Original Article

In vitro Regeneration of *Taverniera Abyssinica* A. Rich: A Threatened Medicinal Plant

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Abstract

In vitro germination, callus induction and plant regeneration has been established for dingetegna, Taverniera abyssinica. The best in vitro germination of seeds and vigorous seedlings growth as a prerequisite for the development of tissue culture methods was obtained on Murashige and Skoog medium supplemented with 12 g 1^{-1} phytoagar without sucrose. Light green compact calli from node, petiole and shoot meristem explants were efficiently induced on Gamborg medium containing 0.90 or 1.80 µM dichlorophenoxyacetic acid (2,4-D) combined with 2.22 µM 6-benzylaminopurine (BAP), and supplemented with 30 g 1^{-1} sucrose and 5 g 1^{-1} phytagel. Callus initiation from shoot meristems and nodes was faster and occurred with a higher frequency than callus initiation from petiole and leaf segments ($P<0.05$). A high frequency of shoot regeneration (100%) was obtained upon transfer of calli onto regeneration medium containing 8.88 µM BAP combined with 1.14 µM indoleacetic acid (IAA). Regenerated shoots were transferred to rooting medium, which turned out to be optimal when half strength B5 medium was supplemented with 9.84 µM indolebutyric acid (IBA). Upon transfer to glasshouse, 86% survived and grew vigorously. The development of in vitro regeneration protocol for T. abyssinica provides the possibility to preserve endangered germplasm from the increasingly devastating man-made environmental conditions. Moreover, the method established can be used for micropropagation and genetic improvement of this medicinally important species.

Abbreviations: BAP – 6-benzaylaminopurine; 2,4-D – dichlorophenoxyacetic acid; IAA – indole-3-acetic acid; IBA – indole-3-butyric acid; NAA - α-naphthalenacetic acid; TDZ – 1-phenyl-3-(1,2,3-thiadiazol-5-yl) ure.

Keywords: Callus induction, Dingetegna, germination, growth regulators, medicinal plant.

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INTRODUCTION

Taverniera abyssinica A. Rich., a medicinal plant species belongs to the Fabaceae family commonly known under the Amharic name as *dingetegna*, literally meaning, remedy against sudden illness. The species is known to occur in Northeast Africa and Southeast Asia. It 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 (Thulin, 1990). Due to over-exploitation, *T. abyssinica* is now found as a remnant of isolated and scattered populations in the East Shewa Zone and Tigray region of Ethiopia (Kelbessa et al., 1992; Kibebew and Addis, 1996; Addis, 2003).

According to ethno-botanical information, *T. abyssinica* has been traditionally used for the treatment of various diseases in Ethiopia. A small bundle of the roots are chewed and the juice swallowed for immediate relief of fever, discomfort and pain. The root extracts are used, locally, as antipyretic and analgesic (Dange et al., 1990). Nematicidal and antimicrobial properties have confirmed the rational basis behind the ethno-botanical use of the species (Stadler et al. 1994).

The medicinal importance of *T. abyssinica* has been recognized by the findings of different chemical compounds isolated from the rootstocks. These chemical compounds include four isoflavonoid derivatives and a new pterocarpan; 3, 4-dihydroxy-9-methoxy therocarpan (Naomesi et al., 1990). Consequently, pharmacological studies were conducted using rootstock extracts on rats made hypothermic with yeast injection (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).

