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Double Haploids in *Brassica napus* L. Breeding Programs

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Abstract
The recovery of doubled haploid plants from anther or microspore culture is a constraint in breeding programs. In *Brassica napus* the extent of spontaneous diploids varied from 4 to 38 percent. The conventional diploidization of juvenile haploids with colchicine is labour intensive and requires a generation for seed multiplication. This study examines the potential from *in vitro* diploidization of microspores and increasing the efficiency of doubled haploid breeding programs.

Introduction
Haploid embryos of *B. napus* were first obtained from anther culture (Thomas and Wenzel 1975; Keller and Armstrong, 1978). The development of the isolated microspore culture technique in rapeseed by Lichter, 1982 enabled high frequencies of embryogenesis and the rapid production of homozygous doubled haploid (DH) lines. Studies on early morphogenesis of microspores *in vitro* have shown symmetrical division taking place before embryogenesis (Nitsch, 1972; Fan *et al*., 1988; Zaki and Dickinson, 1990). Heat treatment of microspores shortly before first pollen mitosis lead to a first symmetrical division in *B. campestris* (Hamaoka *et al*., 1991) and in *B. napus* (Zaki and Dickinson, 1991), causing a higher rate of embryo regeneration. Recently, other factors like N-starvation (Ferrie and Keller, 1997) and a short term colchicine treatment of isolated microspores of *B. napus* were reported to result in a highly significant increase in embryo regeneration (Zaki and Dickinson, 1991; Iqbal *et al*., 1994). Plants regenerated from microspore-derived embryoids (MDEs) can be haploid, diploid or polyploid. From *B. napus* microspore culture it is reported that 70-95 percent of the plants regenerated from MDEs are haploid (Lichter *et al*., 1988; Chen and Beversdorf, 1992; Mollers *et al*., 1994). Conventional chromosome doubling techniques involve colchicine treatment of whole plants in the greenhouse. Immersion of roots in a colchicine solution is most common (Gland, 1981). Other alternatives are injecting a 0.2 percent solution of colchicine into the secondary buds (Lichter *et al*., 1988), applying a cotton swab soaked in colchicine to the buds (Gland, 1981), or treatment of whole *in vitro* plantlets (Mathias and Robbelen, 1991). However, besides being laborious, these treatments share the disadvantage that the induction of chromosome doubling in rapeseed rarely exceeds 50-70 percent, which includes spontaneous diploidizations. In addition, problems with chimeras frequently occur. As a consequence, seed set is usually reduced and seed harvest is delayed for several weeks. Thus, an additional generation for multiplication may become necessary to allow seed quality analyses and yield trials in the field.

The recently developed *in vitro* treatment of freshly isolated *Brassica napus* microspores with colchicine or other potent mitotic inhibitors leads to a high diploidization rate of 80 -90 percent of the regenerating plantlets. The treatment is performed with a low dosage of 10-100 mg/l of colchicine for a limited duration of up to 3 days (Chen *et al*., 1994; Mollers *et al*., 1994; Hansen and Andersen, 1996). Plantlets regenerated from the so treated microspores can be transferred to the greenhouse without further laborious cytological investigations; however, this implies that losses of up to 20-30 percent of the plantlets due to haploidy or polyploidy have to be taken into account. Alternatively, using a flow-cytometric equipment the ploidy level of the MDEs can be determined as early as 4 weeks after microspore culture. Ploidy can be determined from single detached cotyledons 5-10 mg in fresh weight so that the remainder of the embryoids can be regenerated to plants. This saves labour and space capacities in the tissue culture lab and in the greenhouse. As a positive side effect, the colchicine treatment improves embryogenesis and revealed no negative effect on the further development of the embryos (Zaki and Dickinson, 1991; Iqbal *et al*., 1994). The objectives of this study was to evaluate the overall changes and contributions in the DH breeding programme through *in vitro* chromosome doubling in isolated microspore culture of *Brassica napus*.

