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IN VITRO REGENERATION OF THE CROATIAN ENDEMIC SPECIES *IRIS ADRIATICA* TRINAJSTIĆ EX MITIĆ

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Plant regeneration via somatic embryogenesis and organogenesis was achieved in leaf base and ovary culture of the Croatian endemic *Iris adriatica* Trinajstić ex Mitić. Callus induction from leaf base explants occurred in the dark on three media with MS mineral solution containing 4.52 μM dichlorophenoxyacetic acid (2,4-D), 4.83 μM naphthaleneacetic acid (NAA), 0.46 μM kinetin (Kin), 5% sucrose and 200 mg L^{-1} casein hydrolysate. The media differed only in vitamin and/or proline content. Calli from ovary culture were achieved on MS medium containing 45.25 μM 2,4-D. The mean percentage of callus induction from leaf base explants was 18.9%, with no significant differences between media, and 27.3% from ovary sections. All embryogenic calli were formed on MS media containing 0.45 μM 2,4-D, 4.44 μM benzyladenine (BA) and 0.49 μM indole-3-butyric acid (IBA) under low light intensity (25 μE m-2s-1). Transfer of embryogenic calli to hormone-free medium enabled the development of mature somatic embryos on the surface of 6.0% of induced calli produced from leaf base explants and 4.0% of those from ovary sections. Genotype had the main effect on plant regeneration efficiency in *Iris adriatica*.

Key words: *Iris adriatica*, leaf base culture, ovary culture, somatic embryogenesis.

INTRODUCTION

The genus *Iris* includes over 300 species, spread mostly across the northern temperate zone (Schulze, 1988). Most of its European as well as Croatian taxa belong to sect. *Iris* (=Pogoniris), comprising rhizomatous irises with bearded outer tepals (Mathew, 1981).

Fifteen *Iris* taxa are distinguished in Croatia (Nikolić, 2008a), three of which grow only under cultivation, and the rest are native. Five native species are endemic: *I. adriatica* Trinajstić ex Mitić, *I. croatica* I. Horvat et M. Horvat, *I. illyrica* Tomm., *I. pseudopallida* Trinajstić and *I.* × *rotschildii* Degen. Two of them are strictly endemic to Croatia: *I. adriatica* and *I.* × *rotschildii.* The distribution area of *I. adriatica* is limited to central Dalmatia – the vicinity of the towns of Zadar, Šibenik, Split, Drniš and Unešić – as well as the islands of Ciovo, Brac and Kornati. Populations are generally small, and some have disappeared or declined. For this reason *I. adriatica* is listed in category NT (near threatened) in the *Flora Croatica Red Book* (Nikolić, 2008b). The taxonomic position of *I. adriatica* was clarified only recently (Mitić, 2002): it is a diploid (2n=16) (Trinajstić et al., 1980), rhizomatous dwarf plant of the *I. pumila* complex.

Due to its flower color (Fig. 1), *I. adriatica* has potential as an ornamental, especially when grown in pots (Vršek et al., 2004); pot culture also improves other dwarf irises, including taxa of the *I. pumila* complex (Schulze, 1988). In view of its decorativeness and its rarity in nature, an efficient propagation method for *I. adriatica* should be established. As is common in irises, propagation by splitting rhizomes is slow (Hussey, 1975; Jéhan et al., 1994). Micropropagation in vitro might be the method of choice. Various iris species have been

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Fig. 1. *Iris adriatica* Trinajstić ex Mitić – flower colors: yellow **(a)**, violet **(b)** and red **(c)** perigonium (photo M. Mitić)

propagated through organogenesis or somatic embryogenesis, using explants from the leaf base (Gozu et al., 1993; Shibli and Ajlouni, 2000; Jevremović and Radojević, 2006), mature zygotic embryos (Radojević and Subotić, 1992; Boltenkov et al., 2007), ovary sections (Laublin and Cappadocia, 1992) and root sections (Laublin et al., 1991).

Propagation of *I. adriatica* via in vitro plant regeneration has not been reported. Here we undertook to develop a protocol for regeneration from leaf base explants and ovary sections as a method for efficient propagation in vitro for this species.

