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Controlling Secondary Somatic Embryogenesis in Persian Oak (*Quercus brantii* L.) Using Hormonal Compounds and Media

E. Faizy[1], A. Moradi[1*], and A. Masoumi Asl[1]

ABSTRACT

Using immature embryos that undergo somatic embryogenesis, we studied the effects of different hormonal compounds and media on controlling secondary somatic embryogenesis (SSE) in Persian oak (*Quercus brantii* L.). To this end, we focused on the immature embryos that were subjected to several treatments including chilling (at 4 ℃) period and SH, MS, 2,4-D, IBA, BAP, and glutamine concentrations in 5 separate sequential experiments. The results showed that, by extending chilling period to 8-weeks, SSE induction was reduced (68.75%). In different MS concentrations or MS containing PGRs, the lowest globular embryo (66%) and the secondary embryo induction (87.5%) were observed in embryos treated with MS+IBA+BA. Adding 0.75 mg L^{-1} glutamine to MS resulted in a decrease in the secondary somatic embryogenesis (56.25%). Among MS and SH media, 1/2 SH almost entirely controlled this phenomenon (6.25%). The highest maturation progression was obtained in the SH+glutamine treatment, which had the highest conversion to plantlet percentage (100%) and vigor index of plantlets (51.93) compared to the use of SH alone. We found that nutrient and PGR concentration were critical in embryo maturation and conversion percentage and stop the embryo induction cycle that plays a major role in secondary embryogenesis.

Keywords: Conversion percentage, Plant growth regulators, Tissue culture, Macronutrient concentration

INTRODUCTION

Oak genus is an endemic plant in the northern hemisphere and includes deciduous and evergreen species living in cold, semi-tropical, and tropical areas. Oak is distributed over 5 continents including Asia, North Africa, Europe, North, South, and Central America (Aldrich and Cavender-Bares, 2011). Zagros forests, with a 5-million-hectare area and the largest forest ecosystem in Iran, have a significant contribution to the conservation of water and soil in this area. This area had about 10 million hectares of forest in the past, but today has shrunk due to overexploitation. There are four oak species in the Zagros forests, with the Persian oak being the most important one. Reforestation and enrichment of these forests are essential for oak species (Zolfaghari, 2009).

The oak achene is used as bird's diet. Moreover, its tannin compounds are used in skin tanning industry, albumin and alkaloids coagulation, reducing irritation, pain, and oral inflation, and relieving catarrh and bronchitis. Oaks multiplication is done through seed, and their asexual multiplication through cutting and grafting is very difficult. However, the oak seeds are recalcitrant and acorn storage for a long time is not possible considering its sensitivity to storage conditions (Mohan Jainne and Haggman, 2007). These factors threaten the life and regeneration of this forest tree.

[1] Department of Agronomy and Plant Breeding, College of Agriculture, Yasouj University, Yasouj, Islamic Republic of Iran.
[*] Corresponding author; e-mail address : amoradi@yu.ac.ir

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The somatic embryogenesis is a method for plant regeneration that has a high potential for mass production of this useful plant (Das *et al.*, 1993; Vonarnold *et al.*, 1994). Through this process, and under suitable condition, embryogenic cells are induced and produced from a somatic cell. In addition, during the somatic embryogenesis, a series of morphological and biochemical changes occur and a bipolar structure is produced that does not have a vascular junction with maternal tissue (Solis Ramos, 2012). Production of somatic embryos is reported for different species of *Quercus* genus such as cork oak (Bueno *et al*, 2000; Pintos *et al*, 2008, 2009), *Qercus rubra* L. (Vengadesan *et al*, 2009; Martinez *et al*, 2014), *Quercus ilex* L. (Mauri and Manzanera, 2003; Pintos *et al*, 2013; Barra-Jimenez *et al*. 2014), and *Quercus robur* L. (Toribio *et al*, 2004).

Secondary somatic embryogenesis is a phenomenon in which a new somatic embryo is produced from the primary somatic embryo (Raemakers *et al*., 1994; Vasic *et al.*, 2001). Compared to the primary somatic embryogenesis, the secondary one has advantages such as a high multiplication rate, independence of an explant source, and repeatability. Furthermore, embryogenicity can be maintained for prolonged periods by repeated cycles of the secondary embryogenesis (Raemakers *et al.*, 1994). In order to control the secondary somatic embryogenesis in *Quercus ilex*, Mauri and Manzanera (2003) cultured somatic embryos in different media including SH, 1/2 SH, MS, and 1/2 MS at several sucrose concentrations and observed that 1/2 SH was the best media for controlling the somatic embryogenesis. Moreover, they successfully controlled the secondary embryogenesis at high concentration of sucrose. In addition, Fernandez-Guijarro *et al.* (1994) reported stratification for a 30-days controlled secondary embryogenesis.

