



Regeneration of *Bellevalia pseudolongipes* Karabacak & Yıldırım Under *in Vitro* Conditions

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Abstract

Profitable viable *in vitro*, multiplication of endemic bulbous geophyte *Bellevalia pseudolongipes* is challenging and is desired for commercial exploitation and conservation. This study aimed to multiply this plant species using double-scale explants using 1/4 (0.5 cm), 1/6 (0.33 cm), or 1/8 th (0.25 cm) longitudinally sliced bulb scale tissues lying in the 1st, and 2nd, clusters of *B. Pseudolongipes* mother bulbs. These were cultured on MS; White and Gamborg B5 media enriched with 1.11 µM - 5.44 µM BAP and 1.34 µM NAA (5 combinations) to compare the regeneration potential of fresh and 2-month stored bulb scale explants. Bulblet induction was observed on all concentrations of BAP-NAA on all culture media. MS medium modified with 1.11 µM BAP and 1.34 µM NAA induced maximum number of 3.56 bulblets explant on 0.25 cm wide two scale explants. The regeneration capability of White and Gamborg B5 medium was less compared to bulblets induced on MS medium. The biggest bulbs were observed on Gamborg B5 medium, which differed significantly from the bulblets induced on White and MS media. Treatment of regenerated bulblets with sucrose improved bulblet diameter by ~2 mm. The bulblet diameter varied between 0.42-0.83 cm. The bulblets were rooted on MS medium without using any auxin. Furthermore, the study revealed the potential of 2 scale explants from the outer cluster for multiplication and laid the groundwork for future breeding and tissue culture-based multiplication to conserve the species. The experimental results provide a useful reference for the efficacy of bulb scales for regeneration.

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1. Introduction

The genus *Bellevallia* is represented by 11 endemic species in the flora of Türkiye (Anonymus, 2025; Karabacak et al., 2014; 2015; Şahin et al., 2016; Pınar et al., 2016). The percentage of endemism within the genus is 45.83%. The rare herbaceous, perennial ornamental, and endemic plant species face a constant problem of adaptation, and depletion due to increasing uncertainty in terms of conserving germplasm with dramatic changes in the climate and emission of greenhouse gasses in environment. Besides their ornamental importance, all species in the genus *Bellevallia*, have importance for anti-inflammatory properties from the compounds found in root extracts (Hosseini et al., 2022; Saraçoğlu, 2024; Yolbaş, 2024a,b). The species is *B. pseudolongipes* largely grows in Siirt and Mardin provinces of Türkiye, where, flowering occurs from April to May. Besides their ornamental importance, this species has importance for its anti-inflammatory properties from the compounds found in root extracts (Saraçoğlu, 2024; Yolbaş, 2024a,b). It has a chromosome number of $2n = 12$ (Karabacak et al., 2014). No statistics or information about on-farm or in vitro multiplication, and maintenance is available (Karabacak et al., 2014, 2015). This situation has resulted in high levels of genetic erosion in many plant species (Öztürk et al., 2025). Intime and serious efforts can protect this species from vulnerability from extinction (Foden et al., 2019). Several countries and international organizations have established open-air and in vitro gene banks to stop the vanishing of rare and endemic germplasm, landraces, and endangered species. A major constraint towards using in vitro gene banks is the conservation of material by freezing the germplasm in latent form (Gowthami et al., 2025). About 1700 gene banks are used to conserve 60.000 species are working the world over with huge operating costs with many bottlenecks (Malik et al., 2025). There is a consensus that the developing multiplication protocols for disease-free tissue-cultured plants can serve the purpose (Engelmann and Engels, 2002). This plant species will adapt

