Analysis of the effectiveness of callusogenesis on media developed on the basis of the RZ medium

Yulia K. Goncharova[1,3 *], *Svetlana A.* Vereshchagina[1], *Viktoria V.* Simonova[1], *Sergey V.* Garkusha[1], and *Karina S.* Krikun[2]

[1]FGBNU "Federal Scientific Center of Rice", 3, Belozerny village, Krasnodar, 350921, Russia
[2]FSBEI of HE "Kuban State Agrarian University named after I.T. Trubilin" 13, Kalinina str., Krasnodar, 350044, Russia
[3]LLC "Aratai", participant of the Skolkovo Innovation Center, 143026, Russia, Moscow

Abstract. There is still no consensus on the composition of nutrient media for various genotypes, and the improvement of their composition is still an urgent problem, the solution of which can significantly speed up and facilitate the breeding process, both in traditional and heterotic breeding. In this regard, the main goal of the study was to compile new variants of nutrient media and establish the relationship between changes in the components of the medium and the efficiency of callus formation. Previously, we showed the advantage of the RZ nutrient medium (Raina, Zapata.1997) over the N nutrient medium (Nitsch, Nitsch 1969) for domestic varieties and hybrids, but to further increase the efficiency of callusogenesis, 5 variants based on the RZ nutrient medium were developed. The nutrient medium R1 was characterized by a reduced content of CaCl2·2H2O by almost three times, however, the results obtained on it were significantly better than on the initial nutrient medium RZ.

1 Introduction

Different genotypes require different amounts and ratios of growth regulators [9, 13, 16]. Rice of the indica subspecies requires higher concentrations of exogenous hormones than rice of the japonica subspecies [1, 5, 15]. Since the first works on rice tissue culture, most researchers have used 2,4D as a dedifferentiator for the induction of rice callus. However, Chen S. and Lin M. found that 2,4D has an inhibitory effect on organogenesis in the culture of rice anthers [6, 7, 16]. Recently, several new nutrient media have been proposed for the cultivation of rice anthers, which differ from the generally accepted medium by the salt content of N6: increased KNO3, KH2PO4, MgSO4·7H2O, MnSO4·4H2O, ZnSO4·7H2O, H3BO3, CaCl2·2H2O and a lower content of (NH4)2SO4, as well as a higher content of vitamins and growth regulators (Table. 1) [3, 4, 10]. The use of only the ammonium or nitrate form of nitrogen led to a decrease in the efficiency of the cultivation of anthers.

* Corresponding author: yuliya_goncharova_20@mail.ru

The change in the concentration of other salts did not give a significant effect on the yield of regenerants [11, 12, 14]. Summarizing the above, we can conclude that there is still no consensus on the composition of nutrient media for various genotypes, and improving their composition is still an urgent problem, the solution of which can significantly speed up and facilitate the breeding process, both in traditional and heterotic breeding [2, 8, 17]. In this regard, the main purpose of the study was to compile new variants of nutrient media and establish the relationship of changes in the components of the medium with the effectiveness of callus formation.

Table 1. Composition of standard nutrient media used in the work

Code	Component	RZ, mg/l	N6, mg/l	MS, mg/l
1	2	3	4	5
ST 1	KNO_3	3134	2830	1900
ST 2	$MgSO_4 \cdot 7H_2O$	370	185	370
	$MnSO_4 \cdot 4H_2O$	22,3	4,4	22,3
	$ZnSO_4 \cdot 7H_2O$	8,6	1,5	8,6
	$(NH_4)_2SO_4$	320	463	1650
ST 3	KH_2PO_4	540	400	170
	KI	0,8	0,8	0,83
	H_3BO_3	6,2	1,6	6,2
ST 4	$CaCl_2 \cdot 2H_2O$	440	166	400
ST 5	$FeSO_4 \cdot 7H_2O$	27,9	27,8	27,8
	$Na_2EDTA \cdot 2H_2O$	37,3	37,3	37,3
	$Na_2MOO_4 \cdot 2H_2O$	0,25		0,25
	$CuSO_4 \cdot 5H_2O$	0,025		0,025
	$CoCl_2 \cdot 6H_2O$	0,025		0,025
Vitamins	thiamine HCl	2	1	0,4
	pyridoxine HCl	2	0,5	0,5
	nicotinic acid	2	0,5	0,5
	glycine	2	2	2
Hormones	2,4D	0,5	2	
	kinetin	0,5	0,5	
	NAA	2		
Maltose		40g		
Sucrose			30-60g	
Agar		5g	7-8g	

