Theor Appl Genet (1989) 77:489–494

Inheritance patterns of erucic acid content in populations of *Brassica napus* microspore-derived spontaneous diploids

J. Siebel* and K.P. Pauls

Crop Science Department, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Received September 30, 1988; Accepted December 6, 1988
Communicated by G. Wenzel

Summary. The levels of erucic acid and other fatty acids in seeds of microspore-derived spontaneous diploid plants from crosses between low and high erucic acid parents were examined. The analysis confirmed that erucic acid is simply inherited and is determined by two genes that act in an additive manner. The effects of the genes for erucic acid on the levels of the other fatty acids was also determined and many significant correlations were found. In particular, erucic acid levels were negatively correlated with oleic acid and linoleic acid levels. The study also illustrates several advantages of using haploidy to analyze the inheritance of agronomically important traits. In particular, the number of phenotypic classes is smaller in androgenic populations and differences between classes are greater than in an F_2 population.

Key words: Microspore culture – Spontaneous diploids – Erucic acid – Inheritance – *Brassica napus*

Introduction

Because high levels of erucic acid in the cooking and salad oil extracted from rapeseed (*Brassica napus* and *Brassica campestris*) have been associated with health problems (Beare et al. 1963), a major effort was made in the 1960s to develop low erucic acid rapeseed (LEAR) varieties. The development of LEAR lines was facilitated by the demonstration that the fatty acid composition of the seed is controlled by the genotype of the embryo rather than the maternal plant (Downey and Harvey 1963). This allowed the genetic analysis of populations to be based on the fatty acid analysis of single seeds or half seeds (Downey and Harvey 1963) on F_1 plants.

Although low erucic acid varieties are required for the production of edible oils, there is considerable interest in high erucic acid varieties for utilization as feed stocks for chemical industries. High erucic acid oils are excellent lubricants and the fatty acid can be modified in numerous ways to produce water repellents, plasticizers, waxes and surface-active agents.

The erucic acid content of seeds of the amphidiploid species *Brassica napus* ($2n=38$) has been shown to be governed by two genes which display no dominance and which act in an additive fashion (Downey and Craig 1964; Dorrell and Downey 1964; Harvey and Downey 1964; Stefansson and Hougen 1964). It has also been determined that multiple alleles occur at each locus (Dorrell and Downey 1964; Stefansson and Hougen 1964; Krzymanski and Downey 1969; Jonsson 1973, 1974, 1977a, b, 1978). At least five alleles govern the erucic acid in *Brassica*, including: e, E^a, E^b, E^c and E^d which contribute $<1\%$, 10%, 15%, 30% and 3.5% erucic acid, respectively. Thus, levels of erucic acid can be fixed at a large number of values ranging from $<1\%$ to $>60\%$ (Krzymanski and Downey 1969; Jonsson 1977a).

Brassica napus has been shown to be responsive to anther (Thomas and Wenzel 1975; Keller and Armstrong 1978) and to microspore culture techniques (Lichter 1982; Chuong and Beversdorf 1985). Haploidy techniques have been used in breeding programs to rapidly fix traits in homozygous condition. This has resulted in a savings of several years of variety development time in barley, wheat and tobacco, and in other species (Morisson and Evans 1988). Another significant advantage of androgenesis is that the genetic ratios in the populations

* Current address: King Agro Inc., P.O. Box 1088, Chatham, Ontario, Canada N7M 5L6

of doubled haploid plants that are produced from F_1s are much simpler than those seen in an F_2 population. Consequently, smaller populations of doubled haploids need to be produced to obtain rare genotypes, particularly those in which a number of recessive traits are combined (Choo et al. 1985).

In this study, the inheritance of erucic acid was examined in populations of spontaneous diploid plants produced from microspore cultures initiated from three F_1s. In addition, the effects that the genes determining erucic acid levels had on the levels of the other fatty acids in the seeds were determined.

Materials and methods

Plant material

The donor plants for microspore culture were two F_1 hybrids obtained from crosses between individual plants of the spring *Brassica napus* L. cultivars Regent (canola) and Golden (rapeseed) and their reciprocals. A cross between a highly embryogenic, canola breeding line G231 and the cultivar Reston (high erucic, low glucosinolate) was also used to initiate androgenic cultures.

