Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Creation of rice doubled haploids with low amylose content using *in vitro* anther culture

Criação de plantas duplo-haploides de arroz com baixo teor de amilose usando espécies de cultura de anteras *in vitro*

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Abstract

In vitro androgenesis is a unique model for producing homozygous doubled haploid plants. The use of haploid biotechnology accelerates to obtain of doubled haploid plants, which is very important in rice breeding. The purpose of this work is to improve the production of doubled haploids in rice anther culture *in vitro* and selection of doubled haploid plants with valuable traits. The study the influence of nutrient media on the production of calli and plant regeneration processes in anther culture of 35 rice genotypes was revealed a significant influence of nutrient media on callus production. It was shown that the addition to culture medium phytohormones ratio with high level of cytokinin (5.0 mg/L BAP) and a low level of auxin (0.5 mg/L NAA), supplemented with amino acid composition promotes high production of green regenerated plants (68.75%) compared to albino plants (31.25%). As a result, doubled haploid lines of the glutinous variety Violetta were selected, which characterized by a low amylose content variation (from 1.86 to 2.80%). These doubled haploids are superior to the original variety in some yield traits and represent valuable breeding material.

Keywords: rice, *in vitro* anther culture, callus, plant regeneration, doubled haploids.

Resumo

A androgênese *in vitro* é um modelo exclusivo para a produção de plantas duplo-haploides homozigotas. O uso da biotecnologia de haploides acelera a obtenção de plantas duplo-haploides, o que é muito importante para o melhoramento do arroz. O objetivo do presente trabalho é aprimorar a produção de haploides duplicados na cultura de anteras de arroz *in vitro* e a seleção de plantas duplo-haploides com características proveitosas. O estudo da influência do meio nutritivo na produção de calos e nos processos de regeneração de plantas na cultura de anteras de 35 genótipos de arroz revelou uma influência significativa do meio nutritivo na produção de calos. Foi observado que a adição ao meio de cultura de uma proporção de fitormônios com alto nível de citocinina (5,0 mg/L BAP) e baixo nível de auxina (0,5 mg/L NAA), quando suplementada com uma composição de aminoácidos, promove uma alta produção de plantas regeneradas verdes (68,75%) em comparação com plantas albinas (31,25%). Como resultado, foram selecionadas linhas de plantas duplo-haploides da variedade glutinosa Violetta, que são caracterizadas por uma baixa variação no conteúdo de amilose (de 1,86 a 2,80%). Esses duplos-haploides são superiores à variedade original em algumas características de rendimento e representam um valioso material de propagação.

Palavras-chave: arroz, cultura de anteras *in vitro*, calo, regeneração de plantas, duplos-haploides.

1. Introduction

The breeding of new varieties in self-pollinating plants comprises three key features: the creation of variation by crossbreeding, stabilization by self-pollination or backcrossing over several generations, and the selection of the desired recombinants. At present, in rice breeding along with traditional breeding, biotechnological approaches are used, enabling to increase the efficiency of the selection process. The most common approach for producing haploids and doubled haploids of rice is induced androgenesis in anther culture.

Niizeki and Oono first reported the production of haploids from rice anther culture in 1968 (Niizeki and Oono, 1968). The haploid biotechnology technique is used to accelerate the breeding process, allowing to obtain a stable plant per a single generation (Mishra and Rao, 2016; Lantos et al., 2023), therefore in rice farming and in

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Received: March 26, 2024 – Accepted: May 24, 2024

breeding of other agricultural crops the method of anther and microspore culture is employed to stabilize promising fissile plant populations (IRRI; Naik et al., 2017). Typically, 6-8 generations are needed with traditional (conventional) breeding methods to obtain homozygous lines (Grewal et al., 2011). Compared to the selection of hybrid lines, the selection of haploid plants based on marker traits is 5-6 times more efficient (Howes et al., 1998; Pattnaik et al., 2020). Haploid plants are of great interest to breeders, as they are used to accelerate the production of doubled haploid homozygous lines (Datta, 2005), the so-called "pure lines", with all the genes accountable for agronomic traits fixed in a homozygous state.

