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IN VITRO PROPAGATION OF ENDEMIC, ENDANGERED PLANT SPECIES *AUBRIETA OLYMPICA* BOISS.

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ABSTRACT

Turkey has a rich biological diversity due to its geographical feature. Uludağ mountain is one of the Important Plant Areas of Turkey. Thus, native plant species are threatened due to various environmental factors and human damage. The micropropagation possibility for *Aubrieta olympica* Uludağ endemic threatened plant species, were investigated in this study. The effect of different concentrations of agar and different levels of pH were evaluated on shoot development. During the proliferation stage, the best development occured at 0.6% agar and pH 5.7-5.8. Shoot tips and internode explants were the best source for the highest shoot induction of *A.olympica* species. The best results for shoot formation were obtained with 0.5 ppm Kinetin and plant growth regulators free Murashige and Skoog medium. Elongated shoots were rooted in the Murashige and Skoog medium supplemented with different concentrations of indole-3-butyric acid, indole-3-acetic acid, and also a medium without plant growth regulators. However, *A.olympica* shoots spontaneously rooted on plant growth regulators free medium. Shoots which developed roots were transferred to greenhouse, successfully. The results of this study could be utilized for conservation purposes of endemic and endangered plant species.

Keywords: *Micropropagation, Uludağ, Plant growth regulators.*

ENDEMİK, NESLİ TEHLİKE ALTINDA OLAN *AUBRIETA OLYMPICA* BOISS. BİTKİ TÜRÜNÜN *IN VITRO* ORTAMDA ÇOĞALTILMASI

ÖZ

Türkiye coğrafik özelliklerinden dolayı zengin bir biyolojik çeşitliliğe sahiptir. Çalışma alanımız olan Uludağ, Türkiye'nin Önemli Bitki Alanlarından birisidir. Bununla birlikte, doğal bitki türlerimizin nesilleri, çeşitli çevresel ve insan faktörlerinden dolayı tehlike altındadır. Çalışmamızda, Uludağ endemiği olan ve nesli tehlike altında olan *Aubrieta olympica* türünün doku kültürü şartlarında çoğaltılma imkanı araştırılmıştır. Farklı agar konsantrasyonları ve farklı pH değerlerinin sürgün gelişimi üzerine etkileri değerlendirilmiştir. Çoğaltma aşaması süresince en iyi sürgün gelişimi % 0,6 agar ve pH 5,7-5,8'de meydana gelmiştir. *A. olympica* bitkisinde sürgün rejenerasyonu için en iyi eksplant kaynağının sürgün ucu ve internod olduğu tespit edilmiştir. En iyi sürgün rejenerasyonu 0,5 ppm Kinetin ve büyüme düzenleyici içermeyen içeren Murashige and Skoog besin ortamlarında elde edilmiştir. Yeterli uzunluğa ulaşmış sürgünler farklı konsantrasyonlarda indol-3-bütirik asit, indole-3-asetik asit ve büyüme düzenleyici içermeyen besin ortamında köklendirilmiştir. Bunun yanında, *A.olympica* sürgünleri bitki büyüme düzenleyici içermeyen besin ortamında kendiliğinden köklenmiştir. Köklenen sürgünlerin sera şartlarına başarılı bir şekilde adaptasyonu sağlanmıştır. Bu çalışma sonuçları, diğer endemik ve nesli tükenmekte olan bitkilerde yapılacak olan koruma çalışmalarına temel oluşturacaktır.

Anahtar kelimeler: *Çoğaltma, Uludağ, Bitki büyüme düzenleyiciler.*

1. INTRODUCTION

Aubrieta olympica Boiss., belonging to the Brassiaceae family, is an endemic and endangered perennial species. This natural populations are located Uludag mountain in Turkey. Uludağ mountain is the highest mountain in the Marmara region, including Thrace and the north-western side of the Anatolian peninsula. The climate of the mountain changes from the base to the alpine belt and resulting a rich plant diversity. On account of this high plant diversity, Uludag mountain is one of the Important Plant Areas (IPAs) of Turkey [1].

