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Full Length Research Paper

Optimization of micropropagation and establishment of cell suspension culture in *Melissa officinalis* L.

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***Melissa officinalis* L., due to its useful application in medicine, is being paid more attention. In order to establish a stable regeneration system with 4 landraces collected from different climate in Iran, major parameters such as regeneration rate, rooting percentage, shooting induction, proliferation rate, fresh and dry weight as a biomass of cells were investigated. Statistical analysis of results showed that BAP in combination with NAA had the highest regeneration in shoot tips explants. NAA in combination with IAA and kinetin had the best response to callus induction. Also 1 mgl/l NAA had a higher response to rooting than other auxins used. 2,4-D at 1.0 mg/l and BAP at 0.5 mg/l showed the highest production of fresh and dry weight, 5.48 and 0.407 g, respectively, that is approximately 20 times the initial weight of callus. 2,4-D (1 mg/l) and BAP (0.5 mg/l) had the highest cells number.**

Key words: *Melissa officinalis*, regeneration, micropropagation, cell suspension, plant cell biomass.

INTRODUCTION

Lemon balm (*Melissa officinalis* L.) is a medicinal herb, native to Northern Mediterranean regions (Tavares et al., 1996). Maximum essential oils is located in top third of plant (0.39%) (Gbolade and Lockwood, 1992). Major components are citral (neral+geranial) representing 48% of the essential oil, followed by citronellal with 39.47% and β-caryohyllene with 2.37% (Tavares et al., 1996). Essential oils from lemon balm are used as an anti-tumeral agent and a potential remedy or prevention for cancer (Turhan, 2006). The volatile oils from lemon balm may also be used as an anti-virus agent and contains anti-herpes simplex virus type 2 (HSV-2) substances (Turhan, 2006). Rapid clonal propagation of *M. officinalis* for obtaining essential oils is necessary; therefore, traditional methods are not efficient, because the obtained population is not homozygote and only the upper part of plant body has much effective compounds. So, micropropagation can be an effective alternative for mass production of selected genotypes. In order to obtain a homozygote population along with the supply cell suspension culture, micro-propagation is required. However *M. officinalis* grow easily, but its population is not homozygote, more over upper parts of plant body contain essential oils, and so its cultivation in farms (traditional method) is not economical. Tavares et al. (1996) also reported micro-propagation in *M. officinalis* using cotyledonary nodes as explants. Gbolade and Lockwood (1992) also used shoot tips excised from field grown plants for micro-propagation. As micro-propagation is affected by genotype, there are not many reports on micro-propagation with various land-races from different climate origins, as well as the effect of culture media types. Little is known about different explants derived from various seedling ages and there have not been reports associated with cell biomass and cell growth. Also little information is known about *in vitro* production of secondary metabolite. Suspension cell culture is a prerequisite for extraction of these worthwhile meta-bolites. For the establishment of cell suspensions, the characteristics of the inoculums (density, age and growth phase) are also important for growing improvements

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Abbreviations: PGRs, Plant growth regulators; **BAP,** 6-benzylaminopurine; **IAA,** indole-3- acetic acid; **IBA,** indole-3-butyric acid; **KIN,** kinetin; **NAA,** α- naphthalene acetic acid; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **SFC,** shoot forming capacity; **TDZ,** thidiazuron; **HSV-2,** herpes simplex virus type 2; **MSN,** means shoot number.

(Smolenskaya et al., 2007). In the process of establishing suspension cell culture of *M. officinalis*, a suitable explants and culture media were selected for callus induction from the selected explants. Hence, the aim of this study was to investigate the effects of plant growth regulators, media and physiological age of seedling on micro-propagation of these landraces in order to obtain efficient shoot regeneration and rooting. It is also an important purpose of this study to establish the condition necessary to get appropriate survival rate of those plantlets in acclimatization stage. We also finally tried to cell suspend culture of locally grown *M. officinalis* with those established conditions to produce useful essential oil.

MATERIAL AND METHODS

Seed sterilization procedure

Viable seeds of *M. officinalis* obtained from botanical garden of Uromia, Hamedan, Ghazvin and Rasht, (Provinces in Iran) were washed with 70% ethanol for 1 min, subsequently sterilized with 2.5% sodium hypochlorite for 10 min, and then rinsed 4 times (10 min) in sterile distilled water.

