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Inhibition of persian walnut (*Juglans regia* L.) microcuttings browning by utilizing different methods

Abdollah Ehteshamnia*, Mansour Gholami

Department of Horticultural Sciences, Faculty of Agriculture, Bu-Ali Sina University, Hamedan, Iran

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Key words: activated charcoal, ascorbic acid, browning, polyvinyl pyrrolidone, subculture.

Abstract

Microcuttings browning and eventual death of the tissue during the initial stage of walnut tissue culture is a frequent problem. In this research, three different experiments were conducted to inhibition microcuttings browning. In first experiment, the effect DKW, DKW and MS culture medium fortified with activated charcoal (AC) and ascorbic acid (AA) on the initiation medium for two Persian walnut cultivars chandler and Jamal were evaluated. Results of this experiment indicated that DKW medium fortified with AC were optimum medium than MS and DKW Supplemented with AC and AA. In second experiment, the subculturing thrice of two walnut cultivars microcuttings at an interval of 48 hrs in DKW basal medium and DKW medium containing AC and AA were studied. The microcuttings subculturing in DKW medium containing AC was better than subculturing in DKW medium containing AA but there was no significant difference between them. In third experiment, the microcuttings were soaked for 2 h before culture by following solutions: soaked in distilled water, polyvinyl pyrrolidone (PVP) and AA for two walnut cultivars. Microcuttings soaked in PVP had suitable establishment than AA and distilled water. Finally, this research indicated in most cases successful control of Persian walnut microcuttings browning could be achieved by different combinations of these methods.

***Corresponding Author:** Abdollah Ehteshamnia ✉ ab.ehteshamnia@gmail.com

Introduction

Walnut tree (*Juglans regia* L.), belongs to family Juglandaceae, is an important temperate nut crop. Walnuts rank third in nut production after cashews and almonds (FAOSTAT, 2011). During the last decade, the worldwide walnut production was doubled, probably reflecting on the increase in consumers demand for this nut (Christopoulos and Tsantili, 2011). The first attempts at walnut micropropagation utilized existing medium formulations which were suitable for other woody plants. Because woody plants are still often very difficult to culture; many different types of medium have been employed. In previous researches done at walnut micropropagation, different culture medium have been used, such as Driver and Kuniyuki (DKW) (Driver and Kuniyuki, 1984), Murashige and Skoog (MS) (Murashige and Skoog, 1962), B5 (Gamborg *et al*., 1968), Cheng (C) (Cheng, 1978), NGE (Sanchez-Zamora, 2006) and woody plant medium (WPM) (Lloyd and McCown, 1981) with varying results. Micropropagation has the immense advantage of rapidly generating a large number of genetically identical plants in a much shorter time than could be achieved by conventional propagation methods. But these techniques receive a set back by certain physiological processes which hinders the success of new technique, particularly in perennial fruit crops.

Explants browning and eventual death of the tissue during the initial stage of walnut culture is a frequent problem. One of the major obstacles associated with *in vitro* multiplication of walnut mature material is the phenolic compound exudation from the cut surface of the explants. Oxidation of these compounds caused lethal browning of explants and culture medium. Oxidation of phenolic compounds released from the cut ends of explants by polyphenoloxidases, peroxidases cause lethal browning of explants and culture medium (Bhat and Chandel, 1991). In some species the establishment of explants frequently requires special procedures to escape or avoid problems associated with oxidation of polyphenols (Ashutosh *et al.,* 2003). Difficulties reported on tissue culture of *Juglans* species were mainly in the initiation phase due to the detrimental effect of phenolic browning beside the low multiplication rate and cultures decline in the proliferation phase. Supplementing the initial medium with different additives that can prevent the production of phenolics or can remove inhibitory phenolic substances from the medium, such as antioxidants, chelate-forming materials or adsorbents (Block and Lankes, 1996; Pan and van Staden, 1998; Dobránszki *et al*., 2000 a,b,c; Sharma *et al*., 2000; Thomas, 2008). In this study, efficient shoot multiplication method from nodal segments of walnut (*Juglans regia* L.) using different nutrient medium, antioxidants, absorbent, different pretreatments and subculturing of microcuttings were evaluated. This study aimed to counteract and declining browning to enhance growth and multiplication of cultures. This research was made to control browning of cultures to get successful establishment of two Persian walnut microcuttings.

