



RESEARCH ARTICLE

REVISED Evaluation of the efficiency of the optimal combination of hormonal, physical and biological stimuli in the induction and growth of callus in *Lavandula angustifolia* Mill

[version 3; peer review: 1 approved, 2 approved with reservations]

Previously titled: "Evaluation of the synergistic efficiency of hormonal, biological and physical stimuli in the induction and growth of callus in lavender"

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Abstract

Background

Lavender is a crucial source of secondary metabolites with medicinal and industrial importance. Its exploitation faces challenges due to the limited availability of raw materials and their sensitivity to climate. Tissue culture techniques, particularly callus culture, represent the most sustainable method for the continuous production of these active compounds.

Methods

This study focused on developing an effective protocol for inducing and stimulating callus cultures in lavender plants. Callus formation was stimulated from leaf tissue has cultured on MS medium supplemented with different concentrations of 2, 4-D (0, 1, 2 and 3) mg L⁻¹. The cultures were incubated under light or dark conditions. In the multiplication: calli (Cultured in MS medium containing 1 mg, 2,4-D incubated in the dark) was transferred to MS medium containing different 2,4-D (0, 1, 2 and 3 mg.L⁻¹) and BA (0,1 and 2 mg.L⁻¹). The

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Any reports and responses or comments on the

multiplied callus (Cultured in MS medium containing 1 mg L^{-1} 2, 4-D and 2 mg L^{-1} BA) was transferred into two nutrient media, MS and B5, each supplemented with different levels of chitosan (0, 50, 100, and 200 mg.L^{-1}) along with combination of 1 mg.L^{-1} 2, 4-D and 2 mg.L^{-1} BA.

article can be found at the end of the article.

Results

The results indicated that the optimal concentration for callus induction was 1 mg L^{-1} of 2,4-D, with the highest callus formation rate 95% recorded under dark conditions. Among all treatments, the combination of 2, 4-D 1 mg L^{-1} and BA 2 mg L^{-1} was the most effective for sustainable callus growth. Regarding the nutrient medium, B5 was more efficient than the MS medium. For the biostimulants, Chitosan at 100 mg L^{-1} recorded the highest efficiency in both fresh and dry callus weights.

Conclusions

This study underscores the necessity of integrating hormonal, nutritional and environmental factors for optimal callus production.

Keywords

Lavender, induction callus, biostimulants, hormonal, physical, biological.



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REVISED Amendments from Version 2

This revised version incorporates substantial improvements in response to the reviewers' comments. The Introduction has been expanded to provide a clearer scientific background and a more comprehensive discussion of the roles of plant growth regulators, physical factors, culture media, and chitosan in callus induction and growth. The Discussion has been strengthened by incorporating additional recent references and providing deeper interpretation and comparison with previous studies. The manuscript has also undergone extensive language editing to improve clarity, coherence, and scientific style. Minor revisions were made throughout the text to enhance readability and consistency. These revisions improve the overall quality of the manuscript without altering the experimental design, results, or conclusions.

Any further responses from the reviewers can be found at the end of the article

Introduction

Lavender (*Lavandula angustifolia* Mill.) is a member of the Lamiaceae family of flowering plants and is the most widely cultivated of the family, which also has over 39 other genera (Vârban *et al.*, 2022; Kumar *et al.*, 2025). The varied secondary metabolites in lavender provide a broad range of industrial uses in the pharmaceutical, cosmetic, and food industries (Crisan *et al.*, 2023). The medicinal value of lavender has been well established over the centuries, considering the wide range of advanced herbal diseases and its other numerous pharmacological attributes, including antimicrobial, antifungal, antispasmodic and other active antioxidant (Hassiotis and Vlachonassios, 2025) as well as digestive function regulating (Firoozzei *et al.*, 2021) properties. It is also used to treat gastrointestinal disorders, cardiovascular diseases, and respiratory and urinary disorders, anxiety, depression, and sleep disorders (Kumar *et al.*, 2025), mood swings (Mehrabian *et al.*, 2022), chronic pain (Pehlivan and Karadakovan, 2019), restless legs syndrome (Ghasemi *et al.*, 2021), migraines (Yuan *et al.*, 2021), and behavioural disorders in dementia patients. (Scuteri *et al.*, 2019) as well as improving physical symptoms such as blood pressure and respiratory rate (Contrada *et al.*, 2021).

There are difficulties in the plant based pharmaceutical industry related to the sourcing of raw materials attributable to the isolation of active compounds being arduous due to plants being low in active compounds or active compounds being affected by climatic conditions. Under these circumstances, the cultivation of plant tissues offers exciting possibilities, as it promotes the incessant active compounds synthesis irrespective of the season. This facilitates the overcoming of climatic and geographical limitations, hence aiding in the supply of growing the demand for medicinal and aromatic plants. This is one of the refined and improved strategies in the biotechnological domain that aims or is directed towards the amplified generation of secondary metabolites relevant for the industry (Ozyigit *et al.*, 2023). This necessitates the adjustment and calibration of every single component in every single process of culture tissue namely, nutrient medium, the different and varied growth hormones, and the different types of stimulants to maximise biomass and secondary metabolites (Adil *et al.*, 2025). The technology of callus culture is one of the sustainable methods for the valuable plant compounds and for the preservation of the habitat of the medicinal plants (Abed *et al.*, 2020; Babich *et al.*, 2020; Alrawi *et al.*, 2022). Compounds can be derived from callus tissue in a lab setting without having to use whole plants, and this method can be commercially scaled by employing bioreactors for compound bioprocessing. In vitro callus tissue development depends on a number of factors; for example, a plant's genetics, the plant organ type, and the composition of the nutrient medium and the growth regulators (Miri and Roughani, 2018). Furthermore, callus culture technology depends on the growth and sustained production and the qualitative and quantitative production of active compounds. The optimum environment must also be available. The importance of growth regulators during callus formation is significant because, in some cases, a tissue may be unresponsive or may even die if the medium's hormone levels are set higher or lower than the tissue's internal level (Yegorova *et al.*, 2020). Previous research indicates that the callus formation is more pronounced when the nutrient medium is enhanced with growth regulators, specifically auxins and cytokinins (Jabr and Mohsen, 2024). The optimization of the hormonal milieu is only attained with the moderators acting synergistically within set ranges. Compared to using each of them separately (Tilkat and Onay, 2009). The balance between auxins and cytokinins in the nutrient medium is a key determinant that stimulates cell division and initiates callus formation.

Despite the central role of plant growth regulators in controlling cellular differentiation and morphogenesis, callus formation is influenced by multiple interacting factors, including hormonal signals (Thiry *et al.*, 2026), physical conditions, culture medium composition, and biostimulants (Pasternak *et al.*, 2024). Among physical factors, light plays a key role in regulating cellular reprogramming through its effects on auxin stability, hormonal balance, and primary metabolism, thereby influencing dedifferentiation efficiency (Cioć *et al.*, 2022; Miguel, 2025). However, these responses vary considerably among plant species (Liu *et al.*, 2025), which limits the generalization of results. Culture medium composition is another critical factor governing morphogenic responses. Even minor changes in ionic balance, organic additives, or nitrogen sources can lead to noticeable shifts in growth patterns (Okao *et al.*, 2025). Therefore,

culture media should be considered dynamic systems whose effectiveness depends on species-specific physiological requirements rather than fixed formulations. Biostimulants have recently gained attention as supplementary regulators of plant growth responses. Chitosan, a deacetylated form of chitin, has been widely studied due to its ability to trigger defense-related pathways and influence secondary metabolism (Javed *et al.*, 2025; Iber *et al.*, 2022). However, its mode of action in relation to hormonal signaling and environmental interactions is still not fully understood, particularly under in vitro conditions. Overall, callus formation results from the interaction of several factors rather than a single regulatory pathway (Liu *et al.*, 2025). Most studies still examine these factors separately, which limits understanding of their combined effects. Although *Lavandula spp.* are economically and pharmacologically important as sources of essential oils and bioactive compounds, the regulatory mechanisms controlling callus induction and stability in vitro remain poorly defined. This study therefore investigates the combined effects of plant growth regulators, light conditions, culture medium composition, and chitosan on callus formation and growth in lavender.

Materials and methods

Plant materials

Lavender seeds were obtained from a specialised seed production company Al-Wadi seed Company located south of the capital Baghdad, Iraq. The seeds were stored in a laboratory at a temperature of $15^{\circ}\pm 2^{\circ}\text{C}$ until use.

Establishment of tissue culture

Medium preparation

The culture medium used in the present study consisted of half MS salts (Murashige and Skoog, 1962), medium supplemented with 30 g L^{-1} sucrose as a carbon source. Gibberellic acid was added to break seed dormancy at a concentration of 0.5 mg L^{-1} . The pH of the medium was adjusted to 5.7–5.8, and agar at 7 g L^{-1} was used as a solidifying agent. The prepared medium was dispensed into culture vials, with each vial containing 10 mL of medium. All culture vessels were autoclaved at 121°C and 15 psi for 15 min for sterilization. The medium was then allowed to solidify in the vials prior to inoculation.

The medium was distributed into glass culture vessels 10 ml vessel. The containers were sealed with tight fitting lids and sterilised in an autoclave Model ABC at a temperature of 121°C and a pressure of 1.04 kg cm for 15 minutes. The sterilised medium was stored at 25°C until use.

