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DIHAPLOID PRODUCTION IN *BRASSICA NAPUS* L. BY IN VITRO ANDROGENESIS[1]

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Summary. Anthers of three cultivars and one hybrid of *Brassica napus* were cultured according to Keller and Armstrong (1978) method. Embryo yields varied widely depending on individual anthers plantings and the cultivar used. Cytological studies and morphological observations of regenerated plants revealed the presence of 50% haploids. The remaining plants had diploid, tetraploid and mixoploid chromosome numbers. Diploidization of haploids by colchicine treatment of excised axillary shoots was applied. Using this technique, seeds were obtained from each colchicinated unfertile plant. A preliminary progeny analysis of androgenetic plants showed high degree of homozygosity.

The use of in vitro cultures of anthers in plant breeding and genetic research has several advantages including the possibility of efficient selection from F_1 derivatives as well as a rapid development of homozygous lines (Hoffmann et al., 1982). Rape-seed (*Brassica napus*) is one of very few important crop plants which can be successfully grown by in vitro techniques. The culture of this species anthers under appropriate conditions resulted in a high frequency of microspore-derived embryos which could regenerate plants (Keller 1983). Cytological studies of rape-seed androgenetic plants revealed the presence of haploids, diploids and higher ploidy levels (Keller, Armstrong, 1978; Chiang Shiong, Ingram, 1982). Morphological analyses of anther-derived diploids displayed homozygosity indicating that these plants originated from haploid microspores, probably due to the process of endomitosis and/or nuclear fusion (Keller et al. 1975).

This report describes the results of the recent studies on haploid induction in three varieties and one hybrid of *Brassica napus*, which were cultivated in the country. Special attention was focussed on the chromosome doubling procedure of anther-derived haploids.

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MATERIAL AND METHODS

The *Brassica napus* material used in this work included two winter varieties (Jet Neuf and Janpol), one spring variety (Erglu), and one F_1 winter hybrid population (592) selected from lines low in erucic acid and glucosinolates. Seeds of F_1 hybrid were kindly provided by Prof. Dr. hab. J. Krzymański from the Department of Oil Plants of the Institute of Plant Breeding and Acclimatization in Poznań.

The winter types were vernalized in the seed stage by keeping them on the moist sand at 2 - 4°C for 7 weeks. Plants maintained in the greenhouse were grown in the soil: peat mix (1:1), containing nutrient supplementation of N-P-K sufficient for active growth. During the winter months the day length was artificially extended to 16 hs.

Buds were collected from healthy plants prior to flower emergence. They were disinfected with 70% alcohol for 1 - 2 min and then floated on sterile water. It was found that the common use of calcium hypochloride was unnecessary. Anthers' were taken in the uninucleate microspore stage according to the relation between the morphological characteristic of the bud and ontogenesis of pollen (Keller et al. 1975). They were planted on the B_5 medium of Gamborg and co-workers (1968), modified by Keller and Armstrong (1977) with the addition of 10% sucrose, 0.8% agar, 800 mg/l glutamine, 100 mg/l serine and 0.1 mg/l of both 2.4-D and NAA.

At first the anthers were incubated in darkness at 30°C for 14 days. After this treatment the cultures were transferred to 25°C also in darkness until the emergence of embryos. Anther-derived embryos were initially kept in continuous light at 25°C for a week to permit greening and were transferred to the same medium as described above, with sucrose level reduced to 2% and in the absence of hormonal substances.

Normal embryos, regenerated directly into plants, were incubated on the embryo culture medium until an adequate root system developed. The majority of embryos were grown abnormally with elongated or swollen hypocotyls and massive cotyledons. In these cases shoots and roots were induced by cutting the embryogenic tissue and culturing it on the Murashige and Skoog (1962) medium with 2% sucrose, 5×10^{-6}M BAP, 10^{-7}M NAA and 0.8% agar. Part of secondary embryos obtained from hybrid 592 were cultured on the same medium but without plant growth substances.

Plantlets with active root system were planted in compost:verniculite mix (1:1) in 7.5 cm plastic pots and covered by beaker for 1 - 2 weeks to facilate hardening. They were kept at 22 - 25°C for 16 hs of photoperiod, before transferring to greenhouse and potting in soil.

The unfertile haploid plants were diploidized with colchicine. Three young secondary axillary shoots were cut off from each haploid plant and placed to the vial with 0.05% colchicine solution containing 1.5% dimethylsulfoxide (DMSO) for 18 hs at 25°C in darkness. After the treatment the cuts were rinsed with water, planted to the soil and kept moist. Under the condition sufficient for active growth the explants were rooted two weeks later and the fertile or partly fertile plants were regenerated.

