



Induction of Somatic Embryogenesis from Immature Cotyledons in 'Tombul' Hazelnut

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Abstract: Effects of 6-benzyladenine (BA) in combination with auxins either 2,4-dichlorophenoxyacetic acid (2,4-D) or α -naphthaleneacetic acid (NAA) on the induction of somatic embryogenesis in different cotyledon maturity stages of open-pollinated seeds of 'Tombul' hazelnut cultivar were investigated. Immature cotyledon explants were collected 45 and 30 days before harvest on July 1 and July 15 respectively. Initial Murashige and Skoog (MS) basal medium with half-strength of macronutrients containing 1.0 mg L⁻¹ BA, 0.5 mg L⁻¹ NAA and 250 mg L⁻¹ L-glutamine resulted in the highest embryogenesis (66.7%) and the highest number of somatic embryo (5.5) per cotyledon explant. The cotyledons collected 45 days before the harvest were more inductive than those collected 30 days before the harvest on embryogenesis.

Key Words: *Corylus avellana*, somatic embryo, cotyledon maturity, 6-benzyladenine, 2,4-dichlorophenoxyacetic acid, α -naphthaleneacetic acid

'Tombul' Fındık Çeşidinde Olgunlaşmamış Kotiledonlardan Somatik Embriyo Oluşumu

Öz: Açıkta tozlanan 'Tombul' fındık çeşidinde, 6-benziladenin' in (BA), 2,4- diklorofenoksiasetik asit (2,4-D) ve Naftalenasetik asit (NAA) ile kombinasyonlarının ve kotiledon olgunlaşma düzeyinin somatik embriyo oluşumu üzerine etkileri incelenmiştir. Olgunlaşmamış kotiledon eksplantları hasattan 45 gün (1 Temmuz) ve 30 gün (15 Temmuz) önce alınmıştır. En yüksek somatik embriyo oluşumu (%66.7) ve eksplant başına en yüksek somatik embriyo sayısı (5.5) 1.0 mg·L⁻¹ BA, 0.5 mg·L⁻¹ NAA ve 250 mg·L⁻¹ L-glutamin ilave edilmiş ½ makro element içeren Murashige ve Skoog (MS) temel besin ortamından elde edilmiştir. Somatik embriyo oluşumu üzerine hasattan 45 gün önce alınan kotiledonların 30 gün önce alınanlara göre daha etkili olduğu belirlenmiştir.

Anahtar Kelimeler: *Corylus avellana*, somatik embriyo, kotiledon olgunluğu, 6-benziladenin, 2,4-diklorofenoksi asetik asit, α -naftalenasetik asit

Introduction

The hazelnut (*Corylus avellana* L.) is one of the world's major nut crops, and Turkey has long been the leading producer and exporter of hazelnut (Thompson et al. 1996). Turkey covered 73.7% of world hazelnut production (848,000 tons) and 84.7% of export (600,000 tons) in 2008 (Hazelnut Council 2008). The main hazelnut cultivar in Turkey is 'Tombul', which contributes 25–30 % of the total production. It is mainly grown in Giresun province and neighboring provinces along the Black Sea coast and the nuts are classified as Giresun (or Premium) quality (Alasalvar et al. 2003). This cultivar has been famous for centuries due to its distinctive taste, aroma, high oil content (~61%), and easily and quickly removable brown skin during roasting. Therefore, there is high demand for Giresun

quality 'Tombul' hazelnut in local markets and in exportation. 'Tombul' is a partly self-compatible cultivar that the percent cluster set could be as high as 44% (Mehlenbacher and Smith 1991). This is a valuable trait in orchards where the conditions for pollination are not in favor.

Somatic embryogenesis from seed parts could be useful tool for clonal propagation and cryopreservation of derived hybrid and apomictic seeds from a breeding program using biotechnological or conventional techniques in fruit and nut crops. Generally, somatic embryos which are genetically identical and often pathogen-free, could be used for mass propagation and genetic transformation

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(Tulecke and McGranahan 1985, Gray 1987), germplasm preservation and a source of protoplasts (Boucaud et al. 1994, Merkle 1997, Jimenez 2001), derivation of somaclonal variants, production of alkaloids, *in vitro* screening and selection, and basic biochemical, physiological and morphological studies, (Prece 2003, McCown 2003, Hiraoka et al. 2004, Klimaszewska et al. 2005, D'Onofrio and Morini 2006, Traore and Guiltinan 2006). However, for these applications to be technically and economically feasible, it is essential to optimize the system variables to obtain high multiplication rates of quality embryos (Traore and Guiltinan 2006).

