Trakia Journal of Sciences, No 2, pp 162-168, 2014

Available online at:
http://www.uni-sz.bg

ISSN 1313-7050 (print)
ISSN 1313-3551 (online)

Original Contribution

HIGH-FREQUENCY DEVELOPMENT OF LEAF-DERIVED PRE-EMBRYOS IN HAZEL (*CORYLUS AVALLENA*)

C. Ebrahimi[1], M. Solouki[1], M. Omidi[2], M. Forootan[1*]

[1]Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Zabol, Zabol, Iran
[2]Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Tehran, Karaj, Iran

ABSTRACT

Common hazel (*Corylus avallena*) is extensively cultivated in North America, Northwest Asia and Europe; consumed both in raw and processed forms, as well as in pharmaceutical industries. The latter makes it a valuable species for *in vitro* propagation. Several studies have reported embryogenesis from different plant organs. This study is focused on producing embryos from petiole and leaf-derived callus cultures.Leaf ($1\times1cm^2$ segments) and petiole (1cm) cuttings were surface sterilized and subcultured in MS medium containing gradient combinations of 2,4-D+BAP, cultures refreshed every four weeks. Each growth regulator combination was replicated three times, with five cutting in each container. The calli were then moved to MS media (both solid and liquid forms) supplemented with gradient levels of 2,4-D+BAP, IBA+BAP, and IBA+Kin for embryogenesis stage; maintained under standard growth chamber conditions. The highest frequency of pre-embryo structure was obtained under 0.1 $mg.l^{-1}$ 2,4-D+0.1 $mg.l^{-1}$ BAP and 0.1 $mg.l^{-1}$ IBA+0.1 $mg.l^{-1}$ Kin for petiole and leaf cultures, respectively. Leaf cutting expressed significantly higher frequency of pre-embryo structure compared to petioles, where all samples expressed formation of pre-embryos. Reducing the nitrate level to 1/4-strength increased the pre-embryo formation, however embryo development was not observed in low-nitrate treatments. Except for BAP-based media, all other embryo-development media resulted in re-appearance of necrotic and/or non-differentiated callus tissue structure. It is therefore postulated that maintaining leaf cutting explants in BAP supplemented media is a promising method to obtain embryos from leaf-derived callus cultures. The feasibility of different approaches was discussed.

Keywords: *Corylus avallena*, pre-emryo, embryogenesis, callus

INTRODUCTION

Common hazelnut (*Corylus avellana L.*) is a nut species habituated in Northwest Asia (Turkey, Azerbaijan, Georgia, Iran), North America (USA), and Europe (Italy, Spain, France, Poland) (1, 2). The nut is popular for the edible dried kernel; not to mention the kernel is extensively used worldwide in chocolate industry, confectionary, bakery, and food products manufacture. Further to kernel consumption, hazel leaves are also consumed as oedema as consequence of its astringency, vasoprotective, anti-oedema properties (3). More in traditional medicine for the treatment of hemorrhoids, phlebitis and lower members'interestingly, the *Corylus* cell culture is reported to be a source of antimitotic agents (4)

Hazels are commonly propagated vegetatively, where commercial propagation is performed mostly via suckers, as well as cuttings and layering (5). However, conventional propagation strategies are known to be time consuming in hazelnut, not to mention microbial contamination will remain a barrier to rapid growth of the lines (6). Therefore, *in vitro* techniques are taken into consideration as an alternative.

***Correspondence to**: *Massih Forootan, Tell number: 0016154756185, Email: massihforootan@gmail.com, Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Zabol, Zabol, Iran*

Micropropagation has shown to be a method for rapid propagation of clones. A well-established method include maintaining juvenile explants originated from grafted branches in modified DKW medium supplemented with IBA (7). It has been reported that the success on such approaches highly relies on the juvenility of the selected explants, namely aged tissues will express reduced initiation and multiplication rate, as well as low shoot elongation (6). This problem can be overcome by supplementing culture medium with cell division enhancer compounds (e.g. polyamines) (8). Alternatively, rapid growth of elite clones can be achieved exploiting undifferentiated tissue, namely regenerating plants from callus. Further to rapid propagation of clones (9), the undifferentiated tissues of *Corylus* is reported to contain Taxol, an antimitotic agent, reported to be capable of suspending metaphase-anaphase transition (4). In fact, although Taxol is originally reported in yew (*Taxus* spp), hazel callus culture is reported to have stringer antimitotic effects compared to that of yew (4).

