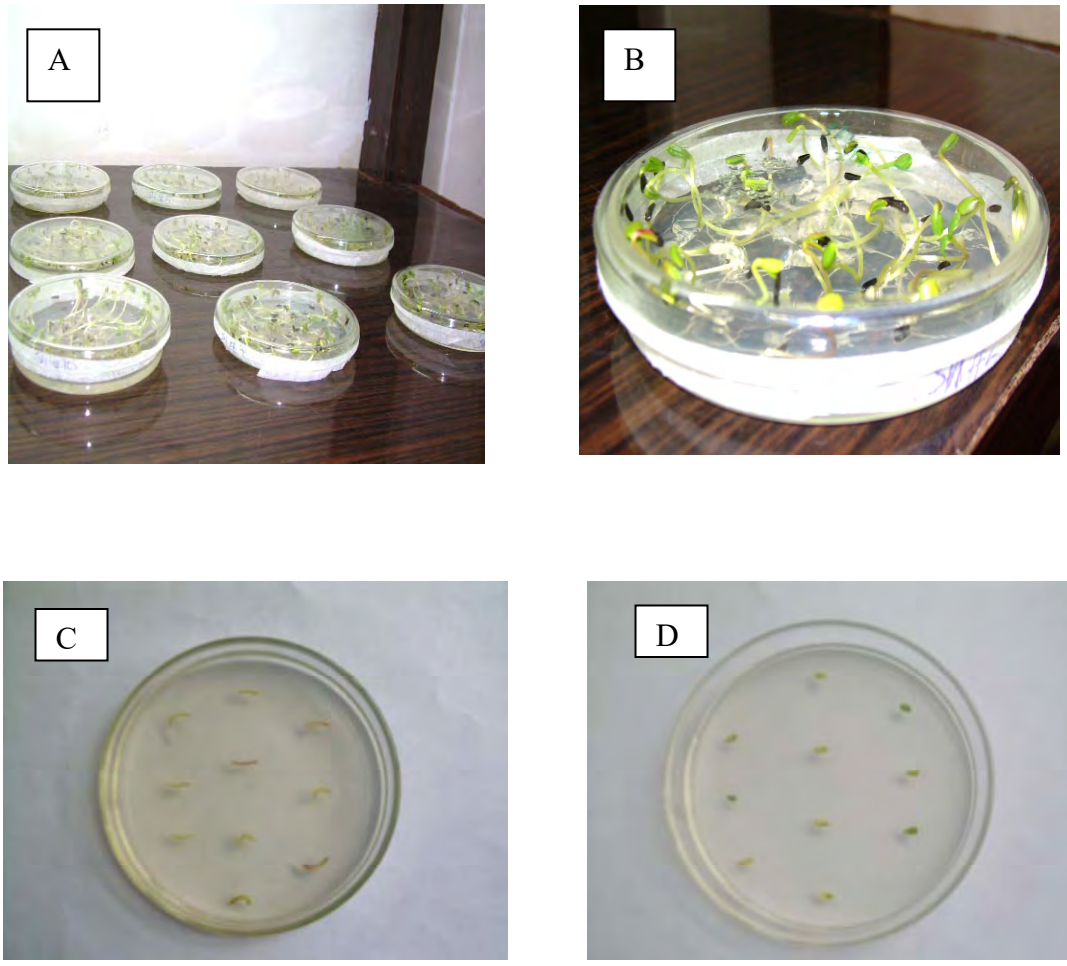


Figure 1. Seedlings of *G. abyssinica* after five days of seed culture (A) All three genotypes (B) Variety Shambu (C) Hypocotyl segments (D) Cotyledons

5.2. Callus induction and proliferation

Calli were formed from both cotyledons and hypocotyl segments of the three varieties (Fogera, Shambu and Esete) of niger. Response of explants to culture was observed within two weeks on callus induction medium. Well developed calli were observed in all three genotypes after three weeks of culturing (Fig. 2A). Different explants showed different color of callus. In all genotypes calli derived from cotyledons showed deep green color (Fig. 2C) whereas calli from hypocotyls segments showed various colors such as green and pale (Fig. 2B), brown (Fig. 2D).