T. abyssinica is one of the ten medicinal plants sold in all the 19 markets surveyed, including the capital city, Addis Ababa (Kloos et al., 1978). Although the traditional medicinal values (Kloos et al., 1978; Dange et al., 1990).), the chemical extracts, their effect (Naomesi et al., 1990; Stadler et al., 1994) and the pre-germination treatments of *T. abyssinica* have been reported, no *in vitro* regeneration or micropropagation technique has so far been developed for this threatened medicinal plant. Currently, rootstocks of wild *T. abyssinica* plants are widely harvested for satisfying local markets throughout Ethiopia. Consequently, extensive and uncontrolled exploitation of this species, combined with the decline in natural regeneration, has led to the drastic depletion of the wild species. Although *T. abyssinica* is a perennial valuable medicinal plant, yet no commercial production under field conditions has been established. Recently, the tissue culture technique *i.e.* micropropagation has expanded its scope and potential on commercial scale. Micropropagation is suitable for the rapid and large-scale clonal multiplication of elite germplasm. *In vitro* micropropagation techniques would constitute an approach for the systematic germplasm conservation and genetic improvement. The *in vitro* regeneration system developed in this research program will rapidly provide large numbers of uniform, pest and disease free plants for investigations of the efficacy of medicinal components in dingatenya and potential commercial production of standard, consistent, and safe preparations.

The main objectives of this study were to (1) establish efficient *in vitro* seed germination *protocol*; and (2) develop *in vitro* regeneration method using apical shoot meristems, nodes, petioles, and leaf explants.

MATERIALS AND METHODS

Plant material

Mature pods of *T. abyssinica* were: (1) obtained from Getachew Addis (Ethiopian Health and Nutrition Research Institute (EHNRI)], during the second week of August, 2005; and also collected from Melka Konture area, Kersana Malima district, East Shoa zone, Oromia (65 km from Addis Ababa to Butajira) at an altitude of about 2100 m.a.s.l. during March 2005. The pods were transported to Germany on 28 August 2005, and consequently the experiments were conducted at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany.

In vitro seed germination

After preliminary experimental trials, the effect of seed polymorphism was observed on germination, and the seeds were categorized into white-large and brown-small ones after removal of the husk using a scalpel. The seeds were soaked in hot water (70 ^{o}C) for 15, 20, 25, 30 or 35 minutes, surface sterilized with 70% ethanol for 3 minutes, and 10% sodium hypochlorite solution for 5 minutes and rinsed three times using sterile double distilled water in a laminar flow hood. The germination medium consisted of MS minerals and vitamins[10] supplemented with 6, 8, 10, 12, 14, 16 g l^{-1} phytoagar (Sigma, St Louis MO, USA) but without sucrose. The pH was adjusted to 5.75, medium was autoclaved at 121 ^{o}C for 20 minutes, and then cooled down in a water bath to about 50 ^{o}C. Approximately the 100 ml medium was then dispensed in 100 mm x 150 mm glass culture vessels. Twelve seeds per vessel were cultured with 10 replicates per treatment. All cultures were inoculated at 25 ^{o}C under cool fluorescent light (40 µmol m^{-2} s^{-1}, 16 h photoperiod). Seed germination indicated by at least radicle emergence was recorded at days 5, 10, 15, 20, 25 and 30.

Callus induction

Two-month-old *in vitro* germinated seedlings were used as sources of explants. Stem segments (0.5 cm) including a node, petiole segments (0.5 cm), leaf segments as well as shoot (apical and lateral) meristems were used for callus induction. The explants were inoculated in 9 cm Petri plates containing MS (Murashige and Skoog, 1962) or B5 (Gamborg and Witter, 1990) media supplemented with auxin (0.90 µM, 1.80 µM and 2, 4-D, 5.37 µM NAA) in combination with cytokinin (2.22 µM BAP), sucrose (30, 60 g l^{-1}), and phytagel (3, 5, 7 g l^{-1}) (Sigma, St Louis MO, USA). Fifteen explants per plate were cultivated with 7 replicates per treatment. The plates were incubated either in the dark or in the light (control) for 3 weeks. Then, all explants were transferred to fresh media and incubated for another 3 weeks under light conditions. Data on the percentage of callus-forming per explants as well as fresh and dry callus weight was scored after 6 weeks of culture.

