# IDENTIFICATION OF THE DIVERSITY OF CULTIVATED PLANTS AND THEIR WILD RELATIVES FOR SOLVING FUNDAMENTAL AND APPLIED PROBLEMS

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# Intracallus cytotypic variation of regenerated *Oryza sativa* L. plants in the *in vitro* androgenesis

**Marina V. Ilyushko**

*Federal Scientific Center of Agricultural Biotechnology of the Far East named after A.K. Chaika, Ussuriysk, Russia*

***Corresponding author:*** Marina V. Ilyushko, ilyushkoiris@mail.ru

**Background.** The *in vitro* androgenesis has proven to be a reliable method for obtaining doubled haploids for many plant species. In the breeding of *Oryza sativa* L., *in vitro* culture of anthers is used, which go through the stage of callus formation followed by regeneration. The ratios between cells of different types and green regenerated plantlets on the callus might not coincide, since not all genetic disorders that accumulate in the *in vitro* culture at the cellular level can go through the stage of morphogenesis and cannot always lead to regeneration. The objective of the study was to assess the frequency of intracallus cytotypic variability of regenerated *O. sativa* plants in the *in vitro* androgenesis.
**Materials and methods.** We studied regenerated plantlets obtained on *O. sativa* calli through the *in vitro* androgenesis of thirty $F_1$ and $F_2$ hybrids. According to their morphological features, regenerated plants were divided into five cytotypic groups: haploids, doubled haploids, aneuploids, tetraploids, and plants that died in the early stages of growth and development.
**Results.** The experiment employed 409 calli with multiple regenerations. Only haploid plants were formed on 79 calli, only doubled haploids on 60 calli, only aneuploids on three calli, only dead plantlets on one callus, and only tetraploids on two calli. It was established that 265 (64.8%) calli were polymorphic. The calli polycytotypicity with two types of regenerated plants, excluding the dead ones, was 138 pcs. (33.7%), with three types 62 pcs. (15.2%), and with four types 7 pcs. (1.7%). The differences between the calli of the $F_1$ and $F_2$ hybrids were highly significant in the haploids, doubled haploids, tetraploids, and dead regenerated plants ($p < 0.0002$). An increase in the number of regenerated plantlets per callus occurred due to the presence of haploid plants; the correlation coefficient was $r = 0.81$ ($p < 0.05$).
**Conclusion.** The intracallus cytotypic variability of *O. sativa* in the *in vitro* androgenesis was 64.8%, which was comparable to the proportion of the calli with different cell ploidy.

***Keywords:*** *in vitro* androgenesis, intracallus variability, calli polycytotypicity, regeneration

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# ИДЕНТИФИКАЦИЯ ГЕНЕТИЧЕСКОГО РАЗНООБРАЗИЯ КУЛЬТУРНЫХ РАСТЕНИЙ И ИХ ДИКИХ РОДИЧЕЙ ДЛЯ РЕШЕНИЯ ФУНДАМЕНТАЛЬНЫХ И ПРИКЛАДНЫХ ПРОБЛЕМ

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## Внутрикаллусная цитотипическая изменчивость регенерантов *Oryza sativa* L. в андрогенезе *in vitro*

**М. В. Илюшко**

*Федеральный научный центр агробиотехнологий Дальнего Востока им. А.К. Чайки, Уссурийск, Россия*

