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Research article

In vitro propagation of four native cultivars of *Juglans regia* L. in Albania using various types of cytokinins

Matilda MYRSELAJ (DELIJA)[1], Valbona SOTA[1,*], Ilirjana BITRI[1], Efigjeni KONGJIKA[2]

[1]Department of Biotechnology, Faculty of Natural Sciences, University of Tirana, Albania

[2]Section of Natural and Technical Sciences, Academy of Sciences of Albania, Tirana, Albania

*Corresponding author email: valbona.sota@fshn.edu.al

Abstract: The aim of this study is to develop an efficient micropropagation protocol for four native cultivars of *Juglans regia* L. in Albania and to determine the optimal cultivar that responds better under *in vitro* conditions. Zygotic embryos of Përmet, Korça, Peshkopi and Tropoja cultivars were excised and germinated under *in vitro* conditions. After pre-treatment with ascorbic acid solution, the excised embryos were inoculated in MS basal media PGRs free where oxidative stress symptoms and proliferation rate in terms of biometric parameters were evaluated. The new shoots were cultivated in MS nutrient media and, in this stage, three types of cytokinins (BAP, kinetin and zeatin) at 2 mg l^{-1} each combined with NAA at 0.1 mg l^{-1} were tested for plantlets regeneration via subcultures. It resulted that the cultivar is an important factor that highly affect the growth parameters in specific conditions of cultivation. Regarding tissue browning rate, Korça cultivar reacted better, meanwhile, Tropoja and Përmeti cultivars were the ones which gave the highest proliferation response during the first stage of organogenesis induction. From an overall point of view, kinetin and zeatin resulted the most suitable cytokinins for the micropropagation of walnut cultivars studied in this research. Mass production of the desired clones for some specific native walnut cultivars in Albania is important for conservation and utilisation of these biotechnological products for various purposes.

Keywords: walnut cultivars, micropropagation, PGRs ratio, basal media, oxidative stress.

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Introduction

Juglans regia L. is an economically important fruit not only in Albania, but for the entire Mediterranean region, as an autochthonous and native species in this geographical area. It is included in the Red Book of Albania as an endangered plant species (EN) (Vangjeli et al., 1995).

Walnuts are widely used around the world, especially for their nutritional value. In addition, walnuts contain many ingredients which have a great effect in the treatment of hypertension, neurological disorders, cancer, etc. (Girzu et al., 1998; Aryapak & Ziarati, 2014; Xiaoying et al., 2014). All these qualities justify the use of walnut on a large scale and precisely for these reasons it is considered as a very important species that should be in the focus of scientific research to identify cultivars with greater values and greater adaptability on various cultivation conditions. In addition, walnuts are widely used in the wood industry for the production of quality furniture (Pirayesh et al., 2012), as well as in the production of dyes that can be used in the textile industry or beyond (Tutak & Benli, 2011).

In Albania, the walnut is a widespread plant species, thus enabling the existence of a large number of cultivars which have high adaptability in several environmental factors depending on the geographical region where they grow. Due to this heterogeneity, it is important to identify elite cultivars which can be included in large-scale cloning and conservation programs. *In vitro* methodologies that involve the use of various plant explants, as well as zygotic embryos, allow mass production and obtaining of homogeneous plant material that can be used for various

purposes (Kongjika et al., 2002; Raghavan & Srivastava, 1982; Ramming, 1990). Furthermore, multiplication by seeds is much slower due to the maturation phase of the zygotic embryo, which is associated with a decrease in production yield, or receiving production at a later period.

Numerous reports in the literature present successfully stabilized protocols for micropropagation of *Juglans regia* L. both in terms of the initial explants used (Zekaj et al., 2000; Kaur et al., 2006; Leslie & McGranahan, 1992) or in terms of different culture media and of a specific hormonal ratio or type used etc. (Driver & Kuniyuki, 1984; Saadat & Hennerty, 2002; Toosi & Dilmagani, 2010). From this large number of reports, it is evident that the stabilization of an effective micropropagation protocol for walnuts, in itself is greatly influenced by the selected cultivar as the suitability of different cultivars to a treatment is quite different (Scaltsotiannes et al., 1997; Payghamzade & Kazemitabar, 2010; Payghamzade & Kazemitabar, 2011).

The aim of this study is to select between four native walnut cultivars in Albania the one that responds better under *in vitro* conditions and also to identify among three types of cytokinins tested the one that is the most effective for mass production of walnut plantlets.

