GCIRCBUL10*06

Genotypic effects on microspore culture in a breeding program for high erucic acid content of Brassica napus

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Summary. Results of microspore culture of rapeseed with high erucic acid content are presented. Embryoid yield of different hybrids and parents was compared. Genotypes with buds mainly containing microspores in late uninucleate stage at isolation showed the best embryoid yield. But homogeneous microspore populations were not obtained for all the genotypes tested. Microspore viability after isolation and during the first days in culture was similiar for all genotypes. Nevertheless, hybrids usually formed more embryoids than their parents. Regeneration of plants did not depend on the embryoid yield as a whole, so that different genes may be responsible for these two traits.

Key words: *Brassica napus* - microspore culture - *in vitro* - embryoid formation - regeneration

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Introduction

For about a decade, microspore culture has been successfully used in rapeseed as a technique for the production of haploid plants (LICHTER, 1982). Nevertheless, there are some difficulties that hamper its application in practical breeding programs. For example, success is strongly affected by physiological state of the donor plants and their genotype. Many attempts have been made to enhance embryoid yield by modification of factors that influence the regeneration of microspore plants, e.g., growing conditions of donor plants (KELLER *et al.*, 1987a), time and method of bud harvest (CHUONG *et al.*, 1988; TAKAHATA *et al.*, 1991), media composition (GLAND *et al.*, 1988; LICHTER, 1985; KELLER *et al.*, 1987b), microspore stage (PECHAN and KELLER, 1988; KOTT *et al.*, 1988a, b), pretreatment and incubation of microspores (KELLER and

(1) This text was not presented in Copenhagen.

ARMSTRONG, 1983; CHUONG and BEVERSDORF, 1985). Most of these experime been carried out with only few, well reacting genotypes. Other materials often show inferior reaction in microspore culture (OHKAWA *et al.*, 1987). Among the factors mentioned above the genotype of donor plants holds a key position concerning culture success (CHUONG *et al.*, 1988; KELLER *et al.*, 1987a).

The cessation of culture growth often observed seems to depend on toxic substances that are released in different concentrations by cultivated microspores, depending on their genotype (KOTT *et al.*, 1988a, b). The reason may be the composition of a microspore population. In anthers of genotypes that react poorly, a fairly large variation of stages was observed (KOTT *et al.*, 1988a). Such cultivars seem to be less suitable for application of microspore culture particularly in a commercial breeding program unless the culture conditions can be further optimized. The present study aimed at the development of a microspore culture technique for the production of doubled haploid, high erucic acid lines. For that purpose, different parental lines and F_1 hybrids were screened for their plant regeneration capacity.

Materials and methods

The basic plant material was derived from winter rapeseed (*B. napus*) cultivars 'Bridger', 'Synra' and 'Marcus' by selfing. Selection was carried out for high erucic acid content in the seed oil. Crosses were carried out between high erucic S_1-progeny and hybrid plants were selected as microspore donor plants (table 1).

Two trials were carried out in a time interval of four weeks. Donor plants were germinated and grown for two weeks, vernalized (9 weeks, 2°C) and further propagated in growth chambers at 20/15°C (day/night 16/8h, 22,000 lux). After four weeks the temperature was reduced to 13/7°C.

Due to the fact that optimum bud size may differ between genotypes, the microspore stage was determined by staining with hematoxylin (KINDIGER and BECKETT, 1985). Furthermore, the viability of young pollen in anthers, freshly isolated microspores and microspores, that were incubated for 4 days was examined by the staining method of ALEXANDER (1969).

