Creating doubled rice haploids with pyriculariosis resistant genes

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Abstract. Combining classical selection methods with molecular marking and gamete cell technology, it is possible to obtain constant source material with pyriculariosis resistant genes in a short time, which will significantly reduce the time of the selection process. The aim of the study was to assess the responsiveness to culture of anthers in vitro of domestic selection rice samples with pyriculariosis resistant genes. Data are given on the callusogenic and regenerative ability of 10 rice genotypes with pyriculariosis resistant genes in anther culture in vitro. Calli with morphogenetic potential were obtained, and 540 genetically stable (homozygous) androgenic lines were rapidly created on the basis of selectively valuable samples of Russian selection that possess the specified characteristics and carry pyriculariosis resistant genes.

1. Introduction
Rice is the most important food culture in the world, which is fed by more than 3 billion people. An increase in the epiphytotics of rice pyriculariosis is observed in all rice-growing regions of the world. Currently, fungicidal treatments only in the Russian Federation are used in 90% of rice-growing areas, because pyriculariosis affects more than 13% of rice crops. Losses of grain yield are 100-104 thousand tons. In recent years, areas treated with fungicides from pyriculariosis have been increasing in arithmetic progression: if in 2006 in the Krasnodar Krai it amounted to 3.2 thousand hectares of rice systems, then at present it is more than 203 thousand hectares.

Areas treated with fungicides from pyriculariosis also increase in all rice-sowing countries of the world, so in China, 70% of the cultivated area is cultivated twice during rice vegetation. The current situation speaks of environmental pollution of both rice-growing regions and rice production. This dictates the urgent need to reorient breeding strategies towards greening the industry, reviewing varietal policies, creating and introducing rice varieties that can produce high yields under conditions of non-pesticidal technologies. In relation to rice growing, this is the creation of varieties resistant to the causative agent of pyriculariosis, the cultivation of which, reducing the use of fungicides, ensures the food safety of rice products. Creation of rice varieties with long-term resistance to the fungal parasite Magnaporthe grisea is impossible without the search for an effective selection algorithm for the accumulation (pyramidation) of these genes on the genetic basis of elite rice plasma.

In developed rice-growing countries (Australia, Western Europe, the USA, Japan, China), progress in rice growing is carried out both due to biogenic and technogenic factors of intensification. The most important of them is the use of modern breeding methods when creating new varieties. In foreign rice breeding programs, biotechnological approaches and techniques, primarily molecular marking of
Economically important genes, and, in particular, resistance to pyriculariosis, are used very widely, for example, IRRI (Philippines); CIRAD (France) et al. There are also genes that determine broad spectrum resistance and are an important genetic resource for selection. These genes include Pi-1, Pi-2, Pi-33 (Deng Y. et al., 2006). Numerous studies show that the listed genes show the greatest effect at joint action (Girish Kumar K. et al., 2000; Correa-Victoria F.J. et al., 2003).

Marker control of target resistance genes in the foreground selection during each backcrossing, combined with the rapid stabilization of the obtained valuable genotypes due to experimental haploidy, significantly increases the efficiency and speed of selection work (from 8-10 years of traditional selection to 4-5 years), allows to introduce widely varieties of new generation with specified properties (high yield, good quality of cereal and long-term resistance to pyriculariosis), which is of great importance in the conditions of a highly competitive agribusiness environment.

A series of lines carrying resistance genes were created at the All-Russian Rice Research Institute using DNA marker selection and phytopathological testing on the genetic basis of local selection varieties. This set of lines is of great value for the selection of rice varieties that resistant to pyriculariosis, adapted to the agroclimatic conditions of the Krasnodar region and having a high level of indicators. The main difficulty in obtaining a significant amount of F$_1$ hybrid seeds during hybridization with domestic varieties is the different duration of the growing season of the lines - donors and recipient forms. In this regard, it is necessary to work out sequential actions when creating rice varieties with long-term resistance to a fungal parasite based on an integrated approach that combines classical selection with postgenomic and cellular technologies.

Plants carrying resistance genes must be used both for performing backcrosses, for the obtaining of subsequent generations, and for creating homozygous lines using experimental haploidy.

For breeding purposes, it is preferable to introduce hybrid progeny F$_1$ into the culture in vitro immediately after backcrossing, since it carries the hereditary information of both parents and at the same time the selection process is accelerated [1, 2].

The production of doubled haploids (DH plants) is an important link in classical plant breeding and in basic research. It occupies a leading position in breeding programs to accelerate the process of creation of highly productive hybrids and varieties of agricultural plants. Modern production requirements dictate the need to use the method of producing androgenic haploids to accelerate the breeding process of varieties with target genes [3, 4, 5, 6, 7, 8].

