

## Callus induction and plant regeneration of *Iris dichotoma* Pall. in endangered species

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Received: 23 August 2012 / Accepted: 1 September 2012  
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**Abstract** *Iris dichotoma* Pall. is an important endangered plant belonging to the family *Iridaceae*. A method was developed for the rapid micropropagation of *I. dichotoma* through plant regeneration from leaf, rhizome, and root explant-derived calli. Leaf, rhizome, and root segments were cultured on Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D; 0–3.0 mg·L<sup>-1</sup>) for callus induction. Callus production was highest at 1.0 mg·L<sup>-1</sup> 2,4-D, where 73.8% and 45.5% of cultured rhizome and root cuttings, respectively, produced calli. The viable calli were maintained at an induced concentration of 2,4-D (3.0 mg·L<sup>-1</sup>). They were then transferred to MS medium supplemented with various concentrations of 2,4-D (0–3.0 mg·L<sup>-1</sup>) in combination with 6-benzyladenine (BA: 0, 1.0 and 3.0 mg·L<sup>-1</sup>) for adventitious shoot regeneration. The addition of a low concentration of 2,4-D into BA-containing medium significantly increased the frequency of shoot regeneration in leaf, rhizome, and root-derived calli. The highest number of adventitious shoots (26.4 per callus) formed at 0.5 mg·L<sup>-1</sup> 2,4-D and 1.0 mg/l BA. For rooting of the shoots, half-strength MS medium supplemented with different concentrations of indole 3-butyric acid (IBA) 0–3.0 mg·L<sup>-1</sup> was tested. The optimal results were observed using half-strength MS medium supplemented with 1.0 mg·L<sup>-1</sup> IBA, on which 98% of the regenerated shoots developed roots with an average of 3.5 roots per shoot within 45 days.

The plantlets raised *in vitro* were acclimatized and transferred to soil with 95% success. This *in vitro* propagation protocol will be useful for conservation and mass propagation of this endangered plant.

**Keywords** acclimatization, callus, *in vitro*, *Iris dichotoma*, proliferation

### Introduction

The genus *Iris* includes over 300 species, spread mostly across the northern temperate zone (Schulze 1988). *Iris* (=Pogoniris) comprises rhizomatous irises with bearded outer tepals (Mathew 1981). Four genera and 31 species of *Iris* are distributed across South Korea. Five native species are critically endangered: *Iris dichotoma*, *I. koreana*, and *I. odaesanensis*. The distribution area of *I. dichotoma* is limited to Daecheong-do and the islands of Korea. Populations are generally small, and some have disappeared or declined. For this reason, the South Korean government has designated the species as “Threatened to extinct: the first grade (I) for preservation” (Lee and Choi 2006). Lee and Choi (2006) treated it as critically endangered at the regional level according to the IUCN (2001) Red List criteria (Gärdenfors et al. 2001).

The natural mode of reproduction in the *I. dichotoma* species limits the large-scale production of valuable plants (Wang et al. 1999). Reproduction of this species using seeds is rarely carried out due to problems with germination, low seed production, capacity for cross-pollination, and the long juvenile period in plant development. Furthermore, similar to vegetative reproduction, the time required to obtain sufficient quantities of planting stock is 4–5 years. On the other hand, the cell and tissue culture method has become popular in iris reproduction, since it considerably increases plant multiplication factors (Shimazu et al. 1997; Wang et

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al. 1999a; Shibli and Ajlouni 2000) and improves the quality of valuable planting stock (Baruch and Quak 1966; Mielke and Anderson 1989). The developed clonal reproduction techniques are used as an alternative method of conserving rare iris species (Radojevic and Subotic. 1992; Shibli and Ajlouni 2000).