Media preparation and direct regeneration

Seeds were placed on ½ strength MS (Murashige and Skoog, 1962) medium supplemented with 3% sucrose and 0.7% agar to germinate. The first experiment was designed to evaluate the performance of two cytokinins, that is, BAP and KIN for shoot proliferation and shoot tips selected as explant. They were placed on MS and B5 media supplemented with 0.7% agar and various concentrations of cytokinins including BAP at 1.0, 1.5, 2.0 and 3.0 mg/l and Kinetin at 0.5, 1.5, 2.0 and 3.0 mg/l. The multiplication potential of shoot tips was evaluated in the primary culture and two successive subcultures at 3–weekly intervals. Percentage of explants producing shoots multiplication rate (that is, mean number of shoots per explants at the end of the culture period) and average length of shoot after two successive subcultures (3 weeks each) were investigated. The cultivation was continued on MS and B5 media supplemented with 3 mgl/l BAP and 1.5 mg/l NAA for 5 months. In other experiments, Hypocotyls were used as explants. These were excised from 10, 15, 20 and 25 day-old seedlings from different landraces. All explants cultured on MS and B5 media to evaluate the effect of cytokinins/auxin combined with callus induction, growth and multiplication rate, frequency of shoot forming (that is, the percentage of explants forming adventitious shoots), the shoot forming capacity (SFC) index, (defined as the number of shoot per explants) × (% explants forming shoot)/100 (Martinez-pulido et al., 1992) and additional treatment without growth regulators were introduced as control. The explants were inoculated in darkness at 25 ± 2°C for 20 days. Each treatment contains 4 replicates with 20 explants per replication (plates). Obtained callus were inoculated in MS and B5 media supplemented with various concentrations of cytokinin BAP at 0, 0.5, 1 and 1.5 mg /l, kinetin at 0, 0.5, 1, 1.5 and 2 mg /l to induce shoots.

Indirect shoot induction

After determination of the best treatment for callus induction, they were subsequently placed on Kinetin for shooting. Then these calli with shoots were transferred to MS and B5 media without growth regulators, to continue shoot elongation. After 20 days, these elongated shoots were excised from callus and placed separately in glass jars containing 35 mg/l of media until shoot were approximately 2 cm long.

Determination of culture medium for callus induction

For callus induction from *M. officinalis*, the explants were excised from only the hypocotyls of 20 days old seedlings and cultivated on MS supplemented with various concentrations of 2,4-D at 0.0 - 3 mg/l, NAA at 0.0 - 2 mg/l, BAP at 1 - 2 mg/l and KIN at 0.5 mg/l to evaluate the comparative responses of these PGRs on callus growth. The pH of all media adjusted to 5.8 with 0.1 N NaOH before autoclaving at 121°C. Each treatment was replicated five times, and each replicate consisted of 20 explants. Callus induction percentage, callus volume (Hooker and Neebers Index) and days of callus induction were evaluated. Hypocotyle-drived calli were transferred to a fresh medium of the same composition as previously used for callus induction. They were sub-cultured every 2 weeks. Statistical analyses were carried out using analyses of variance (ANOVA) and mean scores were compared using Duncan test ($p = 0.01$).

Rooting and acclimatization

For rooting, the elongated shoots were transferred to glass jar containing 50 mg/l media. The media were supplemented with various auxins (IBA, IAA and NAA) in different concentrations for 1 month, using 20 shoot replicates per treatment. After this period, the number of root per plant (shoot) and mean shoot length (cm) were evaluated. Subsequently, rooted plants were sub cultured on MS medium for 30 days. All experiments were analyzed on Complete Randomized Design (CRD) by SAS 9. Acclimatization were carried out in a greenhouse (25°C and 80% relative humidity), in 10 cm plastic pots in a mixture of garden soil : peat : perlite (ratio: 1:1:1).

Cell suspension culture and growth measurement

Cell suspension culture was performed by sub culturing every 12 days using 1.0 g cells (the optimum cell inoculums size) in 200 ml conical flask containing 35 ml IM. The medium contain various concentrations of 2, 4-D at 0.0 - 2 mg/l, NAA at 1.0 - 2.0 mg/l and BAP at 0.5 mg/l. Cell suspension cultures were initiated by shaking 35 g of friable callus at 110 rpm in the dark at 27°C. After 48 h, separate cells were aggregated. The average weight of the cell biomass taken from five cell suspension culture flasks for each treatment was taken after 26 days. The cells were harvested by filtering the suspension culture through filter paper (What man No. 1, diameter 90 mm). The cells were dried for 24 h with a 60°C hot air and weighed.

