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Inclusion of Polyamines in the Medium Improves Shoot Elongation in Hazelnut (*Corylus avellana* L.) Micropropagation

Mehmet Nuri NAS
Kahramanmaraş Sütçü İmam University, Faculty of Agriculture, Department of Horticulture,
46100 Kahramanmaraş - TURKEY

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Abstract: Buds from newly developed shoots obtained from forced outgrowth of mature field-grown hybrid hazelnut trees (*Corylus avellana* L.) were cultured in vitro on Murashige and Skoog [MS (1962)] medium and a modified Driver and Kuniyuki [DKW (1984)] medium containing 6.7 µM, 11.1 µM or 15.5 µM of N-6 benzyladenine (BA) supplemented with or without a combination of polyamines (0.2 mM of putrescine + 0.2 mM of spermidine + 0.05 mM of spermine). The effects of culture medium and BA were found to be insignificant on explant response. Polyamines were found to have a strong effect on both shoot elongation and on number of buds per shoot. Polyamines stimulated mean shoot elongation by 83% and increased the mean number of buds per shoot by 41% compared to when they were not used. In the presence of polyamines, shoot elongation continued up to 4.0 cm while in the absence of polyamines shoot elongation only reached 2.0 cm. Results indicate that polyamines in the culture medium could ease the establishment of cultures and enhance the morphogenic capacity of mature explants.

Key Words: *Corylus avellana*, culture establishment, putrescine, shoot length, spermidine, spermine.

Fındık (*Corylus avellana* L.) Mikro Çoğaltımında Ortama İlave Edilen Poliaminler Sürgün Uzunluğunu Arttırmaktadır

Özet: Bu çalışmada bahçede büyüyen, verime yatmış melez fındık (*Corylus avellana* L.) bitkilerinden alınan dormant çelikler sürme çözeltisine konulmuş ve çeliklerin sürmeleri sağlanmıştır. Dormant çeliklerin sürmesi sonucu meydana gelen taze sürgünlerden alınan gözler 6.7 µM, 11.1 µM veya 15.5 µM N-6 benziladenin (BA) ile poliaminler (0.2 mM putresin + 0.2 mM spermidin + 0.05 mM spermin) içeren veya içermeyen Murashige ve Skoog [MS (1962)] ortamı ve modifiye Driver ve Kuniyuki [DKW (1984)] ortamı üzerine kültüre alınmıştır. Kültür ortamı ile BA'nın eksplant reaksiyonları üzerine olan etkileri önemsiz bulunmuştur. Ortama ilave edildiklerinde, poliaminlerin sürgün uzunluğu ve sürgün başına tomurcuk sayısı üzerine çok etkili oldukları gözlenmiştir. Ortama ilave edildiklerinde, poliaminlerin ortalama sürgün uzunluğunu %83 ve ortalama sürgün başına tomurcuk sayısını %41 arttırdığı gözlenmiştir. Poliamin içeren ortam üzerindeki bazı sürgünlerin 4.0 cm'ye kadar, poliamin içermeyen ortam üzerindeki sürgünlerin ise en fazla 2.0 cm'ye kadar büyüyebildikleri gözlenmiştir. Bulgular otama ilave edildiklerinde poliaminlerin eksplantların kültüre alınmalarını kolaylaştırabileceğini ve morfojenik kapasitelerini arttırabileceğini göstermiştir.

Anahtar Sözcükler: *Corylus avellana*, kültüre alma, putrescine, sürgün uzunluğu, spermidine, spermine

Introduction

Hazelnut is one of the most important nut crops (Mehlenbacher, 1994). Since its fruits are used widely in confectionery and cosmetics, many countries have promoted hazelnut production to avoid the high cost of importing it to meet domestic demand. Recently, in addition to its known uses, hazelnut has been proposed as an important agroforestry crop that could be an alternative oil source to soybean (P. Rutter, personal com.). The increasing demand for hazelnut can only be met by a rapid distribution of standard or new cultivars selected from breeding programs.

