Regular Article
pISSN: 2288-9744, eISSN: 2288-9752
Journal of Forest and Environmental Science
Vol. 39, No. 3, pp. 150-154, September, 2023
https://doi.org/10.7747/JFES.2023.39.3.150

JFES
Journal of Forest and Environmental Science

In vitro Organogenesis and Propagation of *Heloniopsis orientalis* Thunb

Jong-Woo Nam[1], Yoon-Kyung Choi[2], Kyeong-Mi Cho[2], Young-Been Kim[2], Sung Hwan Yim[3,4] and Kee Hwa Bae[2,*]

[1]Department of Applied Biology, Kangwon National University, Chuncheon 24341, Republic of Korea
[2]Seedvault Center, Baekdudaegan National Arboretum, Bonghwa 36209, Republic of Korea
[3]Department of Biology, Kyungpook National University, Daegu 41566, Republic of Korea
[4]Research Institute for Dok-do Ulleung-do Island, Kyungpook National University, Daegu 41566, Republic of Korea

Abstract

Heloniopsis orientalis (Liliaceae) is an important horticultural crop native to Korea. Under natural conditions, germination is poor and plant growth is delayed. Therefore, we have developed a vegetative propagation method to produce plants with vigorous growth characteristics *via* tissue culture. The regenerated shoots were then initiated directly from leaf explants on an MS medium containing either 0.5 to 2.0 mg/L 2,4-D or 1.0 to 3.0 mg/L BA. Healthy plantlets with adventitious roots were formed on the medium supplemented with 1.0 mg/L BA (81%). BA triggered callus initiation without caulogenesis or rhizogenesis, and callus formation was better on the half-strength MS medium than on the full-strength medium. This *in vitro* propagation protocol will be useful for conservation, as well as for mass propagation.

Key Words: *Heloniopsis orientalis*, *In vitro*, propagation, conservation

Introduction

Heloniopsis orientalis (Liliaceae), a perennial evergreen herb, grows under closed canopies in wet soils and rich organic matter (Lee 1982; Min 2000a). Destruction of the forest and an increase in the annual mean air temperature have decreased the size of this species habitat. In the natural population, *H. orientalis* normally produces 3,000 seeds per year, but only a few new plants are recruited in this manner (Min 2000b).

Plant tissue culture is a powerful alternative technique for conservation and propagation of plants, especially for those that are rare and difficult to propagate by conventional methods (Shimazu et al. 1997; Wang et al. 1999a; Shibli and Ajlouni 2000) and improves the quality of valuable planting stock (Baruch and Quak 1966; Mielke and Anderson 1989). The developed techniques for clonal reproduction are used as an alternative way to conserve rare iris species (Radojevic and Subotic 1992; Shibli and Ajlouni 2000). *In vitro* propagation of tissue of monocotyledons is complicated by their low regenerative capacity compared to dicotyledons (Wang and Nguyen 1990; Kawase et al. 1991). These paper showed that the selection of organ or tissue as an explant is important in the development of plant reproduction through explants.

Numerous studies demonstrated that the hormonal composition of the medium is the most important factor for *in vitro* regeneration of Liliaceae (Radojevic et al. 1987; Laublin and Cappadocia 1992; Radojevic and Subotic 1992; Gozu et al. 1993; Jehan et al. 1994; Shimizu et al.

Received: May 31, 2023. Revised: June 23, 2023. Accepted: June 23, 2023.

Corresponding author: Kee Hwa Bae

Seedvault Center, Baekdudaegan National Arboretum, Bonghwa 36209, Republic of Korea
Tel: +82-54-679-2768, Fax: +82-54-679-0960, E-mail: khbae7724@koagi.or.kr

1996; Wang et al. 1999b). Various Liliaceae 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; Boltenkov et al. 2004), ovary sections and root sections (Laublin and Cappadocia 1992), but they reported nothing concerning about directed organogenesis from *H. orientalis*.

However, the propagation of *H. orientalis* by *in vitro* plant regeneration has a few reported (Yun et al. 2000; Cha et al. 2002). 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 plant.