Somatic embryos can be a resource of pathogen-free plant material, a potential tool for clonal propagation, germplasm conservation, and physiological studies (2). Somatics embryos are reported derived from hazelnut seeds (4), cotyledonary leaves (10, 11), cotyledons (12). The present study reports production of pre-embryos from leaf-derived callus. This approach, if established successfully, can lead to large-scale production of somatic embryos, providing the groundbase for a large-scale elite population.

MATERIAL AND METHODS

Plant material

Leaves and petioles were collected during March-May from young *Corylus avellana* trees cultivated at experimental farm of College of Agriculture, University of Tehran at Karaj, Iran. The plant material was washed under running tap water for approximately l h. They were then surface sterilized with 70% ethanol for 30 s, followed by immersing in 0.1% $HgCl_2$ solution for 3-4 min. Then they were rinsed with in sterile double distilled water two to three times. For leaves, 1×1 cm^2 segments were cut from the young leaves prior to subculture. For petioles, 1cm pieces were cut out before culture.

Explant subculture

Leaf and petiole explants were cultured on MS medium supplemented with 3% sucrose, 0.7% agar, and gradient levels of 2.4-D (0.5, 1, 2 $mg.l^{-1}$) in combination of gradient levels of BAP (0.5, 1, 2 $mg.l^{-1}$) with pH adjusted to 5.8. The media was sterilized by being autoclaved at 1.5 $kg.cm^{-1}$ and 121°C for 20 min. All treatments were replicated three times; namely three petri dishes, with five explants in each petri dish.. Cultures were maintained in darkness at 25±1°C; and moved to fresh medium every four weeks. The rate of callus induction, as well as callus wet and dry weight were recorded after eight months.

Somatic embryogenesis

Friable calli were transferred to same media, with growth regulators altered as follow: i) The 0.5, 1, and 2 $mg.l^{-1}$ levels of 2,4-D replaced with 0.1, 0.2, and 0.4 $mg.l^{-1}$, respectively. The 0.5, 1, and 2 $mg.l^{-1}$ levels of BAP replaced with 0.1, 0.2, and 0.4 $mg.l^{-1}$, respectively. ii) The 0.5, and 1 $mg.l^{-1}$ levels of 2,4-D replaced with 0.1 and 0.2 $mg.l^{-1}$ of IBA, respectively. The 0.5, 1, and 2 $mg.l^{-1}$ levels of BAP replaced with 0.1, 0.2, and 0.4 $mg.l^{-1}$ of Kin, respectively. Cultures were maintained in darkness for four weeks at 25±1°C, followed by maintenance in 16h photoperiod, same thermal conditions. The percentage of petri dishes containing pre-embryo tissue was recorded as raw data, and treatments were compared using analysis of variances using MSTAT-C software.

RESULTS

Callus induction

All leaf explants showed callus formation, disregarding the type and level of supplemented growth regulator. Calli appeared two weeks after initial subculture, differed in color from green to light yellow.

For petiole explants, calli initiated three weeks after subculture. Callus induction rate ranged from 60% to 100% among different growth regulator levels **(Figure 1).** At BAP 1 $mg.l^{-1}$, 2 $mg.l^{-1}$, 4 $mg.l^{-1}$, the callus initiation tend to decrease when 2,4-D level increased. However, the decrease rate was more rapid at 4 $mg.l^{-1}$ BAP. The callus initiation rate showed fluctuation at low level (0.5 $mg.l^{-1}$) of BAP **(Figure 2-a).**

