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In Vitro Regeneration of Walnut (*Juglans regia L.*) From Embryo Culture and Histological Analysis of Leaf Epidermal Structures In *in vitro* Derived Plantlets

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***Abstract* -The present research aims the optimization of *in vitro* regeneration of an Albanian cultivar of walnut (*Juglans regia* L.) from Korca region. Except it is an autochthonous species of Albania, walnut is cultivated massively for its importance and multiple values. Due to the dormant embryo, the multiplication and consequently the increase of the production yield in many cases are slowed down. These problems might be overcome by tissue cultures techniques. Except the optimization of micropropagation for this Albanian walnut cultivar, evaluation of some histological characteristics of *in vitro* derived leaves was performed. For micropropagation purposes, initially were cultured mature zygotic embryos in three different basal media: DKW, MS and WPM. The new plantlets were regenerated in MS nutrient medium containing NAA 0.1 mg l^{-1} and three different cytokinins (2 mg l^{-1}each), were tested for mass production of clones, especially, BAP, kinetin and zeatin. Zygotic embryos showed a high ability to undergo organogenesis, and it was observed that inoculation in DKW and MS media didn't had any statistical difference. New shoots were highly responsive during subculture stage in all the three cytokinins tested, but the highest results in the monitored biometric parameters was observed in kinetin containing media. The nail polish procedure was performed for making leaf impressions on the glass slide, followed by the evaluation of microscopic fields. It was clearly observed a simultaneous development of stomata, trichomes and normal communication between cells in a self-regulatory manner in *in vitro* developed plantlets.**

Keywords—walnut micropropagation, embryo culture, cytokinins types

I. INTRODUCTION

Establishment of a tissue culture protocol for clonal regeneration is an essential prerequisite for the potential applications of clonal propagation, genetic transformation and preservation of plant germoplasm, especially in economically important fruit trees and woody plants. Persian walnut (*Juglans regia* L.), a member of Juglandaceae family, is a native woody plant and largely cultivated over the whole Mediterranean region since ancient times. It is an autochthonous species of Albania and based in IUCN criteria, this specie is considered Endangered (EN) (Vangjeli *et al.*, 1995).

Its massive cultivation is related to its importance and multiple values. For this reason, it is largely used especially for its nutritive values in the food industry. Walnut kernels contain high percent of essential oils which serves as antioxidants and also have anti-inflammatory and anticancer effects (Aryapak and Ziarati, 2014; Xiaoying et al., 2014). Also, walnut is also used in the wood processing industry (Vassiliou and Voulgaridis, 2005; Pirayesh et al., 2012), as well as the chemical industry mainly for paints (Bukhari et al., 2017; Tutak and Benli, 2011).

The several uses of this species (alimentary, industrial, forestry and medicinal), as well as the socio-economic impact, justifies the need to optimize walnut cultivation and rapid regeneration of desired clones. In general, walnut is propagated by seeds, but, due to the dormant embryo, the multiplication and consequently the increase of the production yield in many cases are slowed down. These problems might be overcome by tissue cultures techniques. Using zygotic embryos as primary explants has its advantages due to their juvenile nature that allow rapid regeneration of walnut plantlets.

Several protocols have been developed for regeneration of *Juglans regia* L. using different basal media and testing different types and/or concentrations of PGRs for in vitro regeneration of desired clones (Driver and Kuniyuki, 1984, Saadat and Hennerty, 2002; Toosi and Dilmagani, 2010; Kaur et al. 2006 etc.). Payghamzade and Kazemitabar, (2011) in their review for walnut micropropagation, have mentioned a great number of reports for this topic and from this detailed analyses it can be said that cultivar is an important factor that greatly affects the regenerative ability of explants exposed to certain physico-chemical conditions. The effect of different walnut cultivars on their ability for in vitro rigeneration was also reported from other authors (Scaltsotiannes et al., 1997; Payghamzade and Kazemitabar, 2010)

This study aimed to develop an efficient plant regeneration system using zygotic embryos as primary explants for an

important Albanian walnut cultivar from Korça region. Histological characteristics of leaf epidermis from *in vitro* derived plantlets were also observed.

