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IN VITRO CULTURE OF *ALTHAEA OFFICINALIS* L. (BIG MALLOW): OPTIMIZATION OF GERMINATION, SHOOT MULTIPLICATION AND CALLUS INDUCTION

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Abstract

Althaea officinalis L. is a medicinal plant valued for its mucilaginous polysaccharides and bioactive compounds. Efficient in vitro propagation systems are essential for producing uniform plant material and supporting conservation and phytochemical studies. This work aimed to optimize in vitro germination, shoot multiplication, and callus induction using MS medium supplemented with different plant growth regulators. Seeds sterilized with ethanol and mercuric chloride achieved 90% germination on basal MS medium within eight days. Supplementation with GA_3 enhanced seedling elongation. Shoot multiplication was most efficient on MS medium containing BAP and IAA, producing vigorous shoots with extended subculture intervals. Rooting was successfully induced with IBA. Callus formation occurred on MS medium supplemented with either BAP + NAA or 2,4 D + BAP; the latter produced a compact white callus. Regenerated plants acclimatized successfully in a mixture of soil:perlite 1:1. The results confirm that A. officinalis responds strongly to in vitro culture conditions and provide a reliable protocol for micropropagation and callus production.

Key words*: Althaea officinalis, micropropagation, gibberellic acid, BAP, callus induction.*

INTRODUCTION

Althaea officinalis L. (big mallow) is a perennial medicinal species widely used in traditional and modern phytotherapy. Its therapeutic value is attributed to mucilaginous polysaccharides, polyphenols, flavonoids, and volatile compounds with antioxidant, anti-inflammatory, and wound healing properties (Al Snafi, 2013; Pașca et al., 2016; Albahri et al., 2023). The mucilage fraction, composed of glucuronic acid, galacturonic acid, rhamnose, and galactose, is of particular pharmacological interest (Ionkova & Alfermann, 1994).

Phytochemical analyses have identified numerous compounds in *A. officinalis*, including tetradecane, α pinene, hexadecane, 2 hexenal, dodecane, β ionone, and trans geranylacetone (Shahsavari & Derikvand, 2022). Given its medicinal importance, developing efficient in vitro propagation systems is essential for producing uniform plant material, conserving germplasm, and supporting biotechnological applications.

Previous studies have examined seed germination (Younesikelati et al., 2016), artificial seed technology (Naz et al., 2018), and shoot proliferation using BAP (Mujib et al., 2017; Naz et al., 2015). However, protocols vary considerably, and further optimization is needed to improve reproducibility and plant vigor.

The present study aimed to refine in vitro culture techniques for *A. officinalis*, focusing on seed germination, shoot elongation and multiplication, and callus induction.

Despite previous studies, no comprehensive protocol integrating germination, elongation, multiplication, rooting, and callus induction has been reported for A. officinalis. This study aims to fill this gap.

MATERIALS AND METHODS

Plant Material

Seeds of *Althaea officinalis* L. (big mallow) were obtained from a commercial supplier (www.semintelegumeflori.ro; lot PL530/12/210/3473A). Seeds were visually inspected and selected based on uniformity, absence of mechanical damage, and consistent size. Only intact, healthy seeds were used for *in vitro* culture experiments. All procedures were conducted under aseptic conditions in a laminar

airflow cabinet to prevent microbial contamination.

Surface Sterilization Procedure

A multistep sterilization protocol was applied to ensure complete decontamination of the seed surface:

Pre-washing:

Seeds were rinsed thoroughly under running tap water for 10 minutes to remove dust and surface debris.

Initial rinse:

Seeds were washed twice with sterile distilled water.

Ethanol treatment:

Seeds were immersed in 70% (v/v) ethanol for 30 seconds to disrupt microbial membranes.

Mercuric chloride treatment:

Seeds were transferred to a 0.1% (w/v) $HgCl_2$ solution supplemented with three drops of Tween-80 as a surfactant. Exposure time was 3-5 minutes, with gentle agitation to ensure uniform contact.

Final rinses:

Seeds were rinsed 4-5 times with sterile distilled water to remove all traces of $HgCl_2$.

Sterilized seeds were immediately inoculated onto culture media to prevent desiccation.

