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An investigation of tissue culture and co-cultures of different explants in *Calendula officinalis*

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Abstract

Two experiments were conducted separately to investigate the effects of the medium and explant types on the callus induction and regeneration of *Calendula officinalisas* a medicinal plant. In the first experiment, explants were placed on a ¼ MS medium on three levels of NAA (1-Naphthaleneacetic acid) and KIN (Kinetin) in sterile conditions. The parameters, time to callus initiation, callus volume, and the percentage of callus induction were measured to evaluate the callus condition in this experiment. Variance analysis of the results on the first experiment showed that there was no considerable difference in terms of callus induction in mediums with a combination of hormones, while the effect of explant source on these traits was significant. In the second experiment (co-culturing of different explants), main effects of cotyledon, hypocotyl and meristem explants as single cultures, and also effects of cotyledon and meristem, hypocotyl and meristem explants as co-cultures in the two mediums, KIN + NAA and TDZ (Thidiazuron) + IBA (Indole-3-butyric acid), were studied. The results of the second experiment showed that co-cultured explants have a better regeneration. It also indicated a better condition for traits related to the root of the co-culture system. Moreover, the impact of the two explants, hypocotyland meristem, together onrootingandregenerationwas remarkable.

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Introduction

Calendula officinalis belongs to the Asteraceae (Compositae) family, which is an annual herbaceous plant and has branched stiff, stems (Bernath, 2000). This plant is cultivated for the production of pharmace uticalsand active in gredientsin flowers, and particularly in petals (Martin, 2005). Duke (1985) stated that the flowers of *Calendula officinalis* contain carotenoids, saponins, cholesterol, sterols and lauric acid. Martin (2005) refers to antibacterial and antioxidant properties of its essential oils. Oil obtained from *Calendula officinalis* helps to heal rough, in flamed and itchy skin and it is also helpful to improve eczema and itching. Currently one of the main uses of this plant is in the treatment of inflammatory and skin diseases (Paplo *et al*., 2002).

Physiological conditions of the plant can be altered by co-culturing of various explants of a plant or calluses of different plant species (Martin, 2005). Production of embryo genic, organ genic or nonorganogenic calluses and cultures from different tissues of a plant or callus cultures of the same genotype indicates heterogeneity in internal physiological condition in constituent cells of each of them (Paploe *et al*., 2002). Identification of these factor scan lead to causing epigenetic changes in callus cultures. Tissue culture is the manipulation of plant growth in carefully controlled conditions, and auxin and cytokinin are of great importance in these manipulations (Anna and Wirginig, 2002). Use of this method for production of the same amount or even more than the whole plant of pharmaceutical ingredients has been progressed and applied dramatically in recent years (Ramachandra and Rauishankar, 2002). In another study on *Calendula officinalis* by Coco *et al*. (2004), it was reported that hypocotyl and cotyledon had the most adventitious shoot regeneration on MS medium containing TDZ and IBA. They also stated that TDZ is effective on the improvements in morphogenesis and organogenesis of cultured explants. (Tripathi, 2003). Grezelak and Wirnia (2002) studied on callus induction and the formation of *Calendula officinalis* cell and stated that growth regulators such as 2,4-D and KIN and IAA produce the highest amount of callus.

Materials and methods

Plant materials: *Calendula officinalis* seed was prepared from local populations of this plant in Hamedan where it is reproduced and presented commercially in botanic garden Hamedan Bu-Ali Sina University. First the seed surface was disinfected using 96% ethanol for 30 seconds and then rinsed with distilled water and then put in a sodium hypochlorite solution (NaCl with 1% active chlorine). After that a few drops of liquid soap (mixture containing anionic surfactants) were added increase the adsorption. Then they were dried on several layers of filter paper after three times washing in sterile distilled water (Bilia *et al*., 2002).

To prepare the explant, germination medium containing ¼ MS, 15 g/L sucrose and 8 g/L agar without growth regulators at PH = 5.8 was corrected by diluted sodium. Then it was sterilized at 125°C for 30 min under pressure using an autoclave and was distributed in 9 cm Petri-dishwith about 15 ml per petri dish. Seed was transferred to germination environment under complete sterile conditions and petri dishes were placed in a growth chamber at 25 ± 2°C. When the seedlings produced two cotyledons, it was time to prepare the explants. Cutting the sterile grown seedlings each with two cotyledon leaves, rootlet, hypocotyl, meristem, and cotyledon explants were prepared and they were cultured in Petri dishes on MS medium with NAA on three levels (0.5, 1, 2 mg l) and KIN on three levels (2, 4, 6, ppm). After placing the explants in each petri dish, they were completely sealed with Para film and then they were transferred to the growth chamber in 16 h of light and 8 h of darkness at 25 ± 2°C. On callus induction phase, after keeping the cultures in the mentioned conditions for four weeks, time of callus induction (time to initiate callus induction), the mean of callus volume, percentage of callus induction was evaluated to analyze callus induction. Callosum volume assessment was performed using Hookeroni ordinal scale. In Hooker's method, callus volume is

determined based on the size of 23 different circlesin which the smallest circle with a diameter of 1 mm indicates the minimum volume and the largest circle with adiameter of 23 mm represents the maximum volume of callus (Bilia *et al.*, 2002).

