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NUCLEAR DNA CONTENT IN RICE (*Oryza sativa* L.) REGENERANTS DERIVED FROM ANTHER CULTURE in vitro

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Abstract

Rice is an important food crop grown in the south of the Russian Far East. Therefore, breeding new varieties with high harvest and crop quality is relevant. Anther in vitro culture is successfully applied in breeding programs in rice-growing countries, including Russia. In anther in vitro culture, flow cytometry is applicable to select haploid, dihaploid and polyploid regenerants. Cytological studies show genome variations from haploids to hexaploids in plant tissue in vitro culture, and also chromosome changes which result in aneuploidy or endopolyploidy leding to an inconstant nuclear DNA content. In the work, we followed the aims i) to evaluated nuclear DNA content by flow cytometry in an androgenic rice regenerant population, and ii) to estimate the applicability of the combination of two approaches, the anther in vitro culture technique and flow cytometry, in rice breeding. A total of 1099 regenerants from in vitro anther culture of a single F_2 (UkrNIIS 3435 × Ukr 96) rice (*Oryza sativa* L. ssp. *japonica* Kato) hybrid plant were separated into four groups with regard to morphological features. Haploids were sterile plants with very small flowers, dihaploids were fertile plants, tetraploids were the plants with very few large seeds, an expressed keel and the ribbed floral scales. Also, there were the plants without seeds which flowers were normal in size but formed two or more sterile panicles. In the last group of the regenerants the plants died during early development. A total of 176 regeneranats were estimated by flow cytometry. It was revealed that nuclear DNA content varied greatly (*Cv* = 32 %) in the plants without seeds. This group seems to include plants with double set of chromosomes, triploids, tetraploids, and pentaploids. Additionally, in this group we found the regenerants with endopoyploidy since five of the plants had two nuclear DNA content peaks like those for haploids and diploids. In 23 plants nuclear DNA content approximated to dihaploid chromosome set and averaged 2.00 pg. Obviously, an aneuploidy characteristic of rice anther in vitro cultures could lead to aliquant changes in chromosome set in the regenerants, causing a loss of fertility. The dihaploid and tetraploid plants were low variable (*Cv* of 10.5 and 5.3 %) and had nuclear DNA content of 1.88 and 3.75 pg, respectively, whereas the haploids were high variable (C*v* = 29 %) with an average nuclear DNA amount of 0.89 pg. Our findings indicate that flow cytometry, together with production index, may be applied to reveal tetraploid regenerants and to remove haploids in rice breeding. That allows avoiding ex vitro trials of unpromising regenerants.

Keywords*:* *Oryza sativa* L., anther culture in vitro, flow cytometry, regenerant, haploid, dihaploid, tetraploid

The soil and climatic conditions of Russian Far East differ from the conditions of the South of Russia, where the main cultivation areas of rice are located. The nearest neighboring provinces of China, which have achieved significant success in rice breeding, use seeding technologies with a significant part of manual labor, which distinguishes them from the rice cultivation technology adopted in Russia [2]. Therefore, it is very difficult to borrow the varieties from the Western

regions of Russia and China for cultivation in the Primorsky Krai. In the State Register of Selective Breeding Results of the Russian Federation in the 12th zone, there are only rice varieties of Far Eastern breeders (http://www.gossort.com); therefore, the programs on the creation of rice varieties for the Far East of Russia should be continued.

To speed up selection and create new parental material, the anther culture is successfully used in vitro [3, 4]. Some varieties of rice in our country were selected by this method [5]. The biotechnology methods in rice breeding were also used in the Far East [6], including research to create varieties of rice using the anther culture in vitro; in particular, the basic elements of the technology of the androclinic haploidy for the Far Eastern varieties and hybrids of rice are optimized [7]. According to different estimates, 29-72% of rice regenerants obtained in the anther culture in vitro are productive dihaploids [3, 8-10]. They have a particular breeding value. Others (up to 60%) are seedless regenerants, often haploids which either are rejected or require manipulations to double their chromosome sets. Morphological features are mainly used to identify the type of the regenerant: haploid rice plants are characterized by smaller vegetative organs, increased bushiness, small flowers, and sterility [11]. Before identification, a routine procedure of growing the regenerants, bringing to the stage of flowering and ripening of seeds is carried out.

