Micro-propagation of *Mentha pulegium* L. through high-frequency shoots-tip and nodal explants culture

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Abstract

The purpose of this study was to develop a biotechnological technique to standardize micro-propagation procedures in *Mentha pulegium* L. (*Lamiaceae*) via direct and indirect organogenesis from nodal and shoot tip explants, recurrent production of biomass and phyto-chemical study. The protocols are designed to provide optimal axillaries-buds multiplication using MS and WPM basalts salts mediums. The shoot-tip explants were more responsive (100%) than the nodal ones (42.1%). The MS medium promoted a significant shoot culture growth compared to WPM one. The most effective medium for shoots proliferation rate (100%) and shoot number (14 shoots.explants^{-1}) was MS medium supplemented with 0.5 mg.l^{-1} BAP. The rate of callus induction was positively correlated with concentration of 2,4-D and explants type. Callus proliferated predominantly from the shoot-tip explants (95%) and sporadically from the nodal explants (80%). The viable and organogenic calli were maintained at reduced concentration of 2,4-D (0.1 mg l^{-1}). These calli were transferred to MS medium supplemented with various concentrations of BAP (0.5-2 mg.l^{-1}) for shoot regeneration. The regeneration rate was highest at 1 mg.l^{-1} BAP, where 79% and 87% of cultured callus developed from nodal and shoot-tip explants, respectively. During the multiplication stage, the rate of micro-shoots regeneration was identified to maintain the same level for 24 month without loss of vigor. Rooting in *M. pulegium* micro-shoots from direct and indirect organogenesis occurred spontaneously without the addition of PGRs in the culture medium. Regenerated plantlets were acclimatized and transferred to pot containing commercial potting mix, which grew normally with a survival rate of 95% and without any phenotype aberration neither in the vegetative stage nor in the floral one.

Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid; BA: 6-benzyladenime; BAP: benzylarninopurine; IAA: indoleacetic acid; IBA: indole 3- butyric acid; Kin: kinetin; NAA: naphthalene acetic acid; PGR: plant growth regulators.

Key words: *Mentha pulegium* L., tissue culture, growth regulators, callus, shoots regeneration.

Introduction

The pennyroyal (*Mentha pulegium* L., *Lamiaceae*) is a short-lived perennial mint and native species of Europe, North Africa, minor Asia and near East (Chalchat *et al.*, 2000). This species is generally known for different properties (Badisa *et al.*, 2003; Shirazi *et al.*, 2004; In-Sook RIM and Cha-Ho JEE, 2006). At the chemical level, this specie is characterized by a high content of essential oils (4% of dry weight) (Farah *et al.*, 2001). Many studies related to morphological and chemical aspects show evidence of polymorphic and biological properties variations (Panizzi *et al.*, 1993, Lorenzo *et al.*, 2002; El-Ghorab, 2006; Cook *et*

al., 2007; Mkaddem *et al.*, 2007; Mahboubi and Haghi, 2008), related to geographical and ecological distribution of *M. pulegium*. To achieve high productivity, homogeneity and good quality of the pennyroyal products, it is necessary to control and optimize culture conditions.

Plant regeneration via *in vitro* culture biotechnologies is regarded as a key factor for realization of mass propagation of elite and standardized plants in the optimized environment conditions (Murch *et al.*, 2000; Saxena, 2001; Peter *et al.*, 2005). However, many studies reported some physiological disorder in many mint micro-plants that affect both regeneration of healthy plantlets and secondary metabolites production (Banthorpe and Brown, 1990).

This study aimed to develop *in vitro* culture techniques that would be used to ensure mass production of healthy plantlets, and to standardize plant materials for biochemical studies. In this paper, we report a reproducible and rapid method for the *in vitro* multiplication of *M. pulegium* through direct and indirect organogenesis from nodal and shoot-tip explants, followed by successful establishment of regenerated plant in soil.