Double haploid populations are widely used in molecular genetic studies when marking and localizing gene locus of economically valuable traits, creating disease-resistant lines, and increasing genetic diversity [9, 10].

The purpose of this study is to determine the responsiveness of the studied domestic breeding genotypes with target genes for resistance to pyriculariosis Pi-b, Pi-ta, Pi-1, Pi-2, Pi-33, Pi-z to anther culture in vitro, production of morphogenic callus lines and accelerated creation by the method of experimental haploidy of genetically stable (homozygous) DH lines with high morphological and genetic evenness based on selection-valuable samples possessing predetermined characteristics and carrying genes with a wide spectrum of resistance to pyriculariosis also samples with specific genes for resistance to pathogen.

2. Material and methods
To obtain doubled haploids, we used rice seed samples of Oryza sativa L. subspecies japonica with pyriculariosis resistance genes Pi-b, Pi-ta, Pi-i, Pi-2, Pi-33, Pi-z from the collection of the All-Russian Rice Research Institute (Table 1).
### Table 1. Samples with pyriculariosis resistance genes to create DH lines.

| No. | Catalog number | Sample name | Gene availability |
|-----|----------------|-------------|-------------------|
| 1.  | 04072          | Snowflake   | Pi-b, Pi-ta       |
| 2.  | 02890          | Mutant 744-82 | Pi-ta             |
| 3.  | 0590           | without a number | Pi-zt             |
| 4.  | 01717          | without a number | Pi-z              |
| 5.  | 04437          | L.9         | Pi-1, Pi-2, Pi-33 |
| 6.  | 04636          | Lm3         | Pi-1, Pi-33       |
| 7.  | 04434          | B 33-38.7   | Pi-1, Pi-33       |
| 8.  | 04438          | L.5         | Pi-1, Pi-2, Pi-33 |
| 9.  | 04433          | B 33-38.6   | Pi-b, Pi-z        |
| 10. | 04435          | L.6         | Pi-1, Pi-2, Pi-33 |

In the spring-summer period, donor plants were grown under the conditions of a vegetation site, in the autumn-winter period in artificial climate chambers at 18-20°C, relative humidity 70%. The selection of the biological material (immature panicles) was carried out in the morning.

Panicles with anthers were preliminarily kept at a low positive temperature of 8–10°C for ten to fourteen days to stimulate the transition of most microspores to the single-core stage and increase their life expectancy due to the formation of specific temperature shock proteins. At the same time, the destruction (disintegration) of the anther wall is also delayed, which has a detrimental effect on the development of isolated microspores in culture [11].

Before landing of anthers on artificial culture media, panicles were sterilized with a commercial solution “Belizma” for 10 minutes, washed three times in sterile distilled water. All manipulations were performed under aseptic conditions of the laminar box.

Blades basic agar medium was used to inoculate anthers (Blaydes, 1966), and Murashige and Scoogy (MS, 1962) for callus passivation. The composition of the media included macro and micro salts, Fe-chelate, organic additives (vitamins and amino acids), agar-agar and sugars. The medium was autoclaved at 1.2 atmosphere for 20-25 minutes. To stimulate cell determination and induce callusogenesis, auxin 2,4-D at a concentration of 2.0 mg/L was introduced into the Blades medium. For cell proliferation and plant regeneration in MS medium, 1.0 mg/L α-NAA (α-naphthylacetic acid) and 5.0 mg/L kinetin were used.

150 anthers of each genotype were inoculated in triplicate. When working, the sterility rules developed for the cultivation of cells and tissues were observed.

Anthers were cultured in a thermostat at a temperature of 25 ± 20°C, relative humidity of 50% in the dark for 20-30 days before the formation of calli, then calli were cultivated in the photo period 12 hours - day (5 thousand lux), 12 hours - night before the emergence of seedlings, which, for rooting, were transferred to a hormone-free medium MS.

Serial histological sections were used to study the development of microspores and callus on nutrient medium. The study and microphotography were carried out on an MBI-6 microscope.

The method of squeezed preparations was used for counting chromosomes in the roots of rice regenerants and determining the ploidy of test plants. The macerated root stained with acetocarmine was examined using an MBI-6 microscope, 40x and 70x lenses with water immersion.

Haploid plants were transferred to the diploid level using the method of rudimentary panicles, in which panicles of 0.5-0.7 cm in size were singled out under sterile box conditions and transferred to nutrient medium to stimulate form-building processes.