Thus, Uludag is most famous centers for winter sports in Turkey. In alpine environments, natural or human disturbances are very common. The species habitat has been damaged construction of hotels, ski areas, excessive human activities and use of artificial snow [2-4]. For this reasons, *A. olympica* species habitat have been damaged and this species is classified as Endangered (EN) according to IUCN classification and it is under threat of extinction according to Europe Important Plant Areas Project (IPAs) [5, 6].

A. olympica species grows on rocks poor in soil and prefers calcareous habitats, especially within rocky clefts. The species is wide spread on the mountain at altitudes over 2000 m (2000-2450 m). This plant is perennial herb; stems are prostrate, leaves are oblanceolate, dentate, the lower frequently subopposite, sepals saccate, petals violet, 11-19 mm and fruits are oblong, flattened. Endemic to Uludağ. Flowering specimens of this species can be seen with the rocky clefts together with other endemics (ie. *Senecio hypochionaeus* Boiss. var. *hypochionaeus*, *Papaver pilosum* Sibth. & Sm., etc) at the higher reaches of the mountain in June-August [5, 7].

In recent years, with the increasing withitness of extinction risks, *ex situ* conservation has been more important. *Ex situ* conservation provide recover and rehabilitation of threatened species and their reintroduction into natural habitats under appropriate conditions [8-11].

A. olympica is rare endemic plant species which is under threat of extinction. The development of a viable micropropagation protocol, is an effective and rapid method for clonal propagation and conservation of many rare and endemic species [12-16]. Up until today, no study has been initiated on *in vitro* propagation of *A. olympica*. Therefore, in the present research it is aimed to develope a successful *in vitro* regeneration system for *A. olympica* from shoot tips and internodes under *in vitro* culture conditions.

2. MATERIALS AND METHODS

Mature seeds of the *A. olympica* were collected from alpine belt between 2000 and 2400 m of Uludağ Mountain during July-August 2010. Seeds were air dried at room temperature and sealed in sample bags till further use. Seeds of *A. olympica* was surface sterilised in 0.5, 1 and 2% sodium hypochlorite (NaOCl) with a few drops of the surfactant Tween-20 for 10 min, then rinsed three times with sterile distilled water. Seeds germinated in a growth chamber at 21/16 ^{o}C and 16/8 h photoperiod on Murashige and Skoog (MS) medium supplemented with 3% sucrose [17]. In order to study the effect of different agar concentrations on shoot multiplication, MS medium was modified using different concentrations (0.6, 0.7, 0.75, 0.80%) of agar. The pH of the medium was adjusted between 5.7-7.0 (5.7, 5.8, 6.0, 6.5, 7.0) and autoclaved at 121 ^{o}C and 1.1 atm for 20 min. Seeds were aseptically transferred into Magenda vessels containing MS medium [18]. Each trial consisted of five magenda dish containing ten seeds. Three replicates were used for each treatment.

Seeds germinated after 9-15 days. *A. olympica* shoot tips and internode explants were removed from 30 days old *in vitro* germinated seedlings (Figure 1a). Full strength MS basal medium with different concentrations of 6-benzylamino purine (BAP) (1, 2, 4, 6, 8 ppm) and kinetin (Ki) (0.5, 1, 2, 3 ppm) were used for *in vitro* multiplication responses. Plant growth regulator free medium was used as control. Subculture was achieved three times for eight weeks.

The regenerated shoots (2 to 3 cm) were excised and induvidually transferred into MS medium without plant growth regulators (control) or with various concentrations of indole-3-butyric acid (IBA) (0.5, 1, 1.5, 2 ppm) and indole-3-acetic acid (IAA) (0.5, 1, 1.5, 2 ppm) to test the rooting potential. The number of roots per shoot, root lengths and rooting percentage were determined after 8 weeks from the culture initiation. Rooted plantlets were acclimatized in a growth chamber and transferred to 16 cm pots containing 2:1 torf and perlite, and were grown under greenhouse conditions.