Materials and Methods
The spring rape genotypes ' Duplo', ' Janetzki', WP27 and the F_1 hybrid (Janetzki x Duplo) were grown in the greenhouse as donor plants. When the first buds appeared the plants were transferred to a growth chamber with a 16 h photoperiod, a day/night temperature of 12°C/8°C, and a relative humidity of 80 percent. The plants were fertilized fortnightly with a 0.2 percent solution of commercial fertilizer (N:P:K = 15:11:15) and watered as required. Flower buds 3 to 4 mm in length were collected from the primary and the lateral inflorescence. Isolation and culture of the microspores were carried out under sterile conditions on a laminar flow bench as follows. The buds were surface sterilized for 5 min in a 0.5 percent

calcium hypochlorite solution with periodic agitation and rinsed thrice in sterile deionized water. A sepal was removed from the buds under a binocular microscope and those having a petal : anther length ratio of 0.50: 0.75 were selected for microspore culture. The buds were placed in a metal sieve basket of diameter 240 mm with 25 ml of the induction medium NLN 82, as described by Gland *et al*. (1988) and crushed gently with the flat end of a pestle to release the microspores. The sieve containing debris from the buds was removed and the suspension of microspores passed through a second nylon sieve of diameter 40 mm. The microspore suspension was centrifuged at 100 x g for 5 min and the turbid supernatant discarded. The pellet of microspores was suspended in NLN medium and centrifuged as before. After discarding the now clear supernatant, the microspore pellet was resuspended in NLN medium. The microspores were plated at a density of 2-3 bud equivalents per ml of NLN medium. The microspores were cultured in plastic petri-dishes (55 x 15 mm) containing 30 ml of a 1 percent sterile suspension of active charcoal per ml of NLN medium. The microspore cultures were then incubated at 30°C for 5 days in darkness and thereafter transferred to a shaker (90 rpm) under light and 25°C thereafter. Embryoids were observed after 7 days and in some genotypes after 14 days of culture. Fresh NLN medium was exchanged every week and the density of embryoids reduced to maintain vigor.

Colchicine treatment

Microspores: A stock solution of colchicine (0.05%) in NLN medium was prepared and filter-sterilized using a bacterial filter (Sartorius NML, pore size 0.2 mm). Appropriate volumes from the stock solution were added to the induction medium to give the desired final concentrations of 10, 50 and 100 mg/l colchicine; respectively. After the colchicine treatment the microspores were washed twice by centrifuging the microspore suspension with fresh NLN medium and discarding the supernatant. The microspores were then resuspended in NLN medium and incubated.

Haploid plants: Potted microspore derived plants in good vigor were freed from soil, the roots clipped back and placed in a 0.3 percent solution of colchicine for 2 hours. Thereafter they were washed in running water and repotted and left under shade to recover and produce new shoots.

Regeneration of embryoids to plants: Embryoids at the cotyledonary stage (3 to 5 mm) were transferred to a regeneration medium modified with 400 mg/l magnesium sulphate, no hormones and solidified with 0.8 percent agar. Each petri dish (55 x 15 mm) contained 10 embryoids in 25 ml medium. The embryoids were maintained at 25°C and a 16 h photoperiod for regeneration to plants. After development of roots and normal leaves, the plantlets were transferred to peat soil and kept under a moist plastic hood for 2 to 3 weeks. The established plants were then transplanted in a mixture of compost, peat and sand (3:2:1) using a series of plastic pots with increasing growth.

Data analysis: The percentage data was transformed using 'arc sine'. The treatment parameters were analysed as a single factor by analysis of variance and treatment differences were determined using Duncan's multiple range analysis.

Results

The *in vitro* culture of microspores produced microspore derived embryoids after 3-4 weeks of culture. However, the extent of spontaneous diploidy was low, ranging from 5-37 (Fig. 1) thus requiring colchicine treatment to produce double haploid plants. By using colchicine in the induction medium, doubled haploid embryoids were produced. A colchicine concentration of 50 mg/l for 24 h produced the maximum diploidy of the embryoids (Table 1) though not significantly different from 25 mg/l. Plants from the embryoids were raised in the greenhouse and the effect of *in vitro* diploidization was determined on plant regeneration, ploidy of the plants and greenhouse performance.

Regeneration of embryoids to plants: To regenerate plants, morphologically well developed embryoids at the

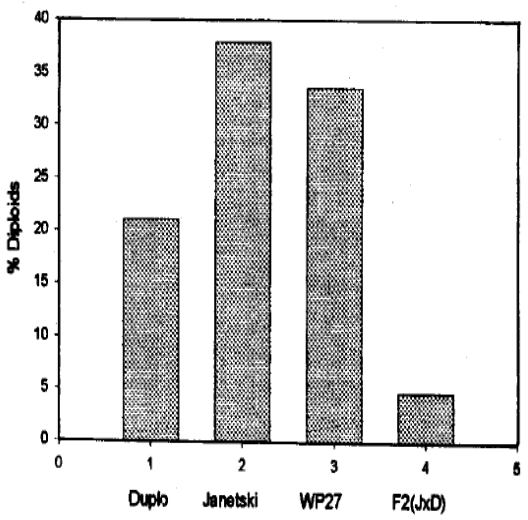

Fig. 1: The frequency of spontaneous diploids of microspore derived embryoids from 4 genotypes of *B. napus*.

