PLANT REGENERATION FROM SUSPENSION CULTURES OF *IRIS PUMILA* L.

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Abstract

A protocol for efficient plant regeneration of *Iris pumila* L. was developed *via* somatic embryogenesis and/or organogenesis from suspension cultures. Induction of embryogenic calli was achieved by leaf-base culture of *in vitro* grown plants on solid Murashige and Skoog (MS) medium supplemented with 3 % sucrose and (in mgL^{-1}): inositol 100, pantothenic acid 10, nicotinic acid 5, vitamin B_1 2, vitamin B_6 1, casein hydrolysate 250, proline 250, 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (1.0 mgL^{-1}, each). Cell suspensions were established and maintained in MS liquid medium with the same content of 2,4-D and kinetin as used for induction and proliferation of embryogenic calli. After three subcultures stable suspension cultures were successfully established and maintained by subculturing every 3 weeks. The suspension cultures were initially composed of single cells, bi-, three and multicellular proembryos and cell aggregates. In prolonged suspension cultures (6-8 weeks) three types of embryogenic calli were observed: yellow, compact (Type I); yellow-green, friable (Type II) and white, friable (Type III). The effect of cytokinins (zeatin 0.05; 0.1; 0.2 and / or 6-benzylaminopurine-BAP, 0.1 and 1.0 mgL^{-1}, respectively) on plant regeneration of these three types of calli were investigated. Friable (Type II and III) suspension derived calli have the highest morfogenic potential. During the regeneration process, two different regeneration pathways were observed: somatic embryogenesis and/or organogenesis dependent on used cytokinin. Germination of normally developed somatic embryos was achieved on MS solid medium without hormones. Potted plants of *I. pumila* grew normally and flowered.

1. Introduction

The genus *Iris* includes over 300 species (fam. Iridaceae, Monocotyledons), many of them are horticulturally and pharmaceutically important. Like other ornamental monocotyledonous species with bulbs or rhizomes, irises are generally propagated vegetatively, but with a very low propagation rate.

Iris pumila L (2n=32) is a dwarfish, perennial, herbaceous species, with attractive, variously colored flowers. Its natural habitats in Yugoslavia are restricted to northern and eastern Serbia. Since it is a rare species, it is the subject of strict protection. In addition to their ornamental value, *I. pumila* contains xanthones, substances important for pharmacy. The decorative and biological properties of the *I. pumila* prompted us to initiate their vegetative propagation by *in vitro* cell cultures.

In recent years, number of studies have appeared regarding *in vitro* propagation of some *Iris* species such us *I. hollandica* (Hussey, 1976), *I. ensata* (Yabuya *et al*., 1991), *I. pumila* (Radojevic *et al*., 1987), *I. pseudocorus*, *I. versicolor* (Laublin *et al*., 1991), *I. setosa* (Radojevic and Subotic 1992), *I. sibirica* (Subotic and Radojevic, 1995), *I. pallida* (Gozu *et al*.,1993). Plant regeneration from cell suspensions and protoplasts was achieved only for *Iris germanica* (Shimizu *et al*., 1996, 1997, Wang *et al*., 1999, a, b). In *I. pumila*, Radojevic *et al*., (1987) induced embryogenic calli from mature zygotic embryos, but

embryogenic calli lost their morphogenic potential after many years.

The objective of this study was the improvement of *in vitro* plant regeneration of *I. pumila via* somatic embryogenesis and/or organogenesis using leaf base of *in vitro* grown plants. In addition, we would like to establish an efficient and reproducible plant regeneration protocol from suspension-cultured cells of *I. pumila* that would be suitable for mass propagation of this iris as ornamental and /or pharmaceutically valuable species.

2. Materials and methods

2.1. Plant material

Explants for induction of somatic embryogenesis were shoots of *I. pumila* that has been multiplied many years on media for organogenesis described earlier by Radojevic and Subotic (1992).

