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Synthesis and fertility of xBrassicoraphanus and ways of transferring Raphanus characters to Brassica

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Abstract

About 250 intergeneric hybrids with the genome constitution AR, AARR or ARR were obtained from over 15 000 crosses between Brassica campestris (AA or AAAA, female parent) and Raphanus sativus (RR or RRRR). The poor crossability was shown to be a consequence of various breeding barriers. The diploid and tetraploid hybrids were studied in form, fertility, chromosome association at meiosis and crossability both among the hybrids and in crosses with the parental species and Brassica napus. ×Brassicoraphanus (AARR) was backcrossed twice with B. campestris (AA), and was also crossed and backcrossed with B. napus (AACC). Form and chromosome association at meiosis in AAR and AACR hybrids were studied.

Two populations of ×Brassicoraphanus (AARR) were propagated and studied in more detail, partly for possible suitability as a new crop. Most plants were highly sterile because of breeding barriers, which were similar to those in the original intergeneric crosses. The increase in fertility and chromosomal stability were studied in the first three generations of the populations.

Free descriptors: Brassica campestris, Raphanus sativus, incongruity, breeding barriers, embryo culture, intergeneric hybrids, ×Brassicoraphanus, fertility, mating system, introgression, Brassica napus, allosyndesis, forage crop.

Contents

1 General introduction

1.1 RATIONALE

Like most other cruciferous crops, *Brassica* spp. are hosts to the beet eelworm *(Heterodera schachtii* Schm.) (den Ouden, 1954; Steele, 1965), which is distributed all over the world (Stelter, 1973) and is the most serious nematode of sugar-beet *(Beta vulgaris* L.) in Europe (Heijbroek, 1979). In the Netherlands, for example, a quarter of the sugar-beet area has been infected by this nematode, especially in the south-west and south-east (Heijbroek, 1979). Problems arise mainly from too narrow crop rotations. The growing of sugar-beet is economically attractive to the farmers, and they will try to continue with such rotations, despite disadvantages from a phytosanitary point of view.

Under present circumstances, no place is left for *Brassica* crops in rotations with beets, unless resistant varieties are bred with preferably the ability to reduce the population density of the eelworm. In the genus *Brassica*, however, no resistance has been found sofar (Baukloh, 1976; Lubberts & Toxopeus, 1982). In the 1960s, reports appeared in German farming journals on resistance in *Raphanus sativus* L. var. *oleiformis*, the oil-seed radish. The early contradictory reports were reviewed by Hirling (1976). With oil-seed radish the eelworm usually multiplied slowly, but in a few reports rather quickly. In recent years, the presence of resistance in *Raphanus* has been confirmed by Baukloh (1976) and Toxopeus & Lubberts (1979). Baukloh (1976) studied the inheritance and assumed a single dominant gene for resistance.

The genus *Brassica* includes major crops for fodder, oil-seed production, and green manure, some of which might fit quite well into crop rotations with sugar-beet. So a programme was started on intergeneric hybridization between *Brassica* spp. and nematode-resistant forms of *R. sativus* with the ultimate goal of transferring resistance to *Brassica*. A second argument for this programme was the presence of resistance to club-root *(Plasmodiophora brassicae* Woron.) in oil-seed radish.

1.2 SPECIES AND ALLOPOLYPLOID HYBRIDS

The programme on intergeneric hybridization includes the species *R. sativus* (2n = 18, RR), *B. campestris* (2n = 20, AA) and *B. napus* (2n = 38, AACC). Their genome constitutions and interrelationships are outlined in

Figure 1 (Mizushima, 1980). *B. napus* is a natural allotetraploid and is composed of two genomes of *B. campestris* and two of *B. oleracea* ($2n = 18$, CC). Table 1 enumerates the subspecies or botanical varieties distinguished in these species. According to Helm (1957), *R. sativus* comprises five botanical varieties, which include various vegetables and agricultural crops. The best known in Europe are radish, giant radish, black radish, and oil-seed or fodder radish. The latter is however mainly used as green manure. Mougri-radish is grown in south-east Asia, especially for its leaves and young pods (Banga, 1976). For *B. campestris*, the classification of Olsson (1954) has been followed. He distinguished various subspecies, which were classified by other taxonomists as separate species. *B. campestris* contains many oriental leafy vegetables, and several root, oilseed and fodder crops. *B. napus* also includes major root, oilseed and fodder crops, although its domestication started only a few hundred years ago (Toxopeus, 1974a; McNaughton, 1976). Since the discovery of the genome constitution of *B. napus* (U, 1935), this species has often been resynthesized from its diploid ancestors. This has increased genetic variation in the existing crops and even resulted in a heading type called 'Hakuran' (Nishi, 1980). More detailed information on the four species in the present study is found in Boswell (1949), Herklots (1972), Simmonds (1976) and Nishi (1980).

Intergeneric hybrids between *R. sativus* and *Brassica* species have a long and well known history. At present various meiotically stable allopolyploids are known (Fig. 1). Plants with the genome constitution RRCC were already obtained by Karpechenko (1927; 1928) and named *Raphanobrassica*. Some years later, Terasawa (1932) was the first to produce allotetraploids with the genome constitution AARR, which he called *Brassicoraphanus*. In this study, the names ×*Raphanobrassica* and ×*Brassicoraphanus* are used for plants with the genome constitution RRCC and AARR, respectively, irrespective of the female parent in the original crosses. According to the international rules for nomenclature, those names should be preceded by multiplication sign (International Bureau for Plant Taxonomy and Nomenclature, 1980). A less known type is the allohexaploid with the constitution AACCRR, which was recently produced by Clauss (1978).

1.3 SCOPE OF THE RESEARCH PROGRAMME

The presence of resistance to the beet eelworm in *R. sativus* was the main reason for starting a programme on intergeneric hybridization in order to transfer this trait to the genus *Brassica*, especially to *B. campestris* and *B. napus*. Those species include most of the major arable *Brassica* crops grown in the Netherlands. Figure 1 indicates that various ways for transferring genes from *Raphanus* to *Brassica* may be feasible.

Table 1. Latin and common English names of subspecies and botanical varieties in
Raphanus sativus L., Brassica campestris L. and Brassica napus L.

Species	Subspecies/ botanical variety	Common name(s)
R. sativus L.	var. gayanus Webb.	wild radish
	var. oleiformis Pers.	oil-seed or fodder radish
	var. mougri Helm	Mougri-radish or rat-tailed radish
	var. niger (Miller) Pers. sensu lat.	black radish, giant radish, Japanese radish
	var. sativus Helm	radish
B. campestris L.	ssp. eu-campestris Olsson	wild type
	ssp. oleifera (Metzg.) Sinsk.	turnip-rape
	ssp. rapifera (Metzg.) Sinsk.	turnip
	ssp. chinensis (L.) Makino	Chinese mustard
	ssp. pekinensis (Lour.) Olsson	Pe-tsai, Chinese cabbage
	ssp. narinosa (Bailey) Olsson	
	ssp. nipposinica (Bailey) Olsson	
	ssp. dichotoma (Roxb.) Olsson	toria
	ssp. perviridis Bailey	tendergreen
	ssp. trilocularis (Roxb.) Olsson	yellow-seeded sarson
B. napus L.	var. napobrassica (L.) Peterm.	swede
	ssp. oleifera (Metzg.) Sinsk.	oil-seed and fodder rape
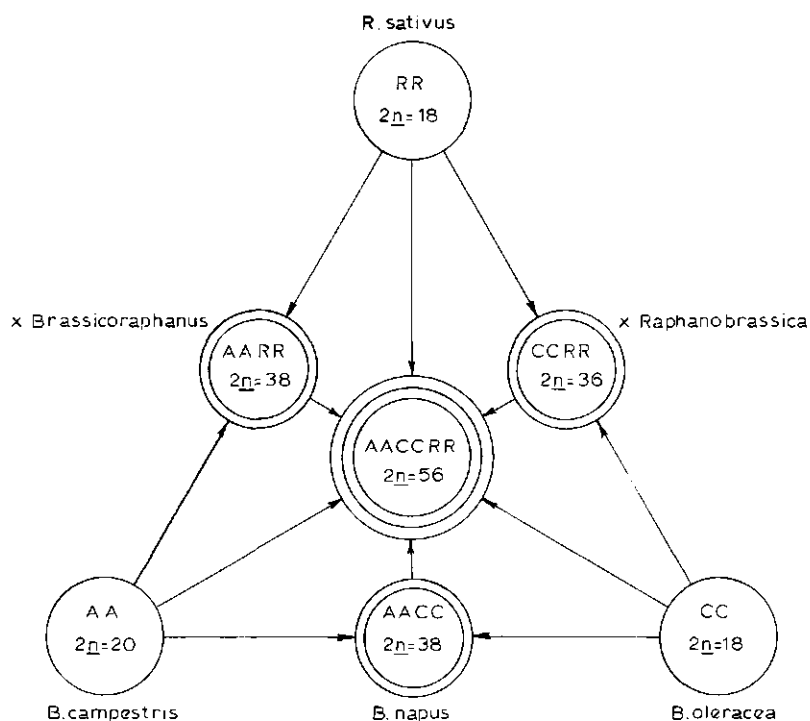
Figure 1. Genome designations, chromosome numbers and interrelationships of Raphanus
sativus, some Brassica species and allopolyploid hybrids.

Direct introgression into *B. napus* by crossing this species with *R. sativus* was apparently difficult, although some hybrids have been obtained (see for instance Yarnell, 1956). A more promising first step was the cross *B. campestris* × *R. sativus*, because (1) the crossability is better (Yarnell, 1956) and (2) the resulting allotetraploid hybrids may be a bridge between *R. sativus* and *B. napus*. So all efforts were concentrated on this particular cross (Chapter 2).

Most initial hybrids grew vigorously and suggested that the allotetraploid hybrids (= ×*Brassicoraphanus*) might be suitable as a fodder crop, combining rapid growth and beet eelworm and club-root resistance of oil-seed radish with the better palatability of the *Brassica* parent. A prerequisite for successful selection within such a 'new species' is the availability of a broad genetic variation, i.e. to produce hybrids involving many genotypes of the parental species. So the nature of breeding barriers and ways of improving crossability between *B. campestris* and *R. sativus* were studied (Chapter 2 and 3).

Several properties of the primary hybrids, e.g. form, fertility, crossability and meiosis are described in Chapter 4 and 5. Possible causes of sterility and improvement of fertility in ×*Brassicoraphanus* are given in Chapter 6 and 7.

We tried to transfer *Raphanus* genes to *Brassica*, in two ways. The first approach was to produce in *B. campestris* monosomic addition lines carrying an extra *Raphanus* chromosome. The intention was to test such lines for resistance to various diseases and afterwards to transfer the resistance genes from separate *Raphanus* chromosomes in a more directed attempt into the *B. campestris* genome (Chapter 8). The second approach was a backcross programme of ×*Brassicoraphanus* with *B. napus* as the recurrent parent (Chapter 9).

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2 Crosses between Brassica campestris and Raphanus sativus

2.1 INTRODUCTION

Since the 1920s several research workers, especially from Japan have
reported the hybridization of *B. campestris* and *R. sativus* (Table 2).
Terasawa & Shimotomai (1928) obtained the first diploid hybrids. More
reports on successful hybridization followed. Initially the crosses were
carried out at the diploid level only but later autotetraploid forms of
either one or both species have been used as well, especially during the
last two decades (Table 2). Crossability was poor in all combinations.
The best results were nevertheless obtained from crosses between diploid
forms, using *B. campestris* as the female parent. In those crosses, there
was marked variation in the number of hybrids produced per pollination
(Table 2).

As far as known, the primary hybrids reported by various authors
(Table 2) had nearly always the expected genome constitution, but some
deviant plants were found in $2x \times 2x$ crosses with *R. sativus* as the fe-
male parent. U et al. (1937), Morris & Richharia (1937) and Mizushima
(1950a) obtained among their hybrids five allotriploid plants comprising
two genomes of *Raphanus* and one of *Brassica*. These plants probably arose
from a fertilized $2n$ egg cell.

Apart form an interest in the intergeneric hybrids as such, the
crosses mentioned in Table 2 have been made mainly for two reasons: to
study the causes of matromorphy (Nishi et al., 1964; Tokumasu, 1965;
1970a; Opeña & Lo, 1978) and to get information on interspecific incom-
patibility (Kakizaki, 1925; Becker, 1951; Hinata et al., 1974). Usually
progeny of crosses between *B. campestris* and *R. sativus* consist partly
of matromorphic plants. Tokumasu (1965; 1970a) showed that such plants
were not completely homozygous as Nishi et al. (1964) assumed. Matromor-
phy in Cruciferae is a kind op diploid parthenogenesis and has been ob-
served in many interspecific and intergeneric crosses within this family
(Eenink, 1974).

This chapter presents results of intergeneric crosses between diploid
and tetraploid forms of *B. campestris* and *R. sativus*. The latter species
was always used as the pollen donor, because in this direction the cross-
ability was much better than in the reciprocal (Table 2). Many accessions
of *B. campestris* were used in order to minimize the chance of using poor-
ly crossable genotypes only. The male parents varied less, being concen-

Table 2. List of reported crosses between diploid and tetraploid forms of
Brassica campestris (AA and AAAA) and Raphanus sativus (RR and RRRR).

Cross	Number of pollinations	Number of hybrids	Number fraction hybrids to pollinations (%)	Source
AA × RR	33	0	0	Kakizaki (1925)
	90	6	6.7	Terasawa & Shimotomai (1928)
	428	12	2.8	Morris & Richharia (1937)
	217	0	0	Becker (1951)
	903	167	18.5	Mizushima (1952)
	8 003	263	3.3	Nishi et al. (1964)
	286	31	10.8	Tokumasu (1970b)
	82	0	0	Hinata et al. (1974)
	13 287	44	0.3	Opeña & Lo (1978)
	2 111	13	0.6	Namai (1980)
	24	0	0	Takeshita et al. (1980)
RR × AA	39	0	0	Kakizaki (1925)
	30	0	0	Terasawa & Shimotomai (1928)
	?	≥ 2	0.2	U et al. (1937)
	413	2	0.5	Morris & Richharia (1937)
	328	0	0	Becker (1951)
	4 775	2	0.04	Nishi et al. (1964)
	417	0	0	Tokumasu (1965)
	1 111	0	0	Namai (1980)
	19	0	0	Takeshita et al. (1980)
AA × RRRR	?	?	2	Ellerström (pers. commun.)
RRRR × AA	?	?	0	Ellerström (pers. commun.)
AAAA × RR	481	0	0	Nishi et al. (1964)
RR × AAAA	471	0	0	Nishi et al. (1964)
AAAA × RRRR	58	0	0	Tokumasu (1970a)
RRRR × AAAA	132	0	0	Tokumasu (1970a)

Remark: Intergeneric hybrids were also reported by Morris (1936), Nishiyama (1946),
Hosoda (1946; 1947) Mizushima (1950a), Clauss (1978), McNaughton & Ross (1978),
Ellerström (1978), Olsson & Ellerström (1980), Namai et al. (1980).

trated to fodder radish because of the requirement of combined resistance
to beet eelworm and club-root. Some attempts were made to improve the re-
sults of these intergeneric crosses by culture of immature embryos in
vitro.

2.2 MATERIAL AND METHODS

The diploid and tetraploid genotypes of *B. campestris* and *R. sativus*
used for intergeneric hybridization are listed in Table 3. The fodder
radishes 'Siletta', 'Levana', 'Palet' and RS14 were chosen because they
were partially resistant to beet eelworm. In most tests for resistance

Table 3. Description, ploidy and origin of Brassica and Raphanus accessions used for intergeneric crosses.

Designation	Species/subspecies or botanical variety.	Description	Ploidy	Origin *
	B.campestris			
A1	ssp. rapifera	inbred line BC183	2x	1
A2	ssp. rapifera	inbred line BC182	2x	1
A3	ssp. rapifera	inbred line BC171	2x	1
A4	ssp. rapifera	inbred line BC172	2x	1
A5	ssp. rapifera	inbred line BC181	2x	1
A6	ssp. rapifera	inbred line BC176	2x	1
A7	ssp. rapifera	inbred line BC184	2x	1
A8	ssp. rapifera	cv. Trofee	2x	2
A9	ssp. oleifera	boterzaad	2x	1
A10	ssp. pekinensis		2x	1
A11	ssp. chinensis	Shan-Jue-Man-Tsien-Tsaj	2x	3
A12	ssp. chinensis		2x	1
A13	ssp. chinensis	S_1 line from A12	2x	
AA1	ssp. rapifera	cv. Novitas	4x	4
AA2	ssp. rapifera	cv. Taronda	4x	5
AA3	ssp. rapifera	cv. Tigra	4x	6
AA4	ssp. pekinensis	2243	4x	7
AA5	ssp. nipposinica	2245	4x	7
AA6	ssp. perviridis	2240	4x	7
AA7	ssp. narinosa	2260	4x	7
AA8	ssp. chinensis	2242	4x	7
	R.sativus			
R1	var. oleiformis	cv. Siletta	2x	8
R2	var. oleiformis	cv. Levana	2x	8
R3	var. mougri	Rs 4.01	2x	1
R4	var. niger	Ra 74/65 China 1956:Kanton 35	2x	3
R5	var. oleiformis	Rs 6.04	2x	1
R6	var. niger	Ra 113/64 Japanese white New Delhi	2x	3
RR1	var. oleiformis	cv. Palet	4x	8
RR2	var. oleiformis	RS14	4x	9

*] 1. Stichting voor Plantenveredeling (SVP), Wageningen, The Netherlands.
    2. Zwaan & De Wiljes, Zaadteelt en Zaadhandel BV, Scheemda, The Netherlands.
    3. Zentralinstitut für Genetik und Kulturpflanzenforschung, Gatersleben, DDR.
    4. Cebeco-Handelsraad, Rotterdam, The Netherlands.
    5. Zelder BV, Ottersum, The Netherlands.
    6. Kon. Zaadteelt en Zaadhandel Sluis en Groot BV, Enkhuizen, The Netherlands.
    7. Swedish Seed Association, Svalöv. Sweden.
    8. Kon. Kweekbedrijf en Zaadhandel D.J. van der Have BV, Kappelle, The Netherlands.
    9. Scottish Plant Breeding Station, Pentlandfield, United Kingdom.

usually some multiplication of this nematode occurred, but to a relatively low degree (Toxopeus, pers. commun.). Fodder-radish varieties often have a high degree of resistance to club-root (Crute et al., 1980). The German variety Siletta was resistant to many isolates of club-root (Toxopeus, 1974b). The fodder radishes 'Levana' and 'Palet', are direct derivatives from 'Siletta' and have probably the same genes for resistance.

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Most of the plant material was grown in rather well conditioned green-houses at temperatures varying from 15 to 20 °C and a daylength between 14 and 17 h. The plants were grown in pots of capacity about 2½ litres. The intergeneric crosses were made by hand in the period from January to May of 1975 and 1976. Open flowers and young flower buds were removed from the inflorescences and subsequently the rest of the buds was emasculated. The inflorescences, with an average of about 15 buds were bagged. They were pollinated between 13:00 and 15:00 immediately after emasculation in 1975 and two to three days after emasculation in 1976 by putting a small amount of a pollen mixture onto the stigmas with a small soft brush. Such mixtures were composed of almost equal amounts of fresh pollen collected from 25 to 40 plants per accession. At the moment of pollination in 1976, the oldest bud was marked and buds and open flowers of each inflorescence were counted. Three to four weeks later the developed siliquae were counted.

At harvest, the total number of siliquae and the number of seeds were counted for each inflorescence. The largest diameter of each seed was determined as a measure of seed size. Large seeds were germinated in Petri dishes on wet filter paper at 23 °C in the dark. Small and shrivelled seeds were first disinfected for 20 min. in aqueous sodium hypochlorite of mass concentration 3 g/l, followed by thorough rinsing in sterilized water. The disinfected seeds were then also germinated at 23 °C in the dark but in small Petri dishes on a solid nutrient medium of the composition given by Guha & Maheswari (1964).

Harberd's method of embryo culture (1969;1971) was used. The developmental stages of the cultured embryos were established according to the drawings of Wilmar & Hellendoorn (1968) for *B. oleracea*. Initially all developed ovules were cultured, whether an embryo was present or not, but only ovules with a visible embryo have been cultured since 1976.

The distinction between hybrids and matromorphic plants was feasible on the basis of plant morphology, as was confirmed by counting chromosomes in root-tip or meiotic cells. The root-tips were treated and squashed for counting the chromosomes according to the method of Jochemsen & Młyniec (1974). For counting in meiotic cells young buds were fixed in a solution of three parts by volume of aqueous ethanol (volume fraction of ethanol 0.96) and of one part glacial acetic acid and placed at 4 °C for a few days. The buds were then transferred to aqueous ethanol (volume fraction of ethanol 0.7) and kept in storage at 4 °C for a few weeks. The buds were subsequently stained by Snow's method (1963) for about 14 h at 60 °C.

In a small experiment in 1977 the possibility was tested of using genetically male-sterile *B. campestris* plants for intergeneric hybridization. Four male-sterile F_2 plants from a cross between a male-sterile plant from accession A5 and a plant of A11 were transplanted into iso-

lation cages with flowering plants of *R. sativus*. Honey-bees were used
for pollination.

2.3 RESULTS

2.3.1 *Setting of siliquae and seeds*

Setting of siliquae was assessed for all the crosses in 1976 three to
four weeks after pollination and at harvesting. The results for $2x \times 2x$,
$2x \times 4x$, $4x \times 2x$ and $4x \times 4x$ are summarized in Table 4 for each *Brassica*
accession, irrespective of pollinator. After 21-28 days the *Brassica* ac-
cessions differed very much in setting of siliquae. The ploidy of the
pollinators had apparently no large effect (Table 4).

A low proportion of siliquae shrivelled away after initial development
and were not harvested (Table 4). The remainder only partly contained
seeds. Those seeds gave sometimes rise to matromorphs, especially in
crosses in which the number ratio of developed siliquae to pollinated
flowers was comparatively low, as on A8, A9, AA2, AA3 and AA4.

2.3.2 *Seed size*

Nearly all seeds obtained from the crosses in 1975 and 1976 were

Table 4. Number fraction of siliquae and of siliquae with seeds to pollinations, and
number ratio of seeds per pollination in the crosses made in 1976 between diploid and
tetraploid forms of Brassica campestris and Raphanus sativus (Tables 5, 6 and 7). The
setting of siliquae was assessed 21-28 days after pollination and at harvest. The column
subheadings indicate the genome constitution of the pollinator.

Female parent	Number fraction of siliquae to pollinations (%)				Number ratio of siliquae with seeds to pollinations (%)		Number ratio of seeds to pollinations	
	after 21-28 days		at harvest					
	RR	RRRR	RR	RRRR	RR	RRRR	RR	RRRR
B. campestris (2x)								
A8	29	20	18	19	10	16	0.18*	0.35*
A9	22	26	22	23	10	23	0.16*	0.45*
A13	80	69	72	61	27	13	0.37	0.22
B. campestris (4x)								
AA2	9	13	9	13	4	8	0.08*	0.12*
AA3	2	3	2	3	1	2	0.02*	0.02
AA4	5	6	5	1	3	0	0.03*	0
AA5	49	45	15	25	0	5	0	0.06
AA6	48	53	31	47	4	17	0.04	0.20
AA7	51	48	16	14	3	1	0.03*	0.01*
AA8	43	40	33	33	1	3	0.01	0.05

*] Seeds gave mostly rise to matromorphs.
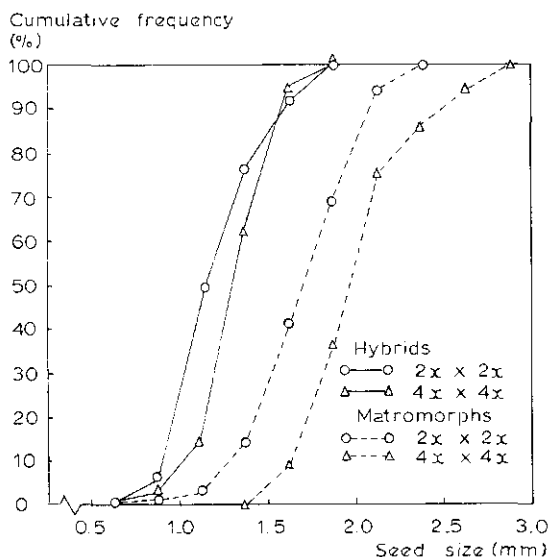
Figure 2. Cumulative frequency distribution of seed size of hybrid and matromorphic seeds obtained from 2x × 2x and 4x × 4x crosses between Brassica campestris and Raphanus sativus.

measured. The seeds were mostly well-filled and were ovoid-spherical, although some were shrivelled. The seed size of hybrid and matromorphic seeds obtained from $2x \times 2x$ and $4x \times 4x$ is presented in Figure 2. The hybrid seeds were on average smaller than seeds yielding matromorphic plants, but there was to some extent an overlap in seed size of hybrids and matromorphs, especially for $2x \times 2x$ crosses. Matromorphic seeds from $4x \times 2x$ and $2x \times 4x$ crosses were similar in size to that of $4x \times 4x$ and $2x \times 2x$ crosses, respectively. The few hybrid seeds from those crosses were also smaller and were at most 1-2 mm in diameter.

