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Original Research Paper

In vitro culturing of hop (*Humulus lupulus* L.) genotypes from the Republic of Tatarstan

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Abstract: Common hop (*Humulus lupulus* L.) is a rich source of phytochemicals for treating various diseases. This necessitates an increase in the number of studies aimed at finding out the effect of different growing conditions on the growth and development of hop plants, adapted to the conditions of various geographical regions for its effective utilization in different spheres of life. Growing plants using *in vitro* cultivation techniques promotes their rapid growth for various purposes. The results of *in vitro* culturing of common hop from seeds collected in the Republic of Tatarstan, regeneration of plantlets from nodal explants, and callus induction from explants obtained from *in vitro* cultures are presented in this study. Despite the incision of the seed coats, a very low germination effect was observed with mechanical scarification of hop seeds. A relevant effect of the BAP to IAA ratio on the morphogenetic potential of nodal culture growth and of roots was revealed. Successful induction of callus from leaf explants of *in vitro* generated plantlets under a 16-hours lighting regime was also achieved using BAP, with IAA, NAA or 2,4-D. Thus, the culture growth dynamics of common hop, adapted to the territory of the Republic of Tatarstan, under *in vitro* conditions have been shown.

Keywords: common hop; seeds; nodal explants; callus; phytohormones; tissue culture.

1. Introduction

Common hop (*Humulus lupulus* L.), which belongs to the Cannabaceae family, is known in history as a crop used in brewing, being responsible for the characteristic beer aroma and bitterness (Bocquet et al., 2018). In addition to its traditional use in beer production, it has long been used for medicinal purposes (Dostálek et al., 2017; Zanoli and Zavatti, 2008). Hop is known for its rich content of amino acids, polyphenols, and volatile acids (Carbone and Gervasi, 2022; Kobus-Cisowska et al., 2019), making it a promising source for industrial use in the quest to obtain bioactive compounds of plant origin (Fadaka et al., 2021). Owing to its phytochemical profile, hop has antimicrobial, anti-carcinogenic, antioxidant, and anti-inflammatory properties making it useful for the treatment of some diseases (Abiko et al., 2022; Bocquet et al., 2018; Cermak et al., 2017; Di Sotto et al., 2018; Kontek et al., 2021).

Particular attention is drawn to the flavonoid xanthohumol, found mainly in hop, which is known for its potential importance in the treatment of tumor, microbial and viral diseases. Various studies focusing on the antitumor, anti-aging, and other therapeutic properties of xanthohumol have shown positive effects under both *in vitro* and *in vivo* conditions (Elrod, 2018; Neumann et al., 2022; Tejero et al., 2024; Zugravu et al., 2022).

It has been reported that hop seeds have a low germination rate (Liberatore et al., 2018; Suciu et al., 1977) due to the presence of resins in its seed coat, preventing it from imbibing water, which makes

sowing it with traditional methods challenging. To overcome the challenge of poor seed germination, the biotechnological approach of growing seeds on nutrient medium under *in vitro* conditions, coupled sometimes with seed pretreatment procedures, have been used to enhance germination and produce aseptic seedlings in some plant species (Liberatore et al., 2018; Souza et al., 2016; Thokozani et al., 2011).

Plant tissue culture methods make it possible to obtain large amounts of plant tissues and biomass in culture for further *in vitro* studies and also aseptic planting materials within a short time frame for medicinal, agricultural, and industrial purposes (Engelmann, 2011; Negash, 2014; Norouzi et al., 2022; Wawrosch and Zotchev, 2021). Also, callus cultures, consisting of a mass of unorganized plant tissue and cells, have gained popularity as an alternative source of plant material for starting suspension cultures, the production of biologically active molecules, genetic transformation, and plant breeding (Baldi et al., 2006; Efferth, 2019; Norouzi et al., 2022; Rout et al., 2011).

Over the past three decades, common hop cultivation and other research related activities, such as disease and pest management, breeding, the application of biotechnology methods to enhance cultivation, and the use of hop resources and capacity building for hop related research, have declined in the Russian Federation (Bychkova et al., 2024; Khlynovskiy et al., 2023). In 2021 domestic production in the entire Russian Federation amounted to about 2% of the industrial demand for beer production alone, making the brewing industry entirely dependent on hop imports (Khlynovskiy et al., 2023). However, the interest in hop cultivation in the Russian Federation has increased over the last few years, posing particular attention on how tissue culturing methods can be used to enhance the production of healthy planting materials, conserve their genetic resources, and also maximize the production of biologically active substances of hop under *in vitro* conditions (Bychkova et al., 2024; Chandran et al., 2020; Khlynovskiy et al., 2023).

The status of hop production in the Republic of Tatarstan does not deviate from the federal picture. Apart from a few plantations in the Republic, hop grows mainly as wild plants, serving as decorative plants and for the use of its decoction in folk medicine as a pain reliever and sedative (Encyclopedia Tatarica, accessed online on 28 May, 2025).

Research studies on *in vitro* hop culturing have focused on micropropagation, conservation of their genetic resources, improvement of the crop, its genetic stability, and eradication of viruses (Bychkova et al., 2024; Clapa and Hârta, 2021; Farago et al., 2009; Gashenko et al., 2019; Kastritskaya et al., 2014; Khlebova et al., 2021; Machado et al., 2018; Mafakheri and Hamidoghli, 2019; Reed et al., 2003). The studies on hop *in vitro* cultivation highlighted differences in plant growth induced by the culture media composition and genotypes.

