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Efficient *in vitro* Regeneration System for Conservation of *Levisticum officinale*: A Rare Medicinal Plant

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ABSTRACT

One of the benefits of in vitro culture is conservation of endangered species. In this study, an efficient protocol for plant regeneration from callus cultures derived from leaves, petioles and roots was developed for Levisticum officinale a wild endangered medicinal plant. The effect of various concentrations of cytokinins on plant regeneration was examined. The calli derived from leaf, petiole and root were cultured on MS media supplemented with 6-Benzylaminopurine(BAP) and Kinetin (0.5-2.0 mg/l) along with 0.1 mg/l naphthalene acetic acid (NAA). Somatic embryos were only observed when leaf derived calli cultured on MS medium supplemented with BAP. The most effective regeneration response was observed when leaf derived calli were cultured in media supplemented with BAP. It was found root formation was only seen in presence of Kinetin and application of BAP was failed for root formation.

KEY WORDS: *Levisticum officinale*, Regeneration, Cytokinin, Somatic embryo

1. INTRODUCTION

Levisticum officinale Koch. (Lovage), belongs to Apiaceae family, is a perennial plant originating from West Asia (Iran) [1] and growing in many European countries and North America [2]. It grows as a wild medicinal plant in a limited area of Hezar Mountains situated in south East of Iran. Different parts of the plant being strongly aromatic and have been used in different futures such as perfumery, food, beverage and tobacco industries. The roots have also been used in medicine for centuriesand possessing carminative and spasmodic activity [3]. Recently several studies reported positive health effects of this plant such as anti-inflammatory, anti-tumor, anti-thrombotic activity and positive effect against neurological disorders [4].

Due to its multipurpose importance, locals have been extensively collected the plant and sell in market for their more income. However, low seed maturation which was the reason ofstiff competition in eradication of whole vegetative parts anddisruption of reproductive stage, recent drought and presence of seed dormancy restricted propagation of this important medicinal plant. Overall, all mentioned items caused this plant endangered and rare in its natural habitat. So, genetic conservation of Lovage is necessary to protect this plant as well as propagation and domestication.Biotechnological approaches such as tissue culture and micropropagation system offer an excellent solution for rapid and mass propagation and thereby conservation of rare and endangered medicinal plants [5].

Micropropagation provides an opportunity to provide a large number of uniform plants around the year. In this technique all generated plants origin from one cell, so it is a powerful tool for genetic improvement of valuable plants [6]. In somatic embryogenesis pathway, somatic cells change to structures which seem zygotic embryos. These are bipolar and without vascular connections to the parental tissue [7]. Plant propagation via somatic embryogenesis is a useful tool to obtain large number of plants in a short time and limited space using a small piece of plant material and with minimal impact on the native populations [8]. The main advantages of medicinal plants micropropagation are: higher rate of multiplication, availability of plants all year rounds, selection and production of clones with suitable characters, mass production of secondary metabolites, conservation of rare and endangered plants[9].Callus culture is a well-known type of tissue culture techniques which is used for micropropagation and plant regeneration.

There is only one report on somatic embryogenesis of this species using root segments for large-scale multiplication [10]. The aim of this work was to introduce an in vitro regeneration method for *L. Officinale* using different explants and different plant growth regulators.

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2. MATERIALS AND METHODS

The mature seeds of *L. officinale* were collected from natural habitat of Hezar Mountains of Kerman situated inSouth East of Iran. The seeds were raised into seedlings aseptically, as described before [11].The four-week old healthy seedlings were used as the source of explants.

Briefly different explants (leaf of 5 mm^2, root and petiole of 10 mm) were excised sterile seedlings. Explants were cultured on sterile MS medium fortified with different of auxin (a-naphthalene acetic acid (NAA), 2, 4-dichlorophenoxyacetic acid (2, 4-D), Indole butyric acid (IBA) and Picloram (Pi) for callus induction. Induced calli were subcultured into fresh media every 30 days, up to two times. Blackened and dead parts of the callus were removed during every subculture. The callus induction efficiency was evaluated by callus induction percentage and degree of callus, required time for beginning of callus initiation.

Plant regeneration from callus cultures

To evaluate the plant regeneration, induced calli were transferred to MS medium supplemented with 0.1 mg/l NAA along with different concentrations (0, 0.5, 1.0 and 2.0 mg/l) of 6-benzyl amino purine (BAP) or Kinetin. Calli were subcultured every 30 days in fresh medium. After 90 days of culture, the following parameters were recorded to evaluate regeneration efficiency: number of shoot, number of roots, shoots and root length was calculated. Regeneration frequency was calculated as the percentage of calli that produced shoots after 5 weeks.

Statistical analysis

For regeneration experiment three replicate were carried out. All collected data were analyzed using SAS software version 9.3. The means and the differences within the treatments were compared using one-way analysis of variance (ANOVA) and the differences among means were compared by Duncan. Results were expressed as the mean ± SE and differences in mean values were considered to be statistically significant at $p < 0.05$.