However, application of biotechnology in plant breeding programs requires efficient in vitro regeneration procedures (Naing *et al.*, 2013). Although the secondary somatic embryogenesis is a desirable method of plant regeneration and has many applications including plant transformation, breeding haploid using multiplication, wild species breeding, and breeding of genetically incompatible species (Raemakers *et al.*, 1994), it is considered a problem due to its role in restricting embryo maturation and synthetic seed. Therefore, controlling the secondary embryogenesis is of great importance. In the present study, we aimed to investigate the effect of the hormone, chilling time, and culture media on the somatic embryogenesis and control of the secondary embryogenesis in the Persian oak.

MATERIALS AND METHODS

To study the somatic embryogenesis of the Persian oak, immature seeds were collected from Boyer Ahmad region, Iran, in August 2015, and transferred to the central laboratory of the Agriculture Department of Yasouj University. Plant material was first washed with distilled water, followed by removing the internal and external shells of seeds. Seeds were then rinsed in ethanol 70% (w/v) for 15 min followed by immersion in sodium hypochlorite 2% with few drops of tween 20 for 5 min.

Murashige and Skoog (MS) and Schenk and Hildebrandt (SH) media as well as 2,4-D (2,4-Dichlorophenoxy acetic acid), IBA (Indol-3-Butyric Acid), BAP (Benzyl amino purine), and glutamine as plant growth regulator, and somatic embryos explant were used in the experiment. To prepare MS medium, 34.43 g MS powder was solved in 1000 cc distilled water, its pH adjusted to 5.7, and then autoclaved at 120 ℃ for 15 min.

For the somatic embryogenesis induction in the Persian oak, immature zygotic embryos were cultured in MS medium containing three 2,4-D concentrations (0.25, 0.5, 0.75 $mg.L^{-1}$). After 4 weeks treatment in this condition, the samples were transferred to new MS with three glutamine concentrations (0.25, 0.5, 0.75

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mg.L^{-1}). Somatic embryos obtained from previous experiments were transferred to free hormone MS for maturation. After one week, the secondary somatic embryogenesis was observed.

To control the secondary somatic embryogenesis, five consecutive tests were performed. At the end of each experiment, the amount of different embryos and the secondary embryogenic percentages were measured.

In order to evaluate the effect of stratification on the secondary somatic embryogenesis, 80 immature somatic embryos in four replications were cultured in MS media and then exposed to 4 ℃ in darkness for 0, 2, 4, 6, and 8 weeks. In the next step of the control of the secondary somatic embryogenesis, 80 immature somatic embryos in four replications were cultured in MS, 1/2 MS, 1/4 MS, MS+2,4-D (0.05 mg.L^{-1}), and MS+BAP (0.1 mg.L^{-1}) + IBA (0.5 mg.L^{-1}). Explants were then cultured and incubated at 25 ℃ in darkness for 4 weeks.

In the third experiment, the effect of glutamine on control of the secondary somatic embryogenesis was evaluated. For this purpose, 48 immature somatic embryos in four replications were cultured in MS containing 0, 0.25, and 0.75 mg.L^{-1} glutamine, where glutamine was added to culture media before autoclave. Explant was then cultured and incubated at 25 ℃ in darkness for 4 weeks. In the next step, 80 immature somatic embryos in four replications were cultured in MS (control), 1/2MS, SH, 1/2 SH, and MS+ glutamine (0.5 mg.L^{-1}), where glutamine was added after autoclave. Explants were cultured and incubated at 25 ℃ in darkness for 4 weeks. In the last test, 64 immature somatic embryos in four replications were cultured in SH (control), 1/2 SH, 1/4 SH, and SH+ glutamine (0.5 mg.L^{-1}) that was added after autoclave. Explants were cultured and incubated at 25 ℃ in darkness for 4 weeks.

Similarly, activated charcoal was used to control phenolic compounds in all experiments. The following parameters were evaluated at the end of all tests: the number of globular, heart, and torpedo embryos and percentage of the secondary embryogenesis.