Material and Methods

Plant material, collection and sterilization: As primary explants were used zygotic embryos isolated from mature dried seeds of walnut trees collected from four natural habitats of Korça, Peshkopi, Përmet and Tropoja region. For stabilizing aseptic cultures, the seeds were treated with $HgCl_2$ 0.01% for 20 min before and after removing their tegument. After that, soaking in ethanol 70% for 30 sec. and rinsing three times with H_2Od were performed.

Pretreatment with ascorbic acid solution: After sterilization, the explants were pre-treated with ascorbic acid solution 200 mg l^{-1} for 15 min. to avoid excessive browning as a result of oxidative stress. Their response was evaluated in three levels of the browning rate, as it follows: 0 when slight signs of oxidative stress occurrence were observed; 1 when moderate signs of oxidative stress occurrence were observed; 2 when severe signs of oxidative stress occurrence were observed.

Germination and proliferation of zygotic embryos: After sterilisation, the zygotic embryos were isolated under aseptic conditions in a laminar flow and were inoculated in MS (Murashige & Skoog, 1962) PGRs free media, combined with 3% sucrose and 0.7% agar. pH value was established in 5.6. Biometric parameters such as leaves number, shoots number, shoots length (cm) after 3 weeks of culture were measured and compared between cultivars.

Subculture stage: The shoots of walnut cultivars obtained from the proliferation stage were further exposed to subculture nutrient media where the effect of three different cytokinins, specifically BAP (6-benzylaminopurine), kinetin and zeatin, at a concentration of 2 mg l^{-1} each, was tested. In all cases MS media was used, combined with 1-Naphthaleneacetic acid (NAA) (0.1 mg l^{-1}), 3% sucrose and 0.7% agar. pH value was established in 5.6.

In all stages of the experiment, the cultures were incubated in a 25° ± 2°C temperature and in a 16 h light/24 h photoperiod with cool, white fluorescent light of intensity 43.4 μmol m^{-2} s^{-1}.

Statistical analyses: For each stage of micropropagation, at least 30 explants were inoculated and the experiments were repeated at least twice. Biometric parameters such as leaves number, shoots number, shoots length (cm) after 3 weeks of culture were measured and compared between cultivars and in all the three cytokinins types tested, All biometric data are presented as mean ± standard deviation and/or standard error mean in an Oneway Anova Chart. For the statistical analyses was used the statistical evaluation program JMP 7.0.

Results and Discussions

Oxidative stress occurrence

Pre-treatment with ascorbic acid solution 200 mg l^{-1} for 15 min. resulted in the reduction of oxidative stress in which walnut explants undergo during the first days of *in vitro* culture. This procedure was applied for the four cultivars of walnut and the variability in the browning rate response was evaluated. From continuous observations from the first day of inoculation and during the following days, it results that in general all varieties respond satisfactorily to this treatment. The best result in terms of the browning rate is given by Korça cultivar with the lowest average value of 0.6, a value which represents a different statistical difference compared to the value of the browning rate obtained by the other three cultivars. There were observed no significant differences in the browning rate values

between Peshkopi, Përmet and Tropojë cultivars, respectively 0.86, 0.90 and 0.93 (Fig. 1).

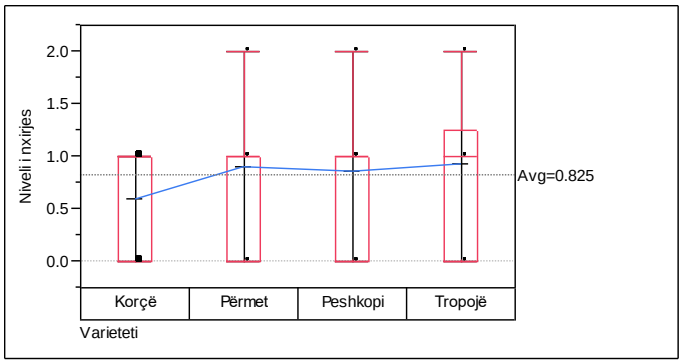

Figure 1. Variability on the response of different cultivars in terms of occurrence of oxidative stress

Walnut explants show a high ability to produce phenolic compounds under *in vitro* conditions. Excision of embryos from the surrounding tissues, as well the controlled physical and chemical factors and/or treatment with various sterilizing agents, can cause the excessive browning of these types of explants due to oxidative stress occurrence. This can lead to a low germination potential and to other difficulties on successfully stabilizing the first stage of micropropagation. In this regard, it is important to apply protocols that can reduce the symptoms of oxidative stress. In this study, pre-treating with ascorbic acid solution at 200 mg l^{-1} for 15 min. greatly decreases the excessive browning of the explants, although, as mentioned above, there are observed differences between responses in different cultivars. Despite this, in all cases, the number of explants that were successfully stabilized in the first step of micropropagtion was high.

