THE UTILIZATION OF MICROSPORE CULTURE AND MICROSPORE-DERIVED DOUBLED-HAPLOIDS IN A RAPESEED (*BRASSICA NAPUS* L.) BREEDING PROGRAMME

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The potential to increase the efficiency of plant breeding programmes through the use of doubled haploids has led to extensive research into the development of reliable and efficient haploidy techniques. In *B. napus*, a haploidy system based on microspore culture has the greatest potential for general breeding applications. Research conducted at the University of Guelph during the past four years has resulted in development of a microspore haploidy system suitable for both large-scale doubled haploid line production and for *in vitro* mutagenesis and selection. This report details some of the results of our ongoing research, and of two years of application of microspore culture in a commercial-scale, rapeseed breeding programme.

Doubled Haploid Line Production:

Producing doubled haploid (DH) lines through microspore culture involves three steps: (1) production of haploid embryos from isolated microspores, (2) regeneration of plants from the embryos, and (3) doubling the chromosomes of regenerated haploids to produce DH plants. The efficiency of DH line production is a function of both the conversion efficiency (output/input) and the time involved at each step.

Embryo yields reported from microspore culture have averaged <30 per anther, with considerable variation in yield among genotypes (Keller and Armstrong, 1983; Chuong and Beversdorf, 1985). Plant regeneration frequencies in the range of 5 to 20% have been reported by the same authors. Our studies show a strong correlation between the synchrony of microsporogenesis, the stage of microspore development at time of culture, and embryo yields (Table 1). In genotypes with a high degree of developmental synchrony (i.e. SV), by using anthers with a high proportion of uninucleate microspores we have obtained yields of up to 1300 embryos per anther (Kott *et al.*, submitted a). Placing cultures on a rotary shaker when embryos are first visible results in rapid, uniform embryo development, and production of a high proportion of torpedo-shaped and cotyledonary embryos within 21 days of isolation. Chemical analysis suggests that the release of toxic compounds from binucleate microspores which burst in culture may be the cause of low embryo yields and lack of morphological uniformity in genotypes with asynchronous development (i.e. OAC Triton) (Kott *et al.*, submitted b). Using liquid B_5 (Gamborg *et al.*, 1968) medium supplemented with 0.1 mgL^{-1} GA_3, or solid B_5 supplemented with 0.1 mgL^{-1} GA_3 and 0.4 mgL^{-1} benzyl adenine, we have obtained regeneration frequencies in excess of 40% in a number of genotypes (Charne, 1986; Polsoni *et al.*, submitted).

The procedures reported for chromosome-doubling of *B. napus* haploids involve either topical application of colchicine to axillary buds of

Table 1. Relationship between anther length, microspore developmental stage, and embryo yields for a genotype with a high degree of synchrony in microsporogenesis (SV) and one with a low degree of synchrony (OAC Triton).

	Anther length (mm)	Microspore developmental stage: Descriptive†	Numerical (x+sd)††	Embryo yields* (per anther)
SV	1.4	EU - MU	1.07 ± .24	0
	1.8	MU - LU	2.10 ± .30	0
	2.2	LU - VG	3.02 ± .10	79
	2.4	LU - VG	3.04 ± .20	1299
	2.7	LU - VG	3.94 ± .24	580
	2.9	VG - VSS	4.26 ± .37	0
OAC Triton	1.4	EU - LU	1.93 ± .32	0
	1.9	EU - LU	2.14 ± .39	0
	2.2	MU - VG	2.47 ± .57	2
	2.4	MU - VG	2.58 ± .57	51
	2.7	MU - VSS	3.83 ± .78	0
	3.1	LU - VSS	4.23 ± .69	0

† Descriptive abbreviations for range of microspore developmental stage found in a sample of 100 microspores, where EU = early uninucleate, MU = mid-uninucleate, LU = late uninucleate, VG = vegetative and generative nucleii present (binucleate), and VSS = vegetative and two sperm nucleii present.

†† Mean developmental score with standard deviation (± sd) for a sample of 100 microspores, based on arbitrarily assigned values for each stage, as follows: EU = 1, MU = 2, LU = 3, VG = 4, and VSS = 5.

* Embryo yields given are from a sample culture.

cuttings taken from haploid plants (Wenzel et al., 1977; Thompson, 1979; Hoffman et al., 1982), or immersion of rooted cuttings in a colchicine solution (Cardy, 1986). The ability to produce large numbers of haploids with relative ease allows us to colchicine-treat haploid plants directly, reducing the time required to produce DH seed by ca. two months. Immersing the roots of haploids at first flower in a 0.1% colchicine solution for five hours yields doubled sectors on >80% of plants within two months (D. Charne, unpublished data).

Diploid plants are obtained directly from microspore culture at variable frequencies. While the origin of these "spontaneous" diploids is unknown, they could arise from unreduced gametes, or from endomitosis during the first few proembryo divisions (Keller and Armstrong, 1977). We have observed considerable variation in diploid frequencies within individual genotypes (Table 2). Because spontaneous diploids offer the most rapid route to pure line production provided they are homozygous, we are currently investigating factors affecting their production with a view to maximizing their frequency. Both phenotypic uniformity within head rows and isozyme analysis of self-seed from SD plants are being used to assess the homozygosity of SD lines. Several hundred spontaneous diploid lines grown in head rows in the field in 1986 showed no phenotypic variation. Of

46 SD lines screened from a Topas x Hanna cross, only one showed heterozygosity for the glucose phosphate isomerase (GPI) enzyme (Charne et al., submitted). While these results suggest that SD lines are homozygous, and should be useable in lieu of DH lines in breeding applications, other SD populations are currently being screened, and correlated to data on phenotypic uniformity in field plots.

