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Effects of different concentrations of growth regulators on the *in vitro* callusogenesis of *Lavandula angustifolia* Mill

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Abstract
Lavandula angustifolia Mill. is an important ornamental and medicinal plant, widely valued for its essential oil and horticultural applications. Efficient *in vitro* propagation requires the optimisation of explant type and plant growth regulator combinations to ensure reliable callus induction. This study aimed to evaluate the callogenic potential of leaf and stem explants of *L. angustifolia* under different auxin–cytokinin regimes. Explants were cultured on Murashige and Skoog medium supplemented with various concentrations and combinations of 6-benzylaminopurine (BAP), α-naphthaleneacetic acid (NAA), and kinetin. Callus induction frequency was assessed after four weeks of cultivation. Leaf explants exhibited a higher callus induction frequency, reaching up to 100%, indicating superior regenerative responsiveness, whereas stem explants produced a significantly greater callus biomass. The most effective hormonal combination for callus formation was 1.5 mg l^{-1} BAP combined with 1.0 mg l^{-1} NAA. The results demonstrate that both explant type and growth regulator composition critically influence callusogenesis in *L. angustifolia*, providing a practical basis for improving *in vitro* propagation protocols for horticultural production and further biotechnological applications.

Keywords: *Lavandula angustifolia*, callus induction, plant growth regulators, BAP, NAA, *in vitro* culture

Introduction

Plants with various medicinal properties are widely cultivated in Eastern Europe. One of the most important is lavender, which has aromatic properties and is important in medicine [Brailko *et al*. 2017] [1]. Lavender is an aromatic plant, a member of the Noterlina family. Plants in this family accumulate large quantities of essential oils. The plant in question is perennial, ornamental and aromatic. The stems of this plant reach about 60 cm and are steep and branched. The leaves are linear, greyish-green in colour, narrow, with narrow, overlapping margins. The inflorescence is bell-shaped and has 3-8 flowers per panicle. It is mainly grown in open fields with acid-free and well-drained soil. The main reason for its mass cultivation is the essential oil it contains [Najar *et al*. 2019] [7].

Given that Lithuania is located in a temperate climate zone, most lavenders cannot grow in such harsh conditions, but true lavender (*Lavandula anustifolia* Mill) and Italian lavender (*Lavandula stoechas* L) are some of the few that can be cultivated in our country.

Lavender can be propagated by seed and vegetatively. Propagating by seed may save money, but in this way the offspring will, with each generation, move away from the qualities of the parent plant. Vegetative propagation is the most efficient way to propagate lavender, but propagating these plants outdoors will not prevent diseases and pests. Modern biotechnology allows us to ensure high quality, infection-free and identical offspring to the mother plant by *in vitro* micropropagation [Brailko *et al*. 2017] [1].

Plants propagated *in vitro* are characterised by their phytosanitary quality, extent of rejuvenation, vigour and uniformity [Kimura *et al*. 2023] [4]. Totipotency is the property of somatic plant cells to express genetic information required for the development of individual plant organs or the whole organism [Ezhova 2003] [3]. The main advantages of *in vitro* propagation are that we have the possibility to regulate the relative humidity and temperature of the air and to use media with specific growth regulators, which allows us to produce a high-quality and identical progeny of the mother plant. Such *in vitro* propagation can be carried out on common herbaceous plants as well as on those with a perennial stem. By conducting *in vitro* studies on plants or their cells, we can ensure that the test subject, whose cells have a nucleus or are alive, will be able to initiate dedifferentiation (callus formation) and begin to form organogenic tissues [Ezhova 2003] [3]. *In vitro* propagation of plants can produce high quality clones with the characteristics of the mother plant, but we must also be aware that this method is not cheap, but the end result is a more productive and higher quality production that will repay the investment [Chaimae *et al*. 2020] [2].

Phytohormones are needed to control *in vitro* plant cells, tissues, organs, or entire plant crops according to the desired results: maintain vitality, grow, initiate organogenesis (the formation of organs, for example, the formation of a stem with leaves or roots), and so on. Without phytohormones, plant growth could not be controlled [Vivanco and Flores 2024; Kimura *et al*. 2023] [4, 9].

