AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 3, pp. 554-566
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

In vitro cultures

UDC 633.18:575:57.085.23

doi: 10.15389/agrobiology.2023.3.554eng
doi: 10.15389/agrobiology.2023.3.554rus

INTRA-CALLUS VARIABILITY FOR RICE BLAST RESISTANCE GENES IN *Oryza sativa* L. INDICATED BY GENETIC ANALYSIS OF ANDROGENIC DOUBLED HAPLOIDS

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The authors declare no conflict of interests
Final revision received February 27, 2023
Accepted April 03, 2023

Abstract

In vitro culture of cells and tissues of agricultural crops can be conditionally divided into two groups, those to generate a genetically modified initial breeding material and those for mass cloning of existing forms and varieties. Androgenesis in vitro makes it possible to redirect the microspore development from the gametophytic to the sporophytic pathway with the formation of doubled haploids (DHs) in diploid species or the fixation of dihaploids (polyhaploids) in tetraploid species for their wide use in plant breeding. The variability of plants derived from anther or microspore cultures of one donor plant has been studied to a greater extent at the genomic and chromosomal level, since researchers and breeders are primarily interested in spontaneous chromosome duplication and, as a result, completely homozygous fertile offspring. In this work, for the first time, the frequency of intra-callus genetic variability for *Pi* family blast resistance genes (two and three genes) was estimated using rice (*Oryza sativa* L.) doubled haploids (DHs) obtained via androgenesis in vitro of hybrid plants. No significant increase in intra-callus genetic variability was shown with an increase in the number of detected genes. The intra-callus variability frequency in androgenesis in vitro in rice was studied in order to determine the genetic homogeneity degree of doubled haploids (DHs) from one anther. Studies were carried out on doubled haploids obtained in androgenesis in vitro of thirteen F_1 hybrids and one F_2 hybrid of rice *O. sativa*. Molecular genetic analysis of 1271 plants (83 callus lines) was performed to reveal resistance/susceptibility alleles of the genes *Pi-z*, *Pi-b*, *Pi-1*, *Pi-2*, *Pi-ta* for rice blast-resistance to *Pyricularia oryzae* Cav. [*Magnaporthe grisea* (Hebert Barr.)]. In doubled haploids, one to four blast-resistance genes were identified depending on the presence of heterozygotes in the original hybrids. When determining one gene in DHs, the frequency of variable callus lines accounted for 24.0 %. For two genes, polymorphism occurs among 47.7 % of calli. For three genes, 62.5 % of callus lines were polymorphic. No more than four combinations of rice blast resistance gene alleles are present in one callus line. There are no differences in the monomorphic callus lines frequency detected for one, two and three genes (χ^2 = 0.21-0.95, p = 0.33-0.65). With the same combination of two resistance gene alleles, up to 66 plants were formed, and with the same combination of three genes alleles, up to 18 plants were produced per callus line. There was no dependence of polymorphism on the number of doubled haploids in the callus line. The correlation coefficients between the number of DHs and the number of alleles for one, two and three genes in the combination accounted for $r = -0.14$, $r = 0.25$, and $r = -0.35$ ($p < 0.05$). Genetic analysis of rice doubled haploids revealed a low intra-callus genetic variability during in vitro androgenesis due to gametoclonal variability. Thus, the polymorphic callus lines frequency is high, but with a limited set of allele combinations of rice blast resistance genes among DHs. There is true cloning of rice doubled haploids within the callus lineage in androgenesis in vitro. However, due to the DHs polymorphism within one callus, it is expedient to select lines of doubled haploids as breeders usually do. This work is relevant for optimizing the breeding process, including haploid technology.

