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Anther Culture as a Breeding Tool in Rape

II. Progeny Analyses of Androgenetic Lines and Induced Mutants from Haploid Cultures

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Summary. Progeny analysis of androgenetic plants from inbred rape-seed (*Brassica napus*) shows that selective growth of microspores can occur in cultured anthers. The property of privileged growth in culture seems to be linked to such characters as flowering time and seed glucosinolate content which can be analyzed in regenerated plants. This type of selection and the fact that more variability is visible in regenerants from different microspores than in the progeny of the highly inbred anther donor line, demonstrates the higher degree of homozygosity in the doubled amphihaploids of *B. napus*. Furthermore, it is shown that haploid genomes of rape may be mutable. Thus it is possible to obtain several different homozygous lines from a single microspore. A system of haploid embryoids arising from single cells of the primary microspore regenerant has also been used to produce experimentally induced mutants. It is demonstrated that recessive mutations can be obtained in a homozygous state in doubled haploid regenerants from mutagenized haploid single cells.

Key words: Anther culture – *Brassica napus* – Mutagenesis – Rape-seed

Introduction

Rape-seed (*Brassica napus*) is the most productive oil plant of the northern temperate zone. Its economic value could be further increased if high yielding oil- and forage-types could be developed which have low contents of toxic glucosinolates. This would make possible the extensive use of the high quality seed protein and of the green fodder. Furthermore, *Brassica napus* is one of the very few important crop plants which can be successfully established in tissue culture and probably one of the first examples of crop improvement by the combination of classical plant breeding methods and modern in vitro-techniques (Hoffmann 1980).

Many of these in vitro-techniques have recently been described. The production of haploid plants of androgenetic origin has been reported by several authors (Thomas and Wenzel 1975; Wenzel et al. 1977; Keller and Armstrong 1977, 1978; Hansson 1978; Renard and Dosba 1980; Lichter 1981). Regeneration in stem explants has been shown by Kartha et al. (1974) in diploid tissue and by Stringham (1977) in haploids. Furthermore, stem embryogenesis has been induced from somatic embryoids (Thomas et al. 1976) and this system of mass regeneration of single tissue cells has been utilized in mutagenesis (Hoffmann 1978) and resistance selection (Sacristán and Hoffmann 1979). Plant production from callus cultures of different origin and from isolated protoplasts has also been obtained for both ploidy levels (Kartha et al. 1974b; Thomas et al. 1976; Stringham 1979; Sacristán 1981). An improved protoplast method has been described by Kohlenbach et al. (1982). *Brassica napus* protoplasts have been used in fusion experiments for the successful creation of hybrid cells (Kartha et al. 1974a; Hoffmann et al. 1980).

In this paper we report the results from several field tests with androgenetic doubled haploids, including the analysis of biochemical quality characters, and the production of homozygous mutant populations from mutagenized haploid tissue cultures.

* Part of the material analyzed in this work was produced under the guidance of Emrys Thomas when he was one of the project leaders of the "Projektgruppen Haploide in der Pflanzenzüchtung" at the Max-Planck-Institut für Pflanzengenetik in Ladenburg. His untimely death at the age of 36 (May 23, 1981), which overtook him at his new place of work at Harpenden, prevented him seeing the completion of this paper. It is dedicated to the memory of this distinguished and restless researcher and highly-valued colleague

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Materials and Methods

The anther donor material of *Brassica napus,* the anther culture technique and plant production from androgenetic embryoids have been described previously (Wenzel et al. 1977). Only the method of diploidization was altered. Instead of applying colchicine with small cotton wool plugs to debuded axils of leaves, the colchicine (0.2% aqueous solution) was injected into the buds with a 0.45 mm needle. The volume depended on the receptivity of the tissue. This method is very simple and efficient: in an experiment with 21 stable haploid plants, e.g., a haploid genotype which did not show spontaneous chromosome doubling for many years, we dispensed 306 injections and eventually obtained 31 sprouts with seeds. The average success rate was probably higher but was not regularily monitored. In one case chromosome doubling could not be induced at all.

