UDC 581.143.5:633.11:577.182.76:632.954 https://doi.org/10.7124/FEEO.v34.1640

NITOVSKA I. O.[1]✉, PANASENKO A. S.[1], REDKO V. Yu.[2], ZADOROZHNA M. I.[2], MORGUN B. V.[1]
[1] Institute of Cell Biology and Genetic Engineering, NAS of Ukraine,
Ukraine, 03143, Kyiv, Akademika Zabolotnoho str., 148, ORCID: 0000-0002-8976-5725, 0009-0007-9802-2679, 0009-0003-4606-0156, 0000-0001-7041-6894
[2] National University of "Kyiv-Mohyla Academy",
Ukraine, 04655, Kyiv, Hryhoriia Skovorody str., 2
✉ *iranit@ukr.net, (067) 598-44-03*

DETERMINATION OF PHOSPHINOTHRICIN AND PAROMOMYCIN SELECTIVE CONCENTRATIONS FOR OBTAINING TRANSGENIC SPELT PLANTS

Aim. To determine the selective concentrations of phosphinothricin and paromomycin for the selection of transgenic plants of spelt wheat. ***Methods.*** Shoot apical meristem culture, mature embryo culture, *Agrobacterium*-mediated genetic transformation. ***Results.*** Isolation and cultivation of shoot apical meristems of seedlings from three spelt genotypes and mature embryos from three other genotypes were carried out. A high frequency (from 80 to 100 %) of callus induction from explants was observed. It was shown that the addition of 5 mg/l of phosphinothricin or 100 mg/l of paromomycin to the culture medium almost completely inhibited plant regeneration compared to the control. After *Agrobacterium*-mediated genetic transformation of calli with a vector containing the phosphinothricin-N-acetyltransferase gene, regeneration of spelt shoots for one genotype was observed on a selective medium with 5 mg/l phosphinothricin. ***Conclusions.*** The selective concentrations of herbicide and antibiotic for obtaining transgenic spelt wheat plants with the corresponding marker genes are 5 mg/l for phosphinothricin and 100 mg/l for paromomycin.

Keywords: *Triticum spelta* L., spelt wheat, shoot apical meristem culture, mature embryo culture, plant genetic transformation.

Common wheat (*Triticum aestivum* L.) plays a vital role in maintaining global food security and resources, accounting for a fifth of total calorie and protein consumption [1]. Despite its widespread distribution and annual growth in production volumes, wheat is vulnerable to many biotic and abiotic factors, posing a serious threat to its cultivation in the future. Therefore, researchers are increasingly turning their attention to ancient cereals, in particular spelt (*T. spelta* L.), as this type of wheat is most closely related to common wheat. Spelt has a lower yield (70–80 % of the yield of bread wheat), husk-covered grains complicating sowing and threshing procedures, but its grain has a higher nutritional value [2]. The plant is resistant to diseases and undemanding to growing conditions, freely interbreeding with tetraploid and hexaploid species. Such features make spelt an attractive target for both organic farming and biotechnological research.

To obtain *in vitro* biotechnological plants of cereal crops, immature or mature embryos [3, 4], as well as shoot apical meristems [5, 6] and the callus collected from them, are most often used as explants, as they have a high regeneration potential. Although mature embryos (ME) and shoot apical meristems (SAM) usually have a lower morphogenetic potential compared to immature embryos (IE), the use of these explants in biotechnological projects is attractive because they can be obtained from seeds throughout the year without greenhouse use. To facilitate the selection of transgenic plants, selectable marker genes are used to ensure plant resistance to antibiotics or herbicides. Among them, the phosphinothricin-N-acetyltransferase (*pat*) gene, which provides resistance to phosphinothricin, and the gene of neomycin phosphotransferase II (*npt*II), which inactivates aminoglycoside antibiotics, such as kanamycin, paromomycin, neomycin, and others, are the most commonly used [7]. In the study, selective concentrations of the phosphinothricin and the paromomycin were determined for the biotechnological production of spelt plants in mature embryos and shoot apical meristems cultures.