better to physical and environmental conditions and could have better interaction and sustainability with surrounding life forms and facilitate conservation (Chapman et al., 2012). Once the species is adapted, and starts growing its management is comparatively easy if local farmers are involved in the process the help of local bodies like conservation of *Allium tuncelianum* at Tunceli province (Kızıl et al., 2014) and Kavlica wheat at Kars province of Türkiye (Ilhan, 2021). Although most of the researchers are using MS medium for shoot regeneration like Özel et al. (2007) in *Ornithogalum oligophyllum*, Özel et al. (2008) *Muscari macrocarpum* *Ornithogalum ulophyllum*, Özel et al. (2009), Özel et al. (2015), preferred MS medium while working on muscari species. Komai et al. (1996) has studied in vitro plant regeneration of spinach, using Nitsch, MS, White, B5 and SH media containing 0.00876 M sucrose, 10 µM 1-naphthalinacetic acid (NAA) and 0.1 µM gibberellic acid, and found positive influence of the constitution of media on regeneration and embryogenesis. Therefore, it was desired to compare the efficiency of MS, White, and Gamborg B5 in the current study. Fresh bulbs of *B. pseudolongipes* contain large number of metabolites that are prone to mitochondria-generated ATP-based respiratory oxidation of organic acids. Their reaction with oxygen release reactive oxygen species (ROS) (Zhang et al., 2025). These have negative influence on cellular functioning, signaling after the bulbs are sectioned during optimization of the width and number of bulb scales in an explant Very few literature is available about in vitro regeneration of other *Bellevallia* species (Yolbaş, 2024a,b; Saraçoğlu, 2024). No previous study mentions tissue culture-based multiplication of *B. pseudolongipes*. Therefore, the study aimed to develop an in vitro multiplication protocol for *Bellevallia pseudolongipes* using 2 or 3 bulb scales of 1/8, 1/6 and 1/4 width of bulb as explant by culturing them on MS, White, and Gamborg B5 media for conservation purposes.

2. Material and Methods

2.1. Collection of the plant material

B. pseudolongipes bulbs were collected after a study of the literature describing complete identification keys (Karabacak et al., 2014, 2015; Şahin et al., 2016). The voucher specimens of the collected plant species were deposited in the herbarium of the Department of Horticulture of the Van Yüzüncü Yıl University of Türkiye. Both fresh and 2-month stored bulbs of *B. pseudolongipes* were used in the study. The stored bulbs were kept in a clean, dark, and airy place after removing their leaves and roots with a sterile scalpel blade.

2.2. Tissue culture studies

MS (1962), White (1963), and Gamborg B5 media (1968), supplemented with 8 g l⁻¹ agar (Duchefa) amended with 0.88 M (30 g l⁻¹) sucrose was used in the study. MS medium was developed by Murashige and Skoog (1962) while trying to culture tobacco plant. This medium is most popular medium used for tissue culture of several plants to date. White medium was developed by White (1963) for the establishment of tomato root culture. White medium contains Mg while MS medium does not contain any Mg. It has a higher MgSO₄ concentration. Nitrate concentration is 19 %, which is less compared to that found in MS medium. It contains less K and N as compared to MS medium. The Gamborg B5 medium (1968) was used for callus and cell suspension culture of groundnut and is made up of inorganic salts, vitamins and carbohydrates. It has a higher nitrate and potassium concentrations and a less ammonia, which improves soybean root and callus formation, with a role in cell growth. It contains small amounts of K, Ca and N compared to the MS medium. Gamborg B5 medium contains a small amount of Mg. Double distilled water

was used to prepare respective cultures showing concentrations of 1-.11 to 4.44 µM BAP and 1.34 µM NAA (Table 1) and written on labels pasted on each glass bottle before autoclaving Table 1. The pH of each medium was adjusted individually to 5.8 using 1N NaOH or 1N HCl. The sterilization was performed by adjusting the autoclave pressure to 1.45 kPa at 121 °C for 21 minutes. All cultures were kept under white fluorescent light at 24±1 °C for 16 hours of light and 8 hours of darkness.