Donor plants for microspore culture were grown from the F_1 hybrid seed. Randomly chosen seeds of each cross were planted in Jiffy-7 peat pellets and were transferred after 2 weeks to 15-cm (6″) plastic pots containing MetroMix (W. R. Grace and Co., Ajax, Ontario) potting medium. The plants were grown in an evironmentally controlled growth room under a 16-h photoperiod and a 22 °C day/18 °C night temperature regime. Grolux Wide Spectrum and Cool White fluorescent lamps (Sylvania Canada, Drummondville, Canada) supplied a photosynthetic photon flux density of 250 $\mu M\ m^{-2}\ sec^{-1}$ at bench level. The plants were watered as required and 20:20:20 (N:P:K) fertilizer was applied twice weekly at a rate of 1 $g\ L^{-1}$.

Microspore culture techniques

Buds 4–5 mm in length were removed from the terminal and the upper two axillary racemes of donor plants that had approximately five open flowers on the terminal raceme. The buds were surface-sterilized in a 5.7% hypochlorite solution for 10 min and were subsequently rinsed three times (for 5 min) with autoclaved, deionized-distilled water. Intact anthers were removed from buds which had petal-to-anther ratios between 1:2 and 2:3.

The anthers were placed in the liquid B5 medium and macerated with a teflon rod to release the microspores. The resulting suspension was filtered through 63 μm and 44 μm Nytex filters and the filtrate was centrifuged at 1,000 rpm for three min to pellet the microspores. The supernatant was removed and the microspores were resuspended in B5 wash and centrifuged at 1,000 rpm for 3 min. This step was repeated three times. The final microspore pellet was resuspended in a modified Nitsch and Nitsch (1967) liquid NLN medium (Lichter 1982; Chuong and Beversdorf 1985) to give a microspore density of approximately 204,000 microspores ml^{-1}. One ml of microspore suspension was added to 60 × 15 mm Falcon plates, which already contained 1.5 ml of liquid NLN, to give a final microspore density of 81,600 microspores ml^{-1}. Microspore cultures were incubated in the dark at 32 °C for 3 days followed by 19 days at 25 °C (Chuong and Beversdorf 1985).

Embryo culture plant regeneration and selfed seed production

After 22 days, the cotyledonary embryos (Chuong and Beversdorf 1985) that were produced in the microspore cultures were transferred to solid B5-H medium containing 2% sucrose and 0.8% agar without hormones (Gamborg et al. 1968). Additional cotyledonary embryos, which had previously been too small, were transferred onto solid B5-H from the microspore culture plates after 25 days. After 4 weeks, plantlets that had developed normal shoots were transferred to flats containing MetroMix and covered with a clear plastic lid. Additional plantlets were produced by explanting abnormally-growing embryos onto fresh B5-H. These were also transferred to the growth room.

At flowering, the spontaneous diploids were bagged to produce selfed seed

Field seed increase

Selfed seed from the microspore-derived spontaneous diploid lines was planted in the field in 1-m rows with an intra-row spacing of 10 cm and an inter-row spacing of 50 cm. A randomized complete block design with three blocks was used. The following were planted: 13 lines from the cross designated Regent-2 × Golden-2 (R2 × G2), 16 lines from the cross designated Regent-14 × Golden-20 (R14 × G20) and 47 lines from the cross Reston × G231 (Re × G231), along with their respective parents and the check varieties Westar and Global.

Fatty acid analysis

The seeds from each headrow were ground to a fine meal. For each headrow, a 200-mg sample of meal was weighed into a vial, and fatty acid methyl esters were produced by adding 2 ml of hexane, 0.15 ml of sodium methoxide ($NaOCH_3$) and 0.55 ml of anhydrous ether (Shehata et al. 1970). After 10 min the supernatant was decanted into another vial and 1 ml of dilute hydrochloric acid was added. After another 10 min, 0.5 ml of the upper layer of the supernatant was removed and the fatty acid profiles were determined by gas-liquid chromatography using a Hewlett-Packard 5710A GLC with a stainless steel column packed with GP 10% SP-2330 on 100/200 chromosorb W AW (Chromatrographic Specialties Inc., Brockville, Canada).