Materials and methods

Aseptic culture

The plant material was derived from mature plants collected in spontaneous sites (N of Morocco). Axillary-buds explants were rinsed with soapy water (with Tween-20) for 2 min and washed with distilled water. The explants were sterilized with 70% (v/v) ethanol for 10 s and sterilized in a distilled water solution containing 7% (w/v) $Ca(ClO)_2$ and two drops of Tween-20 during 20 min. After repeated wash with sterile distilled water (4 times), the explants were placed in tube cultures containing sterile nutriment medium.

Rod-forming

In this experiment, the effect of different cytokinins on shoot-forming was examined. Two types of cytokinin (BA and kinetin, all supplemented 0.25-0.5-1.0 $mg.l^{-1}$) were added to the MS medium (Murashige and Skoog, 1962). Two medium formulations were tested to determine shoot-proliferation and development extent. These media comprised Murashige and Skoog (MS) and woody plant medium (WPM) (Lloyd and Mc Cown, 1980), all supplemented with 0.5$mg.l^{-1}$ BA.

Callus induction and shoot regeneration

For callus induction, shoot-tip and nodal explants were cultured on MS medium supplemented with various concentrations of 2,4-D (0.5-2.0 $mg.l^{-1}$). Calluses were sub-cultured on MS fresh medium at 4 weeks intervals.

After two subcultures, the calluses were transferred to regeneration medium supplemented with different concentrations of BA (0.5-1 $mg.l^{-1}$). For regeneration, a minimum of 30 cultures were raised for each treatment and all experiments were repeated there times.

Mass shoot-propagation and root-forming

The micro-shoots regenerated by direct and indirect organogenesis from explants of *M. pulegium* were placed on full and half- strength MS medium supplemented/or not with 0.5 $mg.l^{-1}$ BA.

After two subcultures, the micro-shoots were transferred to rooting medium, which consisted of full and half-strength MS medium supplemented or not with different concentrations of IBA or NAA (5-1 $mg.l^{-1}$).

To raise sterile cultures, the basal medium of all study conditions was supplemented with 3% sucrose (w/v) (Sigma) and gelled with 0.7% (w/v). The pH of all the media was adjusted to 5.8±0.02 with 1N HCl or 1NaOH before autoclaving during 20 min at 121°C and 15 Pa. The cultures were incubated at 25°C under 16/8h day/night photoperiod using cool white florescent light (40 µmol.m^{-2}.s^{-1}).

Acclimatization of regenerated plants

The rooted plantlets regenerated by direct and indirect organogenesis were transferred to pot containing commercial-potting mix and grown under covered greenhouse conditions, and finally transfer to field.

Statistical analysis

The data obtained were processed using ANOVA analysis, statistical comparisons and Ducan's multiple-range tests at $P < 0.05$.

Results

Plant regeneration via direct and indirect shoot-organogenesis has been achieved from all type of explants. Explants type influenced direct organogenesis (Table 1), and shoot-tip

Table 1. Effect of different supplements/concentrations on cultures of shoot-tip and nodal explants of *M. pulegium* un MS medium.

