

REDUCING OXIDATIVE STRESS ON ZYGOTIC EMBRYOS OF WALNUT (*JUGLANS REGIA* L.) UNDER *IN VITRO* CONDITIONS BY THEIR PRETREATMENT WITH ASCORBIC ACID

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ABSTRACT

Persian walnut (*Juglans regia* L.) is an autochthonous species of Albania and considered endangered (EN) based on IUCN criteria. It is important to find and develop successful protocols for *in vitro* propagation. A major problem in this regard is the oxidative stress that explant undergo in nutrient media after their isolation. The purpose of this study is to find a suitable method for preventing polyphenolics exudation by pre-treatment of zygotic embryos with different concentrations of ascorbic acid and different exposing time in this solution. It was observed that browning rate, is a parameter highly affected by the concentration of ascorbic acid solution and exposing time. Explants from Control group show a high rate of browning and this parameter is significantly different from all the other treatments tested. Exposing time does not have any significant effect when using 150 mg/l ascorbic acid. Furthermore, treating with 200 mg/l ascorbic acid for 10 min. does not have any significant effect with none of the treatments using 150 mg/l ascorbic acid. It was observed from the results, the groups that showed high values of browning rates, had lower values of proliferation rates. It was evident that treating with 200 mg/l ascorbic acid for 20 min, gave a lower proliferation rate (82.2 %) in comparison with treatment using 200 mg/l ascorbic acid for 15 min (88.8). This may be related to the side effects that occur when the treatment lasts for a longer time.

Keywords: Persian walnut, *in vitro* propagation, oxidative stress, ascorbic acid.

INTRODUCTION

Persian walnut (*Juglans regia* L.) is grown as autochthonous specie in Albania and based in IUCN criteria, this specie is considered Endangered (EN) (Vangjeli *et al.*, 1995). It is represented by some elite cultivars adapted to different environmental conditions. The walnut is not important only for nutritive values, but this specie is characterized for containing compounds that have antihypertensive, neuroprotective, anticancer properties etc. (Girzu *et al.*, 1998).

For years the production has originated from seeds causing heterogeneity and adaptation to local environmental conditions. These characteristics of variability and adaptation to abiotic and biotic stresses for years make walnut (*Juglans regia* L.) an autochthonous plant in our country. Conservation of genetic material and development of clones for elite varieties with economic values in danger of extinction, are the main reasons that make walnut the object of *in vitro* propagation techniques. Micropropagation is a suitable method for obtaining a large quantity of genetically homogeneous and healthy plant material which can be used for planting (Kongjika *et al.*, 2002, Damiano *et al.*, 2008). Some authors (Cossio & Minotta, 1983; 2000) have reported on micropropagation and zygotic embryo culture of several nut cultivars.

A major problem in walnut micropropagation, is oxidative stress observed during the first stage of *in vitro* culture. In such situation is observed the browning or yellowing of nutrient medium due to the exudation of phenolics into the medium. Oxidative stress is observed in many micropropagation protocols which can lead to shoot necrosis and mortality of the explant/cultures due to the absorption of polyphenolic substances (Thorpe and Harry, 1997). It was found that the phenol synthesis of plant tissues strongly depends on environmental conditions. The frequency of explant browning due to phenols thus also varies according to the season.

There are reported several protocols which can reduce significantly the oxidative stress of the explants, such as pretreatment with ascorbic acid and/or citric acid prior explant inoculation in the nutrient media (Farooq *et al.*, 2002), transferring explants onto fresh medium (Hutchinson, 1989), adding antioxidants as ascorbic acid, citric acid, PVP etc. in the nutrient media (Wang *et al.*, 1994 Modgil *et al.*, 1999) etc.

The present study involved investigations on the effect of pretreatments with ascorbic acid for the avoidance of oxidative stress of walnut zygotic embryos under *in vitro* conditions. Various concentrations and/or exposure time in ascorbic acid solution were tested.

