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HORTICULTURAL STUDIES

The Effect of Antioxidants on Micropropagation of Avocado by Nodal Segments

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Abstract

Tissue culturing, which is an alternative method to clonal reproduction of avocado (*Persea americana* Mill.) has started to become widespread in recent years. However, the browning of tissue which is caused by oxidation of phenolic compounds is one of the most important factors limiting success for the in vitro reproduction. Therefore, in this study, the effects of different antioxidants (activated charcoal (AC), ascorbic acid (ASA), citric acid (CA) alone or in combination with each other on browning and regeneration of avocado shoots, grown under in vitro conditions, were investigated. In the study, MS (Murashige and Skoog) nutrient medium with 1 mg L^{-1} BAP, 0.1 mg L^{-1} GA_3 and 3% sucrose was used in all treatments. Moreover, antioxidants alone or combined with each other with different concentrations were used in the nutrient media in all treatments except the control. As a result of the research, the lowest browning intensity and the highest survival rate values were shown in the application of 100 mg L^{-1} ascorbic acid (ASA) alone and in the combination of 60 mg L^{-1} ascorbic acid (ASA) and 40 mg L^{-1} citric acid (CA). Furthermore, maximum shoot length (2.8 cm) and maximum leaf number (6.4) in terms of survival rate of the explants, shoot length and number of leaves were determined in MS nutrient medium containing a combination of 60 mg L^{-1} ascorbic acid (ASA) and 40 mg L^{-1} citric acid (CA).

1. Introduction

Avocado (*Persea americana* Mill.) is the most economically important crop in the Lauraceae family. It is widely grown in many tropical and subtropical parts of the world (Morton, 1987). The popularity of avocado has increased over the last few decades and became a very important commercial crop to cultivate recently in many countries of the world (Silva and Ledesma, 2014). Worldwide production of avocado almost doubled in the last 10 years due to the increase in consumption. Thus, the trade of avocado reached over 6.4 million tons in 2018 (FAO, 2018). In 2018, the largest avocado producers were Mexico (2.1 million tons), Dominican Republic (644 thousand tons), Peru (504 thousand tons), Indonesia (410 thousand tons), and Colombia (326 thousand tons) (FAO, 2018).

Commercial cultivation of avocado has been generally accomplished by vegetative propagation (grafted) (Ben-Ya'acov and Michelson, 1995). However, seed-propagated rootstocks showed high genetic variation and trees were not true-to-type. Hence, seedling rootstocks are not suitable for commercial purposes. Clonal rootstocks are vegetatively propagated by the Frolich & Platt double grafting method, and has been used commercially for more than 40 years. However, this method is an expensive, time consuming and laborious (Frolich and Platt, 1972; Ernst, 1999). Micropropagation is an essential part of clonal

propagation and more advantageous than the traditional propagation methods (Thorpe, 2007). However, in vitro techniques are considered as an alternative method for seedling production of avocado, in vitro propagation of avocado is associated with several problems as do other woody plants (Barceló-Muñoz and Pliego-Alfaro, 2012; Hiti-Bandaralage et al., 2017). One of the most important problem is browning, it occurs at the initial establishing stage of in vitro culture of avocado due to leaching of phenolic substances and secondary metabolites from the cut surfaces of explants (Schall, 1987).