Field tests have been carried out from 1977 to 1980 at Ladenburg. Selfing and crossing were performed either in isolation chambers or by bagging the flowers. The material was precultured in Jiffy strips and transplanted in early April. The test plots had a size of 150×150 cm. The distance between the plants was 30 cm, which resulted in 16 plants per plot.

Photometrical analysis of the total glucosinolate content was carried out after Lein (1972a). Determination of the content of single glucosinolates was performed using the gas chromatography method of Thies (1977). The fatty acid composition of some samples was measured according to the method of Thies (1971). Usually, the appearance of erucic acid was only controlled by thin layer chromatography. The extraction of fatty acids was performed as described by Thies (1971) and samples were run on paraffin impregnated cellulose plates (Merck 5716) in acetic acid. Staining of the fatty acids was carried out in an iodine vapor.

Photometrical estimation of myrosinase (thioglucoside glucohydrolase, E.C. 3.2.3.1.) activity was performed as described by Lein (1972), and analysis of the multiple forms of the enzyme was carried out according to the method of Davis (1964) using a disc acrylamide gel system. The separation system used was No 6 of Maurer (1968) and the electrode buffer was borate-NaOH at pH 8.3 Staining of myrosinase isozymes was performed as described by MacGibbon and Allison (1970) with sinigrin. Additionally, we used glucoiberin in our experiments. As an early screening, a quick and simple test was carried out with callus or tissue pieces from stem embryo cultures. Homogenates from about 100 mg of material in 1 ml M/15 phosphate buffer, pH 7, were centrifuged and mixed with 1 mg ascorbic acid and 5 mg glucotropaeolin. The presence of myrosinase was indicated by an increase in turbidity caused by the precipitation of benzylisothiocyanate.

For the induction of mutants, stem embryogenic tissue of stable haploid lines (ca. 2 cm^2 expansion) was mutagenized. The treatment was such that, on average, out of 10 pieces mutagenized only one plantlet regenerated. For the X-irradiation we used the Philips Radifluor 120. The dose was 120 Gy at 120 kV and 5 mA^1. EMS (ethyl methanesulfonate) was applied as a suspension of 1 g per 100 ml (1 h) and ethidiumbromide as a 2.5 mg per 100 ml solution (3 hs). To avoid chimerism only secondary embryoids derived from surviving cells were isolated, propagated and used for seed production.

1 The instrument is not well suited for this load because the cooling system is not efficient enough for the long irradiation time which is necessary to obtain the wanted dose

Results

In the experimental series reported here a total of 66 plants were grown from 78 androgenetic macroscopic structures. From these, 59 plants were analyzed in the field: 45 were descended from "homozygous" anther donor lines (inbred line 24/72 and the commercial varieties 'Tower' and 'Egra') and 14 from F_1 hybrids. Later on, by carefully monitoring all culture parameters and the genetic background of the donor material, the induction rate of the embryoids could be steadily increased (Fig. 5; see also Lichter 1981) in new experimental series. These data will be published elsewhere.

Derivatives from Homozygous Anther Donor Plants

After selfing, 36 A_1 plants produced homozygous progeny (referred to as the A_2 generation); the offspring of the other 9 plants produced heterozygous A_2 populations. Most of these heterozygous progenies were rather vigorous and parental phenotypes were included. The variability amongst the lines was, however, much more distinct than the variability in progenies of the selfed anther donor lines, which shows that these plants were not of somatic origin. Twenty-eight of the 36 non-segregating lines showed a similar phenotype as the parent, while 8 revealed growth depression and partial sterility. Amongst the latter heterozygosity could not be completely excluded but vigorous plants were missing (in contrary to the 9 heterozygous populations). Between the 28 vigorous lines clear differences concerning leaf shape (Wenzel et al. 1977) and colour, flower type, pod shape and size were detected. These variations were much more distinct than differences within the progeny of selfed anther donor material, demonstrating quite a high amount of residual heterozygosity in classical inbred material. All 28 androgenetic homozygotes flowered, on average, 1 week later than the parental plants (Fig. 1), which indicated that during microspore culture selection took place.

