Journal of **A**pplied **B**iotechnology **R**eports

Original Article

Optimization of Callus Induction in Pennyroyal (*Mentha pulegium*)

Elaheh Darvishi[1], Elham Kazemi[2, 3], Danial Kahrizi[1*], Sayed Reza Chaghakaboudi[4], Yavar Khani[1]

Abstract

Findings in relation to the inhibitory effects of natural substances against microorganisms showed that herbal products may be alternatives to synthetic and chemical drugs due to their significant therapeutic effects. The pennyroyal (*Mentha pulegium*) as an important medicinal plant, which belongs to the Lamiaceae family is applied in the pharmaceutical and food industries. In recent years, plant tissue culture techniques have appeared as a powerful tool for the micropropagation and breeding of many plant species. The aim of current study was to find the best medium composition for callus induction in *M. pulegium*. For this purpose, three explants (leaf, root and stem) of *M. pulegium* were cultured on MS medium supplemented with BAP (0.5 and 1.0 mg/L) and 2,4-D (0.0, 1.0, 2.0 and 4.0 mg/L) in a factorial experiment. The results showed that the leaf explant had the highest effect on callus induction (9.65 mm in callus diameters at 28 days after culture) and hormone levels of 1 mg/L 2,4-D and BAP-free were identified as the most efficient concentrations for callus growth rate (0.29 mm/d). Also statistical analysis demonstrated that among interaction effects the leaf explant in 1 mg/l 2,4-D and BAP-free was found as the highest effect for callus induction (12.35 mm in callus diameter at 28 days after culture).

1. Agronomy and Plant Breeding Department, Razi University, Kermanshah, Iran
2. Science and Research Branch, Islamic Azad University, Kurdistan, Iran
3. Social Security Organization, Kermanshah, Iran
4. Environmental Protection Office, Kermanshah, Iran

* **Corresponding Author**
Danial Kahrizi
Agronomy and Plant Breeding Department, Razi University, Kermanshah, Iran
E-mail: dkahrizi@yahoo.com

Submission Date: 6/10/2014
Accepted Date: 7/14/2014

Keywords: *Mentha pulegium*, Callus Induction, Medicinal Plants, Explant, Hormone

Introduction

Medicinal plants are an important source of drugs that had been used thousands years ago. Herbal therapy is a branch of traditional medicine in the last hundred years and has had a significant role in determining the treatment of diseases. Many chemical agents are made using plant material [1]. With the advent of synthetic drugs and replacing herbal medicines with them, the medicinal plants gradually faded. Then chemical drugs and antibiotics were used in the treatment of a wide variety of diseases due to the high speed of treatment. Due to harmful side effects of chemical drugs for human health, in recent years, attention and request of people to use herbal medicines to treat diseases has increased. As a third to a half of pharmaceutical products in United States of America are plant-derived [2]. Resistance to chemical drugs and adverse effects of drugs are a threat to human health; however people with low immune are more vulnerable. In recent years, researches on the effects of natural inhibition against microorganisms showed that herbal products should be alternatives to synthetic drugs and may have a significant therapeutic effect [3].

Pennyroyal (*Mentha pulegium*) as an important medicinal plant belonging to Lamiaceae family, has numerous applications in pharmaceutical and food industries. The pennyroyal plant is herbaceous and shrub height to 60 cm that grow in around springs. All aerial parts of the plant have medicinal properties. This medicinal plant is used as analgesic, anti-bacterial, fungicide, stomach tonic and etc. The main constituent of pennyroyal is the volatile oil pulegone. Other ingredient of pennyroyal contains menthone, (α-) and (β-), pinene and caryophyllene [4].

