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

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Variability of morphological features and nuclear DNA content in haploids and doubled haploids of androgenic callus lines of rice (*Oryza sativa* L.)

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The work is relevant for understanding evolutionary processes in plant species. Twelve callus lines with multiple regeneration of haploids and doubled haploids were obtained in F_1 hybrids of *Oryza sativa* L. through *in vitro* androgenesis. Intracallus variability of the morphological features of haploids was often accompanied by a decrease in the values of morphological features with an increase in the serial number ($p < 0.05$). The number of panicles on a plant and the number of flowers on a panicle on two callus lines in the second or third group were increased. No variability was detected in five callus lines, i.e., such a phenomenon was not a rule. The nuclear DNA content of doubled haploids in four groups of the same callus line was 1.03–1.09 pg, and for haploids it was 0.53–0.58 pg. Intracallus variability of nuclear DNA content was detected between groups of haploids of the same line and among doubled haploids of the same line. Significant differences were found between the haploids of one callus line and the three other callus lines of the Sadko × Kuboyar hybrid towards an increase of nuclear DNA content ($p < 0.0015$). The theoretical possibility of the appearance of intraspecific variability among plants with a small number of chromosomes is considered. A scheme of genomic reorganization is proposed for such species: initial plant (2n) → aneuhaploid plants (n + 1) → megasporogenesis and microsporogenesis of the 0-n type, formation of fertile pollen (n + 1) → diploid plant (2n + 2).
Aneuhaploid evolution explains the intraspecific variability of chromosome numbers among plant species with low ploidy. Aneuploid technologies can help in the artificial formation of new polyploid crops, and rice is given a primary role.

Keywords: *in vitro* androgenesis, flow cytometry, intracallus variability, aneuhaploid plant evolution

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

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Изменчивость морфологических признаков и содержания ядерной ДНК гаплоидов и удвоенных гаплоидов в андрогенных каллусных линиях риса (*Oryza sativa* L.)

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Работа актуальна для понимания эволюционных процессов у видов растений. Исследование проведено на гибридах F_1 риса (*Oryza sativa* L.). Получено 12 каллусных линий с множественной регенерацией (более 60 растений на линии) гаплоидов и удвоенных гаплоидов. Регенерантные растения одной каллусной линии, полученной из одного пыльника риса, разделяли на две–четыре группы в зависимости от объема выборки в порядке их дифференциации на каллусе и высадки на среду укоренения. В первую группу входили растения с порядковым номером 1–30, во вторую – 31–60, в третью – 61–90, в четвертую – 91–120. Измеряли биометрические показатели и содержание ядерной ДНК. Между группами гаплоидов и удвоенных гаплоидов внутри каллусной линии выявлены различия по одному, двум или трем морфологическим признакам ($p < 0,05$). Внутрикаллусная изменчивость признаков гаплоидов чаще сопровождалась уменьшением значений морфологических признаков с увеличением порядкового номера. На пяти каллусных линиях не обнаружено изменчивости, то есть такое явление не является правилом. Среднее содержание ядерной ДНК удвоенных гаплоидов в четырех группах одной каллусной линии составило 1,03–1,09 пг, у гаплоидов – 0,53–0,58 пг. Внутрикаллусная изменчивость содержания ядерной ДНК выявлена между группами у гаплоидов на одной линии и среди удвоенных гаплоидов одной линии. Обнаружены достоверные отличия гаплоидов одной каллусной линии от трех других каллусных линий гибрида Садко × Кубояр по содержанию ядерной ДНК в сторону увеличения ($p < 0,0015$). Рассматривается теоретическая возможность появления внутривидовой изменчивости среди растений с небольшим числом хромосом. Предлагается схема геномных преобразований у таких видов: исходное растение (2n) → анеугаплоидные растения (n + 1) → мегаспорогенез и микроспорогенез по типу 0-n, формирование фертильной пыльцы (n + 1) → диплоидное растение (2n + 2).
Анеугаплоидная эволюция объясняет внутривидовую изменчивость чисел хромосом среди видов растений с низкой плоидностью.

