



In vitro propagation of *Iris reichenbachii* Heuff. and clonal fidelity of regenerated plants

Sladjana JEVREMOVIĆ^{1*}, Milena LOJIĆ¹, Zoran JEKNIĆ², Milana TRIFUNOVIĆ-MOMČILOV¹, Dragana ANTONIĆ¹, Marija PETRIĆ¹, Angelina SUBOTIĆ¹ and Ljiljana RADOJEVIĆ¹

1 Institute for Biological Research "Siniša Stanković", University of Belgrade, Bulevar despota Stefana 142, 11060 Belgrade, Serbia

2 Oregon State University, Department of Horticulture, ALS4017, Corvallis, OR 97331, USA

ABSTRACT: An efficient propagation protocol by somatic embryogenesis and organogenesis of Balkan endemic iris, *Iris reichenbachii*, was achieved and clonal fidelity of regenerated plants evaluated. Both regeneration pathways were induced at the same time in zygotic embryo culture on Murashige & Skoog (MS) medium supplemented with 0.5-5.0 mM 2,4-dichlorophenoxy acetic acid (2,4-D) as the sole hormone. Embryogenic calli were further maintained on medium supplemented with 2,4-D and kinetin (Kn; 0.5 and 5.0 mM, respectively). Organogenic calli were selected and further cultured on MS media supplemented with 1-naphthaleneacetic acid (NAA) and benzyladenine (BA; 0.5 and 4.5 mM, respectively) for shoot initiation. Somatic embryos germinated and shoots rooted on MS plant growth regulator-free medium. Plants regenerated by both processes were successfully acclimatized in greenhouse conditions and flowered in the following flowering season. Some alterations in flower morphology were detected among plants regenerated by organogenesis. Flow cytometric analysis revealed that plants with altered morphology of flowers had the same ploidy level and genome size as plants collected from the natural habitat. A tetraploid plant was observed in the population of plants regenerated by somatic embryogenesis induced at a high concentration of 2,4-D (10.0 mM).

KEYWORDS: Iris, somatic embryogenesis, organogenesis, flowering, flow-cytometry

ABBREVIATIONS:

2,4-D	2,4-dichlorophenoxyacetic acid
BA	6-benzyladenine
DAPI	4,6-diamidino-2-phenylindole.2HCl)
Kn	kinetin
NAA	1-naphthaleneacetic acid
MS	Murashige and Skoog

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INTRODUCTION

The subject of this study was *Iris reichenbachii* Heuffel 1853, an endemic iris which grows in several localities on the Balkan peninsula, $2n = 24, 48$; *Syn: I. bosniaca* G. Back 1960, *I. balkana* Janka, 1960, *I. skorpilii* Velenovsky, *I. serbica* Pančić, 1856. Besides *I. reichenbachii*, there is only one other species from the genus *Iris* which

is regarded as endemic to the Balkan Peninsula, the Southern Adriatic iris (*Iris pseudopallida* Trinajstić) (Šilić 1991). This is a dwarf iris, with yellow flowers mainly, belonging to the section of small bearded irises (*Pumilae*) which is used in breeding programs of dwarf irises. Iris flowers display a highly specialized morphology, different from other monocotyledonous plants. The perianth is not differentiated into the calyx

*correspondence: sladja@ibiss.bg.ac.rs; jevremovic.sladjana@gmail.com

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and corolla. Instead, all the perianth elements can be similar in appearance, named tepals. The three outer, petals, hang down and are known as falls and the other three are called standards, standing upright. The upper surface of the falls can be equipped with a beard consisting of short fine hairs, in the "bearded" irises.

Propagation of horticulturally valuable *Iridaceae* species by tissue culture has been elaborated and documented very well by ASCOUGH *et al.* (2009). Using a cell and tissue culture method has become popular in iris reproduction as it considerably increases plant multiplication factors and improves the quality of valuable planting stock. Also, plant regeneration by tissue culture is increasingly used for biodiversity conservation of rare and endemic species, which represent an important component of plant genetic resource management (PENSE 2013). Irises can be regenerated by organogenesis *via* formation of meristems on calli and further activation of axillary meristems and somatic embryogenesis on solid or liquid media (HUSSEY 1976; LAUBLIN *et al.* 1991; SHIMIZU *et al.* 1997). The best responses for efficient induction of morphogenesis of iris *in vitro*, as for other monocotyledonous plants, are shown by those that are close to the meristematic state in mother plants, like leaf bases, flower parts or zygotic embryos (JEVREMOVIĆ *et al.* 2006b). Developed regeneration systems are used for clonal reproduction as an alternative way to conserve some rare, endangered or endemic iris species (SHIBLI & AJLOUNI 2000; AL-GABBEISH *et al.* 2006, JEVREMOVIĆ *et al.* 2008; 2009).