cotyledonary stage were transferred from the liquid induction medium to regeneration medium with 3 percent sucrose four weeks after microspore culture. The embryoids developed roots and a shoot emerged from between the cotyledons, which produced secondary leaves. However, the direct regeneration of a plantlet from an embryo was inconsistent. Fertility of the plants: The plants from the

Table 1: Influence of colchicine treatment of microspores for 24 hours on the diploidization of embryoids.

Colchicine concentration mg/l	Percent Diploids	
	Duplo	Janetzki
25	71.8 ab	82.9 a
50	85.0 b	89.3 a
100	80.5 b	76.2 a

Means followed by a common letter are not significantly different at the 5 percent level.

Table 2: Frequency of diploids determined at the embryoid and plant level.

Treatment	n	Diploids (%)		
		Embryoids	Plants	Mean No. of pods
Duplo 100 C	73	72	74	27 ± 6
Janetzki 100 C	63	76	89	13 ± 3

C - colchicine mg/l - 24 h. ± standard deviation

Table 3: Time required (in weeks) for the different stages to produce DH lines by the conventional and the improved method.

Stage	Conventional	Improved
Microspore culture of F1 to MDE	3-4	3-4
Regeneration to plants	6-8	4
Hardening in greenhouse	2	2
Flowering and seed maturation	12-16	10-12
Seed increase in greenhouse	12-16	-
Total time in weeks	35-46	19-24

colchicine treated microspores were morphologically similar to seed raised plants of the respective varieties. Fertile flowers were produced in three months from transfer to the greenhouse. Pod set from open pollination for Duplo' ranged from 16 to 36 pods and for Janetzkir from 8 to 18 pods per plant (Table 2).

Plants from microspore culture without colchicine in the induction medium were predominantly haploid with small pale coloured leaves and thin stems. The flowers were small with shrunken anthers producing very little or no pollen. Pod set was rare and if at all had few seeds. These plants were routinely treated with colchicine in the greenhouse, which produced diploid shoots emerging from new axillary buds. These shoots eventually produced fertile flowers and pod set.

Other ploidy levels: The treatment of microspores *in vitro* with colchicine produced a few tetraploid and mixoploid embryoids. This was dependent on the genotype, concentration and duration of colchicine in the culture medium of the microspores. Of the four genotypes tested, two produced only haploid and diploid embryoids while in cv. Duplo 0.45 percent of the embryoids were tetraploid and cv. Janetzki produced 1.42 percent tetraploids (Fig. 2).

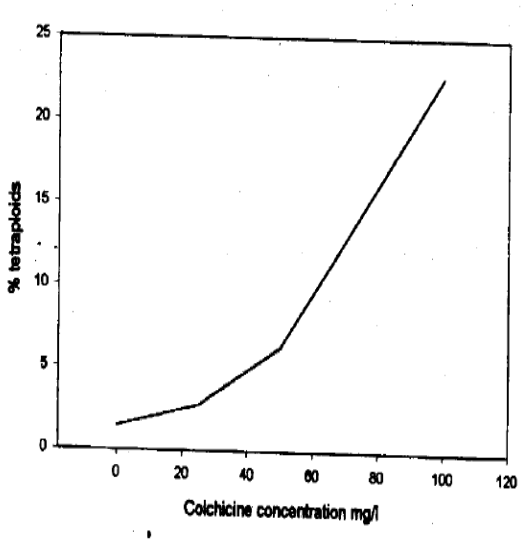

Fig. 2: Effect of a 24 h treatment of microspores with different colchicine concentrations on the frequency of tetraploid embryoids in the cv. Janetzski.

Some plants were observed with large flowers and producing profuse pollen. However, pod set was low or nil in these flowers. Flow cytometric analysis of their leaves showed, 6 of the plants were tetraploid and one was mixoploid (haploid and tetraploid).

Diploidization *in vitro*: Microspore derived embryoids were raised to plants from *in vitro* colchicine treated (Improved method) and non-treated microspore cultures (Conventional method). The latter were haploid plants raised in the greenhouse and treated with colchicine by the root dip method. The duration in time for the Improved and Conventional method for the different stages in the production of DH plants from microspore culture to seed harvest in the greenhouse is compared in Table 3.

