Plant Cell, Tissue and Organ Culture (PCTOC) (2026) 164:31
https://doi.org/10.1007/s11240-026-03345-x

ORIGINAL ARTICLE

Cytokinin-mediated shoot induction and liquid culture-enhanced rooting in Turkish hazelnuts (*Corylus avellana* L.)

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Received: 13 October 2025 / Accepted: 6 January 2026 / Published online: 26 January 2026
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Abstract

Three commercially important Turkish hazelnut (*Corylus avellana* L.) cultivars ('Tombul', 'Çakıldak', and 'Foşa') were evaluated to optimize an in vitro propagation protocol. The effects of different cytokinins; benzyladenine (BA), zeatin (ZT), meta-topolin (mT), and thidiazuron (TDZ) were tested at 1, 2, and 3 mg L^{-1} in the presence of 0.01 mg L^{-1} indole-3-butyric acid (IBA). TDZ at 1 mg L^{-1} promoted high shoot multiplication, whereas higher TDZ concentrations reduced shoot proliferation and were associated with increased hyperhydricity, shoot tip necrosis, and callus formation. BA supported high shoot multiplication and elongation at specific concentrations, and mT also resulted in long shoots with minimal physiological disorders. Callus formation was most pronounced under TDZ treatments, reaching 100% at 2 and 3 mg L^{-1} in all cultivars. For rooting, micro-shoots were cultured on media containing 1 or 2 mg L^{-1} IBA solidified with different gelling agents or maintained as liquid culture. Rooting responses differed among cultivars and were influenced by both IBA concentration and rooting system. High rooting percentages, root numbers, and root lengths were obtained in liquid medium and in phytagel, and plant agar solidified media supplemented with 1 or 2 mg L^{-1} IBA, whereas bacto agar generally resulted in lower rooting performance. Overall, 'Foşa' exhibited lower rooting responses compared with 'Tombul' and 'Çakıldak'.

Key message

Cytokinin type and concentration determine shoot proliferation and callus formation in hazelnut, whileoptimized IBA levels in liquid culture improve in vitro rooting efficiency.

Keywords Micropropagation · Adventitious shoot regeneration · Woody plants

Abbreviations

BA	Benzyladenine
CK	Cytokinin
mT	meta-topolin
IBA	Indole-3-butyric acid
NaOCl	Sodium hypochlorite
NRM	Nas and Read medium
PGR	Plant growth regulator
PTFE	Polytetrafluoroethylene
STN	Shoot tip necrosis
TDZ	Thidiazuron
ZT	Zeatin
2iP	2-isopentenyl adenine

Communicated by Cristian Silvestri

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Introduction

The European hazelnut (*Corylus avellana*, family Betulaceae) is one of the most economically important nut crops worldwide, valued for both its commercial importance and health benefits. Türkiye is the leading global producer of hazelnuts, with an average annual production of 650.000 tons (FAOSTAT 2022), and is recognized as a major center of hazelnut genetic diversity (Gürcan et al. 2010; Botta et al. 2019). Hazelnut cultivation in Türkiye is expanding, particularly in the western Black Sea region, where new

plantations are increasingly being established (Erdogan 2017). As global demand for hazelnuts grows, the need for the rapid, large-scale propagation of true-to-type, disease-free cultivars is increasing.

Türkiye possesses a rich pool of hazelnut genetic resources. Among the most widely cultivated and commercially valuable cultivars are 'Tombul', 'Çakıldak', and 'Foşa' (Erdogan 2017; Kurt and Karakaya 2025). High-yielding clones have emerged as a variety candidate through selection studies with the 'Tombul' variety (Taylan et al. 2023). However, the limited number of studies on the clonal propagation of these elite cultivars with superior traits poses a significant barrier to the establishment of new orchards using high-quality plant material. The production of genetically uniform seedlings from these cultivars is critical for the successful establishment of commercial orchards.

In Türkiye, the most common propagation method is via suckers due to its low cost and minimal labor requirements (Beyhan et al. 2018). However, this approach is associated with several limitations, including weak root system development and the risk of producing plants that are not genetically identical to the mother plant (Beyhan et al. 2018). In addition, traditional sucker-based propagation may facilitate the transmission of soil-borne and systemic diseases and is insufficient to meet the growing demand for large quantities of high-quality planting material (Silvestri et al. 2019). Micropropagation offers a reliable alternative by enabling year-round, large-scale clonal propagation under controlled conditions. Plants produced via tissue culture have demonstrated comparable growth performance, yield, and canopy volume to those propagated by grafting or own-rooting, while providing greater uniformity and genetic fidelity (Traini et al. 2024). Nevertheless, despite the increasing interest in hazelnut biotechnology, the broader application of micropropagation and advanced breeding tools remains constrained by the lack of efficient and reproducible regeneration protocols applicable across diverse genotypes (Ferrucci et al. 2023). In this context, micropropagation has emerged as a strategic approach for the large-scale production of woody fruit species in Europe, including hazelnut, where commercial in vitro systems are increasingly adopted to ensure the supply of uniform and pathogen-free planting material despite existing technical and economic constraints (Sota et al. 2025).

Cytokinin (CK) type and concentration are key factors in shoot organogenesis (Šmeringai et al. 2023; Skoog and Miller 1957). Benzyladenine (BA) is the most commonly used cytokinin for shoot multiplication and elongation in hazelnut (Hand et al. 2014; Yahyaoui et al. 2021; Hemmati-Gougeh et al. 2025). Other CKs, such as zeatin (ZT) (Bacchetta et al. (2008) (Clapa et al. 2023),; 2-isopentenyl adenine (2iP), kinetin (Thomson and Deering 2011), and meta-topolin (mT) (Clapa et al. 2023; Sgueglia et al. 2019) have also been tested, often with cultivar-dependent responses. While high cytokinin levels promote shoot proliferation and elongation, they can also lead to undesirable effects such as hyperhydricity and shoot necrosis (STN) (Ivanova and Van Staden 2011; Surakshitha et al. 2019).

Gelling agent sources significantly influence tissue culture success due to differences in purity, composition, and gelling properties (Salehi et al. 2017; Bornman and Vogelmann 1984; Debergh 1983). Agar, although commonly used, is one of the most expensive components of culture media. As a result, various studies have explored alternative gelling agents and concentration adjustments to reduce production costs. The in vitro rooting stage is particularly critical for successful acclimatization and plant establishment; however, limited research has focused on the influence of gelling agent type or the differences in rooting efficiency between liquid and semi-solid media at this stage Nicholson et al. (2020).

