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Boron and Cytokinin Combinations Modulate Shoot Tip Necrosis and Improve Subculture Efficiency in Grapevine Micropropagation

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Abstract

This study was conducted to determine the effects of different combinations of BAP (6-Benzylaminopurine) and boric acid (H_3BO_3) on the morphological development during the initial and first subculture stages of the Ekşi Kara grape cultivar under *in vitro* conditions. The evaluated parameters included shoot formation rate, number of shoots per explant, shoot length, fresh shoot weight, leaf area, number of nodes, and shoot tip necrosis. The findings indicated that BAP applications had notable effects particularly on shoot formation, shoot length, and fresh shoot weight. The highest fresh shoot weight (2.934 g) was obtained from the combination of 1.0 mg/L BAP and 1.18 mg/L boric acid, which also led to the formation of multiple shoots (6 shoots per explant). PCA analysis revealed a strong association between this treatment and the parameters representing shoot development. While boric acid alone had limited influence, its effectiveness increased when combined with appropriate BAP doses. The results suggest that specific combinations of plant growth regulators and micronutrients may enhance morphological development during the *in vitro* propagation process.

Keywords: *In vitro* culture, BAP, Boric acid, Micropropagation, Shoot development, Morphological parameters

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INTRODUCTION

In vitro plant tissue culture is a commonly employed biotechnological method that enables the large-scale propagation of plants under sterile and precisely controlled conditions (Campos et al., 2021). This technique plays a crucial role in conserving genetic resources, producing pathogen-free plantlets, and propagating species that are difficult to reproduce using traditional methods (Kim et al., 2023; Ekinci et al., 2024; Özdemir Memiş and Sağlam, 2024). In economically important species such as grapevine (*Vitis vinifera* L.), micropropagation allows for the production of large numbers of healthy plants; however, this process may be limited by certain physiological disorders (Surakshitha et al., 2019). One of the most common of these is shoot tip necrosis (STN), characterized by browning of the apical region, followed by cell death and growth arrest (Thomas, 2000; Teixeira da Silva et al., 2020).

The occurrence of STN is influenced by a complex interaction of factors including plant genotype, nutrient availability, composition of the culture medium, frequency of subculturing, and ventilation conditions inside the culture vessels during *in vitro* propagation (Isah, 2015; Teixeira da Silva et al., 2020). Low levels of calcium (Ca^{2+}) and magnesium (Mg^{2+}) in shoot tissues have been associated with impaired ion transport, while boron (B) deficiency has also been reported to exacerbate STN (Isah, 2015; Day and Aasim, 2020; Vera-Maldonado et al., 2024). In particular, susceptible grapevine cultivars such as 'Red Globe' have shown widespread STN symptoms following subculture (Surakshitha et al., 2019).

Plant growth regulators, especially cytokinins, play a significant role in the control of STN. 6-Benzylaminopurine (BAP), a commonly used cytokinin in *in vitro* culture, may affect STN incidence depending on its concentration (Bairu et al., 2009; Bairu et al., 2011). While some studies report that BAP deficiency slows down cell division in the shoot apex and promotes necrosis, others suggest that high concentrations of BAP can also increase the incidence of STN (Vieitez et al., 1989; Piagnani et al., 1996; Surakshitha et al., 2019). These conflicting findings highlight the importance of optimizing BAP levels. Furthermore, in grapevine cultures, BAP is recommended to be evaluated in combination with minerals such as

calcium and boric acid, as their interaction has been shown to play a critical role in suppressing STN (Kundan Kishore et al., 2015; Liu et al., 2024).

Ekşi Kara is an autochthonous grape cultivar of Konya, Türkiye, recognized for its regional adaptation and valuable genetic traits, making it a promising candidate for conservation and *in vitro* propagation studies (Kara and Yazar, 2020). In this study, the effects of various combinations of boron and BAP (6-Benzylaminopurine) on *in vitro* subculture performance and the incidence of shoot tip necrosis (STN) were investigated in the grapevine cultivar Ekşi Kara. For this purpose, three concentrations of boron and three doses of BAP were applied in combination, and their influence on culture efficiency was assessed. Within the scope of the study, it was intended to elucidate the role of the boric acid–cytokinin interaction in regulating *in vitro* physiological responses and the development of STN, as well as to determine an optimized culture medium composition that can minimize STN occurrence.

