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IN VITRO CULTURE OF FOREIGN AND LOCAL *PANICUM VIRGATUM* AND *PANICUM MILIACEUM* CULTIVARS

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ABSTRACT

The development of effective methods for obtaining sterile cultures and identification of explants with high morphogenetic potential are the basis for the implementation of biotechnological methods used in plant breeding programs. Local and foreign varieties of *P. miliaceum* and *P. virgatum* were used to improve the qualitative and quantitative characteristics of *Panicum*. *Panicum* seeds provided a 100% yield of sterile explants and were found to be more effective than sprouts. The callus-forming potential of *Panicum* seeds was high: Kormovoe variety 2014 were 97%, Forestburg variety were 94% and cotyledon explants were 29%. Intensive morphogenic callus formation was observed on MS basal medium containing B5 vitamins without exogenous hormons. In addition, our experiments attempted the rotation of *Panicum* culture on solid and liquid media for the induction of embryonic structures and increased regeneration potential. Combined surface and deep culturing of callus tissues led to an increased frequency of embryonic structures. The results of these experimental studies can be used for further development of genetic engineering techniques, for structural transfers in embryogenic tissues, and for the regeneration of transgenic plants.

Keywords: *P. miliaceum*, *P. virgatum*, induced morphogenesis of *Panicum* in tissue culture

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INTRODUCTION

The homeland of cultivation of proso millet (*Panicum miliaceum*) is considered to be China, from where it spread to Central, East and South-West Asia, Transcaucasia. Significant acreage is known in countries such as Mongolia, Korea, Southeast Russia, Afghanistan, Pakistan, India, and southern Europe. According to the famous works of N. I.Vavilov *Panicum miliaceum L*. - common proso millet, *Panicum italicum L*. (*Setaria italica* (L.) *Beauv*.) – Mohar or Tenai, and the other millet species have origin from the Chinese center of origin of cultivated plants [1, 2].

As stated by FAO (Food and Agriculture Organization), the area under millets in the world recorded to 36.3 million hectares; in Kazakhstan, the area of millet crops is 1.7 million hectares, cultivated in Akmola, Kostanay, Pavlodar, Aktobe and West Kazakhstan districts.

According to the Institute for agricultural market conditions, the share of consumption of grains, the consumption of proso millet ranks 5th after rice, buckwheat, hercules and peas. In addition, millet takes one of the first places in taste and nutritional qualities least allergenic crops. Millet culture has a number of valuable agronomic and biological properties. The nutritional value amounts to 12% of protein, 3.5% of fat, 81% of starch.

One of the modern uses of millet is the processing and food industry. For example, due to the significant content of starch in the grain, millet is also used in alcohol production. *Panicum virgatum* L. is one of the most promising crops for the U.S. bioenergy due to the high yield of biomass [4, 5]. Besides that species such as *Panicum virgatum* are increasingly used in horticulture, which creates unusual shades of color and texture in the landscape design. Thus, the area of use of the switchgrass culture is diverse and attracts attention that culture does not require special agrotechnical techniques. Different ecotypes and morphotypes are characterized by the quality of feedstock in biomass. For example, the morphotype of the stem has significant advantages over leafy morphotype for the quality of raw materials [6]. In the work of Qinghua Yang with co-authors, the qualitative characteristics of switchgrass culture are discussed in detail [7]. Switchgrass has more than 400 species, for example, the main species of common switchgrass are: *Vittellinum Flavum, Subflavum Coccineum Subcoccineum ,Album Aureum, Sanquineum Grizeum, Dacicum Brunneum*.

All this increases the interest to a biotechnological improvement of switchgrass cultures to increase its bioenergy value, which usually requires cultivation of in vitro cultures.

In 1980-1990, as for many other cultures, protocols were developed for organogenesis and somatic embryogenesis from tissues and cells of various explants of in vitro, optimization of nutrient mediums as for superficial and deep cultivation [9-13]. When discussing the development of switchgrass tissues culture, special attention was paid to the species of explants and the effectiveness of regeneration from different types of original culture [14, 15].

