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Full Length Research Paper

Improved *in vitro* culture and micropropagation of different *Melissa officinalis* L. genotypes

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Melissa officinalis L. **is widely cultivated in the world and Iran as well, due to its useful application in medicine. In order to establish a stable and high frequency of regeneration system with 4 landrace collected from different climate in Iran, major parameters such as regeneration rate, rooting percentage, shooting and proliferation rate 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 the higher response to rooting than other's used auxins. Explants derived from Hamedan landrace had the highest potential for regeneration. This is the first report on high frequency *in vitro* regeneration in four diverse landraces of *M. officinalis* selected from various climates regions in Iran.**

Key words: *Melissa officinalis*, regeneration, micropropagation, Iran.

INTRODUCTION

Lemon balm (*Melissa officinalis* L.) is a medicinal herb, native to northern Mediterranean regions (Tavares et al., 1996). Average essential content in the top third part is 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 of lemon balm are used as an anti-tumeral agent as a potential for cancer remedy or prevention (Turhan, 2006). The volatile oils of lemon balm may also be used as an anti-virus agent and contains as 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. So, micro-propagation can be an effective alternative for mass production of selected genotypes. In order to obtain a homozygote population and along with to supply cell suspension culture, micro-propagation is required. However *M.* officinalis grow easily, but this population is not homozygote, more ever top one-third part of it, is contained essential oils, so cultivated in farm (traditional method) is not economically. 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 affected by genotype, there is not many reports on micro-propagation from various landraces with different climate origins, as well as the effect of culture media types. Little is known about different explants derived from various seedling ages. Iran is producing *M. officinalis* all the year round and thus it is cultivated in most of its regions (Zargari, 1990) Hence, for the first time, we established a rapid and efficient *in vitro* regeneration of different Iranian's *M. officinalis* landraces using shoot tips and hypocotyledon axes explants. We studied the effect of some growth regulators, media and various seedlings age on micropropagation of these landraces in order to obtain high shoot regeneration and high rooting percentage as well as appropriate survival rate in acclimatization stage.

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MATERIAL AND METHODS

Seed sterilization procedure

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

Media preparation and direct regeneration

For germination, seeds were placed on ½ strength MS (Murashige and Skoog, 1962) medium supplemented with 3% sucrose and 0.7% agar. The cultures were maintained at 25 ± 2°C with 16-h day light with intensity of 10 - 20 µmol/ms during a different period of 10, 15, 20 and 25 days. The first experiment was performed to evaluate the performance of two cytokinins including BAP and kinetin for shoot proliferation. For this purpose, shoot tips selected as explants, were placed on MS and B5 media supplemented with 0.7% agar and different cytokinins with their varied concentrations including BAP (1.0, 1.5, 2.0 and 3.0 mg/l) and kinetin (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. The parameters investigated were, the 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 week each). The cultivation was continued on MS and B5 media supplemented with 3 mgl/l BAP and 1.5 mg/l NAA for 5 month. In other experiment, 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 combination on 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 was introduced as a control. The explants were inoculated in darkness at 25 ± 2°C for 20 days. Each treatment contains 4 replicate with 20 explants per replication (plates). Obtained callus were inoculated in of MS and B5 media supplemented with different types of cytokinin with various concentrations (BAP: 0, 0.5, 1 and 1.5 mgl/l), kinetin (0, 0.5, 1, 1.5 and 2 mgl/l) to induce shoots.

In direct shoot induction

After determination of the best treatment for callus induction, subsequently, placed on kinetin for shooting. Then these calli with shoot 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.

Rooting and acclimatization

For rooting, the elongated shoots were transferred to glass jar containing 50 mgl^{-1}media. The media supplemented with various auxins (IBA, IAA and NAA) in different concentration for 1 month, using 20 shoot replicates per treatment. After these period, the number of root per plant (shoot), mean shoot length (cm) were evaluated. Subsequently, rooted plants were sub cultured on MS medium for 30 days. All experiments were analyzed Complete Random Design (CRD) by SAS 9. Acclimatization were carried out in a greenhouse (25°C and 80% relative humidity), in10 cm plastic pots in a mixture of: garden soil: peat: perlite (ratio: 1: 1: 1).

