

PLANT REGENERATION OF SOUTHERN ADRIATIC IRIS BY SOMATIC EMBRYOGENESIS

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Abstract — A simple protocol has been developed for plant regeneration by somatic embryogenesis of Southern Adriatic iris (*Iris pseudopallida* Trinajstić), an endemic species of the Balkan Peninsula. Somatic embryogenesis was induced in zygotic embryo culture on media supplemented with 2,4-dichlorophenoxy acetic acid (2-10 mgL⁻¹) as the sole plant growth regulator, where both embryogenic calli and somatic embryos were induced. Subsequent decrease of 2,4-D in the media promoted formation of somatic embryos. Developed somatic embryos germinated on medium without growth regulators. The regenerated plantlets had diploid chromosome number. Planted plantlets acclimatized very well under greenhouse and garden conditions.

Key words: Zygotic embryo culture, somatic embryogenesis, *Iris pseudopallida*

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INTRODUCTION

Southern Adriatic iris (*Iris pseudopallida* Trinajstić – Syn.: *I. pallida* auct. Dalm. & Montenegr., non L.) – belongs to the genus *Iris*, subgenus *Iris* (bearded irises), section *Iris* (eupogons, the true bearded irises), and series Pallideae. This series consists of the cultivated species *I. pallida*, very important for the perfume industry and three indigenous and endemic Alpine-Dinaric species: *I. cengualti* Ambr. (endemic in the Alpine region), *I. illirica* Tomm. (endemic to the Northern Adriatic Coast), and *I. pseudopallida* Trinajstić (endemic to the Southern Adriatic littoral) (Mitić et al., 2001). Besides the Southern Adriatic iris, there is only one more species from the genus *Iris* which is endemic to the Balkan Peninsula, *I. reichenbachii* (Šilić, 1991).

Plant propagation by *in vitro* tissue culture is a powerful tool for *ex situ* conservation of many endangered and endemic irises (Radojević et al., 1987; Shibli and Ajlouni, 2000; Al-Gabbeish et al., 2006). Somatic embryogenesis of iris species was induced for the first time in zygotic embryo culture of Dutch iris (Reuther, 1975). Several protocols for

in vitro regeneration by somatic embryogenesis of irises with botanical or horticultural value have been reported until now. The auxin 2,4-dichlorophenoxy-acetic acid (2,4-D) in combination with a cytokinin (mainly kinetin) was found to be the most potent inducer of somatic embryogenesis for many irises (Jevremović et al., 2006a). In previous studies, 2,4-D alone was used for induction of somatic embryogenesis in zygotic embryo culture of *Iris germanica* (Reuther, 1975, 1977) and bulb-scale culture of *I. hollandica*.

Plant regeneration by organogenesis of Southern Adriatic iris has been reported recently (Jevremović et al., 2008). There are no data on regeneration of this endemic species by somatic embryogenesis.

This study was conducted to develop a valuable protocol for plant regeneration by somatic embryogenesis as a contribution to preservation of this endemic Balkan iris.

MATERIAL AND METHODS

Seeds of *I. pseudopallida* Trinajstić, obtained from Mt. Orjen, Montenegro, were surface-sterilized with

30% commercial bleach solution with 4% active NaOCl for 30 min. After rinsing with distilled water, seeds were placed on the basal medium for two days as a test of sterility. The basal medium (BM) was composed of MS mineral solution (Murashige and Skoog, 1962), 3% sucrose, 0.7% agar, and (in mgL⁻¹): inositol 100, nicotinic acid 5, pantothenic acid 10, vitamin B₁ 2, vitamin B₂ 1, casein hydrolyzate 250, and L-proline 250, pH 5.8. Zygotic embryos were isolated from seeds and placed on media A supplemented with different concentrations of 2,4-D as listed in Table 1. Six weeks later, the percentage of morphogenesis induction was recorded. Formed calli from each zygotic embryo were divided in half and subcultured on the same medium or medium A₃ supplemented with 1 mgL⁻¹ of 2,4-D. The number of somatic embryos per 1 g of embryogenic calli was counted eight weeks later. Somatic embryos were germinated on BM medium. The cultures were grown at 25±2°C under conditions of a 16-h photoperiod, a "Tesla" (Pančevo, Serbia) fluorescent tube providing light of 50 μmoles · m⁻², and subcultured at 4-week intervals. Plantlets were potted and transferred to greenhouse conditions.

