An in vitro anther culture method for creating rice dihaploids resistant to prolonged flooding

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Abstract. An assessment was made of the possibility of obtaining dihaploids by the method of anther culture *in vitro* to accelerate breeding for resistance to prolonged flooding of rice with water. The object of the study is F2 rice hybrids of the Federal State Budgetary Scientific Institution, Agrarian Research Center "Donskoy" (ARC "Donskoy") rice breeding and seed laboratory, obtained by crossing the best varieties in terms of economically valuable traits with samples carrying genes for resistance to prolonged flooding with water. Basic nutrient media with the optimal composition of nutrients and growth hormones that stimulate callus and morphogenesis were used. Cultivation of anthers revealed large genotypic differences between the samples. In terms of responsiveness to neoplasms, 1/3 of the number of plants showed a positive result, the rest did not give calli. The most responsive to the formation of calli were hybrid combinations: 5009/2 – 84 pcs., 5010/2 – 94 pcs., 4565/3 –85 pcs., 4641/2 – 69 pcs. They also showed the ability to morphogenesis. Androgenic plants were obtained from 13 hybrid combinations, their share was 1.03% of the total number of inoculated anthers. 30 green regenerated lines were obtained from four rice hybrids, differing in visual morphological assessment: 5009/2 – 5 pcs., 5010/2 – 5 pcs., 4565/3 – 2 pcs., 4641/2 – 18 pcs. The isolated lines are characterized by good responsiveness in anther culture *in vitro*, carry genes for resistance to prolonged flooding, and can be used in rice breeding programs using DG technologies.

1 Introduction

Using traditional breeding methods, many varieties of agricultural crops have been created. crops adapted to a variety of growing conditions. At the same time, increasing biotic and abiotic stressors, population growth and a decrease in land for agricultural purposes stimulate the intensification of breeding work to create plant varieties with higher yields and resistance [1, 22].

Rice is one of the main food sources for more than half of the world's population and is the third largest grain producer. Rice grain production is closely related to environmental changes. Unfavorable climate conditions hinder the growth of rice yields. The impact of

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environmental change on rice cultivation is already being seen, but it is not yet fully understood how long-term and short-term exposure to environmental factors will affect the growth of rice plants. Breeders are constantly looking for biotic and abiotic stress tolerance mechanisms to increase yields under these stressful conditions. Great importance is attached to increasing resistance to flooding, since the peculiarities of the natural conditions of rice cultivation in the tropics and subtropics are associated with complete flooding of rice crops for a short period of several days to two weeks, due to heavy rainfall. Flooding or flooding is one of the major environmental stressors affecting many artificial and natural ecosystems around the world. Increased frequency and duration of heavy rainfall due to climate change adversely affects plant growth and development, eventually leading to plant death if it persists for several days. As a result, crops of unstable varieties fall out, yield loss occurs.

During vegetative growth, rice uses various flood tolerance strategies. Genetic variability in plant response to flooding includes various schemes: 1) dormancy, which makes it possible to withstand a long time under water, 2) a strategy of rapid stem elongation with changes in plant structure and metabolism [1].

Dormancy is observed in rice varieties with the Sub1A-1 locus, which limit starch mobilization and thus produce less ethanol and other fermentation products, as well as affect the processes associated with aging [1-3].

The researchers also identified two additional loci associated with flood resistance, Snorkel1 and Snorkel2. Plants with these genes exhibit rapid growth and elongation of the coleoptile during development under anaerobic conditions, which is called the “tube effect” [3].

The Sub1, Snorkel1,2 loci belong to the genes that control flood resistance, which manifests itself in waiting for the onset of an unfavorable factor. Another type of flood resistance includes plants that can avoid adverse environmental conditions. The genes that can control this property include AG1 and AG2. They contribute to the rapid anaerobic germination of plants, which allows rice leaves to be above the surface of the water and receive enough oxygen for their further development.

As the environment changes over time, it can also affect the species composition of pathogens, pests and weeds in ecosystems.

Another aspect of the use of resistance to flooding, and the most relevant for Russia, is the fight against weeds due to a deep layer of water, which weeds cannot overcome and die [4].

This leads to such positive effects as: the use of herbicides is unnecessary, the cost per unit of production is reduced, the resulting products are of higher quality and can be used in the production of products for baby and diet food, and there is no environmental damage, since there is no need to treat crops with herbicides [4-5].

The most malicious weeds of rice fields are varieties of millet. Millets are very sensitive to flooding during germination and in the germination phase, so they can be dealt with quite effectively by increasing the water layer to 20–30 cm at the initial stage of plant development. However, this technique has a significant drawback – a strong thinning of rice seedlings (up to 50% or more), caused by a lack of oxygen, due to an increase in the water level in the fight against weeds and field vegetation. This phenomenon can be minimized by using deep flood resistant rice varieties [3-4].

