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Study of Somatic Embryogenesis Potential of Male Florets and Pistillate Flowers of Persian Walnut (*Juglansregia* L.)

M. Farsi[*1],K. Vahdati[1],M. Lotfi[1],D. Hassani[2], M. Mirmasoumi[3]*
1.Department of Horticultural Sciences, College of Abouraihan, University of Tehran, Tehran, Iran
2.Department of Horticulture, Seed and Plant Improvement Institute, Karaj, Iran
3.Department of Biology, College of Sciences, University of Tehran, Tehran, Iran

Abstract
Male florets and pistillate flowers of cvs.Chandler and Hartley of Persian walnut were cultured on modified Murashige and Skoog medium (MS) supplemented with 0.05 mg l^{-1} biotin, 0.5 mg l^{-1} folic acid, 100 mg l^{-1} glutamine and different concentrations of Naphthalene acetic acid (NAA) and Kinetin (KIN) and for pistillate flowers culture, these concentrations were 0.25, 0.5, 1 and 2 mg l^{-1}). After four weeks, the percentage of callogenesis of male florets in cv. Chandler was more than cv. Hartley. In the sixth and tenth weeks, the rate of callogenesis of male florets was depended on cultivars, the ratio of NAA: KIN and interaction between them; so in the sixth week, the highest rate of callogenesis was obtained in the higher ratio of NAA: KIN. In the tenth week, the effect of cultivars was not significant and the highest rate of callogenesis of male florets was obtained in treatments 4 (2.5 mg l^{-1} NAA with 1.25 mg l^{-1} KIN), 5 (1.25 mg l^{-1} NAA with 1.25 mg l^{-1} KIN), 7 (2.5 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) and 8 (1.25 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) of cv. Chandler. By culturing pistillate flowers on modified MS medium, nodular compact calli formed on the upper and lower part of flowers. During the next weeks, callogenesis occurred in sepals and leaflets of pistillate flowers and then stigmas and styles swelled and formed nodular calli. Similar to male florets, the rate of callogenesis in pistillate flowers depended on cultivars, the ratio of NAA: KIN and interaction between them. In the sixth and tenth weeks, the highest rate of callogenesis of cv. Chandler pistillate flowers was obtained in treatments including high or same concentrations of NAA: KIN. Callogenesis of pistillate flowers of cv. Hartley was achieved from different ratios of NAA: KIN. The lowest rate of callogenesis in the sixth and tenth weeks was obtained in the low concentrations of NAA and KIN. With increase of callogenesis in treatments 3 (0.5 mg l^{-1} NAA with 2 mg l^{-1} KIN), 6 (1 mg l^{-1} NAA with 1 mg l^{-1} KIN), 10 (1 mg l^{-1} NAA with 0.5 mg l^{-1} KIN), 11 (0.5 mg l^{-1} NAA with 0.5 mg l^{-1} KIN), 12 (0.25 mg l^{-1} NAA with 0.5 mg l^{-1} KIN), 14 (1 mg l^{-1} NAA with 0.25 mg l^{-1} KIN), 15 (0.5 mg l^{-1} NAA with 0.25 mg l^{-1} KIN) and 16 (0.25 mg l^{-1} NAA with 0.25 mg l^{-1} KIN) of cv. Chandler and treatments 9 (2 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) and 11 (0.5 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) of cv. Hartley, the tissues of pistillate flowers turned into masses of nodular calli similar to embryogenic ones.

Keywords: Callus, Catkins, Male florets, Pistillate flowers, Somatic embryo, Walnut

Introduction

Somatic embryogenesis is defined as a process in which a bipolar structure, resembling a zygotic embryo, develops from a non-zygotic cell without vascular connection with the original tissue (von Arnold et al. 2002). Somatic embryos from Persian walnut (*Juglans regia* L.) are being used for clonal propagation (Tulecke and McGranahan1985), production of interspecific hybrids (McGranahan et al. 1986), triploids from endosperm (Tulecke et al. 1988) and introduction of specific genes (McGranahan et al. 1988). Somatic embryogenesis has been induced from immature walnut cotyledons (Tulecke and McGranahan1985; McGranahan et al. 1987; Cornu1988; Cornu1989; Deng and Cornu1992;Neuman et al. 1993), zygotic embryos (McGranahan et al. 1986; Cornu1989; Aly et al. 1992) and gelatinous endosperm (Tulecke et al. 1988). In our knowledge, there is no report of somatic embryogenesis from maternal tissues of walnut. Somatic embryos from these tissues are genetically identical. The process of somatic embryogenesis can continue for years, producing large numbers of genetically identical embryos. These can then be germinated to produce genetically identical seedling (Romas1988). Embryogenesis potential of different maternal tissues of walnut such as ovules, anthers and leaves were investigated (Aly et al. 1992; Vahdati 2002; Jariteh2004; Bayat2007; Farsi 2011). Vahdati (2002) cultured anthers of Persian walnut some cultivars on different combinations of plant-growth-regulators and obtained nodular calli in the media including 1 mg l^{-1} KIN, 2 mg l^{-1} NAA and 0.01-1 mg l^{-1}Thidiazuron (TDZ). He reported that use of 2,4-Dichloroxyacetic acid (2,4-D) in the medium, caused that calli became friable and non-embryogenic. Jariteh(2004) used different concentrations of NAA with 6-Benzyladenine (BA) for anther culture of some cultivars of Persian walnut but no embryo was formed.

Also Bayat(2007) cultured anthers of Persian walnut

* **Corresponding author:**Email: minafarsi@alumni.ut.ac.ir

Abbreviations
2,4-D 2,4-Dichloroxyacetic acid
BA 6-Benzyladenine
DKW Driver and Kuniyuki walnut medium
KIN Kinetin
MS Murashige and Skoog medium
NAA Naphthalene acetic acid
NN Nitsch and Nitsch medium
TDZ Thidiazuron

cvs. Chandler and Hartley and genotype Z60 on MS medium(Murashige and Skoog 1962) including different concentrations of 2,4-D and TDZ and stated that the volume of calli was effected by TDZ concentration and the most of calli became friable. In addition, cultivar influenced the texture of callus; so calli derived from anthers in cv. Chandler became more nodular than other cultivars.

For inducing somatic embryogenesis from leaf explants of Persian walnut, different concentrations of plant-growth-regulators (NAA with BA (Vahdati 2002; Farsi et al. 2012); 2,4-D with KIN (Jariteh2004); 2,4-D with TDZ; NAA with TDZ and NAA with KIN (Bayat2007; Frasi2011)) were used; the calli became nodular and similar to embryogenic ones just in treatments had the combination of NAA and KIN. Therefore, in this study, we investigated the effect of different concentrations of KIN and NAA on the embryogenic potentials of other explants such as male florets and pistillate flowers of *J. regia* L. cvs. Chandler and Hartley.

Materials and methods

Explants source and preparation

Immature walnut catkins with 1.5-2 cm length were collected in late March from walnut cvs. Hartley and Chandler from the walnut experimental orchard of Kamal shahr station of Seed and Plant Improvement Institute, Karaj, Iran. At this stage of catkin development, the male florets have not been opened and the anthers were yellow-greenish. Also pistillate flowers of these cultivars were collected 1-2 days before the opening of the stigmas in late April. Catkins and pistillate flowers were placed in plastic bags and brought back to the lab on the ice pieces. Catkins and pistillate flowers were rinsed with sterile distilled water 4-5 times each for 5 minutes. Then they were disinfected by immersing in 70% ethanol for 60 seconds, followed by 10% (v/v) bleach (0.6% sodium hypochlorite) containing 1 drop Tween-20 to ensure wetting for 15 minutes, and they rinsed thoroughly for 4 washes of sterile distilled water. Male florets from the middle part of each catkin were dissected on a moistened filter paper and then transferred to sterile petri dishes containing moistened filter papers. After sterilization, the pistillate flowers were transferred to petri dishes with moistened filter paper.