Concentration ($mg.l^{-1}$)	Culture responding (%)	Shoot-tip and nodal morphogenesis after 4 weeks			Number of shoots.explant^{-1}	Shoot length (cm)
		Shoot	Callus	Callus and shoot		
Shoot-tip						
BA						
0.0	0 ± 0.00 m	0 ± 0.00b	0 ± 0.00d	0 ± 0.00d	0 ± 0.00g	0 ± 0.00 l
0.25	56.8 ± 0.173j	100±0.00a	0 ± 0.00d	0 ± 0.00d	9.1 ± 0.121c	6.0 ± 0.034b
0.5	100 ± 0.00a	100±0.00a	0 ± 0.00d	0 ± 0.00d	14.0 ± 0.04a	10 ± 0.033a
1.0	95.0 ± 0.014b	0 ± 0.00b	89.7± 0.103b	10.3±0.017c	6.02 ± 0.063e	3.0 ± 0.040g
Kin						
0.0	0 ± 0.00m	0 ± 0.00b	0 ± 0.00d	0 ± 0.00d	0 ± 0.00g	0 ± 0.00 l
0.25	47.5 ± 0.063h	100±0.00a	0 ± 0.00d	0 ± 0.00d	6.0 ± 0.023e	4.1 ± 0.08d
0.5	82.0 ± 0.02d	100±0.00a	0 ± 0.00d	0 ± 0.00d	13.4 ± 0.136b	5.7 ± 0.013c
1.0	84.0 ± 0.051c	0 ±0.00b	100± 0.00a	0 ± 0.00d	0 ± 0.00g	0 ± 0.00 l
Nodal explant						
BA						
0.0	0 ± 0.00m	0 ± 0.00b	0 ± 0.00d	0 ± 0.00d	0 ± 0.00g	0 ± 0.00 l
0.25	41.01±0.034k	100± 0.00a	0 ± 0.00d	0 ± 0.00d	5.54 ± 0.0057j	3.41± 0.0057f
0.5	42.1 ± 0.063g	100± 0.00a	0 ± 0.00d	0 ± 0.00d	7.87± 0.0115d	3.7 ± 0.0288e
1.0	57.9 ± 0.015f	0 ± 0.00b	0 ± 0.00d	100± 0.00a	4.97 ± 0.104h	2.71 ± 0.018k
Kin						
0.0	0 ± 0.00m	0 ± 0.00b	0 ± 0.00d	0 ± 0.00d	0 ± 0.00g	0 ± 0.00 l
0.25	22.7± 0.034n	100± 0.00a	0 ± 0.00d	0 ± 0.00d	5.02 ± 0.023h	3.27±0.0057j
0.5	25.0 ±0.024 l	100± 0.00a	0 ± 0.00d	0 ± 0.00d	5.8 ± 0.034f	3.2 ± 0.0144h
1.0	60.2 ± 0.017e	0 ± 0.00b	19.40±0.017b	80.6±0.057b	5.76 ± 0.011f	3.08± 0.011g

Values represent a mean of seven replicates each within 24 explants per treatment. Values followed by the same letter in each column are similar ($P < 0.05$).

explants responded efficiently than nodal ones. The *in vitro* propagation of shoots was also influenced by cytokinins type, and BA was significantly more efficient than kin at the same concentrations for promoting growth. BA at 0.5 $mg.l^{-1}$ appeared to be

optimal for rapid explants response and shoot forming (Table 1, Figure 1).

After 4 weeks of culture, nodal and shoot-tip explants were able to regenerate most vigorously on MS medium supplemented with 0.5 mg.l^{-1} BA, with highest regeneration rates: 100% for shoot-tip explants and 82% for the nodal ones; more than 10 cm of length in shoots cultured on MS medium supplemented with 0.5 mg.l^{-1} BA and 5.2 cm of length in micro-shoots developed in MS medium supplemented with 0.5 mg.l^{-1} kin (Table 1, Figure 1).