The germinability of the seeds was related to seed size. The seeds not germinating or whose seedlings died before the cotyledon stage were on average smaller than the germinated hybrid seeds and were probably mainly hybrid seeds. The respective ratios of seeds not germinating to hybrid seeds in $2x \times 2x$, $4x \times 2x$, $2x \times 4x$ and $4x \times 4x$ were approximately 1, 7, 18 and 1, indicating that especially in $4x \times 2x$ and $2x \times 4x$ the germinability of hybrid seeds was poor.

2.3.3 *Production of hybrids and matromorphs*

The results of all intergeneric crosses made in 1975 and 1976 are given in Table 5, 6 and 7. Table 5 lists the crosses between diploids. In 1975 nearly all available plants from most *Brassica* accessions were crossed as females with the *Raphanus* accessions, R1 and R3. Usually each

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Table 5. Results of crosses between diploid forms of <u>Brassica campestris</u> (♀) and <u>Raphanus sativus</u> in 1975 and 1976.

Cross	Number of pollinations	Number of female plants		Number of hybrids	Number of matromorphs	Number ratio of hybrids to pollinations (%)	
		tested	yielding hybrids			average	maximum
1975							
A1 × R1	488	18	4	12	21	2.5	17.1
R3	267	13	4	8	17	3.0	11.9
A2 × R1	207	8	0	0	27	0	0
R3	205	8	0	0	6	0	0
A3 × R1	429	17	3	6	25	1.4	7.4
R3	462	17	2	6	48	1.3	8.0
A4 × R1	249	10	0	0	8	0	0
R3	283	10	2	2	11	0.7	3.3
A5 × R1	443	18	0	0	12	0	0
R3	400	17	2	3	11	0.8	7.4
A6 × R1	364	15	2	2	3	0.5	6.3
R3	344	14	3	4	3	1.2	15.0
A7 × R1	423	18	2	4	7	0.9	10.0
R3	432	17	3	11	21	2.5	36.0
A10 × R1	44	2	0	0	0	0	0
A11 × R1	346	14	3	3	20	0.9	5.6
R3	295	13	0	0	30	0	0
A12 × R1	102	1	1	4	0	3.9	3.9
1976							
A8 × R1	239	10	0	0	41	0	0
A9 × R1	105	7	1	1	16	1.0	5.0
A13 × R1	182	11	10	44	8	24.2	43.8
R2	129	4	3	18	1	14.0	122.2
Total	6438			128	336	2.0	122.2

plant was pollinated with both pollinators on the same day and an almost
equal number of pollinations was made for each pollinator. The number
ratio of hybrids per pollination was low in both years and ranged from 0
to 24.2 % between crosses. A large variation in hybrid production was
also found within the *Brassica* accessions (Table 5). Usually a low pro-
portion of the female plants actually gave rise to hybrids (Table 5). In
1975, no striking differences were observed between the two pollinators
in production of hybrids from crosses with various *Brassica* accessions
(Table 5). Crosses of A13 gave rise to a substantial number of hybrids;
nearly all plants from that accession produced some hybrids in crosses
with the accessions R1 and R2. A13 was an S_1 line obtained by selfing a
particular A12 plant, which in 1975 showed a good fruit-set and good pro-
duction of hybrids, i.e. number ratio of hybrids to pollinations (cross-
ability) 3.9 % in crosses with R1. The results with A13 suggest that se-
lection within *B. campestris* may give rise to a considerable increase in
crossability. If we exclude the results of crosses with A13, the mean
crossablility over the two years was about 1 %. The crossability of the

Table 6. Results of crosses between autotetraploid forms of <u>Brassica campestris</u> (♀) and <u>Raphanus sativus</u> in 1975 and 1976.

Cross		Number of pollinations	Number of female plants		Number of hybrids	Number of matromorphs	Number ratio of hybrids to pollinations (%)	
			tested	yielding hybrids			average	maximum
1975								
AA1 ×	RR2	629	29	0	0	1	0	0
1976								
AA2 ×	RR1	138	8	0	0	12	0	0
	RR2	133	8	0	0	18	0	0
AA3 ×	RR1	73	6	0	0	0	0	0
	RR2	128	7	1	1	1	0.8	5.0
AA4 ×	RR1	121	7	0	0	0	0	0
	RR2	26	2	0	0	0	0	0
AA5 ×	RR1	39	6	3	3	1	7.7	25.0
	RR2	107	10	1	1	0	0.9	9.1
AA6 ×	RR1	455	18	13	39	5	8.6	53.3
	RR2	74	6	3	6	1	8.1	30.0
AA7 ×	RR1	158	8	0	0	3	0	0
	RR2	52	6	0	0	0	0	0
AA8 ×	RR1	398	18	2	12	2	3.0	57.9
	RR2	68	6	0	0	0	0	0
Total		2599			62	44	2.4	57.9

best A13 plant was even more than 100 % (Table 5).

Table 6 lists crosses between tetraploids. The mean crossability was slightly better than between diploids; on average 2.4 % with a range 0-8.6 %. Four tetraploid accessions of *B. campestris* gave no hybrids at all; of those accessions that produced hybrids, usually only a few plants did so, AA6 being a welcome exception. Female plants with good crossability were found in AA5, AA8 and especially AA6.

Crosses between diploid forms of *B. campestris* and tetraploid *R. sativus* gave rise to 5 hybrids from 3132 pollinations on about 130 *Brassica* plants (Table 7). The mean crossability was only 0.16 %.

In crosses with tetraploid forms of *B. campestris* as female parents and diploid forms of *R. sativus* as pollinator, crossability was poor too (Table 7). In total, 1411 pollinations on several *Brassica* plants gave rise to one hybrid only, which amounts to a crossability of 0.07 %.

Matromorphs were frequently found in $2x \times 2x$ and $2x \times 4x$ crosses and less frequently in $4x \times 2x$ and $4x \times 4x$ crosses (Table 5, 6 and 7). The respective number ratios of matromorphs to pollinations were 5.2, 8.1, 1.6 and 1.7 %. So diploid forms of *B. campestris* gave rise to matromorphs more easily than tetraploid forms.

Table 7. Results of 2x × 4x and 4x × 2x crosses between Brassica campestris (♀) and Raphanus sativus in 1975 and 1976.

Cross	Year	Number of pollinations	Number of female plants		Number of hybrids	Number of matromorphs
			tested	yielding hybrids		
2x × 4x						
A1 × RR2	1975	441	17	0	0	52
A2 × RR2	1975	213	8	0	0	12
A3 × RR2	1975	413	17	0	0	44
A4 × RR2	1975	189	8	0	0	3
A5 × RR2	1975	464	18	0	0	12
A6 × RR2	1975	353	15	2	2	3
A7 × RR2	1975	437	18	0	0	21
A8 × RR1	1976	69	4	0	0	15
RR2	1976	99	7	0	0	40
A9 × RR1	1976	31	2	0	0	14
A11 × RR2	1975	319	14	0	0	30
A13 × RR1	1976	38	4	0	0	1
RR2	1976	66	7	2	3	7
Total		3132		.	5	254
4x × 2x						
AA1 × R3	1975	727	29	0	0	7
AA2 × R1	1976	97	7	0	0	8
AA3 × R2	1976	161	7	0	0	3
AA4 × R1	1976	58	5	0	0	2
AA5 × R1	1976	9	1	0	0	0
R2	1976	32	6	0	0	0
AA6 × R1	1976	107	12	1	1	0
AA7 × R1	1976	10	1	0	0	0
R2	1976	27	4	0	0	1
AA8 × R1	1976	114	12	0	0	1
R2	1976	69	6	0	0	0
Total		1411			1	22

2.3.4 *Use of genetic male-sterility*

To avoid time-consuming hand-pollination, use of genetically male-sterile plants of *B. campestris* was tested in 1977, though on a limited scale. Four male-sterile plants grown in four isolation cages together with a flowering diploid or tetraploid accession of *Raphanus* gave rise to a considerable number of small seeds (Table 8). Unfortunately these cages were not completely pollen proof and some undesired cross-pollination occurred from flowering plants of *B. carinata* (BBCC) growing quite near to the cages. Therefore deviant plants with the genome constitution ABC were recovered as well as the expected intergeneric hybrids and matromorphs.

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Table 8. Results of crosses between male-sterile plants of <u>Brassica campestris</u> (2x) and diploid or tetraploid forms of <u>Raphanus sativus</u> (1977). Plants were grown in isolation cages and were pollinated by bees.

Parent No	Pollinator	Number of seeds		Number of				
		obtained	sown	plants	hybrids			matromorphs
						true	false*	
1	R4	156	15	12	0	10		2
2	R5	2	2	0	0	0		0
3	R6	70	15	9	8	0		1
4	RR1	15	15	5	1	3		1

*] Number of hybrids with the genome constitution ABC obtained by illegitimate pollination.

Table 9. Setting of siliquae, ovules and hybrid and matromorphic embryos in crosses in 1976 between diploid and tetraploid forms of <u>Brassica campestris</u> (AA and AAAA) and <u>Raphanus sativus</u> (RR and RRRR), as well as the developmental stage of the hybrid embryos 3-4 weeks after pollination.

Female parent	Number of					Embryo stage of hybrid embryos**									Number ratio of hybrid embryos to pollinations (%)
	polli-nations	siliquae	developed ovules	embryos*		?	G	H	LH	ET	T	LT	WS	M	
				M	H										
AA × RR															
A8	61	18	24	14	7					3	4				11
A9	109	24	37	21	8		2	1	2	3					7
A13	62	54	88	7	46			4	4	2	1	2	4	29	74
AA × RRRR															
A8	54	10	21	16	2		1	1							4
A9	84	32	64	64	0										0
A13	25	18	8	0	2	1							1		8
AAAA × RR															
AA5	126	50	10	1	5	1						1		3	4
AA6	164	78	59	2	29	2	10	7	3	2			2	3	18
AA7	14	10	1	0	0										0
AA8	106	57	41	0	18	1	6	9			1		1		17
AAAA × RRRR															
AA5	310	137	57	3	29	1	1	1	1	5	3		2	15	9
AA6	251	131	187	14	119	2	12	23	19	18	12	2	5	26	47
AA7	97	94	42	1	20		3	3	2	3	5		2	2	21
AA8	175	81	128	6	61	1	15	25	7	3	4	1	1	4	35

*] M matromorphic embryo; H hybrid embryo.
**] ? unidentified; G globular; H heart + early heart; LH late heart; ET early torpedo; T torpedo; LT late torpedo; WS walking stick; M mature I + II (Wilmar & Hellendoorn, 1968).

2.3.5 *Culture of embryos*

Embryo culture depends first on the availability of sufficient hybrid embryos. Table 9 summarizes data from intergeneric crosses made in 1976

for embryo culture. There were large differences between female parents in number ratio of developed ovules to pollinations, and number ratios of matromorphic and hybrid embryos to pollinations. Siliquae nearly always contained one or more developed ovules, which in general were rather small and often milky or even brown and shrivelled. Embryos obtained from such ovules were classified as hybrid embryos. However, large ovules were found too, especially in crosses with A8 and A9. These ovules contained large embryos, which were classed as matromorphic. As far as plants were raised from these ovules, the large ones gave exclusively matromorphs and the small ones hybrids. Not all developed ovules had a developed embryo.

Success of culturing embryos in vitro further depends on the size and condition of the embryos. The hybrid embryos cultured in 1976 differed very much in developmental stage (Table 9). Classification of the embryos by the scheme of Wilmar & Hellendoorn (1968) was usually possible except for a few embryos of irregular shape. The developmental stage ranged from globular to mature I + II. The great majority of the embryos had already reached their final stage, as judged from the condition of the ovules. This means that the development usually stopped before embryo maturity.

The results of culture of embryos from crosses between *B. campestris* and *R. sativus* in vitro are summarized in Table 10. In 1975 the technique was tested on embryos from crosses of A10 and A12 with 'Siletta'. The developmental stages of those embryos ranged from globular to mature. The proportion of plants raised from embryos was about 31 %, including hybrids and matromorphs. In 1976, all embryos mentioned in Table 9 were cultured in vitro. Hybrids and matromorphs were raised from $2x \times 2x$ and $4x \times 4x$ crosses and only matromorphs from $2x \times 4x$ crosses (Table 10). The matromorphs were obtained from comparatively large embryos. On average, 11 % of all embryos cultured in 1976 gave rise to a plant. The number ratio of hybrids to pollinations was unaccountably low in comparison to the hybrid production without embryo culture (Table 5, 6 and 7). Initially most embryos grew fairly well in the liquid medium, but afterwards many turned yellow and died.

2.3.6 *Genome constitution and number of chromosomes in the primary hybrids*

The chromosome number of 140 primary hybrids was determined (Table 11). Intergeneric crosses between diploids gave only plants with 19 chromosomes, although several hybrids had root-tip cells with doubled number of chromosomes. Apparently all embryos from these crosses had the expected genome constitution AR.

Crosses between tetraploids gave rise to hybrids, whose chromosome number ranged from 35 to 40. About 48 % had 38 chromosomes, the expected

Table 10. Summary of the results of culture of embryos obtained from crosses between diploid and tetraploid forms of <u>Brassica campestris</u> (AA and AAAA) and <u>Raphanus sativus</u> (RR and RRRR). The embryos were isolated 21-28 days after pollination.

Cross	Year	Number of pollinations	Number of embryos cultured	ovules cultured*	Number of hybrids	Number of matromorphs	Number ratio of hybrids to pollinations (%)
AA × RR							
A10 × R1	1975	69	1	39	0	1	0
A12 × R1	1975	191	132	76	38	2	19.9
A8 × R1	1976	61	21		0	1	0
A9 × R1	1976	109	29		0	3	0
A13 × R1	1976	36	36		7	1	19.4
R2	1976	26	17		3	1	11.5
Total		492	236		48	9	9.8
AA × RRRR							
A8 × RR1	1976	37	8		0	0	0
RR2	1976	17	10		0	1	0
A9 × RR2	1976	84	63		0	19	0
A13 × RR1	1976	8	1		0	0	0
RR2	1976	17	1		0	0	0
Total		163	83		0	20	0
AAAA × RR							
AA5 × R1	1976	6	1		0	0	0
R2	1976	120	5		0	0	0
AA6 × R1	1976	164	31		0	0	0
AA7 × R2	1976	14	0		0	0	0
AA8 × R1	1976	106	18		0	0	0
Total		410	55		0	0	0
AAAA × RRRR							
AA5 × RR1	1976	143	20		0	0	0
RR2	1976	167	12		2	1	1.2
AA6 × RR1	1976	207	108		4	2	1.9
RR2	1976	44	15		2	0	4.5
AA7 × RR1	1976	34	10		0	3	0
RR2	1976	63	11		0	0	0
AA8 × RR1	1976	175	67		2	0	1.1
Total		833	243		10	6	1.2

*] Ovules with no visible embryo.

Table 11. Chromosome numbers and probable genome constitutions of primary hybrids from crosses between diploid and tetraploid forms of <u>Brassica campestris</u> (AA and AAAA) and <u>Raphanus sativus</u> (RR and RRRR).

Cross	Number of hybrids classified	Genome constitution and chromosome number								
		AR	ARR		AARR					
		19	28	29	35	36	37	38	39	40
AA × RR	85	85								
AA × RRRR	6		3	1				2		
AAAA × RR	1						1			
AAAA × RRRR	48				1	1	12	23	10	1
Total	140	85	3	1	1	1	13	25	10	1

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euploid chromosome number of plants with the genome constitution AARR. Chromosome numbers deviating from 38 might result from fusion of aneuploid gametes derived from either of the two parents, especially the female parent.

The $2x \times 4x$ crosses resulted in two tetraploid and four triploid hybrids with probable genome constitutions AARR and ARR, respectively. One of the triploids was hyperploid ($2n = 29$). The two allotetraploids were presumably derived from a fertilized $2n$ egg cell.

The $4x \times 2x$ crosses gave rise to one hybrid with 37 chromosomes. A plausible explanation for this unexpected chromosome number is fusion of an aneuploid egg-cell and a $2n$ male gamete.

2.4 DISCUSSION

In this study, the crossability between diploid forms of *B. campestris* and *R. sativus* was intermediate to that found by other workers (Table 2). The mean crossability at the tetraploid level, however, was better than in previous reports. The results of $2x \times 4x$ and $4x \times 2x$ crosses were poor in comparison to those of crosses at the diploid and tetraploid level. So there is apparently an effect of ploidy on crossability in this intergeneric cross. Such effects have been described in various interspecific and intraspecific crosses among Cruciferae (e.g. Howard, 1942b). In the crosses studied, the optimum ratio of the genome number in embryo and endosperm is apparently 2 to 3. The occurrence of an unexpected high proportion of allotetraploids among the hybrids from $2x \times 4x$ and $4x \times 2x$ supports this view.

The viable seed from intergeneric crosses consisted of hybrid and matromorphic ones. Usually small seeds gave rise to hybrids, as was earlier reported by Nishi et al. (1964) and Tokumasu (1970b). Plump seeds resulted in matromorphs.

Many more matromorphs were obtained per pollination in crosses on diploid forms of *B. campestris* than on tetraploids. Although illegitimate self-pollination and cross-pollination cannot be precluded, the diploid forms seemed to have a better parthenogenetic ability of $2n$ eggs than the tetraploids after pollination with *Raphanus*.

Clear-cut differences between and within *B. campestris* accessions existed in crossability with *Raphanus*. Only a small proportion of the female plants used in this study produced hybrids. Variation in crossability could be caused by variation in environmental conditions. However as the female plants were grown and treated in a similar way, there must be genotypic variation in crossability. The finding that nearly all plants from an S_1 line (A13), which had been obtained by selfing a plant with good crossability, also had a good crossability, supports this view and proves that selection among the female parents for improved crossability

could be effective. The effect of pollinator genotype on crossability, was less pronounced. Possible genotypic variation within the male accessions, however, may have been masked by the use of bulked pollen.

The production of large numbers of hybrids is time-consuming. Efficiency could be improved by the use of genetically male-sterile plants of *B. campestris*. To facilitate large-scale production of hybrids it might be worth trying to combine male-sterility with a good genetically determined crossability.

Another interesting approach is embryo culture. Development of hybrid embryos mostly stops before embryo maturity. The results of embryo culture in this study, however, were disappointing, although in 1975 it considerably increased the number of hybrids raised per pollination.

3   Nature of breeding barriers

3.1 INTRODUCTION

Only a few brief reports exist on breeding barriers in the progamic
phase of crosses between *B. campestris* and *R. sativus*. Oelke (1957) ob-
served that pollen of several unnamed cruciferous species was inhibited
on radish stigmas. One of those species was *B. campestris* spp. *rapifera*
(Sampson, 1962). Its pollen germinated but did not penetrate the radish
stigma. Reciprocal crosses between the two species were studied at the
Scottish Plant Breeding Station by fluorescence microscopy. 'Pollen ger-
mination and growth were fairly good but very few ovules started devel-
opment' (Scottish Plant Breeding Station, 1971, p. 28).

Self-incomptability in *B. campestris* and *R. sativus* is governed by the
sporophytic action of one *S*-allelic series (reviewed by Hinata & Nishio,
1980). In contrast to Lewis & Crowe (1958), Sampson (1962) observed that
most crosses between self-incompatible species in Cruciferae were incom-
patible, except in the bud stage of flowers. Oelke (1957) had also found
that bud pollination had a beneficial effect in crosses between kohlrabi
(*B. oleracea* var. *gongyloides* L.) and radish, using the former as pis-
tillate parent. Thus bud pollination seemed an attractive method for
overcoming interspecific breeding barriers.

The present study was intended to clarify the nature of breeding bar-
riers during the progamic phase of fertilization in crosses between di-
ploid and tetraploid forms of *B. campestris* (♀) and *R. sativus* and to
test the significance of bud-pollination.

3.2 MATERIAL AND METHODS

Part of the 1976 crossing programme was an experiment on breeding bar-
riers. Plant material, growing conditions and crossing methods were as
described in Chapter 2, flowers being pollinated 2-3 days after emascu-
lation when some flower buds had reached the open-flower stage and the
rest were still in the bud stage. The oldest bud of each inflorescence
was marked and three days later the third pistil below and above the
marked pistil were fixed in Carnoy solution (6 volumes of aqueous etha-
nol (volume fraction 0.96), 3 volumes of chloroform and 1 volume of gla-
cial acetic acid) and stored in a refrigerator. The rest of the pistils
of each inflorescence were left on the plants and used for embryo cul-

ture afterwards. The results of these cultures are reported in Table 10.

The fixed pistils were subsequently softened in NaOH solution of substance concentration 1 mol/l for 1 h at 60 °C and stained in an aqueous solution with mass concentration of aniline blue 2g/l and K_3PO_4 20 g/l. The pistils were gently squashed in a drop of glycerol and studied under a fluorescence microscope.

In all pistils, the proportions of germinated pollen and the proportions of pollen grains with a pollen tube that had penetrated the stigma were counted among a random group of about 50 pollen grains per stigma. Although usually over 1000 pollen grains were applied on each stigma, mostly a few hundred grains were still present and sometimes even less than 50. The reduction in number of pollen grains was probably, at least in part, caused by the loss of ungerminated pollen during fixation, maceration and staining. In all pistils, the pollen tubes were counted in the transitional area between style and ovary and the ovules fertilized, i.e. the ovules with a pollen tube in their micropyle, were counted for each ovary.

3.3 RESULTS

Intergeneric incompatibility was studied in $2x \times 2x$, $2x \times 4x$, $4x \times 2x$ and $4x \times 4x$ crosses between *B. campestris* and *R. sativus*. In all crosses, typical incompatibility reactions occurred on the stigmas similar to incompatibility reactions after self-pollination. Usually more than 80 % of the pollen still present on a stigma had germinated. Germination was somewhat better on stigmas pollinated in the open-flower stage than in the bud stage. The pollen tubes were mostly short and strongly fluorescent, because of callose formation (Fig. 3A). Callose deposits were usually also observed in many stigma papillae. Such reactions apparently prevented the penetration of pollen tubes into the style. However a certain degree of penetration was usually observed after bud and open-flower pollination. Some abnormalities occasionally occurred within stigma papillae, such as branching of pollen tubes and inhibition of pollen tube growth (Fig. 3B).

No barrier seemed to exist in the style, and one or more pollen tubes entered the ovary in nearly all pistils, irrespective of the mode of pollination (Table 12). A high proportion of pistils in all crosses had more than ten pollen tubes per style. Bud pollination did not increase the number of pollen tubes per style. The results for the various crosses indicate that there was no marked effect of ploidy on penetration of pollen tubes into the style.

In the ovaries, most pollen tubes usually grew fairly straight to the bottom, where growth became erratic. Usually only a few pollen tubes grew in the direction of an ovule and penetrated through the micropyle (Table 13).

