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EFFECT OF VARIOUS CYTOKININS ON CALLUS DEVELOPMENT AND THE FREQUENCY OF SHOOT REGENERATION IN FLAX CALLUS CULTURE

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This research explores the role of cytokinins – specifically 6-benzylaminopurine (BAP), thidiazuron (TDZ), and zeatin (ZEA) – in promoting flax callus development and shoot regeneration, using two distinct linseed genotypes. The main objective is to determine how different cytokinins influence callus growth and regeneration rates in flax, aiming to identify optimal conditions for enhanced shoot formation. Calli derived from hypocotyls of the linseed varieties Zaporozhsky Bogatyr and Evrika were cultivated on N_6 media supplemented with BAP, TDZ, or ZEA at a concentration of 1 mg/L. Growth characteristics and shoot regeneration rates were assessed after four weeks. The study found that TDZ positively influenced callus development, while zeatin notably boosted the rate of shoot regeneration, particularly in the Evrika variety, which showed a threefold increase in shoot formation frequency. Overall, TDZ enhanced callus growth, and zeatin significantly improved shoot regeneration, highlighting the importance of cytokinin type and genotype in effective flax regeneration strategies.

Key words: callus, benzylaminopurine, thidiazuron, zeatin, regeneration, linseed.

Introduction. The genus *Linum* comprises around 200 species though in agriculture the primary species used are *Linum usitatissimum* (known as common flax) and *Linum narbonense* (Narbonne Flax). Other species such as *L. bienne, L. angustifolium, L. hispanicum,* etc. are mainly used in scientific projects to incorporate valuable traits into new flax varieties. Several *Linum* species exhibit high ornamental value, most notably *L. perenne, L. narbonense*, and particularly *L. grandiflorum* (Lyakh, 2013; Tork et al., 2022).

Linum usitatissimum holds significant economic and ecological value, it is primarily cultivated for its seeds, which are processed to produce linseed oil and incorporated as a high-fiber food ingredient. Flaxseed is also included in animal feed due to its nutritional benefits. Flax fibers are employed in textiles to make linen as they are exceptionally strong, durable, and have a natural luster. Its strength makes it resistant to wear and tear, moreover flax linen is breathable, absorbent, and has a crisp, textured feel. Flax is important as a rotational crop as well, due to its benefits in soil health and pest management. Its roots help break up compacted soil and improve soil structure (McGregor, 2007; Hossain & Hossain, 2014; Jansen & Riemens, 2017).

In addition to these uses, the plant's potential in biotechnological applications is noteworthy. The ability to cultivate flax cells *in vitro* is essential for numerous research and industrial purposes. Various *in vitro* techniques provide a controlled environment to study and manipulate plant growth and development. *In vitro* callus culture, a technique that allows applying selection pressure to thousands of cells, can

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play a significant role in enhancing Linum varieties, helping to select genotypes with important characteristics (Efferth, 2019).

Regeneration in callus culture is a crucial technique in plant biotechnology that allows for the propagation of plants from undifferentiated cell masses. As callus cells are totipotent, they have the potential to differentiate into any cell type. This characteristic is essential for regenerating whole plants from a single cell or tissue sample. Plants obtained via callus culture can possess new characteristics that allows for the manipulation of plant genetics, facilitating the development of new varieties with desirable traits.

Plant regeneration *in vitro* can occur spontaneously, however, it predominantly relies on the addition of exogenous hormones (Bernula et al., 2020). Cytokinins, such as benzylaminopurine, kinetin, zeatin, thidiazuron, and others, whether of natural or artificial origin, play a pivotal role in the regeneration process of callus cultures. They promote cell division, dedifferentiation and differentiation, which are essential for the formation of shoots and roots from callus tissue. Additionally, cytokinins influence the expression of genes involved in various plant hormone signaling pathways (Lu et al., 2022; Šmeringai et al., 2023).

Different cytokinins influence regeneration efficiency and quality in diverse ways. Investigating their effects on specific plant species and desired outcomes is crucial for determining which cytokinins are most effective for optimizing plant tissue culture. To advance plant tissue culture techniques, it is essential to understand the distinct roles and effects of various cytokinins. Some data are available on the effects of phytohormones, including cytokinins, on the induction of regeneration in specialized *Linum* organs (Dedicova et al., 2000; Mundhara & Rashid 2006; Janowicz et al., 2012). However, data on the comparative effects of different cytokinins on regeneration processes in flax cell cultures are lacking.

