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Effects of Plant Growth Regulators on Shoot Multiplication of *Crepis bithynica* (Asteraceae): An Endangered Rare Species in Türkiye

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Abstract

Aim of study: In this study, the effects of plant growth regulators (PGRs) on shoot multiplication of *Crepis bithynica* Boiss. (Asteraceae), an endangered rare species in Türkiye were investigated.

Area of study: The plant samples were collected from Kastamonu Ilgaz Mountain National Park.

Material and method: The nodal segments were used as explant and they were cultured on Murashige and Skoog (MS) basal media supplemented with different plant growth regulators. Morphological changes of propagated plants were monitored during 21 days *in vitro* cultures.

Main results: All plant growth regulators induced shoot growth and development without callus formation. Additionally, the highest shoot number and length of shoot per explant were achieved in MS media supplemented with 4.4 µM 6-BA plus 0.5 µM NAA, and 4.9 µM 2iP plus 0.5 µM IBA, respectively.

Highlights: This study is the first study on the effects of PGRs in shoot multiplication of *C. bithynica*, and the results obtained can be used micropropagation for the species.

Keywords: *Crepis bithynica*, Plant Growth Regulators, Shoot Multiplication, *Ex Situ* Conservation

Bitki Büyüme Düzenleyicilerinin *Crepis bithynica* (Asteraceae)'nın Sürgün Çoğaltımı Üzerine Etkileri: Türkiye'de Tehlike Altında Nadir Bir Tür

Öz

Çalışmanın amacı: Bu çalışmada, bitki büyüme düzenleyicilerinin (PGRs) Türkiye'de tehlike altında nadir bir tür olan *Crepis bithynica* Boiss. (Asteraceae)'nın sürgün çoğaltımı üzerine etkileri araştırılmıştır.

Çalışma alanı: Bitki örnekleri, Kastamonu Ilgaz Dağı Milli Parkı'ndan toplanmıştır.

Materyal ve yöntem: Nodal segmentler eksplant olarak kullanıldı ve bunlar farklı bitki büyüme düzenleyicileri ile desteklenmiş Murashige and Skoog (MS) temel besiyerlerinde kültüre edildi. Çoğaltılan bitkilerdeki morfolojik değişmeler 21 gün boyunca *in vitro* kültürlerde izlendi.

Temel sonuçlar: Tüm bitki büyüme düzenleyicileri kallus oluşturmaksızın sürgün büyüme ve gelişmesini teşvik etmişlerdir. Bununla birlikte, eksplant başına en yüksek sürgün sayısı ve sürgün uzunluğu, sırasıyla 4.4 µM 6-BA ile 0.5 µM NAA ve 4.9 µM 2iP ile 0.5 µM IBA içeren MS besiyerlerinde elde edilmiştir.

Araştırma vurguları: Bu çalışma, *C. bithynica*'nın sürgün çoğaltımında PGR'lerin etkileri üzerine ilk çalışmadır ve elde edilen sonuçlar türün mikroçoğaltımı için kullanılabilir.

Anahtar Kelimeler: *Crepis bithynica*, Bitki Büyüme Düzenleyicileri, Sürgün Çoğaltımı, *Ex Situ* Koruma

Introduction

Crepis bithynica Boiss. (Asteraceae) is a Balkan subendemic species distributed in Asia Minor (Bithynian Olympus) and in the Balkan Peninsula (Yurukova-Grancharova & Dimitrova, 2006). According to Inceer & Aksu Kalmuk (2019), *C. bithynica* is a rare endangered species growing in alpine regions of Türkiye (Figure 1).

Plant growth regulators (PGRs) have a significant role for physiological process *in vitro* cultures (Nowakowska et al., 2019). During the stage of the shoot multiplication, a first step in micropropagation, the

interactions of cytokinins with high concetrations and auxins with low concentrations control morphogenesis (Su et al., 2011). In the literature, *in vitro* shoot multiplications of *Crepis* species are limited to few taxa (Corral et al., 2010; Mitrofanova et al., 2020, 2021), and no studies on the effects of PGRs on shoot multiplication of *C. bithynica* have been carried out till now. The aim of this work is fill up to this gap in the literature.