In addition to these attributes, in the last two tests where the secondary embryogenesis was successfully controlled, the percentage of conversion to plantlet and vigor index of plantlet was also assessed. For this purpose, after incubation at 25 ℃, immature embryos were exposed to 4 ℃ for 6 weeks in darkness, and were then cultured in suitable media for germination. Conversion percentage (germination percentage) and plantlet vigor were calculated according to the following formulas:

GP (%) = n / N $\times$ 100 (1) Jefferson and Penachchio (2003)

$$SVI = [GP \times (SHL + RL)] \div 100$$

(2) Abdul-baki and Anderson (1970)

Where, GP: germination percentage, n: number of germinated seeds, N: number of seeds, SVI: seedling vigor index, SHL: Shoot Length, and RL: Root Length.

Design and Statistical Analysis

All the experiments were organized based on completely randomized design CRD design with 4 replications, and in each replication, 4 samples were used. Analysis of the experimental data was done using SAS 9.2, and the means were compared using Duncan's multiple range test (DMRT) at 0.05 probability level.

RESULTS AND DISCUSSION

Our results showed that 100% secondary somatic embryos were obtained from hormone-free MS. A similar behavior has been reported in other species, e.g., *Hovenia dulcis* Thunb**.** (Yang *et al.*, 2013), *Panax ginseng (*Kim *et al*., 2012), *Q. rubra* (Gingas, 1991), and *Q. suber* (El Maataoui *et al.*, 1990) (Figure1).

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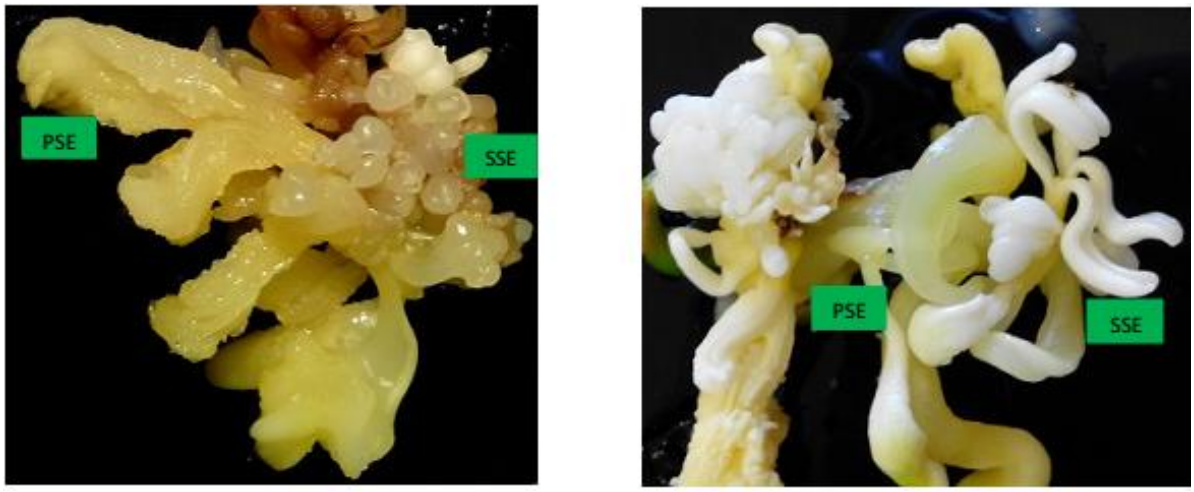

Figure 1. Secondary Somatic Embryogenesis (SSE) and Primary Somatic Embryos (PSE) observed in immature somatic embryos cultured in MS media.

Effect of Stratification Period on SSE

The progression of embryonic developmental stages from globular to heart, and then a globular-like form are prerequisite of embryo maturation. Results showed that the number of globular and heart shape embryos increased with increasing chilling period to two weeks, and then decreased with increasing the time of stratification such that the highest number of torpedo embryos (12.75) was observed in an 8-weeks stratification that had no significant differences with the 6-weeks one. Therefore, the lowest secondary somatic embryogenesis percentage (68.75%) was observed in the 8-week stratification (Table 1). The increase in the number of torpedo-shaped embryos, along with the decrease in the number of globular and heart embryos with increasing stratification period indicates a positive effect of chilling on the control of the secondary embryogenesis phenomena. The positive and significant relationship between embryo maturation and duration of chilling has also been reported elsewhere (Deng and Comu, 1992). In a research on cork oak, Bueno *et al.* (1992) reported that 5 weeks stratification was efficient in controlling the secondary embryogenesis and embryo maturation. Also, Fernandez-Guijarro *et al.* (1994) reported a significant difference between 30 days and non-stratified embryos of cork oak. However, conflicting reports of the negative role of chilling in the control of the secondary embryogenesis are also available (Gingas and Lineberger, 1989). It seems that cold treatment induces expression of genes with GA biosynthesis pathway. The production of endogenous GA increases the rate of maturation so that stratification period is probably one of the factors that decreased the secondary somatic embryogenesis (Bewley *et al.*, 2013).