Preliminary results of vegetative regeneration by tissue culture of *I. reichenbachii* have been reported earlier by JEVREMOVIĆ & RADOJEVIĆ (2000). Zygotic embryos represent very potent explants for regeneration of *I. reichenbachii* as well as for many other iris species (JEVREMOVIĆ *et al.* 2006a; JEVREMOVIĆ *et al.* 2006b, JEVREMOVIĆ *et al.* 2013). Induction of the regeneration process by somatic embryogenesis and organogenesis at the same time has been reported in many protocols of iris vegetative propagation *in vitro* (RADOJEVIĆ & SUBOTIĆ 1992; SHIMIZU *et al.* 1997; WANG *et al.* 1999). Plant regeneration by organogenesis of Southern Adriatic iris has been reported recently (JEVREMOVIĆ *et al.* 2008) as well as somatic embryogenesis (JEVREMOVIĆ *et al.* 2009). However, there are no reports on the clonal fidelity of Balkan endemic irises regenerated by culture *in vitro*.

In this study we applied the protocol for *in vitro* plant regeneration *via* somatic embryogenesis and organogenesis in zygotic embryo culture of Balkan endemic iris which can be used for biodiversity conservation. In addition, we estimated the clonal fidelity of regenerated plants *via* genome size and ploidy level by flow cytometry and chromosome count analysis of plants collected in nature and regenerated *in vitro*.

MATERIAL AND METHODS

Plant material: Seeds of *I. reichenbachii* were collected from Mountain Suvobor, (at a height of ca. 830 m) West Serbia during August 2002-2004 and stored at room temperature until use. Seeds were rinsed with tap water one hour before the sterilization procedure. Surface sterilization was done using commercial bleaching solution with 0.4% NaOCl for 20 min, rinsed 3 times with sterile water for 5 min each. Zygotic embryos were isolated aseptically from the seeds under a binocular microscope by cutting the end with a sharp razor and pressing the middle of the seed and placed on either basal medium without plant growth regulators or seven induction media containing 2,4-dichlorophenoxyacetic acid (2,4-D). Basal medium was composed of MURASHIGE & SKOOG (1962) mineral solution (MS) containing 7.0 g L⁻¹ agar, 30.0 g sucrose, nicotinic acid 5.0 mg L⁻¹, pantothenic acid 10.0 mg L⁻¹, vitamin B₁ 2.0 mg L⁻¹, vitamin B₆ 1.0 mg L⁻¹, L-proline 250.0 mg L⁻¹ and casein hydrolysate 250.0 mg L⁻¹. Induction media were supplemented with increasing concentrations of 2,4-D (0.05, 0.1, 0.5, 1.0, 5.0, 10.0 and 15.0 mM). Five to ten embryos were incubated in each Petri dish filled with 25 ml of medium and cultured for 6 weeks. For each induction treatment 50 seeds were used. The experiment was repeated twice. Cultures were grown in a 24 ± 2°C temperature regime and 16/8h photoperiod with 50 mmol m⁻² light intensity.

Somatic embryogenesis: Following induction of morphogenesis, white, friable embryogenic calli with somatic embryos were selected and further subcultured on Petri dishes with 25 ml of basal MS medium supplemented with 2,4-D and kinetin (Kn, 0.5, 5.0 mM, respectively). The number of somatic embryos per 1g of embryogenic calli was measured after three successive subcultures for four weeks.

Organogenesis: Calli with green organogenic nodules and proliferating shoots were selected and cultured on basal MS medium supplemented with 1-naphthaleneacetic acid (NAA) and 6-benzyladenine (BA; 0.5 and 4.5 mM, respectively). Shoot multiplication index was determined as the increase of shoot number after four weeks of subculturing on the above medium. Ten single shoots were cultivated per one culture vessel (700 ml) containing 100 ml of medium and the multiplication index was calculated after three successive subcultures.

