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## Effect of Light Intensity and Quality on Rice *Oryza sativa* L. Regeneration from Callus Generated through In Vitro Androgenesis

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**Abstract**—To optimize the process of in vitro androgenesis, the influence of light intensity and quality on regeneration processes of the rice *Oryza sativa* L. callus was studied. The work was carried out based on plants of the Kaskad and Almaz varieties and the F2 hybrid Rassvet × Oxy 2x. To light calli on a regeneration medium, two types of LED lamps were used: with white light (color temperature 6500 K, which corresponds to the spectrum of midday sunlight) and violet light (emitting wavelengths of 400, 430, 660, and 730 nm). Experimental options were as follows: white light, 4200 lux; white + violet light in the 3/1 ratio, 3900 lux; violet light, 3600 lux; and intense white light, 7500 lux. The light intensity and quality equally affect the induction of morphogenic callus and the number of regenerants per callus. Spontaneous chromosome duplication occurs during the light stage of callus development. The quality of lighting has an ambiguous effect on the formation of doubled haploids of different genotypes. Intense white light contributes to the formation of highly ploid plants in all studied genotypes: 44.4–54.0% of doubled haploids and 1.2–38.9% of tetraploids. When being lighted using violet lamps, the Almaz variety and the hybrid produce more than 60.0% of doubled haploids, while the number of doubled haploids in the Kaskad variety decreases to 26.1–31.8%. To obtain a guaranteed high (49.4%) number of doubled haploids, it is advisable to use intense white type of lighting.

**Keywords:** *Oryza sativa*, in vitro androgenesis, lighting, callus, regeneration, doubled haploids

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### INTRODUCTION

In vitro androgenesis is the process of the formation of sporophytic plants from an immature gametophyte that leads to the formation of haploids. Spontaneous doubling during one generation results in the formation of completely homozygous lines of doubled haploids, which makes it possible to accelerate the selection process of many important food crops by several years [1–3]. Well-known laboratories on different continents support large-scale induction of haploids, and some of them even offer the production of regenerant plants to order at an affordable price to intensify the breeding process [3]. Mass work allows the optimization of existing approaches for in vitro androgenesis. In addition, within all plant species, the presence of difficult-to-cultivate promising genotypes is possible, for which it is necessary to search for new types of action on the gametophyte to launch the sporophyte program for the development of immature microspores [4, 5].

Light has a decisive influence on morphogenetic responses in in vitro culture [6]. However, there is a small number of works devoted to the study of the effect of lighting on in vitro androgenesis [7]. The influence of the quality and intensity of lighting on

donor plants in the in vitro anther culture of cereals has been established [3, 8, 9]. Callus formation in various cultures is induced in the dark or under very low light [3, 10, 11]. For rice, lighting in different modes of day and night is permissible, but callus formation is reduced in this case [11]. The negative effect of blue light on the callus formation in *Citrus clementine* Hort ex Tan. has been noted [7]. After the transfer of callus to the regeneration medium, weak artificial lighting is recommended [10]. For wheat, combined lighting of callus during the regeneration period was proposed: 15 days of darkness, then 7 days of weak light, then high-intensity lighting [8].

In the world and in the Russian Federation, significant success has been achieved in the breeding of rice *Oryza sativa* L. using in vitro androgenesis [10, 12, 13]; a number of reviews on the features of the creation of doubled haploids of culture have been published, which indicates a significant interest in this area of research [11, 12, 14]. However, the question of the effect of lighting on the already induced callus transferred to the regeneration medium has not been sufficiently studied.

