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CALLUS FORMATION AND PLANT REGENERATION IN ANTHER CULTURE OF WHEAT (TRITICUM AESTIVUM L.)

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Šesek, S., Borojević, K. and Radojević, Lj. (1985): *Callus formation and plant regeneration in anther culture of wheat (Triticum aestivum* L.). – Glasnik Instituta za botaniku i botaničke bašte Univerziteta u Beogradu, Tom XIX, 23–30.

Anthers with uninuclear microspores originating from 19 genetically divergent wheat genotypes were cultured in four induction media A_1–A_4 containing MS mineral solution (Murashige and Skoog, 1962), 4.5 and 5.0 sucrose, 0.7% agar, 10% potato extract and varying concentrations of vitamins, auxins and kinetin. Androgeneous calluses were formed depending on both genotype and nutrient medium composition.

Cultivation of calluses during four passages was performed using media supplemented with cytokinins and in some genotypes rhizogenous calluses were formed. Only calluses of wheat genotype „Veery–4 x Novosadska Jara" regenerated four albino plants with haploid chromosome number.

Key words: *Triticum aestivum* L., wheat genotypes, anther culture, callus formation, plant regeneration, albino plant.

Ključne reči: *Triticum aestivum* L., genotipovi pšenice, kultura antera, formiranje kalusa, regeneracija biljke, albino biljka

INTRODUCTION

Since the first publication appeared concerning haploid plant production by *in vitro* anther culture (Guha and Maheswari, 1964), numerous investigators con-

centrated their efforts on these problems and soon after that, haploid plants of many plant species have been obtained. Although several research groups were dealing with *in vitro* anther culture of wheat, O u y a n g *et al.* (1973) were the first who published the results on this topics. In their experiments, about 3% of anthers formed calluses and 10% of the calluses gave green plants. However, at present, it is possible to obtain more than 60% of androgeneous calluses and more than one third of them is capable to regenerate green haploid plants (H u *et al.*, 1983).

Individual phases of microsporogenesis have been investigated in order to determine the most suitable stage of pollen development for the anther culture. Only anthers with microspores during the phase from tetrade separation to the beginning of postmeiotic division are suitable for the isolation. O u y a n g *et al.* (1973) recommended middle uninuclear stage as the most suitable one, while S c h a e f f e r *et al.* (1979) used anthers with microspores in both middle and late uninuclear stage.

It has been observed that pollen capability for androgenesis depends on genetic characteristics of individual genotypes (S h i m i d a and M a k i n o, 1975; B a j a j, 1977; B e v e r s d o r f and B i n g h a m 1977; L a z a r *et al.*, 1984). H u and S h a o (1981) found that significantly better results were achieved with anthers of F_1 or F_2 hybrids, than with anthers of pure lines or cultivars of wheat. Besides genotype, the nutrient medium composition also strongly influences haploid production from the wheat anther culture. Concentrations of mineral constituents, vitamins, auxins to cytokinin balance, and especially the amount of sucrose in the nutrient medium, influence both formation and differentiation of the callus, *i.e.* affects its ability to regenerate plants (O u y a n g *et al.*, 1973; S c h a e f f e r *et al.*, 1979). It has also been found that 10 – 20% potato extract stimulates the process of androgenesis, resulting in better quality of embryos and higher percentage of haploid plants (C h u a n g *et al.*, 1978; D e B u y s e r and H e n r y, 1980). Investigations concerning the effects of temperature pretreatment on androgenesis in wheat microspores, showed that exposure of anthers to 4–5°C before or after inoculation, accelerate the process of embryonic callus formation (S c h a e f f e r *et al.*, 1979; H u *et al.*, 1983).

Taking into account all above mentioned factors influencing androgenesis, we investigated the possibilities of callus and haploid plants formation from *in vitro* anther cultures of several genotypes, obtained by crossbreeding of some genetically divergent wheat lines with wheat cultivars of Novi Sad.

MATERIAL AND METHODS

Anthers of F_1 generation isolated from 15 genotypes obtained by crossbreeding in which genetically divergent hybrids were included, as well as anthers from four different wheat (*Triticum aestivum* L.) cultivars were used. Plant material which was obtained in the field under normal environmental conditions, is listed in Tab. 1.

