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Increased Induction and Chromosome Doubling of Androgenetic Haploid Rye

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Summary. Further progress of studies aimed at increasing production of androgenetic *Secale cereale* plants via the culture of anthers is described. Two culture media initially developed for rice and wheat anther culture have been shown to have pronounced influence on rye. It has been possible to increase the average percentages of responsive anthers (i.e. those producing embryoids or calluses) from 0.26 % to 10 % with a maximum in certain experiments of over 40 %. Of nearly 400 plants produced in 1976, 1/4 are green and can be grown further by transfer to potting compost; 3/4 are albino. Stable green haploid lines were present amongst the plants, and after vegetative propagation of the lines representative samples have been treated with colchicine resulting in diploid, triploid and tetraploid plants. The influence of the genetic background of the donor plants on the success rate of anther culture and on the percentage of albino formation is discussed.

Key words: Anther Culture - Rye - Haploids - Plant Breeding

Introduction

In previous papers (Thomas and Wenzel 1975; Thomas et al. 1975) we reported upon the production of embryoids and calluses from microspores cultured within anthers of *Secale cereale*. Although these results were reproducible throughout the year, we were faced with several problems. Firstly, the number of anthers which gave rise to macroscopic calluses or embryoids never reached 1 %. Secondly, although some haploid albino plants were obtained, the first green plants were shown by genetical analysis of the second selfed anther derived generation (A_2) not to be homozygous, a fact which led to the proposal that these first seven green lines originated from aberrant, possibly unreduced, microspores (Wenzel et al. 1976). In this paper we report further progress in increasing the percentage of anthers capable of undergoing androgenesis and in establishing stable green haploid and probably homozygous diploid lines.

Materials

The experimental material used consisted of the tetraploid variety 'Tero' (v. Lochow Petkus, Bergen Germany), a self-fertile diploid inbred line (obtained from H. Kuckuck, Hannover) and 10 diploid F_1 hybrids. These hybrids included mutants (of descendants of the cross *S. cereale* × *S. vavilovii;* see Kuckuck 1976) crossed with commercial varieties. The particular hybrids, lines and varieties used in the described experimental series are listed in Table 2.

Plant material was grown throughout the year in a temperature-controlled greenhouse, maintained at 20 to 25° C and supplemented in winter with artificial illumination. The distance of the lights from the top

Table 1. Induction medium for microspore development of *Secale cereale* within the anther (modified N6 medium; Hu, pers. commun.; Yin et al. 1976)

component	mg/l	component	mg/l
KNO_3	2,830	glycine	2.0
KH_2PO_4	400	thiamine HCl	1.0
$(NH_4)_2SO_4$	463	pyridoxine HCl	0.5
$CaCl_2 \cdot 2H_2O$	166	nicotinic acid	0.5
$MgSO_4 \cdot 7H_2O$	185	glutathione	30.0
NaFeEDTA	10^{-4} M	ascorbic acid	10.0
$MnSO_4 \cdot 4H_2O$	4.4	glucose	50,000.0
$ZnSO_4 \cdot 7H_2O$	1.5	2,4-D	2.0
H_3BO_3	1.6	kinetin	0.5
KJ	0.8		
active carbon	2,000.0	pH	5.8

Table 2. Percentages of development of macroscopic structures in best single experiments according to the genome, the medium and the time of cold pretreatment

No.	F_1 hybrid	days of cold pretreatment		medium A: modified N6			medium B: potato extract		
				number of		%	number of		%
		med.A	med.B	anthers plated	developing structures		anthers plated	developing structures	
1	Mut 570×'Kustro'	9	6	600	9	1.5	396	38	9.6
2	Mut 543×M I	10	10	432	26	6.0	120	6	5.0
3	St. 333×M II	8	7	300	23	7.7	156	17	10.9
4	(*S. vavilovii* × *S. cereale*) ×'Perolo'	9	7	216	19	8.8	336	48	14.3
5	Mut 543×'Kustro'	11	11	192	19	9.9	192	16	8.3
6	Mut 504×M II	9	7	228	22	9.6	180	28	15.6
7	Mut 511×M II	7	7	276	30	10.9	228	44	19.3
8	Mut 530×M II	6	5	648	91	14.0	300	92	30.7
9	Mut 543×M II	10	8	228	39	17.1	168	66	39.3
10	Mut 530×M I	7	7	192	69	35.9	156	68	43.6

