

Micropropagation of Yeheb from Shoot Tip: An Endangered Multipurpose Shrub

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Abstract

The study focuses on *in vitro* propagation of an endangered species of *C. edulis*. *In vitro* propagation has advantages to address low seed viability and over exploitation problems. Thus, the objective of this study is to develop micropropagation method from shoot tip explant. To do so, seeds were de-coated and disinfected using 5% (v/v) Clorox and cultured on growth regulators-free solid MS medium. Shoots were initiated on MS medium containing BAP alone or in combination with IBA. Then, initiated shoots were multiplied on solid MS medium supplemented with various concentration and combinations of BAP and TDZ alone and in combination with IBA or NAA respectively. Shootlets were cultured on full, half and one-third strength MS medium containing 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l IBA and NAA for root induction. As a preliminary test for rooting, B₅ and WPM media, both supplemented with 0.5, 1.0, 2.0 mg/l IBA, were used. Shootlets were cultured on full strength MS medium containing higher concentration of 3.0 and 5.0 mg/l IBA and 1.0 g/l activated charcoal for four days and then transferred into growth regulators-free MS medium for a month for root induction as preliminary test. The highest percentage of shoot induction (70.8%) was obtained on medium containing 0.5 mg/l BAP in combination with 0.01 mg/l IBA. The highest number of shoots (4.2 ± 0.85) produced per explant was achieved on MS multiplication medium containing 2.0 mg/l BAP. None of the shoots induced roots in any of different MS salt strengths and other media. As conclusion, this is the first try of micropropagation and the existing result is valuable for cryopreservation of this endangered plant.

Keywords: *Cordeauxia edulis*; *in vitro*; micropropagation; plant growth regulators; shoot multiplication; shoot tip culture

Abbreviations: BAP = 6-benzyl Amino Purine; B₅ = Gamborge medium; IBA = Indole -3-Butyric Acid; MS = Murashige and Skoog; NAA = α -naphthalene Acetic Acid; PGRs = Plant Growth Regulators; SATG = Somali Agricultural Technical Group; SPSS = Statistical Package for Social Science; TDZ = Thidiazuron; WPM = Woody plant medium

1. Introduction

1.1. Introduce the Problem

Yeheb (*Cordeauxia edulis*, Hemsl.) is the only species (monotypic) in the genus *Cordeauxia* (International Board for Plant Genetic Resources, & Royal Botanic Gardens, Kew, 1984) and it is a

wild plant with multipurpose usage. Its bush is valuable as a building material since it is not attacked by termites and source of firewood for the native people. As the result, the resident people cut down the bushes to bring with them (Yusuf, 2010). *C. edulis* is an important dry season fodder for camels and goats. When the leaves of other neighboring plants fall, especially during the dry period, it remains evergreen. Hence, it serves as the source of feed for animals (Yusuf, Teklehaimanot, & Rayment, 2012). *C. edulis* is one of a hopeful species that has been endangered because of over use in times of crop scarcity in Eastern Ethiopia (Asfaw & Tadesse, 2001). This species included among minor crops that are in threat of economic loss and is mentioned as endangered in 1997 by IUCN (Holsinger, 2005).

Cordeauxia edulis produces seeds, but grows slowly especially in the seedling stage while it is establishing its huge root system. Up to 80% germination can be obtained (Yusuf, Teklehaimanot & Gurmu, 2013), but seed storage behavior is questionable. Viability is completely lost after four months of open storage at room temperature (Johansson, 2006). The seeds are also vulnerable to insect attack especially at higher temperature and should not be stored for extended period. To prevent seeds from being attacked by insects, freshly picked seeds are roasted or boiled to kill insects by the local people. In this form, the seeds may fetch a higher price on the market, but this practice makes obtaining viable seeds difficult (Johansson, 2006).

In general, the populations of *C. edulis* have declined due to deterioration of the vegetation caused by widespread human intervention, natural states of the plant including biological factors and climate changes that always accompanied the process of deforestation.

So far, there is no research accomplishments reported with regards to micropropagation of *C. edulis*. An *in situ* conservation, management and utilization, biochemical analysis as well as preliminary assessment of the phenology are among research efforts with regard to this species. Some of those results imply the need for further comprehensive protection of the natural stands and expansion of its cultivation. In *C. edulis*, *in vitro* culture allows plant propagation within a relatively short period of time, to obtain clones with known characteristics, and to genetically improve the species. However, *in vitro* establishment of woody plants in general and tropical trees in particular is often difficult. The most frequently encountered problems are culture contamination by endogenous bacteria and browning of explants (Machado *et al.*, 1991).