Materials and methods

T. spelta seeds of the variety Europa (VNIS) and breeding lines Nos. 851, 853, 4093, 4114, 4130 (kindly provided by the Institute of Plant Physiology and Genetics of the National Academy of Sciences of Ukraine) were used in the work. To intro-

duce spelt into *in vitro* culture, surface sterilization of grains was carried out according to the method [6], using potassium permanganate, silver nitrate, and ethanol. Before sterilization, the grains were freed from the husks. Sterilized seeds germinated on MS medium [8] without growth regulators in the lighting regime with a 16-hour photoperiod at a temperature of 24°C for three days. Mature embryos were isolated from freshly sterilized seeds of breeding lines Nos. 4093, 4114, 4130. Shoot apical meristems were isolated from 3-day-old seedlings of spelt of the Europa variety and breeding lines Nos. 851 and 853. For callus induction, isolated explants were placed on modified N6 medium [9], which contained 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg/l dicamba, and 0.1 mg/l abscisic acid, or MS medium containing Morel's vitamins [10], 30 g/l sucrose, 5 mg/l $AgNO_3$, 2 mg/l 2,4-D, and grown in the dark at 27°C for two to three weeks. Callus formation frequency (CFF) was calculated as a percentage of explants that formed callus to the total number of explants. The obtained calli were planted on regeneration media (MS with 1 mg/l 6-benzylaminopurine (BAP) and 0.1 mg/l naphthylacetic acid (NAA), or with 0.25 mg/l BAP, or without phytohormones), containing phosphinothricin in concentrations of 3, 5, or 10 mg/l, or paromomycin in concentrations of 100, 150, or 200 mg/l, and without selective agents as a control and were cultured under the above conditions for a month. The experiment was performed three times.

Agrobacterium-mediated genetic transformation of calli obtained in the shoot apical meristems culture was carried out using the pCB203 vector [11] according to [12]. In days the calli were placed on the medium used in their induction, containing 500 mg/l of the antibiotic cefotaxime to inhibit the bacteria growth. After 4 days of cultivation on the bacteriostatic medium, calli were transferred to selective regeneration MSR medium (MS with 1 mg/l BAP and 0.1 mg/l NAA) containing 5 mg/l phosphinothricin and were cultured under the above conditions for a month. Calli, for which all steps of the transformation procedure were performed, except cultivation with bacterial suspension, served as a negative control.

Results and discussion

The use of the indicated method of grain surface sterilization led to 100 % sterility of the plant material. Seed germination was more than 95 % of the total number of processed grains, which is a fairly high indicator. A high frequency of callus formation was observed (Table 1), similar to other studies [4, 13–16].

During the cultivation of calli on media containing different concentrations of phosphinothricin, it was shown that the lowest tested concentration of the herbicide, 3 mg/l, partially suppressed the regeneration of plants from calli (Fig. 1). The application of an herbicide concentration of 5 mg/l almost completely inhibited the regeneration of plants of all studied genotypes. Under cultivation of calli on a medium containing 10 mg/l of phosphinothricin, the greening of calli and plant regeneration were absent. The results did not depend on which medium was used for callus induction. Therefore, the selective concentration of phosphinothricin for obtaining transgenic spelt plants with the selectable marker gene of phosphinothricin-N-acetyltransferase (*pat*) is 5 mg/l of the medium.

Table 1. Callus formation frequency (CFF) from different spelt explants on the two media

Genotype	Explant type	Medium	CFF, %*
Europa	SAM	N6	98.0 ± 1.9
		MS	96.7 ± 3.0
851	SAM	N6	99.6 ± 0.7
		MS	99.0 ± 1.8
853	SAM	N6	96.3 ± 4.5
		MS	97.3 ± 2.9
4130	ME	N6	92.3 ± 11.1
		MS	97.5 ± 5.0
4114	ME	N6	92.8 ± 10.4
		MS	98.1 ± 2.1
4093	ME	N6	80.5

Note. * data presented with standard deviation.