2.3. Surface sterilization

Thereafter, healthy and unbruised bulbs were sorted and treated with 5 solutions of 1, 2, 3, 4, and 5% NaOCl-Ace[®] Turkey for optimizing sterilization procedure in a Laminar flow cabinet with dripping of 1.5 ml L⁻¹ of Tween 20 to sterilize them for 15, 25, 35, 45 and 60 minutes (165 explants) each. Rinsing was carried out by passing through horizontally shaken sterile distilled water for 3 × 3 minutes. Sterilization was also performed by treatment with 30% hydrogen peroxide for 20, 30, 40, and 50 min each followed by rinsing with distilled water for 3 × 3 min in the 2nd experiment.

2.4. Selection of the explants

The bulbs had an approximate circumference of 2 cm each. These sterilized bulbs were dried on sterile absorbent/drying papers. The bulbs contained 1-7 or 8 bulb scales. Generally, outer and middle clusters were made up of 3 scales each. The scales in the middle clusters were comparatively thinner and juvenile. The 1-2 scales in the inner cluster were immature and not fully developed. They were made up of 1 or rarely 2 papery scales. 1st leaf scale of all bulbs was removed after sterilization and before making vertical slices to obtain 2 – 3 scale explants (Figure 1).

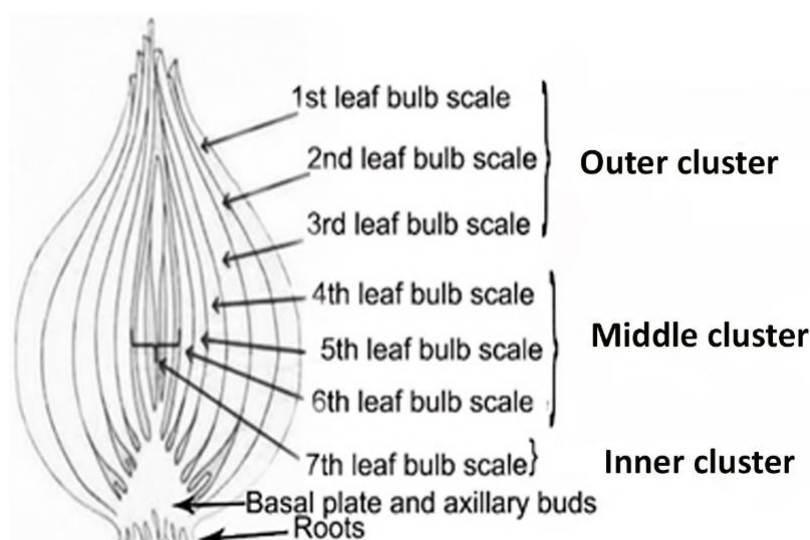


Figure 1. Bulb scales in each of outer, middle, and inner clusters of *B. pseudolongipes* Explants were taken in the following way

Two scale explants

- ✓ Two scales (scale 2 and 3 from outer cluster)
- ✓ Scale 3 from outer cluster, scale 1 from middle cluster
- ✓ Scale 2, 3 from middle cluster

Three scale explants

- ✓ Scales 1,2,3 from the middle cluster
- ✓ Scale 2, 3 from outer cluster and scale 1 from middle cluster,
- ✓ Scale 3 from outer cluster and scale 1,2 from middle cluster

No explant was taken from the inner cluster because the explant scales were very thin and

juvenile. The dried bulbs were sliced vertically into 4 (with a width of about 0.5 cm width and length of 1 cm), 6 (with a width of about 0.33 cm and length of 1 cm), and 8 (with width of about 0.25 cm and length of 1 cm) slices (Figure 2) to obtain double, and triple bulb scales from outer, and middle clusters in a tunic attached at the basal plate. Five explants were cultured in each Petri dish (replication). It was noticed that each bulb had a variable thickness of the scale lying in the outer clusters (1 to 0.8 mm), middle clusters (0.7-1.7 mm), and inner clusters (0.03-0.05 mm.).



