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SATI UZUN

ALİ İRFAN İLBAŞ

ARİF İPEK

NEŞET ARSLAN

SURENDRA BARPETE

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Efficient in vitro plant regeneration from immature embryos of endemic *Iris sari* and *I. schachtii*

Satı UZUN[1,*], Ali İrfan İLBAŞ[2], Arif İPEK[3], Neşet ARSLAN[4], Surendra BARPETE[4]

[1]Department of Field Crops, Faculty of Agriculture, Erciyes University, Kayseri, Turkey
[2]Department of Agronomy, Faculty of Agriculture, Kyrgyzstan-Turkey Manas University, Bishkek, Kyrgyzstan
[3]Department of Biology, Faculty of Science, Çankırı Karatekin University, Çankırı, Turkey
[4]Department of Field Crops, Faculty of Agriculture, Ankara University, Ankara, Turkey

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Abstract: Plant tissue culture is an efficient technique for conserving endemic plant species. A reproducible in vitro regeneration protocol was developed for the endemic *Iris sari* and *Iris schachtii* in the present study. The highest number of shoots per explant was obtained on Murashige and Skoog (MS) medium supplemented with 0.5 mg/L thidiazuron (TDZ) plus 0.5 mg/L α-naphthaleneacetic acid (NAA) and 1 mg/L TDZ plus 0.5 mg/L NAA, whereby 96.88% and 100% shoot induction with 9.55 and 11.34 shoots per explant of *Iris sari* and *Iris schachtii* were recorded, respectively. Regenerated shoots were successfully rooted on MS medium with either 1 mg/L indole-3-butyric acid (IBA) or 1 mg/L IBA plus 0.2 mg/L NAA. Rooted shoots were transferred to pots containing either a peat-soil-sand (1:1:1) mixture or a hydroponic culture containing Hoagland solution to acclimatize the regenerated plants to the greenhouse chamber. Approximately 90% of plants were transferred ex vitro successfully.

Key words: Endemic species, immature embryo, in vitro regeneration, *Iris sari*, *Iris schachtii*

1. Introduction

Environmental pressures such as agriculture, erosion, overgrazing of meadows, and urbanization cause significant loss of plant species and their habitats. Therefore, special attention should be paid to protect and preserve the endemic flora that is normally found in limited areas (Fay, 1992; Sarasan et al., 2006). Conservation of endemic species by in vitro plant tissue culture has attracted the attention of researchers (Mallon et al., 2010; Piovan et al., 2010; Corral et al., 2011). In vitro techniques make the rapid and reliable production of a large amount of individuals from a small amount of plant material possible, and thus are preferred as an efficient way for conservation of endemic plants and wild populations (Corral et al., 2011). In addition, they play a major role in the production of plant material required for genetic transformation, virus elimination, rejuvenation of mature plant material, and the acquisition of industrial raw material (Endress, 1994; Hasançebi et al., 2011).

The genus *Iris* belongs to the family Iridaceae, which contains 43 species in Turkey, 13 of which are endemic (Aslan and Karataş, 2012). *Iris sari* and *Iris schachtii* are presented in the 'least concern' category in the Red Data Book of Turkish Plants (Ekim et al., 2000). Both species have potential as ornamental plants with their attractive flowers. A recent trend of using wild plants for ornamental purposes by collecting them from their natural habitats could lead to the extinction of wild plants (Prado et al., 2010). *Iris* species can be propagated from seeds, bulbs, or rhizomes with a low propagation speed like other geophytes (Jevremovic and Radojevic, 2002; Boltenkov et al., 2007). Therefore, in recent years, a number of studies have reported in vitro propagation of *Iris* species such as *I. ensata* (Yabuya et al., 1991; Boltenkov et al., 2007), *I. germanica* (Shimizu et al., 1997; Wang et al., 1999a, 1999b), *I. nigricans* (Shibli and Ajlouni, 2000), *I. pumila* (Jevremovic and Radojevic, 2002), *I. ensata*, *I. setosa*, *I. sanguinea* (Boltenkov and Zarebno, 2005), *I. hollandica* (Fidalgo et al., 2005), *I. atrofusca*, *I. petrana*, *I. vartanii* (Al-Gabbiesh et al., 2006), and *I. adriatica* (Keresa et al., 2009) from a range of explants.