MATERIALS AND METHODS

Nodal explants of the 'Ekşi Kara' were obtained from field-grown plants during the active growth period, located in the clone comparison vineyard at the Faculty of Agriculture, Selçuk University. Single-node segments were isolated and sterilized under aseptic conditions within a laminar flow cabinet. The sterilization procedure consisted of immersing the explants in 70% ethanol for two minutes, followed by 12% sodium hypochlorite for 15 minutes. Residual disinfectants were removed by rinsing the explants three times with sterile distilled water. The shoot development phase was initiated by culturing nodal explants on Murashige and Skoog (MS) medium containing 3% (w/v) sucrose and solidified with 0.7% (w/v) agar (Kara et al., 2022). Explants were exposed to nine application combinations consisting of BAP at concentrations of 0, 0.5, and 1.0 mg/L, each combined with boric acid at 0, 1.18, or 2.17 mg/L. The initial culture phase lasted 12 days under incubation conditions of 25 ± 1°C, with a 16-hour light / 8-hour dark photoperiod and a light intensity of approximately 4000 lux (Surakshitha et al., 2019; Al-Aizari et al., 2020).

Following this period, explants were transferred to the first subculture stage, where the same treatment combinations were reapplied and maintained for an additional four weeks under identical conditions. In the initial culture phase, parameters such as shoot formation rate (%) and number of nodes were assessed. During the first subculture, observations included shoot formation rate (%), shoot number (per plant), shoot length (cm), number of nodes, fresh shoot weight (g), leaf area (cm^2), and the incidence of shoot tip necrosis (STN) was expressed as the % of explants showing visible necrosis symptoms at the apical shoot tips. Leaf area measurements were performed using the Portable Adobe Photoshop software (Katabuchi, 2015). Each treatment was applied in three replicates, with 15 explants per replicate. All morphological observations were recorded at the end of each culture stage for statistical analysis (Kara and Yazar, 2020; Ekinci et al., 2024).

Data analysis

Statistical evaluation of the experimental data was carried out using Tukey's HSD test ($p < 0.05$) in JMP software (version 13) to determine significant differences among treatment means. To further explore the relationships between measured parameters, hierarchical cluster analysis (HCA) and principal component analysis (PCA) were conducted using R software (v4.1.1; R Foundation for Statistical Computing).

RESULTS AND DISCUSSION

Initial stage

In the initial culture stage, different combinations of BAP and boric acid did not lead to significant differences in shoot formation rate or the number of shoots per plant. Shoot formation was generally high across all treatments (94.17–100%), and no treatment induced the formation of multiple shoots. These results suggest that plant responses at this stage were relatively uniform, and the applied growth regulators had limited influence on morphogenetic activity during early development (Table 1).

First subculture

Shoot formation rate (%)

Statistical analysis revealed that BAP applications had a significant effect on shoot formation rate ($p < 0.05$), whereas boric acid doses and the BAP × boric acid interaction did not result in statistically significant differences for this parameter. In the first subculture stage, shoot formation rate remained high across all treatments, ranging from 66.67% to 100.00%. In hormone-free treatments, the rates were comparatively lower (66.67–68.89%), while combinations containing 0.5 and 1.0 mg/L BAP achieved rates between 96.00% and 100.00%. The mean shoot formation rates according to BAP doses were 68.15% at 0 mg/L, 97.33% at 0.5 mg/L, and 100.00% at 1.0 mg/L. These findings indicate that shoot formation during the subculture phase was significantly enhanced by BAP applications, while boric acid alone had no substantial effect (Table 2).

Fresh shoot weight (g)

Fresh shoot weight was one of the most responsive morphological parameters to the interaction between BAP and boric acid. At 0.5 mg/L BAP, shoot weight progressively increased with boric acid supplementation. In the absence of boric acid at this level, shoot weight remained moderate, whereas the addition of 2.17 mg/L boric acid led to a notable increase in biomass. The highest shoot weight (2.93 g) was recorded with the combination of 1.0 mg/L BAP and 1.18 mg/L boric acid, indicating that 1.0 mg/L BAP was more effective than 0.5 mg/L in promoting shoot growth across most boric acid levels. This treatment also resulted in the formation of an average of six shoots per explant (6 shoots/explant), while all other treatments produced only one shoot per explant (1 shoot/explant). These findings suggest that the observed increase in biomass was driven by both enhanced shoot proliferation and improved individual shoot growth (Table 2).