Composition of the media

Male florets and pistillate flowers of these cultivars were cultured on modified MS medium including 0.05 mg l^{-1} biotin, 0.5 mg l^{-1} folic acid, 100 mg l^{-1} glutamine and 3% sucrose supplemented with different concentrations of NAA and KIN. For male florets culture, the concentrations of NAA and KIN were 0.5, 1.25 and 2.5 mg l^{-1}(Table 1). Also these concentrations for pistillate flowers culture were 0.25, 0.5, 1 and 2 mg l^{-1}(Table 2). Semisolid media were gelled with 2 g l^{-1}Gelrite. The pH was adjusted to 5.6 before sterilization by autoclaving at 121°C for 20 min. For male florets culture, each petri dish was containing 5 male florets and for pistillate flowers culture, each one including 2 pistillate flowers. 4 replicates were used for each treatment. Explants were subcultured every three weeks and exposed with plant-growth-regulators for 5-6 months.

Culture conditions

Petri dishes were maintained in a growth chamber under a 16-h photoperiod provided by the cool white fluorescent lamps (approximately 30 µmol $m^{-2}s^{-1}$) at 25±2 ºC.

Statistical analysis

The main goal of culturing male florets and pistillate flowers was obtaining somatic embryos, but at the first repeat of these experiments, no embryo was formed. In the second repeat, other factors such as percentage of callogenesis of male florets in the first month, rate of callogenesis of male florets and pistillate flowers in the sixth and tenth weeks, quality of calli and formation or non-formation of somatic embryo were recorded. Data were recorded by callus scoring (1: weak, 2: medium, 3: good, 4: very good and 5: excellent).

In male florets culture, differences in two cultivars in combination with nine hormonal treatments and in pistillate flowers culture effect in two cultivars in combination with 16 hormonal treatments were investigated in a factorial experiment.

Analysis of variance was carried out and means of the studied variables were compared using Duncan's multiple range test (DMRT) (Duncan 1955).

Results

Male florets culture

During the first week, most of the male florets opened (Fig 1a) and a small portion of them turned black and died soon after culturing on the media. In the second week, the bracts, sepals and receptacles of male florets swelled and in the third and fourth weeks the green compact calli formed (Fig 1b).

The percentage of callogenesis of male florets was investigated in the fourth week. All effects including the effects of cultivars, the effects of hormonal treatments and the interaction effects were significant at 1% level (Table 3). The percentage of callogenesis of cv. Chandler was higher than cv. Hartley. The highest percentage of callogenesis was observed in hormonal treatments 1 (2.5 mg l^{-1} NAA with 2.5 mg l^{-1} KIN), 2 (1.25 mg l^{-1} NAA with 2.5 mg l^{-1} KIN) and 3 (0.5 mg l^{-1} NAA with 2.5 mg l^{-1} KIN) of cv. Chandler and in hormonal treatments 1 (2.5 mg l^{-1} NAA with 2.5 mg l^{-1} KIN) to 9 (0.5 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) of cv. Hartley (Fig 2).

In the sixth week, callogenesis was mostly observed on the receptacle, bracts and sepals of male florets (Fig1c). Anthers turned greenish-cream, brown or black. Also in the sixth week, effect of cultivars at 5% level and effect of hormonal treatments and the interaction effect between cultivars and hormonal treatments at 1% level on the rate of callogenesis of male florets were significant (Table 3). The rate of callogenesis of cv. Hartley was higher than cv. Chandler. The highest and lowest rate of callogenesis were obtained, respectively, in hormonal treatment 7 (2.5 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) and 2 (1.25 mg l^{-1} NAA with 2.5 mg l^{-1} KIN) of cv. Chandler.

In the tenth week, the most of old calli turned brown or black especially in hormonal treatment 1 (2.5 mg l^{-1} NAA with 2.5 mg l^{-1} KIN) of cv. Chandler and all hormonal treatments of cv. Hartley (Fig 1d). The new semi-compact calli emerged from male florets lobes (Fig 1e). The most types of calli were semi-compact with irregular small or big nodes and greenish-brown color (Fig 1f) (Table 4).

In the tenth week, effect of hormonal treatments and the interaction effect between cultivars and hormonal treatments on the rate of callogenesis of male florets were significant at 1% level but effect of cultivars was not significant (Table 3). The highest rate of callogenesis was achieved in hormonal treatments 4 (2.5 mg l^{-1} NAA with 1.25 mg l^{-1} KIN), 5 (1.25 mg l^{-1} NAA with 1.25 mg l^{-1} KIN), 7 (2.5 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) and 8 (1.25 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) of cv. Chandler and the lowest one was obtained in hormonal treatment 2 (1.25 mg l^{-1} NAA with 2.5 mg l^{-1} KIN) and 6 (0.5 mg l^{-1} NAA with 1.25 mg l^{-1} KIN) of this cultivar.

In the fourth month, the volume of old calli increased and the cotton-like tissues were formed on their surface (Fig 1g). Compact or semi-compact nodular calli grew on the surface of male florets which had become black (Fig 1h). The most types of new calli were semi-compact with light green color (Table 4). Also some of them had cotton-like tissues and small nodes (Table 4). At the end of the sixth month, no embryo was formed from these calli.

Pistillate flowers culture

During the first week, the pistillate flowers swelled and their volume increased; also sepals and leaflets surrounding the stigmas swelled and separated apart (Fig 5a).

In the second week, most of the pistillate flowers except treatments 12 (0.25 mg l^{-1} NAA with 0.5 mg l^{-1} KIN), 15 (0. 5 mg l^{-1} NAA with 0.25 mg l^{-1} KIN) and 16 (0.25 mg l^{-1} NAA with 0.25 mg l-1 KIN) of cv. Chandler were elongated and bulky (Fig 5b). The length of stigmas and styles, especially in the pistillate flowers of cv. Hartley increased (Fig 5c). With the volume increase of pistillate flowers, longitudinal slot occurred on the surface of involucre and sepal bases (Fig 5d).

At the end of third week, callogenesis occurred in hormonal treatments 1 (2 mg l^{-1} NAA with 2 mg l^{-1} KIN), 2 (1 mg l^{-1} NAA with 2 mg l^{-1} KIN), 3 (0.5 mg l^{-1} NAA with 2 mg l^{-1} KIN), 4 (0.25 mg l^{-1} NAA with 2 mg l^{-1} KIN), 6 (1 mg l^{-1} NAA with 1 mg l^{-1} KIN), 9 (2 mg l^{-1} NAA with 0.5 mg l^{-1} KIN), 10 (1 mg l^{-1} NAA with 0.5 mg l^{-1} KIN), 11 (0.5 mg l^{-1} NAA with 0.5 mg l^{-1} KIN), 12 (0.25 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) of cv. Chandler and in hormonal treatments 1 (2 mg l^{-1} NAA with 2 mg l^{-1} KIN), 2 (1 mg l^{-1} NAA with 2 mg l^{-1} KIN), 4 (0.25 mg l^{-1} NAA with 2 mg l^{-1} KIN), 7 (0.5 mg l^{-1} NAA with 1 mg l^{-1} KIN) and 9 (2 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) of cv. Hartley and the nodular calli formed at the bottom of pistillate flowers (Fig 5e). Sepals and leaflets that had been swelled turned to callus-like tissues (Fig 5f).

In the fourth and fifth weeks, nodular and compact calli were formed on different parts of the involucre and sepal bases (Fig 5g). Some of these calli were similar to warts (Fig 5h) and had significant growth in hormonal treatments 3 (0.5 mg l^{-1} NAA with 2 mg l^{-1} KIN), 4 (0.25 mg l^{-1} NAA with 2 mg l^{-1} KIN), 6 (1 mg l^{-1} NAA with 1 mg l^{-1} KIN), 7 (0.5 mg l^{-1} NAA with 1 mg l^{-1} KIN), 8 (0.25 mg l^{-1} NAA with 1 mg l^{-1} KIN), 14 (1 mg l^{-1} NAA with 0.25 mg l^{-1} KIN) and 15 (0.5 mg l^{-1} NAA with 0.25 mg l^{-1} KIN) of cv. Chandler and in hormonal treatments 1 (2 mg l^{-1} NAA with 2 mg l^{-1} KIN) and 5 (2 mg l^{-1} NAA with 1 mg l^{-1} KIN) of cv. Hartley.

The nodular calli in hormonal treatments 9 (2 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) to 16 (0.25 mg l^{-1} NAA with 0.25 mg l^{-1} KIN) of cv. Chandler, were very similar to embryogenic ones (Fig 5i). Also each part of pistillate flowers was separated and formed nodular and compact calli individually (Fig 5j).