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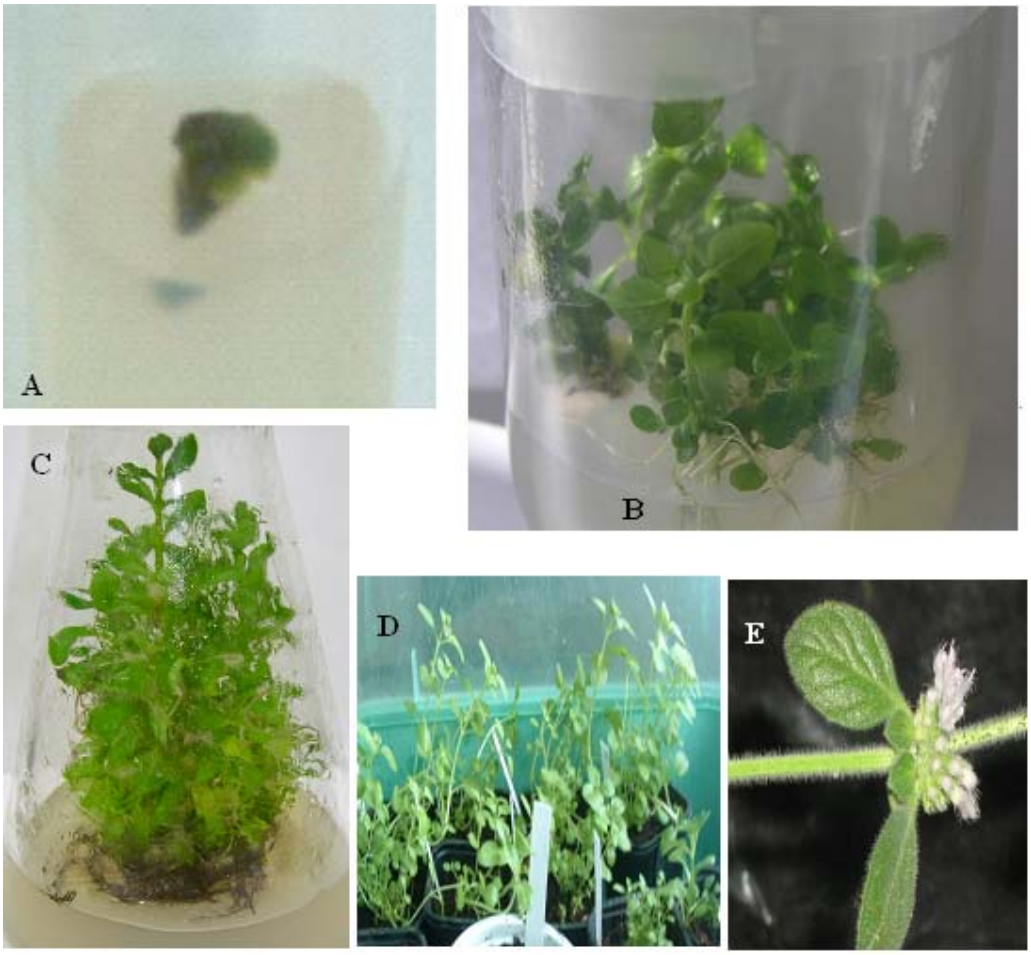

Figure 1. Shoot-tip cultures of *M. pulegium*. **(A)** Explant showing morphogenesis. **(B)** Shoot-tip explants with multiple shoot development on MS medium supplemented with 0.5mg.l^{-1} BA after 4 weeks of culture. **(C)** Elongation and roots development of micro-shoots sub-cultured on half-MS medium without auxins. **(D**) Regenerated plants in the greenhouse. (**E**) Flowering stage.

The number of shoots formed per culture was higher at 0.5 mg.l^{-1} of BA (more than 14 shoots.explant^{-1}) than kinetin at the same concentration (more than 7 shoots.explant^{-1}). The cytokinins (BA and kin) presence at higher concentrations (1 mg.l^{-1}) in the case of shoot-tip and nodal explants did not promote shoot production, when cultures responded callus induction. The MS medium promoted healthy and significant shoot-culture growth than the WPM one (Table 2). In terms of shoot length, there were no considerable differences between WPM and MS media. Owing to abnormal caulogenic response in WPM medium, further experiments were carried out using MS salts medium.

Table 2 Comparison of morphological parameters of *M. pulegium*, micro-propagated on MS and WPM salts supplemented with 0.5 mg. l^{-1} BA.

Parameters	MS salts		WPM salts	
	N	St	N	St
Number of shoots	7.8±0.005c	14.0±0.115a	7.1±0.057d	13.6±0.014b
Plant height (cm)	5.7±0.057b	10.0±0.017a	5.8±0.0115b	10.0±0.081 a
Leaves	Green, large		Dark green, stunted	
Culture responding (%)	42.0±0.051b	100±0.00a	41.2± 0.001b	100±0.00a

Values represent a mean of seven replicates each within 24 explants per treatment. Values followed by the same letter in each column are similar ($P < 0.05$). N: Nodal explants; St: Shoot tip explants.