Buds with microspores in a uninucleate or early binucleate stage were surface sterilized in 96% ethanol (10s) and 2% NaOCl (8min) and subsequently rinsed in sterile water. Six buds were sampled and transferred to a centrifuge tube; 8ml of NLN medium (pH 6.0; GLAND *et al.*, 1988) were added and buds were crushed with a glass stick. The suspension was filtered through a steel filter and washed by centrifugation (10min, 100g). The pellet was resuspended in 3ml of fresh medium, which was tested at four pH-values (pH 5.8, 6.0, 6.2, 6.4). Aliquots were poured into two petridishes (35mm each). Two of these petridishes were set into a large one (9cm) and sealed

with nescofilm. The microspores were incubated at 30°C and 1,000lux light intensity. After one day in culture the medium was changed by centrifugation and a third petridish with sterile water was added to avoid evaporation.

For regeneration, MS medium (2% sucrose, pH 6.0, MURASHIGE and SKOOG, 1962) was used. Embryoids were transferred to solid medium when they were about 4mm in lenght or when earlier stages became brown or yellowish in liquid medium. Calli were transferred onto fresh medium monthly and shoots were rooted on WL20 medium (NICHTERLEIN *et al.*, 1991). Plantlets were set into soil and further grown in the greenhouse.

For statistical evaluation of the data, an analysis of variance was carried out with SPSS PC+, even though the requirement of homogeneity of variances was not fulfilled.

Results

Microspores in an optimum uni- or early binucleate stage were found in buds of 3.5 to 4mm length. One group of the genotypes (e.g., K26, K32, K50) had microspores of nearly the same stage in one bud, whereas buds of others (e.g., sf08, sf45, K66) contained microspores in up to four stages. Genotypic differences concerning the optimal lenght of the buds could not be observed. The same applied to the viability of microspores. Nearly 100% of the microspores of each genotype seemed to be vital before and after isolation. However, approximately 40% of the microspores died during the first four days in culture.

After isolation, the microspore suspensions of some genotypes again appeared very homogenous as compared to others. No significant relationship was found between the number of divisions after four days in culture and embryoid yield (SPEARMAN's rank-correlation-coefficient: 0.099 and 0.375, first and second trial, respectively). The initiation of microspore division is a prerequisite for subsequent embryoid formation. However, it was neither possible to predict the number of embryoids nor to predict the final success of microspore culture in general from the number of divisions during the first days of incubation. Furthermore, embryoid yield was strongly affected by genotype (table 2), but did not depend on the pH of the incubation medium.

In some cases, especially if large numbers of embryoids appeared in one petridish, these embryoids showed an abnormal anthocyanin production. However, this did not lead to significant negative effects on further embryoid development. After embryoids had been transferred to solid medium, they started growing very rapidly. Secondary shoots were harvested from these calli and were rooted on WL20 medium. Direct embryogenesis, that means direct regeneration of a plant from an embryoid, was observed only in a few cases. These plantlets seemed to be weaker than those obtained from secondary shoots, so that the latter procedure was preferred.

Table 1: Donor plant material and final results of microspore culture

Genotype	Embryoid yield	LSD (0.10)[1]	Regeneration rate (%)[2]
sf08 (Synra)	61	ab	34.7
sf11 (Marcus)	27	ab	23.5
sf43 (Bridger)	19	a	50.0
sf44 (Bridger)	88	abc	33.3
sf45 (Bridger)	4	a	0.0
K03 (sf08 x sf11)	326	cd	23.2
K12 (sf11 x sf08)	64	ab	1.8
K19 (sf08 x sf43)	236	abcd	14.1
K24 (sf43 x sf08)	276	bcd	19.7
K26 (sf08 x sf44)	373	d	36.9
K32 (sf44 x sf08)	343	d	19.2
K41 (sf08 x sf45)	166	abcd	16.8
K49 (sf45 x sf08)	63	ab	18.8
K50 (sf11 x sf43)	922	e	36.2
K66 (sf43 x sf11)	53	ab	0.0
K68 (sf11 x sf44)	124	abcd	5.5
K78 (sf44 x sf11)	192	abcd	14.0
K84 (sf11 x sf45)	218	abcd	10.9
K89 (sf45 x sf11)	230	abcd	24.8