Shoot regeneration

All calli were transferred to shoot induction media during 3rd week of 2 passages. B5 and K4N[11] media, supplemented with BAP (2.22, 4.44, 6.66, 8.88 µM) or TDZ (2.21, 4.42, 6.63, 8.84 µM) alone or in combination with IAA (1.14 µM). Light green, compact callus was selected and distributed onto ten different shoot regeneration media (Table 6). Three replicates comprising at least 20 calli

(about 0.5 cm in diameter) were cultured in each medium. After 6 weeks of culture on shoot regeneration media, the percentages of shoot forming calli and the number of shoots per callus were recorded**.**

Rooting

The regenerated shoots were cultured on half-strength B5 media supplemented with IBA (2.46, 4.92, 7.38, 9.84 µM) for root induction. At least three replicates with 20 shoots each were conducted for each of these media. The percentage of root-forming shoots, and the number and length of roots per shoot were scored after one month of culture on rooting medium.

Acclimatization

Twenty five plantlets were removed from the medium. The roots were carefully washed with running tap water to remove the phytagel. The plantlets were then planted into pots (4.5 cm x 3.5 cm) filled with a mixture of loam and sandy soil in equal proportions and acclimatized for 3 months under natural diffuse sunlight and 70% humidity in a glasshouse.

Statistical analysis

In all experiments, each treatment consisted of three replicate culture plates randomly assigned throughout the growth chamber and each experiment was repeated at least twice. Frequency of shoot regeneration was observed after 10, 14, and 20 days of culture. Data were analyzed using SPSS.ANOVA (version 13) followed by Tukey Honest Significant Difference Test; was run for detecting significant differences among means. Test for ANOVA assumptions (i.e., homogeneity of variance was run using Tukeys' homogeneity test).

RESULTS

Seed germination

The germination of *T. abyssinica* seeds was influenced by the availability of water as was adjusted by the concentration of phytogar (Table 1). Concentrations of 6 and 16 g 1^{-1} resulted in poor germination. The best germination (96 $\pm$ 0.6%) was obtained at a concentration of 12 g 1^{-1} after 30 days of seed incubation. The germination of *T. abyssinica* seeds was also influenced by seed polymorphism as was adjusted by hot water (70 ^{o}C) treatments for different times (Table 2). No seed viability was obtained with large-white or small-brown seeds under control treatment. Scarification of brown-small seeds for 15 to 35 minutes and large-white seeds for <20 or > 40 minutes resulted in poor germination. The best germination (95.3 ± 0.34%) was obtained with large–white seeds treated with hot water (70 ^{o}C) for 25 minutes 28 days after seed incubation (Table 2). All subsequent experiments were performed using this treatment for seed germination as a source of explants.

Table 1. Effect of phytogar concentration and seed polymorphism on seed germination of *T. abyssinica*

Phytoagar concentration ($g1^{-1}$)	Germination %				
Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
16 ± 0.6^{e}	21 ± 0.6^{e}	28 ± 0.6^{d}	31 ± 0.6^{f}	35 ± 0.7^{e}	41 ± 0.3^{f}
27 ± 0.6^{d}	33 ± 0.9^{c}	44 ± 0.6^{c}	57 ± 0.6^{d}	65 ± 0.9^{c}	68 ± 0.6^{d}
38 ± 0.6^{b}	56 ± 0.6^{a}	64 ± 1.8^{a}	74 ± 0.6^{b}	78 ± 0.6^{b}	85 ± 0.5^{b}
43 ± 0.6^{a}	57 ± 0.6^{a}	66 ± 0.6^{a}	79 ± 0.6^{a}	85 ± 0.6^{a}	96 ± 0.6^{a}
33 ± 0.6^{c}	43 ± 0.6^{b}	57 ± 0.6^{b}	61 ± 0.7^{c}	68 ± 0.6^{c}	72 ± 0.5^{c}
13 ± 0.6^{f}	23 ± 0.6^{d}	30 ± 0.6^{d}	38 ± 0.6^{e}	43 ± 0.6^{d}	47 ± 0.6^{e}

Means with standard deviations within the same column followed by different letters (a-f) are significantly different ($p<0.05$).