In this regard, it has become necessary to obtain specific information on the *in vitro* culturing and callus development pattern of wild hop, adapted to the conditions of the Republic of Tatarstan. Such information will be useful for the establishment of hop cultures *in vitro*, aimed at uncovering their biochemical potential, and for creation of an *in vitro* system targeted at the biosynthesis and extraction of its biologically active substances to meet emerging demands. The aim of this study was to determine the effect of scarification on seed germination processes and to determine the optimal nutrient medium for *in vitro* culturing and callus induction of common hop (*Humulus lupulus* L.), collected in the Republic of Tatarstan.

2. Materials and Methods

The experiments were carried out at the Department of Botany and Plant Physiology of the Kazan Federal University, Russia.

2.1. In vitro culture initiation from seed explants

Seeds of wild growing hop, collected in the Republic of Tatarstan, were used as primary explants

for *in vitro* culture initiation. The seeds were separated from the inflorescences and soaked in a soapy solution with simultaneous shaking on an orbital shaker for 60 minutes. After washing, all the seeds were sterilized with 70% ethyl alcohol for 30 seconds. Further surface sterilization of the seeds was carried out with a 20% solution of commercial bleach "Belizna" containing 5.25% sodium hypochlorite (NaOCl) with 2 drops of Tween 80 for 15 minutes. The seeds were then washed with sterilized distilled water until traces of the bleach disappeared. Half of the sterilized seeds were mechanically scarified under aseptic conditions by incision of the seed coats prior to culturing all the seeds in hormone-free MS (Murashige and Skoog, 1962) culture medium. The cultures, after four weeks, were evaluated visually by the frequency of seed germination and culture contamination.

2.2. Determination of the effect of phytohormones on morphogenetic growth in vitro

Nodal explants excised from the *in vitro* developed shoots were used to study the morphogenetic potential. About 1 cm long nodal explants were cultured in 20 mm × 200 mm test tubes filled with 15 mL aliquots of MS medium with 30 g/L of sucrose and containing 17 combinations of N^6-benzylaminopurine (BAP) and indole acetic acid (IAA) concentrations to obtain the following 17 treatments: a control treatment (the base media contained 0.0 mg/L of BAP and IAA); 16 treatments obtained combining four BAP concentrations (0.25, 0.5, 0.75, or 1.0 mg/L) and four IAA concentrations (0.0, 0.1, 0.25, or 0.5 mg/L). After six weeks of cultivation, the following data were recorded: number of new developed microshoots per explant, number of nodes formed on microshoots per explant, length of developed shoots, number of roots per explant, and length of the roots. All the cultures were grown in a phytotron "Lia-3" brand at a temperature of +26±2 ºC, relative humidity higher than 70%, and a 16-hour photoperiod with illumination of 3000 lux.

2.3. Determination of the effect of phytohormones and light regime on the induction of callus cultures

Furthermore, the effect of phytohormones and lighting regime on the establishment of callus cultures of common hop was studied. Leaf fragments of about 1 cm^2 and 1 cm long root sections obtained from *in vitro* hop cultures were cultured on MS medium containing 13 combinations of BAP, 2,4-dichlorophenoxyacetic acid (2,4-D), IAA, and naphthalene acetic acid (NAA) concentrations to obtain the following 13 treatments: a control treatment (the base media contained 0.0 mg/L of BAP, 2,4-D, IAA, and NAA); four treatments obtained combining two BAP concentrations (0.5 or 1.0 mg/L) and two 2,4-D concentrations (0.5 or 1.0 mg/L); four treatments obtained combining two BAP concentrations (0.5 or 1.0 mg/L) and two IAA concentrations (0.5 or 1.0 mg/L); four treatments obtained combining two BAP concentrations (0.5 or 1.0 mg/L) and two NAA concentrations (0.5 or 1.0 mg/L). Half of the petri dishes with both explants were kept in an incubator at +26±2 ºC in the dark. The other half of the cultures were kept in a phytotron "Lia-3" brand at +26±2 ºC, relative humidity of at least 70% and 16-hour photoperiod with illumination of 3000 lux. After six weeks of culturing, percent callus induction in the explants per culture medium was accessed. The developed callus was transferred onto the same medium as the induction medium and maintained at 16-hour photoperiod with illumination of 3000 lux

2.4. Experimental design and Statistical analysis

The hop seed surface sterilization and germination experiments were conducted in four replications, with an average of 25 seeds in each replication. The morphogenetic growth experiments were conducted in four replications with 5 explants in each replication. The callus induction experiments were carried out in three replications with 10 explants per replicate. The data were statistically analyzed using ANOVA at a significance level of $p<0.05$ using the GraphPad Prism 8.4.3 software package. When statistically significant differences were observed, mean values were compared using the Tukey HSD test.

3. Results

3.1. In vitro culture initiation from seed explants

After four weeks of culturing the hop seeds, following double sterilization with 70% ethyl alcohol for 30 seconds and 20% commercial bleach solution for 15 minutes, 7.8±1.5% microbial contaminations were observed in the cultures. Generally, a low germination rate in the hops seeds was observed under *in vitro* conditions. After mechanical scarification of the sterilized seeds, about 9.9±3.6% of the seeds germinated. In the control group without mechanical scarification, 9.6±2.4% seed germinated.

