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Control of Shoot Tip Necrosis and Plant Death during in Vitro Multiplication of Pistachio Rootstock UCB1 (*Pistacia integrima* × *P. atlantica*)

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Abstract

Pistachio is one of the most important nuts and its mass production using tissue culture techniques is of great importance. Micro-propagation has many complications. Contamination is one of the critical limitations in the establishment stage. This study focused on different NaClO and $HgCl_2$ concentrations and exposure times. Shoot tip necrosis (STN) is one of the most common problems during large-scale *in vitro* propagation of UCB1 rootstock. This physiological disorder is associated with the deficiency of calcium and boron content in the medium. Thus, in order to prevent STN, in this study, explants were cultured in varying concentrations of calcium using multiples of concentrations of calcium chloride (1x, 1.5 x and 2x), and boric acid (1x, 2x and 3x) in Murashige and Skoog (MS) medium with Gamborg's vitamins containing 30 gl^{-1} sucrose, 2 mgl^{-1} BA and 6.2 gl^{-1} agar. To evaluate the effects of various concentrations of calcium and boron on STN, the experiment was conducted as a factorial on completely randomized design. Results showed that soaking explants in 15% NaClO for five minutes followed by soaking in 0.01% HgClO for seven minutes efficiently removed contamination. It was also revealed that increasing calcium concentration enhanced proliferation rate and decreased STN. Moreover, doubling boric acid decreased the rate of necrosis while, tripling its concentrations increased the rate of necrosis. Increasing the concentration of boric acid also decreased proliferation. Finally, the lowest necrosis rate (17%) was obtained in the treatment containing 3x calcium chloride concentrations in combination with 2x boric acid concentration. In contrast, maximum rate of necrosis was obtained in the treatment containing 1x calcium chloride concentration in combination with 1x boric acid. Finally, in order to improved proliferation of shoot deferent, concentrations of NH_4NO_3 and KNO_3 were used. The maximum proliferation rate of shoot was obtained for 2280 mgL^{-1} KNO_3 as well as 1320 mgL^{-1} $NH4NO_3$.

Keywords: Boric acid, Calcium chloride, Contamination, Micro-propagation, MS medium, Shoot tip necrosis, UCB1 rootstock.

Introduction

Most of the pistachio rootstocks are produced from seeds. UCB-1 hybrid rootstock is produced from the seed of a controlled cross between a *P. atlantica* female and a *P. integerrima* male. Clonal propagation of this rootstock is also necessary, since it produces identical genotypes.

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In vitro propagation of this rootstock is difficult because of problems such as bacterial contamination and shoot-tip necrosis (STN). STN disorder occurs in the in vitro multiplication, elongation or rooting stages of some species such as wild cherry (Druart *et al.*, 1981), *Pistacia vera* (Barghchi and Alderson, 1985) and sour cherry (Borkowska and Michalczuk, 1985). Sha *et al.* (1985) found apical necrosis in actively growing shoot cultures of birch, apple, redwood, elm, rhododendron and potato. Vieitez *et al.* (1989) suggested that necrosis of the actively growing shoot-tips may be because of a deficiency of nutrients or growth regulators owing to a delay in restoring their efficient distribution.

Machado *et al.* (2014) found that the middle and basal portion had higher Ca^{2+} content than apical portion and they decreased STN using 1320 mg/l $CaCl_2.2H_2O$.

Some reasons that cause STN include nutrient deficiency (Ca and B), concentration of cytokinins, medium type and its nutrient concentration, aeration, gelling agent, pH of the medium and subculture period (Bairu *et al.*, 2009). Barghchi and Alderson (1996) found that Ca and B deficiency are of the most common reasons for STN, which results in less quality shoots and increased production cost. Srivastava and Joshi (2013) revealed that increasing $CaCl_2$ concentration to 18mM in MS medium decreased in vitro STN disorder with no effect on the growth of the explants. Hence, the present work was undertaken to overcome the in vitro contamination and STN disorder.

Materials and Methods

This study was conducted in the tissue culture laboratory of Horticultural Sciences of Faculty of Agriculture of Tarbiat Modares University in 2014.

Plant material and initiation of culture and shoot multiplication

Single node explants were prepared of pot plants of UCB1 in a greenhouse. Explants were placed under tap water for 120 minutes.