RESULTS AND DISCUSSION

Proliferation of shoot tip explants

Shoot tip explants were cultured on MS and B5 media supplemented with different concentration of BAP and kinetin with 1 mgl/l NAA to evaluate their effect on *Melissa* shoot multiplication. During 30 days, on all tested media, shoots developed directly from meristematic explants (shoot tips) and indirectly through redifferentiation of callus, which often was induced at the base of shoot tips. First, the best cytokinin type must be known, subsequently its effect with different types of auxins. BAP showed better response than kinetin (Table 1). This is coinciding with the previous observations that the increase of BAP concentration up to 1 mgl/l gave greatest efficiency in shoot number (Tavares et al., 1996). Sato et al. (2005) reported that 8.8 µmol BAP in 11.42 µmol caused increase 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 3 mgl-1 BAP, (82 - 90%) of explants showed shoot proliferation with 3.2 - 4.1 shoots per explant (average) during 30 days (Table 2). This fact can be explained that cytokinins especially at the high concentration overcome apical dominance and promote shoot formation (cheverrigary and Fracaro, 2001). The multiplication rate was highest (3.44 ± 0.25 cm) when 3 mgl^{-1} BAP in combination with 1 mgl/l NAA was used. Presence of NAA promotes shoot elongation as reported by Barrueto et al. (1999). The statistical differences in the response of shoot tips in term of shoot multiplication rate on the media were significant ($p \leq 0.05$). Therefore, MS supplemented with BAP 3 mgl/l in combination with 1 mgl/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. But in our study higher BAP to 3 mg/l induced more and long shoot that is consistence with Gulati and Jaiwal, 1994 which reported a direct relationship between the number of shoots and their elongation in M*. officinalis*. In high levels of cytokinin and auxin (4, 4.5 mgl/l BAP) and (2.5, 3 mgl/l NAA) cased no shooting response (Table 3). This is also reported by Lobna et al. (2008) which showed the use of high cytokinin levels as one of the most effective methods to reduce shoot growth in *Paulownia kowakamii.* It is worth mentioning that, both root and shoot were produced from shoot tips at concentration of 3 mgl/l BAP in combination with 1 mgl/l NAA. These adventitious shoot were vigorous and long high. We indicated that the medium containing 1 mg/l NAA and 3 mg/l BAP was the most effective for *M.*

Table 1. Comparison BAP and Kin in various concentrations.

BAP(mg/l)	KIN(mg/l)	Explants producing shoot%	Mean no. shoot per exp	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 ($p \leq 0.05$).

Table 2. Effect of BAP (mg/l) and different auxins (IAA, NAA, IBA) on mean number of shoot per explants.

BAP	IAA	MSN	IBA	MSN	NAA	MSN
0	0	0.26 ± 0.001^{j}	0	0.28 ± 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 ($p \leq 0.05$).

Table 3. The Comparison of different explants for induction shoots in various concentrations.

Explants	BAP	NAA	Explants. No	Explants producing shoot%	MSN+ SE	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 ($p \leq 0.05$).