Pretreating with antioxidants, or adding antioxidants in the nutrient media to prevent polyphenols synthesis in excessive amounts, is also reported by other authors in different plant species and especially in walnut. Kaur et al. (2006) found as effective the addition of ascorbic acid (100 mg l^{-1}) in the nutrient media for the micropropagation of five Indian walnut cultivars. Meanwhile, Yari et al. (2014) reported that even the simultaneous addition of various antioxidants in the nutrient media (ascorbic acid, citric acid and polyvinylpyrrolidone at 100 mgl^{-1} each of them) resulted successful in preventing tissue browning of *J. regia* L. shoots of two genotypes from Iran. As a pretreatment protocol, Kepenek and Kolağasi (2016) mentioned that rinsing with a mixture of polyvinylpyrrolidone (500 mg l^{-1}), cysteine (20 mg l^{-1}) and ascorbic acid (5 mg l^{-1}) was successful for overcoming the problems related to oxidative stress.

Organogenesis induction during the proliferation stage

Organogenesis for each cultivar, regardless of the basal medium used, is observed one week after inoculation. Zygotic embryos of all cultivars exhibited a high organogenic response by developing organs from both basal and apical meristems (Fig. 2 a, b). After a period of two weeks in these growing conditions, the formation of complete plants is observed (Fig. 2 c).

The results indicate that there are significant differences regarding the germination and proliferation rate of zygotic embryos in the first stage of micropropagation. These differences are highly affected by the cultivar (Fig. 3). As it can be observed, for leaves number parameter, Përmet and Tropoja cultivars show the highest value, specifically 4.30 and 4.41, and there are not observed significant differences among them. Meanwhile, for Dibra and Korça cultivars, the values for this parameter are lower, specifically 3.30 and 3.25.

The same tendency is observed even for shoots length parameter, where the differences are statistically significant. The highest value is given from Përmet and Tropoja cultivars, specifically 1.14 and 1.01. Meanwhile, for shoots number, although there is a slight difference between cultivars where Përmet cultivar gave the best result (2.05) it can be said that there are not observed significant differences between all the four cultivars.

The genotype, is an important factor that highly affect the growth parameters in specific conditions of cultivation. In this study, for the first stage of micropropagation, zygotic embryos excised from nuts of Përmet and Tropoja cultivars, were distinguished from the two other cultivars on their organogenic response. These differences come as a result of the interaction of external factors with internal genetic and hormonal ones. Different reactions on proliferation or plant *in vitro* regeneration rate from different cultivars when they are grown under the same conditions have been reported by many other authors (Kaur et al., 2006; Sánchez-Zamora et al., 2006; Kepenek & Kolağasi, 2016) etc.

In this study MS medium is used for proliferation of walnut zygotic embryos for four native walnut cultivars in Albania. Others reports also found as effective the use of MS basal medium for stabilizing *in vitro* cultures of

walnut (Cossio & Minolta, 1983; Gruselle & Boxus, 1990; Kaur et al., 2006; etc.).

The effect of different types of cytokinins during subculture stage

During subculture stage a high rate on organogenesis induction is observed (Fig. 2 d, e), but this response differs significantly and is affected not only by the type of cytokinins used, but also by the walnut cultivar. In this regard, for this stage of micropropagation, in addition to identifying the most effective cytokinin for each cultivar, the effect of the same cytokinin is compared between the four cultivars (for all the three cytokinins tested).