Materials and Methods

Mature seeds of *H. orientalis* were collected from Hwangak mountain in Korea, and they were sown on seed beds prepared in a greenhouse at the Kongju National University. Mature seeds of *H. orientalis* were scarified by immersion in 70% EtOH for 1 min and then sterilized with commercial bleach 1% (v/v) (5% of sodium hypochlorite) with a few drops of Tween-20 (Sigma, USA) for 30 min. The seeds were washed 5 times in sterile water and placed into petri dishes containing 20 mL 1/3MS medium (Murashige and Skoog 1962, Modified D668, Duchefa, Netheland) without plant growth regulators supplemented 9 g/L plant agar (Duchefa, Netheland) 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 carried out on triplicates of 30 seeds.

After leaf, rhizome, and root segments were cut into 10 mm in sizes, they were cultured on MS medium supplemented with 0, 0.5, 1.0, and 3.0 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) and a-Naphtalene acetic acid (NAA). All media were supplemented with 30 g/L sucrose and solidified with 8.0 g/L plant agar, and then adjusted to 5.8 pH before autoclaving at 121°C for 20 min. The culture room was maintained at 25±1°C in darkness. The frequency of callus induction was evaluated after 12 weeks of culture. Thirty explants (each leaf, rhizome, and root) were incubated for each treatment and repeated 3 times.

Calli subcultured on the same medium for two generations, were used for inducing adventitious bud. Calli were transferred onto MS medium supplemented with sucrose (30 g/L) and solidified with agar (8 g/L); pH 5.7; added with 0, 0.5, 1.0, and 2.0 mg/L 2,4-D and 0, 0.5, 1.0, 3.0 mg/L BA. Calli were maintained under cool white fluorescent lights (56 μmol/m^{-2}/s) on a 16 h photoperiod at 25°C. After 8 weeks, adventitious shoot induction was eval-

Table 1. Effect of 2,4-D and NAA on callus formation and adventitious bud formation from root, rhizome and leaf explants of *H. orientalis* on MS medium including 30 g/L sucrose after 12 weeks of culture

PGR's (mg/L)		Frequency of callus					
		CF (%)			LC (mm)		
2.4-D	NAA	LF	RZ	RT	LF	RZ	RT
0		0	0	0	0	0	0
0.5		41±3.2	67±5.8	44±3.2	2.2±0.2	3.1±0.4	2.1±0.4[a)]
1.0		48±4.8	77±3.4	52±1.8	2.8±0.3	3.4±0.5	2.5±0.5
3.0		36±4.2	58±2.3	36±2.9	2.1±0.4	3.2±0.4	2.8±0.4
	0	0	0	0	0	0	0
	0.5	0	0	0	0	0	0
	1.0	0	0	0	0	0	0
	3.0	0	0	0	0	0	0

CF, Callus formation; LC, Length of callus; ARF, Adventitious root formation; LAR, Length of adventitious root; LF, Leaf; RZ, Rhizome; RT, Root.

[a)]Data are the means±SD, of three experiments.

uated and expressed as shooting frequency and number of adventitious shoot per callus. The experiments were performed on 20 calli for each callus line, and repeated twice.

Adventitious buds were transferred to WPM (Woody Plant Medium), half-strength WPM, one-third strength WPM medium and MS, half-strength MS, and one-third strength MS medium for the growth of plantlets. The culture room was maintained at 25±1°C with a 16 h photoperiod under 56 μmol/m^{-2}/s cool white fluorescent light. Plantlet conversion rate was evaluated by counting plantlets with well-developed leaves and roots after 4 weeks of culture. Plantlet height was evaluated by measuring average length of shoots and roots after 4 weeks of culture. Four germinated embryos were transferred onto each plastic square culture vessels (7.2 cm×7.2 cm×10 cm). Each experiment was performed 5 times.

All data were analyzed using ANOVA and expressed as means±standard error (SE). Each experiment contained 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 $p \leq 0.05$ (SAS 2001).

