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In Vitro Shoot Proliferation via Immature Embryos of Iris kirkwoodiae Chaudhary

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***ABSTRACT: In vitro** techniques can be successfully used for protection of endemic ornamental plants and the geophytes that have wide natural distribution in Turkey. **Iris kirkwoodiae** Chaudhary belonging to Iridaceae family has a geophyte spectacular flowers, and is widely distributed in some mountainous regions in Turkey. The aim of this study was to investigate the possibilities of effective vegetative propagation of **Iris kirkwoodiae** Chaudhary which is a geophyte of Turkey with tissue culture techniques. Therefore, immature embryos of **Iris kirkwoodii** Chaudhary plant were used as explant source and used for **in vitro** propagation. The highest survival rate of immature embryos was 58% on MS medium containing 0.5 mg l^{-1} NAA and number of shoots per explant was the best on MS medium containing 2.0 mgl^{-1} BAP + 2.0 mgl^{-1} NAA with 23.3. Developed plantlets from immature embryos were acclimatized with 20% survival rate 4 weeks after transfer to pots.*

***Key words**; **Iris kirkwoodiae** Chaudhary, immature embryos, **in vitro** shoot proliferation.*

Iris kirkwoodiae Chaudhary Olgunlaşmamış Embriyoları ile In Vitro Sürgün Çoğaltımı

***ÖZ:** In vitro teknikler endemik süs bitkilerinin yanı sıra Türkiye'de doğal yayılış alanına sahip geofitlerin korunması amacıyla da başarılı bir şekilde kullanılabilmektedir. Iridaceae familyasına ait olan **Iris kirkwoodiae** Chaudhary, gösterişli çiçeklere sahiptir ve Türkiye'de bazı dağlık bölgelerde yayılış göstermektedir. Bu çalışmanın amacı, Türkiye'nin geofiti olan **Iris kirkwoodiae** Chaudhary'nin doku kültürü teknikleriyle etkili vejetatif çoğaltım olanaklarının araştırılmasıdır. Bu sebeple, **Iris kirkwoodiae** Chaudhary bitkisine ait olgunlaşmamış embriyolar eksplant kaynağı olarak **in vitro** şartlarda çoğaltımı gerçekleştirmek için kullanılmışlardır. Çalışma sonuçlarında, olgunlaşmamış embriyoların en yüksek canlılık oranı % 58 ile NAA 0,5 mgl^{-1} içeren MS ortamında ve en iyi çoğaltım oranı eksplant başına 23,3 sürgün ile 2,0 mgl^{-1} BAP+ 2,0 mgl^{-1} NAA içeren MS ortamında gözlenmiştir. Olgunlaşmamış embriyolardan gelişen bitkicikler saksılara aktarımdan 4 hafta sonra %20 canlılık oranıyla aklimatize edilmiştir.*

***Anahtar kelimeler: Iris kirkwoodiae** Chaudhary, olgunlaşmamış embriyolar, **in vitro** sürgün çoğaltımı.*

INTRODUCTION

The *Iris* genus, which is a member of the *Iridaceae* family, is a rhizome, bulbous, and rarely tuber-forming plant. It is known that there are 65 genera and 2025 species belonging to the *Iridaceae* family in the world (Asgough *et al*., 2009). The genus is widely distributed from the temperate zone to the subarctic zone in the Northern Hemisphere (Shibata, 1998). Genetic plant diversity is represented by 56 taxa of endemic species with 23 belonging to the *Iridaceae* family in our country (Güner *et al*., 2012; Erken *et al*., 2009). The distinctive and spectacular flower arrangements, colors and leaf arrangements are indicative of the high suitability for the flower industry (Uzun *et al*., 2014). *Iris* species have a wide range of applications in the construction of plants for use in the treatment of upper respiratory tract diseases and in cosmetics industry, for which perfumes, soaps, etc. (Wang *et al*., 1999; Jevremović and

Radojević, 2002; Al-Gabbiesh *et al*., 2006; Francescangeli, 2009; Nasircilar and Deniz, 2014). Significant improvements have been recorded in the plant breeding sector with biotechnological methods and these revolutionary innovations are continuing rapidly. *In vitro* production techniques also provide important steps in the provision of initial breeding materials.