In the sixth week, all effects including effect of cultivars, effect of hormonal treatments and the interaction effect between them on the rate of callogenesis of pistillate flowers were significant at 1% level (Table 5). The highest rate of callogenesis was obtained in hormonal treatments 1 (2 mg l^{-1} NAA with 2 mg l^{-1} KIN) and 9 (2 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) of cv. Chandler and hormonal treatments 1 (2 mg l^{-1} NAA with 2 mg l^{-1} KIN), 2 (1 mg l^{-1} NAA with 2 mg l^{-1} KIN), 3 (0.5 mg l^{-1} NAA with 2 mg l^{-1} KIN), 5 (2 mg l^{-1} NAA with 1 mg l^{-1} KIN), 6 (1 mg l^{-1} NAA with 1 mg l^{-1} KIN), 7 (0.5 mg l^{-1} NAA with 1 mg l^{-1} KIN), 8 (0.25 mg l^{-1} NAA with 1 mg l^{-1} KIN), 9 (2 mg l^{-1} NAA with 0.5 mg l^{-1} KIN), 10 (1 mg l^{-1} NAA and 0.5 mg l^{-1} KIN), 11 (0.5 mg l^{-1} NAA with 0.5 mg l^{-1} KIN), 12 (0.25 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) of cv. Hartley (Fig 6). The lowest rate of callogenesis was achieved in hormonal treatment 16 (0.25 mg l^{-1} NAA and 0.25 mg l^{-1} KIN) of cv. Chandler (Fig 6).

In the second month, the volume of pistillate flowers increased and the involucre and sepal bases of them became friable and opaque (Fig 5k). With increase of callogenesis, pistillate flowers were converted into callus masses (Fig 5l). Also compact or semi-compact calli were formed on the leaflets, sepals, stigmas and styles of pistillate flowers (Fig 5m). The wart-shaped calli increased on the surface of involucre and sepal bases and became larger than before (Fig 5n). The most types of calli were irregular compact nodes with green or greenish-brown color (Table 6).

In the tenth week, all effects (effects of cultivars, effects of hormonal treatments and the interaction effects between them) on the rate of callogenesis of pistillate flowers were significant at 1% level (Table 5). The highest rate of callogenesis was obtained in hormonal treatments 1 (2 mg l^{-1} NAA with 2 mg l^{-1} KIN), 6 (1 mg l^{-1}NAA with 1 mg l^{-1} KIN) and 9 (2 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) of cv. Chandler and hormonal treatments 1 (2 mg l^{-1} NAA with 2 mg l^{-1} KIN), 2 (1 mg l^{-1} NAA with 2 mg l^{-1} KIN), 3 (0.5 mg l^{-1} NAA with 2 mg l^{-1} KIN), 5 (2 mg l^{-1} NAA with 1 mg l^{-1} KIN), 6 (1 mg l^{-1} NAA with 1 mg l^{-1} KIN), 10 (1 mg l^{-1} NAA with 0.5 mg l^{-1} KIN), 11 (0.5 mg l^{-1} NAA with 0.5 mg l^{-1} KIN), 13 (2 mg l^{-1} NAA with 0.25 mg l^{-1} KIN) and 15 (0.5 mg l^{-1} NAA with 0.25 mg l^{-1} KIN) of cv. Hartley and the lowest one was

achieved in treatment 16 (0.25 mg l^{-1} NAA with 0.25 mg l^{-1} KIN) of cv. Chandler (Fig 7).

In the third month, the volume of calli increased and the involucre and sepal bases of pistillate flowers became more friable and opaque than before. Also increase the volume of nodular calli made them friable. The new nodular calli formed on the surface of leaflets, sepals, stigmas and styles (Fig 5o).

In the fourth month, the cotton-like structures increased on the surface of old calli of cv. Hartley (Fig 5p) (Table 6). The most types of calli were irregular compact and semi-compact small or big nodes with green or greenish-brown color (Table 6).

Also with increase of callogenesis in hormonal treatments 3 (0.5 mg l^{-1} NAA with 2 mg l^{-1} KIN), 6 (1 mg l^{-1} NAA with 1 mg l^{-1} KIN), 9 (2 mg l^{-1} NAA with 0.5 mg l^{-1} KIN), 10 (1 mg l^{-1} NAA with 0.5 mg l^{-1} KIN), 11 (0.5 mg l^{-1} NAA with 0.5 mg l^{-1} KIN), 12 (0.25 mg l^{-1} NAA with 0.5 mg l^{-1} KIN), 14 (1 mg l^{-1} NAA with 0.25 mg l^{-1} KIN), 15 (0.5 mg l^{-1} NAA with 0.25 mg l^{-1} KIN) and 16 (0.25 mg l^{-1} NAA with 0.25 mg l^{-1} KIN) of cv. Chandler and hormonal treatments 9 (2 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) and 11 (0.5 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) of cv. Hartley, the tissues of pistillate flowers turned into the masses of nodular calli which were very similar to embryogenic ones (Fig 5q); but after 6 month no embryo was formed from these calli.

Table 1. Hormonal treatments including different combinations of NAA (Naphthalene acetic acid) with KIN (Kinetin) used for inducing somatic embryogenesis from male florets of Persian walnut cvs. Chandler and Hartley

NAA (mg l^{-1})	KIN (mg l^{-1})		
	2.5	1.25	0.5
2.5	1	2	3
1.25	4	5	6
0.5	7	8	9

Table 2. Hormonal treatments including different combinations of NAA (Naphthalene acetic acid) and KIN (Kinetin) used for inducing somatic embryogenesis from pistillate flowers of Persian walnut cvs. Chandler and Hartley

NAA (mg l-1)	KIN (mg l-1)			
	2	1	0.5	0.25
2	1	2	3	4
1	5	6	7	8
0.5	9	10	11	12
0.25	13	14	15	16

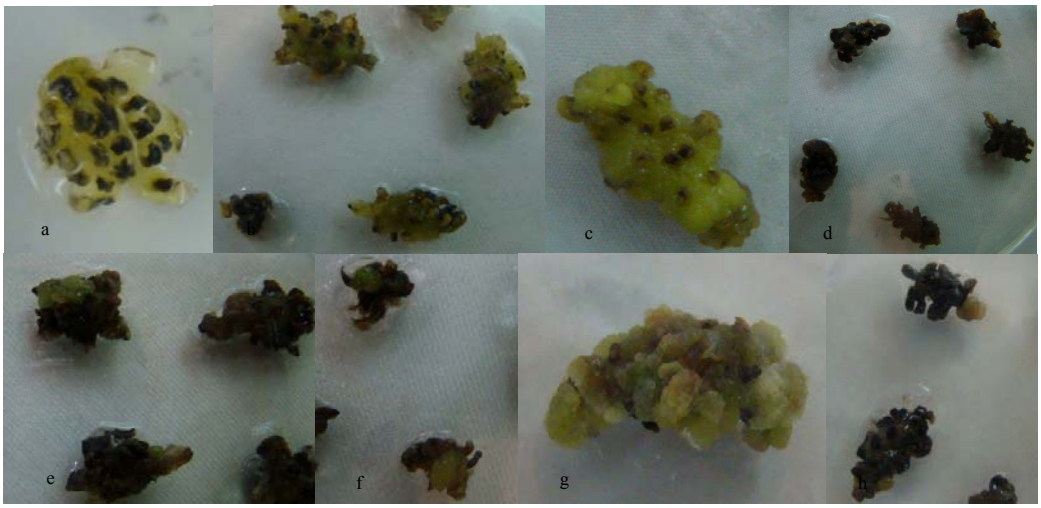

Fig 1.(**a**) Male florets of Persian walnutcv. Chandler opening in treatment 9 (0.5 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) during the first week (**b**) Formation of green calli from bracts, sepals and receptacel ofPersian walnutcv. Hartley in treatment 1 (2.5 mg l^{-1} NAA with 2.5 mg l^{-1} KIN) during the fourth week (**c**)Increase of green compact calli from bracts, sepals and receptacel of cv. Chandler in treatment 4 (2.5 mg l^{-1} NAA with 1.25 mg l^{-1} KIN) in the sixth week (**d**) Darkly colored old calli in treatament 7 (2.5 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) in the tenth week (**e**) Increase of semi-compact calli from male florets lobes of cv. Hartley in treatment 4 (2.5 mg l^{-1} NAA with 1.25 mg l^{-1} KIN) in the tenth week (**f**)Formation of calli with small or big nodes from male florets of cv. Hartley in treatment 7 (2.5 mg l^{-1} NAA with 0.5 mg l^{-1} KIN) (**g**) Increase of semi-compact calli with cotton-like tissues from male floret of cv. Chandler in treatment 2 (1.25 mg l^{-1} NAA with 2.5 mg l^{-1} KIN) (**h**)Formation of compact nodular calli on the surface of male florets of cv. Chandler in treatment 8 (1.25 mg l^{-1} NAA with 0.5 mg l^{-1} KIN)