Nevertheless, the technique of culturing isolated anthers and microspores has not yet gained extensive application in practical breeding due to the genotype-dependence of the regeneration process and the low frequency of obtaining green regenerant plants. Different responsiveness in anther culture is characteristic of rice varieties, species and subspecies: the maximum responsiveness is observed in glutinous rice, followed by *japonica* subspecies, *japonica/indica* hybrids, *indica/indica* hybrids and *indica* subspecies (Medhabati et al., 2009; Lantos et al., 2023). The variation in this trait within the subspecies is also quite significant.

Haploid plants possess a range of advantages in breeding work. Haploids contain a single set of chromosomes, allowing breeders to observe mutations directly as plants are examined. The polyploidization of haploids results in doubled haploids characterized by absolute homozygosity, which enables stabilization of valuable hybrid breeding lines and generation of "pure lines".

The use of rice doubled haploids makes it feasible to increase the efficiency of the breeding process and accelerate the generation of genetically stable lines. The application of *in vitro* androgenesis technique enables to accelerate the production of homozygous doubled haploids, and select beneficial lines of desired traits (resistance to diseases, drought, cold and grain quality, etc.). Biotechnology of obtaining doubled haploids is considered to be one of the most environmentally sound and entirely harmless, which enables overcoming the shortfalls of the classical breeding methods.

Therefore, the aim of this study is to obtain doubled haploids in rice anther culture and select valuable lines for the breeding process.

2. Materials and Methods

2.1. Plant materials

All the research in this work was conducted in a Green House and at the experimental fields of the Institute of Plant Biology and Biotechnology (IPBB). A total of 35 genotypes were taken as research subjects: 8 varieties (*Bakanasski*, *Marzhan*, *Madina*, *Barakat*, *Regulus*, *Anahit*, *Viola* and *Violetta*) as well as 24 hybrid lines (F_1 *italica*, F_1 *zeravschanica*, F_3 BR-3, F_3 BR 5, F_3 BR-6, F_3 BR-8, F_3 BR-12, F_3 BR-14, F_3 BR-16, F_3 BR-17, F_3 D23, F_4 GS 207, F_5 D22, F_5 D23, F_5 D24, F_5 D26, F_5 BR 3, F_6 SGP-177, F_6 GS 189, F_6 SPE 39, F_6 KS 6-8, F_6 GS 195, F_6 GS 207 and F_6 GS 208) and 3 collection rice specimens (*Kuramochi*, A-5-1, SSP-5-6) from the world selection.

2.2. Growing conditions of donor plants

Rice genotypes donor plants for haploid technology were grown up to the boot stage under IPBB greenhouse conditions. Plants were fertilized with ammophos at the tillering phase at the rate of 50 g/m^2.

2.3. Collection and pre-treatment of plant materials

The panicles were sampled at the booting phase 2-3 days before the inflorescences were swept out (in the booting phase), representing the uninucleate stage of pollen development. The cut panicles were placed in vessels with water and subjected to cold treatment of +4 °C for 5-7 days. Cold pretreatment is known to promote synchronization of cell division and maintain the viability of embryogenic microspores.

2.4. In vitro techniques of anthers isolation and sterilization

The experiments were conducted according to the standard procedure for *in vitro* cell and tissue culture in compliance with aseptic cultivation conditions. The panicles were surface sterilized with 70% ethanol in a laminar box before planting on nutrient medium.

For callus induction, anthers were passaged onto N6 (Chu et al., 1975) and Blades' (Bl) (Blaydes, 1966) nutrient media supplemented with 2.0 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) and as a carbohydrate source was used maltose at a concentration of 90.0 g/L. The anthers were cultured in the dark at 25 ± 2 °C. Callus appearance was observed on the 20th day of anther cultivation.

2.5. Plant regeneration and acclimatization

On reaching callus diameter greater than 3 mm, calluses were transferred to Murashige and Skoog (MS) (Murashige and Skoog, 1962) regeneration medium with high concentration of BAP (5.0 mg/L) and low concentration of NAA (0.5 mg/L), supplemented with 500.0 mg/L casein hydrolysate and 250.0 mg/L of proline to stimulate plant regeneration, according to our previously work on plant tissue culture (Amirova et al., 2022). Nutrient medium was autoclaved with no added phytohormones. As the temperature of sterile hormone-free medium was lowered to 70 °C, vitamins and hormones were added through sterile Millipore membrane filters of 0.22 µm diameter.