The objective of this study was to develop an efficient and reproducible micropropagation protocol for three commercially important Turkish hazelnut cultivars by optimizing culture establishment, shoot multiplication, rooting, and acclimatization stages. The ultimate goal is to support large-scale production of uniform, high-quality hazelnut plants suitable for commercial orchard establishment.

Materials and methods

Plant material

One-year-old *C. avellana* plants of the cultivars 'Tombul', 'Çakıldak', and 'Foşa', previously propagated in vitro and grown in 5-liter pots under greenhouse conditions, were used as explant sources. To maintain plant health, a fungicide solution (Aldeid®, containing 200 g/L Azoxystrobin and 125 g/L Difenoconazole) was applied biweekly at a concentration of 1 mlL^{-1}. Additionally, plants were fertilized monthly with 6 gL^{-1} of a 20–20–20 N-P-K fertilizer supplemented with micronutrients (Grow up®). In March, all branches were pruned, leaving only the main trunk (Hand et al. 2016). Actively growing shoots that emerged from the pruned plants were harvested in May and used as explants.

Surface sterilization

The apex of the shoots and leaves were removed, leaving the first four nodes below the apex. The shoots were then washed under tap water for 30 minutes. After washing, they were cut into segments approximately 3 cm in length, each containing a single node. Nodal explants were surface-sterilized

by immersion in 70% ethanol for 30 seconds, followed by treatment with 1% sodium hypochlorite (NaOCl) solution for 10 minutes. Explants were then rinsed three times with sterile distilled water. To minimize tissue damage caused by disinfectants, the basal ends of the explants were trimmed by approximately 1 cm on sterile filter paper prior to culture, as described by Yahyaoui et al. (2021).

Culture establishment and growth conditions

Sterile nodal explants were transferred to culture tubes (Sigma, C5916) containing 5 mL of NRM medium (Nas and Read 2004), supplemented with 2 mg L^{-1} BA and 0.6% plant agar (Duchafe), with the pH adjusted to 5.7. Cultures were incubated at 23 ± 2 °C under a 16-hour photoperiod with a light intensity of 40 µmol m^{-2} s^{-1}. In all experiments, plants were maintained under the same culture conditions. After 4 weeks, the explants were transferred to 300 mL vessels (n = 5) for shoot multiplication. Subculturing was performed every 4 weeks by transferring the cultures to fresh medium.

Shoot multiplication

After six months of continuous culture, uniform and healthy plantlets were selected. Axillary shoot segments measuring approximately 10–15 mm in length and containing two nodes were excised and transferred to NRM medium supplemented with 0.01 mg L^{-1} IBA and varying concentrations (1, 2, or 3 mg L^{-1}) of BA, ZT, mT, or TDZ (Table 1). ZT, TDZ, and IBA were filter-sterilized using 0.22 µm pore-size PTFE filters (Isolab) and added to the medium after it had cooled. Following three weeks of incubation, cultures were subcultured onto fresh medium and maintained for an additional three weeks, resulting in a total treatment duration of six weeks. At the end of this period, the number of shoots and the length of the longest shoot were recorded. Observations of hyperhydricity, STN, and basal callus formation were also documented.

Rooting

For in vitro rooting, healthy microshoots measuring 3–4 cm in length were selected. The shoots were cultured on NRM medium supplemented with IBA at concentrations of 1 or 2 mg L^{-1} and solidified with different gelling agents. The semi-solid media included bacto agar (Merck, 6 g L^{-1}), plant agar (Duchafe, 6 g L^{-1}), and Phytagel (Sigma, 1.75 g L^{-1}), while a liquid medium without gelling agents was also tested. The pH of all media was adjusted to 5.7 prior to autoclaving. Following autoclaving and cooling, filter-sterilized IBA was added to each medium. For semi-solid cultures, 50 mL of medium was dispensed into 300 mL culture vessels, with five shoots placed per vessel. In the liquid treatment, 2 mL of medium was transferred into sterile culture tubes (Sigma, C5916), and one shoot per tube was placed after removing its basal leaves. After four weeks of culture,

Table 1 Effects of cytokinin type and concentration on hyperhydricity, shoot tip necrosis, and callus induction in nodal explants of three hazelnut cultivars

Cytokinins	Hyperhydricity (%)			Shoot tip necrosis (%)			Callus (%)		
	Tombul	Çakıldak	Foşa	Tombul	Çakıldak	Foşa	Tombul	Çakıldak	Foşa
1 mg L^{-1} BA	0 ± 0 e	0 ± 0 d	3.33 ± 3.33 e	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0c	0 ± 0 c	16.67 ± 3.33 c
2 mg L^{-1} BA	3.33 ± 3.33 e	3.33 ± 3.33 d	6.67 ± 3.33 e	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c	23.33 ± 3.33 c
3 mg L^{-1} BA	10 ± 6.67 de	13.33 ± 6.67 c	16.67 ± 10 de	0 ± 0 c	3.33 ± 3.33 c	6.67 ± 3.33 c	6.67 ± 3.33 c	0 ± 0 c	20 ± 0 c
1 mg L^{-1} ZT	0 ± 0 e	0 ± 0 d	0 ± 0 e	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c	23.33 ± 3.33c
2 mg L^{-1} ZT	0 ± 0 e	0 ± 0 d	3.33 ±3.33 e	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c	16.67 ± 3.33 c
3 mg L^{-1} ZT	3.33 ± 3.33 e	3.33 ± 3.33 d	6.67 ± 3.33 e	0 ± 0 c	0 ± 0 c	0 ± 0 c	6.67 ± 3.33 c	3.33 ±3.33 c	23.33 ± 6.67 c
1 mg L^{-1} mT	0 ± 0 e	0 ± 0 d	0 ± 0 e	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 d
2 mg L^{-1} mT	0 ± 0 e	0 ± 0 d	0 ± 0 e	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c	3.33 ± 3.33 d
3 mg L^{-1} mT	0 ± 0 e	0 ± 0 d	0 ± 0 e	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c	6.67 ± 3.33 d
1 mg L^{-1} TDZ	33.33 ± 3.33 c	46.67 ± 6.67 b	50 ± 5.77 c	23.33 ± 3.33 b	23.33 ±6.67 b	23.33 ±3.33 b	50 ± 5.77 b	56 ± 6.67 b	73 ± 5.77 b
2 mg L^{-1} TDZ	53.33 ± 6.67 b	60 ± 5.77 ab	63.33 ± 13.33 bc	33.33 ± 3.33 ab	31.03 ± 0 ab	43.33 ± 6.67 ab	100 ± 0 a	100 ± 0 a	100 ± 0 a
3 mg L^{-1} TDZ	80 ± 11.55 a	73.33 ± 6.67 a	86.67 ± 3.33 a	50 ± 5.77 a	50 ± 10 a	56.67 ± 6.67 a	100 ± 0 a	100 ± 0 a	100 ± 0 a