3.2. Effect of phytohormones on morphogenetic growth in vitro

After six weeks of cultivation of hops nodal explants, the number of microshoots developed per explant in all variants of the BAP and IAA concentrations used ranged from 1.75 to 2.9 (Figure 1). Even though the highest number of microshoots developed per unit of nodal explant was on MS medium containing only 0.5 mg/L BAP, no statistically significant differences in the mean number of microshoots were observed among all the concentrations of phytohormones used.

The number of nodes developed per cultured explant ranged from 8.4 on MS medium with 1.0 mg/L BAP in combination with 0.1 mg/L IAA to 17.8 with 0.5 mg/L BAP in combination with 0.25 mg/L IAA. This last combination of growth regulators gave a number of nodes significantly higher than 1.0 mg/L BAP + 0.1 mg/L IAA and the control (Figures 1, 2B, 2C, and 2A). In fact, the number of nodes developed on hormone-free MS medium (Figure 2A) was similar to that which was observed on media with 1.0 mg/L BAP + 0.1 mg/ IAA (Figure 2C). A significantly lower number of developed nodes, compared to the combination 0.5 mg/L BAP + 0.25 mg/L IAA was also observed in the culture medium containing 0.25 mg/L BAP + 0.5 mg/L IAA. The other combinations gave a number of nodes not significantly different from 0.5 mg/L BAP in combination with 0.25 mg/ IAA (Figures 1 and 2D).

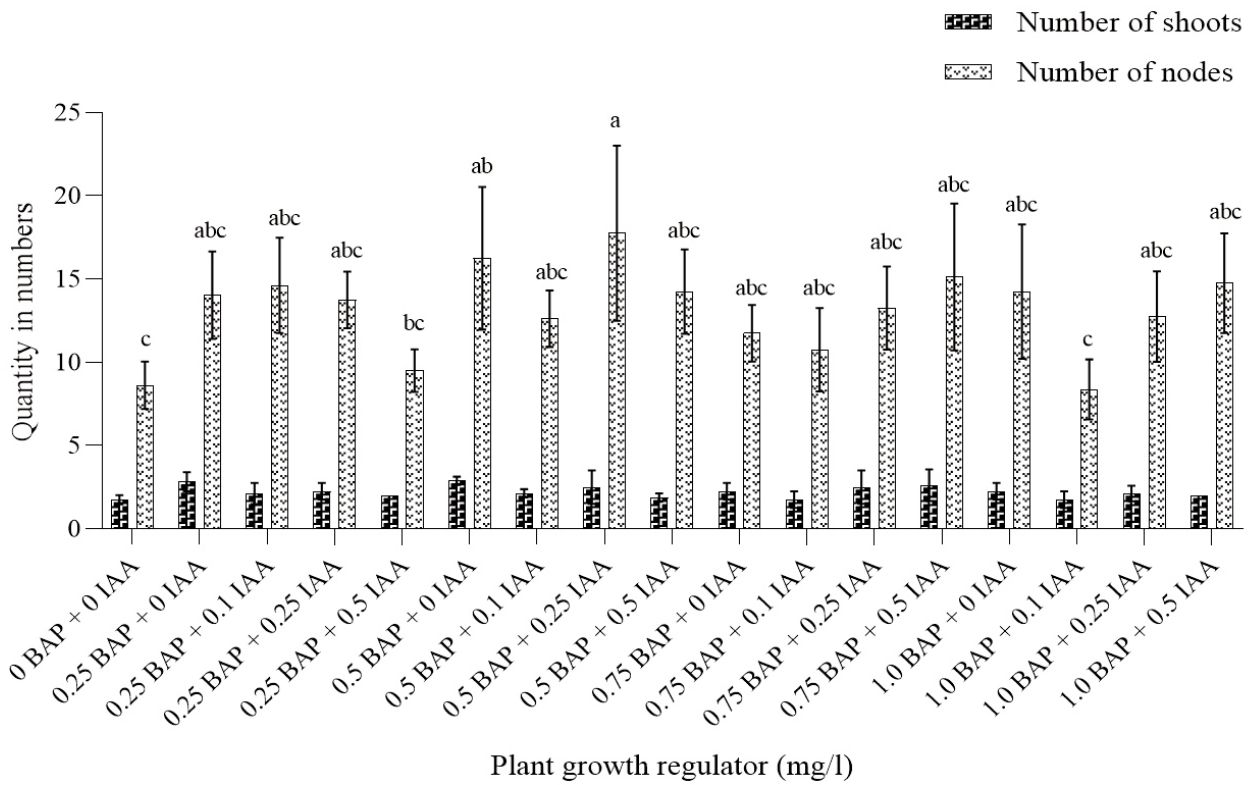

Figure 1. Effect of BAP and IAA on the number of microshoots and nodes of *H. lupulus* L., under *in vitro* growth conditions. Means values with different letters differ statistically at $p < 0.05$. Error bars indicate the standard deviations of the means.

