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Plant Regeneration of *Iris koreana* Nakai through Organogenesis for Ex-situ Conservation

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Abstract

Iris koreana (Iridaceae) is an endangered plant native to Korea. In order to develop an *in vitro* propagation method, we investigated the effect of 2,4-dichlorophenoxy acetic acid (2,4-D) and a-naphthalene acetic acid (NAA) on callus induction in different *I. koreana* tissues. In addition, we also investigated the effect of 2,4-D and Benzyl aminopurine (BA) treatments on adventitious shoot induction in viable calli and the effect of indole-3-butyric acid (IBA) on root formation in viable shoots. We found that callus production was highest with 1.0 mg/L NAA (94.4% cultured rhizome explants), and adding low concentrations of 2,4-D to BA containing media significantly increased the frequency of shoot primordial formation. The best rooting results were obtained with 1.0 mg/L IBA, on which 98% of regenerated shoots developed roots and produced an average of 7.4 roots within 45 days. This *in vitro* propagation protocol will be useful for conservation, as well as for mass propagation.

Key Words: *Iris koreana*, callus, *in vitro*, proliferation

Introduction

The genus *Iris* comprises over 300 species, which are spread mostly across the northern temperate zone. *Iris* comprises rhizomatous irises with bearded outer tepals. Four genera and 31 species of *Iris* are distributed across South Korea. *Iris koreana* 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).

This species is rarely propagated by seed, owing to its capacity for cross-pollination, low seed production, problems with germination, and long juvenile period. Similar to vegetative reproduction, the time required to obtain sufficient quantities of planting stock is 4-5 years. On the other hand, cell and tissue culture methods have become popular in iris reproduction, since they considerably increase plant multiplication factors (Shimazu et al. 1997; Wang et al. 1999a; Shibli and Ajlouni 2000) and improve the quality of planting stock (Baruch and Quak 1966; Mielke and Anderson 1989). Therefore, clonal reproduction techniques are used in the conservation of rare iris species (Radojevic and Subotic 1992; Shibli and Ajlouni 2000).

However, the *in vitro* propagation of tissue from monocotyledons is complicated by lower regenerative capacity than that of dicotyledons (Wang and Nguyen 1990), and the regenerative capacity of plants in the Iridaceae is reportedly lower than that of plants in the Amaryllidaceae, Araceae, and Liliaceae (Hussey 1975). Hussey (1975) also

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demonstrated that the selection of organs or tissues 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 *in vitro* iris regeneration. For instance, flower tissues have been used for the direct regeneration of *I. ensata* (Ichihashi and Kato 1986; Kawase et al. 1991), and the perianth tube and upper ovary proved to be the most effective tissues for the microreproduction of *I. ensata* cultivars (Kawase et al. 1995). The patterns of shoot and organogenic callus formation in young stem cultures were studied in cultivars and wild-type *I. ensata* (Yabuya et al. 1991).

In addition, numerous studies have demonstrated that the hormonal composition of the culture 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; Jéhan et al. 1994; Shimizu et al. 1996; Wang et al. 1999b). The morphogenetic capacity of explant tissues can be induced by varying the compositions of phytohormones in the medium, which can result in the production of organogenic calluses and the differentiation of accessory buds via organogenesis or embryoidogenesis, respectively.

Owing to its rarity and ornamental value, an efficient propagation method for *I. koreana* should be established. As is common in irises, propagation of the species by splitting rhizomes is slow (Hussey 1975; Jéhan et al. 1994). Therefore, *in vitro* micropropagation might be more suitable. Various iris species have been propagated through organogenesis or somatic embryogenesis, using explants from leaf bases (Gozu et al. 1993; Shibli and Ajlouni 2000), mature zygotic embryos (Radojevic and Subotic 1992; Boltenkov et al. 2004), ovary sections (Laublin and Cappadocia 1992), and root sections (Laublin et al. 1991).

