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# Open the Windows toward Somatic Embryogenesis of Leaf Explants of Persian Walnut (*Juglans regia* L.)

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**Abstract:** Leaves from *in vitro* proliferating shoots of cvs. Chandler and Hartley of Persian walnut were cultured on MS and NN media supplemented with BA and NAA. Three light conditions including darkness, low light intensity and modified light intensity were used. Most of the calli were friable and non-embryonic in all three conditions. Rate of callogenesis depended on cultivar, culture medium, light conditions, and interaction between them. In the sixth week, all individual effects and interaction effects were significant and the highest rate of callogenesis were achieved from cv. Hartley on NN medium in the darkness and low light intensity conditions and the lowest one was in cv. Chandler on MS medium in the low light intensity and on NN medium in the modified light intensity conditions. However, in the tenth week, just individual effects of cultivars, light conditions, and media were significant and rate of callogenesis in Hartley, NN medium and dark conditions were more than Chandler, MS medium and other light conditions. Three months after culturing of leaf explant on plant-growth-regulator-free media, gradually, frequency of callogenesis decreased. Browning of explants and calli was more in the MS media, cv. Chandler and darkness or low light intensity conditions. At the end of five months after initial culture, no embryo was formed from these calli.

**Keywords:** Callus, Walnut, Light Conditions, Culture Medium, Cultivar.

## INTRODUCTION

Somatic embryogenesis is the capacity of somatic or non-sexual (*i.e*. all cells other than the gametes) plant cells to form embryos by a process resembling zygotic embryogenesis. It thus leads to the formation of a bipolar structure with a root/shoot axis and a closed vascular system [28]. Somatic embryos (SE) from walnut are being used for clonal propagation, production of interspecific hybrids and introduction of specific genes [18, 20, 31]. Somatic embryogenesis has been reported from immature walnut cotyledons [4, 5, 9, 19, 22, 30, 34]. Some also have used zygotic embryos to obtain SE [1, 5, 18]. Triploid *J. regia* was regenerated from gelatinous endosperm [32].

In our knowledge, there is no report of somatic embryogenesis from maternal tissues of walnut. Embryos from these tissues are genetically identical because they are derived from altered development of somatic (non-sexual) cells, without any sexual recombination [28]. Somatic embryos were derived from stem and leaf explants of *Quercus robur* L. [7] and leaf explants of *Castanea sativa* Mill. [6] by using MS medium supplemented with benzyladenine (BA), 1- naphthaleneacetic acid (NAA) and 500 $mg.l^{-1}$ casein hydrolysate (CH) as the source of reduced nitrogen. Other factors involve in inducing somatic embryogenesis are culture medium and light conditions. The same results were obtained from leave culture of

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*Azadirachta excelsa* (Jack) on MS [21] or NN [23] media [27].

The effect of culture media on induction of somatic embryogenesis was investigated from leaf explants of *Cydonia oblonga* Mill. [13] The most frequency of callogenesis was obtained on both MS and NN media and the highest rate of embryo formation was occurred on MS medium.

Das *et al*. [8] reported calli from leaf disk culture of *Vitis vinifera* L. in the dark conditions, differentiated into pro-embryos and embryos only when kept under conditions of low light intensity (15 µE $m^{-2}$ $s^{-1}$) for 2 weeks before being transferred to high light intensity (60 µE $m^{-2}$ $s^{-1}$).

Also Gatica *et al*. [16] emphasized that average of somatic embryos per leaf explants of *Coffea arabica* L. cvs Caturra and Catuaí depend on light conditions. The highest number of embryoids in cv. Caturra was in the light conditions and in cv. Catuaí was in the darkness.

In addition, by leaf culture of Bulgarian spray carnation cvs. Fea and Rossitzain in the dark and light conditions, induction of embryogenic calli were observed from both of them. In case of cv. Fea, embryogenic callus induced in the dark conditions was able to form embryos. In contrary, calli of cv. Rossitza induced in the light conditions were more competence for somatic embryo formation [15].

So in this study, the influence of culture medium, light conditions and cultivar were investigated on induction leaf explants of *J. regia* L. cvs. Chandler and Hartley.