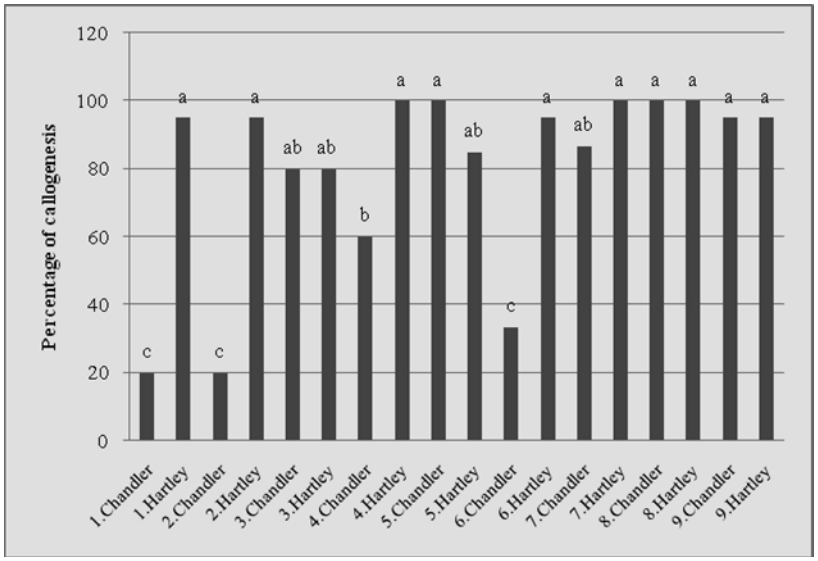

Fig 2. Interaction effects between two cultivars (Chandler and Hartley) and nine hormonal treatments on the percentage of callogenesis of Persian walnut male florets in the fourth week
Nine hormonal treatments include: 1 (2.5 mg l^{-1} NAA: 2.5 mg l^{-1} KIN), 2 (1.25 mg l^{-1} NAA: 2.5 mg l^{-1} KIN), 3 (0.5 mg l^{-1} NAA: 2.5 mg l^{-1} KIN), 4 (2.5 mg l^{-1} NAA: 1.25 mg l^{-1} KIN), 5 (1.25 mg l^{-1} NAA: 1.25 mg l^{-1} KIN), 6 (0.5 mg l^{-1} NAA: 1.25 mg l^{-1} KIN), 7 (2.5 mg l^{-1} NAA: 0.5 mg l^{-1} KIN), 8 (1.25 mg l^{-1} NAA: 0.5 mg l^{-1} KIN) and 9 (0.5 mg l^{-1} NAA: 0.5 mg l^{-1} KIN); for example: 1. Chandler: cv. Chandler in treatment 1 (2.5 mg l^{-1} NAA: 2.5 mg l^{-1} KIN)

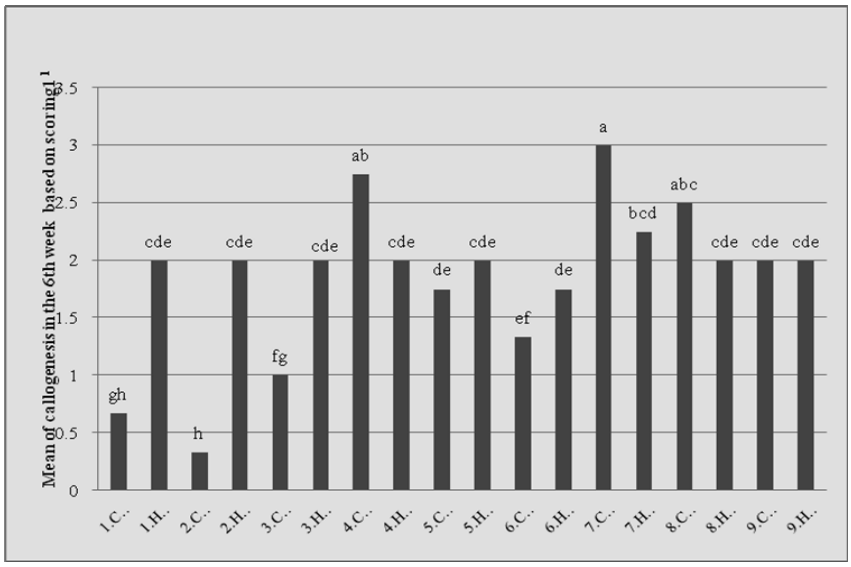

Fig 3. Interaction effects between two cultivars (Chandler and Hartley) and nine hormonal treatments on the rate of callogenesis of Persian walnut male florets in the sixth week
Nine hormonal treatments include: 1 (2.5 mg l^{-1} NAA: 2.5 mg l^{-1} KIN), 2 (1.25 mg l^{-1} NAA: 2.5 mg l^{-1} KIN), 3 (0.5 mg l^{-1} NAA: 2.5 mg l^{-1} KIN), 4 (2.5 mg l^{-1} NAA: 1.25 mg l^{-1} KIN), 5 (1.25 mg l^{-1} NAA: 1.25 mg l^{-1} KIN), 6 (0.5 mg l^{-1} NAA: 1.25 mg l^{-1} KIN), 7 (2.5 mg l^{-1} NAA: 0.5 mg l^{-1} KIN), 8 (1.25 mg l^{-1} NAA: 0.5 mg l^{-1} KIN) and 9 (0.5 mg l^{-1} NAA: 0.5 mg l^{-1} KIN); for example: 1. Chandler: cv. Chandler in treatment 1(2.5 mg l^{-1} NAA: 2.5 mg l^{-1} KIN)
[1] Calli scoring: 1: weak, 2: medium, 3: good, 4: very good

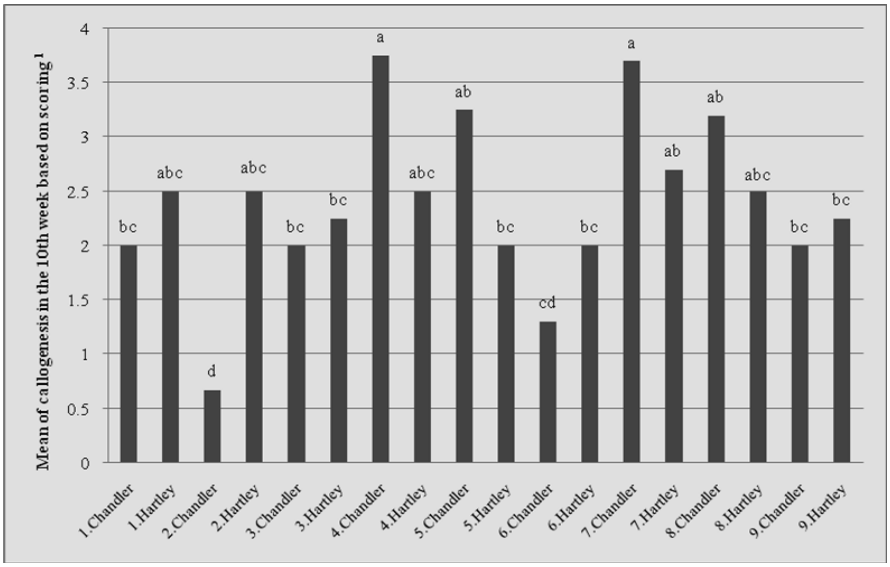

Fig 4. Interaction effects between two cultivars (Chandler and Hartley) and nine hormonal treatments on the rate of callogenesis of Persian walnut male florets in the tenth week
Nine hormonal treatments include: 1 (2.5 mg l^{-1} NAA: 2.5 mg l^{-1} KIN), 2 (1.25 mg l^{-1} NAA: 2.5 mg l^{-1} KIN), 3 (0.5 mg l^{-1} NAA: 2.5 mg l^{-1} KIN), 4 (2.5 mg l^{-1} NAA: 1.25 mg l^{-1} KIN), 5 (1.25 mg l^{-1} NAA: 1.25 mg l^{-1} KIN), 6 (0.5 mg l^{-1} NAA: 1.25 mg l^{-1} KIN), 7 (2.5 mg l^{-1} NAA: 0.5 mg l^{-1} KIN), 8 (1.25 mg l^{-1} NAA: 0.5 mg l^{-1} KIN) and 9 (0.5 mg l^{-1} NAA: 0.5 mg l^{-1} KIN); for example: 1. Chandler: cv. Chandler in treatment 1(2.5 mg l^{-1} NAA: 2.5 mg l^{-1} KIN)
[1] Calli scoring: 1: weak, 2: medium, 3: good, 4: very good