Previously, somatic embryogenesis from zygotic embryo tissues has been achieved in hazelnuts. As in other fruit and nut crops, the embryogenic potential of the explants was dependent on genotype, source and developmental stage of explants, salt formulation and combinations of growth regulators in basal medium (Perez et al. 1983, Thompson et al. 1996, Centeno et al. 1997, Rodriguez et al. 2000). Endogenous hormonal content and somatic embryogenic capacity of cotyledons collected in August (immature) and in September (mature) from 'Casina' and 'Negretta' which are late maturing hazelnut cultivars compared to Turkish cultivars were investigated by Centeno et al. (1997). In general, MS (Murashige and Skoog 1962) or T (Tulecke and McGranahan 1985) basal medium, and BA and/or kinetin as cytokinins, and IBA or 2,4-D as auxins were used in somatic embryogenesis studies. Among the auxins, the most frequently used was 2,4-D (49%) followed by NAA (27%), indole-3-acetic acid (IAA) (6%), IBA (6%), Picloram (5%) and Dicamba (5%) in plants (Jimenez 2001). The effects of NAA on induction of somatic embryos in hazelnut have not been investigated to our knowledge.

The objective of this study was to improve somatic embryogenesis from cotyledons of open-pollinated immature seeds by identifying the best stage for cotyledon maturity and the appropriate growth regulator combinations of cytokinin (BA) with auxins (2,4-D and NAA) on MS basal medium in hazelnuts.

Materials and Methods

Plant material: Immature cotyledons of open-pollinated seeds of 'Tombul' hazelnut were used as explant source. 'Tombul' is early maturing cultivar compared to European cultivars. Thus, developing immature seeds were collected on July 1 and 15 in 2001 and 2002, from the hazelnut collection plot

maintained at the Hazelnut Research Institute, Giresun, Turkey. The sampling dates are approximately 45 and 30 days before the harvest date, respectively. In hazelnuts, pollination period begins at about early December and continues through several weeks. Thus, we did not use "weeks after anthesis" to define the precise maturity level of cotyledons in the open-pollinated trees. The seeds were cracked out by hand and surface sterilized by immersion in a solution of sodium hypochlorite (3% active chlorine) for 25 min and rinsed three times with sterile distilled water. Seed coat and embryonic axes of the seeds were removed under aseptic conditions, and cotyledon explants were placed on the initial medium in petri dishes (100 x 10 mm).

Media and culture conditions: The MS basal medium (Murashige and Skoog 1962) with half-strength of macronutrients, containing 30 g L⁻¹ sucrose and 7 g L⁻¹ Oxoid agar was used in the experiments. For initial cultures, the basal medium was supplemented with BA (0.0, 1.0 or 2.0 mg L⁻¹) in combination with 2,4-D or NAA (0.0, 0.1, 0.5, 1.0 or 2.0 mg L⁻¹) and 250 mg L⁻¹ L-glutamine. All of the plant growth regulators were added to media before autoclaving at 121 °C for 20 min. The pH was adjusted to 5.7 before adding agar, sucrose and autoclaving. The explants were cultured on the initial medium for 4 weeks and then subcultured two times at 4 weeks intervals on a MS basal medium without plant growth regulators and L-glutamine. All of the cultures were incubated at 25 °C in the dark. The number of explants that formed somatic embryos and the number of somatic embryos per explant were determined at the end of the subcultures.

Experimental design and statistical analysis: The experiments were conducted according to completely randomized design with factorial combinations of sampling date, BA concentration, and auxin type and concentration. Each treatment consisted of four dishes with five explants per dish. Statistical analyses were performed using analysis of variance (ANOVA) in Minitab software (MINITAB Inc.). The means were separated by Duncan's multiple range test ($P \leq 0.05$). The percent data was transformed into angle values prior to analysis.

