Adventitious shoot regeneration and in vitro flowering of *Anthemis xylopoda* O.Schwarz, a critically endangered Turkish endemic

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**Abstract:** A protocol has been developed for the direct induction of adventitious shoots from leaf explants of in vitro raised *Anthemis xylopoda* O.Schwarz, a critically endangered Turkish endemic. The Murashige and Skoog (MS) media supplemented with N\textsuperscript{6}–benzyladenine (BA), kinetin (KIN), and thidiazuron (TDZ) were used in regeneration experiments. BA at 0.5 mg L\textsuperscript{-1} concentration was chosen as the optimal treatment because it yielded the maximal number of adventitious shoots with the best quality (6.70 ± 1.05 shoots/explant). The highest mean of maximum shoot length was observed in MS media containing 0.2 mg L\textsuperscript{-1} BA (4.30 ± 0.48 cm). Regenerated shoots rooted best on ½MS medium containing 0.5 mg L\textsuperscript{-1} indole-3-butyric acid (IBA). Flower buds also occurred during rooting. For this study, the existence of auxins in the medium was essential for in vitro flower bud induction. IBA was found to be more efficient than indole-3-acetic acid (IAA) in the induction of flower buds of *A. xylopoda*. The maximum flowering percentage was obtained when the shoots were cultured on ½MS medium containing 1.0 mg L\textsuperscript{-1} IBA. Flowers produced in vitro were morphologically normal and yellow.

**Key words:** *Anthemis xylopoda*, endemic plant, adventitious shoot regeneration, in vitro flowering

**Kritik olarak tehdit altında Türkiye endemiği *Anthemis xylopoda* O.Schwarz’ın adventif sürgün rejenerasyonu ve in vitro çiçeklenmesi

Özet: Kritik olarak tehdit altında Türkiye endemiği *Anthemis xylopoda* O.Schwarz’in in vitro üretilmiş sürgünlerinin yaprak eksplantlarından doğrudan adventif sürgün oluşumu için bir yöntem geliştirilmişdir. Rejenerasyon denemelerinde N\textsuperscript{6}–benzyladenine (BA), kinetin (KIN) ve thidiazuron (TDZ) ilaveli Murashige ve Skoog (MS) besi ortamları kullanılmıştır. 0,5 mg L\textsuperscript{-1} BA konsantrasyonu iyi kalitedeki adventif sürgünlerin en yüksek sayısını vermesi nedeni ile optimum uygulama olarak seçilmiş (6,70 ± 1,05 sürgün/eksplant). En yüksek maksimum sürgün boyu ortalaması 0,2 mg L\textsuperscript{-1} BA içeren MS ortamında gözlenmiştir. Rejenere olmuş sürgünler en iyi 0,5 mg L\textsuperscript{-1} indol-3-bütik asit (IBA) ilaveli ½ MS ortamında köklenmiştir. Köklenme sırasında çiçek tomurcukları da oluşmuştur. Bu çalışmadı, in vitro çiçek tomurcuklarının oluşumu için ortamda oksinlerin varlığı gerekliydi. *A. xylopoda’nın* in vitro çiçek tomurcuklarının oluşumunda IBA, indol-3-asetik asit (IAA) daha verimlidi. Adventif sürgünler 1,0 mg L\textsuperscript{-1} IBA içeren ½MS ortamına kültüre edildiklerinde en yüksek çiçeklenme yüzdesi elde edilmişti. In vitro üretilmiş çiçekler morfolojik olarak normal görüümüldü vearsi renklidiydi.

**Anahtar sözcükler:** *Anthemis xylopoda*, endemik bitki, adventif sürgün rejenerasyonu, in vitro çiçeklenme
Introduction

Turkey has an enormous phytodiversity due to its geological, geographical, and ecological properties. One third of the species in the Turkish flora are known to be endemic and many of them require proper management practices to conserve their germplasm because of their rarity and threatened existence. Besides conventional methods of propagation, plant tissue culture techniques are an excellent option for the study and the conservation of endemic, threatened, and endangered species (1,2). In this case, seeds are used as explants since they are representative of the genetic structure of the target population to be conserved. Additionally, the in vitro germination of seeds allows the yield of a large number of aseptic explants to be inoculated in tissue culture (3).