However, the propagation of *I. koreana* by *in vitro* plant regeneration has not been reported. Therefore, in the present study, we aimed to develop a protocol for regeneration from leaf, rhizome, and root explant sections as an efficient *in vitro* propagation method that could significantly help in the multiplication and conservation of this endemic iris.

Materials and Methods

Seeds of *I. koreana* were scarified by immersion in 70% EtOH for 1 min and then sterilized by immersion in 1% (v/v) commercial bleach (5% sodium hypochlorite) with a few drops of Tween-20 (Sigma, St. Louis, MO, USA) for 30 min. The seeds were washed five times in sterile water and transferred to petri dishes that contained 20 mL solid hormone-free 1/3 MS medium (Murashige and Skoog 1962), under cool white fluorescent lights (56 μmoL/m^{-2}/s) on a 16 h photoperiod or in the dark at 25°C. The germination test was performed in triplicate, with 30 seeds in each replicate. Seeds collected in Kongju National Unviersity green house.

Two-week-old seedlings were used as an explant source, and explants (5-10 mm in length) from leaf, rhizome, and root (n=60 each) were placed on 1/2 MS basal medium that contained 0, 0.1, 0.5, 1.0, or 3.0 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) or 0, 0.1, 0.5, 1.0, or 3.0 mg/L a-naphthalene acetic acid (NAA). The media were also supplemented with sucrose (30 g/L), solidified with agar (8 g/L), and adjusted to a pH of 5.7. The explants were incubated under cool white fluorescent lights on a 16 h photoperiod at 25°C and subcultured on fresh media every 4 weeks.

Calli that were subcultured on the same medium for two generations were used for the induction of adventitious shoot formation. Briefly, the calli were transferred onto 1/2 MS medium supplemented with 30 g/L sucrose, solidified with 8 g/L agar, and adjusted to a pH of 5.7. The medium was also supplemented with 0.1, 0.5, 1.0, or 3.0 mg/L 2,4-D and 1.0 or 3.0 mg/L Benzyl aminopurine (BA). The calli were maintained under cool white fluorescent lights (56 μmoL/m^{-2}/s) 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 in duplicate, and each replicate included 20 calli from each callus line.

For root induction, individual shoots of 3-4 cm in length were placed in test tubes that contained 10 mL 1/2 MS medium supplemented with 30 g/L sucrose, solidified with 8 g/L agar, and adjusted to a pH of 5.7. The auxin indole-3-butyric acid (IBA) was assayed at 0, 0.5, 1.0, and 3.0 mg/L. After 4 weeks, rooting was evaluated and expressed as rooting frequency, root number, and the longest root length per plantlet.

All data were analyzed using analysis of variance

(ANOVA) and expressed as means±standard error (SE). To examine significant differences among the treatments, multiple comparison tests were performed using Duncan's multiple range test at $\alpha \leq 0.05$ (SAS).

Results and Discussion

In *I. koreana*, callus formation varied significantly, depending on explant type (Table 1). Rhizome explants yielded the earliest signs of callus formation, after 4 weeks of culture, whereas the leaf and root explants did not start to generate callus until after 5 weeks of culture. The calli produced on 1/2 MS medium with 1.0 mg/L 2,4-D were compact, globular, and yellowish (Fig. 1c), and those produced on 1/2 MS medium with 1.0 mg/L NAA were globular and dark yellowish (Fig. 1b). However, the explants cultured on control medium failed to produce callus (Fig. 1a). In addition, the rhizome explants exhibited 90.4% callus formation after 8 weeks, whereas the leaf and root explants exhibited significantly lower rates of callus induction (8.1 and 39.4%, 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 mg/L NAA and 0.5 mg/L BA (Boltenkov et al. 2004). In monocots the induction and proliferation of callus are reportedly slow and difficult processes, compared to other iris species (Geier 1990; Zheng et al. 1998; Luciani et al. 2006; Liu 2020). In the present study, the largest callus (5.8 mm) was achieved using 1/2 MS medium with 3.0 mg/L NAA, whereas the