Materials and methods

Plant Materials

Newly grown shoots of two Persian walnut cultivars (*Juglans regia* L.), `Chandler' and `Jamal' were collected in early May 2013 from selected mature trees growing in Walnut Research Station in Toyserkan, Iran. Trees were managed in terms of nutrition, pruning, irrigation, pests and diseases, similarly.

Sterilization of microcuttings

Shoots were cut into nodal segments (uninodal microcuttings with a length between 3 and 5 cm). Then were washed with tap water for 20±05 min and surface-disinfected by immersion in 70% (v/v) ethanol/water solution for 30 s followed by 1.5% (w/v) sodium hypochlorite fresh solution with two drops of Tween 20 per 100 ml for 20 min, followed by three rinses in sterile deionized water.

In this research, three different experiments have been conducted:

Experiment 1

The experiment was performed as a factorial experiment with three factors. These factors including three different nutrient medium ($\frac{1}{2}$DKW, DKW and MS) as factor A; additives substance in basal medium including AC (2 g/l) and AA (150 mg/l) as factor B; and 'Jamal' and 'Chandler' two Persian walnut (*Juglans regia* L.) cultivars as factor C. The treatments were arranged in a completely randomized design with three replications, with each replication containing 20 microcuttings.

Experiment 2

The experiment was performed as a factorial experiment with two factors. These factors including three different subculturing of microcuttings thrice, at an interval of 48 hrs in DKW basal medium, DKW medium which was containing AC (2 g/l) and DKW medium which was containing AA (150 mg/l) as factor A and 'Jamal' and 'Chandler' two Persian walnut (*Juglans regia* L.) cultivars as factor B. Treatments were arranged in a completely randomized design with four replications, with each replication containing 20 microcuttings.

Experiment 3

In this experiment, the immediate effects of anti-browning pre-treatments of microcuttings were tested on the initiation phase. The experiment was performed as a factorial experiment with two factors. These factors including prior to culturing, microcuttings were soaked for 2 hours in one of the following solutions, soaked in distilled water, soaked in antioxidant (AA 150 mg/l) and soaked in PVP (500 mg/l) as factor A and 'Jamal' and 'Chandler' two Persian walnut (*Juglans regia* L.) cultivarsas as factor B. Treatments were arranged in a completely randomized design with four replications, with each replication containing 20 microcuttings. The immediate effects of pre-treatments were tested in the initiation phase.

Culture conditions

Nodal segments of two cultivars (Jamal and Chandler) were used as microcuttings. Microcuttings (3 to 5 cm in length) were cultured on basal medium consisted of Driver and Kuniyuki (Driver and Kuniyuki, 1984) Walnut (DKW) macro- and microelements and vitamins, supplemented with 3% (w/v) sucrose and without any growth regulators. AC and PVP added to medium before autoclaving but AA is not stable at high temperature and added to medium after autoclaving by filtration sterilization. The medium was solidified with 0.8% (w/v) Sigma agar and autoclaved at 121°C and 1.06 kg/cm² pressure for 15 min after adjusting the pH to 5.8 with 0.1 N HCl or 0.1 N KOH. Microcuttings were kept at 22 ±1 ◦C and 16 h light: 8 h dark Photoperiod (provided by cool-white fluorescent light). The cultures were maintained 30 days at the same conditions. Observations of the extent of browning were recorded. The rate of each treatments efficiency for the inhibition of browning in each experiment recorded with followed scores: -: Not effective for the inhibition of browning, +: low efficiency, and ++: high efficiency.

