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Development of an effective technique for callus formation from mature embryos of *Triticum spelta* L. and *Triticum aestivum* L.

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Purpose. Introducing *in vitro* culture and obtaining calluses from mature embryos of 3 samples of spelt wheat with further quantitative comparison of their callusogenesis with 2 samples of soft wheat. **Methods.** For this study, 5 samples of hexaploid wheat were taken: 3 spelt and 2 soft wheat. Grain sterilization was performed with 96% ethyl alcohol and 5% sodium hypochlorite solution. Mature embryos were taken as *in vitro* explants. For callusogenesis, three types of Murashige and Skoog (MS) nutrient media with different component composition were used. The explants were cultured in the dark along 21 days. **Results.** The optimal conditions for the induction of tissue culture of *Triticum spelta* L. and *Triticum aestivum* L. from mature embryos were developed. The callus, obtained from the different samples, grown on the three types of modified MS nutrient medium did not differ morphologically. The spices of 'Europa' spelt, 'Bunchuk' and 'Elegiya Myronivska' soft wheat, regardless of the medium composition, differ by high callus formation efficiency, while the explants of 'Zorya Ukrainy' spelt produce slow callus formation. **Conclusions.** Mature embryo explants formed tissue culture of 3 spelt specimens and of 2 soft wheat specimens. It was found that the most effective for callus formation from the explants of mature soft wheat and spelt germs was the MS nutrient medium with 3% sucrose, supplemented by 2 mg/l of 2,4-D and 10 ml/l of argentum nitrate. The efficiency of callusogenesis on the 21st day of cultivation, depending on the sample, varied between 80.2% and 100.0%. The tested samples differed in their ability to form calluses on nutrient media with different component composition.

Keywods: *spelt; soft wheat; in vitro culture; callus; explants.*

Introduction

Wheat (*Triticum aestivum* L.) and spelt (*Triticum spelta* L.) are important industrial crops. It is known that in the world today the soft wheat sown areas cover about 220 million hectares and the annual grain harvest is about 749 million tons [1]. At the same time spelt, in contrast to soft wheat, occupies a narrow niche and is often in demand on the organic market. With regard to the economic spread of soft wheat and the demand for organic products, it is promising to create new varieties of soft wheat and spelt that will have improved economic value such as increased yield, resistance to lodging, biotic and abiotic stresses. Classical genetics does not have enough time to meet the modern needs of creating new promising plant material, so it is advisable to use biotechnological and genetic engineering approaches to solve this problem. It is important to obtain tissue culture *in vitro* of the original sample, to transfer the genes of economically valuable features and to create plant material with improved properties. Therefore, the first stage of this work is the introduction of the original working sample in culture *in vitro*.

It is known that plant regeneration *in vitro* is both direct and indirect. For the indirect type of *in vitro* regeneration, the callus formation stage is important. In the future, the cal-

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lus will regenerate into plant through organogenesis or somatic embryogenesis. Indirect regeneration is quite typical of cereal crops. Therefore, the formation of an organogenic callus is very important and necessary step in the development of an effective spelt regeneration system. In view of this, the efficacy of the callusogenesis stage for obtaining the spelt callus was studied.

For today, there is virtually no data on the effectiveness of spelt callusogenesis, as it is less studied than soft wheat, for which is known many approaches to callus production. Introduction to culture *in vitro* should begin with the selection of the type of explant. In most cases, the apical meristems of young seedlings [2], immature embryos [3] and mature embryos [4] are used as explants for soft wheat.

For the induction of callus formation, one can use the ratio of growth factors, artificial auxins and cytokinins, in which more auxins, namely 2,4-dichlorophenoxyacetic acid (2,4-D) [3, 5], picloram, 1-naphthylacetic acid (NOC) , thidiazuron (TDZ), indole-3-acetic acid (IOC), etc. and less (or even absent at all) cytokinins - kinetin [3], 6-benzylaminopurine (BAP), etc. In addition to reducing the risk of tissue culture contamination by various microorganisms, argentum nitrate can also be used in addition to antibiotics and fungicides [6].