Ключевые слова: андрогенез *in vitro*, проточная цитометрия, внутрикаллусная изменчивость гаплоидов и удвоенных гаплоидов, анеугаплоидная эволюция растений

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Introduction

The polyploidy phenomenon is widespread in many angiosperm families (Grant, 1981; Stebbins, 1999; Levin, 2019). Some plant families are up to seventy percent polyploid (Grant, 1981; Stebbins, 1985). The fact that polyploidy plays an important role in the emergence of families does not mean that it contributed to progressive evolution – it is a complicating force that produces countless variations on old themes without new major deviations (Stebbins, 1966; 1999). The increased genome numbers create a rigid genetic system that has tactical advantages in the already mastered severe conditions, but it is absent from the strategic plan, when evolutionary plasticity is required (Khokhlov et al., 1976). Polyploid species have higher extinction rates than their diploid cousins (Levin, 2019). It has also been shown on yeast that haploid populations are more adaptable to changing environmental conditions than diploid ones (Zeyl et al., 2003). Progressive evolution usually proceeds at the lowest possible ploidy level, hence the constant change of ploidy levels within the boundaries of the biologically optimal ploidy range (Khokhlov et al., 1976). V. Grant (1981) considers evolution from diploid to tetraploid and higher polyploid to be largely irreversible. Nevertheless, there are few examples of reverse plant evolution (depolyploidization) from tetraploids to polyhaploids (Raven, Thompson, 1964; de Wet, 1971; Khokhlov et al., 1976), called dihaploids in the modern scientific literature (Jauhar et al., 2009).

Plant species plurality with non-multiple intraspecific changes in chromosome numbers are known, and the majority of them are highly ploid (Agapova et al., 1990, 1993). Depolyploidization of such species proceeds through the successive loss of individual chromosomes and the aneuploid plant formation (Khokhlov et al., 1976). However, this pathway does not explain the occurrence of variability in species with a small basic number of chromosomes, such as *Crepis pannonica* (Jacq.) C. Koch (2n = 8, 9, 10, 11), *Erysimum amurense* Kitag. (2n = 12, 14), *Antyllis lachnophora* Juz. (2n = 10, 12), *Primula nutans* Georgi (2n = 20, 22), *Aquilegia parviflora* Ledeb. (2n = 14, 16), and others (Agapova et al., 1990, 1993; Takhtajan, 2009). Elimination of a chromosome or a pair of chromosomes at the diploid level would lead to the loss of a genome part with irreversible consequences. It should be noted that such species are relatively scarce in comparison with depolyploidized highly ploid species (Agapova et al., 1990, 1993).

It is believed that spontaneous haploidy is a rare phenomenon; however, it is constantly encountered in many plant species. Usually the frequency of haploidy does not exceed 0.1%, but it can reach 15% (Kunakh, 1995). Haploid flowering plants are rare in nature, since they are less viable and die soon after germination (Kirillova, 1966). Under experimental conditions many exceptions have been described, when mono- and polyhaploids are not inferior to the original diploids in a number of characteristics. In some cases, haploid plants were more powerfully developed than the original diploid forms (Khokhlov et al., 1976). There is an opinion that haploid androgenesis as a particular form of haploidy can lead to the intensification of the morphogenesis process (Khokhlov et al., 1976).

In vitro culture is a stressor for plants (Kunakh, 1998). Various deviations from the chromosome diploid set by cytology and flow cytometry have been found in callus cells (Kunakh, 1998; Barow, Jovtchev, 2007; Ochatt, 2008). Not all genetic disorders that accumulate in *in vitro* culture at the cellular level can pass through the morphogenesis stage, and it is not always possible to obtain regenerants and their offspring (Kuznetsova et al., 2006; Barow, Jovtchev, 2007; Ochatt, 2008). This fully applies to haploid technologies. There is evidence of cytological changes in regenerants obtained in *in vitro* androgenesis, with corresponding phenotypic manifestations, often leading to the death of plants or their sterility (Kasha et al., 2001; Zagorska et al., 2004; Cistué et al., 2006). Aneuhaploids have been recorded in a number of cultivated species (Khokhlov et al., 1976; Tyrnov, 2005). There are cases when in a haploid, derived from polyploidy-origin species, a number of chromosomes is less than in typical cases. The existence of monoploids without one or several chromosomes is apparently practically impossible (Khokhlov et al., 1976; Kunakh, 1995; Wu et al., 2018). V. S. Tyrnov (2005) highlights the specific role of haploidy and the karyotype reorganization possibility based on aneuhaploids.