*In vitro* propagation of the tissue of monocotyledons is complicated by their low regenerative capacity compared to dicotyledons (Kamo et al. 1990; Wang and Nguyen 1990). Analysis of the regenerative capacity in some ornamental monocotyledons has demonstrated that it is lower in *Iridaceae* than in *Amaryllidaceae*, *Araceae*, and *Liliaceae* (Hussey 1975). In this paper, it is shown that the selection of organ or tissue as an explant is important in the development of plant reproduction in callus culture. Tissues of generative organs have been used in most studies of iris regeneration *in vitro*. For instance, flower tissues were used for direct regeneration of *I. ensata* (Ichihashi and Kato 1986; Kawase et al. 1991). The perianth tube and upper ovary proved to be the most convenient for microreproduction of *I. ensata* cultivars (Kawase et al. 1995). The patterns of shoot and organogenic callus formation in young stem culture were studied in cultivars and wild type *I. ensata* (Yabuya et al. 1991).

Numerous studies have demonstrated that the hormonal composition of the medium is the most important factor for the *in vitro* regeneration of irises (Radojevic et al. 1987; Laublin et al. 1991; Radojevic and Subotic 1992; Gozu et al. 1993; Jehan et al. 1994; Shimizu et al. 1996; Wang et al. 1999b). The morphogenetic capacity of explant tissues can be induced by varying the composition of phytohormones in the medium for the production of organogenic calluses and differentiation of accessory buds *via* organogenesis or embryoidogenesis. In view of its decorativeness and rarity in nature, an efficient propagation method for *I. adriatica* should be established. As is common in irises, propagation by splitting rhizomes is slow (Hussey 1975; Jehan et al. 1994); thus, micropropagation *in vitro* might be the method of choice. Various iris species have been propagated through organogenesis or somatic embryogenesis, using explants from the leaf base (Gozu et al. 1993; Shibli and Ajlouni 2000), mature zygotic embryos (Radojević and Subotić 1992; Boltenev et al. 2004), ovary sections (Laublin and Cappadocia 1992), and root sections (Laublin et al. 1991).

Propagation of *I. dichotoma* *via in vitro* plant regeneration has not been reported. In this study, we undertook to develop a protocol for regeneration from leaf, rhizome, and root explant sections as a method for efficient propagation *in*

*vitro* for this species. Those techniques would help significantly in the multiplication and conservation of this endemic *Iris* species.

## Materials and methods

### Plant materials and culture conditions

Seeds of *I. dichotoma* were scarified by immersion in 70% EtOH for 1 minute and then sterilized with commercial bleach 1% (v/v) (5% of sodium hypochlorite) with a few drops of Tween-20 (Sigma, USA) for 30 minute. The seeds were washed five times in sterile water and placed into petri dishes containing 20 ml solid hormone-free 1/3 MS medium (Murashige and Skoog 1962) under cool white fluorescent lights ( $56 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) on a 16 h photoperiod or in the dark at 25°C. The germination test was carried out on triplicates of 30 seeds.

### Effect of explant type and growth regulators on callus induction

Half strength MS basal medium was used to induce callus induction and growth. Two-week-old seedlings were used as explant source. Explants (5–10 mm in length) from the leaf, rhizome and root were placed on the media containing 0, 0.1, 0.5, 1.0, and 3.0  $\text{mg}\cdot\text{L}^{-1}$  2,4-dichlorophenoxy acetic acid (2,4-D) or 0, 0.1, 0.5, 1.0, and 3.0  $\text{mg}\cdot\text{L}^{-1}$  *a*-naphthalene acetic acid (NAA) supplemented with sucrose (30  $\text{g}\cdot\text{L}^{-1}$ ) and solidified with agar (8  $\text{g}\cdot\text{L}^{-1}$ ); the pH of the medium was adjusted to 5.7. The explants, 60 for each explant type, were incubated under cool white fluorescent lights on a 16 h photoperiod at 25°C and subcultured on fresh media every 4 weeks.

### Adventitious shoot induction and multiplication

Calli subcultured on the same medium for two generations, were used to induce adventitious shoots. They were transferred onto 1/2 MS medium supplemented with sucrose (30  $\text{mg}\cdot\text{L}^{-1}$ ) and solidified with agar (8  $\text{g}\cdot\text{L}^{-1}$ ), pH 5.7, added with 0.1, 0.5, 1.0, and 3.0  $\text{mg}\cdot\text{L}^{-1}$  2,4-D and 1.0, 3.0  $\text{mg}\cdot\text{L}^{-1}$  BA. Calli were maintained under cool white fluorescent lights ( $56 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) on a 16 h photoperiod at 25°C. After 8 weeks, shoot induction and multiplication were evaluated and expressed as shooting frequency and number of shoots per callus. The experiments were performed on 20 calli for each callus line, and repeated twice.