Mature germs from switchgrass seeds are identified as the most effective. In Y. Varalaxmi's et al work, described in more detail the introduction of explants into culture, cultivation modes, and manipulations with growth regulators. For example, when cultivating explants on Murasige and Skoog medium (MS) with a low concentration of 2,4-Dichlorophenoxyacetic acid (2,4-D) and kinetin (Kn), the frequency of embryoid yield is high, and the regeneration potential is 40 shoots per callus. When transferring shoots to a medium containing 1-naphthylacetic acid (NAA), 90% of rooted plants were obtained, which produced seed offspring [16].

In addition, the introduction of explants in the culture is putting a strong focus on further ways of morphogenesis, in particular, the induction of somatic embryogenesis, as the most effective way for direct regeneration.

Santha B, Seetharama N. showed, as non-embryogenic, friable, primary calluses quickly differentiated into pale yellow embryogenic calluses, within 2 weeks from inflorescences of switchgrass. After that, embryogenic calluses were separated from the primary culture and cultured on the environment for the proliferation and development of globular structures before the development of somatic embryoids. It should be noted that the frequency of formation of embryogenic structures increased with subsequent subcultivation [17].

Despite of a large number of works, there is no universal protocol for all types of millet, in addition, even varieties requiring optimization of *in vitro* cultivation. The purpose of these studies is to create and optimize cultural conditions and to obtain a sufficient amount of callus, embryogenic tissue and plant regeneration for successful transformation of creating genetically modified plants of millet varieties by using *in vitro* methods.

MATERIALS AND METHODS

The following switchgrass seeds were used as the initial plant material of *Panicum virgatum*: Pathfinder, Forestburg, Trailblazer, Shawnee, purchased from USDA, ARS, Plant Genetic Resources Conservation Unit (Griffin, Georgia); Kazakhstan varieties of millet Kormovoye 2014 that belongs to *Panicum miliaceum* species, suggested by the selectionist Kobernitsky V. I.(Shortandy) LLP « Research-Production Center for Grain Farming named after A.I. Barayev » , The Yarkoye-6 variety which is provided by Aktobe cultivation center. Seeds of donor plants are shown in figure 1.

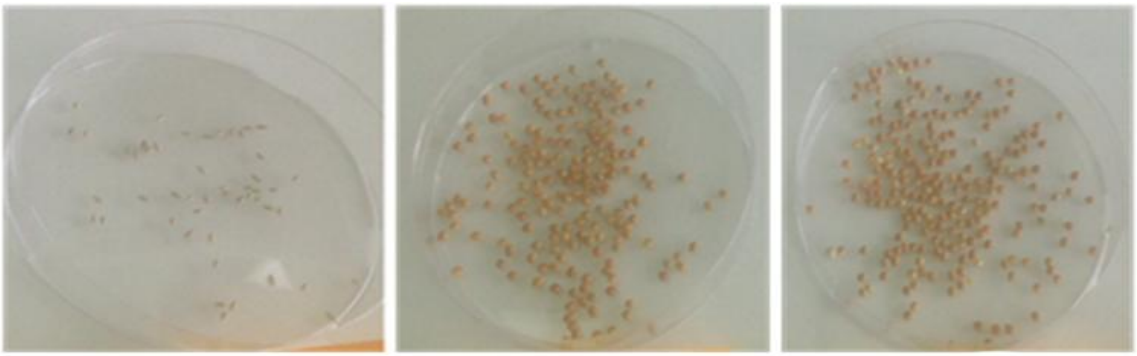


Fig. 1. The seeds of donor plants of millet

With the aim of obtaining aseptic plant material disinfection of seeds of millet was carried out. As a sterilizing agent disinfectant «Belizna» was used, containing as an active substance of 5% sodium hypochlorite with the addition of 1-2 drops of Twin-20.

The seed sterilization process included the following stages: 1) pre-sterilization (washing seeds with tap water followed by treatment with 70% of ethanol for 1 min); 2) sterilization in a solution of 5% sodium hypochlorite; 3) post-sterilization (4-5-fold washing with sterile distilled water); 4) transfer of sterilized seeds in Petri dishes to nutritious Murasige and Skuga (MS).