2 Materials and methods

The selection of panicles was carried out in the morning hours, based on cytological observations of the stage of development of microspores and morphological features: the distance between the flag and the subsequent leaf, the intensity of color of the flower and

anthers. Panicles of rice in the leaf vagina, with microspores in the late unicellular/early bicellular phase of development, were cut 2-3 days before sweeping, after treatment with alcohol for sterilization, placed in a glass of water, covered to prevent dehydration. Stored in the refrigerator at a temperature of 7-90C for 7-12 days. Rice panicles were sterilized using 4% sodium hypochlorite (NaClO) for 20 minutes and washed three times with sterile water autoclaved at 1.5 atmospheres for 1 hour. All the rules of sterility developed for tissue cultivation were observed in the work. The manipulations were carried out under aseptic conditions in boxing. Bactericidal lamps were used to sterilize the box, while partial sterilization of the instrument took place. There are anthers in the box for a medium pre-autoclaved for 20-25 minutes at 1.2 atmosphere. 100 anthers were planted in Petri dishes, incubated at a temperature of 24-260 C in the dark, callus counting began on day 30, and was carried out after 10 days. To increase the efficiency of the analysis, the results were divided into groups according to the number of callus. The first group is from 1 to 5 callus per Petri dish, the second is from 5 to 10, the third is from 10 to 15 pieces, the fourth is more than 15 pieces.

3 Results and discussion

Previously, we showed the advantage of the RZ nutrient medium over the N nutrient medium (Nitsch, Nitsch 1969) for domestic varieties and hybrids, but 5 variants based on the RZ nutrient medium were developed to further improve the efficiency of callus formation (Table 2).

Table 2. Differences in the composition of the nutrient media used

Nutrient medium	$CaCl_2 \cdot 2H_2O$	NAA	2,4D	$(NH_4)_2SO_4$	$MgSO_4 \cdot 7H_2O$
RZ	**440**	**2**	**0,5**	**320**	**370**
R1	**150**	**2**	0,5	320	370
R2	440	-	0,5	320	370
R3	440	2	**2**	320	370
R4	440	2	0,5	**231,5**	370
R5	440	2	0,5	320	**185**

Anthers of various short-grained and medium-grained varieties of domestic breeding were planted on all variants of nutrient media, the results are presented in Table 3.

Table 3. Variants of nutrient media, amount of work, reliability of differences in nutrient media for callus formation

Nutrient medium	Average value, pcs.	Number of Petri dishes, pcs.	Standard deviation, pcs.	Average error, pcs.
Rz	6,46	53	4,20	0,52
R1	8,59	22	4,60	0,41
R2	7,67	25	5,88	1,21
R3	7,57	28	4,84	0,91
R4	7,47	27	4,69	1,04
R5	7,48	21	4,93	1,07
Average value	7,60	349	4,99	

The nutrient medium R1 was characterized by a reduced content of CaCl2·2H2O by almost three times (Fig. 1), however, the results obtained on it were significantly better than on the nutrient medium RZ, the number of cups with a high callus content increased, and the callus formation on it was significantly higher than on other variants of nutrient media.

