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Full Length Research Paper

Somatic embryogenesis and plant regeneration in *Cnidium officinale* Makino

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The development of an efficient protocol for somatic embryogenesis and plant regeneration from immature flower cultures of *Cnidium officinale* Makino is reported. Embryogenic callus and somatic embryos induced from immature flower of *C. officinale* was cultured on medium supplemented with 2,4-D and BAP. The highest induction frequency of embryogenic callus (68%) and number of somatic embryos per explants (12.3) was obtained on MS medium containing 1.0 mg/l 2,4-D and 0.1 mg/l BAP. The addition of cytokinins (BAP and Kinetin) and GA_3 in MS medium improved the plantlet conversion and shoot growth from somatic embryo culture of *C. officinale*. The optimal concentration of 0.1 mg/l BAP and 3.0 mg/l GA_3 was most suitable for the highest conversion rate of plantlet (74 and 75%) and shoot growth (3.6 and 4.1 cm). The rooted plants were hardened and transferred to soil with an 80% survival rate. The continuous production of *C. officinale* regenerated plants via somatic embryogenesis could be used as a possible micropropagation system and plant transformation.

Key words: *Cnidium officinale* Makino, embryogenic callus, micropropagation, plant regeneration, somatic embryogenesis.

INTRODUCTION

Cnidium officinale Makino is a perennial herb of the family Umbelliferae, and an important traditional oriental medicinal plant in China, Japan and Korea. Cnidii Rhizoma, the dried rhizome of *C. officinale*, is widely used as a traditional oriental medicine for treatment of replenishing the blood, tonicity, headaches, women's diseases, stagnated blood, and inflammation of the lymphatic gland.

Chemical investigation of *C. officinale* revealed that Cnidii Rhizoma contains a variety of bioactive chemical constituents including senkyunolide (Yamagishi and Kaneshima, 1977), pregnenolone, coniferyl ferulate, hydroxyphthalides (Kobayashi et al., 1984), acidic polysaccharide (Tomoda et al., 1992), essential oil (Choi et al., 2001; Kapetanos et al., 2008), glucan (cnidirhan SI) (Tomoda et al., 1994a), beta-heteroglucan (cnidirhan SIIA) (Tomoda et al., 1994b), falcarindiol (Kim et al., 2003a), and other compounds.

C. officinale and its main active components have been reported to exhibit various pharmacological and biological activities including antiangiogenic (Kwak et al., 2002), anticancer (kim et al., 2003b); anti- complementary (Tomoda et al., 1994a; Tomoda et al., 1994b), antidiabetic (Jeong et al., 2005), Immunological (Tomoda et al., 1992), acaricidal (Kwon and Ahn, 2002; Kwon and Ahn, 2003) and insecticidal activities (Tsukamoto et al., 2005).

The usual propagation of *C. officinale* is by division of the tuberous roots and up to now it is the only way to regenerate of this species as it can not produce seed

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Table 1. Effect of 2,4-D and BAP on the induction frequency of embryogenic callus and somatic embryos from immature flower of *Cnidium officinale* after 5 weeks in culture.

Growth regulators (mg/l)	Embryogenic callus* (%)	Number of somatic embryos per explant**
2,4-D 0.5	32	5.7 ± 1.1
2,4-D 0.5 + BAP 0.1	46	7.9 ± 1.2
2,4-D 0.5 + BAP 0.5	43	10.4 ± 1.2
2,4-D 1.0	46	6.5 ± 0.9
2,4-D 1.0 + BAP 0.1	68	12.3 ± 1.4
2,4-D 1.0 + BAP 0.5	57	9.2 ± 1.1
2,4-D 2.0	18	2.1 ± 0.3
2,4-D 2.0 + BAP 0.1	32	2.6 ± 0.4
2,4-D 2.0 + BAP 0.5	24	4.3 ± 0.5

*From 100 immature flower explants tested.