The anthers were isolated under sterile conditions and percentage of contamination was about 3. Approximately 5.0 cm long spikes were separated from the leaf sheath, sterilized for 7 min in 0.1% mercury chloride solution and washed five times with sterile distilled water. Anthers, 0.8 – 2.0 mm long, enclosing uninuclear microspores (Fig. 1) were inoculated. During the initial period of culture, anthers were exposed to low temperature (4 – 5°C) for 64 hrs, representing the first part of temperature pretreatment, which was shown to stimulate androgenesis of microspores (S c h a e f f e r *et al.*, 1979). After that, the cultures covered with aluminium foil to provide complete

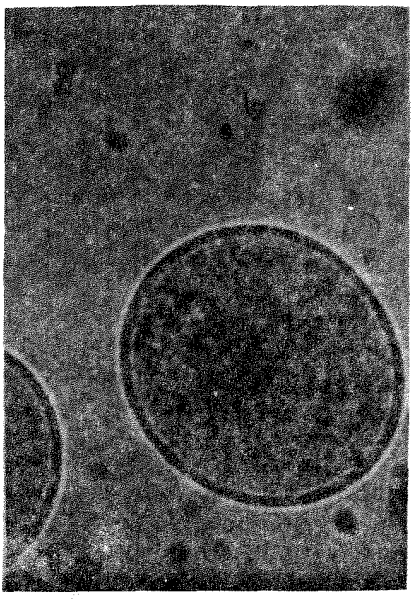

Fig. 1. – Microspores in uninuclear stage of development (x 2 600).

Fig. 2. Rhizogenous callus of the wheat genotype „Veery–4 x Neretva" in B_3 nutrient medium (x 4.5).

darkness, were transferred into a laboratory thermostat and kept for 5 days at 26°C. Later, the cultures were grown at 24 – 28°C under the fluorescent light tubes for varying periods of time. During the cultivation in induction nutrient media, the cultures were exposed to 800 – 1 000 lx for 8 hrs. After the first transfer, the cultures were exposed to 1 500 lx for 16 hrs a day.

Four different induction media, designated A_1 – A_4 were used. Medium A_1 contained mineral solution after Zhuan and Jia (1980) and the three others (A_2 – A_4) MS mineral solution (Murashige and Skoog, 1962). Concentration of sucrose was 4.5% and 5.0%, that of agar 0.6% and 0.7%. Media A_2 – A_4 contained 10% potato extract (PE). Concentration of vitamins and growth stimulators in the media used is given in Table 2.

Nutrient media, use for callus differentiation, necessary for plant regeneration (media B_1 – B_3) contained decreased amount of sucrose (3%) and an increased concentration of PE (20%). These media also contained less auxins and more cytokinins in comparison with the induction nutrient media (Tab. 3).

RESULTS AND DISCUSSION

The results presented in Table 1 demonstrate significant differences in percentage of androgeneous callus formation of eight wheat genotypes of F_1 generation when anthers were cultured in A_1 and A_2 nutrient media. It can be seen that these differences are related to both nutrient medium composition and wheat genotype. In medium A_1 which contained somewhat lower auxin concentration (1.5 mg · l^{-1} 2,4–D and 0.5 mg ·

Tab. 1. The effect of nutrient medium on the androgeneous callus formation and plant of different genotypes of Triticum aestivum L.

Nutrient medium	A_1		A_2		A_3		A_4		B_1	B_2	B_3	
									Number of calluses and regenerated plants in subcultures			
Genotype	Number of isolated anthers	Formed calluses N° %	Number of isolated anthers	Formed calluses N° %	Number of isolated anthers	Formed calluses N° %	Number of isolated anthers	Formed calluses N° %	I	II	III	IV
NS–722xJugoslav.	296	5 1.7	245	46 18.8					34c	16c	7rc	1c, 1LP
(NS–58–97xAA)xNz.	287	1 0.3	294	72 24.5					21c	13c	8rc	–
Žitnica x SO–8123	283	2 0.7	285	48 16.8					37c	17c	2rc	–
NS–559 x Jugoslav.	287	10 3.5	290	5 1.7					5c	–	–	–
WW 33618 x Žitn.	231	6 2.6	273	19 7.0					17c	8c	4rc	–
(AA x Top) x Nizija	216	14 6.4	246	20 8.1					21c	8c	2rc	–
Hays x Jugoslavija	267	1 0.4	249	0					1c	1c	–	–
Hays x Ličanka	261	0 –	249	0 –					–	–	–	–
Centurk					444	9 2.0			4c	4c	–	–
Kite					513	6 1.2			–	–	–	–
Chris					470	0 –			–	–	–	–
Jarka					288	15 5.2			3c	1c	–	–
Veery–4 x Neretva					473	206 43.5			100c	48c	14rc	4c, 4LP
Veery–4 x Dugoklasa					243	28 11.5			15c	4c	–	–
Pavon–76 x Novjara							422	13 3.1	4c	3c	–	–
Pavon–76 x Neretva							536	30 5.6	15c	2c	–	–
Verry–4 x Novjara							534	37 6.9	5c	2c	1rc	4A1
Pavon–76 x Dugokl.							494	4 0.8	2c	–	–	–
Pavon–76 x Jarka							415	8 1.9	2c	–	–	–

c=callus; rc=rhizogenous callus; LP=green leaf primordia; AL= albino plants.