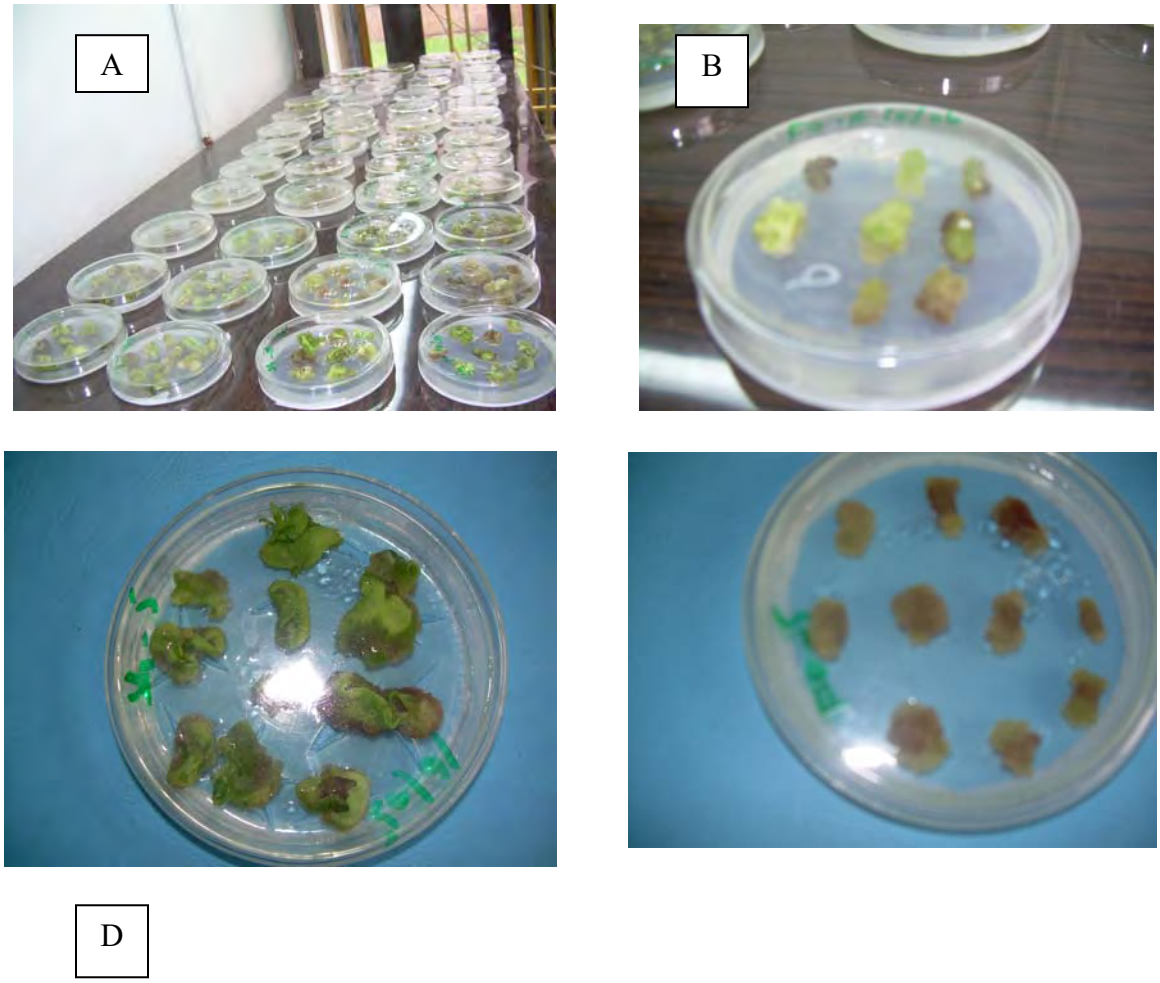


Figure 2. Induced calli after three weeks of culturing. (A) calli derived from all genotypes. (B) green callus from hypocotyls (Variety Fogera). (C) deep green callus from cotyledons (Variety Shambu). (D) brown callus from hypocotyls (Variety Fogera)

Different concentrations of growth regulators showed different callus induction frequency regardless of explant type and genotype. MS media supplemented with 0.5 mg/l NAA in combination with 1 mg/l BAP showed maximum percentage of callus induction in almost all genotypes using hypocotyls explants (Table 4). Callus induction frequency decreases with further increasing the concentration of NAA and BAP (Fig. 3). Besides, 2 mg/l IAA in combination with 1 mg/l BAP was found to be the next effective combination in callus induction (Table 4). However, there was a decrease in the percentage of callus responding with increase in the concentration of IAA (Fig. 4).

Explants cultured on growth regulator-free MS medium showed very low (<20%) response of callus induction. Furthermore, the calli were smaller in size and showed delayed response in cell proliferation.

Significant differences for callus induction were observed among different concentrations and combinations of growth regulators (Table 4). However, the frequency of callus induction was not significantly different between cotyledon and hypocotyl of a cultivar and among the cultivars (Table 5).

Table 4. Effects of different concentrations of growth regulator combinations on callus induction percentage of the three varieties.

Growth regulator combinations (mg/l)									
				Fogera		Shambu		Esete	
Tre't	IAA	NAA	BAP	Hypo	Cotyl	Hypo	Cotyl	Hypo	Cotyl
1	-	0.1	1.0	91.67^{ab}	63.33^{bcd}	80.00^{bc}	56.67^{cd}	80.00^{ab}	86.67^{a}
2	-	0.5	1.0	98.33^{a}	83.33^{ab}	98.33^{a}	76.67^{ab}	95.00^{a}	80.00^{ab}
3	-	1.0	1.0	86.67^{ab}	73.33^{abc}	86.67^{ab}	70.00^{abc}	81.67^{ab}	58.33^{bcd}
4	-	0.1	5.0	75.00^{abc}	43.33^{d}	76.67^{bc}	66.67^{abc}	81.67^{ab}	66.67^{abc}
5	-	0.5	5.0	75.00^{abc}	56.67^{cd}	80.00^{bc}	68.33^{abc}	80.00^{ab}	51.67^{cd}
6	-	1.0	5.0	48.33^{de}	58.33^{cd}	78.33^{bc}	60.00^{bc}	78.33^{ab}	58.33^{bcd}
7	2.0	-	1.0	95.00^{a}	91.67^{a}	88.33^{ab}	83.33^{a}	81.67^{ab}	88.33^{a}
8	2.0	-	2.0	66.67^{bcd}	75.00^{abc}	66.67^{c}	66.67^{abc}	66.67^{b}	70.00^{abc}
9	3.0	-	1.0	53.33^{cde}	66.67^{bcd}	43.33^{d}	73.33^{abc}	43.33^{c}	50.00^{cd}
10	5.0	-	1.0	53.33^{cde}	61.67^{bcd}	33.33^{d}	80.00^{a}	38.33^{c}	55.00^{cd}
11	5.0	-	2.0	46.67^{de}	53.33^{cd}	40.00^{d}	56.67^{cd}	43.33^{c}	66.67^{abc}
12	10.0	-	2.0	28.33^{ef}	53.33^{cd}	30.00^{de}	41.67^{d}	30.00^{cd}	36.67^{de}
13		Control		18.33^{f}	11.67^{e}	15.00^{e}	11.67^{e}	16.67^{d}	13.33^{e}

Means followed by the same letters in the same column are not significantly different at 5 % level of probability.

Table 5. Mean callus induction percentage using the three genotypes.

Genotypes	Explants	Mean of callus induction (%)
Fogera	Cotyledons	61.00[a]
	Hypocotyls	64.34[a]
Shambu	Cotyledons	62.44[a]
	Hypocotyls	62.82[a]
Esete	Cotyledons	60.13[a]
	Hypocotyls	62.82[a]

Means followed by the same superscript (a) in the column are not significantly different at 5 % level of probability.