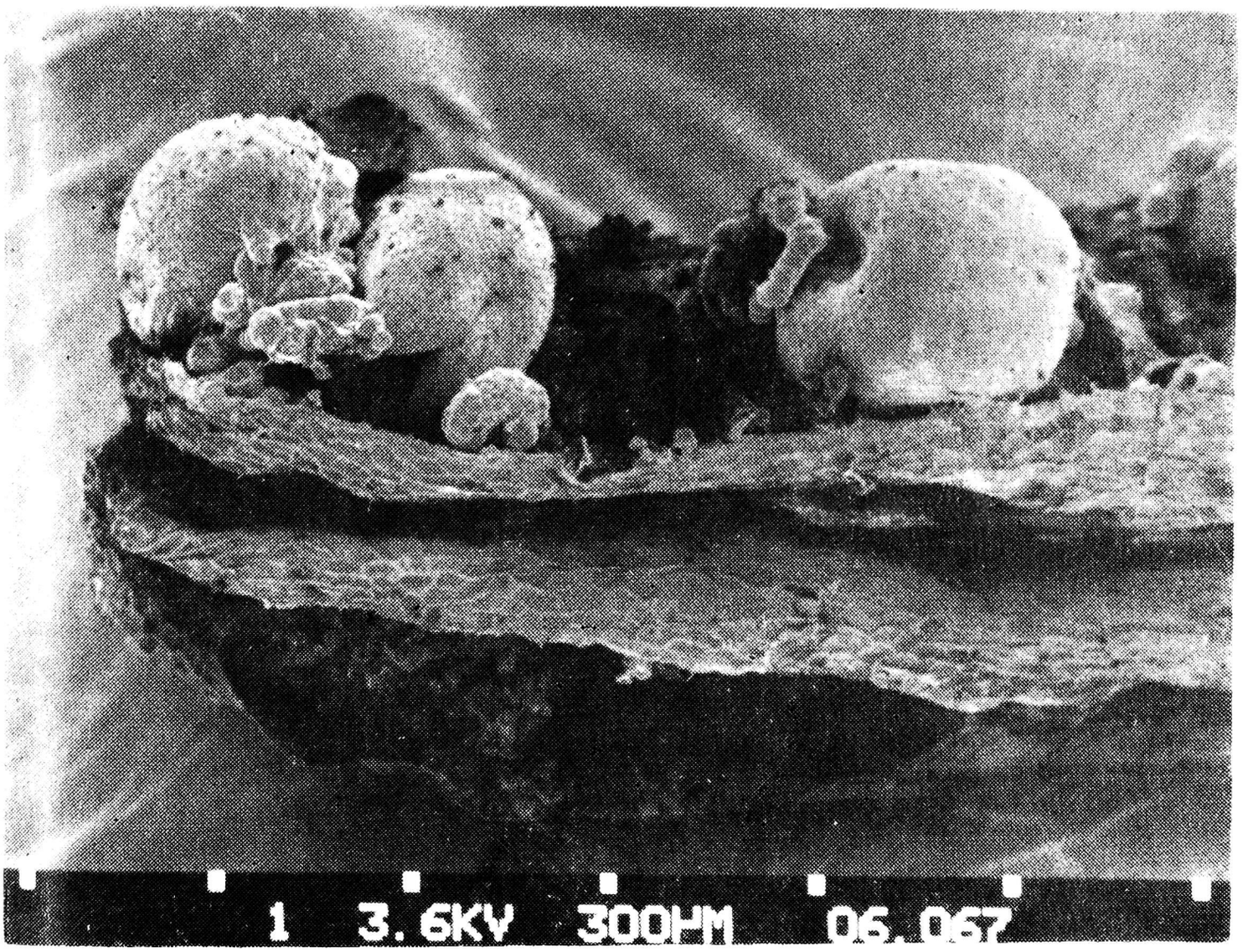

Fig. 1. A scanning electron microscope view of several embryos emerging from a cultured *Brassica napus* anther (×78). Photographed by Dr. J. Macewicz

The ploidy level of regenerates was examined in the root tip cells. They were pretreated in 0.05% colchicine for 2 hs, fixed in acetic ethanol for one day and squashed in aceto-orceine.