Each treatment had three replicates containing five explants in each culture vessel. Collected data were subjected to one-way analysis of varience (ANOVA, SPSS for Windows 18.0) and the post hoc analysis was performed using Duncan's multiple range test. Graphs were prepared using SPSS.

3. RESULTS AND DISCUSSION

The most important and rather diffucult aspect of the *in vitro* techniques is the requirement to carry out various operations under aseptic conditions [19]. Prevention of contamination of tissue culture media is important for the whole process of plant propagation. So sterilization of seeds before *in vitro* culture is obligatory. In this study, there were no contamination observed at all level of NaOCl concentrations on the seeds of *A. olympica*. The results presented in Table 1 show clearly that the increasing NaOCl concentration reduced % of seed germination in this species. 0.5% NaOCl treatment was selected as the optimum concentration for *A. olympica* seeds. Similar findings were obtained by other previous studies on rare, endemic and endangered plant species [12, 13, 20].

Table 1. Effects of different concentrations of NaOCl on decontamination and germination of *A. olympica* seeds.

Treatments	% of seeds germinated	% of seeds contaminated
0.5% NaOCl x 10 min.	96.67 ± 3.33 a*	0.00
1.0% NaOCl x 10 min.	80.67 ± 0.42 b	0.00
2.0% NaOCl x 10 min.	80.33 ± 1.33 b	0.00

* Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P < 0.05$), ± SE.

The pH of the culture medium is an important factor for proliferating shoots *in vitro* [21]. Medium pH is extremely important, as it influences the uptake of nutrients and plant growth regulators by regulating their solubility in the culture media [22]. Our results indicated that the number of shoots per explant and shoot length increased the pH from 5.7 to 6.0 (Table 2). Although shoot proliferation was highest on medium at pH 6.0, high pH levels caused serious abnormalities and shoots were non-viable. For this reason, a better performance in all parameters on shoot development was found at pH 5.7 and 5.8. Similar finding were obtained by Karim *et al*. (2007) [21], Ebrahim and Ibrahim (2000) [23], Gurel and Gulsen (1998) [24], and Finn *et al* (1991) [25].

Table 2. Effect of pH value on shoot formation of *A. olympica* shoot tips cultured for sixty days on MS basal medium supplemented with benzyladenine (2 mg/l).

pH value	Number of shoot per explant	Shoot length (mm)
5.70	3.50 ± 0.17 a *	18.17 ± 0.73 b
5.80	3.73 ± 0.12 a	17.57 ± 0.30 b
6.00	3.87 ± 0.12 a	20.37 ± 0.32 a
6.50	2.50 ± 0.05 b	16.83 ± 0.44 b
7.00	2.23 ± 0.25 b	15.00 ± 0.28 c

* Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P < 0.05$), ± SE.

Agar concentration is important in determining culture response. Concentrations of agar in the medium can effect the culture growth and shoot development. The lowest agar concentration (0.60%) induced the the number of explants forming shoots (2,57 ± 0,88 shoot/explant) while shoot length increased by increasing agar concentration to 0.75% (18,67 ± 0,88) . However, healtly plant growth was obtained at low agar concentration (Table 3). Low agar levels have been reported to promote shoot proliferation in several culture systems [24, 26] (Gurel & Gulsen, 1998; Suthar *et* al., 2011). According to Abdoli *et al*. (2007) [27], average number of shoots per explant had increased at low concentration of agar such as 0.6 %.

Table 3. Effect of agar concentration on shoot formation of *A. olympica* shoot tips cultured for sixty days on MS basal medium supplemented with benzyladenine (2 mg/l).

Agar concentration %	Number of shoot per explant	Shoot length (mm)
0.60	2.57 ± 0.88 a *	12.00 ± 0.58 b
0.70	2.00 ± 0.00 b	17.17 ± 0.73 a
0.75	2.13 ± 0.88 b	18.67 ± 0.88 a
0.80	1.92 ± 0.44 b	13.83 ± 0.93 b

* Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P < 0.05$), ± SE.