Browning of the explant usually depends on the phenolic compounds that are secondary metabolites and the quality of the total phenols in the explant tissues (Ozyigit, 2008). Phenolic compounds frequently cause browning and are well known to be inhibitory to the plant's cellular growth (Monaco et al., 1995). Due to browning, the tissues of the explant turned necrotic and died within a few days after inoculation. Different treatments have been investigated to eliminate the browning problem with varying efficiency from species to species (Saenz et al., 2010). For instance, low temperature and dark incubation, have been used to reduce browning in the explants of avocado (Barceló-Muñoz et al., 1999; Castro et al., 1995) and pear (Poudyal et al., 2008). Besides, antioxidants have been commonly used in tissue culture media to improve cell growth and development and have many roles in the plants physiological processes (Shao et al., 2007). They play an important role in the adsorption of phenolic compounds. Therefore, antioxidants should be considered as one of the most important factors for an effective control of browning (George, 1996). Adding the antioxidants (activated charcoal (AC), ascorbic acid (ASA), citric acid (CA), polyvinylpyrrolidone (PVP) and etc.) to the nutrient medium is among the most commonly used treatment for tissue browning in woody plant species (Ahmad et al., 2013). Using antioxidants in browning control has been demonstrated in several species such as guava (Zamir et al., 2004), mango (Chandra et al., 2003), pomegranate (Singh and Patel, 2016), pear (Poudyal et al., 2008), banana (Munguatosha et al., 2014), and hazelnut (Shirazi et al., 2020). In addition, different antioxidants have been used to reduce browning in avocado tissue culture medium. Several researchers have reported the addition of PVP (Dalsaso and Guevara, 1989; Ahmed et al., 2001; O'Brien et al., 2020) and melatonin (O'Brien et al., 2020) alone in the medium to control browning of cultured explants of avocado. Pre-treatment with different antioxidants is one of the ways to remove phenols or reduce the media browning (Krishna et al., 2008). Pre- treatment of different antioxidants was found to be quite efficient, not only for inhibition of browning, but also for prevention of leakage of phenolic compounds into avocado (Castro et al., 1995; Wessels et al., 1996), guava (Ahmad et al., 2016), longan (Hong-bin, 2008) and mango (Krishna et al., 2008). Therefore, various antioxidant compounds were selected in this study.

According to previous published literature, there is still no exact treatment to control of the avocado nodal segment completely. Based on that, the objective of the study was to investigate the effects of various antioxidants (AC, ASA, CA) and their combinations (ASA+CA, AC+ASA, AC+CA) on the browning of nodal segments and the survival rate of the explants, shoot length and number of leaves in avocado.

2. Materials & Methods

The experiment was carried out in the Tissue Culture Laboratory in Batı Akdeniz Agricultural Research Institute in Antalya province in Turkey in August, 2020.

2.1. Plant material

Shoots having at least five nodal segments were collected from 3-4 years old grafted plants grown in the glasshouse. Explants were taken in early August of 2020 and before sterilization, all leaves were removed from the shoots.

2.2. Surface sterilization

Shoots were washed under tap water for 30 minutes, after that, they were submerged with 70% (v/v) ethanol for a minute followed by rinsing with sterile distilled water three times in a laminar air-flow cabinet. Finally, the twigs were treated with 5% (v/v) sodium hypochlorite with three drops of tween-20 for 3 minutes and then rinsed three times with sterile distilled water. The sterilized explants were cut into 1-2 cm nodal segments with axillary buds aseptically.

2.3. Culture conditions and media

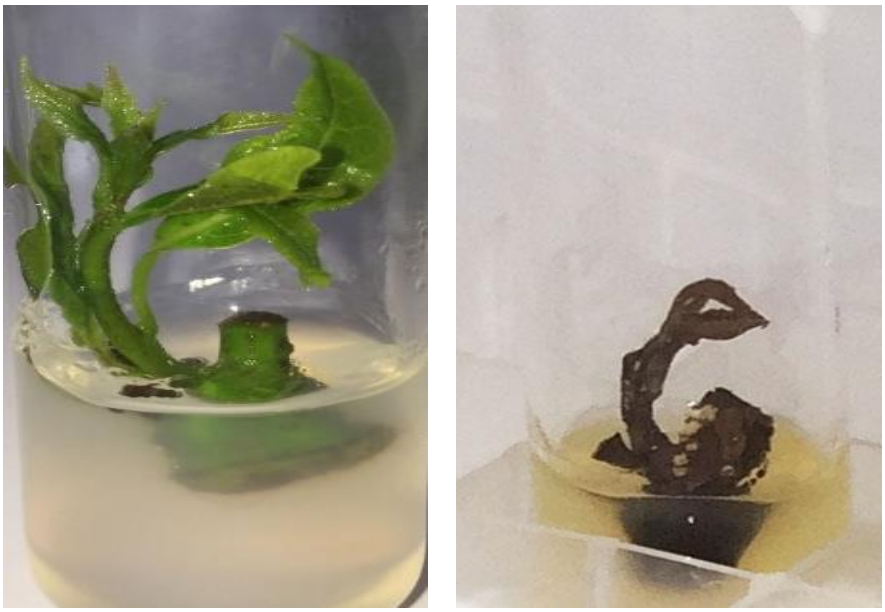
As a nutrient medium, Murashige and Skoog (MS) basal medium was used in the experiment. Also, gibberellic acid (GA_3) at 0.1 mg L^{-1} and benzylaminopurine (BAP) at 1 mg L^{-1} were added to the MS basal medium in all treatments. In addition, various antioxidants (AC, ASA and CA) with different concentrations and combinations were added to the medium (Table 1).

