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Effects of different calcium concentrations on *in vitro* shoot and root development in Gök Üzüm (*Vitis vinifera* L.) cultivar

Gök Üzüm (Vitis vinifera L.) çeşidinde in vitro sürgün ve kök gelişimine farklı kalsiyum konsantrasyonlarının etkisi

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ABSTRACT

In this study, the effects of different calcium ($CaCl_2 \cdot 2H_2O$) concentrations on shoot development, shoot tip necrosis (STN), and rooting were investigated in the Gök Üzüm (*Vitis vinifera* L.) cultivar under *in vitro* conditions. During the first subculture, although shoot formation was promoted in the 1 mg L^{-1} BAP treatment, the STN rate was found to be high. Culture media containing 120, 180 and 240.0 mg L^{-1} $CaCl_2 \cdot 2H_2O$ provided significant increases in parameters such as shoot length, number of nodes and leaf area compared to MS and BAP media. In addition, STN formation was completely eliminated at 180 and 240.0 mg L^{-1} calcium levels. The hormone-free MS0 medium gave the lowest values in all parameters. The suppression of STN was associated with the stabilizing effect of Ca^{2+} ions on the cell wall and the maintenance of plasma membrane integrity. In addition, Ca^{2+} plays an important role as a signaling element in plant physiology and influences hormonal regulations, particularly those related to auxin transport. During the rooting the highest root fresh weight was obtained with 240 mg L^{-1} $CaCl_2 \cdot 2H_2O$, indicating the contribution of calcium to root tissue development and integrity. The findings revealed that calcium enhances plantlet quality both morphologically and physiologically. Consequently, culture media enriched with 180–240 mg L^{-1} $CaCl_2 \cdot 2H_2O$ have the potential to increase the efficiency of grapevine micropropagation by promoting shoot development, reducing STN, and supporting root formation. Further studies are recommended to comprehensively evaluate the roles of different calcium sources and application strategies in plant tissue culture.

Key Words: Micropropagation, calcium, shoot tip necrosis, shoot development, plantlet quality

ÖZ

Bu çalışma kapsamında, Gök Üzüm (*Vitis vinifera* L.) çeşidinde *in vitro* koşullarda farklı kalsiyum ($CaCl_2 \cdot 2H_2O$) konsantrasyonlarının sürgün gelişimi, sürgün ucu nekrozu (STN) ve köklenme üzerine etkileri araştırılmıştır. Birinci altkültür aşamasında, 1 mg L^{-1} BAP uygulamasında sürgün oluşumunun teşvik edilmesine rağmen STN oranı yüksek bulunmuştur. 120, 180 ve 240.0 mg L^{-1} $CaCl_2 \cdot 2H_2O$ içeren kültür ortamları ise; sürgün uzunluğu, boğum sayısı ve yaprak alanı gibi parametrelerde MS ve BAP ortamlarına göre anlamlı artış sağlamıştır. Ayrıca, 180 ve 240.0 mg L^{-1} kalsiyum düzeylerinde STN oluşumu tamamen ortadan kalkmıştır. Hormon içermeyen MS0 ortamı ise tüm parametrelerde en düşük değerleri vermiştir. STN'nin baskılanması, Ca^{2+} iyonunun hücre duvarı stabilitesi ile plazma membranı bütünlüğünü koruyucu etkisiyle ilişkilendirilmiştir. Bunun yanında, Ca^{2+} bitki fizyolojisinde önemli bir sinyal iletim elemanı olarak görev yapmakta ve özellikle oksin taşınımıyla ilişkili hormonal düzenlemeleri etkilemektedir. Köklenme aşamasında ise 240 mg L^{-1} $CaCl_2 \cdot 2H_2O$

dozunda en yüksek kök ağırlığı elde edilmiş; bu da kalsiyumun kök dokularının gelişimi ve bütünlüğüne katkı sağladığını göstermiştir. Elde edilen bulgular, kalsiyumun hem morfolojik hem de fizyolojik açıdan bitkicik kalitesini artırdığını ortaya koymaktadır. Sonuç olarak, 180–240 mg L^{-1} $CaCl_2 \cdot 2H_2O$ içeren kültür ortamlarının sürgün gelişimini teşvik ederek, STN'yi azaltarak ve kök gelişimini destekleyerek asma mikroçoğaltımında verimliliği artırma potansiyeline sahip olduğu kaydedilmiştir. Gelecek çalışmalarda, farklı kalsiyum kaynaklarının ve uygulama stratejilerinin bitki doku kültürü süreçlerindeki rolünün daha kapsamlı biçimde değerlendirilmesi önerilmektedir.

Anahtar Kelimeler: Mikroçoğaltım, kalsiyum, sürgün ucu nekrozu, sürgün gelişimi, bitkicik kalitesi

Introduction

Grapevine (*Vitis vinifera* L.) is one of the oldest and most economically significant fruit crops globally, widely used for wine, table grapes, and raisins (OIV, 2023). Viticulture plays a vital role in global agriculture, with ongoing efforts to enhance yield and quality through cultivar development and innovative approaches. According to FAOSTAT (2023), grapes are cultivated on approximately 6.6 million hectares worldwide, yielding about 72.5 million tons annually; Türkiye ranks sixth in vineyard area with 377,848 hectares and seventh in grape production with nearly 3.4 million tons. Among local cultivars, 'Gök Üzüm' is an ancient cultivar traditionally grown in Konya, Türkiye, known for its strong ecological adaptation and role as a pollinator, especially for 'Ekşi Kara' (Kara et al., 2016; Kara et al., 2017). Due to its genetic and agronomic value, research on its propagation is crucial for sustaining traditional viticulture.