MATERIALS AND METHODS

Plant material: As primary explants were used zygotic embryos isolated from mature dried seeds of walnut trees from natural habitats of Korça region.

Explant sterilization procedures: Before sterilization, dried seeds were left for 12- 24 hours in tap water in order to alleviate the embryos isolation. Then was realized double sterilization with $HgCl_2$ 0.01% for 20 min before and after removing seeds tegument. After that, the explants were rinsed three times with sterilized H_2O_d, followed by the final treatment with ethylic alcohol 70% for 30 sec.

Organogenesis induction: Three types of basal media were tested: MS (Murashige and Skoog, 1962), WPM (Lloyd and McCown, 1981) and DKW (Driver and Kuniyuki, 1984). In all cases, the media was without phytohormones or PGRs, combined with 3% sucrose and 0.7% agar. pH value was established in 5.6.

Subcultures stage: The regenerated shoots from the previous stage were inoculated in multiplication media mass production. In these stage, the effect of three different cytokinins (2 mg l^{-1}each) was tested, specifically BAP (6-benzylaminopurine), kinetin and zeatin. 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.

Physical conditions in growth room: The cultures in every developmental stage were incubated in a 25° ± 2° C temperature and in a 16 h light/24 h photoperiod.

Histological evaluation of upper and lower leaf epidermis: For evaluation of leaf epidermis characteristics were used samples from 4 weeks old plantlets in subculture stage. The nail polish procedure was performed for making leaf impressions on the glass slide, followed by the evaluation of microscopic fields using a 10x ocular magnification and a 40x objective magnification. For the calibration of microscopic fields was used the micrometer objective (10 µm) in the above specifications of magnification. Presence and type of stomata and trichomes in the upper and lower epidermis, and presence of calcium oxalate crystals was evaluated in *in vitro* derived leaf material.

Data elaboration and statistical analyses: For each stage of micropropagation were inoculated at least 30 explants and the experiments were repeated at least twice. Data collections in experiment were subjected to analyses of variance and evaluated by computer using the statistical evaluation program JMP 7.0. All biometric data are presented as mean ± standard deviation in an Oneway Anova Chart. All photomicrographs for epidermal characteristics were made using Olympus trinocular microscope equipped with Microgiciel camera.

RESULTS AND DISCUSSION

Organogenesis induction

After sterilisation, zygotic embryos were isolated and inoculated in organogenesis induction media under aseptic conditions (Fig. 1a). Organogenesis under the influence of different basal media is observed one week after inoculation. Although in all cases the media is PGRs free, the mature zygotic embryos show a high ability to undergo organogenesis developing in this stage organs from both apical and basal meristem (Fig. 1b).

As observed in Figure 2, the differences in growth parameters are significant. Regarding shoots length and shoots number, proliferation in DKW or MS media does not show any significant difference regarding shoots length and shoots number. Meanwhile the lowest value for both parameters, significantly different is observed during proliferation of zygotic embryos on WPM media. Regarding leaves number, this parameter is not affected by basal media because are observed not significant differences.

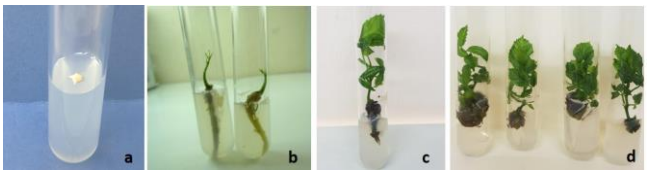

Figure 1. Different stages of walnut micropropagation from zygotic embryos **a)** isolation and inoculation of zygotic embryo **b)** organogenesis induction and proliferation of basal and apical meristem **c)** full plantlets recovery **d)** multiplication in subculture stage