Therefore, the objective of our study was to evaluate the impact of cytokinins such as benzylaminopurine (BAP), zeatin (ZEA), and thidiazuron (TDZ) on regeneration rates and callus development in flax callus cultures.

Materials and Methods. Two modern varieties of cultivated flax (Linum usitatissimum L.) of different origins, designated as Zaporozhsky Bogatyr and Evrika, were utilized in this study. The seeds were surface sterilized using a 5% Chloramine B (N-chloro-4-toluenesulfonamide) solution, thoroughly rinsed with sterile water, and placed in Petri dishes lined with filter paper moistened with distilled water to promote germination. After a 10-day germination period, hypocotyls were excised from the seedlings, segmented into 5-7 mm pieces, and cultured on Murashige and Skoog (Murashige & Skoog, 1962) basal medium (MS) supplemented with 1.5 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/L 6-Benzylaminopurine (BAP) to induce callus formation.

After four weeks, the obtained calli were separated from the initial explants and subcultured for an additional four weeks on N6 medium supplemented with BAP and NAA, each at a concentration of 0.1 mg/L, to promote further multiplication. Following this period, the multiplicated calli were subdivided into 3-4 mm clusters, and transferred to fresh N6 medium (Chu, 1978) enriched with the amino acids glutamine, asparagine, and serine. This medium was further supplemented with 0.05 mg/L α-Naphthaleneacetic acid (NAA) and various cytokinins. The cytokinins used included both synthetic compounds BAP and thidiazuron (TDZ) and the natural compound zeatin (ZEA), all at a concentration of 1 mg/L.

The calli were placed in Petri dishes, with seven calli per dish, and each treatment was replicated ten times. The cultures were maintained under a 16-hour photoperiod at a temperature of 25 ± 1°C for four weeks. Post-cultivation, the calli were evaluated for color (chlorophyll presence indicated by green color versus white), degree of development, and regenerative capacity. The number of calli exhibiting active growth and those forming regenerated shoots were recorded.

Statistical analysis of the differences between treatments was performed using the Student's t-test in Microsoft Excel to assess the significance of the observed variations.

Results and Discussion. During the experiment, the behavior of flax calli cultured on media supplemented with different cytokinins exhibited distinct variations, though most calli continued to grow actively. By the conclusion of the study, the callus tissue resulting from cultivation was predominantly characterized by a compact structure, often displaying distinctly defined globules. Nevertheless, friable calli, which readily disintegrated into fragments when touched, were also observed across all experimental variants.

The study demonstrated that different cytokinins had varying effects on chlorophyll synthesis, as evidenced by the differing intensities of green coloration in the calli. This variation was further reflected in the growth parameters of the callus as well as its regenerative capabilities. As illustrated in Table 1, the inclusion of TDZ in the nutrient medium generally had a more favorable impact on callus development compared to BAP and ZEA. Specifically, in the Zaporozhsky Bogatyr variety, the incidence of chlorophyll-deficient calli was nearly three times lower on the medium supplemented with TDZ than on media with BAP and ZEA, concurrently leading to an increased frequency of calli exhibiting active growth.

Table 1

Effects of phytohormones of cytokinin type on callus development (2024)

Phyto-hormone	Calli tested, pcs.	Ratio of calli with different color, %		Ratio of calli with different growth rate, %	
		white	green	no active growth	active growth
Evrika					
BAP	70	27.14[a]	72.85[a]	38.57[a]	61.43[a]
TDZ	70	11.43*[b]	88.57*[b]	22.86*[b]	77.14*[b]
ZEA	70	15.71	84.29	27.14	72.86
Zaporozhsky bogatyr					
BAP	70	30.00[a]	70.00[a]	35.71[a]	64.29[a]
TDZ	70	11.43**[b]	88.57**[b]	20.00*[b]	80.00*[b]
ZEA	70	31.43[a]	68.57[a]	37.14[a]	62.86[a]

*, ** - Differences between the treatments are significant at $p<0.05$ and $p<0.01$, respectively. Significant differences within genotype are designated with different letters.

In contrast, significant differences in the Evrika variety were observed only when comparing TDZ with BAP. Regarding regeneration induction, TDZ

demonstrated efficacy comparable to BAP but was significantly less effective than zeatin (Table 2).