The evolutionary changes of the most significant deviation, the most divergent aberrant populations occur under environmental stress conditions (Takhtajan, 1983). The saltation chromosome reorganization can even lead to speciation (Lewis, 1966). *In vitro* cultivation is human creation of environmental stress for plants. The aim of the study was to simulate the conditions under which changes in the DNA content in a haploid rice (*Oryza sativa* L.) population are possible during *in vitro* androgenesis and to describe the haploid evolution scheme with cytotypic variability.

Materials and methods

The studies were carried out on the rice (*Oryza sativa* L.) F_1 hybrids: Sadko × Kuboyar, Magnat × Dolinny, and Dubrava × Atlant. The original plants were grown in a climatic chamber in spring at the 21°C temperature, 5000 lux illumination, 70% humidity, and photoperiod of 16/8 h. The technique of anther cold pretreatment, and anthers, calli, and regenerants in *in vitro* culture were discussed by M. Ilyushko et al. (2018).

Green R_0 regenerants with a well-developed root system were transplanted to pots and grown under controlled conditions in a cultivation room up to seed formation in the doubled haploids. Haploid plants were characterized by small flowers and sterility. Regenerant plants of the same callus line derived from one rice anther were divided into 2–4 groups (depending on the sampling volume) in the order of their differentiation on the callus and transplanting onto a rooting medium. The registered morphological features included plant height (cm), length of the main panicle (cm), number of flowers per main panicle (pieces), number of panicles per plant (pieces), and the number of seeds per main panicle (pieces) for doubled halpoids.

The content of nuclear DNA was determined using flow cytometry. For haploids and doubled haploids R_0 freeze-dried straw leaves stored for 6–9 months at – 80°C were used. The seeds of the doubled haploids R_1 germinated; in the three–four leaves phase the leaf was lyophilized and used for analysis without storage. The sample preparation procedure was described by M. Ilyushko et al. (2018). Isolated nuclei of *Ficus benjamina* L. with a known DNA content 2C = 1.07 pg were used as the reference (Skaptsov et al., 2016). Peaks with no less than 1000 detectable particles were used. The fluorescence data of isolated nuclei were recorded on a Partec CyFlow PA flow cytometer with a laser radiation source (λ = 532 nm) in duplicate. The data obtained were processed using the Statistica v.10.0 software.

The following statistical treatments were carried out: the analysis of variance (ANOVA) was used to characterize mor-

phological traits, while differences in traits in the groups were identified using Tukey's test. To determine the significance of differences in the mean values of DNA content between groups within the callus line, the Wilcoxon z-test was used, which was applicable for small samples.

Results

1286 rice anthers were introduced into *in vitro* culture; callus formation was 9.8%. Callus lines with multiple regenerations were selected for the study. A total of 483 plants were analyzed for biometric features and 224 for the nuclear DNA content.

Intracallus differences were revealed between the groups of haploids and doubled haploids in one, two, or three morphological features ($p < 0.05$) (Table 1).

The average nuclear DNA content for doubled haploids was 1.03–1.09 pg (Table 2). In three haploid plants of callus lines 36.2.1 and 214.1.1 of the Sadko × Kuboyar hybrid, the nuclear DNA content was typical for a diploid plant (1.05–1.16 pg), although the plant height (26–42 cm) and flower size were characteristic of haploids, and the number of flowers per panicle was small (7–16 pcs.). These plants were excluded from the further analysis.

Intracallus variability in the nuclear DNA content was found between groups for haploids in line 124.2.2 and doubled haploids in line 39.1.2 R_0 (see Table 1). These lines have a very low standard deviation (Fig. 1, 2), which made it possible to experience significant differences in values between plant groups at $p < 0.05$.

A weak correlation among doubled haploids was found between plant height and DNA content ($r = 0.54$ at $p < 0.05$).

Table 1. Intracallus morphological variability of haploids and doubled haploids in androgenic callus lines of rice (*Oryza sativa* L.)

Таблица 1. Внутрикаллусная морфологическая изменчивость гаплоидов и удвоенных гаплоидов в андрогенных каллусных линиях риса (*Oryza sativa* L.)