Table 1. Effects of bap and boric acid on shoot formation rate and number of shoots per plant in the initial stage*

	BAP (mg/L)	Boric Acid (mg/L)	Value	Mean (BAP)	Mean (Boric Acid)
Shoot Formation Rate (%)	0	0	94.17 ± 8.25 a	97.41 ± 5.15 A	98.70 ± 3.89 *A*
	0	1.18	100.00 ± 0.00 a		100.00 ± 0.00 *A*
	0	2.17	96.11 ± 6.74 a		98.70 ± 3.89 *A*
	0.5	0	100.00 ± 0.00 a	100.00 ± 0.00 A	98.70 ± 3.89 *A*
	0.5	1.18	100.00 ± 0.00 a		100.00 ± 0.00 *A*
	0.5	2.17	100.00 ± 0.00 a		98.70 ± 3.89 *A*
	1.0	0	100.00 ± 0.00 a	100.00 ± 0.00 A	98.70 ± 3.89 *A*
	1.0	1.18	100.00 ± 0.00 a		100.00 ± 0.00 *A*
	1.0	2.17	100.00 ± 0.00 a		98.70 ± 3.89 *A*
Number of Shoots (per explant)	0	0	1.00 ± 0.00 a	1.00 ± 0.00 A	1.00 ± 0.00 *A*
	0	1.18	1.00 ± 0.00 a		1.00 ± 0.00 *A*
	0	2.17	1.00 ± 0.00 a		1.00 ± 0.00 *A*
	0.5	0	1.00 ± 0.00 a	1.00 ± 0.00 A	1.00 ± 0.00 *A*
	0.5	1.18	1.00 ± 0.00 a		1.00 ± 0.00 *A*
	0.5	2.17	1.00 ± 0.00 a		1.00 ± 0.00 *A*
	1.0	0	1.00 ± 0.00 a	1.00 ± 0.00 A	1.00 ± 0.00 *A*
	1.0	1.18	1.00 ± 0.00 a		1.00 ± 0.00 *A*
	1.0	2.17	1.00 ± 0.00 a		1.00 ± 0.00 *A*

Different lowercase letters indicate significant differences among BAP × boric acid interactions ($p < 0.05$, Tukey's HSD test). Uppercase letters represent BAP main effects; *italic uppercase letters* indicate boric acid main effects.

Number of shoots (per explant)

In the first subculture stage, a significant increase in shoot number was observed only in the treatment combining 1.0 mg/L BAP and 1.18 mg/L boric acid, which induced multiple shoot formation with an average of six shoots per explant (6 shoots/explant). In all other treatments, the number of shoots remained constant at one shoot per explant (1 shoot/explant). This result highlights the synergistic effect of high BAP concentration and moderate boric acid supplementation in promoting shoot proliferation (Table 2; Figure 1).

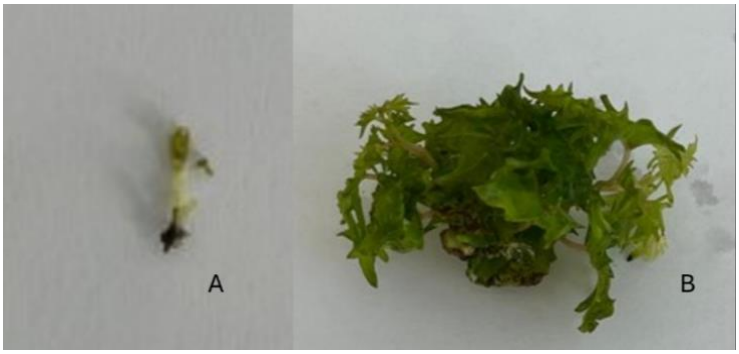

Figure 1. Shoot development in response to different bap and boric acid concentrations during the subculture stage. (a) shoot growth observed in the treatment without bap and boric acid (bap 0 mg/l + boric acid 0 mg/l). (b) increased shoot number and leaf development in the treatment with 1.0 mg/l bap + 1.18 mg/l boric acid.

Shoot length (cm)

Shoot length was significantly influenced by the applied concentrations of BAP and boric acid. The highest value (2.38 cm) was recorded in the treatment combining 1.0 mg/L BAP with 1.18 mg/L boric acid. This was followed by other combinations involving 0.5 and 1.0 mg/L BAP, such as 0.5 mg/L BAP + 2.17 mg/L boric acid (2.23 cm) and 1.0 mg/L BAP + 0.0 mg/L boric acid (2.24 cm), which were also statistically placed in the upper group. In contrast, treatments without BAP resulted in considerably shorter shoots, ranging from 0.61 to 0.69 cm. These findings indicate that both increasing BAP concentration and boric acid supplementation promoted shoot elongation. However, a notable suppression of shoot length was observed with the combination of 0.5 mg/L BAP and 1.18 mg/L boric acid (0.17 cm), suggesting that while boric acid may have a stimulatory effect at certain doses, it can also inhibit growth when combined in suboptimal ratios (Table 2).