Besides, 3% sucrose (w/v), 7% agar (w/v), and 100 mg L^{-1} myo-inositol were included to media. The pH of the medium was adjusted to 5.7 before the addition of agar and autoclaved at 121°C for 20 min under 1.0 atm. After surface sterilization, nodal segments were cultured on 15 ml semi-solid medium as 1 explant per test tube. Explants were cultured on initiation media for four weeks and then were transferred to sub-culture media. Cultures

Table 1. The concentration and combination of antioxidants used in the study

AC (mg L^{-1})	ASA (mg L^{-1})	CA (mg L^{-1})	ASA+CA (mg L^{-1})	AC+ASA (mg L^{-1})	AC+CA (mg L^{-1})
0	0	0	0	0	0
150	50	20	30+20	1000+50	1000+20
250	100	40	60+40	500+100	500+40
500	150	60	90+60	250+150	250+60
1000	200	80	120+80	150+200	150+80

AC: activated charcoal, ASA: ascorbic acid and CA: citric acid



a b

Figure 1. Green, healthy and vigorous shoot obtained in the basal medium with the 60+40 mg L^{-1} combination of ASA+CA (a) and dead shoots due to the presence of phenolic compounds in the control treatment (b).

were incubated in a growth room at 25±2°C with a 16-h photoperiod for eight weeks.

2.4. Investigation criteria

2.4.1. Browning intensity: Browning intensity was observed four weeks after culturing explants in the initiation media. The number of died explants due to the phenolic compounds were evaluated for each treatment. Browning intensity was evaluated on an arbitrary browning scale ((++++) = intense browning, (+++) = moderate browning, (++) = low browning, (+) = very low browning) (Singh and Patel, 2016).

2.4.2. Survival rate of the explants (%): Survival rate was recorded four weeks after the sub-culturing of explants. The number of green, healthy and vigorous shoots were counted for each treatment and the survival percentage were calculated.

2.4.3. Shoot length (cm): The average shoot length was recorded four weeks after sub-culturing. Shoot length was measured from the node to the tip of the shoot at the time of subculturing.

2.4.4. Number of leaves: The number of leaves was recorded four weeks after sub-culturing. The total number of leaves were counted per shoot and the percentage was calculated.

2.5. Data analysis

The experiment was established according to completely randomized design with three replicates and 4 explants for each replicate. The data were subjected to analysis of variance using the SAS software version 9.00. The least significant difference (LSD) method was used to test the difference between treatments and $p \leq 0.05$ was considered statistically significant.

3. Results & Discussion

The effects of different antioxidants alone or in combination on browning intensity are shown in Table 2. It was found that the use of all antioxidants both alone and together reduces the intensity of browning according to the control treatment. Increasing the concentration of some antioxidants used alone in the experiment decreased the intensity of browning, while some increased the browning intensity (Figure 1 a, b). For instance, it was found that when the concentration of AC exceeds 500 mg L^{-1}, the intensity of browning increased. It is thought that this situation occurs when AC increases the uptake of plant growth regulators in the nutrient medium. Unlike AC, the intensity of browning decreased as the concentrations of other antioxidants in the

Table 2. The effects of different antioxidant concentrations and combinations on browning intensity, survival rate of the explants, shoot length and number of leaves

