International Journal of Biosciences (IJB)
ISSN: 2220-6655 (Print) 2222-5234 (Online)
Vol. 1, No. 6, p. 110-118, 2011
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

In vitro regeneration of niger (*Guizotia abyssinica* L.F.) Cass.)

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Received: 17 November 2011
Revised: 25 November 2011
Accepted: 28 November 2011

Key words: *In vitro* regeneration, *Guizotia abyssinica*, rooting, acclimatization.

Abstract

Callus induction and shoot formation from hypocotyls and cotyledons of *G. abyssinica* has been achieved in this experiment. 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. The frequency of callus induction was influenced by the concentrations and types of growth regulators. Highest percentage of shoot formation 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.

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Introduction

Guizotia abyssinica is stout, erect, moderately branched annual herb with attractive yellow flowers and small black glossy seeds. It 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 (Baagøe, 1974). All of them are native to tropical Africa, and five are found in Ethiopia. *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 (Dagne, 1994).

It is indigenous to Ethiopia and one of the most important oil crops of the country. The seed contains edible oil which is important for human consumption. The oil has fatty acid composition typical for seed oils of the compositeae. About two third of the total edible oil in Ethiopia is obtained from niger seed (Rilay and Belayneh, 1989). The oil is used to prepare various types of foods, paints and soaps, and as an illuminant. In Europe, the seed is used in the preparation of animal feed and is especially known in the feed cage birds. The oil of the seeds is also used in rheumatism (Duke, 1983). Traditionally, niger sprouts mixed with garlic is used to cure cough. Its oil is also used in birth control and, cooked with spices in the treatment of syphilis (Belayneh, 1991). Plants are also used as a bee plant due to its attractive yellow flower.

Ethiopia is one of the major niger producing countries in the world. However, the yield of niger is considerably low (Belayneh, 1986) mainly due to its cultivation on very shallow soil of poor fertility, lack of suitable package practices, 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 maturity. These problems necessitated the application of biotechnological tools in addition to conventional plant breeding in order to improve the yield and the nutritional or oil content of the crop.

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 cell must be accessible to gene transfer by whatever technique is chosen (gene transfer methods).

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). 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). However, *In vitro* regeneration of niger has not been reported with Ethiopian genotypes. Therefore, this work was the first attempt to develop plant regeneration protocols for three Ethiopian genotypes of niger.

Materials and methods

Establishment of aseptic seedlings, culture media and culture conditions

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. The culture medium consisted of MS salts and vitamins containing 3 % (w/v) sucrose, and various concentrations of Indol-3-Acetic acid (IAA), α-Naphthalene Acetic Acid (NAA) and 6-Benzylaminopurine (BAP) were prepared. The pH of the solution was adjusted to 5.7. Then, 0.7% (w/v) agar was added into the solution and the media were autoclaved at 121^{o}C for 15 minutes.

Callus induction and proliferation

In this experiment, the effects of twelve combinations of IAA, NAA and BAP concentrations 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 seedling three to four hypocotyl segments and the two cotyledons were used. Growth regulators free MS basal medium was used as control.The cultures were kept in the dark at a temperature of 21$\pm$2^{o}C.

Shoot regeneration and multiplication

After two subcultures carried out every three weeks on the original callus induction medium, well developed and non-friable calli were isolated and transferred onto MS media containing various concentrations of growth regulators in order to examine the regeneration potential of each genotype. Furthermore, growth regulator free MS basal medium was used as a control. The cultures were transferred to continuous cool white fluorescent light (40µmolm^{-2}s^{-1}) at a temperature of 25$\pm$ 2^{o}C. The regenerated shoots were separated and cultured onto media containing 0.5 mg/l BAP for further multiplication of shoots. Multiple shoots were transferred onto growth regulators free MS basal medium for elongation.

Rooting and acclimatization

The isolated shoots (3-8 cm long) were collected and transferred to MS medium supplemented with Indol-3-Butyric acid (IBA) at different concentrations (0.5, 1, and 2 mg/l). Similarly, 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 and polythene plastic were gradually removed after one week and completely removed after two weeks in order to acclimatize plants.

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.