Traditional hazelnut propagation techniques are time-consuming and are not satisfactory in commercial propagation. Micropropagation has been considered as an alternative to traditional hazelnut propagation techniques. Micropropagation will allow rapid propagation and distribution of elite lines on a commercial scale (Diaz-Sala et al., 1990a; Yu and Reed, 1995). It will also be important for a rapid increase of

* Correspondence to: mnurinas@ksu.edu.tr

new cultivars or clones resistant to eastern filbert blight (*Anisogramma anomala*), which is of great concern in the U.S. (Mehlenbacher, 1994) and may pose a threat to Europe in the future (Sansavini, 1994).

Proven mature hazelnut tissues are the best choice for clonal propagation. However, unlike juvenile tissues, mature tissues have reduced morphogenic capacity and they are recalcitrant to in vitro culture. Low initiation and multiplication rates, insufficient shoot elongation and high rates of microbial contamination are the main factors limiting the success of micropropagation of selected adult trees (Diaz-Sala et al., 1990a; Yu and Reed, 1995).

In order to minimize the recalcitrance problem, the most juvenile tissues (e.g., basal sprouts) from adult trees are used and source plant materials are subjected to rejuvenation treatments (Bhojwani and Razdan, 1996; Diaz-Sala et al., 1994). The main objective of treatments applied to source plant material is to increase the hormonal (growth regulator) content of explants to levels similar to those in juvenile tissues.

In plants, polyamines are involved in a wide range of important processes including cell division, protein synthesis and DNA replication, and play important roles in various morphogenic responses (Bais and Ravishankar, 2002). In general, polyamines are found in higher amounts in juvenile tissues than in mature ones. Using mature, forced-outgrowth, and micropropagated plant material, Rey et al. (1994) found levels of polyamines to be higher in juvenile tissues than in adult ones. High levels of free putrescine were correlated with the ease of culture establishment observed with juvenile tissues. Adult tissues containing low levels of putrescine were difficult to establish in tissue culture (Diaz-Sala et al., 1990b). Furthermore, it has been reported (Bais et al., 2000) that exogenous application of polyamine (putrescine) may reduce the production of unwanted ethylene and enhance morphogenesis. Therefore, the objective of this study was to explore whether the morphogenic capacity of mature hazelnut tissues could be increased by the inclusion of polyamines into the culture medium.

Materials and Methods

Plant material

Dormant twigs of field grown unpruned mature hybrid hazelnut genotypes [E-093-S, E-295-S, G-029-N (8 to 10-year-old stock plants)] were used as the explant source. These hybrid hazelnut genotypes were developed by pollinating female European hazelnuts (*Corylus avellana* L.) with wild American hazelnuts (*C. americana* Marsh.) resistant to eastern filbert blight (pers. com. P. Rutter, Badgersett Research Farm, Canton, MN, USA). Twigs, 35-45 cm long, were surface disinfested with 15% (v/v) commercial bleach (0.78% sodium hypochlorite) plus 10 drops of Tween 20 l^{-1} for 15 min and rinsed under running tap water for 5 min. After surface disinfestation, both the apical and basal 0.5-1.0 cm ends of twigs were cut. Axillary bud outgrowth was forced by immersing the basal end of twigs in forcing solution (Read and Yang, 1987) containing 8-hydroxyquinoline citrate at 200 mg l^{-1} + 2% (w/v) sucrose + gibberellic acid (GA_3) at 10 mg l^{-1}. The basal 0.5 cm ends of the cuttings were pruned and the solutions were replaced twice a week. Newly developed shoots were harvested within 2 to 3 weeks when they had 3 or more leaves.

After defoliation and stipule removal, new shoots were surface disinfested with 70% (v/v) ethanol for 5 min, rinsed once with sterile distilled deionized water for 5 min, soaked in 12% (v/v) bleach + 10 drops of Tween 20 l^{-1} for 15 min and rinsed with 70% (v/v) ethanol for 30 s. This was followed by three 5-min rinses in sterile distilled deionized water.

