Original scientific paper
DOI: /10.5513/JCEA01/20.1.2126
Journal of Central European Agriculture, 2019, 20(1), p.415-430

Plant regeneration and clonal fidelity assessment of subendemic species *Iris illyrica* Tomm. originated from Croatia

Regeneracija biljaka i procjena klonske vjernosti subendemske vrste *Iris illyrica* Tomm. porijeklom iz Hrvatske

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Abstract

The present study was undertaken to evaluate efficiency of callogenesis and regeneration by somatic embryogenesis of the subendemic Iris species *Iris illyrica* from Croatia and to select highly regenerative donor plants/genotypes. Leaf base segments were used as explants. Callogenesis and somatic embryogenesis were induced on MS media supplemented with: (1) 4.52 μM 2,4-dichlorophenoxyacetic acid (2,4-D) + 4.83 μM 1-naphthaleneacetic acid (NAA) + 0.46 μM kinetin (Kin); (2) 4.52 μM 2,4-D + 4.6 μM Kin; (3) 13.4 μM NAA + 2.3 μM Kin. Transfer of embryogenic calli onto hormone-free medium enabled the development of mature somatic embryos. Frequency of callogenesis was influenced by the donor plant. Among 15 donor plants tested, 3 of them exhibited high regeneration capability and produced 87 regenerants. Seven morphological traits were observed in order to assess phenotypic variability of flowering regenerants. Regenerants with higher values of fall and standard width and length show potential for further breeding of new varieties of Illyrian iris. Flowering and non-flowering regenerants had the same ploidy level as donor plants. Also, the nuclear DNA content (2C = 12.936 ± 0.038 pg) of this species was estimated for the first time using flow cytometry.

Keywords: auxins, explant browning, flow cytometry, Iris, leaf base explants, somaclonal variation, somatic embryogenesis

Sažetak

Ovo istraživanje provedeno je s ciljem procjene uspješnosti kalogeneze i regeneracije somatskom embriogenezom hrvatske subendemske vrste *Iris illyrica,* te odabira regenerabilnih donorskih biljaka/genotipova. Kao eksplantati korištene su baze listova. Kalogeneza i somatska embriogeneza inducirane su na MS mediju uz dodatak (1) 4,52 μM 2,4-diklorofenoksioctene kiseline (2,4-D) + 4,83 μM α-naftalenoctene kiseline (NAA) + 0,46 μM kinetina (Kin); (2) 4,52 μM 2,4-D + 4,6 μM Kin i (3) 13,4 μM NAA + 2,3 μM Kin. Prijenosom embriogenih kalusa na medij bez dodatka regulatora rasta omogućen je razvoj zrelih somatskih embrija. Na uspješnost kalogeneze značajno su utjecale donorske biljke. Među 15 testiranih donorskih biljaka, tri su pokazale veliku sposobnost regeneracije i proizvele 87 regeneranata. U svrhu procjene fenotipske varijabilnosti cvatućih regeneranata, mjereno je sedam morfoloških karakteristika. Regeneranti s utvrđenom većom širinom i dužinom donjeg i gornjeg lista perigona u odnosu na matične biljke predstavljaju potencijalno vrijedan genetski materijal za selekciju novih kultivara Ilirske perunike za hortikulturnu primjenu. Cvatući i necvatući regeneranti imali su jednak stupanj ploidnosti kao i donorske biljke. Također, po prvi puta je procijenjen sadržaj jezgrine DNA (2C = 12,936 ± 0,038 pg) ove vrste upotrebom protočne citometrije.