In order to find the best decontamination procedure, different concentrations (5, 10, 15 and 20%, v/v) of NaClO in various explants soaking times (5, 10 and 20 minutes) were used. In addition, different concentrations (0.01, 0.1, 0.5 and 0.25%) of $HgCl_2$ in various explants soaking times (3, 5 and 7 minutes) were evaluated. Sterilized explants were immersed in 100 mg/l ascorbic acid.

Modified MS medium containing Gamborg's vitamins, 3% sucrose and 6.2 g/l agar, 0.5 mg/l 6-benzyle adenine (BA) and 0.05 mg/l indole-3-butyric acid (IBA) and 0.05 mg/l gibberellic acid (GA) was used for culture establishment. In order to prevention of phenol exudation, explants were inoculated diagonally at an angle of approximately 60°.

Obtained shoots were sub-cultured in MS medium consisting $CaCl_2.2H_2O$ (1, 1.5 and 2 times of MS) and boric acid 1, 2 or 3 times of MS (Table 1). Finally, in order to improve shoot proliferation modified concentrations of NH_4NO_3 (1520, 1900 and 2280 mgL-1) and KNO_3 (1320, 1650 and 1980 mgL-1) in MS Medium containing Gamborg's vitamins, 3% sucrose and 6.2 g/l agar, 1.5 mg/l 6-benzyle adenine (BA) were used.

Table 1. combination various concentration of $CaCl_2$ and H_3BO_3 in basal MS medium

Treatment		H_3BO_3		
		1X	2X	3X
$CaCl_2$	1X	T1	T2	T3
	1.5X	T4	T5	T6
	2X	T7	T8	T9

Cultures were incubated in a growth room with light at 40µmol m^{-2} S^{-1} photosynthetic photon flux density (fluorescent lamps, 75 W) at plant level and a photoperiod of 16 hours of light and 8 hours of darkness at 25±2 °C. Factors such as shoot number per explant, apical necrosis percent, lost leaves and vitrification percent were evaluated.

Statistical analyses

Experiments were conducted with at least five replications, each including three explants per treatment. Completely randomized design based on factorial arrangement was used for all experiments. The data was statistically analyzed using SAS 9.2 (SAS Institute Inc., 2008). The significance of difference among means was carried out using Least Significant Difference (LSD) test at $P < 0.05$.

Results

Culture establishment contamination

NaClO concentration and soaking time were significantly ($P < 0.01$) more effective on decontamination of cultures, while their interaction was not significant. Increasing NaClO concentration decreased culture contamination percent so that the highest (57%) and lowest (3%) contamination percent were observed in concentrations of 5 and 20%, respectively. Increasing both the concentration and soaking time decreased contamination in that the lowest contamination (0%) was detected in 20% NaClO for 10 and 20 minutes. The highest contamination (63%) was related to the lowest NaClO concentration for five minutes.

There were no significant differences among $HgCl_2$ concentrations and soaking times effects on bacterial contamination. The highest contamination rate (33%) was related to 0.01% $HgCl_2$ for three minutes and the lowest one was related to 0.1% $HgCl_2$ for seven minutes. Additionally, although there was no significant difference among three used soaking times in 0.01% concentration, decontamination decreased from 33% to 20% with increasing soaking time.

Proliferation

Multifactorial analysis of variance showed that different concentrations of calcium chloride and boric acid had significant effects on the proliferation rate. However, the interaction between them had no significant effects on in vitro multiplication of UCB1 rootstock (Table 2). The results indicated that an increase in concentration of calcium chloride proliferation resulted in an increase in the maximum (an average of 6.2 shoots per explant) and minimum (an average of 1.7 shoots per explant) proliferation rate, which were obtained in heights (2X) and lowest (1.5X) calcium chloride concentration, respectively (Table 3). As seen in Table 3, there were not significant differences between the concentration of 1.5X and 2X calcium chloride. Although increasing calcium chloride had a positive effect on the proliferation rate versus increasing the concentration of boric acid, proliferation rate decreased with increasing boric acid concentration from 1X to 2X and 3X, an average of 2.6, 2.3 and 1.9 shoots per explant were obtained, respectively (Table 3).

