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Research Note

Somatic embryogenesis and plant regeneration of lovage (*Levisticum officinale* Koch)

Woo Tae Park[1], Yong Kyoung Kim[1], Md. Romij Uddin[1], Nam Il Park[1], Su Gwan Kim[2], Sook Young Lee[2,*] and Sang Un Park[1,]**

[1]Department of Crop Science, Chungnam National University, 79 Daehangno, Yuseong-Gu, Daejeon, 305-764, Korea
[2]Medical device clinical center, Chosun University, 375 Seosuk-Dong, Dong-Gu, Gwangju, 501-759, Korea

***Corresponding author: seedbank2001@hanmail.net; supark@cnu.ac.kr**

Abstract

Lovage (*Levisticum officinale* Koch.), whose leaves and seeds are used to flavor food, also has medicinal uses. In order to establish a multiple micropropagation and genetic transformation protocol for lovage, a reliable and highly efficient method for the regeneration of intact plants from *in vitro* culture is essential. In the current study, a rapid protocol was developed for somatic embryogenesis and plant regeneration from lovage leaf and stem callus cultures using 2,4-dichlorophenoxyacetic acid (2,4-D). We found that the stem source was more effective for induction of both embryogenic calluses and somatic embryos. Nevertheless, in both cases, 2,4-D at 0.1 mg/l was optimally effective for maximum induction of embryogenic calluses and somatic embryos per explant. After germination of somatic embryos, regenerated plantlets were recovered on medium containing half-strength Murashige and Skoog (MS) salts and vitamins without plant growth regulator. This system for continuous production of *L. officinale* somatic embryos and regenerated plants could be used for micropropagation of other species as well as for the regeneration of transgenic plants.

Key words: *Levisticum officinale*, lovage, embryogenic callus, plant regeneration, somatic embryogenesis, micropropagation
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; MS, Murashige and Skoog

Introduction

Lovage (*Levisticum officinale* Koch.) is a perennial herb of the family Umbelliferae, and its leaves and seeds are used to flavor food, especially in South European cuisine. Lovage has been used for medicinal purposes as early as the 14th century. The lovage root is used as a diuretic drug and, as such, may be available from pharmacies (Yarnell, 2002). All parts of the lovage plant contain essential oils, and of these, ligustilide**,** a naturally occurring phthalide, is the most abundant bioactive ingredient in the essential oils contained in the roots of *L. officinale* (Gijbels et al., 1982; Penka and Kocabova, 1962; Segebrecht and Schilcher, 1989). *L. officinale* exhibits various pharmacological and biological activities, including estrogenic (San Martin, 1958), apoptotic (Bogucka-Kocka et al., 2008), and antimycobacterial (Schinkovitz et al., 2008) activities. Steward et al. (1958) originally observed plant regeneration by somatic embryogenesis from cultured carrot cells. In somatic embryogenesis, somatic cells develop by division to form complete embryos analogous to the development of zygotic embryos. The bipolar structure of the somatic embryo contains both shoot and root meristems. As the embryos develop, they progress through the distinct globular, heart, torpedo, cotyledonary, and mature structural stages. Somatic embryogenesis can occur directly from cells of the explant tissue without an intervening callus phase. However, the indirect embryogenesis pathway, where somatic embryos are induced and develop from a proliferated callus, is generally more common (Pierik, 1987; Rashid, 1988). A reliable and highly efficient method for the regeneration of intact plants from *in vitro* culture is essential for establishing a multiple micropropagation and genetic transformation protocol for lovage. In this paper, we describe the first development of a method for high-frequency somatic embryogenesis and plant regeneration of *L. officinale.*

Materials & methods

Seed sterilization and germination

For preparing plant materials, seeds of *L. officinale* were purchased from Otto Richter and Sons Limited (Goodwood, Canada) and stored at 4°C. The seeds were surface-sterilized with 70% (v/v) ethanol for 30 s and 2% (v/v) sodium hypochlorite solution for 10 min, then rinsed three times in sterilized water. Ten seeds were placed on 25 ml of agar-solidified culture medium in Petri dishes (100 × 15 mm). The basal medium consisted of MS (Murashige and Skoog, 1962) salt and vitamin medium solidified with 0.7% (w/v) agar. The MS salt and vitamin medium was adjusted to pH 5.8 before adding the agar and was then sterilized by autoclaving at 121°C for 20 min. The seeds were germinated in a growth chamber at 25°C under standard cool white fluorescent tubes with a flux rate of 35 μmol s^{-1} m^{-2} and a 16-h photoperiod.