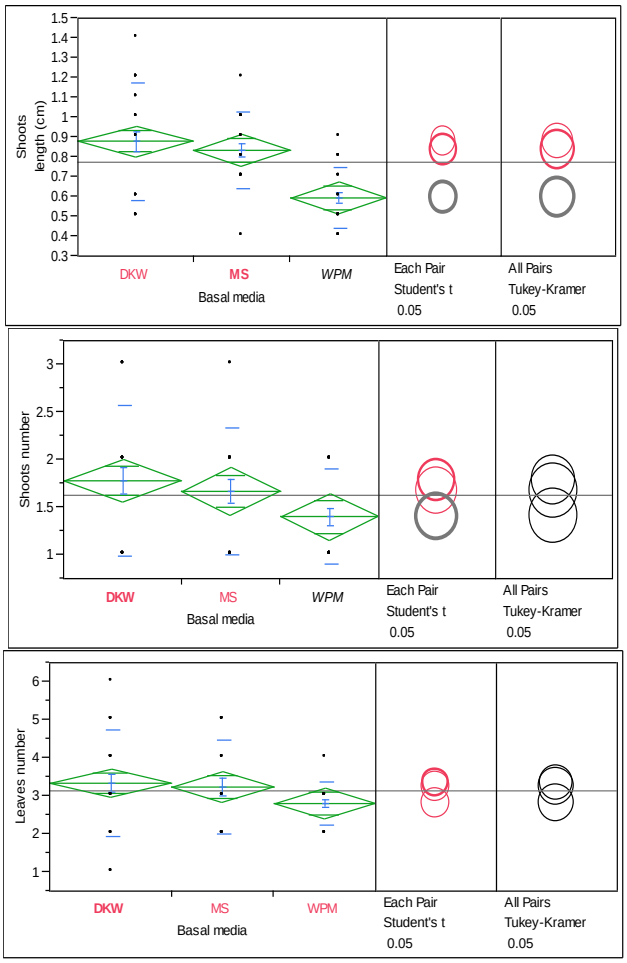

Figure 2. Biometric parameters measured during proliferation of zygotic embryos in three basal media a) shoots length b) shoots number c) leaves number

Subculture stage

20 days old plantlets obtained from proliferation stage (Fig. 1c), were inoculated in subculture media for further multiplication. From the results obtained on proliferation

stage where cultivation in MS media gave high values in all parameters measured (although not statistically different with cultivation in DKW media), MS basal media is chosen for *in vitro* regeneration of plantlets via subcultures. In this case, with the aim of establishing an efficient protocol for subculture stage, the effect of three types of cytokinins was tested. It can be said that in all cases, adding a cytokinin in the nutrient media in a percentage by 2 mg l^{-1} results effective in mass production of clones from walnut plantlets (Fig. 1d).

As it can be observed from Figure 3, the type of cytokinin highly affects plantlets regeneration in this stage. Regarding shoots length, regeneration from kinetin containing media gives the best value (2.44 cm) in comparison with the cultivation in BAP or zeatin containing media (specifically 1.40 and 1.72). The positive effect of kinetin is also observed for shoots number parameter (5.20) comparing to the effect of zeatin and BAP for this parameter, specifically 3.65 and 3.40. In both cases, the results are statistically significant. Leaves number is a parameter which does not show any significant difference during cultivation in kinetin or zeatin containing media (specifically 11.10 and 8.40), despite that, cultivation in kinetin gives a higher amount of biomass in general. Meanwhile, cultivation in BAP containing media gives the lowest value for leaves number parameter (6.20), statistically significant with the other types of cytokinins used.