Keywords: *Oryza sativa*, doubled haploids, intra-callus genetic variability frequency, blast resistance genes

In vitro cultures of cells and tissues of agricultural crops can be divided into two groups according to their intended purpose, i.e., those for obtaining genetically modified initial breeding material and for mass cloning of existing forms and varieties. Anther culture (androgenesis in vitro) allows you to switch the microspore development program from the gametophytic path to the sporophytic one. Spontaneous doubled haploids (DHs) appeared in diploid species or dihaploids (polyhaploids) fixed in teraploid species are widely used in plant breeding [1-3]. Variation among plants regenerated from gametes is commonly called gametoclonal [1, 4]. Strictly speaking, these regenerants are not clones of the donor plant genotype because of the lack of complete genetic identity due to recombinations that occur during the microspore formation. In fact, each microspore is a new genotype, albeit with the same combination of genes. In addition, variability can be induced by the in vitro cultureing itself [4, 5].

The variability generated in the culture of anthers or microspores from one donor plant has been studied to a greater extent at the genomic and chromosomal level, since researchers are primarily interested in spontaneous chromosome duplication and, as a consequence, completely homozygous fertile offspring. The proposed main mechanisms of genomic duplication are endoreduplication, nuclear fusion, endomitosis, and C-mitosis [6, 7]. D.E. Daghma et al. [8] used time-lapse imaging technology and detected only nuclear fusion in barley anther culture. Indirect methods have shown the emergence of regenerants from unreduced 2*n* gametes [9]. The origin of wheat haploids from a single cell has been proven [10]. In direct embryogenesis from microspores, all plants a priori differ genotypically. As a result, among the androgenic regenerants of one donor plant, multiple and non-multiple changes in the number of chromosomes [11-13], molecular genetic variability [14], variability in valuable traits [11, 15] and resistance to pathogens [13, 16, 17] have been identified.

In in vitro anther culture, rice *Oryza sativa* L. undergoes an additional stage of callus formation from which regenerant plants derive [18, 19]. During microsporogenesis, a highly vacuolated microspore of cereals undergoes symmetrical mitotic division with the formation of two equal cells which undergo the stage of callus formation by mitosis in vitro and implement different morphogenesis pathways [20]. In this case, additional variability of cells and regenerants arises, associated with the phenomenon of somaclonal variability [21]. Haploid, diploid, tetraploid, hexaploid and octaploid cells were identified in rice calli. Long passages reduced the number of haploid cells and the cells with high ploidy levels [22]. During prolonged calli culture, the proportion of diploid and high ploidy regenerants increases and deviations from the normal distribution of agronomically important rice traits [23] and from expected segregation for molecular markers [24] occur. Moreover, if the culture of barley microspores produces up to 50 regenerants per anther [25], then the rice callus, if formed, in some cases produces several hundred haploid plants or more than 120 doubled haploids [26]. There are calli (17%) with small numbers (up to 18) of tetraploids or seedless non-haploid regenerants [27]. On the rice variety Cascade, intracallus morphological variability of plants derived from calli with multiple regeneration was investigated ex vitro. The study revealed a significant variability of haploids and monomorphism of doubled haploids for biometric parameters [28]. However, there were several callus lines of the hybrid rice plant F_1, where, among the doubled haploids of one callus line, variability in awning and anthocyanin coloring of the stem was demonstrated, and molecular genetic variability

was detected in the rice genes *Pi-ta* and *Pi-ta2* for resistance to *Pyricularia oryzae* Cav. [*Magnaporthe grisea* (Hebert Barr.)] [29]. Theoretically, a callus line can be formed by one or more microspores. In rice, more than 1000 pollen grains mature in the anther [30]. Studying the frequency of intra-callus variability occurrence provides information on the degree of genetic homogeneity of doubled haploids from the same anther.

The variability of plants obtained via the culture of anthers or microspores from one donor plant has been studied to a greater extent at the genomic and chromosomal level, since researchers are primarily interested in spontaneous chromosome duplication and, as a consequence, completely homozygous fertile offspring. However, to optimize the selection process, including haploid technology (especially with heterotic selection), it is important to assess genetic homogeneity of regenerated plants and to understand the patterns of its change.

In this work, for the first time, we assessed the frequency of intra-callus genetic variability for two and three blast resistance genes of the *Pi* family in rice *O. sativa* doubled haploids derived from hybrid plants of various crossing combination by androgenesis in vitro. It was shown that there was no significant increase in intra-callus genetic variability with an increase in the number of detected genes.

