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EFFECT OF SYNTHETIC AUXIN LIKE GROWTH REGULATORS ON CALLUS REGENERATIVE ABILITY OF COMMON WHEAT VC. ZYMOYARKA

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The aim of the study was to determine the dependence of morphogenetic reactions of wheat callus tissues to content of syntetic growth regulators of auxin nature (picloram, dicamba) in the nutrient medium.

Apical meristems of *Triticum aestivum* wheat were the primary explants for callusogenesis. Basic culture medium MS supplemented by vitamins of Gamborg, dicamba at different concentrations (0.2, 0.4, 0.6 mg/l), and picloram (0.16; 0.25; 0.5 mg/l) was used for regeneration. It was established that dicamba at a concentration of 0.2 mg/l is the most effective for production of regenerants. It was also observed that at the concentration of 0.16 mg/l picloram there are the formation of the greatest number of morphogenic zones (60%) and a significant amount of plant-regenerants. Increased concentrations of picloram to 0.25 mg/l and 0.5 mg/l caused a decrease in the number of morphogenic islands: in the first case, 10%, and the second — 36.4%. Among the described options the MS medium supplemented with 0.5 mg/l 6-benzylaminopurine and 0.16 mg/l picloram was the most effective. Shoots obtained from callus culture were capable to form roots in vitro and adapt to septic conditions. Regenerated plants when cultivated in greenhouse showed high viability (over 75%) and reached the generative phase.

Key words: growth regulators, picloram, dicamba, *Triticum aestivum*, *in vitro* culture.

Modern biotechnology techniques based on the use of plant tissues play an important role in creating crops with valuable features, as well as in improvement of their agronomic characteristics. The *in vitro* culture of the plant cells and tissues has caused great interest lately, because it gives an ability to study the physiological and genetic processes during the grade improvement through increase in genetic diversity [1]. To improve food properties of wheat many attempts were made to pick the *in vitro* cultivation conditions [2].

Despite the fact that many methods are available for biotechnological modification at the cellular level; their use in the process of improving crops is often being complicated by features of regenerating whole plants [3]. Regeneration of the monocotyledons is limited by low morphogenetic potential, which, in some cases, cannot give fertile plants [4]. In addition, it is known that the intensity of callus induction and regeneration of plants in the tissue culture mostly depend on the content of growth regulators in the culture medium and the type of explants. It is believed that the best sources of explants for tissue culture are the immature embryos and also apical meristem, mature embryos, inflorescences, mesocotyl, seeds and young leaves [5]. Also, one cannot ignore the fact that hormonal balance is an important factor that influences the *in vitro* initiation and plant regeneration [1].

Usually active auxin growth regulators are used as stimulants of the callus and root induction [6]. However, regeneration of shoots and somatic embryos from callus cultures also occurs via the auxin, although the leading role belongs to cytokines [7]. The combination of these substances in certain ratio is important in the initiation of morphogenetic processes *in vitro* [8–11].

Effect of synthetic auxin active growth regulators, namely picloram and dicamba, for regeneration from callus cultures that derived from apical meristem of wheat is considered for the first time. Analysis of the

literature showed that these plant hormones are usually used as auxiliary components, so their morphogenetic effect was not described. The study emphasized on their leading role in the regeneration of bread wheat. Besides, one cannot leave out the fact that this kind of study was first conducted in wheat *T. aestivum* c. v. Zymoyarka which is strategically important for further work towards genetic transformation of cereals and properly selected conditions of regeneration are the success in obtaining valuable biotechnologically modified plants.

In view of the above, the aim of our study was to determine the dependence of morphogenetic callus tissue reactions of wheat on the content of synthetic growth regulators auxin nature (picloram, dicamba) in the culture medium.