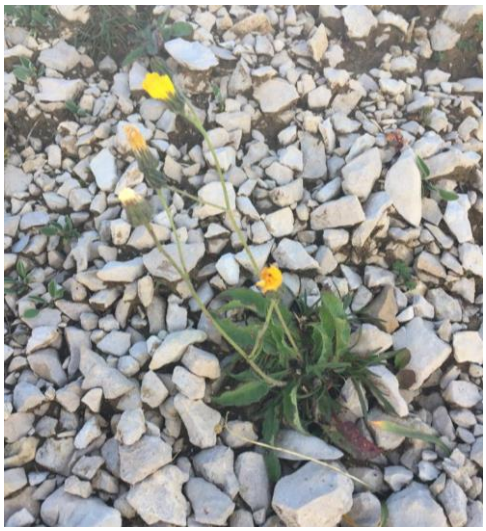

Figure 1. Habit of *Crepis bithynica*

Material and Method

Plant Material

The achenes were collected from natural population in Ilgaz Mountain National Park, Kastamonu province in Türkiye (1900-2000 m, 16.08.2014, *Inceer* 1115, KTUB). *In Vitro Achene Germination*

The surface sterization of the achenes of *C. bithynica* was done with 70% ethanol and then soaked 3% sodium hypochlorite and finally washed with sterilized distillated water. Later, they were germinated on Murashige and Skoog (MS) (Murashige & Skoog, 1962) basal medium including vitamins supplemented with 4.7 µM kinetin (KIN) (Inceer et al., 2022).

Shoot Multiplication

MS medium containing vitamins as well as 2% (w/v) sucrose and 0.8% (w/v) phyto agar were used for shoot multiplication. The filter 0.22 µm was used for the sterilization of PGRs and then the PGRs (KIN, 2iP-6-(y,y-dimethylallylamino)-purine, IBA-indole-3-butyric acid) were added to the cooled media after autoclaving. 6-BA (6-benzylaminopurine) and NAA (α-naphthalene acetic acid) were added to the culture medium before autoclaving. Before autoclaving, pH of MS media was adjusted to 5.8 and the sterilization was carried out 20 min at 121°C under 1.1 kPa pressure. Nodal explants were transferred to seven different MS media (Table 1). Culture conditions in the growth room were adjusted at 24 ± 2°C under a 16 h photoperiod at a photosynthetic flux density of 50 µmol m^{-2} s^{-1} with cool daylight fluorescent lamps (Inceer et al., 2022).

Data Analysis

Each treatment has three replicates containing 16 plants. The data were evaluated with analysis of variance (one-way ANOVA) in SPSS.

Table 1. Effects of different cytokinins and auxins on shoot multiplication of *C. bithynica*.

	Cytokinin (µM)			Auxin (µM)					
Control	KIN	6-BA	2iP	IBA	NAA	Shoot number	Shoot length (mm)	Leaf number	Callus
0.0	0.0	0.0	0.0	0.0	0.0	1.25 ± 0.45a	24.05 ± 2.49d	30.56 ± 2.71c	nd
	4.7			0.5		1.13 ± 0.34a	19.94 ± 2.35b	42.25 ± 2.24e	nd
		4.4		0.5		1.63 ± 0.50b	17.86 ± 1.36a	42.50 ± 2.58e	nd
			4.9	0.5		1.25 ± 0.45a	28.98 ± 2.75e	26.44 ± 1.93b	nd
	4.7				0.5	1.19 ± 0.40a	22.62 ± 2.29cd	37.56 ± 1.63d	nd
		4.4			0.5	1.75 ± 0.45b	23.57 ± 2.29d	51.25 ± 2.49f	nd
			4.9		0.5	1.25 ± 0.45a	21.65 ± 1.86c	16.75 ± 2.38a	nd

The mean value followed by the same letter at each treathment was not significantly different at $p < 0.05$, nd: not detected

Monitoring Morphological Changes in Shoots

The shoots of *C. bithynica* were monitored and photographed to characterize morphological changes based on the combinations of cytokinins and auxins in MS media. In addition, the time needed for its growth and development in the *in vitro* cultures was recorded.