### 3. Results

Before bringing under the cultivation, a cytological assessment of the microspore state was carried out and the relationship between their development and morphological traits of the panicle was compared. Based on cytological studies, panicles located in the leaf sheath were selected for which the distance between the flag and the second leaf corresponded to 5-7 cm (2–3 days before selective sweeping),
while the floral scales had a light green color, the length of the anthers and stamen threads had a ratio of 1/3 to 1/2 the length of the floral scales (Figure 1).

**Figure 1.** Rice panicle in the leaf sheath.

At the same time, microspores were at the unicellular or early bicellular stage (Figure 2, 3) and were able in vitro to switch from the gametophytic path of development and proceed to abnormal development, as a result of which plants developed from calli or embryoids.

**Figure 2.** Cross section of rice anther with microspores at the unicellular and bicellular stages, 100x.

**Figure 3.** Rice microspores in the unicellular (A) and bicellular (B) stages, 100x.

Only individual microspores proceeded to abnormal development; on the 5th day of cultivation, their nuclei were unlimitedly divided with the formation of microcallus, which was formed inside the anther on 10-15 days after inoculation on nutrient medium. At an early stage of development (inside the anther socket), callus aggregates were represented by random clusters of cells (Figure 4A).

On the surface of cracked anthers, callus appeared on 25–40 days, which depended on the genotype of the studied sample (Figure 4B). Differentiation of newly formed cells led to an increase in their mass and the formation of different types of tissue, as a result of which, in vitro induced callus masses were characterized by a high degree of heterogeneity, even when they were obtained from the same donor genotypes. This was manifested in the morphological and structural diversity of calli. Different morphotypes of callus cultures and, as a result, a different number of viable regenerants were obtained from one explant.
Accounting of the callus quality and quantity was carried out for each sample. Induction of callusogenesis on a medium containing auxin 2,4-D at a concentration of 2.0 mg/l was observed in all studied rice genotypes. Variability in terms callus forming was genetically determined. As can be seen from Table 2, the indicators of callusogenesis differed between the studied samples, they also varied between different plants - donors of the same sample.

Table 2. The effect of the Oryza sativa L. genotype on callusogenesis in the culture of isolated anthers, %.

| Sample No | Callusogenesis, % | Callusogenesis, % |
|-----------|-------------------|-------------------|
| 04072     | 10.1              | 26.3              |
| 02890     | 1.6               | 11.5              |
| 0590      | 4.2               | 10.2              |
| 01717     | 3.4               | 13.8              |
| 04437     | 1.3               | 7.5               |
| 04636     | 6.7               | 12.9              |
| 04434     | 6.5               | 16.1              |
| 04438     | 0.8               | 5.0               |
| 04433     | 4.6               | 20.8              |
| 04435     | 0.8               | 7.0               |

When analyzing the average indicators of callusogenesis, the maximum result was shown for sample No. 04072 (18.2%), the minimum - 2.9% was observed for sample No. 04438 (Figure 5).
Figure 5. Callusogenesis of rice samples with pyriculariosis resistance genes.

Variability in terms of regeneration also depended on the genotype. Table 3 presents the minimum and maximum indicators of regeneration from calli of different plants of one sample (Table 3).

**Table 3.** The influence of the Oryza sativa L. genotype on the regeneration of isolated anther culture.

| Sample No | Regeneration,% |
|-----------|----------------|
|           | Min | Max |
| 04072     | 1.6 | 7.8 |
| 02890     | 0.3 | 3.5 |
| 0590      | 0.5 | 3.5 |
| 01717     | 0.8 | 3.0 |
| 04437     | 1.0 | 1.4 |
| 04636     | 0.9 | 5.7 |
| 04434     | 0.3 | 8.1 |
| 04438     | 0.3 | 2.7 |
| 04433     | 1.1 | 4.7 |
| 04435     | 0.1 | 3.5 |

The average regeneration rates ranged from 1.8% to 4.8% (Figure 6). The maximum values for this feature were noted in images No. 04434 and 04072 (4.2 and 4.8%, respectively). The remaining samples were characterized by a lower frequency of morphogenesis and yield of green seedlings.
Figure 6. Regeneration of callus rice samples with pyriculariosis resistance genes.

The most responsive in vitro anther culture according to the complex of callus formation/regeneration traits were samples No. 04072, 04434 and 04433 (18.2/4.8%; 11.6/4.2% and 12.7/2.9 %, respectively) (Figure 7).

Figure 7. Regeneration of in vitro rice anther culture.