The aim of this study was to investigate the callogenic potential of various *L. angustifolia* explants and evaluate the effects of different growth regulator concentrations on *in vitro* morphogenesis**.**

Materials and Methods

The experiment was prepared and carried out at the Vytautas Magnus University, Faculty of Agronomy, Institute of Biology and Plant Biotechnology, Agrobiotechnology Laboratory. Research period 2021-2022. The subject of the study was the work carried out on true lavender (*Lavandula angustifolia* Mill.); as well as growth

regulators NAA (1-Naphthaleneacetic acid) BAP (6 – Benzylaminopurine) and kinetin (6- Furfurylaminopurin).

Type of explant: leaves (Fig. 1) and stem segments of the true lavender (*Lavandula angustifolia* Mill.) were used in the study.

Fig 1: Callus induction from leaf explants of *Lavandula angustifolia* Mill. under in vitro conditions (Agrobiotechnology Laboratory, Kaunas, Lithuania, 2021–2022)

Sterilisation of explants: cuttings from the donor plant are soaked in distilled water for 10 minutes. They were then immersed for a short time (20 seconds) in 70 % ethyl alcohol, after which the next step was a 1 % sodium hypochlorite solution containing 1 drop of polysorbate Tween- 20. The explants were soaked in this solution for 15 minutes. After these treatments, the plants were treated with laminare using autoclaved distilled water (3 times for 5 min each). After the sterilisation procedure for lavender explants, they are placed on nutrient medium [Murashige and Skoog 1962] [6], sealed in a Petri dish and placed in an isolation cabinet. In these cabinets, the plants are provided with optimal conditions and are not exposed to ex *vitro* factors such as heat, water shortage, frost, etc. In this room, the plants receive optimum light intensity (50 µmol m^{-2} s^{-1}), optimum photoperiodicity (16 hours day, 8 hours night), a temperature favourable for the most intensive growth and development (22 °C during the day and 18 °C at night), and a humidity of 75%.

For studies on selected plant parts (stem segments and leaves), we need to use MS [Murashige and Skoog 1962] [6] nutrient medium without growth regulators (control) and MS medium containing the growth regulators selected for the study (Table 1).

Table 1: Concentrations of growth regulators used in the test (NAA (1-Naphthaleneacetic acid) BAP (6 – Benzylaminopurine) and kinetin (6- Furfurylaminopurin)

Variants	Test 1	Test 2	Test 3	Test 4
0	-	-	-	-
1	0.5 mg l-1 BAP+ 0.5 mg l-1 NAA	0.2 mg l-1 BAP + 0.5 mg l-1 NAA	2.0 mg l-1 BAP + 0.5 mg l-1 NAA	0.5 mg l-1 BAP + 0.5 mg l-1 KIN +1.0 mg l-1 NAA
2	1.0 mg l-1 BAP+ 0.5 mg l-1 NAA	0.2 mg l-1 BAP + 1.0 mg l-1 NAA	2.0 mg l-1 BAP + 1.0 mg l-1 NAA	1.0 mg l-1 BAP + 0.5 mg l-1 KIN + 1.0 mg l-1 NAA
3	1.5 mg l-1 BAP+ 0.5 mg l-1 NAA	0.2 mg l-1 BAP + 1.5 mg l-1 NAA	2.0 mg l-1 BAP + 1.5 mg l-1 NAA	1.5 mg l-1 BAP + 0.5 mg l-1 KIN +1.0 mg l-1 NAA

Weighing of the mass of the callus was carried out in the course of the study. This was done every 28 days, weighing all variants with repetitions and averaging between repetitions. Statistics of Fig. 2-5 show means and their standard deviations (x ± SD). The reliability of differences between samplings was assessed by the dispersion analysis with further evaluation of the least significant difference (*LSD*) using the Statistica 5 software package.