During the callogenesis stage, the explants of *M. pulegium* cultured on MS medium supplemented with various concentrations of 2,4-D (0.5-2 mg.l^{-1}) developed calluses that have either a soft or a compact textures (Figure 3A). Calluses were induced in about 10 days of culture.

Percentage of cultures responding in callus formation from nodal and shoot-tip explants of *M. pulegium* increased proportionally to 2,4-D concentration. The maximum response was observed on MS medium supplemented with 2 mg.l^{-1} of 2,4-D, with a callus production from nodal and shoot-tip explants of 95% and 80% respectively (Figure 2). Comparatively, shoot-tip explants responded better than the nodal ones in term of percentage of cultures producing callus. Calluses were sub-cultured on MS fresh medium with reduced 2,4-D (0.1 mg.l^{-1}) at 4-5 weeks intervals to maintain their viability and activity.

For plant regeneration, calluses were transferred to the MS medium supplemented with different concentrations of BA (0.5, 1, 1.5 and 2 mg.l^{-1}). The regeneration frequency of shoots was influenced by the callus

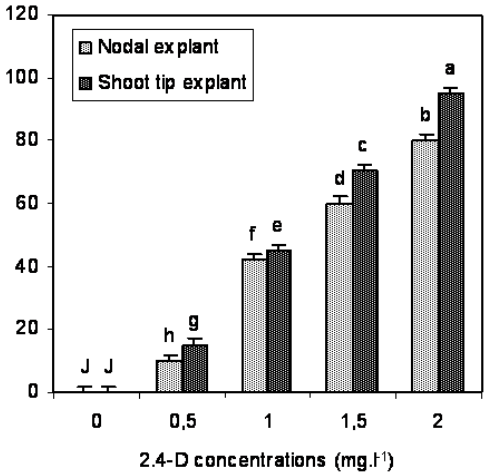

Figure 2. Percentage of responding in callus formation from nodal and shoot-tip explants of *M. pulegium*. The explants were cultured on MS medium supplemented with 2,4-D (0.25, 0.5 and 1 mg.l^{-1}). Cultures periods: 6 weeks of three independent experiments. Values followed by the same letter in each column are similar ($P < 0.05$). Bars represent the standard deviation.

Table 3. Influence of MS medium formulations and PGR concentrations on *M. pulegium* rooting.

	Half- MS salt					Full- MS salt				
	Shoot height (cm)	Rooting (%)	Roots number.explant^{-1}	Root length (cm)	Callusing (%)	Shoot height (cm)	Rooting (%)	Roots number.explant^{-1}	Root length (cm)	Callusing (%)
PGR-free	12.0±0.042a	100±0.0a	14±0.85a	7±0.426a	0±0.00b	12.01±0.07a	100±0.00a	10.2±0.27b	5.1±0.04b	0±0.00b
IBA (mg l^{-1})										
0.25	6.3±0.09b	100±0.0a	4.02±0.036c	4.5±0.27c	100±0.00a	5.91±0.16c	100±0.0a	4±0.042c	3.3±0.01e	100±0.00a
0.5	5.8±0.07d	0±0.00b	0±0.00f	0.0±0.00j	100±0.00a	5.83±0.07d	0±0.00b	0±0.00f	0±0.00j	100±0.00a
1.0	5.7±0.042e	0±0.00b	0±0.00f	0.0±0.00	100±0.00a	5.8±0.07d	0±0.00b	0±0.00f	0±0.00j	100±0.00a
NAA (mg l-1)										
0.25	5.41±0.011f	100±0.0a	3.7±0.034d	3.8±0.16d	100±0.00a	5.4±0.038f	100±0.00a	3.6±0.011e	3.1±0.05f	100±0.00a
0.5	4.71±0.057 l	0±0.00b	0±0.00f	0.0±0.00j	100±0.00a	4.9±0.04j	0±0.00b	0±0.00f	0±0.00j	100±0.00a
1.0	4.7±0.037 l	0±0.00b	0±0.00f	0.0±0.00j	100±0.00a	4.82±0.25k	0±0.00b	0±0.00f	0±0.00j	100±0.00a

Values represent a mean of seven replicates each within 24 explants per treatment. Values followed by the same letter in each column are similar ($P < 0.05$). PGR: Plant growth regulator.