Ključne riječi: auksini, eksplantati baze lista, irisi, protočna citometrija, smeđenje eksplantata, somaklonska varijabilnost, somatska embriogeneza

Introduction

The genus *Iris* L. includes over 300 species, and is distributed mostly across the northern temperate zone (Schulze, 1988). The exceptional variety of flower and leaf shape, size and colour make this group an ideal resource for developing new floricultural crops (Ascough et al., 2009). Most of the European as well as the Croatian taxa of the genus *Iris* belong to the section Iris (=Pogoniris), composed of rhizomatous irises with bearded outer tepals (Mathew, 1981). In Croatia, 15 Iris taxa are present (Nikolić, 2018) and 12 of them are native. *Iris illyrica* Tomm. is a subendemic species distributed along the northern Adriatic, with the largest populations in the northern part of the Croatian coast (Mitić and Cigić, 2007). The main obstacle for possible commercialization of cultivated plants of *I. illyrica* is their slow rhizomes multiplication rate (10 plants/year; Jéhan et al., 1994). *In vitro* propagation has potential for rapid multiplication of true-to-type genotypes. According to Jevremović et al. (2006), in the genus *Iris*, 11 species have been micropropagated through somatic embryogenesis.

Since monocot cells differentiate early and quickly and therefore lose their morphogenetic potential (Krishnaraj and Vasil, 1995), explants source is one of the most important factors in the induction of morphogenetic response (Jevremović et al., 2006). In general, only parts of the plant that are close to meristematic tissue *in vivo* can respond to *in vitro* treatments (Bhaskaran and Smith, 1990; Jevremović et al., 2006). Leaf bases, used as explants source, are suitable parts for the induction of morphogenesis *in vitro* of several Iris species: *I. pallida* Lam. (Gozu et al., 1993), *I.*

nigricans Dinsm. (Shibli and Ajlouni, 2000), *I. pumila* L., *I. reichenbachii* Heuff. (Jevremović et al., 2006) and *I. adriatica* Trin. ex Mitić (Kereša et al., 2009).

It is well known that plant cell growth *in vitro* and regeneration of whole plants from plant cells is a vegetative process. It includes mitotic divisions only and should not cause any variability. Nevertheless, it has been demonstrated that during mitotic divisions, changes in somatic cells occasionally occur which can cause clonal progeny variability. This phenomenon is called somaclonal variability (Larkin and Scowcroft, 1981). Although it depends on many factors, synthetic auxins (2,4-D and NAA) are most frequently associated with genetic abnormalities such as polyploidy, changes in chromosome number or DNA sequence (Bouman and De Klerk, 2001; Bairu et al., 2011). Polyploidy and changes in chromosome number can be detected by flow cytometry or by chromosome counting. However, flow cytometric assay has some important advantages over chromosome counting. It is a convenient and rapid method, it does not require dividing cells, sample preparation requires only a few milligrams of tissue and it can detect mixoploidy (Doležel, 1997). The aim of this study was to detect the influence of donor plants and medium constitution on the induction of callogenesis and somatic embryogenesis, and to assess clonal fidelity of regenerants using phenotypic and flow cytometry analysis of subendemic species *I. illyrica* originated from Croatia, as potentially the largest source of native donors of Illyrian irises.

Materials and methods

Preparation of explants and callus induction

Thirty plants of *I. illyrica* were collected during the flowering period (early May 2007) on a natural habitat, on the rocky pasture between Senj and Karlobag (voucher specimen deposited in the live Iris collection of the Botanical Garden Zagreb, no. 2007/5). They were grown for 1 year in pots in the greenhouse under natural light regime. Fifteen randomly-chosen greenhouse-grown plants of Illyrian iris used in this experiment were designated as II1 – II15. Because of the allogamous nature of irises, they could be considered as different genotypes. Emerging sprouts (2–4 cm long) with a small piece of rhizome attached were taken from each donor plant. Sprouts were sterilised according to a procedure described by Kereša et al. (2009). Leaf bases were cut into small squares and cultured on callus induction medium (CIM) which consists of Murashige and Skoog (MS) (Murashige and Skoog, 1962) salts and vitamins, 0.25% Phytagel (Sigma, St Louis, MO, USA), 5% sucrose, 200 $mg*L^{-1}$ casein hydrolysate, 290 $mg*L^{-1}$ L-proline and one of the following plant growth regulators constitution (treatments): CIM1 = 4.52 μM dichlorophenoxyacetic acid (2,4-D) + 4.83 μM naphthaleneacetic acid (NAA) + 0.46 μM kinetin (Kin); CIM2 = 4.52 μM 2,4-D + 4.6 μM Kin; CIM3 = 13.4 μM NAA + 2.3 μM Kin. In all media, pH was adjusted to 5.8 before autoclaving at 121 °C and 100 kPa for 20 min. Each treatment x donor plant combination was performed in 60x15 mm Petri plates containing 5 explants. Explants were subcultivated on these media in the dark at 24 °C for 2 months in 2-week intervals, after which callogenesis was recorded.

