ROLUL VENTILAȚIEI, CALCIULUI ȘI BORULUI ÎN REDUCEREA NECROZEI ȘI VITRIFICĂRII VÂRFURILOR LĂSTĂRILOR LA PORTALTOII DE FISTIC ‘BADAMI’ ȘI ‘UCB1’ OBȚINUȚI *IN VITRO*

ROLE OF VENTILATION, CALCIUM AND BORON ON SHOOT TIP NECROSIS AND HYPERHYDRICITY REDUCTION OF *IN VITRO* ROOTSTOCKS OF PISTACIO ‘BADAMI’ AND ‘UCB1’

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Abstract

Shoot tip necrosis (STN) is one of the main physiological disorders in the micropropagation of pistachios. In the current study, the effects of $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ at 196 mg/L and 291 mg/L, H_3BO_3 at 196 mg/L and 291 mg/L, and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ at 2,980 mg/L on STN and hyperhydricity reduction of *Pistacia vera* L., ‘Badami’ and ‘UCB1’ rootstocks were assessed, compared to the MS standard medium containing 3% sucrose, 0.7% agar supplemented with benzyladenine (BA) (1.5 mg/L), indole butyric acid (IBA) (0.1 mg/L). For ventilation parameter, filter container vessels with a 50- μm microporous polypropylene membrane (Pardis®) were used. Based on the results, an increase in calcium chloride content of the MS standard medium prevented hyperhydricity in the UCB1 rootstock, whereas it increased STN, yellow leaves, decreasing the multiplication of shoots in the ‘Badami’ rootstock. The results also showed that increasing boric acid from 196 mg/L up to 291 mg/L decreased STN in the UCB1 rootstock and increased this disorder by 37% in the ‘Badami’ rootstock. Ventilation showed no significantly reducing effect on the percentage of STN in the regenerated shoots of the ‘Badami’ rootstock, whilst it decreased the STN of the ‘UCB1’ rootstock to the lowest percentage. For the ‘Badami’ rootstock, $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ at 196 mg/L led to the highest proliferation rate, shoot height, shoot diameter, and leaf number, but for the ‘UCB1’ rootstock, an increase in the concentration of $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ up to 291 mg/L under ventilated conditions resulted in an increase in proliferation, shoot height, and shoot diameter.

Cuvinte cheie: necroza lăstarilor, micropropagarea fisticului, portaltoi ‘Badami’, hibrid ‘UCB1’

Key words: shoots die-back, pistachio micropropagation, ‘Badami’ rootstock, ‘UCB1’ hybrid

1. Introduction

Pistacia vera L. is a subtropical fruit tree belonging to the *Anacardiaceae* Family. ‘UCB1’ has been introduced as a standard hybrid (*P. atlantica* X *P. integerrima*) rootstock of pistachio plants. It has been well known for both being a resistant rootstock to *Verticillium* wilt and good and uniform growth power (Morgan et al., 1992). On the other hand, ‘Badami’ (*Pistacia vera* L.) has been introduced both as a resistant rootstock to salinity, drought stress and the best rootstock for the Iranian commercial pistachio cultivar in terms of both quantity and quality of production (Esmaeilpour, 2000). The genus *Pistacia* can be propagated either sexually by seeds or asexually by tissue culture methods.

Obviously, the propagation of a superior genotype by seeds on a large scale is time-consuming since in some features, plants show great variation so that the produced plant would be quite different from the mother plant. Barghchi and Alderson (1982, 1989) first reported the use of tissue culture methods for the rapid clonal propagation of pistachios. Based on the literature, the micropropagation of pistachio plants has been discussed from different aspects. Most of the studies have especially focused on medium optimizing, explant growth stage, and propagation methods, including somatic embryogenesis, shoot tip *in vitro* regeneration, and adventitious organogenesis (Barghchi and Alderson, 1982; Bustamante-Garcia, 1984; Martinelli et al., 1988; Abousalim, 1990; Yang and Ludders, 1993; Onay et al., 1995, 1996; Onay, 2000; OzdenTokatli et al., 2005; Tilkat, 2013; Benmahioul et al., 2009; Benmahioul and Dorion, 2012). In addition, the regenerated pistachio shoots show some sensitivity to *in-vitro* conditions: They could be affected by some physiological disorders, such as STN and hyperhydricity. STN, in the micropropagation of pistachio plants, is one of the main physiological disorders needing to be avoided. The senescence and death of tissues in the apical buds close to the apex of regenerated shoots

