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In vitro propagation and conservation of *Diaphanoptera khorasanica* (Caryophyllaceae), a threatened endemic and potential ornamental species in northeast of Iran

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Abstract

Tissue culture methods provide tools to supplement traditional methods for collection, propagation and preservation of endangered plant species. In this study, *in vitro* propagation of *Diaphanoptera khorasanica* Rech.f., a rare and threatened plant species with limited distribution range and population was investigated. This species has a potential as an ornamental plant. Single node explants were provided from both adult and seedling sources. Several disinfection treatments were tried to permit selection of a suitable method. Different growth regulators were used for establishment, proliferation and rooting stages. Explants showed the highest establishment percentage after 5 min treatment with 1% sodium hypochlorite (NaOCl), cultured in MS medium containing 2.2 µM 6-benzylaminopurine (BAP) and 2.4 µM indole-3-butyric acid (IBA). The highest proliferation of explants from both adult and seedling source explants was obtained from media supplemented by BA treatment in contrast to TDZ. No significant differences were found between different concentrations of BAP and TDZ. Proliferated shoots in TDZ were longer and had more internode length and less vitrification, in comparison with those in BAP. *In vitro* rooting of proliferated shoots just induced in liquid half-strength MS medium and rooting was not observed in solid medium. The shoots that originated from adult plants gave rise to the highest rooting rate with 4.8µM α-naphthalene acetic acid (NAA) and 2.4 µM, but NAA rooted plantlets showed higher survival percentage in acclimatization step. This study was aimed towards developing an efficient protocol for *in vitro* propagation of *D. khorasanica* and conservation of this vulnerable species.

***Keywords*:** micropropagation, proliferation, nodal explant, preservation

Introduction

World biodiversity is declining at an unprecedented rate. During the period of 1996–2011, a total of 9098 plant species were added to the International Union for the Conservation of Nature (IUCN) red list of threatened species (IUCN, 2011a). During this period, there was also an increase of over 60% in the number of plants recorded as critically endangered. This is alarming and immediate conservation actions are required to protect these species. Although species conservation is achieved most effectively through the management of wild populations and natural habitats (*in situ* conservation), *ex situ* techniques can be used to complement *in situ* methods and, in some instances, may be the only option (Maunder et al., 1998; Ramsay et al., 2000). Biotechnological methods are now as essential components of plant genetic resource management (Benson et al., 2000), and they are becoming increasingly powerful for the conservation of rare and endangered plants.

In vitro propagation can offer considerable benefits for the rapid cultivation of endangered plant species, which have a limited reproductive capacity and exist in threatened habitats (Fay, 1994). In addition to micropropagation, plant tissue culture provides means for conservation of genetic resources. This is especially critical for rare and threatened endemic plant species as this method allows establishing cultures from a minimal amount of starting plant material for further multiplication (Benson et al. 2000). *In vitro* techniques have been found to be useful in the propagation of a large number of threatened plants (Amo-Marco and Lledo, 1996; Dhar and Joshi, 2005).

Diaphanoptera Rech.f. (Caryaphyllaceae: Silenoideae) is a regionally endemic genus which, comprises of six local or highly narrow endemic species in its distribution range, *D. khorasanica* Rech.f. and *D. stenocalycina* Rech.f. & Schiman-

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Czeika in northeast of Iran, *D. transhyrcana* (Preobr.) Rech.f. and Schiman-Czeika in west of Turkmenistan, *D. lindbergii* Hedge and Wendelbo, *D. afghanica* Podlech and *D. ekbergii* Hedge and Wendelbo in the north and northeast of Afghanistan (Rechinger and Schiman-Czeika, 1988). *Diaphanoptera* species are usually cushion-form herbal chamaephytes growing in different habitats, including plains with moderately saline soils and from low mountain steppes up to high mountain stony slopes.