Therefore, it is essential to enhance the effort for *in vitro* propagation to prevent from extinction and develop profit for the local people in contribution to poverty reduction. Thus, the main objective of this study were to develop micropropagation method for this endangered species from shoot tip explants as alternative approach to address problems of seed viability and over exploitation. Different methods for germination, shoot initiation as well as shoot multiplication were examined.

1.2. Literature Review

Cordeauxia edulis has multiple stems, long root system that reaches the deep moisture and makes the bush stay green all year round and the taproot, which is up to three meters long, has small secondary rhizomes close to the surface (National Academy of Science [NAS], 1979). The lateral roots extend at 10 to 40 cm under the soil surface. There are many red glands on the underside of the leaves and stem. The leaves have an extremely thick cuticle and mesophyll consisting of palisade cells with lateral walls capable of folding in a concertina like way (Brink & Belay, 2006).

Hemsley in 1907 named the yeheb, a bushy Caesalpiniaceae from the Amherstieae tribe, after Captain Cordeaux, who first obtained botanical samples of it in the Ogaden area, Ethiopia, in regions near to Somalia in the ex-Italian territories. It is a bush well adapted to semi-arid regions. It is also exotic in Israel, Kenya, Sudan, Tanzania and Yemen (Ali, 1988). It blooms during the rainy season once or twice if the rainfall is abundant. The flowers are yellow and the plant starts to produce pods at an age of three to four years, which contains one to four seeds (NAS, 1979).

Cordeauxia edulis is found to grow at an altitude of 100-1000 meter on sands locally called 'haud' (Booth & Wickens, 1988). Its native area is characterized by red colored sandy soil which is extremely low in nitrogen (Drechsel & Zech, 1988). The area where it grows is one of the most important livestock producing areas in Somalia (Somali agricultural technical group [SATG], 2004). The fodder value of the leaves is comparable to other tropical tree legumes (Drechsel & Zech, 1988). Two forms of *C. edulis* are known; the smaller Suley from Northern Somalia and the taller and more common Moqley (Soderberg, 2010). Suley is pale green with large leaflets, stem thickness, and the pods contain several smaller seeds and have a bit higher protein and fat contents while Moqley is dark green and have small leaflets, stem thickness, and the pods contain one large seed and less protein and fat contents (Yusuf, 2010).

It is a famine food from the dry savannas of Somalia and Ethiopia and relied through recent warfare in the Ogaden desert district to the coverage that it became highly scarce. These are only a few of the potentially economic species awaiting investigation (Ayensu, 1983). Similarly, it has been a staple food of nomadic groups in Somalia and Ethiopia (Cannon, May & Jackson, 2009). It is grouped among potentially valuable crop plants and is the wild edible plant in Ethiopia (Lulekal, Asfaw, Kelbessa & Van-Damme, 2011). Somalis prefer it to staples such as corn and sorghum. It also has high demand by the urban people among other wild fruit plants. For example, a tin full of nuts is sold for 4000 Somali Shillings (i.e. 0.25 US dollar) in 2001 (Drechsel & Zech, 1988).

2. Material and Methods

2.1. The plant Materials and Explant Source

Matured seeds of *C. edulis* were obtained from Somali Region Pastoral and Agro Pastoralist Research Institute (SORPARI), 630 km South-east of Addis Ababa, which is found in Somali Regional State. The current investigations were carried out at Plant Tissue Culture Laboratory of the Institute of Biotechnology, Addis Ababa University, Ethiopia.

2.2. Surface Sterilization of Seeds

The seeds were washed repeatedly using running tap water and detergent (OMO) following soaking and de-coating of them. The seeds from which seedlings were obtained for *in vitro* culture sterilized by 70% ethanol for 1 min and rinsed over again with sterile distilled water followed by sterilization using 5% concentration of Clorox (NaOCl, 5.25% of available chlorine) and 2-3 drops of Tween-20 for 5 min. Finally, it was rinsed four times with sterile distilled water.