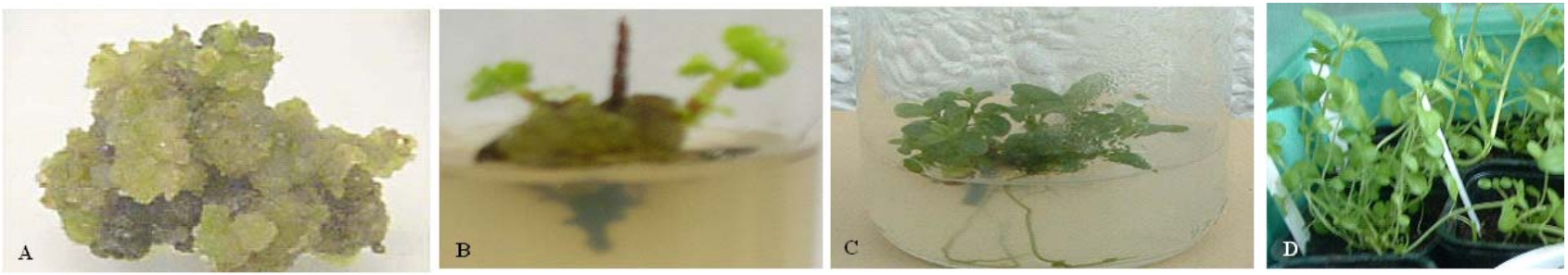

Figure 3. *In vitro* organogenesis in *M. pulegium*. **(A)** Callus induction from shoot-tip and nodal explants cultured on MS medium supplemented with 2 mg.l^{-1} 2,4-D, after 6 weeks of culture; Nodular and green callus. **(B)** Induction of organogenesis from nodal and shoot-tip explants, derived from callus of *M. pulegium* cultured on MS medium supplemented with 1 mg.l^{-1} BA, after 6 weeks of sub-culture. **(C**) Root initiation from *in vitro* derived shoots, after 10 days of sub-culture on half- MS medium. **(D)** Regenerated plants in the greenhouse.

source, subculture in a fresh medium and BA concentration. Our preliminary investigations showed that BA induced a better regeneration response than kinetin. With increased BA concentration (1 $mg.l^{-1}$), the callus generated on the medium supplemented with 2,4-D (2 $mg.l^{-1}$) and sub-cultured in fresh MS with reduced concentration of 2,4-D (0.1 $mg.l^{-1}$), and showed an increase regeneration response (Table 3) as well as the highest number of shoots per responding callus, except at the highest concentration of BA (1.5 and 2 $mg.l^{-1}$). On the other hand, such response wasn't observed in 4 weeks-callus on initial medium without sub-culture in fresh medium (MS- PGR free).

Among the various concentrations of BA (0.5, 1, 1.5 and 2 $mg.l^{-1}$) and origin of callus tested, the callus produced from shoot-type explants and sub-cultured in MS supplemented with 1 $mg.l^{-1}$ BA showed better response in term of shoot regeneration (87%) and shoots number per callus (8) than the callus produced from nodal explants (79% of regeneration rate, and 5 $shoots.callus^{-1}$) (unpublished data).