Mut = Mutants arisen from F_{10} *S. cereale* × *S. vavilovii;* M I = Leningrad rye; M II = Bulgarian short stalk mutant (all kindly provided by H. Kuckuck; the varieties were obtained by v. Lochow Petkus)

of the plants was critical for the formation of good flowering spikes. This was kept at a minimum of 1 m. During artificial illumination, the CO_2 content of the air was increased tenfold. The vitality of the microspores was carefully monitored by fertilization and pollen tube growth tests, according to the method described by Pfahler (1965). Nevertheless, in some rye types, the production of pollen grains of larger circumference (possibly indicative of their unreduced nature; Wenzel et al. 1976), was increased in comparison to field grown plants.

Methods and Results

Cold Pretreatment

It was found that by subjecting anthers to a cold treatment before plating onto agar it was possible to increase the number of anthers undergoing microspore development. This finding is in agreement with that of Nitsch and Norreel (1973) for *Nicotiana* and *Datura* and of Picard and de Buyser (1975) for *Triticum*. In order to harvest axenic anthers, the greenhouse grown spikes were dissected and precultured under sterile conditions (Thomas et al. 1975) in the liquid medium given in Table 1. This preculture medium lacked hormones and active carbon (culture conditions: 26°C with a 12 h light circle of 7,000 lux). Once the microspores in the anthers of the central florets had reached the stage of the first mitosis, the spikes were transferred to a dark refrigerator and maintained at 6°C. The length of the cold treatment required for maximum anther response depended upon the genotype of the donor plant. Although we found that an average of 6 to 10 days was optimal (Table 2), it was possible to induce embryogenesis or callus formation from microspores even after 30 days of cold pretreatment. In the first experimental series using this cold pretreatment routinely, the average rate of macroscopic structure development could be increased from 0.26 % (from 20,000 anthers plated) to 0.50 % (from 28,000). We believe there are three possible causes for the effect of cold treatment: 1.) As a result of cold storage, weak or non-viable anthers and microspores are killed leaving only vigorous material. We ourselves exert a positive selective pressure for such viable material by plating out only material which, as demonstrated by acetocarmine staining of representative samples, is highly active. 2.) At 6°C increased numbers of microspores are arrested at or during the first microspore mitosis which gives an increased possibility of development on subsequent culture. 3.) Cold treatment may provide a non-specific shock resulting in the establishment of endogenous cellular conditions which permit development in the desired manner.

Induction of Microspore Development

In the present series of experiments, the medium given in Table 1 was tested. This was modified from

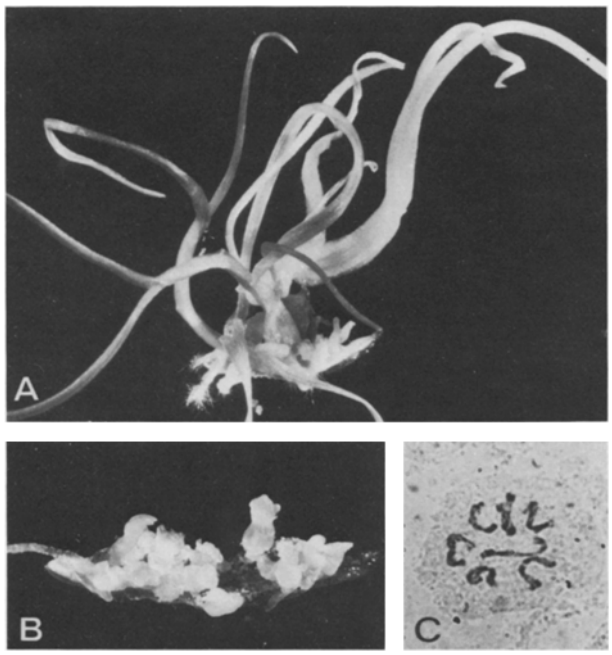

Fig. 1. A. 8 green and 1 albino plantlets developing from the *Secale cereale* anther shown in B, together with 22 further macroscopic structures; C. haploid chromosome number (2n = 7) in root tips of a green androgenetic rye plant growing in potting compost (they descend from the particular cross Mut 530 × M II)