2.2. Medium for embryogenic callus induction

Leaf bases (length 1 cm) were cultured 6-8 weeks on solid medium **A** consisting of MS mineral solution (Murashige and Skoog, 1962), 0.7 % agar, 3 % sucrose and (in mgL^{-1}): inositol 100, pantothenic acid 10, nicotinic acid 5, vitamin B_1 2, vitamin B_6 1, casein hydrolysate 250, proline 250, 2,4-D and kinetin (1.0 mgL^{-1}, each). Induced calli were subcultured monthly and embryogenic calli were selected.

2.3. Medium for initiation and maintenance of embryogenic suspensions

Cell suspensions were initiated by transfering 1-2 g embryogenic calli in 50 ml liquid medium **A** with the same amount of 2,4-D and kinetin (1.0 mgL^{-1}, each), as the ones used for somatic embryogenesis induction. Stable suspension cultures were successfully established after three subcultures and maintained by subculturing every 3 weeks. In prolonged suspension cultures (6-8 weeks) three types of embryogenic calli were observed: yellow, compact (Type I), yellow-green, friable (Type II) and white, friable (Type III). After filtration of cell suspensions through sieves (200μ) and prolonged cultures, three types of calli were also observed and permanently maintained.

2.4. Media for plant regeneration from suspension derived calli

Suspension derived calli were manually selected and cultured on media **B** supplemented with MS mineral solution and additives like **A** solid medium but with different combinations of hormones (in mgL^{-1}): $\mathbf{B_0}$= hormone free; $\mathbf{B_1}$ = BAP 0.1; $\mathbf{B_2}$= BAP 1.0; $\mathbf{B_3}$= Zeatin (Zea) 0.05; $\mathbf{B_4}$= Zea 0.1; $\mathbf{B_5}$=Zea 0.2; $\mathbf{B_6}$= Zea 0.05 + BAP 0.1; $\mathbf{B_7}$=Zea 0.05 + BAP 1.0; $\mathbf{B_8}$= Zea 0.1+ BAP 0.1; $\mathbf{B_9}$= Zea 0.1 + BAP 1.0; $\mathbf{B_{10}}$=Zea 0.2 + BAP 0.1; $\mathbf{B_{11}}$=Zea 0.2 + BAP 1.0.

Further development of somatic embryos until cotyledonary stage was achieved on the same media. Germination of somatic embryos was achieved on MS solid medium without hormones.

2.5. Culture conditions

The media were adjusted to pH 5.8 before sterilization. All culture media were autoclaved at 114 ^{0} C and 0.07 MPa for 30 min. Zeatin was filter-sterilized and added after autoclaving. Cultures were grown at 25 ± 2 ^{0} C, with 16 h photoperiod from "Tesla" fluorescent tubes providing a light intensity of 50 μmol $m^{-2}s^{-1}$. Suspension cultures were maintained on a rotatory shaker.

Regenerated plants of *I. pumila* that originated from somatic embryos were grown in a greenhouse in a mixture of sand and soil (3:1). Fully developed plantlets were

transferred to a greenhouse for flowering.

2.6. Analysis of data

Results are expressed as mean ± standard error (SE). The results were analyzed according to analysis of variance (ANOVA), using completely randomized design. Values were transformed using log transformation procedures. Separation of means was tested using Fisher's Least Significant Difference (LSD, $\alpha = 0.05$).

3. Results

Leaf bases of *in vitro* grown shoots of *I. pumila* were cultured for 8 weeks and friable, nodular, yellow or white calli were obtained (Fig. 2.1). Induction rate was 95 % and fresh callus weight was 259.1 ± 22.0 mg *per* leaf base. After the first subculture, compact organogenic calli with green nodules were also formed. Calli types were separated and embryogenic calli were used for establishment of suspension cultures.