Tissue cultures were initiated using explants consisting of apical shoot tips and nodal shoot segments containing 1 or 2 axillary buds. Single explants were cultured in baby food jars (135 mm^3) containing 30 ml of medium. The experiment was a completely randomized design with factorial treatments (medium, genotype, polyamine and BA). For each genotype, the experiment was started with at least 6 replications. To reduce oxidation, cultures were kept in darkness for the first 20-24 h (Bonga and Aderkas, 1992). The cultures were maintained at 23 ± 1 °C under cool white fluorescent light (28 $\mu mol s^{-1} m^{-2}$) for 16 h per day.

Culture medium

For culture establishment the following culture media were used:

MS1: Murashige and Skoog (1962) salts and vitamins supplemented with 22.2 µM of N-6 benzyladenine (BA) + 0.04 µM of indole-3-butyric acid (IBA) + the following

polyamines: 0.4 mM of putrescine + 0.4 mM of spermidine and 0.1 mM of spermine.

MS2: MS1 with no polyamines.

DKW1: Driver and Kuniyuki (1984) medium as altered by Yu and Reed (1995) and supplemented with plant growth regulators and polyamines as in MS1 medium.

DKW2: DKW1 with no polyamines.

Glucose at 30 g l^{-1} was used as the carbon source (Yu and Reed, 1993). After the pH was adjusted to 5.8, 6 g l^{-1} of Difco Bacto-Agar was added to the medium. The media were autoclaved at 121 °C and 1.4 kg cm^{-2} for 25 min. If polyamines were to be added to a medium, the medium was first autoclaved, and then filter (0.2 µm) sterilized polyamines were added to the medium when it became lukewarm.

Explants that survived initial culture for 30 days and were not contaminated were cut into single node explants containing an axillary bud. For multiplication explants were cultured on the same initiation culture medium with 3 BA concentrations (6.7 µM, 11.1 µM and 15.5 µM). In the multiplication medium if polyamines were to be added to a medium their concentrations were reduced to half of that used in the establishment culture (putrescine = 0.2 mM, spermidine = 0.2 mM, spermine = 0.05 mM). After 30 days explants were recultured on fresh multiplication medium.

Statistical analysis

Following two 30-day culture periods on multiplication medium, data (shoot length and numbers of buds per shoot) were recorded and the experiment was terminated. Statistical analysis of data was performed using the SAS general linear model procedure (GLM; SAS Institute Inc., 2001). Medium, genotype, polyamine and BA were considered as fixed effects. Separation of treatment means was done by Fisher's LSD test at P = 0.05.

Results and Discussion

The initial responses of explants were tissue browning, contamination, bud growth, shoot elongation, and leaf expansion. Superficial and/or endogenous contamination was the main factor limiting culture establishment. Explant contamination started after the fourth day of culture initiation and persisted until the third subculture. Contamination frequency varied from 30 to 90%, depending on genotype. Because of high contamination rates genotype E-093-S was excluded. Apical shoot tips usually had lower contamination rates compared to the lower portion of the stems. However, shortly after disinfestation and excision most of the apical shoot tip explants died. Another limiting factor in culture establishment was browning of explants. The explants that were not contaminated turned from green to brown and subsequently died. Similar limitations in micropropagation of mature hazelnut have been reported by other researchers (Diaz-Sala et al., 1990a, Yu and Reed, 1995;), and are known to be common in most woody plants (Preece and Compton, 1991).

The general linear model for the effects of factors on shoot elongation and on number of buds per shoot is presented in Table 1. Analyzing the significant effect of genotype x medium on shoot elongation, DKW medium seemed to be preferable to MS medium (Figure 1). However, in general, shoot lengths and numbers of buds per shoot observed on both DKW medium (0.9 ± 0.1 cm shoot length and 2.2 ± 0.2 buds) and on MS medium (0.8 ± 0.1 cm shoot length and 2.0 ± 0.2 buds) were insufficient for practical use. The insufficient shoot lengths observed on DKW and MS media indicate that these 2 media are suboptimal for hazelnut micropropagation and that they need to be further improved.

