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Full Length Research Paper

In vitro propagation of *Arabis drabiformis* Boiss. (Brassiaceae) an endemic rare species of Uludağ mountain (Bursa-Turkey)

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***In vitro* methods were widely used in the conservation of the threatened plants in recent years and it is clear that this trend is increasingly applied to more plant species facing risk of extinction. This study aimed to standardise a simple and proper micropropagation system for relic endemic plant species *Arabis drabiformis by* using shoot tip and internode explants. Shoot tip and internode explants were excised from 30 to 40 day old *in vitro* germinated seedlings and cultured on Murashige and Skoog (MS) media containing different combinations of benzylamino purine (BAP) and kinetin (Ki). MS medium supplemented with 2.0 ppm BAP was the most effective for shoot formation. Elongated shoots were successfully rooted in the MS medium that contained 0.5 ppm indole-3-butyric acid (IBA). Propagation and growth of plantlets under greenhouse condition were managed successfully.**

Key words: *Arabis drabiformis*, *in vitro* propagation, shoot regeneration.

INTRODUCTION

Besides the conventional propagation methods, endemic and threatened plants can be efficiently conserved with various *ex vitro* and *in vitro* cultural methods which have low impact on wild populations with a minimum requirement of plant material (Cuenca, 1999; Rao, 2004; Fay, 1992). *Arabis drabiformis* Boiss. (Brassiaceae) is an endemic species of Uludağ mountain in Turkey. The species habitat has been damaged by heavy recreational and winter sports activities. In alpine environments, natural or human-induced disturbances are fairly common. Human impact on alpine areas has been continuously increased as a result of development of resorts and recreational activities (Urbanska and Fattorini, 2000; Pintar et al., 2009). *A. drabiformis* species is classified as vulnerable (VU) according to the IUCN classification (Güleryüz, 1998) and it is under threat of extinction according to Europe Important Plant Areas (IPAs) Project.

Uludağ mountain has a rich flora containing numerous endemic species and several well-distinguished vegetation types which vary from Mediterranean to Euro-Siberian and Alpine type. Because of this high plant diversity, Uludağ mountain is one of the IPAs of Turkey (Güleryüz et al., 2010). *A. drabiformis* spp. grows on sandy-loam and calcareous soils of the Uludağ mountain. This plant is a perennial herb with a stout woody stock; the stems are erect, glabrous and unbranced; leaves are all basal, oblanceolate and setosepilose; petals are white and fruits are siliquae. Species is distributed on alpine pastures (*Acantholimon ulucinum* and *Festuca punctoria* community) at the altitudes above 1900 m. *A. drabiformis* shares its habitats with another endemic species such as *Veronica caespitosa* var. *caespitosa*, *Astragalus hirsutus* Vahl., *Astragalus sibthorpianus*, *Festuca punctoria*, *Linum olympicum*, *Muscari baurgaei* and *Thymus bornmuelleri*. The flowering time is in June and August (Güleryüz, 2000).

A. drabiformis seeds exhibit strong dormancy characteristics that are consistent with the enviromental conditions in its habitat. The development of an efficient regeneration protocol can assist the conservation of this rare/endemic plant species which is under threat of

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Table 1. Effects of different concentrations of NaOCl on decontamination and germination of *Arabis drabiformis* seeds.

Treatment	Percentage of seeds germinated	Percentagbe of seeds contaminated
0.5% NaOCl × 10 min	19.05 ± 2.38[c]*	34.57 ± 2.38[a]
1.0% NaOCl ×10 min	66.67 ± 1.51[a]	4.76 ± 1.51[c]
2.0% NaOCl × 10 min	51.33 ± 0.93[b]	21.14 ± 0.8 [b]

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

extinction. Compared to slow conventional propagation methods, tissue culture represents an important potential method used to effectively propagate *A. drabiformis*.