The method of flow cytometry in the study of plants has become widespread relatively recently [12]. In the anther culture in vitro, it is used mainly to separate the fractions of regenerants into haploid, dihaploid, triploid plants, etc. [13]. The data on a clear multiple increase in chromosome sets were presented; the peak value of every set is considered as a constant value [13-16]. Cytological studies of plants in cell and tissue culture in vitro indicate the existence of not only genomic variations from haploids to hexaploids [3, 4, 11, 17, 18] but also chromosomal changes leading to aneuploidy [3, 12, 18, 19] and endopolyploidy [12, 20]. In this case, the size of the genome cannot be a constant value.

In this paper, the parameters of the variability of the nuclear DNA content in different groups of regenerants are described first for the rice obtained in the anther culture in vitro (haploids, duplicated haploids, tetraploids, and non-haploid seedless forms).

The work objective was to characterize the population of rice regenerants obtained in the anther culture in vitro, according to the content of nuclear DNA by the flow cytometry method and to assess the effectiveness of the combination of these methods in breeding with rice.

Techniques. The hybrid F_2 (UkrNIIS 3435 × Ukr 96) of rice (*Oryza sativa* L. ssp. *japonica* Kato) obtained in Primorskii Agricultural Research Institute was grown on the vegetative plot in 2014.

Before introduction to in vitro culture, rice anthers were exposed to low positive temperatures (5 °C) for 7 days by placing a panicle in the cylinder with water. Further on, the anthers were placed in the inductive nutrient solution N_6 [21] with 2,4-dichlorophenoxyacetic acid (2.0 mg/l) and cultured in the dark at a temperature of 25-27 °C to form calli with the size of 1-5 mm. For secondary differentiation of shoots, the calli were transferred in N_6 nutrient solution with sucrose (6%), 6-benzylaminopurine and kinetin (1 mg/l) and cultured at an illumination of 4000 lux, a temperature of 22-25 °C and a photoperiod 16/8 h. For rooting of regenerants, the Murashige and Skoog medium (MS) was used with the half mineral composition of macro salts in the variation by Yu.K. Goncharova [18].

Regenerants with a well-developed root system were planted in pots and kept growing in the conditions of a cultural room (4000 lux, a temperature of

22-25 °C and a photoperiod 16/8 h) till the formation of seeds. According to morphological characteristics, all regenerants were divided into five groups: haploids (sterile plants with very small flowers); dihaploids (plants with seeds); tetraploids (plants with few very large seeds, an expressed panicle and ribbing on the floral scales); plants with no seeds (formed flowers of normal size but did not form seeds on two or more panicles); plant, dead at the early stages of growth and development. The leaves were exposed to lyophilization and stored in a freezer at –80 °C.

The DNA content was measured by the flow cytometry method. From one to four haploid and dihaploid plants from each callus line were used, 52 plants with no seeds referred to the non-haploid group, and 10 tetraploids, the 176 plants altogether. Lyophil-dried leaves (1-2 cm^2) were graded by a blade in a Petri dish with 1 ml of chilled Tris-$MgCl_2$ buffer containing 0.2 M Tris-base, 4 mM $MgCl_2 \cdot 6H_2O$ (Russia) and 0.5% Triton X-100 with addition of β-mercaptoethanol (1 μl/ml) (Serva, Germany), 50 μg/ml of propidium iodide (Biotium, USA) and 50 μg/ml of ribonuclease (Sintol, Russia) [22]. The samples were filtered through the CellTrics nylon membrane with a pore size of 50 μm (Sysmex Europe GmbH, Germany). *Ficus benjamina* L. nuclei with the known DNA content of 2C = 1.07 pg [23] isolated with Tris-$MgCl_2$ buffer were used as an external standard. The mean value (*M*) of the standard peak was recorded three times per the day of the study; then, it was averaged for further calculations. The mean value (*M*) of the sample peak was recorded in singlicate at the same settings of the fluorescence-based cytometry device for the sample and the standard (the same voltage on the photomultiplier tube); the peaks with at least 1000 detectable particles were used. The fluorescence data of isolated nuclei were recorded on the fluorescence-based flow cytometry device Partec CyFlow PA (Partec GmbH, Germany) with a laser radiation source (λ = 532 nm). The signals were recorded in the logarithmic representation of the fluorescence results (the logarithmic scale) [24].