Explants (n = 30) were cultured on NRM medium supplemented with 0.01 mg L^{-1} IBA and different cytokinin types and concentrations for two consecutive subcultures (3 weeks each). Values represent means ± SE. Within each column, means followed by the same letter are not significantly different according to Tukey's HSD test ($p \leq 0.05$)

the rooting percentage, number of roots per explant and length of the longest root were recorded for all treatments.

Acclimatization

Following root induction, plants were transferred to paper pots filled with a 2:1:1 (v/v/v) mixture of peat, coco peat, and perlite (Fig. 1). The pots were placed in plastic containers with lids that remained closed for the first two weeks to maintain high humidity. During this period, the lids were opened daily for three minutes to allow gradual ventilation. After two weeks, the lids were progressively opened, and by the end of the fourth week, the acclimatized plants were transplanted into 2-liter pots containing a 3:1 (v/v) peat-to-perlite mixture and moved to greenhouse conditions. During acclimatization, the plants were fertilized weekly with a full-strength Hoagland nutrient solution (Hoagland and Arnon 1938) for four weeks. Thereafter, fertilization continued weekly using a 20–20–20 NPK fertilizer (Grow up®) at a concentration of 1 mg L^{-1}.

Statistical analysis

All experiments were conducted using a completely randomized design (CRD) with three replications, each consisting of 10 plants ($n=30$). For each treatment, a total of 30 plants were used, with five plants placed per culture jar, resulting in two jars per replication across three replications. Percentage data were arcsine-transformed prior to analysis. All data were subjected to analysis of variance (ANOVA) using SPSS software (version 18.0; SPSS Inc., Chicago, USA). Mean comparisons were performed using Tukey's Honest Significant Difference (HSD) test at a significance level of $p<0.05$.

Results

Shoot multiplication and growth

Different cytokinin types and concentrations significantly influenced shoot multiplication (Fig. 2a; Fig. 3; Table 2).

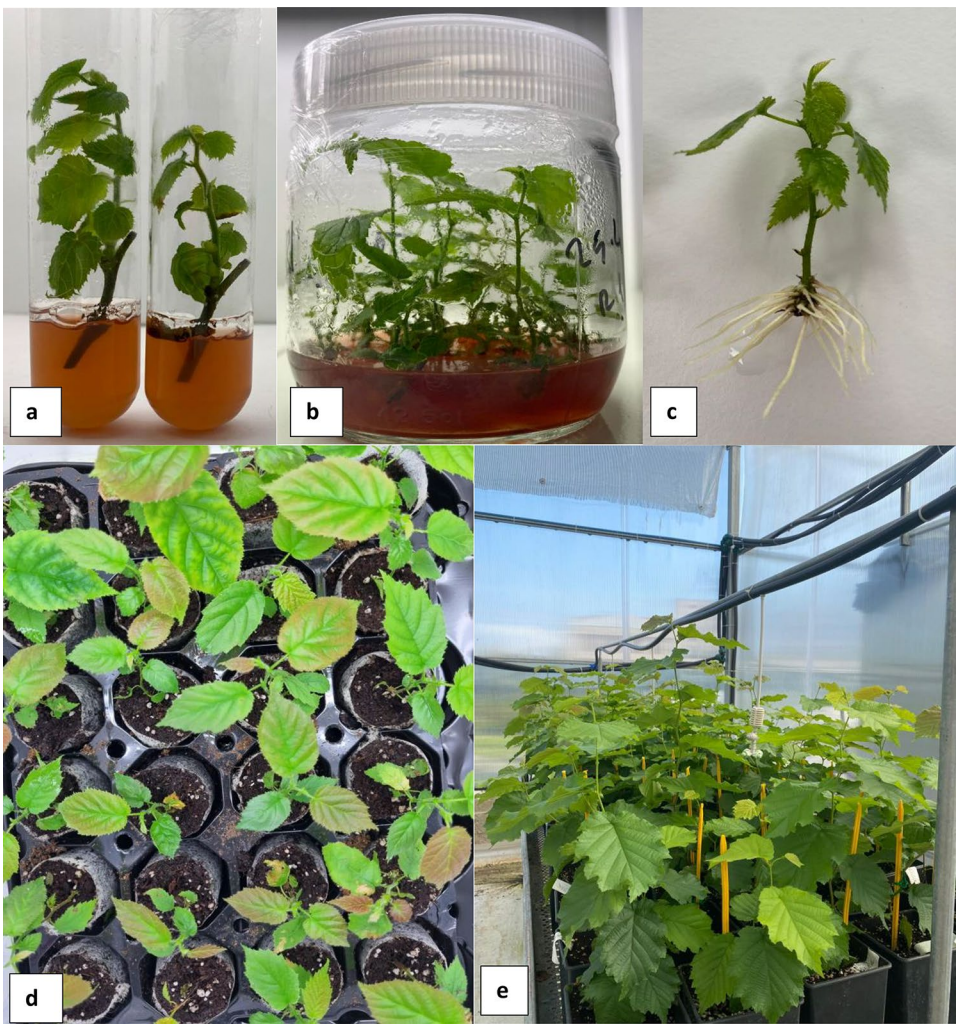
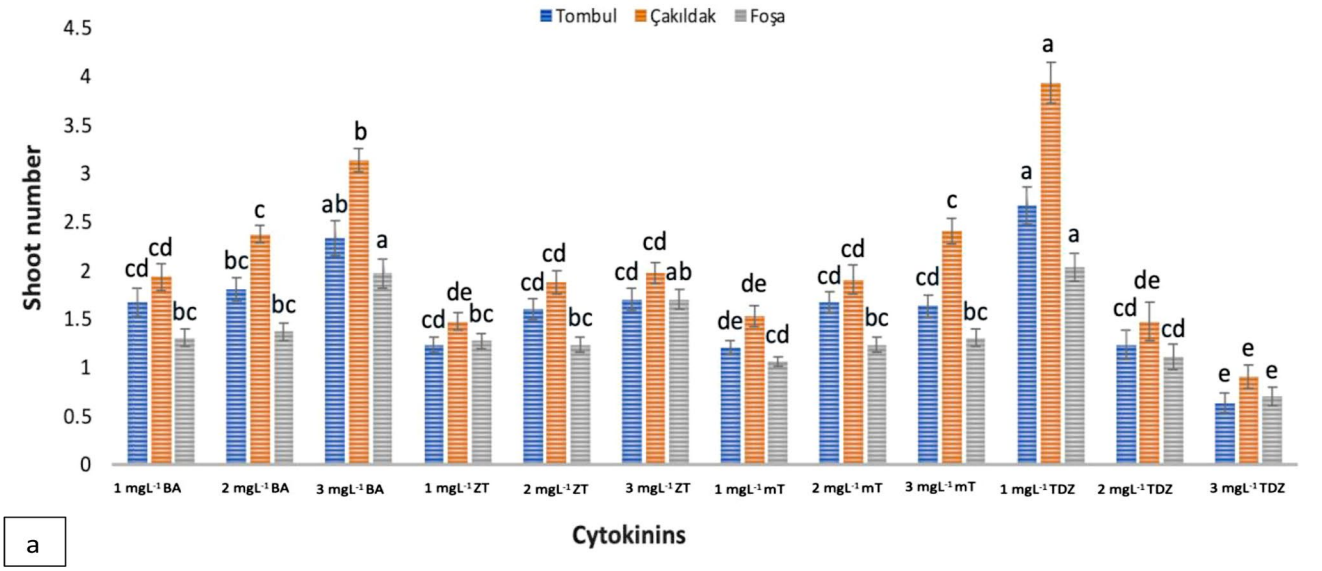