## Root induction

For root induction, individual shoots of 3–4 cm in length, were placed into a test tube containing 1/2 MS medium (10 ml) supplemented with sucrose ( $30 \text{ g}\cdot\text{L}^{-1}$ ) and solidified with agar ( $8 \text{ g}\cdot\text{L}^{-1}$ ); pH 5.7. The auxin indole-3-butyric acid (IBA) was assayed at 0, 0.5, 1.0, and  $3.0 \text{ mg}\cdot\text{L}^{-1}$ . After 4 weeks, rooting was evaluated and expressed as rooting frequency, root number, and the longest root length per plantlet.

## Acclimatization

Well-developed, plantlets were selected and removed from the culture tubes. The roots were cleared of medium with water to prevent contamination. The plantlets were potted in sterilized universal planting soil and transferred to a greenhouse for acclimatization. Pots were shielded with plastic covers to initially maintain the plants at high humidity, and the plantlets were acclimatized by gradually opening the covers. After 1 month, they were completely uncovered and hardened to the greenhouse.

## Statistical analysis

All data were analyzed using analysis of variance (ANOVA), and expressed as means  $\pm$  standard error (SE). Each experiment comprised three replications with at least 200 seeds per replication. To examine significant differences among the treatments, multiple comparison tests were then performed by Duncan's multiple range test at  $\alpha \leq 0.05$  (SAS).

## Results and discussion

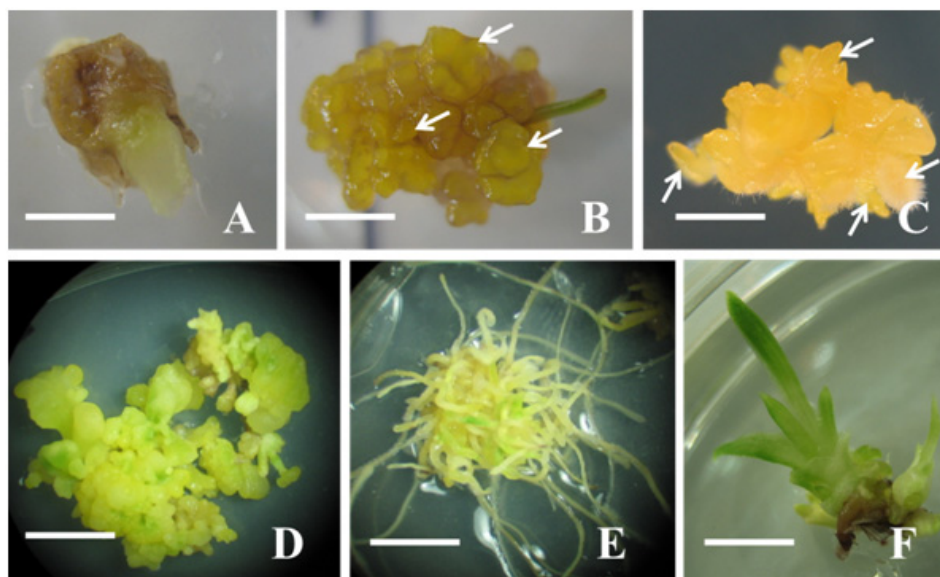
### Effects of explant type and growth regulators on callus induction