In the present study, rapid and reliable protocols for in vitro shoot regeneration, rooting, and acclimatization of endemic *I. sari* and *I. schachtii* were developed. The study presents the first in vitro shoot regeneration of *I. sari* and *I. schachtii*, which can be valuable for commercial production and germplasm conservation of both endemic species.

* Correspondence: saticocu@yahoo.com

2. Materials and methods

2.1. Surface disinfection of plant materials

I. sari and *I. schachtii* plants were collected from the Erzurum and Ankara provinces of Turkey and planted in the experimental fields of the Agricultural Faculty at Ankara University, Ankara (Turkey). Immature pods containing immature zygotic embryos approximately 1 mm in length were harvested, washed with running tap water, and then surface sterilized with 50% commercial bleach [5% (v/v) sodium hypochloride, ACE, Turkey] for 10 min, washed 3 times with sterile distilled water for 5 min each, and blotted onto tissue paper.

2.2. Shoot regeneration from zygotic immature embryos

Immature pods were dissected under sterile conditions and the seed was squeezed using forceps until the immature zygotic embryo was released. Immature embryos were then placed onto Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose, 0.7% plant agar (w/v, Duchefa, the Netherlands), 6-benzylaminopurine (BAP; 1, 2, or 4 mg/L), thidiazuron (TDZ; 0.5 or 1 mg/L), or kinetin (KIN; 2 or 4 mg/L), with or without 0.5 mg/L α-naphthaleneacetic acid (NAA). Explants were placed in 100 × 10 mm petri dishes containing 25 mL of culture medium and incubated in the growth chamber. Cultures were transferred to fresh media at 4-week intervals. Each treatment contained 6 explants for *I. schachtii* and 8 explants for *I. sari*, with 4 replicates. The frequency of shoot regeneration and mean number of shoots per explant was determined after 12 weeks.

2.3. Rooting of the regenerated shoots

In vitro regenerated shoots that originated from immature embryos were rooted on 1 mg/L indole-3-butyric acid (IBA) alone or in combinations with 0.2 or 0.4 mg/L NAA. Plant growth regulator (PGR)-free MS medium was also used as control. For rooting experiments, 10 regenerated shoots were used and each treatment was replicated 4 times. Data on root induction ratio, mean number of roots per shoot, and mean root length were recorded after 2 months of culture.

The pH levels of all media were adjusted to 5.6–5.8 before autoclaving for 20 min at 121 °C. All cultures were grown at 24 ± 2 °C with a 16-h photoperiod.

2.4. Acclimatization

For acclimatization, healthy plantlets with well-developed root and shoot systems were chosen. They were removed from the culture media, washed in running tap water to remove agar, and then transferred to plastic pots containing either sterile soil-sand-peat (1:1:1) or hydroponic culture containing Hoagland solution. Each vessel or pot was covered with a transparent polyethylene bag to create high relative humidity; afterwards, the bags were gradually opened by perforating small holes in the bag. After 10 days the bags were opened completely. Transplanting plants were maintained in the growth chamber at 24 ± 2 °C with a 16-h photoperiod.

2.5. Statistical analysis

The data were evaluated by analysis of variance (ANOVA) and the differences between means were compared by Duncan's multiple range tests. Data given in percentages were subjected to arcsine ($\sqrt{X}$) transformation (Snedecor and Cochran, 1967) before statistical analysis.

3. Results

3.1. Shoot regeneration from zygotic immature embryos

In the present study, shoot regeneration response was observed in all culture media irrespective of MS medium supplemented with or without NAA in *I. sari* and *I. schachtii* (Table 1). Morphogenic green callus appeared from the explants after 2 weeks and development of shoots was observed after 4–5 weeks of culture (Figure 1a), followed by well-developed shoots after 12 weeks (Figures 1b and 1c).

Significant variations in the frequency of shoot regeneration were determined depending on PGRs ($P < 0.01$). The frequency of shoot regeneration ranged from 78.12%–100% and 70.84%–100% in *I. sari* and *I. schachtii*, respectively (Table 1). The highest shoot frequency was obtained from all media except 1 mg/L TDZ, 2 mg/L KIN, and 4 mg/L KIN. Inclusion of NAA used with BAP, TDZ, or KIN resulted in increased shoot regeneration frequency compared to use alone.