The goal of the study is to determine the effect of the intensity and quality of lighting on the regenera-

**Table 1.** Number of calli with green regenerants in in vitro androgenesis in rice *Oryza sativa* L. under different types of lighting

Indicator	Type of lighting			
	white	white + violet	violet	white intense
Kaskad variety				
Total, pcs.	30	34	30	36
Number of calli with green regenerants, pcs.	17	20	16	17
Proportion of calli with green regenerants, %	57	59	53	47
$\chi^2$	0.03...0.94; at $p > 0.33$			
Almaz variety				
Total, pcs.	16	14	16	16
Number of calli with green regenerants, pcs.	4	6	4	5
Proportion of calli with green regenerants, %	25	43	25	31
$\chi^2$	0.00...1.07; at $p > 0.30$			
Hybrid Rassvet × Oxy 2x				
Total, pcs.	14	14	13	14
Number of calli with green regenerants, pcs.	8	11	8	8
Proportion of calli with green regenerants, %	57	79	62	57
$\chi^2$	0.00...1.47; at $p > 0.23$			

tion processes in the androgenic callus of rice for the creation of doubled haploids and their subsequent use in the breeding process.

## METHODS

The work was carried out based on plants of Kaskad and Almaz rice varieties as well as F<sub>2</sub> hybrid Rassvet × Oxy 2x. We introduced 1016, 1002, and 348 anthers into the culture in vitro, respectively. Donor plants were grown in a climatic chamber at a temperature of 21°C, a humidity of 60%, a light intensity of 15000 lux, a daylight mode of 16 h, and a night mode of 8 h. Cold treatment of anthers, cultivation of anthers, calli and regenerants in vitro were carried out according to the methods described earlier [15], and their transfer to a nutrient medium for regeneration was carried out in the order presented in [16].

To light calli on a regeneration medium, two types of LED lamps were used: with white light (color temperature 6500 K, which corresponds to the spectrum of midday sunlight) and violet light (emitting wavelengths of 400 nm, 430 nm, 660 nm, and 730 nm) [17]. The experiment scheme provided for four options: white light, 4200 lux; white light + violet light in a ratio of 3/1, 3900 lux; violet light, 3600 lux; white intense light, 7500 lux. The lighting intensity was recorded with a Yu116 luxmeter. To avoid shading, the racks

with test tubes were placed at a distance of at least 30 cm from one another.

Green R0 regenerants with a developed root system were transplanted into pots and continued to be grown in the culture room. The order of dividing green regenerants into groups is indicated in [20].

Statistical parameters (mean values of the trait ( $\bar{x}$ ), coefficient of variation ( $C_v$ ), nonparametric criteria for the significance of differences in the Kruskal-Wallis variation series (H), and chi-square ( $\chi^2$ )) were calculated using the Statistica program.

## RESULTS AND DISCUSSION

Callus formation was 12.8% in the Kaskad variety, 6.2% in the Almaz variety, and 15.8% in the Rassvet × Oxy 2x hybrid, respectively.

There was a tendency towards an increase in the number of calli with green regenerants under the combined type of lighting. In the white light + violet light option, the maximum number of them in the experiment was formed using all the studied genotypes. However, the differences between the options are not significant (Table 1). The cultures that form callus in in vitro androgenesis, including rice [10], do not have a significant gametoclonal variability of doubled haploids of one anther. Mitotic division of callus results in true cloning of a limited number of genotypes (often only one) formed on the anther [18]. In in vitro androgenesis, it is important to obtain as many calli with morphogenetic responses as possible since this expands the variety of regenerants required for selection.

In the experiment, 2868 green regenerants were formed. The type of lighting did not affect the average number of regenerants per callus (Table 2). The exception was the Almaz variety, in which the value of this indicator under white and violet types of lighting was 17.5 and 13.3 pcs., respectively, which is significantly ( $p = 0.04$ ) higher than in case of the combined (4.5 pcs.) and white intense lighting (3.6 pcs.).