In *I. dichotoma*, callus formation varied significantly depending on explant type (Table 1). Rhizome part the earliest signs of callus formation after 4 weeks of culture, while leaves and roots started to generate callus from cut surfaces after 5 weeks of culture. Calli were compact, globular, and yellowish on 1/2MS medium with  $1.0 \text{ mg}\cdot\text{L}^{-1}$  2,4-D (Fig. 1C), and globular and dark yellowish on 1/2MS medium with  $1.0 \text{ mg}\cdot\text{L}^{-1}$  NAA (Fig. 1B); the control exhibited no response (Fig. 1A). The rhizomes showed 90.4% callus formation after 8 weeks. However, root and leaves exhibited a significantly lower callus induction with 8.1 and 39.4% callus formation respectively (Table 1). Conversely, a passaged *I. ensata* culture was obtained from the globular callus formed after the development of the embryos at the stem base induced by  $2.0 \text{ mg}\cdot\text{L}^{-1}$  NAA and 0.5 mg/l BA (Boltenkov et al. 2004). Callus induction in monocots requires long time for its initiation (Geier 1986). It was reported that the induction of callus was difficult and the proliferation of initiated callus was very slow and somehow difficult to maintain compared to other iris species (Zheng et al., 1998; Luciani et al., 2006). The highest callus size was achieved when  $3.0 \text{ mg}\cdot\text{L}^{-1}$  NAA on 1/2MS medium (5.8 mm) was used in comparison with  $0.1 \text{ mg}\cdot\text{L}^{-1}$  2,4-D on 1/2MS medium (3.2 mm) (Table 1). Callus formation from plates also varied significantly depending on plant growth regulators and their combinations (Table 1). The formation of morphogenic callus

**Table 1.** Effect of 2,4-D and NAA on callus formation and its length derived from root, rhizome and leaf explants of *I. dichotoma* on 1/2 MS medium including  $30 \text{ g}\cdot\text{L}^{-1}$  sucrose after 4 weeks of culture

Treatment ( $\text{mg}\cdot\text{L}^{-1}$ )	Callus formation (%)			Length of callus (mm)		
	Leaf	Rhizome	Root	Leaf	Rhizome	Root
2,4-D 0	0	0	0	0	0	0
2,4-D 0.1	6.6	73.8	45.5	3.2 $\pm$ 0.2	3.5 $\pm$ 0.4	3.1 $\pm$ 0.4
2,4-D 0.5	9.0	73.7	26.7	3.8 $\pm$ 0.3	3.6 $\pm$ 0.5	3.5 $\pm$ 0.5
2,4-D 1.0	10.2	58.4	21.3	5.1 $\pm$ 0.4	5.6 $\pm$ 0.4	3.8 $\pm$ 0.4
2,4-D 3.0	4.2	58.4	18.8	5.3 $\pm$ 0.5	5.4 $\pm$ 0.6	5.1 $\pm$ 0.6
NAA 0	0	0	0	0	0	0
NAA 0.1	7.5	75.3	51.4	5.5 $\pm$ 0.5	5.4 $\pm$ 0.2	5.3 $\pm$ 0.3
NAA 0.5	11.6	76.6	34.2	5.8 $\pm$ 0.5	5.6 $\pm$ 0.4	5.4 $\pm$ 0.4
NAA 1.0	8.1	90.4	34.9	5.4 $\pm$ 0.4	5.4 $\pm$ 0.5	5.5 $\pm$ 0.3
NAA 3.0	7.4	75.3	32.5	5.8 $\pm$ 0.6	5.8 $\pm$ 0.5	5.4 $\pm$ 0.1

\*Data are the means  $\pm$  SD, of three experiments. Different alphabetical letters are significantly different according to Duncan's multiple range test at  $\alpha \leq 0.05$ .



**Fig. 1.** Plant regeneration from callus derived from *in vitro* cultured explant types of *I. dichotoma*. A: Rhizome part on control medium after 4 weeks culture, B: Rhizome part on 1/2 MS medium including  $1.0 \text{ mg}\cdot\text{L}^{-1}$  NAA after 8 weeks culture, C: Rhizome part on 1/2 MS medium including  $1.0 \text{ mg}\cdot\text{L}^{-1}$  2,4-D after 8 weeks culture, D: Callus regeneration on 1/2 MS medium with  $0.5 \text{ mg}\cdot\text{L}^{-1}$  BA and  $1.0 \text{ mg}\cdot\text{L}^{-1}$  2,4-D after 8 weeks of culture, E: Rooting form callus on 1/2 MS medium without plant growth regulators after 8 weeks of culture, F: In vitro shoots formed