D. khorasanica, the type species of the genus, has broad winged ribs on its calyx and thus is easily distinguished from the others (figure 1). This species, described in 1940, had been known until the publication of the Flora Iranica only from its *locus classicus* in serpentine hills of Robate Sefid between Mashhad and Torbate Heydarieh. According to Rechinger and Schiman-Czeika (1988) its re-collection through repeated travelling to the type locality and its surrounding area had been unsuccessful in 1948, 1975 and 1977. After the publication of Flora Iranica, more populations of *D. khorasanica* have been discovered in different areas of Khorassan-Kopedagh Mountains and mainly collected by and preserved in Herbarium of Ferdowsi University of Mashhad (FUMH). Because of the limited recorded localities, this species has been considered as vulnerable (Jalili and Jamzad, 1999) and faces a high risk of extinction in the wild in the medium-term future; therefore, instantly *in situ* and *ex situ* conservation is needed. Furthermore, since as low seed set makes seed propagation of this species difficult, *in vitro* multiplication might be used as an attractive alternative method for the reintroduction of this threatened species into the natural environment and reducing the risk of extinction (Chandra et al., 2006).

Taking these considerations into account the objectives of this study were to: (1) establish a simple and rapid micropropagation system for *D. khorasanica*, and (2) to assess the efficiency of the system in producing *in vitro* plantlets.

Materials and Methods

The distribution map of all the species of *Diaphanoptera* is mostly based on herbarium records in FUMH and geo-referencing of the distribution data in Flora Iranica (Rechinger and Schiman-Czeika, 1988) by DIVA-GIS 7.3 software. The species were evaluated for threat status and red-listed using IUCN categories and criteria (IUCN, 2011b).

Two kinds of plant resources were used in this experiment in order to investigate the possibility of *in vitro* propagation of *D. khorasanica* including both adult and seedling plants.

In vitro culture of single nodes from adult plants

Young shoots (20 cm long) were collected from *D. khorasanica* plants in Kalat, Razavi-Khorasan Province in May 2009. The Collected shoots were washed with running tap water for 1 h and surface sterilized with 70% ethanol (for 30 seconds), and different concentrations plus time intervals of sodium hypochlorite (NaOCl) and mercury chloride ($HgCl_2$). After rinsing three times with distilled water, the terminal buds and leaves were removed. Single node explants, about 1.5 cm long, were cultured in Murashige and Skoog (MS) basal medium (Sigma-Aldrich, USA; pH adjusted to 5.8) with 2.2 µM 6-benzylaminopurine (BAP) and 1 µM IBA.

After four weeks of culture, the number of surviving explants, explants with developing axillary shoots and shoots per nodal explants were recorded. For the proliferation stage, developed axillary shoots were cultured in MS medium supplemented with BAP (2.2, 4.4, 8.8 µM) or TDZ (0.5, 1.1, 2.2 µM). The number of shoots per explant and shoot length were recorded after four weeks. Rooting of shoots was examined in two phases. In the first experiment, IBA and NAA (1.2, 2.4, and 4.8 µM) treatments were used in solidified ½ MS medium with 8 gr/l Agar. In the second one, shoots were either put in liquid $^1/_2$ MS medium with the same treatments of the first experiment or pulse treated for 15 seconds in 25 or 50 or 100 µM concentrations of IBA or NAA. Shoots were put on filter-paper bridge in liquid medium. Rooted shoots were gently washed with distilled water and transferred to plastic cups with drainage holes containing a 1:1 mixture of perlite and fine sand for acclimatization stage. For the initial seven days, plantlets were covered with a transparent cup and irrigated with a half-strength MS solution (sucrose free). After seven days, plantlets were exposed to the atmosphere and irrigated with both distilled water and a half-strength MS solution. Cultures were maintained at 24±1ºC and illuminated by white fluorescent tubes (40 µmol m-2 s-1) 16 h per day. Fifteen replicates were used for each treatment.

In vitro culture of single nodes from seedlings

Explants were obtained by germinating seeds collected from a natural population, namely, during June 2009. Seeds were surface sterilized with 70% ethanol (30 s), 1% NaOCl for 20 min followed by

three washes with sterile distilled water. Seeds were then germinated on the one-half strength hormone-free MS medium under a 16-h photoperiod provided by fluorescent light. Shoots formed after 5 weeks of culture. Uniform shoots were selected from *in vitro* seedlings and nodal stem segments with a pair of auxiliary buds excised and cultured in MS medium containing 2.2 µM BAP and 1 µM IBA. Proliferation and rooting treatments were done using similar treatment and methods used for adult source explants.