the N6 medium which was developed for rice anther culture by the Institute of Botany, Peking (Hu, pers. commun.; Yin et al. 1976). In the presence of active carbon (0.1 to 0.5 %; Anagnostakis 1974), ascorbic acid (10 mg/l) and glutathione (30 mg/l), it was possible to increase the average induction rate (i.e. the average of all hybrids used and receiving 5 to 10 days of cold pretreatment) from 0.5 to 3 % with a maximum (most successful hybrid at optimal pretreatment) of 30 % (Table 2). Especially the tripeptide glutathione (γ-glutamyl-cysteyl-glycine), which has an unspecific effect on many detoxification processes as well as some regulatory functions (Meister 1975) was very effective. Since it was autoclaved before use, experiments with filtersterilized glutathione are now in progress. The number of structures developing per anther also increased compared with the Nitsch and Nitsch medium (1969) used in early experiments (Wenzel and Thomas 1974). Further, the relationship of embryoids to calluses was pushed to the embryoid side.

By changing to the Chinese potato extract medium, originally composed by the 301 Research Group at the Institute of Genetics in Peking for wheat anther culture (Anonymous 1976), a further increase could be achieved. This medium simply consists per litre of 90 g sucrose, 10^{-4}M NaFeEDTA, together with the liquid extract of 200 g potatoes. The fresh potatoes (unpeeled, but the eyes removed) were cut into small cubes, boiled for 30 min in 400 ml of water, squeezed in this water and then from the macerate the liquid is separated by filtering through a Büchner funnel. The beneficial influence of the extract could be further increased by boiling the potatoes in the sugar solution and pressing the macerate with a hydraulic press. Replacement of the sucrose by glucose was also beneficial in some rye types. The phytohormone concentration used was not critical. Good results could be obtained by addition of 1.75 mg/l 2,4-D and 0.5 mg/l kinetin. 2,4-D concentrations above 5 mg/l decreased the response. The pH was normally 6.2 and required no further adjustment. Under the influence of this medium the average anther response reached 10 % with a maximum for certain experiments of over 40 % (Table 2). Optimally up to 30 structures per anther were formed (Fig. 1, A and B), especially when potatoes 2 to 3 months after harvesting were used for preparing the extract. It turned out, however, that there is a strong influence of the potato variety used: A comparison between extracts prepared from the different varieties 'Culpa' and 'Secunda' (Ragis, Brockhöfe, Germany) showed that 1,075 anthers of 11,376 (9.5 %) responded on media with 'Culpa' extract, while on 'Secunda' media only 3.9 % (364 from 9,456) showed androgenesis. In a parallel experiment with the defined salt medium, 7.3 % of the plated anthers became androgenetic. This means that only after screening the potato quality, will the undefined potato medium be definitively better than the defined salt medium. Furthermore, the quality of the potatoes depends on the storage time; therefore frozen extracts, prepared at the optimal storage time (2 to 3 months after harvesting) should be used. It has already been shown that deep-freezing is not harmful in relation to microspore response. In further test experiments extracts such as malt, corn meal, tomato fruit, rice (purchased from DIFCO, Detroit, USA) were inactive on the cultured rye anthers. Commercial