Materials and methods. Doubled haploids of *Oryza sativa* L. subspecies *japonica* Kato generated by in vitro androgenesis were studied for F_1 rice hybrids Lugovoi × Maratelli 5A (L×5A), Rassvet × (Oxy 2x × Dary 23) (P×O×23), Almaz × [(Maratelli 5A × Boyarin) × Maratelli 5A] (4P), 242-01 × Rassvet (242×P), Dolinny × Magnat (D×M, two plants); Dubrava × Viola (Db×V), Dolinny × Maratelli 5A (D×5A, two plants); Almaz × Magnat (A×M), Kaskad × [(Dary 8 × Hayayuki) × Slavutich] (K×3R). Lugovoi × [(Dary 8 × Khayayuki) × Slavutich] (L×3R), Kaskade × (Auguazta × Othello No. 1) (K×2P) and hybrid F_2 Rassvet × Oxy 2x (P×O). The original plants of the hybrids vegetated in containers on the growing site until the panicle collection. Before introducing anthers into in vitro culture, F_1 and F_2 plants heterozygous for rice blast resistance genes of the *Pi* family were selected by molecular labeling.

The anthers were cold treated at 5 °C for 7 days. N6 medium was used to induce callus formation [31], a modified N6-based nutrient medium [32] for regeneration. For rooting, regenerants were planted onto MS medium with half the mineral composition modified by Yu.K. Goncharova [33]. Anthers were cultured in the dark at 25-27 °C, calli and regenerants were grown in a culture room at 25 °C, 5000 lux lighting and 16 h daylight.

2-5 mm callus aggregates (calli) were transplanted from the induction nutrient medium to the regeneration medium with a 7 day interval and assigned a serial number.

R_0 regenerants with a developed root system were planted in vegetation pots and grown in a culture room until seeds formed.

DNA was isolate from fresh plant leaves by salting-out method [34]. The DNA concentration was determined in a volume of 1 μl (a BioSpec-nano spectrophotometer, Shimadzu, Japan).

In PCR, the nucleotide sequence of the forward and reverse primers, the annealing temperature, and the size of the target product of the studied genes were as described [32, 33]. The reaction was run in a 25 μl reaction mixture containing 10× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μl of forward and reverse primers, 1 unit. Taq DNA polymerase (Synthol LLC, Russia) and 70-120 ng of DNA of the studied samples. Temperature response profiles varied among genes.

PCR protocol for the *Pi-1* and *Pi-2* genes was 5 min at 94 °C for initial denaturation; 35 cycles: denaturation for 30 s at 94 °C, annealing for 30 s at 56 °C, elongation for 35 s at 72 °C; 3 min at 72 °C for final elongation. For the *Pi-z*, *Pi-b* and *Pi-ta* genes: initial denaturation for 1 min at 96 °C; 35 cycles including for denaturation 15 s at 94 °C, annealing for 30 s at 60 °C, elongation for 2 min at 72 °C; final elongation for 5 min at 72°C. Amplification was run in 3 repetitions (an MJ Mini thermal cycler, Bio-Rad, USA). Plants of differentiator varieties and known varieties with resistance alleles of the target genes served as control.

Amplification products were separated electrophoretically in a 1.4% agarose gel based on 0.5× TBE buffer (an SE-1 electrophoresis chamber, OOO Helicon Company, Russia; an Elf-4 power source, DNA-Technology LLC, Russia). Bands were pre-stained with 1.0% ethidium bromide for UV-visualization (the Gel Doc XR+ gel documentation system, Bio-Rad, USA).

Characterization of the callus lines of the hybrid was based on the mean (*M*), maximum (max) and minimum (min) number of doubled haploids per callus. The frequency of intra-callus genetic variability was expressed as the proportion of polymorphic calli (%) for one gene in hybrids P×O×23, P×O, K×3P, L×3P (one or two combinations of alleles). In hybrids L×5A, 4P, 242×P, K×2P, D×5A(1), D×5A(2), which were analyzed for two genes, the proportion of calli (%) with one to four allele combinations was determined. In hybrids 242×P, D×M(1), D×M(2), Al×M, one to eight expected combinations of alleles for three genes were identified and the proportion of polymorphic calli (%) was calculated. Differences in the frequency of monomorphic callus lines detected by one, two and three genes were determined by the χ^2 test. The correlation coefficient (r) between the number of doubled haploids and the number of allele combinations per callus was calculated. The obtained data were processed in the Statistica 10 program (StatSoft, Inc., USA).