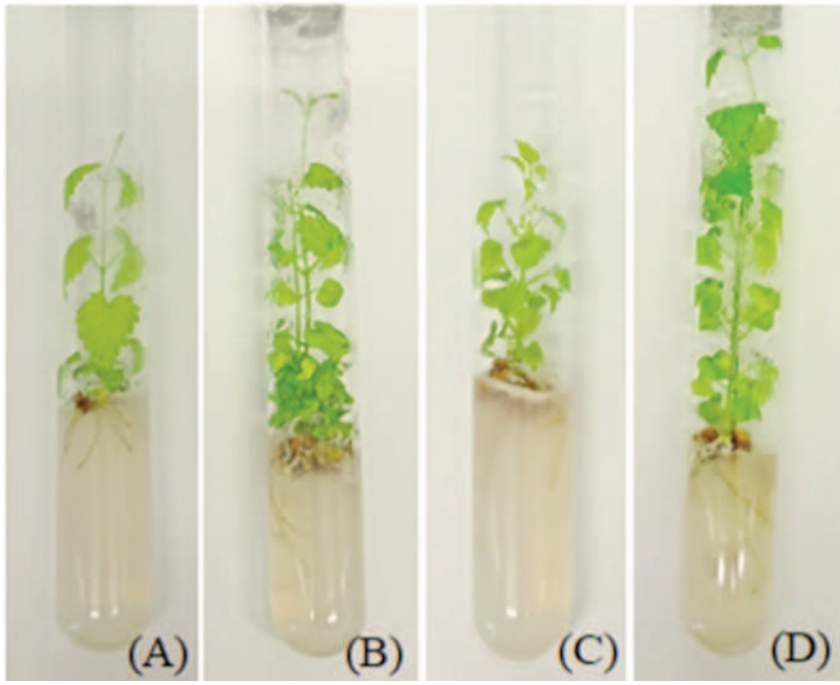

Figure 2. Morphogenetic growth of *H. lupulus* L. on different MS media: **(A)** hormone-free medium; **(B)** with 0.5 mg/L BAP in combination with 0.25 mg/L IAA; **(C)** with 1.0 mg/L BAP in combination with 0.1 mg/L IAA; **(D)** with 0.75 mg/L BAP in combination with 0.25 mg/L IAA.

The length of microshoots developed from the nodal explants ranged from 50.5 mm to 132.0 mm (Figure 3). Statistically significant differences were observed between the means of microshoot length. The lowest microshoot length was found on medium with 1.0 mg/L BAP + 0.1 mg/L IAA and in the control.

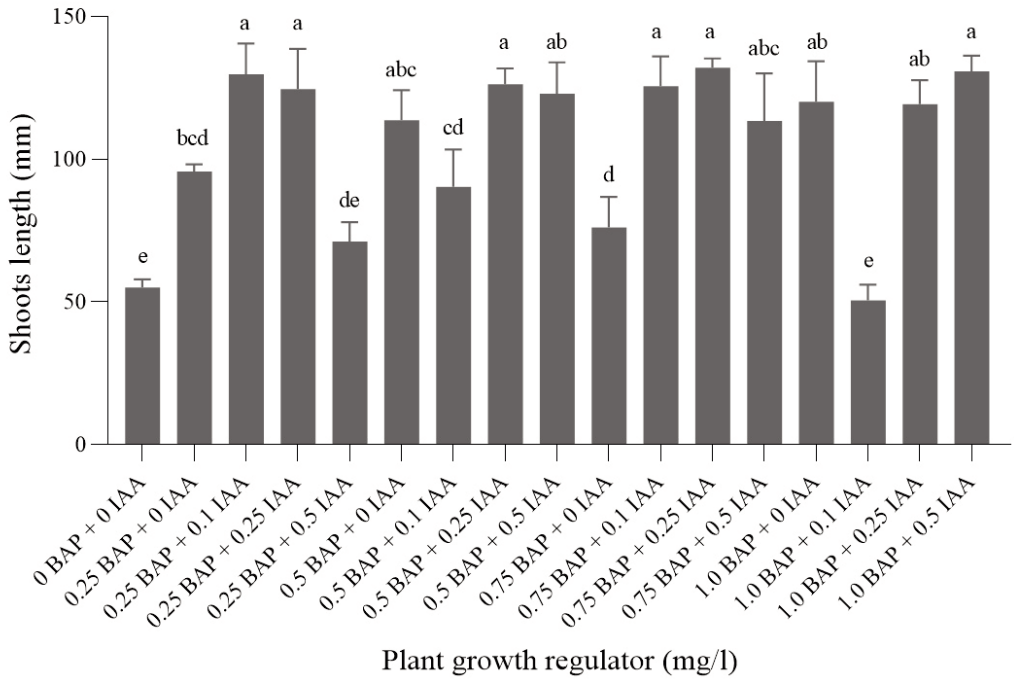

Figure 3. Effect of BAP and IAA on shoot length of *H. lupulus* L. under *in vitro* growth conditions. Mean values with different letters differ statistically at $p < 0.05$. Error bars indicate the standard deviations of the means.

Simultaneous development of roots and shoots were observed in all cultures on all the media used. The mean number of roots developed per explant ranged from 7.4 to 14.3 (Figure 4). The highest number of roots was formed on the culture media with 0.5 mg/L BAP + 0.25 mg/L IAA, 0.75 mg/L BAP + 0.1 mg/L IAA, and 1.0 mg/L BAP + 0.5 mg/L IAA which were the combinations also giving significantly higher number of roots than that observed with a BAP concentration of 1.0 mg/L in combination with 0.1 mg/L IAA and on the medium supplied with 0.5 mg/L BAP + 0.1 mg/L IAA.