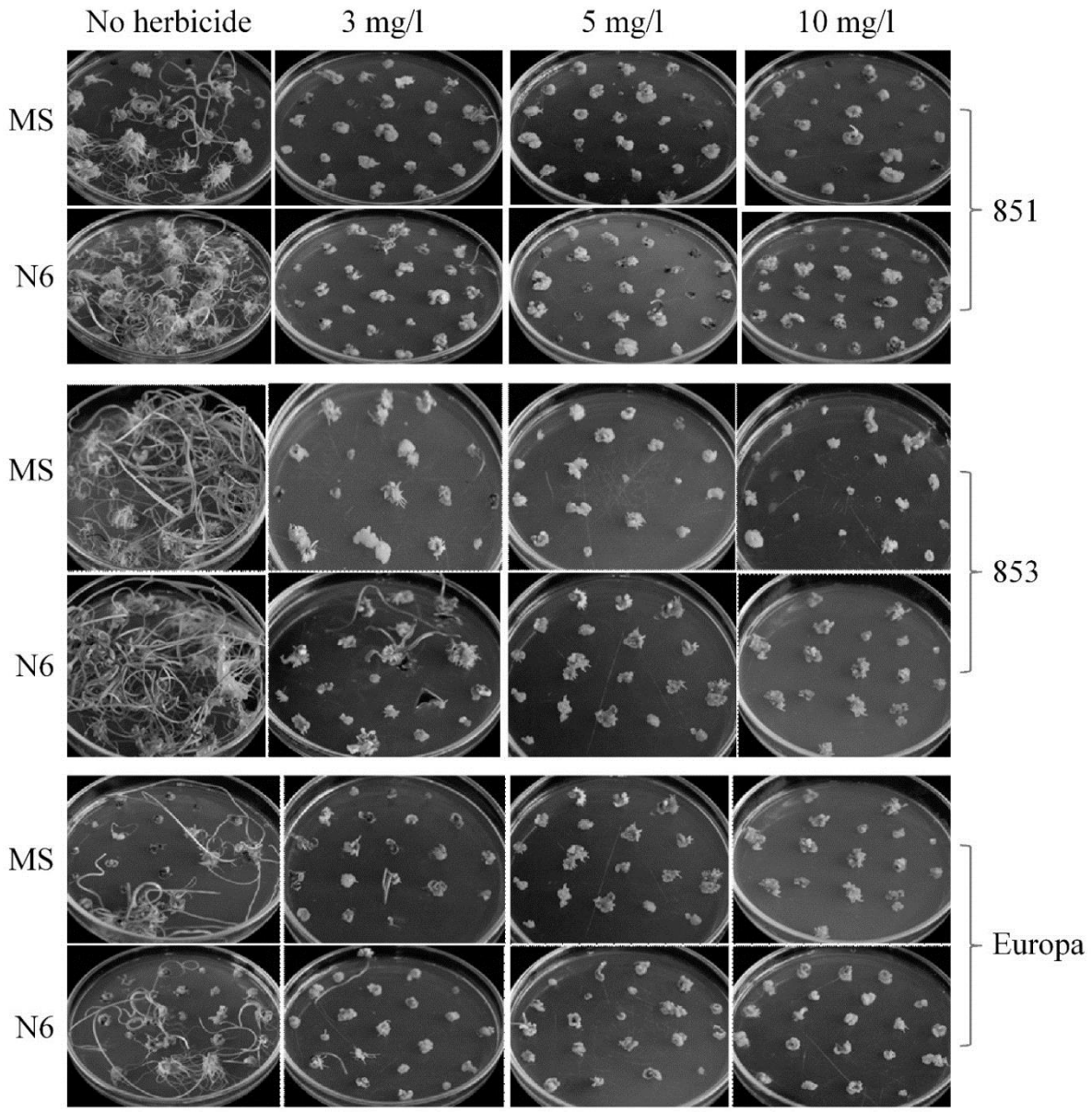

Fig. 1. The appearance of calli formed from the shoot apical meristems of spelt seedlings (variety Europa and breeding lines Nos. 851 and 853) on modified media for callogenesis (MS and N6), one month after planting on regeneration media MSR, which contained phosphinothricin in concentrations 3, 5, or 10 mg/l and no herbicide as a control.