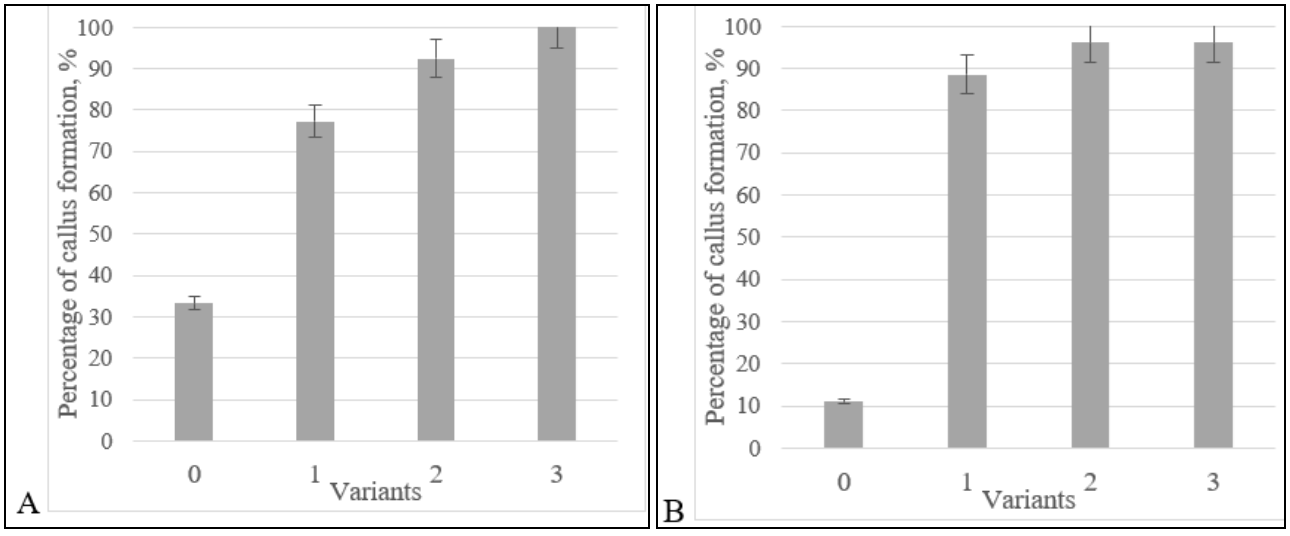

Fig 2: Effect of different concentrations of 6-benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA) on the frequency of callusogenesis in *Lavandula angustifolia* explants: (A) leaf explants ($LSD_{0.01}$ = 3.521); (B) stem explants ($LSD_{0.01}$ = 2.421). Treatments correspond to *Test 1* (Table 1).

Results and Discussion

In our studies with true lavender explants (leaves), we found that the best results were obtained when the nutrient medium was supplemented with 1.5 mg l^{-1} cytokinin BAP + 0.5 mg l^{-1} auxin NAA (Fig. 2, A). As the cytokinin level in the combination was gradually reduced, the incidence of callusogenesis also decreased. Significant differences only occurred when the cytokinin concentration was reduced to

0.5 mg l^{-1}, when the frequency of callus induction dropped to 77.3% with statistical significance. The second explant type chosen for the study was true lavender stem segments (Fig. 2, B) The lowest incidence of callusogenesis was 11.1%, which was statistically significant in the control (MS). When the nutrient medium was enriched with 1.5 mg l^{-1} cytokinin BAP and 0.5 mg l^{-1} auxin NAA, 96.2% callus formation was recorded. Auxins have many different functions in plants. They promote cell elongation and growth, partly also cell division, induce the formation of adventitious roots [Ngomuo *et al*. 2013] [8]. This group of growth regulators is also responsible for the formation of plant roots [Vivanco and Flores 2024] [9].

In a subsequent study, lavender leaves were cultured with constant concentrations of the cytokinin BAP and variable concentrations of auxin NAA. True lavender leaf explants formed the least amount of callus (33.3%) in MS medium without growth regulators (Fig. 3, A). In media supplemented with growth regulators, the frequency of callus formation varied depending on the changing concentration of auxin NAA in combination with a constant (0.2 mg l^{-1}) amount of cytokinin BAP. The best results (73.5%) were obtained when the nutrient medium was supplemented with the maximum concentration (1.5 mg l^{-1}) of auxin NAA in combination with 0.2 mg l^{-1} BAP. The next study was carried out on stem segments of true lavender. The explants of the tested lavender stem segments showed the lowest frequency of callosogenesis (11.1%) in the medium without growth regulators (control). Gradually increasing the auxin content in combination with the constant cytokinin BAP, the frequency of callusogenesis also tended to increase. The highest frequency was recorded (96.2%) with 0.2 mg l^{-1} cytokinin BAP in combination with 1.5 mg l^{-1} auxin (Fig. 3, B). Cytokinins induce the development of apical and adventitious buds, promote cell division, weaken apical dominance, participate in chloroplast formation, regulation of leaf growth, maintenance of leaf functional activity, and inhibition of senescence [Marković *et al*. 2023] [5].