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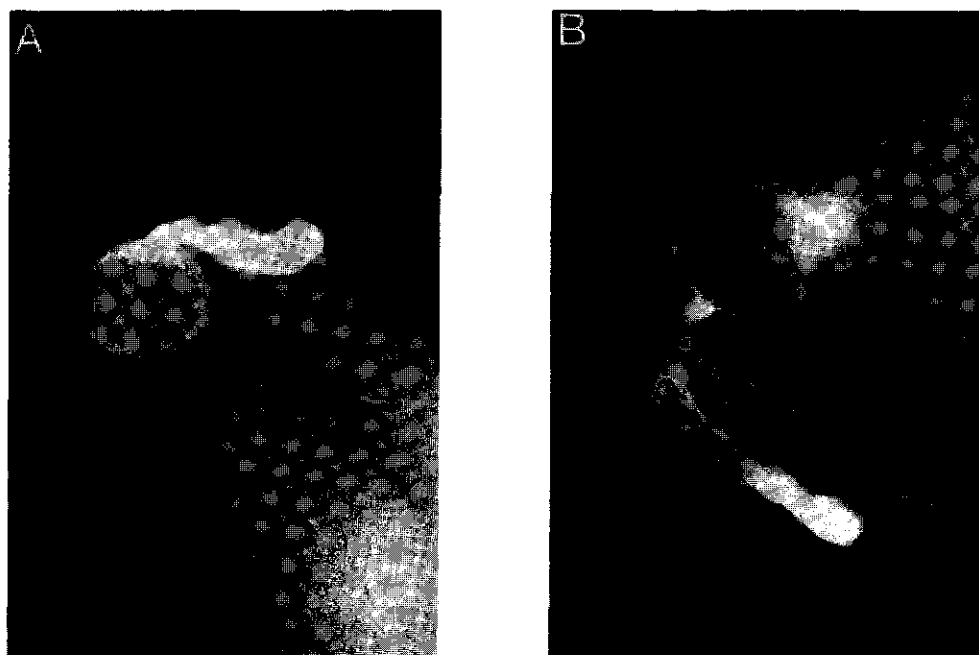
Figure 3. Incompatibility reactions in the progamic phase of crosses between Brassica
campestris and Raphanus sativus (ultraviolet fluorescence).
A. Inhibition of growth of pollen tube on a stigma papilla (×490).
B. Inhibition of growth of pollen tube within a stigma papilla (×490).

Table 12. Number of pollen tubes entering the ovary after bud and open-flower
pollination in crosses between diploid and tetraploid forms of Brassica campestris
(AA and AAAA) and Raphanus sativus (RR and RRRR). The data are based on observations
in the same pistils as in Table 13.

Cross	Mode of pollination	Number of pistils	Number of pistils		
			with no tubes	with 1-9 tubes	with ≧10 tubes
AA × RR	Bud	13	1	5	7
	Flower	13	1	6	6
AA × RRRR	Bud	12*	0	3	7
	Flower	13	1	4	7
AAAA × RR	Bud	18	1	5	13
	Flower	17	1	5	11
AAAA × RRRR	Bud	44	4	12	28
	Flower	44	2	9	33

*] Data from two pistils missing.

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Table 13. Number of fertilized ovules and the number of pistils in which fertilization
was observed in crosses between diploid and tetraploid forms of <u>Brassica campestris</u>
(AA and AAAA) and <u>Raphanus sativus</u> (RR and RRRR).

Cross		Number of pistils observed	Number of ovules observed[*]	Fertilized ovules		Pistils with ovules fertilized [**]	
				number	proportion(%)	number	proportion(%)
<u>AA</u>	× <u>RR</u>						
A8	× R1	9	203	11	5.4	3	33
A9	× R1	17	362	17	4.7	8	47
<u>AA</u>	× <u>RRRR</u>						
A8	× RR1/RR2	12	285	9	3.2	5	42
A9	× RR1	13	278	12	4.3	8	62
<u>AAAA</u>	× <u>RR</u>						
AA6	× R1	19	424	14	3.3	10	53
AA8	× R1	16	276	17	6.2	7	44
<u>AAAA</u>	× <u>RRRR</u>						
AA4	× RR1	9	141	2	1.4	2	22
AA5	× RR1	5	108	1	0.9	1	20
AA6	× RR1	27	637	30	4.7	15	56
AA6	× RR2	6	130	5	3.8	3	50
AA7	× RR1/RR2	12	248	23	9.3	9	75
AA8	× RR1	29	512	17	3.3	11	38

[*] Only easily visible ovules in each ovary were assessed.

[**] Fertilization is underestimated because some ovules were not visible.

Occasionally erratic growth of pollen tubes was also observed near the en-
trance to the micropyle. The proportion of ovules fertilized was low, 0.9
to 9.3 %. In many pistils, no fertilization was observed, and the number
of fertilized ovules per pistil averaged less than two in the best cross
(Table 13).

3.4 DISCUSSION

In the progamic phase of the intergeneric crosses studied, there were
obvious barriers to breeding: poor penetration of pollen tubes into the
stigmas and erratic growth of the pollen tube within the ovaries. The
first barrier was not fully effective and in most crosses a considerable
number of pollen tubes reached the ovaries. These observations agree
quite well with those at the Scottish Plant Breeding Station (1971). The
poor development of ovules observed in Scotland and in this study (Ta-
ble 9) seems to be caused primarily by scanty fertilization. Apparently
the ovules failed to attract pollen tubes. Such a breeding barrier was
also observed in interspecific crosses in *Arabidopsis* (Berger, 1968). A
rather high proportion of fertilized ovules apparently gave rise to de-
veloped ovules in which an embryo frequently was present (Table 9). Se-
lection within *B. campestris* for improved crossability might be achieved

by selection for genotypes with a relatively good fertilization as de-
tected by ultraviolet microscopy or for genotypes with a high proportion
of developed ovules or embryos per pollination.

In the progamic phase, there were no striking differences between $2x$
$\times$ $2x$, $2x \times 4x$, $4x \times 2x$ and $4x \times 4x$ crosses for the number of pollen tu-
bes observed in the style and success of fertilization. The effect of
ploidy on crossability (Section 2.4) apparently came after fertilization
and not before it.

Bud pollination was certainly not an effective method of circumven-
ting the breeding barriers on the stigma, as would be expected from the
results of Sampson (1962), because there was no marked difference be-
tween bud and open-flower pollination in degree of the incompatibility
reactions on the stigma and penetration of pollen tubes into the stigma.

A role of the S-locus in interspecific incompatibility or incongru-
ity as defined by Hogenboom (1973; 1975) was assumed, for instance by
Sampson (1962), but should be queried, because the activities of that
locus are found in stigmas of open flowers and older buds. Perhaps the
buds studied were too old and the S-locus was already operative. However
the results of Namai (1980), who recently studied the effect of age of
buds in crosses between *B. campestris* and *R. sativus*, indicate that
crossability of younger buds is even less.

4 Primary allodiploid AR hybrids and their progeny

4.1 INTRODUCTION

Several authors (Table 2) have obtained AR hybrids from crosses be-
tween diploid forms of *B. campestris* and *R. sativus*. Terasawa & Shimo-
tomai (1928), Tokumasu (1970b) and Opeña & Lo (1978) have described such
hybrids from crosses between oriental vegetable forms of *Brassica* and
common radish or Japanese radish. The plants were intermediate between
the parental species in many respects with a leaf rosette in the vege-
tative stage and hardly show any thickening of the roots, if at all. The
white and purple flower of *R. sativus* proved to be dominant over the
yellow of *Brassica*. Clauss (1978), however, obtained only yellow-flower-
ed hybrids from similar crosses. The siliquae of the hybrids consisted
of two parts as in *Brassica*, i.e. a valvate part and a rather large in-
dehiscent part, the beak. Both parts contained ovules (Terasawa & Shi-
motomai, 1928; Tokumasu, 1970b). Tokumasu (1970b) observed also that the
hybrids grew vigorously in the later stages of development.

Various authors studied meiotic chromosome behaviour in such hybrids
(Terasawa & Shimotomai, 1928; Richharia, 1937; U et al., 1937; Mizushi-
ma, 1944b; 1950a; Tokumasu, 1970b). The data presented vary considera-
bly. According to U et al. (1937), Terasawa found a maximum of five bi-
valents per cell. In their own hybrids, U et al. (1937) counted no more
than three bivalents and Richharia (1937) even found a maximum of eight
bivalents per cell. Tokumasu (1970b) observed six bivalents per cell at
most and also found a low frequency of trivalents and quadrivalents. The
causes of the differences in degree of chromosome association are un-
known, but might be in part genetic.

Another intriguing aspect of allodiploid hybrids is their fertility.
Morris (1936) and Tokumasu (1970b) studied male fertility. Morris found
that all hybrids studied produced a considerable amount of diploid ger-
minable pollen. Pollen stainability varied considerably from day to day
and probably from plant to plant. Tokumasu (1970b) also noticed a rather
high proportion of stainable pollen, varying from 4 to 30 % with a mean
of 11 %. However seed fertility of the hybrids, as determined by inter-
mating, was very poor. Terasawa & Shimotomai (1928) obtained some F_2
plants, which differed in form and flower colour. Also Morris (1936) and
Mizushima (1952) obtained F_2 plants. However Tokumasu (1970b) did not
succeed in obtaining a progeny from F_1 hybrids.

The crossability of the diploid hybrids with the two parental species, *B. campestris* and *R. sativus* was equally poor (Terasawa & Shimotomai, 1928; Tokumasu, 1970b). Only Terasawa & Shimotomai (1928) obtained a few completely sterile plants from backcrossing F_1 hybrids as females with the *Brassica* parent. Backcrosses with *R. sativus* failed.

This chapter presents observations on various aspects of the diploid F_1 hybrids obtained in this study, such as form, meiotic association of chromosomes, pollen and seed fertility. Also reported are chromosome number and fertility of F_2 plants, attempts to double the chromosome number of F_1 hybrids and crosses of F_1 hybrids with *B. campestris*, *R. sativus* and *B. napus* respectively.

4.2 MATERIAL AND METHODS

All hybrids studied were obtained from the crosses between the diploid accessions of *B. campestris* and *R. sativus*. All hybrid plants, as far as was known, had 19 chromosomes and the genome constitution was probably AR. The plants will be referred to as AR hybrids or *rapifera* hybrids, for instance, referring to the *Brassica* parent in the original intergeneric crosses. The hybrids were grown in the same way as their parents, and under similar conditions, but most were transplanted into soil of a heated greenhouse just before flowering. Non-bolting plants were first placed in a cold greenhouse for about 40 days in order to induce flowering.

Pollen stainability was assessed on fresh pollen collected from various flowers by a method of Sass (1964) with a random sample of about 200 pollen grains per plant. Some slides were also used for measuring pollen grain size. The methods used for studying mitosis and meiosis have been described in Section 2.2. All data on the constitution of sporad cells were obtained from anthers squashed in a drop of propiono-iron-alum haematoxylin (Henderson & Lu, 1968).

Several AR hybrids were vegetatively propagated from cuttings, culture in vitro of stem and petiole explants (Kartha et al., 1974) or of axillary buds. Propagation by cuttings, which were treated with a talcum powder containing indolebutyric acid (IBA) at a substance content of 5g/kg, gave usually rise to weak and small plants. The two methods of culture in vitro resulted in well developed plants. The overall results of propagation through stem and petiole explants, however, were poor; some shoot formation occurred on a culture medium of Murashige & Skoog (1962) containing 6-benzylamino purine (BA) and 1-naphthaleneacetic acid (NAA) at substance concentrations of 10 and 0,5 mmol/m³, respectively. Culture conditions and medium for in vitro culture of axillary buds were the same as in Kartha et al. (1974). The method gave relatively good results on a medium containing BA at 5 mmol/m³ and NAA at 1 mmol/m³.

Cuttings of various AR hybrids were transplanted in isolation cages (2.5 m × 2.5 m) placed outside and intercrossed with the help of honey-bees to obtain an F_2 generation. Subsequently, the F_2 plants were multi-plied in a similar way in cages in a greenhouse.

All axillary buds of rooted cuttings of several AR-hybrids were treated with colchicine in four substance concentrations ranging from 2 to 6 g/l. The colchicine treatments were carried out according to the traganth-slime method of Schwanitz (1949) at about 18 °C and with high air humidity.

4.3   RESULTS

4.3.1 *Form of AR hybrids*

Intergeneric crosses between *B. campestris* and *R. sativus* gave rise to hybrids and matromorphs. The distinction between the two types proved to be easy and was usually possible in an early developmental stage. For example, hybrids usually had a whitish hypocotyl and their first leaf was pubescent. In contrast, matromorphs from crosses between *B. campes-tris* ssp. *chinensis* and fodder radish, for instance, had plain green and more robust hypocotyls and glabrous leaves. The hybrids subsequently de-veloped a leaf-rosette with several slightly pubescent leaves (Fig. 4A). The shape of the leaves was intermediate, but very variable, ranging from simple to pinnate. Hybrids usually had slightly pubescent petioles, which were sometimes slightly red from anthocyanins. Some hybrids from crosses with turnip showed a swollen turnip-like root. On the whole, the hybrids proved to be vigorous, although a few were stunted or even deformed.

The transition from vegetative to generative stage did not require cold treatment, except for some hybrids originating from crosses with stubble turnip, a form of *B. campestris* ssp. *rapifera* that needs cold for flower induction. The flowering time of AR hybrids was intermediate between that of the parental species.

In the generative stage, hybrids usually had an erect main stem with a terminal inflorescence (Fig. 4B). Afterwards many lateral branches with secondary inflorescences developed and the plants reached a height of 1.3-1.7 m. The indeterminate flowering observed in AR hybrids, probably caused by a high degree of sterility, resulted finally in a wild dense bunch of branches. On the stem and lateral branches, a slight pubescence and some anthocyanin coloration was occasionally present.

The inflorescences of AR hybrids were more similar to *B. campestris* than to *R. sativus* in number of flowers, flower size and the position of flowers and flower buds (Fig. 4C). The buds were usually slightly pubescent. Most AR hybrids had white or purple flowers with clear-cut veins, like *Raphanus*, but some plants had plain white flowers or even

Figure 4. Hybrids with the genome constitution AR from the cross *Brassica campestris* ssp. *chinensis* × *Raphanus sativus* cv. Siletta.
A. Vegetative stage.
B. Generative stage.
C. Inflorescence.

yellow flowers, like *Brassica*. In total, about 11 % of all flowering AR
hybrids were yellow-flowered. The flowers mostly had striking veins, but
a few had plain yellow flowers. In a few hybrids with white or purple
flowers, sectorial chimaeras for flower colour were observed; usually a
small sector of a petal being yellow.

Some AR hybrids showed some setting of small bivalved siliquae with a
large beak.

4.3.2 *Chromosome association at meiosis*

Early meiotic stages in AR hybrids were quite regular but in diaki-
nesis and metaphase I, frequent univalent formation disturbed meiosis
(Table 14). Data presented in the Table 14 should be interpreted with
caution. Analysis of chromosome association in such hybrids was difficult,
because the high frequency of univalents sometimes hindered recognition
of meiotic stages (metaphase I and anaphase I, for example). It also pro-
ved difficult to distinguish between true bivalents and secondary asso-
ciations of univalents.

No chromosome association was observed in 67 % of all pollen mother
cells studied in six AR hybrids. Cells with one or two bivalents occur-
red in 21 % and 8 % of the pollen mother cells, respectively, and tri-
valents in 2.6 % of the cells. The maximum number of bound chromosomes
was eight in a cell with four bivalents. Most bivalents were rod-shaped
(Fig. 5A, B), and all trivalents were chains. The differences in chromo-

Table 14. Distributions of meiotic configurations in diakinesis or metaphase I in six
AR hybrids from crosses between <u>Brassica campestris</u> ssp. <u>chinensis</u> and <u>Raphanus sativus</u>
cv. Siletta.

Configurations*	Number fractions (%) of named hybrids						Number of cells	
	EC119	EC186	EC209	EC274	EC314	75.1040-9	Observed	Expected**
19 I	78.4	63.9	62.0	75.5	65.7	62.8	361	336
17 I + 1 II	15.7	26.4	24.0	13.8	21.2	24.4	115	158
16 I + 1 III		1.4		1.1	2.0	1.7	7 }	37
15 I + 2 II	5.9	5.6	12.0	6.4	10.1	8.7	44 }	
14 I + 1 II + 1 III			2.0	1.1		1.7	5 }	6
13 I + 3 II		1.4		1.1	1.0		3 }	
12 I + 2 II + 1 III		1.4				0.6	2 }	1
11 I + 4 II				1.1			1 }	

	Total number of cells							
	51	72	50	94	99	172		538

*] I, II and III are symbols for univalents, bivalents and trivalents, respectively.
**] Expectation based on a Poisson distribution for a mean number of 0.47 bivalents
   per cell.

Figure 5. Chromosome association, dyad formation and stainable pollen in AR hybrids
(2n = 19).
A. Pollen mother cell at metaphase I with 15 I + 2 II (×1540).
B. Pollen mother cell at metaphase I with 17 I + 1 II (×1540).
C. Sporad stage with dyads and one tetrad (×550).
D. Stainable pollen (×550).

Table 15. Frequency distribution of pollen stainability in AR hybrids from crosses between <u>Brassica campestris</u> and <u>Raphanus sativus</u>. Data from 1975.

Pollen stainability (%)	Number of plants	Number fraction of plants (%)
0	53	62
1 - 10	16	19
11 - 20	8	9
21 - 30	6	7
31 - 40	2	2
Total	85	

some association between the AR hybrids were only small.

The mean number of bivalents per cell in the six AR hybrids was 0.47 (one trivalent calculated as two bivalents). The frequency distribution of the number of bivalents observed per cell deviates significantly from a Poisson distribution (P < 0.01) for μ = 0.47, because of an evident excess of cells with exclusively univalents and cells with one trivalent or two bivalents. Chromosome association is apparently not simply a matter of chance; some chromosomes probably pair more easily than others.

4.3.3 *Male and seed fertility*

The majority of AR hybrids were completely male-sterile, but 38 % of the plants studied produced some stainable pollen grains (Table 15, Fig. 5D). All AR hybrids from crosses with male-sterile turnips, for instance, were partly male-fertile. The density function of proportion of pollen grains stained showed that two plants produced over 30 % stainable pollen. Stainable pollen was usually ovoid-spherical and had a diameter of 35.2 µm (mean for 5 AR hybrids).

Cytokinesis in AR hybrids was irregular, resulting in various types of sporad : besides normal tetrads, monads, dyads, triads, pentads and hexads (Table 16). The size of the cells in a sporad usually varied considerably, but dyads usually contained two cells of equal size (Fig. 5C). Relatively high frequencies of dyads occurred in several *chinensis* hybrids and in nearly all *rapifera* hybrids. Most of these plants also had a relatively high proportion of stainable pollen. So dyad formation is prerequisite but not a guarantee for pollen stainability. One *rapifera* hybrid, 75.1283-1, also produced and exceptionally high number of monads (47 out of 98).

The high proportion of dyads in many AR hybrids can be explained by the occurrence of restitution nuclei at interkinesis, as such nuclei were observed mainly in plants with a high dyad production or a high pollen stainability.

The well filled and stainable pollen of AR hybrids were germinable.

Table 16. Distribution of sporads with different number of cells per sporad in some
AR hybrids originating from crosses between Brassica campestris ssp. chinensis or ssp.
rapifera and Raphanus sativus, cv. Siletta or var. mougri.

Plants	Total number of sporads	Number of sporads						Number fraction of dyads (%)	Pollen stainability (%)
		1	2	3	4	5	6		
chinensis hybrids									
EC119	87	0	15	10	52	10	0	17	0
EC138	58	0	1	6	48	1	2	2	1
EC186	129	2	32	8	84	2	1	25	6
EC189	74	2	58	7	7	0	0	78	24
EC200	65	2	12	0	48	3	0	18	0
EC263	137	0	43	21	71	2	0	31	7
EC274	63	0	0	8	44	11	0	0	0
EC277	69	0	4	5	52	7	1	6	0
EC282	68	1	25	2	40	0	0	37	32
EC305	57	0	20	7	30	0	0	35	11
EC314	55	3	23	2	27	0	0	42	17
75.1040-9	72	0	5	6	60	1	0	7	0
75.1088-1	94	2	6	2	81	3	0	6	0
rapifera hybrids									
75.1283-1	98	47	49	1	1	0	0	50	2
75.1308-2	66	0	34	9	23	0	0	52	8
75.1310-2	72	1	37	11	22	1	0	51	6
75.1427-3*	79	0	51	8	20	0	0	65	20
75.1671-1	56	0	1	1	53	1	0	2	0
75.1682-5*	62	0	41	1	20	0	0	66	25
Total	1461	60	457	115	783	42	4	31	8

*] The marked AR hybrids originated from R. sativus var. mougri.

In crosses of AR hybrids as male parents with B. campestris ssp. chinensis and B. napus, for example, germinated pollen could be demonstrated by ultraviolet microscopy. Two days after pollination, pollen tubes were often observed in the style and some of them had even reached the micropyle of an ovule.

Seed fertility of AR hybrids was poor. Many attempts were made to propagate such hybrids by intercrossing, even with culture of immature embryos in vitro (Table 17). The considerable efforts with intercrossing of AR hybrids resulted in only three plants. However, propagation in isolation cages of some AR hybrids or cuttings of them still gave rise to several more seeds (Table 18). In total, 36 % of the hybrids produced seeds in this way.

4.3.4 Crossability of AR hybrids with various species

The results of crosses between AR hybrids and B. campestris, B. napus and R. sativus, respectively were poor (Table 17). From crosses of AR hybrids (2n = 19) with B. campestris (2n = 20) as pollinator, only one hybrid was obtained with 47 chromosomes. The reciprocal cross gave only

Table 17. Numbers of seeds and embryos obtained by intercrossing AR hybrids and by crossing AR hybrids with Brassica campestris (AA), Raphanus sativus (RR), Brassica napus (AACC) and AARR hybrids. All embryos were cultured in vitro.

Cross		Number of pollinations	Number of		Number of		Number of plants	
			seeds	plants obtained	embryos cultured	plants obtained	hybrids	matromorphs
AR	× AR	ca 2500	5	2	11	1	3	0
AR	× AA	ca 350	1	1	2	0	1	0
AR	× AACC	120	4	2	3	1	3	0
AA	× AR	ca 900	13	4	26	4	0	8
RR	× AR	66	0	0	1	0	0	0
AACC	× AR	811	105	84	15	4	1	87
AARR	× AR	ca 1000	12	6	2	0	6	0
AR	× AARR	ca 250	3	0	0	0	0	0

matromorphs, some of which were raised by embryo culture.

Pollination of AR hybrids by *B. napus* gave three very similar hybrids, all derived from one particular *rapifera* hybrid. The plants had a stem like that of forage rape and leaves and petioles were pubescent. In the generative stage, these plants produced only a few flowers, which were completely white. Unfortunately the chromosomes of these hybrids were not counted. The reciprocal crosses gave an impressive number of plants, partly raised by embryo culture. Only one plant was a true hybrid with $2n = 55$; the others were matromorphs. The hybrid was obtained from a small seed; it grew irregularly, had white flowers and even produced stainable pollen.

Crosses of fodder radish (♀) and AR hybrids were made on a small scale, resulting in only one embryo (Table 17). Culture of this embryo in vitro failed.

Table 18. Seed production in the F_1 and F_2 generation of two populations of AR hybrids. The F_1 and F_2 plants from each population were propagated in cages and pollinated by bees.

