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# Plant Regeneration of *Iris germanica* L. from Shoot Apexes via an Improved Somatic Embryogenesis Protocol

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***Abstract.*** **An improved three-stage protocol for plant regeneration via somatic embryogenesis of the horticulturally important plant *Iris germanica* L. was developed using shoot apex segments as explants. At the first stage of the experiment, 60% of callus was obtained from shoot apex segments of *I. germanica* on Murashige and Skoog's (MS) medium supplemented with 4.52 μM 2,4-dichloropheoxyacetic acid (2,4-D) and 0.44 μM 6-benzyladenine (6-BA). When nonembryogenic calli were subcultured on MS medium with 11.31 μM 2,4-D and 0.44 μM 6-BA, maximum frequency of embryogenic callus (66.0%) was obtained. At the second stage, the treatment of 9% (w/v) sucrose resulted in the optimum somatic embryo (SE) formation (70.0%). More than 90.0% of SEs germinated with bipolar structure and regenerated into plantlets on plant growth regulator-(PGR)free MS medium during the third stage. Regenerated plantlets were successfully acclimatized in greenhouse environment with little somaclonal variation. Histological study showed that somatic embryogenesis stages were asynchronous and SEs developed from the surface and inner tissue of embryogenic calli.**

The genus *Iris* L., belonging to the family *Iridaceae*, encompasses more than 300 species of herbaceous perennials. These plants are commonly used as ornamental plants in gardens and heavy metal phytoremediation (Han et al., 2013; Huang et al., 2003a, 2011; Kim et al., 2009). *I. germanica* L., which is regarded as one of the most horticulturally important tall bearded irises, has been cultivated ever since ancient time with hundreds of commercially valuable cultivars (Huang et al., 2003b; Jeknic et al., 1999). In addition, the rhizomes of some *I. germanica* cultivars contained an essential oil composed partly of irones (Jehan et al., 1994). These violet-scented ketonic compounds have been frequently used in cosmetics and perfumes (Gozu et al., 1993).

The cultivation of *I. germanica* is limited, thus failing to meet the ever increasing demand of the raw materials. Although *I. germinica* is traditionally propagated through splitting the rhizomes or by seeds, these methods are not efficient in producing sufficient seedlings (Jehan et al., 1994; Simonet, 1932). Besides, conventional breeding for iris's genetic improvement is hampered by the high degree of incompatibility between species (Jeknic et al., 1999).

Somatic embryogenesis is the fastest system of plant regeneration and generally considered to be prerequisite for genetic transformation (Jeknic et al., 1999; Karami et al., 2006). Therefore, several protocols for iris regeneration via somatic embryogenesis from a variety of explant types have been developed (Jevremović and Radojević, 2006; Kim et al., 2009; Laublin et al., 1991; Radojević et al., 1987; Radojević and Subotić, 1992; Shibli and Ajlouni, 2000), including *I. germanica* regenerated from leaf bases, apices, sepals, petals, and ovaries (Jehan et al., 1994). Jehan et al. (1994) found that the optimal medium for obtaining embryogenic callus was MS medium supplemented with 2.9 g/L proline. The frequency of embryogenic callus induction was 10% and 95% of embryos coverted into the plantlets. Therefore, the low efficiency of proline to improve embryogenic callus in *I. germanica* and that of plant regeneration in other *Iris* species has hindered the establishment of a suitable transformation system (Jeknic et al., 1999). To date, genetic transformation based on regeneration system via somatic embryogenesis is not available in any kind of *Iris* species. Therefore, the search for a method to improve embryogenic callus production and plant regeneration is imperative.

Somatic embryogenesis also provides a model system for the study of early events in plant embryo development (Zimmerman, 1993). In monocotyledonous plants, the morphology of embryos varies depending on the species, especially of the cotyledon which is also called scutellum (Hartmann et al., 1997). However, detailed information concerning morphological or histological changes in SEs of *I. germanica* has not been reported yet.