Hot water(70^{o}C) /minute	Germination %					
	Day 7		Day 14		Day 21	
	White-large seeds	Small-brown seeds	White-large seeds	Small-brown seeds	White- large seeds	Small-brown seeds
Control	-	-	-	-	-	-
15	11.4 ± 0.31^{b}	-	23.5 ± 0.03^{a}	15.3 ± 0.54^{a*}	31.2 ± 0.78^{a}	18.5 ±2.51^{a}
20	20.8 ± 0.43^{c}	17.4 ± 0.87^{a*}	30.7 ± 0.04^{b}	15.6 ± 0.65^{a}	40.3 ± 0.34^{b}	25.6 ± 1.34^{b}
25	63.4 ±1.32^{e}	20.6 ± 0.65^{a}	75.8 ± 0.67^{d}	25.4 ± 0.23^{b}	83.5 ± 0.62^{d}	32.9 ± 1.34^{cd}
30	42.6 ±1.44^{d}	23.4 ± 0.76^{a}	55.4 ±1.04^{c}	30.2 ±1.12^{bc}	65.6 ± 0.32^{c}	35.6 ± 0.53^{d}
35	12.9 ± 1.54^{b}	30.7 ± 0.09^{b}	20.9 ±1.23^{a}	35.8 ± 1.34^{c}	34.4 ± 0.65^{a}	30.5 ± 0.35^{c}

Means with standard deviations within the same column followed by different letters (a-e) are significantly different ($p<0.05$).

Callus induction

All explants responded well except from leaf segments. Light green compact callus was obtained upon culture in the dark on B5-based medium supplemented with 2, 4-D and BAP. The highest frequency (94.4 ± 0.8%) of callus induction was obtained from shoot meristem explants followed by petiole (90.6 ± 0.6%) cultured on B5 medium supplemented with 1.80 µM 2, 4-D, 2.22 µM BAP, 30 g 1^{-1} sucrose and 5 g 1^{-1} phytagel. The data on callus formation from node, petiole and shoot meristem explants are presented in Tables 3, 4 and 5, respectively. Culture on MS media resulted only in the formation of dark brown calli and the accumulation of polyphenols in the medium. Explants exposed to light during culture did not form callus at all (data not shown) while culture on medium supplemented with 1.80 µM NAA generally resulted in light brown callus not capable of forming shoot buds (Table 3, 4, 5). Media containing 1.80 µM 2, 4-D appeared to be superior in producing light green callus formation compared with that containing 0.90 µM. However, 30 g 1^{-1} sucrose and 5 g 1^{-1} Phytagel turned out to be best suited for regenerable callus formation than the other concentrations of these compounds tested (Table 3, 4, 5). Under these conditions, also shoot bud formation was most abundant. The highest callus fresh weight (554.3 ± 4.2 mg) resulted from petiole segments (Table 3, 4, 5).

Table 3. Effects of medium modifications on callus formation from node explants of *T. abyssinica* recorded after 6 weeks of culture

Particularities media	Callus formation (%)	Callus colour	Callus fresh weight (mg)	Callus dry weight (mg)
Standard *	73.4 ±2.60^{b}	Light green	400.2 ± 3.30^{b}	59.2 ± 4.21^{b}
0.90 µM 2,4-D	83.3 ± 0.82^{a}	Light green	484.4 ± 5.81^{a}	69.6 ± 3.90^{a}
1.80 µM NAA	63.5 ±1.23^{c}	Light brown	252.7 ± 4.52^{d}	57.3 ± 4.34^{b}
60 g1^{-1} sucrose	74.6 ± 0.91^{b}	light green	326.3 ± 3.92^{c}	59.4 ± 4.23^{b}
3 g1^{-1} phytagel	43.8 ± 1.12^{d}	light green	194.4 ± 2.03^{e}	57.7 ± 4.22^{b}
7 g1^{-1} phytagel	34.4 ± 0.63^{e}	light green	193.4 ± 1.74^{e}	58.3 ± 4.41^{b}

* Standard conditions: B5 minerals and vitamins, 0.90 µM 2, 4-D, 2.22 µM BAP, 30gl^{1} sucrose, 5g 1^{-1} phytagel Means with standard deviations within the same column followed by different letters (a-e) are significantly different ($p<0.05$).