Data analysis

The data were analyzed by SAS (SAS Institute, Inc., Cary, NC) software. Mean values were separated according to Duncan's multiple range test at probability of 0.05 level. Percentage data were log transformed to achieve normal distribution.

Results

Biogeography and conservation

The distribution map of this species shows that they are mainly local endemics or distribute in a highly confined area, except for *D. khorasanica* which has a relatively wider distribution range in Khorassan-Kopedagh floristic province (figure 2). All species evaluated as threatened but in different categories. *D. khorasanica* is re-examined as a vulnerable species and the other species are red-listed as endangered or critically endangered plant species for the first time in the World based on IUCN criteria (table 1).

Adult source explants

Based on the results, 1% NaOCl for 5 min showed the best effect in disinfection of explants. Explants showed the highest establishment rate (less contamination and higher growth) after a 1% NaOCl disinfection treatment for 5 min in MS medium containing 2.2 µM BAP and 2.4 µM IBA. Bud break and growth of shoots were noticed in the nodal explants in the presence of both BAP and TDZ cytokinins in MS medium. The higher multiplication rates were obtained with BAP treatments with only significant differences between 2.2 µM BAP, and 1.1 µM TDZ based on shoot number and between 2.2 and 4.4 µM BAP and 1.1 and 4.4 µM TDZ based on the mean shoot length. Less vitrification percent was observed in different concentrations of TDZ, in comparison with BAP (table 2). Callus formation was seen in some explants in spite of IBA elimination in proliferation phase. Proliferated shoots showed very low root formation (less than 5%) in the solid ½ MS medium (figure 4). The highest rooting percentage was observed in liquid medium with the highest rate in 4.8 µM NAA and 2.4 µM IAA, and no evidence of root initiation or development was observed in pulse treated shoots (table 3). Root formation was observed later in NAA treated shoots, but shoots were stronger and more aggregate and subsequently less injured during transfer to acclimatization phase, so they showed higher acclimatization percentage (table 3) in comparison with rooted shoots with IAA and IBA treatments.

Seedling source explants

A high germination (80%) was observed after 2 or 3 weeks in ½ MS medium. Single nodes from one month old seedling showed good establishment in MS medium containing 2.2 µM BAP and 2.4 µM IBA. In proliferation phase, higher proliferation (based on shoot numbers) was observed in BAP treatments compared to TDZ ones, but only 8.8 µM BAP showed a significant difference with 1.1 and 4.4 µM TDZ. The percentage of vitrified shoots was higher (table 2) in medium containing BAP than the TDZ treatments (figure 3) and significant differences were found between BAP and TDZ treatments. The acclimation percentage of rooted shoots was lower in the seedling source explants, but still higher acclimation was recorded on the 4.8 µM NAA treated shoots (table 3).