Figure 2. Width of Bulb scales of *B. pseudolongipes* taken as explant

These Petri dishes were wrapped with stretch film after culturing explants and kept under white fluorescent light at 24 ± 1 °C under 16 hours light illumination. 1 × MS, White, and Gamborg B5 media (Merck Germany) powders were used in the

regeneration experiments and prepared according to the concentrations described above. Each experiment used 45 explants divided equally into nine replications containing 5 explants each after pouring respective media into 100×10 mm Petri dishes

using one factorial experiment and repeated thrice. The statistical data were subjected to analysis of variance with the help of “SPSS 26 for Windows” computer software, and the Duncans Multiple Ranges test was used to compare the developing regeneration differences among the means.

3. Results and Discussion

3.1. Surface sterilization

Fungal (*Fusarium oxysporum* and *Asparagus racemosus*) contamination was observed on the bulb scales after sterilization with any concentration of NaOCl for any duration of time. The contamination percentage on the explants varied between 10-100% using 25-100% commercial bleach for 10-30 minutes (data not shown). It is well known that the tissue culture environment is highly prone to the multiplication of several microorganisms like bacteria, filamentous, and nonfilamentous (yeasts) fungi in the culture medium. The infected bulbs were checked daily to find the potential initiation of fungi at the end of each day under a stereo microscope against sharp light. Efforts to stop the multiplication of these biological contaminants on the explants or cultures failed in agreement with Da Silva et al. (2015). They changed the regeneration process, by inducing the necrosis on the explants in corroboration with Da Silva et al. (2016); Niedz and Bausher (2002); Cassells (2020), who suggested the release of proteins, metabolites, and oxidation of organic phenolic acids ending up with changed pH and composition of the culture medium. This resulted in extensive colonization of the contaminants and death of the explants within a few days. A shift from NaOCl to H₂O₂ showed its high potential to disinfect the explants. No contamination was noted using 30 % H₂O₂ with a wide and rapid antimicrobial and antifungal action (Wen et al., 2025). No visible damage was inflicted on the health of the explants (Hardstaff et al., 2025).

3.2. Regeneration on 0.25, 0.33 and 0.5 cm wide and 1 cm long vertically sliced 2 scales taken from fresh and stored bulbs

The aim of taking 0.25, 0.33 and 0.5 cm

wide vertically sliced twin bulb scales was the optimization of the best width of the scales for highest bulblet induction. The 2 (0.5 cm wide) and 3 (0.33 cm wide) bulb scales taken from fresh bulb scales were prone to necrosis due to the oxidation of bulb exudates. There was an increase in the induction of these exudates as the width of the explants decreased from 0.5 cm to 0.25 cm. Contrarily, the 2 (0.5 cm wide) and 3 (0.33 cm wide) bulb scales taken from stored bulb scales were less prone to necrosis. It is well established that fresh bulbs contain a large number of metabolites that are prone to mitochondria-generated ATP-based oxidation of organic acids, which release reactive oxygen species (ROS) and negatively impact cellular functioning and signaling. This also weakens the defense system of the plants leading to their death by producing hydroxyl radicals, H₂O₂, singlet oxygen, ozone, and superoxides (Roche et al., 2025). A few *bellevialia* species are studied to report precise and reliable information about their production, their short half-life or their role in targeting of any molecules to date. It is presumed that H₂O₂ and superoxide are good candidates for ROS generation in *B. pseudolongipes* cellular mitochondria in agreement with Roche et al. (2025). This was confirmed and approved when the stored bulbs showed reduced or no necrosis is well established that fresh bulbs contain large number of metabolites that are prone to mitochondria generated ATP based oxidation of organic acids, which release reactive oxygen species (ROS) and negatively impact cellular functioning, and signaling. This also weaken defence system of the plants leading to their death by producing hydroxyl radicals, H₂O₂, singlet oxygen, ozone and superoxides. Any *bellevialia* species is not studied to report precise and reliable information about their production, their short half-life or their role in targeting of any molecules till todate. This was confirmed and approved when the stored bulbs showed reduced or no necrosis. Regardless of the width of the longitudinally sliced bulbs, they exudated internal metabolites through sliced or injured edges depending on the width of the explants. These were sticky. These hindered

regeneration and were prone to necrosis. Two months of storage promoted bulblet regeneration on the scales taken from outer clusters. This practice may have slowed down the oxidation of metabolites in the explants. It is well known that the phenolic acid contents of bulbs decrease regularly during storage (Abdelgawad et al., 2025). This storage period was not effective on the bulb scales embedded in the middle and inner clusters; which were embedded in the bulbs below outer clusters and were not in direct contact with outside air (oxygen). Therefore 0.5 cm wide two-scale explants taken from the outer clusters were preferred in the study. The results are

described in detail in the following paragraphs.