MATERIALS AND METHODS

PREPARATION OF LEAF BASE EXPLANTS AND CALLUS INDUCTION

In 2001, thirty *Iris adriatica* plants were collected at flowering (early spring) from Bilice near the town of Šibenik and grown for six years in pots in the greenhouse under a natural light regime. Plants were shown to have different genotypes, as their perigonium color may vary from yellow to yellow suffused with brown, yellow suffused with violet, violet, or red. During February 2007, emerging sprouts (2–4 cm long) with a small piece of rhizome attached were collected from 23 randomly chosen greenhouse-grown plants without destroying them. Sprouts were thoroughly washed under tap water and immersed in 70% ethanol for 2 min, and then in 5% sodium hypochlorite with 0.1% Tween 20 for 10 min. After two rinses with sterile water, one or two outer leaves were removed under aseptic conditions. Sprouts were shortened to 2 cm and sterilized again by rinsing in 70% ethanol for 1 min, and in 1.5% sodium hypochlorite with 0.1% Tween 20 for 25 min. Finally the sprouts were rinsed three times in sterile water. Leaf bases (white parts of leaves proximate to the rhizome) were cut into 6–9 mm^2 segments and cultured in the dark at 24°C on three callus induction media. Medium A_1 contained MS salts and vitamins (Murashige and Skoog, 1962) 0.25% Phytagel (Sigma), 5% sucrose, 200 mg L^{-1} casein hydrolysate, 4.52 μM dichlorophenoxyacetic acid (2,4-D), 4.83 μM naphthaleneacetic acid (NAA) and 0.46 μM kinetin (Kin). Media A_2 and A_3 had the same ingredients as A_1 except that A_2 additionally contained 290 mg L^{-1} L-proline, and A_3 contained Gamborg's B_5 vitamins instead of MS vitamins (Gamborg et al., 1968). The pH of all media was adjusted to 6.0 prior to autoclaving. Six explants were placed in two 60×15 mm Petri dishes for each medium × donor plant combination (two replicates; 828 explants in total).

OVARY PREPARATION AND CALLUS INDUCTION

Immature flowers (3–5 cm long) were removed from seven different plants and sterilized in 70% ethanol for 1 min, then in 3% sodium hypochlorite with 0.1% Tween 20 for 12 min and rinsed 3 times in sterile water. Each ovary was sliced into three or four explants under a stereo dissecting microscope, placed on MS induction medium containing 3.5% sucrose, 200 mg L^{-1} casein hydrolysate and 45.25 μM 2,4-D (A_4 medium), and cultured in the dark at 24°C. Six to 15 ovary explants were taken from each plant, depending on the available floral axes.

After four weeks on induction media, explants with induced calli were subcultured on B medium containing MS salts and vitamins supplemented with 0.25% Phytagel, 3.5% sucrose, 200 mg L^{-1} casein hydrolysate, 0.45 μM 2,4-D, 4.44 μM benzyladenine (BA) and 0.49 μM indole-3-butyric acid (IBA). Cultures were maintained on B medium under low light intensity (25 μE $m^{-2}s^{-1}$) under a 16 h photoperiod. The percentage of embryogenic calli was determined after five weeks. Embryogenic calli were then subcultured on hormone-free C_1 medium containing MS salts and vitamins, 0.25% Phytagel, 2% sucrose and 200 mg L^{-1} casein hydrolysate. Clusters of mature somatic embryos were divided into smaller pieces and placed on the same medium (C_1).

Three months after the beginning of the experiment, germinated somatic embryos were transferred to Erlenmayer flasks on hormone-free C_2 medium containing MS salts and vitamins, 0.25% Phytagel and 3.5% sucrose. Somatic embryo germination and transfer to C_2 medium took place over about two months. Developed plants were transferred to pots, hardened and grown in growth chamber under a 16 h photoperiod at 40 μE $m^{-2}s^{-1}$ light intensity.

STATISTICAL ANALYSIS

The efficiency of callus induction and embryogenic callus production was expressed as the ratio (%) of

responsive to total explants; the level of embryogenic expression was defined as the ratio (%) of calli yielding mature somatic embryos to the total number of induced calli. ANOVA and Duncan's multiple range test were used for statistical analyses.

RESULTS

CALLUS INDUCTION

After three to four weeks in culture, sections cut from the base of immature leaves formed yellow/white calli along their edges (Fig. 2a). After enlargement and swelling, ovary sections also produced calli, but spread out over their surface.