Origin population	Generation	Number of plants		Number of seeds
		tested	producing seeds	
rapifera hybrids	F_1	21	9	45
	F_2	24	10	53
chinensis hybrids	F_1	15*	4	23
	F_2	15	10	441

*] 23 cuttings

4.3.5 *Somatic and meiotic doubling of chromosome number*

Various methods were used to double the chromosome number of AR hybrids. The first method, colchicine treatment of axillary buds of 90 rooted cuttings from 28 AR hybrids, resulted in doubling in 32 cuttings. Recognition of such doubled sectors proved rather easy, because flowers were larger and pollen was usually more stainable. A treatment with colchicine solution of mass concentration 4 g/l (in traganth slime) gave the best results, but differences from other colchicine concentrations were small.

Another approach for doubling the chromosome numbers of AR hybrids was to intercross AR and AARR hybrids. Doubling was expected in this way by meiotic nuclear restitution in the AR hybrids. Table 17 shows that no plants were obtained if AR hybrids were used as female parent. The reciprocal cross gave rise to six vigorous hybrids. Two of these plants had 38 chromosomes and one plant had 36; the chromosomes of the others were not counted.

Chromosome doubling occurred by chance in a stem explant of one AR hybrid cultured in vitro.

4.3.6 *Progeny of crosses between AR hybrids*

Seeds from crosses between AR hybrids were produced by hand-pollination (Table 17) or insect pollination (Table 18). Of the seeds, about 64 % germinated and most of those gave rise to viable plants. The offspring of such crosses varied widely in chromosome number (Table 19). However, a remarkably high proportion of the plants had about 57 chromosomes, especially if obtained from crosses between *chinensis* hybrids. Such hexaploids probably had the genome constitution AAAARRR. The remaining plants mostly had aneuploid chromosome numbers and were genetically less balanced than the hexaploid plants.

The progeny of crosses between AR hybrids also varied widely in form, not surprisingly in view of the variation in chromosome number. Some

Table 19. Somatic chromosome numbers in progeny of AR hybrids produced by intercrossing chinensis hybrids and rapifera hybrids respectively.

Population	Chromosome number															Number of plants classified
	29	31	35	36	37	38	39	40	46	52	54	55	56	57	58	
rapifera hybrids	2	1	1	2	1	2	3	1		1				5	1	20
chinensis hybrids	2								1		1	1	2	6		13
Total	4	1	1	2	1	2	3	1	1	1	1	1	2	11	1	33

Figure 6. Two hexaploid plants with probable genome constitution AAARRR obtained by intercrossing AR hybrids derived from crosses between stubble turnip (2x) and Raphanus sativus (2x).

plants showed serious growth irregularities, but others grew vigorously, especially the hexaploids (Fig. 6). The hexaploid plants usually had plump flower buds, large white or purple flowers, and produced some stainable pollen.

For propagation, most offspring of *rapifera* and *chinensis* hybrids was transplanted into three isolation cages with bees for pollination (Table 18). Over 50 % of the offspring produced some seed. The seed production per plant was 2.2 and 29.4 in the offspring of *rapifera* and *chinensis* hybrids, respectively. Most of the seed-producing plants were hexaploids with about 57 chromosomes.

4.4 DISCUSSION

The form of AR hybrids obtained by other authors (Table 2) and of the ones in the present study were similar. Some of the present hybrids, however, had yellow flowers, as reported by Clauss (1978). In hybrids with white and purple flowers, the synthesis of flavonoids was apparently suppressed, as in *R. sativus* (Bonnet, 1978). This suppressor system is perhaps inactivated in the yellow-flowered hybrids by one or more genes

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from the A genome. Interaction between genes of the two genera also occurred in partially male-fertile hybrids, which originated from crosses between male-sterile turnips and *R. sativus*. Pollen sterility in turnip proved to be inherited in a simple monofactorial and recessive way (Dolstra, unpublished). So occurrence of pollen fertility in AR hybrids of male-sterile turnips indicated that the effect of the gene for pollen sterility in these hybrids is compensated by gene(s) of *Raphanus*.

Only a low degree of chromosome association was found at meiosis in AR hybrids. In comparison with the results of Richharia (1937) and Tokumasu (1970b), the frequency of cells with only univalents was high and the maximum number of associated chromosomes per cell low. Genotypic and environmental variation may account for such differences. In extensive studies on chromosome association in interspecific hybrids and haploids, Mizushima (1980) found a maximum of two autosyndetic chromosome pairs in the A and R genome together. If that estimate is correct, the maximum number of allosyndetic chromosome pairs noticed in the present AR hybrids is two. Higher values have been found (Mizushima, 1980).

A remarkable phenomenon is that a high proportion of the AR hybrids produced a considerable proportion of stainable pollen. The main cause is the occurrence of dyads in the sporad stage, probably by the formation of restitution nuclei in interkinesis, as suggested by Morris, (1936). However, Tokumasu (1970b) observed low frequencies of dyads in three hybrids with a rather good pollen stainability. The mechanism of formation of restitution nuclei is still obscure. A possible cause might be a poorly developed spindle as a consequence of scanty pairing of chromosomes. However, variation in the degree of dyad formation was not related to the degree of chromosome association.

Intercrossing of AR hybrids gave a very poor seed set. The only feasible method of obtaining seed was to grow the hybrids in isolation and to use bees for pollination. A remarkable number of F_1 plants produced in this way had a high number of chromosomes compared with the results of Terasawa & Shimotomai (1928). Such plants with about 57 chromosomes were frequently found and presumably had the genome constitution AAARRR. Fusion of 4n egg cells and unreduced male gametes may give rise to such plants. A hybrid with 47 chromosomes from a cross between an AR hybrid (2n = 19) and *B. campestris* (2n = 20) possibly also originated by fusion of a hypoploid 4n gamete and a normally reduced *Brassica* gamete. In Cruciferae, the occurrence of 4n gametes was reported earlier for haploids of *B. napus* (Heyn, 1974).

Chromosome doubling by colchicine treatment with the traganth-slime method of Schwanitz (1949) was possible, but had the disadvantage that the doubled sectors mostly were rather small. As primary AARR hybrids have a poor fertility (Section 5.3.1), seed production on such doubled sectors often failed because of a shortage of flowers.

The production of viable unreduced pollen by many AR hybrids makes it tempting to double the chromosome numbers of these hybrids meiotically by using them as pollinator in crosses with AARR hybrids. However, such crosses gave rather poor results, probably because of the poor fertility of the AARR hybrids used. The use of more fertile ×*Brassicoraphanus* may improve the seed-set considerably.

5 xBrassicoraphanus: form, fertility and crossability with various species

5.1 INTRODUCTION

No published data were available on the agricultural value of ×*Brassicoraphanus*, although such allotetraploid hybrids have been produced in various ways. Mizushima (1968) expected that synthesis of ×*Brassicoraphanus* by crossing turnips and radish could be a promising way of obtaining a new root crop.

Allotetraploids with the genome constitution AARR were first found in an F_4 generation of some AR hybrids (Terasawa, 1932). The common methods of synthesis were doubling of the chromosome number of AR hybrids by colchicine treatment (Mizushima, 1950b; Tokumasu, 1976; Section 4.3.5) and crossing of tetraploid forms of the two parental species (Nishiyama, 1946; Tokumasu, 1970b; Olsson & Ellerström, 1980; McNaughton & Ross, 1978; Section 2.3). Less attractive methods are $2x \times 4x$ crosses between *B. campestris* and *R. sativus*, although it was possible to obtain hybrids in this way (Section 2.3.6). McNaughton (Scottish Plant Breeding Station, 1976, p. 16) tried to produce ×*Brassicoraphanus* indirectly by crossing *B. napocampestris*, an artificial hexaploid with the genome constitution AAAACC, with autotetraploid forms of *R. sativus* (RRRR). In the subsequent generations of the AARRC hybrids, a gradual loss of the C genome was expected. Many hundreds of pollinations gave only one seed.

The form of the AARR hybrids was poorly described. In many respects such hybrids were known to be intermediate between the parental species and similar to the corresponding AR hybrids (Terasawa, 1932). An undesired trait of such plants was the poor fertility (Mizushima, 1950b; Tokumasu, 1976; Olsson & Ellerström, 1980).

The crossability of ×*Brassicoraphanus* as a female parent with the diploid parental species was rather poor, but seeds were obtained (Terasawa, 1933; Nishiyama & Inomata, 1966; Kato & Tokumasu 1979; 1980). The reciprocal crosses gave no seeds at all (Nishiyama & Inomata, 1966). The failure of such crosses was due either to the death of hybrid embryos or to abnormal development of the endosperm and inhibition of embryo development (Nishiyama & Inomata, 1966). Kato & Tokumasu (1979) reported that ×*Brassicoraphanus* with white flowers showed much more cross-affinity with white-flowered *R. sativus* than with diploid yellow-flowered *B. japonica* Sieb. (= *B. campestris* ssp. *nipposinica*). The reverse was found for yellow-flowered ×*Brassicoraphanus*. Pollination of white flowered ×*Brassico-*

raphanus with tetraploid *R. sativus* gave rise to 1.1 seeds per pollination, whereas pollination with tetraploid *B. japonica* gave no seed at all (Kato & Tokumasu, 1978).

Terasawa (1933) tested the crossability of ×*Brassicoraphanus* with *B. napus* (AACC), *B. cernua* (2n = 36, AABB) and *B. oleracea* (CC), using the *Brassica* spp. as pollinators. An exceptionally high seed-set was obtained from crosses with *B. napus*, a rather good seed-set with *B. cernua* and no seed with *B. oleracea*.

The form and fertility of primary allotetraploid hybrids was studied and the crossability with various species was tested.

5.2 MATERIAL AND METHODS

The primary allotetraploid hybrids described below arose directly from the intergeneric crosses mentioned in Tables 6 and 10. The data presented on seed-set of primary AARR hybrids, however, also include crosses with 'doubled' AR hybrids (Section 4.3.5). The parental material of ×*Brassicoraphanus* used in crosses with various species consisted in 1976 and 1977 of primary hybrids and in 1978 of plants from the second generation. Sometimes the various hybrids are below called, for instance, *chinensis* or *perviridis* hybrid, referring to the *Brassica* parent in the original intergeneric cross.

Most accessions of the various species crossed with ×*Brassicoraphanus* were mentioned in Table 3, except for two varieties of spring oil-seed rape. Seed of 'Zephyr', a Canadian variety, was kindly supplied by Dr. G.R. Stringam (Research Station Canada Department of Agriculture, Saskatoon, Canada) and seed of 'Tantal', a French variety, by Zelder BV (Ottersum, Netherlands).

Plant material was treated normally, except that the AARR plants were transplanted before flowering into the soil of a heated greenhouse. Crossing technique and other methods were the same as in previous experiments. In 1976, flower buds were mostly pollinated immediately after emasculation but, since 1977, two or three days after emasculation, when some buds had opened. The reason for this change of technique was the questionable significance of bud pollination in intergeneric hybridization.

5.3 RESULTS

5.3.1 *Form and fertility of primary hybrids*

Growth habit of F_1 AARR hybrids was similar to that of AR hybrids. In the vegetative stage, all had a leaf-rosette, irrespective of the *Brassica* parent used (Fig. 7). Leaf shape was intermediate but varied widely. Leaves and petioles were less pubescent than of *Raphanus*. Hairiness pro-
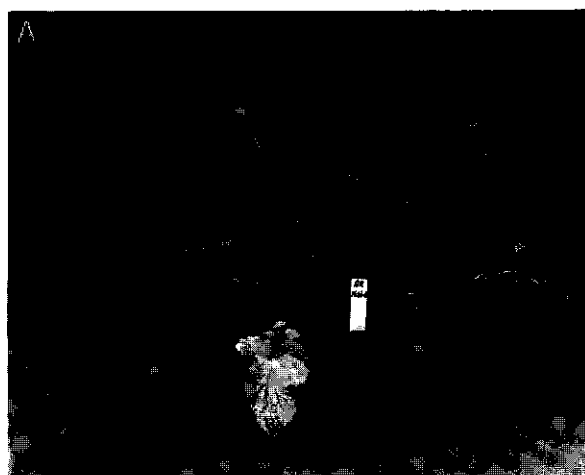
Figure 7. Allotetraploid hybrids from crosses of a tetraploid fodder radish, RS14.
A. With a diploid inbred line of turnip (♀).
B. With tetraploid turnip cv. Tigra (♀).
C. With a tetraploid form of Brassica campestris ssp. perviridis (♀).

ved to be an excellent marker in early stages of development to distin-
guish hybrids from matromorphs in crosses between oriental vegetable
forms of *B. campestris* and *R. sativus*, because the former were glabrous.

Similarity in growth habit to AR hybrids was also remarkable in the
generative stage. All hybrids had a main flower stalk, which later pro-
duced many lateral branches. The flowers were somewhat larger than in
tetraploid *B. campestris* and were white or purple. However, two hybrids,
both with 39 chromosomes, had yellow flowers. Most flowers, whatever the
colour, had petals with strikingly dark veins, although plants with
plain white or plain yellow flowers occurred too. Variation in the ex-
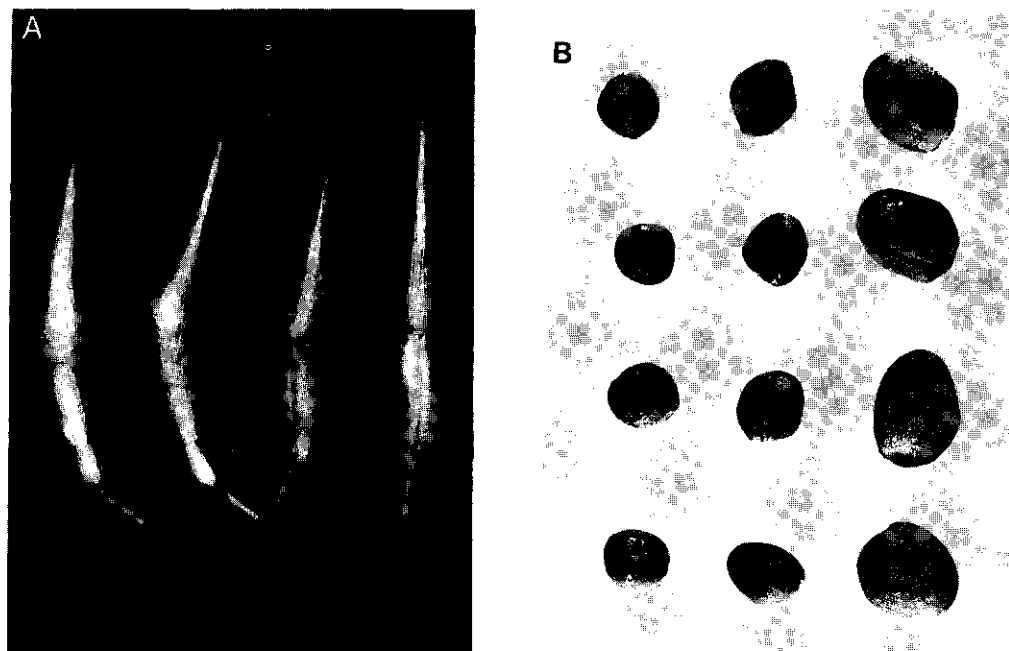tent of flowering was frequently observed. Some plants produced only a

Figure 8. Siliquae and seeds of F_1 hybrids with genome constitution AARR.
A. Siliquae of a hybrid from the cross <u>Brassica</u> <u>campestris</u> ssp. <u>chinensis</u> (4x) and fodder radish cv. Palet.
B. Seeds of B. <u>campestris</u> ssp. <u>perviridis</u> (left row), an AARR hybrid (middle row) and <u>Raphanus</u> <u>sativus</u> cv. Palet (right row).

few flowers, if at all; whereas the flower buds generally dried and dropped prematurily, others flowered abundantly. Siliquae of ×*Brassicoraphanus* were intermediate between those of the parents (Fig. 8A). Each consisted of a dehiscent bivalved part with a septum and a rather large indehiscent part, the beak or rostrum. The mean number of ovules per pistil was about 19, with some in the beak.

The fertility of F_1 AARR hybrids will be described in more detail in Section 7.3.2. In summary, the hybrids showed a large variation in pollen stainability and varied widely in setting of siliquae. The seed-set, on the other hand, was very poor in crosses between AARR hybrids; on average 0.008 seed per pollination. Usually, these seeds were rather well-filled and were similar in diameter and 1000-grain weight to *B. campestris* (Fig. 8B).

5.3.2 *Crossability with Brassica campestris, Raphanus sativus and Brassica napus*

×*Brassicoraphanus* was used extensively in crosses with the parental species and *B. napus* (Table 20). The crosses in which diploid forms of

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Table 20. Results of crosses of ×Brassicoraphanus (AARR) with Brassica campestris (AA and AAAA), Raphanus sativus (RRRR) and Brassica napus (AACC).

Cross	Year	Number of				seeds		hy-brids	matro-morphs
		female plants	seed produ-cing plants	polli-nat-ions	sili-quae	obta-ined	sown		
AARR × AA									
chinensis hybrids × B.c.ssp. chinensis	1976	4	1	ca 85	34	19	19	9	
	1977	4	0	90	21	0	0	0	
chinensis hybrids × B.c.ssp. oleifera	1976	1	1	ca 6C	7	5	5	3	
perviridis hybrids × B.c.ssp. chinensis	1977	6	1	112	6	4	4	3	
	1976	1	0	7	0	0	0	0	
rapifera hybrids × B.c.ssp. chinensis	1976	3	0	16	2	0	0	0	
rapifera hybrids × B.c.ssp. oleifera	1976	1	0	ca 110	0	0	0	0	
AARR × AAAA									
perviridis hybrids × B.c.ssp. chinensis	1977	1	0	19	6	0	0	0	
chinensis hybrids × B.c.ssp. chinensis	1977	2	0	19	0	0	0	0	
perviridis hybrids × B.c.ssp. perviridis	1978	6	0	113	0	0	0	0	
AARR × RRRR									
perviridis hybrids × 'Palet'	1976	1	1	12	6	1	1	1	
	1978	8	0	98	25	0	0	0	
AARR × AACC									
chinensis hybrids × 'Zephyr'	1977	5	3	51	13	20	10	9	
'Tantal'	1977	3	1	27	1	1	0	0	
perviridis hybrids × 'Zephyr'	1977	12	6	185	52	115	48	39	
'Tantal'	1977	6	1	48	7	7	0	0	
rapifera hybrids × 'Zephyr'	1977	2	1	19	1	1	0	0	
'Tantal'	1977	4	0	83	0	0	0	0	
AAAA × AARR									
B.c.ssp. perviridis × perviridis hybrids	1978	4	0	94	50	0	0	0	
RRRR × AARR									
'Palet' × perviridis hybrids	1978	6	0	70	1	0	0	0	
AACC × AARR									
'Zephyr' × perviridis hybrids	1977	8	8	134	33	208	99	0	98
'Zephyr' × chinensis hybrids	1977	8	8	215	63	256	132	4	118

B. campestris were used as pollinators gave an average of 0.06 seed and
0.03 hybrid per pollination. In 1976, only one chinensis hybrid produced
seed in crosses with B. campestris ssp. chinensis and ssp. oleifera.
This plant originated from an AR hybrid, whose chromosome number had
doubled during regeneration of plantlets in vitro from stem explants. In
total, this plant gave rise to 12 backcross hybrids. In addition, four
hybrids from crosses between this plant and B. campestris ssp. chinensis
(2x) were obtained by culture of 43 embryos in vitro (from 13 pollina-
tions only). In 1977, only one perviridis hybrid produced seed.

Crosses between ×Brassicoraphanus (female) and tetraploid forms of B.
campestris (male) gave very few siliquae and no seed (Table 20). The re-

ciprocal crosses, however, resulted in a much better setting of siliquae, though they were seedless. Crosses between ×*Brassicoraphanus* (female) and tetraploid forms of *R. sativus* (male) gave good setting of siliquae and one seed, but the reciprocal crosses yielded only one siliqua and no seed. In both sets of reciprocal backcrosses, the reciprocals differ distinctly in setting of siliquae, but in opposite directions.

In crosses between ×*Brassicoraphanus* (female) and *B. napus*, especially 'Zephyr', a much better seed-set was obtained; on average about 0.35 seed per pollination. Several females gave seed, although there was wide variation between females in the setting of siliquae and seeds. The mean seed production of the best female in crosses with rape was 3.4 seed per pollination. The seeds from these crosses were larger than from intercrossing ×*Brassicoraphanus* and were on average 2.2 mm in diameter. The reciprocal cross, AACC × AARR, gave rise to even more seed per pollination, on average 1.3. However nearly all 220 plants raised from these seeds turned out to be matromorphs. Only four plants were hybrids and arose from small seeds with a diameter less than 2.0 mm.

5.4 DISCUSSION

The primary AARR hybrids mostly had a well balanced, rather vigorous and regular growth. The growing conditions, however, do not permit far-reaching conclusions about the agronomic value of ×*Brassicoraphanus*, although the vigour of the hybrids indicated potential as a forage crop. The lesser hairiness than fodder radish would make them more palatable for cattle. Morphological characteristics, like abundant flowering and a rather high potential seed production per siliqua indicate that ×*Brassicoraphanus* could yield a much larger number of seed than fodder radish but the seed size is much smaller than of fodder radish. Threshing difficulties, as in *R. sativus*, can be expected to some extent in ×*Brassicoraphanus*, because some ovules are in the indehiscent beak of the siliquae. Given the appearance of the hybrids and the large diversity in *B. campestris* and *R. sativus*, the creation of attractive and variable source material for the production of a new forage crop, ×*Brassicoraphanus*, is possible. The main hindrance in achieving this goal is the very poor fertility of newly synthesized hybrids.

Most crosses between ×*Brassicoraphanus* (female) and diploid forms of *B. campestris* failed to set seed. Only two white-flowered hybrids produced seed; one plant even had an excellent setting of siliquae and seeds. The differences observed in crossability were certainly not related to the flower colour of ×*Brassicoraphanus* as such, as suggested by Kato & Tokumasu (1979). The two yellow-flowered plants set fruit poorly and gave no seed at all.

Reciprocal crosses of ×*Brassicoraphanus* with tetraploid forms of *B.*

campestris and of *R. sativus* were nearly all unsuccessful. The differences in setting of siliquae were remarkable; crosses with *R. sativus* (4x) gave the best results with ×*Brassicoraphanus* as female parent. The reverse held true for crosses with *B. campestris (4x)*. These contrasts in fruit setting might reflect the effect of selection for better crossable genotypes in *B. campestris* and *R. sativus*, which probably occurred during the synthesis of ×*Brassicoraphanus*.

Setting of siliquae and of hybrid seed were quite good in crosses between ×*Brassicoraphanus* (female) and *B. napus*. The reciprocal crosses also resulted in a good seed-set, but most seeds gave rise to matromorphs. Although some matromorphs might have originated from illegitimate selfings, matromorphy must occur frequently in the rape variety 'Zephyr'. The occurrence of hexaploid hybrids in offspring of these crosses further supports this view (Table 36). Strikingly large numbers of matromorphs were also obtained in crosses between *B. napus* and AR hybrids (Table 17). So AR and AARR hybrids apparently have an excellent ability to induce parthenogenesis in *B. napus*. *R. sativus* probably has a similar ability in rape (McNaughton & Ross, 1978).

The reciprocal difference in production of hybrid seed in crosses between ×*Brassicoraphanus* and *B. napus* was strikingly similar to a classic example of unilateral incompatibility, namely the cross between *B. oleracea* and *R. sativus*. In both combinations, crosses are more difficult with species having a C genome for female parent; *B. napus* (AACC) and *B. oleracea* (CC), respectively.

The good seed-set of ×*Brassicoraphanus* plants after pollination by *B. napus* showed that the poor fertility of ×*Brassicoraphanus* was not caused by a disturbed megasporogenesis.