Statistical Analysis

All statistical analyses were performed using the programme GLM on SAS (Anonymous, 1988). The cultures were maintained 30 days on the same conditions and the data were analyzed using analysis of variance (ANOVA) to calculate statistical significance, and the mean±SE (standard error). Differing significantly were determined using Duncan's multiple range test at $p < 0.05$ level.

Results

Experiment 1

This experiment was conducted to find the optimum nutrient medium ($\frac{1}{2}$ DKW, DKW and MS) fortified with additives substance (AC and AA) for shoot multiplication of two Persian walnut cultivars (Table 1). The results of this experiment showed the DKW medium fortified with AC was optimum medium. Establishment of microcuttings was better in DKW

basal medium than other medium. Significant difference was observed for main shoot length and shoot fresh weight between DKW with MS and ½ DKW medium, and no significant difference was observed for Leaf number on shoots and shoot number on microcuttings between DKW and MS medium. Micro-shoots on ½ DKW were thinner, had less leaf expansion and were less green than those on DKW and MS medium (Fig. 2).

Table 1. In vitro growth and browning of different cultivars of *J. regia* L. 30 day after inoculation on different nutrient media fortified with two additives substances[a].

Media	Main shoot length (cm)	Shoot fresh weight (g)	Leaf number on shoots	Shoot number on explants	Tissue browning[b]
DKW	3.66±0.09 a	0.77±0.04 a	9.87±0.99 a	3.50±0.53 a	+
MS	3.52±0.15 b	0.64±0.04 b	9.12±0.64 ab	2.62±0.74 ab	+
½ DKW	3.40±0.20 c	0.61±0.04 b	8.62±0.92 b	2.50±0.76 b	++
Additives substance					
AC	3.62±0.17 a	0.70±0.08 a	9.67±0.98 a	3.00±0.74 a	++
AA	3.43±0.15 b	0.65±0.07 b	8.75±0.75 b	2.75±0.87 a	+
Cultivar					
Chandler	3.56±0.22 a	0.68±0.08 a	9.33±0.89 a	3.00±0.74 a	+
Jamal	3.49±0.15 a	0.67±0.08 a	9.08±1.08 a	2.75±0.87 a	+
Interaction	$P < 0.05$	NS[c]	NS	NS	

[a] Means ± standard error followed by the same letter are not significantly different ($P < 0.05$).

[b] -: Not effective for the inhibition of tissue browning, +: low efficiency, and ++: high efficiency.

[c] Not significant ($P < 0.05$). AA = ascorbic acid; AC= activated charcoal.

All three nutrient medium (½ DKW, DKW and MS) fortified with AC obtained better result than combination medium with AA in both cultivars. The best results for main shoot length, shoot fresh weight and Leaf number on shoots were observed on cultures fortified with AC in both cultivars and significantly better than cultures fortified with AA. There was no significant difference between medium fortified with AC and AA for shoot number on microcuttings (Table 1).

The graph for main shoots length rate of microcuttings in different medium, additives substances and different cultivars, illustrated in Fig. 1. results showed medium fortified with AC promoted shoot elongation in all medium for Chandler and Jamal cultivars except ½ DKW medium for Jamal cultivar that obtained longer main shoot when fortified with AA than AC.

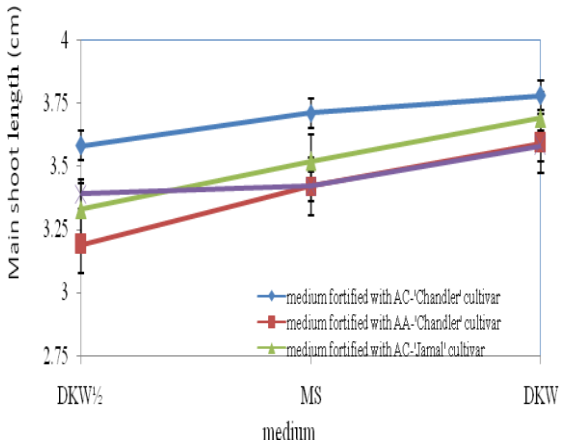

Fig. 1. Explants main shoot length rate of two Persian walnut cultivars (*J. regia* L.) 30 day after inoculation on different media fortified with AC and AA. AA= ascorbic acid; AC= activated charcoal.