RESULTS AND DISCUSSION

In all the cultivars of rape-seed tested after 6 - 10 days in culture, many of planted anthers contained divided microspores. Unfortunately groups of few cells frequently died inside the exine. It was revealed that the embryos emerged during 15 - 32 days of treatment (Fig. 1). It was found that in anthers of Erglu and hybrid 592 a single production of embryos prevailed, and in the cases of Jet Neuf and Janpol it was a mass production from a few anthers in the series. Embryo yields of individual anther

Table 1. Production of embryos by cultured anthers in three rape-seed cultivar and in one hybrid line of *B. napus* L.

Time of experiments	*B. napus*	Number of				Range of embryo yield in series (per cent)
		anthers cultured	series*	embryogenic anthers (total) per cent	embryos (total) per cent	
August – September 1981	Jet Neuf cv.	408	2	16 (3.9)	51 (12.5)	7.8 – 47.9
September 1981	Erglu cv.	396	4	9 (2.3)	11 (2.8)	1.0 – 3.7
January – February 1982	Janpol cv.	696	3	24 (3.5)	96 (13.8)	5.8 – 25.0
July – October 1982	Hybrid line (592)	2316	12	67 (2.9)	133 (5.7)	1.0 – 9.2

* The anthers were planted in different numbers and days depending on the available bud material.

plantings varied widely being consistently higher in Jet Neuf and Janpol than in Erglu and hybrid 592 (Table 1). An anther culture of Jet Neuf variety using the same technique as in this report (Keller, Armstrong 1978), was also carried out by Chiang Shiong and Ingram (1982). In their experiment the frequency of the embryo emergence ranged only from 0.7 to 1.2%. The higher production of Jet Neuf embryos 12.5% in these studies might be rather due to physiological conditions of donor plants than due to genotypic differences in selected anthers.

The developmental stages of embryos were observed to range from small globular to fully differentiated forms. Approximately 5 - 10% of the normal embryos survived and developed directly into plantlets after transferring them on the B_5 medium free of hormones and containing 2% sucrose. The remaining types showed different abnormality, such as fusion of cotyledons, elongated or swollen hypocotyls, albinism and the lack of bipolar structure. Similar observations in the anther culture of rape-seed were made by authors (Thomas, Wenzel 1975; Keller, Armstrong, 1977).

The transference of abnormal embryos according to Keller, Armstrong (1977) to the Murashige and Skoog medium supplemented with BAP and NAA caused the initiation of shoot meristems in 40 - 60% of Jet Neuf, Janpol and Erglu explants, particularly on the surface of a swollen hypocotyl. The frequency of secondary

embryogenesis was stimulated up to 95% in abnormal embryos of hybrid 592 after being transferred to the Murashige and Skoog medium in the absence of hormonal substances. In these conditions the explants swelled and cellus with multiple shoot--buds developed. After 2 - 4 weeks of culturing about 50% of explants produced entire plantlets. The remaining calluses regenerated shoots and roots after the next transfer to a fresh medium. This finding confirmed the results of Chiang Shiong and Ingram (1982) who obtained large numbers of secondary embryos using a similar procedure.

Androgenetic plantlets were subsequently potted, those of the winter type vernalized in a cooling room and grown to maturity in the greenhouse. Of the total 291 embryos obtained, 158 plants were regenerated directly from the embryos or indirectly from secondary explants (Jet Neuf — 71, Erglu — 8, Janpol — 22 and hybrid 592 — 57 plants). A great variation in morphological traits was observed between individual plants during growth. This concerned differences in the leaf size and shape in the height, branching, flower size and degree of sterility. Plants with small sterile flowers were identified and a cytological analysis showed the chromosome number equal to 19. The general cytological and morphological observations of 101 anther-derived plants of Jet Neuf, Erglu and Janpol revealed the presence of 51 haploids, 24 diploids, 1 tetraploid and 25 mixoploids with sterile and fertile flowers on the same plant. The ploidy level of the remaining 57 plants derived from the anthers of hybrid 592 has not been estimated up to the moment.

In order to restore fertility of haploid plants a chromosome doubling should be made. For this purpose colchicine was used with a varying success (Wenzel et al. 1977; Hoffmann et al. 1982). In this study diploidization by colchicine treatment of excised axillary shoots was used (see Methods). As a result of the experiments with 39 haploid plants out of 121 colchicinated axillary shoots 92 fertile plants were regenerated (average 76%). Though not each colchicinated axillary shoot could be doubled in the chromosome number, the seeds were derived finally from each originally haploid embryo.

A preliminary progeny analysis of androgenetic plants obtained from three varieties of *Brassica napus* showed a high degree of the phenotypic homozygosity. At present, 68 of the winter type dihaploid lines are under the field test. The remaining materials are propagated in order to receive a sufficient number of seeds for further experiments.