The primary objective of this study is to establish an efficient plant regeneration system via somatic embryogenesis of *I. germanica* from shoot apexes and study morphological and histological changes of the SEs.

## Materials and Methods

*Plant materials.* The seedlings of *I. germanica* 'India Chief' were grown and maintained in the Iris Germplasm Resources Nursery of the Institute of Botany, Jiangsu Province and the Chinese Academy of Sciences (32°30' N and 118°50' E). Shoot apex segments of 2-year-old plants were collected and rinsed with running tap water for 1 h. They were then surface sterilized with 75% (v/v) ethanol for 45 s, 0.1% (w/v) mercuric chloride for 10 min, followed by six rinses with sterile distilled water.

*Embryogenic callus induction.* The shoot apexes were cut into thin lamellas of ≈2 mm in length as explants and placed on MS medium (Murashige and Skoog, 1962) supplemented with different concentrations of 2,4-D (2.26, 4.52, 6.78, or 9.04 μM) and 0.44 μM 6-BA. All media were supplemented with 3% (w/v) sucrose and 0.6% (w/v) agar (Sigma-Aldrich, St. Louis, MO). The pH was adjusted to 5.8 with 1 N NaOH or HCl before autoclaving at 121 °C for 20 min. All the experiments were carried out in flasks (6 × 6 cm) containing 40 mL of medium each. All cultures were incubated in growth room at 25 °C in darkness. The frequency of callus and embryogenic callus induction were evaluated 8 weeks after the culture.

To increase the frequency of embryogenic callus formation, nonembryogenic calli were subcultured on MS medium supplemented with different concentrations of 2,4-D (2.26, 6.78, 11.31, or 15.84 μM), 0.44 μM BA, and 3% (w/v) sucrose. All cultures were incubated in growth room at 25 °C under a 12 photoperiod with 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ cool-white fluorescent lights. The frequency of embryogenic callus formation was evaluated 4 weeks after the culture.

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*Somatic embryo induction.* To assess the effect of sucrose concentration on SE induction, embryogenic calli were transferred to PGR-free MS medium with (1.5%, 3%, 6%, and 9% sucrose). All cultures were incubated under a 12 photoperiod. As detailed above, the frequency of SE formation was recorded 8 weeks after the culture.

*Germination and plant regeneration.* SEs were separated from each other and transferred to half-strength or full strength MS medium with 3% (w/v) sucrose for germination and plant regeneration. All cultures were incubated under a 12 photoperiod. The frequency of embryo germination was recorded 2 weeks after the culture. The frequency of embryo conversion and plant height were evaluated 5 weeks after the culture.

*Acclimatization.* Plantlets with well-developed roots and leaves were washed with running tap water to remove the culture media and transferred to plastic pots (30 cm × 30 cm) containing a mixture of garden soil, peat, and perlite (1:1:1, v/v/v). After planting, they were covered with a shading net cutting 90% of light under greenhouse condition and irrigated with tap water twice a week. The frequency of plantlets survived were recorded 8 weeks after the transference.

*Histological examination.* To study the ontogeny and development of the embryogenic cells, embryogenic calli and SEs were fixed in the FAA solution (formaldehyde: acetic acid: ethanol, 1:1:18, v/v/v) for 24 h, dehydrated through a graded ethanol series and embedded in paraffin wax. The 10-µm-thick sections were cut by a rotary microtome, stained with safranin-fast green and observed under the light microscope (Nikon Eclipse 50i).

*Statistical analysis.* All experiments were replicated once after a 2-week interval. Each experimental treatment contained five explants per flask and five flasks per treatment. Data were analyzed by one-way analysis of variance (ANOVA) and treatment means were tested by Ducan's multiple range test ($P < 0.05$) using SPSS v. 17.0. Data expressed as percentages were previously transformed according to arcsin $(x/100)^{0.5}$.