Results

The large difference between the erucic acid content of the Regent-2 parent, which had 0.1%, and the Golden-2 parent, which had 42.0%, is apparent in Fig. 1. The R2 × G2 microspore-derived lines were distributed into three classes containing 0%–3%, 21%–30% and 33%–48% erucic acid in their seeds. The frequencies fit a 1:2:1 segregation ratio ($P > 0.25$). Several R2 × G2 lines that had higher levels of erucic acid than the Golden parent and a few lines equal to the Regent parent were also present.

The erucic acid contents of the seeds of the Regent-14 and Golden-20 parents were 0.0% and 37.3%, respectively (Fig. 2). The R14 × G20 lines were divided into classes containing 0%–3%, 15%–30% and 35%–45% erucic acid in their seeds. A Chi-square test ($P > 0.75$) indicated that a 1:2:1 ratio fit the distribution of the R14 × G12 spontaneous diploid lines in the low-,

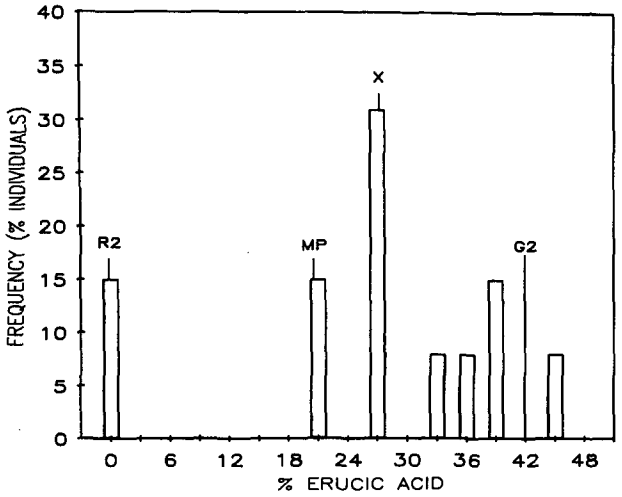

Fig. 1. Frequency distribution of erucic acid levels in seeds of 13 microspore-derived lines obtained from Regent-2 × Golden-2 (R2 × G2). The mean erucic acid levels of the parents grown in the same test are given, Regent (R2) = 0.1%, Golden (G2) = 42.0%. The midparent value (MP) was 21.1% while the population mean (X) was 27.9%

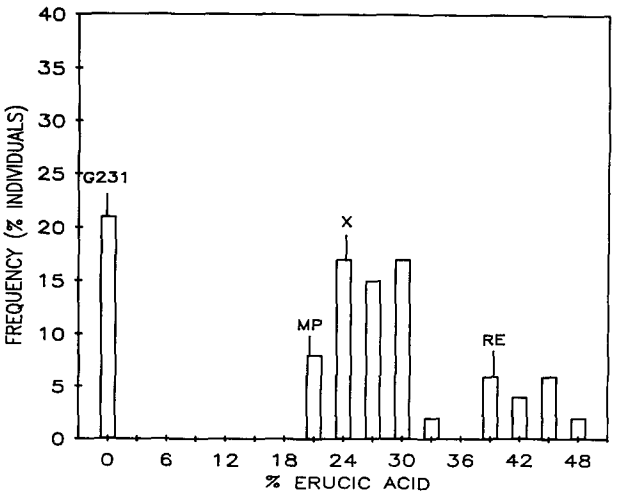

Fig. 3. Frequency distribution of erucic acid levels in seeds of 47 microspore-derived lines obtained from the Reston × G321 (Re × G321) cross. The mean of the parents grown in the same test are given, G231 (G231) = 0.3%, Reston (RE) = 41.6%. The midparent value (MP) was 21.0% while the population mean (X) was 25.3%.

