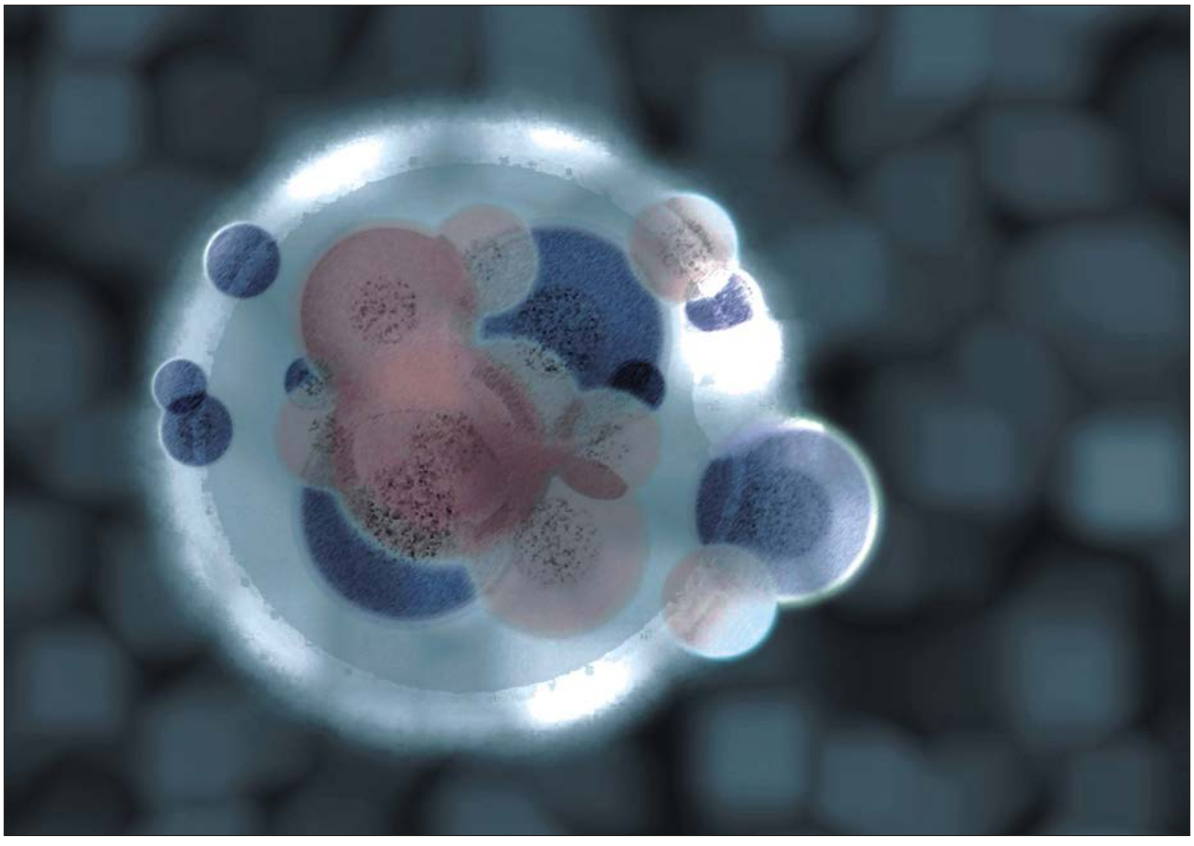

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In vitro Propagation of Endangered *Iris* Species

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Abstract: Calli from *Iris petrana* and *I. atrofusca* were produced from flower bases, leaf bases and anthers. The flower bases responded positively to the culture conditions and gave the best results. Callus induction medium was Murashige and Skoog (MS) supplemented with 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 μM Kinetin and 4.5 μM 1-naphthaleneacetic acid (NAA). Formation of green structures was noticed on calli of both species after transferring calli to callus culture medium (MS supplemented with 4.5 μM N^6-benzyladenine (BA), 0.45 μM 2,4-D and 4.5 μM NAA). Embryo-like structures were appeared after transferring calli to embryogenesis induction medium (MS supplemented with 4.5 μM 2,4-D, 0.5 μM Kinetin, 4.5 μM NAA and 300 mg L^{-1} proline). Both green and albino plantlets were observed after calli were transferred to regeneration medium {MS supplemented with 4.5 μM BA, 0.45 μM 2,4-D and 0.49 μM indol-3-butyric acid (IBA)}.

Key words: Callus culture, growth regulators, propagation of *Iris* sp.