***Автор, ответственный за переписку:*** Марина Владиславовна Илюшко, ilyushkoiris@mail.ru

**Актуальность.** Андрогенез *in vitro* зарекомендовал себя как надежный способ получения удвоенных гаплоидов для многих видов растений. В селекции *Oryza sativa* L. используется культура пыльников *in vitro*, которые проходят стадию каллусообразования с последующей регенерацией. Соотношение клеток разного типа и зеленых регенерантов на каллусе может не совпадать, так как не все генетические нарушения, которые накапливаются в культуре *in vitro* на клеточном уровне, могут пройти этап морфогенеза и не всегда могут привести к регенерации. Целью исследования являлось изучение частоты внутрикаллусной цитотипической изменчивости регенерантов *O. sativa* в культуре пыльников *in vitro.*
**Материалы и методы.** Исследовали регенеранты, полученные на каллусах риса *O. sativa* в андрогенезе *in vitro* тридцати гибридов $F_1$ и $F_2$. По морфологическим признакам регенеранты разделяли на пять цитотипических групп: гаплоиды, удвоенные гаплоиды, анеуплоиды, тетраплоиды и растения, погибшие на ранних этапах роста и развития.
**Результаты.** В эксперименте использовано 409 каллусов с множественной регенерацией. На 79 каллусах образовались только гаплоидные растения, на 60 – только удвоенные гаплоиды, на трех – только анеуплоиды, на одном – только погибшие, на двух – только тетраплоиды. Полиморфными оказались 265 (64,8%) каллусов. Полицитотипичность каллусов с двумя типами регенерантов без учета погибших составляет 138 шт. (33,7%), с тремя типами – 62 шт. (15,2%), с четырьмя типами – 7 шт. (1,7%). Различия между каллусами гибридов $F_1$ и $F_2$ высокозначимы по гаплоидам, удвоенным гаплоидам, тетраплоидным и погибшим регенерантам ($p < 0,0002$). Увеличение числа регенерантов на каллус происходит за счет гаплоидных растений, коэффициент корреляции $r = 0,81$ ($p < 0,05$).
**Заключение.** Внутрикаллусная цитотипическая изменчивость риса *O. sativa* в андрогенезе *in vitro* составляет 64,8%, что сопоставимо с долей каллусов с различной плоидностью клеток.

***Ключевые слова:*** андрогенез *in vitro*, внутрикаллусная изменчивость, полицитотипичность каллусов, регенерант

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## Introduction

*In vitro* androgenesis has proven to be a reliable method for producing doubled haploids (DH) for many plant species (Seguí-Simarro et al., 2021). The method is used in genomics and gene mapping, and is quite effective in crop breeding programs, since DH are completely homozygous (Germana, 2011; Niazian, Shariatpanahi, 2020). The haploid technology of the *in vitro* androgenesis was first described in 1964, and currently allows the production of haploids and doubled haploids for a large number of cultivated species (Seguí-Simarro et al., 2021). It is based on the ability of a microspore to switch the development program from the gametophytic path to the sporophytic path with the formation of a haploid and subsequent spontaneous doubling of chromosome sets (Niazian, Shariatpanahi, 2020).

For haploid induction in the *in vitro* androgenesis, two main approaches are used: isolated microspore culture, and anther culture (Niazian, Shariatpanahi, 2020; Seguí-Simarro et al., 2021). The first method makes it possible to obtain a plant of embryoidogenic origin from a single microspore. With the second method, one anther can provide up to several dozen plants developed from microspores (Seguí-Simarro et al., 2021). In both cases, callus formation is possible under certain conditions (Seguí-Simarro et al., 2021). However, a callus develops from a single microspore genotype in the microspore embryoidogenesis, and it can be formed by one or several genotypes of microspores in anther culture (Chen C.C., Chen C.M., 1980).

In general, the protocols for obtaining haploids/DH in many plant species have been well studied, and their features have been described (Germana, 2011; Niazian, Shariatpanahi, 2020). Callus-free methods are considered preferable. There are a number of species where isolated microspore culture for DH embryoidogenesis has not yet been successful (Seguí-Simarro et al., 2021). These include *Oryza sativa* L. (Sarao, Gosal, 2018). Despite the fact that this species was among the first to be responsive to the *in vitro* androgenesis (Niizeki, Oono, 1968), for a long time only anther culture was possible for *O. sativa* (Sarao, Gosal, 2018). In 1995, a report on the microspore *in vitro* embryoidogenesis in rice appeared (Ogawa et al., 1995); however, for a long time no one reproduced this result. The research group of S. Tajedini et al. published a protocol for obtaining a few haploids in the microspore *in vitro* culture (Tajedini et al., 2022). However, for breeding purposes, mass production of doubled haploids is needed for subsequent selection of the best ones (Goncharova, 2018). Thus, anther culture is still widely used in the *in vitro* androgenesis for the selection of the widespread sought-after species *O. sativa*. Moreover, rice anthers go through the stage of callus formation, followed by regeneration (Goncharova et al., 2019; Sakhina et al., 2020; Lantos et al., 2022).