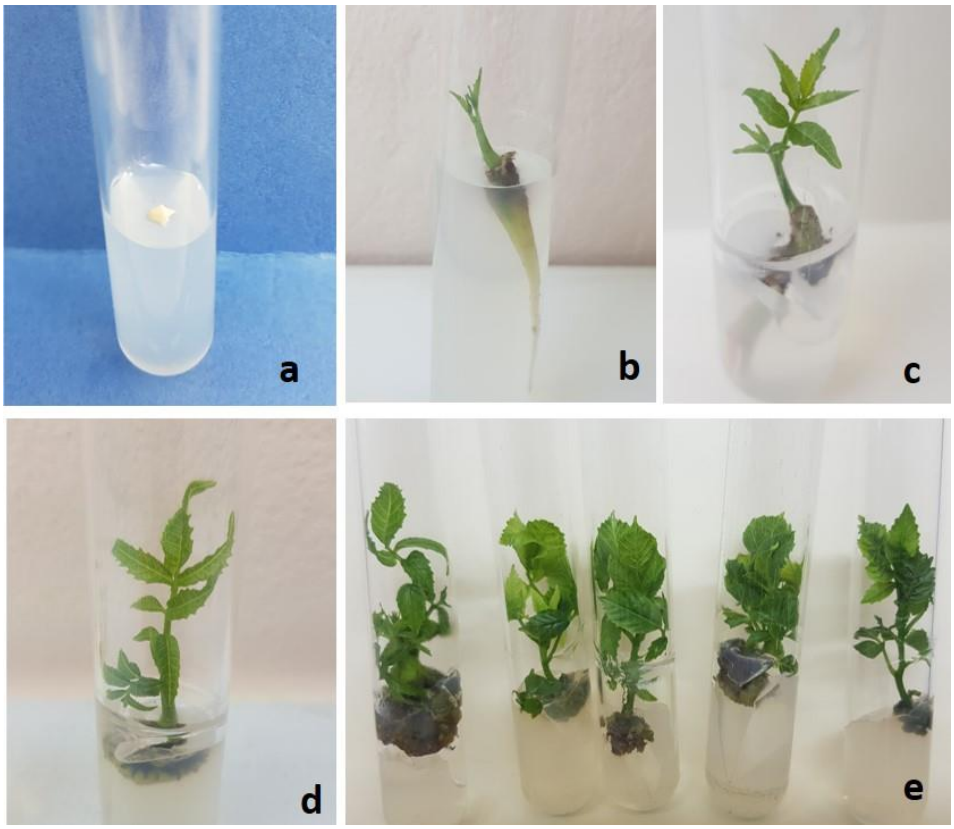

Figure 2. Micropropagation of *Juglans regia* L. cultivars: **a, b, c)** Organogenesis induction from zygotic embryos **d, e)** multiplication of plantlets via subcultures

Table 1. Comparison of biometric data between four walnut cultivars during cultivation in different types of cytokinins

Cytokinins	Cultivars	Leaves number (means ± Std Err)	Shoots number (means ± Std Err)	Shoots length (means ± Std Err)
BAP	Korça	6.20 ± 0.81^{bc}	3.40 ± 0.41^{bc}	1.40 ± 0.14^{c}
	Përmet	5.00 ± 0.40^{c}	2.80 ± 0.20^{c}	1.99 ± 0.07^{b}
	Peshkopi	9.75 ± 1.41^{a}	4.10 ± 0.25^{ab}	2.11 ± 0.11^{ab}
	Tropoja	7.37 ± 0.59^{b}	4.21 ± 0.24^{a}	2.48 ± 0.15^{a}
Kinetin	Korça	11.10 ± 1.10^{a}	5.25 ± 0.43^{a}	2.44 ± 0.13^{a}
	Përmet	6.70 ± 0.65^{b}	2.90 ± 0.26^{b}	2.15 ± 0.14^{ab}
	Peshkopi	8.55 ± 1.12^{ab}	4.45 ± 0.51^{a}	2.20 ± 0.21^{ab}
	Tropoja	7.87 ± 0.88^{b}	4.33 ± 0.41^{a}	2.54 ± 0.15^{a}
Zeatin	Korça	8.40 ± 0.95^{ab}	3.65 ± 0.45^{b}	1.71 ± 0.18^{b}
	Përmet	9.35 ± 1.04^{a}	5.7 ± 0.55^{a}	3.09 ± 0.21^{a}
	Peshkopi	6.25 ± 0.74^{b}	3.35 ± 0.50^{b}	1.60 ± 0.16^{b}
	Tropoja	10.19 ± 0.95^{a}	5.30 ± 0.46^{a}	2.67 ± 0.20^{a}

Note: Comparisons for each pair using Student's t ($\alpha = 0.05$). Levels not connected by the same letter for the same biometric parameter monitored within a cytokinin type are significantly different between them

