Effect of various concentrations of different growth regulating hormones on callus weight and the amount of thymol of *Thymus daenensis*

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Abstract

Thymus daenensis is a perennial plant belonging to *Lamiaceae* family that is a species endemic to Iran. Due to the indiscriminate use of this plant which possesses medicinal values, *T. daenensis* tissue culture is suggested to increase the quality and quantity of its effective compounds. Callus cultures using leaf and stem explants were subjected to different hormonal treatments. Ten-millimeter explants of leaves and stems were cut up from sterile seedlings. MS medium was used with different concentrations of naphthalene acetic acid, benzyl amino purine, kinetin and 2,4-D (mg per liter) in which callus formation was observed. The highest level of thymol (28/1335 micrograms per gram) was recorded in calluses derived from leaf explants whereas the lowest level was observed in calluses obtained from stem explants. Also the highest levels of thymol, i.e. 65/1313 and 30/1322 micrograms, were observed in treatments of 0.4 NAA+3 BAP and 0.8 NAA+6 BAP mg per liter, respectively. Moreover, the interaction of effects of different treatments of calluses and different concentrations of hormones showed that the highest level of thymol (38/1345 micrograms per gram) was obtained from the treatment of NAA+1.5 BAP 0.2 mg per liter in the calluses derived from leaf explants. In addition, the maximum callus weight (22.1 grams) was related to the treatment of 0.8 NAA+6 BAP milligrams per liter. Results of the callus culture of *T. daenensis* to enhance the quality and quantity of its active ingredients showed that different explants and hormones with different concentrations and as well as their interactions had a significant effect on the level of thymol and callus weight ($p \leq 0.01$).

Key words: *Thymus daenensis*; benzyl amino purine; thymol; kinetin

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Introduction

Thymus daenensis is a perennial plant belonging to *Lamiaceae* family (Haji Akhondi et al., 2011). *Thymus* includes 215 species of herbaceous and small shrubs in the world, of which 14 species are distributed in Iran (Daneshvar et al., 2009; Ghasemi, Pirbalouti, Karimi, Yousefi, Golparvar, 2011). *Thymus daenensis* in Iran is an endemic species that grows mainly in the highlands of the Zagros

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Mountains (Rahimmalek et al., 2009; Nickavar and Esbati, 2012). Infusion of the aerial parts of thymus species has long been used in traditional medicine as tonic, carminative, antispasmodic, anti-inflammatory, expectorant agent and also for colds (Ghasemi, Pirbalouti, Rahimmalek, Malekpoor, Karimi, 2011; Ghasemi, Zarei, Torki, 2010; Mozaffarian, 2006; Ourmazd and Chalabian, 2010). Plant tissue culture in the laboratory is an important tool to achieve impossible goals that do not exist outside of the laboratory (Sayed Tabatabaei and Omidi, 2008; Soltani Pol et al., 2010). Callus culture is one of the techniques of tissue culture in which a differentiate tissue is removed to produce a mass of undifferentiated cells called the callus *in vitro* (Rahimmalek and Goli, 2013; Shabnum and Wagay, 2011).

Shabnum and Wagay (2011) in their study on the callus induction of *lemon balm* medicinal plant (*Melissa officinalis* L.) used various hormonal treatments of 2,4-D and BAP. Their results showed that the best hormonal treatment to produce the highest quality callus with considerable volume and weight of the hatch rate was1mg per liter of 2,4-D plus 1mg per liter BAP. Optimization of callus culture of *Zataria multiflora* Boiss was conducted by Francoise et al. (2007) to increase Rosmarinic acid production (RA) as secondary metabolites by changing the culture conditions. Callus induction from culturing of stem node of thyme on MS medium was performed with the hormonal combination of 2,4-D 0/5 mg/1 and kinetin 1mg/l (Francoise et al., 2007). The study of tissue culture and Organogenesis was carried out in medicinal plant of *Salvia nemorosa* on MS medium with different levels of the hormone (Ourmazd and Chalabian, 2010).

Given the importance of thyme as a medicinal plant with aromatic properties of this genus and the fact that natural resources of thyme to meet human needs are not enough (Smith, 2006), the implementation of techniques of tissue culture for the protection and reproduction of local species of *Thymus* with high quality is an important area for research. In this study, the effects of different hormonal treatments of callus formation have been investigated on callus weight and the amount of thymol obtained from calluses.

Materials and Methods

The study consisted of two phases: The first step was the formation of sterile seedling and the second step involved callus formation of sterile seedling explants. Sterilized seeds were cultured in MS medium without hormones. After 4 weeks, the produced seedlings were used as a sterile herbal source for continuing experiments. In the second phase, prepared explants from all parts of sterile plants (leaves, roots, stems) were used for callus induction with different hormonal treatments (Table 1). The explants were subcultured with an interval of 15 days.

The data collected in the study were submitted to ANOVA using SPSS to investigate the effects of different organs and hormones on callus weight and also interaction of effects of organs and hormone concentrations on thymol content.