Results and Discussion

The immature cotyledons collected on July 1 were more embryogenic than the cotyledons collected on July 15 (Fig. 1) and resulted in higher number of embryos per cotyledon explant (Fig. 2). However, the the interactions including sampling date such as

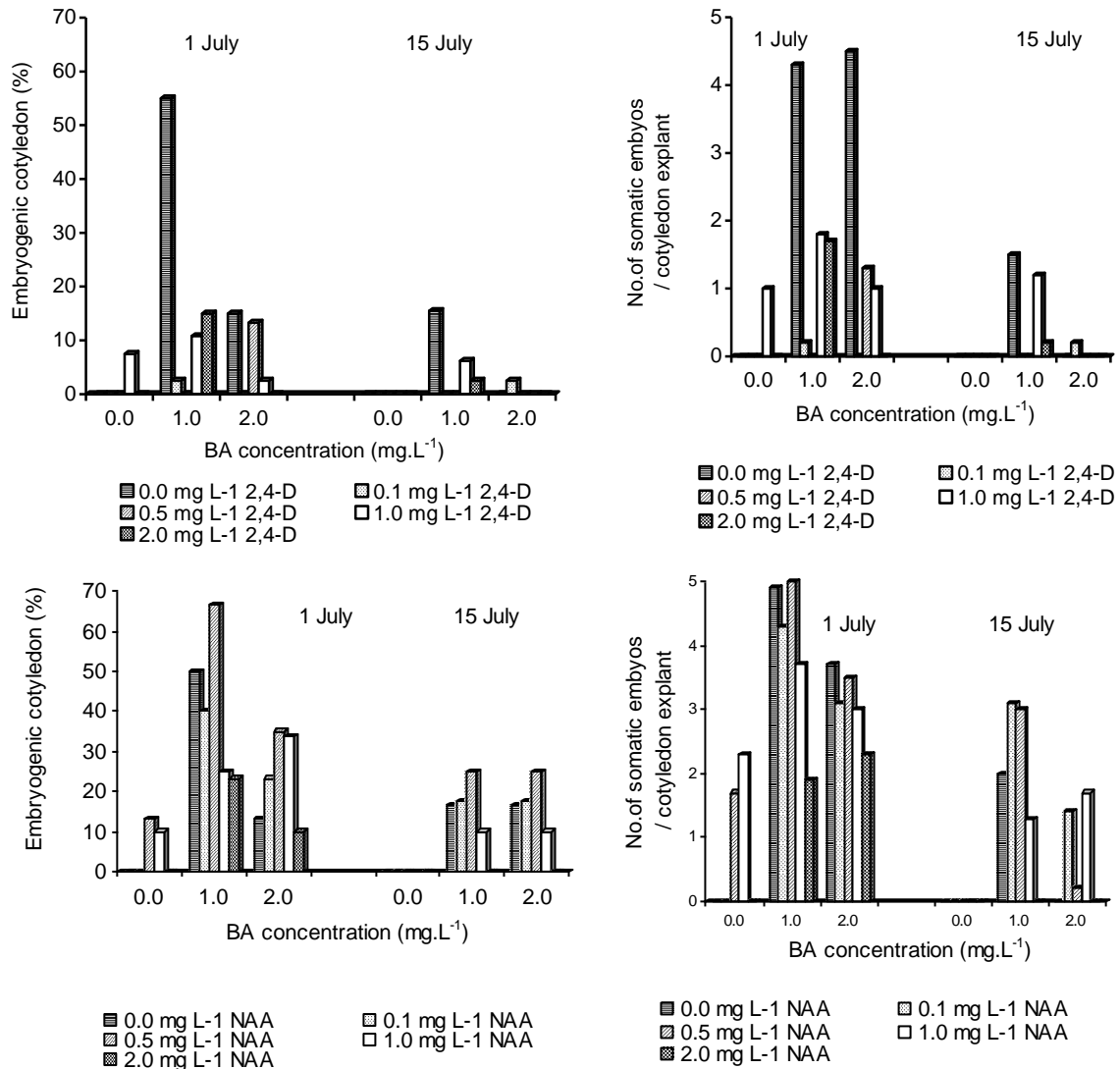


Figure 1. Effect of 2,4-D (above) and NAA (below) with BA on induction of somatic embryogenesis across two sampling dates on July 1 and 15 in 'Tombul' hazelnut.