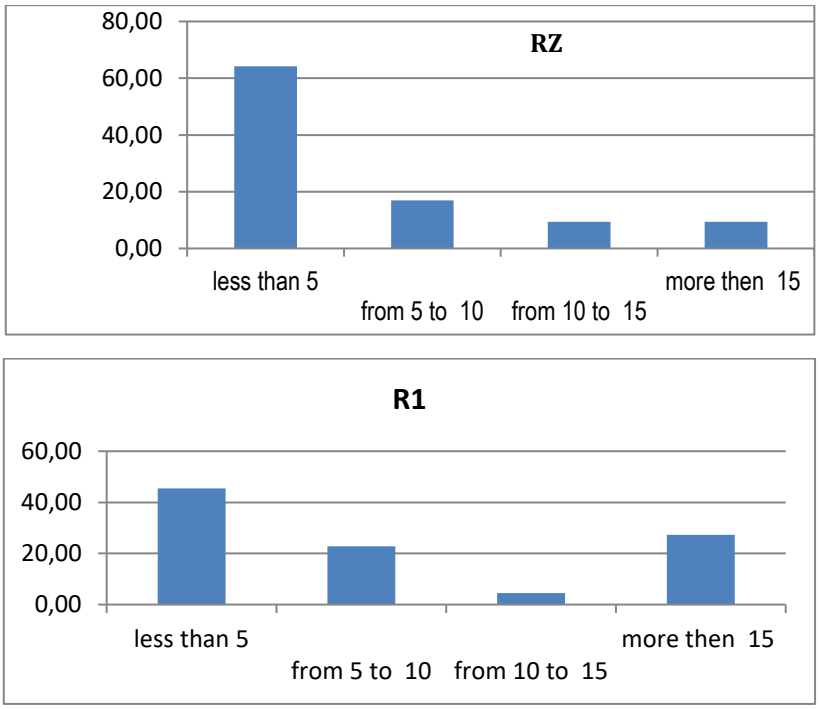

Fig. 1. Callus formation on nutrient media RZ and R1 (%)

Nutrient media R2 and R3 were superior to other variants of nutrient media in callus formation, with the exception of R1, however, due to a small sample, this difference was not significant, in future work it will be necessary to repeat these variants with a larger sample. On the nutrient medium R3, the number of cups with a callus of more than 15 pcs. almost doubled, on the nutrient medium R2, this indicator increased 3 times (the number of cups with a callus of more than 15 pcs.) (Fig. 2).

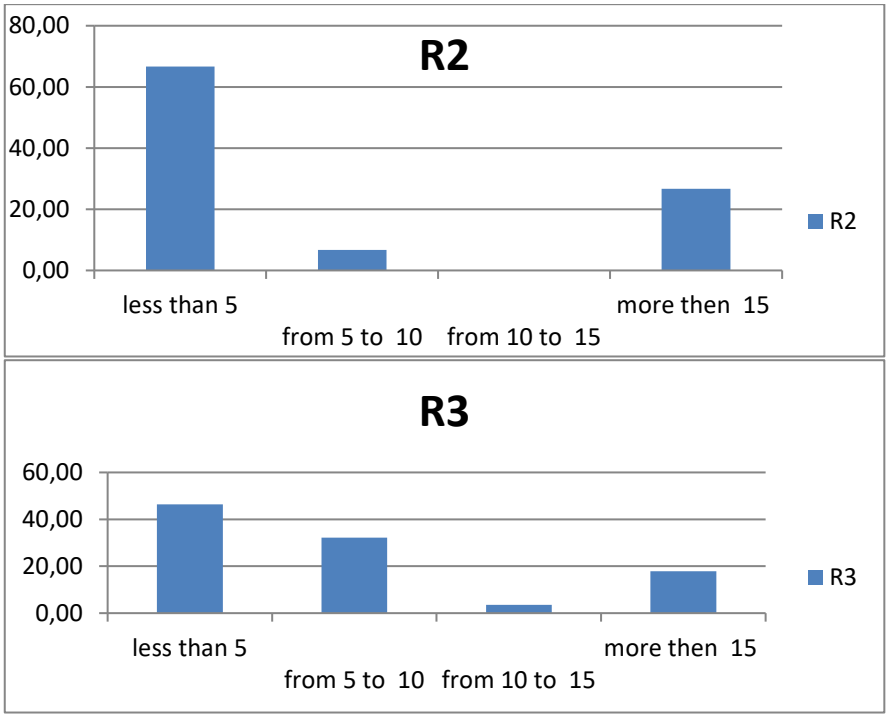

Fig. 2. Callus formation on nutrient media R2 and R3 (%)

Analysis of the composition of nutrient media showed that the nutrient medium R2 was characterized by the absence of naphthyl acetic acid NAF (0), that is, the combination of a decrease in the amount of hormones increased the yield of callus. The nutrient medium R3 was characterized by an increased content of 2,4D, the result on it surpassed others on this nutrient medium with the exception of the nutrient media R2 and R1.