In this experiment, Chandler and Jamal cultivars were not significantly different with together. Efficiency rate of each treatment for the inhibition of tissue browning (Table 1) showed present treatments

on this experiment had a variable effect for the inhibition of tissue browning in both cultivars. However AA and AC treatments alone had low (+) and high (++) efficiency for the inhibition of tissue browning, respectively. Also, different $\frac{1}{2}$ DKW, DKW and MS medium treatments alone had high (++), low (+) and low (+) effective for the inhibition of tissue browning, respectively.

Experiment 2

Results of this experiment (Table 2) indicated that subculturing of microcuttings thrice, at an interval of 48 hrs in DKW medium containing AC was better than subculturing in DKW medium containing AA. But significant difference was not observed between them. The subculturing of microcuttings thrice, at an interval of 48 hrs in DKW medium containing AC was not significantly different from subculturing of microcuttings thrice, at an interval of 48 hrs in DKW medium containing AA. But both of them were significantly better than sub culturing of microcuttings thrice, at an interval of 48 hrs in DKW basal medium. In two traits, Main shoot length and Leaf number on shoots, Jamal cultivar was significantly different from Chandler cultivar that these traits higher in Jamal cultivar. In other traits, these two cultivars were not significantly different together. Efficiency rate of each treatment for the inhibition of browning (Table 2) showed present treatments in this experiment had a low efficiency (+) on Chandler cultivar and high efficiency (++) on Jamal cultivar for the inhibition of tissue browning. However DKW medium fortified with AC, AA and basal medium had high (++), low (+) and not effective (-) for the inhibition of tissue browning, respectively.

Table 2. Responses of two Persian walnut (*J. regia* L.) explants 30 day after inoculation to subculturing of explants on different media[a].

The subculturing of explants: thrice, at an interval of 48 hrs in:	Main shoot length (cm)	Shoot fresh weight (g)	Leaf number on shoots	Shoot number on explants	Tissue browning[b]
DKW medium	3.09±0.10 b	0.54±0.05 b	7.50±2.27 b	1.87±0.83 b	-
DKW medium with AA	3.51±0.15 a	0.71±0.05 a	9.00±1.69 a	3.00±0.75 a	+
DKW medium with AC	3.62±0.10 a	0.73±0.06 a	9.38±0.74 a	3.25±0.71 a	++
Cultivar					
Chandler	3.33±0.23 b	0.64±0.10 a	8.17±1.95 b	2.75±0.96 a	+
Jamal	3.48±0.28 a	0.68±0.10 a	9.08±1.62 a	2.67±0.98 a	++
Interaction	NS[c]	NS	$P < 0.05$	$P < 0.05$	

[a] Means ± standard error followed by the same letter are not significantly different ($P < 0.05$).

[b] -: Not effective for the inhibition of tissue browning, +: low efficiency, and ++: high efficiency.

[c] Not significant ($P < 0.05$). AA = ascorbic acid; AC= activated charcoal.

Experiment 3

In third experiment, the microcuttings were soaked in different solution for 2 h before culture to allow leaching out of phenolic compounds. Microcuttings soaked in PVP were more successful than other two anti-browning pretreatments for Main shoot length, Shoot fresh weight and Leaf number on shoots. Although microcuttings soaked in AA were also successful on rate of cultures establishment than microcuttings soaked in distilled water. In this experiment Chandler cultivar was significantly better than Jamal cultivar. However, significant difference was not observed for shoot number on microcuttings (Table 3). Efficiency rate of each treatment for the inhibition of browning (Table 3) showed present treatments in this experiment had a high efficiency (++) on Chandler cultivar and low efficiency (+) on Jamal cultivar for the inhibition of tissue browning. However pretreatments soaked microcuttings for 2 hours in distilled water, PVP and AA had low (+), high (++) and low (+) efficiency for the inhibition of tissue browning, respectively.