Seed sterilization

The seeds were sterilised and thoroughly washed under running tap water for 10 minutes to remove surface debris. The healthy seeds were transferred to a laminar air cabinet, then a 3% sodium hypochlorite solution was added for surface sterilization for 15 minutes with continuous stirring. The seeds were rinsed with distilled water for 5 minutes, and the washing process was repeated three times to ensure complete removal of the sterilizing agent (Al-Alwani and Mohammed, 2022).

Seed germination

The sterilised seeds were planted on the prepared nutrient medium. The planted seeds were then transferred to the growth chamber at a temperature of 25°C and a light intensity of 1000 lux for 16 hours of light and 8 hours of darkness (Sekhi *et al.*, 2025). After four weeks, the seedlings were transferred to MS medium (hormone-free) and incubated under the same conditions for four weeks, seedlings ready for use were obtained for callus induction experiments. The chemicals listed in Table 1 were used in this study.

Experimental design

The experiment was conducted in three successive phases, each designed to achieve a specific objective. Each subsequent phase built upon the results of the previous phase (Figure 1), to evaluate callus production efficiency and the growth and enhancement of biomass production. The following experiments were conducted.

(1) Callus induction

A nutrient medium based on full MS salts strength was prepared, with varying concentrations of the growth regulator dichlorophenoxyacetic acid (2,4-D) (0, 1, 2 and 3) mg L^{-1} . Then 30 g L^{-1} of sucrose was added to the medium as a carbon source and 7 g L^{-1} of agar as a stabilising agent. The nutrient medium was then sterilised according to the standard protocol. After preparing the media The third leaf from the growing tip (the four weeks-old healthy tissue culture lavender

Table 1. Reagents used in the study, including the amount used, supplier information, and catalogue numbers.

Reagent item	Quantity	Supplier	Catalogue numbers
MS.	4.74 g. L ⁻¹	Duchefa Biochemie	M0221
Gamborg B5 medium	3.92 g. L ⁻¹	Duchefa Biochemie (Holland)	G0209
Dichlorophenoxyacetic acid	(0,1,2 and 3) mg. L ⁻¹	Duchefa Biochemie (Holland)	D0911
Benzyladenine	BA (0,1 and 2) mg. L ⁻¹	CDH (India)	022625
Sucrose	30 g. L ⁻¹	CDH (India)	030299
Chitosan (Medium MW) extrapure, 150-500 m.Pas, 75% DA	(0,50,100, 200) mg. L ⁻¹	CDH (India)	046555
Agar Agar	7 g. L ⁻¹	Duchefa Biochemie (Holland)	P1001.1000

plants) have used as explants. The leaves tissue were cut into a square pieces measuring 0.5*0.5 cm² by a sterile scalpel on the clean bench and afterword inoculated on the MS medium the specified concentrations of the growth regulator 2,4-D. The cultures were incubated in a growth chamber with controlled conditions at a constant temperature of 25 C. To investigate the effect of light on callus induction, the cultures were exposed to two different lighting regimes complete darkness and 1000 lux light intensity under a cyclic light regime 16 hours of light 8 hours of darkness. At the end of the four-week incubation period, data were collected by measuring Induction rate the percentage of tissue sections in which callus was formed, Fresh weight and Dry weight.

(2) Callus Proliferation

Calli (150 mg in fresh weight), induced from **leaves** tissue on the MS medium containing 1 mg.L⁻¹ 2,4-D for 4 weeks, were used. These calli were transferred to the MS medium supplemented with 30 g.L⁻¹ sucrose, 7g.L⁻¹ agar and 2,4-D at 0, 1, 2 and 3 mg.L⁻¹ in combination with 0, 1 and 2 mg.L⁻¹ BA. The cultures were incubated in complete darkness at a constant temperature of 25°C for four weeks. At the end of the incubation period, data were collected by measuring the fresh weight and then the dry weight of the callus growth.

(3) The role of nutrient components and chitosan in promoting callus growth

Based on the best combination was reported in the previous experiment, 150 g of 4-week-old propagated calli (cultured on MS medium supplemented with a combination of 1 mg of 2,4-D and 2 mg of BA) were transferred to two media (B5 and MS). Each media was supplied with four concentrations of chitosan (0, 50, 100, and 150) mg, with the addition of 1 mg.L⁻¹ of 2,4-D and 2 mg of BA, 30 g sugar and 7 Agar. After a four-week incubation period, growth rates were determined by measuring the fresh and dry weights of the callus formed.

Statistical analysis

The data were statistically analysed using GenStat statistical software (Gen 12 ed) (Payne *et al.*, 2009) according to a completely randomised design (C.R.D.), with ten replicates for each treatment. The treatments were arranged in a factorial arrangement. The means were compared on LSD test after partitioning the variance components via fisher's test by using probability ≤ 0.05 whereas, the callus percentage was analysed using originPro 2025b (origin 2025).

Results and discussion

The effect of light conditions and 2,4-D concentrations on callus formation

Statistical analysis, as shown in Figure 1 revealed that the growth regulator 2,4-D had a significant effect on callus induction. It was found that the optimal concentration for callus formation from leaf tissue was 1 mg L⁻¹ of 2,4-D added to the nutrient medium, which reached 95%, while an increase in concentration above this level led to a significant decrease in the rate of callus formation.

On the other hand, lighting had a relatively limited effect on callus induction compared to the effect of 2,4-D growth regulator concentration. The highest callus production values were recorded under complete darkness, and all auxin containing treatments outperformed their counterparts grown under lighting conditions (Figure 1).

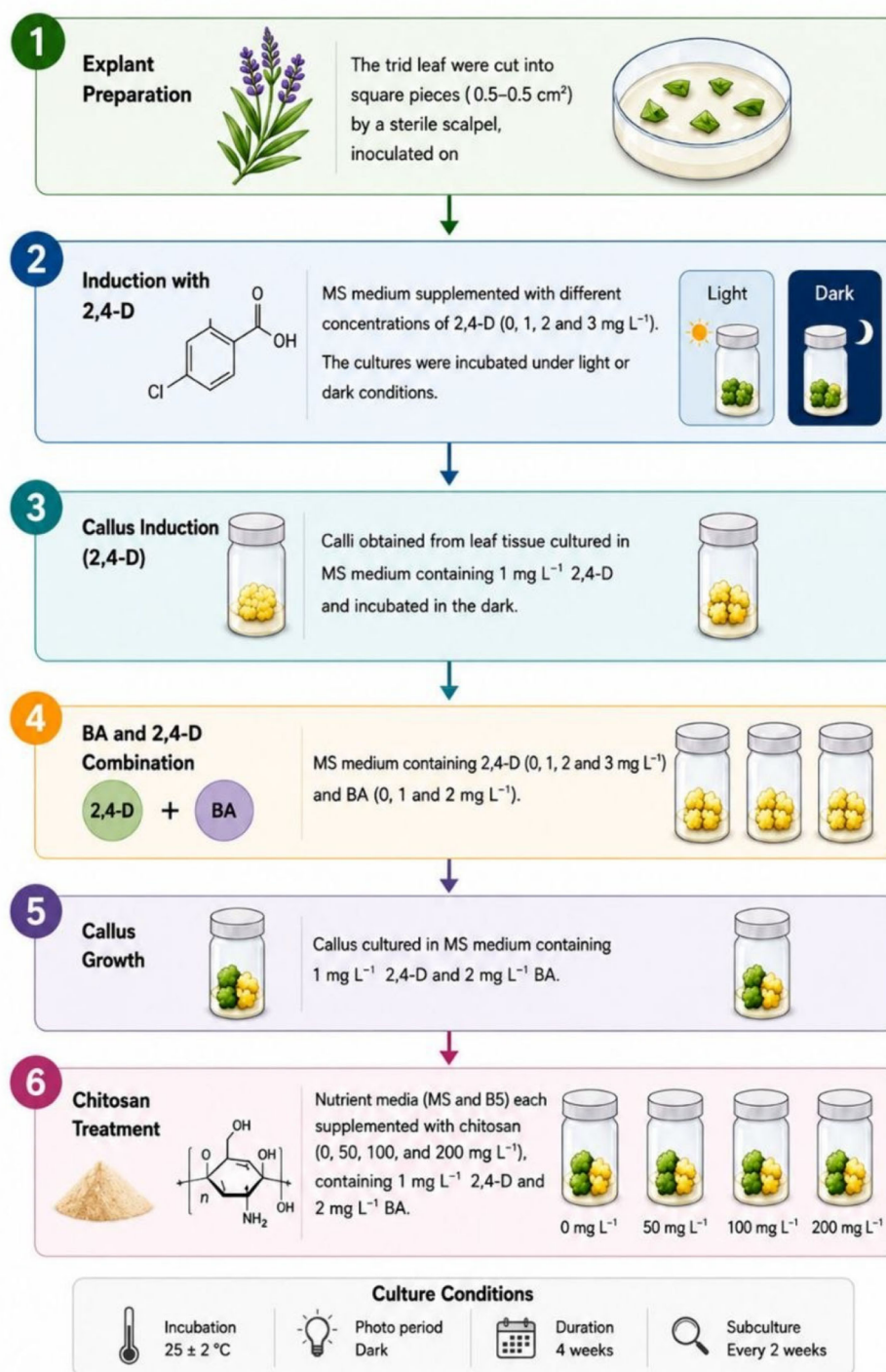


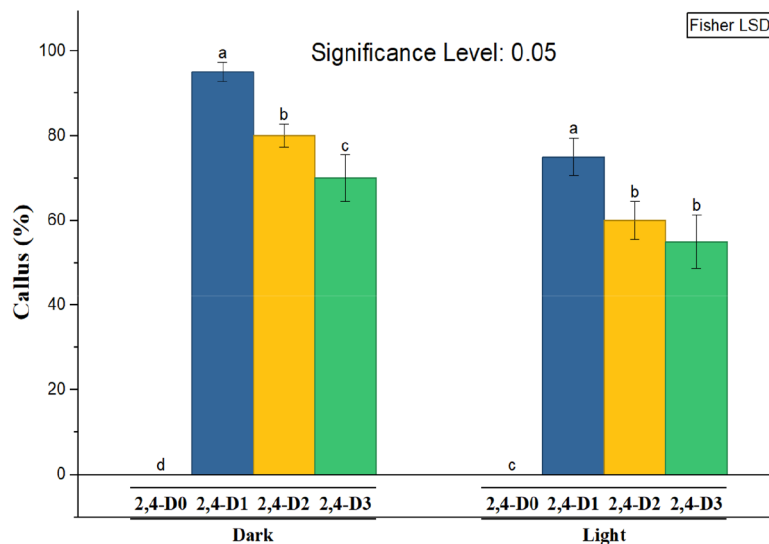
Figure 1. Three phase factorial design for in vitro callus induction proliferation and elicitor-mediated growth stimulation.