Results

In Vitro Achene Germination

Sterilization process for achenes of *C. bithynica* was reached 100% success. There was no microbial contamination *in vitro* cultures. Besides, the germination rate was 50%.

Shoot Multiplication

The shoot multiplication of *C. bithynica* in MS medium containing PGRs and without PGRs was successfully achieved after 21 days. The best multiplication results with average shoot number (1.75 ± 0.45) as well as leaf number (51.25 ± 2.49) per explant for *C. bithynica* were determined on the medium supplemented with 4.4 µM 6-BA plus 0.5 µM NAA (Table 1). However, MS medium containing kinetin yielded with low shoot number values, but there was only significant difference between kinetin and 6-BA applications. Besides, the lowest mean leaf number (16.75 ± 2.38) per explant in *C. bithynica* was observed on medium containing 4.9 µM 2iP plus 0.5 µM NAA. In addition, the medium containing 4.9 µM 2iP plus 0.5 µM IBA yielded the highest shoot elongation value with 28.98 ± 2.75 mm, whereas the lowest shoot elongation value with 17.86 ± 1.36 mm was recorded with 4.4 µM 6-BA plus 0.5 µM IBA combination. Besides, significant differences were determined in between these and other applications. No callus formation was detected in cultures.

Change of Plantlets Morphology

The leaf growth and development of plantlets have continued until end of the 21 days in MS media supplemented with PGRs (Figures 2-4). The leaf shape of the plantlets was developed end of 21 days. As seen in Figures 2-4, the leaves are oblanceolate, dentate to deeply pinnatifid with several pairs of ± triangular lobes, apex acute or obtuse. However, yellowing and browning in some young leaves appeared in end of 7 days, and these morphological changes increased in end of 21 days. Likewise, the yellowing and the browning in leaves of this species were observed during subculturing.

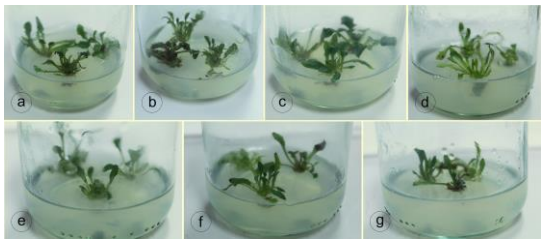

Figure 2. Morphological changes at 7 days *in vitro* culture of *Crepis bithynica*; (a) Control, (b) KIN + IBA, (c) 6-BA + IBA, (d) 2iP + IBA, (e) KIN + NAA, (f) 6-BA + NAA, (g) 2iP + NAA

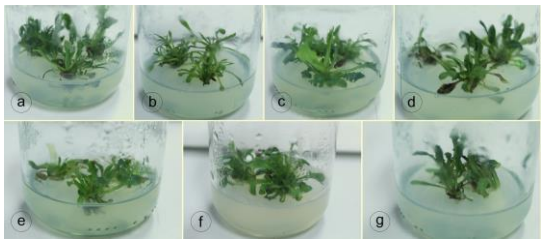

Figure 3. Morphological changes at 14 days *in vitro* culture of *Crepis bithynica*; (a) Control, (b) KIN + IBA, (c) 6-BA + IBA, (d) 2iP + IBA, (e) KIN + NAA, (f) 6-BA + NAA, (g) 2iP +NAA

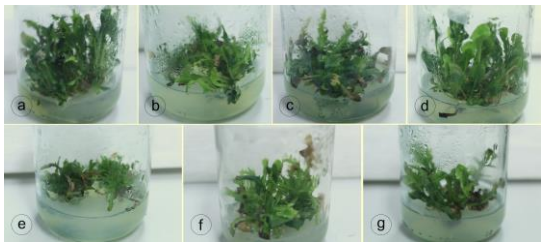

Figure 4. Morphological changes at 21 days *in vitro* culture of *Crepis bithynica*; (a) Control, (b) KIN + IBA, (c) 6-BA + IBA, (d) 2iP + IBA, (e) KIN + NAA, (f) 6-BA + NAA, (g) 2iP + NAA