Table 3. Responses of two Persian walnut cultivars 30 day after inoculation to different anti-browning pretreatments of explants [a].

Anti-browning pretreatments: Soaked explants for 2 hours in	Main shoot length (cm)	Shoot fresh weight (g)	Leaf number on shoots	Shoot number on explants	Tissue browning[b]
Distilled water	3.25±0.16 b	0.53±0.30 c	4.00±1.07 c	1.62±0.74 b	+
PVP	3.71±0.07 a	0.69±0.06 a	9.00±1.93 a	3.00±0.76 a	++
AA	3.19±0.11 b	0.59±0.07 b	5.75±1.91 b	2.75±0.71 a	+
Cultivar					
Chandler	3.46±0.23 a	0.63±0.09 a	6.92±2.75 a	2.58±0.99 a	++
Jamal	3.32±0.29 b	0.57±0.08 b	5.58±2.50 b	2.33±0.89 a	+
Interaction	NS[c]	NS	NS	NS	

[a] Means ± standard error followed by the same letter are not significantly different ($P < 0.05$).

[b] -: Not effective for the inhibition of tissue browning, +: low efficiency, and ++: high efficiency.

[c] Not significant ($P < 0.05$). AA = ascorbic acid; PVP = polyvinyl pyrrolidone.

Discussion

Result of first experiment showed taking into account all the variables studied, the most appropriate culture medium for the in vitro walnut shoot multiplication was the Driver and Kuniyuki walnut (DKW) medium fortified with AC can be optimum medium (Fig. 1 and Fig. 2). Considering that minerals macroelements can be a limit factor for growth of walnut microcuttings (Amiri, 2004), comparing the amounts of macroelements in culture medium shows that $\frac{1}{2}$ DKW medium is infirm medium than DKW and MS mediums. DKW medium have Ca^{2+}, Cl^{-}, Mg^{2+} and NO_3^{-} more than MS medium, but less K^{+}, NH_4^{+} and So_4^{2-} (Toosi and Dilmagani, 2010). DKW and MS medium are high salt medium than $\frac{1}{2}$ DKW medium, certainly. This point indicated Persian walnut microcuttings needs a strong medium than infirm medium for *in vitro* nodal segments growth and proliferation. Similar results by Bourrain *et al.*, (2000), Tetsumura *et al.*, (2002), Bosela and Michler, (2008), Toosi and Dilmagani, (2010) for walnut multiplication using DKW medium has been reported (Bonga and Aderkas, 1992; Bourrain, 2000; Saadat and Hennerty, 2002; Tetsumura *et al.*, 2002). In other hand, with increasing salt concentration in medium, increased production of phenolic compounds on medium then browning extended (Senoviratne and Wijesekara, 1996). However the release of phenolic component into the culture medium and browning (Table 1) appeared in the DKW and MS medium higher than $\frac{1}{2}$ DKW medium but, $\frac{1}{2}$ DKW medium don't advised for initial steps in vitro culture of walnut. Because microcuttings on $\frac{1}{2}$ DKW were thinner, had less leaf expansion and were less green than those on DKW and MS medium (Fig. 2).

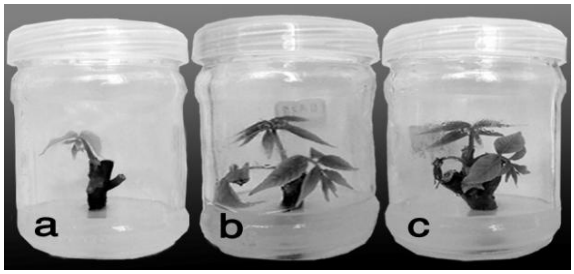

Fig. 2. Images of Persian walnut explants 30 day after culture incubation on different basal nutrient media fortified with AA. A) 1/2 DKW medium B) DKW medium and C) MS medium.