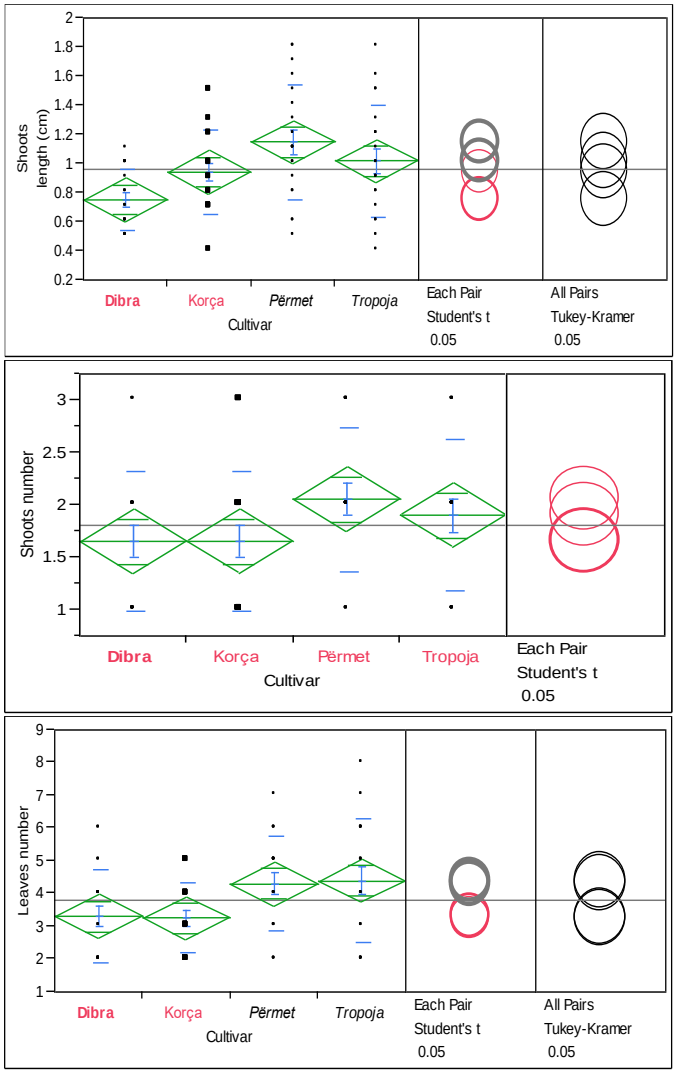

Figure 3. Biometric parameters measured during proliferation of zygotic embryos of four walnut cultivars a) leaves number b) shoots length (cm) c) shoots number

Comparison between walnut cultivars for their organogenesis response during cultivation in different types of cytokinins

From the results, it is clearly observed that different cultivars show different organogenic response during their cultivation in different cytokinins containing media. In this study, it is evidencied that when the shoots are cultivated in BAP, the cultivar that responds better regarding shoots number and shoots length parameter is Tropoja cultivar, specifically 4.21 and 2.48. Meanwhile, regarding leaves number parameter Peshkopi cultivar gives the highest value (9.75) which is significantly different from the three other cultivars.

For the cultivation in kinetin containing media Korça cultivar is the one who responded better for all the three biometric parameters measured, although there are not observed significant differences between this cultivar and Tropoja one for shoots length and shoots number parameter. Even Peshkopi cultivar is similar to the formers regarding shoots number parameter.

It is observed almost the same tendency for the results given in zeatin containing media. Even in this case, in general, Tropoja and Përmet cultivars are the one who gave the best values, and there are not observed significant differences for leaves number parameter (10.10 and 9.35), for shoots number parameter (5.30 and 5.70) and for shoots length (cm) parameter (2.67 and 3.09).

Identification of the most effective cytokinin within a cultivar depending on its organogenic response

As mentioned above, different cultivars react differently depending on the nutrient media used, especially as it is evidenced in this study, from the type of cytokinin used. In this context, it is important to evaluate the effect of different cytokinins within a cultivar to identify the most effective one in each case. In the Figures 4, 5 and 6 are given the variability charts for the effect of the three cytokinins tested and compared within a cultivar (for all the four cultivar under study) for biometric parameters as: leaves number, shoots number and shoots length (cm). For all the estimated biometric parameters, for Korça cultivar, kinetin results the mos appropriate cytokinin which gives the highest value specifically 5.25 for shoots number, 11.1 for leaves number and 2.44 for shoots length. These values show significant differences with the specific values given from the cultivation in BAP or zeatin containing media. Results from organogenic responses for Peshkopi cultivar show that regarding shoots number there are not observed significant differences between all the three cytokinins tested in this study. Meanwhile, for leaves number parameter, that value is higher when is used the cytokinin BAP, specifically 9.75. There are not observed significant differences between the results given from the shoots cultivated in BAP or kinetin containing media regarding shoots length parameter.