Germination and rooting: For germination 50 isolated somatic embryos (3-5 mm) from each induction treatment were placed on 25 ml MS basal medium in Petri dishes and cultured for four weeks. The experiment was replicated three times. For rooting, 50 single shoots

(4–5 cm) were transferred to 20 mm long test tubes with 5 ml of MS basal medium and cultured for four weeks.

Acclimatization: Plantlets obtained by both somatic embryogenesis and organogenesis were removed from culture vessels and washed with sterile water to remove culture medium. Plants were potted in a mixture of peat and perlite (3:1) and grown in greenhouse conditions until plants flowered, after two years.

Determination of ploidy level and chromosome counting: Ploidy level of flowering *I. reichenbachii* plants was determined by flow cytometry analysis according to the method reported in DOLEŽEL *et al.* (1992). Nuclei staining was done with DAPI (4,6-diamidino-2-phenylindole.2HCl) and flow cytometric analysis was performed with a PAS flow-cytometer (Partec) with *Vicia faba* as an internal standard. For chromosome counting, vigorously growing root tips were pre-treated with 2-hydroxy quinoline, fixed and stained with DAPI or acetocarmine. Chromosome number was observed using light and fluorescent microscopy (Carl Zeiss AxioVision microscope, Zeiss, Germany).

Data analysis: Data were analysed using one-way analysis of variance (ANOVA) and means were compared using LSD at the 5% level of probability.

RESULTS AND DISCUSSION

Somatic embryogenesis and organogenesis. In many protocols reported for *in vitro* propagation of irises, regeneration by both somatic embryogenesis and organogenesis occurred (RADOJEVIĆ & SUBOTIĆ 1992;

SHIMIZU *et al.* 1997; WANG *et al.* 1999; ASCOUGH *et al.* 2009). Previous preliminary results on mature zygote embryo culture of *I. reichenbachii* identified that induction of morphogenesis was very efficient on MS medium containing only 2,4-D. Also, addition of Kn in the culture medium during the induction process increased the frequency of organogenic calli (JEVREMOVIĆ & RADOJEVIĆ 2000). The same results were obtained in zygotic embryo culture of *I. germanica* (REUTHER 1975; 1977) as well as in bulb scale culture of *I. hollandica* (FIDALGO *et al.* 2005). For induction of morphogenesis in our experiments, we used graded concentrations of 2,4-D varying from 0 to 15 mM. During the induction process, three types of calli could be distinguished based on their color and consistency (Fig. 1a). Formation of white embryogenic and green organogenic calli was 2,4-D dependent (Table 1.). Induction of embryogenic calli with somatic embryos occurred on medium supplemented with 2,4-D in concentrations higher than that mM. The highest number of somatic embryos forming per gram of embryogenic calli was observed on medium supplemented with a relatively low concentration of 2,4-D (1.0 mM). All other protocols for induction of morphogenesis in many iris species have required at least 4.5 mM or even 45 mM and higher concentrations of 2,4-D accompanied with NAA and Kn (JEVREMOVIĆ *et al.* 2006a).

Organogenic calli with adventive shoots were formed when 2,4-D was added in medium at a concentration of 1 mM while on medium with higher concentrations of 2,4-D sporadically and non regenerative green callus was formed (Table 2.). After induction, selected organogenic callus was measured

Table 1. Effect of 2,4-D on somatic embryogenesis in zygotic embryo culture of *I. reichenbachii*

Culture media (2,4-D in mM)	SOMATIC EMBRYOGENESIS			
	Frequency (%)	Nº of somatic embryos / g	Germination (%)	Acclimatization (%)
0	0.0	0.0 ^{d*}	-	-
0.05	0.0	0.0 ^d	-	-
0.1	0.0	0.0 ^d	-	-
0.5	20.0	33.0 ± 2.8 ^b	67.9	81.2
1.0	23.3	71.1 ± 9.9 ^a	50.0	61.4
5.0	33.3	40.7 ± 11.1 ^b	46.8	64.6
10.0	30.6	15.3 ± 4.6 ^c	45.0	75.0
15.0	31.7	10.7 ± 2.5 ^c	34.0	-