Table 3. Androgenetic lines produced in 1976 from different *Secale cereale* types

anther donor plant	lines in vitro			ploidy level							green clones[b] growing in potting compost
				checked	1×		1×+2×[a]	2×		4×[a]	
	Σ	green	albino		green	alb.	green	green	alb.	green	
F_1 No. 1[c]	8	1	7	2	-	-	-	-	2	-	-
F_1 No. 2	11	0	11	3	-	2	-	-	1	-	-
F_1 No. 3	20	1	19	4	-	2	1	-	1	-	1
F_1 No. 4	31	18	13	8	2	-	-	4	2	-	10
F_1 No. 5	10	5	5	4	1	-	-	-	3	-	1
F_1 No. 6	9	0	9	-	-	-	-	-	-	-	-
F_1 No. 7	15	0	15	2	-	2	-	-	-	-	-
F_1 No. 8	182	64	118	70	7	15	3	12	33	-	35
F_1 No. 9	100	5	95	4	-	-	-	-	4	-	-
F_{10} *S. cereale* × *S. vavilovii*	1	1	0	1	-	-	1	-	-	-	1
4×variety 'Tero'	3	2	1	2	-	-	-	1	-	1	-
Σ	390	97	293	100	10	21	5	17	46	1	48
					31			63			

[a] according to the method of estimating the ploidy level for albinos by nucleoli counts, these groups are counted as diploids
[b] each green clone represents an average number of 50 plants
[c] the number corresponds to Table 2

potato extract preparations (DIFCO) used in equivalent concentrations caused only a 3 % anther response in comparison to 10 % with freshly prepared medium, possibly due to the potato variety from which they were prepared. Currently, extracts from effective potatoes are being fractionated in an attempt to identify the active compounds.

As active carbon was detrimental in combination with the potato medium, the agar is boiled now with charcoal and used for medium preparation after removal of the charcoal by passing through a hot Büchner funnel.

Regeneration of Calluses and Plantlets

When embryoids, formed from microspores within anthers, break out from the anthers and come into direct contact with the culture medium, the presence of 2,4-D and the high sucrose level tend to induce callus formation or even death of many of the structures. To prevent this, transference to a second culture medium is necessary. An early transfer after 4 weeks, i.e. before the embryoids become visible, could not, however, prevent callus formation, although most calluses seem to descend from originally preformed embryoids. Routinely macroscopic structures formed after 6 to 8 weeks incubation in the dark are removed from the anthers and transferred to 1.6 cm 24-holed multidishes (Costar, Cambridge, USA) in which each culture hole contains 0.75 ml modified N6 medium with 20 g/l glucose, or potato extract medium with 2 % sucrose, both lacking growth hormones. The dishes are then incubated under light of 3,000 lux (16 h/day). Under these conditions approximately 65 % of the macroscopic structures survive (half of them stay as embryoids). 20 % of the surviving embryoids produced plantlets capable of further growth, whereas only 3 % of the surviving calluses formed plantlets spontaneously.

Table 3 summarises the number of plantlets produced from microspores of 9 F_1 hybrids and two further anther donor types plated during 1976. For all types except 'Tero', approximately 30,000 anthers were plated (for 'Tero' the number plated was 9,000). As it can be seen albinism is one of the most striking

Fig.2. Phenotype of 2 clones (right and left) of androgenetic rye descended from different embryoids of the same F_1 hybrid (*S. vavilovii* × *S. cereale*) × *S. cereale* var. 'Perolo', confirming meiotic segregation (in the centre plants of the anther donor type of corresponding age)

problems in *Secale* microspore derived plantlets. About 75 % are chlorophyll deficient. From the plantlets so far produced it can be seen that some hybrids produce higher rates of green plants (F_1 Nos.4 and 5), and others, predominantly albino ones (F_1 No.9). It may be that in certain situations albinism is correlated with the genotype of the anther donor material, in which case albinism would be genetically based. Such a strong influence of the genome is also demonstrated in the appearance of quite a high percentage (up to 5 %) of albinos segregating in sexually produced F_2 generations from anther donor F_1 hybrids. However, in two instances from the same callus, green and albino plantlets arose, either documenting chimaerism or demonstrating the influence of physiological factors. The influence of the genome is further demonstrated by different percentages of microspore plantlets produced by different hybrids (Tables 2 and 3). The hybrids yielding large numbers of macroscopic structures and later of microspore plantlets do possess a common pollinator - the Bulgarian short stalk mutant M II (obtained by H. Kuckuck). Most of the plantlets so far obtained, including green haploids (2n = 7; Fig. 1C) capable of growth in potting compost, were derived predominantly from parental stocks containing this M II genome. This corresponds with the results of conventional breeding work with these stocks. Lines M I, M II and Mut 530 are also superior parents in normal crossing programms (Kuckuck, pers. commun.). Figure 1 shows a cultured anther of such a hybrid which has produced 8 green, 1 albino plantlets and 22 calluses.

