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In Vitro Propagation Potential via Somatic Embryogenesis of the Two Maturing Early Cultivars of European Chestnut (*Castanea sativa* Mill.)

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Abstract

Turkey ranks the third in the production of chestnuts in the world having an important place both in domestic and global markets. However, the chestnut production and the number of trees have been diminishing in recent years. Therefore, *in vitro* propagation of the chestnut, in addition to the classical propagation techniques, should be applied. Especially the propogation of the early maturing cultivars and production of the quality chestnuts will provide a better income to the producer. Here, somatic embryo production and regeneration from the immature cotyledons of the early maturing cultivars of the European chestnut (*Castanea sativa* Mill), Hacıbiş and Karamehmet, were studied using the somatic embryogenesis, one of the *in vitro* propagation techniques. To induce the somatic embryogenesis, 168 different combinations were applied to both cultivars. The somatic embryogenesis rate in Hacıbiş cultivar, in which the interactions were observed among the applications, was found to be 9.9% while it was 11.1% for the Karamehmet cultivar. Dessication, cold treatment, gibberellic acid (GA_3) and benzyladenine (BA) + naphthaleneacetic acid (NAA) applications were performed on the regeneration of the somatic embryos, and 40% conversion to plant was obtained with desiccation together with BA + NAA supplementation to the medium.

Keywords

Early Maturing Cultivars, *in Vitro* Propagation, Somatic Embryogenesis, Germinating

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1. Introduction

The genus *Castanea* (chestnut) belongs to the *Fagaceae* family with its 13 different species, and it is found in the natural flora of the northern hemisphere. Among the chestnut species, the origin of European chestnut (*Castanea sativa* Mill.) is Anatolia, and it also spreads throughout the Southern Europe, Northern Africa, and Caucasia [1]-[4].

According to the FAO 2013 statistics, Turkey ranks the third in the world with 60.019 tonnes of chestnut production after China (925 thousand tonnes) and South Korea (70 thousand tonnes), and has a share of 5.2% in the global production (2 million tonnes) [5]. However, the chestnut production has been dropping off due to the common diseases in recent years [6]. In 2013, The General Directorate of Forestry reported an 83.497 ha chestnut forests in Turkey [7]. The tannin-rich, dark coloured, and durable wood of this economically important species is used in the forestry, as well as its flowers and fruits are used in beekeeping and the food industry, respectively [8].

The early maturing chestnut cultivars Hacıibiş and Karamehmet are grown in the Marmara Region in Turkey [2]. The important factors affecting the earliness are genetic make-up, cultural applications, and the environmental pressure [2] [9]. The goal in the earliness is to bring the vegetation and maturation time of the crop to the most proper period considering the geographical conditions of the region, hence to obtain high efficiency and high quality products. The earliness and early maturation also gain importance in terms of the pest control, reduction of the production costs, and early appearance in the market [10].

Somatic embryogenesis from the zygote-originated tissues is an important potential for the clonal propagation of the hybrids obtained via hybridization breeding [11]. In the present study, somatic embryo production from the immature cotyledons of the seeds belonging to the early maturing cultivars, Hacıibiş and Karamehmet, of the European chestnut (*C. sativa* Mill) was investigated. The effects of basal media, combinations of plant growth regulators (PGRs), silver nitrate ($AgNO_3$), L-glutamine, casein hydrolyzate, and solidifying substances on the somatic embryogenesis as well as the roles of gibberellic acid (GA_3), dessication, cold treatment, benzyldenine (BA), and BA + auxin applications on the plant regeneration were studied.

2. Materials and Methods

2.1. Plant Material

The early maturing cultivars of European chestnut (*C. sativa* Mill) Hacıibiş and Karamehmet were used as plant material in this research. The trees grew in the chestnut collection parcel in Atatürk Central Horticultural Research Institute, Yalova, Turkey and in the gardens of two manufacturers located at the skirts of the Uludağ Mountain (Bursa-Turkey).

For the first step of somatic embryogenesis,explants were obtained from the cotyledons of immature seeds because of their high levels of auxin and cytokinin levels. For 3 yr, burs (fruiting heads) were manually harvested 7 wk (50 d) after the female flowers were in full bloom, between the end of July and the beginning of August (with slight seasonal variation), and were divided into their fruits [12].

2.2. Sterilization

Immature fruits were separated from the bur and suspended for 15 min in 20% (v/v) commercial sodium hypochlorite solution containing a few drops of Tween 20. This was followed by five rinses with sterile distilled water (dH_2O) for 5 min (with agitation). The immature fruits were opened in a laminar flow cabinet and cotyledonary explants, about 0.5 cm^2 in size, were excised using a scalpel.