Effect of illumination and 2,4-D concentrations on callus fresh weight (g)

The results of statistical analysis according to the data shown in Table 2 showed a statistically significant difference in the fresh weight values of callus induced from lavender leaf tissue, where the fresh weight of callus differed according to concentration (0, 1, 2 and 3) mg L⁻¹ of 2,4-D added to the MS nutrient medium. The auxin concentration of 1 mg L⁻¹ showed the highest significant value for callus fresh weight, which reached 0.796 g. An increase in auxin concentration

Table 2. Effect of illumination and 2,4-D concentrations on callus fresh weight (g).

Light conditions	2,4-D (mg.L ⁻¹)				Mean
	0	1	2	3	
Dark	0.000 d	0.830 a	0.752 b	0.647 c	0.557 a
Light	0.000 d	0.762 b	0.723 bc	0.624 c	0.527 b
Mean	0.000 d	0.796 a	0.737 b	0.635 c	

LSD_{0.05} (Interaction) = 0.014; LSD_{0.05} (Main effect) = 0.010.**Figure 2.** Effect of light conditions and 2,4-D concentrations on callus formation. X-axis (Abscissa: 2,4-D concentrations): 0, 1, 2 and 3 mg L⁻¹ and illumination type: Dark and light, y-axis (ordinate): Callus percentage. Error bar: SEM.

above this level led to a gradual decrease in the rate of callus formation, indicating that high auxin concentrations have an inhibitory effect on the callus formation process. It should be noted that the auxin free treatment 0 concentration did not show any visible or statistical response in callus formation, confirming the pivotal role of auxin in initiating induction and stimulating cell division in plant leaf tissues. As for the effect of lighting, the results showed a clear superiority of callus growth under conditions of complete darkness for all concentrations studied, with the average fresh weight values under these conditions showing a remarkable superiority of 0.557 g compared to their counterparts grown under lighting conditions, which reached 0.527 g. The effect of the light factor can be observed in Figure 3, where the callus appeared dark greenish to gray (friable, granular, compact), while in the dark, the callus appeared Pale yellowish to green (compact dense less granular). Callus grown in darkness was also larger and more developed, making it suitable for further experiments on callus cultures.

It can be concluded that the process of callus induction from lavender leaf tissue requires a delicate balance between the optimal concentration of auxin and suitable environmental conditions, emphasising the complex interaction between hormonal and environmental factors in regulating growth and differentiation processes in plant cells. The results also show that the highest fresh weight of callus was achieved at a concentration of 1 mg L⁻¹ of regulator 2,4-D when grown under dark conditions, reaching 0.830 g. While no value was recorded for fresh weight of callus (0 g) in treatments free of auxin under dark or light conditions. This confirms the importance of auxin as a basic factor in callus formation.

Effect of illumination and 2,4-D on callus dry weight of callus (g)

Effect of auxin on dry weight of callus the results of statistical analysis shown in Table 3. Indicate a clear variation in biological response when treating lavender (*Lavandula angustifolia* Mill.) leaf tissues with different concentrations of the growth regulator 2,4-D. A concentration of 1 mg L⁻¹ of 2, 4-D recorded the highest significant dry weight of callus 0.064 g. An increase in concentration to 2 mg L⁻¹ resulted in a significant decrease in dry weight 0.058 g, while a

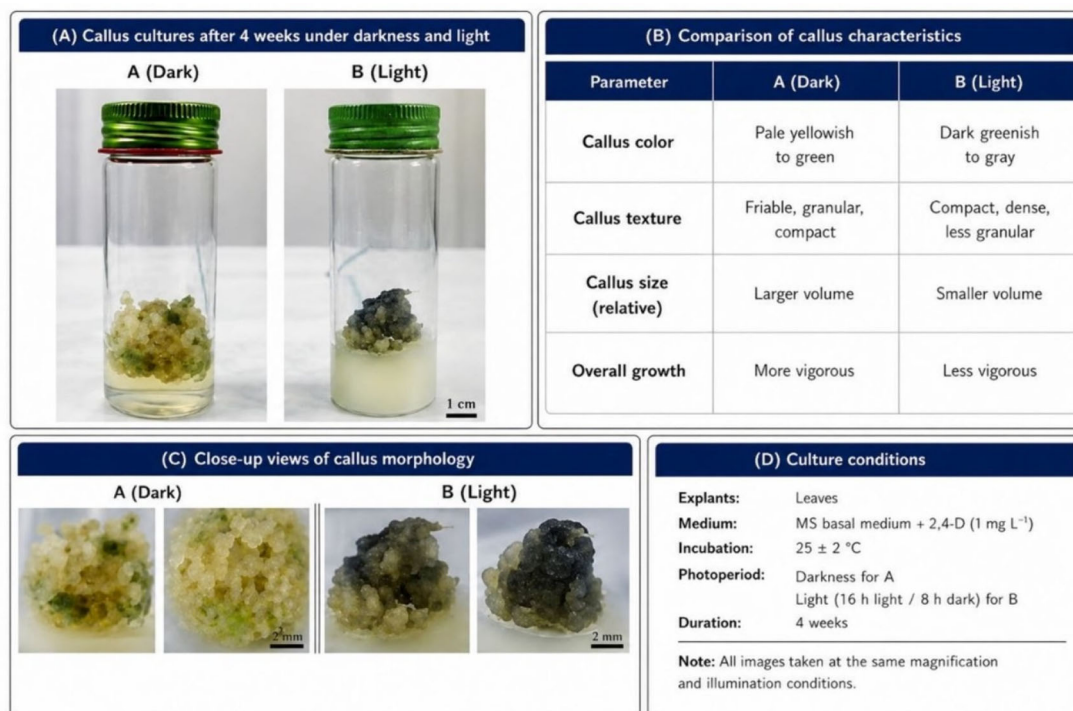


Figure 3. Effect of light and darkness on callus induction and growth from lavender leaves in vitro. (A): Callus cultures after 4 weeks under darkness and light. (B): Comparison of callus characteristics. (C): Close-up views of callus morphology. (D) Culture conditions.

Table 3. Effect of light conditions and 2,4-D concentrations on callus dry weight (g).

Light conditions	2,4-D (mg L ⁻¹)				Mean
	0	1	2	3	
Dark	0.000 e	0.068 a	0.059 b	0.048 c	0.044 a
Light	0.000 e	0.061 b	0.057 bc	0.044 d	0.040 b
Mean	0.000 d	0.064 a	0.058 b	0.046 c	

LSD_{0.05} (Interaction) = 0.002; LSD_{0.05} (Main effect) = 0.001.

concentration of 3 mg L⁻¹ recorded a decrease of 0.046 g. The auxin free treatment did not show any visible or statistical response in callus formation, confirming the pivotal role of auxin in the process of induction and initiation of cell division.

Statistical analysis showed a significant interaction between auxin concentration and lighting conditions. Treatments under lighting conditions recorded an average dry weight of 0.040 g, while treatments in complete darkness significantly exceeded this with an average dry weight of 0.044 g.

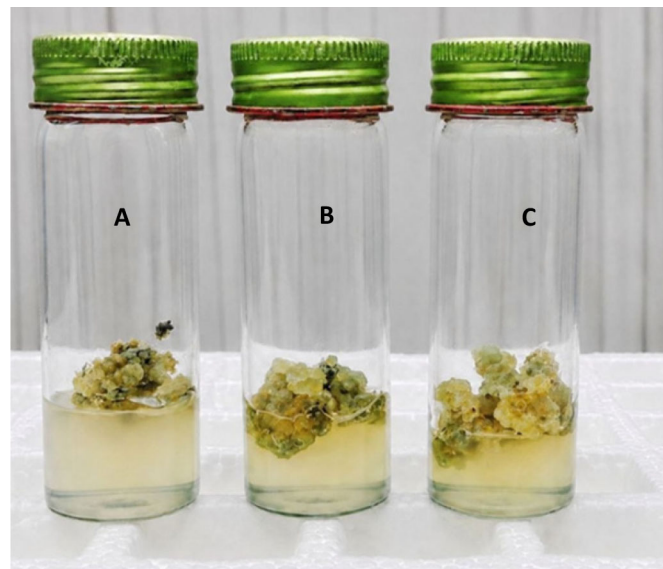
Effect of 2,4-D and BA on fresh weight of callus (g)

The results in Table 4, Showed that adding synthetic auxin of type 2,4-D at different concentrations to the nutrient medium had a significant statistically significant effect on the fresh weight of the callus formed. A clear response to the concentrations added to the nutrient medium was observed, with the low concentration 1 mg L⁻¹ outperforming all other treatments, recording the highest significant value for fresh weight of 0.920 g. With an increase in hormone concentration to 2 and then 3 mg L⁻¹, a gradual and significant decline in growth efficiency was observed, with the fresh weight of callus decreasing to 0.811 g and 0.632 g, respectively. This decrease indicates the possibility of an inhibitory effect of high concentrations of auxin on the process of development and growth.