From the data, it can be observed that for Tropoja cultivar, shoots number and shoots length (cm) are not affected by the type of the cytokinin used, whereas for the leaves number parameter, the highest rate is given from the shoots cultivated in zeatin containing media, specifically 10.19.

For Përmet cultivar, the highest values for all the biometric parameters monitored are given from the shoots

cultivated in zeatin containing media, specifically 3.09 for shoots length, 5.7 for shoots number and 9.35 for leaves number. In all cases, significant differences in comparison to the values obtained from the cultivation in BAP and kinetin containing media are observed.

As can be seen from the obtained data, the four cultivars react very differently during cultivation in nutrient media containing different types of cytokinins. But, in addition to the cultivar, it is noticed that the type of cytokinin for a certain cultivar greatly affects the regenerative potential of plantlets from shoots cultivated in culture. From a general point of view it can be said that Tropoja cultivar is the one that generally reacts better from all the other cultivars to these *in vitro* cultivation conditions. While Korça cultivar is distinguished for the highest regenerative potential in nutrient medium with kinetin content. Përmet cultivar gives a very satisfactory answer in the nutrient medium combined with zeatin, while that of Peshkopi does not present any difference to be emphasized regardless of the type of cytokinin used. On the other hand, the cytokinin BAP at 2 mg l^{-1} resulted not very effective in micropropagation of these native cultivars of walnut in Albania, because, in general, there were observed the lowest values of the monitored biometric parameters. This result is in contrast with the one reported by Yari et al. (2014), in which BA combined with IBA results in *in vitro* regeneration of walnut plants of two Iranian cultivars. Other authors also reported the efficiency of BA on walnut in vitro propagation (Saadat & Hennerty, 2002; Rodriguez et al., 1982). Other reports also that found as effective the use of BA for *J. regia* micropropagation (Fernandez et al., 2000; Sanchez-Zamora et al., 2006; Kaur et al., 2006 etc.). Meanwhile, Kepenek & Kolağasi, (2016) mentioned as effective for walnut shoots regeneration the use of TDZ. In our study, the use of zeatin results much more effective than the use of BAP as a cytokinin. In their report, Bosela and Michler (2008) also found that zeatin (5–25μM) gave high responses rate for the *in vitro* propagation of nodal segments of black walnut.

Taking into account the very different reports of the reaction of different cultivars in terms of the used explants or the components of the nutrient medium, it is necessary to stabilize an effective micropropagation protocol of the cultivars of interest in order to produce homogeneous plant stocks. These can be included in conservation, selection and / or genetic improvement programs.