Materials and Methods

The apical meristem of wheat *T. aestivum* c. v. Zymoyarka provided by the Institute of Plant Physiology and Genetics of the National Academy of Sciences of Ukraine was used as an initial explant for callus induction. The choice of source material for the cultivation was caused by its availability throughout the year in large quantities and the absence of seasonal influences on the tissue culture [2]. To obtain aseptic donor seedlings of apical meristem the seeds were consistently sterilized using 1% $KMnO_4$ solution for 3 min, 1% solution of $AgNO_3$ — 2 min, 96% ethanol — 1 min and washed three times with sterile distilled water [12, 13]. After sterilization, the seeds were germinated at 24 °C and 16-h photoperiod on a hormone-free MS medium for 3 days [14]. Apical meristems were separated from the obtained 3-day-old seedlings and placed on the modified MS medium for callus induction, which contained 2 mg/l 2,4-D and 10 mg/l $AgNO_3$, vitamins of Gamborg [15] and cultured at 26 °C in the dark for 18 days (Fig. 1).

The formed 18-day old callus tissues had been transferred to regeneration MS medium, supplemented by vitamins of Gamborg and growth regulators, which are shown in Table 1. It was cultured at 24 °C and 16-h photoperiod.

Shown the variable components only that were used in media for regeneration. They were compared with the control medium: in the case of dicamba — medium MS, supplemented with vitamins of Gamborg, 10 mg/l $AgNO_3$ and 1 mg/l BAP; if picloram — MS medium supplemented with vitamins of Gamborg, 10 mg/l $AgNO_3$ and 0.5 mg/l BAP was used.

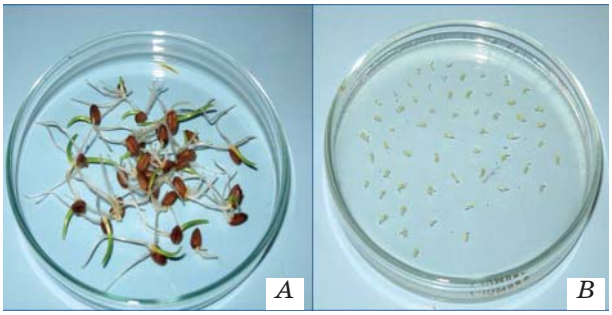

Fig. 1. **Seedling of the wheat cultivar Zymoyarka on the 3rd day of germination (*A*) and separated from their apical meristem (*B*)**

In the experiment the shoot regeneration features of 325 callus samples on media with dicamba (Duchefa Biochemie, 3,6-dichloro-2-methoxybenzoic acid), and 327 — with picloram (Duchefa Biochemie, 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid) were investigated. The explants were cultured in Petri dishes (50 pcs. in a cup). The quantity of the morphogenic callus samples was noted on the 18th day of cultivation. The analysis of plant regeneration from 20th to 35th day was performed every 5 days. MS medium for regenerating supplemented with vitamins of Gamborg, 10 mg/l $AgNO_3$ and BAP (1 mg/l and 0.5 mg/l depending on the version of the experiment) was used as a control. The percentage of shoot formation was determined as the ratio of the number of explants that formed the regenerated plants, to the total number of explants.

The acquired regenerated plants were separated from callus and planted on MS culture medium with two times reduced sucrose content, supplemented with 0.1 mg/l NAA (a-naphthylacetic acid) for the rhysogenesis initiation. Rooting lasted for 2 weeks at a 24 °C and 16-hour photoperiod. Regenerants with well-developed root system were adapted to septic conditions using sphagnum moss as a primary adaptive substrate. Plants were subjected to quenching by increasing the

Table 1. **Composition of the culture MS media for the shoot regeneration from callus of wheat cultivar Zymoyarka**

10 mg/l $AgNO_3$, vitamins of Gamborg		
1 mg/l BAP + dicamba		0.5 mg/l BAP + picloram
1	0.2 mg/l	0.16 mg/l
2	0.4 mg/l	0.25 mg/l
3	0.6 mg/l	0.5 mg/l

outdoor exposure from 20 min per day till the noctidial stay throughout 2 weeks. Accustomed to non-sterile conditions regenerants were transplanted to an augmentation of yield, which consisted of peat, sod land and sand in the ratio 2:1:1 and cultivated in a greenhouse under conditions of natural light and temperature 22–28 °C.