Explants with induced calli were then subcultured on the B medium (as CIM with 3.5% sucrose and without L-proline) supplemented with 0.45 μM 2,4-D, 4.44 μM

benzyladenine (BA) and 0.49 μM indole-3-butyric acid (IBA) for further development of somatic embryos, as previously described by Shibli and Ajlouni (2000) and Kereša et al. (2009), and maintained under low light intensity (25 $\mu E*m^{-2}*s^{-1}$) and 16/8 h photoperiod for 5 weeks. Embryogenic calli were subcultured on hormone-free medium containing MS salts and vitamins, 0.25% Phytagel, 2% sucrose and 200 $mg*L^{-1}$ casein hydrolysate (C medium). Efficiency of somatic embryogenesis was calculated as percentage of calli producing clusters of torpedo-shaped somatic embryos/induced calli. Clusters of mature (torpedo-shaped) somatic embryos were divided into smaller pieces and placed onto the same medium, in Erlenmeyer flasks for germination. Developed plants were transferred into pots, acclimatized in a chamber under a 16/8 h light/dark photoperiod, light intensity 40 $\mu E*m^{-2}*s^{-1}$, and then moved to the greenhouse.

Phenotypic variability assessment

Morphological measurements were made 2 years after regeneration and acclimatisation, at the peak flowering of regenerants and donor plants. Seven traits were measured (Table 1) and phenotypic variability among regenerants was evaluated. Morphological traits were measured for regenerants and donor plants. For each characteristic, minimal, maximal and average values are presented and compared with values measured on the donor plant.

Table 1. Description of traits measured in donor plants and regenerants of Illyrian iris

Character	Abbreviation	Description/remarks
Fall length (cm)	FL	Measured from the base to the highest point of fall
Fall width (cm)	FW	Measured at its broadest place
Standard width (cm)	SW	Measured at its broadest place
Standard length (cm)	SL	Measured from the base to the highest point of standard
Width of longest leaf (cm)	WLL	Measured at its broadest place
Height of longest leaf (cm)	HLL	Measured from the base to the highest point of leaf
Stem height (cm)	SH	From soil-surface to base of fall

Ploidy stability analysis using flow cytometry

The DNA ploidy level and DNA content of both donor plants and regenerants of Illyrian iris were analysed by flow cytometry analysis according to the method reported in Doležel et al. (1992). Two years after regeneration and acclimatisation, both flowering and non-flowering regenerants as well as donor plants of *I. illyrica* were analysed. For each plant, a 1 cm^2 portion of the leaf blade of young leaf tissue was used for sample preparation. The total DNA amount in the leaf nuclei was assessed by flow cytometry using *Vicia faba* L. (2C = 26.9) as an internal standard. Two types of staining were used: PI (propidium iodide) and DAPI (4',6-diamidino-2-phenylindole).

When PI was used for DNA staining, nuclear suspensions were prepared according to Doležel et al. (1989). Plant tissue from both the sample and standard species (*V. faba*) was chopped using a sharp razor blade in a Petri dish containing 2–3 mL of buffer solution. The resulting homogenate was filtered through a 30-µm nylon filter to remove large debris. Nuclei were stained with 25 $\mu g*mL^{-1}$ propidium iodide (PI; Fluka, Buchs, Switzerland), and 50 $\mu g*mL^{-1}$ RNase (Sigma, St Louis, MO, USA) was added to the nuclear suspension to prevent staining of double-stranded RNA.

For staining with DAPI, nuclei of the sample and standard species (*V. faba*) were released in 0.4 ml of nuclei extraction buffer (0.1 M Tris, 2 mM $MgCl_2$, 0.1 M NaCl, 0.05% (v/v) Triton X-100, pH 7.0). The suspension was filtered through a 30-µm nylon sieve. A 3–4 fold volume of staining buffer containing 0.1 $mg*ml^{-1}$ of DAPI (4',6-diamidino-2-phenylindole) in 0.4 M Na_2HPO_4 x 12 H_2O was added.