For plant regeneration, induced calli were transferred to MS regeneration medium (Amirova et al., 2022) and cultured under a 16 /8 h of light and dark photoperiod. Then, after two weeks of cultivation on MS regeneration medium, the regenerated plants were transferred to MS hormone-free rooting medium to promote root system development. After one month of cultivation on hormone-free medium, rooted plants were rinsed from the nutrient medium under running water and placed in vessels with tap water for adaptation to *in vivo* from *in vitro*. Then plants with well-developed root system were transferred

to soil-peat mixture upon 2-3 weeks of adaptation and grown in a Green House.

Haploid plants that appeared to be infertile were observed among the regenerants. Seeds from each regenerated plant were collected separately. The seed progeny of one regenerated plant (P0) was considered as a new line.

2.6. Stem node method for spontaneous chromosome duplication in haploid plants

Plant diploidization was performed using the method of stem nodes (Kharchenko et al., 1997), which is where meristematic cells are located that can be regenerated into a whole plant. As a result of the active *in vitro* meristem growth, a spontaneous chromosome doubling occurs. To achieve this, stems of haploid plants were cut from stem nodes and transplanted onto MS medium containing 2.0 mg/L of 2,4-D and 0.5 mg/L BAP for plant regeneration and regenerant plants were obtained.

2.7. Cytogenetic study of mitosis

The roots of regenerants were used for cytological analysis of chromosome counting. The material was fixed in the morning hours in a fresh Carnoy's reagent (in 3:1 ratio of ethanol and glacial acetic acid, respectively), repeatedly rinsed in ethyl alcohol, stained using 2% acetocarmine solution (Pausheva, 1988).

2.8. Cytogenetic examination of meiosis

Rice panicles that had not come out of the flag leaf sheath served as samples for cytogenetic studies. Fixation was performed in the morning hours in freshly prepared Carnoy's reagent (3 parts of 96% ethyl alcohol: 1 part of glacial acetic acid), where they were preserved for 12-24 hours. The material was then bathed in 96% ethyl alcohol for 1 hour, 80% ethyl alcohol I for 1 hour, 80% ethyl alcohol II for 1 hour, 70% ethyl alcohol I for 1 hour, 70% ethyl alcohol II for 1 hour, 70% ethyl alcohol III for 1 hour, and left for storage in a fresh portion of 70% ethyl alcohol. Anther staining was performed in a 2% acetocarmine solution prepared by a standard technique for staining microsporocytes in cereals (Pausheva, 1988).

All the captions of mitosis and meiosis were photographed using a video camera on a "MEIJI" microscope coupled with TV Capture Card video recorder at 100x magnification.

2.9. Analysis of amylose content in rice grain

The amilose content in rice grains was determined by the commonly known Giuliano method (Juliano, 1971). For this, 1 ml of ethanol (96%) and 9 ml of 1N NaOH were added to 100 mg of ground rice flour. Then the mixture was heated in a water bath (100 °C, 10 min) and the volume was brought to 100 mL with distilled water by constant stirring. Then, 1 mL of 1N acetic acid and 2 mL of iodine reagent ($KI+J_2$) were added to 5 mL of each sample mixture thoroughly stirring, and the volume of the samples was adjusted to 100 ml with distilled water. After that, the samples were left in the dark for 20 minutes. The amylose content was measured at λ=620 nm on a Genesys10 UV (Thermo Scientific, USA) spectrophotometer.

2.10. Statistical analysis

The productivity of regenerant plants was evaluated according to biometric indicators of the main elements of the crop structure (bushiness, pcs; plant height, cm; panicle length, cm; number of grains from the main panicle, pcs; weight of seeds from the main panicle, g; weight of 1000 seeds, g and etc.). Statistical parameters including the mean value and standard deviation using Microsoft Excel 2021 were used to characterize agronomic traits.

An analysis of variance (two-way ANOVA) was performed to compare the mean values over two or more groups of data. Data processing was performed in the Microsoft Excel 2021 application using a data analysis package.

3. Results

A study of the effect of two nutrient media N6 and Blades (Bl) with 2.0 mg/L 2,4-D on the induction of calli from anthers of 35 rice genotypes showed differences in the effect of culture media and the response of genotypes to callus formation. (Table 1).

