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ORIGINAL ARTICLE

High frequency plant regeneration system for *Nymphoides coreana* via somatic embryogenesis from zygotic embryo-derived embryogenic cell suspension cultures

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Abstract Culture conditions were established for high frequency plant regeneration via somatic embryogenesis from cell suspension cultures of *Nymphoides coreana*. Zygotic embryos formed pale-yellow globular structures and calluses at a frequency of 85.6% when cultured on half-strength Murashige and Skoog (MS) medium supplemented with 0.3 mg l^{-1} of 2,4-D. However, the frequency of pale-yellow globular structures and white callus formation decreased slightly with an increasing concentration of 2,4-D up to 10 mg l^{-1} with the frequency rate falling to 16.7%. Cell suspension cultures were established from zygotic embryo-derived calluses using half-strength MS medium supplemented with 0.3 mg l^{-1} of 2,4-D. Upon plating onto half-strength MS basal medium, over 92.3% of cell aggregates gave rise to numerous somatic embryos and developed into plantlets. Regenerated plantlets were successfully transplanted into potting soil and achieved full growth to an adult plant in a growth chamber. The high frequency plant regeneration system for *Nymphoides coreana* established in this study will be useful for genetic manipulation and cryopreservation of this species.

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Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BA	6-Benzyladenine
MS	Murashige and Skoog (1962)
NAA	α-Naphthaleneacetic acid

Introduction

Aquatic plants are very useful as resources for the recovery of the ecosystem by purifying the water (Brix and Schierup 1989; Zimmels et al. 2004). However, lots of aquatic plants are endangered because of habitat destruction. Therefore, emergent programs for protection and preservation of endangered plant species are required. *Nymphoides coreana* (H. Lév.) Hara (Menyanthaceae) is an aquatic flowering plant growing in lakes, ponds, and fallow fields (Cook 1996). *N. coreana* is characterized by long stolons producing new plantlets, floating and oval-orbicular leaves, and showy white flowers about 1.5 cm in diameter (Choi 2007). This species is widely distributed in Korea, Japan, China, and east Russia (Choi 2007; Shibayama and Kadono 2008; Yan 1983). In South Korea, it is found rarely in the Gangwon Province and Jeju Island (Hyun 2001; Yang et al. 1990). Furthermore, it is listed as a threatened species because of habitat loss (Lee et al. 2005).

Tissue culture and in vitro plant regeneration systems may provide an alternative mean for mass proliferation and ex situ conservation of endangered plant species. A few tissue culture studies of *Nymphoides* have been reported

including shoot organogenesis from petiole explants (Jenks et al. 2000) and somatic embryogenesis from the floral stem of *Nymphoides indica* (Oh et al. 2007). Therefore, this study describes the culture conditions for high frequency plant regeneration via somatic embryogenesis from seed-derived cell suspension cultures of *Nymphoides coreana*.

Materials and methods

Plant material and culture condition

Mature seeds of *Nymphoides coreana* Hara were supplied from the plant systematics laboratory of Ajou University. Seeds were surface-sterilized in 70% (v/v) ethanol for 1 min followed by a 0.4% (v/v) sodium hypochlorite solution for 20 min with occasional agitation. They were rinsed four times with sterile distilled water. Seeds were stored at 4°C until needed. The culture medium used throughout the experiments consisted of half-strength MS (Murashige and Skoog 1962) inorganic salts, 0.4 mg l^{-1} thiamine HCl, 100 mg l^{-1} myo-inositol, 30 gl^{-1} sucrose, and 4 gl^{-1} Gelrite. The pH of all media was adjusted to 5.8 with 1 N NaOH before autoclaving. Finally, 25-ml aliquots of medium were dispensed into plastic Petri dishes (87 × 15 mm).

Effects of 2,4-D on embryogenic callus formation

To examine the effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on embryogenic callus and somatic embryo formation from the seeds of *Nymphoides coreana*, surface-sterilized seeds (approximately less than 1 mm in length) were longitudinally dissected with forceps and scalpel in the laminar flow clean bench. Zygotic embryos (approximately less than 1 mm in length) were collected from the dissected seeds and placed onto half-strength MS medium containing 0, 0.1, 0.3, 1, 3, and 10 mg l^{-1} of 2,4-D. Each treatment consisted of ten explants per dish with three replicates. Unless mentioned otherwise, all cultures were incubated at 25°C in the dark. After 4 weeks of culture, the frequency of explants producing pale-yellow globular structures and calluses was counted for each treatment.