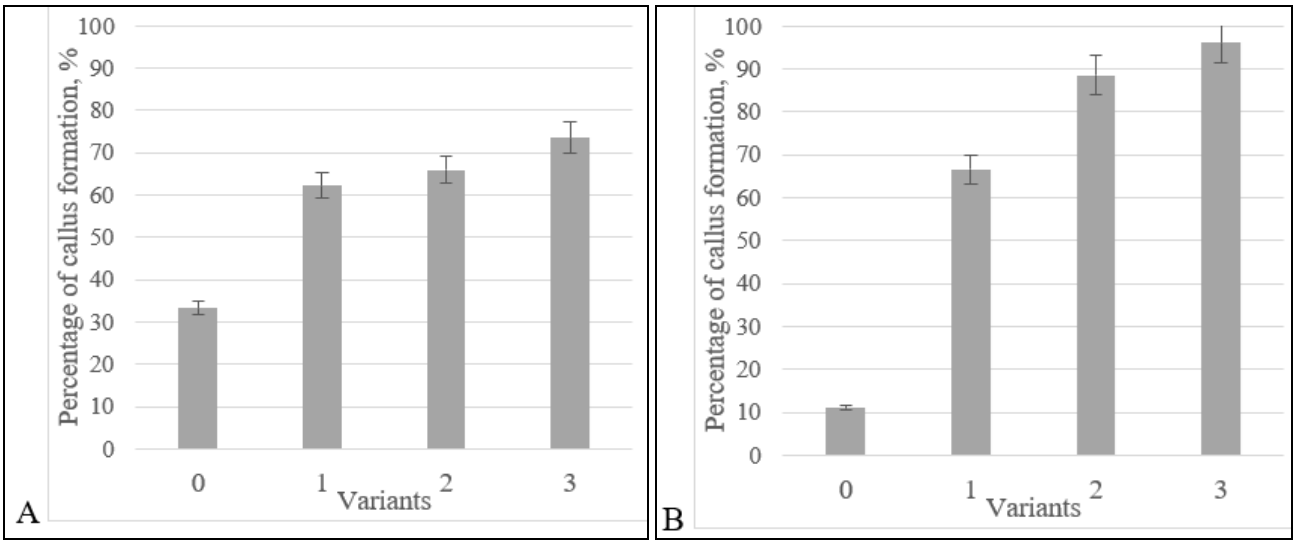

Fig 3: Effect of constant 6-benzylaminopurine (BAP) concentration combined with variable α-naphthaleneacetic acid (NAA) concentrations on the frequency of callusogenesis in *Lavandula angustifolia* explants: (A) leaf explants ($LSD_{0.01}$ = 2.321); (B) stem explants ($LSD_{0.01}$ = 2.521). Treatments correspond to *Test 2* (Table 1)