Effects of MS Media and Hormonal Treatment on SSE

Our results showed that MS concentration alone did not have any significant effect on control of the secondary somatic embryogenesis such that the signs of embryo maturation (torpedo embryo) were not obvious in these treatments (Table 2). However, Adding hormonal compounds, 2,4-D, or IBA + BAP to the medium improved this process. Moreover, the lowest percentage of the secondary somatic embryogenesis was observed in the pro-embryos treated with MS+IBA+BAP and almost no 2,4-D. Continued secondary

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Table 1. Effect of stratification period on globular, heart, torpedo embryos and secondary somatic embryogenesis of Persian oak (*Quercus branti* L.).

Stratification period	Globular embryo	Heart embryo	Torpedo embryo	Secondary embryogenesis (%)
0	85.50[a]	13.50[a]	7.5[b]	100.00[a]
2 week	67.00[a]	12.00[ab]	8.75[b]	100.00[a]
4 week	51.50[b]	9.75[b]	9.75[b]	100.00[a]
6 week	44.50[b]	12.5[a]	12.75[a]	81.25[ab]
8 week	40.50[b]	11.00[ab]	12.5[a]	68.75[b]

Means comparison was done with Duncan multiple range test and in each column data with the same letter are not significantly different at 0.05 probability level.

Table 2. Effect of MS and hormonal treatment on globular, heart, torpedo embryos and secondary somatic embryogenesis of Persian oak (*Quercus branti* L.).

media culture	Globular embryo	Heart embryo	Torpedo embryo	Secondary embryogenesis (%)
MS	73.00[b]	11.75[b]	5.00[b]	100.00[a]
½ MS	67.25[b]	10.25[b]	6.00[b]	100.00[a]
¼ MS	39.00[c]	4.5[c]	1.25[c]	100.00[a]
MS + 2,4-D	83.00[a]	14.5[a]	8.5[a]	100.00[a]
MS+ IBA+BAP	66.00[b]	16.00[a]	9.75[a]	87.5[b]

Means comparison was done with Duncan multiple range test and in each column data's with the same letter are not significantly different at 0.05 probability level.

embryogenesis was also reported on a hormone-free medium in a study on *Brassica napus* (Burbulis *et al*., 2007). A previous study suggests that auxin reduction leads to organogenesis, although 2,4-D and NAA alone or in combination with Kin are essential for continuity of callus induction (Valizadeh and Kazemi Tabar, 2009).

Also, in a study on *Morus alba*, Agarwal *et al.* (2004) used BAP in 4 concentrations (0, 0.02, 0.05, and 0.10 $mg.L^{-1}$) and sucrose in 2 levels (3% and 6%) and then reported the lowest secondary somatic embryogenesis (25.26 %) in BAP 0.02 $mg.L^{-1}$ + sucrose 3%. Also, Yang *et al.* (2013) investigated the effect of five BA concentration (0, 0.1, 0.5, 1.00, and 2.00 $mg.L^{-1}$), and reported that, in 2.00 $mg.L^{-1}$, the secondary somatic embryogenesis was decreased to less than the control treatment. In contrast to the present results, Fernandez-Guijarro *et al*. (1994) reported that adding BAP to media had no positive effect on control of the secondary somatic embryogenesis and germination improvement in cork oak. A similar result was also noticed for *Q. ilex*. Some other comparative results on the induction and maturation of secondary embryogenesis are presented in Table 3.