On the nutrient medium R4, the content of (NH4)2SO4 c 320 grams was reduced to 31.5, as on the nutrient medium C [9], while callus formation increased compared to the initial version of the nutrient medium. On the nutrient medium R5, the content of MgSO4·7H2O was reduced from 370 grams to 185, as on the nutrient medium C, while callus formation also increased compared to the initial version of the nutrient medium (Fig. 3).

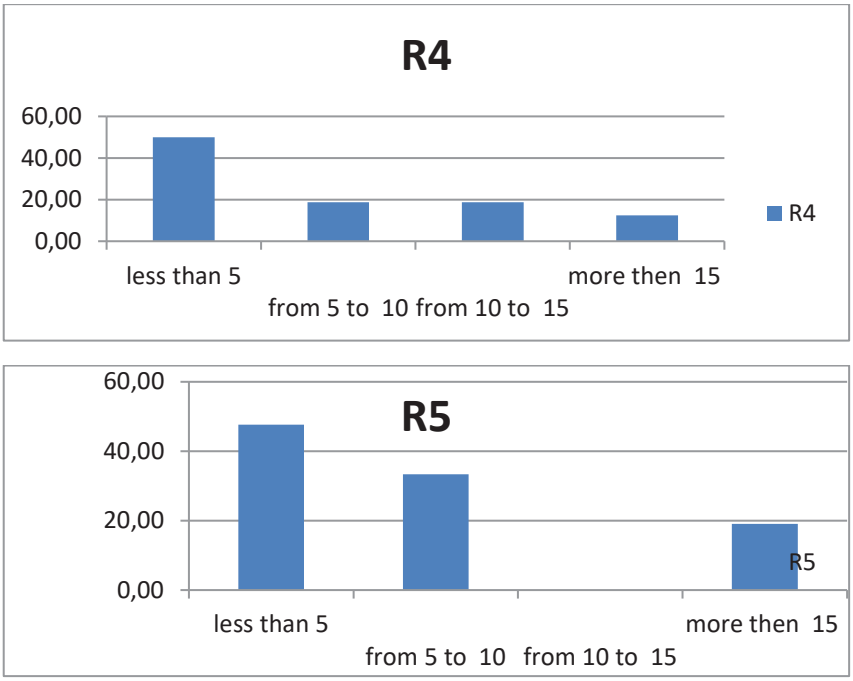

Fig. 3. Callus formation on nutrient media R4 and R5 (%)

From all the above, it was concluded that all variants of nutrient media showed a positive effect, its maximum value was on the nutrient medium R1 with a three-fold reduced content of CaCl2 ·2H2O.

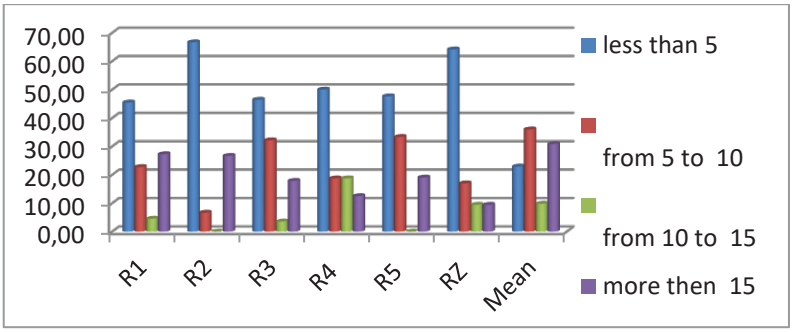

Fig. 4. Callus formation on the developed nutrient media in comparison with the RZ standard

Nutrient media R2 and RZ are the most versatile, most of the genotypes form a callus on these nutrient media. Nutrient media R1, R2, are more specific and allow you to get a large amount of callus in a smaller number of variants. For domestic short-grain and medium-grain varieties, among the developed nutrient media, the most suitable were the nutrient media R5, RZ, R1. Moreover, the nutrient media R1 and R5 did not show good results in all combinations, the nutrient medium RZ was suitable for a larger number of samples.

Significantly, due to small samples, the nutrient medium R1 differed for each variant of the nutrient media, but a positive effect was noted in all 5 variants.