INTRODUCTION

Iris is the largest and most complicated genus of Iridaceae, which includes over 300 species. The name of the genus is recorded in Theophrastus, being the Greek name of the goddess of the rainbow (Mathew, 1985). The genus *Iris* is originated in Japan and in the Mediterranean (Rasoul, 1984). However, the species of this plant are more concentrated in the south of equator (Feinbrun-Dothan, 1986; Rodionenko, 1987). The genus *Iris* has been reported to contain volatile oils and can be used for medicinal purposes (Williams and Harborne, 1985).

Iris grows naturally in the northern regions of Jordan including Mafraq, Wadi Shueib, Salt, Ajloun, Jordan Valley and the southern regions including Tafila, Karak and Shobak (Kareem and Qor'an, 1988). In Jordan, *Iris* is considered as a wild perennial herbaceous plant that subjected to strict protection. Ten species of *Iris* were confirmed to occur in Jordan, nine of which are perennial herbaceous plants growing in nature: *I. aucheri*, *I. regis-uzziae*, *I. edomensis*, *I. postii*, *I. vartanii*, *I. nigricans*, *I. petrana*, *I. atrofusca* and *I. atropurpurrea*. The tenth species, *I. germanica*, is a cultivated one. *I. petrana*, *I. atrofusca* and, *I. vartanii* are considered as rare endemic plants (Al-Eisawi, 1986; Al-Khader, 1997; Al-Eisawi *et al*., 2000).

Plant tissue culture in its broad sense refers to the culturing of all plant parts under aseptic conditions. Perhaps, the most significant benefit of this technique has been the increasing utility of cell and organ culture in enhancing clonal propagation (Carlson, 1973; Murashige, 1974; Narayanaswamy, 1977; Nickel and Torrey, 1969; Vasil and Vasil, 1972). Most herbaceous plants are propagated *in vitro* by shoot culture (George, 1996), leaf sections (Torres, 1988), or meristem culture (Jungnickel and Zaid, 1992).

Nutrient media for plant tissue culture systems have been designed to enable the plant to grow and develop in a completely artificial microenvironment (Sutter, 1996). Different types of media were used in the initiation and culture of callus of so many plant species; Murashige and Skoog medium (MS medium) supplemented with a combination of an auxin and cytokinine was effective in callus induction and growth (Pierik, 1987).

Macronutrients, micronutrients, vitamins and plant growth regulators are the key constituents in changing the composition of any media used in initiating and growth of calli (Decendit and Merillon, 1996). Also, other factors can influence the callus induction ability of the selected explants; physical factors (light, humidity and temperature) and genetic constitution of the plant are standing examples (Caponetti, 1996).

Callus is a relatively undifferentiated non-organized tissue consisting primarily of parenchyma cells, which usually arises on wounds of differentiated tissues and organs (Allan, 1991). Callus tissue from different plant species may differ in structure, color and growth habit. Regeneration of adventitious organs (shoots and roots) can take place from callus. Cell and organ cultures serve also as sources for pharmaceuticals and other plant

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constituents (Tabata, 1977). Callus was obtained from Iris leaf bases, flower pieces or rhizome apices and the best explants were flower pieces. The induction medium used to obtain embryogenic callus was MS media (Jehan *et al*., 1994). Somatic embryogenesis was achieved from callus, cell suspension and protoplast culture of *I. nigricans* (Shibli and Ajlouni, 2000).

Numerous species of the Iridaceae family were vegetatively propagated by *in vitro* tissue culture since classical vegetative propagation of irises using rhizomes or bulbs gives limited number of plants (Hussey, 1975, 1976a, b; Reuther, 1975, 1977; Radojevic *et al*., 1985; Laublin and Cappadocia, 1990; Laublin *et al*., 1991; Radojevic and Subotic, 1992; Gozu *et al*., 1993; Shimizu *et al*., 1997). In addition to commercial purposes, micropropagation of some endemic iris species was performed with the aim of preserving them, as in the case of *I. pumila* (Radojevic *et al*., 1987).

The objectives of this study were to propagate and save some important threatened species of the genus *Iris* that grown in Jordan by using tissue culture technique.

MATERIALS AND METHODS

Plant materials: Samples of three threatened *Iris* species (*I. petrana*, *I. atrofusca* and *I. vartanii*) were collected from different areas of Jordan (Al-Salt, Al-Ghor, Al-Tafila, Humrat Al-Sahen and Wadi-Shueib). To establish enough mother stock cultures from these species, bulbs were cultivated in 3:1 (peat moss: perlite) mixture and grown in the green house at 22°C day and 16°C night temperatures with a 16 h photoperiod.

Media preparation: The media and concentrations of growth regulators used in this study were adopted from Shibli and Ajlouni (2000) and presented in Table 1. Stock solutions of MS medium and growth regulators were prepared and stored in the refrigerator.

