J. Appl. Genet. 40(3), 1999, pp. 165-174

The influence of various in vitro culture conditions on androgenetic embryo induction and plant regeneration from hexaploid triticale (× *Triticosecale* Wittm.)

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Abstract. The effect of sucrose and maltose in culture media PII, C_{17} and MN_6 on androgenetic embryo formation was investigated in seven F_2 triticale hybrid progenies. In all genotypes, the highest number of androgenetic embryos (89.8-320.6/100 anthers) was formed on medium C_{17} containing maltose. Also the highest number of green plants (6.17/100 embryos) was regenerated from embryos obtained on PII with sucrose. The effect of the physical environment (temperature, light) in the first week of embryo culture on the regeneration medium was tested. The highest rate of green plants per 100 embryos (2.5-11.8) was obtained from incubation at 22°C in the dark.

Key words: androgenetic embryos, incubation temperature, media, plant regeneration, triticale.

Introduction

In triticale (×*Triticosecale* Wittm.), as in other crops, the practical application of anther culture technique to breeding is still limited by the low frequency of haploid green plants produced from cultured anthers. The success rate depends on two different components: embryoid production from microspores and green plant regeneration from those embryoids (CHARMET, BERNARD 1984).

For many years, sucrose has been the primary sugar used in culture media. However, in recent studies on haploid production other sugars have been tested for their effect on androgenesis induction in triticale (GLAND-ZWERGER et al. 1994, KARSAI et al. 1994, KARSAI, BEDÖ 1997), wheat (OTANI, SHIMADA 1993,

Received: September 1998. Accepted: August 1999.
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NAVARRO-ALVAREZ et al. 1994), and barley (SCOTT et al. 1995), and differences between sucrose and maltose have been analysed. In the culture medium, sugar serves as a carbon source and as an osmotic regulator. The different results reported for the influence of maltose and sucrose on embryoid production have been explained as an effect of medium osmolality (ZHOU et al. 1991).

The aim of the present work was to compare the effect of maltose and sucrose in three induction media on androgenetic response and production of haploid plants from different genotypes in triticale. The effect of temperature and light intensity in the first week of embryo culture in the regeneration medium was also tested.

Material and methods

F_2 hybrid progenies of hexaploid triticale from seven cross combinations were used as anther donor plants: F_2-1 (Lasko × DED 1048/91), F_2-2 (Presto × Bogo), F_2-3 (Moreno × CHD 477/91), F_2-4 (Moreno × DED 52/90), F_2-5 (Vero × Bogo), F_2-6 (Nemo × Bogo), F_2-7 (Nemo × Dalo). The hybrids were obtained from the "Danko" Plant Breeding of Choryń, Poland. The plants were grown in the greenhouse. The spikes were cut at the uninuclear stage of microspore development. Before being plated on the medium, the spikes were kept at 4°C for 6-9 days in the mineral salt medium N_6 (CHU et al. 1975). After sterilization in 5% calcium hypochlorite, anthers at the mid-uninucleate stage were plated on induction media:

I: PII (CHUANG et al. 1978) + 0.5 mgL^{-1} 2.4-D;

II: PII (CHUANG et al. 1978) + 0.5 mgL^{-1} 2.4-D + 90 g/l maltose (instead of sucrose);

III: C_{17} (WANG, CHEN 1983);

IV: C_{17} (WANG, CHEN 1983) + 90 gL^{-1} maltose (instead of sucrose);

V: MN_6 (CHU, HILL 1988);

VI: MN_6 (CHU, HILL 1988) + 90 gL^{-1} maltose (instead of sucrose).

The anthers were incubated in darkness at 30°C. After 4 weeks, the embryos developed from microspores on media I, II, III V and VI were transferred to a regeneration medium 190-2 (ZHUANG, XU 1983) and illuminated for 16 h/day at 22°C.

The androgenetic embryos obtained on medium C_{17} with maltose were divided into three groups and during the first week of regeneration the cultures were kept at: (1) 22°C in 16/8 h light /dark; (2) 22°C in the dark; and (3) 4°C in the dark. After one week, the embryos were incubated at 22°C and 16/8 h light/dark. Green plantlets were transferred into pots.

