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Induction of callus and somatic embryogenesis from cotyledon and leaf explants of Yeheb (*Cordeauxia edulis* Hemsl)

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Abstract: 'Yeheb' (*Cordeauxia edulis* Hemsl) is a multipurpose and evergreen shrub and endemic to southeastern corner of Ethiopia and Somalia. It is adapted to low and irregular rainfall and survives a very long dry season. It has enormous economic and food security roles to the pastoralist of Somali Region State in Ethiopia. However, the plant is threatened with extinction due to over exploitation and its' poor natural regeneration capacity. The aim of this was to explore the potential for in vitro rapid regeneration of 'yeheb' from cotyledon and leaf explants on Murashige and Skoog (MS) media supplemented with 1.0 – 8.0 mg l^{-1}concentrations of 2, 4-D for callus induction and 2.0 and 3.0 mg l^{-1}concentration of N6-benzylaminopurine (BAP), thidiazuron (TDZ) and kinetin (Kin) with combination of 4.0 mg l^{-1} of 2, 4-Dichlorophenyl acetic acid (2, 4-D) for embryo induction. The result of these studies revealed that the highest percentage of callus induction (89%) were obtained from both leaf and cotyledon explants on MS media supplemented with 4.00 and 8.00 mg l^{-1} 2, 4-D, respectively. The highest percentage of embryo regeneration responses (88.89 and 77.78%) were obtained from leaf and cotyledon explants on same media: MS media supplemented by 3.00 mg l^{-1} TDZ +4.00 mg l^{-1} 2, 4-D. As a conclusion; this is the first attempt for callus and embryo *in vitro* regeneration of *C. edulis* and permissible result for mass propagation and cryopreservation.

Keywords: Callus, Cotyledon, Embryo, Explants, In Vitro

1. Introduction

Cordeauxia edulis Hemsl, belongs to the family *Leguminosae* and the subfamily *Caesalpinioideae* and is locally known as 'Yeheb' [1]. It is among the priority edible wild food plants in Ethiopia [2]. The species is a much branched ever green shrub or small tree up to 2.5m height, and is endemic to restricted localities in eastern Ethiopia and parts of central Somalia [1].

'Yeheb' is a multi-purpose plant where most parts of the plant are used. The seeds are edible and eaten fresh, roasted, boiled or dried. The seed of the species is potentially a valuable protein source with high sugar and fat contents. It has high energy value (0.39 - 1.87 MJ/Kg). The leaves are also rich in energy (5.59 – 5.86 MJ/Kg dry matter) [3]. In addition, leaves have been used to dye cloths, calico and wool since the cordeauxiaquinone forms vividly colored and insoluble combinations with many metals [4]. Another major use of the species is its contribution of up to half of the biomass in the area that make it important dry season browse to camel and goat. The estimated average forage production is 325-450 kg/ha (1.4-2 kg/plant) [5]. In semi-arid and arid areas the species represent an economical interest. As it is adapted to low and irregular rainfall and survives a very long dry season, it could indeed represent an enormous advantage in the fight against hunger. The development of cultivation of such plants for the Sahelian zone could also constitute an interesting food supplement in an area poor in protein supply [6]. The species

constitutes the staple food of the pastoralist of Somali region in Ethiopia. Moreover, the nut is sold on the market and even exported to the coastal cities of Somalia.

Even if the species has such and many other uses and has a potential to play a role in ensuring food security in the region, the plant is threatened with extinction due to overexploitation of the shrub by long term heavy grazing pressure, harvesting of seeds, cutting and fire. In addition, erosion, drought and war in the region has led to poor or none natural regeneration [4, 7 and 8].

Some reference [9 and 10] reported the decline and progressive destruction of the stands of *C. edulis* due to over grazing, and had recommended protection and use of the plant. Likewise [11 and 12] reported that *C. edulis* plant is in great danger of extinction and speedily narrowing distribution area because of the increase in population and their herds. Unlike many other plants, 'yeheb' shrubs flowers just before the onset of rains and the seeds mature when the plant moisture content is at its peak [5]. 'Yeheb' seeds have been reported not to retain viability for more than a few months, even if they are stored under ideal conditions and the recommendation has therefore been to sow them immediately. Some pilot studies made regarding vegetative propagation but finalized without greater success [12]

Hence, tissue culture technique is a power full tool for mass multiplication and germplasm conservation for this valuable species which is on the verge of extinction. *In vitro* regeneration via somatic embryogenesis has been success in many species, such as dahlia [13] canola [14], roselle [15], papaya [16] and etc. Nevertheless, limited work has been done so far vis-à-vis *in vitro* regeneration from callus in *C. edulis*.

