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Effects of plant growth regulators and explant on callus induction in pennyroyal (*Mentha pulegium* L.)

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Abstract. Plants are the most important sources of medicine. In present era the large number of drugs in use derived from plants. The mint species have a great importance, both medicinal and commercial. The *Mentha pulegium* as an important medicinal plant and belongs to Lamiaceae family has numerous applications in pharmaceutical and food industries. Tissue culture is an age-old practice for *in vitro* regeneration of plants, especially the medicinally valuable plant or plants that are difficult to propagate in natural environment. The aim of current study was to find the best medium composition for callus induction in *M. pulegium*. For this purpose, two explants (leaf and hypocotyl) of *M. pulegium* were cultured on MS medium supplemented with BA (0.0, 0.5 and 1.0 mg/L), NAA (0.5, 1.0 and 2.0) and 2,4-D (2.0 and 4.0 mg/L) in a factorial experiment. The results showed that there were not significant differences ($p<0.01$) among explants types for callus induction percentage and growth rate. The hormone levels of BA-free and 1 mg/L NAA (86.00%), 0.5 mg/l BA and 0.5 mg/l NAA (86.00%), 0.5 mg/l BA and 1 mg/l NAA (92.00%) and 1 mg/l BA and 2 mg/l NAA (88.00%) had the highest effects on callus induction percentage. In addition the hormone levels of BA-free and 1 mg/L NAA identified as the most efficient concentrations (0.25 mm/d) for callus growth rate. Also statistical analysis demonstrated that among interaction affects the hypocotyl explant in BA-free and 1 mg/L NAA was the more efficient effect (0.29 mm/d) for callus induction.

Key words: *Mentha pulegium,* callus Induction, medicinal plant, explant, hormone.

Introduction

Plants are the most important sources of medicine. In present era the large number of drugs in use derived from plants (Satyavati et al. 1976).

The genus *Mentha* includes 25–30 species that grow in the temperate regions of Eurasia, Australia and South Africa. The mint species have a great importance, both medicinal and commercial (Hadjlaoui et al. 2009). The *Mentha pulegium* L. belongs to the *Mentha* genus commonly known as pennyroyal (Mahboubi & Haghi 2008). The pennyroyal as an important medicinal plant has numerous applications in pharmaceutical and food industries. The pennyroyal plant is herbaceous and shrub height to 60 cm that grow in around springs (Darvishi et al. 2014). It is native species of Europe, North Africa and in Asia Minor and near East. The flowering aerial parts of *Mentha pulegium* L. has been traditionally used for its antiseptic for treatment of cold, sinusitis, cholera, food poisoning, bronchitis and tuberculosis, and also as antiflatulent, carminative, expectorant, diuretic, antitussive, menstruate (Mahboubi & Haghi 2008). The main constituent of pennyroyal is the volatile oil pulegone. Other ingredient of pennyroyal contains menthone, (α-) and (β-), pinene and caryophyllene (Zargari 2011).

Tissue culture is an age-old practice for *in vitro* regeneration of plants, especially the medicinally valuable plant or plants that are difficult to propagate in natural environment (Atal & Kapoor 1989). This method is also used for studying the plant development at the molecular level thereby artificially increasing the plant output molecules (Dhar et al. 1968). In vitro propagation is an important tool for rapid multiplication of medicinal plants (Atal & Kapoor 1989, Yew et al. 2010) as well as for the extraction of secondary metabolite products. Plant growth and developmental processes require the action and cross talk of phytohormones including auxins and cytokinins (Bajguz & Piotrowska 2009). Auxins and cytokinins interact in the control of many central developmental processes in plants, particularly in apical dominance and root and shoot development. It was clearly documented that auxin may regulate cytokinin level and metabolism and vice versa (Nordstrom et al. 2004). By adjusting phytohormone concentration in the medium, differences in amount, rate and growth patterns of explants can be observed (Pierik 1987, Ekiz, & Konzak 1997). Generally, high concentration of auxins and low cytokinins in the medium promote abundant cell proliferation with the formation of callus (Shah et al. 2003).

In tissue cultures of *M. pulegium*, few studies have been carried out to date, but there are some reports on this and other species of *Mentha* genus that some of them are mentioned in the following. Darvishi *et al*. (2014) studied the tissue culture of *Mentha pulegium* and in their investigation the MS medium containing two levels of BAP (0.0 and 0.5 mg/l), four levels of 2,4-D (0, 1, 2 and 4 mg/l) and three explant type stem, leaf and root were used for callus production. They reported that leaf explants, BAP-free and 1 mg/L 2,4-D is the most efficient combination for callus induction . Rech & Pires (1986) studied the tissue culture of six species of *Mentha* and in their investigation the lateral buds were used as explants from one-year stock plants. Xu et al. (2009) investigated the callus induction in *Mentha haplocalyx*. They reported that leaf explant, ½MS medium, 1.5 mg/L BA and 1.5 mg/L NAA is the most efficient combination for callus induction. Samantaray et al. (2012) were studied callus induction in *Mentha spicata* for comparison of media and 10 hormone levels. They reported that MS medium that was supplemented with 2.5 mg/L 2,4-D indicated as the best composition for callus induction percentage and callus weight. A high frequency and rapid regeneration protocol was developed from shoot tip and nodal explants of *Mentha piperita* L.

on MS medium supplemented with either BAP or zeatin by Ghanti et al. (2003).