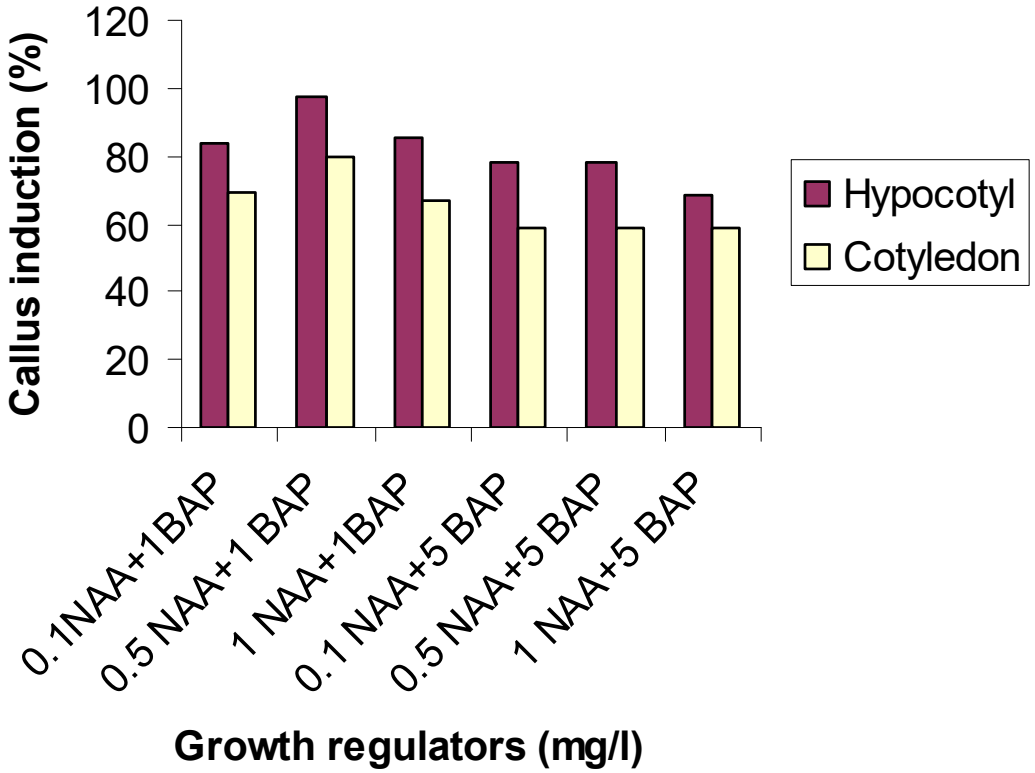


Figure 3. The effect of different concentrations of NAA and BAP on callus induction using the three genotypes.

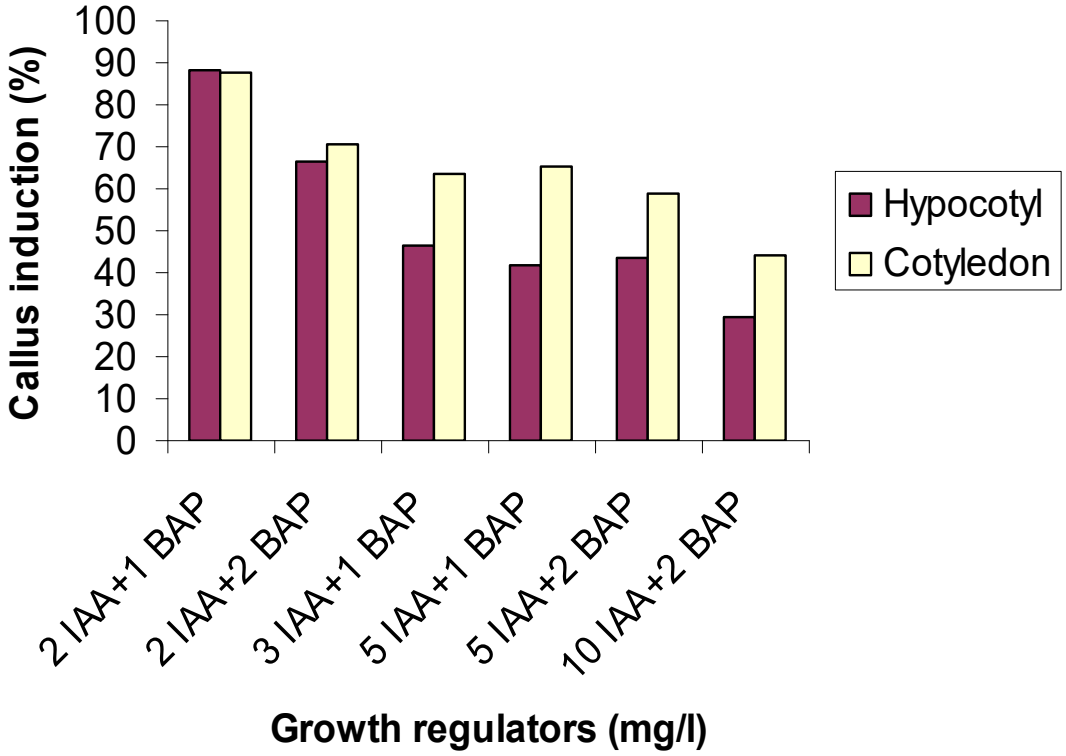


Figure 4. The effect of different concentrations of IAA and BAP on callus induction using the three genotypes.

5.3. Shoot regeneration and multiplication

Shoots started to appear on shoot regeneration media within 2-3 weeks in culture. Green callus obtained from cotyledon explants produced more number of shoots than pale green or brown colored callus (Fig. 5A, B). The maximum shoot regeneration frequency (60%) was observed in medium supplemented with 3 mg/l IAA in combination with 1 mg/l BAP from cotyledons of Fogera variety (Fig. 6A). The maximum mean shoot number per explant (20.3) also obtained from cotyledonary explant supplemented with 0.1 mg/l NAA and 1 mg/l BAP (Table 7).

Fogera genotype showed more shoot formation compared to the two genotypes and significant differences were observed between the three genotypes in terms of shoot regeneration. Similarly, significant differences between hypocotyls and cotyledons of a genotype and among the genotypes were observed (Table 6). Cotyledons were found to be the most suitable explant for regeneration of shoots in all three varieties of niger.