By the end of the second culture period on multiplication medium, shoot elongation usually ceased and shoot tips showed necrosis. The discontinuity of shoot elongation and episodic growth are common problems in newly established woody plant cultures. It usually persists until explants are fully adjusted to the in vitro environment, which may take several subcultures to several years depending on the species, the age and the source of explants, and the culture medium (McCown and McCown, 1987). The response of explants was similar at different BA concentrations tested (Figure 2). During the course of the experiment only a limited number of explants displayed proliferation (data not shown). Thus, the potential multiplication rates were mainly determined by the number of buds per shoot that could be cut into single bud explants and recultured. The ineffectiveness of BA was probably because the tested concentrations were

Table 1. GLM analysis of the mean shoot length (S. Length) and number of buds per shoot (Buds). Gen.: Genotype, Med.: Medium, Pol.: Polyamine.

		Sum of Squares		F-value	
Source	DF	S. Length	Buds	S. Length	Buds
Gen.	1	0.64	2.05	1.64^{NS}	1.21**
Med.	1	0.39	1.15	0.98 ^{NS}	0.68 ^{NS}
Gen. x Med.	1	1.70	1.70	4.34**	1.00 ^{NS}
BA	2	0.03	1.81	0.00 ^{NS}	0.53 ^{NS}
Gen. x BA	2	0.07	2.54	0.09 ^{NS}	0.75 ^{NS}
Med. x BA	2	1.58	9.28	2.00 ^{NS}	2.74 ^{NS}
Gen. x Med. x BA	2	1.25	10.1	1.59 ^{NS}	2.98 ^{NS}
Pol.	1	3.71	11.6	9.44***	6.83**
Gen. x Pol.	1	0.00	0.92	0.00 ^{NS}	0.55 ^{NS}
Med. x Pol.	1	0.95	3.85	2.42 ^{NS}	2.27 ^{NS}
Gen. x Med. x Pol.	1	1.00	1.49	2.52 ^{NS}	0.88 ^{NS}
BA x Pol.	2	0.04	1.18	0.06 ^{NS}	0.35 ^{NS}
Gen. x BA x Pol.	2	0.45	1.54	0.57 ^{NS}	0.45 ^{NS}
Med. x BA x Pol.	2	0.40	2.16	0.50 ^{NS}	0.64 ^{NS}

, *: Significant at P = 0.05 and P = 0.005 levels of probability, respectively. ^{NS}: Not significant at P = 0.05 level of probability.

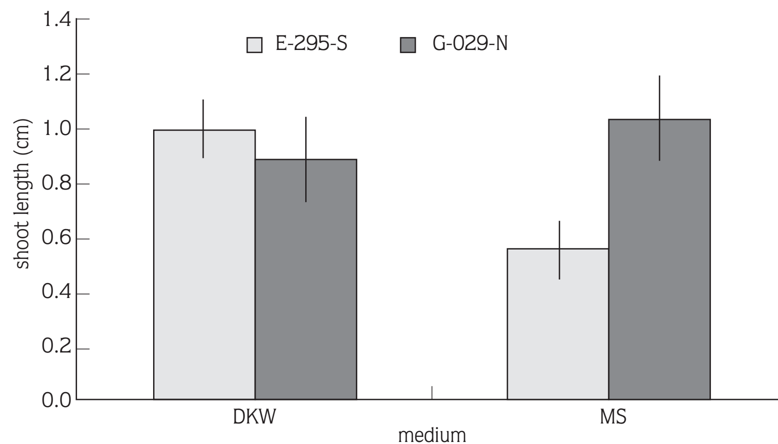

Figure 1. Shoot lengths of 2 hybrid hazelnut genotypes cultured on Driver and Kuniyuki (DKW) and Murashige and Skoog (MS) media. Means represent data across media with and without polyamines. Bars represent standard errors.

not high enough to stimulate cell division. Similar results were previously reported by Anderson (1983), who found BA to be ineffective in shoot multiplication at concentrations of 1.5, 2.5 and 5.0 mg l^{-1}.