2.3. Evaluation of Germination Rate

After washing, 45 seeds out of 90 were soaked in water and seed coat was removed. The germination rate of the seeds was investigated in three ways using both coated and de-coated seeds. In the first experiment, the seeds were sown on wet filter papers on Petri dishes. In the second experiment, seeds were sterilized and cultured on growth regulators-free MS (Murashige & Skoog, 1962) medium. In the third treatment, the seeds were sown in pots containing soil and sand with 2:1 proportion in green house. In each experiment, 15 coated or de-coated seeds were used.

2.4. Shoot Initiation from Shoot Tip Explant

After two weeks of culture, the shoot tips were collected from *in vitro* germinated seedlings on MS medium and used for shoot initiation. Old leaves and root parts were removed and the shoot tips were trimmed to 1 cm length in aseptic condition and cultured immediately on MS medium containing BAP (0.5, 1.0 or 2.0 mg/l) alone or in combination with IBA (0.01, 0.01 or 0.1 mg/l). MS medium contained 3% sucrose. The pH of the medium was adjusted to 5.8 and solidified using 8 g/l agar. Growth regulators-free MS medium was used as a control.

The explants were cultured in Magenta GA-7 culture vessels each containing 40 ml solid MS medium. There were four explants per culture vessel with six replications. The culture vessels were then sealed with Parafilm, labeled and maintained in culture room under 16 h photoperiod at 25 ± 2 °C and light intensity of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$. Unless otherwise indicated, all the cultures were maintained under these conditions. The cultures were transferred into fresh media within two weeks by removing the black or brown accumulations at the bottom tip of the explants and maintained under dark conditions for three days to reduce the phenolic compounds from the explants till the secretion reduced. After three days, the cultures were maintained in the culture room under the conditions indicated above.

Contamination was checked within three days interval. Percentage of shoots initiated and died was calculated. All changes in growth were carefully observed and recorded.

2.5. Shoot Multiplication

After 35 days of development on the culture initiation medium, young and vigorous microshoots were cultured in Magenta GA₇ culture vessel containing 50 ml shoot multiplication full strength MS medium supplemented with different concentrations of BAP (0.5, 1.0, 1.5, 2.0 or 3.0 mg/l) or BAP (0.5, 1.0, 1.5, 2.0, 3.0 or 5.0 mg/l) in combination with IBA (0.01, 0.1, or 0.5 mg/l). The other composition was TDZ (0.5, 1.0 or 1.5 mg/l) or TDZ (0.5, 1.0, 1.5, 2.0, 3.0 or 5.0 mg/l) in combination with NAA (0.01, 0.1, 0.5 or 1.0 mg/l). The growth regulators-free MS medium was used as control.

For each treatment, a total of 30 explants were used. There were five explants per culture vessel with 6 replications. The culture vessels were properly sealed, labeled and randomly placed in culture room. Shoot number and length were recorded after 30 days.

2.6. Root Induction

The *in vitro* proliferated shootlets were used for root induction and cultured on full, half and one-third strength MS media supplemented with 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l NAA or IBA. Likewise, WPM and B₅ basal media containing 0.5, 1.0 and 2.0 mg/l IBA were used as a preliminary test. The root induction treatment was also tested on full strength MS medium supplemented with 3.0 and 5.0 mg/l IBA using 1.0 g/l activated charcoal for 4 days and then it was transferred into growth regulators-free full strength MS medium for a month as preliminary test. Growth regulators-free MS medium was used as control. The shoots were kept at darkness for 3 days and then transferred to culture room. The shoots were examined for root induction after a month.

2.7. Statistical Analysis

The rate of germination, the number and percentage of initiated shoots, effect of different concentrations of growth regulators on shoot number, shoot length and the rooting conditions from rooting treatments were recorded. The recorded data were subjected to one way analysis of variance (ANOVA) and the variables that showed significant difference were compared by least significance difference (LSD) at 5% probability level. SPSS version 20 statistical software at probability ($\alpha < 0.05$) were used to analyze the whole data.

3. Results

3.1. Seed Germination

When the seeds were transversally cut at the tip, germination was enhanced. The seeds sown at 45° orientation on MS medium had better germination percentage.

Maximum number (73.3%) of de-coated seeds germinated on MS basal medium in 6-7 days after seed sowing, but intact seeds didn't show any sign of germination. Seeds were sown in pots

containing soil and sand (2:1) in greenhouse, showed poor germination. In contrast, 53% of the de-coated seeds were germinated on filter paper within a week whereas none of the seeds with seed coat germinated (Table 1). The aerial parts of the seedlings showed stunted growth and the leaves were folded. The roots showed rapid growth and it was very long relative to its shoot system (figure 1).