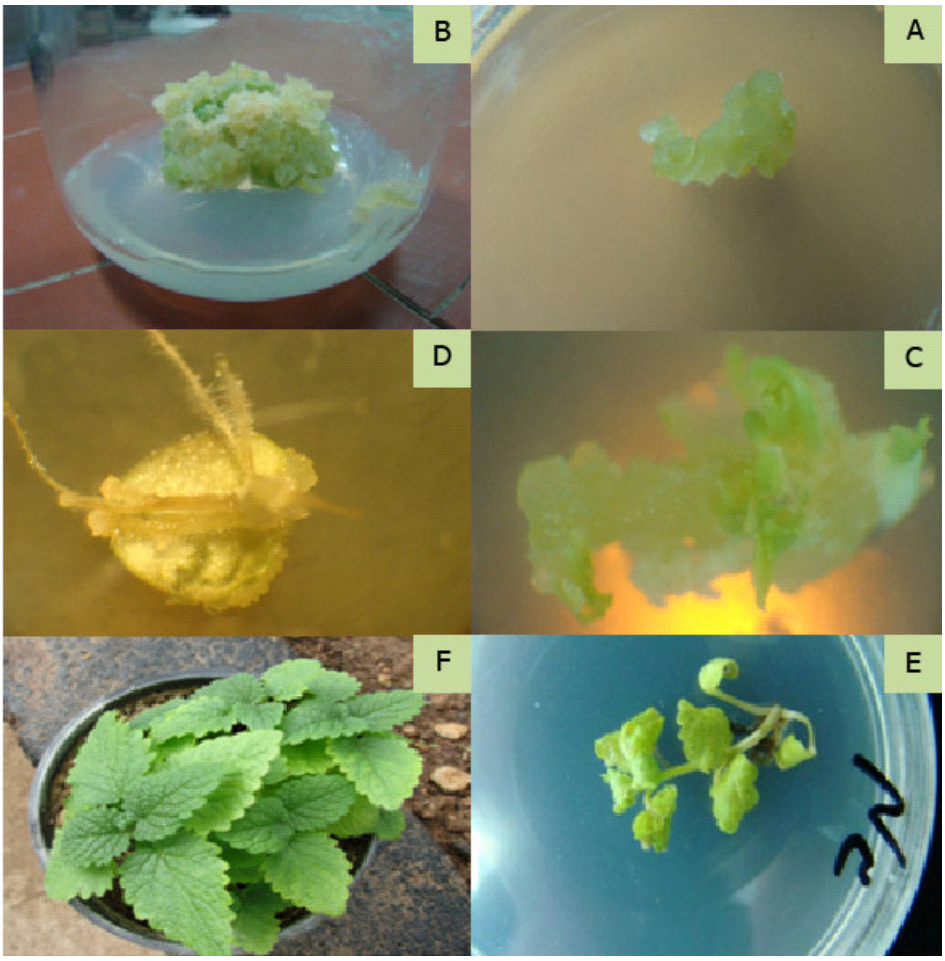

Figure 1. (A) Indirect regeneration in hypocotyls explants in *Melissa officinalis*. (B) Callus induction derived from hypocotyls explants. (C) Proliferation callus for indirect regeneration. (D) Indirect regeneration from obtained callus. (E) Rooting induction from shoot tips base. (F) Indirect shoot derived from hypocotyls multiplicate and acclimatized.

officinalis shoot regeneration. In the higher concentration of 3 mg/l was not increased significantly (Figure 1). In these conditions, the number of shoots (1.67 per explants) was significantly higher ($p \leq 0.05$) than obtained on the medium containing only BAP. It has been already reported for other plants, including those from laminaceae family, that shoot regeneration is promoted on media containing both auxin and cytokinin (Rout and Das, 1997). 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). MS medium can produce more shoot per explants and regeneration rate as well as B5 (Table 7). This result can be attributed to the No3/NH4 on MS and B5 media (66:34 and 50:50, respectively for MS and B5). This ratio is an important parameter on nitrogen uptake and pH regulation during plant tissue culture (George, 1993). It can relate to the interaction between these growth regulators. It has not been reported that shoots of *M. officinalis* obtained by shoot-tips proliferation, showed hyperhydricity symptoms, but in our study with increasing of both cytokinins (BAP, kinetin) the hyperhydricity, that is, slightly swollen, lighter green and translucent tissue was increased. This is agreed with the previous findings of Nobre (1996) who also observed hyperhydricity under high concentration of TDZ and BAP in Lavendula streechas. Described results indicated that MS medium supplemented with 2 - 3 mg/l BAP in combination with 1 mg/l NAA is recommended for *M. officinalis* shoot initiation from shoot tip explants. Some earlier studies have not been 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. There was no significant difference between 10 and 20 day-old seedling in term of shoot length in all landraces (Table 5). According to obtained results, Hamedan landrace with (3 - 3.75 ± 0.29 cm) was the best among other landraces in for multiplication rate (Table 6). Genotype types can influence on essential oils as well as

Table 4. The effect of different GRs concentrations on callus volume, callus percentage and days to callus 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 seedling age (day) with BAP (3mg/l) and NAA (1/5 mg/l) on some investigated parameters.