“Embryoidogenic callus” was mainly obtained by anther culturing of samples No. 04072 and 04434. When staining of sections of such calli, continuous accumulation of dye was noted, which indicates an intense concentration of proteins, division and high growth activity of all callus cells (Figure 8).
Samples No. 01717, 02890, 0590, 04433, 04636 and 04435 mostly induced loose light brown, granular callus masses with large cells, which were characterized by low ability to morphogenesis. In them, the dye accumulated foci, only in small zones of cell growth (Figure 9).

Non-morphogenic dark brown, hydrated, with large shapeless cells of different sizes, calli were characteristic for samples No. 04437 and 04438.

Viable rice regenerants from morphogenic types of callus were formed according to the type of embryoidogenesis and hemorrhogenesis. In embryoids/gems, a coleoptile, shoot growth points with buds of leaves and a meristem of the germinal root were laid (Figure 10).
For regenerants obtained in the anther culture was identified ploidy for early detection of plants with a haploid set of chromosomes and timely transfer to the diploid level. Haploid set of chromosomes (12n) had 15% of regenerants (Figure 11), 2% of plants had chromosomal abnormalities.

Regenerants with a spontaneously doubled set of chromosomes (DH) accounted for 83% of all viable plants obtained through anther culture; they are rooted in liquid nutrient medium (Figure 12) and planted in vessels with soil to adapt to ex vitro conditions.
Figure 12. Rooting of viable rice regenerant plants.

4. Discussion
In relation to the studied samples, 5 main callus morphotypes were distinguished, No. 1-4 were characterized as morphogenic (to varying degrees) and the 5th non-morphogenic:
1. matte, light shades, fine-grained, medium density;
2. rounded, white, light yellow, medium density;
3. dense, white, fine-grained (nodular);
4. light brown, granular, loose, with large cells;
5. dark brown, hydrated, with large shapeless cells of different sizes.

The callus morphology directly affected on the regenerative ability. The highest regenerative ability was possessed by a dense, white, fine-grained nodular (nodulated) callus of a white or light yellow shade with white inclusions. Its characteristics corresponded to "embryoidogenic callus". This callus consisted of round, small cells with a dense cytoplasm and a large nucleus, which is characteristic of meristem cells.

Rounded, white, light yellow, medium density callus also had a sufficiently high regenerative ability. The cells of his tissues were slightly larger, and the nuclei were less visible. Such callus aggregates were formed in sample No. 04636.

The callus transfer of different morphotypes to the regeneration medium revealed sharp differences in their morphogenetic properties. The maximum yield of regenerants was given by young 10-day calli. There were few such callus aggregates, mainly larger transplants (5-10 mm) were transferred to the regeneration medium to stimulate morphogenesis. The callus cultivation for 30 days ensured the highest yield of regenerants in relation to the number of initial subcultured calli. With an increase in the duration of cultivation (up to 50-60 days), the proportion of healthy callus tissue decreased due to necrotization, and as a result, the morphogenetic potential decreased.

In morphogenic callus at the initial stage of morphogenesis along the periphery of the callus, foci of undifferentiated meristematic cells were formed in the form of protrusions. Growth points appeared in the meristem, then growth zones (large cells with a large rounded nucleus and a noticeable nucleolus arranged in rows), which exuded above the surface of the protrusions with tubercles. In these zones, the rudiments of a new plant organism appeared: germ-like structures - callus embryoids (secondary embryoids) or gems (buds).

The morphogenetic potencies of the studied rice samples were realized only through callus tissues in the following ways: embryoidogenesis (the formation of somatic embryoids of calli), hemogenesis.
(bud formation), histogenesis (tissue formation) and hemorrhogenesis (simultaneous formation of a bud and a root).

According to their responsiveness to the anther culture, the studied samples are allocated in 3 groups:
- highly responsive: No. 04072 and 04434;
- medium responsive: No. 01717, 02890, 0590, 04433, 04636 and 04435;
- poorly responsive: No. 04437 and 04438.

Genetically stable (homozygous) androgenic rice regenerants based on 10 selection-valuable samples with target genes for resistance to pyriculariosis were obtained from morphogenic calli accelerated by the in vitro anther culture method. The formation of a database containing microsatellite (or SNP) profiles of doubled haploids, as well as selection-valuable rice samples created on their basis, has begun.

5. Results
The use of digaploid rice lines in the future will help to intensify the breeding of this valuable cereal crop in the Krasnodar Krai and will significantly reduce the time to create varieties with target genes, thereby increasing the efficiency of the breeding process. The created digaploid lines can be used in environmental tests of rice-growing zones of the Russian Federation.

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