Table 1. Germination percentage of *C. edulis* seeds on different germination media

Medium	Seeds	Germination time (day)	Germination (%)
Soil	Intact seeds	20-25	20
	De-coated seeds	-	0.0
Filter paper	Intact seeds	-	0.0
	De-coated seeds	8-10	53
MS medium	Intact seeds	-	0.0
	De-coated seeds	6-7	73.3

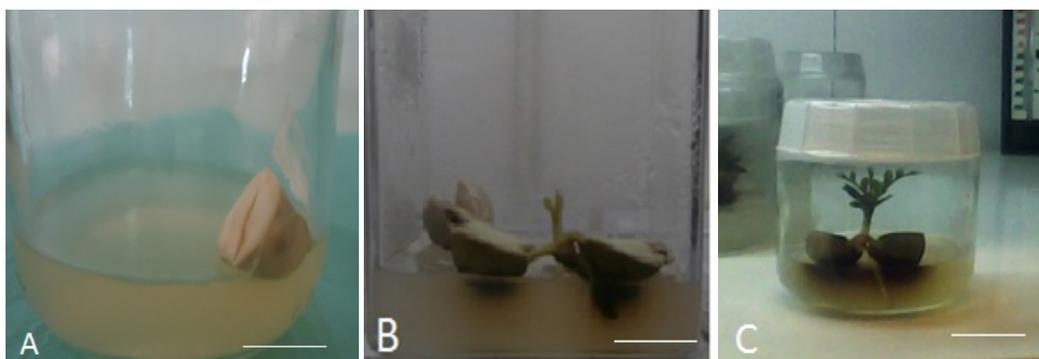


Figure 1. Germination and seedling appearance of *C. edulis* Seed culture on MS medium (A); New emerging seedling (B); Two-week-old seedling (C). Bars represent 2 cm

3.2. Shoot Initiation from Shoot Tip Explant

Shoot tips were started to show sign of new microshoots after 10 days on initiation medium. The growth of newly emerging shoots was clearly observed after 15 days. Shoots were differentiated into either single or multiple shoots.

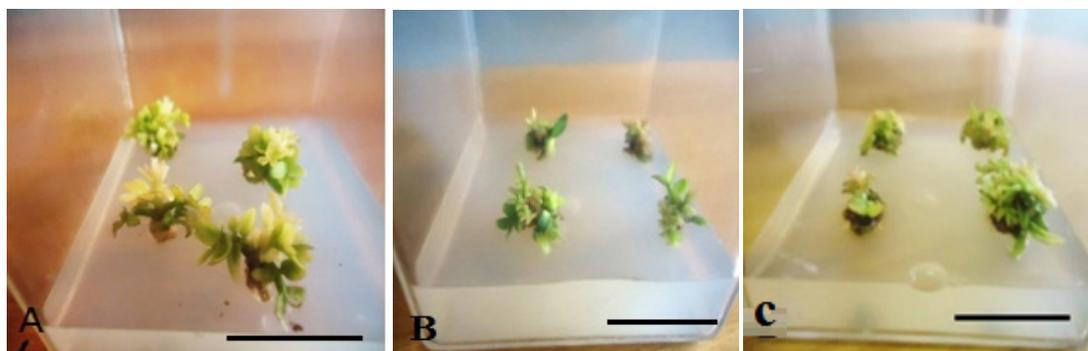


Figure 2. Shoot initiation from shoot tip explants of *C. edulis* on MS medium containing different concentrations of BAP in combination with IBA. 0.5 mg/l BAP and 0.01 mg/l IBA (A); 1.0 mg/l BAP and 0.01 mg/l IBA (B) and 2.0 mg/l BAP (D). Bars represent 2 cm

After 35 days of growth on initiation medium, 70.8% of explants were initiated on MS initiation medium containing 0.5 mg/l BAP in combination with 0.01 mg/l IBA. These shoots were greenish-brown in color, but some with whitish leaves and independently survived when sub-cultured, fast in growth and taller than others (figure 2A). The result revealed that 62.5% of the explants induced shoots in the initiation medium supplemented with 2.0 mg/l BAP. They produced relatively short, but higher number of shoots than others (figure 2B&C). Only 8.3% of shoots were initiated on growth regulators-free MS medium. Medium containing the rest of growth regulator concentrations resulted in low number of survived shoots with different features like callus formation at the bottom end of the explants, production of excessive phenolic compounds, folded and brittle leaves and very dwarfed shoots.