It is known that to obtain calluses from mature embryos, you can use the basic MS medium and after three weeks of cultivation proceed it on the MS medium with 2 mg/l of 2,4-D [7]. The combination of 2,4-D with concentration of 2 mg/l and argentum nitrate with concentration of 10 mg/l are used for the callus formation from leaf explants [8]. The combination of 2,4-D (1.5 mg/l) and kinetin (0.5 mg/l) is used to obtain callus culture from wheat anthers on the nutrient medium C-17 [9]. Artificial auxin 2,4-D with concentration of 2 mg/l as part of the MS base medium can be used to induce callus formation of soft wheat [4, 10] and durum wheat [11]. MS-based growth medium with 2.5% sucrose and half of MS salinity (1/2 MS medium) with the 2 mg/l content of 2,4-D causes callus formation of soft wheat after two weeks of cultivation [12]. Increasing the concentration of 2,4-D to 3,0-3,5 mg/l has a positive effect on the formation of calluses from explants of mature grains [13]. The use of 2,4-D with concentration of 1 mg/l without the use of other growth regulators leads to the formation of compact calluses, but increases rhizogenesis and subsequently adversely affects on the quality of formed embryoids. For the formation of embryogenic callus the most optimal concentration of 2,4-D is 2 mg/l [14]. In his work, Baday S. J. S. [15] noted that for the induction of callusogenesis from explants of mature embryos, the most optimal concentrations of 2,4-D and kinetin in the MS base medium are 2.5 and 0.9 mg/l, respectively. It is also known that argentum nitrate with the concentrations of up to 10 mg/l contributes to the formation of somatic durum wheat embryoids for explants of immature embryos [16].

The purpose of the study was to introduce mature embryo explants into *in vitro* culture, to obtain calluses of 3 spelt specimens and to compare the effectiveness of their callusogenesis with 2 specimens of soft wheat.

Materials and methods of research

Five samples of hexaploid wheat were selected for the study: soft spring wheat - *Triticum aestivum* L. cultivar 'Elegiya Myronivska' (sample No. 1), winter - *Triticum aestivum* L. cultivar 'Bunchuk' (No. 2), winter spelt - *Triticum spelta* L. varieties of 'Alberta' (No 3), 'Europa' (No 4) and 'Zorya Ukrainy' (No 5). We chose these samples because 'Bunchuk' and 'Elegiya Myronivska' varieties are national standards for soft wheat and 'Zorya Ukrainy' variety for spelt. Two other spelt samples - 'Europa' and 'Alberta' were taken to compare the spelt data obtained. The seed samples were obtained from the collection of All-Ukrainian Scientific Institute of Breeding LLC.

Separated from the grain mature embryos were taken as explants for introduction into culture *in vitro*. The grains were sterilized with 96% ethyl alcohol for 5 min, 5% sodium hypochlorite for 10 min and three times washed in sterile distilled water. For easier separation of mature embryos from the grain, 2 hours pre-soaking in sterile distilled water was performed.

For the induction of callusogenesis we used three types of modified nutrient medium Murashige and Skoog (MS) [17]: MS No.1 supplemented by 1 mg/l of 2,4-D, 0,5 mg/l of picloram, 150 mg/l of ceftriaxone and 3% sucrose; MS No.2 supplemented by 2 mg/l of 2,4-D, 10 mg/l of AgNO3, 150 mg/l of ceftriaxone and 3% sucrose; MS No.3 supplemented by 2 mg/l of 2,4-D, 0,5 mg/l of kinetin, 150 mg/l of ceftriaxone, 2% sucrose and vitamins for nutrient medium [18].

The explants were uniformly (45-55 items) placed on Petri dishes with appropriate nutrient medium and cultured in the dark at 25°C for 21 days. 5 replicates were used for each

sample and type of culture medium. The intensity and nature of callusogenesis was assessed every 7 days. This took into account the size of calluses, their structure, color, frequency of formation and nature of growth.

The effectiveness of callus formation was determined by the formula (K / E) Ч 100, where, K is the number of explants on which the callus was formed, E is the total number of cultivated explants [19].

Results and Discussion

For *in vitro* culture, mature embryos of spelt and soft wheat were cultivated on three types of nutrient media to induce callusogenesis. We obtained *in vitro* culture of 'Alberta', 'Europa', 'Zorya Ukrainy' spelt tissues and soft wheat of 'Bunchuk' and 'Elegiya Myronivska'. The findings of callus formation efficiency are presented in Table 1.