Fig. 1 Micropropagation of *Corylus avellana* 'Çakıldak' cultivar. (**a**) In vitro establishment of axillary buds. (**b**) Shoot multiplication on NRM medium supplemented with 3 mg L^{-1} BA and 0.01 mg L^{-1} IBA after 6 weeks of culture. (**c**) Rooted shoots developed in liquid NRM medium containing 2 mg L^{-1} IBA. (**d**) Rooted plantlets acclimatized for 4 weeks in trays containing a 3:1 (v/v) peat:perlite mixture. (**e**) Acclimatized hazelnut plants growing in the greenhouse

In the presence of a constant 0.01 mg L^{-1} IBA, shoot proliferation generally increased with increasing cytokinin concentration; however, the extent of the response varied depending on cytokinin type and cultivar. TDZ at 1 mg L^{-1} promoted high shoot multiplication in all cultivars, yielding 2.67, 3.93, and 2.03 shoots per explant in 'Tombul', 'Çakıldak', and 'Foşa', respectively. Among the other treatments, 3 mg L^{-1} BA resulted in high shoot multiplication in 'Tombul' and 'Foşa', while 3 mg L^{-1} ZT was particularly effective in 'Foşa'. Overall, TDZ, BA, and ZT were effective in promoting shoot proliferation, although optimal responses were achieved at different concentrations. In contrast, increasing the TDZ concentration to 3 mg L^{-1} markedly reduced shoot multiplication across all cultivars ('Tombul': 0.63, 'Çakıldak': 0.90, 'Foşa': 0.70), indicating an inhibitory effect at higher concentrations.

Shoot elongation varied depending on cytokinin type, concentration, and cultivar (Fig. 2b; Fig. 3; Table 2). mT treatments resulted in increased shoot length in all cultivars, with the highest values generally observed at 3 mg



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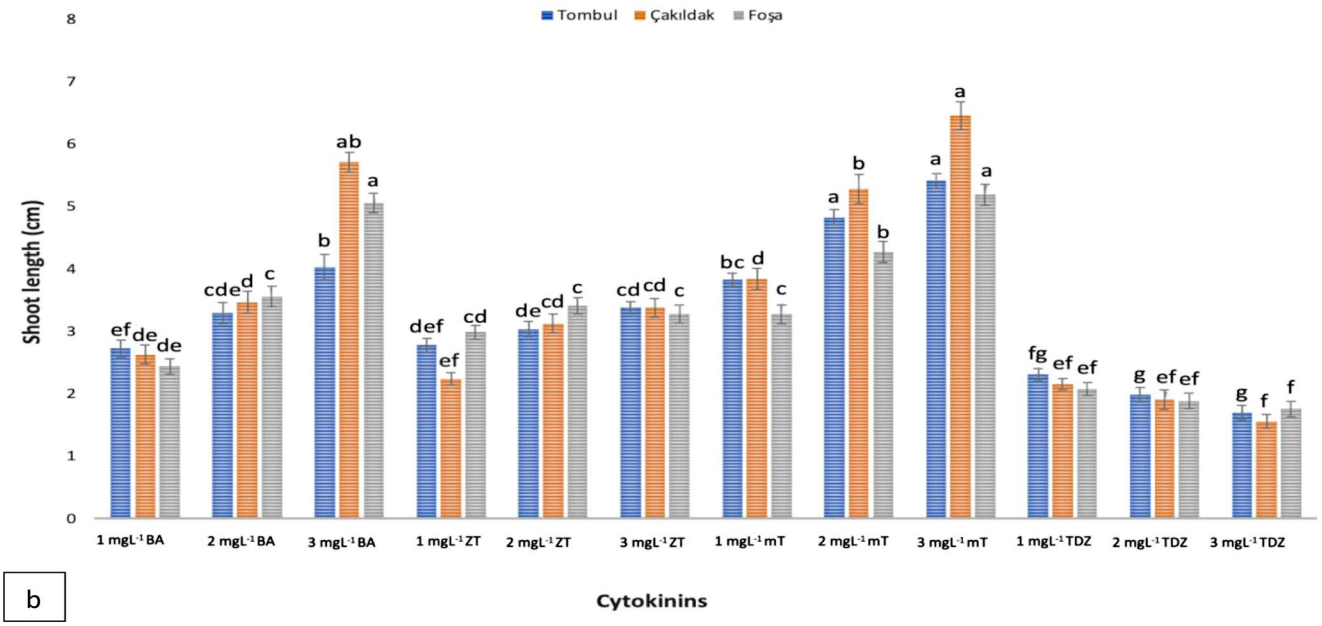