Counting of cell number

Cells were counted after maceration of cell suspension with 12% chromic acid at 60°C for 10 min, every other day, for 26 days. The cells were counted to determine the proliferation of cells in various days.

RESULTS AND DISCUSSION

Proliferation of shoot tip explants

During the 30 days, adventitious shoot developed directly

Table 1. Comparison of BAP and Kin in Explants producing shoot%, Mean No. shoot per exp, Shoot high and Shoot-forming capacity (SFC).

BAP (mg/l)	KIN (mg/l)	Explants producing shoot%	Mean number of shoot per experiment	Shoot high	Shoot-forming capacity (SFC)
Control	-	45	0.478^{f}	0.21^{e}	0.2
1	-	68	1.15^{d}	0.57d	0.6
1.5	-	73	1.59^{c}	0.72^{c}	1.2
2	-	80	3.43a	1.72^{a}	2.4
3	-	85	3.44^{a}	1.73^{a}	3.1
-	0.5	55	0.75^{e}	0.25^{e}	0.4
-	1.5	63	1.490^{c}	0.63^{cd}	0.7
-	2	66	1.493^{c}	0.66^{cd}	1.1
-	3	73	2.453^{b}	1.29^{b}	1.8

Means followed by the same letter are not significantly different using Duncan's multiple range tests ($p \leq 0.05$).

Table2. Effects of BAP (mg/l) and various other auxin growth regulators on adventitious shooting of *M. officinalis*.

BAP (mg/l)	IAA (mg/l)	MSN	IBA (mg/l)	MSN	NAA (mg/l)	MSN
0	0	0.26 ± 0.001^{j}	0	o.28 ± 0.003^{j}	0	o.35 ± 0.003^{l}
0	0.5	0.5 ± 0.005^{i}	0.5	0.58 ± 0.002^{i}	0.5	0.64 ± 0.001^{k}
0	1	0.57 ± 0.003^{i}	1	0.62 ± 0.003^{i}	1	0.68 ± 0.002^{k}
1	0	0.74 ± 0.002^{h}	0	0.88 ± 0.004^{h}	0	0.92 ± 0.004^{j}
1	0.5	0.87 ± 0.003^{g}	0.5	0.95 ± 0.010^{h}	0.5	1.2 ± 0.022^{i}
1	1	0.95 ± 0.011^{g}	1	1 ± 0.013^{h}	1	1.4 ± 0.024^{h}
1.5	0	1.35 ± 0.007^{f}	0	1.5 ± 0.019^{g}	0	1.65 ± 0.008^{g}
1.5	0.5	1.68 ± 0.005^{e}	0.5	1.62 ± 0.010^{g}	0.5	2.035 ± 0.019^{f}
1.5	1	1.75 ± 0.006^{e}	1	1.89 ± 0.011^{f}	1	2.095 ± 0.018^{f}
2	0	2.51± 0.007^{d}	0	2.57 ± 0.003^{e}	0	2.75 ± 0.026^{e}
2	0.5	2.83 ± 0.003^{c}	0.5	2.92 ± 0.009^{d}	0.5	3.37 ± 0.020^{d}
2	1	3.09 ± 0.038^{b}	1	3.2 ± 0.039^{c}	1	3.68 ± 0.008^{c}
3	0	3.12 ± 0.010^{b}	0	3.31 ± 0.007^{bc}	0	3.75 ± 0.013^{c}
3	0.5	3.51 ± 0.009^{a}	0.5	3.43 ± 0.021^{b}	0.5	4.2 ± 0.028^{b}
3	1	3.55 ± 0.009^{a}	1	3.85 ± 0.020^{a}	1	5 ± 0.056^{a}
3.5	1	3.1 ± 0.003^{b}	1	3.32 ± 0.003^{bc}	1	4 ± 0.004b^{c}
4	1	2.85 ± 0.002^{c}	1	3.2 ± 0.006^{b}	1	3.53 ± 0.004^{c}

MSN: Means shoot number. Data are from four independent experiments. Means followed by the same letter are not significantly different using Duncan's multiple range tests ($p \leq 0.05$).