Many of medicinal plants have limited natural habitats and only grow in certain geographical and environmental conditions. With regard to this matter, collecting them is facing problems. In addition excessive harvesting may also be associated with risk of extinction germplasm. Hence attentions are focused on the use of biotechnology strategies to increase production and productivity of medicinal plants. Using of biotechnology and sciences such as genetics, biology, biochemistry and strategies using cell, tissues and organs culture and genetic engineering of plants to produce the drug is able to increase the quantity and quality [5-6]. In recent years, the tissue culture techniqes as an effiecient and powerfull tools has been applied for plant micropropagation and breeding [7]. Plant tissue culture has numerous applications in the field of medicinal plants; such as rapid and mass plant multiplication, off-season and year round medicinal plants production, pathogen-free plants production, enhance performance and yield, protect endangered species and the in vitro production of secondary metabolites [8, 5, 7]. In in-vitro culture, the primary source of drugs in controlled and sterile conditions, independent of the environment,

All rights reserved for official publication of Baqiyatallah university of medical sciences©

increased production of the desired compounds and produce new compounds with methods of tissue culture and cell culture is possible [9].

In tissue cultures of *M. pulegium*, few studies have been carried out to date, but there are some reports on other species of Menthe genus that some of them are mentioned in the following. Rech and 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 [10]. Chakraborty and Chattopadhyay (2008) induced the menthone production in pennyroyal cell culture [11]. 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 [12]. Callus induction was studied in *Mentha spicata* for comparison of media and 10 hormone levels by Samantaray et al. (2012) [13]. They reported that MS medium supplimented with 2.5 mg/L 2,4-D indicated as the best composition for callus induction percent and callus weight.

Due to the many uses of this medicinal plant in industry and its economic importance, proliferation and expansion of this plant as in vitro or in greenhouse conditions is important [7]. 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

Medicinal plant pennyroyal (*M. pulegium*) was used as plant material in current 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.

Seed sterilization and germination

Seeds of pennyroyal (*M. pulegium*) were surface-disinfected with 70% ethanol for 1 min, surface sterilized with 1.5% sodium hypochlorite for 7 min, thoroughly washed with sterile distilled water four times and were blot-dried briefly in the laminar flow hood. The sterilized seeds were then placed on to MS medium (Murashige and Skoog, 1962) [14] 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 plant elongation.

Callus induction

The MS medium containing 0.7% agar and 3% sucrose was used as basal medium for callus induction experiment. 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 compared for callus production indices in a factorial experiment based on completely randomized block design in three replications. The explants were cultured on medium with different hormone balances including eight compositions [(0 mg/L BAP + 0 mg/L 2,4-D), (0 mg/L BAP + 1 mg/L 2,4-D), (0 mg/L BAP + 2 mg/L 2,4-D), (0 mg/L BAP + 4 mg/L 2,4-D), (0.5 mg/L BAP +0 mg/L 2,4-D), (0.5 mg/L BAP +1 mg/L 2,4-D), (0.5 mg/L BAP + 2 mg/L 2,4-D) and (0.5 mg/L BAP + 4 mg/L 2,4-D)]. The cultures were incubated in dark at 25°C conditions. The callus diameter (in mm) was measured in two times (21 and 28 days after explant culture) and growth rate (in mm/d) was recorded in 28 days after explant culture. Analysis of variance and mean comparison (Duncan's test) for above traits was performed by MSTATC software.

Results

Explant type

Analysis of variance results (Table 1) demonstrated that there is significant different ($p<0.01$) among explant types for callus diameters in first and second measurements (21 and 28 days after culture). This effect was not significant differences for callus growth rate. The statistical analysis of data (Table 2) showed that the leaf explant had the highest effects on callus diameter (8.62 and 9.65 mm in first and second measurements respectively). Mean comparison indicated that there is not significant differences among explant types for callus growth rate. The lowest rank for callus diameter was related to leaf explant (6.67 and 7.80 mm in first and second measurements respectively).