Culture Media Preparation

All media were prepared using Murashige and Skoog (MS) basal salts and vitamins. Media were supplemented with 3% (w/v) sucrose and solidified with 0.7% (w/v) agar. The pH was adjusted to 5.7 ± 0.1 prior to autoclaving at 121°C for 20 minutes. Plant growth regulators (PGRs) were added to the media after cooling to approximately 50°C.

***In Vitro* Germination and Seedling Elongation**

Sterilized seeds were placed individually on basal MS medium to assess germination capacity. To evaluate the effect of gibberellic acid (GA_3) on seedling elongation, three additional media were prepared:

MS + 0.5 mg/L GA_3

MS + 1.0 mg/L GA_3

MS + 1.5 mg/L GA_3

Seeds were incubated in a growth chamber at 24 ± 2°C under a 16/8 h light/dark photoperiod with a light intensity of approximately 40-50 µmol m^{-2} s^{-1}. Germination percentage was recorded after eight days, and shoot length was measured after five weeks. Each treatment included n = 10 replicates, with one seed per culture vessel.

Shoot Multiplication

Nodal segments (0.8-1.0 cm) excised from in vitro-grown seedlings were used as explants for shoot multiplication. Three hormonal combinations were tested:

MS + 3 mg/L BAP + 0.5 mg/L IAA

MS + 3 mg/L BAP + 1 mg/L GA_3

MS + 3 mg/L BAP + 1 mg/L GA_3 + 0.5 mg/L IAA

Explants were cultured in 350 mL culture jars containing 40 mL of medium. Cultures were maintained under the same environmental conditions described for germination. Shoot proliferation, the number of shoots per explant, internode length, and overall vigor were evaluated after four weeks. Subculture intervals were gradually extended to eight weeks as shoot vigor increased. Each treatment included n = 10 explants.

Root Induction

Elongated shoots (3-4 cm) were transferred to MS medium supplemented with 1.5 mg/L indole-3-butyric acid (IBA) to induce rooting. Root initiation and development were monitored over a two-week period. Parameters recorded included rooting percentage, the number of roots per shoot, and root morphology. Rooting experiments were conducted with n = 10 shoots per treatment.

Callus Induction

Callus formation was induced from hypocotyl and cotyledon explants excised from in vitro seedlings. Two hormonal combinations were tested:

Cytokinin-auxin balance:
MS + 1 mg/L BAP + 1 mg/L NAA

High-auxin induction:
MS + 5 mg/L 2,4-D + 2.5 mg/L BAP + 2 g/L casein hydrolysate

Explants were placed on callus induction media and incubated in the dark at 24 ± 2°C for the first 10 days to promote dedifferentiation. Cultures were then transferred to low-light conditions (10-15 µmol m^{-2} s^{-1}). Callus characteristics

(color, texture, compactness, and growth rate) were recorded after three weeks.

Acclimatization of Regenerated Plants

Rooted plantlets were gently removed from the culture vessels, and agar residues were washed off with lukewarm sterile water. Plants were transferred to Magenta boxes containing a soil:perlite mixture (1:1). During the first week, humidity was maintained by covering the containers with transparent lids. Gradual ventilation was introduced to harden the plants. After two weeks, acclimatized plants were transferred to greenhouse conditions and monitored for survival and growth.

RESULTS

Seed germination and elongation

The sterilization protocol applied to *A. officinalis* seeds proved highly effective, yielding a 100% contamination-free rate across all replicates. This ensured that subsequent developmental responses were attributable solely to the culture conditions rather than microbial interference. When inoculated onto basal MS medium, seeds initiated visible swelling within the first 48 hours, followed by radicle emergence. By day eight, germination reached 90%, demonstrating that *A. Officinalis* seeds possess strong intrinsic viability and respond favourably to MS basal salts without the need for exogenous growth regulators (Figure 1).



Figure 1. *Althaea officinalis* plantlets germinated *in vitro* on basal MS medium eight days after inoculation

Seedlings developed uniformly, producing well-formed hypocotyls and cotyledons. The early establishment of vigorous plantlets provided a reliable source of explants for subsequent micropropagation and callus induction experiments.