Effect of Glutamine on Control of SSE

Applying different concentrations of glutamine resulted in a better control of the secondary embryogenesis than two previous tests (Table 4). Glutamine treated concentrations of 0.75 and 0.25 $mg.L^{-1}$ induced the lowest secondary embryo percentage. In addition, no glutamine (zero concentration) treatment showed the highest progress in the secondary embryogenesis, although the number of torpedo embryos was very low compared with globular and heart shape embryos. Addition of organic nitrogen from sources such as glutamine has been reported to have a positive effect on the somatic embryogenesis and maturation

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Table 3. Examples of secondary somatic embryogenesis.

Species	*Explant of Primary somatic embryos*	*Medium*		*Reference*
		Induction	Maturation	
Quercus suber	leaves of epicormic shoots	MS, SH, $N_{30}K$, BTM	½ SH, WPM	Naouar and Lamarti, (2014)
Quercus robur	leaves of forced epicormic shoots	MS+BA+NAA	MS+ sorbitol	Mallon *et al*. (2012)
Aesculus hippocastanum L.	anther	MS+ 2,4-D +kinetin	MS+ active charcoal, ABA, PEG and mannitol	Calic *et al*. (2005)
Cinnamomum camphora L.	immature zygotic embryos	MS+ NAA +malt extract	MS+thidiazuron (TDZ) or ABA	Shi *et al*. (2010)

Table 4. Effect of glutamine concentration on globular, heart, torpedo embryos and secondary somatic embryogenesis in Persian oak (*Quercus branti* L.).

Glutamine concentration	Globular embryo	Heart embryo	Torpedo embryo	Secondary embryogenesis (%)
0	61.00[a]	19.00[c]	9.5[a]	87.5[a]
0.25 mg.l-1it	48.5[ab]	50.00[a]	5.25[b]	68.75[ab]
0.75 mg.l-1it	40.75[b]	24.5[b]	2.25[c]	56.25[b]

Means comparison was done with Duncan multiple range test and in each column data's with same letter are not significantly different at 0.05 probability level.

(Robichaud *et al*., 2004; Zouine and Hadrami, 2007). Similar to what was observed in the present experiment, Rai *et al* (2009) cultured the somatic embryos of guava (*Psidium guajava* L.) in MS supplemented with glutamine (0, 0.34, 0.68 and 1.36 mM). Results showed that 0.68 mM glutamine increased maturation percentage.

Effect of MS and SH medium on control of SSE

The secondary somatic embryogenesis of samples cultured in the SH medium was controlled better than that of MS medium (Figures 2 and 3). The lowest secondary embryogenesis percentage and the highest conversion percentage (Table 5) were observed in 1/2SH that had no significant difference with SH and MS+glutamine (0.25 $mg.L^{-1}$); whereas MS concentrations were not effective and MS and 1/2 MS produced 100% secondary embryogenesis. According to our findings, it is important to note that types of basal medium used was also highly supporting for induction of secondary embryogenesis, which is supported by the results of Naing *et al.* (2013) on *Chrysanthemum*.

In addition, the lowest number of globular, heart, and torpedo embryos were observed in 1/2SH medium that had no significant differences with SH medium (Table 5). It seems that a decline in macronutrients is effective in improving maturation and decreasing the secondary

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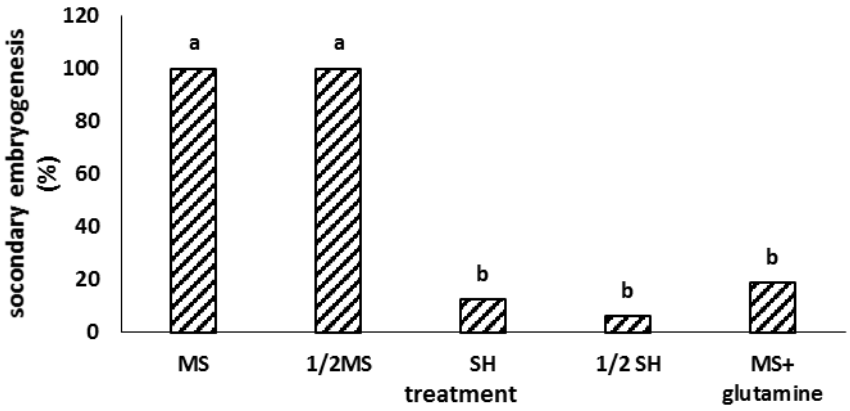

Figure 2. Effect of different medium on secondary embryogenesis percentage in Persian oak (*Quercus branti* L). Means comparison was done with Duncan multiple range test and columns with the same letter are not significantly different at 0.05 probability level.

somatic embryogenesis; in the line with our result in this experiment. Similar to this result, in a research on holm oak, Mauri *et al*. (2003) reported the highest maturation rate and lowest secondary embryogenesis rate in ½ SH.