At present, there are no released varieties in Russia that would meet these requirements. Therefore, the problem of creating such varieties of rice is relevant, as it will reduce the cost of producing a unit of production, reduce grain losses during harvesting, improve the quality of the resulting products, and also reduce the pesticide load on the ecosystem [3]

In modern breeding, an important direction is the improvement and creation of fundamentally new genotypes of agricultural plants with single, group or complex

resistance to biotic and abiotic stress factors of the environment, while maintaining and increasing their productivity and quality [6]. For rice, it has recently become very important to create vigorously growing varieties that can withstand prolonged flooding with water. This allows you to deal with hydrophytic weeds without the use of herbicides. In Asia, varieties have been found that have genes for this resistance. It is possible to transfer these genes to the geneplasm of Russian varieties. To accelerate this process, it is necessary to use the methods of androgenesis.

A rational combination of classical breeding methods with biotechnological methods allows solving the tasks set in a shorter time [7].

Therefore, in addition to the classical methods of creating new rice varieties, biotechnological approaches such as androgenesis and haploidy are widely used [7]. Rice-growing countries of the world have long used the cultivation of anthers on an artificial nutrient medium in breeding work [9-11]. The use of anther culture in breeding work allows you to quickly obtain homozygous plants that are resistant to various harsh environmental conditions, including drought, soil salinity, extreme temperatures and diseases. Cultivation of anthers on an artificial nutrient medium makes it possible to obtain rice haploids and homozygous dihaploids in 1-2 years. Extensive work on this technique is being carried out in Federal State Budgetary Scientific Institution Federal Research Center for Rice [12-14] and Primorsky Research Institute of Agriculture [15-16].

The technique for creating regenerative rice lines in anther culture is necessary for their mass production from promising hybrids, which makes it possible to speed up the breeding process by breeding a homozygous constant breeding material - dihaploids. The success of obtaining dihaploids with a combination of breeding-valuable traits, as in any breeding work, is due to a fairly high amount of material being worked out [17]. Of the hundreds of lines tested in the field, only a few are distinguished by economically valuable traits [18].

In order to provide hundreds and thousands of lines in the culture of anthers *in vitro*, it is necessary to obtain a large percentage of regeneration of green buds and plants in each hybrid combination [19]. The indicators of regenerative capacity vary greatly depending on the hybrid and on the genotype, i.e. even within the same hybrid combination, different plants provide different rates of callus formation and regeneration of green plants: from very low to very high. Thus, it is necessary to cover the maximum possible number of hybrid plant genotypes for introduction into *in vitro* culture [20].

It is recommended to take hybrids F_1 or F_2, later generations of hybrids go through several recombination cycles, which is undesirable. For breeding purposes, it is preferred to introduce the first hybrid progeny into *in vitro* culture, for several reasons. Firstly, it contains equally the hereditary information of both parents. Second, the use of hybrids F_2 increases the duration of selection by one year. However, there are several arguments in favor of hybrids. F_2. In some years, the F1 hybrid progeny does not produce callus formation; in this case, only hybrids of the next generation can be used for the anther culture. In addition, rice hybrids F_2 have the higher frequency of callus formation than F_1 [21].

The high yield of green buds and plants regeneration depends on the composition of nutrient media for the cultivation of anthers and calluses; therefore, many researchers continue to search for more effective nutrient media and other cultivation conditions for this purpose [22].

Creation of rice breeding lines by the method of *in vitro* anther culture, resistant to prolonged flooding with water for herbicide-free cultivation technologies, is relevant.

The purpose of the study is to obtain rice dihaploids by *in vitro* anther culture to accelerate breeding for resistance to prolonged flooding with water.

2 Materials and method

2.1 Donor plants and growth conditions

The object of the study was hybrids of the second generation of rice (donors of the target genes Sub1, Snorkel1,2, AG1 and AG2) obtained by crossing the best varieties in terms of economically valuable traits with samples carrying genes for resistance to prolonged water flooding. Cultivation of rice plants was carried out according to the technology of cultivation of crops in this soil-climatic zone. The physiology of the donor plant is an important success factor in rice anther culture. Anthers collected from panicles grown in the field are significantly better in anther culture than anthers collected from plants grown in a greenhouse [4, 23].

2.2 Panicle selection and pretreatment

Sampling was carried out in the fields of the ARC "Donskoy" Rice Breeding and Seed Laboratory in Proletarsk, Rostov Region in 2022. Rice panicles (anther donors) were collected in the field in the morning, in clear weather, since after rain they can be infected .

A morphological trait suitable for the selection of shoots was the distance from the ear of the flag leaf to the ear of the next leaf, which should be from 5 to 10 cm. Each sample was marked with a label, the cut end was placed in water and delivered to the laboratory [5].

Selected shoots were subjected to surface sterilization, that is, treatment with 96% alcohol for 3 minutes, which removes external infection. After that, they were placed in vessels with water, covered with a plastic bag and exposed to low positive temperatures (5°C) for 7-10 days in a refrigerator (Liebherr-Hausgeraete).