from meristematic explants (shoot tips) and through differentiation of calli from the base of shoot tips. Shoot tips responded to BAP better than to Kinetin (Table 1). This coincided with the previous observations; the increase of BAP concentration up to 1 mg/l gave the greatest efficiency in shoot number (Tavares et al., 1996). Sato et al. (2005) reported that 8.8 µmol BAP in 11.42 µmol caused an increase in proliferation rate in shoot tip explants in *M. officinalis*. In general, MS medium supplemented with NAA and BAP were more effective in promoting shoot development than those supplemented with Kinetin. On the media containing 3mgl/l BAP, 82 - 90% of explants showed shoot proliferation (Table 2). This fact can be explained that cytokinins especially at the high concentration overcome apical dominance and promote shoot formation (Echeverrigary and Fracaro, 2001). The presence of NAA promotes shoot elongation as reported by Barrueto et al. (1999).

The statistical differences in the response of shoot tips in terms of shoot multiplication rate on the media were significant ($p \leq 0.05$). Therefore, MS supplemented with 3 mg/l BAP in combination with 1 mg/l NAA had better response than B5 (3.5 in comparison with 2.98). Tavares et al. (1996) also reported that, higher concentration of BAP induced more but smaller shoots, suggesting an inverse relation between the number of shoots and their elongation. Although higher BAP to 3 mg/l induced more and long shoots in our study, this is consistent with Gulati

Table 3. Comparison of effect of various combinations of plant growth regulators on shoot induction from various different explants.

Explants	BAP (mg/l)	NAA (mg/l)	Number of explants	Explants producing shoot%	MSN	Shoot-forming capacity (SFC)
Shoot tip	1	0.5	73	65	1.18 ± 0.0131 ^{c}	0.6
	1.5	0.5	75	70	1.30 ± 0.0320^{c}	1.05
	2	0.5	74	78	2.3 ± 0.041^{b}	1.79
	3	0.5	72	82	3.2 ± 0.0665^{a}	2.62
Leaf segment + petiole	1	0.5	73	35	0.82 ± 0.0165^{c}	0.2
	1.5	0.5	76	43	0.92 ± 0.0125^{c}	0.3
	2	0.5	74	56	1.37 ± 0.0599^{b}	0.6
	3	0.5	75	62	1.67 ± 0.0329^{a}	1.08

Means followed by the same letter are not significantly different using Duncan's multiple range tests ($p \leq 0.05$).

and Jaiwal, (1994) which reported a direct relation between the number of shoots and their elongation in *M. officinalis*. In BAP at 4, 4.5mg/l and NAA at 2.5, 3mg/l, shooting response was not observed. This is also reported by Lobnas et al. (2008) who showed the use of high cytokinin levels as one of the most effective methods to reduce shoot growth in *Paulownia kowakamii.*

We indicated that the medium containing 1 mg/l NAA and 3 mg/l BAP was the most effective for *M. officinalis* shoot regeneration. The higher concentration of 3 mg/l was not significantly increased in the shoot number (Table 2). In these conditions, the number of shoots (1.67 per explants) was significantly higher ($p \leq 0.05$) than that obtained in the medium containing only BAP. The best results with respect to shoot regeneration in *M .officinalis* were also obtained on medium supplemented with IAA and BA (Gbolade and Lockwood, 1992). This result can be attributed to ratio of NO_3/NH_4 on MS and B5 media (ratio of NO_3/NH_4 is 66:34 and 50:50, respectively for MS and B5). This ratio is an important parameter on nitrogen uptake and pH regulation during plants tissue culture (George, 1993).

In our study on increase of both cytokinins (BAP and Kinetin), the hyperhydricity, that is, slightly swollen, lighter green and translucent tissue, was increased. This is in agreement with the previous findings of Nobre (1996) who also observed hyperhydricity under high concentration of thidiazuron (TDZ) and BAP in *Lavendula streechas.* Effect of various concentrations of BAP and NAA showed that the shoot tip has the better response to evaluated parameters than leaf segment (Table 3). Described results indicated that MS medium supplemented with 2 - 3 mg/l BAP in combination with 1 mg/l NAA was recommended for *M. officinalis* shoot initiation from shoot tip explants. Some earlier studies have not reported proliferation via shoot tip from seedling with various old-days in 4 landraces but our study recognized that 15 days-old seedling had the greater multiplication rate (2.2 - 3 ± 0.72) than other treatments (Table 5). This is because knowing the physiological age for proliferation of explants excised from seedling is important.