Samples were analysed with a Partec PAS flow cytometer operating at 488 nm for PI staining and 372 nm for DAPI staining. For each sample 5,000–7,000 nuclei were analyzed.

To estimate the ploidy level, the position of G_0/G_1 peak of the sample on a histogram was compared with the internal reference plant with known ploidy. The size of the nuclear genome of *I. illyrica* was estimated according to the following formula: *I. illyrica* 2C nuclear DNA content [pg] = ((*I.illyrica* G_0/G_1 peak mean / *Vicia faba* G_0/G_1 peak mean) x 26.9).

Data analysis

The experiment was set up with 15 donor plants and 3 callus induction media. Explants of each genotype were set up in 3 replicates of 5 explants on each growing medium. A total of 15 explants per treatment and donor plant were used. Logistic regression was used to model the effects of genotype and media on the number of produced calli (on CIM1–CIM3 media), number of produced somatic embryos (on C medium) and number of plantlets regenerated from each calli with somatic embryos (before acclimatization). Logistic regression is used to explain the relationship between one dependent binary variable and one or more nominal, ordinal, interval or ratio-level independent variables. Success of callogenesis and embryogenesis was coded as binary variable for each explant (1 when callus culture and SE were induced and 0 for lack of callogenesis and somatic embryos induction). Statistical analysis was carried out using PROC LOGISTIC in SAS software Version 9.4 (SAS Institute Inc., Cary, NC, 2004).

The results of flow cytometry analysis were acquired using the FLOMAX software (Partec, Münster, Germany). Seven thousand nuclei per sample were measured and at least 4 repetitions of separate nuclear isolation were made for each species. Flomax1 software (Partec, Münster, Germany) was used to calculate the positions of G_0/G_1 peaks of standard species (*V. faba*) and Illyrian iris.

Results and discussion

Callus induction and somatic embryogenesis

Three callus induction treatments were used in this experiment (CIM1, CIM2 and CIM3). Regardless of which medium was used, browning of explants in the establishment phase of the experiment caused great loss of explants. Specifically, 46% of all explants (data not shown) turned brown in colour (Figure 1a) and therefore were eliminated from the experiment within the first 2 subcultivations. A possible explanation is the release of undesirable phenolic compounds when explants were first placed onto a growth medium. This is in agreement with findings of Chuanjun et al. (2015), who have reported that Phalaenopsis hybrid 'Red Sky' readily undergoes browning in tissue culture which caused explant death and failure of regeneration. Since problems with explant browning were not yet reported in Iris plant tissue culture, methods for avoiding explant browning were not in the protocol of this experiment.

However, responsive explants formed calli 4–6 weeks after culture establishment (Figure 1b). Calli first started to appear near vascular bundles (Figure 1c). This is typical for monocots, where first cell divisions start near vascular tissue probably because of a high level of endogenous plant regulators (Vasil, 1987). Results of callus induction efficiency from leaf base explants of *I. illyrica* indicate that donor plant (genotype) significantly influenced *in vitro* response for callogenesis (Table 2). Average percentage of callus induction across all treatments ranged from 2.2–31.1% for different donor plants (Table 3). In genus *Iris*, the importance of genotype on *in vitro* culture ability was already reported by Laublin et al. (1991) and Kereša et al. (2009). According to Laublin and Cappadocia (1992), all genotypes of *Iris pseudacorus* responded positively at the callus induction phase and regenerated on all media tested, while all genotypes of *Iris setosa* had low callus response. Moreover, preselection of donor plants with high regenerative capacity *in vitro* was also found to be a prerequisite for efficient regeneration via somatic embryogenesis in *Trifolium pratense* L. (Radionenko et al., 1994) and *Medicago sativa* L. cultivars (Zare et al., 2009). There were substantial differences among treatments (CIM1–3) in efficiency of callus induction (Tables 2 and 4). Treatment CIM1 is identical to the hormone constitution used in studies of Jéhan et al. (1994), Shibli and Ajlouni (2000), Al-Gabbiesh et al. (2006) and Kereša et al. (2009) for callus induction of different Iris species, and mostly resulted in good callogenesis either from leaf bases or flower explants. CIM2 medium was used by Jéhan et al. (1994) and Laublin and Cappadocia (1992), who have observed the best callogenesis of ovary explants on this medium. Jéhan et al. (1994) have reported that these 2 plant growth regulator combinations (as in treatments CIM1 and CIM2) resulted in undifferentiated callus on 50% of the cultured flower explants. Plant growth regulator constitution as in CIM3 treatment was previously used by Kamo et al. (1990) in *Gladiolus* species.

