

Development of an *in vitro* propagation protocol for *Lathyrus undulatus* Boiss.: A threatened species endemic to Türkiye

Original Article

Abstract:

Lathyrus undulatus Boiss. is a threatened plant species endemic to Türkiye from the Fabaceae family. The present study represents the first attempt to develop an *in vitro* propagation protocol for the conservation of this species by evaluating the effects of several cytokinins and auxins on organ development. Nodal segments from adult individuals were cultured in Murashige and Skoog (MS) medium with 6-benzylaminopurine (BAP), thidiazuron (TDZ), and *N*⁶-(2-isopentenyl)adenosine (2IP), and generated *in vitro* shoots were cultured on full-, half-, and quarter-strength MS medium with 3-indoleacetic acid (IAA), 1-naphthaleneacetic acid (NAA), and indole-3-butyric acid (IBA). BAP treatments produced axillary shoots with a higher number of nodes than the other treatments tested. TDZ treatments were superior to other cytokinins tested for shoot production from nodal segments, while 2IP treatments were superior for triggering shoot elongation. Rooting experiments demonstrated that *L. undulatus* is a problematic species to root *in vitro*. Shoots cultured on hormone-free MS medium and its diluted variants did not exhibit the rhizogenesis response. Likewise, shoots were found to be unresponsive to IBA treatments. NAA treatments triggered a rooting response in full- and half-strength culture medium, while IAA induced a limited rooting response in half- and quarter-strength culture medium. In the rooting phase, NAA reached a maximum root-induction rate of only 20%, which should be studied further to increase the response and ensure enhanced integration into the soil for conservation purposes. Our findings indicated that while adenine-type cytokinins are superior at inducing shoot elongation, phenylurea-type cytokinins should be favored for multiple shoot induction in *L. undulatus*.

Key words:

auxins, cytokinins, *ex situ* conservation, micropropagation, nodal segment

Apstrakt:

Razvoj protokola za *in vitro* razmnožavanje vrste *Lathyrus undulatus* Boiss.: ugrožene vrste endemične za Tursku

Lathyrus undulatus Boiss. je ugrožena biljna vrsta iz porodice Fabaceae, endemična za Tursku. Ovo istraživanje predstavlja prvi pokušaj razvoja protokola za *in vitro* razmnožavanje ove vrste u cilju njenog očuvanja, kroz ispitivanje efekata različitih citokinina i auksina na razvoj biljnih organa. Nodalni segmenti uzeti sa adultnih biljaka gajeni su na Murashige i Skoog (MS) podlozi sa 6-benzilaminopurinom (BAP), tidiazuronom (TDZ) i *N*⁶-(2-izopentenil)adenozinom (2IP), dok su *in vitro* dobijeni izdanci dalje kultivisani na punoj, polovičnoj i četvrtinskoj MS podlozi sa 3-indolsirćetnom kiselinom (IAA), 1-naftalensirćetnom kiselinom (NAA) i indol-3-buternom kiselinom (IBA). Tretmani sa BAP-om rezultirali su formiranjem aksilarnih izdanaka sa većim brojem nodusa u odnosu na ostale ispitivane tretmane. Tretmani sa TDZ-om pokazali su se superiornim za indukciju izdanaka iz nodalnih segmenata, dok su tretmani sa 2IP-om bili najefikasniji u podsticanju izduživanja izdanaka. Eksperimenti ožiljavanja pokazali su da je *L. undulatus* vrsta koju je teško ožiliti u *in vitro* uslovima. Izdanci gajeni na MS podlozi bez hormona i njenim razblaženim varijantama nisu pokazali odgovor u vidu rizogeneze. Takođe, izdanci nisu reagovali na tretmane sa IBA. Tretmani sa NAA izazvali su formiranje korena na punoj i polovičnoj podlozi, dok je IAA indukovala ograničen odgovor na polovičnoj i četvrtinskoj podlozi. U fazi ožiljavanja, NAA je obezbedila maksimalnu stopu indukcije korena od svega 20%, što zahteva dalja istraživanja radi povećanja uspešnosti ožiljavanja i boljeg prilagođavanja biljaka zemljištu u svrhu očuvanja vrste. Dobijeni rezultati ukazuju da su citokinini adeninskog tipa efikasniji u podsticanju izduživanja izdanaka, dok citokinine fenilurejskog tipa treba favorizovati za indukciju većeg broja izdanaka kod vrste *L. undulatus*.