Stage A: The duration from microspore culture to embryoids with 2 cotyledons is independent of colchicine in the culture medium. Morphological differences were not observed in the embryoids between the two methods of culture.

Stage B: The embryoids from the conventional microspore culture however produced callus or secondary embryoids along the stem while only a few embryoids underwent direct embryogenesis. This indirect regeneration of plants contributed to non-synchronous development and maturation and delayed regeneration by 2-4 weeks. The diploid embryoids of the improved method underwent direct embryogenesis with a low incidence of callus formation.

Stage C: Was similar to both methods of culture.

Stage D: In the conventional method the roots of haploid plants were clipped and dipped in colchicine solution. New diploid shoots developed from which fertile flowers and seed set occurred after 12-16 weeks. Some plants did not survive the colchicine treatments. The plants from the improved method developed and matured with flowering and seed set (Table 3).

Stage E: The DHs from the improved method produced sufficient pods, thereby avoiding an additional generation for seed increase. DHs from the conventional method set seed from flowers that developed from the new sectors produced after colchicine treatment. These diploid shoots produced fewer flowers and pod set.

The significant savings in time are in Stages B, D and the avoidance of an additional generation to produce seeds in E. A saving of about 19 weeks and of the extra resources (colchicine treatment, repotting, washing of roots etc.) and costs associated with stage D is possible by *in vitro* diploidization at the microspore stage.

Discussion

Regeneration of embryoids to plants: A major drawback in DH breeding programs in *Brassica napus* in particular and others in general is the difficulty and delay in regeneration of embryoids to plants. On transfer to a regeneration medium, most of the embryoids fail to develop shoots from the apical meristem and instead form a callus and undergo secondary embryogenesis. Previous regeneration of *B. napus* embryoids also confirms these findings. In anther culture derived embryoids of *B. napus* only 10 percent developed directly into plants (Naleczynska and Krzymanski, 1987).

Beversdorf *et al*. (1987) reported 30 percent regeneration, while according to Kott and Beversdorf (1990) this varies from 8 -50 percent. However, Sieble and Pauls (1989) obtained less than 5 percent plants directly from the embryoids. In our study colchicine treatment of microspores produced embryoids from which direct regeneration varied from 30-40 percent and reduced callusing and secondary embryogenesis. This was associated with an increase in shoot formation directly from the apical meristem. This suggests an association between diploidy of the embryoids and direct plant development, whereas Sieble and Pauls (1989) showed that 80 percent of the plants that developed directly from embryoids were haploid. However, in wheat anther culture, treatment of anthers with colchicine reduced the number of abnormal albinos significantly from 14 to 2 percent (Barnabas *et al*., 1991).

Diploidization *in vitro* and *in vivo*: The production of homozygous lines using doubled haploids by conventional chemical doubling requires one year compared to the 5 years by the pedigree method (Beversdorf *et al*., 1987). This study was an attempt to improve chromosome doubling in conventional DH breeding programs by inducing double haploidy at the microspore stage.

The ultimate objective of colchicine treatment of haploid plants is to cause chromosome doubling to produce fertile flowers. For this the sub-epidermal cells of haploid plants at the new growing point should be diploidized following colchicine treatment, since this layer of cells gives rise to the gametophytic cells (Jensen, 1974). In the conventional scheme of chromosome doubling the colchicine treatment of juvenile haploid plants is immediately followed by a retardation of growth. The recovery or survival of plants from this method is usually low at 30 percent (Jensen, 1974). For the breeder this is a delayed and non-synchronous regeneration of his material and possibly loss of valuable recombinants. The new sectors that arise following colchicine treatment develop mericlinal haploid/diploid cyto-chimeras where different cell layers at the growing point are of different chromosomal ploidy. For practical purposes the fertility of flowers is the most useful indicator of haploid or diploid plants. However, due to the cytochimeras and inefficiency of colchicine treatment the new fertile flowers are few and seed set is poor. In tobacco colchicine treatment of haploid plants yielded 30-40 percent diploids, 20-30 percent polyploids and the rest were haploid (Burk *et al*., 1972). Thus seeds for quality analysis and evaluation of DHs are taken from the second generation after chromosome doubling. In contrast, the DHs from the improved method in this study yielded sufficient pods thereby avoiding an additional generation for seed increase. The seeds are sufficient to screen for fatty acids, glucosinolates and for field testing without seed multiplication.

