



Clonal fidelity of *Iris sibirica* plants regenerated by somatic embryogenesis and organogenesis in leaf-base culture – RAPD and flow cytometer analyses



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ABSTRACT

Efficient protocols, safe from somaclonal variation, were developed for regeneration of *Iris sibirica* plants via organogenesis and somatic embryogenesis from leaf-base explants cultivated on Murashige and Skoog media supplemented with thidiazuron (TDZ, 1.0 mg/l) or 2,4-dichlorophenoxyacetic acid (2,4-D, 1.0 mg/l). The morphogenic response and callus formation efficiency differed significantly between 2,4-D (80.9%) and TDZ (67%) morphogenesis induction treatments. TDZ induced only organogenic calli, while calli obtained with 2,4-D were composed of three types differing in color and consistency: white, friable – embryogenic calli (4.5%, 3.8 mg/explant), green, compact – organogenic calli (12.4%, 48.4 mg/explants) and yellow – non-regenerative calli (77.3%, 254.4 mg/explant). The cultivation of embryogenic calli on medium with 2,4-D and Kinetin resulted in further development of somatic embryos (54 embryos/g of calli) which germinated with a frequency of 62% after being transferred to a medium without plant growth regulators. Stable shoot cultures were established by transferring organogenic calli with shoot primordia to media with 0.1 mg/l α -naphthaleneacetic acid (NAA) and 1.0 mg/l 6-benzylaminopurine (BA), while further cultivation on media of the same composition (TDZ or 2,4-D) resulted in the reduced growth and rhizogenesis, respectively. The TDZ induction treatment resulted in higher number of shoots per explant (7.9) than the 2,4-D treatment (4.3). After successful rooting and *ex vitro* acclimatization, plants were grown in the field and flowered to seed production. Flow cytometry, chromosome counting and random amplification of polymorphic DNA (RAPD) analysis indicated no evidence of genetic variation in plants regenerated via somatic embryogenesis or organogenesis. The results suggest that established protocols are safe for use in genetic transformation procedures or large-scale production of true-to-type *I. sibirica* plants.

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1. Introduction

Siberian Iris (*Iris sibirica* L.) is a perennial monocotyledonous plant and a horticulturally valuable member of the *Iridaceae* family of Monocot plants (Goldblatt and Manning, 2008). It is a rhizomatous plant, up to 120 cm tall with long narrow leaves and monochasial cymose inflorescence with 1–7 blue-lilac actinomorphic flowers (Szöllösi et al., 2010, 2011). It is native to wet fields and mountain regions of the Northern Hemisphere and listed as an endangered and protected species in

many countries such as Poland (Kostrakiewicz and Wróblewska, 2008) and Hungary (Szöllösi et al., 2011). *I. sibirica* plants, like *Iris pallida* and *Iris germanica*, accumulate in their roots essential oils, whose constituent terpenoid ketone-irone with strong violet-like scent is widely used in perfumery and cosmetics (Aslanyants and Marshavina, 1979; Gozu et al., 1993; Jéhan et al., 1994). Also, flowers of *I. sibirica* are the source of color used for painting on silk (Para and Baratti, 1992; Marner et al., 1995). Because of its beauty, *I. sibirica* is widely grown as ornamental garden plant or cut flower.

Creation of new varieties of horticultural value is the main objective in breeding programs of many ornamental plants. Due to poor fruit setting, low germination (Simonet, 1932) and vegetative propagation rate (10 plants/year, Jéhan et al., 1994) the genetic improvement of iris species by conventional and mutation techniques is a time- and labor-consuming process. Recently, particle bombardment and *Agrobacterium*-mediated transformation have become the most popular and powerful approaches employed for the creation of horticulturally valuable characteristics such as novel flower colors, leaf shapes, dwarf forms, prolonged

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, 6-benzyladenine; BM, basal medium; DAPI, 4,6-diamidino-2-phenylindole-2HCl; MS, Murashige and Skoog mineral solution; NAA, α -naphthaleneacetic acid; OI-2,4-D, organogenesis induced by 2,4-D; OI-TDZ, organogenesis induced by TDZ; PGR, plant growth regulators; RAPD, random amplification of polymorphic DNA; SEM, scanning electron microscopy; TDZ, thidiazuron (1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea)

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cut-flower vase-life, resistance to abiotic or biotic stresses such as viruses/viroids, pathogens and insects (Teixeira da Silva et al., 2013). Up to date, genetic transformation in genus *Iris* has been reported only for *I. germanica*, where stable overexpression of the *Lilium lancifolium* capsanthin-capsorubin synthase (*Llccs*) gene in callus tissue resulted in color change from yellow to red-orange (Jeknić et al., 1999, 2012). Genetic transformation of *I. sibirica* can also be an attractive way to create novel flower colors or to increase capacity for secondary metabolite production or content of essential oils.

There are numerous studies concerning *in vitro* regeneration of different members of the genus *Iris*, such as *I. germanica* (Reuther, 1975, 1977; Jéhan et al., 1994), *I. pallida* (Gozu et al., 1993; Jéhan et al., 1994), *Iris pumila* (Radojević et al., 1987), *Iris hollandica* (Fidalgo et al., 2005), *Iris setosa* (Radojević and Subotić, 1992), *Iris pseudocorus* (Laublin and Cappadocia, 1992) and *Iris versicolor* (Laublin et al., 1991). Initial results concerning successful *I. sibirica* shoot induction were achieved from flower parts with 6-benzyladenin (BA) and Kinetin as cytokinins and α -naphthaleneacetic acid (NAA) as auxin (Meyer, 1984; Asao et al., 1993). An alternative regeneration pathway of *I. sibirica* via parallel induction of somatic embryogenesis and organogenesis in mature zygotic embryo culture was obtained on medium supplemented with high concentration of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D; 5.0 mg/l) and Kinetin (1.0 mg/l, Subotić and Radojević, 1995). Recently, Jevremović et al. (2013) have reported the induction of somatic embryogenesis with an even higher concentration of 2,4-D (10 mg/l).

In vitro culture conditions, especially high concentration of 2,4-D act as a stress factor that can induce instability in cultured cells, tissues and organs, a phenomenon known as somaclonal variation (Larkin and Scowcroft, 1981; Gyulai et al., 2003; Bairu et al., 2011). This phenomenon refers to genetic and phenotypic changes in qualitative and quantitative traits of plants regenerated by *in vitro* culture. The synthetic auxin 2,4-D or unbalanced concentrations of auxins and cytokinins in the culture medium are often associated with genetic abnormalities such as polyploidy, changes in chromosome number or DNA sequence (Swartz, 1991; Bairu et al., 2011). Thus, a regeneration system safe from somaclonal variation is a fundamental need for producing true-to-type transgenic plants.