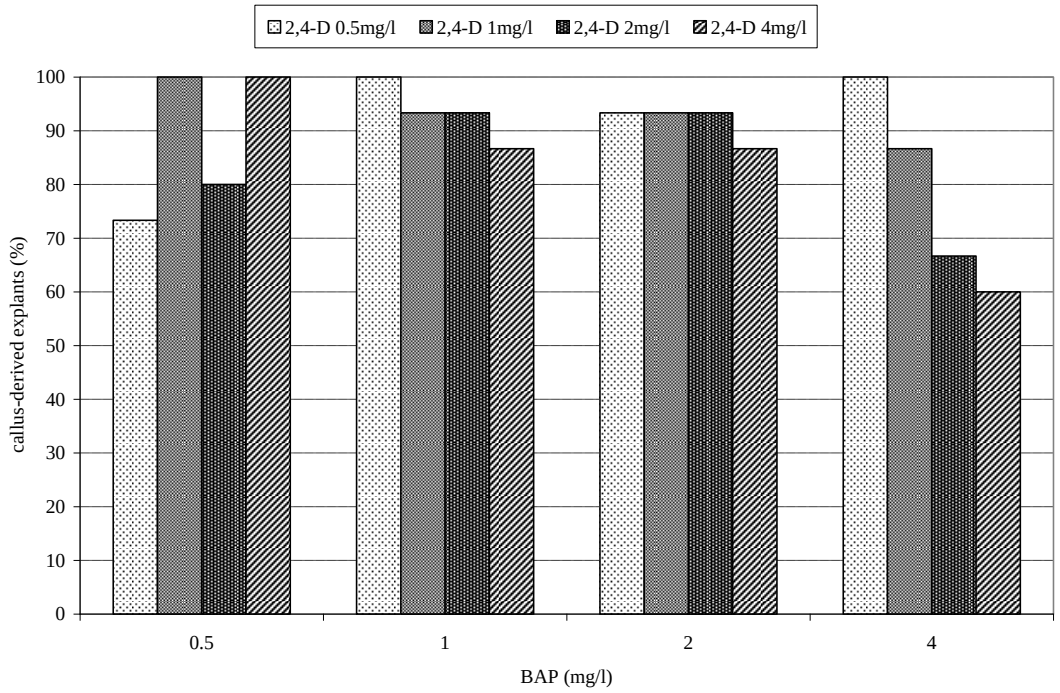


Figure 1. The callus initiation percentage from petiole explants of *C. avellana* , cultured in gradient levels of growth regulators.

(a) (b) (c)

Figure 2. Callus formation in *C. avallena* explants. a) Callus initiated from petiole explants. b) Callus greening in pre-embryo formation medium. c) Callus culture in pre-embryo medium exposed to light.

Pre-embryo formation

Pre-embryo structure was developed both from leaf- and petiole-derived explants **(Figure 3);** however no embryogenesis was observed in the explants. Pre-embryo structures emerged in dark condition; the calli turned red when moved to 16h light regime **(Figure 2-b, c).** The frequency of calli developing pre-embryo structure was higher in leaf-derived calli compared to petiole-derived ones (91% in leaves compared to 50% in petioles). In both cases, the growth regulator levels influenced the frequency of embryo-developing calli.

For petiole-derived pre-embryos, where media was supplemented with 2,4-D and BAP, the frequency of calli containing pre-embryo showed correlation with 2,4-D level when BAP concentration was 0.2 $mg.l^{-1}$. The MS+0.2 $mg.l^{-1}$BAP+0.4 $mg.l^{-1}$2,4-D showed the highest percentage of calli developing pre-embryo structure **(Figure 4).**