3.3. Regeneration of bulblets on the explants

All explants showed 100% induction on 3 regeneration media containing different concentrations of BAP + 1.34 μ M NAA (Table 1). Several effects of all 3 culture media against induction of the number of bulblets per explant and their diameter were noted. Micro-macro elements and vitamins in each culture medium affected regeneration differently in line with Bagherian-Shamasbi et al. (2025) and Barpete et al. (2016). They are described below.

Table 1. Regeneration on two scale explants using MS medium modified with different doses of BAP and NAA on double leaf scale explants of *B. pseudolongipes*

	Bulblet induction percentage (%)	Number of bulblets per explant	Bulb diameter
BAP (μ M)	1.11	1.11	1.11
	2.22	2.22	2.22
	3.33	3.33	3.33
	4.44	4.44	4.44
NAA (μ M)	1.34	1.34	1.34
	1.34	1.34	1.34
	1.34	1.34	1.34
	1.34	1.34	1.34
1×MS medium	100.00*	3.56a**	0.25d**
	100.00	1.00d	0.91a
	100.00	2.21c	0.77c
	100.00	2.42b	0.83b
1×White medium	100.00*	2.26b	1.51 c**
	100.00	2.27 b	0.49c
	100.00	2.21b	1.53b
	100.00	2.57b	1.72a
1×Gamborg B5 medium	100.00*	1.44c	1.34 b**
	100.00	1.61b	1.35b
	100.00	1.79a	1.79a
	100.00	1.82a	1.85a

*All values in a column were similar, therefore no statistical analysis was performed.

**All values shown with small letters in a single column were separated by different letters are significantly different at 0.01 level of significance using Duncan Multiple range test LSD test

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Plant was noted on MS medium containing 1.11 μ M BAP + 1.34 μ M NAA, and Gamborg B5 containing 4.44 μ M BAP + 1.34 μ M NAA, respectively. The maximum number of bulblets (4.71 pieces) and bulb diameter (1.42 cm) per explant were obtained from White medium containing 2.22 μ M BAP + 1.34

μ M NAA.

3.4. MS medium

The bulblet induction ranged 1.00-3.56 per explant (Table 1). These explants showed a considerably sharp necrosis during induction, which had depressing and inhibitory effects on

bulblet regeneration. Bulblet diameter ranged 0.25-0.91 cm. The largest bulblets were collected and induced on MS medium containing 2.22 μ M BAP and 1.34 μ M NAA.

3.5. White medium

Two scale explants induced 2.21-2.57 bulblets per explant and a difference of 0.49-1.72 cm in the bulblet diameter (Table 1). The largest bulblets on White medium were noted on 4.44 μ M BAP and 1.34 μ M NAA (Figure 4).

3.6. Gamborg B5 medium

The explants induced 1.44-1.82 bulblets per explant (Table 1). The bulblet's diameter had a difference of 1.34-1.85 cm. The largest bulblets were monitored on Gamborg B5 medium containing 4.44 μ M and 1.34 μ M NAA. Tissue culture characterize one of the

alternative techniques that needs consideration of several factors highlighting success that differ in plant species depending on their endogenous level of phytohormones, their concentrations, combinations age type of explant, culture medium; and use of techniques, that influence regeneration on the explants. Endogenous factors have significant roles in physiological growth and rejuvenation (Mulyati et al., 2025). Plants undergo a vegetative growth phase, which includes a juvenile and adult phase with unique growth patterns and structures. Endogenous factors, such as hormones and genetic factors play significant roles during growth of plants. The timing of these changes depends on the species. The maximum diameter was noted on the MS medium containing 4.44 μ M BAP + 1.34 μ M NAA (Figure 3).