In vitro multiplication and cryopreservation techniques for endangered plant species make possible their reintroduction into the natural environment (Wochock, 1981) and reduce the risk of extinction (Nadeem et al., 2000; Chandra et al., 2006). However, no study exists about the *in vitro* regeneration protocol of *A. drabiformis* spp. Therefore, this study was aimed to develop a successful *in vitro* regeneration systems for *A. drabiformis* from shoot tips and internodes under *in vitro* culture conditions.

MATERIALS AND METHODS

Mature seeds of *A. drabiformis* were collected from subalpine belt between 2100 and 2300 m of Uludağ mountain during August 2009. Laboratory experiments started after drying and cleaning of the seeds. Surface sterilization of seeds was carried out with 0.5, 1 and 2% NaOCl solution containing a few drops of the surfactant Tween-20 for 10 min followed by three rinsing steps with sterile distilled water. Seeds germinated in a growth chamber at 21/16°C and 16/8 h photoperiod on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and 0.6% agar. The nutrient medium was adjusted to pH 5.8 prior to autoclaving at 121°C and 1.1 atm for 20 min. Seeds were aseptically transferred into Magenda vessels containing media with 0 (control) and 100 ppm of gibberellic acid (GA_3) (filter-sterilised and added to the growth media after autoclaving) (Kocaçalışkan, 2008). Each trial consisted of five magenda dish containing ten seeds. Three replicates were used for each treatment.

Seeds germinated after 7 to 13 days. Shoot tip and internode explants were removed from 30 days old *in vitro* germinated seedlings and were initially placed on MS media with various concentrations of BAP (0, 1, 2, 4, 6 and 8 ppm) and Ki (0.5, 1, 2 and 3 ppm) for eight weeks. Subculture was achieved three times.

The regenerated shoots (2 to 3 cm) from shoot tips and internodes were excised and individually transferred into MS medium without plant growth regulators (control) or with various concentrations of IBA (0.5, 1, 1.5 and 2 ppm) in 50 ml Magenda vessels to test the rooting potential. The number of roots per shoot, root lengths and rooting percentage were determined after eight weeks from the culture initiation. Rooted plantlets were acclimatized in a growth chamber and were transferred to 16 cm pots containing 2:1 torf and perlite, and were grown under green house conditions for nine months.

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

RESULTS

Sterilization is the most important stage in the tissue culture experiments. In this study, the frequencies of germinated and contaminated seeds changed significantly with the different concentrations of sodium hypochlorite. One percent sodium hypochlorite treatment was selected as the optimum concentration for the decontamination and germination of *A. drabiformis* seeds (Table 1). The sterilization procedure for seeds was satisfactory. However, *A. drabiformis* seeds germinated in *in vitro* conditions without GA_3 (62.33% germination) and addition of 100 ppm GA_3 increased the germination response to 89.33% .

Shoot tips and internode segments were excised from 30 to 40 days old seedling (Figure 1) and they were cultured on the MS media supplemented with different concentrations of BAP and Ki. The influence of different concentrations of BAP and Ki plant growth regulators and various explant types on the shoot differentiation of *A. drabiformis* are given in Table 2. Shoot tips were the best source for the highest shoot induction as compared to internode (Figure 2). All explants developed shoots after eight weeks from the culture initiation in all concentrations of BAP and Ki. Parameters of shoots and leaves were significantly influenced by plant growth regulators. For both shoot tips and internode explants, the highest number of shoots per explant, shoot length, number of leaf per explant and percentage of shooted explant were obtained in the MS medium supplemented with 2.0 ppm BAP. As a result, the best shoot multiplication was also achieved in a media containing 2.0 ppm BAP. When BAP and Ki growth regulators were compared with each other in both explant types, it was found that BAP was more effective than Ki on shoot parameters.