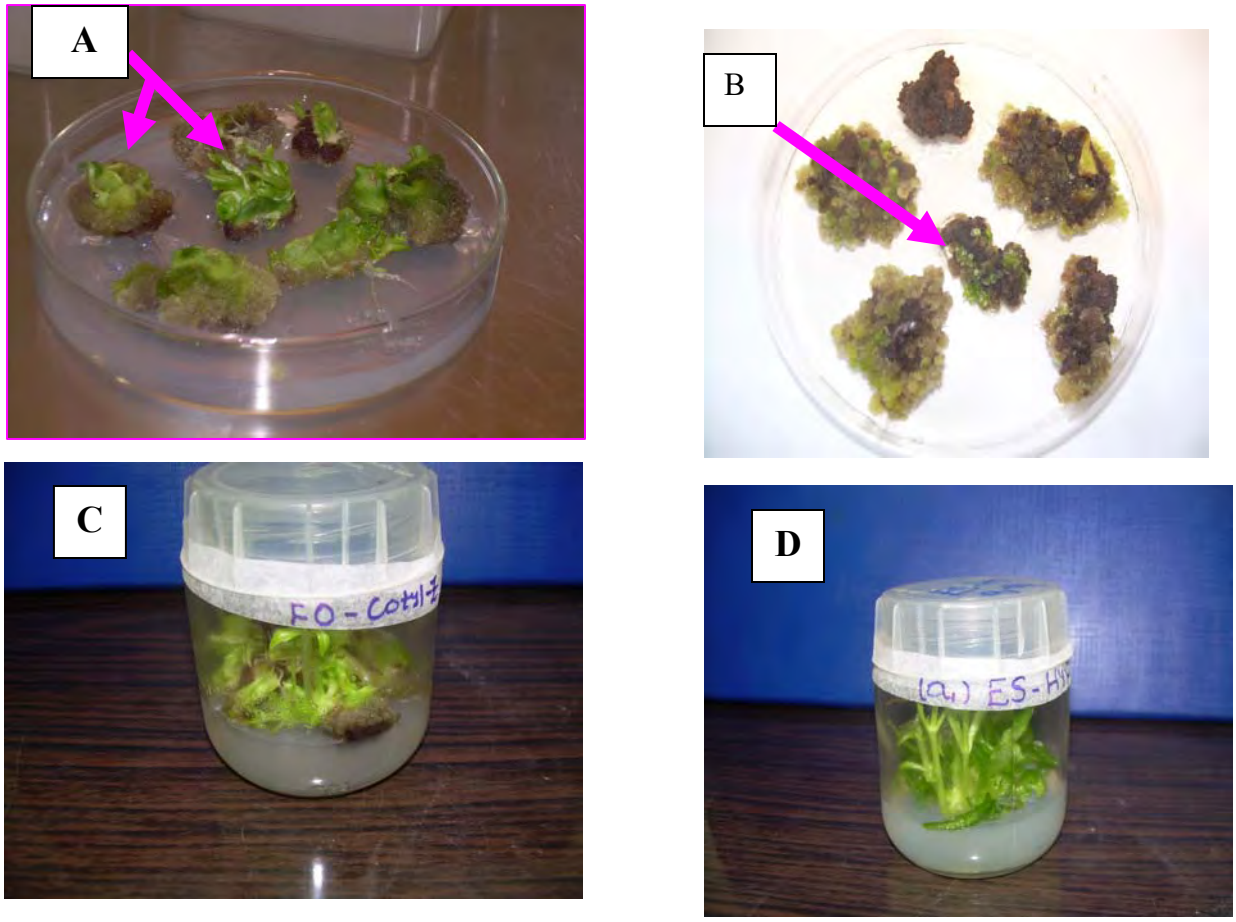


Figure 5. Shoot formation and multiplication. (A) Shoot buds derived from green callus (Variety Shambu) (B) From brown callus (Variety Fogera) (C) Multiple shoot (Variety Fogera) (D) Multiple shoot (Variety Esete). Arrows indicate shoot buds.

Table 6. Relationship between source of explants and shoot formation in *G. abyssinica.*

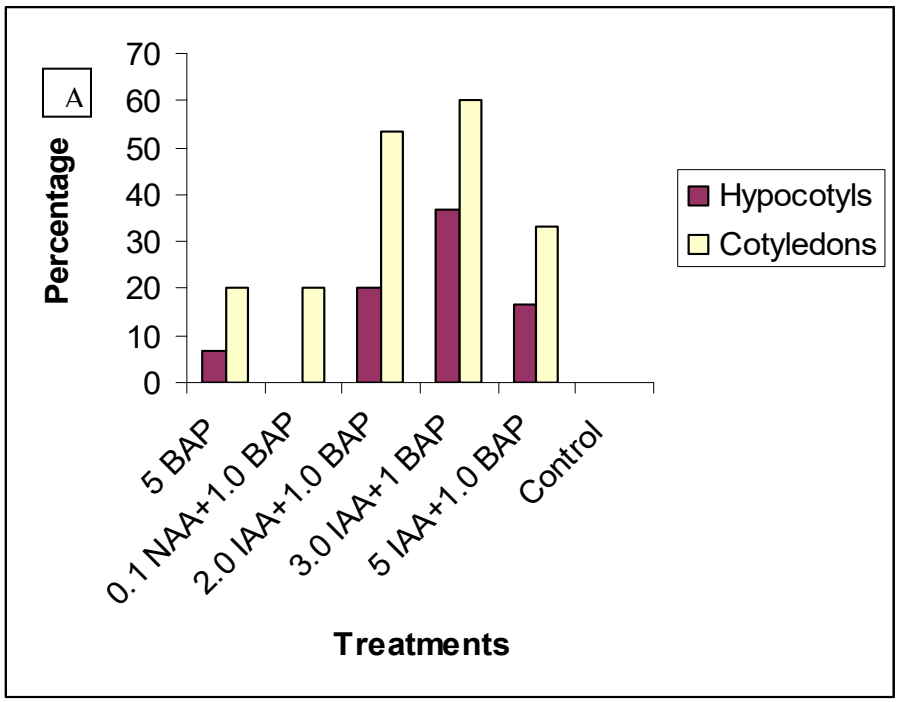
Varieties	Explants	Mean of shoot formation (%) ± SE
Fogera	Hypocotyls	16.0 ± 3.88^{a}
	Cotyledons	37.3 ± 5.39^{A}
Shambu	Hypocotyls	7.3 ± 1.82^{ab}
	Cotyledons	28.7 ± 5.15^{AB}
Esete	Hypocotyls	6.7 ± 1.87^{b}
	Cotyledons	14 ± 2.89^{B}