The experiment was carried out in a complete randomized design with three replications. The number of androgenetic embryos per 100 anthers and the number of green and albino plants per 100 androgenetic embryos were recorded.

Statistical analysis

Two-factor analysis of variance was performed to study the effects of genotypes, induction media, and medium × genotype interaction on androgenetic response traits, as well as to estimate the effect of genotypes, physical conditions and genotype × physical condition interaction on plant regeneration rate. Since the embryos obtained on C_{17} medium with maltose were intended for the next step of the experiment concerning the influence of physical conditions on plant regeneration, only the data for five induction media (I, II, III, V, VI) were included in the statistical analysis concerning the effect of induction medium on plant regeneration. Before variance analyses the data (with the exception of the number of androgenetic embryos per 100 anthers) were transformed by the arc sin $\sqrt{x}$ to normalize the distribution. Comparison of means for media, genotypes and medium × genotype was done by means of Least Significant Difference, calculated separately for individual sources of variation.

Results

Embryogenic structures were observed after 20-30 days of culture. Mean frequencies of androgenetic embryos and plants regenerated from seven genotypes on three induction media containing either maltose or sucrose are given in Table 1. Analysis of variance showed significant influence of genotypes, induction media and genotype × medium interaction on androgenetic embryo production (Table 2).

For all the genotypes tested, the highest number of embryogenic structures were obtained on medium C_{17} containing maltose (89.8-320.6/100 anthers, mean over genotypes – 223.7; Table 3). On the original medium C_{17} with sucrose the frequency of embryogenic structures was markedly lower (45.1-152.4/100 anthers, mean – 90.5). Of the three media tested, the lowest embryo production was observed on medium MN_6. The MN_6 and PII media with maltose showed a lower embryo production than the respective media with sucrose (Table 3). Only for one genotype (F_2-3) the frequency of embryoids was somewhat higher on medium PII with maltose than on the original PII with sucrose. Androgenetic embryo production was found to be also dependent on genotypes. Means for particular genotypes (over media) showed that F_2-6 may be distinguished as the genotype with the highest embryo production (Table 3)

The frequency of green plants on the regeneration medium reflected the frequency of embryo induction. Analysis of variance showed that the effect of genotype was significant for green plant regeneration, and the effect of induction medium was significant for albino plants (Table 2). The number of green and albino plants per 100 androgenetic embryos obtained from seven genotypes on five induction media (PII + sucr., PII + malt., C17 + sucr., MN6 + sucr., MN6 + malt.) is presented in Tables 4 and 5. The number of green plants regenerated from

Table 1. Effect of media and genotypes on androgenetic embryo induction and plant regeneration

Geno-type	Medium	Anthers plated	Andro-genetic embryos	Green plants	Albino plants	Green/al-bino plants
F_2 - 1	PII+sucr.	601	1179	42	405	0.10
	PII+malt.	601	508	13	135	0.10
	C_{17}+sucr.	601	415	11	108	0.10
	C_{17}+malt.	601	1927	67	346	0.19
	MN_6+sucr.	601	251	4	102	0.04
	MN_6+malt.	601	62	0	2	0.00
F_2 - 2	PII+sucr.	859	523	27	190	0.14
	PII+malt.	859	562	20	156	0.13
	C_{17}+sucr.	859	1309	45	286	0.16
	C_{17}+malt.	859	2307	76	444	0.17
	MN_6+sucr.	859	510	20	77	0.26
	MN_6+malt.	859	151	13	32	0.41
F_2 - 3	PII+sucr.	669	237	8	65	0.12
	PII+malt.	669	395	9	50	0.18
	C_{17}+sucr.	669	302	7	2	3.50
	C_{17}+malt.	669	601	14	61	0.23
	MN_6+sucr.	669	257	1	30	0.03
	MN_6+malt.	669	141	1	9	0.11
F_2 - 4	PII+sucr.	648	460	21	34	0.62
	PII+malt.	648	364	8	94	0.09
	C_{17}+sucr.	648	489	3	87	0.03
	C_{17}+malt.	648	1153	30	134	0.22
	MN_6+sucr.	648	180	4	17	0.24
	MN_6+malt.	648	8	0	1	0.00
F_2 - 5	PII+sucr.	621	758	33	175	0.19
	PII+malt.	621	394	10	98	0.10
	C_{17}+sucr.	621	593	22	102	0.22
	C_{17}+malt.	621	1131	88	209	0.42
	MN_6+sucr.	621	662	24	98	0.25
	MN_6+malt.	621	263	17	28	0.61
F_2 - 6	PII+sucr.	648	1412	151	287	0.53
	PII+malt.	648	649	54	153	0.35
	C_{17}+sucr.	648	879	32	137	0.23
	C_{17}+malt.	648	1787	158	319	0.50
	MN_6+sucr.	648	868	62	134	0.46
	MN_6+malt.	648	213	16	14	1.14
F_2 - 7	PII+sucr.	504	347	13	93	0.14
	PII+malt.	504	382	0	106	0.00
	C_{17}+sucr.	504	306	5	44	0.11
	C_{17}+malt.	504	1264	33	181	0.18
	MN_6+sucr.	504	171	5	27	0.19
	MN_6+malt.	504	121	5	10	0.50