In first experiment, almost, once culture in DKW medium containing AC was successful than medium containing AA, also in second experiment the medium subculture thrice, at an interval of 48 hrs in DKW medium containing AC than AA achieved better result on cultures establishment and regeneration (Fig. 3). This topic indicated activated charcoal is a strong adsorbent (Bin Zhou *et al.*, 2010). Medium containing AC effectively prevented explants browning by adsorbing phenolics, and by providing a dark environment caused inactivating

polyphenoloxidase (PPO) and peroxidase (POD), resulting in increased explants survival and organogenesis (Tisserat, 1979; Pan and van Staden, 1998; Nguyen *et al.*, 2007; Thomas, 2008). Similar results have been reported Sharada *et al.*, (2003), Prajapati *et al.*, (2003) and Dibax *et al.*, (2005) about positive influences of adding activated charcoal to the culture medium that prevented the effect of leached phenolics that hindered regeneration.

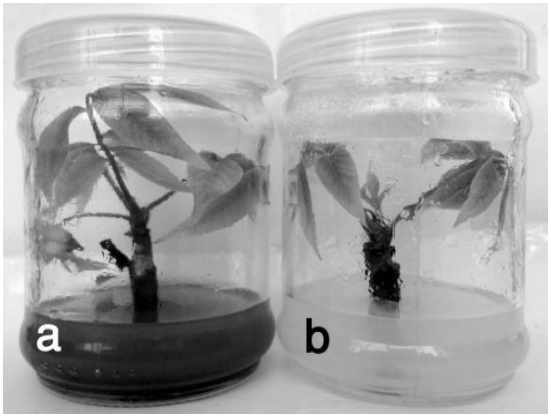

Fig. 3. Difference between DKW basal nutrient media fortified with AC and AA on explants growth and proliferation 30 day after incubation in second experiments. A) Media fortified with AC. B) Media fortified with AA.

In other hand, ascorbic acid is known to decay rapidly in plant tissue culture medium. Ascorbic acid is oxidized by reactions catalyzed by Cu (II) and Fe (III) (Elmore *et al.*, 1990), both of which are component of Driver and Kuniyuki Walnut medium. On the basis of our results, we conclude that treating with AA had a suitable result for decrease browning in medium and microcuttings establishment, also. Elmore *et al.*, (1990) used AA as antioxidant and anti-browning agent in plant cell, tissue and organ culture. Furthermore, Strosse *et al.*, (2004) reported that adding ascorbic acid to the medium inhibited the exudation of phenols in banana tissue culture.

Our results showed that treating the 2 h soaked nodal segments with PVP solution before culture eliminated browning in explants and allowed leaching out of phenolic compounds. In similar research, in trees and woody plants, studies have suggested that antioxidants, such as PVP, can be added to the medium to reduce the oxidation, and thus browning, in cultured tissue (Gupta *et al.*, 1980; Tyagi *et al.*, 1981; Zhong *et al.*, 1995).

Conclusion

Concluding from the obtained results, in all experiments studied in this research, efficiency rate of each treatment for the inhibition of browning showed present treatments had a varying success. Results indicated low efficiency for both cultivar in first experiments, low efficiency for Chandler cultivar and high efficiency for Jamal cultivar in second experiments, and high efficiency for Chandler cultivar and low efficiency for Jamal cultivar in third experiments for the inhibition of browning, respectively. Therefore, AC and AA and PVP are useful and effective in managing the problem of phenolics and improving regeneration in *Juglans regia* L. but, the efficiency of treatments is highly sampling time and genotype dependent and in most cases successful control of explants browning could be achieved by different combinations of these methods.

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