Issues of the intracallus variability of regenerated *O. sativa* plants in anther culture were studied *in vitro* at the morphological and genetic level, as well as nuclear DNA content (Ilyushko et al., 2021, 2022, 2023). It is known that most calli are polymorphic and variable, but with a small set of morphotypes and genotypes on one callus. The issue of the intracallus cytotypic variability of regenerated plants in anther culture has not been discussed. C. C. Chen and C. M. Chen reported that rice calli, obtained *in vitro* from a single microspore in anther culture, contained haploid, doubled, triploid, and aneuploid cells (Chen C.C., Chen C.M., 1980). The presence of certain types of cells in the callus does not mean their regeneration. The ratios between different cell types in a callus and green regenerated plantlets on a callus might not coincide, since not all genetic disorders that accumulate in *in vitro* culture at the cellular level can go through the stage of morphogenesis and cannot always lead to regeneration (Kuznetsova et al., 2006). The research objective was to study the frequency of intracallus cytotypic variability of regenerated *O. sativa* plants in anther *in vitro* culture.

## Materials and methods

We studied green regenerated plantlets obtained through the *in vitro* androgenesis of $F_2$ rice hybrids (*O. sativa*) from the following crossing combinations: R × D × 67 (two plants), D × S × H (three plants), and K × V × K in 2017; $F_1$ hybrids of combinations D × M, 4P, L × 5A, 242 × R, Db × V, Db × At, R × O × 23, Abc, and A × M in 2018; 2P × L (two plants), L × 3P (four plants), K × 2P (two plants), D × M (3), D × 5A (two plants), and D × 257 in 2020; K52 × 9 × D (three plants) in 2021. A total of 30 hybrids were used as donor plants. Table 1 shows the hybrid combinations' breakdown. The method of obtaining and growing regenerated plants was described previously (Ilyushko et al., 2018, 2023).

Callus aggregates (calli) measuring 2–5 mm were removed from the anthers at intervals of seven days. A callus line was considered as all callus aggregates of one anther.

On the basis of their morphological features, regenerated plants were divided into five cytotypic groups: haploids – H (sterile plants with very small flowers); doubled haploids – DH (plants with seeds); aneuploids – An (formed flowers of normal size but did not form seeds on two or more panicles); tetraploids – TH (plants with few very large seeds, a pronounced keel, and ribbing on the flower scales); and plants that died at the early stages of growth and development – L. The error in assigning regenerated plants to the corresponding cytotype on the basis of their morphological characteristics, when compared with data on nuclear DNA content, was 4.5% (Ilyushko et al., 2018).

Differences in the polycytotypicity of the calli obtained from the $F_1$ and $F_2$ hybrids were determined using the chi-square ($\chi 2$) test. ANOVA (Fisher's F-test) was employed to identify differences between the calli in the regenerated plants belonging to different cytotypes. To identify differences between the calli within the $F_1$ and $F_2$ hybrids, ANOVA (Kruskal–Wallis H-test) was used. This criterion was chosen because of a small number of regenerated calli ($n \geq 4$) on some hybrids (see Table 1). The correlation coefficient (r) was calculated between the total number of green regenerated plantlets per callus and plants of five cytotypes. Statistical data processing was carried out using the Statistica 10 software (StatSoft, Inc., USA).