Therefore, the aim of this study was to develop the basic protocols for the establishment of callus culture and induction of somatic embryogenesis from cotyledon and leaf explants of *C. edulis*.

2. Materials and Methods

2.1. Description of the Experimental Area

The experiment was conducted at the Plant Biotechnology Laboratory of Holetta Agricultural Research Center (HARC). The center is located 29 km west of Addis Ababa at an altitude of 2400 meter above sea level, 9^0 00'N latitude, 38^0 30'E longitude.

2.2. Experimental Material

2.2.1. Explant Selection

As the shrubs had not produced seed during the experimental period due to lack of rain in the region, the seeds that were full size and dried were collected from local market of 'Boh', Warder Zone of Ethiopia Somali Regional State (ESRS) in June, 2011. Healthy seeds were selected carefully and used for this study.

2.2.2. Explant Preparation

The seeds used for this experiment were washed under running tap water for 30 min. This was followed by immersing 70% (v/v) ethanol for 3 min, and later rinsed three times (3 min each) with sterile distilled water. After sterilization, seeds were soaked in sterile distilled water for 12 hr. The seed coats were then removed and subjected to surface disinfection with 5.00 % sodium hypochlorite for 5 min and then rinsed three times (3 min each) with sterile distilled water. Sterile seeds were inoculated in Murashige and Skoog (MS) media for germination.

Both cotyledon and leaf were used as explants for this experiment. Cotyledon explants were obtained from 21 days old seedlings grown in aseptically. Leaf explants were also obtained from aseptically grown seedlings.

2.3. Callus Induction

Callus induction studies were carried out by culturing the sterile cotyledon and leaf explants of *C. edulis* on MS media supplemented by different concentration of 2, 4-D (0.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 8.0 mg l^{-1}). Media contained 500 mg l^{-1} of casein hydrolysate (C7290, Sigma), 3% sucrose, and 0.7% agar. The media were adjusted to pH 5.7 after addition of the plant growth hormone, but prior to adding agar. The media was later poured into magenta (40ml) before autoclaving at 120 ^{0}C for 15 minutes. One explant was placed per magenta, and each treatment had nine explants. All the cultures were incubated in a growth room adjusted at room temperature of 25 ± 2^0C in the dark. The morphology, degree of callogenesis and days of callus formation were recorded after 8 weeks of culture.

2.4. Embryo Induction

Those calli produced on cotyledon and leaf explants were transferred to MS media supplemented with 2.0 and 3.0 mg l^{-1}concentrations of N6-benzylaminopurine (BAP), thidiazuron (TDZ) and kinetin (Kin) with combination of 4.0 mg l^{-1} of 2, 4-D and free plant growth regulator (as control), each type and level of concentration of cytokinin were combined with the Auxin to use as treatment. Media contained 500 mg l^{-1} of casein hydrolysate (C7290, Sigma), 3% sucrose, and 0.7% agar. The media were adjusted to pH 5.7 after addition of the plant growth hormone, but prior to adding agar. The media were later poured into magenta (40 ml) before autoclaving at 120^0C for 15min. One explant was placed per magenta, and each treatment had nine explants. All the cultures were incubated in a growth room adjusted at room temperature of 25 ± 2^0C in the dark. Data on embryo regeneration percentage was recorded from both explants after 8 weeks after transferred to embryo induction media.

2.5. Experimental Design and Data Analysis

Treatments in all the experiments were arranged in a completely randomized design (CRD) with three replications. The data was subject for analysis of variance (ANOVA) using SAS [16] and significant differences among mean values were compared using Duncan's Multiple Range Test (DMRT) at $p<0.05$. Logarithmic transformation was used for percentages data to fulfill the normality test before doing analysis of variance.

3. Results and Discussion

3.1. Callus Induction

The analysis of variance result revealed that MS media supplemented by different concentration of 2, 4-D had a highly significant effect ($p<0.01$) on percentage of callus induction on both explants. It was possible to induce callus successfully from both explants used on media supplemented by 4.00, 5.00, 6.00 and 8.00 mg l^{-1} of 2, 4-D. Low concentration of 2, 4-D (1.00, 2.00 mg l^{-1}) and control (hormone free) did not induce callus. Leaf explants were better than cotyledon explants in terms callus induction (Table 1).