Mean number of shoots per explant varied significantly depending on species, PGRs, and the interactions between species and PGRs ($P < 0.01$). The highest number (9.55) of shoots per explant in *I. sari* was achieved on MS medium supplemented with 0.5 mg/L TDZ plus 0.5 mg/L NAA. The maximum of 11.34 shoots per explant of *I. schachtii* was observed on medium with 1 mg/L TDZ plus 0.5 mg/L NAA. Comparing effects of PGRs, TDZ proved to be more efficient than BAP and KIN on shoot multiplication in *I. sari.* On the other hand, TDZ and BAP produced more shoots than KIN in *I. schachtii* (Table 1). The addition of 0.5 mg/L NAA with all concentrations of BAP, TDZ, and KIN exerted positive effects on shoot proliferation in both species. However, the combination of TDZ plus NAA promoted a considerable increase in the number of shoots per explant relative to that induced by combinations of BAP plus NAA and KIN plus NAA in both species.

3.2 Rooting of the regenerated shoots and acclimatization

Root development was observed on all rooting mediums with or without auxins; most of the shoots were rooted within 2 months (Figures 1d and 1e). While rooting percentage and mean root length were significantly affected by PGRs ($P < 0.01$), mean number of roots per shoot was

Table 1. Effect of plant growth regulators (PGRs) on shoot regeneration of *I. sari* and *I. schachtii.*

PGRs (mg/L)		Frequency of shoot regeneration (%)			Mean number of shoots per explant		
BAP	NAA	*I. sari*	*I. schachtii*	Mean	*I. sari*	*I. schachtii*	Mean
1	-	87.50	100.00	93.75 ab*	3.55 fg*	7.63 de*	5.59 fg*
1	0.5	100.0	100.00	100.00 a	5.58 bcde	9.58 bc	7.58 bc
2	-	81.25	95.8	88.54 ab	3.75 efg	8.54 cd	6.15 def
2	0.5	96.88	95.83	96.35 ab	5.63 bcd	8.79 bcd	7.21 cd
4	-	81.25	100.00	90.63 ab	4.00 defg	9.63 abc	6.81 cde
4	0.5	100.0	87.50	93.75 ab	5.25 cdef	9.17 bcd	7.21 cd
TDZ							
0.5	-	93.75	95.83	94.79 ab	7.05 b	9.96 abc	8.50 b
0.5	0.5	96.88	95.83	96.35 ab	9.55 a	10.45 ab	10.00 a
1	-	87.50	87.50	87.50 bc	4.70 cdefg	5.75 fg	5.23 fgh
1	0.5	96.87	100.00	98.44 ab	5.85 bc	11.34 a	8.59 b
KIN							
2	-	78.12	70.84	74.48 c	4.00 defg	4.42 g	4.21 h
2	0.5	84.38	91.67	88.02 ab	4.00 defg	5.37 fg	4.69 gh
4	-	90.62	83.34	86.98 bc	3.25 g	4.83 fg	4.04 h
4	0.5	93.75	87.50	90.62 ab	5.00 cdefg	6.33 ef	5.67 efg
Mean*		90.63	92.26	--	5.08 b	7.99 a	--

*: Values within a column or row followed by different letters are significantly different at the 0.05 probability level of Duncan's multiple range test.

affected by PGRs and interaction (species and PGRs, $P < 0.05$). The lowest rooting frequency was recorded on MS medium devoid of auxins in both species (52.50%). Root inductions of *I. sari* and *I. schachtii* (95% and 92.5%, respectively) were promoted with 1 mg/L IBA, with mean root lengths observed as 7.10 and 5.88 cm, respectively (Table 2). Maximum mean number of roots per shoot of *I. sari* (4.37) and *I. schachtii* (4.80) was recorded on MS medium supplemented with 1 mg/L IBA and 1 mg/L IBA plus 0.2 mg/L NAA, respectively. The presence of 0.4 mg/L NAA with IBA resulted in decreased rooting frequency and mean root length.