Results and discussion

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. 1B). Different concentrations of growth regulators showed different callus induction frequency regardless of explant type and genotype (Table 1). 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. Callus induction frequency decreases with

further increasing the concentration of NAA and BAP (Table.1). Besides, 2 mg/l IAA in combination with 1 mg/l BAP was found to be the next effective combination in callus induction. However, there was a decrease in the percentage of callus responding with increase in the concentration of IAA. 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 as well as between cotyledon and hypocotyl of a cultivar. However, the frequency of callus induction was not significantly different among the cultivars.

Table 1. 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	0.0	0.1	1.0	91.67^{ab}	63.33^{bcd}	80.00^{bc}	56.67^{cd}	80.00^{ab}	86.67^{a}
2	0.0	0.5	1.0	98.33^{a}	83.33^{ab}	98.33^{a}	76.67^{ab}	95.00^{a}	80.00^{ab}
3	0.0	1.0	1.0	86.67^{ab}	73.33^{abc}	86.67^{ab}	70.00^{abc}	81.67^{ab}	58.33^{bcd}
4	0.0	0.1	5.0	75.00^{abc}	43.33^{d}	76.67^{bc}	66.67^{abc}	81.67^{ab}	66.67^{abc}
5	0.0	0.5	5.0	75.00^{abc}	56.67^{cd}	80.00^{bc}	68.33^{abc}	80.00^{ab}	51.67^{cd}
6	0.0	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	0.0	1.0	95.00^{a}	91.67^{a}	88.33^{ab}	83.33^{a}	81.67^{ab}	88.33^{a}
8	2.0	0.0	2.0	66.67^{bcd}	75.00^{abc}	66.67^{c}	66.67^{abc}	66.67^{b}	70.00^{abc}
9	3.0	0.0	1.0	53.33^{cde}	66.67^{bcd}	43.33^{d}	73.33^{abc}	43.33^{c}	50.00^{cd}
10	5.0	0.0	1.0	53.33^{cde}	61.67^{bcd}	33.33^{d}	80.00^{a}	38.33^{c}	55.00^{cd}
11	5.0	0.0	2.0	46.67^{de}	53.33^{cd}	40.00^{d}	56.67^{cd}	43.33^{c}	66.67^{abc}
12	10.0	0.0	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 2. Mean number of shoots per explant produced from cotyledons and hypocotyls of *G. abyssinica* (Varieties: Fogera, Shambu and Esete)

PGR 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 different letters in the same column are significantly different at 5 % level of probability.

Table 3. Effect of explant source on 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 between hypocotyls whereas capital letters indicate between cotyledons of the three varieties.

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 98.3 % 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 (Table 1). 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.

Table 4. 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
Control	10.00^{d}	1.67±0.33^{b}	10.00^{c}	0.83±0.31^{c}	6.67^{d}	1.33±0.42^{c}
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^{c}	1.83±0.31^{b}	16.67^{bc}	1.17±0.48^{c}	13.33^{c}	1.67±0.33^{c}

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

Shoot regeneration and multiplication genotype effect

The effect of genotypes also plays an important role on response of oil crop to various plant tissue culture techniques (Tang *et al.*, 2003). Every species require studies to find the right ratio of growth regulators for optimum physiological development *in vitro* (Taji and Williams, 1996). Herbaceous species require different media and culture conditions than woody species (McCown, 1986). In this study, the effect of genotype was statistically significant and the maximum mean number of shoots per explant (20.3) was significantly higher in Fogera variety (Table 2). 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.