Seedling age (day)	BAP (3mg/l)		NAA (1/5 mg/l)	Shoot height
	Explants .No	Mean No. shoot per exp	Regeneration rate%	
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 ($p \leq 0.05$).

other useful compound (Turhan, 2006). *M. officinalis* is native Mediterranean region, so it sounds, Hamedan has similar climates to it and it seems that this plant is adaptive to this condition.

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). But in our study, hypocotyls were 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 does not obtained. Hypocotyls explants had not shooting under these conditions. Adventitious shoot regeneration was not promoted when kinetin or BAP used alone. Morphogenesis response of *M. officinalis* was depending 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 mgl/l, NAA = 1.5mgl/l and kinetin = 0.5 mgl/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 = 1 mgl/l hypocotyls explants exhibit callus with adventitious shoots. The mean number of shoot per explants was rather low at BAP 3 mgl/l in combination with NAA 0.5 mgl/l (1.67 ± 0.32). 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 are expected, because high cytokinin application has been reported as a one of the factors involved in shoot hyperhydricity during in vitro culture of several plant species. Hamedan landrace had good response than others landrace to callus induced adventitious shoot. A different response of the genotype to *in vitro* condition, especially to growth regulators, has been reported in many plant species (Bajaj, 1986).

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 8). 96% rooting were obtained after 25 days with 1 mg/l NAA,

Table 6. Landrace's response in (BAP = 3mg/l and NAA = 1mg/l) for shoot formation and NAA (1/5 mg/l) for rooting percentage on some evaluated parameters.

Population	BAP	NAA	NAA
	MNS	RR (%)	RP (%)
Hamadan	3.2 ± 0.0294[a]	85	83
Ghazvin	1.8 ± 0.0135[c]	78	74
Rasht	2 ± 0.0445[c]	68	73
Uromia	2.5 ± 0.0125[b]	86	76

MNA: Mean Number Shoot per Explants, RR: regeneration rate, RP: rooting percentage. Data are from four independent experiments. Means followed by the same letter are not significantly different using Duncan's multiple range tests ($p \leq 0.05$).

Table 7. Effect of MS (BAP = 0, 1, 1/5 and 3 mg/l with NAA = 1 mg/l) and B5 media (BAP = 0, 1, 1/5 and 3 mg/l with NAA = 1 mg/l) on mean number shoots per explant and regeneration rate.

Treat	MS (mg/l)				B5 (mg/l)				SEM	Significant
	0	1	1.5	3	0	1	1.5	2		
MNS	0.499[d]	1.294[c]	2.689[b]	3.500[a]	0.488[d]	1.25[c]	2.402[bc]	2.998[b]	0.0798	***
RR (%)	45[e]	70[c]	76[b]	83[a]	32[f]	64[d]	72[c]	78[b]	2	***
EX (%)	46[e]	68[d]	80[b]	85[a]	42[e]	65[d]	70[c]	81[b]	1.965	***
SFC	0.22[g]	0.87[e]	2.15[c]	2.98[a]	0.18[g]	0.78[f]	1.69[d]	2.43[b]	0.117	***

MNA: mean number shoot per explants, RR: regeneration rate, Ex. %: explants producing shoot, SFC: shoot frequency capacity. Data are from four independent experiments. Means followed by the same letter are not significantly different using Duncan's multiple range tests ($p \leq 0.05$).

Table 8. Effect of auxins on the number of roots, root Length (cm) and shoot elongation obtained from 4WK of culture on MS medium.

NAA	IBA	IAA	Root NO. per explants+ SE	Root height+ SE	Shoot height+ SE	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 ($p \leq 0.05$).

while IBA at the same time and concentration induced rooting in 64% of shoots. In above treatments, in average 3.32 ± 0.045 cm roots were developed per shoot. Similar report has been reported for *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) reported in *Eucalyptus* that activated charcoal has an inhibitor effect on rooting. Roots developed also in auxin-free medium (43% rooted shoots after 25 days).

Conclusion

This study has shown that *M. officanalis* is a species in which micro-propagation can be achieved by various methods. These methods include production direct rege-

neration from shoot tips, adventitious shooting through organogenesis via callus cultures and indirect shoot formation from hypocotyls explants. Only 6 - 8 week 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.

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