Tissue culture techniques have become increasingly important in viticulture, owing to their capacity to eliminate pathogens, maintain genetic uniformity, and enable rapid large-scale propagation under sterile conditions (Dutt et al., 2006; Gammoudi et al., 2022; Dias et al., 2013; Sağlam et al., 2023). In grapevine, cultivar-specific protocols have been developed to fully exploit these advantages, especially in economically valuable varieties (Kassa and Feyissa, 2020; Ekinci et al., 2024).

Shoot tip necrosis (STN) is a frequent physiological disorder that adversely affects *in vitro* propagation of woody plant species, including grapevine (Bairu et al., 2009; Surakshitha et al., 2019; Teixeira da Silva et al., 2020). This condition is initially characterized by browning at the apical shoot tip, followed by downward progression that may result in the complete degeneration of shoots and consequently reduces the efficiency of micropropagation. The occurrence of this disorder is linked to several factors such as medium composition, types and concentrations of plant growth regulators, frequency of subculturing, and restricted transpiration caused by high humidity in closed culture vessels. Among these factors, calcium plays a critical role due to its involvement in maintaining membrane stability, strengthening cell walls, and regulating intracellular signaling processes, particularly in meristematic tissues (Hirschi, 2004; Hepler, 2005). The application of calcium in the form of calcium chloride has been reported to alleviate shoot tip necrosis in various plant species by contributing to structural integrity and hormonal regulation (Barghchi and Alderson, 1996; Piagnani et al., 1996; Surakshitha et al., 2019). However, excessively high calcium concentrations may aggravate the symptoms in certain cultivars, indicating that optimal levels must be carefully adjusted (Bairu et al., 2009).

The effects of cytokinins, particularly benzyladeninepurine (BAP), on shoot tip necrosis have been extensively studied. Although BAP is commonly used to promote shoot proliferation, it has been reported to induce or exacerbate shoot tip necrosis in certain species and genotypes depending on its concentration (Piagnani et al., 1996; Bairu et al., 2009). In some cases, cytokinin deficiency leads to reduced cell division in the apical meristem and subsequent necrosis, whereas in others, particularly high concentrations of BAP can worsen these symptoms. Therefore, managing shoot tip necrosis largely depends on maintaining a balance

between cytokinin levels and calcium availability. Recent studies have shown that optimizing calcium and boron levels along with balanced phosphate nutrition improves shoot quality and reduces apical degeneration in grapevine cultures (Al-Aizari et al., 2020; Teixeira da Silva et al., 2020). These findings highlight the importance of tailoring culture media formulations to specific species and genotypes for successful *in vitro* shoot proliferation and healthy plantlet development.

In this study, five treatments were evaluated: hormone-free Murashige and Skoog (MS0) medium; MS medium supplemented with 1 mg L^{-1} benzylaminopurine (BAP); and MS medium containing 1 mg L^{-1} BAP in combination with additional calcium at concentrations of 120.0, 180.0, and 240.0 mg L^{-1}. The effects of these treatments on subculture success, rooting, and the quality of the plantlets were investigated with the aim of improving micropropagation efficiency in the Gök Üzüm grape cultivar.

Materials and Method

Plant material consisted of explants collected from the Gök Üzüm grapevine cultivar obtained from the clone comparison vineyard at the Faculty of Agriculture Selçuk University during the active growth period. Single-node microcuttings were excised from these plants and subjected to surface sterilization under aseptic conditions in a vertical laminar flow cabinet. The sterilization procedure involved sequential immersion of nodal explants in 70% ethanol for 2 minutes, followed by treatment with 12% sodium hypochlorite (NaOCl) for 15 minutes. Explants were then rinsed three times with sterile distilled water to remove any residual disinfectants. Following surface sterilization, nodal explants were cultured on Murashige and Skoog (MS) medium supplemented with 3% (w/v) sucrose and solidified with 0.7% (w/v) agar. At this stage, five treatment combinations were evaluated: hormone-free MS medium (MS 0), MS supplemented with 1 mg L^{-1} BAP, and MS enriched with 1 mg L^{-1} BAP in combination with calcium chloride at concentrations of 120.0, 180.0, and 240.0 mg L^{-1}. The explants were maintained in this initial culture phase for a total of 13 days under growth room conditions set to 25 ± 1°C, with a 16-hour light/8-hour dark photoperiod and approximately 4000 lux light intensity (Kara and Yazar, 2020).

After the initial phase, explants were transferred to the first subculture stage, where the same treatment combinations were reapplied. This phase lasted for four weeks and was carried out under the same environmental conditions as the initial culture (Al-Aizari et al., 2020; Teixeira da Silva et al., 2020). Following four weeks of incubation in the subculture phase, regenerated shoots were transferred to the rooting medium composed of MS supplemented with 1 mg L^{-1} indole-3-butyric acid (IBA) (Ekinci et al., 2024). Thus, no cytokinin was present during the rooting stage, and differences among treatments at this phase reflect carry-over effects from earlier shoot culture conditions. During the initial culture phase, the evaluated parameters included shoot formation rate (%), number of nodes, shoot length, and number of shoots per explant. In the first subculture, assessments focused on shoot formation rate (%), number of shoots per explant, shoot length, number of nodes, and incidence of shoot tip necrosis. At the rooting stage (after six weeks of rooting), measurements included root fresh weight, shoot fresh weight, shoot length, number of nodes, internode length, internode diameter, rooting rate (%), root length, root number, leaf area, and number of shoots. (Kara and Yazar, 2020; Ekinci et al., 2024). Leaf area measurements were conducted using scanned leaf images analyzed with Photoshop Portable Sfx (Gomes et al., 2015). Each treatment was replicated three times with 15 plantlets per replicate.

