

AN EXAMPLE OF CLONAL MICROPROPAGATION
OF ENDEMIC BULBOUS ORNAMENTAL PLANTS:
BELLEVALIA TAURI

Ayşe Gul Nasircilar¹, Ozgul Karaguzel^{2✉}, Semra Mirici³,
Ozkan Eren⁴

Received on December 15, 2024

Presented by H. Najdenski, Member of BAS, on March 25, 2025

Abstract

Bellevalia tauri Feinbrun which has great potential as an ornamental, is an endemic bulbous plant of the *Asparagaceae* family. This study, one of a very limited number of in vitro culture studies in *Bellevalia* species, investigated the in vitro clonal propagation potential of immature zygotic embryos and twin bulb scale explants of *B. tauri*. Surface sterilization of immature embryos was performed using commercial bleach and ethanol. In addition to these, hot water applications were also tested in bulb scale explants to overcome the heavy contamination. Both explant types were cultured in MS medium containing different ratios of BAP and NAA after sterilization. Although there was no contamination after sterilization in immature embryos, it was quite difficult to overcome contamination in bulb scale explants despite different sterilization methods. The in vitro regeneration method also differed according to the type of explant used in the study. New bulb formation was achieved by direct organogenesis from bulb scale explants and by indirect organogenesis after callus formation from cultured immature embryos. The highest callus and bulblet formation from immature embryo explant was obtained on medium supplemented with 2.0 mg L⁻¹ BAP + 0.25 mg L⁻¹ NAA medium. In this medium, percentage of explants forming callus and number of bulblets per explant were determined as 100% and 3.43, respectively. As a result of the chromosome analysis performed to determine whether plant growth regulators have an effect on the chromosome number and morphology of *B. tauri* in tissue culture, no anomalies were detected in the chromosome number and structure.

This study was supported by the Scientific and Technical Research Council of Türkiye (TUBITAK, Project No. TOVAG-105O246). The authors wish to thank TUBITAK for the financial support.

<https://doi.org/10.7546/CRABS.2025.04.04>

Key words: *Bellevalia tauri* Feinbrun, immature embryo, in vitro propagation, chromosome analysis

Abbreviations: 6-Benzylaminopurine (BAP), Murashige and Skoog (MS), Naphthaleneacetic acid (NAA), PGRs (plant growth regulators)

Introduction. The genus *Bellevalia* Lapeyr., which belongs to the family *Asparagaceae*, subfamily Scilloideae, tribe Hyacinthaceae includes spring flowering bulbous geophytic ornamental plants. This genus, which has about 74 species worldwide, has 25 species in Türkiye, 15 of which are endemic [1, 2]. *B. tauri* is a perennial ornamental bulb species that is endemic to Türkiye, flowers from April to May, and is found only in a few locations in the Mediterranean region at altitudes of 700–1300 m [3]. Besides being an alternative to exotic hyacinths for its showy flowers, it has potential for medicinal use [4]. The species has been classified as Lower Risk-conservation dependent (LRcd) in the Red Data Book of Turkish Plants [5].

Many ornamental geophytes used for potted plant production, landscaping or cut flower production have an important commercial value [6]. However, the natural propagation rate of most geophytes is very slow [7, 8] and conventional propagation methods offer limited multiplication of them [9]. In vitro propagation techniques are an alternative to conventional methods for geophyte propagation, increasing the multiplication rate of desired genotypes and providing plant material to be free of viruses and other pathogens [8]. In vitro propagation has been previously reported for many species of the family *Asparagaceae* for conservation and mass production from various explants and nutrient media [10]. In vitro culture of three members the genus of *Bellevalia* has also been achieved: *B. romana* [11], *B. sarmatica* [12] and *B. dubia* [13]. *B. tauri*, which has got beautiful purple flowers, can also be used for ornamental purposes. Cytological studies show that chromosome number of *B. tauri* is $2n=8,16$ [3]. Geophytes with low chromosome number can be used to determine the effects of the plant growth regulators and tissue culture conditions on the chromosome number and morphology. Due to the horticultural interest in *B. tauri* as an ornamental plant and its low chromosome number, this species is a suitable material for in vitro studies. In this paper, the in vitro bulblet regeneration potential of *B. tauri* and the effects of tissue culture conditions on the chromosome number of the in vitro plants were investigated.