Callus induction from leaf base explants was unaffected by medium composition (Tabs. 1a,b), but varied significantly between explants collected from different plants ($p < 0.05$) (Tab. 1a). The mean percentage of callus induction across plants was 18.9%, with values ranging from 4.2% to 51.1% (Tab. 2).

Four out of seven plants produced friable calli from ovary sections on A_4 medium. The mean percentage of callus induction from ovary sections across plants was 54.5%, with values ranging from 26.7% to 84.6%.

EMBRYOGENIC CALLUS

Calli induced on all media were mostly undifferentiated. Subculture (without separation from explants) onto B medium resulted in an increase of callus mass and the formation of globular embryo structures (Fig. 2b,c). The development of embryogenic calli on leaf base explants was not significantly influenced by the induction medium though differentiation did seem more distinct in calli induced on the proline-enriched A_2 medium (Tab. 1a,b). Instead, donor plant was found to be the critical factor for embryogenic callus formation ($p < 0.01$) (Tab. 1a). The mean percentage of embryogenic callus formation across plants was 7.0%, with values ranging from 0.0% to 38.0% for different plants (Tab. 2). Seven out of 23 plants used for leaf base explant preparation did not produce embryogenic calli.

SOMATIC EMBRYO DEVELOPMENT

Somatic embryos started to develop on B medium (Fig. 2c) and continued their development after transfer to C_1 medium. About 6.0% of the calli induced from leaf base explants developed mature somatic embryos, that is, allowed complete embryogenic expression (Fig. 2d). Germination of somatic embryos (Fig. 2e) was improved by dividing larger clusters of mature embryos and placing them in contact with the medium. Some somatic embryos developed roots but

TABLE 1. ANOVA for percentage of callus induction and embryogenic callus formation from leaf base explants, and average values from three different culture media

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Source of variation	Callus induction	Embryogenic callus formation
Donor plant	*	**
Medium	ns	ns

ⓑ

Medium	Callus induction (%)	Embryogenic callus (%)
A_1	18.6	6.7
A_2	19.9	9.1
A_3	18.9	5.4

ns – not significant; * – significant at $p<0.05$; ** – significant at $p<0.01$

TABLE 2. Effect of donor plant on callus induction and embryogenic callus formation from leaf base explants

Donor plant	Callus induction (%)	Embryogenic callus (%)
1	51.1 a	38.0 a
2	18.1 bc	0.0 c
3	30.8 abc	16.6 b
4	14.7 bc	12.3 b
5	29.2 abc	16.1 b
6	16.9 bc	9.2 b
7	11.9 bc	9.5 b
8	11.8 bc	0.0 c
9	17.8 bc	13.3 b
10	21.6 bc	6.7 b
11	13.9 bc	6.1 b
12	38.3 abc	0.0 c
13	16.9 bc	0.0 c
14	12.0 bc	2.6 b
15	11.8 bc	9.2 b
16	4.2 c	4.2 b
17	10.3 c	5.1 b
18	13.8 bc	2.2 b
19	16.7 bc	7.0 b
20	29.2 abc	0.0 c
21	12.6 bc	0.0 c
22	21.9 bc	2.6 b
23	9.1 c	0.0 c
Mean	18.9	7.0

Values with the same letter in the same column do not differ significantly at $p<0.05$.

