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EFFECTS OF CYTOKININS IN *CORYLUS AVELLANA* L. MICROPROPAGATION

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Abstract

This study was carried out to investigate the effect of three cytokines [6-benzyladenine (BA), meta-Topoline (mT), and zeatin (Z)] on the in vitro propagation of C. avellana. The culture medium used in all in vitro culture stages was McCown's Woody Plant Medium (WPM) supplemented with 100 mg/l Sequestrene 138.3% (w/v) sugar, solidified with 5 g/l. Plant agar and pH 5.8. At the initiation stage, single-node mini cuttings were inoculated on WPM medium supplemented with 2 mg/l Z, and the initiation percentage was 66.66%. The highest proliferation rate (2.21 ± 0.11) was observed on the culture medium supplemented with 4 mg/l BA followed by the culture medium supplemented with 4 mg/l Z (1.93 ± 0.03). The shortest shoots were measured on WPM medium with 4 mg/l BA, with an average length of 1.81 ± 0.08 cm, and the longest shoots (3.46 ± 0.02 cm) were obtained on WPM medium with 4 mg/l Z. The in vitro rooting percentage was 81.66 ± 0.39% when using the WPM medium with 1 mg/l Z. The rooted plants obtained in vitro showed an acclimation rate of 97.66%, and out of these, 96% survived under greenhouse conditions.

Key words*: Barcelona hazelnut, 6-benziladedine, meta-Topoline, zeatin, proliferation rate*

INTRODUCTION

The hazelnut is considered a recalcitrant species under *in vitro* growth conditions because it exhibits a low rate of initiation and multiplication, insufficient shoots elongation, and high rates of microbial contamination (Yu & Reed, 1995; Nas, 2004; Nas & Read, 2004; Prando et al., 2014: Ellena et al., 2018; Sgueglia et al., 2019; Silvestri et al., 2020; Neda et al., 2020). Many basal media and their modifications have been used for hazelnut micropropagation: Anderson medium (Anderson, 1984) (Yu & Reed, 1993), DKW (Driver & Kunijuki, 1984) (Yu & Reed, 1993; Nas, 2004; Tegg et al., 2016; Ellena et al., 2018), Murashige and Skoog medium (MS) (Murashige & Skoog, 1962) (Nas, 2004; Silvestri et al., 2020), WPM (Lloyd and McCown, 1980) (Clapa et al., 2019; Kiran et al., 2021).

Furthermore, several studies have been conducted with the aim of developing tissue culture media to improve the micropropagation systems for different hazelnut genotypes (Bacchetta et al., 2008; Hand and Reed, 2014; Hand et al., 2014; Akin et al., 2017).

Among plant growth regulators (PGRs), cytokinins played a crucial role in shoot development (Wu et al., 2021). Thus, exogenous and endogenous cytokinins used in different concentrations in plant tissue culture were considered closely associated with shoot organogenesis (García-Ramírez, 2023). Consequently, some cytokinins were tested in order to obtain maximum values recorded on proliferation rates during the *in vitro* multiplication stage of hazelnut. In several studies, N6 benzyladenine (BA) was used at different concentrations for *in vitro* multiplication at hazelnut cultivars such as Montebello (Daminano et al., 2005), Dorris, Jefferson, and Sacajawea (Hand & Reed, 2014), Dorris, Wepster and Zeta (Akin et al 2017), Carrello, Ghirara, Minnulara, and Panottara (Sgueglia et al., 2019) and also local hazelnuts varieties collected from different locations in Turkey (Kiran et al., 2021). Other cytokinins used in hazelnut micropropagation

were: kinetin, iso-pentenyladenine (IPA) (Thomson and Deering, 2011), and zeatin (Bacchetta et al., 2005; Thomson & Deering, 2011).