Figure 2. Effect of 2,4-D (above) and NAA (below) with BA on number of somatic embryo per cotyledon explant across two sampling dates on July 1 and 15 in 'Tombul' hazelnut.

a-- sampling date x BA concentration x auxin type x auxin concentration

b) - sampling date x BA concentration x auxin type

c) - sampling date x auxin type x auxin concentration were not significant (Table 1). This is likely due to short time interval between sampling dates. Centeno et al. (1997) investigated the endogenous plant growth regulator content of cotyledons in different

developmental stages in immature (August 5) and mature (September 8) hazelnut seeds. They did not find a significant difference between the ABA content of cotyledons neither at early nor late developmental stages, but the IAA content of cotyledons at early stage was higher than that of the cotyledons at late stage. They also found similar total cytokinin content at both collection dates, although the ratio of iP-type/Z-type cytokinins was very different.

Table 1. Effect of sampling date, and combinations of BA with auxins (2,4-D or NAA) on the induction of somatic embryogenesis from immature cotyledons in 'Tombul' hazelnut

Plant Growth Regulator Concentration	45 days before harvest		30 days before harvest		
	Cotyledon explants with somatic embryos (%)	No. of somatic embryo / cotyledon explant	Cotyledon explants with somatic embryos (%)	No. of somatic embryo / cotyledon explant	
Control	0 g*	-**	0 d	-	
BA	1 mg L ⁻¹	52.5 ab	4.6 ab	16.6 ab	2.7 bc
	2 mg L ⁻¹	13.3 cdef	3.7 abc	0 d	-
2,4-D	0.1 mg L ⁻¹	0 g	-	0 d	-
	0.5 mg L ⁻¹	0 g	-	0 d	-
	1 mg L ⁻¹	7.5 efg	2.0 cd	0 d	-
	2 mg L ⁻¹	0 g	-	0 d	-
NAA	0.1 mg L ⁻¹	0 g	-	0 d	-
	0.5 mg L ⁻¹	13.3 defg	2.5 bcd	0 d	-
	1 mg L ⁻¹	10.0 defg	3.5 abc	0 d	-
	2 mg L ⁻¹	0 g	-	0 d	-
1 mg L ⁻¹ BA	0.1 mg L ⁻¹ 2,4-D	2.5 fg	1.0 d	0 d	-
	0.5 mg L ⁻¹ 2,4-D	0 g	-	0 d	-
	1 mg L ⁻¹ 2,4-D	10.8 defg	2.8 bcd	6.3 bcd	2.4 cd
	2 mg L ⁻¹ 2,4-D	15.0 defg	2.5 bcd	2.5 cd	1.00 d
	0.1 mg L ⁻¹ NAA	40.0 abc	4.3 abc	17.5 ab	4.1 ab
	0.5 mg L ⁻¹ NAA	66.7 a	5.5 a	25.0 a	4.5 a
	1 mg L ⁻¹ NAA	25.0 bcde	3.8 abc	10.0 abc	2.0 cd
	2 mg L ⁻¹ NAA	23.3 bcde	1.9 cd	0 d	-
2 mg L ⁻¹ BA	0.1 mg L ⁻¹ 2,4-D	0 g	-	2.5 cd	1.0 d
	0.5 mg L ⁻¹ 2,4-D	13.3 defg	2.0 cd	0 d	-
	1 mg L ⁻¹ 2,4-D	2.50 fg	4.0 abc	0 d	-
	2 mg L ⁻¹ 2,4-D	0 g	-	0 d	-
	0.1 mg L ⁻¹ NAA	23.3 bcde	3.1 bcd	10.0 abc	2.8 bc
	0.5 mg L ⁻¹ NAA	35.0 bcd	4.7 ab	5.0 cd	1.0 d
	1 mg L ⁻¹ NAA	33.7 bcd	4.0 abc	18.8 ab	2.3 cd
	2 mg L ⁻¹ NAA	10.0 defg	3.5 abc	0 d	-

* Means followed by the same letter within each column are not significantly different at P ≤ 0.05

** No detection

The interaction among BA concentration x auxin type x auxin concentration for both the percentage of embryogenic cotyledons and the number of embryo per cotyledon explant was significant, ($P = 0.001$ and 0.005 , respectively) (Table 1).