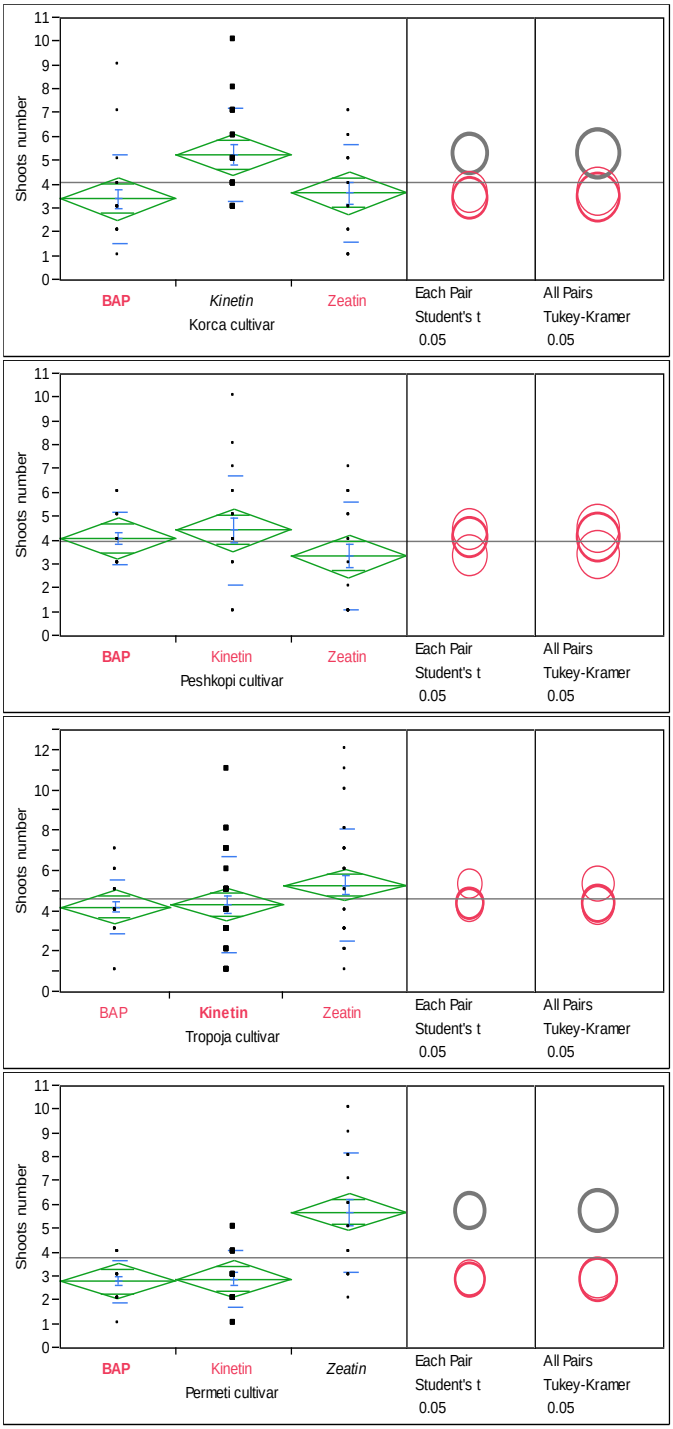

Figure 4. Oneway analysis for the effect of the three cytokinins tested on shoots number parameter within each walnut cultivar

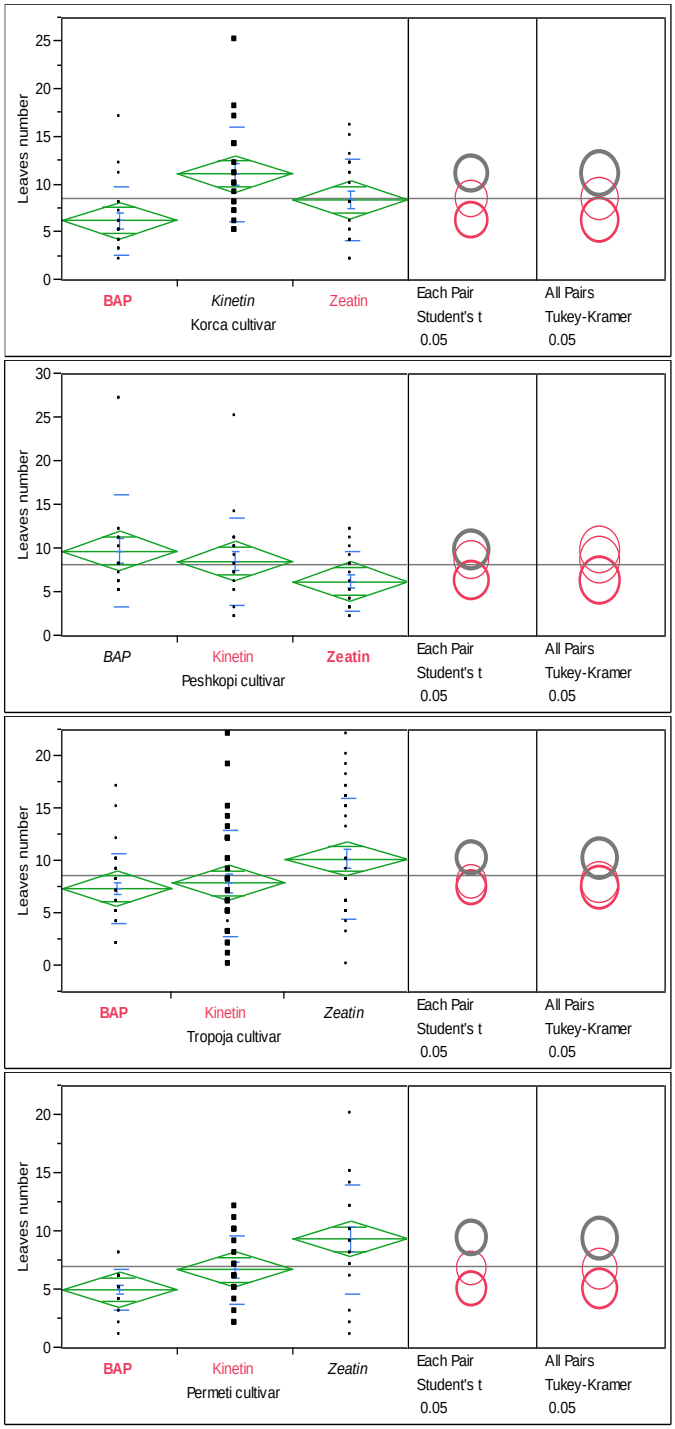

Figure 5. Oneway analysis for the effect of the three cytokinins tested on leaves number parameter within each walnut cultivar