Conversely, some studies have shown that the choice of iron source significantly influences the success of hazelnut *in vitro* culture. Fe-EDTA represents the most used iron source in hazelnut micropropagation (Silvestri et al., 2020). Sequestrene 138 has also been a source of iron successfully used in *in vitro* culture of hazelnut (Yu & Reed, 1993; Yu & Reed, 1995; Thomson & Deering, 2011; Hand & Reed, 2014; Hand et al., 2014; Clapa et al., 2019).

Although in recent years, there have been significant advancements in the development of protocols for micropropagation of *C. avellana*, proliferation rates have remained relatively low. Therefore, the aim of this study was to investigate the influence of three cytokines (BA, mT, and Z) used in the in vitro multiplication stage of C. avellana and to develop an efficient and reproducible micropropagation protocol adapted for the Barcelona hazelnut variety.

MATERIALS AND METHODS

Barcelona cv. was used for this study. All the experiments were carried out using McCown's Woody Plant Medium (WPM) supplemented with 100 mg/l Sequestrene 138.3% (w/v) sugar, and solidified with 5 g/l Plant agar. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH and/or 0.1 N HCl before adding the gelling agent and autoclaving at 121ºC for 20 min. The *in vitro* cultures were incubated in the growth chamber at 16 h photoperiod, 32.4 µmol $m^{-2}s^{-1}$ light intensity (Philips CorePro LEDtube 1200 mm 16W865 CG, 1600lm Cool Daylight) and temperature of 23 ± 3°C. The chemicals used were purchased from Duchefa Biochemie B.V., Haarlem, The Netherlands.

In vitro culture initiation

Young plants (Figure 1a), with juvenile growths, were used for the initiation of *in vitro* cultures. In April, shoot fragments measuring 5-7 cm in length with a 1-1.5 mm diameter were harvested. After removing the leaves, mini-cuttings were washed thoroughly, first with running tap water and the distilled water with three drops of Twen 20 for 10 minutes on a magnetic stirrer plate to eliminate all the dust and impurities. After that, the shoot fragments were disinfected with a bleach solution of 20% ACE (Procter and Gamble, București, Romania; <5% active ingredient) for 20 min followed by triple-rinse with sterile distilled water. The single-node explants of hazelnut were inoculated on WPM medium supplemented with 2 mg/l Z and gelled with 5 g/l (w/v) Plant agar in glass test tubes (11.5 × 2 cm ø) containing 5 ml sterile medium (Figure 1 b, c.). In the *in vitro* initiation stage, 60 explants were inoculated into the culture media and, after one month of culture, the shoot growing percentage and contamination rate was calculated.

In order to establish and provide plant stock for subsequent *in vitro* multiplication experiments, the regenerated shoots were further multiplied at two month intervals through two passages on WPM medium supplemented with 1 mg/l Z. In the stabilization stage, 720 mL (v/v) culture jars (13.5 × 9 cm ø) with screw caps were used as culture vessels. The screw caps were fitted with ventilation holes (4 mm ø) and with an autoclavable plastic sponge (18 mm × 18 mm). In each culture jar, 100 mL (v/v) of sterile medium was dispensed and ten explants (1-1.5 cm in length, containing 2-3 nodes) were inoculated.

In vitro shoots multiplication

In the multiplication stage, the effect of three cytokinins was examined: 6-benzyladenine (BA), meta-Topoline (mT), and zeatin (Z). Each was added to the culture medium at a concentration of 4 mg/l before autoclaving. In each jar (similar to those described above) containing 100 ml of medium, 10 mini cuttings with 2-3 nodes from the culture medium supplemented with 1 mg/l Z were inoculated. After two months of culture, the shoot length and proliferation rate were calculated.

In vitro rooting and acclimatization

Rooting was tested *in vitro* on WPM supplemented with 1 mg/l Z and after three months, the percentage of rooting, the average shoot length, the average no. of roots, and roots length were measured and calculated from 108 plantlets (3 jars × 12 plantlets/jar in 3 replicates). Subsequently, *in vitro* rooted plants

were acclimatized in a floating hydroponic system according to the method described by Clapa et al (2013) for one week. Then, the plantlets were planted in a mix of peat and perlite (3:1, v/v) (Klasmann, TS3 Medium Basic Standard, with pH = 6) in mini-greenhouses (Versay, T1, sizes 39 × 25 × 7.5 cm, PVC). The percentage of acclimated plants was then recorded after 20 days of culture in mini-greenhouses.