are also one of the major symptoms of shoot die-back. STN could result in low-quality shoots, and any propagator is involved with this issue at the beginning of proliferation process. Apical dominance occurs when the shoot apex inhibits the growth of lateral buds. By the appearance of STN because of apical dominance elimination, secondary shoots grow from axillary buds (Barghchi and Alderson, 1996). Major and elongated shoots are firstly affected by STN, followed by shoots of lower parts as well as very small shoots might be influenced by this disorder, however, small shoots are usually healthier and stronger (Barghchi and Alderson, 1996). Many researchers have carried out studies for diminishing STN. According to the literature, the Ca^{2+} concentration in a medium has been shown to have a strong correlation with STN, meaning that it decreases when the calcium content increases in the medium STN (Sha et al., 1985; Qiguang et al., 1985; Piagnani et al., 1996). Ca^{2+} , known as the main component in the structure of plant cell walls, plays a key role in maintaining membrane strength (Marschner, 1995; Machado et al., 2014). Additionally, the main structural function of Ca^{2+} has been proved to synthesize new plant cell walls and middle lamellae that separates newly divided cells (Taiz and Zeiger, 2006). Mirabbasi and Hosseinpour (2014) also verified a low percentage of STN in the medium with very high doses of calcium. Moreover, McIlrath and Skok (1964) stated that increasing in the concentration of medium calcium chloride significantly decreased STN without any negative effects on proliferation and shoot elongation in sunflower and tobacco plants. Nezami et al. (2015) also declared that STN, as a physiological disorder, is related to calcium and boron deficiencies. Their results further revealed that increasing the calcium concentration decreased STN and enhanced the proliferation rate in the UCB1 rootstock. Likewise, Abousalim and Mantelli (1994) showed that increasing the medium boron level decreased STN in *P. vera*. On the other hand, the heavy concentration of boron led to less proliferation, shorter micro-shoots, and consequently, more necrosis. Considering the boric acid content of medium, Nezami et al. (2015) also showed that double boron decreased STN, while tripling its concentration increased STN and, consequently, decreased the proliferation rate. Furthermore, Nezami et al. (2015) reported the lowest necrosis rate in the treatment containing calcium chloride concentrations in combination with boric acid. Similarly, Barghchi and Alderson (1996) defined STN as a physiological as well as mineral disorder associated with calcium and boron deficiencies. They stated that calcium chloride could be the best source to reduce STN without any adverse side effects in either shoot multiplication or elongation. They also found that adding boron in the medium could reduce the shoot multiplication rate (Barghchi and Alderson, 1996). Barghchi and Alderson (1996) further reported that ventilation or increasing air exchange had not any significant effect on STN. There are a few reports on ventilation and the effects of these variable environmental conditions on the shoot proliferation of pistachio plants. For example, Roberts et al. (1994) reported that plantlets grown in common vessels with 95% relative humidity showed the lower transpiration rate. It has also been well documented that water and nutrient uptake as well as plant growth are stimulated by the high transpiration rate (Roberts et al., 1994). Ventilation can also be effective in some other plant-related physiological disorders (Zobayed et al., 2000; Hassankhah et al., 2014).

In the current study, we investigated the STN associated with the Ca^{2+} concentration from different sources, boron concentration as well as vessel ventilation, trying to highlight their importance in hyperhydricity and STN reduction of *in-vitro* regenerated shoots of pistachio plants and the promotion of health of these plants.