Interaction of different concentrations of NH_4NO_3 on shoot length was significant ($p < 0.01$). The highest shoot length (2.31 cm) was attributed to the highest used concentrations i.e. 2280 mg/l NH_4NO_3 and the lowest length (0.9 cm) was obtained in lowest used concentrations i.e. 1520 mg/l KNO_3 + 1320 mg/l NH_4NO_3.

Table 2. Analysis of variance of the effect of various concentrations of calcium chloride and boric acid on proliferation, necrosis and hyperhydricity according to mean squares

Source of variations	Degrees of freedom	Proliferation	Necrosis	Number of necrotic leaf	Hyperhydricity
$CaCl_2$	2	3.32**	0.09**	3.49**	0.05**
H_3BO_3	2	1.99**	0.05**	2.11**	0.03**
$CaCl_2 \times H_3BO_3$	4	0.28*	0.01 *	0.39**	0.002^{ns}
Error	32	0.12	0.007	0.03	0.005

Table 3. Effect of different concentrations of calcium chloride and boric acid on proliferation rate, necrosis and hyperhydricity

Treatment	Number of microshoot per explant	Necrosis (%)	Number of necrosis leaf	Hyperhydricity (%)
T1	1.86c	0.42a	2.33a	0.18c
T2	1.79c	0.29abc	1.68b	0.22bc
T3	1.66c	0.38ab	2.28a	0.29abc
T4	2.93a	0.21bc	1.14c	0.25abc
T5	2.66ab	0.19c	1.09c	0.27abc
T6	1.86c	0.32 abc	1.65b	0.36ab
T7	3.06a	0.19c	0.93c	0.33ab
T8	2.73ab	0.17c	0.85c	0.34ab
T9	2.20 bc	0.30abc	1.91b	0.37a

Results of NH_4NO_3 and KNO_3 on proliferation showed that there was a significant difference ($p < 0.05$) among the interaction of the various concentrations of NH_4NO_3 and KNO_3 on shoot proliferation and proliferation. An increase in the concentration of KNO_3 enhanced proliferation rate and decreased in its concentration from 1980 to 1320 reduced proliferation. The highest proliferation rate was detected in 2280 mg/l KNO_3 and 1320 mg/l NH_4NO_3 treatment. The lowest proliferation rate was obtained in 1980 mg/l NH_4NO_3 and 1980 mg/l KNO_3 (Table 4).

Table 4.Effect of different concentration of KNO_3 and NH_4NO_3 on proliferation rate, necrosis, virtification and shoot length

KNO_3 (mg/l)	NH_4NO_3 (mg/l)	Proliferation rate	Necrosis (%)	Virtification (%)	Shoot length (cm)
2280	1980	2.28^{c}	10.00^{a}	5.00^{c}	2.31^{a}
	1650	2.81^{b}	0.00^{b}	0.00^{d}	1.79^{b}
	1320	2.99^{a}	0.00^{b}	0.00^{d}	$1.68b^{c}$
1900	1980	1.84^{e}	10.00^{a}	10.00^{b}	1.60^{c}
	1650	2.25^{c}	10.00^{a}	5.00^{c}	1.41^{d}
	1320	1.98^{d}	10.00^{a}	5.00^{c}	1.08^{e}
1520	1980	1.06^{g}	10.00^{a}	15.00^{a}	1.30^{d}
	1650	1.54^{f}	10.00^{a}	5.00^{c}	$1.02e^{f}$
	1320	1.13^{g}	10.00^{a}	10.00^{b}	0.90^{f}

Shoot tip necrosis

The multi-factorial analysis of variance indicated that different concentrations of calcium chloride and boric acid had significant effects on shoot tip necrosis but their interaction had no significant effect on STN (Table 2). This study showed that an increase in calcium chloride concentration resulted in a decrease in shoot tip necrosis. The lowest rate of necrosis (22%) was observed in highest concentration and the highest necrosis percentage (36%) was observed in lowest concentration (Table 3). The application of different concentrations of boric acid showed different results. Increasing the concentration from 1X to 2X decreased the necrosis rate. However, increasing the concentration to 3X resulted in increased shoots tip necrosis. Necrosis rate in 1X, 2X and 3X concentrations were 33%, 27% and 21%, respectively (Table 3). The interaction between calcium chloride and boric acid showed that the lowest necrosis (17%) was obtained in the medium that contained 2X calcium chloride in combination with 1.5X boric acid. The highest necrosis were obtained in the medium that contained 1X calcium chloride and 1X boric acid and in the medium that contained 1X calcium chloride and 3X boric acid, respectively (Table 3).