After three subcultures stable suspension cultures were successfully established and maintained by subculturing every 3 weeks. The suspension cultures were initially composed of single cells, bi-, three, and multicellular proembryos and cell aggregates (Figs. 2.2 and 2.3). In prolonged suspension cultures (6-8 weeks) three types of embryogenic calli were observed: yellow, nodular, and compact (Type I); yellow-green, friable (Type II) and white, friable (Type III). Representation of these callus types were 10-15 %, 20-25 % and 60-70 % for Type I, Type II, and Type III respectively. Further differentiation of Type I suspension derived calli were observed only on B_0 (hormone free) and $\mathbf{B_{3\text{-}5}}$ media supplemented with zeatin (0.05, 0.1 and 0.2 mg L^{-1} respectively). The average number of somatic embryos *per* one nodule was 9.0 ± 0.3 ($\mathbf{B_0}$), 8.1 ± 0.3 ($\mathbf{B_3}$), 23.6 ± 1.4 ($\mathbf{B_4}$) and 17.2 ± 0.6 ($\mathbf{B_5}$). Friable calli (Type II and Type III) had higher morphogenic potential then compact (Type I) on tested media supplemented with zeatin and/or BAP. Type II callus had the greatest embryogenic potential on media supplemented with Zea ($\mathbf{B_{3\text{-}5}}$ media) and BAP in lower concentration ($\mathbf{B_1}$ medium, Table 1, Fig. 2.4). White callus (Type III) had the greatest embryogenetic potential comparing with other suspension derived calli (Table 2). Organogenic callus was also observed on $\mathbf{B_0}$-$\mathbf{B_{11}}$ media during differentiation of friable calli (Fig. 1; Fig. 2.5). The appearance of organogenic calli on tested media during further differentiation of white callus (Type III) depended on the kind of applied cytokinin. The percentage of organogenic calli was higher on $\mathbf{B_1}$ and $\mathbf{B_2}$ media supplemented only with BAP (0.1 or 1.0 mgL^{-1}), or on tested media with BAP (media $\mathbf{B_7}$, $\mathbf{B_9}$ and $\mathbf{B_{11}}$; Fig. 1).

Plant conversion from somatic embryos was on MS solid medium without hormones. Potted plants of *I. pumila* grew normally and flowered. Eighteen months after potting, plants obtained from somatic embryos *via* suspension culture of *I. pumila* were mutually compared and no somaclonal variations were found (Fig. 2.6).

4. Discussion

The leaf base as explant for induction of somatic embryogenesis of irises has been earlier reported for *I. pallida* (Gozu *et al*., 1993). Jehan *et al*., (1994) for the first time mentioned the use of *in vitro* plantlets of *I. germanica*, that originated from somatic embryos as the initial explants. We used leaf bases of *in vitro* shoots of *I. pumila* that were multiplied many years, as material for embryogenic callus formation. The use of *in vitro* plants for induction of somatic embryogenesis has many advantages. There were no troubles with sterilization of plant material or seasonal variation depending on the stage of leaf development (Gozu *et al*., 1993). We achieved a higher callus induction rate on media with 2,4-D and kinetin (1.0 mgL^{-1}, each) compared to *I. germanica* hybrids (Shimizu *et al*., 1997).

Light intensity was a determinant factor for a successful induction phase, since

only explants cultured at low intensity could be subcultured (Laublin *et al*., 1992). Mayer *et al*., (1975), working with *I. hollandica*, observed that only explants kept in the dark would respond with callus formation, but not in light. Our results suggested that the light had no influence on induction of embryogenic calli in leaf base culture and establishment of suspension culture.

In herbaceous monocots, embryogenic suspension cultures are very difficult to establish and to maintain. For irises they have been obtained only for a few commercial hybrids of *I. germanica* (Shimizu *et al*., 1997; Wang, *et al*., 1999 a, b). In earlier reports of plant regeneration from cell suspension cultures of *Iris* authors mentioned that further differentiation of suspension derived calli occured *via* both somatic embryogenesis and organogenesis. Shimizu *et al*., (1997) measured the number of shoots that originated from suspension derived calli *via* organogenesis. In their study, different plant hormones like GA_3 and kinetin had an effect on somatic embryogenesis but had no effect on shoot development. Shimizu *et al*., (1997) also concluded that the GA_3 promoted somatic embryogenesis, whereas kinetin was inhibitory. Our results suggest that both cytokinins tested (zeatin and/or BAP) influence further differentiation of friable suspension derived calli of *I. pumila*. In addition, these two cytokinins had different effects on the formation of organogenic calli especially of white callus (Type III); zeatin promotes somatic embryogenesis, whereas BAP promotes organogenesis. This supports the observations of other authors that application of two cytokinins during differentiation of embryogenic calli may have different effects on somatic embryogenesis and organogenesis (Levi and Sink, 1991).