Table 2

Effects of phytohormones of cytokinin type on shoot regeneration rate (2024)

Phyto-hormone	Calli tested, pcs.	Frequency of regenerating calli, %		Frequency of regenerating calli, %		Total frequency of regenerating calli, %
		white callus	green callus	callus without active growth	callus with active growth	
Evrika						
BAP	70	1.43	5.71	1.43	5.71	7.14**
TDZ	70	1.43	7.14	1.43	7.14	8.57*
ZEA	70	2.85	21.44	4.28	20.01	24.29
Zaporozhsky bogatyr						
BAP	70	1.43	1.43	1.43	1.43	2.86*
TDZ	70	0	1.43	0	1.43	1.43*
ZEA	70	2.85	8.58	4.28	7.15	11.43

*, ** - Differences between the treatments with ZEA and TDZ, and ZEA and BAP are significant at $p<0.05$ and $p<0.01$, respectively

Zeatin exhibited effects similar to BAP concerning callus development, with no significant differences observed in the number of chlorophyll-deficient calli or the proportion of calli with active growth. However, zeatin notably enhanced regeneration processes, markedly enhancing shoot formation frequencies compared to the addition of either BAP or TDZ. This was particularly evident in the Evrika variety, where the frequency of calli producing regenerated shoots was over three times higher when zeatin was included in the medium compared to the respective treatments with BAP or TDZ.

BAP had a weaker effect on callus development compared to TDZ. When assessing regeneration processes, effect of BAP was comparable to TDZ, but it significantly underperformed in shoot regeneration compared to zeatin.

There is evidence in the literature that regeneration processes depend on the size of the explant. Cells from explants that are too small are often unable to synthesize sufficient amounts of metabolites, primarily hormones, which are necessary to induce organogenesis. Additionally, larger explants typically have a higher regenerative potential because they contain more cells capable of responding to hormonal signals (Thorpe, 2007; George et al., 2008). In our study, according to the data obtained, the appearance of regeneration loci was independent of callus size. Both callus with inactive growth (3-4 mm) and active growth (8-14 mm) were observed to induce shoots. This suggests that the artificial addition of phytohormones was sufficient to stimulate regeneration processes even in calli of smaller size.

It should be noted that the amount of photosynthetic pigments determining the green coloration of calluses did not affect their ability to regenerate. Shoot formation

was observed on both green and chlorophyll-defective calli. This indicates that the amount of photosynthetic pigments in the cells was not crucial for inducing regeneration processes.

According to numerous scientific data the influence of genotype is often a determining factor in the development of any biotechnological techniques (Raghavan, 2019; Chen & Mette 2018; Yaroshko et al., 2023). This highlights the importance of working with genetically diverse material. A study on two flax varieties, Evrika and Zaporozhsky Bogatyr, demonstrated different responses of these two genotypes in *in vitro* culture. The frequency of regenerating callus in the Evrika variety was two times higher than in the Zaporozhsky Bogatyr variety (Figure 1). However, the general regularities of the effect of cytokinin-type hormones on callus growth and development and on regeneration processes were preserved.

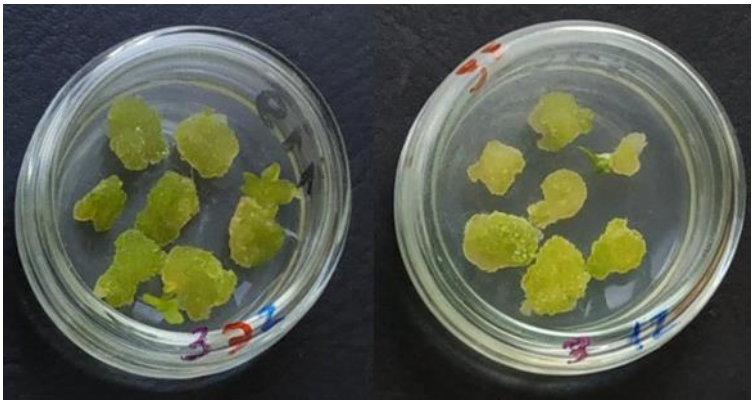

Fig. 1. **Shoot regeneration in the Evrika (left) and Zaporozhsky Bogatyr flax varieties (right) on the medium with 1 mg/L zeatin**

The data presented indicate that the frequency of regeneration of adventitious shoots in callus culture is notably low, with a maximum of 14.29% observed in the Evrika variety. Existing literature reports significantly higher frequencies of shoot regeneration in various crop species, including flax, reaching up to 80-100% in certain genotypes (Yildiz & Özgen, 2006; Janowicz et al., 2012). However, such high frequencies have been achieved only when utilizing specialized organs such as hypocotyls or cotyledons for cultivation. The diminished frequency of shoot regeneration in callus tissue is likely attributed to genetic and hormonal imbalances arising from cell dedifferentiation.