Means followed by different letters in the same column are significantly different at 5 % level of probability. Small letters indicate the difference b/n hypocotyls whereas capital letters indicate b/n cotyledons of the three varieties

Table 7. Mean number of shoots per explant produced from cotyledons and hypocotyls of *G. abyssinica* (Varieties: Fogera, Shambu and Esete)

Growth regulator combinations (mg/l)			Mean number of shoots per explant ± SE					
			Fogera		Shambu		Esete	
IAA	NAA	BAP	Hypo	Cotyl	Hypo	Cotyl	Hypo	Cotyl
-	-	0.5	11.00±2.52^{b}	11.6±2.73^{ab}	6.33±1.45^{ab}	3.33±0.67^{ab}	6.33±1.45^{a}	3.67±0.88^{a}
-	0.1	1.0	0.00±0.00^{c}	20.33±1.20^{a}	3.67±1.67^{ab}	6.33±1.20^{ab}	0.00±0.00^{b}	0.00±0.00^{b}
2.0		1.0	9.33±0.88^{b}	12.00±1.53^{ab}	7.67±2.33^{a}	9.33±2.90^{a}	7.67±0.88^{a}	4.33±0.88^{a}
3.0		1.0	11.33±2.40^{b}	10.33±3.39^{b}	4.00±1.00^{ab}	3.67±3.33^{ab}	3.67±0.88^{ab}	6.33±0.88^{a}
5.0		1.0	19.33±1.86^{a}	18.33±1.45^{ab}	0.00±0.00^{b}	4.00±1.00^{ab}	3.33±1.20^{ab}	6.67±0.67^{a}
Control			0.00^{c}	0.00^{c}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}

Means followed by the same letters in the same column are not significantly different at 5 % level of probability.



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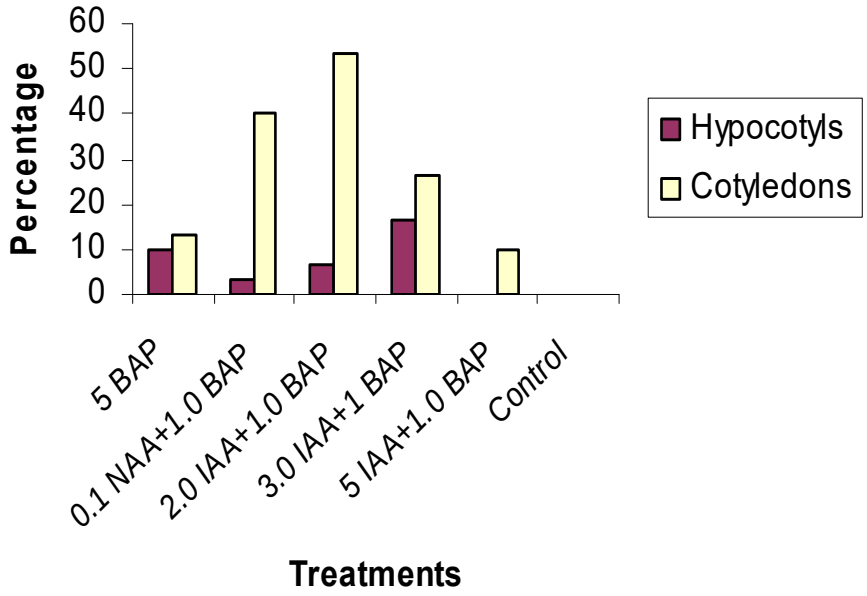

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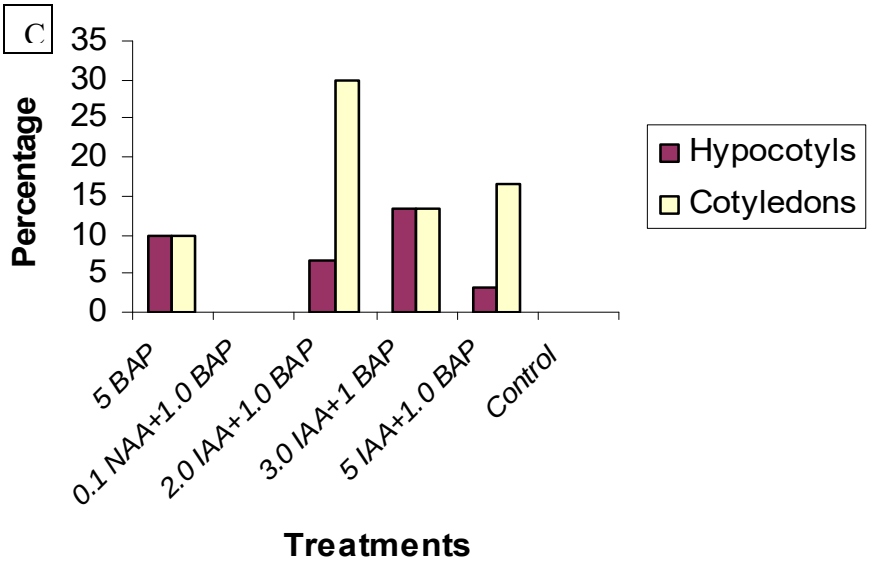

Figure 6. Effect of explant type, growth regulators and genotypes on shoot formation. (A) Var. Fogera (B) Var. Shambu (C) Var. Esete

The concentration of treatments is expressed in mg/l & percentage refers to % of shoot formation

5.4. Rooting

Elongated and well developed shoots transferred onto rooting medium showed variation in rooting potential with different concentration of IBA (Table 8). Maximum average percentage of root formation (96.67 %) was obtained from media supplemented with 0.5 mg/l IBA almost for all varieties (Table 8). At this concentration, an average number of 12 roots per shoot were produced for variety Esete, 10 for Shambu and 11 for Fogera. Furthermore, the shoots produced from this concentration were longer than the other treatments (Fig. 7 B, C).