At the stage of processing with using of the solution of 5% of sodium hypochlorite, 3 variants of sterilization were tested which differed in the duration of exposure to the disinfectant «Belizna»: 30 minutes, 45 minutes and 60 minutes. Seeds and 2-week switchgrass seedlings were used as explants to obtain the primary callus. Explant isolation was performed under laminar-box conditions using a sterile scalpel. Isolated segments of explants in the form of cotyledons were cut into segments 4-5 mm long and sterilely transferred to Petri dishes with MS modified nutrient medium: macroelements and microelements of MS, vitamins of Gamborg B5, for solid nutrient media used phytogel (0,3%), as a carbon source maltose was used (30g/l).

Incubation of explants was performed in the thermostatic conditions in the dark at 26°C. Morphogenic calluses were transferred after 35-40 days of cultivation into 3 variants of nutrient media for embryoidogenesis, differing in the content of mineral substances and hormonal composition shown in Table 1.

Table 1 - Composition of culture medium for embryogenesis

№	Components of culture medium	Culture medium options		
		MSBOK	MSP	AA
Macroelements (mg / l)				
1	Ammonium nitrate (NH_4NO_3)	1650	1650	-
2	Calcium chloride ($CaCl_2 \cdot 2H_2O$)	440	440	440
3	Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	370	370	370
4	Potassium hydrophosphate (KN_2PO_4)	170	170	170
5	Potassium chloride (KCl)	-	-	2940
6	Potassium nitrate (KNO_3)	1900	-	-
7	Magnesium chloride ($MgCl_2 \cdot 6H_2O$)	1000	-	-
Microelements (mg / l)				
8	Boric acid (H_3BO_3)	6.2	6.2	6.2
9	Cobalt chloride ($CoCl_2 \cdot 6H_2O$)	0.025	0.025	0.025
10	Copper(II) sulfate ($CuSO_4 \cdot 5H_2O$)	0.025	0.025	0.025
11	Iron(II) sulphate ($FeSO_4 \cdot 7H_2O$)	27.8	27.8	27.8
12	Manganese(II) sulphate ($MnSO_4 \cdot 4H_2O$)	22.3	22.3	22.3
13	Potassium iodide (KI)	0.83	0.83	0.83
14	Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$)	0.25	0.25	0.25
15	Zinc sulphate ($ZnSO_4 \cdot 7H_{2O}$)	8.6	8.6	8.6

16	$FeSO_4 \cdot 7H_2O$	27.95	27.95	27.95
17	Na_2-EDTA	37,3	37,3	37,3
Vitamins (mg / l)				
18	Myo-Inositol	100	100	100
19	Nicotinic acid	1.0	0.5	0.5
20	Pyridoxine · HCL	1.0	0.5	0.5
21	Thiamine · HCL	10.0	0.1	0.1
Amino acids (mg / l)				
22	Asparagine	-	-	266
23	Glutamine	-	-	877
24	Glycine	-	-	75
25	Arginine	-	-	228
26	Proline	-	2000	-
Sugar (mg / l)				
27	Sucrose	-	-	30000
28	Maltose	-	30000	-
29	Glucose	30000	-	-
Hormones (mg / l)				
30	2,4-D	-	5.0	2.0
31	Kinetin	-	-	0.2
32	GA3	-	-	0.2
33	BAP	-	1.0	-

6-benzylaminopurine (BAP) (0,2 mg/l) and artificial analogue of auxin - 2,4-D (3 mg/l) were used as growth regulators.

RESULTS AND DISCUSSION

In expanding the scope of switchgrass culture, as discussed above, methods *in vitro* are of great importance. An important point in the initial stages of the work is the introduction of explants into the culture of cells, tissues, and organs and obtaining a large number of sterile culture.

In our experimental studies, the 45-minute exposure proved to be the most effective and provided 100% yield of sterile explants while maintaining high seed viability. It should be noted that the 60-minute incubation of seeds in solutions of a sterilizing agent was also effective, but the seeds partially lost their germination.

As an additional source of nitrogen, amino acids L-Proline (500 mg/l) and casein hydrolysate (300 mg/l) were added to the medium. Amino acids, introduced into the nutrient medium in addition to nitrates, that have a stimulating effect on the growth of tissue culture.