Consequently, the assessment of somaclonal variation of *in vitro* regenerated plants requires a wider approach and application of several methods to assess possible alterations at different levels. Because of its high accuracy and rapidity, flow cytometry and chromosome counting are widely used to assess changes in ploidy and chromosome number as the most common tissue culture-induced aberrations (Sharma et al., 2007; Mohanty et al., 2008; Fiuk et al., 2010; Prado et al., 2010). Recently, several DNA markers have been successfully used to assess the genomic stability of regenerated plants: restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD) and microsatellite markers (Hu et al., 2008; Bairu et al., 2011; Singh et al., 2013). RAPD has been one of the most commonly used methods because it is simple, rapid and cost-effective. It has proven to be a suitable molecular technique to detect genetic variation that occurs during *in vitro* regeneration of different plant species such as grape (Yang et al., 2008), cotton (Jin et al., 2008), potato (Vargas et al., 2008), chrysanthemum (Miñano et al., 2009), date palm (Ahmed et al., 2009), olive (Peyvandi et al., 2009), cowpea (Sivakumar et al., 2011), jojoba (Kumar et al., 2011), *Viola patrinii* (Chalageri and Babu, 2012) and *Limonium sinensis* (Dam et al., 2013). Except for chromosome counting (Laublin et al., 1991; Laublin and Cappadocia, 1992; Radojević and Subotić, 1992), no studies have been performed to check the genetic stability and uniformity of *Iris* sp. plants regenerated by tissue culture and to our best knowledge none concerning the genetic fidelity of *I. sibirica* plants regenerated *in vitro*.

The aim of this study was to develop an efficient protocol, safe from somaclonal variation, for *in vitro* regeneration of *I. sibirica* via somatic embryogenesis and organogenesis from leaf-base explants, that can be

used in future experiments for producing true-to-type *in vitro* regenerated plants. An effort was made to reduce the probability of somaclonal variation using lower concentration of plant growth regulators individually in the morphogenesis induction treatments.

2. Material and methods

2.1. Germination of seeds

Seeds of *I. sibirica*, obtained from Jelitto (Germany, IA167, 44007) were used as starting material for the establishment of aseptic cultures. After several trials with seed sterilization procedures, the following method was chosen: seeds (200) were imbibed in distilled water for 2 days before the removing of seed coats. Naked seeds were immersed in a 2% (v/v) solution of commercial antifungal agent Venturin (active molecule captan 480 g/l, Župa, Chemical Industry, Kruševac, Serbia) for 1 day. The following day, seeds were rinsed in tap water (for 1 h), then in 20% (v/v) commercial bleaching solution with 4% NaOCl (2×15 min) and distilled water (3×5 min). Sterilized seeds were placed on basal medium (BM) consisting of mineral salts (Murashige and Skoog, 1962), 3% (w/v) sucrose, 0.7% (w/v) agar (Torlak, Serbia) and inositol (100 mg/l), nicotinic acid (5.0 mg/l), pantothenic acid (10 mg/l), vitamin B₁ (2.0 mg/l), vitamin B₆ (1.0 mg/l), without plant growth regulators (PGR). After 2 days of cultivation in the growth chamber at 24 ± 2 °C under a 16 h/8 h photoperiod provided by cool-white fluorescent tubes ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$), seeds germinated and seedlings were used as a source of explants for regeneration procedures *in vitro*. Non-sterilized seeds (85) were sown in soil, germinated and used as a control in further cytogenetic and molecular analyses.

2.2. Tissue culture

Leaf bases (1 cm) of 7-day-old seedlings were used as initial explants for the induction of morphogenesis (Fig. 1). Only one leaf-base explant could be obtained from each seedling (Fig. 2a). Explants (10/Petri dish, 5 replicates) were cultivated on BM without PGR or BM supplemented with thidiazuron (TDZ, 1.0 mg/l) or 2,4-D (1.0 mg/l) for the induction of morphogenesis *in vitro*. All cultures were grown in a growth chamber at 24 ± 2 °C under a 16 h/8 h photoperiod provided by cool-white fluorescent tubes ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$). After 6 weeks of cultivation, efficiency of callus production was calculated as number of explants that produced calli/total number of explants and the fresh weight of produced calli per each explant was measured under sterile conditions in a laminar hood using a fine balance (Chyo MK-200B).

2.2.1. Somatic embryogenesis

Different types of calli (embryogenic, organogenic and non-regenerative) occurring on explants cultured on BM supplemented with 2,4-D, were separated under binocular and their weights were measured. Embryogenic and non-regenerative calli were further subcultured on BM supplemented with 0.1 mg/l 2,4-D and 1.0 mg/l Kinetin (6-furfurylaminopurine) for further development of somatic embryos (Fig. 1). The number of somatic embryos per gram of embryogenic calli was measured after 3 successive 4-week subcultures. Isolated somatic embryos, 3–5 mm long were placed in Petri dishes with BM without PGR (50 embryos per Petri dish), where they germinated after 4 weeks.

2.2.2. Organogenesis

The number of shoot primordia per explant was measured after 6 weeks of cultivation on media with 2,4-D (Organogenesis induction, OI-2,4-D) or TDZ (OI-TDZ). Organogenic nodules and shoot primordia formed on organogenic calli (OI-2,4-D or OI-TDZ) were further cultured either on BM of the same composition or on BM supplemented with NAA (0.1 mg/l) and BA (1.0 mg/l) for shoot multiplication (Fig. 1). The shoot multiplication index was determined as the number of shoots

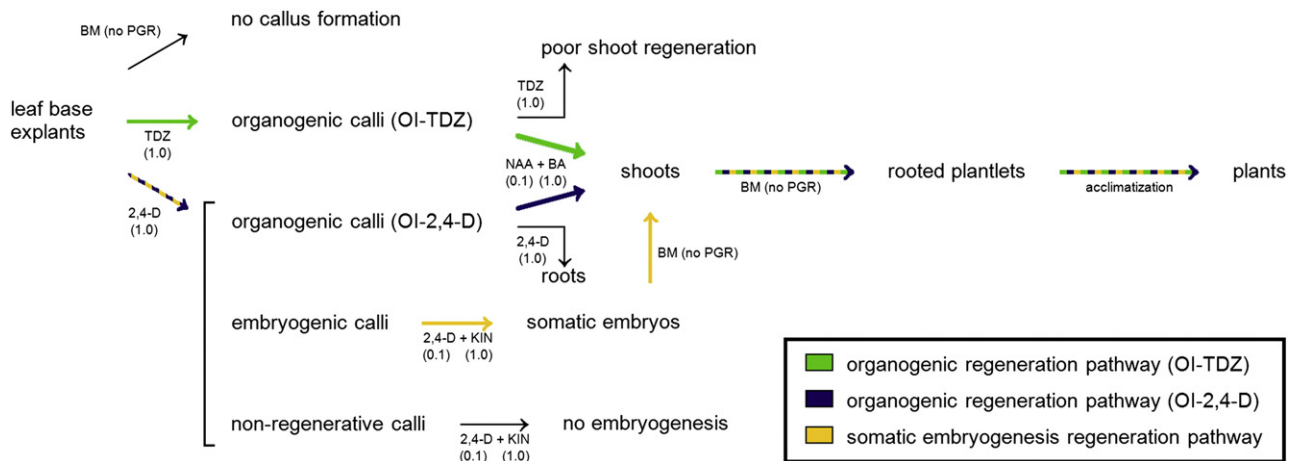


Fig. 1. Regeneration of *Iris sibirica* from leaf-base explants using various plant hormones in the regeneration media. Numbers in brackets correspond to concentration of plant growth regulators supplemented to the media, given as (mg/l). The three successful regeneration pathways are indicated in the diagram by respectively colored arrows. Organogenesis occurred from calli obtained on regeneration media supplemented with either TDZ (OI-TDZ) or 2,4-D (OI-2,4-D), while somatic embryogenesis occurred only from calli grown on media with 2,4-D. Organogenic calli were further cultured on medium supplemented with NAA and BA for shoot multiplication, while embryogenic and non-regenerative calli on medium supplemented with 2,4-D and Kinetin for further development of somatic embryos. Shoots regenerated either from organogenic calli or from somatic embryos subsequently underwent rooting and acclimatization in the greenhouse.

after 4 weeks/initial shoot number per culture vessel; 10 single initial shoots were cultivated per culture vessel (700 ml) containing 100 ml medium and the multiplication index was calculated after 3 successive subcultures.