2.3. Somatic Embryo Initiation

In order to induce initial stage somatic embryos: 1) Murashige and Skoog medium (MS) [13]; 2) Driver and Kuniyuki Walnut Medium (DKW) [14]; or 3) Woody Plant Medium (WPM) [15] were evaluated. These basal media were tested in combination with seven different plant growth regulator (PGR) combinations; a) 6-benzyladenine (BA 1 mg/L) + kinetin (KIN 2 mg/L) + indole-3-butyric acid (IBA 0.01 mg/L; b) BA (1 mg/L) + 1-phenyl-3-(1,2,3-thiadiazol-5-yl; TDZ, 0.1 mg/L) + IBA (0.01 mg/L); c) KIN (2 mg/L) + TDZ (0.1 mg/L) + IBA (0.01 mg/L); d) BA (1 mg/L) + KIN (2 mg/L) + 2,4-dichlorophenoxyacetic acid (2,4-D 0.02 mg/L); e) BA

(1 mg/L) + TDZ (0.1 mg/L) + 2,4-D (0.02 mg/L); f) KIN (2 mg/L) + TDZ (0.1 mg/L) + 2,4-D (0.02 mg/L); or g) BA (1 mg/L) + α-naphthaleneacetic acid (NAA 1 mg/L) and organic additives (L-glutamine (250 mg/L) or casein hydrolizate (1000 mg/L) and $AgNO_3$ (0 mg/L - 1.7 mg/L). Media was solidified with Gelrite™ (2.1 g/L) or agar (6 g/L-Fluka™). Media supplemented with sucrose (30 g/L) and Gelrite™ (2.1 g/L), and the pH was adjusted to 5.7 before autoclaving at 121˚C for 20 min. These 168 different combinations were used to test the response for both the Hacıbiş and Karamehmet chestnut cultivars. The PGR combinations were decided according to previous studies [12] [16].

The rate of somatic embryogenesis (%) was calculated dividing the number of total cotyledon explants generated somatic embryos to the number of total cotyledon explants planted onto the initiation media, and the resulting number was multiplied with 100.

The average number of somatic embryos (number/cotyledon explant) was calculated dividing the number of embryos (at the stage of cotyledon) to the number of total explants.

2.4. Initiation Cultures

The cotyledon explants were placed horizontally on the media under aseptic conditions. Each of the Petri dish contained five explants, and was cultured for a period of 4 wk in a growth chamber at 25˚C ± 1˚C in the dark. In order to induce growth of somatic embryos, after 4-wk culture to initiate somatic embryogenesis, cotyledon explants were sub-cultured on the same media without PGRs, $AgNO_3$ or L-glutamine, for a total of four times at 4-wk intervals onto the basal media with the same supplementation.

2.5. Regeneration of the Somatic Embryos

In regeneration experiments, 30 g/L sucrose and 6 g/L agar were added to DKW or half-strength MS basal salts (1/2 MS). The somatic embryos were used at the stage of the cotyledon derived from the cotyledon explants in the regeneration experiments. The somatic embryos (five pieces/Petri dish) were placed in a vertical position so that the root tip of the root/epicotyl axis was in contact with the media. All the cultures were incubated for a period of 4 wk 25˚C ± 1˚C temperature, 16 h light (35 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and 8 h dark for regeneration. Three different trials were conducted for regeneration of the somatic embryos.

2.5.1. Desiccation and GA_3 Treatment

For the desiccation procedure, a Nalgene® desiccator (1.5 L capacity) was sterilized by autoclaving with 40 ml saturated $MgCl_2 \cdot 6H_2O$ solution and then, in a laminar air flow cabinet, 20 somatic embryos at the cotyledon stage were placed in Petri dishes without media (four pieces per plate), and were placed in the desiccator. The desiccator was wrapped with stretch film and kept in the growth chamber at 25˚C ± 1˚C in the dark for 4 d. After treatment with or without desiccation, somatic embryos were cultured on DKW basal media containing 0, 1, 3, 5, 7, or 9 mg/L GA_3.

2.5.2. BA Applications with Desiccation and Cold Treatment

These treatment conditions were performed according to Corredoira *et al*. [17]. In the trials, the somatic embryos were desiccated and/or cold treated, and then were placed on media (DKW containing 438 mg/L L-glutamine, 30 g/L sucrose, and 2.1 g/L gelrite, pH 5.7) supplemented with either BA (0.1 mg/L) alone, BA (0.1 mg/L) + NAA (0.1 mg/L), or BA (0.1 mg/L) + IBA (0.1 mg/L). Cold treatment was conducted at 4˚C, in the dark for 8 wk. Fifteen somatic embryos were transferred to the Petri dishes (100 × 10 mm) containing 40 mL of DKW basal media with 30 g/L sucrose and 2.1 g/L gelrite at pH 5.7.