Ključne reči:

auksini, citokinini, *ex situ* očuvanje, mikropropagacija, nodalni segment

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Introduction

The Fabaceae (Leguminosae) family is one of the plant families with the highest biodiversity in angiosperms, with nearly 19,500 species in 770 genera, including many crops of agricultural and economic value (Azani et al., 2017). The Fabaceae family shows high diversity in all three main tropical vegetation types: dry forests, tropical rainforests, and woody savannas (Yahara et al., 2013). Legumes play a role in biological nitrogen fixation, which can meet most of the soil's nitrogen demand and increase nitrogen resources. The main threats to family members are overgrazing, climate change, haymaking, land reclamation, dam construction, erosion, and urbanization (Aydin-Kandemir & Demir, 2021). The members of the genus *Lathyrus* are predominantly centered in the Mediterranean region, while 25 of 79 *Lathyrus* taxa are endemic to Türkiye (Genç et al., 2021). Endemic plants are vulnerable to anthropogenic threats and natural disturbances, thereby facing a higher risk of extinction (Coelho et al., 2020). *Lathyrus undulatus* is one of those endemic plant species to Türkiye, which is listed as vulnerable (VU) in the Red Data Book of Turkish Plants (Ekim et al., 2000) and as endangered (EN) in The IUCN Red List of Threatened Species with a decreasing trend of its natural populations (Rowe et al., 2019). Thus, suitable propagation and conservation methods are necessary for *L. undulatus*.

Plant tissue culture techniques, allowing the propagation of organs, tissues, or cells under controlled aseptic conditions in the laboratory, have been widely used to propagate endangered plant species for conservation purposes (Wochok, 1981; Phillips & Garda, 2019). Adding plant growth regulators (PGRs) to a culture medium can enhance the efficacy of the medium on *in vitro* propagation. In this context, this study aims to develop an *in vitro* propagation protocol for the endemic and threatened *L. undulatus* using nodal explants obtained from mature individuals. Because the chemical structure of PGRs profoundly impacts developmental responses, this study evaluated the comparative effects of adenine- and phenylurea-type cytokinins, as well as indole- and naphthalene-based auxins, to optimize the propagation protocol for *L. undulatus*. In the literature, *in vitro* propagation efforts on *Lathyrus* species are limited to *L. odoratus* (Ochatt et al., 2010), *L. sativus* (Barpete et al., 2014), *L. cicera* (Li et al., 2016), *L. latifolius* (Hosoki and Honda, 1995), *L. chrysanthus* (Kahramanogullari et al., 2013), *L. pratensis* (Klčová & Gubišová, 2003), *L. japonicus* (Debnath et al., 2001), and *L. ochrus* (Saglam, 2012). However, to the best of our knowledge, there is no report on the *in vitro* propagation of *L.*

undulatus. Also, besides their utilization as food sources by humans and animals, *Lathyrus* species exhibit anti-inflammatory, antimicrobial, and cell membrane stabilization properties (Heydari et al., 2018). Thus, *in vitro* propagation strategies should be developed to enable biodiversity conservation and sustainable use of threatened *Lathyrus* species for medicinal purposes. In this context, the present study describes the first *in vitro* propagation protocol for *L. undulatus* by evaluating the effects of PGRs.

Materials and Methods

Explant source and surface sterilization

Shoot samples were collected from three blooming *L. undulatus* individuals in the Çınarlıdere Recreation Area (Derince, Kocaeli, Türkiye) in June 2016. No individual plant was collected by being completely removed from the wild. The explants were washed with tap water, and the leaves were removed before surface sterilization with 1% (v:v) NaOCl for 15 min. Then, they were thoroughly rinsed with sterile distilled water at least three times. The explants were cut into segments having at least one node and were transferred to Murashige and Skoog's (MS) medium (Murashige & Skoog, 1962) supplemented with 1.0 mg L⁻¹ 6-benzylaminopurine (BAP; Caisson Labs, B001) for generating plant material for shoot multiplication experiments.

Shoot multiplication, rooting, and acclimatization

The effects of cytokinins in shoot multiplication were tested in the MS medium supplemented with BAP, N⁶-(2-isopentenyl)adenosine (2IP; Duchefa Biochemie, D0934), and thidiazuron (TDZ; Duchefa Biochemie, T0916) at 0.25, 0.5, 1.0, and 2.0 mg L⁻¹ concentrations.