Results. The compositions of the media used are given in Table 1.

1. Compositions of nutrient media for in vitro androgenesis of rice (*Oryza sativa* L.)

Ingredient	Concentration per 1 l medium		
	induction, N_6	regeneration, $N_{6\text{-}pk}$	rooting, MS
Macrosols, ml	50.0	50.0	25.0
Microsalts, ml	1.0	1.0	1.0
Iron chelate, ml	5.0	5.0	5.0
Thiamine, mg	10.0	1.0	Absent
Pyridoxine, mg	0.5	0.5	Absent
Nicotinic acid, mg	0.5	0.5	Absent
Glycine, mg	2.0	2.0	Absent
2,4-Dichlorophenoxyacetic acid, mg	2.0	Absent	Absent
Naphthylacetic acid, mg	Absent	Absent	0.25
6-Benzylaminopurine, mg	Absent	1.0	Absent
Kinetin, mg	Absent	1.0	Absent
Sucrose, g	30.0	60.0	20.0
Agar-agar, g	8.0	8.0	8.0
pH	5.8	5.8	5.8

Plants of differentiator varieties and known varieties with resistance alleles of target genes were used as controls (Table 2).

Molecular genetic analysis confirmed the heterozygous state for alleles of blast resistance genes (Table 3) in hybrid plants. From the anthers of hybrid plants, 83 callus lines with three or more DHs were formed (the term “callus line” means all callus aggregates formed from one anther). A total of 1314 doubled rice haploids were obtained and 1271 plants were analyzed (96.7% of all DHs). One callus line produced up to 70 doubled haploids (see Table 3).

2. Molecular markers to identify blast resistance genes in androgenic doubled haploids of rice (*Oryza sativa* L.)

Gene	Marker		Primer nucleotide sequence 5′→ 3′	Resistance allele size, bp	Primer annealing temperature, °C	Reference	Control variety
Pi-1	Rm224	F	ATCGATCGATCTTCACGAGG	158	56	[32]	Magnat
		R	TGCTATAAAAGGCATTCGGG				
Pi-2	Rm527	F	GGCTCGATCTAGAAAATCCG	233	56	[32]	Magnat
		R	TTGCACAGGTTGCGATAGAG				
Pi-b	Pi-b	F	CATCAACGAAGTCCAGCTCA	490	60	[32]	Oxy 2x
		R	CCGCGCTATCTTGTACATTC				
		R	CTCAGCATATGTGGCAGCTC				
Pi-ta	Pi-ta	F1	GCCGTGGCTTCTATCTTTACCTG	270	60	[32]	Differentiator CD 8 Pi No 4
		F2	TTGACACTCTCAAAGGACTGGGAT				
		R1	ATCCAAGTGTTAGGGCCAACATTC				
		R2	TCAAGTCAGGTTGAAGATGCATAGA				
Pi-z	Z56592	F	GGACCCGCGTTTTCCACGTGTAA	292	60	[33]	Maratelli 5A
		R	AGGAATCTATTGCTAAGCACGAC				

3. Characterizqtion of androgenic callus lines with doubled haploids (DHs) derived from rice (*Oryza sativa* L.) hybrids with different blast resistance genes