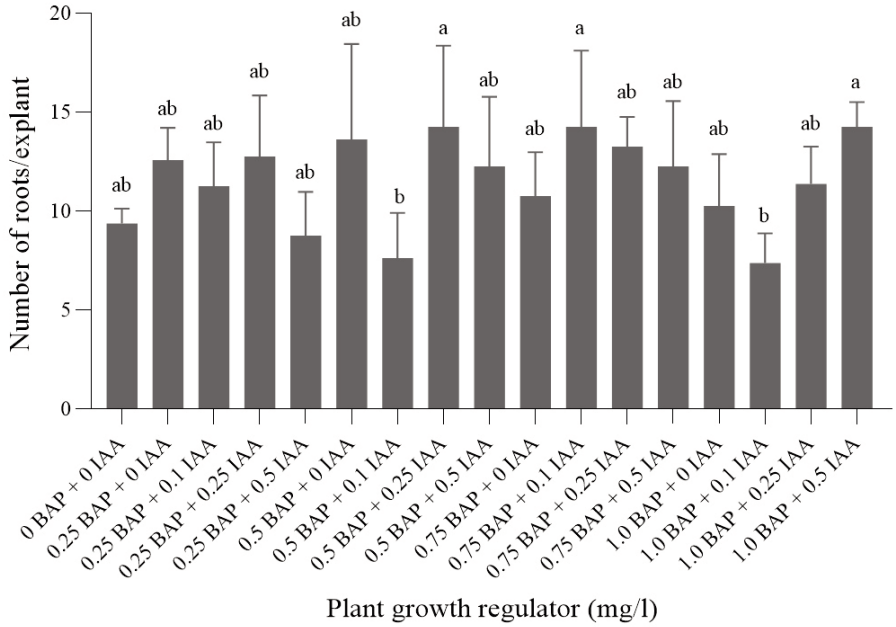

Figure 4. Effect of BAP and IAA on number of roots in *H. lupulus* L. *in vitro* cultures. Mean values with different letters differ statistically at $p < 0.1$. Error bars indicate the standard deviations of the means.

Some differences in mean length of roots per explant were statistically significant and the values found ranged from 27.1 to 65.8 mm (Figure 5). The length of roots developed on the medium with 0.5 mg/L BAP + 0.25 mg/L IAA was significantly higher than the root length on hormone-free medium, 0.25 mg/L BAP + 0.5 mg/L IAA, 0.5 mg/L BAP + 0.1 mg/L IAA, 0.75 mg/L BAP + 0.0 mg/L IAA, 0.75 mg/L BAP + 0.1 mg/L IAA, 0.75 mg/L BAP + 0.5 mg/L IAA and 1.0 mg/L BAP + 0.1 mg/L IAA.

3.3. Effect of phytohormones and light regime on the induction of callus cultures

Callus induction was not observed in all the root explants cultured and also on hormone-free MS medium. Overall, callus was induced at a much higher frequency under light conditions (up to 100%) compared to darkness (up to 73%) (Figure 6).

In the 16-hours light conditions, callus developed from leaves on all the media with phytohormones. Although 100% callus induction rate was achieved in light on MS media containing 1 mg/L BAP + 0.5 mg/L 2,4-D or + 0.5 mg/L IAA, there was no statistically significant differences between them and the treatment with 0.5 mg/L BAP + 1.0 mg/L 2,4-D, 0.5 mg/L BAP + 1.0 mg/L IAA, 0.5 mg/L BAP + 0.5 mg/L NAA, 0.5 mg/L BAP + 1.0 mg/L NAA, 1.0 mg/L BAP + 0.5 mg/L NAA and 1.0 mg/L BAP + 1.0 mg/L NAA . Under light conditions, the effect of BAP in combination with 2,4-D or IAA at the ratios of 0.5 mg/L to 1.0 mg/L was similar. When BAP + 2,4-D or IAA were applied at the same concentration, a similar callus induction effect was also observed.

Under dark conditions, callus was induced only on the edges of leaf explants after the six weeks of observation (Figure 7B). Callus formation was not observed on three of the media apart from the hormone-free medium.