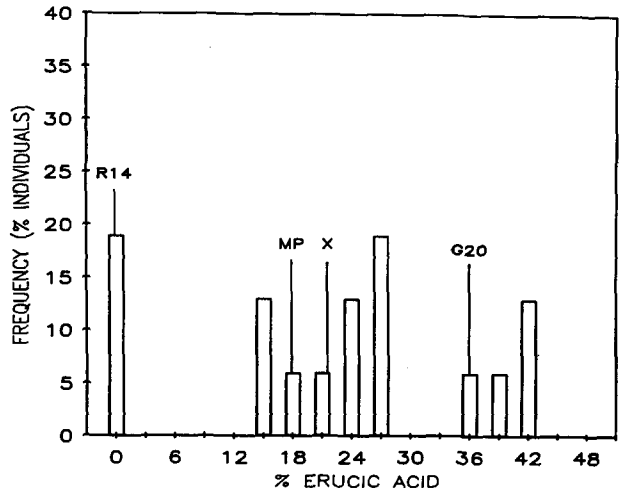

Fig. 2. Frequency distribution of erucic acid levels in seeds of 16 microspore-derived lines obtained from Regent-14 × Golden-20 (R14 × G20). The mean erucic acid levels of the parents grown in the same test are given, Regent (R14) = 0.0%, Golden (G20) = 37.3%. The midparent value (MP) was 18.6% while the population mean (X) was 23.3%

medium- and high-erucic acid classes, respectively. Several lines that transgressed the high parent and several lines that did not contain erucic acid were present in the R14 × G20 spontaneous diploid population.

The G321 and Reston parents had erucic acid levels of 0.3% and 41.6%, respectively (Fig. 3). The Re × G321 lines had erucic acid levels that placed them into three classes with 0%–3%, 21%–36% and 39%–51% erucic acid. The lines were distributed among these classes in a 1:2:1 ratio with a Chi-square probability of >0.25. Twenty percent of the Re × G321 lines had an erucic acid content similar to the low parent. Approximately 15%–20% of the Re × G231 lines had higher erucic acid levels in their seeds than the high parent.

Figure 4 illustrates the relationships between the levels of erucic acid and the other fatty acids in the three populations. In general, high levels of erucic acid were associated with low levels of palmitic acid (16:0), oleic acid (18:0) and linoleic acid (18:2). Linolenic acid (18:3) levels increased with increasing concentrations of erucic acid in the populations derived from the Regent × Golden crosses, but decreased in the lines derived from the Reston × G321 cross (Fig. 4). The negative correlations between erucic acid levels and other fatty acid levels in Re × G321 were statistically significant (Spearman correlation coefficient significant at 1% or 5% level). This was also true for the R2 × G2 and R14 × G20 populations, except that the correlations between erucic acid and linolenic acid were positive and nonsignificant.

Discussion

The analysis of fatty acid distributions in populations of androgenic lines initiated from the three crosses used in the present study confirmed that erucic acid is simply inherited. The clear 1:2:1 class segregation for low-, intermediate- and high-erucic acid lines, respectively, in the three populations support previous proposals that