Adventitious root formation is very important for the vegetative propagation and a key step in micropropagation systems. An efficient modification on rooting may cause high frequency of rooted shoots and a high-quality root system. Regenerated shoots (~ 10 mm length) were excised and readily rooted in the MS media supplemented with different concentration of IBA to induce the development of roots and to reduce the duration of root induction (Figure 3). The number of roots per shoot, root length and the percentage of rooting were changed significantly with different concentrations of IBA.

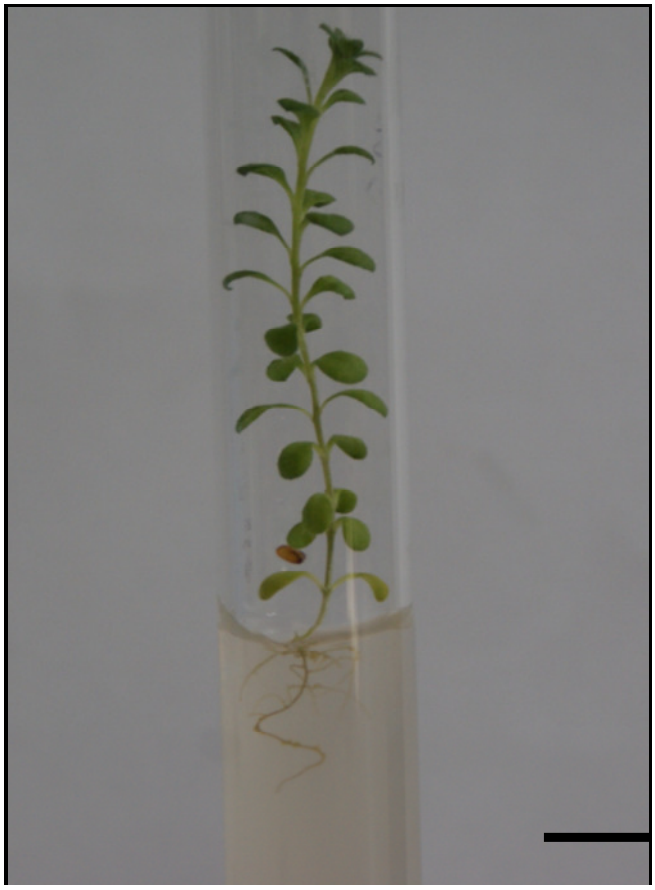

Figure 1. 30 to 40 days old seedling of *A. drabiformis* cultured on MS medium (bar 0.5 cm).

Figure 2. Shoot regeneration from shoot tips of *A. drabiformis* (bar 1 cm).

The highest number of root, root length (mm) and rooting percentage were obtained from the MS medium supplemented with 0.5 ppm IBA (Table 3).

Well rooted shoots were rinsed with sterilized water to remove residual rooting medium and were transplanted to pots containing 2:1 mixture of torf and perlite, and were grown in a growth chamber (Figure 4). The survival rate of regenerated plantlets transferred to greenhouse was 80%. These plants were phenotypically normal and showed no abnormality in their morphology.

DISCUSSION

This study represents, for the first time, a simple and proper methodological procedure for the tissue culture and micropropagation of *A. drabiformis*, a rare plant endemic to Uludağ/Bursa. Contamination is a major obstacle for *in vitro* culture of plants. It is particularly important when the material source is often limited and usually located in the wilds. Contamination from internal sources can be potentially serious during cultivation experiments, because of the plant tissues hosting endophytic bacteria or fungi (Sarasan et al., 2006). The use of seeds is generally a preferable approach to conserve a species, because it helps to conserve the genetic variation in the species (Özel et al., 2006).

Sterilization of plant materials (explants/seeds) before subjecting them for *in vitro* propagation is essential for the production of clean *in vitro* plantlets and ensures the reduction of the contaminants as well as high survival rate of explants (Srivastava et al., 2010). In our study, different concentrations of NaOCl were tested and the frequencies of germinated and contaminated seeds varied significantly.