We can conclude that the described protocol yielded high rates of plant regeneration from suspension cultures of *I. pumila* and could be a useful method for mass propagation for different biotechnological purposes of this iris species without destroying the natural habitat.

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Table 1. Influence of BAP and/or zeatin on differentiation of yellow-green (Type II) suspension derived calli of *I. pumila*. Average number of somatic embryos *per* 0.5 g of calli.

BAP (mgL^{-1})	Z E A T I N ($mg\ L^{-1}$)			
	0	0.05	0.1	0.2
0	8.5 ± 0.6 a	190.7 ±13.9 d	78.0 ± 9.9 c	32.7 ± 3.3 b
0.1	67.7 ±12.9 b	8.7 ± 0.3 a	4.3 ± 0.3 a	4.7 ± 0.3 a
1.0	20.2 ± 1.5 a,b	0 a	5.0 ± 0.6 a	0 a

a,b,c,d significant differences at 5% level of LSD test.

Table 2. Influence of BAP and/or zeatin on differentiation of white (Type III) suspension derived calli of *I. pumila*. Average number of somatic embryos *per* 0.5 g of calli.

BAP (mgL^{-1})	Z E A T I N ($mg\ L^{-1}$)			
	0	0.05	0.1	0.2
0	19.5 ± 0.4 a	22.0 ± 8.7 a,b	141.7 ± 2.7 f	55.2 ± 2.6 c,d
0.1	48.3 ± 3.1 b,c	62.5 ± 4.6 d	16.5 ± 0.8 a	43.2 ± 2.9 c
1.0	43.7 ± 1.7 c	15.0 ± 1.0 a	131.0 ± 5.2 e	29.0 ± 1.0 b

a,b,c,de, f significant differences at 5% level of LSD test.