After transfer of explants producing callus onto B medium, asynchronous development of somatic embryos was observed, which is in agreement with findings of Radojević et al. (1987), Radojević and Subotić (1992) and Fidalgo et al. (2005). Donor plants that were prone to somatic embryo formation formed embryo-like structures at calli surface in a relatively short period. In most cases, visible embryo-like structures and globular embryos (Figure 1b, d, e) appeared simultaneously with calli formation. Hormone-free C medium induced formation of torpedo-shaped somatic embryos on 19.67% of induced calli of Illyrian iris (Figure 1f). Calli originating from CIM1 and CIM2 that contained 2,4-dichlorophenoxyacetic acid (2,4-D) had better embryogenesis response (Table 4). This result is consistent with Jevremović et al. (2006), who reported that auxin 2,4-D in combination with a cytokinin (mainly kinetin) was the most potent inducer of somatic embryogenesis for many iris species. Once clusters of mature SE developed, they continued to grow on hormone-free C medium and multiple new embryos arose from existing ones. Despite the small number of calli with clusters of mature SE observed, the 3 most responsive donor plants regenerated 87 plants (Table 5, Figure 1h). After acclimatisation, regenerated plants were transferred to the greenhouse and high establishment (96%) was achieved.

Table 2. Analysis of effects for callus induction using the logistic regression model

		Callogenesis	
Effect	DF	χ^2	Pr>χ^2
Treatment	2	4.62	0.099
Donor plant	14	30.47	0.006

DF - degrees of freedom; χ^2 – chi-square statistic; Pr>χ^2 – probability of acceptance of null hypothesis

Beside the explant browning that impaired callus formation during the establishment phase, abberant embryo formation (Figure 1g) is another factor that decreased regeneration success. Some donor plants were prone to embriogenic calli formation but somatic embryos proliferated, swelled irregularly and no plantlets with well-developed shoots and roots were obtained from them (Tables 3 and 5). The same problem in other Iris species was described by Gozu et al. (1993) and Kim et al. (2009). Fasciated/swelled somatic embyos, which lack an apical meristem as a vital factor in conversion to plantlets, could result from diaxial fusion of two embryos, as found by Suhasini et al. (1996).

Table 3. Efficiency of callogenesis as associated with donor plant

Donor plant	Callogenesis (%)
II 14	31.1
II 2	15.6
II 9	15.6
II 5	11.1
II 1	8.9
II 6	8.9
II 10	8.9
II 15	8.9
II 12	6.7
II 4	4.4
II 8	4.4
II 3	2.2
II 7	2.2
II 11	2.2
II 13	2.2
Average (%)	8.9