Hybrid	Callus line number	Sampling volume (pcs)	Mean values (M) of morphological indices:					ANOVA
			plant height (cm)	main panicle length (cm)	main panicle number of flowers (pcs)	panicle per plant (pcs)	seeds per main panicle (pcs)	
Haploids								
Sadko × Kuboyar	36.2.1	22	42.1^{a}	7.5	69.7	2.2	–	F = 2.82, p = 0.04
		30	37.6^{b}	7.6	67.9	1.8	–	
	101.2.2	15	44.0	7.5	63.7	2.5	–	F = 1.88, p = 0.07
		30	41.2	7.8	58.8	2.1	–	
		20	39.1	8.2	68.9	2.2	–	
	124.2.2	26	45.7^{a}	7.8	62.5^{a}	2.2	–	F = 6.92, p = 0.000001
		24	43.6^{ab}	7.8	77.5^{b}	1.9	–	
		18	37.6^{c}	8.2	66.6^{ab}	2.6	–	
	214.1.1	25	45.6	7.1^{a}	59.6	2.5^{a}	–	F = 5.16, p = 0.0002
		23	43.0	7.7^{b}	64.8	1.8^{b}	–	
Magnat × Dolinny	150.1.1	27	49.6^{a}	10.0	94.7	1.0	–	F = 4.12, p = 0.006
		26	42.2^{b}	9.4	84.5	1.0	–	
Dubrava × Atlant	459.1.2	29	40.9	9.0	112.8^{a}	1.3	–	F = 3.04, p = 0.003
		28	43.8	9.6	157.0^{b}	1.4	–	
		28	44.0	9.5	161.4^{bc}	1.6	–	
Doubled haploids								
Magnat × Dolinny	39.1.2	29	77.1^{a}	10.5	41.6	1.2	7.7^{a}	F = 2.10, p = 0.01
		28	69.5^{b}	10.7	38.1	1.2	10.5^{ab}	
		28	71.8^{ab}	10.3	34.4	1.2	11.9^{ab}	
		24	71.7^{ab}	10.5	38.2	1.1	14.4^{b}	

Note: ^{abc} – Tukey's test at $p < 0.05$

Примечание: ^{abc} – критерий Тьюки при $p < 0,05$

Table 2. **Intracallus variability of nuclear DNA content in haploids and doubled haploids in androgenic callus lines of rice (*Oryza sativa* L.)**

Таблица 2. **Внутрикаллусная изменчивость содержания ядерной ДНК гаплоидов и удвоенных гаплоидов в андрогенных каллусных линиях риса (*Oryza sativa* L.)**

Hybrid	Callus line number	Sampling volume (pcs)	Mean values (M) of nuclear DNA content:	
			R_0, pg	R_1, pg
Haploids				
Sadko × Kuboyar	36.2.1	10	0.55	–
		9	0.54	–
	101.2.2	9	0.55	–
		10	0.54	–
		10	0.54	–
	124.2.2	10	0.58*	–
		9	0.57*	–
		10	0.57	–
	214.1.1	10	0.55	–
		10	0.56	–
Magnat × Dolinny	150.1.1	10	0.57	–
		10	0.56	–
Dubrava × Atlant	459.1.2	11	0.52	–
		9	0.53	–
		10	0.53	–
Doubled haploids				
Magnat × Dolinny	39.1.2	10	1.04	1.07
		10	1.03*	1.07
		9	1.04	1.08
		10	1.04*	1.09

* – Intracallus significant differences at $p < 0.05$

* – Внутрикаллусные различия достоверны при $p < 0,05$

A very weak negative correlation was detected between the number of flowers per panicle and the DNA content (r = –0.25, $p < 0.05$).

There are significant differences ($p = 0.0001$) in the nuclear DNA content between the doubled haploids R_0 and R_1. In R_0 regenerants the average value was 1.04 pg. The average value among regenerates of the first generation R_1 was higher – 1.09 pg, standard deviation (see Fig. 2). This happened due to three plants with callus differentiation numbers 56, 74, and 92, where the nuclear DNA content was 1.19, 1.20, and 1.31 pg, respectively. When these plants were excluded from the calculations, the average DNA and the standard deviation decreased to 1.07 pg; however, significant differences between the samples of doubled haploids R_0 and R_1 remained.

Significant differences were found between callus line 124.2.2 haploids and three other callus lines of the Sadko × Kuboyar hybrid in terms of the nuclear DNA content upward ($p < 0.0015$) (see Fig. 1).