The regenerated shoots were transferred to quarter-, half-, and full-strength MS media, each supplemented with 3-indoleacetic acid (IAA; Sigma-Aldrich, 57333), 1-naphthaleneacetic acid (NAA; Sigma-Aldrich, N0640), and indole-3-butyric acid (IBA; Sigma-Aldrich, 57310) at 0.25, 0.5, 1.0, and 2.0 mg L⁻¹ concentrations. The concentration ranges for the PGRs were determined based on the works of Ochatt et al. (2010) and Barpete et al. (2014). The hormone-free quarter-, half-, and full-strength media were used as the control.

After the plants' roots had fully grown, they were removed from the culture containers, and the medium residue was removed from their roots. The plants were then transferred to the pots containing a 70:30 (v:v) combination of peat and perlite. The pots were covered with clear plastic bags with air holes for a week to complete the hardening process.

Culture media and environmental conditions

All medium variants were solidified with 7 g L⁻¹ agar (Duchefa Biochemie, P1001) and supplemented with 30 g L⁻¹ sucrose. The pH of the medium was adjusted to 5.7 - 5.8 before autoclaving. The cultures were incubated in the plant growth chamber at 23 ± 1 °C under 16-h light 8-h dark photoperiod under 80 µmol m⁻² s⁻¹ photon flux density. The parameters were measured at the end of an incubation period of 4 weeks.

Statistical analysis

All experiments were replicated three times, and each replicate contained at least 20 and 10 explants in the shoot multiplication and rooting phase, respectively. The treatments were conducted in a completely randomized design. The data were given as mean ± standard error. Levene's test was used to check the homogeneity of variances. The data were transformed where appropriate. Comparison of means was analyzed using Duncan's multiple range test at a significance level of $p < 0.05$ following an analysis of variance (ANOVA) through IBM SPSS Statistics 22 software. Following the approach of Kıymaz & Acemi (2024), the means were subjected to min-max normalization into [0, 1], and hierarchical cluster analysis supported with a heat map was used to visualize the relationships between the results. The cluster analysis based on Euclidean distance and the complete-linkage clustering method was created using the Interactive Clustered Heat Map Builder (Ryan et al., 2019).

Results

Shoot multiplication

The highest frequency of shoot multiplication was found in the medium with 0.25 and 2.0 mg L⁻¹ BAP. The TDZ treatments reached the maximum shoot induction ratio at 1.0 mg L⁻¹, while the maximum ratio from the 2IP treatments was found at 1.0 mg L⁻¹ concentration (Fig. 1A). The cytokinin treatments altered the shoot production from the nodal explants. The control medium gave 2.7 ± 0.2 shoots per explant, while TDZ treatments at 0.25 and 2.0 mg L⁻¹ slightly increased shoot production to 2.8 ± 0.3 and 2.9 ± 0.3 shoots per explant, respectively. The rest of the treatments decreased the number of shoots produced per explant. The minimum shoot number (1.5 ± 0.03) was found in the medium with 1.0 mg L⁻¹ BAP (Fig. 1B). The control group induced a mean shoot length of 2.5 ± 0.02 cm, shorter than all BAP and 2IP treatments. The maximum shoot elongation (6.5 ± 0.1 cm) was found in the explants cultured in the medium with 0.25 mg L⁻¹ 2IP, which gave statistically similar results to BAP treatment

at 0.5 mg L⁻¹. TDZ treatments gave statistically the same results and induced the formation of shorter shoots than the control group (Fig. 1C). The shoots that emerged from the nodes on the explants showed a significant variation in node numbers in response to cytokinin treatments. The BAP treatments greatly increased node production on the *in vitro* shoots, resulting in shorter internodes. The maximum node number was 3.3 ± 0.3 per shoot, which emerged on the medium with 1.0 and 2.0 mg L⁻¹ BAP. The node production results from all of the BAP treatments were statistically the same. The TDZ and 2IP treatments also gave higher node numbers than the control. However, they were relatively close to the control (0.8 ± 0.1) and each other, ranging from 1.2 to 2.0 nodes per shoot (Fig. 1D). General appearance of the species and the images of the cultures representing *in vitro* shoot development are given in Fig. 2A-E.