The data processing was done with Statistica 10.0 ("StatSoft, Inc., USA.) The mean values (*M*) and standard errors of means (±SEM) are presented in the tables. Histograms of the relative DNA content were constructed with Flowing Software 2 (Perttu Terho, Finland) at the standard settings with the determination of the number of events (nuclei), the coefficient of variation (*Cv*, %), the mean value (*M*) and the median peak (*Me*). To determine the significance of differences between the mean values of the nuclear DNA content in the groups, Student *t*-criterion was used; the correlation coefficient and the *t*-criterion were calculated at the significance level of 5%.

1. Callus formation and regeneration in the anther culture of F_2 plants (UkrNIIS 3435 × Ukr 96) of rice (*Oryza sativa* L. ssp. *japonica* Kato) in vitro

Indicator	Value
The number of inoculated anthers, pcs.	240
Calli formation, %	37.5
The number of planted calli, pcs.	90
Calli with regenerants, pcs./%	72/80
Calli with green regenerants, pcs./%	39.0/43.3
The number of green regenerants per callus, pcs.	14.9

Results. The frequency of callus formation in the anther culture of the studied hybrid was 37.5% (Table 1). Some anthers began to form callus very early, 18 days after inoculation. With the weekly transfer of callus aggregates to the regeneration medium for 6 weeks, up to five or six passages were obtained. All of them were considered in our experience as a single callus. However, on some passages of callus, for example, on the first and third ones, green buds were not formed sometimes, while green regenerants have been formed on others. At the same time, the haploidic regenerants only may be located at the callus of the

one passage; the doubled haploids only may be located on the other callus. The callus aggregates with different combinations of green regenerants were most often: dihaploids and haploids; dihaploids, tetraploids, and dead plants.

2. Characterization of groups of regenerants obtained from F_2 plants (UkrNIIS 3435 × Ukr 96) of rice (*Oryza sativa* L. ssp. *japonica* Kato) in anther culture in vitro

Indicator	Haploids	Dihaploids	Tetraploids	Plants with no seeds	Dead plants
Total, pcs.	348	494	10	58	189
The number of regenerants per callus, pcs.					
M	9.2	13.0	0.3	1.5	5.0
±SEM	12.1	26.5	0.7	4.0	6.6
Percent of the total number of regenerants	31.7	45.0	0.9	5.3	17.2
Total number of regenerants per callus, pcs.	45	126	3	22	32

The calli formed 1099 green regenerants (Table 2). After the determination of the nuclear DNA content, it was found that the three plants, referred to dihaploids, have the parameters of tetraploids (3.78; 3.83; 3.86). Two seedless plants with a small genome similar to haploids were also found (1.34; 1.05), and three haploids with the DNA content close to that of the diploid plants (2.13; 2.16; 2.05). To characterize the rice regenerant population, these plants were referred to tetraploids, haploids, and plants with no seeds correspondingly. The error in the reference of regenerants to the desired plants fraction according to the morphological characteristics was 4.5%.