**Values represent the mean ± standard deviation of somatic embryos per explant.

even after flowering. Some studies have reported *in vitro* plant regeneration of *C. officinale* from the culture of shoot tip and Inflorescence explants for multiple propagation (Pant et al., 1996; Cho et al., 2000) . However, the plant regeneration efficiency was low and not reliable. A reliable and highly efficient method for the regeneration of intact plants from *in vitro* culture is essential to establish a multiple micropropagation system and genetic transformation protocol for *C. officinale.* In this paper, we describe the development of a rapid method for high frequency somatic embryogenesis and plant regeneration from immature flower culture of *C. officinale.*

MATERIALS AND METHODS

Induction of embryogenic callus and somatic embryos

Immature flowers of *C. officinale* were taken from greenhouse grown plants at Agricultural Experimental Farm, Chungnam National University, Korea. Immature flowers were surface- sterilized by a 10 s immersion in 70% (v/v) ethanol and for 10 min in aqueous solution of 1% (v/v) sodium hypochlorite containing a few drops of tween 20. After three washes in sterilised water, each immature flower was cut aseptically and placed on the medium in the Petri dish (100 x 25 mm). Petri dish contained approximately 25 ml of culture medium. Ten immature flowers were cultured in each Petri dish. The basal medium consisted of salts and vitamins of Murashige and Skoog (MS) medium (1962) and solidified with 0.7% (w/v) Phytagar. The pH of medium was adjusted to 5.8 before adding Phytagar. The media were sterilised by autoclaving at 1.1 $kgcm^{-2}$ (12°C) for 20 min. Cultures were maintained in a growth chamber in the dark at 25°C.

For embryogenic callus and somatic embryo induction from imamture flowers, the basal medium was supplemented with different combinations and concentrations (0.5, 1.0 and 2.0 mg/l) of 2,4-D (2,4-dichlorophenoxyacetic acid) and (0.1 and 0.5 mg/l) BAP (6-benzylaminopurine).

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 MS salts and vitamins, and contained various concentrations of BAP, Kinetin and GA_3 (gibberellic acid). 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.

Production of hardened plantlets

Before transferring regenerated plantlets to pots, the plantlets were subcultured on half-strength MS salts and vitamins, in the absence of growth regulators, and cultured for 1 month. The medium was solidified with 3 g/l Gelrite and dispensed at 30 ml per Magenta box and four plantlets were cultured in each box. Regenerated plantlets were incubated at 25 ± 1° 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. After five weeks, the plantlets were washed with sterile water to remove Gelrite, transferred to pots containing autoclaved vermiculite, and covered with polyethylene bags for one week to maintain high humidity. The plants were then transferred to soil and maintained in a growth chamber with a 16-h photoperiod, and a night/day temperature of 18/20°C for 2 weeks. These hardened plants then transferred to the greenhouse.

RESULTS AND DISCUSSION

A simple and effective protocol has been developed for the *in vitro* somatic embryogenesis and plant regeneration of *C. officinale*. The immature flowers were cultured onto solid MS medium containing different combinations and concentrations of 2,4-D and BAP for the induction of embryogenic callus and somatic embryos. BAP treatment increased the percentage of friable calli that developed embryogenic callus when added to 2,4- D containing medium. The presence of 2,4-D alone was less effective than a combination of 2,4-D and BAP for embryogenic callus and somatic embryo induction (Table 1). Induction of embryogenic callus and somatic embryos was highest on MS medium containing 1.0 mg/l 2,4-D and 0.1 mg/l BAP after 5 weeks of culture. These results suggest that the presence of BAP in the induction medium is crucial