Table 4. Efficiency of somatic embryogenesis as associated with CIM treatments

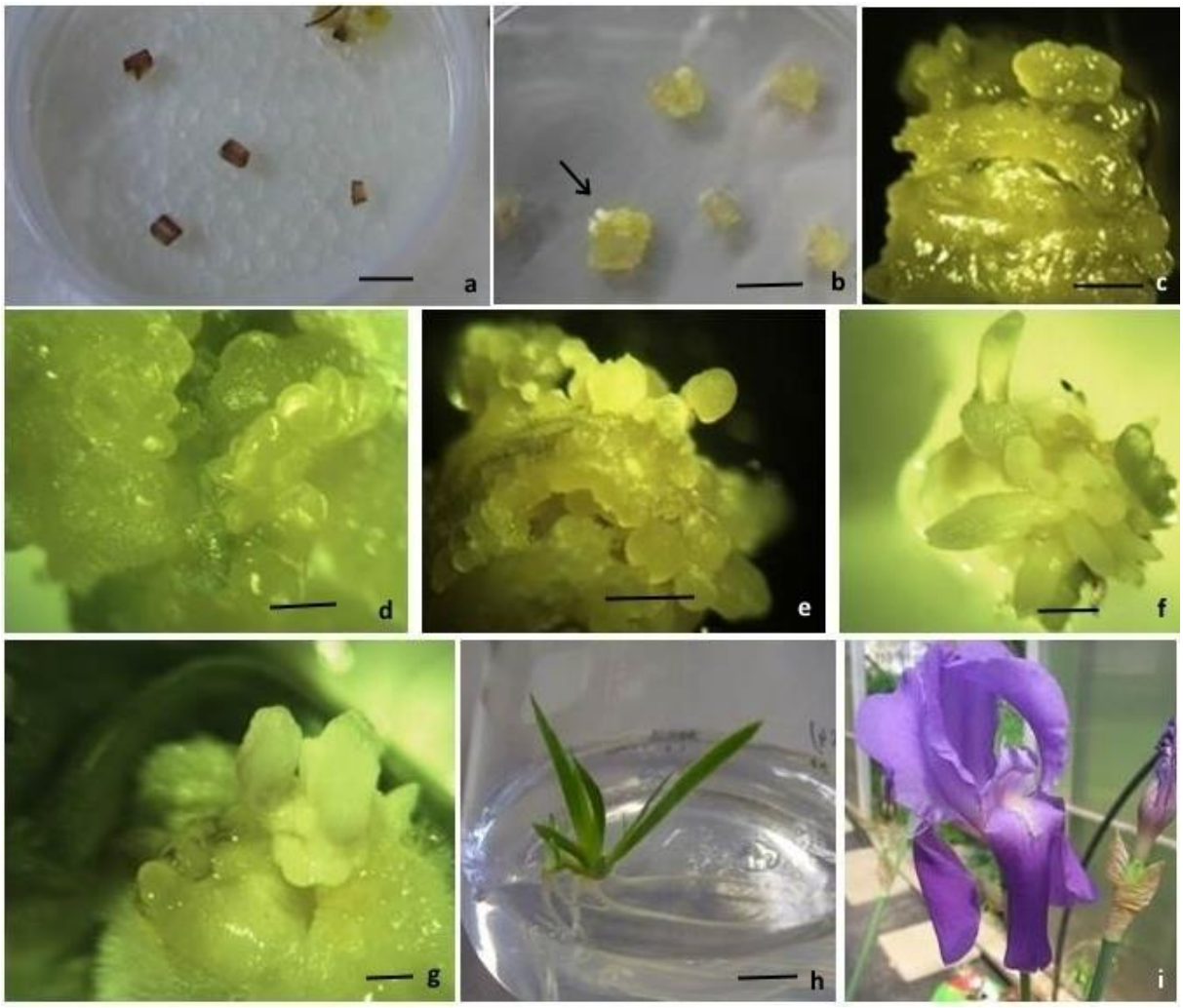
Treatment	Calli-forming somatic embryo clusters (%)
CIM1	21.74
CIM2	25
CIM3	13.64

Table 5. Number of regenerated plants as associated with CIM treatments and donor plant

Donor plant	Treatment		
	CIM1	CIM2	CIM3
II 1	42	0	0
II 15	21	0	0
II 5	21	3	0
II 6	1	0	0

Phenotypic variability assessment

Among 85 regenerated and acclimatised plants, 7 did not flower 2 years after regeneration. Altogether, 81 plants were measured: 78 regenerants and 3 mother plants.This could be attributed to the rejuvenation which appears as an epigenetic change of plants propagated *in vitro* (Smulders and de Klerk, 2011). Juvenile plants are unable to flower, even when the conditions are optimal. After some time, depending upon the species, plants become adult and capable of flowering. On flowering regenerants, 7 morphological traits (Table 1) were observed in order to assess phenotypic variability.



a) explant browning in the establishment phase (bar = 1 cm), b) callus induction (arrow shows formation of somatic embryo, bar = 1 cm), c) calli first formed on vascular tissue (bar = 0.1 cm), d) embryo-like structures (bar = 0.1 cm), e) globular somatic embryos (bar = 0.5 cm), f) cluster of mature somatic embryos and asynchronous formation of somatic embryos (bar = 0.2 cm), g) abnormal/swelled somatic embyos (bar = 0.1 cm), h) plantlet of Illyrian iris (bar = 1 cm), i) Illyrian iris regenerant in the bloom.