Iris kirkwoodiae Chaudhary, which is called "Maras Kurtkulagi" in Turkish, grows naturally in the Kahramanmaras, Gaziantep and Hatay regions which also show the most geophyte distribution (C6 region) in Turkey. Kahramanmaras-Ahir Mountain is located at the junction between the Mediterranean and Iranian-Turanian floristic regions, at the meeting point of the northern part of the Taurus Mountains. Ahir mountain is one of the most studied mountains with its rich flora and 122 taxa are endemic to Turkey (Anonim, 2015).

Iris species are vegetatively propagated through bulbs (bulbous iris) or splitting of rhizomes (rhizomatous iris). Most of the bulbous *Iris* do not produce more than five daughter bulbs annually (Shibli and Ajlouni, 2000). In rhizomatous *Iris*, the splitting rhizome gives a maximum 10 plants per year (Je´han *et al*., 1994). Furthermore, the propagation of *Iris* species through seedlings is known to be difficult due to poor fruit set and a very low seed germination rate (Simonet, 1932).

Plant tissue culture is a powerful alternative technique for conservation and propagation of plants, especially for those that are rare and difficult to propagate by conventional methods. Therefore, *in vitro* multiplication of *I. kirkwoodiae* Chaudhary will have great valuable for commercial production and *ex situ* germplasm conservation.

The present study, which aimes to supply *in vitro* multiplication of *Iris kirkwoodiae* Chaudhary with plant tissue culture techniques, is the first report for the establishment of high frequency shoot proliferation system via immature embryos in *Iris kirkwoodiae* Chaudhry.

MATERIAL and METHODS

Surface sterilization of plant materials

Immature capsules containing immature embryos, belong to *Iris kirkwoodiae* Chaudhary plants were harvested from the Kahramanmaras-Ahir Mountain of Turkey, in May 2010 and were conserved in the Biotechnology Laboratory of Kahramanmaras Sutçu Imam University, and used in this study. Immature capsules were washed with running tap water and surface sterilized with ethanol of 70% for 1 min, then immersed in NaOCI solution of 25%. The capsules were kept in the solution for 25 min and then washed 3 times with sterile distilled water. Finally, capsules were dried on tissue paper.

Culture of immature embryos

Capsules containing immature seeds were opened under sterile conditions and the immature embryos were excised from the seeds by squeezing with forceps and scalpel. Explants were placed onto MS (Murashige and Skoog, 1962) basal medium supplemented with 2% (w/v) sucrose, 0.7% plant agar (w/v), consisting of different concentrations of 6-benzylaminopurine (BAP; 0.0, 0.5, 1.0 and 2.0 mg l^{-1}) and α-naphtaleneacetic acid (NAA; 0.0, 0.5, 1.0 and 2.0 mg l^{-1}). A total of, 16 different medium combinations were tested. These media were supplemented with growth regulators and adjusted to pH 5.7 prior to autoclaving at 120 ^{0}C for 20 min. Immature embryos were placed in petri dishes (10x100 mm) containing culture medium of 20 ml and incubated in a growth chamber at 24±1°C and dark condition. After 20 days, petri dishes were transferred to light (3000 lux) and all cultures were incubated at 24±1°C under cool white fluorescent light with 16-h (day)/8-h (night) photoperiods. Cultures were subcultured in fresh nutrient media at the and of 6 weeks. The frequency of survival rate 8 weeks after beginning of the culture and shoot regeneration and mean number of shoots per explant after 18 weeks were determined.

Statistical analysis

In this study, sixteen different combinations of plant growth regulators such as BAP and NAA were tested. Each combination had 3 replicates, each replicates consisted of 4 explants. Data was statistically analysed using the JMP 8.0. Means were separated according to the least significant difference (LSD) test at the 0.05 level of probability. The angle transformation values were calculated for the data in percentage (%). As a

result survival rate of embryos and number of shoots per explant were calculated (Steel and Torrie, 1980; Yurtsever, 1984).

RESULTS

Survival rate of immature embryos

Immature embryos were placed on MS medium supplemented with different combinations of BAP and NAA for 8 weeks. No contamination was observed. Eight weeks after, survival rate was observed from immature embryos belonging to *Iris kirkwoodiae*. The highest survival rate was 7 viable explant per 12 immature embryo (58%) on MS medium supplemented with 0.5 mg l^{-1} NAA (Table 1). Immature embryos that were cultured in medium containing 1.0 mg l^{-1} NAA, 2.0 mg l^{-1} NAA and 2.0 mg l^{-1} BAP+0.5 mg l^{-1} NAA developed only callus. Hovewer, these callus were not embryogenic callus.