Measurement of seedling vigor showed that the highest seedling vigor was observed in SH medium that had a significant difference with 1/2 SH, SH, and MS+ glutamine media (Table 5). Mauri and Manzanera (2003) also observed the highest fresh weight in mature embryos in SH medium and reported that nutrient concentration has a significant role in the control of the secondary embryogenesis. One of the most distinct differences between the two basal media is the concentration of $NH4^+$ ion, which is 20.6 mM in MS and 2.6 mM in SH. It seems that the high concentration of $NH4^+$ ion in MS medium inhibits maturation of embryos. This finding is concordant with the results of similar studies in North American ginseng (Zhou and Brown, 2006).

Effect of different concentration of SH medium on control of SSE

Finally, the secondary somatic embryogenesis was completely controlled in the media that contained SH, ½ SH, ¼ SH, and SH+ glutamine. The highest conversion percentage was observed in 1/2 SH and SH+ glutamine (0.5 $mg.L^{-1}$) that had no significant difference with SH medium (Figure 4). With decreasing SH concentration, conversion percentage decreased such that the lowest conversion percentage was observed in 1/4

Table 5. Effect of different medias on globular, heart, torpedo embryos, secondary somatic embryogenesis and seedling vigor of Persian oak (*Quercus branti* L.).

Treatment	Globular embryo	Heart embryo	Torpedo embryo	Conversion percentage	Plantlet Vigor index
MS	84.25^{a}	11.5^{b}	4.5^{b}	0.00^{b}	0.00^{d}
1/2 MS	72.00^{b}	12.25^{b}	5.25^{b}	0.00^{b}	0.00^{d}
SH	83.00^{ab}	14.25^{a}	7.5^{a}	87.5^{a}	32.01^{a}
1/2 SH	8.75^{c}	0.5^{c}	0.75^{c}	93.75^{a}	24.69^{b}
MS+ GLN	15.75^{c}	1.25^{c}	2.00^{c}	81.25^{a}	17.59^{c}

Means comparison was done with Duncan multiple range test and in each column data's with same letter are not significantly different at 0.05 statistical level.

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(a) (b)

Figure 3. Embryos conversion to plantlet in 1/2SH (a) and SH (b) media.

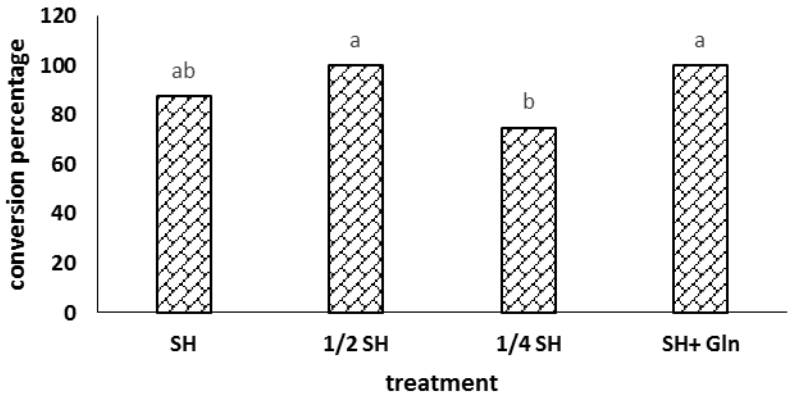

Figure 4. Means Comparison of the effect of different concentration of SH and SH+ glutamine on conversion percentage of Persian oak (*Quercus branti* L). Means comparison was done with Duncan multiple range test and columns with same letter are not significantly different at 0.05 probability level.

SH (Figure 4). In SH media, somatic embryos grew to 40-60 mm in length at maturity in 6 weeks (Figure 3b), providing a prerequisite base for strong plants. The highest plantlet vigour (51.93) was achieved in SH +Gln (0.5 $mg.L^{-1}$) medium (Figure 5). The nutrient concentration is critical in embryo conversion percentage. A significantly lower seedling vigor was observed when macronutrient concentration was reduced to a 1/4 SH. In this case, a significant interaction between macronutrient concentration and medium phase was recorded.