Many plant species have very specific *in vitro* requirements for multiplication and therefore, substantial variation is observed in the culture medium formulations. A range of cytokinins (BAP, kinetin, 2-iP, zeatin and TDZ) commenly used in micropropagation studies [28-32]. The effect of various concentrations of PGRs (BAP and Ki) in shoot tips and internode explants of *A. olympica* were studied (Table 4). Shoot tips and internode explants were the best source for the highest shoot induction. The highest number of shoots were obtained in 0.5 ppm Ki both in shoot tips and internode explants, on the other hand other shoot parameters were enhanced by PGRs-free MS medium (control) (Fig. 1b). This effect was also described in various species [33-34].

Table 4. Influence of different concentrations BAP and Ki and various explant types on shoot formation of *A. olympica*.

Explant	BAP (ppm)	Ki (ppm)	Number of shoots /explant	Shoot length (mm)	Number of leaves /explant	Shooted explant %
Shoot tip	0	0	3.16 ± 0.16 b *	35.16 ± 0.39 a	39.97 ± 0.55 a	100.00 ± 0.00 a
	1	0	1.95 ± 0.16 cd	16.00 ± 0.39 c	15.90 ± 0.49 e	94.07 ± 0.58 c
	2	0	2.20 ± 0.16 c	18.27 ± 0.39 b	22.70 ± 0.32 c	93.83 ± 0.47 c
	4	0	2.00 ± 0.16 cd	15.07 ± 0.39 cd	14.30 ± 0.38 f	96.76 ± 0.43
	6	0	1.57 ± 0.16 de	14.60 ± 0.39 de	10.07 ± 0.26 g	86.50 ± 0.87 d
	8	0	1.17 ± 0.16 e	14.50 ± 0.39 de	10.30 ± 0.35 g	94.20 ± 0.55 c
	0	0.5	4.10 ± 0.16 a	15.08 ± 0.39 cd	24.50 ± 0.36 b	83.33 ± 0.53 e
	0	1	2.93 ± 0.16 b	10.47 ± 0.39 f	19.23 ± 0.34 d	66.50 ± 0.44 g
	0	2	2.90 ± 0.16 b	14.10 ± 0.39 de	23.73 ± 0.50 bc	80.03 ± 0.54 f
	0	3	2.80 ± 0.16 b	13.55 ± 0.39 e	23.17 ± 0.41 c	83.17 ± 0.60 e
İnternode	0	0	2.50 ± 0.22 bc	34.47 ± 0.34 a	41.10 ± 0.56 a	100.00 ± 0.00 a
	1	0	3.07 ± 0.22 ab	20.10 ± 0.34 b	24.80 ± 0.47 b	96.47 ± 0.29 b
	2	0	2.20 ± 0.22 c	17.37 ± 0.34 c	20.00 ± 0.32 d	73.43 ± 0.41 f
	4	0	1.50 ± 0.22 d	13.33 ± 0.34 e	11.73 ± 0.43 e	93.67 ± 0.44 c
	6	0	1.20 ± 0.22 d	11.07 ± 0.34 f	9.77 ± 0.58 f	63.50 ± 0.51 ı
	8	0	1.30 ± 0.22 d	9.43 ± 0.34 g	11.27 ± 0.33 e	63.40 ± 0.29 ı
	0	0.5	3.37 ± 0.22 a	14.77 ± 0.34 d	24.63 ± 0.50b	83.47 ± 0.38 d
	0	1	2.60 ± 0.22 bc	13.17 ± 0.34 e	24.13 ± 0.38 bc	66.37 ± 0.35 h
	0	2	2.83 ± 0.22 abc	13.70 ± 0.34 e	22.97 ± 0.52 c	70.53 ± 0.26 g
	0	3	2.83 ± 0.22 abc	13.00 ± 0.34 e	23.87 ± 0.33 bc	80.10 ± 0.21 e

* Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P< 0.05$), ± SE

In vitro regenerated shoots of *A. olympica* showed different behaviour during the processes of rooting. Thus, the number of roots per shoot, root length and the percentage of rooting have changed significantly with different concentrations of IBA and IAA (Table 5, Fig. 1c). However, compared to the control and IBA, the best root length was

obtained 0.5 mg IAA (32,90 ± 0,52). Besides, the highest number of root and rooting percentage were obtained from the PGRs-free medium. There are several reports on suitability of PGRs-free medium for rooting [33, 35-37].