Table 1. Effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on callus and embryogenic callus induction of *Iris germanica*.

| Plant growth regulators (µM) | | Percentage of callus formation (%) | Percentage of embryogenic callus formation (%) |
|---|---|---|---|
| 2,4-D | Benzyladenine | | |
| 2.26 | 0.44 | 24.0 ± 7.5 b | 4.0 ± 4.0 a |
| 4.52 | 0.44 | 60.0 ± 8.9 a | 20.0 ± 6.3 a |
| 6.78 | 0.44 | 28.0 ± 4.9 b | 8.0 ± 4.9 a |
| 9.04 | 0.44 | 44.0 ± 4.0 ab | 20.0 ± 6.3 a |

Each value represents the mean ± SE. Different letters within the same column indicate significantly differences by Duncan's multiple range test ($P < 0.05$); $n = 50$ per explant.

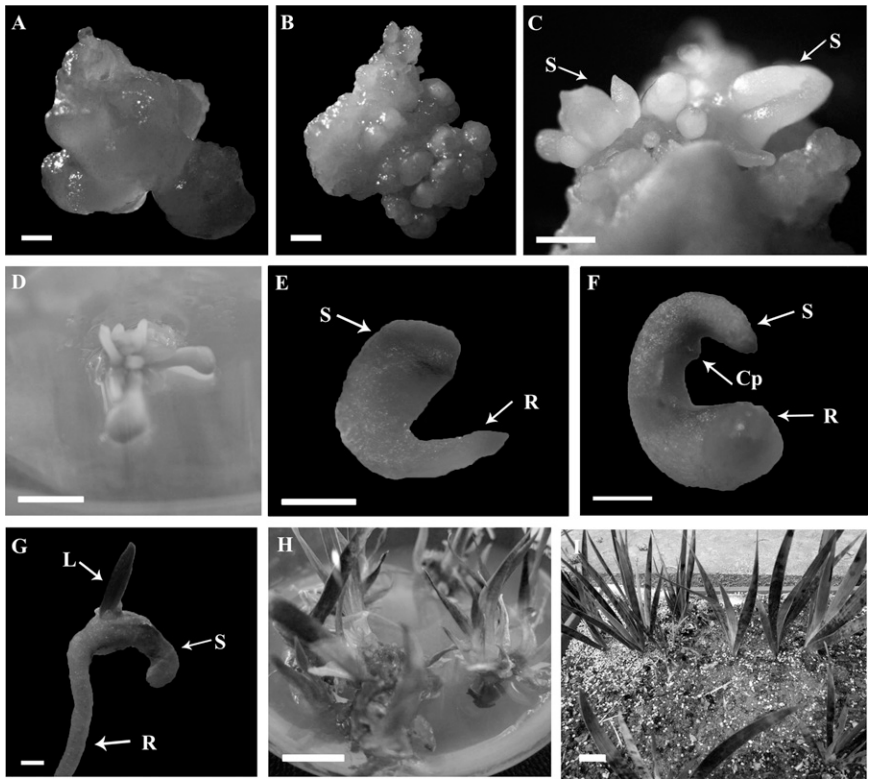


Fig. 1. Somatic embryogenesis from shoot apex segments of *Iris germanica*. (**A**) Nonembryogenic callus were induced on MS medium with 2.26 µM 2,4-D. (**B**) Embryogenic callus were induced on MS medium with 11.31 µM 2,4-D. (**C**) Scutellar stage of embryos were induced on PGR-free MS medium with 3% sucrose (arrows). (**D**) Clusters of scutellar embryos. (**E**) Primary root developed from radicle on full strength MS medium (arrows). (**F**) Coleoptile developed within another week (arrows). (**G**) Leaf emerged from coleoptile (arrows). (**H**) Regenerated plantlets with well-developed leaves and roots. (**I**) Accilimatized plantlets in the greenhouse. MS = Murashige and Skoog's; 2,4-D = 2,4-dichlorophenoxyacetic acid; PGR = plant growth regulator; S = scutellum; R = root; Cp = cleoptile; L = leaf. Bars = A–G = 1 mm; H–I = 1 cm.