The rooted and acclimatized hazelnuts plantlets were then transplanted into pots (VQB 9 x 9 x 9.5 black, SC BLONDY ROMANIA SRL 540390 Tg-Mures, Romania) containing peat-based potting mix (Klassman TS3) and kept in greenhouse conditions (21 ± 4°C), under natural photoperiod conditions. The survival rates (%) under greenhouse conditions were calculated after 30 days.

Data Analysis

The *in vitro* experiments were carried out in a completely randomized design (CRD) and one-way ANOVA was performed to check the differences between the experimental variants. When the null hypothesis was rejected, Tukey's HSD test ($p < 0.05$) was used to determine the differences between the means. The values presented are means ± S.E.

RESULTS AND DISCUSSIONS

Previous research has shown that the *in vitro* initiate potential of hazelnuts cultivars is generally difficult due to microbial contaminants and decontamination techniques (Bacchetta et al., 2008; Sgueglia et al., 2019; Silvestri et al., 2020). In a study conducted by Andrés et al. (2002) endogenous levels of indole-3-acetic acid, abscisic acid, and cytokinins (Z-type: dihydrozeatin, dihydrozeatin riboside, zeatin, and zeatin riboside; iP-type: N6 -isopentenyl adenine and N6-isopentenyl adenosine), were determined in leaves of hazelnut. The aforementioned study shows that the ratios of iP-type/Z-type cytokinins were low in the analysed samples from autumn and spring leaves, while they were high in the juvenile and forced outgrowth samples. Thus, in our study, young plants, with juvenile growths, were used for the initiation of *in vitro* cultures and the cytokinin used was zeatin. Our results show that during the initiation stage, when single-node micro-cuttings were inoculated on WPM medium supplemented with 100 mg/l Sequestrene 138 and 2 mg/l Z, 66.66% (Figure 1 b, c) of the explants were viable, 21.66% did not show any development or growth, and 11.66% were infected with contaminants.

Previous studies have demonstrated an improvement in the regeneration potential of hazelnut cultivars within micropropagation systems. In a study conducted by Silvestri et al. (2020), an efficient *in vitro* establishment stage was performed for the Tonda Gentile Romana hazelnut variety. The researchers utilized a half-strength MS medium supplemented with 20 g/L sucrose, 6 mg/l 6-benzyl aminopurine, 0.1 mg/l naphthalene acetic acid, 0.1 mg/l thidiazuron, and 0.55% plant agar. Different concentrations of $CuSO_4 \cdot 5H_2O$ (0.0, 1.25, 2.5, and 5 mg/L) were added to the medium. The addition of copper sulfate was found to significantly reduce bacterial contamination in the culture medium. Specifically, 2.5 mg/l $CuSO_4 \cdot 5H_2O$ resulted in a significant decrease in contamination frequency during the establishment phase while maintaining a high rate of bud sprouting. On the other hand, the highest concentration (5.0 mg/l) of copper sulfate significantly reduced contamination rates but had a negative impact on bud sprouting. Therefore, this concentration was deemed unsuitable for the purpose of the study.

Sgueglia et al. (2019) conducted a study in which they utilized axillary buds taken from 1-year-old twigs of mature plants. The buds were subjected to decontamination treatments involving sodium hypochlorite and sodium merthiolate for either 35 or 40 minutes. The researchers observed that the 40+40 minute treatment positively reduced explant contamination in all cultivars. However, it also led to a higher incidence of necrosis overall, particularly in the Carrello cultivar (50%) and the Panottara cultivar (46.7%). In terms of bud survival, the 40+40 minute treatment yielded the highest rates in Minnulara (26.7%) and Ghirara (20%), while the 35 + 35 minute treatment was more effective for Carrello (33.3%) and Panottara (23.3%).