Table 3. Analysis of variance of cultivars and hormonal treatments on callogenesis of male florets of Persian walnut

S.O.V.	Percentage of callogenesis in the fourth week		Callogenesis in the sixth week		Callogenesis in the tenth week	
	df	Mean square	df	Mean square	df	Mean square
hormonal treatments	8	1315.9515**	8	1.5297**	8	2.2227**
cultivars	1	8751.0886**	1	1.05882*	1	0.4853ns
hormonal treatments× cultivars	8	2358.1972**	8	11.7626**	8	1.9363**
error	49	276.1905	50	0.1600	48	0.5104
cv(%)	-	19.9547	-	21.2500	-	29.2874

ns, *, and **, respectively, represents non-significant difference, and significant differences on %5 and %1 levels

Table 4. Texture and color of calli from male florets of Persian walnutcvs. Chandler and Hartley on modified MS medium under nine hormonal treatments in the tenth week and fourth month

No. treatments	Callus in the tenth week		Callus in the fourth month	
	Texture	Color	Texture	Color
1.Chandler	Semi-compact	Greenish-cream	Semi-compact with cotton-like tissues and small nodes	Light green
1.Hartley	Semi-compact	Light green and greenish-cream	Semi-compact	Green and light greenish-cream
2.Chandler	Semi-compact	Greenish-cream	Semi-compact with irregular nodes and cotton-like tissues	Light green
2.Hartley	Semi-compact	Light green, greenish-cream	Semi-compact	Light greenish-cream
3.Chandler	Semi-compact	greenish-cream	Semi-compact and small nodes	Light cream Light green
3.Hartley	Semi-compact	Light green, green and greenish-cream	Semi-compact and small nodes	Light green
4.Chandler	Semi-compact and small compact nodes	Glossy green and greenish-brown	Semi-compact with cotton-like tissues	Light green
4.Hartley	Semi-compact small nodes	Greenish-cream and	Irregular big nodes with	Green

		greenish-brown	cotton-like tissues	
5.Chandler	Semi-compact with the cotton-like tissues and irregular compact big nodes	Green and greenish-brown	Irregular big nodes with cotton-like tissues	Green
5.Hartley	Semi-compact	Greenish-cream	Semi-compact	Cream
6.Chandler	Semi-compact	Greenish-brown	Semi-compact with small nodes	Glossy light green
6.Hartley	Semi-compact	Greenish-cream	Semi-compact	Green
7.Chandler	Semi-compact and irregular small or big nodes	Greenish-brown	Semi-compact with cotton-like tissues	Light green
7.Hartley	Semi-compact	Greenish-cream	Semi-compact with cotton-like tissues	Green
8.Chandler	Semi-compact and irregular small or big nodes	Greenish-brown and light green-cream	Semi-compact with cotton-like tissues	Light green
8.Hartley	Semi-compact small nodes	Glossy green and greenish-cream	Semi-compact with cotton-like tissues	Light green
9.Chandler	Semi-compact small nodes	Greenish-brown and glossy green	Semi-compact with cotton-like tissues and small nodes	Light green
9.Hartley	Semi-compact small nodes	Glossy green	Semi-compact and small nodes	Cream

Nine hormonal treatments include: 1 (2.5 mg l^{-1} NAA: 2.5 mg l^{-1} KIN), 2 (1.25 mg l^{-1} NAA: 2.5 mg l^{-1} KIN), 3 (0.5 mg l^{-1} NAA: 2.5 mg l^{-1} KIN), 4 (2.5 mg l^{-1} NAA: 1.25 mg l^{-1} KIN), 5 (1.25 mg l^{-1} NAA: 1.25 mg l^{-1} KIN), 6 (0.5 mg l^{-1} NAA: 1.25 mg l^{-1} KIN), 7 (2.5 mg l^{-1} NAA: 0.5 mg l^{-1} KIN), 8 (1.25 mg l^{-1} NAA: 0.5 mg l^{-1} KIN) and 9 (0.5 mg l^{-1} NAA: 0.5 mg l^{-1} KIN); for example: 1. Chandler: cv. Chandler in treatment 1 (2.5 mg l^{-1} NAA: 2.5 mg l^{-1} KIN)

Fig 5. (**a**)Swollen different parts of pistillate flower of Persian walnut cv. Chandler in treatment 3 (0.5 mg l^{-1} NAA: 2 mg l^{-1} KIN) during the first week (**b**) Elongated and swollen pistillate flower of Persian walnut cv. Hartley in treatment 2 (1 mg l^{-1} NAA: 2 mg l^{-1} KIN) in the second week (**c**)Increase of stigma and style length in pistillate flower of cv. Hartley in treatment 1 (2 mg l^{-1} NAA: 2 mg l^{-1} KIN**)** in the second week (**d**) Occurred longitudinal slot on the surface of involucre and sepal bases of cv. Chandler pistillate flower in treatment 9 (2 mg l^{-1} NAA: 0.5 mg l^{-1} KIN) (**e**) Formation of nodular calli at the bottom of cv. Chandler pistillate flower in treatment 7 (0.5 mg l^{-1} NAA: 1 mg l^{-1}

KIN) during the thirth week (**f**) Swollen sepals and leaflets of cv. Chandler pistillate flower in treatment 1 (2 mg l^{-1} NAA: 2 mg l^{-1} KIN) turned into callus-like tissues in the thirth week (**g**) Formation of nodular calli on the different parts of the involucre and sepal bases of cv. Hartley in treatment 7 (0.5 mg l-1 NAA: 1 mg l^{-1} KIN) during the fourth and fifth week(**h**)Formation of wart-shape calli on the surface of involucare and sepal bases of cv. Chandler pistillate flower in treatment 4 (0.25 mg l^{-1} NAA: 2 mg l^{-1} KIN) during the fourth and fifth weeks(**i**) Increase of nodular calli similar to embryogenic ones in cv. Chandler pistillate flower in treatment 9 (2 mg l^{-1} NAA: 0.5 mg l^{-1} KIN) (**j**) Callogenesis of separated parts of cv. Chandler pistillate flower in treatment 7 (0.5 mg l^{-1} NAA: 1 mg l^{-1} KIN) (k)Friable and opaque calli of involucre and sepal bases of cv. Hartley pistillate flower in treatment 5 (2 mg l^{-1} NAA: 1 mg l^{-1} KIN) during the second month (**l**) Conversion of cv. Chandler pistillate flower into callus masses in treatment 2 (1 mg l^{-1} NAA: 2 mg l^{-1} KIN) during the second month(**m**) Formation of compact or semi-compact calli from different parts of cv. Chandler pistillate flowers in treatment 6 (1 mg l^{-1} NAA: 1 mg l^{-1} KIN) in the second month(**n**) Increase of wart-shaped calli on the surface of involucre and sepal bases of cv. Chandler pistillate flower in treatment 5 (2 mg l^{-1} NAA: 1 mg l^{-1} KIN) during the second month(**o**) Increase of nodular calli in different parts of cv. Chandler pistillate flower in treatment 3 (0.5 mg l^{-1} NAA: 2 mg l^{-1} KIN) in the third month (**p**) Increase of cotton-like structures on the surface of old calli of cv. Hartley pistillate flower in treatment 7 (0.5 mg l^{-1} NAA: 1 mg l^{-1} KIN) in the fourth month(**q**) Conversion of cv. Chandler pistillate flower into masses of nodular calli similar to embryogenic ones in treatment 10 (1 mg l^{-1} NAA: 0.5 mg l^{-1} KIN) in the fourth month