Table 1

The efficiency of callus formation from mature embryos of 3 samples of spelt and 2 samples of soft wheat after 21 days of growth on nutrient media of different component composition

Cultivar	The efficiency of callus formation (%), $\bar{x} \pm S$		
	MS medium No.1	MS medium No.2	MS medium No.3
'Elegiya Myronivska'	95,0 ± 7,0	99,2 ± 1,7	91,6 ± 10,5
'Bunchuk'	96,6 ± 4,9	98,4 ± 3,5	94,8 ± 4,8
'Alberta'	86,4 ± 8,6	86,2 ±7,8	76,0 ± 17,1
'Europa'	97,8 ± 4,9	100,0 ± 0,0	98,0 ± 4,4
'Zorya Ukrainy'	72,0 ± 16,3	80,2 ± 19,7	64,0 ± 15,3

Note. $\bar{x}$ – mean, S – mean sampling error.

It is shown that the test specimens differ in their ability to form a callus. The highest percentage of callusogenesis was observed with nutrient medium MS No. 2. It varied within 80.2-100.0% for 21 days of growth for different samples (Table 2). Thus, on the 7th day of cultivation, the percentage of callusogenesis efficiency was not less than 59.8%, and on the 21st day - it already exceeded 80.2%. For this nutrient medium, the most effective callus formation was found for the sample of 'Europa' spelt for 21 days of growth - 100% (Table 2). The lowest efficiency of callus formation was in the sample of the spice variety 'Zorya Ukrainy'. On the 7th day of callusogenesis it was 59.8%, and on the 21st day - 80.2% (Table 2). Wheat specimens of 'Bunchuk', 'Elegiya Myronivska' and 'Alberta' spelt showed average callusogenesis efficiencies compared to the above. On the 7th day of growth on the nutrient medium MS No. 2, these samples showed the following results: 90,2; 95.0 and 75.0%, respectively (Table 2). On the 21st day of cultivation, the average efficiency of callusogenesis increased to 99.2; 98.4 and 86.2%, respectively (Table 2). Based on the results obtained, it can be assumed that 2,4-D with the concentration of about 2 mg/l and argentum nitrate with the average concentration (about 10 mg/l) have the positive effects on the efficiency of callusogenesis.

Hussein K. Zaire Al-Kaaby et al. [6] note that argentum nitrate plays a role in the formation of embryogenic callus. High concentrations (more than 10 mg/l) of this agent are capable of causing an inhibition of callus formation as well as low (less than 5 mg/l). Argentum nitrate with the concentration of 5-10 mg/l has a positive effect on the formation of embryogenic callus [6].

It was found that the addition of 2,4-D with concentration of 2 mg/l into the nutrient medium leads to 97.6–100.0% range of mature embryos callus formation depending on the sample [20].

Table 2

The dynamics of callus formation of 3 spelt specimens and 2 specimens of soft wheat on MS No. 2 nutrient medium along 21 days of growth

Cultivar	The efficiency of callus formation (%), $\bar{x} \pm S$		
	7th day of culturing	14th day of culturing	21st day of culturing
'Elegiya Myronivska'	90,2 ± 10,1	92,8 ± 5,0	99,2 ± 1,7
'Bunchuk'	95,0 ± 4,5	98,4 ± 3,5	98,4 ± 3,5
'Alberta'	75,0 ± 11,7	77,8 ± 10,6	86,2 ± 7,8
'Europa'	95,0 ± 5,0	97,2 ± 4,7	100,0 ± 0,0
'Zorya Ukrainy'	59,8 ± 13,4	68,6 ± 14,3	80,2 ± 19,7

Note. $\bar{x}$ – mean, S – mean sampling error.

The next nutrient medium suitable for receiving callus was MS No.1 (Table 3). On the 7th day, average values of callus formation efficiency varied within 64.2-96.6%; and on the 21st day of cultivation - 72,0-97,8% (Table 3). Similar to MS No.2 medium, MS No.1 showed the most efficient callusogenesis for Europa spelt on the 21st day with the average callusogenesis value 97.8% (Table 1), which is slightly lower than during cultivation on MS No.2 (100%) (Table 1). Spelt 'Zorya Ukrainy' showed again the lowest efficiency of callusogenesis - 72.0% on the 21st day of cultivation (Table 3), which was even less than in the case with MS No.2 nutrient medium (80.2%) (Table 1).

Parmar et al. [21] noted that picloram with the concentrations of 2 mg/l and 4 mg/l causes 100% induction of callus formation. Picloram with the concentration higher than 4 mg/l increases the percentage of regeneration (68.0% versus 63.0%).