b

Fig. 2 Effect of cytokinin type and concentration on shoot proliferation and elongation of three hazelnut cultivars ('Tombul', 'Çakıldak', and 'Foşa') cultured for six weeks on NRM medium supplemented with 0.01 mg L^{-1} IBA, following two subcultures (three weeks each). (**a**) Mean number of shoots per explant. (**b**) Mean length of the longest shoot per explant. Data represent means ± SE (n = 30). Different lowercase letters above the bars indicate significant differences among treatments according to Tukey's HSD test (α = 0.05)



Fig. 3 Representative examples illustrating the best and poorest shoot proliferation responses of the 'Tombul' cultivar cultured for six weeks on NRM medium supplemented with 0.01 mg L^{-1} IBA and various cytokinin types and concentrations. (**a**) 3 mg L^{-1} BA (**b**) 3 mg L^{-1} ZT (**c**) 3 mg L^{-1} mT (**d**) 1 mg L^{-1} TDZ, and (**e**) 3 mg L^{-1} TDZ showing extensive callus formation and shoot tip necrosis (STN). (bar = 1 cm)

Table 2 Best treatments for shoot induction, shoot elongation, callus formation, and in vitro rooting in three hazelnut cultivars

Cultivar	Response parameters	Best treatments
Tombul	Shoot induction	3 mgL^{-1} BA; 1 mgL^{-1} TDZ
	Shoot elongation	2–3 mgL^{-1} mT
	Callus formation	2–3 mgL^{-1} TDZ
	Rooting	Plant agar + 2 mgL^{-1} IBA Bacto agar + 2 mgL^{-1} IBA Phytagel+ 1–2 mgL^{-1} IBA Liquid medium + 1–2 mgL^{-1} IBA
Çakıldak	Shoot induction	1 mgL^{-1} TDZ
	Shoot elongation	3 mgL^{-1} BA; 3 mgL^{-1} mT
	Callus formation	2–3 mgL^{-1} TDZ
	Rooting	Plant agar + 2 mgL^{-1} IBA Phytagel+ 1–2 mgL^{-1} IBA Liquid medium + 1–2 mgL^{-1} IBA
Foşa	Shoot induction	3 mgL^{-1} BA; 3 mgL^{-1} ZT; 1 mgL^{-1} TDZ
	Shoot elongation	3 mgL^{-1} BA; 3 mgL^{-1} mT
	Callus formation	2–3 mgL^{-1} TDZ
	Rooting	Plant agar + 2 mgL^{-1} IBA Bacto agar + 2 mgL^{-1} IBA Phytagel+ 1–2 mgL^{-1} IBA Liquid medium + 1–2 mgL^{-1} IBA

Treatments listed represent cytokinin types, concentrations, and rooting medium that produced the best responses ($p \leq 0.05$), highlighting genotype-dependent differences and providing practical guidance for protocol optimization

L^{-1} mT (5.40 cm in 'Tombul', 6.45 cm in 'Çakıldak', and 5.18 cm in 'Foşa'). However, shoot elongation obtained with 3 mg L^{-1} mT was similar to that achieved with 3 mg L^{-1} BA in 'Çakıldak' and 'Foşa'. In addition, in 'Tombul', shoot lengths at 2 mg L^{-1} mT were comparable to those recorded at 3 mg L^{-1} mT. Overall, both mT and BA supported increased shoot elongation, with comparable responses observed at specific concentrations. In contrast, TDZ treatments resulted in shorter shoots in all cultivars, and shoot length decreased progressively with increasing TDZ concentration. The shortest shoots were observed at 3 mg L^{-1} TDZ, measuring 1.69 cm in 'Tombul', 1.55 cm in 'Çakıldak', and 1.75 cm in 'Foşa'.

Hyperhydicity and STN

Hyperhydricity levels varied depending on cytokinin type, concentration, and cultivar (Table 1). TDZ resulted in the highest hyperhydricity values across all cultivars, with an increase observed as concentration increased. At 3 mg L^{-1} TDZ, hyperhydricity reached 80% in 'Tombul', 73.33% in 'Çakıldak', and 86.67% in 'Foşa'. Lower TDZ concentrations also induced substantial hyperhydricity across all cultivars.

BA treatments resulted in low to moderate hyperhydricity, with values increasing with concentration in all cultivars.

ZT induced only low levels of hyperhydricity, generally not exceeding 6.67%, whereas no hyperhydricity was recorded in any cultivar under mT treatments at any concentration.

STN was primarily associated with TDZ treatments and increased with concentration (Table 1). At 1 mg L^{-1} TDZ, STN values were uniform across cultivars (23.33%), rising to 31.03–43.33% at 2 mg L^{-1} TDZ and reaching the highest levels at 3 mg L^{-1} TDZ (50% in 'Tombul' and 'Çakıldak', and 56.67% in 'Foşa').

In contrast, BA treatments resulted in very low STN, with only limited symptoms (up to 6.67%) observed at the highest BA concentration in 'Foşa'. No STN was observed under any ZT or mT treatment in any cultivar.

Callus formation

Cytokinin treatments differed markedly in their effects on callus formation among the three hazelnut cultivars (Table 1). TDZ induced the highest levels of callus formation in all cultivars, with a clear increase as concentration increased. At 2 and 3 mg L^{-1} TDZ, callus formation reached 100% in 'Tombul', 'Çakıldak', and 'Foşa'. At 1 mg L^{-1} TDZ, callus formation was also substantial, ranging from 50% in 'Tombul' to 73% in 'Foşa'.