Discussion

The nuclear DNA content of 1.03–1.09 pg in doubled haploids is consistent with the data of other rice researchers at the diploid level, where the value in the main set of *O. sativa* chromosomes varies within 0.91–1.00 pg (Bennet, Smit, 1991; Bai et al., 2012).

The differences in morphological features were revealed between the groups of haploids and doubled haploids obtained from hybrid plants within the callus line ($p < 0.05$). This was not observed among doubled haploids obtained from varietal plants. Variability of intracallus morphological features in varietal haploids was always accompanied by a decrease in the values of morphological features with an increase in the serial number (Ilyushko, Romashova, 2019). In some callus lines (459.1.2 and 124.2.2) in the second or third group in hybrid haploids the number of panicles per plant and the number of flowers per panicle increased. In general,

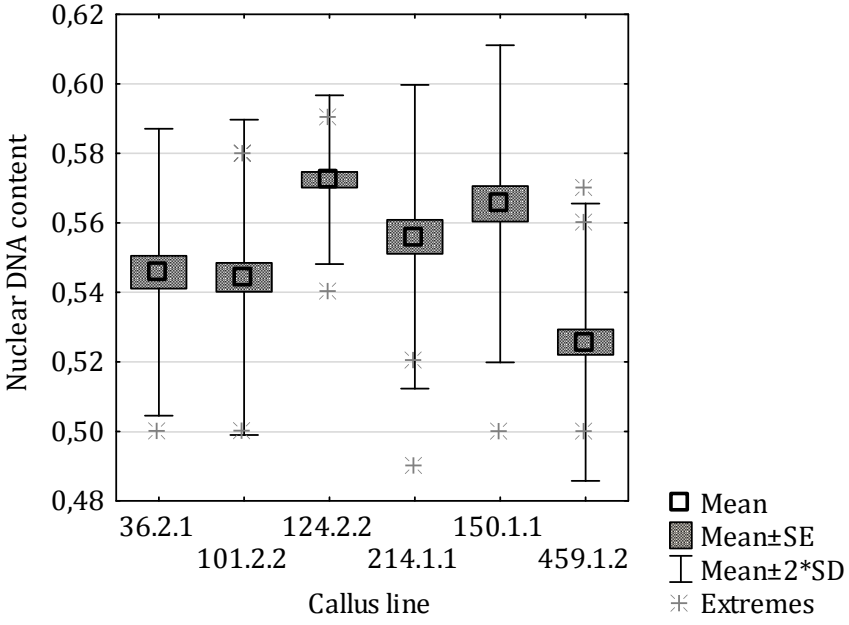

Fig. 1. **Nuclear DNA content in haploids of *Oryza sativa* L. androgenic callus lines**

Рис. 1. **Содержание ядерной ДНК гаплоидов в андрогенных каллусных линиях риса (*Oryza sativa* L.)**

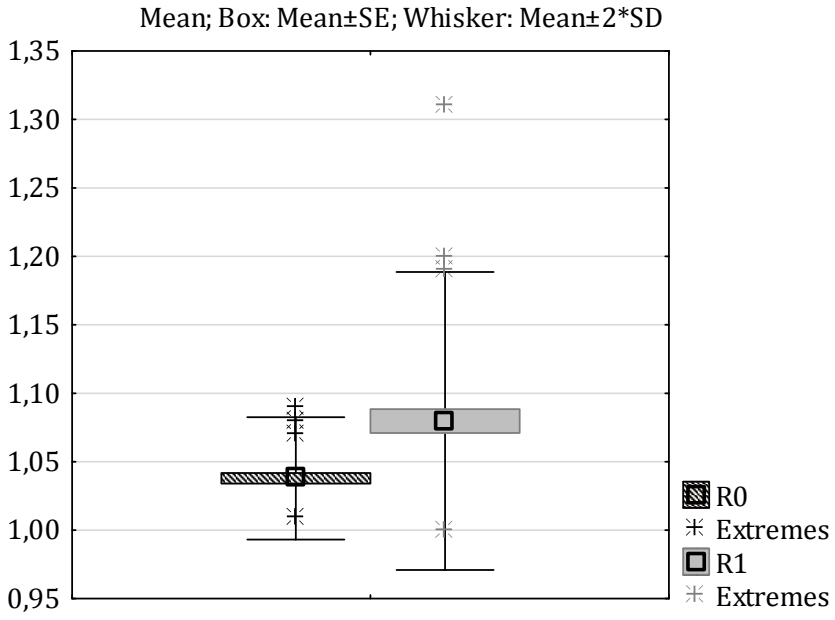

Fig. 2. **Nuclear DNA content in androgenic callus line 39.1.2 of the Magnat × Dolinny hybrid (*Oryza sativa* L.): R0 – doubled haploids R_0, R1 – doubled haploids R_1**