In the end, it is not so much the total number of regenerants that is important for the breeder as the yield of spontaneously doubled haploids. Their share among different researchers varies from 25 to 95% [1, 19]. The type of lighting influenced the value of this indicator in different directions (see Fig. 1). This was most clearly manifested under violet lighting, when the Almaz variety and the hybrid were noted to have the highest number of doubled haploids (67.9 and 63.8%, respectively) and the Kaskad variety has the lowest number of them (26.1%). Under white and combined lighting, a significant variation in the proportion of doubled haploids among rice regenerants persisted: 34.3–52.8 and 31.8–62.7%, respectively. A more stable and high number of doubled haploids was formed under intense white lighting: 44.3–54.0%. Pairwise comparison using  $\chi^2$  confirmed a significant

excess of the number of doubled haploids under violet lighting over white lighting in the Almaz variety and the hybrid ( $p < 0.04$ ). In the Kaskad variety, intense white and white lighting resulted in a significantly greater formation of doubled haploids than under both types of lighting with violet LEDs ( $p < 0.00001$ ).

The studies of the effect of lighting with the same LED lamps on in vitro microcloning of virus-free potatoes [17] showed an ambiguous effect of the quality of light (white and combined) on the intensity of reproduction of 14 varieties, on the basis of which it was concluded that it was necessary to individually select lighting for each genotype. At the same time, vegetative propagation of potatoes allows repeated studies with the same variety. In the course of rice breeding, scientists often deal with a unique hybrid genotype, the regenerants of which must be transferred to a homozygous state, and there is no possibility of replicating the experiment. In the best case, there remain several seeds of the same hybrid combination that are not identical either. Therefore, to achieve a guaranteed high, albeit not maximum, but stable result, it is preferable to use white intense lighting, under which doubled haploids account for half of green regenerants.

Our data confirm the opinion of other researchers [1, 19] about the dependence of the ability for spontaneous doubling of the number of chromosomes on the genotype. In the experiment with violet lighting, the proportion of doubled haploids in the Kaskad variety (21.6%) was lower ( $p = 0.00001$ ) than in the Almaz and hybrid varieties by 46.3 and 42.2%, respectively. Under combined lighting, 31.8% of doubled haploids were formed on the Kaskad variety, which is 30.9% less than in the hybrid ( $p = 0.00001$ ).

In addition to spontaneous doubling of chromosomes in androgenic calli, chromosomal changes of a different order occur: triploid, tetraploid, pentaploid, and aneuploid cells are formed [20], which leads to the formation of tetraploids and seedless plants [1, 18, 21]. The type of lighting influenced the formation of such regenerants in the same way in different directions. Tetraploid forms were observed in the Kaskad variety under white lighting, but they were absent under violet lighting,  $p = 0.0043$  (see Fig. 1). Intense white lighting promoted a multiple increase in chromosomes with the formation of tetraploid plants (5.7%); under three other types of lighting, their number was significantly less (0.0–1.4%,  $p < 0.0006$ ). The number of seedless plants under white intense lighting (1.2%) was less than under violet (4.8%) and white + violet lighting (11.7%). No tetraploids were revealed among the regenerants obtained from a hybrid plant under white lighting. Under white intense lighting, the proportion of seedless plants (19.5%) was significantly larger than in other options of the experiment by 9.4–12.5% ( $p < 0.01$ ). This indicates the genotypic dependence of polyploid and aneuploid changes in in vitro androgenesis.

No tetraploid plants were recorded in the Almaz variety. The proportion of seedless plants was much higher than in the variants with other genotypes and

**Table 2.** Number of regenerants on calli in in vitro androgenesis in rice *Oryza sativa* L. under different types of lighting

Indicator	Type of lighting			
	white	white + violet	violet	white intense
Kaskad variety				
Average number of regenerants per callus, pcs.	17.9	14.7	19.4	10.7
Maximum number of regenerants per callus, pcs.	128	65	73	71
H for all calli	2.49; at $p = 0.48$			
H for calli with green regenerants	3.56; at $p = 0.31$			
Almaz variety				
Average number of regenerants per callus, pcs.	3.8	1.3	3.9	1.6
Maximum number of regenerants per callus, pcs.	31	5	28	12
H for all calli	0.25; at $p = 0.97$			
H for calli with green regenerants	8.62; at $p = 0.04$			
Hybrid Rassvet × Oxy 2x				
Average number of regenerants per callus, pcs.	9.2	13.0	15.8	13.2
Maximum number of regenerants per callus, pcs.	37	54	75	86
H for all calli	1.73; at $p = 0.19$			
H for calli with green regenerants	2.1; at $p = 0.55$			

reached 7.6–38.9% (see Fig. 1). Under intense white lighting, it significantly increased in comparison with white and violet lighting ( $p < 0.008$ ).