Materials and methods. Plant material, explant preparation and surface sterilization. In the study, bulbs and fruits of *Bellevalia tauri* were used as plant material and bulb scales and immature embryos were used as explants. Plants with fruits were collected from their natural habitats in Antalya province of Türkiye in May. After removing the fruit capsules, bulbs were stored in the dark at 5 °C for 6 weeks. Fruit capsules containing immature embryos were washed in detergent and dipped in 95% ethanol for 3 min and surface disinfected in 80% commercial bleach for 20 min. After six weeks, for the sterilization of the bulbs,

the roots and the outer scales were removed, and several surface sterilizations were done. These sterilization protocols were as follows: (i) The bulbs were washed in detergent and dipped in 95% ethanol for 3 min and surface disinfected in 20%, 40% or 80% commercial bleach for 20 or 30 min. (ii) Sterile bulbs were cut longitudinally into four sections and again the surface was sterilized in 20% commercial bleach for 5, 10, 15 min. (iii) After surface sterilization in commercial bleach, the bulbs were soaked in hot tap water (50 °C) for 1 h as described in [14]. Thereafter, both bulbs and fruits were rinsed three times with sterile distilled water.

After surface sterilization immature seeds were removed from fruit capsules. Immature embryos were extracted from immature seeds by squeezing with a pair of forceps. The bulbs of *B. tauri* were longitudinally cut into four sections. Bulb scale explants consisting of twin scale segments attached to a thin segment of the basal plate were isolated.

Culture medium and growth conditions. The disinfected both types explants were implanted on to the MURASHIGE and SKOOG's [15] medium with 3% (w/v) sucrose and 0.7% (w/v) agar (Sigma type A) supplemented with BAP alone in three concentrations (1.0, 2.0, 4.0 mg L⁻¹) or in combination with two concentrations of NAA (0.25, 0.5 mg L⁻¹), resulting in nine variants of media containing plant growth regulators. The pH of all media was adjusted to 5.7 before autoclaving at 121 °C for 20 min. The cultures were kept at 25 ± 1 °C under 16-h light (40 μmol m⁻² s⁻¹).

Bulblet proliferation medium. Explants and explants with bulblets were subcultured every month onto fresh medium. The bulblets regenerated on bulb scale explants and the immature zygotic embryos were separated from the explants and transferred to the MS medium free of PGRs for rooting. Rooted bulblets were washed under running tap water to remove the agar.

Cytological analysis. For cytological analysis, the root tips of in vitro induced bulblets were cut and put into α-bromonaphthalene (4–5 drops of α-bromonaphthalene were added into 250 cm³ distilled water and shaken) for 16 h at room temperature. Then root tips were put into glacial acetic acid (%99.9) for 30 min [16]. After that root tips were stored in 70% ethanol in +4 °C. Before staining, root tips were kept in 60% hydrochloric acid for 11 min for hydrolysis, stained Feulgen for 1 h, and squashed in 45% glacial acetic acid [17].

Statistical analysis. Each treatment had three replicates of five explants for each explant type. Analysis of variance was administered to analyze the multiplication rates with SAS 9.1 program.

Results and discussion. Different plant parts and organs such as basal leaf portions, bulb basal plate, root tips [9], inflorescence stalk [7], and immature zygotic embryos [18] have been used as explants in previous studies for in vitro propagation of geophytes. In the present study aimed at the in vitro propagation of *B. tauri*, immature zygotic embryos and bulb twin scales were used as explants.