*Data followed by different letters are significantly different at the 5% level

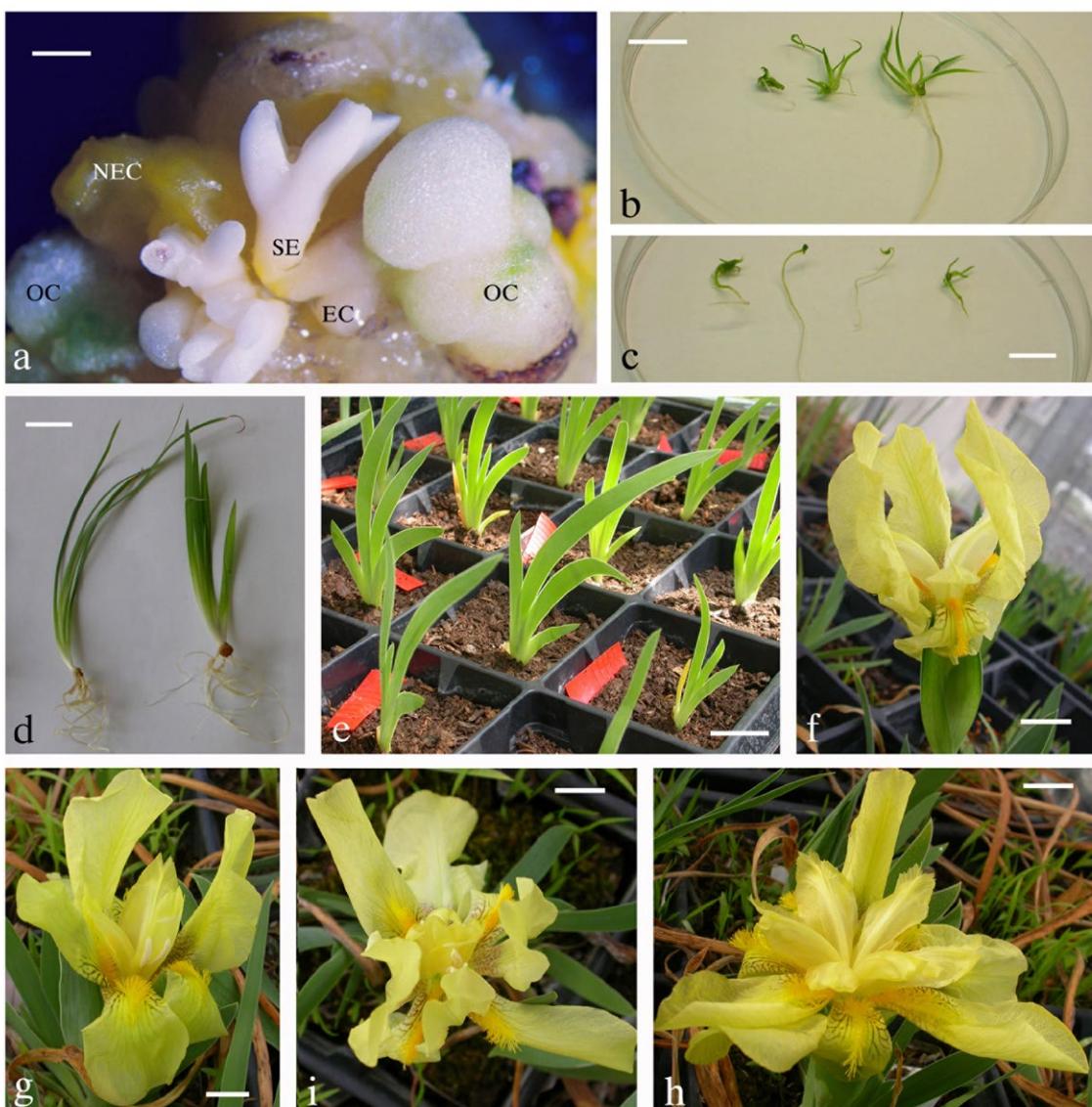


Fig. 1 Plant regeneration of *Iris reichenbachii* by somatic embryogenesis and organogenesis. **a** Induction of organogenic (OC), non-embryogenic (NEC) and embryogenic (EC) with somatic embryos (SE) on media supplemented with 1.0 mM 2,4-D, bar = 1 cm. **b** Germination of somatic embryos, bar = 1 cm. **c** Asynchronous germination of somatic embryos, bar = 1 cm. **d** Rooted plantlets obtained by organogenesis, bar = 1 cm. **e** Acclimatized iris plantlets after one year of growth under greenhouse conditions, bar = 1 cm. **f** Plant regenerated by somatic embryogenesis with normal flower morphology, bar = 1 cm. **g-h** *I. reichenbachii* plants regenerated by organogenesis with changed number of flower parts, bar = 1 cm.

and subcultured on medium for shoot multiplication (NAA and BA; 0.5 and 0.45 mM, respectively). Successful *de novo* formation and multiplication of shoots was obtained when organogenic calli were cultured for four weeks on this media (Table 2).