2.3. Rooting and acclimatization of regenerated plants

For shoot rooting, 50 single shoots (4–5 cm) obtained either through somatic embryogenesis or organogenesis (OI-2,4-D and OI-TDZ), were transferred to 20 mm-long test tubes with 5 ml of BM without PGR and cultured for 4 weeks. After rooting, plants were potted in a mixture of peat and perlite (3:1), acclimatized and grown in greenhouse conditions. Plants were planted in the field 1 year later.

2.4. Scanning electron microscopy (SEM)

Samples of calli and somatic embryos were subjected to scanning electron microscopy (SEM) on JOEL JSM-6460LV microscope at the Institute for Biology, University of Novi Sad, Serbia without previous preparation.

2.5. Flow cytometry

The ploidy level of greenhouse-grown *I. sibirica* plants regenerated by somatic embryogenesis and organogenesis (OI-2,4-D) from the same initial explant and by OI-TDZ was determined by flow cytometry according to the method of Doležel et al. (1992). Since seedlings were destroyed during leaf-base excision, they could not be preserved as mother plants for further analyses. Instead of them, plants derived from seeds were used as control in flow cytometry and analyses of genetic uniformity of regenerated plants. A 1 cm² portion of the leaf blade of plants grown in greenhouse was cut and placed into 0.4 ml nuclei extraction buffer (0.1 M Tris, 2 mM MgCl₂, 0.1 M NaCl, 0.05% (v/v) Triton X-100, pH 7.0). After filtration through a 50 µm nylon sieve (Partec Cell Trics Disposable filter units, No. 04-0041-2317), 2.0 ml staining solution containing 0.1 mg/ml dye DAPI (4,6-diamidino-2-phenylindole-2HCl) was added. Flow cytometry was performed with a PAS flow cytometer (Partec) with an argon laser (488 nm, 20 mW) and *Vicia faba* as an internal standard. At least 2000–5000 nuclei were analyzed per sample.

2.6. Chromosome counting

For chromosome counting, 10 mm-long root tips were collected from 10 randomly selected greenhouse-grown *I. sibirica* plants regenerated by organogenesis (OI-TDZ) or somatic embryogenesis and organogenesis (OI-2,4-D) from the same initial explant and plants derived from seeds. Root tips were treated with 2 mM 8-hydroxyquinoline at room temperature for 4–5 h, fixed in Carnoy's solution (ethanol: glacial acetic acid 3:1) for 24 h at 4 °C and stored dehydrated in 70% (v/v) ethanol. Fixed roots were hydrolyzed for 15 min at 60 °C and subsequently stained with 1% (w/v) aceto-orcein for 10 min. Chromosome number was observed using light microscopy (Carl Zeiss AxioVision microscope, Zeiss, Germany).

2.7. RAPD analysis

RAPD analysis was performed on greenhouse-grown *I. sibirica* plants regenerated by organogenesis (OI-TDZ) or somatic embryogenesis and organogenesis (OI-2,4-D) from the same initial explant and plants derived from seeds. Total genomic DNA was isolated from 250 mg of frozen leaves of 30 randomly chosen plants of each group, using the standard CTAB method according to Zhou et al. (1994). The quality of genomic DNA was confirmed by electrophoresis in 0.8% (w/v) agarose gel containing ethidium bromide (0.5 µg/ml) in 1 × TBE buffer (Sambrook et al., 1989). The concentration of DNA was determined by a UV-vis Recording Spectrophotometer (UV-2501 PC, Shimadzu) and each sample was diluted to 100 ng/µl. Samples were stored at 4 °C for further analysis.

Three RAPD primers: OPD-8, OPD-13 and OPB-12 (OPERON technology Alameda, Canada; Table 1) were used for the amplification of the total DNA. DNA amplification was performed in a volume of 30 µl reaction mixture containing: 0.75 µg template DNA, 0.5 µM random primers (Applied Biosystems), 0.75 mM dNTPs, 3 units Taq polymerase AmpliTaq Gold® (Applied Biosystems), 3 µl 10 × PCR buffer and 13.6 µl sterile distilled water. All PCR reactions were performed in a PCR thermal cycler (Techne Genius) and consisted of an initial denaturation step (4 min at 95 °C); followed by 40 cycles of denaturation (30 s at 95 °C), annealing (45 s at 36 °C) and elongation (1 min at 72 °C); and final extension (5 min at 72 °C). Amplified DNA fragments were separated by electrophoresis in 1.5% (w/v) agarose gels. Bands were visualized and recorded using a gel documentation system (Gel Explorer Ultra