*Data for green and albino plants regenerated from androgenetic embryos obtained on induction medium C_{17}+malt. were pooled over various conditions applied during regeneration culture (see Material and methods and Table 6).

Table 2. Analysis of variance for effect of induction medium and genotype on androgenetic response traits in triticale

Source of variation	Androgenetic embryos/100 anthers		Green plants/100 embryos		Albino plants/100 embryos	
	d.f.	MS	d.f.	MS	d.f.	MS
Medium(M)	5	997.97**	4	0.03397	4	0.38921**
Genotype (G)	6	201.29**	6	0.06426**	6	0.09256
M×G interaction	30	49.02**	24	0.01015	24	0.07587
Error	82	17.96	68	0.01513	68	0.07365

** significant at P<0.01

Table 3. Androgenetic embryos per 100 anthers obtained from the studied triticale genotypes on various induction media

Genotype	Medium						Mean
	PII+sucr.	PII+malt.	C_{17}+sucr.	C_{17}+malt.	MN_6+sucr.	MN_6+malt.	
F_2 - 1	196.2c	84.5b	69.1ab	320.6d	41.8ab	10.3a	120.4d
F_2 - 2	60.9a	65.4a	152.4b	268.6c	59.4a	17.6a	104.0cd
F_2 - 3	35.4a	59.0a	45.1a	89.8a	38.4a	21.1a	48.21a
F_2 - 4	71.0b	56.2ab	75.5b	177.9c	27.8ab	1.2a	68.3ab
F_2 - 5	122.1bc	63.4ab	95.5ab	182.1c	106.6ab	42.4a	102.0cd
F_2 - 6	217.9c	100.2ab	135.6b	275.8c	134.0b	32.9a	149.4e
F_2 - 7	68.8a	75.8a	60.7a	250.8b	33.9a	24.0a	85.7bc
Mean	110.3c	72.1b	90.5bc	223.7d	63.1b	21.4a	

Means within a row followed by the same letter were not significantly different at P = 0.05

Table 4. Green triticale plants regenerated from 100 androgenetic embryos depending on genotype and induction medium

Genotype	Induction medium*					Mean
	PII+sucr.	PII+malt.	C_{17}+sucr.	MN6+sucr.	MN6+malt.	
F_2-1	3.60a	2.80b	2.70	1.37a	0.00a	2.09a
F_2-2	5.53b	4.27b	3.80b	4.47b	9.87d	5.59b
F_2-3	6.20b	2.37b	2.27ab	0.33a	0.40a	2.31a
F_2-4	5.30b	2.40b	0.43a	2.67ab	0.00a	2.16a
F_2-5	6.57b	3.23b	3.70b	3.47b	9.83d	5.36b
F_2-6	12.70c	11.70c	3.77b	7.03c	5.30b	8.10c
F_2-7	3.27a	0.00a	2.97b	2.7a	7.57c	3.17a
Mean	6.17a	3.82a	2.80a	3.06a	4.71a	

*C_{17}+ maltose induction medium was excluded (see Materials and methods)
Means within a column followed by the same letter were not significantly different at P = 0.05.