MATERIALS AND METHODS

Plant material: The mature seeds were taken from walnut trees of Permeti district. In order to establish the most appropriate protocol for *in vitro* cultivation, as primary explants were used zygotic embryos.

Explant isolation and sterilization: Before sterilization, the seeds were left for 12- 24 hours in tap water in order to alleviate the embryos isolation. Then was realized double sterilization with HgCl₂ 0.01% for 20 min before and after removing seeds tegument. Thereafter the explants were rinsed out three times with sterilized H₂O (Muriithi *et al.*, 1993). Zygotic embryos were isolated from mature seeds under aseptic conditions using a stereo microscope.

Pretreatments with ascorbic acid:

Sterile explants were soaked in ascorbic acid solution under aseptic conditions using the laminar air flow hood. 6 treatments were tested differing from concentration of ascorbic acid solution and/or exposure time (Table 1). After each treatment the explants were rinsed three times in sterile distilled water.

Table 1. Exposure time and concentrations of ascorbic acid solutions

Treatment	Ascorbic acid concentration	Exposing time
Control	0 mg/l	-
I	150 mg/l	10 min.
II	150 mg/l	15 min
III	150 mg/l	20 min
IV	200 mg/l	10 min
V	200 mg/l	15 min
VI	200 mg/l	20 min

Nutrient media for *in vitro* cultivation: For organogenesis induction, MS basal media PGRs free (Murashige and Skoog, 1962) was used, meanwhile during subculture stage in MS media was added 1mg/l of 6-Benzylaminopurine (BAP) and 0.1 mg/l of 1-naphthaleneacetic acid

(NAA). In all cases the nutrient media was combined with sucrose 3% and agar 0.7%. The pH of the media was adjusted to 5.6 prior to autoclaving.

Physical parameters in growth room: The explants in every developmental stage were grown in the growth chamber at temperature of $25^{\circ} \pm 2^{\circ}$ C in a 16 h light/24 h regime with cool, white fluorescent light.

Measurements and browning level analyses: For each treatment were used 30 explants and all experiments were repeated three times. All data were collected and analyzed after 15 days of culture. To determine the level of explant browning in culture in order to make the comparison between treatments, the calculation according to Table 2 was used. Experimental data was elaborated by Tukey-Kramer, Student's methods and the analyse of variance (ANOVA) with JMP 7.0 statistical software.

Table 2. Determination of the browning level

Browning rate	Observation of the oxidative stress symptoms
2	Slight explant browning
1	Moderate explant browning
0	Severe explant browning (which led to explant death)

RESULTS AND DISCUSSION

Observations and measurements were taken 15 days after explant inoculation in each treatment, including control. Explants in different treatments and/or control show different levels of browning (Fig. 1). Results for explant browning rate, contamination and survival percentage for the explants in all treatments is presented in Table 3.



Figure 1. Various levels of browning due to oxidative stress in *Juglans regia* L. explants

Regarding browning rate, it could be observed that this parameter is highly affected by the concentration of ascorbic acid solution and exposing time. Explants from Control group show a high rate of browning, specifically 1.63, which could be considered as a severe oxidative stress that cause the death of most of the explants inoculated in the nutrient media. The browning rate for Control group is significantly different from all the other treatments tested (Table 3; Graphic 1).