Table 4. Effects of medium modifications on callus formation from petiole explants of *T. abyssinica* recorded after 6 weeks of culture

Particularities of media	Callus formation (%)	Callus colour	Callus fresh weight (mg)	Callus dry weight (mg)
Standard *	81.7 ± 0.6^{d}	Light green	450.1 ± 3.3^{c}	60.0 ± 4.32^{a}
0.90 µM 2,4-D	90.6 ± 0.6^{e}	Light green	554.3 ± 4.2^{d}	60.7 ± 4.93^{b}
1.80 µM NAA	46.4 ± 0.6^{b}	Light brown	435.5 ± 3.5^{c}	60.3 ± 4.94^{a}
(without 2,4-D) no BAP				
60 $g1^{-1}$ sucrose	74.3 ± 2.3^{c}	light green	370.4 ± 4.5^{b}	60.9 ± 4.95^{a}
3 $g1^{-1}$ phytagel	35.4 ± 1.6^{a}	light green	232.6 ± 2.5^{a}	59.1 ± 4.22^{a}
7 $g1^{-1}$ phytagel	43.5 ± 1.1^{b}	light green	251.7 ± 2.7^{a}	59.4 ± 4.33^{a}

*) Standard conditions: B5 minerals and vitamins, 0.90 µM 2, 4-D, 2.22 µM BAP, 30 gl^{1} sucrose, 5 $g1^{-1}$ phytagel Means with standard deviations within the same column followed by different letters (a-e) are significantly different ($p < 0.05$).

Shoot regeneration

Shoot buds were formed from the light green callus after 3 to 4 weeks of culture. At the end of the callus induction period, the first leaf primordial appeared. The K4N-based media turned out not to be suitable for shoot formation (data not shown). While both shoot formation frequency and shoot number per callus increased with increasing concentration of BAP, no shoots were formed on the media supplemented with TDZ (Table 6). On B5 medium (Gamborg and Witter, 1990) supplemented with 1.14 µM IAA and 8.88 µM BAP, shoots were formed from the calli derived from both node and shoot meristem explants. The maximum number of shoots (5.6 ± 0.4%) were regenerated from callus cultured on B5 medium supplemented with 8.88 µM BAP and 1.14 µM IAA.

Table 5. Effects of medium modifications on callus formation from shoot meristem explants of *T. abyssinica* recorded after 6 weeks of culture

Particularities of media	Callus formation (%)	Callus color	Callus fresh weight (mg)	Callus dry weight (mg)
Standard *	92.3 ± 1.2^{a}	Light green	372.2 ± 3^{b}	60.3 ± 3.0^{a}
0.90 µM 2,4-D	$94 .4 \pm 0.8^{a}$	Light green	454.3 ± 3^{a}	63.7 ± 3.0^{a}
1.80 µM NAA	54.5 ± 1.5^{c}	Light brown	342.6 ± 4^{d}	62.7 ± 2.0^{a}
(without 2,4-D) no BAP	-	-	-	-
60 $g1^{-1}$ sucrose	76.6 ± 2.3^{b}	light green	357.5 ± 3^{c}	62.7 ± 3.0^{a}
3 $g1^{-1}$ phytagel	55.6 ± 2.1^{c}	light green	227.4 ± 4^{f}	61.9 ± 3.0^{a}
7 $g1^{-1}$ phytagel	48.4 ± 1.4^{d}	light green	238.3 ± 4^{e}	60.8 ± 2.8^{a}

*) Standard conditions: B5 minerals and vitamins, 0.90 µM 2, 4-D, 2.22 µM BAP, 30 g l^{1} sucrose, 5 g 1^{-1} phytagel

Means with standard deviations within the same column followed by different letters (a-f) are significantly different ($p<0.05$).