Leaf area (cm²)

Leaf area varied significantly in response to the different combinations of BAP and boric acid. The largest leaf area (1.94 cm²) was obtained from the treatment with 1.0 mg/L BAP and 0.0 mg/L boric acid, followed closely by 0.5 mg/L BAP + 2.17 mg/L boric acid (1.80 cm²). These combinations were statistically superior to others. In contrast, all treatments without BAP resulted in minimal leaf expansion (0.06–0.07 cm²). The results clearly show that BAP plays a key role in promoting leaf development, with higher BAP doses generally leading to increased leaf area. Interestingly, the addition of boric acid at 2.17 mg/L also contributed positively to leaf area when combined with 0.5 mg/L BAP, suggesting a potential synergistic effect. However, the combination of 1.0 mg/L BAP and 1.18 mg/L boric acid resulted in a moderate leaf area (0.67 cm²), indicating that not all BAP–boric acid interactions were equally beneficial for this parameter (Table 2).

Number of nodes (per plant)

The number of nodes was significantly influenced by the different combinations of BAP and boric acid. The highest value (3.75 nodes) was observed in the treatment with 1.0 mg/L BAP and 1.18 mg/L boric acid, followed by other combinations containing 0.5 or 1.0 mg/L BAP with moderate to high levels of boric acid. In contrast, treatments without BAP resulted in significantly fewer nodes, ranging from 1.89 to 2.00. These findings suggest that increasing the BAP concentration plays an important role in enhancing nodal development. Moreover, the presence of boric acid, especially at 1.18 mg/L and 2.17 mg/L, appeared to strengthen this effect when applied together with higher BAP concentrations. The results indicate a combined influence that improves stem elongation and morphological differentiation during the subculture stage (Table 2).

Shoot tip necrosis (%)

Shoot tip necrosis was significantly affected by both BAP and boric acid applications. The highest levels of necrosis (44.44% to 61.11%) were observed in treatments without BAP, particularly when combined with 0.0 or 2.17 mg/L boric acid. In contrast, necrosis was completely absent (0.00%) in several treatments containing 0.5 or 1.0 mg/L BAP with 1.18 or 2.17 mg/L boric acid, indicating a significant reduction in tip necrosis under these conditions (Table 2; Figure 2).

Figure 2. Shoot Tip Necrosis Observed Under The Treatment of BAP (0.5 mg/L) + Boric Acid (0 mg/L).

These results suggest that BAP has a critical role in mitigating shoot tip necrosis, and its effectiveness is enhanced by the presence of boric acid at suitable concentrations. Especially, the combination of 1.0 mg/L BAP and 1.18 mg/L boric acid not only promoted growth but also completely eliminated tip necrosis, highlighting the potential of this combination to improve both morphological quality and physiological integrity of the shoots during the subculture stage (Table 2; Figure 2).

HCA

The clustering results presented in the heatmap illustrate the classification of different BAP and boric acid dose combinations based on morphological parameters. The treatment with 1.0 mg/L BAP + 1.18 mg/L boric acid, located in Group D, showed the highest values across all traits and clearly stood out, particularly in terms of shoot number, fresh shoot weight, shoot length, and leaf area. Treatments in Groups E and F, which involved hormone-free media (0 mg/L BAP), were separated due to their low biomass and growth values, with notably high levels of shoot tip necrosis. Intermediate treatments, such as those in Groups B and C (e.g., 0.5 or 1.0 mg/L BAP + 2.17 mg/L boric acid), demonstrated positive effects by showing high values for certain parameters. Meanwhile, treatments in Group A, including 1.0 mg/L BAP + 0.0 mg/L boric acid and 0.5 mg/L BAP + 2.17 mg/L boric acid, supported growth with strong performance in shoot length and leaf area. This classification clearly indicates that increasing the BAP dose generally had a positive effect, and when combined with boric acid, it often resulted in synergistic outcomes (Figure 3).

PCA

The PCA results revealed a clear separation among the seven treatment combinations (P1–P7), each representing distinct BAP and boric acid levels. P3 (1.0 mg/L BAP + 1.18 mg/L boric acid) was positioned furthest from the origin, indicating its strong influence on overall variation. This treatment corresponded to the highest values in shoot formation rate (100.00%), fresh shoot weight (2.934 g), shoot number (6.00 per plant), shoot length (2.38 cm), and number of nodes (3.75), while completely eliminating shoot tip necrosis (0.00%), highlighting its superior morphogenetic effect. Conversely, P1 (0.0 mg/L BAP + 0.0 mg/L boric acid) clustered separately and was characterized by the lowest fresh shoot weight (0.106 g), reduced shoot length (0.63 cm), limited leaf area (0.06 cm^2), and the highest shoot tip necrosis (44.44%), reflecting minimal growth promotion and physiological stress. P2 (0.5 mg/L BAP + 2.17 mg/L boric acid) was moderately distant, showing notable values for shoot length (2.23 cm) and leaf area (1.80 cm^2), suggesting partial efficacy. The remaining combinations (P4–P7) exhibited intermediate positions, aligning variably with growth parameters. Overall, PCA effectively distinguished treatment responses and emphasized the synergistic impact of optimal BAP and boric acid concentrations, particularly in P3 (Figure 4).