Chromosome observation was carried out on root tips according to a procedure involving pretreatment with 8-hydroxyquinoline. The root tips (1 cm) were collected from regenerated plants, pretreated for 4-5 h with 0.002 M 8-hydroxyquinoline at room temperature, and subsequently kept at 4°C for 24 h. The roots were then fixed in Carnoy fixative (3: 1 acetic acid and ethyl alcohol) mixture for another 24 h. Preservation was done by immersion of root tips in 70% alcohol and hydrolyzation in

Table 1. Composition of media used for somatic embryogenesis induction.

Medium	Components (2,4-D in mgL ⁻¹)
BM (base medium)	growth regulator-free (BM)
A ₁	BM + 0.1
A ₂	BM + 0.5
A ₃	BM + 1.0
A ₄	BM + 2.0
A ₅	BM + 3.0
A ₆	BM + 5.0
A ₇	BM + 10.0

1N HCl at 60°C for 10 min. After short rinsing in distilled water (2 x 5 min), root tips were stained with 1% aceto-orceine. Squash slides were viewed and counted using a light microscope (Leica, Leitz DMRB, Germany). Samples of embryogenic calli were subjected to scanning electron microscopy (SEM) on a JOEL JSM - 6460LV microscope at the Institute of Biology, University of Novi Sad, without any previous preparation.

Data were subjected to analysis of variance (ANOVA) to assess treatment differences. All experiments were repeated three times. Data are presented as means ± standard error. Significance of differences between means was tested by LSD (least significant differences).

RESULTS

Isolated zygotic embryos of *I. pseudopallida* were cultured under conditions of eight treatments for induction of morphogenesis *in vitro* (Table 2). At lower concentrations of 2,4-D (media A₁-A₃), only organogenic (rhizogenic) calli were formed, while on media with higher 2,4-D content, yellow nodular embryogenic calli were formed (Fig. 1a). At the surface of embryogenic calli, embryo-like structures arise which have a narrow connection with the embryogenic nodule (Fig. 1b). The average number of embryo-like structures per gram of embryogenic calli is presented in Table 2. Formation of somatic embryos depended on the concentration of 2,4-D in the nutrient media. The highest number of somatic embryos was observed on medium A₄ supplemented with 2.0 mgL⁻¹ of 2,4-D, the lowest on medium A₇ with 10.0 mgL⁻¹ of 2,4-D.

In the next subculture, embryogenic calli from each zygotic embryo were divided in half and cultured on the same medium or A₃ medium supplemented with 1.0 mgL⁻¹ of 2,4-D. Results are summarized in Table 3. The average number of embryo-like structures did not increase after prolonged culture at high concentrations of 2,4-D. However, the same trend was maintained: formation of embryo-like structures was 2,4-D-dependent, higher concentrations of 2,4-D giving lower formation of embryo-like structures. A considerably higher number of somatic

Table 2. Effect of different concentrations of 2,4-D in BM medium on *in vitro* morphogenesis of Southern Adriatic iris. * Each value represents the mean \pm standard error. The difference between values followed by the same letter is insignificant at $P \leq 0.01$ as analyzed by the LSD test.

Medium	% of explants with response	Morphogenic response	
		Description	N ^o of somatic embryos per 1 g of embryogenic calli
BM	60.0	Germination	0.0 ^{a*}
A ₁	70.0	Organogenic calli	0.0 ^a
A ₂	83.3	Organogenic calli	0.0 ^a
A ₃	83.3	Organogenic calli	0.0 ^a
A ₄	70.0	Embryogenic calli	2.7 \pm 0.1 ^d
A ₅	100	Embryogenic calli	2.5 \pm 0.3 ^{b, c}
A ₆	100	Embryogenic calli	2.2 \pm 0.2 ^{b, c}
A ₇	100	Embryogenic calli	1.7 \pm 0.2 ^b