Explant co-culturing: In the co-culture of various explants, main effects of leaf, cotyledon, hypocotyl and node cotyledon explants as single cultures, cotyledon and hypocotyls, cotyledon and meristem, hypocotyl and meristem, and cotyledon, hypocotyl and meristemas co-cultures were modified on MS medium with half strength nitrates, 2 mg/ l Kin, 1 mg / l NAA, 0.75 mg/l TDZ, 0.5 mg/l IBA, 30 gr sucrose and 8 gr agar (Pout and Samantaras, 2000). After 4 weeks, attributes such as callus volume, percentage of callus induction, time to callus initiation, percentage of shoot regeneration, number of shoots, number of leaflets, percentage of root regeneration, number of roots, root length, time to root regeneration, and percentage of regeneration till branching were measured. The experiment was conducted in a factorial randomized complete block design and the statistical analysis of data was done using SAS software making the necessary conversions.

Results and discussion

According to Table 1, it was observed that the effect of explant source on time to callus induction (days), percentage of callus induction and callus size on the surface was significant at the 5% level. It indicates that various explants differ significantly in the rate of callus induction and growth amount and it could show different genetic control of these explants. On the other hand, the effect of medium on callus induction and growth in the explants was not significant in any of studied attributes. This could indicate that the used hormonal compounds do not make considerable changes in the mentioned attributes. Therefore it is necessary to change the ratio or types of hormonal compounds depending on our purpose in further experiments.

Table 1. Analysis of variance for evaluated characteristics in callus induction experiment.

		Means square		
Source	DF	Time to callus initiation	Percentage of callus	Callus volume
Explant	3	67.38*	2139.83*	43.07*
Culture media	2	45.36 ns	637.52 ns	0.14 ns
Explant × Culture media	6	45.36 ns	1477.49*	16.61 ns
Error	36	21.87	582.12	9.36

*, ** and ns significant at p ≤ 0.05, p≤ 0.01 and non-significant, respectively.

Table 2. Mean comparison of explant and media effects on evaluated characteristics in callus induction experiment.

Source		Time to callus initiation	Callus volume	Percentage of callus
	Root	13.75[a]	8.45[b]	63.13[b]
Explant	Cotyledon	8.57[b]	12.22[a]	70.43[b]
	Meristem	8.57[b]	12.81[a]	100[a]
	Hypocotyls	8.5[b]	10.10[b]	82.5[ab]
	0.5 NAA + 2 KIN	7.9[b]	10.86[a]	85.5[a]
Culture media	1 NAA + 4 KIN	9.75[ab]	10.9[a]	73.58[a]
	2 NAA + 6 KIN	12.75[a]	10.52[a]	77.5[a]

To analyze the time to callus initiation, different explants according to Table 2, the obtained means show that the root explant has started the callus induction later than the other explants. In analyzing the table of means comparison, it was specified that cotyledon and meristem explants differed significantly with other explants with the highest scores in callus volume. It can also be noted about the

percentage of callus induction that callus induction of meristem explant with the highest percentage of callus was significantly different from other explants. For hormonal compounds, explants had the highest callus induction period in (2 NNA + 6 KIN) medium. It is also specified that the different mediums used for percentage of callus induction and callus volume do not make a statistically significant difference (Table 2).

Table 3. Analysis of variance for evaluated traits in explant co-culturing experiment.

Source of variation	DF	Percentage of callus	Callus volume	Time to callus initiation	Percentage of shoot regeneration	number of shoot	Number of leaf	Percentage of regeneration	Time to shoot regeneration	Time to root regeneration	Percentage of root regeneration	Number of root	Length of root
Explant	6	1224.75*	43.68*	31.9^{ns}	7385.19*	2.75*	40.43*	1753.9*	56.69^{ns}	58.63^{ns}	693.73^{ns}	141.3^{ns}	1.2^{ns}
Culture media	1	5425.98*	438.55*	78.66*	82.71^{ns}	0.36^{ns}	19*	2689.8*	3.12^{ns}	383.39*	11911.3*	996.61*	27.2*
Explant × Culture media	6	441.9^{ns}	5.48^{ns}	11.65^{ns}	86.76^{ns}	0.08^{ns}	5.03^{ns}	466*	27.11^{ns}	51.86^{ns}	305.7^{ns}	58.49^{ns}	3.8^{ns}
Error	28	456.09	5.96	15.74	98.85	0.43	3.26	176.14	34.90	36.09	564.22	200.05	3.42

*, ** and ns significant at p ≤ 0.05, p≤ 0.01 and non-significant, respectively .