6 xBrassicoraphanus: sterility factors and mating system

6.1 INTRODUCTION

Poor fertility is a normal phenomenon in early generations of ×*Brassi-coraphanus* (Section 7.3.2). Small-scale cytogenetic studies have shown that meiosis in this hybrid is mostly regular: usually only bivalents were found at metaphase I (Terasawa, 1932; Mizushima, 1944b, 1950b; Nishiyama & Inomata, 1966; Tokumasu, 1976). Tokumasu (1976), for example, found only bivalents in 68 % of the pollen mother cells examined; the other cells contained some univalents or multivalents. He concluded that the frequency of meiotic irregularities was too low to cause such poor fertility. Nishiyama & Inomata (1966) showed that meiotic irregularities might reduce fertility in later generations. They examined meiosis in two strains originating from the amphidiploids produced by Terasawa (1932) and found that one strain with highly fertile pollen and high seed-set had regular meiosis and the other strain with pollen of low fertility had slightly irregular meiosis (a few univalents, sometimes fragments). In general, meiosis in ×*Brassi-coraphanus* gave rise to balanced gametes, which were often viable. For example, the primary AARR hybrids usually produced functional and normally reduced egg cells, as indicated by the good crossability with *B. napus* (Section 5.3.2).

So sterility in ×*Brassicoraphanus* must be caused by barriers in the progamic phase and after fertilization. Kato (1971) showed that 50 to 70 % of all embryo sacs (in a few plants of probably an advanced generation) had been fertilized five days after pollination. Fifteen days after pollination, the embryos had reached the globular or early heart stage. Subsequently, the development of embryos was retarded in comparison to that in the parental species and many embryos stopped growing and degenerated.

Tokumasu (1976) and Kato & Tokumasu (1976) observed a sudden increase in fertility in association with a change in flower colour from white to yellow. The authors concluded that a sterility gene controlling the development of embryos (or endosperm) was closely linked to the white-flower gene and that the increase in fertility was caused by an interchange between a *Brassica* and a *Raphanus* chromosome. This interchange finally resulted in a duplication-deficiency type of plant, lacking the sterility gene as well as the white-flower gene. However, the sterility of the white-flowered plants may also be caused by a discordance between the

cytoplasm of *Brassica* and the white-flower gene (or rather genes linked to it) from *Raphanus*. Recently the same authors suggested that there must be still other genes affecting fertility (Tokumasu & Kato, 1980).

Sterility is a serious and poorly understood problem. So various aspects of it were studied: the chromosomal behaviour in meiosis; the occurrence of breeding barriers; the presence and significance of self-incompatibility.

6.2 MATERIAL AND METHODS

Meiosis was examined in G_1 and G_2 plants from the PH and CH population of ×*Brassicoraphanus* (Section 7.2). The cytological techniques were described in Section 2.2.

Breeding barriers and extent of self-incompatibility in ×*Brassico-raphanus* were tested in an experiment with seven PH plants and five CH plants of G_2. About five buds of four inflorescences on each of these plants were emasculated and bagged. Three days later, two inflorescences of each PH and CH plant were pollinated with bulked pollen from all available PH or CH plants, respectively. At the same time, the two remaining inflorescences were selfed. The pistils of those inflorescences were fixed two days after pollination and later studied by ultraviolet micros-copy for pollen germination, penetration of pollen tubes into the style, the number of pollen tubes per style and the number of ovules with a pol-len tube in the micropyle (Section 3.2). The whole test was repeated three weeks later.

Six G_2 plants of the PH population and three G_2 plants of the CH popu-lation were tested separately for setting of siliquae and plant-to-plant variation in number of ovules developed per siliqua and development of embryos. A total of 60 to 80 emasculated flowers per plant were pollina-ted with bulked pollen from several PH or CH plants, respectively. Thirty days later, the developed siliquae and ovules were counted and the deve-lopmental stage of all embryos was determined according to the scheme of Wilmar & Hellendoorn (1968).

6.3 RESULTS

6.3.1 *Meiosis*

Meiosis in ×*Brassicoraphanus* was similar to that in the diploid par-ental species and showed almost exclusively bivalents (Fig. 9A, B). A random sample of euploid G_1 plants from the PH and CH population showed bivalents only in 79 and 87 % of the pollen mother cells, respectively, and for G_2 plants these figures were 91 and 94 % (Table 21).

The main irregularity in meiosis of euploid plants ($2n = 38$) was the
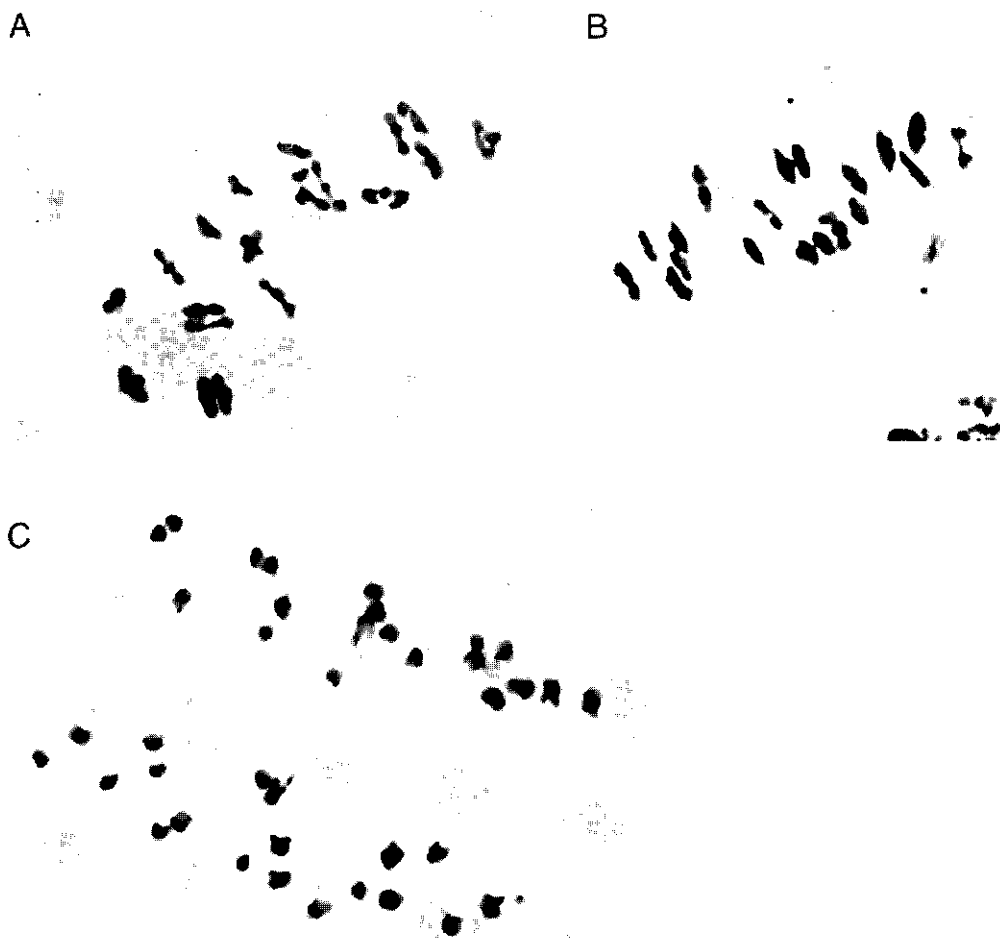
Figure 9. Meiosis in ×Brassicoraphanus (AARR hybrids).
A., B. Pollen mother cells at metaphase I with 19 II (×1540).
C. Pollen mother cell at anaphase I (×1090).

occurrence of univalents. Two univalents were found in about 13 % of all
cells examined and four in only one cell. The only other irregularity,
multivalents, occurred sporadically; only 1.4 % of all cells examined
had a quadrivalent, usually of chain configuration.

Aneuploid plants showed more meiotic irregularities than euploid ones.
The hypoploid plants ($2n = 37$) had one univalent and 18 bivalents in about
82 % of the cells (Table 22). Most of the remaining cells had three or
five univalents. Multivalents were observed sporadically, as in the euploids.
Hyperploid plants ($2n = 39$) showed trivalents in about 25 % of the cells
studied, but most cells contained one, three or even five univalents as
well as bivalents (Table 23). Quadrivalents were not noticed.

Table 21. Chromosome association at diakinesis and metaphase I in pollen mother cells of euploid G_1 and G_2 plants ($2\underline{n} = 38$) from the PH and CH population of ×Brassicoraphanus.[1]

Plant	Number of cells examined	Number of cells with *				
		19II	18II + 2I	17II + 4I	17II + 1IV	1IV + 16II + 2I
PH population/G_1						
PH3	50	42	6		2	
PH5	25	22	2			1
PH6	25	22	3			
PH9	25	22	3			
PH18	25	22	3			
PH10	16	5	11			
PH12	9	1	8			
PH15	25	18	7			
76.2079-2	7	6		1		
76.2257-4	25	21	3		1	
75.2326-1	25	19	5		1	
76.2256-1A	25	22	2		1	
76.2256-1B	25	22	3			
Total	307	244	56	1	5	1
PH population/G_2						
PH2-1	25	22	2		1	
PH3-1	22	18	4			
PH5-1	25	23	2			
PH7-1	25	24	1			
PH7-3	25	22	3			
PH9-6	25	25				
Total	147	134	12		1	
CH population/G_1						
CH1	25	24	1			
CH2	25	21	4			
CH3	25	20	3		2	
76.2104-2	25	22	3			
Total	100	87	11		2	
CH population/G_2						
CH3-4	25	22	3			
CH4-3	25	25				
CH4-6	50	47	3			
Total	100	94	6			

*] I, II, III and IV are symbols for univalents, bivalents, trivalents and quadrivalents, respectively.

At anaphase I, the homologous chromosomes separated synchronously and were usually regularly distributed to the poles (Fig. 9C). Laggards and misdivisions of chromosomes were observed, though rarely (Table 24). About 88 % of all pollen mother cells in the euploid plants showed an equal chromosome distribution (19/19). Like the first meiotic division, the second meiotic division was quite regular with occasional laggards and misdivisions.

The high frequency of pollen mother cells with univalents in the aneuploid, probably monosomic and trisomic, plants resulted in more half chro-

Table 22. Chromosome association at diakinesis and metaphase I in pollen mother cells of hypoploid G_1 and G_2 plants ($2\underline{n} = 37$) from the PH and CH population of ×Brassicoraphanus.

Plant	Number of cells examined	Number of cells with *			
		18II + 1I	17II + 3I	16II + 5I	other **
PH population/G_1					
PH1	25	24	1		
PH4	25	24	1		
PH14	25	23	1		1
76.2147-2	13	7	4	1	1
76.2229-1	25	25			
76.2229-12	4	1	3		
76.2237-7	25	2	14	9	
76.2251-2	25	23	2		
Total	167	129	26	10	2
CH population/G_1					
CH-5	25	20	5		
76.2104-7	25	23	1		1
76.2202-2	25	25			
Total	75	68	6		1
CH population/G_2					
CH2-1	14	12	1		1

*] I, II, III and IV are symbols for univalents, bivalents, trivalents and quadrivalents, respectively.
**] Other chromosome configurations, i.e.
1 III + 15 II + 4 I, 1 IV + 16 II + 1 I, or 1 III + 16 II + 2 I

Table 23. Chromosome association at diakinesis and metaphase I in pollen mother cells of hyperploid G_1 and G_2 plants ($2\underline{n} = 39$) from the PH and CH population of ×Brassicoraphanus.

Plant	Number of cells examined	Number of cells with*			
		18II + 1III	19II + 1I	18II + 3I	17II + 5I
PH population/G_1					
PH8	26	2	16	6	2
PH11	25	6	18	1	
76.2154-5	25	8	14	3	
76.2237-11	25	2	9	12	2
Total	101	18	57	22	4
CH population/G_1					
CH7	25	5	20		
76.2104-3	2	1	1		
Total	27	6	21		
CH population/G_2					
CH1-1	25	10	14	1	
CH4-5	18	7	8	3	
Total	33	17	22	4	

*] I, II, III are symbols for univalents, bivalents, trivalents respectively.

Table 24. Chromosome distribution at late anaphase I and metaphase II in pollen mother cells of G_1 plants from the PH and CH population of ×Brassicoraphanus.

Plants with 2n = 38		Plants with 2n = 37		Plants with 2n = 39	
distribution	number of cells	distribution	number of cells	distribution	number of cells
19 : 19	169	19 : 18	64	19 : 20	29
18 : 20	16	20 : 17	1	19½: 19½	8
19 : 18*	4	18 : 18*	9	19 : 19*	3
17 : 21	1	18½: 18½	17	Other	1
Other	2	Other	13		
Total number of cells examined	192		104		41
Number of laggards per cell	0.021		0.13		0.07
Number of misdivisions per cell	0.016		0.26		0.20

*] plus one laggard

mosomes and laggards per cell in the subsequent meiotic stages than in euploid plants. The probable cause of half chromosomes was centromere misdivision in the univalents. In the euploids, rather high frequencies of unbalanced gametes were produced, because of the occurrence of univalents. Most univalents were included in one of the anaphase groups of chromosomes.

Univalent formation was apparently under genetic control, because most G_1 plants with many univalents were derived from the same Brassica plant, i.e. PH10 and PH12 in Table 21, plant 76.2237-7 in Table 22 and plant 76.2237-11 in Table 23. Not surprisingly, the half-sib families derived from these plants contained a high frequency of aneuploid plants.

6.3.2 *Barriers in the progamic phase*

Stigmas of the cross-pollinated flowers of G_2 plants from the CH and PH population always showed serious incompatibility reactions two days after pollination. Strong ultraviolet fluorescence due to callose deposits occurred in many stigma papillae (Fig. 10A), and pollen grains mostly failed to germinate or produced a short ultraviolet-fluorescent pollen tube. Only few tubes actually penetrated the stigma. The final result of these incompatibility reactions was a poor penetration of pollen tubes into style and ovary. The number of tubes counted per style varied from 0 to 22 and was on an average about 10. It was nearly always less than
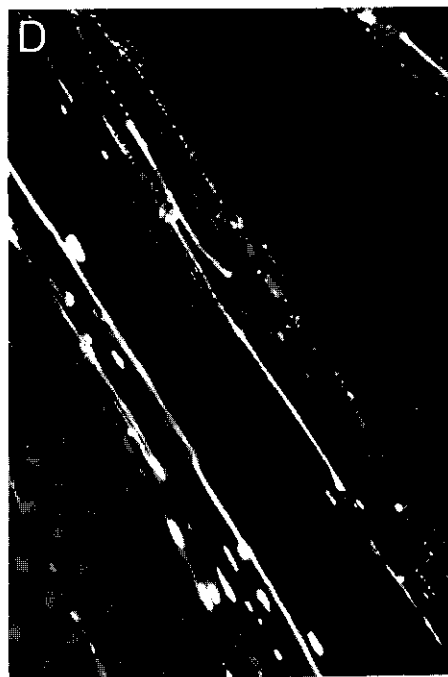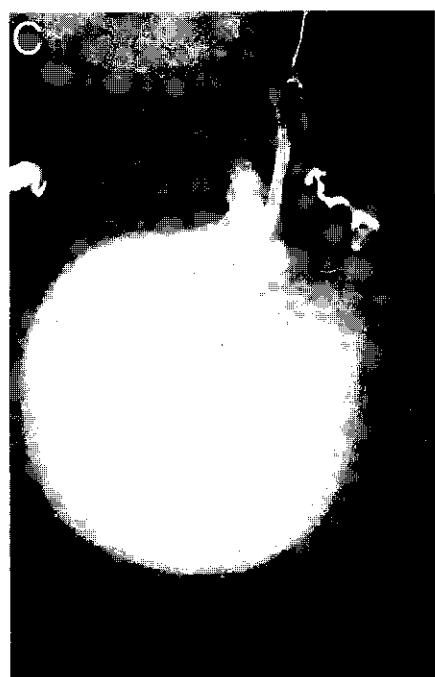
Figure 10. Ultraviolet fluorescence photographs of the progamic phase in
×Brassicoraphanus 2 days after pollination.
A. Callose in stigma papillae after cross-pollination (×100).
B. Callose in stigma palillae after self-pollination (×24).
C. Fertilized ovule (×100).
D. Bundle of pollen tubes in the style after self-pollination (×100).

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the number of ovules in an ovary (about 19). Significant differences were observed between individual plants (Table 25). In a few plants, however, there was rather large variation between the test dates, for instance plant PH9-4.

The pollen tubes that penetrated apparently grew unhindered through the style and nearly all reached the ovary. Many tubes subsequently entered an ovule through the micropyle. Such ovules with a tube in their micropyle were classed as fertilized, although it was unknown whether fertilization had occurred (Fig. 10C). There were large and significant differences in the number of fertilizated ovules per pistil between PH plants but not between the CH plants. Number of fertilized ovules per pistil ranged from 1.2 in PH7-2 to 14.9 in PH3-2. Except for PH9-4, this number was nearly equal on the two evaluation dates. The low number for PH9-4 in the second test was obviously associated with the few pollen tubes in the style at that date; it accounted completely for the difference between the overall means of 6.3 and 4.9 fertilized ovules per ovary (against 5.5 and 5.3 respectively if the values for PH9-4 were excluded).

Table 25 shows a large variation in the ratio of number of fertilized ovules and number of pollen tubes in the style, i.e. the fertilization

Table 25. Results of ultraviolet-fluorescence studies on breeding barriers in the progamic phase of fertilization after cross-pollination in the PH and CH population of ×Brassicoraphanus. These tests were repeated after three weeks. Each value represents the mean of 4 or 5 flowers.

Plant	Number of pollen tubes per style (A)		Number of fertilized ovules per ovary (B)*		Fertilization ratio (B/A)	
	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
PH population						
PH3-2	14.60	17.50	14.00	15.75	0.96	0.91
PH5-1	4.75	11.00	3.25	5.00	0.80	0.42
PH7-1	9.80	6.25	6.00	3.00	0.57	0.50
PH7-2	11.80	11.60	1.40	1.00	0.12	0.08
PH7-3	12.20	11.75	11.00	10.00	0.89	0.83
PH9-2	5.00	8.00	2.00	2.40	0.47	0.31
PH9-4	17.40	2.80	17.00	1.40	0.98	0.36
CH population						
CH1-1	11.00	17.75	5.60	5.50	0.52	0.30
CH4-3	6.50	12.40	3.00	3.60	0.47	0.28
CH4-4	5.60	12.25	3.60	3.25	0.64	0.37
CH6-1	6.80	5.60	5.20	3.00	0.78	0.60
CH7-1	9.75	-	3.75	-	0.39	-
Mean	9.60	10.63	6.32	4.90	0.63	0.47
Least significant difference (0.05)	3.36	5.70	2.80	2.96	0.27	0.26

*] Fertilized ovule was defined as ovule with pollen tube in its micropyle.

ratio. This variation did not merely reflect variation in the number of
pollen tubes in the style because the plant means for these traits were
not significantly correlated (r = 0.18). The fertilization ratio and the
number of pollen tubes in the style were apparently two independent
causes of variation in number of fertilized ovules per ovary. This agrees
with the observation that in plants with poor fertilization the orienta-
tion of pollen tube growth within the ovary was often disturbed as in
the original intergeneric crosses.

6.3.3 *Barriers after fertilization*

In a sample of ×*Brassicoraphanus* plants, the setting of siliquae and
the development of ovules and embryos were assessed thirty days after
cross-pollination. By then, nearly all embryos had reached the final size
and stage. The setting of siliquae ranged from 38 % in CH4-4 to 97 % in
PH7-3 (Table 26). The plants also differed in mean length and outer ap-
pearance of the siliquae (Table 26). Small siliquae were often yellow
green and sometimes slightly shrivelled. Other fruits, mainly the larger
ones, were normal green and often swollen. A positive correlation of
0.65 was found for plants between setting and average length of siliquae.
The variation in both characteristics probably reflected variation in
the number of fertilized ovules, because both traits were significantly
correlated with the number of developed ovules per siliqua (r = 0.93 and
r = 0.65, respectively).

Table 26. Development of siliquae, ovules and embryos on G_2 plants from the
PH and CH population 30 days after pollination with bulked pollen mixture
from various plants of the PH or CH population, respectively.

Plant	Number of polli- nations	Number of siliquae obtained	Mean length of sili- quae (cm)	Number of ovules developed per sili- qua	Number of ovules collapsed after initital development	Number of embryos per siliqua
PH population						
PH3-2	82	77	4.3	6.6	5.2	1.3
PH4-4	62	44	3.7	2.0	1.2	0.6
PH7-2	62	37	1.7	0		0
PH7-3	63	61	2.5	1.7	0.6	0.9
PH9-4	62	51	2.3	0.7	0.6	0.04
PH12-2	61	54	4.3	7.5	1.9	3.8
CH population						
CH4-4	61	23	1.7	0.4	0.4	0
CH4-6	64	59	5.1	8.0	7.6	0.3
CH6-1	86	46	2.8	1.2	1.2	0.02
Mean			3.2	3.1	2.3	0.77

The trait developed ovules per siliqua (Table 26) included all initially developed ovules. There was enormous plant-to-plant variation in it. On the whole, the number of ovules developed per siliqua was lower than the number of ovules with a pollen tube in their micropyle (Table 25). A high proportion of the initially developed ovules later collapsed and shrivelled (Table 26).

Most unshrivelled ovules contained an embryo, but about 26 % of them contained no embryo. The number of embryos per siliqua was only 0.77, which was far less than the number of initially developed ovules. However individual plants differed widely; PH12-2 produced 3.8 embryos per siliqua, whereas in other plants not a single embryo could be detected.

The developmental stage of all embryos was classified according to the system of Wilmar & Hellendoorn (1968). Although this system was devised for Brussels sprouts, it could be readily applied to ×*Brassicoraphanus*. The number of embryos isolated per plant differed greatly, but nearly all plants had an almost continuous frequency distribution of the embryos over the various developmental stages, indicating that there is not a specific stage at which growth and development stopped. The differences in frequency distribution between plants suggest that some variation existed in the degree of inhibition of growth and development (contrast PH7-3 and PH12-2). The condition of the ovules indicated that most embryos had already stopped developing or completed development. Under normal conditions, only the ovules with embryos in the walking stick or mature I + II stage would probably give rise to viable seed (Table 27).

Table 27. Classification by the scheme of Wilmar & Hellendoorn (1968) of all embryos obtained from the crosses mentioned in Table 25.

Plant	Number of embryos	Developmental stage*						
		G	H	ET	T	LT	WS	M
PH population								
PH3-2	97	4	46	25	16	1	3	2
PH4-4	26	6	7	4	5	3	1	0
PH7-3	55	2	8	13	16	6	6	4
PH9-4	2	1	0	0	1	0	0	0
PH12-2	203	17	90	62	26	6	1	1
CH population								
CH4-6	18	0	0	7	3	5	0	3
CH6-1	1	0	0	0	1	0	0	0
Total	402	30	151	111	68	21	11	10

*] G globular stage; H heart + early heart stage; ET early torpedo stage; T torpedo stage; LT late torpedo stage; WS walking stick stage; M mature I + II.

6.3.4. *Mating system*

The parental species of ×*Brassicoraphanus* are predominantly outbree-
ders, because of their self-incompatibility systems accompanied by insect
pollination. ×*Brassicoraphanus* plants also attract insects, but there
was no published data on whether they are self-incompatible. To study
this critical aspect of fertilization, two series of selfings were made
on the same plants as used for tests on breeding barriers in the proga-
mic phase of fertilization. The mean number of ovules fertilized per ova-
ry with selfing and cross-pollination were presented in Table 28. Four
plants were completely self-incompatible (Fig. 10B), and three, PH3-2,
PH7-2 and CH4-3, had a much smaller number of pollen tubes per style after
selfing than after cross-pollination. Absence of pollen tubes in the style
could be caused by a poor pollen germinability. However, pollen stainabil-
ity was not related to self-incompatibility and germinated pollen grains
were observed on selfed stigmas of all plants examined by ultraviolet
microscopy. So such plants were probably truly self-incompatible, as would
be expected for allotetraploids derived from two species with a sporophytic
incompatibility system. In contrast, five plants were fully self-compatible.
In these plants, the number of pollen tubes per style after selfing was
even higher than that after cross-pollination (Fig. 10D). However, the
progamic barrier on the stigma (Section 6.3.2) was observed in all stig-

Table 28. Comparison of ×Brassicoraphanus plants from the PH and CH popula-
tions for number of pollen tubes per style and number of ovules fertilized
per ovary after self-pollination and after cross-pollination. Each value
represents the mean of 8-10 flowers.