2.5.3. Pre-Treatment of Somatic Embryos with Desiccation, Cold Temperature, or GA_3

In the second trial, the aim was to determine whether the somatic embryos respond better when they were pre-treated with desiccation, cold (cold treatment was conducted at 4˚C, in the dark for 8 wk), or GA_3 (3 mg/L for 10d), before being cultured on DKW with BA + NAA (0.1 mg/L each). The effect of the same media with or without 438 g/L L-glutamine were tested [17]. The media used contained 30 g/L sucrose and 6 g/L agar at pH 5.7. The regeneration response of somatic embryos for different treatments and/or pre-treatments was determined via analysis of the percentage of embryos showing root generation (percentage), shoot generation (percentage), and plantlet generation (percentage) after 4 wk.

2.6. Experimental Design and Data Analysis

In this study, all trials were established according to Coincidence Pattern Experimental Design (Minitab Inc. 814-238-3280 WS112102553). The effects of five different applications (basal media × PGR combinations × organic matters × $AgNO_3$ × solidifying substances) were investigated on the somatic embryogenesis of two early cultivars Hacıibiş and Karamehmet. The experiments were repeated five times in each case, with five explants per treatment. All trials were repeated throughout two years.

The trials of somatic embryo regeneration were evaluated by analyzing the interactions among "cultivars × desiccation × GA_3" in the first trial, "cultivars × BA applications" in the second trial, and "cultivars × pre-application combination × BA + NAA" in the third trial. The experiments were repeated five times in each case, with five explants per treatment. Each experiment was repeated at least twice.

Data obtained from somatic embryogenesis and regeneration experiments were calculated as the average of repeats using the analysis of variance (ANOVA) in Minitab Package Program, and controlled with F test ($P \leq 0.05$, 0.01, and 0.001). Significant differences were determined depending on 5% error limit with the Duncan test, and the differences were indicated with letters. In statistical analyses, transformed angle values were utilized for the percentage data.

3. Results

The effects of basal media, combinations of plant growth regulators (PGRs), organic substance, $AgNO_3$, and solidfying substance on somatic embryo formation as well as the impacts of desiccation, cold treatment, and GA_3 were shown in **Figure 1**.

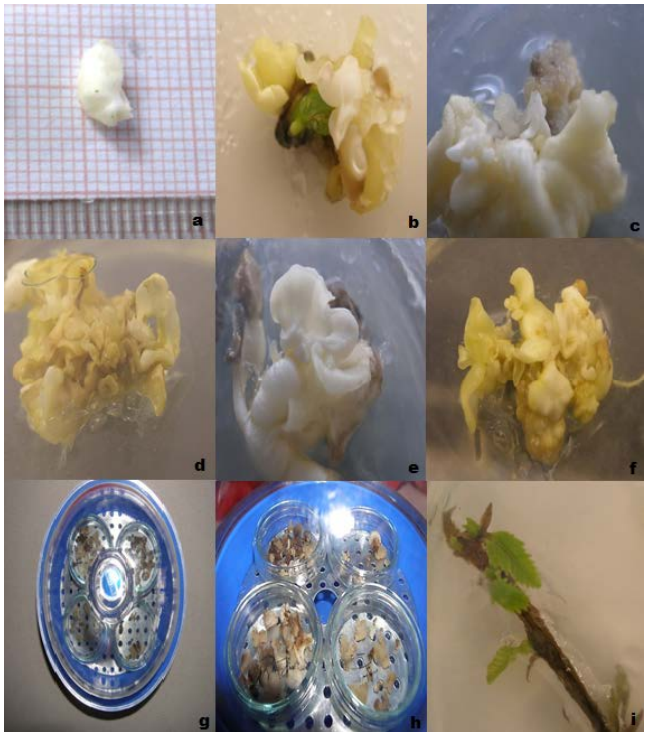

Figure 1. Somatic embryo formation of early chesnut cultivars Hacıibiş and Karamehmet. (a) Somatic embryo of Hacıibiş at the cotyledon stage. (b-c) Somatic embryos of Hacıibiş chestnut growing on DKW basal medium containing L-glutamine, solidified with gelrite, and supplemented with PGR combination KIN (2 mg/L) + TDZ (0.1 mg/L) + IBA (0.01 mg/L). (d) Somatic embryos of Hacıibiş chestnut growing on DKW basal medium containing casein hydrolizate, solidified with gelrite, and supplemented with PGR combination BA (1 mg/L) + KIN (2 mg/L) + IBA (0.01 mg/L). (e-f) Somatic embryos of Karamehmet chestnut growing on DKW basal medium containing L-glutamine, solidified with agar, and supplemented with PGR combination BA (1 mg/L) + KIN (2 mg/L) + IBA (0.01 mg/L). (g-h) Desiccation of somatic embryos using MgCl2.6H2O solution in Nalgene® desiccator. (i) A regenerating plantlet growing on germination medium [BA (0.1 mg/L) and NAA (0.1 mg/L)] originating from a somatic embryo of *C. sativa* Mill. cv. "Hacıibiş" after desiccation and cold pre-treatments.