In contrast, BA, ZT, and mT resulted in low callus formation. In 'Tombul' and 'Çakıldak', BA induced little or no callus formation across concentrations, whereas in 'Foşa', BA treatments resulted in callus formation ranging from 16.67% to 23.33%. ZT showed a similar pattern, with no callus formation in 'Tombul' and 'Çakıldak' at 1 and 2 mg L^{-1}, while 'Foşa' exhibited limited callus development (16.67–23.33%) across ZT concentrations. mT produced minimal callus formation, with no callusing observed in 'Tombul' and 'Çakıldak', and only low levels (0–6.67%) recorded in 'Foşa'. Overall, among the cytokinins tested, TDZ resulted in substantially higher callus formation than BA, ZT, or mT across all cultivars.

In vitro rooting

Rooting success was influenced by both the gelling agent and IBA concentration, with responses differing among cultivars (Fig. 4a; Table 2). In 'Tombul', the highest rooting percentage was obtained in the liquid medium supplemented with 2 mg L^{-1} IBA. Comparable rooting levels were also achieved in plant agar supplemented with 2 mg L^{-1} IBA, phytagel supplemented with either 1 or 2 mg L^{-1} IBA, liquid medium supplemented with 1 mg L^{-1} IBA, and bacto agar supplemented with 2 mg L^{-1} IBA.

In 'Çakıldak', the highest rooting percentages were recorded in liquid medium supplemented with either 1 or 2 mg L^{-1} IBA, as well as in phytagel supplemented with 1 or 2 mg L^{-1} IBA. High rooting success was also observed in plant agar supplemented with 2 mg L^{-1} IBA, whereas bacto agar resulted in lower rooting percentages, particularly at the lower IBA concentration.

In 'Foşa', rooting was lower overall compared with 'Tombul' and 'Çakıldak'. The highest rooting success was obtained in liquid medium and phytagel supplemented with either 1 or 2 mg L^{-1} IBA. Plant agar supplemented with 2 mg L^{-1} IBA, as well as bacto agar supplemented with 2 mg L^{-1} IBA, supported moderate rooting, while bacto agar combined with 1 mg L^{-1} IBA resulted in the lowest rooting percentage.

Overall, high rooting percentages were achieved using several gelling agents when supplemented with appropriate IBA concentrations, and the optimal rooting conditions varied depending on the cultivar.

Root number varied depending on both the rooting medium and IBA concentration (Fig. 4b). In all three cultivars, the highest numbers of roots per shoot were obtained in the liquid medium supplemented with 2 mg L^{-1} IBA. This response was particularly pronounced in 'Çakıldak', where liquid medium combined with 2 mg L^{-1} IBA resulted in substantially greater root production than all semi-solid media.

In 'Foşa' high root numbers were also observed in plant agar and bacto agar supplemented with 2 mg L^{-1} IBA, as well as in phytagel and liquid media supplemented with 1 or 2 mg L^{-1} IBA. In 'Tombul', phytagel and plant agar supplemented with 2 mg L^{-1} IBA supported higher root numbers than the corresponding treatments with 1 mg L^{-1} IBA. In contrast, bacto agar generally resulted in lower root numbers across cultivars, especially at the lower IBA concentration.

Root length was strongly influenced by the rooting system and IBA level (Fig. 4c). In all cultivars, long roots were obtained in the liquid medium supplemented with 2 mg L^{-1} IBA. In 'Foşa', similarly long roots were also produced in phytagel supplemented with 2 mg L^{-1} IBA, indicating comparable root elongation between these two systems. In contrast, liquid medium containing 1 mg L^{-1} IBA and other semi-solid media generally resulted in shorter roots.

Overall, in vitro rooting responses varied among cultivars and were affected by both the gelling agent and IBA concentration. High rooting percentages, root numbers, and root lengths were obtained in liquid medium as well as in phytagel- and plant agar–solidified media supplemented with 1 or 2 mg L^{-1} IBA, whereas bacto agar generally resulted in lower values. Overall, 'Foşa' exhibited lower rooting performance than 'Tombul' and 'Çakıldak'.