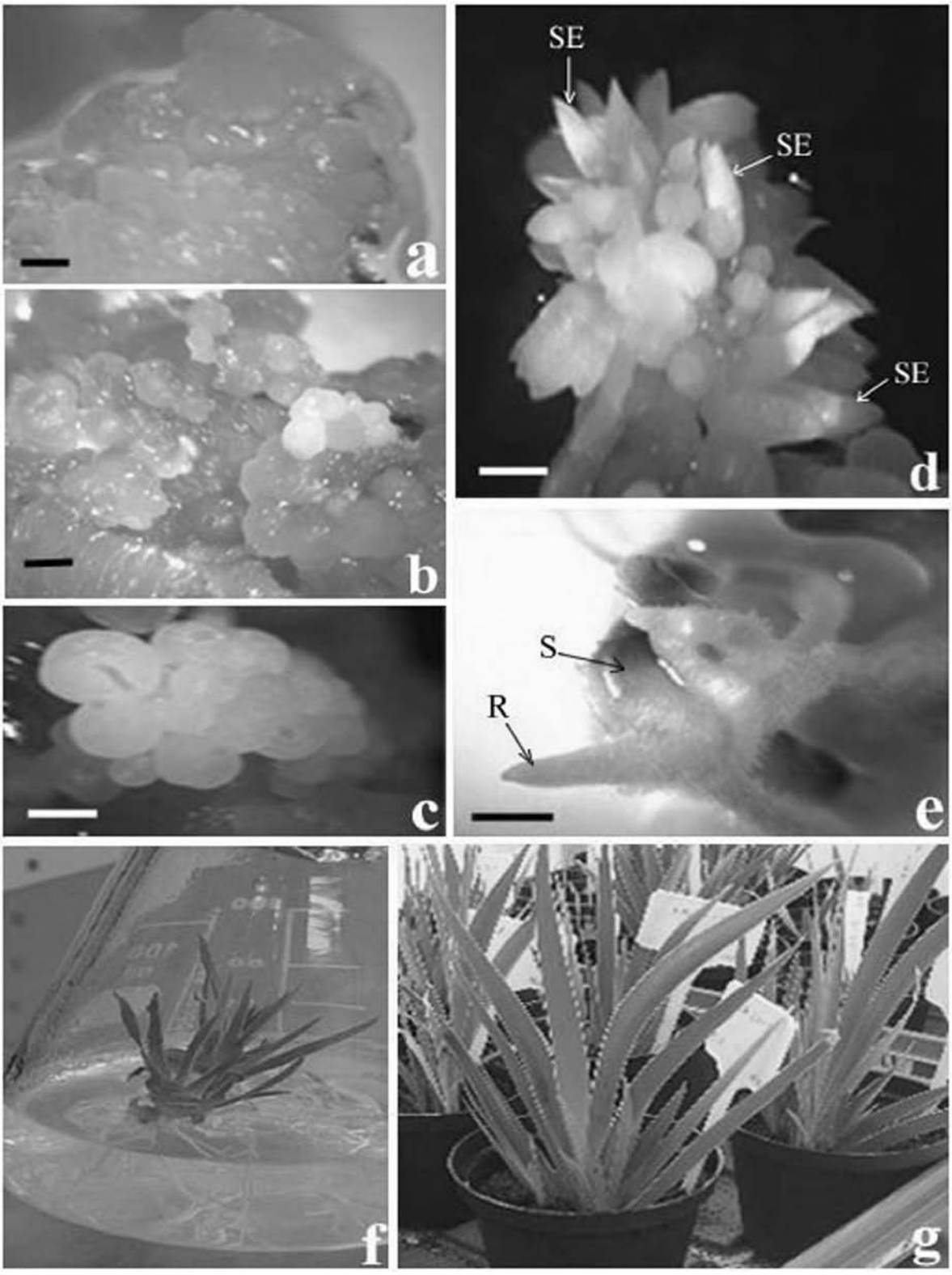

Fig. 2. Plant regeneration from *Iris adriatica* leaf base explants. **(a)** Callus induced after 4 weeks of culture, **(b)** Embryogenic callus after 8 weeks of culture, **(c)** Embryo-like structures, **(d)** Cluster of mature somatic embryos. SE – mature somatic embryos, **(e)** Germination of somatic embryos. S – shoot; R – root, **(f, g)** Plants regenerated in vitro. Bar = 1mm in (a–d) and 5 mm in (e).

then miscarried during shoot development. Only two out of 24 calli induced from ovary sections were embryogenic; one of them produced mature somatic embryos developing in three plants. Sporadically, shoots developed directly from calli by organogenesis. They were successfully rooted in C_2 medium.

Somatic embryos transferred to Erlenmayer flasks containing C_2 medium rooted abundantly and frequently produced new microshoots (Fig. 2f). Altogether, 330 plantlets were produced through somatic embryogenesis. Plants were potted in peat and successfully hardened in a growth chamber (Fig. 2g).

DISCUSSION

Iris adriatica is a dwarf iris with attractive yellow, violet or red flowers. It has potential as an ornamental plant, but its decline in nature prevents further extensive collecting, and natural vegetative propagation by rhizomes is slow. Generative propagation, although possible, would be difficult for mass propagation. For species like this, micropropagation is essentially the only efficient way to produce plants for research and industry. Irises are also a potential subject for genetic transformation aimed at slowing down their rapid flower senescence, and this would require regeneration of whole plants from genetically transformed cells.