Table 2. Effects of bap and boric acid interactions on *in vitro* morphological parameters of shoots (first subculture phase)*

	BAP (mg/L)	Boric Acid (mg/L)	Value	Mean (BAP)	Mean (Boric Acid)
Shoot Formation Rate (%)	0	0	68.89 ± 3.85 a	68.15 ± 2.94 B	88.30 ± 15.18 *A*
	0	1.18	66.67 ± 0.00 a		87.56 ± 16.14 *A*
	0	2.17	68.89 ± 3.85 a		89.63 ± 15.67 *A*
	0.5	0	96.00 ± 6.93 a	97.33 ± 5.29 A	88.30 ± 15.18 *A*
	0.5	1.18	96.00 ± 6.93 a		87.56 ± 16.14 *A*
	0.5	2.17	100.00 ± 0.00 a		89.63 ± 15.67 *A*
	1.0	0	100.00 ± 0.00 a	100.00 ± 0.00 A	88.30 ± 15.18 *A*
	1.0	1.18	100.00 ± 0.00 a		87.56 ± 16.14 *A*
	1.0	2.17	100.00 ± 0.00 a		89.63 ± 15.67 *A*
Fresh Shoot Weight (g)	0.0	0	0.106 ± 0.00 f	0.11 ± 0.00 C	0.22 ± 0.11 *B*
	0.0	1.18	0.113 ± 0.00 ef		1.09 ± 1.09 *A*
	0.0	2.17	0.112 ± 0.00 ef		0.16 ± 0.05 *C*
	0.5	0.0	0.118 ± 0.01 d	0.21 ± 0.02 B	0.22 ± 0.11 *B*
	0.5	1.18	0.206 ± 0.00 cd		1.09 ± 1.09 *A*
	0.5	2.17	0.227 ± 0.01 c		0.16 ± 0.05 *C*
	1.0	0.0	0.357 ± 0.02 b	1.14 ± 1.35 A	0.22 ± 0.11 *B*
	1.0	1.18	2.934 ± 0.01 a		1.09 ± 1.09 *A*
	1.0	2.17	0.134 ± 0.00 e		0.16 ± 0.05 *C*
Number of Shoots (shoots/explant)	0	0	1.00 ± 0.00 b	1.00 ± 0.00 B	1.00 ± 0.00 *B*
	0	1.18	1.00 ± 0.00 b		2.67 ± 2.55 *A*
	0	2.17	1.00 ± 0.00 b		1.00 ± 0.00 *B*
	0.5	0	1.00 ± 0.00 b	1.00 ± 0.00 B	1.00 ± 0.00 *B*
	0.5	1.18	1.00 ± 0.00 b		2.67 ± 2.55 *A*
	0.5	2.17	1.00 ± 0.00 b		1.00 ± 0.00 *B*
	1.0	0	1.00 ± 0.00 b	2.67 ± 2.55 A	1.00 ± 0.00 *B*
	1.0	1.18	6.00 ± 1.00 a		2.67 ± 2.55 *A*
	1.0	2.17	1.00 ± 0.00 b		1.00 ± 0.00 *B*
Shoot Length (cm)	0.0	0.0	0.63 ± 0.02 d	0.64 ± 0.05 C	1.71 ± 0.81 *A*
	0.0	1.18	0.69 ± 0.07 d		1.08 ± 1.00 *C*