Among the three nutrient media studied, MS No.3 was the least effective for callus forma-

Table 3

The dynamics of callus formation of 3 spelt specimens and 2 specimens of soft wheat on MS No.1 nutrient medium along 21 days of growth

Cultivar	The efficiency of callus formation (%), $\bar{x} \pm S$		
	7th day of culturing	14th day of culturing	21st day of culturing
'Elegiya Myronivska'	78,6 ± 26,2	95,0 ± 7,0	95,0 ± 7,0
'Bunchuk'	96,6 ± 4,9	96,6 ± 4,9	96,6 ± 4,9
'Alberta'	74,4 ± 13,1	82,6 ± 3,9	86,4 ± 8,6
'Europa'	92,8 ± 6,3	94,0 ± 7,1	97,8 ± 4,9
'Zorya Ukrainy'	64,2 ± 17,9	67,6 ± 12,5	72,0 ± 16,3

Note. $\bar{x}$ – mean, S – mean sampling error.

Table 4

The dynamics of callus formation of 3 spelt specimens and 2 specimens of soft wheat on MS No.3 nutrient medium along 21 days of growth

Cultivar	The efficiency of callus formation (%), $\bar{x} \pm S$		
	7th day of culturing	14th day of culturing	21st day of culturing
'Elegiya Myronivska'	85,0 ± 7,4	85,0 ± 7,4	91,6 ± 10,5
'Bunchuk'	89,8 ± 8,0	92,0 ± 5,1	94,8 ± 4,8
'Alberta'	65,2 ± 24,1	66,2 ± 23,3	76,0 ± 17,1
'Europa'	91,0 ± 15,1	98,0 ± 4,4	98,0 ± 4,4
'Zorya Ukrainy'	47,2 ± 20,1	49,2 ± 22,2	64,0 ± 15,3

Note. $\bar{x}$ – mean, S – mean sampling error.

tion. On the 7th day of cultivation, the callusogenesis efficiency varied within 47.2–91.0% and on the 21st day 64.0–98.0%. In general, the cultivars 'Europf' and 'Zorya Ukrainy' have shown the ability to form callus on nutrient media with different components. The 'Europa' spelt sample showed the highest callus formation efficiency - 98.0% on the 21st day. Spelt 'Zorya Ukrainy' was the least effective in calluses forming - 64.0% on the 21st day (Table 4). It can be assumed that the ability to make a callus for specimens of the spelt varieties 'Europa' and 'Zorya Ukrainy' is a genetically determined trait that will manifest equally on the different nutrient media under different cultivation conditions.

It is known that low concentrations of kinetin (1 mg/l) can positively affect on callus formation of hexaploid wheat [3]. However, in our study, the nutrient medium MS with this growth regulator with the concentration of 0.5 mg/l showed the lowest percentage of callusogenesis efficiency - 47.2%, while for MS No.1 - 72.0% and MS No.2 - 80,2% on the 21st day. For the induction of callus formation from explants of immature embryos also use the base MS medium with casein hydrolyzate (300 mg/l), L-glutamine (500 mg/l), L-proline (500 mg/l) and with 2,4-D (mg/l) and kinetin (0,5 mg/l) as the growth regulators. If you add 2–10 mg/l of argentum nitrate to this nutrient medium, it positively contributes to callus formation (81.7–95.0% callus formation frequency) and reduces necrosis (11.7–1.7% callus necrosis rate), depending on the sample [22]. Very often, for the callus formation induction from explants of mature wheat germs, a nutrient medium with 2,4-D (2 mg/l) is used without additional auxins and cytokinins [4].

In general, calluses of different specimens and those, formed on different nutrient media did not differ morphologically. Calluses of 'Europa' spelt and 'Bunchuk' soft wheat are presented in figures 1 and 2 respectively. Up to the 14th day of growth on the nutrient medium calluses had a loose structure and were white or sometimes light yellow in color. The sizes of calluses mostly varied from 3 to 8 mm, for most of the samples (excepting the sample of 'Zorya Ukrainy' spelt) they were 3-4 mm after 7 days of growth, and closer to 21st day of observations they reached 6-8 mm.

During the study, it was observed that some specimens of spelt and soft wheat may efficiently or inefficiently form calluses that do not depend on the nutrient medium they grow on.