Colchicine is toxic to animal cells at concentrations that have no measurable effect on plant cells. Protection of persons handling the colchicine and disposal of large volumes of colchicine solutions is a 'routine evil' in diploidization. In large DH breeding programs colchicine treatment requires the process of washing the roots free of soil, colchicine treatment of plants, washing and repotting the plants which is a laborious process. Colchicine treatment of microspores requires 500 µl of a 0.05 percent solution for a single microspore culture, while treatment of 30 haploid plants by the conventional method require 150 ml of a 0.3 percent solution. This is a considerable saving of resources in the long term and reduces the exposure and contact with toxic colchicine by green house personnel.

Application of DH lines in plant. breeding: The rapid use of DH lines requires the primary DH plants to yield enough seeds for quality analysis and for an observation plot in the field. This amount of seed is only obtained regularly from DH plants following early diploidization of microspores. Direct diploidization of microspores has in addition the advantage that in the greenhouse plant development and

seed harvest is synchronized. Following the standard DH method an additional generation for seed multiplication is usually necessary.

Theoretically the use of DH lines should be less efficient in crosses where one of the parents is not adapted or lacks required quality traits. In such materials pedigree selection in early segregating generations is relatively easy whereas unselected DH lines would contain a large proportion of unacceptable types. If, on the contrary, crosses among relatively narrow elite material are performed, visual selection in early generations of a pedigree program is difficult and DH lines have the advantage that the genetic variance among them is higher than in any other generation. There is still some debate on the question which generation should be used for the production of DH lines. In most cases Fl derived DH lines are used to get a maximal gain in time, but DH production from F2 or F3 plants will allow for more recombination and moreover some preselection of the material (Snape, 1997).

Selection *in vitro*: Besides providing completely homozygous lines, microspore culture of B anpus offers the possibility of *in vitro* selection to improve the efficiency of the DH breeding programme. The present attempts to increase single fatty acids through mutation breeding is based on changes in the recessively inherited genes. Such genes can be effectively selected in DH lines. Development of appropriate screening methods of *in vitro* embryos would improve the efficiency of the DH program by reducing the field testing of unnecessary genotypes. In *B. napus*, seeds from a single DH plant from the improved method is sufficient for screening glucosinolate content, fatty acid composition and for planting. Using a segregating population of MDEs Albrecht *et al*. (1995) showed that an efficient selection of genotypes with a low and a high erucic acid content in the seeds, respectively, can already be performed by analysis of the fatty acid composition of one cotyledon, dissected *in vitro* at an early stage of the development.

The *in vitro* diploidization of microspores in *Brassica napus* enables a saving of 16-22 weeks besides enabling testing for seed quality prior to field testing. It also enables synchronous development of plants in the greenhouse and normal seed set. The use, disposal and exposure of colchicine is considerably reduced. Further improvements in *in vitro* screening of embryoids for quality traits shbuld increase the efficiency of the DH breeding program.

References

Albrecht, S., C. Mollers and G. Robbelen, 1995. Selection *in vitro* for erucic acid content in segregating populations of microspore derived embryoids of *Brassica napus*. Plant Breeding, 114: 210-214.

Barnabas, B., P.L. Pfahler and G. Kovacs, 1991. Direct effect of colchicine on the microspore embryogenesis to produce dihaploid plants in wheat (*Triticum aestivum* L.). Tag. Theor. Applied. Genet., 81: 675-678.

Beversdorf, D.D., D.G. Charne, L.S. Kott, P.V. Chuong, L. Polsoni and J. Zilka, 1987. The utilization of microspore culture and microspore-derived doubled-haploids in a rapeseed (*Brassica napus* l.) breeding programme. Proceedings of the 7th International Rapeseed Congress, May 11-14, 1987, Poznan, Poland.

Burk, L.G., G.R. Gwynn and J.F. Chaplin, 1972. Diploidized haploids from aseptically cultured anthers of *Nicotiana tabacum* a colchicine method applicable to plant breeding. J. Heredity, 63: 355-360.

Chen, J.L. and W.D. Beversdorf, 1992. Production of spontaneous diploid lines from isolated microspores following cryopreservation in spring rapeseed (*Brassica napus* L.). Plant Breed., 108: 324-327.

Chen, Z.Z., S. Snyder, Z.G. Fan and W.H. Loh, 1994. Efficient production of doubled haploid plants through chromosome doubling of isolated microspores in *Brassica napus*. Plant Breed., 113: 217-221.