Table 4. Effect of 2,4-D and BA on fresh weight (g) of callus.

BA (mg L ⁻¹)	2,4-D (mg L ⁻¹)			Mean
	1	2	3	
0	0.838 c	0.728 d	0.628 f	0.731 c
1	0.880 b	0.843 c	0.636 ef	0.786 b
2	1.042 a	0.863 bc	0.634 ef	0.846 a
Mean	0.920 a	0.811 b	0.632 c	

LSD_{0.05} (Interaction) = 0.018; LSD_{0.05} (Main effect) = 0.010.

**Figure 4.** Illustrates the effect of benzyl adenine at a concentration of (A: 0 mg.L-1 , B: 1 mg.L-1 and C: 2 mg.L-1) added to MS nutrient medium on the growth of callus generated from leaf tissue after four weeks.

On the other hand, the results also showed statistically significant differences between the different concentrations of cytokinin benzyladenine (BA) added to the nutrient medium. The effect of BA was characterised by a positive linear relationship, where the growth rate of callus measured by fresh weight increased significantly with increasing hormone concentration (Figure 4). Growth peaked at a concentration of 2 mg L⁻¹, achieving the highest fresh weight value of 0.846 g. In contrast, the treatment containing a concentration of 0 mg L⁻¹ free of cytokinin recorded the lowest fresh weight value of 0.731 g, highlighting the vital importance of cytokinin in supporting cell division and callus formation. The most significant scientific importance of this study lies in evaluating the complementary and reciprocal effects of these two hormones when used together. The results showed a clear superiority of some combinations over others. The addition of a combination of auxin 2,4-D at a concentration of 1 mg L⁻¹ in conjunction with cytokinin BA at a concentration of 2 mg L⁻¹ resulted in the highest total value of 1.041 g. This result reflects a synergistic effect between the two hormones, each enhancing the effect of the other, leading to optimal stimulation of growth and development processes. In contrast, the combination of auxin 2,4-D at a concentration of 3 mg.L⁻¹ with cytokinin at a concentration of 0 mg.L⁻¹ recorded the lowest fresh weight value, which was 0.628 g. This poor performance confirms the negative effects of high auxin concentrations in the absence of a balancing factor such as cytokinin, supporting the hypothesis that the balance between these two hormones, rather than the presence of only one, is the decisive factor in directing plant cell growth and differentiation in tissue cultures.

Effect of 2,4-D and BA on dry weight of callus (g)

The results in Table 5 showed a clear response when auxin was added at different concentrations to the nutrient medium. The concentration of 1 mg L⁻¹ significantly outperformed all other treatments, recording the highest dry weight of callus at 0.064 g. This result indicates that this concentration is optimal for stimulating cell division and hypertrophy, which positively affects biomass accumulation. However, a gradual and significant decline in growth efficiency was observed with increasing auxin concentration. Dry weight decreased to 0.060 g at a concentration of 2 mg L⁻¹ and to 0.044 g at a

Table 5. Effect of 2,4-D and BA on dry weight (g) of callus.

BA (mg L ⁻¹)	2,4-D (mg L ⁻¹)			Mean
	1	2	3	
0	0.065 ab	0.055 cd	0.041 f	0.054 c
1	0.062 bc	0.060 bc	0.042 ef	0.055 bc
2	0.070 a	0.065 ab	0.050 de	0.062 a
Mean	0.065 a	0.060 b	0.044 c	

LSD_{0.05} (Interaction) = 0.002; LSD_{0.05} (Main effect) = 0.001.

concentration of 3 mg L⁻¹. This decrease indicates that higher concentrations of auxin may have an inhibitory or toxic effect on cells, hindering their growth and limiting callus formation, which is common in plant responses to growth regulators when the optimum limit is exceeded.

The results showed statistically significant differences in the dry weight of callus when different concentrations of cytokinin were added. The effect of cytokinin was characterised by a positive linear relationship, with the dry weight of induced callus increasing significantly with increasing hormone concentration. This effect peaked at a concentration of 2 mg L⁻¹, achieving the highest dry weight value of 0.062 g. This confirms the vital role of cytokinin in regulating cell division and differentiation, which is a complementary and important factor in the callus induction process.

The greatest scientific value of this study lies in assessing the combined and complementary effects of auxin and cytokinin hormones, as it is known that the balance between them is the main driver of plant cell growth. In this context, the results showed a significant advantage for the combination of auxin at a concentration of 1 mg.L⁻¹ with cytokinin at a concentration of 2 mg.L⁻¹. This combination achieved the highest dry weight value for callus 0.070 g. This outstanding performance is attributed to the synergistic effect between the two hormones, where auxin at its optimal concentration 1 mg.L⁻¹ stimulates cell division and callus formation, while cytokinin at its optimal concentration 2 mg L⁻¹ promotes cell division which together leads to optimal cell mass enlargement and higher dry matter accumulation.

In contrast, the combination with the highest concentration of auxin 3 mg.L⁻¹ and a complete absence of cytokinin 0 mg L⁻¹ recorded the lowest dry weight value, at 0.041 g. These results confirm that the success of callus induction and its maximum growth measured by dry weight depend critically on the selection of the optimal concentration of each plant regulator separately and more importantly, on the selection of the optimal combination of the two. The interaction between auxin and cytokinin is what directs the final physiological response of plant tissues.

Effect of nutrient medium components and chitosan on callus fresh weight (g)

The results of statistical analysis, shown in Table 6, revealed a clear variation in the components of different nutrient media in stimulating callus growth. The comparison showed that the nutrient medium B5 significantly outperformed the other media, recording the highest average fresh weight of callus induced from leaf tissue, with a value of 2.197 g. In contrast, the MS medium showed the lowest fresh weight growth rate for the same type of callus, with a value not exceeding 1.233 g.

As for the results related to the analysis of the effect of chitosan concentration, presented in Table 6. They showed statistically significant differences between the four concentrations studied (0, 50, 100 and 200) mg L⁻¹ was observed that all tested concentrations of chitosan significantly outperformed the control group (Figure 5), confirming the positive effect of this substance in stimulating soft weight gain in callus. This effect followed a clear pattern of proportionality,

Table 6. Effect of nutrient medium components and chitosan on callus fresh weight (g).

Medium components	Chitosan (mg. L ⁻¹)				Mean
	0	50	100	200	
MS	1.000 f	1.206 e	1.904 c	0.824 g	1.233 b
B5	1.892 c	2.374 a	2.392 a	2.131 b	2.197 a
Mean	1.446 d	1.790 c	2.148 a	1.477 d	

LSD_{0.05} (Interaction) = 0.060; LSD_{0.05} (Main effect) = 0.042.

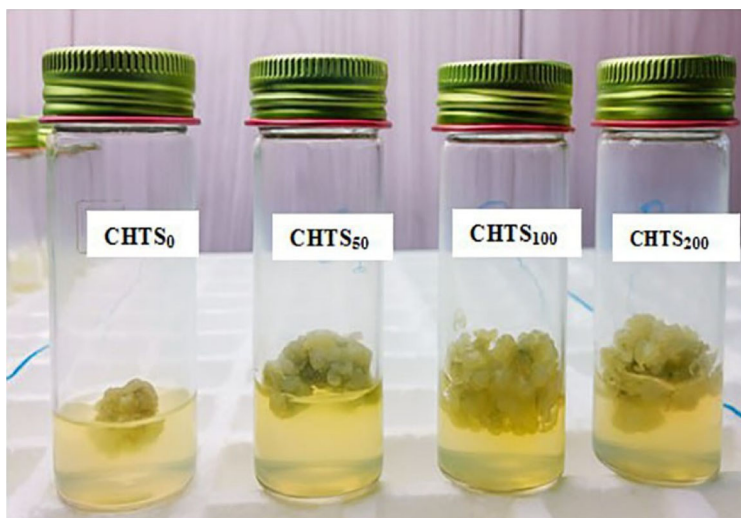


Figure 5. Effect of chitosan concentrations added to medium B5: CHTS0: without chitosan, CHTS50: 50 mg chitosan per liter, CHTS100: 100 mg chitosan per liter and CHTS200: 200 mg chitosan per liter on callus growth of lavender leaves, after four weeks.

as the rate of soft weight gain in calves increased significantly with increasing chitosan concentration, reaching its highest value at a concentration of 100 mg L^{-1} , with a peak of 2.148 g. Performance reversed significantly when the highest concentration 200 mg L^{-1} , where the fresh weight of the callus decreased compared to the optimal concentration 100 mg L^{-1} , indicating that the positive effect of chitosan is at its maximum at a specific concentration, and that increasing the concentration beyond this limit may lead to adverse results.

Effect of nutrient medium components and chitosan on dry weight of callus (g)

The results of statistical analysis, as shown in Table 7, revealed a clear variation in the performance of nutrient medium components in terms of their effect on the dry weight of callus. The results showed a marked superiority of the B5 diet in achieving the highest average dry weight of callus induced from leaf tissue, recording a value of 0.103 g. In contrast, the nutrient medium MS recorded the lowest dry weight, with a value of 0.076 g, indicating that the composition of the nutrient medium B5 was more suitable and effective in promoting growth and dry matter accumulation in callus compared to the medium MS.