2. Material and methods

Pistachio shoot explants from two rootstocks (Badami and UCB1) were firstly cultured on the MS medium (Murashige and Skoog, 1962) containing 3% sucrose, 0.7% agar supplemented with BA (1.5 mg/L), and Indolil-3-butyric acid (0.1 mg/L). The pH of the medium was adjusted to 5.7 before autoclaving (at 121 °C for 20 min.). Next, vessel filters made from 50- μm microporous polypropylene membranes (Pardis Co, Iran®) were used to assess the effect of ventilation. Each culture vessel contained 40 mL medium. The treatments consisted of (i) $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ at 196 mg/L and 291 mg/L in respect of MS, (ii) H_3BO_3 at 196 mg/L and 291 mg/L in respect of MS, and (iii) $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ at 2,980 mg/L in respect of the MS standard medium. Proliferation percentage, shoot length (cm), shoot number per explant, leaf number, shoots diameter (mm), as well as STN and hyperhydricity (%) were evaluated in two rootstocks after 45 days. In this experiment, the cultures were exposed to the light intensity of 30 $\mu\text{m}^{-2}\text{s}^{-1}$ supplied by white fluorescent lamps with a 16h photoperiod. The experimental design was a randomized complete block (RCB) one with four replications of 40 explants per repetition. Eventually, to analyze the data, we performed the analysis of variance (ANOVA) followed by the Duncan's multiple range test at $p \leq 0.01$ and $p \leq 0.05$.

3. Results and discussions

3.1. Proliferation Rate

The results showed that different concentrations of salts had a significant effect on the proliferation rate. The maximum proliferation rate, an average of 8.5 shoots per explant, was related to the 'UCB1' rootstock treated with $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ (291 mg/L) (Figure 1). For the 'Badami' rootstock, $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ (196 mg/L) resulted in the maximum proliferation rate (an average of 5.75 shoots per explant) (Figure 1). Moreover, ventilation had a significant effect on the proliferation rate of both rootstocks and the ventilated conditions resulted in an increase in the proliferation rate (Table 1).

3.2. Shoot height (mm)

There is no significant difference between $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ (291 mg/L) and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in terms of the shoot height of the 'UCB1' rootstock. Increasing the concentration of H_3BO_3 from 196 mg/L to 291 mg/L increased the height of regenerated shoots from 6.34 to 7.23 mm for the 'Badami' rootstock (Figure 2). In 'UCB1', the concentration of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (196 mg/L) was more beneficial for the shoot height than both concentrations of H_3BO_3 . According to the results, ventilation had a significant effect on the proliferation rate of both rootstocks. The ventilation treatment increased the height of the regenerated shoots (Table 1). For the 'Badami' rootstock, the ventilated conditions resulted in an increase in the shoot height by 11.2 mm (Table 1).

3.3. Shoot Diameter

Figure 3 shows that increasing the concentration of H_3BO_3 from 196 mg/L to 291 mg/L increased the shoot diameter in the 'Badami' rootstock, whereas with increasing the H_3BO_3 concentration from 196 mg/L to 291 mg/L resulted in average shoot diameters of 0.25 mm and 0.21 mm for the UCB1 rootstock. The shoot diameter in the MS standard media was also more than media containing $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and different H_3BO_3 concentrations (Figure 3). Moreover, based on the result (Table 1), the ventilation treatment had a significant overall positive effect on the shoot diameter in both rootstocks.

3.4. Leaf Number

Our results also indicated that increasing the concentration of $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ from 196 mg/L to 291 mg/L had no significant effect on the leaf number in both rootstocks (Figure 4). $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, as a calcium source, also had a negative effect on the leaf number of the 'Badami' rootstocks (Figure 4). The findings, moreover, showed that different concentrations of H_3BO_3 and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ did not induce significant differences with the MS standard medium in terms of the leaf number of the 'Badami' rootstock. However, ventilation had no statistically significant effect on the number of leaves in 'Badami', while a positive effect was observed in 'UCB1' (Table 1).