The studies were performed independently in triplicate by the following general scheme:

1) the same culture media were used;
2) the same physical factors (temperature, lighting, etc.) were applied;
3) the time to collect data was agreed.

The results were statistically processed using Microsoft Excel. To confirm the accuracy of the results described above the studies were performed in triplicate. This approach provided the reproducible conditions of experiment, because in all three cases the differences between readings of regeneration were not observed. For authenticity the expected least significant difference (NCI), which at $P < 0.05$ was 3.23, and the relative error of experiment (5.4%) were calculated.

Results and Discussion

According to the literature [16] cells in the G1-phase dominate in the early stages of cultivation that causes the transition to specialize callus tissue. For induction of morphogenesis it is advisable to use young callus, which reached sufficiently large size (5–7 mm) with cells that are actively dividing, and which generally provides the ultimate realization of regenerative potential of wheat.

Regenerative ability of cereal plants depends on the availability of formed callus morphogenic zones [17]. On the 15th day of callus culturing under light conditions, the appearance of green cells was noted. This callus was attributed to the morphogenic type. Later, some of them formed the regenerated plants. Development of shoots began on the third week of cultivation.

In the experiment to determine the impact of dicamba on the regenerative ability of wheat callus it was discovered that the investigated growth regulator generally promotes the formation of regenerants. However, the high concentration of dicamba (0.6 mg/l) slows down the growth of callus, reducing the frequency of formation of morphogenic zones and thus regeneration compared to the lower concentration (Table 2). Besides, the initiation of the regenerative processes had started two times later (Fig. 2).

It is shown the average frequency of regeneration in % (with error) for each of the studied concentrations of growth regulators. The regeneration media were compared with each other and the most effective one was determined. This turned out MS medium supplemented with vitamins of Gamborg, 10 mg/l $AgNO_3$, 0.5 mg/l BAP and 0.16 mg/l picloram.

As the concentration was reduced, the acceleration of the morphogenic zones and shoot-regenerants formation had been detected. Visual observations also confirmed

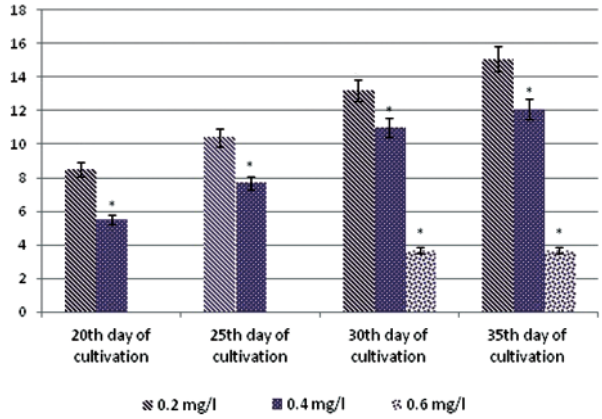

Fig. 2. **Influence of different concentrations of dicamba on regenerative ability (%) of callus cultures of wheat cultivar Zymoyarka**
The control is not shown. The impact of concentration of growth regulator is compared only. Hereinafter, * $P < 0.05$.

Table 2. **The frequency of callus formation and regeneration**

0.5 mg/l BAP + picloram			1 mg/l BAP + dicamba		
Concentration, mg/l	% of morphogenesis	% of regeneration	Concentration, mg/l	% of morphogenesis	% of regeneration
0.16	60±4.2*	35.5±2.0*	0.2	91.4±4.3*	15.1±0.9*
0.25	40±1.7	25.1±1.1	0.4	89.1±4.0	12.1±1.0
0.5	36.4±1.9	25±1.1	0.6	25±1.2	3.7±0.2

the improvement of the physiological state of cultures. It should be noted that the formation of a large number of morphogenic sites was observed on culture medium supplemented with 0.4 mg/l dicamba, though organogenesis in this case took place mainly as rhyzogenesis, described in the literature [18]. The shoot rate of formation in the callus under the influence of 0.4 mg/l dicamba has gradually increased within 15 days, and the on the last stage of observation the regeneration had almost stopped (Fig. 3).