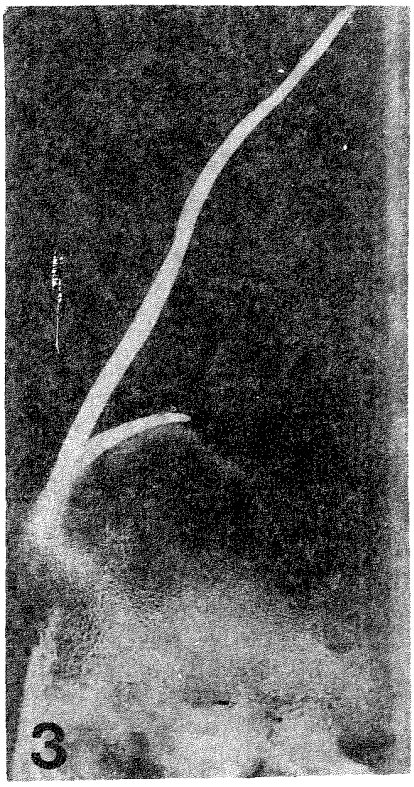

Fig. 3. – Haploid plant regenerated from the callus of the wheat genotype „Veery–4 x Novosadska jara" in B_2 nutrient medium (x 4).

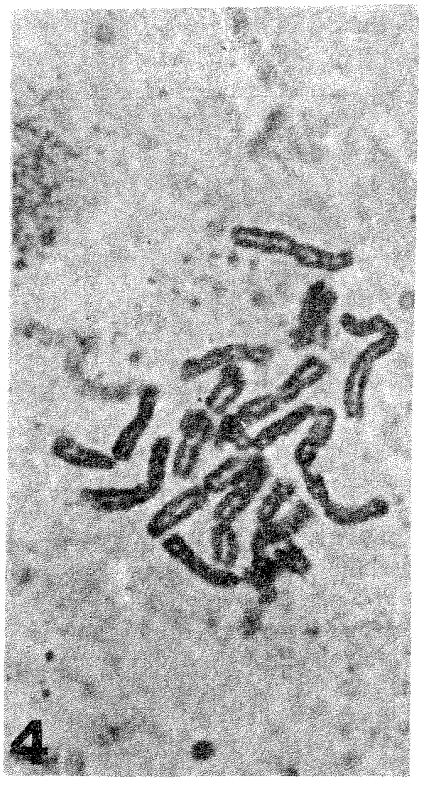

Fig. 4. – Karyotype with haploid chromosome number (n = 21) regenerated from the callus of the wheat genotype „Veery–4 x Novosadska jara" (x 4 000).

Tab. 2. – Composition of induction nutrient media for anther culture of wheat

Substrate	A_1	A_2	A_3	A_4
Mineral solution	Z+FeEDTA	MS+FeEDTA	MS+FeEDTA	MS+FeEDTA
Agar	0.7%	0.6%	0.7%	0.7%
Sucrose	4.5 %	5%	5%	5%
Inositol	100 mg l^{-1}	100 mg l^{-1}	100 mg l^{-1}	100 mg l^{-1}
Nicotinic acid	0.5 mg l^{-1}	5.0 mg l^{-1}	–	5.0 mg l^{-1}
B_1	1.0 mg l^{-1}	1.0 mg l^{-1}	1.0 mg l^{-1}	1.0 mg l^{-1}
B_6	0.5 mg l^{-1}	–	0.5 mg l^{-1}	–
Glutamine	–	500 mg l^{-1}	500 mg l^{-1}	500 mg l^{-1}
Glycine	2.0 mg l^{-1}	–	–	–
Kinetin	0.5 mg l^{-1}	–	–	0.4 mg l^{-1}
2,4–D	1.5 mg l^{-1}	3.0 mg l^{-1}	2.0 mg l^{-1}	3.0 mg l^{-1}
NAA	0.5 mg l^{-1}	–	0.5 mg l^{-1}	–
PE	–	10%	10%	10%
pH = 5.8				