Table 5. Albino triticale plants regenerated from 100 androgenetic embryos depending on genotype and induction medium

Genotype	Induction medium*					Mean
	PII+sucr.	PII+malt.	C17+sucr.	MN6+sucr.	MN6+malt.	
F_2-1	36.8b	27.5b	26.0b	49.4b	0.00a	27.9a
F_2-2	35.2a	34.4a	22.4a	14.7a	19.2a	25.2a
F_2-3	34.2b	12.7ab	0.60a	11.7ab	23.5ab	16.5a
F_2-4	7.70ab	27.8b	21.4b	9.1ab	0.00a	13.2a
F_2-5	52.5a	27.6a	19.7a	13.7a	12.6a	25.2a
F_2-6	22.5a	31.1a	16.1a	16.2a	25.5a	22.3a
F_2-7	50.1b	31.3ab	14.8ab	8.5a	8.3a	22.6a
Mean	34.2b	27.5ab	17.3a	17.6a	12.7a	

Means within a row followed by the same letter were not significantly different at P = 0.05.
*C_{17}+ maltose induction medium was excluded (see Material and methods).

100 embryos was the highest for medium PII + sucr. (3.27-12.70 depending on genotype, on average 6.17), and the lowest for medium C_{17} + sucr. (0.43-3.80 depending on genotype, on average 2.80). For comparison, in the same regeneration conditions (22°C, 16/8 h light/dark) 2.2-5.5 green plants per 100 androgenetic embryos were regenerated. Among the studied genotypes from the embryos obtained on C_{17} + malt. F_2-6 was found to give the highest frequency of green plants (3.77-12.70/100 embryos depending on induction medium, on average 8.10). The frequency of albino plants was dependent mainly on induction medium (Table 2). From embryos obtained on PII + sucr. and PII + malt., 34.2 and 27.5 albino plants per 100 embryos were regenerated, respectively. From embryos developed on other induction media, less then 20 albino plants per 100 embryos were recorded (Table 5).

Table 6. Analysis of variance for effect of physical conditions and genotypes on plant regeneration in triticale

Source of variation	d.f.	Mean square	
		Green plants	Albino plants
Physical condition (P)	2	0.026*	0.012
Genotype (G)	6	0.035**	0.032*
P×G interaction	12	0.008	0.011
Error	40	0.009	0.015

* significant at P = 0.10
** significant at P = 0.01

The effect of embryo culture conditions on plant regeneration is presented in Table 7. Within the group of embryos obtained from the best induction medium (C_{17}), the frequency of green plants was influenced (but only at P = 0.1; Table 6) by temperature and light conditions during the first week of embryo culture on

Table 7. Efficiency of plant regeneration from androgenetic embryos obtained on medium C_{17} + malt. in different regeneration conditions

Geno-type	22°C, 16 h light				22°C dark				4°C dark			
	Androgenetic embryos No.	Green plants No.(%)	Albino plants No.(%)	Green/ albino plants	Androgenetic embryos No.	Green plants No.(%)	Albino plants No.(%)	Green/ albino plants	Androgenetic embryos No.	Green plants No.(%)	Albino plants No.(%)	Green/ albino plants
F_2 - 1	653	19(2.9)	110(16.8)	0.17	636	29(4.6)	127(20.0)	0.23	638	18(2.8)	99(15.5)	0.18
F_2 - 2	769	17(2.2)	100(13.0)	0.17	769	27(3.5)	120(15.6)	0.23	769	11(1.4)	94(12.2)	0.12
F_2 - 3	185	5(2.7)	32(17.3)	0.16	240	6(2.5)	15(6.5)	0.4	185	5(2.7)	14(7.6)	0.36
F_2 - 4	429	14(3.3)	44(10.3)	0.32	361	14(3.9)	51(14.1)	0.28	363	2(0.6)	39(10.7)	0.05
F_2 - 5	379	21(5.5)	61(16.1)	0.34	371	40(10.8)	74(19.9)	0.54	381	27(7.1)	74(19.4)	0.37
F_2 - 6	490	14(2.9)	73(14.9)	0.19	612	72(11.8)	117(19.1)	0.62	685	68(9.9)	129(18.8)	0.53
F_2 - 7	463	14(3.0)	70(15.1)	0.20	388	12(3.1)	63(16.2)	0.03	413	7(1.7)	46(11.1)	0.15