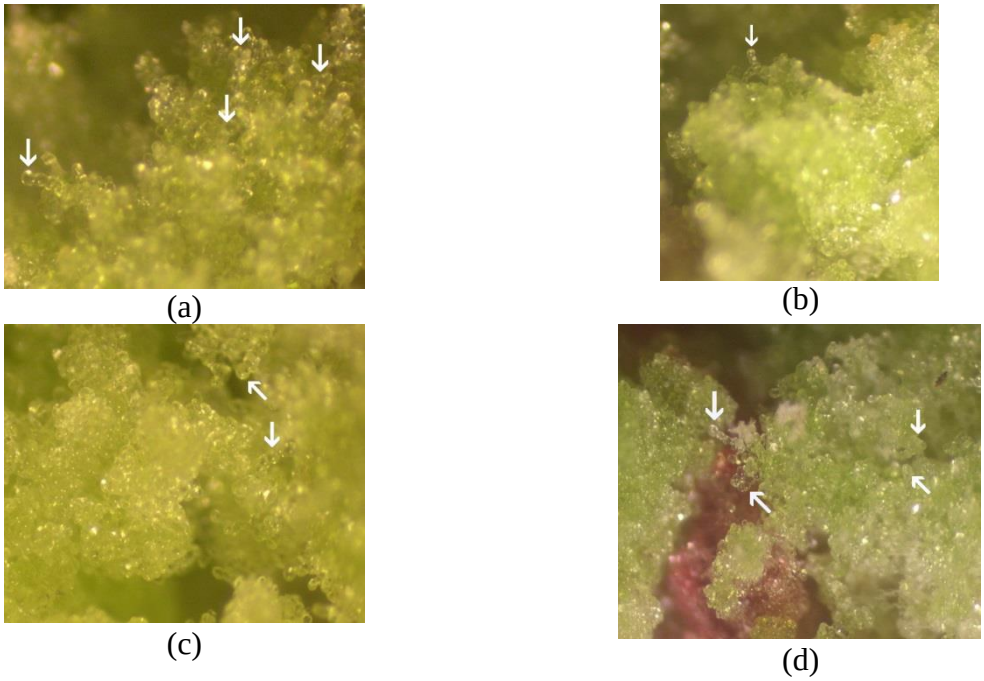


Figure 3. Formation of pre-embryo structure in leaf-derived callus cultures of *C. avellana* explants. a-c in dark condition. d) in light regime.

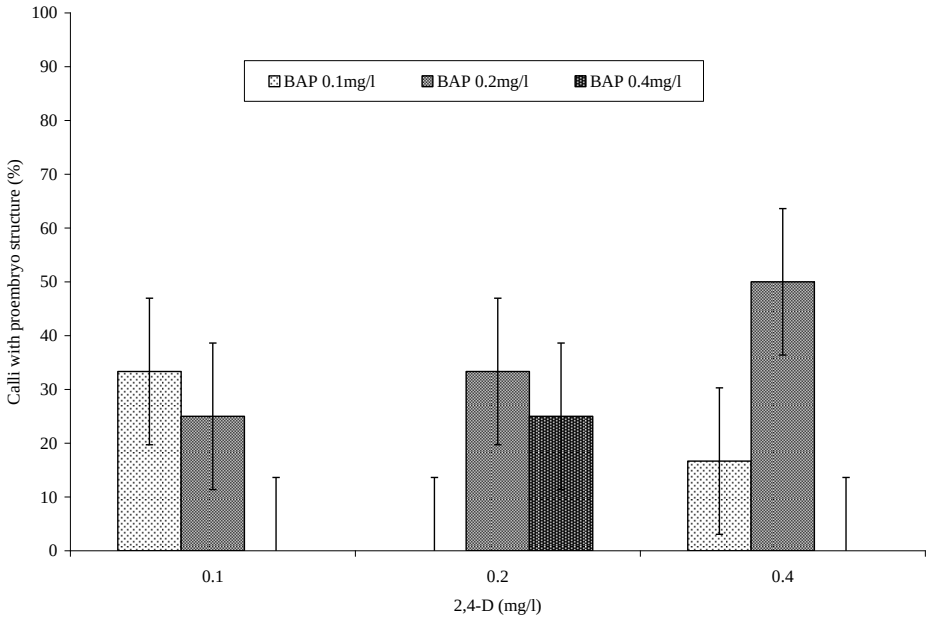


Figure 4. The frequency of pre-embryo structure developing in petiole-derived calli in *C. avallena* under gradient levels of growth regulators. I-bars indicate the least significant difference (α=0.05)

Pre-embryo development from leaf-derived calli was investigated using three different growth regulator supplements: *i*) In 2,4-D + BAP containing cultures, higher concentration (0.4 $mg.l^{-1}$) of BAP resulted reduction in developing pre-embryo structure in all 2,4-D levels except for 0.4 $mg.l^{-1}$ 2,4-D which lowest BAP level (0.1 $mg.l^{-1}$) showed the lowest pre-embryo development **(Figure 5-a).** *ii*) In IBA + Kin containing cultures, the highest pre-embryo

development was related to media which both IBA and Kin were supplemented at the highest concentrations. At 0.2 $mg.l^{-1}$ concentration of IBA, the pre-embryo structure frequency was positively correlated with Kin level **(Figure 5-b).** *iii*) In IBA + BAP containing cultures, less fluctuation in pre-embryo frequency was observed among growth regulator levels compared to above mentioned cultures. No significant difference was observed among IBA levels used; except for when BAP was at the highest concentration used **(Figure 5-c).**

Further to growth regulators, reducing the nitrate to 1/4-strength increased the development of pre-embryo structure. Although, the calli started browning in reduced nitrate level, they preserved the capability of developing pre-embryo structure in subcultures.