3. RESULTS

According to previous study, all three used explants including root, leaf and petiole could successfully produce callus in media supplemented with different auxins (2, 4-D, NAA, IBA and Picloram). It was proved calli were different in percentage, morphology and texture [11]. In the present study, the regeneration ability of induced calli was evaluated. To achieve this aim, obtained calli were sub cultured on MS medium fortified with 0-2 mg/l BAP or Kinetin in combination to 0.1 mg/l NAA. Cultures were incubated in growth cabinet at 25±1°C under 16-h photoperiod. There were significant differences between regeneration frequency and the number of shoots per callus depends on source of explant. Whitish spots started emerging on the various calli on the 4th weeks (figure1 a).

Results showed that shoot regeneration was observed in all used media whereas root formation was just observed in media containing Kinetin (fig.1c-d). Finally after 90 days mature plantlets were observed (fig1. f).

Regeneration of calli derived from root explants

Results of regeneration of root derived calli revealed that the largest frequency of regeneration percentage (20.66%) was observed in MS medium supplemented with 2 mg/l Kinetin along with 0.1 NAA. In this medium the highest shoot number (7.66) and mean of shoot length (37.33mm) was produced too. According to the table 1 there was not any significant difference between different applied concentrations of kinetin on number of produced roots, but the treatment that yielded the greater root length was 1 mg/l Kinetin (Table.1).

Table 1.Indirect shoot regeneration from callus obtained through root explants

Plant growth regulator	C (mg/L)	Regeneration Frequency (%)	Mean number of shoots	Mean shoot length (mm)	Mean number of roots	Mean root length (mm)
NAA(0.1mg/l) +K	0.5	$13.00^{c}\pm0.57$	$2.00^{d}\pm0.08$	$11.10^{e}\pm0.85$	$1.05^{a}\pm0.00$	$3.05^{b}\pm0.05$
	1.00	$15.67^{bc}\pm0.53$	$2.80^{d}\pm0.57$	$22.33^{b}\pm1.52$	$1.38^{a}\pm0.57$	$5.33^{a}\pm0.55$
	2.00	$20.66^{a}\pm2.08$	$7.66^{a}\pm0.57$	$37.33^{a}\pm2.51$	$1.05^{a}\pm$	$2.38^{c}\pm0.50$
NAA(0.1mg/l) +BAP	0.5	$15.33^{bc}\pm1.52$	$3.66^{c}\pm0.28$	$14.16^{cd}\pm0.76$	NR	NR
	1.00	$17.66^{ab}\pm2.51$	$6.16^{b}\pm0.65$	$16.66^{c}\pm1.25$	NR	NR
	2.00	$12.66^{c}\pm2.51$	$2.33^{d}\pm0.40$	$12.05^{de}\pm0.91$	NR	NR

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range tests ($P \geq 0.05$).
NR= No response was observed

Regeneration of calli derived from leaf explants

According to previous study, the culture of leaf explants on MS medium supplemented with 2, 4-D and NAA caused the formation of globular-like structures, called protocorm like bodies (PLBs) on adaxial surface of the calli. In this study, leaf-derived calli were sub-cultured on MS medium supplemented with 1.0 mg/l BAP and 0.1 mg/l NAA for two times with one month interval. Our observations showed that somatic embryos were visible on the surface of callus (fig. 1b).The six weeks later somatic embryos matured and shoot formation was started (fig.1d). The results obtained from leaf derived calli showed that the highest frequency of regeneration which was 27.66%, the highest shoot length which was 61.25 mm and shoot number (11.96) was observed on MS medium supplemented with 2.0 mg/l BAP and 0.1mg/l NAA. Also, it was found that root formation was only seen in presence of Kinetin and application of BAP was failed for root formation (fig. 1e). Our data revealed that there was not any significant difference between various concentration of Kinetin on mean number of root, but the highest length of root was obtained when 1 mg/l Kinetin + 0.1 mg/l NAA was used (Table.2).

Fig 1. Somatic embryogenesis and plant regeneration in *Levisticum officinale* Koch.
(a) induced calli from leaf segments, (b) Somatic embryos on the surface of leaf derived calli, globular and hurt shape embryos are showed by arrows, (d) Cotyledonary stage, (d)
(e) young plantlet. (f) mature Plants.

Table 2.Indirect shoot regeneration from callus obtained through leaf explants.

Plant growth regulator	C (mg/L)	Regeneration Frequency (%)	Mean number of shoots	Mean shoot length (mm)	Mean number of roots	Mean root length (mm)
NAA(0.1mg/l) +K	0.5	9.00 ^{d}±1.00	2.66^{d}±0.57	26.16^{d}±1.25	1.05^{ab}±0.01	9.05b±1.00
	1.00	13.0 ^{c}±1.73	10.00^{b}±1.00	45.87^{c}±3.23	1.71^{a}±0.57	13.55a±1.50
	2	12.33^{c}±1.50	5.00^{c}±1.00	11.00 ^{e}±1.00	1.51^{a}±0.44	10.38b±1.52
NAA(0.1mg/l) +BAP	0.5	24.00^{b}±1.70	10.00^{b}±1.00	51.00^{b}±3.00	NR	NR
	1.00	26.00^{ab}±2.00	10.83^{ab}±1.44	57.48^{a}±2.81	NR	NR
	2.00	27.66^{a}±2.50	11.96^{a}±1.06	61.25^{a}±1.25	NR	NR