Rooting

The percentage of root-forming shoots and the number of roots per shoot increased with the concentration of IBA within a range of 2.46 to 9.84 µM. Of these, 9.84 µM produced the highest percentage (98%) of roots and root number (4.0 ± 0.44), but the longest root (3.0 ± 0.3 cm) was obtained at 7.38 µM. Shoots grown using higher IBA concentrations seemed to form shorter roots, yet this effect was not statistically significant (Table 7).

Acclimatization

Approximately 25 plantlets were harvested and transferred to greenhouse; 86% plants survived with vigorous growth within 5 months after planting while the rest (14%) showed aberrant phenotypes and stunted growth (Figure1).

Table 6. Effect of growth regulators in B5 media on shoot formation from calli of *T.* Abyssinica

Growth regulator(s) (µM)	Node		Petiole		Shoot meristem	
	Shoot-forming callus (%)	Shoot number/ callus	Shoot-forming callus (%)	Shoot number/ callus	Shoot-forming (%)	Shoot number/ callus
2.22 BAP	51	2.4 ± 0.3^{d}	43	3.9 ± 0.3d	39	4.0 ± 0.4^{c}
4,44 BAP	63	3.0 ± 0.4^{c}	48	4.7 ± 0.3^{c}	45	4.1 ± 0.3^{c}
6.66 BAP	64	4.0 ± 0.9^{b}	55	5.3 ± 0.4^{a}	65	5.3 ± 0.3^{b}
8.88 BAP	73	4.0 ± 0.5^{b}	71	4.8 ± 0.4^{b}	85	5.1 ± 0.5^{b}
2.21 TDZ	-	-	-	-	-	-
4.42 TDZ	-	-	-	-	-	-
6.63 TDZ	-	-	-	-	-	-
8.84 TDZ	-	-		-	-	-
8.88 BAP + 1.14 IAA	100	5.6 ± 0.4^{a}	76	4.6 ± 0.3^{b}	100	6.8 ± 0.5^{a}
8.84 TDZ + 1.14 IAA	-	-	-	-	-	-

Values within the same column followed by different letters (a-d) are significantly different ($p<0.05$).

Table 7. Effect of IBA concentration in half-strength B5 media on rooting of shoots derived from *T .abyssinica* calli

IBA concentrations (µM)	Node			Petiole			Shoot meristem		
	Root-forming shoots (%)	Root number	Root length (cm)	Root-forming shoots (%)	Root number	Root length	Root-forming shoots (%)	Root number	Root length
2.46	24^{a}	$3.3 \pm 0.39^{a*}$	3.2 ± 0.9^{a}	33^{d}	$3.0 \pm 0.38^{a*}$	2.7 ± 0.3^{a}	50^{d}	3.2 ± 0.27^{b}	$2.9 \pm 0.3^{a*}$
4.92	36^{b}	3.4 ± 0.37^{a}	2.5 ± 1.0^{a}	56^{c}	3.1 ± 0.38^{a}	2.5 ± 1.2^{a}	75^{c}	4.0 ± 0.36^{a}	2.6 ± 0.3^{a}
7.38	47^{c}	3.5 ± 0.33^{a}	2.5 ± 0.9^{a}	68^{b}	3.3 ± 0.30^{a}	3.0 ± 1.2^{a}	88^{b}	4.2 ± 0.35^{a}	3.1 ± 0.3^{a}
9.84	98^{d}	3.6 ± 0.41^{a}	2.2 ± 1.1^{a}	77^{a}	4.0 ± 0.44^{a}	2.3 ± 0.9^{a}	100^{a}	4.3 ± 0.25^{a}	2.6 ± 0.3^{a}

*) Values within the same column n followed by different letters (a-d) significantly different ($p<0.05$).