Figure 3. Bulblet induction on 2 scale explants of *B. pseudolongipes*

It was observed that both the number and the bulblet diameter per explant increased with the direct regeneration was noted on two scale leaf explants on White medium (Table 1). The number of bulbs per explant varied between 1.27-1.82 with the maximum number of (1.82) bulblets per explant. This showed that the regeneration potential of White medium was inhibitive and the the number of bulblets induced on this medium was significantly lower compared to the regeneration potential of the explants when they were cultured on MS medium. The explants cultured on white medium induced maximum diameter on bulblets using 4.44 μ M BAP + 1.34 μ M NAA (Table 1). A parallel increase in the bulblets induction per explant was observed with each increase in the concentration of BAP during regeneration. The bulblet diameter varied

between 0.42 - 0.83 cm. The maximum bulblet diameter was observed on the medium containing 4.44 μ M BAP + 1.34 μ M NAA. Tissue culture is a technique that depends on factors like phytohormones, concentrations, photoperiod, explant age, and culture medium type. Plants undergo a vegetative growth phase, with unique patterns and structures. Endogenous factors like hormones and genetics play significant roles in plant development. Both vegetative and generative growth are distinct processes. The regeneration potential of white medium is inhibitive, with lower number of bulblets induced compared to MS medium. The maximum bulblet diameter was observed on the medium containing 4.44 μ M BAP + 1.34 μ M NAA.

4. Conclusion

Tissue culture is a technique that depends on factors like phytohormones, concentrations, photoperiod, explant age, and culture medium type. Endogenous factors like hormones and genetics play significant roles in plant development. Bulblet induction was noted on every treatment regardless of the culture medium and plant growth regulator combination treatments. The MS medium induced largest number of bulblets per explant followed by induction of bulblets on White and Gamborg B5 medium. The regeneration potential of white and Gamborg B5 medium was inhibitive, with lower number of bulblets induced compared to MS medium. Whereas, the largest bulbs were noted on Gamborg B5 medium which were sharply different compared to the bulblets induced on White and MS medium. This is the first successful tissue culture report of *Bellevia pseudolongipes*. A

comparison among the three culture media showed no differences among the three in terms of bulblet regeneration percentage. However, the maximum number of bulblets were induced on MS medium. The maximum size of the bulblet was induced on Gamborg B5 medium. Regardless of the induction of bulblets, all bulbs are rooted on MS medium without using any auxin for rooting (Figure 4). Similarly, roots were induced on garlic (Zel et al., 1997) *Allium cepa* after sucrose addition. According to Gao et al. (2018), that the specific signaling of sucrose may affect bulb formation, swelling, and rooting. They used the exclusion method to confirm the above results and hypothesis, and observed that only a small portion of sucrose is converted by cell wall invertase and enters cells via hexose transporters, with a certain effect on bulb growth and development via hexose or hexose signaling systems.



Figure 4. Rooting of *B. pseudolongipes* bulbs on MS medium

In some plants, it is possible to provide root formation in bulbs or shoots without auxin treatment. Dushimimana et al. (2025) has reported induction of rooting without using auxin. The study demonstrates the potential of *B. pseudolongipes* to survive and adapt to rapid environmental changes. It shows bulblet regeneration on double-scale leaves using MS, White, and Gamborg B5 media, with a 2mm increase in bulblet diameter after sucrose treatment. The study will lay the groundwork for future tissue culture studies. Furthermore, this study will help reveal and open ways to the commercial propagation of this species. Future research should explore physical and genetic

factors limiting the multiplication of the species.

Declaration of Author Contributions

The authors declare that they have contributed equally to the article. All authors declare that they have seen/read and approved the final version of the article ready for publication.

Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

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