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In vitro propagation of *Verbascum biledschikianum* Bornm, an endemic species of Türkiye

Sinem Gül Kepenek [1], Nurşen Çördük [2,*], Ebru Cambaz [3], Aylin Yilmaz [4] and Özer Yilmaz [4]

[1] *Department of Biology, Faculty of Arts and Sciences, Çanakkale Onsekiz Mart University, Çanakkale, Türkiye.*
[2] *Department of Biology, Faculty of Sciences, Çanakkale Onsekiz Mart University, Çanakkale, Türkiye.*
[3] *Department of Biology, School of Graduate Studies, Çanakkale Onsekiz Mart University, Çanakkale, Türkiye.*
[4] *Department of Biology, Faculty of Arts and Sciences, Uludağ University, Bursa, Türkiye.*

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Abstract

Verbascum biledschikianum is the local endemic plant belonging to the Scrophulariaceae family and classified in the vulnerable category. This study aims to establish and optimize a rapid and reliable protocol for the propagation *V. biledschikianum*. Leaf explants from *in vitro* grown seedlings were embedded in solid MS medium supplemented with various concentrations and combinations of cytokinins and auxins at a density of ten explants per plate. All cultures were maintained in the growth chambers at 25 ±2°C under 16/8 h photoperiod with 72 µmol m^{-2} s^{-1}. The mean number of shoots per explants and percentage of explants forming shoots were recorded after 4 weeks in culture. MS medium supplemented with 2 mg/L benzylaminopurine and with 3 mg/L benzylaminopurine + 1 mg/L naphthalene acetic acid gave the highest number of explants per explants. Root induction was achieved on an MS medium containing 1 g/L of activated charcoal.

Keywords: Mullein; Adventitious Shoot; Plant Growth Regulators; Propagation

1. Introduction

Verbascum biledschikianum Bornm., is the local endemic species which has a restricted distribution in northwest Anatolia between the border of Bursa and Bilecik, Türkiye [1]. It is referred to as Bilecik Sığırkuyruğu, is a biennial plant, reaching heights of 65-105 cm, with a short and densely hairy (tomentose) or hairless surface. [2]. The species is classified as a vulnerable (VU) category by the Red Data Book of Turkish Plants [3].

In vitro techniques offer the possibility of propagating a wide range of plant species. Efficient and rapid plant propagation in *in vitro* cultures under optimized conditions is still in demand to obtain biomass for phytochemical and biological analyses [4]. *In vitro* studies in *Verbascum species* include plant regeneration by organogenesis and somatic embryogenesis in *V. sinuatum* L. [5], *in vitro* culture of *V. thapsus* [6], shoot proliferation and callus induction in *V. thapsus* [7], *in vitro* shoot culture in *V. eriophorum* Godr [8], enhancement of secondary metabolite accumulation using elicitors in callus culture of *V. thapsus* [9], determination of secondary metabolite quantities such as verbascoside, isoverbascoside, luteoseptoside A and B through callus and cell suspension cultures in *V. thapsus* [10], and secondary metabolite production through callus culture of *V. scamandri* [11]. To the best of our knowledge, there is no report on the *in vitro* propagation of *V. biledschikianum*. Hence, the aim of this study was to develop an improved protocol for propagation of *Verbascum biledschikianum*.

* Corresponding author: Nurşen Çördük.

2. Materials and Methods

2.1. Plant material and sterilization

The seeds of *Verbascum biledschikianum* Bornm. were obtained from the Bursa Uludağ University Faculty of Arts and Sciences Herbarium (BULU), Türkiye. The seeds were sterilized by dipping in 5% (v/v) sodium hypochlorite for 20 minutes then rinsed three times with sterile ddH_2O. The germination was achieved in darkness at 25±2°C and 50±5% humidity in a plant growth chamber. Further growth and development of the germinated plants were maintained in *in vitro* culture vessels under aseptic conditions. Germinated plants were transferred to MS medium with 3% sucrose and 0.8% phytoagar once every 4 weeks. All plants were kept in the growth chamber at a light intensity of 72 μmol $m^{-2}s^{-1}$.