## Results

From one to five callus aggregates developed on one rice anther. The experiment involved 266 callus lines with multiple regenerations, i.e., four or more green plants per callus were obtained. In some cases, regenerated plants belonging to different cytotypes were formed on callus aggregates of one anther (Table 2). For example, only doubled haploids developed on the first callus, only haploids and one dead plant were formed on the second callus in callus line 68.2 of the K × 2P(1) hybrid. This finding confirmed the possibility of forming one rice anther callus by several microspores (Chen C.C., Chen C.M., 1980) and became the basis for considering each callus aggregate separately (Fig. 1). Two calli were formed on 61 callus lines, three calli on 12 callus lines, four

**Table 1.** **Rice hybrids (*Oryza sativa* L.) and the sample size in the *in vitro* androgenesis**
**Таблица 1.** **Гибриды риса *Oryza sativa* L. и объем выборки в андрогенезе *in vitro***

| Hybrid combination | Hybrid code | Hybrid generation | Number of calli, pcs. |
|---|---|---|---|
| Romanika × (Dariy 122 × 9167) | R × D × 67(15) | $F_2$ | 10 |
| | R × D × 67(16) | $F_2$ | 4 |
| Don 4237 × (Szorvasi 70 × Heyludjan) | D × S × H(5) | $F_2$ | 18 |
| | D × S × H(7) | $F_2$ | 11 |
| | D × S ×H(8) | $F_2$ | 30 |
| Kitaetz × (VNIIR 3223 × Kenzo) | K × V × K | $F_2$ | 8 |
| Dolyniy × Magnat | D × M(1) | $F_1$ | 5 |
| Almaz × [(Maratelli 5A × Boyarin) × Maratelli 5A | 4P | $F_1$ | 26 |
| Lugovoy × Maratelli 5A | L × 5A | $F_1$ | 12 |
| 242-01 × Rassvet | 242 × R | $F_1$ | 11 |
| Dubrava × Viola | Db × V | $F_1$ | 6 |
| Dubrava × Atlant | Db × At | $F_1$ | 4 |
| Rassvet × (Oxy 2x × Dariy 23) | R × O × 23 | $F_1$ | 10 |
| Almaz × Magnat | A × M | $F_1$ | 5 |
| Austral ×718-5 × (Oxy 2x × Dariy 23) | Abc | $F_1$ | 4 |
| Khankayskiy 52 × (Ungi №9 × Dolynniy) | K52 × 9 × D(1) | $F_1$ | 11 |
| | K52 × 9 × D(2) | $F_1$ | 15 |
| | K52 × 9 × D(3) | $F_1$ | 8 |
| (Auguszta × Otello №1) × Lugivoy | 2P × L(2) | $F_1$ | 6 |
| | 2P × L(3) | $F_1$ | 53 |
| Lugovoy × [(Dariy 8 × Khayauki) × Slavutich] | L × 3P(1) | $F_1$ | 10 |
| | L × 3P(2) | $F_1$ | 4 |
| | L × 3P(3) | $F_1$ | 12 |
| | L × 3P(5) | $F_1$ | 9 |
| Kaskad × (Auguszta × Otello №1) | K × 2P(1) | $F_1$ | 72 |
| | K × 2P(4) | $F_1$ | 9 |
| Dolynniy × Magnat | D × M(3) | $F_1$ | 4 |
| Dolynniy × Maratelli 5A | D × 5A(1) | $F_1$ | 11 |
| | D × 5A(2) | $F_1$ | 17 |
| Dolynniy × Szr 257 | D × 257 | $F_1$ | 4 |
| Total | | | 409 |