In this study, callus induction of pennyroyal (*M. pulegium*) has been studied. For this purpose different explants and hormone levels were compared.

Materials and Methods

Plant Materials

The pennyroyal (*Mentha pulegium*) was used as plant material in present research. The seeds were collected from around Kermanshah (Latitude: 34° 18'N, longitude: 047°30'E at an elevation of 1374 m above sea level) in the west of Iran. The scientific name was identified in Zagros Bioidea Co., Razi University Incubator, Kermanshah, Iran.

Seed sterilization and germination

The Seeds of pennyroyal were surface sterilized with 70% ethanol for 1 min and then 1.5% sodium hypochlorite for 7 min, thoroughly washed with sterile distilled water four times in the laminar flow hood. The sterilized seeds were then placed on to MS (Murashige and Skoog, 1962) medium containing 0.7% agar and 3% sucrose without any plant growth regulator (PGR) and incubated at 25°C under the 16-h light/8-h dark photoperiod for germination and stock plant elongation.

Callus induction

The MS medium containing 0.7% agar and 3% sucrose was used as medium for callus induction experiment. In this experiment the different concentrations of plant growth regulators (PGRs) including NAA (0.5, 1 and 2 mg/l), BA (0.0, 0.5 and 1 mg/l) and 2,4-D (2 and 4 mg/l) and two plant explant type (leaf and hypocotyl) were compared for callus induction indices in a factorial experiment based on randomized completely design with five replications. The explants were cultured on medium with different hormone balances including fifteen compositions [(0 mg/L BA + 0.5 mg/L NAA), (0 mg/L BA + 1 mg/L NAA), (0 mg/L BA + 2 mg/L NAA), (0 mg/L BA + 2 mg/L 2,4-D), (0.0 mg/L BA +4 mg/L 2,4-D), (0.5 mg/l BA+ 0.5 mg/l NAA), (0.5 mg/l BA+ 1 mg/l NAA), (0.5 mg/l BA+ 2 mg/l NAA), (0.5 mg/L BA +2 mg/L 2,4-D), (0.5 mg/L BA + 4 mg/L 2,4-D), (1 mg/l BA+ 0.5 mg/l NAA), (1 mg/l BA+ 1 mg/l NAA), (1 mg/l BA+ 2 mg/l NAA), (1 mg/L BA + 2 mg/L 2,4-D) and (1 mg/L BA + 4 mg/L 2,4-D)]. The cultures were incubated in dark at 25°C conditions. The callus diameter (mm) was measured in five times (35, 40, 45, 50 and 55 days after explants culture) and growth rate (in mm/d) and Callus Induction Percentage (CIP) were recorded in 55 days after explant culture. Analysis of variance and mean comparison (Duncan's Multiple Range Test) for above traits was performed by MSTATC software.

Results

Explant type: Analysis of variance results (Table 1) demonstrated that there were not significant differences ($p<0.01$) among explants types for callus induction percentage and growth rate.

Hormone concentration: Analysis of variance results (Table 1) indicated that there are significant differences ($p<0.01$) among hormone levels for callus induction percentage and callus growth rate. The mean comparison (Table 2) showed that the hormone levels in BA-free and 1 mg/L NAA, 0.5 mg/l BA and 0.5 mg/l NAA (86.00%), 0.5 mg/l BA and 0.5 mg/l NAA (86.00%), 0.5 mg/l BA and 1 mg/l NAA (92.00%) and 1 mg/l BA and 2 mg/l NAA (88.00%) had the highest effects on callus induction percentage. Furthermore the hormone level in BA-free and 1 mg/L NAA had the highest effects (0.25 mm/d) on callus growth rate.

Interaction effects of explant type × hormone concentration: Analysis of variance results (Table 1) indicated that there is not significant differences ($p<0.05$) among interaction effects of explant type × hormone concentration for callus induction percentage and these effects are significant for callus growth rate. Mean comparison for interaction effects (Table 3) demonstrated that among interaction effects, the hypocotyl explant in BA-free and 1 mg/L NAA was the more efficient effect (0.29 mm/d) for callus induction.

Table 1. Mean squares for explant and hormone effects on callus induction percentage and callus growth rate in *M. pulegium*.

Source of variation	CIP	CGR
Explant (E)	193.835 ^{ns}	0.001 ^{ns}
Hormone (H)	1037.910 **	0.017 **
E × H	93.458 ^{ns}	0.008 *
Error	96.148	0.004
CV	5.54	8.32

Where * and ** significant differences in 0.05 and 0.01 level respectively, ns: Non- significant, CGR: callus growth rate, CIP: callus induction percentage and CV: coefficient of variations.