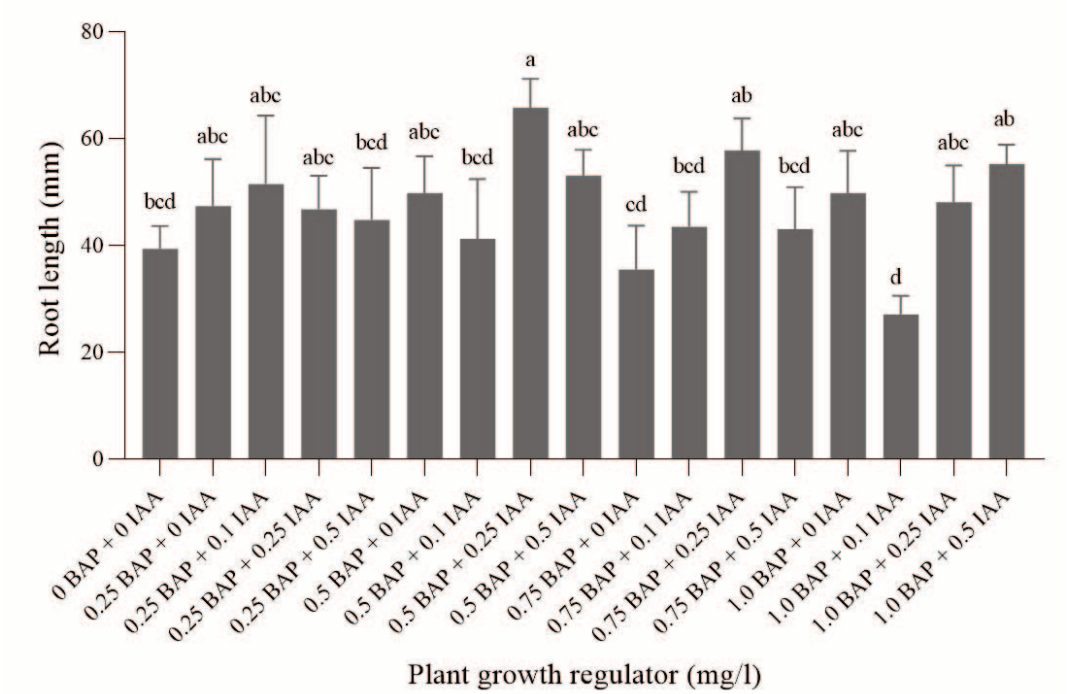

Figure 5. Effect of BAP and IAA on *H. lupulus* L. root length under *in vitro* conditions. Mean values with different letters differ statistically at $p < 0.05$. Error bars indicate the standard deviations of the means.

After subculturing under light conditions, callus of creamish yellow colour with slightly greenish zones and friable consistency was obtained (Figure 7C).

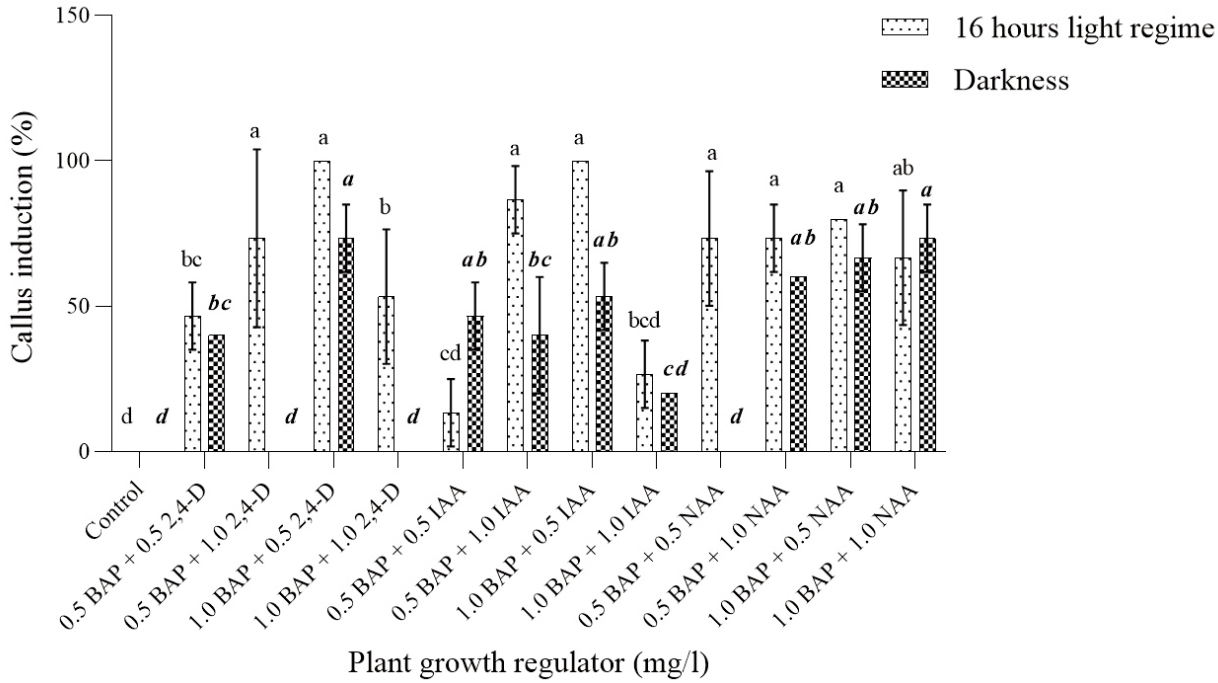

Figure 6. Effect of phytohormones and light regime on frequency of callus induction in *H. lupulus* L. leaf explants. For the treatment "Control", the medium included 0.0 mg/L of BAP, 2,4 D, IAA, and NAA. Mean values with different letters differ statistically at $p < 0.05$. Letters in italics indicate the means for cultures grown in darkness. Error bars indicate the standard deviations of the means.