Hybrid	Number of callus lines	Genes	Number of DHs		
			M	min	max
P×O×23	2	*Pi-ta*	10.5	7	14
P×O	20	*Pi-b*	16.5	4	69
K×3P	3	*Pi-ta*	5.3	5	6
Л×3P	4	*Pi-ta*	13.0	3	25
Л×5A	6	*Pi-z, Pi-ta*	8.8	3	22
4P	9	*Pi-z, Pi-ta*	15.8	5	43
242×P	1	*Pi-1, Pi-2*	31.0	31	31
K×2P	21	*Pi-2, Pi-ta*	16.8	4	70
Д×5A(1)	3	*Pi-2, Pi-ta*	14.3	3	26
Д×5A(2)	4	*Pi-2, Pi-ta*	17.5	3	55
242×P	3	*Pi-z, Pi-1, Pi-2*	8.3	3	17
Д×M(1)	1	*Pi-1, Pi-2, Pi-ta*	20.0	20	20
Д×M(2)	1	*Pi-1, Pi-2, Pi-ta*	15.0	15	15
A×M	3	*Pi-z, Pi-1, Pi-2*	5.7	4	8
Дb×B	2	*Pi-z, Pi-1, Pi-2, Pi-ta*	5.0	3	7

N o t e. *M*, min, max — the mean, minimum and maximum number, respectively, of doubled haploids in a callus line.

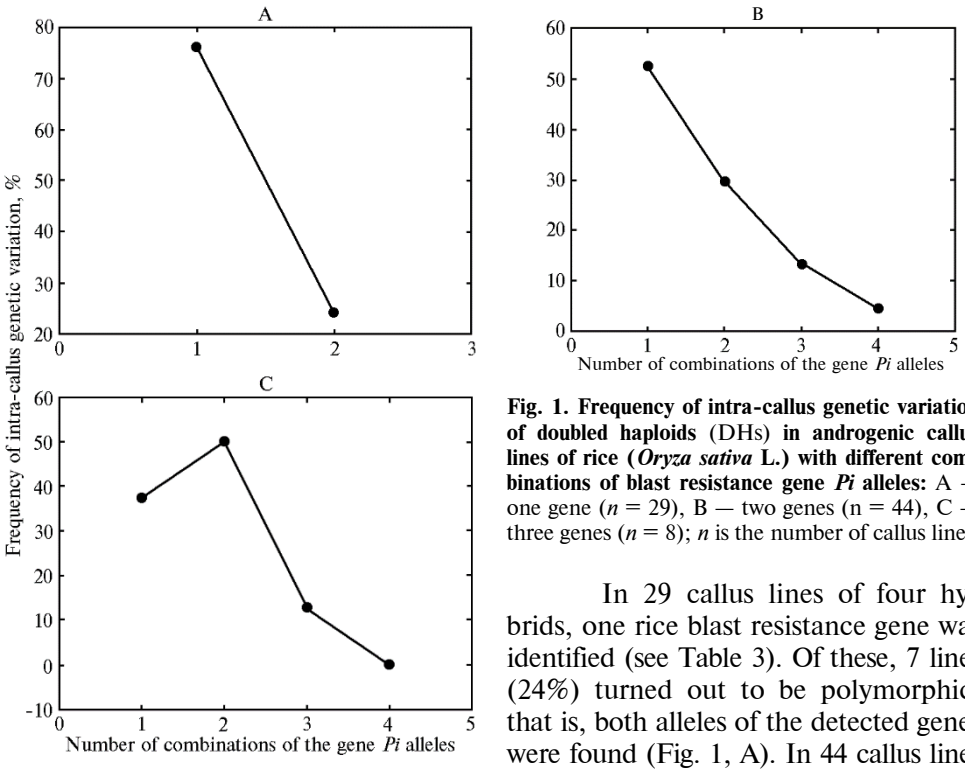

Fig. 1. Frequency of intra-callus genetic variation of doubled haploids (DHs) **in androgenic callus lines of rice (*Oryza sativa* L.) with different combinations of blast resistance gene *Pi* alleles:** A — one gene ($n = 29$), B — two genes (n = 44), C — three genes ($n = 8$); n is the number of callus lines.