## Results

*Callus induction.* Callus, yellowish in color was induced from the explants in all culture media and 2,4-D had a significant impact on the frequency of callus formation at 8 weeks after the culture. Among different media, explants cultured on medium with 4.52 µM 2,4-D displayed the best response with the highest frequency of callus formation of 60.0% (Table 1). Meanwhile, two types of calli could be distinguished based on texture. One type, which was most commonly induced, had a soft and gelatinous appearance and was considered as the nonembryogenic callus (Fig. 1A). The other type, which was considered as the embryogenic callus, presented a friable and nodular structure (Fig. 1B). Only a few embryogenic calli were induced with the maximum of 20.0% and there were no significant differences among the induction treatments (Table 1).

*Embryogenic callus conversion.* When nonembryogenic calli were subcultured on MS medium with different concentrations of 2,4-D (2.26, 6.78, 11.31, or 15.84 µM), they started to convert into embryogenic calli over a 2-week incubation period. Significant increase in the frequency of embryogenic callus induction was observed with the increasing concentration of 2,4-D up to 11.31 µM (Table 2). However, higher concentration of 2,4-D (15.84 µM) exerted an inhibitory effect on embryogenic callus conversion, lowering its frequency to 40.0%.

*SE induction.* When embryogenic calli were transferred to PGR-free MS medium with different sucrose concentrations (1.5%, 3%, 6%, or 9%), SEs were induced and developed into scutellar stage on the surface of the explants 8 weeks after the culture (Fig. 1C). All scutella were in an opaque white color and in clusters (Fig. 1D). There were significant differences in SE formation among different sucrose concentrations. Only 8% SEs were induced on medium containing 1.5% sucrose (Table 3). Some embryogenic calli even converted into nonembryogenic calli or became necrotic. The optimum sucrose concentration was 9%, resulting in the highest SE formation of 70.0%.

*Germination of SEs.* When the isolated embryos were transferred to half-strength or full-strength MS medium, they started to germinate by developing primary root from

Table 2. Effect of 2,4-dichloropheoxyacetic acid (2,4-D) on the conversion of embryogenic callus of *Iris germanica*.

| Plant growth regulators (μM) | | Percentage of embryogenic callus |
|---|---|---|
| 2,4-D | Benzyladenine | formation (%) |
| 2.26 | 0.44 | 24.0 ± 2.4 c |
| 6.78 | 0.44 | 56.0 ± 4.0 a |
| 11.31 | 0.44 | 66.0 ± 4.0 a |
| 15.84 | 0.44 | 40.0 ± 3.2 b |

Each value represents the mean ± SE. Different letters within the same column indicate significantly differences by Duncan's multiple range test ($P < 0.05$); $n = 50$ per explant.

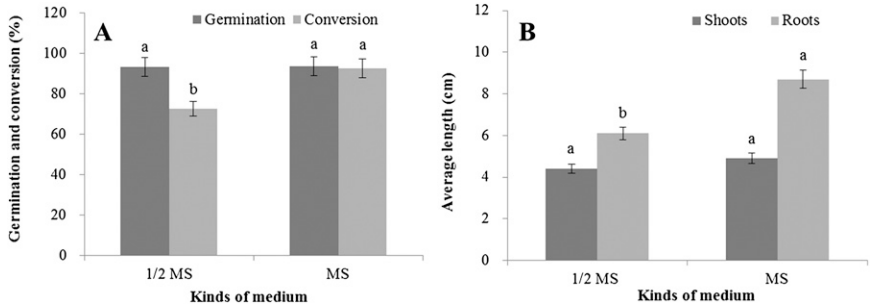


Fig. 2. Effect of medium on the germination and conversion of somatic embryos (**A**) and the growth of plantlets (**B**) of *Iris germanica*. Vertical bars represent the standard error of the mean. Different letters indicate significantly differences by Duncan's multiple range test ($P < 0.05$); $n = 50$ per explant.