Plant	Pollen stainability	Number of pollen tubes per style		Number of ovules fertilized per ovary	
	(%)	crossed*	selfed	crossed*	selfed
PH population					
PH3-2	74	16.05	0.20	14.88	0.00
PH5-1	66	7.88	0.00	4.13	0.00
PH7-1	39	8.03	8.10	4.50	6.20
PH7-2	64	11.70	1.75	1.20	0.00
PH7-3	61	11.98	0.00	10.50	0.00
PH9-2	28	6.50	0.00	2.20	0.00
PH9-4	62	10.10	13.90	9.20	10.10
CH population					
CH1-1	33	14.38	15.45	5.55	5.70
CH4-3	63	9.45	1.50	3.30	1.00
CH4-4	37	8.93	9.45	3.43	3.35
CH6-1	28	6.20	0.00	4.10	0.00
CH7-1	55	9.75	10.10	3.75	2.50
Mean	51	10.08	5.04	5.56	2.40

*] Data from Table 25.

mas, whether self- or cross-pollinated. So self-pollination of self-incompatible plants only further reduced penetration of pollen tubes into the style.

6.4 DISCUSSION

In the populations studied, sterility had several causes. The main factors were incompatibility reactions on the stigma, disorientation of pollen-tube growth within the ovary, poor setting or growth of siliquae, and poor growth and arrested development of embryos. All these factors do not operate equally in all plants or have the same impact on fertility. A disorientation of pollen-tube growth, for example, was absent in some plants. The major cause of sterility was undoubtedly poor growth and arrested development of embryos, probably by malfunctioning of endosperm, as concluded by Kato & Tokumasu (1976).

The sterility factors were quite similar to the breeding barriers observed in the original intergeneric crosses. In terms of the theory of incongruity (Hogenboom 1973; 1975), this means that the barrier and penetration capacity of ×Brassicoraphanus matched poorly. So residual factors of the incongruity system of B. campestris and R. sativus were at least partially responsible for sterility. The situation in such newly established allotetraploids is probably like a door with a double lock: the barrier capacities of B. campestris and R. sativus. Fertilization only occurs if the key, the combined penetration capacities of those species within ×Brassicoraphanus, fits both locks. A consequence of this hypothesis is that selection for easier crossable genotypes within the parental species of ×Brassicoraphanus may also have a positive effect on fertility of allotetraploids derived from such genotypes.

Chromosome association in meiosis agreed quite well with that described by Terasawa (1932), Mizushima (1950b) and Nishiyama & Inomata (1966). The only irregularities were a certain degree of uni- and multivalent formation. Tokumasu (1976), in contrast, found many more multivalents per pollen mother cell. Since one of his F_1 hybrids was a mixoploid (2x/4x) with a diploid sector producing stainable pollen, his F_2 plants may have originated from a 4x × 2x cross. Such an origin would give a more satisfying explanation for the remarkably high frequency of multivalents. The degree of univalence in ×Brassicoraphanus was far less than in octoploid and hexaploid triticale (Scoles & Kaltsikes, 1974), so that aneuploidy in ×Brassicoraphanus seems a less important problem. There was some genetic variation in this characteristic of ×Brassicoraphanus and so selection against plants with a comparatively high degree of desynapsis should reduce the frequency of aneuploids.

Fertility problems in the newly established populations are probably under genetic control and at least some genetic variation seems to exist

in all components of sterility. A simple breeding programme to accumul-
ate fertility factors should improve fertility, as must have happened in
the extensive breeding programmes of ×*Raphanobrassica* (McNaughton, 1973;
Ellerström & Zagorscheva, 1977; Iwasa & Ellerström, 1981). The question
remains whether the populations contain sufficient genetic variation for
all sterility factors. If not, structural changes in the karyotype (e.g.
loss of a chromosome segment carrying a sterility gene) could be benefi-
cial (Tokumasu, 1976). However, a serious disadvantage of such changes
is the possible loss of useful genetic information. For instance, intro-
duction into the present populations of ×*Brassicoraphanus* of the defi-
ciency that was described by Tokumasu (1976) to improve the fertility
would probably soon result in completely homozygous populations for this
deficiency, because of the selective advantage of removal of a sterility
factor. Its genetic load, however, is unknown and so introduction into
the breeding programme should be avoided.

   Mizushima (1950b) reported that ×*Brassicoraphanus* was self-sterile.
The present results, however, show that such allotetraploids could be
self-compatible, despite the presumed presence of a one-locus sporo-
phytic self-incompatibility system in the parental species of ×*Brassi-
coraphanus*. The occurrence of self-compatible plants has also been ob-
served in other artificial cruciferous allotetraploids derived from
self-incompatible diploid species, e.g. in ×*Raphanobrassica* (Howard,
1942a). The occurrence of self-compatible plants in our populations indi-
cates that the self-incompatibility systems of the parental species were
sometimes entirely or partially eliminated. The presence of self-incom-
patible plants does not necessarily imply that self-incompatibility mech-
anisms of the parental species were actively involved; poorly matching
barrier and penetration capacities could be responsible as well. If so,
improvement in matching between barrier and penetration capacities should
increase the proportion of self-fertile plants.

7 Improvement in fertility of xBrassicoraphanus

7.1 INTRODUCTION

Primary allotetraploid hybrids derived from crosses between *B. campestris* and *R. sativus* were poorly fertile or produced no seeds at all (Hosoda, 1946, 1947; Mizushima, 1950b, 1952; Tokumasu, 1976; Olsson & Ellerström, 1980). In my hybrids, for example, the mean seed-set per pollination was only about 0.008. Mizushima (1950b) even failed to obtain any seeds at all. In the subsequent generations of newly established *xBrassicoraphanus*, Olsson & Ellerström (1980) observed deterioration and extinction by the fifth generation. Other authors, however, obtained rather fertile strains of *xBrassicoraphanus* (Terasawa, 1932; Tokumasu, 1976; Tokumasu & Kato, 1980).

Terasawa (1932) reported the occurrence of allotetraploids in the F_4 of a cross between *B. campestris* ssp. *chinensis* (2x) and *R. sativus* (2x). Most of these plants were completely fertile. In 1966, two strains of Terasawa's material still existed (Nishiyama & Inomata, 1966). One of them (2n = 38) had highly fertile pollen and a high seed-set. Another fertile strain of *xBrassicoraphanus* produced by Tokumasu (1976) showed a sudden increase in fertility in F_3 derived from two F_1 hybrids of which the chromosome number was doubled with colchicine. The fertility in this strain gradually improved even further in the subsequent generations (Kato & Tokumasu, 1976). Recently the same authors obtained a new rather fertile strain from an allodiploid of a cross between a triploid *B. japonica* (AAA) and diploid *R. sativus* (RR). One plant obtained by open-pollination (possibly with the fertile strain produced earlier as the pollen donor) was an allotetraploid and produced about three seeds per siliqua.

All existing strains of *xBrassicoraphanus* with a rather good fertility have a narrow genetic basis. In contrast, the hybrids obtained in the present study have a much broader origin. Improvement in fertility is prerequisite for exploitation of any agricultural value of *xBrassicoraphanus*. So fertility improvement and chromosomal stability were studied in early generations of two populations composed of primary hybrids.

7.2 MATERIAL AND METHODS

The two *xBrassicoraphanus* populations coded PH and CH, were composed of primary allotetraploids. The PH population consisted of 48 *perviridis*

Table 29. Size of the PH and CH population in the three
generations studied and the number of isolation cages used
in each generation.

Generation	Population	Number of isolation cages	Number of plants*
G_1	PH	3	48
	CH	1	14
G_2	PH	3	82(-17)
	CH	1	30(-9)
G_3	PH	6	180
	CH	1	25

*] In brackets the number of plants eliminated because of
   highly deviant chromosome numbers.

hybrids from crosses between *B. campestris* ssp. *perviridis* (4x) and the
tetraploid fodder radish accessions 'Palet' and RS14 (Tables 6 and 10).
The CH population was composed of 14 *chinensis* hybrids derived from
crosses between *B. campestris* ssp. *chinensis* (4x) and 'Palet' (Tables 6
and 10).

Both populations were propagated for three generations in isolation
cages (Table 29). In each cage, random mating was stimulated by honey-
bees for almost the whole flowering period. The generations G_2 and G_3 of
the two populations included all half-sib families obtained in the pre-
vious generation. To avoid inbreeding as much as possible, the same num-
ber of seedlings was raised for each half-sib family, if sufficient seeds
were available, and for PH equally distributed over the cages. So PH in
G_1, G_2 and G_3 consisted of 3, 3 and 6 subpopulations, respectively, where-
as CH was propagated as one population. Some plants with exceptionally
high or low chromosome numbers were excluded from G_2 and multiplied sepa-
rately (Table 29). If possible, these plants were replaced by other plants
from the same half-sib families.

The isolation cages used for propagation were 2.5 m × 2.5 m or 3 m ×
3 m and were mounted in a heated greenhouse (18 °C) for G_1 and G_2, and
outdoors for G_3. The plants were raised in pots of about 2,5 litres and
were transplanted into the cages before flowering. The main flowering
period was from February to June in G_1 and G_2 from June to August in G_3.

Four fertility traits, i.e. pollen stainability, number of siliquae
per plant, number of seeds per plant and seed production per siliqua,
were studied in the first three generations of the populations. Pollen
stainability was usually assessed twice at a time interval of several
weeks with lactophenol acid fuchsin by the method of Sass (1964). Pollen
preparations from one flower were used for G_1; about 500 pollen grains
were examined per flower. In G_2 and G_3, a random sample of 200 grains
was examined in bulked pollen from about five flowers.

The remaining fertility traits were monitored at harvest, again for each plant separately. In G_1 and G_2, all siliquae developed were harvested, counted and shelled by hand. All seeds were then counted and the number of seeds per siliqua was calculated. The same procedure was used for G_3 when the number of siliquae per plant was less than 100. If more than 100 siliquae were present in G_3, all siliquae were weighted and seed production per siliqua was estimated in a random sample of 100 siliquae, which was also weighted in order to estimate the total number of siliquae.

Chromosomes were counted at meiosis or mitosis of G_1 and G_2 plants.

7.3 RESULTS

7.3.1 *Chromosome numbers*

The chromosomes of 46 G_1 plants and 118 G_2 plants from the PH and CH populations were counted (Table 30). In G_1, the chromosome numbers ranged from 35 to 39 and among G_2 plants from 34 to 58. Both populations in G_2, had a remarkable bimodal frequency distribution for chromosome number with maxima at 38 and 57. More than 12 % of G_2 plants classified had over 50 chromosomes. In G_2, the frequency of euploid plants ($2n$ = 38 or 57) was higher than in G_1 but the proportion of plants with 38 chromosomes had hardly increased. The number of hypoploid plants, especially in the PH population, was much higher than the number of hyperploid plants, especially in G_2.

The frequency of chromosome numbers in offspring of all seed-producing G_1 plants from the two populations studied is listed in Table 31. G_1 plants with 37 chromosomes gave predominantly euploid plants ($2n$ = 38), like the euploid G_1 plants. Nearly all hypoploid plants in the half-sib progenies of euploid G_1 plants were derived from PH10 and PH12 (Table 31). The chromosome number in offspring of G_1 plants with 39 chromosomes was quite variable. Most aberrant PH plants were derived from the hybrid PH11, which originated from a cross on the same *Brassica* plant as PH10 and PH12, two hybrids that showed a comparatively high frequency of univalents.

Table 30. Frequency of chromosome numbers in G_1 and G_2 of the PH and CH population.

Genera-tion	Popula-tion	Number of plants	Chromosome number														
			34	35	36	37	38	39	40	41	43	45	52	54	56	57	58
G_1	CH	10				3	5	2									
	PH	36		1	1	11	16	7									
G_2	CH	32		1		2	15	4	1	1	2					4	2
	PH	86	1	5	9	12	47	1	2				1	1	1	5	1

Table 31. Frequency of chromosome numbers in half-sib families of G_1 plants with various numbers of chromosomes in the PH and CH population.

Seed parents (G_1 plants)			Chromosome number G_2 plants*																Number of G_2 plants
Chromosome number	Population	Number of plants	34	35	36	37	38	39	40	41	43	45	52	54	56	57	58	
37	CH	1		1			1											2
	PH	3			2	3	11											16
38	CH	4				1	9	3		1		1						15
	PH	11		4	6	3	28		1					1		3		46
				(3)	(6)	(3)	(1)							(1)		(1)		
39	CH	1					3	1			1	1				3	1	10
	PH	2	1	1	1	4	7	1	1				1			2	1	20
			(1)	(1)	(1)	(1)	(2)						(1)			(2)	(1)	
unknown	CH	1				1	2									1	1	5
	PH	2				2	1								1			4

*] In brackets the number of plants from PH10, PH11 and PH12.

In a few half-sib families, G_2 plants with 52-58 chromosomes were found. Of the 25 families raised, only 7 gave rise to such aberrant plants.

The occurrence of hexaploid and highly aneuploid plants in G_2 seemed a serious threat to the chromosomal stability of the PH and CH population. Therefore, all G_2 plants with more than 39 and less than 37 chromosomes were excluded from the two populations. Some aneuploids, however, were not eliminated because their chromosome number was not established properly before transplantation into the isolation cages.

In G_3 of the PH and CH populations, no hexaploids were detected: morphological observations, measurements of pollen size and checks of chromosome number in a few suspects revealed no hexaploids.

7.3.2 Changes in four fertility traits

Changes in pollen stainability, setting of siliquae, seed production and seed-set per siliqua were studied in the first three generations of the PH population (Fig. 11) and CH population (Fig. 12). The results were expressed as cumulative-frequency distributions. The actual number of plants, on which the graphs are based, may differ slightly from each other because of missing data and because a few plants died prematurely and so provided no data. The three generations of the two populations were successively studied in a period of three years. So differences between generations can be attributed to some extent to year influences.

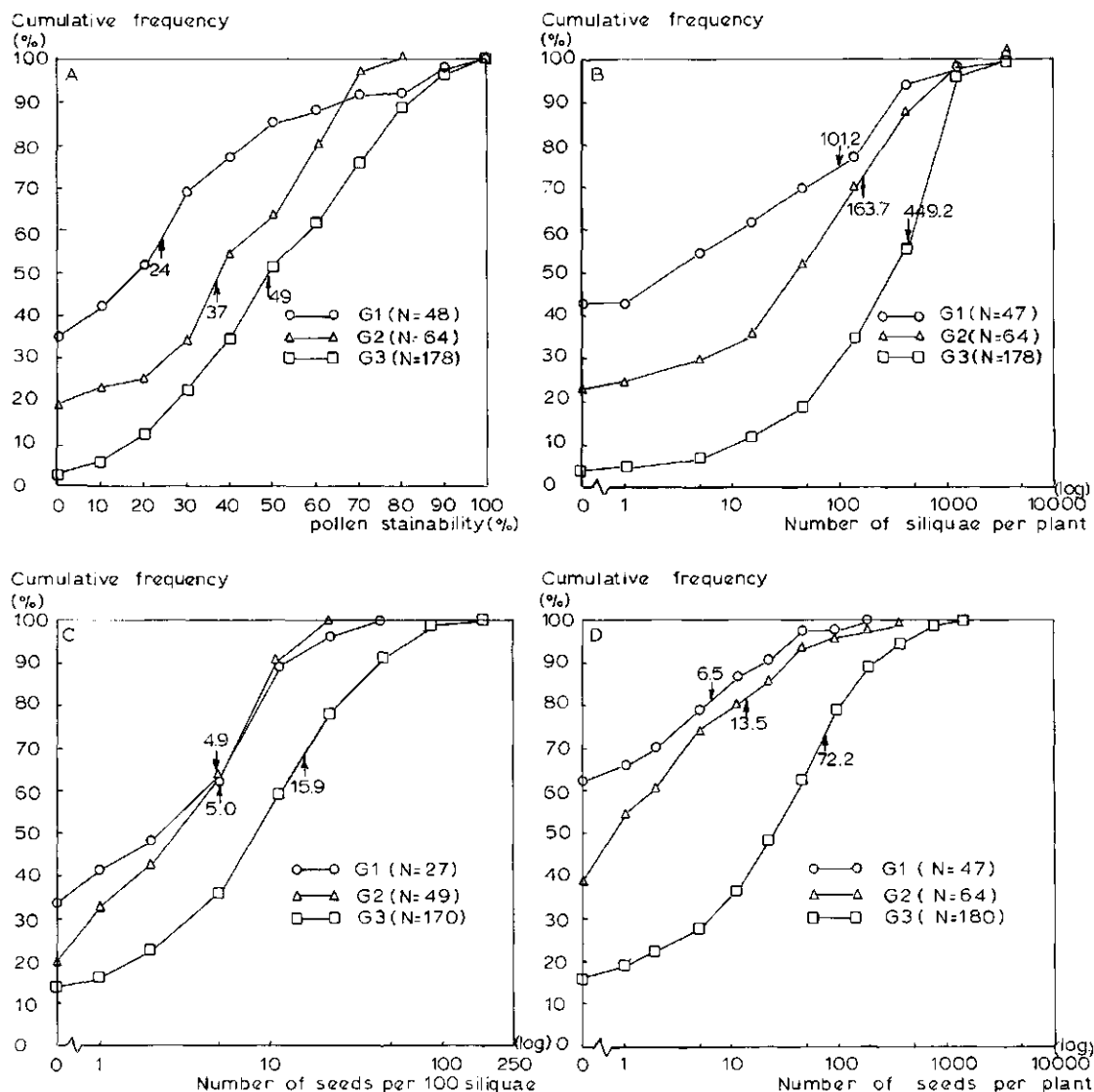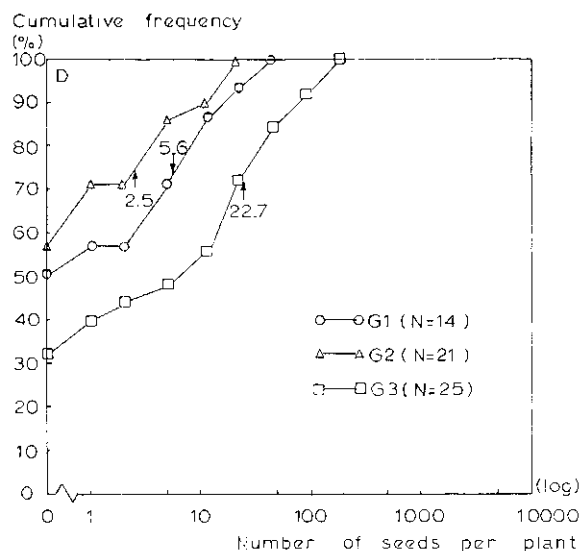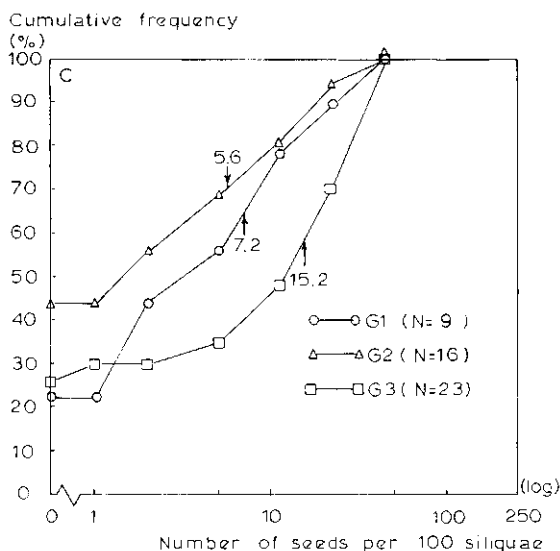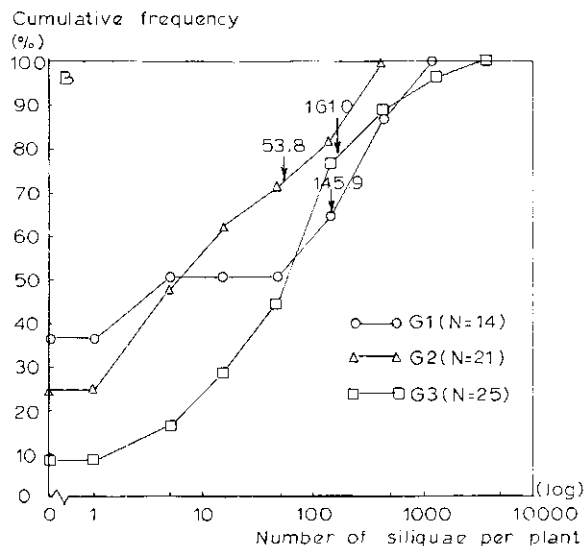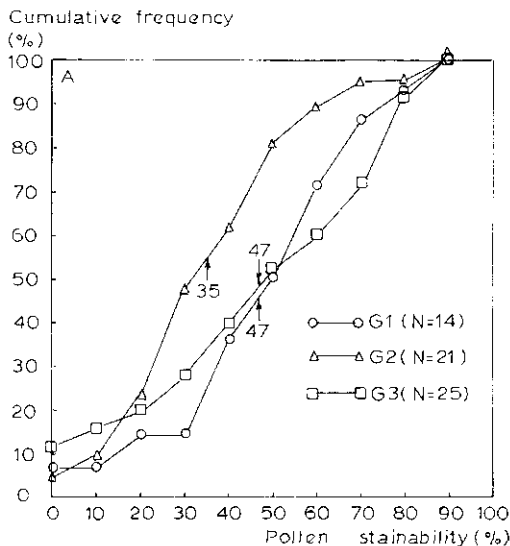The plant-to-plant variation in pollen stainability was very large

Figure 11. Cumulative frequency distributions of various fertility traits in the first three generations of the PH population of ×Brassicoraphanus.
A. Pollen stainability (%).
B. Number of siliquae per plant.
C. Number of seeds per 100 siliquae.
D. Number of seeds per plant.
The population means are marked with an arrow. N = number of plants in the generation.

(Fig. 11A and 12A). About 35 % of G_1 plants of the PH population pro-
duced no stainable pollen at all. This male-sterility had various causes,
such as the absence of pollen shedding or even of flowers in a few plants.
In the subsequent generations, the proportion of male-sterile plants grad-

Figure 12. Cumulative frequency distributions of various fertility traits in the first three generations of the CH populations of ×Brassicoraphanus.
A. Pollen stainability (%).
B. Number of siliquae per plant.
C. Number of seeds per 100 siliquae.
D. Number of seeds per plant.
The population means are marked with an arrow. N = number of plants in the generation.

ually decreased and the mean pollen stainability of the PH population improved from 24 % in G_1 to 49 % in G_3. The CH population had a higher pollen stainability initially, mainly because of fewer male-sterile plants; there was no improvement in subsequent generations; the G_2 even showed a decline.

On the whole, pollen stainability was highly variable. In a separate test, a coefficient of variation of about 30 % was found. So data must be interpreted cautiously.

The PH population also showed an increase in the three successive generations in the number of siliquae harvested per plant (Fig. 11B). The proportion of plants without any siliquae at all decreased considerably. The same was true for the CH population (Fig. 12B). The G_1 plants of the PH population produced only few siliquae. However a striking improvement occurred in the next two generations by a reduction in the proportion of plants without siliquae and plants with few siliquae. The maximum production in the respective generations was 1482, 1386 and 1947 siliquae per plant. The data on the CH population are less indicative because fewer plants were available in each generation. However, G_2 showed a serious drop in production of siliquae per plant, though recovering to level of G_1 followed in the next generation.

The most crucial trait is undoubtedly seed production. The PH and CH populations had very low fertility in G_1 and produced only 6.5 and 5.6 seeds per plant, respectively (Fig. 11D and 12D, respectively). In the following generations of the PH population, a considerable improvement occurred, the G_3 plants producing about 72 seeds per plant. The frequency distributions for seed production in the PH population showed a striking reduction in the proportion of completely seedless plants in successive generations. The last generation showed a marked shift towards a higher productivity. The CH population, on the other hand, had a setback in seed production in G_2 parallel to the decrease in production of siliquae, but the results of G_3 were much better, even than G_1.