Rooting treatments

It was found that the rhizogenesis response is difficult to trigger in *L. undulatus* cultures *in vitro*. Most *in vitro* shoots cultured on a rooting medium did not respond. Thus, the root induction frequency was found at lower percentages. The shoots cultured on hormone-free MS medium and its 2- and 4-fold diluted variants did not show a rhizogenesis response. The highest root induction ratio was 20% from the full- and half-strength medium with 1.0 mg L⁻¹ NAA. Regardless of the medium strength, none of the IBA treatments induced a rhizogenic response. IAA-supplemented full-strength medium and NAA-supplemented quarter-strength medium also did not induce rooting. However, the limited effect of NAA in rooting makes it advantageous over other auxins tested for rooting in *L. undulatus*. The root production performance in the cultures was calculated over the mean root numbers. The highest root number (3.0 ± 0.58) was found in the shoots cultured on half-strength medium with 0.5 mg L⁻¹ NAA, and the other NAA treatments that induced rhizogenesis gave a single root per shoot. The half-strength medium with 1.0 and 2.0 mg L⁻¹ IAA gave 1.5 ± 0.29 and 1.3 ± 0.17 roots, while the other IAA treatments that induced rhizogenesis gave a single root. The auxin treatments greatly influenced root elongation in *L. undulatus*. The longest root (7.1 ± 0.58 cm) was found in the quarter-strength medium with 0.5 mg L⁻¹ IAA. The efficacy of low-concentration NAA on root elongation increased when the medium strength was reduced to half. A concentration-dependent decrease in root length was found in NAA-supplemented half-strength medium (Fig. 1E). The images of the cultures from the *in vitro* root development and soil transfer phases are