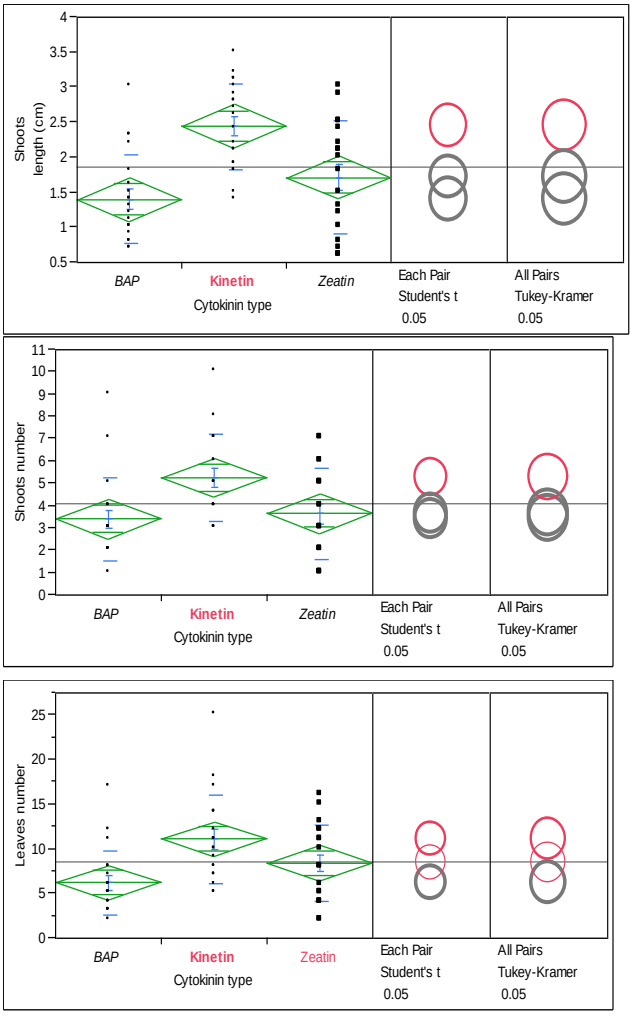

Figure 3. Biometric parameters measured during subculture stage in where three types of cytokinins are tested a) shoots length b) shoots number c) leaves number

From the obtained results, it is clear that for Korca walnut cultivar, it is possible to stabilise successfully the first stage of micropropagation via mature zygotic embryos. These explants, show a high ability to undergo organogenesis in PGRs free media, due to the fact that the embryo has a considerable size and is in an autotrophic phase. As reported by other authors (Raghavan, *2003*), there is no specific need for additional amounts of PGRs in the nutrient media for a large broad of wild plants. Stabilizing *in vitro* cultures from zygotic embryos was reported as a successful method, especially for walnut as a recalcitrant species (Leal et al., 2007; Leslie and McGranahan, 1992; Kaur et al., 2006 etc.).

Driver and Kuniyuki, (1984) reported DKW media as an effective one for in vitro walnut plantlets regeneration. But, in our study, are not observed significant differences when using MS or DKW basal media. Similar to this, many Kaur et al. (2006) reported the use of MS basal media as very efficient in walnut clones production via *in vitro* techniques.

Regarding PGRs type and/or ratio for walnut shoots multiplication, there are reported several protocols with a wide variety of results. Cytokinins play an important role on cell division, explant proliferation and lateral buds formation. For shoot multiplications in different plant species, a variety of cytokinins such as is found as effective for this purpose. In our study, the use of kinetin resulted the most effective cytokinin in comparison to BAP and zeatin, Other authors also reported the necessity of a high ratio cytokinin : auxin for optimization of walnut micropropagation (Yari et al., 2014; Zarghami and Salari, 2015).

Histological evaluation

Four weeks after subculture stage, from the obtained plantlets regenerated in kinetin containing media, were isolated leaves for histological investigations.

Epidermis structure: The upper and lower epidermis is almost similar to each other in shape but differ slightly in size where the cells of the lower epidermis are smaller. The upper epidermis is formed of polygonal isodiametric cells with slight thick cellulosic, beaded anticlinal walls, they are covered by thin striated cuticle (Fig. 4a). The lower epidermis is similar to the upper epidermis but the cells are smaller in size and have thick beaded anticlinal walls (Fig. 4b).