Derivatives from F_1 Anther Donor Plants

The offspring of the self-fertilized anther donor plants (F_2) showed strong segregation. From 14 microspore-derived plants only 7 gave a homogeneous progeny (A_2) while another 7 produced a heterogeneous A_2. Of the former lines, 4 showed growth depression and poor seed set while the other 3 revealed vigorous growth. Two of these three were comparable with the donor F_1, and the remaining one was even superior. Amongst 200 plants of the segregating donor F_2 no plant

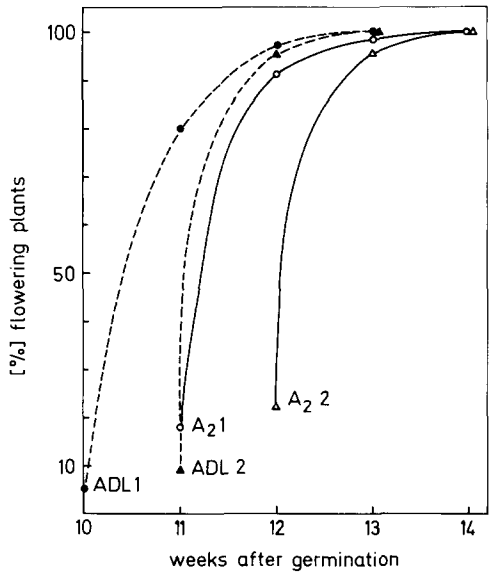

Fig. 1. Percentage of flowering plants between 10 and 14 weeks after germination: ADL 1 = anther donor line 1 (homozygous line 24/72), ADL 2 = anther donor line 2 (commercial varieties Tower and Egra), A_2 1 and A_2 2 = corresponding androgenetic progenies. The progenies (A_2) of the microspore-derived plantlets (A_1) flowered on average 1 week later than the parent (ADL). The same result was obtained in the A_3

similar to the latter androgenetic rape was present with respect to yield and such phenotypic characters as size and shape.

Glucosinolate and Fatty Acid Content

The corn quality of the homozygous donor material was already very good, i.e., nearly free of glucosinolates and erucic acid. Most of the androgenetic lines had a much higher glucosinolate content than the starting material. These lines had doubled up spontaneously during such early stages in culture that only shoots with fertile flowers were formed. The remaining lines proved to have a glucosinolate content as low as the parent and, surprisingly, all of these developed either predominantly or exclusively haploid shoots via stem embryogenesis (Fig. 2). Here again the genotypes of the microspores possessed a different regeneration capacity, which was probably coupled with some physiological property in a unexpected selection of a regeneratable microspore population. Although a strong selection for an increased glucosinolate content took place in the genotypes which showed an early spontaneous diploidization, the fatty acid quality remained stable in all cases. This is worth mentioning because the appropriate genes have been found to be closely linked (Röbbelen 1975). Table 1 compares typical analytical data from the inbred anther donor line 24/72 and two androgenetic derivatives of the different types described.

Using F_1 material for anther culture a glucosinolate selection could not be examined, probably because of the small number of evaluable seed samples. Nevertheless, the above mentioned superior line must have been doubled with colchicine and showed a reduced glucosinolate content (72 µM; donor F_2 151 µM).