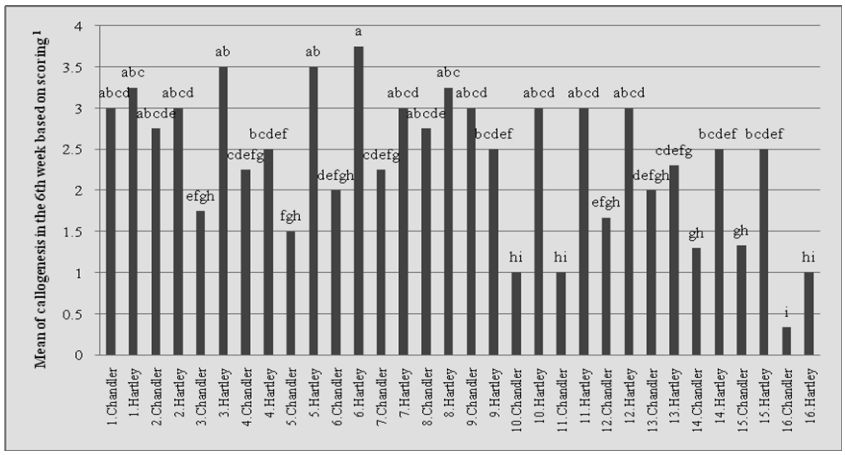

Fig 6. Interaction effects between two cultivars (Chandler and Hartley) and sixteen hormonal treatments on the rate of callogenesis of Persian walnut pistillate flowers in the sixth week
Sixteen hormonal treatments include: 1 (2 mg l^{-1} NAA: 2 mg l^{-1} KIN), 2 (1 mg l^{-1} NAA: 2 mg l^{-1} KIN), 3 (0.5 mg l^{-1} NAA: 2 mg l^{-1} KIN), 4 (0.25 mg l^{-1} NAA: 2 mg l^{-1} KIN), 5 (2 mg l^{-1} NAA: 1 mg l^{-1} KIN), 6 (1 mg l^{-1} NAA: 1 mg l^{-1} KIN), 7 (0.5 mg l^{-1} NAA: 1 mg l^{-1} KIN), 8 (0.25 mg l^{-1} NAA: 1 mg l^{-1} KIN), 9 (2 mg l^{-1} NAA: 0.5 mg l^{-1} KIN), 10 (1 mg l^{-1} NAA: 0.5 mg l^{-1} KIN), 11 (0.5 mg l^{-1} NAA: 0.5 mg l^{-1} KIN), 12 (0.25 mg l^{-1} NAA: 0.5 mg l^{-1} KIN), 13 (2 mg l^{-1} NAA: 0.25 mg l^{-1} KIN), 14 (1 mg l^{-1} NAA: 0.25 mg l^{-1} KIN), 15 (0.5 mg l^{-1} NAA: 0.25 mg l^{-1} KIN), 16 (0.25 mg l^{-1} NAA: 0.25 mg l^{-1} KIN); for example: 1. Chandler: cv. Chandler in treatment 1 (2 mg l^{-1} NAA: 2 mg l^{-1} KIN)
[1] Calli scoring: 1: weak, 2: medium, 3: good, 4: very good

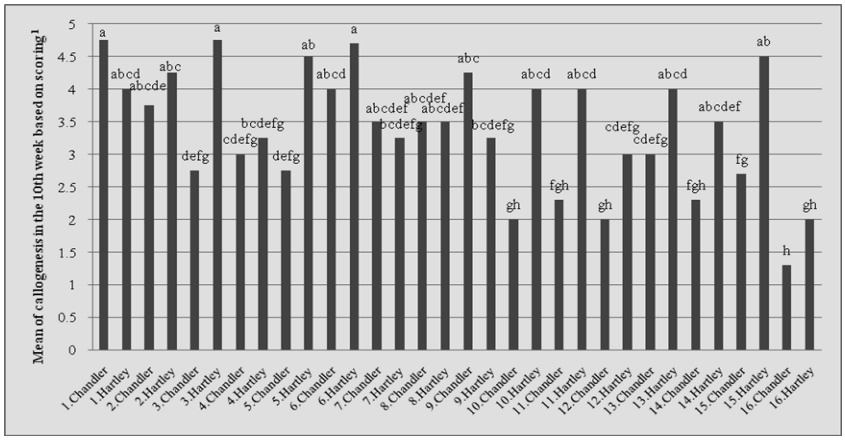

Fig 7. Interaction effects between two cultivars (Chandler and Hartley) and sixteen hormonal treatments on the rate of callogenesis of Persian walnut pistillate flowers in the tenth week. Sixteen hormonal treatments include: 1 (2 mg l^{-1} NAA: 2 mg l^{-1} KIN), 2 (1 mg l^{-1} NAA: 2 mg l^{-1} KIN), 3 (0.5 mg l^{-1} NAA: 2 mg l^{-1} KIN), 4 (0.25 mg l^{-1} NAA: 2 mg l^{-1} KIN), 5 (2 mg l^{-1} NAA: 1 mg l^{-1} KIN), 6 (1 mg l^{-1} NAA: 1 mg l^{-1} KIN), 7 (0.5 mg l^{-1} NAA: 1 mg l^{-1} KIN), 8 (0.25 mg l^{-1} NAA: 1 mg l^{-1} KIN), 9 (2 mg l^{-1} NAA: 0.5 mg l^{-1} KIN), 10 (1 mg l^{-1} NAA: 0.5 mg l^{-1} KIN), 11 (0.5 mg l^{-1} NAA: 0.5 mg l^{-1} KIN), 12 (0.25 mg l^{-1} NAA: 0.5 mg l^{-1} KIN), 13 (2 mg l^{-1} NAA: 0.25 mg l^{-1} KIN), 14 (1 mg l^{-1} NAA: 0.25 mg l^{-1} KIN), 15 (0.5 mg l^{-1} NAA: 0.25 mg l^{-1} KIN), 16 (0.25 mg l^{-1} NAA: 0.25 mg l^{-1} KIN); for example: 1. Chandler: cv. Chandler in treatment 1 (2 mg l^{-1} NAA: 2 mg l^{-1}
[1] Calli scoring: 1: weak, 2: medium, 3: good, 4: very good, 5: excellent.

Table 5. Analysis of variance of cultivars and hormonal treatments on callogenesis of pistillate flowers of Persian walnut

S.O.V.	Callogenesis in the sixth week		Callogenesis in the tenth week	
	df	Mean square	df	Mean square
hormonal treatments	15	2.4626**	15	2.8483**
cultivars	1	27.8758**	1	12.9614**
hormonal treatments× cultivars	15	0.8183**	15	1.4756**
error	77	0.3030	72	0.4143
cv (%)	-	22.8146	-	18.6997

ns, *, and **, respectively, represents non-significant difference, and significant differences on %5 and %1 levels

Table 6.Texture and color of calli from pistillate flowers of Persian walnut cvs. Chandler and Hartley on modified MS medium under sixteen hormonal treatments in the second and fourth months

No. treatments	Callus in the second month		Callus in the fourth week	
	Texture	Color	Texture	Color
1.Chandler	Irregular compact big nodes	Greenish-brown	Semi-compact tissues with irregular semi-compact big nodes	Green and greenish-brown
1.Hartley	Irregular compact big nodes	Greenish-brown	Semi-compact tissues with cotton-like structures and irregular compact and semi-compact big nodes	Green and greenish-brown
2.Chandler	Irregular compact big nodes	Greenish-brown	Semi-compact tissues with irregular compact and semi-compact big nodes	Green, greenish-brown and brown
2.Hartley	Irregular compact big nodes	Greenish-brown	Semi-compact tissues with cotton-like structures and irregular compact and semi-compact big nodes	Green and greenish-brown
3.Chandler	Irregular compact big nodes	Greenish-brown	Semi-compact tissues with irregular compact and semi-compact small or big nodes	Light green, green and greenish-brown
3.Hartley	Irregular compact big nodes	Greenish-brown	Semi-compact tissues with cotton-like structures and irregular compact and semi-compact big nodes	Green and greenish-brown
4.Chandler	Irregular compact big nodes	Greenish-brown	Semi-compact tissues with irregular compact and semi-compact big nodes	Greenish-brown
4.Hartley	Irregular compact big nodes	Greenish-brown	Semi-compact tissues with cotton-like structures and irregular compact and semi-compact big nodes	Green and greenish-brown
5.Chandler	Irregular compact big nodes	Greenish-brown	Semi-compact tissues with semi-compact big nodes	Light green, green and greenish-brown
5.Hartley	Irregular compact big nodes	Greenish-brown	Semi-compact tissues with cotton-like structures and irregular compact and semi-compact big nodes	Green, greenish-brown and brown
6.Chandler	Irregular compact big nodes	Greenish-brown	Semi-compact tissues with irregular compact and semi-compact big nodes	Light green, green and greenish-brown
6.Hartley	Irregular compact big nodes	Greenish-brown	Semi-compact tissues with cotton-like structures and irregular compact and semi-	Green and greenish-brown