3.3. Shoot Multiplication

Table 2. Percentage of shoots producing multiple shoots, number and length of shoots per explants that were cultured on MS medium containing different concentrations of BAP, IBA, TDZ and NAA

PGR (mg/l)				Explants with multiple shoots (%)	Mean No. Shoots per explant	Mean length of Shoot (cm)
BAP	IBA	TDZ	NAA			
0.0	0.0	0.0	0.0	0.0	1.00 ± 0.0 ^j	0.82 ± 0.15 ^{eh}
0.5	0.0	0.0	0.0	86.7	2.960 ± 0.77 ^c	0.89 ± 0.24 ^{cef}
1.0	0.0	0.0	0.0	93.3	3.210 ± 0.68 ^b	0.89 ± 0.19 ^{ce}
1.5	0.0	0.0	0.0	96.7	3.00 ± 1.10 ^{bc}	0.80 ± 0.20 ^{fghi}
2.0	0.0	0.0	0.0	100	4.20 ± 0.85 ^a	0.86 ± 0.19 ^{eg}
3.0	0.0	0.0	0.0	60.0	2.12 ± 1.07 ^{dc}	0.85 ± 0.11 ^{eh}
0.5	0.01	0.0	0.0	96.7	3.93 ± 0.94 ^a	1.06 ± 0.21 ^a
1.0	0.01	0.0	0.0	70.0	2.24 ± 0.99 ^d	0.99 ± 0.19 ^{ab}
1.5	0.1	0.0	0.0	83.3	3.04 ± 0.89 ^{bc}	0.92 ± 0.21 ^{bc}
2.0	0.1	0.0	0.0	83.3	2.76 ± 1.15 ^c	0.96 ± 0.21 ^{bc}
3.0	0.5	0.0	0.0	86.7	3.00 ± 1.20 ^{bc}	0.79 ± 0.19 ^{ghi}
5.0	0.5	0.0	0.0	50.0	1.72 ± 0.68 ^{efg}	0.79 ± 0.17 ^{ghi}
5.0	1.0	0.0	0.0	30.0	1.46 ± 0.66 ^{fgh}	0.69 ± 0.15 ^{jl}
0.0	0.0	0.5	0.0	56.70	1.88 ± 0.72 ^{df}	0.65 ± 0.14 ^{kl}
0.0	0.0	1.0	0.0	40.0	1.63 ± 0.65 ^{fgh}	0.55 ± 0.14 ^m
0.0	0.0	1.5	0.0	0.0	1.00 ± 0.0 ^j	0.60 ± 0.08 ^{lm}
0.0	0.0	0.5	0.01	26.70	1.40 ± 0.50 ^{ghj}	0.71 ± 0.11 ^{ik}
0.0	0.0	1.0	0.01	23.30	1.38 ± 0.62 ^{ghj}	0.70 ± 0.14 ^{il}
0.0	0.0	1.5	0.1	36.70	1.52 ± 0.59 ^{fgh}	0.63 ± 0.12 ^{klm}
0.0	0.0	2.0	0.1	53.30	1.65 ± 0.62 ^{fg}	0.62 ± 0.15 ^{klm}
0.0	0.0	3.0	0.5	23.30	1.29 ± 0.46 ^{hj}	0.60 ± 0.13 ^m
0.0	0.0	5.0	0.5	10.0	1.17 ± 0.38 ^{hj}	0.75 ± 0.13 ^{hij}

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

The highest mean number of shoots per explant (4.20±0.85) was obtained on MS medium containing 2.0 mg/l BAP (figure 3A). Higher concentration of BAP (above 5.0 mg/l) in combination with IBA (above 0.5 mg/l IBA) produced lower number of shoots compared to

cultures with lower or moderate concentrations of these growth regulators. In most cases, number of shoots per explant was higher on medium containing only BAP than those containing combination of BAP and IBA. Explants cultured on higher concentration of TDZ (1.5 mg/l) had significantly lower mean number of shoots per explant than all other treatments (figure 3H). In general, TDZ alone or in combination with NAA at all used concentrations induced lower shoots. No multiplication of explants was observed on PGRs-free medium and only shoots with very slow or no growth was obtained (Table 2).