Figure 1. Plant regeneration from *Iris illyrica* leaf base explants

For most characteristics, average values of morphological traits were higher in regenerated plants than in the donor plant (Table 6). Higher vigour of regenerants compared with donor plant could be attributed to the influence of plant growth regulators used during *in vitro* growth. However, regenerants with outstandingly different values of some morphological traits could have the genetic changes found by Dey et al. (2015) in analysing superior regenerants of *Cymbopogon winterianus* Jowitt. Superior regenerants, which have higher values of fall and standard width and length, were detected and could be used in further breeding.

DOI: /10.5513/JCEA01/20.1.2126

Table 6. Measurements of morphological traits for each donor plant and its regenerants

Trait* (cm)	Donor plant 1				Donor plant 2				Donor plant 3			
	Regenerants			Donor plant	Regenerants			Donor plant	Regenerants			Donor plant
	Min	Max	Average		Min	Max	Average		Min	Max	Average	
FL	5.3	8.7	7.4	6.7	6.1	8.9	7.5	7.6	5.7	8.5	7	6.5
FW	2.6	6.9	3.7	3.3	3.2	4.5	3.9	3.7	3.1	4.8	3.7	3.3
SW	5.1	8.6	7.1	6.5	6	9.5	7.2	7.3	3.6	8.4	6.4	6.4
SL	2.5	4.5	3.5	3.1	2.5	7.1	3.8	3.7	2.8	6.5	3.8	3.3
WLL	12.7	35.7	22.7	12.3	11	29.6	20.6	20.4	15.5	34.7	26.1	16.5
HLL	1	2.5	1.7	1.1	0.8	2.2	1.7	1.8	0.7	2.5	1.8	0.9
SH	7.5	34.5	20.8	27.3	9.6	44.1	24.3	29	8.3	34.3	19.9	12.5

*Abbreviations as in Table 1; min - minimum, max – maximum.

Ploidy stability

The use of flow cytometry (FCM) for nuclear DNA content analysis is an excellent method for assessing clonal fidelity (Loureiro et al., 2007), while correlation between nuclear DNA content and ploidy level makes flow cytometry suitable for estimation of DNA ploidy level and mixoploidy and aneuploidy detection (Doležel, 1997). Moreover, FCM is more convenient, rapid, it does not require dividing cells and sample preparation requires only a few milligrams of tissue as compared to chromosome counting (Doležel, 1997).

In this experiment, 2 protocols were used: one based on 4',6'-diamidino-2-phenylindole (DAPI) and the other on propidium iodide (PI) staining. A fluorochrome used to estimate nuclear DNA content must bind selectively and stoichiometrically to DNA. Both methods are equally reliable, while both fluorochromes (PI and DAPI) are known to specifically bind to DNA. However, DAPI binds to AT base pairs and therefore lower values for absolute genome size analysis are found (Bohanec, 2003).

The ploidy level of donor plants as well as flowering and nonflowering regenerants was characterised by analysing small pieces of leaves from donor plants and regenerated plantlets using flow cytometry analysis. Since estimation of DNA content in absolute units requires internal standardization (Doležel et al., 2007), in this experiment *Vicia faba* was used. Genome size (2C) of *V. faba* is 26.9 pg DNA. It can be seen on representative flow-cytometry histograms as the peak on channel 430 (Figure 2). Flow cytometry analysis showed that ploidy levels were stable in all regenerated plants. Namely, all analysed plants had a single peak at channel 200, which demonstrated the presence of homogenous 2C nuclei, without any detectable aneuploidy (Figure 2).