the regeneration medium. The highest percentage of green plants (2.5-11.8/100 androgenetic embryos, depending on genotype) was obtained from the culture at 22°C in the dark. The green to albino plants ratio was also the best in these conditions, but the number of albino plants was always higher than that of green ones. The percentage of green plants regenerated at 4°C in the dark varied from 0.6 to 9.9, whereas at 22°C in 16/8 h light/dark photoperiod the percentage varied from 2.2 to 5.5%.

Discussion

The present study clearly demonstrated the importance of the induction medium for androgenetic embryo formation. The selected media, PII, C_{17} and MN_6, were used previously in various modifications for wheat and triticale androgenesis. KARSAI et al. (1994) studied the effect of pH, gelling agent and maltose concentration in, medium MN_6 for triticale and wheat, and found that a higher maltose concentration significantly increased the percentage of embryogenic structures in triticale. MARTINEZ-GARCIA et al. (1992) used media PII and N_6 with sucrose for various genotypes of triticale and reported a better response for anther culture on PII. ŚLUSARKIEWICZ-JARZINA and PONITKA (1997) compared media PII and MN_6 with sucrose for other triticale genotypes and also reported a better response on medium PII. EL-MAKSOUD and BEDÖ (1993) tested medium PII with sucrose and medium MN_6 containing either sucrose or maltose for wheat and observed that the original medium PII was the best for embryoid induction and plant regeneration.

In this work, in all the genotypes studied, maltose increased the efficiency of embryo production in medium C_{17}, but not in medium PII and MN_6. The worst results were obtained for MN_6 with maltose. OTANI and SHIMADA (1993) investigated the effect of various sugars in medium C_{17} on pollen embryo formation and obtained similar results. The authors observed the greatest pollen embryo formation and green plant regeneration on the medium containing maltose. KARSAI et al. (1994) reported maltose-increased efficiency of triticale androgenesis on medium MN_6. However, EL-MAKSOUD and BEDÖ (1993) replaced sucrose by maltose and observed a lower wheat anther culture response in MN_6. Genetic factors are also major determinants in the ability to regenerate green plants in triticale (CHARMET, BERNARD 1984, MATVEENKO et al. 1994). The results presented in this paper suggest that medium × genotype interaction can also have a considerable effect on pollen embryo formation in triticale anther culture.

The physical environment (temperature, light intensity and photoperiod) can also affect embryo and plantlet induction frequencies. Up to now, only cold pretreatment prior to culture, or change of temperature during anther culture in order to increase embryo production were analysed (MCGREGOR, MCHUGHEN 1990). The influence of light on green/albino plants ratio was also investigated

(BERNARD 1980). ZIEGLER et al. (1990) reported that in wheat significantly more green plants were produced when light was absent during the differentiation process, than under low light conditions. In our study, the number of green plants was higher when the first week of incubation of the androgenetic embryos in the regeneration medium took place in the dark.

In conclusion, the results of our study suggest that the number of androgenetic embryos and regenerated green plants in triticale can be increased by using the induction medium C_{17} containing maltose instead of sucrose and by applying the appropriate physical conditions (22°C in the dark) during the first week of culture on the regeneration medium.

Acknowledgements: The authors wish to thank Jan BOCIANOWSKI, M. Sc. (Institute of Plant Genetics, Polish Academy of Sciences, Poznań, Poland) for his help in statistical calculations.

REFERENCES

BERNARD S. (1980). *In vitro* androgenesis in hexaploid triticale: determination of physical conditions increasing embryoid and green plant production. Z. Pflanzenzüchtg. 85: 308-321.

CHARMET G., BERNARD S. (1984). Diallel analysis of androgenetic plant production in hexaploid triticale (× *Triticosecale* Wittmack). Theor. Appl. Genet. 69: 55-61.