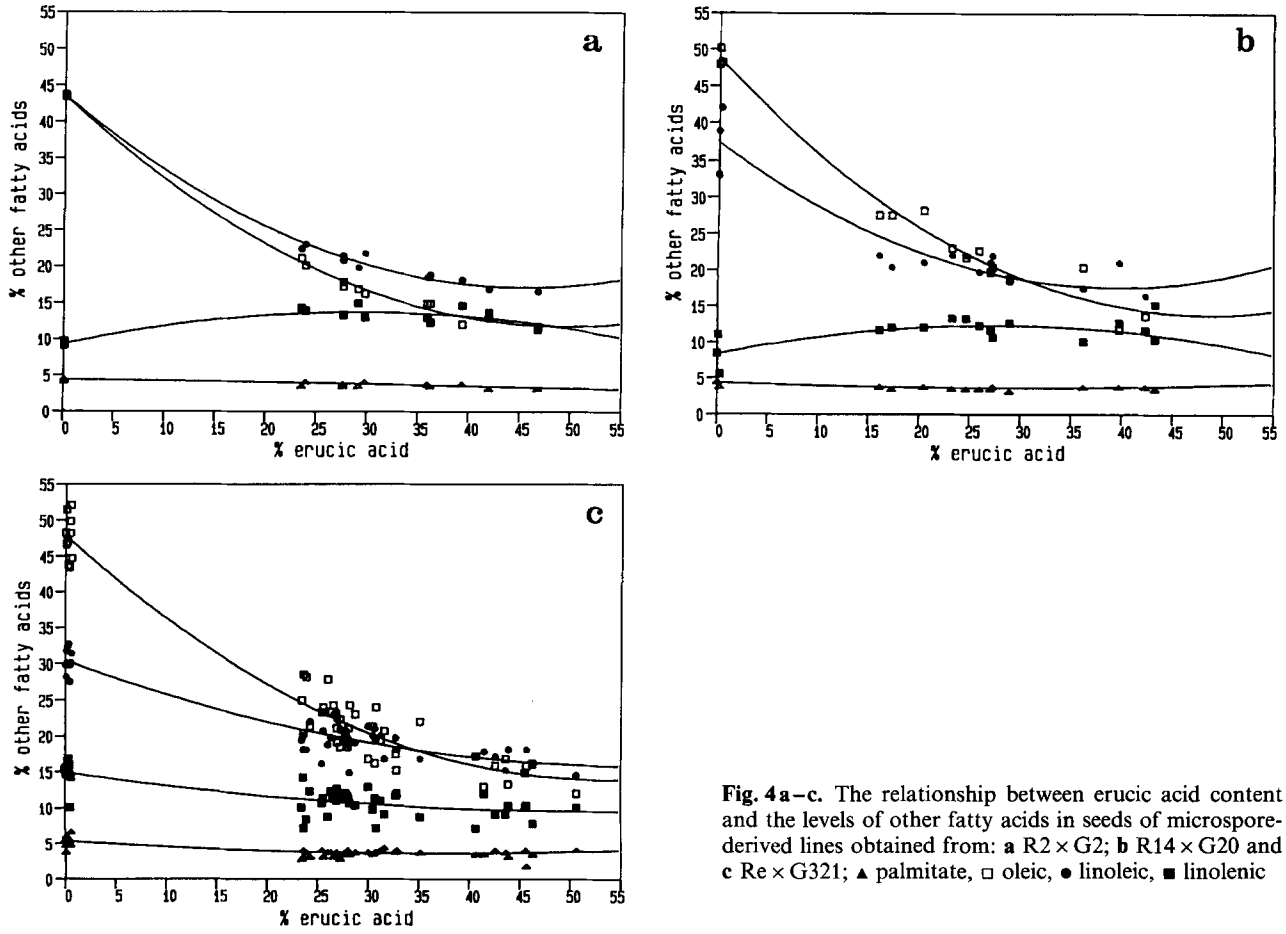

Fig. 4a–c. The relationship between erucic acid content and the levels of other fatty acids in seeds of microspore-derived lines obtained from: **a** R2 × G2; **b** R14 × G20 and **c** Re × G321; ▲ palmitate, □ oleic, ● linoleic, ■ linolenic

Table 1. Postulated genotypes, expected ratios and erucic acid contents of androgenic lines obtianed from Regent × Golden and Reston × G321 crosses and F_2 lines from Liho × Golden Cross (Harvey and Downey 1964)

Genotype	Erucic acid [+] (%)	Expected ratios in F_2 or androgenic lines
Androgenic lines		
aabb	0	1
aaBB, AAbb	18–20	2
AABB	36–40	1
F_2 seeds*		
aabb	0	1
Aabb, aaBb	9–10	4
AAbb, AaBb, aaBB	18–20	6
AaBB, AABb	27–30	4
AABB	36–40	1

* Taken from Harvey and Downey (1964)
[+] Assuming that each gene contributes 9%–10%

the erucic acid content in seeds of *Brassica napus* is governed by two genes acting in an additive manner (Harvey and Downey 1964). Table 1 shows the expected genotypes, the amounts of erucic acid per class and the expected ratio between classes in the doubled haploid populations, assuming that each allele acts equally to contribute ca. 9%–10% erucic acid (Harvey and Downey 1964). The same information for an F_2 population obtained from a cross between Liho and Golden, taken from Harvey and Downey (1964), is also shown in Table 1. In the present study, the slight deviation of erucic acid contents from proposed levels in the intermediate- and high-erucic acid classes may be a reflection of an environmental influence on the levels of this fatty acid. Harvey and Downey (1964) suggested that the environment may markedly affect the content of erucic acid especially in plants with large amounts of erucic acid in the seed oil. In their study this environmental influence was reflected in a wide range of erucic acid levels found in the F_1 and G_1 (Golden parent) populations.

