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Induced androgenesis in *Phleum pratense*

Z. BRODA, S. MIKOŁAJCZYK

Department of Genetics and Plant Breeding, August Cieszkowski Agricultural University of Poznań

Abstract. Producing homozygous lines in anther culture of timothy (*Phleum pratense* L.) is yet insufficient. With using *in vitro* culture techniques for obtaining double haploid plants of timothy we can do it in a short time. In monocotyledons one of the methods for the induction of haploids is androgenesis (anther culture and isolation microspores). The most important factors influencing the androgenic capacity are: genotype, pre-treatment of spikes or anthers and medium. The present investigation centered on the factors influencing haploid plant production efficiency via induced embryogenesis.

K e y w o r d s : androgenesis, anther culture, genotype, *Phleum pratense*, timothy, medium, anther treatment

1. Introduction

Haploids are one of the most important problems of plant cell cultures both in theoretical and practical aspects. The simplicity of the anther culture method developed by MAHESHVARI et al. (1980) and the great number of haploids obtainable are of great advantage. Moreover, the gene pool of the species is enriched by the variability that appears fragmentally among regenerants. Despite the considerable results obtained with anther cultures of timothy, tobacco, rice, maize and potatoes (PULLI and GUO, 2003; GUO et al., 1999; WANG and HU, 1984) research in grasses, and especially in timothy, is yet insufficient.

The present investigation centered on the factors influencing haploid plant production efficiency via inducted embryogenesis.

2. Material and Methods

Twenty timothy (*Phleum pratense* L.) genotypes were used as initial material origin from variety Skaut: genotypes Sk_1 – Sk_{10} and variety Bartovia: genotypes Ba_1 – Ba_{10}. Ten seeds were sown into plastic pots filled with a 1:1 peat/sand mixture. After emergence three timothy plants per pot were left to grow. When the plants reached 10 cm in height, they were cut to about 4 cm, followed by 2-3 repetitive cuts. When the plants had shooted enough after the last cutting, they were transferred into cool room at $+4^{o}C$ and

vernalized for 10-12 weeks. After vernalization, the last plants were moved to a greenhouse under the following growth conditions 18°C/13°C day/night temperature and 16 h photoperiod. Spikes emerged 4-5 weeks later.

When the timothy heads were about 3-4 cm out of the sheaths, the developmental stage of the microspores was examined microscopically by acetocarmine staining. The optimum development stage was between the very late uninucleate and the binucleate stages, when 50% of microspores were at the binucleate stage. In the uninucleate stage the microspore should be round with clearly visible nucleus. In the binucleate stage microspores should be round with some starch grain deposits (IMMONEN and ANTTILA, 1998). If the spikes need cold pretreatment, they could be harvested as a slightly earlier microspore development stage to uninucleate stage. The spikes were cut, labeled, bagged and placed in a bottle containing a sufficient amount of water and then they were transferred to a cool room at 4°C in darkness for a cold pretreatment of 2-4 weeks. After the cold pretreatment, the timothy spikes were used for anther culture. The spikes were surface sterilized with 20% sodium hypochlorite on a shaker for 15 min and then washed several times with sterilized distilled water. The anthers were then transferred on Petri dishes containing various solid induction media. The basic mineral media of MURASHIGE and SKOOG (1962), NITSH and NITSH (1967), GAMBORG et al. (1968) and BLAYDES (1966) supplemented with various concentrations of 2 mg l^{-1} kinetin, 0,3 mg l^{-1} zeatin, 2 mg l^{-1} 2ip, 2 mg l^{-1} BAP, 2 mg l^{-1} 2, 4-D, 2 mg l^{-1} IAA and 2 mg l^{-1} NAA were tested.

The anthers were treated with low temperatures (4-10°C) for 2-4 days and also were gamma-irradiated 1, 2, 4, 8, Gy immediately before they were placed on nutrient medium. The anthers were kept in growth chamber with regulated parameters: temperature 25°C ±2°C in the dark up to 10-15 days and in 3000 lux light intensity and 16/8 day night photoperiod. The regenerants obtained were placed on B5 medium (GAMBORG et al., 1968) without supplements and were transferred to sterile perlite for 20 days. Afterwards they were planted in soil and grown under greenhouse conditions. Chromosome numbers of the obtained regenerants were determined on squash preparations from root meristems stained with acetocarmine.