In 29 callus lines of four hybrids, one rice blast resistance gene was identified (see Table 3). Of these, 7 lines (24%) turned out to be polymorphic, that is, both alleles of the detected genes were found (Fig. 1, A). In 44 callus lines of six hybrids, two *Pi* genes were detected (see Table 3). Of the four possible combinations of resistance and susceptibility gene alleles, all four were found in only two callus lines (see Fig. 1, B); more than half of the calli were monomorphic. In eight callus lines of four hybrids, alleles for three genes were identified (see Table 3). Theoretically, eight combinations of alleles are possible for three genes. However, we found the largest number of combinations (three) in doubled haploids of one callus line; three out of eight calli were monomorphic (see Fig. 1, B). In the Db×B hybrid, two callus lines with four blast resistance genes were formed (see Table 3). In both lines, doubled haploids had only two combinations of Pi resistance/susceptibility gene alleles out of a possible 15 (Table 4). There were no differences in the frequency of monomorphic

callus lines detected for one, two and three genes ($\chi 2 = 0.21$-0.95, $p = 0.33$-0.65).

4. Examples of intra-callus variability for blast resistance genes in doubled haploids (DHs) derived from androgenic callus lines of rice (*Oryza sativa* L.) hybrids

Hybrid	Callus line	Number of doubled haploids.	Gene combination
By two genes:			
K×2P	95.2	6	*Pi-2* (–), *Pi-ta* (–)
		3	*Pi-2* (–), *Pi-ta* (+)
K×2P	99.2	66	*Pi-2* (+), *Pi-ta* (+)
		4	*Pi-2* (–), *Pi-ta* (–)
D×5A(2)	112.2	55	*Pi-2* (+), *Pi-ta* (+)
By three genes:			
Al×M	564.2	1	*Pi-z* (+), *Pi-1* (+), *Pi-2* (–)
		1	*Pi-z* (–), *Pi-1* (+), *Pi-2* (–)
		3	*Pi-z* (–), *Pi-1* (+), *Pi-2* (+)
242×P	582.2	17	*Pi-z* (–), *Pi-1*(–), *Pi-2* (+)
By four genes:			
Db×B	610.2	1	*Pi-z* (+), *Pi-1* (+), *Pi-2* (–), *Pi-ta* (+)
		6	*Pi-z* (–), *Pi-1* (+), *Pi-2* (+), *Pi-ta* (–)

N o t e. «+» — a resistance allele, «–» — a susceptibility allele.

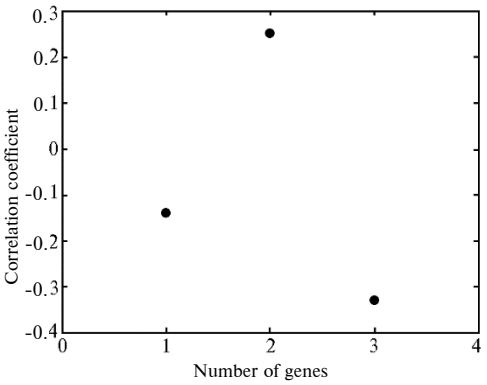

Fig. 2. Correlation coefficients (r) between the number of blast resistance genes and the number of doubled haploids (DHs) in androgenic callus lines of rice (*Oryza sativa* L.) hybrids: 1 — one identified gene ($n = 29$), 2 — two identified genes ($n = 44$), 3 — three identified genes ($n = 8$); n is the number of callus lines. The correlation coefficients are statistically significant at $p < 0.05$.

In the experiment, we used callus lines with multiple regeneration (see Table 3). There was no dependence of polymorphism on the number of doubled haploids in the callus line (Fig. 2). Insignificant negative correlation coefficients indicate that the search for polymorphic callus lines is best carried out among lines with a small number of doubled haploids. Multiple regeneration is more likely to result in the replication of identical DHs genotypes.

Nineteen callus lines formed from two to four callus aggregates with green regenerants. Analysis of the data from callus line 415.2 of the L×5A hybrid showed that the resistance alleles of the two genes *Pi-z* and *Pi-ta* were detected in all doubled haploids of the first callus aggregate, and the second callus aggregate had the resistance allele *Pi-z* and the susceptibility allele *Pi-ta*. This means that callus aggregates are initiated by different immature microspores, indicaying the phenomenon of gametoclonal variability.

Figure 3 illustrates the production of regenerated rice plants from calli based on androgenic doubled haploids.