Effect of auxins and cytokinins on direct bulblet regeneration from the bulb scales explants. The use of storage organs of geophytes as an explant source is often associated with heavy contamination from soil pathogenic fungi and bacteria for in vitro propagation of geophytes [7]. Therefore, various surface sterilization methods such as soaking in commercial bleach and ethanol as well as hot water application as an alternative application were applied to overcome the contamination in our study. It has been found that hot water treatment completely prevents fungal contamination in almond tissue culture. As a result of pre-treatment with 50 °C hot water, 88% of the explants survived without contamination or necrosis [19]. In our study, although there was no contamination in the explants treated with hot water, none of them regenerated and all of them turned brown and died. Although hot water application significantly reduced contamination in *Lilium* bulbs, browning and deterioration were observed in the explants at the end of one week similar to our study. The temperature of the water used in *Lilium* was relatively lower (44–45 °C) [20] and probably for this reason, regeneration occurred in some of the explants. In all applications, heavy bacterial and fungal contamination was observed when commercial bleach in different concentrations was used as another sterilization method in bulb scale explants. On the other hand, in the method where sterilized bulbs were cut into four pieces and re-sterilized, although there was no contamination, the explants turned brown and died in a similar way to hot water application. Due to the contamination, a limited number of bulblets were obtained from the bulb scale explants (Fig. 1) and statistical analysis could not have been done for this explant type.

Effect of auxins and cytokinins on indirect bulblet proliferation from the immature zygotic embryo. Contamination from the underground storage organ was a limiting factor in geophyte micropropagation and to overcome this, alternative explant sources such as inflorescence stalk [7] and immature zygotic embryos [17] have been used. Auxins and cytokinins are widely used for callus formation, which provides information about the in vitro regeneration potential of different plant species. Callus is used for effective regeneration of different ornamental plants as well as other plant species [21]. Consistent with this,

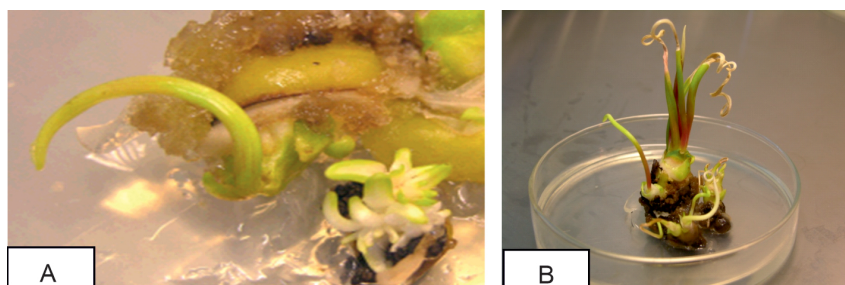


Fig. 1. Bulblet formation and regenerated bulblets from two scale explants

T a b l e 1

Callus and bulblet formation from immature
zygotic embryo explants of *B. tauri*

Plant growth regulators (mg L ⁻¹)		Explants with callus formation (%)	Number of bulblets per explant (mean)
BAP	NAA		
1	0.25	100.0 ^a	2.33 ^{abc} ± 0.11
2	0.25	100.0 ^a	3.43 ^a ± 1.33
4	0.25	56.7 ^{bc}	1.40 ^{dc} ± 0.57
1	0.5	73.3 ^{bc}	1.06 ^{dc} ± 0.11
2	0.5	100.0 ^a	1.00 ^d ± 0.00
4	0.5	83.3 ^{ab}	1.13 ^{dc} ± 0.11
1	—	93.3 ^{ab}	1.46 ^{bdc} ± 0.00
2	—	86.7 ^{ab}	2.73 ^{ab} ± 0.46
4	—	80.0 ^{abc}	1.60 ^{bcd} ± 0.34

*LSD_{0.05-1.5}

the use of auxin and cytokinin also induced callus formation of *B. tauri*. Callus formation was obtained at different rates (56.7–100%) depending on the auxin and cytokinin concentrations in all media. The highest callus formation was obtained in 1–2 mg L⁻¹ BAP + 0.25 mg L⁻¹ NAA and 2 mg L⁻¹ BAP + 0.5 mg L⁻¹ NAA media, and all explants formed callus.