3. Results and Discussion

Two or tree weeks after cultivation timothy anthers swelled and burst open. Pale-yellow to light green friable or grain callus originated from the interior in some of them. Callus induction and growth intensity depended on the previous treatments, the genotype and the nutrient medium. Therefore, we focused our attention on those factors.

Nutrient medium. Medium composition is widely known to be one of the most important factors affecting callus formation and induction at *in vitro* embryogenesis. Different species and even varieties have certain requirements regarding nutrients and especially hormones.

The best callus induction and callus development in almost all clones were observed on BLAYDES (1966) nutrient medium. The highest percentage of anthers yielding callus was observed on BLAYDES medium with 2 mg l^{-1} kinetin or 2 mg l^{-1} BAP for Sk_1 – 64%, 63,8% and Ba_7 – 59,4%, Ba_1 – 64,7% respectively (Table 1). Compared to all tested

nutrient media based on Blaydes, those containing 1 mg l^{-1} 2ip and 500 mg l^{-1} meso-inositol were the most favorable for induction of embryogenesis from the anther derived callus.

Table 1. Influence of genotype and cytokinins on callus induction in anther cultures of *Phleum pratense* on Blaydes nutrient medium – each of variations containing 2,4-D and NAA (2 mg l^{-1})*

Genotypes	Cytokinins tested (2mg l^{-1})				Growth rate**
	Kinetin	2ip	BAP	Zeatin	
Anthers producing callus (%)					
Sk_1	64.0	49.7	63.8	56.0	6
Sk_2	20.5	19.0	12.4	8.4	4
Sk_3	17.2	13.0	13.7	12.3	4
Sk_4	28.8	1.2	6.8	14.0	3
Sk_5	22.3	14.3	0.7	3.7	3
Sk_6	7.4	4.8	14.8	5.6	3
Sk_7	58.6	39.4	40.9	36.8	6
Sk_8	14.7	34.0	3.7	37.2	5
Sk_9	17.8	10.6	20.1	14.7	4
Sk_{10}	13.4	9.6	18.5	16.5	4
Ba_1	59.0	50.1	64.7	59.6	6
Ba_2	21.5	18.7	13.5	9.3	4
Ba_3	18.2	14.2	12.8	13.2	4
Ba_4	27.4	0.7	7.1	14.8	4
Ba_5	21.1	16.3	1.2	4.3	3
Ba_6	6.4	5.1	15.6	5.1	2
Ba_7	59.4	40.8	40.3	38.7	5
Ba_8	16.7	35.1	7.4	34.2	4
Ba_9	17.4	11.4	25.4	15.1	4
Ba_1	14.8	10.7	19.6	17.4	4

* 400 anthers were tested per variant of Blaydes nutrient medium
** The growth rate is given in six grades scale: 1 – very low, 2 – low, 3 – moderate, 4 – good, 5 – very good, 6 – high

Genotype. The genotype is of particular importance among the factors influencing pollen embryogenic capacity. The efficiency of androgenesis depends on species, cultivar and single genotype. The genus *Nicotiana* is a remarkable example. The high embryogenic ability of *Nicotiana tabacum* is well known.

Table 1 shows the effect of the genotype on callus induction and its development on Blaydes nutrient medium supplemented with 2,4-D, NAA and kinetin. The data show that varieties Skaut clone Sk_1 and Bartovia clones Ba_1, Ba_7 exhibit the highest percentage of anthers producing callus – 64%, 59,0% and 59,4% respectively. They also have the best callus development which is presented in Table 1 with the highest mark 5 and 6.

Anther treatment. Treatment of flower spikes or anthers with chemical and physical factors prior to cultivation *in vitro* is particularly important for some species. Reference data about the influence of high and low temperature shocks are numerous. The low temperature was used most frequently for inducing of androgenesis in many species, especially for the cereals: wheat (MARSOLAIS et al., 1984), barley (POWELL, 1988) and oats (KIVIHARJU and PEHU, 1998).