	0.0	2.17	0.61 ± 0.01 d		1.41 ± 0.70 *B*
	0.5	0.0	2.26 ± 0.01 b	1.55 ± 1.04 B	1.71 ± 0.81 *A*
	0.5	1.18	0.17 ± 0.00 d		1.08 ± 1.00 *C*
	0.5	2.17	2.23 ± 0.04 b		1.41 ± 0.70 *B*
	1.0	0.0	2.24 ± 0.04 b	2.01 ± 0.46 A	1.71 ± 0.81 *A*
	1.0	1.18	2.38 ± 0.02 a		1.08 ± 1.00 *C*
	1.0	2.17	1.40 ± 0.03 c		1.41 ± 0.70 *B*
Leaf Area (cm^2)	0.0	0.0	0.06 ± 0.00 e	0.07 ± 0.00 C	0.86 ± 0.84 *A*
	0.0	1.18	0.07 ± 0.00 e		0.33 ± 0.27 *B*
	0.0	2.17	0.06 ± 0.00 e		0.83 ± 0.77 *A*
	0.5	0.0	0.59 ± 0.07 c	0.88 ± 0.71 B	0.86 ± 0.84 *A*
	0.5	1.18	0.24 ± 0.02 d		0.33 ± 0.27 *B*
	0.5	2.17	1.80 ± 0.08 b		0.83 ± 0.77 *A*
	1.0	0.0	1.94 ± 0.00 a	1.08 ± 0.65 A	0.86 ± 0.84 *A*
	1.0	1.18	0.67 ± 0.07 c		0.33 ± 0.27 *B*
	1.0	2.17	0.63 ± 0.04 c		0.83 ± 0.77 *A*
Number of Nodes (per explant)	0.0	0.0	1.94 ± 0.10 c	1.94 ± 0.12 B	2.68 ± 0.72 *A*
	0.0	1.18	2.00 ± 0.00 c		2.78 ± 0.80 *A*
	0.0	2.17	1.89 ± 0.20 c		2.94 ± 0.87 *A*
	0.5	0.0	2.89 ± 0.19 ac	3.02 ± 0.52 A	2.68 ± 0.72 *A*
	0.5	1.18	2.59 ± 0.36 bc		2.78 ± 0.80 *A*
	0.5	2.17	3.59 ± 0.36 ab		2.94 ± 0.87 *A*
	1.0	0.0	3.22 ± 0.84 ab	3.44 ± 0.58 A	2.68 ± 0.72 *A*
	1.0	1.18	3.75 ± 0.22 a		2.78 ± 0.80 *A*
	1.0	2.17	3.33 ± 0.58 ab		2.94 ± 0.87 *A*
Shoot Tip Necrosis (%)	0.0	0.0	44.44 ± 19.25 a	51.85 ± 13.03 A	38.52 ± 17.25 *A*
	0.0	1.18	50.00 ± 0.00 a		16.67 ± 25.00 *B*
	0.0	2.17	61.11 ± 9.62 a		20.37 ± 30.93 *B*
	0.5	0.0	33.33 ± 0.00 a	11.11 ± 16.67 B	38.52 ± 17.25 *A*
	0.5	1.18	0.00 ± 0.00 b		16.67 ± 25.00 *B*
	0.5	2.17	0.00 ± 0.00 b		20.37 ± 30.93 *B*
	1.0	0.0	37.78 ± 26.94 a	12.59 ± 23.20 B	38.52 ± 17.25 *A*
	1.0	1.18	0.00 ± 0.00 b		16.67 ± 25.00 *B*
	1.0	2.17	0.00 ± 0.00 b		20.37 ± 30.93 *B*