The positive effects of cold pretreatment on callus induction are to slow down the aging of the anther wall, enhance the synchronous division of pollen grains, and release substances necessary for androgenesis, mainly amino acids and shockothermal proteins [1]. Some researchers talk about the stimulating effect of low-temperature shock on the androgenic response. Cold pretreatment at 8°C for 14 days is most effective for anther cultivation for some rice subspecies indica, japonica and intersubspecies hybrids [3], and pretreatment at 10°C for 7–9 days has a positive effect on rice subspecies indica [5]. There is also evidence that cold pretreatment at 12 C for 5 days gives the best results for callus induction and plant regeneration in 13 indica rice genotypes [10]. Other authors [11] found a positive effect on androgenesis in the indica subspecies with a two-day pretreatment cycle at 10°C. The exposure of rice anthers and calluses to high temperatures during meiosis easily leads to the formation of albino plants, suggesting that the optimal temperature seems to depend on the genotype [1]. Thus, to enhance androgenesis, the most common is the use of a preliminary low-temperature stress of sufficient duration.

2.3 Panicle sterilization

Determining the stage of development of microspores Before introducing into the culture, flag leaves were removed from panicles, twigs with spikelets were selected according to morphological characteristics with pollen grains in the stage of middle and late mononuclear microspores, the rest were removed. The twigs were placed in sterile gauze and fixed loosely with a thread. Then the sterilizing solution of 5% sodium hypochlorite was immersed for 10 minutes. Then the panicles were washed three times in sterile distilled water.

2.4 Determination of the stage of development of microspores

It is believed that the stage of development of microspores has a great influence on the androgenic response. The desired stage of microspores is determined by the morphological characteristics of the plant, which are strongly associated with the stage of pollen development, as well as by the cytological method. At the same time, 2-3 spikelets were isolated from each panicle, from its middle part, to determine the stage of microspore development. To do this, the spikelets were placed on a glass slide and the anthers were removed using dissecting needles. Next, the anther was cut across and the microspores were squeezed out. Then two drops of acetocarmine were added, heated over an alcohol lamp and left for staining (10-15 minutes). After that, the preparation was covered with a cover slip and examined under a microscope (optical microscope ADF U300FL) (see Fig. 1a). The most suitable stage of development of microspores is from the late single-nuclear to early binuclear stage. However, the middle uninuclear stage of microspore development has been identified as optimal for an efficient androgenic response [1].

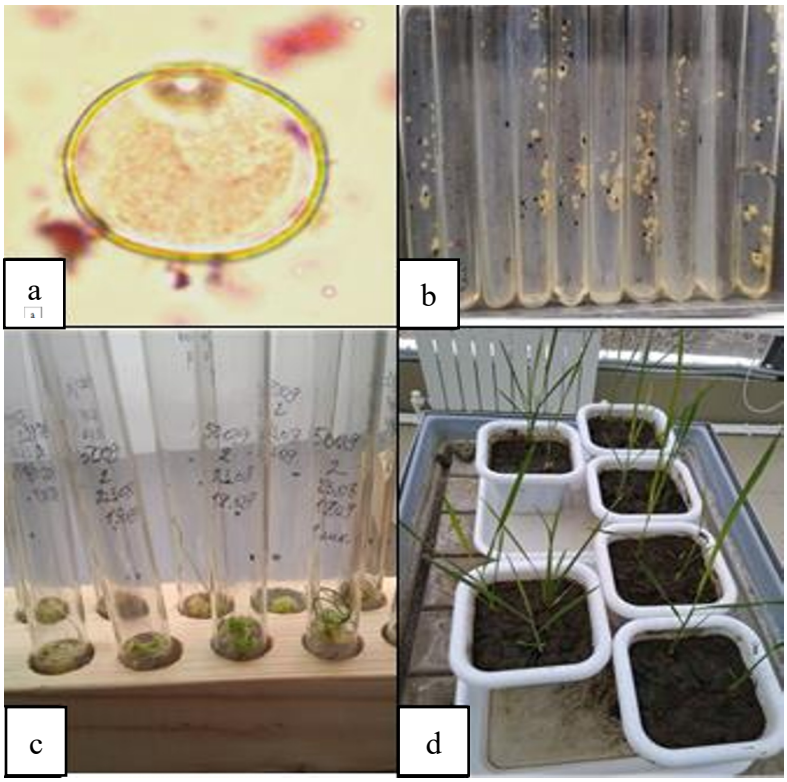

Fig. 1 Stages of obtaining rice regenerative plants in anther culture *in vitro*:
a – microspores in the middle uninuclear stage; **b** – neoplasms on anthers;
c – morphogenesis on calli; **d** – growing regenerative plants in the soil.

2.5 Preparing the environment

Androgenesis *in vitro* is influenced not only by physical, but also by various chemical factors. Media N6, MS, B5, Potato-2 are the most widely used base media for anther culture. In these studies, for the induction of callusogenesis from rice anthers, Blades

medium containing 2.0 mg/l 2,4-D, 30 g/l sucrose and 8 g/l agar was used as the base medium. For regeneration, morphogenic calli were transplanted onto Murashige and Skoog (MS) base medium with 1.0 mg/l NAA, 5.0 mg/l kinetin, 20 g/l sucrose, and 8 g/l agar (Table 1). The prepared nutrient medium was poured into test tubes with a diameter of 20 mm, autoclaved (steam sterilizer Tuttnauer 3870 ELV) for 15 minutes at a temperature of 121°C and a pressure of 0.9-1.0 atm. Immediately after sterilization, tubes with hot medium were placed at an angle of about 30° to obtain a slant agar and left to solidify [7, 14].