[1] the same letter indicates the same group of means
[2] shooting embryoids on regeneration medium within 8 weeks

Table 2: Results of analysis of variance[1]

Source of variation	D.F.	M.S.	F-value	probability
main effects	22	6761.80	3.749	<0.001
genotype	18	7357.99	4.079	<0.001
pH-value	3	1912.58	1.060	0.370
replication	1	2981.26	1.653	0.202
error	91	1803.72		
total	113	2769.01		

[1] the second trial was treated as a replication of the first

Usually, hybrids showed a better embryoid yield than their parents (table 1). However, if one cross direction showed a better average response, this was due to extremely well reacting buds in one replication. Only genotype K50 showed a reproducibly superior reaction and it was also better than the reciprocal cross in both trials. Differences between genotypes were statistically significant in some cases.

Colchicine treatment for chromosome doubling was carried out by different methods. Subsequently, analyses of the doubled haploid (DH) lines and comparisons with conventional breeding lines are scheduled.

Discussion

The identification of flower buds in an optimum stage is a critical factor for microspore culture success. In the present investigation, the microspore stage could easily be identified by staining with hematoxylin. No substantial differences were found between the genotypes concerning the length of buds that contained microspores in an optimum stage, so that appropriate buds could be selected by estimating their size. PECHAN and KELLER (1988) tried to establish correlations with other parameters - such as anther size or anther/petal ratio - with embryoid yield. They were able to differentiate between buds with higher or smaller amounts of embryogenic microspores even by bud size. However, it was not possible to predict embryoid yield.

The death of many microspores during incubation time has been observed repeatedly (LICHTER, 1983; DESLAURIERS *et al.*, 1991). Older microspores produce toxins and inhibitors during culture (KOTT *et al.*, 1988a, b). The classification of a substance as 'inhibiting' or 'promoting' depends on its concentration. Whereas KOTT *et al.* (1988b) reduced the embryoid yield by adding conditioned medium to the culture, HUANG *et al.* (1990) raised the embryoid production of lower density microspore cultures by using medium in which 30-40,000 microspores had been incubated for several days.

Although potential toxins that are produced by aborted microspores should at least have been diluted by changing the medium after one day in culture, a difference between the genotypes was observed in the present case. KOTT *et al.* (1988a) reported different reactions of buds that contained microspores in nearly the same or in several stages, respectively. One of their genotypes had a very homogeneous microspore population and reacted well in culture. The other contained microspores in up to four stages, i. e. fewer microspores in an optimum stage, so that finally a smaller embryoid yield was obtained. Genotypic variation for microspore stage in individual buds has also been described in *B. oleracea* (DUIJS *et al.*, 1992).

In our studies, genotypes with homogeneous microspore populations, e. g. hybrid K50, produced more embryoids than genotypes with buds, in which microspores at different stages were found. For these genotypes the most suitable stage for culture was probably not always present; PECHAN and KELLER (1988) assumed that microspores are capable of undergoing embryogenesis at less than 2% of the total bud elongation time.

As a consequence, several steps are necessary in order to homogenize the microspore stage. Lower growing temperatures for the donor plants can support an equal development, as experienced by DUNWELL *et al.* (1985), KELLER *et al.* (1987a) and BAILLIE *et al.* (1992). Further possibilities to obtain homogeneous microspore populations are sorting of microspores after isolation by flow cytometry (DESLAURIERS *et al.*, 1987, 1991; PECHAN *et al.*, 1988) or fractionation using 'Percoll' density gradients (FAN *et al.*, 1987). Low density microspore cultures can also be stimulated by using medium previously conditioned with higher microspore density (HUANG *et al.*, 1990). A next step would be to use medium conditioned by well reacting genotypes to incubate microspores of obstinate genotypes. By this procedure, toxic substances of microspores in unsuitable pollen stages may be excluded. Promoting substances on the other hand can be supplied via the conditioned medium.