3. Nuclear DNA content in the regenerants population obtained from F_2 plants (UkrNIIS 3435 × Ukr 96) of rice (*Oryza sativa* L. ssp. *japonica* Kato) in the anther culture in vitro

Indicator	Haploids	Dihaploids	Tetraploids	Seedless plants
The number of plants, pcs.	61	50	13	52
The DNA content, pg:				
M	0.887	1.881	3.752	3.046
±SEM	0.033	0.028	0.055	0.135
min	0.606	1.438	3.442	1.717
max	1.636	2.188	4.197	4.380
Cv, %	29.0	10.5	5.30	32.0
Relatively to the mean value of haploids		2.12	4.23	3.43

N o t e. *Cv* — the coefficient of variation; 1pg of DNA = 978 million bp [29]. The differences between the mean values of the nuclear DNA content in the groups are significant at p = 0.05.

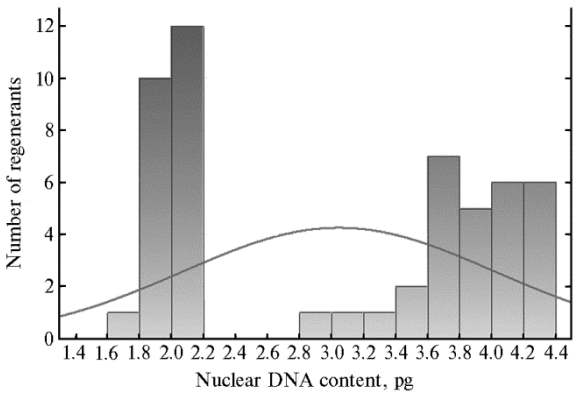

Fig. 1. Nuclear DNA content in seedless non-haploid regenerants obtained from F_2 plants (UkrNIIS 3435 × Ukr 96) of rice (*Oryza sativa* L. ssp. *japonica* Kato) in the anther culture in vitro The curve shows normal distribution.

The group of plants with no seeds was high variable in the DNA content in cell nuclei (*Cv* = 32%) (Table 3). This group probably includes plants with the double set of chromosomes, triploids, tetraploids, and pentaploids (Fig. 1). Endopolyploidy is observed in this group of regenerants, which is characterized by the peak of diploid nuclei (2×), the peak of the tetraploid nuclei (4×), combined with the G_2 peak of the mitosis stage of the diploid nuclei and the G_2 peak of the mitosis stage of the tetraploid nuclei (Fig. 2, C) [25, 26]. In the detection of the isolated nuclei, the five plants had double-vertex in the areas of haploids and diploids (see Fig. 2),

23 plants had the set of chromosomes close to that of the dihaploid regenerants (2.00 on the average). The phenomenon of aneuploidy, typical of the culture of rice anthers in vitro [18], led to the multiple changes in the chromosomal sets of regenerants, which did not allow plants to form seeds. In the groups of dihaploids and tetraploids, variability was insignificant (see Table 3). One endopolyploidy regenerant was found among dihaploids. In general, these results are comparable with the results of other authors, according to which the nuclear DNA content in the main set of chromosomes in the *O. sativa* rice varies from 0.91 up to 1.00 pg [27, 28].