Myrosinase Content

Myrosinase, an enzyme contained in *Brassica* plants, hydrolyzes mustard oil glucosides, thus generating toxic products. No plant could be detected with a reduced activity of seed myrosinases, neither did the activity increase when selection for high glucosinolate content occurred. Staining with sinigrin or glucoiberin resulted in 5 and 6 different bands, respectively, of multiple forms of the enzyme and the pattern was the same in all plants. No differences were observed even when anther donor plant seeds, roots and leaves with samples from the corresponding androgenetic plants were com-

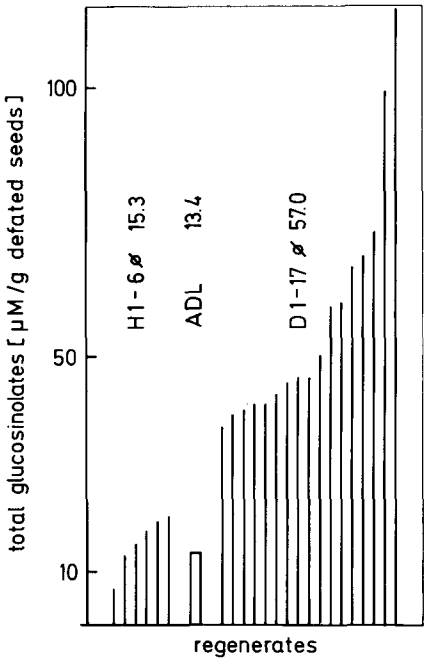

Fig. 2. Content of seed glucosinolates in progenies (A_2) from 23 androgenetic derivatives (A_1) of the inbred anther donor line 24/72 (ADL). Progenies H 1–6 originated from regenerants which had sporadic or exclusively haploid shoots, while progenies D 1–17 originated from regenerants which displayed early spontaneous chromosome doubling and developed exclusively diploid flowers. The difference between the groups H and D is highly significant ($t_{21} = 4{,}53$; t_{21} for P 0.001 = 3,82)

Table 1. Glucosinolate and fatty acid content of an inbred anther donor line (ADL) and 2 androgenetic progenies. Stem embryo cultures H had developed predominantly haploid shoots while D had doubled spontaneously and gave exclusively fertile flowers

Plant	Glucosinolates (μM/g defated seeds)					
	Gluconapin	Glucobrassicanapin	Progoitrin	2-OH-4-pentenylglucosin.	Total	
ADL	3.0	0.3	10.0	0.1	13.4	
H	1.5	0.3	4.6	0.2	6.6	
D	48.6	1.7	58.5	5.9	114.7	
	Fatty Acids (% total)					
	satur. fatty acids	Oleic acid	Linolenic acid	Linoleic acid	Erucic acid	Rest
ADL	6.5	55.5	23.9	11.0	0.5	2.6
H	7.4	44.7	31.7	12.8	0.8	2.6
D	6.7	64.5	17.8	7.7	0.5	2.8

Fig. 3 A–C. **A** shows several test plots, each representing the A_2 generation of androgenetic derivatives. Homogeneous lines as well as heterogeneous lines which show strong growth depression are visible. **B** a homogeneous A_2 derived from the inbred anther donor line (24/72). **C** a heterogeneous A_2 generation derived from the same, largely homogeneous anther donor line. This segregation can not be explained with unreduced microspores

pared. The number of myrosinase bands did not change in haploids and homo- or heterozygous diploids and, therefore, analysis of multiple forms of myrosinases was not used for the investigation of these data.

For more than two years extensive work has been conducted studying the application of isoenzyme patterns for distinguishing homo- and heterozygosity (Hoffmann and Button, unpublished). In addition to

myrosinases, esterases and peroxydases have been predominantly used in these studies. Contrary to the results of several authors using other plant species we obtained ambiguous data in amphidiploid *B. napus*.

Segregating Plants of Microspore Origin

The occurrance of heterogeneous A_2 generations (Fig. 3) from F_1 anther donor material could be explained as being due to the regeneration of unreduced microspores (Wenzel et al. 1976), unless normal vegetative tissue had been cultured. Random sample analysis revealed that regeneration of unreduced microspores also occurred during experiments with *B. napus*. However, most of the cases in question could not be explained in this manner.