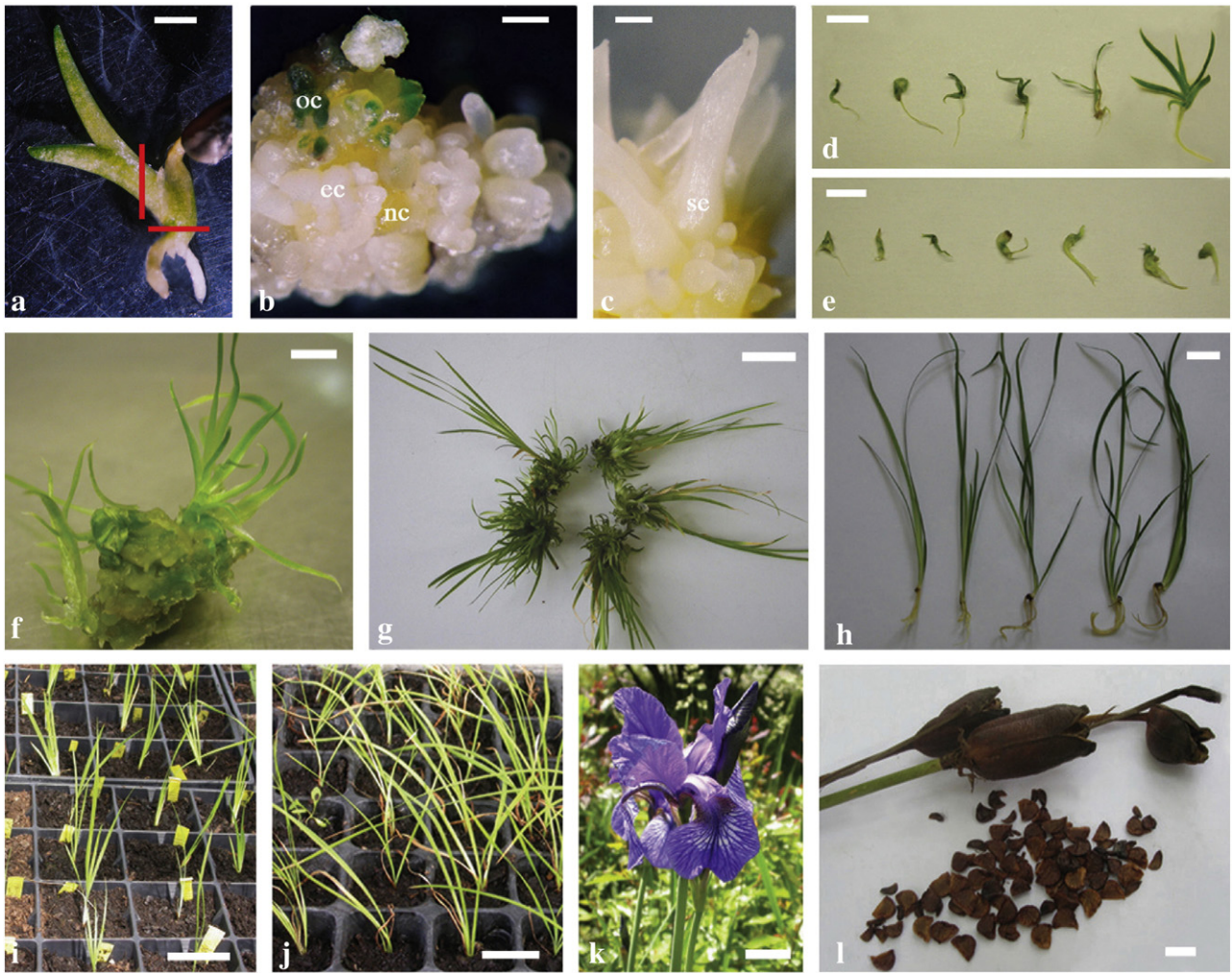


Fig. 2. Plant regeneration of *I. sibirica* by somatic embryogenesis and organogenesis in leaf-base culture. Leaf-base of 7-day-old seedling used as initial explants – red mark is place of cutting, bar 5 mm (a) Induction of calli on basal medium (BM) supplemented with 1.0 mg/l 2,4-D; ec – embryogenic calli, nc – non-regenerative calli, oc – organogenic calli, bar 1 mm (b). Detail of embryogenic calli with somatic embryos (se) at different stages of development, bar 10 mm (c). Normal germination of somatic embryos, bar 10 mm (d). Abnormal somatic embryo germination, bar 10 mm (e). Adventitious shoot formation on organogenic calli formed on BM medium supplemented with 1.0 mg L⁻¹ TDZ, bar 1 mm (f). Shoot multiplication by axillary shoot proliferation on BM medium supplemented with 0.1 mg/l NAA and 1.0 mg/l BA, bar 10 mm (g). Plantlets obtained by organogenesis in the rooting phase, bar 20 mm (h). Plantlets regenerated by organogenesis grown in the greenhouse, bar 20 mm (i). Plantlets regenerated by somatic embryogenesis grown in the greenhouse, bar 20 mm (j). Flowering plant after two years of field growth, bar 20 mm (k). Fruit and seeds of *I. sibirica* plants regenerated by tissue culture, bar 20 mm (l).

Lum). The size of the amplicons was estimated by comparison to the 100 bp DNA ladder (Serva).

2.8 . Data analysis

All data were subjected to statistical analysis using the computer program STATGRAF. 4.2 (STSC Inc. and Statistical Graphics Corporation, 1985–1989, USA) and percentage data were arcsine-transformed prior to statistical analysis. The experiment for shoot regeneration and rooting was replicated three times. Data were analyzed using the least significant difference test (LSD). DNA amplification with each primer was repeated at least twice to confirm the reproducibility of the results.

Table 1

List of decamer primers used for RAPD analysis of genetic variability of *Iris sibirica* plants, their base sequence and G + C (%) content.

Primer	DNA sequence	G + C content (%)
OPD-8	5'-GTGTGCCCCA-3'	70
OPD-13	5'-TCGTACCCC-3'	70
OPB-12	5'-CCTTGACGCA-3'	50

Only reproducible bands were considered for analysis. Specific amplification products (bands) were scored as present (1) or absent (0) at a particular position.

3 . Results

3.1 . Plant regeneration through somatic embryogenesis and organogenesis

3.1.1 . Callus formation

Sterilized, naked seeds germinated with 76.38% frequency two days after cultivation and seedlings were used as a source of explants for regeneration procedures. After 6 weeks of culture, leaf-base explants of *I. sibirica* cultivated on BM supplemented with 2,4-D or TDZ developed calli with a frequency of 80.9% and 66.7% respectively, while no callus formation was observed on BM without PGR (Fig. 1). However, BM without PGR supported growth of leaf-base explants into shoots with 36% frequency. This result was expected due to preservation of intact shoot apical meristem in explants during excision from seedlings (Fig. 2a). Calli produced on explants cultured on BM supplemented with 2,4-D were composed of three types of calli differing in color and consistency. White, friable calli (4.5%, 3.8 mg/explant) with developed

somatic embryos at different developmental stages on the callus surface were designated as embryogenic (ec, Fig. 2b). The compact green calli (12.4%, 48.4 mg/explant) with shoot primordia were designated as organogenic (oc), and the third, most abundant group were the yellow calli (77.3%, 254.4 mg/explant) designated as non-regenerative (nc, Fig. 2b). Leaf-base explants cultured on BM supplemented with TDZ developed only green organogenic calli.

3.1.2. Somatic embryogenesis

Most somatic embryos produced on embryogenic calli were in the globular stage of development. The next 4-week subculture on medium with 2,4-D and Kinetin resulted in further development of somatic embryos (54 embryos/g of calli). Somatic embryos developed asynchronously, thus embryos from the globular to the cotyledonary stage could be observed at the same time on the same callus (Figs. 2c; 3a,b). In order to achieve a higher germination rate, single somatic embryos were transferred to BM without PGR where they germinated with a frequency of 62% after 4 weeks (Fig. 2d). Apart from normally growing embryos, 10% showed abnormal morphology with asynchronous development of shoot and root meristem during the germination process (Figs. 2e, 3c).

3.1.3. Organogenesis

Shoot primordia which developed on green organogenic calli (OI-TDZ, 7.9 shoots/explant) and were subsequently cultured on the same medium, remained very small and were not useful for the establishment of shoot cultures. On the other side, green organogenic calli obtained after cultivation of *I. sibirica* leaf bases on induction medium supplemented with 2,4-D (OI-2,4-D, 4.3 shoots/explant) and further cultured on the medium of the same composition became rhizogenic. Stable shoot cultures were established by transferring organogenic nodules with shoot primordia (OI-2,4-D or OI-TDZ) to BM supplemented with 0.1 mg/l NAA and 1.0 mg/l BA. Initially, *de novo* formation of adventitious shoots was observed in callus culture (Fig. 2f), subsequently shoot cultures were established and maintained by development of axillary shoots (Fig. 2g). No significant differences in mean number of axillary shoots per explant were observed between OI-2,4-D and OI-TDZ plants (Fig. 4a).

3.2. Rooting and acclimatization of regenerated plants

Shoots obtained through somatic embryogenesis or organogenesis were successfully rooted on BM without PGR (Fig. 2h). No significant differences in rooting efficiency were observed between OI-2,4-D and OI-TDZ plants (Fig. 4c). The other two measured parameters, plant height (Fig. 4b) and root number (Fig. 4d) were also similar in both

groups of analyzed plants, contrary to the root length that was significantly higher in OI-TDZ plants (Fig. 4e).