DISCUSSION

Callus initiation

At low levels of BAP, the increase of 2,4-D level resulted in fluctuation in callus initiation frequency; while when BAP level increased, callus initiation frequency shifted to linear patterns. This pattern alteration hypothesizes that higher levels of BAP is more appropriate for callus initiation, and proportionally lower 2,4-D levels will enhance it. Similar reports have recommended high cytokinin together with low auxin for means of callus initiation increase (e.g. 13, 14). However, it is noteworthy that to conclude an accurate model for the role of growth regulators in *C. avellana*, a larger statistical population will be required to confirm this hypothesis, however similar reports from other studies from a wide range of species can be a reliable support for this hypothesis.

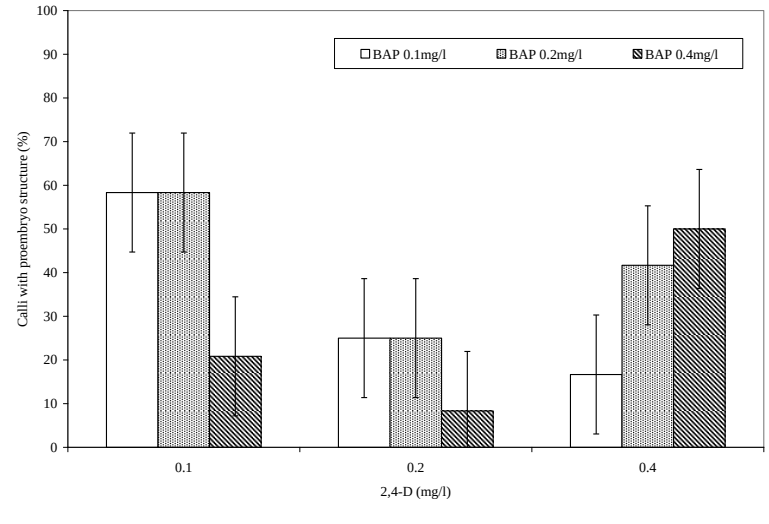
Pre-embryo formation

The pre-embryo structure formation has responded differently to the growth regulator regime, while in IBA+BAP containing media the IBA increase resulted in an increase in pre-embryo frequency, the 2,4-D+BAP and IBA+Kin containing media, the growth regulator levels interacted, so that higher pre-embryo frequency was obtained when hormones were both at their highest or lowest levels. The interaction therefore suggests that the hormone level proportion may influence the pre-embryo formation, as equally as the hormone type. Growth regulator interaction has been reported for 2,4-D and BAP (15, 16) and IBA and Kin (16); supporting the hypothesis that the auxin/cytokinin ratio plays important role in morphogenesis. The interaction is reported to be correlated with auxin/cytokinin antagonistic cell signaling system; in which one signaling system represses the other (17). On the contrary, IBA and BAP have been reported to have no interactive effect (18, 19). Likewise, the present study suggests that IBA and BAP affect the pre-embryo formation independently or with negligible suppressive interaction in *C. avellana*. Again it is a distinct possibility that the results of the present study are influenced by the sample size used for this study, and generalizing the results may demand investigating a population of larger size.