Plantlets with well-developed roots were transferred to either plastic pots containing a 1:1:1 mixture of peat-soil-sand (Figures 2a and 2b) or hydroponic culture containing Hoagland solution (Figures 2c and 2d) and kept in a growth chamber under a 16-h light photoperiod. After 3 months, the survival rates of *I. sari* and *I. schachtii* were 56% and 61% in peat-sand-soil mixture and 89.5% and 91.89% in hydroponic culture, respectively (data not shown).

4. Discussion

Efficient shoot regeneration has been previously reported from immature embryos of geophytes (Mirici et al., 2005; Uranbey, 2010; Nasırcılar et al., 2011). Immature embryos are suitable explants for in vitro shoot regeneration and they may prevent overharvesting of the parent plant from natural populations.

The cytokinins BAP and KIN, and cytokinin-like substance TDZ (alone or in combination with auxin), are commonly used to induce shoot regeneration in plant tissues (Özcan et al., 1996; Çöçü et al., 2004; Aasim et al. 2013) and especially to promote organogenesis from immature explants (Özcan et al., 1992; Özcan et al., 1996; Mirici et al., 2005; Uranbey 2010).

According to the results of the current study with *I. sari* and *I. schachtii*, immature embryo explants showed better response when cultured on MS medium supplemented with TDZ plus NAA. The MS medium with 0.5 mg/L TDZ plus 0.5 mg/L NAA and 1 mg/L TDZ plus 0.5 mg/L NAA produced the largest number of regenerated shoots per explant. Although TDZ has been proven to be effective for in vitro regeneration of various plants (Çöçü et al., 2004; Erişen et al., 2011; Nasırcılar et al., 2011), Shibli and Ajlouni (2000) reported that embryogenesis did not occur with TDZ treatment in *Iris nigricans*.

The lowest shoot regenerations were obtained from 2 mg/L or 4 mg/L KIN-containing media for both species,