Treatments	Concentration (mg L^{-1})	Browning intensity	Survival rate of the explants (%)	Shoot length (cm)	Number of leaves
AC	0	++++	16.67 b*	1.4 b	3.2 b
	150	++	58.33 a	1.5 ab	3.4 ab
	250	++	58.33 a	1.6 ab	3.5 ab
	500	++	66.67 a	1.7 a	3.6 a
	1000	+++	41.67 ab	1.4 b	3.2 b
$LSD_{5\%}$			26.259	0.215	0.372
ASA	0	++++	16.67 c	1.4 c	3.2 d
	50	+++	41.67 bc	1.9 b	4.9 b
	100	+	83.33 a	2.6 a	6.0 a
	150	++	75.00 ab	2.4 a	5.7 a
	200	++	66.67 ab	1.8 bc	4.4 c
$LSD_{5\%}$			31.070	0.417	0.453
CA	0	++++	16.67 b	1.4 c	3.2 b
	20	+++	41.67 ab	1.6 bc	3.4 b
	40	++	75.00 a	2.2 a	5.3 a
	60	++	66.67 a	2.0 ab	5.1 a
	80	++	66.67 a	1.5 c	3.4 b
$LSD_{5\%}$			37.136	0.384	0.479
ASA+CA	0	++++	16.67 b	1.4 d	3.2 d
	30+20	+++	41.67 b	1.8 c	4.5 c
	60+40	+	83.33 a	2.8 a	6.4 a
	90+60	++	75.00 a	2.2 b	5.2 b
	120+80	++	75.00 a	1.9 bc	4.8 bc
$LSD_{5\%}$			28.765	0.318	0.616
AC+ASA	0	+++	16.67 c	1.4 c	3.2 c
	1000+50	+++	41.67 b	1.7 bc	3.2 c
	500+100	++	75.00 a	1.9 ab	4.1 b
	250+150	++	66.67 a	2.2 a	5.2 a
	150+200	++	58.33 ab	1.6 bc	3.5 bc
$LSD_{5\%}$			23.487	0.433	0.662
AC+CA	0	++++	16.67 b	1.4 c	3.2 d
	1000+20	+++	41.67 ab	1.8 ab	4.1 b
	500+40	++	66.67 a	2.1 a	5.0 a
	250+60	++	58.33 a	1.6 bc	3.6 cd
	150+80	+++	41.67 ab	1.8 ac	4.0 bc
$LSD_{5\%}$			26.259	0.384	0.492

(++++) = Intense browning, (+++) = Moderate browning, (++) = Low browning, (+) = Very low browning.
* Represent significant differences ($p<0.05$) among treatments.

experiment increased. For example, increasing concentrations of ASA and CA reduced the intensity of browning. In the use of antioxidants with each other, the best results in terms of browning intensity were found in 60+40 mg L^{-1} combination of ASA and CA. In combinations of AC with other antioxidants (AC+ASA and AC+CA), when the concentration of AC was decreased and the concentration of ASA and CA was increased, browning intensity reduced. When the research findings were evaluated in terms of browning, it was determined that ASA is the best antioxidant when using antioxidants alone and using the ASA+CA combination is the best antioxidant treatment when using them together. The results of this study were similar to some previous researches. In these studies, it has been reported that the application of AC, ASA, CA together to avocado explants gave better results than the application alone in terms of browning intensity (Nel et al., 1983; Pliego-Alfaro and Murashige, 1987; O'Brien et al., 2020). Moreover, in the study conducted by Munguatosha et al. (2014), it was stated that adding 100 mg L^{-1} ASA in the nutrient media decreased the browning in bananas. Also, in the study performed by Ndakidemi et al., (2014) about *Brachylaena huillensis* (Asteraceae), it was reported that the best results were obtained in terms of browning intensity when 200-250 mg L^{-1} ASA was added to the medium.