Shoot induction from hypocotyls

Preliminary experiments using different explants of *M. officinalis* showed that cotyledonary nodes and leaves from 10 days-old seedlings were more suitable for regeneration than others such as hypocotyledons (Tavares et al., 1996). Nevertheless, in our study, hypocotyls excised from 20 days–old seedlings had appropriate response to callus induction. In the first experiment, the explants were cultured on MS and B5 media without growth regulators, but desirable results were not obtained. Hypocotyle explants had no shooting under these conditions. Adventitious shoot regeneration was not promoted when Kinetin or BAP was used alone. Morphogenesis response of *M. officinalis* depended on explants and type of growth regulators used in culture media (Bajaj, 1986; Kool et al., 1999). Callus induction was obtained when IAA = 1.5 mg/l, NAA = 1.5 mg/l and KIN = 0.5 mg/l were used. This callus was a pale yellow, friable, with or without small green globules (Table 4). After 25 days of incubation on Kinetin at 1 mg/l, hypocotyls explants exhibited callus with adventitious shoots. The SFC % (shoot forming capacity) index value was 1.08. No shoot formation from intact leaf was observed under this condition. The shoot hyperhydricity frequency was higher (25%) on MS and B5 media containing higher concentration of BAP. These results were expected because higher cytokinin application has been reported as one of the factors involved in shoot hyperhydricity during *in vitro* culture of several plant species.

Effect of PGRs on establishment of callus culture

Explants from hypocotyle significantly produced more callus than those from cotyledonary leaf and shoot tips base (Table 4). The remarkable callus induction was obtained in MS medium containing 2,4-D at 1 mg/l, NAA at 1mg/l and KIN at 0.5 mg/l. This coincides with some other reports by Smolenskaya et al. (2007). The exclusive presence of auxins in the medium, regardless of its

Table 4. The effect of various different combinations of PGRs on rate of callus induction, callus volume and period required for the induction.

GRs concentration	Callus volume	Callus induction (%)	Days to callus induction
Control	0^{f}	0	0
2,4-D, NAA, KIN =1,1,0/5	$17/80^{a}$	80	$3/9^{a}$
2,4-D, NAA, KIN =1/5,1,0/5	$11/50^{dbc}$	70	$12/7^{cde}$
2,4-D, NAA, KIN =2,1,0/5	$9/20^{dec}$	64	$10/6^{b}$
2,4-D,IAA,BAP =1,1,0/5	$13/20^{b}$	68	$13/6^{ef}$
2,4-D,IAA,BAP =1/5,1,0/5	$11/70^{bc}$	58	$11/65^{bcd}$
2,4-D,IAA,BAP =2,1,0/5	11^{dbc}	55	$12/6^{cde}$
2,4-D, BAP =1,0/5	$8/60^{de}$	45	$11/5^{bcd}$
2,4-D, BAP =1/5,0/5	10^{dc}	50	$13/2^{def}$
2,4-D, BAP =2,0/5	$9/40^{dec}$	62	$11/1^{bc}$
NAA,BAP =1,0/5	$6/80^{e}$	50	$13/1^{def}$
NAA,BAP =1/5,0/5	$10/1^{dc}$	35	$16/9^{f}$
NAA,BAP =2,0/5	$8/90^{dec}$	28	$16/9^{f}$

Means followed by the same letter are not significantly different using Duncan's multiple range tests ($p \leq 0.05$).

Table 5. Effect of age of seedling on number of shoot, rate of regeneration and height of shoot.

Seedling age (day)	BAP (3 mg/l)		NAA (1/5 mg/l)	
	Number of explants	Mean number of shoot per experiment	Regeneration rate%	Shoot height (cm)
10	68	2.3 ± 0.788^{ab}	72	1.2 ± 0.0111^{c}
15	73	2.5 ± 0.072^{a}	84	1.8 ± 0.015^{a}
20	65	2.4 ± 0.085^{ab}	80	1.7 ± 0.0127^{a}
25	71	2.2 ± 0.0125^{b}	64	1.6 ± 0.0316^{b}

Data are from four independent experiments. Means followed by the same letter are not significantly different using Duncan's multiple range tests ($p \leq 0.05$).

concentration, was not sufficient for the callus formation. Thus, the combination of these PGRs could induce callus. The reason for it may be the synergistic effects of auxins and cytokinins. This is in agreement with Gopi and Vatsala (2006).