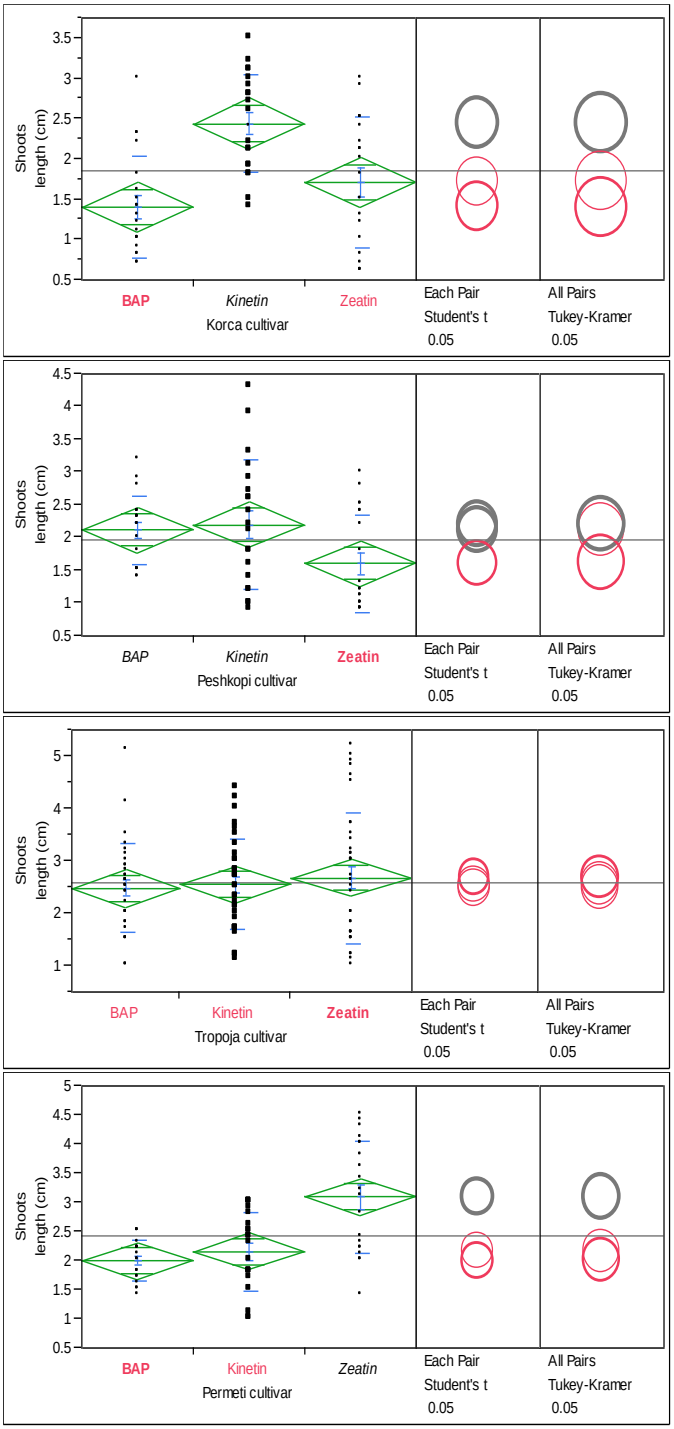

Figure 6. Oneway analysis for the effect of the three cytokinins tested on shoots length (cm) parameter within each walnut cultivar

Conclusions

To overcome the problems caused by oxidative stress in walnut zygotic embryos grown under *in vitro* conditions, the explants should be treated with ascorbic acid solution at a concentration of 200 mg l^{-1} for 15 min. Micropropagation of these walnut cultivars, results effective, but media composition in terms of the type of cytokinins used is depended on the cultivar. From the four cultivars, Tropoja one results the most optimal for the plantlets regeneration under in vitro conditions. From an overall point of view, kinetin and zeatin resulted the most suitable cytokinins compared to BAP for the most of biometric parameters evaluated in this study. Mass production of the desired clones of walnut for some specific walnut cultivars in Albania, is important for conservation and utilisation of these biotechnological products for various purposes.

Ethical Approval

The authors declare that no need to ethical approval.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Funding Statement

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