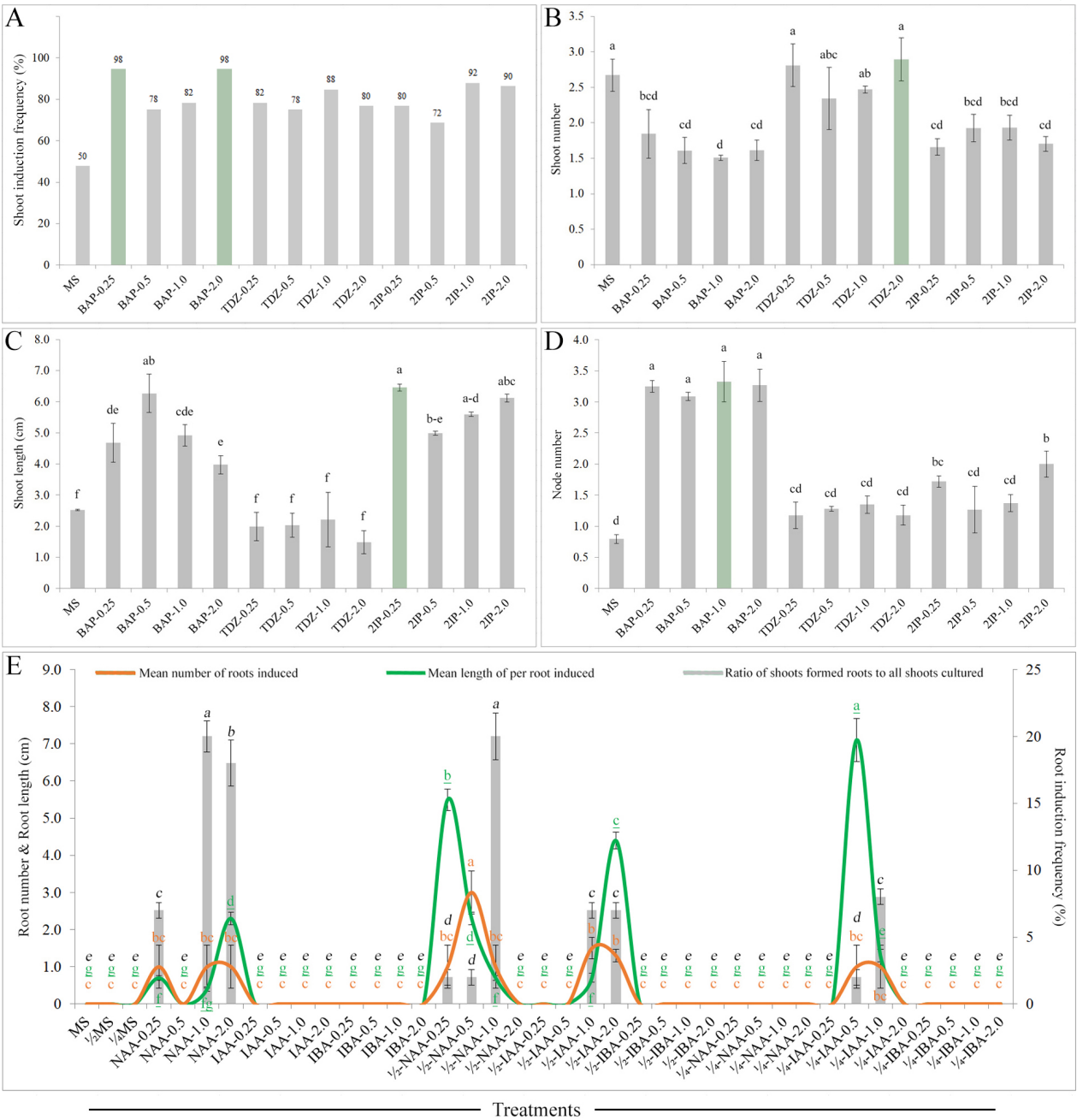


Fig. 1. The effects of plant growth regulators on *in vitro* organ formation and development in *Lathyrus undulatus*. The impacts of 6-benzylaminopurine, thidiazuron, and *N*⁶-(2-isopentenyl)adenosine on shoot induction rate (A), shoot production (B), shoot elongation (C), node number (D), and effects of 3-indoleacetic acid, 1-naphthaleneacetic acid, and indole-3-butyric acid on rhizogenesis (E). The means having the same letters are not significantly different by Duncan's multiple range test ($p < 0.05$).

given in Fig. 2F-I.

Hierarchical cluster analysis of normalized data

The hierarchical cluster analysis was performed only on shoot-related parameters because the rhizogenesis response was very low, and most treatments did not induce roots. The treatments are grouped into two main clusters. The control and TDZ treatments were grouped in the first main cluster, while the second main cluster was divided into two subgroups. The

first subgroup of the second main cluster included all 2IP treatments, while the other subgroup had all BAP treatments. Thus, the analysis showed that the cytokinins tested on shoot multiplication induced distinctive effects from each other on shoot number, shoot length, and node production (Fig. 3).