Rooted plantlets obtained through organogenesis (OI-2,4-D or OI-TDZ) (Fig. 2i) and somatic embryogenesis (Fig. 2j), 50 from each group, were planted and successfully acclimatized to greenhouse conditions (Fig. 4f). After one year, plants were planted in the field and in the following season they flowered (Fig. 2k) and set seeds (Fig. 2l). Regenerated plants displayed morphological and reproductive characteristics similar to seed-derived plants (data not shown).

3.3. Flow cytometry and chromosome counting analyses

The ploidy level of *I. sibirica* plants derived from seeds and those regenerated by somatic embryogenesis and organogenesis (OI-2,4-D or OI-TDZ) was determined by flow cytometry using a 1 cm² portion of the leaf blade of plants grown in greenhouse conditions. The flow cytometry profiles indicated stability of ploidy level in leaf cells of regenerated plants (Fig. 5b,c). A similar nuclear DNA content and total number of peaks were found in plants derived from seeds taken as a diploid control (Fig. 5a).

The results obtained by flow cytometry were, in general, consistent with those obtained by chromosome counting (Fig. 5d–f). Having demonstrated a diploid number of chromosomes ($2n = 2x = 28$) in the root-tip cells of the regenerated *I. sibirica* plants, the chromosome counting analysis verified that numeric chromosomal aberration (*i.e.* aneuploidy) has not occurred during organogenesis and somatic embryogenesis, comparing to plantlets derived from initial stock seeds.

3.4. RAPD analysis

RAPD markers were used to estimate the genetic stability at the DNA level of *I. sibirica* plants regenerated by organogenesis and somatic embryogenesis. The efficiency and specificity of OPD-8, OPD-13 and OPB-12 primers were tested in a previously optimized RAPD protocol for *I. sibirica* plants derived from seeds. Banding patterns obtained with each primer were specific and reproducible, clearly pointing out the differences at the DNA level between individual plants (Fig. 6; Table 2). Based on this result, we concluded that OPD-8, OPD-13 and OPB-12 primers were adequate and sufficient for the assessment of clonal fidelity of *I. sibirica* plants regenerated *in vitro*. Plants regenerated through one regeneration pathway (OI-TDZ, OI-2,4-D or somatic embryogenesis) did not differ between themselves as evidenced by the absence of polymorphic bands in their RAPD profiles (Figs. 7; 8; Table 2). Plants originated from the same explant showed identical RAPD profiles regardless of regenerative pathway (OI-2,4-D and somatic embryogenesis) (Table 2). On the other hand, plants regenerated *via* organogenesis

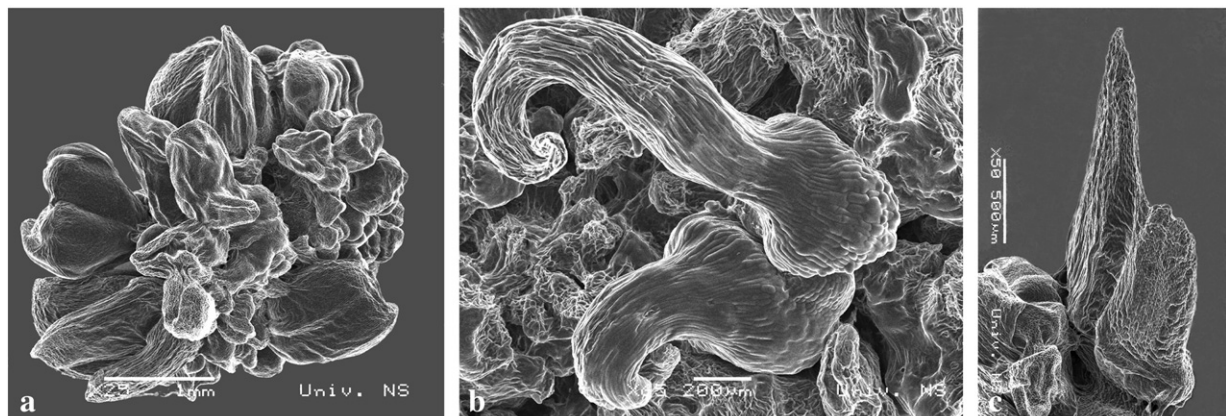


Fig. 3. Scanning electron microscopy of somatic embryogenesis of *I. sibirica* in leaf-base culture. Embryogenic calli with somatic embryos in different stages of development (a). Somatic embryos at the cotyledonary stage (b). Asynchronous germination of somatic embryos (c).

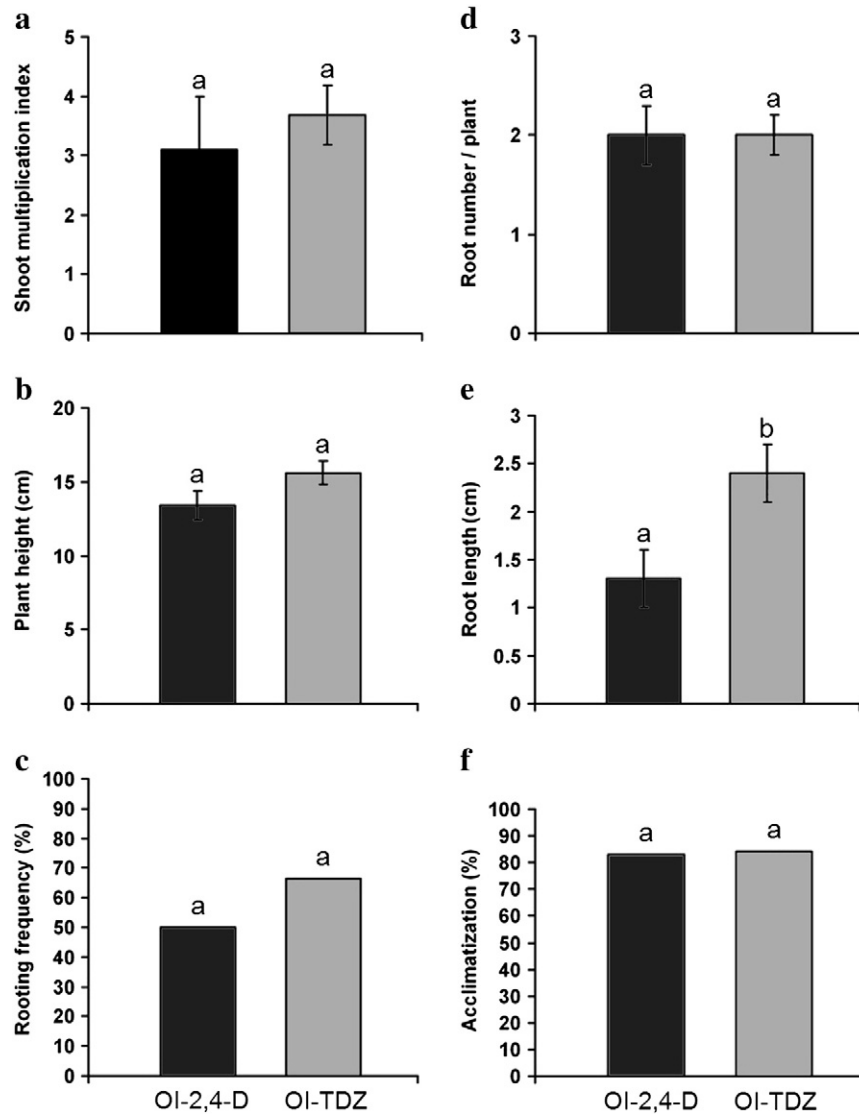


Fig. 4. Plant multiplication, rooting efficiency and acclimatization of *I. sibirica* plants regenerated via organogenesis induced by 2,4-D (OI-2,4-D) or TDZ (OI-TDZ). Shoot multiplication index obtained on BM medium supplemented with NAA and BA (a). Shoot and root development on BM medium without plant growth regulator: average plant height, rooting frequency, average root number and root length, respectively (b–e). Acclimatization frequency (f).