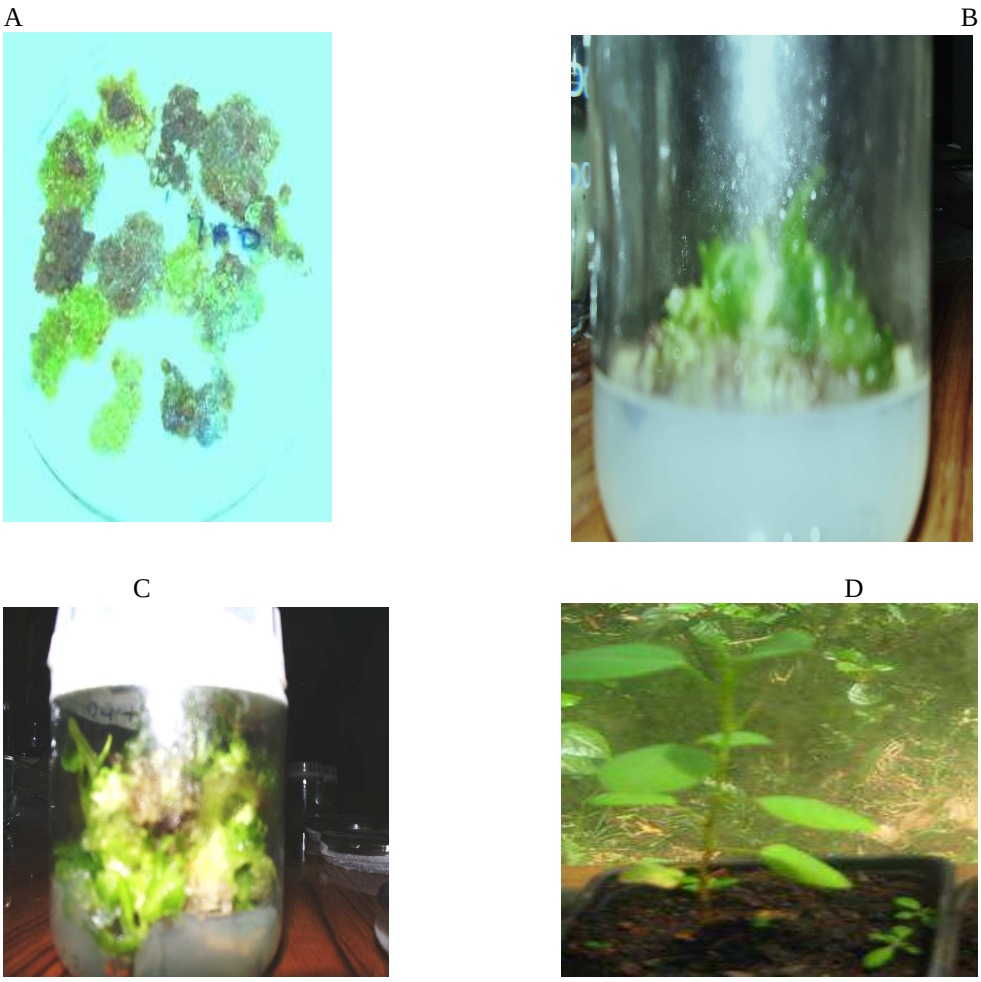

Figure 1. *In vitro* regeneration of *T. abyssinica*

A. callus induction after 6 weeks on B5 medium supplemented with 1.80 µM 2, 4-D, 2.22 µM BAP, 30 g l^{-1} sucrose and 5 g l^{-1} phytagel; B. shoot regeneration after 9 weeks on B5 medium supplemented with 1.14 µM IAA and 8.88 µM BAP, formed from all calli derived from node and shoot meristem explants; C**.** root formation on B5`medium supplemented with 9.84 µM IBA; and D. seedlings acclimatization under greenhouse condition. *Bars*; a) 3.7 mm, b) 1 cm, c) 3.5 cm, d) 4.5 cm

Legends to Figures

Figure 1. Three month-old *T. abyssinica* that was regenerated from callus, rooted and planted in greenhouse.