Table 1. Mean squares for callus diameters and callus growth rate in *M. pulegium*

Source of variation	CD_1	CD_2	GR
Replication	0.49 ^{ns}	1.36 ^{ns}	0.008 ^{ns}
Explant (E)	22.91**	20.64**	0.002 ^{ns}
Hormone (H)	81.06**	77.27**	0.032**
E × H	1.55 ^{ns}	2.27*	0.012 ^{ns}
Error	1.17	1.16	0.007
CV	14.23	12.44	13.05

Where * and ** significant differences in 0.05 and 0.01 level respectively, ns: Non- significant, GR: growth rate, CD: callus diameters in first measurement (21 days after explant culture) and CD2: callus diameters in second measurement (28 days after explant culture).

Table 2. Mean comparison for effect of explant type on callus diameters and callus growth in *M. pulegium*

Explant	CD_1 (ml)	CD_2(ml)	GR(ml/d)
Stem	8.62 ^{a}	9.65 ^{a}	0.15 ^{a}
Leave	6.67 ^{c}	7.80 ^{c}	0.16 ^{a}
Root	7.56 ^{b}	8.59 ^{b}	0.15 ^{a}

Where GR: growth rate, CD: callus diameters in first measurement (21 days after explant culture) and CD2: callus diameters in second measurement (28 days after explant culture). Similar letters in each column hadn't any significant statistical difference in 0.05 levels.

Hormone concentration

Analysis of variance results (Table 1) indicated that there are significant differences ($p<0.01$) among hormone levels

for callus diameters in first and second measurements (21 and 28 days after culture) and callus growth rate. The statistical analysis of data (Table 3) showed that the hormone level in 1 mg/L 2,4-D and BAP-free had the highest effects on callus diameter (8.60 and 10.66 mm in first and second measurements, respectively).

Table 3. Mean comparison for effect of 2,4-D concentration on callus diameters and callus growth for seven-day in *M. pulegium*

2,4-D concentration (mg/L)	CD_1(ml)	CD_2(ml)	GR(ml/d)
BAP (0) +2,4-D (0)	2.68 ^{c}	4 ^{b}	0.18 ^{b}
BAP (0) + 2,4-D (1)	8.60 ^{b}	10.66 ^{a}	0.29 ^{a}
BAP (0) + 2,4-D (2)	8.84 ^{ab}	9.83 ^{a}	0.14 ^{b}
BAP (0) + 2,4-D (4)	9.83 ^{a}	10.59 ^{a}	0.10 ^{b}
BAP (0.5) +2,4-D (0)	2.93 ^{c}	3.94 ^{b}	0.14 ^{b}
BAP (0.5) + 2,4-D (1)	9.59 ^{ab}	10.32 ^{a}	0.12 ^{b}
BAP (0.5) + 2,4-D (2)	9.58 ^{ab}	10.45 ^{a}	0.14 ^{b}
BAP (0.5) + 2,4-D (4)	8.89 ^{ab}	9.68 ^{a}	0.13 ^{b}

Where GR: growth rate, CD1: callus diameters in first measurement and CD2: callus diameters in second measurement. Similar letters in each column hadn't any significant statistical difference in 0.05 levels.

The mean comparison indicated that the hormone level in 1 mg/L 2,4-D and BAP-free had the highest effects on callus growth rate (0.29 mm/d). The calluses that had been derived from this medium showed more voluminous, embryogenic, granular, crisp white, with the high proliferation and fast growth (Fig. 1).

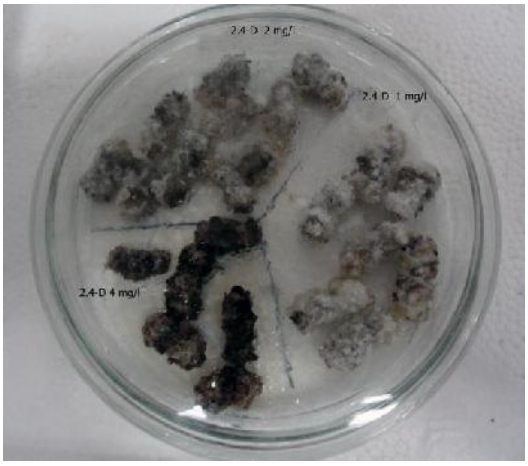

Figure 1. Callus leaf explant-derived in different 2,4-D concentrations. Callus browning is caused by increasing 2,4-D concentration. For better comparison, all calluses derived from different 2,4-D concentrations have been collected in one petridish.