2.2. Medium and culture conditions

The basal medium was Murashige & Skoog [12] basal medium (MS, M0222, Duchefa Biochemie B.V, The Netherlands). All media were supplemented with 3% (w/v) sucrose (S0809, Duchefa Biochemie B.V, The Netherlands), 100 mg/L ascorbic acid (180476, AFG Bioscience, USA), and 50 mg/L citric acid (910.046, Isolab Chemicals, Germany). The solidification of the medium was achieved by adding 0.8% (w/v) phyto agar (P1003, Duchefa Biochemie B.V, The Netherlands). All media pH was adjusted to 5.75 prior autoclaving for 15 min at 121°C. The filter-sterilized 1mL/L Plant Preservative Mixture (PPM; Plant Cell Technology, USA) were added to media before dispensing in petri plates. Medium was supplemented with various concentrations and combinations of cytokinins [Benzylaminopurine (6-BAP: B0904, Duchefa Biochemie), Kinetin (KIN: K0905, Duchefa Biochemie)] and auxins [Naphthalene Acetic Acid (NAA: N0903, Duchefa Biochemie), 2,4-Dichlorophenoxyacetic acid (2,4 D: D0911 Duchefa Biochemie)].

All *in vitro* culture experiments were conducted in a plant growth chamber set to a 16/8-hour photoperiod, 25±2°C, with a light intensity of 72 μmol $m^{-2}s^{-1}$, and a relative humidity of 55-60%.

2.3. Induction of adventitious shoots from leaf explants

Sterile seeds were transferred to a MS medium containing 3% sucrose and 0.8% phyto agar for germination and placed in a plant growth room in the dark. After germination, the seedlings were transferred to the plant growth room with a 16/8-hour photo period. Leaf explants, derived from 15-week-old germinated seedlings of *V. biledschikianum*, were cultured on MS medium with different concentrations of plant growth regulators (PGRs), including 0, 0.5, 1.0, 2.0, and 3.0 mg/L of cytokinin (6-BAP, KIN), and 0, 0.5, and 1.0 mg/L auxin (NAA, 2,4 D). Leaf explants of approximately 0.5x0.5 cm in size were cut and placed 5 in each petri dish in the shoot induction medium, with each trial set up with 3 replicates.

2.4. Shoot multiplication, elongation and rooting of shoots

All cultures were retained for four weeks, then the adventitious shoots were sub-cultured onto the MS medium with the same formulations as the shoot induction medium to facilitate shoot multiplication within eight weeks of culture. The mean number of shoots per explant in different media was calculated, and each experiment included three replications of 5 explant per treatment in the 12^{th} week of culture. Root induction was established in media such as, MS without plant growth regulators, MS with 1 g/L charcoal activated (102183, Merck, Germany), MS with 1 mg/L NAA, and MS with 2 mg/L NAA+ 1 g/L charcoal activated.

3. Results and Discussion

21 different media were compared as shoot induction media. Enlargement and swelling were not observed in any leaf explants cultured for 28 days in 21 different MS media containing different plant growth regulators for shoot induction. When the explants are cultured, morphological swelling and an increase in volume are observed due to increased cell division induced by both endogenous and exogenous plant growth regulators. However, we did not observe these changes during the 4 weeks of cultivation of this species. No differentiation was observed in explants cultured on MS medium without plant growth regulators. Explants in some media (0.5 mg/L BAP, 2 mg/L BAP+0.5 mg/L NAA, 2 mg/L BAP+1 mg/L NAA, 3 mg/L BAP, 0.5 mg/L KIN+ 0.5 mg/L 2,4 D, 2 mg/L KIN, 3 mg/L KIN+1 mg/L 2,4 D) were completely browned and neither callus nor shoot formation was observed in these explants. However, callus formation was observed in the cut areas of explants in some media despite browning. Callus tissues were induced in MS media containing different concentration of KIN and 2,4 D (0.5 mg/L KIN+1 mg/L 2,4 D, 1 mg/L KIN+1 mg/L 2,4 D, 2 mg/L KIN+0.5 mg/L 2,4 D, 2 mg/L KIN+1 mg/L 2,4 D, 3 mg/L KIN, 3 mg/L KIN+0.5 mg/L 2,4 D). Among the media containing different concentrations of KIN, and 2,4 D, shoot induction occurred only in MS medium with 0.5 mg/L KIN (Medium 11). Callus tissues and adventitious shoot primordia were formed around the wound edges of leaf explants cultured on