**Table 2.** **Examples of the cytotypic responsiveness of *Oryza sativa* L. in androgenic callus lines *in vitro***

**Таблица 2.** **Примеры цитотипической отзывчивости риса *Oryza sativa* L. в андрогенных каллусных линиях *in vitro***

| Hybrid | Callus line | Callus unit number | Number, pcs. | | | | |
|---|---|---|---|---|---|---|---|
| | | | H | DH | An | L | TH |
| **Different cytotypic responsiveness** | | | | | | | |
| 2P × L(3) | 31.1 | 1 | 13 | 5 | 0 | 1 | 0 |
| | | 2 | 0 | 14 | 0 | 2 | 0 |
| | | 3 | 0 | 2 | 11 | 1 | 0 |
| | | 4 | 0 | 0 | 5 | 1 | 0 |
| | | 5 | 1 | 13 | 3 | 1 | 0 |
| K × 2P(1) | 68.2 | 1 | 0 | 31 | 0 | 1 | 0 |
| | | 2 | 12 | 0 | 0 | 1 | 0 |
| K × 2P(1) | 70.2 | 1 | 0 | 15 | 0 | 0 | 0 |
| | | 2 | 23 | 0 | 0 | 1 | 0 |
| 4P(1) | 418.2 | 1 | 0 | 0 | 7 | 2 | 1 |
| | | 2 | 0 | 15 | 0 | 0 | 0 |
| **Same cytotypic responsiveness** | | | | | | | |
| D × 5A(2) | 113.1 | 1 | 36 | 0 | 0 | 5 | 0 |
| | | 2 | 22 | 0 | 0 | 1 | 0 |
| 2P × L(3) | 103.1 | 1 | 1 | 19 | 1 | 0 | 0 |
| | | 2 | 2 | 37 | 2 | 2 | 0 |

Note: in Tables 2 and 3, H – haploids, DH – doubled haploids, An – aneuploids, TH – tetraploids, L – dead plants

Примечание: в таблицах 2 и 3 H – гаплоиды, DH – удвоенные гаплоиды, An – анеуплоиды, TH – тетраплоиды, L – погибшие растения.

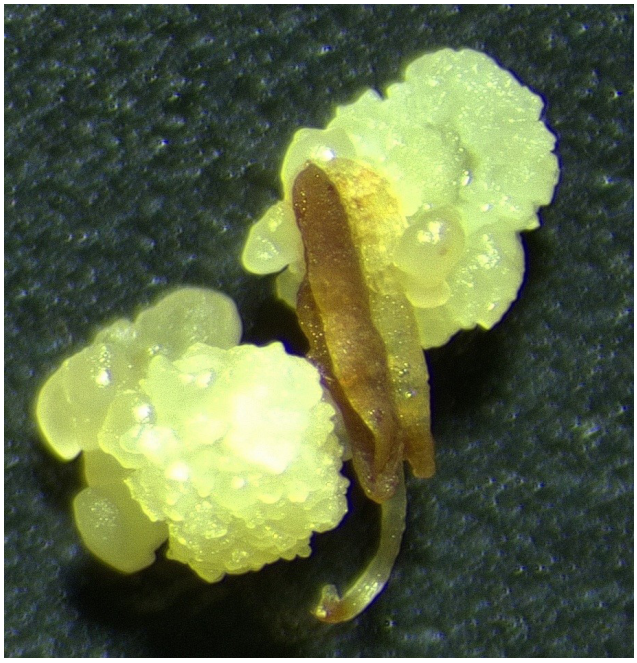

**Fig. 1.** **Calli on an *Oryza sativa* L. anther in the *in vitro* androgenesis**

**Рис. 1.** **Каллусные агрегаты на пыльнике риса *Oryza sativa* L. в андрогенезе *in vitro***

calli on ten callus lines, and five calli were formed on seven callus lines. A total of 409 calli were analyzed.

Only haploid plants were formed on 79 calli, only DH on 60 calli, only An on three calli, only L on one callus, and only TH on two calli. It was established that 265 (64.8%) callus aggregates were polymorphic. The polycytotypicity of the calli with two types of regenerated plants, excluding L, was 138 pcs. (33.7%), with three types 62 pcs. (15.2%), and with four types 7 pcs. (1.7%). A higher number of calli with three and four types of regenerated plants developed on the $F_2$ hybrids, compared to the $F_1$ hybrids. The differences between the $F_1$ and $F_2$ hybrids were significant: $\chi^2 = 9.72$ (at $p = 0.0018$) and $\chi^2 = 70.58$ (at $p = 0.00001$), respectively.

It was calculated that 8172 green regenerated plantlets were formed on calli. On the anthers of the $F_1$ hybrids, 328 callus aggregates were obtained, which formed 5306 regenerated plants. The $F_2$ anthers contained 81 callus aggregates with 2866 regenerated green plants. The highest regeneration rates were detected on the $F_2$ hybrids for H – 162 pcs. per callus, with 121 for DH, 26 for An, and 12 for TH; the highest number of dead plants (27 pcs. for L) was observed on the $F_1$ hybrids. The average rates of regenerated plants per callus for five cytotypes were higher among the $F_2$ hybrids (Fig. 2). The differences between the calli of the $F_1$ and $F_2$ hybrids were highly significant in H, DH, L, and TH ($p < 0.0002$), and in An at $p = 0.055$ (Table 3). There were no differences between the calli within the $F_1$ and $F_2$ hybrids.