A comparison of the Kaskad and Almaz varieties without taking lighting into account makes it evident that there is a difference in their androgenetic responses in vitro: calus formation and regeneration in Kaskad is two and six times higher, respectively. The influence of the genotype on these processes has been proven [10, 11]. The range of variation of traits in regenerants of the same genotype obtained in in vitro culture has not been sufficiently studied. It is known that the level of genetic divergence in somaclonal regenerants of *Pisum sativum* L. depends on the initial genotype [22]. Rice in in vitro androgenesis was revealed to have intracal genetic polymorphism and variability of morphological traits, which vary to different degrees on different anthers of the same hybrid [18]. The genetic instability of the Almaz variety in in vitro culture manifested itself in the formation of a large proportion of seedless plants, among which there were mainly highly ploid genotypes [21]. Apparently, chromosomal abnormalities arose that impeded normal meiotic division during the formation of mega- and microspores, which led to their complete sterility. Highly ploid forms of rice (tetraploids) are characterized by low pollen fertility under normal conditions [23]; they are formed in small quantities in in vitro

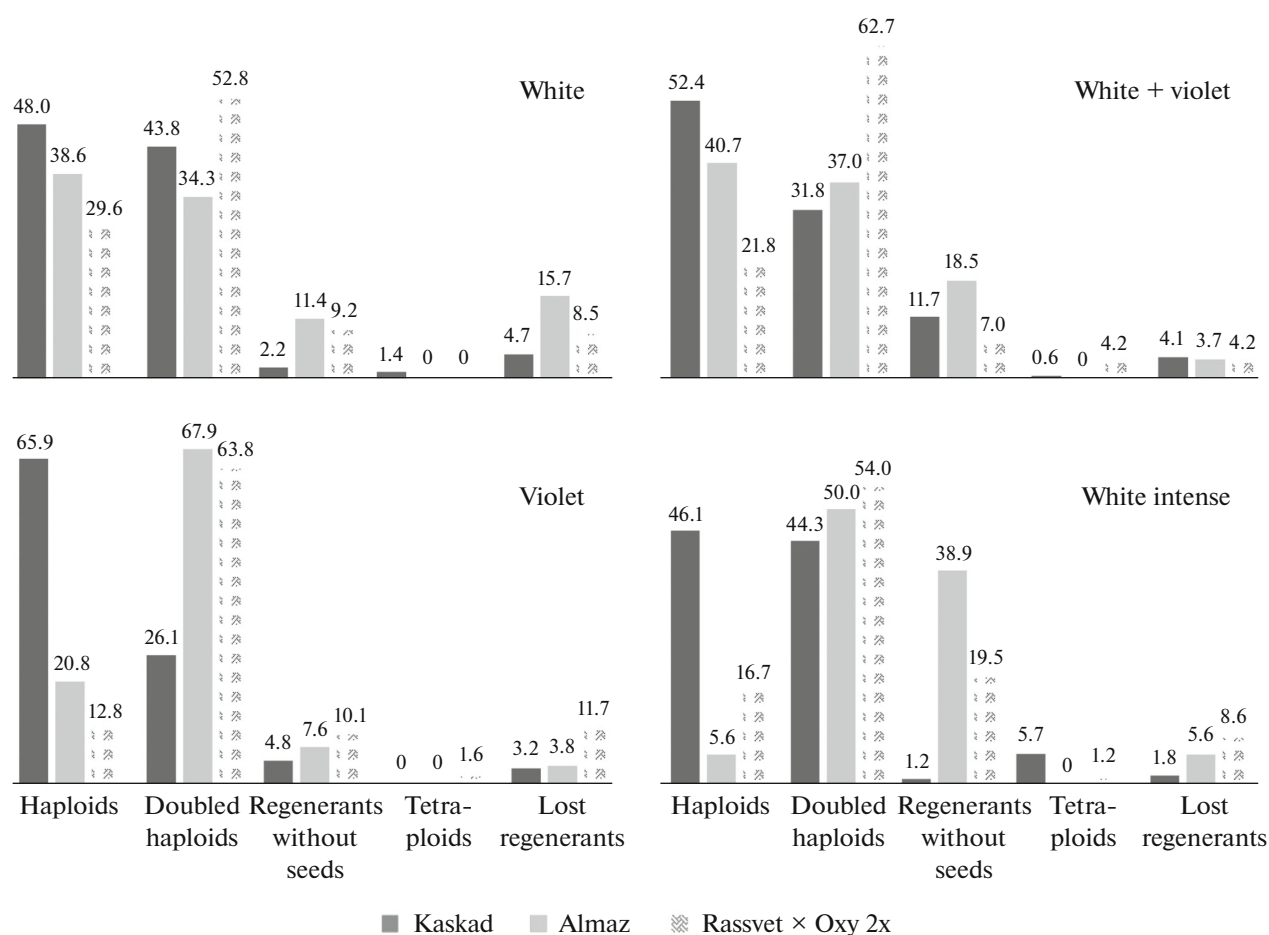


Fig. 1. Regenerants in in vitro androgenesis in rice *Oryza sativa* L. under different types of lighting, %.

androgenesis [16, 21, 24] but not in all cases [1]. Thus, the range of variability of rice regenerants of the Almaz variety is higher than that of the Kaskad variety according to an indirect cytometric estimate. The low intensity of callus formation and morphogenesis may be associated not only with the presence of genes that determine androgenetic responses [25] but also with the cytological characteristics of the donor plant as well as chromosomal transformations that are incompatible with normal mitotic division of callus cells in in vitro culture.