The shoots obtained during the initiation phase of hazelnut *in vitro* culture were subsequently

transferred to the same culture medium used for the initiation stage but with the addition of 1 mg/l Z. On this medium, the shoots exhibited robust growth and successfully developed roots. It is worth mentioning that the inoculums containing apical buds demonstrated higher growth compared to those with axillary buds. Additionally, the results of our study showed that the shoots obtained from apical buds rooted at a 100% rate. This indicates that the mini shoots with apical buds are more suitable for rooting compared to other types of explants in hazelnut micropropagation. (as depicted in Figure 1, panels d and e).

In contrast, the use of mT at a concentration of 4 mg/l did not lead to any proliferation, with only one shoot being generated from each inoculum. This suggests that mT may not be as effective as BA and Z in shoot multiplication in hazelnut cultures. (Figure 2 c, d, and Figure 3).

The highest proliferation rate, recorded as 2.21 ± 0.11, was observed on the culture medium supplemented with 4 mg/l BA (Figure 2 a, b) followed by the culture medium supplemented with 4 mg/l Z (Figure 2 e, f) which generated an average rate of proliferation of 1.93 ± 0.03. However, no significant differences were observed between the mean values of the multiplication rate for these two variants, as depicted in Figure 3. A significant portion of the literature emphasizes the positive outcomes of using BAP for *in vitro* shoot multiplication in hazel plants. For example, the multiplication rate of hazelnut cvs. Nonpareil and Tonda Gentile Romana were 3.2 respectively 3.1, on DKW medium supplemented with 3 mg/l BA (Yu & Reed, 1993). The effect of BA concentration on shoot formation was tested on the cultivar Montebello. Opposite of our results, the highest multiplication rates were obtained using concentrations of 1.5 and 2.0 mg /l BA. The shoots treated with 3.0 mg l^{-1} BA were highly hyperhydric (Damiano et al., 2005).

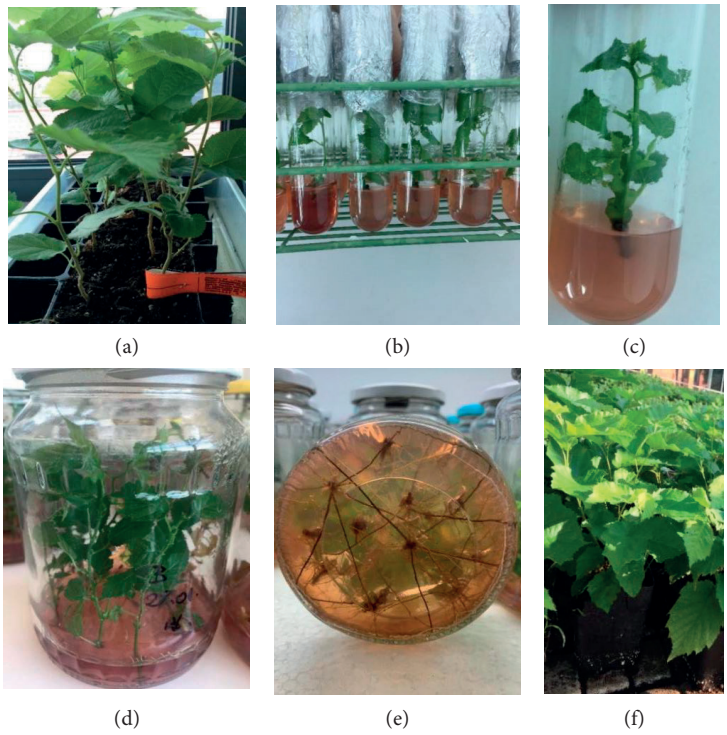

Figure 1. *In vitro* propagation of *C. avellana*, cv. Barcelona: (a) Plants used to initiate *in vitro* culture; (b, c) *In vitro* culture initiation on WPM+ 100 mg/l Sequestrene 138 +1 mg/l Z; (d) *In vitro* shoot proliferation on medium WPM+ 100 mg/l Sequestrene 138 +1 mg/l Z after 12 weeks of incubation; (e) *In vitro* rooting of shoots cultured on WPM+ 100 mg/l Sequestrene 138 +1 mg/l Z; (f) Plants acclimatized in the greenhouse