The practical application of homozygous lines obtained from spontaneously occurring *Brassica napus* haploids (Thomson 1979), and the utilization of androgenetic dihaploids in the production of new cultivars of other crops (Fedak 1976; Oono 1981) would suggest that it has become already possible to use haploids in the breeding programme of rape-seed.

CONCLUSION

The anther culture technique could be used as a valuable breeding tool in *Brassica napus* for a rapid production of homozygous lines in a large number.

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REFERENCES

1. Chiang Shiong L., Ingram D. S. (1982). Production of haploid plants from anther cultures and secondary embryoids of winter oilseed rape *Brassica napus* ssp. *oleifera*. New Phytol. 91: 507 - 516.
2. Fedak G. (1976). Evaluation of doubled haploids in barley. Z. Pflanzenzüchtg. 76: 147 - 151.
3. Gamborg O. L., Murashige T., Thorpe T. A., Vasil I. K. (1976). Plant tissue culture media. In Vitro 12: 473 - 478.
4. Hoffmann F., Thomas E., Wenzel G. (1982). Anther culture as a breeding tool in rape. II. Progeny analyses of androgenetic lines and induced mutants from haploid cultures. Theor. Appl. Genet. 61: 225 - 232.
5. Keller W. A. (1983). Anther culture of *Brassica*. In: J. K. Vasil (ed) "Cell culture and somatic cell genetics". In press.
6. Keller W. A., Armstrong K. C. (1977). Embryogenesis and plant regeneration in *Brassica napus* anther cultures. Can. J. Bot. 55: 1383 - 1388.
7. Keller W. A., Armstrong K. C. (1978). High frequency production of microspore-derived plants from *Brassica napus* anther cultures. Z. Pflanzenzüchtg. 80: 100 - 108.
8. Keller W. A., Rajhathy T., Lacapra J. (1975). In vitro production of plants from pollen in *Brassica campestris*. Can. J. Genet. Cytol. 17: 655 - 666.
9. Murashige T., Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473 - 497.
10. Oono K. (1981). In vitro methods applied to rice. In: "Plant Tissue Culture" (Trevor A. Thorpe ed.) Academic Press 1981.
11. Thomson K. F. (1979). Superior performance of two homozygous diploid lines from naturally occurring polyhaploids in oilseed rape (*Brassica napus*). Euphytica 28: 127 - 135.
12. Wenzel G., Hoffmann F., Thomas E. (1977). Anther culture as a breeding tool in rape. I. Ploidy level and phenotype of androgenetic plants. Z. Pflanzenzüchtg. 78: 149 - 155.

OTRZYMYWANIE DIHAPLOIDÓW U RZEPAKU *BRASSICA NAPUS* L. NA DRODZE ANDROGENEZY IN VITRO

Streszczenie

Przedstawiono wyniki hodowli in vitro pylników rzepaku *Brassica napus* (trzech odmian i linii mieszańcowej) według metody Kellera i Armstronga (1978). Liczba uzyskanych embrionów różniła się znacznie, zależnie od serii przeszczepu pylników, jak również od materiału z którego pochodziły. Obserwacje cytologiczne i morfologiczne zregenerowanych roślin wykazały obecność 50% haploidów. Pozostałe rośliny były diploidalne, tetraploidalne i miksoploidalne. W celu podwojenia liczby chromosomów u haploidów wprowadzono kolchicynowanie odciętych bocznych pędów. Stosując tę technikę otrzymano nasiona z każdej niepłodnej rośliny. Wstępna analiza potomstwa androgennych roślin wykazała wysoki stopień ich homozygotyczności.

ПОЛУЧЕНИЕ ДИ-ГАПЛОИДОВ У *BRASSICA NAPUS* ПУТЁМ АНДРОГЕНЕЗЫ IN VITRO

Резюме

В работе представлены результаты культуры in vitro пыльников *B. napus* трёх сортов и гибридных линий согласно методу Келлера и Армстронга (1978). Число полученных зародышей зависило от перепрививки пыльников и от материала, из которого они происходили. Цитологические и морфологические наблюдения регенированных растений показали наличие 50% гаплоидов. Остальные растения были диплоидные, тетраплоидные и миксоплоидные. С целью удвоения числа хромосом у гаплоидов, произведена обработка колхицином отрезанных боковых побегов. С помощью этой техники были получены семена с каждого неплодоносного растения. Предварительный анализ потомства андрогенных растений показал высокий уровень гомозиготности.