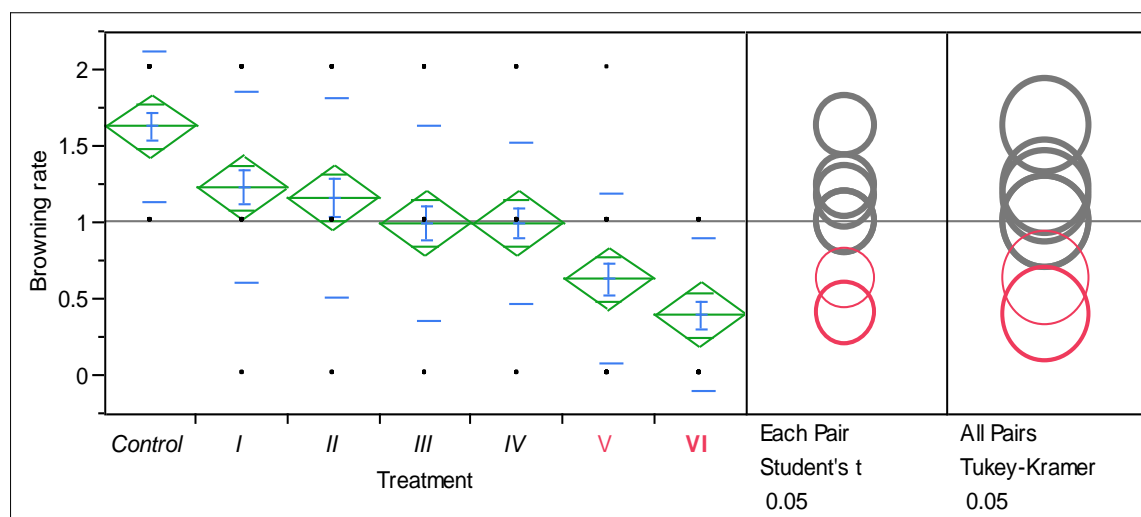
Also, it is observed that treatments using 150 mg/l ascorbic acid, in all cases show higher browning rates in comparison with treatments using 200 mg/l ascorbic acid. In this regard, exposing time does not have any significant effect when using 150 mg/l ascorbic acid. Furthermore, treating with 200 mg/l ascorbic acid for 10 min. does not have any significant effect with none of the treatments using 150 mg/l ascorbic acid.

Table 3. Browning rate, proliferation (%) and contamination (%) of explants in different Treatments compared to Control

Treatment	Browning rate	Contamination (%)	Proliferation (%)
Control	1.63 ± 0.09 ^a	31.1	24.4
I	1.23 ± 0.11 ^b	28.8	33.3
II	1.16 ± 0.12 ^b	28.8	37.7
III	1.01 ± 0.12 ^b	24.4	51.1
IV	1.02 ± 0.10 ^b	20.0	66.6
V	0.63 ± 0.11 ^c	26.6	88.8
VI	0.40 ± 0.09 ^c	17.7	82.2

Note: Levels not connected by same letter are significantly different

From the results it is observed that the treatments with the lower browning rate and statistically different from all the other treatment are those using 200 mg/l ascorbic acid for 15 min. and 20 min. In these cases, the combination of ascorbic acid concentration and exposing time gave the best results for browning rate, respectively 0.63 when treating for 15 min and 0.40 when treating for 20 min. these two treatments are not statistically different between them (Table 3; Graphic 1).