3.1. Somatic Embryogenesis in Hacıibiş Cultivar

A significant interaction among the basal media, combinations of PGRs, organic substance, $AgNO_3$, and solidfying substance could not be observed in Hacıibiş chestnut cultivar. However, the interactions among PGR combination × organic substance × solidifying substance were found to be significant (P = 0.045). In the content of PGR combination × organic substance × solidifying substance interaction in this cultivar, the PGR combination number 3 [KIN (2 mg/l) + TDZ (0.1 mg/l) + IBA (0.01 mg/l)] × L-glutamin × gelrite showed the highest somatic embryogenesis rate with 9.9% (Table 1). However, the PGR combination number 3 ×casein hydrolizate × gelrite provided 7.2% rate, and PGR combination number 1 [BA (1 mg/l) + KIN (2 mg/l) + IBA (0.01 mg/l)] × casein hydrolizate × gelrite provided 8.0% rate, which are not statistically significant, therefore they were grouped together (Table 1). The rates for PGR combination numbers 4, 5, 6, and 7 remained at very low levels (0.3% - 2.2%).

For the Hacıibiş cultivar,when the interaction group of "PGR combination × $AgNO_3$ × solidifying substance" is considered (P = 0.001), among the basal media solidified with gelrite, the PGR combination number 3 provided 9.2% embryogenesis rate with $AgNO_3$, and 8.6% without $AgNO_3$, which are the highest rates. The embryogenesis rate of PGR combination number 2 without $AgNO_3$ (7.7%), and the PGR combination number 1 with $AgNO_3$ (7.1%) were found to be statistically significant. Moreover, in the media solidified with agar, the PGR combination numbers 2 and 3 provided significant results with $AgNO_3$ (4.9%) and without $AgNO_3$ (5.8%), respectively. In this context, low rates (0.3% - 3.1%) were obtained for the PGR combination numbers 4, 5, and 6 containing 2,4-D as auxin, and PGR combination number 7 containing NAA, with two solidifiers and $AgNO_3$ dose (Table 2).

Table 1. Impact of the interaction PGR combination × organic substance × solidifying substance on the rate of the somatic embryogenesis in Hacıibiş chestnut.

PGR		Organic substance			
		L-glutamine	Casein hydrolyzate	L-glutamine	Casein hydrolyzate
		Agar		Gelrite	
1	BA + KIN + IBA	3.2 (4.08)** defghi*	2.3 (2.86) fghi	2.7 (3.11) fghi	**8.0 (8.42) abc**
2	BA + TDZ + IBA	3.0 (4.25) cdefgh	4.1 (5.06) bcdef	**6.1 (7.09) abcd**	5.0 (5.71) bcdefg
3	KIN + TDZ + IBA	**6.0 (7.26) abcde**	3.0 (3.55) efghı	**9.9 (9.85) a**	**7.2 (8.61) ab**
4	BA + KIN + 2,4-D	0.7 (0.89) hi	0.3 (0.44) i	1.4 (1.75) ghi	1.4 (1.70) hi
5	BA + TDZ + 2,4-D	2.2 (3.02) fghi	0.7 (0.89) hi	1.7 (1.50) hi	2.0 (2.66) fghi
6	KIN + TDZ + 2,4-D	1.0 (1.10) hi	1.4 (1.77) fghi	3.3 (3.71) efghi	0.7 (0.89) hi
7	BA + NAA	2.2 (3.10) fghi	2.2 (2.87) fi	1.5 (1.54) hi	0.7 (0.89) hi

BA (1 mg/L), KIN (2 mg/L), IBA (0.01 mg/L), TDZ (0.1 mg/L), 2,4-D (0.02 mg/L), NAA (1 mg/L). *Different letters indicate significant differences between the treatments (P = 0.045). **The numbers in parenthesis represents the angle value of percentages.

Table 2. Effect of PGR combination × $AgNO_3$ × solidifying substance on the rate of somatic embryogenesis in Hacıibiş chestnut.