The micro-shoots regenerated from direct and indirect organogenesis were transferred to full and half strength MS medium supplemented or not with various concentrations of IBA and NAA (0.25 and 1 $mg.l^{-1}$). The micro-shoots produced *in vitro* from nodal and shoot-tip explants were rooted on full and half MS medium without plant growth regulators. The roots obtained on half MS medium were healthier and longer (Table 3, Figure 1C) and no reduction was observed in shoot number or length. Auxins (IBA or ANA) addition in the medium inhibited completely rhizogenesis and micro-shoots growth, they also induced callus from the cut end of shoots (Table 3). The half- MS medium (PGR-free) appeared to be the optimal medium for micro-shoots rooting; 100% of the micro-shoots developed healthy and longer roots with an average of 12 $roots.shoot^{-1}$. Single rooted plantlets were removed from the medium after 5 weeks, transferred to pots containing the commercial potting mix in a covered plastic greenhouse and transferred after to the field. The survival rate following acclimatization was over 95% and plantlets showed a vigorous growth in the field conditions (Figures 1D, 2D and 2C).

Discussion

A method has been developed for the rapid multiplication of *M. pulegium* through direct and indirect regeneration from nodal and shoots-tip explants for better establishment of the plantlets in soils.

The cultures establishment of the explants collected from field-grown plants was thwarted by a high degree of contamination. To control the high incidences of microbial contamination, the calcium hypochlorite $Ca(ClO)_2$ (7%) is used as a sterilizing agent by washing in the appropriate solution for 25 min, followed by several rinses by sterilized water. By using this method, 97% aseptic cultures were obtained; the induction of direct shoot-organogenesis from *M. pulegium* was greatly affected by the explants type. The best response of shoot organogenesis was observed from shoot-tips explants (100%). Our results confirm those reported by Murashige (1974) and Duong Tan Nhut *et al.* (2007). The explants type is favorable for maximum induction of shoot buds in many plant species. Cytokinins contents were shown to be the most critical for multiplication of many medicinal plants (Barna and Wakhlu, 1988; Sharma et Bhavandan,

1993; Mao *et al.*, 1995). BA is one the most effective and affordable cytokinins used in many micro-propagation systems (Mao *et al*., 1995; Rajasubramanian and Pardha Saradhi, 1997; Komalavalli and Rao, 2000; Misic *et al.*, 2006), and in our study, BA was superior to kin for *M. pulegium* micro-propagation. Addition of BA at 0.5 $mg.l^{-1}$ to the medium not only enhanced the proliferation (100% for shoot-tip explants and 80% for the nodal ones) and multiplication rate (more than 14 shoots per shoot-tip explant and 10 shoots per nodal explant), but also favored the proliferation of healthier shoots (more than 10 cm of length), compared to 0.5 $mg.l^{-1}$ BA. The shoots obtained with kin (0.5 $mg.l^{-1}$) were stunted (5.7 cm of length) and leaves of such shoots were small and dark green. The formation of stunted shoots and their fasciation on cytokinins (kin) supplemented medium have been reported in many medicinal-plant species (Rajasubramanian and Pardha Saradhi, 1996; Singh and Sehgal, 1999).

The differential caulogenic responses of cytokinins can be explained by their differential uptake rate as reported in different plants (Blakesy, 1991). However, the use of cytokinins (BA and kin) at higher concentration (1 $mg.l^{-1}$) in the basal medium decreased shoots proliferation in all the treatments and produced callus formation; similar observations were reported by Sarabjeet Suri *et al.,* (1998) and Faisal and Anis (2003). Basal callusing formation has also been shown to limit *in vitro* culture some systems (Lakshmanan *et al*., 1997), due to the action of accumulated auxin at the basal ends especially in the presence of cytokinins (Tao and Verbelen, 1996). Experiments were also showed that MS medium was must better for shoots multiplication than the WPM one. Our results also confirm observations by Llyod and Mc Cwn (1980) and further experiments were carried out using MS basal medium.