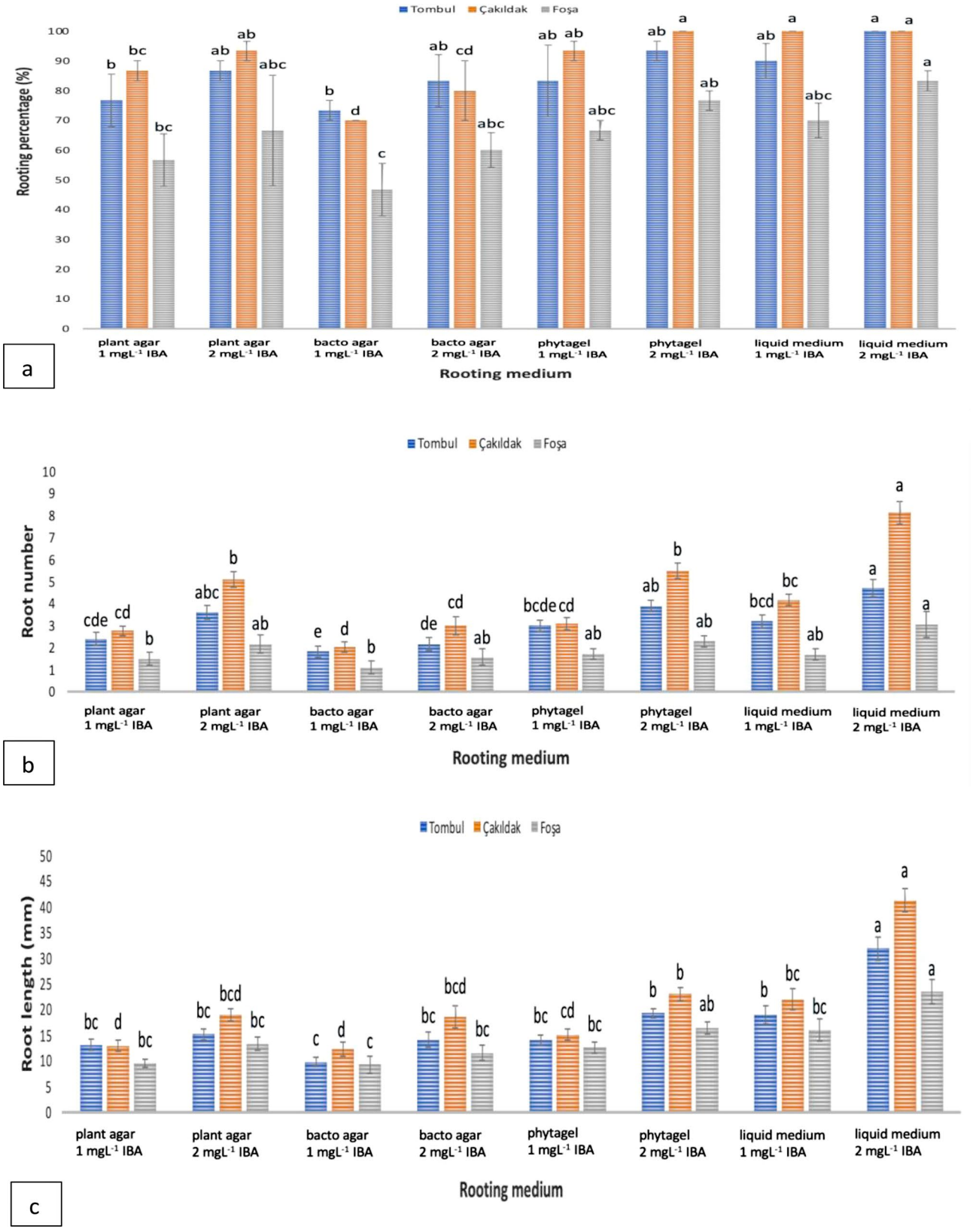

Fig. 4 In vitro rooting of three hazelnut cultivars ('Tombul', 'Çakıldak', and 'Foşa') cultured for 4 weeks on NRM medium supplemented with 1 or 2 mg L^{-1} IBA and solidified with different gelling agents or maintained as a liquid medium. (**a**) Rooting percentage, (**b**) number of roots per micro-shoot, and (**c**) root length. Values represent the effects of gelling agents on rooting performance and are presented as means ± SE ($n = 30$). Different lowercase letters above the bars indicate significant differences among treatments according to Tukey's HSD test ($\alpha = 0.05$)

Discussion

Micropropagation of hazelnut, as with many other woody species, is hindered by their low capacity for regeneration under in vitro conditions. A crucial step in overcoming this limitation is the optimization of plant growth regulators (PGR). Among these, cytokinins play a pivotal role in regulating cell division, promoting shoot initiation, and stimulating axillary bud development (Schaller et al. 2014; Yang et al. 2021). Yet, the effectiveness of cytokinin treatments often varies depending on genotype, highlighting the need for cultivar-specific protocols in hazelnut micropropagation (Hand et al. 2014). Excessive cytokinin levels or the use of unsuitable cytokinin types can trigger physiological abnormalities, including hyperhydricity and STN (Aremu et al. 2012). Therefore, careful selection of cytokinin type and concentration is essential to enhance shoot multiplication while minimizing such adverse effects, ultimately contributing to the development of reliable, genotype-adapted propagation systems. In this study, we evaluated the effects of different cytokinins combined with IBA across three Turkish cultivars, identifying cultivar-dependent variations in organogenic responses.

Shoot multiplication is a critical determinant of the overall efficiency of a micropropagation system. Among the cytokinins tested, BA consistently enhanced both shoot induction and elongation, confirming its role as the most reliable cytokinin for hazelnut culture. These results agree with previous reports showing that BA in the range of 1–5 mg L^{-1} effectively stimulates shoot proliferation in *C. avellana* (Messeguer and Mele 1987; Perez et al. 1987; Yu and Reed 1993; Hand et al. 2014; Akin et al. 2017, 2018; Sgueglia et al. 2019). Among BA treatments, 3 mg L^{-1} BA induced the highest shoot number (3.13 in 'Çakıldak') and also supported shoot elongation, corroborating earlier findings of Yu and Reed (1993) who reported superior shoot multiplication at this concentration. Although ZT supported shoot development, its effects were weaker than BA, in line with earlier studies where BA consistently outperformed ZT, kinetin and 2iP in hazelnut (Messeguer and Mele 1987; Perez et al. 1987; Thomson and Deering 2011). Nevertheless, ZT has been associated with improved bud break and elongation in certain hazelnut genotypes (Messeguer and Mele 1983; Bacchetta et al. 2008), suggesting that its role may depend on genetic background and culture environment.

TDZ, a phenylurea-derived PGR, exhibits both cytokinin- and auxin-like activities and has been successfully applied in various regeneration systems (Murthy et al. 1998; Guo et al. 2011). This synthetic regulator promotes adventitious shoot organogenesis (Cappelletti et al. 2016), somatic embryogenesis (Nautiyal et al. 2023), and callus formation (Chung and Ouyang 2020), and has shown particularly strong effects in woody species (Huetteman and Preece 1993; Novikova and Zaytseva 2018). At low concentrations (<200 µg), TDZ has been reported to induce greater axillary shoot formation compared with many other cytokinins, thereby enhancing shoot proliferation (Huetteman and Preece 1993). In this study, 1 mg L^{-1} TDZ significantly enhanced shoot proliferation in all cultivars, in agreement with Gao et al. (2008), who reported superior multiplication rates with TDZ compared to BA and ZT in hybrid hazelnuts ('Kuixiang'). However, higher concentrations (2–3 mg L^{-1}) resulted in hyperhydricity, STN, and excessive callusing, consistent with physiological disorders previously described in hazelnut and other woody plants (Gao et al. 2008; Cappelletti et al. 2016; Chung and Ouyang 2020).

Cytokinin type and cultivar exerted a significant influence on callus development. TDZ proved to be the most effective inducer, resulting in up to 100% callus formation at 2–3 mg L^{-1}, whereas BA, ZT, and mT elicited only minimal or negligible callogenic responses. TDZ is widely recognized as one of the most potent PGR for promoting callogenesis, and its strong inductive effect has been documented in blueberry (Cappelletti et al. 2016) and *Crocus sativus* (Moradi et al. 2018). The consistently higher responsiveness of the cultivar 'Foşa' across treatments indicates a genotype-dependent variation, corroborating earlier findings by Hand et al. (2014) and Akin et al. (2017), who attributed differences in callus induction to intrinsic variation in hormonal sensitivity and mineral nutrient uptake.

mT is a naturally occurring aromatic cytokinin originally isolated from poplar leaves (*Populus* × *canadensis*) (Strnad et al. 1997). Recently, it has been widely explored as a substitute for conventional cytokinins because it not only supports shoot proliferation but also reduces culture-related disorders such as hyperhydricity and STN (Abdouli et al. 2022). In the present study, mT promoted vigorous, elongated shoots free from hyperhydricity and STN, confirming earlier findings that highlight its benefits for shoot quality and overall plantlet development. The replacement of BA with mT has been shown to alleviate hyperhydricity in several species, including apple (*Malus domestica*) (Abdalla and Dobránszki 2024), pear (*Pyrus communis*) (Lotfi et al. 2020), and *Aloe* (Bairu et al. 2007), as well as other herbaceous and woody plants. Comparable outcomes were reported in hazelnut, where mT outperformed BA in certain cultivars (Sgueglia et al. 2019; Elçiçek 2020). Similarly, Gentile et al. (2017) observed that 2 mg L^{-1} mT not only improved shoot multiplication in *C. colurna* compared to BA but also enhanced rooting and acclimatization efficiency.