Table 1. Composition of media for growing rice anthers

Environment components	**Induction environment (Blaydes, 1966) (mg/L)**	**Environment for regeneration (Muraschige and Skoog, 1964) (mg/L)**
Macro salts		
NH_4NO_3	1000	1650
KNO_3	1000	1900
$Ca(NO_3)_2$ x 4 H_2O	347	440
KH_2PO_4	300	170
$MgSO_4$ x 7 H_2O	35	370
KCl	65	-
Micro salts		
$ZnSO_4$ x 7 H_2O	1.5	8.6
H_3BO_3	1.6	1.6
$MnSO_4$ x 4 H^2O	4.4	6.92
KI	0.8	0.83
Na_2MoO_4 x 2 H_2O	-	0.25
$CuSO_4$ x 5 H_2O	-	0.025
$CoCl_2$ x 6 H_2O	-	0.025
Iron source		
$FeSO_4$ x 7 H_2O	27.8	27.8
Na_2EDTA	37.2	37.2
Vitamins		
Nicotinic acid	0.5	0.5
Pyridoxine HCI	0.5	0.5
Thiamine HCI	0.5	0.5
Other componentss		
myo-Inositol	100	100
Glycine	2.0	2.0
Agar	8.0	8.0
Sucrose	30.0	20.0
Growth regulators		
2,4-D	2.0	-
NAA	-	1.0
Kinetin	-	5.0
pH	6.0	6.0

2.6 Anther inoculation and callus induction

The inoculation of anthers was carried out under aseptic conditions in a laminar box (microbiological safety box BMB-II-"Laminar-S"), which was sterilized with UV lamps. The instruments used were also subjected to partial sterilization. All items needed in the

work were wiped with 96% ethyl alcohol, then they were burned in the flame of an alcohol lamp. Sterile instruments were placed between sheets of thick wrapping paper. The paper was pre-sterilized by dry heat in a drying cabinet (Binder FD23 dry-air sterilizing cabinet) at a temperature of 130°C for 2 hours (from the moment the desired temperature was set).

Hands and the inner surface of the box were periodically wiped with 96% alcohol. In a sterile box, anthers were isolated from spikelets using a scalpel, tweezers, and a dissecting needle and transferred to the medium up to 30 pcs. into a test tube, closed with a foil cap and placed in a thermostat (Binder KV400 thermostat-incubator) with a temperature of 28 ± 2°C and a relative humidity of about 50%. Under such conditions, the anthers turned brown, burst in the longitudinal direction, and callus formed inside the burst anther. The callus mass increased within 30-50 days (Fig. 1, b).

2.7 Planting calluses and plant regeneration

It should be taken into account the morphotypes of the obtained callus cultures. The morphology of calli is closely related to their ability to regenerate plants. In the course of research in the culture of rice anthers, Shevelukha (1996) identified the following types of calli [14]:

1. with meristematic foci, light shades, fine-grained, medium density (morphogenic);
2. globular, white, light yellow, medium density (morphogenic);
3. dense, white, fine-grained (morphogenic);
4. loose, hydrated, with vascular cords (rhizogeneous);
5. brown, granular, friable, with large cells (very low ability to morphogenesis);
6. dark brown, hydrated, with large shapeless cells, of different sizes (non-morphogenic).

When the calli were 1 mm or more in size, they were placed on the Murashige and Skoog regeneration medium poured into test tubes 20 mm in diameter (Fig. 1c). Tubes with callus explants were incubated in an illuminated growth room with a temperature control of 25 ± 2°C, illumination of 2000 Lx, and a photoperiod of 15 h / 9 h. After 15–20 days, regenerated plants were formed in the light. The formation of green shoots was recorded weekly, tubes with darkened calluses and infection were rejected [6, 7].

2.8 Acclimatization of regenerative plants and planting them in the soil

Regenerants with a developed root system and 4–5 leaves, at least 8 cm long, were planted in a pot culture (Fig. 1d). Before acclimatization of plants, their root system was thoroughly washed from the agar medium and left for a day in glasses with water. The temperature of the soil should correspond to the temperature of the nutrient medium; therefore, the soil was preliminarily sieved, sterilized (3 hours in a dry oven at a temperature of +110°C) and moistened. Vessels with plants were placed in a light room, without direct illumination, so that a sharp change in environmental conditions would not adversely affect the plants.

After that, the regenerated plants were transferred to the greenhouse, where they continued their development until flowering, seed formation and maturation. Favorable for rice plants were the following microclimate parameters: daytime temperature 25°C, illumination more than 5000 lx; night temperature 20°C, illumination 0 lx; humidity 70-80%; photoperiod 12 hours [6, 7].

Spent one top dressing with a nutrient solution (1/2 MS). Plants were watered 3-4 times a week, depending on the drying of the soil in the growing vessels.