The frequency distributions of seed-set per siliqua differed only slightly between G_1 and G_2 of the PH population; about 0.05 seed were obtained in both generations (Fig. 11C). The G_3, however, showed a striking improvement. Some plants even had a production of over one seed per siliqua, and the frequency of plants with only seedless siliquae further decreased. The range of seed-set per siliqua in the three successive generations of the CH population was about the same as in G_1 of the PH population. After a setback in seed-set in G_2 by increase in the number of plants with seedless siliquae, the seed-set in G_3 greatly improved.

In summary, seed production in the PH population improved with an increase in siliqua production and seed-set per siliqua. The two traits were only slightly correlated ($r = 0.17$ in G_3), but were both positively correlated with seed production ($r = 0.53$ and $r = 0.70$ in G_3, respectively). Similar relationships were found in G_3 of the CH population. The improvement in fertility of this population was brought about entirely by an improved seed-set per siliqua. The fourth fertility trait, pollen stainability, was independent of seed production ($r = 0.23$ and $r = -0.07$ in G_3 of the CH and PH populations, respectively).

7.3.3 *Effects of chromosome number on fertility*

Chromosome number varied in the G_1 and G_2 of the two populations of
×*Brassicoraphanus* studied. The impact of this variation on various fer-
tility traits is summarized in Table 32. Euploid plants had, on average,
more stainable pollen and produced considerably more siliquae and seeds
than aneuploids. An exception was the class of plants with 36 chromosomes.
Six of those plants, however, were derived from the same G_1 plant, which
had a comparatively good fertility. So the rather good fertility of this
class was probably not caused by a direct effect of chromosome number on
fertility.

In conclusion, aneuploidy had a small negative effect on all fertility
traits. So selection for fertility favours euploid ×*Brassicoraphanus*
plants. Cytological selection for euploidy seems not necessary.

7.4 DISCUSSION

The populations responded fairly strongly to natural selection for im-
proved fertility. The PH population responded with a gradual improvement
in all the traits; the appearance of the plants improved too, resulting
in G_3 of greater uniformity, abundant flowering, and only a few plants of
abnormal form. The CH population, on the other hand, showed less obvious
trends. In G_2, an obvious loss of vigour was accompanied by a serious
setback in all fertility characteristics. The population, however, reco-
vered to some extent in G_3.

A large variation in all fertility traits studied was still present
in G_3 of both populations. Pollen stainability and fruit setting varied
from zero to almost 100 %. In contrast, seed-set per siliqua was always
far below potential. In G_3, for example, the best seed-set observed was
about 1 and 0.3 seed per siliqua in the PH and CH population, respect-

Table 32. Effects of chromosome number on variation in some fertility traits
in G_1 and G_2 of the ×<u>Brassicoraphanus</u> populations PH and CH.

Characteristic	Chromosome number							
	35	36	37	38	39	40	41	45
Number of plants*	1	7	27(2)	80(2)	14	1	1	1
Mean pollen stainability	15	57	28	41	32	13	0	19
Number of siliquae per plant	3	284	75	159	120	0	4	13
Number of seeds per plant	0	9.7	2.5	13.4	8.1	0	0	0
Number fraction of plants with siliquae (%)	100	86	56	81	57	0	100	100
Number of seeds per 100 siliquae	0	4.1	3.6	5.8	5.7	0	0	0

*] In brackets the number of plants, which died before maturity.

ively, whereas the number of ovules per siliqua was about 19. The large variation in setting of siliquae and of seeds per siliqua in G_3 suggested that succeeding generations would further improve.

Prerequisite for commercial use of ×*Brassicoraphanus* is a stable chromosome number. In G_1 and G_2, most plants had about 38 chromosomes, but several plants had an aberrant number. The most striking phenomenon was the occurrence of hexaploid plants with about 57 chromosomes in some G_2 families from both populations. Such plants probably arose from fusion of an unreduced and a reduced gamete, resulting in plants with the genome constitution AAAARRR. The occurrence of unreduced gametes indicates that ×*Brassicoraphanus* may form unreduced diplosporic or aposporic embryosacs as ×*Raphanobrassica* (Ellerström & Zagorscheva, 1977). The hexaploid G_2 plants usually showed only rather small differences in appearance compared to the tetraploid counterparts, such as larger flower buds and flowers, and usually had somewhat larger stainable pollen. In G_3, no hexaploids were detected by visual screening and by measuring pollen size. The occurrence of such hexaploids could badly affect chromosomal stability in newly created populations. The deterioration and subsequent extinction in Ellerström's material (Olsson & Ellerström, 1980) might have been caused by non-reduction.

The other important aspect of chromosomal stability, the aneuploidy, was already present in G_1. In that generation, the occurrence of aneuploid plants was largely enhanced by the use of autotetraploid forms of the parental species in the production of ×*Brassicoraphanus*. In G_2, most plants derived from monosomic and trisomic G_1 plants turned out to be euploid, indicating that balanced gametes and zygotes were fitter and had a selective advantage. However several aneuploids were still found in G_2, probably because of formation of univalents in meiosis.

In summary, sterility in early generations of ×*Brassicoraphanus* is predominantly caused by various breeding barriers, which were genetically determined. Meiotic irregularities like non-reduction and desynapsis probably had no effect on fertility as such, but may give rise to aneuploid offspring with reduced fertility. However non-reduction and desynapsis seem of little consequence.

8 AAR hybrids and introduction of alien chromosomes into Brassica campestris

8.1 INTRODUCTION

Introgression of *Raphanus* genes into *B. campestris* has not yet been accomplished. The aim of the present programme, was to produce monosomic additions in *B. campestris*, each plant having the normal complement of chromosomes plus an extra chromosome from *R. sativus*. The expectation was that such plants would serve as stepping stones for transfer of particular *Raphanus* genes, e.g. resistance to beet eelworm or to club-root.

The method chosen for production of such additions included two steps: production of AAR hybrids and backcrossing with *B. campestris* (AA) as recurrent parent. This chapter reports attempts to achieve the aim and discusses the problems, that may hamper successful transfer of *Raphanus* genes into *B. campestris*.

8.2 MATERIAL AND METHODS

The attempts to produce AAR hybrids were summarized in Table 20. In total, 19 first backcross hybrids (BC_1) including 4 plants produced by embryo culture were obtained from crosses of two primary AARR hybrids, 75.1040-9 ($2n = 38$) and 76.2229-13 ($2n = 39$) with *B. campestris* ($2x$). The allotetraploid, 75.1040-9, originated from doubling in vitro of the chromosome number of a diploid hybrid from a cross between *B. campestris* ssp. *chinensis* ($2x$) and cv. Siletta ($2x$). The other allotetraploid, 76.2229-13, was obtained from a cross between *B. campestris* ssp. *perviridis* ($4x$) and cv. Palet. The AAR hybrids were studied for form, chromosome number, fertility in crosses with *B. campestris* ssp. *chinensis* ($2x$) and in particular for chromosome association in meiosis.

The AAR hybrids and *B. campestris* ssp. *chinensis* ($2x$) were crossed in the winters 1976/77 and 1977/78 in a heated greenhouse, in which all parent plants were grown together. In the first winter, newly opened flowers of the AAR hybrids were hand-pollinated with bulked pollen from the recurrent parent, *B. campestris*, two or three times a week over a period of about three months. In the second winter, the parents were grown together in an isolation cage with bees for pollination.

The second backcross generation (BC_2) was grown in a similar way to the BC_1 generation in 1977/78; in an isolation cage with the recurrent parent and with bees for pollination.
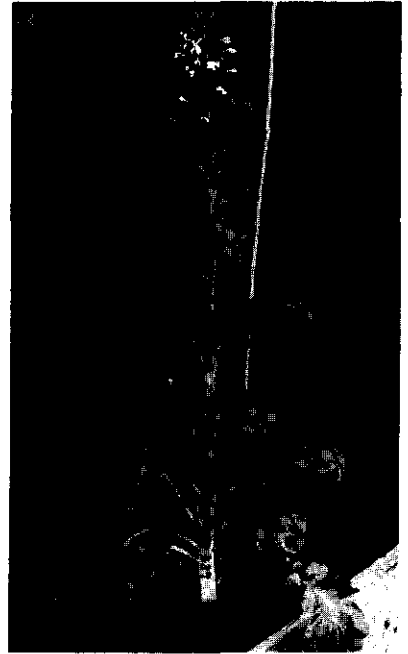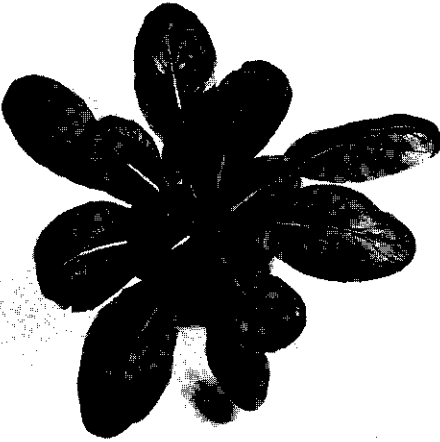
Figure 13. Backcross derivatives of a 'doubled' F_1 hybrid from the cross <u>Brassica</u> <u>campestris</u> ssp. <u>chinensis</u> (2x) × <u>Raphanus</u> <u>sativus</u> cv. Siletta (2x) and <u>B</u>. <u>campestris</u> ssp. <u>chinensis</u> (2x) as the recurrent parent.
A. First backcross derivative (AAR, $2\underline{n} = 29$) in the vegetative stage.
B. First backcross derivative (AAR, $2\underline{n} = 29$) in the generative stage.
C. Second backcross derivative ($2\underline{n} = 25$ plus fragment).

8.3   RESULTS

8.3.1 *Form and chromosome association in AAR hybrids*

   Of 12 BC_1 plants, ten plants had 29 chromosomes and were apparently true allotriploids with the genome constitution AAR. The two remaining plants had 28 chromosomes plus a fragment and 31 chromosomes, respectively. The latter occurred in the progeny of plant 76.2229-13.

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The BC_1 plants derived from crosses between 75.1040-9 (*4x*) and *B. campestris* ssp. *chinensis* or ssp. *oleifera* grew quite vigorously and were in many respects intermediate between the parents. All plants formed a leaf-rosette in the vegetative stage and had shallowly lobed and slightly pubescent leaves (Fig. 13A). The incisions of the leaves were more pronounced in plants from crosses with *B. campestris* ssp. *oleiferea*. In the generative stage, the BC_1 plants initially had an erect main stem with a terminal inflorescence (Fig. 13B) and reached a height of about 1.5 m. Afterwards many secondary branches were formed, resulting in a dense bushy plant. The hybrids derived from plant 75.1040-9 (*4x*) flowered abundantly with plain white flowers like those of plant 75.1040-9.

In contrast, the three BC_1 plants originating from plant 76.2229-13 (*4x*) showed disturbed growth with irregularly shaped leaves. Only one of these plants flowered; the flowers were veined and purple.

None of the AAR hybrids produced stainable pollen, the anthers were already shrivelled before pollen shed. The development of floral parts was apparently quite normal, although poorly developed ovules were observed in a few hybrids. After pollination with pollen from *B. campestris*, a few siliquae developed; they were bivalved with a well developed beak and had a mean length of about 2.6 cm.

Chromosome association at metaphase I of meiosis was studied in three closely related allotriploid plants (Table 33). The presence of a trivalent in a few pollen mother cells and the large proportion of pollen mother cells (86 %) with 10 bivalents and 9 univalents (Fig. 14A) indicate that homologous pairing occurred almost exclusively. However the occurrence of pollen mother cells with eleven bivalents shows that some autosyndetic pairing between R-chromosomes occurred (Fig. 14C). The presence of a few chain-trivalents further suggested that allosyndetic pairing between A and R chromosomes was possible (Fig. 14B; Table 33).

Table 33. *Chromosome association at metaphase I in three AAR hybrids (2n = 29) from crosses between a homozygous* ×Brassicoraphanus *plant (75.1040-9 (4x)) and* Brassica campestris *ssp.* chinensis *(2x).*

Chromosome configurations[*]	Number of cells observed		
	Hybrid 1	Hybrid 2	Hybrid 3
11 I + 9 II	3	1	0
9 I + 10 II	57	34	29
8 I + 9 II + 1 III	1	2	0
7 I + 11 II	7	3	2
Total	68	40	31

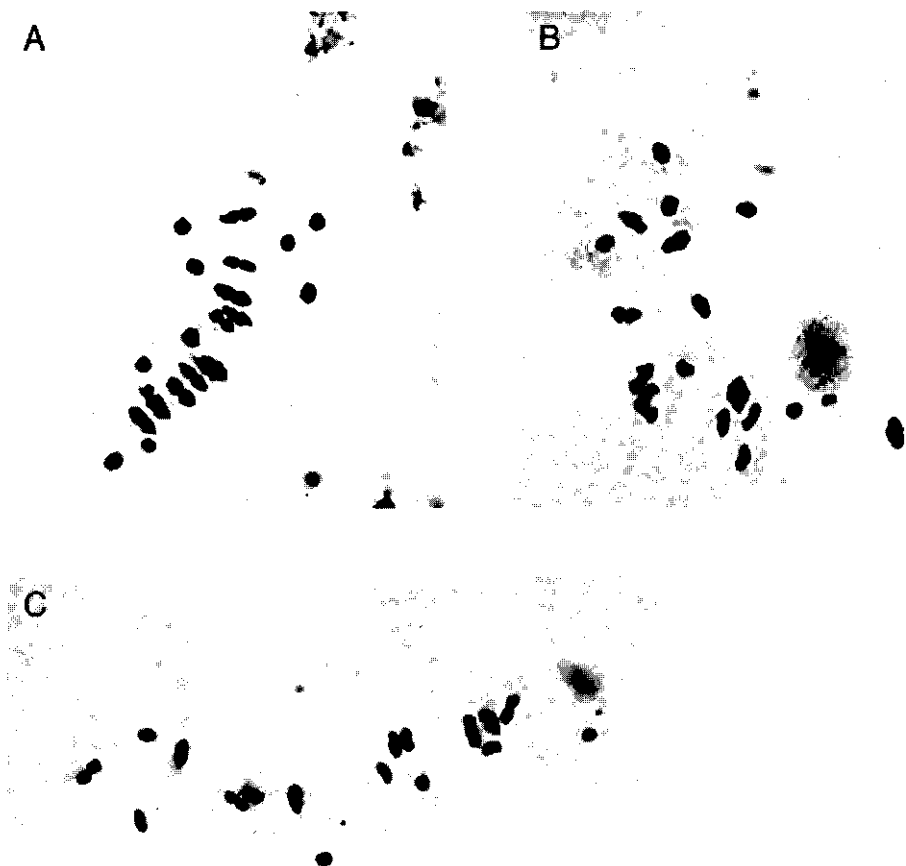[*] I, II and III are symbols for univalents, bivalents and trivalents, respectively.

Figure 14. Chromosome association at metaphase I in AAR hybrids from a cross between
a 'doubled' AR hybrid, 76.1040-9 (4x), and Brassica campestris ssp. chinensis (2x).
A. 10 II + 9 I (×1540).
B. 1 III + 9 II + 8 I (×1540).
C. 11 II + 7 I (×1090).

The differences in chromosome association between the three AAR hybrids
were small. One reason may be that the plants had an identical set of
R and A chromosomes.

8.3.2 *Attempts to introduce alien chromosomes*

In 1977 and 1978, thousands of flowers of the AAR hybrids were pollin-
ated by hand or by bees with pollen from *B. campestris* (2x). The results
of these crosses, i.e. BC_2, are summarized in Table 34. Mostly no siliquae

Table 34. Seed-set on several AAR hybrids using Brassica campestris ssp. *chinensis* (AA) as *pollinator*.

Origin of AAR hybrids	Year	Number of AAR hybrids	Total number of seeds
75.1040-9 (4x) × B.c. ssp. chinensis	1977	11	5
	1978	2	0
75.1040-9 (4x) × B.c. ssp. oleifera	1978	3	27
76.2229-13 (4x) × B.c. ssp. chinensis	1978	3	0

or only a few developed on each plant, except on one AAR hybrid derived from a cross between 75.1040-9 (4x) and *B. campestris* ssp. *oleifera*. Despite considerable efforts, only 32 seeds were produced from six of the plants only.

The BC_2 generation consisted of eleven plants, of which three died before maturity. In 1978, another three BC_2 plants were raised by culture of 26 embryos in vitro. The chromosomes of most full-grown BC_2 plants were counted (Table 35). Most had a few chromosomes less than the original AAR hybrids, but two plants had considerably more chromosomes. So no monosomic additions were obtained. The BC_2 plants were usually weak and flowered poorly. All plants were male-sterile and had white flowers, except two plants with pale yellow flowers. Figure 13C shows the only BC_2 plant with 25 chromosomes (plus a fragment). This plant had rather irregular growth, and most *Raphanus* characteristics were apparently lost.

All BC_2 plants with 29 or fewer chromosomes were backcrossed again with *B. campestris* ssp. *chinensis* (2x). Despite large-scale pollinations, none of these plants produced seeds.

Table 35. Frequency of chromosome numbers in full-grown plants obtained from crosses between AAR hybrids and Brassica campestris (2x) in 1977 and 1978.

Year	Number of plants with 2n =								Total number of plants
	25+f*	26	27	28+f	29	44	48	?	
1977	1			1		1	1		4
1978		1	3		1			2	7
Total	1	1	3	1	1	1	1	2	11

*] f = fragment

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8.4 DISCUSSION

The introduction of single *Raphanus* chromosomes into *B. campestris* proved as yet impossible. My difficulties in obtaining a BC_2 generation were similar to those of Bannerot et al. (1974) and McCollum (1979) in attempts to cross CCR hybrids with *B. oleracea* (CC). My problems might be overcome by using more AAR hybrids and a wider range of pollinators.

In BC_2, the variation in chromosome number was large, as others found for CCR hybrids (Bannerot et al., 1974; McCollum, 1979). Even repeated backcrossing by those authors with *B. oleracea* as recurrent parent did not result in monosomic additions. Bannerot et al. (1974) obtained such an addition by culture in vitro of immature embryos. The same approach may be necessary to obtain additions in *B. campestris*.

Analysis of chromosome association during meiosis in AAR hybrids showed that only homologous A chromosomes paired as a rule. Allosyndetic and non-homologous autosyndetic pairing was rare. The present data confirmed the hypothesis of Mizushima (1980) that the R genome may give rise to one autosyndetic bivalent. The frequency was about 0.086 bivalents per cell. The degree of allosyndetic pairing was presumably even lower but at least one R chromosome seemed sometimes to be bound to an A chromosome. This agrees with the findings on chromosome association in an ARR hybrid by Mizushima (1980). Comparison of chromosome association in AR and AARR hybrids indicates that the degree of allosyndesis in the AAR hybrids was reduced, because of preferential pairing between homologous A chromosomes in the hybrids (Tables 14 and 33). Preferential pairing probably affected especially the degree of allosyndesis, because the degree of autosyndesis within the A genome was already small (Mizushima, 1980) and there was no reason for a decrease in autosyndesis within the R genome.

In conclusion, transfer of *Raphanus* characteristics to *B. campestris* by the method chosen is very difficult and time-consuming, because of difficulties of introducing single R chromosomes into *B. campestris* and the expected low degree of recombination between R and A chromosomes.

9 Ways of transferring Raphanus characteristics to Brassica napus

9.1 INTRODUCTION

Direct transfer of *Raphanus* characteristics to *B. napus* by crossing the two species and backcrossing the F_1 hybrids with the latter is difficult because of the poor crossability between these species (Chopinet, 1942; 1944; Fukushima, 1945; Becker, 1951; Heyn, 1973; Valdivia & Badilla, 1977; McNaughton & Ross, 1978; Rousselle, 1979; Takeshita et al., 1980; my results). Some authors, however, obtained a few hybrids. Takeshita et al. (1980) cultured several ovules from reciprocal crosses in vitro and raised one plant. Crossability could be somewhat better, if tetraploid forms of *R. sativus* were used (Turesson & Nordenskiöld, 1943; Chopinet, 1944). Hybrids from such crosses, however, appear less suitable as starting material for transfer of *Raphanus* genes, because of preferential pairing between R chromosomes and probably a poor crossability with *B. napus*.

Alternative ways for introgression are possible with the various intergeneric hybrids from crosses between *Brassica* species and *R. sativus*, i.e. allopolyploids with the genome constitution CCRR, AARR, or AACCRR. An attractive way is a backcross programme of ×*Raphanobrassica* (CCRR) with *B. napus*, because ×*Raphanobrassica* hybridizes readily with that species (Karpechenko, 1937; Rousselle, 1979). In this way, Stefansson (Winnepeg, Canada, cited by Heyn, 1978) has succeeded in transferring the white-flower trait of radish to rape. Similar work is in progress in Germany (Heyn, 1978) and France (Rousselle, 1979) to transfer fertility-restorer genes from *Raphanus* to rape with cytoplasmic male sterility. Heyn (1978) also crossed such male-sterile rape with an allohexaploid with the genome constitution AACCRR, that had been produced by Chopinet.

As ×*Brassicoraphanus* seemed a good bridge for transfer of *Raphanus* characteristics to *B. napus*, a backcross programme with *B. napus* as recurrent parent was set up with special attention to form, fertility and chromosome association at meiosis of hybrids between ×*Brassicoraphanus* and *B. napus*.

9.2 MATERIAL AND METHODS

Initial material for the backcross programme was 24 male-sterile hybrids from crosses of ×*Brassicoraphanus* (*perviridis* and *chinensis*

hybrids) with 'Zephyr', a Canadian rape variety (Table 20). Before flowering, these plants were transplanted into two isolation cages in a heated greenhouse, in which continuously flowering plants of 'Zephyr', the recurrent parent in the backcross programme, were present. Honeybees were kept in the cages for pollination and so many flowers of the hybrid plants were pollinated with 'Zephyr' pollen. Some embryos were raised in vitro by the method of Harberd (1969, 1971) in order to shorten the life-cycle.

The first backcross generation, partly raised by embryo culture, was grown under isolation in a heated greenhouse together with 'Zephyr'. About 100 hand-pollinations were done on each plant to obtain a second backcross generation.

9.3 RESULTS

9.3.1 *Hybrids from crosses between ×Brassicoraphanus and Brassica napus*

Table 36 summarizes the data on somatic chromosome numbers in hybrids from reciprocal crosses between ×*Brassicoraphanus* and rape. Plants with 38 chromosomes presumably had the expected genome constitution AACR and are called AACR hybrids. A small group of plants from crosses with *B. napus* as female, however, had about 57 chromosomes and probably had the genome constitution AAACCR. Also a plant with 31 chromosomes was found, whose origin was not clear.

Most AACR hybrids grew vigorously and had a balanced habit of growth. The plants exhibited many characteristics of rape, such as an obvious

Table 36. Chromosome numbers of F_1 hybrids of ×Brassicoraphanus × rape 'Zephyr' and setting of siliquae and seeds of the F_1 after pollination with rape 'Zephyr' (BC_1).

Origin of F_1 hybrids	Chromosome number in F_1	Number of F_1 plants	BC_1		
			Number of seed-producing plants	Number of siliquae harvested	Number of seeds
perviridis hybrids × 'Zephyr'	31	1	0	0	0
	38	14	5	129	44
	?	1	1	142	52
chinensis hybrids × 'Zephyr'	38	3	2	61	24
	?	1	1	63	37
'Zephyr' × chinensis hybrids	38	1	1	378	57
	57	1	1	339	102
	58	2	0	0	0

Figure 15. Hybrids from crosses between Brassica napus and AARR hybrids.
A. Offspring of crosses between rape cv. Zephyr and chinensis hybrids in the vegetative
stage. Top row from left to right: rape; hybrid with $2n = 38$; hybrid with $2n = 57$.
Bottom row from left to right; two hybrids with $2n = 58$; rape.
B. AACR hybrid in the generative stage.

stem in the vegetative stage and leaves that were dull green because of
a markedly waxy layer (Fig. 15A). Most AACR hybrids grew rather tall
(upto 2 m) and flowered abundantly (Fig. 15B), but a few did not flower
at all. The flowers of the hybrids were somewhat larger than in ×*Brassi-
coraphanus* and were bright and white. Marked veins, as in ×*Brassicorapha-
nus*, were absent. A few flowers had a yellow sector on the petals. The
siliquae of AACR hybrids were bivalved, as in *Brassica*, and had a length
of about 3 cm, including a beak of about 1.5 cm. The aberrant plant with
31 chromosomes remained small, grew irregularly and did not flower at
all.