embryos was formed after decrease of 2,4-D in the media. The greatest increase was observed after successive transfer of embryogenic calli induced on A₇ induction medium with 10.0 mgL⁻¹ of 2,4-D to 10 times lower 2,4-D concentration (medium A₃, Table 3), where formation of embryo-like structures was six times higher. Decrease of 2,4-D from 2 to 1 mgL⁻¹ (media A₄ \rightarrow A₃) gave a threefold increase of somatic embryo formation. Apart from increasing the number of formed embryo-like structures, this decrease of 2,4-D in the medium allowed further development of somatic embryos, and advanced developmental stages of somatic embryos were observed (Fig. 1c). Formation of somatic embryos was constant in the next subcultures on A₃ medium. Observation by SEM confirmed that somatic embryo structures were classical somatic embryos like those obtained for other irises (Fig. 2a).

Germination of somatic embryos was tested on the hormone-free base medium, where 70% of somatic embryos germinated (Fig. 1d).

Cytological analysis revealed that the plants had 24 chromosomes (Fig. 2b) and had a ploidy level like that of the mother plants.

Regenerated plantlets were transferred to greenhouse conditions, where they successfully acclimatized (81%, Fig. 1e) and attained full physiological maturity.

DISCUSSION

The explant source is one of the most important factors in the induction of a morphogenetic response of *in vitro* culture, especially in monocots, where

Table 3. Influence of 2,4-D decrease in the nutrient medium on somatic embryogenesis induction of Southern Adriatic iris. * Each value represents the mean \pm standard error. Values in same column sign with the same letter are insignificant at $P \leq 0.01$.

Medium	Number of somatic embryos per 1 g of embryogenic calli
A ₄	2.4 \pm 0.3 ^{a, b}
A ₄ \rightarrow A ₃	7.9 \pm 0.9 ^a
A ₅	2.8 \pm 0.5 ^b
A ₅ \rightarrow A ₃	7.7 \pm 0.6 ^a
A ₆	1.8 \pm 0.3 ^{a, b}
A ₆ \rightarrow A ₃	7.8 \pm 0.4 ^a
A ₇	1.6 \pm 0.2 ^a
A ₇ \rightarrow A ₃	9.7 \pm 0.3 ^b