3.5. STN and Hyperhydricity Rate

Different concentrations of $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and H_3BO_3 led to the statistically different percentages of STN and hyperhydricity ($p \leq 0.01$ and $p \leq 0.05$). The concentrations of $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ (196 mg/L and 291 mg/L) and H_3BO_3 (291 mg/L) were also found to be beneficial for the in-vitro shoots of the Badami rootstock since they prevented hyperhydricity (Figure 5). For the 'UCB1' rootstock, $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ at 196 mg/L and 291 mg/L, H_3BO_3 at 291 mg/L, and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ prevented hyperhydricity as well. For the 'UCB1' rootstock, the highest percentage of hyperhydricity was observed for the MS standard medium and H_3BO_3 at 196 mg/L. This study indicated that with raising the H_3BO_3 concentration from 196 mg/L to 291 mg/L, the hyperhydricity rate decreased (Figure 5). The 'Badami' rootstock showed the same pattern as 'UCB1'. The difference was that $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ could not prevent hyperhydricity in the 'Badami' rootstock (Figure 5). Our results also demonstrated that ventilation had no significantly reducing effect on the percentage of hyperhydricity in the regenerated shoots of the 'Badami' rootstock. The lowest percentage of hyperhydricity (0.25%) was observed for the ventilation treatment in the 'UCB1' rootstock (Table 1). Furthermore, this study indicated that maximum STN (75%) for 'UCB1' was observed for the MS standard medium, followed by H_3BO_3 (62%) at 196 mg/L, while for the 'UCB1' rootstock, $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ at 196 mg/L and 291 mg/L effectively prevented STN (Figure 6). For the 'Badami' rootstock, the highest STN percentage was related to the MS standard medium and the $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ treatment (62%) (Figure 6). In addition, the high concentration of H_3BO_3 (291 mg/L) resulted in more STN in the 'Badami' rootstock.

3.6. Discussions

Under tissue culture conditions, the necrosis and death of tissues in the apical buds of the regenerated shoots are known as STN, which results in low-quality shoots. STN and hyperhydricity are the main plant disorders in tissue culture of pistachio (Nezami et al., 2015). Some researchers have suggested the optimization of calcium and boron rates in the medium for preventing these abnormalities (McIlrath and Skok, 1964; Barghchi and Alderson, 1996; Nezami et al., 2015). Because of low mobility of calcium and boron, these two nutrients have been more discussed (Barghchi and Alderson, 1996). It has been well documented that the calcium concentration in the medium has a strong negative correlation with STN (Sha et al., 1985; Qiguang et al., 1985; Piagnani et al., 1996). The results of this study are consistent with those obtained by Qiguang et al. (1985) and Piagnani et al. (1996). McIlrath and Skok

(1964), and Barghchi and Alderson (1996) declared that increasing the calcium chloride content in the medium decreased STN without either shoot multiplication or elongation adverse effects. In addition, the results of this study are in agreement with those of the studies done by McIlrath and Skok (1964) and Nezami et al. (2015) concerning 'UCB1'. Furthermore, for the 'Badami' rootstock, they showed that increasing the calcium chloride content in the MS standard medium increased STN, yellow leaves, and decreased the shoot multiplication, which is in contrast to what we observed in this study. In the current study, for 'UCB1', increasing the calcium chloride content of MS standard medium prevented hyperhydricity. This is strongly in line with Nezami et al. (2015), who stated that double boric acid decreased STN. Another finding of the present study was that adding boron to the medium reduced the shoot multiplication rate in the 'Badami' rootstock under non-ventilated conditions. Barghchi and Alderson (1996) also reported the same results. Nezami et al. (2015) reported a low necrosis rate in the treatment containing different concentrations of calcium chloride combined with a given concentration of boric acid. Contrarily, in the present study, we found that the lowest necrosis rates were related to $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ at 196 mg/L for the 'Badami' rootstock and $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ at 196 mg/L and 291 mg/L for the 'UCB1' rootstock. Considering calcium and its positive effect, we acquired the same results as Penel et al. (1984) as well as Carbonell and Jones (1984), but our positive results are related to the genotype. Mirabbasi and Hosseinpour (2014) also showed that low STN was seen in the medium with high doses of calcium. In a similar study on *P. vera* STN, Abousalim and Mantell (1994) indicated that increasing the boron concentration prevented STN in the regenerated shoot. However, high level of boron showed negative effects on the proliferation rate and micro-shoot elongation, while showing increasing effect on necrosis. These negative responses were not observed in the current study. Furthermore, in line with the study done by Barghchi and Alderson (1996), we noticed that ventilation had no significantly reducing effect on the percentage of STN in the regenerated 'Badami' shoots.