Among all the three NaOCl concentrations, 1% NaOCl treatment gave the maximum germinated and healtly seedlings. The results confirm the previous studies on various rare and/or endemic plants namely, *Gentiana cerina, Gentiana corymbifera, Astragalus tmoleus* var. *tmoleus, Stachys tmolea, Sideritis sipylea, Digitalis cariensis, Thymus sipyleus, Thermopsis turtica, Silene sangaria*, etc. (Morgan et al.,1997; Yürekli and Baba, 1995; Dayan, 2006; Erdoğan, 2010).

Many plant species have very specific *in vitro* requirements for multiplication and therefore, substantial variation is observed in the culture medium formulations. Specific growth regulators or supplements greatly enhance regeneration and growth in many cases (Sarasan et al., 2006; Babaoğlu et al., 2001). Maximum shoot multiplication in rare endemic *A. drabiformis* shoot tips

Table 2. Influence of different concentrations BAP and Ki on MS medium and various explant types on shoot formation of *A. drabiformis*.

Explant	BAP (ppm)	Ki (ppm)	Number of shoot/explant	Shoot length (mm)	Number of leaves/explant	Shooted explant (%)	Necrosis explant (%)
Shoot tip	0	0	1.73 ± 0.08^{l}*	25.50 ± 0.50^{c}	27.80 ± 0.44^{g}	84.07 ± 0.55^{d}	6.50 ± 0.35^{c}
	1	0	6.93 ± 0.45g	27.67 ± 0.31^{b}	54.23 ± 0.41^{f}	91.27 ± 0.54^{c}	0.00
	2	0	18.58 ± 0.18^{a}	35.20 ± 0.26^{a}	316.17 ± 0.62^{a}	100.00 ± 0.00^{a}	0.00
	4	0	16.27 ± 0.25^{b}	25.00 ± 0.30^{d}	278.10 ± 0.55^{b}	93.63 ± 0.41^{b}	0.00
	6	0	14.30 ± 0.20^{c}	17.68 ± 0.18^{e}	176.03 ± 0.58^{c}	83.63 ± 0.54^{d}	10.13 ± 0.38^{b}
	8	0	2.40 ± 0.10^{h}	12.70 ± 0.18^{h}	21.13 ± 0.35^{h}	53.17 ± 0.60^{f}	33.17 ± 0.56^{a}
	0	0.5	1.42 ± 0.07^{l}	10.00 ± 0.20^{l}	16.07 ± 0.58^{l}	73.63 ± 0.45^{e}	0.00
	0	1	10.50 ± 0.50^{d}	13.88 ± 0.16^{fg}	119.20 ± 0.46^{d}	100.00 ± 0.00^{a}	0.00
	0	2	8.67 ± 0.23^{f}	13.48 ± 0.08^{g}	97.10 ± 0.59^{e}	73.60 ± 0.56^{e}	0.00
	0	3	9.12 ± 0.10^{e}	14.30 ± 0.30^{f}	98.50 ± 0.35^{e}	93.07 ± 0.56^{b}	0.00
İnternod	0	0	1.50 ± 0.12^{h}	19.93 ± 0.21^{c}	18.17 ± 0.60^{f}	76.50 ± 0.40^{d}	0.00
	1	0	2.65 ± 0.12^{g}	17.53 ± 0.21^{d}	16.27 ± 0.45^{g}	73.37 ± 0.35^{e}	0.00
	2	0	17.10 ± 0.12^{a}	30.30 ± 0.21^{a}	301.87 ± 0.45^{a}	100.00 ± 0.00^{a}	0.00
	4	0	11.35 ± 0.12^{c}	23.43 ± 0.21^{b}	51.63 ± 0.37^{e}	83.50 ± 0.36^{c}	6.73 ± 0.33^{c}
	6	0	12.68 ± 0.12^{b}	15.23 ± 0.21^{f}	161.33 ± 0.69^{b}	76.97 ± 0.30^{d}	10.13 ± 0.35^{b}
	8	0	1.40 ± 0.12^{h}	16.37 ± 0.21^{e}	17.57 ± 0.35 ^{fg}	59.90 ± 0.46^{h}	20.23 ± 0.15^{a}
	0	0.5	1.37 ± 0.12^{h}	7.33 ± 0.21^{h}	10.33 ± 0.35^{h}	66.57 ± 0.41^{g}	0.00
	0	1	10.30 ± 0.12^{d}	13.50 ± 0.21^{g}	108.63 ± 0.41^{c}	100.00 ± 0.00^{a}	0.00
	0	2	7.20 ± 0.12^{f}	13.40 ± 0.21^{g}	91.67 ± 0.34^{d}	70.40 ± 0.51^{f}	0.00
	0	3	8.42 ± 0.12^{e}	13.73 ± 0.21^{g}	92.93 ± 0.29^{d}	93.50 ± 0.51^{b}	0.00