Callusogenesis processes were observed on both nutrient media. Thus, the greatest number of calluses was obtained from hybrids: F_6 KS 6-8 – 56 calli, F_1 *Italica* – 52, F_3 BR-8 – 40, F_6 SPE 39 – 32 calli. The *Violetta* variety was the most responsive, with 72 calli obtained from it. *Bakanasski* and *Barakat* varieties had 64 and 36 calli, respectively. Some rice genotypes appeared unresponsive in anther culture, varieties like *Anahit*, *Marzhan*, *Regulus* and *Viola* formed no structures *in vitro* (Table 1).

Notably, on N6 medium supplemented with 2.0 mg/L of 2,4-D, anther calluses were obtained in most of the genotypes studied. Thus, comparison of two nutrient media N6 and Blades showed that for 17 genotypes out of 35 genotypes studied, N6 medium was the most favorable for callus induction, while 12 genotypes were responsive on Blades medium (Table 1). The conducted analysis of variance (two-way ANOVA) revealed a significant effect of nutrient media on the number of induced calli ($p<0.05$) in the *in vitro* rice anthers culture (Table 2).

It should be noted that the induced calli differ in color (from white to light yellow) and in the structure of the tissue (from dense compact to soft moist and loose structure).

As a result of transferring all the obtained calli to MS regeneration medium containing 5.0 mg/L BAP, 0.5 mg/Ll NAA, 500.0 mg/L casein hydrolyzate, 250.0 mg/L proline and 30.0 g/L maltose was able to achieve plant regeneration in rice anther culture. So, higher rate of regenerants obtained from the glutinous *Violetta* variety with 13 regenerant plants, 11 of which were green plants and 2 albino plants (over 15.38% of shootings had lethal albino mutation). 6 plants were obtained from the calli of the F_3 BR-8 hybrid, with 5 green plants and 1 albino plant (16.66% of plantlets with a lethal albino mutation). Regenerant plants of *Violetta* variety were selected for further breeding procedure, as they yielded the highest number of green plants. In addition, the *Violetta* variety is featured by its low amylose content, which is a characteristic of interest to generate new glutinose lines of rice.

Table 1. Calli induction and plant regeneration of rice *in vitro* anther culture.

Genotype	Nutrient media for callus induction				Plant regeneration	
	N6 with 2.0 mg/L 2,4-D		Blaydes (Bl) with 2.0 mg/L 2,4-D		Number of transferred calli, pcs	Number of green plants/albino plants, pcs
	Number of anthers, pcs	Number of obtained calli, pcs	Number of anthers, pcs	Number of obtained calli, pcs		
Hybrids						
F_1 *italica*	420	52	180	-	52	0/1
F_1 *zeravschanica*	180	24	100	8	32	-
F_3 BR-3	220	8	140	12	20	-
F_3 BR 5	300	20	-	-	20	1/2
F_3 BR -6	140	24	-	-	24	0/1
F_3 BR-8	160	40	-	-	40	5/0
F_3 BR 12	80	8	-	-	8	-
F_3 BR-14	-	-	120	12	12	-
F_3 BR-16	120	8	-	-	8	-
F_3 BR-17	120	16	200	16	32	-
F_3 D23	100	-	100	-	-	-
F_4 GS 207	80	4	180	-	4	-
F_5 D22	-	-	60	4	4	-
F_5 D23	-	-	120	20	20	-
F_5 D24	80	-	60	-	-	-
F_5 D26	60	-	240	4	4	-
F_5 BR 3	240	-	-	-	-	-
F_6 SGP -177	240	16	80	-	16	-
F_6 GS 189	-	-	60	4	4	0/2
F_6 SPE 39	-	-	320	32	32	-
F_6 KS 6-8	460	56	220	24	80	2/1
F_6 GS 195	-	-	40	8	8	-
F_6 GS 207	140	20	-	-	20	-
F_6 GS 208	-	-	140	12	12	-
A-5-1	-	-	180	20	20	-
SSP-5-6	80	12	-	-	12	1/0
Varieties						
Kuramochi	180	-	-	-	-	-
Violetta	1400	72	380	-	72	11|2
Viola	220	-	260	-	-	-
Madina	320	24	-	-	24	-
Bakanasski	460	64	40	-	64	2/1
Anahit	160	-	-	-	-	-
Marzhan	240	-	-	-	-	-
Barakat	360	36	-	-	36	1/0
Regulus	120	-	160	-	-	-
Total:	6580	504	3380	176	680	22/10

Table 2. Analyses of the effect of culture media and genotype on induced callus number of rice by Two-way ANOVA.

	df	MS	F	P-value	F crit
Culture media	1	75,347.11*	4,242.62	0,041.27	3,908.74
Genotype	34	16,119.22	0,907.64	0,617.37	1,513.38
Interaction	34	11,864.26	0,668.05	0,914.72	1,513.38
Within	140	17,759.56			

df: the Degrees of Freedom. MS = Mean Square. Significance at *p < 0.05. F crit - α level (0.05). P-value (< 0.05).