An increase in the number of regenerated plants per callus occurred due to the presence of haploid plants, the correlation coefficient was r = 0.81 ($p < 0.05$). There was a tendency for the number of regenerated plants per callus to increase from the number of doubled haploids r = 0.55 and dead plants r = 0.44 ($p < 0.05$).

## Discussion

The formation of several calli on a single anther does not mean that they originate from different microspores. Therefore, the same cytotypic responsiveness of regenerated plants was revealed on calli of different orders in many cases due to the division of one callus into several aggregates under the *in vitro* conditions (see Table 2). It was noted that the calli of 2–3 microspores most often developed on one anther. However, multiple induction of microspores (up to nine) and callus formation on only one microspore can occur in *O. sativa* (Chen C.C., Chen C.M., 1980). The cited work was performed on the rice cultivar 'Tainan' obtained through self-pollination. Quite often, *in vitro* haploid techniques are tested on plant cultivars that are homozygous for most genes. However, when we reach the stage of working with hybrids that are heterozygous for a large number of genes, the effectiveness of the protocols for the *in vitro* androgenesis is significantly reduced or even absent due to low callus formation and/or regeneration rates. In such cases, it is customary to refer to the genotypic dependence of androgenic responses *in vitro* in plants (Germana, 2011; Murovec, Bohanec, 2012; Sarao, Gosal, 2018). Many authors point to the higher responsiveness of $F_2$ hybrids compared to $F_1$ hybrids in the *in vitro* androgenesis (Murovec, Bohanec, 2012; Sarao, Gosal, 2018), the heterozygosity of which is also distinguishable. This experiment confirmed a similar pattern; the regeneration of all cytotypes was higher in the $F_2$ hybrids. Thus, the actual number of microspores with calli on one anther of first-generation hybrids may be very limited. The results of the genetic analysis of androgenic doubled haploids using *Pi* genes demonstrated that from one to three microspores were induced, four in very rare cases (Ilyushko et al., 2023).