Table 3. Effect of sucrose concentrations on the induction of somatic embryos of *Iris germanica*.

| Sucrose (% w/v) | Percentage of somatic embryos matured into scutellar stage |
|---|---|
| 1.5 | 8.0 ± 3.7 c |
| 3 | 46.0 ± 5.1 b |
| 6 | 40.0 ± 5.5 b |
| 9 | 70.0 ± 5.8 a |

Each value represents the mean ± SE. Different letters within the same column indicate significantly differences by Duncan's multiple range test ($P < 0.05$); $n = 50$ per explant.

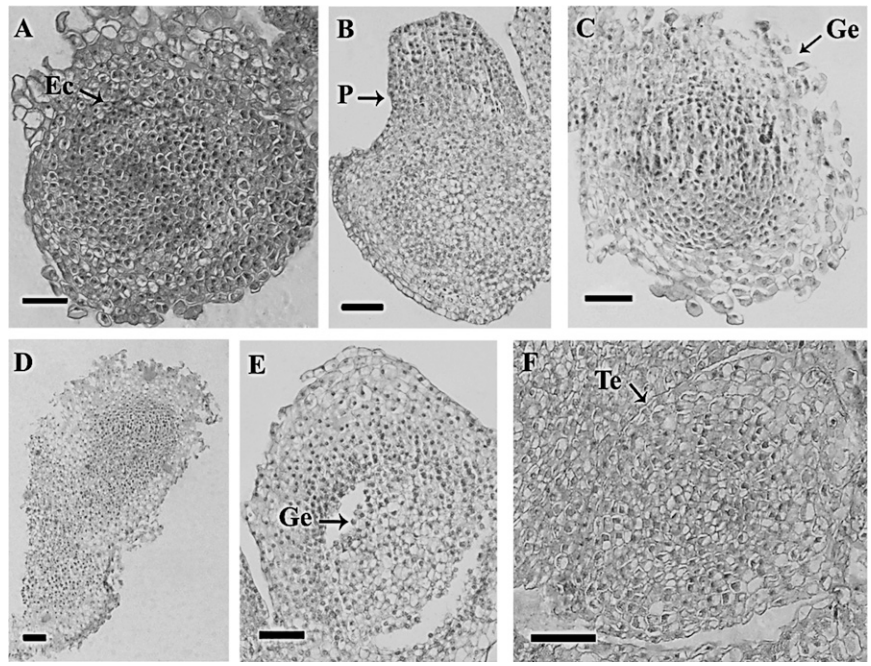


Fig. 3. Histological sections of embryogenic calli and somatic embryos of *Iris germanica*. (**A**) The cells of embryogenic callus with dense cytoplasm and conspicuous nucleus (arrow). (**B**) The protuberance on the embryogenic callus (arrow). (**C**) Globular somatic embryo developed on the surface of embryogenic callus (arrow). (**D**) Torpedo somatic embryo developed on the surface of embryogenic callus. (**E**) Globular somatic embryo developed from the inner tissue of embryogenic callus (arrow). (**F**) Torpedo somatic embryo developed from the inner tissue of embryogenic callus (arrow). Ec = embryogenic callus; Ge = globular embryo; P = protuberance; Te = torpedo embryo. Bars = 100 μm.

radicle within 1 week after the culture (Fig. 1E). Then green coleoptiles developed within the following week (Fig. 1F). Thus, the germinated embryos presented the bipolar structure and germination frequency was more than 90.0% regardless of the culture medium (Fig. 2A).

*Plant regeneration.* Five weeks after the culture, primary leaf emerged from the slit of the coleoptile (Fig. 1G). SEs converted into plantlets with well-developed roots and leaves on both culture media (Fig. 1H). Conversion rate was significantly increased to 92.5% on full-strength MS medium compared with half-strength MS medium (Fig. 2B). The average length of roots was also remarkably higher on full-strength MS medium, while that of shoots showed no difference between the two treatments (Fig. 2B). Therefore, full-strength MS medium was a better choice for the growth of plantlets of *I. germanica*. The frequency of survival of the converted plantlets was 60.0% 8 weeks after the acclimation in artificial soil in greenhouse. Phenotypic variation was not observed between regenerated plantlets and naturally growing plants (Fig. 1I).