HU C.C., HILL R.D. (1988). An improved anther culture method for obtaining a higher frequency of pollen embryoids in *Triticum aestivum* L. Plant Sci. 55: 175-181.

CHU C.C., WANG C.C., SUN C.S., HSU C., YIN K.C., CHU C.Y., BI F.Y. (1975). Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci. Sin. 18: 659-668.

CHUANG C.C., OUYANG J., CHIA H., CHOU S.M., CHING C.K. (1978). A set of potato media for wheat anther culture. In: Proc. China-Australia Plant Tissue Culture Symp., Peking 1978: 51-66.

EL-MAKSOUD M.M.A., BEDÖ I. (1993). Genotypes and genotype × medium interactive effects on androgenic haploid production in wheat (*Triticum aestivum* L.). Cereale Res. Commun. 21(1): 17-24.

GLAND-ZWERGER A., KREFT I. (1994). Production of doubled haploid lines through anther culture in barley and triticale. In: Proc. International Colloquium on Impact of Plant Biotechnology on Agriculture (Javornik B., Bohanec B., eds.), Rogla, Slovenia, December 5-7, 1994: 9-14.

KARSAI I., BEDÖ Z., HAYES P.M. (1994). Effect of induction medium, pH and maltose concentration on *in vitro* androgenesis of hexaploid winter triticale and wheat. Plant Cell Tiss. Organ Cult. 39: 49-53.

KARSAI I., BEDÖ Z. (1997). Effect of carbohydrate content on the embryoid and plant production in triticale anther culture. Cereale Res. Commun. 25(2): 109-116.

MARTINEZ GARCIA C., MARTIN SANCHES J.A., SIN CASAS E. (1992). Plant regeneration from anther culture in six hexaploid triticale varieties and their F_1-hybrids. In: Livre

des Résumé de Posters. Book of Poster Abstracts. XIIIth EUCARPIA Congress, July 6-11, 1992, Angers-France: 189-190.

MATVEENKO S.N., KHOTYLEVA L.V., KAMINSKAYA L.N. (1994). Genetic analysis of the effectiveness of triticale anther culture *in vitro*. Genetika 30(9): 1238-1242.

MCGREGOR L. J., MCHUGHEN A. (1990). The influence of various cultural factors on anther culture of four cultivars of spring wheat (*Triticum aestivum* L.) Can. J. Plant Sci. 70: 183-191.

NAVARRO-AVAREZ W., BAENZIGER P.S., ESKRIDGE K.M., SHELTON D.R., GUSTAFSON V.D., HUGO M. (1994). Effect of sugars in wheat anther culture media. Plant Breeding 112: 53-62.

OTANI M., SHIMADA T. (1993). High frequency of pollen embryo formation in *Triticum aestivum* on maltose containing medium. Cer. Res. Commun. 21(1): 11-15.

SCOTT P., LYNE R.L., AP REES T. (1995). Metabolism of maltose and sucrose by microspores isolated from barley (*Hordeum vulgare* L.). Planta 197: 435-441.

ŚLUSARKIEWICZ-JARZINA A., PONITKA A. (1997). Effect of genotype and media composition on embryoid induction and plant regeneration from anther culture in triticale. J. Appl. Genet. 38: 253-258.

WANG P., CHEN Y. (1983). Preliminary study on production of high of pollen H_2 generation in winter wheat grown in the field. Acta Agron. Sin. 9: 283-284.

ZHUANG J.J., XU J. (1983). Increasing differentiation frequencies in wheat pollen callus. In: Cell and Tissue Culture Techniques for Cereal Crop Improvement, Science Press. Beijing: 431.

ZHOU H., ZHENG Y., KONZAK C. (1991). Osmotic potential of media affecting green plant percentage in wheat anther culture. Plant Cell Rep. 10: 63-66.

ZIEGLER G., DRESSLER K., HESS D. (1990). Investigations on the anther culturability of four German spring wheat cultivars and the influence of light on regeneration of green vs. albino plants. Plant Breeding 105: 40-46.