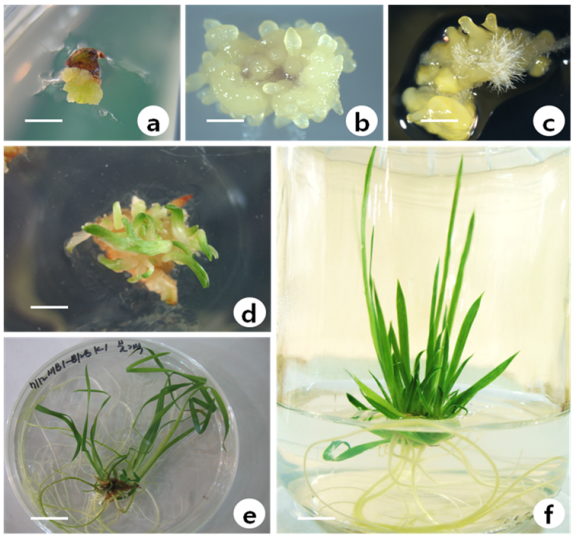

Fig. 1. Plant regeneration from callus derived from *in vitro* cultured explants type of *I. koreana*. (a) Leaf explant on 1/2 MS medium with 0.5 mg/L 2,4-D after 8 weeks of culture. Bar=4.3 mm. (b) Rhizome explant on 1/2 MS medium with 1.0 mg/L NAA after 8 weeks of culture. Bar=4.6 mm. (c) Root part on 1/2 MS medium with 1.0 mg/L 2,4-D after 8 weeks culture. Bar=4.6 mm. (d) Shoot regeneration on 1/2 MS medium with 0.5 mg/L BA and 1.0 mg/L 2,4-D after 8weeks of culture. Bar=14 mm. (e) Rooting on 1/2 MS medium with 1.0 mg/L IBA after 8 weeks of culture. Bar=24 mm. (f) Further growth of shoots and roots in 1/2 MS medium without plant growth regulators after 8 weeks of culture. Bar=28 mm.

Table 1. Effect of 2,4-D and NAA on the formation and length of callus derived from root, rhizome, and leaf explants in *Iris koreana*

Treatment (mg/L)	Callus formation (%)			Length of calli (mm)*		
	Leaf	Rhizome	Root	Leaf	Rhizome	Root
2.4-D 0	0	0	0	0	0	0
2.4-D 0.1	16.3	78.8	44.5	3.1±0.2	3.1±0.4	3.5±0.4
2.4-D 0.5	19.0	71.7	28.3	3.4±0.3	3.5±0.5	3.6±0.5
2.4-D 1.0	13.2	54.4	20.1	5.4±0.4	3.8±0.4	5.6±0.4
2.4-D 3.0	14.2	51.4	17.2	5.4±0.5	5.1±0.6	5.4±0.6
NAA 0	0	0	0	0	0	0
NAA 0.1	17.5	71.3	52.2	5.1±0.5	5.3±0.3	5.4±0.2
NAA 0.5	14.6	78.6	31.2	5.7±0.5	5.4±0.4	5.6±0.4
NAA 1.0	13.1	94.4	32.9	5.3±0.4	5.5±0.3	5.4±0.5
NAA 3.0	11.4	76.3	33.5	5.5±0.6	5.4±0.1	5.8±0.5

2,4-D, 2,4-dichlorophenoxy acetic acid; NAA, a-naphthalene acetic acid (NAA).
*Data are the means±SE (n=3).

smallest was observed on 1/2 MS medium with 0.1 mg/L 2,4-D (3.2 mm). The formation of morphogenic callus in cultures of *I. pumila*, *I. pseudacorus*, and *I. virginica* embryos also required 2,4-D (Radojevic et al. 1987).