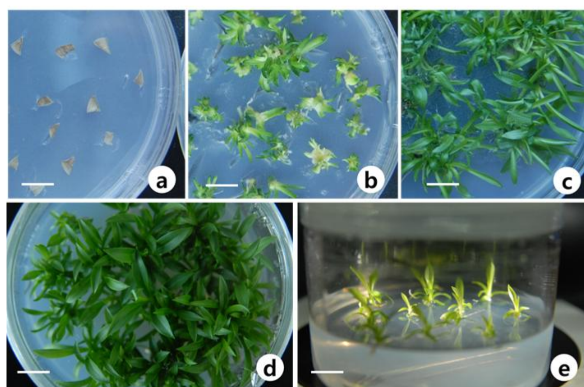

Fig. 1. Plant regeneration from callus derived from *in vitro* cultured explants type of *H. orientalis*. (A) Leaf explant on 1/2MS medium with 0.5 mg/L 2,4-D after 8 weeks of culture. Bar=4.3 mm. (B) Rhizome explant on 1/2MS medium with 1.0 mg/L NAA after 8 weeks of culture. Bar=4.6 mm. (C) Further growth C of 4 weeks of culture. Bar=4.6 mm. (D) Shoot regeneration on 1/2MS medium with 0.5 mg/L BA and 1.0 mg/L 2,4-D after 8 weeks of culture. Bar=14 mm. (E) *In vitro* regeneration on 1/2MS medium without plant growth regulators after 8 weeks of culture. Bar=28 mm.

Results and Discussion

Callus formation is varied significantly depending on kind of explant of *H. orientalis*. rhizome explant showed 77% callus formation after 12 weeks of culture (Table 1). However, roots and leaves exhibited a significantly lower callus induction with 51 and 33% callus formation respectively (Table 1). Non-treated 2,4-D or NAA was not response all explants (Fig. 1a) but callus were induced from rhizome explant on MS medium with 2,4-D after 12 weeks of culture (Fig. 1b).

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 mg/L α-naphthylacetic acid and 0.5 mg/L BA (Boltenkov et al. 2004). 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 1.0 mg/L 2,4-D on MS medium (3.4 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 in a culture of *I. pumila* (Radojevic et al. 1987), *I. pseudacorus*, and *I. virginica* embryos also required 2,4-D. It was also reported to be the optimal concentration of 2,4-D for callus formation compared to other species (Myers and Simon 1999; Luciani et al. 2006).

For determination of adventitious shoot induction from callus, both types (compact and friable ones) were transferred to 2,4-D and BA supplemented with MS medium and placed under illumination. Twenty to 25 days after transference, only the compact calli turned greenish (partially green) and several adventitious shoot regenerated on the calli (data not shown). The differentiated multiple shoots were divided and transplanted onto the same medium (Fig. 1c). The highest adventitious shoot induction rate was obtained in 1.0 mg/L 2,4-D and 1.0 mg/L BA (Table 2). 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 elongation. After 4 weeks of culture, most of the compact calli started to turn to light green. Most of the callus at the early stage developed many yellow-

Table 2. Effect of 2,4-D with BA on adventitious shoot induction from rhizome derived callus segments of *H. orientalis* MS medium including 30 g/L sucrose after 8 weeks of culture

PGR's (mg/L)		Adventitious shoot induction (%)	No. of adventitious shoot/petri-dish (mm)
2,4-D	BA		
0	0	0	0
0.5	0.5	54±3.6 (fg)	121.4±1.9[a] (h)
	1.0	59±4.6 (f)	158.1±2.9 (d)
	3.0	41±5.8 (h)	144.7±8.9 (e)
1.0	0.5	77±7.1 (b)	211.1±6.1 (b)
	1.0	81±2.9 (a)	228.9±5.9 (a)
	3.0	74±8.8 (c)	209.4±4.6 (bc)
2.0	0.5	68±5.6 (d)	127.6±9.3 (g)
	1.0	69±2.3 (de)	132.2±4.6 (f)
	3.0	54±5.6 (fg)	113.2±8.4 (i)

Different alphabetical letters are significantly different according to Duncun's multiple range test at $p < 0.05$.
[a]Data are the means±SD, of three experiments.