			compact big nodes	
7.Chandler	Irregular compact small or big nodes	Green	Irregular compact small or big nodes	Greenish-brown and brown
7.Hartley	Irregular compact big nodes	Greenish-brown	Semi-compact tissues with cotton-like structures and irregular compact and semi-compact big nodes	Green and greenish-brown
8.Chandler	Irregular compact small or big nodes	Light green	Semi-compact tissues with irregular compact and semi-compact small or big nodes	Greenish-brown and brown
8.Hartley	Irregular compact big nodes	Greenish-brown	Semi-compact tissues with cotton-like structures and irregular compact and semi-compact big nodes	Green and greenish-brown
9.Chandler	Irregular compact small or big nodes	Green	Semi-compact tissues with irregular compact and semi-compact small or big nodes	Light green, green and greenish-brown
9.Hartley	Irregular compact big nodes	Greenish-brown	Semi-compact tissues with cotton-like structures and irregular compact and semi-compact small or big nodes	Light green, green and greenish-brown
10.Chandler	Irregular compact big nodes	Light green	Irregular compact and semi-compact small or big nodes	Light green, green and greenish-brown
10.Hartley	Irregular compact small or big nodes	Green	Semi-compact tissues with cotton-like structures and irregular compact and semi-compact small or big nodes	Light green, green and greenish-brown
11.Chandler	Compact small nodes	Light green	Irregular compact small nodes	Light green, green and greenish-brown
11.Hartley	Irregular compact big nodes	Green	Semi-compact tissues with cotton-like structures and irregular compact small nodes	Light green, green and greenish-brown
12.Chandler	Compact small nodes	Light green	Irregular compact small nodes	Light green, green and greenish-brown
12.Hartley	Compact small nodes	Green	Semi-compact tissues with compact small nodes	Green and greenish-brown
13.Chandler	Irregular compact small or big nodes	Light green	Irregular compact small or big nodes	Light green, green and greenish-brown
13.Hartley	Compact small nodes	Green	Semi-compact tissues with cotton-like structures and irregular compact and semi-compact small or big nodes	Green and greenish-brown
14.Chandler	Irregular compact small or big nodes	Light green	Irregular compact small or big nodes	Light green, green and greenish-brown
14.Hartley	Irregular compact big nodes	Green	Semi-compact tissues with cotton-like structures and irregular compact small nodes	Green and greenish-brown
15.Chandler	Compact small nodes	Light green	Irregular compact small nodes	Light green, green and greenish-brown
15.Hartley	Compact small nodes	Green	Semi-compact tissues with cotton-like structures and irregular compact small nodes	Greenish-brown
16.Chandler	Compact small nodes	Light green	Irregular compact small nodes	Light green, green and greenish-brown
16.Hartley	Compact small nodes	Green	Irregular compact small nodes	Greenish-brown

Sixteen hormonal treatments include: 1 (2 mg l^{-1} NAA: 2 mg l^{-1} KIN), 2 (1 mg l^{-1} NAA: 2 mg l^{-1} KIN), 3 (0.5 mg l^{-1} NAA: 2 mg l^{-1} KIN), 4 (0.25 mg l^{-1} NAA: 2 mg l^{-1} KIN), 5 (2 mg l^{-1} NAA: 1 mg l^{-1} KIN), 6 (1 mg l^{-1} NAA: 1 mg l^{-1} KIN), 7 (0.5 mg l^{-1} NAA: 1 mg l^{-1} KIN), 8 (0.25 mg l^{-1} NAA: 1 mg l^{-1} KIN), 9 (2 mg l^{-1} NAA: 0.5 mg l^{-1} KIN), 10 (1 mg l^{-1} NAA: 0.5 mg l^{-1} KIN), 11 (0.5 mg l^{-1} NAA: 0.5 mg l^{-1} KIN), 12 (0.25 mg l^{-1} NAA: 0.5 mg l^{-1} KIN), 13 (2 mg l^{-1} NAA: 0.25 mg l^{-1} KIN), 14 (1 mg l^{-1} NAA: 0.25 mg l^{-1} KIN), 15 (0.5 mg l^{-1} NAA: 0.25 mg l^{-1} KIN), 16 (0.25 mg l^{-1} NAA: 0.25 mg l^{-1} KIN); for example: 1. Chandler: cv. Chandler in treatment 1 (2 mg l^{-1} NAA: 2 mg l^{-1} KIN)

Discussion

In this study embryogenic potential of walnut male florets and pistillate flowers were investigated. The research indicated that whole flowers are suitable explants for establishing embryogenic cultures because of their ease of collection and excision compared to anthers and ovaries; dissection can be done without a stereomicroscope and damaging the explants during excision is unlikely (Gambino et al. 2007).

Jariteh(2004) and Bayat (2007) used cold pre-treatment to induce somatic embryogenesis from walnut anthers; so they stored walnut catkins at 4 °C for 10 days. Also, Farsi (2011) used cold pre-treatment to induce somatic embryogenesis from male florets and pistillate flowers of some cultivars of Persian walnut. She stated that cold pre-treatment caused the male florets and pistillate flowers became sensitive to bleach and some of them turned black and died soon after culturing on the media. In addition the required time for sterilization with bleach decreased that led to an increase of fungal infection. But in this study, explants were cultured without cold pre-treatment that led to increase of the sterilization time with bleach and followed by decrease of fungal infection.

We used MS medium (Murashige and Skoog1962) which over 70% of successful cases have reported the use of this medium or the derivatives thereof (Jain et al. 2000). These media contain high level of ammonia and nitrate salts (Jain et al. 2000). Often the ratio of nitrate and ammonia in the medium is important for somatic embryogenesis induction (Neids1994).

In this study, 0.05 mg l^{-1} biotin and 0.5 mg l^{-1} folic acid were added to the MS medium. The research showed that these vitamins increased the cell division (von Arnold et al. 2002). Somatic embryogenesis initiation of loblolly pine (*Pinustaeda*L.) was improved by supplementing the initiation medium with 0.5 mg l^{-1}folic acid and 0.05 mg l^{-1} biotin. These vitamins increased the percentage of explants with extruded tissues that continued the initiation process to form embryogenic tissues (Pullman et al. 2005). El-shiatyet al. (2004) reported that, the most active medium for callus initiation and growth was MS medium supplemented with glutamine at 50 mgl^{-1} or biotin at 5 mg l^{-1} concentration. Cultures of walnut species have declined (growth slowed and tissues became brown or black) when not transferred to fresh medium at regular intervals. The healthiest walnut tissue cultures were those that were transferred to fresh medium weekly (Preece et al. 1995). In this study, 2-3 days after culturing of the male florets and pistillate flowers, they swelled and only a small portion of them became black. Subcultures were done every 3 weeks without any negative effect on the explants.

Initiation of somatic embryogenesis was improved by use of nitrogen in the form of amino acid such as glutamine (Martinelli et al. 1993; Vidal et al. 2009). Positive effect of glutamine on the induction of embryogenesis from anthers and ovaries of grapevine (*Vitis* sp.) genotypes was proved by Kikkert et al. (2005). Vidal et al. (2009) stated that somatic embryogenesis initiation of grapevine anthers and ovaries was reduced in the absence of glutamine. Supplementing culture media with glutamine at a high concentration (50 or 100 mg l^{-1}) gave the highest number of embryos and shoots in date palm (*Phoenix dactylifera* L.) (El-shiaty et al. 2004). In this study, 100 mg l^{-1} glutamine was added to MS medium; calli of the male florets and pistillate flowers of Persian walnut were mostly nodular and very similar to embryogenic ones.