As for the results shown in Table 7, Statistical analysis revealed statistically significant differences between the different concentrations of chitosan included in the study (0, 50, 100 and 200) mg L^{-1} . It was observed that all concentrations treated with chitosan significantly outperformed the control group, to which no concentration of the substance was added 0 mg L^{-1} . This superiority confirms the stimulating and positive effect of chitosan on callus growth.

The results also revealed a positive correlation between increased chitosan concentration and the dry weight of induced callus, with values increasing steadily with higher concentrations, peaking at a concentration of 100 mg L^{-1} . The average dry weight of the callus reached its highest value, amounting to 0.096 g. A decline in this positive effect was also observed when using the highest concentration 200 mg L^{-1} , where the average dry weight decreased to 0.091 g. This decrease indicates that the stimulating effect of chitosan follows an optimum pattern, achieving maximum growth response, and that exceeding this concentration may have an inhibitory or less effective effect on the accumulation of dry matter in callus.

Table 7. Effect of nutrient medium components and chitosan on dry weight of callus (g).

Medium components	Chitosan (mg. L^{-1})				Mean
	0	50	100	200	
MS	0.068 e	0.071 d	0.087 c	0.081 cd	0.076 b
B5	0.101 b	0.103 ab	0.106 a	0.102 ab	0.103 a
Mean	0.084 c	0.087 b	0.096 a	0.091 ab	

LSD_{0.05} (Interaction) = 0.002; LSD_{0.05} (Main effect) = 0.001.

Discussion

Growth regulators, especially auxins, are key factors in stimulating callus formation. The addition of auxin to the nutrient medium elicited a response in the leaf tissues of lavender plants, stimulating callus formation. The added auxin induces fundamental changes in the plant cells in contact with the medium and stimulates the initiation of cell division. During this stimulation phase, constructive processes such as protein synthesis and DNA replication are activated, followed by a series of changes in cellular activity that culminate in cell division and the formation of a callus mass covering most parts of the plant fragment.

The response of plant tissue varies depending on the concentration of auxin in the nutrient medium. An increase in concentration promotes callus formation until the optimum concentration of 1 mg L^{-1} is reached, which achieved the highest callus formation rate 95%, in addition to an increase in fresh and dry weight. Conversely, exceeding this optimal concentration, as observed at concentrations of 2 and 3 mg L^{-1} , leads to adverse effects that inhibit growth. The results also showed that light acted as an inhibitory factor for callus growth induced from lavender leaves, whereas darkness provided more favorable conditions for callus induction and growth (Bojko *et al.*, 2024). This may be attributed to improved auxin stability under dark conditions, reduced photo-oxidative stress, and reduced early cell differentiation, thereby supporting cellular dedifferentiation and enhancing callus proliferation (Bojko *et al.*, 2024). In contrast, light conditions may negatively affect auxin stability, thereby reducing its effectiveness and contributing to the lower level of callus formation observed under light. Light also had a clear effect not only on growth but also on the morphological characteristics of the callus. Callus cultured under light conditions was characterized by a dark green to gray color and a brittle, granular, and compact texture, whereas callus induced under dark conditions appeared pale yellow to green with a compact, dense, and less granular structure (Figure 3). It was also observed that callus grown in darkness was larger and more developed compared to that grown under light conditions, which gives it an advantage in this study. However, the effect of light on callus growth is complex and may vary depending on the plant species and culture conditions. Similar responses have been reported in several plant species, confirming the regulatory role of darkness in morphogenic responses during *in vitro* culture (Keykha *et al.*, 2014; Suhartanto *et al.*, 2022; Bojko *et al.*, 2024). It is important to note that continuing to supplement the nutrient medium with auxin at the optimal concentration in order to increase callus production may inhibit growth due to auxin accumulation, necessitating the addition of cytokinin. Cytokinin plays an important role in sustaining callus growth, and previous studies indicate that the interaction between auxins and cytokinins at different concentrations is a decisive factor in achieving optimal hormonal balance (Jabr and Mohsen, 2024; Tilkat and Onay, 2009). In this regard, the treatment consisting of 1 mg L^{-1} auxin and 2 mg L^{-1} benzyladenine BA recorded the highest fresh and dry weight values, indicating that it is a suitable combination for sustained callus growth. This can be explained by the ability of cytokinins, especially benzyladenine, to promote nutrient uptake, stimulate cell division and inhibit protein degradation, which supports the division process when the appropriate hormonal balance is achieved. Cytokinins also promote the synthesis of RNA, proteins, and enzymes within the cell. Overall, the increase in soft and dry weight of callus reflects changes in cellular components such as carbohydrates, proteins and amino acids associated with the process of division and growth (Bulya *et al.*, 2023). Therefore, the balance between auxins and cytokinins in the nutrient medium remains a key determinant of continued cell division and callus growth.

In addition to plant growth regulators, the basal culture medium significantly influenced both fresh and dry biomass accumulation, where Gamborg's B5 medium showed superior performance compared with MS medium. This difference is mainly attributed to variations in the nutritional composition of the two media. Gamborg's B5 medium provides higher levels of potassium nitrate along with additional nitrogen sources such as ammonium sulfate, which contribute to improved nitrogen assimilation, protein biosynthesis, and enhanced morphogenic potential in plant tissue cultures (Yin *et al.*, 2017; Bari *et al.*, 2022; Riba, 2026). In addition, its relatively higher thiamine concentration may support key enzymatic functions and metabolic pathways required for active cell proliferation and tissue differentiation (Fitzpatrick *et al.*, 2020; Vollmer *et al.*, 2023; Riba, 2026). Overall, plant tissue responses to culture media are highly species-dependent, suggesting that Gamborg's B5 medium may be more suitable than MS medium for specific plant species (Kurmi *et al.*, 2020; Riba, 2026).

Bioactive chitosan was also used as a callus growth stimulator, and the results were consistent with previous studies on the role of chitosan in stimulating growth (Pirbalouti *et al.*, 2017; Byczyńska, 2018; El-Khateeb *et al.*, 2018; Atteya *et al.*, 2023). Chitosan treatments recorded significant increases in growth indicators and a positive correlation was observed between chitosan concentration up to a certain level and callus growth, with the highest fresh and dry weight values recorded at a concentration of 100 mg L^{-1} . This stimulation is attributed to the biological properties of chitosan, which enhances the absorption of key nutrients such as nitrogen and phosphorus, thereby supporting metabolic processes and the accumulation of organic matter. This was reflected in an increase in fresh weight due to higher water and organic matter content and dry weight due to the accumulation of proteins and carbohydrates, resulting in improved biomass of the callus. Stimulation must be limited and sensitive to concentration; at a concentration of 200 mg L^{-1} , growth indicators

decreased significantly, and this decrease can be explained physiologically by ionic disturbance and inhibition of nutrient absorption.

Conclusion

Callus formation in *Lavandula angustifolia* is influenced by several interacting factors, including plant growth regulators, light, culture medium, and chitosan. These factors work together and affect how cells dedifferentiate and develop into callus. Both light conditions and nutrient composition of the medium play an important role in this process, while chitosan contributes to improving overall plant physiological responses. In general, successful callus induction requires considering all these factors together as one system rather than separately. This helps improve the efficiency and stability of tissue culture in lavender and supports its use in micropropagation and secondary metabolite production.

Data and software availability

All raw data files underlying the results of this study are available on OSF: **Raw data for lavender study**, <https://doi.org/10.17605/OSF.IO/AMV4Z> (Almehemdi 2025).

This project contains the following underlying data:

- Lavenderdata1.xlsx

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

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Version 3

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Duha Mysire Majeed 

Plant Biotechnology, Al-Nahrain University / Biotechnology Research center, Baghdad, Iraq

I have reviewed the revised version and the authors have adequately addressed my comments. I have no further comments.

Competing Interests: I have previously collaborated with one of the authors, Mohammed A. Mohammed, as a co-author on a published research article in 2020. This collaboration was limited to that publication and there is no ongoing collaboration related to the current manuscript. I have had no direct contact with the authors regarding this article, and the manuscript was not discussed with them. I confirm that I am able to assess this work impartially.

Reviewer Expertise: Plant Biotechnology, Plant Tissue Culture, Molecular Biology, Plant Genetic Engineering

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Reviewer Report 06 July 2026

<https://doi.org/10.5256/f1000research.202213.r493248>

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Tassanai Jaruwattanaphan 

Kasetsart University, Bangkok, Thailand

The authors answered well to the concerns I addressed in my earlier review on Version 1. The taxonomic inconsistency has been resolved, the Y-axis scaling error in figure 1 has been corrected, chitosan characterization has been added, the use of GA₃ has been clarified, a helpful workflow diagram has been included, and the terminology has been revised from “synergistic” to “optimal combination”, all of which have significantly improved the clarity and consistency of the manuscript.

I would like to draw the authors' attention to two points from my previous report where the response letter describes a revision that I could not locate in the current text. It is possible these were simply missed during the final edit, so I mention them for the authors' convenience:

- Auxin mechanism (previous Comment 13). The Discussion still reads that “the reduced callus growth under light can be attributed to the reduced effectiveness of auxin.” I'd suggest either adding a supporting reference or slightly rewording this sentence to reflect a more precise physiological mechanism.
- B5 medium superiority (previous Comment 14). The Discussion still attributes B5's better performance generally to “the difference in the composition of the two media.” A brief note on which specific components (e.g., nitrogen source ratio, vitamins) likely drive this difference, with a supporting citation, would strengthen this section. I noticed another reviewer of this version raised a similar observation, so this may simply need one more pass.