The present study also aimed at a quantification of the influence of genotype on culture success in a practical breeding program. It could be demonstrated that genotype influenced embryoid yield significantly. An interesting aspect is the different embryoid yield of the three inbred lines selected from 'Bridger'. Similiar results were reported by ASLAM *et al.* (1990), where several *B. campestris* lines selected from one plant brought different success in anther culture. Subsequently, self pollination of well reacting lines again produced superior lines - an indication of genetic control of culture suitability.

Pronounced genotypic effects were reported in many publications (e.g., PECHAN and KELLER, 1988; CHUONG *et al.*, 1988; DUNWELL *et al.*, 1983, 1985). An improvement of culture capacity of hybrids seems to be feasible by crossing with well reacting genotypes (CHUONG and BEVERSDORF, unpubl., cit. in CHUONG *et al.*, 1988; SCHOLZ, 1987).

Our results confirm the superior reaction of hybrids as compared to their parents. It has been speculated that self pollinating plant species may be more suitable for tissue culture techniques than cross pollinating species because of the absence of lethal genes (MANDAL, 1987). *B. napus* has an intermediate position between self- and cross-fertilisation. Although pronounced inbreeding depression is generally not observed after self pollination, the hybrids in the present study proved to be better donor plants than their parents. Similiar reactions were also observed by JACOBSEN and SOPORY (1978) in potato, where hybrids showed a better suitability for anther culture than their parents. One single genotype with 30-40% embryoid production could be selected. The authors assumed a positive combination of culture suitability.

Heritability of tissue culture ability seems to be effected only by a few genes (HENRY and DE BUYSER, 1985; UHRIG, 1985). In wheat anther culture 1B/1R translocation lines proved to be more suitable than lines without the translocated segment, although most of the rye cultivars are non responsive in tissue culture (FOROUGHI-WEHR and ZELLER, 1990). For rapeseed, SIEBEL and PAULS (1989) did not find any reciprocal effects in their experiments. Therefore, they supposed the nucleus to be the site of genes responsible for culture success. In the present study the cross direction sometimes had an effect on embryoid yield, so that reciprocal effects may be expected, depending on the material.

Regeneration by direct embryogenesis caused some problems in our studies. Direct plant formation led to weaker plants than regeneration via secondary shoots. This reaction is generally known for microspore culture of rapeseed (LICHTER, 1983, 1985; KELLER and ARMSTRONG, 1983; NEUHAUS *et al.*, 1987). MATHIAS (1988) reported a direct plant formation of 50% by using a double-layer culture medium. Young embryoids are susceptible to any kind of stress. Therefore, direct regeneration may be raised by cold treatment or drying (COVENTRY *et al.*, 1988). After a cold treatment, 98% of the embryoids directly regenerated plants. POLSONI *et al.* (1988) obtained 100% of direct regeneration by individual transfer of single embryoids of the germplasm line G231 to solid medium. However, it will probably be difficult to obtain such a high success rate with other breeding materials.

The regeneration rate of the different genotypes does not depend on the yield of embryoids as a whole. Even genotypes with low embryoid yield were able to regenerate similiar rates of shoots, as soon as they were transferred to solid medium. Therefore, shoot formation may be influenced by other genes than embryoid formation. Corresponding results were reported by SIEBEL and PAULS (1989).

Sometimes a discolouration of leaves was observed on rooting medium. In some cases these leaves turned yellowish and became necrotic. Together with the violet colour of embryoids that occasionally appeared in petridishes, these observations may have been due to a deficiency of sulphur (BAUMEISTER and BURGHARDT, 1972; BERGMANN, 1988). Symptoms of deficiency disappeared after transfering shoots to fertilized soil and had no negative effect on culture. Nearly all plants survived the transfer to greenhouse conditions. After vernalisation plants were colchicine treated and will be available for further analysis.

Acknowledgements

Thanks are due to Miss Annette Feil for excellent technical assistance.

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