Рис. 2. **Содержание ядерной ДНК в андрогенной каллусной линии 39.1.2 гибрида риса (*Oryza sativa* L.) Магнат × Долинный: R0 – удвоенные гаплоиды R_0, R1 – удвоенные гаплоиды R_1**

variability of intracallus morphological features in callus lines with multiple regeneration may happen due to two reasons: a) the callus line formation by several immature anther microspores, each of which is a separate genotype, and b) the regenerant somaclonal variability, explained by transposition explosions, the activation of mobile genetic elements occurring both at the chromosomes number and structure level and at the gene level (Scowcroff, 1985; Kunakh, 1998). Earlier, we detected morphological and genetic variability among doubled rice haploids in five callus lines of the same hybrid (Ilyushko et al., 2020) and revealed intracallus genomic changes among rice regenerants (Ilyushko, Romashova, 2020). These experiments included callus lines, where intracallus morphological polymorphism of haploids and doubled haploids was observed. However, five callus lines with multiple regeneration were obtained, where no variability was found, i.e., the phenomenon is not a rule. The callus lines are likely initiated by one microspore (Ilyushko et al., 2020; Ilyushko, Romashova, 2020).

There are significant differences in the nuclear DNA content between the doubled haploids R_0 and R_1. To determine the DNA content in R_0 regenerants, straw leaves were used,

and young plants in the four-leaf phase were used in the R_1 regenerates; the average value was higher – 1.09 pg. The cytotypic variability of three R_1 plants with large differentiation numbers on the callus (56, 74, 92) was probably facilitated by a longer stay of plants on a nutrient medium with hormones, which in some cases leads to morphological and genetic variability (Ilyushko et al., 2020). R_1 regenerants underwent seed reproduction; however, an increased DNA content is detected in the leaves of three plants relative to the entire group of plants.

In rice *in vitro* culture, amplification of highly repetitive DNA sequences from several to several thousand times is possible (Zheng et al., 1987, Kikuchi et al., 1987), accompanied by aneuploid chromosome changes in some varieties (Zheng et al., 1987; Wu et al., 2018). At the same time, Y. Wu et al. (2018) documented that the gain of chromosomes is more prevalent over their loss. Similar changes were fixed in the regenerated corn and flax (Kunakh, 1998). This is a probable way of increasing the DNA content in the haploids of callus line 124.2.2 and individual doubled haploids R_1. It does not mean a mandatory change in the number of chromosomes; it requires cytological confirmation. Nevertheless, calli of haploid origin, especially from anthers, contain a small number of aneuploid cells (Kunakh, 1998), and the cases of aneuhaploid plant appearance are known (Khokhlov et al., 1976, Tyrnov, 2005).

Thus, a theoretical basis appeared to suggest the existence of flowering plant species' aneuhaploid evolution. It is shown graphically in Figure 3. In rare aneuhaploid plants that are formed under stress conditions, 0-n-type microsporogenesis is possible; its frequency in diploid flowering plants is very low, but in some cases 2n gametes with a somatic number of chromosomes occur with a high frequency – 14–36% (Tsatsenko, Mosunov, 2008), and in haploid plants n-gametes lead to the formation of fertile pollen up to 34% (Khokhlov et al., 1976). The 0-n-type megasporogenesis also occurs in haploids: up to 28% of normal embryo sacs are recorded in various species. Despite the fact that haploid plants are extremely sterile, when pollinated with normal pollen from diploid plants, they produce seeds, although in insignificant quantities (Khokhlov et al., 1976). This is indirectly confirmed by the fact that few (two to three) plants with single seeds appear on callus androgenic lines with multiple regeneration of haploids (Ilyushko, Romashova, 2019), which are probably formed through megasporogenesis and microsporogenesis according to the 0-n type with subsequent self-pollination. Aneuhaploid evolution explains the intraspecific variability of chromosome numbers among plant species with low ploidy.