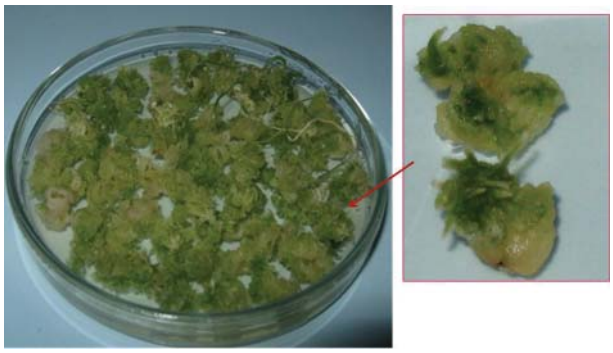

Fig. 3. **Organogenesis of cultured wheat callus on MS regeneration medium supplemented with 0.4 mg/l dicamba**

The lowest content of dicamba (0.2 mg/l) in the medium was found to be the most effective for getting regenerants. At each stage of regeneration throughout the whole 20 days the emergence of new shoots was noticed, therefore the callus had kept morphogenic capability until the end of the experiment (Fig. 2). At the later stages of the study the regeneration slowed down and callus gradually lost the ability of morphogenesis.

Similar to experiment with the use of dicamba, we found that low concentrations of picloram in the culture medium had positive effect on morphogenetic processes in the wheat callus. Thus, at the lowest concentration (0.16 mg/l) of the substance the highest regenerative activity had been observed: the formation of the greatest number of morphogenic sites (up to 60%) and a significant number of plant regenerants on the 30^{th}–35^{th} day of cultivation (Fig. 4).

It should be noted that after the 30^{th} day of cultivation the regenerant quantity increase had stopped. This phenomenon suggests that the regeneration potential of wheat callus had been exhausted during the aforementioned period.

The increase in concentrations of picloram to 0.25 mg/l and 0.5 mg/l causes a decrease in the number of morphogenetic zones: in the first case by 10%, and in the second — by 36.4%. At high concentrations of the studied regulator, necrotic processes in the large regions of the callus and reduced regeneration activity were spotted. Thus, increasing the concentration of picloram in the culture medium adversely affects morphogenetic capability and regeneration of shoots of wheat.

To confirm the positive effect of selected concentrations of picloram (0.16 mg/l) not only the 18-day calli but the older, such as 30-days old ones were tested, for which the same pattern had been observed. However, the plant regenerants formation rate was lower compared to the previous version (Fig. 5).

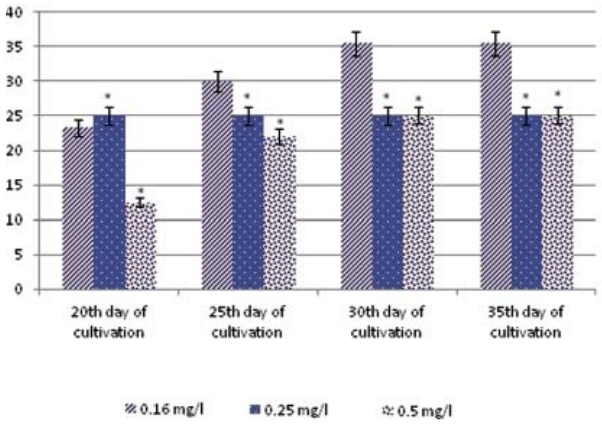

Fig. 4. **Rate of formation of plant regenerants (%) on the MS culture medium supplemented with different concentrations of picloram**

Similarly, Fig. 2 shows the regenerative ability of callus cultures of wheat (%) depending on the content of different concentrations of picloram in combination with 0.5 mg/l BAP.