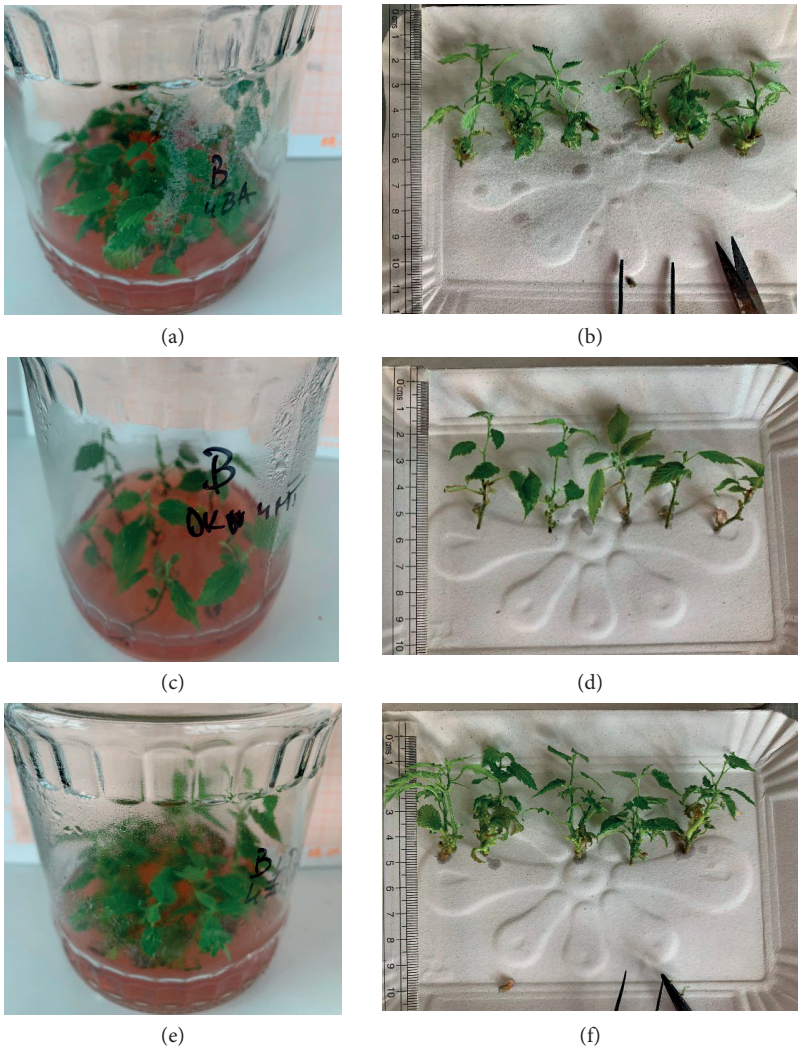

Figure 2. *In vitro* shoot proliferation of *C. avellana*, cv. Barcelona on WPM medium supplemented with 100 mg/l Sequestrene 138 and different cytokinins and gelled with 5 g/l Plant agar after three months of incubation: (a, b) Shoot proliferation on media containing 4 mg/l BA; (c, d) Shoot proliferation on media containing 4 mg/l mT; (e, f) Shoot proliferation on media containing 4 mg/l Z.