The whole spikes of study clones of Skaut and Bartovia varieties were gamma-irradiated before anthers were placed on nutrient medium. Beside that, studies on callus and embryo induction under combined treatment with low temperatures and gamma rays were carried out (Table 2). Treatment with low temperature and low doses of gamma rays stimulated callus formation and embryogenesis in anther cultures. Anthers treated with 4°C for 4 days and 1 Gy developed callus less than 10 days. Callus of the other genotypes emerged after 15–20 days. Combined treatment with 10°C for 2 days and 8 Gy resulted in the highest callus formation. In this case, 70% of the anthers produced callus but no embryogenesis was observed. The best embryogenesis was observed after gamma

Table 2. Influence of temperature and gamma rays on callus and embryos formation in anther cultures of *Phleum pratense**

Genotypes	Treatments	Anthers producing callus				Calli showing embryogenesis
	T°	Days	Gy	Number	%	
Sk_1	4	2	1	158	39.5	3.2
Sk_2	4	4	1	274	68.5	6.7
Sk_3	4	2	1	115	28.7	3.4
Sk_4	10	4	1	120	30.0	2.4
Sk_5	10	2	2	218	54.5	4.7
Sk_5	10	2	4	283	70.7	4.7
Sk_6	10	2	8	210	52.5	4.2
Sk_7	4	2	1	241	60.2	4.1
Sk_8	4	4	1	0	0	0
Sk_9	10	4	1	16	4.0	0
Sk_{10}	4	4	4	235	58.7	4.3
Ba_1	4	4	4	194	48.5	1.4
Ba_2	10	2	4	161	40.2	?
Ba_3	4	2	1	264	66.0	2.8
Ba_4	10	2	8	250	62.5	2.1
Ba_5	10	4	1	117	29.2	1.8
Ba_6	4	4	2	105	26.2	2.4
Ba_7	10	2	4	106	26.5	0
Ba_8	10	2	8	270	67.5	0
Ba_9	10	2	4	271	67.7	1
Ba_{10}	10	4	4	273	68.2	0

*400 anthers were tested per genotype

ray treatment with 1 and 4 Gy. The highest percentage of embryogenesis resulted from combining treatment with 4°C for 4 days and 4 Gy was Sk_2 – 6,7% and Ba_2 – 4,3% respectively (Table 2).

Cytological characteristic of regeneration plants. Over five hundred plants were obtained as a result of the embryogenesis induced in the anther callus of clones from two varieties – Skaut and Bartovia (Table 3). The cytological analysis of the root meristem of the produced plants showed considerable variation in the chromosome number. Most of the regenerants were hexaploid 2n = 6x = 42 – clones $Sk_{1–10}$ 36,9% and $Ba_{1–10}$ 35,4%, mixoploids 40,8% and 46,8% respectively. Haploids n = 3x = 21 plants obtained $Sk_{1–10}$ – 22,3%, $Ba_{1–10}$ 17,8% (Table 3).

Table 3. Ploidy level of the regenerated timothy plants

Genotypes	Number of hexaploids (double haploids) 2n = 6x = 42	Number of haploids n = 3x = 21	Number of mixoploids
Sk_1	15	7	12
Sk_2	20	12	17
Sk_3	16	9	21
Sk_4	17	8	15
Sk_5	19	10	20
Sk_6	21	14	20
Sk_7	16	5	18
Sk_8	–	–	–
Sk_9	–	–	–
Sk_{10}	18	11	16
$Sk_{1–10}$	**126 (36.9%)**	**76 (22.3%)**	**139 (40.8%)**
Ba_1	10	6	16
Ba_2	12	8	15
Ba_3	17	9	18
Ba_4	16	7	20
Ba_5	12	6	14
Ba_6	15	7	12
Ba_7	–	–	–
Ba_8	–	–	–
Ba_9	1	–	–
Ba_{10}	–	–	–
$Ba_{1–10}$	**72 (35.4%)**	**36 (17.8%)**	**95 (46.8%)**
$Sk_{1–10}$ + $Ba_{1–10}$	**198 (36.4%)**	**112 (20.6%)**	**234 (43.0%)**

Several authors explain the wide range of ploidy levels of androgenic plants with endomitosis and nucleus convergence which were observed during early stages of pollen development in anthers cultivated *in vitro* (KASHA et al., 2001). INDRIANTO et al. (1999) showed that ploidy level could depend on spikes or anthers pretreatment. These proces-

ses are well described in many cytological studies (NIŻECKI and KATI, 1973; GUO and PULLI, 2000).