Next, *Agrobacterium*-mediated genetic transformation of calli obtained from shoot apical meristems was carried out using the pCB203 vector, which contained the β-glucuronidase (*uid*A) gene under the control of the promoter and the first intron of the maize ubiquitin gene and the phosphinothricin-N-acetyltransferase (*pat*) gene under the control of bacterial nopaline synthase promoter [11]. After transformation, regeneration of plants of breeding line No. 853 was observed with a frequency of 35.3%, which was 3.5 times higher compared to the control (10%). The Europa variety and breeding line No. 851 were found to be sensitive to bacterial contamination (Fig. 2). They were characterized by a complete absence of callus greening and shoot regeneration, although the beginning of regeneration was observed in the control. Therefore, the spelt breeding line No. 853, which had the highest regeneration potential (data not shown), was the least sensitive to contamination by the bacterium *A. tumefaciens* and showed a high regeneration frequency on the selective medium after *Agrobacterium*-mediated transformation. Breeding line No. 853 is promising to obtain transgenic spelt plants

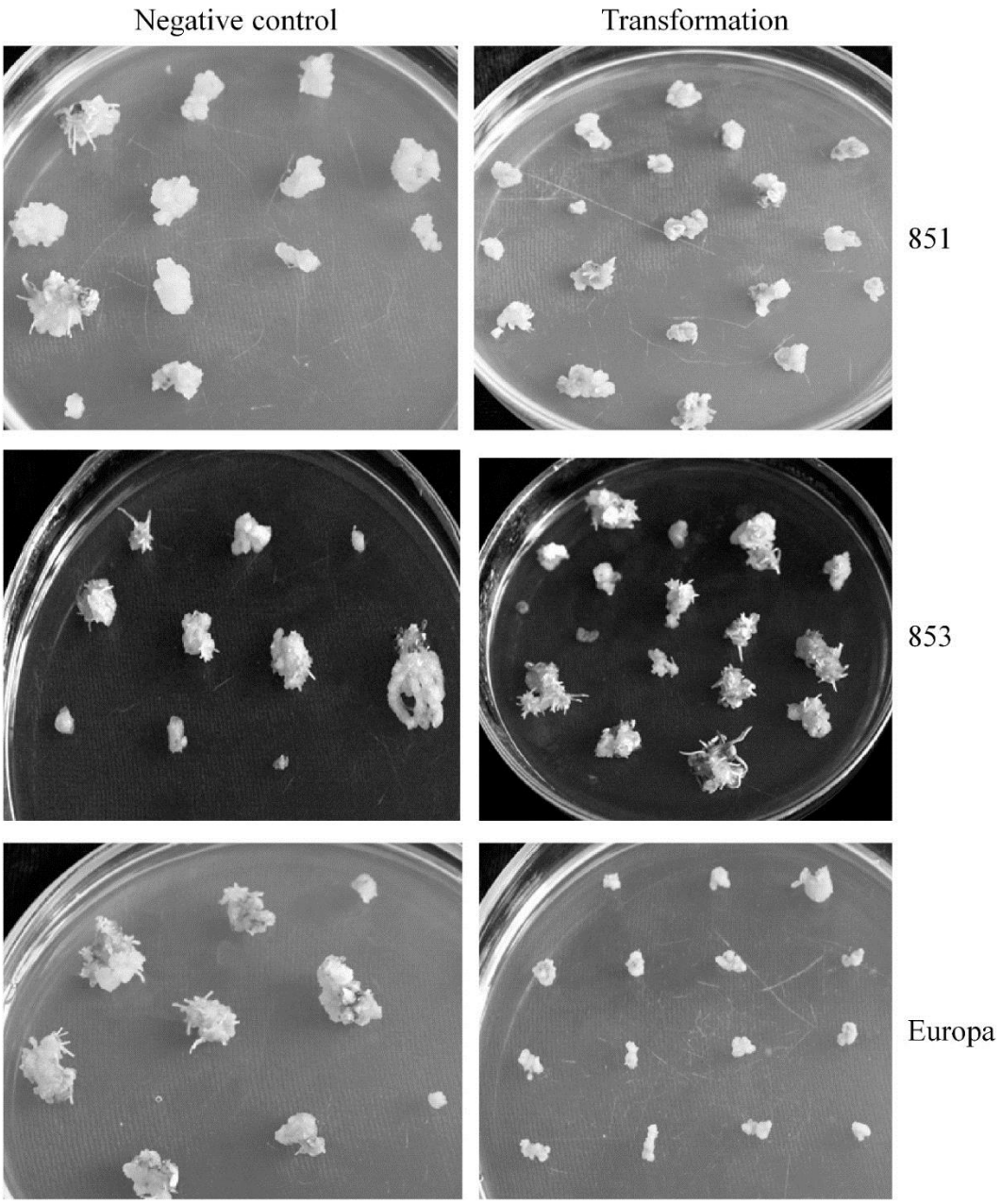

Fig. 2. The appearance of spelt calli (variety Europa and breeding lines Nos. 851 and 853) after *Agrobacterium*-mediated transformation following 4-week cultivation on the regeneration selective medium compared to the control.

During testing the effect of different concentrations of the antibiotic paromomycin on spelt plant regeneration, it was shown the use of the lowest concentration (100 mg/l) completely inhibited the greening of calli and the shoot regeneration from them, except line No. 4114, for which from callus obtained on a modified medium N6, regeneration of one plantlet was observed (Fig. 3). Compared to another aminoglycoside antibiotic kanamycin, paromomycin has a milder effect on plant tissues and did not have an irreversible effect on the regeneration potential of plant cells, which makes it possible to select regenerants in sufficient quantities [17]. Plant regeneration from tobacco leaves [17] and maize calli [18] were observed using 100 mg/l of paromomycin. Then the concentration of 100 mg/l is expected to be nontoxic to spelt callus. Therefore, the selective concentration of the antibiotic paromomycin for the production of transgenic spelt plants with the neomycin phosphotransferase II marker gene is 100 mg/l of the medium.