Table 2. Haploid and spontaneous diploid frequencies in microspore-derived populations from several lines and hybrids of spring canola.

Genotype	Population size	Haploids		Diploids	
		Number	Percentage	Number	Percentage
SV	93	68	73.1	25	26.9
RegentxWestar F_1	52	45	86.5	7	13.5
	117	86	73.5	31	26.5
	60	36	60.0	24	40.0
WestarxHanna F_1	103	99	96.1	4	3.9
RegentxTopas F_1	79	52	65.8	27	34.2
	71	57	80.3	14	19.7
TopasxHanna F_1	78	65	83.3	13	16.7
	44	28	63.6	16	36.4
Regent	37	28	75.7	9	24.3

Field trials are planned for summer, 1987, to compare the frequency distributions of DH, SD, and single seed descent (SSD) lines produced from the same spring canola hybrids for several quantitative traits (seed yield, maturity, protein and oil percentage, and fatty acid composition). A similar approach is being used to compare three generations of DH lines produced from a Regent x Topas cross, to detect any changes in agronomic performance and quality characters associated with successive cycles of haploidy. The results of these studies will provide us with estimates of the effect on genetic gain from the use of DH and/or SD lines in long-term rapeseed improvement.

The use of microspore culture in the University of Guelph rapessed breeding programme in both 1985-86 and the current year has given us important preliminary data on resource requirements and costs associated with doubled haploid line production. Given the frequencies of embryogenesis, regeneration, and chromosome-doubling cited earlier in this paper, the current haploid conversion rate in the rapeseed microspore culture system is 1,000 cotyledonary embryos x 90% survival x 30% regeneration x 80% doubling = ca. 216 doubled haploid lines, for an overall efficiency of 21%. The time required to produce DH lines for both spring and winter B. napus is shown in Table 3 (including seed increase generation); these figures are based on current averages from the Guelph programme. The resulting effect on the length of the breeding cycle is summarized in Table 4. Approximately 4,000 doubled haploid lines will be produced in the Guelph programme during the current year, at a cost of ca. $6.00 Cdn. per line.

Table 3. Approximate time required to produce doubled haploid lines using microspore culture.

	Time (in months):	Spring Types	Winter Types
1.	Embryogenesis	1.0	1.0
2.	Regeneration	1.5	1.5
3.	Plantlet to flowering	1.0	3.5
4.	Colchicine-treatment to DH seed harvest	3.0 - 5.0	3.0 - 5.0
5.	Seed increase generation (indoors)	3.5	6.0
	Total Time:	10 to 12 mos.	15 to 17 mos.

Table 4. Comparison of breeding cycles in spring and winter rapeseed using pedigree breeding (PED), single seed descent (SSD), and microspore culture (HAP).

	Spring Rapeseed			Winter Rapeseed		
	PED	SSD	HAP	PED	SSD	HAP
Stage I†	0.5††	0.5	0.5	1.0	1.0	1.0
Stage II	5.0	1.5	1.0	5.0	3.0	1.5
Stage III	3.0	3.0	3.0	4.0	4.0	4.0
Totals:	8.5	5.0	4.5	10.0	8.0	6.5

† Stage I is from selection of parents to flowering of F_1, Stage II is time required for production of homozygous (or near-homozygous) lines, and Stage III is performance evaluation of line (does not include time in cultivar licensing trials).

††All times are in years; all figures are approximate.

In Vitro Mutagenesis and Selection:

A prototype mutagenesis/selection system using haploid embryos as the selection unit has been developed for use with Brassica napus in the Crop Science Department at the University of Guelph. Use of a whole bud blender isolation technique (Polsoni et al., submitted) allows rapid and efficient extraction of very large microspore populations. Two to three hundred flower buds of ca. 4.5 mm length are homogenized in a mechanical blender, then microspores are separated by seiving, and washed prior to culturing. Using this procedure, we can process 200 to 250 million microspores at a time, which produce many hundred thousand haploid embryos within three weeks of isolation.

Following isolation, and prior to culture, microspores are exposed to gamma radiation or sodium azide at a level which reduces embryogenesis by 50% (LD_{50}), and is potentially mutagenic. Following mutagenesis, microspores are cultured in Lichter's (1982) medium, as modified by Polsoni et al. (submitted). Early cotyledonary stage embryos are transferred to

large flasks containing liquid B_5 medium with a selection agent present at approximately 110% of the minimum lethal dosage. The flasks are placed on a rotary shaker in the light for seven to ten days, during which time embryos germinate while exposed to the selection agent. Surviving green embryos are sub-cultured onto regeneration medium to allow plantlet formation; haploid regenerates are colchicine-doubled, then self-pollinated to produce S_1 seed. The S_1 generation is then screened with the selection agent to determine expression of the mutation at the whole plant level, and to eliminate "escapes" (i.e. non-mutants which survived exposure to the selection agent in culture).

To date, we have used this prototype system in conjunction with our rapeseed breeding programme to select for resistance to two herbicides, chlorsulphuron and glyphosate. We are currently modifying our selection techniques to accommodate selection for resistance to other herbicides and specific mycotoxins, as well as altered fatty acid composition and ultra-low glucosinolate content. The *in vitro* mutagenesis/selection system developed at Guelph is attractive for breeding applications because very large embryo populations can be handled efficiently and inexpensively; both recessive and dominant mutations are easily identified in haploid tissue, and desirable mutants can be easily doubled with colchicine to produce fertile diploids.

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