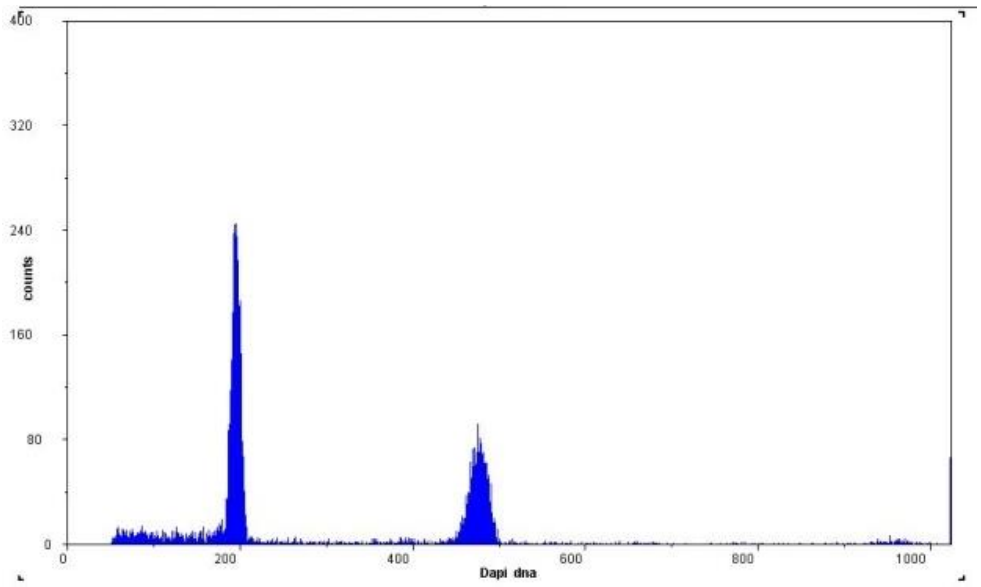


Figure 2. Representative flow-cytometry histogram of DAPI stained nuclei isolated from *Iris illyrica* leaf tissue showing G1/G0 peak (channel 200) of the diploid samples and G1/G0 peak (channel 430) of the diploid standard *Vicia faba*

The determination of total DNA content of *I. illyrica* Tomm. donor plants from PI and DAPI stained cells was 12.936 ± 0.038 and 11.151 ± 0.027 pg/2C, respectively. Non-flowering regenerants could not be declared as somaclones because causes for not-flowering could be physiological. Moreover, flow cytometry analysis could not distinguish flowering and non-flowering regenerants. 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). Several growth regulators, such as 2,4-dichlorophenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA), BAP (6-benzylaminopurine) and synthetic phenylurea derivatives (4-CPPU, PBU and 2,3-MDPU) have been most frequently considered to be responsible for genetic variability (Krishna et al., 2016). Doses of the synthetic auxins 2,4-D and NAA used in this experiment did not have an effect on alterations in ploidy levels in regenerated plantlets as revealed by flow cytometry analysis.

Conclusions

In conclusion, results of callogenesis induction efficiency from leaf base explants of *I. illyrica* indicate that genetic background is an important factor determining *in vitro* response and therefore, the preselection of donor plants with high regenerative capacity *in vitro* is a prerequisite for efficient regeneration via somatic embryogenesis. Among 15 donor plants tested, 3 responsive donor plants were identified, which regenerated 87 plants. Three plant growth regulators constitutions tested did not significantly influence induction of callogenesis and somatic embryogenesis. However, the vast majority of plants regenerated from calli induced on CIM1 medium supplemented with 2,4-D and NAA. Measurements of 7 morphological traits revealed regenerants with higher values of fall and standard width and length that could be used in further breeding. *In vitro* conditions and plant growth regulators used for callogenesis and SE induction in this study did not cause changes in ploidy level as revealed by flow cytometry analysis. In order to reveal whether regenerants with superior morphological traits are genetically different from the mother plant, clonal fidelity assessment at the molecular level should be performed. For this purpose, Amplified Fragment Length Polymorphisms (AFLP) could be used to assess DNA mutations and Methylation Sensitive Amplified Polymorphisms (MSAP) to evaluate the influence of epigenetic changes.

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