The combination of auxins and cytokines does not only induce more friable callus, the number of days taken for callus induction were also shorter than other treatments (ie fast growing calli). The types of callus observed in 2,4-D at 1 mg/l, in NAA at 1 mg/l and in KIN at 0.5 mg/l were green yellowish and friable, so these types of callus could be used either for indirect regeneration or suspension cell culture. Also, the results showed that two types of auxins with cytokinins have more effect than one auxin with cytokinins. For instance, the effect of both NAA and BAP or 2,4-D and KIN induced soft and white calli were not appropriate for suspension cell culture. Also, hyperhydricity was much in these treatments. However, NAA at 1 mg/l, 2,4-D at 1 mg/l and KIN at 0.5 mg/l had the highest percentage callus induction, but the higher concentration did not cause the induction of much callus (Table 4). As can be seen in Table 6, the medium containing 2,4-D, rather than NAA, was generally more effective for inducing callus. Some previous reports showed that either 2,4-D or NAA can be used as the utter source for induction of callus (Smolenskaya et al., 2007). For *M. officinalis,* these two PGRs had to be used in combination with cytokinins for optimum callus induction from hypocotyle. This is in accordance with shamsardakani et al. (2003) who reported that the combination of auxins and cytokinins can induce compact callus. Also, Gopi and Vatsala (2006) reported that for induction and growth callus, the adjustment of the levels of auxins and cytokinins were necessary.

Root induction in regenerated shoots

After 25 days, regenerated shoots were excised and transferred into MS rooting medium. Auxins (IBA, NAA and IAA) induced rooting in *M. officinalis* (Table 6). 96% of rooting was obtained after 25 days of putting explants on MS medium supplemented with 1 mg/l NAA, while IBA at the same concentration induced the rooting in 64% of

Table 6. Effect of auxins on the number and length of roots formed and height of shoot in MS medium.

NAA (mg/l)	IBA (mg/l)	IAA (mg/l)	Root number per explants	Root length	Shoot length	% Rooting
0	0	0	0.5 ± 0.016^{g}	0.75 ± 0.010^{g}	0.75 ± 0.005^{g}	43
0.5	-	-	1.2 ± 0.015^{e}	2.1 ± 0.039^{d}	1.5 ± 0.023^{e}	68
0.75	-	-	3 ± 0.045^{b}	3.2 ± 0.071^{b}	2.13 ± 0.045^{c}	94
1	-	-	3.2 ± 0.045^{a}	4 ± 0.0109^{a}	3.3 ± 0.049^{a}	96
-	0.5	-	1 ± 0.026^{f}	0.75 ± 0.10^{g}	1.04 ± 0.037^{fg}	53
-	0.75	-	1.25 ± 0.027^{e}	1.25 ± 0.037^{f}	1.06 ± 0.129^{fg}	58
-	1	-	1.5 ± 0.023^{d}	2.2 ± 0.051^{d}	1.84 ± 0.035^{d}	64
-	-	0.5	1.2 ± 0.032^{e}	1.5 ± 0.014^{e}	1.24 ± 0.015^{ef}	64
-	-	0.75	1.5 ± 0.016^{d}	2.4 ± 0.025^{c}	2.23 ± 0.280^{bc}	75
-	-	1	2 ± 0.033^{c}	3.2 ± 0.071^{b}	2.52 ± 0.052^{b}	73

Means followed by the same letter are not significantly different using Duncan's multiple range tests ($p \leq 0.05$).

Table 7. Cell biomass of *M. officinalis* after 26 days culture in liquid MS medium supplemented with different concentration of 2,4-D (0.0-2 mg/l) and NAA (0.0-2 mg/l).

2,4-D	NAA	BAP	Fresh weight	Dry weight
0	-	0	0^{f}	0^{e}
1	-	0.5	5.48^{a}	0.407^{a}
1.5	-	0.5	5.17^{b}	0.347^{b}
2	-	0.5	4.15^{d}	0.267^{c}
-	1	0.5	3.74^{e}	0.215^{d}
-	1.5	0.5	4.76^{c}	0.352^{b}
-	2	0.5	4.33^{d}	0.280^{c}

*Means within column followed by same letter are not significantly different (Duncan p= 0.01).

the shoot. In the earlier stated treatments, roots with averages of 3.32 ± 0.045 cm were developed per shoot (Table 6). A similar report has been unraveled on *M. officinalis* shoots, in which root formation required the presence of NAA in the culture medium (Tavares, 1996). The rooting ability of *M. officinalis* was inhibited in medium containing activated charcoal. This is consistent with Barrueto et al. (1999) who reported in Eucalyptus that activated charcoal has an inhibitor effect on rooting. Roots were developed further in auxin-free medium in 43% rooted shoots after 25 days of cultivating explants onto rooting medium.