The rooting stage represents a crucial factor for the successful acclimatization of plantlets. Previous studies have shown that IBA is among the most effective auxins for inducing root formation in hazelnut (Yu and Reed 1995;

Damiano et al. 2005; Elçiçek 2020). In the present study, supplementation with 2 mg L^{-1} IBA generally resulted in the highest rooting percentage, root number, and root length across cultivars, which is in line with earlier reports (Damiano et al. 2005; Bacchetta et al. (2008). Root formation in hazelnut is known to be strongly genotype-dependent (Yu and Reed 1995; Bacchetta et al. (2008), and substantial variation in response to IBA among cultivars has been documented (Bassil et al. 1992; Latawa et al. 2016). Consistent with these findings, our results showed that 'Çakıldak' produced the highest number of roots, followed by 'Tombul' and 'Foşa'.

Medium composition was also found to play a decisive role. The liquid medium supported superior root induction compared to solidified media, achieving 100% rooting in 'Tombul' and 'Çakıldak', with root elongation up to 41 mm. Similar enhancements in root formation and elongation have been reported in temporary immersion systems Nicholson et al. (2020), highlighting the potential of liquid-based culture approaches for efficient hazelnut micropropagation. Comparable trends have also been described in other woody and herbaceous species such as *Malus domestica*, *Betula lenta*, and *Musa* spp., where liquid media promoted higher root production than agar-based substrates (Gangopadhyay et al. 2002; Shukla et al. 2020). In contrast, Latawa et al. (2016) reported that the physical state of the medium (liquid or solid) did not significantly influence root development in *C. avellana* under conditions with or without FeEDDHA supplementation.

Solidifying agents are another factor influencing rooting efficiency in vitro. Agar, a polysaccharide derived from seaweeds, has long been the most common gelling agent due to its affordability and gel strength (Gamborg et al. 1968). Gellan gum derivatives such as Phytagel™, on the other hand, are bacterial polysaccharides produced by *Pseudomonas elodea* (Saha and Datta 2022; Karamchand et al. 2023; Sworn 2009). Beyond simply solidifying the medium, these agents affect nutrient diffusion, thereby influencing morphogenetic responses and the growth of cultured explants (Das et al. 2015). To date, however, no comprehensive study has examined the role of different gelling agents specifically in hazelnut rooting. In the present work, phytagel proved more effective than plant agar and bacto agar. Similarly, Mohamed et al. (2021) observed that gellan gum and bacto agar significantly enhanced root formation in rice compared with agar. Conversely, Sulusoglu (2014) reported that bacto agar was more effective than agar and Oxoid gelatine in promoting in vitro rooting of a pear rootstock.

Overall, this study revealed pronounced genotype-dependent differences among the three hazelnut cultivars with respect to shoot induction, shoot elongation, callus formation, and in vitro rooting. Similar genotype-dependent responses have been widely reported in hazelnut and other woody species, where endogenous hormone balance, tissue competence, and developmental sensitivity play key roles in determining in vitro morphogenesis (Pérez-Jiménez et al. 2014; Elmoreigi et al. 2025). In the present study, the strong stimulation of shoot multiplication by TDZ at lower concentrations, together with increased hyperhydricity and STN at higher doses, is consistent with previous reports indicating that TDZ exhibits high cytokinin activity and may disrupt normal developmental regulation in sensitive genotypes (Dewir and Nurmansyah Naidoo 2018; Novikova and Zaytseva 2018). In contrast, the stable shoot growth and absence of physiological disorders observed under mT treatments suggest a more balanced cytokinin action, which has been associated with improved shoot quality in woody plants (Abdouli et al. 2022; Abdalla and Dobránszki 2024). Differential responses to BA, mT, and ZT further support earlier findings demonstrating cultivar-specific cytokinin sensitivity in hazelnut (Bacchetta et al. (2008) (Thomson and Deering 2011; Sgueglia et al. 2019).; Moreover, cultivar-dependent differences in rooting performance indicate genotype-specific auxin sensitivity and distinct hormone uptake dynamics between liquid and semi-solid culture systems. Collectively, these results highlight the importance of incorporating physiological and genotype-specific responses when optimizing in vitro propagation protocols for hazelnut.

Conclusion

This study shows that shoot multiplication, shoot growth, physiological responses, and callus formation in hazelnut are strongly influenced by cytokinin type, concentration, and cultivar. TDZ was effective for shoot induction and callus formation but was also associated with increased hyperhydricity and STN at higher concentrations. BA and mT supported shoot development and promoted shoot elongation with fewer physiological disorders. Rooting responses were affected by both IBA concentration and rooting system, with liquid medium and selected semi-solid media supplemented with 1 or 2 mg L^{-1} IBA supporting higher rooting percentages, root numbers, and root lengths. Overall, the results demonstrate clear genotype-dependent responses among the three hazelnut cultivars and provide an optimized basis for in vitro propagation.

Author contributions M.S. designed and conducted the micropropagation experiments, collected and interpreted the data, and wrote the manuscript. D.B. conducted the aseptic tissue experiments and collected the data. All authors have read and agreed to the published version of the manuscript.

Funding Open access funding provided by the Scientific and Technological Research Council of Türkiye (TÜBİTAK). This work was

supported by the General Directorate of Agricultural Research and Policies (TAGEM), Türkiye.

Data availability The authors confirm that the data will be available on request.

Declarations

Ethical approval Not applicable.

Conflict of interest The authors have no conflict of interest to declare.

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