Different lowercase letters indicate significant differences among BAP × boric acid interactions ($p < 0.05$, Tukey's HSD test). Uppercase letters represent BAP main effects; *italic uppercase letters* indicate boric acid main effects.

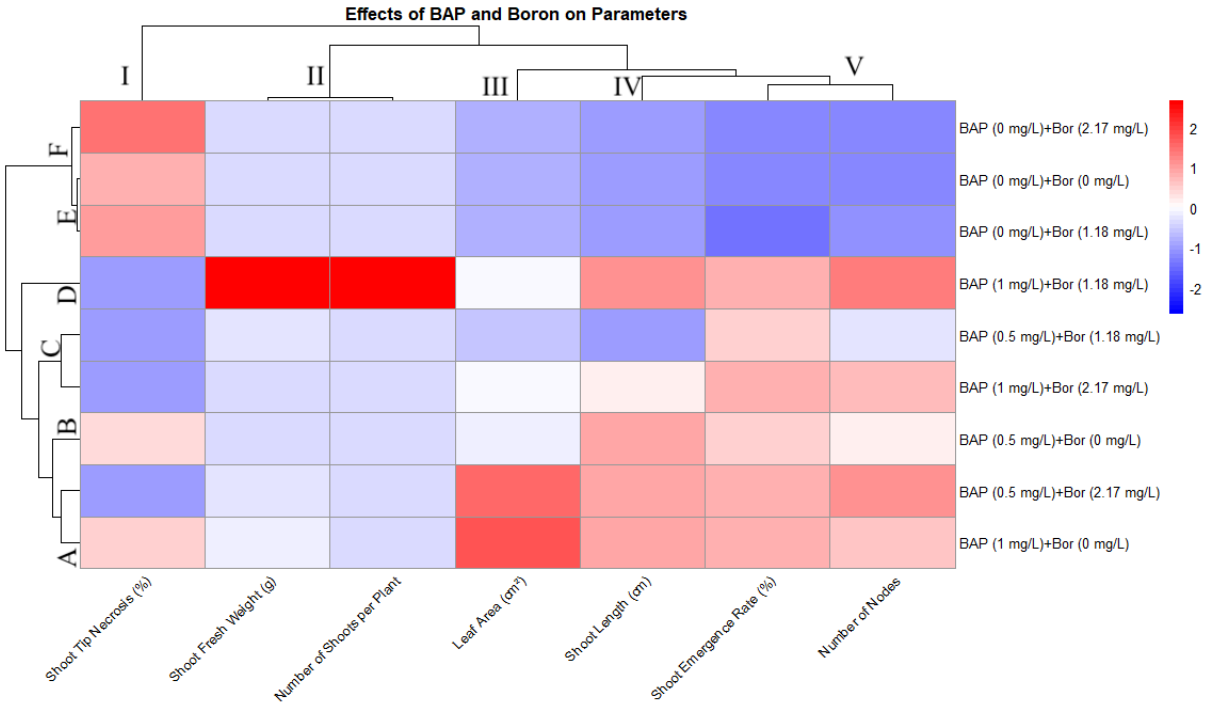

Figure 3. Heatmap showing the hierarchical clustering of different bap and boric acid combinations based on seven morphological parameters in the first subculture stage. treatments are grouped (a–f) according to similarity, and five major parameter clusters (i–v) are indicated. Red tones represent higher values, while blue tones indicate lower values.

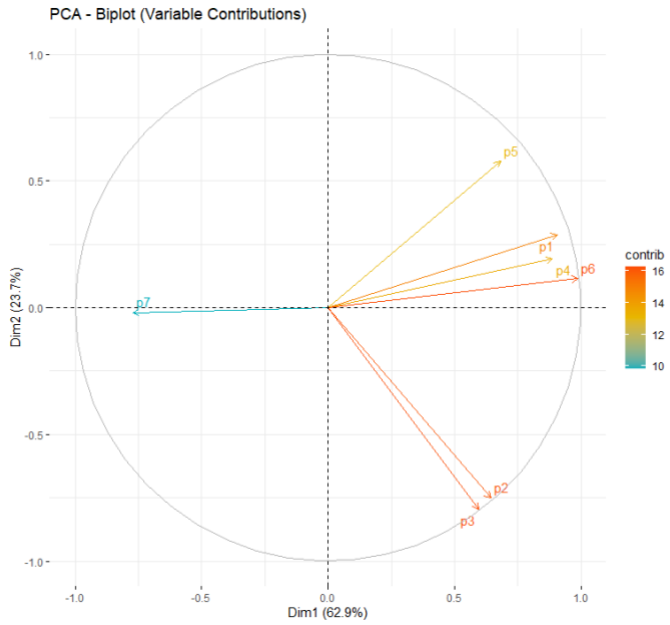

Figure 4. Principal component analysis (pca) biplot showing the distribution of treatments (p1–p7) based on morphological parameters in the first subculture stage. The analysis illustrates the relationships among shoot formation rate, fresh shoot weight, number of shoots per plant, shoot length, leaf area, number of nodes, and shoot tip necrosis.

This study clearly demonstrates that the interaction between BAP and boric acid plays a decisive role in regulating *in vitro* shoot development and minimizing physiological disorders such as shoot tip necrosis (STN) in the grapevine cultivar 'Ekşi Kara'.

In the initial culture stage, no significant differences were observed among treatments for shoot formation rate or shoot number. This uniform response may be attributed to the intrinsic regenerative capacity of the explants at the onset of culture, possibly supported by endogenous hormonal reserves and initial nutrient sufficiency (Sharma et al., 2011; Us-Camas et al., 2014). Additionally, the short duration of the initial stage (12 days) may have been insufficient for exogenous BAP or boric acid applications to exert visible morphological effects (Sojková et al., 2016; Abdalla et al., 2022). The low metabolic

activity and stress adaptation of explants during this phase may also have contributed to the lack of treatment-specific differentiation (Pazuki et al., 2017; Trivedi and Joshi, 2021).

In contrast, the first subculture stage revealed marked and statistically significant responses to BAP and boric acid treatments. Shoot formation rate, shoot length, number of nodes, fresh shoot weight, and leaf area all increased notably in response to higher BAP concentrations, particularly at 1.0 mg/L. BAP-induced improvements in shoot development have also been reported in other studies. This shift likely reflects an increase in metabolic activity and hormonal sensitivity of explants following the initial adaptation phase, which may have enabled more effective perception and utilization of exogenous BAP and boric acid (Parsa and Wallace, 1980; Bairu et al., 2009).