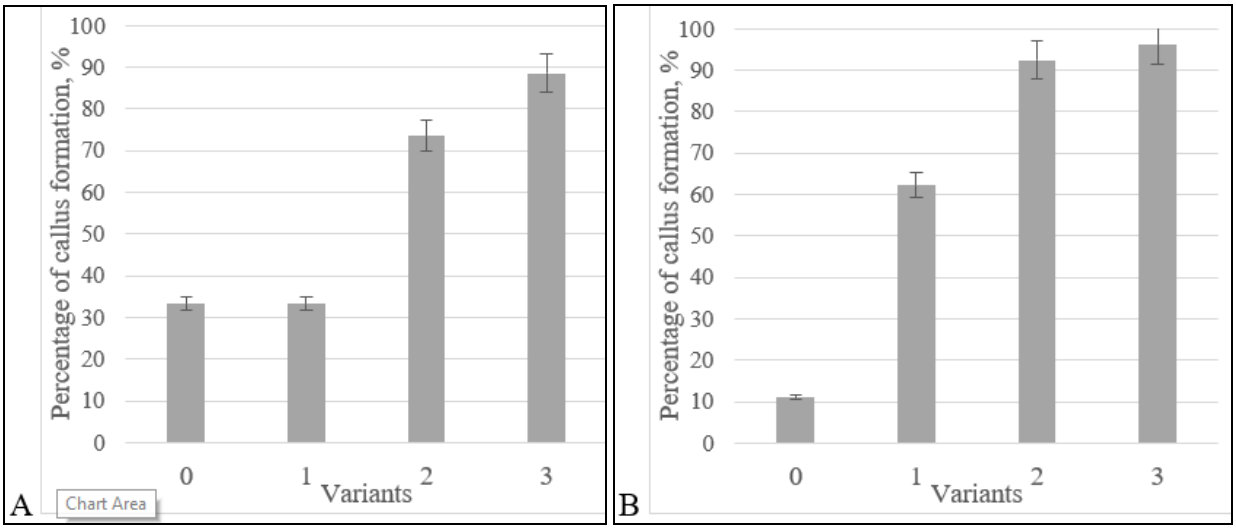

Fig 4: Effect of constant 6-benzylaminopurine (BAP) concentration combined with different α-naphthaleneacetic acid (NAA) concentrations on the frequency of callusogenesis in *Lavandula angustifolia* explants: (A) leaf explants ($LSD_{0.01}$ = 3.535); (B) stem explants ($LSD_{0.01}$ = 3.521). Treatments correspond to *Test 3* (Table 1)

The following studies, using lavender of different explant types, were carried out with a combination of growth regulators with constant concentrations of BAP and variable concentrations of NAA (Fig. 4, A). Lavender leaves formed the highest amount of callus (88.6%) in medium with 2 mg l^{-1} cytokinin BAP and 1.5 mg l^{-1} auxin NAA supplement. Gradually decreasing the auxin concentration resulted in a statistically significant decrease in the frequency of callus formation. In the tests on lavender stem segments (Fig. 4, B), the medium with the lowest level (0.5 mg l^{-1}) of auxin NAA had the lowest frequency of callusogenesis (62.3%). When the effect of auxin concentration on callus formation in stem segments was evaluated, it was found that the highest callus formation frequencies were observed when the cytokinin combination was supplemented with 1 mg l^{-1} and 1.5 mg l^{-1} auxin NAA. No significant differences were found when comparing these treatments.