Somatic embryo germination and shoot rooting. Isolated somatic embryos were germinated on basal MS medium after four weeks. The best germination of somatic embryos (67.9%) was observed when somatic embryogenesis was induced on medium containing 0.5 mM 2,4-D. Germination gradually decreased when higher concentrations of 2,4-D were used in the

induction medium. Besides normally-formed plantlets (Fig. 1b) with synchronously formed shoots and roots, 10% of embryos aborted with cessation of differentiation of root or shoot meristems (Fig. 1c). Somatic embryos of *I. reichenbachii* germinated without any plant growth regulators or other additional treatments to break dormancy. The only necessity was separation of somatic embryos from each other and transferral of separated embryos to new hormone-free medium. The same phenomenon was also observed for other irises (SHIBLI & AJLOUNI 2000, KIM *et al.* 2009)

Rooting of *I. reichenbachii* shoots obtained by organogenesis varied from 22.8 to 76.9%. The best

Table 2. Effect of 2,4-D treatment on organogenesis induction in zygotic embryo culture of *I. reichenbachii*

Culture media (2,4-D in mM)	ORGANOGENESIS		
	Frequency (%)	Callus/zygotic embryo (mg)	Multiplication index**
0	80	germination	-
0.05	100	71.3 ± 23.2 ^{a,b} *	0.6 ± 0.2 ^c
0.1	100	81.5 ± 39.4 ^{a,b}	1.8 ± 0.2 ^b
0.5	36.6	145.3 ± 92.9 ^a	3.2 ± 0.2 ^a
1.0	36.6	40.7 ± 15.0 ^{a,b}	3.0 ± 0.4 ^a
5.0	30.0	6.0 ± 2.0 ^b	3.1 ± 0.8 ^a
10.0	3.0	0.2 ± 0.1 ^b	0.0 ^d
15.0	3.0	0.2 ± 0.1 ^b	0.0 ^d

*Data followed by different letters are significantly different at the 5% level. ** Multiplication index represents the average number of *de novo*-formed shoots/callus, recorded for three successive subcultures on media with NAA and BA (0.5 and 0.45 mM, respectively)

Table 3. Rooting and acclimatization of *Iris reichenbachii* shoots derived by organogenesis

Induction (2,4-D in mM)	Rooting				Acclimat. (%)
	Freq. (%)	Shoot height (cm)*	Root length (cm)*	Nº of roots*	
0.1	76.9	11.4 ± 0.7 ^a	11.1 ± 1.5 ^a	3.6 ± 0.5 ^a	75.6
0.5	53.3	10.1 ± 0.4 ^a	8.5 ± 1.9 ^a	4.4 ± 0.8 ^a	86.9
1.0	55.5	10.0 ± 0.8 ^a	9.7 ± 1.8 ^a	3.9 ± 0.7 ^a	82.4
5.0	22.8	9.5 ± 0.7 ^a	9.4 ± 0.9 ^a	3.8 ± 0.3 ^a	84.0

* Data are mean ± standard error for 50 plants. Data followed by different letters are significantly different at the 5% level following a LSD test.

rooting frequencies were observed on shoots derived at lower concentrations of 2,4-D in the induction treatment. The frequency decreased when higher applications of 2,4-D in the induction medium were applied, the same as for germination of somatic embryos (Table 3.). When morphological characteristics of rooted shoots (mean shoot and root length, average number roots per shoot) were compared, there were no significant differences among regenerated plants regarding the induction treatment (Fig. 1d).

Acclimatization and clonal fidelity of regenerated plants. Germinated somatic embryos and rooted shoots were potted in greenhouse conditions and acclimatized (Fig. 1e). The frequency of acclimatization ranged from 61.4 to 81.2% for plantlets regenerated *via* somatic embryogenesis and 75.6 to 86.9% *via* organogenesis. These plants flowered in the following flowering season. Most of these plants had the same shape, number of

flower parts and color as the mother plants (Fig. 1f), though among plants regenerated by organogenesis several types of modifications occurred. Plants were observed with 4 falls and stamens and 3-4 standards (Fig. 1g, i) or with all flower parts doubled (Fig. 1h). Clonal fidelity of regenerated irises has already been confirmed for many irises. Some alterations in chromosome number and ploidy level have been recorded previously (INOUE *et al.* 2006), but changes in number of flower parts has not been recorded to date. Flow cytometry analysis revealed that *I. reichenbachii* plants with mutated flowers had the diploid number of chromosomes, so the changes in flower part number was not due to a change of ploidy levels (Fig 2 a-c). Flowers of most angiosperms consist of four types of specialized organs (sepals, petals, stamens and carpels) that are arranged in a series of concentric rings or whorls under the control of several classes of regulatory genes (A, B, C). According to the ABC model of floral development, the expression of the A gene alone leads to the production