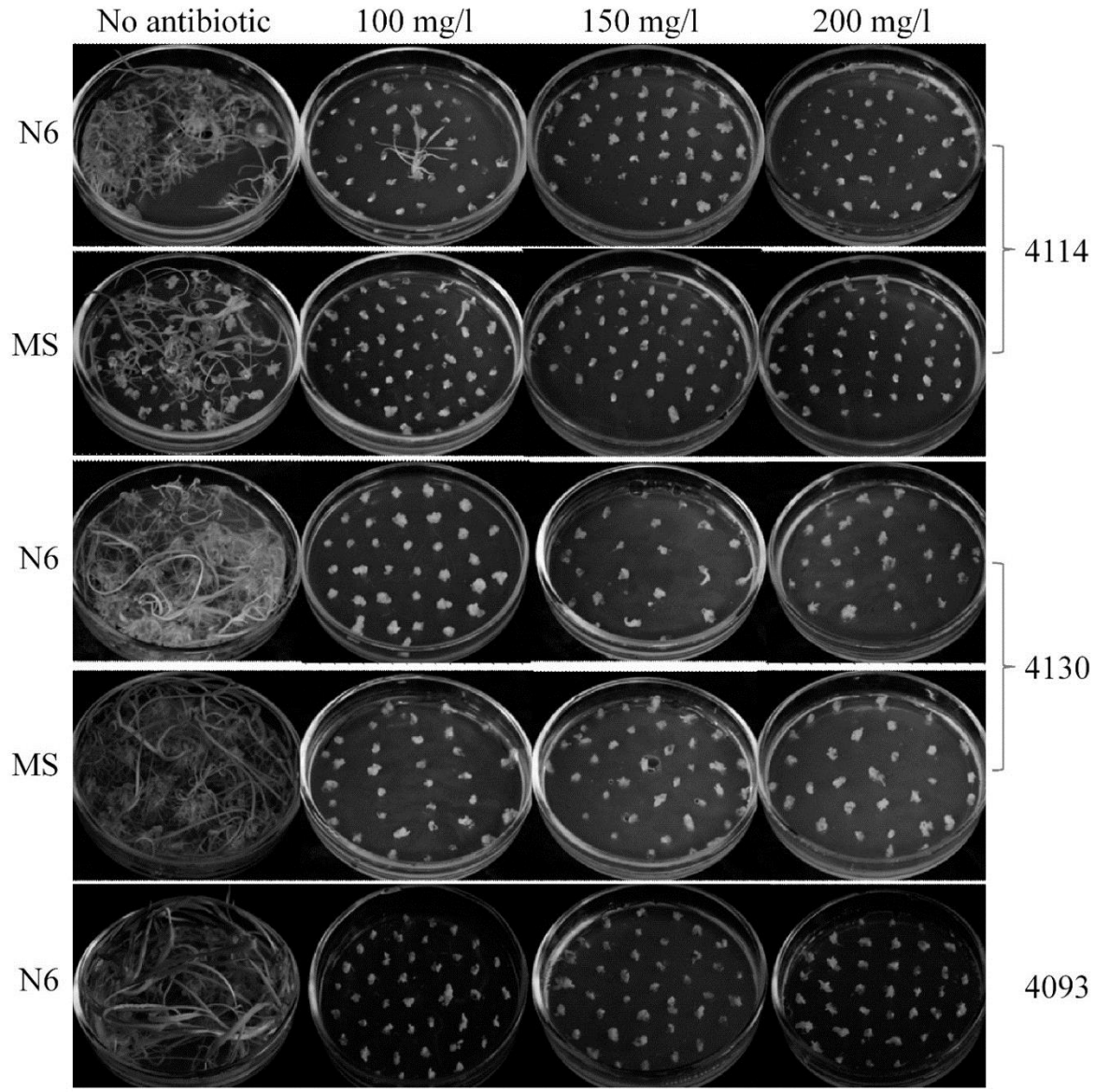

Fig. 3. The appearance of calli obtained in the mature embryos culture of spelt (breeding lines Nos. 4093, 4114, 4130) on modified media for callogenesis (MS and N6), one month after planting on regeneration media that contained paromomycin in concentrations of 100, 150, or 200 mg/l and no antibiotic as a control.

Conclusions

As a result of the conducted research, it was shown the applied technique for surface sterilization of spelt seeds is effective and ensures the getting of sterile material in its entirety. All studied spelt genotypes had a high callus formation frequency from explants placed on two different callus induction media. No significant difference in callus formation frequency was found among genotypes and explants. The determined selective concentrations of phosphinothricin and paromomycin to obtain transgenic plants with the corresponding marker genes were 5 mg/l for the herbicide and 100 mg/l for the antibiotic. Testing of the specified conditions to receive transgenic plants using *Agrobacterium*-mediated transformation showed their compliance with the tasks. Breeding line 853 highlighted as promising for production transgenic spelt plants using the shoot apical meristem culture.

This work was supported by the Project 0120U103770 "Biotechnology Development for Plants of the Genus Triticum to Increase Their Yield" of the Target Comprehensive Interdisciplinary Scientific Research Program of the National Academy of Sciences of Ukraine and by the Project 0123U100462 "Study of Vital Activity Peculiarities of Biotechnological Plants after Genomic Modifications" of the National Academy Agrarian Sciences of Ukraine.