The effects of using different antioxidants alone or in combination with each other on the survival rate of the explants are given in Table 2. The survival rate increased compared to the control in all antioxidant treatments. It is clearly seen in Table 2 that there is a relationship between the survival rate and browning intensity. It has been determined that as the density of browning increased, the survival rate decreased. When AC is used among the antioxidants, the highest survival rate was determined at the concentration of 500 mg L^{-1} with 66.67%. When another antioxidant, ASA, was used

alone, the best result was obtained at a concentration of 100 mg L^{-1} with 83.33%, followed by 150 mg L^{-1} with 75% and 200 mg L^{-1} with 66.67%, respectively. As in ASA, when used above a certain concentration in CA too, the survival rate decreased. The highest survival rate in CA was 75% at a concentration of 40 mg L^{-1}. When antioxidants were used together, the highest survival rate was found at 60+40 mg L^{-1} concentration from ASA + CA combination. This was followed by 500+100 mg L^{-1} AC+ASA combination with 75% and 500+40 mg L^{-1} AC+CA combination with 66.67%. As a result of our findings, the highest survival rate was determined with 83.33% as a result of using ASA alone and ASA+CA together. The findings in the present study were similar to previously published results. In a study carried out by O'Brien et al. (2020), it was reported that the survival rate in avocado increased in the media containing 100 and 250 mg L^{-1} AA. In the study conducted by Ahmad et al. (2016), it was found that using AC, ASA and CA alone increased the survival rate in guava.

The effects of using different antioxidants alone or in combination with each other on shoot length are given in Table 2. As can be seen in Table 2, it has been found that shoot length varied between 1.4-1.7 cm when using AC alone. Moreover, in the use of ASA, the highest shoot length was obtained as 2.6 cm with 100 mg L^{-1} concentration, followed by 2.4 cm with 150 mg L^{-1} and 1.9 cm with 50 mg L^{-1}. Besides, when using CA alone the highest shoot length was 2.23 cm with 40 mg L^{-1}. When antioxidants were used together, the best results in terms of shoot length were found with a combination of 2.8 cm and 60+40 mg L^{-1} ASA+CA. In other combinations, the best results were obtained at 2.2 cm with 250+150 mg L^{-1}, AC+ASA and 2.1 cm with 500+40 mg L^{-1} AC+CA. As a result of our findings, the highest shoot length was obtained from the media containing ASA when antioxidants were used alone and ASA+CA combination when used together. In a study conducted by Al-Mayahi et al. (2016) on persimmon, it was reported that the highest shoot length was obtained by combining ASA with salicylic acid (SA). In the study conducted by Jakhar et al. (2019) on guggul (*Commiphora wightii*), the highest shoot length was obtained in the medium where 150-200 mg L^{-1} AC were added, different from our results.

When the effect of the treatments on the number of leaves was examined, it was found that the use of all antioxidants alone or together with each other was found to be statistically significant as in the shoot length, except for the use of AC alone, (Table 2). In addition, it is clearly seen in Table 2 that there is a relationship between shoot length and the number of leaves, as well as the relationship between browning density and the survival rate. The number of leaves has been found to be between 3.2 and 3.6 when using AC alone. The best result in terms of leaf number of ASA, another antioxidant, was obtained as 6.0 at a concentration 100 mg L^{-1}, followed by 5.7 with 150 mg L^{-1} and 4.9 with 50 mg L^{-1}. The effect of CA on the leaf number changed between 3.4-5.3. In the use of antioxidants with each other, the best treatment for the number of leaves as well as the number of shoots and the survival rate was obtained in the combination of ASA+CA at a concentration of 60+40 mg L^{-1}. In other antioxidant combinations, the highest number of leaves was found as 5.2 in the combination of 250+150 mg L^{-1} AC+ASA and 5.0 in 500+40 mg L^{-1} AC+CA. When the results were examined in terms of number of leaves, the best results were obtained in 100 mg L^{-1} ASA used alone and 60+40 mg L^{-1} ASA+CA in combination with each other. Al-Mayahi et al. (2016) reported that the highest number of leaves of persimmon was obtained with the combination of ASA+SA.

4. Conclusion

As a result of the study, it was observed that browning of survival rate of the explants, shoot length and number of leaves in avocados can be prevented by adding antioxidants to the plant nutrient media. The best results, in order to prevent browning, were obtained by the addition of 100 mg L^{-1} ASA and the combination of 60+40 mg L^{-1} ASA+CA to the nutrient medium and statistically significant differences were found in these treatments compared to the control group. Moreover, the best result in terms of shoot length and leaf number was obtained from the 60+40 mg L^{-1} ASA+CA combination. In future studies, it is recommended to try different antioxidant combinations in different plant nutrient media to prevent browning in avocado plants under *in vitro* conditions.

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