Among the three cytokinin-type phytohormones examined, zeatin exhibited the highest efficacy in inducing adventitious shoot regeneration in flax, surpassing TDZ and BAP. The superior performance of zeatin is further supported by research conducted on other plant species (Yadav & Sticklen, 1995; Farooq et al., 2017; García-Fortea et al., 2020). These studies demonstrated that Z and its derivatives have been shown to be highly effective as a natural plant growth regulator in various *in vitro* culture applications. Cytokinins, including zeatin, are known to regulate various physiological processes in plants, such as cell division and differentiation. They are essential for the establishment of adventitious shoot regeneration systems. Presumably, zeatin's superior performance in inducing adventitious shoot regeneration can be

attributed to its structural advantages, enhanced ability to induce cell division, high metabolic stability, synergistic interactions with auxins, and specific effectiveness in certain plant species. At the same time, some studies in the literature indicate that TDZ may be effective or even superior to ZEA in promoting shoot regeneration (Hosokawa et al., 1996; Dedicova et al., 2000). However, our investigations revealed that TDZ did not result in a higher frequency of shoot regeneration compared to ZEA. Instead, TDZ was found to be effective in sustaining the growth and condition of callus tissue.

Conclusions. The research investigates the influence of various cytokinins on the development of flax callus and the regeneration of shoots. The study revealed that thidiazuron in the nutrient medium generally had a more favorable impact on callus development compared to benzylaminopurine and zeatin. Calli grown on TDZ-supplemented medium exhibited chlorophyll deficiency at much lower frequencies than those grown on media with BAP and ZEA, which corresponded with an increased frequency of calli exhibiting active growth. Zeatin exhibited effects similar to BAP concerning callus development. Zeatin significantly enhanced the regeneration processes, resulting in higher frequencies of shoot formation compared to the addition of either BAP or TDZ. When evaluating other cytokinins for their ability to induce regeneration, the effects of BAP were found to be comparable to those of TDZ. The impact of genotype on regeneration rates was significant.

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ВПЛИВ РІЗНИХ ЦИТОКІНІНІВ НА РОЗВИТОК КАЛУСУ ТА ЧАСТОТУ РЕГЕНЕРАЦІЇ ПАГОНІВ У КАЛУСНІЙ КУЛЬТУРІ ЛЬОНУ

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Linum usitatissimum **має значну економічну та екологічну цінність, його вирощують заради насіння, яке переробляють для виробництва лляної олії та високоякісного волокна. Макуха використовується на корм**

тваринам або як харчовий інгредієнт з високим вмістом клітковини. З огляду на широке сільськогосподарське та промислове значення, льон також став об'єктом біотехнологічних досліджень, спрямованих на покращення його адаптивних та продуктивних характеристик для забезпечення сталого виробництва. Одним із можливих підходів у цьому контексті є використання калусної культури, яка забезпечує контрольовану систему для вивчення процесів росту та диференціювання. Регенерація в калусній культурі є ключовим методом у рослинній біотехнології, оскільки дозволяє отримувати та розмножувати рослини з недиференційованих клітинних мас. В даній роботі досліджується роль цитокінінів, зокрема 6-бензиламінопурину (BAP), тідіазурону (TDZ) та зеатину (ZEA), у стимулюванні розвитку калусу льону та регенерації пагонів. Використовували два різних за походженням генотипи льону – Запорізький богатир та Еврика. Головною метою було визначення того, як різні цитокініни впливають на ріст калусу та швидкість регенерації у льону, з метою визначення оптимальних умов для кращого формування пагонів. Калуси, отримані з гіпокотилів двох вказаних сортів льону, культивували на середовищі N_6, доповненому BAP, TDZ або ZEA у концентрації 1,0 мг/л. Характеристики росту та швидкість регенерації пагонів оцінювали через чотири тижні культивування у стандартних умовах. Дослідження показало, що TDZ позитивно впливав на розвиток калюсу, тоді як зеатин помітно підвищував швидкість регенерації пагонів, особливо у сорту Еврика, у якого відмічали трикратне збільшення частоти формування пагонів. Загалом, TDZ посилював ріст калусу, а зеатин значно покращував регенерацію пагонів, що підкреслює важливість врахування типу цитокініну та генотипу в ефективних стратегіях регенерації льону.

Ключові слова: калус, бензиламінопурин, тідіазурон, зеатин, регенерація, льон.