When the concentration of IBA exceeds 0.5 mg/l, root forming frequency was significantly reduced (Table 8). Shoots transferred onto growth regulator-free media (control) showed the least root forming frequency (6.67%) as well as average number of roots (0.83) per explant (Table 8).

The frequency of rooting per shoot was significantly different among the different concentrations of IBA but not between genotypes (Fig.8).

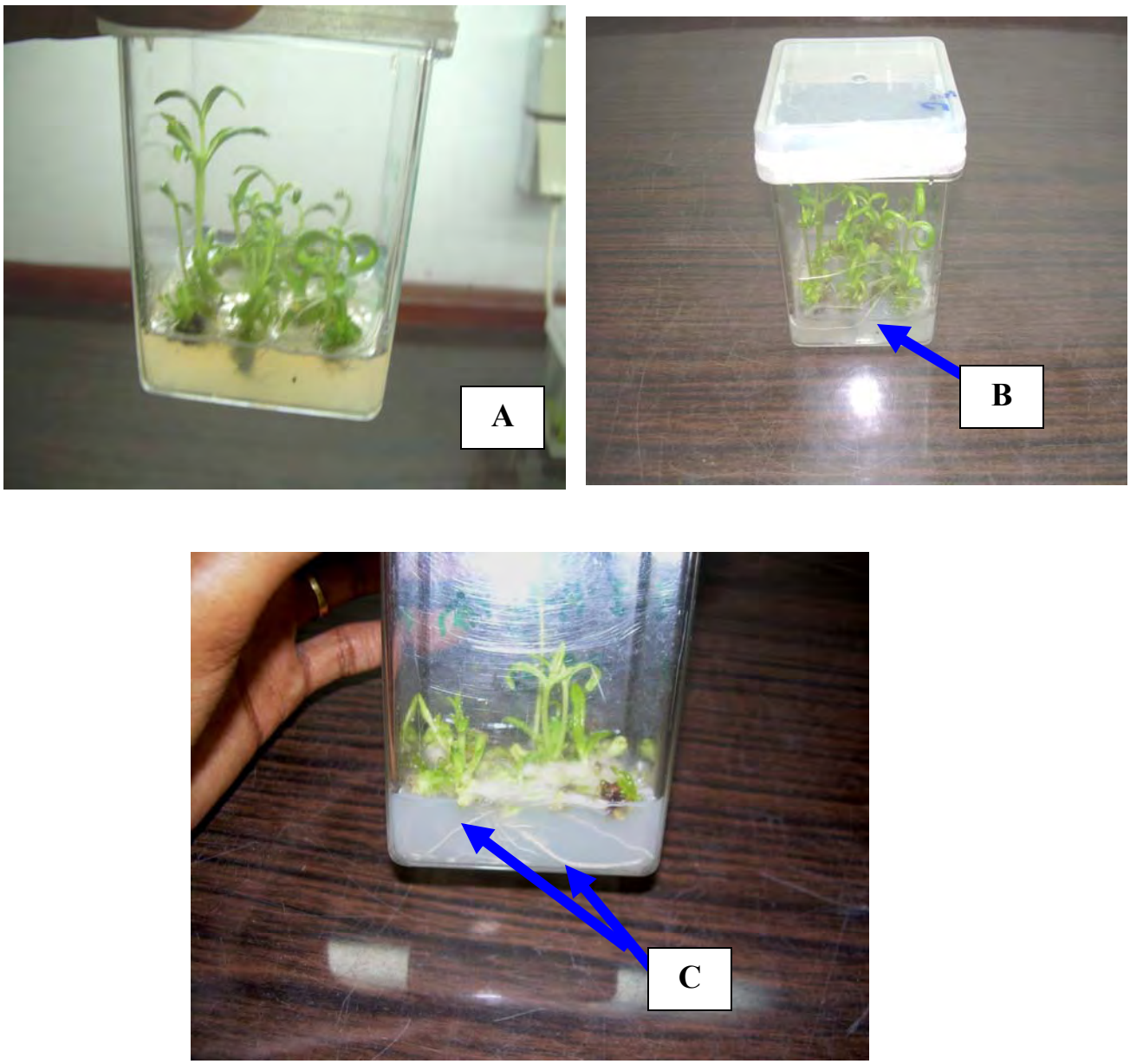

Figure 7. Effect of IBA on rooting (A) elongated shoot transferred to fresh rooting media (B) roots after ten days of culturing (C) multiple root formation after two weeks

Table 8. Effect of different concentrations IBA on rooting using the three genotypes

Growth regulators (mg/l)	Genotypes					
	Fogera		Shambu		Esete	
	Rooting (%)	Mean No. of root per explant ±SE	Rooting (%)	Mean No. of root per explant ±SE	Rooting (%)	Mean No. of root per explant ±SE
0.5 IBA	96.67^{a}	11.33±2.50^{a}	96.67^{a}	10.5±1.71^{a}	93.33^{a}	12.17±3.77^{a}
1.0 IBA	43.33^{b}	2.33±0.49^{b}	36.67^{b}	2.67±0.67^{b}	36.67^{b}	2.17±0.48^{b}
2.0 IBA	23.33^{bc}	1.83±0.31^{b}	16.67^{bc}	1.17±0.48^{b}	13.33^{bc}	1.67±0.33^{b}
Control	10.00^{c}	1.67±0.33^{b}	10.00^{c}	0.83±0.31^{b}	6.67^{c}	1.33±0.42^{b}

Means followed by the same letters in the same column are not significantly different at 5 % level of probability.