(OI-TDZ) differed from plants regenerated through organogenesis (OI-2,4-D) and somatic embryogenesis (Figs. 7; 8; Table 2), confirming their origin from leaf-base explants of different initial seedlings. In the OPD-13 RAPD pattern of plants regenerated by somatic embryogenesis and organogenesis (OI-2,4-D) (Fig. 7; lanes 7–10) we noted the presence of two bands (800 bp and 600 bp) and the absence of the 750 bp band in comparison to the OI-TDZ plants pattern (Fig. 7; lanes 1–6). In the OPD-8 RAPD pattern of plants regenerated via somatic embryogenesis and organogenesis (OI-2,4-D) (Fig. 8; lanes 7–10) the presence of a 650 bp band and the absence of 550 bp, 450 bp and 150 bp bands, as well as the absence of bands in the case of the OPB-12 primer, was noticed in comparison to the OI-TDZ plants pattern (Fig. 8; lanes 1–6). The total number of amplification products generated with all 3 primers was: 24 for the plants derived from seeds, 18 for the OI-TDZ plants, 12 for the OI-2,4-D plants and 12 for the plants regenerated by somatic embryogenesis. The highest (29) and the lowest (15) total number of bands (the 4 groups of plants altogether) was obtained by OPD-8 and OPB-12 primers, respectively.

4. Discussion

Up to date, a large number of protocols for somatic embryogenesis and organogenesis have been developed for different genotypes of *Iris* sp. (Hussey, 1975; Reuther, 1977; Radojević et al., 1987; Laublin et al., 1991; Fidalgo et al., 2005). An optimal regeneration method for each particular genotype has been determined by searching for the optimal hormonal composition of the culture medium and type of explants (reviewed by Ascough et al., 2009). Here, we established two efficient protocols for *in vitro* regeneration of *I. sibirica* from leaf-base culture using low concentration of only one growth factor (2,4-D or TDZ) in the morphogenesis induction medium.

Successful regeneration of iris species from leaf-base explants was reported by several authors (Gozu et al., 1993; Shimizu et al., 1997; Shibli and Ajlouni, 2000; Jevremović et al., 2013). In this study, leaf bases proved to be suitable explants for *I. sibirica* regeneration as organogenic and embryogenic calli developed in most of the explants. Leaf-base advantage is their availability throughout the year in contrast

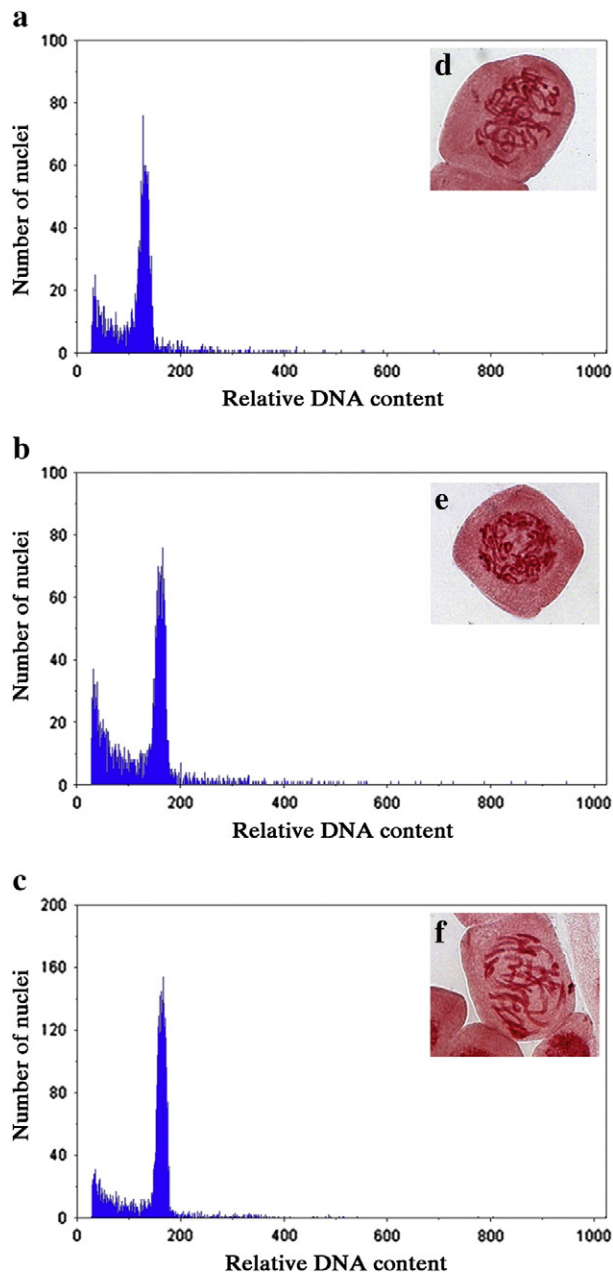


Fig. 5. Flow cytometry analysis and chromosome counts of *I. sibirica* plants. Flow cytometry profiles of plants derived from seeds (a), regenerated from leaf-base explants via somatic embryogenesis induced with 1 mg/l 2,4-D (b) and organogenesis induced with 1 mg/l 2,4-D (Ol-2,4-D) (c). Root-tip cells with visible chromosomes ($2n = 2x = 28$) of plants derived from seeds (d), regenerated by somatic embryogenesis induced with 1 mg/l 2,4-D (e) and organogenesis induced with 1 mg/l 2,4-D (Ol-2,4-D) (f).

to flower parts that have been found as the best explants in many species of genus *Iris* (Gozu et al., 1993; Fidalgo et al., 2005; Ascough et al., 2009).

Several factors are proved to affect the morphogenic response and callus formation efficiency *in vitro*, especially the type and concentration of plant growth regulators used for the morphogenesis induction (Ikeuchi et al., 2013; Smith, 2013). In accordance with this, the callus formation efficiency differed significantly in two PGR treatments reaching 80.9% for 2,4-D and 67% for TDZ. Callus formation was never observed in leaf-base explants cultured on medium without plant growth regulators, indicating that callus formation could not be attributed to the *in vitro* culture-induced stresses. The TDZ treatment induced only organogenic calli, while parallel induction of somatic

embryogenesis and organogenesis in the same explant was achieved on medium supplemented with 2,4-D. Since the regenerative response depends on the remodeling of the chromatin and the release of morphogenic programs previously blocked by chromatin-mediated gene silencing (Fehér, 2006), the distinction between the induction of embryogenesis and organogenesis in the same explant may provide an experimental system for studying the developmental biology of these two regenerative pathways. The capability of 2,4-D in activating the embryogenic pathway may be related to its capacity to induce expression of stress genes which have been shown to contribute to the cellular reprogramming of somatic cells toward embryogenesis (Kitamiya et al., 2000).