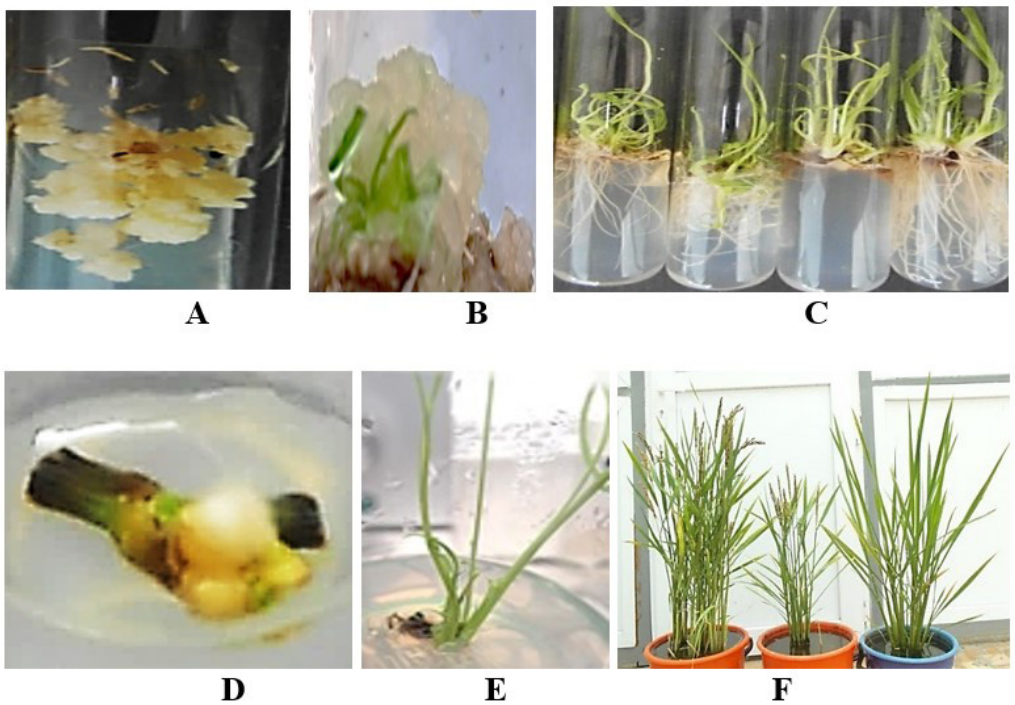

Figure 1. *In vitro* rice anther culture: (A) Callus induction from anthers; (B) Plant regeneration from calluses on the MS medium with 5.0 mg/L of BAP, 0.5 mg/L of NAA, 500.0 mg/L of casein hydrolysate, 250.0 mg/L of proline; (C) Rooting of regenerated plants on the phytohormone free MS medium; (D) Stem nodes of glutinous rice cultivated on regeneration medium; (E) Plant regeneration; (F) Regenerated plants on the soil in Green House.

Figure below (Figure 1A-C) illustrates callus formation from anthers and the regeneration of plants. For spontaneous diploidization of haploids, plant stem nodules (Figure 1D) consisting of multiple vascular tissues were then placed on MS medium supplemented with 2.0 mg/L of 2,4-D. Growth points appear from the stem nodes, giving rise to new green shootings (Figure 1D). Then the plantlets were rooted on a hormone-free MS medium and the rooted plantlets were transferred to the ground and grown under Green House conditions (Figure 1E and F).

Cytological analysis was performed to ascertain the ploidy of regenerants obtained from the *Violetta* variety versus the original variety. Chromosome counting via cytological squash preparation for ploidy confirmed a diploid set of chromosomes (2n=24) in both control (Figure 2A and B) and experimental (Figure 2C and D) specimens.