PGR		$AgNO_3$			
		0.0 mg/L	1.7 mg/L	0.0 mg/L	1.7 mg/L
		Agar		Gelrite	
1	BA + KIN + IBA	4.1 (5.45) bcdef*	1.4 (1.55) fgh	2.3 (3.10) defgh	**7.1 (8.11) abc**
2	BA + TDZ + IBA	3.5 (4.34) cdefg	**4.9 (6.27) abcde**	**7.7 (8.56) ab**	3.7 (4.77) bcdefg
3	KIN + TDZ + IBA	**5.8 (6.80) abcd**	3.2 (4.11) defgh	**8.6 (9.61) a**	**9.2 (10.17) a**
4	BA + KIN + 2,4-D	0.3 (0.45) h	0.7 (0.89) gh	1.3 (1.77) fgh	1.3 (1.77) fgh
5	BA + TDZ + 2,4-D	2.1 (2.56) efgh	1.0 (1.33) gh	1.9 (1.99) efgh	2.0 (2.04) fgh
6	KIN + TDZ + 2,4-D	1.3 (1.54) fgh	1.0 (1.33) gh	2.0 (2.22) efgh	2.0 (1.99) efgh
7	BA + NAA	1.2 (1.77) fgh	3.1 (4.15) defgh	1.7 (1.98) fgh	0.3 (0.44) h

BA (1 mg/L), KIN (2 mg/L), IBA (0.01 mg/L), TDZ (0.1 mg/L), 2,4-D (0.02 mg/L), NAA (1 mg/L) *Different letters indicate significant differences between the treatments (P = 0.001). **The numbers in parenthesis represents the angle value of percentages.

3.2. Somatic Embryogenesis in Karamehmet Cultivar

In the Karamehmet cultivar of the chestnut, only the interaction between "basal medium × PGR combination" was found to be significant in terms of the somatic embryogenesis rate and the number of the somatic embryos (P = 0.000) (Table 3). DKW basal medium × PGR combination number 1 provided 11.1% somatic embryogenesis rate. Besides, DKW basal medium × PGR combination number 2 provided 9.2% rate, and these two values took part in the same statistical group.

Although 7 different PGR combinations were used for the Karamehmet cultivar, any somatic embryogenesis was induced by the WPM basal medium because of its lower nitrogen content with respect to DKW and MS media. No positive result was obtained also with MS medium PGR combination numbers 3, 4, 5, 6, and 7, as well as DKW medium PGR combination number 7.

3.3. Regeneration Experiements

The impact of gibberellic acid (GA_3) and desiccation applications on root, shoot, and plantlet regeneration from somatic embryos was examined in Hacıibiş and Karamehmet early cultivars. The interactions among "cultivar ×

Table 3. Percentage of somatic embryogenesis (%) in Karamehmet chestnut cultivars and the interaction between basal medium × PGR on the number of the somatic embryos per explants.

	PGR Combinations	Somatic embryogenesis (%)		Somatic embryos (embryos/explants)	
		DKW			
1	BA + KIN + IBA	**11.1 (12.08)****	**a***	**0.46 ± 0.07**	**a**
2	BA + TDZ + IBA	**9.2 (10.26)**	**ab**	**0.41 ± 0.07**	**ab**
3	KIN + TDZ+IBA	8.0 (9.11)	bc	0.33 ± 0.06	b
4	BA + KIN + 2,4-D	3.0 (4.12)	def	0.14 ± 0.04	cdefg
5	BA + TDZ +2,4-D	7.0 (7.41)	cd	0.22 ± 0.05	cd
6	KIN + TDZ + 2,4-D	4.0 (4.79)	def	0.16 ± 0.05	cdef
7	BA + NAA	2.5 (3.28)	efg	0.13 ± 0.04	defgh
		MS			
1	BA + KIN + IBA	5.0 (5.95)	de	0.21 ± 0.04	cde
2	BA + TDZ + IBA	7.1 (7.56)	cd	0.24 ± 0.05	c
3	KIN + TDZ+IBA	2.2 (2.99)	efg	0.12 ± 0.04	defgh
4	BA + KIN + 2,4-D	1.6 (1.63)	fg	0.07 ± 0.03	efgh
5	BA + TDZ +2,4-D	0.7 (0.96)	g	0.04 ± 0.02	gh
6	KIN + TDZ + 2,4-D	1.2 (1.55)	fg	0.05 ± 0.03	fgh
7	BA + NAA	3.0 (3.50)	efg	0.12 ± 0.04	efgh
		WPM			
1	BA + KIN + IBA	0.0 (0.00)	g	0.0 ± 0.0	h
2	BA + TDZ + IBA	0.0 (0.00)	g	0.0 ± 0.0	h
3	KIN + TDZ+IBA	1.2 (1.36)	fg	0.05 ± 0.02	fgh
4	BA + KIN + 2,4-D	0.0 (0.00)	g	0.0 ± 0.0	h
5	BA + TDZ +2,4-D	0.0 (0.00)	g	0.0 ± 0.0	h
6	KIN + TDZ + 2,4-D	0.0 (0.00)	g	0.0 ± 0.0	h
7	BA + NAA	0.0 (0.00)	g	0.0 ± 0.0	h