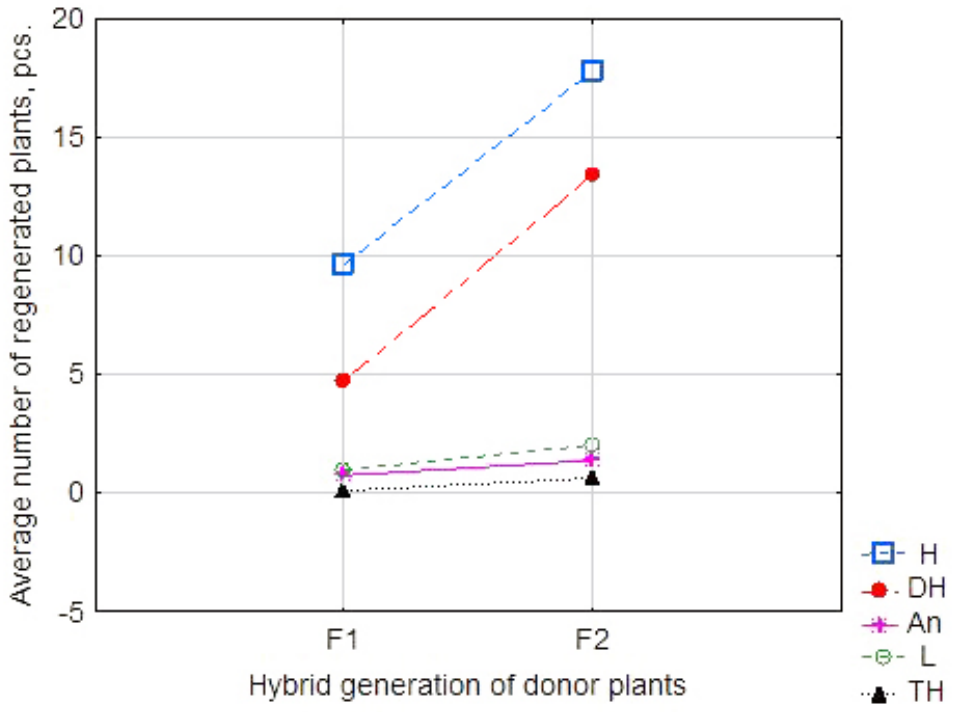


**Fig. 2. The number of regenerated plants on the *in vitro* androgenic calli of the first ($F_1$) and second ($F_2$) generation hybrids (*Oryza sativa* L.): H – haploids, DH – doubled haploids, An – plants without seeds, L – dead, and TH – tetraploids**

**Рис. 2. Численность регенерантов на андрогенных каллусах *in vitro* гибридов первого ($F_1$) и второго ($F_2$) поколений *Oryza sativa* L.: H – гаплоиды, DH – удвоенные гаплоиды, An – растения без семян, L – погибшие, TH – тетраплоиды**

**Table 3.** **Regeneration of the *in vitro* androgenic calli of *Oryza sativa* L.**
**Таблица 3.** **Регенерация *in vitro* андрогенных каллусов *Oryza sativa* L.**

| Index | Type of regenerated plants | | | | |
|---|---|---|---|---|---|
| | H | DH | An | L | TH |
| **$F_1$ hybrids, number of calli: 328 pcs.** | | | | | |
| Average number per callus, pcs. | 9.63 | 4.76 | 0.76 | 0.97 | 0.06 |
| Highest number per callus, pcs. | 81 | 47 | 20 | 27 | 3 |
| Share of the total, % | 59.6 | 29.4 | 4.7 | 6.0 | 0.4 |
| **$F_2$ hybrids, number of calli: 81 pcs.** | | | | | |
| Average number per callus, pcs. | 17.84 | 13.44 | 1.40 | 2.05 | 0.65 |
| Highest number per callus, pcs. | 162 | 121 | 26 | 13 | 12 |
| Share of the total, % | 50.4 | 40.0 | 3.9 | 5.8 | 1.9 |

Note: the differences between $F_1$ and $F_2$ are significant for H, DH, L, and TH at $p < 0.0002$, and for An at $p = 0.055$
Примечание: различия между $F_1$ и $F_2$ достоверны по H, DH, L и TH при $p < 0,0002$, по An при $p = 0,055$

The proportion of calli with different cell ploidy is 67.4% (Chen C.C., Chen C.M., 1980), and the proportion of calli with different cytotypes of regenerated plants is the same – 64.8%. Thus, in rice, cell ploidy is a good predictor of calli cytotypic variability in the *in vitro* androgenesis. However, the necessary mass production of regenerated plants, especially DH, requires simple, cheap methods for diagnosing the cultivated material. Conventional flow cytometry and cytological screening methods are expensive, time-consuming and labor-intensive, while morphological discrimination of different cytotypes of *O. sativa* plants is quite reliable (Singh et al., 2023). A high number of dead plants (up to 6.0%) were formed among the regenerated ones (see Table 3), which is the rule in the *in vitro* androgenesis (Goncharova, 2018). However, it is impossible to determine the cytotype of nonviable forms by appearance without special diagnostics. Apart from the dead plants, 50.6% of the calli manifested polycytotypicity, forming haploids, doubled haploids, aneuploids, and tetraploids in various combinations.

Haploid tissue is characterized by significant genetic stability. The rapid rate of cell division and short mitotic index make it more competitive with respect to polyploid, binuclear and aneuploid cells (Tyrnov, Davoyan, 1976), therefore the proportion of haploids affects the overall regenerative capacity of calli (r = 0.81) and accounts for more than half of all the green regenerated plantlets (see Table 3).