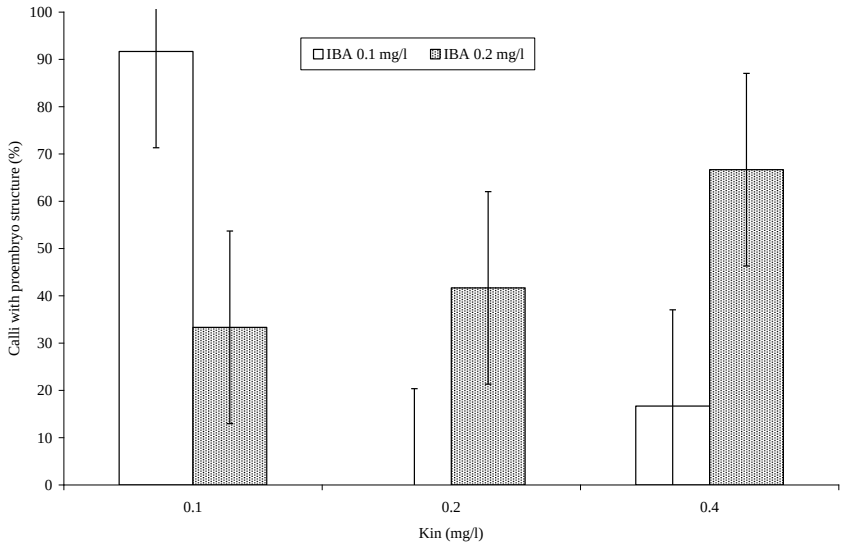
Another bottleneck the present study encountered was the absence of embryogenesis. Previous studies report that pre-embryo tissue contains a specific endoplasmic reticulum structure in which ribosomes are bound as arrays (20). The frequency of this structure will decrease when pre-embryo structure develops into embryos; which can be influenced by hormone level alterations (21). The hormone level alteration did not result in embryogenesis in present study. It is possible that further time (namely further subculturing) is required for embryo development. If the pre-embryo successfully develops into embryos, a valuable groundbase for breeding *Corylus* germplasm will be achieved.

CONCLUSION

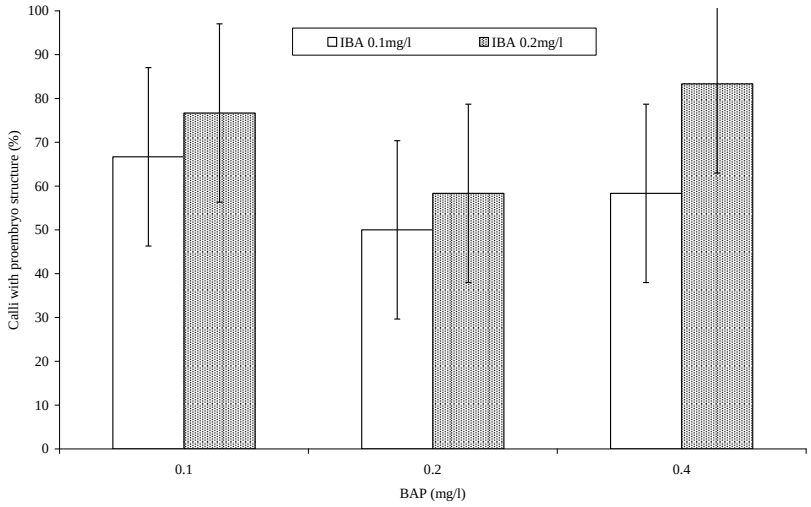
The present study demonstrated general superiority of leaf for means of callus initiation over petioles. Furthermore, the results lead to the conclusion that BAP based media provide the most promising conditions for callus generation. Thus, among the growth regulator combinations used for pre-embryo formation, the combination of IBA and BAP seems to be the most suitable one, due to insignificant interaction between them. This study also suggests that callus-derived embryogenesis is likely to be a time consuming procedure and thus similar studies demand protracted timeframes.



(a)



(b)



(c)

Figure 5. The frequency of pre-embryo structure developing in leaf-derived calli in *C. avallena* under gradient levels of growth regulators. I-bars indicate the least significant difference (α=0.05)

Abbreviations: 2,4-D: *2,4-dichlorophenoxyacetic acid* BAP: *6-benzylaminopurine* IBA: *indole butyric acid* Kin: *Kinetin* MS: *Murashige & Skoog*