I'd also appreciate confirmation that F-values and p-values were included in Tables 2–5 as described in the response letter, as this wasn't fully clear to me from the version I reviewed.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant germplasm conservation, plant molecular genetics, plant tissue culture, and cytogenetics.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 08 Jul 2026

MOHAMMED ABDULGAFOR

We sincerely thank the reviewer for the careful re-evaluation of our revised manuscript and for the positive assessment of the improvements made. We greatly appreciate your recognition that the taxonomic inconsistency, Figure 1 scaling, chitosan characterization, clarification of GA₃ application, inclusion of the workflow diagram, and the revised terminology have substantially improved the quality and clarity of the manuscript.

We also thank you for pointing out the remaining issues that were unintentionally

overlooked during the final revision.

Response to Comment 13 (Auxin mechanism):

We have revised the Discussion to provide a more accurate explanation of the physiological mechanism underlying the reduced callus growth under light conditions. The statement has been reworded to avoid oversimplification, and an appropriate supporting reference has been added to strengthen this interpretation.

Response to Comment 14 (B5 medium superiority):

We agree with the reviewer's suggestion. The Discussion has been expanded to explain that the superior performance of B5 medium is likely associated with differences in nutrient composition, particularly the nitrogen source ratio and vitamin formulation, compared with MS medium. Relevant supporting references have also been incorporated to support this explanation.

F-values and p-values in Tables 2–5:

Thank you for requesting clarification. We confirm that the F-values and corresponding p-values have now been clearly presented in Tables 2–5 as indicated in our response letter. We carefully reviewed the final version to ensure that these statistical values are included and correctly displayed.

We sincerely appreciate your constructive comments and careful review. Your valuable suggestions have helped us further improve the scientific quality, clarity, and accuracy of the manuscript.

Competing Interests: The authors declare that they have no competing interests.

Author Response 08 Jul 2026

MOHAMMED ABDULGAFOR

We sincerely thank the reviewer for the careful re-evaluation of our revised manuscript and for the positive assessment of the improvements made. We greatly appreciate your recognition that the taxonomic inconsistency, Figure 1 scaling, chitosan characterization, clarification of GA₃ application, inclusion of the workflow diagram, and the revised terminology have substantially improved the quality and clarity of the manuscript.

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We sincerely appreciate your constructive comments and careful review. Your valuable suggestions have helped us further improve the scientific quality, clarity, and accuracy of the manuscript.

Competing Interests: The authors declare that they have no competing interests

Reviewer Report 01 July 2026

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Huda Enaya Mahood

University of Al-Qadisiyah, Al Diwaniyah, Iraq

The manuscript addresses an interesting topic with potential value for plant tissue culture research. However, several sections require substantial revision.

1. The introduction does not fully match the study title. The role of physical stimuli is not sufficiently discussed, despite being a key component of the research. Please include a brief background on physical stimuli and clearly align the study objective with the title.
2. The Materials and Methods section is generally well-organized, but some scientific and linguistic points need correction.

The details of the culture medium are lengthy and unnecessary (such as transferring the solution to the graduated cylinder and boiling at 100°C). These could be shortened, and the focus could be on the essential information.

Sterilization: The phrase "according to the method used by the researcher" is inappropriate. It would be better to state that it was performed according to a published protocol or to describe the method directly.

The experimental design is good, but the number of replicates per experiment and the number of plant parts (explants) used per replicate should be specified.

Results: This section is the weakest part of the manuscript, not because the results are bad, but because the way they are presented and discussed needs significant improvement.

Discussion

The numerical results are repeated; the discussion should focus on interpretation rather than mere presentation.

The discussion of the effect of darkness (physical stimulus) is too brief, even though the title focuses on physical stimuli. It should be expanded and linked to previous studies.

The reason for the superiority of B5 over MS was not adequately discussed; the authors simply stated the general phrase "due to the difference in composition." It would be better to clarify which components of B5 might be responsible for this and cite references.

(The reason for the reduced callus growth under light can be attributed to the reduced effectiveness of auxin). This statement needs a reference or rewording.

The conclusion is clear, but it mainly summarizes the results. Please strengthen it by highlighting the main significance of the study and ensuring that it reflects all aspects of the work, including the effect of the physical and biological stimuli.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant Biotechnology

I confirm that I have read this submission and believe that I have an appropriate level of

expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 05 Jul 2026

MOHAMMED ABDULGAFOR

Response to Reviewer

We sincerely thank the reviewer for the careful evaluation of our manuscript and for the constructive comments, which have significantly improved its scientific quality. All comments were carefully considered, and the manuscript has been revised accordingly. Our point-by-point responses are presented below.

Comment 1: *The introduction does not fully match the study title. The role of physical stimuli is not sufficiently discussed, despite being a key component of the research. Please include a brief background on physical stimuli and clearly align the study objective with the title.*

Response: Thank you for this valuable suggestion. The Introduction has been revised to include a dedicated background describing the role of physical stimuli, particularly light and darkness, in regulating callus induction and growth through their influence on endogenous hormones, cellular dedifferentiation, and morphogenesis. In addition, the study objective has been rewritten to better reflect the title by emphasizing the integrated evaluation of hormonal, physical, and biological stimuli.

Comment 2: *The details of the culture medium are lengthy and unnecessary.*

Response: We agree with the reviewer. The description of culture medium preparation has been shortened by removing unnecessary procedural details while retaining all essential information required for reproducibility.

Comment 3: *Sterilization: The phrase "according to the method used by the researcher" is inappropriate.*

Response: Thank you for pointing this out. The sterilization procedure has been revised by removing this phrase and replacing it with a clear description of the protocol used, together with the appropriate reference.

Comment 4: *The number of replicates and explants per replicate should be specified.*

Response: This information has now been added to the Materials and Methods section. The number of biological replicates and the number of explants used in each replicate are clearly stated.

Comment 5: *The Results section is weak because of the way the results are presented and discussed.*

Response: We appreciate this important observation. The Results and Discussion have been extensively revised to improve clarity, organization, and scientific interpretation. The discussion now emphasizes the biological significance of the findings rather than simply describing the numerical results.

Comment 6: *The numerical results are repeated; the discussion should focus on interpretation.*

Response: We agree. Repetitive descriptions of numerical values have been removed, and the Discussion has been rewritten to focus on interpreting the results in relation to previous studies and the underlying physiological mechanisms.

Comment 7: *The discussion of the effect of darkness is too brief.*

Response: Thank you for this suggestion. The discussion concerning the influence of darkness has been substantially expanded. It now explains the physiological basis of darkness-induced callus formation, including its effects on auxin stability, cellular

dedifferentiation, and morphogenic responses, and is supported by additional relevant literature.

Comment 8: *The superiority of B5 over MS was not adequately discussed.*

Response: We appreciate this comment. The Discussion has been expanded to explain the likely reasons for the superior performance of Gamborg's B5 medium, including differences in nitrogen sources, nitrate/ammonium balance, vitamin composition, and their influence on cellular metabolism and biomass accumulation. Appropriate references have also been added.

Comment 9: *The statement regarding reduced auxin effectiveness under light requires a reference or rewording.*

Response: Thank you for identifying this issue. The statement has been revised and is now supported with appropriate references describing the influence of light on auxin metabolism and signaling.

Comment 10: *The conclusion mainly summarizes the results.*

Response: We agree with the reviewer. The Conclusion has been rewritten to emphasize the broader scientific significance of the study, highlighting the importance of integrating hormonal, physical, nutritional, and biological stimuli for optimizing callus production in *Lavandula angustifolia* and its potential applications in plant biotechnology and secondary metabolite production.

Once again, we sincerely thank the reviewer for the insightful comments and valuable suggestions, which have substantially improved the quality, clarity, and scientific rigor of the manuscript.

Competing Interests: The authors declare that they have no competing interests

Reviewer Report 29 June 2026

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Duha Mysire Majeed 

Plant Biotechnology, Al-Nahrain University / Biotechnology Research center, Baghdad, Iraq

The authors have satisfactorily addressed the concerns raised in my previous review. The manuscript has been improved in terms of methodology description, statistical analysis, discussion, and overall presentation. I consider the revised version suitable for indexing.

Competing Interests: I have previously collaborated with one of the authors, Mohammed A. Mohammed, as a co-author on a published research article in 2020. This collaboration was limited to that publication and there is no ongoing collaboration related to the current manuscript. I have had no direct contact with the authors regarding this article, and the manuscript was not

discussed with them. I confirm that I am able to assess this work impartially.

Reviewer Expertise: Plant Biotechnology, Plant Tissue Culture, Molecular Biology, Plant Genetic Engineering

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 08 May 2026

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Tassanai Jaruwattanaphan 

Kasetsart University, Bangkok, Thailand

This manuscript investigates the effects of 2,4-D, BA, light conditions, culture medium (MS versus B5), and chitosan on callus induction and growth in lavender. The main topic of the study is relevant to the field of plant biotechnology, and the presented results, especially positive effects of 1 mg L⁻¹ 2,4-D in dark conditions, along with 2 mg L⁻¹ BA, B5 medium, and 100 mg L⁻¹ chitosan on callus production, are considered acceptable. However, the manuscript needs major revision to be considered scientifically accurate and acceptable for indexing.