Table 1. *Effect of 2, 4-D on callus induction on cotyledon and leaf explants of 'yeheb'*

Explants	2, 4-D (mg l^{-1})	Days to induce callus	Percentage of callus induction (%)	Callus size	Callus color
Cotyledon	0.00	60	0 ± 0.00^{c}	-	-
	1.00	60	0 ± 0.00^{c}	-	-
	2.00	60	0 ± 0.00^{c}	-	-
	3.00	60	11 ± 0.19^{c}	+	-
	4.00	44-46	44 ± 0.20^{b}	++	White
	5.00	45-43	56 ± 0.20^{b}	++	White
	6.00	44-45	67 ± 0.19^{ab}	+++	White
	8.00	40-42	89 ± 0.19^{a}	++++	Yellow
Mean			38.10		
CV (%)			2.06		
Leaf	0.00	60	0 ± 0.00^{d}	-	-
	1.00	60	0 ± 0.00^{d}	-	-
	2.00	58-60	44 ± 0.20^{bc}	+	White
	3.00	46-48	67 ± 0.19^{b}	+	White
	4.00	37-38	89 ± 0.19^{a}	++++	White
	5.00	37-38	44 ± 0.19^{bc}	+++	White
	6.00	37-38	33 ± 0.00^{c}	+	White
	8.00	35-36	33 ± 0.00^{c}	+	White
Mean			44.44		
CV (%)			1.61		

Means with same letter (s) in the same column are not significantly different at 1% according to Duncan's Multiple Range Tests (DMRT). CV= coefficient of variation (%), Callus color data was recorded after 2 month of inoculation, +=0.01mm size callus.

3.1.1. The Effect of 2, 4-D on Cotyledon Explants

The result reviled in Table 1 indicated that the highest percentage of callus induction (89%) was observed on MS media supplemented by 8.00 mg l^{-1} 2, 4-D. While poor (11%) or no response was observed on media supplemented with 3.00 mg l^{-1} and less concentration of 2, 4-D. Similar result was reported by [17] on *Vigna radiata*. Bigger callus size with shorter period of time was observed, when 2, 4-D concentration increase from 4.00 to 8.00 mg l^{-1}. The highest concentration (8.00 mg l^{-1}) of 2, 4-D produced the biggest callus size within 40-42 days after inoculation. Lower concentration (4.00 mg l^{-1}) of 2, 4-D resulted in the least callus induction. Callus production from cotyledon explants of woody plants was reported on *Parkia biglobosa* [18] and *Fraxinus pennsylvanica* [19].

3.1.2. The Effect of 2, 4-D on Leaf Explants

The two highest callus induction percentage (89% and 67%) was recorded on MS media supplemented with 4.00 and 3.00 mg l^{-1} 2, 4-D, respectively. While poor (33%) or no response was observed on media supplemented with 8.00 and 6.00 mg l^{-1} or 1.00 mg l^{-1} and free 2, 4-D. Similar biggest callus size was obtained on 4.00 mg l^{-1} of 2, 4-D within 37-38 days of after inoculation, while lower callus size was observed on the concentration 2.00, 3.00, 6.00, and 8.00mg of 2, 4-D (Table 1).

Generally callus production was observed only at the cut edges of both explants and on the abaxial surface, even when placed upside down. This result showed that callus formation is affected among other factors is orientation of the explants on the culture medium [20]. Reference [21] also reported similar observation with the leaf explants of *Cuphea ericoides*.

3.2. Embryo induction

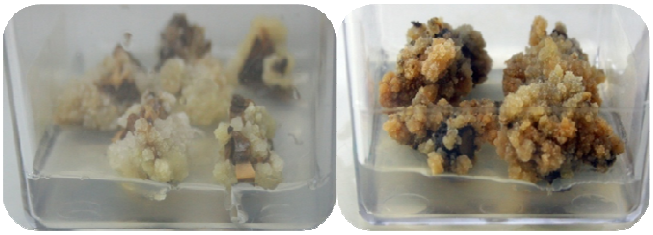
The analysis of variance revealed that the effect of plant growth hormone had highly significant effect on embryo regeneration from both types of explant. The mean callus derived from leaf explants (47.61±0.16) resulted in highest embryo regeneration percentage than callus derivative from cotyledon explants (34.92±0.16) (Table 2 and Fig. 1a and b).