According to the table of variance analysis (Table 3), it can be observed that percentage of callus induction, callus volume, percentage of shoot regeneration, number of shoots, number of leaves and percentage of regeneration among the main effects of explants, and also percentage of callus induction, callus volume, time to callus initiation, percentage of shoot regeneration, number of roots, and root length about the source of the main effect of medium, there is a significant differenceat 5% level. However, the medium × explant interaction for all traits except percentage of callus induction was non-significant at the 5% level.

Table 4. Mean comparison of experimental factors on callus induction and regeneration of culture.

xperimental factors		Percentage of callus	Callus volume	Time to callus initiation	Percentage of shoot regeneration	number of shoot	Number of leaf	Percentage of regeneration	Time to shoot regeneration	Time to root regeneration	Percentage of root regeneration	Number of root	Length of root
	C (Cotyledon)	83.33^{bc}	13.7^{a}	10^{a}	3.33^{d}	0.33^{d}	0.66^{d}	0^{d}	3^{b}	4.66^{b}	33.33^{bc}	5.5^{b}	1.83^{ab}
	I (Hypocotyled n)	50^{d}	5.29^{f}	5.16^{b}	0^{d}	0^{e}	0^{e}	0^{d}	0^{c}	5.16^{b}	16.67^{d}	1.33^{b}	1.5^{b}
	M (Meristem)	81.67^{bc}	6.91^{e}	9.66^{a}	96.66^{a}	1^{c}	4^{c}	40^{a}	8^{a}	10^{a}	40^{b}	5.66^{b}	1.5^{b}
xplant	C+H	93.5^{a}	9.58^{d}	6^{b}	2^{d}	0.16^{ed}	0.33^{ed}	2^{d}	3.33^{b}	11^{a}	28.83^{c}	6.66^{b}	2.25^{ab}
	C+M	89.5^{ab}	11.0^{c}	6^{b}	47.5^{b}	1.5^{b}	5.5^{b}	25^{b}	4.87^{b}	9.62^{a}	29.75^{c}	15.1^{a}	2.5^{a}
	H+ M	10^{c}	12.4^{b}	6.2^{b}	46.6^{b}	1^{c}	4^{c}	38.6^{a}	8.8^{a}	11.6^{a}	50.6^{a}	6^{b}	2.1^{ab}
	C+H+M	81^{bc}	12.84^{ab}	5.2^{b}	40^{c}	1.8^{a}	6.8^{a}	11.4^{c}	3.4^{b}	5.6^{b}	24.8^{cd}	12.8^{a}	1.8^{ab}
ulture edia	1NAA+2KIN	90.21^{a}	13.4^{a}	5.74^{b}	34.26^{a}	0.91^{a}	3.56^{a}	24.08^{a}	4.78^{a}	10.6^{a}	47.65^{a}	12.3^{a}	2.63^{a}
	0.5IBA+0.75TDZ	67.73^{b}	6.24^{b}	8.26^{a}	33.52^{a}	0.73^{b}	2.42^{b}	7.78^{b}	4^{a}	5.47^{b}	12.21^{b}	2.47^{b}	1.13^{b}

Analyzing the comparison of the mean values (Table 4); it was observed that the highest mean was related to percentage of callus induction between cotyledon and hypocotyl explants in the co-cultures. While for callus volume and time to callus induction, single cultures had the highest mean value. Single-culturing also resulted in explant increasing for the percentageof shoot regeneration, percentage of regeneration, time to shoot regeneration, and time to root regeneration. On the other hands, for other attributes, co-culturing of mentioned explants led to explant increasing in the mentioned attribute. Overall, results of the second experiment showed that single-culturing of the explant is better than co-

culturingin the callus induction stage, but the co-cultured explants showed better regeneration. Also for the traits associated with root, systems of co-culturing of explants showed a better condition than single-culturing of explants. Impact of meristem and hypocotyl explants together on rooting and regeneration was remarkable. The results of the second experiment indicated that the regeneration increase in the single-culturing is related to a specific matter in the young explants. Identification of these factors can lead to a change in the improvement of callus culture condition. Moreover, resultsof this study can be used in allelopathic researches and alchemical, protoplast culture, selection of mutant cells, and induction or control of somaclonal variations (rapid growth).

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