Z = mineral solution (Zhuan and Jia, 1980):
MS = mineral solution (Murashige and Skoog, 1962):
PE = Potato extract

Tab. 3. – Nutrient media for differentiation and plant regeneration from androgeneous wheat callus

Substrate	B_1 *1st passage*	B_2 *2nd passage*	B_3 *3rd–4th passage*
Mineral solution	MS+FeEDTA	MS	MS+FeEDTA
Agar	0.7 %	0.7%	0.7%
Sucrose	3.0%	3.0%	4.0%
Glutamine	146 mg l^{-1}	146 mg l^{-1}	146 mg l^{-1}
Nicotinic acid	1 mg l^{-1}	–	–
B_1	–	1 mg l^{-1}	–
Kinetin	0.5 mg l^{-1}	–	0.2 mg l^{-1}
Zeatin	–	0.1 mg l^{-1}	0.1 mg l^{-1}
IAA	0.5 mg l^{-1}	0.3 mg l^{-1}	1.0 mg l^{-1}
NAA	1.0 mg l^{-1}	1.0 mg l^{-1}	–
PE	20%	20%	20%
pH = 5.8			

l^{-1} NAA) and no PE, 0.3 to 6.4% (mean 1.8%) of anthers formed androgeneous calluses. In medium A_2, containing not only higher concentrations of 2,4–D (3.0 mg $\cdot l^{-1}$), but also 500 mg $\cdot l^{-1}$ of glutamine and 10% PE, 1.7 to 24.5% (mean 10%) of the anthers formed androgeneous calluses. Anthers isolated from the genotype „Hays x Yugoslavia" and „Hays x Ličanka" had the poorest regeneration capacity on both media. About 3.5% of anthers of the genotype „NS–559 x Yugoslavia" formed androgeneous calluses in medium A_1, while this percentage was much lower (1.7%) when the anthers of the same genotype were grown in medium A_2. Only 0.3% anthers of the wheat genotype „(NS–58–97xAA)xNz" formed androgeneous calluses in medium A_1, but 24.5% anthers of the same genotype gave androgeneous calluses when cultured in medium A_2. These results strongly suggest the dependence of callus formation on both wheat genotype and composition of the nutrient medium.

Anthers of the wheat cultivar „Chris" formed no androgeneous calluses. Percentage of androgeneous callus forming anthers of all other wheat genotypes examined throughout this work and cultured in induction nutrient media A_3 and A_4 varied from 0.8% (genotype „Pavon–76 x Dugoklasa" to 43.5% (genotype „Veery–4 x Neretva"), (Tab. 1).

Further cultivation of androgeneous calluses, 35 – 40 days after the isolation proceeded in nutrient media for callus differentiation and plant regeneration (media B_1 – B_3; Tab. 3). After the first transfer, some wheat genotypes expressed retarded development in medium B_1, even necrosis of androgeneous calluses appeared, while approximately 45% of the calluses grew intensively, forming green homogeneous nodules with the buds or leaf primordia. During the second transfer, these calluses were cultured for 20 – 25 days in B_2 nutrient medium, containing no 2,4–D. Approximately 30% of the calluses were rhizogenous. These calluses grew very intensively and formed numerous roots (Fig. 2). During the fourth transfer, most of the calluses gradually developed necrosis starting from the green nodules and after that, necrosis spread over the entire tissue.

Rhizogenous calluses were transferred in B_3 nutrient medium supplemented with kinetin (0.2 mg $\cdot l^{-1}$) and zeatin (0.1 mg $\cdot l^{-1}$) in order to induce plant regeneration. Six calluses obtained from the anthers of three different wheat genotypes regenerated plants.

An anther of the genotype „Veery–4 x Novosadska jara" produced a well developed, compact, androgeneous callus, with numerous differentiated leaf primordia. After the culture in B_3 nutrient medium, this callus was divided into four segments and each regenerated one well developed albino plant (Fig. 3) with haploid chromosome number (Fig. 4.). These plants survived for about 20 days in B_3 nutrient medium. Androgeneous calluses of genotypes „NS–722 x Yugoslavia" (one callus) and „Veery–4 x Neretva" (four calluses) gradually developed necrosis, especially in nodular parts, but nevertheless the other parts formed roots. Culture of rhizogenous calluses proceeded in B_3 nutrient medium. These calluses showed abundant rhizogenesis even after six months of continuous transfers.