As a result of the data obtained, it was found that the formation of the primary callus tissue of switchgrass is observed from the seeds. At the same time, active tissue proliferation from cotyledon explants and seeds was observed for 10-15 days. On 28-30 days, the primary callus mass was formed in the volume that allowed its sub-cultivation. Figure 2 shows the processes of callus formation from different explants.

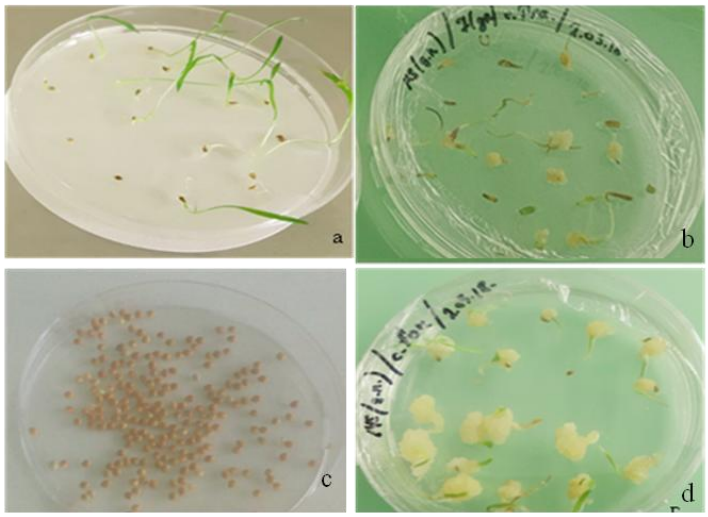


Fig. 2. Formation of cotyledon explants callus tissue (a,b) and seeds (c,d) in culture *in vitro*

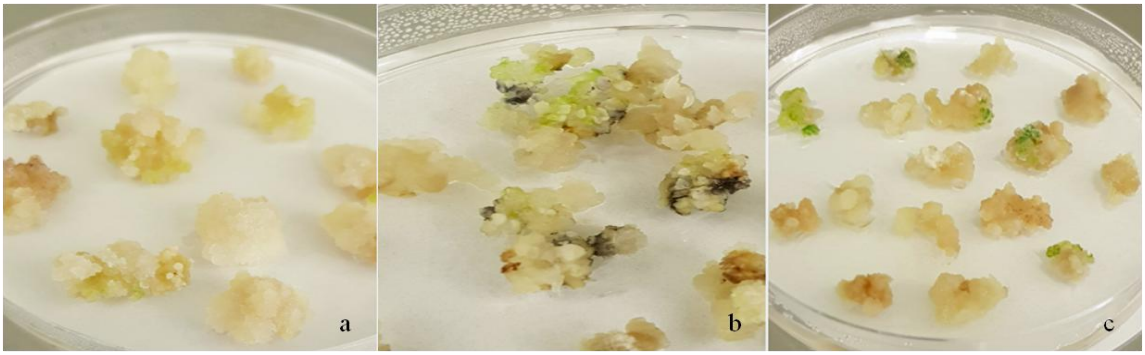
Callus-forming ability and intensity of callus development is an important point of further successful work on tissue and organ culture. As a result of research, was determined the ability for callusogenesis of varieties of local and foreign selection. The results shown in Table 2 demonstrate that when 14-day-old seedlings and switchgrass seeds were cultivated, the maximum frequency of callus formation was observed when using switchgrass seeds as an explant (97%), whereas in the cotyledon explants, the callus formation was 29%.

Table 2-Frequency of callus formation of 14 day-old seedlings and switchgrass seeds.

№	Varieties	Types of explants	Number explants (PCs)	Callus formation (primary), PCs	Callus formation, %
1	Pathfinder	seedlings	100	9	9±10,17
		seeds	33	18	55±3,05
2	Shawnee	seeds	37	26	70±2,15
3	Trailblazer	seedlings	42	12	29±6,36
		seeds	53	36	68±3,18
4	Forestburg	seeds	18	17	94±0,24
5	Yarkoye-6	seeds	62	59	95±1,05
6	Kormovoye 2014	seeds	67	65	97±0,27