The results on the interaction of NH_4NO_3 and KNO_3 on shoot tip necrosis showed significant differences ($p < 0.05$). The lowest shoot tip necrosis (0.00%) was found in 2280 mg/l KNO_3 + 1650 or 1320 mg/l NH_4NO_3 with significant difference toward the rest treatments (10%) (Table 4).

Number of necrosis leaf

Variance analysis showed that calcium chloride, boric acid and the interaction between them had significant effects on the number of necrosis leaf of UCB1 rootstock (Table 2). The results revealed that the number of necrosis leaf was associated with shoot tip necrosis, and the highest number of necrosis leaf was observed in micro-shoot that show highest shoot tip necrosis. Increasing the calcium chloride concentration not only decreased necrosis but also decreased the number of necrosis leaf and the lowest necrosis rate (1.2 leaves per micro-shoot). The highest rate of necrosis (2 leaves per micro-shoot) was obtained in highest and lowest concentration of calcium chloride, respectively (Table 3). As seen in Table 3, there were not significant differences between different concentrations of boric acid. The lowest (1.2 leaf per micro-shoot) and highest (1.9 leaf per micro-shoot) number of necrosis leaf were observed in 1.5X and 3X concentrations, respectively (Table 3). Also, the lowest and highest number of necrosis leaf were obtained in treatment contain 2X calcium chloride in combination of 1.5X boric acid and 1X calcium chloride in combination with 1X boric acid, respectively (Table 3).

Hyperhydricity percentage

Analysis of variance showed that various concentration of calcium chloride and boric acid and the interaction between them had significant effects on the hyperhydricity of micro-shoots of UCB1 rootstock (Table 2). This study indicated that with an increase in calcium chloride resulted in an increase in the hyperhydricity rate. The highest percent of hyperhydricity (34%) was observed in the in highest calcium chloride concentration (2X) A decrease in calcium chloride to 1.5X and 1X resulted in 29% and 22% hyperhydricity, respectively (Table, 3). Also, the highest concentration boric acid resulted in the highest hyperhydricity. Decreasing the concentration from 2X to 1.5X and 1X resulted in a decrease in hyperhydricity from 34% to 27% and 22% (Table 3).

The results showed that increasing NH_4NO_3 enhanced vitrification rate but increasing KNO_3 decreased it. The lowest vitrification rate was in 2280 mg/l KNO_3 + 1320 or 1650 mg/l NH_4NO_3 and

increasing NH_4NO_3 (1980 mg/l) enhanced vitrification rate.

Discussion

NaClO significantly affected culture decontamination. The concentration and explant soaking time effects showed significant differences on culture decontamination. An increase in the concentration and soaking time can have serious effects on explants and result in necrosis of explants. Thus, determining the ideal concentration that results in the lowest necrosis and the highest decontamination is critical. On the other hand, soaking time is also important in explant browning. Although increasing soaking time decreased contamination, non-significant differences among concentrations indicated that using the lowest concentration is reasonable. Using concentration of 15% for five minutes was selected as the best treatment. Tilkat *et al*. (2013) stated that increasing NaClO concentration from 5% to 20% decreased contamination from 15% to 5% but decreased vitality in 10% NaClO was 70% and in 20% NaClO was 16%. In their study (who's study?), browning was increased with increasing NaClO concentration as well, from 65% and 78% in 5% and 20% NaClO concentrations, respectively. In fact, this can be attributed to the effect of NaClO on destruction of cell wall and phenolic compounds exudation. Browning of explants is greatly affected by disinfection materials. Hence, it is critical to consider both the concentration, vitality and browning of explants. Soaking time was also effective in decontamination. Using 10% NaClO for 5 and 40 minutes decreased contamination to 43% and 100%. An increase in the time of exposure decreased viability as the highest and lowest viability were attained in 5 and 30 minutes. There is no report on disinfection of pistachio explants.