Discussion

To the best of our knowledge, this study presents

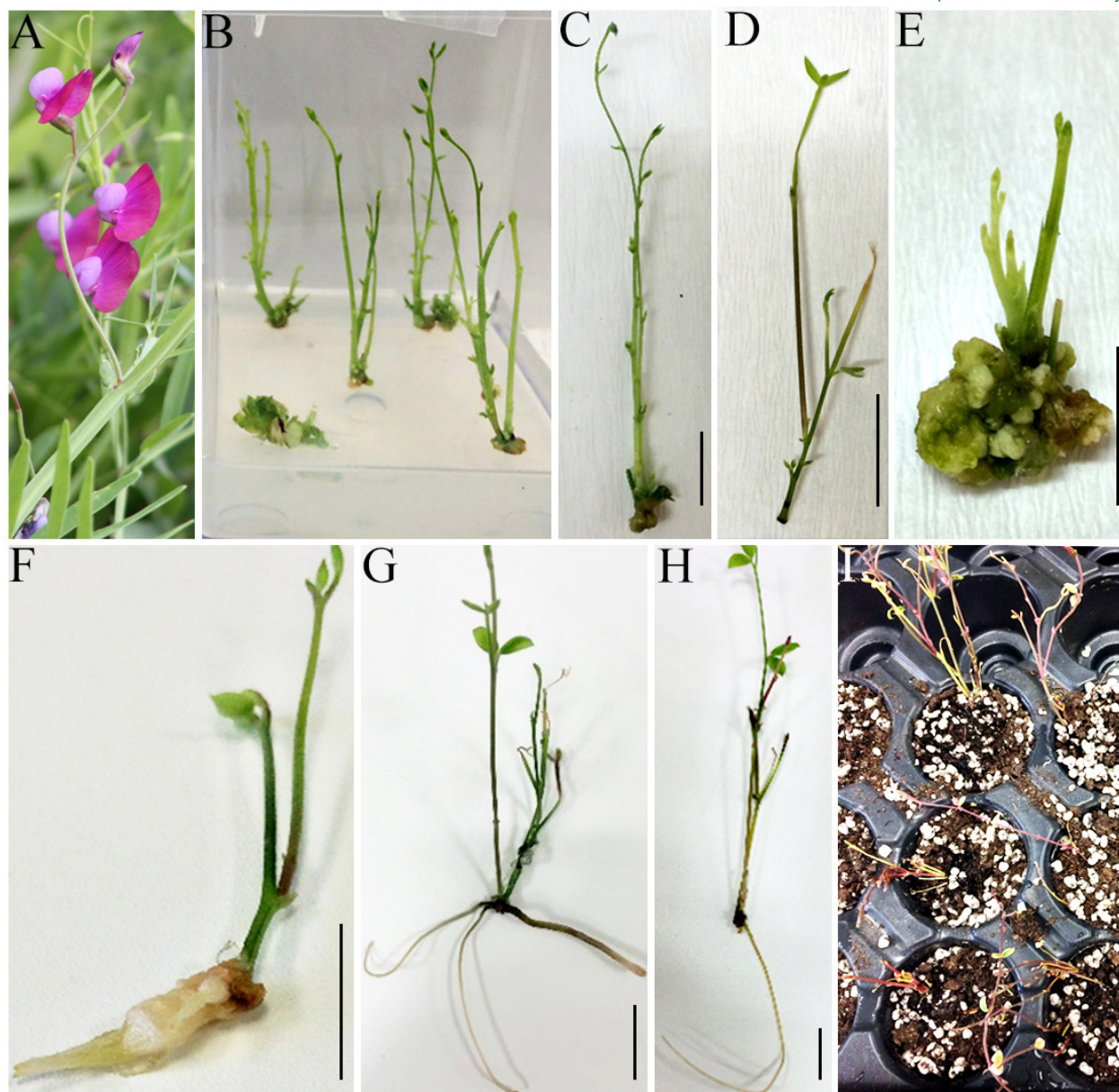


Fig. 2. Appearance of *Lathyrus undulatus* in nature and during *in vitro* propagation. The panels represent the general appearance of the species in nature during flowering period (A), view of the culture during *in vitro* shoot multiplication (B), the shoot development in the full-strength MS medium with 2.0 mg L⁻¹ 6-benzylaminopurine (C), 0.5 mg L⁻¹ N⁶-(2-isopentenyl)adenosine (D), 2.0 mg L⁻¹ thidiazuron (E), and the root development in the full-strength MS medium with 1.0 mg L⁻¹ 1-naphthaleneacetic acid (F), half-strength MS medium with 0.5 mg L⁻¹ 1-naphthaleneacetic acid (G), quarter-strength MS medium with 0.5 mg L⁻¹ 3-indoleacetic acid (H), and acclimatized plants to soil (I). The scale bars represent 1 cm.

the first *in vitro* propagation protocol for *Lathyrus undulatus*, demonstrating distinct responses of shoot multiplication and rooting to different classes of cytokinins and auxins and providing a preliminary protocol for the *ex situ* conservation of this threatened endemic species. To establish the primary cultures, nodal segments from mature individuals have been used to propagate plant species with recalcitrant seeds, including endemics and threatened plants (Acemi et al., 2013; Hernández-García et al., 2021).

Similarly, in our study, nodal segments obtained from adult individuals were used to propagate *L. undulatus*, which is an endangered species endemic to Türkiye. In our study, the mean shoot induction ratios of BAP treatments were superior to those of the other cytokinins tested. The high bud-breaking ability of BAP has been reported from various species, ranging from the mulberry tree (Zhang et al., 2021) to a macrophyte species, water speedwell (Shahzad et al., 2011). Our study also demonstrated