4 Conclusions

Nutrient media R2 and RZ are the most versatile, most of the genotypes form a callus on these nutrient media. The nutrient medium R1 is more specific and allows obtaining a large amount of callus from a smaller number of samples.

The nutrient medium R1 is characterized by a reduced content of CaCl2·2H2O by almost three times than the standard RZ.

Due to the less significant effect on the callus formation of other variable components of the environment, their reliable influence has not been established, although their positive effect has been noted, a further increase in the sample is required to confirm the result obtained.

References

1. Yu.K. Goncharova, *The use of the anther culture method in rice breeding* (2012)
2. Yu.K. Goncharova, *Method of fixing the heterosis of hybrids*, Patent for invention No. 2759222 C2, 11.11.2021. Application No. 2019140920 dated 11.12.2019
3. Yu.K. Goncharova, E.M. Kharitonov, N.Y. Bushman, S.A. Vereshchagina, *Comparative analysis of the effectiveness of nutrient media for the induction of callus formation in rice hybrids*, Reports of the Russian Agricultural Academy, **6**, pp. 6-9, (2013)
4. M.V. Ilyushko, M.V. Skaptsov, M.V. Romashova, *The content of nuclear DNA in rice regenerants (ORYZA SATIVA L.) obtained in the culture of anthers IN VITRO*, Agricultural Biology, **53(3)**, pp. 531-538 (2018)
5. M.V. Ilyushko, *Creation of source material for rice breeding by the method of anther culture IN VITRO in the Russian Far East*, In the collection: The role of agricultural science in ensuring food security of the Far Eastern region. Collecting.
6. C.C. Chen, *In vitro development of plant from microspores of rice IN VITRO*, National Library of Medicine, **13**, pp. 484-489 (1977)
7. Y. Chen, *Anther and pollen culture of rice Haploids of higher plants in vitro*, China academic publishers, pp. 3-25 (1986)
8. J.S. Croser, M.M. Lulsdorf, P.A. Davies, H.J. Clarke, K.L. Bayliss, N. Mallikarjuna, K.H.M. Siddique, *Toward doubled haploid production in the Fabaceae: progress, constraints, and opportunities*, Critical reviews in plant sciences, **25**, pp. 139-157 (2006)
9. Y.K. Goncharova, S.A. Vereshagina, S.V. Gontcharov, *Nutrient media for double haploid production in anther culture of rice hybrids*, Plant Cell Biotechnology and Molecular Biology, **21**, 23-24, pp. 1215-1223 (2020)
10. Z.P. Lentini, R.C. Martinez, W.M. Rosa, *Androgenesis of highly recalcitrant rice genotypes with, maltose and silver nitrate*, Plant Science, **110**, 127-138 (1995)
11. M.W. Nabors, J.W. Heyser, O.A. Dykes, E.D. Mott, *Long-duration, high-frequency plant regeneration from cereal tissue cultures*, Planta, **157**, pp. 385 (1983)
12. S. Ochatt, C. Pech, R. Grewal, C. Coreux, M. Lulsdorf, L. Jacas, *Abiotic stress enhances androgenesis from isolated microspores of some legume species (Fabaceae)*, Journal of plant physiology, Doi: 10.1016/j.jplph. 2009.01.011. (2009)
13. S.K. Raina, F.J. Zapata, *Enhanced anther culture efficiency of indica rice (Oryza sativa L.) through modification of the culture media*, Plant Breed, **116**, pp. 305-315 (1997)
14. E.G. Savenko, Zh.M. Machine, V.A. Glazyrina, L.A. Shundrina, *Cyto-histological aspects of haploid androgenesis when obtaining haploids/doubled haploids in rice*

(Oryza sativa L.) anther culture in vitro, E3S Web of Conferences, **285**, https://doi.org/10.1051/e3sconf/202128502033 (2021)

15. T.D. Silva, *Indica rice anther culture: can the impasse be surpassed?* Plant Cell Tiss. Organ Cult, **100**, pp. 1-11 (2010)
16. L.S. Zhuo, H.M. Si, S.H. Cheng, Z.X. Sun*, Phenylacetic acid stimulation of direct shoot formation in anther and somatic tissue cultures of rice (Oryza sativa L.)*, Plant Breeding, **115**, pp. 295-300 (1996)