BA (1 mg/L), KIN (2 mg/L), IBA (0.01 mg/L), TDZ (0.1 mg/L), 2,4-D (0.02 mg/L), NAA (1 mg/L). *Values followed by different letters indicate significant differences between the treatments (P = 0.000). **The numbers in parenthesis represents the angle value of percentages.

desiccation × GA_3 dosage", "cultivar × desiccation", "cultivar × GA_3 dosage", and "desiccation × GA_3 dosage", and the differences among the cultivar, desiccation, and GA_3 dosage were also studied. However, no statistically significant interactions were observed (**Table 4**). In both cultivars, after desiccation on media containing GA_3 with different dosages, no regeneration of root, shoot, or plantlets was observed. However, when desiccation was omitted (control embryos), the regeneration was observed when the media were supplemented with GA_3 at 3 mg/L in Hacıibiş cultivar and 5 mg/L in Karamehmet cultivar (**Table 5**). Nevertheless, the germination rates were still relatively low, between 20% and 40%. Furthermore, none of the treatments tested in this experiment had significant effects on development of roots, shoots or regeneration of plantlets (**Table 4** and **Table 5**).

The supplementation of BA in the media did not significantly affect the regeneration rates of the somatic embryos of Hacıibiş and Karamehmet early cultivars after the desiccation and cold treatments (cultivar × BA) (P = 0.982). Furthermore, the differences among the cultivars were also not significant (P = 0.787). On the other hand, the differences between the control and the BA, BA + NAA, and BA + IBA treatments were found to be statistically significant (P = 0.000). All PGRs were used at a concentration of 0.1 mg/L. The impacts of the applications on root, shoot, and plantlet regeneration of the cultivars are presented in **Table 6**.

Table 4. P values for the regeneration rates in the desiccation and GA_3 applications and in European chestnut somatic embryos.

Origin	Degree of freedom	P value		
		Root	Shoot	Plant reg.
Cultivar	1	0.728	1.00	0.681
Desiccation	1	0.215	0.220	1.00
GA_3 dosages	5	0.645	0.681	0.681
Cultivar × desiccation	1	0.728	1.00	1.00
Cultivar × GA_3 dosages	5	0.404	0.325	0.325
Desiccation × GA_3 dosages	5	0.645	0.681	0.681
Cultivar x desiccation × GA_3 dosages	5	0.404	0.325	0.325

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 5. Impact of desiccation and GA_3 applications on the rates of regeneration of chestnut somatic embryos.

GA_3 (mg/L)	Root reg. (%)	Shoot reg. (%)	Plant reg. (%)	Root reg. (%)	Shoot reg. (%)	Plant reg. (%)
	Hacıibiş			Karamehmet		
	Control					
0	0.0	0.0	0.0	0.0	0.0	0.0
1	0.0	0.0	0.0	0.0	0.0	0.0
3	**20.0**	**20.0**	**20.0**	0.0	0.0	0.0
5	0.0	0.0	0.0	**40.0**	**20.0**	**20.0**
7	0.0	0.0	0.0	0.0	0.0	0.0
9	0.0	0.0	0.0	0.0	0.0	0.0
	Desiccation					
0	0.0	0.0	0.0	0.0	0.0	0.0
1	0.0	0.0	0.0	0.0	0.0	0.0
3	0.0	0.0	0.0	0.0	0.0	0.0
5	0.0	0.0	0.0	0.0	0.0	0.0
7	0.0	0.0	0.0	0.0	0.0	0.0
9	0.0	0.0	0.0	0.0	0.0	0.0

The highest rate of the root formation (22.0%) was obtained using media supplemented with either BA + NAA or BA + NAA. There was no response (*i.e.*, 0.0% root regeneration) from all other treatments tested (**Table 6**). The highest rate of shoot formation (24.0%) was obtained from the same two applications. Although 8.0% shoot formation was obtained with media supplemented with BA, this value was not significant as compared to the control or to the embryos on media supplemented with BA + IBA, where no shoot regeneration was obtained (**Table 6**).