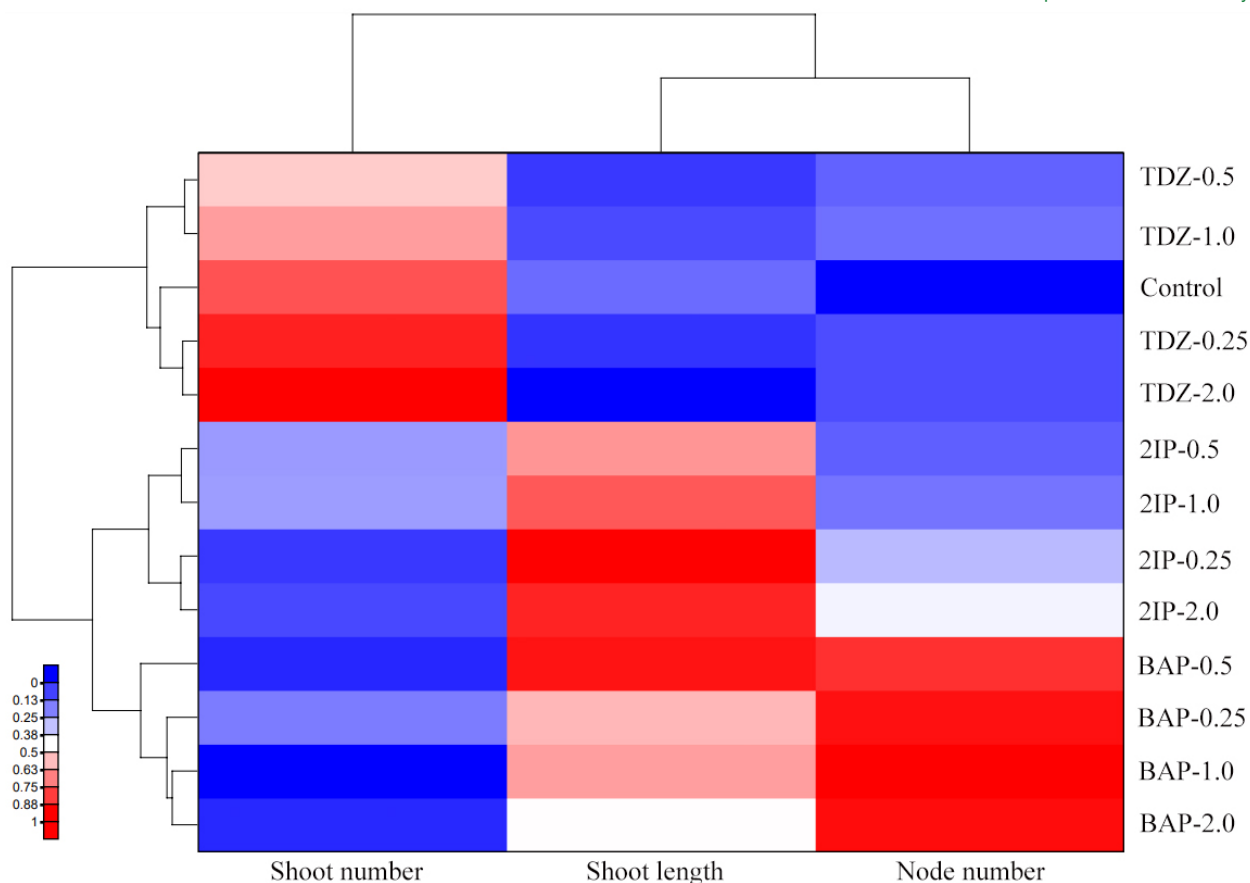


Fig. 3. A hierarchical clustering heat map created using the normalized developmental data, displaying similarities between the cytokinin treatments

and validated the cytokinin's efficiency on lateral bud dormancy release. The shoot production, however, was reduced in BAP- and 2IP-supplemented medium, whereas TDZ treatments resulted in more shoots per explant in *L. undulatus*. Although the shoot induction rate was lower in the control than in the cytokinin-supplemented medium, the shoot number per node was statistically the same as in the medium with 0.25 and 2.0 mg L⁻¹ TDZ. Thus, the finding showed the ability of TDZ in shoot production from nodes in *L. undulatus*. Similar findings were also reported from ornamental snapdragon (Kıymaz & Acemi, 2024) and strawberry (Li et al., 2021). The shoot elongation was triggered after 2IP treatments, although these treatments reduced the shoot production per node. Furthermore, the TDZ treatments did not show their shoot production efficacy in elongating the shoots. On the other hand, Klčová & Gubišová (2003) found that BAP treatments were better than TDZ treatments in shoot elongation in *L. pratensis*. A similar finding was also reported from rose cultivars. The researchers found that TDZ treatment inhibited shoot elongation and attributed the findings to the suppression of the bioactive gibberellic acid biosynthesis genes' expression and slightly increased expression of the