## MATERIALS AND METHODS

*Plant material and culture condition*

Shoots of *J. regia* cv. Chandler and Hartley were propagated *in vitro* by nodal cuttings and maintained on DKW medium [10] supplemented with 3% sucrose, 1 $mg.l^{-1}$ of benzylaminopurine (BAP) plus 0.01 $mg.l^{-1}$ of indole-3-butyric acid (IBA) and solidified with phytagel (2.1 $g.l^{-1}$) [24].

Shoots were micro-propagated every month. Jars were kept in a growth chamber for a 16-hour photoperiod (60 $\mu molm^{-2}s^{-1}$) at 25±2 ºC.

*Explant preparation*

Leaves around 1 cm were collected from the second to the fourth node of the apical portion of shoots and cut across the midvein by scalpel blade. Leaf halves were placed with the abaxial side down on the culture medium.

*Composition of the media*

The leaf explants of two cultivars were initially cultured on MS or NN media supplemented with 1 $mg.l^{-1}$ BA, 1 $mg.l^{-1}$ NAA, 500 $mg.l^{-1}$ casein hydrolysate, 3% sucrose and 6% agar [6]. After four weeks, the cultures were transferred to the basal media supplemented with 0.1 $mg.l^{-1}$ BA and 0.1 $mg.l^{-1}$ NAA. Four weeks later, the cultures were transferred to basal media without growth regulators and subsequent subculture to fresh medium every month. The pH was adjusted to 5.6 before sterilization by autoclaving at 121°C for 20 min.

*Culture conditions*

Three light conditions containing darkness, low light intensity (15 µmol $m^{-2}s^{-1}$ of cool white fluorescent lamps) and modified light intensities [first month: darkness, then low light intensity (15 µmol $m^{-2}$ $s^{-1}$) of cool white fluorescent lamps for four weeks and finally high light intensity (60 µmol $m^{-2}$ $s^{-1}$) of cool white fluorescent lamps] were used. The explants were kept in a growth chamber with 16 hour photoperiod at 25±2 ºC.

## DATA ANALYSIS

In the first experiment, four replicates each with six leaf explants were used for each treatment (Data was not recorded). In the second repeat, eight replicates each with six leaf explants were used for

each treatment. The weight of calli was taken on 6th week and 10 of initiation.

Effect of 2 media in combination with 2 cultivars and 3 light conditions were investigated in a factorial experiment. Analysis of variance was carried out using the General Linear Model procedure of the computing statistical program package SAS [25]. Means of the studied variables were compared with Duncan's multiple range test (DMRT) [11].

## RESULTS

After 2 weeks of culture, friable or compact calli formed along the midveins or margins of leaf explants (Figure 1 A, B and C). A small portion of explants became black and died soon after culturing on the media. Callogenesis increased in the fourth week. Also color, texture and origin of calli were variable between treatments (Table 1). The most types of calli were semi-compact and friable. In the dark conditions, calli became more friable than low light intensity. In the low light intensity, calli had irregular small or big nodes. The calli on NN medium was more compact than MS medium. Colors of calli were different among treatments.

In the 6th week, callogenesis of leaf explants increased by decreasing concentration of plant growth regulators and calli were evident in all part of leaf explants (Figure 2 A and B). Most of the calli were friable on both media. Browning of leaf explants and calli increased particularly in the leaf explants of cv. Chandler on MS medium in the dark conditions (Figure 2 C). Frequency of callogenesis decreased with transferring explants from dark conditions to low intensity light.

Also in the sixth week, all effects (excluding the interaction effect between cultivars and light conditions) were significant at 1% level (Table 2). The highest frequency of callogenesis was observed in cv. Hartley on NN medium in the dark and low light intensity conditions and the lowest one was in cv. Chandler on MS medium in the low light intensity and on NN medium in the modified light intensity conditions (Figure 3).