There was very low induction of somatic embryos in the absence of BA in the medium containing auxins (Fig 1). However, 1.0 mg·L⁻¹ BA alone resulted in 52.5% and 16.6% of embryogenic cotyledon as an average of two sampling dates (Table 1). The embryogenic potential of explants is closely associated with their contents of both natural and exogenously applied plant growth regulators (PGRs) (Centeno et al. 1997). In practice, the initiation of embryogenic cells requires *in vitro* culture of appropriate explant on a medium that contains specific PGRs such as auxin and cytokinin-like (Gray 1987). In particular, the auxin to cytokinin ratio appears to be the most important factor in embryo induction (D'Onofrio and Morini 2006). To induce somatic embryogenesis in the plants, BA was the most effective and commonly used cytokinin (57%), followed by kinetin (37%), zeatin (Z) (3%) and thidiazuron (3%) (Gray 1987, Rodriguez et al. 2000, Jimenez 2001). In hazelnut, the use of iP type cytokinin was suggested by Centeno et al. (1997) for the stimulation of cell division prior to somatic embryogenesis.

The percentage of embryogenic cotyledons was the highest (66.7%) when 1.0 mg L⁻¹ BA plus 0.5 mg L⁻¹ NAA for 4 weeks was used (Fig 1) in the first cotyledon sampling date. In general, NAA induced more somatic embryos than 2,4-D (Fig 2). Concentrations of 0.1 – 1.0 mg L⁻¹ NAA plus 1.0 and 2.0 mg L⁻¹ BA gave over 15% cotyledon explants with somatic embryos (Table 1). Rodriguez et al. (2000) reported that indirect embryogenesis was obtained on 2,4-D (0.02 – 0.2 mg·L⁻¹) in combination with kinetin (0.02 – 0.2 mg L⁻¹) and/or BA (0.02 mg L⁻¹). However, we found that concentrations of 2,4-D (0.1, 0.5, 1.0 and 2.0 mg L⁻¹) gave lower (0.0% - 15.0%) cotyledon explants with somatic embryos in presence of BA in the medium. In addition to cytokinins, auxins are also required to induce the formation of embryogenic cells possibly by initiating differential gene activation, and appear to promote an increase of embryogenic cell populations through repetitive cell division while simultaneously suppressing cell differentiation and growth into embryos (Gray 1987). Among the auxins Rodriguez et al. (2000) suggested the use of IBA for direct and 2,4-D for indirect somatic embryogenesis in hazelnuts. While NAA is the second most frequently used growth regulator for somatic embryogenesis, however, there is no information on the use of NAA in hazelnuts.

The number of embryo per embryogenic cotyledon explant on medium without BA or BA plus 2,4-D was low in general (Table 1). However, number of somatic embryos was higher on medium containing BA alone and BA plus NAA than those of the other combinations. Plant growth regulator combinations including 0.5 mg L⁻¹ NAA had significantly higher number of embryos than that of the others. The

highest number of somatic embryo (5.5 and 4.5 / explant) were obtained from immature cotyledons collected on July 1 and July 15, respectively, at 1 mg L⁻¹ BA plus 0.5 mg L⁻¹ NAA combination. Auxins of 2,4D and NAA did not result in somatic embryo formation on immature cotyledons collected on July 15 when they were used alone. Our results are similar to that of Berros et al. (1997) in which number of somatic embryo per embryogenic cotyledon explant ranged from 1.5 to 4.2. Actual PGR concentration is important for an optimum response because the concentrations that are too low may not trigger the inductive events, and concentrations that are too high, particularly when considering phenoxy-auxins, may become toxic (Gray 1987). Perez et al. (1983) achieved embryogenesis in 60% of the explants over two 20-day culture steps in the presence of IBA (1.0 mg L⁻¹) plus BA (0.1 mg L⁻¹) and IBA (0.1 mg L⁻¹) plus BA (1.1 mg L⁻¹).

As a conclusion, immature cotyledons of 'Tombul' hazelnut collected at early developmental stages, especially 45 days before harvest maturity, have high embryogenic potential. The use of NAA in initial medium, previously not reported in hazelnuts, induced embryogenesis more than 2,4-D in which the ratio of embryogenic cotyledons and the number of embryo per embryogenic cotyledon significantly increased. The highest values for both embryogenesis and number of embryo were obtained on the initial MS medium, with half-strength of macronutrients, containing 1.0 mg·L⁻¹ BA plus 0.5 mg·L⁻¹ NAA and 250 mg·L⁻¹ L-glutamine for 4 weeks, followed on basal medium without growth regulators and L-glutamine for 8 weeks. In this experiment, the subsequent proliferation for secondary embryogenesis on the basal medium was successfully maintained.

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