Table 3. Effect of various kinds of medium on the conversion of adventitious shoot of *H. orientalis* on 1/2MS medium including 30 g/L sucrose after 4 weeks of culture

Various kinds of medium	Conversion into plantlet (%)	Length of shoot (cm)	Length of root (cm)
WPM	97.6±1.7* (a)	8.7±1.8* (d)	5.1±1.8* (f)
1/2WPM	97.2±2.3 (a)	10.4±0.8 (c)	5.4±2.1 (e)
1/3WPM	97.1±3.5 (a)	11.2±2.1 (b)	6.1±1.9 (b)
MS	94.4±5.1 (a)	12.8±3.1 (a)	6.2±1.3 (a)
1/2MS	95.8±4.2 (a)	12.8±1.4 (b)	5.7±2.3 (bc)
1/3MS	92.5±4.2 (a)	12.4±4.8 (c)	5.9±3.5 (cd)

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

ish green globular structures. Calli formed numerous shoots when they were cultured on MS medium supplemented with different concentrations of BA and 2,4-D (Table 2). Adventitious shoot of *H. orientalis* were transferred to various media (WPM, 1/2WPM, 1/3WPM, MS, 1/2MS, and 1/3MS) to investigate the conversion into plantlets. After 4 weeks of culture, more than 90% of the adventitious shoot converted into plantlets with well-developed leaves and roots in all media (Fig. 1d-e). However, there was a remarkable difference on the growth of plantlets among the six media (Table 3).

The length of shoots and roots of plantlets was the longest in the MS medium. Therefore, MS medium was the most effective for growth of plants in *H. orientalis*. BA plays a key role in shoot regeneration *in vitro* (Ayabe et al. 1995; Ayabe et al. 1998; Guo et al. 2005; Xu et al. 2008). In the present experiment, BA could induce shoot regeneration at the rate of 100% when cultured on medium with 1.0 or 3.0 mg/L BA, although the highest BA concentration at 3.0 mg/L appeared to show a suppressive effect on shoot differentiation (Table 2). These results agree with the reports of Luciani et al. (2006), where BA could induce shoot regeneration from callus, but were different from the observations of Myers and Simon (1999) who found that BA alone did not induce shoot regeneration. we established high frequency plant regeneration *via* callus induction in *H. orientalis*. This protocol can be applied to mass propagation of this endangered endemic species and can be applied to molecular breeding by genetic transformation in *H. orientalis*.

Acknowledgements

This study was carried out with the support of R&D Program for Forest Science Technology (Project No. 2021398B10-2325-CA02) provided by Korea Forest Service (Korea Forestry Promotion Institute).

References

Ayabe M, Sumi S. 1998. Establishment of a novel tissue culture method, stem-disc culture, and its practical application to micropropagation of garlic (*Allium sativum* L.). Plant Cell Rep 17: 773-779.

Ayabe M, Taniguchi K, Sumi S. 1995. Regeneration of whole plants from protoplasts isolated from tissue-cultured shoot primordia of garlic (*Allium sativum* L.). Plant Cell Rep 15: 17-21.

Baruch ER, Quak F. 1966. Virus free plants of iris "Wedgewood" obtained by meristem culture. Neth J Plant Pathol 72: 270-273.

Boltenkov EV, Rybin VG, Zarembo EV. 2004. Cultivation of Iris ensata Thunb. callus tissue. Prikl Biokhim Mikrobiol 40: 244-251.

Cha HC, Park HJ, Min BM. 2002. Plant regeneration and morphology during *in-vitro* organogenesis from *Heloniopsis orientalis* (Thunb.) C. Tanaka. J Plant Biol 45: 56-61.

Gozu Y, Yokoyama M, Nakamura M, Namba R, Yomogida K, Yanagi M, Nakamura S. 1993. *In vitro* propagation of *Iris pallida*. Plant Cell Rep 13: 12-16.

Guo DP, Zhu ZJ, Hu XX, Zheng SJ. 2005. Effect of cytokinins on shoot regeneration from cotyledon and leaf segment of stem mustard (*Brassica juncea* var. *tsatsai*). Plant Cell Tiss Organ Cult 83: 123-127.

Jéhan H, Courtois D, Ehret C, Lerch K, Pétiard V. 1994. Plant regeneration of *Iris pallida* Lam. and *Iris germanica* L. *via* somatic embryogenesis from leaves, apices and young flowers. Plant Cell Rep 13: 671-675.