After transferring the explants to plant-growth-regulator-free media, volume of calli increased lightly. Some of calli became brown; particularly in the explants taken from cvs. Chandler and Hartley on MS medium in the dark conditions. Rate of callogenesis decreased when explants were transferred from low light intensity to high light intensity and some of them became brown. In the tenth week, interaction effect between cultivars, media and light conditions were not significant; but effect of cultivars and light conditions at 1% level and effect of media at 5% level were significant (Table 2). Callogenesis in cv. Hartley (Figure 4) cultured on NN medium under dark conditions (Figure 5) was more than cv. Chandler cultured on MS medium under other light conditions.

The leaf explants remained on plant-growth-regulator-free media for three months. Gradually, frequency of callogenesis decreased. Browning of explants and calli was more in the MS media, cv. Chandler and darkness or low light intensity conditions. At the end of five months after initial culture, no embryo was formed from these calli.

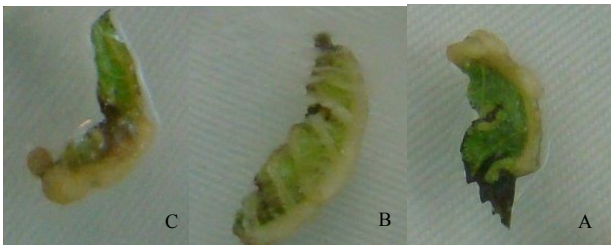


Fig.1. A-C Callogenesis from leaf explants of *J. regia* cvs. Hartley and Chandler two weeks after initial culture. **A.** Callogenesis along midvein of cv. Chandler on NN medium in low light intensity. **B.** Creamy semi-compact calli along veins of cv. Chandler on MS medium in the darkness. **C.** Callogenesis along midvein of cv. Hartley on MS medium in the low light intensity.

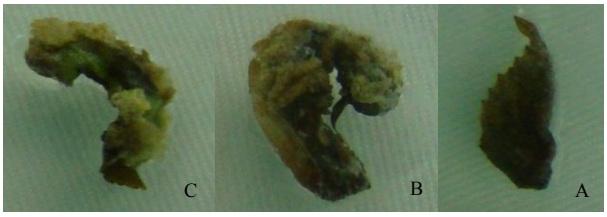


Fig. 2. A-C Increase of callogenesis from leaf explants of *J. regia* cvs. Hartley and Chandler after transferring to media with low concentrations of plant-growth-regulators. **A.** Increase of friable calli from leaf explants of cv. Chandler on NN medium in the dark conditions. **B.** Increase of friable and crispy calli from leaf explants of cv. Hartley on MS medium in the low light intensity. **C.** Increase of browning in calli from leaf explants of cv. Chandler on MS medium in the darkness condition.

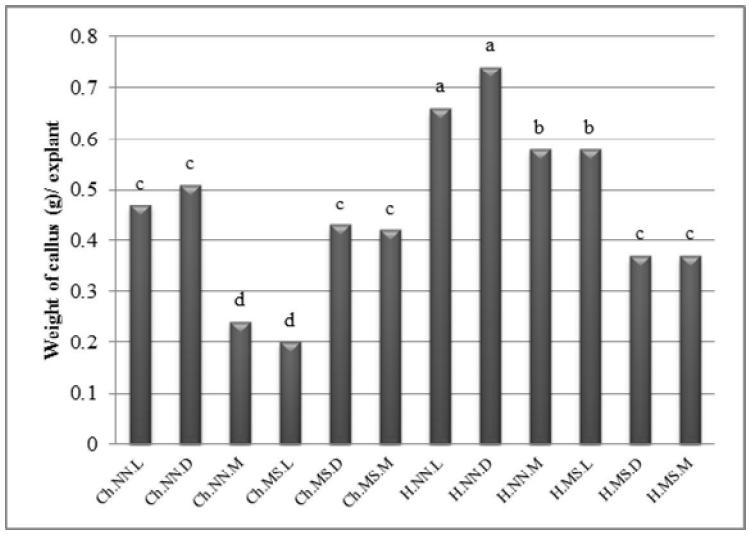


Fig. 3**.** Interaction effects between cultivars, culture media and light conditions on the callogenesis of leaf explants of Persian walnut cultivars in the sixth week. Ch: Chandler, H: Hartley, NN: Nitsch and Nitsch medium, MS: Murashige and Skoog medium, L: Low light intensity, D: Darkness, M: Modified light intensity (For example: Ch.NN.L: cv. Chandler on NN medium in the low light conditions).