*Histological structure of somatic embryos.* The cells of embryogenic callus had dense cytoplasm and conspicuous nucleus, representing strong meristematic activity (Fig. 3A). As the culture progressed, protuberance emerged on the surface of embryogenic callus, showing the initial development of SEs (Fig. 3B). Somatic embryogenesis stages were asynchronous as both globular and torpedo stages of SEs formed simultaneously (Fig. 3C and D). SEs could also develop from the inner tissue of embryogenic callus (Fig. 3E and F). Vascular connections were not observed in the torpedo stage of the embryos (Fig. 3D and F).

## Discussion

Although the regeneration protocol of *I. germanica* via somatic embryogenesis has been previously reported, it was restricted by the low efficiency of proline to induce embryogenic callus (Jehan et al., 1994). In this study, addition of 2,4-D to culture medium was found to enhance embryogenic callus formation significantly. 2,4-D, one of the auxins, has been known for promoting somatic embryogenesis in many plant species (Bano et al., 1991; Chen et al., 2014; Jevremović and Radojević, 2006; Kim et al., 2009; Laublin et al., 1991; Limanton-Grevet and Jullien, 2000; Manrique-Trujillo et al., 2013; Prado and Berville, 1990; Tremblay, 1990). In some species, 2,4-D is effective not only for the induction of somatic embryogenesis but also for the maturation of SEs in *Helianthus annus* (Prado and Berville, 1990), *Asparagus officinalis* (Limanton-Grevet and Jullien, 2000), *Picea glauca* (Tremblay, 1990), and *Valeriana jatamansi* (Chen et al., 2014). However, in this study, 2,4-D was effective in somatic embryogenic induction phase but failed to stimulate the further growth of embryos. In our preliminary experiment, the embryos became swollen and were neither able to separate from each other nor convert into plantlets with an elongated culture time on medium containing 2,4-D (data not shown). Similar observations have been reported in *I. pseudacorus* (Kim et al., 2009). Therefore, it could be suggested

that the removal of 2,4-D was required for the maturation of embryos in *I. germanica*. Similar to these results have been reported in *I. pumila* (Jevremović and Radojević, 2006) and *I. pseudacorus* (Kim et al., 2009).

Sucrose, as one of the most commonly used carbohydrate sources in plant tissue culture, has been known to play a key role in the formation and maturation of SEs (Fuentes et al., 2000). Sucrose at 0.2 M (6%, w/v) was found to produce the maximum number of embryos of *I. nigricans* (Shibli and Ajlouni, 2000). Meanwhile, Kim et al. (2009) found that 0.22 M sucrose (6.6%, w/v) induced the highest frequency of SEs of *I. pseudacorus*. In this study, the frequency of SEs formation increased with the increasing concentrations of sucrose. Similar results have been reported in other plant species (Karami et al., 2006; Lou et al., 1996; Nakagawa et al., 2001). It has been reported that sucrose can serve not only as a carbon source but also as an osmotic regulator during somatic embryogenesis (Biahoua and Bonneau, 1999; Litz, 1986). It is commonly known that high sugar concentration may impact the cell osmolarity in somatic embryogenesis. Thus, it could be suggested that high sucrose concentrations in this study might act as providing nutrition and regulating osmolarity. It should also be noted that 1.5% (w/v) sucrose inhibited the formation of SEs with aberrant embryos, which was consistent with observations reported in *Dianthus caryophyllus* (Karami et al., 2006). Therefore, it could also be suggested that an osmotic effect of sucrose is required for normal development of SEs.