In many cases, rape plantlets derived from microspores possessed abnormally swollen stems. Such plantlets produce many thousands of secondary embryoids from single cells of the epidermis (Thomas et al. 1976). The secondary embryoids give rise to tertiary embryoids and so on, and, after several passages, plantlets are formed. One such haploid plantlet, obtained from the F_1 anther donor, was doubled with colchicine and selfed. As expected, no segregation occurred. From the same androgenetic line about 50 spontaneous diploids were also selfed and the seed material was collected and pooled as usual. Plants (A_2) grown from this bulk showed drastic segregation. This can not be explained as being due to the regeneration of unreduced microspores, as haploid plants were obtained from the same stem embryo progeny. Furthermore, the progenies of 11 of 14 randomly selected single plants (A_3) did not segregate further, which would happen in the F_3 (3 plants were sterile/selfsterile). Eight of the 11 populations obtained, showed good seed setting and were analyzed for their glucosinolate content. The results are given in Table 2. The content is again generally reduced. Surprisingly, the level of 2-OH-4-pentenyl-glucosinolate, was unusually high.

Unreduced microspores could also not explain the occurrance of heterogeneous progenies from homozygous anther donors. Amongst these segregating offsprings sterile or reduced fertile forms are quite frequent. In an otherwise homogeneous population of a 'Tower' (spring-type variety) progeny a winter-type appeared which required cold for flowering. The analysis of these segregants showed a dramatic increase in glucosinolate content (Table 2). A similar result was obtained in experiments using mutagens.

Table 2. Glucosinolate content of an anther donor F_1 hybrid (F_1 ADL) and single plant progenies from a heterogeneous microspore-derived A_2 population (A_3 1–8) and glucosinolate content of the anther donor variety "Tower" (T ADL) and a summer-type (A_3 S) and a winter-type (A_3 W) progeny from a heterogeneous microspore-derived A_2 population

Plant	Glucosinolates (μM/g defated seeds)				
	Gluconapin	Glucobrassicanapin	Progoitrin	2-OH-4-pentenylglucosin.	Total
F_1 ADL	27.6	9.7	107.6	5.9	150.8
A_3 1	2.3	7.6	41.2	28.1	79.2
A_3 2	4.5	9.7	48.2	19.2	81.6
A_3 3	5.0	2.6	34.8	15.9	58.3
A_3 4	2.4	1.8	30.1	21.3	55.6
A_3 5	6.0	3.2	34.8	20.0	64.0
A_3 6	8.1	8.3	45.3	26.8	88.5
A_3 7	2.9	8.7	41.8	37.1	90.5
A_3 8	10.8	3.4	54.1	11.9	80.2
T ADL	4.2	0.2	8.9	0.0	13.3
A_3 S	2.7	0.2	11.3	0.0	14.2
A_3 W	11.5	14.3	52.7	15.0	93.5

Induced Mutants from Haploid Cultures

The fact that large numbers of embryoids are produced from single haploid cells of the epidermis not only offers a rapid propagation system, but also allows the possibility of subjecting haploid embryogenic tissue to mutagenic agents to obtain recessive mutations in a homozygous state after diploidization (Fig. 4).

Chemical treatment had a strong effect on the regenerated plants (M_1). Most of them showed poor growth and reduced fertility, or were sterile. The majority of the M_2 were homogeneous, demonstrating that indeed the M_1 originated from single mutated cells. Only 10% of the population revealed heterogeneity. X-rays did not have a comparable effect. From an experiment with the stem embryogenic culture H (for analytical data see Table 1) 25 embryos could be regenerated and the M_2 could be analyzed. All populations except one were homogeneous, the exception was a winter-type which segregated from this one isolate. The glucosinolate content of all the different spring-types was unchanged when compared with the control embryo (6.6 μM). Again the winter-type variant, after vernalization, showed an increased content (80.0 μM). Morphologically, all the homogeneous populations looked exactly like the progeny of the control embryo with 2 exceptions: one population developed light yellow flowers and the other flowers with deformed petals (Fig. 4) which were otherwise regular. Both mutations were recessive and segregated from the backcross in a much smaller ratio than 1 : 3.