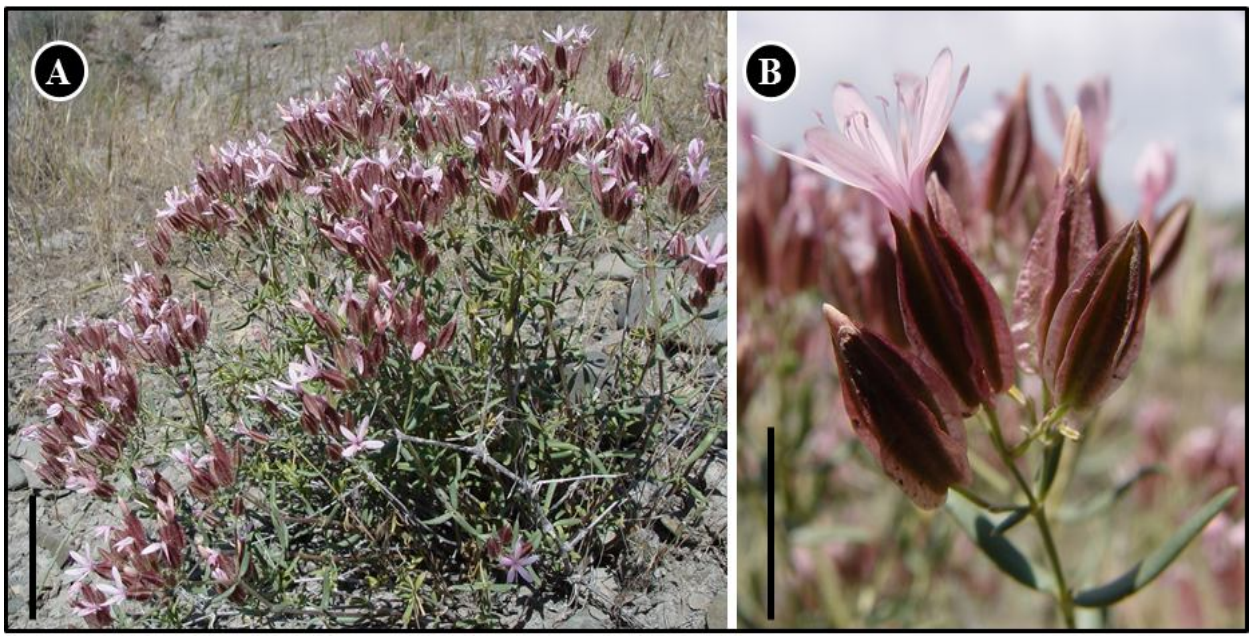

Figure 1. *Diaphanoptera khorasanica* in its natural habitat in Fereizi region, northern foothills of Binalood mountain range, Razavi Khorassan. A: lax cushion-form habit, scale bar = 5 cm; B: close-up view of the flowers, scale bar = 1 cm.