For similar reasons immature embryos were cultured on MS medium supplemented with different combinations of NAA and BAP (Table 1) in our study and no contamination was observed from in vitro cultured immature zygotic embryo. QADIR et al. [10] reported that NAA and BAP, either alone or in combinations with other PGRs were widely used for shoot regeneration of in vitro propagation of some medicinally important plant species of family Asparagaceae. LUPI et al. [11] found that the combination of NAA and BAP was more effective than 2,4-D for callus growth and plant regeneration from mesocotyl explants of *Bellevalia romana*. Moreover, 2,4-D suppressed the shoot bud development in the same type of callus in their study. Different combinations of NAA and BAP were also used for the in vitro propagation of *Bellevalia sarmatica* [12]. The most limiting factor that restricts the study with rare and threatened endemic plants is the fact that the number of these plants in nature is limited. *Bellevalia tauri* is found only in several localities in nature and also heavy contamination ratio restricted the establishment of further experiments with different kind of plant growth regulators in this study. So, only different combinations of NAA and BAP which were generally the best plant growth regulators for the in vitro propagation of bulbous plants were used in our study. Most explants formed callus and shoot regeneration appeared on this callus in one month (Fig. 2A, Table 1). Some explants formed shoot directly. Within 4–5 months, some shoots developed into small bulblets

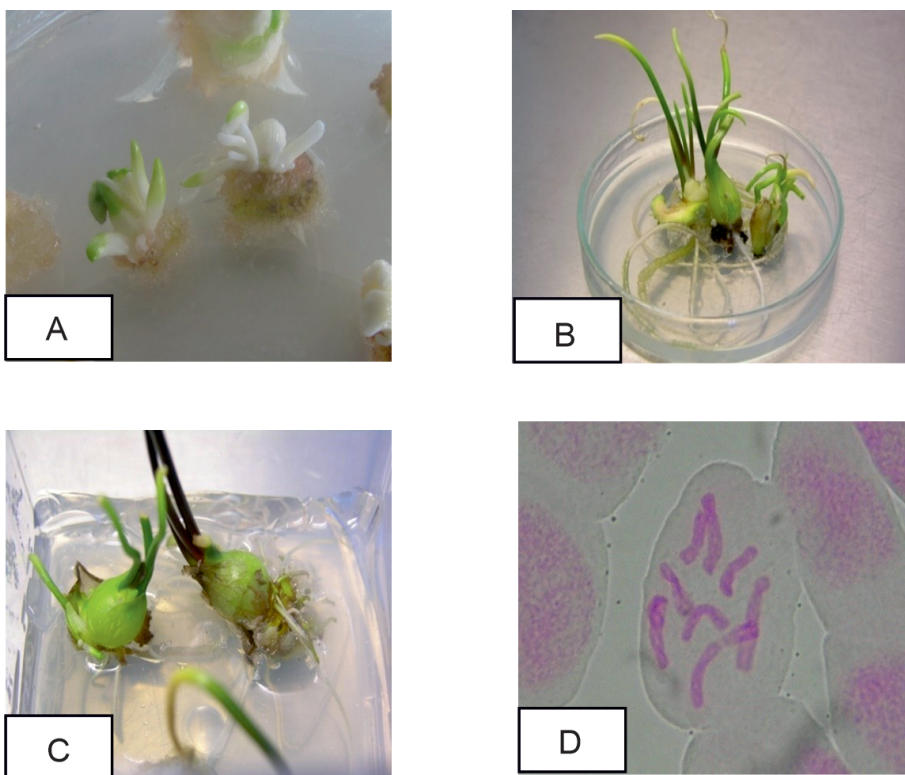


Fig. 2. (A) Callus and shoot formation from immature zygotic embryo; (B) Rooted bulblet on MS medium supplemented with 2 mg L^{-1} BAP + 0.25 mg L^{-1} NAA; (C) Root formation in the regeneration medium; (D) Chromosome number of *B. tauri* produced under in vitro conditions is $2n=8$

(Fig. 2B). There was significant difference between the mean number of bulblets per explant grown on media supplemented with different concentrations of PGRs (Table 1). Concerning the propagation coefficient, our best results were in the media containing 2 mg L^{-1} BAP used in combination with low concentration of NAA or alone.