The genus *Anthemis*, belonging to the family *Asteraceae*, is represented by 50 species in Turkey (4), with an endemism of nearly 54%. *Anthemis xylopoda* O.Schwarz, a stenoendemic of Turkey falls within the CR (Critically Endangered) category in the Red Data Book of Turkey (5). This species is only known to have 2 populations found on the Çıplak Mountain and the Mahmut Mountain in western Turkey. According to the ongoing local ex situ conservation studies, the number of individuals in these populations is prone to decrease under natural conditions. Habitat destruction is one of the main factors threatening the existence of *A. xylopoda* (6).

Uzel et al. (7) have recently reported the chemical composition and antimicrobial activity of the essential oils isolated from air-dried leaves and flowers of *A. xylopoda*. In their studies on *A. xylopoda* the major components were borneol, 1,8-cineole, and 2,5,5-trimethyl-3,6-heptadien-2-ol for the essential oil from flowers and borneol, 1,8-cineole, α,β-thujone, 2,5,5-trimethyl-3,6-heptadien-2-ol, and carvacrol for the essential oil from leaves. Borneol was the major constituent of both oils studied. Their results showed that the oils of *A. xylopoda* exhibited significant antimicrobial activity. These results have the potential to create increased interest about this critically endangered species. This kind of interest may also threaten the natural stands of *A. xylopoda*. Therefore, there is an urgent need to look for alternative ways of propagation. At this point, in vitro propagation is a feasible alternative for the rapid multiplication and maintenance of the germplasm (8).

Recently Erdağ and Emek studied the in vitro shoot propagation of this species through axillary branching from in vitro germinated seeds (9). Our primary aim in the present study was to develop an efficient protocol for direct differentiation of shoots from leaf segments of *A. xylopoda* without intervening callus. The development of an efficient regeneration protocol can assist in the conservation of this critically endangered plant as well as in the future with transformation studies. In addition, we report the in vitro flowering of the *A. xylopoda* plantlets. The present work can be regarded as the first report on in vitro developed flowers of Turkey. In this preliminary study, we investigated the influence of auxins on the in vitro flowering of plants regenerated from leaf explants that could potentially be used for the extraction of active compounds for pharmaceutical purposes.

Materials and methods

The seeds of *A. xylopoda* collected from the wild were acquired from the Ege University Botanical Garden & Herbarium Research and Application Center. Shoots were obtained in aseptic conditions through axillary shoot branching from in vitro germinated seeds following the procedure reported earlier (9). Fully developed leaves, measuring about 1.0-1.5 cm in length, were excised from in vitro proliferated shoots of *A. xylopoda*. Each leaf was cut into two portions and explants were placed either abaxially or adaxially in contact with the regeneration media. The regeneration medium used contained MS salts, vitamins (10) and 3% (w/v) sucrose; this basal medium was used as the control. Plant growth regulators tested included various cytokinins like N⁶-benzyladenine (BA) (0.1, 0.2, 0.5, 1.0, and 2.0 mg L⁻¹), kinetin (KIN) (0.1, 0.2, 0.5, 1.0, and 2.0 mg L⁻¹), and thidiazuron (TDZ) (0.005, 0.01, and 0.05 mg L⁻¹). Culture vessels used were 190 mL glass jars containing 30 mL of medium and 100 mL Erlenmeyer flasks containing 25 mL of medium; both were sealed with 4 layers of aluminum foil. Regenerating explants were subcultured with fresh medium of the same composition at an interval of 4 weeks. After 8 weeks,
the well-developed regenerated shoots in vitro at 2-4 cm were excised and transferred to MS and ½MS medium (100 mL Erlenmeyer flask containing 25 mL of medium) with or without different concentrations (0.5, 1.0, and 1.5 mg L\(^{-1}\)) of auxins (IBA and IAA) for root induction.

In all experiments, nutrient media were adjusted to pH 5.8 using 0.1 M NaOH and 0.1 M HCl. Agar-agar (Sigma) was added (0.8%) before autoclaving at 105 kPa for 15 min at 121 °C. Cultures were incubated at 24 ± 2 °C under a light regime of 16 h photoperiod by cool-white fluorescent lamps.

Twenty explants per treatment were tested on each adventitious shoot regeneration and rooting medium. Experiments were carried out at least 3 times using a completely randomized design. Results were analyzed comparing the variance and the means using Duncan’s multiple range test at P < 0.05.