2.9 Plant ploidy diagnostics

The ploidy of the obtained regenerated plants at the stage of growth and development (before seed formation) was identified by morphological features such as plant height and leaf size. Plants with normal morphological features were considered doubled haploids. Haploid plants [23] had low plant height and narrow leaves. The tetraploids were very tall with large leaves. Simultaneously, the content of nuclear DNA in different groups of regenerated animals was determined. The DNA content was determined using a GuavaMuse flow cytometer. Green regenerative plants obtained from calluses were evaluated. The plant material (leaves) was freeze-dried. Sheet Sample of 1-2 cm^2 minced with a blade in a Petri dish in 1 ml of chilled Tris-$MgCl_2$ buffer. Buffer contained 0.2 M Tris base, 4 mM $MgCl_2$ x $6H_2O$ and 0,5 % Triton X-100 with addition of β-mercaptoethanol (1 µl/ml), 50 µg/ml propidium iodide and 50 µg/ml RNase. The samples were filtered through a nylon membrane filter with a pore size of 50 µm. The nuclei of Ficus benjamina L. isolated in a similar way with a known DNA content of 2C = 1.07 pg were used as an external standard [24, 25].

2.10 Molecular genetic assessment of the presence of flood resistance genes

The genomic DNA of isolated samples was extracted from freshly cut leaves by the CTAB method with homogenization (Bertin Precellys 24 homogenizer). Homogenization in 2 stages of 30 seconds, speed - 3000 rpm. The quantity and quality of the isolated DNA were assessed on a spectrophotometer (Implen Nanophotometr NP80). The isolated DNA was placed in an amplifier (Rotorgene 6000, Corbett Research, Australia) for polymerase chain reaction (PCR) and a multiple increase in the number of copies of the molecular marker of the target gene. After the amplification reaction, the samples were placed in the gel electrophoresis chamber. The gel was prepared on the basis of 0.5x TBE buffer (100 ml) and agarose (2 g). 3–5 µl of a dye containing bromophenol blue, xylene cyanol FF, and orange G were added to each tube with a sample after PCR. First, they were vortexed for several seconds, then centrifuged for 1 minute. The prepared samples were applied to the wells of the frozen agarose gel. A colored molecular weight marker was also applied to the gel. The duration of electrophoresis is from 30 minutes to 2 hours, depending on the expected size of the amplificates. After electrophoresis, the gel plates were placed for 20-30 minutes in a solution of ethidium bromide for staining. The gel was then removed from the fluorescent dye solution, gently washed in a cuvette, and photographed under ultraviolet light using a Bio-Rad GelDoc XR+ instrument [26].

2.11 Observations and statistical analysis

The analysis of the results included an assessment of the effectiveness of the anther culture according to the following characteristics: the number of new growths/100 anthers, the number of all regenerants/100 anthers, the number of green regenerants/100 anthers and the Number of all regenerants/100 new growths. Mathematical and statistical data processing was carried out in MS Excel.

3 Results and discussion

Rice anther culture is a two-stage process of initial development of calli and subsequent regeneration of green plants from embryogenic calli [13, 25]. Features of androgenesis in hybrid combinations of rice were studied by cultivating anthers on Blades induction medium.

As a result of the experiment, 12604 anthers of hybrids were extracted from hybrid rice panicles and planted on an induction nutrient medium in 26 crossing combinations (68 panicles). The maximum number of anthers was planted from hybrid 5016/2 - 339 pieces, and the minimum - 4773/1 - 47 pieces.

When anthers of all genotypes were cultivated on an induction medium, neoplasms appeared from them - embryo-like structures (single embryoids and polyembryoids) and calluses. When cultivated on a regeneration medium, as a rule, calli and part of the embryo-like structures remained unchanged. Some embryo-like structures developed roots, while others developed single seedlings or clusters of seedlings. The appearance of calli and embryo-like structures began on the 30th–33rd day from the moment the anthers were planted on the nutrient medium. Embryo-like structures included dense or slightly transparent, well-defined neoplasms. Callus formation continued for another four weeks; the anthers were on the medium for two months.

As follows from the data presented, not all hybrid combinations of rice showed the ability to form calluses and embryo-like structures. The value of callus formation in the anther culture varied significantly both between hybrid combinations and in different plants from one hybrid combination, which is apparently due to genotypic differences, as well as the action of external factors (explant quality, cultivation conditions, etc.). A total of 716 neoplasms were obtained, on average 10 pcs. per plant, taking into account non-responsive ones (table 2).

In terms of responsiveness to neoplasms, 60% of the studied material (39 panicles) showed a positive result, 40% (29 pcs.) did not give calli. The most responsive to the formation of calluses were hybrid combinations 5009/2 – 84 pcs., 5010/2 – 94 pcs., 4565/3 – 85 pcs., 4641/2 – 69 pcs. (table 2). The same samples showed the ability to morphogenesis, the remaining combinations formed a non-morphogenic callus (in some cases up to 100%). Combinations 5007, 5006, 5011 and 4585 showed no response to *in vitro* culture.