Polyamines, however, were found to be very effective in both shoot elongation and in increasing the number of buds per shoot (Figure 3). Polyamines stimulated mean shoot elongation by 83% (0.6 ± 0.1 vs. 1.1 ± 0.1 cm)

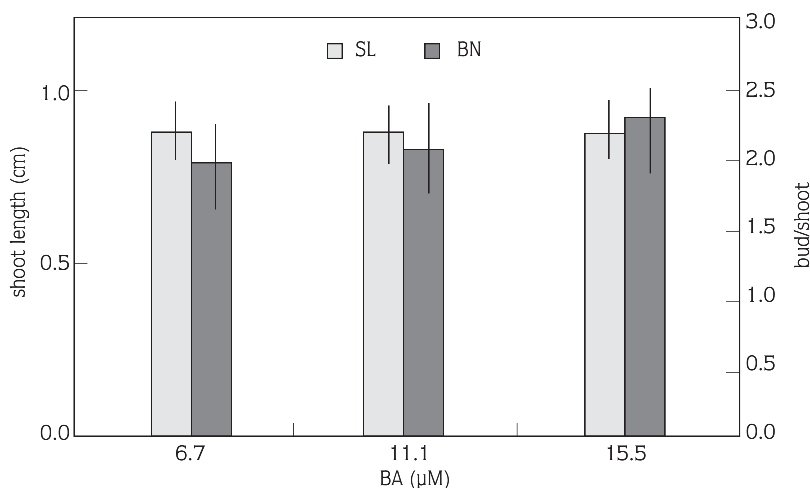

Figure 2. Mean shoot lengths (SL) and mean numbers of buds per shoot (BN) observed at different levels of BA. Means represent data across genotypes E-295-S and G-029-N cultured on Driver and Kuniyuki (DKW) and Murashige and Skoog (MS) media with and without polyamines. Bars represent standard errors.

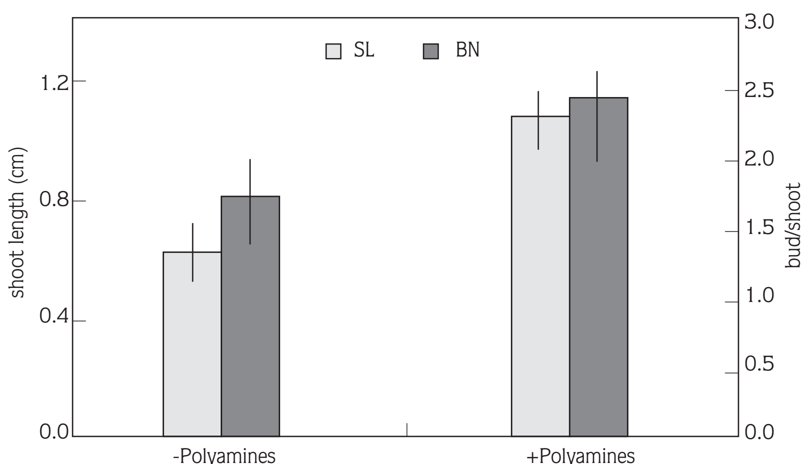

Figure 3. Mean shoot lengths (SL) and mean numbers of buds per shoot (BN) observed on medium with (+ polyamines) and without (- polyamines) polyamines. Means represent data across genotypes E-295-S and G-029-N cultured on Driver and Kuniyuki (DKW) and Murashige and Skoog (MS) media. Bars represent standard errors.

and increased mean number of buds per shoot by 41% (1.7 ± 0.2 vs. ± 2.5 ± 0.2 buds) compared to when they were not used. Only 14% of explants cultured on medium without polyamines underwent ≥1.0 cm shoot elongation while 36% of explants cultured on medium containing polyamines underwent ≥1.0 cm shoot elongation (data not shown). These results are in agreement with those of Diaz-Sala et al. (1990b) and Rey et al. (1994). The enhancing effects of polyamines on shoot elongation and on number of buds per shoot could be ascribed to their stimulatory effect on cell division (Bais and Ravishankar, 2002) and/or to their inhibitory effect on ethylene production (Bais et al., 2000).

Conclusion

The establishment of culture is one of the most difficult stages in the micropropagation of mature woody plants. In addition to high contamination and death rates, unsatisfactory growth usually limits the success of micropropagation studies. The results presented here indicate that polyamines in the culture medium could ease the establishment of cultures and enhance the morphogenic capacity of mature explants. Therefore, to increase potential multiplication rates and alleviate insufficient shoot length, we suggest the addition of these growth regulators into the culture medium.

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