Root production is extremely important for the regeneration of *I. germanica*. In this study, the embryos germinated and formed the primary root with bipolar structure on PGR-free regeneration medium, which was in accordance with the results in many *Iris* species (Kim et al., 2009; Radojević et al., 1987; Reuther, 1977). However, Laublin et al. (1991) demonstrated that the SEs of *I. pseudacorus*, *I. setosa*, and *I. versicolor* did not form roots, despite the presence of a well-defined root meristem, unless they were transferred to an indole-3-butytric acid (IBA)-containing medium. Therefore, it could be suggested that rooting problem in *Iris* species could be overcome either by transferring the mature embryos to PGR-free medium or supplementing medium with IBA.

To investigate the medium for plant regeneration, Kim et al. (2009) cultured embryos on various kinds of medium and revealed that half-strength MS medium was the most effective for growth of plants in *I. pseudacorus*. In that study, both the length of plantlet shoots and roots were the longest in the half-strength MS medium. However, in this study, full-strength MS medium was better for plant regeneration with higher conversion rate of plantlets and longer length of roots.

The histological investigation showed that SEs could develop from both the surface and inner tissue of embryogenic callus. It was the first report on such histological features of the SEs for the genus *Iris* L. Histological study also indicated that the somatic embryogenesis stages were asynchronous. Similar observations have been reported in *I. pumila* (Jevremović and Radojević, 2006) and *D. caryophyllus* (Karami et al., 2006). Abscisic acid (ABA) is known to foster the synchronized development of SEs (Ammirato, 1974). Kim et al. (2009) cultured globular SEs on medium supplemented with various concentrations of ABA, and found that there were no significant differences for embryo maturation in *I. pseudacorus*. Whether ABA can promote synchronous SE development in *I. germanica* needs to be further investigated.

In conclusion, the induction of embryogenic callus could be controlled by the addition of 2,4-D, while SE formation depended on high sucrose concentration. Therefore, an efficient plant regeneration protocol via somatic embryogenesis of *I. germanica* was established and could be applied to micropropagation and genetic transformation. As it generally takes 2 years for the regenerated plantlets of *Iris* species to flower, data concerning the floral phenotypic variability are not available and will be evaluated later.

## Literature Cited


Ammirato, P.V. 1974. Effects of abscisic acid on the development of somatic embryos from cells of caraway (*Carum carvi* L.). Bot. Gaz. 135:328–337.

Bano, Z., S. Rajarathnam, and B.D. Mohanty. 1991. Somatic embryogenesis in cotyledon culture of tea (*Thea sinensis* L.). J. Hort. Sci. 66:465–470.

Biahoua, A. and L. Bonneau. 1999. Control of *in vitro* somatic embryogenesis of the spindle tree (*Euonymus europaeus* L.) by the sugar type and the osmotic potential of the culture medium. Plant Cell Rpt. 19:185–190.

Chen, R., M. Zhang, J. Lü, X. Zhang, J.A.T. da Silva, and G. Ma. 2014. Shoot organogenesis and somatic embryogenesis from leaf explants of *Valeriana jatamansi* Jones. Sci. Hort. 165:392–397.

Fuentes, S.R., M.B. Calheiros, J. Manetti-Filho, and L.G. Vieira. 2000. The effects of silver nitrate and different carbohydrate sources on somatic embryogenesis in *Coffea canephora*. Plant Cell Tissue Organ Cult. 60:5–13.

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

Han, Y.L., S.Z. Huang, H.Y. Yuan, J.Z. Zhao, and J.G. Gu. 2013. Organic acids on the growth, anatomical structure, biochemical parameters and heavy metal accumulation of *Iris lactea* var. *chinensis* seedling growing in Pb mine tailings. Ecotoxicology 22:1033–1042.

Hartmann, H.T., D.E. Kester, J.F.T. Davies, and R.L. Geneve. 1997. Plant propagation, principles and practices. 6th ed. Prentice Hall, NJ.

Huang, S.Z., Y.L. Han, M.Y. Xie, W.M. Guo, and Y. Gu. 2003a. The study and exploitation of Chinese ornamental iris resources. Chinese Wild Plant Resources 22:4–7 [in Chinese].