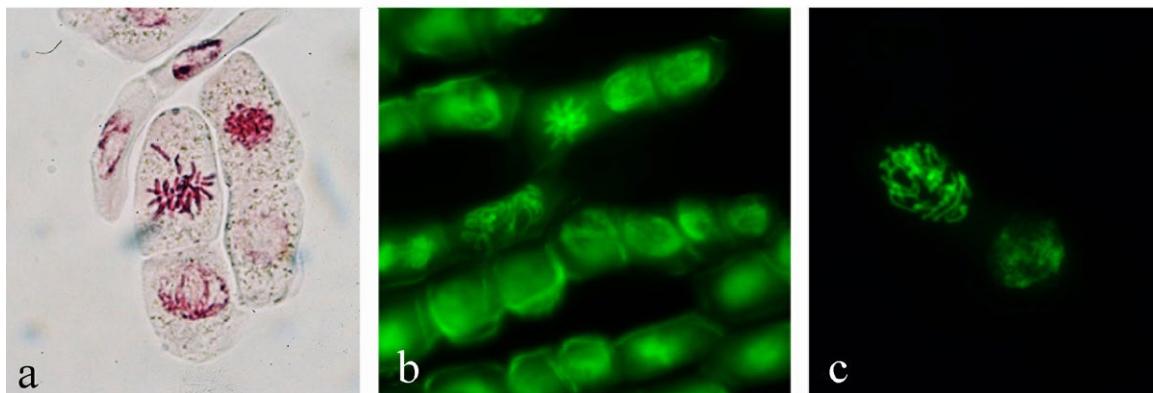


Fig. 2 Chromosome number analysis of *I. reichenbachii* regenerated plants **a** Root-tip cells with chromosomes of plants regenerated by somatic embryogenesis stained with acetocarmine **b, c** Root-tip cells with chromosomes of plants regenerated by somatic embryogenesis stained with DAPI.

Table 4. DNA content in leaf samples in *I. reichenbachii* of different origin.

Origin of plant material	Induction (2,4-D in mM)	CV* (%)	DNA content (pg/2C)**
Plants collected in the natural habitat	-	3.10 ± 0.2	11.18 ± 0.1
Plants regenerated by organogenesis	0.1-5.0	2.99 ± 0.1	11.04 ± 0.1
Plants regenerated by somatic embryogenesis	1.0	4.14 ± 0.5	11.73 ± 0.3
Plants regenerated by somatic embryogenesis	5.0	2.77 ± 0.7	11.12 ± 0.9
Plants regenerated by somatic embryogenesis***	5.0	2.83 ± 0.3	21.92 ± 0.3

* CV Coefficient of variation ** DNA content was calculated according to the formula reported in DOLEŽEL (2007). *Vicia faba* was used as the internal standard (nuclear DNA content = 26.90 pg), *** Data for a single plant are presented. Other means are for 10 randomly-chosen regenerated plantlets.

of sepals, coexpression of the A+B or A+C function leads to the production of petals and stamens, respectively and expression of the C function alone induces the formation of carpels (RUNNING 2006). For monocotyledonous plants, the modified ABC model has developed which supposes that the role of B class genes is shifted and they also regulate the first whorls (KANO 2006). The observed alterations in flower morphology of *I. reichenbachii* plants derived by organogenesis might be a consequence of mutations of the B class of genes for floral development or some other epigenetical variations or even both. Changes in flower morphology have been found only within plants derived by organogenesis *via* adventive bud formation. The possible explanation of this phenomenon could be the origin of somatic embryos and adventive buds of irises. Adventive buds of irises are formed as *de novo* formed shoot meristems on organogenic calli and have a multicellular origin while the unicellular origin of somatic embryos was documented earlier (REUTHER 1977; RADOJEVIĆ & SUBOTIĆ 1992; JEVREMOVIĆ & RADOJEVIĆ