To prepare one liter of medium, inorganic and organic solutions, sucrose and agar were mixed as recommended and sterilized by autoclave at 121°C and 15 psi for 20 min. Then, the medium was poured into 9-cm Petri dishes (about 20-25 mL in each). After solidification, Petri dishes were kept in refrigerator until using.

Culture system: The collected plant samples were washed with running tap water. Different explants (flower bases, leaf bases, sepals and petals) were excised and immersed in 70% ethanol for 1-2 min. These explants were then longitudinally sectioned into 5-6 mm segments. The small segments were surface sterilized for 15 min with 5% (v/v) sodium hypochlorite solution, supplemented with 1% (v/v) Tween 20 and rinsed three times with sterile distilled water. Anthers that also used as explants were excised and sterilized by soaking in 70% ethanol for 1 min.

Sterilized explants were transferred to the surface of Callus Induction Medium (CIM). Cultures were maintained under dark conditions at 27±1°C. Some explants (flower bases and leaf bases) were transferred to fresh (CIM) medium because of browning that caused by phenolic exudations. Calli were maintained by sub-culturing on fresh (CIM) as needed.

The formed calli were transferred after about 3 weeks from (CIM) to Callus Culture Medium (CCM) and maintained in dark with continuous sub-culturing on the new medium as needed.

After about four weeks, friable callus were transferred from (CCM) to Embryogenesis Induction Medium (EIM), where embryogenesis was observed. Embryogenic calli fragments were then transferred to Regeneration Medium (RM) and incubated under 16 h light.

The following data were recorded: frequency of explants producing calli, frequency of calli producing embryo-like structures, frequency of calli producing green structures, frequency of calli producing green plantlets and frequency of calli producing albino-plantlets.

Callus initiation frequency was measured as the number of explants producing calli divided by the total number of explants cultured, embryo-like structures frequency as the number of calli producing embryo-like structures divided by the total number of explants cultured, green structures frequency as the number of calli producing green structures divided by the total number of explants cultured, green plantlets frequency as the number of calli producing green plantlets divided by the total number of explants cultured, albino plantlets frequency as the number of calli producing albino plantlets divided by the total number of explants cultured.

All the data were transformed using the $(\text{arcsine } \%)^{1/2}$ transformation for statistical analysis. Data were analyzed as a complete random design with three replications (Steel and Torrie, 1960).

Table 1: Names and components of media used in this study

Components	Media
MS medium+4.5 μM 2,4-D+0.5 μM Kinetin+4.5 μM NAA	Callus Induction Medium (CIM)
MS medium+4.5 μM BA+0.45 μM 2,4- D+4.5 μM NAA	Callus Culture Medium (CCM)
MS medium+4.5 μM 2,4-D+0.5 μM Kinetin+4.5 μM NAA+300 mg L^{-1} proline.	Embryogenesis Induction Medium (EIM)
MS medium+4.5 μM BA+0.45 μM 2,4- D+0.49 μM IBA	Regeneration Medium (RM)

RESULTS

In spite of substantial efforts to induce callus from different explants (flower bases, leaf bases, anthers, sepals and petals), only flower bases were found to respond positively to the culture conditions comparing to the other explants, especially in inducing callus formation and in sustaining faster callus growth. More than 5600 flower bases from *I. atrofusca*, *I. petrana* and *I. vartanii* were cultured on CIM. Callus initiation was observed in cultures within 2-3 weeks (Fig. 1).

The frequencies of studied characters are reported in (Table 2). The results showed different responses for callusing. Regarding genotypes, *I. vartanii* produced no calli on CIM. In contrast, *I. atrofusca* and *I. petrana* were particularly responsive. The frequency of *Iris* flower bases producing calli were 0.00% for *I. vartanii*, 43% for *I. atrofusca* and 58% for *I. petrana*; these differences were statistically significant (Table 3).

Calli fragments that were transferred to (EIM) produced white embryo-like globular structures within two weeks (Fig. 2). These structures grew rapidly and new ones continued to appear on the callus surface, as well as on previously formed structures. Within 3 more weeks, clusters of structures at various stages of development could be found on the same callus (Fig. 3). The frequency of *Iris* embryo-like structures ranged from 0.00% for *I. vartanii* to 1.6% for *I. atrofusca*, while *I. petrana* produced 0.9% (Table 2) and the differences were statistically significant (Table 3).