Distribution of stomata: In the upper epidermal surface is observed absence of stomata and subsidiary cells. These leaves result hypostomatic (with stomatal complex only in the lower epidermal surface) (Fig. 4 a, b).

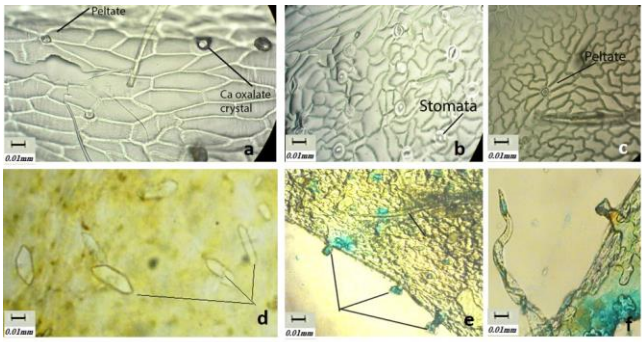

Figure 4. Histological evaluation in the upper and lower leaf epidermis **a)** upper leaf epidermis with peltate trichomes and Ca oxalate crystals **b)** Lower epidermis with anomocytic stomatal comples **c)** peltate glandular trichomes **d)** prismatic Ca oxalate crystals in the upper epidermis **e)** capitate trichome, non-glandular trichome and presence of Ca oxalate crystals in the upper epidermis **f)** capitate trichome, non-glandular trichome and Ca oxalate crystals in the lower epidermis

Stomatal type: In the lower epidermal surface the stomatal complex type is anomocytic (subsidiary cells enclose radially the guard cells) (Fig. 4b).

Type of trichomes: trhere are observed two types of glandular trichomes: capitate and peltate. Capitate glands consist in a unicellular head and multicellular stalk (1 to 2 cells) (Fig. 4d). Peltate glands are present consisting on a 1 – 2 cell secretory head and 6 – 8 peripheral cells (Fig. 4c).

The non-glandular trichomes are unicellular conical, with an extended body that ends with a sharp apex. These trichomes are observed in both epidermis but with a higher frequency in *the upper one* (Fig. 4c, e).

Crystalline structures: Numerous spherical and prismatic crystals of calcium oxalate are observed in both epidermis (Fig. 4 a, d, f).

The fact that the leaves are hypostomatic is attributed as ecological adaptation to avoid loss of excess water through the stomata at the upper leaf surfaces. The stomatal complex type is considered a very important feature in taxonomic decisions. The derived result is in agreement with the reports in some dicotyledonous plants (Zekaj, *et al.*, 2007), which are characterized by anomocytic stomata. This type is seen in most of the fruit trees, and is very variable in herbaceous plants.

Regarding the type of trichomes, this charachteristic in plants is in a close relation with environment conditions and vary in dependence of the conditions in which the plant is disposed (Zekaj, *et al.*, 2007).

In this study, it is clearly distinguished the presence of calcium oxalate crystals in *in vitro* derived plantlets. According to Horner and Wagner (1995), the formation of calcium oxalate crystals is a result of an increase in calcium concentration levels. In this conditions, calcium is deposited outside the cell, in a non-toxic and non-soluble form, which absorbs and reflect light. This survey shows clearly a simultaneous development of stomata, trichomes and normal communication between cells in a self-regulatory manner in *in vitro* developed plantlets.

CONCLUSIONS

Micropropagation of walnut cultivar from Korca region can offer an efficient technique for its clonal propagation. The use of zygotic embryos as primary explants provides an efficient induction of organ formation through direct organogenesis. When comparing trheee basal media for organogenesis induction, there are observed no differences between MS and DKW one. Regarding new plantlets regeneration via subcultures, it can be concluded that addition of kinetin in the nutrient media at 2 mg l^{-1} in combination with NAA at 0.1 mg l^{-1} was the most effective combination. Another important conclusion is the development of stomata, trichomes and normal communication between cells in a self-regulatory manner in *in vitro* developed plantlets.

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