The samples studied differed in their speed of callus formation and their growth. The rate of callusogenesis can be estimated by the effectiveness of callus formation on the 7th day of cultivation. In particular, the best and quickest growth was typical for calluses formed by the specimens of 'Europa' spelt - 92.8% (MS No.1), 95.0% (MS No.2), 91.0% (MS No.3) and soft wheat 'Bunchuk' - 96.6% (MS No.1), 95.0% (MS No.2), 89.8% (MS No.3); slowest – 'Zorya Ukrainy' spelt - 64.2% (MS No.1), 59.8% (MS No.2), 47.2% (MS No.3). Samples of 'Alberta' spelt and of 'Elegiya Myronivska' soft wheat were characterized by a moderate rate of callus formation, namely 74.4% (MS No.1), 75.0% (MS No.2), 65.2% (MS No.3).) - 'Alberta' spices and 76.6% (MS No.1), 90.2% (MS No.2), 85.0% (MS No.3) - 'Elegiya Myronivska' spices.

In addition to callus formation, induced organogenesis was observed in some cases. Some specimens, including the 'Alberta' and 'Zorya Ukrainy' spices (Figs. 1, 3), on different types of nutrient media, on the 6th -7th days of cultivation, formed light yellow shoots that reached 45 mm by 21 day. It should be noted that the explants from which shoots sprouted continued to form calluses. The number of explants from which shoots were formed was less than 10%.

The results obtained of the effectiveness of spelt callus formation are the first stage for the development of *in vitro* methods for introducing spelt samples into the culture, creating an effective system for their regeneration and subsequent production of biotechnological

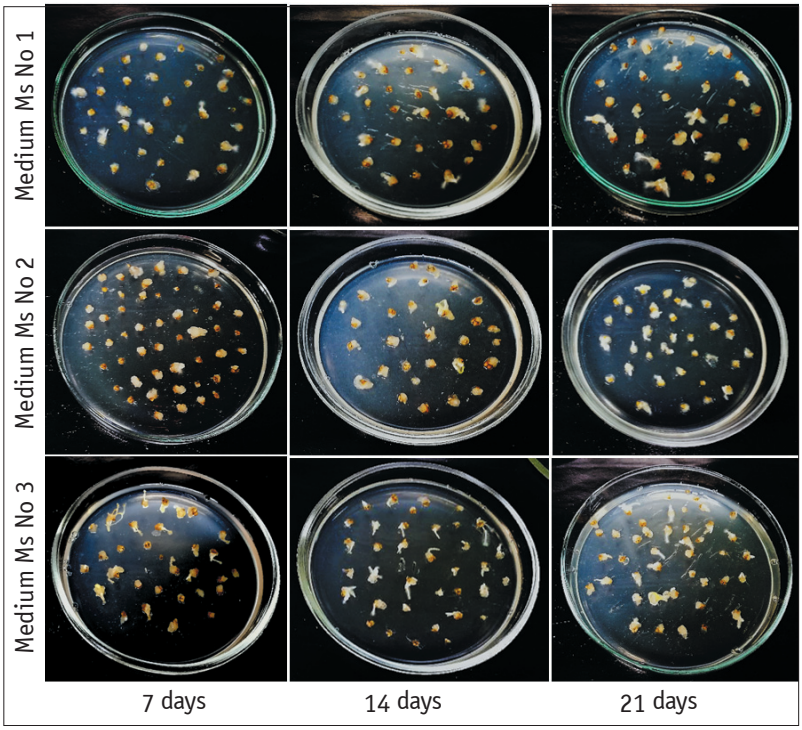

Fig. 1. Formation and growth dynamics of callus specimens of *T. spelta* L. 'Europa' on different types of nutrient medium