Data analysis

The experimental data were statistically analyzed using Tukey's HSD test ($p < 0.05$) in JMP version 13 to identify significant differences

among treatments. Additionally, hierarchical cluster analysis (HCA) was performed using R software (v4.1.1, R Foundation for Statistical Computing) to examine the correlations among variables.

Results and Discussion

Initial culture

Shoot formation and initial growth parameters during the initial culture phase

During the initial culture, shoot formation rate, number of nodes, shoot length, and number of shoots per explant were evaluated to assess the early morphogenetic responses of grapevine explants. Statistical analysis revealed that all parameters except shoot number showed significant differences among the applications ($p < 0.05$).

The shoot formation rate reached 100% in both the BAP (1 mg L^{-1}) and 180 mg L^{-1} calcium applications, followed closely by 240 mg L^{-1} (97.22%) and 120 mg L^{-1} (95.24%). In contrast, the MS (0) medium resulted in a significantly lower formation rate of 80.56%. These results indicate that both cytokinin and calcium play a beneficial role in shoot induction, with calcium applications performing similarly or slightly better than BAP (Table 1).

Regarding the number of nodes, the highest values were observed in the 240 mg L^{-1} (3.44) and 180 mg L^{-1} (3.42) calcium applications, comparable to the BAP treatment (3.33). The 120 mg L^{-1} application supported moderate nodal development (2.33), while MS (0) produced the fewest nodes (1.67). These results suggest that calcium, particularly at higher concentrations, may enhance nodal differentiation during early shoot development (Table 1).

Shoot length was also significantly affected by the applications. The longest shoots were observed in the 180 mg L^{-1} calcium application (2.68 cm), followed by 240 mg L^{-1} (2.59 cm), BAP (2.57 cm).and 120 mg L^{-1} (2.55 cm). Shoots formed in MS (0) medium were considerably shorter (1.89 cm), confirming the supportive role of both BAP and calcium in promoting shoot elongation (Table 1).

In contrast, the number of shoots per explant remained constant (1.00) across all treatments, with no statistically significant differences. This uniformity indicates that none of the applications induced multiple shoot formation at this early stage, and the observed effects were mainly limited to initiation and elongation rather than proliferation (Table 1).

Table 1. Effects of different calcium concentrations on shoot formation rate, shoot length, number of nodes, and number of shoots per explant in '*Gök Üzüm*' during the initial culture phase*

Applications	Shoot Formation Rate (%)	Shoot Length (cm)	Number of Nodes (per explant)	Number of Shoots (per explant)
MS (0)	80.56 ± 4.81b	1.89 ± 0.37b	1.67 ± 0.58b	1 ± 0.0a
BAP (1 mg l^{-1})	100.00 ± 0.00a	2.57 ± 0.07a	3.33 ± 0.58a	1 ± 0.0a
Ca (120 mg l^{-1})	95.24 ± 4.12a	2.55 ± 0.04a	2.33 ± 0.58ab	1 ± 0.0a
Ca (180 mg l^{-1})	100.00 ± 0.00a	2.68 ± 0.04a	3.42 ± 0.52a	1 ± 0.0a
Ca (240 mg l^{-1})	97.22 ± 4.81a	2.59 ± 0.05a	3.44 ± 0.51a	1 ± 0.0a

*Means followed by different letters in the same column are significantly different according to Tukey's HSD test ($p < 0.05$).

Shoot formation and morphological development during the first subculture phase

During the first subculture phase, the responses of grapevine explants to different treatments were evaluated in terms of shoot formation rate, number of shoots per explant, shoot length, and number of nodes. Statistical analysis revealed that all parameters were significantly affected by the treatments ($p < 0.05$, Table 2).

Shoot formation rate reached the highest value (100%) with the application of 180 mg L^{-1} calcium, followed by 240 mg L^{-1} Ca (94.44%), BAP and 120 mg L^{-1} Ca treatments (both 83.33%). The lowest rate was recorded in MS (0) medium (55.56%). These findings indicate that both BAP

and calcium positively influenced shoot formation, with 180 mg L^{-1} Ca showing the most prominent effect.

Number of shoots per explant was highest in the 120 mg L^{-1} Ca treatment (2.83), followed by 180 mg L^{-1} (2.58), 240 mg L^{-1} (2.52), and BAP (1.83). The lowest value (1.00) was obtained from MS (0) medium. These results demonstrate the promotive effect of calcium on shoot proliferation.

Shoot length was also significantly enhanced in the 120 and 180 mg L^{-1} Ca applications (2.63 cm), while lower values were observed in 240 mg L^{-1} (2.38 cm), BAP (2.04 cm), and MS (0) (1.24 cm). These results confirm the stimulatory role of Ca^{2+} in shoot elongation.