Extensive work has been performed in selecting for cold resistance in spring-type cultures in vitro. For this

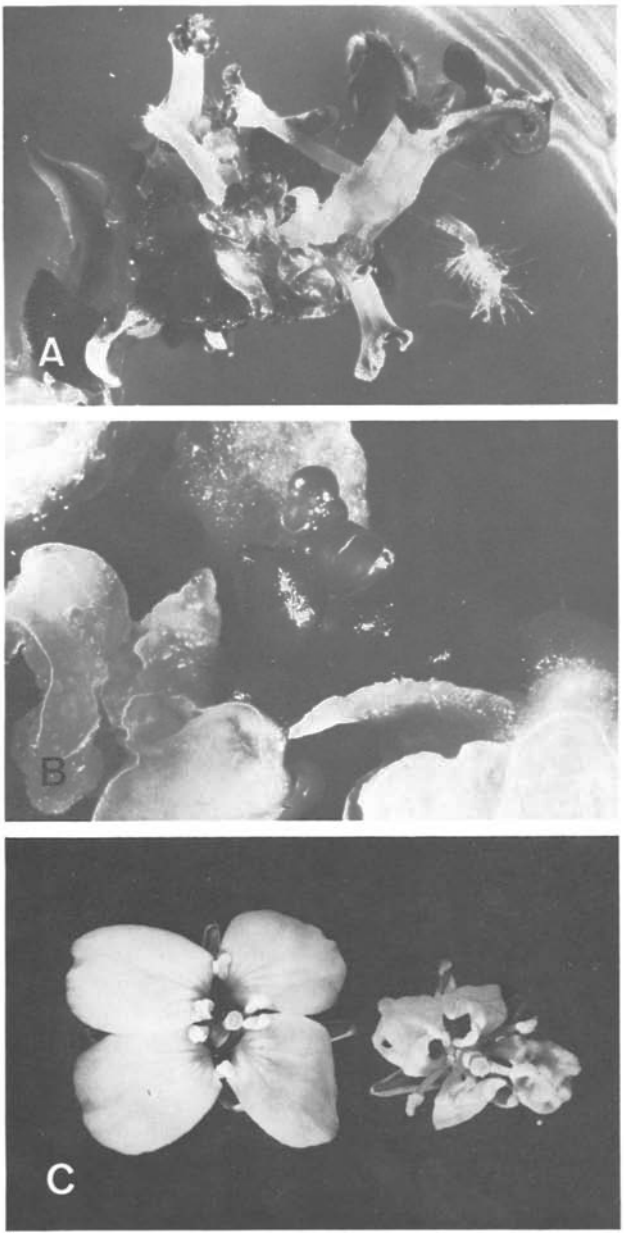

Fig. 4A–C. Mutation breeding with stem embryogenic tissue cultures utilizing the regeneration of single haploid cells. **A** a piece of stem embryogenic tissue derived from a primary microspore-embryo of *Brassica napus*. **B** a piece of stem embryogenic tissue about 2 weeks after mutagenic treatment with EMS. The tissue is mainly dead but a surviving haploid cell regenerates a green shoot, giving rise directly to a homozygous mutant. **C** the deformed, but fertile, flower (control on the left) is a recessive mutation which has been obtained immediately in the homozygous stage by X-irradiation of haploid stem embryogenic tissue followed by colchicine treatment

purpose mutagenized and unmutagenized cultures were treated with different cold shock regimes ranging from −7 °C for 24 hs to −38 °C for 70 minutes. In some cases the treatment was repeated with secondary cultures from surviving tissue until in vitro resistance was obtained. Ninety-eight populations from this experiment were tested and none of them required vernalization or showed tolerance to natural winter conditions.