Although a comparative study evaluating the regeneration capacity of several monocotyledonous ornamental species ranked Iridaceae last (Hussey, 1975), several authors have reported sufficient or efficient regeneration in various *Iris* species, using embryogenesis or organogenesis after callus induction on solid agar medium (Radojević and Subotić, 1992; Gozu et al., 1993; Shibli and Ajlouni, 2000; Jevremović and Radojević, 2006) or in suspension culture (Shimizu et al., 1997; Wang et al., 1999; Shibli and Ajlouni, 2000; Jevremović and Radojević, 2002).

All callus induction media (for leaf base explants) used in this research had the same hormone constituents – 4.52 μM 2,4-D, 4.83 μM NAA and 0.46 μM Kin; that is almost identical to the hormone combination used in the work of Shibli and Ajlouni (2000) and Al-Gabbiesh et al. (2006), differing only in the presence of proline or in vitamin content. Jéhan et al. (1994) showed that proline promotes embryogenic callus formation. Our medium A_3 contained Gamborg B_5 instead of MS vitamins. We chose Gamborg B_5 vitamins because the thiamine concentration is 100 times higher than in MS. It is known that thiamine is essential for plant cell growth (Gamborg et al., 1968), so we thought it might benefit callus induction. In spite of all these differences, the media tested did not significantly influence callus and embryo production.

Irises are allogamous species and can be propagated generatively as well as vegetatively. Each plant collected from nature can potentially possess a different genotype, especially when taken from wider areas (as was the case here). According to perigonium color, the plants we used belonged to at least several different genotypes. Tissue culture response is known to be highly genotype-dependent (Sears and Deckard, 1982; Kereša et al., 2004), so we expected to find differences in callogenesis and embryogenesis between our different donor plants, and indeed we found significant differences in callus induction ($p < 0.05$) and embryogenic callus formation ($p < 0.01$) between plants. All the plants formed the calli on leaf base explants, some with very low efficiency, but only some of them progressed further and developed embryogenic calli on B medium. Genotype-dependent responses have also been found in ovary culture of some apogon garden irises (*I. pseudacorus* L., *I. setosa* Pall. and *I. versicolor* L.) (Laublin and Cappadocia, 1992) and leaf base and rhizome explant culture of *I. pallida* Lam. and *I. germanica* L. (Jéhan et al., 1994).

Transfer of embryogenic calli to C1 medium enabled development of somatic embryos to maturity, that is, embryogenic expression. The level of successful embryogenic expression (6.0%) we obtained in callus culture from leaf base explants obtained is higher than reported in *I. pallida* and *I. germanica* (2.0–4.0%) (Jéhan et al., 1994), and lower than from root culture of *I. versicolor* and *I. pseudacorus* (14.3–85.0% depending on medium) (Laublin et al., 1991).

Some of our somatic embryos failed to develop shoots. The same was reported in plant regeneration of *I. setosa* (Radojević and Subotić, 1992): prolonged culture caused asynchronous development of shoot and root meristem, so that the embryos developing only several long roots.

As we did not study the plants anatomically, the nature of the shoot-like structures that appeared sporadically on calli remains undetermined. However, as some shoots appeared very early, some even during callus induction when embryogenic calli were still absent, conceivably they originated by true organogenesis, and not by early germination of somatic embryos as found in *I. pumila* tissue culture (Jevremović and Radojević, 2006). Other authors have reported concomitant embryogenesis and organogenesis in iris tissue culture (Shimizu et al., 1997; Shibli and Ajlouni, 2000).

Although we used a relatively small number of explants produced from ovary, once again the donor plant has been shown to be very important to the tissue culture response. Some plants showed very high efficiency of callus induction (84.6%), even higher than that from leaf explants; others did not respond at all. Embryogenic callus formation was low, how-

ever, and ended with only three regenerated plants. This outcome probably can be improved through optimization of the plant growth regulator concentration in the regeneration medium.

This is the first report on regeneration via somatic embryogenesis and organogenesis of *Iris adriatica* Trinajstić ex Mitić, a Croatian endemic dwarf iris. Callus induction and development of somatic embryos to maturity were achieved from leaf base explants and from ovary sections. Callus induction and embryogenic callus production differed significantly between the explants from different donor plants. On the other hand, all the different media formulas appeared equally effective. With leaf base explants as starting material, the level of somatic embryogenesis was high enough for fast, large-scale production of plants suitable for ornamental cultivation. This method can also be applied for regeneration of genetically transformed plants.

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