In this study, we also established an efficient indirect regeneration protocol using nodal and shoot-tip explants derived from callus of *M. pulegium*. The induction of callus growth and subsequent organogenesis was performed for both types of explants. Various concentrations of 2,4-D (0.5, 1.5 and 2 $mg.l^{-1}$) were used for callus induction from both nodal and shoot-tip explants. 2,4-D is the most effective auxin to led a rapid cell proliferation (Gamborg *et al.*, 1968; George and Sherrington, 1984). Callus induction from both nodal and shoot-tip explants was maximal on MS medium supplemented with 2 $mg.l^{-1}$ of 2,4-D. Furthermore, we noted a significant improvement in callus viability rate by sub-culturing of callus on fresh medium with reduced concentration of 2,4-D (0.1 $mg.l^{-1}$) at 4-5 weeks intervals. This result corroborated other studies having showed that the low concentration of 2,4-D was employed for further maintenance of organogenic calli (Thomas and Maseena, 2006).

Many studies related to the *in vitro* organogenesis underline the importance of anxin/cytokinin ratio in the culture medium (Satheek and Bhavandan, 1988; Dias *et al.*, 2002). In contrast, our results showed that callus derived from shoot-tip and nodal explants of *M. pulegium* could regenerate on MS medium supplemented with only BA (1 $mg.l^{-1}$).

Previous studies reported the relative importance of explants type for *in vitro* plant regeneration via indirect organogenesis (Mantell and Hugo, 1989; Sharma and Rajam, 1995; Ramarosandratana and Staden, 2003).

The regeneration frequency and the number of shoots per callus varied in nodal and shoot type of explants derived from callus. The callus produced from shoot explants type showed a better response (92%) than the callus produced from the nodal explants (41%) (Unpublished data).

For a further cultures proliferating, shoots were sub-cultured every 4 weeks on fresh medium. This mode of multiplication ensured a continuous supply of shoots for a longer period of time, and no reduction was observed in shoots number or length. Sub-culturing of micro-shoots decreases the effects of competition of the developing shoots for nutriments (Upadhyay *et al.*, 1989; Rout *et al.*, 1999).

During the rooting stage, the micro-shoots regenerated by direct and indirect organogenesis were rooted better in half- MS PGR-free medium than the full- MS PGR-free one. Effectiveness of half MS macro-salts has been reported in many medicinal-plant species (Husain and Anis, 2006; Bouhouche and Ksiksi, 2007). High rooting percentage (100%), longer roots (6-7 cm), large roots- number (12-14 roots.micro-shoot^{-1}) and normally developed roots were established on half-MS medium (PGR- free), whereas full-MS medium (PGR-free) induced abnormal roots. In contrast, many micro-propagation protocols used auxins such as IBA or NAA to induce rooting (Hussey, 1980; Wakhlu and Barna, 1989; Arikat *et al.*, 2004). The auxins use in the rooting medium inhibited roots formation and promoted excessive amount of callus formation; Heloir *et al.* (1997), Sarabjeet Suri *et al.* (1997), Mei-Chun Lu (2005) and Duong Tan Nhut *et al*. (2007) also reported that auxins does not yield roots but produced callus formation. It is also confirmed that the need of auxins for rooting induction is not constant since after root initiation (high auxin requirement), the root- primordium outgrowth requires a low concentration, and a continuous high auxin concentration will inhibit the root elongation (Sarabjeet Suri *et al.*, 1999; Duong Tan Nhut *et al.*, 2007). Rooted plantlets were successfully transferred *ex vitro* in order to acclimatize the plants. In certain plant species, establishment of tissue culture raised plants under greenhouse, and later often very poorly in field. This is mainly attributed to the inability of such plants to tolerate different types of stresses such as excessive water loss (Grout, 1975; Sutter and Langhans, 1979; Krishna *et al.*, 2005). In this study, the primary acclimatization stage under covered plastic greenhouse conditions prevents water loss during the initial stages of plant growth and the survival rate was 95%. Following acclimatization in the greenhouse, regenerated plantlets transferred to field conditions were uniform and identical to the donor plant with respect to growth characteristics in the vegetative stage and the floral one.

In summary, the micro-propagation system developed in this study produced masses of healthy plant materials that would suitable for commercial or biochemical research applications.

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