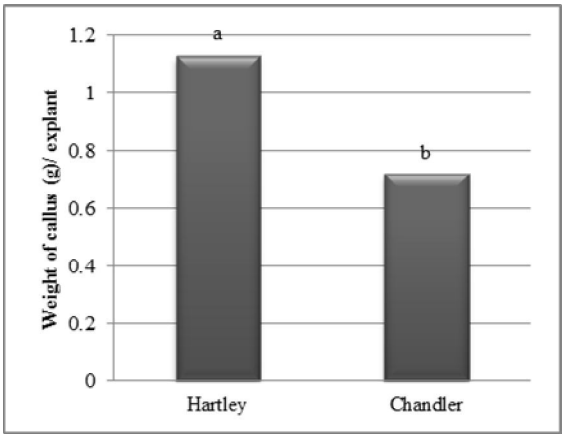


Fig. 4. Comparison effect of Chandler and Hartley cultivars of Persian walnut on the callogenesis of leaf explants in the tenth week.

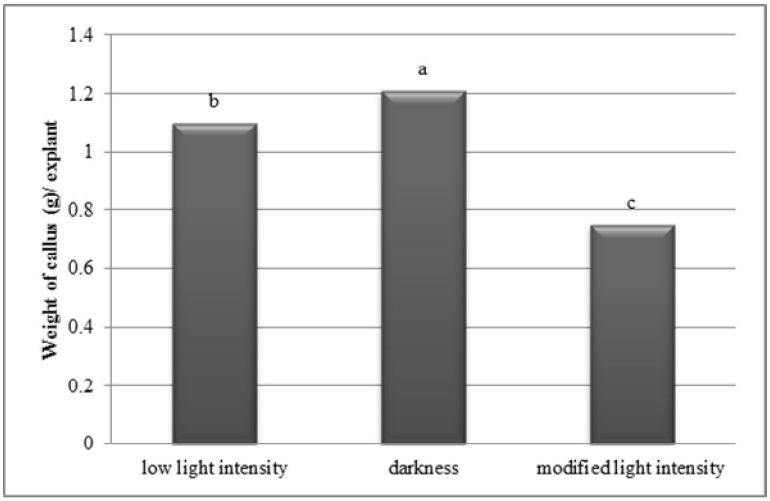


Fig. 5. Comparison effect of three light conditions including darkness, low and modified light intensity on the callogenesis of leaf explants of Persian walnut cvs. Chandler and Hartley in the tenth week.

Table 1. Texture, color and origin of the calli from leaf explants of cvs. Chandler and Hartley on NN and MS media under three light conditions including darkness, low and modified light intensity in the fourth week.

| No. Treatments | Callus | | |
|---|---|---|---|
| | Texture | Color | Origin |
| 1 Ch.NN.L | Irregular compact nodes | Whitish-creamy | Midveins |
| 2 Ch.NN.D | Semi-compact and friable | Creamy | Midveins and margins |
| 3 Ch.NN.M | Semi-compact and friable | Whitish-creamy | Midveins |
| 4 Ch.MS.L | Semi-compact and friable | Whitish-creamy | Midveins |
| 5 Ch.MS.D | Semi-compact and friable | Creamy | Midveins |
| 6 Ch.MS.M | Semi-compact and friable | Creamy | Midveins |
| 7 H.NN.L | Irregular compact and friable nodes | White | Midveins and margins |
| 8 H.NN.D | Compact | Whitish-creamy | Midveins |

| | | | |
|---|---|---|---|
| 9 H.NN.M | Compact | Whitish-creamy | midveins and margins |
| 10 H.MS.L | Irregular compact nodes | Whitish-creamy | midveins and margins |
| 11 H.MS.D | Semi-compact and friable | Creamy | midveins |
| 12 H.MS.M | Semi-compact and friable | Creamy | midveins |

Ch: Chandler, H: Hartley, NN: Nitsch and Nitsch, MS: Murashige and Skoog, L: Low light intensity, D: Darkness, M: Modified light intensity (For example: Ch.NN.L: cv. Chandler on NN medium in the low light conditions).