The highest mean shoot length (1.06 ± 0.21 cm) was obtained on MS medium containing 0.5 mg/l BAP in combination with 0.01 mg/l IBA while the lowest mean shoot length (0.55 ± 0.14 cm) was obtained on the medium containing 1.0 mg/l TDZ (figure 3B&I respectively). When the concentration of BAP alone increased to 3.0 mg/l, the shoots became dwarf, folded and bushy in appearance. The presence of IBA over BAP was better for shoot length than other media compositions. However, the higher concentration of BAP in combination with IBA (5.0 mg/l BAP with 1.0 mg/l IBA) reduced shoot length than other BAP and IBA combinations at lower concentrations. Almost all of the explants cultured on MS media supplemented with TDZ alone or in combination with NAA showed the lowest percentage of explants with multiple shoots (Table 2).

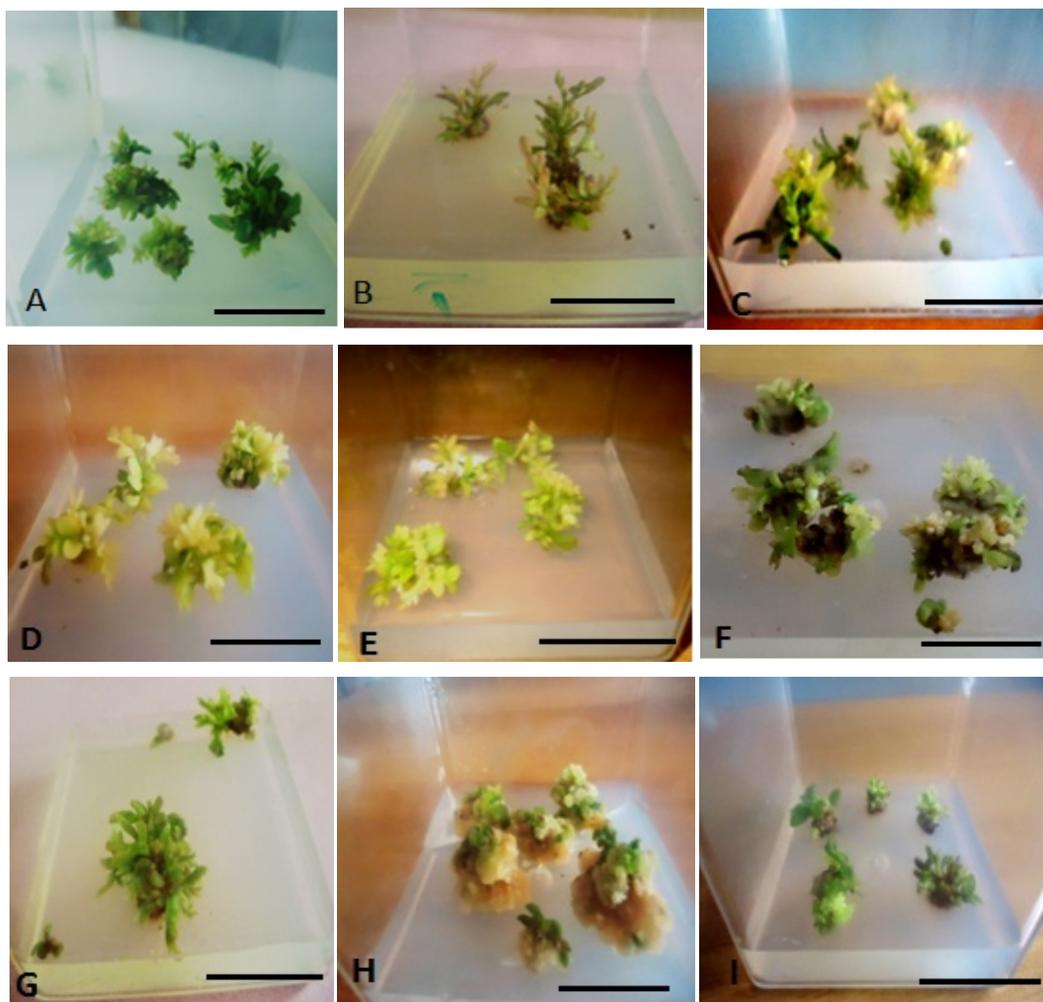


Figure 3. Multiple shoots of *C. edulis* on different multiplication media. 2.0 mg/l BAP (A, B, C); 3.0 mg/l and 0.01 mg/l IBA (D, E); 3.0 mg/l BAP (F, G); 1.5 mg/l TDZ (H); 5.0 mg/l BAP and 0.5 mg/l IBA (I). Bars represent 2 cm

3.4. Root Induction

None of the shootlets appeared to root on a given medium strength and with respective auxin concentrations. Relatively, shootlets cultured on MS rooting media containing NAA developed callus. As none of the shoots rooted, there was no any experiment conducted for acclimatization.