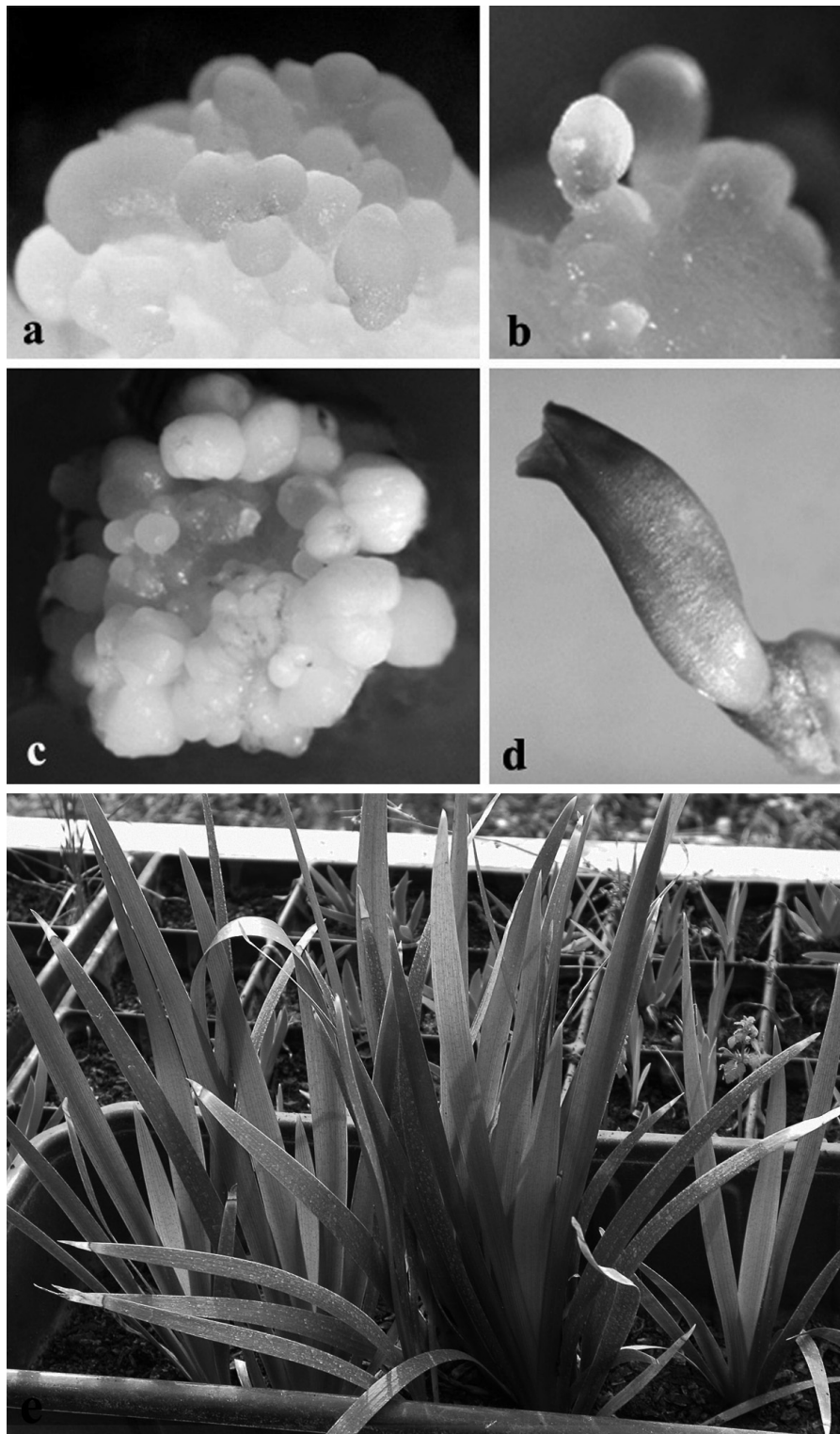


Fig. 1. Somatic embryogenesis of Southern Adriatic iris. a) nodular embryogenic calli; b) globular embryogenic-like structures; c) somatic embryos formed on embryogenic calli after A7 → A3 change of media; d) germination of somatic embryo on hormone-free medium; e) plantlets in greenhouse conditions.