Effect of callus inoculation density on cell culture establishment

The cells of *M .officinalis* were found to grow well in liquid medium containing 1 mg/l 2,4-D, 1 mg/l NAA and 0.5 mg/l KIN. This indicated that 1 g inoculums in 35 ml culture medium gave the best growth (Table 7). The maximum increase of cell biomass with the initial inoculums of 0.75 g was almost 5.48 g after 18 days of culture.

Cell suspension culture

2,4-D at 1.0 mg/l and BAP at 0.5 mg/l showed the highest fresh and dry weight, that is, 5.48 and 0.407, respectively. This is approximately 20 times higher than the initial weight of callus. The control samples did not show response towards PGRs. It demonstrated that the presence of PGRs in the suspension cell culture is crucial. Smolenskaya et al. (2007) reported that the presence of growth regulators and their proportions are most important factors for the sustained culturing of cell lines. It is worthwhile to note that higher concentration (2 mg/l) of 2,4-D had no good response to PGRs and fresh weight was 4.15; it is indicated that appropriate concentration of 2,4-D is 1 mg/l for obtaining the highest fresh and dry weight in *M. officinalis* (Table 7). In the pattern of *M. officinalis,* cells in MS medium supplemented with 1.0 mg/l 2,4-D and the lag phases were short (6 days) for both fresh weight and dried weight growth curve as well as in the cells counting (Figure 1). Also the maximum dry weight (0.407 g) of the culture occurred 15 days after inoculation followed by a gradual decline of growth (Figure 1). The difference could be interpreted as a