In haploid breeding, it is important to generate the greatest possible variety of doubled haploids per plant. This can be achived through active callus formation and a large number of induced immature microspores. For species capable of direct embryogenesis, microspore culture provides up to 50 regenerants per anther [25]. Rice is a species that forms callus during androgenesis in vitro [18], and inoculation of microspores on nutrient media is unsuccessful [37, 38]. When ripe, the anther of rice opens on both terminal parts [39]. We have repeatedly observed the beginning of callus formation in vitro at opposite ends of the anther. Subsequently, these callus aggregates grew to a size suitable for transplantation into a regeneration nutrient medium. Visually, they can be mistaken for a single callus aggregate. In general, a limited number of polymorphic callus lines with three or

more gene combinations was noted (see Fig. 1, B, C). Possibly, the induction of callus formation on one microspore inhibits the development of neighboring microspores, but does not interfere with their development at the terminal ends.

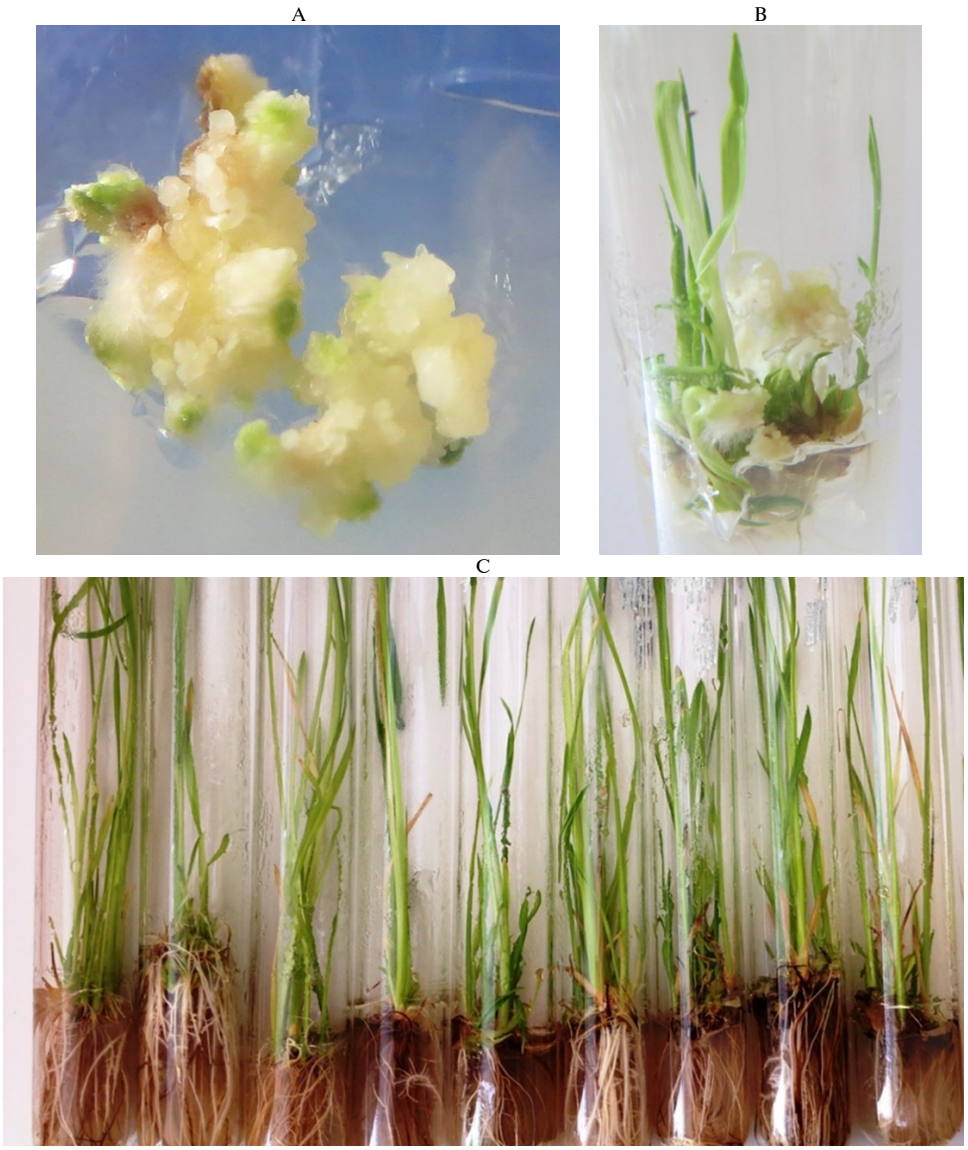

Fig. 3. Plants regenerated from androgenic calli in the studied rice (*Oryza sativa* L.) hybrids: A — androgenic callus at the stage of morphogenesis, B — multiple regeneration on androgenic callus, C — obtained regenerant plants.

The second and subsequent callus aggregates of one anther are often, but not always, identical in the combination of genes of doubled haploids in the first aggregate. For example, callus line 35.1 of hybrid L×3P is formed by two callus aggregates, they contain 25 DHs of the same genotype (resistance allele of the *Pi-ta* gene), and callus line 112.2 of hybrid D×5A(2) is formed by four callus aggregates with 55 genotypically identical doubled haploids (resistance alleles of both genes). This indicates true clonal micropropagation of doubled haploids both for one callus aggregate and for different callus aggregates from the same anther. The number of regenerants of different ploidy per the callus aggregate indirectly supports the cloning. Up to 18 tetraploids are formed per callus, up to 125 doubled

haploids, and up to 349 haploids [26, 27]. The mitotic index of haploid cells exceeds this parameter in doubled haploids and tetraploids [40], which promotes rapid micropropagation of haploids in the callus culture. In addition, the previous process of cell fusion or endomitosis reduces the number of cells involved in the formation of doubled haploids. Cell fusion can occur under certain research protocols at the initial stage during the pretreatment of anthers and microspores [41] and immediately before the formation of regenerants with a high level of ploidy (doubled haploids, triploids, tetraploids, etc.) in the light stage of callus culture [38].