Is the study design appropriate and is the work technically sound? **Partly**

- The three experimental stages are not clearly related as the settings adjusted in phase 1 were not explicitly taken to phases 2 and 3. Thus, it is impossible to evaluate the alleged synergistic effects. The explant description is also not detailed enough, as key characteristics such as leaf age, location and size are not included.
- Furthermore, the callus development experiment lacks a zero-auxin control and the chitosan experiment does not state the plant growth regulators in the basal media.
- The manuscript refers to *Lavandula angustifolia* Mill. in the Introduction but uses *Lavandula* spp. in the Results. This inconsistency is taxonomically unacceptable and must be corrected throughout.
- The authors could define sequential experimental design and possibly include a basic workflow diagram illustrating how each phase was related.

Are sufficient details of the methods and analysis provided to allow replication by others? **Partly**

- Methodological information is partially adequate for reproduction. Basic culture conditions are presented; however, numerous important aspects are omitted.
- Inadequate description of explant preparation (leaf age, location, size and developmental stage are not provided)
- There is no characterization of chitosan; degree of deacetylation (DD%) and molecular

weight should be given since they have a substantial influence on biological activity.

- While the sterilizing approach (3% NaOCl for 15 min) may be too severe, there is no information on contamination or explant survival rates. No mention of strategies to reduce oxidative browning either.
- The use of GA₃ in germination media is not supported or referred to; however, it may affect explant physiology and subsequent induction of callus.
- The physiological condition and origin of the 150 mg callus inoculum employed in later phases, such as treatment source, callus age, and subculture history, are unknown.

Is the statistical analysis and its interpretation appropriate? **Partly**

- The statistical approach (CRD analyzed by ANOVA with LSD at $p \leq 0.05$) is generally appropriate, and the use of ten replicates per treatment is commendable.
- However, ANOVA outputs are not reported. Tables include only treatment means and LSD_{0.05} values, without F-values or p-values for main effects and interactions.
- Pairwise comparison letters are absent from all tables, making treatment differences difficult to interpret.
- Figure 1 contains a major scaling error, as the Y-axis exceeds 100% despite representing callus formation percentage.
- Error bars are not defined (SD or SE), and the large variation observed in some treatments is not discussed.

Are the conclusions drawn adequately supported by the results? **Partly**

- Overall, indicated trends are generally consistent with provided data; however, several findings are misleading or inadequately supported.
- The notion of “synergistic efficiency” has no justification. Most factors were tested in isolation in the studies, and no real cross-factor interaction analysis was performed.
- Physiologically, the concept of auxin action is incorrect. Auxin is mostly related to cell division and dedifferentiation in callus induction, not to decreased cell elongation.
- The reason given for the advantage of B5 medium is too superficial and should mention precise compositional differences between B5 and MS media.
- The introduction highlights the synthesis of secondary metabolites, but no metabolite analysis was done; hence, the data do not support these assertions.
- Only biomass measurements were taken to determine callus quality, and essential features such as texture, color, viability, and oxidative browning were not investigated.

Comments on Figures

- Figure 1 contains a critical Y-axis error (scale to 200%) that must be corrected. Error bar definitions must be stated, and the source of high variability acknowledged.
- Figure 2 is of insufficient quality for publication. The image is low resolution, includes distracting background elements, does not specify which 2,4-D treatment is shown, and lacks a scale bar.
- Figure 3 does not specify whether images are from MS or B5 medium.
- No photographic documentation is provided for the 2,4-D + BA experiment.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant germplasm conservation, plant molecular genetics, plant tissue culture, and cytogenetics.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 21 May 2026

MOHAMMED ABDULGAFOR

Response to the Reviewer

Authors' Response to the First Reviewer's Report (Tassanai Jaruwattanaphan)

We would like to express our sincere appreciation to the esteemed reviewer for the valuable time devoted to the careful evaluation of our manuscript, as well as for the constructive and insightful comments provided. The reviewer's detailed observations have been highly valuable in improving the scientific quality, clarity, and overall rigor of the manuscript. In response to the reviewer's suggestions and recommendations, we have thoroughly revised the manuscript and carefully addressed all comments raised. Below, we provide a detailed point-by-point response outlining the revisions made accordingly.

First: Study Design and Technical Soundness

Comment 1: *The relationship between the three experimental phases was unclear, making it difficult to assess the synergistic effect.*

Response:

We sincerely appreciate the reviewer's insightful comment regarding the clarity of the experimental design. In response, we have revised the Materials and Methods section by incorporating a new subsection entitled Sequential Experimental Design to better clarify the relationship among the three experimental phases and the rationale underlying their progression.

Specifically, the study was structured in a hierarchically linked and sequential manner.

Phase 1 aimed to identify the optimal concentration of **2,4-D** under light conditions. Based on the outcomes of this phase, the most effective concentration (**1 mg/L 2,4-D**) was selected and subsequently fixed for the following experiments conducted under dark conditions.

This sequential approach was intentionally designed to ensure experimental consistency and to facilitate a clearer evaluation of the cumulative and synergistic effects among the study variables.

In **second stage**, the induced callus obtained from explants cultured on Murashige and Skoog (MS) medium supplemented with 1 mg/L 2,4-D under dark conditions was subsequently transferred to media containing different combinations of auxin and cytokinin (BA) to determine the optimal hormonal combination for callus growth and maintenance.

In **third stage**, the callus induced under the previously optimized hormonal conditions (**1 mg/L 2,4-D + 2 mg/L BA**) was transferred to experimental media in which this hormonal combination was maintained as a fixed parameter. This phase was specifically designed to evaluate and compare the effects of different nutrient media and varying concentrations of **chitosan** added to the culture medium.

To further improve the clarity of the experimental workflow and the interrelationship among the study phases, we have incorporated a workflow diagram (Figure 1) into the revised manuscript. This newly added figure visually illustrates the sequential progression of the experimental stages and the transitions between them, thereby facilitating a clearer understanding of the integrated and synergistic evaluation of the investigated factors

Comment 2: *The description of the leaflet is not sufficiently detailed (leaf age, location, size).*

Response:

We sincerely thank the reviewer for this valuable and insightful observation. In response, we have substantially expanded the description of the explant source in the Materials and Methods section to provide greater experimental precision and reproducibility.

Comment 3: *The absence of an auxin-free control treatment in the callus development experiment.*

Response:

We sincerely thank the reviewer for this important observation. The primary objective of the experiment was to evaluate callus proliferation and maintenance, rather than callus induction. During the preliminary callus induction phase (Phase 1), we observed that explants cultured on auxin-free medium failed to initiate callus formation, indicating that auxin supplementation was essential for successful callus induction in this species. Furthermore, an auxin-free medium, combined with the addition of cytokinin, leads to the formation of vegetative shoots; for this reason, the (0 auxin) treatment was excluded from the study.

Comment 4: *The growth regulators used in the base media for the chitosan experiment were not specified.*

Response:

We appreciate the reviewer for highlighting this point. In response, we have revised the **Materials and Methods** section to explicitly specify the plant growth regulator composition used during the chitosan experiment.

Comment 5: *The taxonomic discrepancy between **Lavandula angustifolia** in the Introduction and **Lavandula spp.** in the Results.*

Response:

We sincerely thank the reviewer for identifying this inconsistency. We fully acknowledge that maintaining taxonomic accuracy and consistency throughout the manuscript is essential. In response, the scientific nomenclature has been carefully reviewed and standardized across all sections of the manuscript. The species name has been consistently corrected to *Lavandula angustifolia* **Mill.**, which is the actual species utilized in all

experimental procedures.

Second: Methods and Replication Details

Comment 6: *The characterization of chitosan is missing (degree of deacetylation and molecular weight).*

Response:

We sincerely appreciate the reviewer for highlighting this important methodological detail. In response, the physicochemical characteristics of the chitosan used in the study have now been explicitly included in the Materials and Methods section to enhance methodological transparency and reproducibility.

Comment 7: *The sterilization protocol was harsh (3% NaOCl for 15 min), and no information was provided regarding contamination or tissue browning.*

Response:

We sincerely thank the reviewer for this important observation. We acknowledge that the sterilization conditions may appear relatively stringent; however, the selected protocol was established based on a series of preliminary optimization experiments as well as previously published studies involving similar plant materials and tissue culture conditions.

Comment 8: *The use of GA3 in the germination medium and its potential residual effect on the explants was not addressed.*

Response:

We appreciate the reviewer for raising this valuable point. We would like to clarify that **GA₃ (0.5 mg/L)** was used exclusively during the seed germination stage to promote dormancy breaking and enhance germination of sterilized seeds. Importantly, GA3 was not included in any subsequent tissue culture media used for callus induction or proliferation experiments.

Third: Statistical Analysis and Interpretation

Comment 10: *The ANOVA tables do not include F-values and p-values, and no multiple comparison letters are presented.*

Response:

We sincerely thank the reviewer for this valuable observation, which has significantly contributed to improving the statistical clarity of the manuscript. In response, all relevant tables (**Tables 2–5**) have been comprehensively revised to include the calculated **F-values** and corresponding **p-values** obtained from the analysis of variance (**ANOVA**) for both the main effects and interaction effects.

Additionally, to facilitate interpretation and enable clearer identification of statistically significant differences among treatment means, multiple comparison letters (a, b, c, etc.) have been incorporated based on the Least Significant Difference (**LSD**) test at a significance level of **p ≤ 0.05**. These annotations have been added adjacent to the reported means to improve readability and statistical transparency.