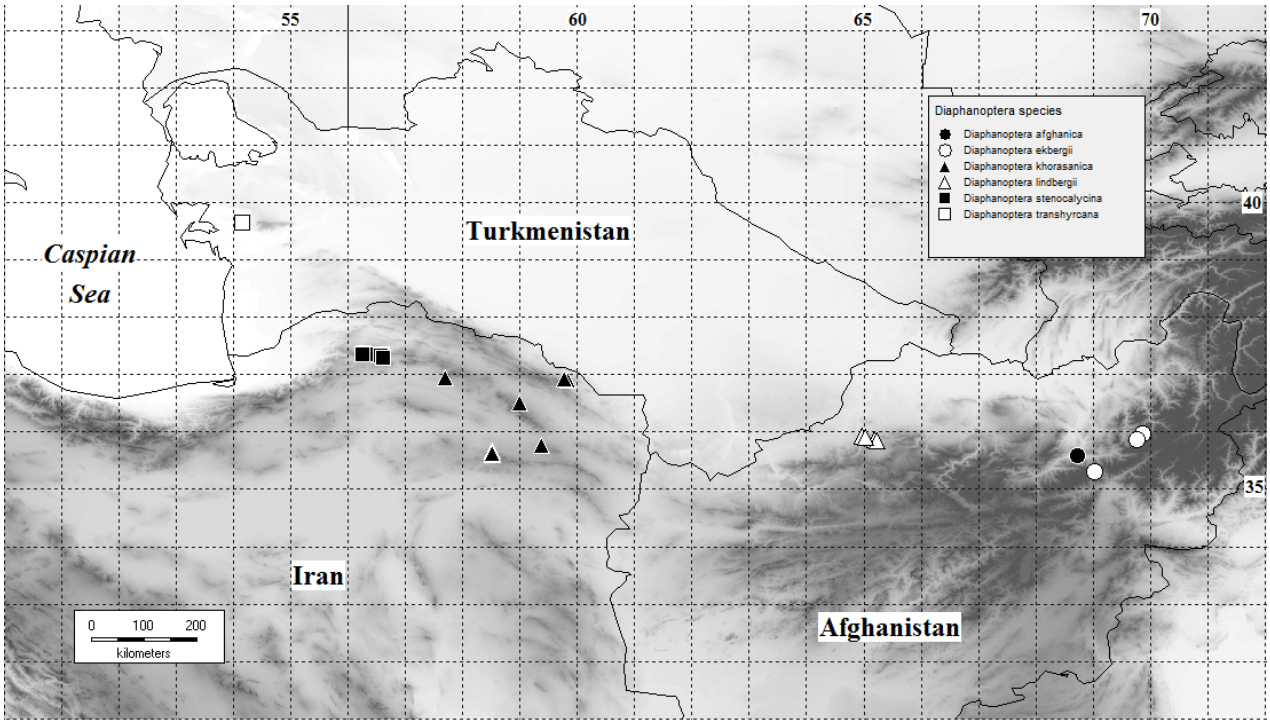

Figure 2. Distribution map of *Diaphanoptera* species in the World.

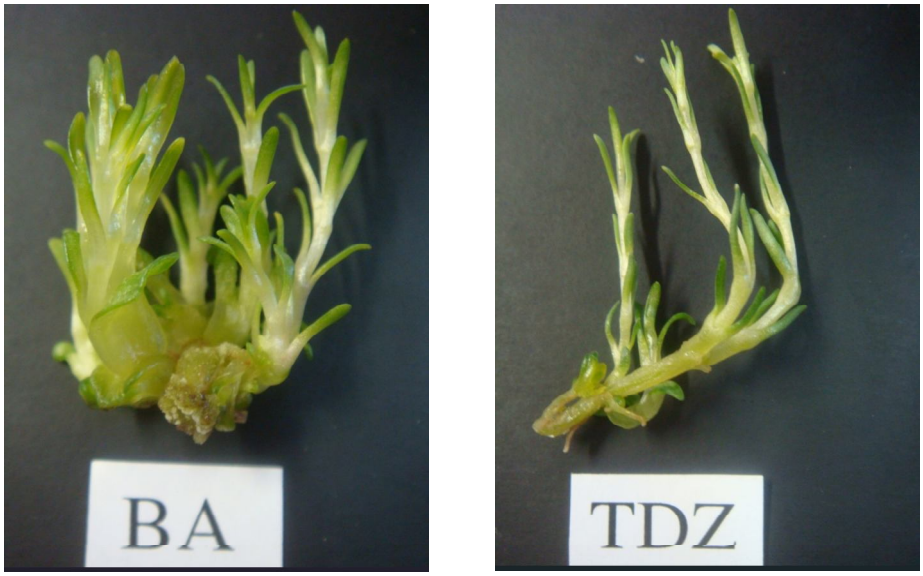

Figure 3. Comparison of proliferated shoots from adult plant in BAP and TDZ treatments.

Figure 4. Rooted shoot in liquid ½ MS medium and 4.8 µM NAA treatment.

Table 1. Threat status and biogeography of *Diaphanoptera* species. (Abbreviations: CR: Critically Endangered, EN: Endangered, VU: Vulnerable, IT: Irano-Turanian, KK: Khorassan-Kopetdagh, Afgh.: Afghanistan).

Species	Threat status (Criteria)	Habitat	Chorotype
D. khorasanica	VU (B1+B2ab (i, iv))	low to middle mountain steppes	IT ^{KK}
D. stenocalycina	CR (B2 bc (i, iii))	plains with saline soils	IT ^{KK}
D. transhyrcana	CR (B1+B2 a,b (iii))	low mountain steppes	IT $^{Aralo\text{-}Caspian}$
D. afghanica	CR (B1+B2 a,b (iii))	valley plains	IT $^{Afgh.}$
D. lindbergii	EN (B1+B2 a,b (iii))	low mountain steppes	IT $^{Afgh.}$
D. ekbergii	EN (B1+B2 a,b (iii))	high mountain stony steppes	IT $^{Afgh.}$

Table 2. Shoot proliferation results from adult and seedling sources of *Diaphanoptera khorasanica*.