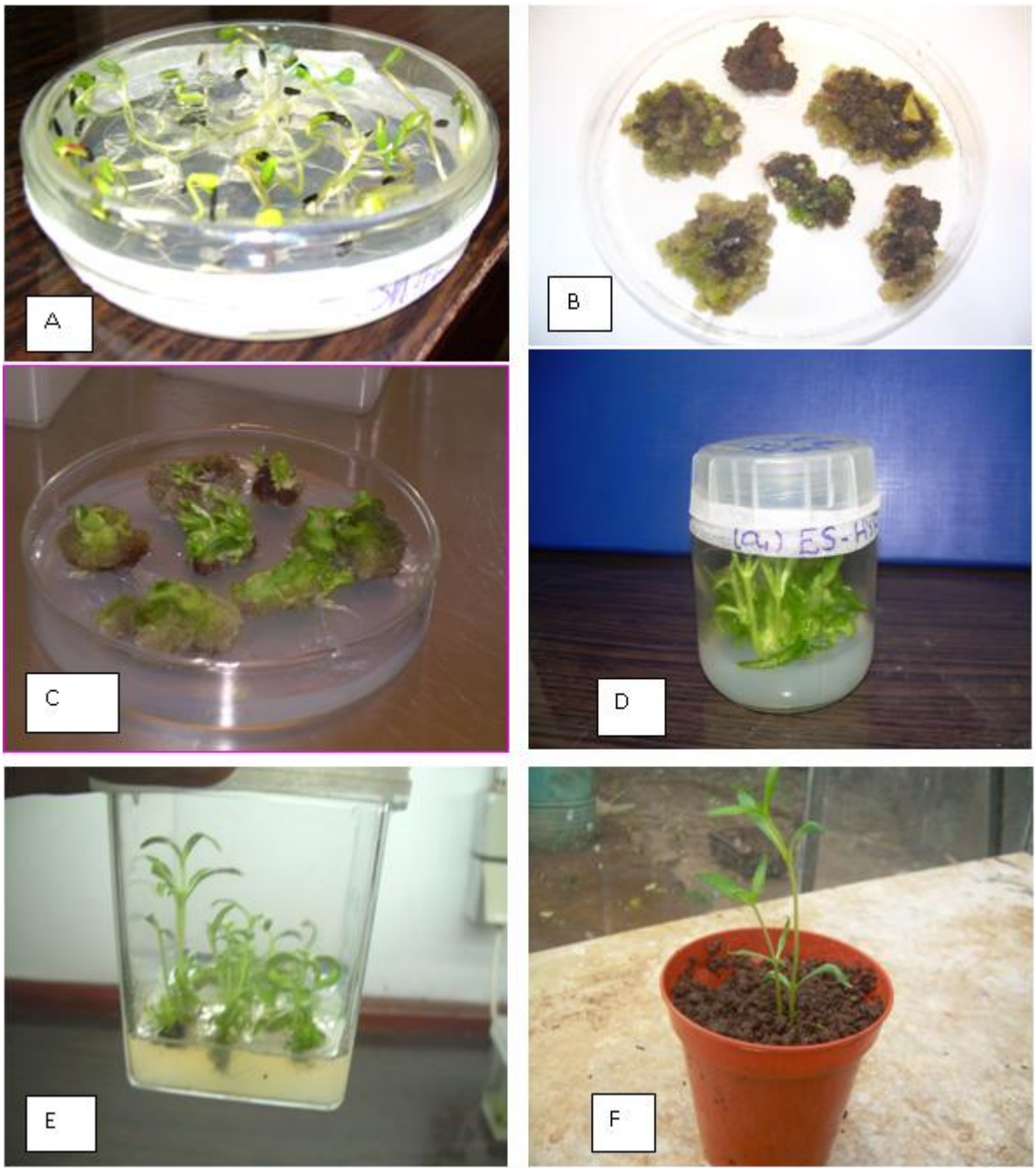

Fig. 1. *In vitro* regeneration of *G. abyssinica*. A. Aseptically germinated seed. B. Induced calli after three weeks of culturing, *C.* Shoot formation, D. Shoot proliferation, E. Shoot elongation and rooting, F. Acclimatized plantlets.

The effect of growth regulators

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 percentage of shoot formation. 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 (Table 2). Similarly, the maximum number of shoot per explant from media supplemented with 0.1 mg/l NAA alone was reported to be 28.2 (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. 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.

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 (Table 3). 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. 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.

Rooting and acclimatization

Elongated and well developed shoots transferred onto rooting medium showed variation in rooting potential with different concentration of IBA (Table 4). Among the different concentrations of IBA tested, the highest rooting (96.7%) percent and maximum 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). 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 and stronger than the other treatments. 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). The result of this experiment showed consistency in all genotypes and there was no significant difference among them. But, the frequency of root formation was significantly different among the treatments. When the concentration of IBA exceeds 0.5 mg/l, root forming frequency was significantly reduced (Table 4). 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 4).

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. The percentage of survival rate of shoots after three weeks of transfer to soil was 65% which was better than that of the result (61.36%) achieved from the previous study on niger (Sujatha, 1997). All plants had normal leaf development and did not show any detectable differences in morphology with the donor plant.

Acknowledgement

The Authors are grateful to Holetta Agricultural Research Center for providing seed materials. The authors also wish to thank Prof. Legesse Negash for

allowing his tissue culture laboratory with all the necessary facilities.

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