4. Discussion

4.1. Seed Germination

In MS medium, 73.3% of de-coated seeds were germinated. This may be due to narrow temperature variation in the culture room compared to green house. In contrast, coated seeds didn't germinate *in vitro*. This was suggested as hard seed coat may hamper water uptake, resulting in little or no germination in low moisture conditions. Similarly, successful germination (91±2.7%) of de-coated seeds of a tree plant, African cherry (*Prunus africana*) in the laboratory (Negash, 2004).

Intact seeds that were sown in pot containing soil and sand (2:1) in green house, showed poor germination (20%). However, up to 80% germination can be obtained from its habitat (Yusuf *et al.*, 2013).

Viable seeds germinate well and give rise to healthy seedlings in the presence of their own microflora including that of the soil (Bradbeer, 1994).

When seeds were soaked in water before sowing, enhanced its moisture content and arrived at highest germination. As Liew (2003) and Johansson (2006), the proportion of germination and viability of *C. edulis* seeds were highly dependent on seed moisture content. In plants, DNA injury was repaired in the embryo and restored early in imbibitions and can be vital for germination performance (Waterworth *et al.*, 2010).

4.2. Shoot Initiation from Shoot Tip Explant

For shoot initiation, MS medium containing lower concentration of BAP in combination with IBA (0.5 mg/l BAP in combination with 0.01 mg/l IBA) were initiated highly (70.8%) followed by 2.0 mg/l BAP (62.5%). The result thus conclusively showed that lower concentration of cytokinins (BAP) in combination with auxin (IBA) was essential for shoot initiation of shoot tip explants of *C. edulis*. Similarly, study on a deciduous tree, *Prunus amygdalus*, indicated that relatively low auxin and cytokinin levels were used during the initiation stage of shoot tip cultures (Rugini & Verma, 1983).

The initiated shoots on MS medium supplemented with 0.5 mg/l BAP and 0.01 mg/l IBA were significantly taller than those initiated on 2 mg/l BAP. The possible reason may due to the addition of auxin (IBA) into cytokinin (BAP) reduced the cytokinin effect which suppressed the apical dominance. According to Aasim, Khawar, Sancak and Ozcan (2009), addition of IBA to BAP gave significantly longer shoots, but lower in number as they were tested on micropropagation of a legume crop, fenugreek. But, results on *Amygdalus communis* (deciduous fruit tree) showed that when BAP and IBA concentrations increased, the length of the shoots decreased significantly (Gurel & Gulsen, 1998).

At the beginning of shoot initiation, the production of phenolic compounds from the explants was a problem for *in vitro* propagation of *C. edulis*. Release of phenolic compounds is common in tree plants (McCown, 2000). The problem was reduced by frequent transfer of shoots into new medium within 15 days through washing by sterile distilled water and preventing from direct florescence light 3-4 days starting from culture. Likewise, the result on a fruit tree, *apple*, revealed that dark treatment of initiated shoots for 4 days reduces the release of phenolic compounds from the explants. Moreover, transfer of the cultured shoots to new fresh medium is important to reduce the risk of death of explants (Demsachew, 2011).

4.3. Shoot Multiplication

In shoot multiplication investigation, BAP was found best for shoot multiplication from shoot tip culture of *C. edulis*. Other leguminous tree species where BAP induced shoot multiplication has been reported are, *Albizzia lebbeck* (Upadhyaya & Chandra, 1983) and *Prosopis laevegata* (Buendía-González, Orozco-Villafuerte, Cruz-Sosa, Chavez-Avila & Vernon-Carter, 2007).