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

Table 3. Effect of different concentrations of IBA on rooting of *in vitro* regenerated shoots after 8 weeks of rooting treatments.

Growth regulator (ppm)		Number of roots/shoot	Root length (mm)	Rooting (%)
IBA	0.0	6.50 ± 0.28^{bc}*	18.06 ± 0.30^{b}	83.50 ± 0.32^{b}
	0.5	20.90 ± 0.26^{a}	22.93 ± 0.26^{a}	100.00 ± 0.00^{a}
	1.0	7.00 ± 0.26^{b}	16.40 ± 0.26^{e}	83.17 ± 0.43^{b}
	1.5	6.07 ± 0.28^{cd}	11.90 ± 0.26^{d}	74.53 ± 0.26^{c}
	2.0	5.63 ± 0.27^{d}	7.90 ± 0.26^{e}	67.83 ± 0.33^{d}

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

and internodes was achieved with the inclusion of 2 ppm BAP. BAP also significantly induced the auxiliary shoots as compared to Ki. Among the cytokinins, the superior effect of BAP over Ki on multiplication was reported by Anish et al. (2008) and Gümüşçü et al. (2008). The concentrations of BAP in the medium influenced the final shoot numbers. Results corroborate the reports in a number of species investigating the effectiveness of BAP in plant tissue cultures (Pevalek-Kozlina et al.,1999; Bhatia et al., 2002; Gangaprasad et al., 2005). On the other hand, BAP affected the levels of necrosis. Poor growth or abnormal shoots was very common at high concentrations of BAP as mentioned earlier in the literature (Gümüşçü et al., 2008; Onay, 1996). As a result, our study shows that culture medium with specific growth regulator concentrations affected the organogenesis. Shoot tips and internodes can be used for rapid clonal propagation with optimized culture medium and BAP also plays a major and distinctive role in the induction of shoot multiplication by organogenesis.

Adventitious root formation is very important for vegetative propagation and it is a key step in micropropagation systems. An efficient modification on rooting may cause high frequency of rooted shoots and a high

Figure 3. Shoots growth in 0.5 ppm IBA rooting media (bar 1 cm).

Figure 4. *In vitro* plantlet after 4 week of the transfer into plastic pot (bar 1 cm).

quality root system. The percentage of rooting, root length (mm) and the number of roots per shoot was changed significantly in *A. drabiformis* with different concentrations of IBA. The best rooting was achieved in the MS medium containing 0.5 ppm IBA. Similarly, IBA as a synthetic auxin was reported to induce adventitious rooting for a wide range of different species, and was preferred based on the results of prior studies (George et al., 2008; Mikulík, 1999; Pretto and Santarém, 2000; Prasad et al., 2004). Rooted plants were transplanted into pots in greenhouse for further acclimatization studies. Plants matured successfully in nine months.

Conclusion

Conclusively, this study describes a proper and simple micropropagation system for *A. drabiformis* for the first time. The regeneration system described here can be successfully used in studies dealing with *in vitro* preservation of *A. drabiformis* spp. and other genetic manipulation studies where appropriate.

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