The highest potential for plant regeneration was observed in anthers of genotype „Veery–4 x Novosadska jara". Plant regeneration from the calluses of this genotype was achieved in a relatively short period of time, *i.e.* for approximately 30 days. Regeneration of the plants of genotypes „NS–722 x Novosadska jara" and „Veery–4 x Neretva" began with the development of apical meristem with a pair of leaf primordia, but rapid necrosis of the calluses of these two genotypes prevented further differentation and development of these structures into the plants.

On the basis of the results presented in this paper following conclusions could be pointed out: a. Formation of androgeneous calluses and plant regeneration depend on the composition of the nutrient medium and on wheat genotype (Tab. 1). b. Differentiated callus originated from the anthers of wheat genotype „Veery–4 x Novosadska jara" regenerated four albino plants with haploid chromosome number after brief cultivation in B_3 nutrient medium.

REFERENCES

Bajaj, Y. P. S., (1977): *In vitro* induction of haploids in wheat (*Triticum aestivum* L.) – Crop Improv. 4: 54–64.

Beversdorf, W. D. Bingham, E. T., (1977): Degrees of differentiation obtained in tissue culture of *Glycine* species. – Crop Sci. 17: 307–311.

Chuang, C. Ouyang, T., Chia, H., Chou, S., Chang, C., (1978): A set of potato media for wheat anther culture. – Proc. Symp. Plant Tissue Culture, Peking. pp 51–56.

De Buyser, J., Henry, Y., (1980): Comparaison de differents milieux utilises en culture d'antheres *in vitro* chez le Ble tendre. – Can. J. Bot. 58: 997–1000.

Guha, S., Maheshwari, S. C., (1964): *In vitro* production of embryos from anthers of *Datura*. – Nature 204: 497.

Hu, H., Shao, O., (1981): Advances in plant cell and tissue culture in China. – Advances in Agronomy, 34: 1–34.

Hu, H., Hi, Z., Jing, J., Wang, X., (1983): Production of wheat pollen–derived aneuploid plants through anther culture. Cell and Tissue Culture Techniques for Cereal Crop Improvement. – Sc. Press, Beijing, China. pp. 173–182.

Lazar, M. D., Schaeffer, G. W., Baenziger, P. S., (1984): Cultivar x environment of callus and polyhaploid plants from anther cultures of wheat. – Theor. Appl. Genet. 67: 273–277.

Murashige, T., Skoog, F., (1962): A revised medium for rapid growth and bioassay with tobacco tissue cultures. – Physiol. Plant. 15: 473–497.

Ouyang, T., Hu, H., Chuang, C., Tseng, C., (1973): Induction of pollen plants from anthers of *Triticum aestivum* L. cultured *in vitro*. – Sci. Sin. 16 (1): 79–90.

Pan, C. L., Kao, K. H., (1978): The production of wheat pollen embryo and the influence of some factors on its frequency of induction. – Proc. Symp. Plant Tissue Culture, Peking, pp. 133–142.

Schaeffer, G.W., Baenziger, P. S., Worley, J., (1979): Haploid plant development from anthers and *in vitro* embryo culture of wheat. – Crop Science 19: 697–702.

Shimada, T., Makino, T., (1975): *In vitro* culture of wheat. III. Anther culture of the A genome aneuploids in common wheat. – Theor. Appl. Genet. 46: 407–410.

Zhuang, J., Jia, X. (1980): Studies on the differentiation of pollen calli of wheat. – Ann. Rep Genet. Acad. Sin. pp. 70–72.

Rezime

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FORMIRANJE KALUSA I REGENERACIJA BILJAKA U KULTURI ANTERA PSENICE (TRITICUM AESTIVUM L.)

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Antere sa jedarnim mikrosporama iz 19 genetski divergentnih genotipova pšenice kultivisane su na četiri indukcione podloge A_1–A_4 koje su sadržale MS mineralni rastvor (Murashige i Skoog, 1962), 4,5 i 5% saharozu, 0,7% agar, 10% ekstrakt krompira, različite koncentracije vitamina, auksina i kinetina. Androgeni kalus se formirao u zavisnosti od genotipa i hranljive podloge.

Sukcesivno gajenje kalusa tokom četiri pasaža, nastavljeno je na podlogama obogaćenim citokininima (podloge B_1 – B_3), što je kod pojedinih genotipova dovelo do formiranja rizogenih kalusa. Jedino su kalusi genotipa „Veery–4 x Novosadska jara" regenerisali četiri albino biljke sa haploidnim brojem hromozoma.