The combination of 1.0 mg/L BAP and 1.18 mg/L boric acid yielded the most favorable outcomes across nearly all parameters. This treatment also completely suppressed shoot tip necrosis, suggesting a synergistic interaction between cytokinin and boric acid in promoting both morphogenesis and physiological stability. Boric acid may have contributed to cellular membrane integrity and auxin transport, while BAP stimulated active cell division and bud activation, leading to enhanced overall growth responses and reduced stress symptoms (Grunewald and Friml, 2010; Teixeira da Silva et al., 2020).

It is noteworthy that shoot multiplication was observed only under the P3 treatment (1.0 mg/L BAP + 1.18 mg/L boric acid), which produced an average of six shoots per explant, while all other combinations resulted in only one shoot per explant. This suggests that a precise hormonal–micronutrient balance is required to overcome apical dominance and initiate axillary bud development, which was not achieved under suboptimal combinations (Bairu et al., 2011).

The suppression of shoot tip necrosis in BAP and boric acid-treated groups further confirms the role of these compounds in mitigating stress-induced tissue damage. BAP might modulate cytokinin-mediated stress responses, while boric acid could alleviate physiological disorders through its stabilizing effect on cell walls and membranes (Bairu et al., 2009). Particularly, the absence of necrosis in several combinations containing 0.5–1.0 mg/L BAP and moderate boric acid concentrations points to an effective balance that supports shoot health (Beckman, 2000; Gaspar et al., 2002). In contrast, hormone-free media or suboptimal combinations led to high necrosis rates and weak growth. This may result from hormonal deficiency or ionic imbalance, which fails to support the regenerative demand of the explants during the subculture phase (Teixeira da Silva et al., 2020).

The classification results from the hierarchical clustering and PCA analyses supported these findings by separating the most effective treatment, P3 (1.0 mg/L BAP + 1.18 mg/L boric acid), from all other combinations due to its superior performance. These multivariate analyses underline the importance of balanced growth regulator and micronutrient combinations in improving *in vitro* culture outcomes. Such clustering patterns reflect the cumulative impact of multiple morphological improvements and provide statistical confirmation of the synergistic effects observed (Bairu et al., 2009; Bairu et al., 2011; Teixeira da Silva et al., 2020).

Overall, the data suggest that while the initial stage of culture is primarily influenced by intrinsic factors and baseline culture conditions, the subculture phase is more responsive to external inputs. This temporal variation in responsiveness highlights the necessity of stage-specific medium optimization, particularly during later culture phases where exogenous signals are more efficiently perceived (Vieitez et al., 1989; Gaspar et al., 2002). Therefore, optimization of hormonal and nutritional factors is particularly critical during the later phases of micropropagation to ensure robust growth and minimize physiological disorders (Pazuki et al., 2017; Trivedi and Joshi, 2021).

CONCLUSION

This study demonstrated that the interaction between boric acid and BAP plays a critical role in regulating *in vitro* shoot development and physiological disorders, particularly shoot tip necrosis (STN), in the grapevine cultivar ‘Ekşi Kara’. While the initial culture stage showed limited responsiveness to treatments, significant differences were observed during the subculture phase. BAP application, especially at 1.0 mg/L, markedly enhanced shoot formation rate, shoot length, fresh biomass, and leaf area. The combination of 1.0 mg/L BAP with 1.18 mg/L boric acid (P3) yielded the most favorable outcomes, promoting prolific shoot development while completely eliminating STN symptoms. PCA and HCA analyses supported these findings by clearly distinguishing this treatment group due to its superior performance across all morphological parameters. In contrast, hormone-free treatments (e.g., P1) exhibited poor growth and the highest incidence of necrosis. These results underscore the importance of optimizing cytokinin and micronutrient concentrations to improve micropropagation efficiency and shoot quality. The findings may guide the development of improved *in vitro* protocols for grapevine propagation, with potential applicability to other genotypes prone to STN. However, further studies are required to validate the long-term stability of these improvements and to investigate their influence under *ex vitro* and field conditions.

Compliance with Ethical Standards

Peer Review

This article has been peer-reviewed by independent experts in the field using a double-blind review process.

Conflict of Interest

The author declares that there is no conflict of interest.

Author Contribution

The author solely conceived, designed, and conducted the study, analyzed the data, and wrote the manuscript.

Ethics Committee Approval

Ethical approval was not required for this study.

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