4. Conclusions

In conclusion, we can claim that appropriate concentrations of $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ could prevent STN. Furthermore, the results of this study proved that the optimized MS medium containing $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ could result in the lowest hyperhydricity rate and produce healthy plantlets.

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Tables and Figures

Table 1. Vegetative growth parameters in two pistachio rootstocks, 'Badami' and 'UCB1', cultured in the MS medium, supplemented with BA (1.5 mg/L), IBA (0.1 mg/L), exposed or not to ventilation conditions

Treatments	Proliferation		Shoot height (mm)		Shoot diameter (mm)		Leaf number (n)		STN (%)		Hyperhydricity (%)	
	Badami	UCB1	Badami	UCB1	Badami	UCB1	Badami	UCB1	Badami	UCB1	Badami	UCB1
No ventilation	2.66 b	6.54 b	6.95 b	6.22 b	0.41 b	0.25 b	6.29 a	7.29 b	41 a	50.00 a	12 a	25.00 a
Ventilation	3.00 a	7.50 a	8.98 a	8.64 a	0.57 a	0.41 a	7.00 a	9.16 a	25 a	25.00 b	16 a	4.10 b
Ventilation (b)	*	*	*	**	**	**	n.s	**	n.s	*	n.s	**

**Means followed by the same letters are not significantly different ($p \leq 0.01$).
 *Means followed by the same letters are not significantly different ($p \leq 0.05$).

Fig. 1. Proliferation rate in two pistachio rootstocks ('Badami' and 'UCB1') cultured in the MS medium, supplemented with BA (1.5 mg/L), IBA (0.1 mg/L), exposed to different concentrations of calcium and boron compared to the MS standard medium

Fig. 2. Shoot length (mm) in two pistachio rootstocks ('Badami' and 'UCB1') cultured in the MS medium, supplemented with BA (1.5 mg/L), IBA (0.1 mg/L), exposed to different concentrations of calcium and boron compared to the MS standard medium

Fig. 3. Regenerated shoot diameter (mm) in two pistachio rootstocks ('Badami' and 'UCB1') cultured in the MS medium, supplemented with BA (1.5 mg/L), IBA (0.1 mg/L), exposed to different concentrations of calcium and boron compared to the MS standard medium

Fig. 4. Leaf number of two pistachio rootstocks ('Badami' and 'UCB1') cultured in the MS medium, supplemented with BA (1.5 mg/L), IBA (0.1 mg/L), exposed to different concentrations of calcium and boron compared to the MS standard medium

Fig. 5. Percentage of hyperhydricity in two pistachio rootstocks ('Badami' and 'UCB1') cultured in the MS medium, supplemented with BA (1.5 mg/L), IBA (0.1 mg/L), exposed to different concentrations of calcium and boron compared to the MS standard medium

Fig. 6. Percentage of STN in two pistachio rootstocks ('Badami' and 'UCB1') cultured in the MS medium, supplemented with BA (1.5 mg/L), IBA (0.1 mg/L), exposed to different concentrations of calcium and boron compared to the MS standard medium