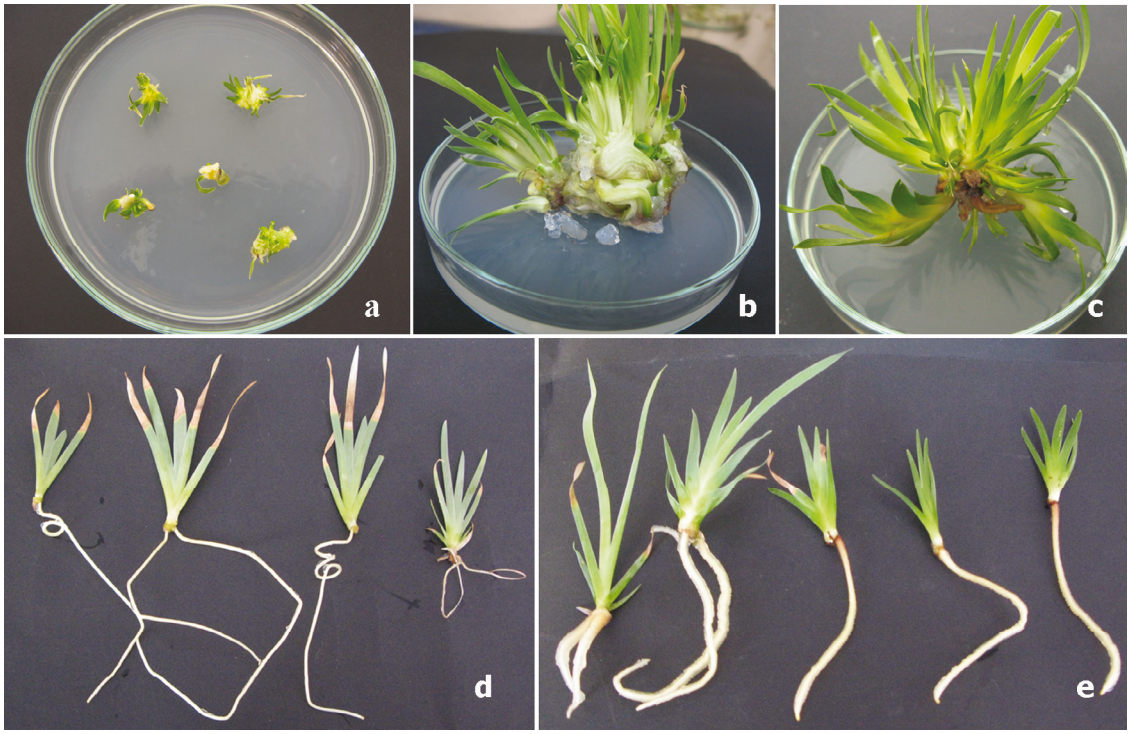


Figure 1. In vitro propagation and rooting of *I. sari* and *I. schachtii:* (a) shoot initiations from callus after 1 month of culture; (b) multiple shoots formation *I. sari* and (c) *I. schachtii* after 3 months of culture; (d) root formation of *I. sari* and (e) *I. schachtii*.

in line with the findings of Shimizu et al. (1997), who reported that KIN had an inhibitory effect on somatic embryogenesis but no effect on shoot development in *I. germanica*. On the other hand, KIN was generally used in the induction of embryogenic callus in irises (Gozu et al., 1993; Shimizu et al., 1996; Wang et al., 1999a, 1999b). This variation may stem from genotype and concentration or combination of PGRs. Boltenkov and Zarembo (2005) reported that plant species and PGRs play an important role in the establishment of regeneration.

In treatments including BAP, shoot regeneration response changed with species and *I. schachtii* was found to be better than *I. sari*. The promotive effect of BAP alone or in combination with NAA-inducing callus and adventitious bulblet regeneration was previously reported in other *Iris* species (Kawase et al., 1995; Suzhen et al., 1999; Shibli and Ajlouni, 2000; Boltenkov and Zarembo, 2005; Boltenkov et al., 2007).

The effectiveness of MS basal medium supplemented with 1 mg/L IBA was demonstrated for better root development with 95% and 92.5% successful rooting in *I. sari* and *I. schachtii*, respectively. As observed by Laublin et al. (1991), IBA-containing medium allowed rapid development of roots in *Iris* species. Half-strength MS

Table 2. Effects of IBA and NAA combinations on in vitro rooting of *I. sari* and *I. schachtii*.

PGRs (mg/L)		Root induction (%)			Mean number of roots per shoot			Mean root length (cm)		
IBA	NAA	*I. sari*	*I. schachtii*	Mean	*I. sari*	*I. schachtii*	Mean	*I. sari*	*I. schachtii*	Mean
-	-	52.50	52.50	52.50 c*	3.29 a*	2.00 b*	2.65 b*	4.44	4.68	4.56 c*
1	-	95.00	92.50	93.75 a	4.37 a	3.45 a	3.91 a	7.10	5.88	6.49 a
1	0.2	82.50	92.50	87.50 a	3.96 a	4.80 a	4.38 a	6.10	5.54	5.82 ab
1	0.4	77.50	77.50	77.50 b	3.14 a	4.29 a	3.72 a	5.16	4.43	4.79 bc
Mean		78.88	78.75	--	3.69	3.64	--	5.70	5.13	---

*: Values within a column followed by different letters are significantly different at the 0.05 probability level of Duncan's multiple range test.



Figure 2. Acclimatization of in vitro regenerated plantlets: (a) *I. sari*, (b) *I. schachtii*, (c and d) hydroponic culture of regenerants.

plus 0.5 mg/L IBA were reported to have better impacts on rooting growth of *Iris lactea* Pall. var. *chinensis* (Meng et al., 2009). However, with shoots cultured in medium without PGRs, rooting success was only 52.5% in both species. Boltenkov et al. (2007) suggested that PGRs were required for root initiation in *Iris ensata*.

Acclimatization is a critical stage and often associated with slow growth and significant plant loss. Wang et al. (1999b) stated that plant survival and growth after transfer from in vitro culture to ex vitro were affected by potting substrate in *I. germanica*. In the present study, hydroponic culture yielded better survival rates than a 1:1:1 peat-soil-sand mixture. Similarly, Zapata et al. (2003) observed the best growth, as well as the maximum survival percentage of plants acclimatized in the hydroponic system, in *Curcuma longa* L.

In conclusion, an efficient and rapid in vitro regeneration protocol was developed for *I. sari* and *I. schachtii*, followed by high frequency of rooting and acclimatization protocols. These results could be useful for the propagation and conservation of these endemic species.

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