Table 1
MS medium with different concentrations of NAA, BAP, 2,4-D, Kin

Kin Hormone Milligrams per liter	2,4-D Hormone Milligrams per liter	Culture medium
0	0	MS
0/5	2	MS
1	4	MS
0.25	1	MS
0.25	1	MS
BAP	NAA	
0	0	MS
3	0.4	MS
6	0.8	MS
1.5	0.2	MS

Results

The effect of organ and hormones on callus weight

Initially, root, stem and leaf explants were used for callus induction. That callus formation was observed in different concentrations of NAA, BAP, 2,4-D, Kinetin of leaves and stems. But root explants rarely and partially formed callus. On the other hand, they

gradually turned black and died after vaccination. Stem and leaf explants on MS medium without hormone (controls) did not form any callus. Analysis of variance shows that the impact of different explants, hormones with different concentrations, and their interaction effects on callus weight were significant at p≤0.01 (Table 2).

Comparison of the means revealed that the maximum (862.0 g) and minimum weights of callus were related to leaf and stem explants. Furthermore, the highest callus weight (22.1 g) was related to the treatment of 0.8 NAA+6 BAP milligrams per liter and the lowest was related to the control treatment. Also, based on the results of interaction of various organs and hormone treatments with different hormone concentrations, the highest callus weight (87.1 g) was related to the treatment of 0.2 NAA+1.5 BAP mg and leaf explants.

Interaction organs and hormone concentrations on thymol content

The impact of different explants, hormones with different concentrations, and their interaction effects on the amount of thymol (p≤0.01) was significant (Table 3).

According to the results of comparison of the mean, the maximum amount of thymol (28.1335 micrograms per gram) was related to leaf explants and the lowest was related to stem explants. Also, the maximum amount of thymol (65.1313 and 30.1322 mg/g) was related to 0.4 NAA+3 BAP and 0.8 NAA+6 BAP mg per liter respectively. Based on the results of interaction of various organs and hormone treatments with different concentrations, the highest amount of thymol (38.1345 micrograms per gram) was related to the treatment of 1.5 NAA+0.2 BAP mg per liter at calluses obtained from leaf explants**.**

Discussion

The results of callus induction from explants showed that after several cultivation of the explants in MS medium with different concentrations of NAA, BAP, 2,4-D, and Kinetin callus formed. The stem and leaf explants on MS medium without hormone (controls) did not form any callus. This is consistent with a series of studies reported by various researchers (Asvad et al., 2011; Amini et al., 2013; Chaturvedi et al., 2007; Iranbakhsh et al., 2007; Iranbakhsh and Ebadi, 2011; Nickavar and Esbati, 2012; Zarinpanje et al., 2011). Soltanpoor et al. (2011) observed callus induction in *Melissa officinalis L* in an experiment using various hormonal treatments of 2,4-D and BAP.

Based on the results of interaction of different organs of *Thymus* and different concentrations of hormones, the maximum weight of callus was related to the treatment of 0.2 NAA+1.5 BAP mg per liter and leaf organ. This is consistent with the results of callus induction of *Melissa officinalis* L leaves treated with 2,4-D and BAP (Shabnum and Wagay, 2011). Ormazdi and Chalabi (2011) in their study on tissue culture of *Salvia nemorosa* and micropropagation on MS medium with different concentrations of hormones found that leaf explants and apical meristem formed call uses on MS medium containing of NAA plus BAP and some calluses

Table 2
Analysis of variance2: different treatments of organs and hormones with different concentration on callus weight

Callus Weight (gr)	Degrees of Freedom	Sources of Changes
**44/4	1	organ
**1/65	6	treatment
*0/89	6	organ * treatment
0/411	39	mistake
11/45		Coefficient of variation

** Significant difference at level of 1% , statistically significant difference in the level of 5%,n.s no significant difference

Table 3
Analysis of variance of different treatments of calluses obtained from stem and leaf explants and hormones with different concentrations of thymol

Thymol (m gr)	Degrees of freedom	Sources of changes
**65.14	1	Organ
**36038.98	6	Treatment
**323.60	6	organ * treatment
1.53	39	mistake
1.20		Coefficient of variation

** significant difference at 1% level,* significant difference in the level of 5%,n.s no significant difference

started organogenesis in the medium containing of BA plus Kinetin.

The results of the callusing of *Thymus daenensis* to enhance the quality and quantity of thymol showed that the impact of different explants, hormones with different concentrations, and their interaction effects were significant on the thymol content. In fact, the highest amount of thymol (38.1345 micrograms per gram) was related to the treatment of 1.5 NAA+0.2 BAP mg per liter at calluses obtained from leaf explants**.** Hassan poor et al. (2007) in their studies for optimization of the callus medium of *Zataria multiflora* Boiss tried to increase Rosmarinic acid production (RA) as secondary metabolites by changing the culture conditions and using various hormonal treatments (2,4-D, Kinetin and NAA) on leaf explants. Optimization of tissue culture of *Silybum marianum* was evaluated to generate medicinal silymarin using various hormonal treatments (2,4-D, Kinetin and NAA) on leaf and petiole explants (Arekhi et al., 2012). This is in line with the findings of the present study, i.e., increasing the quality and quantity of active ingredients through callusing in the *Thymus daenensis*.

The findings of our study are consistent with those of another study by Pirian and Piri (2014). Who investigated the effects of applying different concentrations of BA and 2,4-D hormones on the roots of *Portulaca oleracea* for production of callus to enhance secondary metabolites. Pirian and Piri (2014) found that BA in combination with 2,4-D was the best treatment for callus and accumulation of secondary metabolites in callus was higher than that in seedlings *in vitro*.

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