Rooting of in vitro proliferated bulblets. Bulblets without roots were transferred into hormone free MS medium for rooting. In this experiment most of the bulblets rooted in all regeneration media containing PGRs (Fig. 2C).

Effect of growth regulators on chromosome number. The genus *Bellevalia* is easily distinguished by its large chromosome based on $X=4$. Chromosome numbers of *B. tauri*, which grows in the different mountains of west Antalya, are determined as $2n=8$ or $2n=16$ [1]. In this study, the bulbs and immature embryos of *B. tauri* were collected in the Calbali mountain as the explant sources. The chromosome number of *B. tauri*, which grows at these localities, was counted by JOHNSON and BRANDHAM [22] as $2n=8$. In the present study chromosome num-

ber of in vitro *B. tauri* plants was counted to detect the chromosome number changes. The root tips of in vitro produced bulblets were used for cytological analysis. It was found that the bulblets were diploid with normal chromosome number ($2n=8$) (Fig. 2D) when compared with the chromosome number of source plant.

Conclusion. In this study, immature embryo and bulb scale explants of *Bellevia tauri* Feinbrun were used for rapid in vitro propagation. The best result of 3.43 bulbs per explant was obtained from immature embryo explants on MS medium containing 2 mg L^{-1} BAP and 0.25 mg L^{-1} NAA. The results of chromosome analysis showed that the chromosome number of in vitro produced bulblets was $2n=8$. In conclusion, this study described a successful regeneration of *B. tauri* by direct organogenesis and indirect organogenesis using immature embryos. To complete the in vitro micropropagation protocol of the species, further experiments will be conducted on ex vitro adaptation and acclimatization of the in vitro obtained plants. The present study could be useful for the in vitro propagation of other endemic and endangered plant species.

REFERENCES

- [1] JOHNSON M. A. T. (2003) Polyploidy and karyotype variation in Turkish *Bellevia* (Hyacinthaceae), Bot. J. Linn. Soc., **143**(1), 87–98.
- [2] JAFARI A., J. VAEZI, M. M. FORGHANIFARD et al. (2021) A revised infrageneric classification of *Bellevia* Lapeyr. (Asparagaceae: Scilloideae) based on molecular analysis, Phytotaxa, **525**(1), 70–84.
- [3] WENDELBO P. (1984) *Bellevia*. In: Flora of Turkey and the East Aegean Islands (ed. P. H. Davis), Vol. 8, Edinburgh, Edinburgh University Press, 264–274.
- [4] SENTÜRK M., R. BINZET (2021) Some dicotyl endemic plants of Mersin province with ornamental plant potential, Euroasia Journal of Mathematics, Engineering, Natural & Medical Sciences, **8**(16), 79–92, (in Turkish).
- [5] EKIM T., M. KOYUNCU, M. VURAL et al. (Eds) (2000) Red Data Book of Turkish Plants. Pteridophyta and Spermatophyta, Ankara, Barışcan Ofset.
- [6] DE HERTOGH A., J. M. SCHEPEEN, M. LE NARD et al. (2013) Globalization of flower bulb industry. In: Ornamental Geophytes, CRC Press, 1–16.
- [7] ZIV M., H. LILIEN-KIPNIS (2000) Bud regeneration from inflorescence explants for rapid propagation of geophytes *in vitro*, Plant Cell Reports, **19**, 845–850.
- [8] YASEMIN S., M. A. BERUTO (2024) A review on flower bulb micropropagation: Challenges and opportunities, Horticulturae, **10**(3), 284.
- [9] ESCOBAR L. H., M. JORDAN, E. OLATE et al. (2008) Direct and indirect *in vitro* organogenesis of *Leucocoryne purpurea* (Alliaceae) a Chilean ornamental geophyte, Propag. Ornam. Plants, **8**(2), 59–64.
- [10] QADIR J., S. SINGH, Z. A. KALOO (2017) A review on *in vitro* propagation of some medicinally important plant species of family Asparagaceae, Int. J. BioSci. Technol., **10**(1), 1–12.