References

1. Erenstein O., Jaleta M., Mottaleb K. A., Sonder K., Donovan J., Braun H. J. Global trends in wheat production, consumption and trade. In: Reynolds M. P., Braun H. J. (eds) Wheat Improvement. Springer, Cham. 2022. doi: 10.1007/978-3-030-90673-3_4.
2. Babenko L. M., Hospodarenko H. M., Rozhkov R. V., Pariy Y. F., Pariy M. F., Babenko A. V., Kosakivska I. V. *Triticum spelta*: Origin, biological characteristics and perspectives for use in breeding and agriculture. *Regul. Mech. Biosyst*. 2018 May 14. 9 (2). P. 250–257. Retrieved from: https://medicine.dp.ua/index.php/med/article/view/436.
3. Sidorov V. A. Plant tissue culture in biotechnology: recent advances in transformation through somatic embryogenesis. In Biochemistry and Biotechnology for Modern Medicine. Edited by S. Komisarenko. Kyiv : Publishing House Moskalenko O. M. NAS of Ukraine, 2013. P. 44481–44505. doi: 10.15407/biotech6.04.118.
4. Alikina O., Chernobrovkina M., Dolgov S., Miroshnichenko D. Tissue culture efficiency of wheat species with different genomic formulas. *Crop Breeding and Applied Biotechnology*. 2016. Vol. 16, Is. 4. P. 307–314. doi: 10.1590/1984-70332016v16n4a46.
5. Sticklen M. B., Oraby H. F. Shoot apical meristem: a sustainable explant for genetic transformation of cereal crops. *In Vitro Cell. Dev. Biol. – Plant*. 2005. Vol. 41, Is. 3. P. 187–200. doi: 10.1079/IVP2004616.
6. Bavol A. V., Dubrovna O. V. Lyalko I. I. Plant regeneration from shoot tips of wheat. *The Bulletin of Vavilov Society of Geneticists and Breeders of Ukraine*. 2007. Vol. 5, Is. 1–2. P. 3–10. [in Ukrainian]
7. Shrawat A. K., Lörz H. *Agrobacterium*-mediated transformation of cereals: a promising approach crossing barriers. *Plant Biotechnol. J.* 2006. Vol. 4. P. 575–603. doi: 10.1111/j.1467-7652.2006.00209.x.
8. Murashige T., Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant*. 1962. Vol. 15. P. 473–497. doi: 10.1111/j.1399-3054.1962.tb08052.x.
9. Pescitelli S. M., Sukhapinda K. Stable transformation via electroporation into maize Type II callus and regeneration of fertile transgenic plants. *Plant Cell Rep*. 1995. Vol. 14. P. 712–716. doi: 10.1007/BF00232653.
10. Morel G., Wetmore R. H. Fern callus tissue culture. *Am. J. Bot.* 1951. Vol. 38, Is. 2, P. 141–143. doi: 10.1002/j.1537-2197.1951.tb14804.x.
11. Gorbatyuk I. R., Shcherbak N. L., Bannikova M. O., Velykozhon L. H., Kuchuk M. V., Morgun B. V. Establishing transgenic wheat plants of cv. Zymoyarka resistant to the herbicide phosphinothricin *in vitro*. *Fiziol. Rast. Genet*. 2016, Vol. 48, Is. 1. P. 65–74. doi: 10.15407/frg2016.01.065. [in Ukrainian]
12. Sidorov V., Duncan D. *Agrobacterium*-mediated maize transformation: immature embryos versus callus. Methods in Molecular Biology: Transgenic Maize. M. Paul Scott (ed.). USA : Humana press, 2009. P. 47–58. doi: 10.1007/978-1-59745-494-0_4.
13. Ozgen M., Birsin M. A., Benlioglu B. Biotechnological characterization of a diverse set of wheat progenitors (*Aegilops* sp. and *Triticum* sp.) using callus culture parameters. *Plant Genetic Resources: Characterization and Utilization*. 2017. 15 (1). P. 45–50. doi: 10.1017/S1479262115000350.
14. Kyriienko A. V., Shcherbak N. L., Kuchuk M. V., Parii M. F., Symonenko Yu. V. *In vitro* plant regeneration from mature embryos of amphidiploid spelt *Triticum spelta* L. *In Vitro Cellular & Developmental Biology – Plant*. 2021. doi: 10.1007/s11627-021-10158-4.
15. Kyriienko A., Parii M., Kuchuk N., Symonenko Yu., Shcherbak N. Elaboration of an effective method of callusogenesis induction from mature germs of *Triticum spelta* L. and *T. aestivum* L. *Plant Varieties Studying and Protection*. 2019. Vol. 15, Is. 3. P. 259–266. doi: 10.21498/2518-1017.15.3.2019.181084. [in Ukrainian]
16. Kyriienko A. V., Parii M. F., Symonenko Yu. V., Kuchuk M. V., Shcherbak N. L. Callus induction from shoot apical meristem in *Triticum spelta* L. and *T. aestivum* L. *Factors in Experimental Evolution of Organisms*. doi: 10.7124/FEEO.v25.1169. [in Ukrainian]
17. Nitovska I. O., Avilov I. D., Morgun B. V. The positive effect of antibiotic paromomycin compared with kanamycin for selection of transgenic plants with *npt*II gene on the example of *Nicotiana tabacum*. *Factors in Experimental Evolution of Organisms*. 2015. Vol. 17. P. 270–273.
18. Nitovska I. O., Morgun B. V., Abraimova O. Ye., Satarova T. M. Glyphosate selection of maize transformants containing CP4 *epsps* gene. *Factors in Experimental Evolution of Organisms*. 2020. Vol. 26. P. 239–244. doi: 10.7124/FEEO.v26.1273.