Table 1. *In vitro* rooting and acclimatization data recorded in micropropagated plants of *C. avellana* cv. Barcelona

In vitro shoots and roots morphometry			*In vitro* rooting (%)	*Ex vitro* acclimatization (%)	Survival under greenhouse conditions (%)
Length of shoots (cm)	No. of roots	Length of roots (cm)			
6.35 ± 0.25	3.65 ± 0.36	2.10 ± 0.04	81.66 ± 0.39	97.66	96

Values shown are means ± SE.

Regarding the average length of the proliferated shoots, the shortest shoots were observed when using WPM medium supplemented with 4 mg/l BA, measuring an average length of 1.81 ± 0.08 cm. On the other hand, the longest shoots (3.46 ± 0.02 cm) were obtained when utilizing a WPM medium supplemented with 4 mg/l Z (Figure 3). Another study (Bacchetta et al., 2005) showed

that zeatin (1 mg/L) was more suitable than BAP (0.5 mg/L) for shoot elongation.

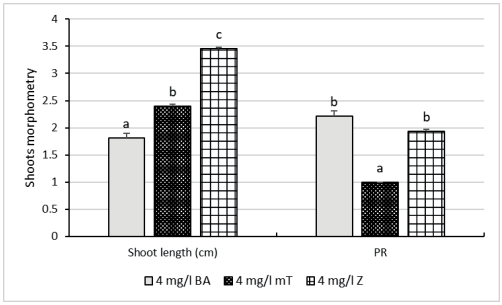

Figure 3. The effect of 6-benzylidene (BA), metatopolin (mT), and zeatin (Z) treatments on shoot proliferation in *C. avellana* cv. Barcelona after 12 weeks of *in vitro* culturing. Different lowercase letters above the bars indicate significant differences between the means of the same parameter according to Tukey's HSD test ($p<0.05$)

Therefore, the WPM culture medium supplemented with 100 mg/l Sequestrene 138 and 4 mg/l Z is the most suitable for shoot proliferation in the Barcelona hazelnut variety as it promotes the growth of longer shoots compared to 4 mg/l BA.

For the *in vitro* rooting of hazelnut shoots, different culture media were supplemented with indole-3-butyric acid (IBA), indoleacetic acid (IAA), or naphthaleneacetic acid (NAA) at various concentrations and combinations. In their study, Damiano et al. (2005) demonstrated that the best rooting response in the Montebello hazelnut variety was achieved with 9.8 μM (2 mg/l) of IBA, resulting in a rooting percentage of 79%. At similar concentrations of IAA and NAA, the rooting percentages were lower (71% and 65%, respectively).

In our study, on the WPM medium with 1 mg/l Z, 81.66 ± 0.39 % of the shoots were *in vitro* rooted while the average number of roots per plantlet was 3.65 ± 0.36 and the average root length was 2.10 ± 0.04 cm (Table 1). The plantlets rooted *in vitro* had a 97.66% acclimatization rate (in floating hydroculture followed by acclimatization in a mix of peat and perlite in mini-greenhouses) and 96% of the plants survived in greenhouse conditions (Table 1; Figure 1 d, e, f).

Our results show that among the tested cytokines, zeatin can be used in all stages of *in vitro* multiplication in the Barcelona hazelnut variety. This can be explained by the fact that juvenile hazelnut tissues as well as those with forced growth show a high morphogenetic potential, suggesting that the ratio of iP-type/Z-type cytokinins may be a good index of the *in vitro* potential of the hazelnut (Andrés et al., 2002).

CONCLUSIONS

Our research has highlighted that the WPM culture medium supplemented with 100 mg/l Sequestrene 138 and 1-4 mg/l Z can be used in all in vitro culture stages for hazelnut (*Corylus avellana* L.) cv. Barcelona. In the initiation stage, a 66.66% initiation rate was achieved on the WPM culture medium supplemented with 2 mg/l Z. The longest shoots, measuring 3.46 ± 0.02 cm, were obtained on the WPM culture medium supplemented with 4 mg/l Z, and an in vitro rooting percentage of 81.66 ± 0.39% was observed when using the WPM medium with 1 mg/l Z.

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