In terms of the effects of BA + NAA application in combination with desiccation, cold, or GA_3 pre-treatments, on root, shoot, and plantlet regeneration of the somatic embryos of both chestnut cultivars, only pre-application combinations followed by the BA + NAA treatment (average of the cultivar responses) were statistically significant ($P = 0.050$). No difference was observed between "cultivar × pre-application combinations × BA + NAA" ($P = 0.986$). Only somatic embryos treated with BA + NAA regenerated into plantlets (**Table 6**; **Figure 1(i)**), whereas treatments using PRG-free media failed to produce regenerated somatic embryos. Furthermore, the highest rates of regeneration (28.8% - 40.0%) were obtained for treatments using desiccation (desiccation only, desiccation + cold, or desiccation + GA_3 applications). The differences among the rates of these applications, however, were not statistically significant. The results revealed that without cold or GA_3 pre-applications, root, shoot, and plantlet regeneration could be obtained in somatic embryos cultured on media supplemented with BA + NAA following a 4d desiccation period (**Table 7**).

4. Discussion

The aim of "earliness" in agriculture is to obtain high yield with quality product in the proper time period. In addition to the classical production techniques, *in vitro* propagation of the European chestnut (*C. sativa* Mill), a valuable tree in agriculture and forestry, has an important potential. Development of a standard protocol both in

Table 6. The effects of BA on the rates of regeneration in the chestnut somatic embryos.

Application	Root reg. (%)		Shoot reg. (%)		Plant reg. (%)	
Control	0.0	b*	0.0	b	0.0	b
BA	0.0	b	8.0	b	0.0	b
BA + NAA	**22.0**	**a**	**24.0**	**a**	**22.0**	**a**
BA + IBA	0.0	b	0.0	b	0.0	b

BA (0.1 mg/L), IBA (0.1 mg/L), NAA (0.1 mg/L). *Different letters indicate significant differences between treatments.

Table 7. The effects of BA + NAA with pre-application on the rates of the regeneration in the chestnut somatic embryos.

Application		Root reg. (%)	Shoot reg. (%)	Plant reg. (%)	Plantlet/ Emb.**	Root reg. (%)	Shoot reg. (%)	Plant reg. (%)	Plantlet/ Emb.**
		Control BA (0.0 mg/L) + NAA (0.0 mg/L)				BA (0.1 mg/L) + NAA (0.1 mg/L)			
Desicc.	**(-)**								
Cold	**(-)**	0.0 c*	0.0 c	0.0 c	0/45	**20.0 b**	**20.0 b**	**20.0 b**	**9/45**
GA_3	**(-)**								
Desicc.	**(+)**								
Cold	**(-)**	0.0 c	0.0 c	0.0 c	0/45	**40.0 a**	**40.0 a**	**40.0 a**	**18/45**
GA_3	**(-)**								
Desicc.	**(+)**								
Cold	**(+)**	0.0 c	0.0 c	0.0 c	0/45	**40.0 a**	**40.0 a**	**40.0 a**	**18/45**
GA_3	**(-)**								
Desicc.	**(+)**								
Cold	**(-)**	0.0 c	0.0 c	0.0 c	0/45	**28.8 a**	**28.8 a**	**28.8 a**	**13/45**
GA_3	**(+)**								

*Different letters indicate significant differences between treatments ($P = 0.000$). **The number of the plantlet/embryo.

classical and *in vitro* production, and emphasizing its results with repetitive and diverse trials has an importance in terms of time and economy. The present study was conducted for the optimization of an *in vitro* propagation protocol for the two early maturing cultivars of European chestnut, Hacıibiş and Karamehmet.

In the literature, varying results have been reported for the somatic embryogenesis and regeneration in chestnut [4] [12] [17]-[27]. In this study, the somatic embryogenesis rates in the Hacıibiş and Karamehmet cultivars were found to be 6.0% - 9.9% and 9.2% - 11.1%, respectively. These results are similar to those of Vieitez *et al*. [20], and higher than the findings of Carraway and Merkle [22], Xing *et al*. [23] and Corredoira *et al*. [16]. The better results might be due to the utilization of DKW medium as well as its optimum combination with PGRs, L-glutamine and $AgNO_3$.

The transformation of somatic embryos into plants for chestnut genotypes is a limiting step in all *in vitro* culture systems [4] [17] [25]. The need for pre-germination applications such as cold, partial desiccation, and GA_3 to overcome dormancy in many kinds of chestnut somatic embryos has been a demonstrated [4] [12] [17] [27] [28]. The effect of desiccation was exhibited in our study, especially when it was used together with BA + NAA supplemented medium. Desiccation plays a role in breaking dormancy in mature embryos [29], and stimulates the necessary programs for maturation and germination [30] as well as it decreases internal ABA levels, changes the sensitivity towards ABA, and increases the ability of embryos to germinate [31]. The GA_3 stimulates regeneration of somatic embryos via activation of hydrolytic enzymes such as α-amylase and reactivation of growth activity [32]. Corredoira *et al*. [17] also state the advantage of BA utilization increasing shoot growth in axial meristems. Similarly, we showed that the supplementation of the regeneration media with BA + NAA PRGs facilitated the formation of root, shoot, and recovery of plantlets (**Table 6**).