Table 2. Rice Anther Cultivation Results (2022)

№	№ of the sample	№ of the plant	Anthers inoculated, pcs.	Number of neoplasms, pcs.	Non-morphogenic callus, pcs.	Total regenerated plants, pcs.	Plants are green, pcs.	Albino plants, pcs.
1	5022	1	243	4	4	0	0	0
		2	275	0	0	0	0	0
		3	92	0	0	0	0	0
2	5103	2	259	20	16	4	0	4
		4	245	2	2	0	0	0
		5	110	0	0	0	0	0
3	5007	1	214	0	0	0	0	0
		3	152	0	0	0	0	0
		4	114	0	0	0	0	0
4	5005	1	299	37	34	3	0	3
		2	86	0	0	0	0	0
		3	225	1	1	0	0	0
5	5029	3	270	0	0	0	0	0
		5	132	1	1	0	0	0
		8	304	1	1	0	0	0
		10	284	0	0	0	0	0
6	5006	1	277	0	0	0	0	0

		2	189	0	0	0	0	0
		5	120	0	0	0	0	0
7	5093	1	194	1	1	0	0	0
		3	82	0	0	0	0	0
		4	272	0	0	0	0	0
8	5019	1	251	8	5	3	0	3
		2	126	3	2	1	0	1
		3	289	1	1	0	0	0
9	5003	1	306	0	0	0	0	0
		3	258	1	1	0	0	0
10	5009	1	212	0	0	0	0	0
		2	119	84	67	17	5	12
		4	278	12	12	0	0	0
11	5010	1	183	0	0	0	0	0
		2	277	94	87	7	5	2
12	5011	1	271	0	0	0	0	0
		3	186	0	0	0	0	0
13	5008	1	86	3	3	0	0	0
		2	184	0	0	0	0	0
		3	279	21	21	0	0	0
14	5020	1	243	26	15	11	0	11
		2	47	0	0	0	0	0
		3	210	13	13	0	0	0
15	5018	1	132	1	1	0	0	0
		2	298	5	4	1	0	1
		3	297	23	20	3	0	3
16	4565	2	82	3	3	0	0	0
		3	195	85	82	3	2	1
		5	245	46	43	3	0	3
17	4773	1	47	4	4	0	0	0
		2	114	0	0	0	0	0
		3	59	1	1	0	0	0
18	5016	2	339	1	1	0	0	0
		3	120	0	0	0	0	0
		4	216	0	0	0	0	0
19	4758	1	209	4	2	2	0	2
20	5021	1	248	62	37	25	0	25
		2	112	3	1	2	0	2
		3	140	0	0	0	0	0
21	4641	1	193	5	4	1	0	1
		2	194	69	48	21	18	3
22	5017	1	51	4	2	2	0	2
		2	255	2	1	1	0	1
		3	195	42	32	10	0	10
23	4526	1	80	10	3	7	0	7
24	4688	1	85	12	9	3	0	3
		2	117	0	0	0	0	0
		3	57	0	0	0	0	0
25	4617	1	83	1	1	0	0	0
26	4585	1	104	0	0	0	0	0
		2	94	0	0	0	0	0
Sum		69	12604	716	586	130	30	100
Average		2.5	185.35	10.53	8.6	1.91	0.44	1.47
Minimum		1	47	0	0	0	0	0

Maximum	4	339	94	87	25	18	25

It was established that not only hybrid combinations, but also individual plants within the same combination differed in terms of the ability to form callus. For example, in sample 5103, anthers from three plants were planted on a nutrient medium: 259, 245, and 110 pcs. Of these, 20, 2 and 0 calli were formed, respectively, but only 4 regenerated plants appeared from the first one. From sample 4641, an equal number of anthers was taken from two plants: 193 and 194 pcs. However, the former had 5 calluses, while the latter had 69, that is, 14 times more. The former subsequently developed only one regenerated plant, which was albino, while the latter had 18 green plants and 3 without chlorophyll. This indicates the presence of genetic factors that significantly affect the ability of cells to callus formation and regeneration.

Green seedlings and albinos developed from the formed neoplasms during cultivation on a regeneration medium; structures developing according to the type of rhizogenesis and structures with no development were also noted.

The ability of calli to morphogenesis was assessed by plant regeneration. The studied rice samples formed 130 regenerated plants according to 14 hybrid combinations. Of these, only 30 plants were green. 4 samples stood out that formed regenerated plants without chlorophyll defects in leaves – 5009/2 – 5 pcs., 5010/2 – 5 pcs., 4565/3 – 2 pcs., 4641/2 – 18 pcs. Albino plants died in the early stages of development, as they were not capable of photosynthesis and, accordingly, of the autotrophic type of nutrition. The largest number of albinos was formed in sample 5021/1 – 25 pcs.

As a result of evaluating the efficiency of rice hybrid anther culture, it was found that the largest number of neoplasms per 100 cultivated anthers (76.9) was observed in sample 5009/2, which significantly exceeded the average value in the experiment (table 3).