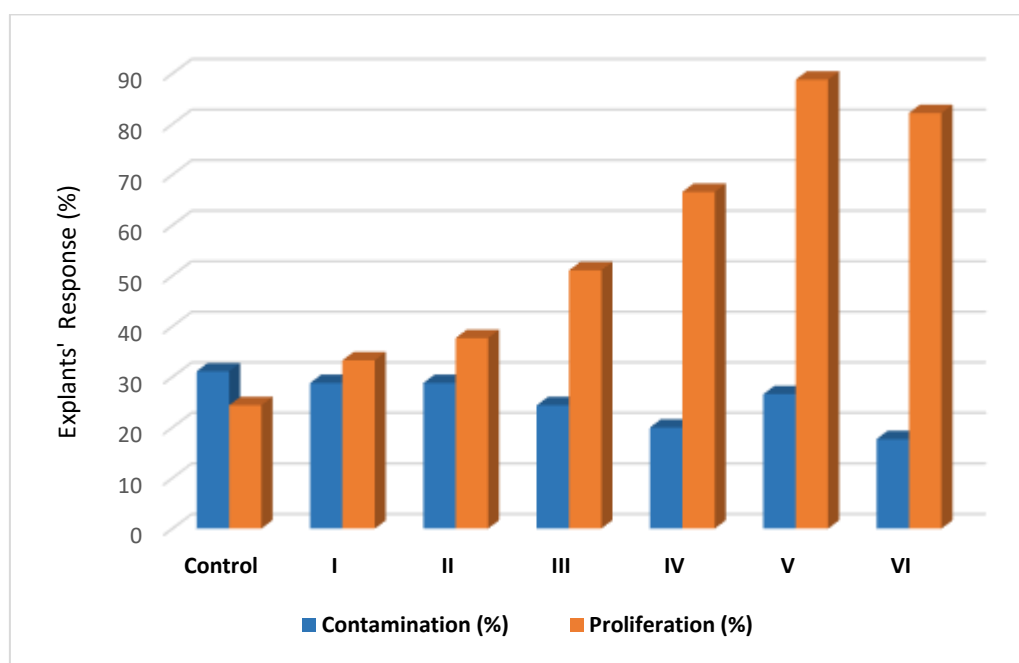


Graphic 1. Oneway Analysis for evaluation of Browning rate in different Treatments and Control group

Regarding contamination rate, for all treatments and control group was followed the same procedure for stabilization of aseptic conditions. Ascorbic acid does not play any role as

disinfectant or sterilizing agent. For these reasons differences in contamination rates between groups might be as a result of contamination during mechanical isolation of zygotic embryos and/or other procedures performed for their inoculation in nutrient media.

Proliferation rate is a parameter highly affected by the oxidative stress that explants undergo in culture. As it can be observed from the results, the groups that show high values of browning rates, have lower values of proliferation rates (Table 3). In this content, it is clearly observed that the Treatments V and VI using 200 mg/l ascorbic acid for 15 and 20 min. respectively, have the highest proliferation rates, 88.8 and 82.2, due to the avoidance of polyphenolic oxidation of the explants (Graphic 2). Also, it can be observed that treating with 200 mg/l ascorbic acid for 20 min, gives a lower proliferation rate (82.2 %) in comparison with treatment using 200 mg/l ascorbic acid for 15 min (88.8). This may be related to the side effects that occur when the treatment lasts for a longer time.



Graphic 2. Contamination and proliferation rates in various treatments for oxidative stress avoidance compared to Control

The germination of zygotic embryos and roots development was observed after 3 - 5 days of cultivation, meantime the leaves developed after 7 – 8 days. Because of juvenile properties, the embryos possess a great regeneration potential and therefore are considered optimal explants for *in vitro* micropropagation purposes (Fig. 2 a, b, c).

In many cases, when using mature zygotic embryos, PGRs or phytohormones are not necessary because the embryo has a considerable size and is in an autotrophic phase. As reported by other authors (Raghavan *et al.*, 1982), there is no specific need for additional amounts of PGRs in the nutrient media for a large broad of wild plants. In many cases, cultivation of zygotic embryo is used to avoid successfully post zygotic incompatibility in many woody plants (Ramming, 1990).

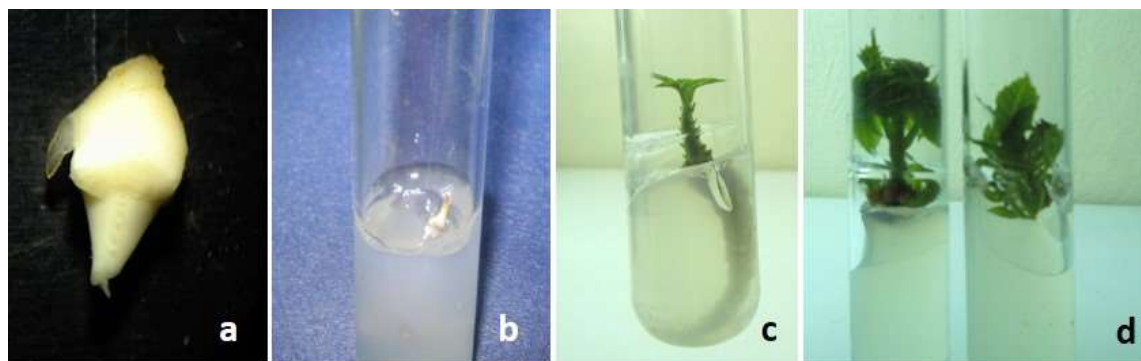


Figure 2. Micropropagation of *Juglans regia* L.: **a; b)** Fully isolated zygotic embryo **c)**