2006). The detrimental effect of 2,4-D in tissue culture is very well documented (BAIRU *et al.* 2011). It is well known that high concentrations of the synthetic auxin 2,4-D act as a stress factor that can induce genetic and phenotypic changes in qualitative and quantitative traits of plants regenerated *in vitro*. This phenomenon is known as somaclonal variation (BAIRU *et al.* 2011). On the other hand, in the population of plants regenerated by somatic embryogenesis we found one plant with a two-fold higher genome size than all other analysed plants either collected from nature or regenerated by tissue culture (Table 4.). This plant was regenerated by somatic embryogenesis that was induced in zygote embryo culture on a medium supplemented with 10.0 mM 2,4-D. Evidence for the production of tetraploid plants of *I. pseudacorus* was presented by LAUBLIN *et al.* (1991). Protoplast culture of *I. fulva* revealed the production of diploids, triploids, tetraploids and hexaploids while plants regenerated by cell suspensions were all diploids (INOUE *et al.* 2006). In this study, the observed alterations in genomic size of the *I.*

reichenbachii plant regenerated by somatic embryogenesis might not be a consequence only of cultivation in tissue culture and application of 2,4-D. One of the reasons might be the genetic basis of donor plants as natural collections of *I. reichenbachii* are known to have plants with 24 and 48 chromosomes very frequently (MITRA 1956).

CONCLUSION

Zygotic embryo culture is a useful method for *in vitro* propagation, especially for endemic plant species because it can be applied to many genotypes and can be used to keep as much biodiversity as possible. Our presented protocol is efficient and safe as simple application of 2,4-D (0.5-5.0 mM) induced plant regeneration *via* somatic embryogenesis and organogenesis and whole plantlets were obtained. Some phenotypic changes in flower morphology occurred in plants regenerated by organogenesis while changes in ploidy level were found among plants regenerated by somatic embryogenesis induced on medium supplemented with 10.0 mM 2,4-D. The somaclonal variations observed can be very useful for further investigation of flower development of irises as well as breeding of dwarf irises.

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Botanica SERBICA



REZIME

In vitro propagacija *Iris reichenbachii* Heuff. i klonalna identičnost dobijenih biljaka

Slađana JEVREMOVIĆ, Milena LOJIĆ, Zoran JEKNIC, Milana TRIFUNOVIĆ -MOMČILOV, Dragana ANTONIĆ, Marija PETRIĆ, Angelina SUBOTIĆ, Ljiljana RADOJEVIĆ

Prikazan je efikasan protokol za propagaciju somatskom embriogenezom i organogenezom Balkanske endemične perunike, *Iris reichenbachii* i procenjena je klonalna identičnost dobijenih biljaka. Oba načina za regeneraciju u uslovima *in vitro* su postignuta u kulturi zrelih zigotskih embriona na Murashige & Skoog (MS) hranljivoj podlozi obogaćenoj sa 2,4-dihlorofenoksi sirćetnom kiselinom 0.5-5.0 mM (2,4-D) kao jedinim regulatorom rastenja. Dobijeni embriogeni kalusi su dalje gajeni na hranljivoj podlozi sa 2,4-D i kinetinom (Kn; 0.5 odnosno 5.0 mM). Formirani organogeni kalusi su dalje gajeni na MS hranljivoj podlozi obogaćenoj sa α-naftilsirćetnom kiselinom (NAA) i benziladeninom (BA; 0.5 odnosno 4.5 mM) kada je došlo do formiranja izdanaka. Kljanje somatskih embriona kao i ožiljavanje formiranih izdanaka postignuto je na MS hranljivoj podlozi bez biljnih regulatora rastenja. Biljke dobijene na oba načina su dalje uspešno aklimatizovane na uslove gajenja u stakleniku i cvetale su sledeće godine u proleće. Uočene su neke promene u morfologiji cvetova kod biljaka dobijenih procesom organogeneze. Na osnovu flow-citometrijske analize pokazano je da su biljke sa izmenjenom morfologijom cvetova imale isti nivo ploidnosti i veličinu genoma kao biljke iz prirode. U populaciji biljaka regenerisanih procesom somatske embriogeneze koja je indukovana na podlozi sa visokom koncentracijom 2,4-D (10.0 mM) jedna biljka je bila tetraploidna.

Ključne reči: perunka, somatska embriogeneza, organogeneza, cvetanje, flow-citometrija