genes related to gibberellic acid catabolism (Çelikel et al., 2021). Furthermore, Sağlam (2012) reported the efficacy of combined BAP and NAA treatments in inducing shoot formation from cotyledon node explants of *L. ochrus*. Among the cytokinins tested in our study, 2IP and BAP are adenine-type cytokinins, while TDZ is a phenylurea-type cytokinin. Therefore, the efficacy of BAP and 2IP treatments on shoot elongation in our study reflects the superiority of adenine-type cytokinins over phenylurea-type cytokinins on shoot elongation in *L. undulatus*. The present results are in line with reports on medicinal *Sophora tonkinensis* (Jana et al., 2013) and *Butea monosperma* (Yarra et al., 2016), which also support the efficacy of 2IP and BAP on shoot elongation, respectively. Although 2IP and BAP induced longer shoots, only BAP treatments favored the production of more nodes per shoot, which is an advantageous condition for shoot multiplication through nodal cultures. An improvement in node number after *in vitro* BAP treatment was also reported from peppermint by Santoro et al. (2013).

In the rooting phase of *in vitro* cultures, the root induction can be stimulated by applying proper auxins, the effects of which could vary among

species cultured. In our study, we compared the effects of indole- (IAA and IBA) and naphthalene-based (NAA) auxins. IAA, IBA, and NAA were tested, and shoots did not respond to any of the IBA treatments tested in full-, half-, and quarter-strength MS medium. However, IBA treatments were reported to induce rooting in *L. latifolius* (1.1-1.7 roots per shoot) by Hosoki & Honda (1995). Klčová & Gubišová (2003) also reported rooting in full- and half-strength MS medium, and IBA-supplemented medium in *L. pratensis*. IBA has been stated as more stable than IAA and NAA, with lower level toxicity to the cultures, and it has a strong control on root initiation (Yunita & Nugraha, 2021). However, our study on *L. undulatus* showed that NAA exhibited superior responses over IBA and IAA in root initiation. Thus, the effects of those auxins are species-specific. The root production increased when the strength of the culture medium was reduced to half in the presence of 0.5 mg L⁻¹ NAA. The enhancement of root production due to reduced medium strength was also demonstrated in *Spathiphyllum* (Dönmez et al., 2022). Nonetheless, the efficacy of NAA on rooting in *L. undulatus* was inhibited when the medium strength was reduced to a quarter. Interestingly, only the IAA-supplemented medium induced rooting when the strength was reduced to a quarter. Thus, our rooting results also suggested a dynamic relationship between culture medium strength and auxin type tested. Also, it was found that as the culture medium strength decreased, the root lengths increased. In the other two *Lathyrus* species, *L. sativus* and *L. cicera*, researchers reported similar rooting responses to our study in *L. undulatus*, finding NAA as an effective auxin in rooting but not exceeding 60% rooting rate (Li et al., 2016). Ochatt et al. (2010) also described an *in vitro* propagation protocol for another *Lathyrus* species, *L. odoratus*, reporting that BAP and NAA are suitable for shoot multiplication and rooting, respectively.

The recalcitrancy of rooting under *in vitro* conditions in another *Lathyrus* species, *L. sativus*, was also declared by Barpete et al. (2014). The researchers suggested IAA for rooting in the species. Therefore, although NAA was found to be more advantageous than other auxins tested on *L. undulatus* in our study, it is clear that further studies should be conducted to enhance rooting. The suggested studies may involve testing the effects of carbon sources, other auxins, or different culture media. Also, the low rooting response observed in *L. undulatus* may be associated with species-specific limitations in rhizogenic competence (Barpete et al., 2014). Root induction is regulated not only by exogenously supplied auxins but also by the endogenous hormonal balance within the

tissues. Therefore, the limited effectiveness of the auxins tested may reflect unfavorable endogenous auxin-cytokinin interactions in the cultured shoots (Lakehal & Bellini, 2019).

Conclusion

In conclusion, our results suggested that phenylurea-type cytokinins should be preferred in multiple shoot induction, while adenine-type cytokinins can induce shoot elongation better. However, other cytokinins, or combinations thereof, should also be tested to improve the efficiency of shoot multiplication. Our study also demonstrated that *L. undulatus* is one of the hard-to-root species under *in vitro* conditions. A synthetic auxin, NAA, should be preferred over natural auxins to enhance rooting, but with limited efficiency. Considering that root development is one of the significant factors that directly affect the acclimatization of the species to the soil, further experiments should be designed with a special emphasis on rooting in *L. undulatus*. Lastly, the present study characterized the developmental effects of three cytokinins and auxins in *L. undulatus*, a threatened and endemic species to Türkiye, that have not been reported in earlier studies.

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