The 2,4-D is used more often in combination with other plant growth regulators (Gozu et al., 1993; Jéhan et al., 1994; Radojević and Subotić, 1992; Shimizu et al., 1997; Shibli and Ajlouni, 2000; Fidalgo et al., 2005; Jevremović et al., 2008) for morphogenesis induction in *Iris* sp. In this study, we demonstrated that efficient regeneration of *I. sibirica* could be achieved by 2,4-D induction treatment alone and in concentration five- and ten-fold lower than used in previous studies in *I. sibirica* (Subotić and Radojević, 1995; Jevremović et al., 2013).

Sequential changing of the hormonal composition by further decreasing the concentration of 2,4-D and the introduction of Kinetin in the regeneration medium improved the embryogenic potential of *I. sibirica*. Our results confirmed previous findings that multiple PGR sequences are essential for development of somatic embryos in *Iris* species (Ascough et al., 2009; Radojević and Subotić, 1992; Laublin et al., 1991).

The conversion of somatic embryos into plantlets is an important step for the application of somatic embryogenesis in breeding and improvement programs. In our work, a satisfactory conversion frequency of somatic embryos was achieved in the absence of PGRs contrary to the previous report for *I. sibirica* (Subotić and Radojević, 1995) and some other iris species, where addition of auxin and cytokinin (Radojević and Subotić, 1992), gibberelic acid (Shimizu et al., 1997) or abscisic acid (Fidalgo et al., 2005) was necessary for embryo conversion. Asynchronous development of shoot and root meristem in *Iris* sp. embryos has been well documented previously (Radojević et al., 1987; Radojević and Subotić, 1992; Fidalgo et al., 2005). Although in one of the previous studies only 18% of *I. hollandica* somatic embryos eventually developed into normal plantlets (Fidalgo et al., 2005), we found that only 10% of the *I. sibirica* embryos showed abnormal development upon transfer to BM without PGR.

Although the sole synthetic auxin 2,4-D has shown very effective, successful regeneration in *I. sibirica* could be as well induced by cytokinin alone. Up to now, there have been no literature data about the effect of TDZ on *I. sibirica* morphogenesis and generally it has been rarely used for the regeneration of other iris species (Ascough et al., 2009). An unsuccessful attempt of *I. nigricans* somatic embryo regeneration was reported by Shibli and Ajlouni (2000), while organogenic calli and adventitious shoots were successfully induced in zygotic embryos culture of *Iris pseudopallida* (Jevremović et al., 2008). Leaf-base explants of *I. sibirica* were twice as responsive to the same concentration of TDZ (1.0 mg/l). TDZ was more effective than other cytokinins in promoting the adventitious shoot formation in many plant species (Zhang et al., 2001; Espinosa et al., 2006). It seems that TDZ modulates the level of endogenous auxins *via* enhanced accumulation and translocation, thus providing an optimal endogenous auxin/cytokinin ratio within the cultured tissue (Jones et al., 2007). Although some authors reported that TDZ could be used to stimulate somatic embryogenesis (Visser et al., 1992; Murthy et al., 1995, 1998) or simultaneous production of shoots and somatic embryos (Bates et al., 1992), here we report evidence only for the effects of TDZ on callus and shoot formation.

TDZ-induced shoots remained very small after prolonged culture on the same medium, indicating the inhibitory effect of TDZ on *I. sibirica* shoot elongation. The deleterious effect of the continuous presence of TDZ on the growth and multiplication has been documented in several

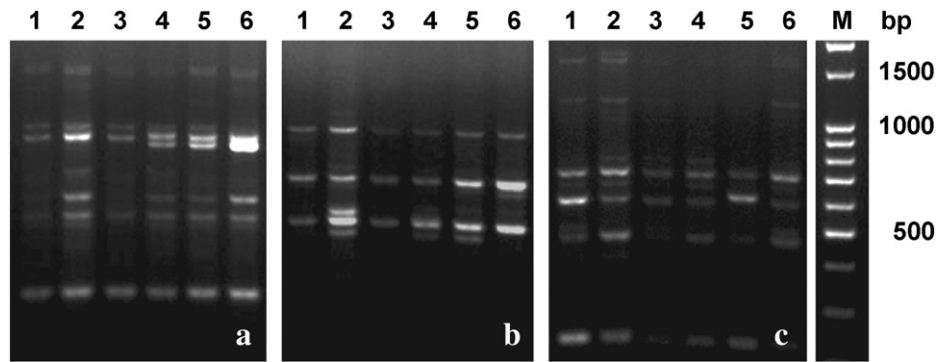


Fig. 6. RAPD profiles of *I. sibirica* seed-derived plants obtained with primers: OPD-8 (a), OPD-13 (b), OPB-12 (c). Lane M: DNA Standard 100 bp Ladder (Serva).

plant species including *I. pseudopallida* (Faisal et al., 2005; Jevremović et al., 2008; Jahan and Anis, 2009; Parveen and Shahazad, 2010; Ahmed and Anis, 2012). To solve this problem, a two-stage culture procedure including a secondary medium lacking TDZ, with or without growth regulators was applied in all these species. Among all the media compositions investigated, BA (1 μ M) along with NAA (0.5 μ M) was found to be the most effective (Kim et al., 1997). The efficiency of BA and NAA on TDZ-exposed explants for maximum shoot induction and elongation was also confirmed in our work.

The type, concentration and combination of plant growth regulators in the culture medium are the major factors affecting the incidence of somaclonal variation (Bairu et al., 2011). The synthetic auxin 2,4-D is commonly considered to be responsible for genetic changes, especially alterations in chromosome number and ploidy levels in plants regenerated through callus formation. It is possible that 2,4-D affects ploidy levels by triggering unorganized cell growth and disturbing cell cycle control, leading to DNA synthesis and endoreduplication (Neelakandan and Wang, 2012). Contrary to numerous plant species regenerated through callus formation using 2,4-D alone or in combination with other growth regulators (Nehra et al., 1992; Jin et al., 2008; Godo et al., 2010; Pinto et al., 2010; Prado et al., 2010), we report no altered ploidy level in *I. sibirica* plants obtained by described protocols. Flow cytometry, as one of the approaches commonly used to verify the absence of chromosomal aberration (Vujović et al., 2012; Escobedo-Gracia Medrano et al., 2014), confirmed that relative content of DNA in plants regenerated via indirect somatic embryogenesis and organogenesis, remained unchanged in comparison to the plants germinated from the original *I. sibirica* seeds. Apparently, the DNA level remained unaffected by callogenesis and *in vitro* conditions (including PGRs present in the culture media) to which the plants were subjected in this study.

Table 2

The size and number of scored bands produced by RAPD analysis.