A comparison of genetic ratios in F_2 versus androgenic populations (Table 1) illustrates some of the advantages of utilizing haploidy to examine the genetics of simply-inherited traits. Because the androgenic popula-

tions represent gametic ratios in a homozygous form, the number of genotypes is smaller than observed in an F_2 population. The androgenic population contains only the aaBB and AAbb genotypes (which express the same phenotype) and the homozygous recessive (aabb) and homozygous dominant (AABB) genotypes. In comparison, an F_2 population from the same F_1 would contain five classes of individuals with respect to their erucic acid content. In addition, class separations are greater in androgenic populations, making it easier to distinguish classes than in F_2 populations.

Strong negative correlations between erucic acid and the C18 fatty acids were demonstrated, confirming previous findings (Craig 1961; Stefansson et al. 1961; Stefansson and Hougen 1964; Downey and Craig 1964; Jonsson 1973; Jonsson and Persson 1983; Stefansson 1983). The strong correlations reflect the fact that oleic acid is a common precursor for the biosynthetic pathways leading to C22 fatty acids and the highly-unsaturated C18 fatty acids. Therefore, a block in the chain elongation process to erucic acid would leave more oleic acid available for desaturation to linoleic acid and linolenic acid. As previously reported (Kondra and Thomas 1975), both negative (Regent × Golden) and positive correlations (Reston × G231) were found in this study between linoleic and linolenic acid levels, although positive correlations have been most common (Thies 1968; Stefansson and Storgaard 1969; Röbbelen and Thies 1973; Jonsson and Persson 1983) and are the strongest in this study. Since oleic and linoleic acids both increase in amount at the expense of erucic acid, positive correlations between these two C18 fatty acids are not surprising, although strong negative correlations have been reported in previous studies (Stefansson and Storgaard 1969; Kondra and Thomas 1975).

Work with doubled haploid spring barley lines (Kasha and Reinbergs 1975; Park et al. 1976; Reinbergs et al. 1978; Song et al. 1978; Choo and Reinbergs 1979; Choo et al. 1982; Friedt and Foroughi-Wehr 1983) has demonstrated the efficiency of haploidy for fixing desirable genes of qualitatively and quantitatively inherited characteristics and has indicated the advantages of haploidy in the study of inheritance, linkage and quantitative genetics. In a similar way, the results of the present study illustrate several advantages of using haploidy to investigate the inheritance of simply inherited traits in *B. napus*.

Acknowledgements. This research was funded by the Natural Sciences and Engineering Research Council of Canada in the form of an industrial postgraduate scholarship to J. Siebel, and by the National Research Council under the Programme for Industry/Laboratory Projects Arrangement No. CA910-4-0080/B-56 with King Agro Inc. We would like to thank Dr. W. D. Beversdorf for helpful discussions.

References

Beare JL, Campbell JA, Youngs CG, Craig BM (1963) Effects of saturated fat in rats fed rapeseed oil. Can J Biochem Physiol 41:605–612

Choo TM, Reinbergs E (1979) Doubled haploids for estimating genetic variances in the presence of linkage and gene association. Theor Appl Genet 55:129–132

Choo TM, Reinbergs E, Park SJ (1982) Comparison of frequency distributions of doubled haploid and single seed descent lines in barley. Theor Appl Genet 61:215–218

Choo TM, Reinbergs E, Kasha KJ (1985) Use of haploids in breeding barley. Plant Breed Rev 3:219–252

Chuong PV, Beversdorf WD (1985) High frequency embryogenesis through isolated microspore culture in *Brassica napus* L. and *Brassica carinata* Braun. Plant Sci 39:219–226

Craig BM (1961) Varietal and environmental effects on rapeseed. III. Fatty acid composition of 1958 varietal tests. Can J Plant Sci 41:204–210

Dorrel DG, Downey RK (1964) The inheritance of erucic acid content in rapeseed (*Brassica campestris*). Can J Plant Sci 44:499–504

Downey RK, Craig BM (1964) Genetic control of fatty acid biosynthesis in rapeseed (*Brassica napus* L.). J Am Oil Chem Soc 41:475–478

Downey RK, Harvey BL (1963) Methods of breeding for oil quality in rape. Can J Plant Sci 43:271–275

Friedt W, Foroughi-Wehr B (1983) Field performance of androgenetic doubled haploid spring barley from F_1 hybrids. Z Pflanzenzücht 90:177–184