Apart from 10 haploid green lines so far found, four plants were detected where during propagation haploid and later diploid plants arose. Either the macroscopic structures these plants originated from were already composed of mixed material coming from different microspores, or spontaneous doubling has occurred. Within the albino plantlets such mixed populations were not detected, since no root tip chromosomes but rather leaf nucleoli were counted. This method allows only a quick screening into haploids or diploids (see review Nitzsche and Wenzel 1977).

Plants cloned from the haploid green plants so far obtained have been diploidized by treating the roots of the young plantlets for 5 h with 0.5 % aqueous colchicine as described by Jensen (1976) for barley haploids. The survival rate of the anther-derived plantlets was about 50 %. Nearly 20 % of the surviving plants showed an altered chromosome number, yielding diploid, triploid and tetraploid clones. In the first diploidized offspring, a high rate of complete sterility appeared, possibly due to chromosome aberrations.

Since the F_1 hybrid anther donor plants are heterozygous for self-fertility, a certain number (theoretically 50 %) of the diploid plants carried the recessive allele and were self-sterile, documenting their meiotic origin. From these, however, seeds could be obtained by cross-pollination. The presence of the self-fertile plants makes a quick propagation via selfing possible. Figure 2 shows the variability of diploid A_1 plants originating from different embryoids of the same F_1 hybrid, in this particular case from the hybrid (*S. vavilovii* × *S. cereale*) × *S. cereale* var. 'Perolo'. Their different phenotype demonstrates meiotic segregation and their microspore offspring. Such material should allow a very effective selection, for instance for alkylresorcinol-poor lines (Hoffmann and Wenzel 1977), immediately delivering material of economic importance.

Discussion

It has been possible to produce viable green haploid plants from microspores of rye by closely monitoring the preculture of the anther donor plants, by

altering the media used for the culture of anthers and by screening different genotypes. Since the plant material varies rather widely, not only during different seasons, but also under quite controlled conditions (due to the outbreeding character of rye), not too small numbers of anthers must be plated to ensure significant results at each stage in macroscopic structure production. Furthermore, to determine the optimum conditions for plantlet production from the macroscopic structures again requires the production of large numbers of the structures. The ability to handle high numbers is, consequently, one important prerequisite for confirmation of the conditions leading to haploid plant formation via anther culture. Using rye, one can now concentrate on specific conditions for increasing the number of different lines for testing whether such plants can make a useful contribution to applied plant breeding.

The genome of the parents used for production of the F_1 hybrid anther donor plants appears to be very important in determining the subsequent success rate of anther culture. An increase of up to 500 % in microspore development could be observed in hybrids possessing the pollinator M II as one of the parents in hybrid production. As there are other hybrids of high commercial value but yielding only a few embryoids, it might be necessary first to make a second cross with a father inheriting a good regeneration capacity, and then to start anther culture followed by an extended screening for desired genotypes.

Although we still face numerous problems such as albinism, it has now been demonstrated that green haploid plant production is possible from microspores of rye. It remains to be shown that the homodiploids derived from them are advantageous for applied breeding programms. The recent results obtained with androgenetic homodiploid rice lines in applied breeding (Yin et al. 1976) and also those of Reinbergs et al. (1976) with barley clearly demonstrate that homozygous lines produced by unconventional methods in very short time periods deliver material which is at least comparable to that produced by conventional breeding techniques.

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