Effect of GA_3 on shoot length

Supplementation of MS medium with gibberellic acid (GA_3) significantly influenced seedling elongation. After five weeks of culture, clear differences in shoot length were observed among treatments (Table 1). The 0.5 mg/L GA_3 treatment produced the most pronounced elongation, with shoots reaching an average of 13.5 cm (Figure 2).



Figure 2. *Althaea officinalis* seedling elongated to 13.5 cm on MS medium supplemented with 0.5 mg/L GA_3 after five weeks

Seedlings exhibited elongated internodes, expanded leaves, and an overall vigorous appearance.

At 1.0 mg/L GA_3, shoot elongation remained enhanced (12 cm) but was slightly reduced compared to the 0.5 mg/L treatment. Interestingly, the highest concentration tested (1.5 mg/L) resulted in noticeably shorter shoots (8 cm), suggesting that excessive GA_3 may inhibit elongation or induce physiological stress. Seedlings at this concentration displayed thicker stems and smaller leaves, indicating a shift in the growth balance.

Table 1. Effect of gibberellic acid on shoot length after five weeks

GA_3 concentration (mg/L)	Shoot length (cm)
0.5	13.5
1.0	12.0
1.5	8.0

These results confirm that *A. officinalis* is highly responsive to GA_3, but optimal elongation occurs at lower concentrations. The highest elongation occurred at 0.5 mg/L GA_3.

Shoot multiplication

Nodal segments excised from in vitro seedlings responded strongly to cytokinin-rich media. The

combination of 3 mg/L BAP with 0.5 mg/L IAA produced the highest multiplication rate, with explants generating multiple axillary shoots within four weeks. Shoots were compact, with short internodes and a dense foliar system, characteristics desirable for micropropagation.
When GA_3 (1 mg/L) was added to BAP, shoot proliferation still occurred, but the morphology shifted toward elongated shoots with fewer lateral branches. This suggests that GA_3 promotes elongation at the expense of lateral bud activation. The medium containing BAP + GA_3 + IAA produced intermediate results, with moderate elongation and acceptable shoot numbers (Figure 3 a, and c).



Figure 3. Shoot multiplication on MS medium supplemented with:
(a) 3 mg/L BAP + 0.5 mg/L IAA;
(b) 3 mg/L BAP + 1 mg/L GA_3 + 0.5 mg/L IAA;
(c) 3 mg/L BAP + 1 mg/L GA_3.

A notable observation was a progressive increase in the subculture interval. Initially, explants required transfer every four weeks; however, as shoots adapted to the optimized medium, the interval extended to eight weeks due to their vigorous and sustained growth. This represents a practical advantage for large-scale propagation, reducing labor and resource inputs.

Rooting

Root induction was successfully achieved on MS medium supplemented with 1.5 mg/L IBA. Within two weeks, shoots developed thick, well-branched root systems. Roots were white, robust, and exhibited a strong geotropic response. The rooting percentage approached 100%, indicating that A. officinalis is highly amenable to auxin-induced rhizogenesis (Figure 4).



Figure 4. Root induction on MS medium supplemented with 1.5 mg/L IBA

Rooted plantlets displayed no signs of hyperhydricity or physiological disorders, confirming the suitability of the chosen auxin concentration.

Callus induction

Two types of callus were obtained:
- Green, friable callus on MS + BAP (1 mg/L) + NAA (1 mg/L) (Figure 5).
- Compact white callus on MS + 2,4 D (5 mg/L) + BAP (2.5 mg/L) + casein hydrolysate (2 g/L). The latter produced the most vigorous callus mass (Figure 6).

Callus formation varied markedly depending on the auxin-cytokinin balance in the medium. On MS medium containing 1 mg/L BAP and 1 mg/L NAA, explants produced a green, friable callus with visible meristematic nodules. This callus

type expanded moderately and retained chlorophyll, suggesting partial differentiation.