Table 5. Effect of different growth regulators (IBA & IAA) on rooting of *in vitro* regenerated shoots after 8 weeks of rooting treatments.

Medium	IBA (ppm)	IAA (ppm)	Number of roots/shoot	Root length (mm)	Rooting (%)
MS	Control		20.90 ± 0.49 a	28.57 ± 0.46 b	100.00 ± 0.00 a
	0.5	0.0	11.90 ± 0.52 de	20.80 ± 0.26 d	88.37 ± 0.59 c
	1.0	0.0	11.17 ± 0.44 ef	12.70 ± 0.47 g	82.30 ± 0.47 e
	1.5	0.0	7.90 ± 0.26 gh	9.97 ± 0.38 h	73.27 ± 0.43 h
	2.0	0.0	8.90 ± 0.38 g	16.67 ± 0.44 e	70.00 ± 0.46 i
	0.0	0.5	14.20 ± 0.44 c	32.90 ± 0.52 a	91.53 ± 0.26 b
	0.0	1.0	10.07 ± 0.38 f	12.63 ± 0.45 g	88.90 ± 0.26 c
	0.0	1.5	7.70 ± 0.44 gh	14.80 ± 0.40 f	78.27 ± 0.33 g
	0.0	2.0	7.97 ± 0.26 gh	22.53 ± 0.26 c	80.37 ± 0.58 f

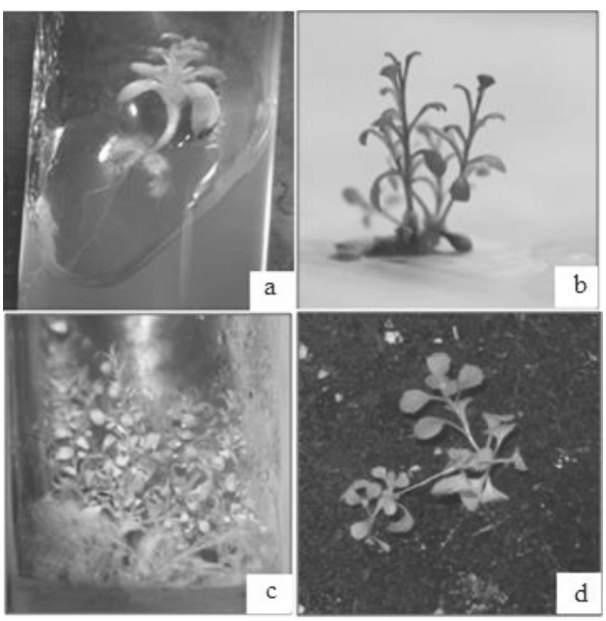

Fig. 1 Seed germination, shoot regeneration, rooting and acclimatization of *A. olympica* **a.** 30-40 days old seedling cultured on MS medium **b.** *In vitro* shoot regeneration from a shoot tip explant cultured on 0,5 ppm Ki **c.** Microshoots rooting on PGRs-free MS medium **d.** Acclimatized plantlets grown in a greenhouse.

Finally, rooted plantlets were transferred for acclimatization to pots containing 2:1 mixture of torf and perlite, and grown in a growth chamber. The survival rate of regenerated *A. olympica* plantlets transferred to greenhouse was 75% (Fig. 1d). Acclimatized plantlets were healthy and well developed when tranferred to soil.

4. CONCLUSIONS

As a result, *in vitro* techniques are a potentially useful technique for growing rare, threatened endemic plants. We have described first time here a proper and simple micropropagation system for *A. olympica.* The regeneration system reported here can be successfully used in studies dealing with *in vitro* conservation of *A. olympica* and it may also be applied on related species belonging to the same genus *Aubrieta.*

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