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Harvey BL, Downey RK (1964) The inheritance of erucic acid content in rapeseed (*Brassica napus* L.). Can J Plant Sci 44:104–111

Jonsson R (1973) Breeding for low erucic acid content in summer turnip rape (*Brassica campestris* L. var *annua* L.). Z Pflanzenzücht 69:1–18

Jonsson R (1974) Breeding for low erucic acid content in winter turnip rape (*Brassica campestris* L. var *biennis* L.). Z Pflanzenzücht 73:259–268

Jonsson R (1977a) Erucic acid heredity in rapeseed (*Brassica napus* L. and *Brassica campestris* L.). Hereditas 86:159–170

Jonsson R (1977b) Breeding for improved oil and meal quality in rape and turnip rape. Hereditas 87:205–218

Jonsson R (1978) Erucic acid heredity in rapeseed. Proc 5th Int Rapeseed Conf, Organizing Committee of 5th International Rapeseed Conference, Malmo

Jonsson R, Persson C (1983) Breeding for improved fatty acid composition in rapeseed. Proc 6th Int Rapeseed Conf, Organizing Committee of 6th International Rapeseed Conference, Paris

Kasha KJ, Reinbergs E (1975) Utilization of haploids in barley. In: Gaul H (ed) Barley genetics III, Thiemig, München, pp 307–315

Keller WA, Armstrong KC (1978) High frequency production of microspore-derived plants from *Brassica napus* anther culture. Z Pflanzenzücht 80:100–108

Kondra ZP, Thomas PM (1975) Inheritance of oleic, linoleic and linolenic acids in seed oil of rapeseed (*Brassica napus*). Can J Plant Sci 55:205–210

Krzymanski J, Downey RK (1969) Inheritance of fatty acid composition in winter forms of rapeseed, *Brassica napus*. Can J Plant Sci 49:313–319

Lichter R (1982) Induction of haploid plants from isolated pollen of *Brassica napus*. Z Pflanzenphysiol 105:427–434

Morrison RA, Evans DA (1988) Haploid plants from tissue culture: new plant varieties in a shortened time frame. Biotech 6:684–690

Nitsch C, Nitsch JP (1967) The induction of flowering in vitro in stem segments of *Plumbago indica* L. I. The production of vegetative buds. Planta 72:355–370

Park SJ, Walsh EJ, Reinbergs E, Song LSP, Kasha KJ (1976) Field performance of doubled haploid barley lines in comparison with lines developed by the pedigree and single seed descent methods. Can J Plant Sci 56:467–474

Reinbergs E, Song LSP, Choo TM, Kasha KJ (1978) Yield stability of doubled haploid lines of barley. Can J Plant Sci 58:929–933

Röbbelen G, Thies W (1973) Inheritance of polyenoic fatty-acids and their selection in oil seeds. Genetics 74:S231

Shehata AY, DeMan JM, Alexander JC (1970) A simple and rapid method for the preparation of methyl esters of fats in milligram amounts for gas chromatography. Can Inst Food Technol J 3:85–89

Song LSP, Park SJ, Reinbergs E, Choo TM, Kasha KJ (1978) Doubled haploid vs the bulk plot method for production of homozygous lines in barley. Z Pflanzenzücht 81:271–280

Stefansson BR (1983) The development of improved rapeseed cultivars. In: Kramer JKG, Sauer FD, Pigden WJ (eds) High and low erucic acid rapeseed oils: production usage chemistry and toxilogical evaluation. Academic Press, Don Mills, pp 143–159

Stefansson BR, Hougen FW (1964) Selection of rape plants (*Brassica napus*) with seed oil practically free from erucic acid. Can J Plant Sci 44:359–364

Stefansson BR, Storgaard AK (1969) Correlations involving oil and fatty acids in rapeseed. Can J Plant Sci 49:573–580

Stefansson BR, Hougen FW, Downey RK (1961) Note on the isolation of rape plants with seed oil free from erucic acid. Can J Plant Sci 41:218–219

Thies W (1968) Die Biogenese von Linol- und Linolensäure in den Samen höherer Pflanzen, insbesondere Raps und Rubsen, als Problem der Ölpflanzenzüchtung. Angew Bot 42:140–154

Thomas E, Wenzel G (1975) Embryogenesis from microspores of *Brassica napus*. Z Pflanzenzücht 74:77–81