Fan, Z., K.C. Armstrong and W.A. Keller, 1988. Development of microspores *in vivo* and *in vitro* in *Brassica napus* L. Protoplasm, 147: 191-199.

Ferrie, A.M.R. and W.A. Keller, 1997. Culture media components and their effect on *Brassica* microspore embryogenesis. Proceedings of the 10th Crucifer Genetics Workshop, September 23-27, 1997, Rennes, France, pp: 157.

Gland, A., 1981. Doubling chromosomes in interspecific hybrids by colchicine treatment. Eucarpia Cruciferae Newslett., 6: 20-22.

Gland, A., R. Lichte and H.G. Schweiger, 1988. Genetic and exogenous factors affecting embryogenesis in isolated microspore culture of *Brassica napus*. Plant Physiol., 132: 613-617.

Hamaoka, Y., Y. Fujita and S. Iwai, 1991. Effects of temperature on the mode of pollen development in anther culture of *Brassica campestris*. Physiol. Plant., 82: 67-72.

Hansen, N.J.P. and S.B. Andersen, 1996. *In vitro* chromosome doubling potential of colchicine, oryzalin, trifluralin and APM in *Brassica napus* microspore culture. Euphytica, 88: 159-164.

Iqbal, M.C.M., C. Mollers and G. Robbelen, 1994. Increased embryogenesis after colchicine treatment of microspore cultures of *Brassica napus* L. J. Plant Physiol., 143: 222-226.

Jensen, C.J., 1974. Chromosome Doubling Techniques in Haploids. In: Haploids in Higher Plants Advances and Potential, Kasha, K.J. (Ed.). University of Guelph, Guelph, pp: 153-190.

Keller, W.A. and K.C. Armstrong, 1978. High frequencey production of microspore derived plants from *Brassica napus* anther cultures. Z Pflanzenzucht, 80: 100-108.

Kott, L.S. and W.D. Beversdorf, 1990. Enhanced plant regeneration from microspore-derived embryos of *Brassica napus* by chilling, partial desiccation and age selection. Plant Cell Tissue Organ. Cult., 23: 187-192.

Lichter, B., 1982. Induction of haploid plants from isolated pollen of *B. napus*. Z. Pflanzenphysiol., 105: 427-434.

Lichter, R., E. Groot, D. Fiebig, R. Schweiger and A. Gland, 1988. Glucosinolates determined by HPLC in the seeds of microspore derived homozygous lines of rapeseed (*Brassica napus* L.). Plant Breed., 100: 209-221.

Mathias, R. and G. Robbelen, 1991. Effective diploidization of microspore derived haploids of rape (*Brassica napus* L.) by *in vitro* colchicine treatment. Plant Breed., 106: 82-84.

Mollers, C., M.C.M. Iqbal and G. Robbelen, 1994. Efficient diploidization of *Brassica napus* by colchicine treatment of microspores and regeneration of doubled haploid plants. Euphytica, 75: 95-104.

Naleczynska, A. and J. Krzymanski, 1987. Regeneration and ploidy level of plants from anther cultures of winter rapeseed. Proceedings of the 7th International Rapeseed Congress, Volume 1, May 11-14, 1987, Poznan, pp: 103-106.

Nitsch, J.P., 1972. Haploid plants from pollen. Z. Pflanzenzuchtung, 67: 3-18.

Sieble, J. and K.P. Pauls, 1989. Alkenyl glucosinolate levels in androgenic populations of *Brassica napus*. Plant Breed., 103: 124-132.

Snape, J.W., 1997. Application of Doubled Haploid Lines in Plant Breeding and Genetical Research: Current Issues and Approaches. In: Advances in Biometrical Genetics, Krajewski, P. and Z. Kaczmarek (Eds.). Institute of Plant Genetics, Polish Academy of Sciences, Poznan, ISBN: 9788385583165, pp: 35-46.

Thomas, E. and G. Wenzel, 1975. Embryogenesis from microspores of *Brassica napus*. Z. Pflanzenzuchtg, 74: 77-81.

Zaki, M.A.M. and H.G. Dickinson, 1990. Structural changes during the first divisions of embryos resulting from anther and free microspore culture in *Brassica napus*. Protoplasma, 156: 149-162.

Zaki, M.A.M. and H.G. Dickinson, 1991. Microspore-derived embryos in *Brassica*: The significance of division symmetry in pollen mitosis I to embryogenic development. Sexual Plant Reprod., 4: 48-55.