- [11] LUPI C., A. BENNICI, D. GENNAI (1985) In vitro culture of *Bellevalia romana* (L.) Rchb. I. Plant regeneration through adventitious shoots and somatic embryos, *Protoplasma*, **125**(3), 185–189.
- [12] SEDCENCO M., C. NINA, C. DOINA, F. ALEXANDRU (2011) The conservation of species *Bellevalia sarmatica* (Georgi) Woronov by the vitroculture method, *J. Plant Develop.*, **18**, 11–15.
- [13] CANHOTO J. (2018) Somatic embryogenesis for cloning the endangered species *Bellevalia dubia*, *In Vitro Cellular & Developmental Biology, Plant*, **54**(1), 67.
- [14] ULRICH M. R., F. T. DAVIES, Y. C. KOH et al. (1999) Micropropagation of *Crinum* ‘Ellen Bosanquet’ by tri-scales, *Scientia Horticulturae*, **82**, 95–102.
- [15] MURASHIGE T., F. SKOOG (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures, *Physiol. Plant.*, **15**, 473–497.
- [16] ELCI Ş. (1994) Research methods and observations in cytogenetics, *Yuzuncu Yıl Universitesi Publications*, No: 18, 68–69, Van (in Turkish).
- [17] FEINBRUN-DOTHAN N. (1938) A monographic study on the genus *Bellevalia* Lapeyr., *Palestine J. Bot.*, **1**, 337–409.
- [18] MIRICI S., I. PARMAKSIZ, S. OZCAN et al. (2005) Efficient *in vitro* bulblet regeneration from immature embryos of endangered *Sternbergia fischeriana*, *Plant Cell Tiss. Organ Cult.*, **80**, 239–246.
- [19] SHEKAFANDEH A., M. GHASEMI (2009) Effect of hot-water and cold treatments on reducing contamination in almond tissue culture, *J. Appl. Horticulture*, **11**(2), 143–145.
- [20] LANGENS-GERRITS M., M. ALBERS, G. JAN DE KLERK (1998) Hot-water treatment before tissue culture reduces initial contamination in *Lilium* and *Acer*, *Plant Cell Tiss. Organ Cult.*, **52**, 75–77.
- [21] MEHBUB H., A. AKTER, M. A. AKTER et al. (2022) Tissue culture in ornamentals: cultivation factors, propagation techniques, and its application, *Plants*, **11**(23), 3208, <https://doi.org/10.3390/plants11233208>.
- [22] JOHNSON M. A. T., P. E. BRANDHAM (1997) New chromosome numbers in petaloid monocotyledons and in other miscellaneous angiosperms, *Kew Bulletin*, **52**(1), 121–138.

¹Department of Mathematics and Science Education, Faculty of Education,
Akdeniz University, Antalya, Türkiye
e-mail: nasircilar@akdeniz.edu.tr

²Department of Horticulture, Faculty of Agriculture,
Recep Tayyip Erdogan University, Rize, Türkiye
e-mail: ozgul.karaguzel@erdogan.edu.tr

³Department of Mathematics and Science Education, Faculty of Education,
Gazi University, Ankara, Türkiye
e-mail: semramirici@gazi.edu.tr

⁴Department of Biology, Faculty of Science Literature, Adnan Menderes University, Aydın, Türkiye
e-mail: ozkaneren@adu.edu.tr