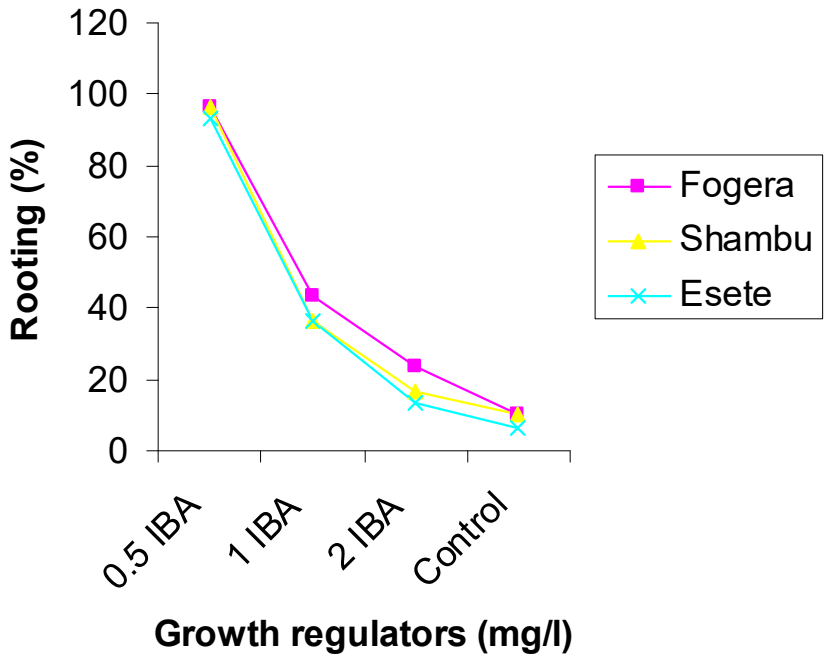

Figure 8. Rooting frequency among the three varieties of *G. abyssinica*

5.5. Acclimatization

Shoots with four to five fully expanded leaves and well-developed roots were hardened after subsequently transferred to a mixture of loam soil and sand in green house (Fig. 9). The percentage of survival rate of shoots after three weeks of transfer to soil was 65%. All plants had normal leaf development and did not show any detectable differences in morphology with the donor plant.

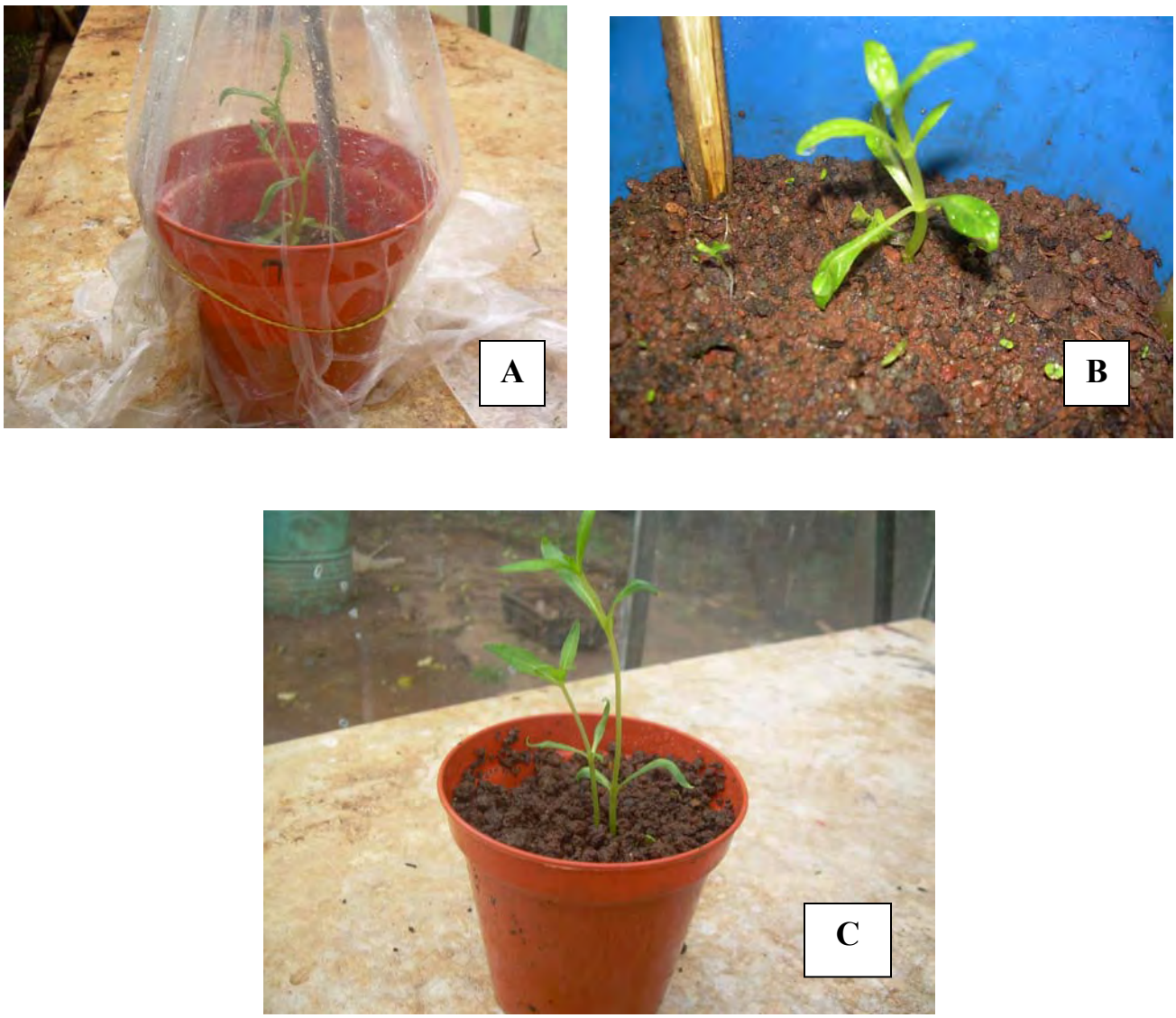

Figure 9. *In vitro* regenerated plantlets of *G. abyssinica* acclimatized in green house (A) After one week of transfer into soil mix. (B) After two weeks (C) After three weeks

6. Discussion

6.1. Establishment of callus culture

Successful development of efficient genetic transformation system requires an effective callus induction and regeneration protocols. In this study, the optimum callus induction protocol was developed from both hypocotyl and cotyledon explants of niger. Sarvesh *et al*. (1993) reported maximum (80%) callus induction from cotyledonary explant of niger cultured on MS media supplemented with 0.5 mg/l BAP in combination with 0.1 mg/l NAA. In addition, 93.3% callus induction was reported by Sujatha (1997) using leaf segments, but in this study a maximum of 98.33 % callus induction was observed from hypocotyl segments of both Fogera and Shambu varieties cultured on media supplemented with 0.5 mg/l NAA in combination with 1 mg/l BAP. In this experiment, 1 mg/l BAP and increasing the concentration of NAA from 0.1 mg/l to 0.5 mg/l increases callus induction frequency in all varieties. However, callus induction started declining when the concentration of NAA exceeds 0.5 mg/l. In general, the degree of callus induction varied depending on the concentration and type of growth regulator combinations. In contrast, callus induction frequency was not significantly affected by the type of explants (hypocotyl segments and cotyledons) and varieties in this experiment. However, cotyledon explants gave the maximum callus induction percent when cultured on media containing 2 mg/l IAA and 1 mg/ l BAP although not significantly differ with NAA.