After 12 weeks of root initiation, rooted plantlets were removed from the culture and acclimatized as described by Erdağ and Emek (9).

**Results and discussion**

After being cultured for 15 days on MS medium supplemented with different concentrations of BA, KIN, or TDZ, the leaf explants of *A. xylopoda* began to swell and produce granular structures on the margins of their cutting surfaces and whole leaf surfaces. Two weeks later, the granular structures developed green shoots without callus formation. The formation of shoot buds occurred either on the adaxial or on the abaxial surface, whichever was in contact with the medium. The orientation of the explant on the medium was not an effective factor in this study.

Shoot regeneration occurred in response to all growth regulator combinations tested. A non-morphogenic response was developed from leaf explants in MS medium without growth regulators. TDZ treatments resulted in shoot formation in 100% of explants with an average of 9-10 shoots per explant. The shoots developed abnormally because of hyperhydricity and did not elongate (less than 0.5 cm in length). Increased TDZ concentrations (0.005, 0.01, and 0.05 mg L\(^{-1}\)) decreased the number of shoots per explant (respectively 9.8 ± 0.42, 4.3 ± 0.49, 3.1 ± 0.47). Erdağ and Emek (9) reported that axillary shoot proliferation was prompted on an MS medium supplemented with TDZ, but vigorous callus formation at bases of shoots was observed with no hyperhydricity in their study. Hyperhydricity is a frequent problem in tissue culture studies and limits the growth and multiplication in vitro and establishment ex vitro (11,12). The occurrence of hyperhydric shoots was affected by explant type, plant growth regulators, and the gelling agent in the medium, as well as the type of ventilation closure used (13). In this study, the hyperhydricity may depend on the correlation between explant type and plant growth regulator type. Therefore, because of the occurrence of abnormal shoot formation, this cytokinin was not used in further experiments.

BA and KIN induced healthy adventitious shoots. BA was more effective than KIN in inducing adventitious shoots. Cytokinins are generally recognized as critical for the production of shoot primordia under in vitro condition and a number of examples in the literature show the beneficial effect of BA (also observed in our study) over other cytokinins for shoot multiplication (14). Moreover, BA was evaluated as an effective cytokinin for shoot multiplication in some Asteraceae members such as *Gerbera jamesonii* (15), *Centaurea junoniana* (16), *Tagetes erecta* (17), and *Achillea filipendulina* (18). The number of shoots increased with the increase in concentration of BA or KIN from 0.1 to 0.5 mg L\(^{-1}\) and decreased when higher concentrations (1.0 and 2.0 mg L\(^{-1}\)) were used (Table 1) (Figure 1). BA at 0.5 mg L\(^{-1}\) concentration was chosen as the optimal treatment because it yielded the maximal number of adventitious shoots showing the best quality (6.70 ± 1.05 shoots/explant) (Figure 2). The highest mean for maximum shoot length was observed in the MS media containing 0.2 mg L\(^{-1}\) BA (4.30 ± 0.48 cm).

After 2 subculturings, the shoots regenerated directly from leaf explants were excised from the parent culture and transferred to the MS and ½MS medium supplemented with or without different concentrations of (0.5, 1.0, and 1.5 mg L\(^{-1}\)) IAA or IBA for rooting. Rhizogenesis was achieved in all tested media. The rooting process was very slow and most roots emerged after 8 weeks of culture; however, the
results were recorded after 12 weeks. In the media with the IAA treatment, a little callus formation occurred on the bottom of the shoots (Figure 3). Results obtained using the rooting media containing auxin were very similar, without significant differences between them (Table 2). The highest value for rooting was obtained in the ½MS medium containing 0.5 mg L⁻¹ IBA (Figure 4).

Flower buds also occurred during rooting. In the present study, the supply of exogenous growth regulators in the medium was essential for flower bud induction. It is known that the plant growth regulator requirement of plants for in vitro flowering varies. Empirical guides show that exogenous auxin could act as a principal floral inhibitor (19) but in the present study IAA and IBA in the medium did not inhibit in

Table 1. Effect of different concentrations of BA and KIN on adventitious shoot regeneration from leaf explants of A. xylopoda.