4. Conclusions

- The success achieved in inducing haploids gives opportunities for fundamental research and for the solution of practical plant breeding problems.
- Improving embryogenesis and plant regeneration in timothy at the hexaploid level is a complex and very slow process. Induced haploids in this species provide fast development of new more productive and high-grade forms.
- Particular attention should be paid to increase of the efficiency of *in vitro* haploid and double haploid production, to the factors influencing induced androgenesis, and to the genetic nature of the produced timothy plants. Induced androgenesis in timothy could be applied widely in breeding programs only after the development of easily carried out and effective methodology for obtaining a great number of stable haploids and double haploids.

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Summary

The anthers of twenty timothy genotypes were used for the induction of androgenesis and plant regeneration. They originated from variety Skaut: genotypes Sk_1 – Sk_{10} and from variety Bartovia: genotypes Ba_1 – Ba_{10}. The highest rate of induction of androgenesis was observed on Blaydes medium with 2 mg l^{-1} kinetin or 2 mg l^{-1} BAP for genotype Sk_1 – 64%. The best rate of embryogenesis was observed when anthers were treated of gamma ray with radiation dose 1 and 4 Gy. The highest percentage of embryogenesis resulted from combining pretreatment anthers with 4ºC for 4 days and 4 Gy was Sk_2 – 6.7%. Chromosome counting of plantlets regenerated showed that most of the regenerants were allohexaploid 2n = 6x = 42 – clones $Sk_{1–10}$ 36.9% and $Ba_{1–10}$ 35.4%. Haploids n = 3x = 21 plants obtained from genotypes $Sk_{1–10}$ – 22.3% and 17.8% from genotypes $Ba_{1–10}$.

Indukowana androgeneza w *Phleum pratense*

Z. BRODA, S. MIKOŁAJCZYK

Katedra Genetyki i Hodowli Roślin, Akademia Rolnicza im. A. Cieszkowskiego w Poznaniu

Streszczenie

Pylniki dwudziestu genotypów tymotki były wykorzystane do indukowania androgenezy i regeneracji roślin. Pochodziły one z odmiany Skaut: genotypy Sk_1 – Sk_{10} i z odmiany Bartovia: genotypy Ba_1 – Ba_{10}. Najwyższą wartość indukcji androgenezy zaobserwowano na pożywce Blayds'a z 2 mg l^{-1} kinetyny lub 2 mg l^{-1} BAP dla genotypu Sk_1 – 64%. Najlepszą efektywność androgenezy zaobserwowano, kiedy pylniki były traktowane promieniowaniem gamma w dawce 1 i 4 Gy. Najwyższy procent embriogenezy otrzymano w kombinacji traktowania wstępnego pylników temperaturą 4°C przez 4 dni i 4 Gy dla genotypu Sk_2 – 6,7%. Liczenie chromosomów u zregenerowanych roślin wykazało, że większość regenerantów była alloheksaploidami 2n = 6x = 42 – klony

$Sk_{1–10}$ – 36,9% i $Ba_{1–10}$ 35,4%. Haploidalnych roślin n = 3x = 21 otrzymanych z genotypów $Sk_{1–10}$ było 22,3% i 17,8% z genotypów $Ba_{1–10}$.

Recenzent – Reviewer: *Andrzej Joachimiak*

Adres do korespondencji – Address for correspondence:
Prof. dr hab. Zbigniew Broda
Katedra Genetyki i Hodowli Roślin, Akademia Rolnicza im. A. Cieszkowksiego w Poznaniu
ul. Wojska Polskiego 71C, 60-625 Poznań
e-mail: broda@au.poznan.pl