Sample	Primer	Number of bands			Size range (bp)
		Monomorphic	Polymorphic	Total	
Plants derived from seeds	OPD-8	5	4	9	350–1500
	OPD-13	3	2	5	500–1000
	OPB-12	4	6	10	250–1800
Plants regenerated by TDZ-induced organogenesis (OI-TDZ)	OPD-8	8	0	8	150–850
	OPD-13	5	0	5	450–1200
	OPB-12	5	0	5	250–1000
Plants regenerated by 2,4-D-induced organogenesis (OI-2,4-D)	OPD-8	6	0	6	300–850
	OPD-13	6	0	6	450–1200
	OPB-12	0	0	0	–
Plants regenerated by 2,4-D-induced somatic embryogenesis	OPD-8	6	0	6	300–850
	OPD-13	6	0	6	450–1200
	OPB-12	0	0	0	–

Although flow cytometry is usually considered an efficient and reliable method (Doležel et al., 2012; de Oliveira et al., 2013), additional screening of DNA ploidy level is always desirable because of the potentially high background noise or low accessibility of the dye to nuclear DNA due to high concentration of secondary metabolites in the analyzed cells (Loureiro, 2007). Thus, we used chromosome counting in root-tip cells to confirm the stability of ploidy level in embryo- and shoot-derived *I. sibirica* plants and showed the presence of diploid sets of chromosomes, same as in seed-derived plants. The unchanged chromosome number in shoots regenerated via indirect embryogenesis and organogenesis was previously reported in *I. setosa* (Radojević and Subotić, 1992), *I. versicolor* and *I. pseudacorus* (Laublin et al., 1991; Laublin and Cappadocia, 1992).

Beside ploidy level changes, other types of genetic differences, such as DNA polymorphism, could also be expected in *in vitro* derived plants. RAPD analysis has been the most widely used to estimate the genetic fidelity at the DNA level. The disappearance or appearance of a new band in a RAPD profile is usually a consequence of DNA damage and/or mutation that results in the inability of the primer to anneal to a previous binding site (Cenkci et al., 2010). Changes in RAPD profiles as a result of presence of 2,4-D in the callus induction medium, alone or along with other growth regulators, were frequently found in callus cultures or regenerated plants of many species, e.g. *Cereus peruvianus* (Mangolin et al., 2002), potato (Bordallo et al., 2004), *Curcuma amada* Roxb. (Prakash et al., 2004), *Cymbopogon flexuosus* (Bhattacharya et al., 2008), papaya (Homhuan et al., 2008) and cotton (Jin et al., 2008). Also, there are several reports about TDZ affecting the DNA stability of plants propagated *in vitro* (Sarmast et al., 2012; Roy et al., 2012; Khattab et al., 2014). In contrast, the RAPD analysis performed in our study detected no polymorphism within plants regenerated through the same regeneration pathway, whereas the RAPD analysis with the same decamer primers revealed considerable polymorphism within iris plants derived from seeds. Moreover, no difference was found between organogenic and embryogenic plants originating from the same explant. Differences found between 2,4-D embryogenic and organogenic plants on one side and TDZ organogenic plants on the other, are the consequence of different starting leaf-base explants (different genotypes) which is in accordance with a high amount of polymorphism detected among seed-derived *I. sibirica* plants. The high variability in RAPD profiles of plants obtained from seeds has been already reported for *I. setosa* (Artyukova et al., 2001) and also for three *Iris aphylla* populations from Poland (Wroblewska et al., 2003) where higher variability was revealed within (77.2%) than between populations (22.8%).

The primers chosen for RAPD analysis of *I. sibirica* had been previously successfully used for the analysis of genetic variability of numerous *Iris* species (Kozyrenko et al., 2002; Artyukova et al., 2001; Al-Gabbiesh et al., 2006) confirming the applicability of RAPD methods in the genus *Iris*. Moreover, Makarevitch et al. (2003) showed that only four different primers (OPD-8, OPD-11, OPD-13 and OPB-12) were sufficient for the successful analysis of phylogenetic relationships of 22

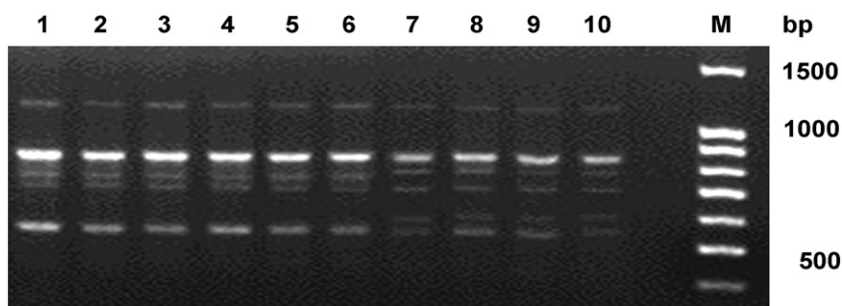


Fig. 7. RAPD profiles of *in vitro* regenerated *I. sibirica* plants obtained with the primer OPD-13. Lanes 1–6: plants regenerated by 1 mg/l TDZ-induced organogenesis (OI-TDZ), lanes 7–10: plants regenerated by 1 mg/l 2,4-D-induced somatic embryogenesis, lane M: DNA Standard 100 bp Ladder (Serva).

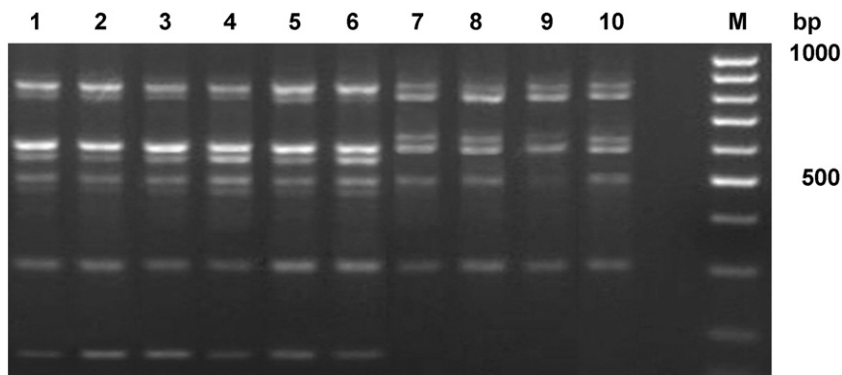


Fig. 8. RAPD profiles of *in vitro* regenerated *I. sibirica* plants obtained with the primer OPD-8. Lanes 1–6: plants regenerated by 1 mg/l TDZ-induced organogenesis (OI-TDZ), lanes 7–10: plants regenerated by 1 mg/l 2,4-D-induced somatic embryogenesis, lane M: DNA Standard 100 bp Ladder (Serva).

species of this genus. Out of three primers screened, two (OPD-8 and OPD-13) produced specific and reproducible RAPD patterns in all groups of analyzed *I. sibirica* plants, while one (OPB-12) gave no amplicons for somatic embryo-derived plants and plants regenerated via organogenesis (OI-2,4-D), similarly to the results of Al-Gabbiesh et al. (2006) where DNA of only five out of seven analyzed *Iris* species had been amplified by OPB-12.

In conclusion, we have developed simple and efficient regeneration protocols for *I. sibirica* plants via organogenesis and somatic embryogenesis using leaf-base culture. Identical ploidy level, chromosome number and RAPD banding patterns of plants obtained by each regeneration pathway suggest that developed protocols can be used for safe regeneration of both genetically modified and untransformed *I. sibirica* plants, as well as for large-scale production of true-to-type plants of particular genotype selected for improved characteristics.

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