6.2. Shoot formation

6.2.1. The effect of Explants

This experiment compared the regeneration potential of two explants (hypocotyls and cotyledons), based on the percentage of shoot formation and average shoot number per explant in three varieties. The result revealed that shoot formation frequency from cotyledon was significantly higher than those from the hypocotyl explants in all varieties (Fig. 7). This result was in agreement with the result achieved from the previous study on niger cv. Ootacamund (Sarvesh *et al*., 1993). Therefore, cotyledons were found to be more responsive to shoot regeneration, indicating that cotyledons believed to be more suitable for transformation experiments. These differences may be due to different nutrient requirements of the explants for optimal shoot regeneration. The maximum average number of shoots (20.3) per explant was also observed in cotyledon explants cultured on media containing 0.1 mg/l NAA in combination with 1 mg/l BAP (Table 7). According to Khehra and Mathias (1992), the most important factors for shoot regeneration were explant type and genotype. In addition, the concentration and combination of growth regulator also plays an important role in shoot regeneration.

6.2.2. Genotype effect

Every species require studies to find the right ratio of growth regulators for optimium physiological development *in vitro* (Taji and Williams, 1996). Herbaceous species require different media and culture conditions than woody species (McCown, 1986). The effect of genotypes also plays an important role on response of oil crop to various modern technologies like plant tissue culture (Tang *et al*., 2003). In this study, the effect of genotype was statistically significant and the maximum percentage of shoot regeneration (60%) and mean number of shoots per explant (20.3) was significantly higher in Fogera variety. Therefore, one has to focus on this genotype in order to improve the productivity of niger through modern techniques as it is more suitable for *in vitro* regeneration. In genetic transformation, higher number of plantlets from a single explant is very important since the chance of regeneration of the treated cell or tissue would be higher.

6.2.3. The effect of growth regulators

In other study on niger, the maximium number of shoot per explant (28.2) was reported from media supplemented with 0.1 mg/l NAA alone (Sarvesh *et al*., 1993). In same study, the maximum percentage (81%) of shoot regeneration was obtained from media supplemented with 2 mg/l IAA in combination with 1 mg/l BAP. In this experiment, although media supplemented with 0.1 mg/l NAA in combination with 1 mg/l BAP was found to be the best combination in terms of average number of shoots (20.3) per explant for variety Fogera using cotyledon explant, 3 mg/l IAA in combination with 1 mg/l BAP gave the highest (60%) shoot formation (Fig. 7). Furthermore, 5.0 mg/l IAA combined with 1 mg/l BAP gave high mean

number of shoots for explants, cotyledon (19.3) and hypocotyl (18.3) using Fogera variety. Shoot regeneration capacity of the callus increases with significant increase of the concentration of IAA especially in Fogera variety, but it started to decrease in all genotypes when the concentration of IAA exceeds 3 mg/l (Fig.7).

No shoot has been developed from callus cultured on growth regulator free medium. This indicates that plant growth regulator plays a significant role for differentiation of shoots.

6.3. Rooting and acclimatization

Among the different concentrations of IBA tested, the highest rooting (96.67%) percent and maximium number of roots per shoots were obtained from rooting media that contained 0.5 mg/l IBA. Similar result has been observed with previous study regarding root formation frequency (Sujatha, 1997). The result of this experiment showed consistency (Fig. 9) in all genotypes and there was no significant difference among them. But, the frequency of root formation was significantly different among the treatments (Table 7).

In addition to high percentage and high number of roots per shoots, roots obtained from media supplemented with 0.5 mg/l IBA were found to be relatively longer (Fig. 8 B, C). This may enhance the survival rates of the shoots because a well developed root system are needed to increase the percentage of successfully acclimatized plantlets (Ohki *et al*., 1991).

Plantlets transferred to growth regulator free medium showed minimum percentage of rooting and reduced number of roots per shoot.

The survival rate (65%) of the transferred shoots of this study was better than that of the result (61.36%) achieved from the previous study on niger (Sujatha, 1997).

7. Conclusions and recommendations

7.1. Conclusions

The result of this study shows that

- Optimizing the concentrations and combinations of growth regulators is very important for *in vitro* regeneration of niger

- Callus induction from hypocotyl segments of niger is best on MS medium supplemented with 0.5 mg/l NAA in combination with 1 mg/l BAP although shooting was found best using cotyledon explants for all genotypes.

- IAA at a concentration of 2 mg/l in combination with 1 mg/l BAP also favours callus induction from both cotyledons and hypocotyl segments.

- Callus induction, shoot formation and rooting are influenced by the concentrations and combinations of growth regulators, genotypes and type of explants.

- Green callus regenerates better than brown callus.

- Rooting of niger shoots is best in medium supplemented with 0.5 mg/l IBA.

7.2. Recommendations

There are various activities that should be performed in the future. Therefore, future work should include:

- The improvement of survival rate of plantlets in the field condition. Further investigations are required in order to enhance the efficiency of acclimatization.

- Molecular genetics study of the regenerated shoots to assess genetic stability and screening at green house condition.

- Genetic transformation studies using the developed protocol.

- Study the karyotype and polyploidy level of regenerated plantlets samples of niger.

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Declaration

I, the undersigned, declare that this Thesis is my original work and has not been presented for a degree in any other University. All sources of materials used for the Thesis have been duly acknowledged.

Name: Tesfaye Disasa

Signature: _____

This Thesis has been submitted for examination with my approval as a University advisor.

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Tileye Feyissa (PhD)