<table>
<thead>
<tr>
<th>Plant Growth Regulators (mg L⁻¹)</th>
<th>Explant forming shoots (%) Mean ± SD</th>
<th>Number of shoots/explant Mean ± SD</th>
<th>Shoot length (cm) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA</td>
<td>KIN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>-</td>
<td>70 ± 0.47 abcd</td>
<td>1.40 ± 0.51 e</td>
</tr>
<tr>
<td>0.2</td>
<td>-</td>
<td>65 ± 0.49 bcd</td>
<td>2.20 ± 0.63 d</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
<td>85 ± 0.37 ab</td>
<td>6.70 ± 1.05 a</td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>100 ± 0.00 a</td>
<td>3.20 ± 0.78 c</td>
</tr>
<tr>
<td>2.0</td>
<td>-</td>
<td>80 ± 0.41 abc</td>
<td>1.20 ± 0.42 e</td>
</tr>
<tr>
<td>-</td>
<td>0.1</td>
<td>40 ± 0.50 e</td>
<td>1.20 ± 0.42 e</td>
</tr>
<tr>
<td>-</td>
<td>0.2</td>
<td>50 ± 0.51 cd</td>
<td>1.40 ± 0.51 e</td>
</tr>
<tr>
<td>-</td>
<td>0.5</td>
<td>60 ± 0.50 bcd</td>
<td>4.00 ± 0.94 b</td>
</tr>
<tr>
<td>-</td>
<td>1.0</td>
<td>60 ± 0.50 bcd</td>
<td>2.25 ± 0.79 d</td>
</tr>
<tr>
<td>-</td>
<td>2.0</td>
<td>50 ± 0.51 cd</td>
<td>1.30 ± 0.48 e</td>
</tr>
</tbody>
</table>

The experiments were repeated 3 times, each experiment consisting of 20 replicates. Data were collected after 8 weeks of culture. Means within a column followed by the same letter are not significantly different at 0.05 level according Duncan’s multiple range test.
vitro flower bud formation (except 0.5 mg L\(^{-1}\) IAA). Regenerants subcultured on the MS and ½MS supplemented with or without 0.5 mg L\(^{-1}\) IAA failed to flower in vitro. The regenerated plantlets that were transferred onto the IAA (1.0 and 1.5 mg L\(^{-1}\)) and IBA (0.5, 1.0, and 1.5 mg L\(^{-1}\)) media showed flowering. The first flower buds were visible after 2 successive subcultures; however, the results were recorded after 3 subcultures. While the plantlets which were transferred to the media supplemented with IBA produced new shoots and flower buds, plantlets in

![Figure 3. A little callus formation occurred on the bottom of the shoots on ½MS medium supplemented with 1.5 mg L\(^{-1}\) IAA.](image)

![Figure 4. Rooted plantlet on ½MS medium containing 0.5 mg L\(^{-1}\) IBA.](image)

### Table 2. Rooting and flowering of *A. xylopoda* on MS and ½ MS media supplemented with various auxins.

<table>
<thead>
<tr>
<th>Treatments (mg L(^{-1}))</th>
<th>Rooting (%)</th>
<th>Flowering response</th>
<th>Flower induction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>15 c</td>
<td>No flowering response</td>
<td>0 d</td>
</tr>
<tr>
<td>½MS</td>
<td>20 bc</td>
<td>No flowering response</td>
<td>0 d</td>
</tr>
<tr>
<td>MS + IAA (0.5)</td>
<td>50 ab</td>
<td>No flowering response</td>
<td>0 d</td>
</tr>
<tr>
<td>½MS + IAA (0.5)</td>
<td>55 ab</td>
<td>No flowering response</td>
<td>0 d</td>
</tr>
<tr>
<td>MS + IAA (1.0)</td>
<td>50 ab</td>
<td>Flowering response</td>
<td>10 d</td>
</tr>
<tr>
<td>½MS + IAA (1.0)</td>
<td>55 ab</td>
<td>Flowering response</td>
<td>20 bcd</td>
</tr>
<tr>
<td>MS + IBA (0.5)</td>
<td>55 ab</td>
<td>Flowering response</td>
<td>10 d</td>
</tr>
<tr>
<td>½MS + IAA (1.5)</td>
<td>55 ab</td>
<td>Flowering response</td>
<td>14 cd</td>
</tr>
<tr>
<td>MS + IBA (0.5)</td>
<td>55 ab</td>
<td>Flowering response</td>
<td>20 bcd</td>
</tr>
<tr>
<td>½MS + IBA (0.5)</td>
<td>80 a</td>
<td>Flowering response</td>
<td>40 b</td>
</tr>
<tr>
<td>MS + IBA (1.0)</td>
<td>55 ab</td>
<td>Flowering response</td>
<td>36 bc</td>
</tr>
<tr>
<td>½MS + IBA (1.0)</td>
<td>50 ab</td>
<td>Flowering response</td>
<td>65 a</td>
</tr>
<tr>
<td>MS + IBA (1.5)</td>
<td>55 ab</td>
<td>Flowering response</td>
<td>25 bcd</td>
</tr>
<tr>
<td>½ MS + IBA (1.5)</td>
<td>50 ab</td>
<td>Flowering response</td>
<td>40 b</td>
</tr>
</tbody>
</table>