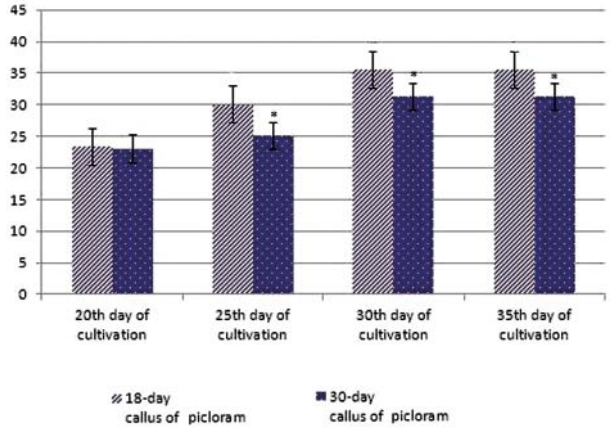

Fig. 5. **Regenerant formation (%) in the 18 and 30-days old callus on medium supplemented with 0.16 mg/l picloram**

Comparing the above experiments with both growth regulator options investigated, the regenerative MS medium supplemented with 0.5 mg/l BAP and 0.16 mg/l picloram had proven to be the most effective (Fig. 6).

In addition to the active formation of meristematic cells, regeneration of shoots and roots in a given environment, the overall physiological condition of the callus during all phases of the experiment was satisfactory. Importantly, in the later stages of cultivation (30–35 days) these parameters remained at a stable high level. The dicamba presence in the culture medium provided relatively lower regeneration potential, and a smaller number of morphogenic zones. Much of the callus remained non morphogenic, moving in the stationary phase of growth and showing signs of aging.

Research shows that stems derived from callus cultures can form *in vitro* roots and adapt to septic conditions. Adapted regenerated plant, cultivated under greenhouse conditions had shown high viability (over 75%) and reached the generative stage of development (Fig. 7).

The results are also consistent with the statement that the highest capacity for callus morphogenesis is characteristic for the callus of compact structure and slow growth speed [19]. Therefore, in the current study, the same 18-day callus was used that had all the characteristics mentioned above, and was sufficiently large (5–7 mm) and still quite young, to ensure the best possible regeneration potential of wheat.

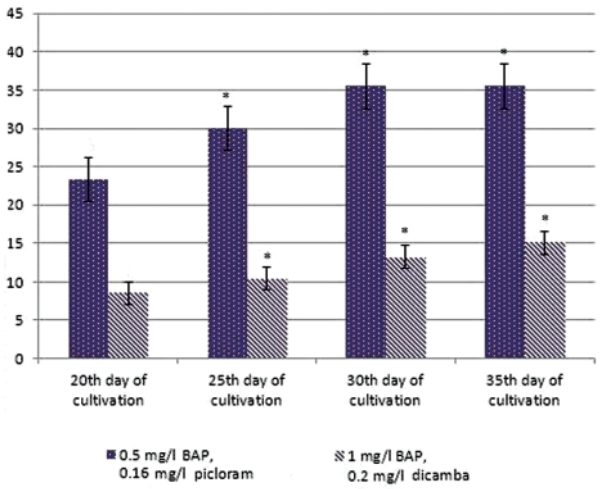

Fig. 6. **Comparative characteristic of the callus regenerative capacity (%) on MS nutrient medium supplemented with 0.5 mg/l BAP in combination with 0.16 mg/l picloram and 1 mg/l BAP in combination with 0.2 mg/l dicamba**
Because the effects of 0.16 mg/l picloram and 0.2 mg/l dicamba were compared between each other the control is not shown in this figure.