In general, the issue of the total yield of spontaneously doubled haploids attracts the interest of researchers, since it significantly affects the overall yield of DH. It ranges from 30 to 40% in rice, reaching 72–95% in some cases (Germana, 2011; Sarao, Gosal, 2018; Sartbaeva et al., 2018). Many researchers consider the third stage of doubled haploid obtainment to be the induced chromosome duplication by antimitotic substances with establishing the *in vitro* culture or treating haploids *ex vitro*. Even in this case, a significant increase in the yield of doubled haploids is not observed; still, the proportion of doubled haploids remains about 35% (Hooghvorst et al., 2018). It seems to us that the problem lies not in the methods of induced chromosome duplication, but in the imbalance of chromosome sets in haploid plants, incapable of duplication followed by mitotic division. Studies on the haploid morphological variability and variability in nuclear DNA content revealed significant differences both among haploids of one hybrid and within the callus line (Ilyushko et al., 2018, 2022). Apparently, spontaneous chromosome duplication in the *in vitro* androgenesis of rice reaches the maximum or almost the maximum possible rates in existing protocols. Subsequent manipulations to increase the yield of DH are not justified due to the laboriousness and danger when working with antimitotic substances.

Regardless of *in vitro* anther culture, *O. sativa* is capable of producing tetraploid plants whose fertility remains insufficiently high for cultivar production due to cytogenetic defects in meiosis (Luan et al., 2009; Chen L. et al., 2019). It is considered possible to increase the fertility of tetraploid rice through selection (Tu et al., 2007), i.e., increased homozygosity. The generation of a hybrid ($F_1$ or $F_2$) affects the overall yield of regenerated plants with a multiple set of chromosomes in the *in vitro* androgenesis – the number of DH and TH is higher among $F_2$ hybrids (see Figure 2). Many authors noted the genotypic dependence of the intensity of the spontaneous chromosome duplication in the *in vitro* androgenesis (Germana, 2011; Sartbaeva et al., 2018). And in this sense, hybrids of the first and second generations, even in the transmission of the same hybrid combination, are genotypically distinguishable, and, therefore, respond differently in *in vitro* anther culture. In the context of allotetraploidy of *O. sativa* (subspecies *japonica* and *indica*), there is a predominance of chromosome number over chromosome loss, and rice has a clear tendency to be in the aneuploid stage (Wu et al., 2018). Variations in aneuploidy in rice are extensive (Ilyushko et al., 2018; Wu et al., 2018), and provide optimism towards polyploid speciation in crop plants (Wu et al., 2018). Anther culture *in vitro* can become a reliable assistant in achieving this goal, considering aneuploid morphogenesis among hybrids in a range of 3.9–4.7%.

## Conclusions

1. The intracallus cytotypic variability of *O. sativa* in the *in vitro* androgenesis was 64.8%, which was comparable to the proportion of calli with different cell ploidy. Apart from

the dead plants, 50.6% of all the calli formed from two to four types of regenerated plants in various combinations.

2. The $F_2$ hybrids formed a higher number of haploids, doubled haploids, tetraploids, and dead regenerated plants than the $F_1$ hybrids.

3. A higher number of calli with three and four types of regenerated plants developed on the $F_2$ hybrids than on the $F_1$ hybrids.

4. The overall increase in the number of regenerated plantlets per callus occurred mainly due to the presence of haploid plants ($r = 0.81$, $p < 0.05$).

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***Information about the author***

**Marina V. Ilyushko**, Cand. Sci. (Biology), Leading Researcher, Federal Scientific Center of Agricultural Biotechnology of the Far East named after A.K. Chaika, 30 Volozhenina St., Timiryazevsky Settlem., Ussuriysk 692539, Russia, ilyushkoiris@mail.ru, https://orcid.org/0000-0001-7042-8641

**Информация об авторе**

**Марина Владиславовна Илюшко**, кандидат биологических наук, ведущий научный сотрудник, Федеральный научный центр агробиотехнологий Дальнего Востока им. А.К. Чайки, 692539 Россия, Уссурийск, пос. Тимирязевский, ул. Воложенина, 30, ilyushkoiris@mail.ru, https://orcid.org/0000-0001-7042-8641

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