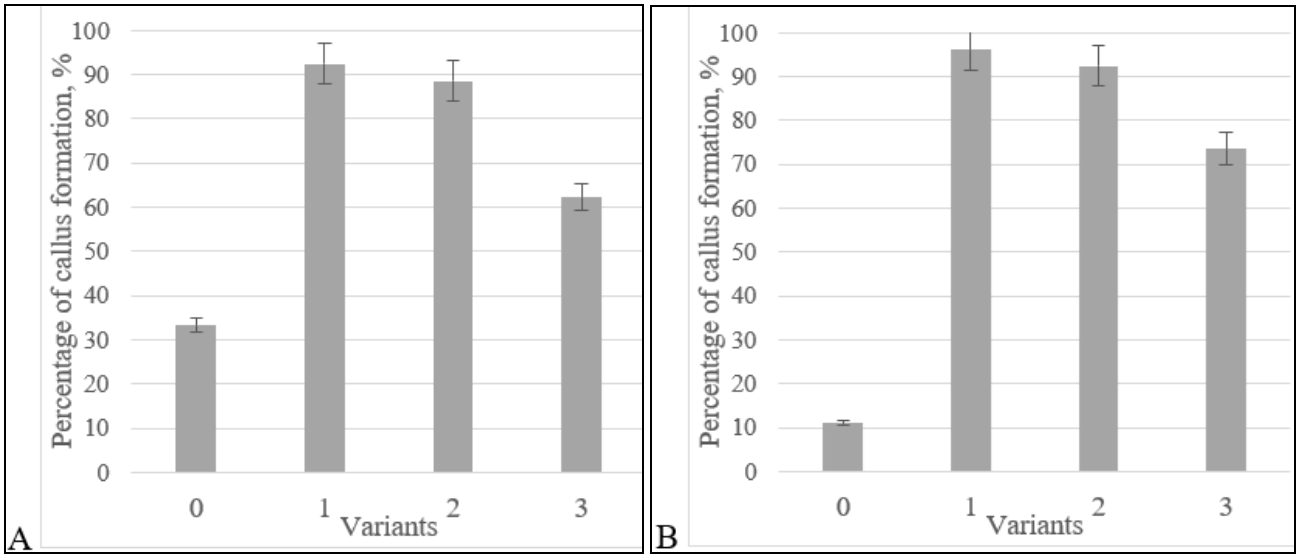

Fig 5: Effect of different concentrations of 6-benzylaminopurine (BAP) combined with kinetin (KIN) and α-naphthaleneacetic acid (NAA) on the frequency of callusogenesis in *Lavandula angustifolia* explants: (A) leaf explants ($LSD_{0.01}$ = 2.358); (B) stem explants ($LSD_{0.01}$ = 2.657). Treatments correspond to *Test 4* (Table 1).

For the latest studies on the frequency of callusogenesis, nutrient media supplemented with combinations of growth regulators with variable levels of the cytokinin BAP and constant concentrations of KIN and NAA were used. When the effect of cytokinin BAP concentration on the frequency of callus formation in lavender leaves was evaluated, it was found that the highest callus frequencies were observed in nutrient media with growth regulators containing 0.5 mg l^{-1} and 1 mg l^{-1} of cytokinin BAP in a combination of 0.5 mg l^{-1} KIN and 1 mg l^{-1} NAA. The nutrient medium supplemented with 1.5 mg l^{-1} cytokinin BAP had substantial differences and showed 31% less callus formation (Fig. 5, A). In the stem segment tests (Fig. 5, B), we observed that the media supplemented with 1 mg l^{-1} and 0.5 mg l^{-1} cytokinin BAP had the highest frequency of callus formation and no significant differences, but increasing the cytokinin BAP concentration to 1.5 mg l^{-1} in combination with 0.5 mg l^{-1} KIN and 1 mg l^{-1} NAR reduced the frequency of callus formation by an average of 12 %, which was statistically reliable. Cytokinins, namely 6-benzylamino purine (BAP) and kinetin (KIN), have the ability to reduce the dominance of apical meristems, which allows the induction of adventitious shoots from the meristematic explant under study [Kimura *et al*. 2023; Ngomuo *et al*. 2013] [4, 8].

It is hypothesised that the formation and development of leaves *in vitro* had a significant positive influence on the IAR and kinetin. It can be theorised that the amount of endogenous phytohormones was determined by the genotype of the plant and thus influenced the dependence of organogenesis on the content of exogenous hormones. Scientific literature indicates that the effect of one phytohormone on the metabolism of another is not direct, but rather a consequence of the functional effect on the activity of tissues.

Conclusions

The results of this study demonstrate that callus induction in *Lavandula angustifolia* Mill. is strongly influenced by both the type of explant and the composition of plant growth regulators in the culture medium. Leaf explants showed a higher callogenic responsiveness, achieving the highest callus induction frequencies, whereas stem explants were characterised by the formation of a larger callus biomass, indicating different morphogenetic potentials of the analysed tissues. The most effective hormonal treatment for callus formation was the combination of 1.5 mg l^{-1} BAP and 1.0 mg l^{-1} NAA, confirming the importance of a balanced auxin–cytokinin ratio for successful *in vitro* morphogenesis. Increasing auxin concentration in the presence of a constant cytokinin level generally promoted callus formation in both explant types, while higher cytokinin concentrations tended to reduce callogenic efficiency. The inclusion of kinetin in combination with BAP and NAA affected callus induction in a concentration-dependent manner, highlighting the need for precise optimisation of multi-hormonal systems. Overall, the findings provide a reliable experimental basis for the improvement of *in vitro* propagation protocols and further biotechnological applications of *L. angustifolia*.

Authorship contribution statement: R.M., N.B. and R.A. designed the experiments; R.M., N.B. and R.A. performed the experiments and analyzed the data, assisted by R.M.; R.M., R.A. and N.B. drafted the manuscript; R.A. revised the manuscript & editing and provided materials.

Data availability: All data generated or analyzed during this study are included in this published article (and its Supplementary information files).

Declaration of competing interest: The authors declare no conflict of interest

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