Table 2. Mean comparison for effect of hormonal level on callus induction percentage and callus growth rate in *M. pulegium*.

Hormonal level (mg/L)	CIP (%)	CGR (mm/d)
BA (0) + NAA (0.5)	74.00 ab	0.24 ab
BA (0) + NAA (1)	86.00 a	0.25 a
BA (0) + NAA (2)	66.00 abcd	0.19 abc
BA (0) + 2,4-D (2)	44.00 cd	0.14 cd
BA (0) + 2,4-D (4)	4.00 f	0.06 e
BA (0.5) + NAA (0.5)	86.00 a	0.18 abc
BA (0.5) + NAA (1)	92.00 a	0.19 abc
BA (0.5) + NAA (2)	74.00 ab	0.13 cde
BA (0.5) + 2,4-D (2)	38.00 de	0.15 cd
BA (0.5) + 2,4-D (4)	12.00 ef	0.10 dc
BA (1) + NAA (0.5)	72.00 abc	0.12 cde
BA (1) + NAA (1)	76.00 ab	0.16 bcd
BA (1) + NAA (2)	88.00 a	0.14 cd
BA (1) + 2,4-D (2)	48.00 bcd	0.12 cde
BA (1) + 2,4-D (4)	0.00 f	0.11 cde

Where CIP: callus induction percentage and GR: growth rate. Similar letters in each column hadn't any significant statistical difference in 0.05 levels.

Discussion

After recording of data related to callus induction percentage and callus growth rate, the statistical analysis of data including analysis of variance and mean comparison were carried out. The characters for callus induction including the callus induction percentage (%) and growth rate (in mm/d) were analyzed. The effects of hormone concentration and explant type on these characters are explained separately.

There was a not significant difference among explant types for callus induction percentage and callus growth rate.

Table 3. Mean comparison for interaction effects of explant × hormone concentrations on callus growth rate in *M. pulegium*.

Hormonal level (mg/L)	GR(mm/d) in leaf explant	GR(mm/d) in hypocotyl explant
BA (0) + NAA (0.5)	0.21 abcd	0.27 ab
BA (0) + NAA (1)	0.21 abc	0.29 a
BA (0) + NAA (2)	0.19 abcde	0.20 abcde
BA (0) + 2,4-D (2)	0.11 cdef	0.18 abcde
BA (0) + 2,4-D (4)	0.05 f	0.08 ef
BA (0.5) + NAA (0.5)	0.21 abc	0.15 bcdef
BA (0.5) + NAA (1)	0.20 abcd	0.18 abcde
BA (0.5) + NAA (2)	0.15 cdef	0.12 cdef
BA (0.5) + 2,4-D (2)	0.22 abc	0.09 def
BA (0.5) + 2,4-D (4)	0.08 ef	0.12 cdef
BA (1) + NAA (0.5)	0.12 cdef	0.13 cdef
BA (1) + NAA (1)	0.20 abcd	0.13 cdef
BA (1) + NAA (2)	0.18 abcde	0.11 cdef
BA (1) + 2,4-D (2)	0.10 cdef	0.14 cdef
BA (1) + 2,4-D (4)	0.05 f	0.18 abcde

Where GR: growth rate. Similar letters in the tables showed that hadn't any significant statistical difference in 0.05 levels.

This result is corresponded with analysis of variance results. Darvishi et al. (2014) reported that there were significant differences ($p<0.01$) among explant types for callus diameters. This effect was not significant for callus growth rate.

The hormone levels in BA-free and 1 mg/L NAA, 0.5 mg/l BA and 0.5 mg/l NAA, 0.5 mg/l BA and 1 mg/l NAA and 1 mg/l BA and 2 mg/l NAA had the highest effects on callus induction percentage. The hormone level in BA-free and 1 mg/L NAA had the highest effects on callus growth rate. The calluses that had been derived from this medium showed more voluminous, embryogenic, and granular, with the high proliferation and fast growth. These calluses are suitable for cell suspension culture. In addition subculture of explants to new medium increases the production rate of callus and cell mass.

Xu *et al*. in 2009 investigated the 1.5 mg/L BA and 1.5 mg/L NAA for callus induction in *Mentha haplocalyx*. They reported that NAA is the most efficient combination for callus induction. The NAA has been the commonly and widely auxin in plant tissue culture. However it is defined as the use of high doses of this auxin leads increase in cell division and cell elongation. Our results have been showed that increase in the concentration of this hormone was a factor in the rapid degradation of explants. This result is consistent with the results of Xu *et al*. (2009). There were not significant effects among other hormone levels. In current research, we found that interaction effects of explant type × hormone concentration for callus induction percentage measurements were not significant and these effects are significant for callus growth rate.

It is concluded that among interaction effects the hypocotyl explant in 1 mg/l NAA and BAP-free was the more efficient effect for callus induction; it is recommended that this composition be used for callus production and cell suspension culture in pennyroyal (*Mentha pulegium*).

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