The fastest callus growth rate was obtained in media with either 0.5 or 1.0 mg/L BA and 1.0 mg/L 2,4-D, whereas BA concentrations higher than 2.0 mg/L inhibited callus proliferation and promoted shoot formation. In this sense, our results demonstrae that 2,4-D induces callus formation and also, maintains callus proliferation, in agreement with previous report (Boltenkov et al. 2004; Liu 2020).

Proliferated compact calli were transferred to 1/2 MS medium with different BA and 2,4-D concentrations to investigate their potential for shoot regeneration (Table 2). After 4 weeks of culture, most of the compact calli began to turn light green (Fig. 1d), and the calli under the control treatment was rooting (Fig. 1e). At the early stage, most of the calli developed yellowish green globular structures, and the calli formed numerous shoots when cultured on 1/2 MS medium with different concentrations of BA and 2,4-D (Table 2; Fig. 1f). Previous studies have reported that BA plays a key role in *in vitro* shoot regeneration (Ayabe et al. 1995; Ayabe and Sumi 1998; Guo et al. 2005; Xu et al. 2008; Liu et al. 2020), and in the present study, BA induced shoot regeneration at a rate of 100%, when cultured on media with 1.0 or 3.0 mg/L BA, although 3.0 mg/L BA appeared to suppress shoot differentiation (Table 2). These results agree with the reports of Luciani et al. (2006), who found that BA could induce shoot regeneration from calli, but differed from the observations of Myers and Simon (1999), who found that BA alone failed to 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 IBA to investigate root induction. After 4 weeks of culture, 90% root induction was observed, with well-developed leaves and roots in all media (Fig. 2b-c). The plantlet roots were longest in the 1/2 MS medium with 1.0 mg/L IBA (Table 3). 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. However, the calli-regenerated shoots also produced roots on growth regulator-free medium.

Therefore, we conclusively established a method for high-frequency plant regeneration via callus induction in *I.*

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. koreana* on 1/2 MS medium including 30 g/L sucrose after 4 weeks of culture

Treatment (mg/L)	No. roots	Length of roots (mm)*
IBA 0	4.3 ± 1.2^{d}*	3.4 ± 0.4^{d}
IBA 0.5	4.8 ± 1.8^{bc}	7.1 ± 1.0^{b}
IBA 1.0	7.4 ± 0.9^{a}	13.4 ± 1.1^{a}
IBA 3.0	6.7 ± 1.1^{b}	6.8 ± 0.3^{c}

*Data are the means±SE (n=3). Different alphabetical letters indicate means that are significantly different according to Duncun's multiple range test at $\alpha \leq 0.05$.

Table 2. Effect of 2,4-D and BA on the formation and length of adventitious shoots derived from root, rhizome, and leaf explants in *I. koreana*

Treatment (mg/L)		Shoot formation (%)			Length of shoots (mm)*		
BA	2,4-D	Leaf	Rhizome	Root	Leaf	Rhizome	Root
0	0	0	0	0	0	0	0
1.0	0.1	94	91	91	11.2±1.5	11.5±1.4	13.4±1.2
	0.5	91	98	94	12.8±1.1	11.6±1.3	13.8±1.4
	1.0	100	100	100	15.4±1.2	14.6±1.2	13.8±1.3
	3.0	100	100	100	15.8±1.3	13.4±1.1	15.8±1.2
3.0	0.1	100	100	100	14.2±1.1	13.5±1.3	13.2±1.1
	0.5	100	100	100	13.7±1.8	15.6±1.4	13.6±1.3
	1.0	100	100	100	15.8±1.2	16.6±1.5	13.7±1.5
	3.0	100	100	100	15.5±1.1	16.4±1.1	15.8±1.1

*Data are the means±SD, of three experiments.

koreana. This protocol can be applied to the mass propagation of the species and to molecular breeding of *I. koreana* by genetic transformation.

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