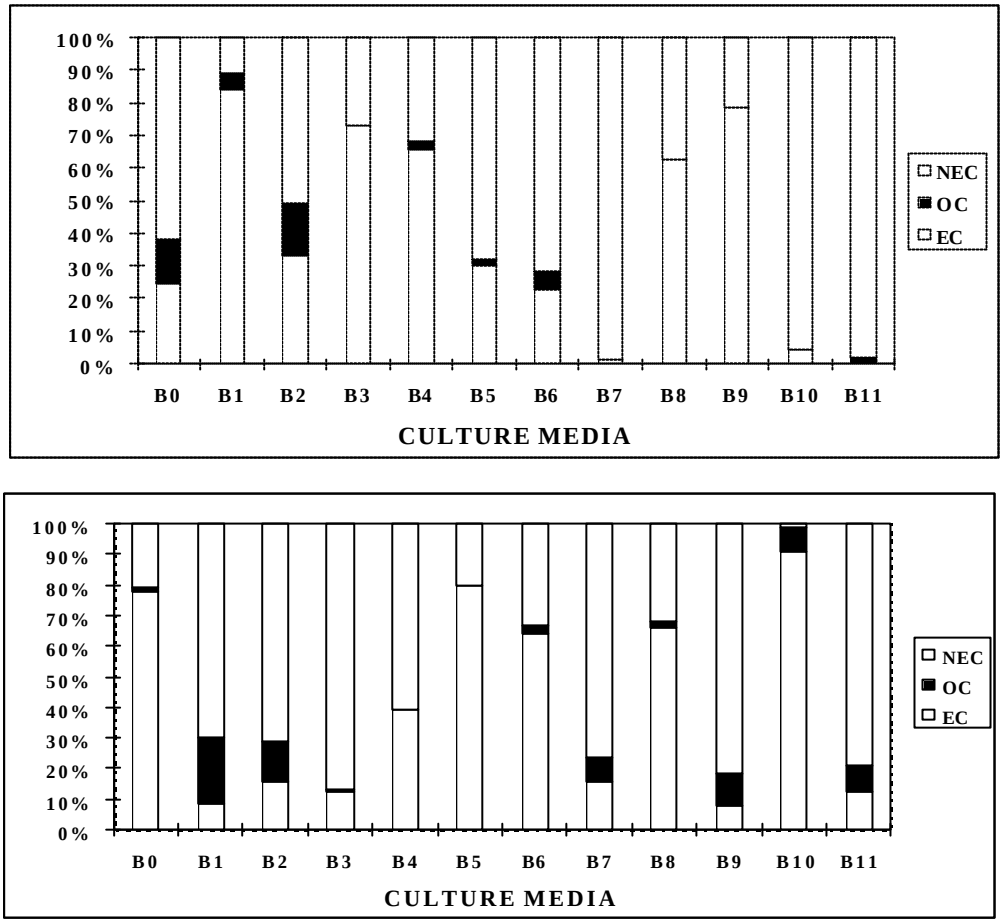


Figure 1. Morphogenic potential of friable suspension derived calli of *I. pumila*. Values represents percent of embryogenic (EC), organogenic (OC) and nonembryogenic calli (NEC) after one month of culture on media supplemented with cytokinins of yellow-green (Type II, upper figure) and white (Type III, lower figure).

Figure 2. Plant regeneration from suspension derived calli of *I. pumila* (See next page)

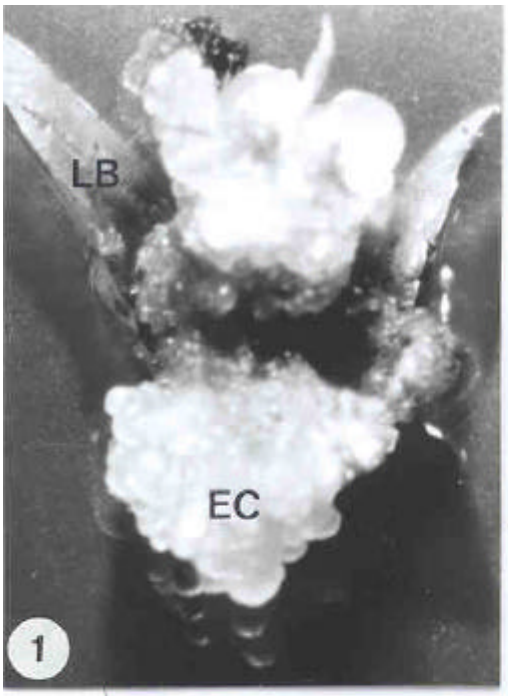
1 Formation of embryogenic callus (EC) at the leaf-base (LB) when cultured on A solid medium; x21.

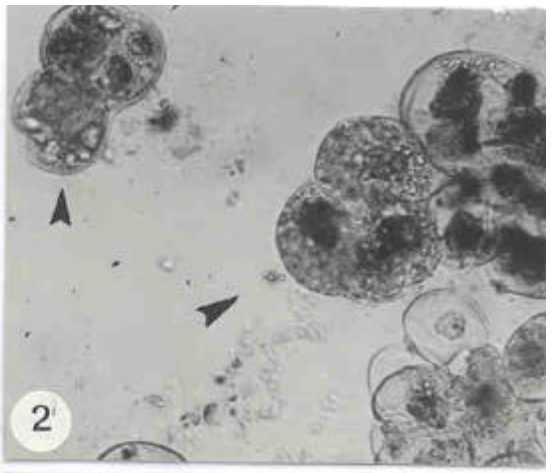
2-3 Three-celled proembryo (arrows, Fig. 2) and proembryonic mass (arrow, Fig. 3) originating from suspension culture; x500; x250.

4 Differentiation of somatic embryos (SE) from Type II callus on B_4 medium; x32.

5 Plantlet formation from Type III callus on B_7 medium supplemented with zeatin and BAP (0.1; 1.0 mg, respectively). Note formation of organogenic callus (OC), x3.2.