Heterosis Between Homodiploids

The degree of homozygosity in doubled haploids should theoretically be 100%, a situation which can not be obtained by selfing. Three androgenetic lines were used in crossing experiments. One of the lines (C 10, colchicine-doubled) was extracted from the inbred anther donor while the two others (C 58, colchicine-doubled; 73, spontaneously-doubled) originated from the F_1 hybrid. Although the genetic diversity was accordingly low, hybrid vigour could be demonstrated using plant height as a guiding principle. The androgenetic parents had the following data: C 10/131 cm, C 58/122 cm and 73/133 cm. The data from all crosses performed were as follows: C 10 × C 58 = 152 cm, C 58 × C 10 = 164 cm, C 10 × 73 = 170 cm, 73 × C 10 = 180 cm and C 58 × 73 = 179 cm.

Discussion

Selective Growth of Microspores in Cultured Anthers

If the parents of an F_1 hybrid contain characters of value to plant breeding programmes, then success in inducing a high frequency of plant formation from microspores of this hybrid should rapidly uncover important gene combinations and should lead to the production of homozygous lines in a single breeding step. However, this will only be true if high numbers of androgenetic plants can be obtained and no "in vitro selection pressure" allows only special genotypes to grow. Figure 5 shows a cultured anther with several embryoids or embryoid-like structures in competition for the limited space. A maximum number of 40 of these structures can develop but not all of them will survive. This raises the question whether only a very special class of microspores, containing something like a regeneration capacity gene, have a chance to survive or whether representative growth is still guaranteed by random selection of a position effect. Our results indicate that genetic selection can occur and that the "regeneration capacity gene" may be closely linked with characters such as late flowering which can be observed in the field. If in vitro selection pressure and breeding aims are congruent this may be profitably used. This must not only concern such obvious characters as vigorous growth but also the content of certain metabolites. However, if representative regeneration of micro-

Fig. 5. Embryoids or embryoid-like structures developing from a single anther of *Brassica napus*. The anther itself is only just visible because of the large number of growing structures. The limited space during the early phase of embryoid development raises the question whether individual representative growth of the different microspore genotypes is still guaranteed. The results obtained indicate in vitro-selection

spores is desired, then the problem of in vitro-selection could be reduced to some extend by culturing isolated microspores. Unfortunately, this technique has not been successfully developed for *B. napus* or any other crop. There are already preliminary observations of the heritability of regeneration capacity (Wenzel and Uhrig 1981); probably, such linkage can be overcome by sufficient recombination and the character itself can be combined with other useful traits.

Variability from Anther Cultures

In the past, tissue culture techniques have been normally discussed as a tool for the clonal propagation of plants. Today it is a modern trend to sell tissue culture methods as a tool for the production of variability in plants (see Larkin and Scowcroft 1981). In our experiments we found heterogeneous plant populations segregating from a single haploid cell. This new variability, which can only be explained by spontaneous mutations, need not be caused by tissue culture manipulations but, more probably, by the haploid condition of certain genotypes themselves (perhaps in combination with in vitro-growth). One of the most important tasks in rape breeding is to increase the variability. Natural variability is already relatively low in this young species and has been further decreased by plant breeders during selection for zero-erucic acid. Because of the low genetic diversity, hybrid lines of *B. napus* show relatively small heterotic effects (Röbbelen 1975). In this respect our finding that several different homozygous genotypes can be produced from a single cultured microspore of a certain genotype may be of some significance for rape breeding. On the other hand, this is not a general method for the production of variability. We have regenerated homogeneous populations from haploid stem embryo cultures and diploid embryos have never been observed to yield a spectrum of mutants. Eventually, a drastically changed phenotype appeared which was usually sterile (probably due to chromosomal mutation). This phenomenon has also been observed from time to time in our experiments with other plant species and regenerants from callus cultures or isolated protoplasts which otherwise lead to clonal propagation of plants. However, the whole phenomenon of drastic damage of the genome of cultured cells is something which has to be overcome, rather than something to be used in plant breeding.

Another interesting observation is the finding of more variability in regenerants from different microspores than in the highly inbred anther donor line. This variability is obviously not new variability. It demonstrates the higher degree of homozygosity in the doubled haploids in comparison to inbred lines of amphidiploid *Brassica napus*. This is probably even more significant in an amphidiploid than in a true diploid plant species.

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