Fig. 7. **Rooting (*A*) and adaptation of plants-regenerants to the soil conditions (*B, C*)**

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ВПЛИВ СИНТЕТИЧНИХ АУКСИНОПОДІБНИХ РЕГУЛЯТОРІВ РОСТУ НА РЕГЕНЕРАЦІЙНУ ЗДАТНІСТЬ КАЛЮСУ М'ЯКОЇ ПШЕНИЦІ СОРТУ ЗИМОЯРКА

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Метою дослідження було встановити залежність морфогенетичних реакцій калюсних тканин пшениці від вмісту в живильному середовищі синтетичних регуляторів росту ауксинової природи (піклорам, дикамба).

Первинними експлантами для калюсогенезу слугували апікальні меристеми пшениці *Triticum aestivum*. Для регенерації використано базове живильне середовище MS, доповнене вітамінами за Гамборгом, різними концентраціями дикамби (0,2; 0,4; 0,6 мг/л) та піклораму (0,16; 0,25; 0,5 мг/л). Встановлено, що для одержання регенерантів найефективнішою є дикамба в концентрації 0,2 мг/л. За концентрації 0,16 мг/л піклораму спостерігається утворення максимальної кількості (до 60%) морфогенних осередків, а також значної кількості рослин-регенерантів. Збільшення концентрації піклораму до 0,25 мг/л та 0,5 мг/л спричинювало зменшення кількості морфогенних зон: у першому разі на 10%, а в другому — на 36,4%. Серед описаних варіантів найефективнішим є середовище MS, доповнене 0,5 мг/л 6-бензиламінопурину і 0,16 мг/л піклораму. Одержані з культури калюсу пагони здатні утворювати корені *in vitro* та адаптуватися до септичних умов. Рослини-регенеранти за культивування в умовах захищеного ґрунту виявляють високу життєздатність (понад 75%) і досягають генеративної стадії розвитку.

Ключові слова: регулятори росту, піклорам, дикамба, *Triticum aestivum*, культура *in vitro*.

ВЛИЯНИЕ СИНТЕТИЧЕСКИХ АУКСИНОПОДОБНЫХ РЕГУЛЯТОРОВ РОСТА НА РЕГЕНЕРАЦИОННУЮ СПОСОБНОСТЬ КАЛЛУСА МЯГКОЙ ПШЕНИЦЫ СОРТА ЗИМОЯРКА

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Целью исследования было установить зависимость морфогенетических реакций каллусных тканей пшеницы от содержания в питательной среде синтетических регуляторов роста ауксиновой природы (пиклорам, дикамба).

Первичными эксплантами для каллусогенеза были апикальные меристемы пшеницы *Triticum aestivum*. Для регенерации использована базовая питательная среда MS, дополненная витаминами по Гамборгу, различными концентрациями дикамбы (0,2; 0,4; 0,6 мг/л) и пиклорама (0,16; 0,25; 0,5 мг/л). Установлено, что для получения регенерантов наиболее эффективной является дикамба в концентрации 0,2 мг/л. При концентрации 0,16 мг/л пиклорама наблюдается образование максимального количества (до 60%) морфогенных очагов, а также значительного количества растений-регенерантов. Увеличение концентрации пиклорама до 0,25 мг/л и 0,5 мг/л вызывало уменьшение количества морфогенных зон: в первом случае на 10%, а во втором — на 36,4%. Среди описанных вариантов наиболее эффективной является среда MS, дополненная 0,5 мг/л 6-бензиламинопурина и 0,16 мг/л пиклорама. Полученные из культуры каллуса побеги способны образовывать корни *in vitro* и адаптироваться к септическим условиям. Растения-регенеранты при культивировании в условиях защищенного грунта проявляют высокую жизнеспособность (более 75%) и достигают генеративной стадии развития.

Ключевые слова: регуляторы роста, пиклорам, дикамба, *Triticum aestivum*, культура *in vitro*.