$HgCl_2$ was as effective in decontamination. Without it, all of the explants were contaminated. In order to decrease the internal contamination of pistachio explants, Benmahioul *et al*. (2009) used 0.1% $HgCl_2$ and significantly decreased contamination rate.

High concentrations of $HgCl_2$ were also effective in decontamination but vitality of explants was decreased.

UCB1 is known as an appropriate rootstock for pistachio. UCB1 is widely used by growers because of its good resistance to verticillium wilt (Morgan and Epstein, 1992) and other appropriate traits. In order to establish high density orchard, developing an efficient micro-propagation protocol of UCB1 is one of the important requisitions. Optimization of the multiplication stage is vital for commercial micro-propagation of this plant. Several factors such as plant growth regulator, medium culture and agar concentration influence micro-propagation process (Abdoli *et al*., 2007). UCB1micro-shoots in proliferation stage are involved with shoot tip necrosis and hyperhydricity. Some factors such as cytokinin concentration (Oliveira *et al.,* 2010), type of medium culture (Scheidt *et al.,* 2011; Silva *et al.,* 2012), cultivar and agar concentration (Abdoli *et al.,* 2007; Carvalho *et al.,* 2013) are the reasons of these abnormalities. One of the strategies to overcome this problem is to modify the composition of the culture medium (Machado *et al.,* 2014). Optimization of calcium and boron in the culture medium is very important in the prevention of these abnormalities many researchers have suggested that the deficiency of some nutrients such as B and Ca because of low mobility may be the cause of STN (Barghchi and Alderson, 1996), as indicated in this study.

The results of this study are in concurrence with those of Barghchi and Alderson (1996) and McIlrath and Skok (1964), which indicated that increasing calcium chloride significantly decreased necrosis and increasing $CaCl_2$ up to 24 mM had no negative effect on proliferation and elongation. The positive effects of

calcium may be associated with its roles in cell differentiation (Penel *et al*., 1984), enzyme activation (Carbonell and Jones RL, 1984) and membrane permeability (Kohle *et al*., 1985). Therefore, disruption of normal metabolism and tissue growth due to calcium deficiency may lead to symptoms of shoot tip necrosis. Increasing calcium content in the medium was also associated with increasing chloride concentration. However, increasing chloride concentration had no negative impact on in vitro growth of micro-shoots, which is consistent with Barghchi and Alderson (1996). Similar to the results of this study, Barghchi and Alderson (1996) reported that increasing boron concentration up to 200 mM significantly decreased STN rate and decreased proliferation rate. Boron deficiency alone or in combination with the lack of calcium can cause necrosis (Barghchi and Alderson, 1996). Abousalim and Mantelli (1994) reported that increasing boric acid concentration up to 200 mM decreased STN in *P. vera* but very high concentration of boron resulted in a decrease in the proliferation rate and length of micro-shoot and caused necrosis, which was also observed in this study. Mirabbasi and Hosseinpour (2014) reported that the lowest STN was obtained in a ME medium, which may be due to very high doses of calcium.

The results of modifications in NH_4NO_3 and KNO_3 concentrations showed that increasing KNO_3 concentration increased proliferation rate and increasing NH_4NO_3 decreased proliferation rate. Increasing KNO_3 resulted in improving shoot tip necrosis, which may be due to improved and accelerated vegetative growth and refining Ca absorb. In addition, although rising NH_4NO_3 increased shoot length, an increase in NH_4NO_3 caused shoot tip necrosis.

Vitrification occurrence is under the influence of different factors such as concentration and quality of used minerals. NH_4NO_3 is greatly affects vitrification rate. We also observed that increasing NH_4NO_3 concentration enhanced vitrification rate. Decreasing vitrification rate was also observed with an increase in KNO_3 concentration. These results were in accordance with the study by Partfit and Almehdi (1991), which showed that an increase in KNO_3 in basal medium of DKW enhanced proliferation rate, leaf number and quality of obtained plantlets.

Conclusions

The results clearly showed that determining the appropriate concentration of calcium chloride and boric acid was crucial for a successful proliferation phase in various pistachio rootstocks and cultivars. Overall, the results of the current study indicated that optimized MS medium contain 3x calcium chloride concentrations in combination with boric acid 2X concentration, which resulted in the lowest necrosis rate (17%).

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