Number of nodes was highest with 180 mg L^{-1} Ca (3.33), followed by 120 and 240 mg L^{-1} (both 2.33), BAP (1.92), and MS (0) (1.33). These findings suggest that higher calcium concentrations promote shoot segmentation more effectively than hormone-free conditions (Table 2).

Table 2. Effects of different calcium concentrations on shoot formation rate, number of shoots, shoot length, and number of nodes per explant in 'Gök Üzüm' during the first subculture phase*

Applications	Shoot Formation Rate (%)	Number of Shoot (per explant)	Shoot Length (cm)	Number of Nodes (per explant)
MS (0)	55.56 ± 9.62b	1.00 ± 0.00c	1.24 ± 0.04d	1.33 ± 0.58b
BAP (1 mg l^{-1})	83.33 ± 8.33a	1.83 ± 0.14b	2.04 ± 0.03c	1.92 ± 0.14b
Ca (120 mg l^{-1})	83.33 ± 4.12a	2.83 ± 0.08a	2.63 ± 0.04a	2.33 ± 0.58ab
Ca (180 mg l^{-1})	100.0 ± 0.00a	2.58 ± 0.14a	2.63 ± 0.05a	3.33 ± 0.58a
Ca (240 mg l^{-1})	94.44 ± 4.81a	2.52 ± 0.13a	2.38 ± 0.02b	2.33 ± 0.58ab

*Means followed by different letters in the same column are significantly different according to Tukey's HSD test ($p < 0.05$).

In addition to these morphological traits, shoot tip necrosis was also assessed. The highest incidence was observed in MS (0) (38.91%) and BAP (30.44%) applications. In contrast, a substantial reduction was observed in all calcium-enriched media. The 120 mg L^{-1} calcium application resulted in a low necrosis rate of 5.55%, while both the 180 mg L^{-1} and 240 mg L^{-1} applications completely eliminated the disorder (0.00%). These results clearly demonstrate the preventive effect of calcium against shoot tip necrosis, likely due to its role in maintaining membrane stability and meristem integrity (table 3).

Table 3. Effect of different calcium concentrations on shoot tip necrosis (%) in 'Gök Üzüm' during the first subculture stage*

Applications	Shoot Tip Necrosis (%)
MS (0)	38.91 ± 4.83a
BAP (1 mg l^{-1})	30.44 ± 4.72a
Ca (120 mg l^{-1})	5.55 ± 4.81b
Ca (180 mg l^{-1})	0.00± 0.00b
Ca (240 mg l^{-1})	0.00 ± 0.00b

*Means followed by different letters in the same column are significantly different according to Tukey's HSD test ($p < 0.05$).

Rooting stage

Effects of calcium on shoot development during rooting stage

During the rooting, all evaluated shoot development parameters were significantly influenced by calcium supplementation ($p < 0.05$).

For shoot length, the longest shoots were recorded in the treatment with 120 mg L^{-1} calcium (8.98 cm), followed by 180 mg L^{-1} (7.42 cm) and 240 mg L^{-1} (6.76 cm). These values were significantly greater than those observed in the BAP-only medium (4.37 cm) and the hormone-free control (0.67 cm). These results indicate that the presence of calcium, especially at moderate concentrations, substantially promotes shoot elongation during the rooting period (Table 4).

In terms of shoot fresh weight, the highest value was observed under the 240 mg L^{-1} calcium treatment (2.66 g), indicating a considerable improvement in biomass accumulation compared to all other treatments. The 180 mg L^{-1} and 120 mg L^{-1} calcium concentrations supported moderate shoot fresh weights (1.12 g and 0.86 g, respectively), while much lower values were recorded in the BAP (0.34 g) and control (0.25 g) groups. These findings suggest that increased calcium availability enhances physiological development and contributes to shoot mass formation (Table 4).

For number of shoots per explant, the 180 mg L^{-1} calcium treatment was the most effective, with an average of 7.33 shoots. The treatments with 240 mg L^{-1} and 120 mg L^{-1} calcium followed with 3.33 and 1.92 shoots, respectively. In contrast, the BAP and control treatments resulted in only one shoot per explant, indicating minimal shoot proliferation in the absence of calcium. These results highlight the promotive effect of calcium on axillary shoot initiation, particularly when applied at optimal levels (Table 4).

Table 4. Effects of different calcium concentrations on shoot length, shoot fresh weight, and number of shoots per explant in 'Gök Üzüm' during the rooting stage*

Applications	Shoot length (cm)	Shoot fresh weight (g)	Number of shoots (per explant)
MS (0)	0.67 ± 0.07d	0.25 ± 0.04d	1.00 ± 0.00c
BAP (1 mg l^{-1})	4.37 ± 0.08c	0.34 ± 0.03d	1.00 ± 0.00c
Ca (120 mg l^{-1})	8.98 ± 0.53a	0.86 ± 0.02c	1.92 ± 0.14bc
Ca (180 mg l^{-1})	7.42 ± 1.06ab	1.12 ± 0.15b	7.33 ± 1.53a
Ca (240 mg l^{-1})	6.76 ± 1.19b	2.66 ± 0.09a	3.33 ± 0.58b

*Means followed by different letters in the same column are significantly different according to Tukey's HSD test ($p < 0.05$). The 'BAP' group refers to plantlets previously cultured on BAP-supplemented shoot induction medium; all rooting was performed on MS + IBA medium for all groups.