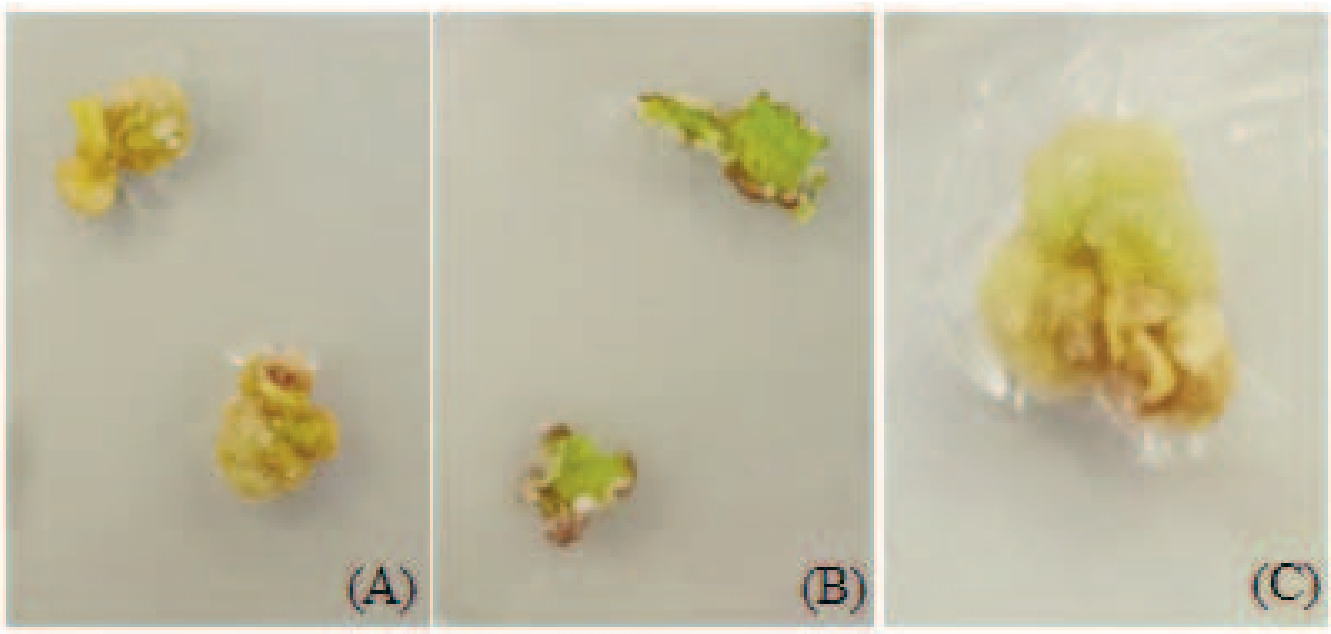

Figure 7. Induction and maintenance of *H. lupulus* L callus of leaf origin on MS medium with 1.0 mg/l BAP in combination with 0.5 mg/l IAA: **(A)** under 16-hours light regime; **(B)** in darkness; **(C)** callus maintenance under 16-hours light regime.

4. Discussion

Surface sterilization of explants before *in vitro* culturing is a key step for ensuring the production of aseptic cultures. The low level of contamination observed in the cultures in this work is clear evidence that the surface sterilization procedure was effective. Successful sterilization of explants has also been achieved in other *in vitro* plant studies using 70% ethyl alcohol and bleach in the range of 10 to 20% (Davoudpour et al., 2020; Felek et al., 2015; Firoz et al., 2016; Mihaljevic et al., 2013).

According to Liberatore et al. (2018) and Suciu et al. (1977), hops have a low seed germination rate as was also observed in the current study. The use of seed pretreatment measures such as mechanical scarification improved germination in both *in vitro* and *in vivo* studies in some plants (Cho et al., 2020; Liberatore et al., 2018; Nejad et al., 2022). However, in our experiments, incision of the seed coats prior to culturing was not found to be more effective as compared to the control treatment (without incision). Liberatore et al. (2018) reported that soaking hop seeds in gibberellic acid was better for breaking seed dormancy and increasing germination, but further studies on other genotypes are required to address this aspect.

The composition of culture medium, particularly the type and concentration of phytohormones, plays a key role in determining the growth characteristics of plants cultivated under *in vitro* conditions (George et al., 2008; Phillips and Garda, 2019). The number of microshoots, as well as the number of nodes developed *in vitro* by plants growing vertically is an important indicator for the selection of an optimal culture medium for clonal micropropagation (Bychkova et al., 2024). In our study, growing the nodal explants of hops on the MS medium with or without BAP and IAA combinations did not result in significant differences in the numbers of microshoot developed. However, the presence of phytohormones in the culture medium was important for node development, as evidenced by the weak effect observed on the hormone-free MS medium.

In addition, the BAP to IAA ratio also played a crucial role in determining the morphogenetic characteristics of the *in vitro* hop cultures. MS medium with 1.0 mg/L BAP + 0.1 mg/L IAA induced low node formation similarly to the hormone-free medium and this response might be due to the tenfold increase in the ratio of BAP to IAA. Indeed, cytokinins play a key role in cell proliferation and apical

meristem activity (Kieber and Schaller, 2018). In addition, the low number of developed nodes observed on MS medium containing 0.25 mg/L BAP + 0.5 mg/L IAA might be due to the higher auxin to cytokinin ratio.

Clapa and Hârta (2021) reported for *H. lupulus* L. cv. 'Cascade' that the best multiplication rate was also noted on a modified MS medium containing BAP (0.5 mg/L) compared to other cytokinins, as in our experiments. Another study on the 'Cascade' variety in a different geographical location showed higher stem length, and number of shoots and nodes on MS medium supplemented with 1.0 mg/L BAP + 0.1 mg/L IAA, which could be due to the specific growing environment (Ha et al., 2024). However, a genotype dependent response could be also hypothesized since in another study on hops, nodal segment proliferation (Mafakheri and Hamidoghli, 2019) was best on MS medium containing 1.0 mg/L BAP + 0.1 mg/L IAA, that conversely induced low growth in our study.