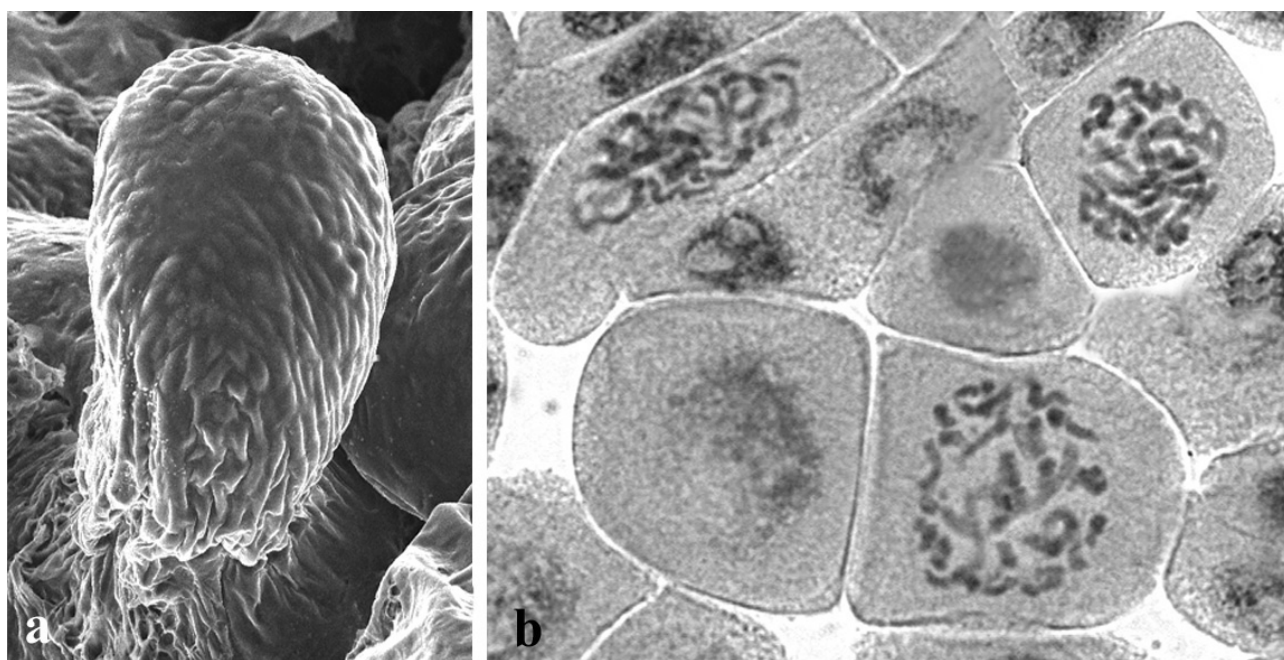


Fig. 2. Morphological and cytological analysis of somatic embryogenesis of Southern Adriatic iris. a) SEM of somatic embryo; b) somatic chromosomes in root tips of regenerated plantlet cells.

cells differentiate early and quickly. Only parts of plants that are close to meristematic tissue *in vivo* can respond to *in vitro* treatment. Zygotic embryos are the most potent explant type for many irises (Jevremović et al., 2006a). Our previous work with other protocols for induction of somatic embryogenesis in irises using 2,4-D and kinetin in the culture media was unsuccessful. In the current study, we found that somatic embryogenesis of Southern Adriatic iris is only possible to achieve with 2,4-D as the sole hormone treatment in the culture media. The embryogenic callus induction rate was 70-100%, depending on concentration of 2,4-D in the media. Similar results were observed for a commercial hybrid of *I. hollandica* Hort., cv. "Bronze Queen", where the highest induction rate (82%) was achieved on media supplemented with 9 μM of 2,4-D.

The significance of concomitant decrease of 2,4-D in the culture medium for enhancement and further growth and differentiation of embryo-like structures into normally developing somatic embryos for many species is very well documented in the literature (Jaminéz, 2005). An inhibitory effect of high concentrations of 2,4-D on embryogenic callus

formation of irises has been observed in cell suspension as well as protoplast culture of black iris (Shibli and Ajlouni, 2000).

The main step of *in vitro* propagation is adaptation of plantlets to external conditions. Plants of Southern Adriatic irises grew well when transferred to *ex vitro* conditions and flowered in the next blooming season.

Cytological analysis performed for many irises revealed diploid chromosome number, but some abnormalities like tetraploids were also reported. The genetic stability of Southern Adriatic iris regenerated by somatic embryogenesis has been confirmed in the current study by chromosome analysis.

In conclusion, we here report a simple protocol for somatic embryogenesis that can be an efficient method for mass propagation of Southern Adriatic iris. With this study, we close a series of reports dealing with the induction and plant regeneration of Balkan endemic irises by tissue culture. Plant regeneration by somatic embryogenesis and organogenesis of *I. reichenbachii* was reported earlier (Jevremović

et al., 2006a, 2006b). The described protocols could be used for *ex situ* conservation with destruction of the natural habitat of these endemic irises.

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РЕГЕНЕРАЦИЈА ЈУЖНО-ЈАДРАНСКОГ ИРИСА СОМАТСКОМ ЕМБРИОГЕНЕЗОМ

СЛАЂАНА ЈЕВРЕМОВИЋ, АНГЕЛИНА СУБОТИЋ, МИЛАНА ТРИФУНОВИЋ и МАРИЈА НИКОЛИЋ

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Једноставан протокол за регенерацију биљка процесом соматске ембриогенезе развијен је за јужно-јадрански ирис (*Iris pseudopallida* Trinajstić), ендемит Балканског полуострва. Соматска ембриогенеза је индукована у култури "зрелих" зиготских ембриона на хранљивој подлози обогаченој 2,4-дихлорофенокси сирћетном киселином (2,4-D), (као јединим регулатором растења), где

су ембриогени калус и соматски ембриони формирали. Смањење концентрације 2,4-D у хранљивој подлози доводи до повећања броја формираних соматских ембриона. Клијање соматских ембриона је постигнуто на подлози без регулатора растења. Регенерисане биљчице су имале диплоидан број хромозома. Аклиматизација биљака је била добра у условима стакленика и баште.