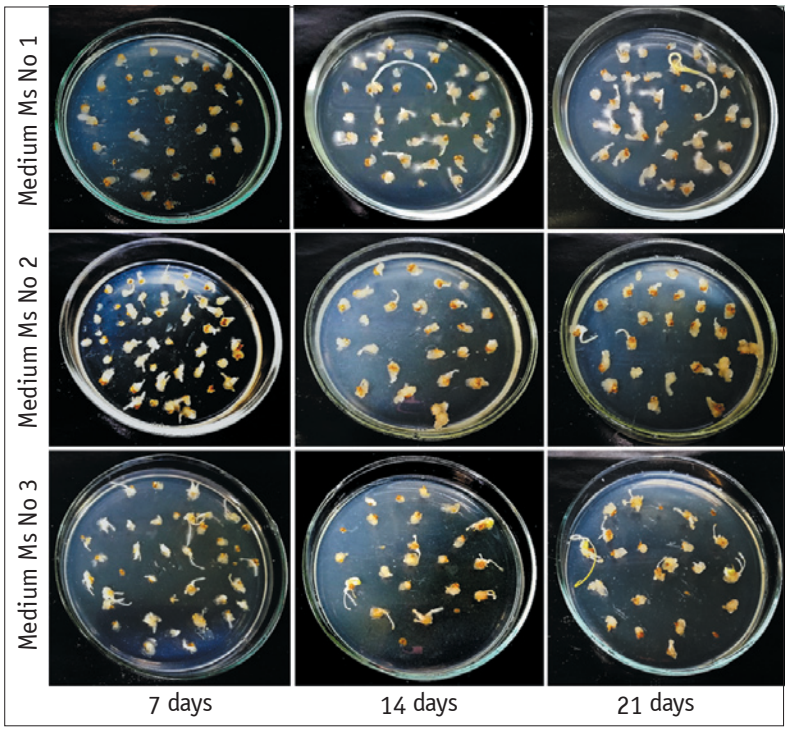

Fig. 2. Formation and dynamics of callus growth of *T. aestivum* L. 'Bunchuk' sample on different types of nutrient medium

plants (genetically modified plants, plants with edited genome, etc.).

Conclusions

An *in vitro* tissue culture was obtained for 3 specimens of spelt and 2 specimens of soft wheat using mature embryos as explants. It was found that the most effective for callus formation of spelt and soft wheat mature embryos explants was MS nutrient medium supplemented by 2 mg/l of 2,4-D, 10 ml/l of argentums nitrate and 3% sucrose. The efficien-

cy of callusogenesis of different samples on the 21st day of culturing was 80.2-100.0%.

It was shown that the tested samples differed in their ability to form calluses from mature embryos on nutrient media with different component composition. Some specimens showed genetic predisposition to callus formation from mature embryos irrespective of the modified composition of the MS medium, including spelt 'Europa', soft wheats of 'Bunchuk' and 'Elegy Mironovska', while explants of 'Zorya Ukrainy' spelt showed the slow formation of the callus.

The most effective callus formation was in the sample of the spelt 'Europa', the rate of which on the 7th day fluctuated within 91,0-95,0%.

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Цель. Введение в культуру *in vitro* и получение каллюса от зрелых зародышей 3 образцов спельты и сравнение эффективности их каллюсогенеза с 2 образцами пшеницы мягкой. **Методы**. Для работы взяты 5 образцов гексаплоидной пшеницы – 3 спельты и 2 пшеницы мягкой. Поверхностную стерилизацию зерна проводили в 96% этиловом спирте и 5% растворе гипохлорита натрия. В качестве эксплантов использовализрелые зародыши. Для калюсогенеза использовали три типа питательных сред MS с различным компонентным составом. Экспланты культивировали в темноте 21 день. **Результаты.** Подобраны оптимальные условия для индукции культуры тканей *Triticum spelta* L. и *T. aestivum* L. из зрелых зародышей. Полученные каллюсы из разных образцов, которые выращивали на трех типах питательных сред MS, не отличались между собой морфологически. Наблюдали генетическую предрасположенность к каллюсообразованию образцов спельты сорта 'Європа' и пшеницы мягкой сортов 'Бунчук' и 'Елегія Миронівська' независимо от состава среды MS в то время, как на эксплантах спельты сорта 'Зоря України' происходило медленное формирование каллюса. **Выводы.** Получено культуру тканей 3 образцов спельты и 2 образцов пшеницы мягкой с использованием в качестве эксплантов зрелых зародышей. Установлено, что наиболее эффективной для каллюсообразования из эксплантов зрелых зародышей пшеницы мягкой и спельты была питательная среда, дополненная 2 мг/л 2,4-Д, 10 мл/л нитрата серебра и содержащая 3% сахарозы. Среднее значение эффективности каллюсогенеза при этом составляло 80,2–100,0% на 21 сутки выращивания. Исследуемые образцы отличались между собой способностью формировать каллюс на питательных средах с различным компонентным составом. Впервые в Украине исследована эфективность каллюсогенеза спельты.

Ключевые слова: *спельта; пшеница мягкая; культура in vitro; каллюс; экспланты.*

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