Table 3. Evaluation results of anther culture efficiency, isolated rice samples (2022)

№ of the sample	**№ of the plant**	**Number of neoplasms/100 anthers**	**Number of all regenerants /per 100 anthers**	**Number of green regenerants/100 anthers**	**Number of all regenerants/per 100 neoplasms**
5009	2	70.6*	14.3*	4.2	20.2
5010	2	33.9	2.5	1.8	7.5
4565	3	43.6	1.5	1.0	3.5
4641	2	35.6	10.8	9.3*	30.4*
Average value		45.9	7.3	4.1	15.4
Standard deviation		17.0	6.3	3.7	12.3

Note: * – significant difference from the mean
5009 (Inbara-3 x Innovator) x Contact (AG1 gene)
5010 (Inbara-3 x Novator) x Contact (AG1 gene)
4565 IR-64 x Tycoon (Sub1A gene)
4641 (Inbara-3 x Contact) x Khao Hlan On (Sk2, AG2 genes)

Morphogenesis and the ability of neoplasms to form seedlings, including green and albino ones, were evaluated on the basis of "the number of all regenerants per 100 planted anthers." More seedlings regenerated on the basis of the hybrid combination 5009/2, which was significantly higher than the average value. In general, accessions 5009/2 and 4641/2 produced more regenerants, 14.3 and 10.8, respectively.

Of practical interest are green seedlings, so the most important indicator of anther culture is the feature "the number of green regenerants per 100 isolated anthers". According to this trait, sample 4641/2 had a significantly high value (9.3). The indicators of other genotypes were at the level of the average value.

To assess the ability of neoplasms to regenerate seedlings, the number of all regenerants per 100 neoplasms was determined. The maximum value for this trait was in the hybrid combination 4641/2 (30.4). On average for genotypes, neoplasms also regenerated well in sample 5009/2 (20.2). The rest of the combinations formed less seedlings, but within the experimental average.

Sample 4641 was obtained by stepwise hybridization of the deep-flooded variety Inbara-3 with the Russian early ripe variety Contact, and then the fifth generation line was used in crossing with the vigorous variety Khao Hlan On carrying the Sk2 and AG2 genes. These genes allow seeds to germinate quickly and plants to vigorously navigate the water column. Apparently, this ability also affected the regenerative ability of callus cells, sharply increasing the number of regenerative plants.

The anther culture plays a significant role in the DG technologies of many cereal crops. Dihaploid (DG) plants allow the combination and fixation of desired genes of valuable parental genotypes, and are also used in the creation of populations for the study of genetic linkage and gene mapping. Therefore, works on the study of responsiveness to androgenesis *in vitro* and the development of methodological nuances are of great importance. Significant differences in the efficiency of anther culture can be explained by the difference in the origin of the studied genotypes. In general, combinations 5009/2 and 4641/2 show a trend towards higher anther culture efficiency. Indicators of androgenesis *in vitro* have a genotypic dependence.

In most rice plants obtained through anther culture, induced duplication of chromosomes occurs (spontaneous diploidization) [26]. This is confirmed in our studies (table 4). The resulting green regenerated plants were divided into 3 groups according to the content of nuclear DNA: haploids, doubled haploids and tetraploids.

Table 4. The content of nuclear DNA in the population of rice regenerants obtained from hybrids of the second generation in an anther culture *in vitro*

Indicator	**Haploids**	**Doubled haploids**	**Tetraploids**
Number of plants, pcs.	14	12	4
DNA content, pg:			
M	0.901	1.880	3.762
±SEM	0.012	0.023	0.048
min	0.790	1.654	3.590
max	1.112	2.015	3.960
Cv, %	8.3	9.6	10.0

All plants within their group were characterized by insignificant variability in DNA content in cell nuclei. Our data are comparable with the results of other authors, according to which the content of nuclear DNA in the main set of chromosomes in rice O. sativa varies from 0.91 to 1.00 pg [24, 26]. The ratio of the average values of the content of nuclear DNA in dihaploids and haploids was not a multiple of two. This may indirectly indicate the loss of some parts of chromosomes in haploids during cultivation, which leads to changes in the morphotype of regenerants.

As follows from the results obtained in this work, between rice hybrids of the second generation,. carriers of the target genes Sub1A, Snorkel1,2, AG1 and AG2, there are strongly pronounced differences in the manifestation of signs of androgenesis in the anther culture. At the same time, the low ability for androgenesis in these hybrids could depend on

many factors. Thus, in particular, the success of cultivating objects has been proven depending on the correct choice of the nutrient medium and the thoroughness of its preparation. Neoplasm initiation in rice anther culture was assessed using different media (Blades, N6, Gamborg, White) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), in combination with α-naphthaleneacetic acid (NAA), kinetin (Kin) or 6-benzylaminopurine (BAP). It has been established that the composition of the Blades nutrient medium for the cultivation of rice anthers is most consistent with the type of nutrition of this crop. Studies have shown that the rate and frequency of callus induction can be increased up to 27.9%, and even higher in some genotypes [6, 27]. These results are consistent with the studies carried out in our work, namely, the inclusion of 2.0 mg/l 2,4-D in the induction medium made it possible to obtain up to 70% of neoplasms per 100 cultivated anthers.