Induction of embryogenic callus and somatic embryos

Leaves and stems of *L. officinale* were cut into pieces approximately 0.7 × 0.7 cm and 0.7 cm in length, respectively, from plants grown *in vitro* that had been cut aseptically at the ends. Explants were placed on medium (approximately 25 ml) in 100 ×25 mm Petri dishes. Seven

Table 1. Effect of 2,4-D on the induction frequency of embryogenic callus and somatic embryos from leaves of *Levisticum officinale* after 6 weeks in culture.

2,4-D (mg/l)	Embryogenic callus* (%)	Number of somatic embryos per explant**
0.0	0	0
0.1	45	5.9 ± 0.7
0.5	32	2.3 ± 0.3
1.0	22	0
2.0	0	0

*From 100 leaf explants tested.
**Values represent the mean ± standard deviation of somatic embryos per explant

Table 2. Effect of 2,4-D on the induction frequency of embryogenic callus and somatic embryos from stems of *Levisticum officinale* after 6 weeks in culture.

2,4-D (mg/l)	Embryogenic callus* (%)	Number of somatic embryos per explant**
0.0	0	0
0.1	82	16.5 ± 1.5
0.5	74	7.3 ± 0.5
1.0	65	0
2.0	0	0

*From 100 leaf explants tested.
**Values represent the mean ± standard deviation of somatic embryos per explant

explants were cultured in each Petri dish on basal medium consisting of MS medium solidified with 0.7% (w/v) Phytagar (Gibco, Carlsbad, CA) that had been sterilized by autoclaving at 1.1 kg cm^{-2} (121 °C) for 20 min. The pH of the MS medium was adjusted to 5.8 before adding Phytagar. For embryogenic callus and somatic embryo induction from the culture of leaves and stems, the basal medium was supplemented with different concentrations (0.0, 0.1, 0.5, 1.0, and 2.0 mg/l) of 2,4-D (2,4-dichlorophenoxyacetic acid). Cultures were maintained in a growth chamber in the dark at 25°C.

Conversion of somatic embryos and plant regeneration

Mature somatic embryos were transferred to regeneration media to promote somatic embryo conversion and plant development. The regeneration medium consisted of half strength of MS salts and vitamins. Isolated somatic embryos and regenerated plantlets were incubated at 25 °C in a growth chamber with a 16 h photoperiod under standard cool white fluorescent tubes (35 µmol s^{-1} m^{-2}) for 5 weeks.

Results and discussion

Based on our findings, we believe that a simple and effective protocol has been developed for the *in vitro* somatic embryogenesis and plant regeneration of *L. officinale*. We investigated the effects of different concentrations of 2,4-D on the induction frequency of embryogenic calluses and somatic embryos from leaves and stems of *L. officinale* after 6 weeks in culture. Embryogenic calluses and somatic embryos were induced from both leaves (Table 1) and stems (Table 2) of *L. officinale* using 2,4-D. The stem source was more effective for both induction of embryogenic calluses and somatic embryos. Nevertheless, in both cases, a 2,4-D concentration of 0.1 mg/l induced the highest number of embryogenic calluses and somatic embryos per explant. At this concentration, approximately 45% of leaves produced embryogenic calluses, with an average of 5.9 somatic embryos per explant. Moreover, 82% of stems produced embryogenic calluses with an average of 16.5 somatic embryos per explant. Increasing the concentration above 0.1 mg/l decreased the induction frequency and number of somatic embryos. At a concentration of 2 mg/l, embryogenic calluses and somatic embryos were not induced in either leaves or stems of *L. officinale*. The frequency of induction of embryogenic calluses was almost doubled after applying 2,4-D at 0.1 mg/l to stems of *L. officinale* compared with the induction frequency in leaves. In stems, the number of somatic embryos produced (16.5) was almost three times more than from leaves (5.9). Therefore, we conclude that stems are a superior explant for regeneration of *L. officinale*. Under the microscope, various stages of somatic embryo development could be observed (Fig 1).