As for rice, Y. Wu et al. (2018) assume the creation of aneuploid synthetic species. This is especially true for cultivated species with unsuccessful polyploidy. Rice has relatively stable tetraploid formation (He et al., 2010), including that in anther *in vitro* culture (Ilyushko, Romashova, 2020). However, there are no commercial polyploid cultivars due to low pollen fertility (He et al., 2010). Aneuploid technologies can help in the artificial formation of new polyploid crops, and rice is given a primary role (Wu et al., 2018).

Conclusions

The existing concepts of aneuploid evolution are strongly associated with polyploid species, in which a high percentage of duplicated genes makes possible the loss of a part of DNA and even individual chromosomes without a drop in viability. Diploid plant species are able to follow the aneuploid path of development with an increase in DNA content, including acquisition of additional chromosomes. This study clearly demonstrated an increase in nuclear DNA content in 19 haploids of the *O. sativa* androgenic callus line. The results obtained are the theoretical basis for the creation of synthetic aneuhaploid cultivated plant species using modern biotechnological *in vitro* methods, and can explain the emergence of intraspecific variability in chromosome numbers among wild plant species with low ploidy.

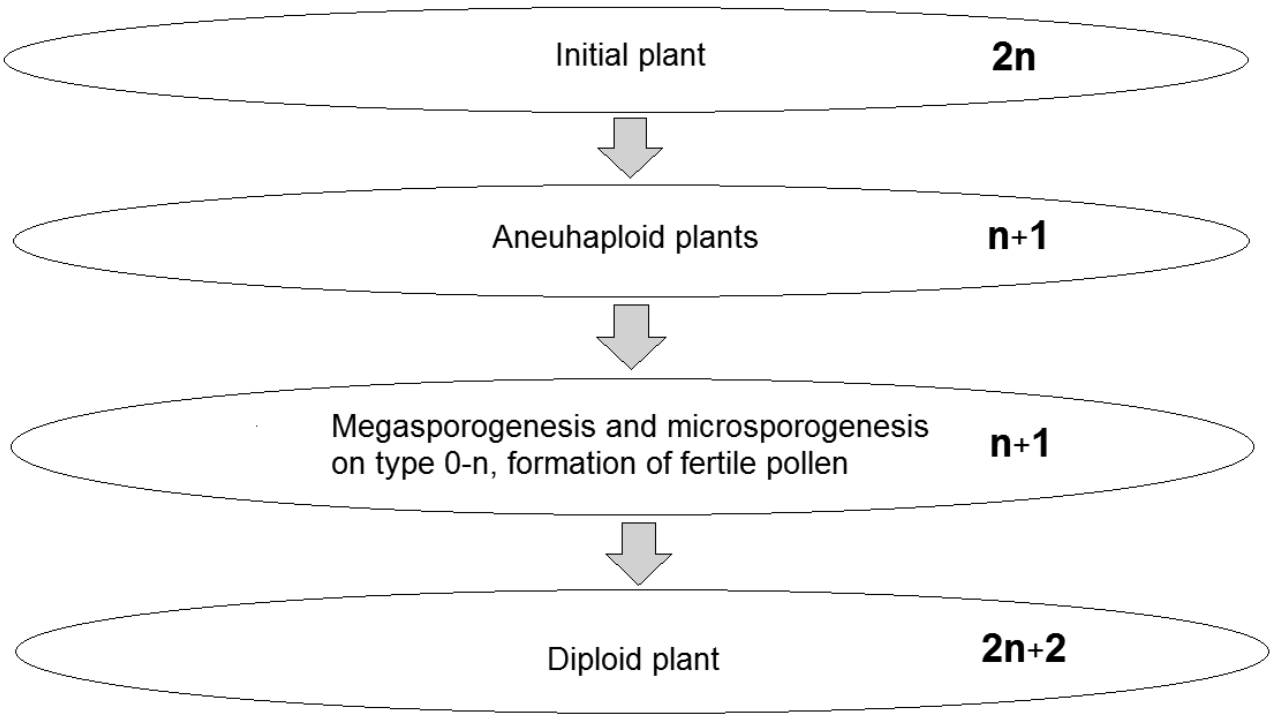

Fig. 3. Diagram of aneuhaploid plant evolution

Рис. 3. Схема анеуплоидной эволюции растений

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