Shoots and roots regeneration after organogenesis induction **d)** Multiplication via subcultures
The regenerated plantlets from Stage I were inoculated in subculture medium. Most of explants reacted normally giving a great number of leaves and lateral shoots (Fig. 1d). Except new shoots formation, during subcultures was observed callus formation in shoot basis and new shoots were developed from this callus. Micropropagation coefficient resulted very high and this is due to the presence of BAP cytokinin, whose function is apical dominance interruption and new shoots formation.

Juglans regia propagation is object of a lot of studies in regard to find a universal nutrient media specific for this specie or to test the efficiency of different explants for its *in vitro* propagation (Rios Leal et al., 2007; Kepeneka and Kolařasib, 2016; Kaur et al., 2006; Zekaj et al., 2000).

The major problem of establishing *in vitro* cultures from zygotic embryos of walnut is the oxidative stress that explants undergo in culture after their isolation. Therefore the objective of this study was to develop an effective protocol for avoidance of polyphenolics synthesis and their accumulation in the nutrient media. This is a common problem in many species of fruit trees where the browning or yellowing of the cut surfaces and media is induced which subsequently leads to the death of the explants (Hu and Wang, 1983; Chikezie, 2012; Kaur et al., 2006). It was observed that the most critical phase being the first 24 to 48 h in culture.

In the present study, was used pre-treatment of the explants with various concentrations of ascorbic solution and/or exposing time. In this study, the lower rate of browning was observed when 200 mg/l ascorbic acid was used for 20 or 15 min. Kepeneka and Kolařasib, (2016) reported as effective rinsing in an antioxidant aqueous solution [polyvinylpyrrolidone (PVP) (500 mg/l), cysteine (20 mg/l) and ascorbic acid (AA) (5 mg/l)]. In many other reports, antioxidants such as ascorbic acid or citric acid were included in the nutrient media to prevent oxidative stress in walnut (Kaur et al., 2006; Zekaj et al., 2000), in apple (Wang et al., 1994; Modgil et al., 1999) etc.

According to literature (Hutchinson, 1989), growth of explants is strongly inhibited by the accumulation of toxic substances in the medium near the cut ends of the explants and this is more pronounced in the case of semi-solid medium. In such cases the most widely used method is the frequent transfer of explants to fresh medium. Leslie and McGranahan, (1998) reported as a successful method to avoid polyphenolics exudation in walnut explants frequent transferring in fresh media.

In the other hand, the activity of enzymes involved in the oxidation of polyphenols to quinones can be reduced by physical factors such as light and temperature. It was found that not only the etiolating of stock plants but also of the initiated cultures minimizes the incidences of browning. Incubation of cultures at low temperature ($\approx 5^{\circ}\text{C}$) was found to be more effective. Establishment of shoot tip explants of Fuji apple cultivars were affected by both temperature and the dark treatment during the first phase of culture initiation (Wang et al., 1994).

CONCLUSIONS

In vitro propagation of walnut via zygotic embryos offers a great potential for rapid propagation of this endangered species from Permeti district. It is possible to avoid phenolic oxidation of the explants by pretreatment with ascorbic acid solution. It was achieved a low browning rate when using 200 mg/l ascorbic acid solution for 15 min. and 20 min. Proliferation rate is a parameter highly affected by the oxidative stress that explants undergo in culture. The highest percentage of proliferation rate was obtained when treating with 200 mg/l ascorbic acid for 15 min (88.8). Micropropagation coefficient resulted very high and this is due to the presence of BAP cytokinin, whose function is apical dominance interruption and new shoots formation.

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