Overall, calcium-enriched media significantly improved all shoot-related parameters during the rooting stage when compared to the standard BAP medium without calcium. Among the calcium treatments, 180 mg L^{-1} was the most effective in enhancing shoot proliferation, while 120 mg L^{-1} was more favorable for shoot elongation. These outcomes emphasize the critical role of calcium in supporting *in vitro* plantlet development under rooting conditions.

Morphological traits related to internodal and foliar development

During the rooting stage, node number, internode length, internode diameter, and leaf area were all significantly influenced by the applications ($p < 0.05$).

The highest node number was observed in the 120 mg L^{-1} calcium application (11.0), which was significantly greater than those recorded in the MS (0) (1.92) and BAP (7.43) media. Although 180 mg L^{-1} (8.0) and 240 mg L^{-1} (7.0) calcium applications also promoted nodal development, their effects remained lower than the 120 mg L^{-1} level. These findings indicate that moderate calcium supplementation can enhance nodal differentiation more effectively than BAP alone (Table 5).

Internode length was also positively affected by calcium. The longest internodes were formed in plantlets treated with 240 mg L^{-1} calcium (1.0 cm), followed by 180 mg L^{-1} (0.86 cm) and 120 mg L^{-1} (0.78 cm). These values were significantly higher than those measured in the BAP (0.59 cm) and MS (0) (0.16 cm) applications, indicating that calcium facilitates internodal elongation during the rooting stage (Table 5).

In terms of internode diameter, the thickest stems developed under the 240 mg L^{-1} calcium application (0.25 cm), showing a significant increase compared to BAP (0.12 cm) and MS (0) (0.13 cm). The applications of 120 mg L^{-1} Ca (0.17 cm) and 180 mg L^{-1} Ca (0.16 cm) resulted in intermediate values. These results suggest that calcium contributes to increased stem robustness, particularly at higher concentrations (Table 5).

The most substantial leaf area was recorded in the 240 mg L^{-1} calcium group (2.85 cm^2), followed by 120 mg L^{-1} (2.27 cm^2) and 180 mg L^{-1} (1.37 cm^2). In contrast, BAP (0.40 cm^2) and MS (0) (0.25 cm^2) applications resulted in markedly smaller leaf surfaces. These results reflect calcium's potential to enhance foliar development, likely through improved cell expansion and physiological activity (Table 5).

All measured morphological parameters exhibited statistically significant improvements in response to calcium-enriched applications when compared to the standard BAP medium. The 120 mg L^{-1} calcium application proved to be the most effective in enhancing node formation, whereas the 240 mg L^{-1} concentration resulted in the greatest increases in internode length, stem diameter, and leaf area. These results clearly demonstrate the beneficial role of calcium in supporting structural and foliar development during the rooting phase of *in vitro*-propagated grapevine plantlets.

Table 5. Effects of different calcium concentrations on number of nodes, internode length, internode diameter, and leaf area of '*Gök Üzüm'* plantlets during the rooting stage*

Applications	Number of nodes (per plantlet)	Internode length (cm)	Internode diameter (cm)	Leaf area (cm^2)
MS (0)	1.92 ± 0.14c	0.16 ± 0.02d	0.13 ± 0.01cd	0.25 ± 0.03d
BAP (1 mg l^{-1})	7.43 ± 0.58b	0.59 ± 0.06c	0.12 ± 0.03d	0.40 ± 0.04d
Ca (120 mg l^{-1})	11.0 ± 1.0a	0.78 ± 0.01b	0.17 ± 0.01b	2.27 ± 0.07b
Ca (180 mg l^{-1})	8.0 ± 1.0ab	0.86 ± 0.05b	0.16 ± 0.01bc	1.37 ± 0.05c
Ca (240 mg l^{-1})	7.0 ± 1.0b	1.0 ± 0.05a	0.25 ± 0.02a	2.85 ± 0.08a

**Means followed by different letters in the same column are significantly different according to Tukey's HSD test ($p < 0.05$). The 'BAP' group refers to plantlets previously cultured on BAP-supplemented shoot induction medium; all rooting was performed on MS + IBA medium for all groups.

Root development parameters during the rooting stage

Root fresh weight, rooting rate, root length, and root number were significantly influenced by the different applications ($p < 0.05$).

The MS (0) medium, which lacked both growth regulators and calcium supplementation, resulted in the lowest values across all root parameters. Root fresh weight was limited to 0.39 g, the rooting rate was 13.33%, root length averaged 1.09 cm, and only 1.67 roots were formed per plantlet. These results emphasize the inadequacy of hormone-free medium in supporting root induction and development (Table 6).

In contrast, the BAP (1 mg L^{-1}) application achieved full rooting (100%) and showed a moderate root fresh weight of 1.38 g, root length of 3.26 cm, and the highest root number among all treatments (6.00). This indicates that while BAP enhances rooting efficiency and lateral root formation, it provides limited stimulation for root elongation and biomass accumulation compared to calcium-enriched media (Table 6).

Among the calcium-supplemented applications, 120 mg L^{-1} calcium led to the highest root length (16.27 cm) and a substantial root fresh weight of 3.19 g, although root number (2.67) remained lower than in the BAP treatment. The 180 mg L^{-1} calcium application resulted in improved root biomass (4.35 g), slightly shorter roots (13.51 cm), and a higher root number (4.48). The 240 mg L^{-1} calcium application produced the highest root fresh weight overall (4.54 g), a root length of 14.82 cm, and a comparable root number (4.75). All calcium treatments, except for 240 mg L^{-1}, achieved 100% rooting, while the latter maintained a high rate of 96.67% (Table 6).