In rice, anther development is known to occur as it does in all cereals with the formation of exothecium, endothecium, middle layers and tapetum with orbicules, as well as sporogenic tissue – microsporocytes, microspores and pollen grains (Zhang et al., 2011). After transplanting plants into the soil in the booting phase, panicles were picked for cytological analysis, which revealed mature fertile pollen grains in the examined plant-regenerants (Figure 2E and F), as direct evidence of the appearance of spontaneous diploidization in *in vitro* culture.

A seed generation was obtained from regenerated plants grown in the conditions of the IPBB Green House. New doubled haploid lines were biochemically studied and yield structure was analyzed. Biochemical analysis of the amylose content revealed the wide range of variation in the amylose content (from 1.86 to 4.5%) in the population of doubled haploids obtained from the glutinous *Violetta* variety. All the doubled haploid lines of intra-varietal types derived from *Violetta* variety belong to the low amylose group based on amylose content.

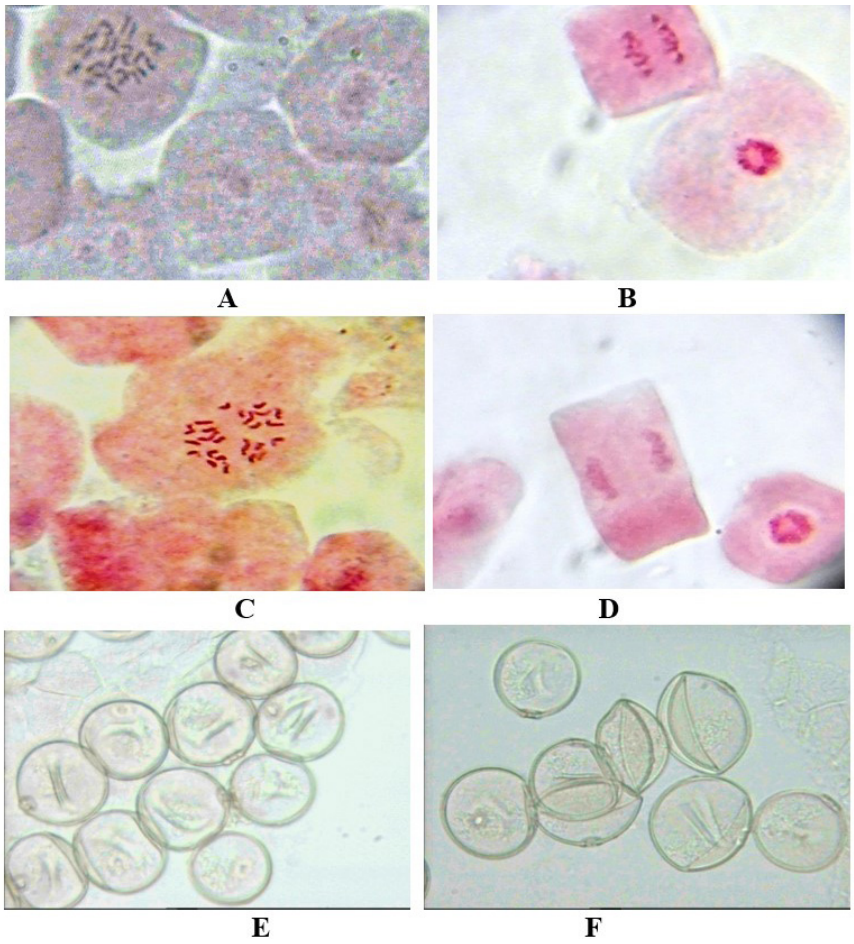

Figure 2. Cytological examination of regenerated plants: (A) and (B) Cytological examination of mitosis (at metaphase and anaphase stages) in root tip cells of glutinous rice cv. *Violetta* (control); (C) and (D) Regenerated plant of cv. *Violetta*; (E) and (F) Mature fertile pollen grains of the regenerated plants.

The results of amylose content assessment revealed that 6 out of 11 lines of regenerant plants were found to have low amylose content (below 3%), and these lines can be recommended for further breeding. Amylose content in cereals is a trait of polygenic nature and is only stabilized by conventional breeding in F_6-F_7 generations. With haploid biotechnology applied, this trait was stabilized by 2 years instead of 6-8 years in case of conventional breeding, i.e. it was reduced by 4-6 years, confirming once again the importance of involving haploid biotechnology in breeding process.