Shoot regeneration from viable immature embryos

In the study, the shoot regeneration rates of embryos surviving and developing were determined at the end of three subcultures (Table 1). Plant growth regulators had statistically significant effect on the percentage of explants producing shoots and the mean number of shoots per explant of embryos (Figure 1). In our study, BAP and NAA were used as the plant growth regulators for shoot regeneration from immature embryos. The highest number of shoots per explant (23.3 shoots/explant) were obtained on the MS media supplemented with 2.0 mg l^{-1} BAP + 2.0 mg l^{-1} NAA (Table 1). All of the embryos formed green coloured compact callus about 2-4 weeks after culture initiation in all media tested. Embryogenic clusters from immature embryos were visible after 8-10 weeks on compact calli. Shoot proliferation were seen on explants 12 weeks after culture initiation (Figure 2a-c). It has been determined that there was a tendency for callus formation in explants with increasing NAA concentrations. The root formation occured in the medium containing only auxin (0.5 mg l^{-1} NAA), but not shoot formation. Also, root formation showed on developed shoots on MS media supplemented with 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA after three sub-culture (Figure 2d-e). Well-developed and rooted plantlets were transferred into potting mixture of sterilised peat-perlite (2:1) after 16 weeks (Figure 2f).

Table 1. Survival rate of embryos (%) (after 8 weeks) from immature embryos and shoots growth (after 18 weeks) on MS medium suplemented with various concentrations of BAP and NAA.
Çizelge 1. Çeşitli BAP ve NAA konsantrasyonları eklenen MS ortamında embriyoların canlılık oranları (%) (8 hafta sonra) ve olgunlaşmamış embriyolardan sürgün gelişimi (18 hafta sonra).

Plant growth regulators (mg l^{-1}) Bitki büyüme düzenleyicileri (mg l^{-1})		Survival rate of embryos (%) Embriyo canlılık oranları (%)	Mean number of shoots per explants Eksplant başına düşen ortalama sürgün sayısı (adet)*	
BAP	NAA			
0.5	-	8.3	1.16	f
1.0	-	16.6	1.16	f
2.0	-	25	15.83	bc
-	0.5	58.3	1.5	f
-	1.0	25	1.0	f
-	2.0	8.3	1.0	f
0.5	0.5	50	9.8	de
0.5	1.0	16.6	11.6	cd
0.5	2.0	25	6.0	ef
1.0	0.5	33.3	11.5	cd
1.0	1.0	16.6	10.6	cde
1.0	2.0	25	18.6	ab
2.0	0.5	8.3	1.0	f
2.0	1.0	8.3	1.0	f
2.0	2.0	50	23.3	a
Control		16.6	1.16	f

*Means followed by different letters in same column are significantly different at 0.05 level of significance.
* Aynı sütunda farklı harflerle gösterilen ortalamalar arasındaki fark 0.05 seviyesinde önemlidir.

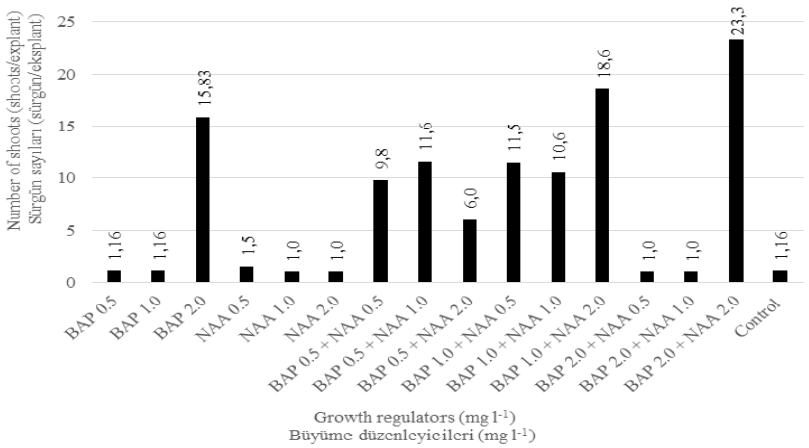

Figure 1. Shoot regeneration from immature embryos after 18 weeks on MS media supplemented with various concentrations of BAP and NAA.
Şekil 1. Çeşitli BAP ve NAA konsantrasyonları eklenen MS ortamlarında 18 hafta sonra olgunlaşmamış embriyolardan sürgün rejenerasyonu.