Table 2**.** Analysis of variance of cultivars, culture media and light conditions on callogenesis of leaf explants of Persian walnut.

| | Callogenesis in the sixth week | | Callogenesis in the tenth week | |
|---|---|---|---|---|
| | df | Mean square | df | Mean square |
| a | 1 | $0.2910^{**}$ | 1 | $1.4591^{**}$ |
| b | 1 | $0.2071^{**}$ | 1 | $0.0803^{*}$ |
| c | 2 | $0.3854^{**}$ | 2 | $0.6686^{**}$ |
| a×b | 1 | $0.0670^{**}$ | 1 | $0.0002^{ns}$ |
| b×c | 2 | $0.0481^{**}$ | 2 | $0.0010^{ns}$ |
| a×c | 2 | $0.0269^{*}$ | 2 | $0.0471^{ns}$ |
| a×b×c | 2 | $0.1045^{**}$ | 1 | $0.0543^{ns}$ |
| error | 38 | 0.0059 | 32 | 0.0169 |
| cv (%) | - | 16.6792 | - | 13.3383 |

a: effect of cultivars, b: effect of culture media, c: effect of light conditions, a×b: interaction effect between cultivars and culture media, b×c: interaction effect between culture media and light conditions, a×c: interaction effect between cultivars and light conditions, a×b×c: interaction effect between cultivars, culture media and light conditions. $^{ns}$, *, and **, respectively, represents non-significant difference, and significant differences on %5 and %1 levels.

## DISCUSSION

The combination of NAA and BA was required for induction of somatic embryogenesis in *Quercus robur* L. [7, 12, 14, 29] and *Castanea sativa* Mill. [6].

Cuenca *et al.* [7] cultured leaf explants of four different *Q. robur* stands in different locations and reported that somatic embryogenesis depended on origin of plant. They reported somatic embryos formation from two out of the four studied provenances. In our experiment, two cultivars were used and somatic embryos were not obtained from them. In the future studies, potential of somatic embryogenesis of other walnut cultivars should be investigated.

Also they observed adventitious root formation after 8–10 weeks of culture in three out of the four provenances. But root formation was at a low frequency due to the inhibitory effect of BA in the medium. They stated rhizogenesis was related to media containing NAA (2–4 $mg.l^{-1}$) in combination with BA (0.5–1.0 $mg.l^{-1}$). In our study, adventitious root formation was not occurred; probably due to the same concentrations (1.0 $mg.l^{-1}$) of NAA and BA. Vahdati [33] cultured leaf explants of *Juglans regia* L. on MS medium supplemented with different levels of NAA and BA and observed adventitious root formation in the high concentrations of NAA in combination with low concentration of BA. He reported that all of the calli were friable and non-embryonic.

In our study, texture, color and origin of calli were different among treatments. In the dark conditions, calli were more friable than low light intensity in the first month; so the intensity of light affect on

texture of calli. In addition, most of calli on NN medium were more compact than MS medium.

Avilés *et al*. [2] cultured the adult leaves of *J. regia* on MS, DKW, WPM [17], BTM [3] and reported that color and consistency of calli depend on the culture medium. In DKW medium, the calli were abundant, compact and brownish-yellow in color. In BTM and WPM media, the calli presented a nodular appearance; while only in WPM medium the calli presented a greater degree of browning and like our results, in MS medium, the calli were more friable than other media. BTM and WPM media presented a higher proportion of nodular calli than MS and DKW media. This could be because BTM and WPM have lower amount of macronutrients than DKW and MS medium, in particular, lower concentrations of Ca and N.

Also we compared concentrations of nutrients in NN and MS media. The concentration of Ca and N was higher in MS medium than NN medium (Table 3); so it caused most of the calli in MS medium became friable.