CONCLUSION

Culture temperature has shown some effects on the secondary embryo control and maturation of Oak embryo. Our results showed that free hormone MS is suitable for embryo proliferation (the secondary somatic embryogenesis). Moreover, we found that an 8-weeks stratification stimulated maturation and hampered the secondary somatic embryogenesis. Adding IBA+BAP or glutamine to MS was also effective on the

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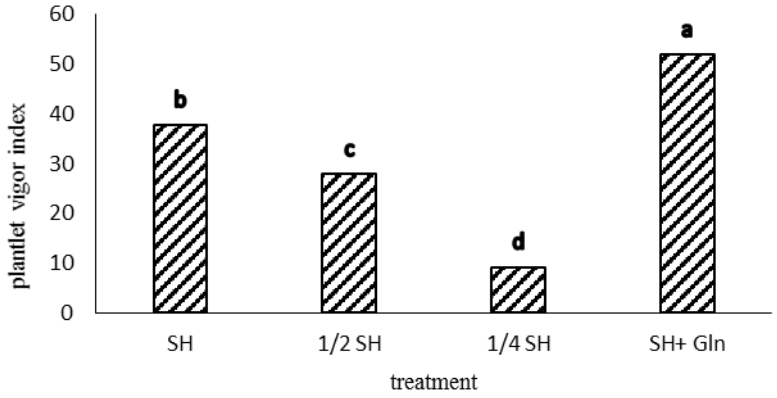

Figure 5. Means Comparison effect of different concentration of SH and SH+ glutamine media on plantlet vigor index in Persian oak (*Quercus branti* L). Means comparison was done with Duncan multiple range test and columns with same letter are not significantly different at 0.05 probability level.

control of the secondary embryogenesis. Finally, the reduction of macronutrient concentration to half-strength for SH was found as the most efficient treatment in both improving the maturation rate of somatic embryos and reducing the frequency of the secondary embryogenesis.

In this work, we reported the optimal conditions necessary for the control of the secondary somatic embryogenesis and plantlet conversion in the Persian oak. The protocol established in this study will be helpful for the conservation and large-scale vegetative propagation of the Persian oak.

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کنترل جنین زایی ثانویه در بلوط ایرانی (*Quercus branti* L.) تحت تأثیر ترکیبات هورمونی و محیط کشت

ا. فیضی، ع. مرادی، ا. معصومی اصل

چکیده

به منظور بررسی تأثیر محیط کشت و ترکیب هورمونی بر کنترل جنین‌زایی ثانویه در بلوط ایرانی (*Quercus branti* L.) از جنین‌های زیگوتی نابالغ استفاده شد. بدین منظور، جنین‌های نابالغ در ۵ آزمایش جداگانه متوالی در معرض تیمارهای مختلف شامل دوره های مختلف سرمادهی (۴ درجه سانتی‌گراد)، محیط های کشت SH، MS، غلظت‌های مختلف هورمون‌های 2,4-D، BAP، IBA و ویتامین گلوتامین قرار گرفتند. نتایج نشان داد که با افزایش طول دوره سرمادهی به ۸ هفته، القاء جنین زایی ثانویه کاهش یافت (۶۸/۷۵ درصد). در میان غلظت‌های مختلف MS یا MS حاوی تنظیم کننده

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رشد گیاهی، کمترین میزان جنین کروی (۶۶ درصد) و القاء جنین‌زایی ثانویه (۸۷/۵ درصد) در تیمار MS+IBA+BA مشاهده شد. افزودن ۰/۷۵ میلی گرم در لیتر گلوتامین به محیط MS منجر به کاهش جنین‌زایی ثانویه شد (۵۶/۲۵ درصد). در میان محیط‌های کشت MS و SH، تیمار SH ½ به طور کامل (۶/۲۵ درصد) این پدیده را کنترل کرد. بیشترین میزان بلوغ جنین و کنترل جنین زایی ثانویه در تیمار SH+گلوتامین به‌دست آمد که بالاترین تبدیل گیاهچه (۱۰۰ درصد) و شاخص بنیه گیاهچه (۵۱/۹۳) را در مقایسه با کاربرد SH به تنهایی داشت. نتایج کلی نشان‌دهنده این موضوع است که غلظت مواد غذایی و تنظم‌کننده رشد در بلوغ جنین و تبدیل گیاهچه دارای اهمیت زیادی هستند و جنین‌زایی ثانویه را از طریق کنترل چرخه جنین‌زایی کنترل می‌کنند.

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