Compared to BAP, which primarily promoted rooting rate and root number, calcium applications provided a more balanced enhancement of both root biomass and length, indicating their superior role in structural root development. Notably, 120 mg L^{-1} calcium was most effective in stimulating root elongation, while 240 mg L^{-1} promoted the greatest increase in root mass. Both 180 mg L^{-1} and 240 mg L^{-1} applications supported high root numbers, similar

to those obtained with BAP, while also contributing to greater root system robustness (Table 6).

These results underline the beneficial role of calcium in promoting a well-developed root system during *in vitro* rooting. While BAP alone was effective in triggering root initiation, calcium supplementation, particularly at 180 and 240 mg L^{-1}, was more advantageous in enhancing overall root quality in terms of weight, length, and structure.

Table 6. Effects of different calcium concentrations on root fresh weight, rooting rate, root length, and number of roots per plantlet in 'Gök Üzüm' during the rooting phase.*

Applications	Root fresh weight (g)	Rooting rate (%)	Root length (cm)	Number of roots (per plantlet)
MS (0)	0.39 ± 0.01e	13.33 ± 5.77b	1.09 ± 0.04d	1.67 ± 0.58b
BAP (1 mg l^{-1})	1.38 ± 0.01d	100.0 ± 0.00a	3.26 ± 0.25c	6.00 ± 1.00a
Ca (120 mg l^{-1})	3.19 ± 0.01c	100.0 ± 0.00a	16.27 ± 1.05a	2.67 ± 0.58b
Ca (180 mg l^{-1})	4.35 ± 0.09b	100.0 ± 0.00a	13.51 ± 1.11b	4.48 ± 0.50a
Ca (240 mg l^{-1})	4.54 ± 0.04a	96.67 ± 5.77a	14.82 ± 0.74ab	4.75 ± 0.43a

**Means followed by different letters in the same column are significantly different according to Tukey's HSD test ($p < 0.05$). The 'BAP' group refers to plantlets previously cultured on BAP-supplemented shoot induction medium; all rooting was performed on MS + IBA medium for all groups.

Hierarchical cluster analysis (HCA)

The hierarchical cluster analysis (HCA) presented in the heatmap, based on data from the first subculture stage, reveals distinct groupings among the effects of different calcium treatments and control media on key morphological parameters of 'Gök Üzüm' plantlets. The 180 mg L^{-1} $CaCl_2 \cdot 2H_2O$ treatment formed a separate cluster, reflecting the most favorable impact with significantly enhanced shoot length, number of nodes, shoot formation rate, and number of shoots, along with the lowest shoot tip necrosis (STN) rate. This highlights a strong positive morphogenetic response to this calcium dose during the early culture stage. The 120 mg L^{-1} treatment also showed moderate improvements, especially in shoot elongation, while 240 mg L^{-1} occupied an intermediate position, indicating partial enhancement. In contrast, MS (0) and BAP controls clustered together, associated with reduced morphological development and high shoot tip necrosis (STN) incidence, suggesting suboptimal growing conditions. Notably, shoot tip necrosis (STN) clustered independently and inversely with growth parameters, emphasizing its antagonistic role in shoot quality. Overall, the HCA findings clearly demonstrate that calcium supplementation, particularly at 180 mg L^{-1} $CaCl_2 \cdot 2H_2O$, supports shoot development and reduces physiological stress in grapevine plantlets during the first subculture phase (Figure 1).