Callus formation in rice during androgenesis in vitro contributes to the production of a large number of doubled haploids, but with a limited number of genotypes. The increase in genetic variability of DHs may be facilitated by somaclonal variability inherent in in vitro rice anther culture [21, 29], as in any culture of plant cells and tissues [5]. This is rather an undesirable phenomenon in in vitro androgenesis, since it is not clear whether somaclonal cell variants arise before cell fusion followed by mitotic division and regeneration or after this event. During regeneration after fusion, the cell that arose as a somaclonal variant multiplied and participated, along with other groups of cells, in the formation of doubled haploids. Hence the mixoploidy of plants occurs in androgenesis in vitro [11, 42]. The appearance of heterozygous doubled haploids is even possible, which contradicts the idea of haploid selection, where the goal is to obtain homozygous seed offspring. Although it is generally accepted that heterozygosity in androgenesis in vitro is a sign of proliferation of somatic tissues of the anther walls [1], the absence of this phenomenon has been proven in rice, that is, callus is always formed from immature microspores [43]. The tendency to somaclonal variability in diploid cells of various crops is genetically determined [44]; obviously, this pattern also applies to haploid cells. And since each microspore of an anther and plant represents a separate genotype, the somaclonal variability may differ in callus lines initiated by different anthers.

Thus, based on analysis of rice doubled haploids (DHs), we estimated the frequency of intra-callus genetic variation in in vitro androgenesis driven by gametoclonal variation. When one gene is detected in DHs, the frequency of occurrence of variable callus lines is 24.0% of all studied lines, for two genes polymorphism occurs among 47.7% of calli, for three genes 62.5% of callus lines are polymorphic. One callus line has no more than four combinations of rice blast resistance gene alleles. Consequently, the frequency of occurrence of polymorphic callus lines is high, but the range of gene allele combinations among DHs is limited. There is true cloning of doubled rice haploids within a callus line in androgenesis in vitro. Nevertheless, due to the polymorphism that occurs in regenerants within the same callus, the selection of lines of doubled haploids, adopted by breeders, is rational.

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