Interaction effects of explant type × hormone concentration

Analysis of variance results (Table 1) indicated that there are significant differences ($p<0.01$) among interaction effects of explant type × hormone concentration for callus diameter in second measurements (28 days after culture) and these effects are not significant for callus diameter in first measurements (21 days after culture) and callus growth rate.

Statistical analysis and mean comparison for interaction effects (Table 4) demonstrated that among interaction effects the leaf explant in 1 mg/L 2,4-D and BAP-free had the more efficient effect on callus induction (12.35 mm in callus diameters at 28 days after culture).

Table 4. Mean comparison for interaction effects of explant × hormone concentrations on callus diameters in second measurement in *M. pulegium*

Explant	Hormonal level/ (mg/L)	CD_2 (ml)
Stem	BAP (0) + 2,4-D (0)	4.33 ^{g}
	BAP (0) + 2,4-D (1)	12.35 ^{a}
	BAP (0) + 2,4-D (2)	10.86 $^{a-d}$
	BAP (0) + 2,4-D (4)	11.53 ^{ab}
	BAP (0.5) + 2,4-D (0)	4.63 ^{g}
	BAP (0.5) + 2,4-D (1)	11.20 $^{a-c}$
	BAP (0.5) + 2,4-D (2)	11.17 $^{a-d}$
	BAP (0.5) + 2,4-D(4)	11.17 $^{a-d}$
Leaf	BAP (0) + 2,4-D (0)	3.76 ^{g}
	BAP (0) + 2,4-D (1)	10 $^{b-e}$
	BAP (0) + 2,4-D (2)	7.40 ^{f}
	BAP (0) + 2,4-D (4)	9.70 $^{b-e}$
	BAP (0.5) + 2,4-D (0)	3.58 ^{g}
	BAP (0.5) + 2,4-D (1)	4.40 $^{c-e}$
	BAP (0.5) + 2,4-D (2)	9.06 $^{d-f}$
	BAP (0.5) + 2,4-D (4)	9.53 $^{b-e}$
Root	BAP (0) + 2,4-D (0)	3.90 ^{g}
	BAP (0) + 2,4-D (1)	9.62 $^{b-e}$
	BAP (0) + 2,4-D (2)	11.25 $^{a-c}$
	BAP (0) + 2,4-D (4)	10.53 $^{a-d}$
	BAP (0.5) + 2,4-D (0)	3.60 ^{g}
	BAP (0.5) + 2,4-D (1)	10.37 $^{a-e}$
	BAP (0.5) + 2,4-D (2)	11.11 $^{a-d}$
	BAP (0.5) + 2,4-D (4)	8.36 ^{ef}

Where CD2: callus diameters in second measurement. Similar letters in each column hadn't any significant statistical difference in 0.05 levels.

Discussion

After recording of data related to callus diameter and growth rate, the statistical analysis of data including analysis of variance and mean comparison were carried out. Three characters for callus induction including the callus diameter (in mm) was measured in two times (21 and 24 days after explant culture) and growth rate (in mm/d) were analyzed. The effects of hormone concentration and explant type on these characters are explained separately.

Leaf explant showed the highest effects on callus diameter in first and second measurements. The calluses that had been derived from this medium demonstrated more voluminous, embryogenic, granular, crisp white, with the high proliferation and fast growth. Thus these callus types are appropriate for cell suspension culture. There was a not significant difference among explant types for callus growth rate. This result is corresponded with analysis of

variance results.The statistical analysis of data showed that the lowest rank for callus diameter was related to leaf explant.