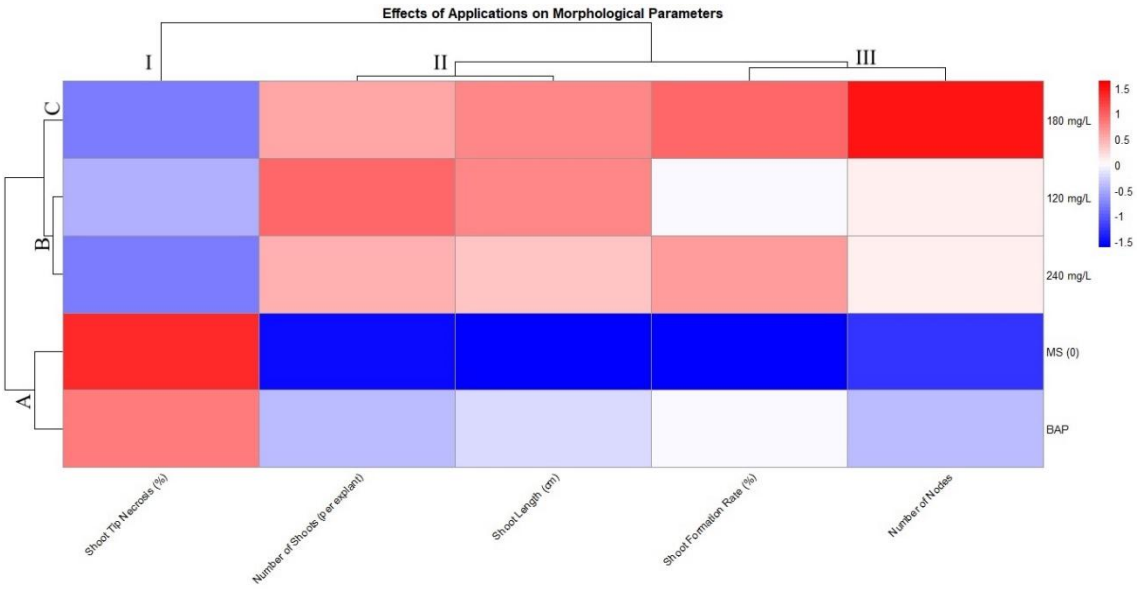

Figure 1. *Hierarchical Cluster Analysis (HCA) of the first subculture stage based on morphological parameters including shoot tip necrosis (STN), number of shoots per explant, shoot length, shoot formation rate, and number of nodes in Gök Üzüm (Vitis vinifera L.) plantlets cultured on MS medium supplemented with different concentrations of calcium ($CaCl_2 \cdot 2H_2O$).*

The hierarchical cluster analysis (HCA) of the rooting stage parameters revealed a clear separation among treatments based on their effects on root and shoot development. The application of 240 mg L^{-1} $CaCl_2 \cdot 2H_2O$ formed a distinct cluster (C), characterized by the highest shoot fresh weight, root fresh weight, root length, internode length, and leaf area, suggesting that this concentration most effectively enhanced both root biomass and shoot characteristics. The 120 mg L^{-1} Ca treatment, placed in cluster B, showed moderate improvement in rooting rate, root number, and shoot length, indicating its stimulatory role in root initiation and elongation. Conversely, the MS (0) and BAP treatments, grouped in cluster A, exhibited the lowest values for most parameters, particularly in root-related traits, underscoring their limited effectiveness in supporting plantlet development during the rooting stage. These findings support the role of calcium in promoting both structural and functional quality of root systems and overall plantlet vigor during *in vitro* rooting (Figure 2).

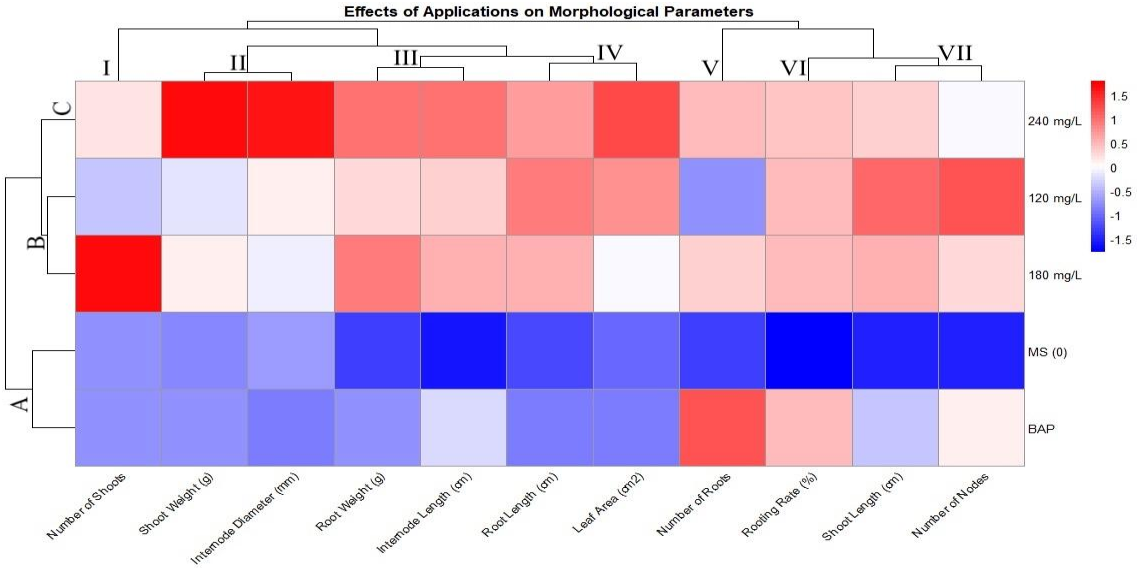

Figure 2. Hierarchical Cluster Analysis (HCA) based on 13 morphological parameters evaluated during the rooting stage of 'Gök Üzüm' (*Vitis vinifera* L.) plantlets cultured on MS medium supplemented with different concentrations of calcium ($CaCl_2 \cdot 2H_2O$).

The findings revealed that particularly the 180 and 240 mg L^{-1} Ca treatments significantly enhanced morphological parameters such as shoot length, number of nodes, leaf area, and root fresh weight, demonstrating superior performance compared to the BAP treatment.

Calcium applications not only suppressed STN formation but also promoted shoot development. Notably, in the first subculture stage, 120 and 180 mg L^{-1} Ca doses resulted in the highest values for both shoot length and number of nodes. An increase in the number of nodes is a critical factor in nodal micropropagation, as it enhances the number of segments available for multiplication (Teixeira da Silva and Dobránszki, 2013). The increase in shoot length may be attributed to the stimulatory role of Ca^{2+} on cell elongation. Specifically, Ca^{2+} contributes to cell wall elasticity and stabilizes the plasma membrane, thus supporting elongation (White and Broadley, 2003).