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range tests ($P \geq 0.05$).
NR= No response was observed

Regeneration of calli derived from petiole explants

To determine the shoot regeneration potential of petiole segments, effect of different concentrations of Kinetin and BAP were examined. Results showed that Kinetin was more efficient than BAP. The results revealed that the effect of different concentrations of Kinetin on regeneration frequency was the same, while 1 mg/l Kinetin had the most positive effect on shoot number and shoot length. The maximum number of shoot (5.56mm) and shoot length (21mm) was seen in medium fortified with 1mg/l Kinetin. There was not any significant difference between three used concentrations of kinetin on root length but 2mg/l kinetin produced less number of roots (table.3).

Table 3.Indirect regeneration from callus obtained through petiole explants.

Plant growth regulator	C (mg/L)	Regeneration frequency (%)	Mean number of shoots	Mean shoot length (mm)	Mean number of roots	Mean root length (mm)
NAA(0.1mg/l) +K	0.5	16.67^{a}±1.15	5.00^{ab}±0.01	17.75^{b}± 1.2	1.55^{a}±0.40	6.33^{a}±1.00
	1.00	18.00^{a}±2.00	5.56^{a}±0.51	21^{a}±2.64	1.65^{a}±0.47	7.21^{a}±0.76
	2.00	17.00^{a}±2.00	4.33^{bc}±0.57	17.5^{b}±1.50	0.55^{b}± 0.04	6.55^{a}± 1.50
NAA(0.1mg/l) +BAP	0.5	12.66^{b}±0.66	2.00^{d}±0.01	10.33^{d}±0.57	NR	NR
	1.00	10.66^{b}±1.15	4.66^{b}±0.57	13.74^{c}±1.72	NR	NR
	2.00	11.33^{b}±1.10	3.66^{c}±0.57	7.11^{e}±1.16	NR	NR

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range tests ($P \geq 0.05$).
NR= No response was observed

DISCUSSION

Cytokinins play an important role in plant growth and take part in the maintenance of meristem [12]. In tissue cultures, cytokinins are required for plant cell division. They are necessary for regulation of the synthesis of proteins which are involved in the formation and function of the mitotic spindle apparatus. Also they contribute in division of cell nuclei. n this study regeneration traits were influenced by source of explants, type and concentration of plant growth regulators. Among three explants (root, leave, petiole), leaves showed the best responses in comparison to root and petiole. Somatic embryos were observed in leaf derived calli. Somatic embryos are bipolar structures containing both shoot and root meristems. These structures pass through the distinct globular, heart, torpedo, cotyledonary and mature structural stages. Somatic embryogenesis can occur directly or indirectly. In direct embryogenesis embryos directly create from explants cultured on medium supplemented with proper plant growth regulator without callus induction. However, the indirect embryogenesis in which embryos induce through an intervening callus phase is generally more common [13].

According to the results the highest regeneration frequency (27.66%), shoot number (11.96) and shoot length (61.25 mm) was achieved for leaf explants. The less responses were seen when calli from petiole segments were evaluated for plant regeneration. Statistical analysis proved differences in regeneration between two types of applied cytokinins (BAP and Kinetin). Regard to data obtained from leaf derived calli, it was found BAP was superior to Kinetin. However, it was observed that by increase in the concentration of BAP rate of regeneration frequency was increased as well as number and length of produced shoot. This difference between root, petiole and leaf explant response to BAP and kinetin is probably a reflection of different levels of endogenous growth regulators in the explant sources or different tissue sensitivities to these plant growth regulators [14].

The effectiveness of interactive effect of BAP and NAA on multiple shoot differentiation has also been demonstrated in *Polygonatum verticillatum* [15] and *Elaeagnus angustifolia* [16]. Similarly Singh et al (2013) reported MS medium supplemented with BAP along with NAA was the most effective medium for regeneration of *Naringi crenulata* from leaf explants [17].

Results of our study are in agreement with those studies which demonstrated that low concentrations of NAA in combination with BAP were successful for proliferation and shoot differentiations [18]. Although somatic embryogenesis is related to exogenous auxins inside the medium but the effect of BAP in combination with NAA in plants such as Lovage shows the unique effect of BAP and maybe is related to effect of BAP on gene activity. Interestingly, root induction was observed in media containing Kinetin. It was proved that kinetin was more effective than BAP for root induction. Among different applied concentration of kinetin, concentration of 1mg/l caused the best responses. There was not any significant difference between concentrations of 1mg/l and 2 mg/l. The negative effects of cytokinins on shoot rooting have been reported [19]. Also it was showed root formation *of Lilium longifolium* was inhibited by BAP [20].

In conclusion, *L. officinale* presented a good response in terms of in direct somatic embryogenesis. Our results demonstrated that leaf derived callus has high potential to induce somatic embryo, embryo maturation, and regeneration of plantlets in a medium fortified with BAP.

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