Huang, S.Z., Y.L. Han, S.L. Yu, J.G. Gu, and L.L. Zhang. 2011. The effect of lead and copper on the growth and physiological response of water flower *Iris pseudacorus* L. Fresenius Environ. Bul. 20:2246–2250.

Huang, S.Z., Y.L. Han, Y.G. Zhang, and W.M. Guo. 2003b. Breeding dwarf plants of *Iris germanica* L. through hybridization. J. Nanjing Agr. Univ. 26:21–25 [in Chinese].

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

Jeknic, Z., S.P. Lee, J. Davis, R.C. Ernst, and T.H. Chen. 1999. Genetic transformation of *Iris germanica* mediated by *Agrobacterium tumefaciens*. J. Amer. Soc. Hort. Sci. 124:575–580.

Jevremović, S. and L.J. Radojević. 2006. Establishment of efficient regeneration protocol from leaf explants of *Iris pumila* shoots cultured *in vitro*. Sci. Hort. 108:100–103.

Karami, O., A. Deljou, M. Esna-Ashari, and P. Ostad-Ahmadi. 2006. Effect of sucrose concentrations on somatic embryogenesis in carnation (*Dianthus caryophyllus* L.). Sci. Hort. 110:340–344.

Kim, T.D., C.H. Ahn, K.H. Bae, and Y.E. Choi. 2009. The embryogenic competency and morphological changes during somatic embryogenesis in *Iris pseudacorus*. Plant Biotechnol. Rpt. 3:251–257.

Laublin, G., H.S. Saini, and M. Cappadocia. 1991. *In vitro* plant regeneration via somatic embryogenesis from root culture of some rhizomatous irises. Plant Cell Tissue Organ Cult. 27:15–21.

Limanton-Grevet, A. and M. Jullien. 2000. Somatic embryogenesis in *Asparagus officinalis* can be an *in vitro* selection process leading to habituated and 2, 4-D dependent embryogenic lines. Plant Physiol. Biochem. 38:567–576.

Litz, R.E. 1986. Effect of osmotic stress on somatic embryogenesis in *Carica* suspension cultures. J. Amer. Soc. Hort. Sci. 111:969–972.

Lou, H., M. Tamaki, and S. Kako. 1996. Influence of sucrose concentration on *in vitro* morphogenesis in cultured cucumber cotyledon explants. J. Hort. Sci. 71:497–502.

Manrique-Trujillo, S., D. Díaz, R. Reaño, M. Ghislain, and J. Kreuze. 2013. Sweetpotato plant regeneration via an improved somatic embryogenesis protocol. Sci. Hort. 161:95–100.

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

Nakagawa, H., T. Saijyo, N. Yamauchi, M. Shigyo, S. Kako, and A. Ito. 2001. Effects of sugars and abscisic acid on somatic embryogenesis from melon (*Cucumis melo* L.) expanded cotyledon. Sci. Hort. 90:85–92.

Prado, E. and A. Berville. 1990. Induction of somatic embryo development by liquid culture in sunflower (*Helianthus annuus* L.). Plant Sci. 67:73–82.

Radojević, L.J., O. Sokić, and B. Tucić. 1987. Somatic embryogenesis in tissue culture of iris (*Iris pumila* L.). Acta Hort. 212:719–723.

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

Reuther, G. 1977. Embryoide differenzierungsmuster im kallus der Gattungen *Iris* und *Asparagus*. Ber. Dtsch. Bot. Ges. 90:417–437.

Shibli, R.A. and M.M. Ajlouni. 2000. Somatic embryogenesis in the endemic black iris. Plant Cell Tissue Organ Cult. 61:15–21.

Simonet, M. 1932. Recherches cytologiques et genetiques chez les *Iris*. Édition du Bulletin bioligique de la France et de la Belgique 66:255–444.

Tremblay, F.M. 1990. Somatic embryogenesis and plantlet regeneration from embryos isolated from stored seeds of *Picea glauca*. Can. J. Bot. 68:236–242.

Zimmerman, J.L. 1993. Somatic embryogenesis: A model for early development in higher plants. Plant Cell 5:1411.