**Table 2.** Effect of 2,4-D with BA on adventitious shoot formation and its length derived from explants of root, rhizome and leaf in *I. dichotoma* on 1/2 MS medium including  $30 \text{ g}\cdot\text{L}^{-1}$  sucrose after 4 weeks of culture

Treatment ( $\text{mg}\cdot\text{L}^{-1}$ )		Adventitious shoot formation (%)			Length of shoot (mm)		
2,4-D	BA	Leaf	Rhizome	Root	Leaf	Rhizome	Root
0	0	0	0	0	0	0	0
0.1		98	98	100	$12.2\pm 1.2$	$13.5\pm 1.4$	$13.1\pm 1.4$
0.5	1.0	100	100	100	$13.8\pm 1.3$	$13.6\pm 1.5$	$13.5\pm 1.5$
1.0		100	100	100	$15.1\pm 1.4$	$15.6\pm 1.4$	$13.8\pm 1.4$
3.0		100	100	100	$15.3\pm 1.5$	$15.4\pm 1.6$	$15.1\pm 1.6$
0.1		100	100	100	$13.2\pm 1.2$	$13.5\pm 1.4$	$13.1\pm 1.4$
0.5	3.0	100	100	100	$13.8\pm 1.3$	$13.6\pm 1.5$	$13.5\pm 1.5$
1.0		100	100	100	$15.1\pm 1.4$	$15.6\pm 1.4$	$13.8\pm 1.4$
3.0		100	100	100	$15.3\pm 1.5$	$15.4\pm 1.6$	$15.1\pm 1.6$

\*Data are the means  $\pm$  SD, of three experiments. Different alphabetical letters are significantly different according to Duncun's multiple range test at  $\alpha \leq 0.05$ .

in a culture of *I. pumila* (Radojevic et al. 1987), *I. pseudacorus*, and *I. virginica* embryos also required 2,4-D. This concentration was also reported to be the optimal concentration for callus formation compared to other iris species (Myers and Simon 1999; Luciani et al. 2006).

#### Plantlet production and soil transfer

The highest callus growth rate was obtained in either  $0.5$  or  $1.0 \text{ mg}\cdot\text{L}^{-1}$  BA and  $1.0 \text{ mg}\cdot\text{L}^{-1}$  2,4-D, while BA concentration higher than  $2.0 \text{ mg}\cdot\text{L}^{-1}$  inhibited callus pro-

liferation and promoted shoot formation. However, the best callus growth was obtained in the presence of  $1.0 \text{ mg}\cdot\text{L}^{-1}$  2,4-D, indicating that 2,4-D played a key role in callus proliferation (data not shown). In this sense, our results showed that 2,4-D not only induced callus initiation, but also maintained callus proliferation and agreed with previous results observed in iris callus culture (Boltenkov et al. 2004). Proliferated compact calli were transferred to 1/2MS medium supplemented with different BA and 2,4-D concentrations under light conditions to investigate their potential for shoot regeneration (Table 2). After 4 weeks of culture, most of