Establishment of embryogenic cell suspension culture

To establish embryogenic cell suspension culture, initial calluses were used. For the initiation of a cell suspension culture, the calluses (approximately 1 g), maintained on half-strength MS medium containing 0.3 mg l^{-1} of 2,4-D, were carefully disintegrated with sterile forceps and transferred to a 250-ml Erlenmeyer flask containing 20 ml of half-strength MS liquid medium containing 0.3 mg l^{-1} of 2,4-D. The culture was maintained in a gyratory incubator (100*g*) at 25°C in the dark. After 2 weeks of incubation, 20 ml of fresh half-strength MS liquid medium containing 0.3 mg l^{-1} of 2,4-D was added. After a further 2 weeks of incubation, 5 ml of cell suspension was transferred to a 250-ml Erlenmeyer flask containing 50 ml of half-strength MS liquid medium containing 0.3 mg l^{-1} of 2,4-D. Cell suspension cultures were subcultured at 4-week intervals.

Plant regeneration from embryogenic cell suspension culture

To regenerate whole plants, cell aggregates (1–2 mm in size) were collected from 2-week-old cell suspension cultures using a stainless steel mesh (pore size 1 mm) and rinsed with liquid half-strength MS basal medium. Twenty cell aggregates were plated onto half-strength MS basal medium per Petri dish. After 4 weeks of incubation in the light (30 μmol m^{-2} s^{-1} from cool-white fluorescent lamps with a 16-h photoperiod), the frequency of somatic embryo formation was determined by counting the number of green regenerated plantlets in five Petri dishes. Plantlets developed from somatic embryos were transplanted into potting soil (vermiculite:perlite, 3:1 mixture), and maintained in a growth chamber (25°C day/22°C night, 80 μmol m^{-2} s^{-1} from cool-white fluorescent lamps with a 16-h photoperiod and 50–70% RH). After emergence of new leaves from transplanted plant, the pot was filled by tap water for growing of mature plants in a growth chamber.

Results and discussion

Culture conditions were established for high frequency plant regeneration via somatic embryogenesis from embryogenic cell suspension cultures of *Nymphoides coreana* (Fig. 1). Zygotic embryos cultured on half-strength MS medium containing 2,4-D. Pale-yellow globular structures were beginning to form on the surface of the zygotic embryo after 2 weeks of culture (Fig. 1a). After 4 weeks of culture, pale-yellow globular structures and white friable calluses formed on the entire surface of zygotic embryos (Fig. 1b). When transferred to half-strength MS basal medium, these pale-yellow structures developed into multiple somatic embryos after 4 weeks of culture. These results indicated that the initial pale-yellow structures were globular or torpedo stage of the somatic embryo.

Embryogenic cell suspension cultures were established from zygotic embryo-derived calluses using half-strength MS medium containing 0.3 mg l^{-1} of 2,4-D. After two rounds of subculture at 2-week intervals, cell suspension

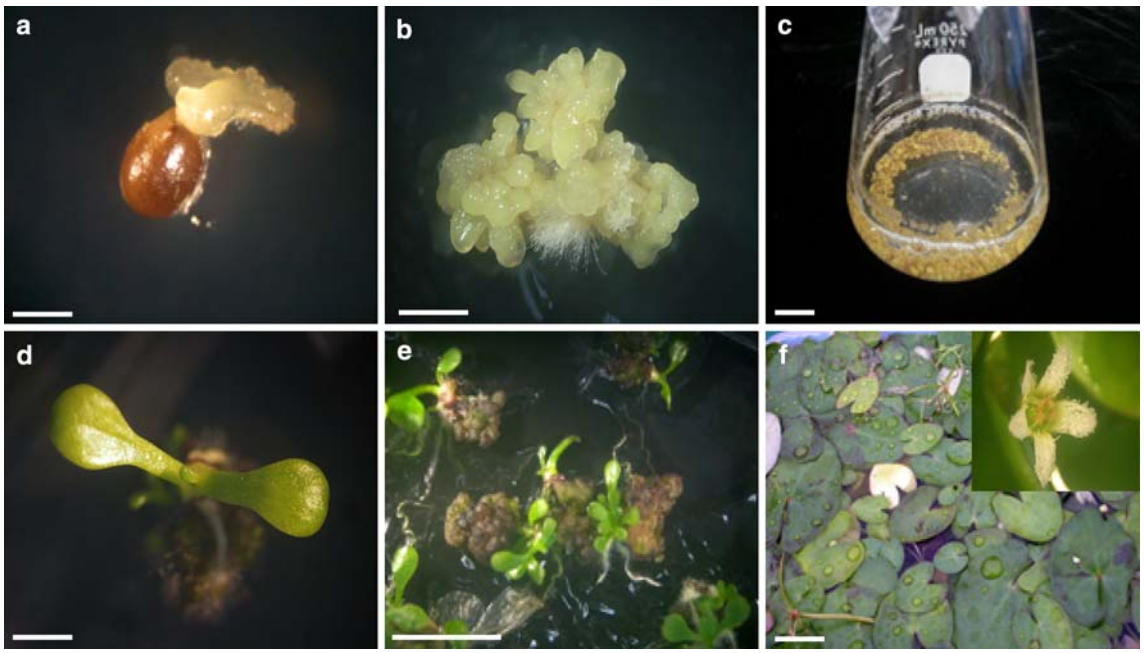

Fig. 1 Plant regeneration of *Nymphoides coreana* via somatic embryogenesis. **a** Emergence of zygotic embryo from mature seed; **b** formation of pale-yellow globular structures from zygotic embryo; **c** establishment of embryogenic cell suspension cultures from zygotic embryo-derived calluses; **d** development of somatic embryos from cell aggregates; **e** numerous plantlets regeneration from somatic embryos onto half-strength MS basal solid medium in the light. **f** Successful acclimatization of regenerated plants and enlarged view of flowering from regenerated plants. *Scale bars* (**a**, **b**, **d**) 1 mm, (**e**) 5 mm, (**c**, **f**) 2 cm