MS medium with BAP and NAA (Medium No 2, 3, 4, 5, and 10) after 4 weeks of culture (Figure 1). It was observed that the biomass of the callus tissue with shoot primordium was very low during the 4th week of culture.

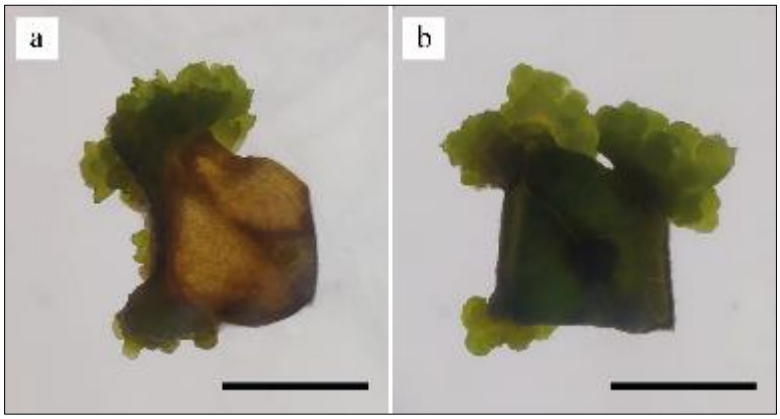

Figure 1 Callus tissues and adventitious shoot primordia on leaf explants of *V. biledschikianum* (*scale bar*=1 cm)

In our study, we observed indirect shoot formation from leaf explants of *V. biledschikianum*. According to the results, BAP and NAA combination were more effective for induction of shoot primordium than KIN and 2,4 D in *V. biledschikianum*. The differentiation of the callus tissue into the shoot primordium was not observed in the explants cultured on these media except MS medium with 0.5 mg/L KIN (Medium 11). However, it was observed that the number of shoots formed in this medium was very low. This response was similar to the previously published report on *V. sinuatum* L. In the study, BA was reported to be more effective than Kin, with the highest number of shoots on medium containing 1 mg/L BA in *V. sinuatum* L. [5].

When different combinations of BAP and NAA were compared with each other, callus tissues and adventitious shoots primordium were formed around the wound edges of leaf explants cultured on MS medium with 0.5 mg/L BAP, 0.5 mg/L BAP+0.5 mg/L NAA, 1 mg/L BAP+1 mg/L NAA, 2 mg/L BAP and 3 mg/L BAP+1 mg/L NAA. When we compared the MS media with various concentrations and combinations of plant growth regulators, the maximum shoot formation was observed in MS medium with 2 mg/L BAP and MS medium with 3 mg/L BAP and 1 mg/L NAA. Similarly, a study by other researchers demonstrated that more shoot development was obtained with 13.32 µM BA and 5.37 µM NAA in *V. thapsus* [6].

After 4 weeks, calli and shoots were transferred to media containing the same concentrations of plant growth regulators for shoot multiplication. Small adventitious shoots and clustered shoots were observed during 8 weeks of culture (Figure 2). The number of shoots per explant and the shoot regeneration rate were calculated to determine the most suitable shoot induction medium (Table 1). The callus tissue and shoot primordia were allowed to multiplication by subculturing. Subculturing was found to be effective in the multiplication of callus tissues and shoot primordia of *V. biledschikianum*.