Discussion

Crepis has rarely been tissue culture studies untill now: it has been carried out *C. novoana* (Corral et al., 2010), *C. purpurea* (Mitrofanova et al., 2020), *C. callicephala*

and *C. vesicaria* subsp. *bivonana* (Mitrofanova et al., 2021). Here, we investigated the effects of the combinations of selected PGRs on shoot multiplication as a first step in the conservation of *C. bithynica* for the first time. The best results in the shoot multiplication of *C. bithynica* were achieved in the medium supplemented 4.4 µM 6-BA plus 0.5 µM NAA, and 4.9 µM 2-iP plus 0.5 µM IBA, respectively without callus formation. Our results show that these PGRs have more essential effect than other applications for shoot multiplication of *C. bithynica*.

It is known that the species within Asteraceae have low germination rate in the achenes (Inceer et al., 2022). Similarly, the germination rate in the achenes of *C. bithynica* is relatively low in the *in vitro* culture. However, seedlings obtained from achene germination in the medium are healthy and likely explants. The low germination rate in this species may be due to enforced domancy, reflecting adaptation to its habitat in alpine region, which is stressful environment because of harsh climatic conditions, such as low temperatue, snowfall and summer drought.

The shoot proliferation was successfully achieved after 21 days of the culture. From all tested combinations, the highest shoot number (1.75 ± 0.45) and leaf number (51.25 ± 2.49) were observed on 4.4 µM 6-BA plus 0.5 µM NAA. These findings agree with the previous report for *Crepis novoana* (Corral et al., 2010). Similar results were reported from *Senecio macrophyllus* (Trejgell et al., 2010) (Asteraceae). On the contrary, the highest shoot elongation was yielded with 4.9 µM 2iP plus 0.5 µM IBA. The uptake and recognition of PGRs by cells or mechanism of action of PGRs may give different response for shoot growth and development (Sujatha & Reddy, 1998; Kim et al., 2001; Trejgell et al., 2010)

However, the using 4.7 µM kinetin plus 0.5 µM IBA as well as 4.4 µM 6-BA plus 0.5 µM IBA yielded in the lowest shoot number and length, respectively. In addition, the presence of 4.9 µM 2iP plus 0.5 µM NAA in the medium strongly inhibited the leaf number of the shoots. It is concluded that these treatments were not suitable for regeneration of *C. bityhynica*.

The data obtained from shoot multiplication showed that PGRs used in the study controlled the growth and development of the shoots in MS media during 21 days. Our results showed that the plantlets have a similar leaf morphology with mother plants in native population (Lamond, 1975). On the other hand, we observed intense yellowing and browning in the shoots after 21 days of the culture. As point out by Silvia et al. (2020), the necrosis in the shoot multiplication is a physiological response and disorder due to several *in vitro* factors, such as nutrient deficiency, concentration and type of PGRs, possibly humidity and hyperhydricity. The cuttings of nodal explants in subcultures as well as high humidity and poor ventilation *in vitro* cultures due to widening the lamina of the leaf after 21 days may increase ethylene production and cause yellowing and darkening *in vitro* cultures of this species. Further studies may help to understand the physiological process *in vitro* cultures of this species.

Conclusions

This is the first study on the effects of PGRs on shoot multiplication of *C. bithynica*. The results obtained from shoot multiplication may be used in future micropropagation of this species. Additionally, furher studies such as rooting of shoots and aclimatization of plantlets would be helpful in micropropagation of *C. bithynica* for *ex situ* conservation.

Ethics Committee Approval

N/A

Peer-review

Externally peer-reviewed.

Author Contributions

Conceptualization: H.I.; Investigation: T.E., H.I.; Material and Methodology: T.E., H.I.; Supervision: H.I.; Visualization: T.E., H.I.; Writing-Original Draft: H.I.; Writing-review & Editing: H.I.; Other: All authors have read and agreed to the published version of manuscript.

Conflict of Interest

The authors have no conflicts of interest to declare.

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