The hormone level in 1 mg/L 2,4-D and BAP-free had the highest effects on callus growth rate. The calluses derived from this medium showed a more voluminous, embryogenic, granular, crisp white, 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.

Samantaray et al. in 2012 suggested the 2.5 mg/L 2,4-D for callus induction in Mentha spicata. This result may be due to the use of different species obtained [13]. The 2,4-D has been the commonly and widely auxin in plant tissue culture. However it is defined that the use of high doses of this auxin leads an increase in cell division and cell elongation. Our results have been showed that an increase in the concentration of this hormone was a factor in the rapid degradation of explant (Fig. 1). This result is not consistent with the results of Aniel Kumar et al. (2010) [15]. There were not significant effects among other hormone levels.

In current research, we found that interaction effects of explant type × hormone concentration for callus diameter in second measurements (28 days after culture) was significant and these effects are not significant for callus diameter in first measurements (21 days after culture) and callus growth rate.

Conclusion

Statistical analysis and mean comparison for interaction effects demonstrated that among interaction effects the leaf explant in 1 mg/l 2,4-D 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*).

Acknowledgments

Thanks to Biotechnology Unit of Razi University Incubator for all supports.

References

1. Clark, A.M., Natural products as a resource for new drugs. *Pharm Res*, 1996, Vol. 13, pp. 1133-1141.
2. Molsaghi, M., Moieni, A., Kahrizi, D., Efficient protocol for rapid *Aloe vera* micropropagation. *Pharm Biol*, 2014, Vol. 52(6), pp.735-739.
3. Nassiri Asl, M., Hosseinzadeh, H., Review of pharmacological effects of *Glycyrrhiza sp*. and its bioactive compounds. *Phytother Res*, 2008, Vol. 22, pp. 709-724.
4. Zargari, A., Medecinal Plant, 2011.Tehran University, Press.
5. Mulabagal, V., Tsay, H.S., Plant cell cultures an alternative and efficient source for the production of biologically important secondary metabolites. Int. *J Appl Sci Eng*, 2004, Vol .2, pp. 29-48.
6. Kumar, J., Gupta, P.K., Molecular approaches for improvement of medicinal and aromatic plants. *Plant Biotech Rep*, 2008, Vol. 2, pp. 93-112.
7. Kahrizi, D., Arminian, A., Masumi Asl, A., In vitro Plant Breeding. 2011, Razi University. Press.
8. Tripathi, L., Tripathi, J.N., . Role of biotechnology in medicinal plants. Trop. *J Pharm Res*, 2003, Vol. 2, pp. 243-253.
9. Bourgaud, F., Gravot, A., Milesi, S., Gontier, E., Production of plant secondary metabolites: a historical perspective. *Plant Sci*, 2001, Vol. 161, pp. 839-851.
10. Rech, E., Pires, M., Tissue culture propagation of Mentha spp. by the use of axillary buds. *Plant cell reports*, 1986, Vol. 5, pp. 17-18.
11. Chakraborty, A., and Chattopadhyay, S. 2008. Stimulation of menthol production in *Mentha piperita* cell culture. In Vitro Cell Dev-Pl. 44: 518-524.
12. Xu, Y., Wei Lin, L., Cheng Yuan, L., Yan, L., Induction and multiplication of leaf callus from two cultivars of *Mentha haplocalyx*. *J Plant Resour Environ*, 2009, Vol.18, pp. 84-88.
13. Samantaray, A., Sial, P., Kar, M., Micro-propagation and biochemical analysis of Spear Mint (*Mentha spicata*). *Indian J Innov Dev*, 2012, Vol. 1, pp. 489-493.
14. Murashig, E.T., Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*, 1962, Vol. 15(3), pp. 473-497.
15. Aniel Kumar, O., Tata, S.S., Rupavati, T., In vitro induction of callusogenesis in chilli peppers (*Capsicum annum L.*). *Int J Curr Res*, 2010, Vol. 3, pp. 42-45.