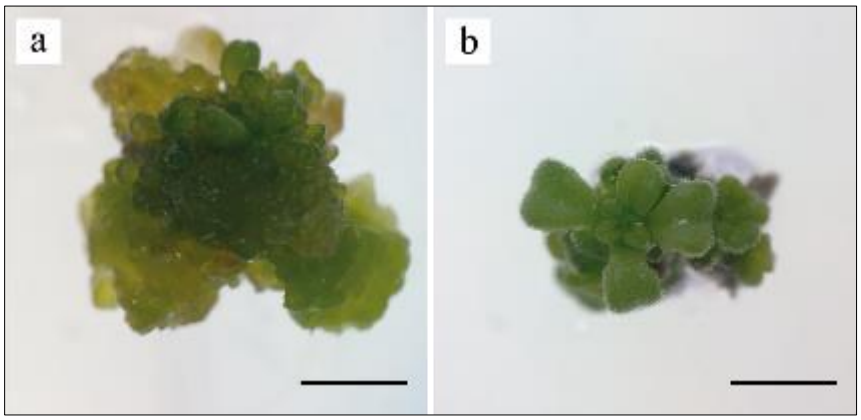

Figure 2 The adventitious shoots on MS medium containing PGRs in 8 weeks of culture (***scale** bar*=0.5 cm)

Table 1 Effect of MS medium composition and growth regulators on shoot multiplication from shoot tip explants of *V. biledschikianum* after 12 weeks of *in vitro* culture (-: No development, +++: Little shoot development, ++++: Good shoot development, ++++: Intensive shoot development)

Medium No	Plant Growth Regulators (mg/L)				Number of shoots per explants
	6-BAP	NAA	KIN	2,4 D	
2	0.5	0.5			+
3	0.5	1			+++
4	1	1			+
5	2				++++
10	3	1			++++
11			0.5		+

The effect of different media for the establishment of root induction from shoots was investigated. We used different media for root induction of adventitious shoots of *V. biledschikianum*. Root induction was established in MS without plant growth regulators, MS with 1 g/L charcoal activated, MS with 1 mg/L NAA, and MS with 2 mg/L NAA+1 g/L charcoal activated. Micro shoots were carefully excised from the shoot-clump and individually transferred to root induction media. Rooting was not obtained on MS medium without plant growth regulators. For root induction, MS medium with 1 g/L activated charcoal was more effective than MS medium with either 1 mg/L NAA or a combination of 2 mg/L NAA and 1 g/L activated charcoal. (Figure 3). All propagated plants exhibited normal growth and were fully developed by the end of the culture.

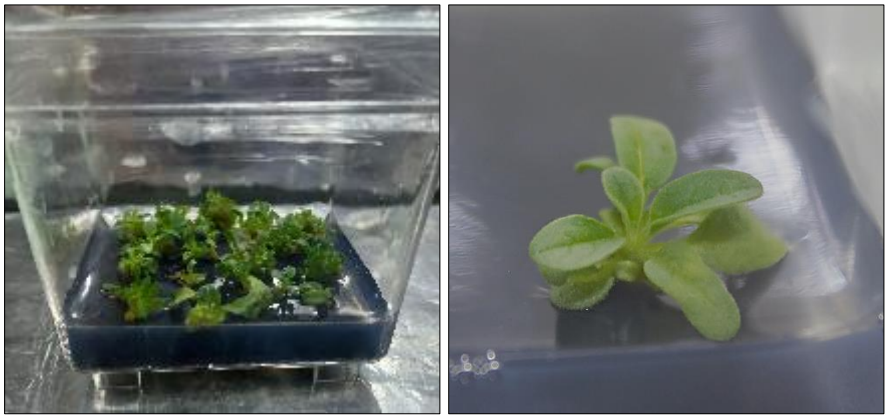

Figure 3 Shoot elongation and root induction of *V. biledschikianum*

4. Conclusion

The effects of different concentration and combination of plant growth regulators on shoot induction in *V. biledschikianum* were investigated. The best results for shoot regeneration frequency of *V. biledschikianum* in our study were obtained by MS media supplemented with 2 mg/L BAP and MS medium with 3 mg/L BAP + 1 mg/L NAA. The obtained shoots were rooted in MS medium with 1 g/L charcoal activated. In this study, we established an effective propagation system for *V. biledschikianum* via indirect organogenesis, using leaf as a source of explants for shoot induction.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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