cultures were actively proliferated (Fig. 1c). When transferred to half-strength MS basal medium, 92.3% of the suspension cultured-cell aggregates were developed into somatic embryos after 4 weeks of incubation in the light (Fig. 1d). These somatic embryos were successfully converted into plantlets and rooted well without any rooting treatments and subculture (Fig. 1e). Rooted plantlets were successfully transferred to potting soil and acclimatized. After 2 months of incubation in a growth chamber, plantlets grew into mature plants with flowering and seed setting (Fig. 1f).

The effect of 2,4-D concentration on embryogenic callus formation from zygotic embryos. Zygotic embryos formed pale-yellow globular structures and white calluses at a frequency of 85.6% when cultured on half-strength MS medium supplemented with 0.3 mg l^{-1} of 2,4-D (Fig. 2). The frequency of white callus formation was 58.3% when cultured on half-strength MS medium supplemented with 0.1 mg l^{-1} of 2,4-D. However the frequency of white callus formation sharply decreased at 16.7% when the concentration of 2,4-D was increased up to 10 mg l^{-1} (Fig. 2). These results were similar to those of floral stem cultures of *Nymphoides indica* via somatic embryogenesis (Oh et al. 2007). Based on these results, we suggested that a low concentration of 2,4-D was suitable for embryogenic callus induction and proliferation from zygotic embryo of *Nymphoides coreana*. We have already established plant

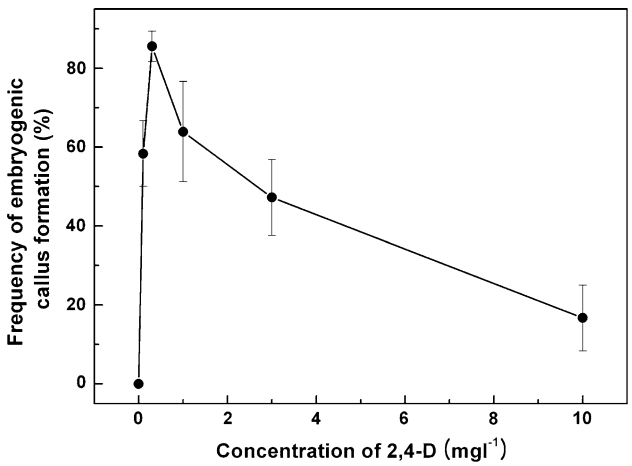

Fig. 2 Effect of 2,4-D on embryogenic callus formation from zygotic embryos of *Nymphoides coreana*. Each treatment consisted of ten explants with three replicates. *Vertical bars* SD

regeneration systems via zygotic embryo-derived embryogenic cell suspension cultures for aquatic plants such as *Ranunculus kazusensis* (Min et al. 2007) and *Brasenia schreberi* (Oh et al. 2008). These plants also formed embryogenic calluses from zygotic embryos when cultured on the medium supplemented with a low concentration of 2,4-D. Therefore, we suggest that a low concentration of 2,4-D is suitable plant growth regulator for embryogenic

callus induction and proliferation from zygotic embryo cultures of aquatic plants.

In preliminary studies, we examined the germination rate of a mature seed of *Nymphoides coreana*. A mature seed of *Nymphoides coreana* had a nil germination rate without any treatment (cold, growth regulators). However, the germination rate was over 90% after seed coat removal. In this study, we established for the first time a high frequency plant regeneration system via somatic embryogenesis of aquatic *Nymphoides coreana*. This plant regeneration system will be useful for mass propagation and restoration of these endangered plants. To ensure the maintenance of threatened plants, more genetic resources from various wild populations of plants should be considered for in vitro cultures (Sarasan et al. 2006). The distribution of *Nymphoides coreana* is rare in a restricted region of Korea (Hyun 2001; Yang et al. 1990). Nevertheless, there is no information on the genetic variation of this species in Korea. Thus, we suggest that the genetic diversity of the wild and cultured populations of *N. coreana* should be assessed for establishing conservation strategies (Kim et al. 2008; Shibayama and Kadono 2008). Our in vitro culture system for *N. coreana* can be expanded to include a wide variety of rare and endangered aquatic plants possessing a high level of genetic diversity. Furthermore, the cell culture system could be applied for genetic manipulation and long-term preservation of genetic resources via cryopreservation systems.

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