НІТОВСЬКА І. О.[1], ПАНАСЕНКО А. С.[1], РЕДЬКО В. Ю.[2], ЗАДОРОЖНА М. І.[2], МОРГУН Б. В.[1]

[1] Інститут клітинної біології та генетичної інженерії НАН України,
Україна, 03143, м. Київ, вул. Академіка Заболотного, 148
[2] Національний університет «Києво-Могилянська академія»,
Україна, 04070, м. Київ, вул. Григорія Сковороди, 2

ВИЗНАЧЕННЯ СЕЛЕКТИВНИХ КОНЦЕНТРАЦІЙ ФОСФІНОТРИЦИНУ ТА ПАРОМОМІЦИНУ ДЛЯ ОТРИМАННЯ ТРАНСГЕННИХ РОСЛИН СПЕЛЬТИ

Мета. Визначити селективні концентрації гербіциду фосфінотрицину та антибіотику паромоміцину для відбору трансгенних рослин пшениці спельти. ***Методи.*** Культура апікальних меристем пагонів, культура зрілих зародків, *Agrobacterium*-опосередкована генетична трансформація. ***Результати***. Проводили ізоляцію та культивування апікальних меристем пагонів проростків трьох генотипів спельти та зрілих зародків від трьох інших генотипів. Спостерігали високу частоту (від 80 до 100 %) індукції калюсу з експлантів. Показано, що внесення 5 мг/л фосфінотрицину або 100 мг/л паромоміцину в живильне середовище практично повністю

пригнічувало регенерацію рослин, порівнюючи із контролем. Після *Agrobacterium*-опосередкованої генетичної трансформації калюсів вектором, що містив ген фосфінотрицин-N-ацетилтрансферази, спостерігали регенерацію пагонів спельти генотипу № 853 на селективному середовищі із 5 мг/л фосфінотрицину. ***Висновки.*** Селективні концентрації гербіциду та антибіотику для отримання трансгенних рослин пшениці спельти з відповідними маркерними генами становлять 5 мг/л для фосфінотрицину та 100 мг/л для паромоміцину.
Ключові слова: *Triticum spelta* L., пшениця спельта, культура апікальних меристем пагонів, культура зрілих зародків, генетична трансформація рослин.