Ability of callus to produce green structures (Fig. 4) varied in relation to different species. *I. atrofusca* was the most responsive and yielded 0.09%, followed by *I. petrana* (0.04%), while no results were observed for *I. vartanii* (Table 2). The ability of callus to produce green plantlets varied among genotypes and the frequency was found to be fairly low in all *Iris* species. *I. petrana* yielded 0.04% after transferring the embryo-like structures and the green structures onto regeneration medium (Fig. 5). No positive results were observed with the other two species *I. atrofusca* and *I. vartanii* (Table 2). Albino-plantlets

Fig. 1: Callus formation from flower base of *Iris petrana* on callus culture medium

Fig. 2: Embryo-like structure formation from flower base of *Iris petrana*, two weeks after transferring callus to embryogenesis induction medium

formation was observed after transferring calli to RM (Fig. 6). Albinos ranged from 0.00% for species *I. atrofusca* and *I. vartanii* to 0.08% for *I. petrana* (Table 2).

Callus induction frequency from leaf bases and anthers (Fig. 7) are reported in (Table 4). *I. petrana* was the only genotype that produced calli from these two explants. Leaf bases induced 0.06% calli, while anthers induced 1.30%. The differences were not significant

Table 2 Frequency (%) of five characters for three *Iris* species

Iris species	No of cultured flower bases	(%)of flower bases producing calli	(%) of flower bases producing embryo-like structures	(%) of flower bases producing green structures	(%) of flower bases producing green plantlets	(%) of flower bases producing albino-plantlets
I petrana	2700	58 00	0 90	0 04	0 04	0 08
I atrofusca	2400	43 00	1 60	0 09	0 00	0 00
I vartanii	375	0 00	0 00	0 00	0 00	0 00

Table 3 Mean squares and degree of freedom (df) from analyses of variance for the frequency (%) of five characters for the flower bases of three *Iris* species

Source of variation	df	(%) of flower bases producing calli	(%) of flower bases producing embryo-like structures	(%) of flower bases producing green structures	(%) of flower bases producing green plantlets	(%) of flower bases producing albino-plantlets
Treatment	2	2083 964**	40 808**	0 702	0 364	1 456
Error	6	-	-	-	-	-

** Significant at p = 0 01

Table 4 Frequency (%) of two characters for three *Iris* species

Iris species	No of cultured leaf bases	(%) of leaf bases producing calli	No of cultured anthers	(%) of anthers producing calli
I petrana	1600	0 06	153	1 30
I atrofusca	1000	0 00	56	0 00
I vartanii	100	0 00	100	0 00

Fig. 3: Embryo-like structures formation from flower base of *Iris atrofusca*, six weeks after transferring callus to embryogenesis induction medium

Fig. 4: Green structure formation from flower base of *Iris petrana* on callus culture medium

Fig. 5: Green plantlets formation from flower base of *Iris petrana* on regeneration medium

(Table 5). Hundreds explants of sepals and petals were cultured from the three species but no positive results were obtained.

Table 5 Mean squares and degree of freedom (df) from analyses of variance for the frequency (%) of two characters for the leaf bases and anthers of three *Iris* species

Source of variation	df	(%) of leaf bases producing calli	(%) of anthers producing calli
Treatment	2	0 3640	0 3640
Error	6	-	-

Fig. 6: Albino plantlets formation from flower base of *Iris petrana* on regeneration medium

Fig. 7: Callus formation from anthers of *Iris petrana* on callus culture medium

DISCUSSION

In the present study several trials were carried out to propagate *Iris* tissues from different organs of *I. petrana, I. atrofusca*. However, all attempts were futile except those performed using flower bases. These results were in agreement with Jehan *et al*. (1994) who reported that flower pieces were the best explants for somatic embryogenesis. Also, successful calli propagation was reported from the leaf base of *I. nigricans* (Shibli and Ajlouni, 2000). The only regeneration state was obtained from bases of immature flowers, probably due to the

juvenile state of the explants and perhaps also due to sterilization problems of the other explants, as 95% of the immature flowers can be correctly decontaminated.

Seven weeks after start culturing, the first embryos were formed at the surface of the embryogenic calli. These structures were similar to the somatic embryos described by Reuther (1977) with other *Iris* species and by Jehan *et al.* (1994) with *I. pallida* and *I. germanica* and by Radojevic and Subotic (1992) with *I. setosa*. Three weeks later, these embryos developed into plantlets. *Iris* plantlet was regenerated via somatic embryogenesis; this has the advantage of not destroying the original plant. *I. petrana* had the ability to convert embryos into plantlets better than *I. atrofusca*. This result indicated that flower bases capable of producing plantlets were genotypic dependent. Although, the frequency of green plantlets produced from flower bases was low (0.04%) but this was the first successful attempt and this percentage could be improved in the future by optimizing the culture conditions.

I. vartanii needs more work on the components of CIM, especially the kinds and concentrations of growth regulators in order to enhance callus formation. Effect of genotypes on albino production was clearly recognizable. Formation of albinos is a common phenomenon in plant tissue culture; this could be due to retard in plastids development.

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