DISCUSSION

Optimal water availability provided by appropriate concentration of gelling agent is one of the most important factors for *in vitro* germination of seeds either to obtain sterile seedlings for tissue culture or to

produce plantlets for its conservation (Anjali et al., 2000). This study indicates that MS minerals supplemented with 12 g l^{-1} phytoagar showed the highest percentage (96 ± 0.6%) seed germination of *T. abyssinica* after 30 days of inoculation. Similarly, numerous published studies have provided evidence that *in vitro* germination of *P. Africana* seeds can strongly depend on the concentrations of the gelling agent (Negash, 1992) which influences not only the water status but also mineral and carbohydrate availability (Erica, 2000).

Jurado et al. (2001) have reported that the characteristics of *Tama Lipan thorns cub* seed polymorphism including seed size, shape and colour play a major role in percentage and rate of germination. Similarly, significant difference resulted in percentage and rate of germination between white-large and brown-small seeds. The highest percentage germination (95.4%) was obtained with white-large compared to small-brown, which germinated poorly even with increased scarification time.

In this study, the maximum amount of light green compact callus was obtained from shoot meristem and petiole explants cultured on B5 media containing different concentrations of 2, 4-D in combination with BAP. According to previous reports, 2, 4-D in combination with BAP proved to be effective plant growth regulators for callus induction fro many medicinal plant species (Aniali et al., 2000; Chen et al., 2000; Manickam et al., 2000; Azad et al., 2004; Vinod et al., 2004). Successful callus induction resulted from the combined interaction of plant genotype, plant physiological condition, explant source as well as the nutritional and regulatory conditions provided by the specific medium (Vinod et al., 2004). The poor induction of callus in contrast to other explants leaf responded least in this experiment. This may be at least in part due a relatively highly advanced cell and tissue differentiation status in the leaves. In *Spurious virginicus,* young leaf tissue as well as shoot meristem, node and petiole explants frequently produced callus, yet the type of callus varied and the leaves produced the least amount (Straub et al., 1992).

Browning of both explants and media was the major impediment when *T. abyssinica* explants were cultured in MS-based media containing the same supplements as of the B5 medium. This significant difference between B5 and MS was probably due to the relatively high concentration of ammonium ions and their high proportion among the total nitrogen provided by the MS medium which is not tolerated by quite a number of plant species explants (Murashige and Skoog, 1962; Ang and Chan, 2003).

The promoting effect of BAP on shoot initiation is in agreement with results on other medically important plant species such as in *Spilanthes acmella* (Ang and Chan, 2003; Ayan et al., 2004)), in *Hemidesmus indicus (*Siddique et al., 2003, in *Guizotia abyssinica (*Nikam and Shitole, 1993) as well as in *Anogeissus sericea* (Kaul et al., 1992). Plant regeneration in *T. abyssinica* appeared to occur via organogenesis rather than through somatic embryogenesis. This was supported by the observation that adventitious roots were only formed from well developed shoots after being placed on rooting medium.

Summary

I*n vitro* plant regeneration protocol was established and this allows production of multiple plantlets per single seed via callus induction. The value of such basic protocol for *T. abyssinica* is many-fold. Above all, it

can be used to preserve germplasm of this endangered species of high medicinal importance. Callus or suspension cultures may be used to identify and comprehensively analyze the function and significance of the bioactive metabolites as well as to produce biomass potentially employed for the extraction of the medically relevant substances. Furthermore, a rapid and efficient method of plant multiplication is of particular importance in perennial species such as *T. abyssinica*. Also, the method established will enable diverse approaches to improve genetic this species genetically.

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