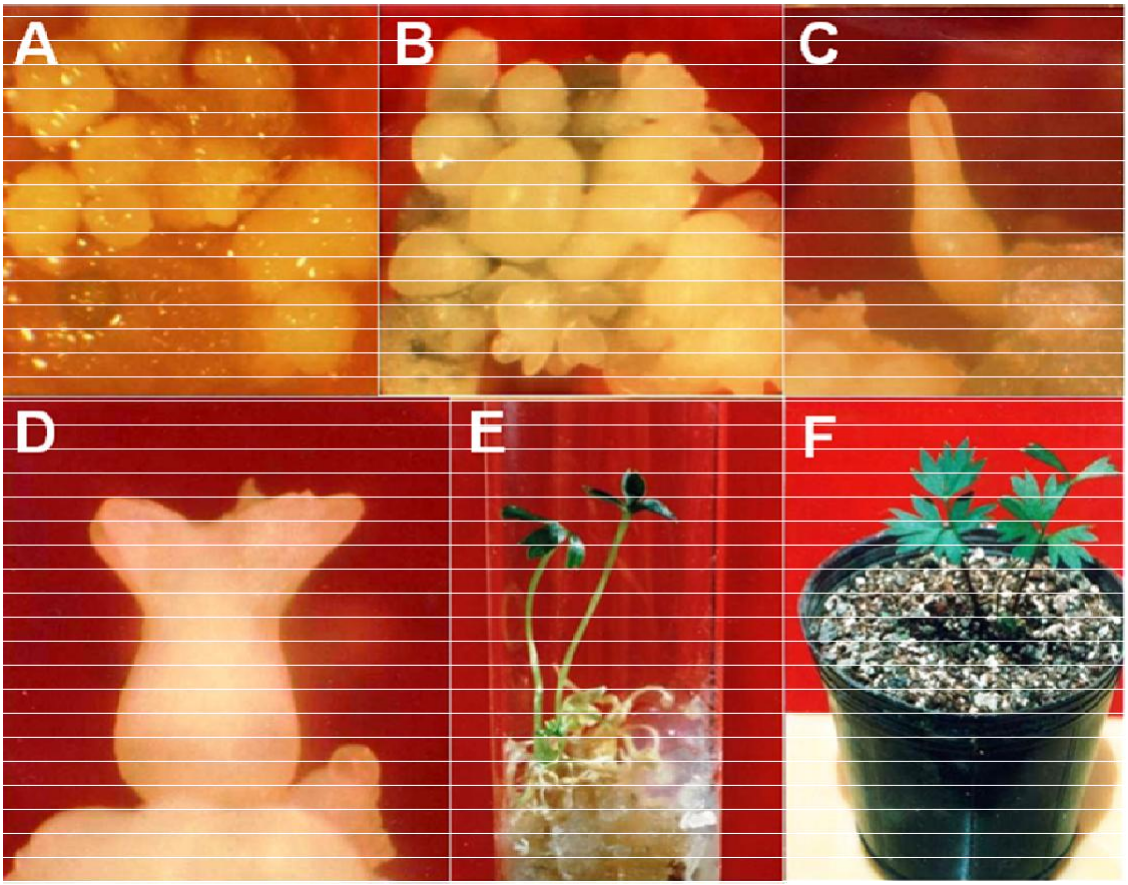

Figure 1. Somatic embryogenesis and plant regeneration in *Cnidium officinale* Makino. embryogenic callus (A), numerous globular (B), torpedo (C), and cotyledonary somatic embryos are shown developing on the surface of immature flower cultured on solid MS medium supplemented with 1.0 mg/l 2,4-D and 0.1 mg/l BAP. Plantlets (E) were regenerated on solid MS medium containing 0.1 mg/l BAP. The regenerated plant grew in the pot (F). Magnification: A - D, x15; E, x1; F, x0.5.

for the high frequency expression of somatic embryogenesis in *C. officinale*.

Under a microscope, various stages of somatic embryo development could be observed (Figure 1). The immature flower explant cultured on medium containing 1.0 mg/l 2,4-D and 0.1 mg/l BAP developed yellowish embryogenic callus within 2 to 3 weeks (Figure 1A). Small globular somatic embryos (Figure 1B) were visible within 3 weeks. The establishment of embryogenic cultures was followed by the development of torpedo (Figure 1C) and cotyledonary (Figure 1D) stages of somatic embryos within 4 to 5 weeks.