Explant source								
	Adult				Seedling			
Cytokinin (µM)	Mean Shoot Number	Mean Shoot Length (cm)	Mean Number of Nodal Segment	Vitrification%	Shoot Number	Mean Shoot Length (cm)	Mean Number of Nodal Segment	Vitrification%
BAP								
2.2	5^{ab}	1.1^{bcd}	1.8^{ab}	67 b	2^{ab}	0.8^{b}	2.5^{a}	58 b
4.4	4.4^{abc}	1.6^{a}	2.1^{ab}	85 a	1.7^{abc}	0.9^{b}	3^{a}	76 a
8.8	4.6^{abc}	1.4^{ab}	1.8^{ab}	91 a	2.3^{a}	0.9^{b}	2.6^{a}	80 a
TDZ								
1.1	2.6^{c}	0.9^{d}	2.1^{ab}	0 e	1.5^{b}	1.9^{a}	3^{a}	0 d
2.2	3.4^{bc}	1.4^{abc}	2^{ab}	3.2 d	1.8^{ab}	1.4^{ab}	3.2^{a}	0 d
4.4	2.8^{bc}	1^{cd}	2.4^{a}	4.8 c	1.3^{b}	1.34^{ab}	2.6^{a}	4 c

Table 3. Rooting percentage from adult and seedling sources of *Diaphanoptera khorasanica*.

Auxin	Concenteration (µM)	Adult source		Seedling source	
		Rooting %	Acclimatization %	Rooting %	Acclimatization %
IAA	1.2	34 d	12 e	18 de	7 d
	2.4	56 a	18 c	25 ab	8 cd
	4.8	45 bc	18 c	22 bc	8 cd
IBA	1.2	23 g	10 f	15 f	7 d
	2.4	26 f	10 f	16 ef	8 cd
	4.8	42 c	15 d	20 cd	9 c
NAA	1.2	30 e	17 cd	17 ef	9 c
	2.4	48 b	26 b	22bc	12 b
	4.8	60 a	32 a	27 a	16 a

Discussion

D. khorasanica is a vulnerable species in spite of finding more populations in different mountain ranges of Khorassan-Kopetdagh after the Flora Iranica. None of populations of *D. khorasanica* are located in National Parks or Protected Areas of Khorassan; therefore, there is no *in situ* conservation for this species and more efforts are needed to preserve the remaining threatened populations.

The results showed that it was possible to propagate *D. khorasanica* using both adult and seedling sources. Shoots showed different percents of vitrification depending on cytokinin type and concentration that was in agreement with the other reports (Casas et al., 2010; Kharrazi et al., 2011). Vitrification is one of the most influential issues of the other species of Caryophyllaceae family such as *Dianthus caryophyllus* (Saher et al., 2005; Fernandez-Garcia et al., 2008; Casas et al., 2010) and *Colobanthus quitensis* (Zuniga et al., 2009). Up to 60% loss of plants has been reported in

micropropagation of different species because of vitrification (Pâques, 1991). The physiological basis of this changes is not clear although some have suggested the role of oxidative stress (Saher et al., 2004), changes in pectin methylation pattern (Fernandez-Garcia et al., 2008) or incomplete development of cuticular waxes (Olmos and Helin, 1998). The vascular connections at the root-to-shoot interface are important for the function of the vascular system and the viability and survival of the plants after transplanting. Smith et al. (1991) reported that the vascular connections between new *in vitro* roots and the micro cutting stems of maple, birch, and apple were often discontinuous since the *in vitro* roots were often subtended by callus or emerged from callus.

At the rooting phase, *D. khorasanica* shoots showed high sensitivity to the culture medium regarding to be solid or liquid and growth regulator; therefore, providing the optimized conditions for rooting is recommended. In spite of good rooting of shoots, this species showed low acclimatization percentage in *ex vitro* conditions (table 3). The possible reason might be the succulent nature of this species leaves that causes more intensification and vitrification of them during rooting phase in liquid mediumThe other possible reason for low acclimatization of rooted shoots might be callus formation at the end of these shoots. Consequently it is suggested that for obtaining normal plantlets with minimum vitrification rate, low concentrations of TDZ is more suitable. Complimentary experiments are under way to optimize the acclimatization step.

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