Furthermore, our study demonstrated that leaf area was expanded by calcium supplementation. This is crucial for increasing photosynthetic capacity, accelerating metabolic activity, and enhancing overall plant performance. The literature also supports that Ca^{2+} improves leaf morphology by maintaining cell wall stability and membrane integrity, thus promoting the development of broad and healthy leaves (Hepler, 2005; Martin et al., 2007; Naaz et al., 2014; Teixeira da Silva et al., 2020). Therefore, $CaCl_2$-enriched media contribute not only to the number of shoots but also to the formation of functional leaves and nodal structures during micropropagation.

During the first subculture period, STN ratios recorded in hormone-free MS and BAP media were relatively high, at 38.91% and 30.44%, respectively. However, these rates significantly decreased in calcium-containing media and were completely eliminated at 180 and 240 mg L^{-1} concentrations. These results indicate that Ca^{2+} plays a critical role in preventing STN by maintaining cell wall stability and plasma membrane integrity. In addition, previous studies have shown that cytokinin type and concentration can also influence physiological disorders such as shoot tip necrosis and hyperhydricity (Kadota and Niimi, 2003). In our study, BAP was kept constant at 1 mg L^{-1}, and the observed differences primarily reflect the effects of calcium supplementation. Similar findings in the literature suggest that Ca deficiency triggers STN and that supplementation can alleviate this physiological disorder (Thakur and Kanwar, 2011; Teixeira da Silva and Dobránszki; 2013 Ahmed and Palta, 2017).

STN development was also found to be influenced by culture duration. Observations made during the fourth week of subcultures showed higher STN rates in MS and BAP treatments, indicating that prolonged culture duration may exacerbate this physiological disorder. Srivastava and Joshi (2013) reported that STN rates increased from 62% at two weeks to 90% at four weeks; similarly, Ahmed and Palta (2017) observed STN rates of 56% at day 15 and 75% at day 25. This increase may be attributed to factors such as nutrient depletion, ethylene accumulation, and reduced transpiration during extended culture periods.

Beyond its structural functions, Ca^{2+} is known to play vital physiological roles. As a ubiquitous secondary messenger in plant cells, Ca^{2+} is involved in the regulation of hormone signaling. Vanneste and Friml (2013) reported a bidirectional interaction between Ca^{2+} and auxin, where auxin signaling triggers Ca^{2+} influx, which in turn affects auxin transport. Ozgen et al. (2011) demonstrated that adding a calcium chelator (EGTA) to a Ca-sufficient medium induced shoot tip necrosis (STN) and axillary shoot formation, whereas the addition of Sr^{2+} (Ca^{2+} analog) restored apical dominance. Similarly, the complete elimination of STN observed at 180 and 240 mg L^{-1} $CaCl_2 \cdot 2H_2O$ concentrations highlights the structural and physiological role of Ca^{2+} in maintaining shoot meristem integrity and preventing apical injury.

In the rooting stage, $CaCl_2 \cdot 2H_2O$ applications positively influenced not only root formation but also the overall quality of plantlets. The highest root biomass and robust root morphology were observed at 240 mg L^{-1} $CaCl_2 \cdot 2H_2O$, reflecting the role of calcium in enhancing structural integrity in root tissues. Conversely, long but thin roots developed under 120 mg L^{-1} $CaCl_2 \cdot 2H_2O$ indicate that this dose promotes cell elongation in roots. Ca^{2+} contributes to both division and differentiation in meristematic root cells and supports cell wall formation, thereby improving water and nutrient uptake (White and Broadley, 2003; de Freitas et al., 2012).

Additionally, Ca^{2+} contributes to xylem formation and water transport regulation, helping to maintain physiological balance and improve stress tolerance (Hepler, 2005). Bairu et al. (2009) also reported that calcium treatments improve root morphology and overall plantlet quality *in vitro*. In conclusion, this study demonstrates that $CaCl_2 \cdot 2H_2O$ supplementation not only enhances shoot development and controls STN formation but also improves nodal and leaf growth and supports the structural and functional integrity of the root system. Thus, calcium application contributes holistically to improving the overall quality of 'Gök Üzüm' plantlets during *in vitro* propagation.

Conclusion

This study demonstrated that different concentrations of calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) significantly influence the micropropagation performance of the Gök Üzüm grape (Vitis vinifera L.) cultivar under *in vitro* conditions. Specifically, the 120, 180, and 240 mg L^{-1} Ca treatments promoted notable improvements in shoot and root development compared to the BAP-supplemented medium. While 120 mg L^{-1} particularly enhanced shoot number, shoot length, node number, and root length, the 180 and 240 mg L^{-1} concentrations were more effective in eliminating shoot tip necrosis and improving root biomass. Furthermore, these treatments effectively eliminated shoot tip necrosis (STN), emphasizing the structural and physiological roles of Ca^{2+} in maintaining cellular integrity and hormonal regulation. The enhanced root morphology observed at 240 mg L^{-1} and the formation of longer roots at 120 mg L^{-1} Ca support the dual role of calcium in both root thickening and elongation. Overall, calcium chloride dihydrate supplementation not only enhanced shoot and root development but also improved the structural and physiological quality of *in vitro*-derived plantlets. These findings suggest that optimizing calcium levels in the culture medium can significantly enhance the success of grapevine micropropagation protocols.

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Conflict of Interest

The author declares that there is no conflict of interest.

Author Contributions

K.Y. designed and conducted the experiments, collected and analyzed the data, interpreted the results, and prepared the manuscript. The author has read and approved the final version of the manuscript.

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