6 Plants of *I. pumila* after 18 months of acclimation, obtained from suspension culture x3.2.


LB
EC
1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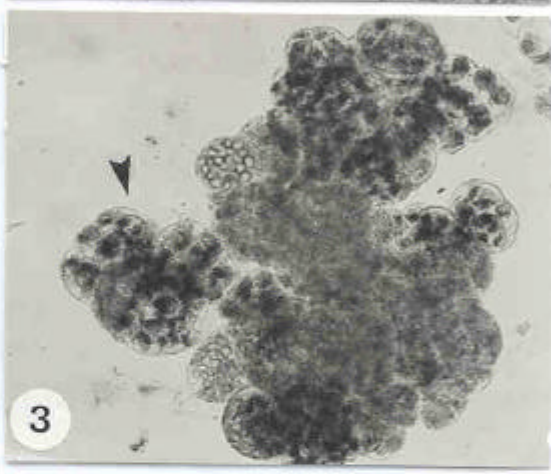
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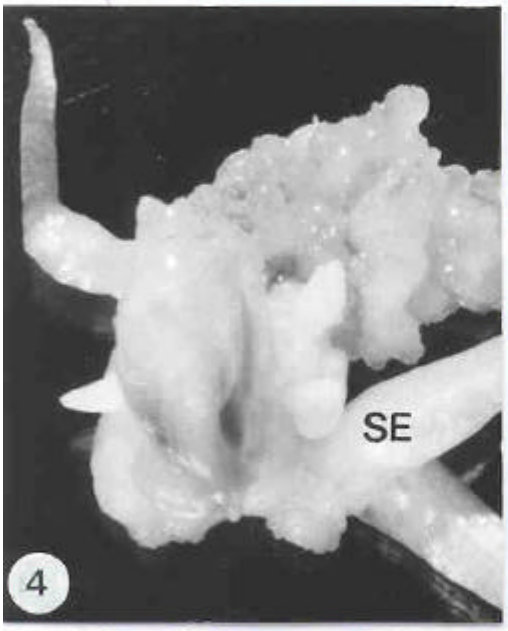
SE
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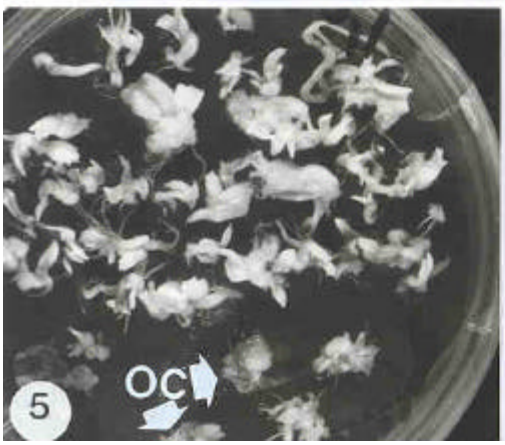
OC
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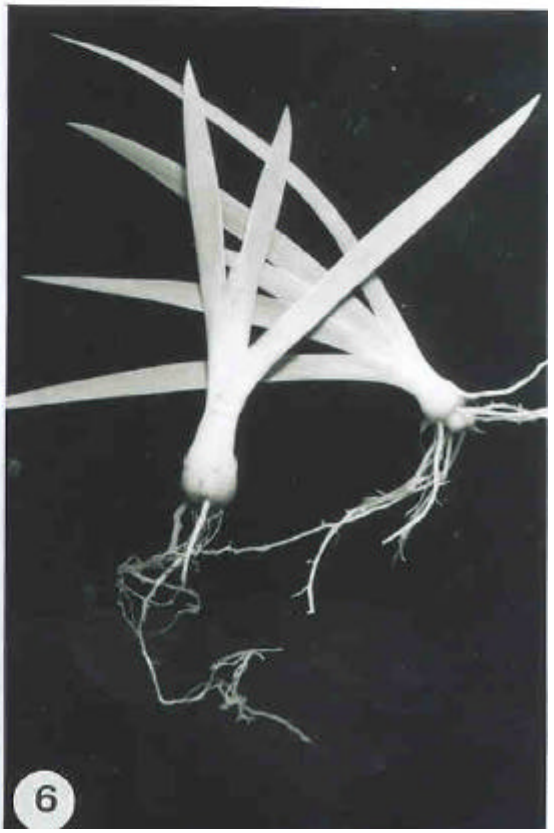
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