A comparative analysis of yield structure elements of glutinous doubled haploids and initial variety *Violetta* grown under IPBB Green House and field conditions was carried out. Thus, the results of studies of glutinous doubled haploids grown in Green House conditions of IPBB demonstrated an exceeded indices of economically valuable traits, with the exception of plant height and empty grain per panicle (Table 3).

The structure analysis of yield elements indicated that the obtained doubled haploid lines compared to the original glutinous *Violetta* variety exceeds the most economically important yield traits (bushiness, number of grains per panicle, weight of seeds from the main panicle, etc.) (Table 3), while the presence of undesirable trait "empty grains" cannot be avoided, which makes it essential to select the most fertile panicles for further breeding. No significant differences were found in other parameters.

Field tests of *Violetta* variety and its derived doubled haploids in field conditions of Balkhash district of Almaty region demonstrated that doubled haploid lines are featured by favorable bushiness and tallness (Table 3). As seen in Table 3, doubled haploids are almost at the level of control variety in "number of grains per main panicle" trait and are slightly inferior to the initial variety in "weight of 1000 seeds" trait. One of the advantages of glutinous doubled haploid analogues grown in the field over the main *Violetta* variety is that the plants are leveled off in height, in the length of the panicle. Doubled haploids were more leveled off compared to the control. Plant height averaged 68.3 ± 4.1 in the control variety, while the deviation in the doubled haploids was less than in the control and reached 76.4 ± 2.1 (Table 3). The panicle length in doubled haploids was more leveled in contrast to the control. No pyriculariosis lesions and lodging were observed in the selected doubled haploids in field conditions.

Table 3. Characteristics of economically important traits of *Violetta* variety and doubled haploids.

Traits	in Green House		in field	
	Violetta. control	Violetta. doubled haploid	Violetta. control	Violetta. doubled haploid
Bushiness, pcs	2.5 ± 0.4	10.1 ± 1.1	4.2 ± 0.5	5.6 ± 0.5
Plant height, cm	72.8 ± 5.1	71.5 ± 4.4	68.3 ± 4.1	76.4 ± 2.1
Panicle length, cm	11.8 ± 1.5	14.3 ± 1.3	11.6 ± 1.3	22.6 ± 1.9
Number of grains from the main panicle, pcs	42.7 ± 1.7	67.1 ± 5.6	119 ± 10.6	119.8 ± 8.3
Empty grain per panicle, pcs	16.2 ± 1.7	26.6 ± 4.1	8.0 ± 0.7	13.3 ± 1.2
Weight of seeds from the main panicle, g	1.3 ± 0.4	1.5 ± 0.1	2.6 ± 0.5	2.1 ± 0.4
Weight of 1000 seeds, g	25.4 ± 0.7	23.7 ± 1.7	24.0 ± 3.1	20.1 ± 2.6

4. Discussion

Hence, combining the methods of conventional breeding and advanced biotechnology (experimental haploidy) significantly increases the productivity and speed of generation of constant forms. The findings confirm that callus induction depends on the nutrient medium composition and is controlled by the genotype, i.e. there are responsive and non-responsive genotypes.

Two types of calli were obtained in the rice anther culture: morphogenic white compact and non-morphogenic light yellow loose calli. In the literature, white dense, compact rice calli are characterized to be capable of embryoidogenesis and regeneration of whole plants, while yellowish calli of loose consistency, in most cases, are non-regenerative (Pérez Bernal et al., 2014).

Of the total number of calli transferred to the regeneration medium, 32 regenerant plantlets were obtained, including 22 green plants (68.75%) and 10 albino plants (31.25%) (Table 1). As can be observed, a high percentages of green regenerant plant yield have been achieved in this work due to the use of specific nutrient media at different stages: induction of calli from pollen, development of the whole plants and rooting of the regenerated plants in pollen culture *in vitro*.