As indicated in Table 2, the highest percentage of embryo regeneration response was obtained from media supplemented by 3 mg l^{-1} TDZ +4 mg l^{-1} 2, 4-D on both cotyledon (77.78 ±0.19) and leaves (88.89 ±0.19) explants after eight week of cultured (Fig 2a and b) followed by 3 mg l^{-1} BAP +4 mg l^{-1} 2, 4-D and 2 mg l^{-1} TDZ + 4 mg l^{-1} 2, 4-D; while none embryogenic response was observed from hormone free (control) medium (Table 2). Reference [15] reported similar observation on cotyledon explants of Hibiscus sabdariffa on MS media supplemented with TDZ + 2, 4-D had better embryo regeneration potential than BAP + 2, 4-D or Kin + 2, 4-D

Table 2. *Effect of cytokinins and auxin concentration on embryo development*

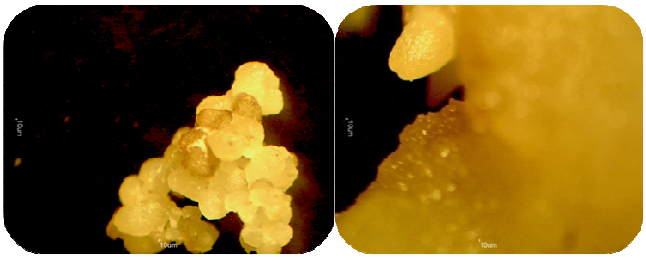
Plant growth hormone	Embryo regeneration response from callus (%)	
	Cotyledon	Leaf
0.00	0±0^{d}	0± 0^{e}
2 mg l^{-1} BAP + 4 mg l^{-1} 2, 4-D	33.33 ±0^{bcd}	44.44 ±0.19^{cd}
2 mg l^{-1} Kin + 4 mg l^{-1} 2, 4-D	22.22 ±0.38^{bcd}	22.22 ±0.19^{de}
2 mg l^{-1} TDZ + 4 mg l^{-1} 2, 4-D	44.44 ±0.19^{abc}	55.56 ±0.19^{bc}
3 mg l^{-1} BAP + 4 mg l^{-1} 2, 4-D	55.56 ±0.19^{ab}	77.78 ±0.19^{ab}
3 mg l^{-1} Kin + 4 mg l^{-1} 2, 4-D	11.11 ±0.19^{cd}	44.44 ±0.19^{cd}
3 mg l^{-1} TDZ + 4 mg l^{-1} 2, 4-D	77.78 ±0.19^{a}	88.89 ±0.19^{a}
Mean	34.92±0.16	47.61±0.16
CV (%)	8.10	5.50

Means with same letter (s) in the same column are not significantly different at 1% according to Duncan's Multiple Range Tests (DMRT). CV= coefficient of variation (%).



(a) (b)

Figure 1. *Embryo induction media supplemented with 3 mg l^{-1} TDZ + 4 mg l^{-1} 2, 4-D before 4 weeks of culture a) on cotyledon explant; b) on leaf explant*



(a) (b)

Figure 2. *Embryo induction on media supplemented with 3 mg l^{-1} TDZ + 4 mg l^{-1} 2, 4-D after 8 week of culture a) from leaf explant (10x magnified); b) from cotyledon (10x magnified).*

4. Conclusion

'Yeheb' (*Cordeauxia edulis* Hemsl.) is a multi-purpose plant where most parts of the plant are useable. Even if the species has multitude uses and has a potential to play a role in ensuring food security in the region, the plant is threatened with extinction due to overexploitation, this it has led to poor or none natural regeneration. This study is the foremost protocols for indirect *in vitro* regeneration of *C. edulis* from cotyledon and leaf explants. It has also indicated callogenic capacity of cotyledon and leaf explants of *C. edulis* and the possibility of inducing somatic embryogenesis from the induced calli. Further research will be needed in order to advance the embryoids to plantlets and their establishment in the field. In a nut shell, this is the first attempt and found a permissible result to rescue this rare, endemic, and endangered species by mass and continuous plantlet production within short period of time through in vitro propagation. In addition it is used as a baseline for *ex situ* conservation through cryopreservation.

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