In the sixth week, individual effect of cultivars, culture medium, light conditions and interaction effect of them (excluding interaction effect of cultivar and light condition which was significant at 5% level) were significant at 1% level. This means that they had strong effect on callogenesis. Comparison of individual effects with interaction effects between factors indicated despite callogenesis of cv. Chandler, NN medium and dark conditions were higher than cv. Hartley, MS medium and low and modified light intensity, but the same result was obtained when cv. Chandler cultured on both MS and NN media in the darkness. This indicated that interaction effect between factors can influence results of individual effects and determine the final results.

Te-Chato and Rungnoi [27] reported same result by culturing leaves of *Azadirachta excelsa* (Jack) Jacobs on both MS and NN media. Also in our experiment, when interaction effects between factors compared with each other, MS and NN media had the same results in some cases (Figure 3).

Iantcheva *et al*. [15] reported that frequency of callogenesis of leaf explant from Bulgarian spray carnation cultivars (Fea and Rossitza) depends on light conditions and the callus formation in the darkness was more than illumination. Also in our study, light conditions affected on callogenesis and frequency of callogenesis in dark conditions was more than other ones.

With comparison interaction effect between cultivars, culture media and light conditions in the sixth and tenth weeks, results showed that interaction effect of factors just in the sixth week became significant. In this experiment, we decreased concentration of plant growth regulators after first month and used the plant growth regulator free media after second month. So the concentrations of plant growth regulators in the sixth and tenth weeks were not equal. Maybe concentrations of plant growth regulator decreased or increased effect of cultivar, culture medium and light conditions on callogenesis. For confirming this statement, we should do another experiment which do not decrease concentration of plant growth regulator then analyze the data.

In the tenth week, individual effects have more influence on the rate of callogenesis than interaction effects. Effects of cultivars and light conditions were significant at 1% level but effect of media was significant at 5% level. This showed that cultivar and light conditions versus culture medium have more effect on the rate of callogenesis. Increase of light intensity decreased the rate of callogenesis and did not induce somatic embryogenesis. The main goal of this study was inducing somatic embryos from leaf explants of Persian walnut but by transferring explant to plant growth regulator free media, most of the calli

became friable and non-embryonic. In contrary, somatic embryos were observed from friable calli of leaf explants of *Quercus robur* L. in the plant growth regulator free media [7]. They reported the frequency of embryogenesis from leaf explants was lower than zygote embryos. Also in walnut, somatic embryos were produced from immature cotyledons [4, 5, 19, 30, 33], zygote embryos [1, 4, 18] and endosperm [32].

Production of somatic embryos indirectly from an intermediate callus was difficult [26]. In *Juglans regia*, the indirect process is more difficult to achieve than direct regeneration and in the future studies, effect of another factors such as other media, cultivars, explants and plant growth regulators for direct somatic embryogenesis should be investigated.

Table 3.Composition of MS and NN media used in callogenesis of walnut leaf explants. Value expressed in $g.l^{-1}$

| Constituent | MS [21] | NN [23] |
|---|---|---|
| $NH_4NO_3$ | 1.65 | 0.720 |
| $KNO_3$ | 1.9 | 0.950 |
| $CaCl_2.2H_2O$ | 0.44 | 0.166 |
| $MgSO_4.7H_2O$ | 0.370 | 0.185 |
| $KH_2PO_4$ | 0.170 | 0.068 |
| KI | 0.00083 | - |
| $H_3BO_3$ | 0.0062 | 0.01 |
| $MnSO_4.4H_2O$ | 0.0223 | 0.025 |
| $ZnSO_4.7H_2O$ | 00.0086 | 0.01 |
| $Na_2MoO_4.2H_2O$ | 0.00025 | 0.00025 |
| $CuSO_4.5H_2O$ | 0.000025 | 0.000025 |
| $CoCl_2.6H_2O$ | 0.000025 | - |
| $FeSO_4.7H_2O$ | 0.0278 | 0. 0278 |
| $Na_2EDTA.2H_2O$ | 0. 0373 | 0. 0373 |
| *myo*-Inositol | 0.1 | 0.1 |
| Nicotinic acid | 0.0005 | 0.0005 |
| Pyridoxine.HCl | 0.0005 | 0.0005 |
| Thiamine.HCl | 0.0005 | 0.0005 |
| Glycine | 0.002 | 0.002 |
| Sucrose | 30 | 30 |

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