The small group of hexaploids consisted of one well balanced and two
irregularly shaped plants (Fig. 15A). The former had 57 chromosomes, was
similar to a related AACR hybrid and flowered abundantly. The two other
plants had 58 chromosomes, flowered poorly and had plain white flowers,
although one plant also produced some yellow flowers. Only the hexaploid
with 57 chromosomes yielded siliquae, whose size and shape were similar
to those of the AACR hybrids.

All F_1 hybrids were completely male-sterile and, after pollination

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with pollen from rape, set siliquae and seed poorly: about half the AACR hybrids and one hexaploid yielded seed (Table 36). However a considerable number of seeds was obtained, because of the large-scale pollinations. Most productive were the two F_1 hybrids with 'Zephyr' as female parent.

The total number of siliquae harvested and of seeds per hybrid were somewhat reduced, because 53 siliquae of AACR hybrids and 40 siliquae of the hexaploid with $2n = 57$ were used for embryo culture. The respective numbers of embryos obtained were 49 and 17. Most embryos had reached the walking-stick stage (Wilmar & Hellendoorn, 1968) or were mature already. In total, 21 and 5 plants, respectively, were obtained by embryo culture.

9.3.2 *Chromosome association in AACR hybrids*

Meiosis in AACR hybrids ($2n = 38$) was analysed in a few pollen mother cells (Table 37). If only homologous pairing were to occur, ten bivalents (two A genomes) and 18 univalents (one C and one R genome) would be expected. The degree of association, however, was higher, the mean numbers per cell of bivalents, trivalents and quadrivalents being 10.59, 0.75 and 0.02, respectively. Consequently the number of univalents per cell, 14.48, was less than expected. The number of chromosome configurations exceeded 10 in 87 % of all pollen mother cells with a maximum of 13 biva-

Table 37. Chromosome association in metaphase I of two AACR hybrids ($2\underline{n} = 38$) from crosses between ×<u>Brassicoraphanus</u> and rape.

Chromosome configurations*	Number of pollen mother cells		
	Plant 1	Plant 2	Total
18 I + 7 II + 2 III	1	0	1
18 I + 10 II	0	1	1
17 I + 9 II + 1 III	1	0	1
16 I + 8 II + 2 III	1	0	1
16 I + 9 II + 1 IV	1	0	1
15 I + 7 II + 3 III	0	1	1
16 I + 11 II	8	1	9
15 I + 10 II + 1 III	4	2	6
14 I + 9 II + 2 III	0	2	2
13 I + 8 II + 3 III	0	1	1
14 I + 12 II	5	4	9
13 I + 11 II + 1 III	2	3	5
12 I + 10 II + 2 III	0	3	3
12 I + 13 II	2	0	2
11 I + 12 II + 1 III	0	1	1
Total	25	19	44

*] I, II, III and IV are symbols for univalents, bivalents, trivalents and quadrivalents, respectively.

lents and multivalents. This number indicates the degree of autosyndesis and allosyndesis within and between the C and R genome, respectively. The number of trivalents in a cell ranged up to 3. An increasing number of multivalents in a cell was associated with a reduction in number of bivalents in that cell. So most trivalents probably resulted from pairing between one C or R chromosome and one pair of A chromosomes.

9.3.3 *First backcross generation*

A few BC_1 plants derived from AACR and AAACCR hybrids were raised, partly by embryo culture (Table 38). Both types of hybrid gave rise to highly variable offspring. Some plants showed slight abnormalities, such as deformed leaves, but all flowered. The flowers were usually white, although in the first backcross generation derived from AACR and AAACCR hybrids some were yellow (Table 38). Also white flowers with a yellow sector occurred occasionally in the first backcross generation, even more frequently than in the parent hybrids.

A promising phenomenon was the occurrence of some partly male-fertile plants in the first backcross generation (Table 38). One plant, derived from the only fertile AAACCR hybrid, even had a pollen stainability of 59 %. The seed set of the first backcross generation was low in crosses with rape.

9.4 DISCUSSION

Introgression of *Raphanus* characteristics into rape by repeated back-crossing of *×Brassicoraphanus* with rape as the recurrent parent is probably feasible. Major drawbacks are low fertility in successive backcross generations, and rather infrequent association between *Raphanus* and *Brassica* chromosomes. The adapted method for large-scale crossing of AACR

Table 38. Flower colour, pollen fertility and seed fertility in BC_1 plants from about 100 hand-crosses of either AACR or AAACCR hybrids with Brassica napus 'Zephyr' and raised from seed or by embryo culture.

Cross	Number of flowering plants	Mode of culture	Flower colour		Number of		
			white	yellow	plants with some stainable pollen	plants producing seed	BC_2 seeds
AACR x AACC	15	embryo	12	3	2	5	20
	14	seed	10	4	1	9	41
AAACCR x AACC	4	embryo	2	2	1	2	75

and AAACCR hybrids with rape by growing hybrids and rape together in an isolation cage proved to be useful for production of a first backcross generation of a reasonable size, despite poor fertility of F_1 hybrids. The infertility in successive backcross generations, which probably will be less severe, may be overcome in a similar way.

Successful transfer of *Raphanus* genes depends mainly on the pairing ability between *Raphanus* and *Brassica* chromosomes. The chances for pairing and exchange between R and A chromosomes in AACR hybrids are probably as small as in AAR hybrids because of preferential pairing between homologous A chromosomes. The rather high frequency of multivalents was probably mainly due to pairing between A and C chromosomes, because in AAC hybrids similar numbers of multivalents occurred (Namai, 1976; 1978) and in AAR hybrids only a few. On the other hand, AACR hybrids are more favourable for exchange between R and C chromosomes. The frequency of pairing between such chromosomes is difficult to estimate because the degree of autosyndesis in the R and C genome is unknown. On the basis of detailed genomic analyses, Mizushima (1980) estimated that the maximum number of autosyndetic pairs was two in the C genome and one in the R genome. The bivalent frequency in the AACR hybrids due to non-homologous autosyndesis was probably low, because such pairing only occurs sporadically in AAR and AAC hybrids (my results; Namai, 1976; 1978). Consequently most pairing involving C or R chromosomes, on average at least 1.34 bivalents per pollen mother cell, was probably caused by allosyndesis. The number of cells examined was too small to justify conclusions about the maximum number of *Raphanus* chromosomes that could pair with a C chromosome. However the observation of up to 9 bivalents in CR hybrids indicates that several R chromosomes are capable of pairing allosyndetically (Harberd & McArthur, 1980; Namai, 1976; 1980; McNaughton, 1973).

10 Prospects

10.1 RESISTANCE TO BEET EELWORM

The absence of resistance to beet eelworm in *Brassica* and its presence in some fodder radish varieties was the main motive for the present research programme. Little was known about the inheritance and mechanism of the resistance when the programme started. Baukloh (1976) presumed simple monofactorial dominant inheritance.

Toxopeus & Lubberts (1979) showed that the multiplication rate of beet eelworm was low on most forms of *R. sativus*. *Raphanus* populations responded well to selection for resistance and Toxopeus and his colleagues at the Foundation for Agricultural Plant Breeding (SVP), Wageningen, have produced populations on which the nematode multiplied much slower than on the original stock (Lubberts & Toxopeus, 1982). Recent studies have shown that resistance results from a shift in the sex ratio of the eelworm in favour of the males (Veerman, 1981) The effect of growing partially resistant fodder radish populations on beet eelworm populations in the soil is being studied in the Netherlands.

The present populations of ×*Brassicoraphanus* also showed more resistance than *Brassica* species. The cyst production per plant, however, was highly variable and the multiplication rate of the eelworm was on average considerably higher than for the *Raphanus* parents. To improve resistance, selection for resistance is proceeding in those populations. The experience with *Raphanus* suggests that considerable improvement is possible.

10.2 ×BRASSICORAPHANUS

In synthesizing a new forage crop, ×*Brassicoraphanus*, several problems arose such as the presence of breeding barriers between the parental species, poor fertility in the first three generations and occurrence of aneuploid and hexaploid plants. Extensive studies have shown that such difficulties hamper the rapid establishment of fertile and broadly based populations. However production of such populations is likely to be possible by selection for improved fertility in the available breeding populations and resynthesis of ×*Brassicoraphanus* in order to broaden the genetic basis. Resynthesis might be facilitated by exploitation of the genetic variation in crossability in *Brassica campestris* and *Raphanus sativus*, use of male-sterile *B. campestris* plants in crosses and improvement of

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embryo-culture techniques for such crosses.

The agricultural value of ×*Brassicoraphanus* could not yet be assessed, because the number of seeds available did not permit field trials. However growth habit and life-cycle are reasons for optimism on suitability as a forage crop. However yield and qualitative aspects like palatability, digestibility, crude protein content and possible occurrence of toxic constituents must be tested as for ×*Raphanobrassica* (McNaughton, 1979).

In summary, ×*Brassicoraphanus* might become a productive forage crop, highly resistant to beet eelworm, powdery mildew (*Erysiphe cruciferarum*) and to club-root. In the agricultural systems of North Western Europe, such a crop might fit into the same niche as stubble turnips, fodder rape and fodder radish, catch crops grown for forage production or green manuring.

10.3 INTROGRESSION

First steps for transfer of *Raphanus* traits to *B. campestris* and *B. napus* were made but introgression of resistance to beet eelworm and club-root have not yet been accomplished because of the long-term nature of such aims and the problems encountered.

Transfer of *Raphanus* genes to *B. campestris* proved to be difficult, because production of alien monosomic additions failed. Only with considerable efforts such additions might be obtained. Alien chromosomes in such plants, however, would pair only sporadically with *Brassica* chromosomes, if at all. So the way chosen has proved unattractive.

The prospects for introgression of *Raphanus* genes into *B. napus* were more promising. ×*Brassicoraphanus* proved a useful bridge between *R. sativus* and *B. napus*, because the crossability with *B. napus* was rather good and the conditions for exchange between *Raphanus* and *Brassica* chromosomes were more favourable in the AACR hybrids. However difficulties in achieving backcrosses restricted success to some extent.

Finally increased knowledge of the mechanism of resistance to the beet eelworm made transfer far less feasible than it seemed to be at the start of the programme. The partial and possibly polygenic character of resistance will hamper transfer to *B. campestris* and *B. napus*.

Summary

In Dutch agriculture, cruciferous arable crops are of little signifi-
cance, although the genus *Brassica* does comprise attractive crops like
rape. The small interest is not surprising in areas with an emphasis on
cultivation of sugar-beets, because beet eelworm (*Heterodera schachtii*)
is able to multiply on *Brassica* crops. Breeding of cruciferous crops with
resistance to this nematode would remove a major objection to such crops.
This consideration was the main motive for the present programme on in-
tergeneric cruciferous hybrids.

The investigations were directed to transfer of genes from fodder ra-
dish (*Raphanus sativus*) to forage rape or oil-seed rape (*Brassica napus*)
and stubble turnip (*Brassica campestris*), especially genes for resistance
to beet eelworm and club-root, and to production of a fertile artificial
allotetraploid, ×*Brassicoraphanus*. Such hybrids from the cross *B. cam-
pestris* × *R. sativus* could have agricultural value as a forage crop, by
combining the rapid growth, resistance to beet eelworm and club-root of
fodder radish and palatability of foliage of *B. campestris*.

On a large scale, $2x \times 2x$, $4x \times 4x$, $4x \times 2x$ and $2x \times 4x$ crosses be-
tween *B. campestris* (AA or AAAA) and *R. sativus* (RR or RRRR) were made,
always with the former species as female parent. This resulted in 0.020,
0.024, 0.001 and 0.002 hybrid per pollination, respectively: in total,
196 hybrids. Moreover, another 58 hybrids were obtained by embryo cul-
ture.

The poor crossability between the two species resulted from poor fer-
tilization and arrested embryo growth and development. On the stigma,
growth and penetration of most pollen tubes into stigma papillae were
disturbed. Such events limited considerably the number of pollen tubes,
penetrating the style. Even so ten or more pollen tubes per style were
counted in most pistils. Bud pollination had no positive effect on this
number. Poor fertilization was predominantly due to erratic growth of
pollen tubes within the ovary. In aggregate, 1 to 2 ovules per pistil
were fertilized. The number of developed ovules in $2x \times 2x$ and $4x \times 4x$
crosses with rather easily crossable accessions of *B. campestris* was al-
most as high. The ovules were small and often contained an embryo with
arrested growth (Table 9). However some ovules were bigger and contained
a normally developed embryo. The embryos from those ovules gave rise only
to matromorphs, and the embryos from small ovules only to hybrids.

For large-scale production of hybrids, culture of embryos in vitro

seemed attractive, since otherwise a great many embryos died before maturity. Results of embryo culture, however, were on the whole rather poor. A larger effect was obtained by selection of more easily crossable geno types of *B. campestris*. The laborious work of crossing could be cut down by using genetic male-sterile plants of *B. campestris*.

The genome constitution of the hybrids was AR, ARR or AARR. The crosses $2x \times 4x$ and $4x \times 2x$ gave rise to a few AARR hybrids. The intergeneric hybrids were in general vigorous, formed a leaf rosette at the vegetative stage and had white, purple or yellow flowers, which were mostly distinctly veined.

Despite deranged meiosis with only 0.47 bivalents per cell, 38 % of all AR hybrids still produced some stainable pollen by formation of $2n$ gametes, probably as a consequence of formation of restitution nuclei during interkinesis. Intercrossing of AR hybrids resulted in few offspring with about half hexaploids (AAAARRR). Intercrossing of such offspring gave rise to a few seeds.

In some of the AR hybrids, the number of chromosomes was doubled by colchicine treatments of rooted cuttings or by crossing of AR hybrids with AARR hybrids (meiotic doubling).

The AARR hybrids were highly sterile; crosses between such hybrids resulted in 0.008 seed per pollination. The low fertility was not caused by meiotic irregularities, but by breeding barriers, which were very similar to the barriers in the original intergeneric crosses. So the incongruity systems of *B. campestris* and *R. sativus* were apparently still active in *×Brassicoraphanus*. Some plants proved self-compatible, i.e. the number of pollen tubes in the style after self-pollination was no less than after cross-pollination.

Two populations of *×Brassicoraphanus* were established and exposed for three generations to natural selection. Both populations improved for pollen stainability, production of siliquae and seeds, and seed-set per siliqua. However fertility was still low after three generations. The best population gave 72 seeds per plant. Variation in fertility traits was still large, so that a considerable improvement was still expected in later generations.

Beside good fertility, a stable number of chromosomes is prerequisite for commercial use of *×Brassicoraphanus*. The occurrence of hexaploids (AAAARRR) and the formation of univalents endanger chromosomal stability. The danger will probably decrease as the fertility of *×Brassicoraphanus* improves.

The AR and AARR hybrids were crossed with various species (Tables 17 and 20). *×Brassicoraphanus* was backcrossed twice with *B. campestris* to produce a series of monosomic additions in *B. campestris*. Unfortunately, the BC_2 plants did not have the desired number of chromosomes and were completely sterile. *×Brassicoraphanus* and *B. napus* hybridized readily, if

the intergeneric hybrids were used as female parent. The reciprocal crosses resulted mainly in matromorphs and only some hybrids, mostly hexaploid, with the genome constitution AAACCR. The F_1 hybrids (AACR and AAACCR) were subsequently twice backcrossed with *B. napus*. Studies of chromosome association in the meiosis of AAR and AACR hybrids showed, that the former types were rather promising for the introgression of *Raphanus* genes into a *Brassica* species.

Samenvatting

In de Nederlandse landbouw is de teelt van kruisbloemige landbouwge-
wassen van geringe betekenis, hoewel het geslacht *Brassica* aantrekkelijke
landbouwgewassen omvat, zoals koolzaad. In streken met een intensieve
teelt van suikerbieten is de geringe belangstelling voor landbouwcruci-fe-
ren echter niet zo verwonderlijk, omdat het bietecystenaaltje (*Heterodera
schachtii*) zich op *Brassica*-gewassen vermeerdert. Het kweken van rassen
van landbouwcruciferen met resistentie tegen dit aaltje zal naar verwach-
ting een belangrijke beperkende factor voor de teelt van dergelijke ge-
wassen wegnemen. Deze overweging vormde het hoofdmotief voor het verwe-
zenlijken van een soortkruisingsprogramma.

Het onderzoek was gericht op problemen die samenhangen met de over-
dracht van genen uit bladramenas (*Raphanus sativus*) naar bladkool of
koolzaad (*Brassica napus*) en stoppelknol (*Brassica campestris*), in het
bijzonder genen voor resistentie tegen het bietecystenaaltje en knolvoet,
en het produceren van een fertiele kunstmatige allotetraploid, ×*Brassi-
coraphanus*. Zulke hybriden uit de kruising *B. campestris* x *R. sativus*
kunnen naar verwachting landbouwkundige waarde hebben als voedergewas
door combinatie van de snelle groei en resistentie tegen het bietecysten-
aaltje en knolvoet van *R. sativus* met de smakelijkheid van *B. campestris*.

Tussen *B. campestris* (AA of AAAA) en *R. sativus* (RR of RRRR) zijn op
grote schaal $2x \times 2x$, $4x \times 4x$, $4x \times 2x$ en $2x \times 4x$ kruisingen gemaakt;
steeds met de eerstgenoemde soort als moeder. Dit leverde respectieve-
lijk 0,020, 0,024, 0,001 en 0,002 hybriden per bestuiving op; in totaal
196 hybriden. Toepassing van embryocultuur leverde nog eens 58 hybriden.

De slechte kruisbaarheid tussen de twee soorten was het gevolg van
(1) een slechte bevruchting en (2) een geremde embryogroei en -ontwikke-
ling. Op de stempel waren de groei en de penetratie in de stempelpapil-
len van de meeste pollenbuizen in sterke mate gestoord. Dergelijke reac-
ties veroorzaakten een aanzienlijke beperking van het aantal pollenbui-
zen, dat de stijl wist binnen te dringen. Toch werden in het grootste
deel van de stampers tien of meer pollenbuizen per stijl geteld. Knop-
bestuiving had geen positieve invloed op dit aantal. De slechte bevruch-
ting werd vooral veroorzaakt door een gestoorde oriëntatie van de pollen-
buisgroei in het vruchtbeginsel. Gemiddeld werden 1 à 2 zaadknoppen per
stamper bevrucht. Het aantal uitgegroeide zaadknoppen was bijna net zo
groot in $2x \times 2x$ en $4x \times 4x$ kruisingen met goed kruisbare *B. campestris*-
herkomsten. De zaadknoppen waren klein en bevatten vaak een embryo, waar-

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van de groei meestal voortijdig stagneerde (Tabel 9). Sommige zaadknoppen waren echter aanzienlijk groter en bevatten een normaal ontwikkeld embryo. Uit deze grote zaadknoppen werden door embryocultuur uitsluitend matromorfen verkregen en uit de kleine zaadknoppen alleen hybriden.

Voor grootschalige produktie van hybriden leek in vitro opkweek van hybride-embryo's aantrekkelijk, omdat erg veel embryo's in een onvolgroeid stadium afsterven. De resultaten van embryocultuur waren echter over het geheel nogal teleurstellend. Betere resultaten werden verkregen door de selectie van goed kruisbare *B. campestris*-genotypen. Het tijdrovende kruisingswerk kon voorts worden vermeden door genetisch mannelijk steriele *B. campestris*-planten te gebruiken.

De genoomsamenstelling van de verkregen hybriden was AR, ARR of AARR. Het was opmerkelijk dat $2x \times 4x$ en $4x \times 2x$ kruisingen enkele AARR-hybriden opleverden. De geslachtshybriden waren over het algemeen groeikrachtig, vormden in het vegetatieve stadium een bladrozet en hadden witte, paarse of gele bloemen, die meestal duidelijk geaderd waren.

Ondanks een volledig gestoorde meiose met gemiddeld slechts 0,47 bivalenten per cel produceerde toch nog 38 % van alle AR-hybriden enig kleurbaar stuifmeel. Dit hield verband met het ontstaan van $2n$-gameten waarschijnlijk als gevolg van de vorming van restitutiekernen tijdens de interkinese. Onderlinge kruising van AR-hybriden leverde een kleine nakomelingschap op, die voor iets meer dan de helft uit hexaploiden (AAAARRR) bestond. Onderlinge bestuiving van de planten uit deze nakomelingschap gaf een klein aantal zaden.

Van een deel van de AR-hybriden werd het aantal chromosomen verdubbeld door (1) okselknoppen van bewortelde stekken met colchicine te behandelen en (2) door kruising van AR-hybriden met AARR-hybriden (meiotische verdubbeling).

De AARR-hybriden waren in hoge mate steriel; onderlinge kruisingen leverden gemiddeld 0,008 zaden per bestuiving op. De geringe fertiliteit werd niet veroorzaakt door onregelmatigheden in de meiose, maar door kruisingsbarrières die veel overeenkomst vertoonden met de barrières in de oorspronkelijke geslachtskruising. Dit wijst erop dat in ×*Brassicoraphanus* de incongruentiesystemen van *B campestris* en *R. sativus* nog actief zijn. Een deel van de planten bleek bij zelfbestuiving zelf-compatibel, d.w.z. het aantal pollenbuizen in de stijl was na zelfbestuiving niet lager dan na kruisbestuiving.

Twee populaties van ×*Brassicoraphanus* zijn samengesteld en zijn gedurende drie generaties min of meer overgelaten aan natuurlijke selectie. Beide populaties vertoonden verbetering van pollenfertiliteit, hauw- en zaadproduktie en zaadzetting per hauw. Na drie generaties was het fertiliteitsniveau echter nog laag. De beste populatie leverde gemiddeld ongeveer 72 zaden per plant op. De variatie voor de verschillende fertiliteitskenmerken was nog groot, zodat naar verwachting de fertiliteit in de volgende generaties nog aanzienlijk zal verbeteren.

Naast een goede fertiliteit is een stabiel chromosoomaantal een nood-
zakelijke voorwaarde voor commercieel gebruik van ×*Brassicoraphanus*. Het
ontstaan van hexaploiden (AAARRR) en de vorming van univalenten zijn een
potentieel gevaar voor de chromosomale stabiliteit. Naar verwachting wor-
den deze gevaren minder, indien de fertiliteit van ×*Brassicoraphanus* ver-
betert.

De AR- en AARR-hybriden zijn met diverse soorten gekruist (Tabel 17
en 20). ×*Brassicoraphanus* is tweemaal met succes teruggekruist met *B. cam-
pestris*, teneinde in *B. campestris* een reeks monosome addities te produ-
ceren. De T_2-planten hadden evenwel niet het gewenste aantal chromosomen
en waren volledig steriel. Kruising van ×*Brassicoraphanus* met *B. napus*
verliep opvallend goed met de geslachtshybride als moeder. De reciproke
kruisingen leverden daarentegen hoofdzakelijk matromorfen op en slechts
enkele, meest hexaploide hybriden met de genoomsamenstelling AAACCR. De
F_1-hybriden (AACR en AAACCR) werden vervolgens tweemaal met succes terug-
gekruist met *B. napus*. Studies van de chromosoomassociatie in de meiose
van AAR- en AACR-hybriden tonen aan, dat de laatstgenoemde typen vrij
kansrijk zijn met betrekking tot de introgressie van *Raphanus*-genen in
een *Brassica*-soort.

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