Effects of different combinations and concentrations of plant-growth-regulators were investigated on somatic embryogenesis induction of Persian walnut. Vahdati (2002) cultured anthers of Persian walnut in some cultivars on DKW medium (Driver and Kuniyuki1984) supplemented with different combinations of 2,4-D, NAA, BA, KIN and TDZ. The calli became friable and non-embryogenic in the media including 2,4-D. He reported that nodular calli similar to embryogenic ones were achieved in the media with combinations of NAA, KIN and TDZ. Rodriguez and Sanchez-tames (1981) used the different concentrations of 2,4-D, IBA, NAA in combination of KIN and BAP for somatic embryogenesis induction of Persian walnut cotyledons and roots segments. Adventitious roots were obtained in the medium including 5 mg l^{-1} NAA and 0.5 mg l^{-1} KIN.

In three experiments, Bayat (2007) cultured the leaves and petioles of some cultivars of Persian walnut. The root formation depended on cultivar and hormonal treatment; so only three cultivars formed the adventitious roots in the media including NAA (0.5, 1 and 4 mg l^{-1}). She reported that nodular calli were formed just in the media containing NAA (0.5, 1 and 2 mg l^{-1}) in combination with KIN (0.1, 0.5, 1, 2 mg l^{-1}). These calli were transferred to maturation medium including 1 mg l^{-1} ABA; but after two months only white and translucent masses appeared on the nodular calli. In this study root formation did not occur from male florets and pistillate flowers of Persian walnut cvs. Chandler and Hartley. Maybe organogenesis depends on cultivar, the type of explant, and concentration and combination of plant-growth-regulators.

In coconut (*Cocus nucifera* L.) (Verdeil et al. 1994; Verdeil and Buffrad-Morel 1995), a gradual reduction in 2,4-D concentrations induced formation of normal somatic embryogenesis. Farsi (2011) cultured the male florets and pistillate flowers of Persian walnut cvs. Chandler and Hartley on NN medium (Nitsch and Nitsch1969) supplemented with different concentrations of NAA and KIN (0.25, 0.5, 1, 2 mg l^{-1}). In the second month, the male florets and pistillate flowers with nodular calli were transferred to media including 0.1 mg l^{-1} NAA and KIN. The volume of calli increased and some of calli became brown. In the third month, the explants were transferred to a hormone-free NN medium. After 3 months, most of the calli became brown and no embryo was formed. Mostly maintaining calli in the auxin free media for a long time caused that calli do not produce somatic

embryos (Chawla2000). The duration of explant exposure to a plant growth regulator is an important factor for induction as well as for the production of normal somatic embryos (Jain et al. 2000). Somatic embryogenesis initiation was reported by continuous presence of plant-growth-regulators in papaya (Fitch and manshardt1990) and pomegranate (Raj Bhansali1990). Therefore in this study, male florets and pistillate flowers of Persian walnut were exposed to the different concentrations of NAA and KIN for 6 months. Callogenesis occurred in various parts of explants at different times. In the fourth week, individual effect of cultivars, hormonal treatments and their interaction effects on the percentage of callogenesis of male florets were significant at 1% level. This means that they had strong effect on the percentage of callogenesis of male florets. Also in the sixth and tenth weeks, individual effect of cultivars and interaction effect of cultivars and hormonal treatments on the rate of callogenesis of male florets were significant at 1% level but the effect of cultivars was significant at 5% level in the sixth week and non-significant in the tenth week. So the effect of hormonal treatments on the rate of callogenesis of male florets appeared to be more important than effect of cultivars.

In addition, of significant interaction effect of cultivars and hormonal treatments at 1% in both sixth and tenth weeks we can state that the effect of hormonal treatments on the rate of callogenesis was influenced by cultivars. Similarly, the rate of callogenesis of pistillate flowers depends on cultivars, hormonal treatments and interaction between them. However, the effect of cultivars on the rate of callogenesis of pistillate flowers was significant at 1% in both sixth and tenth weeks.

The ratio of auxin to cytokinin stimulated embryogenic callus initiation. Also the auxin to cytokinin ratio that stimulates embryogenic callus formation is, however, not universal for all species (Furmanek and Banas2011); for example, birch (*Betulapendula* Roth.) cultures had an ability to form embryos even on media containing higher concentration of auxin (2.4-D) (Kurten et al. 1990). The same auxin and cytokinin ratio stimulated in the greater number of globular somatic embryos in poplar (*Populus* spp.) (Michler and Bauer 1991).Embryogenic mass culture in nordmann fir (*Abiesnordmanniana*) was initiated by cytokinin (BA and KIN) alone and auxin found to be inhibitory (Norgaard and Krogstrup1991). Also in this study, the rate of callogenesis of male florets and pistillate flowers depended on the ratio of auxin to cytokinin and the highest rate of callogenesis from these explants was obtained in the higher ratio of NAA to KIN. The length and volume of the pistillate flowers increased at the higher concentrations of cytokinin to auxin and the tissues of these pistillate flowers turned into the callus-like structures; so the rate of callogenesis was increased in the higher ratio of cytokinin to auxin.

In addition, the concentrations of NAA and KIN and their ratio had an important role in the rate of callogenesis of male florets and pistillate flowers, so that the lowest rate of callogenesis in both types of explants was obtained in the lowest concentrations of these hormones. Also, the rate of callogenesis was influenced by cultivar so that the highest rate of callogenesis in pistillate flowers was achieved in cv. Chandler in sixth and tenth weeks and in male florets was obtained in cv. Hartley in the sixth week, but by continuous exposure of male florets with plant-growth-regulators, the rate of callogenesis of both cultivars was being the same.

An interesting point of callogenesis of male florets and pistillate flowers was the interaction effect of different concentrations of NAA and KIN; for example in the sixth week, the highest rate of callogenesis of male florets was obtained in treatments 4 including 2.5 mg l^{-1} NAA and 1.25 mg l^{-1} KIN and 7 including 2.5 mg l^{-1} NAA and 0.5 mg l^{-1} KIN of cv. Chandler or in the tenth week, the highest rate of callogenesis of pistillate flowers was achieved in treatments 1 including 2 mg l^{-1} NAA and 2 mg l^{-1} KIN and 6 including 1 mg l^{-1} NAA and 1 mg l^{-1} KIN of cv. Hartley. These results revealed that the highest concentrations of plant-growth-regulators do not always cause the highest rate of callogenesis and other factors such as interaction effect between plant-growth-regulators influence the results.

Also investigation of interaction effect between cultivars and hormonal treatments on the rate of callogenesis indicated that the finale results were influenced by them. For instance, in the tenth week, the rate of callogenesis of pistillate flowers of cv. Chandler in treatment 6 (1 mg l^{-1} NAA: 1mg l^{-1} KIN) and pistillate flowers of cv. Hartley in treatment 10 (1 mg l^{-1} NAA: 1 mg l^{-1}) were similar.

The most important part of callogenesis was the quality of calli. In the first months, calli from male florets were mostly compact but by continuous exposure of calli with plant-growth-regulators, most of them especially in the high concentrations of NAA and KIN, became friable and non-embryogenic and cotton-like tissues appeared on their surface. Similarly, the calli of pistillate flowers were compact and similar to emberyogenic ones in the first months but the calli and the involucre and sepal bases of pistillate flowers especially in cv. Hartley became semi-compact after the fourth month. The rate of calli increased in the higher concentrations of plant-growth-regulators but these calli became non-embryogenic in the last months.

Also the nodular calli of pistillate flowers increased in the treatments had low concentrations of plant-growth-regulators. In these treatments, the rate of callogenesis was in the low level in the first months but after fourth month the whole tissues of pistillate flowers turned into the masses of nodular calli which were very similar to embryogenic ones but after 6 month these calli became semi-compact and no embryo was formed from them.

In present study, duration of male florets and pistillate flowers exposure to plant-growth-regulators was not determined and by continuous exposure of explants to the plant-growth-regulators, nodular calli became

friable and non-embryogenic. In mango (*Mangiferaindica* L.), the temporal effect of 2,4-D on induction of somatic embryogenesis from nucellus explants was investigated. Among 0, 7, 14, 21, 28, 35, 42, 49, 56 and 63 days of pulse treatment, a minimum of 7-14 days pulse with 2,4-D was necessary for induction of somatic embryogenesis. Optimum level of induction was achieved with 28 days of pulse treatment while embryogenesis was continued but in the least efficiency with the maximum of 56 days pulse treatment (Lad et al. 1997). Similarly, in the future study, an experiment should be done to determine the best hormonal treatment period for induction of somatic embryogenesis from male florets and pistillate flowers of Persian walnut.

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