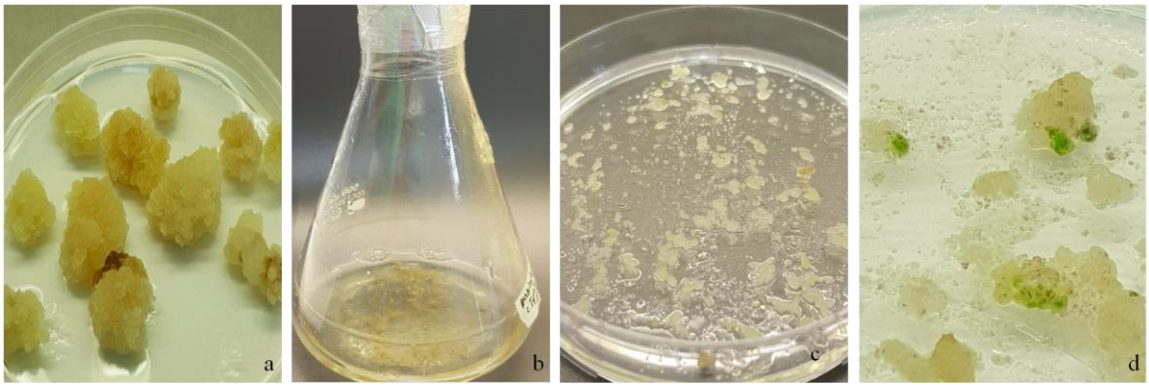
After 35-40 days of culturing in the medium, the calluses were transferred on 3 variants of culture media for embryogenesis, as shown in figure 3

In addition, there is a direct regeneration from the callus tissues without long processes of passage on the AA medium. It is necessary to focus that it is extremely important for the culture, as long-term subcultivation leads to a loss of morphogenic potential of cell culture, and is not desirable for further regeneration.



a) MSP; b) MSBOK; c) AA **Fig. 3**. Morphogenic calluses in a nutrient medium

The next stage of experimental studies is the introduction of calluses into suspension culture for effective induction of embryogenic tissues in switchgrass culture and accelerated production of plants through direct regeneration. Figure 4 shows the phased passaging of callus tissue from the surface method of cultivation to the liquid nutrient medium. At the same time, the nutrient medium was updated after 2 weeks, and after 4 weeks was transferred to the agar of 3-variant mediums: AA, MSP, and MSBOK [18].



a) callus culture; b) suspension culture; c) on the agar medium; d) morphogenic callus tissue
Fig. 4. Surface and deep cultivation of switchgrass callus

Two weeks later, an intensive growth of morphogenic tissues was observed on the MSBOK medium, which was not detected on other variants of media. On average, per 10 ml of the inoculum, when transferred to a solid nutrient medium, the percentage of well-structured morphogenic tissue was 33% on the 20th day of cultivation.
Apparently, as well as for other *Panicum virgatum* cultures, the alternation of superficial and deep cultivation determines the growth of morphogenic tissue.

Thus, according to the experimental scheme of the initial stages of research, various types of explants have been introduced into the culture and found that the most effective form of seeds are switchgrass. For the successful continuation of research on genetic engineering developments at this stage, protocols of nutrient media have been developed. An effective medium for the cultivation of millet tissues are probably MSBOK and AA. In addition, we modified the above media by adding different concentrations of hormones and changing the mineral basis of these media. A positive stage of research can be considered a successful alternation of various modes of cultivation. The final results will be obtained with statistical conformation from experimental studies.

CONCLUSION

In experimental studies, the initial stage of experiments on the introduction of switchgrass into the culture *in vitro* was successfully carried out. In particular, the protocols of nutrient media have been developed, at the same time the modification of media for the induction of embryogenic tissues has been carried out, which is an important point of cell and organ culture studies. In addition, it was found that there is a direct regeneration of callus tissue without passages at the AA medium. The callus-forming capacity of foreign varieties ranged from 9-94%, and local varieties had a high potential for this indicator of 95-97%.

The skilful combination of superficial and deep culturing of callus tissues has led to an increase in the frequency of embryonic structures. It was noted that 33% of well-structured morphogenic tissue were obtained in 10 ml of inoculate when transferred to a solid nutrient medium.

The results of these experimental studies can be used for further genetic engineering development, as for the transfer of the structure in embryogenic tissue, as well as for the regeneration of transgenic plants.

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