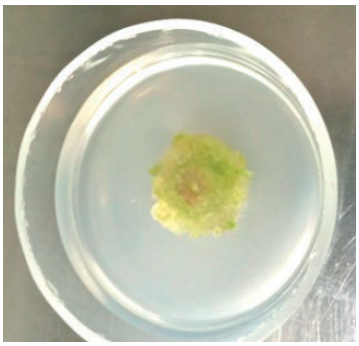

Figure 5. Green callus induced on MS medium containing 1 mg/L BAP and 1 mg/L NAA

In contrast, the medium supplemented with 5 mg/L 2,4-D, 2.5 mg/L BAP, and 2 g/L casein hydrolysate induced a compact, white callus with rapid proliferation. The callus mass increased substantially within three weeks, forming dense, homogeneous tissue. Casein hydrolysate appeared to enhance callus vigor, likely due to its amino acid content.

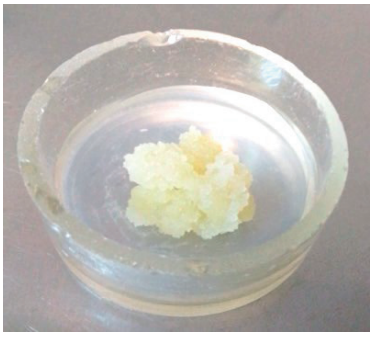

Figure 6. Compact white callus induced on MS medium supplemented with 5 mg/L 2,4-D, 2.5 mg/L BAP, and 2 g/L casein hydrolysate

The distinct callus morphologies indicate that *A. officinalis* tissues can be directed toward different developmental pathways depending on hormonal composition, offering flexibility for future applications such as secondary metabolite production or somatic embryogenesis.

Acclimatization

Regenerated plants were transferred to Magenta boxes containing a soil:perlite mixture (1:1) exhibited high survival rates (Figure 7).



Figure 7. Acclimatized *Althaea officinalis* plant in a soil:perlite (1:1) substrate.

Within two weeks, plantlets produced new leaves and displayed strong root anchorage. No chlorosis or wilting was observed, indicating a successful transition from in vitro to ex vitro conditions.

Plants maintained vigorous growth over the following month, confirming the robustness of the micropropagation protocol and the physiological stability of the regenerated individuals.

DISCUSSIONS

The present study provides a comprehensive evaluation of the in vitro behavior of *Althaea officinalis* L., demonstrating that this species responds strongly and consistently to controlled culture conditions. The high germination rate obtained on basal MS medium (90%) confirms the intrinsic viability of the seeds and supports previous findings that *A. officinalis* seeds do not require complex hormonal supplementation for successful germination (Younesikelati et al., 2016). The absence of contamination following the sterilization protocol further ensured that developmental responses were attributable solely to the culture environment.

The influence of gibberellic acid on seedling elongation was particularly evident. GA_3 at 0.5 mg/L produced the most vigorous elongation, while higher concentrations resulted in progressively shorter shoots. This dose-dependent response aligns with the

well-established role of GA_3 in promoting cell elongation and internode expansion. Similar patterns have been reported in other species, such as Dietes bicolor, where moderate GA_3 concentrations enhanced germination and seedling vigor, while excessive levels inhibited elongation (Silva et al., 2020). The reduced shoot length observed at 1.5 mg/L GA_3 in our study may reflect a hormonal imbalance that disrupts endogenous growth regulation, suggesting that *A. officinalis* is particularly sensitive to supra-optimal GA_3 levels.

Shoot multiplication was strongly influenced by the cytokinin–auxin balance. The combination of 3 mg/L BAP and 0.5 mg/L IAA produced the highest multiplication rate and the most compact, morphologically stable shoots. This result is consistent with previous studies reporting that BAP is the most effective cytokinin for inducing axillary shoot proliferation in *A. officinalis* (Naz et al., 2015; Mujib et al., 2017). The addition of GA_3 to BAP-containing media resulted in elongated shoots with fewer lateral branches, indicating that GA_3 shifts the developmental balance toward elongation rather than proliferation. This observation reinforces the importance of carefully adjusting cytokinin–auxin ratios to achieve the desired morphogenic response.

Root induction using 1.5 mg/L IBA was highly successful, producing thick, well-branched root systems. IBA is widely recognized as one of the most effective auxins for rhizogenesis in dicotyledonous species, and the strong rooting response observed here confirms its suitability for *A. officinalis*. The absence of hyperhydricity or physiological abnormalities further indicates that the hormonal environment was well-balanced and that the shoots were physiologically competent for rooting.