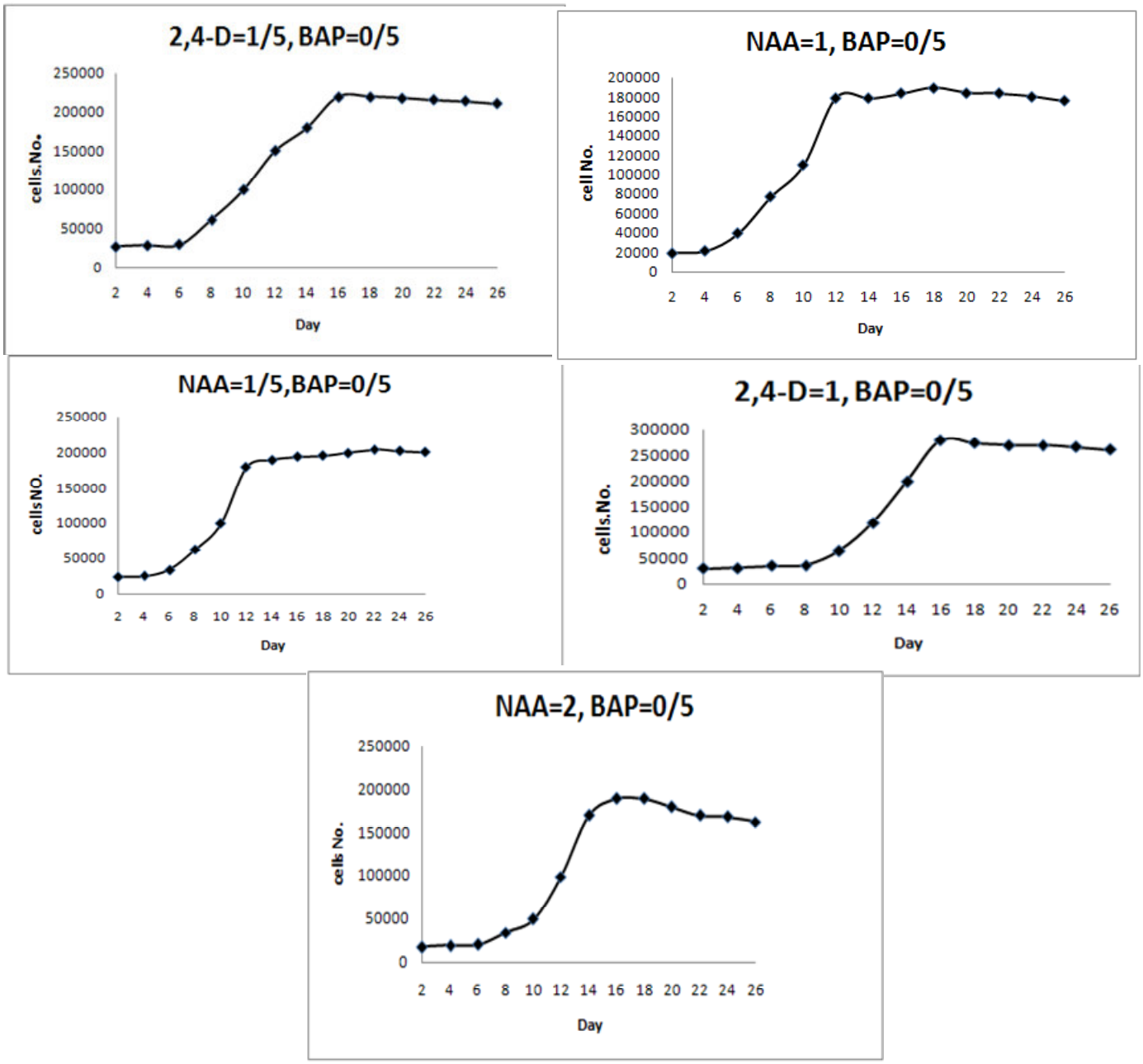

Figure 1. Cell counting during 26 days in various concentration of plant GRs.

consequence of the dissimilation of intracellular stored carbohydrates and the increase of the water content in the cells. Smolenskaya et al. (2007) reported that NAA is a common auxin which ensured the growth and viability of cell cultures, but in our study, this was not necessary for the cell culture of *M. officinalis*. The results indicated that cells of *M. officinalis* could grow well in MS supplemented with 2,4-D and BAP.

Replacement of 2,4-D with NAA in the culture medium had a remarkable effect on growth of cells, also the maximum increase in the number of the cells was observed. It seems that the presence of NAA in the suspension culture retards cell division but promotes cell elongation, which is accompanied by endorduplication of nuclear DNA. 2,4-D and NAA as a two important auxins, exert opposite actions on division and elongation of the cells (Smolenskia et al., 2007). The reason for their action may be related to the fact that these two auxins activated different pathway controlling cell division and cell elongation. In addition, the signal transmission from the putative receptor of 2,4-D involves heterotrimeric G-proteins (Smolenskia et al., 2007). In general, NAA

impedes growth while promoting cell elongation and in the presence of 2,4-D only active growth of the cells was observed. The cell cultures were maintained by sub culturing 1.0 g of cell inoculums every 2 weeks into 35 ml of the maintenance medium. The supplied cell suspension cultures were used as the material source for the production of the secondary metabolites study in the next research activity. Bajaj (1986) reported that there is a certain relationship between cytophysiological characteristics (cell counting, fresh weight etc) and level of synthesized metabolite in some medicinal plants. The counting of cell numbers during 26 days with 12% trioxide chrome was done. There is a direct relation between fresh weight and cell number. However, fresh and dry weights are indication of biomass, but cell counting is more accurate than cells volume pack (CVP) and fresh weight. At the first 8^{th} that is, lag phase, the numbers of the cells were not increased, but the size of the cells was large. On the 15^{th} day, the cell number was high (290000 cells per 1 cube centimeter). These cells characteristics were few. It is because of this that proliferation of the separated cell was done. From 18th to 20th, the stationary phase started as a rule in the middle of the subculture; later, individual cells and small cell aggregates perished.

Conclusion

This study showed that *M. officinalis* is a species of which micropropagation of plantlets can be achieved by various methods including direct regeneration from shoot tips, adventitious shooting via organogenesis of callus and indirect shoot formation from hypocotyle explants. Only 6 -8 weeks is required for the whole course of plant regeneration. This protocol would be useful for clonal propagation and genetic transformation. *M. officinalis* contained various secondary metabolites with biological activities. Also, establishment of cell suspension culture for the production of secondary metabolites is very important. The different methods utilized for measuring cells culture (cell counting, fresh and dry weight) of *M. officinalis* are in agreement and have overlapped with one other.

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