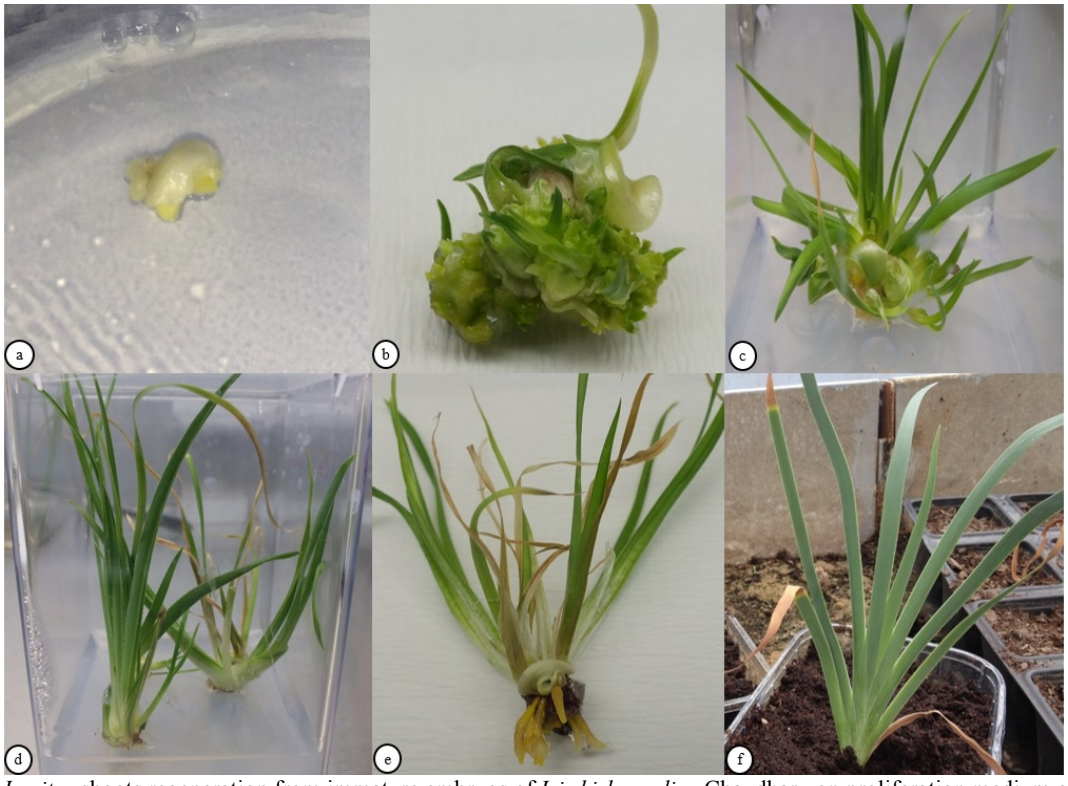

Figure 2. *In vitro* shoots regeneration from immature embryos of *Iris kirkwoodiae* Chaudhary on proliferation medium containing 2.0 mg l^{-1} BAP +2.0 mg l^{-1} NAA, (a) development of embryogenic callus 10 days after culture, (b) prolific shoot regeneration 8 weeks after culture, (c) development of shoots from explant 12 weeks after culture, (d) development of shoots on medium containing 0.5 mg l^{-1} BAP +0.5 mg l^{-1} NAA, after 14 weeks in culture (e) rooting of shoots on medium containing 0.5 mgl^{-1} BAP +0.5 mg l^{-1} NAA, after 14 weeks in culture, (f) well-developed plantlets of 8 weeks after tranfer into potting mixture peat-perlite.
Şekil 2. *Iris kirkwoodie* Chaudhary olgunlaşmamış embriyolarından *in vitro* sürgün rejenerasyonu, 2.0 mgl^{-1} BAP +2.0 mgl^{-1} NAA içeren çoğaltım ortamlarında (a) kültüre alındıktan 10 gün sonra embriyojenik kallus gelişimi, (b) kültüre alındıktan 8 hafta sonra sürgün rejenerasyonu, (c) kültüre alındıktan 12 hafta sonra eksplantlardan sürgünlerin gelişimi, (d) kültüre alındıktan 14 hafta sonra 5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA içeren ortamda eksplantlardan sürgünlerin gelişimi, (e) kültüre alındıktan 14 hafta sonra 5 mg l^{-1} BAP +0.5 mg l^{-1} NAA içeren ortamda sürgünlerin köklenmesi, (f) torf-perlit karışımı içeren saksılara aktarıldıktan 8 hafta sonra iyi gelişen bitkicikler.