In the course of the work, the quantity and quality of calli were recorded for each hybrid combination. Morphogenic calli were light, dull, compact, had green chlorophyll-containing areas, which were zones of morphogenesis. This is confirmed in other works by other authors [28]. Thus, callus cultures are characterized by different morphotypes and a high degree of heterogeneity, even if they were obtained from the same donor genotypes and under the same cultivation conditions. This is manifested in the morphological and structural heterogeneity of these tissues.

A systematic study of the needs of rice in anther culture showed that the component composition of the Murashige and Skoog medium, supplemented with hormones, better supports unorganized callus growth and causes the induction of morphogenesis, which leads to the formation of calli with a high level of organogenesis. Activation of morphogenesis processes and further development of neoplasms are stimulated by the use of 1.0 mg/l α-naphthylacetic acid (NAA) in combination with 5.0 mg/l kinetin in the MS regeneration medium. So, for example, samples with a low regenerative capacity (0.31–0.72%); with medium (6.07–7.61%) and high (21.65–27.9%) [7, 28].

The results are confirmed in our studies. Among the isolated hybrid combinations of rice, the number of all regenerants per 100 neoplasms reached a maximum of 30%, including green ones - from 1 to 9%, i.e. samples showed low and medium regenerative capacity.

Analysis of the results showed that the intensity of the processes of callus formation and regeneration of rice plants is determined not only by the mineral and organic composition of nutrient media, the nature and concentration of the phytohormone, but to a large extent by genetic factors. Genotypes with a high ability to callusogenesis were not always optimal for the induction of morphogenic callus and plant regeneration, since callus formation and regeneration are controlled by different genetic mechanisms.

It is known that the signs of neoplasm induction, regeneration and frequency of green seedlings are regulated by different genes and are inherited independently. Therefore, in further work, it is possible to use the parental forms of these hybrids in crosses as donors of valuable alleles of high responsiveness to androgenesis *in vitro*.

The assessment of the level of ploidy in regenerants is the most important key step in the application of androgenesis in a breeding program. Therefore, the method of determining ploidy should be chosen wisely, taking into account the savings in time and cost. In this study, morphological evaluation was found to be reasonably reliable in distinguishing diploids from other ploids, and was performed quickly and easily. Although flow cytometry is an attractive approach for assessing the ploidy level of regenerants, its use is still limited in many laboratories due to the high cost of equipment and the higher cost of each analysis [27, 29]. In addition, this method cannot distinguish homozygous doubled haploids from heterozygous diploid plants grown *in vitro* [30]. The flow cytometry method in combination with morphological assessment can be used in rice breeding to

identify the ploidy of regenerants, as well as to cull haploids in order to exclude the stage of growing unpromising forms under ex vitro conditions.

Since the resulting regenerative plants at this stage have a poor development, we were not able to directly determine the resistance to flooding, so PCR analysis was performed followed by electrophoresis. As a result of molecular genetic evaluation, it was found that plants had flood resistance genes: from the combination (Inbara-3 x Novator) x Contact (5009/2; 5010/2) – AG1 gene, from the combination IR-64 x Magnat (4565/ 3) – Sub1A gene, from the combination (Inbara-3 x Contact) x Khao Hlan On (4641) – Sk2, AG2 genes.

After obtaining seeds from rice regenerants, they will be tested against a provocative background, namely under flooding conditions. The obtained DG lines of rice based on hybrids will be evaluated in the field for a set of economically valuable traits, and the best samples will be included in the breeding process.

4 Conclusion

For the induction of callusogenesis, 12604 pcs. were planted on an induction nutrient medium anthers by 26 hybrid combinations represented by 68 plants, resulting in 716 neoplasms, including 586 non-morphogenic calluses, 130 regenerated plants, of which 100 are albinos and 30 are green.

Cultivation of anthers on nutrient media revealed large genotypic differences between the samples. In terms of responsiveness to neoplasms, 60% of panicles showed a positive result, the rest did not give calli. The most responsive to the formation of calli were hybrid combinations: 5009/2 – 84 pcs., 5010/2 – 94 pcs., 4565/3 – 85 pcs., 4641/2 – 69 pcs. The same samples showed the ability to morphogenesis.

The studied rice samples formed 130 regenerated plants according to 14 hybrid combinations. The proportion of androgenic regenerants is 1.03% of the total number of inoculated anthers.

30 green regenerated lines were obtained from four rice hybrids, differing in visual morphological assessment: 5009/2 – 5 pcs., 5010/2 – 5 pcs., 4565/3 – 2 pcs., 4641/2 – 18 pcs.

Distinguished lines characterized by good responsiveness in anther *in vitro* culture, carry genes for resistance to prolonged flooding (Sub1A, Sk2, AG1, AG2) and can be used in rice breeding programs using DG technologies. Based on the obtained androgenic plants, which will have chromosome-doubling, dihaploid lines will be formed, which will later be included in the work on studying the manifestation of economically valuable and adaptive traits.

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