Kawase K, Mizutani H, Yoshioka M, Fukuda S. 1991. Propagation of Iris by tissue culture. 2. Culture of flower organs. J Jpn Soc Hortic Sci 60: 436-437.

Laublin G, Cappadocia M. 1992. *In vitro* ovary culture of some apogon garden Irises (*Iris pseudacorus* L., I. setosa Pall., I. versicolor L.). Bot Acta 105: 319-322.

Laublin G, Saini HS, Cappadocia M. 1991. *In vitro* plant regeneration *via* somatic embryogenesis from root culture of some rhizomatous Irises. Plant Cell Tiss Organ Cult 27: 15-21.

Luciani GF, Mary AK, Pellegrini C, Curvetto NR. 2006. Effects of explants and growth regulators in garlic callus formation and plant regeneration. Plant Cell Tiss Organ Cult 87: 139-143.

Mielke KA, Anderson WC. 1989. *In vitro* bulblet formation in Dutch Iris. HortScience 24: 1028-1031.

Min BM. 2000a. Population dynamics of *Heloniopsis orientalis* C. Tanaka (Liliaceae) in natural forests- annual life cycle. J Plant Biol 43: 189-196.

Min BM. 2000b. Population dynamics of *Heloniopsis orientalis* C. Tanaka (Liliaceae) in natural forests- sexual reproduction. J Plant Biol 43: 208-216.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497.

Myers JM, Simon PW. 1999. Regeneration of garlic callus as affected by clonal variation, plant growth regulators and culture conditions over time. Plant Cell Rep 19: 32-36.

Radojević L, Sokic O, Tucic B. 1987. Somatic embryogenesis in tissue culture of Iris (*Iris pumila* L.). Acta Hortic 212: 719-724.

Radojević L, Subotić A. 1992. Plant regeneration of *Iris setosa* Pall. Through somatic embryogenesis and organogenesis. J Plant Physiol 139: 690-696.

Shibli RA, Ajlouni MM. 2000. Somatic embryogenesis in the endemic black Iris. Plant Cell Tiss Organ Cult 61: 15-21.

Shimizu K, Nagaike H, Yabuya T, Adachi T. 1997. Plant regeneration from suspension culture of *Iris germanica* 1. Plant Cell Tiss Organ Cult 50: 27-31.

Shimizu K, Yabuya T, Adachi T. 1996. Plant regeneration from protoplasts of *Iris germanica* L. Euphytica 89: 223-227.

Statistical Analysis System. 2001. User's Guide: Statistics, Version 8.2. SAS Institute, Cary, NC.

Wang WC, Nguyen HT. 1990. A novel approach for efficient plant regeneration from long-term suspension culture of wheat. Plant Cell Rep 8: 639-642.

Wang Y, Jeknic Z, Ernst RC, Chen THH. 1999a. Efficient plant regeneration from suspension-cultured cells of tall bearded *Iris*. HortScience 34: 730-735.

Wang Y, Jeknic Z, Ernst RC, Chen THH. 1999b. Improved plant regeneration from suspension-cultured cells of *Iris germanica* L. 'Skating Party'. HortScience 34: 1271-1276.

Xu Z, Um YC, Kim CH, Lu G, Guo DP, Liu HL, Bah AA, Mao Aining. 2008. Effect of plant growth regulators, temperature and sucrose on shoot proliferation from the stem disc of Chinese jiaotou (*Allium chinense*) and *in vitro* bulblet formation. Acta Physiol Plant 30: 521-528.

Yi CB. 1980. Illustrated Flora of Korea. Hyangmunsa, Seoul.

Yun SY, Lee MS, Lim SC, Shin JD. 2000. Micropropagation of *Heloniopsis orientalis* (Thunb.) C. Tanska *in vitro*. Korean J Plant Tiss Cult 27: 197-202.

Zheng S, Henken B, Sofiari E, Jacobsen E, Krens FA, Kik C. 1998. Factors influencing induction, propagation and regeneration of mature zygotic embryo-derived callus from *Allium cepa*. Plant Cell Tiss Organ Cult 53: 99-105.