All (100%) of the explants produced the maximum mean number of shoots (4.2 ± 0.85) on MS medium containing 2.0 mg/l BAP. This might be due to its concentration fit to stimulate the tissue to metabolize the endogenous hormones and induce the production of endogenous hormones for the induction of multiple shoots. The result on micropropagation of legume crop, *Cicer artium*, showed that 2.0 mg/l BAP was more effective for proliferation of shoots (6.0 ± 0.3) (Ugandhar, Venkateshwarlu, Sammailah, & Reddy, 2012). Similarly, the highest percentage (83.3%) of explants from *Albizia lebbeck* developed shoots on MS medium containing 2 mg/l BAP (Mamun *et al.*, 2004).

Even though shoots cultured on medium supplemented with 2 mg/l BAP produced the maximum mean number of shoots, the shootlets lacked homogeneity in size and morphology. The possible reason could be the occurrence of two or more species in the genus of *C. edulis*. All suitable requisites such as shoot number, length and multiplication rates were determined by genotype as they verified on deciduous tree, Chest nut (*Castanea sativa*) (Vieitez, Sanchez, Garcia-Nimo & Ballester, 2007). The relevance of PGRs for every explants of every species is different (Faisal, Ahmad & Anis, 2005; Siddique & Anis, 2008; Ahmad, Faisal, Anis & Aref, 2010).

When the concentration of BAP in combination with IBA increased above 3.0 mg/l BAP and 0.5 mg/l IBA, the rate of shoot multiplication was reduced and formed callus like appearance at the bases of the explants. This could be due to the inhibitory effect of IBA. This is in line with the report of tissue culture of deciduous tree, *Amygdalus communis*, an extensive amount of callus was developed when 3 mg/l BAP in combination with 1 mg/l IBA was used and caused the shoots to become unviable during sub-cultures (Gurel & Gulsen, 1998).

The mean length of shoots (1.06 ± 0.21) was found best on MS medium supplemented with 0.5mg/l BAP in combination with 0.01 mg/l IBA than other PGRs concentrations. Similarly, a combination of BAP (0.5 mg/l) and IBA (0.09 mg/l) is suitable for good shoot length of shoots as they tested on ornamental fruit tree, *Pyrus calleryana* (Karimpour, Davarynejadur, Bagheri & Tehranifar, 2013).

The increasing concentration of BAP towards 3 mg/l on MS medium showed decreasing in length of shootlets. This may be due to the suppression of apical dominance that reduced shoot length and leads to the production of multiple shoots. Similar result on *Pyrus calleryana* was found as higher BAP concentrations led to lower shoot lengths (Karimpour *et al.*, 2013).

4.4. Root Induction

None of the shootlets appeared to root on a given medium strength and with respective auxin concentrations. Micropropagation of *Albizia lebbeck*, the highest percentage (90%) of the shoots induced roots in full MS medium containing 1.0 mg/l IBA (Demsachew, 2011). But in our case, none of the shoots induced roots at similar concentration. In view of this, Bonga and von Aderkas (1992) added that the requirement and concentration of auxin for *in vitro* root induction are species specific. *In vitro* micropropagation of munamuna (*Clinopodium odorum*) revealed that 90% of the explants induced roots in growth regulators-free half strength MS medium (Diaz, Palacio, Figueroa, & Goleniowski, 2012). On the other hand, the shoots of woody tree, *Taxodium distichum*, entirely failed to form root in half strength growth regulators-free MS medium (Abou Dahab, Habib, El-Bahr & Gabr, 2010). In our study also shoots that were cultured on full, half or one-third strength growth regulators-free MS medium didn't induce root. Similarly, in the experiment on the endemic argan tree, *Argania spinosa*, the root induction was failed on MS medium regardless of the hormone used (Nouaim, Mangin, Breuil, & Chaussod, 2002).

5. Conclusion

In conclusion, cutting the de-coated seeds transversally enhanced the *in vitro* germination and culturing seeds at 45° orientation was found to enhance *in vitro* germination of seeds of *C. edulis*. *In vitro* germinated seedlings are important for further experimental process of micropropagation. The highest shoot initiation percentage of explants cultured on MS medium containing 0.5 mg/l BAP in combination with 0.01 mg/l IBA and Maximum number of shoots cultured on MS medium containing 2.0 mg/l BAP was obtained from shoot tip explants. When the elongated shoots transfer to the root induction medium, none of the shootlets appeared to root on a given medium strength and with respective auxin concentrations. Thus, further research activities is needed induce the root. In general, this is the first attempt of micropropagation of *C. edulis* and the current result is vital for cryopreservation of this endangered multipurpose plant. In addition, it used as effort for further research activities.

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