The experiments were repeated 3 times, each experiment consisting of 20 replicates. Data were recorded after 3 subcultures in induction. Mean percentages within a column followed by the same letter are not significantly different at 0.05 level according Duncan’s multiple range test.
IAA medium developed only reproductive shoots (Figure 5). This result is probably due to a correlation between vegetative growth and the plant growth regulator stimulating in vitro flowering; however, this hypothesis requires further investigation. In this study, the existence of auxins in the medium was essential for the in vitro induction of *A. xylopoda* flower buds. The essentiality of auxins for flower bud induction and development has been reported for a few plants such as *Pisum sativum* (20), *Vigna radiate* (21), and *Vigna mungo* (22). Between the 2 kinds of auxin used in the experiment, IBA was found to be more efficient than IAA in the induction of in vitro flowering of *A. xylopoda*. Similar findings were obtained for another Asteraceae member *Pentanema indicum* (23). However, the formation of flower buds depended on the concentration of the applied plant growth regulators. The maximum flowering percentage was obtained when the shoots were cultured on ½MS medium containing 1.0 mg L\(^{-1}\) IBA (Table 2) (Figure 6).

The flowering percentage increased when the shoots cultured on ½MS medium with plant growth regulators. The MS medium is characterized by its high concentration in mineral salts in general and nitrogen in particular and it is known as a floral inhibitor (24). A high concentration of nitrogen in the MS media inhibited flowering and promoted vegetative growth, where the vegetation most probably competed more efficiently for carbohydrates from the medium (25). According to the floral nutrient diversion hypothesis, C/N ratios increase in buds during flower induction (26). In the present investigation using ½MS medium enhanced in vitro flowering in *A. xylopoda*. This result shows an accordance with the floral nutrient diversion hypothesis. Many studies have reported that using ½MS mineral medium or a reduced nitrogen level enhanced in vitro flowering in geophytes like *Bambusa vulgaris, Dendrocalamus giganteus, D. strictus* (27), *Cymbidium* (28), *Doritis* (29), and *Panax gingseng* (30), and in annual herbaceous species like *Orichophragmus violaceus* (31).

Flowers produced in vitro were morphologically normal and yellow, although the structures of the female and male gametophytes were not examined in detail and no seed testing was conducted. Therefore, further research is required to study fruiting and seed physiology.

Rooted and flower buds bearing plantlets were gradually acclimatized under conditions described by Erdağ and Emek (9) and transferred to ex vitro conditions. The survival percentage of plantlets was approximately 50%.

Figure 5. In vitro flowering on ½MS medium with 1.0 mg L\(^{-1}\) IAA (tube diameter is 25 mm).

Figure 6. In vitro flowering on ½MS medium with 1.0 mg L\(^{-1}\) IBA (arrows = flower buds).
To conclude, we report an efficient protocol on adventitious shoot regeneration of *A. xylopoda*, an endemic and critically endangered species from Turkey. Micropropagation techniques are an important aid for the ex situ conservation of rare, endemic, or threatened plants. A reliable and efficient regenerating protocol is also a prerequisite for the genetic engineering of these species. In addition, we report the in vitro flowering of *A. xylopoda* plantlets. In vitro flowering provides an ideal experimental system for studying the biological mechanism of flowering as well as in vitro formed flowers that have already been derived from differentiated organs can accumulate various substances of pharmaceutical importance.

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**References**

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