In the research, 20 plants growing and rooted plants were transferred to greenhouse conditions. Four of the 20 *Iris kirkwoodiae* Chaudhary plants, which were grown in pots including peat: perlite (2:1) mix and acclimating to external conditions continued their growth and adapted to greenhouse. These plants after a period of 15 months started to flowering without any treatment.

DISCUSSION

Embryos, one of the least time-consuming and easier explants in geophytic plants, have been used as explant resources in *in vitro* micropropagation studies, where proliferation with seed takes a long time or otherwise requires some chemical applications to break dormancy. High frequency of shoot regeneration has been studied in recent years from immature embryo explants of many geophytes (Mirici *et al*., 2005; Ipek *et al*., 2009; Uranbey, 2010a; Uzun *et al*., 2014). The use of immature embryos for induction of regeneration or propagation has many advantages. These explants which have high regeneration productivity may be an alternative explant source for the multiplication of geophytes (Mirici *et al*., 2005). The results of the study showed that immature embryos stimulated shoot regeneration. Previous studies relating to shoot proliferation in geophytes indicated that the addition of plant growth regulators to the basal nutrient medium supported shoots and bulblet regeneration of many geophytes (Ulrich *et al*., 1999; Wawrosch, 2001; Mirici *et al*., 2005; Uranbey, 2010a).

The cytokinins such as BAP, KIN (alone or in combination with auxin) are generally used to promote shoot proliferation in plant tissues (Ozcan *et al*., 1996; Çöçü *et al*., 2004; Aasim *et al*., 2013; Uzun *et al*., 2014) and especially to promote organogenesis from immature plant materials (Ozcan *et al*., 1992; Ozcan *et al*., 1996; Mirici *et al*., 2005; Uranbey, 2010b; Uzun *et.al*., 2014).

In the present study, immature embryos of *Iris kirkwoodiae* Chaudhary plants were cultured on MS medium supplemented with 16 different concentrations and combinations of BA and NAA in order to optimize propagation MS medium containing BAP and NAA combination more potent in stimulating shoot proliferation compared to only NAA concentration. When the shoots were subcultured, after 12 weeks a remarkable advance of shoot was observed in MS medium enriched with 2.0 mg l^{-1} BAP and 2.0 mg l^{-1} NAA, 1.0 mg l^{-1} BAP and 2.0 mg l^{-1} NAA, respectively. Both concentrations of NAA in combination with BAP did not affect the regeneration response and number of bulblets per explant of oriental lily (Kumar *et al*., 2007). However, this study showed that shoot regeneration was more significant on MS medium containing 0.5 or 1.0 mg l^{-1} BAP combinations with 0.5,1.0 or 2.0 mg l^{-1} NAA using immature embryo. Nasircilar and Deniz, (2014) also reported that the best freguencies of shoot formation from immature embryos of *Iris pampyhlica* were cultured on MS medium supplemented 2.0 mg l^{-1} BAP + 0.25 mg l^{-1} NAA. This present study also gave the similar result, but the concentrations of 2.0 mg l^{-1} BAP and 2.0 mg l^{-1} NAA was slightly changed. The interaction on the plants of plant growth regulators which applied by adding to the nutrient media may be different between species and even between explant types used (Boltenkov and Zarembo; 2005). The BAP-NAA combination, which is frequently used in geophyte ornamental plants, may be suitable for micropropagation (Kawase *et al*.,1995, Suzhen *et al*.,1999; Shibli and Ajlouni, 2000, Boltenkov *et al*., 2005, Boltenkov and Zarembo, 2005; Kapoor *et al*., 2009; Uzun *et al*., 2014). In *in vitro* studies, cytokinin is necessary for plant cells because of regulator effect on protein synthesis (George *et al*., 2007) BAP is the most effective and low priced cytokinin for tissue culture studies (Pattnaik and Chand, 1997, Sevindik *et al*., 2017).

According to the findings, micropropagation of *Iris kirkwoodiae* Chaudhary plant was successfully achieved and very promising results were obtained. As it can be seen in the study output, it also contains data that can be used for *in vitro* proliferation of other species of *Iris* L. genus. Moreover, this protocol, in which immature embryos are used as explant source can be applied for rapid propagation of other geophytes species with potential for ornamental plants and also could be a useful method for germplasm conservation of valuable plant species.

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