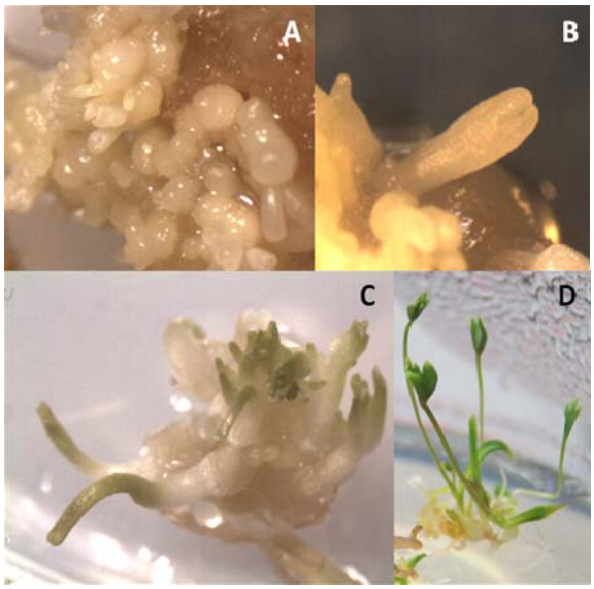

Fig 1. Somatic embryogenesis and plant regeneration in *Levisticum officinale* Koch. Numerous (A) globular and heart, (B) torpedo, and (C) cotyledonary somatic embryos are shown developing on the surface of a stem cultured on solid MS medium supplemented with 0.1 mg/l 2,4-D. Plantlets (D) were regenerated on solid half-strength MS medium without plant hormones. Magnification: A–C, ×15; D, ×1.

The stem explants cultured on medium containing 0.1 mg/l 2,4-D developed yellowish embryogenic calluses within 2–3 weeks. Small globular and heart-stage somatic embryos (Fig 1A) were visible within 4 weeks. The establishment of embryogenic cultures was followed by the development of torpedo (Fig 1B) and cotyledonary (Fig 1C) stages of somatic embryos within 5–6 weeks. Somatic embryos at the cotyledonary stage of development were selected for conversion and shoot growth. Mature somatic embryos were transferred to regeneration media to promote plant development and cultured in growth chamber for 5 weeks. Before transferring regenerated plants to pots, the plantlets were subcultured on half-strength MS salts and vitamins in the absence of growth regulators for 1 month (Fig 1D). Plantlets were then transferred to pots containing autoclaved vermiculite, covered with polythene bags to maintain high humidity, and kept at 25°C in a growth chamber for 1 month. After 1 week, the bags were perforated, and the plants were transferred to soil. Plant tissue culture plays an important role in plant regeneration and micropropagation. The term

"regeneration" has been broadly used in the context of tissue culture to refer to the production of whole plants from cells, tissues, organs, meristems, or zygotic embryos cultivated *in vitro*. There are two major systems of plant regeneration: organogenesis and somatic embryogenesis. These systems are defined based on the developmental stages through which a whole plant is regenerated (Phillips and Hubstenberger, 1995). Many plant species can be regenerated and propagated by the production of somatic embryos derived from diverse explant tissues (Ammirato, 1989 Miroshnichenko et al., 2009; Zhang et al., 2009). The system described here for continuous production of *L. officinale* somatic embryos and regenerated plants without loss of morphogenetic capacity could be a model for micropropagation systems for other species as well as for the regeneration of transgenic plants.

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