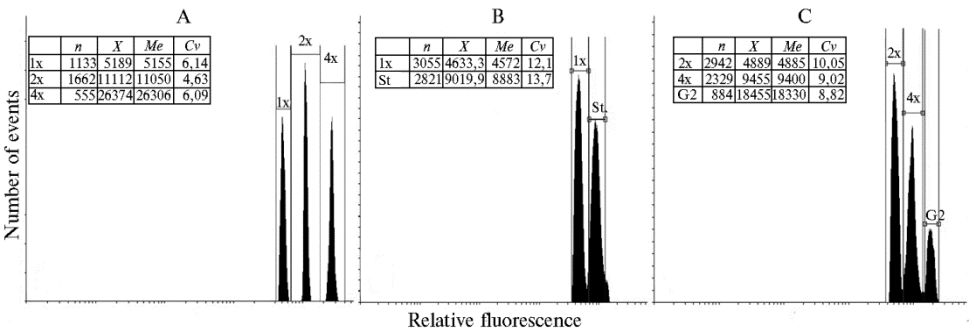

Fig. 2. Histograms constructed in the study of the ploidy and the relative DNA content among regenerants obtained from F_2 plants (UkrNIIS 3435 × Ukr 96) of rice (*Oryza sativa* L. ssp. *japonica* Kato) in the anther culture in vitro: A — consensus histogram of regenerants with 1×, 2×, and 4× ploidy, B — histogram containing regenerant peaks with 1× ploidy and the standard peak (St.), C — histogram of an endopolyploid regenerant; *n* — the number of events, *M* — the mean of the peak, *Me* — peak median, *Cv* — coefficient of variation, %.

The group of haploidic plants was highly variable in the nuclear DNA content (*Cv* = 29%). The maximum values of the haploids were higher than the minimum values of dihaploids. S.I. Maletskii et al. [29], by studying the variability of sugar beet plants (*Beta vulgaris* L.), also found higher epiplastomic and epigenetic instability in haploid genomes compared to dihaploids.

Haploid plants, derived from one callus line, differed habitually. First, strong, tall plants with large panicles and the significant number of sterile flowers were formed. The last formed plants were small with single flowers on a short panicle.

In the anther culture in vitro, the viability of haploids and dihaploids is expressed by the number of "harmful" genes, which they got as a result of meiosis [30]. The chromosomal changes occurring during the callus and regenerants cultivation in vitro [3, 18-20] lead to changes in the morphotype of plants and their viability. The plants that died in the early stages of development were probably haploids, the genotype of which contained a lot of lethals, semilethals, and sublethals [30]. The ratio of the average values of the nuclear DNA content in dihaploids and haploids was not divisible by two (see Table 3). This may indicate the loss of some parts of chromosomes in haploids indirectly during the cultivation, which led to the changes in the regenerants' morphotype.

We found no relationship between the duration of cultivation (up to 6 months) and the deviation from the average amount of the nuclear DNA in haploids (r = –0.09 at p = 0.05). The ratio of the average values of the nuclear DNA content in tetraploids and dihaploids was equal to two. Productive tetraploids, probably, arose by means of the multiple increase in the number of chromosomes in the basic set, but even in this case, they formed a small number of seeds, from one to five. According to the information provided by S.S. Guchenko (a personal communicaton), all the tetraploids of R_1 sprouted, developed, two of

them formed panicles but were sterile. Among seedless plants, 23 samples had the DNA content typical for tetraploids (see Fig. 1), but they turned out to be sterile. Low pollen fertility is associated with significant cytological changes in autotetraploid rice plants [31].

As is shown in this work and some foreign publications, the method of flow cytometry is accurate enough to identify aneuploid plants. However, the studied aneuploid plants mainly had a significant genome size with large chromosomes [22, 32]. Most of the fluorescence-based cytometry devices have the standard error of measurement equal to 2.5-5.0%. The average chromosome size for *O. sativa* is ~ 0.04 pg, which is within the limits of the standard error of fluorescence-based cytometry, whereas, for example, for *Triticum aestivum* L., the average size is ~ 0.82 pg, for *Lolium perenne* L. ~ 0.39 pg [33, 34]. Therefore, in addition to the flow cytometry technique, microscopy techniques should be used in the study of aneuploidy of plants with small genomes.

Thus, in the groups of sterile rice plants (haploids and seedless non-haploids) obtained in the anther culture in vitro, the content of the nuclear DNA is variable within considerable limits, while the fertile regenerants (dihaploids and tetraploids) have stable nuclear DNA content. The flow cytometry method together with the productivity assessment can be used in rice breeding to identify tetraploid regenerants, as well as for the purpose of haploid culling to exclude the stage of growing futile forms in ex vitro conditions.

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