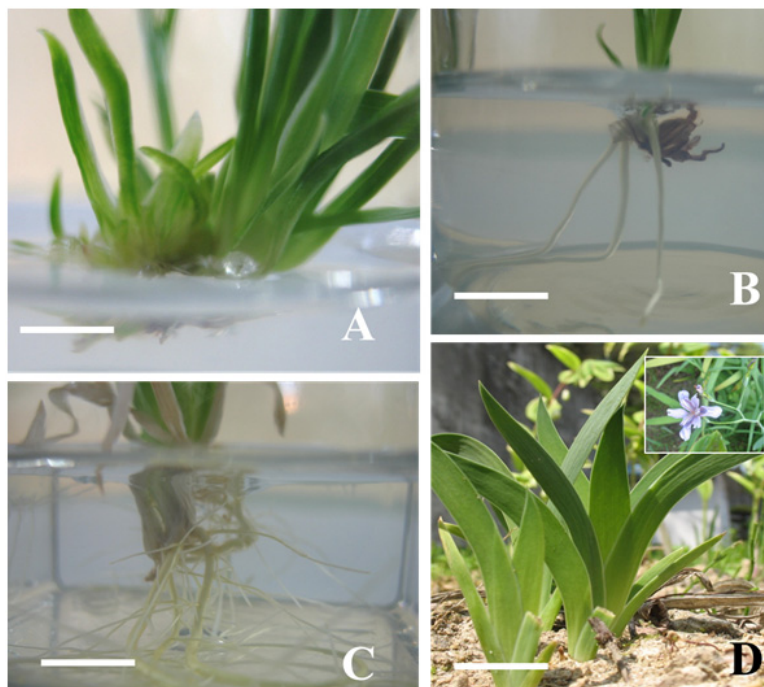
the compact calli started to turn to light green (Fig. 1D), and control was rooting from calli after 4 weeks of culture (Fig. 1E). Most of the calli at the early stage developed many yellowish green globular structures. Calli formed numerous shoots when they were cultured on 1/2MS medium supplemented with different concentrations of BA and 2,4-D (Table 2) (Fig. 1F). BA plays a key role in shoot regeneration *in vitro* (Ayabe et al. 1995; Ayabe and Sumi 1998; Guo et al. 2005; Xu et al. 2008). In the present experiment, BA induced shoot regeneration at the rate of 100% when cultured on medium with 1.0 or 3.0 mg·L<sup>-1</sup> BA, although the highest BA concentration at 3.0 mg·L<sup>-1</sup> appeared to show a suppressive effect on shoot differentiation (Table 2). These results agreed with the reports of Barandiaran et al. (1999) and Luciani et al. (2006), where BA could induce

shoot regeneration from calli, but were different from the observations of Myers and Simon (1999), who found that BA alone did not induce shoot regeneration. Regenerated shoot-derived calli were transferred to 1/2 MS medium with 0, 0.5, 1.0, and 3.0 mg·L<sup>-1</sup> IBA to investigate the root induction into plantlets. After 4 weeks of culture, more than 90% root induction into plantlets was observed, with well-developed leaves and roots in all media (Fig. 2B-C). The length of roots of plantlets was the longest in the 1/2 MS medium with 1.0 mg·L<sup>-1</sup> IBA (Table 2). Shoot regeneration was enhanced by the combinations of BA and 2,4-D compared with the use of control. Root formation varied with different NAA concentrations. In this sense, root formation increased with higher NAA concentrations and callus growth was suppressed after rooting under light conditions.

**Table 3.** Effect of 2,4-D and NAA on callus formation and its length derived from explants of root, rhizome and leaf in *I. dichotoma* on 1/2 MS medium including 30 g·L<sup>-1</sup> sucrose after 4 weeks of culture

Treatment (mg·L <sup>-1</sup> )	No. of root	Length of root (mm)
IBA 0	3.1±1.2*d	4.4±0.4d
IBA 0.5	4.1±1.8bc	8.1±1.0b
IBA 1.0	6.1±0.9a	12.8±1.2a
IBA 3.0	4.7±1.1b	7.9±0.3c

\*Data are the means ± SD, of three experiments. Different alphabetical letters are significantly different according to Duncun's multiple range test at  $\alpha \leq 0.05$ .



**Fig. 2.** Plant regeneration from callus derived from *I. dichotoma* A: Shoot multiplication on 1/2 MS medium without plant growth regulators after 8 weeks of culture B: Rooting on 1/2 MS medium without plant growth regulators after 8 weeks of culture C: Rooting on 1/2 MS medium with 1.0 mg·L<sup>-1</sup> IBA after 8 weeks of culture D: Plants survived *ex vitro* (Flowering of plantlets transferred to soil)

However, those calli regenerated shoots also produced roots on growth regulator-free medium (data not shown). When the converted plantlets were acclimated in artificial soil and then transferred to greenhouse, about 96.4% of the plants survived 2 months after transfer (Fig. 2D) and produced flowers after maturing (Fig. 2D).

We conclusively established high-frequency plant regeneration via callus induction in *I. dichotoma*. This protocol can be applied to mass propagation of this endangered endemic species and can be applied to molecular breeding by genetic transformation in *I. dichotoma*.

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