Somatic embryos at the cotyledonary stage of development were selected to optimize conditions for conversion and shoot growth. A comparison of conversion and shoot growth efficiencies for somatic embryos cultured on MS medium containing different cytokinins (BAP and Kinetin) and GA_3 are shown in Tables 2 and 3. The concentrations of BAP (0.1 mg/l) and Kinetin (0.5 mg/l) increased the frequency of conversion and the growth of shoots, whereas concentrations above 0.5 mg/l were inhibitory (Table 2). GA_3 treatment was found to increase conversion efficiency to levels that were similar to those observed for cytokinins, but GA_3 was more effective than cytokinins at promoting shoot growth (Table 3). Addition of 3 mg/l GA_3 to the culture medium led to a 60% increase in conversion efficiency and a 78% increase in shoot growth compared to somatic embryos cultured in the absence of growth regulators (control). These results suggest that low concentrations of a cytokinin (BAP or Kinetin) and 3 mg/l GA_3 are optimal for the promotion of conversion and shoot growth in somatic embryos of *C. officinale*.

Before transferring regenerated plantlets (Figure 1E) to pots, the plantlets were subcultured on half-strength MS medium without plant growth regulators, and cultured for 1 month. The rooted plants were hardened and transferred to soil with an 80% survival rate where they grew normally (Figure 1F) and flowered within 4 months.

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

Table 2. Effect of cytokinins on the conversion frequency and shoot growth of somatic embryos of *Cnidium officinale* after 4 weeks in culture.

Cytokinins (mg/l)	Conversion* (%)	Shoot length** (cm)
Control	47	2.3 ± 0.3
BAP 0.01	52	2.5 ± 0.4
BAP 0.05	57	2.8 ± 0.3
BAP 0.1	74	3.6 ± 0.6
BAP 0.5	59	3.3 ± 0.4
BAP 1.0	41	1.8 ± 0.2
Kinetin 0.01	49	2.4 ± 0.3
Kinetin 0.05	56	2.6 ± 0.3
Kinetin 0.1	67	3.2 ± 0.4
Kinetin 0.5	69	3.5 ± 0.5
Kinetin 1.0	45	2.1 ± 0.3

*From 100 somatic embryos at the cotyledonary stage tested.
**Values represent the mean ± standard deviation of 50 shoots.

Table 3. Effect of GA_3 (gibberellic acid) on the conversion frequency and shoot growth of somatic embryos of *Cnidium officinale* after 4 weeks in culture.

Gibberellic acid (mg/l)	Conversion* (%)	Shoot length** (cm)
Control	47	2.3 ± 0.3
GA_3 0.1	51	2.9 ± 0.4
GA_3 0.5	55	3.3 ± 0.4
GA_3 1.0	67	3.8 ± 0.5
GA_3 3.0	75	4.1 ± 0.6
GA_3 5.0	71	3.9 ± 0.4

*From 100 somatic embryos at the cotyledonary stage tested.
** Values represent the mean ± standard deviation of 50 shoots.

as 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. Many plant species can be regenerated and propagated by the production of somatic embryos derived from diverse explant tissues (Ammirato, 1989). The superiority of combination treatment with 2,4-D and BAP for embryogenic callus and somatic embryo induction has also been reported for *Ocimum basilicum* (Gopi and Ponmurugan, 2006), saffron (Rajabpoor et al., 2007), and *Lilium longiflorum* var. ceb-dazzle (Khosravi et al., 2007).

The improvement of plantlet conversion and shoot growth from somatic embryos with treatment of a cytokinin (BAP or Kinetin) has been reported for *Agave sisalana* (Nikam et al., 2003), Australian fan flower (Wang and Bhalla, 2004), cotton (Sun et al., 2006), and *Pennisetum glaucum* (Arockiasamy et al., 2006). The promotion of plantlet conversion and shoot growth from somatic embryos with treatment of a GA (gibberellic acid) has also been reported for *Gossypium hirsutum* (Kumria et al., 2003), *Cryptomeria japonica* (Igasaki et al., 2003), and carrot (Tokuji and Kuriyama, 2003).

The continuous production of *C. officinale* somatic embryos and regenerated plants without loss of morphogenetic capacity is important since this technique could be used as a possible micropropagation system, and for the regeneration of transgenic plants.

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