A direct relationship was observed between the number of nodes and the length of microshoots developed. Machado et al. (2018) reported the highest microshoot length on a modified hormone-free MS medium containing 30 mg/L of sucrose compared to another medium containing 1 mg/L BAP and 20 g/L sucrose. Conversely, microshoot length on hormone-free MS media with 30 g/L sucrose was lower compared to media with the phytohormones tested in our study. A similar weak effect was induced by media with 1.0 mg/L BAP + 0.1 mg/L IAA and 0.25 mg/L BAP + 0.5 mg/L IAA both on the length of the microshoot and the number of nodes. In general, longer microshoots were formed after six weeks of *in vitro* culturing as also previously observed in the cv. 'Cascade' (Clapa and Hârta, 2021) and after eight weeks in the cv. 'Columbus' (Machado et al., 2018).

The number and length of developed roots showed the worst i*n vitro* growth effect on the medium with 1.0 mg/L BAP + 0.1 mg/L IAA, which followed the pattern of number of shoots developed and shoot length. Hop genotypes of American origin also showed such effect on root and shoot development parameters on media containing BAP and IAA (De-Souza-et al., 2022). It can be hypothesized that the observed similar effects of the different media on the growth patterns of root and shoot are the result of mutual signaling between the two plant systems (George et al., 2008; Puig et al., 2012; Shabala et al., 2015). In addition, it can be hypothesized that root development was not fully dependent on the action of added phytohormones, since root length and number on hormone-free MS medium were not the lowest.

Callus cultures are useful for achieving constant biomass production, organ and tissue regeneration, maintenance of plant genetic resources, production of biologically active substances, and providing explants for growing suspension cultures (Rout et al., 2011; Efferth, 2019).

This study revealed that the explant type and the use of phytohormones and light supplementation were all important factors for allowing the induction of callus cultures of a hop landrace growing in the Republic of Tatarstan.

The type of explant used played a significant role in the induction of hop callus. This was demonstrated by the total absence of callus induction from root explants irrespective of the growth regulators and light conditions used. The absence of callus induction in roots may be due to the effect of the morphogenetic potential of the different explants. Generally, explants of different types excised from the same source plant have showed varying responses to callus initiation (Farhadi et al., 2017; Trojak-Goluch et al., 2015). Although callus was initiated from both stem and petiole explants of *H. lupulus* cv. 'Iunga', differences in the regeneration potential of their calli were detected (Trojak-Goluch et al., 2015). Moreover, endogenous hormone levels could have affected the behavior of root explants in the present study. Phytohormones added to the culturing medium have played a significant role in the morphogenetic growth patterns of cultures and callus in different plants (Chakravarthi et al., 2010; Mostafa et al., 2020).

Although all plant cells have the potential for cell differentiation (Efferth, 2019; Fehér, 2019), creation of the optimal chemical and physical growth conditions has a direct effect on callus growth. Callus initiation was dependent on the presence of phytohormones in the culture medium. The leaf explants induced varying percentages of callus on media containing phytohormones under light and dark condi-

tions. Auxins and cytokinins added to culture medium work together to induce callus formation through the stimulation of cell division (Pasternak et al., 2000; Bano et al., 2022).

The concentration ratio of BAP, IAA, and 2,4-D in the MS medium has an effect on the rate of callus induction. Under light conditions, a ratio from 0.5 to 1 mg/L obtained combining BAP with either 2,4-D or IAA for in light was necessary for obtaining high callus induction in the hop genotype studied in our experiments. A clear trend in the effect of phytohormone combinations on callus formation in darkness was not observed in this study. Conversely, callus induction rate in leaf and internode explants of three Slovenian hop genotypes was highest under 16-hours light conditions on MS medium containing 2 mg/L BAP + 2 mg/L 2,4-D, while a 2 mg/L BAP + 2 mg/L NAA combination induced the best results in the dark (Pšenáková et al., 2009). The different effects observed in the present study could be due to a genotype specific response and the lower phytohormone concentration used compared to the study of Pšenáková et al. (2009). Hop genotypes exhibited a wide variation of responses under different experimental conditions (Bychkova et al., 2024).

The *in vitro* established plantlets and obtained callus cultures have provided the initial material for further *in vitro* studies aimed at the establishment of suspension cultures and the biosynthesis and determination of biologically active substances of the hop wild genotype of the Republic of Tatarstan.

5. Conclusions

In this study, the wild hop genotype of the Republic of Tatarstan was successfully cultivated under *in vitro* growth conditions. This allowed to obtain (a) fully developed plantlets in culture vessels starting from seeds as primary explants and (b) callus cultures starting from leaves.

Although the surface sterilization procedure used for hop seeds was largely effective, a low germination rate of the hop seeds was recorded, regardless of the application of scarification. In the *in vitro* cultures, a relevant positive effect of the BAP to IAA concentration ratio was revealed on the development of shoots from nodal explants. Roots developed simultaneously with shoots and their formation was not fully dependent on the presence of phytohormones in the growth medium. It was revealed that callus induction was only successful from leaf explants derived from *in vitro* cultures in comparison to root explants. Moreover for a vigorous callus initiation, it was necessary to grow the cultures under a 16-hours light supply with illumination of 3000 lux.

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Conflicts of Interest: The authors declare no conflict of interest.

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