REFERENCES

1. Mehlenbacher, S. A., Hazelnuts (Corylus). *Acta Horticulturae: Genetic Resources of Temperate Fruit and Nut Crops* 290: 791-838, 1991.
2. Aygun, A., San, B. and Erdogan, V., Induction of somatic embryogenesis from immature cotyledons in Tambul Hazelnut. *Tarim Bilimleri Dergisi* 15(2): 113-118, 2009.
3. Oliveira, I., Sousa, A., Morais, J. S., Ferreira, I. C. F. R., Bento, A., Estevinho, L. and Pereira, J. A., Chemical composition, and antioxidant and antimicrobial activities of three hazelnut (*Corylus avellana* L.) cultivars. *Food and Chemical Toxicology* 46(5): 1801-1807, 2008.
4. Bestoso, F., Ottaggio, L., Armirotti, A., Balbi, A., Damonte, G., Degan, P., Mazzei, M., Cavalli, F., Ledda, B. and Miele, M., In vitro cell cultures obtained from different explants of *Corylus avellana* produce Taxol and taxanes. *BMC biotechnology* 6(1): 45-55, 2006.
5. Andres, H., Fernández, B., Rodríguez, R. and Rodríguez, A., Phytohormone contents in *Corylus avellana* and their relationship to age and other developmental processes. *Plant Cell, Tissue and Organ Culture* 70(2): 173-180, 2002.
6. Díaz-Sala, C., Rey, M. and Rodríguez, R., In vitro establishment of a cycloclonal chain from nodal segments and apical buds of adult hazel (*Corylus avellana* L.). *Plant Cell, Tissue and Organ Culture* 23(3): 151-157, 1990.
7. Yu, X. and Reed, B. M., A micropropagation system for hazelnuts (Corylus species). *HortScience* 30(1): 120-123, 1995.
8. Nas, M. N., Inclusion of polyamines in the medium improves shoot elongation in hazelnut (*Corylus avellana* L.) micropropagation. *Turkish Journal of Agriculture and Forestry* 28(3): 189-194, 2004.
9. Gao, X. H., Liu, J. N. and Ling, Q., Tissue culture and rapid propagation of hybrid hazelnut (*Corylus heterophylla * C. avellana*). *Acta Horticulturae* 771: 207-211, 2008.
10. Berros, B., Alvarez, C. and Rodriguez, R., Effect of putrescine-synthesis inhibitors on somatic embryogenesis in hazelnut. *Angewandte Botanik* 71(3/4): 90-93, 1997.
11. Perez, C., Rodriguez, R. and Sanchez-Tames, R., Regulation of asexual embryogenesis in filbert cotyledonary nodes. Morphological variability. *Plant Science*, 45(1): 59-64, 1986.
12. Centeno, M. L., Rodriguez, R., Berros, B. and Rodriguez, A., Endogenous hormonal content and somatic embryogenic capacity of *Corylus avellana* L. cotyledons. *Plant Cell Reports* 17(2): 139-144, 1997.
13. Werner, E. T., Milanez, C. R. D., Mengarda, L. H. G., Vendrame, W. A. and Cuzzuol, G. R. F., Culture media, growth regulators and nitrogen sources in callus formation regulation of Brazilwood (Caesalpinia echinata Lam.). *Acta Botanica Brasilica* 24(4): 1046-1051, 2010.
14. El Abidine Triqui, Z., Guedira, A., Chlyah, A., Chlyah, H., Souvannavong, V., Haicour, R. and Sihachakr, D., Effect of genotype, gelling agent, and auxin on the induction of somatic embryogenesis in sweet potato (Ipomoea batatas Lam.). *Comptes Rendus Biologies* 331(3): 198-205, 2008.
15. Carimi, F., Zottini, M., Formentin, E., Terzi, M. and Lo Schiavo, F., Cytokinins: new apoptotic inducers in plants. *Planta* 216(3): 413-421, 2003.
16. Mezzetti, B., Savini, G., Carnevali, F. and Mott, D., Plant genotype and growth regulators interaction affecting in vitro morphogenesis of blackberry and raspberry. *Biologia Plantarum* 39(1): 139-150, 1997.
17. Muller, B. and Sheen, J., Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. *Nature* 453(7198):1094-1097, 2008.
18. Tricoli, D. M., Maynard, C. A. and Drew, A. P., Tissue culture of propagation of mature trees of Prunus serotina Ehrh. I. Establishment, multiplication, and rooting in vitro. *Forest Science* 31(1): 201-208, 1985.
19. De Vries, D. and Dubois, L. A. M., The effect of BAP and IBA on sprouting and adventitious root formation of Amanda'rose single-node softwood cuttings. *Scientia Horticulturae* 34(1-2): 115-121, 1988.
20. Vujicic, R., Radojevic, L. J. and Neskovic, M., Orderly arrangement of ribosomes in the embryogenic callus tissue of *Corylus avellana* L. *Journal of Cell Biology* 69(3): 686-692, 1976.
21. Radojevic, L., Vujicic, R. and Neskovic, M., Embryogenesis in tissue culture of *Corylus avellana* L. *Zeitschrift fur Pflanzenphysiologie* 77(1): 33-41, 1975.