In this report, 22% - 24% plantlets were obtained via PGR application, which increased to 28.0% - 40.0% when using pre-applications such as desiccation, cold and GA_3 (**Table 7**). Moreover, BA + NAA treatments were found to be essential to achieve plantlet regeneration, irrespective of the type of pre-treatment applied (**Table 7**). Corredoira *et al*. [17] obtained a regeneration percentage of 23% with BA + NAA application for somatic embryos in the European chestnut which were cold-treated at 4˚C for 8 wk and desiccated in laminar flow cabinet for 2 h. Similarly, Carraway *et al*. [22] achieved 23% regeneration using BA + NAA applications on somatic embryos that were treated with cold at 4˚C for 12 wk and desiccated in a cabin with laminar air flow for 2 h. Moreover, Andrade and Merkle [25], using American chestnut, have provided regeneration of 47% rate when cooling at 4˚C for 0, 6, and 12 wk in the somatic embryos. However, Robichaud *et al*. [24] succeeded in germination of same type of somatic embryos at 8.78% using cold treatment (4˚C) for 4 wk. In this report, a 40% rate was obtained for the somatic embryo germination on medium supplemented with BA+NAA when desiccation was pre-applied. Utilization of desiccation, GA_3, and cold treatments on the medium supplemented with BA + NAA resulted in plant regeneration from somatic embryos. Although the germination rates obtained with desiccation only and desiccation plus cold or GA_3 was found to be significant, application of cold or GA_3 together with desiccation did not increase the rate. However, Corredoira *et al*. [17] reported that the highest level of regeneration was observed using the media with BA (0.1 mg/L) + NAA (0.1 mg/L) and the cold application.

5. Conclusion

Desiccation treatment together with BA + NAA supplementation to the medium was shown to be optimum condition for the somatic embryo formation and regeneration of the chestnut early cultivars Hacıibiş and Karamehmet. The *in vitro* somatic embryogenesis is an important technique for propagation of the early cultivars and development of novel genotypes.

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Supplementary Table

Table 1. Murashige and Skoog (MS), Driver and Kuniyuki Walnut Medium (DKW) and Woody Plant Medium (WPM) media content.

(A) MACRO ELEMENTS	g/mol	MS Medium	DKW Medium	WPM Medium
		mg/L	mg/L	mg/L
Ammonium nitrate (NH_4NO_3)	80.04	1650	1416	400
Potassium nitrate (KNO_3)	101.11	1900	-	-
Calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$)	147.02	440	149	96
Calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$)	236.15	-	1968	-
Zinc nitrate hexahydrate ($Zn(NO_3)_2{\cdot}6H_2O$)	297.48	-	26.68	-
Potassium sulfate (K_2SO_4)	174.27	-	1559	-
Potassium dihydrogen phosphate (KH_2PO_4)	136.09	170	265	-
Magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$)	246.48	370	740	370
(B) FERROUS				
Sodium-ferrous EDTA ($C_{10}H_{12}N_2FeNa$)	367.1	36.7	-	-
Sodium EDTA (Na_2EDTA)	372.24	-	45.4	37.3
Ferrous sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$)	278.02	-	33.8	27.8
(C) MICRO ELEMENTS				
Manganese sulfate monohydrate ($MnSO_4{\cdot}H_2O$)	169.02	16	33.4	22.3
Zinc sulfate heptahydrate ($ZnSO_4{\cdot}7H_2O$)	287.54	8.6	-	8.6
Nickel sulfate hexahydrate ($NiSO_4{\cdot}6H_2O$)	262.90	-	0.05	-
Boric acid (H_3BO_3)	61.83	6.2	4.8	6.2
Potassium iodide (KI)	166.01	0.83	-	-
Potassium dihydrogen phosphate (KH_2PO_4)	136.09	-	265.0	-
Sodium molybdate dihydrate ($Na_2MoO_4{\cdot}2H_2O$)	241.95	0.25	0.39	0.25
Copper sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$)	249.68	0.025	0.25	0.25
Cobalt chloride hexahydrate ($CoCl_2{\cdot}6H_2O$)	237.9	0.025	-	-
(D) VITAMINS				
Myo-Inositol ($C_6H_{12}O_6$)	180.16	100	100	100
Glycine ($C_2H_5NO_2$)	75.07	2	2	2
Thiamin chloride hydrochloride ($C_{12}H_8C_{l2}N_4OsxH_2O$)	337.27	0.1	2	1
Nicotinic acid ($C_6H_5NO_2$)	123.11	0.5	1	0.5
Pridoksol hydrochloride ($C_8H_{12}ClNO_3$)	205.64	0.5	-	0.5