Spontaneous haploid genome duplication (SHGD) leading to the formation of doubled haploids, triploids, and tetraploids in in vitro androgenesis is believed by some authors to occur at very early stages of embryogenesis [1], while others think that SHGD is possible at various stages of in vitro cultivation, including callus formation, callus differentiation, and embryogenesis [12]. SHGD mechanisms remain controversial [1, 26]. After the transfer of callus from darkness to light, its significant growth occurs, which is followed by morphogenesis. The revealed differences in the proportion of doubled haploids and tetraploids under different types of lighting in the two varieties and the hybrid are evidence in favor of a rather

late SHGD process at the stage of callus differentiation.

A decisive role in choosing a technology for creating cultivated plants is played by economic efficiency. LED lamps have obvious advantages over traditional fluorescent lamps, including their price [6, 17]. Among the variety of LEDs, phytolamps with limited wavelengths (violet light) are twice as expensive as white light lamps. The absence of obvious advantages in the experimental options for the studied varieties and hybrids allows us to recommend the use of lighting of any type. In this case, the use of white light lamps is economically justified for large-scale use in in vitro androgenesis.

Thus, the studied options for the intensity and quality of lighting have the same effect on the values of indicators, such as the number of morphogenic calli and regenerants per callus. Spontaneous duplication of chromosomes takes place, including during the light stage of callus development. The quality of lighting affects the formation of doubled haploids of different genotypes ambiguously. Intense white light promotes the formation of highly ploid plants. To obtain a guaranteed high, although not maximum number of productive regenerants, it is advisable to use a white

intense type of lighting. In this case, spontaneous doubling occurs in half (49.4%) of all regenerants. In the presence of several seeds of one difficult-to-cultivate hybrid combination of rice, it is advisable to illuminate the callus of some of them in in vitro androgenesis with violet light. In large-scale studies, it is economically feasible to use white high-intensity lighting.

#### COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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