Callus induction varied significantly depending on the hormonal composition of the medium. The combination of BAP and NAA (1 mg/L each) produced a green, friable callus, suggesting partial differentiation and the presence of chlorophyll-retaining tissues. In contrast, the medium containing 2,4-D, BAP, and casein hydrolysate produced a compact, rapidly proliferating white callus. This response is consistent with the strong callus-inducing capacity of 2,4-D, which promotes dedifferentiation and cell proliferation (Hosseini et al., 2017; Görmez et al., 2019). The addition of casein hydrolysate likely enhanced callus vigor by providing amino acids and peptides that support rapid cell division. The distinct callus morphologies observed in this study highlight the developmental plasticity of *A. officinalis* tissues and suggest potential applications in secondary metabolite production, somatic embryogenesis, or genetic transformation.

Acclimatization of regenerated plants was highly successful, with plantlets exhibiting rapid adaptation to *ex vitro* conditions. The use of a soil-to-perlite mixture (1:1) provided adequate aeration and moisture retention, supporting root establishment and shoot development. The high survival rate indicates that the *in vitro*-derived plants were physiologically stable and capable of transitioning to natural growth conditions without stress symptoms.

Overall, the results of this study demonstrate that *A. officinalis* is an excellent candidate for in vitro propagation and callus culture. The optimized protocol presented here offers several advantages: high germination rates, efficient shoot multiplication, reliable rooting, and robust callus induction. These findings contribute to the growing body of knowledge on medicinal plant biotechnology and provide a solid foundation for future research aimed at large-scale propagation, conservation, and secondary metabolite production in *A. officinalis*. Further studies may explore the biochemical profiles of callus tissues, the potential for somatic embryogenesis, and the influence of alternative growth regulators or elicitors on metabolite accumulation.

CONCLUSIONS

The present study establishes a reliable and efficient *in vitro* propagation system for *Althaea officinalis* L., a medicinal species of considerable pharmacological importance. The high germination rate obtained on basal MS medium demonstrates that the species does not require complex hormonal supplementation for early development, making it highly suitable for in vitro culture. The addition of gibberellic acid significantly enhanced seedling elongation, with 0.5 mg/L GA_3 identified as the optimal concentration. This finding is particularly relevant for laboratories aiming to generate

elongated seedlings for explant preparation or physiological studies.

Shoot multiplication was most effectively achieved on MS medium supplemented with 3 mg/L BAP and 0.5 mg/L IAA, producing compact, vigorous shoots with extended subculture intervals. This optimized hormonal combination offers a practical advantage for large-scale propagation by reducing labor and resource inputs. The successful rooting of shoots on MS medium containing 1.5 mg/L IBA further confirms the suitability of this protocol for generating complete plantlets ready for acclimatization.

Callus induction was strongly influenced by the auxin–cytokinin balance. The combination of 2,4-D, BAP, and casein hydrolysate produced compact, rapidly proliferating white callus, highlighting the potential of *A. officinalis* tissues for applications such as secondary metabolite production, somatic embryogenesis, or genetic transformation. The ability to generate distinct callus types depending on hormonal composition underscores the developmental plasticity of this species and opens avenues for future biotechnological studies.

The high survival rate of regenerated plants during acclimatization demonstrates the physiological stability and robustness of the *in vitro*-derived material. This confirms that the optimized protocol is suitable not only for laboratory propagation but also for producing healthy plants capable of thriving under *ex vitro* conditions.

Overall, the results of this study provide a comprehensive and reproducible protocol for the in vitro propagation and callus induction of *A. officinalis*. The optimized system offers significant potential for germplasm conservation, large-scale propagation, and future research on the biosynthesis of valuable secondary metabolites. Further investigations may focus on the biochemical characterization of callus tissues, the induction of somatic embryos, and the use of elicitors to enhance the production of pharmacologically active compounds.

Taken together, these results provide a solid foundation for future applications in medicinal plant biotechnology, including large-scale propagation for industry, the conservation of valuable germplasm, and the targeted production of bioactive metabolites through callus-based or cell-suspension systems.

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