The yield of green plants is commonly shown in many studies to be very low compared to albino plants in androgenesis *in vitro* (Makowska and Oleszczuk, 2014). The albinism has been one of the unsolved challenges in anther and microspore culture *in vitro* of cereals. It has been hypothesized that at the initial stage of microspores, plastids are in a state of metamorphosis, gearing for their role in gametophyte generation. Microsporial cells formed under cold treatment do not have gametophytic features. Callus arising from those cells give rise to albinos. The occurrence of albino plants is a function of the donor plant genotype and cultivation conditions (Gajecka et al., 2021). A study of albino wheat regenerants derived from anther culture revealed loss of chloroplast DNA sites at 80% or more of the total content. The occurrence of mutations in genes controlling chlorophyll development, impaired pollen development and mutations in chloroplast DNA (Gajecka et al., 2021; Valikhanova and Rakhimbaev, 1989; Yamagishi, 2002), as well as in the nuclear genome during *in vitro* cultivation may affect the formation of albino plants (Larsen et al., 1991). Some hybrid lines failed to give rise to green regenerated plants, as shown in Table 1. Hybrids F_1 *italica*, F_1 *zeravshanica*, F3 BR-6 and F6 GS 189 showed no green regenerants obtained. These haploids or homozygous doubled haploids probably have numerous lethals, semi-lethals, or subvitales in their genotypes that have been inherited from the hybrid genotype (Ilyushko, 2019).

Supplementation of plant growth regulators in different concentrations and proportions to nutrient media enables the regulation of the endogenous plant hormone synthesis, contributing to callus induction and plant regeneration in tissue culture *in vitro* (Saeedpour et al., 2021). The combination of cytokinin and auxin in a 10:1 ratio has previously been shown to stimulate morphogenesis in plant tissue culture in vitro, promoting massive plant regeneration (Amirova et al., 2022). In this study, this technology has been tested for the rice anthers culture. This resulted in the largest number of green regenerant plants. In the present work, media containing auxin (2.0 mg/L 2,4-D) was used for callus induction, which is frequently used to induce cell elongation that promotes callus formation. And for plant regeneration was used, a specific MS medium with high level of BAP (5.0 mg/L) and low level of NAA (0.5 mg/L) supplemented with 500.0 mg/L of casein hydrolysate, 250.0 mg/L of proline, and 30.0 g/L of maltose, which increase the production of green plants. It can be observed from the references that another combination (high level of auxin and low level of cytokinin) is mainly used for plant regeneration for *in vitro* androgenesis.

It is also important to note that in practice, to overcome sterility, haploid regenerants are converted into diploid level by treating with colchicine or prolonged cultivation of meristems of haploid regenerants on dedifferentiating medium (the method of rudimentary panicles or stem nodes). The frequency of diploid formation is known to increase to 79% when treated with colchicine (50-250 mg/L), while the rate of diploidization does not exceed 53.8% when not subjected to colchicine. Furthermore, it is known that the high concentration of colchicine can lead to increase albino plants production (Hu and Liang, 1979). Colchicine has been noted to possess toxicity and does not always provide a positive outcome. Thus, treating haploids with colchicine resulted in 62-80% plant death

and 14-22% of plants remained haploid. Diploidization efficiency for this method was low and averaged about 16%. The chosen method of spontaneous diploidization of stem nodes was favored due to the challenges encountered with the application of colchicine for the diploidization (Saeedpour et al., 2021) of rice haploids. Therefore, as colchicine can have an adverse effect on the production of fertile plants from haploids, in this study the stem node method was employed for diploidization of rice haploids.

5. Conclusion

The widespread application of biotechnological methods is limited by the high yield of chlorophyll-deficient plant regenerants and research aimed to improving the techniques of rice haploid biotechnology is ongoing. This research achieved a high yield of green regenerated plants (68.75%) in rice anther culture, yet the occurrence of albino plants is present. The study demonstrates the whole cycle of doubled haploids generation in rice *in vitro* anther culture – from cultured anthers on artificial nutrient media to callus formation, as well as obtaining viable androgenic green regenerated plants from callus to doubled haploids and testing them in field and Green House conditions. Aligned digaploid lines with a very low amylose content (from 1.86 to 2.80%) representing valuable breeding material has been selected in the rice anthers culture and involved in the breeding process. The obtained data can be applied to to improve varieties and stabilize valuable hybrid lines, as well as for genetic transformation to generate transgenic doubled haploid plants resistant to a huge number of stress factors, which allows to sustainable development in the face of global climate change scenarios.

Acknowledgements

This research was funded by the Science Committee of the Ministry of Science and Higher Education of the Republic of Kazakhstan Grant AP14869300 and Program-targeted financing BR 18574149.

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