Comment 11: *There is a major error in the Y-axis scale of Figure 1 (exceeding 100%), the error bars are not defined, and the high variability is not discussed.*

Response:

Thank you for this important observation. Figure 1 has been revised to correct the Y-axis scaling so that the callus formation percentage does not exceed 100%. In addition, the type of error bars has now been clearly defined in the figure legend as standard error (SEM).

Fourth: Supporting the Results and Conclusions

Comment 12: *There is no justification for the term “synergistic efficiency” because the factors were tested in isolation and without a true interaction analysis.*

Response:

We sincerely thank the reviewer for this insightful and scientifically important observation. We fully acknowledge that the use of the term “synergistic efficiency” was not sufficiently justified within the framework of the experimental design, as the investigated factors were evaluated in a sequential manner rather than through a full factorial interaction analysis. In response, we have carefully revised the terminology throughout the **title, main text, and conclusions** to ensure greater conceptual precision and alignment with the actual study design. Accordingly, the revised title now reads:

*“Evaluating the Efficiency of an Optimal Combination of Hormonal, Biological, and Physical Stimuli in Inducing and Promoting Callus Formation in **Lavandula angustifolia** Mill.”*

Comment 13: *The physiological concept of auxin action is incorrect.*

Response:

We sincerely appreciate the reviewer for identifying this conceptual inaccuracy. In response, the relevant statements in both the **Introduction** and **Discussion** sections have been revised to reflect a more accurate physiological interpretation of auxin function in plant tissue culture.

Comment 14: *The justification for the superiority of B5 medium is superficial.*

Response:

We sincerely thank the reviewer for this valuable observation, which enabled us to substantially strengthen the interpretation of our findings. In response, the **Discussion** section has been expanded to provide a more comprehensive physiological and nutritional justification for the superior performance of **B5 medium** relative to **MS medium** in supporting callus growth.

Comment 15: *The claim regarding secondary metabolite production is not supported by experimental analysis.*

Response:

We sincerely thank the reviewer for this important observation and fully acknowledge that the original wording may have unintentionally overstated the scope of the present findings. Since no direct biochemical or metabolomic analyses were conducted to evaluate secondary metabolite production, we agree that such claims should not be presented as experimentally validated outcomes of this study.

Comment 16: *Callus quality assessment was limited to weight measurements only.*

Response:

We sincerely appreciate the reviewer for this valuable suggestion, which has strengthened the evaluation of callus performance in the revised manuscript. In response, we have expanded the assessment of callus quality beyond biomass measurements by incorporating a **supplementary qualitative evaluation** of callus morphology under the most effective treatment conditions.

Fifth: Figures and Image Quality

Comment 17: *Figure 1 (Y-axis scale, error bars).*

Response:

This issue has been fully addressed as described in **Comment 11**. Briefly, the **Y-axis scale** has been corrected to range from **0–100%**, and the **error bars** have been explicitly identified in the figure legend as representing the **standard error of the mean ($\pm$ SE)**.

Comment 18: *Figure 2 is of low quality, has a blurred background, does not specify the treatment, and lacks a scale bar.*

Response:

We sincerely thank the reviewer for this important observation. In response, **Figure 2** has

been completely replaced with a **high-resolution image (300 DPI)** to improve visual clarity and presentation quality. The revised image was prepared using a **uniform background** to minimize visual interference and enhance specimen visibility

Comment 19: *Figure 3 does not specify whether the images correspond to MS or B5 medium.*

Response:

We appreciate the reviewer for highlighting this lack of clarity. In response, the revised **Figure 3** has been updated to include **direct labels within each image panel**, positioned in the upper-right corner to clearly indicate the treatment condition (e.g., "**B5 + Chitosan 100 mg/L**").

Comment 20: *There is no photographic documentation of the 2,4-D + BA experiment.*

Response:

We sincerely thank the reviewer for this valuable suggestion. In response, we have incorporated a **new** figure (Figure 4) into the revised manuscript, presenting representative photographic documentation of the optimized hormonal combination (1 mg/L 2,4-D + 2 mg/L BA).

This figure illustrates the morphological characteristics and quality of the resulting callus, including the **vigorous, sustainable, and morphologically uniform callus structure** that was subsequently selected for use in later experimental stages

We sincerely hope that the revisions, clarifications, and additional analyses incorporated into the revised manuscript have adequately addressed all of the reviewer's insightful comments and concerns. We are deeply grateful for the reviewer's constructive feedback, which has significantly contributed to improving the scientific rigor, clarity, and overall quality of the manuscript.

We trust that the revised version now meets the required scientific and publication standards, and we remain fully committed to implementing any further revisions or recommendations that may be suggested.

**Sincerely,
The Authors**

Competing Interests: Thank you for your kind reminder. I confirm that I have no conflict of interest.

Reviewer Report 27 April 2026

<https://doi.org/10.5256/f1000research.191471.r476656>

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Duha Mysire Majeed

Plant Biotechnology, Al-Nahrain University / Biotechnology Research center, Baghdad, Iraq

This study investigates the combined effects of hormonal, environmental, and biological factors on callus induction and growth in lavender. The topic is relevant and of interest in plant tissue

culture; however, several aspects require improvement to enhance the scientific quality of the manuscript.

Major comments:

1. The manuscript requires substantial language editing to improve clarity and scientific writing style. Several sentences are repetitive and lack precision.
2. The novelty of the study is limited, as it mainly focuses on optimization using well-established factors such as 2,4-D, BA, nutrient media, and chitosan. The authors should better highlight the originality of their contribution.
3. The experimental design is not sufficiently detailed. The authors should clearly describe the explant type, treatment structure, and factorial design to ensure reproducibility.
4. The statistical analysis needs to be strengthened. The manuscript lacks detailed ANOVA results, p-values, and a clear explanation of statistical significance.
5. The discussion section should be improved by providing deeper interpretation of the results and better comparison with recent literature.

Minor comments:

- Ensure consistency in units and formatting (e.g., mg L⁻¹).
- Improve figure quality and labeling.
- Correct grammatical and typographical errors.

Conclusion:

The conclusions are generally supported by the results but are somewhat overstated and should be moderated.

Recommendation: Major Revision**Is the work clearly and accurately presented and does it cite the current literature?**

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: I have previously collaborated with one of the authors, Mohammed A. Mohammed, as a co-author on a published research article in 2020. This collaboration was limited to that publication and there is no ongoing collaboration related to the current manuscript. I have

had no direct contact with the authors regarding this article, and the manuscript was not discussed with them. I confirm that I am able to assess this work impartially.

Reviewer Expertise: Plant Biotechnology, Plant Tissue Culture, Molecular Biology, Plant Genetic Engineering

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 30 Apr 2026

MOHAMMED ABDULGAFOR

Response to Reviewer Comments

We sincerely thank the reviewer for their careful evaluation of our manuscript and for providing constructive and insightful comments that have helped us improve the quality of the work.

Comment 1: Language editing

- We appreciate this important remark. The manuscript has undergone professional language editing prior to submission; however, we acknowledge that some sections may still require further refinement. Accordingly, we have carefully revised the text to improve clarity, reduce repetition, and enhance the overall scientific style.

Comment 2: Limited scientific novelty

- We thank the reviewer for this valuable observation. While the study employs established factors such as 2,4-D, BA, nutrient media, and chitosan, its novelty lies in the integrated evaluation of multiple interacting variables within a single experimental framework. Specifically, the originality of this study is reflected in: The combined assessment of hormonal, environmental (light/dark), and biological (chitosan) factors and their interactive effects on callus induction and growth in lavender. Demonstrating that plant responses to identical growth regulator concentrations vary depending on tissue type and treatment combinations, which may explain inconsistencies reported in previous studies. We have revised the Introduction and Discussion sections to more clearly highlight these contributions.

Comment 3: Experimental design details

- We appreciate the reviewer's concern regarding reproducibility. The manuscript has been revised to provide clearer and more detailed descriptions of the experimental design. Specifically: The explant type (leaf tissue) is now explicitly stated. The treatment structure has been clarified, including: Four concentrations of 2,4-D (0, 1, 2, 3 mg L⁻¹) under two light conditions. A factorial combination of 2,4-D (1, 2, 3 mg L⁻¹) and BA (0, 1, 2 mg L⁻¹). Additional clarification has been added regarding replication and experimental setup. We believe these revisions sufficiently improve reproducibility.

Comment 4: Statistical analysis

- We thank the reviewer for highlighting this point. The statistical methodology has been clarified in the revised manuscript, including: The use of a Completely Randomised Design (CRD) with ten replicates. A factorial arrangement of treatments. Mean separation using the LSD test at $P \leq 0.05$. P-values have been included within the results where appropriate. To maintain manuscript conciseness, full ANOVA tables were not included in the main text; however, we are willing to provide them as supplementary material upon request.

Comment 5: Discussion of the results

- We appreciate this constructive suggestion. The Discussion section has been revised to provide a deeper interpretation of the findings and stronger linkage to existing literature. Due to the limited number of studies specifically addressing lavender callus induction under similar combined conditions, we have incorporated relevant and recent studies from related species within the same family (Lamiaceae), while clearly acknowledging this limitation. This also underscores the importance of our study in addressing a gap in the literature. Additional minor comments All minor issues raised by the reviewer have been addressed: Units and formatting (e.g., mg L^{-1}) have been standardised. Figures have been improved in clarity and labeling. Grammatical and typographical errors have been corrected. Conclusion We have revised the conclusion to ensure it accurately reflects the results without overstatement, in line with the reviewer's recommendation.

Competing Interests: No competing interests were disclosed.

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